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PURIFICATION, RADIOIMMUNOASSAY, AND DISTRIBUTION OF HUMAN BRAIN 14-3-2 PROTEIN (NERVOUS-SYSTEM SPECIFIC ENOLASE) IN HUMAN TISSUES

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Summary

Human 14-3-2 protein, a nervous-system specific enolase (EC 4.2.1.11) isoenzyme, has been purified from human brain and a sensitive radioimmunoassay has been developed for its detection. A systematic survey of human organs has shown that immunoreactive nervous-system specific enolase is present in all human organs but at levels less than 3% of those found in human brain, with especially low levels in liver, kidney and skeletal muscle, and with the highest levels in adrenal and large intestine. In all organs immunoreactive nervous-system specific enolase occurs in two forms representing the heterodimer and homodimer forms of the enzyme, and in all tissues except brain the heterodimer predominates. The presence of nervous-system specific enolase in other organs is unlikely to be explicable by innervation alone since significant quantities are found in red blood cell haemolysates. Tissues which contain amine precursor uptake and decarboxylation cells, for which the protein has been claimed to be a specific molecular marker, do not contain significantly higher levels of immunoreactive nervous-system specific enolase than other tissues. Both the heterodimer and homodimer forms of the enolase appear to be expressed at low levels in all tissues.

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Introduction

It has been known for several years that the mammalian nervous system contains several proteins at much higher concentrations than those found in other organs [1]. One such protein, initially named 14-3-2 protein [2], was later shown by degeneration studies [3] and immunohistochemical evidence [4,5] to be localized in neurones and was redesignated neurone-specific protein [6,7]. Independently of work on 14-3-2 protein Rider and Taylor [8] detected a new immunologically distinct form of enolase in rat brain and proposed that nervous tissue contains three enolase isoenzymes with the subunit composition $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$. Two of the three forms of enolase in rat brain (i.e. the $\alpha\gamma$ and $\gamma\gamma$ dimers) were shown to cross-react with antiserum against bovine 14-3-2 protein [9], and it has therefore been proposed that 14-3-2 protein be redesignated neurone-specific enolase [5]. However, recent immunocytochemical evidence has indicated that NSE is not confined to neurones but is also present in islet cells in the pancreas, C-cells in thyroid, and chromaffin cells in the adrenal cortex and it has been suggested that neurone-specific enolase is a marker for cells of the amine precursor uptake and decarboxylation system [10].

The ability to define tissue specificity of an enzyme depends on the sensitivity of the assay system used. Assays based on electrophoresis followed by activity staining [11] or on immunoprecipitation of enzyme activity are relatively insensitive and do not detect inactive enzyme protein. Recent reports have indicated at least one nervous-system specific isoenzyme (creatine kinase) may exist in other tissues in an immunologically active but enzymatically inert form [12], such 'silent' isoenzymes can conveniently be detected by immunoassay. Furthermore, the distribution of tissue-specific isoenzymes is known to show considerable interspecies variation [13]. For the above reasons we have investigated the distribution of nervous-system specific enolase in human tissue by means of a sensitive radioimmunoassay.

Materials and Methods

Purification procedure

Human brain material was obtained within 12 h post mortem and stored deep frozen at −20°C. All purification procedures were performed at 4°C. Frozen brain was homogenized with extraction buffer (100 mM potassium phosphate buffer pH 7.0 containing 12 mM 2-mercaptoethanol, 1 mM EDTA) and centrifuged at 20 000 × g for 1 h. The supernatant was pumped through a 20 × 4 cm DEAE-cellulose column and the column washed with extraction buffer until the A_{280} of the eluate fell below 0.1 units. Soluble acidic proteins bound to the column were then eluted in one step with 1.0 M KCl in the same buffer. After dialysis overnight against deionized water solid ammonium sulphate was added to a final concentration of 50%. The precipitate was removed by centrifugation as above and the supernatant was dialysed against deionized water and lyophilized. The lyophilized 50% supernatant proteins were then redissolved in 100 mM potassium phosphate buffer pH 7.2 containing 0.4 M ammonium sulphate, 1 mM EDTA, 10 mM 2-mercapto-

