Purification of Choline Acetyltransferase from Rat and Cow Brain†

Rick L. Ryan‡ and William O. McClure*

ABSTRACT: Choline acetyltransferase was extensively purified from rat cerebra and bovine caudate nuclei. The initial steps in each purification were essentially those described by Malthe-Sørenssen et al. [Malthe-Sørenssen, D., Eskeland, T., & Fonnum, F. (1973) *Brain Res. 62*, 517–522]. Rat cerebral choline acetyltransferase was solubilized, fractionated by precipitation with acetic acid and with ammonium sulfate, and chromatographed on carboxymethyl-Sephadex C-50. Further purification was obtained by sequential chromatography on an affinity column of coenzyme A (CoA)–Sepharose and on hydroxylapatite to yield a final product with a specific activity of 40 μmol min^{-1} mg^{-1}. The yield was 14%, with an overall purification of 18 300-fold. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated that the enzyme was 68% pure. The first three steps of the purification of rat choline acetyltransferase were used in an identical manner for purification of the enzyme from bovine caudate nuclei. In this case, chromatography on carboxymethyl-Sephadex C-50 resulted in the separation of two peaks of enzyme activity. In order of their elution, these peaks accounted for 75–80% (Bov I) and 20–25% (Bov II) of the eluted activity. Purification of Bov I by using affinity chromatography on CoA–Sepharose and chromatography on hydroxylapatite generated a final product with a specific activity of 58.8 μmol min^{-1} mg^{-1}. A yield of 18% was obtained with an overall purification of 21 500-fold. The final preparation was estimated to be 80% pure. Bov II was purified further only by affinity chromatography. The specific activities of these preparations are the highest yet reported for choline acetyltransferase from a mammalian source. Although highly purified, the enzyme is not yet homogeneous. The findings are discussed with respect to the results obtained by other investigators, some of whom claim to have purified choline acetyltransferase to homogeneity.

Choline acetyltransferase (choline *O*-acetyltransferase, EC 2.3.1.6) is the enzyme responsible for the biosynthesis of the neurotransmitter acetylcholine. Efforts to obtain information with regard to the molecular properties and in vivo site of action of this enzyme have yielded contradictory results (Rossier, 1975, 1976a,c; Eng et al., 1974; Chao, 1975; McGeer et al., 1974). These discrepancies can be attributed, in part, to the use of choline acetyltransferase preparations of variable and questionable purity.

The purification of choline acetyltransferase from mammalian sources has been undertaken by many investigators (Malthe-Sørenssen et al., 1973; Chao & Wolfgram, 1973; Wenthold & Mahler, 1975; Roskoski et al., 1975; Singh & McGeer, 1974; Rossier, 1976b; Glover & Potter, 1971). Unfortunately, the preparations of enzyme which have been obtained have differed greatly with respect to their observed electrophoretic purity and specific activities. The purification of electrophoretically homogeneous enzyme has been reported from bovine brain (Chao & Wolfgram, 1973) and human brain (Singh & McGeer, 1974). The specific activities of the preparations were respectively 1.45 and 0.012 μmol min^{-1} mg^{-1}. Using the methods of Singh & McGeer (1974), Roskoski et al. (1975) have reported the purification to homogeneity of choline acetyltransferase from bovine brain, human brain, and human placenta. In this case, enzyme from each source had an equal specific activity of 0.039 μmol min^{-1} mg^{-1}. It is noteworthy that this specific activity is 37-fold lower than that reported by Chao & Wolfgram (1973) for the bovine enzyme, although both preparations are supposedly homogeneous. Each of the preceding three groups (Chao & Wolfgram, 1973; Roskoski et al., 1975; Singh & McGeer, 1974) has undertaken immunologic experiments using their preparation of choline acetyltransferase. Monospecific antisera were claimed to be elicited in each case.

Others have purified the enzyme to even higher specific activities but have not obtained homogeneous material. Both Malthe-Sørenssen et al. (1973) and Wenthold & Mahler (1975) reported preparations with specific activities of ~4 μmol min^{-1} mg^{-1}. In neither case was the enzyme homogeneous. Rossier (1976b) has used column chromatography on an immunoabsorbent to purify rat brain choline acetyltransferase to a specific activity of 20 μmol min^{-1} mg^{-1}. This preparation was also heterogeneous. During the preparation of this paper, Malthe-Sørenssen et al. (1978) reported purification of the bovine enzyme to a specific activity of 25–30 μmol min^{-1} mg^{-1} but pointed out that even their material is heterogeneous. While it is true that homogeneous enzymes from different mammalian sources need not display identical specific activities, differences of more than 500-fold are surprising. The low and varied enzymatic activities which characterize preparations of this enzyme suggest that choline acetyltransferase has not yet been purified from mammalian sources. As a result, the task of purifying the enzyme from rat and bovine brains was undertaken, with the intention of obtaining efficient and reproducible methods with which to isolate highly purified enzyme. The present report describes the utilization of an affinity column employing coenzyme A as a ligand to achieve this end.

Materials and Methods

Assay of Choline Acetyltransferase. Choline acetyltransferase activity was monitored essentially as described by Fonnum (1969) (assay I) and Schrier & Schuster (1967) (assay II). Assay I was used for rapid measurement of the acetylase throughout the chromatographic procedures. The incubation mixture consisted of (final concentrations) 0.4 mM [^{3}H]acetyl-CoA, 1 mM EDTA, 10 mM choline chloride, 300

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801 (R.L.R.), and the Section of Cellular Biology, Department of Biological Sciences, University of Southern California, Los Angeles, California 90007 (W.O.M.). *Received June 22, 1979.* This work was supported, in part, by the National Institute of Neurological and Communicative Diseases and Stroke (NS-09082 and NS-13215), the Nelson Research and Development Co., Irvine, CA, and the Alfred P. Sloan Foundation. A preliminary account of this work has been published (Ryan & McClure, 1976).

‡ Taken from a thesis submitted to the University of Illinois for the Ph.D. degree. Present address: Armour-Dial Research Center, Scottsdale, AZ.

0006-2960/79/0418-5357$01.00/0

mM NaCl, 50 mM sodium phosphate buffer, 0.1 mM eserine sulfate, and 0.5 mg/mL bovine serum albumin (BSA).[1] Following the addition of enzyme, the final volume was 0.1 mL and the pH was 7.4. Assays were carried out at 37 °C in 15-mL conical glass centrifuge tubes. For termination of the reaction, 7 mL of 10 mM sodium phosphate buffer, pH 7.0, was added to each tube, followed by the immediate addition of 1.0 mL of 3-heptanone containing 25 mg of sodium tetraphenylboron. Each solution was vigorously shaken on a vortex mixer and centrifuged to separate the aqueous and organic phases. A 0.1-mL volume of the organic phase, which contained newly synthesized acetylcholine as an ion pair with the tetraphenylboron anion, was transferred to a scintillation vial containing 5 mL of scintillation fluid (Anderson & McClure, 1973). Radioactivity was determined in a Beckman LS-230 liquid scintillation spectrometer equipped with automatic external standardization. In some cases, radioactivity data were transferred on punched paper tape to a PDP-8/e minicomputer, with which corrections and calculations could be performed.

