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An acetylcholinesterase sensitive to sulfhydryl inhibitors

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SUMMARY

In contrast to acetylcholinesterases from mites, Mediterranean fruit fly heads, or bovine red blood cells, acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) of aphids was sensitive to *p*-chloromercuribenzoic acid (PCMB) and to 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The inhibition by DTNB was diminished by increasing the concentration of substrate and completely prevented by reduced glutathione. These results indicate that acetylcholinesterase from aphids contains an essential SH group located at the active site or at an area greatly influencing this site. The enzyme preparation from aphids contains at least two esterases, one a true acetylcholinesterase, the other enzyme probably being a pseudocholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8). Only the true acetylcholinesterase was sensitive to DTNB and to PCMB. The resemblance of aphid acetylcholinesterase to another component of the cholinergic system, the electroplax of the electric eel, is discussed with respect to SH inhibition.

Determination of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) activity in the green peach aphid *Myzus persicae* by the method of Ellman *et al.*[1] could be carried out only if the 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) reagent was added at the end of the reaction period instead of during the reaction. Because of the interference of DTNB with the acetylcholinesterase determination a sulfhydryl(SH)-sensitive group was suspected to be present at the active site of aphid acetylcholinesterase.

The postsynaptic membrane-bound acetylcholine receptor of electroplax of the electric eel *Electrophorus electricus* contains an SH-sensitive group at the acetylcholine binding site[2], but sensitivity of acetylcholinesterase to SH inhibitors has not yet been reported. In view of the possible unique properties of aphid acetylcholinesterase, we

Abbreviations: DTNB, 5,5-dithio-bis-2-nitrobenzoic acid; PCMB, *p*-chloromercuribenzoic acid.

studied the effect of SH inhibitors (DTNB and *p*-chloromercuribenzoic acid) on this enzyme. The results were compared with those obtained under identical conditions with acetylcholinesterases of spider mites, Mediterranean fruit flies and bovine erythrocytes.

Materials

The green peach aphid *Myzus persicae* (Sulz.) was reared on Chinese cabbage. A 5- to 7-day-old population containing alatiform and apteriform larvae was used. Methods for the rearing of the organophosphorus-sensitive "Amirim" strain of the carmine spider mite *Tetranychus cinnabarinus* (Boisduval) are described elsewhere[3]. The Mediterranean fruit fly (Medfly) *Ceratitis capitata* Wiedeman, was obtained from the Biological Control Laboratory of the Citrus Marketing Board, Rehovot, and fed in our laboratory on sugar and water only.

A soluble preparation of acetylcholinesterase from bovine erythrocytes, glutathione (GSH), oxidised glutathione (GSSG), *p*-chloromercuribenzoic acid (PCMB) and iodoacetate was obtained from Sigma Chemical Co. or Aldrich Chemical Co. Other details of materials are described in previous publications[4-6].

Preparation of particulate acetylcholinesterase from aphids

Two hundred and forty aphids were ground three times in a Potter–Elvehjem homogenizer in 6 ml of 0.134 M ice-cold sodium potassium phosphate buffer (pH 7.2) for 30 s and cooled on ice between grindings. The homogenate was filtered through ten layers of gauze and centrifuged at 9000 × *g* at 2 °C for 10 min. The particles were resuspended in 2.2 ml of the buffer solution.

Determination of acetylcholinesterase from aphids by the method of Hestrin[7]

A 0.1-ml sample of the particulate preparation (corresponding to about ten aphids) was incubated with 0.05 ml of the inhibitor at 30 °C for 10 min. Afterwards, 0.1 ml of 0.004 or 0.006 M acetylcholine were added and the reaction continued for 1 h. At the end of this period, unhydrolyzed acetylcholine was determined by the method of Hestrin[7].

Determination of acetylcholinesterase from aphids by the method of Ellman et al.[1]

A 0.1-ml sample of the aphid particulate preparation was diluted with 0.4 ml of 0.067 M sodium potassium phosphate buffer (pH 7.5) to maintain appropriate buffer concentration and pH. The reaction mixture and conditions of reaction were as described elsewhere[4]. However, DTNB was not included in the reaction mixture (except when used as inhibitor), but added only at the end of the 20-min reaction period in 0.05 ml of 0.067 M sodium potassium phosphate buffer (pH 7.5) to give a final concentration of $6 \cdot 10^{-4}$ M DTNB. This concentration of DTNB terminated the reaction completely when either acetylthiocholine or propionylthiocholine served as substrates. However, with butyrylthiocholine as substrate, the reaction continued in the presence of $6 \cdot 10^{-4}$ M DTNB. Unless otherwise stated, the reaction mixture minus substrate was preincubated

with the inhibitor, DTNB, PCMB or malaoxon (*O, O*-dimethyl *S*-(1,2-dicarbethoxy) ethylphosphorothiolate) for 10 min. Protein was determined by the method of Lowry *et al.*[8], using crystalline serum albumin as reference.

