Title

Interaction of the antileprosy drug dapsone and its metabolite monoacetyldapsone with human serum albumin and mouse serum albumin

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INTERACTION OF THE ANTILEPROSY DRUG DAPSONE AND ITS METABOLITE MONOACETYLDAPSONE WITH HUMAN SERUM ALBUMIN AND MOUSE SERUM ALBUMIN

by

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INTRODUCTION

"The capacity of drug molecules to enter into specific combinations with proteins poses for pharmacology its most fundamental task--to comprehend the intimate nature of drug actions in terms of these molecular interactions (1)."

Avram Goldstein

Dapsone (4,4' diaminodiphenylsulfone,DDS) is currently the most efficacious drug in the treatment of leprosy (2). After oral administration of DDS, there is rapid acetylation to monoacetyldapsone (4-amino-4'-acetamidodiphenylsulfone,MADDS) (MADDS is also deacetylated to DDS) (3,4,5,6). DDS acetylation represents a genetic polymorphism similar to that for isoniazid acetylation. Rapid acetylators of DDS are distinguished from slow acetylators only by the ratio of plasma concentrations of MADDS to that of DDS (6).

In examining the excretion of DDS and MADDS, both drugs have the same half-time. The mean half-time of DDS and MADDS after administration of each compound to 2 subjects of each phenotype was 21 hours (6). Very small amounts of both compounds could be detected in the urine. A study by Linderstrom - Lang has shown DDS to be strongly bound to bovine serum albumin (7). Glazko, in a preliminary study of binding of DDS and MADDS to human serum albumin, has demonstrated high affinity of both compounds to albumin with MADDS the more strongly bound (8). Biggs and Levy (9)

examined the human plasma protein binding of DDS and MADDS in both rapid and slow acetylators. When binding was examined *in vivo* or *in vitro* at therapeutic concentrationsof the drug, DDS was 70% to 80% bound and MADDS was 98% to 100% bound. The plasma of rapid and slow acetylators of DDS bound the drugs to the same degree.

Levy et al (10) have demonstrated that DDS and MADDS also bind significantly in mouse plasma. MADDS behaves similarly in both undiluted and diluted human and mouse plasma; however, DDS has been shown to behave somewhat differently. In undiluted human and mouse plasma binding appears identical. If the plasma is diluted (1.0 gm%) the percent binding for human plasma remains high (50% - 70%); however, the percent binding for mouse plasma drops significantly (70% to 5%).

The molecular interactions between the plasma proteins and drugs are of basic importance. These interactions influence the distribution of drugs in the body and their access to sites of action, of metabolism, and of excretion. Quantitively, the most important constituent in terms of nonspecific binding of drug molecules of the plasma proteins is albumin.

It is the intent of this project to examine the binding of DDS and its metabolite MADDS to human serum albumin and to explore the difference in binding of DDS by mouse serum albumin and human serum albumin.

Materials and Methods

A. Materials

1. DDS - K and K Laboratories, Inc., Hollywood, California.
2. MADDS - Parke, Davis, and Company, Ann Arbor, Michigan
3. Dialysis tubing - A. H. Thomas Company, Philadelphia, Pennsylvania.
4. Human Serum Albumin - Hyland Laboratories, Costa Mesa, California.
5. Dextran - Nutritional Biochemical, Cleveland, Ohio.

B. Methods

1. Isolation and Purification of Mouse Serum Albumin.

Mouse serum albumin was fractionally precipitated with ammonium sulfate at 5°C (11). Initially a saturated ammonium sulfate solution was added to an equal volume of mouse serum to produce 50% saturation. The resulting precipitate consisting of gamma, alpha 1, alpha 2, beta 1, and beta 2 globulins was removed by centrifugation. At 62% saturation, produced by adding more saturated ammonium sulfate, the albumin precipitated and was also collected by centrifugation.

