Biochem. J. (1984) **217**, 675–683
Printed in Great Britain

Characterization of the multiple forms of hydroxymethylbilane synthase from rat spleen

D. Clive WILLIAMS
Department of Biochemistry, Trinity College, Dublin 2, Ireland

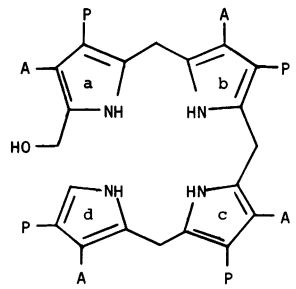
(*Received 4 July 1983/Accepted 30 September 1983*)

1. Phenylhydrazine treatment induced hydroxymethylbilane synthase activity (EC 4.3.1.8) in rat spleen, erythrocytes and liver by 40-fold, 7.5-fold and 6-fold respectively. 2. Five multiple forms of the enzyme were resolved by DEAE-cellulose chromatography. In the presence of phenylmethanesulphonyl fluoride only three forms, two major and one minor, were resolved by the fractionation, suggesting that two of the original forms arose by proteolytic modification. Heat treatment (70°C) in the presence of proteinase inhibitor converted one of the major forms into the other major form. Product isomer analysis suggested that this heat-labile form represented an enzyme–substrate covalent intermediate and not a hydroxymethylbilane synthase–uroporphyrinogen III synthase complex. 3. Identical elution profiles and kinetic properties of the enzymes from rat spleen and erythrocytes suggested that the enzyme isolated from spleen was possibly from stored erythrocytes. 4. Sephadex G-75 chromatography of the heat-stable DEAE-cellulose-purified form yielded pure enzyme as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The M_r was found to be 43000 ± 1500. 5. Initial-velocity studies on all enzyme forms showed a hyperbolic dependence of velocity on substrate concentration, demonstrating the existence of a displacement-type mechanism. For the heat-stable form $V_{max.}$ varied with pH as a typical bell-shaped curve, indicating that two ionizable groups with pK values of 7.4 and 8.8 are important for catalysis. K_m decreased with decreasing pH on the acid side of the pH optimum, suggesting the absence of ionization of a group with pK7.4 in free enzyme or substrate.

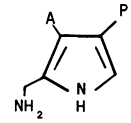
Hydroxymethylbilane synthase [formerly named porphobilinogen deaminase or porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] catalyses the formation of hydroxymethylbilane (1) and four molecules of ammonia from four molecules of porphobilinogen (2) (Battersby *et al.*, 1979*a*,*b*; Burton *et al.*, 1979; Scott *et al.*, 1980). The hydroxymethylbilane can be converted into the cyclic tetrapyrrole uroporphyrinogen III (3) with reversal of ring d by uroporphyrinogen III synthase (EC 4.2.1.75, formerly called co-synthase) (Battersby *et al.*, 1979*a*; Jordan *et al.*, 1979).

It has been established from single-turnover experiments with labelled porphobilinogen (Battersby *et al.*, 1979*c*; Jordan & Seehra, 1979; Seehra & Jordan, 1980) that the order in which the four porphobilinogen rings are added by hydroxymethylbilane synthase is ring a followed by ring b, ring c and finally ring d (see structures 1 and 3). Under normal conditions no intermediates between porphobilinogen and uroporphyrinogen III are liberated into solution, hydroxymethylbilane synthase preferring to synthesize tetrapyrrole by a mechanism involving only enzyme-bound species (Anderson & Desnick, 1980; Berry *et al.*, 1981; Jordan & Berry, 1981; Williams *et al.*, 1981), with uroporphyrinogen III synthase rapidly converting hydroxymethylbilane into uroporphyrinogen III (Battersby *et al.*, 1979*a*). Hydroxymethylbilane synthase has been completely purified from several sources, including spinach (Higuchi & Bogorad, 1975), *Rhodopseudomonas spheroides* (Davies & Neuberger, 1973; Jordan & Shemin, 1973) and human erythrocytes (Anderson & Desnick, 1980), and partially purified from several sources, including *Euglena gracilis* (Williams *et al.*, 1981) and bovine liver (Sancovich *et al.*, 1969).

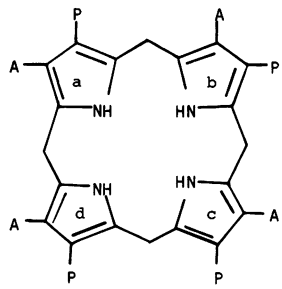
Multiple forms of the enzyme have been reported by Anderson & Desnick (1980) and Jordan & Berry (1981) from human erythrocytes and



(1)



(2)



(3)

A = CH_2-CO_2H

P = CH_2-CH_2-CO_2H

Rhodopseudomonas spheroides respectively, representing stable enzyme–substrate covalent intermediates. Multiple forms of human erythrocyte enzyme on DEAE-cellulose chromatography and gel electrophoresis have also been reported by Miyagi *et al.* (1979); however, the relationship (if any) to those reported by Anderson & Desnick (1980) has not been established.

