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The Interaction of the Phosphorothioate Insecticides Chlorpyrifos and Parathion and Their Oxygen Analogues with Bovine Serum Albumin

L. G. SULTATOS,[1] K. M. BASKER, M. SHAO,[2] AND S. D. MURPHY[3]

Department of Pharmacology and Experimental Therapeutics, Louisiana State University Medical Center, New Orleans, Louisiana 70112, Department of Anesthesiology, University of Texas Medical School of Houston, Houston, Texas 77025, and Department of Environmental Health SC-34, University of Washington, Seattle, Washington 98195

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SUMMARY

The distribution and subsequent toxicity of hazardous chemicals can be influenced by their interactions with plasma proteins. In the present study reversible binding of the phosphorothioate insecticides chlorpyrifos and parathion to fatty acid-free bovine serum albumin (BSA) was examined using the technique of equilibrium dialysis. Computer analyses of the binding data revealed that chlorpyrifos and parathion each bound reversibly to a single class of binding sites on BSA, with apparent K_D values of 3.4 ± 0.1 and 11.1 ± 0.3 μM, respectively. Additionally, the maximal number of binding sites for each insecticide per molecule of BSA was one. Displacement studies using both chlorpyrifos and parathion indicated that each was a competitive inhibitor of the other's binding, suggesting that they were bound to the same site. Incubation of chlorpyrifos oxon or paraoxon with a 1% solution of BSA resulted in limited, EDTA-insensitive formation of 3,5,6-trichloro-2-pyridinol or *p*-nitrophenol, respectively. Pretreatment of BSA with 5 mM paraoxon, chlorpyrifos oxon, or 1 mM diisopropylfluorophosphate did not alter this activity, suggesting that these reactions resulted from an esterase-like capacity of BSA, and not from phorphorylation of BSA by these oxons.

INTRODUCTION

Organosphosphate compounds have been shown to interact with plasma albumin by several different mechanisms. The reactive phosphorofluoridate, DFP,[4] is known to phosphorylate the hydroxyl group of a tyrosine residue of albumin (1, 2), whereas incubation of the phosphate paraoxon with albumin resulted in formation of *p*-nitrophenol, raising the possibility of an esterase-like activity of albumin toward paraoxon (3). The less reactive phosphorothioates, such as parathion, can interact reversibly with albumin, probably as a result of hydrophobic interactions (4, 5). Each of these three types of interactions between organophosphates and albumin could potentially influence the acute mammalian toxicity of these chemicals. Phosphorylation and hydrolysis by albumin could constitute, respectively, non-enzymatic and enzymatic detoxification pathways, whereas weak binding to albumin might influence the distribution and biotransformation, and consequently the degree and duration of action of these insecticides. Additionally, such reversible binding to albumin might represent a potential protective mechanism for transport in blood of those organophosphates which are substrates for plasma A-esterases.

Although these types of interactions between albumin and certain organophosphates have been identified, little is known regarding the frequency with which they occur, or the degree to which each influences the acute mammalian toxicity of these chemicals. The present study identifies and characterizes the interactions of BSA with the two phosphorothioate insecticides parathion and chlorpyrifos, as well as with their corresponding oxygen analogues, paraoxon and chlorpyrifos oxon (Fig. 1).

METHODS

Chemicals. The following analytical grade chemicals were furnished by Dow Chemical Company (Midland, Mich.); chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothionate], chlorpyrifos oxon [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphate], and 3,5,6-trichloro-2-pyridinol. Parathion was supplied by American Cyanamid Company (Princeton, N. J.). Paraoxon was synthesized by mixing 27.8 g (0.2 mole) of *p*-nitrophenol (Fisher Scientific Company, Fair Lawn, N. J.), 21.2 g (0.2 mole) of anhydrous sodium carbonate (Sigma Chemical Company, St. Louis, Mo.), and 150 ml of acetone, following by heating to mild reflux (58°) with stirring. A total of 34.4 g (0.2 mole)

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[1] Department of Pharmacology and Experimental Therapeutics, Louisiana State University Medical Center. Recipient of National Institute of Environmental Health Sciences Fellowship ES 05223.

