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V. M. Pavelkić, K. S. Krinulović, J. Z. Savić, M. A. Ilić

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Malathion-Induced Inhibition of Human Plasma Cholinesterase Studied by the Fluorescence Spectroscopy Method[1]

V. M. Pavelkić[a], K. S. Krinulović[a], J. Z. Savić[a], and M. A. Ilić[b]

[a] *Department of Physical Chemistry, Institute of Nuclear Sciences Vinča, Belgrade, Serbia*

[b] *Institute of General and Physical Chemistry, Belgrade, Serbia*

e-mail: vesnap@vin.bg.ac.yu

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Abstract—The in vitro effect of technical grade malathion was assessed via the kinetic parameters of human plasma butyrylcholinesterase (BChE) using N-methylindoxyl acetate as a substrate for BChE. An inhibitor kinetics study demonstrated the existence of a biphasic inhibition curve, indicating high- and low-affinity binding sites of malathion. The IC_{50} values as calculated from the experimental inhibition curves were 1.33×10^{-9} and 1.48×10^{-5} M for the high- and low-affinity binding sites, respectively; Hill's analysis gave 1.29×10^{-9} and 1.38×10^{-6} M. The Cornish–Bowden plots and their secondary plots indicated that the nature of inhibition was of mixed type with the predominant competitive character of both affinity binding sites.

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INTRODUCTION

Butyrylholinesterase (BChE) is a non-specific cholinesterase found in plasma and tissues of many vertebrates at various levels. It can hydrolyze esters of choline [1–3]. The physiological function of BChE remains a puzzle. It has no known specific natural substrate although it is capable of hydrolyzing acetylcholine (ACh), but at a slower rate of hydrolysis than acetylcholinesterase [4, 5] (AChE). BChE has a structure similar to AChE and the action of both enzymes is a multistep process. Reaction schemes are similar and involve the formation of a reversible enzyme-substrate complex, acetylation of the catalytic site, and its rapid hydrolysis into acetic acid, choline, and regenerated enzyme [6, 7]. BChE acts as a scavenging enzyme in the detoxication of natural compounds [8]. Organophosphorous compounds (OPs), which are largely used in agriculture as pesticides, inhibit cholinesterase activity [9–11].

Malathion, an organophosphate insecticide commonly used to control mosquitoes and other flying insects, is far more toxic to insects than to mammals, but it can be absorbed through the permeable membranes of the human body after inhalation and oral or dermal exposure and is able to damage the nervous and immune systems [12]. The principal toxicological effect of malathion is cholinesterase inhibition. The major metabolites of malathion (mono- and dicarboxylic acid derivatives) and malaoxon as a minor metabolite can also affected cholinesterase activity.

The aim of this work was to investigate the mechanism of technical grade malathion-induced inhibition of BChE using the sensitive fluorimetric method based on N-methylindoxyl acetate as a substrate for BChE determination. The choice of the substrate enables us to assay enzyme quantities on the order of 10^{-4} UI/ml of human blood plasma cholinesterase by the direct initial reaction rate method as reported in [13, 14] (the unit of BChE activity is defined as that degrading 1 μmol of the substrate per minute at 25°C, pH 7.0). The method of improved kinetic spectrophotometric Ellman procedure based on enzyme-catalysed hydrolysis of the thiocholine substrate was used as a control [15–17].

EXPERIMENTAL

Materials and Methods

All reagents were of analytical grade. Butyrylthiocholine iodide and N-methylindoxyl acetate were purchased from Fluka Chemical Co. 5,5'-dithiobis-(2-nitro)benzoic acid (DTNB) was obtained from Merck. Heparin (sodium salt) used as an anti-coagulant of blood was purchased from Sigma Aldrich Co. Malathion E-50, diethyl (dimethoxythiophosphorylthio) succinate, purchased from "Zorka–Šabac" Holding Company as a commercial insecticide, CAS number 121-75-5, technical grade, was used without further purification (95% purity). The content of malaoxon (malathion oxidation product) was 100 ppm. As it was previously reported, malaoxon exhibits a similar pattern of inhibition of cholinesterase as malathion, but it is much more potent inhibitor of cholinesterase than malathion [18–21]. Malathion degrades rapidly in the environment. In water solution, at pH 7, malathion is stable for few days [22]. To prevent the degradation of malathion and formation of toxic degradation products, all solutions were prepared daily.

