Vinyl Phosphate Insecticide Sorption to Proteins and Its Effect on Cholinesterase I_{50} Values

A. C. BOYER

The two vinyl phosphate insecticides, 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (SD 8447) and 2-chloro-1-(2,4-dichlorophenyl)-vinyl diethyl phosphate (SD 7859), are sorbed to mammalian blood plasma and homogenates of housefly (*Musca domestica*) heads. The I_{50} values for the inhibition of the cholinesterase present in these preparations were determined for both compounds. The sorption data were used to correct the observed I_{50} values. The corrected I_{50} values represent the concentration of compound available for 50% inhibition of the enzyme, rather than the actual concentration of the inhibitor.

Cohen and Warringa (*2*) showed that ox red cell suspensions contained large amounts of DFP (diisopropyl phosphofluoridate)-binding proteins, which considerably increased the concentration of DFP required for 50% inhibition of red cell acetylcholinesterase. More recently, Van Asperen and Dekhuijzen (*7*) have studied the inhibition of mouse brain cholinesterase and proposed the reversible binding of the organophosphate insecticide DDVP (2,2-dichlorovinyl dimethyl phosphate) to some proteinaceous material in mouse brain homogenates. These findings indicate that a protein-insecticide interaction could reduce the concentration of the insecticide available for cholinesterase inhibition. If this is true, a correlation may exist between the binding of cholinesterase inhibitors to proteins and the toxicity of the compound to mammals.

A study was therefore undertaken to measure the binding of the two vinyl phosphate insecticides, 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate (SD 8447) and 2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate (SD 7859), to mammalian blood plasma and homogenates of housefly (*Musca domestica*) heads. These compounds were selected primarily because of their low toxicity to mammals. This paper considers the effect which the protein-insecticide interaction has on the inhibition of cholinesterase contained in the above preparations.

Reagents

The β-isomers of 1,2-C^{14}-SD 8447 and SD 7859 were prepared with specific activities of 0.9 and 1.0 mc. per mmole, respectively. The β-isomer is arbitrarily designated as the one in which the phosphate group and the largest group on the second vinyl carbon are cis. Configurations of the individual isomers were assigned from NMR and infrared spectra following methods used earlier for Phosdrin insecticide as described by Stiles *et al.* (*5*). However, the configuration of the β-isomers of these two compounds is not unequivocal. C^{14}-Methoxy-labeled SD 3562 (crotonamide, *N,N*-dimethyl-3-hydroxy-*cis*-, dimethyl phosphate), SD 9129 (crotonamide, *N*-methyl-3-hydroxy-*cis*-, dimethyl phosphate), and SD 8447 insecticides were prepared with specific activities of 1.2, 0.2, and 1.0 mc. per mmole. The nonlabeled β-isomers of SD 8447 and SD 7859 were synthesized according to the method described by Whetstone *et al.* (*6*) and assayed by the colorimetric phosphorus determination method of Saliman (*3*).

Filtrates of fly head homogenates containing cholinesterase were prepared at 2° C. by homogenizing 25 housefly heads per ml. in a 0.45*M* NaCl solution containing 0.01*M* KH_2PO_4–KOH buffer, pH 8.0. The solution was filtered under reduced pressure to remove debris. Blood plasma preparations containing cholinesterase were prepared by centrifuging heparinized whole blood at 2° C. for 15 minutes at 10,000 G. The supernatant was used as such. These preparations were used for both the cholinesterase inhibition and sorption studies. No dilution of these preparations was made.

A Model 3003 Packard Tri-Carb Scintillation Spectrometer was used for isotope counting. The scintillation solution consisted of 2.5 parts (by volume) absolute ethanol, 7.1 parts toluene, and 0.4 part Liquifluor (Nuclear Chicago, Des Plaines, Ill.).

