MOLECULAR PHARMACOLOGY, 7, 611-619

Insensitivity of Acetylcholinesterases to Organophosphorus Compounds as Related to Size of Esteratic Site

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(Received April 2, 1971)

SUMMARY

The activity patterns of sensitive and organophosphorus-insensitive acetylcholinesterases of mites were tested with a homologous series of three acylthiocholines. The activities of organophosphorus-sensitive enzymes from two mite strains were higher with propionylthiocholine than with acetylthiocholine, and decreased sharply with butyrylthiocholine. On the other hand, the organophosphorus-insensitive acetylcholinesterases from three resistant mite strains displayed decreasing activity in the following order: acetylthiocholine > propionylthiocholine > butyrylthiocholine. An essentially similar difference between sensitive and insensitive acetylcholinesterases was also observed when $1/K_m$ or maximal velocities were determined for the three acylthiocholine substrates. Consequently, with propionylthiocholine as substrate, the activities, $1/K_m$ values, and maximal velocities of organophosphorus insensitive enzymes were much lower than those of organophosphorus-sensitive acetylcholinesterases.

In view of these results and of the considerably lower extent of inhibition of mite acetylcholinesterase by the *O*,*O*-diethyl analogue of malaoxon, as compared to that by malaoxon (*S*-(1,2-dicarbethoxymethyl *O*,*O*-dimethyl)phosphorothioate) itself, it is suggested that the esteratic site of the organophosphorus-sensitive mite enzyme is wide enough to accommodate the *O*,*O*-dimethylphosphoryl residue of malaoxon but not an *O*,*O*-diethylphosphoryl residue. On the other hand, the esteratic site of organophosphorus-insensitive acetylcholinesterase is not wide enough to accommodate even an *O*,*O*-dimethylphosphoryl residue.

The pattern of activities of organophosphorus-sensitive acetylcholinesterases from aphids, roaches, and Mediterranean fruit flies, and of organophosphorus-insensitive acetylcholinesterase from toad brains, was also tested with the three acylthiocholines. With the sensitive enzymes only the pattern of $1/K_m$ values was similar to that of the sensitive enzyme of mites for the three acylthiocholines tested.

INTRODUCTION

Organophosphorus-insensitive acetylcholinesterases have been reported in organophosphorus-resistant strains of spider mites (1-3) and cattle ticks (4). In addition to their lower affinity for organophosphorus compounds, acetylcholinesterase preparations from resistant strains were also less active with the substrates acetylcholine (2, 4) and acetylthiocholine (1) than were otherwise identical preparations from organophosphorus-susceptible strains.

In a previous communication (3) we reported that low acetylcholinesterase activity and organophosphorus insensitivity

This research was supported in part by Grant PL 480 from the United States Department of Agriculture.

in spider mites are not always correlated. In the present investigation, acetylcholinesterase activity patterns with the substrates acetylthiocholine, propionylthiocholine, and butyrylthiocholine differed considerably in organophosphorus-resistant and -susceptible mite strains. Thus, from the behavior of organophosphorus-sensitive and -insensitive mite acetylcholinesterases toward this homologous series of acylthiocholines, the relative size of the esteratic sites in these enzymes could be inferred. An extension of the study to acetylcholinesterases from other sources suggested a correlation between the size of the esteratic site on the enzyme and insensitivity to organophosphorus compounds.

MATERIALS AND METHODS

Materials. The iodides of acetyl-, propionyl-, and butyrylthiocholine were obtained from Calbiochem or from Sigma Chemical Company; 5,5′-dithiobis(2-nitrobenzoic acid), from Aldrich Chemical Company; purified malaoxon (racemic mixture), as a gift from American Cyanamid Company; and the D- and L-stereoisomers of the *O,O*-diethyl analogue of malaoxon (5), as a gift from Dr. W. C. Dauterman, North Carolina State University, Raleigh.

Mite strains. Strains of the carmine spider mite *Tetranychus cinnabarinus* were collected from various regions in Israel and reared in the laboratory. Methods of rearing the mites, the acaricide tests, and resistance indices for malathion are described elsewhere (6). The Amirim and Wadi Ara strains were organophosphorus-susceptible while Ayelet Hashahar XII, Neve Ur I, and Neve Ur II were resistant. A quantitative relationship between their toxicity to malathion *in vivo* and sensitivity to malaoxon *in vitro* has been reported previously (3). The Neve Ur II strain was recently collected from the same region as the Neve Ur I strain; it has a higher malathion resistance than the former.

