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Molecular Weight Determinations of Soluble and Membrane-bound Fractions of Choline *O*-Acetyltransferase in Rat Brain[1]

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Abstract

Three fractions of choline *O*-acetyltransferase (ChAT) (EC 2.3.1.6) were solubilized from a nerve ending fraction of rat forebrain using three sequential washes of an increasingly chaotrophic nature (100 mM sodium phosphate, pH 7.4; 500 mM NaCl; 2% Triton DN-65) as previously described (Benishin, C. G., and P. T. Carroll (1983) J. Neurochem. *41:* 1030–1039). The molecular weights of the soluble (NaP) and membrane-bound fractions (NaCl and 2% Triton DN-65) of ChAT, following partial purification, were determined using either gel filtration on Sephadex G-200, G-100 Superfine, or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by "Western blotting" and immunochemical visualization of ChAT with four different anti-ChAT monoclonal antibodies (Ab8, Ab9, 4D7, and 1E6). Results obtained with gel filtration indicated that the NaP- and Triton DN-65-solubilized fractions of ChAT had molecular weights in the range of 73,000 to 78,000, whereas the NaCl-solubilized fraction of ChAT had a molecular weight in the range of 230,000 to 240,000. Results obtained with SDS-PAGE and Western blotting indicated that all three fractions of ChAT were composed of the same nonidentical subunits.

Choline *O*-acetyltransferase (EC 2.3.1.6; ChAT) catalyzes the formation of acetylcholine (ACh) in central cholinergic neurons. Although the majority of this enzyme is believed to be soluble and to exist in the cytoplasm of central cholinergic nerve terminals (Fonnum, 1968), some of it appears to be membrane bound (Smith and Carroll, 1980; Benishin and Carroll, 1981). Following hypoosmotic rupture of central cholinergic nerve terminals prepared from mouse or rat brain, soluble ChAT binds ionically to membranes and can be removed by repeated washes of the synaptosomal fraction with 100 mM sodium phosphate buffer (pH 7.4). Two membrane-bound fractions can then be solubilized by consecutive washes with 500 mM NaCl and 2% Triton DN-65 (Benishin and Carroll, 1983). The enzyme fractions removed by high salt and detergent differ from each other and from the soluble fraction in the following respects: pH profile, capacity to acetylate homocholine, sensitivity to heat inactivation, differential loss of the enzyme forms in the hippocampus following interruption of the septohippocampal pathway, and rate of development in neonatal brain (Benishin and Carroll, 1983, 1984).

The objective of the present study was to determine whether these three fractions of ChAT also differed in molecular weight. To accomplish this objective, two procedures were used: (*1*) gel filtration in Sephadex G-100 Superfine and G-200, and (*2*) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by "Western blotting" and immunochemical visualization of ChAT with four different monoclonal antibodies (Ab8, Ab9, 4D7, and 1E6). The results indicated that the molecular weight of the native form of the high salt-soluble fraction of ChAT clearly exceeded the molecular weights of the sodium phosphate- and detergent-soluble fractions of ChAT. However, when the molecular weights of these ChAT fractions were determined on denaturing gels, none of these three fractions could be distinguished from each other. Instead, they all appeared to consist of the same nonidentical subunits.

Materials and Methods

Bovine serum albumin (BSA), thyroglobulin, catalase, aldolase, ovalbumin, chymotrypsinogen A, blue dextran 2000, and Sephadex G-200 and G-100 Superfine were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), pepstatin, soybean trypsin inhibitor, leupeptin, aprotinin, antipain dihydrochloride, benzamidine hydrochloride, benzethonium chloride, choline bromide, eserine sulfate, acetyl-CoA, Triton DN-65, Nonidet P-40 (NP-40), and naive rat IgG were from Sigma Chemical Co. (St. Louis, MO). SDS low and high molecular weight protein standards and Bio-Beads SM-2 were from Bio-Rad Laboratories (Richmond, CA). Spectrafluor and [acetyl-1-^{14}C]acetyl-CoA (specific activity, 51 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Hyamine hydroxide was obtained from Research Products International Corp. (Mt. Prospect, IL). Nitrocellulose paper was obtained from Schleicher & Schuell (Keene, NH). Anti-ChAT monoclonal antibodies Ab8 and Ab9 were kindly provided by Dr. Bruce Wainer (University of Chicago, Chicago, IL). Horseradish peroxidase (HRP) and rabbit anti-rat antibody were purchased from Cappel Laboratories (Cochranville, PA). Anti (rat ChAT)-monoclonal antibodies 1E6 and 4D7 were kindly provided by Dr. Paul Salvaterra (Beckman Res. Inst., Duarte, CA). Extracti-Gel D was purchased from Pierce Chemical Co. (Rockford, IL). Silver stain kit was obtained from Gelcode System Products (South Heven, MI). Other reagents were analytical grade.

Enzyme assay

The ChAT activity of brain samples was determined by the method of McCaman and Hunt (1965), as modified by Fonnum (1969). To start the reaction, 2 μl of an enzyme sample were added to 20 μl of substrate solution of the following composition: 234 mM NaCl, 0.0375% bovine plasma albumin, 17 mM $MgCl_2$, 84 mM sodium phosphate buffer (NaP), pH 7.4, 150 μM eserine, 340 μM [^{14}C]acetyl-CoA (specific activity, 2 mCi/mmol), and 10 mM choline bromide. Following either a 10- or a 15-min incubation at 39°C, samples were placed in ice water. Ice cold sodium tetraphenylboron (150 μl) in 3-heptanone (5 mg/ml) was then added to stop the reaction and extract the [^{14}C]ACh (Tucěk, 1978). The Organic extract (100 μl) was then added to 11 ml of Scintillant (1 ml of hyamine hydroxide-methanol, and 10 ml of toluene containing Spectrafluor) and counted for ^{14}C-acetylated products.

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ChAT purification

a. Three fractions of ChAT were initially solubilized from the forebrains of rats or the caudates of bovine brain as described by Benishin and Carroll (1983). Male King rats (King Animal Laboratories, Oregon, WI) were killed by decapitation. Brains were quickly removed and placed in ice-cold 0.32 M sucrose. Subcellular fractions were then prepared as described previously (Benishin and Carroll, 1983). Brains were homogenized in 15 vol of ice-cold 0.32 M sucrose and then centrifuged at 1,000 × *g* for 10 min. The resulting supernatant was spun at 17,000 × *g* for 15 min. The second supernatant was discarded, and the synaptosomal pellet was homogenized in 6 vol of ice-cold glass distilled water and then centrifuged at 100,000 × *g* for 60 min. The supernatant was discarded and the pellet was initially homogenized in 3 vol of 100 mM NaP, pH 7.4, to solubilize ionically bound nerve ending ChAT. The homogenate was then centrifuged at 100,000 × *g* for 30 min, and the supernatant containing the "soluble" or "cytoplasmic" fraction of ChAT was saved for assay. The pellet was washed a second and third time with NaP buffer (pH 7.4), but these washes removed negligible ChAT activity and were discarded. The resulting NaP-washed pellet was then homogenized in 3 vol of 500 mM NaCl and centrifuged at 100,000 × *g* for 30 min. The supernatant contained the NaCl-solubilized ChAT and was saved for assay. The pellet was then homogenized in 3 vol of 5 mM NaP buffer, pH 7.4, containing 2% Triton DN-65 and allowed to set at −20°C for 12 to 16 hr. This homogenate was then thawed and centrifuged at 100,000 × *g* for 60 min. The supernatant contained the Triton DN-65-solubilized ChAT. Triton DN-65 (2%) solubilized approximately 70% of the membrane-bound ChAT from the pellet. Sodium phosphate (100 mM, pH 7.4)-, NaCl (500 mM)-, and Triton DN-65 (2% in 5 mM NaP buffer, pH 7.4)-soluble fractions of ChAT were then fractionated with ammonium sulfate. The precipitates obtained from 40 to 60% and 0 to 60% ammonium sulfate fractions of NaP- and NaCl-soluble ChAT, respectively, were redissolved in a minimal volume of citrate/phosphate buffer (10 mM disodium phosphate neutralized to pH 7.2 with 1 M citric acid and containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol) and were dialyzed overnight against two 1-liter changes of the same buffer. The Triton DN-65-solubilized ChAT was fractionated with ammonium sulfate either before or after the removal of the Triton DN-65 by affinity chromatography using Extracti-Gel D or Bio-Beads SM-2. The precipitate obtained from a 0 to 60% ammonium sulfate fraction was then redissolved in a minimal volume of citrate/phosphate buffer and dialyzed against two 1-liter changes of the same buffer overnight. Undissolved materials were removed by centrifugation at 39,000 × *g* for 30 min. The clear supernatants of the three ChAT fractions were then applied to either a 1 × 30 cm column of Sephadex G-200 or a 1.6 × 70 cm column of G-100 Superfine equilibrated in the citrate/phosphate buffer. The flow rate of both columns was 2 ml/hr. Appropriate fractions were pooled and concentrated in an Amicon concentrator (Minicon B-15). The partially purified ChAT, stored in the same buffer at 0°C, was stable for several months. The specific activities of the NaP-, NaCl-, and Triton DN-65-soluble fractions of ChAT were 60, 30, and 20 milliunits/mg of protein, respectively. The specific activities of the NaP-, NaCl-, and Triton DN-65-soluble fractions of ChAT prior to gel filtration were 5, 2, and 1.5 milliunits/mg of protein, respectively. Units are defined as micromoles of ACh produced per min^{-1} ml^{-1}.

