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PREPARATION AND CHARACTERIZATION OF TWO ISOZYMES OF CHOLINE ACETYLTRANSFERASE FROM SQUID HEAD GANGLIA

I. PURIFICATION AND PROPERTIES

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Summary

Two isozymes of choline acetyltransferase (Acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) have been isolated and purified from squid head ganglia. Each isozyme contains multiple isoelectric forms with isoelectric points ranging from pH 5.0 to 6.2. The isozymes differ in their affinities for cellulose phosphate on column chromatography, as well as in their heat stabilities and in their capacities to be activated by salt. Both isozymes are stabilized by sucrose and by sulfhydryl-protecting reagents such as mercaptoethanol and dithiothreitol.

Introduction

Although the enzyme choline acetyltransferase (Acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) has been purified from diverse species [1—15], many of the characteristics of this enzyme remain confusing and obscure.

Considerable data pointing to the existence of multiple forms and/or isozymes of choline acetyltransferase have accumulated recently. Malthe-Sørenssen & Fonnum [16] separated the enzymes from both cat brain and rat brain into three distinct peaks of activity by isoelectric focusing, and White and Wu [9] showed multiple isoelectric forms of choline acetyltransferase exist in human brain. Both groups of workers suggest that the more acidic forms are aggregates, whereas the forms with the higher isoelectric points may be either isozymes [16] or conformational isomers [9]. Chao and Wolfgram [15] have shown that bovine brain choline acetyltransferase aggregates during $(NH_4)_2SO_4$ fractionation.

Prempeh et al. [17] have speculated that the peculiar heat denaturation and

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renaturation shown by choline acetyltransferase isolated from squid head ganglia implies the existence of isozymes. It has been postulated that the biphasic heat denaturation curves obtained with choline acetyltransferases from various species [3,5,18—21] indicate the presence of two isozymes with different heat stabilities.

This paper describes the purification from squid head ganglia of two isozymes, of choline acetyltransferase, each of which has multiple isoelectric forms. A preliminary report of this work has appeared [7].

Materials

Loligo pealei (native Atlantic squid) were purchased either from The Woods Hole Marine Biological Laboratory (Woods Hole, Mass.) or Abramo Fish Co. (Boston, Mass.). *Loligo opalescens*, caught in the Pacific near Monterey, were purchased from Globe Fish Co. (Boston, Mass.). The resins used for ion-exchange chromatography were obtained from the following companies: carboxymethyl-cellulose and cellulose phosphate, Whatman, W. and R. Balston, Ltd. (England); AG 1-X8 (chloride form) and hydroxyapatite, Bio-Rad Labs. (Richmond, Calif.).

[1-^{14}C] Acetyl coenzyme A (approx. 50 μCi/mol) and Aquasol, universal liquid scintillation counting cocktail, were purchased from New England Nuclear (Boston, Mass.).

The lithium salt of acetyl-CoA was obtained from P-L Biochemicals (Milwaukee, Wisc.). Dithiothreitol, choline chloride and carnitine were purchased from Sigma Chemical, Co. (St. Louis, Mo.). Ampholines were purchased from LKB Produckter (Sweden). Bovine serum albumin (Cohn Fraction V) was obtained from Nutritional Biochemical Co. (Cleveland, Ohio). Neostigmine bromide (prostigmine) was purchased from Hoffman-La Roche (Nutley, N.J.). Collodion bags and Collodion Bag Apparatus were obtained from Schleicher and Schuell, Inc. (Keene, N.H.).

All other chemicals were purchased from Fisher Scientific Co. (Boston, Mass.).

Methods

The assay used to measure choline acetyltransferase activity was essentially that of Schrier and Shuster [20]. For quantitation, the enzyme was usually diluted such that a 0.1 ml aliquot contained enough activity (0.0003 unit) * to convert approx. 10% (1200 cpm) of the trace-labeled acetyl-CoA to acetylcholine during a 10 min assay. However, even under conditions where up to 25% of the substrate was consumed, the reaction was essentially linear with time for at least 40 min. Full activity was maintained both before and during assay by the use of a solution containing 1 mg/ml bovine serum albumin, $1.0 \cdot 10^{-3}$ M EDTA and $1.0 \cdot 10^{-4}$ dithiothreitol as diluent. To the 0.1 ml aliquot of enzyme, a 0.1 ml aliquot of a substrate mixture containing the following reagents was added: $3.0 \cdot 10^{-4}$ M acetyl-CoA (the lithium salt, trace labeled with 0.01 μCi [1-^{14}C] acetyl-CoA/0.1 ml), 0.10 M choline chloride, 0.05 M potas-

* 1 unit of choline acetyltransferase is that amount of enzyme which is capable of producing 1 μM of acetylcholine per min.

sium phosphate buffer, pH 7.0, $1.0 \cdot 10^{-4}$ M neostigmine bromide, $5.0 \cdot 10^{-4}$ M EDTA, $5.0 \cdot 10^{-5}$ M dithiothreitol, 0.55 mg/ml bovine serum albumin, and 0.30 M NaCl. Incubation was at 32° C, and the reaction was stopped by imersion of the tubes containing the incubation mixture in an ice/water bath.

Determinations of protein concentrations for samples in $1.0 \cdot 10^{-4}$ M dithiothreitol or no dithiothreitol were performed according to the method of Lowry et al. [22]. Protein concentrations in samples containing dithiothreitol at a concentration of $1.0 \cdot 10^{-3}$ M were measured by using a modification of the Lowry technique developed by Geiger and Bessman [23].

Dialysis tubing was prepared for use by soaking it in 0.1 M EDTA for at least 3 days at room temperature. Immediately prior to use, the tubing was removed from the EDTA solution and washed exhaustively with distilled water. Thick-walled (0.002 inch), 1/4 inch diameter tubing was used for dialyses in which pressure would be exerted against the tubing (i.e. removal of high concentration of sucrose and/or salt) and for small volumes. Thin-walled (0.001 inch), 7/8 inch diameter tubing was used for dialyses in which no pressure would be exerted against the tubing, and for large volumes. Concentration of large volumes of enzyme solutions was performed by covering the dialysis tubing with solid sucrose overnight at 4° C. Small volumes were concentrated under vacuum in a collodion bag in a Collodion Bag Apperatus.

Cellulose phosphate ion-exchange resin was prepared according to the manufacturers instructions, and was finally equilibrated with 0.03 M potassium phosphate buffer, pH 6.0. Hydroxyapatite was equilibrated with 0.03 M potassium phosphate buffer, pH 6.8, prior to use.

0.02% NaN_3 was used as a preservative both for adsorbents and for sucrose-containing buffers.

