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Rat hepatic uroporphyrinogen III co-synthase

Purification and evidence for a bound folate coenzyme participating in the biosynthesis of uroporphyrinogen III

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Rat hepatic uroporphyrinogen III co-synthase was isolated and purified 73-fold with a 13% yield by $(NH_4)_2SO_4$ fractionation and sequential chromatography on DEAE-Sephacel, Sephadex G-100 (superfine grade) and folate–AH-Sepharose 4B. The purified co-synthase has an M_r of approx. 42000, and is resolved into two bands, each possessing co-synthase activity, by polyacrylamide-gel electrophoresis. A factor was dissociated from the purified co-synthase. Results of both microbiological and competitive protein-binding assays suggest that it is a pteroylpolyglutamate. The isolated pteroylpolyglutamate factor was co-eluted with authentic N^5-methyltetrahydropteroylheptaglutamate on DEAE-Sephacel. Uroporphyrinogen III is formed by co-synthase-free preparations of uroporphyrinogen I synthase in the presence of tetrahydropteroylglutamate. Tetrahydropeteroylheptaglutamate is also able to direct the formation of equivalent amounts of uroporphyrinogen III at a concentration approximately one-hundredth that of tetrahydropteroylmonoglutamate. These results suggest that a reduced pteroylpolyglutamate factor is associated with rat hepatic uroporphyrinogen III co-synthase, and that this may function as a coenzyme for the biosynthesis of uroporphyrinogen III.

The mechanism of formation of Uro III, the physiological precursor of haem, from the pyrrole PBG has yet to be fully resolved. The conversion of PBG into Uro III has been studied in preparations from various sources, including avian and human erythrocytes, rabbit reticulocytes, mouse spleen, cow liver, *Rhodopseudomonas spheroides*, *Chlorella* and higher plants (Cornford, 1964; Levin, 1971; Batlle & Rosetti, 1977), and is believed to be mediated by two enzymes, Uro I synthase (EC 4.3.1.8) and Uro III co-synthase. The heat-labile Uro III co-synthase alone cannot form uroporphyrinogen, but is able to direct the synthesis of the III isomer without functioning as a uroporphyrinogen isomerase (Levin & Coleman, 1967).

Levin (1968*b*) has reported that Uro-III co-synthase activity disappears during reaction with Uro I synthase and the substrate PBG under conditions where Uro III co-synthase is not measurably thermolabile, suggesting the possible participation of a substance as a cofactor. Sancovich *et al.* (1969) have isolated an ultrafiltrable factor from partially purified cow liver porphobilinogenase preparations (an enzyme complex consisting of the two enzymes Uro I synthase and Uro III co-synthase) that simulates uroporphyrinogen formation from PBG. Piper & van Lier (1977) have isolated a pteroylpolyglutamate factor from rat hepatic cytosol that activates Uro I synthase and may serve as a coenzyme for this step of haem biosynthesis. Wider de Xifra *et al.* (1980) have demonstrated both biochemical and clinical recovery in acute intermittent porphyria patients after short-term treatment with folic acid. Other work by Battersby *et al.* (1978, 1979*a*,*b*,*c*, 1980), Scott *et al.* (1976,

Abbreviations used: Uro I, uroporphyrinogen I; Uro III, uroporphyrinogen III; PBG, porphobilinogen; 5-CH_3-H_4PteGlu$_5$, 5-methyltetrahydropteroylpentaglutamate; 5-CH_3-H_4PteGlu$_6$, 5-methyltetrahydropteroylhexaglutamate; 5-CH_3-H_4PteGlu$_7$, 5-methyltetrahydropteroylheptaglutamate.

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1980), Burton *et al.* (1979) and Jordan *et al.* (1979, 1980) has indicated that a C_1 transfer may be involved in the intramolecular rearrangement whereby hydroxymethylbilane, the product of the Uro I synthase-catalysed reaction, is re-arranged to Uro III in the presence of Uro III co-synthase.

These observations, together with reports of decreased drug metabolism, cytochrome *P*-450 concentrations and haem content in folate-deficient rats (Parke, 1978) and guinea pigs (Clement *et al.*, 1981), suggest the possible regulation of the conversion of PBG into uroporphyrinogens by a folate molecule, and have prompted us to investigate the possible association of a folate component with rat hepatic Uro III co-synthase.

Experimental

Materials

Chemicals. Folic acid, ascorbic acid, saponin, gelatin, neutral activated charcoal, 2-mercaptoethanol, bovine serum albumin (type V), glycine, reagent-grade Trizma base, dithiothreitol, riboflavin 5'-phosphate, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride, Bromophenol Blue and Coomassie Brilliant Blue G and R were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. PBG, uroporphyrin I octamethyl ester, uroporphyrin III octamethyl ester and uroporphyrin I dihydrochloride were purchased from Porphyrin Products, Logan, UT, U.S.A. Enzyme-grade $(NH_4)_2SO_4$ and ultra-pure sucrose were obtained from Schwarz–Mann, Orangeburg, NY, U.S.A. Bacto Lactobacilli broth AOAC, folic acid casei medium and chicken pancreas acetone-dried powder were purchased from Difco Laboratories, Detroit, MI, U.S.A., and *Lactobacillus casei* (A.T.C.C. 7469) was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. [3',5',7,9-^{3}H]Folic acid (potassium salt) (20–70Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL, U.S.A. $NaBH_4$ and 'HPLC-grade' n-heptane and acetone were obtained from MCB Manufacturing Chemists, Cincinatti, OH, U.S.A., and 'HPLC-grade' acetic acid was purchased from J. T. Baker, Phillipsburg, NJ, U.S.A. Electrophoresis-purity acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethylethylenediamine, ammonium persulphate and Dowex 50W-X8 (Cl^- form) were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Sephadex G-100 (superfine grade), DEAE-Sephacel, AH-Sepharose 4B and gel-filtration calibration kits ('low-molecular-weight' range) were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. PtO_2 was obtained from K and K Laboratories, Plainview, NY, U.S.A. Pteroylpentaglutamate, pteroylhexaglutamate and pteroylheptaglutamate standards were generously given by Dr. E. L. R. Stokstad. All other laboratory reagents were of analytical grade and were purchased from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A., or Fisher Scientific, St. Louis, MO, U.S.A.