Other animals. The Mediterranean fruit fly (Medfly) *Ceratitis capitata*, obtained from the Biological Control Laboratory of the Citrus Marketing Board, Rehovot, was fed in our laboratory on sugar and water only. The American cockroach *Periplaneta americana* was collected from an infested building at Ness-Ziona. The green peach aphid *Myzus persicae* was reared on Chinese cabbage in our laboratory. The toad *Bufo viridis* was collected in Afula.

Preparation of acetylcholinesterases of mites, Medfly, and roach. Preparation of mite homogenate and particulate acetylcholinesterases and solubilization of the particulate enzyme by sonication have been described elsewhere (3). The same procedure was used for heads of Medflies (1 head/ml) and of roaches (0.5 head/ml), and for brains of toads (10 mg/ml). The homogenates of Medfly or roach heads were filtered through 10 layers of gauze before centrifugation.

Preparation of particulate acetylcholinesterase of aphids. One hundred fifty aphids were ground three times in a Potter-Elvehjem homogenizer in 7.5 ml of 0.134 M ice-cold sodium potassium phosphate buffer (pH 7.2) for 30 sec and cooled on ice between grindings. The homogenate was filtered through 10 layers of muslin and centrifuged at 9000 × *g* at 2° for 10 min. The particles were resuspended in 1.5 ml of the buffer solution. A 0.1-ml sample of this particulate preparation was taken for acetylcholinesterase determination.

Determination of enzyme activity. Acetylcholinesterase was measured by the method of Ellman *et al.* (7). Particulate acetylcholinesterase was used for the experiments on inhibition by malaoxon, since this enzyme preparation was 10–30% more sensitive to malaoxon than the one obtained from solubilized particles. Solubilized particles were used for determination of enzyme activities with various substrates to eliminate a permeability barrier factor.

Only particulate acetylcholinesterase was used with aphid, since sonication of particles, even under moderate conditions (3), destroyed the enzyme activity. Because 5,5′-dithiobis(2-nitrobenzoic acid) at 3×10^{-4} M inhibited the acetylcholinesterase of aphids completely, this reagent was added after completion of the reaction.

Aliquots of particles or solubilized particles taken for acetylcholinesterase determination were as follows: 0.5 ml corresponded to 100 mites, to 1/14 Medfly head, to 1/80 roach

head and to 1/100 toad brain. For aphids, 0.1 ml of particles corresponded to about 10 aphids; to maintain appropriate phosphate buffer concentrations and pH, 0.4 ml of 0.067 M phosphate buffer (pH 7.5) was added per 0.1-ml sample. In the inhibition experiments in which the organophosphorus compounds were added in 5% acetone solutions, the reaction mixtures contained acetone at 0.5% final concentration. In all other experiments no acetone was present. A more detailed description of the reaction mixture was given in a previous paper (3).

RESULTS AND DISCUSSION

Activity of mite acetylcholinesterase with a homologous series of acylthiocholines, and organophosphorus sensitivity. Acetylcholinesterases from sensitive mite strains had different activity patterns with acylthiocholines of various acyl chain lengths than those of resistant strains (Tables 1 and 2). The activity of the organophosphorus-sensitive enzyme increased when the propionyl radical was substituted for the acetyl radical, but decreased with butyryl substitution (Table 1). This indicates that the esteratic site of the sensitive enzyme from mites is wide enough to accommodate a propionyl but not a butyryl residue. By analogous deduction from the comparative size of acyl and phosphoryl residues (Fig. 1), the esteratic site of organophosphorus-sensitive mite acetylcholinesterase should be large enough to fit an *O*-methylphosphoryl radical but not the *O*-ethyl radical. This conclusion was also supported by the decline in sensitivity of mite acetylcholinesterase toward the *O,O*-diethyl analogue of malaoxon, as compared to malaoxon itself, while an essentially opposite effect was found with the enzyme from Medfly heads (Table 2). Voss and Matsumura (2) have previously reported that acetylcholinesterase from spider mites also decreased in sensitivity toward the malaoxon analogues, ranging from *O,O*-dimethyl to *O,O*-dibutyl.

