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PURIFICATION OF SOLUBLE GLUTAMINASE FROM PIG BRAIN

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Abstract—A relatively simple method for the purification of the soluble form of glutaminase from pig brain is described. Because the soluble and membrane-bound forms of the enzyme differ in their sensitivity to activation by phosphate ions and by preincubation in buffers containing phosphate and borate, the apparent yield and recovery of the purified enzyme varied with the assay and preincubation conditions. The recovery of the soluble enzyme was about 25 per cent and the purification was more than 49,000 fold, but the apparent yield and purification were about one order of magnitude less than this when glutaminase activity was assayed in the total absence of phosphate, because the activity of the soluble enzyme constitutes only some 10 per cent of the total activity present in pig brain homogenates as measured under these conditions. The purified enzyme appeared to be homogeneous by the criterion of polyacryamide gel electrophoresis in the presence of sodium dodecyl sulphate. The subunit molecular weight and amino acid composition of the purified enzyme are presented and studies of the specificities of the soluble and membrane-bound enzymes indicated that they are both specific glutaminases.

The enzyme glutaminase (EC 3.5.1.2) catalyses the hydrolysis of L-glutamine to form L-glutamate and ammonium ions. A phosphate-activated glutaminase has been purified to homogeneity by Svenneby *et al.* [1], although in a very low yield. This procedure used the ability of the enzyme to aggregate in phosphate–borate buffers, a process which was reversed by transferring the enzyme to Tris–HCl buffer, to purify the enzyme by a series of centrifugation steps. A phosphate-activated glutaminase has also been purified from rat kidney by Curthoys *et al.* [2] who also utilised the aggregation–disaggregation behaviour in successive gel-filtration steps. The kidney enzyme has been reported to be immunologically identical to that from pig brain [3], although structural differences between the enzymes from the two sources have also been observed [1,4].

The enzyme is believed to play an important, although as yet incompletely understood, role in the formation and transport of the neurotransmitters glutamate and γ-aminobutyrate in the brain (see e.g. [28]). A regulatory function for glutaminase may be indicated by the sensitivity of its activity to activation or inhibition by a wide variety of endogenous and exogenous effectors, including phosphate, borate, thyroxine and bromothymol blue. The observation that the soluble and membrane-bound enzymes differ in their subcellular distribution in brain, as well as in their responses to effectors [5], suggests that they may play different roles, but several studies on this aspect have been made difficult by failure to distinguish completely between them. In order to obtain a clearer understanding of the differences between these two forms, it is necessary to study them in isolation and this paper describes the purification of the soluble form to apparent homogeneity by a procedure based on a combination of those of Svenneby [6] and Curthoys *et al.* [2] and a determination of some of its properties.

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MATERIALS

Membrane-bound glutaminase was prepared from pig brain mitochondria by the method previously described [5].

Rabbit muscle aldolase (EC 4.1.2.13), beef liver catalase (EC 1.11.1.6), *E. coli* β-galactosidase (EC 3.2.1.23), ox liver glutamate dehydrogenase (EC 1.4.0.3), rabbit muscle pyruvate kinase (EC 2.7.1.40), rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig heart malate dehydrogenase (EC 1.1.1.37), NADH, L-asparagine and 2-oxoglutarate, were obtained from C. F. Boehringer und Soehne, Mannheim GmbH, Germany. Bovine serum albumin (Fraction V), dithiothreitol (DTT) and γ-L-glutamyl 4-nitroanilide were obtained from BDH Chemicals Ltd., Poole, Dorset, England. L-2-amino 3-ureido propionic acid (albizzin) (recrystallised twice from aqueous ethanol before use) and nitroblue tetrazolium were obtained from Koch-Light Laboratories, Colnbrook, Bucks, England. L-glutamic acid γ-mono-hydroxamate, D-glutamine, L-glutamine and phenazine methosulphate were obtained from Sigma (London) Ltd., London, England. L-glutamic acid was obtained from Serva GmbH and Co., Heidelberg, F.D.R. Amido black 10B and coomassie brilliant blue were obtained from G. T. Gurr and Co. Ltd., High Wycombe, Bucks, England. Sepharose 4B was obtained from Pharmacia, Fine Chemicals Ltd., Uppsala, Sweden. Megathura crenulata hemocyanin was obtained from Mann Research Labs. Inc., New York, U.S.A. *O*-Diazoacetyl-L-serine (azaserine)recrystallised twice

from 90% ethanol before use) was obtained from Calbiochem, San Diego, CA, U.S.A.

2-oxoglutarate dehydrogenase complex from *E. coli* (EC 1.2.4.2) and tobacco mosaic virus were gifts from Dr. R. A. Harrison.

Lipids were extracted from pig brain by the method of Bligh and Dyer [7].