Animals. Male Sprague–Dawley rats (180–200g) were obtained from Sasco, Omaha, NE, U.S.A.

Methods

Uro I synthase purification. The enzyme was purified from rat liver as described previously (Piper & van Lier, 1977). This method employed heat treatment of hepatic cytosol (55°C for 5min), $(NH_4)_2SO_4$ fractionation, DEAE-Sephacel chromatography with a 0–0.4M-KCl gradient and Sephadex G-100 gel chromatography. The enzyme preparations used for this study represented 900–1000-fold purifications from hepatic cytosol and were stable for several weeks when stored at −70°C. All enzyme preparations were assayed for Uro III co-synthase activity before use. Preparations were consistently devoid of Uro III co-synthase activity.

Uro I synthase assay. The activity of Uro I synthase was measured by the method of Strand *et al.* (1972). Enzyme was incubated for 45min at 37°C in the presence of 20μmol of Tris/HCl buffer, pH7.8, and 40nmol of PBG in a volume of 400μl. Reactions were stopped by addition of 1.5ml of 2M-$HClO_4$/95% (v/v) ethanol (1:1, v/v), the mixtures were diluted to 3.0ml and centrifuged to remove protein, and the uroporphyrin in the supernatants was measured fluorimetrically and compared with a Uro I standard by using an Aminco–Bowman spectrophotofluorimeter with excitation and emission wavelengths of 405 and 595nm respectively. One unit of Uro I synthase activity was defined as the amount necessary to catalyse the formation of 1nmol of Uro I/h.

Preparation of folate–AH-Sepharose 4B. Folate–AH-Sepharose 4B was prepared by coupling with folate and AH-Sepharose 4B in the presence of carbodi-imide by using the method of Kamen & Caston (1975). AH-Sepharose 4B (4.7g) was swollen and washed with 0.5M-NaCl and further with distilled water on a sintered-glass filter. The washed gel was resuspended in 20ml of distilled water. To this suspension 50ml of folic acid solution (containing 125mg of folate), pH7.0, and 250mg of 1(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride were added, and the mixture was incubated with gentle shaking in the dark for 18h. After incubation, the gel was washed with water, followed by alternating washes with 0.5M-NaOH and 0.5M-HCl, and finally by an exhaustive wash with 50mM-Tris/HCl buffer, pH7.6. The resulting gel, equilibrated with 50mM-

Tris/HCl buffer, pH7.8, was packed into the column (2.0cm × 5.5cm) and used as folate–AH-Sepharose 4B for affinity chromatography.

Uro III co-synthase purification. Rats were killed by decapitation, livers were perfused *in situ* with 0.9% NaCl (4°C) and 25% (w/v) homogenates were prepared in 50mM-Tris/HCl buffer, pH7.8, containing 0.1mM-dithiothreitol by using a hand-operated glass (Dounce) homogenizer with the loose pestle. Tris/HCl buffer (50mM, pH7.8, containing 0.1mM-dithiothreitol) was used throughout the enzyme purification studies. All operations were conducted in the cold-room at 4°C unless otherwise stated. Homogenates were centrifuged at 9000*g* for 20min at 4°C, and the supernatants were removed and centrifuged at 105000*g* for 1h to obtain the hepatic cytosol fraction. The cytosol fraction was adjusted to 60% saturation with $(NH_4)_2SO_4$ and centrifuged at 12000*g* for 10min, the pellet was discarded, and the remaining supernatant was adjusted to 90% saturation with $(NH_4)_2SO_4$ and centrifuged at 12000*g* for 10min. The pellet was suspended in and dialysed against buffer. This enzyme preparation was subjected to chromatography on a DEAE-Sephacel column (2.0cm × 25cm). Columns were equilibrated with buffer, and the enzyme was eluted with a linear gradient of 0–0.25M-KCl in buffer. The eluted fractions containing the enzyme were combined and concentrated with an Amicon model 52 ultrafiltration unit, with a YM-10 filter. The concentrated enzyme preparation was then subjected to gel chromatography on a Sephadex G-100 (superfine grade) column (2.5cm × 60cm) that had been equilibrated with buffer. The eluted fractions containing the enzyme were combined and subjected to affinity chromatography on a folate–AH-Sepharose 4B column (2.0cm × 5.5cm). Columns were equilibrated at 4°C in the dark with 50mM-Tris/HCl buffer, pH7.8, containing 0.2mM-dithiothreitol, and the enzyme was eluted with a linear gradient of 0–1M-KCl in buffer. After elution of the enzyme, columns were washed successively with 50mM-Tris/HCl buffer, pH7.8, and 0.2M-acetic acid. The washed columns were re-equilibrated with the 50mM-Tris/HCl buffer, pH7.8, containing 0.2mM-dithiothreitol, and stored in the dark at 4°C for subsequent use. The collected fractions were assayed for Uro III co-synthase activity, and the active fractions were pooled and concentrated by using Amicon CF25 Centriflo ultrafiltration membranes. The concentrated enzyme preparations were stable for several weeks when stored at −70°C, and were used as the enzyme source for further experiments. All Uro III co-synthase preparations were determined to be free of Uro I synthase activity by fluorimetric assay.

