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SEPARATION BY DIFFERENT METHODS OF SOLUBLE PROTEINS ISOLATED FROM SYMPATHETIC SPLENIC NERVES

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SUMMARY

The soluble proteins of sympathetic splenic nerves were separated by high-performance liquid chromatography (HPLC), sodium dodecyl sulphate (SDS)–HPLC on TSK-GEL-SW columns and SDS–polyacrylamide gel electrophoresis (PAGE). With HPLC the main fractions had apparent molecular weights M_r 150,000, 75,000–85,000, 27,000 and < 5000, with SDS–HPLC the M_r values were 75,000, 13,500 and < 5000 and with SDS–PAGE the M_r were 75,000, 55,000, 45,000 and < 12,500. Of the proteins eluted after electrofocusing, the bands of pH 4.0–5.2 showed a single peak of M_r 75,000 in all separation methods used; in the range pH 5.8–6.5, proteins with M_r 150,000 were found by HPLC, 75,000 by SDS–HPLC and 55,000 and 45,000 by SDS–PAGE. The M_r 150,000 fraction in the range pH 5.8–6.5 showed DOPA decarboxylase (E.C. 4.1.1.28) activity. The results provide new information about the soluble proteins of sympathetic nerve.

INTRODUCTION

The determination of protein polypeptides by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the separation of proteins by gel chromatography are complicated by the different mobilities of the proteins. The use of porous silica gel columns (TSK-GEL-SW) for high-performance liquid chromatography (HPLC) gives good results in short times[1,2]. Proteins isolated from adrenal medulla granules separated by SDS-PAGE showed different fractions with and without 2-mercaptoethanol in the samples[3].

In this work, high speed gel filtrations of proteins isolated from the sympathetic splenic nerve on porous silica gel columns with (SDS–HPLC) and without SDS in the mobile phase were compared with the fractions resulting from SDS–PAGE and analytical electrofocusing methods. Takagi[4] has differentiated between the results of SDS–HPLC (TSK-G-SW column) and SDS–PAGE with defined protein-kits. The proteins isolated from sympathetic nerves show some differences in SDS–HPLC and SDS–PAGE.

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EXPERIMENTAL

Materials

The chemicals used were of analytical grade: sodium phosphate, sodium chloride, sodium dodecyl sulphate and 2-mercaptoethanol were obtained from E. Merck (Darmstadt, G.F.R.); dithiothreitol (DTT) from Sigma; protein-standard-kits from Pharmacia and Bio-Rad Labs; Sephadex from Pharmacia; Coomassie blue from LKB or Serva blue G and Servalyt from Serva (Heidelberg, G.F.R.) and ampholine from LKB.

Instrumentation

An HPLC-Varian 5000 with UV-detector (280 nm) and intregrator CDS 111 was employed. For electrophoresis and electrofocusing, a LKB 2117-multiphor system was used in all experiments. The columns for HPLC were TSK G 3000 SW, LKB (600 × 7.5 mm) or Varian (500 × 7.5 mm), with pre-columns (75 respectively 100 × 7.5 mm). The mobile phase was 0.10 *M* sodium phosphate buffer of pH 6.8 and 0.1 *M* NaCl with a flow-rate of 0.5 ml/min for the LKB columns and 1.0 ml/min for the Varian columns. The same columns and flow-rates were used in the experiments with SDS (0.1%) and DTT (2.5 m*M*). The operating temperature was 20 ± 2°C. A Rheodyne injector with a sample loop of 25 or 100 μl was used.

Sample preparation

The soluble proteins from sympathetic splenic nerve were prepared according to a modification of the method of Schümann and Burger[5]. The supernatant obtained after centrifugation at 12,000 *g* was ultra-centrifuged at 230,000 *g* for 60 min without a sucrose gradient. The supernatant was used for separation of the soluble protein content. For HPLC determination, the samples were diluted to a concentration of 1 mg/ml protein and 25 or 100 μl of this solution were injected. In experiments with SDS the protein samples were treated with 0.5% SDS and 0.4% DTT and heated for 5 min at 100°C. For slab gel electrophoresis the protein concentration was 5 mg/ml. SDS-PAGE was carried out according to the method of Fehrnström and Moberg[6].

