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PURIFICATION AND IMMUNOCHEMICAL PROPERTIES OF CHOLINE ACETYLTRANSFERASE FROM HUMAN BRAIN

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Choline acetyltransferase (CAT) was purified to homogeneity from 363 g of human neostriatum by means of ammonium sulfate and protamine sulfate fractionation, followed by chromatography on DEAE-Sephadex, hydroxyapatite, phosphocellulose, and agarose–hexane–Co A columns. The final product migrated as a single component on 7.5% gels with or without SDS. It had a molecular weight of 66,000 daltons and a specific activity of 7.3 μmol acetylcholine formed per milligram protein per minute. Antibodies prepared in rabbits gave single precipitin lines against this protein on Ouchterlony immunodiffusion and immunoelectrophoresis plates. The CAT–anti-CAT IgG complex migrated as a single band on gel electrophoresis, establishing the monospecificity of the antibodies. Strong cross-reactivity to the IgG was obtained with CAT from rat, rabbit, and guinea pig, but only weak reactivity with chicken. Fab fragments were prepared from the rabbit IgG and were used to stain CAT-containing neurons in the spinal cord and nerve endings at the neuromuscular junction using the PAP technique.

INTRODUCTION

It is appropriate that this special issue in honor of J. H. Quastel should provide information on cholinergic cells in brain and some new details of the purification of choline acetyltransferase (CAT, EC 2.3.1.6), the synthesizing enzyme for acetylcholine (ACh). Quastel and associates in 1936 were the first to demonstrate the synthesis by brain tissue of "choline ester," which was indistinguishable from acetylcholine (23). Despite this

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early beginning, knowledge of cholinergic mechanisms in the mammalian central nervous system remains relatively obscure, partly because it has been difficult to define the anatomy of central cholinergic neurons. Immunohistochemical localization of CAT is the most promising technique for achieving this objective. Pure enzyme is required for this purpose as well as for the detailed study of its properties.

There have been a number of reports on purification of CAT from brain tissues of vertebrates and invertebrates in recent years. Some enzyme preparations with low specific activity have been reported to be homogeneous on gel electrophoresis (5, 24, 32), while other preparations with much higher specific activity have been reported to be heterogeneous (12, 18, 26). Although homogeneous CAT preparations with very high specific activity have been obtained from squid head ganglia (13), *Torpedo californica* (3), and *Drosophila melanogaster* (7), the usefulness of these preparations for the immunohistochemical localization of CAT in the mammalian CNS is uncertain because of low cross-reactivity of mammalian anti-CAT antibodies with submammalian CAT (28, 33).

Antibodies to CAT from different mammalian species have been produced by immunization of rabbits (8, 18, 25, 27, 31–33), and some immunohistochemical localizations of CAT in the CNS have been reported (8, 11, 14, 20). Questions have, however, been raised about the purity of enzyme preparations of lower specific activity, and therefore about the specificity of antibodies produced to them (26–28). We now report more detailed studies on CAT purification from human brain and on the monospecificity of antibodies produced in rabbits. Fab fragments from these antibodies have provided good immunohistochemical detail of CAT-containing neurons in mammalian brain.

EXPERIMENTAL PROCEDURE

Materials

Human brains were obtained from various hospitals in the Vancouver area within 48 hr after death. The caudate and putamen nuclei were dissected out on ice and stored at −20°C until used for purification.

[^{14}C]Acetyl-coenzyme A was obtained from New England Nuclear Corporation (Lachine, Quebec). Acetyl-CoA, choline chloride, eserine sulfate, and Triton X-100 were obtained from Sigma Chemical Co. Amberlite CG-50 was purchased from Rohm and Haas Co. Bovine serum albumin, catalase, cytochrome *c*, protamine sulfate, and Carbowax were obtained from Schwartz and Mann Research Laboratories. Hydroxyapatite (Bio-Gel HTP) and phosphocellulose (Cellex-P) were from Bio-Rad Laboratories, Richmond, California. DEAE-Sephadex (A-25) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Agarose–hexane–CoA type 5 was purchased from P. L. Biochemicals, Milwaukee, Wisconsin. Acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N*-tetramethylethylenediamine, and β-mercaptoethanol were obtained from Eastman Kodak Co., Rochester, New York. Coomassie brilliant blue G-250 was purchased from SERVA Feinbiochemica, Heidelberg. Mercury papain came from Worthington Biochemical Corp. Goat antirabbit IgG and rabbit PAP were from Cappel Laboratories. Complete and incomplete Freund's adjuvant were from Miles Laboratories. Unless otherwise stated, all common laboratory chemicals were reagent grade and used without further purification.

Assay Procedures

CAT was assayed radiochemically according to the method of Singh and McGeer (34) with minor modification. The reaction mixture contained 50 mM sodium phosphate buffer, pH 7.4, 12.5 mM choline chloride, 2 mM EDTA, 0.9% sodium chloride, 0.1 mM eserine sulfate, 50 μM [^{14}C]acetyl-CoA (21,000 cpm/min/assay of a specific activity of 4.5×10^6 dpm/μmol), and enzyme preparation in a final volume of 80 μl. After phosphocellulose chromatography, the assay was done without eserine sulfate in the reaction mixture, and 10 μg of bovine serum albumin was added to protect the enzyme activity during assay. The mixture was incubated at 37°C for 20 min. Termination of the reaction with acid, separation of the acetylcholine on Amberlite CG-50 columns, and counting of the radioactivity were as previously reported (34). One unit of enzyme activity is defined as 1 μmol ACh formed per minute.

