Comparison of the Action of an Organophosphorus Insecticide and Its Metabolite on Chloride and Sulfate Transport in Erythrocyte Membrane

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The effect of the organophosphorus insecticide methylparathion and its main metabolite methylparaoxon on chloride and sulfate equilibrium exchange in pig erythrocytes was investigated using an isotope labelling technique. Efflux of both radioactive isotopes with time followed a single exponential. Methylparathion and methylparaoxon inhibited the chloride equilibrium exchange in erythrocyte ghosts in a dose- and time-dependent manner. There was no difference between effects evoked by these two compounds. Methylparathion and methylparaoxon inhibited sulfate efflux from resealed ghosts. The effect was also dose- and time-dependent. Again, there was no difference between the action of both agents. Dixon analysis revealed a non-competitive character of the inhibition of the exchange of both anions with apparent K_i values 183 and 184 μM for methylparathion and methylparaoxon, respectively in the case of chloride transport; for sulfate exchange these values were 675 and 648 μM. It was suggested that structural similarity between the parent agent and its metabolite accounts for their identical effects. Methylparathion and methylparaoxon might inhibit the anion exchange indirectly by changing the fluidity of the erythrocyte membrane or directly by binding to the band 3 protein and evoking conformational changes that lead to the inhibition of the anion transport. The insecticides, due to their ability to phosphorylate, might also disturb some regulation processes in the band 3 protein and affect anion transport in this way.

Introduction

Organophosphorus insecticides are known to be powerful toxicants due to their ability to phosphorylate the serine residues of the active site of acetylcholinesterase, an enzyme that terminates the action of acetylcholine by catalyzing its hydrolysis (Taylor, 1990). Inhibition of acetylcholinesterase produces a variety of toxic effects culminating in death, usually due to respiratory failure (Gallo and Lawryk, 1991). The majority of organophosphorus insecticides, except phosphates and phosphorothiolates, inhibit little acetylcholinesterase in pure state unless they are activated (Eto, 1974).

Organophosphorus insecticide, due to their lipophilic nature, can interact with lipid-rich biomembranes (Antunes-Madeira et al., 1981, 1994). Besides acetylcholinesterase they can affect some non-specific membrane proteins including cation transporting enzymes, namely $(Ca^{2+}+Mg^{2+})$-ATPase (Antunes-Madeira and Madeira, 1982; Deshpande and Swany, 1989; Luo and Bodnaryk, 1988; Blasiak, 1995c) and $(Na^{+}+K^{+})$-ATPase (Dierkes-Tizek et al., 1984; Robineau et al., 1991; Imamura and Hasegawa, 1984; Blasiak, 1995b). The question on possible effects of organophosphorus insecticides on the anion transport system in the membrane is still open. This subject is worth studying because few proteins have as many diverse important biological functions as the band 3 protein, responsible for anion transport in the erythrocyte membrane (Wang, 1994; Passow, 1986). On the other hand organophosphorus insecticides are commonly used chemicals (Barnett and Rodgers, 1994) so the determination of their non-specific (i.e. not connected with anticholinergic action) effects are of interest and are important to evaluate their environmental safety.

The purpose of this study was (1) to determine an effect of the organophosphorus insecticide methylparathion on chloride and sulfate transport in erythrocyte membrane and (2) to compare the

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action of the parent organophosphorus insecticide and its metabolite methylparaoxon. Chemical structure of these compounds is displayed in Fig. 1. The insecticide is one of the most common used around the world (Dearfield *et al.*, 1993; Dolara *et al.*, 1994).

Materials and Methods

Preparation of erythrocytes

Pig heparinized blood was centrifuged at $2000 \times g$, the serum and the buffy coat were removed and the red cells were washed three times at 4 °C in 166 mM NaCl. The packed cells were resuspended with 130 mM NaCl, 1 mM Na_2SO_4, 20 mM EDTA (pH 7.4) at a hematocrit of 10%. The anion transport inhibitor H_2DIDS was added to a part of the cell to a final concentration of 60 mM.