b. Three fractions of rat brain ChAT were also initially solubilized as described by Ryan and McClure (1979), with minor modifications. Male King rats (King Animal Laboratories) were killed by decapitation. Brains were quickly removed and homogenized in 15 vol of ice-cold 25 mM NaP buffer, pH 7.4, with a Teflon pestle at 840 rpm, with 20 up-and-down strokes. This homogenate was then centrifuged at 20,000 × *g* for 1 hr (first NaP wash). The pellet was washed a second time with the same buffer and centrifuged at 20,000 × *g* for 1 hr, but this second wash did not solubilize a significant amount of ChAT activity and was therefore discarded. The pellet was then homogenized in 3 vol of 500 mM NaCl (NaCl-solubilized ChAT). Further homogenizations of the pellet in 5 vol of 2% Triton DN-65 in 5 mM NaP, pH 7.4, after the 500 mM NaCl wash solubilized the majority of the ChAT activity remaining in the pellet. The supernatants from the NaCl and Triton DN-65 washes were then fractionated with ammonium sulfate. The supernatant from the first NaP wash was adjusted to pH 4.5 with 50% acetic acid added over a 1-hr period. The solution was stirred for 50 min at 4°C and centrifuged at 20,000 × *g* for 1 hr. The supernatant was then adjusted to pH 6.0 with 1 M NaOH and then fractionated with ammonium sulfate. The pellets from 40 to 60% ammonium sulfate fractions for NaP-, NaCl-, and Triton DN-65-solubilized ChAT were resuspended in 10 mM citrate/phosphate buffer, pH 7.2, containing 0.1 mM dithiothreitol and 0.1 mM EDTA, dialyzed against the same buffer overnight before use, and stored at 0°C. The specific activities of the ChAT fractions solubilized by NaP, NaCl, and Triton DN-65 were 20, 14, and 12 milliunits/mg of protein, respectively.

c. Three fractions of ChAT were also solubilized in the presence of proteolytic enzyme inhibitors. Rat brains (~5 gm) were homogenized in 75 ml of 0.32 M ice-cold sucrose containing the following proteolytic enzyme inhibitors: EDTA, 0.1 mM; EGTA, 0.1 mM; PMSF, 0.1 mM; pepstatin, 1 μg/ml;

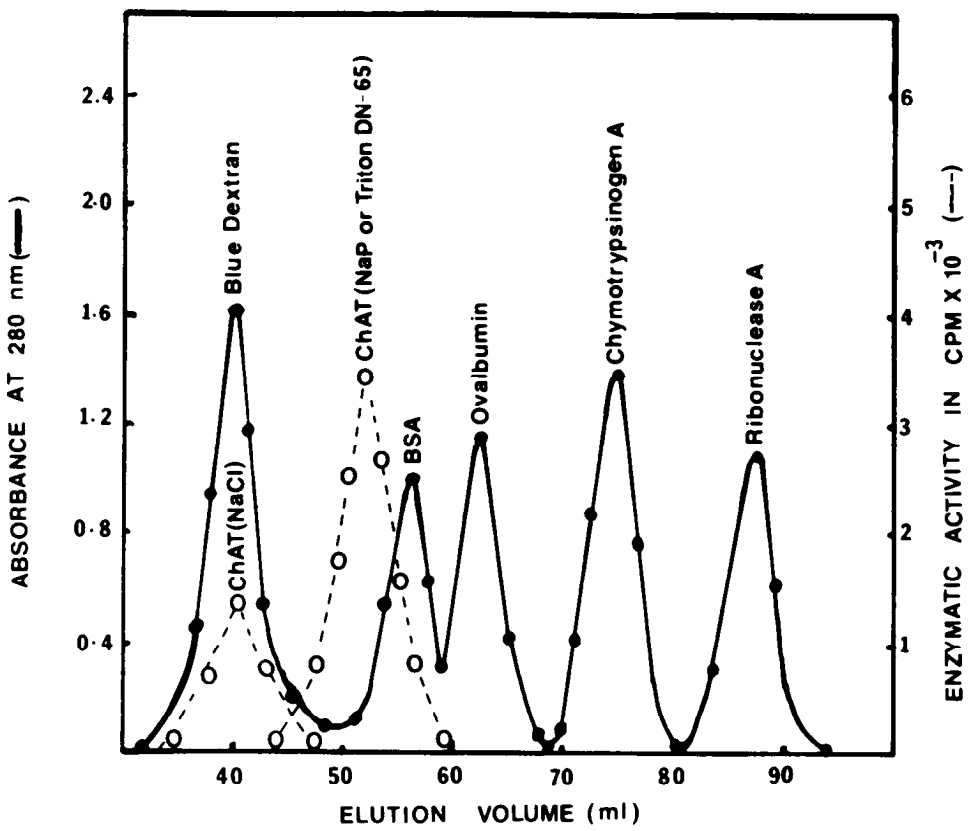

Figure 1. Gel filtrations of rat brain ChAT fractions and globular proteins with known molecular weights on Sephadex G-100 Superfine. A 1.6 × 70 cm column was equilibrated with citrate/phosphate buffer (10 mM disodium phosphate neutralized to pH 7.2 with 1 M citric acid and containing 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol). Standards used were albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). Proteins were eluted with the same buffer at a flow rate of 2 ml/hr.