Uro III co-synthase assay. The activity of Uro III co-synthase was measured by incubating the enzyme for 45min at 37°C in the presence of 20μmol of Tris/HCl buffer, pH7.8, 2nmol of PBG, 24μmol of KCl and 0.25 unit of Uro I synthase in a volume of 400μl. Reactions were stopped by plunging the mixtures into an ice/water bath at 0°C. Samples were then frozen in a solid-CO_2/ethanol bath, freeze-dried and esterified in 0.4ml of methanolic 4M-HCl. The mixtures of uroporphyrin I and III methyl esters were centrifuged at 10000*g* for 1min to remove any protein precipitates, neutralized with 1M-Na_2CO_3, extracted into chloroform, washed once with 0.1M-Na_2CO_3 and then twice with deionized water, filtered to remove any particulate matter, and analysed by high-pressure liquid chromatography by the method of Bommer *et al.* (1979). All analyses were performed on a Perkin–Elmer series 3 liquid chromatograph with recycle accessory utilizing a Perkin–Elmer model 204-A fluorescence spectrophotometer with excitation and emission wavelengths of 400 and 624nm respectively, and a Waters Associates μPorasil column (3.9mm × 30cm). Sample recovery after esterification, centrifugation, neutralization, extraction, washes, and filtration. was 81.8±2.4%. One unit of Uro III co-synthase activity was defined as the amount necessary to give 50% Uro III under the specified reaction conditions.

Determination of M_r. The M_r of the purified Uro III co-synthase preparation (folate–AH-Sepharose 4B step) was estimated by gel filtration on a Sephadex G-100 (superfine grade) column (2.5cm × 23cm) previously equilibrated at 4°C with 50mM-Tris/HCl buffer, pH7.8, containing 0.2M-KCl. The marker proteins used were bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), chymotrypsinogen A (M_r 25000) and ribonuclease A (M_r 13700). The void volume was determined with Blue Dextran 2000.

Polyacrylamide-disc-gel electrophoresis. Polyacrylamide-disc-gel electrophoresis of the purified enzyme was conducted according to the procedure of Davis (1964) in 7.5% polyacrylamide gels in Tris/glycine buffer, pH8.3. A constant current of 2mA per gel was applied until the protein migrated into the separation gel; then the current was increased to 3mA per gel, and the gel was maintained at 4°C for 45min. Gels were fixed in 12.5% (w/v) trichloroacetic acid for 30min and stained in Coomassie Brilliant Blue R250 (1:250 dilution of an aqueous 1% solution with 12.5% trichloroacetic acid) for 30min.

Electrophoretic elution of protein from gel slices was carried out by the method of Braatz & McIntire (1977) with 30% (v/v) glycerol in the electrophoresis buffer (25mM-Tris/HCl/0.2M-glycine, pH8.3). Before elution, the gels containing

protein to be eluted were aligned with duplicate stained gels and the appropriate gel sections were cut out with a clean razor blade.

Enzyme activity on gels was detected by incubating the appropriate gel slices for 45min in the presence of 20μmol of Tris/HCl buffer, pH7.8, 5nmol of PBG, 24μmol of KCl and 0.25 unit of Uro I synthase in a volume of 400μl. Reactions were stopped by plunging the mixtures into an ice/water bath at 0°C, gel slices were removed, and the mixture of reaction products (Uro I and Uro III) was frozen, freeze-dried, esterified and analysed by high-pressure liquid chromatography as described above.

Microbiological assay for folates. The microbiological assay for folate with the use of *L. casei* was modified from the methods of Waters *et al.* (1961) and Herbert (1966). Growth of the organisms was determined by measurement of turbidity at 660nm after incubation in a water bath at 37°C for 20h.

Competitive protein-binding assay for folate. The assay of folate by competitive protein binding was a modification of the method of Mortensen (1976), in which the extraction procedure was eliminated and 0.1M-Tris/HCl, pH9.3, was used as the reaction buffer. Folic acid was used as the standard. The binding protein was purified from milk by the method of Rothenberg *et al.* (1972).

Preparation of γ-glutamyl carboxypeptidase (conjugase). Conjugase was isolated from chicken pancreas acetone-dried powder and endogenous folates were removed by modifications of the methods of Bird *et al.* (1965) and Mims & Laskowski (1945). All conjugase preparations were determined to be folate-free by microbiological assay with *L. casei* before use.

Dissociation of folate component from Uro III co-synthase. Purified rat hepatic Uro III co-synthase preparations (folate–AH-Sepharose 4B step) were heated for 10min at 95°C in the presence of 50mM-Tris/HCl buffer, pH7.8, containing 0.2M-2-mercaptoethanol. Heated preparations were cooled on ice, centrifuged at 12000*g* for 10min, and the supernatants stored at 4°C in the dark until analysis.

Hydrolysis of folate component with conjugase. Conjugase was prepared from chicken pancreas acetone-dried powder as described above. The reaction mixture contained 0.10ml of the folate component dissociated from the purified Uro III co-synthase (60μg of protein), 0.05ml of conjugase (28μg of protein), and 0.50ml of 0.1M-Tris/HCl buffer, pH7.8, containing 20mM-$CaCl_2$ and 2% (w/v) sodium ascorbate, in a total volume of 0.65ml. All mixtures were incubated at 32°C for 6h, stopped by heating for 10min at 95°C, centrifuged at 12000*g* for 10min, and the supernatants retained for assay of folate content.

Preparation of 5-methyltetrahydropteroylpolyglutamates. 5-CH_3-H_4PteGlu$_5$, 5-CH_3-H_4PteGlu$_6$ and 5-CH_3-H_4PteGlu$_7$ were synthesized from the respective pteroylpolyglutamates by the Suzuki & Wagner (1980) modification of the method of Blair & Saunders (1970). Authentic PteGlu$_5$, PteGlu$_6$ and PteGlu$_7$ prepared by the solid-phase synthetic method (Krumdieck & Baugh, 1969) were generously given by Dr. E. L. R. Stokstad.

DEAE-Sephacel chromatography of pteroylpolyglutamate standards and the folate component from Uro III co-synthase. Approx. 1pmol of each of the synthesized standards (5-CH_3-H_4PteGlu$_5$, 5-CH_3-H_4-PteGlu$_6$ and 5-CH_3-H_4PteGlu$_7$) was mixed and chromatographed on a column (2.0cm × 6.3cm) of DEAE-Sephacel previously equilibrated with 10mM-potassium phosphate buffer, pH6.0, containing 10mM-2-mercaptoethanol. The pteroylpolyglutamate standards were eluted with a linear gradient of 0–1.0M-KCl in buffer. Pteroylglutamate activity of the eluate was assayed after conjugase treatment by the microbiological method with *L. casei* as described above. In a second experiment, identical quantities of each of the synthesized standards were mixed with 600μl of the folate component dissociated from Uro III co-synthase (160μg of protein) and the mixture was chromatographed and assayed for pteroylglutamate activity as described above.