[2] Department of Anesthesiology, University of Texas Medical School at Houston.

[3] Department of Environmental Health, University of Washington.

[4] The abbreviations used are: DFP, diisoprophylfluorophosphate; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; HSA, human serum albumin.

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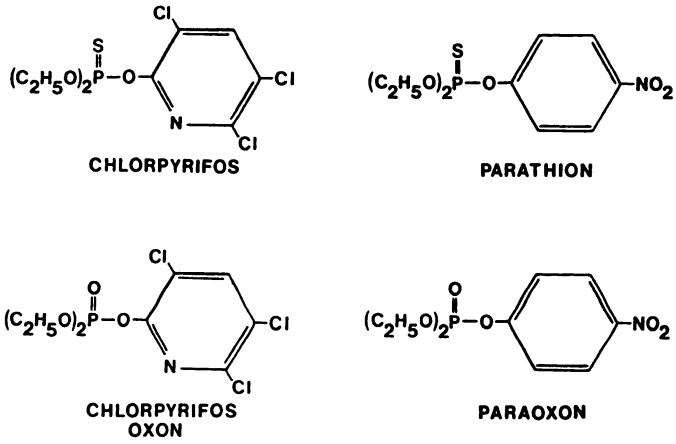

FIG. 1. *Structures of chlorpyrifos, parathion, and their oxygen analogues.*

of diethyl phosphorochloridate (Aldrich Chemical Company, Milwaukee, Wisc.) was added dropwise over 30 min. The batch was refluxed overnight, cooled, filtered, and stripped of acetone at reduced pressure. The resulting oil was then vacuum-distilled. Paraoxon had a boiling point of 167° at 0.2 mm Hg. Recovery was 47%, and the purity was greater than 99% as indicated by thin-layer chromatography (6) and HPLC (7) analyses.

Binding studies. Binding of chlorpyrifos or parathion to fatty acid-free BSA (99% pure, essentially fatty acid-free prepared from essentially globulin-free albumin; Sigma Chemical Company), was evaluated by equilibrium dialysis. A molecular weight of 66,700 was assumed for BSA (8). Preliminary studies indicated that chlorpyrifos and parathion bound extensively to the dialysis cells (Technilab Instruments, Ind., Pequannock, N. J.) as well as to numerous types of dialysis membranes. These problems were overcome by lining each cell with aluminum foil (Reynolds Metals Company, Richmond, Va.), and by using 20/32 dialysis membrane from Union Carbide Corporation (Chicago, Ill.) (neither parathion nor chlorpyrifos bound significantly to this membrane). Membranes were pretreated as described by Chignell (9). Dialysis was performed using a 1% solution of BSA in 50 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl, at 37° with an incubation time of 24 hr. Preliminary studies indicated that equilibrium was achieved in this system in 16–20 hr. Concentrations of free ligand were analyzed by HPLC following extraction with ethyl acetate (7). Binding data were analyzed as described by Fletcher *et al.* (10).

Hydrolysis reactions. Hydrolysis of paraoxon or chlorpyrifos oxon by a 1% solution of BSA at 37° was monitored by measuring formation of *p*-nitrophenol or 3,5,6-trichloro-2-pyridinol, respectively, by HPLC techniques (7). Incubations were carried out at 37° for 15 min at pH 7.4 in a total volume of 1 ml unless otherwise indicated. In all experiments, the rates of spontaneous hydrolyses of the oxygen analogues were determined in the absence of BSA, and were subtracted from the corresponding samples containing BSA. However, at no time did spontaneous hydrolysis exceed 5% of BSA-mediated hydrolysis. Studies of the *p*-nitrophenol inhibition of paraoxon hydrolysis were performed using a Cary 219 UV-Vis recording spectrophotometer in the dual beam mode at 412 nm. Paraoxon was added to 3 ml of 1% BSA in the sample position, whereas only 1% BSA occupied the reference position. Subsequent addition of *p*-nitrophenol was made to both the sample and reference cuvettes so that no change resulted in the difference spectrum from addition of *p*-nitrophenol. This same experimental approach could not be used with chlorpyrifos oxon and 3,5,6-trichloro-2-pyridinol, since these two compounds have nearly identical UV-Vis spectra.