METHODS

Glutaminase assay. Glutaminase was assayed by a modification of the method of Kvamme *et al.* [8]. The standard assay mixture contained, in a total of 1 ml, 100 mM triethanolamine hydrochloride–NaOH buffer pH 7.4, 5 mM phosphate, 0.1 mM EDTA 3 mM 2-oxoglutarate, 0.1 mM NADH, 10 U glutamate dehydrogenase (0.5 mg protein in 50 per cent glycerol) 5 mM glutamine and enzyme sample. The oxidation of NADH was monitored at 340 nm at 30°.

Where the properties of soluble and membrane-bound glutaminase were compared, three sets of assay conditions were used: assay A—the glutaminase sample was dialyzed into 100 mM Tris–HCl buffer pH 7.4 (30°), containing 1 mM EDTA and 0.05% (v/v) 2-mercaptoethanol and was assayed in the standard assay mixture without phosphate; assay B—the glutaminase sample was dialyzed as for assay A and was assayed in the presence of 5 mM phosphate; assay C—the glutaminase sample was dialyzed into 50 mM phosphate–15 mM borate–NaOH buffer, pH 7.4, containing 1 mM EDTA and 0.05% (v/v) 2-mercaptoethanol and was assayed in the presence of 5 mM phosphate [5].

One unit (U) is defined as the amount of enzyme which catalyses the production of 1 μmole of product in 1 min.

Protein estimation. Protein was estimated by the method of Lowry *et al.* [9] using bovine serum albumin as a standard. The concentration of pure soluble glutaminase was estimated by measuring the absorbance at 280 nm and assuming that $E^{1\%}_{280}$ was 10 in a 1 cm path-length cuvette.

Spectroscopic measurements. Fluorescence spectra were measured at 30° in an Aminco–Bowman spectrofluorometer using 0.5 ml of 8.6 μg/ml soluble glutaminase. u.v. spectra were measured in a Beckman DKIIA ratio recording spectrophotometer using 1 ml of 0.188 mg/ml soluble glutaminase.

Amino acid analysis. Samples of the enzyme were dialysed exhaustively against glass-distilled water and freeze-dried. They were then dissolved in 0.2 ml 1.0 M Tris–HCl buffer, pH 8.2, 0.05 ml 0.1 M dithiothreitol, and 0.15 ml water containing 0.4 g recrystallised urea. The solutions were sealed under N_2 and incubated at room temperature for 15 min. Urea (0.1 g) and 0.1 M iodoacetate (0.2 ml), neutralised with NaOH, were then added and the mixtures were sealed under N_2 and incubated in the dark at room temperature for 1 hr before the carboxymethylation was terminated by the addition of 1 drop of 2-mercaptoethanol to each sample. The samples were then exhaustively dialyzed against distilled water and freeze-dried. Three 0.5 g samples were then hydrolyzed for 24, 48 and 72 hr at 150° with 0.4 ml of 4.0 M methane sulphonic acid and a small crystal of tryptamine in evacuated tubes and then analyzed in a Beckman automatic amino-acid analyzer.

Calibration of Sepharose 4B columns. The following molecular weight markers were used: megathura crenulata hemocyanin mol. wts 7,500,000, 3,700,000, 814,000 [10]; *E. coli* β-galactosidase, 540,000 [11]; rabbit muscle pyruvate kinase, 237,000 [12]; rabbit muscle lactate dehydrogenase, 140,000 [13]; pig heart malate dehydrogenase, 70,000 [14]. The void volume was measured using tobacco mosaic virus and a calibration curve was constructed by the method of Andrews [14].

Assays for other enzyme activities.

The hydrolysis of L-asparagine was assayed at 30° in a total volume of 10 ml containing 100 mM Tris–HCl buffer, pH 7.4, 0.1 mM EDTA, 10 mM L-asparagine and enzyme sample. The liberation of NH_4^+ was followed using an ammonium electrode [5]. A similar method was used to assay for the hydrolysis of D-glutamine, L-albizzin and L-azaserine with a 10 mM concentration of each of these compounds replacing asparagine.

The hydrolysis of L-γ-glutamyl-*p*-nitroanilide and L-γ-glutamyl transpeptidase activity were assayed by a modification of the method of Orlowski and Meister [15]. The assay mixtures contained in a total volume of 1.0 ml, 100 mM Tris–HCl buffer pH 9.0, 0.1 mM EDTA, 5 mM L-γ-glutamyl-*p*-nitroanalide and enzyme sample, ± 10 mM $MgCl_2$ and ± 20 mM glycylglycine. The release of *p*-nitroaniline was followed spectrophotometrically at 410 nm and at 30°.

Hydrolysis of γ-glutamyl hydroxamate was assayed by a modification of the method of Lipmann and Tuttle [16]. The assay mixture contained in a total volume of 1.0 ml, 100 mM Tris–HCl buffer, pH 7.4, 0.1 mM EDTA, 3 mM γ-glutamyl hydroxamate and enzyme sample. The mixture was incubated at 30° for 30 min before the reaction was stopped by the addition of 2 ml of 8% (w/v) $FeCl_3$, 10% (w/v) trichloroacetic acid in 0.5 N HCl. The solution was filtered and the absorbance was determined at 535 nm. A standard curve was constructed using γ-glutamylhydroxamate.