For preparative flat-bed electrofocusing in a granulated gel[7], 40–80 mg protein were used on the flat-bed Sephadex for separation. After focusing (16 h) in a stable pH-gradient (4–9, Servalyt) the protein fractions were separated and eluted with 0.1 *M* phosphate buffer (pH 7.0) (5 × 5 ml) and, after desalting, subsequently concentrated on Amicon membrane filter PM 10 and subjected to HPLC or SDS electrophoresis.

Calibration curves

For accurate determination of apparent molecular weights, M_r, protein-standard-kits were used. Fig. 1 shows the calibration curve for the HPLC determination of proteins. The calibration-kit was from Bio-Rad Labs. The molecular weights of the proteins ranged between M_r 670,000 and 1350. For SDS–HPLC the protein calibration curve was constructed with the aid of a kit from Pharmacia, the molecular weight range being 94,000–14,400 (Fig. 2). In both calibration curves the molecular weights were plotted semi-logarithmically *versus* their retention times.

For construction of the calibration curve in SDS–PAGE (5% gel), kits with low- and high-molecular-weight proteins (Bio-Rad Labs.) were used, M_r between

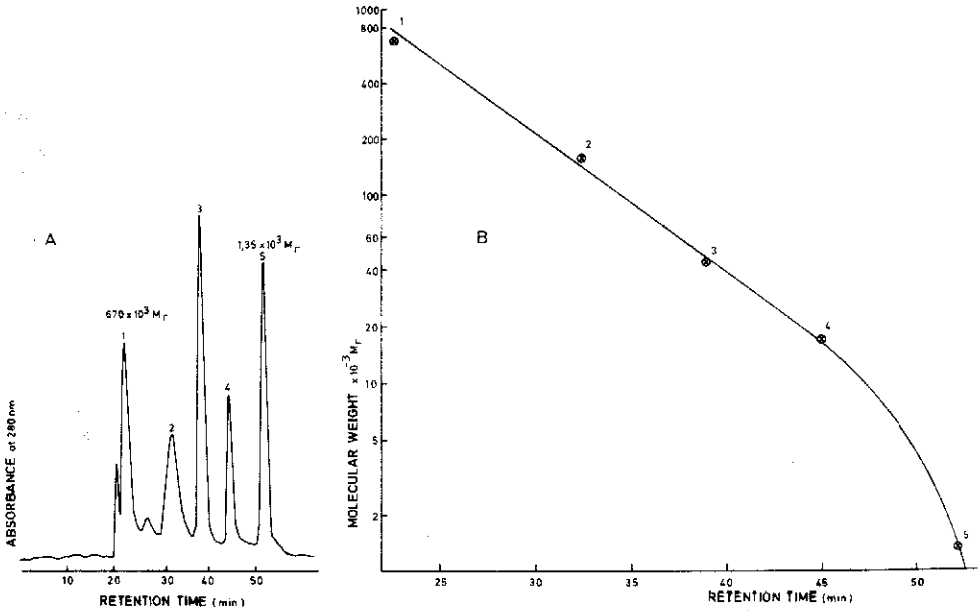


Fig. 1. A, Elution pattern of calibration proteins (Bio-Rad Labs.) estimated by HPLC with an LKB TSK-GEL 3000 SW column (600 × 7.5 mm). The proteins were dissolved in 2.0 ml of mobile phase and 10 µl were injected. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 6.8) and 0.1 *M* NaCl. Flow-rate: 0.5 ml/min. The vial contained: 1, thyroglobin (bovine), M_r 670,000; 2, γ-globin (bovine), M_r 158,000; 3, ovalbumin (chicken), M_r 44,000; 4, myoglobin (horse), M_r 17,000; 5, vitamin B_{12}, M_r 1350. B, Plot of molecular weights M_r of the calibration proteins *versus* their retention times estimated by HPLC on the TSK-GEL 3000 SW column.

200,000 and 14,300. The molecular weights were calculated from the mobilities of the proteins relative to bromophenol blue (Fig. 3).