The reaction mixture of assay II was the same as that used for assay I, except in the case of highly purified enzyme preparations when no eserine sulfate was included. Assays were carried out at 37 °C in 10 × 75 mm glass test tubes, the final volume being 0.1 mL. For termination of the reaction, 0.1 mL of 0.15 M $HClO_4$ was added to the assay tube, after which the tube was mixed for 5 s on a vortex stirrer and placed on ice. After a number of samples were collected, a 0.1-mL aliquot from each acidified solution was applied to a 0.5 × 5.0 column of Dowex 1-X8 chloride (200–400 mesh) formed over a glass wool plug in a 4.5-in. Pasteur pipet. Each column was then washed with six 0.2-mL portions of distilled water, and the effluents were collected in a 25-mL glass scintillation vial. Radioactivity was determined after adding 12 mL of scintillation fluid (Anderson & McClure, 1973). Control experiments using [^{3}H]acetylcholine chloride were carried out to quantitate the elution of acetylcholine through the Dowex column. In each case, 97–100% of the [^{3}H]acetylcholine was recovered.

Acetyl-CoA Synthesis and Purification. The basic procedure described by Simon & Shemin (1953) was used for synthesis of acetyl-CoA. One hundred milligrams of CoA was dissolved in 30 mL of 0.1 M $NaHCO_3$, and the solution was chilled to 4 °C. While this was being stirred, a commercially prepared solution (0.5 mL) of benzene containing 25.5 mg of [^{3}H]acetic anhydride (New England Nuclear) was added to the buffered CoA solution. After 15 min, maintaining thorough stirring, 15 μL of unlabeled acetic anhydride was added to ensure completion of the reaction. Five minutes later, 2 M acetic acid was added to the reaction mixture until the pH dropped to 4.5, after which the solution was shell-frozen and lyophilized to near dryness. The acetylated CoA was purified according to the method of Jones & Nelson (1968). Briefly, the free and acetylated forms of CoA were separated by chromatography on DEAE-cellulose. Fractions of the peak of [^{3}H]acetyl-CoA were pooled, lyophilized, and desalted on Sephadex G-25 in glass-distilled water. The final pH of the [^{3}H]acetyl-CoA solution was 4.5. Aliquots of this solution were stored frozen in breakseal vials at −20 °C.

The degree of acetylation of the final product was determined by following the loss of acetyl-CoA catalyzed by phosphotransacetylase (Stadtman, 1957). By comparison of the amount of acetyl-CoA susceptible to this enzyme with the total amount of all forms of CoA, determined by absorbance at 259 nm, a purity of at least 97% was assigned to each batch of acetyl-CoA. Because some tritium compounds display special problems of instability (Oldham, 1968), the [^{3}H]acetyl-CoA was routinely checked for changes in specific radioactivity. Losses of specific radioactivity were no greater than 5% for periods up to 6 months, after which time new [^{3}H]acetyl-CoA was prepared. Individual preparations of [^{3}H]acetyl-CoA varied in specific activity from 155–255 mCi/mol, depending upon the specific radioactivity of the [^{3}H]acetic anhydride used in the acetylation reaction.

Synthesis of CoA–Sepharose. Sepharose 4B was activated with cyanogen bromide by the method of March et al. (1974). The coupling of coenzyme A to activated Sepharose was carried out according to a slight modification of the method of Chibata et al. (1974). To a solution of 60 mg of CoA dissolved in 70 mL of 0.1 M NaOAc, pH 6.0, was added 15 mL of activated Sepharose 4B which had been previously washed with the same buffer. After the mixture was shaken at 4 °C for 16 h, 2 mL of 1 M ethanolamine hydrochloride, pH 7.0, was added and the shaking was continued for another 4 h. The coupled Sepharose was then washed on a sintered glass funnel with 100 mL of 1 M NaCl, followed by 200 mL of H_2O. Next, 100 mL of a solution of 1% 2-mercaptoethanol was slowly passed over the Sepharose, and the salt and water washes were repeated. CoA–Sepharose (CoA–S) columns packed from this material were used repeatedly up to 3 weeks. After each experiment, the column was washed with 2 M guanidine hydrochloride and stored in 0.1 M NaOAc. By use of CoA labeled with tritium, the amount of CoA coupled to the Sepharose 4B was determined. Typically, 6.5 mg of CoA was covalently bound to 12 mL of the packed Sepharose 4B.

The chemical nature of this column is not well-defined. Although Chibata et al. (1974) felt that the linkage to CoA was through the 6-NH_2 group, Mautner (1977) feels that the SH group is involved.

Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method of Fairbanks et al. (1971), except that 5% sodium dodecyl sulfate was used. Gels were stained with Coomassie blue (Fairbanks et al., 1971) at 540 nm with a Gilford linear transport device. Prior to electrophoresis, samples of choline acetyltransferase were reduced by boiling in the presence of 5% 2-mercaptoethanol for 5 min.

Determination of Protein. Protein was routinely determined by the method of Lowry et al. (1951). The fluorescamine method of Udenfriend et al. (1972) was used for measuring small amounts of protein (0.1–5 μg).

Determination of the Specific Activity and Purity of the Enzyme following Hydroxylapatite Chromatography. The specific activity of the hydroxylapatite pool of choline acetyltransferase could not be determined with fluorescamine due to the presence of ammonium sulfate. The specific activity of this pool was determined in the following manner. Following polyacrylamide gel electrophoresis in sodium dodecyl sulfate of the concentrated pooled fractions, the gel was scanned with a microdensitometer. Areas corresponding to peaks of protein were cut from the paper. The total area was weighed and defined as the total protein in the gel. The peak of protein corresponding to the molecular weight of this enzyme, as

[1] Abbreviations used: BSA, bovine serum albumin; CPED, elution buffer containing citrate, phosphate, EDTA, and dithioerythritol; CPED + C, CPED buffer containing choline; PED, elution buffer containing sodium phosphate, EDTA, and dithioerythritol; PED + G, PED buffer containing glycerol; PEDS, elution buffer containing sodium phosphate, EDTA, dithioerythritol, and sucrose; CoA–S, affinity matrix of Sepharose with covalently bound coenzyme A; Bov I and Bov II, two forms of choline acetyltransferase found in bovine caudate nuclei.

Table I: Purification of Choline Acetyltransferase from Rat Cerebra[a]

step	vol (mL)	protein (mg)	total act. (μmol min^{-1})	sp act. (μmol min^{-1} mg^{-1})	yield (%)	purifn (x-fold)
crude supernatant	1788	6079	13.40	0.0022	100	1
pH 4.5 supernatant	1755	2703	4.43	0.0037	74	1.7
40-60% ammonium sulfate fraction	136	861	8.47	0.0098	63	4.5
CM-Sephadex	32.2	54.4	5.14	0.094	38	43
Sepharose-CoA	*b*	0.498	3.83	7.69	28	3496
hydroxylapatite	*b*	*c*	1.88	40.2	14	18280

[a] Details of the experimental procedures are presented in the text. The purification was carried out by using 250 g of rat cerebra. [b] The volumes of the choline acetyltransferase pools after affinity chromatography and hydroxylapatite chromatography were omitted because variable portions of the CM-Sephadex pool were applied to the CoA-Sepharose column as needed. The total yield was calculated from the sums of the recovered choline acetyltransferase from each hydroxylapatite pool. [c] Protein could not be chemically determined after hydroxylapatite chromatography (see Materials and Methods).

determined by gel filtration, was assumed due to choline acetylase. This protein peak was actually the only major peak remaining after chromatography on hydroxylapatite. The weight of paper representing the acetylase peak, expressed as a percent of the total paper weight, yields a measure of the purity of the enzyme. The purity after chromatography on hydroxylapatite, compared to the purity preceding this step, yields the relative purification. This purification factor was then used to approximate the specific activity of the choline acetyltransferase after chromatography on hydroxylapatite.