[1] The article is published in the original.

Blood specimens used to assess the precision and accuracy of the methods and to give a wide range of plasma cholinesterase activity were obtained from 20 healthy individuals who had no known exposure to cholinesterase inhibitors. The 120 blood samples used to derive "normal" reference interval were obtained from healthy voluntary blood presenters of both sexes aged from 25 to 50 years. Blood taken by vein puncture was collected into 5 ml tubes containing Na-heparin as anticoagulant. Erythrocytes were separated from plasmas by centrifugation (3000 rpm, 10 min, 25°C; Tehtnica, Centric 322 PLC centrifuge). 1 ml of a plasma was diluted by the addition of 10 ml 0.05 M phosphate buffer, pH 7.2.

The spectrophotometric measurements were performed on a UV-VIS LKB Spectrophotometer, Biochrom—ULTROSPEC II, and the fluorescence excitation and emission spectra were obtained on a Perkin-Elmer, 3000 fluorescence spectrometer.

Substrate Characterization

The fluorescence excitation and emission spectra of 2 mM N-methylindoxyl acetate were recorded in phosphate buffer at pH 7.2. The fluorescence excitation spectra showed two maxima at 395 and 425 nm. The emission spectra obtained with excitation at λ_{ex} = 395 nm had a maximum at λ_{em} = 485 nm. The intensity of fluorescence emission, I_f, which is a consequence of spontaneous hydrolysis of N-methylindoxyl acetate, was followed at 485 nm and a linear function of I_f vs. time was observed over the concentration range from 5×10^{-5} to 3×10^{-3} M of N-methylindoxyl acetate. The rate of the fluorescence reaction was proportional to the fluorescence quantum yield (Φ_F), i.e., to the ratio between photons absorbed and photons emitted through fluorescence. To simplify further calculation of butyrylcholinesterase activity, we approximated the fluorescence quantum yield of the reaction using the correlation between emission and absorption. The quotient between the slopes of the emission and absorption lines gave the proportionality factor F calculated as

$$F = \text{slope}_{em}/\text{slope}_{ex} = 4.5 \times 10^3/25.5 \times 10^3 = 0.176. \quad (1)$$

For further butyrylcholinesterase activity determination, we used this value.

The plasma separated from whole blood was diluted with 154 mM NaCl (1 : 10) and 0.1 ml was used for further analyses. The working solution also contained 0.1 ml of 10 mM N-methylindoxyl acetate and 1.8 ml phosphate buffer (pH 7.2). After excitation at 395 nm, a change in characteristic fluorescence emission was measured at 485 nm, and activity was calculated by the equation

$$a_{BChE} = (\Delta I_f/\Delta t)F \times 10, \quad (2)$$

where a_{BChE} (in UI/ml of plasma) is the activity of BChE, F = 0.176 is the proportionality factor, and factor 10 was used because of sample dilution. As a reference method for the evaluation of enzymatic activity, the improved Ellman procedure [17, 23] based on the enzyme-catalyzed hydrolysis of thiocholine substrate was used.