ethanol, and chromatographed on a 2.5 × 90 cm Sephadex G-100 column. The column was eluted with the same buffer and fractions containing enolase activity (Peak II, Fig. 1, see Results) were pooled, dialysed against deionized water and lyophilized. The lyophilized powder was then redissolved in 50 mM sodium acetate-acetic acid buffer, pH 5.5 containing 1 mM $MgCl_2$ and chromatographed on a 20 × 1 cm column of DEAE-Sephadex-A25. The column was washed with 50 ml of 0.1 M NaCl in the same buffer and eluted with a 0.1 M—0.4 M NaCl gradient in the same buffer. Fractions containing enolase activity were pooled, dialysed against deionized water containing 1 mM $MgCl_2$ and lyophilized.

Enolase assay

Initial experiments showed that human brain enolase activity was maximal at pH 7.0 and 1 mM $MgCl_2$. Activity was measured in 50 mM Tris-HCl, pH 7.0 with 1 mM $MgCl_2$ either by the coupled assay at A_{340} [14] or by the direct assay at A_{240} [15], essentially identical results being obtained by either method. Electrophoresis on cellulose acetate strips and staining for enolase activity were performed as described elsewhere [16].

Polyacrylamide gel electrophoresis

This was performed by the method of Davis [17], sodium dodecyl sulphate gel electrophoresis was performed by the method of Weber and Osborn [18].

Molecular weight estimations

(a) Gel filtration. This was performed with the same Sephadex G-100 column described in the purification procedure by the method of Andrews [19], using bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c* as standards. The molecular weight of the purified protein was also determined on an 80 × 1 cm column of Ultrogel ACA 54 equilibrated with 50 mM Tris-HCl, pH 7.4 containing 100 mM $MgCl_2$, using the same protein standards.

(b) Sucrose density gradient centrifugation. This was performed by the method of Martin and Ames [20]. 1 mg of purified nervous-system specific enolase was layered onto a 20 ml sucrose gradient (5—15% in 50 mM Tris-HCl, pH 7.0 containing 0.1 M $MgCl_2$) and centrifuged at 98 000 × *g* for 24 h. Fractions were collected from the bottom of the tube with a peristaltic pump. Cytochrome *c*, bovine serum albumin, and lactate dehydrogenase were used as standards.

(c) Guanidine HCl column chromatography. This was performed by the method of Fish et al. [21] in 6 M guanidine HCl using a 3 × 25 cm Biogel A5M column with bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c* as standards.

Production of antiserum

Antiserum to human nervous-system specific enolase was produced in sheep by multiple intradermal injections of 1—2 mg of the purified protein emulsified with Freunds complete adjuvant. Injections were repeated at approximately monthly intervals and the sheep were beld ten to fourteen days after injection.

Antisera were tested for the presence of antibodies by neutralisation of enolase activity and by ability to bind iodinated human nervous-system specific enolase protein.

Radioimmunoassay procedure

(a) Iodination of human nervous-system specific enolase. 10 μg of the purified protein was iodinated by the method of Bolton and Hunter [22] using (*N*-succinimidyl-3,4-hydroxy-5-[^{125}I]iodophenyl propionate) (Radiochemical Centre, Amersham). The iodinated product was separated at 4°C on a 1 × 15 cm column of Sephadex G-25 equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 0.25% gelatine. A typical iodination produced a 30% incorporation of the total radioactivity into the protein. Fractions from the Sephadex column containine the iodinated protein were diluted in single strength veronal buffer (Buffer 1), pH 8.1, containing 10.3 g/l sodium barbitone, 5 g/l bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, U.K.), 5 g/l NaCl, and 0.1 g/l sodium azide to give approximately 25 000 cpm/ml. The diluted tracer (specific activity approximately 3000 dpm/ng) was stored in aliquots at −20°C.

