Acetyl-CoA:choline O-acetyltransferase (EC 2.3.1.6) has been purified approximately 17,000-fold from rat brain. The purification protocol included acid and NH₄SO₄ precipitation, followed by chromatography on CM-Sephadex, phenyl-Sepharose, Sephadex G-150, and blue dextran Sepharose. Two peaks of enzyme activity were detected after blue dextran Sepharose chromatography containing an overall yield of approximately 4% of the starting enzyme activity. The final specific activity of the blue dextran fractions was 70 to 80 μmol of acetylcholine formed min⁻¹ mg⁻¹ of protein⁻¹. Sodium dodecyl sulfate electrophoresis of the blue dextran fractions revealed three closely spaced proteins with a molecular weight of approximately 87,000. Tryptic peptide maps were prepared for each of the gel bands and indicated that they contained nearly identical primary structure.

Choline acetyltransferase¹ is the biosynthetic enzyme responsible for production of the important central and peripheral neurotransmitter acetylcholine. The enzyme is widely distributed in neural and non-neural tissue from a variety of species and has been the subject of several recent reviews (1-3).

Mammalian choline acetyltransferase has been prepared to varying degrees of purity by several groups. A wide and often conflicting range of molecular, kinetic, and immunological properties has been reported by various laboratories for the mammalian brain enzyme which has resulted in considerable controversy (4, 5). Several groups have reported electrophoretically homogeneous choline acetyltransferase preparations from bovine (6), human (7), and mouse (5) brain. The specific activities of these preparations are low (0.012 to 1.45 μmol of acetylcholine produced min⁻¹ mg⁻¹) compared to other studies where electrophoretic heterogeneity has been reported. Rosier (8) and Ryan and McClure (9) have prepared partially purified choline acetyltransferase from rat brain with specific activities of 20 and 40 μmol min⁻¹ mg⁻¹ and estimated a purity of 20 to 68% by scanning Coomassie blue-stained polyacrylamide gels. Malthe-Sörenssen et al. (10) and Ryan and McClure (9) have prepared choline acetyltransferase from bovine caudate nuclei to specific activities of 25 to 58.8 μmol min⁻¹ mg⁻¹, with the latter group estimating 80% purity. In addition, Malthe-Sörenssen et al. (10) and Rosier (11) have demonstrated immunological heterogeneity for their enzyme preparations while Singh and McGeer (7) and Chao (5) have claimed production of monospecific antiserum to low specific activity choline acetyltransferase preparations.

Several groups have observed multiple molecular forms of mammalian brain choline acetyltransferase. Malthe-Sörenssen and Fonnum (12) have reported three stable isoelectric focusing forms of rat brain choline acetyltransferase (pI = 7.4, 7.8, and 8.3). Froissart et al. (13) have recently reported multiple isoelectric focusing forms of rat brain choline acetyltransferase in crude homogenates and in addition have shown a shift to a predominately low pI (6.3) form at high protein concentrations, which they have attributed to aggregation. Malthe-Sörenssen et al. (10) and Ryan and McClure (9) have demonstrated two forms of choline acetyltransferase from bovine caudate following chromatography on CM-Sephadex. Chao (5), on the other hand, has not observed any multiple forms of choline acetyltransferase from mouse brain following purification and has suggested that multiple forms or aggregates of choline acetyltransferase may be induced artifactualy following ammonium sulfate fractionation. Malthe-Sörenssen et al. (10) and Ryan and McClure (9) have not confirmed this observation. It is interesting to note that Chao and Wolfram (6) have estimated a molecular weight of 120,000 for native choline acetyltransferase which dissociated into a complex mixture of subunits in its denatured state, while several other investigators have observed a molecular weight for denatured and native choline acetyltransferase of 60,000 to 70,000 (10, 14, 15, 16).