Reduction of pteroylglutamates. Tetrahydropteroyl-monoglutamates and -heptaglutamates were prepared from the corresponding pteroylglutamates by using PtO_2-catalysed reduction with H_2 in 0.1M-Tris/HCl buffer, pH7.2, by the method of Blakley (1957).

Protein. Protein was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Results

Purification of Uro III co-synthase from hepatic cytosol

$(NH_4)_2SO_4$ fractionation (60–90% saturation) was initially employed in order to isolate Uro III co-synthase from hepatic cytosol. This procedure separates Uro III co-synthase from Uro I synthase. Chromatography on DEAE-Sephacel (Fig. 1) results in a 11-fold purification with a 87% yield, and an additional purification step on Sephadex G-100 (superfine grade) (Fig. 2) purified Uro III co-synthase 37-fold with a 39% yield. The final purification step with folate–AH-Sepharose 4B affinity chromatography (Fig. 3) purifies Uro III co-synthase 73-fold with a 13% yield. The purification of the enzyme is summarized in Table 1. The purified Uro III co-synthase is completely devoid of Uro I synthase activity but retains the ability to

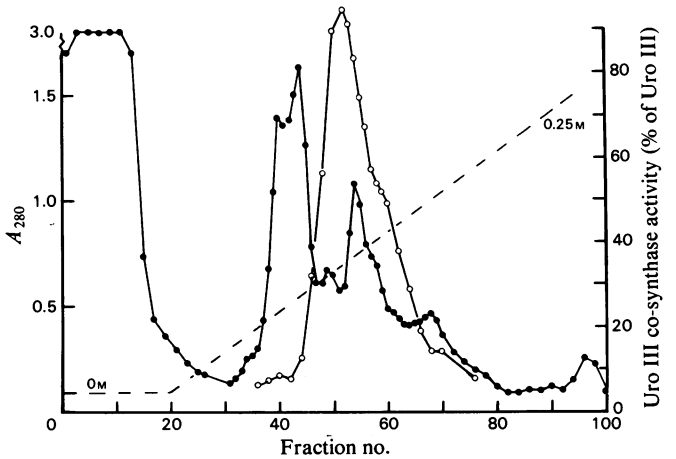

Fig. 1. *Purification of rat hepatic Uro III co-synthase on DEAE-Sephacel*
The enzyme preparation concentrated from the $(NH_4)_2SO_4$ fractionation was applied to a column (2.0cm × 25cm) of DEAE-Sephacel and eluted with a 0–0.25M-KCl gradient in 50mM-Tris/HCl buffer, pH7.8, containing 0.1mM-dithiothreitol. One-hundred 3ml fractions were collected. ●, A_{280}; ○, Uro III co-synthase activity expressed as percentage of total uroporphyrin as the III isomer. The linear gradient of KCl is superimposed on the elution pattern (----). Activities were determined as described in the Experimental section.

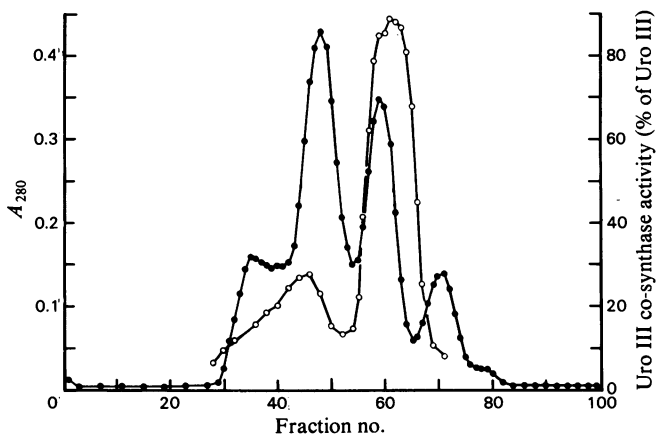

Fig. 2. *Purification of rat hepatic Uro III co-synthase on Sephadex G-100 (superfine grade)*
A concentrated solution of the pooled fractions of the enzyme preparation from DEAE-Sephacel chromatography was applied to a column (2.5cm × 60cm) of Sephadex G-100 (superfine grade). One-hundred 2ml fractions were eluted with 50mM-Tris/HCl buffer, pH7.8, containing 0.1mM-dithiothreitol. ●, A_{280}; ○, Uro III co-synthase activity expressed as percentage of total uroporphyrin as the III isomer. Activities were determined as described in the Experimental section.

direct the synthesis of Uro III by purified Uro I synthase.

Determination of the M_r of Uro III co-synthase

The purified Uro III co-synthase resolved by the final affinity-chromatography step was subjected to chromatography on a calibrated column of Sephadex G-100 (superfine grade) to determine the approximate M_r. As illustrated in Fig. 4, the purified Uro III co-synthase was eluted as a single peak corresponding to an M_r of approx. 42000.