Carnitine acetyltransferase (CtAT) was assayed using the Dowex 1 method of Schrier and Shuster (30) for CAT, except that carnitine was used instead of choline as one of the substrates. The incubation was carried out at 37°C for 30 min. [^{14}C]Acetylcarnitine formed was collected in the column effluent plus 3 × 0.7 ml washes of cold distilled water.

Protein. Protein concentration was determined by measuring the absorbence at 260 nm and 280 nm, using a Beckman model DU spectrophotometer, by Lowry's method, or by the Bio-Rad protein assay method (Bio-Rad Laboratories Technical Bulletin 1051).

Gel Electrophoresis. Disk gel electrophoresis was performed according to Davis' procedure (6). The 7.5% gels were prepared by mixing 10 ml of 50 mM Tris HCl, pH 8.5, or 50 mM sodium phosphate buffer, pH 6.8, 5 ml of acrylamide solution (1.5 g of acrylamide and 40 mg of methylenebisacrylamide), 4 ml of distilled water, 20 μl of TEMED, and 1 ml of ammonium persulfate (15 mg/ml). The electrophoretic buffers were 25 mM Tris HCl, pH 8.5, or 25 mM sodium phosphate buffer, pH 6.8, both containing 50 mM β-mercaptoethanol and 2 mM EDTA. Electrophoresis was done at 1 mA/gel for 30 min until the tracking dye passed the stacking gel and then at 2 mA/gel for 2 hr at 4°C. Some gels were stained, others were sliced into 1-mm slices and 3 slices/fraction were homogenized in 100 μl of 100 mM potassium phosphate buffer, pH 6.8, and incubated for 2 hr at 4°C before assay for CAT activity.

SDS gel electrophoresis using 7.5% SDS gels was carried out according to the method of Weber and Osborn (36). The electrophoretic buffer contained 0.1 M sodium phosphate buffer, pH 7.2, and 0.2% SDS in the buffer. The standard marker proteins used were BSA, catalase, and cytochrome *c*. The protein samples were run initially at 3 mA/gel for 30 min, and 6 mA/gel for 4 hr. The gels were stained with 0.25% Coomassie blue R-250 dissolved in a mixture of methanol–glacial acetic acid–water (1 : 0.2 : 1) for 2–5 hr at room temperature. Destaining was carried out by diffusion against 7.5% acetic acid containing 5% methanol until the background became clear.

Enzyme Purification

All operations were carried out at 0–4°C. Three hundred sixty-three grams of caudate nuclei and putamen dissected out from 24 brains were homogenized in 10 volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA for 2 min. The homogenate was centrifuged at 34,800 *g* for 60 min. The pellet was discarded (32).

Ammonium Sulfate Fractionation. The supernatant was brought up to 35% saturation by addition of solid ammonium sulfate. After stirring for 30 min, the solution was centrifuged at 27,000 *g* for 20 min. The precipitate was discarded. The supernatant was brought up to 60% saturation with solid $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged at 27,000 *g* for 20 min. The precipitate contained about 93% of the CAT activity in the extract. It was dissolved in a minimum amount of 45 mM Tris HCl buffer, pH 7.9, containing 50 mM of β-mercaptoethanol, 2 mM EDTA, and 10% glycerol. These additives were in every buffer used from this stage on.

Protamine Sulfate Precipitation. To the solution from the previous step an equal volume of a 4% protamine sulfate solution was added drop by drop. The mixture was stirred for 20 min and centrifuged at 27,000 *g* for 20 min; the pellet was discarded. The supernatant was dialyzed against 45 mM Tris HCl buffer, pH 7.9, until the solution became clear. The dialyzate was centrifuged at 27,000 *g* for 20 min and the pellet discarded.

DEAE-Sephadex (A-25) Chromatography. The material from the protamine sulfate precipitation was applied to a DEAE-Sephadex column (2.5 × 20 cm), previously equilibrated with 45 mM Tris HCl buffer, pH 7.9. As described by Singh and McGeer (32), the effluent fractions with high CAT activity were pooled and concentrated by ammonium sulfate precipitation. The precipitate was dissolved in 10 mM potassium phosphate buffer and dialyzed against the same buffer.

Hydroxyapatite Chromatography. Material from the previous step was applied to a hydroxyapatite column (2.5 × 14 cm), which had been preequilibrated and washed with 10 mM potassium phosphate buffer, pH 6.8. After the enzyme was absorbed, the column was washed with equilibrating buffer and then with 0.1 M potassium phosphate buffer until the A_{280} reading of the eluents was below 0.1. The enzyme on the column was eluted with a linear potassium phosphate buffer gradient from 0.1 M to 0.5 M at pH 6.8. Fractions of 6 ml were collected at a flow rate of 6 ml/hr (Figure 1). The most active fractions (F 220–233), with a specific activity higher than 0.1 μmol/min/mg protein), were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 6.8.

Phosphocellulose Chromatography. Phosphocellulose was washed with 0.25 M HCl solution, twice with distilled water, once with 0.25 M NaCl–0.25 M NaOH, and then with distilled water until free of chloride. It was equilibrated with 10 mM sodium phosphate

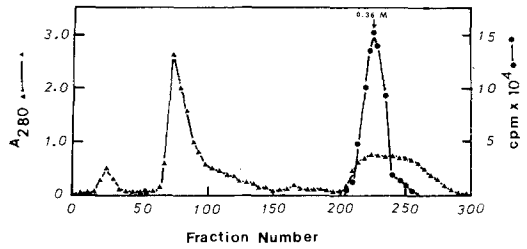

FIG. 1. Elution profile of choline acetyltransferase from hydroxyapatite chromatography. Details are described in the text.