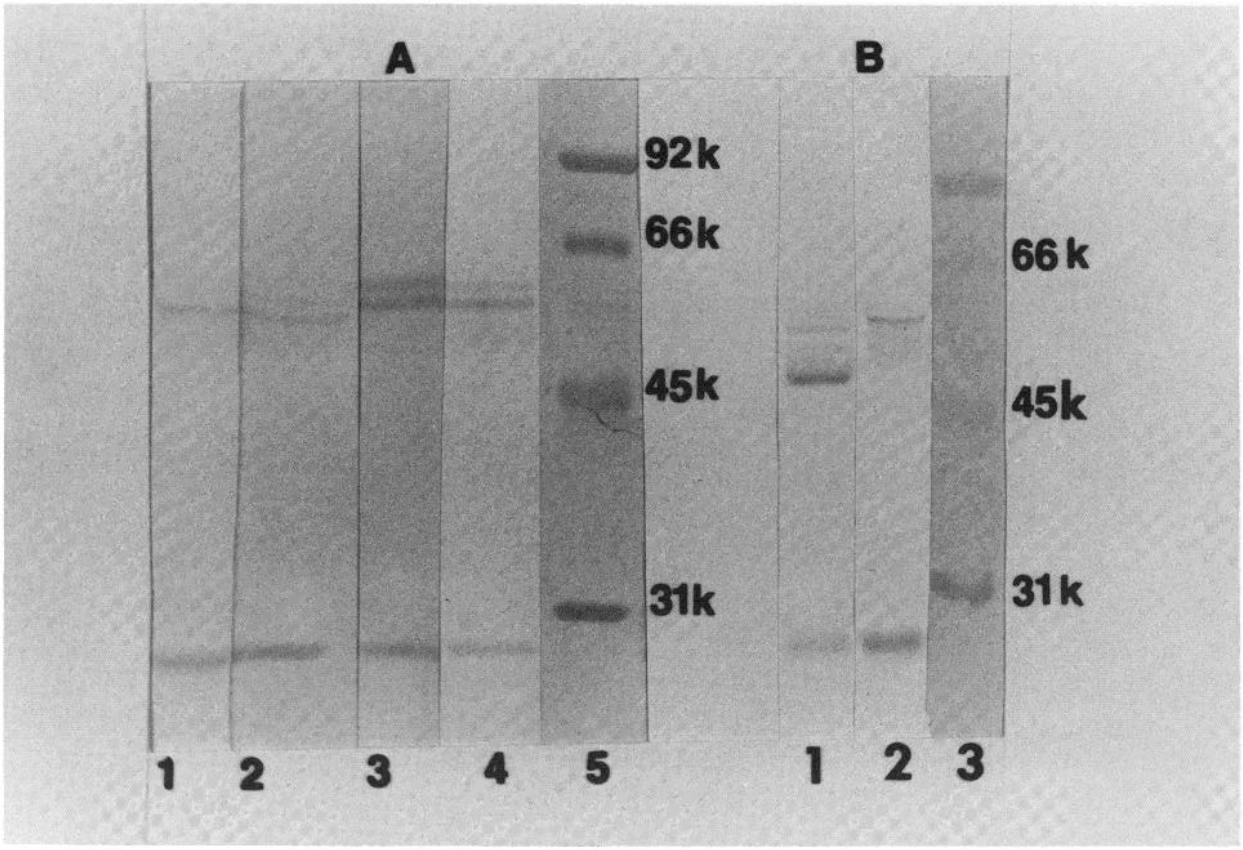

Figure 2. Localization of NaP-soluble ChAT prepared from bovine and rat brain on Western blots using anti-ChAT monoclonal antibodies Ab8 and Ab9. Five to 10 milliunits of Sephadex G-100-purified, NaP-solubilized ChAT from bovine brain were immunoprecipitated with Ab8 and Ab9 (*A*) and from rat brain immunoprecipitated with Ab8 (*B*), electrophoresed in a 1.5- or 3-mm 10% SDS-polyacrylamide gel for 5 hr, blotted onto nitrocellulose paper, and then immunostained with Ab8 or Ab9. *A*, *Lane 1:* Ab8 non-immunoprecipitated; *lane 2:* Ab9 non-immunoprecipitated; *lane 3:* bovine ChAT immunoprecipitated with Ab9; *lane 4:* bovine ChAT immunoprecipitated with Ab8; *lane 5:* low molecular weight marker. *B*, *Lane 1:* rat ChAT immunoprecipitated with Ab8; *lane 2:* Ab8 non-immunoprecipitated; *lane 3:* low molecular weight markers from Bio-Rad blotted onto nitrocellulose paper and stained with amido black.

soybean trypsin inhibitor, 100 μg/ml; leupeptin, 1 μg/ml; aprotinin, 9 Kallikrein units; antipain dihydrochoride, 1 μg/ml; benzamidine hydrochloride, 1 μg/ml; and benzethonium chloride, 1 μg/ml. The proteolytic enzyme inhibitors did not affect the ChAT activity. Three fractions of ChAT were then prepared as previously described (Benishin and Carroll, 1983) using sequential NaP, NaCl, and Triton DN-65 washes of a crude vesicular fraction. These washes were then dialyzed against the citrate/phosphate buffer overnight before use.

d. A soluble fraction of rat brain ChAT, which had initially been prepared by the procedure of Ryan and McClure (1979) and then purified on a monoclonal antibody affinity column, was kindly provided by Dr. Louis B. Hersh, University of Texas Southwestern Medical School (Dallas, TX).

Immunoprecipitation of ChAT

ChAT was immunoprecipitated with an anti-ChAT monoclonal antibody (Ab8 or Ab9), as described previously (Hersh et al., 1984), with minor modifications. Five to 10 milliunits of ChAT activity were first incubated at 4°C for 60 min in the citrate/phosphate buffer (10 mM disodium phosphate, neutralized to pH 7, with 1 M citric acid and containing 0.1 mM EDTA and 7% glycerol) with either 12.5 μg of anti-ChAT antibody (Ab8) or naive rat IgG (as control) in a total volume of 250 μl. Five microliters of rabbit anti-rat antibody (4 mg/ml of antibody protein) were then added to the sample and incubated at 4°C for 24 to 48 hr. The incubation mixture was centrifuged for 5 min and washed with sodium borate buffer, pH 8.3 (0.1 M boric acid, 64 mM NaCl, 5% NP-40). The pellet and an aliquot of supernatant were assayed for ChAT activity. For the SDS-PAGE experiments, the pellet was dissolved in SDS sample buffer (50 mM Tris-Cl, pH 6.8; 10% glycerol (v/v); 2% SDS (w/v); 5% 2-mercaptoethanol (v/v); and 0.001% bromophenol blue (w/v).

Gel electrophoresis

Gel electrophoresis on 1.5-mm-thick slabs of 7.5% acrylamide at pH 4.3 was done according to the method of Reisfeld et al. (1962). Electrophoresis was conducted by applying a current of 60 mA/slab gel for 2 hr. Samples in 0.1 mM NaP buffer, pH 7.4, were loaded in 20% glycerol. SDS-PAGE was performed using 10% polyacrylamide slab gels (16 cm × 18 cm × 3.0 mm), according to the method of Laemmli (1970). ChAT samples were incubated at 90°C for 5 min before gel electrophoresis. Proteins were visualized in the gels using Brilliant Blue R (B0630) or were silver stained. Molecular weights were estimated using the low and high molecular weight protein standards obtained from Bio-Rad.

Extraction of ChAT from native gels after electrophoresis

To avoid possible acid hydrolysis, gels were stained for only 30 min in Brilliant Blue R and destained for less than 1 hr in 50% methanol and 7% acetic acid. The ChAT band on the gel was localized by comparing the immunostained Western blot of ChAT with either Coomassie-stained or unstained gel. The ChAT band was then cut from the gel and the ChAT protein was eluted by incubating the gel slices in 200 μl of SDS-sample buffer for 48 hr. Prior to SDS-PAGE the gel slices in SDS-sample buffers were incubated at 90°C for 5 min and the supernatants were used for SDS-PAGE.

Western blotting and immunochemical visualization of ChAT

Western blotting was performed according to the method of Burnette (1981), as modified and described by Hersh et al. (1984), with minor modifications. After SDS-PAGE, the slab gels (minus the stacking gels) were electroblotted onto nitrocellulose paper (0.45 μm pore size, Schleicher & Schuell) in Tris-glycine buffer (160 mM Tris, 10 mM glycine, 20% methanol, pH 9.4) using a Bio-Rad Trans-Blot Apparatus. Native gels were electroblotted onto nitrocellulose paper in 0.7% acetic acid. Following electroblotting, the nitrocellulose paper was incubated in a 25-ml solution containing 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5% BSA; and 0.2% NP-40 (Ima_1) solution containing 500 μg of Ab8 or Ab9, or 60 μg of 95% 4D7 or 1E6. After this incubation, the nitrocellulose paper was washed four times with a 25-ml