Procedure

Assay of Cholinesterase. Initial rates of acetylcholine hydrolysis were measured by the electrometric continuous titration method (*4*). The apparatus consists of a Beckman Model K automatic titrator equipped with a homemade automatic NaOH delivery unit and a Varian recorder. The assay was run in a thermostatically controlled reaction vessel at 25° C. The assay was performed by incubating either 0.2 ml. of mouse plasma, human plasma, or 0.5 ml. of the filtrate of fly head homogenate in 0.5*M* NaCl for a 2-minute pH and thermal equilibration period. The volume of the mixture was 3.5 ml. The volume of NaCl added to the mixture was appropriately reduced to compensate for the addition of an inhibitor of cholinesterase. Enzymatic activity in the absence of inhibitor was determined

Shell Development Co., Modesto, Calif.

upon the addition of 0.5 ml. of 0.1M acetylcholine iodine. The acetic acid liberated from the hydrolysis of acetylcholine by cholinesterase was automatically and continuously titrated to pH 8.0. When the inhibitory power of SD 8447 or SD 7859 was determined, the mixture was incubated for exactly 4 minutes with the inhibitor before adding acetylcholine. The inhibitor was added to the mixture after the pH and thermal equilibration period. The activity of the enzyme was calculated from the slope of the titration curve as the millimoles of acetylcholine hydrolyzed per minute. For those assays which contained bovine serum albumin (BSA), the BSA was added to the incubation mixture prior to the inhibitor. Four different concentrations of BSA were tested with each concentration of inhibitor. BSA was dissolved in 0.01M KH_2PO_4–KOH buffer, pH 7.0.

Plotting of Cholinesterase Data. Aldridge (*1*) pointed out that the reaction between an irreversible inhibitor and cholinesterase follows the kinetics of a simple first-order reaction when one component (inhibitor) is in considerable excess. Equation 1, derived by Aldridge, was used for the determination of I_{50} values.

$$K = \frac{1}{tI} \ln \frac{100}{b} \qquad (1)$$

I_{50} is the concentration of inhibitor required to give 50% inhibition of the enzyme when the assay condition described above is used. The K of Equation 1 is the bimolecular rate constant, I the concentration of the inhibitor, and b the per cent enzyme activity remaining after t minutes of incubation with the inhibitor. Converting to $\log_{10}$ and plotting $\log_{10}$ of the per cent enzymatic activity *vs.* the concentration of inhibitor will give a straight line with an intercept on the y axis of 2 and a slope of $-(KI/2.303)$. Four or five concentrations of each inhibitor were used for each I_{50} determination.

Sorption of SD 8447 and SD 7859 to Components of Blood Plasma and Homogenates of Fly Heads. 1,2-C^{14}-labeled SD 8447 and SD 7859 were used for estimating the degree of binding of these compounds to blood plasma and fly head nerve tissue by means of equilibrium dialysis. Five different concentrations of a given radioactive compound were placed into dialysis sacks containing either 0.2 ml. of mouse plasma, human plasma, or 0.5 ml. of filtrate of fly head homogenate. The volume was then brought to 3 ml. with 0.1M KH_2PO_4–KOH buffer, pH 7.0. Sacks were placed in tubes containing 17 ml. of the same buffer. The course of dialysis at 2° C. was followed until the radioactivity on the outside of each sack reached a constant value. At this point an equilibrium has been established between the bound and unbound compound. Two-hundred-fifty-microliter aliquots from the inside and outside of each sack were then counted, and the total amount of compound bound to the cellular material at a given equilibrium concentration was calculated.

Quenching of counts was determined by the addition of a standard solution of C^{14}-benzoic acid to each sample vial. A maximum of 3% quenching was observed. The appropriate quench correction was applied to each sample counted.

The equilibrium dialysis data were plotted in accordance with Freundlich's equation

$$\log \frac{x}{m} = \log K + \frac{1}{n} \log C \qquad (2)$$

to give the isotherm for the sorption of SD 8447 and SD 7859 to the various tissue preparations. For the present experiments, x denotes the micromoles of compound bound, m the volume of solution containing the sorbent, C the molarity of compound in solution at equilibrium, n an empirical constant usually greater than unity, and K the ratio of x/m when C is equal to unity. Therefore, K represents the following expression:

$$\frac{\text{Micromoles of compound sorbed}}{\text{milliliters of sorbent}} \times (\text{molarity of compound})^{-\frac{1}{n}}$$