RESULTS

The soluble proteins isolated from bovine sympathetic splenic nerves show main fractions with molecular weights, M_r, of 75,000, 55,000, 45,000 and <12,000 as separated by SDS–PAGE with 2-mercaptoethanol. Without 2-mercaptoethanol the M_r 75,000 and 55,000 fractions show combined peak in the range of M_r 68,000 (Fig. 4A and B).

The proteins from the same preparations when separated by HPLC on the TSK-GEL 3000 SW column show different elution patterns with and without SDS. Without SDS the proteins were separated in fractions with molecular weights M_r of 150,000, 75,000–85,000, 27,000 and with different fractions of <5000. The first void peak (Fig. 5A) contains high-molecular-weight proteins (M_r >670,000) which were not separated. In the presence of SDS, after the first void peak, the proteins were found in fractions with apparent molecular weights M_r of 75,000, 13,500 and <5000 (SDS–HPLC) (Fig. 5B). The behaviour of soluble proteins of the sympathetic nerves under these experimental conditions is summarized in Table I.

A comparison between the apparent molecular weights of the proteins with

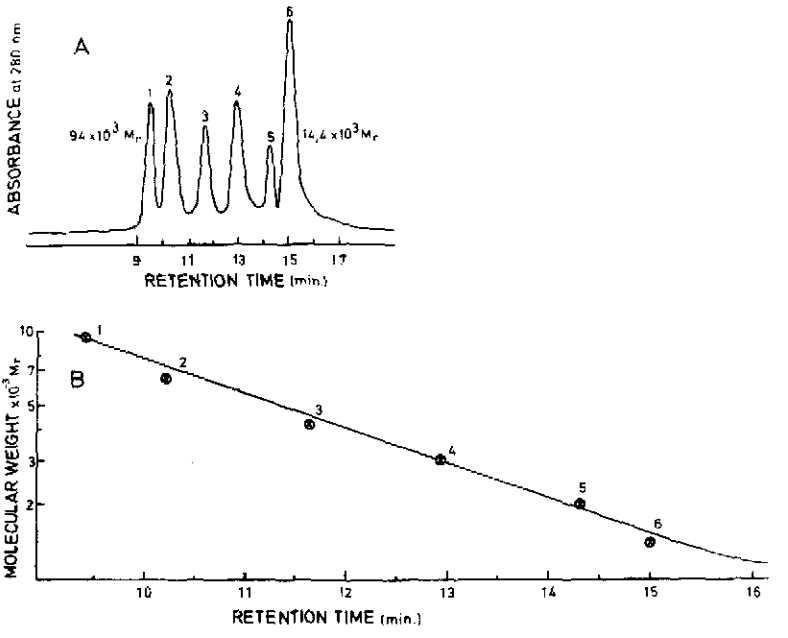
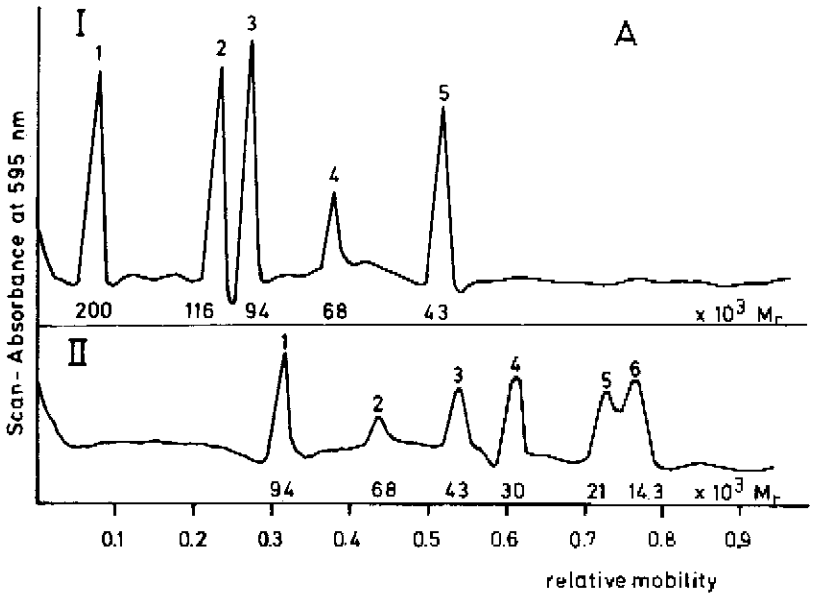