General. All purification steps were performed at 0–4 °C. Centrifugations were carried out in plastic bottles at 20000*g* for 20 min unless otherwise noted. Dialysis tubing was purified by boiling in 0.1 mM EDTA for 60 min and soaking in glass-distilled water for two periods of 12 h each. Buffers containing citrate were made up by dissolving the stated concentrations of the basic form of the buffer salt, and any added components, and titrating to the desired pH with citric acid. All other buffers were made up by dissolving the stated concentrations of the acidic form of the buffer salt, and any added components, and titrating to the desired pH with NaOH. Measurements of pH were carried out with a Metrohm/Brinkman Model 103 pH meter equipped with a large combination glass electrode (Metrohm EA120) and standardized with Beckman buffers. Samples were not diluted before measurement of pH, even when high concentrations of protein were present.

Results

Purification of Rat Brain Choline Acetyltransferase. A summary of the purification is given in Table I. Rat brains were excised from 20–23-day-old female rats (Holtzman). After light blotting to remove exterior fluids, the portion of the brain anterior to the cerebrellum was dissected free from the rest of the brain, frozen in liquid nitrogen, and stored at −20 °C.

Initial Solubilization. Two hundred and fifty grams of whole rat brain cerebra was thawed and minced with scissors in 25 mM sodium phosphate buffer, pH 7.4 (25 g of cerebra per 10 mL). Following the addition of 90 mL more of buffer, the slurry was homogenized with a Teflon pestle driven at 3000 rpm in a smooth glass mortar. After 20 strokes at full speed, another 100 mL of buffer was added and the procedure repeated. Centrifugation for 1 h yielded a turbid reddish supernatant (crude supernatant, Table I) which was quickly decanted from a tan jellylike precipitate.

Acid Precipitation. The supernatant was adjusted to pH 4.5 with 50% acetic acid (~50 mL for a 250-g preparation) added over a 45-min period. After gentle stirring for another 50 min, the solution was centrifuged to give a clear reddish supernatant and a firm tan pellet. The pellet was discarded. Precise standardization of the pH meter and exact adherence to the procedure were required to achieve reproducible results at this step. Failure to carry out this step carefully resulted in greatly reduced yields in later steps.

Ammonium Sulfate Precipitation. The supernatant was titrated to pH 6.0 with 1 M NaOH. Solid ammonium sulfate was added to a saturation of 40% without adjusting the pH. After the ammonium sulfate had completely dissolved, the solution was stirred for 30 min. The suspension was centrifuged, and the pellets were discarded. The supernatant was made 60% saturated with ammonium sulfate, again without adjusting the pH, and stirred another 30 min. Following centrifugation, the pellets were resuspended in 10 mM citrate–sodium phosphate buffer, pH 7.2, which contained 0.1 mM EDTA and 0.1 mM dithioerythritol (CPED buffer, 0.5 mL/g of cerebra). The resulting amber solution was dialyzed overnight against 150 volumes of CPED buffer.

CM-Sephadex Chromatography. The dialyzed enzyme preparation was loaded onto a carboxymethyl-Sephadex C-50 column (2.5 × 16 cm) which had been preequilibrated with CPED buffer. After being washed with 30–35 mL of CPED buffer, the column was eluted with a linear gradient from 0 to 0.25 M NaCl in the same buffer (Figure 1), using a gradient volume of 600 mL pumped at 20 mL/h. Fractions containing an activity of 18.5 nmol of acetylcholine min^{-1} mL^{-1} were pooled and concentrated by ammonium sulfate at 60% saturation (no pH adjustment). After centrifugation, the pellet was resuspended in a minimal volume (~6 mL) of CPED buffer and desalted on Sephadex G-25 preequilibrated with CPED buffer containing 120 mM choline chloride (CPED + C). The resulting enzyme solution was divided into aliquots and frozen at −20 °C, under which conditions it was stable for at least 8 weeks.

Sepharose–CoA Chromatography. A thawed aliquot (less than 2 units) was applied to a CoA–S column (10–12-mL bed volume) preequilibrated with CPED + C buffer. After being washed with 35–40 mL of the equilibration buffer, the enzyme was eluted with a linear gradient from 60 mL of CPED + C buffer to an equal volume of CPED + C buffer containing 1.5 M NaCl (Figure 2). The flow rate was maintained at 10 mL/h. A symmetrical peak of enzyme activity, centered at 0.35 M NaCl, was obtained. Fractions possessing the majority of the activity were pooled, and solid sucrose was added to the pool to a final concentration of 10%. The addition of sucrose was necessary to stabilize the activity of the enzyme throughout the subsequent hydroxylapatite chromatography. The stabilizing effect of sucrose on choline acetyltransferase has been previously reported (Schuster & O'Toole, 1974).

Hydroxylapatite Chromatography. The sucrose solution was applied to a hydroxylapatite column (Clarkson Labora-

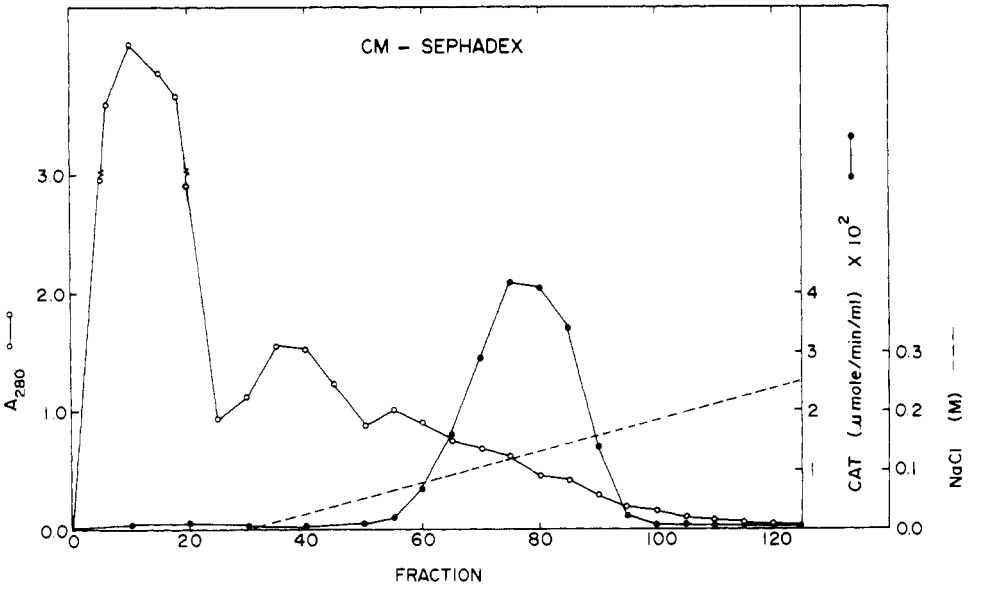

FIGURE 1: Chromatography of rat choline acetyltransferase on CM-Sephadex C-50. The dialyzed solution of enzyme from step 3 (~120 mL) was applied to a column of CM-Sephadex (2.4 × 16 cm). Enzyme was eluted with a linear gradient of NaCl (---) in CPED buffer. Enzymatic activity in individual fractions was measured by assay I (●). Protein was monitored by absorbance at 280 nm (○).