Electrophoresis of Uro III co-synthase

The 37-fold-purified Uro III co-synthase preparation [Sephadex G-100 (superfine grade) step] showed five bands on polyacrylamide-gel electrophoresis at pH8.3 (Fig. 5, track 1). This enzyme preparation was divided into two peaks, desig-

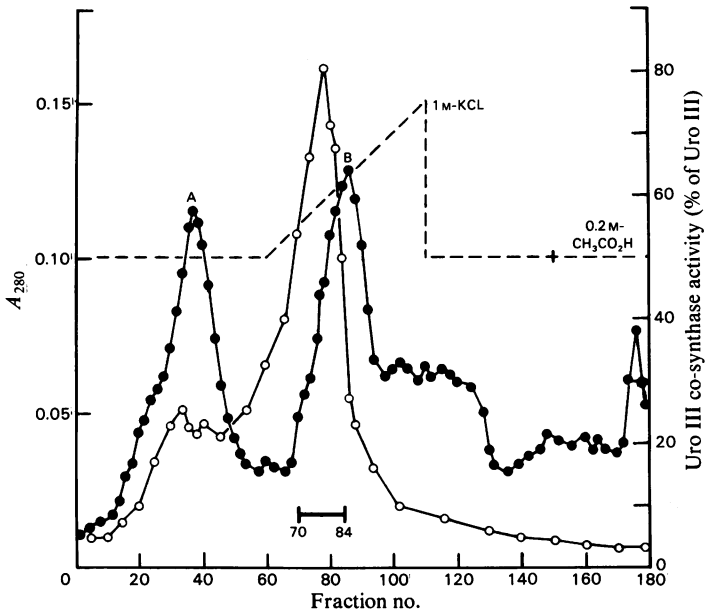

Fig. 3. *Purification of rat hepatic Uro III co-synthase on folate–AH-Sepharose 4B*
Pooled fractions of the enzyme preparation from the Sephadex G-100 (superfine grade) purification step were applied to a folate–AH-Sepharose 4B column (2.0cm × 5.5cm) and eluted with a linear gradient of 0–1.0M-KCl in 50mM-Tris/HCl buffer, pH7.8, containing 0.2mM-dithiothreitol. The active fractions (——) were pooled and concentrated as described in the Experimental section. Fractions of volume 1 ml were collected. ●, A_{280}; ○, Uro III co-synthase activity expressed as percentage of total uroporphyrin as the III isomer. The linear gradient of KCl is superimposed on the elution pattern (----). Activities were determined as described in the Experimental section.

Table 1. *Purification of Uro III co-synthase from rat liver*
One unit of activity is defined as the amount of the enzyme necessary to give 50% Uro III under the specified reaction conditions as described in the Experimental section.

Fraction	Protein: Concentration (mg/ml)	Protein: Total (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
60–90%-satn. $(NH_4)_2SO_4$	13.8	622.0	18598	30	100	1
DEAE-Sephacel	5.5	49.2	16285	331	87.6	11.1
Sephadex G-100 (superfine grade)	0.6	6.6	7296	1099	39.2	36.8
Folate–Sepharose	0.4	1.2	2500	2174	13.4	72.7

nated A and B, by further purification with the use of folate–AH-Sepharose 4B affinity chromatography (Fig. 3). After concentration with Centriflo CF25 ultrafiltration membranes, peak A (very little Uro III co-synthase activity) and the pooled active fractions of peak B (73-fold-purified Uro III co-synthase preparation) were subjected to polyacrylamide-gel electrophoresis. Peak A was resolved into two protein bands, corresponding to the two lower bands of the Sephadex G-100 (superfine grade) Uro III co-synthase preparation (Fig. 5, track 2), and the pooled concentrated active fractions of peak B were resolved into two bands, corresponding to two of the upper, slower-migrating, bands of the Sephadex G-100 (superfine grade) Uro III co-synthase preparation (Fig. 5, track 3).

The two bands associated with the pooled concentrated active fractions of peak B, designated B_1 and B_2 (Fig. 5, track 3), were each assayed for Uro III co-synthase activity by incubating the appropriate gel slices with the substrate PBG and Uro I synthase as described in the Experimental section. Both bands had Uro III co-synthase activity (expressed as percentage Uro III formation), and the sum of the activities of bands B_1 and B_2 was similar to the activity of an uncut gel containing both bands and a similar amount of protein (Table 2).

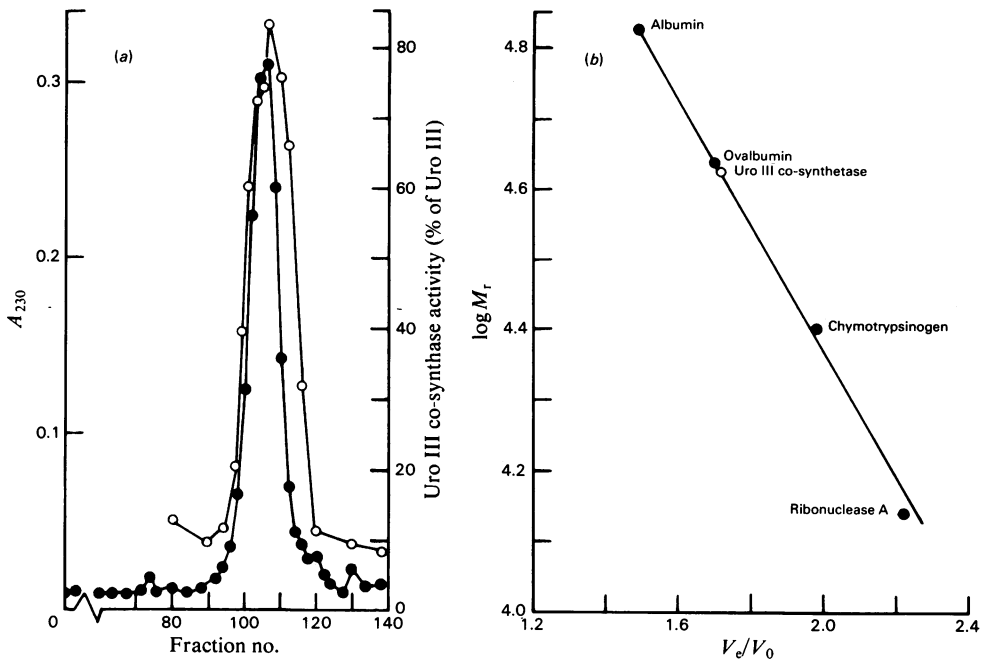

Fig. 4. *Determination of the M_r of rat hepatic Uro III co-synthase*

(*a*) Chromatography of purified Uro III co-synthase (folate–AH-Sepharose 4B step) on a calibrated column of Sephadex G-100 (superfine grade). Concentrated Uro III co-synthase preparation (400 μg) was applied to a Sephadex G-100 (superfine grade) column (2.5cm × 25cm) and eluted with 50mM-Tris/HCl buffer, pH7.8, containing 0.2M-KCl. Fractions of volume 1ml were collected. ●, A_{230}; ○, Uro III co-synthase activity expressed as percentage of total uroporphyrin as the III isomer. Activities were determined as described in the Experimental section. (*b*) Plot of relative mobility against logM_r for determination of Uro III co-synthase M_r. Purified Uro co-synthase (400 μg of protein) and 5mg of each protein standard were applied to a column (2.5cm × 23cm) of Sephadex G-100 (superfine grade). Elution of Uro III co-synthase and standards was by the same procedure as described in (*a*).

