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Reactions of isodimethoate with human red cell acetylcholinesterase

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ABSTRACT

Isodimethoate is a thermal decomposition product that is present in usual pesticide formulations of dimethoate. Owing to its P=O structure the compound is a direct anticholinesterase agent whose properties, to the best of our knowledge, are presented here for the first time. Isodimethoate shows an inhibition rate constant towards human red blood cell acetylcholinesterase (AChE) of $2.3 \times 10^3\ M^{-1}\ min^{-1}$ (pH 7.4, 37 °C), indicating a somewhat higher potency than found with omethoate, the CYP450-mediated active metabolite of pure dimethoate. Isodimethoate-inhibited AChE shows fast spontaneous reactivation and aging kinetics (half-life 2.3 and 25 min, respectively). The inhibited, non-aged enzyme is readily reactivated by obidoxime ($k_r = 9\ min^{-1}$; $K_D = 0.1$ mM) but hardly by pralidoxime at therapeutic concentrations. Interestingly, isodimethoate hydrolyzes readily in buffered solutions at pH 7.4 and 37 °C with liberation of methylmercaptan (half-life 16 min). Liberation of *N*-(methyl)mercaptoacetamide, the expected leaving group, was not observed. These properties make isodimethoate a hit-and-run agent that renders part of AChE non-reactivatable within a short period of time. The clinical consequences of exposure to or intentional ingestion of isodimethoate-containing dimethoate formulations are a partly untractable AChE shortly after incorporation. In fact, aging of AChE in dimethoate-poisoned patients on admission was much more advanced than expected from the reaction with omethoate. Manufacturers, researching scientists and clinical toxicologists should be aware of this problem.

1. Introduction

It has long been known that oximes are of little value in reactivating acetylcholinesterase (AChE) after dimethoate poisoning. Sanderson and Edson were probably the first who showed that there was no advantage of oxime administration in dimethoate-poisoned rats with the tendency that the benefits of atropine were curtailed by inclusion of pralidoxime. In contrast, there was some benefit of oximes (pralidoxime and trimedoxime) in the case of parathion methyl poisoning [1]. The authors reasoned that the difference may be caused by the slower formation of the phosphorylating metabolite in dimethoate poisoning, as opposed to parathion methyl. During the gradual formation of the phosphorylated enzyme in dimethoate poisoning a larger part was already in the aged state when the toxic syndrome becomes evident. In

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Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); ATCh, acetylthiocholine; AU, absorbance units; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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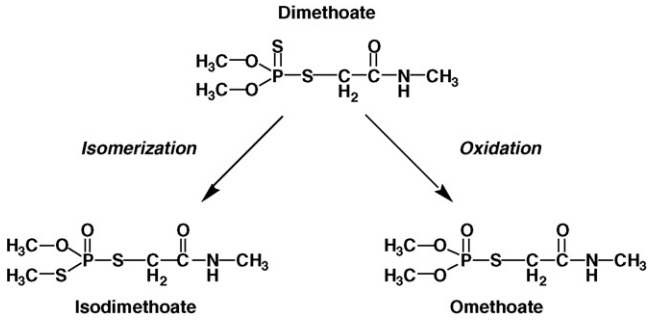


Scheme 1 – Activation of dimethoate to phosphorylating agents.

contrast, the precipitous inhibition of AChE and the arising cholinergic crisis in parathion methyl poisoning occurred earlier when a smaller proportion of AChE was aged. The resistance of inhibited AChE towards pralidoxime or obidoxime treatment was also shown in dogs poisoned with dimethoate [2], and similar observations were made in man [3]. Inhibited AChE of dimethoate-poisoned patients occasionally showed much faster aging than expected from an *O*,*O*-dimethylphosphoryl enzyme and contrasted with the picture seen, e.g., with oxydemeton-methyl poisoning. This strange behavior suggested either an alternative inhibition reaction or the involvement of dimethoate impurities, or both.

It is generally assumed that P=S organophosphorus esters have to be metabolically activated by oxidative desulfuration, mainly by the CYP450 superfamily, leading to the corresponding oxons that render the phosphorus atom more electrophilic (Scheme 1). The corresponding dimethoate metabolite is omethoate [4,5]. Given that liberation of *N*-methyl-mercaptoacetamide as the expected leaving group of omethoate forms *O*,*O*-dimethylphosphoryl–AChE, the inhibited enzyme should be indistinguishable from the enzyme blocked by other *O*,*O*-dimethyl organophosphates [6]. If, however, an *O*-methyl was the leaving group, a completely different enzyme would result that might be much more prone to aging and spontaneous reactivation as seen with other phosphorothiolates [7]. Such a pathway for dimethoate/omethoate has, however, never been detected and seems quite unlikely for chemical reasons.