Fig. 2. A, Elution pattern of calibration proteins (Pharmacia) estimated by SDS–HPLC with a Varian TSK-GEL 3000 SW column (500 × 7.5 mm). The mobile phase was 0.1 *M* sodium phosphate (pH 6.8) and 0.1 *M* NaCl containing 0.1% SDS and 2.5 m*M* DTT; flow-rate 1.0 ml/min. The kit proteins were dissolved in 200 µl 0.1 *M* phosphate buffer (pH 6.8), 30 mg SDS and 10 mg DTT and heated to 100°C for 5 min. The vial contained: 1, phosphorylase *b* (rabbit), M_r 94,000; 2, serum albumin (bovine), M_r 67,000; 3, ovalbumin (egg white), M_r 43,000; 4, carbonic anhydrase (bovine), M_r 30,000; 5, trypsin inhibitor (soy bean), M_r 20,000; 6, 2-lactalbumin (bovine), M_r 14,400. B, Plot of molecular weights of the calibration proteins from Pharmacia *versus* their retention times by SDS–HPLC estimation with TSK-GEL 3000 SW.

and without SDS was made after separation by quantitative electrofocusing using a stable gradient of pH 4–9. After separation, fractions I–IV were eluted with 0.1 *M* phosphate buffer and concentrated on Diaflow-filter. The eluted proteins were reanalyzed by HPLC, SDS HPLC and SDS-PAGE (Fig. 6A–C). Fraction I (isoelectric pH values 4.5–5.2) contains a single protein band with a molecular weight M_r of 75,000 as determined by HPLC as well as by SDS-HPLC and SDS-PAGE. Fraction II (isoelectric pH values 5.2–5.6) contains proteins with different molecular weights M_r of 75,000 (40%) and 150,000 (60%) estimated by HPLC (Fig. 6A). Both may be components of Fractions I and III. In SDS–HPLC the proteins found had M_r 75,000 (75%), 55,000 (20%) and 13,500 (5%). The proteins detected by SDS–PAGE had M_r 55,000 (45%) and 75,000 (25%) and small amounts of 84,000, 45,000 and 27,000. Fraction III (isoelectric pH values 5.8–6.5) contains a protein with molecular weight M_r of 150,000 is detected by HPLC. This protein shows a molecular weight M_r of 75,000 in SDS–HPLC. Two proteins, M_r 55,000 (72%) and 45,000 (28%), were found by SDS-PAGE. Fraction IV (isoelectric pH values 7.0–7.4) contains proteins with molecular weights M_r of 27,000 (90%) and 13,500 (10%) by HPLC estimation. These proteins show molecular weights M_r of 13,500 and $< 12{,}000$ as determined by SDS–HPLC and SDS–PAGE (Fig. 6A–C).