Preparation and determination of particulate acetylcholinesterase from mites or Medfly heads

Preparation of particles from mites and Medfly heads is described elsewhere (Zahavi and Tahori)[4,5]. Acetylcholinesterases from these particles and from bovine

TABLE I

INHIBITION OF ACETYLCHOLINESTERASE FROM *MYZUS PERSICAE* BY VARIOUS SH REAGENTS

Data are average of 3 to 5 experiments. In columns 1 and 2 acetylthiocholine was used as substrate, acetylcholinesterase activity was determined by the method of Ellman *et al.*[1] and specific activity measured in μmoles/mg protein per h was 8.0 and 8.8, respectively. In columns 3 and 4 acetylcholine was used as substrate. Acetylcholinesterase was determined by the method of Hestrin[7] and specific activity was 7.2 and 7.7, respectively.

Addition to reaction mixture (M)	*% Inhibition of acetylcholinesterase activity*			
	(1) Acetylthiocholine $(6 \cdot 10^{-2}$ *M)*	*(2) Acetylthiocholine* $(1.8 \cdot 10^{-3}$ *M)*	*(3) Acetylcholine* $(1.6 \cdot 10^{-3}$ *M)*	*(4) Acetylcholine* $(2.4 \cdot 10^{-3}$ *M)*
DTNB $1 \cdot 10^{-5}$	30 (5)*	21 (8)*		
DTNB $2 \cdot 10^{-5}$	63 (41)*	55 (23)*	55	47
DTNB $4 \cdot 10^{-5}$	83 (60)*	81 (54)*		
DTNB $5 \cdot 10^{-5}$	87		78	68
DTNB $1 \cdot 10^{-4}$	90	87	84	82
DTNB $3 \cdot 10^{-3}$	94	100		
DTNB $5 \cdot 10^{-5}$ + GSH $2 \cdot 10^{-4}$			2 (44)**	13
DTNB $1 \cdot 10^{-4}$ + GSH $2 \cdot 10^{-4}$			12 (63)**	
GSSG $5 \cdot 10^{-4}$			4	
GSSG $2 \cdot 10^{-3}$			30	
GSSG $2.5 \cdot 10^{-4}$ + GSH $1 \cdot 10^{-3}$			18	
Iodoacetate $5 \cdot 10^{-4}$			2	
Iodoacetate $2 \cdot 10^{-3}$			37	
PCMB $2 \cdot 10^{-7}$				15
PCMB $1 \cdot 10^{-6}$				43
PCMB $1.6 \cdot 10^{-6}$				53
PCMB $2 \cdot 10^{-6}$				67
PCMB $4 \cdot 10^{-6}$				88
PCMB $2 \cdot 10^{-5}$				94
PCMB $5 \cdot 10^{-5}$				94
PCMB $1 \cdot 10^{-4}$				94
PCMB $4 \cdot 10^{-4}$				100

* DTNB was added together with substrate without performing the standard 10-min preincubation period.

** The GSH was added after the standard 10-min preincubation period with DTNB.

erythrocytes were determined by the method of either Hestrin[7] or Ellman *et al.*[1] at conditions identical to those used for acetylcholinesterase from aphid particles. A 0.1-ml sample of the particulate preparation corresponding to 200 mites or 0.3 Medfly heads was used in the determination by the method of Hestrin[7] and a sample at half this concentration by the method of Ellman *et al.*[1].

Results and Discussion

Table I shows that aphid acetylcholinesterase was sensitive to PCMB and DTNB and only slightly to GSSG or to iodoacetate. The inhibitory effect of DTNB on acetylcholinesterase could be demonstrated by two methods for acetylcholinesterase determination, that of Ellman *et al.*[1] which uses acetylthiocholine as substrate (columns 1 and 2 in Table I) and that of Hestrin[7] which uses acetylcholine as substrate (columns 3 and 4 in Table I). In the experiments with PCMB, GSSG and iodoacetate, acetylcholinesterase was determined by the method of Hestrin[7] only, since these SH inhibitors interfere with acetylcholinesterase determination by the method of Ellman *et al.*[1].