Isoelectric focusing was performed in an apparatus modified from that used by Macko and Stegemann [24]. The modified apparatus consisted of a piece of polyvinyl tubing (Pharmaseal K57, disposable urinary drainage tube 3/16 inch internal diameter by 60 inches in length) coiled into a horizontal flat spiral containing 3 or 4 turns. Each tube was filled with 20 ml of 1% (by vol.) Ampholine in a 10% (by weight) sucrose solution, and then taped to the bottom of a 9 inch square plastic mouse cage. One end of the tube was immersed in an electrolyte bath holding 0.4% ethanolamine, the other in 0.1% phosphoric acid. Tubing was covered with cold water; isoelectric focusing was in refrigerated room at 4° C. In order to achieve equilibrium, the current was kept at 600 V for 12—16 h, and then at 100 V for 48—72 h. After equilibrium was achieved, the tubing was frozen in a solid CO_2 and ethanol bath (or just completely covered with finely chopped solid CO_2), and the coil was cut into 48 segments along previously marked 2.5-cm divisions. Each of the segments, containing 0.4 ml of solution, was allowed to melt and drain into a small test tube kept in an ice/water bath. The pH values of the fractions were read on a Corning pH Meter fitted with a Thomas Microelectrode.

In some of the initial experiments the tubing was wound around a piece of copper tubing as described by Macko and Stegemann [24], rather than into a flat horizontal spiral. In these experiments the isoelectric points of precipitated material was sometimes shifted as much as 0.5 pH unit, due to the settling of material to the bottom of the coil turn nearest to the isoelectric point of the

material. The change in geometry of the coils eliminated such settling, since each segment of the spirals was at the same height as all of the others. Samples were loaded in 0.01 M potassium phosphate buffer, pH 6.8, containing 20—30% sucrose and 1% Ampholine, by means of a syringe attached to a long piece of thin polyethylene tubing (internal diameter 0.023 inch). The sample was placed approx. 1/3 of the way through the isoelectric focusing tube from the cathodic end.

Results

Purification of choline acetyltransferase

Head ganglia were dissected from partially thawed squid and placed in a dish surrounded by crushed ice. All subsequent procedures were performed at 0—4° C. The volume of the ganglia was measured, and they were immediately homogenized in 10 volumes of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The extraction buffer contained $1.0 \cdot 10^{-3}$ M dithiothreitol and $1.0 \cdot 10^{-3}$ M EDTA, as did the solutions used for later steps. The homogenate was centrifuged at 27 000 $\times g$ for 15 min. The supernatant was decanted and brought to 30% saturation by the addition of solid $(NH_4)_2SO_4$ with stirring. After having been stirred for 20 min, the suspension was centrifuged at 27 000 $\times g$ for 20 min. The supernatant was next brought to 55% saturation with solid $(NH_4)_2SO_4$, and was stirred and centrifuged as already described. The resulting precipitate (ammonium sulfate 30—55 fraction) was dissolved, as were all ammonium sulfate precipitates, in 0.1 M potassium phosphate buffer, pH 7.0. The volume used was equal to the volume of ganglia that was homogenized.

At this stage in purification the total amount of activity routinely appeared to be 110—260% as high as it had appeared immediately after extraction. This material was then re-fractionated with $(NH_4)_2SO_4$. In the second ammonium sulfate precipitation, enzyme isolated from *L. pealei* did not begin to precipitate to a significant degree until the solution had reached 35% saturation, whereas enzyme isolate from *L. opalescens* began to precipitate at $(NH_4)_2SO_4$ concentrations above 33% saturation. Thus, an ammonium sulfate 35—55 fraction was prepared from *L. pealei*, and an ammonium sulfate 33—35 fraction was prepared from *L. opalescens*. The precipitate was dissolved as before, and dialyzed overnight against 25 volumes of 0.01 M potassium phosphate buffer, pH 6.8, containing 20% sucrose. The next day, the dialysate (ammonium sulfate 35—55 or 33—55) was centrifuged at 27 000 $\times g$. It was then diluted by addition of four volumes of 0.02 M potassium phosphate buffer, pH 5.7, to bring the pH of the solution to 6.1, while keeping the buffer concentration between 0.01 and 0.02 M. The enzyme solution was next loaded onto a 50 × 2.7 cm column of cellulose phosphate which had been equilibrated with 0.03 M potassium phosphate buffer, pH 6.0. Over 99% of the choline acetyltransferase activity prepared from *L. pealei* was retained on the resin. The column was eluted with a concave gradient of potassium phosphate, 0.03 M pH 6.0, to 0.3 M, pH 8.0 (700 ml each). The increase in pH of this gradient was nearly linear.

The enzyme prepared from *L. pealei* consistently gave two clearly separated peaks of enzyme activity on cellulose phosphate chromatography. The first

peak (cellulose phosphate 1) began to elute at approx. 0.06 M potassium phosphate, pH 6.80. The second peak of activity (cellulose phosphate 2) began to come off at approx. 0.14 M potassium phosphate, pH 7.15. There was an area of overlap between the peaks (see Fig. 1), but if the fractions in this area were discarded, the method resulted in clear peak separation. When the peak fractions containing cellulose phosphates 1 and 2 were combined separately, and re-chromatographed on cellulose phosphate separately, each peak was re-isolated at the same pH and salt concentration as before (see Fig. 2). Peak 1 began to elute at 0.07 M potassium phosphate, pH 6.8, and peak 2 began to elute at 0.14 M potassium phosphate, pH 7.2.

On the basis of the empirical finding that 2 distinct fractions of choline acetyltransferase could routinely be separated on cellulose phosphate chromatography, the two fractions were designated isozyme 1 (cellulose phosphate 1, eluted at pH 6.8, 0.06 M potassium phosphate) and isozyme 2 (cellulose phosphate 2, eluted at pH 7.15, 0.14 M potassium phosphate).

When a solution containing enzyme isolated from *L. opalescens* was loaded onto cellulose phosphate, only 75% of the activity was retained on the column. Elution of the material that was retained resulted in only one peak of activity which appeared at approximately the same pH and ionic strength as did cellulose phosphate 1. The enzyme isolated from *L. opalescens* was, therefore,

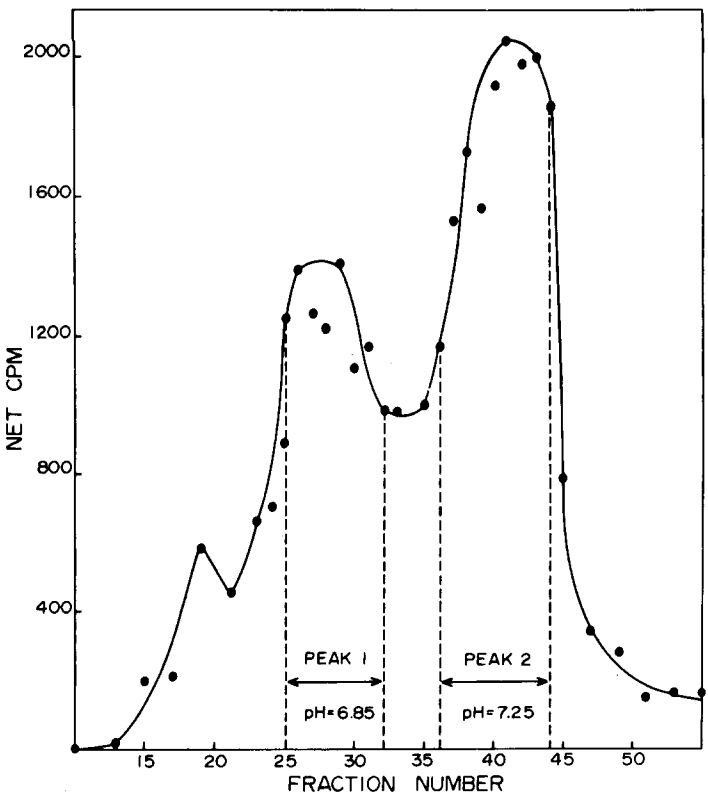

Fig. 1. Elution of squid choline acetyltransferase from cellulose phosphate. Cellulose phosphate 1 began to elute at approx. 0.06 M potassium phosphate, pH 6.80. Cellulose phosphate 2 began to elute approx. 0.14 M potassium phosphate, pH 7.15. The fractions in the peaks indicated were pooled separately. All other fractions were discarded.