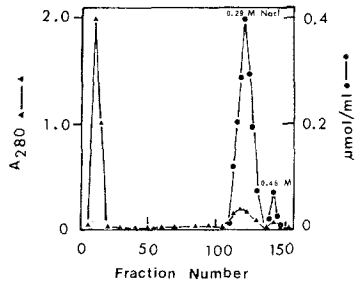

FIG. 2. Elution profile of choline acetyltransferase from phosphocellulose chromatography. Details are described in the text.

buffer, pH 6.8, and packed into the column (1.5 × 16 cm). After application of the dialyzed HTP enzyme, the column was washed with equilibrating buffer until the A_{280} reading was below 0.03. The enzyme in the column was eluted with a linear NaCl gradient from 0.05 M to 0.5 M in the same buffer at a flow rate of 15 ml/hr (Figure 2). CAT was separated in two peaks, one major and one minor. The highly active fractions (F 121–125) around the major peak had a specific activity higher than 0.9 μmol/min/mg protein and were pooled for further purification.

Affinity Chromatography on Agarose–Hexane–CoA Column. In order to protect the enzyme activity, the partially purified CAT was not subjected to prolonged dialysis but was diluted twofold immediately before application to a 10-ml agarose–hexane–CoA column, which had previously been equilibrated and washed with 10 mM sodium phosphate buffer, pH 6.8. After the enzyme was absorbed, the column was washed with 10 mM equilibrating buffer and then eluted stepwise with increasing NaCl concentrations from 0.2 M to 0.5 M in the buffer. CAT activity was eluted with 0.3 M NaCl (Figure 3). Since the enzyme concentration was very low, fractions 52–62 were combined and concentrated 20-fold with Carbowax for protein determination.

Immunochemical Work

Preparation of Antiserum. CAT, having a specific activity of about 7 μmol ACh formed/min/mg protein, was used for immunization of the rabbits according to the method of Harboe and Ingild (10).

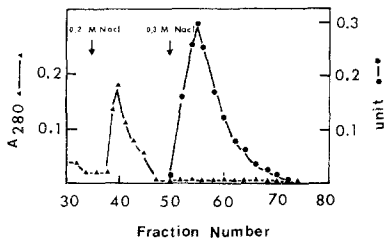

FIG. 3. Elution profile of choline acetyltransferase from agarose–hexane–CoA chromatography. Details are described in the text.

Two young New Zealand white rabbits (1♂, 1♀) weighing 1.5–2 lb were immunized by subcutaneous injection into the infrascapular and neck regions with 100 μg of purified CAT in 10 mM phosphate-buffered saline, pH 6.8, emulsified in complete Freund's adjuvant. The injection was given in the thicker part of the skin above the scapula and the neck and as superficially as possible. Two further injections were given at 14 and 18 days with each rabbit receiving each time 100 μg of CAT emulsified with 50% complete and 50% incomplete adjuvant. Twelve days after the last injection, blood was collected through marginal ear veins and allowed to clot at room temperature for 1 hr and 4°C overnight. The serum was drawn after the clot was centrifuged off at 2500 *g* for 15 min. Preimmune serum was obtained before immunization.

For isolation of immunoglobulins by the method of Harboe and Ingild (10), 25 g of ammonium sulfate were added to 100 ml of antiserum or preimmune serum and the mixture left for 20 hr at room temperature. The mixture was centrifuged at 4000 *g* for 30 min, and the supernatant discarded. The precipitate was dissolved in 25 ml of water and dialyzed at 4°C for 2 × 12 hr against distilled water and 1 × 24 hr against 50 mM sodium acetate buffer, pH 5.0. These dialyses were repeated once. After dialysis, the material was centrifuged at 4000 *g* for 30 min. The supernatant was applied to a DEAE-Sephadex A-50 column (2.5 × 15 cm) equilibrated with the acetate buffer, pH 5.0. The immunoglobulins were recovered in the eluate, which was dialyzed against the 10 mM phosphate-buffered saline, pH 7.2. The immunoglobulins were frozen at −20°C until use.

Immunochemical Studies. Double immunodiffusion studies were carried out on Ouchterlony double-diffusion plates (Hyland Division, Travenol Laboratories) in a moist chamber at room temperature. Antiserum or preimmune serum (10 μl) was placed in the center well, and 10-μl samples of CAT preparations at different stages of purification were placed in the outer wells. The precipitin line usually showed up within 48 hr of incubation. The precipitin patterns were photographed after a week of observation. CAT extracts from guinea pig, rat, and rabbit neostriatum made from 1:10 (w/v) homogenates were concentrated 5 times before being placed in the outer wells. Chicken brain CAT HTP fraction was used for double-diffusion studies.

Immunoelectrophoresis. The technique of Arquembourg (1) was used. The buffer used was 0.1 M sodium barbital, pH 8.3, and the immunoelectrophoresis was carried out at 4°C for 4 hr.

Immunoprecipitin Test. Various amounts of purified CAT (1–40 μg) in 10 mM phosphate buffer, pH 6.8, were added to various volumes of rabbit antihuman CAT immunoglobulins or preimmune globulins used as controls. The reaction mixtures were adjusted to a final volume of 100 μl with 100 mM phosphate buffer and incubated at room temperature for 1 hr and then at 4°C overnight. Portions of the mixtures and the supernates obtained after centrifugation at 12,000 *g* for 20 min were assayed for residual CAT activity. The precipitate was washed 3 times with 10 mM phosphate buffer, pH 6.8, and either assayed for protein content by Lowry's method (17) or was assayed for CAT activity.