Where indicated, 1% BSA solutions were pretreated for 2 hr at 37° with 5 mM paraoxon or chlorpyrifos oxon or with 1 mM DFP. Samples were subsequently dialyzed in buffer a 4° for 2 days in order to remove free organophosphate. Under these conditions, DFP has been shown to phosphorylate at least 95% of those tyrosine residues of albumin capable of reacting with DFP (1).

Because of the high concentrations of BSA required to determine hydrolysis rates accurately, kinetic data were analyzed as described by Dixon (12). This analysis allows determinations of kinetic parameters when a significant fraction of the added substrate is bound to enzyme. Substrate concentrations ranged from about 0.5 K_m to 7 K_m. Turnover numbers, defined as the number of moles of substrate transformed per minute per mole of enzyme, were calculated as described by Segal (13).

RESULTS

Both parathion and chlorpyrifos bound extensively to BSA, although chlorpyrifos had a slightly higher affinity

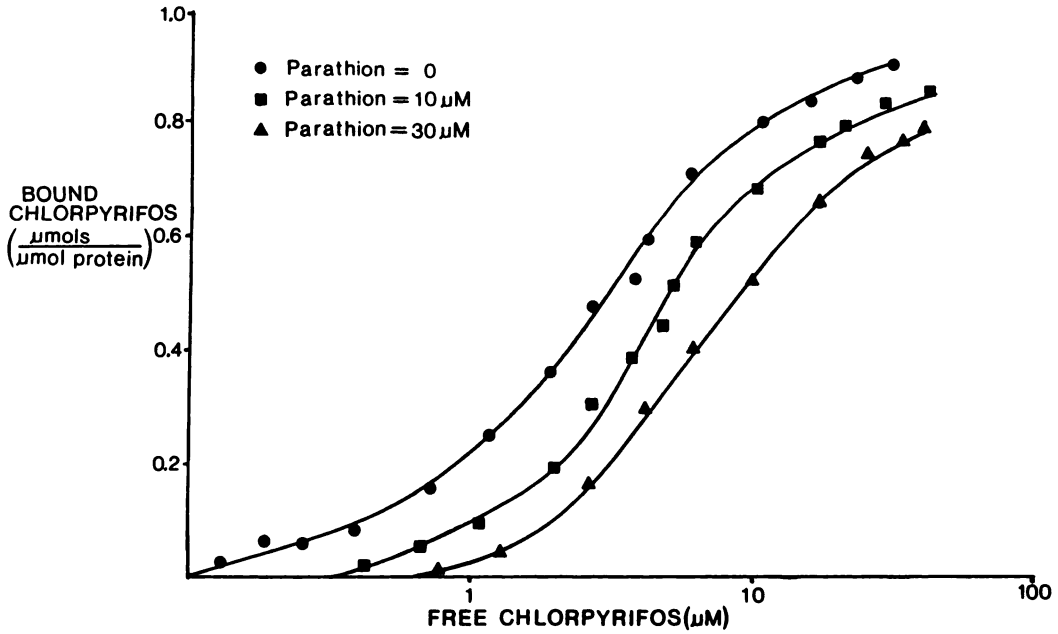

FIG. 2. *Binding curves of the amount of chlorpyrifos bound to BSA plotted against the free chlorpyrifos concentration on log scale, in the absence and presence of parathion*

Each point represents the mean of triplicate samples. See Table 1 for kinetic parameters descriptive of these binding curves.