To remove the ammonium sulfate from the albumin solution, the precipitate was redissolved in an isotonic buffer (0.075M, pH7.4), and the protein solution was dialyzed repeatedly against a phosphate buffer containing dextran. This high molecular weight polysaccharide was used to oppose the osmotic force produced by the albumin.

The purity of the mouse albumin solution was determined by electrophoresis (12). After the first fractionation, residual gamma and beta 1 globulins were still present in the albumin solution. A second fractionation procedure was performed, and the subsequent electrophoretogram displayed a pure solution of mouse serum albumin.

2. Protein Assay

The concentration of albumin was determined by ultraviolet spectrophotometery (13, 14).

The spectrophotometric method involved diluting the mouse serum albumin solution 2,000 fold with a 9.0 gram per liter solution of NaCl. A Beckman Model DU ultraviolet spectrophotometer was used for the absorption measurements. The absorbance was measured at 215 and at 225 nm, the saline diluent solution being used as the blank. The absorbance at 225 nm was substracted from that at 215 nm. The difference multiplied by a factor of 144 gives the protein concentration in the diluted solution expressed in mcg. The procedure was standardized with a solution of egg albumin.

3. Equilibrium Dialysis

The binding of DDS and MADDS to albumin was determined by equilibrium dialysis (1,15,26). Equilibrium dialysis, which directly determines albumin-drug binding and reproduces physiologic conditions reasonably well (27,28,29), was preferred to ultrafiltration, which may produce some error because of accumulation of protein at the membrane surface which in turn adversely affects the ligand-protein bond (16).

The dialysis procedure utilized 27/32 inch cellulose dialysis tubing, which was suspended in deionized water 1/2 hour prior to using to ensure

complete saturation and then blotted dry. The tubing was filled with 3 ml of the albumin solution, and suspended in 12 ml of an isotonic, buffered drug solution in a 50 ml centrifuge tube. The centrifuge tubes were agitated at 5°C for 48 hours.

Preliminary studies were performed to eliminate sources of error in dialysis. Complete equilibration of the drug occured between 24 and 36 hours, and the binding of drug to the dialysis tubing was negligible. Measurements made at the end of the dialyses showed no significant Donnan effect detectable by a change in volume inside the bag, no pH change, and no leakage of protein across the membrane. No evidence of acetylation of DDS to yield MADDS, nor of deacetylation of MADDS to DDS was found in any of the experiments as a result of simultaneous quantitation of both drugs using ethylene dichloride extraction.

In the dialysis procedure three standard protein concentrations were used for human serum albumin (HSA) (0.10 gram percent, 0.50 gram percent, 0.60 gram percent), and two standard protein concentrations were used for mouse serum albumin (MSA) (0.10 gram percent, 0.50 gram percent). In order to achieve wide ranges of values for ν (molar concentration of bound drug/molar concentration of albumin), the drug concentrations were varied as follows:

HSA with MADDS - 1 mcg/ml to 15 mcg/ml

HSA with DDS - 0.5 mcg/ml to 30 mcg/ml

MSA with DDS - 0.5 mcg/ml to 10 mcg/ml

For each protein - drug concentration, the binding was analyzed in duplicate. In all dialyses, the pH was maintained constant at 7.4 except in two studies in which the binding of HSA and DDS was analyzed at pH 9.8 and pH 5.6. The protein binding was determined by the difference in drug concentration between the outside and the inside of the dialysis bag.

The differences between the duplicate measurements of $\bar{\nu}$, the means of which are shown in Tables V, IV, and VI, were used to calculate the standard deviations and 95% confidence limits around each mean value of $\bar{\nu}$. The differences between the duplicate values of $\bar{\nu}$ for each dialysis of HSA with DDS and of MSA with DDS were pooled; the standard deviation and confidence limits for HSA with MADDS were determined separately. The calculations were as follows (17):

$(\text{DIFFERENCE})^2$ = square of the difference between the members of each pair of duplicate determinations of $\bar{\nu}$.

