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Interaction of Insecticides with Human Serum Albumin

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SUMMARY

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The binding of chlorinated hydrocarbon, carbamate, and organophosphate insecticides to human serum albumin was studied at pH 7.0 and 16 and 26° using equilibrium dialysis, difference spectra, and fluorescence. There is one site of higher affinity than the other sites and 4–6 binding sites of moderate affinity. The affinity is inversely related to the aqueous solubility of the compounds. The interaction is primarily hydrophobic as binding is only weakly temperature dependent. Binding gives rise to a long wavelength shift of the tyrosyl and tryptophyl absorption spectra. The protein fluorescence is quenched to varying degrees as a result of the binding. The difference spectra and fluorescence quenching indicate that tyrosine and tryptophan residues are located close to the moderate affinity binding sites, although the possible role of binding-induced conformational changes cannot be ruled out. A red shift in the spectrum of bound carbaryl and carbofuran, and a severalfold decrease in binding in *F* form as compared to that in *N* form for most of the insecticides, suggests that the binding sites are in the regions which are formed by interactions between hydrophobic surfaces of several domains of albumin.

INTRODUCTION

A variety of low molecular weight compounds such as fatty acids, bilirubin, hormones, drugs, and metabolites are carried from their site of absorption to their site of action and elimination by the circulating blood. Some compounds are simply dissolved in serum water, but many others are associated with blood constituents such as albumin, globulins, lipoproteins, polypeptides, and erythrocytes. Binding to plasma proteins has physiological significance in transport, modulation, and inactivation of the compounds and their metabolite activities. It also serves as a protective device in binding and in inactivating potential toxic compounds to which the body is exposed (1). Insecticides are one of the largest groups of toxic chemicals in use today. However, little systematic work has been done on the binding of insecticides with plasma proteins. Beginning with the publication by Moss and Hathway (2) on solubilization of dieldrin by serum, a number of studies have implicated plasma proteins, especially lipoproteins, in transport of chlorinated hydrocarbon insecticides in various animals. Most of these studies have been based on distribution or solubility of insecticides in various plasma fractions, although a few binding constants have been reported (3–6). In an earlier study from this laboratory, Skalsky and Guthrie (7) showed binding of insecticides (DDT,[1] dieldrin, carbaryl, and parathion) to five major plasma protein fractions. Albumin and lipoproteins had two- to three-order higher affinity for all the insecticides and could account for *in vivo* binding. The present investigations have been undertaken to explore the binding of different insecticide classes with human serum albumin and lipoproteins, the relationship between binding and the physicochemical properties of the insecticides, the nature of the interaction, and to characterize the binding sites. The present communication concerns studies with albumin and results with lipoproteins will be communicated separately.

MATERIALS AND METHODS

Chemicals. [^{14}C]DDT (29.7 mCi/mmole), [^{14}C]dieldrin (85 mCi/mmole), [^{14}C]lindane (48 mCi/mmole), [^{14}C]parathion (19 mCi/mmole), and [^{14}C]carbaryl (57 mCi/mmole) were purchased from Amersham/Searle Corporation (Des Plaines, Ill.). [^{14}C]Diazinon (11 mCi/mmole) was a gift from Ciba-Geigy Corporation (Summit, N. J.).

[1] The abbreviations used are: DDT, 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane; dieldrin, 3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphth[2,3-*b*]oxirene; carbaryl, 1-naphthalenol methylcarbamate; parathion, *O,O*-diethyl *O*-(4-nitrophenyl)ester; lindene; (γ isomer) 1,2,3,4,5,6-hexachloracyclohexane; diazinon, *O,O*-diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidyl)-phosphorothioate; Carbofuran, 2,3-dihydro-2,2-dimethyl-7-benzofuramyl methylcarbamate; aldicarb, 2-methyl-2-(methylthio)propionaldehyde-*O*-(methylcarbamoyl)oxime; monocrotophos, 3-(dimethoxyphosphinoxy-*N*-methyl-*cis*-crotonamide; HSA, human serum albumin.