Cross-Reactivity. CAT extracts from rat, rabbit, guinea pig, and chicken were prepared by homogenizing tissue in 10 vol of 50 mM potassium phosphate buffer, pH 7.4, containing 2 mM EDTA. The homogenates were centrifuged at 34,800 *g* for 60 min and the pellets discarded. The supernatants were similarly incubated with antihuman CAT immunoglobulins or preimmune globulins and the levels of inhibition were determined.

Electrophoresis of the Immunoprecipitate. Disk gel electrophoresis was performed on 7.5% gels according to Davis' procedure (6) as already described, except that electrophoresis was done at 2 mA/tube for 1.5 hr at 4°C in 50 mM Tris glycine buffer, pH 8.3. After electrophoresis, some gels were stained for protein and other gels were sliced into 1-mm slices and assayed for residual CAT activity.

Immunohistochemistry

Fab fragments were prepared from the CAT antisera by a previously reported method (22). Cryostat sections of tissues fixed with paraformaldehyde–glutaraldehyde were incubated with these Fab fragments. The tissues were then rinsed, incubated subsequently with goat anti-rabbit IgG, and then with rabbit PAP. After each incubation the sections were washed with phosphate-buffered saline. The tissue-bound PAP was visualized by reacting with a 3,3′-diaminobenzidine solution containing hydrogen peroxide in Tris buffer. In control experiments, rabbit serum collected before immunization and Fab fragments of antiserum to CAT that had been treated with purified human CAT were used instead of the Fab fragments in the above protocol. No positive staining was obtained in these control experiments. The fixation and staining procedures will be described in detail elsewhere (16).

RESULTS

Enzyme Purification and Some Properties. The results of the overall purification are summarized in Table I. The final enzyme preparation was purified about 7734-fold with 4% recovery. The specific activity of the purified CAT is 7.3 μmol ACh formed/min/mg protein. It may be noted that the specific activity did not increase much during the protamine sulfate precipitation; omitting this step, however, resulted in less overall purification and higher 260–280 ratios at later stages.

The enzyme was quite stable if 50 mM β-mercaptoethanol, 2 mM

TABLE I

SUMMARY OF PURIFICATION OF CHOLINE ACETYLTRANSFERASE FROM HUMAN BRAIN

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Homogenate[a]	47.36	50362	0.00094	100	1
Extract	30.32	10829	0.0028	64	3
Ammonium sulfate fractionation	28.14	5114	0.0055	59	6
Protamine sulfate precipitation	26.99	3272	0.0083	57	9
DEAE-Sephadex chromatography	24.15	1373	0.0176	51	19
Hydroxyapatite chromatography	12.31	103	0.12	26	128
Phosphocellulose chromatography	4.26	4.15	1.03	9	1096
Agarose–hexane–CoA chromatography	1.89	0.26	7.27	4	7734

[a] Prepared from 363 g of fresh human caudate nucleus and putamen. One unit of enzyme activity is equal to 1 μmol ACh formed/min at 37°C.

EDTA, and 10% glycerol were included in the buffers at all stages of purification and the protein concentration was above 0.1 mg/ml. The HTP fraction could be stored at −20°C for 2 months with only 10% loss of enzyme activity. The final preparation lost 50% of its enzymic activity 12 hr after elution from the affinity column, and 80% after 24 hr at −20°C. Addition of 10 μg of BSA into the incubation mixture was necessary to protect the activity of the final enzyme preparation during assay; assays run without this concentration of BSA indicated only about 30% of the enzyme activity.

The molecular weight of the human brain enzyme was determined on 7.5% SDS gels to be about 66,000 daltons (R_f = 0.36). The R_fs for the protein standards were: BSA (mol wt 68,000) = 0.35; catalase (mol wt 58,000) = 0.39; cytochrome *c* (mol wt 11,700) = 0.88.

The purified CAT migrated as a single component on 7.5% gels with or without SDS (Figure 4). No carnitine acetyltransferase activity was present in the final enzyme preparation. Enzyme activity was identical if assayed with or without eserine, indicating that the preparation contained no esterases.

Immunochemistry. The rabbit anti-human CAT sera or immunoglobulins gave single precipitin lines on Ouchterlony immunodiffusion and immunoelectrophoresis plates when tested against human CAT at various stages of purification (Figure 5). Preimmune sera did not give any precipitin line at all. A residual CAT activity (cpm 1215 above a blank of less than 100) was found when assaying a sliced agar gel along the precipitin line in a radiochemical assay, indicating that these antibodies were active against CAT.

The activity of the antisera produced was further demonstrated by immunoprecipitation tests. Incubation of anti-CAT immunoglobulins with purified CAT led to a 48–51% inhibition of activity in the mixtures and a 95–98% reduction of activity in the supernate after centrifugation as compared with parallel incubations using preimmune globulins (Figure 6). The precipitated enzyme–antibody complexes still retained about 50% of the initial CAT activity as demonstrated in assays of the washed resuspended pellet (Figure 6).

Some inhibition of CAT activity could be observed after 30 min of preincubation of the enzyme with anti-CAT immunoglobulins at 4°, 23°, and 37°. The degree of inhibition of CAT activity by preincubation with anti-CAT immunoglobulins was dependent on both time and temperature (Figure 7); the lower the temperature, the longer the time of preincubation required to reach about the same level of inhibition.