(b) Assay procedure. The assay was performed in LP3 tubes (Luckham, Burgess Hill, Sussex). 100 μl of standard or unknown nervous-system specific enolase solution was mixed with 100 μl of sheep antiserum (final dilution 1 in 20 000 in Buffer 1) and after incubation for 24 h at 4°C 200 μl of iodinated protein containing approximately 5000 cpm were added. After mixing the tubes were incubated overnight at 4°C and then 100 μl of non-immune sheep serum diluted 1 in 150 in Buffer 1 were added, followed by 100 μl of donkey-anti-sheep IgG (Guildhay, University of Surrey) diluted 1 in 10 in Buffer 1. After incubation at 4°C for 1 h, the tubes were centrifuged at 2000 rev./min in an MSE Coolspin for 30 min, the supernatants were removed and the pellets counted in a Wallach gamma counter.

Preparation of human tissue extracts

Specimens of human organs were obtained within 12—24 h post mortem and immediately frozen at −70°C. Each specimen was then thawed at 4°C overnight and homogenised on ice as a 20% homogenate (w/v) in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM β-mercaptoethanol and 1 mM $MgCl_2$, using a Willems Polytron homogeniser. After centrifugation at 20 000 × *g* for 30 min at 4°C, 5 ml of supernatant was applied to a 1.5 × 7 cm column of DEAE-cellulose. The column was washed with 100 ml of extraction buffer and then eluted with a gradient of 0—0.5 M NaCl in the same buffer. Human erythrocyte haemolysates were prepared by mixing 20 ml of a fresh venepuncture sample with 40 ml of ice-cold saline and centrifuging at 20 000 × *g* for 30 min. The white cell layer was then removed with a pipette and the packed red cells was hed three times in ice-cold saline by centrifugation as above. The red cells were then mixed with six volumes of ice-cold distilled water and left at 4°C for 10 min. After centrifugation at 20 000 × *g* for 30 min, 14 ml of the supernatant were applied to the same DEAE-cellulose column used for other human tissue extracts above, and the column was eluted in an identical manner. Recovery of immunoreactivity and enolase activity on these columns was quantitative.

Amino acid analysis

Samples of the purified brain specific enolase isoenzyme were hydrolysed at 110° C for 24 h in 6 N HCl, the hydrolysate was then evaporated to dryness and dissolved in citrate buffer, Ph 2.2. Amino acid analyses were performed on a Locarte amino acid analyser.

Results and Discussion

Purification procedure

The initial stepwise separation stage on DEAE-cellulose removed rapidly migrating acidic proteins from the total extract, these occurred for approximately 25% of the total soluble proteins. Fractionation of proteins soluble in 50% ammonium sulphate on Sephadex G-100 as shown in Fig. 1 produced three peaks, with enolase activity confined to the second protein peak (peak II). Further purification of peak II on DEAE-Sephadex produced the profile shown in Fig. 2. Table I summarises the recovery of total protein and enolase activity during the purification procedure. Approx. 30 mg of human 14-3-2 protein was obtained from a single whole brain, representing a recovery of approximately 10%. The purified protein showed a single band on SDS electrophoresis while on non-denaturing polyacrylamide gel electrophoresis the protein shows a single band with a very faint diffuse staining behind it, similar profiles are seen with bovine 14-3-2 protein [23].