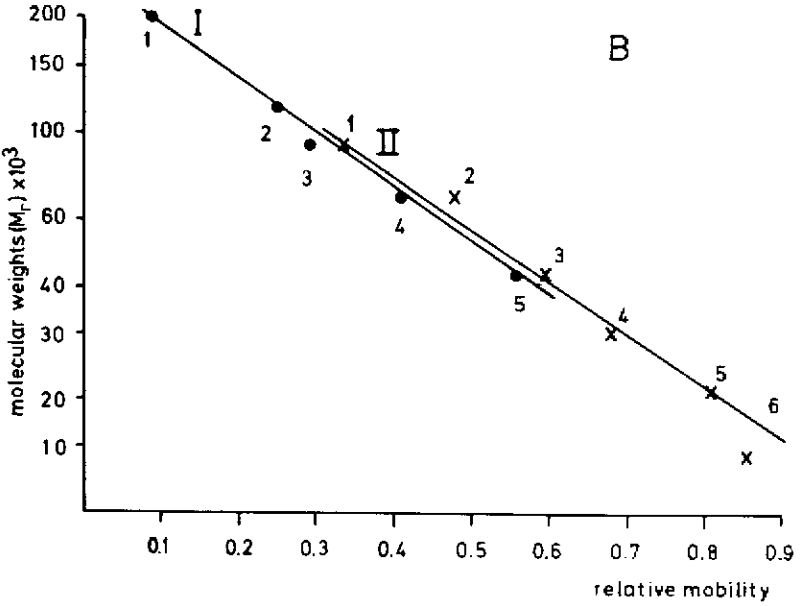


Fig. 3. A, Scan-profile of SDS–PAGE-on a 5% slab gel obtained for the proteins of the Bio-Rad kits by a Quick-Scan densitometer at 595 nm. I, High-molecular-weight standards: 1 = myosin, M_r 200,000; 2 = β-galactosidase, M_r 116,500; 3 = phosphorylase *b*, M_r 94,000; 4 = serum albumin (bovine), M_r 68,000; 5 = ovalbumin, M_r 43,000. II, Low-molecular-weight standards: 1 = phosphorylase *b*, M_r 94,000; 2 = serum albumin (bovine), M_r 68,000; 3 = ovalbumin, M_r 43,000; 4 = carbonic anhydrase, M_r 30,000; 5 = trypsin inhibitor (soy bean), M_r 21,000; 6 = lysozyme, M_r 14,300 . The vial contents were dissolved in phosphate buffer (0.01 *M*; pH 7.1) containing 10 μl 2-mercaptoethanol and 10 μg SDS and heated to 100°C for 5 min. The SDS–PAGE plate was stained with Coomassie brilliant blue (Serva). B, Plot of molecular weights *versus* mobility relative to bromophenol blue for high (I) and low (II) molecular weight standards analyzed by SDS–PAGE (5% gel).