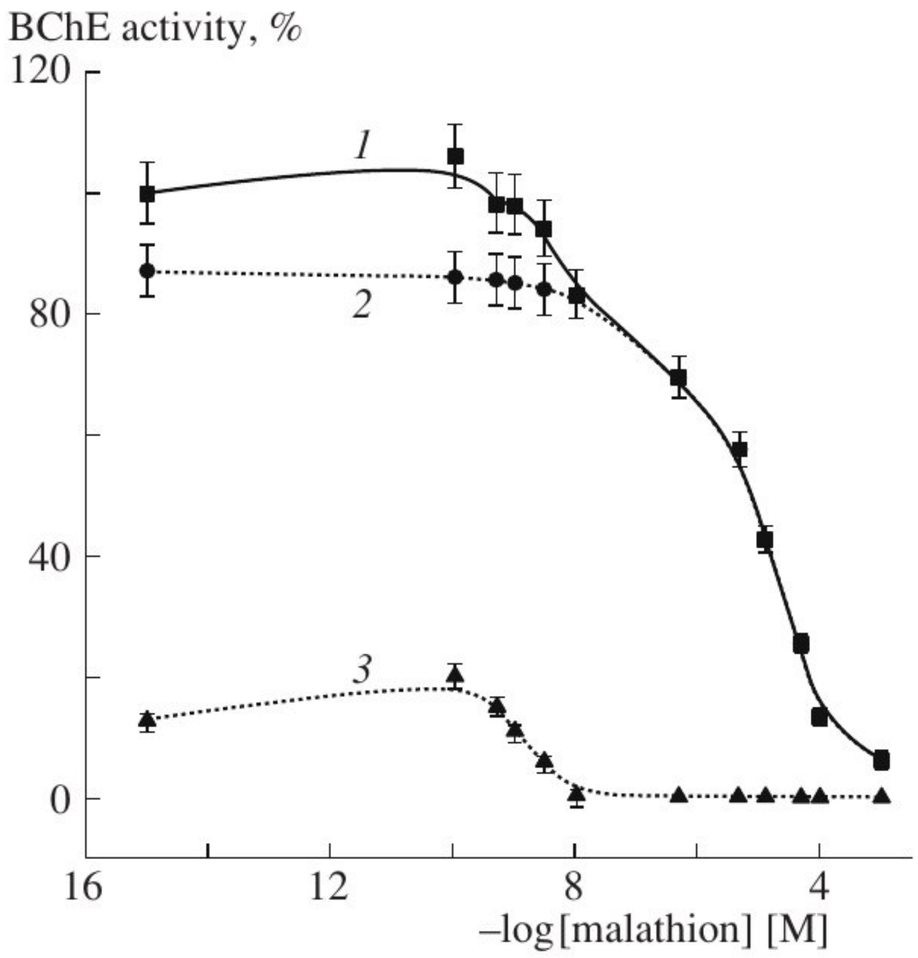

Fig. 1. (*1*) Biphasic inhibition curves of human plasma BChE by malathion, (*2*) low- and (*3*) high-affinity parts of the inhibition curve. The IC_{50} values for the low- and high-affinity sites of the inhibition curves were graphically evaluated for both enzymes, 1.48×10^{-5} and 1.33×10^{-9} M, respectively.

The "normal" reference interval for BChE activity determined by the fluorimetric method ranged from 3.12 ± 0.31 to 6.27 ± 0.63 UI/ml. These results are the mean of three independent determinations. A comparison between the activities obtained by these two procedures with performing a two-population (paired) t-test at a confidence level of 95% showed agreement between the two methods.

RESULTS

The interference of malathion over the concentration range from 1×10^{-10} to 1×10^{-3} M with the activity of serum BChE from human blood was determined using the fluorimetric method. The results presented in Fig. 1 showed that malathion induced the inhibition of BChE activity in a concentration-dependent manner. Biphasic inhibition profile of BChE activity indicated the existence of two binding sites of the inhibitor with different affinities for malathion, i.e., a high-affinity site (the region of lower inhibitor concentrations) and a low-affinity site (the region of higher inhibitor concentrations).

The plot of activity vs. malathion concentration therefore represents the sum of two overlapping sigmoid curves separated by a plateau. The results of mathematical experimental data processing are pre-

