Choline Acetyltransferase and Acetylcholine Levels in *Drosophila Melanogaster*: A Study Using Two Temperature-sensitive Mutants¹

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Abstract

Choline acetyltransferase (ChAT, acetyl-CoA:choline-O-acetyltransferase, EC 2.3.1.6) activity was measured in homogenates prepared from wild type (Canton S) and two temperature-sensitive presumed ChAT structural gene mutants (*Cha*^{ts1} and *Cha*^{ts2}; originally described by Greenspan, R. (1980) *J. Comp. Physiol.* 137: 83-92) of *Drosophila melanogaster*. Wild type flies grown at 32°C for 12 or 24 hr showed increased ChAT activity, whereas *Cha*^{ts1} and *Cha*^{ts2} flies showed a progressive decrease in enzyme activity at 32°C (restrictive temperature) when compared to flies reared at 18°C (permissive temperature). Acetylcholine (ACh) and choline levels were determined in formic acid-acetone extracts of individual fly heads, and the ACh levels showed the same variation with time at 32°C as did the ChAT activity. In contrast, choline levels did not vary in any regular pattern. Acetylcholinesterase (EC 3.1.1.7) activity did not vary during heat treatment (except for *Cha*^{ts2} flies held at 32°C for 24 hr, where a decrease was observed) indicating that this treatment may be specific for ChAT. We conclude that ChAT activity is strongly correlated with ACh levels in *Drosophila* heads and may thus have an important regulatory role in determining the levels of ACh available for physiological function.

We also report on the preliminary characterization of ChAT in both *Cha*^{ts} mutants and compare the biochemical properties to those of wild type enzyme. Isoelectric focusing profiles of ChAT from both *Cha*^{ts} mutants revealed enzymes with altered patterns compared to wild type, indicating that the mutations are most probably in the structural gene. The *in vitro* thermolability of individual isoelectric point forms of ChAT were similar to that of total ChAT activity in a homogenate. The *k_m*'s for choline and acetyl-CoA were similar for enzyme from wild type and *Cha*^{ts1} and differed only 2-fold for *Cha*^{ts2}. Immunoblot analysis of sodium dodecyl sulfate gels using a monoclonal antibody to *Drosophila* ChAT revealed only one 67,000-dalton band for either *Cha*^{ts} mutant, whereas wild type enzyme showed two bands reacting with antibody,

one at 67,000 and the other at 54,000 daltons. Since the monoclonal antibody directly inhibits enzyme activity, it is likely that the epitope it recognizes is at or near the active site of the enzyme.

Drosophila melanogaster offers a favorable biological material for studying acetylcholine (ACh) metabolism in animals (Hall et al., 1979; Hall and Greenspan, 1979). ACh is thought to be the primary sensory neurotransmitter in arthropods (reviewed by Pitman, 1971; Gerschfeld, 1973; Klemm, 1976), and high levels of the biosynthetic enzyme choline acetyltransferase (ChAT, EC 2.3.1.6) are present in insect nervous system (Dewhurst et al., 1970). The gene for ChAT has recently been identified and mapped in *Drosophila*, and two temperature-sensitive alleles have been isolated (Greenspan, 1980). The levels of ChAT activity have been manipulated in intact flies by altering the temperature, and the effects of these manipulations result in physiological and behavioral changes including abnormal electroretinograms, paralysis, and altered courtship patterns (Greenspan, 1980). These *Cha*^{ts} mutants have recently been used to identify a synapse using ACh as a neurotransmitter in the giant fiber pathway mediating the flight response (Gorczyca and Hall, 1983).

The relationship of ChAT activity to ACh levels in the nervous system is complex and controversial. The enzyme reaction involves acetylation of choline using acetyl-CoA. Since choline can also be utilized in other metabolic pathways, most notably for phospholipid synthesis (Jope and Jenden, 1979), regulation of ChAT activity could serve as an attractive and selective target for the *in vivo* control of ACh synthesis. Indeed, ChAT activity has been implicated as a regulatory step for ACh production in a number of early studies (Kaiba and Goldberg, 1969; Glover and Potter, 1971; Morris et al., 1971; Kuhar and Murrin, 1978). Others have argued against ChAT being a regulatory and/or rate-limiting step in ACh production on theoretical as well as practical grounds (Hebb, 1972; Krell and Goldberg, 1975; Hanbrich and Chippendale, 1977; Tuček, 1983) and have advanced a number of alternative hypotheses for the biochemical control point(s) regulating ACh production.

