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EVIDENCE OF A STRONG INTERACTION OF 2,4-DICHLOROPHENOXYACETIC ACID HERBICIDE WITH HUMAN SERUM ALBUMIN

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Summary

The interaction of 2,4-dichlorophenoxyacetic acid herbicide (2,4-D) with human serum albumin (HSA) was studied using fluorescence and differential scanning calorimetry (DSC). Fluorescence displacement of 1-anilino-8-naphtalenesulfonate (ANS) bound to HSA was used to evaluate the binding affinity of 2,4-D to HSA. The binding is associated to a high affinity site of HSA located in the IIIA subdomain. The association constant (K_{ass}) of the herbicide was about 5 μM^{-1}, several times higher than the affinity found for pharmaceutical compounds. This relatively strong interaction with HSA was evidenced by the increase in HSA protein thermostability induced as consequence of herbicide interaction. 2,4-D induces an increase in the midpoint of thermal denaturation temperature from 60.1 °C in herbicide free solution to 75.6 °C in full ligand saturating condition. The calorimetric enthalpy and the excess heat capacity also increased upon 2,4-D binding. To investigate the possibility of other/s system/s of 2,4-D transport in blood, besides of HSA, the interaction of the herbicide with lipid monolayers was explored. No interaction was detected with any of the lipids tested. The overall results provided evidence that high affinity 2,4-D-HSA complex exhibits enhanced thermal stability and that HSA is the unique transport system for 2,4-D in blood.

Key Words: 2,4-dichlorophenoxyacetic acid, albumin-ligand interaction, protein thermostability, differential scanning calorimetry, phenoxyherbicide

Since the mid 1940's the use of herbicides has increased dramatically in an attempt to improve the crop production. Particularly, the family of phenoxyherbicides is being extensively used as effective broad leaf herbicides in agriculture and forestry (1). The extensive use of the herbicides has generated a series of toxicological and environmental problems, particularly in developing countries (2). Several animal species including humans, are exposed to the herbicide in water, soil and vegetation as well as agroindustrial and commercial exposure. There is great concern that

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effects have been reported (3-5).

It has been reported that the central nervous system (CNS) is one of the targets for the toxic effects of phenoxyherbicides (4-8). A variety of neurotoxic effects of 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1) and related phenoxyherbicides have been reported in humans (9-13) and experimental animals (4, 6, 7, 13, 14). Recently, it has been reported that 2,4-D associated neurobehavioural changes in rats can be associated with alterations in the serotoninergic and dopaminergic systems (15-18). Moreover, neonatal rats exposed to 2,4-D showed a delay in CNS development concomitantly with biochemical and neurobehavioural alterations (19).

To exert toxicological effects *in vivo,* the herbicide is transported to the target organ via blood stream. Because of its amphiphilic nature it is transported either by lipoproteins, human serum albumin (HSA) or partitioned to erythrocytes membranes (20).

Previous reports suggested that HSA serves as a carrier protein molecule to transport 2,4-D (21, 22). To our knowledge there is no reported evidence that 2,4-D may be transported by lipoproteins. Further studies on the blood/brain transport of 2,4-D have indicated that efflux from the brain occurs via an active organic acid transport system (23, 24), and that organic acid transport out of the brain is inhibited by high concentrations of 2,4-D (24-27). In addition, the accumulation of 2,4-D into the brain is further facilitated by damage of the blood/brain barrier induced by the herbicide (26, 28, 29).

In this report we characterize the interaction of 2,4-D with HSA using fluorescence and differential scanning calorimetry techniques. The evidence of a strong interaction with the principal serum protein and a poor interaction in a model membrane system at relatively low herbicide concentration suggest that HSA is the main carrier system of 2,4-D in blood.

Materials and Methods

Human serum albumin without fatty acid or globulin (HSA), 1-anilino-8-naphtalenesulfonate (ANS), 2,4-dichlorophenoxyacetic acid (2,4-D), phosphate buffer were from Sigma Chem. Co. (St. Louis, MO, USA). The stock solution (5 or 20 mM) of 2,4-D was dissolved in absolute ethanol. An appropriate small volume (< 50 µl) from the stock solution was directly mixed with HSA solution for differential scanning calorimetry (DSC) and fluorescence or injected beneath a lipid or clean air-water interface for lipid monolayer studies.