Properties of purified nervous-system specific enolase isoenzyme

The purified enolase isoenzyme had a specific activity of 20—60 I.U./mg protein. Molecular weight estimations by Sephadex G-100 chromatography

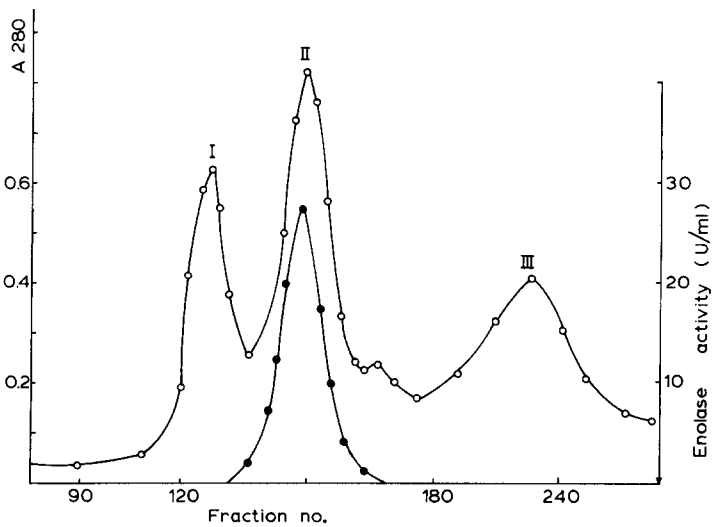

Fig. 1. Sephadex G-100 chromatography of proteins soluble in 50% ammonium sulphate. Approx. 500 mg of 50% ammonium sulphate supernatant proteins were applied in each run. ○———○, A280; ●———●, enolase activity.

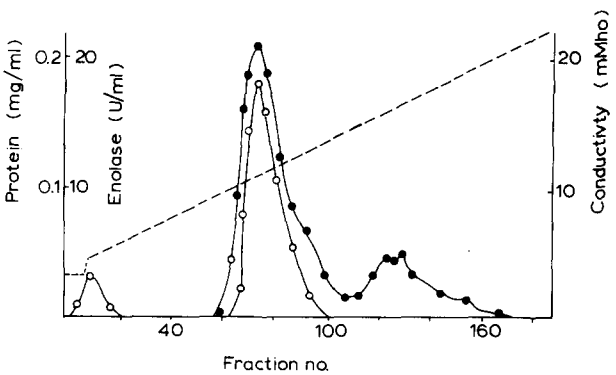

Fig. 2. DEAE-Sephadex chromatography of peak II proteins. Proteins from peak II of Sephadex G-100 chromatography (see Fig. 1) were chromatographed on DEAE-Sephadex A-25. •———•, $A280$; ○———○, enolase activity; ------, conductivity. Protein was measured by the method of Lowry et al. [29].

[19] consistently gave a molecular weight of 62 000. Sucrose density gradient centrifugation [20] gave a molecular weight of approx. 100 000 while subunit molecular weight estimations on dodecyl sulphate polyacrylamide gel electrophoresis [18] consistently gave a value of 50 000. Subunit molecular weight estimations on guanidine HCl columns [21] gave a value of 55 000.

The molecular weight of nervous-system specific enolase isoenzyme (14-3-2 protein) has been the subject of varying reports. The value obtained by sucrose density gradient centrifugation for bovine 14-3-2 protein has been reported as 50 000 and since an identical value was found on dodecyl sulphate polyacrylamide gel electrophoresis it has been proposed that the bovine form of the protein is a single polypeptide chain of 50 000 [23], further studies suggesting either that this single polypeptide chain has extensive internal sequence duplication or that it consists of two identical 25 000 subunits held together by unusually strong non-covalent bonds [24]. The subunit molecular weight of 50 000—55 000 found here is in close agreement with values found previously

TABLE I

RECOVERIES OF PROTEIN AND ENOLASE ACTIVITY DURING PURIFICATION OF HUMAN NERVOUS-SYSTEM SPECIFIC ENOLASE

The results are for a typical purification from a single human brain (1500 g wet wt.). The protein values are the means of quadruplicate determinations ± S.D., the enolase activities are the means of duplicate determinations agreeing to within 10%.