The most popular alternative regulatory and/or rate-determining step involved in ACh production is the sodium- and energy-dependent high affinity choline transport system studied most extensively in mammalian brain synaptosome or slice preparations (Haga, 1971; Yamamura and Snyder, 1973; Kuhar and Murrin, 1978). This hypothesis has been supported by a number of studies which seem to implicate choline availability as the rate-limiting factor in ACh synthesis under a variety of conditions, including manipulation of systemic choline levels (Cohen and Wurtman, 1975; but see Wecker and Schmidt, 1979), inhibition of the high affinity choline transport system by hemicolinium-3 (Guyenet et al., 1973a, b) and the ability of high affinity choline transport capacity to change during nerve stimulation (Collier et al., 1983).

The relationship between high affinity choline transport and acetylation of choline by ChAT is also controversial. Several investigators

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have argued for a tight "physical" coupling (Barker and Mittag, 1975), whereas others have suggested that preferential choline acetylation, following high affinity transport, results from a "kinetic" coupling (Suszkiw and Pilar, 1976; Joep and Jenden, 1977). Other groups have been able to dissociate any apparent coupling between high affinity choline transport and acetylation by ChAT and thus argue for no direct regulatory relationship between these two processes (Marchbanks and Kessler, 1982).

In this paper we report the absolute levels of ACh and choline in wild type Canton S *Drosophila melanogaster*. We also establish a strong correlative relationship between ChAT activity and ACh levels using the two temperature-sensitive single gene mutants described by Greenspan (1980). We provide evidence for the structural gene nature of these mutants by showing their altered isoelectric focusing profiles. The *in vitro* thermolability of the individual isoelectric point (pI) forms of the enzymes from both *Cha*^{ts} mutants is similar to that reported in whole homogenates by Greenspan (1980). In addition, we report the kinetic parameters of ChAT from both mutants and propose a structural model to account for the various pI forms and immunologically identified polypeptides observed on Western blots of wild type and *Cha*^{ts} mutant proteins. A preliminary account of our work has been presented (Salvaterra et al., 1983; Salvaterra and McCaman, 1984).

Materials and Methods

Drosophila stocks. Canton S flies were used as the wild type strain in these studies. Two temperature-sensitive ChAT alleles, *Cha*^{ts1} and *Cha*^{ts2}, were obtained from Dr. Jeff Hall, Brandeis University. In some experiments the mutations were carried over wild type chromosomes with a deletion at the position of the *Cha* gene as described by Greenspan (1980). For other experiments, homozygous *Cha*^{ts1} and *Cha*^{ts2} stocks were prepared by crosses of mutant and wild type flies to neutralize the lethal mutations present on the *Cha*^{ts} chromosomes. All stocks used for the studies of ACh levels, choline levels, and the effect of temperature on ChAT activity were reared for several generations at 18°C before initiation of the experiments.

Temperature shift experiments. Four- or 5-day-old flies of mixed sex (an equal number of male and female flies in most cases) were used. Individual vials contained 6 to 10 flies of known genotype and were kept on a 12-hr light/dark cycle during the course of the temperature shift experiments. For flies held at the restrictive temperature of 32°C for 12 hr, the first 6 hr of dark were followed by 6 hr of light. Flies held at 32°C for 24 hr or at the permissive temperature of 18°C received 6 hr of light, followed by 12 hr of dark and 6 hr of light. Flies transferred from 32°C after 12 hr to 18°C for 12 hr underwent 6 hr of light, 12 hr of dark, and 6 hr of light.

ACh and choline levels. After incubation of flies at the restrictive or permissive temperature for the indicated times, groups of 6 to 10 flies were etherized and decapitated with a scalpel under a dissecting microscope. Individual fly heads were immediately placed in small glass homogenizers along with 100 μ l of N formic acid:acetone mixture (15:85 v/v). After homogenization, the samples were briefly centrifuged (1000 $\times$ g for 5 min),

Figure 1. ChAT activity and ACh levels in *Drosophila* heads after various times of growth at 32°C. Bars represent the mean values for five individual 5-day-old flies for ACh determinations and triplicate assays of five pooled fly heads for ChAT activity. Zero hours at 32° refers to flies grown exclusively at 18°C, the permissive temperature for the mutants. Genotypes are: Canton S, *Cha*^{ts1}/*Cha*^{ts1}; TS₁, *Cha*^{ts1}/*Cha*^{ts1}; TS₂, *Cha*^{ts2}/*Cha*^{ts2}. For further details see "Materials and Methods."