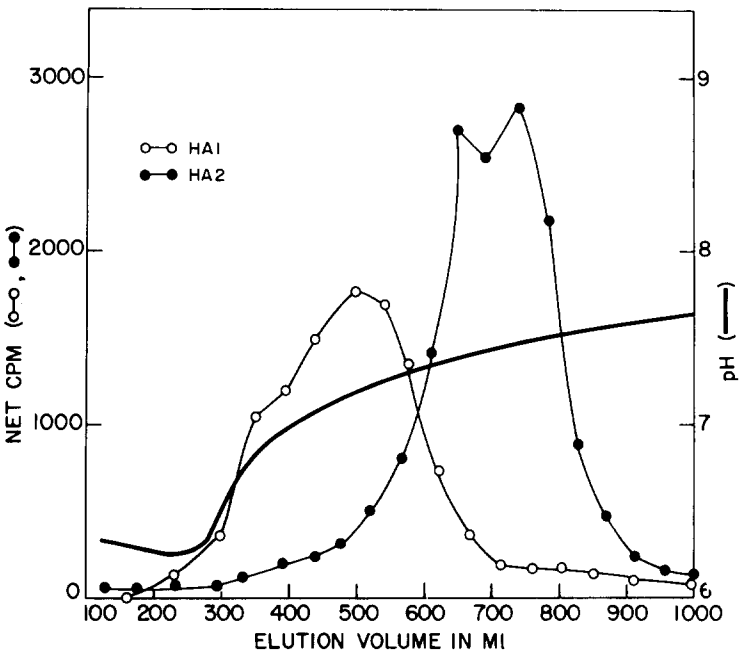

Fig. 2. Re-chromatography of isozymes hydroxyapatite 1 and hydroxyapatite 2 on cellulose phosphate. Prior to their re-chromatography on cellulose phosphate, the specific activity of hydroxyapatite 1 was 130, and that of hydroxyapatite 2 was 480 units/mg. After re-chromatography on cellulose phosphate, the specific activities remained unchanged. Recoveries were 55 and 64% for hydroxyapatite 1 and hydroxyapatite 2, respectively. (The two curves have been aligned on the abcissa in such a way that their pH and potassium phosphate gradients coincide). HA1, HA2: hydroxyapatite 1 and hydroxyapatite 2 respectively.

named cellulose phosphate 1 M (M for Monterey, Calif.) by analogy with isozyme 1. The enzyme fraction that did not adsorb to cellulose phosphate was not retained by the resin even when it was diluted and reapplied to a freshly regenerated column. Both enzyme fractions cross-reacted with antiserum directed against the enzyme fractions isolated from *L. pealei* (Polsky, R. and Shuster, L., unpublished data). Although the fraction that was not adsorbed onto the cellulose phosphate column was never purified, it was presumed to be a second isozyme. Recoveries from cellulose phosphate chromatography ranged from 50 to 80% for enzyme from either species of squid.

The active fractions from the isozyme of *L. pealei* were pooled separately and were purified as separate enzymes from this step. Cellulose phosphate 1 was diluted 3-fold with a solution of $1.0 \cdot 10^{-3}$ M EDTA, and $1.0 \cdot 10^{-3}$ M dithiothreitol in order to adjust the buffer concentration to 0.02 M. The diluted enzyme solution was then loaded directly onto a 10.5×2.6 cm column of hydroxyapatite that had been equilibrated with 0.03 M potassium phosphate buffer, pH 6.8. The column was eluted with a linear potassium phosphate gradient of 0.03—0.3 M buffer, pH 6.8 (700 ml each). Cellulose phosphate 2 was concentrated by overnight dialysis against two volumes of 0.01 M potassium phosphate buffer, pH 6.8, containing 40% sucrose immediately after the pooling of the fractions eluted from cellulose phosphate. Such dialysis resulted in a 3-fold concentration of the enzyme, and a 3-fold reduction in the

buffer concentration. Dilution of this concentrated enzyme to its original volume with 0.01 M potassium phosphate buffer, pH 6.8, resulted in a final potassium phosphate concentration of less than 0.02 M, and a final pH of 6.8. The resulting solution was loaded onto an hydroxyapatite column that had been equilibrated with 0.03 M potassium phosphate buffer, pH 6.8. Elution was done in precisely the same manner as for cellulose phosphate 1. The two enzyme fractions were eluted from hydroxyapatite in approximately the same position.

The tubes containing the bulk of the enzyme activity from each of the enzyme fractions were then pooled separately, as hydroxyapatite 1 and hydroxyapatite 2, and dialyzed overnight against two volumes of 60% sucrose containing $1.0 \cdot 10^{-3}$ M EDTA but no dithiothreitol. The enzyme solutions were then concentrated overnight by dialysis against solid sucrose. Omission of the dithiothreitol during the first dialysis ensures that the dithiothreitol concentration in the final 10-fold concentrated solution will not exceed $1.0 \cdot 10^{-3}$ M. A third overnight dialysis was performed against 10 volumes of 0.01 M potassium phosphate buffer pH 6.8, containing 10% sucrose. The concentrations of sucrose in the enzyme solution was between 15 and 20% after the third dialysis had been completed. This circuitous method of repeated dialysis and concentration was necessary due to the instability of dilute enzyme in the absence of a high concentration of sucrose or protein. The ultimate goal of this procedure was to reduce the volume sufficiently to allow concentration of the enzyme against reduced pressure in a collodion membrane. The enzyme was quite stable to such treatment, and yields were consistently increased by concentration. Since dilute, purified enzyme lost activity rapidly if placed in less than 20% sucrose, concentration was performed with 0.01 M potassium phosphate buffer, pH 6.8, containing 20% sucrose in the bath surrounding the collodion bag. When the volume had decreased sufficiently the enzyme was allowed to concentrate overnight against buffer containing 60% sucrose without vacuum. Such dialysis led to a further 3-fold concentration of the enzyme. If assayed immediately after concentration, the enzyme was much more active (see Table I) than it was if it had stood for even 1 day after concentration. After that initial loss, however, the specific activity remained constant for up to 4 months at 2—4° C. A summary of a typical purification of both isozymes from head ganglia of *L. pealei* is given in Table I. Both sucrose (especially solutions of 20% and higher) and sulfhydryl-protecting reagents (0.5—1.0 mM dithiothreitol or 5—10 mM mercaptoethanol) served to protect enzyme from denaturation at all stages of purification.