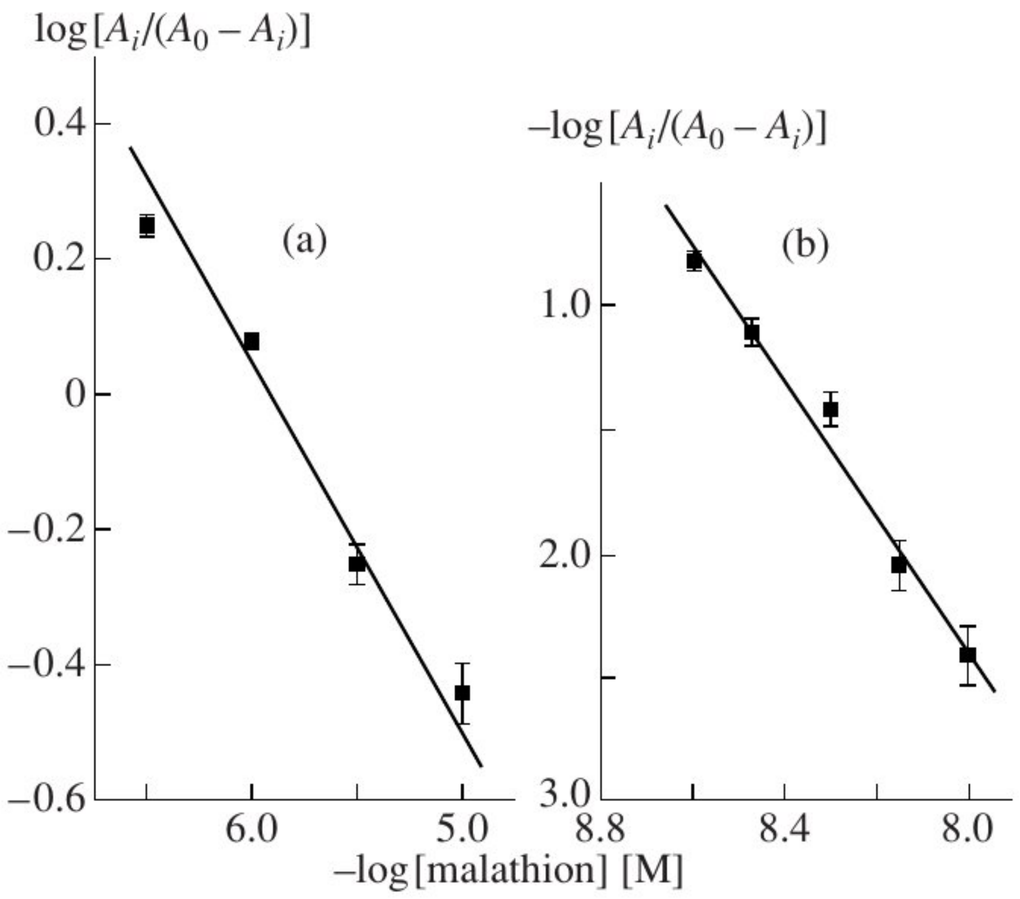

Fig. 2. The Hill plots of malathion inhibition of human plasma BChE for (a) low- and (b) high-affinity parts of the inhibition curve; A_0 and A_i denote the BChE activity in the absence and presence of the inhibitor in the concentrations indicated.

sented in Fig. 1. During data processing, it was assumed that the mass action law was obeyed. The experimental data were fitted by nonlinear regression analysis using the EZ FIT code [24]. The computer program analyzed the data on the assumption of a two-site model. In the first approximation, the half-maximum inhibition concentrations (IC_{50} values) for the high and low inhibitor affinity sites, respectively, were calculated by fitting the experimental results to the sum of two sigmoid curves. The theoretical curves for high- and low-affinity sites are presented in Fig. 1 (dotted curves). The activity of the high-affinity site was obtained by subtracting the calculated low-affinity values from the experimental data. Mathematical analysis of the experimental points gave IC_{50} values (inhibitory concentration that induced the inhibition of 50% enzymatic activity) 1.33×10^{-9} and 1.48×10^{-5} M for the high- and low-affinity inhibitory sites, respectively. The contribution of high- and low-affinity binding sites of BChE toward malathion to the overall specific BChE activity were about 15 and 85% respectively.