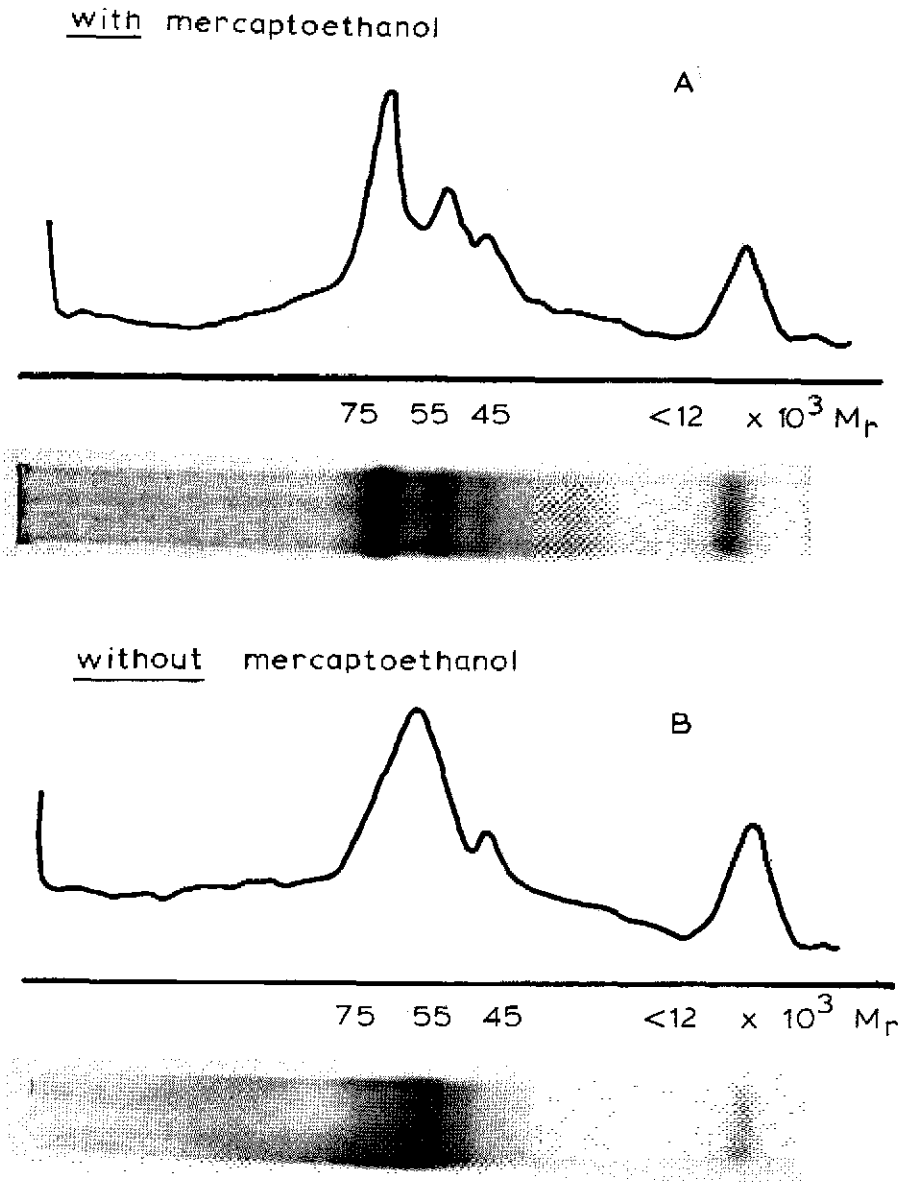


Fig. 4. Separation by SDS–PAGE of soluble proteins isolated from sympathetic splenic nerves. Scan-profile of the Coomassie blue stained fractions and the electrophoretic run with (A) and without (B) 2-mercaptoethanol in SDS containing phosphate sample buffer (0.01 *M*, pH 7.1, heated to 100°C for 5 min). The molecular weights were calculated from the calibration curve in Fig. 3. In B the M_r 75,000 and 55,000 proteins show aggregation ($\approx$ 68,000 M_r).

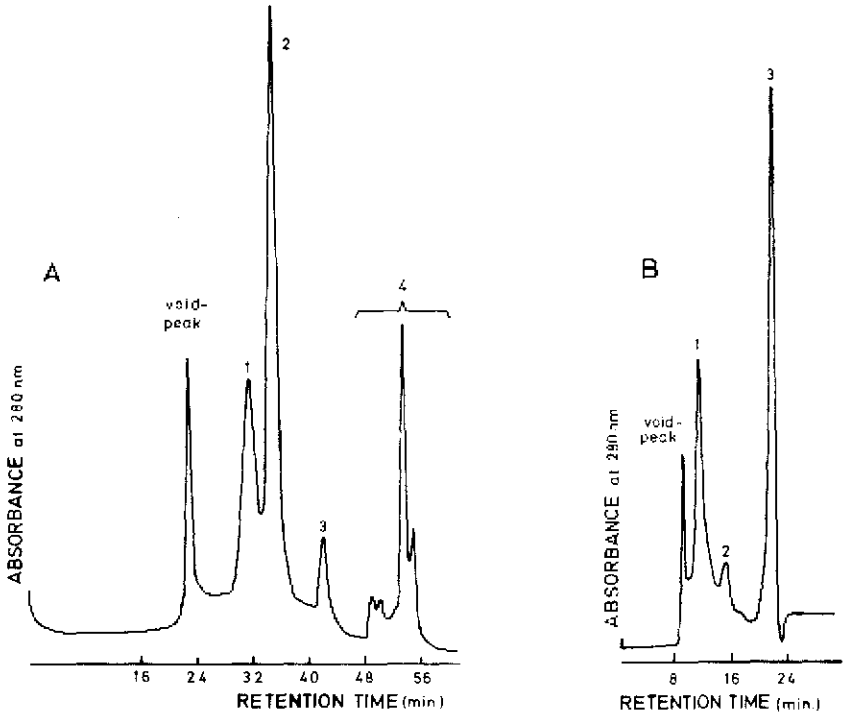


Fig. 5. Separation of soluble proteins isolated from sympathetic splenic nerves by HPLC (A) and SDS-HPLC (B), on a TSK-GEL 3000 SW column. Peaks: A, 1 = M_r 150,000; 2 = M_r 75,000–85,000; 3 = M_r 27,000; 4 = M_r < 5000; B, 1 = M_r 75,000; 2 = M_r 13,500; 3 = M_r < 5000.