The present paper describes the characterization of the multiple forms of hydroxymethylbilane synthase from rat spleen and blood.

Materials and methods

Materials

DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25 and G-75 gels were obtained from Pharmacia, London W.5, U.K. 4-Dimethylaminobenzaldehyde was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Porphobilinogen was prepared by the Rapoport–Wurziger method (Battersby *et al.*, 1977) and was over 95% pure as determined with Ehrlich's reagent (4-dimethylaminobenzaldehyde) by the method of Mauzerall & Granick (1956), by using $\varepsilon_{553} = 57700\,M^{-1}\cdot cm^{-1}$. Phenylhydrazinium chloride (AnalaR) was from BDH Chemicals, Poole, Dorset, U.K., and was recrystallized as the hydrochloride from water before use. Coproporphyrin I dihydrochloride was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Coproporphyrin III was obtained by hydrolysis and decarboxylation or uroporphyrin III octamethyl ester in 0.3M-HCl at 180°C for 4h. The uroporphyrin III octamethyl ester was enzymically prepared from porphobilinogen by using a partially purified hydroxymethylbilane synthase/uroporphyrinogen III synthase fraction from *Euglena gracilis* followed by extraction of the oxidized enzymic product into ethyl acetate/acetic acid (3:1, v/v) (3vol.) and subsequent extraction of the organic phase with 1M-HCl. Esterification was completed (24h) on the dried acid extract by using 5% (v/v) H_2SO_4 in methanol.

Assay of hydroxymethylbilane synthase

Hydroxymethylbilane synthase activity was measured by monitoring porphyrinogen production. Fractions containing enzyme were incubated aerobically in the dark at 37°C for 30min or 1h in 50mM-sodium phosphate buffer (titrated to pH7.5 with NaOH solution) containing porphobilinogen (100μM) in a total volume of 1ml. Trichloroacetic acid (5M) (100μl) was added and the suspension left on ice for 5min. [Addition of acid effects cyclization of any residual hydroxymethylbilane to uroporphyrinogen (Battersby *et al.*, 1979*a*,*b*).]

Iodine solution [10μl of 1% (w/v) I_2 in water with sufficient KI to dissolve the I_2] was added to the suspension to oxidize all porphyrinogens to porphyrins. The suspension was incubated at 37°C for 5min, after which time the excess I_2 was then reduced with 20μl of aq. 2% (w/v) $Na_2S_2O_3$. The protein precipitate was removed by filtering the suspension through a cotton-wool plug held in a Pasteur pipette. The porphyrin content was determined by measuring the A_{406} and by using $\varepsilon_{406} = 528000\,M^{-1}\cdot cm^{-1}$ (Rimington, 1960). For post-DEAE-cellulose enzyme fractions, filtration of assay solutions was found to be unnecessary. 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.

Studies on initial velocities

Hydroxymethylbilane synthase activity was measured by monitoring porphyrinogen production. Enzyme was incubated aerobically in the dark at 37°C for 30 or 60min in 50mM-sodium phosphate buffer, pH7.5, and porphobilinogen (0.2–50μM), in a total volume of 2ml. Trichloroacetic acid (5M) (200μl) was added and the suspen-

sion exposed to sunlight for 20min (addition of acid effects cyclization of any residual hydroxymethylbilane to uroporphyrinogen, which is then oxidized to uroporphyrin). The suspension was filtered through a cotton-wool plug held in a Pasteur pipette, directly into a fluorimeter cuvette. Fluorescence at 597nm, after excitation at 405nm, was measured in a Perkin–Elmer MDF-44B spectrofluorimeter. Porphyrin content was determined from a standard curve of fluorescence of uroporphyrin III in the standard assay solutions. The concentration of a standard uroporphyrin III solution was determined spectrophotometrically by using $\varepsilon_{406} = 528000\,M^{-1}\cdot cm^{-1}$ (Rimington, 1960). A linear fluorescence response was found over the range 1nM–1μM-porphyrin. 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 the incubation times. For pH studies, sodium phosphate buffers were used in the range pH6.0–8.7 and sodium borate buffers in the range pH8.0–10.0.

Stability of the enzyme was demonstrated by incubating it separately at pH6 and pH10, sampling at time intervals and assaying at pH7.5 and 37°C as described.