Early work with P=S organophosphorus esters was carried out without realizing that some of these agents may change spontaneously into compounds with properties very different from their parent forms [8]. Besides of oxidation of the P=S or mercapto sulfur in the leaving group, thiono–thiolo isomerisation (RO–P=S into RS–P=O) readily occurs, particularly at elevated temperatures. The toxicological impact of post-factory chemical alterations was recognized in 1976 when 2800 spraymen using malathion during a malaria eradication program in Pakistan became poisoned with characteristic cholinergic signs and symptoms, which were usually not seen with this comparably safe pesticide. It turned out that isomalathion was one of the contaminants formed upon storage that markedly increased the toxicity of the malathion formulation [9].

Also dimethoate is prone to thermal and photochemical isomerisation with formation of isodimethoate [10,11], an *O*,*S*-dimethyl oxon that is a directly acting inhibitor of AChE. (A revised FAO specification allowed up to 7% of isodimethoate after storage tests [11].) By this isomerisation, a new center of asymmetry at phosphorus is formed. If *N*-methyl-mercaptoacetamide is the leaving group two enantiomeric *O*,*S*-dimethylphosphoryl–AChE molecules may be formed in analogy to the reaction of isoparathion methyl with AChE [12]. The rate of inhibition by isoparathion methyl of AChE from various sources was generally higher with the S_P-isomer compared to the R_P-isomer. There was, however, a marked species difference in both inhibition rate constants and their ratios, being least with human erythrocyte AChE [13]. There also exist remarkable differences between both inhibited enzyme species with regard to post-inhibitory reactions. Thus spontaneous as well as oxime-induced reactivation was generally higher in case of the S_P-isomer, while non-reactivatability proceeded two-times faster with the R_P-isomer [14].

The importance of the shelf-age of dimethoate formulations on its toxicity was suspected in 1964 when Meleney et al. reported the different dose dependence for toxic effects of dimethoate in sheep [15] and by Gaines for rats [16]. The involvement of dimethoate impurities on the enhanced toxicity in the presence of oximes has already been supposed in 1966 when it turned out that oximes were capable of enhancing AChE inhibition only in the case of impure dimethoate samples and it suggested to the authors that omethoate or isodimethoate may react with the oximes to give more potent inhibitors such as phosphoryloximes [17,18]. At that time omethoate and isodimethoate were not available to allow more in-depth studies. As to our knowledge, no further investigations on this topic were carried out subsequently by the above authors.

With regard to the strange observations in dimethoate-poisoned patients [3] and the availability of isodimethoate we undertook an *in vitro* study to elucidate the kinetics of inhibition, spontaneous and oxime-induced reactivation as well as formation of a non-reactivatable enzyme of human erythrocytes. During these experiments we realized that isodimethoate was surprisingly unstable in phosphate buffer, pH 7.4, at 37 °C which prompted an additional study.

2. Materials and methods

Isodimethoate, 93.4% certified purity, (*O*,*S*-dimethyl-*S*-(*N*-methylcarbamoylmethyl) phosphorodithioate; CAS 3344-11-4) was a generous gift from Dr. K. Lystbaek, Cheminova A/S Lemvig, Denmark. Stock solutions (100 and 10 mM) were prepared in acetonitrile and kept at −20 °C. Omethoate (*O*,*O*-dimethyl-*S*-(*N*-methylcarbamoylmethyl) phosphorothioate; CAS 113-02-6), 95.5% certified puritiy, was obtained from Dr. Ehrenstorfer, Augsburg, Germany. Stock solutions (10 mM) were prepared in acetonitrile and stored at −20 °C. The acetonitrile content in all enzyme assays was ≤1% (v/v). *N*-(Methyl)mercaptoacetamide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide (ATCh) and pralidoxime chloride were obtained from Sigma, Taufkirchen, Germany, obidoxime chloride monohydrate from Duphar, Amsterdam,

The Netherlands. All other reagents were at the purest grade available from commercial sources.

Freshly drawn human heparinized blood was centrifuged, plasma discarded and the sediment was washed four times with 0.1 M phosphate buffer, pH 7.4. The hemoglobin content was adjusted to 150 g/l and the erythrocyte preparation was frozen at −20 °C. Aliquots were thawed and completely hemolyzed by sonification (2 × 30 s with 30 s interval) on ice. To mask the reactive SH groups in hemoglobin, the hemolysate was incubated with 6 mM DTNB at 37 °C for 5 min, in order to minimize the blank reaction [19].

2.1. Determination of reaction rate constants

AChE activity was determined by a modified Ellman [19,20] procedure in the presence of 0.45 mM substrate and 0.3 mM DTNB, pH 7.4, 37 °C at 436 nm using a molar extinction coefficient of $11.28 \times 10^3\ M^{-1}\ cm^{-1}$ at 37 °C [21].