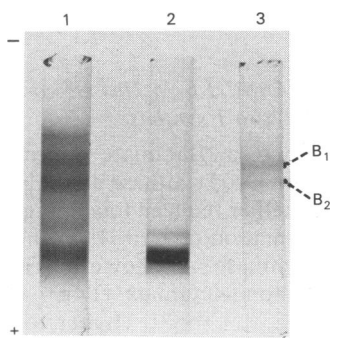

Fig. 5. *Separation of rat hepatic Uro III co-synthase on analytical polyacrylamide-disc-gel electrophoresis*

Track 1, Uro III co-synthase (30 μg of protein) from the Sephadex G-100 (superfine grade) preparation was applied and electrophoresed as described in the Experimental section. Track 2, Uro III co-synthase (16 μg of protein) from the folate–AH-Sepharose 4B peak A concentrated preparation was applied and electrophoresed as described in the Experimental section. Track 3, Uro III co-synthase (11 μg of protein) from the folate–AH-Sepharose 4B co-synthase preparation (pooled, concentrated active fractions of peak B) was applied and electrophoresed as described in the Experimental section. Both bands B_1 and B_2 had Uro III co-synthase activity when assayed as described in the Experimental section.

Folate content of Uro III co-synthase

Preparations of the 73-fold-purified Uro III co-synthase (folate–AH-Sepharose 4B step) were heated in the presence of 2-mercaptoethanol as described in the Experimental section in an attempt to dissociate any coenzymes or cofactors from the native protein. The supernatant obtained from this procedure, after centrifugation to remove any protein precipitates, was assayed for folate content, both before and after conjugase treatment, by the microbiological method with *L. casei* as the test organism and the competitive protein-binding assay. Growth of the folate-dependent organisms and antagonism of [^{3}H]folate binding to folate-binding protein by the dissociated factor indicated that it was a pteroylglutamate derivative (Table 2). Estimated values of ng of factor (as pteroylglutamate)/mg of protein (folate–AH-Sepharose 4B step) by radioassay were in agreement with values obtained by the microbiological assay. The enhanced growth response of the test organisms after treatment of the factor with conjugase (Table 2) indicates that the factor dissociated from Uro III co-synthase is a pteroylpolyglutamate.

To test further the association of a pteroylpoly-

Table 2. *Folate content and activity of rat hepatic Uro III co-synthase purified by polyacrylamide-gel electrophoresis*
Folate was dissociated from Uro III co-synthase (60 μg of protein; folate–Sepharose step) that was eluted from the gel slices, and the microbiological and competitive protein-binding assays for folate were conducted before and after treatment with conjugase as described in the Experimental section. Uro III co-synthase activity was assayed by incubation of the gel slices with PBG and Uro I synthase as described in the Experimental section.

	Folate content before and after treatment with conjugase (ng/mg of protein)				
	Microbiological assay		Radioassay		Uro III co-synthase activity
Fraction	Before	After	Before	After	(% formation of Uro III)
Folate–Sepharose B	14	50	14	15	83.3
Folate–Sepharose B_1	6	36	—	—	64.3
Folate–Sepharose B_2	4	30	—	—	24.7

glutamate factor with Uro III co-synthase, the individual bands B_1 and B_2, obtained by gel electrophoresis of the 73-fold-purified enzyme preparation, were eluted from the polyacrylamide gels as described in the Experimental section and subjected to heat treatment in the presence of 2-mercaptoethanol to dissociate the factor. The supernatants obtained by centrifugation of the heated eluted bands were assayed by the microbiological method, both before and after conjugase treatment, for folate content. Both bands B_1 and B_2 were associated with a pteroylglutamate factor (Table 2), and the enhanced growth of the test organism (*L. casei*) after treatment of the dissociated factor from bands B_1 and B_2 with conjugase again suggests that the factor isolated from Uro III co-synthase is a pteroylpolyglutamate.

Characterization of the pteroylpolyglutamate associated with Uro III co-synthase

Because most of the pteroylglutamate isolated from tissues exists in pteroylpolyglutamate forms (Shin *et al.*, 1974; Scott & Weir, 1976), with reduced pteroyl-pentaglutamates and -hexaglutamates being the predominant forms isolated from rat liver (Houlihan & Scott, 1972; Shin *et al.*, 1972; Brown *et al.*, 1973,1974), the isolated pteroylpolyglutamate factor associated with rat hepatic Uro III co-synthase was chromatographed with the N^5-methyl derivatives of tetrahydropentaglutamate, tetrahydrohexaglutamate and tetrahydroheptaglutamate standards. The N^5-methyl derivatives were chosen because of their stability (Leslie & Baugh, 1974) and predominance in rat liver (Bird *et al.*, 1965; Beck, 1974). Each standard was individually chromatographed on the same DEAE-Sephacel column (2.0cm × 6.3cm) and the conductivity at the point of elution determined. These standard conductivity values were subsequently utilized to identify the respective standards in a mixture of all three. As shown in Fig. 6(*a*), chromatography on DEAE-Sephacel with a linear gradient of 0–1.0M-KCl successfully separates the 5-CH_3-H_4-$PteGlu_5$, 5-CH_3-$H_4PteGlu_6$ and 5-$CH_3H_4PteGlu_7$ standards. When the pteroylpolyglutamate factor dissociated from Uro III co-synthase is chromatographed with the pteroylpolyglutamate standards (Fig. 6*b*), it co-chromatographs with the 5-CH_3-$H_4PteGlu_7$, increasing the growth response of the test organisms approx. 3-fold. These results suggest that the isolated pteroylpolyglutamate factor may be either 5-CH_3-$H_4PteGlu_7$ or a closely related $H_4PteGlu_n$ ($n \geqslant 7$) derivative with similar elution characteristics on a DEAE-Sephacel anion-exchange column.