Figure 2. ACh and choline levels in *Drosophila* heads after various times of growth at 32°C. Bars represent the mean values for four or five individual 6-day-old flies. The last set of bars in each panel (12H, 12L) refers to flies grown for 12 hr at 32°C followed by 12 hr at 18°C to test for recovery. For further details see Figure 1 and "Materials and Methods."

Figure 3. Relationship of ACh, choline, and ChAT activity. Data from Figures 1 and 2 were pooled and tested for correlation by least squares linear regression analysis. ACh versus ChAT activity is highly significant, whereas no significant correlation was found for choline versus ChAT or ACh versus choline.

Figure 4. Thin layer isoelectric focusing of *Drosophila* ChAT. Samples were 50 μl of freshly prepared homogenates applied directly to the gel in adjacent lanes at the position indicated by the line above the genotype. The gel was a 10 × 12.5 cm 5% polyacrylamide slab containing 2% pH 3 to 10 Bio-Rad Biolyte Ampholines. Focusing was at 4°C for 120 min at a constant power of 7 W. After focusing, the gel lanes were separated and sliced into 0.5-cm sections. The pH (a) was determined on two separate lanes, as described under "Materials and Methods," and did not vary by more than 0.05 pH unit. Enzyme activity was determined on duplicate aliquots of each gel slice and the highest value was arbitrarily set equal to 1. Recovery of applied enzyme activity was 50% for Canton S Cha^+/Cha^+ (b) and approximately 20% for Cha^{ts1}/Cha^{df} (c) and Cha^{ts2}/Cha^{df} (d).

and a measured aliquot (80 μl) of the supernatant was removed and placed in a clean tube and reduced to dryness in a vacuum centrifuge. The sample at this point could be stored indefinitely at -20° or -70°C . The dried samples were reconstituted with 0.1 N HCl (20 μl), and at least two aliquots, usually 4 μl, were removed from each sample for subsequent choline and ACh assays, respectively. The HCl solutions along with appropriate choline or ACh standards (freshly prepared in 0.1 N HCl) were again taken to dryness. The microradiometric assays for choline and ACh have been described previously (Goldberg and McCaman, 1973, 1974). The assays of the present study utilized the more recent modifications (McCaman and Stetler, 1977) that provide an enhanced sensitivity, about 30 to 40 fmol. From preliminary tests we determined that choline or ACh measurements in single fly heads, or some smaller fraction thereof, did not require the use of tetraphenylboron

TABLE I
Michaelis constants (K_m) for wild type and mutant ChAT

Genotype	K_m^a	
	Choline	Acetyl-CoA
<i>Cha</i> ⁺ / <i>Cha</i> ⁺	$6.8 \pm 0.4 \times 10^{-3}$	$1.1 \pm 0.2 \times 10^{-4}$
<i>Cha</i> ^{ts1} / <i>Cha</i> ^{ts1}	$6.0 \pm 0.6 \times 10^{-3}$	$1.6 \pm 0.8 \times 10^{-4}$
<i>Cha</i> ^{ts2} / <i>Cha</i> ^{ts2}	$3.4 \pm 1.1 \times 10^{-3}$	$0.8 \pm 0.6 \times 10^{-4}$

^a Values were determined by least squares analysis of two Lineweaver-Burke plots of ChAT activity assayed at 37° C for 15 min. At least five concentrations of choline (from 3×10^{-2} to 2×10^{-3} M) and acetyl-CoA (1.5×10^{-3} to 5.4×10^{-5} M) were assayed in duplicate for each Lineweaver-Burke plot.

TABLE II
Isoelectric points of wild type and mutant ChAT

Genotype	pl ^a
<i>Cha</i> ⁺ / <i>Cha</i> ⁺	4.91 ± 0.15 (9), 5.49 ± 0.11 (9)
<i>Cha</i> ^{ts1} / <i>Cha</i> ^{ts1}	5.46 ± 0.18 (9)
<i>Cha</i> ^{ts2} / <i>Cha</i> ^{ts2}	5.58 ± 0.17 (4), 6.24 ± 0.24 (4)

^a Values represent the mean $\pm$ the standard error.

^b Numbers in parentheses, number of samples.