Fraction	Protein (g)	Enolase activity (I.U.)
Homogenate	101 ± 8	19 700
Soluble protein extract	18 ± 1	13 400
Soluble acidic proteins	3.1 ± 3	4 600
Ammonium sulphate 50% supernatant proteins	0.8 ± 0.1	2 000
Peak II from G-100	0.120 ± 0.1	1 000
Pure nervous-system specific enolase	0.03 ± 0.002	600

with purified cat, rat and human preparations [25]. However, gel filtration on Sephadex G-100 has consistently given a molecular weight of 62 000. We assume that this molecular weight is either due to anomalous filtration of the native protein or possible to formation of an active monomer under the conditions of chromatography. As the apparent molecular weigths of the $\alpha\alpha$ and $\alpha\gamma$ forms of human brain enolase (but not the $\gamma\gamma$ form) have been reported to depend on the presence of magnesium ions [26] we have repeated gel filtration of human nervous-system specific enolase isoenzyme on Sephadex G-100 and also on Ultragel in the presence of magnesium concentrations of up to 100 mM $MgCl_2$, however the apparent molecular weight remained unchanged. Amino acid analysis of a typical preparation of human nervous-system specific enolase isoenzyme is shown in Table II. The amino acid composition is in close agreement with that reported for the human protein prepared by a different method [25].

Radioimmunoassay

The sheep antiserum obtained showed approximately 50% binding of the iodinated tracer at a final dilution of 1 in 20 000, and 50 μl of the neat antiserum produced complete inhibition of 0.03 units of enolase activity. Fig. 3 shows a typical standard curve for the radioimmunoassay, maximum sensitivity is approximately 150 pg per tube (i.e. 1.5 ng/ml), this representing a displacement of 10% of the added tracer. The preincubation stage of the radioimmunoassay increases the sensitivity approximately ten-fold.

Levels in human tissues

The levels of nervous-system specific enolase in human tissues are summarised in Table III. DEAE-cellulose chromatography of whole human brain extract as described in Materials and Methods produced three peaks of enolase activity accounting for 48, 25 and 27% of the total enolase activity in the extract. Cellulose acetate electrophoresis of each peak with a specific stain for enolase activity showed that the first peak represented the least anodic, the second peak the intermediate form and the last peak the most anodic form of the three enolase isoenzymes normally seen in brain extracts [16]. The two most acidic peaks reacted in the radioimmunoassay, the less acidic peak showed

TABLE II

AMINO ACID ANALYSIS OF PURIFIED HUMAN NERVOUS-SYSTEM SPECIFIC ENOLASE

Cysteine and tryptophan were not estimated.

	Mol%		Mol%
Asp	12.5	Met	1.4
Thre	3.9	Ile	5.6
Ser	5.5	Leu	11.0
Glu	10.9	Tyr	2.9
Pro	3.5	Phe	3.3
Gly	8.4	His	1.5
Ala	10.5	Lys	6.7
Val	6.4	Arg	4.6