The inhibitory effect of DTNB was reduced when the concentration of substrate was increased (Table I), or when the standard 10-min preincubation period with the inhibitor was omitted. Reduced glutathione (GSH) also prevented inhibition by DTNB,

TABLE II

INHIBITION OF ACETYLCHOLINESTERASE FROM *MYZUS PERSICAE* BY MALAOXON OR DTNB, AND OF MITE AND MEDFLY HEAD ACETYLCHOLINESTERASE BY MALAOXON WITH THREE ACYLTHIOCHOLINES AS SUBSTRATE

The data are averages of 2 to 4 experiments. The acetylcholinesterase activity was determined by the method of Ellman *et al.*[1].

Substrate (M)	*Acetylcholinesterase from aphids*					
	Activity (μmoles/mg protein per h)	*% Inhibition by malaoxon*		*Activity (μmoles/mg protein per h)*	*% Inhibition by DTNB*	
		$5 \cdot 10^{-8}$ *M*	$1 \cdot 10^{-7}$ *M*		$2 \cdot 10^{-5}$ *M*	$5 \cdot 10^{-5}$ *M*
Acetylthiocholine $6 \cdot 10^{-4}$	5.9	55	74	4.6	54	83
Propionylthiocholine $6 \cdot 10^{-4}$	2.3	51	72	1.8	62	77
Butyrylthiocholine $6 \cdot 10^{-4}$	0.8	6	16	1.2	0	0
Acetylthiocholine $1.8 \cdot 10^{-3}$				5.0	40	85
Propionylthiocholine $1.8 \cdot 10^{-3}$				2.5	58	62
Butyrylthiocholine $1.8 \cdot 10^{-3}$				3.8	0	0

but the GSH had to be present in the preincubation mixture. When GSH was added after the 10-min preincubation period with inhibitor, inhibition was not reversed (Table I).

The sensitivity of acetylcholinesterase of aphids to PCMB and DTNB and the protective effect of glutathione against inhibition indicates that aphid acetylcholinesterase contains an essential SH group located at either the active site or at an area influencing this site. Also since acetylcholine and acetylthiocholine showed some protection against SH inhibitors (Table I) it can be assumed that the sensitive SH group is located at the substrate binding site of the enzyme. Another explanation is possible by assuming that a conformational change, restricting access of the inhibitor to the essential SH group, takes place in the enzyme by binding of the substrate. Such an explanation has already been offered for the protective effect of the substrate against SH inhibitors[9].

A soluble preparation of acetylcholinesterase from bovine red blood cells or particulate preparations of acetylcholinesterases from mite or from Medfly heads, tested at experimental conditions identical to those used in the experiments described in Table I, were not inhibited by up to $6 \cdot 10^{-4}$ M DTNB as determined by the methods of Ellman *et al.*[1] or of Hestrin[7] nor by up to $4 \cdot 10^{-5}$ M PCMB as determined by the method of Hestrin[7]. On the contrary, DTNB caused an increase in activity of these acetylcholinesterases by approximately 10%. These results confirm the unique nature of aphid acetylcholinesterase with respect to sensitivity to SH inhibitors.

TABLE II *(continued)*

Acetylcholinesterase from mites			*Acetylcholinesterase from Medfly heads*		
Activity (μmoles/mg protein per h)	*% Inhibition by malaoxon*		*Activity (μmoles/mg protein per h)*	*% Inhibition by malaoxon*	
	$3.5 \cdot 10^{-8}$ *M*	$7 \cdot 10^{-8}$ *M*		$7.5 \cdot 10^{-9}$ *M*	$2.5 \cdot 10^{-8}$ *M*
1.3	30	51	20.2	33	73
1.5	31	52	16.7	29	67
0.3	36	55	7.5	29	73

Aphid acetylcholinesterase like other insect acetylcholinesterases is inhibited by organophosphorus compounds[10], such as phosphamidon[4], diethyl malaoxon[6] and malaoxon (Table II). Malaoxon is known to act on acetylcholinesterase in a manner similar to other organophosphorus inhibitors[11]. It seems therefore that the sensitive SH group (probably cysteine) present in aphid acetylcholinesterase (Table I) does not substitute for the active center serine group known to be involved in acyl (or phosphoryl) enzyme formation in the mechanism of action (or organophosphorus inhibition) of acetylcholinesterases[12–15].