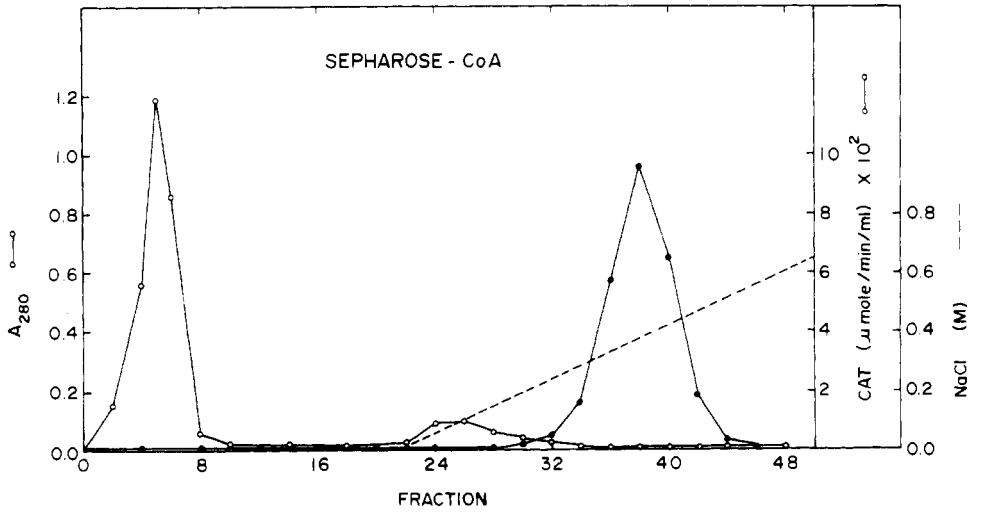

FIGURE 2: Affinity chromatography of rat choline acetyltransferase on CoA–Sepharose. An aliquot of enzyme (2 units or less) was applied to a 1.9 × 3.8 cm CoA–Sepharose column and eluted with a linear gradient of NaCl (---) in CPED + C buffer. Enzymatic activity was measured by assay I (●). Protein was monitored by absorbance at 280 nm (○).

tories; 1.6 × 5.0 cm) which had been preequilibrated with 10 mM sodium phosphate, 1 mM EDTA, and 0.5 mM dithioerythritol in 10% sucrose, pH 7.2 (PEDS buffer). A linear gradient of 0–3.0 M NaCl in PEDS buffer (100 mL total volume) was used to wash the loaded column. Following this wash, the column was washed with 10 mL of PEDS buffer. Choline acetyltransferase was eluted with a linear gradient from 50 mL of PEDS buffer to 50 mL of 100 mM potassium phosphate, 1 mM EDTA, 0.5 mM dithioerythritol, and 10% sucrose in 1.0 M $(NH_4)_2SO_4$, pH 7.2 (Figure 3). The activity eluting between 0.1 and 0.3 M $(NH_4)_2SO_4$ was pooled.

The enzyme thus obtained is quite labile with respect to freezing and dialysis. The addition of BSA partially stabilizes the acetylase activity during dialysis or freezing (Table II). Because of this protective effect, BSA was routinely added at a final concentration of 0.5 mg/mL to solutions of enzyme after hydroxylapatite chromatography. BSA was not added to those choline acetyltransferase preparations to be used for gel electrophoresis.

A summary of the purification of rat cerebral choline acetyltransferase is given in Table I. The final purity of the enzyme, as revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, is shown in Figure 3 (inset). The protein corresponding to a molecular weight of 66 000 (arrow) was assumed to be choline acetyltransferase since gel filtration studies have indicated that the molecular weight of the native enzyme was 65 000 (Ryan, 1976). Although the gel was underloaded, the presence of two staining components in addition to the major band at 65 000 daltons indicates that the enzyme is not homogeneous. By integrating the area under these peaks, the choline acetyltransferase peak was estimated to be ~68% of the total stained material.

Purification of Choline Acetyltransferase from Bovine Caudate Nuclei. Fresh beef brains were obtained from Royal Packing Co. (St. Louis, MO). Caudate nuclei were dissected free immediately after death, frozen in powdered dry ice, and stored at −20 °C. Normally, 250 g of caudate nuclei was used for a preparation. The initial solubilization, acetic acid fractionation, and ammonium sulfate precipitation of the bovine caudate enzyme were carried out exactly as described for the rat brain acetylase.

CM-Sephadex Chromatography. The dialyzed solution of

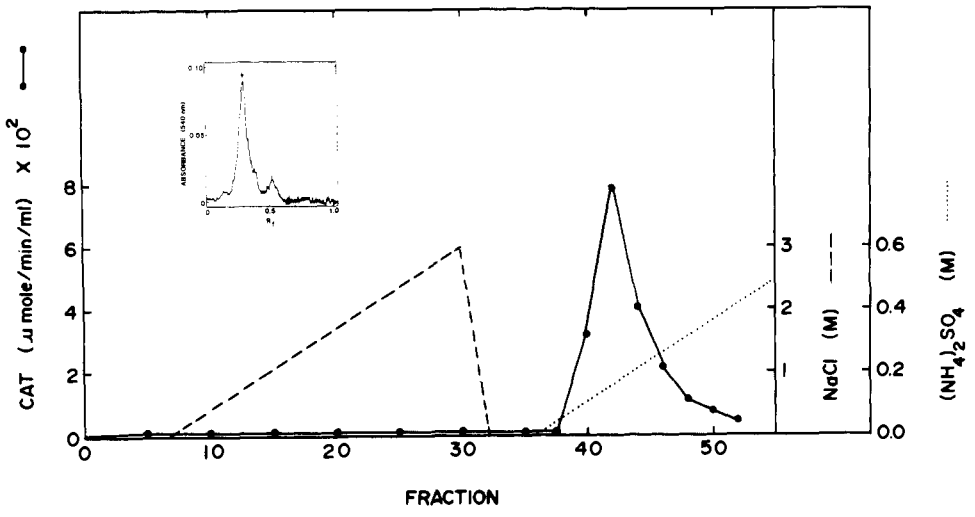

FIGURE 3: Chromatography of rat choline acetyltransferase on hydroxylapatite. Following CoA-S chromatography, the pool of enzyme activity was made 10% in sucrose (w/v) and applied directly to a 1.6 × 5.0 cm column of hydroxylapatite. A gradient of NaCl (---) in PEDS buffer was used to wash the column. Choline acetyltransferase was eluted by a linear gradient of $(NH_4)_2SO_4$ in 100 mM potassium phosphate, 1 mM EDTA, 0.5 mM dithioerythritol, and 10% sucrose, pH 7.2 (···). Enzymatic activity was measured by assay I (●). Inset: gel electrophoresis of rat choline acetyltransferase following hydroxylapatite chromatography. Electrophoresis on polyacrylamide gels in sodium dodecyl sulfate was carried out as described under Materials and Methods. An entire pooled sample from one hydroxylapatite column was applied to a 10% gel. The component at 66 000 daltons (arrow) was assumed to be choline acetyltransferase, on the basis of gel filtration studies with the native enzyme (Ryan, 1976). Using standards of BSA stained with Coomassie blue, it was estimated that the 66 000-dalton component represented ca. 2 to 3 μg of protein.