Figure 5. *In vitro* thermolability of different pI forms of ChAT. The different pI forms of ChAT isolated by polyacrylamide thin layer isoelectric focusing in Figure 4 were tested separately for *in vitro* thermolability. Samples were enzyme activity recovered from gel slices 3 (■) and 8 (○) for Canton S, representing the low and high pI forms of the enzyme; gel slices 7 (●) and 10 (Δ) representing the low and high pH limits of the single pI form observed for *Cha*^{ts1}; and gel slices 7 (▲) and 11 (□) representing the low and high pI forms for *Cha*^{ts2}. Samples were assayed in duplicate for 10 min at 37° C as described under "Materials and Methods."

for prepurification of the tissue extract (Goldberg and McCaman, 1974). The amount of choline and ACh measured in a given extract was directly proportional to the amount of tissue over the range of sample size used for this study (0.002 to 1 head). In two experiments we determined that individual brains dissected free of the head case in *Drosophila* Ringer (thanks to Dr.

K. Ikeda) contained the same amount of ACh as measured in homogenates of whole heads. In five comparative experiments with Canton S flies, no significant differences were detected between male versus female flies raised under identical conditions. Only a few preliminary assays were performed on whole flies, and from these we concluded that: (a) measurement of choline and ACh in these samples required the more cumbersome prepurification procedure with tetraphenylboron, and (b) heads appear to contain about 15% and 35% of the choline and ACh, respectively, present in whole flies.

ChAT and acetylcholinesterase (AChE) activity. For measurements of enzyme activity to be correlated with ACh and choline levels, parallel groups of 6 to 12 flies were etherized and decapitated; then, the heads were pooled and homogenized at 4° C in 200 μ l of 50 mM Tris, pH 7.4, 0.1 M NaCl, 0.5% Triton X-100, using a motor-driven glass-Teflon homogenizer. ChAT activity was determined in triplicate on an aliquot of homogenate by the procedure of Fonnum (1975), as described by Slemmon et al. (1982). AChE activity was determined in separate groups of flies by the assay of Ellman et al. (1961), using acetylthiocholine substrate. All enzyme assays were performed at 37° C under conditions of linearity with time. For ChAT activity in the *Cha*^{ts1} and *Cha*^{ts2} mutants, the activity present at zero time was calculated by correcting for the loss of enzyme activity during the assay incubation at 37° C (usually 10 to 30 min).

Isoelectric focusing. Isoelectric focusing of wild type and mutant ChAT was performed using a thin layer polyacrylamide slab gel and the Bio-Rad 1415 electrophoresis system. The gel (10 $\times$ 12.5 cm) contained 5% acrylamide, 0.15% *N,N'*-methylene-bisacrylamide, and 2% Biolyte Ampholines (either pH 3 to 10 or pH 4 to 6). Samples (25 to 50 μ l) of either homogenates or redissolved polyethylene glycol precipitates (as described by Slemmon et al., 1982) were applied directly to the gel near the cathode. Gels were focused at 4° C using a constant power of 6 to 10 W for 90 to 180 min. Immediately after focusing, gel lanes were separated and sliced into 0.5-cm sections. The individual gel sections were soaked in either 1 ml of 1 M NaCl for pH determination at 4° C or 250 μ l of 50 mM PO₄ buffer, pH 7.4, containing 0.05% Triton X-100, 1 mM dithiothreitol, and 1% bovine serum albumin for enzyme assay on the following day. Twenty-microliter aliquots of the latter samples were assayed in duplicate or triplicate for ChAT activity, and recovery of applied enzyme activity ranged from 10 to 50%.

Immunoblotting. DEAE-purified or redissolved polyethylene glycol precipitates (Slemmon et al., 1982) were electrophoresed in SDS-polyacrylamide slab gels (Laemmli, 1970). The electrophoresed proteins were transferred to nitrocellulose sheets, as described by Towbin et al. (1979), using 250 to 350 mA of current for 12 to 18 hr. The nitrocellulose blots were stained for protein using 0.1% amido black or antigen as described. For detection of ChAT antigen, nitrocellulose blots were first soaked in 3% gelatin or bovine serum albumin in 50 mM Tris-150 mM NaCl, pH 7.4, for 1 hr at 37° C to block nonspecific binding of antibodies. Following several rinses in Tris-NaCl, the nitrocellulose was incubated in a 10 μ g/ml solution of monoclonal anti-*Drosophila* ChAT antibody (Crawford et al., 1982) in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonident P-40 for at least 2 hr at room temperature. The antibody solution was removed and the nitrocellulose was rinsed with several changes of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100. Anti-ChAT antibody was detected by incubating first in a rabbit anti-mouse IgG antibody and rinsing as above, followed by 1×10^6 cpm of [¹²⁵I]protein A. The nitrocellulose was air-dried and radioautographed at -80° C using Kodak x-ray film and a DuPont Cronex intensifying screen.