Our eventual goal is to obtain monospecific antibodies to rat brain choline acetyltransferase to use in immunocytochemical mapping of cholinergic pathways in rodent brain. In order to achieve this goal, a highly purified, well characterized antigen is essential. With this consideration in mind, and in view of the conflicting reports on the molecular nature of mammalian brain choline acetyltransferase, we have developed a procedure for the large scale purification of essentially homogeneous rat brain enzyme. Our purification scheme includes preparative hydrophobic chromatography as well as blue dextran affinity chromatography. We have observed three protein bands after polyacrylamide gel electrophoresis of the final enzyme product in SDS and have characterized them with respect to their tryptic peptide maps.

¹This research was supported by Grant NS 12116 from the National Institutes of Health, National Institute of Neurological and Communicative Disorders and Stroke. This study was presented in part at the Ninth Annual Meeting of the Society for Neuroscience, Atlanta, GA, November, 1979. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

²To whom reprint requests should be addressed.

The abbreviations used are: choline acetyltransferase, acetyl-CoA:choline O-acetyltransferase (EC 2.3.1.6); SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; Con A, concanavalin A.
Rat Brain Choline Acetyltransferase

EXPERIMENTAL PROCEDURES

1. Preparation of Rat Brain Homogenate: The procedure followed was essentially that of Lowry et al. (1). The brain was removed without cooling immediately following death and transferred to a chilled glass homogenizer. The brain was stored in a chilled glass homogenizer at 0°C. The brain was added to the chilled glass homogenizer at 0°C. The brain was then flushed with ice-cold saline and the tissues were homogenized in 4 volumes of 0.01 M phosphate buffer, pH 7.0. The homogenate was then centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

2. Gel Filtration: The tissue homogenate was centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

3. Affinity Chromatography: The tissue homogenate was centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

4. Isoelectric Focusing: The tissue homogenate was centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

5. Disc Electrophoresis: The tissue homogenate was centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

6. Rat Brain Choline Acetyltransferase: The tissue homogenate was centrifuged at 300,000 g for 2 hours at 0°C. The supernatant fraction was used for the preparation of the tissue homogenate.

7. Portions of this paper (including “Experimental Procedures,” Figs. 1 to 4, and additional references) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9605 Rockville Pike, Bethesda, Maryland 20014. Request Document No. 80M-910, cite authors, and include a check or money order for $1.20 per set of photocopies. Full-size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
### Table I

**Summary of rat brain choline acetyltransferase purification**

Data are from a single 150-rat brain preparation. Details of the methods and column elution profiles are included in the miniprint supplement.

| Step | Volume | Total protein | Total activity | Specific activity | Overall yield | Purification |
|------|--------|---------------|----------------|-------------------|---------------|--------------|
| 1.   | ml     | mg            | μmol/min       | μmol/min/mg protein | %             | fold         |
| Crude supernatant | 1,380 | 7,314 | 31.9 | 0.0044 | 87 | 1.4 |
| pH 4.5 supernatant | 1,390 | 4,448 | 27.7 | 0.0062 | 78 | 5.2 |
| 3.   | Dialyzed 40 to 60% (NH₄)₂SO₄ precipitate | 33 | 1,096 | 24.8 | 0.023 | 45 | 56.8 |
| Concentrated CM-Sephadex pool | 30 | 56.7 | 14.3 | 0.25 | 463 |
| Concentrated phenyl-Sepharose pool | 1.45 | 7.25 | 5.5 | 0.76 | 7.2 |
| Concentrated Sephadex G-150 pool | 2.6 | 1.12 | 2.28 | 4.13 | 173 |
| Blue dextran Pool I | 1.0 | 0.012² | 0.94 | 80 | 4² | 18,000 |
| Blue dextran Pool II | 1.8 | 0.0025 | 0.22 | 73 | 16,600 |

⁴ Protein estimate from SDS-gel electrophoresis and comparing Coomassie blue staining intensity with a known amount of bovine serum albumin run on the same gel. See Fig. 6 for details.
³ Sum of yields for blue dextran Pools I and II.