Formation of Uro III by a Uro III co-synthase-free preparation of Uro I synthase

Tetrahydropteroylglutamate was prepared and incubated with Uro I synthase under N_2 in order to determine whether reduced folate might be able to direct the formation of Uro III in the absence of Uro III co-synthase. As shown in Table 3, tetrahydropteroylmonoglutamate (1mM) directed the formation of 23% Uro III. Higher concentrations of tetrahydropteroylglutamate failed to direct the formation of a higher percentage of the III isomer. However, only 9 μM-tetrahydropteroylheptaglutamate was required to direct the formation of 41% Uro III. Because of the scarcity of synthetic pteroylpolyglutamates, it was not possible to evaluate a higher concentration in the reaction mixture. Uro II or IV isomers were not detected in reaction mixtures in the presence of either tetrahydropteroyl-monoglutamate or -heptaglutamate. Furthermore, neither tetrahydropteroyl-monoglutamate nor -heptaglutamate catalysed the formation of Uro I, II, III or IV isomers from the PBG substrate in the absence of Uro I synthase.

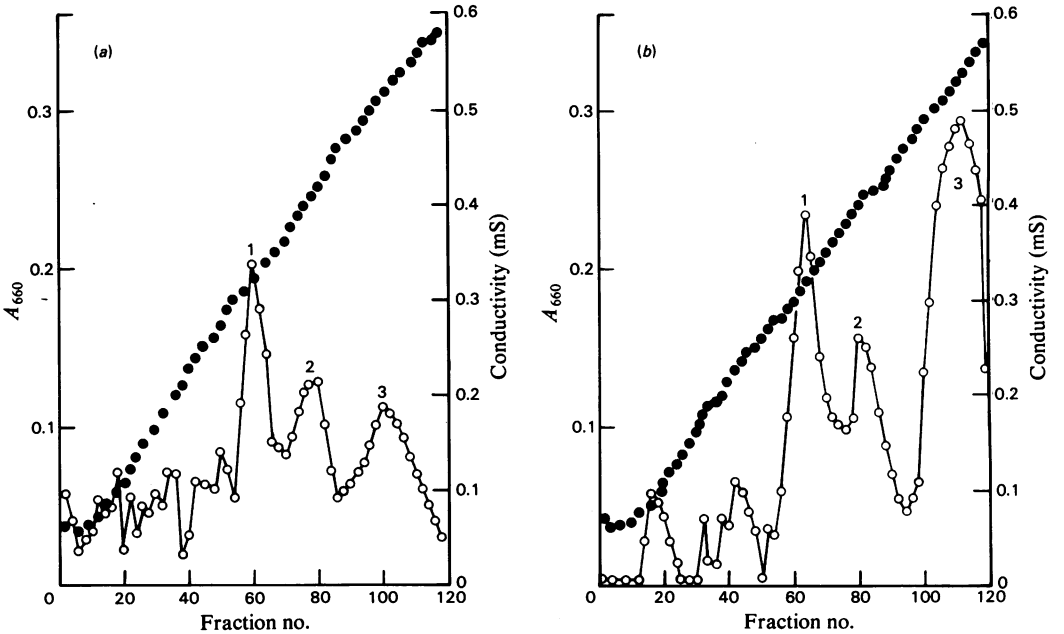

Fig. 6. *Characterization of the folate component of rat hepatic Uro III co-synthase*

(*a*) Chromatography of pteroylpolyglutamate standards on a DEAE-Sephacel column (2.0cm × 6.3cm). A mixture of approx. 1pmol of each standard was applied to the column and eluted with a linear gradient of 0–1.0M-KCl in 10mM-potassium phosphate buffer, pH6.0, containing 10mM-2-mercaptoethanol. Peak 1 is the 5-CH_3-H_4PteGlu$_5$; peak 2 is the 5-CH_3-H_4PteGlu$_6$; peak 3 is the 5-CH_3-H_4PteGlu$_7$. Fractions of volume 2ml were collected. ●, Conductivity; ○, A_{660} from the microbiological assay for folate. Pteroylglutamate activity of the eluted fractions was assayed after conjugase treatment by the microbiological method with *L. casei* as described in the Experimental section. (*b*) Chromatography of pteroylpolyglutamate standards and folate component of Uro III co-synthase on a DEAE-Sephacel column (2.0cm × 6.3cm). A mixture of approx. 1pmol of each standard and 600μl of the folate component dissociated from Uro III co-synthase (160μg of protein) was applied to the column and eluted and assayed for pteroylglutamate activity as described for (*a*). Peak 1 is the 5-CH_3-H_4PteGlu$_5$; peak 2 is the 5-CH_3H_4PteGlu$_6$; peak 3 is a mixture of the 5-CH_3-H_4PteGlu$_7$ and the folate component isolated from Uro III co-synthase. ●, Conductivity; ○, A_{660} from the microbiological assay for folate.

Table 3. *Formation of Uro III from Uro III co-synthase-free Uro I synthase in the presence of reduced folate*

Reaction mixtures were incubated at 37°C under N_2 for 10min in the presence of 20μmol of Tris/HCl buffer, pH7.8, 2nmol of PBG, 24μmol of KCl, reduced folate as indicated below and 0.25 unit of Uro I synthase in a final volume of 400μl.