ANS (Fig. 1) is a fluorescent compound that has a different quantum yield (ϕ_f) in polar and non polar environments. ANS fluorescence is almost completely quenched ($\phi_f = 3 \times 10^{-3}$) in aqueous solution and increases in solvents such as methanol ($\phi_f = 0.21$) or when this probe is bound to the high affinity site of HSA ($\phi_f = 0.67$) (30, 42). This property can be used, in appropriate conditions, to evaluate the affinity constant of a non fluorescence ligand that interacts with the high affinity site of HSA according to:

$$K_{assL} = \frac{(F_{bmax} - F_b)}{X(L_T - F_{bmax} - F_b)} K_{assF} \qquad \text{eq. 1}$$

where F_b is the concentration of bound fluorophore (ANS), in the presence of the total concentration of ligand L_T; F_{bmax} is the concentration of bound fluorophore saturating the HSA in the absence of ligand L (2,4-D); $X = F_b/F_f$, where F_f is the free fluorophore concentration ($F_f = L_T - F_b$). To evaluate the association constant of 2,4-D by ANS displacement, the experiment was

performed by adding increasing amount of 2,4-D (stock ethanolic solution 5 mM) into a fluorometer cuvette containing 1.25 μM HSA in equilibrium with 12.5 μM ANS in 100 mM phosphate buffer, pH 7.4 and 20 °C (30). Fluorescence steady-state spectra were recorded in a SLM 4800C fluorometer using a xenon arc lamp as the light source. The excitation wavelength was set at 390 nm and emission fluorescence monitored in the 420-510 nm range. All fluorescence steady state spectra were corrected by inner filter and dilution effect. The concentration of ANS bound (F_b) was calculated according to (30): $[F_b] = I_{fsample} / \alpha \phi_{fsample}$, where $I_{fsample}$ is the fluorescence intensity of ANS in presence of HSA; the proportional factor $\alpha = I_{fref}/[F]_{ref} \phi_{ref}$ is the relation between the fluorescence intensity of a known concentration of ANS in methanol used as reference. The values of fluoresecence intensities used were taken at 475 nm.

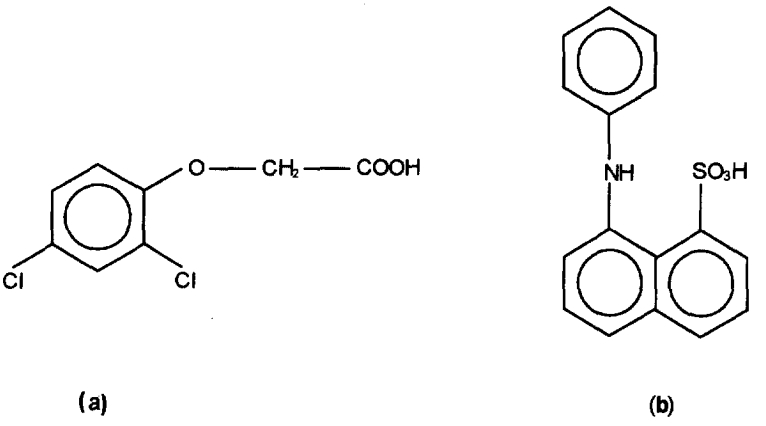

Fig. 1.

Structural formula of 2,4-D and ANS. The non fluorescent herbicide (a) and the fluorescence probe (b) share an aromatic ring that interacts with the high affinity binding site of HSA.

The study of putative surface properties and penetration ability of 2,4-D into lipid monolayers were carried out using procedures and equipment as described previously (31, 32). The lipids tested included natural egg phospatidylcholine and egg phosphatidylethanolamine, dipalmitoylphosphatidylcholine, cholesterol, gangliosides and sphingomyelin. Glycerophospholipids were from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Cholesterol, sphingomyelin and melittin were from Sigma Chem. (St. Louis, MO, USA). Gangliosides were purified from bovine brain as described previously (36). Melittin was further purified as described (38).

HSA denaturation in presence or in absence of 2,4-D was monitored with a MicroCal MC-2D scanning calorimeter with a digital data acquisition system. The calorimetric data were analyzed assuming a reversible non-two state model of denaturation (33) using the software provided by the manufacturer. A possible justification for the applicability of reversible thermodynamics to apparently irreversible processes has been discussed previously (34); for the case in which reversible unfolding is followed by the rate-limited irreversible step. This model was used after checking that no endotherms were found after complete denaturation and that similar thermograms, and similar results were obtained either: (i) at a low scan rate (28,8 and 9.2 $°C.h^{-1}$ than the one usually employed (55 $°C.h^{-1}$ or (ii) at different protein concentration or (iii) by fitting the whole curve using

the first 50 % of the experimental data of the thermogram. All runs were done at a protein concentration of 0.106 mM in 100 mM phosphate buffer, pH 7.4.

