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ACETYLCHOLINESTERASE ISOZYMES FROM THE HOUSEFLY BRAIN

M. E. ELDEFRAWI, R. K. TRIPATHI AND R. D. O'BRIEN
Section of Neurobiology and Behavior, Cornell University, Ithaca, N.Y. (U.S.A.)

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SUMMARY

1. Cholinesterase activity in insect head extracts was investigated by disc electrophoresis followed by spectrophotometric analysis. The supernatant of 100 000 × *g*, 1 h, revealed the presence of four isozymes in the housefly, two in the American cockroach and only one in the southern army worm.

2. The four isozymes, present in the head extract of houseflies, were totally inhibited by 10 μM guthoxon or eserine but were unaffected by 10 μM *p*-chloromercuribenzoate (PCMB) or tri-*o*-cresylphosphate. All four isozymes had higher activity with acetylthiocholine than butyrylthiocholine as substrates; indicating they are isozymes of acetylcholinesterase.

3. Centrifugation at 200 000 × *g* for 4 h precipitated 66% of the acetylcholinesterase originally present in the supernatant of 100 000 × *g* for 1 h but the four isozymes remained soluble and were estimated to have molecular weights of less than 500 000.

4. Two methods of gel staining were used to detect esterase activity, the direct-coloring thiocholine method using acetylthiocholine as a substrate and the modified Gomori method using α-naphthylacetate. The first proved to be effective for detecting cholinesterase activity; but at least two of the eserine-sensitive bands found by the technique of GOMORI[20] were not cholinesterase.

INTRODUCTION

The term "cholinesterase" will be used as a generic term to cover both acetylcholinesterase and butyrylcholinesterase. Cholinesterase from various sources has usually been treated as a single molecular form. Since the pioneering work of MARKERT AND MÖLLER[1] on lactate dehydrogenase isozymes, a large number of enzymes have been shown to exist in multiple molecular forms[2]. A purified preparation of serum butyrylcholinesterase was shown to have two components that were separable electrophoretically, chromatographically and by ultrafiltration[3,4]. These forms were inhibited at different rates by organophosphates. Recently seven molecular forms were isolated

Abbreviation: PCMB, *p*-chloromercuribenzoate.

from serum butyrylcholinesterase, and they were all interconvertible and inhibited by eserine[5]. It was also shown, by kinetic evidence, that serum butyrylcholinesterase, as well as erythrocyte acetylcholinesterase, were each made up of more than one form; varying significantly in their reaction with organophosphates[6]. Three acetylcholinesterase isozymes from heads of face flies, *Musca antumnalis* De Geer[7], three from the nerve cords of the cricket *Acheta domesticus* (L.)[8] and ten from rat retina[9] were also shown electrophoretically.

The brain of the housefly was found to be rich in cholinesterase[10], and its extract was used to measure the potency of organophosphate and carbamate anticholinesterases. The preparations ranged from crude homogenates[10–12] to partially purified extracts[13,14], and the cholinesterase present was always treated as a single form. The present study was designed to investigate mainly the cholinesterase of the housefly brain and to see whether it is present as a single or multimolecular form.

METHODS

Extraction

Heads of houseflies, *Musca domestica* L., Wilson strain, susceptible to insecticides, were harvested by the method of MOOREFIELD[15] and homogenized in a Sorvall Omni-Mixer (Norwalk, Conn.) as a 20% (w/v) aqueous suspension. The homogenate was filtered through cheesecloth and centrifuged at 100 000 × *g* for 60 min at 4°. The supernatant fraction was stored frozen at −18° and used over months without loss of activity.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in the analytical model, Canalco (Rockville, Md.), using 7% separating gel and a Tris–glycine buffer (pH 8.3). The formation of a sample gel was bypassed and the sample, instead, layered on top of a 4% spacer gel[16]. Electrophoresis was performed at 4° and 2.5 mA per column.

Enzyme assay

The gel column was frozen onto the stage of a tissue slicer and sectioned 0.5-mm sections. The enzyme activity in each section was measured by the method of ELLMAN[17] as described previously[18]. A minimum of three runs were performed, each consisting of six gel columns, two of which were randomly selected for sectioning and further testing.