Plotting log x/m *vs.* log C gives a straight line with a slope of $1/n$. The same expression for K holds for those experiments dealing with BSA, except the milliliters of sorbent in the above expression should read micromoles of BSA. A molecular weight of 69,000 was used for BSA. All of the isotherms were linear at the concentration of compounds tested. The range in concentration of SD 8447 and SD 7859 initially added to the dialysis sacks were carefully selected. Concentrations were selected that would give final equilibrium molarities in the range of the concentrations of the compound used for measuring the inhibition of cholinesterase. This was essential since it was not determined whether the sorption isotherm for a given compound and enzyme preparation was linear over a very large range in concentration of compound. The isotherm may depart from linearity at concentrations of compound which are either higher or lower than the range of concentration tested. Erroneously corrected cholinesterase data would then be obtained if the sorption data obtained from the higher or lower concentration range of compound were used for the purpose of correcting cholinesterase data for sorption effects.

Thin-Layer Chromatography of C^{14}-Methoxy Labeled SD 8447. The complex formed between C^{14}-SD 8447 and human plasma was disrupted by adding isopropyl alcohol (IPA) to a concentration of 80%. This mixture was centrifuged and the supernatant combined with IPA washings of the precipitate. The IPA was removed under reduced pressure and the residue dissolved in water. The aqueous solution was extracted with chloroform. The chloroform solution was concentrated and applied to TLC plates. The sample was chromatographed on silica gel G plates with a mixture of 70% acetone and 30% hexane. Radioautograms were prepared to detect the position of radioactivity.

Results

The K and n values obtained from the isotherms for the sorption of SD 8447 and SD 7859 to human and

mouse blood plasma and housefly head homogenates are listed in Table I. As an example, the isotherm for the sorption of SD 7859 to human plasma is shown in Figure 1.

The data of Table I were used to correct cholinesterase inhibition curves as shown in Figure 2. The upper line is the observed inhibition of human plasma cholinesterase by SD 7859. The lower line is the inhibition curve one would obtain if all of the SD 7859 added to the incubation mixture were available for the inhibition of cholinesterase. The corrected curve is obtained by calculation using Equation 2. Selected values of C (concentration of unbound compound when an equilibrium has been established between free and bound compound) and the appropriate K and n values of SD 7859 (Table I) were used to calculate the micromoles of SD 7859 bound per milliliter of human plasma. These data can then be used to obtain the corrected cholinesterase inhibition curve after making the appropriate mathematical corrections in converting from the dimensional units of the equilibrium dialysis data to the dimensions of the cholinesterase inhibition data.

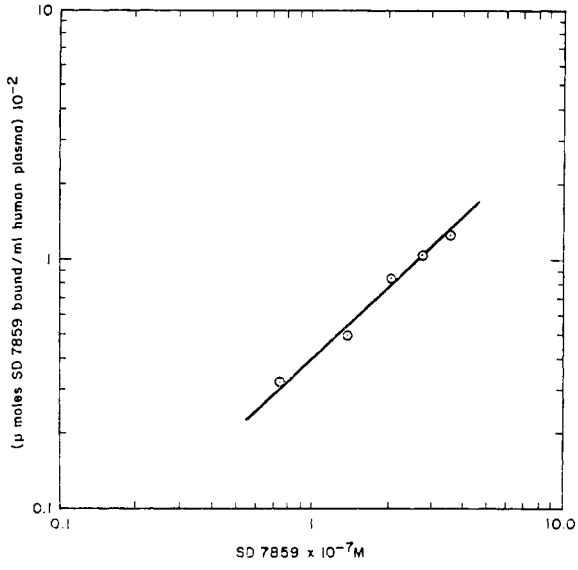

Figure 1. Sorption isotherm of SD 7859 to human plasma

Table I. Sorption Isotherm Constants

Enzyme Source	Mouse Plasma	Human Plasma	Fly Head
K Values			
SD 8447	1.16×10^2	1.21×10^4	1.92×10^{11}
SD 7859	1.37×10^2	1.54×10^4	1.13×10^3
SD 9129	5.37×10^2		
SD 3562	1.33×10^2		
n Values			
SD 8447	1.78	1.13	0.38
SD 7859	1.73	1.06	1.08
SD 9129	0.97		
SD 3562	1.21		