Experiments were done at least in triplicate. Values are mean ± S.E.

Results

Interaction of 2,4-D in the lipid monolayer membrane model system.

The lipid monolayer system has been used extensively as membrane model to measure the ability of amphiphilic compounds to interact with lipids (35). It has been reported that amphiphilic peptides (36-38), fatty acyl-coenzyme A (39) and ANS (40) interact with this lipid model membrane system.

The acid form of the 2,4-D herbicide (Fig. 1) is not very soluble in water and should be transformed in its basic form to increase its solubility. Pure ethanol increases its solubility but it precipitates at concentrations higher than 3-4 M. In spite of its amphiphilic character, 2,4-D has no surface activity when injected beneath a lipid clean air-water interface. No changes were observed in surface pressure when a small aliquot of 2,4-D was injected from an ethanol stock solution into subphase made of 145 mM NaCl pH 7 in the range from 5 to 100 μM of final herbicide concentration. When a few drops of a solvent solution containing 2,4-D was directly spread onto a 145 mM NaCl pH 7 subphase no changes in surface pressure were observed after compressing up to 90 % of the original area of the Langmuir trough. These results indicate that 2,4-D has no intrinsic ability either to partition to air/water interface free of lipids or to form insoluble monolayers. As expected from the above results, 2,4-D was not able to interact with any lipid compressed up to a initial surface pressure of 10 $mN.m^{-1}$ or lower. The interaction of herbicide was also null with melittin, a highly basic charged amphiphilic peptide from the bee venom that forms insoluble monolayers and interacts with lipids by inserting into the membranes (38).

TABLE I
Association constant found for ANS, Diazepam, Aspirin and 2,4-D to the high affinity site of HSA.

Compound	K_{ass} (μM^{-1})
ANS	0.87 ± 0.021
2,4-D	5.10 ± 0.091
Aspirin*	0.02
Diazepam*	0.11

The K_{ass} for ANS_s was evaluated as indicated in ref. 42. The K_{ass} of 2,4-D were evaluated from ANS displacement as described in Materials and Methods. *The value of K_{ass} were taken from ref. 30 and 42. Values were averaged from three experiments.

Interaction of 2,4-D with Human Serum Albumin

a) Fluorescence study with ANS.

HSA is the main plasma protein involved in the transportation of endogenous and exogenous ligands. HSA protein consists of 585 residues and the complete three-dimensional structure has recently been determined by X-ray crystallography (41). Many drugs like diazepam, aspirin, AZT, ibuprofen, and digitoxin are bound to HSA with different affinities (Table I). The more active binding site is located at HSA IIIA subdomain (41) in which is also the binding site of ANS (42). The association constant of ANS (K_{assF}) is 0.87 μM^{-1} (see Table I). Fig. 2 shows the loss in fluorescence intensity when 2,4-D displaces ANS bound to HSA at a final concentration of 2,4-D of 12.5, 25 and 50 μM (Fig. 2). The corresponding average association constant calculated using eq. 1 (see Materials and Methods) is about 5 μM^{-1} (Table I).