**Results**

**Enzyme Purification.**—Table I is a summary of the results of a representative enzyme purification from 150 rat brains. Column elution profiles are included in the miniprint section. This protocol has been repeated several times with similar results. The final product following blue dextran chromatography was obtained in two fractions with similar specific activities. Taken together, they provide an overall yield of 4% with an average specific activity of 77 μmol/min/mg of protein (see below for estimation of protein concentration) and a purification factor of 17,000-fold.

The final products (blue dextran Pools I and II) separated into multiple bands upon SDS electrophoresis. Fig. 5 shows the pattern obtained with Pool I. Three closely spaced bands were observed with electrophoretic mobilities centering about that for bovine serum albumin (BSA) ($M_r = 67,000$). Pool II contained only Bands a and b (gel not shown).

Due to low yields, it was not possible to directly determine the protein concentration of the final fractions by conventional techniques. An estimate was made by running a series of dilutions of albumin on the same SDS gel along with the two enzyme fractions. The gel was stained with Coomassie Brilliant Blue R-250, as described in the miniprint supplement, and scanned with a Joyce, Loebel recording densitometer. The peaks were cut out and weighed on an analytical balance to determine relative areas. The values for BSA have been plotted as peak area, against the quantity of protein loaded on the gel. Arrows, peak areas of choline acetyltransferase Bands a, b, and c (see Fig. 5).

**Fig. 6.** Protein determination of choline acetyltransferase bands after blue dextran Sepharose chromatography. Along with the sample of blue dextran Sepharose Fraction I, shown in Fig. 5, a series of dilutions of BSA were run in alternate lanes on the same SDS gel. The gel was stained with Coomassie Brilliant Blue R-250, as described in the miniprint supplement, and scanned with a Joyce, Loebel recording densitometer. The peaks were cut out and weighed on an analytical balance to determine relative areas. The values for BSA have been plotted as peak area, against the quantity of protein loaded on the gel. Arrows, peak areas of choline acetyltransferase Bands a, b, and c (see Fig. 5). The pattern obtained with Pool I. Three closely spaced bands were observed with electrophoretic mobilities centering about that for bovine serum albumin (BSA) ($M_r = 67,000$). Pool II contained only Bands a and b (gel not shown).
Peptide Mapping—We have used a sensitive radioiodine-labeled peptide mapping technique to examine the primary sequence of the three SDS bands in blue dextran Pool I. Fig. 7, A, B, and C, shows the radioautographs of tryptic peptide maps of Bands a, b, and c from Fig. 5. All three peptide maps are quite similar with only minor differences and thus most likely represent different molecular forms of choline acetyltransferase. The peptide map for BSA run on the same gel is represented in Panel D and serves as a control for the experiment. It is clearly different from the three protein bands purified from rat brain, which therefore are neither rat serum albumin, a protein with similar electrophoretic mobility and primary structure to BSA, nor an artifact of the mapping procedure.

Stability of Enzyme—During purification, it has been noted that the enzyme becomes increasingly labile. Others have achieved partial stabilization of enzyme activity by the addition of exogenous protein such as BSA or small molecules such as sucrose or glycerol. We have had some success with ethylene glycol as a stabilizing agent, and our results are shown in Table II. At higher protein concentrations, the results are even better; e.g., a highly purified concentrated fraction from a phenyl-Sepharose column (0.46 mg/ml of protein, 35% ethylene glycol) lost less than 15% of its activity over a 9-day period on ice.

Hydrophobic Bonding—Initially, it was found that choline acetyltransferase bound to both octyl-Sepharose and phenyl-Sepharose. However, we were unable to recover activity from the octyl-Sepharose using a variety of elution conditions including low ionic strength and ethylene glycol. It was possible, however, to elute the enzyme from phenyl-Sepharose at high concentrations of ethylene glycol. Some of the conditions tested are shown in the experiment summarized in Table III. In this experiment, the enzyme was applied to the column and the elution conditions were tested sequentially. The enzyme was very stable when bound to the column, since almost 24 h elapsed between application and elution with a recovery of over 85% of the initial activity.