The formation of γ-glutamyl hydroxamate from glutamate or glutamine was assayed by a modification of the method of Ehrenfeld *et al.* [17], and the hydroxamate formed was estimated by the method of Lipmann and Tuttle [16]. The reaction mixture contained in a total volume of 1.0 ml, 100 mM Tris–HCl buffer, pH 7.4, 0.1 mM EDTA, 0.5 M hydroxylamine hydrochloride, 10 mM L-glutamine or L-glutamate and enzyme sample. The mixture was incubated at 30° for 30 min before the reaction was stopped by the addition of 2 ml of 8% (w/v) $FeCl_3$, 10% (w/v) trichloroacetic acid in 0.5 N HCl. The mixture was filtered and the absorbance of the filtrate was measured at 535 nm. A standard curve was constructed using γ-glutamyl hydroxamate.

Determination of the stoicheiometry of the L-glutaminase reaction. Soluble or membrane-bound glutaminase (0.1 U) which had been dialyzed into 100 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 0.05% (v/v) 2-mercaptoethanol, was incubated at 30° for 1 hr in a total volume of 1 ml

100 mM Tris–HCl buffer, pH 7.4, containing 0.1 mM EDTA and 10 mM L-glutamine. The reaction was stopped by placing the sample in a boiling water-bath for 3 min. In the case of assays involving the membrane-bound enzyme, the mixture was then centrifuged. Blanks were used in which the glutamine was incubated separately from the mixture and was added after boiling. Glutamine was assayed by hydrolysing it to pyrrolidone carboxylic acid, glutamate and NH_4^+ by boiling for 3 hr in 2 N HCl [18]. The sample was then brought to pH 7.4 by the addition of NaOH and the NH_4^+ concentration was determined. L-glutamate was assayed by the method of Bernt and Bergmeyer [19]. The assay mixture contained in a total volume of 1.0 ml, 0.5 M glycine–hydrazine buffer, pH 9.0, 2 mg NAD^+ and 2 U of glutamate dehydrogenase. The change in absorbance at 340 nm on the addition of the glutamate-containing sample was measured. NH_4^+ was measured by a modification of the method of Kvamme *et al.* [8]. The assay mixture contained, in a total volume of 1.0 ml, 100 mM triethanolamine hydrochloride–NaOH buffer, pH 7.4, 0.1 mM EDTA, 3 mM 2-oxoglutarate, 0.1 mM NADH and 2 U glutamate dehydrogenase. The change in absorbance at 340 nm on the addition of the NH_4^+-containing sample was measured.

Polyacrylamide gel electrophoresis. Electrophoresis in 7% polyacrylamide gels was carried out by the method of Davis [20]. Glutaminase samples were loaded in 10 mM phosphate–3 mM borate, 1 mM EDTA, 0.05% v/v 2-mercaptoethanol adjusted to pH 7.4 with Tris. The gels were maintained at 5° during electrophoresis. Gels were stained for protein in 7% v/v acetic acid containing 1% (w/v) Amido black. Gels to be stained for glutaminase activity were washed twice in 20 ml 100 mM phosphate–NaOH buffer, pH 8.0, for 10 min each at 30°. They were then incubated for 30 min at 30° in the dark in a mixture of 80 mM phosphate–NaOH buffer pH 8.0, 5 mM L-glutamine, 2 mg/ml NAD^+, 4 U/ml glutamate dehydrogenase, 0.4 mg/ml nitro blue tetrazolium, 0.04 mg/ml phenazine methosulphate. A similar stain has been used by Davis and Prusiner [21].

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber and Osborn [24] using half the normal amount of cross linking reagent. The following proteins were used as markers: *E. coli* β-galactosidase, subunit mol. wt 130,000 [11]; *E. coli* 2-oxoglutarate dehydrogenase complex 93,000, 81,000, 59,000 [25]; bovine serum albumin, 68,000 [26]; aldolase, 40,000 [13]; rabbit muscle lactate dehydrogenase, 36,000 [13].

Purification of soluble glutaminase. All steps were carried out at 0–4° except where stated.

Step 1. Preparation of pig brain mitochondria. Pig brains were obtained from freshly slaughtered animals, placed in ice and transported to the laboratory. The brains were defatted and homogenised in 150 g aliquots in 6 vol. of 0.25 M sucrose (adjusted to pH 7.6 with K_2HPO_4) using a Kenwood Chef Liquidiser at full speed for 1 min. The homogenate was centrifuged at 500 *g* for 20 min and the nuclear pellet was reextracted with two volumes of sucrose and centrifuged again. The two supernatants were combined and centrifuged at 10,000 *g* for 30 min and the pellet of mitochondria and synaptosomes obtained was resuspended, so that mitochondria from 1 kg of brains were in a final volume of 1 l, in 50 mM Tris–HCl pH 8.0 (at 0°), 1 mM EDTA, 0.05% v/v 2-mercaptoethanol (Buffer T). The mitochondria were stored frozen.