Inhibition of the CAT activity by increasing amounts of anti-CAT immunoglobulins is shown in Figure 8. The maximum amount of inhibition

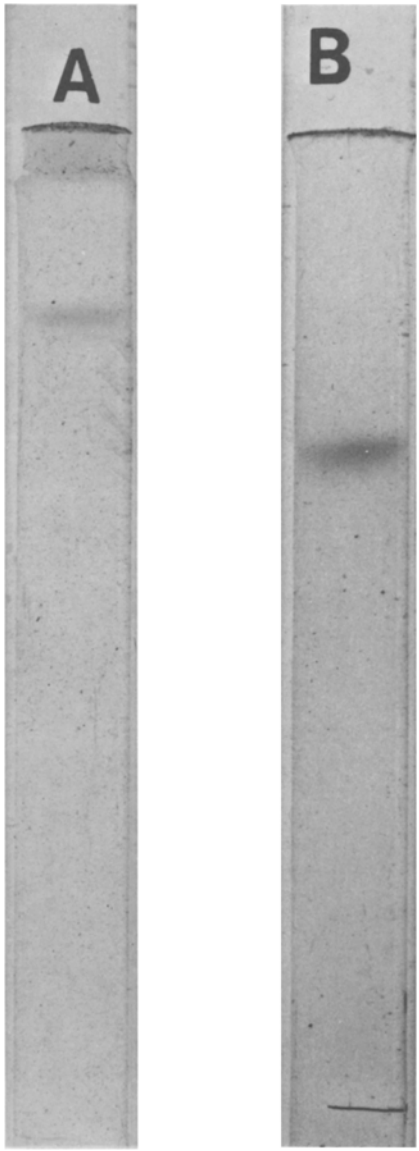

FIG. 4. Disk gel electrophoresis of the purified human CAT. (A) Five micrograms of purified CAT were electrophoresed on 7.5% regular gels without SDS. One gel was stained for CAT and the other gel was cut into 1-mm slices and three pieces were grouped into one fraction and assayed for CAT activity. The CAT activity corresponded to the protein stain band. (B) Ten micrograms of purified CAT were electrophoresed on a 7.5% SDS gel. Details are described in the text.

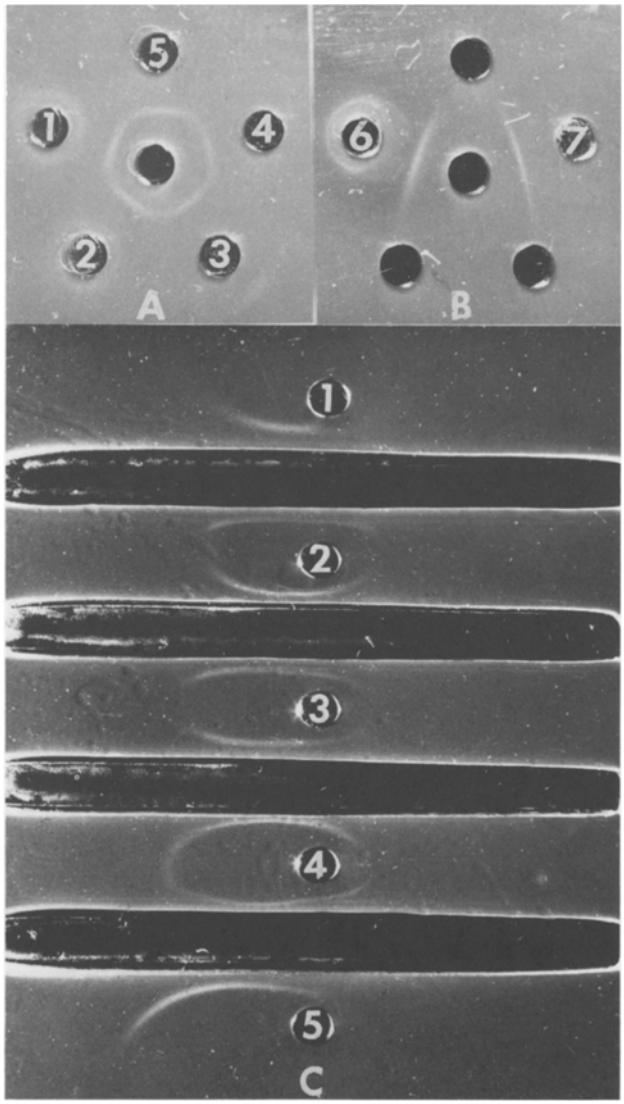

FIG. 5. Ouchterlony double diffusion and immunoelectrophoresis of CAT preparations against the rabbit anti-human CAT serum. The center wells in double-diffusion plates A and B and troughs in immunoelectrophoretic plate C contained 10 μl and 120 μl of antiserum, respectively. The outer wells in plates A and B and wells in plate C contained 10 μl of the CAT preparations. Plates A and C: 1. $(NH_4)_2SO_4$ fraction; 2. DEAE-Sephadex fraction; 3. HTP fraction; 4. PC fraction; 5. agarose–hexane–CoA fraction. Plate B: 6. Guinea pig enzyme; 7. Chicken enzyme. The results from rat and rabbit enzyme were similar to guinea pig and chicken enzyme and are not shown here.