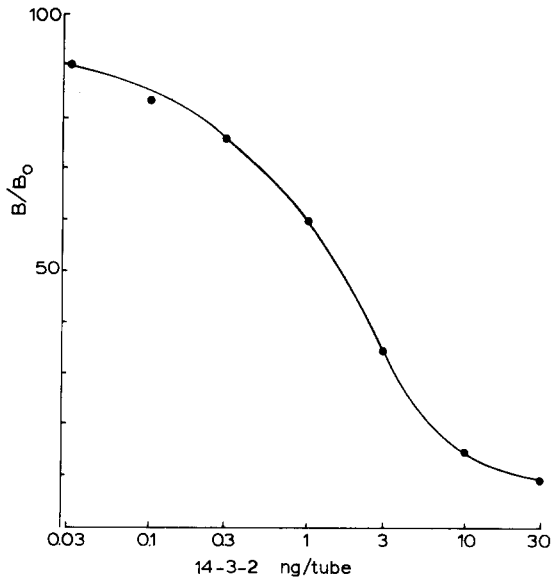

Fig. 3. Standard curve for the radioimmunoassay of human nervous-system specific enolase. Non-specific binding (i.e. binding of the iodinated tracer by the donkey anti-sheep antiserum in the absence of the specific sheep anti-14-3-2 first antibody) amounting to 2.5% has been subtracted initially from each binding value. The points are the mean of duplicate determinations agreeing to within 5%, and are expressed as the percentage binding compared to that seen in the absence of added 14-3-2 (B/B_0). 48% of the added tracer was bound in the absence of added 14-3-2 (B_0).

TABLE III

LEVELS OF THE TWO FORMS OF NERVOUS-SYSTEM SPECIFIC ENOLASE IN HUMAN TISSUES

Alternate fractions were assayed for enolase activity and for immunoreactivity in the radioimmunoassay (e.g. see Fig. 4). The relative amounts of heterodimer and homodimer ($\gamma\gamma$) in each tissue have been calculated from the areas under each peak on the column elution profiles.

Organ	Total enolase activity (I.U./g wet wt.)	% Total activity as heterodimer	% Total activity as homodimer ($\gamma\gamma$)	Immunoreactive heterodimer (μg/g wet wt.)	Immunoreactive homodimer ($\gamma\gamma$) (μg/g wet wt.)
Brain	10.1	25	27	90	207
Adrenal	5.6	3.6	1.2	4.4	3.6
Large intestine	5.1	7.5	1.3	3.5	2.3
Prostate	3.7	5.0	2.0	3.0	1.6
Erythrocytes	1.7	8.0	4.0	2.4	1.4
Small intestine	7.4	1.6	0.3	2.6	1.3
Spleen	10.6	1.4	0.2	3.2	1.1
Uterus	1.5	8.5	1.3	2.6	1.0
Lung	2.6	6.8	0.7	3.9	1.0
Heart	3.6	3.7	0.4	2.9	0.7
Thyroid	3.0	5.2	0.5	3.5	0.7
Ovary	4.0	4.0	0.4	2.0	0.6
Testis	3.3	3.0	0.4	2.5	0.55
Kidney	14.0	0.5	0.05	1.5	0.3
Skeletal muscle	36.5	0.1	0.02	0.5	0.3
Liver	13.5	0.3	0.04	0.8	0.3

less than half the specific activity of the more acidic peak. Fig. 4 shows that both peaks of immunoreactivity diluted out in the radioimmunoassay in parallel with the standard curve, providing further evidence for immunological identity between each peak and the purified 14-3-2 protein used as standard in the assay. Similar profiles were obtained with every organ examined, the results are summarised in Table III. All tissues contained a main unbound peak and two bound peaks eluting in the same positions as the $\alpha\gamma$ heterodimer and the $\gamma\gamma$ homodimer from brain, however unlike brain the two peaks bound to the column represented considerably less of the total enolase activity in the extract. In some tissues (e.g. the uterus and red blood cells), the heterodimer and homodimer together constituted approximately 10% of the total enolase activity, in others (e.g. skeletal muscle, liver and kidney) the two bound forms of the enzyme constituted less than 1% of the total enolase activity. The amount of immunoreactivity in red blood cells is insufficient to account for the levels found in other tissues.

The above results are the first systematic immunological survey of human tissues for nervous-system specific enolase and demonstrate that immunoreactivity can be found in all tissues at levels up to 2—3% of that found in human brain (Table III). Apart from thyroid, liver, adrenal and pituitary [10], no previous immunoassay data for 14-3-2 protein in human tissues are available, and previously in neither rat nor human has the immunoreactivity present in tissues other than liver been shown to chromatograph with enolase activity