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[^{14}C]Nicotine (54 mCi/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.). [^{14}C]Carbofuran (2.85 mCi/mmole) was a gift from FMC Corporation (Homer City, Pa.). [^{14}C]Aldicarb (20 mCi/mmole) was a gift from Union Carbide Corporation (New York, N. Y.), and [^{14}C]monocrotophos (44 mCi/mmole) from Shell Development Company (Modesto, Calif.). The radiochemical purity (greater than 99%) of all the compounds was confirmed by thin-layer chromatography with appropriate solvent systems.

Nonradioactive insecticides were purchased from Chem Service, Inc. (West Chester, Pa.). All other chemicals of ACS grade and solvents of spectroscopy grade were purchased from Fisher Scientific Company (Springfield, N. J.). Cellulose dialyzer tubing (16 mm) was purchased from Arthur H. Thomas Company (Philadelphia, Pa.) and crystallized HSA from Sigma Chemical Company (St. Louis, Mo.).

Methods. Dialyzer tubing was boiled in an excess of distilled water containing 100 μM EDTA to remove impurities. Insecticides were first dissolved in dioxane and then buffer added to achieve a final concentration of dioxane (0.5% v/v). The radioactivity of the insecticide solutions was 100 cpm/μg except in the case of lindane (380 cpm/μg), dieldrin (3750 cpm/μg), and DDT (100,000 cpm/μg).

HSA was defatted by the acid charcoal method of Chen (8). The protein and insecticide solutions were made in 10 mM Tris-HCl buffer, pH 7.0, containing 10 mM NaCl. Aliquots (2.0 ml) of protein solution (15–100 μM) were dialyzed against buffer solution (5.0 ml) containing varying amounts of insecticide for 30 hr on an orbit shaker at 26 ± 0.5° and 16 ± 0.5°. This time interval was found sufficient to attain equilibrium. No appreciable degradation (<1%) of insecticides during equilibrium was detected as judged by appropriate thin-layer chromatography experiments. After completion of equilibrium, 1.0 ml each of protein solution and insecticide solution were pipetted into scintillation vials containing 10 ml of Triton X-100 scintillation liquid (9) and counted on a Packard-Tri Carb Scintillation Spectrometer. Due to limited solubility of DDT and dieldrin in buffer (<0.1 μg/ml), binding was determined from relative solubilities in aqueous solutions with and without added protein. To obtain the solubility in buffer and protein solution, an excess of insecticide was equilibrated with buffer and protein solution (15 μM) for 24 hr at 26 and 16°. The suspensions were centrifuged at 20,000 × *g* for 30 min. The supernatants (1.0 ml) were mixed with 10 ml of scintillation liquid and counted as described earlier. A similar study on solubility of parathion by albumin at various concentrations was also conducted to determine the effect of protein concentration on binding. All experiments were repeated two or more times.

Binding was analyzed by the Scatchard equation, $\bar{\nu}/_{[A]} = K(n - \bar{\nu})$, where $\bar{\nu}$ = moles of ligand bound per mole of protein, n = number of binding sites, K = binding association constant, and $[A]$ = free ligand concentration (10). The Scatchard plots were nonlinear, indicating the presence of multiple classes of binding sites. The plots were analyzed by the graphic parameter fitting method of Feldmann (11) and the parameters were adjusted to obtain the best fit. In the case of monocrotophos, aldicarb, and nicotine, a least squares regression was performed to determine n and K.

Spectral investigations were conducted on a DW-2 Aminco Bowman double beam spectrophotometer using the "tandem cell" arrangement (12) of 4.25 mm cell path length. Difference spectra were obtained by placing the protein solution (40 μM) and the insecticide solution in separate compartments of the cuvette in the reference beam and the protein solution containing insecticide in the same buffer in the sample beam. Spectra were measured at pH 7.0 in the presence of 0.5 M NaCl, 0.8 M 1-butanol, and 0.5 M sorbitol. The difference spectra were also recorded at pH 3.5 in 0.15 M KCl solution without any buffer ion.