Step 2. Extraction. One litre of pig brain mitochondria was thawed and sonicated in 3 aliquots using a Dawe Soniprobe for four 15 sec bursts. The suspension was centrifuged at 20,000 *g* for 30 min. The supernatant was retained and the pellet was resuspended in 500 ml of Buffer T and sonicated and centrifuged as before. The two supernatants were combined. The pellets were resuspended in 500 ml of buffer T and stored frozen as a source of membrane-bound glutaminase [5].

Step 3. Na_2SO_4 precipitation. This step was carried out at room temperature. An equal volume of 2 M Na_2SO_4 was added to the supernatant from step 2 and stirred for 30 min. After centrifugation at 20,000 *g* for 40 min, a floating cake was formed. This was collected by siphoning off and discarding the infranatant and was resuspended in 500 ml of 500 mM phosphate–15 mM borate–NaOH pH 8.0 (at 0°), 1 mM EDTA, 0.05% v/v 2-mercaptoethanol (Buffer P-B) and frozen overnight. It was then thawed and centrifuged at 20,000 *g* for 30 min to remove insoluble material.

Step 4. Removal of lipid using calcium phosphate. The cloudy supernatant from step 3 was mixed with an equal volume of 200 mM Na_2HPO_4. The pH of the mixture was adjusted to 8.5–8.1 using 5 M NaOH and was kept in this range, while 4 M $CaCl_2$ was gradually added with stirring to give a final concentration of 40 mM. The solution was stirred for 1 hr, then the thick white precipitate of calcium phosphate was removed by centrifuging the solution for 5 min at 20,000*g*.

Step 5. $(NH_4)_2SO_4$ precipitation. 20% w/v solid $(NH_4)_2SO_4$ was added to the clear supernatant obtained from Step 4 which was stirred for 30 min. The precipitate was collected by centrifuging at 20,000 *g* for 30 min and was resuspended in 15 ml of Buffer T and dialyzed against 2 litres of Buffer T for 3–4 hr. Any undissolved material was removed by centrifugation at 35,000 *g* for 20 min.

Step 6. Gel filtration in Tris–HCl buffer. The material from step 5 was loaded onto a 3 × 85 cm column of Sepharose 4B equilibrated in Buffer T. The flow rate was 30 ml/hr. The active fractions were pooled as indicated in Fig. 1(a) and concentrated solutions of sodium phosphate and sodium borate (each adjusted to pH 8.0 at 0° were added to give final concentrations of 50 and 15 mM, respectively. The protein was then precipitated with ammonium sulphate as described in Step 5 and was resuspended in 3 ml of Buffer P-B and dialysed against 1 litre of Buffer P-B for 3–4 hr before undissolved material was removed by centrifugation.

Step 7. Gel filtration in phosphate–borate–NaOH buffer. The supernatant from step 6 was loaded onto a 2 × 50 cm column of Sepharose 4B equilibrated in Buffer P-B. The flow rate was 7 ml/hr. The active fractions were pooled as indicated in Fig. 1(b).