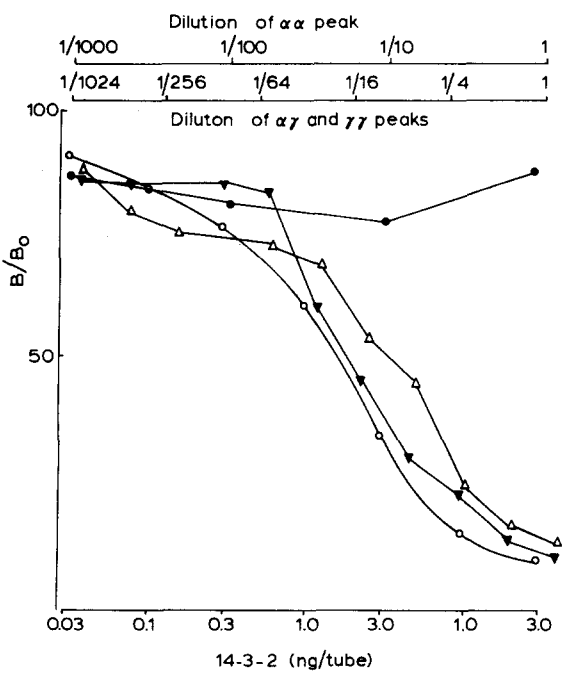

Fig. 4. Immunoreactivity of the three peaks of enolase activity from human brain. ○———○, standard curve; ●———●, first peak ($\alpha\alpha$), ▼———▼, second peak ($\alpha\gamma$); △———△, third peak ($\gamma\gamma$). Each value is the mean of duplicate determinations agreeing to within 5%.

or to be present in more than one form. Previous estimations of the tissue-specificity of nervous-system specific enolase in human organs have come from electrophoretic separation on starch gels followed by activity staining, [26,27], however, these techniques are dependent on detecting enzyme activity and lack the sensitivity of radioimmunoassay.

At least some of the two immunoreactive forms of nervous-system specific enolase detected in human tissues could reflect the normal innervation of each organ. However, red blood cells, with no innervation; contain similar levels of immunoreactivity to other tissues which are innervated. Furthermore the presence of low levels of the heterodimer and homodimer in erythrocytes demonstrates that the expression of the γ subunit can occur in cells with no amine precursor uptake and degradation function.

The predominant form of the enzyme in all tissues (except brain) is the heterodimer, this form of the enzyme could possibly be formed artefactually during the preparation of tissue extracts. In vitro experiments with partially purified, individual isoenzymes from rat brain [28] and human brain [26] have shown that the hybrid dimer $\alpha\gamma$ can dissociate to form $\alpha\alpha$ and $\gamma\gamma$ homodimers and that mixtures of $\alpha\alpha$ and $\gamma\gamma$ homodimers can dissociate to form $\alpha\gamma$ heterodimers when subjected to high salt concentrations and/or freezing and thawing. Immunocytochemical localization of the $\gamma\gamma$ isoenzyme in rat brain has shown a strictly neuronal localization [4,5] however antisera used in the localization presumably did not distinguish between γ subunits contained in $\gamma\gamma$ homodimers from those contained in $\alpha\gamma$ heterodimers. Localization using anti-$\alpha\alpha$ antiserum [5] has shown the $\alpha\alpha$ form to be present in non-neuronal elements with no staining of neurones. However, the anti-serum used had previously been adsorbed with $\alpha\gamma$ heterodimer which would be expected to remove antibodies preferentially directed against the hybrid enzyme. Unadsorbed anti-$\alpha\alpha$ sera did in fact produce faint staining of neurones, suggesting that the $\alpha\gamma$ hybrid may be present in neurones [5]. With the present results human tissues were frozen before analysis and possibly this or the subsequent homogenization procedure led to the formation of the hybrid dimer. However, exactly the same pattern was seen in fresh red blood cells which had not been subjected to freezing or to homogenization but only to gently lysis. This suggests that the heterodimer does exist in vivo in a single cell type along with the respective homodimer forms.

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