Fluorescence measurements of 2 μM protein solution with appropriate insecticide concentration (as indicated in Table 2) were made on a Varian 330 Recording Spectroflurometer using triangular cuvettes. Corrections were made for internal absorption filter effects due to insecticide wherever required (13). Protein solutions were excited at 287 and 297 nm (excitation slit, 3 nm), and emission was measured at 335 nm (emission slit, 5 nm). The fluorescence of HSA was also measured in the presence of parathion over the pH range 3–7.4.

RESULTS

Binding isotherm. Scatchard plots of binding of all the insecticides except DDT and dieldrin to HSA at 26 and 16° are shown in Fig. 1. The results of a Scatchard analysis on the bidning data are presented in Table 1. The binding results can be summarized as follows:[2] (a) There is one site of higher affinity than other sites for most of the insecticides studied. The binding to this site is temperature-dependent for some insecticides, indicating it to be partially enthalpic in nature. (b) There does not appear to be any clear correlation between the number of carbon atoms or methyl groups and the number of primary binding sites. (c) There are four to six moderate affinity binding sites. The binding to these sites does not appear to be temperature-dependent and the free energy of binding is due primarily to an entropy increase upon binding. (d) The affinity of insecticides varies from about 10^6 M^{-1} for DDT to 9.0 M^{-1} for nicotine. (e) The affinity is inversely related to the water solubility of the insecticides. (f) There are probably many sites of low affinity.

Though protein concentrations used differ for various insecticides, the results are comparable, since the preliminary results indicated the binding to be independent of protein concentration in the range studied (15–100 μM). As an example, Fig. 2 shows the binding of parathion to albumin over the protein concentration range. Higher protein concentrations were used in the case of carbofuran, aldicarb, monocrotophos, and nicotine, due to low affinities of these insecticides to HSA.

Spectral studies. Figure 3 shows the difference spectra of carbaryl, carbofuran, aldicarb, diazinon, nicotine (red

[2] Although n was assumed to be 5 in the case of DDT and dieldrin and it was necessary to rely on the relative solubility, these assumptions would not significantly affect the conclusions discussed in the following section.