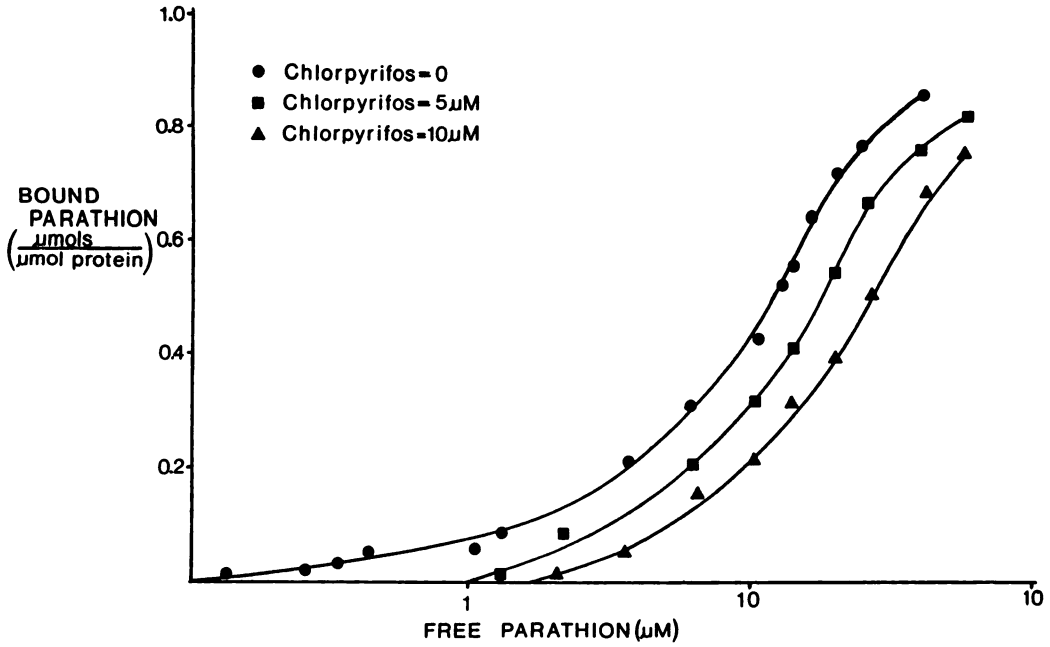

FIG. 3. *Binding curves of the amount of parathion bound to BSA plotted against the free parathion concentration on log scale, in the absence and presence of chlorpyrifos*

Each point represents the mean of triplicate samples. See Table 1 for kinetic parameters descriptive of these binding curves.

than did parathion (Figs. 2 and 3; Table 1). Binding was reversible, since all of either pesticide could be removed from the BSA by repeatedly replacing the BSA-free side of the dialysis cells with unused buffer (data not shown). Computer analysis of the binding data (10) indicated one class of binding sites, with a maximal number of binding sites for either compound of 1 μmole of insecticide per micromole of BSA (Figs. 2 and 3; Table 1), suggesting that each molecule of BSA contained one binding site or region for chlorpyrifos or parathion. Under these conditions, the more general stepwise equilibrium model for the description of macromolecule-ligand interactions reduces to the simpler Scatchard model (10, 14). Additionally, binding studies with both chlorpyrifos and parathion present suggested that each bound to the same site on BSA, since the apparent K_D values increase in the presence of the competing ligand, without altering the maximal number of binding regions (Figs. 2 and 3; Table 1).

TABLE 1

Kinetic parameters describing the reversible binding of the ligands chlorpyrifos and parathion to BSA in the absence and presence of competing ligands[a]

Ligand	Competing ligand	Apparent K_D[b]	Apparent B_{max}
		μM	*μmoles ligand/ μmoles BSA*
Chlorpyrifos	None	3.4 ± 0.1	1
Chlorpyrifos	Parathion, 10 μM	5.2 ± 0.2	1
Chlorpyrifos	Parathion, 30 μM	9.5 ± 0.4	1
Parathion	None	11.1 ± 0.3	1
Parathion	Chlorpyrifos, 5 μM	16.3 ± 0.5	1
Parathion	Chlorpyrifos, 10 μM	24.1 ± 0.7	1

[a] Parameters are descriptive of binding curves in Figs. 2 and 3.

[b] Each value represents the apparent K_D ± standard error of the corresponding curve in Figs. 2 and 3.