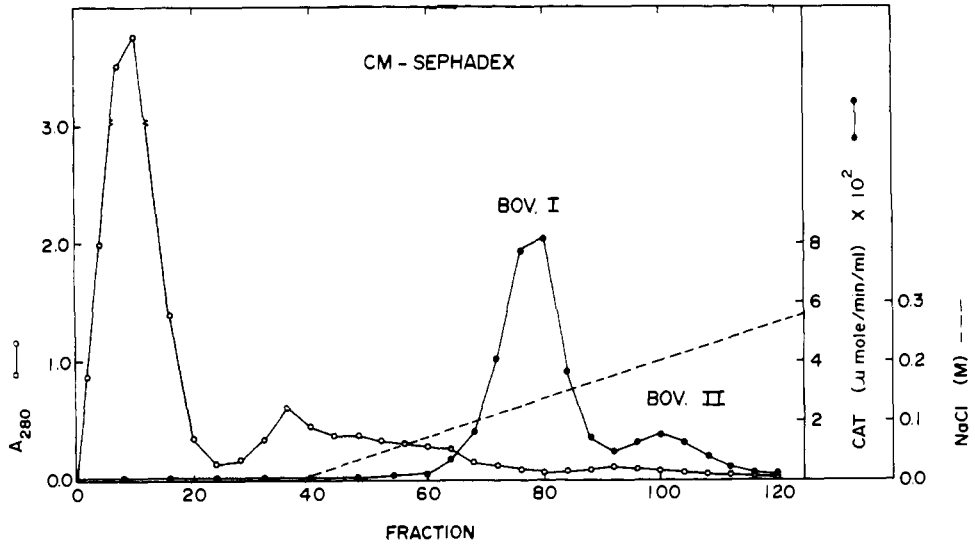

FIGURE 4: Chromatography of bovine choline acetyltransferase on CM-Sephadex C-50. The dialyzed solution of the enzyme from step 3 (~120 mL) was chromatographed on a 2.5 × 16 cm column of CM-Sephadex. The enzyme was eluted with a linear gradient of NaCl (---) in CPED buffer. Enzymatic activity was measured by assay I (●). Protein was monitored by absorbance at 280 nm (○). The two peaks of enzyme activity eluted from the column were defined as Bov I and Bov II, in the order of their emergence.

enzyme after ammonium sulfate precipitation was applied to a CM-Sephadex C-50 column preequilibrated with CPED buffer. After being washed with 30–35 mL of CPED buffer, the enzyme was eluted with a linear gradient from 0 to 0.3 M NaCl in CPED buffer at a flow rate of 20 mL/h (Figure 4). The total volume of the gradient was 700 mL. Enzymatic activity was detected in two peaks, the first (75–80% of the total activity) of which eluted between 0.09–0.17 M NaCl and the second of which eluted between 0.17–0.23 M NaCl. The two peaks will be referred to as bovine I and II (Bov I and Bov II), respectively, in the order of their elution. In an effort to maintain the identity of each peak, we discarded fractions in the trough between Bov I and II when fractions were pooled.

Bov I was concentrated by pressure dialysis (N_2, 3 psi) using a 200-mL Amicon ultrafiltration reservoir and a PM-10 membrane. During the process, most of the NaCl was removed. After a reduction in volume from 170 to 35 mL, choline chloride was added to a final concentration of 100 mM. Aliquots containing no more than 2.0 units of enzymatic activity were frozen at −20 °C. Preparations of Bov II were concentrated (about threefold) and stored in a similar fashion.

Affinity Chromatography of Bov I on CoA-Sepharose. A thawed aliquot of Bov I (less than 2 units) was applied to a 10–12-mL CoA-S column which had been preequilibrated with CPED buffer containing 100 mM choline chloride. After being washed with 35–40 mL of CPED + C buffer, the enzyme was eluted at 10 mL/h with a linear gradient from 60 mL of CPED + C buffer to 60 mL of 1.5 M NaCl in CPED + C buffer (Figure 5). Enzyme activity eluted in a symmetrical peak centered at 0.38 M NaCl. The peak fractions, which contained 75–80% of the eluted activity, were used immediately in the next purification step.

Hydroxylapatite Chromatography of Bov I. Active fractions from the CoA-S column were diluted with an equal

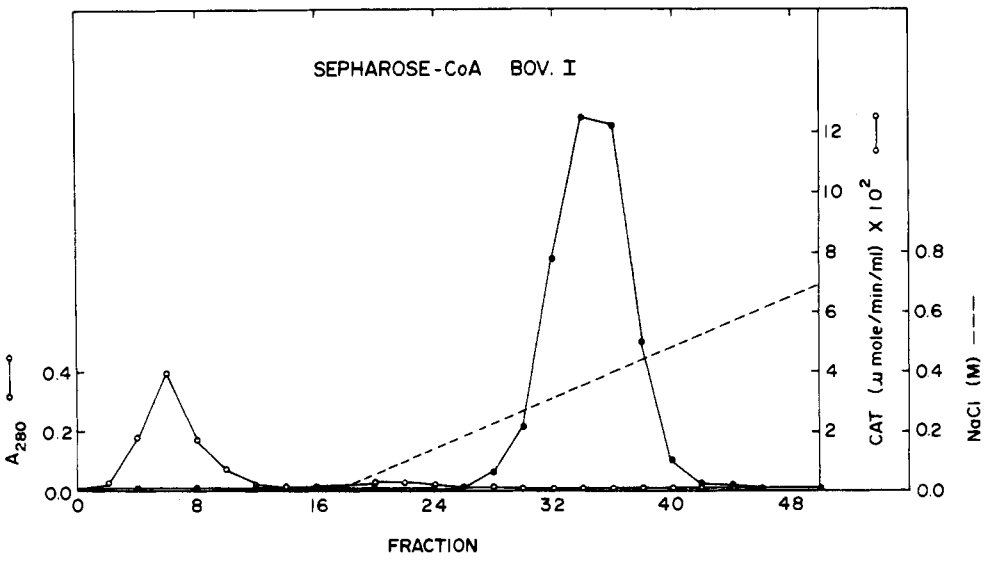

FIGURE 5: Affinity chromatography of Bov I on CoA–Sepharose. An aliquot of the Bov I pool from CM-Sephadex (2 units or less) was chromatographed on a 1.9 × 3.8 cm column of CoA–Sepharose. The enzyme was eluted with a linear gradient of NaCl (---) in CPED buffer containing 100 mM choline. Enzymatic activity was measured by assay I (●). Protein was monitored by absorbance at 280 nm (○).

Table II: Stability to Storage and Dialysis of Rat Choline Acetyltransferase following Hydroxylapatite Chromatography[a]

expt	BSA (mg/mL)	temp (°C)	act. after 24 h (% of initial)
storage of enzyme	0	4	50
	0	−20	6
	0	−70	3
	0.5	4	91
	0.5	−20	86
	0.5	−70	58
dialysis of enzyme, 3 h in tubing A[b]	0	4	29
dialysis of enzyme, 3 h in tubing B[c]	0	4	6
dialysis of enzyme, 1.5 h in tubing A[b]	1.5	4	67
	1.5	4	88

[a] Experimental details are given in the text. Samples were stored for 24 h at the temperature given in the solution in which they emerged from the hydroxylapatite column. Dialysis was carried out as stated vs. a solution of 100 mM potassium phosphate, 1 mM EDTA, 0.5 mM dithioerythritol, and 10% sucrose, pH 7.2. [b] Dialysis tubing A: Union Carbide No. 24. [c] Dialysis tubing B: Spectrapore No. 1.