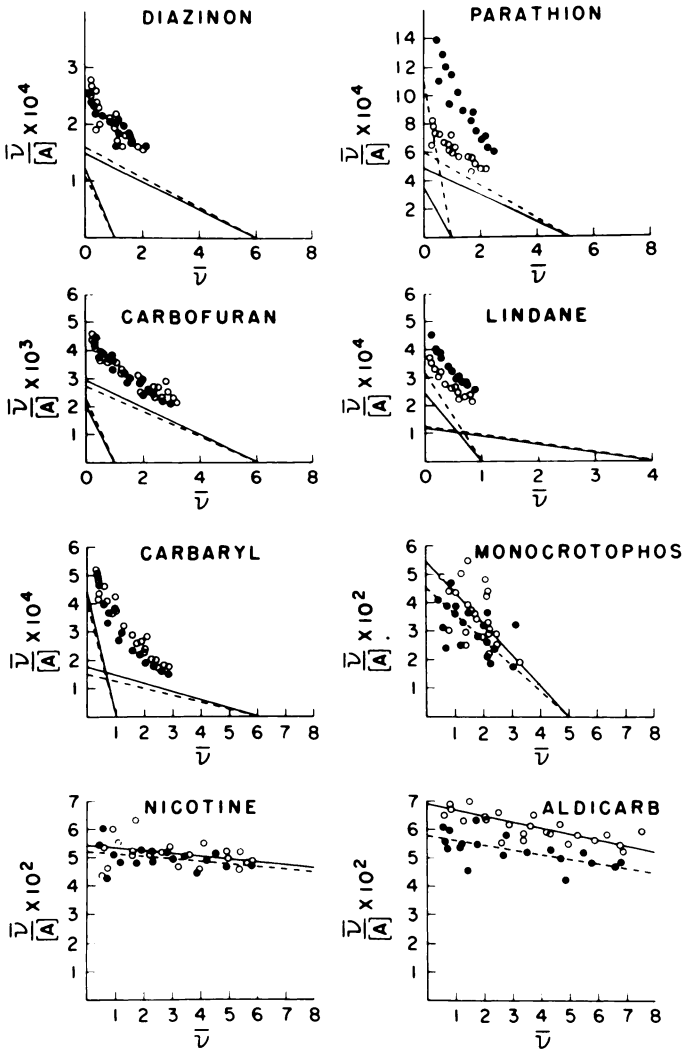

FIG. 1. *Scatchard plots of binding of various insecticides with HSA at 26° (○) and 16° (●) and pH 7.0 (Tris-HCl)*

Binding has been resolved into various classes as represented by *solid lines* (26°) and *broken lines* (16°). These simulated binding curves are calculated from the binding constants presented in Table 1.

shift), and parathion (blue shift) when aqueous solutions of the insecticides and ethanol in separate compartments of the cuvette are placed in the reference beam and ethanol solutions of the same insecticides and buffer in separate compartments are placed in the sample beam. Figure 4 presents the UV difference spectra due to binding of insecticides to the albumin. The following generalizations can be made: (a) Binding causes an apparent red shift in the 270–310 nm region. (b) The peaks are in the range 285–288 nm and are predominantly due to tyrosyl residues of HSA. There is also some contribution from tryptophan to the difference spectra as indicated by a shoulder at 293–295 nm. (c) The similarities in the difference spectra in Figs. 3 and 4 show that the spectral shifts in the spectrum of bound carbaryl and carbofuran contribute significantly to the difference spectra. Comparison of the figures also suggests that the contribution due to the red shift in the spectrum of the other bound insecticides would be small. (d) Butanol decreases and NaCl and sorbitol increase the binding, as indicated by the decrease and increase, respectively, in the magnitude of the difference spectra. There are exceptions in the cases of carbaryl, aldicarb, and nicotine, since high ionic strength decreases the binding. (e) There is a several-fold decrease in the binding at pH 3.5 as compared with that at pH 7.0.

Table 2 presents the quenching of HSA fluorescence due to interaction when excited at 287 and 297 nm. The excitation at 287 nm represents fluorescence due to both tyrosine and tryptophan, whereas excitation at 297 nm results only in tryptophan fluorescence. The results indicate significant quenching of HSA fluorescence due to binding of insecticides at low $\bar{\nu}$. Figure 5 shows that quenching of HSA due to binding of parathion does not change between pH 4.5 and 7.4. However, there is a marked decrease in quenching below pH 4.5.

DISCUSSION

The results indicate the presence of one site of higher affinity than remaining sites and a few sites of moderate affinity for insecticides. Failure to detect discrete binding sites on albumin for carbaryl and parathion in an earlier report (7) was probably due to substantial scatter in the binding isotherm which resulted in the conclusion that the *y*-intercept cannot be differentiated from zero in the Klotz plot. The presence of a few discrete binding sites for pesticides on albumin has been indicated in earlier reports with the association constants of the order 10^4 M^{-1} (5, 6). Our analysis of binding data with DDT and dieldrin is based on the assumption that n is 5. We observed four to six primary sites for other structurally diverse insecticides. Numerous studies have indicated the presence of a few, and usually not greater than 10, high-affinity sites on albumin for a great variety of anionic, cationic, and neutral organic compounds (15). As $\bar{\nu}$ depends on both n and K, the exact value of K in the case of DDT and dieldrin will vary depending on the number of sites available. Whatever are the variations in the value of K for dieldrin and DDT, they will be small and would not affect the lack of temperature dependence of K and the conclusions about the binding phenomena.

Further support to the assumption of discrete binding sites for DDT and dieldrin on albumin comes from binding of structurally similar compounds and from competitive binding studies with DDT metabolites. The compound 2,2′-methylene-bis(3,4,6-trichlorophenol), a biphenyl, binds to bovine serum albumin at five high-affinity sites, and it competitively inhibits the binding of fatty acids.[3] Thyroxine, a zwitterion which is structurally similar to DDT, binds at six primary sites on albumin, and the carboxyl and amino groups are not required for binding (16). The inhibition of binding of 2,2-bis(*p*-chlorophenyl) acetic acid to albumin by chlorophenoxyacetic acid derivatives (17) and thyroxine binding by 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethane (18) also suggest the probability of binding at discrete sites rather than a partitioning phenomenon.