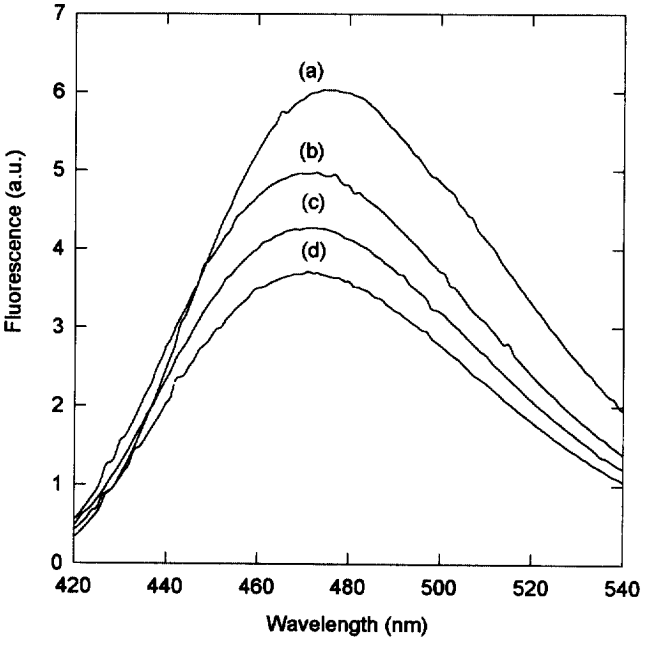

Fig. 2

Displacement of ANS by 2,4-D. Fluorescence steady state spectra of ANS (12.5 μM) bound to HSA (1.25 μM) in absence (a) or in presence of 12.5 μM (b), 25 μM (c) and 50 μM (d) of 2,4-D. Measurements were made in 100 mM phosphate buffer, pH 7.4 and at 20 °C. Data correspond to one of three experiments.

b) Differential scanning calorimetry studies of HSA-2,4-D complex

The effect of 2,4-D on the thermally induced denaturation of HSA was studied at two different herbicide:albumin molar ratios. It is known that the interaction of any ligand with a protein

with considerable affinity induces an increase in the denaturation temperature T_m (temperature of half completion of the denaturation) because the binding is coupled to the unfolding process (43). This model, developed by Shrake and Ross (43), assumes that the equilibrium constant of the *denaturation process* N (Native) ↔ D (Denatured) given by $K_{N\leftrightarrow D}$ is coupled to *binding process* P (protein) + L (Ligand) ↔ P-L (Protein-Ligand complex) given by the K_{ass}. As denaturation takes place, the ratio of free ligand to undenatured protein varies and the T_m increases. As expected according to Shrake and Ross (43) in subsaturing condition of ligand with respect to protein the thermograms are biphasic (not shown) and become unimodal at higher ligand/protein molar ratios

TABLE II
Thermodynamic parameters of thermal denaturation of HSA-2,4-D complex

Condition	ΔH_m (Kcal.mol^{-1})	T_m (°C)	C_{pmax}* (Kcal.mol^{-1}°C^{-1})
HSA	142 ± 2.0	60.1 ± 0.1	21.0 ± 0.29
HSA:2,4-D (1:10)	201 ± 2.6	67.1 ± 0.1	34.9 ± 0.41
HSA:2,4-D (1:50)	274 ± 3.5	75.6 ± 0.2	57.3 ± 0.70

*C_{pmax} is the excess heat capacity at T_m.

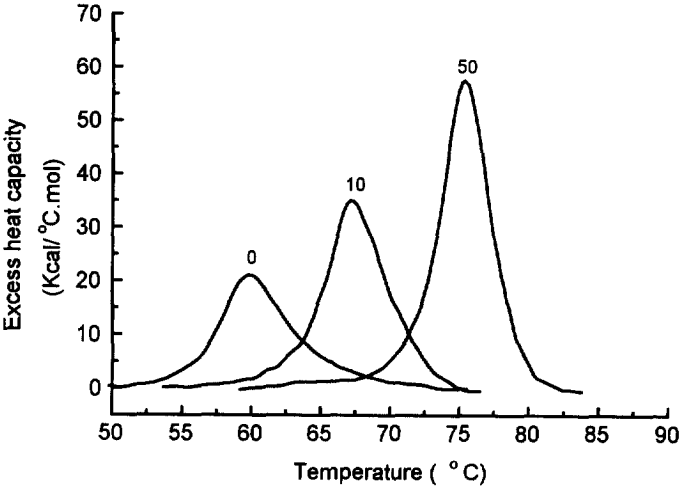

Fig. 3

Differential scanning calorimetry for HSA under different 2,4-D conditions. The thermograms showed are at 2,4-D:HSA molar ratio of 0, 10 and 50. The HSA concentration was 0.106 mM in 100 mM phosphate buffer pH 7.4.

as it was obtained (Fig. 3). The interaction of 2,4-D with HSA takes place with an increase in either the excess heat capacity, the midpoint of denaturation temperature (T_m) and the calorimetry enthalpy of unfolding (ΔH_m), see Table II. The T_m of HSA is shifted about 15 °C in condition of 50:1 herbicide:protein molar ratio (Fig. 3, and Table II). The interaction also takes place with a substantial increment in ΔH_m (Table II). These changes clearly demonstrate the magnitude of binding of 2,4-D to HSA. The absolute values of ΔH_m that we have obtained either for HSA alone or for HSA-2,4-D complex are lower than that previously reported for the same free ligand protein or bound to fatty acids with similar affinities (43). However, the changes in the thermodynamic parameters are in keeping with what it should theoretically expected for HSA that binds a ligand with a K_{ass} in the μM^{-1} range (43).