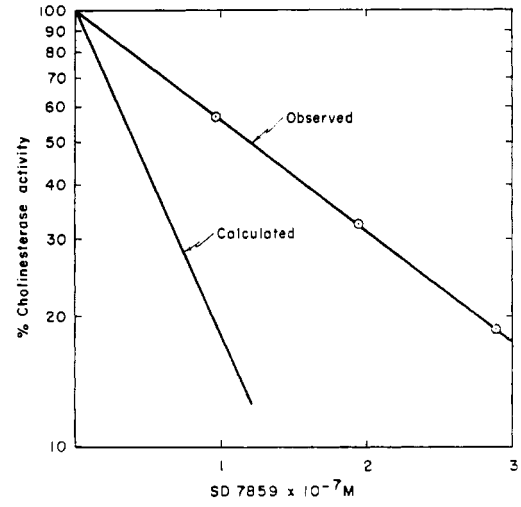

Figure 2. Inhibition of human plasma cholinesterase by SD 7859

These values were used to determine the inhibition values one would obtain if all of the compound were available for cholinesterase inhibition. This is done by locating the molarity of unbound SD 7859 on the SD 7859 axis of Figure 2. A vertical line then is drawn from this point to the observed curve. A horizontal line is then drawn from the observed curve to this vertical line, when the difference in molarity between the unbound concentration of SD 7859 and the observed curve is equal to the concentration of SD 7859 bound. Where the two lines intersect is the per cent inhibition one would obtain if all of the SD 7859 were available for cholinesterase inhibition.

The corrected cholinesterase curves are valid if the amount of compound bound to the sorbent during the cholinesterase inhibition studies is the same as the amount bound as determined from the equilibrium dialysis experiments. The data listed in Table II show the validity of the method. In part 1 of Table II are listed the K and n values for the sorption of SD 8447 and SD 7859 to bovine serum albumin (BSA). If these compounds rapidly reach a binding equilibrium with BSA, then the same K and n values as obtained from cholinesterase inhibition studies should be attained. This was done by measuring the inhibition of fly head cholinesterase in the presence and absence of various concentrations of BSA. In the presence of BSA, less inhibition is acquired at a given concentration of SD 7859. Thus, the per cent cholinesterase activity at a given concentration of SD 7859 is larger, and the point falls above the observed cholinesterase curve. The horizontal distance—difference in SD 7859 concentration—between the per cent activity of the enzyme in the presence of BSA and the observed curve is equal to the amount of SD 7859 bound to BSA. By subtracting the concentration of SD 7859 bound from the actual concentration of SD 7859 added, one obtains the concentration of free SD 7859. These data make possible the construction of the isotherm for the sorption of SD 7859 to BSA and the determination of the K and n

values as given in part 2 of Table II. The K and n values for SD 8447 but not SD 7859 are almost the same as determined from the isotope experiments. The error in the K and n values is due to the fact that some of the compound binds not only to BSA but also to the material in the fly head preparation. When one corrects for this additional binding, one obtains the K and n values given in part 3 of Table II. Since the K and n values given in parts 1 and 3 of Table II are the same, the amount of compound bound to BSA is the same regardless of the method of determination used.

The only anomaly in the K and n values is for the binding of SD 8447 to fly heads (Table I). No obvious explanation is available for this abnormality.

The author assumed, since the equilibrium dialysis experiments were run at 2° C., that little enzymatic modification of these compounds would occur. Nevertheless, this point was checked using C^{14}-methoxy-labeled SD 8447. Data from equilibrium dialysis experiments show that mouse and human whole blood hydrolyzed 7 and 2%, respectively, of the total amount of compound initially added. This was determined by chloroform extraction of aliquots from the dialyzate at equilibrium. Since C^{14}-dimethyl phosphate (hydrolysis product) does not partition to chloroform from an aqueous solution, a simple method is available to measure the amount of catabolism.

Additional experiments have shown that all of the radioactivity sorbed to human plasma is present as SD 8447. This was determined by dialyzing a mixture of human plasma (1.5 ml.) and C^{14}-methoxy-labeled SD 8447 (17 μg. per 5 ml. of 0.01M KH_2PO_4–KOH, pH 7.0) at 2° C. against four 5-liter changes of buffer over a 26 hour period. The control sack at this point contained 0.05 μg. of SD 8447. The experimental sack contained 5.9 μg. and of this, only 0.6 μg. was chloroform extractable. However, a total of 5.3 μg. of SD 8447 could be extracted with chloroform if the nondialyzable material was treated with IPA. Chloroform extracts of the IPA-treated material were chromatographed and contained only SD 8447.