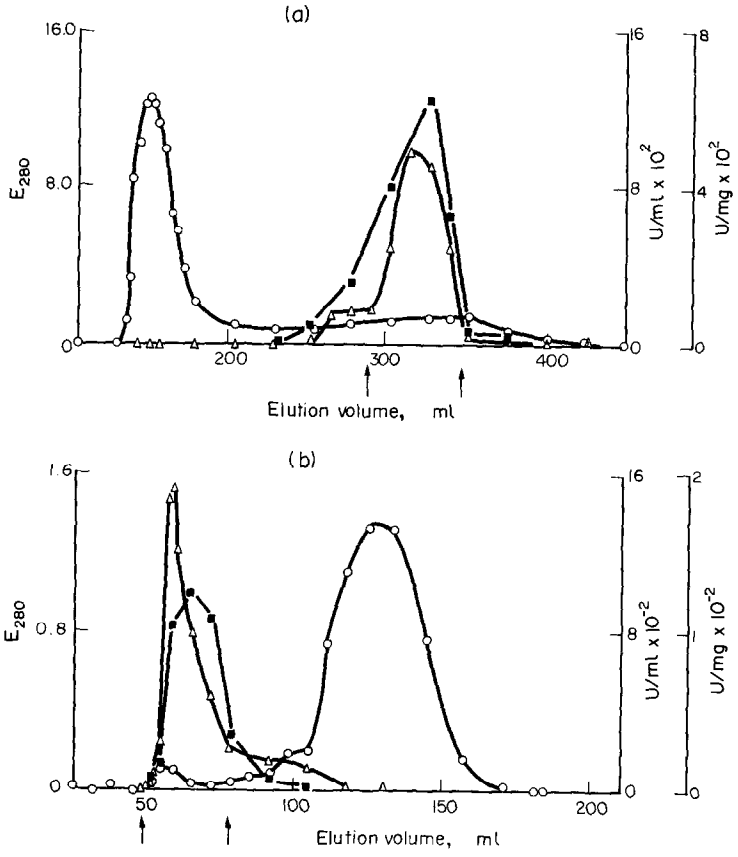

Fig. 1. Gel filtration of soluble glutaminase on Sepharose 4B. The columns were run as described under Methods. The fractions between the arrows were pooled and used for the next step of the purification. (a) Step 6 of the purification: the column was equilibrated in 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% v/v 2-mercaptoethanol. (b) Step 7 of the purification: the column was equilibrated in 50 mM phosphate – 15 mM borate–NaOH, pH 8.0, 1 mM EDTA, 0.05% v/v 2-mercapteothanol. ○ protein concentration (E_{280}), △ glutaminase activity (U/ml), ■ specific activity (U/mg).

Table 1. Purification of soluble glutaminase

Fraction	Volume(ml)	Total protein (mg)	Total glutaminase (U)	Specific activity (U/mg)	Yield (%)	Purification
Homogenate	7000	44,000	1500	0.034	100	1
Step 1	1000	11,000	1160	0.105	77	3.1
Step 2	1500	2500	137	0.055	9.1	1.6
Step 3	500	1100	28	0.025	1.9	0.7
Step 4	1000	400	27	0.067	1.8	2.0
Step 5	15	81	25	0.31	1.7	9.1
Step 6	3.0	22	20	0.91	1.3	27
Step 7	28	0.76	19	25	1.3	740
Step 8	0.7	0.58	13	22	0.9	650
Step 9	0.7	0.22	11	50	0.7	1500

The starting material was 1 kg wet weight of brain. Fractions were dialyzed into 100 mM Tris–HCl pH 7.4, 1 mM EDTA, 0.05% v/v 2-mercaptoethanol before they were assayed for glutaminase activity in the presence of 5 mM phosphate (Assay B).

Table 2. The separation of soluble and membrane-bound glutaminase

Step	Fraction	Total activity (U) (Assay B)	Recovery (%)	Enzyme in Tris assayed *with phosphate* (B)	Enzyme in phosphate-borate assayed with phosphate (C)
				Enzyme in Tris assayed without phosphate (A)	Enzyme in Tris assayed with phosphate (B)
—	Homogenate	1500	100	5.1	1.8
—	Nuclear pellet	206	14	—	—
1	Mitochondrial pellet	1157	77	4.4	2.6
—	Supernatant	54	4	—	—
2	Soluble extract	137	9	2.2	7.1
	Membrane pellet	948	63	6.8	1.4
3	NaSO infranatant	0	0	—	—
	Na_2SO_4 floating cake	130	9	1.5	11.0
	Resuspended frozen soluble Na_2SO_4 cake	28	2	1.6	16.4
	Resuspended frozen in soluble Na_2SO_4 cake	107	7	3.1	1.0
9	Pure soluble glutaminase	11	0.7	1.5	≥11.0*
—	Preparation of membrane-bound-glutaminase	—	—	6.8	1.6

The assays A, B and C are described in more detail in the text.
* This value was found to be dependent on the protein concentration.

Step 8. Centrifugation in phosphate–borate–NaOH buffer. The material from step 7 was centrifuged for 4 hr at 160,000 *g* The pellets were resuspended in ≤1 ml of 100 mM Tris–HCl pH 7.4 (at 30°), 0.1 mM EDTA and this solution was dialyzed overnight against 2 × 1 l of the same buffer.

Step 9. Centrifugation in Tris–HCl buffer. The solution from Step 8 was centrifuged for 20 min at 195,000 *g* and the pellet was discarded. DTT (1 mM) was added to the supernatant and it was stored at 4°.

RESULTS

The purification of soluble glutaminase is summarised in Table 1. The purpose of steps 1–5 was to obtain a reasonably lipid-free solution of protein in a small volume suitable for gel filtration. Most of the purification was obtained in steps 6 and 7. In step 6, when the enzyme was gel filtered on Sepharose 4B in the presence of Tris–HCl buffer, it was well retarded from the void volume of the column and appeared as a peak of protein corresponding to a mol. wt of 224,000 with a shoulder of mol. wt 759,000 as shown in Fig. 1a. In step 7, when the enzyme was incubated in the presence of phosphate–borate–NaOH buffer, it aggregated and was eluted in the void volume of the second Sepharose 4B column with a shoulder of less aggregated material, as shown in Fig. 1b. Steps 8 and 9 gave a slight further purification and concentrated the enzyme.

Since the specific activity of soluble glutaminase was higher when the enzyme was preincubated in phosphate–borate–NaOH buffer than in Tris–HCl buffer [5,6], samples from each stage of the purification were dialyzed into Tris–HCl buffer in order to provide a meaningful measure of the recovery of activity during the purification (Table 1). The total recoveries of glutaminase activity from steps 2 and 3 were very low because the soluble and membrane-bound forms of the enzyme are separated at these stages [5]. That the losses occurring were due to removal of the membrane-bound form of the enzyme was confirmed by the results shown in Table 2, in which the effects of different buffers on the glutaminase activity present during the early stages of the purification are compared with the effects on pure soluble glutaminase and on membrane-bound glutaminase which had been freed of soluble glutaminase [5].