Preparation of resealed ghosts

The cells were centrifuged at $2000 \times g$ at 4 °C and washed twice in ice-cold 166 mM NaCl (Schwoch and Passow, 1973; Glibowicka *et al.*, 1988). A 50% suspension of the cells was hemolyzed with 4 mM $MgSO_4$, 1 mM acetic acid for 5 min at 4 °C. The pH of the mixture was 6.2. A concentrated, ice-cold EDTA was then added to give a final concentration of 20 mM and the mixture was incubated for 5 min on ice, divided into two parts and one part was washed in a sulfate flux medium comprising 108 mM Na_2SO_4, 20 mM EDTA (pH 6.5). The cells were then resuspended in the medium at a hematocrit of 20% and $^{35}SO_4^{2-}$ was added. Specific activity of the samples was 2 μCi/ml (= 7.4×10^4 Bq/ml). The other part was washed in a chloride flux medium consisting of 130 mM NaCl, 1 mM Na_2SO_4, 20 mM EDTA (pH 6.5). The cells were resuspended at a hematocrit of 20% and $^{36}Cl^-$ was added to give a final activity 1.25 μCi/ml

(= 4.6×10^4 Bq/ml). The suspensions were kept on ice for 15 min and then resealed for 45 min at 37 °C.

Measurement of sulfate efflux

The radioactivity labelled ghosts were spun down at $20,000 \times g$ for 5 min at 0 °C and washed three times in the ice-cold sulfate flux medium. The ghosts were incubated for 60 min at 37 °C with an insecticide or its metabolite at a desired concentration before the last washing. The efflux was initiated by mixing the washed packed ghost with the sulfate flux medium precooled to 8 °C at the final hematocrit 2%. 500 μl aliquots of each incubation mixture were withdrawn at scheduled intervals of time and centrifuged in an Eppendorf 5403 table centrifuge at 13,000 rpm for 1 min. 250 μl of the supernatant was added to a scintillation vial containing 2 ml Quickscint 2000. The radioactivity was counted in a LKB Wallac 8100 liquid scintillation counter. Total activity (y_∞) was counted by mixing 250 μl of ghost suspension with scintillation liquid.

Measurement of chloride efflux

Because the $Cl^- - Cl^-$ exchange is much more rapid than that of sulfate, an inhibitor stop method (Ku *et al.*, 1979) was employed in order to measure the chloride efflux. The labelled ghosts were centrifuged at $6000 \times g$ for 15 min at 0 °C and incubated for 60 min at 37 °C in the chloride flux medium containing an insecticide or its metabolite at a desired concentration and centrifuged again 15 min at $6000 \times g$. The $^{36}Cl^-$ efflux was initiated by resuspending 1 vol. of packed cells in 10 vol. of the chloride flux medium at 8 °C. Aliquots (0.7 ml) were taken at 2 s intervals with a repeating syringe and transferred into 0.5 ml of the ice-cold flux medium, containing H_2DIDS at 50 μM which instantly inhibited transport. The cells were centrifuged and the radioactivity of the supernatant was assessed as in the sulfate flux experiment.

Data evaluation

The anion efflux was expressed by the quantity F defined as
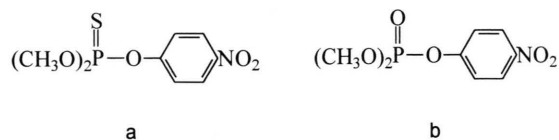
$$F = \frac{y - y_\infty}{y_0 - y_\infty},$$

Fig. 1. Chemical structures of methylparathion (O,O-dimethyl O-4-nitrophenyl phosphorothioate) (a) and methylparaoxon (O,O-dimethyl O-4-nitrophenyl phosphate) (b).

where y and y_0 represent the radioactivity in the supernatant at times t and $t = 0$, respectively; y_∞ is the total radioactivity (Zaki *et al.*, 1975). Because the efflux was measured under conditions where the composition of the media inside and outside the ghosts was identical, it was assumed that it followed a single exponential. The data were thus analyzed as the dependence of $\log F$ on time.

Each experiment was performed in triplicate. One-way analysis of variance was used to evaluate effects evoked by the insecticide or its metabolite. The differences between means were compared using Scheffe's multiple comparison test (Zar, 1974). Two-way analysis of variance (concentration, time) was used to compare effects evoked by the insecticide and its metabolite. No statistically significant interaction was found, so one-way analysis of variance was applied.