Apparently, SD 8447 and SD 7859 are also stable in the presence of fly head homogenates. The same K and n values were obtained for the sorption of these compounds to BSA regardless of whether one measured the sorption directly to BSA or measured the displacement of cholinesterase inhibition in the presence of BSA. With the latter method, one has to determine from equilibrium dialysis data the amount of compound bound to the fly head preparation before determination of the amount of compound bound to BSA is possible, as determined from the cholinesterase data. Extensive metabolism of these compounds does not occur during the equilibrium dialysis portion of this determination since the same K and n values determined from the former method were obtained. The former method involves only the dialysis of compound and BSA mixtures.

Table III lists the observed and corrected I_{50} values for the inhibition of cholinesterase contained in mouse and human plasma and fly head preparations by SD 8447 and SD 7859. For human plasma and SD 8447, the observed and corrected I_{50} values differ by a factor of four. This is significant when one considers that the concentration of SD 8447 available for cholinesterase inhibition is only one fourth of the actual concentration of SD 8447 added. With SD 7859, the factor is three. For mouse plasma, the factor is extremely large; 63 for SD 8447 and 19 for SD 7859. Although both of these compounds are sorbed to fly head homogenates, the amount sorbed has but a small effect on the observed I_{50} values.

The sorption of C^{14}-methoxy-labeled SD 2562 and and SD 9129 insecticides to mouse plasma was also measured by the equilibrium dialysis technique. Both compounds are sorbed to mouse plasma. The K and n values obtained from the isotherms for the sorption of these compounds to mouse plasma are listed in Table I.

Table II. Sorption Isotherm Constants for the Binding of SD 8447 and SD 7859 to BSA

Compound	K	n
	Part 1[a]	
SD 8447	1.57×10^4	0.915
SD 7859	5.10×10^3	0.901
	Part 2[b]	
SD 8447	1.86×10^4	0.896
SD 7859	5.41×10^2	1.020
	Part 3[c]	
SD 8447	1.32×10^4	0.926
SD 7859	5.23×10^3	0.892

[a] Isotope binding of compound to BSA.
[b] Binding of compounds to BSA as determined enzymatically using the fly head preparation. No correction was made for the amount of compound bound to the fly head preparation.
[c] Binding of compounds to BSA as determined enzymatically using the fly head preparation. The values are corrected for the amount of compound to the fly head preparation.

Table III. Observed and Corrected I_{50} Values

Enzyme Source	Mouse Plasma	Human Plasma	Fly Head
SD 8447			
Observed	$6.3 \times 10^{-8}M$	$1.63 \times 10^{-6}M$	$5.0 \times 10^{-8}M$
Corrected	$1.0 \times 10^{-9}M$	$4.0 \times 10^{-7}M$	$4.3 \times 10^{-8}M$
SD 7859			
Observed	$1.43 \times 10^{-7}M$	$1.22 \times 10^{-7}M$	$1.75 \times 10^{-8}M$
Corrected	$7.5 \times 10^{-9}M$	$4.1 \times 10^{-8}M$	$1.15 \times 10^{-8}M$

Discussion

The results presented in Table III show that mammalian blood plasma is not the most desirable source of cholinesterase for structure-activity studies with SD 8447 and SD 7859. Since both compounds sorb to the components of blood plasma, the observed I_{50} value represents the concentration of compound which has to be used to obtain 50% inhibition. The corrected I_{50} values represent the concentration of compound required for 50% inhibition of plasma cholinesterase activity in the absence of protein-insecticide interactions. The difference between the observed and corrected I_{50} values clearly shows that correcting for sorption reduces the concentration of inhibitor required for 50% inhibition.

A far better source of crude cholinesterase was found in homogenates of fly heads. Although sorption of these compounds still occurs, the amount sorbed has but a very small effect on the observed I_{50} values.