TABLE I

BEHAVIOUR OF SOLUBLE PROTEINS OF SYMPATHETIC NERVES UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Values are molecular weights, $M_r \times 10^{-3}$.

A	HPLC	670 + void peak	150	85–75			27		< 5
B	SDS-HPLC			75				13.5	< 5
C	SDS-PAGE with mercaptoethanol			75	55	45			< 12
D	SDS-PAGE without mercaptoethanol			≈68 combined peak					< 12

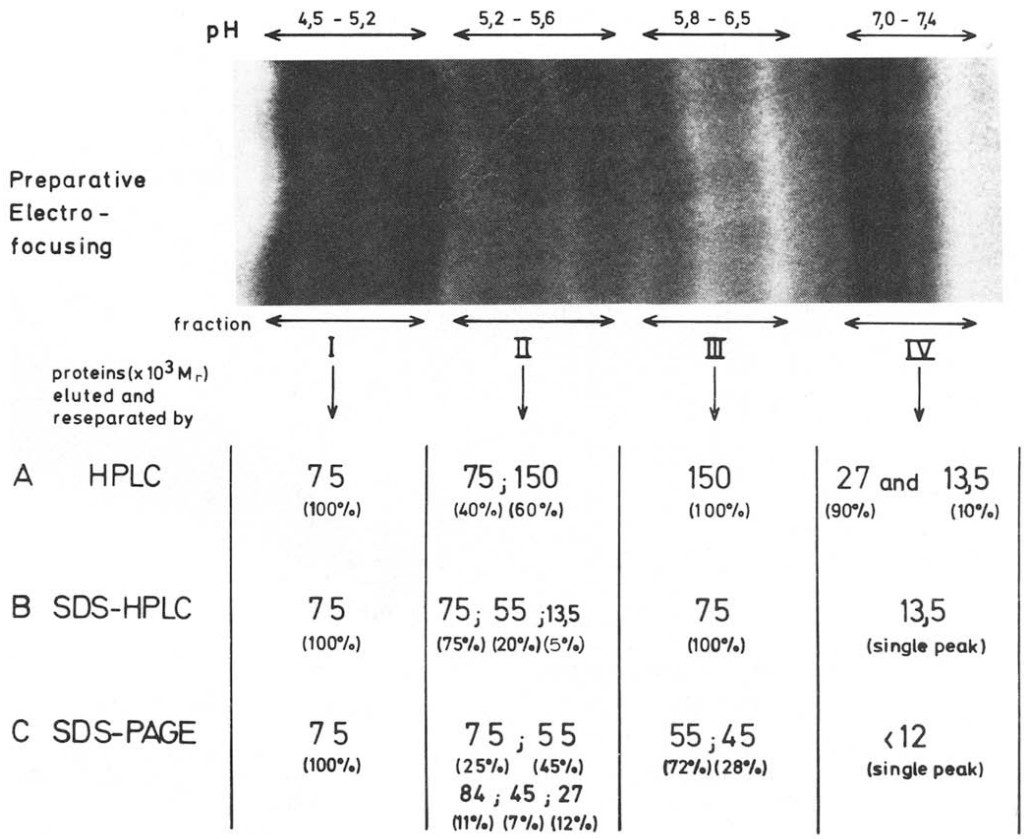


Fig. 6. Preparative flat-bed electrofocusing in stable pH gradients of 4.0–9.0 in granulated Sephadex G-75 gel of the soluble proteins isolated from sympathetic splenic nerves. After electrofocusing for 16 h the proteins of fractions I–IV were eluted with 0.1 *M* phosphate buffer (pH 6.8). The eluted proteins were reanalyzed by HPLC (A), SDS–HPLC (B) and SDS–PAGE (C).