The activity of organophosphorus-insensitive acetylcholinesterase, in contrast, decreased when the acyl moiety of the acylthiocholine substrates was increased by 1 carbon length (Tables 1 and 2). These results and Fig. 1 indicate that the organophosphorus-insensitive enzyme cannot accommodate the propionyl residue of propionylthiocholine or the *O,O*-dimethylphosphoryl residue of malaoxon, thus conferring insensitivity to malaoxon. The esteratic site on the organophosphorus-insensitive enzyme seems to be smaller by about 1 carbon length (1.5 A) than that of the sensitive enzyme. Similar conclusions have already been reached with acylcholines as substrates (2); however, the data obtained with acylthiocholines as substrate are more convincing.

When kinetic parameters such as maximal

TABLE 1

Activity of acetylcholinesterases of organophosphorus-resistant and -sensitive mite strains tested with acylthiocholine esters

Acylthiocholine ester	Concentration	Acetylcholinesterase activity									
		Sensitive strains				Resistant strains					
		Amirim		Wadi Ara		Ayeleth Hashahar XII		Neve Ur I		Neve Ur II	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
	mM	mμmoles/hr/mg protein				mμmoles/hr/mg protein					
Acetylthiocholine	0.1	480	512	545	678	326	436	512	546	340	435
Propionylthiocholine	0.1	615	700	572	797	117	171	294	171	136	157
Butyrylthiocholine	0.1	78	75	68	74	4	9	26	17	8	23
Acetylthiocholine	0.3	735	724	835	992	490	686	747	786	545	660
Propionylthiocholine	0.3	975	1035	1010	1230	230	353	552	332	245	321
Butyrylthiocholine	0.3	135	124	117	144	32	9	55	17	27	0

TABLE 2

Activities of acetylcholinesterases with acylthiocholine esters and sensitivity to organophosphorus compounds

Source of enzyme	ID_{50}[a]			Activity ratio, propionylthiocholine to acetylthiocholine		Activity ratio, butyrylthiocholine to acetylthiocholine	
	dl-malaoxon	*O,O*-Diethylmalaoxon		10^{-4} M substrate	3×10^{-4} M substrate	10^{-4} M substrate	3×10^{-4} M substrate
		d	l				
	M	M	M				
Mites							
Amirim	3×10^{-8}	4.8×10^{-7}	8.6×10^{-7}	1.32	1.38	0.15	0.18
Wadi Ara	4.8×10^{-8}			1.11	1.22	0.12	0.14
Neve Ur I	7.1×10^{-6}			0.44	0.58	0.04	0.05
Ayeleth Hashahar XII	1.2×10^{-5}	1.08×10^{-4}	6.5×10^{-5}	0.38	0.49	0.02	0.04
Neve Ur I	1.7×10^{-5}			0.38	0.47	0.04	0.03
Green peach aphid	2.9×10^{-8}	3.4×10^{-7}		0.50	0.51	0.08	0.17
Medfly head	7.3×10^{-9}	3.8×10^{-9}	1.08×10^{-8}	0.97	0.95	0.33	0.38
American cockroach head	2.4×10^{-8}			0.89	0.84	0.08	0.06
Toad brain	2.8×10^{-7} [b]	8.2×10^{-7} [b]	2.5×10^{-6} [b]	0.11	0.10	0.06	0.02

Inhibition data are averages of at least five experiments.

[a] Concentration of organophosphorus inhibitor causing 50% inhibition. This value was obtained by plotting percentage inhibition against the logarithmic scale of inhibitor concentrations.

[b] Here acetone concentrations were lowered from the standard 0.5% to 0.01% (for explanation, see RESULTS AND DISCUSSION). At 0.5% acetone the ID_{50} for malaoxon was 9.8×10^{-7} M.