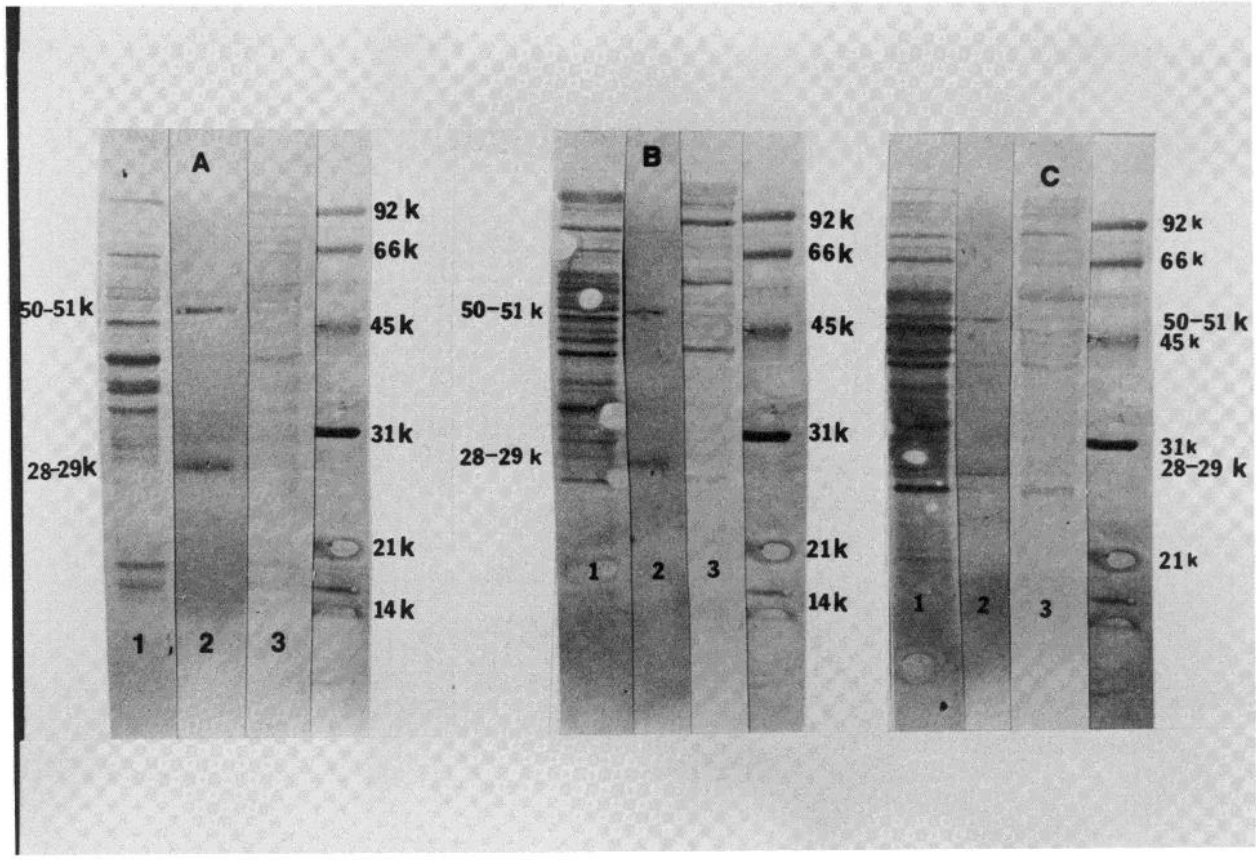

Figure 3. Comparison between the binding of anti-ChAT monoclonal antibody Ab8 and naive rat IgG to Western blots of rat brain ChAT fractions. Partially purified soluble and membrane-bound ChAT fractions from rat brain (2 milliunits) were electrophoresed in a 1.5-mm 12.5% SDS-polyacrylamide gel and blotted onto nitrocellulose paper. *Lanes 1*, Amido black-stained protein bands; *lanes 2*, Ab8-reactive protein bands; *lanes 3*, naive rat IgG-reactive protein bands. On the *right* are standard proteins from Bio-Rad which were blotted onto nitrocellulose paper and then stained with amido black. NaP (*A*)-, NaCl (*B*)-, and Triton DN-65 (*C*)-solubilized ChAT are shown.

solution containing 10 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 0.2% NP-40, 0.25% sodium deoxycholate, and 0.1% SDS (Ima_2) and then washed one time with 25 ml of a solution containing 10 mM Tris and 150 mM NaCl (Western Blot Wash). The monoclonal antibody-ChAT complex was then visualized by incubating the nitrocellulose paper in 25 ml of Ima_1 containing 150 μl of HRP-conjugated rabbit anti-rat antibody for 30 min at room temperature. Following this incubation, nitrocellulose paper was washed four times with 25 ml of Ima_2, followed by one Western Blot Wash. The complex was visualized by incubating the nitrocellulose paper in 50 ml of HRP developing solution containing 15 mg of diaminobenzidine, 0.25 ml of 1 M Tris-HCl, pH 7.4, 50 ml of H_2O, and 9 μl of H_2O_2 for 2 to 5 min as described by Levey et al. (1983). The amount of the sample used for SDS-PAGE was dependent on the specific activity of the enzyme fraction. In general, more than 1 μg of ChAT was used for SDS-PAGE.

Protein determinations

Protein was estimated by the method of Lowry et al. (1951) using BSA as standard.

Molecular weight determinations

The molecular weight was determined on Sephadex G-200 and G-100 Superfine using high and low molecular weight standards from Pharmacia Fine Chemicals. The fraction of the volume of the gel that is available for each protein (K_{av}) was calculated by using the Laurent and Killander (1964) equation, $K_{av} = (Ve - Vo)/V_t - Vo)$, where *Ve* is the volume at which the protein elutes, V_t is the total gel bed volume, and *Vo* is the void volume of column, which was determined by running blue dextran 2000 through the column. The molecular weight of ChAT was also determined by using SDS-PAGE followed by "Western blotting" and immunochemical visualization of ChAT with different anti-ChAT monoclonal antibodies as described earlier. Calibration curves were determined using the high and low SDS molecular weight markers obtained from Bio-Rad.

Results

Gel filtration on Sephadex G-100 Superfine and G-200 was used to determine the native molecular weights of soluble and membrane-bound fractions of ChAT. The chromatographic behavior of partially purified NaP-, NaCl-, and Triton DN-65-solubilized fractions of ChAT on Sephadex G-100 Superfine is illustrated in Figure 1. The column was initially calibrated with standard proteins, and partially purified fractions of ChAT were then loaded onto the column separately. The elution patterns obtained for the NaP- and Triton DN-65-soluble fractions of ChAT indicated that their molecular weights were in the range of 73,000 to 78,000. In contrast, the NaCl-solubilized fraction of ChAT was eluted at the void volume of this column, a result which suggested that its molecular weight exceeded 100,000. Results obtained from gel filtration of the NaP- and Triton DN-65-solubilized fractions of ChAT on Sephadex G-200 again indicated that their molecular weights were in the range of 73,000 to 78,000. Results obtained for the NaCl-soluble fraction of ChAT suggested that its molecular weight was in the range of 230,000 to 240,000. To determine whether the high salt fraction of ChAT might be an aggregate of the NaP-soluble fraction of ChAT, two experiments were performed: (*1*) the NaCl-solubilized ChAT ws dialyzed overnight against the column buffer, and (*2*) 500 mM NaCl was added to the NaP-solubilized ChAT prior to determination of its molecular weight by gel filtration. Neither of these treatments altered the elution patterns of the NaCl- or NaP-soluble ChAT fractions.

Hersh et al. (1984) reported that ChAT prepared from bovine brain consisted of two molecular weight variants of 63 and 68 kilodaltons (Kd). To obtain these results, these investigators immunoprecipitated partially purified ChAT samples with a monoclonal antibody directed against ChAT (Ab8) prior to SDS-PAGE and subsequent Western

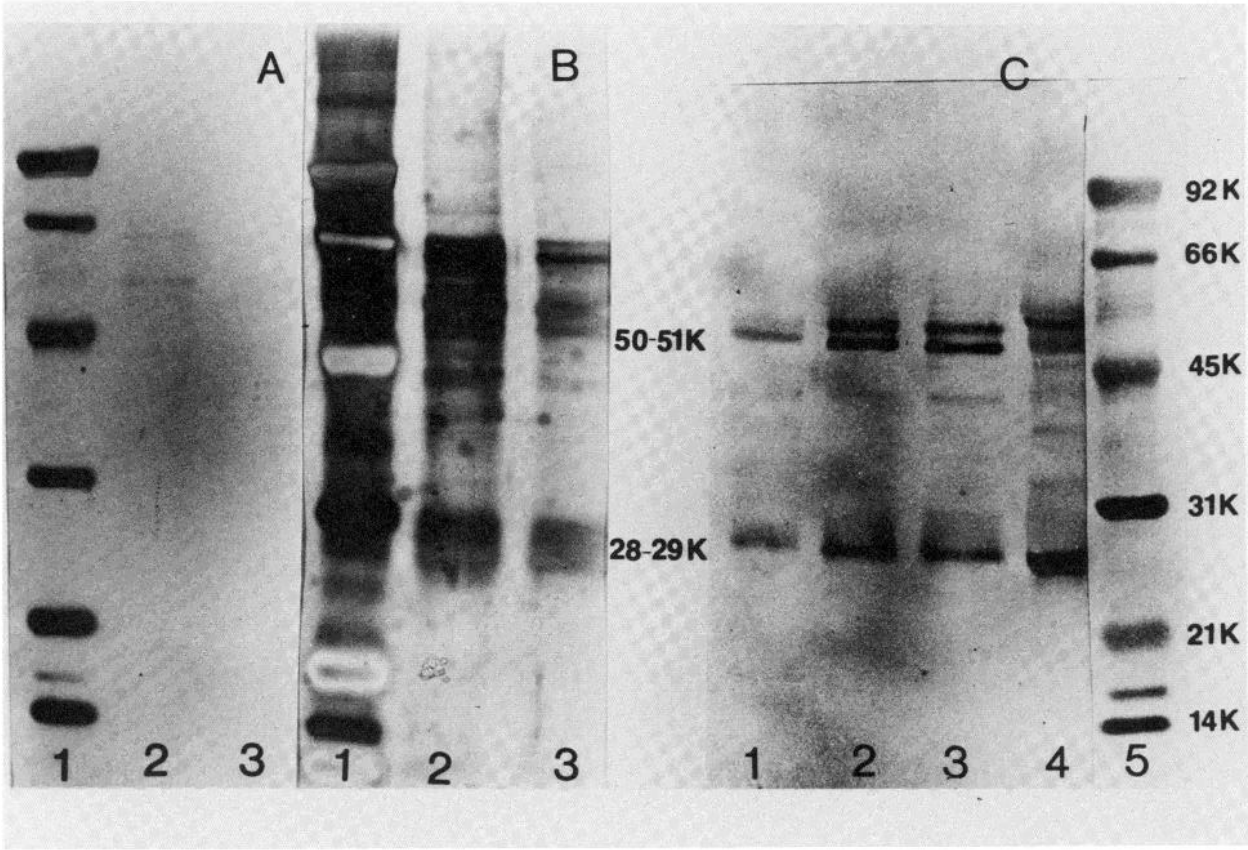

Figure 4. SDS-PAGE and localization of highly purified fraction of NaP-soluble rat brain ChAT on Western blots. *A* and *B*, ChAT from rat brain (2 to 4 milliunits) was electrophoresed in a 3-mm 10% SDS-polyacrylamide gel and stained with Coomassie blue (*A*) and silver stain (*B*). *Lane 1:* Molecular weight markers from Bio-Rad; *lanes 2 and 3:* NaP-solubilized ChAT immunopurified in Dr. Louis Hersh's laboratory. *C*, Sephadex G-100-purified and immunopurified NaP-solubilized ChAT were blotted onto nitrocellulose paper following SDS-PAGE and then immunostained with Ab8. *Lane 1:* Sephadex-purified, NaP-solubilized ChAT; *lane 2:* immunopurified NaP-solubilized (4 milliunits) ChAT; *lane 3:* immunopurified NaP-solubilized ChAT (2 milliunits); *lane 4:* non-immunoprecipitated Ab8; *lane 5:* molecular weight markers from Bio-Rad blotted onto nitrocellulose paper and then stained with amido black.

blotting." Following transfer of the proteins to nitrocellulose paper, the ChAT bands were visualized by immunostaining with monoclonal antibody Ab8 and could be distinguished from the heavy and light chains of the Ab8 used for immunoprecipitation.