The results in Table II indicate that the particulate acetylcholinesterase preparation from aphids contains at least two esterases which hydrolyse choline esters. One esterase seems to be a true acetylcholinesterase hydrolysing acetylthiocholine and to a lesser extent propionylthiocholine, but little butyrylthiocholine. The respective V values, in μmoles/mg protein per h, being 7, 4; 2, 7 and 0, 7; and the respective K_m values ($\times 10^{-4}$), in M, 1, 8; 0, 6 and 1, 5 (ref. 6). This enzyme is inhibited by DTNB or malaoxon and the extent of inhibition was almost the same when acetylthiocholine or propionylthiocholine were used as substrates (Table II). The other enzyme, hydrolysing butyrylthiocholine, was not appreciably inhibited by DTNB or malaoxon (Table II) and is probably a pseudocholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8)[15]. These conclusions are also supported by data showing that acetyl-β-methylcholine and benzoylcholine are hydrolysed in the presence of acetylcholinesterase preparation from aphids[6]. These two substrates were used to distinguish between acetylcholinesterase and pseudocholinesterase[16]. The presence of two enzymes, a true acetylcholinesterase and a butyrylcholinesterase, in the aphid preparation is also indicated by the different shape of the saturation curve for butyrylthiocholine as compared to those for acetylthiocholine or propionylthiocholine: with butyrylthiocholine as substrate an unexpected rise in activity occurred in the plateau region of the saturation curve[6]. A great rise in activity at higher butyrylthiocholine concentration can also be discerned from Table II. Acetylcholinesterase preparations from mites or Medfly heads, in contrast to that from aphids, seem to contain mainly a true acetylcholinesterase. This is indicated from the almost similar extent of inhibition by malaoxon with acetylthiocholine, propionylthiocholine and butyrylthiocholine as substrate (Table II), and from the ability of these preparations to hydrolyse acetyl-β-methylcholine but not benzoylcholine[6]. In conclusion, it seems that the particulate preparation from aphids contains an acetylcholinesterase and a pseudocholinesterase, but only the acetylcholinesterase is sensitive to DTNB.

Opinions differ about the nature of the active sites of acetylcholinesterase and acetylcholine receptor. While some investigators suggested that the two sites are identical[8,9] others presented evidence for their non-identity[20,21]. The sensitivity of the acetylcholine receptor, but not of the acetylcholinesterase from the electroplax of the electric eel to SH inhibitors was used as evidence for the non-identity of the two active sites[2]. Acetylcholinesterase from aphids resembles in this respect the acetylcholine receptor from the electroplax. Also mild sonication destroys the activity of acetylcholinesterase from aphids but does not affect the activity of acetylcholinesterase from other insects[6]. Thus acetyl-

cholinesterase from aphids, similar to the acetylcholine receptor from electroplax[22] seems to be tightly membrane-bound.

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REFERENCES

1 Ellman, G.L., Courtney, D.K., Anders, Jr., V. and Featherstone, R.M. (1961) *Biochem. Pharmacol.* 7, 88–95
2 Karlin, A. (1967) *Biochim. Biophys. Acta* 139, 358–362
3 Tahori, A.S. and Raccah, B. (1970) *J. Econ. Entomol.* 63, 545–548
4 Zahavi, M. and Tahori, A.S. (1970) *Biochem. Pharmacol.* 19, 219–225
5 Zahavi, M. and Tahori, A.S. (1970) *Israel J. Entomol.* 5, 185–191
6 Zahavi, M., Tahori, A.S. and Klimer, F. (1971) *Mol. Pharmacol.* 7, 611–619
7 Hestrin, S. (1949) *J. Biol. Chem.* 180, 249–261
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275
9 Dickinson, F.M. (1972) *Biochem. J.* 126, 133–138
10 O'Brien, R.D. (1960) *Toxic Phosphorus Esters*, pp. 434, Academic Press, New York
11 Chiu, Y.C., Main, A.R. and Dauterman, W.C. (1969) *Biochem. Pharmacol.* 18, 2171–2177
12 Wilson, J.B., Bergmann, F. and Nachmansohn, D. (1950) *J. Biol. Chem.* 186, 781–790
13 Wilson, J.B. (1951) *J. Biol. Chem.* 190, 111–117
14 Schaffer, N.K., May, Jr., S.C. and Summerson, W.H. (1953) *J. Biol. Chem.* 202, 67–76
15 Krupka, R.M. (1964) *Can. J. Biochem.* 42, 677–693
16 Augustinsson, K.B. and Nachmansohn, D. (1949) *Science* 110, 98–99
17 Mendel, B., Mundell, D.B. and Rudney, H. (1943) *Biochem. J.* 37, 473–476
18 Changeux, J.P. (1966) *Mol. Pharmacol.* 2, 369–392
19 Ehrenpreis, S. (1967) *Ann. N.Y. Acad. Sci.* 144, 720–736
20 Nachmansohn, D. (1966) *Ann. N.Y. Acad. Sci.* 137, 877–900
21 Webb, G.D. and Johnson, R. (1969) *Biochem. Pharmacol.* 18, 2753–2761
22 Nachmansohn, D. (1966) *Ann. N.Y. Acad. Sci.* 135, 136–149