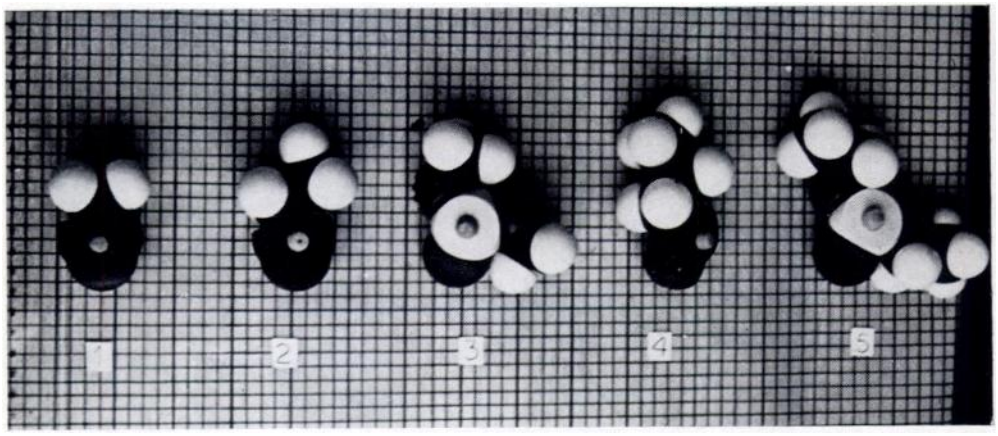

FIG. 1. *Relative sizes of acyl and phosphoryl residues*

Constructed from Corey-Pauling-Koltun (CPK) space-filling atomic models, obtained from Ealing Scientific Company, Ltd., London. *1*, acetyl; *2*, propionyl; *3*, dimethylphosphoryl; *4*, butyryl; *5*, diethylphosphoryl.

velocities (V_{max}) or Michaelis constants (K_m) of the acetylcholinesterases of sensitive and insensitive mites were compared, a characteristic pattern emerged (Table 3). For the sensitive Amirim strain, the lowest K_m was observed for propionylthiocholine, while for the insensitive Ayeleth Hashahar XII strain the K_m of this substrate was higher by a factor of about 2.5 than that of acetylthiocholine. The maximum velocities of the enzyme from the sensitive strain were essentially the same for acetyl- and

TABLE 3

Comparison of maximal velocities (V_{max}) and Michaelis constants (K_m) of acetylcholinesterases for acylthiocholines

All data are averages of three or four experiments. K_m and V_{max} values were obtained by plotting concentration/activity (S/v) against concentration (S) according to the method of Lineweaver and Burk (8).

Source of enzyme	Acetylthiocholine		Propionylthiocholine		Butyrylthiocholine	
	V_{max}[a]	$K_m \times 10^4$	V_{max}	$K_m \times 10^4$	V_{max}	$K_m \times 10^4$
		M		*M*		*M*
Mites						
Amirim	1,130	1.3	1,170	1.1	177	1.82
Ayelet Hashahar XII	920	1.6	550	3.8	153	11.5
Green peach aphid	7,400	1.8	2,700	0.6	700	1.5
American cockroach, head	4,450	0.9	3,950	0.7		
Medfly head	30,300	0.54	30,400	0.32	13,700	0.55
Medfly head[b]	20,200	0.95	17,400	0.45	9,900	1.07

[a] V_{max} values are given in millimicromoles per hour per milligram of protein.

[b] Particulate, not solubilized.

propionylthiocholine, while for butyrylthiocholine they declined sharply. On the other hand, the V_{max} values of the enzyme from the resistant strain decreased gradually with an increase in the acyl chain length of the substrate. The organophosphorus-insensitive enzyme from the resistant mite strain was characterized by lower V_{max} and higher K_m values for propionylthiocholine, but not for acetylthiocholine, than was the sensitive enzyme. This finding again supports the hypothesis that the esteratic site of organophosphorus-insensitive mite acetylcholinesterase is 1 carbon length smaller than that of the sensitive mite enzyme. By a similar technique, i.e., study of the effects of difference in constitution or chain length of a number of substrates on affinity and on rate constants, the sizes of the active sites of papain and carboxypeptidase A were suggested (9, 10). Similarly, a study of the influence of the chain length of a homologous series of fatty acid esters on the V_{max} and K_m values of various esterases revealed that the maximal velocities doubled with each additional carbon atom up to a certain maximum, and dropped sharply thereafter (11, 12).