Kinetics of reactivation was followed continuously in the cuvette as proposed by [22] in the modification of [23]. The latter approach made use of non-linear regression analysis of the absorbance data vs. time using the following equation:

$$d_{Abs} = v_0 \times t + \frac{v_0}{k_{obs}}(e^{-k_{obs} \times t} - 1),$$

with v_0 being the maximal velocity and d_{Abs} the absorbance difference between t_0 and t. In case of oxime-induced reactivation the sample and reference cuvettes contained the oxime along with substrate and DTNB to compensate for oximolysis of ATCh [24–27]. It should be noted that the rate constants obtained by this method are sensitive to the precise time point when reactivation starts. Due to manual mixing of the inhibited aliquot with the cuvette content and some hysteresis of the photometer recorder, we added 10 s reaction time throughout before data were collected.

To follow aging kinetics, AChE in hemolysate with masked sulfhydryl groups was inhibited by 0.5 mM isodimethoate at pH 7.4 and 37 °C. After various time intervals 10 μl aliquots were mixed in the cuvette containing 40 μM obidoxime along with ATCh and DTNB in 3.15 ml. The enzyme activity was read after 4 min reactivation (>10 reactivation half-lives). The reference cuvette contained the same mixture except the enzyme to compensate for the oximolysis of substrate. The reactivatability gradually decreased but stopped at some 20%. This effect was most probably due to the degradation of isodimethoate during the rather long observation period, followed by some spontaneous reactivation. We thus changed our experimental strategy and spiked the inhibition assay repeatedly every 16 min with half the amount of isodimethoate to keep the concentration of the inhibitor fairly constant. With this procedure aging became virtually complete.

Volatile thiols were determined after diffusion into a DTNB-containing trap in miniature Fernbach flasks (outer well 5 cm^2, inner well 0.5 cm^2 surface area, gas volume 10 cm^3), sealed with a serum rubber stopper. To avoid autoxidation of the thiol compounds the flasks were flushed with argon, placed in a gyrotory water bath, protected from light [28], and gently shaken at 37 °C.

2.2. Calculations

Reaction rates were calculated by non-linear regression analysis of the data points using Prism™ Version 3.0 (Graphad Software, San Diego, CA). Numerical solutions of multiple differential equations [29] were performed with Maple 9.0 software (Maplesoft, Waterloo, Canada).

3. Results

3.1. Degradation of isodimethoate

When 100 μM isodimethoate was incubated in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.3 mM DTNB at 37 °C gradual formation of the yellow thiolate anion was observed. The reaction curve followed an exponential association function with a half-life of 16 min (Fig. 1). When the reaction was complete formation of the yellow indicator corresponded to 97 μM isodimethoate. The same result was obtained when isodimethoate was incubated first in plain phosphate buffer for 90 min, followed by the addition of DTNB, indicating that the buffer itself induced isodimethoate degradation. (The rate of isodimethoate degradation was similar using 10 mM phosphate buffer in 0.9% NaCl, pH 7.4.).

To decide whether methylmercaptan or (*N*-methyl)mercaptoacetamide were released, we exploited the different volatility of the potential thiols and placed 0.3 ml 0.2 mM isodimethoate in 0.1 M sodium phosphate buffer in the inner well of a Fernbach flask with 1 ml 0.3 mM DTNB in the outer well and allowed to react at 37 °C. The yellow color in the trap developed steadily and approached 98% of theory if 1 mol isodimethoate had liberated one mol of thiol. The control with plain phosphate buffer in the inner well was negative. The remaining solution in the inner well did no longer react with DTNB, indicating that the reaction was complete. For control, authentic (*N*-methyl)mercaptoacetamide (0.2 mM) was treated the same way. Formation of the yellow product in the outer

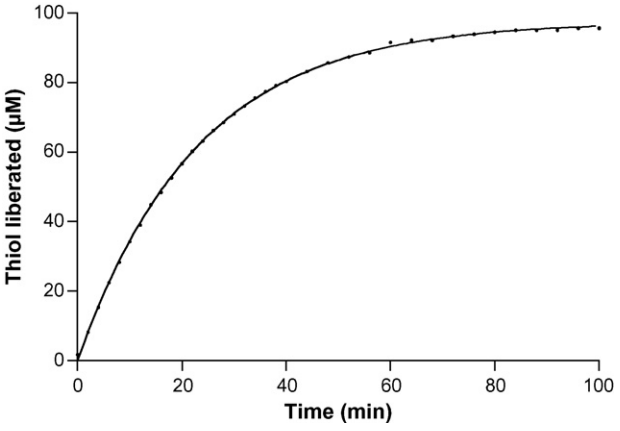


Fig. 1 – Liberation of thiol from isodimethoate. Isodimethoate (100 μM) was incubated in 0.1 M phosphate buffer, pH 7.4, containing 0.3 mM DTNB, at 37 °C. Formation of the yellow indicator was followed at 436 nm and transformed into μM thiol produced upon decomposition of isodimethoate.