To corroborate the results obtained by these investigators and thereby ensure that the "Western blotting" technique was working properly, we prepared the NaP-soluble ChAT fraction from bovine caudate by the procedure of Benishin and Carroll (1983) and partially purified it using ammonium sulfate precipitation and gel filtration to a specific activity of 30 milliunits/mg of protein. Following partial purification, samples of NaP-soluble ChAT were then immunoprecipitated with either Ab8 or Ab9, electrophoresed using SDS-PAGE, blotted onto nitrocellulose, and immunostained with Ab8 or Ab9. The results illustrated in Figure 2*A* indicate that Ab8 reacted with two protein bands at 63 and 65 Kd, a result quite similar to that reported by Hersh et al. (1984). (The molecular weights of the two Ab8-reactive bands were determined by comparing their mobility with the mobility of molecular weight markers which had also been transferred to nitrocellulose, but had been stained with amido black.) These two reactive proteins exceeded the molecular weight of the heavy chain of Ab8 which blotted onto nitrocellulose paper following SDS-PAGE. A reactive band also appeared at 25 to 26 Kd, but this band could not clearly be distinguished from the light chain of Ab8.

It was then of interest to determine whether the molecular weight of Sephadex G-100-purified fractions of NaP-soluble ChAT prepared from rat brain could be determined using the above described technique. The results illustrated in Figure 2*B* indicate that Ab8 reacted with protein bands at 50 to 51 Kd and 28 to 29 Kd, although the latter band could not clearly be distinguished from the light chain of Ab8. In contrast to the results obtained for bovine brain, Ab8 did not react with protein bands of rat brain above the heavy chain of Ab8.

To compare the molecular weights of the three ChAT fractions from rat brain, partially purified fractions of NaP-, NaCl-, and Triton-soluble ChAT were electrophoresed using SDS-PAGE without first being immunoprecipitated by Ab8, blotted onto nitrocellulose paper, and immunostained with either Ab8 or IgG as control. The results indicated that anti-ChAT monoclonal antibody (Ab8) bound to the same two proteins, that is, 28 to 29 and 50 to 51 Kd, on the nitrocellulose paper for all three ChAT fractions (Fig. 3). In contrast, IgG did not stain proteins very intensely in these ranges. It is important to note that the Ab8-reactive bands at 28 to 29 and 50 to 51 Kd on the nitrocellulose paper represented very minor protein bands in the SDS-PAGE of the ChAT fractions blotted and then stained with amido black. Also, staining of the SDS-gel after blotting indicated that all of the proteins, including those in the molecular weight range of 66,000 to 68,000, had been transferred to the nitrocellulose paper (data not shown). It is also important to note that the protein profiles for the NaP-, NaCl-, and Triton-solubilized fractions do not appear similar, yet the same two protein bands reacted with Ab8 in all three fractions.

In the next experiment, we used a soluble fraction of rat brain ChAT, which had been purified in the laboratory of Dr. Louis B. Hersh with a monoclonal antibody affinity column (Ab8), to determine whether Ab8 would still stain protein bands at 28 to 29 and 50 to 5 Kd in a more highly purified sample. The results indicated that Ab8 still reacted with protein bands at 28 to 29 and 50 to 51 Kd in this more highly purified sample (Fig. 4*C*). Also, it appeared that a protein

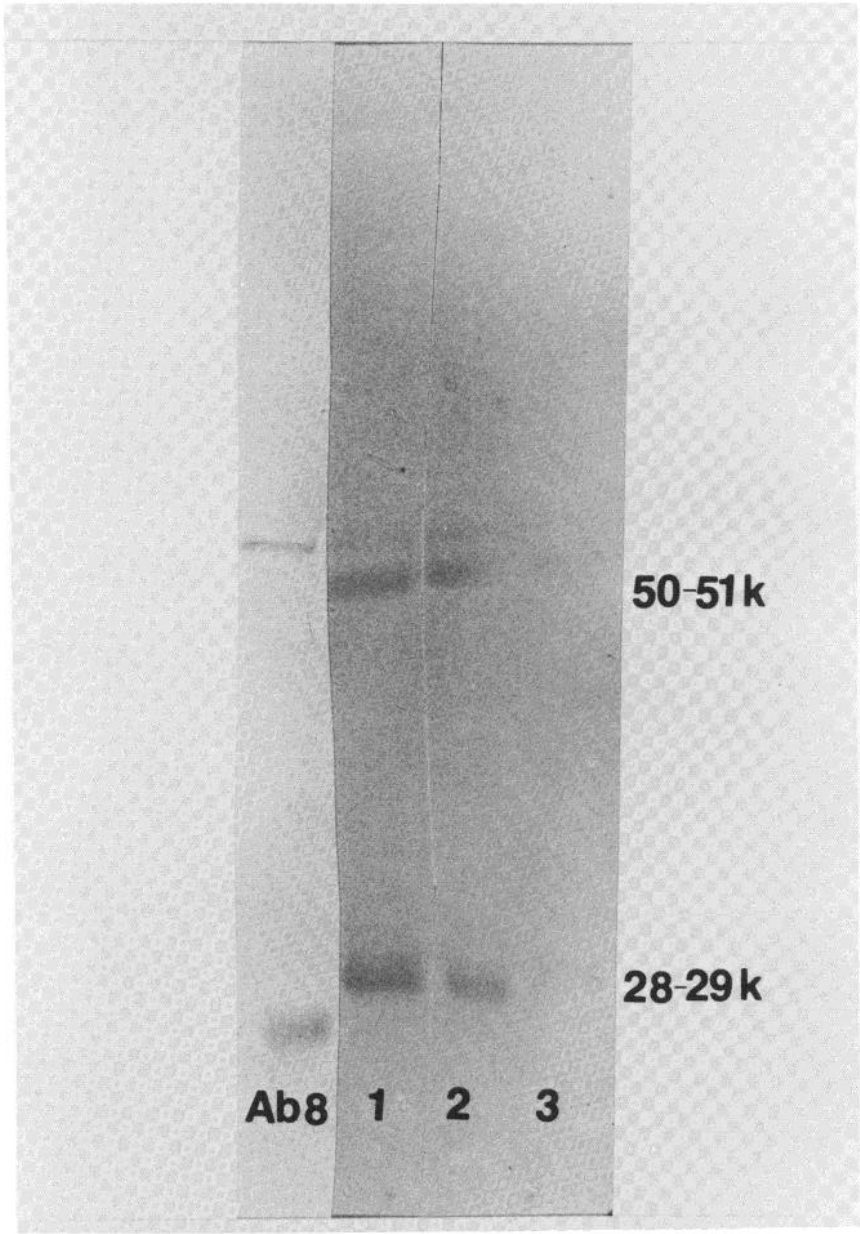

Figure 5. Effect of ChAT concentration on the intensity of the Ab8-reactive protein bands. Different amounts of NaP-solubilized ChAT activity were electrophoresed in a 3-mm 10% SDS-polyacrylamide gel, blotted onto nitrocellulose paper, and then immunostained with Ab8. *Lane 1:* 1.2 milliunits; *lane 2:* 0.5 milliunits; *lane 3:* 0.12 milliunits. On the *left* is Ab8, which was electrophoresed in an SDS-polyacrylamide gel, blotted onto nitrocellulose paper, and then stained with HRP.

band at 54 to 55 Kd also reacted with Ab8. When the protein bands on SDS-gels of this ChAT sample were initially stained with Brilliant Blue R, only two very faint bands at 50 and 63 Kd were evident (Fig. 4*A*). When the gel was then processed with a more sensitive silver stain, more protein bands were evident, including ones at 28 to 29, 50 to 51, and 54 Kd (Fig. 4*B*). It is important to note that several bands in the 62- to 65-Kd range were present in the gel which did not react with Ab8 following their transfer to nitrocellulose paper.