volume of 10 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.2 mM dithioerythritol, and 30% glycerol. A stabilizing effect of glycerol on bovine choline acetyltransferase has been observed by others (Chao & Wolfgram, 1973; Roskoski et al., 1975). The diluted fractions were then applied to a hydroxylapatite (Clarkson) column (1 × 6 cm) preequilibrated with 10 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 0.2 mM dithioerythritol, and 15% glycerol (PED + G buffer). The fractions were diluted and applied to the column one at a time because of the instability upon dilution of choline acetyltransferase at this stage of purification. Following application of the samples, the column was washed with a 60-mL linear gradient of 0–1.0 M NaCl in PED + G buffer to remove inert proteins. Enzymatic activity was then eluted with a linear gradient from 50 mL of PED + G buffer to 50 mL of 100 mM potassium phosphate, 1 mM EDTA, 0.2 mM dithioerythritol, and 15% glycerol in 1 M $(NH_4)_2SO_4$, pH 7.2 (Figure 6). Fractions which eluted between 0.1 and 0.3 M ammonium sulfate were pooled. Recoveries of activity were ~50%. The lability of Bov I to freezing and dialysis was very similar to that of rat choline

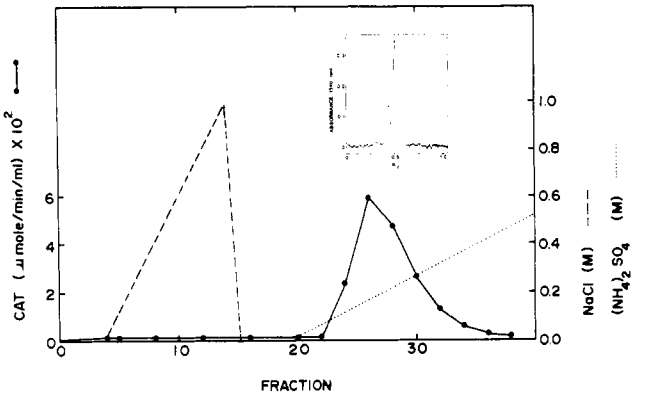

FIGURE 6: Chromatography of Bov I on hydroxylapatite. Following affinity chromatography on CoA–S, the pooled activity was diluted 1:1 with PED buffer containing 30% glycerol and applied directly to a column of hydroxylapatite. The loaded column was washed with a linear gradient of NaCl (---) in the same buffer. Choline acetyltransferase was then eluted with a linear gradient of $(NH_4)_2SO_4$ in 100 mM potassium phosphate, 1 mM EDTA, 1.0 mM dithioerythritol, and 15% glycerol, pH 7.2 (···). Enzymatic activity was measured by assay I (●). Inset: gel electrophoresis of Bov I following chromatography on hydroxylapatite. Electrophoresis was carried out as described under Materials and Methods, using 4.5% gels of polyacrylamide in sodium dodecyl sulfate. An entire pool from one hydroxylapatite column was applied to the gel. The component at 70 000 daltons (arrow) was assumed to be choline acetyltransferase (Ryan, 1976). Using standards of BSA stained with Coomassie blue, it was estimated that the 70 000-dalton component represented ca. 5 to 6 μg of protein.

acetyltransferase. Like the rat enzyme, Bov I could be stabilized by BSA, which was routinely added at this stage to a final concentration of 0.5 mg/mL. Those preparations to be used for gel electrophoresis were examined without the addition of BSA.

Affinity Chromatography of Bov II on CoA–Sepharose. The concentrated pool from CM-Sephadex was thawed and applied to a CoA–S column which had been preequilibrated with CPED buffer containing 100 mM choline chloride. The loaded column was then washed with 35–40 mL of the equilibration buffer before elution of the enzyme was carried out at 10 mL/h with a 120-mL linear gradient of 0–1.5 M NaCl in CPED buffer containing 100 mM choline chloride. Enzyme activity appeared in the gradient at 0.38 M NaCl (Figure 7), almost identical with Bov I. The most active

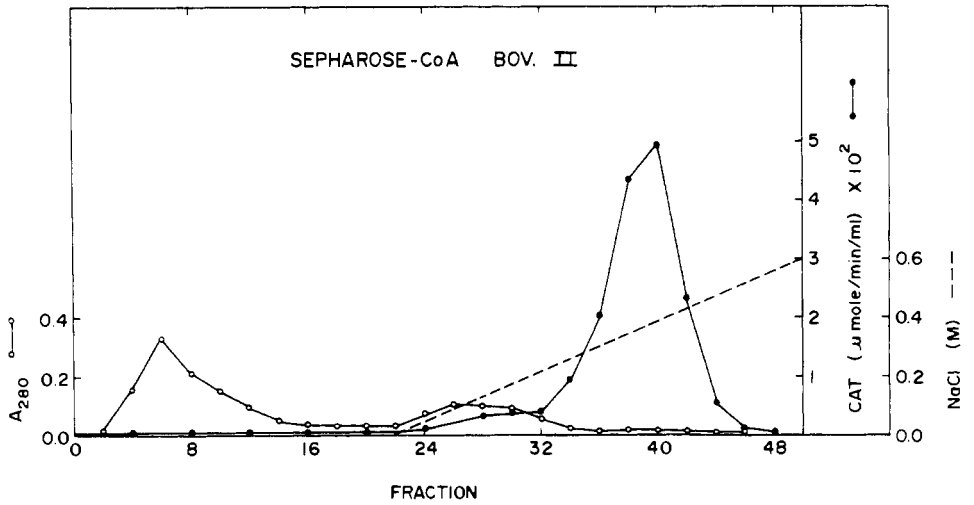

FIGURE 7: Chromatography of Bov II on CoA–Sepharose. The entire pool of Bov II from one column of CM-Sephadex was chromatographed on a column of CoA–Sepharose. The enzyme was eluted with a linear gradient of NaCl in CPED buffer (---) containing 100 mM choline. Choline acetyltransferase was measured by assay I (●). Protein was monitored by absorbance at 280 nm (○).

Table III: Purification of Choline Acetyltransferase from Bovine Caudate Nuclei[a]

step	vol (mL)	protein (mg)	total act. (μmol min^{-1})	sp act. (μmol min^{-1} mg^{-1})	yield (%)	purifn (x-fold)
crude supernatant	1753	5469	13.7	0.0025	100	1
pH 4.5 supernatant	1712	2465	11.9	0.0048	87	1.9
40–60% ammonium sulfate fraction	115	627	10.6	0.0169	77	6.8
CM-Sephadex: I	28	23.5	6.8	0.289	49	116
II	17.6	15.7	0.88	0.050	6	20
Sepharose-CoA: I	*b*	0.354	5.96	16.8	43	6720
II	*b*	0.279	0.67	2.4	4.5	960
hydroxylapatite (I)	*b*	*c*	2.74	54	18	21500

[a] Details of the experimental procedures are presented in the text. The purification was carried out from 250 g of caudate nuclei. Chromatography on CM-Sephadex results in two peaks of activity, referred to here as I and II, in order of their elution. The purification and yield of Bov II are expressed with respect to the crude supernatant. [b] The volumes of the choline acetyltransferase pools after affinity chromatography and hydroxylapatite chromatography were omitted because variable portions of the CM-Sephadex pools were applied to the CoA-Sepharose column as needed. The total yield was calculated from the sums of the recovered choline acetyltransferase from each hydroxylapatite pool. [c] Protein could not be chemically determined after hydroxylapatite chromatography (see Materials and Methods).

fractions were pooled and stored on ice. Recoveries from this step averaged 75%. Because the quantity of Bov II was small with respect to Bov I, further purification was not rigorously attempted at this time.