K_m and $V_{max.}$ values (±S.E.M.) were obtained by direct fitting of the data to a rectangular hyperbola by unweighted non-linear regression by using the method of Wilkinson (1961).

Determination of protein

For routine column monitoring, protein concentration was estimated by measurement of A_{280} by assuming a value for A_{280} of 1.0 for a 1mg/ml solution in a 1cm-path-length cuvette.

Phenylhydrazine treatment of rats

Male rats (Wistar strain) were injected intraperitoneally with 2ml of neutralized freshly prepared phenylhydrazine solution (0.4%, w/v) at 14:00h on day 1 and at 10:00h and 14:00h on day 2. The animals were killed on day 6, and the enlarged spleens and livers were immediately removed and chilled on ice. This procedure is essentially that of Levin & Coleman (1967) for induction of the enzyme in mice. It was noted that the spleens increased in weight from approx. 0.5g to 3.0g by this treatment. In separate experiments, blood was collected from similarly treated animals by cardiac puncture into a heparinized syringe before chilling on ice.

Conditions for preparing buffers containing phenylmethanesulphonyl fluoride

Phenylmethanesulphonyl fluoride (0.1M in acetone) was added to distilled water at 80°C, with vigorous mechanical stirring, to a final concentration of 2mM. This hot aqueous solution was immediately added to 9vol. of cold buffer solution, with vigorous mechanical stirring such that the final phenylmethanesulphonyl fluoride concentration was 0.2mM and the components of the buffer were at their required concentrations. These buffers were cooled to 4°C and used within 4h.

Preparation of homogenates and lysates from rat tissues

All procedures, unless otherwise stated, were performed at 4°C. Spleens and livers, precooled at 4°C, were washed with 10mM-sodium phosphate buffer (titrated to pH7.5 with NaOH) containing 0.25M-sucrose, 1mM-EDTA and, in some specified cases, 0.2mM-phenylmethanesulphonyl fluoride. The tissues were sliced and homogenized in 4vol. of the same buffer by ten passes in a Dounce Teflon-pestle homogenizer. The homogenate was centrifuged at 23000*g* for 1h, and the supernatant was desalted by chromatography on Sephadex G-25 equilibrated with 10mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA and, in some specified cases, phenylmethanesulphonyl fluoride (0.2mM). The protein fractions were collected and pooled.

Chilled blood was diluted 4-fold with 10mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA, 0.9% NaCl and, in some specified cases, phenylmethanesulphonyl fluoride (0.2mM), and centrifuged at 1000*g* for 10min. The cells were then twice resuspended in 10vol. of the same buffer before centrifugation as previously. The washed cells were resuspended in 3vol. of 10mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA and, in some specified cases, phenylmethanesulphonyl fluoride (0.2mM), and incubated with stirring at 4°C for 45min before centrifugation at 23000*g* for 1h. The supernatant was chromatographed on Sephadex G-25 as described above for other supernatants.

DEAE-cellulose chromatography of hydroxymethylbilane synthase from various rat tissues

Enzyme, after Sephadex G-25 chromatography, was applied to DEAE-cellulose (DE-52) columns (either 20cm×2cm for 15–40-rat scale, or 15cm×1cm for two-rat scale) previously equilibrated with 10mM-sodium phosphate buffer, pH7.4, containing 1mM-EDTA and, in some specified cases, phenylmethanesulphonyl fluoride (0.2mM). The column was washed with the same buffer, followed by a linear salt gradient of 0–100mM-KCl in the same buffer (20cm×2cm column, 2×600ml gradient; 15cm×1cm column, 2×250ml gradient). Fractions were collected and assayed for enzyme activity and protein (A_{280}).

Preparation of heat-treated hydroxymethylbilane synthase

The Sephadex G-25 eluate containing protein from phenylhydrazine-treated rats (15) was prepared as described above and divided into 25ml portions in glass boiling tubes of uniform diameter. These tubes were incubated in a water bath at 70°C for 15min before rapid cooling in an ice/water mixture. The suspension (at 4°C) was centrifuged at 23000*g* for 20min, and the pellet was discarded.

Sephadex G-75 chromatography of heat-stable form of hydroxymethylbilane synthase

The pooled fractions of activity from DEAE-cellulose chromatography of heat-treated spleen extract were concentrated in a dialysis sac surrounded by solid sucrose. This concentrated DEAE-cellulose eluate (2ml) was loaded on a Sephadex G-75 column (45cm × 1.5cm) that had been equilibrated with 10mM-sodium phosphate buffer, pH6.7. The column was eluted with the same buffer, and 2ml fractions were collected and assayed for enzyme activity and protein (A_{280}). The enzyme was stored at 0–4°C (unfrozen).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on 15% slab gels with the buffer systems of Laemmli (1970). Bovine serum albumin, ovalbumin, chymotrypsinogen A, cytochrome *c* and β-galactosidase were used as M_r standards. Gels were stained with 1.25% Coomassie Blue R250 in methanol/acetic acid/water (5:1:4, by vol.) and destained in methanol/acetic acid/water (10:4:83, by vol.).