Staining

The direct-coloring thiocholine method for cholinesterase[19], which utilizes acetylthiocholine as a substrate, was used to localize enzyme activity in the gel. Also employed was the GOMORI[20] method, modified by TRIPATHI AND DIXON[21], for determining esterase activity using α-naphthylacetate as a substrate in the gel. The gels were incubated for 1.5 h at room temperature with continuous shaking for cholinesterase and 10 min for esterase activity. Amido black in 7% acetic acid was used to stain proteins. A minimum of ten replicates were made for each test.

TABLE I

CHOLINESTERASE ACTIVITY OF THE EXTRACTS OF HOUSEFLY HEADS

Fraction	*μmoles acetylcholine hydrolyzed/min per ml*	% *Activity*
Total homogenate	0.074	100
Supernatant of 100 000 × *g*, 60 min	.025	34
Supernatant of 200 000 × *g*, 240 min	.010	13.5

RESULTS

Cholinesterase activity in extracts of housefly brain was assayed spectrophotometrically and compared with those in similar extracts of the American cockroach, *Periplaneta americana* (L.), and the southern army worm, *Prodenia eridania* (Cramer). Whereas the head extract of the housefly contained four electrophoretically separable peaks of activity, the American cockroach showed two and the army worm only one (Fig. 1). Extracts from the three species also showed a peak of strong cholinesterase activity that remained on top of the spacer gel, presumably due to particles which were too large to penetrate the gel. These peaks do not appear in Fig. 1, which shows only the separating portion of the gel column. To separate the soluble and particulate fractions present in the housefly head supernatant of 100 000 × *g* for 1 h, the latter was centrifuged at 200 000 × *g* for 4 h. The resultant supernatant, expected to retain molecules with molecular weights below 500 000 (S value below 13.5), contained 40% of the cholinesterase activity as measured by a pH-stat (Table I). Assayed electrophoretically, no activity appeared at the origin, and all activity was in the same four peaks in the gel, indicating that the cholinesterase forms were all relatively small molecules.

To characterize the esterases present in the housefly head supernatant of 100 000

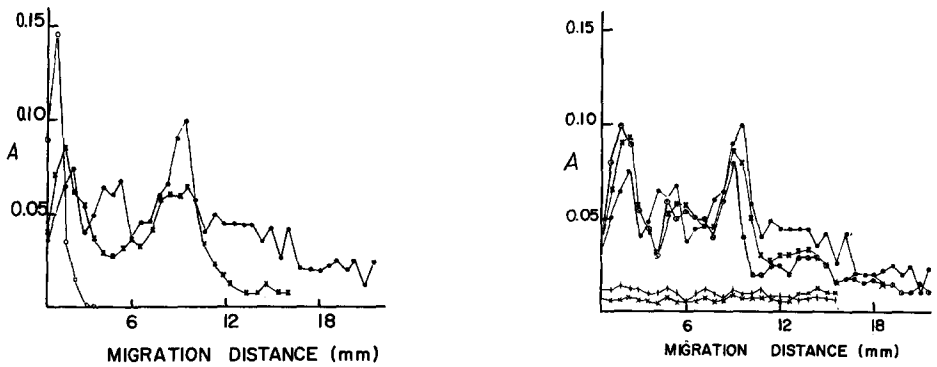


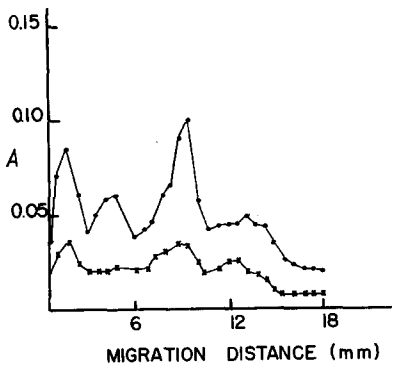
Fig. 1. Spectrophotometric assay of cholinesterase activity in sectioned gel columns containing the electrophoresed supernatants of 100 000 × *g*, 1 h, of head extracts of three insect species. ●—●, Musca; ×—×, Periplaneta; ○—○, Prodenia.

Fig. 2. The effect of various esterase inhibitors on the activity of the acetylcholinesterase isozymes of the housefly brain. ●—●, no inhibitor; ○—○, tri-*o*-cresylphosphate; ×—×, PCMB; ●—●, guthoxon; x—x, eserine.