Table 1. Effect of malathion on K_m and V_{max} of human plasma BChE

[Malathion], M	K_m, mM	V_{max}, μM/min	a, %
0	0.350 ± 0.031	0.102 ± 0.005	100.0
10^{-10}	0.349 ± 0.034	0.108 ± 0.005	106.0
1×10^{-8}	0.358 ± 0.031	0.085 ± 0.004	83.2
1×10^{-7}	0.373 ± 0.030	0.071 ± 0.003	69.4
1×10^{-6}	0.374 ± 0.030	0.044 ± 0.003	42.8
1×10^{-5}	0.376 ± 0.030	0.026 ± 0.002	25.6
1×10^{-4}	0.385 ± 0.029	0.013 ± 0.002	13.4
1×10^{-3}	0.384 ± 0.029	0.006 ± 0.001	6.0

Note: K_m and V_{max} were determined by the corresponding regression equations. The a values are the mean percentages of enzyme activity relative to the corresponding control value from at least three independent measurements.

The Hill analysis of the separate inhibition curves was also performed [25, 26]. The plots for low- and high-affinity inhibitory sites are presented in Figs. 2a and 2b. IC_{50} values obtained by the Hill analysis were 1.38×10^{-6} M for low- and 1.29×10^{-9} M for high-affinity sites and were close to the values obtained by analyzing the inhibitory curve. The Hill analysis yielded a value of the Hill coefficient $n = 0.39$ for low- and $n = 2.70$ for high-affinity sites.

To evaluate the nature of BChE inhibition induced by malathion, the kinetic parameters K_m and V_{max} were determined by varying the concentration of N-methylindoxyl acetate from 0.22 to 3.00 mM. The effect of malathion on the kinetic properties of BChE was determined in the presence of the inhibitor at concentrations from 1×10^{-10} to 1×10^{-3} M. The dependence of the initial reaction rate on substrate concentration in the presence and absence of malathion exhibited a typical Michaelis–Menten kinetics [25]. The kinetic constants K_m and V_{max} were determined from the experimental data. The results obtained from the Lineaweaver–Burk plot (not shown) at a variety of different inhibitor concentrations are presented in Table 1. As can be seen, an increase in malathion concentration decreased V_{max} by 16.8–94% and simultaneously changed the apparent enzyme affinity for the substrate.

Figure 3 presents the Cornish–Bowden plot of [N-methylindoxyl acetate]/V against malathion concentration [27, 28]. At different [S] values, plots yield a series of straight lines where the [I] coordinate of the common intersection point yields K_{iu} (uncompetitive inhibition constant) in accordance with the equation

$$V = \frac{V_{max}[S]}{K_m(1 + [I]/K_{ic}) + [S](1 + [I]/K_{iu})}, \quad (3)$$

in which V is the rate of the reaction in the presence of the inhibitor, V_{max} is the maximum rate, K_m is the Michaelis constant, and K_{ic} (dissociation constant of the enzyme-inhibitor complex) and K_{iu} (dissociation constant of the enzyme–inhibitor–substrate complex) are competitive and uncompetitive inhibition constants, respectively [27, 28].

Any line in the plot of [N-methylindoxyl acetate]/V vs. malathion concentration is represented by transformed Eq. (3), i.e.,

$$\frac{[S]}{V} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_{iu}}\right) + \frac{[S]}{V_{max}}\left(1 + \frac{[I]}{K_{ic}}\right). \quad (4)$$

Any individual line intersects the horizontal axis at $-IC_{50}$ (Fig. 3), i.e., the IC_{50} value changes with the substrate concentrations. For both low- and high-affinity binding sites of malathion on BChE, the K_{ic} value was determined from the plots of $1/IC_{50}$ against V_0/V according to the equation [28]

$$\frac{1}{IC_{50}} = \frac{1}{K_{ic}} + \frac{V_0}{V}\left(\frac{1}{K_{iu}} + \frac{1}{K_{ic}}\right), \quad (5)$$

where V_0/V is the normalized rate of the uninhibited reaction, i.e., the "relative rate" [29].

These plots are straight lines with the slope ($1/K_{iu} - 1/K_{ic}$) and y-intercept $1/K_{ic}$ (Fig. 3). The inhibition constants and IC_{50} values for low- and high-affinity binding sites are presented in Table 2.