Discussion

The rapid penetration and body distribution together with a long half life in blood may allow herbicide toxicity. As 2,4-D has amphiphilic properties, it is reasonable to think that the mechanisms for herbicide transport in blood is one or a combination of several possible carriers and/or reservoirs: lipoproteins, red cells or serum albumin. Red cells have been postulated to fuction as a carrier for thyroid hormones (44). It is well known that serum albumin binds with a relatively high affinity to amphiphilic compounds that have a benzoic acid (or a derivative phenyl ring) as part of its molecular structure. Aspirin, AZT, benzodiazepines, ibuprofen and others are transported by HSA in blood (41). To discount the possibility that 2,4-D is transported either by lipoproteins or red cells, we explored the ability of this herbicide to interact with organized lipid systems at similar concentrations in which substantial interaction with HSA was observed. With this aim, we used the monomolecular lipid film as an artificial membrane model system (36-40). 2,4-D has a very small surface activity per se and was unable to penetrate lipid monolayers set at a initial surface pressure of 10 $mN.m^{-1}$ or below. Furthermore, the ability of 2,4-D to penetrate a protein monolayer was also null. Melittin is a basic peptide that forms insoluble monolayers and it has been reported to interact with negative amphiphilic compounds (38), therefore it can be also discarded as a non-specific mechanism of membrane penetration mediated by basic membrane proteins. Recently, it has been reported that 2,4-D induces alteration in human erythrocytes and in membrane model systems at relatively high concentrations (higher than 100 μM) (20). This paper correlates the considerable erythrocyte membrane alterations observed with an increase in the bilayer width and an decrease in the general polarization of Laurdan fluorescence probe concluding that 2,4-D produces a deep structural perturbation of the hydrophobic region. However, our results indicate a low ability of this herbicide to penetrate lipid monolayers at concentrations lower than 0.1 μM. At higher concentrations near 1 mM the effect of 2,4-D on lipid monolayers was poor and rather inconsistent with an small increase in surface pressure of about 1 or 2 $mN.m^{-1}$ (data not shown). Our results are in keeping with a previous report in which it was concluded that the interaction of 2,4-D with artificial membranes is not associated with changes in orientation of the dipoles of lipids constituting the membrane and the herbicide is rather adsorbed at the non-polar/polar interface [45].

Undoubtedly, 2,4-D herbicide strongly interacts with HSA. It has been demonstrated that ANS interacts with the high affinity site of HSA with an K_{ass} of 0.87 μM^{-1} (30, 42). The high affinity site of HSA localized in the subdomain IIIA works as carrier of amphiphilic derivatives with aromatic groups (41). The association constant K_{ass} of 2,4-D towards HSA can be estimated from fluorescence displacement of ANS probe (30). The K_{ass} calculated value of 5.1 μM^{-1} was several times higher than the values observed for ANS or diazepam (Table I). The relatively strong interaction of 2,4-D with HSA demonstrated in this work provides further evidence that the serum protein is the natural blood carrier of this herbicide in humans and animals as it was postulated

previously (21, 22). Shrake and Ross (43) have reported that the thermostability of HSA can be altered by the presence of ligand with moderate or high affinity. For these cases, the overall profile of the thermogram is shifted to higher temperatures as the ligand:protein molar ratio increases. The midpoint of HSA temperature denaturation is shifted by almost 15 °C indicating a strong thermostabilizing effect induced by 2,4-D binding (Fig. 3). This result is in keeping with the relatively high affinity measured by the decrease in ANS fluorescence due to the displacement of the probe by the binding of herbicide. This interaction also increases the calorimetric enthalpy and the excess heat capacity.

The strong interaction of 2,4-D with human serum albumin has been demonstrated in this work by means of two independently biophysical approaches: fluorescence and differential scanning calorimetry. Since 2,4-D at a low concentration was unable to interact with organized lipid system but it binds to HSA with a relatively high affinity provides further evidences that HSA-2,4-D is the main blood transport system for this herbicide. The fact that the affinity of 2,4-D towards to HSA is several times higher than the affinity found for therapeutic drugs such as aspirin and diazepam tested could imply changes in the bioavailability of these (or others) compounds in humans exposed to herbicide due to a mechanism of competition of these drugs for the high affinity site of HSA.

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