Incubation of chlorpyrifos oxon or paraoxon with 1% BSA resulted in the formation of 3,5,6-trichloro-2-pyridinol or *p*-nitrophenol, respectively (Fig. 4). This activity was not altered by pretreatment of BSA with 5 mM paraoxon or chlorpyrifos oxon, or 1 mM DFP at 37° for 2 hr (data not shown). Additionally, activity was not inhibited by 3 mM EDTA, which excluded the possibility of contamination by A-esterases (data not shown). Formation of 3,5,6-trichloro-2-pyridinol and *p*-nitrophenol was linear for about 60 min and 120 min, respectively, after which hydrolysis of both compounds slowly halted (Fig. 4). Full activity could be restored by extensive dialysis of the BSA for 24 hr at 4°. Hydrolysis of paraoxon by BSA was inhibited by the addition of excess *p*-nitrophenol (Fig. 5).

Temperature and pH profiles indicated that maximal hydrolytic activity occured at 37° and pH 8.0 for both oxons (Figs. 6 and 7). Although the temperature profile in Fig. 6 indicates no hydrolytic activity at 2°, this is somewhat misleading, since the incubation time was only 15 min. Attempts to assess the binding of paraoxon or chlorpyrifos oxon to BSA at 2° failed, since limited hydrolysis still occurred over a period of 24 hr (data not shown).

Kinetic analyses of BSA-mediated hydrolysis indicated marked differences in K_m values, V_{max} values, and turnover numbers between paraoxon and chlorpyrifos oxon (Table 2). However, hydrolysis of both chlorpyrifos oxon and paraoxon by BSA was very inefficient as indicated by low turnover numbers (Table 2).

DISCUSSION

Although binding of parathion to BSA and HSA has been studied by several investigators, reports have been

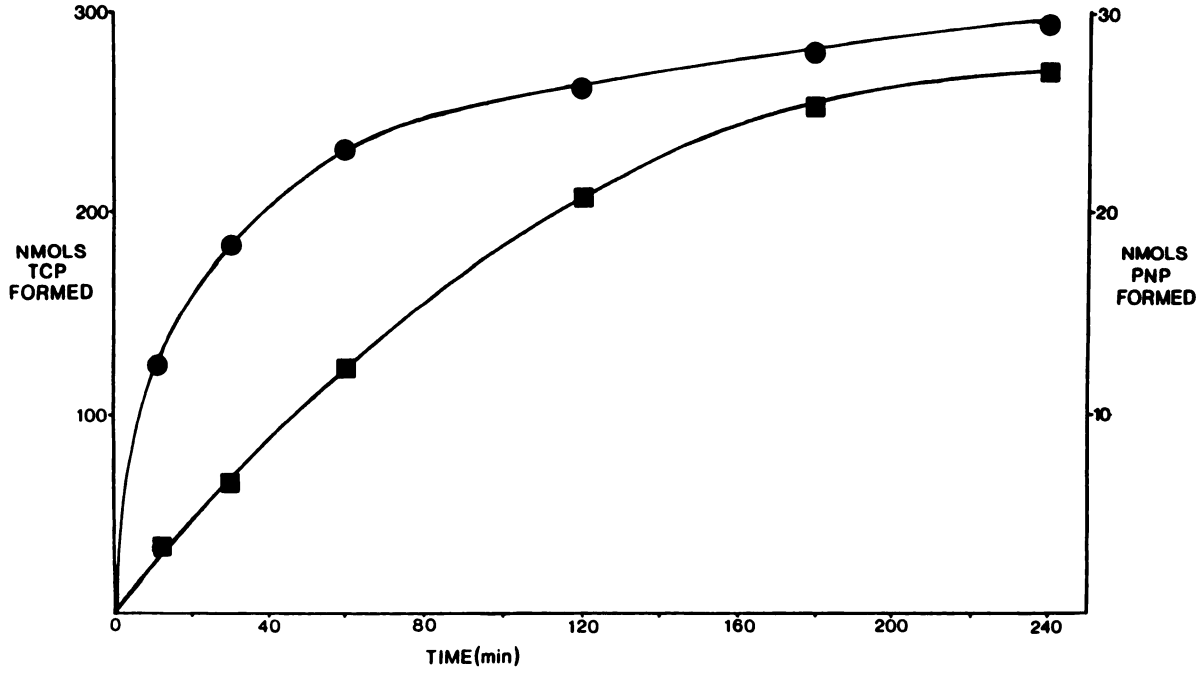

FIG. 4. *Formation of 3,5,6-trichloro-2-pyridinol (TCP) (●——●) from chlorpyrifos oxon and p-nitrophenol (PNP) (■——■) from paraoxon in the presence of 1% BSA*