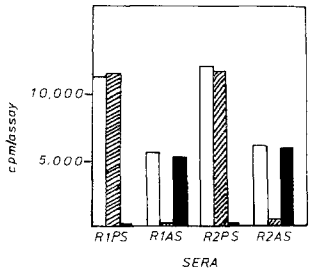

FIG. 6. Comparison of the immunoprecipitation capacity of preimmune serum globulins (PS) and antiserum immunoglobulins (AS) of rabbits 1 (R1) and 2 (R2). CAT (5 μg) was preincubated with 20 μl of preimmune serum globulins or anti-CAT immunoglobulins at room temperature for 1 hr and at 4°C for 20 hr. 20 μl of mixtures and supernatants were assayed for CAT residual activity. ▭ CAT activity in the mixture; ▨ CAT activity in the supernatant; ■ CAT activity in the precipitate.

(about 50%) and precipitation (about 98%) of the activity using 5 μg of purified CAT was obtained with about 20 μl of anti-CAT immunoglobulins.

When increasing amounts of purified CAT were added to a constant amount of anti-CAT immunoglobulins, a characteristic precipitin curve was obtained (Figure 9). The equivalence point is 6 μg antigen/20 μl of antibody. Thus the titer, expressed as the maximum amount of antigen precipitated by 1 ml of antibody, is 0.3 mg/ml. Supernatants at the equiv-

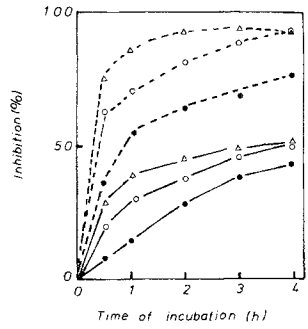

FIG. 7. Effect of temperature on the inhibition of CAT by the antiserum as a function of incubation time. 50 μl CAT PC fraction were incubated at 4°, 23°, and 37°C with 50 μl of anti-CAT immunoglobulins in 0.3 ml of 10 mM phosphate buffer, pH 6.8. 20 μl of the mixtures and supernatants before and after centrifugation at 12,000 *g* for 20 min were assayed for residual CAT activity. Inhibition is expressed as a percentage of the CAT activity found in parallel incubations with preimmune globulins. ——, percent inhibition in the mixtures; – – –, percent inhibition in the supernatants; ●, 4°; ○, 23°; △, 37°C.

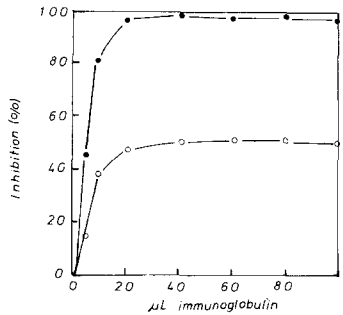

FIG. 8. Inhibition of CAT by increasing amounts of antiserum. 5 μg of purified CAT was incubated with 5, 10, 20, 40, 60, 80, and 100 μl of antiserum in 0.15 ml of 10 mM phosphate buffer, pH 6.8.

alence point showed very little activity, while those on the antigen-excess side of the curve showed CAT activity.

A fivefold concentrate of crude extracts from other mammalian species (rat, rabbit, and guinea pig) and of a chicken CAT HTP fraction produced sharp precipitin lines against rabbit anti-human CAT serum in Ouchterlony immunodiffusion tests (Figure 5). Quantitative evidence also indicated cross-reactivity (Table II). On preincubation with anti-human CAT immunoglobulins, most of the CAT activities of rat, rabbit, and guinea pig appeared in antigen–antibody complexes which could be separated by centrifugation and still retain catalytic activity. The enzyme activity in the chicken extract, on the other hand, was much less inhibited by human anti-CAT, and little catalytic activity was found in the precipitate (Table II).

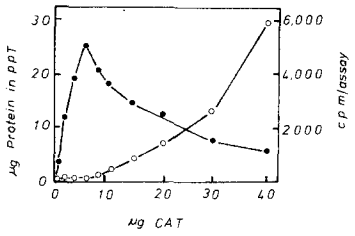

FIG. 9. Titration of anti-human CAT immunoglobulin with purified enzyme. Each assay contained 20 μl of antiserum, 1–40 μg of purified CAT, and 10 mM phosphate buffer, pH 6.8. The reaction mixtures were incubated as described in the text. Total protein was determined on the washed precipitate. Enzyme activities were determined on the supernatants. ●—●, μg protein in the precipitates; ○—○, cpm in the supernatants.

TABLE II
EFFECT OF RABBIT ANTI-HUMAN CAT IMMUNOGLOBULINS ON ACTIVITIES OF CAT EXTRACTED FROM VARIOUS ANIMAL BRAINS

Source of enzyme	Percent inhibition of CAT activity in[a]		CAT activity (cpm) in the precipitate
	Mixture	Supernatant	
Rat neostriatum	56.5	92	1679
Rabbit neostriatum	44	85	1467
Guinea pig neostriatum	41	86	1212
Chicken whole brain	32	36	160

[a] Preimmune serum globulins were used as a control.

Gel electrophoresis of an immunoprecipitate showed that the CAT–anti-CAT IgG conjugate migrated as a single band (Figure 10). The residual CAT activity corresponded with the single protein stained band. This establishes the monospecificity of the anti-CAT immunoglobulin preparation and shows that no IgG fraction exists which reacts with other brain proteins.