DISCUSSION

The present study showed that technical grade malathion inhibited serum BChE in a dose-dependent manner [12]. Moreover, the present study confirmed the existence of two inhibitory processes, assuming the low- ($IC_{50} = 1.48 \times 10^{-5}$ M) and high- ($IC_{50} = 1.33 \times 10^{-9}$ M) affinity binding sites for malathion. The degree of cooperativeness of an enzyme is commonly quantified by references to Hill plots. The Hill plot was a straight line over a wide range of malathion concentrations (Fig. 2).

The Hill coefficient (n) is useful for comparing the properties of enzymes, but it is not derivative from a physical model and does not have a simple physical meaning of the number of binding sites per enzyme molecule. The coefficients evaluated from the Hill analysis [27] of the low- and high-affinity inhibition curve portions fulfilled the relation $1 < n < 3$. From the Hill coefficient values, the conclusion can be drawn that there is cooperative binding of malathion to the high-affinity moiety of the BChE molecule ($n = 2.70$), i.e., inhibitor binding increases the affinity of BChE for further inhibitor binding. On the contrary, the n value below one for low-affinity binding site ($n = 0.39$) confirmed the negative cooperativeness of malathion-induced inhibition, i.e., malathion reduced the affinity of the enzyme for subsequent inhibitor binding.

A kinetic analysis of the substrate dependence of BChE activity in the presence and absence of malathion revealed a mixed nature of enzyme inhibition. The dependence of the initial rate on substrate concentration in the presence of various malathion concentrations showed that malathion induced a decrease in V_{max} with a change in the parent affinity for the substrate (K_m) (Table 1). The inhibition constants K_{ic} (dissociation constant of the enzyme-inhibitor complex) and K_{iu} (dissociation constant of the enzyme-inhibitor-substrate complex) and IC_{50} for low- and high-affinity binding sites were determined for all substrate concentrations according to the procedure suggested by Cornish–Bowden [27, 28]. For high-affinity binding sites,