Step 3 appeared to give little purification of soluble glutaminase but it was found that if this step was omitted the solution was not clarified at step 4 and the ammonium sulphate pellet at step 5 did not redissolve satisfactorily.

The purified enzyme was quite unstable and was found to be most stable in the presence of 1 mM EDTA and 1 mM DTT, in buffer containing 15 mM borate and 50 mM phosphate. It was not stabilised by 10 mM glutamate, 50 mM NH_4Cl, 10 mg/ml bovine serum albumin, 50 mM phosphate, 1 mg/ml brain mitochondrial lipids, storage at −10° in the presence of 50% (v/v) glycerol or storage in liquid nitrogen. The enzyme was more stable in concentrated solution than when diluted. The purified enzyme was routinely stored at 4° in 50 mM phosphate, 15 mM borate, 1 mM EDTA, 1 mM DTT, which had been adjusted to pH 7.4 (at 30°) by the addition of Tris. Under these conditions it had a half-life of about 150 hr when stored at a protein concentration of 0.8 mg/ml, but this value was lower at more dilute protein concentrations, the half-life at a protein concentration of 0.03 mg/ml being about 60 hr under the same conditions. In Tris buffer the enzyme was even less stable, having a half-life of only 26 hr when stored at 4° in 100 mM, Tris–HCl buffer pH 7.4 containing 1 mM EDTA and 1 mM DTT at a protein concentration of 0.8 mg/ml.

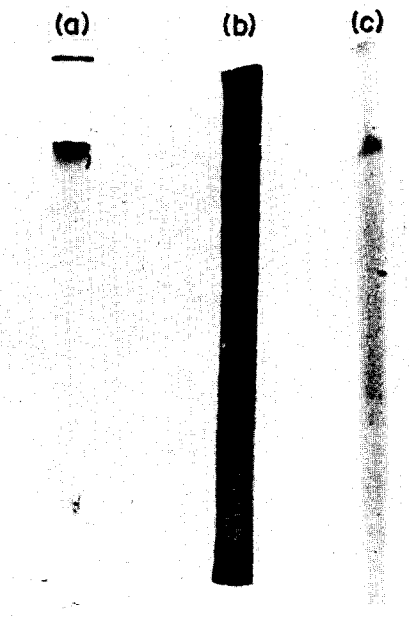

Fig. 2. Polyacrylamide gel electrophoresis of purified soluble glutaminase. The electrophoresis was carried out as described under Methods. (a) Gel stained for protein. (b) Gel stained for glutaminase activity. (The band at the bottom of the gel is the tracking dye bromophenol blue.) (c) Electrophoresis carried out in the presence of sodium dodecyl sulphate and the gel stained for protein.

When purified soluble glutaminase was subjected to electrophoresis in 7% polyacrylamide gels and stained for protein, one major band was detected which was in the same position as the rather diffuse band obtained on staining the gels for glutaminase activity. Typical gels are shown in Fig. 2.

Only one band of protein was detected by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (Fig. 2) suggesting that the preparation of soluble glutaminase is pure and contains a single type of subunit. The subunit molecular weight was estimated to be 73,000 ± 4000 by comparison with the standard markers (Fig. 3).

The stoicheiometries of the reactions catalyzed by the membrane-bound and the purified soluble enzyme were determined in the manner described previously. Under these conditions the soluble

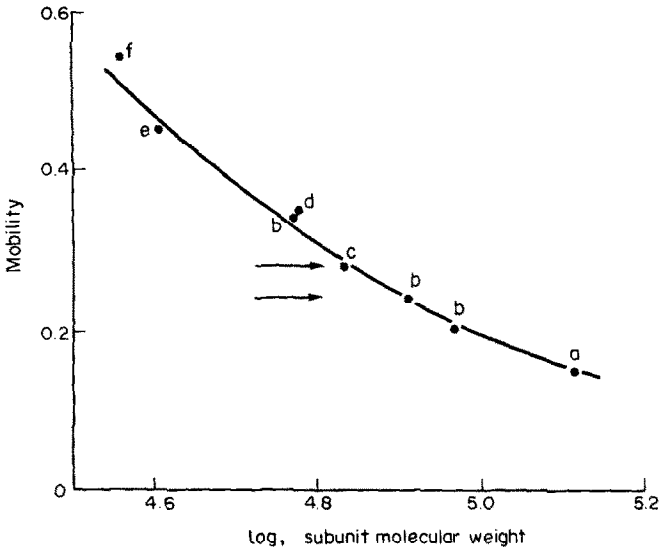

Fig. 3. Determination of the subunit molecular weight of glutaminase by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The electrophoresis was carried out as described under Methods and the molecular weight marker proteins used were (a) β-galactosidase from *E. coli*, (b) 2-oxoglutarate dehydrogenase complex from *E. coli*, (c) bovine serum albumin, (d) catalase from beef liver, (e) aldolase from rabbit muscle, and (f) lactate dehydrogenase from rabbit muscle. The arrows represent the extreme values found for the mobility of soluble glutaminase in five experiments using three different preparations of the enzyme.