Lectin Studies—It was of interest to determine whether choline acetyltransferase is a glycoprotein. This could not be determined directly because of the small amount of choline acetyltransferase protein available; however, binding of the enzyme activity to lectin immobilized on agarose was studied. There was no binding of enzyme activity to either Sepharose-bound Con A or wheat germ agglutinin (data not shown).

### Table II
Partial stabilization of choline acetyltransferase activity with ethylene glycol

| Time after dilution | Buffer | Buffer + ethylene glycol |
|--------------------|--------|--------------------------|
| days               | %      |                          |
| 0                  | 100    | 100                      |
| 1                  | 16     | 74                       |
| 3                  | 0      | 54                       |
| 15                 | 0      | 35                       |

![Fig. 7. Peptide maps of the three protein bands obtained from the SDS-gel electrophoresis of purified choline acetyltransferase. Gel Bands a, b, and c from blue dextran Fraction I (Fig. 5) were treated as described in the miniprint supplement to prepare iodinated tryptic peptides. The peptide sample was applied as a spot in the lower right hand corner of the cellulose thin layer plate. Electrophoresis was from right to left, followed by chromatography from the bottom to the top of the plate (as described in the miniprint supplement). Panels A through C are, respectively, blue dextran Fraction I: Band a, 8 × 10⁶ cpm; Band b, 4 × 10⁶ cpm; Band c, 5 × 10⁵ cpm. Panel D is a map of BSA, 7 × 10⁵ cpm, run on the same SDS gel as the choline acetyltransferase proteins. Panels A, C, and D were exposed for 8 days, while Panel B was exposed for 3 weeks at room temperature. The asterisk in C represents a peptide missing from Band C.](image-url)
TABLE III

| Elution of choline acetyltransferase from phenyl-Sepharose |
|----------------------------------------------------------|
| Elution conditions | Recovery (% of activity loaded) |
|---------------------|--------------------------------|
| Flow through        | 0                              |
| pH 7.2 buffer<sup>4</sup> | 0                              |
| pH 4.9 buffer<sup>5</sup> | 0                              |
| pH 4.9 buffer 50% ethylene glycol | 0                              |
| pH 7.2 buffer 50% ethylene glycol | 86                            |

<sup>4</sup> Citrate (10 mM)/sodium phosphate buffer, pH 7.2, 0.1 mM dithioerythritol, 0.1 mM EDTA.
<sup>5</sup> Acetate (10 mM)/sodium phosphate buffer, pH 4.9, 0.1 mM dithioerythritol, 0.1 mM EDTA.

DISCUSSION

The application of conventional protein purification techniques has not been successful in yielding pure mammalian brain choline acetyltransferase, and so we have searched for other procedures. Hydrophobic chromatography and blue dextran chromatography are recent developments in protein purification and, in our hands, have proven useful for the purification of choline acetyltransferase. The mechanism and significance of protein binding to the active principle in blue dextran, Cibacron-Blue F3Ga, are still uncertain. When the dye is bound to dextran and coupled to agarose, a group-specific affinity resin is made which binds a variety of proteins, including choline acetyltransferase (16, 17). Most of these proteins share a common structural feature called the “denucleotide fold” (18), and the relationship of the dye structure to the structure of various nucleotide-containing enzyme substrates has been studied (19). When the dye is coupled directly to agarose, however, the specificity is relaxed somewhat, and it has been suggested that the structure of the matrix must have some effect, perhaps steric, on the binding specificity (19). There are two common means of eluting proteins from immobilized Cibacron-Blue F3Ga: elevated salt concentration and substrate-related compounds. We have found that, in addition, 50% ethylene glycol will elute choline acetyltransferase from a blue dextran column. This would seem to indicate that choline acetyltransferase binding to blue dextran involves a synergism between hydrophilic and hydrophobic bonds, and that it is sufficient to break one or the other in order to release the bound protein. Since hydrophobic bonds link choline acetyltransferase to phenyl-Sepharose, it became of interest to determine whether there was any biospecificity involved in this case, and we have attempted to elute a phenyl-Sepharose column with acetyl coenzyme A. Concentrations of the substrate up to 4 times that which eluted choline acetyltransferase from the blue dextran were ineffective. It is possible that the binding in this case involved hydrophobic sites on the enzyme, other than those involved in binding the substrate.