Folate	Concentration (μM)	Amount of Uro III formed (%)
Tetrahydropteroyl-monoglutamate	1000	23
Tetrahydropteroyl-heptaglutamate	9	41

Discussion

In the present study rat hepatic Uro III co-synthase has been separated from Uro I synthase and purified 73-fold with a 13% yield, providing a convenient system for the study of this important step of haem biosynthesis. The purified Uro III co-synthase was subjected to chromatography on a calibrated Sephadex G-100 (superfine grade) column, and comparison with standard proteins allowed calculation of an approximate M_r value of 42000. The calculated M_r of rat hepatic Uro co-synthase (42000) is higher than previously reported values for the Uro III co-synthases isolated from bovine liver (30000) or *Euglena gracilis* (30000) (Rossetti *et al.*, 1980). These M_r variations may reflect differences in the enzymes isolated from various sources, or may be due to the degree of aggregation of Uro III co-synthase, as has been suggested by Rossetti *et al.* (1980). Attempts to purify rat hepatic Uro III co-synthase further by utilizing both anion-exchange and cation-exchange resins and PBG–AH-Sepharose 4B affinity columns were unsuccessful. In each instance the protein was eluted as a single peak and no further purification over that obtained

with folate–AH-Sepharose 4B affinity gel was noted.

Electrophoresis of the purified rat hepatic Uro III co-synthase (folate–AH-Sepharose 4B step) showed two bands (B_1 and B_2). Each band was shown to possess Uro III co-synthase activity, and the sum of the individual activities was approximately equal to the activity of an uncut gel containing both bands. These results suggest that Uro III co-synthase may exist in at least two different forms, perhaps as two isoenzymes. Alternatively, the two bands may reflect differences in the net charge of a single protein due to differences in the degree of saturation of charged cofactor-binding sites.

The factor dissociated from rat hepatic Uro III co-synthase stimulated the growth of folate-dependent organisms and antagonized [^{3}H]folate binding to folate-binding protein. Treatment of the isolated factor with conjugase enhanced the growth response of the test organisms. These results suggest that a pteroylpolyglutamate derivative is associated with Uro III co-synthase. It is unlikely that the isolated pteroylpolyglutamate is an impurity associated with the partially purified enzyme preparation, as it was isolated from single bands shown to possess Uro III co-synthase activity that had been eluted from polyacrylamide gels.

The finding that tetrahydropteroylglutamate is capable of directing the formation of Uro III in a Uro III co-synthase-free preparation of Uro I synthase is further evidence that a folate coenzyme participates in the synthesis of the III isomer. Formation of Uro III at a lower concentration of tetrahydropteroylheptaglutamate suggests that polyglutamated folates may be more efficient coenzymes for the synthesis of uroporphyrinogen. These results are not unexpected, since it has been reported that polyglutamated folates function as preferred coenzyme forms for the enzymes methionine synthetase (EC 2.1.1.13), thymidylate synthase (EC 2.1.1.45) and 10-formyltetrahydrofolate:5′-phosphoribosyl-5-aminoimidazol-4-yl-carboxamide formyltransferase (EC 2.1.2.3) (Coward *et al.*, 1975; Dolnick & Cheng, 1978; Baggott & Krumdieck, 1979; Kisliuk *et al.*, 1981).

The data reported in the present paper indicate that a pteroylpolyglutamate is associated with rat hepatic Uro III co-synthase. Such a pteroylpolyglutamate may function as a coenzyme in the postulated C_1 transfer reaction that is thought to occur during the intramolecular re-arrangement whereby the intermediate hydroxymethylbilane is converted into Uro III in the presence of Uro III co-synthase (Scott *et al.*, 1976, 1980; Battersby *et al.*, 1978, 1979*a*,*b*,*c*, 1980; Burton *et al.*, 1979; Jordan *et al.*, 1979). Numerous folate-binding proteins have been reported to be present in a variety of organs, including the liver (Waxman *et al.*, 1977; Colman & Herbert, 1980; Wagner, 1982). Such folate-binding proteins may have a role in storage of reduced pteroylpolyglutamate coenzyme forms for enzymic reactions. Haemoproteins play a central role in drug metabolism, and any decrease in their content is of clinical significance. If a folate derivative is necessary for the conversion of PBG into Uro III, then a depletion of tissue folate content might result in decreased haem synthesis, lowered concentrations of haem and an associated decrease in drug metabolism. Folate deficiency has, in fact, been associated with decreased drug metabolism in individuals on chronic anticonvulsant therapy (Labadarios *et al.*, 1978) and in pregnant women (Blake *et al.*, 1978). Decreased drug metabolism, cytochrome *P*-450 concentrations and haem content have also been reported in folate-deficient rats (Parke, 1978) and guinea pigs (Clement *et al.*, 1981).

Although the regulation of haem biosynthesis is normally governed by the activity of δ-aminolaevulinate synthase, the first and rate-limiting enzyme, under certain conditions other enzymes in the pathway may become rate-limiting (De Matteis, 1975; Elder, 1976). In acute intermittent porphyria, for example, Uro I synthase is thought to become the rate-limiting enzyme for the biosynthesis of haem (Meyer *et al.*, 1972; Strand *et al.*, 1972; Brodie *et al.*, 1977; Moore *et al.*, 1980), and it is also believed that Uro III co-synthase activity may become rate-limiting in congenital erythropoietic porphyria (Levin, 1968*a*). The isolation of a pteroylpolyglutamate factor from rat hepatic cytosol that activates Uro I synthase (Piper & van Lier, 1977) and the reversal of both biochemical and clinical symptoms in acute intermittent porphyria patients after short-term treatment with folic acid (Wider de Xifra *et al.*, 1980) also suggest the possible regulation of the conversion of PBG into uroporphyrinogens by a folate molecule. It is possible that individuals afflicted with acute intermittent porphyria have an impaired ability to supply sufficient reduced pteroylpolyglutamate coenzyme in an appropriate form needed for optimal rates of synthesis of uroporphyrinogen *in vivo*. Further research is required to answer this question. In view of the prevalence of folate deficiency (Blakley, 1969), the role of a folate derivative in the regulation of haem synthesis is of central importance.

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