The studies with defatted albumin have indicated the presence of one very high-affinity site for fatty acids ($K > 10^8$ M^{-1}), anionic detergents, and anions, which is

[3] T. L. Miller, personal communication.

TABLE 1

Binding constants of insecticides for HSA (Tris-HCl, pH 7.0, μm = 0.02)

Ligand	Water[a] solubility		Protein concentration	K_1		K_2		
				N_1		N_2		% RMS[b]
	μg/ml	°C	μM		M^{-1}		M^{-1}	
Chlorinated hydrocarbons								
DDT[c]	0.0026	26	15	5	3.70×10^5 ($\pm 0.40 \times 10^5$)			
		16		5	3.20×10^5 ($\pm 0.30 \times 10^5$)			
Dieldrin[c]	0.07	26	15	5	3.10×10^4 ($\pm 0.40 \times 10^4$)			
		16		5	3.10×10^4 ($\pm 0.30 \times 10^4$)			
Lindane	7	26	30	1	2.5×10^4	4	3.2×10^3	7.2
		16		1	3.2×10^4	4	3.10×10^3	15.2
Carbamates								
Carbaryl	40	26	30	1	4.5×10^4	6	3.00×10^3	14.3
		16		1	4.2×10^4	6	2.50×10^3	7.4
Carbofuran	400	26	60	1	2.0×10^3	6	4.80×10^2	11.3
		16		1	2.2×10^3	6	4.05×10^2	14.5
Aldicarb[d]	6000	26	100	34	2.0×10^1			10.6
				34	1.7×10^1			32.9
Organophosphates								
Parathion	24	26	30	1	3.4×10^4	5	1.00×10^4	11.6
		16		1	1.1×10^5	5	1.20×10^4	5.4
Diazinon	40	26	30	1	1.2×10^4	6	2.4×10^3	15.5
		16		1	1.1×10^4	6	2.55×10^3	15.1
Monocrotophos	Miscible	26	100	5	1.10×10^2			16.6
		16		5	0.90×10^2			35.1
Others								
Nicotine[d]	Miscible	26	100	60	9.0			11.9
		16		60	8.5			6.3

[a] The solubility values are taken from the literature (14) and are for purposes of comparison only.

[b] RMS is the root mean square deviation between the measured value of $\bar{\nu}$ and those calculated by the binding parameters given in the table. Per cent RMS was calculated by multiplying the RMS by 100 and dividing by the mean $\bar{\nu}$.

[c] Results are calculated from relative solubility in aqueous solution with and without added proteins and assuming n to be 5. Results have been expressed as mean ± standard deviation of the mean.

[d] The several-fold decrease in the binding in F form as compared to N form (Fig. 4) suggests the presence of a few sites of higher affinity similar to that observed with other insecticides. However, the experimental scatter in the binding isotherm, along with low affinities, made it difficult to estimate these sites separately.

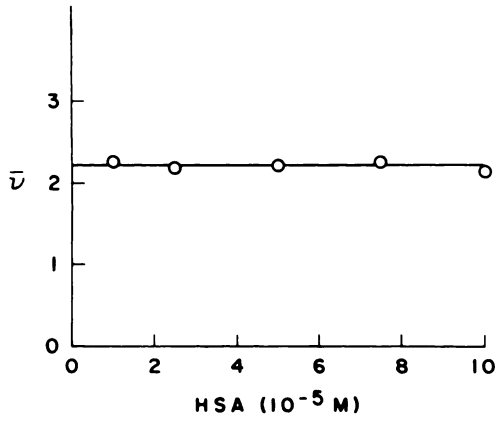

FIG. 2. *Binding parathion to HSA over the concentration range of protein from 1×10^{-5} to 1×10^{-4} M*

The binding was determined by measuring the relative solubility of parathion in aqueous solution with and without added protein.

characterized by significant enthalpy (19). *In vivo*, this site is occupied by fatty acids and would be of little consequence in transport of compounds of lower affinities such as insecticides, since the highest affinity observed in the present study was not more than 10^6 M^{-1}.