Incubations were performed at 37°, with an oxon concentration of 2 mM, in a total volume of 1 ml, at pH 7.4. Each point represents the mean of quadruplicate samples for which the standard deviations did not vary more than 3% from the mean.

conflicting with respect to the nature or characterization of this binding. Initially, Skalsky and Guthrie (15) failed to detect any binding sites for parathion on HSA, whereas a subsequent report from the same laboratory suggested that parathion bound to two classes of binding sites on HSA with maximal numbers of binding sites of one and five molecules of parathion per molecule of HSA (5). In contrast, Mourik and de Jong (4) reported that parathion bound to one class of binding sites on BSA or HSA, and that the maximal number of binding sites per molecule of protein was 0.45. However, the method of analysis of binding data used by Mourik and de Jong (4)

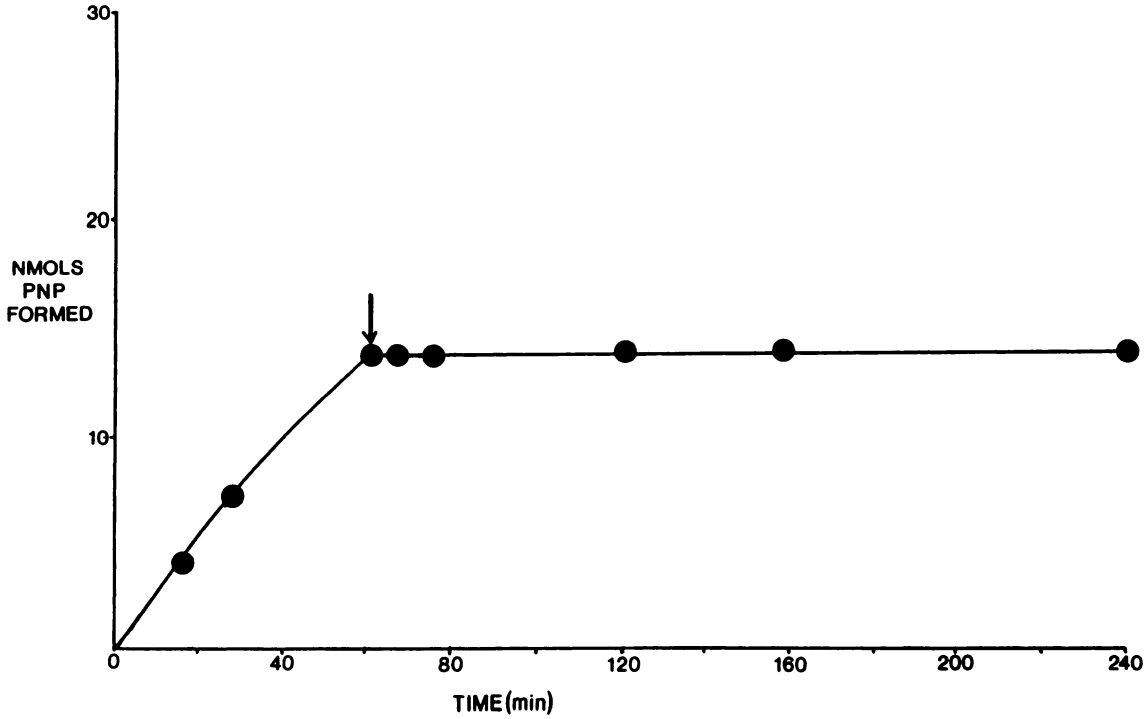

FIG. 5. *Inhibition of hydrolysis of paraoxon by 1% BSA solution by the addition of excess p-nitrophenol (PNP)*

The *arrow* indicates addition of 1 mM PNP. See Methods for details of the procedure. Each point represents the mean of quadruplicate samples for which the standard deviations did not vary more than 3% from the mean.