Results

ChAT, ACh, and choline. Figure 1 shows the levels of ChAT activity and ACh measured in 5-day-old homozygous Canton S and

Figure 6. Western blot of DEAE-purified Canton S ChAT using a monoclonal anti-ChAT antibody. Lanes 1 and 2 are the Coomassie blue-stained 10% polyacrylamide gel, and lanes 3 and 4 are the radioautograph (18-hr exposure) of a nitrocellulose replica of the gel stained as described under "Materials and Methods." Lane 1 shows molecular weight standards with M_r indicated in kilodaltons (K). Lane 2 is the sample (20 μ l) of DEAE-purified ChAT estimated to contain 0.2 mg of ChAT protein based on a final specific activity for pure *Drosophila* ChAT of 500 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg (Slemmon et al., 1982). Lane 3 is the replica of lane 2, and lane 4 is the replica of lane 1 after transfer to nitrocellulose and staining with anti-*Drosophila* ChAT antibody 1G4 (Crawford et al., 1982). The two bands staining in lane 3 have an M_r = 67 K and 54 K. No bands were observed to stain when the monoclonal anti-ChAT antibody was eliminated from the incubation sequence or when an irrelevant monoclonal antibody of the same IgG subclass was substituted for the anti-ChAT antibody (data not shown). Neither band detected by antibody staining in lane 3 was visible by Coomassie staining of lane 2. ChAT represents less than 0.8% of the total protein at this stage of purification.

Cha^{ts1} and *Cha*^{ts2} mutant heads. Both parameters increased about 15% in Canton S flies held at 32°C for 24 hr when compared to flies held at 18°C. In contrast, there was a progressive decrease in ChAT and ACh levels in both *Cha*^{ts1} and *Cha*^{ts2} mutants held at 32°C. The values decreased for both parameters, about 50% for *Cha*^{ts1} and 75% for *Cha*^{ts2} after 24 hr at 32°C. Note also that *Cha*^{ts1} and *Cha*^{ts2} have 25% and 50% less ChAT and ACh initially than do wild type flies. Figure 2 shows data from another experiment in which ACh and choline levels were measured in individual fly heads under the same conditions as in Figure 1. In addition, the recovery of ACh levels to their control values were seen after holding flies at 32°C for 12 hr and then transferring them back to 18°C for 12 hr. ChAT activity also recovered to control levels (data not shown). The temperature-induced changes in ACh and ChAT are thus reversible after 12 hr at 32°C. In contrast to the changes observed for ACh levels and ChAT activity, choline levels did not show a predictable change when flies were held at 32°C for 12 or 24 hr. Choline levels increased 30% to 40% in Canton S and *Cha*^{ts2} flies held at 32°C for 12 hr and in *Cha*^{ts1} flies held at 32°C for 24 hr. Choline levels at other times appeared to remain unchanged from flies held exclusively at 18°C.

In Figure 3 the combined data for ChAT, ACh, and choline were tested for correlation. A strong linear correlation was observed for ChAT versus ACh ($r = 0.992$), whereas no significant correlation was found for ChAT versus choline ($r = 0.450$) or choline versus ACh ($r = 0.372$). The correlation between ACh and ChAT activity held over a range of 8- to 10-fold variation for both parameters.

ChAT activity thus appears to be a good predictor of ACh levels in *Drosophila* heads, whereas choline levels are not.

AChE. The levels of AChE were also determined in flies under conditions identical to those in Figure 2. No change in AChE activity was observed for wild type or *Cha* mutant flies in any group, except for a 30% decrease in AChE activity for *Cha*^{ts2} flies held at 32°C for 24 hr. Variable AChE activity thus is not likely to account for the increased ACh levels observed in Canton S flies held at 32°C for 12 and 24 hr or, more importantly, for the decreased levels of ACh seen in both *Cha* mutants.