The insecticide affinity for albumin was inversely related to their solubilities. This fact, along with the lack of temperature-dependence of the binding at the moderate affinity sites, indicates that favorable energy change due to interaction is largely entropic, which is a general characteristic of hydrophobic interactions (20).

The difference spectra due to binding of insecticides are similar to those produced by the binding of a few equivalents of various alkyl ligands and long-chain fatty acids to albumin (21). The difference spectra show a red shift in the spectrum of tyrosyl and tryptophyl residues as judged by the peaks around 285–287 nm and a shoulder at 293–295 nm due to shielding of these chromophores from contact with aqueous solvent. The difference spec-

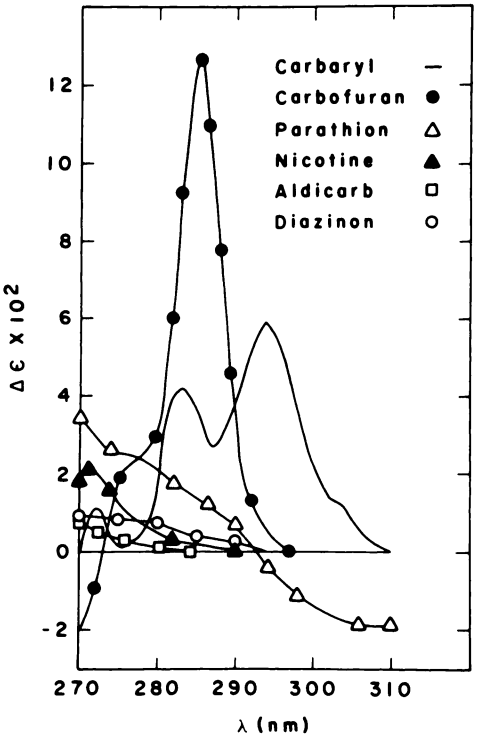

FIG. 3. *Difference spectra of various insecticides when aqueous solutions of insecticides are placed in the reference beam and ethanol solutions of the same insecticides in the sample beam of the spectrophotometer*

The λ_{max} of individual insecticides are carbaryl (281 nm), carbofuran (277.5 nm), parathion (274 nm), diazinon (248 nm), aldicarb (245 nm), and nicotine (262 nm).

tra also show significant contributions due to the red shift in the spectrum of bound carbaryl and carbofuran. These red shifts indicate that the environment of the binding sites is less polar than water. Thus, the difference spectra suggest that these moderate affinity sites are close to, or involve, tyrosyl and tryptophyl residues and that the environment at these sites is less polar than water.

The high ionic strength and the concentrations of butanol and sorbitol used in the present study should not affect the conformation of albumin as suggested by the lack of change in intrinsic viscosity and optical rotation over a wide range of ionic strength at neutral pH (22) and up to 20% (v/v) concentrations of a variety of nonaqueous solvents (23). Any effect on the difference spectra, therefore, would be due to the effect of solvents on the binding. The effect of 1-butanol, sorbitol, and NaCl on the magnitude of the difference spectra further supports the conclusion that interaction is primarily hydrophobic. Polyhydroxyalcohols, such as sorbitol, are known to increase hydrophobic interactions by their effect on water structure, whereas butanol decreases the hydrophobic interactions by its effect on water structure and possibly by competitive interaction with the hydrophobic residues on the protein (24). The lack of decrease in the magnitude of the difference spectra due to 0.5 M NaCl indicates that there is little contribution of ionic interactions in binding except in the case of carbaryl, aldicarb, and nicotine. Among these three insecticides, only nico-