The enzyme isolated from *L. opalescens* behaved in all purification steps, in analogous fashion to isozyme 1. An extract with an initial specific activity of 0.13 unit/mg was purified 75-fold to a final specific activity of 9.1 units/mg, by the procedure described above. Carnitine acetyltransferase has, at times, been mistakenly identified as choline acetyltransferase (White and Wu [9], Shuster, L. and O'Toole, C., unpublished results). When hydroxyapatites 1 and 2 were assayed at pH 7.0, the optimal pH for squid choline acetyltransferase, in the presence of 0.05 M carnitine instead of choline, each isozyme exhibited less than 1% of the activity that it normally exhibited with the same concentration of choline.

TABLE I

PURIFICATION OF CHOLINE ACETYLTRANSFERASE FROM *L. PEALEI* IN 10^{-3} M DITHIOTHREITOL

Enzyme preparation	Specific activity (units/mg)	Protein (mg/ml)	Total protein (mg)	Total volume (ml)	Total activity (units)	Purification (Fold)	Recovery (%)
Crude homogenate	0.15	3.9	1560	400	240	1.0	100
Ammonium sulfate 30-55	0.45	11.2	604	54	270	2.9	114
Ammonium sulfate 35-55	0.73	10.5	315	30	231	4.7	98
Cellulose phosphate 1 [a]	1.0	0.25	56	225	56.2	6.5	24
Cellulose phosphate 2 [a]	2.6	0.15	21	140	54.7	17.0	23
Hydroxyapatite 1 [a]	2.6	0.031	9.6	254	20.8	17.0	9
Hydroxyapatite 2 [a]	11.8	0.0085	2.6	307	31.2	76	13
Hydroxyapatite 1 [b]	7.3	0.17	9.6	46	57.5	47	25
Hydroxyapatite 2 [b]	25.0	0.05	2.6	52	65.0	162	28
Hydroxyapatite 1 [c]	20.8 [e]	2.6	6.5	2.5	137.1 [e]	135	57
	(30.0) [d]				(195.0) [d]	200	(81)
Hydroxyapatite 2 [c]	42.0 [e]	0.8	2.0	2.5	84.0 [e]	273	35
	(58.3) [d]				(116.6) [d]	390	(49)

[a] These values are for unconcentrated fractions assayed immediately after having been eluted and combined.

[b] These values are for fractions that were concentrated with solid sucrose, and kept in concentrated sucrose until the time of assay.

[c] The values given here are for the fractions after they were concentrated against vacuum in a collodion bag, and then overnight against 60% sucrose.

[d] These values represent the activity of the fractions if the activity was determined immediately after the final concentration step had been completed.

[e] These values are the activity found when assay is performed several days after the final concentration step has been completed.

Purification in the presence of phenylmethylsulfonyl fluoride

The existence of two very similar isozymes might possibly be due to limited proteolysis of a single enzyme. It this were the case, use of an inhibitor of proteolysis, such as phenylmethylsulfonyl fluoride [25,26], would be expected to significantly decrease the amount of one of the isozymes. Therefore, extraction of squid head ganglia was performed in the presence of 100 μg/ml (approx. 50 μg/mg protein extracted), according to the method of Steinman and Jakoby [26]. Although incorporation of phenylmethylsulfonyl fluoride into the extraction buffer apparently led to extraction of twice as much activity as normal, the recovery from the second ammonium sulfate preparation was only 40% as high as normal. The enzyme that was recovered was loaded onto a cellulose phosphate column, as described in the previous section. The activity emerged from the column in two peaks at the pH values and buffer concentrations expected for cellulose phosphate 1 and cellulose phosphate 2. 33% of the activity applied to the column was recovered as isozyme 1 and 25% of the activity was recovered as isozyme 1.

Kinetic properties

The K_m values for each of the 2 isozymes of choline acetyltransferase isolated from *L. pealei* were determined with each of the two substrates. The determinations were performed both with and without bovine serum albumin, NaCl, and dithiothreitol, each of which has been reported to either activate or protect choline acetyltransferase [27]. K_m for each substrate was determined in the

TABLE II

K_m (choline) AND K_m (acetyl-CoA) VALUES OF CHOLINE ACETYLTRANSFERASE AS DETERMINED FROM EADIE AND HOFSTEE PLOTS

Prior to use, the concentrated enzyme solutions were stored in 0.01 M potassium phosphate buffer, pH 6.8, containing 60% sucrose. The concentration of sucrose in the final assay mixture was never greater than 0.2%. The largest amount of protein added in the enzyme samples was 4 μg for squid hydroxyapatite 1, 2 μg for squid hydroxyapatite 2, and 1 μg for squid hydroxyapatite M. Due to the variation of V with salt concentration as well as to the great difference between the orders of magnitude of the K_m (choline) and the K_m (acetyl-CoA), it was necessary to use different amounts of enzyme for each K_m determination. 0.0006 unit was used for assays in which choline concentration was varied, and 0.0001 unit was used for assays in which acetyl-CoA concentration was varied. The final concentrations of the additions in the samples assayed with additions were as follows: 0.15 M NaCl, 0.77 mg/ml bovine serum albumin, 0.015 M potassium phosphate buffer, pH 7.0, and 10^{-4} M dithiothreitol. These substances were included in the 100 μl in which the enzyme and the variable substrate were added to all samples, both with and without additions. All samples received a 100-μl aliquot containing $1.0 \cdot 10^{-4}$ M neostigmine bromide and 0.02 M potassium phosphate buffer, pH 7.0, to bring the final concentrations to half those values. Dithiothreitol was included only in the samples assayed with additions. In order to determine the K_m (choline) values, choline was varied from $4 \cdot 10^{-3}$ to $2 \cdot 10^{-5}$ M, while the acetyl-CoA was held constant at $1.5 \cdot 10^{-4}$ M. The K_m (acetyl-CoA) values were determined by varying acetyl-CoA concentration between $2.25 \cdot 10^{-4}$ and $1 \cdot 10^{-5}$ M, while keeping the choline concentration fixed at $1 \cdot 10^{-2}$ M. 0.01 μCi of [1-^{14}C] acetyl-CoA (approx. 12 000 cpm) was used as the radioactive tracer in all determinations. In experiments designed to determine the K_m (choline) values of the isozymes, the tracer was accompanied by a constant amount (30 nmol) of unlabeled acetyl-CoA. When K_m (acetyl-CoA) values were being determined, 0.01 μCi of [1-^{14}C] acetyl-CoA (0.2 nmol) was added per 0.2 ml incubation mixture. To that constant amount of tracer, non-radioactive acetyl-CoA was added in amounts varying from 1.8 to 45.0 nmol. The differences in the specific activities of the acetyl-CoA in the samples used for K_m (acetyl-CoA) determinations were taken into account when calculating reaction velocities. Samples were incubated for 10 min at 32°C.