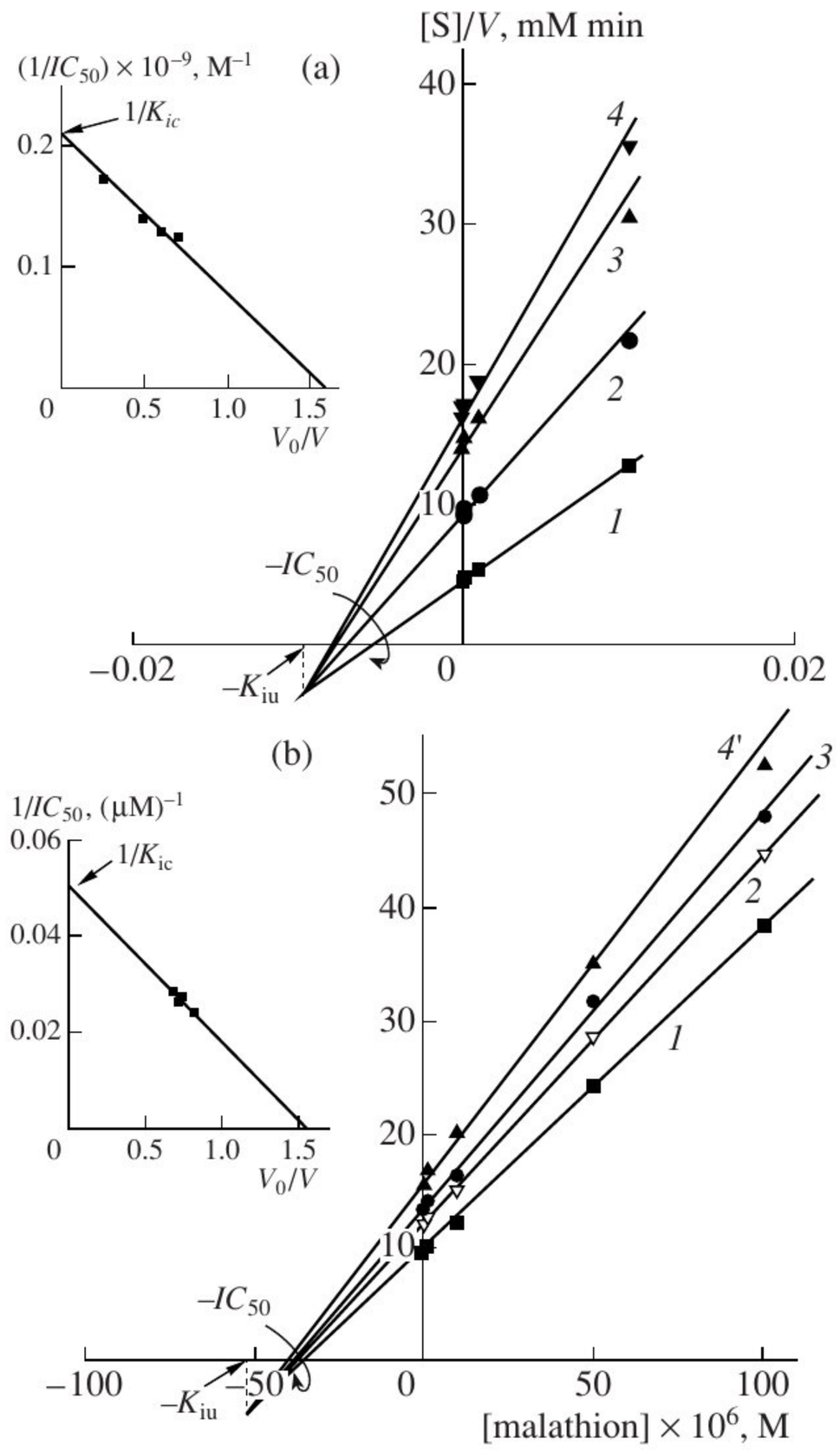

Fig. 3. Inhibition of BChE for (a) high- and (b) low-affinity binding sites at various N-methylindoxyl acetate concentrations ((*1*) 0.50, (*2*) 1.00, (*3*) 1.50, (*4'*) 1.75, and (*4*) 2 mM). The determination of the inhibition constants and type from the dependence of IC_{50} on V_0/V is shown in the inset. As the line in the secondary plot intersects the abscissa axis at a value larger than 1, malathion acts as a mixed inhibitor with a predominant competitive component; the slope is equal to $1/K_{iu} - 1/K_{ic}$.

mixed inhibition with predominantly competitive components was found. The common intersection point that represents the inhibitory constant K_{iu} is below the axis in the plot of [S]/V vs. [I], and abscissa intercepts that represent IC_{50} for particular substrate concentrations increase with [I]. The calculated inhibition constants are $K_i = 9.56 \times 10^{-9}$ M, $K_{ic} = 4.22 \times 10^{-9}$ M, and $K_{iu} = 9.58 \times 10^{-9}$ M. $K_{ic} < K_{iu}$, and inhibition is with a predominant competitive component (Fig. 3). For low-affinity binding sites, the calculated inhibition constants are $K_i = 5.13 \times 10^{-5}$ M, $K_{iu} = 5.23 \times 10^{-5}$ M, and $K_{ic} = 1.98 \times 10^{-5}$ M. $K_{ic} < K_{iu}$, and inhibition is with a predominant competitive component (Fig. 3b). As the

Table 2. Effect of malathion on IC_{50} of human plasma BChE at (I) high- and (II) low-affinity binding sites

[S], mM	$IC_{50} \times 10^9$, M (I)	$IC_{50} \times 10^5$, M (II)
0.50	5.77	5.78
1.00	7.23	7.13
1.50	7.69	7.65
2.0	8.04	7.97

Note: The IC_{50} values were determined from plots of [S]/V vs. [I] against V_0/V. Each point represents the mean of three determinations.

lines in the secondary plots shown in the insets in Fig. 3 intersect the abscissa axis at the value larger than unity [28], we see that malathion acts as a mixed inhibitor with a predominant competitive component.

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