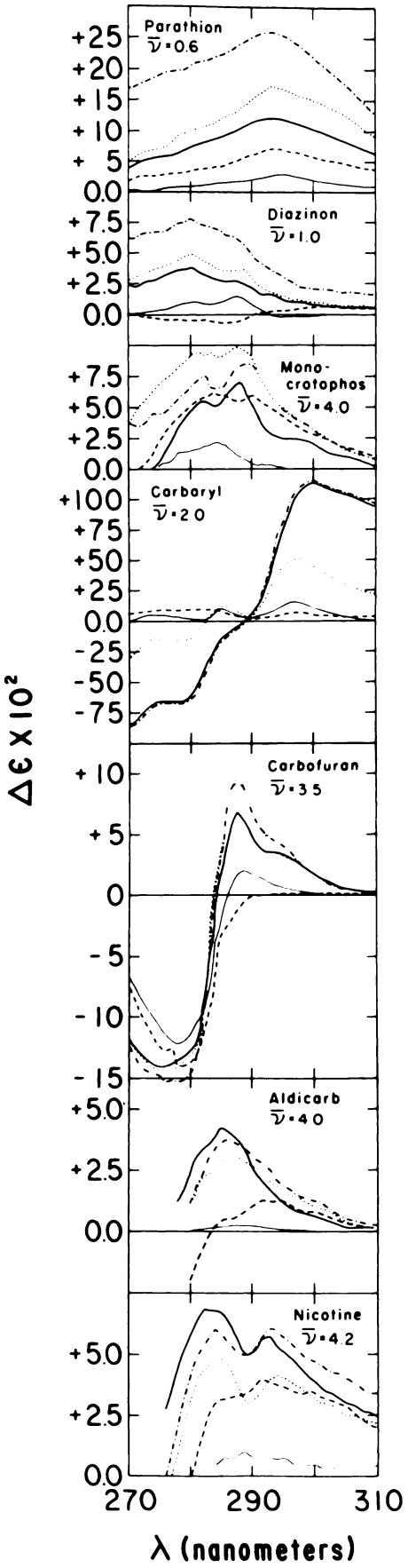

FIG. 4. *Difference spectra of HSA (40 μM) in the UV spectral region (270–310 nm) produced by binding the indicated number of equiva-*

TABLE 2

Relative fluorescence intensity of HSA in the presence of insecticides (26°, Tris-HCl buffer, pH 7.0, of ionic strength 0.02 M)[a, b]

Ligand	$\bar{\nu}$[c]	% Fluorescence intensity (excitation wavelength)	
		287 nm	297 nm
Parathion	2.0	44	44
Diazinon	2.0	40	20
Monocrotophos	3.0	40	50
Carbofuran	4.0	60	83
Aldicarb	4.0	77	30
Nicotine	4.0	44	27

[a] Carbaryl has strong fluorescence and it was not possible to follow the quenching at the concentrations required to achieve $\bar{\nu} = 2$.

[b] HSA at concentration 2 μM was excited at a given wavelength and emission measured at 335 nm in triangular cuvettes. The slit width was 3 nm for excitation and 5 nm for emission. The results represent the average of three estimates with the same protein preparation and are expressed as percentage of the fluorescence intensity of HSA without any insecticide.

[c] The concentrations of ligand to achieve $\bar{\nu}$ was calculated from n and K in Table 1.

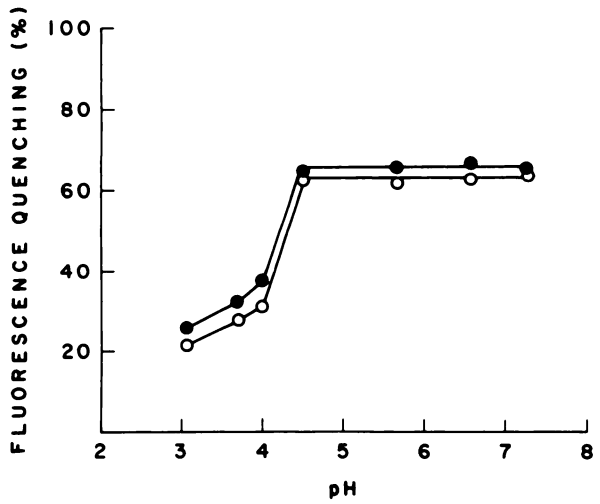

FIG. 5. *Quenching of HSA fluorescence due to binding of parathion between pH 3.5 and 7.4*