Enzyme	Additions (+/—)	K_m (choline) ($M \times 10^{-3}$)	K_m (acetyl-CoA) ($M \times 10^{-5}$)
Hydroxyapatite 1	+	7.6	3.2
	—	8.5	2.2
Hydroxyapatite 2	+	12.5	0.82
	—	5.9	2.3
Hydroxyapatite M	+	19.0	3.2
	—	4.4	not determined

presence of an excess of the other substrate. Ten different substrate concentrations were used to make each K_m determination. The results were plotted according to the method of Eadie and Hofstee [28]. Lines were fitted by the method of least squares with a Wang model 700 Computer-plotter. As shown in Table II, K_m (choline) values of all 3 types of choline acetyltransferase were between $7.6 \cdot 10^{-3}$ and $19.0 \cdot 10^{-3}$ M and the K_m (acetyl-CoA) values were between $0.82 \cdot 10^{-5}$ and $3.2 \cdot 10^{-5}$ M. The K_m (acetyl-CoA) and the K_m (choline) of isozyme 1 seemed to remain fairly constant whether in the presence or absence of the additions. In contrast, the affinity of isozyme 2 for choline appeared to decrease while its affinity for acetyl-CoA increased in the presence of the additions. The values for K_m (choline) are so high, however, regardless of additions, that they are of doubtful significance.

Isoelectric focusing

When a crude 30—55 ammonium sulfate fraction from *L. pealei*, containing both isozymes, was subjected to isoelectric focusing, choline acetyltransferase

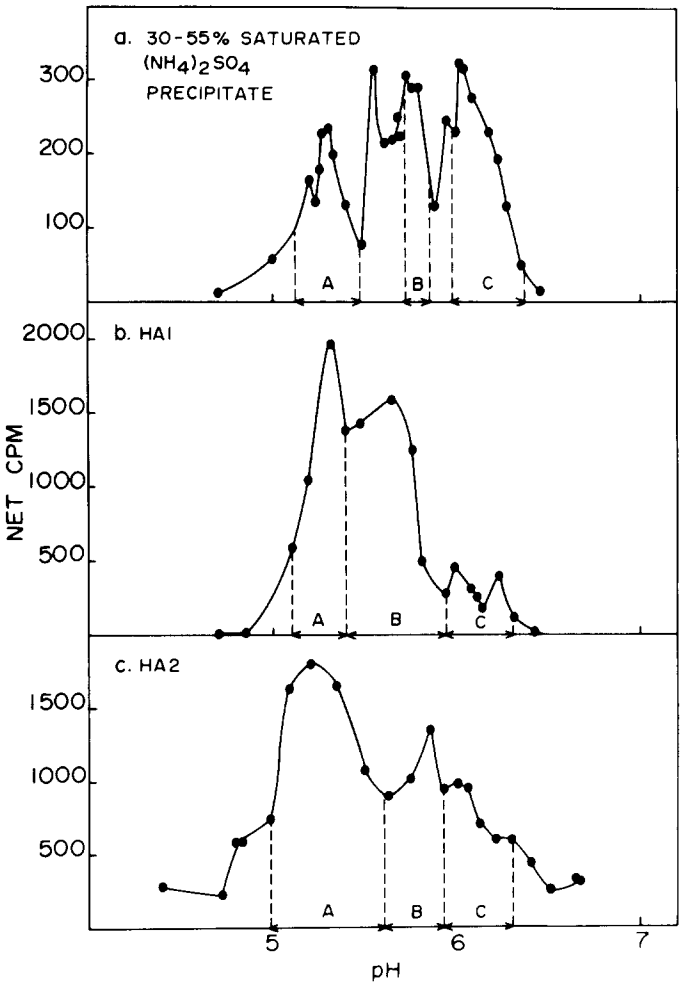

Fig. 3. Comparison of isoelectric focusing pattern of crude and purified choline acetyltransferase. The no. of mg of protein applied, the total number of units applied, the percent of the activity recovered (in fractions A, B, and C collectively), and the ratio of the amounts of the recovered activity found as A, B, and C, respectively, were for each of the experiments shown in this figure as follows: (a) 2.0 mg (ammonium sulfate 30—55 of *L. pealei*): total activity, 0.50 unit; 11% recovery; 10 : 30 : 60; (b) 0.75 mg (isozyme 1); total activity, 7.0 units; 55% recovery; 45 : 45 : 14; (c) 1.2 mg (isozyme 2); total activity, 34.0 units; 30% recovery; 48 : 26 : 26. HA1, HA2: hydroxyapatite 1 and hydroxyapatite 2, respectively.

was resolved into three major active forms. The isoelectric points of these forms were approx. 5.2, 5.7, and 6.2 (see Fig. 3a) *. The different isoelectric forms were arbitrarily designated as follows: the form(s) isoelectric between pH values 5.0 and 5.4 were called A; the form(s) isoelectric between 5.5 and 5.8 were called B; the form(s) isoelectric between 5.9 and 6.2 were called C. Each of the three main peaks described was found to have two components in all experiments performed with crude enzyme preparations.

* In this, and in all subsequent isoelectric focusing experiments, the pH gradients generated by the Ampholine were essentially linear over the pH range of the Ampholine employed. Thus, for ease of interpretation, the pH gradient was taken to be a linear function of fraction number, and all data were plotted with activity represented as a direct function of pH.

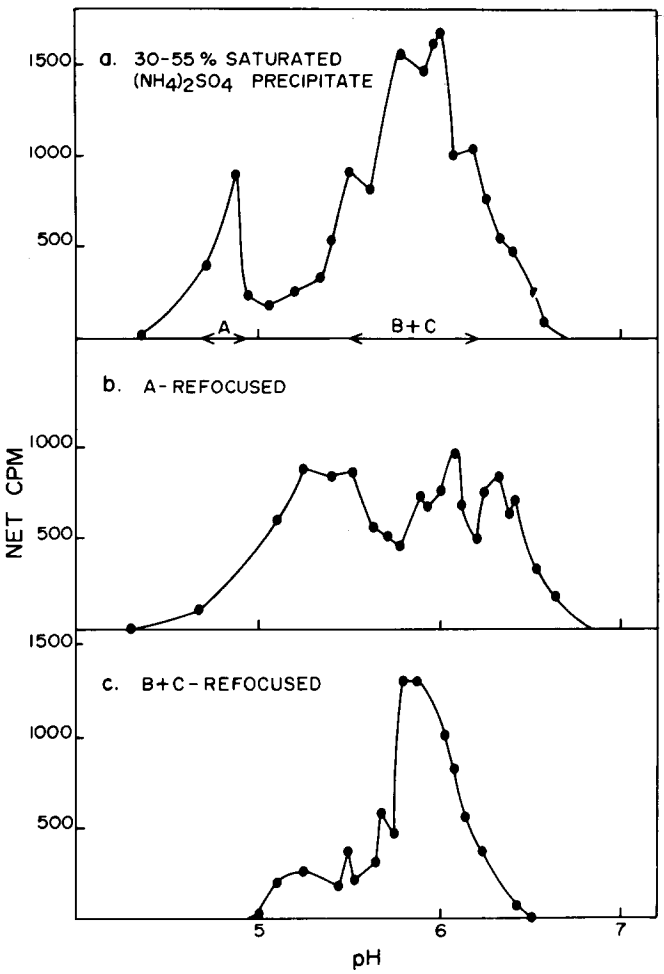

Fig. 4. Refocusing of isoelectric peaks A and (B + C). Legend organized as in Fig. 3. (a) 13.5 mg (ammonium sulfate 30—55 of *L. pealei*); total activity, 2.2 units; 60% recovery; 11 : 39 : 50; (b) not determined; total activity, 0.1 unit; 37% recovery; 26 : 28 : 48; (c) not determined; total activity, 1.0 unit; 26% recovery; 8 : 24 : 68.