Chemicals

$Na_2{}^{35}SO_4$ and $Na^{36}Cl$ were obtained from Amersham (Braunschweig, Germany). H_2DIDS was purchased from Sigma (St. Louis, MO). The organophosphorus insecticide methylparathion (O,O-dimethyl O-4-nitrophenyl phosphorothioate) and its metabolite methylparaoxon (O,O-dimethyl O-4-nitrophenyl phosphate) of purity at least 99.8% were provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany). The insecticides were derived from stock (50 mM) ethanolic solutions to give a final concentration of 25, 50 or 100 μM. Their solubility in the working solutions was checked spectrophotometrically. The control samples received, instead of an insecticide, appropriate volume of ethanol at the final concentration of 0.18%. This concentration of ethanol did not evoke any significant effect on the processes under study.

Results and Discussion

Efflux of both radioactive isotopes *vs.* time followed a single exponential resulted in straight lines in a semi-logarithmic scale due to equilibrium conditions during flux measurements (Fig. 2).

The effect of methylparathion and methylparaoxon on chloride transport in erythrocyte ghosts is displayed in Figs. 2 A and 2 B. The presence of the H_2DIDS inhibited $^{36}Cl^-$ transport by 97%. Due to

technical difficulties of the inhibitor stop method, the first sample was taken 4 s after the initiation of the radioactive efflux and at $t = 0$ the value for the H_2DIDS experiment was used. Both compounds evoked an inhibition of the chloride equilibrium exchange in a dose- and time-dependent manner. There was no difference ($p < 0.05$) between effects caused by methylparathion and methylparaoxon.

Methylparathion and its metabolite inhibited sulfate efflux from resealed ghosts (Figs. 2 C and 2 D, respectively). The effect was also dose- and time-dependent. There was no difference between action of methylparathion and methylparaoxon ($p < 0.05$) at any concentration.

The inhibition of anion fluxes, as indicated by the Dixon plots (Fig. 3), was non-competitive for both methylparathion and methylparaoxon. The values of the apparent inhibition constant K_i are presented in Table I. It can be seen from the table that there were no differences ($p < 0.02$) in the values of K_i between methylparathion and its metabolite for the efflux of both anions.

The organophosphorus insecticide methylparathion and its main metabolite methylparaoxon were non-competitive inhibitors of anion transport in the pig red cell membrane. The effects of these two compounds were qualitatively and quantitatively the same. The identity of the effects evoked by the parent compound and its metabolite suggests that either methylparathion was activated to methylparaoxon or the structural agreement between the parent agent and its metabolite underlies similarity of effects. The first hypothesis is doubtful due to the short time of the experiment and the type of the environment where it was performed. The structural analogy between the insecticide and its metabolite likely explains the lack

Table I. The values of the apparent inhibition constant K_i for methylparathion and methylparaoxon inhibition of the chloride and sulfate equilibrium exchange in pig erythrocyte at 8 °C*.

Anion	K_i [μM]	
	Methylparathion	Methylparaoxon
Chloride	183 ± 21	184 ± 19
Sulfate	675 ± 63	648 ± 70