The emission was measured at 340 nm. Protein solutions were excited at 280 (○) and 300 nm (●). The parathion concentration was 5 10^{-5} M.

tine (with $pK_1 = 6.12$ and $pK_2 = 10.96$) would be positively charged at pH 7.0, and a decrease in binding due to 0.5 M NaCl in this case suggests some contribution from ionic interactions. However, carbaryl and aldicarb are not ionic ligands, and further experimentation will be needed to explain the observed decrease in their difference spectra due to high ionic strength. The small increase in the magnitude of the difference spectra due to high ionic strength in the case of most of the insecticides also indicates binding to be hydrophobic. It is well known that salt at high ionic strength tends to increase the interactions between hydrophobic groups, for example, the salting out effect as seen for solutions of slightly soluble organic molecules in water (25).

A significant decrease in difference spectra at pH 3.5 as compared with that at pH 7.0, and similarity in fluorescence quenching between pH 3.0 and 4.5 due to binding of parathion and the N → F transition, provide further information on the location of the binding sites. The albumin molecule is postulated to be composed of several cylindrical domains, with the presence of many hydrophobic clefts. Some of these hydrophobic areas are significantly disrupted when the pH of albumin is lowered from 4.5 to 3.7 due to expansion of the molecule and separation of the domains [N → F transition (26)]. A similar decrease in binding due to N → F transition has been reported for hydrocarbons (27) and indole compounds (28). These hydrophobic surfaces have been suggested to be high-affinity sites for hydrocarbons, large organic ions, and fatty acids. The insecticides reported herein appear to bind in the same regions.

The observed fluorescence quenching can occur by nonradiative energy transfer as parathion has significant, and carbofuran and nicotine have some, spectral overlap with HSA emission spectra. However, we cannot rule out alternative modes like collisional quenching and conformational changes. Many insecticides are collisional quenchers of varying efficiencies (29). There is also evidence, from optical rotation, of local microdisorganization of the albumin conformation due to binding of various ligands at high-affinity sites (30). Although the mechanism involved in the quenching process cannot be determined with these limited data, the fluorescence quenching along with the red shifts in the difference spectra suggests the presence of tyrosyl and tryptophyl residues at, or near, the binding sites.

We could not obtain binding constants at pH 7.4 and 37°, since some of the insecticides partially hydrolyzed, under these conditions, during the time required for equilibration. However, these results should be applicable to *in vivo* conditions, since results show a very weak temperature-dependence of binding to the physiologically available moderate affinity sites. Also, the small differences between our experimental conditions and the *in vivo* situation regarding pH and ionic strength would not significantly affect the binding, since it is hydrophobic and not influenced by low ionic strength (25). The observed magnitude of insecticide affinities for albumin suggests that a significant portion of water soluble, and probably most of the slightly water soluble, insecticides would be in bound form during their transport in the blood.

lents of insecticide at pH 7.0 Tris-HCl buffer of 0.02 M ionic strength

The symbols are: (—), HSA and the insecticide pH 7.0; (- - -), HSA and insecticide containing 0.8 M 1-butanol; (-·-·-), HSA and insecticide in the presence of 0.5 M sorbitol; (·····), HSA and insecticide in the presence of 0.5 M NaCl; and (——), HSA and insecticides at pH 3.50, 0.15 M KCl. The control in this case is HSA and insecticide at pH 3.5 in reference beam. Note that there is a severalfold difference between the *ordinates* of *panels* representing different insecticides. The $\bar{\nu}$ value will apply only to the control difference spectra, since binding would be affected by ionic strength, presence of butanol and sorbitol, and the pH 3.5.

ACKNOWLEDGMENT

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