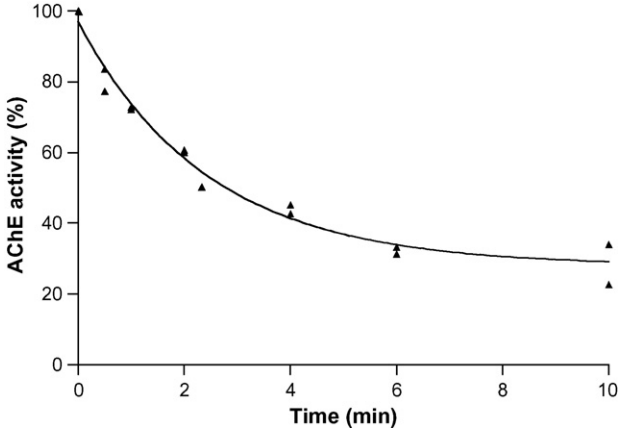


Fig. 2 – Inhibition of AChE by isodimethoate. Human hemolysate with masked sulfhydryl groups was incubated with 0.2 mM isodimethoate at 37 °C. Aliquots (10 μl) were then transferred into a cuvette containing 0.45 mM acetylthiocholine and 0.3 mM DTNB in a total volume of 3.15 ml phosphate buffer, pH 7.4. The enzyme activity was read for 75 s (two experiments). AChE activity refers to percent of control activity.

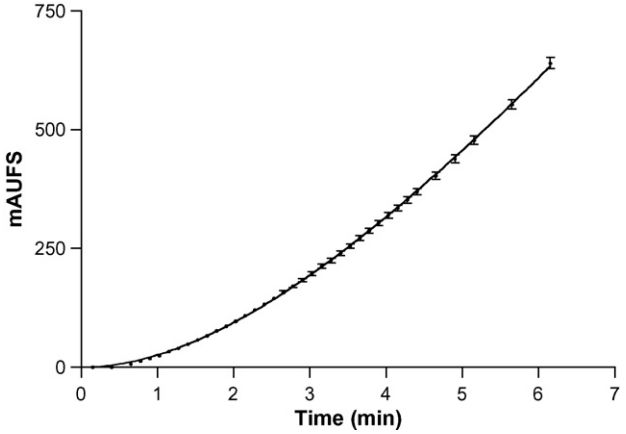


Fig. 3 – Spontaneous reactivation of AChE inhibited by isodimethoate. Human hemolysate mit masked sulfhydryl groups was reacted with 0.5 mM isodimethoate at 37 °C for 6 min. Then 0.01 ml were transferred in a thermostatted cuvette with 3.15 ml of buffer, containing 0.45 mM ATCh and 0.3 mM DTNB. Formation of the yellow indicator was followed continuously at 37 °C and 436 nm over 10 min. To the data points a curve was fitted with $k = 0.3\ min^{-1}$ (means ± S.D., $n = 10$).

well was not detectable, obviously due to the low vapor pressure of (*N*-methyl)mercaptoacetamide. These experiments pointed to hydrolytic cleavage of the thiomethyl group only.

The rapid hydrolysis of isodimethoate ($t_{1/2}$ 16 min) was surprising since isoparathion methyl was found to hydrolyze much more slowly ($t_{1/2}$ 7.4 h, 0.1 M phosphate buffer, pH 7.6, presumably 25 °C) with liberation of *p*-nitrophenol [12]. At any rate the data indicated that reactions with isodimethoate in buffer solutions can be followed only within a narrow time slot to give meaningful results.

3.2. *Inhibition of human erythrocyte AChE by isodimethoate*

Preliminary experiments had shown that AChE was only incompletely inhibited by large molar excess of isodimethoate; after an initial rapid decrease in enzyme activity further inhibition soon halted. A typical experiment is shown in Fig. 2. When the incubation mixture was diluted in plain phosphate buffer and further incubated at 37 °C, the enzyme activity quickly, but incompletely regenerated, pointing to rapid spontaneous reactivation and, presumably, also to rapid aging. These processes were studied separately in the following experiments.

3.3. *Spontaneous reactivation*

Human hemolysate with masked sulfhydryl groups was reacted with 0.5 mM isodimethoate at 37 °C for 5 min, resulting in almost complete inhibition. Then 10 μl was transferred into a thermostatted cuvette with 3.15 ml of buffer containing 0.45 mM ATCh and 0.3 mM DTNB. Formation of the yellow indicator was followed continuously at 37 °C over 6 min. The records showed an accelerating profile, pointing to spontaneous reactivation during the determination process. Drawing tangents on the curve by eye was tedious and dissatisfying. We hence pursued another track. To the data points obtained at 0.25 min intervals a curve was fitted as described in Section 2, yielding a first-order reactivation rate constant of 0.3 min^{-1} (0.27–0.33, 95% CI; $n = 10$; $r^2 = 0.992$). Fig. 3 shows the means ± S.D. and the fitted curve.