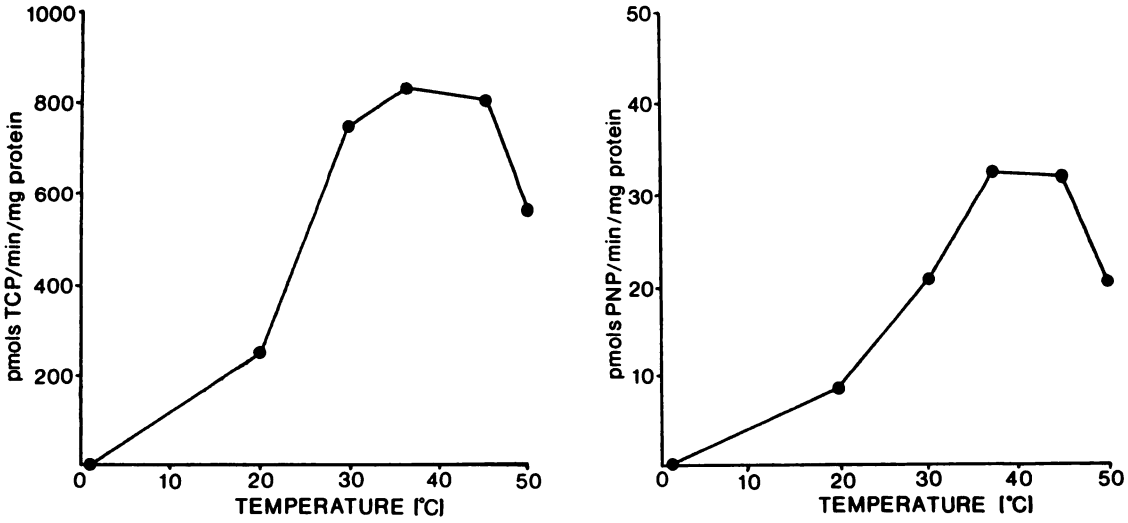

FIG. 6. *Temperature profiles for production of 3,5,6-trichloro-2-pyridinol and p-nitrophenol from chlorpyrifos oxon and paraoxon, respectively, in the presence of 1% BSA*

Incubations were performed for 15 min, with an oxon concentration of 2 mM, in a total volume of 1 ml, at pH 7.4. Each point represents the mean of quadruplicate samples for which the standard deviations did not vary more than 4% from the mean.

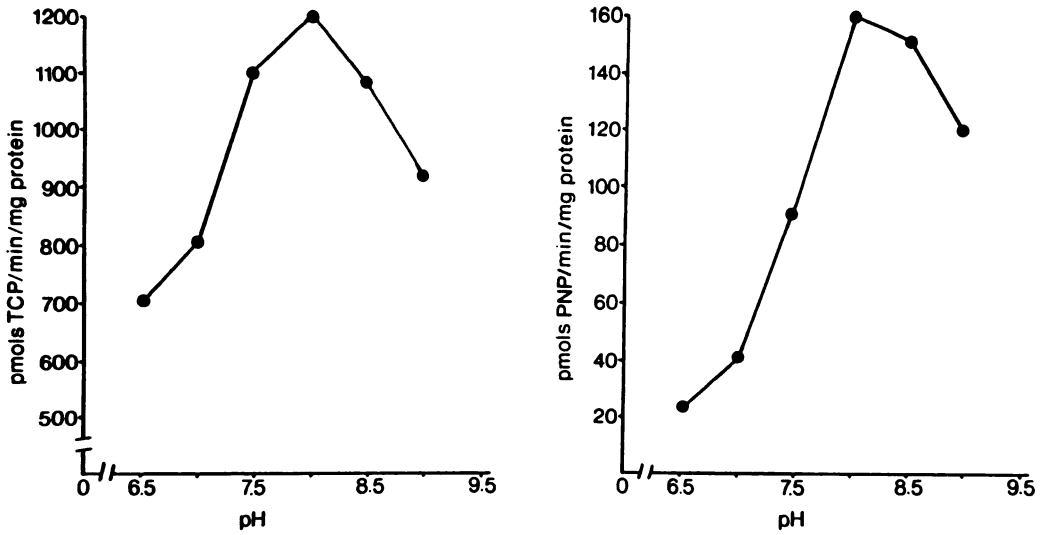

FIG. 7. *pH profiles for production of 3,5,6-trichloro-2-pyridinol and p-nitrophenol from chlorpyrifos oxon and paraoxon, respectively, in the presence of 1% BSA*

Incubations were performed for 15 min at 37°, with an oxon concentration of 2 mM, in a total volume of 1 ml. Each point represents the mean of quadruplicate samples for which the standard deviations did not vary more than 3% from the mean.