enzyme catalyzed the disappearance of approximately 4 mM glutamine and the formation of equivalent amounts of L-glutamate and NH_4^+, whereas the membrane-bound enzyme catalyzed the disappearance of about 3 mM glutamine and the appearance of equivalent amounts of L-glutamate and NH_4^+. The error between duplicate determinations was ± 15%. Thus the reaction catalyzed followed the stoicheiometry of the glutaminase reaction:

L-glutamine ($+H_2O$)→L-glutamate + NH_4^+

in both cases.

The ability of both the soluble and membrane-bound enzymes to catalyze a number of related reactions were determined using the methods described earlier and the results are shown in Table 3, from which it can be seen that neither enzyme preparation has great catalytic activity in any of these reactions.

The amino acid composition of soluble glutaminase was determined as described in the Methods section and is shown in Table 4. The mean residue weight was calculated to be 111.

The ultraviolet absorbance spectrum of soluble glutaminase in Tris–HCl buffer showed no unusual features. The absorbance of the enzyme at 260 and 280 nm was increased by approximately 10 per cent in the presence of phosphate and borate, but was not altered by varying the concentration of Tris–HCl

Table 3. Specificity of soluble and membrane-bound glutaminase

Reaction	Maximum % activity X	Y	Z
L-glutamine + H_2O → L-glutamate + NH_4^+ (phosphate absent)	100	100	100
D-glutamine + H_2O → D-glutamate + NH_4^+	3	5	4
L-asparagine + H_2O → L-aspartate + NH_4^+	0.5	5	3
L-γ-glutamyl hydroxamate + H_2O → L-glutamate + hydroxylamine	2	4	1.6
L-γ-glutamyl-*p*-nitroanilide + $H_2O \xrightarrow{\pm Mg^{2+}}$ L-glutamate + *p*-nitroaniline	0.03	5	0.2
L-albizziin + H_2O → $COOH.NH.CH_2.CH(NH_2).COOH + NH_4^+$ [27]	0.7	7.5	1.6
L-azaserine + H_2O → pyruvate + NH_4^+	0.5	5	3
L-γ-glutamyl-*p*-nitroanilide + glycylglycine $\xrightarrow{\pm Mg^{2+}}$ glutamylglycylglycine + *p*-nitroaniline	0.03	5	0.2
L-glutamine + hydroxylamine $\xrightarrow{\pm Mg,\ ATP}$ L-γ-glutamyl hydroxamate + $2NH_4^+$	0.2	4	1.6
L-glutamate + hydroxylamine $\xrightarrow{\pm Mg,\ ATP}$ L-γ-glutamyl hydroxamate + NH_4^+	0.2	4	8

The assay methods used are described in the text.

X — soluble glutaminase, preincubated in 50 mM phosphate–15mM borate–Tris buffer pH 7.4 (at 30°), 1mM EDTA, 1mM DTT.

Y — soluble glutaminase, preincubated in 100mM Tris–HCl buffer pH 7.4 (at 30°), 1mM EDTA, 1mM DTT.

Z — membrane-bound glutaminase, preincubated in 100mM Tris–HCl buffer pH 7.4 (at 30°), 1mM EDTA, 0.05% (v/v) 2-mercaptoethanol.

Table 4. The amino acid composition of soluble glutaminase. The amino acid composition was determined as described in Methods. The results are expressed as the number of residues per subunit, assuming a subunit mol. wt of 73,000. Each value is the mean of three determinations.

Amino acid	Number of residues
Lysine	39.6
Histidine	17.3
Tryptophan	2.4
Arginine	26.2
Carboxymethylcysteine	11.5
Aspartic acid	71.2
Threonine	29.4*
Serine	51.5*
Glutamic acid	72.2
Proline	27.9
Glycine	58.1
Alanine	48.2
Valine	48.6†
Methionine	12.7
Isoleucine	26.4†
Leucine	57.9
Tryosine	20.7
Phenylalanine	32.3

* Extrapolated to zero hydrolysis time to allow for the partial destruction of these amino acids.

† Only the 72 hr hydrolysis value was used to allow for the slow release of these amino acids.

in the range 10–100 mM. Consequently, where E_{280} measurements were used to estimate the protein concentration the enzyme was always dialyzed into Tris–HCl buffer before measuring the absorbance. The ratio of E_{280}/E_{260} was 1.25 for glutaminase in Tris–HCl buffer and was lower for enzyme in phosphate–borate buffer. A similar behaviour has been reported by Kvamme *et al.* [22]. for the phosphate-actived enzyme from pig kidney.