3.4. *Oxime-induced reactivation*

Reactivation of inhibited AChE was markedly accelerated in the presence of obidoxime. The experimental set-up was similar to the method chosen for spontaneous reactivation. However, the cuvette contained 30 μM obidoxime in addition.

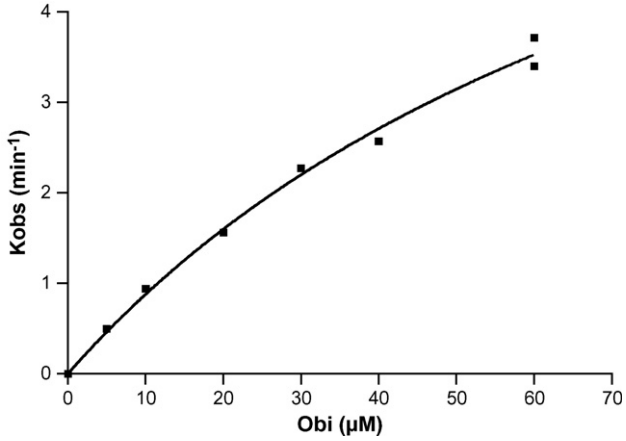


Fig. 4 – Dependence of the observed reactivation rate constant of isodimethoate-inhibited AChE on the obidoxime concentration.

To compensate for the oxime-induced hydrolysis of ATCh, the reference cuvette contained the same mixture except for the inhibited AChE. Analysis of the data revealed a reactivation half-life of 0.31 min. After correction for the spontaneous reactivation, the net reactivation half-life for 30 μM obidoxime was 0.36 min. Analysis of the obidoxime-induced reactivation at different concentrations showed a Michaelis–Menten behavior with K_D = 90 μM (40–143, 95% CI) and k_{rmax} = 8.9 min^{-1} (5.2–12.2). It should be noted, however, that obidoxime concentrations were used within a quite narrow range (5–60 μM), because both the high spontaneous reactivation and the oximolysis of ATCh set their limits (Fig. 4).

When PAM was tested for its reactivating potency, weak effects were noted; at 100 μM PAM the net reactivation half-life was 4.6 min only. Higher concentrations were not tested, because of the annoying oximolysis [23]. It appeared that 100 μM PAM was as effective as about 2 μM obidoxime.

3.5. *Aging of isodimethoate-inhibited AChE*

Aging is usually termed as resistance towards reactivation due to a dealkylation reaction that dramatically reduces the electrophilicity of the phosphorus atom. It can be tested by the gradual inability of an oxime to induce the reactivation. In our first approach AChE was inhibited with 0.5 mM isodimethoate and aliquots were allowed to react with 40 μM obidoxime in the cuvette together with ATCh and DTNB. The enzyme activity was read after 4 min reactivation (>10 reactivation half-lives). The reference cuvette contained the same mixture except the enzyme to compensate for oximolysis of the substrate. The reactivatability gradually decreased with an apparent half-life of some 25 min. Interestingly, the curve approached a plateau at about 20% residual activity (data not shown). This effect was most probably due to the degradation of isodimethoate during the rather long observation period, giving spontaneous reactivation a chance. We thus changed our experimental strategy and spiked the assay repeatedly every 16 min with half the amount of isodimethoate to keep the concentration of the inhibitor fairly constant. In doing so the activity decreased monoexponentially (r^2 = 0.988) and approached the y-axis near zero. The first order rate constant was 0.03 (0.022–0.038; 95% CI) (Fig. 5).

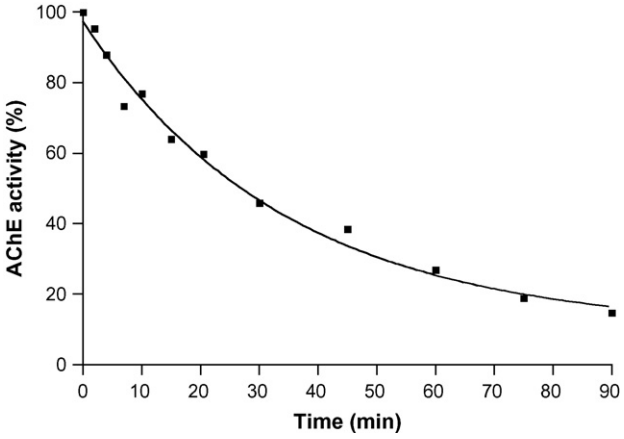


Fig. 5 – Aging (non-reactivatability) of isodimethoate-inhibited human AChE. The loss of reactivatability was tested after incubation of the diluted enzyme with 40 μM obidoxime for 4 min. To keep the intended concentration of 500 μM isodimethoate fairly constant, the mixture was spiked with half the amount every 16 min to compensate for the decomposition of isodimethoate in the buffer medium. The first-order line was fitted with k = 0.03 min^{-1}. AChE activity refers to percent of control activity.