$\times g$ for 1 h, the effect of several inhibitors was studied by preincubating each gel section for 15 min in a final concentration of 10 μM solution of the inhibitor before transferring it to the substrate solution. As shown in Fig. 2, guthoxon and eserine, both inhibitors of butyrylcholinesterase (EC 3.1.1.8) and acetylcholinesterase (EC 3.1.1.7), totally inhibited the four peaks. On the other hand, PCMB, an inhibitor of arylesterase (EC 3.1.1.2), and tri-*o*-cresylphosphate, an inhibitor of aliphatic or carboxyesterase[22,23] (EC 3.1.1.1), had no effect. This indicated that all four forms were cholinesterases. Their activity was tested on different substrates: acetylthiocholine, on which acetylcholinesterase is more active, and butyrylthiocholine, on which butyrylcholinesterase is more active. All four enzyme forms acted as acetylcholinesterases in that they showed higher activity with acetylthiocholine than butyrylthiocholine (Fig. 3). Additional evidence was obtained by use of iso-OMPA at 0.1 mM; this concentration inhibits butyrylcholinesterase but not acetylcholinesterase[24,25] (confirmed experimentally by the pH-stat method for horse serum butyrylcholinesterase and erythrocyte acetylcholinesterase). None of the four housefly enzyme forms was inhibited.

It was of interest to localize the cholinesterase enzymatic activity in the gel with an alternative technique, namely, direct staining of the gel (without slicing) by the two methods mentioned above. Using acetylthiocholine as a substrate, zymograms of the extract of the housefly head showed the same four peaks previously found by the spectrophotometric assay, and the four inhibitors had the same effects (Fig. 4). However, when α-naphthylacetate was used as a substrate, seven zones with esterase activity appeared in the gel. PCMB and tri-*o*-cresylphosphate had no effect on any of these esterases, but guthoxon inhibited five and eserine three of the latter.

When a gel column was split longitudinally, and half was stained using acetylthiocholine as a substrate and the other using α-naphthylacetate, and then the two halves matched, it was shown that the zones with esteratic activity were not quite



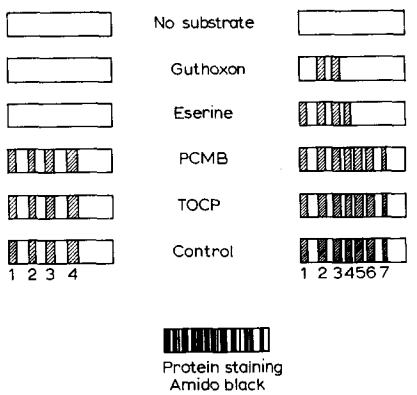


Fig. 3. The activity of the acetylcholinesterase isozymes of the housefly brain on two substrates, acetylthiocholine (●—●) and butyrylthiocholine (×—×).

Fig. 4. The electrophoretic pattern of esterase activity in the housefly brain extract and the effect of four inhibitors. Zymograms on the left are developed with the thiocholine method (acetylthiocholine as a substrate) and on the right with the modified GOMORI[20] method (α-naphthylacetate as a substrate). Gel at bottom is stained with amido black to show protein pattern. TOCP = tri-*o*-cresylphosphate.

superimposed. Staining another gel column with amido black resulted in the appearance of several closely packed proteins. Each stained zone of esteratic activity, shown by acetylthiocholine or α-naphthylacetate, usually covered an area occupied by more than a single protein (Fig. 4).

DISCUSSION

According to MARKERT[26], "once the accepted criteria for defining a collection of molecules as an enzyme have been successfully applied, and if these molecules can be separated into distinguishable types by any means (electrophoretic, chromatographic, solubility, immunochemical... *etc.*), then these separate types represent isozymic forms". The evidence presented above (Figs. 2 and 4) suggests the presence of four cholinesterase isozymes in the brain extract of the housefly. The effect of inhibitors and the higher activity shown by the four forms towards acetylthiocholine compared to butyrylthiocholine (Fig. 3) are evidence that they are in fact acetylcholinesterases. This agrees with earlier reports that the fly head cholinesterase is similar to the acetylcholinesterase of mammals[10,27] and that it is doubtful that a butyrylcholinesterase is present in the housefly. Acetylcholinesterase polymorphism has been observed in other insects. Using the thiocholine staining method on gel columns and the effect of eserine and organophosphate inhibitors, three acetylcholinesterase zones were resolved electrophoretically from the face fly[7] and three from the cricket *Acheta domesticus*[8].