When increasing amounts of NaP-soluble ChAT were subjected to SDS-PAGE, blotted, and reacted with Ab8, the intensities of the protein bands which stained at M_r = 50,000 to 51,000 and 28,000 to 29,000 likewise increased (Fig. 5).

Hersh et al. (1984) reported that the 63- and 68-Kd molecular weight variants of bovine ChAT brain could be the products of proteolytic cleavage; when extracts of bovine brain were prepared in the presence of a variety of proteolytic enzyme inhibitors, a new, higher molecular weight form of the enzyme (73 Kd) was generated. To determine whether the protein bands staining at 28 to 29 and 50 to 51 Kd with Ab8 were the products of proteolysis, rat brain was initially homogenized in the presence of 10 proteolytic enzyme inhibitors prior to the preparation of NaP-soluble ChAT. Ab8 still reacted with protein bands at 28 to 29 and 50 to 51 Kd in this sample (Fig. 6, *lane 1*); also, no new, higher molecular weight reactive band could be visualized. To further test whether the 28- to 29- and 50- to 51-Kd Ab8-reactive bands were the products of proteolysis, a partially purified NaP-soluble fraction of ChAT was electrophoresed on a native gel. Ab8 was run as a control. Following electrophoresis, the samples were transferred to nitrocellulose paper and stained with Ab8. Only one Ab8-reactive band could be visualized for the NaP-soluble ChAT sample (Fig. 7, *lane 2*). Protein which had not entered the gel during electrophoresis did not react with Ab8, suggesting that it did not contain ChAT. In a subsequent experiment, an NaP-soluble fraction of ChAT was run on a native gel, removed from the gel following electrophoresis (the molecular weight marker BSA was used to identify the approximate position of ChAT on a Brilliant Blue R-stained gel), incubated overnight in SDS-sample buffer to denature and elute the ChAT sample, run on SDS-PAGE, and then transferred onto nitrocellulose for immunostaining. The results indicated that Ab8 then reacted with two protein bands

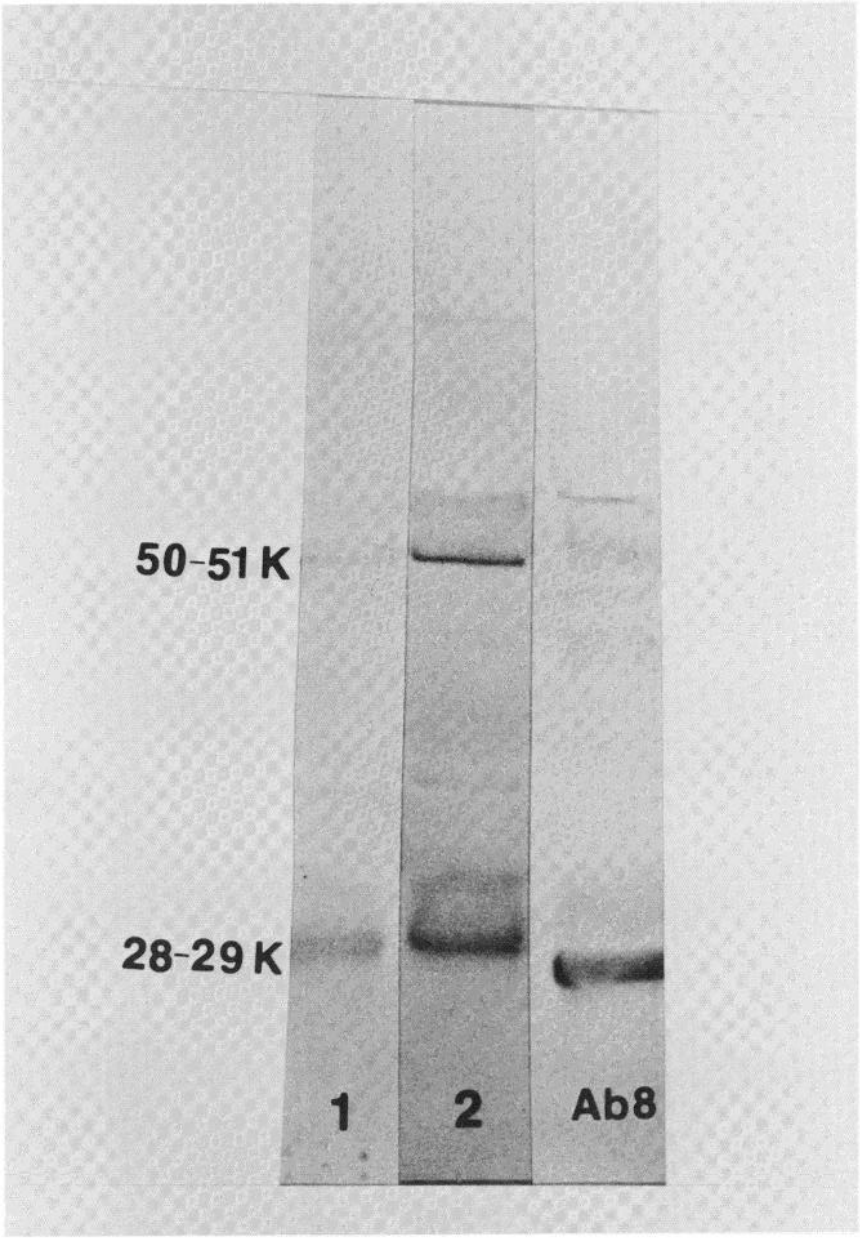

Figure 6. Effect of proteolytic enzyme inhibitors on the Ab8-reactive protein bands in an NaP-soluble ChAT fraction. Two milliunits of NaP-solubilized ChAT, prepared either in the presence of proteolytic enzyme inhibitors (*lane 1*) or according to the procedure of Ryan and McClure (1979) (*lane 2*) or Ab8 (*right*) were electrophoresed in a 3-mm 10% SDS-polyacrylamide gel, blotted onto nitrocellulose paper, and immunostained with Ab8. Details of extraction procedures are given under "Materials and Methods."

rather than one and that these two protein bands were at 28 to 29 and 50 to 51 Kd (Fig. 7, *lane 7*). Additionally, Ab8 then reacted with the heavy and light chains of Ab8 which had been subjected to the identical treatment as the ChAT sample (Fig. 7, *lane 4*).

To determine whether initial homogenization of brain tissue in sucrose might be responsible for the formation of the 28- to 29- and 50- to 51-Kd Ab8-reactive proteins, brain tissue was initially prepared according to the procedure of Ryan and McClure (1979) to avoid the use of sucrose homogenization. Even after this procedure, the Ab8-reactive proteins appeared at 28 to 29 and 50 to 51 Kd. However, a less intensely staining protein band was also evident at 78 Kd (Fig. 6, *lane 2*). However, this protein band was absent in samples which had been immunoprecipitated with Ab8 (data not shown) or which had initially been run on a native gel prior to SDS-PAGE (Fig. 7, *lane 7*).

To provide additional confirmation that the 28- to 29- and 50- to 51-Kd protein bands really represented ChAT, two different anti-ChAT monoclonal antibodies, 1E6 and 4D7, having heavy and light chains somewhat different from those of Ab8, were employed for immunostaining of non-immunoprecipitated ChAT samples. The results indicated that both of these antibodies, like Ab8, reacted with proteins at 28 to 29 and 50 to 51 Kd; their heavy and light chains clearly differed from these positions (Fig. 8). Similar results were obtained when these two antibodies were used to determine the molecular weights of the NaCl- and Triton DN-65-solubilized fractions of rat brain (data not shown).