When isoelectric focusing A, isolated in a similar experiment to the one described above * was refocused, it regenerated all three isoelectric forms of the enzyme (see Fig. 4). These data suggest that A is able to form both B and C. The transition(s) B and/or C to A may also occur, but to a much lesser extent under the conditions employed in our isoelectric focusing procedure, since re-focusing of a combined B plus C fraction produced almost no A (see Fig. 4). Differential ammonium sulfate precipitation (in $1.0 \cdot 10^{-4}$ M dithiothreitol) of extracts of crude homogenates of squid head ganglia made possible

* The shift in apparent isoelectric point of the A fraction used in this experiment was due to the fact that it was isolated in an experiment run in a vertically coiled isoelectric focusing tube. Since the A fractions of crude enzyme preparations were found to precipitate when large protein loads were used, it was possible for the apparent isoelectric points of such fractions to be displaced as much as 0.5 pH unit (see Methods and Fig. 3 (a)).

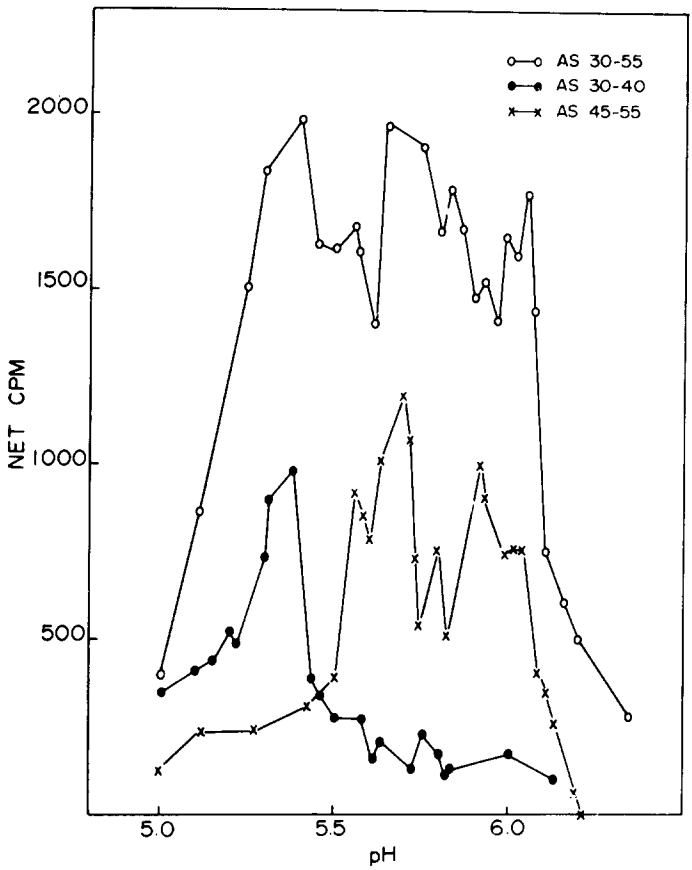

Fig. 5. Isoelectric focusing of ammonium sulfate (AS) fractions prepared from *L. pealei*. Legend organized as in Fig. 3. (a) 3.8 mg; total activity, 2.7 units; 40% recovery; 12 : 41 : 47; (b) 2.9 mg; total activity, 1.7 units; 6% recovery; 68 : 25 : 7; (c) 2.3 mg; total activity, 2.0 units; 14% recovery; 5 : 53 : 32.

the separation of isoelectric focusing A from isoelectric focusing B and C prior to focusing. Focusing of 30—40% and 45—55% saturated ammonium sulfate fractions (ammonium sulfate 30—40 and 45—55, respectively) showed that isoelectric focusing A was precipitated mainly in the 30—40 fraction, whereas B and C predominated in the ammonium sulfate 45—55 fraction (see Fig. 5). All three isoelectric focusing fractions were present in the 40—45% saturated fraction which is not shown.

Isoelectric patterns of purified choline acetyltransferase

Isozymes 1 and 2 from *L. pealei* were each found to separate into the same three active fractions described for the crude fractions, but the peak resolution for each of the fractions was much smoother (see Fig. 3, compare b and c with a). A and B were the predominant isoelectric forms in each.

The focusing pattern of isozyme 1 purified from *L. opalescens* was somewhat different than that for either isozyme purified from *L. pealei;* the isoelectric focusing points of the active isoelectric forms isolated from *L. opalescens* were all displaced 0.4 pH unit toward the alkaline end of the gradient.

Thus, although A, B, and C appeared, they were focused at pH values of 5.5—5.8, 5.85—6.1, and 6.15—6.4, respectively.

A fourth isoelectric fraction consisting of precipitated enzyme protein was isolated from both types of isozyme 1, at a pH just below that at which A focused. Only 1—5% of the total recoverable activity was present in this fraction, but the inactive protein showed immunological identity with active enzyme.

This fraction was called pre-A, and was isoelectric at pH 4.7—4.9 in isozyme 1 isolated from *L. pealei*, and at pH 5.1—5.3 in isozyme 1M isolated from *L. opalescens*.

Recoveries of between 30 and 60% were readily obtainable under favorable conditions of isoelectric focusing. The concentrations of sucrose, protein and dithiothreitol were all found to play crucial roles in enzyme protection and/or stabilization. Recoveries were consistently better in 20% sucrose than in 10% sucrose and with high protein loads as opposed to low protein loads. When protein concentration was decreased, recovery decreased concomitantly: 50—60% recovery for greater than 10 mg, 40% recovery for 3.8 mg and 11% recovery for 2.0 mg (all in 10% sucrose).

About $1.0 \cdot 10^{-4}$ M appeared to be an optimal concentration of dithiothreitol in which to focus squid choline acetyltransferase. Focusing $1.0 \cdot 10^{-3}$ M dithiothreitol eliminated A, whereas concentrations of below $1.0 \cdot 10^{-4}$ M did not afford adequate protection of the enzyme against denaturation during isoelectric focusing.

Stability and activation of purified isozymes 1 and 2

The stabilities to heating of purified isozymes 1 and 2 were determined in the presence of activating (NaCl) and/or stabilizing (bovine serum albumin and sucrose) agents. Hydroxyapatite 1 was found to be more stable to heating than hydroxyapatite 2 in low concentrations of sucrose and NaCl. Both isozymes were greatly stabilized by high concentrations of sucrose. Inactivation was accomplished by preincubation of the enzyme at 37° C for varying lengths of time. After preincubation, 0.1 ml substrate mixture was added to 0.1 ml of each heated enzyme sample, and the resulting 0.2 ml was incubated immediately at 32° C for 10 min. The data obtained from these experiments are shown in Fig. 6.