$$S.D. \pm \sqrt{\frac{(DIFFERENCE)^2}{2N}}$$

WHERE N = # PAIRS OF DUPLICATES

$$C.L. = \pm \frac{S.D.}{\sqrt{2}} \times t_{0.95,\, N-1\, df}$$

FOR DDS:

$$S.D. = \sqrt{\frac{0.0067}{30}} = 0.0143$$

$$C.L. = \bar{\nabla} \pm \frac{0.0143}{1.414} \times 2.00$$

WHERE $\bar{\nabla}$ IS THE MEAN VALUE OF ∇

$$= \bar{\nabla} \pm 0.021$$

FOR MADDS:

$$S.D. = \sqrt{\frac{0.0072}{18}} = 0.0200$$

$$C.L. = \bar{\nabla} \pm \frac{0.0200}{1.414} \times 2.30$$

$$= \bar{\nabla} \pm 0.033$$

The very narrow confidence bands suggest great precision for each measurement of ∇ .

4. DDS, MADDS Fluoresence assay

DDS and MADDS concentrations were determined spectrophotofluorometrically (18, 19). The procedure utilizes the native fluorescence of DDS and MADDS after extraction with ethylene dichloride. All samples were analyzed in duplicate. A blank containing no drug and standards of DDS and MADDS (each 2 mcg/ml) were run with the unknown samples. The extraction procedure was as follows:

1. A 1 ml sample was added to a 50 ml screw-capped culture tube containing 4 gm ammonium sulfate, 25 ml ethylene dichloride (twice redistilled), and 2 ml deionized water. To this were added 2 ml of 5 N sodium hydroxide, and the culture tube was shaken for 15 minutes and centrifuged for 10 minutes at 500 x g.
2. After centrifugation, the organic phase was decanted into a 50 ml screw-capped culture tube containing 1 ml of 0.1 N sodium hydroxide. The solution was shaken for 5 minutes and centrifuged for 5 minutes at 500 x g. The alkali wash was then removed by aspiration.
3. 2 ml of the organic phase were pipetted into a 50 ml culture tube containing 10 ml of 2 N hydrochloric acid. The tubes were shaken for 10 minutes and centrifuged for 5 minutes at 500 x g.
4. 9.5 ml of the hydrochloric acid phase was pipetted into a 50 ml screw-capped culture tube containing 5 ml of 4.5 N sodium hydroxide and 3 ml ethylene dichloride (tube and contents prechilled in an ice bath). The tubes were shaken for 10 minutes and centrifuged

for 5 minutes. The upper aqueous phase was aspirated and the ethylene dichloride phase was used for the fluorometric determination.

The Farrand spectrophotofluorometer was used for the determinations. The instrument was initially standardized and set for maximum activation and fluorescence at 350 nm/450 nm. In analyzing the samples, maximum activation and fluorescence was 295 nm/335 nm for DDS and 295 nm/410 nm for MADDS. The slit arrangement used was 2 mcm to prevent rapid deterioration of the drug. The unknown samples and standards were corrected by the blank determination.

The reproducibility of the fluorescence assay was determined for MADDS and DDS (the determinations for HSA with DDS and MSA with DDS were pooled). The standard deviation and confidence limits were calculated by a method similar to that used for determining the standard deviation and confidence limits of the measurements of ∇ (see pg. 9). The calculations were as follows:

$(\text{DIFFERENCE})^2$ = square of the difference between the members of each pair of duplicate fluorometric determinations both inside and outside the dialysis bag. (Table I, III, V).

FOR DDS.

$$S.D. = \sqrt{\frac{0.8194}{102}}$$

$$= 0.0896$$

$$C.L. = \bar{x} \pm \frac{0.0896}{1.414} \times 1.96$$

$$= \bar{x} \pm 0.14$$

FOR MADDS:

$$S.D. = \sqrt{\frac{0.4915}{72}}$$

$$= 0.0826$$

$$C.L. = \bar{x} \pm \frac{0.0826}{1.414} \times 1.96$$

$$= \bar{x} \pm 0.12$$

where $\bar{X}$ is the mean concentration of DDS or MADDS.