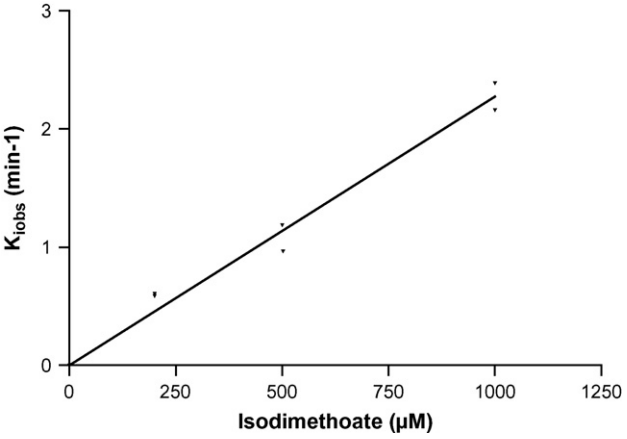


Fig. 6 – Dependence of the observed first-order inhibition rate constant of human AChE on the isodimethoate concentration (individual data points).

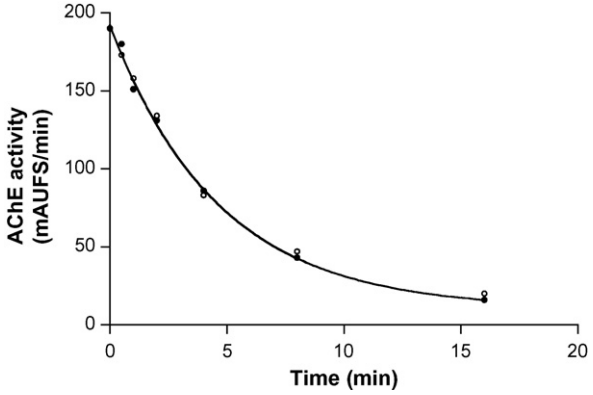


Fig. 7 – Time course of AChE inhibition by 0.1 mM omethoate at pH 7.4 and 37 °C (two experiments).

3.6. *Determination of the kinetic parameters for inhibition of AChE by isodimethoate*

Determination of the inhibition kinetics were complicated by the various simultaneous reactions that proceeded with high velocity. Thus, only high isodimethoate concentrations gave reasonable results, but were limited due to high reaction rates that made handling difficult. In the range between 200 and 1000 μM isodimethoate, a second-order reaction could be assumed without hints of a build-up of appreciable amounts of a Michaelis complex (Fig. 6). The derived second-order rate constant amounted to 2.3 × 10^3 M^{-1} min^{-1} (2.1–2.5; 95% C.I.).

Table 1 – Reaction rate constants of isodimethoate with human AChE at pH 7.4 and 37 °C

Degradation, k_h (min^{-1})	Inhibition, k_i ($M^{-1}\,min^{-1}$)	Aging, k_a (min^{-1})	Spontaneous reaction, k_s (min^{-1})	Obidoxime-induced reactivation	
				k_r (min^{-1})	K_D (mM)
0.043	2.3×10^3	0.03	0.3	9	0.1

3.7. *Inhibition of AChE by omethoate*

For comparison, inhibition of AChE by 100 μM omethoate was determined as described in Section 3.2. The inhibition kinetics followed a clear first-order reaction with $k = 0.22\ min^{-1}$ and showed a plateau when inhibition was by 93% complete (Fig. 7). When the mixture was diluted spontaneous reactivation ensued with k of approx. $0.017\ min^{-1}$, compatible with the observed plateau at steady state. The half-life of the spontaneous reactivation was as observed with other dimethylphosphoryl compounds [30].