Table 1. *Induction of hydroxymethylbilane synthase activity in rat tissues by the administration of phenylhydrazine*

Assays were performed on 'desalted' supernatants. Results are expressed as: total units per organ, or, for blood, per 10ml sample, being the average volume obtainable by this method; specific activities as units of enzyme activity per wet wt. of tissue, or for blood per ml of whole blood. Results are the mean values for 15 animals. Consistent results have been obtained in at least three experiments.

Tissue	Total activity (units)	Specific activity
Spleen		
Normal	12	24
Treated	478	153
Liver		
Normal	10	1.2
Treated	60	8.4
Blood		
Normal	60	6
Treated	440	44

Isomer analysis of uroporphyrinogen products

Enzyme fractions were incubated with 1mM-porphobilinogen in 10mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA for 16h at 37°C in the dark. Incubation mixtures were subjected to sunlight for 30min before extraction with 3vol. of ethyl acetate/acetic acid (3:1, v/v). The organic extract was re-extracted with 1M-HCl. The acidic extract was diluted to 0.3M-HCl before decarboxylation at 180°C for 4h. Coproporphyrin acid isomer analysis was performed by reverse-phase paired-ion chromatography on a Waters Radial-Pak C18 column eluted with methanol/water (11:9, v/v) containing 5mM-tetrabutylammonium phosphate, pH7.5.

Results and discussion

Induction of hydroxymethylbilane synthase by administration of phenylhydrazine

Table 1 shows the yields and specific activities of enzyme from various tissues of normal and phenylhydrazine-induced anaemic rats. Enzyme activities are induced in spleen, blood and liver by 40-, 7- and 6-fold respectively. Enzyme activities are higher in rat tissues than in mouse (Levin, 1968) and ox (cf. ox spleen synthase activity of 1.38 units/g wet wt.; E. Cummins & D. C. Williams, unpublished work). Total activity recoverable from a single rat is similar for spleen and blood. The easier recovery, lack of need for plasma removal and cell washing and easier subsequent cell breakage led me to use spleen as the preferred source of enzyme.

Multiple forms of hydroxymethylbilane synthase on DEAE-cellulose chromatography

Hydroxymethylbilane synthase activity from the spleens of phenylhydrazine-treated rats was resolved into five peaks of activity by chromatography on DEAE-cellulose (Fig. 1), with forms being eluted at 20mM-, 30mM-, 45mM-, 60mM- and 75mM-KCl. Elution positions were consistent in different preparations, and peaks I, II and III, when re-applied to DEAE-cellulose, were eluted at their respective KCl concentrations (results not shown). Enzyme activity from rat erythrocytes was also resolved into five peaks. This elution profile is similar to that found for DEAE-cellulose chromatography of enzyme activity from human erythrocytes, where these forms have been shown to represent native enzyme and charge isomers corresponding to enzyme–substrate (mono-, di-, tri- and tetra-pyrrole) intermediates (Anderson & Desnick, 1980).

When phenylmethanesulphonyl fluoride (0.2mM) was included in buffers, a different elution profile from DEAE-cellulose chromatography

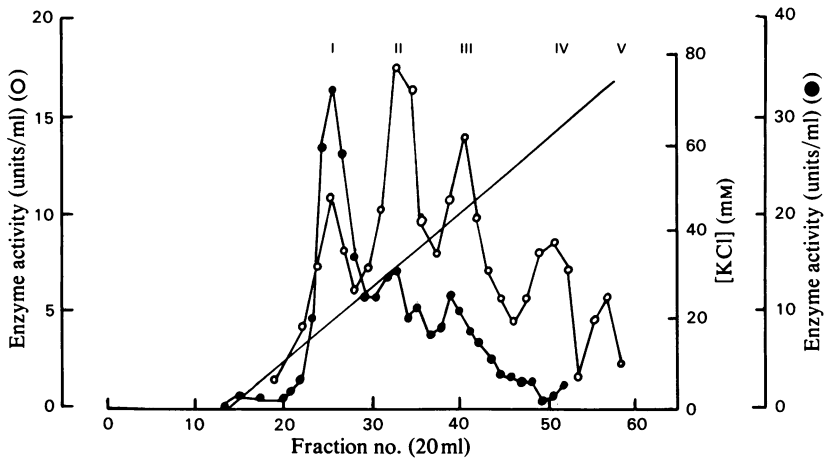


Fig. 1. *DEAE-cellulose chromatography of hydroxymethylbilane synthase from spleen of phenylhydrazine-treated rats*
Enzyme was extracted and chromatographed with (●) or without (○) phenylmethanesulphonyl fluoride in buffers. Heat treatment (70°C) was performed on the preparation obtained in the presence of proteinase inhibitor before the DEAE-cellulose chromatography as described in the text. ——, Concn. of KCl.