* The values of K_i were calculated using the least square method on the basis of the results displayed in Fig. 3. The results are given as mean ± SE.
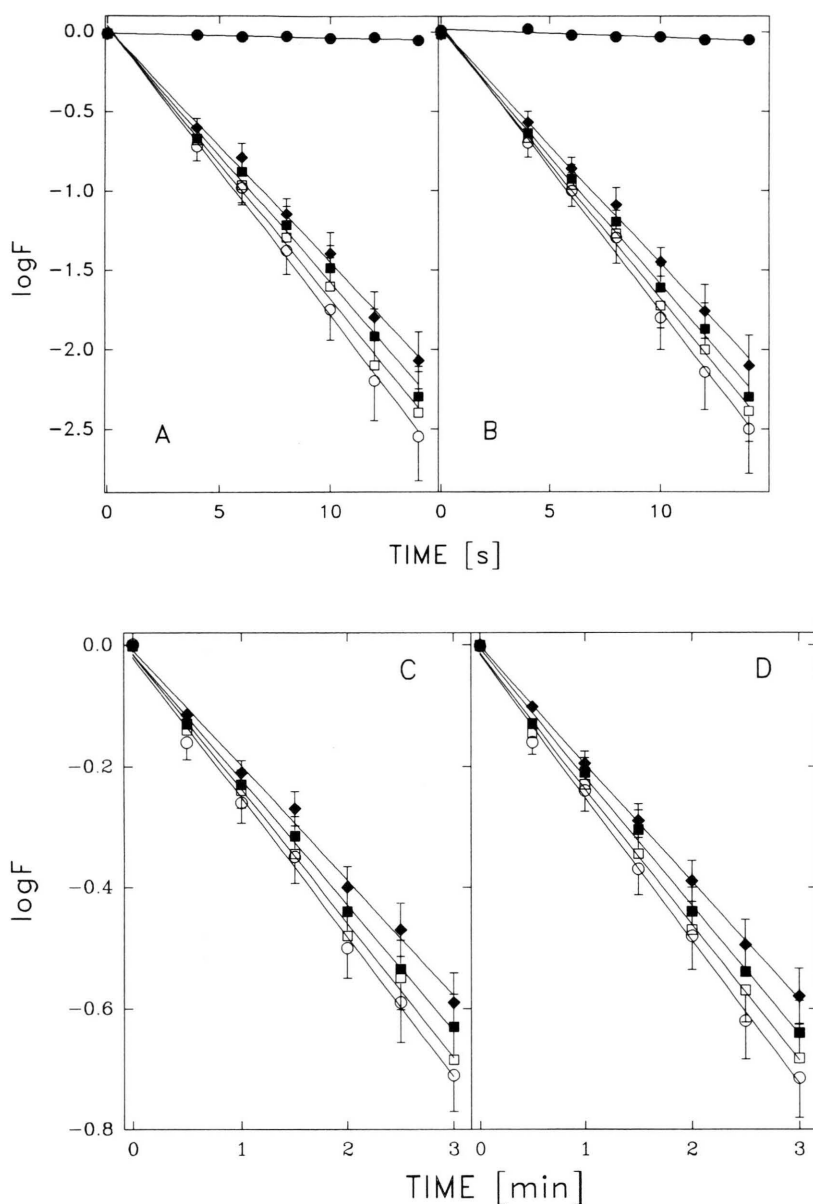
Fig. 2. Effect of methylparathion (A, C) and its metabolite methylparaoxon (B, D) at 25 (□), 50 (■) and 100 (◆) μM on the chloride ($^{36}Cl^-$) (A, B) and sulfate ($^{35}SO_4^{2-}$) (C, D) equilibrium exchange in pig erythrocytes at 8 °C as compared with control (○). In the case of chloride transport the effect of the anion transport inhibitor H_2DIDS was also displayed (●). F is a quantity equal to $(y - y_\infty)/(y_0 - y_\infty)$ where y and y_0 represent the radioactivity at times t and $t = 0$, respectively; y_∞ is the total radioactivity. Each experimental point is a mean of three independent measurements. Error bars represent SE. Regression lines were calculated by means of the least squares method. The R values were in the range 0.914–0.999.

of the differences in its effects on anion transport across the erythrocyte membrane.

The inhibition constant K_i is about five times greater for sulfate than for chloride efflux. This does not necessarily mean that the efflux of chloride is more affected by tested compounds than the efflux of sulfate because these two processes have different character and kinetics (Knauf *et al.*, 1977; Legrum and Passow, 1989).

Anion transport inhibitors can exert their action indirectly, *e.g.* by changing the surface potential or membrane fluidity (Ku *et al.*, 1979). Methylparathion is reported to change the fluidity of various model and native membranes (Blasiak, 1993) so it can be considered as potential agent that affects anion transport indirectly.