The absence of binding to wheat germ agglutinin or Con A makes it unlikely that rat brain choline acetyltransferase is a glycoprotein containing mannose or N-acetylglucosamine residues, but the enzyme could contain other carbohydrate residues.

Multiple forms of choline acetyltransferase from a variety of sources have been demonstrated by a variety of means; however, the molecular nature of this heterogeneity remains unknown. In this discussion, we shall restrict the subject to the rat brain enzyme. Isoelectric focusing has demonstrated at least three forms of the enzyme with the following pI values: pH 7.4 to 7.6, 7.7 to 7.9, and 8.3 (20). DEAE-cellulose chromatography can reportedly separate two forms of choline acetyltransferase (21), and these were shown to have similar molecular weights. Rossier, after polyacrylamide gel electroforesis at pH 4.5, was able to localize choline acetyltransferase activity to a region of the gel containing four protein bands. And finally, Wenthold and Mahler (22) report that chromatography at pH 6 on CM-Sephadex will separate out two peaks of choline acetyltransferase activity. In isoelectric focusing experiments, multiple forms of choline acetyltransferase may be true isoenzymes or, alternatively, they may result from artificial self-aggregation or interaction with other proteins at low ionic strength. For instance, choline acetyltransferase activity which focused at pH 8.3 under certain conditions has been observed to shift to a pI below 7 (20). This shift does not occur when the extract is pretreated to remove acidic proteins, and so it has been interpreted as binding of the choline acetyltransferase to an acidic protein. Others have also found changes in pI which were concentration-dependent and were likewise explained as aggregation phenomena (13). Our observation of three closely spaced protein bands following polyacrylamide gel electrophoresis in SDS indicates three independent molecular forms of protein, since the sample preparation was sufficient to disrupt any aggregation.

For a variety of reasons, it is important to obtain homogeneous choline acetyltransferase and to fully characterize the enzyme. To elicit monospecific antibodies, the immunizing preparation should contain only a single protein. No one has prepared mammalian enzyme which shows only a single band following SDS-gel electrophoresis and, indeed, we were not surprised when our preparation yielded multiple bands. Unfortunately, it has been impossible to recover enzyme activity from the SDS gel and relate it directly to the individual bands. The observation that the three bands we have observed in our final product share so much structure in common, as indicated by the peptide maps, provides strong evidence that they are all choline acetyltransferase isoenzymes.

It would seem more than coincidence that three charge isoenzymes have been described for rat brain choline acetyltransferase, and we purified three polypeptides with closely related SDS-gel electrophoresis mobilities. The relationship between our three bands and the isoelectric focusing forms remains to be investigated. There are at least three explanations for the origin of the multiple SDS gel bands. They may represent post-translational processing of the enzyme molecules or polypeptide chains coded for by different genes, or they may be an artifact resulting from proteolysis after disruption of the brain tissue. When rat brain choline acetyltransferase preparations are treated with trypsin, a new charge isoenzyme is generated (21). Determination of the exact nature of the heterogeneity we observe for rat brain choline acetyltransferase or its possible physiological significance must await further experiments.

Acknowledgments—We wish to thank Mr. Frank Sarinana, Mr. R. Slennon, and Dr. E. Roberts for encouragement and helpful discussions, and Ms. Jill Flanagan for preparing the manuscript.

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Additional references will be found on p. 10613.