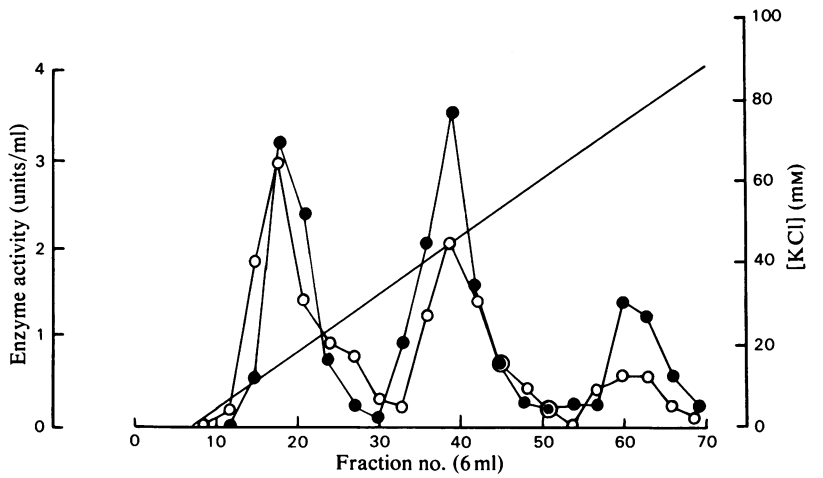


Fig. 2. *DEAE-cellulose chromatography in the presence of phenylmethanesulphonyl fluoride of hydroxymethylbilane synthase from rat spleen and erythrocytes*
Enzyme from spleen (●) and erythrocytes (○) was extracted from phenylhydrazine-treated rats and chromatographed on a DEAE-cellulose column (15cm × 1cm) in the presence of phenylmethanesulphonyl fluoride as described in the text. ——, Concn. of KCl.

was observed for both spleen and erythrocytes (Fig. 2). The major peak II and the minor peak IV were now absent, suggesting that these enzyme forms were the products of serine proteinase action during the purification procedure. Three peaks of enzyme activity were consistently observed when proteinase inhibitor was included in buffers, two major forms being eluted at 20mM-KCl (Peak I) and 45mM-KCl (Peak III) respectively and comprising 85% of recovered activity, and the minor peak (V, 15% of recovered activity). Similar elution profiles were found for enzyme activity from normal (non-phenylhydrazine-treated) rat spleen (Fig. 3), demonstrating that phenylhydrazine treatment induced all three enzyme forms, and for enzyme activity from phenylhydrazine-treated rat erythrocytes (Fig. 2), suggesting that the spleen forms represented trapped (stored) erythrocyte enzyme forms, a finding consistent with the high erythrocyte content of the spleen.

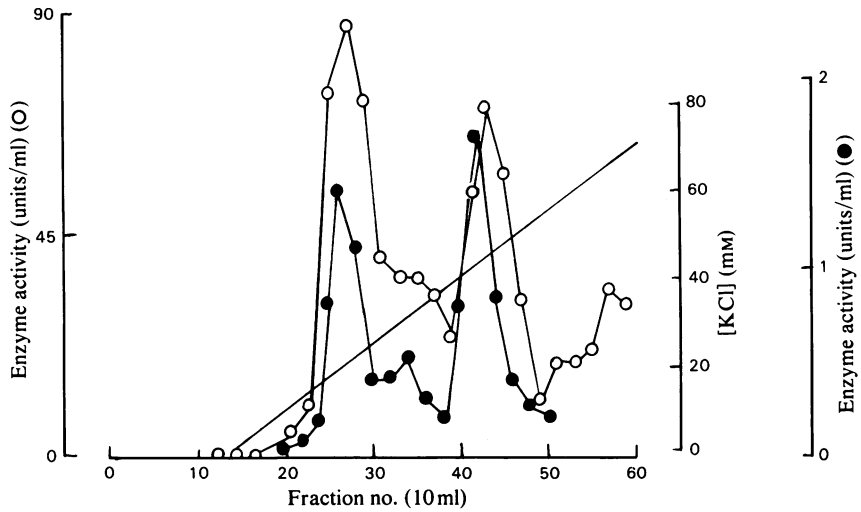


Fig. 3. *DEAE-cellulose chromatography of spleen hydroxymethylbilane synthase from rats with or without phenylhydrazine treatment*

Enzyme was extracted from normal rats (●) and from phenylhydrazine-treated rats (○), and chromatographed on a DEAE-cellulose column (20cm × 2cm) in the presence of phenylmethanesulphonyl fluoride. All experimental details are as in the text. ——, Concn. of KCl.