4. Discussion

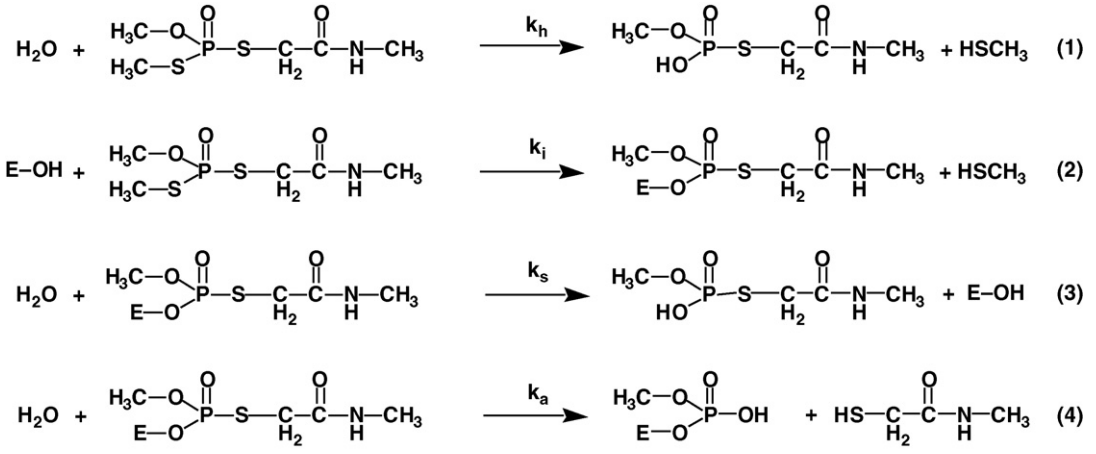
4.1. *Kinetic aspects*

The kinetic data with isodimethoate as shown in Table 1 indicate that all post-inhibitory reactions are unusually fast. When testing the inhibition kinetics for the first time, we were faced with the phenomenon of rapid, but only partial inhibition, a phenomenon that was not easily understood. Fig. 2 illustrates such a reaction with human hemolysate AChE in the presence of 0.2 mM isodimethoate.

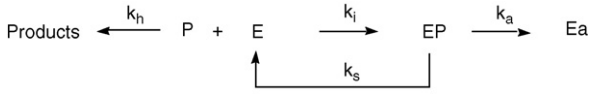
With the individual kinetic data at hand, as shown in Table 1, we can now interpret the reaction course. After the inhibitory phase observed only at the beginning, spontaneous reactivation ensues, leading to a steady state that should be proportional to the reaction rate constants of inhibition and reactivation. Calculation gives an expected inhibition at equilibrium of some 60% at 0.2 mM isodimethoate. The observed equilibrium was attained in that range, apparently justifying our assumption. When we considered the fast aging reaction, however, we were surprised of attaining a quite stable steady state. Rather, we had expected a second slower phase of a steady decrease in activity, because part of the inhibited enzyme should be sequestered from the equilibrium. We tested the inhibitory activity of the assay after various incubation times and found the inhibition fading until no inhibition could be detected at all after 105 min (not shown). This behavior prompted us to look at the stability of isodimethoate in the buffer solution.

Now we know that the degradation half-life of 16 min (Table 1) is causative for the decreasing inhibition, which roughly compensates for aging (half-time 23 min). Scheme 2 tentatively summarizes our observations. For the sake of simplicity we consider the labile thiomethyl group also as the leaving group in the reaction with AChE, although we are well aware that this supposition has to be proved. We also consider the P–S cleavage quite arbitrarily as the aging reaction with loss of the 2-mercapto-(*N*-methyl)acetamide residue. Possibly new MALDI-TOF techniques [31] applied to suitable peptides may shed some more light on the intricate mechanisms, although we are a bit sceptic in light of the lability of the AChE conjugates.

Analysis of the microscopic rate constants of phosphorylation, spontaneous reactivation and aging of the isomerides of thiono products is not without antecedents. The rate constants of isomalathion have been studied with bovine red blood cell AChE at pH 7.4 and 37 °C [7]. The inhibiton rate constant was about 50 times higher with isomalathion $1.25 \times 10^5\ M^{-1}\,min^{-1}$ compared to isodimethoate



Scheme 2 – Simultaneous reactions occurring with AChE and isodimethoate in 0.1 M phosphate buffer, pH 7.4. Reaction (1) hydrolysis with liberation of methylmercaptan. Reaction (2) inhibition of AChE with liberation of methylmercaptan. Reaction (3) spontaneous reactivation giving rise of active enzyme and O-methyl, S-(*N*-methyl)carbamylmethylphosphate. Reaction (4) aging giving rise of monomethylphosphorylated enzyme and 2-mercapto-(*N*-methyl)acetamide.



Scheme 3 – Flow chart showing schematically the simultaneous reactions of the active enzyme E in the presence of the organophosphate P, with regeneration of E from the reversibly inhibited conjugate EP that is prone to aging into Ea.

($2.3 \times 10^3\ M^{-1}\ min^{-1}$). This difference might be caused by the better leaving group (diethylsuccinate) in isomalathion. Correspondingly, malaoxon is a much stronger inhibitor than omethoate ($1 \times 10^6\ M^{-1}\ min^{-1}$ [32] vs. $1.7 \times 10^3\ M^{-1}\ min^{-1}$, this work). Consequently, others have argued that AChE inhibited by isomalathion is an *O*,*S*-dimethylphosphorylated enzyme [7].