The restrictions on size of the insensitive acetylcholinesterase can be partly counteracted by changing the leaving group of the organophosphorus compound. For example, dichlorvos (2,2-dichlorovinyl dimethyl phosphate), which contains the same phosphoryl residue as malaoxon, was nevertheless a much better inhibitor of the insensitive mite acetylcholinesterase than malaoxon, while the enzyme from sensitive mite strains was almost equally well inhibited by the two compounds (3). The ability to accommodate the *O,O*-dimethyl residue probably also depends on the way in which the organophosphorus compound attaches itself to the enzyme. It is suggested that a phosphonate analogue of dichlorvos might be an even better inhibitor of insensitive mite acetylcholinesterase than dichlorvos itself.

Recently some data were published on the activities of organophosphorus-sensitive and -insensitive mite acetylcholinesterases with various thioester substrates (13). Our results agree, in general, with the results obtained in that study, but the organophosphorus inhibitors used by those authors varied in the leaving as well as in the phosphorylating group. This might have caused some discrepancies in the interpretation concerning the role of the size of the phosphorylating group in the inhibition of insensitive mite acetylcholinesterase.

Organophosphorus sensitivity and affinity for acylthiocholines of acetylcholinesterases from animals other than mites. The pattern of activities of the enzymes from Medfly and

roach heads supports the view that sensitivity to an organophosphorus compound is correlated with ability to accommodate a propionyl residue at the esteratic site (Table 2). Although the activity of these enzymes with propionylthiocholine was somewhat lower than with acetylthiocholine, the sharp decline in activity with butyrylthiocholine supports the above view. The activities of aphid acetylcholinesterase with propionyl- and acetylthiocholine were almost equal at low (3×10^{-5} M) concentrations of these compounds (Fig. 2c). At higher concentrations, however, the pattern of activity with the acylthiocholines resembled that of the resistant Neve Ur I mite strain (Table 2). All the organophosphorus-sensitive acetylcholinesterases tested demonstrated a lower K_m value for propionylthiocholine than for acetylthiocholine, while for butyrylthiocholine the K_m value rose again (Table 3). We found the same trend for the K_m values even