Inclusion of a heat-treatment (70°C) step before the DEAE-cellulose chromatography of phenylhydrazine-treated rat spleen (Fig. 1) or blood (results not shown) again resulted in a different DEAE-cellulose elution profile for enzyme activity. A single major peak (I) of activity was observed that comprised 60% of recovered activity, and Peak III was now much diminished, suggesting that heat treatment converted form III into form I. The occurrence of the minor peak V in DEAE-cellulose elution profiles after heat treatment has not been tested for in this work. Isomer analysis of the coproporphyrin derivatives of the enzymic products of forms I, III and V yielded values of 100%, 100% and 97% isomer type I for spleen and 88%, 100% and 98% isomer type I for blood, showing that all forms were substantially free of uroporphyrinogen III synthase. Thus enzyme form III was unlikely to be associated with this known heat-labile protein on DEAE-cellulose chromatography. Conversion of the multiple forms of human erythrocyte hydroxymethylbilane synthase by this heat treatment (70°C) into the single form eluted first from DEAE-cellulose has been achieved in this laboratory (E. Cummins & D. C. Williams, unpublished work). Thus it seems likely that the major form III represented an enzyme–substrate intermediate.

It thus appears that enzyme forms I and III reported here may correspond to forms A and B of human erythrocytes (Anderson & Desnick, 1980). In the present study the relationship of minor form V to minor forms C, D and E of human erythrocytes has not been established.

Purification of heat-stable form of hydroxymethylbilane synthase

Sephadex G-75 chromatography of rat spleen extract that had been subjected to heat treatment and DEAE-cellulose chromatography (Fig. 4) in the presence of phenylmethanesulphonyl fluoride gave a single enzyme form (50% recovery of enzyme activity).

The purity of the enzyme was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and at a loading of 30μg only one band was visible after Coomassie Blue staining. The maximal specific activity obtained for pure enzyme was 1260 units/mg, which is lower than the value of 2300 units/mg reported for pure human erythrocyte enzyme (Anderson & Desnick, 1980) and values of 6000 and 6750 units/mg for purified enzyme from *Euglena gracilis* (Williams *et al.*, 1981) and *Rhodopseudomonas spheroides* (Davies & Neuberger, 1973) respectively. This lower specific activity may represent species variation or some inactivation at the low protein concentration of the Sephadex G-75 eluate.

Enzyme activities are low in mammalian tissue compared with the high activities in microorganisms. However, phenylhydrazine treatment leading to a 40-fold induction of enzyme activity yielded sufficient enzyme for purification and characterization. Spleen was preferred as routine

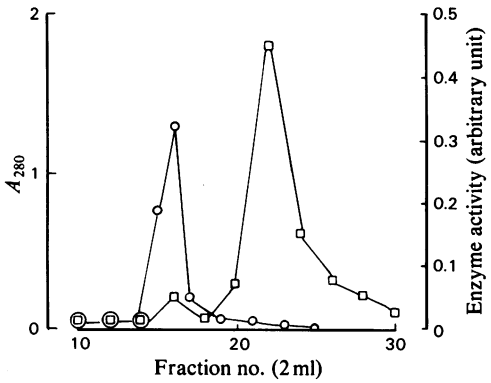


Fig. 4. *Sephadex G-75 chromatography of heat-stable form of hydroxymethylbilane synthase from rat spleen*
Enzyme from spleens of phenylhydrazine-treated rats was extracted, then subjected to heat treatment (70°C) and DEAE-cellulose chromatography in the presence of phenylmethanesulphonyl fluoride before chromatography of DEAE-cellulose peak I on Sephadex G-75 as described in the text. □, Enzyme activity; ○, A_{280}.