No activity was present in dilutions of either isozyme made in the absence of EDTA, and inactivation occurred too rapidly to measure (in less than 30 s) in the absence of 20% sucrose. Even in the presence of 20% sucrose (see C of Fig. 6), each isozyme was inactivated to 33% or less of its original activity within 1 min at 37° C. The addition of NaCl caused inactivation to proceed even more rapidly.

Addition of bovine serum albumin (1 mg/ml), rather than sucrose, stabilized both isozymes (see A of Fig. 6), although its effect was more pronounced on isozyme 1 ($t_{1/2}$ = 6 min) than on isozyme 2 ($t_{1/2}$ = 3 min). Addition of 1 mg/ml bovine serum albumin in conjunction with 20% sucrose caused more stabilization than either agent alone.

Concentrated sucrose had a marked stabilizing effect, as is shown in B of Fig. 6. After 2 h in 60% sucrose, the enzymes still retained 65% of their initial

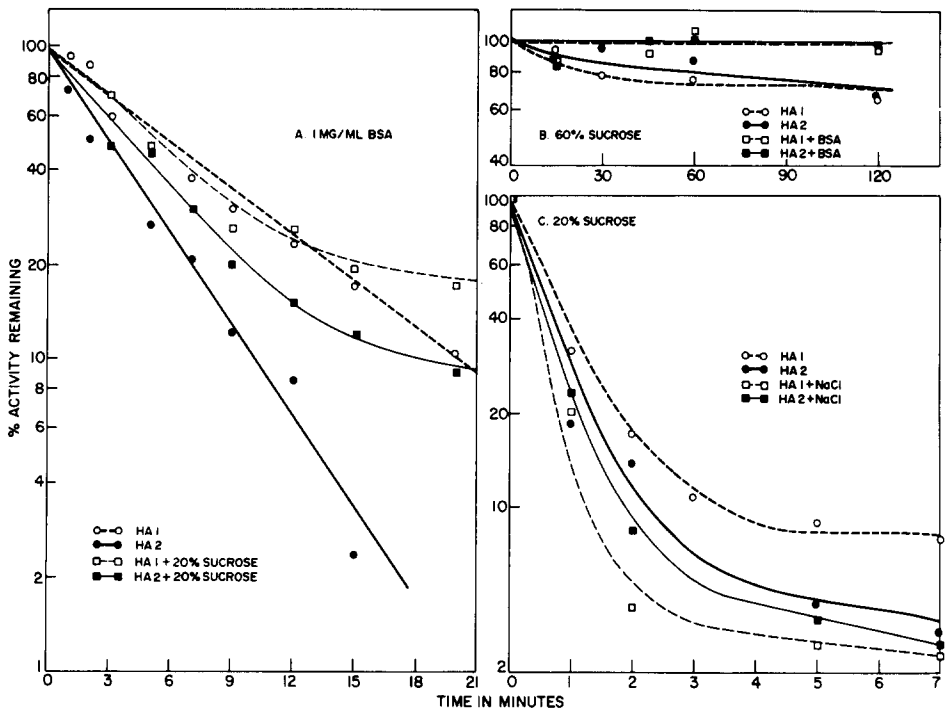

Fig. 6. Heat stabilities of isozymes hydroxyapatite 1 and hydroxyapatite 2. The dithiothreitol concentration was $1.0 \cdot 10^{-4}$ M in the solutions used for incubating the samples used to determine the points represented in a of this figure. The enzyme preparations used in these experiments were stored in 0.01 M potassium phosphate buffer, pH 6.8, containing 60% sucrose prior to use. The assays for all of the points represented on each set of axes were performed on the same amount of enzyme activity. This amount of enzyme was 0.0003 unit for a and c and 0.0006 unit for b. The maximum amount of protein added in the enzyme aliquot was 1.2 μg. Lines were fitted by the method of least squares. HA 1, HA 2: hydroxyapatite 1 and hydroxyapatite 2, respectively. BSA: bovine serum albumin.

activities; there was no difference between hydroxyapatite 1 and hydroxyapatite 2. In 60% sucrose containing 10 mg/ml bovine serum albumin, both isozymes were completely stable for over 2 h at 37° C.

Sucrose was found to stabilize choline acetyltransferase to freezing & storage as well. Either of the isozymes could be stored in 60% sucrose for up to 3 months at 0—4° C with little or no loss of activity, whereas the use of only 20% sucrose resulted in the loss of 60% of the activity. Freezing in either concentration of sucrose led to the loss of an additional 5—10% of the activity.

The activating and stabilizing effects of NaCl and mercaptoethanol either alone or in combination with bovine serum albumin were found to be different for the two isozymes, and among their isoelectric forms as well. The effect of NaCl seems to be due to activation rather than stabilization, since salt has been found to cause inactivation on long-term exposure, or when it was included during mild heating of the enzyme. The other two agents were presumed to exert their effects by protecting the enzymes from denaturation during pre-incubation. The enzymes were considered to be fully activated and protected when all of the additions were present, and results are expressed as percent of that value. Assays were performed on enzyme aliquots that had been pre-incu-

TABLE III

ACTIVATION AND STABILIZATION

The samples used for these experiments were exhaustively dialyzed against 0.01 M potassium phosphate buffer, pH 6.8, containing 60% sucrose and less than 10^{-7} M dithiothreitol. The final sucrose concentration in the pre-incubation mixture was 6%. The concentrations of enzyme protein were all below 0.015 mg/ml during pre-incubation. Pre-incubation was for 30 min in an ice/water bath in a volume of 100 µl. The pre-incubation solution contained either 0.3 M or no NaCl: either 2 mM or no merceptoethanol; either 1 mg/ml or no bovine serum albumin; and 10 mM potassium phosphate buffer, pH 7.0. After the pre-incubation period, 100 µl of a minimal substrate mixture was added. The mixture contained the following reagents: 10 mM potassium phosphate buffer, pH 7.0; 0.10 M choline chloride, $3.0 \cdot 10^{-4}$ M acetyl-CoA, and $1 \cdot 10^{-4}$ M neostigmine bromide. The final incubation mixture, containing 200 µl of solution, was incubated at 32°C for 30 min.

Enzyme preparation	Percent of maximal activity					Maximal activity (cpm)
	Isoelectric form	No additions	NaCl 0.3 M	20 mM mercaptoethanol	NaCl + mercaptoethanol	bovine serum albumin (2 mg/ml) + NaCl + mercaptoethanol
Hydroxyapatite 1	A	10	21	12	43	1980
Hydroxyapatite 1	B	40	44	26	78	750
Hydroxyapatite 1	C	36	25	22	50	415
Hydroxyapatite 2	A	4	13	5	33	2790
Hydroxyapatite 2	B	3	10	4	27	2662
Hydroxyapatite 2	C	15	16	17	30	1030

bated with various combinations of the activating and stabilizing reagents as described in the legend to Table III.