As mentioned above organophosphorus insecticides have strong affinity to membrane lipids. The
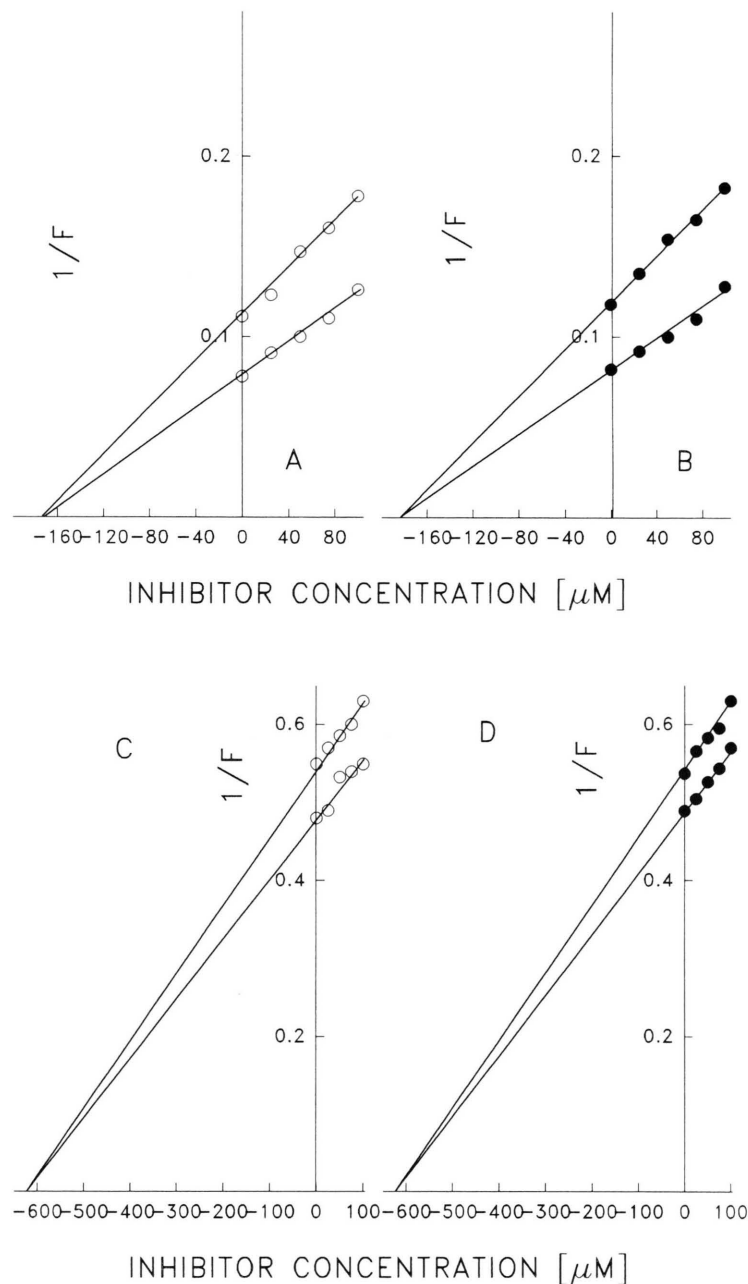
Fig. 3. Dixon plots for chloride (A, B) and sulfate (C, D) equilibrium exchange in pig ghost at 8 °C in the presence of methylparathion (A, C) or methylparaoxon (B, D). See the legend for Fig. 2 for the explanation of the F quantity. Each experimental point is a mean of three independent measurements. Regression lines were calculated by means of the least squares method. The R values were in the range 0.980–0.998. Error bars were omitted to increase the clarity of the figures.

presence of cholesterol in membrane may weaken incorporation of insecticides into it in domain where the sterol is present (Antunes-Madeira and Madeira, 1984, 1987; Blasiak, 1993, 1995 a). The presence of proteins in the membrane may favour insecticides incorporation presumably occurring in lipid–protein boundaries relatively scarce in cho-

lesterol (Silvius *et al.*, 1984). Organophosphorus insecticides can therefore interact directly with membrane proteins including the band 3 protein, responsible for the anion transport in the erythrocyte membrane. This interaction might evoke conformational changes of the band 3 protein leading to the disturbances of erythrocyte anion transport.

The non-competitive type of inhibition suggests that short range forces predominate in the interaction of the inhibitors with the erythrocyte membrane (Schnell, 1972). Most organophosphate compounds has an ability to phosphorylate many biological entities. Phosphorylation of proteins by organophosphorus insecticides consists of covalent attachment of a dialkyl phospho group to a reactive functionality in a protein chain (Matsumura, 1975). The amino terminus of band 3 binds haemoglobin and glycolytic enzymes, regulating glycolysis by a phosphorylation/dephosphorylation mechanism (Wang *et al.*, 1993). Methylparathion and methylparaoxon could therefore disturb some functional properties of the band 3 protein which could be reflected in the changes in anion transport across the erythrocyte membrane.

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