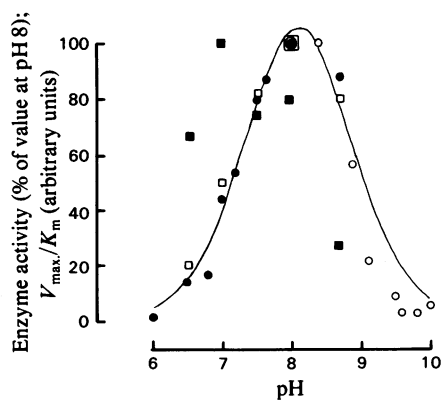


Fig. 5. *Variation of $V_{max.}$, V_{50} and $V_{max.}/K_m$ with pH for heat-stable form (I) of hydroxymethylbilane synthase*
Observed $V_{max.}$ values (□), V_{50} values (velocity at 50 μM-substrate (●, phosphate buffer; ○, borate buffer) and $V_{max.}/K_m$ values (■) are plotted as a function of pH. Velocity values are scaled to value at pH 8.0 equivalent to 100%. $V_{max.}$ at pH 8.0 was 2.4 units/assay. The continuous line is theoretical and is derived from the parameters calculated by weighted regression of the linearized form of eqn. (1) as described in the text.

enzyme source by the criteria of ease of homogenization and lack of need for copious washing procedures.

M_r determination

The M_r value for purified enzyme form I was 43000±1500 by the use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, a value similar to those found for enzyme from other sources, e.g. *Rhodopseudomonas spheroides*, 36000 (Davies & Neuberger, 1973; Jordan & Shemin, 1973), spinach leaves, 38000–40000 (Higuchi & Bogorad, 1975), *Euglena gracilis*, 41000 (Williams *et al.*, 1981), and human erythrocytes, 37000 (Anderson & Desnick, 1980), but different from that reported for human erythrocytes, 25000 (Frydman & Feinstein, 1974).

Studies of initial velocities

These experiments were conducted by measuring the rate of total uroporphyrinogen formation for an incubation time of 30 min over a wide range of porphobilinogen concentrations. Studies were performed on enzyme from normal spleen and from spleen and blood of phenylhydrazine-treated rats before and after the heat-treatment step, and thus representing hydroxymethylbilane synthase/uroporphyrinogen III synthase and hydroxymethylbilane synthase respectively. In all cases a hyperbolic dependence between enzyme activity and substrate concentration was found (results not shown), supporting a sequential displacement mechanism with ammonia release occurring between each substrate-binding step (Williams *et al.*, 1981), this being confirmed by the finding of the hydroxymethylbilane end product (Battersby *et al.*, 1979*a*,*b*) and detection of separable enzyme–substrate complexes (Anderson & Desnick, 1980; Jordan & Berry, 1981).

K_m values obtained for type-III-forming enzyme fractions (2.59±0.29 μM; six determinations) were always consistently higher than K_m values obtained for the type-I-forming enzyme species (1.0±0.11 μM; six determinations). This higher K_m value for uroporphyrinogen III-forming activities can lend support to the interaction of an extra molecular species, perhaps uroporphyrinogen III synthase, with hydroxymethylbilane synthase in these fractions.

Effect of pH on initial velocities

Values of K_m and $V_{max.}$ were obtained by direct fitting to a rectangular hyperbola (Wilkinson, 1961) over a range of pH values (6.5–8.7). Values of velocity (V_{50}) at high substrate concentration (50 μM) were also measured over the range pH 6–10. In order to correct for differences between activities from different enzyme solutions, all pH data have been scaled to the $V_{max.}$ (or V_{50}) values at pH 8.0.

A plot of $V_{max.}$ and V_{50} 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. Values for $V_{max.}$ and V_{50} lie on the same line between pH6.5 and pH8.7, demonstrating that V_{50} is consistent with $V_{max.}$ over this range. At pH values greater than 8.7, V_{50} values lie below the calculated line, suggesting that a substrate concentration of $50\mu M$ is insufficient at these pH values for saturation of enzyme. The pK values determined from weighted regression (assuming constant variance) of the linearized form of the pH-dependence curve (Cleland, 1969):

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

were found to be $pK_A^{ES} = 7.4 \pm 0.1$ and $pK_B^{ES} = 8.8 \pm 0.1$ respectively. The theoretical ionization curve calculated by using these pK values is shown by the continuous line in Fig. 5.

With the use of values only between pH6 and 8.7, pK values are not different to one decimal place.

The effect of pH on an enzyme utilizing four molecules of the same substrate has been considered by Williams *et al.* (1981). Thus, although variation of $V_{max.}$ with pH should reflect ionizations in the complexes of porphobilinogen (S) with the various intermediate forms of the enzyme, without having information about the nature of the rate-limiting step on the overall process, or the possibility of equivalent ionizations throughout the series of partial reactions to enzyme-bound methylbilane, it is not possible to attribute the pK values experimentally obtained to individual reaction steps.

The values for $pK_{A,B}^{ES}$ were compared with values of pK_A^{ES} of 6.1 and pK_B^{ES} of 8.9 calculated for the enzyme from *Euglena gracilis*. Although pK_B^{ES} values agree well, the lower value for pK_A^{ES} in *Euglena gracilis* may reflect enzyme adaptation to a pK value consistent with the acidic cytoplasmic pH found in *Euglena gracilis* (Buetow, 1968).