Behavioral abnormalities. As noted by Greenspan (1980), mutant *Cha* flies raised at elevated temperature showed the behavioral abnormality of paralysis. This paralysis could be related to the levels of ACh or ChAT activity in the flies after shifting them to the restrictive temperature. After 12 hr at 32°C, 14 of 20 *Cha*^{ts2}/*Cha*^{ts2} flies showed abnormal movements similar to those described by Greenspan (1980), whereas all *Cha*^{ts2} flies were paralyzed after 24 hr at 32°C. The *Cha*^{ts1}/*Cha*^{ts1} flies showed no abnormal movements after 12 hr at 32°C, whereas 16 of 22 were paralyzed after 24 hr. Of 12 *Cha*^{ts2}/*Cha*^{ts2} flies grown initially at 32°C for 12 hr and then shifted to 18°C for 12 hr, 10 recovered completely, whereas 2 flies were still partially paralyzed (i.e., legs were moving but flies were not walking or flying). The paralytic behavioral abnormalities thus seem to be present when ACh levels drop below 7 or 8 pmol/head.

Kinetic constants for ChAT. We have determined the Michaelis constants for both substrates of ChAT. The values are shown in Table I. Canton S and *Cha*^{ts1} have similar K_m values for both choline and acetyl-CoA. *Cha*^{ts2} shows K_m 's of approximately one-half the

Figure 7. Western blot of wild type and mutant ChAT. Redissolved polyethylene glycol precipitates of ChAT (Slemmon et al., 1982) were electrophoresed in a 10% polyacrylamide gel. The gel was blotted onto nitrocellulose and stained with monoclonal anti-ChAT antibody 1C8 (Crawford et al., 1982) and [125 I]protein A as described under "Materials and Methods." Samples were calculated to contain 32 ng of Canton S (lane 1) or Cha^{ts1} ChAT (lane 2) and 1 ng of Cha^{ts2} ChAT (lane 3) based on a final specific activity of $500 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for pure ChAT and correcting for temperature inactivation during assay. The molecular weight of the stained bands was determined from the same molecular weight standards used in Figure 6. The radioautograph was exposed for 18 hr.

values for Canton S and Cha^{ts1} for both substrates. Cha^{ts1} thus appears kinetically similar to the wild type enzyme, whereas Cha^{ts2} appears to be a slightly more efficient enzyme.

Isoelectric focusing. We have previously reported two isoelectric focusing forms for partially purified Canton S ChAT (Slemmon et al., 1982). Figure 4 shows the results of isoelectric focusing on freshly prepared homogenate of Canton S flies and compares this pattern to the pattern observed for ChAT from both Cha^{ts} mutants. Canton S has two distinct isoelectric forms, whereas Cha^{ts1} shows only a single form. Isoelectric focusing of Cha^{ts2} most often showed the pattern seen in Figure 4d, with a major and minor form, similar to wild type pattern, but with both forms shifted to a higher pI. In three

experiments only the lower pI form was observed for Cha^{ts2} . The reason for these variable results with Cha^{ts2} is unknown. Table II summarizes the pI's observed in a number of focusing experiments in which different sample or pH gradients were used. Samples included homogenates or polyethylene glycol-precipitated ChAT and were prepared fresh or frozen at -20° or -80°C for 1 day or 3 weeks. Samples were also focused on pH 4 to 6 gradients or pH 3 to 10 gradients. In general, no systematic differences were observed in the pI's or relative amounts of ChAT activity recovered for each pI form, with the exception of the occasional absence of any detectable higher pI form for Cha^{ts2} (three of seven experiments).

In vitro thermolability of high and low pI forms of ChAT. Since Cha^{ts1} showed a pI similar to the high pI form of Canton S, the thermolability of the different pI forms of ChAT was tested after separating them by thin layer polyacrylamide isoelectric focusing. As shown in Figure 5, no significant differences were observed for the temperature lability of the high and low pI forms of Canton S or Cha^{ts2} ChAT. In addition, no differences were noted in the temperature inactivation of Cha^{ts1} ChAT sampled from the high and low pH region of the single ChAT pI form. Different pI forms of ChAT thus have no effect on the *in vitro* thermolability of the enzyme.

Immunoblot analysis of ChAT. We have determined the polypeptides reacting with a monoclonal anti-ChAT antibody (Crawford et al., 1982) by Western blot analysis (Towbin et al., 1979). Figure 6 shows polypeptides staining with antibody at 67,000 and 54,000 daltons for DEAE-purified (Slemmon et al., 1982) Canton S ChAT. Neither band was visible in the Coomassie blue-stained gel of this sample. These two bands correspond in molecular weight to two of the polypeptides we have identified as structurally related proteins in highly purified *Drosophila* ChAT (Slemmon et al., 1982). A third polypeptide at 13,000 daltons present in pure ChAT is not stained by the monoclonal antibody. Since this antibody will directly inhibit ChAT activity, it most probably is recognizing an epitope at or near the active site of the enzyme. The 67,000- and 54,000-dalton proteins therefore probably contain the catalytic site.