The fluorescence emission spectrum of glutaminase in Tris–HCl buffer excited at 280 nm showed a peak at 360 nm. This fluorescence spectrum was not altered by the presence of 10 mM glutamate or 100 mM borate. There was a small increase in fluorescence but no shift of the maximum on the addition of 100 mM phosphate. At concentrations greater than 8.6 μg/ml, self-quenching of the enzyme fluorescence occurred.

DISCUSSION

This purification is based on a combination of the procedure used by Svenneby [6] to purify a phosphate activated glutaminase from pig brain and that devised by Curthoys *et al.* [2] to purify the enzyme from rat kidney. It is apparent from the elution profiles shown in Fig. 1 that the specific activity and molecular weight of soluble glutaminase are higher in the presence of phosphate–borate–NaOH buffer than in the presence of Tris–HCl buffer and this property was used by Svenneby [6] to purify the pig brain enzyme by successive centrifugation steps in different buffers. Curthoys *et al.* [2] used successive gel filtration steps in different buffers to purify the rat kidney enzyme. With the pig brain enzyme we found that the use of centrifugation steps after the gel-filtration steps had the advantage of concentrating the enzyme, and thus rendering it more stable, as well as yielding a small amount of further purification. It is difficult to assess the homogeneity of the native enzyme because of its tendency to aggregate and disaggregate and this may account for the presence of the minor bands seen on polyacrylamide gel electrophoresis and for the somewhat diffuse appearance of the major band (Fig. 2). The presence of a single protein band after electrophoresis in sodium dodecyl sulphate does, however, suggest that the preparation is essentially homogeneous. The subunit mol. wt of 73,000 ± 4000 estimated from the mobility of this band is somewhat larger than the value of 63,900 ± 2300 reported by Svenneby *et al.* [1], and the results obtained in this study also differ from those of Svenneby [6] in that she was unable to detect any enzyme activity after polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate. However, despite these differences, it appears likely from the aggregation–disaggregation behaviour and the effects of different buffer ions on its activity that the soluble enzyme purified here corresponds to the phosphate-activated enzyme previously purified by Svenneby [6]. Electrophoresis in the presence of sodium dodecyl sulphate of the enzyme from rat kidney purified by the method of Curthoys *et al.* gave a number of protein bands in the mol. wt range 57,000–75,000 which may have arisen as the result of partial proteolysis [23].

Calculation of the specific activity of the enzyme during purification is complicated by the effects of different buffer ions on its activity (see Table 2). These effects necessitated the transfer of all samples to the same buffer for activity measurements. The

Table 5. Apparent yield of purified soluble glutaminase under different assay conditions

	Enzyme in Tris assayed without phosphate (A)	Enzyme in Tris assayed with phosphate (B)	Enzyme in phosphate–borate assayed with phosphate (C)
Yield of glutaminase (%)	2.5	0.7	4.4
Yield of glutaminase (U)	7.3	11	120
Final specific activity (U/mg)	33	50	550
Overall purification (fold)	4900	1500	8700

The assays A, B and C for glutaminase are described in more detail in the Methods section.

protein-concentration-dependent instability of the enzyme resulted in the final specific activity being dependent upon the conditions under which the enzyme was stored, although it was reproducible for preparations of the same protein concentration. The final specific activity of 50 U/mg (Table 1) for the purified enzyme in Tris–HCl buffer assayed in the presence of phosphate compares well with that of 106 U/mg reported by Svenneby *et al.* (1973), since the latter preparation was stored at a higher protein concentration and was assayed at a more favourable pH value and phosphate concentration, although at a lower temperature. The entire purification procedure could be completed in 5–6 days, the most time-consuming steps being those involving gel-filtration and dialysis, and was both simpler and quicker than that devised by Svenneby *et al.* [1]. In addition, the yield of enzyme activity per kg of brain was twice that obtained by the method of Svenneby *et al.* [1].

Since pig brain contains two forms of glutaminase, the soluble and membrane-bound enzymes which are activated to different extents by 5 mM phosphate and by preincubation in buffers containing phosphate and borate [5], the value for the final yield of the enzyme obtained, like that of the specific activity, will depend upon the assay conditions as shown in Table 5. The separation of the soluble and membrane-bound enzymes during the early stages of the purification procedure (see Table 2) results in the overall yield of total activity being low, whichever assay conditions are used. It has been estimated, however, that in pig brain no more than 10 per cent of the glutaminase activity determined in the absence of phosphate and without preincubation in phosphate–borate-containing buffer is due to the soluble form of the enzyme [5]. Using this value it can be seen from the data in Table 5 that the total purification achieved is greater than 49,000 fold and that the recovery of the purified soluble enzyme is greater than 25 per cent.

Since a number of enzymes, including amido transferases, non-specific amidases and γ-glutamyl transferases, have some glutaminase activity [27], it was important to investigate the specificity of the purified glutaminase. As can be seen from Table 3, the purified soluble enzyme and the membrane-bound preparation were both specific glutaminases having only small activities in the other systems.

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