Comparing the isozymes present in the brain extracts of the three insect species tested (Fig. 1), it appears that there are specific differences either in the nature of the isozymes or in the manner the brain tissues lent themselves to the extraction procedures.

It was suggested earlier that cholinesterase, present in homogenates of housefly heads, was distributed between a soluble and a particulate fraction[28]; the soluble fractions referred to in these studies were the supernatants of 26 000 × *g* and 50 000 × *g*. The present study showed that even the supernatant of 100 000 × *g* contained 66% of its activity in a particulate form, which sedimented upon further centrifugation (Table I) and failed to penetrate the gel upon electrophoresis. This agrees with recently published data, whereby 73% of the cholinesterase of the aqueous extract of housefly heads was retained in the 105 000 × *g* for 1 h supernatant[29]. The acetylcholinesterase of rat brain[30] and of the electric eel[31] were also found to occur in soluble and particulate forms. The existence of two forms may be due to the fact that acetylcholinesterase tends to form reversible aggregates in solution and distributes itself between them and the soluble fraction in proportions dependent upon the ionic strength and pH of the fluid.

The four isozymes of the housefly brain were present in the supernatant of 200 000 × *g* of 4 h and therefore had a molecular weight of less than 500 000, assuming that the particles were perfect spheres. An earlier determination of the molecular weight of a partially purified housefly cholinesterase was on the order of $3 \cdot 10^6$–$4 \cdot 10^6$ (ref. 13). That figure represents a value closer to the weight of the larger aggregates. A later determination of the soluble form was approx. 160 000 (ref. 32). The molecular weight of acetylcholinesterase extracted and purified from the electric eel Electrophorus was calculated to be 260 000 (ref. 33). It was also found that the latter enzyme

was made of four equal weight subunits, and two subunits were necessary to form a monomer with a single catalytic site; but the enzyme was present naturally in the dimer form[34]. The enzyme tended to form reversible aggregates in solution[31]. These findings raise the possibility that what is seen as isozymes in the present study may be nothing more than variable size aggregates of a single molecular form, separated by the sieving action of the gel. There is also the remote possibility that there exists *in vivo* only a single molecular form, which is acted upon by a proteinase present in the extract; but this is unlikely in view of the fact that all manipulations took place at 4°. To answer these questions, one should separate in pure form each of the proteins and determine their molecular weights, interconvertability in the presence of protein unfolding agents and substrate and inhibitor interactions. Such a goal is presently being pursued starting with their separation by preparative electrophoresis. This has been partially achieved for serum butyrylcholinesterase, whose seven multimolecular forms, separated electrophoretically, were found to be interconvertible either by concentration or by storage for 3 days at 10° (ref. 5).

The α-naphthylacetate method of GOMORI[20] has been used for measuring acetylcholinesterase in gels, on the assumption that the eserine-sensitive esterases were cholinesterases[12,13,35–38]. However, Fig. 4 shows that there are only three eserine-sensitive bands (No. 5, 6 and 7) revealed by α-naphthylacetate, whereas the acetylthiocholine stain reveals four, of which No. 1, 2, and 3 have mobilities clearly different from any of the α-naphthylacetate bands. The spectrophotometric method (Fig. 2) fully confirms the results obtained by gel staining. Consequently, the technique of GOMORI[20] is unsuitable for studying cholinesterases. Bands 6 and 7 must represent eserine-sensitive and PCMB-insensitive esterases which are not cholinesterase.

It has been shown that isozymes of horse serum butyrylcholinesterase and erythrocyte acetylcholinesterase varied significantly in their reaction with organophosphates[3,6]. It is thus possible that we would find similar differences amongst the four acetylcholinesterase isozymes of the housefly. An interesting question would then be whether selection pressure exerted by the use of an anticholinesterase insecticide would favor the dominance of the less sensitive isozymes. There is a precedent: cholinesterase of the Leverkusen organophosphate-resistant strain of mites, *Tetranychus urticae*, was found to be 1000 times less sensitive to organophosphates than that of the parent susceptible strain, and this contributed to its resistance[39]. Once the housefly acetylcholinesterase isozymes are isolated and their interactions with inhibitors studied, it will be interesting to compare them in susceptible and resistant strains.

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