Immunohistochemistry. In the guinea pig spinal cord intense positive reactions were localized in cells of the ventral horn motor neurons (Figure 11), while no cells were found in the apex and head of the dorsal gray column. The nuclei of CAT-staining perikarya in the anterior horn did not stain positively, but there was positive staining of the axon bundles of these cells through the anterior white matter on their way to form the ventral roots. These CAT-immunoreactive motor neurons, which have been shown to stain strongly for cholinesterase and have been visualized in CAT histochemical (16) and previous immunohistochemical studies (8, 20), are known to be cholinergic. Confirmation for the specific localization of immunoreactivity to CAT in cholinergic neurons was obtained by these experiments as well as by the strong staining seen in the nerve endings at the neuromuscular junction of the guinea pig diaphragm (Figure 11). Thus, with this immunohistochemical technique we are able to specifically stain cell bodies, dendrites, axons, and nerve terminals of cholinergic neurons.

The antibodies also gave excellent staining of selected neuronal structures in human, cat, rat, and guinea pig brain. The distribution of positively staining cells, fibers, and probable nerve terminal areas in the rostral forebrain of the rat and guinea pig (16) is in excellent accord with previous indications of cholinergic pathways based on biochemical and physiological studies, as well as acetylcholinesterase histochemistry.

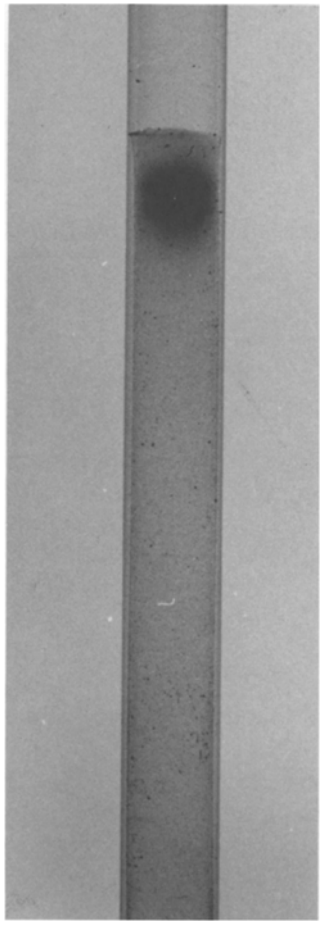

FIG. 10. Disk gel electrophoresis of the immunoprecipitate of human CAT–anti-CAT IgG conjugates; 25 μg of immunoprecipitate were electrophoresed on 7.5% regular gels without SDS. The residual CAT activity corresponding to the protein stain band was found by assaying the gel slices.

DISCUSSION

A homogeneous fraction of choline acetyltransferase has been purified from human caudate nucleus and putamen by a series of procedures involving ammonium sulfate fractionation, protamine sulfate precipitation, and chromatography on DEAE-Sephadex, hydroxyapatite, phosphocellulose, and agarose–hexane–CoA columns. The 7734-fold purified CAT preparation had a specific activity of 7.3 μmol ACh formed/min/mg pro-

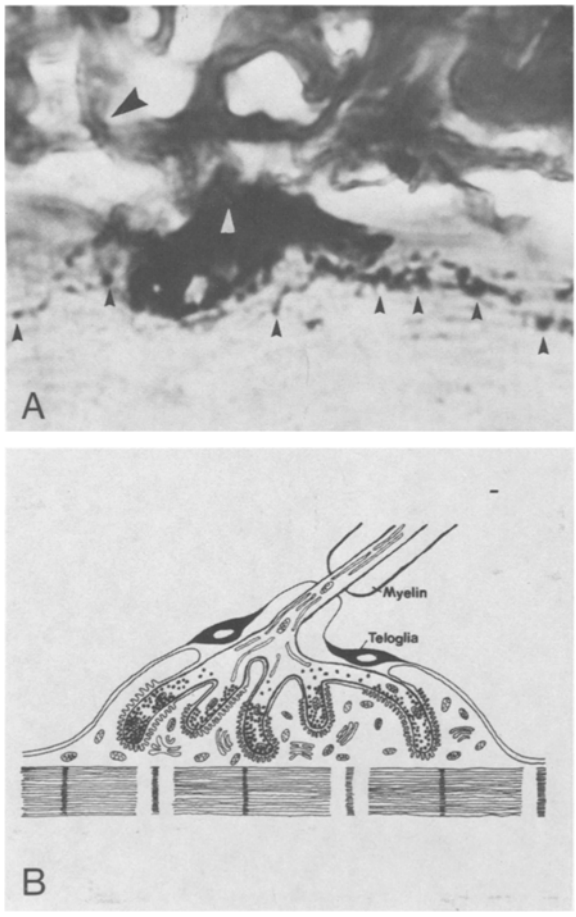

FIG. 11. (A) Choline acetyltransferase-positive staining in the guinea pig diaphragm. Unmyelinated preterminal axon branches (white triangle), which are surrounded by the teloglial cell, are intensely stained, and motor nerve endings (small arrow heads) are also revealed as densely stained dots. Myelinated fibers (large arrow head) are not significantly stained, probably due to poor penetration of the antibodies through the myelin (magnification X450). (B) Schematic diagram of a motor endplate, showing the relationship between the various structures in nerve and muscle. An axis cylinder covered by the myelin sheath is emerging from the sheath, ramifying abruptly under the mantle of the teloglial cell and terminating against the folding surface of the sarcolemna. (C) Low-power magnification of the ventral horn of the guinea pig spinal cord. Motor neurons are positively stained. Note stained reticular network in the background surrounding these cells which probably represents cellular processes. Axons passing through the anterior white matter are also stained. Top is the dorsal (DOR) and right side is the medial (MED) direction of the spinal cord (magnification X150). (All parts reduced 20% for reproduction.)