In view of these results, conclusions should not be drawn concerning the structure-activity relationship of cholinesterase inhibitors when crude preparations of cholinesterase are used. For example, it would be misleading to compare the observed I_{50} values of SD 7859 and SD 8447 in relation to their structure when blood plasma cholinesterase is used as the enzyme source. Table I shows that the K and n values for the sorption of these compounds to mouse plasma are almost identical. This observation is in itself insufficient evidence to justify the use of observed I_{50} values for structure-activity correlations. The observed I_{50} values can be compared only when the value for the ratio of the observed I_{50} value of SD 8447 to SD 7859 is equal to the value for the same ratio of the corrected I_{50} values. Of course, this assumes that these compounds inhibit the enzyme via the same kinetic mechanism. This, however is the same assumption which would be used to compare corrected I_{50} values for the purpose of obtaining structure-activity correlations. However, in the case of residue analysis of insecticides via cholinesterase determinations, protein binding introduces no error in the determination since a calibration curve is always prepared for the desired cholinesterase preparation and the insecticide in question. Thus, whether a compound does or does not bind to proteins in the enzyme preparation is a factor of no consequence since the calibration curve compensates for such factors.

Probably, the sorbent of these compounds is proteinaceous. Both of these compounds sorb to BSA as was shown in Table II. The conclusion that the sorption of these compounds to proteins occurs almost instantaneously seems reasonable. The data of Table II show that the same K and n values were obtained for the sorption of these compounds to BSA as determined by either the isotope equilibrium dialysis technique or the cholinesterase inhibition test. One would not expect to obtain the same K and n values if the sorption was a slow process, for the periods of time involved in the above determinations are grossly different.

The exhaustive dialysis experiment with SD 8447 and human plasma indicates that there is a strong affinity of this compound for the plasma proteins. The proteins had to be denatured before the compound could be removed. However, this does not exclude the possibility of readily reversible sorption in the equilibrium dialysis experiments. Covalent bonds are not likely to be formed since the compound could be recovered after IPA treatment.

One is tempted, also, to correlate the binding of these insecticides to blood plasma with the mammalian oral toxicity of these compounds. The author has discovered that SD 3562, SD 9129, SD 7859, and SD 8447 are sorbed to mouse plasma. The oral LD_{50} values of these compounds for the mouse are 20, 15, 145, and 5000 mg. per kg., respectively. Thus, there appears to be no correlation between whether a compound does or does not bind to mouse plasma and the oral toxicity value. However, an all-or-none effect is probably not the best criterion to use for establishing a relationship between the sorption of these compounds to proteins and the oral toxicity values. Consideration of the role which the sorption of insecticides to proteins plays as one of the factors involved in regulating the over-all toxicity of a compound would be more meaningful. Other factors which regulate the toxicity which results from an insecticide, to mention but a few, are the rate of absorption of the compound from the intestinal tract, the rate of metabolism of the compound, the rate of penetration of the compound to the target site, the rate of excretion of the compound, and the rate of cholinesterase dephosphorylation.

Since the function of blood cholinesterase is unknown, it is difficult to assess how important the sorption of insecticides to blood proteins really is. The in vitro experiments show that the sorption of insecticides to plasma protein reduces the free concentration of insecticide available for cholinesterase inhibition. Sorption of insecticides to proteins could then be looked upon as a safety factor. As a safety factor the sorption of insecticides to proteins or lipid layers in nerve tissue would play an important role in regulating the inhibition of nerve cholinesterase.

Literature Cited

(1) Aldridge, W. N., *Biochem. J.* **46**, 451 (1950).
(2) Cohen, J. A., Warringa, M. G. P. J., *Biochim. Biophys. Acta* **11**, 52 (1953).
(3) Saliman, P. M., *Anal. Chem.* **36**, 112 (1964).
(4) Stein, B., Laidler, K., *Can. J. Chem.* **37**, 1272 (1959).
(5) Stiles, A. R., Reilly, C. A., Pollard, G. E., Tieman, C. H., Ward, L. F., Jr., Phillips, D. D., Soloway, S. B., Whetstone, R. R., *J. Org. Chem.* **26**, 3960 (1961).
(6) Whetstone, R. R., Phillips, D. D., Sun, Y. P., Ward, L. F., Jr., Shellenberger, T. E., Abstracts, p. 12A, 149th Meeting, ACS, Detroit, Mich., April 1965.
(7) Van Asperen, K., Dekhuijzen, H. M., *Biochim. Biophys. Acta* **28**, 603 (1958).

Received for review August 15, 1966. Accepted December 5, 1966.