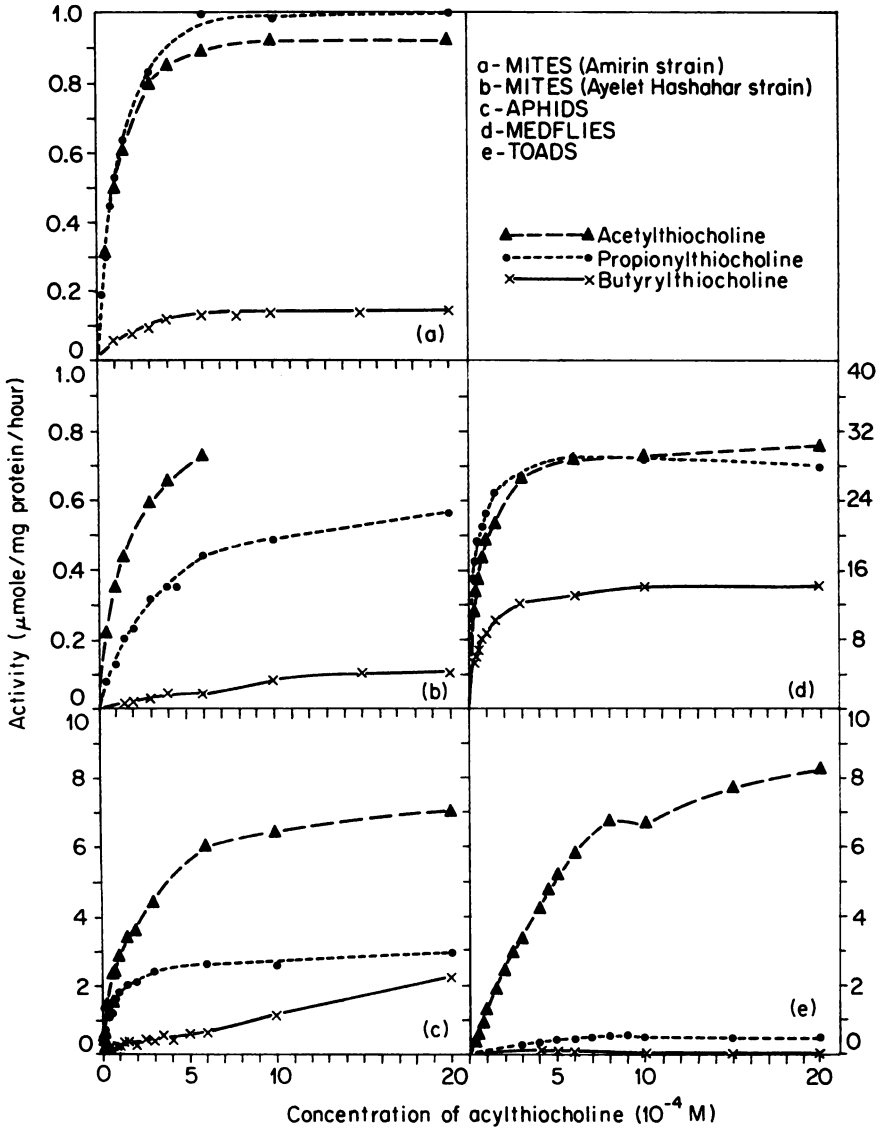

FIG. 2. *Rates of hydrolysis of three acylthiocholines as a function of substrate concentration for acetylcholinesterases obtained from mites, aphids, Medfly heads, and toad brains*

with unsonicated particles from Medfly heads, where the higher K_m values suggest restrictions on penetration of the substrate (Table 3). For the enzyme from Medfly heads, the activity with butyrylthiocholine was comparatively high, with a low K_m value (Tables 2 and 3), indicating that the esteratic site of this enzyme is rather wide. This property is probably correlated with the comparatively high malaoxon sensitivity of this acetylcholinesterase. Following this trend, a higher *O,O*-dialkyl analogue of malaoxon should also be a good inhibitor of the Medfly enzyme (Table 2).

Since the acetylcholinesterase of toad brain was reported to be insensitive to organophosphorus compounds (14), we compared this enzyme with the organophosphorus-sensitive acetylcholinesterases mentioned above. The pattern of activities of the toad brain enzyme with acylthiocholines resembled the one found with the insensitive enzyme of the Ayeleth Hashahar XII mite strain (Fig. 2b and e; Table 2). However, its malaoxon sensitivity was intermediate between those of the sensitive and insensitive mite acetylcholinesterases (Table 2). It should be mentioned that the 0.5% acetone concentration present in all organophosphorus inhibition reaction mixtures inhibited toad brain acetylcholinesterase activity up to 60%. The remaining 40% activity was 32 times less sensitive to malaoxon than was acetylcholinesterase from the sensitive Amirim mite strain. However, when the acetone concentration was reduced to only 0.01%, the sensitivity of this enzyme to malaoxon increased by a factor of 3.5 (Table 2).

In conclusion, it seems that the good correlation between sensitivity to organophosphorus compounds and the pattern of activities (V_{max} values) with the acylthiocholines found for the acetylcholinesterases from mite strains could be demonstrated with these enzymes because they probably resemble each other in other respects; for example, the hydrophobic binding area or anionic sites (15). This is supported by the finding that these acetylcholinesterases display the same stereospecificity for the l-isomer of *O,O*-diethylmalaoxon, in contrast to the enzymes from Medflies, toads (Table 2), and bovine erythrocytes (5), which showed greater sensitivity toward the d-analogue. As for acetylcholinesterases from other sources, malaoxon sensitivity in the insects tested could be correlated with an esteratic site wide enough to bind at least a propionyl residue, as indicated by the finding that the K_m value of propionylthiocholine was the lowest in the homologous series of acylthiocholines.

Some properties of acetylcholinesterase preparations related to their organophosphorus sensitivity and to activities with acylthiocholines. Previous experiments (3) have shown that degradation of malaoxon was not the cause for the 400-fold greater malaoxon insensitivity of the acetylcholinesterase from the Ayelet Hashahar XII mite strain as compared to the Amirim strain (Table 2). Furthermore, when the reaction period was extended from 15 to 60 min, the rate of inhibition by malaoxon or its diethyl analogues did not decrease for the enzyme preparations tested in this study. On the contrary, it always increased in the same experiment by about 25%, indicating that no appreciable degradation of malaoxon or its analogues took place during the reaction. Thus the ID_{50} values for malaoxon or its diethyl analogues presented in Table 2 indeed reflect the sensitivity of the various acetylcholinesterases toward these compounds.