The purification of Bov I and Bov II is summarized in Table III. Polyacrylamide gel electrophoresis of Bov I in the presence of sodium dodecyl sulfate revealed the presence of one major and one minor band (Figure 6, inset). Since gel filtration experiments indicated that the molecular weight of native Bov I was 69 000, the protein peak with a molecular weight of 70 000 (arrow) was assumed to be choline acetyltransferase. From the areas of the peaks of stained protein, Bov I was estimated to be 80% pure (see Materials and Methods).

Discussion

The purification procedure described under Results has consistently yielded preparations of mammalian brain choline acetyltransferase with very high specific activities. This is due largely to the development of an affinity chromatography step with high yield. Both the bovine and rat enzymes were bound to the affinity column with equal effectiveness.

Coenzyme A proved to be an excellent affinity ligand when covalently attached to Sepharose 4B without the use of any spacer group. In fact, the addition of a 10-Å aliphatic spacer between the coenzyme and the solid support resulted in a much less efficient purification of the enzyme (Ryan, 1976). This may be attributed, in part, to hydrophobic interactions with the spacer arm. Hydrophobic interactions between choline acetylase and affinity matrices have also been observed by others (Mautner & Currier, 1977; Malthe-Sørenssen et al., 1978). In support of this argument, the enzyme bound tightly to *n*-heptyl aliphatic chains covalently linked to Sepharose 4B (unpublished experiments). In addition, it is possible that proteins other than choline acetyltransferase could interact with the spacer and prevent efficient binding of the acetylase to the CoA ligand.

In early experiments, solutions of enzyme were applied to the CoA–S column in buffer containing no choline chloride. An improved purification was obtained by including choline in the elution buffer. The effect of choline was maximal at concentrations around 30 mM, but increasing the concentration above 100 mM was not detrimental. Because of the greater ionic strength, 100 mM choline was used for elution of the enzyme. Washing the loaded CoA–Sepharose column with the buffer containing choline released much more nonspecifically bound protein than did the buffer alone, yet no enzymatic activity was liberated. Because of the choline effect, the possibility of eluting the S–CoA column with choline chloride instead of NaCl was tested. However, analysis by polyacrylamide gel electrophoresis showed that the enzyme eluted with choline alone did not differ from that eluted with buffers containing both NaCl and choline.

Of major importance, from a preparative standpoint, is the observation that a large loss of enzymatic activity occurred

if all the material from a 250-g preparation was applied to the affinity column. We have no documented explanation for this phenomenon, and it was not further investigated. The problem was circumvented by simply adding less than 2 units of the enzyme to the column. This amount of activity would be obtained from 75 to 100 g of starting material. Because many physical, chemical, and immunological characterizations of choline acetyltransferase will require milligram quantities of the enzyme, efforts are currently underway to find methods more suitable for large-scale separations.

The characteristics of the hydroxylapatite chromatography presented here differ considerably from those previously reported. For example, Malthe-Sørenssen (1975) used a gradient of 0–0.5 M sodium chloride to elute bovine caudate nuclei choline acetyltransferase from hydroxylapatite, while we found that even 1.0 M NaCl would not elute the enzyme. Several differences between the two procedures could account for the discrepancy. Different conditions of buffers, solvent, and pH were employed and could account for elution of the activity at different concentrations of NaCl. In particular, the instability of choline acetyltransferase following affinity chromatography on CoA–S necessitated the use of glycerol (Bov I) and sucrose (rat) for the ensuing chromatography on hydroxylapatite. Neither was used by Malthe-Sørenssen. In addition, the present procedure utilizes a commercial hydroxylapatite different than that used by Malthe-Sørenssen. Finally, the purity of the Bov I enzyme which was subjected to hydroxylapatite chromatography in the present work was at least 15-fold greater than that used by Malthe-Sørenssen. It is possible that the presence or absence of proteins other than choline acetyltransferase in the two preparations may well influence the chromatographic properties of the enzyme. In support of this argument, our efforts to reproduce the hydroxylapatite chromatography described by Malthe-Sørenssen (1975), using Bov I choline acetyltransferase purified through the CoA–S step, were unsuccessful. The use of hydroxylapatite chromatography has also been previously described by Malthe-Sørenssen et al. (1973) in the purification of the rat enzyme. In this case also, the purification used conditions of pH, solvent, and enzyme purity which differed from those described for this purification, as well as employing a different commercial preparation of hydroxylapatite. Efforts to reproduce the hydroxylapatite chromatography of Malthe-Sørenssen et al. (1973) with rat choline acetyltransferase purified through the CoA–S step were unsuccessful.

The separation of the activity of the bovine enzyme into two peaks following CM-50 chromatography has been previously described by Malthe-Sørenssen (1975). In addition, isoelectric focusing of Bov II (peak B in Malthe-Sørenssen's notation) resulted in the further separation of two peaks of activity. We have not attempted further purification of Bov II. We have observed in a preliminary experiment, however, that Bov II appears to separate into two peaks of activity upon hydroxylapatite chromatography. It is tempting to speculate that the separated activities may correspond to the two species observed by Malthe-Sørenssen. Verification of this conjecture will necessarily require purification of the individual active species and determination of their isoelectric points.

It should be emphasized that the final specific activities of both highly purified rat and Bov I choline acetyltransferases are indirect estimates. Furthermore, the estimates are based upon densitometer tracings of polyacrylamide gels which were, by necessity, loaded with minimal amounts of protein. Following affinity chromatography, the specific activities of both the rat and Bov I enzymes were determined by direct chemical analyses which are quite precise. Even at this early stage, it is apparent that Bov I has a specific activity considerably greater than those values reported for bovine choline acetyltransferase which was previously considered homogeneous (Chao & Wolfgram, 1973; Roskoski et al., 1975). In the present case, the purity of the enzyme was judged to be ~25% at this stage of the preparation. Similarly, the rat enzyme was estimated to be only 10–13% pure after chromatography on CoA–Sepharose.

Even though the final specific activities were indirectly determined, the predicted value for the specific activity of a homogeneous preparation of either the rat or Bov I enzyme would be 60–70 μmol min^{-1} mg^{-1}. This value is in close agreement with the specific activity obtained for the nearly homogeneous enzyme from squid head ganglia (Husain & Mautner, 1973). Because these preparations are from quite different species, this coincidence may be fortuitous. Rossier (1976b) has recently purified rat choline acetyltransferase to a specific activity of 20 μmol min^{-1} mg^{-1} by utilizing immunoabsorbent chromatography. This preparation was estimated by gel electrophoresis in sodium dodecyl sulfate to be ~20% pure. Therefore, a specific activity of ~100 μmol min^{-1} mg^{-1} would correspond to a homogeneous choline acetyltransferase preparation. While this estimate of specific activity is greater than the one presented here, both estimates might well be within experimental error. In any case, additional purification will be required to attain homogeneous enzyme.

In contrast to these results, several investigators have claimed to have purified mammalian brain choline acetyltransferase to electrophoretic homogeneity (Chao & Wolfgram, 1973; Roskoski et al., 1975; Singh & McGeer, 1974). The specific activities of these preparations have varied from 0.015 to 2.5 μmol min^{-1} mg^{-1}. The data presented by some of these investigators indicate that a protein has indeed been purified to homogeneity. In light of the evidence presented here and elsewhere (Rossier, 1976a,b), it appears that choline acetyltransferase is present as a minor contaminant in a nearly homogeneous preparation of some other protein. Physical data and immunohistochemical localizations (Chao, 1975; McGeer et al., 1974; Rossier, 1976c; Roskoski et al., 1975) obtained with these preparations must be judiciously interpreted.