TABLE 2

Kinetic parameters describing hydrolysis of chlorpyrifos oxon and paraoxon by BSA

Substrate	K_m[a]	V_{max}[a]	Turnover no.[a,b]
	mM	*pmoles/min/mg protein*	*min*
Chlorpyrifos oxon	0.41 ± 0.01	895 ± 32	0.0597 ± 0.0035
Paraoxon	1.85 ± 0.10	44 ± 5	0.0029 ± 0.0004

[a] Each value represents the mean ± standard deviation of four experiments.

[b] Defined as the number of moles of substrate transformed per minute per mole of enzyme, under optimal conditions.

and Maliwal and Guthrie (5) has been shown to be unreliable in accurately estimating numbers of binding sites (16). In the present study we found that both parathion and chlorpyrifos bound reversibly to one class of binding sites on BSA, and that each molecule of albumin probably contained one binding region for these insecticides (Figs. 2 and 3; Table 1). Albumin, therefore, might serve as a means of transport of these relatively hydrophobic compounds in blood.

That parathion and chlorpyrifos bound extensively to the dialysis cells, as well as to certain types of dialysis membranes, indicates that interpretation of organophosphate binding data is difficult, if not impossible, unless these problems have been addressed by the investigators. In the present study the dialysis cells were lined with aluminum foil, and a brand of membrane was chosen which did not significantly bind parathion or chlorpyrifos.

Erdos and Boggs (3) reported an EDTA-insensitive production of *p*-nitrophenol from paraoxon by human serum, and concluded that the source of activity was albumin or some factor associated with albumin. In the present study although we cannot entirely rule out the possibility of some other factor associated with BSA as the source of hydrolysis, we can exclude potential contamination of BSA by plasma A-esterases, since EDTA had no effect on hydrolysis rates. Moreover, albumin has been reported to possess esterase-like activity toward a number of compounds such as phenyl acetates and *p*-nitrophenol esters (17), and can biotransform prostaglandin D_2 (18). The EDTA-insensitive formation of 3,5,6-trichloro-2-pyridinol or *p*-nitrophenol observed following incubation of chlorpyrifos oxon or paraoxon, respectively (Fig. 4), could have resulted from either phosphorylation of BSA, or an enzymatic hydrolysis by BSA. Albumin is known to interact with certain compounds by either of these mechanisms (1, 2, 11, 17). Since pretreatment of BSA with either paraoxon or chlorpyrifos oxon did not alter the hydrolytic capacity of BSA with respect to either oxon, it seems unlikely that either oxon phosphorylated BSA. Rather, BSA, although extremely inefficient (Table 2), possessed esterase-like activity toward chlorpyrifos oxon and paraoxon. This enzymatic activity displayed a characteristic temperature and pH profile (Figs. 5 and 6), and was inhibited by excess product in the case of paraoxon (Fig. 5). The hydrolytic capacity of BSA was not altered by pretreatment with 1 mM DFP, suggesting that the tyrosine residue on albumin known to be phosphorylated by DFP (1, 2, 16) was not involved in these hydrolysis reactions.

Although BSA has the capacity to hydrolyze chlorpyrifos oxon and paraoxon, we cannot exclude the possibility that paraoxon and/or chlorpyrifos oxon might reversibly bind to BSA at additional sites, thereby facilitating their transport in blood. Moreover, the inefficient esterase-like activity of BSA with respect to chlorpyrifos oxon and paraoxon suggests that BSA might have the capacity to serve as a carrier in blood for these oxons if enough substrate could remain in the form of enzyme-substrate complex.

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Send reprint requests to: Dr. L. G. Sultatos, Department of Pharmacology and Experimental Therapeutics, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, La. 70112.