Figure 7 shows the results of an immunoblot analysis of redissolved polyethylene glycol precipitates of ChAT from Canton S and both Cha^{ts} mutants. Note that only one band at 67,000 daltons was detected for either Cha^{ts} mutant in contrast to two polypeptides (67,000 and 54,000) for Canton S ChAT. For this gel, an equal amount of ChAT protein, calculated by activity, was electrophoresed for Canton S and Cha^{ts1} , whereas 16-fold less ChAT protein was applied for the Cha^{ts2} mutants to avoid overloading the gel. The polypeptide pattern observed for Cha^{ts1} is clearly different from wild type by the absence of any detectable 54,000-dalton protein, whereas Cha^{ts2} is apparently different from wild type. The presence of a 54,000-dalton component in Cha^{ts2} cannot be ruled out definitively at this time since a smaller amount of ChAT had to be electrophoresed to prevent overloading of the gel with non-ChAT proteins.

Discussion

We have taken advantage of two temperature-sensitive ChAT mutants described initially by Greenspan (1980) to manipulate ChAT activity *in vivo* in adult *Drosophila melanogaster* and to relate ChAT activity to neurotransmitter levels. An increase in ChAT activity for wild type Canton S flies or a decrease for either *ts* mutant was observed for flies reared at 32°C rather than 18°C . Surprisingly, a very strong correlation was observed between ChAT activity and ACh levels. This correlation held over an 8- to 10-fold variation in the absolute amounts of enzyme activity or transmitter content. ChAT activity may thus be an important regulatory and/or rate-limiting step for *in vivo* ACh synthesis. Choline levels did not vary in any regular pattern with respect to either ChAT activity or ACh levels. Thus, the steady-state choline level per se does not seem to participate in the regulation of ACh synthesis in *Drosophila* under the conditions of our experiments. Our observations are in contrast to most studies of the relationship of ChAT activity to ACh levels in vertebrate

Proposed model for *Drosophila* ChAT

Figure 8. Proposed structural model for *Drosophila* ChAT. Native wild type enzyme exists in two forms. One is a single chain 67-kilodalton (K) polypeptide and the other is a noncovalent complex of a 54- and 13-kilodalton polypeptide which could have been generated by a post-translational event (limited proteolysis) occurring at the arrow. These two forms represent different pI forms of ChAT. In SDS-denatured ChAT the 67- and 54-kilodalton peptides stain with active site-directed antibody (solid bars) which should thus contain both the antibody-binding epitope and the enzyme-active site.

nervous system (for review, see Jope, 1979). In these other studies, it has been argued that ChAT activity is far in excess of that necessary to regulate the steady-state levels of ACh (Hebb, 1972; Tucek, 1983). High affinity choline transport into nerve endings seems to represent the key regulatory site for ACh production in several of these other studies (Kuhar and Murrin, 1978). Since we have not directly measured the high affinity choline transport, we cannot directly rule out its significance as a regulatory step in *Drosophila* ACh production. Perhaps the difference between our results and the conclusions reached by others reflects species difference or differences in the specific procedures used to manipulate various potential control sites for ACh production. One obvious difference between our experimental paradigm and those of the other studies is that we rely on no external pharmacological manipulation of uncertain specificity to alter ChAT activity. In our analysis temperature is used as the only procedure to alter ChAT activity. Temperature-dependent alteration in ChAT activity may be relatively specific since we have observed no change in AChE, another important ACh-metabolizing enzyme present in *Drosophila*. Other studies which have sought to inhibit ChAT activity by administering an exogenous ChAT inhibitor (Krell and Goldberg, 1975) have been criticized for their failure to adequately detail side effects of such treatment on AChE activity and/or use-dependent changes in ACh synthesis and release.