DISCUSSION

Different values have been reported for the molecular weights of dopamine-β-hydroxylase (DBH) isolated from the adrenal medulla and the sympathetic nerves, especially by SDS–PAGE[3,8–11]. A fraction of M_r 150,000 separated by SDS–PAGE without mercaptoethanol (Fig. 4, Table I) was not detected in preparations of sympathetic nerve proteins, in contrast to the results obtained by Huber *et al.*[3] and Winkler[10] for adrenal medulla preparations.

Dopamine-β-hydroxylase (DBH) has been characterized as a copper-containing enzyme with a molecular weight M_r of 290,000. The isoelectric values of the marker DBH-"Sigma" showed a similar electrofocusing pattern under the above conditions as the M_r 75,000 protein of fraction I. The latter protein in the pH range of 4.5–5.2, when analysed by HPLC, SDS–HPLC and SDS–PAGE, gave a single fraction. Lagercrantz[11] described DBH as the dominant protein present in the soluble phase of the splenic nerve vesicles. A M_r 75,000 protein estimated with SDS PAGE

was detected as a subunit of DBH by Wallace *et al.*[8]; a dimer of M_r 150,000 was not described by these authors. It is, therefore, assumed that the M_r 75,000 fraction of the soluble proteins in the sympathetic nerve in the pH region of 4.5–5.2 is a subunit of DBH. The enzyme activity of DBH has previously been demonstrated in the soluble and isolated fractions of the sympathetic nerve[9,12,13].

In experiments[14] with $(NH_4)_2SO_4$ fractionation (26–40% saturation) the proteins gave M_r 75,000 and 27,000 fractions when re-separated by HPLC and of 75,000 by SDS–PAGE. A similar fractionation (40–50% saturation) was described for an enzyme assay of dopamine-β-hydroxylase[15]. The acidic pH values of the DBH purified by electrofocusing found by O'Connor *et al.*[16] were in a good agreement with the values of fraction I from Fig. 6 with a maximum density of bands in the pH 4.5 region. In contrast to the previous work[16], a M_r 150,000 protein was not found in this pH 4.5 region when estimated by SDS–PAGE. Instead the M_r 75,000 protein was found which is described by Wallace *et al.*[8] as a subunit of DBH.

Chromogranin A[17,18], another soluble protein, found in high amounts in adrenal medulla preparations, was present only in trace amounts in the sympathetic nerve preparations[11,12]. It has been detected in three forms (M_r 290,000, 74,000 and 33,000) by Helle[19], as M_r 80,000 by Kirshner and Kirshner[20], as two identical "subunits" of M_r 40,600 and 77,000 by Smith and Winkler[17] and as two fractions with M_r 74,000 and 350,000 by Hogue-Angeletti[21]. A definitive conclusion about the composition of chromogranin A cannot be made with the different methods used in this study.

The presence of serum albumin (M_r 68,000) in nerve preparations was discussed[11] and excluded in earlier experiments[9] as a combined peak from fractions of M_r 75,000 and 55,000.

The M_r 150,000 protein of fraction III in the pH region of 5.8–6.5 as estimated by HPLC shows a M_r 75,000 protein when estimated by SDS–HPLC. In contrast to the protein of fraction I (pH 4.5–5.2), this M_r 75,000 protein was separated by SDS–PAGE into M_r 55,000 and 45,000 proteins. Therefore the M_r 75,000 protein of fraction III cannot be a component of DBH as described by Wallace *et al.*[8].

With ammonium sulphate fractionation (10–26% saturation) a M_r 150,000 protein was detected by HPLC. This fraction showed DOPA decarboxylase activity[14]. In SDS–HPLC a M_r 75,000 protein is obtained, whereas with SDS–PAGE a M_r 55,000 and a 45,000 protein could be distinguished.

The elution patterns of the proteins obtained by preparative electrofocusing (pH 5.8–6.5) (Fig. 6) were in agreement with those obtained by ammonium sulphate (10–26%) fractionation.

The molecular weights estimated by HPLC with additional columns (2 × TSK 3000 SW) were not significantly different from the results obtained with single-column techniques. Comparison of the apparent molecular weights from soluble proteins of the sympathetic splenic nerves showed that different results were obtained by HPLC, SDS–HPLC and SDS–PAGE. The use of these methods is helpful in further discrimination of these soluble proteins.

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