In all cases, isozyme 1 retained more than twice as much activity when pre-incubated and assayed with no additions, than did isozyme 2 (see Table III). This was true for unfocused material that had been purified only through the cellulose phosphate step, as well as for all three of the isoelectric fractions of material focused after the hydroxyapatite step. The C forms of both isozymes remained most active in the absence of stabilizing and activating reagents, and were also the least affected by addition of NaCl. A of hydroxyapatite 1, and A and B of hydroxyapatite 2 retained the least activity in the absence of activating and stabilizing reagents, and were the most profoundly affected by the addition of NaCl to the preincubation and incubation mixtures. Although choline acetyltransferase is an SH-containing enzyme, and is more stable during purification when a sulfhydryl-protecting reagent is present, it was almost unaffected by addition of mercaptoethanol alone in this experiment. However, mercaptoethanol acted synergistically with NaCl to cause a greater effect than the sum of the increases caused by either agent alone. Other experiments have shown that the effect of mercaptoethanol was also synergistic with bovine serum albumin.

Discussion

The process developed in this laboratory to purify choline acetyltransferase from the head ganglia of squid, has led to purifications of up to 390-fold over the crude extract. In the past, both Prince [12] and Reisberg [13] purified the squid enzyme approximately 10-fold. The use of both sulfhydryl-protecting

reagents and sucrose made it possible for both Husain and Mautner [11] and ourselves to prepare highly purified choline acetyltransferase from squid head ganglia. The initial steps of the method of Husain and Mautner [11] were based on the procedure developed in this laboratory; the main differences between their procedure and ours was their use of mercurial Sepharose, sulfopropyl Sephadex and styrylpyridinium Sepharose chromatography, and the use of $1.0 \cdot 10^{-4}$ M rather than $1.0 \cdot 10^{-3}$ M dithiothreitol. Husain and Mautner [11] obtained final specific activities of up to 67 units/mg, with a yield of 7%. We obtained final specific activities of up to 58 units/mg, with a yield of over 90%. It is probable that Husain and Mautner [11], by their procedure, selected out only one form of one of the two isozymes of *L. opalescens.*

The pronounced stabilizing effect of sucrose was probably analogous to that observed by Bradbury and Jakoby [30] for the stabilization of aldehyde dehydrogenase by polyhydric alcohols. Sulfhydryl-protecting reagents appear to protect the enzyme from oxidation, as has been shown by many laboratories.

Although the two enzyme fractions isolated from *L. pealei* were empirically identified as isozymes 1 and 2 according to the order in which they eluted on cellulose phosphate chromatography, they also differed from each other in properties such as heat stability, capacity to be salt activated and the presence of precipitated pre-A fraction in isoelectric focusing preparations. The lack of re-equilibration between the fractions when they were re-chromatographed (on cellulose phosphate), indicated that they were not interconvertible [30]. In addition, both isozymes were found in approximately equal amounts, even when purification was conducted in the presence of phenylmethylsulfonyl fluoride. This result suggests that neither fraction was the product of limited proteolysis of the other.

L. opalescens choline acetyltransferase also appeared to contain two isozymes, only one of which, hydroxyapatite 1M, was retained on cellulose phosphate. Both enzyme fractions react with antisera prepared to the isozymes isolated from *L. pealei* (Polsky, R. and Shuster, L., unpublished data). Only the isozyme retained on cellulose phosphate was purified, and its behavior was found to closely resemble that of isozyme 1 isolated from *L. pealei.*

Singh et al. [31] have obtained 2 active fractions of choline acetyltransferase from human neostriatum by fractionation on phosphocellulose. The two enzymes had similar molecular weights (67 000) upon sodium dodecyl sulfate-acrylamide gel electrophoresis, but one of them was more unstable to heat. These data support our findings in studies on squid choline acetyltransferase.

At least three different active isoelectric fractions have been isolated from each of the choline acetyltransferase isozymes. Isoelectric focusing A is identical with the enzyme that precipitates first (ammonium sulfate 30—40) upon narrow-range ammonium sulfate fractionation, and is able to regenerate isoelectric focusing B and C when re-focused. These findings suggest, in accord with the observations of Malthe-Sørenssen and Fonnum [16] and White and Wu [9], that A is the most highly aggregated of the three active isoelectric forms of choline acetyltransferase described. Capacity of isoelectric fractions of choline acetyltransferase to be activated or stabilized was found to vary with the isoelectric focusing point of the fraction. That is, the higher the isoelectric point of the fraction, the more sensitive it is to stabilization by mercap-

toethanol, and the less sensitive to activation by NaCl, and vice versa. Since, upon re-focusing, A can regenerate B and C, it seems unlikely that the differences among the isoelectric forms of the enzyme are due to chemical modifications or to the presence of isozymes.

The results of our experiments using activating and protecting reagents with isoelectric focusing fractions of choline acetyltransferase seem to bear directly on the observations [3,6,12] that aged enzyme loses the capacity to be activated by salt. Three of the six isoelectric fractions isolated from isozymes 1 and 2 were activated by 0.3 M NaCl alone, whereas the other three were unaffected by the presence of the salt. Although none of the fractions, whether affected by NaCl or not, was activated by 20 mM mercaptoethanol alone, simultaneous addition of NaCl and mercaptoethanol had a synergistic effect, and produced at least a 2-fold stimulation of activity. This observation suggests that the loss of ability to salt-activate choline acetyltransferase reported by others [6,12] might actually have been avoided by the use of sulfhydryl-protecting reagents. This conclusion is supported by the fact that Schuberth [3] found that he could restore capacity for salt activation to placental choline acetyltransferase by incubation of the enzyme with 0.5 M cysteamine.

The K_m (acetyl-CoA) of each isozyme in the absence of activating and protecting agents was approx. $2.3 \cdot 10^{-5}$, the same as reported by Prempeh et al. [17] for crude squid enzyme. In the presence of activating and protecting agents, the affinity of isozyme 2 for acetyl-CoA increased 3-fold. Although small, this difference may be significant especially in light of the fact that it is isozyme 2 that is the most susceptible to activation by salt. Prempeh et al. [17] observed, instead, that the affinities of the enzymes from both rat and squid for acetyl-CoA were 2—3-fold decreased in the presence of 0.15 M NaCl. The discrepancy between our results and those of Prempeh et al. [17] may be due to the presence of mercaptoethanol and bovine serum albumin in our activated and protected reaction mixture, and the absence of these reagents in the assay mixture employed by Prempeh et al. [17].

Although the K_m (acetyl-CoA) values of the two isozymes appear to differ in the presence of activating and protecting agents, the effects of these agents cannot be attributing to a change in saturation of the enzyme. Both isozymes were operating virtually at V in assay mixtures containing 0.01 M choline chloride and $1.5 \cdot 10^{-4}$ M acetyl-CoA, either with or without the additions present.

The K_m (choline) values obtained for all 3 types of squid choline acetyltransferase studied indicated an extremely low affinity, on the order of $1 \cdot 10^{-2}$ M. Since choline chloride itself, as well as NaCl and other salts, causes significant stimulation of the reaction velocity above concentrations of $1.0 \cdot 10^{-2}$ M [12], K_m (choline) values of such magnitude may also reflect salt activation by the substrate, in addition to substrate affinity.

Acknowledgements

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