Several interesting features of ACh metabolism and utilization in *Drosophila* deserve comment. The heads of this organism contain the highest reported levels of ChAT activity for any biological source. It may be that in *Drosophila*, and possibly other insects, the numbers of neural elements using ACh are relatively greater than those using other neurotransmitters. We have recently observed the immunocytochemical distribution of ChAT in *Drosophila* CNS (K. Ikeda and P. M. Salvaterra, unpublished observation). The pattern of staining appears to be especially intense in the neuropil of the visual ganglia and other sensory areas such as the antennal complex. This distribution lends support to the long held suspicion that ACh may be a primary sensory neurotransmitter in arthropods. Although ACh synthesis and release have recently been studied in *Drosophila* larvae (Wu et al., 1983), the absolute levels of ACh and choline were not reported. In the present study ACh levels were found to vary from about 20 to 40 pmol/head. Since a *Drosophila* head weighs approximately 0.1 mg, the concentration of ACh would be between 200 and 400 nmol/gm of tissue, or approximately 0.5 mM. This level of ACh is among the highest reported values for tissue ACh concentration and is one or two orders of magnitude higher than the ACh levels reported for vertebrate CNS (Saelens and Simke, 1976). In fact, this level of ACh is similar to the concentration of ACh reported for isolated individual cholinergic somata from various invertebrates (McCaman and McCaman, 1976). The levels of ACh and choline

are about equal in *Drosophila* heads. The ratios of choline to ACh in vertebrate nervous system have been reported to vary from 1:1 to nearly 10:1, depending on the assay, sacrifice, or analysis methods used. In addition, distinct brain regional differences in this ratio have been noted in vertebrates (Saelens and Simke, 1976). Perhaps this reflects the concentration of cholinergic neurons and/or endings in a given region. Choline would be expected to be in all regions of the brain in which it serves its other functional roles not related to neurotransmission, such as phospholipid synthesis. ACh would be expected to be only in brain regions containing cholinergic neurons. It should also be noted that the ratio of ACh to choline has been reported to be 1:1 in individual cholinergic somata (McCaman and McCaman, 1976).

We have recently proposed a model for purified *Drosophila* ChAT (Slemmon et al., 1984) which is summarized diagrammatically in Figure 8. In this model the single structural gene for ChAT (Greenspan, 1980) would code for the synthesis of a single chain polypeptide with $M_r = 67,000$. This 67,000 dalton polypeptide would then undergo post-translational modification by limited proteolysis to generate another form of the enzyme activity consisting of two noncovalently complexed polypeptides with $M_r = 54,000$ and 13,000. Similar tryptic peptides present in all three polypeptides in purified ChAT strongly support this model (Slemmon et al., 1984). In the present study we have presented evidence that both the 67,000- and 54,000-dalton polypeptides present in Canton S ChAT stain with a monoclonal antibody that directly inhibits the enzyme activity. This observation is consistent with the notion that the single-chain 67,000-dalton ChAT and the noncovalent complex of 54,000 + 13,000 daltons are both enzymatically active and most likely represent the two different pI forms we have observed for wild type Canton S ChAT. Unfortunately, we have not been able to establish this point more directly by running two-dimensional gels since isoelectric focusing interferes with the ability of our monoclonal antibodies to recognize ChAT.

Greenspan (1980) has presented evidence that only one gene is present for ChAT in *Drosophila*; therefore, a post-translational modification is likely to account for multiple pI forms. Further support for this hypothesis is the absence of any detectable 54,000-dalton antibody-staining component in the *Cha*^{ts1} mutant and the presence of only a single detectable pI form for *Cha*^{ts1}. We would thus predict that the mutation in *Cha*^{ts1} is a replacement of the particular amino acid residue where proteolysis occurs in wild type ChAT. Although the nature of the *Cha*^{ts1} mutation confers thermolability on the ChAT protein that is produced, lack of proteolytic processing does not in itself lead to a thermolabile enzyme since no differences were noted in the temperature inactivation of the different pI forms of ChAT. As mentioned under "Results," the sensitivity of our immunoblot analysis may not have been adequate to detect the presence of a 54,000-

dalton component in *Cha*^{ts2}. The significance of the proposed limited proteolysis for ChAT function remains unknown. We are attempting to isolate the gene for ChAT from *Drosophila*, and once this is accomplished, it will be possible to perform direct tests on the functional significance of proteolysis.

In summary, we have taken advantage of the production of two temperature-sensitive *Cha* gene mutants to investigate ACh metabolism *in vivo* and to study the structural and biochemical properties of the mutant ChAT gene products. The existence of these conditional mutants makes it possible to regulate neurotransmitter production in a direct and specific manner and should be useful for a variety of future biochemical and physiological studies of ACh metabolism and cholinergic processes by allowing them to come under experimental genetic control (Hall and Greenspan, 1979; Hall et al., 1979).

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