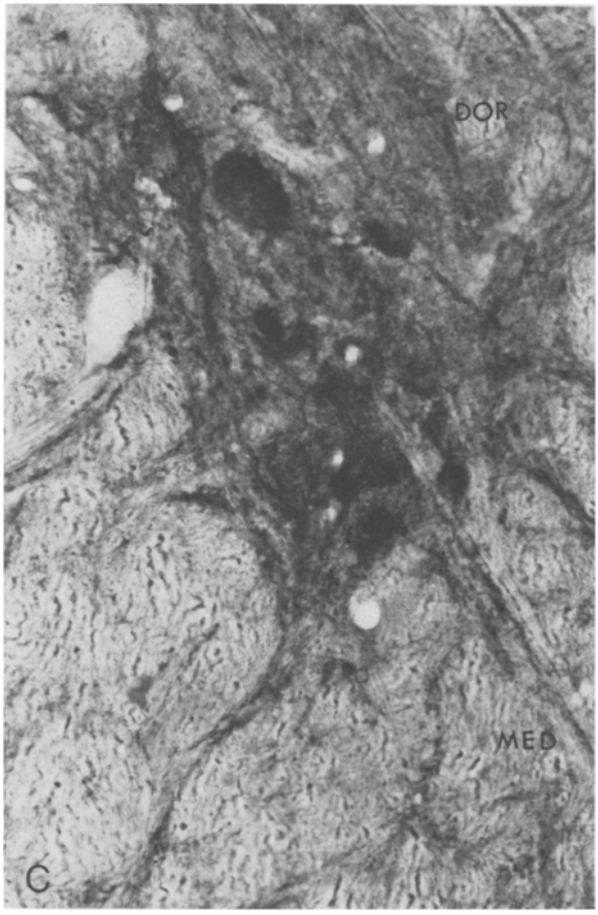

FIG. 11. Continued.

tein and migrated as a single band on 7.5% gels with or without SDS. The present preparation represents the highest specific activity of CAT so far obtained from human brain. This was possible because we used a large amount of starting material and maintained the protein concentration above 0.1 mg/ml until the last step of purification. Driskell et al. (7) have also observed that the stability of CAT from *Drosophila* was preserved as long as the protein concentration was kept above 0.1 mg/ml.

Homogeneous CAT preparations with specific activities of 0.04 and 0.01 μmol ACh formed/min/mg protein have been obtained by Roskoski et al. (24) and Singh and McGeer (32), respectively. On the other hand,

CAT preparations with specific activities as high as 2.3 and 92.7 μmol ACh formed/min/mg protein were obtained from human placenta by Morris (21) and by Hersh et al. (12), and yet they showed heterogeneity on SDS gel electrophoresis. Therefore, it seems doubtful whether there is necessarily a correlation between enzyme purity and specific activity, as has been suggested (26). It also seems unlikely that a protein moving as a single band on gel electrophoresis and possessing CAT activity could have a substantial amount of non-CAT protein as a comigrating impurity (19). That remote possibility has been excluded by developing monospecific antibodies to the CAT in rabbits and then establishing that the immunoprecipitin formed by the CAT–anti-CAT IgG migrates as a single band.

The molecular weight of the human brain CAT as determined by SDS gel electrophoresis was 66,000 daltons, which was in rather close agreement with previous reports of 62,000–69,000 daltons for CAT from mammalian sources. Molecular weights of 62,000 and 67,000 have been reported for preparations from human brain (32, 37), and 67,000 and 68,000 from human placenta (21, 24). Slightly lower values of 58,000 and 60,000 daltons for human placental enzyme were reported by Banns (2). The higher-molecular-weight aggregates (mol wt 125,000) reported by Singh and McGeer (34) from human brain enzyme preparation were not observed in the present studies. CAT aggregates were previously reported from bovine caudate enzyme (5). Banns (2) found that the CAT aggregates were produced by treatment with ammonium sulfate and disappeared after further purification. A minor peak in the phosphocellulose chromatography may represent another molecular form of CAT and confirms the previous report of Singh et al. (35). However, this result is contrary to a recent report that no multiple forms of choline acetyltransferase have been found in extracts of human, mouse, rat, guinea pig, and cat brain (4).

The monospecific anti-CAT IgG readily precipitated the enzyme activity in CAT preparations. The activity of antisera prepared by Malthe-Sørenssen et al. (18) and Rossier (27) were comparable in this respect, although in both instances it was reported that serum albumin or swine immunoglobulins had to be added to the incubation mixture in order to precipitate fully the enzyme activity.

In our experiments, the precipitated enzyme–antibody complexes still retained about 50% of the catalytic activity. Anti-human CAT immunoglobulins also inhibited and precipitated activities of CAT isolated from other mammalian species. In contrast to the previous report of Singh and McGeer (33), these high-titer anti-human CAT preparations even had some cross-reactivity with chicken enzyme. The fact that only about 30%

of the chicken enzyme could be precipitated indicates, however, only a weak antigenic relationship between human and chicken enzyme. Differences between these enzymes are also suggested by their different behavior during purification (Ma and Sung, personal communication).

The immunohistochemical results so far obtained on the spinal cord, neuromuscular junction, and rostral forebrain areas of rodents are a further indication both of the high titer and specificity of the antibodies obtained and of their probable utility in mapping cholinergic structures.

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