The saturation curves for the acetylcholinesterases from mites, aphids, and Medflies are given in Fig. 2a, b, c, and d. No substrate inhibition was observed with concentrations up to 2 mM. Here plotting S/v against S according to Lineweaver and Burk (8) demonstrated a clear linear relationship. The saturation curves for the enzymes from mites and Medflies were almost similar in shape for the three substrates tested (Fig. 2a, b, and d). On the other hand, the saturation curve for butyrylthiocholine displayed a different shape from those for acetyl- and propionylthiocholine for the aphid preparation tested (Fig. 2c).

The saturation curves of toad brain acetylcholinesterase differed from those of other enzyme preparations tested, in that the rise

in activity was slower at low substrate concentrations (compare Fig. 2e with Fig. 2a–d for acetylthiocholine). As already mentioned, the toad brain enzyme was also unusual in that it was inhibited by 0.5% acetone. In plots of $1/v$ vs. $1/S$, according to Lineweaver and Burk (8), no single linear relationship was apparent; two sets of points, each forming a straight line, were obtained for each substrate. Since the kinetics of the saturation curve of toad brain acetylcholinesterase is not sufficiently clear, we have refrained from providing data for the V_{max} and K_m of this enzyme.

Only extensive purification of the acetylcholinesterases tested could confirm that we were dealing with the esteratic site of an acetylcholinesterase reacting with the three substrates, and not with esteratic sites of various cholinesterases. Nevertheless, the assumption made in this study—that the same enzyme hydrolyzes acetyl-, propionyl-, and butyrylthiocholine—was supported by the following experiments with the enzyme preparations from mites and Medflies. Concentrations of malaoxon which produced 50% (Table 2) or about 80% (7×10^{-8} M for mites and 2.5×10^{-8} M for Medflies) inhibition of activity with acetylthiocholine as substrate were inhibitory to almost the same extent with butyrylthiocholine and propionylthiocholine. On the other hand, with the enzyme from aphids, malaoxon caused almost equal inhibition with acetylthiocholine and propionylthiocholine as substrates, but almost none with butyrylthiocholine. These results, together with the different shape of the saturation curve for butyrylthiocholine as compared to those for the acetyl and propionyl derivatives, suggest that in aphids the latter two substrates are hydrolyzed by a different enzyme from the one active against butyrylthiocholine. Voss and Matsumura (2) carried out tests on mite acetylcholinesterase with acetyl-β-methylcholine, benzoylcholine, and eserine, which are known to distinguish between acetylcholinesterase and pseudocholinesterase (16). They showed that the mite enzyme did not hydrolyze benzoylcholine appreciably, in contrast to acetylcholine and acetyl-β-methylcholine. Thus the mite enzyme behaved as a true acetylcholinesterase, although its activity with propionylcholine was higher than with acetylcholine.

We also measured the activities of our acetylcholinesterase preparations from Medfly heads and aphids by the methods of Hestrin (17), using 2 mM acetylcholine, acetyl-β-methylcholine, or benzoylcholine as substrate. While no activity with benzoylcholine could be detected in the acetylcholinesterase preparation from Medfly heads, this substance was still hydrolyzed by the aphid enzyme at about 40% of the rate of acetylcholine. With acetyl-β-methylcholine both preparations showed about 80% of the activity against acetylcholine. These results, together with those of Voss and Matsumura (2), indicate that in the enzyme preparations from Medfly heads and from mites only a true acetylcholinesterase seems to be present, which may be responsible for the hydrolysis of the three acetylcholines. On the other hand, in the aphid preparation, a true acetylcholinesterase as well as a psuedocholinesterase may be present. This pseudocholinesterase would be responsible for the malaoxon-insensitive hydrolysis of butyrylthiocholine by the enzyme preparation from aphids.

ACKNOWLEDGMENTS

The assistance of Mr. I. Rogosick in rearing the mites and aphids and the gifts of the D- and L-stereoisomers of the *O,O*-diethyl analogue of malaoxon by Dr. W. C. Dauterman, and of purified malaoxon by American Cyanamid Company, are gratefully acknowledged. Figure 1 was photographed by Dr. A. Vincze.

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