Discussion

The results obtained in the present study using gel filtration indicated that the molecular weight of the high salt-soluble fraction of rat brain ChAT greatly exceeded the molecular weights of the NaP- and Triton DN-65-soluble fractions of ChAT. Since neither the addition of high salt to the NaP fraction nor dialysis of the high salt-solubilized fraction against NaP buffer altered the molecular weights obtained for these fractions, it would appear that the NaCl-soluble ChAT fraction is not an aggregate of the NaP-soluble ChAT fraction. The molecular weights obtained for the NaP- and Triton DN-65-soluble ChAT fractions were in the range of 73,000 to 78,000. Since gel filtration can accurately determine the molecular weight of a

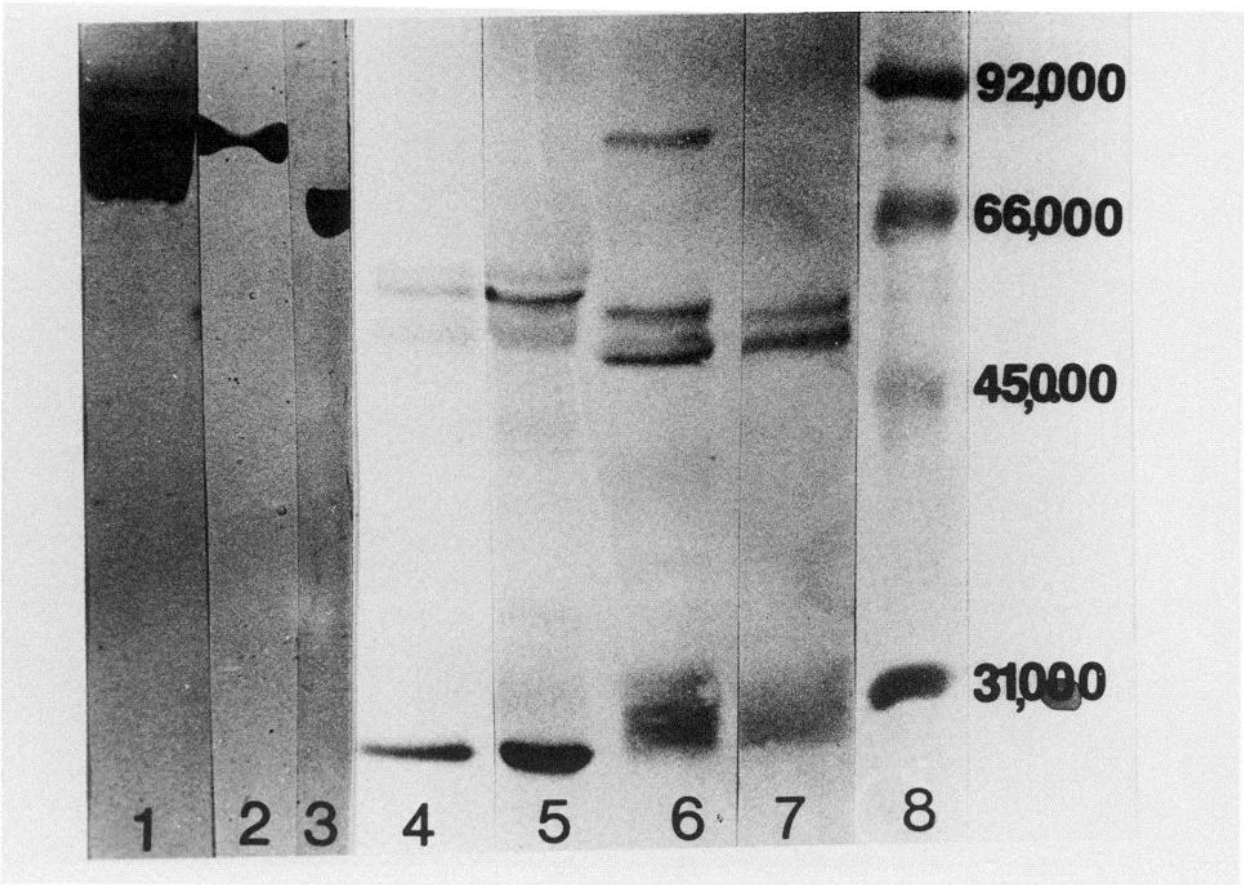

Figure 7. Binding of anti-ChAT monoclonal antibody (Ab8) to Western blot of NaP-soluble rat brain ChAT after electrophoresis in a native gel and subsequent SDS-PAGE. Two to 3 milliunits of NaP-solubilized ChAT (prepared from rat brain, using the procedure of Ryan and McClure (1979)), Ab8, and BSA were electrophoresed in a 1.5-mm 7.5% native polyacrylamide gel at pH 4.3 for 2 hr (Reisfeld et al., 1962), blotted onto nitrocellulose paper, and then immunostained with Ab8 (*lane 1*), NaP-solubilized ChAT (*lane 2*), and BSA (blotted onto nitrocellulose paper and stained with amido black; *lane 3*). In the duplicate native gel, Ab8 and ChAT were eluted from the gels as described under "Materials and Methods" and electrophoresed in a 3-mm 10% SDS-polyacrylamide gel, blotted onto nitrocellulose paper, and immunostained with Ab8 eluted from native gel (*lane 4*), Ab8 (*lane 5*), NaP-solubilized ChAT (*lane 6*), ChAT eluted from native gel (*lane 7*), and low molecular weight markers from Bio-Rad blotted onto nitrocellulose paper and stained with amido black (*lane 8*).

substance to only ±3,000, these two fractions of ChAT could not really be distinguished from each other using this technique.

Our failure to detect a molecular weight difference between soluble and membrane-bound fractions of a neurotransmitter-synthesizing enzyme is not unique; both soluble and membrane-bound forms of dopamine β-hydroxylase are reported to consist of subunits with M_r = 75,000 (Park et al., 1976; Fong et al., 1980).

When the molecular weights of the three fractions of rat brain ChAT were determined using SDS-PAGE followed by Western blotting and immunostaining with anti-ChAT monoclonal antibodies, all three ChAT fractions appeared to be composed of the same two monoclonal antibody-reactive protein bands, that is, 28 to 29 and 50 to 51 Kd. It is important to note that the proteins in the range of M_r = 63,000 to 73,000, which were present in each of the three ChAT fractions, as well as the purified ChAT fraction prepared by Dr. Louis B. Hersh, were transferred to the nitrocellulose paper from the gels but were not immunostained with the monoclonal antibodies used. Therefore, the failure of the anti-ChAT monoclonal antibodies to stain proteins in the range of 63 to 69 Kd, that is, the range normally reported for the molecular weight of rat brain ChAT on denaturing gels (Dietz and Salvaterra, 1980; Ryan and McClure, 1980), cannot be due to poor transfer of proteins from the gel to the nitrocellulose paper. Proteins with high molecular weights in the range of 200,000 were also transferred to the nitrocellulose paper. Therefore, if the high salt fraction of ChAT had not been denatured by SDS treatment and had remained intact, its presence on nitrocellulose paper would have been detected using Ab8.

Although it cannot be conclusively proven that the 28- to 29- and 50- to 51-Kd protein bands represent either fragments or subunits of ChAT, since monoclonal antibodies can react with other proteins with shared antigenic sites, the following lines of experimental evidence suggest that the 28- to 29- and 50- to 51-Kd protein bands do represent ChAT. (*1*) Ab8 could precipitate ChAT out of solution and, when the precipitated ChAT samples were electrophoresed in SDS-PAGE, blotted onto nitrocellulose paper, and then immunostained with Ab8, the same two protein bands at 28 to 29 and 50 to 51 Kd were stained in these samples as in those ChAT samples which had not first been immunoprecipitated with Ab8. (*2*) When Western blots of three fractions of ChAT were immunostained with Ab8 or IgG as a control, Ab8 stained proteins at 28 to 29 and 50 to 51 Kd much more intensely than did IgG. SDS molecular weight markers were not stained with Ab8, suggesting that proteins stained at 28 to 29 and 50 to 51 Kd could not be attributed to nonspecific binding. (*3*) The intensity of the Ab8-stained proteins in the ChAT sample was proportional to the amount of ChAT used for SDS-PAGE. Thus, this result would again suggest that the stains could not be attributed to the heavy and light chains of Ab8. Also, the intensity of the 50- to 51-Kd band was proportional to the amount of ChAT precipitated out of solution by Ab8 prior to SDS-PAGE. (*4*) Immunostaining of Western blots with two other anti-ChAT monoclonal antibodies (4D7 and 1E6), prepared from a different source (mouse immunized with rat ChAT and with heavy and light chains differing from those of Ab8 (rat immunized with bovine ChAT), also