It was tempting to simulate the theoretical course of enzyme inhibition using the following equations as derived from the flow chart in Scheme 3:

$$d(E)(t) = -k_i \times C(t) \times E(t) + k_s \times EP(t);$$

$$d(EP)(t) = -k_a \times EP(t) - k_s \times EP(t) + k_i C(t) \times E(t);$$

with E being the active enzyme, EP the phosphorylated non-aged enzyme, and *C*(*t*) the actual isodimethoate concentration which is prone to simultaneous decomposition:

$$C(t) = C_0 \times \exp(-k_h \times t).$$

The calculated data for E, EP and Ea, the aged enzyme, are plotted in Fig. 8 along with experimental data points. The fit is only partly satisfying. Given, however, that the simultaneously occurring reactions are very fast and influencing each other, the experimental error is presumably quite large. Consequently, the rate constants given in Table 1 should be considered as approximate values. This holds in particular

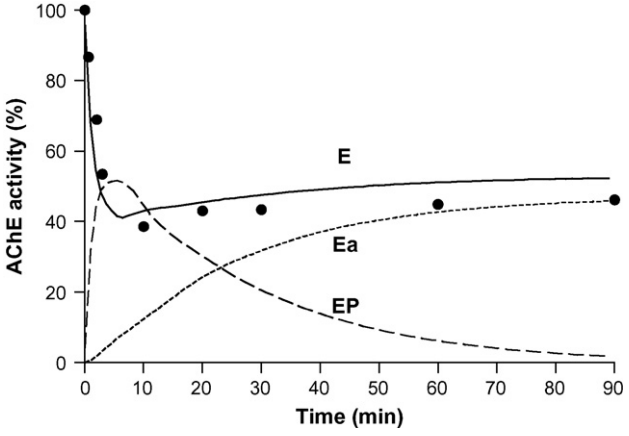


Fig. 8 – Simulated time course of AChE inhibition by 0.2 mM isodimethoate with formation of phosphorylated, but not aged enzyme, EP, and aged enzyme, Ea. The dots represent measured data. AChE activity refers to percent of control activity.

true for the aging and the reactivation constants that are presented with one digit only.

Regardless of these inherent shortcomings, the particularly fast post-inhibitory reactions of isodimethoate, which, to the best of our knowledge, are presented here for the first time, are amazing and should be investigated in more depth with other organophosphorus dithiolates, such as profenofos for which similar reactivities appear to exist [33].

Two aspects deserve separate comments:

(i) We did not find any hints of two isodimethoate enantiomers with different reactivities, as observed with the isoparathion R_P and R_S isomers. We surmise that the preparation used was the racemic form. From this it may be deduced that the reactivities of both isomers are quite similar as are the presumed *O*-methyl, *S*-(*N*-methyl)mercaptoacetamidophosphoryl AChE isomers. In line with this assumption is the homogeneous degradation curve of isodimethoate. While the similarity of the abiotic degradation rates of both enantiomers is easy to understand, it is much less comprehensible that the asymmetric enzyme would not discriminate between the enantiomers. Rather, one enantiomer may be quite unreactive and undergoes degradation, while the other gives homeogeneous reaction rates in inhibition and the post-inhibitory reactions. (We are indebted to one reviewer for these valuable suggestions.)

(ii) Pralidoxime, in contrast to obidoxime, was a very weak reactivator that will not add much to the spontaneous reactivation at therapeutic concentrations. Obidoxime was only tested under conditions of highly diluted enzyme, e.g. 30 pM, where formation of a phosphoryl oxime will not have much consequences. Under therapeutic conditions at about 10 nM AChE the situation may be different [34,35]. However, with respect to the rapid post-inhibitory reactions that will be almost complete before any oxime treatment ensues, this question may be rather academic.

4.2. *Clinical consequences*

If we suppose that some isodimethoate (>4–7%) was present in the formulations used in Sri Lanka and other regions with hot climate [11], we would expect that the contribution of isodimethoate on AChE inhibition cannot be disregarded. Since isodimethoate does not require metabolic activation, AChE at accessible sites will be rapidly phosphorylated to a certain extent with subsequent aging. Inhibition will soon stop when the supply of intact isodimethoate is exhausted and non-aged AChE will quickly regenerate spontaneously. Given that most patients usually present not before 1–2 h post-ingestion [3], we can expect that all these reactions will have come to completion and we will have a good part of aged or active enzyme, but hardly any phosphorylated enzyme that is amenable to reactivation. These patients will present with admission AChE much lower than normal and this fraction will be gradually lowered by the omethoate that is metabolically formed from intact dimethoate. Since spontaneous reactivation is also quite fast with O,O-dimethylphosphoryl-AChE (half-time some 40 min [30]), the potential oxime-induced reactivation will be quite small. Hence the difference between AChE activity *in vivo* and after oxime-induced

reactivation *in vitro* [36] is expected to be small. In fact, this was frequently observed. This behavior may explain the usual clinical failure of oximes in dimethoate poisoning [37]. It is the more suspected in cases were pralidoxime is used as a reactivator, since it appears from this study that therapeutic concentrations of pralidoxime will hardly accelerate the spontaneous reactivation. The situation may be different when pure dimethoate is being used.

We are hesitating to generalize our observations with isodimethoate on the clinical picture seen in dimethoate poisoning, but would like to alert clinical toxicologists to have a closer eye on this problem and to stimulate manufacturers and researchers as well to study the occurrence and the impact of potential degradation products of formulations used in real life. These risks can hardly be ruled out, but should be known at least!

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