The spleen enzyme also showed a similar dependence of K_m with pH (Fig. 5) as did the *Euglena* enzyme, in that K_m decreased with pH on the 'acid' side of the activity pH optimum. This finding has been interpreted (Williams *et al.*, 1981) as an absence of a pK in the free enzyme (substrate-binding enzyme forms) or substrate corresponding to the group with pK approx. 6.1 in *Euglena* enzyme (7.4 in spleen enzyme). Thus, if the pK^{ES} reflects substrate ionization in the ES complex(es), a substrate pK is raised from 3.7 or 4.95 (Dawson *et al.*, 1969) to 7.4 (or 6.1). If this pK^{ES} reflects the ionization of the enzyme portion of the enzyme–substrate complex(es), then the absence of a pK^E in the measured pH range 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.

Thus enzyme from spleen has been shown to be similar to enzyme from other sources in many properties, including M_r, hyperbolic dependence of activity on substrate concentration and nature of ionizable groups.

Grateful acknowledgement is made for the provision of a Medical Research Council Training Fellowship and to Professor K. F. Tipton for advice and facilities in the Department of Biochemistry, Trinity College, Dublin.

References

Anderson, P. M. & Desnick, R. J. (1980) *J. Biol. Chem.* **255**, 1993–1999

Battersby, A. R., McDonald, E., Williams, D. C. & Wurziger, H. K. W. (1977) *J. Chem. Soc. Chem. Commun.* 113–115

Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J., McDonald, E. & Gustafson-Potter, K. E. (1979*a*) *J. Chem. Soc. Chem. Commun.* 316–319

Battersby, A. R., Fookes, C. J. R., McDonald, E. & Matcham, G. W. J. (1979*b*) *Bioorg. Chem.* **8**, 451–464

Battersby, A. R., Fookes, C. J. R., Matcham, G. W. J. & McDonald, E. (1979*c*) *J. Chem. Soc. Chem. Commun.* 539–541

Berry, A., Jordan, P. M. & Seehra, J. S. (1981) *FEBS Lett.* **129**, 220–224

Buetow, D. E. (1968) *The Biology of the Euglena*, vol. 1, p. 288, Academic Press, New York

Burton, G., Fagerness, P. E., Hosozawa, S., Jordan, P. M. & Scott, A. I. (1979) *J. Chem. Soc. Chem. Commun.* 202–204

Cleland, W. W. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–32

Davies, R. C. & Neuberger, A. (1973) *Biochem. J.* **133**, 471–492

Dawson, R. M. C., Elliott, D. C., Elliott, W. M. E. & Jones, K. M. (1969) *Data for Biochemical Research*, p. 299, Oxford University Press, London

Frydman, R. B. & Feinstein, G. (1974) *Biochim. Biophys. Acta* **350**, 358–373

Higuchi, M. & Bogorad, L. (1975) *Ann. N.Y. Acad. Sci.* **244**, 401–418

Jordan, P. M. & Berry, A. (1981) *Biochem. J.* **195**, 177–181

Jordan, P. M. & Seehra, J. S. (1979) *FEBS Lett.* **104**, 364–366

Jordan, P. M. & Shemin, D. (1973) *J. Biol. Chem.* **248**, 1019–1024

Jordan, P. M., Burton, G., Nordlov, H., Schneider, M. M., Pryde, L. M. & Scott, A. I. (1979) *J. Chem. Soc. Chem. Commun.* 204–205

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Levin, E. Y. (1968) *Biochemistry* **7**, 3781–3788
Levin, E. Y. & Coleman, D. L. (1967) *J. Biol. Chem.* **242**, 4248–4253
Mauzerall, D. & Granick. S. (1956) *J. Biol. Chem.* **219**, 435–446
Miyagi, K., Kaneshima, M., Kawakami, J., Nakada, F., Petryka, Z. J. & Watson, C. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6172–6176
Rimington, C. (1960) *Biochem. J.* **75**, 620–623
Sancovich, H. A., Batlle, A. M. C. & Grinstein, M. (1969) *Biochim. Biophys. Acta* **191**, 130–143
Scott, A. I., Burton, G., Jordan, P. M., Matsumoto, H., Fagerness, P. E. & Pryde, L. (1980) *J. Chem. Soc. Chem. Commun.* 384–387
Seehra, J. S. & Jordan, P. M. (1980) *J. Am. Chem. Soc.* **102**, 6841–6846
Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332
Williams, D. C., Morgan, G. S., McDonald, E. & Battersby, A. R. (1981) *Biochem. J.* **193**, 301–310