Acknowledgments

The authors thank Professor Lowell P. Hager for personal support throughout this research and Professor Bonita Katzenellenbogen for generous supplies of starting materials.

References

Anderson, L. E., & McClure, W. O. (1973) *Anal. Biochem. 51*, 173–179.

Chao, L.-P. (1975) *J. Neurochem. 25*, 261–266.

Chao, L.-P., & Wolfgram, F. (1973) *J. Neurochem. 20*, 1075–1081.

Chibata, I., Tosa, T., & Matuo, Y. (1974) *Methods Enzymol. 34*, 267–271.

Eng, L. F., Uyeda, C. T., Chao, L.-P., & Wolfgram, F. (1974) *Nature (London) 250*, 243–245.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fonnum, F. (1969) *Biochem. J. 115*, 465–472.

Glover, V. A. S., & Potter, L. T. (1971) *J. Neurochem. 18*, 571–580.

Husain, S. S., & Mautner, H. G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3749–3753.

Jones, D. H., & Nelson, W. L. (1968) *Anal. Biochem. 26*, 350–357.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Malthe-Sørenssen, D. (1975) in *Cholinergic Mechanisms* (Waser, P. G., Ed.) pp 257–262, Raven Press, New York.
Malthe-Sørenssen, D., Eskeland, T., & Fonnum, F. (1973) *Brain Res. 62*, 517–522.
Malthe-Sørenssen, D., Lea, T., Fonnum, F., & Eskeland, T. (1978) *J. Neurochem. 30*, 35–46.
March, S., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Mautner, H. G. (1977) *CRC Crit. Rev. Biochem.*, 341.
Mautner, H. G., & Currier, S. F. (1977) in *Cholinergic Mechanisms and Psychopharmacology* (Jenden, D. J., Ed.) pp 197–206, Plenum Press, New York.
McGeer, P. L., McGeer, E. G., Singh, V. K., & Chase, W. H. (1974) *Brain Res. 81*, 373–379.
Oldham, K. G. (1968) *Radiochemical Methods of Enzyme Assay: Review 9*, Amersham Buchs, The Radiochemical Center.
Roskoski, R., Jr., Lim, C.-T., & Roskoski, L. M. (1975) *Biochemistry 14*, 5105–5110.
Rossier, J. (1975) *Brain Res. 98*, 619–622.
Rossier, J. (1976a) *J. Neurochem. 26*, 543–548.
Rossier, J. (1976b) *J. Neurochem. 26*, 549–554.
Rossier, J. (1976c) *J. Neurochem. 26*, 555–559.
Ryan, R. L. (1976) Ph.D. Thesis, University of Illinois.
Ryan, R. L., & McClure, W. O. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 1647.
Schrier, B. K., & Schuster, L. (1967) *J. Neurochem. 14*, 977–985.
Schuster, L., & O'Toole, C. (1974) *Life Sci. 15*, 645–656.
Simon, J., & Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.
Singh, V. K., & McGeer, P. L. (1974) *Life Sci. 15*, 901–913.
Stadtman, E. R. (1975) *Methods Enzymol. 3*, 935–938.
Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leingruber, W., & Weigele, M. (1972) *Science 178*, 871–872.
Wenthold, R. L., & Mahler, H. R. (1975) *J. Neurochem. 24*, 963–967.

Partial Purification and Characterization of a Protein Lysine Methyltransferase from Plasmodia of *Physarum polycephalum*†

Malabi Venkatesan and I. Rosabelle McManus*

ABSTRACT: Plasmodia of *Physarum polycephalum* have an active protein lysine methyltransferase (*S*-adenosyl-L-methionine:protein-lysine methyltransferase, EC 2.1.1.43). This enzyme has been purified 40-fold with a 13% yield, and it catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine to the ε-amino group of lysine residues with formation of N^{ϵ}-mono-, N^{ϵ}-di-, and N^{ϵ}-trimethyllysines in a molar ratio of 4:1:1 based on [^{14}C]methyl incorporation into the methylated lysines. The ratio remains unchanged at all stages of the partial purification, as well as after fractionation by sucrose density gradient centrifugation and gel electrophoresis. The rate of protein methylation is time dependent, enzyme concentration dependent, and requires the presence of a sulfhydryl reducing agent for optimal activity. The enzyme has optimal activity at pH 8 and is inhibited by *S*-adenosyl-L-homocysteine and EDTA. Lysine-rich and arginine-rich histones serve as the most effective exogenous protein acceptors; *P. polycephalum* actomyosin is inactive, and chick skeletal myofibrillar proteins are 25% as effective as exogeneous mixed histones as substrates. Lysine, polylysine, ribonuclease A, cytochrome *c*, and bovine serum albumin are not methylated.

Protein methylation is observed in a wide variety of eucaryotic tissues, and many classes of proteins can be methylated, including actomyosins, histones, some ribosomal proteins, fungal and wheat germ cytochrome *c*, flagellin, and opsin (Paik & Kim, 1971, 1975). Protein methyltransferases specific for the N-methylation of lysine residues using *S*-adenosyl-L-methionine as the methyl donor have a wide distribution with the enzyme often associated with nuclei (Kim & Paik, 1965) and utilizing histones (Paik & Kim, 1970) as the preferred protein methyl acceptor. Nochumson et al. (1977) and Durban et al. (1978) have described the purification and characterization of an apparently soluble protein lysine methyltransferase from *Neurospora crassa* that preferentially uses unmethylated horse heart cytochrome *c* as a methyl acceptor.

We have shown previously that myosin prepared from plasmodia of *Physarum polycephalum* contains Lys(Me),[1] Lys(Me_2), Lys(Me_3), and Arg(G-Me_2) in a molar ratio of 1:1:2:2. *P. polycephalum* actin contains predominantly His-(3-Me) and small amounts of Lys(Me) and Lys(Me_2) (Venkatesan et al., 1975). The occurrence of these methylated basic amino acid residues in the contractile proteins of *P. polycephalum* encouraged us to examine further the methylation reactions in *P. polycephalum*. This paper describes the partial purification and characterization of a soluble enzyme which synthesizes protein-bound Lys(Me), Lys(Me_2), and Lys(Me_3) in a ratio of 4:1:1. A preliminary report of some of this work

† From the Department of Biological Sciences, Faculty of Arts and Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received May 31, 1978; revised manuscript received August 3, 1979.* This investigation was supported by research funds provided through grants-in-aid from the National Institutes of Health (AM-15826), the Muscular Dystrophy Association of America, and The United Way Health Research and Services Foundation to I.R.M.

[1] Abbreviations used: Arg(G-Me), N^{G}-monomethylarginine; Arg(G-Me_2), N^{G}-dimethylarginine and $N^{G},N^{G'}$-dimethylarginine; DATD, diallyltartardiamide; His(3-Me), N^{3}-methylhistidine; MBAA, methylated basic amino acids; Lys(Me), N^{ϵ}-monomethyllysine; Lys(Me_2), N^{ϵ}-dimethyllysine; Lys(Me_3), N^{ϵ}-trimethyllysine; SAM, *S*-adenosyl-L-methionine.

0006-2960/79/0418-5365$01.00/0