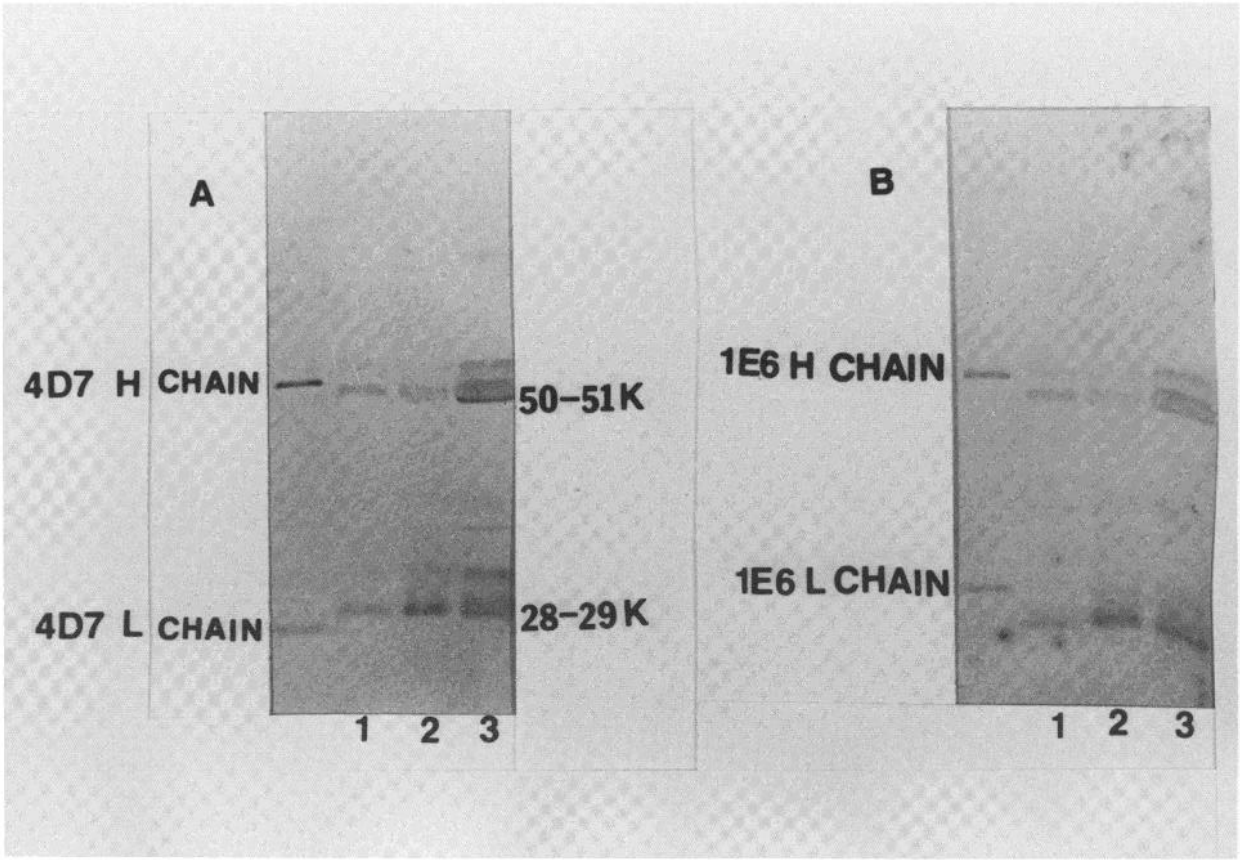

Figure 8. Localization of rat brain ChAT on Western blots using anti-ChAT monoclonal antibodies 4D7 and 1E6: Comparison between enzyme fractions prepared in the presence or absence of proteolytic enzyme inhibitors. Two milliunits of NaP-solubilized ChAT prepared from rat brain, in the presence of proteolytic enzyme inhibitors (*lane 2*), using the procedure of Benishin and Carroll (1983) (*lane 1*), or according to the procedure of Ryan and McClure (1979) (*lane 3*), 1E6, and 4D7 were electrophoresed in a 3-mm 10% SDS-polyacrylamide gel, blotted onto nitrocellulose paper, and immunostained with either monoclonal antibody 4D7 (*A*) or 1E6 (*B*). Details of extraction procedures are given under "Materials and Methods."

stained protein bands at 28 to 29 and 50 to 51 Kd for all three fractions of ChAT. The heavy and light chains of 4D7 and 1E6, which also stained, could be easily distinguished from the protein bands at 28 to 29 and 50 to 51 Kd. These results indicate that both rat brain proteins possibly have similar antigenic determinants which are recognized by the monoclonal antibodies. This situation is also true for the nicotinic ACh receptor of the electric organ of *Torpedo californica* in which a few, but not the majority of monoclonal antibodies, cross-react with some of the same subunits (Tzartos and Lindstrom, 1980), a result which suggests that similar antigenic determinants exist on some of the same subunits. However, some monoclonal antibodies will also react with fragments of these nicotinic receptor subunits, whereas others will not (Conti-Tronconi et al., 1981). Therefore, a subunit or fragment of ChAT could exist which does not react with any of the monoclonal antibodies used in our study. (*5*) Ab8 reacted with the same two protein bands in a highly purified soluble fraction of rat brain ChAT prepared in Dr. Hersh's laboratory. Even in this sample, the major protein band obtained after SDS-PAGE was of M_r = 63,000, yet it did not stain on nitrocellulose paper with Ab8, a result which again suggests that the reactive bands at 28 to 29 and 50 to 51 Kd probably represent ChAT rather than impurities. It is possible that the 63-Kd protein band did not immunostain because it is an endogenous ChAT inhibitor which co-purified with ChAT but did not immunostain following elution of ChAT from the antibody column. This particular molecular weight is in the molecular weight range for endogenous ChAT inhibitors in bovine brain reported by Cozzari and Hartman (1983).

The most difficult issue to resolve in the present study is whether the antibody-reactive protein bands obtained at 28 to 29 and 50 to 51 Kd for rat brain ChAT represent subunits of ChAT or proteolytic breakdown products of ChAT. The following lines of evidence suggest that they represent subunits. (*1*) The presence of 10 proteolytic enzyme inhibitors during the initial homogenization of rat brain did not result in the appearance of a new, higher molecular weight monoclonal antibody-reactive band as was found in the case of bovine brain (Hersh et al., 1984). If proteolytic breakdown were responsible for the generation of the antibody-reactive bands at 28 to 29 and 50 to 51 Kd, it would have to be essentially complete during the extraction of all three ChAT fractions; immunostained bands at $M_r > 54{,}000$ were almost never obtained for any of the three fractions. (*2*) In the case of the NaP- and Triton-soluble fractions of ChAT, the two major ChAT-reactive bands at 28 to 29 and 50 to 51 Kd, when added together, are approximately equal to the molecular weights determined for these ChAT fractions by gel filtration. (*3*) When the NaP-soluble fraction of ChAT was electrophoresed on a nondenaturing gel and then blotted and stained with Ab8, it consisted of only one rather than two protein bands. When a similarly prepared sample was subsequently denatured using SDS, two protein bands were then stained by Ab8; these two bands appeared at 28 to 29 and 50 to 51 Kd.

Although most reports suggest that ChAT does not consist of subunits, there are some which suggest that it does. For example, Chao (1980) reported that bovine ChAT is composed of six identical subunits, whereas Husain and Mautner (1973) reported that ChAT prepared from squid head ganglia is composed of two nonidentical subunits of 51 and 69 Kd. Polsky and Shuster (1976) reported that ChAT from squid head ganglia is composed of two nonidentical subunits with M_r = 27,000 and 56,000. One report suggests that

ChAT from catfish brain is composed of four subunits (Su et al., 1980).

In contrast, it is also possible that a protease not inhibited by any of the 10 proteolytic enzyme inhibitors could have generated the 50- to 51-Kd band from the native form of the enzyme and the 28- to 29-Kd band from the 50- to 51-Kd band. This possibility could account for the cross-reactivity of all three monoclonal antibodies with the 28- to 29- and 50- to 51-Kd protein bands. However, it cannot account for the appearance of only one Ab8-reactive band when ChAT was electrophoresed on a native gel. When the procedure of Ryan and McClure (1979), which does not involve a sucrose homogenization, was used to prepare the ChAT fractions, a minor protein band appeared at 78 Kd. This band could represent ChAT which had not undergone proteolytic breakdown during enzyme extraction. This possibility, however, can probably be discounted because the sample of rat brain ChAT prepared and purified by Dr. Louis B. Hersh was initially extracted using the Ryan and McClure (1979) procedure, and a 78-Kd protein did not immunostain in this sample.

In a couple of rat brain samples, notably the immunopurified sample prepared by Dr. Hersh, Ab8 reacted with a protein band at 54 Kd in addition to ones at 28 to 29 and 50 to 51 Kd. Since the heavy chain of Ab8 is also approximately 54 Kd, it is conceivable that some of the Ab8, in addition to ChAT, was eluted from the immunoaffinity column and that it is the heavy chain of Ab8, rather than ChAT, which is being immunostained at 54 Kd.

In summary, it appears that the high salt fraction of ChAT in its native form has a much higher molecular weight than either of the other two ChAT fractions. Also, the molecular weight of the native form of the soluble fraction of ChAT appears to be quite similar to that of the native form of the detergent-soluble fraction of ChAT. Following denaturation, however, all three fractions of ChAT appeared to be composed of the same nonidentical subunits. Therefore, the NaCl-solubilized fraction of rat brain ChAT may exist as a hexamer in its native state, whereas the other two fractions may exist as dimers in their native state. Thus, it appears possible that some of the ChAT contained in rat brain cholinergic nerve terminals, like acetylcholinesterase, the muscarinic receptor, and the nicotinic receptor, is also composed of subunits.

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