In the fluorometric determination it was found that DDS (295 nm/335 nm) contributes significantly to the fluorescence of MADDS (295 nm/410 nm); and MADDS contributes to the fluorescence of DDS. The mutual contribution requires that the concentrations of DDS and MADDS be calculated using simultaneous equations. The simultaneous equations were also used to detect the possibility of acetylation of DDS to MADDS and deacetylation of MADDS to DDS. The simultaneous equations used were as follows:

$$\text{DDS}_{T335_{nm}} = \text{mcg TRUE DDS} + \left(\frac{\text{NET FL } 295/335 \text{ MADDS}}{\text{NET FL}/\text{mcg } 295/335 \text{ DDS}}\right) \text{TRUE mcg MADDS}$$

$$\text{MADDS}_{T410_{nm}} = \text{mcg TRUE MADDS} + \frac{\text{NET FL } 295/410 \text{ DDS}}{\text{NET FL}/\text{mcg } 295/410 \text{ MADDS}} \quad \text{TRUE mcg DDS}$$

WHERE

$$\text{DDS}_{T335_{nm}} = \text{NETFLUORESCENT INTENSITY AT } 335_{nm} \Big/ \text{NET FL}/\text{mcg } 295/335 \text{ DDS}$$

(NET FLUORESCENT INTENSITY = FLORESCENT INTENSITY WITH CORRECTION FOR BLANK DETERMINATION)

(NET FL/mcg = FLUORESCENT INTENSITY FOR 1.0 mcg WITH CORRECTION FOR BLANK DETERMINATION)

AND

$$\text{MADDS}_{T410_{nm}} = \text{NETFLUORESCENT INTENSITY AT } 410_{nm} \Big/ \text{NET FL}/\text{mcg } 295/410 \text{ MADDS}$$

IF: x = mcg TRUE DDS

AND: y = mcg TRUE MADDS

LET $(A) = \dfrac{\text{NET FL } 295/335 \text{ MADDS}}{\text{NET FL}/\text{mcg } 295/335 \text{ DDS}}$

LET $(B) = \dfrac{\text{NET FL } 295/410 \text{ DDS}}{\text{NET FL}/\text{mcg } 295/410 \text{ MADDS}}$

THEN $MADDS_{T410_{nm}} = Y + (B)X$

AND $DDS_{T\,335\,nm} = X + (A)Y$

BY SUBSTITUTION:

I. $$\frac{FL\ 410_{nm}}{NET\ FL/_{mcg}{}^{295}/_{410}\ MADDS} = Y + (B)X$$

II. $$\frac{FL_{335\,nm}}{NET\ FL/_{mcg}{}^{295}/_{335}\ DDS} = X + (A)Y$$

SOLVING FOR X IN II.

$$X = \left[\frac{FL_{335\,nm}}{NET\ FL/_{mcg}{}^{295}/_{335}\ DDS}\right] - (A)Y$$

AND SUBSTITUTING X IN I.

$$\frac{FL_{410}}{NET\ FL/_{mcg}{}^{295}/_{410}\ MADDS} = Y + (B)\left[\frac{FL_{335\,nm}}{NET\ FL/_{mcg}{}^{295}/_{335}\ DDS} - (A)Y\right]$$

SOLVING FOR Y:

$$Y = \frac{\left[\frac{FL\ 410nm}{NET\ FL/_{ug}\ ^{295}/_{410}\ MADDS}\right] - (B)\left[\frac{FL\ 335nm}{NET\ FL/_{mcg}\ ^{295}/_{335}\ DDS}\right]}{1 - (A)(B)}$$

SOLVING FOR X:

$$X = \frac{\left[\frac{FL\ 335nm}{NET\ FL/_{mcg}\ DDS}\right] - (A)\left[\frac{FL\ 410nm}{NET\ FL/_{mcg}\ ^{295}/_{410}\ MADDS}\right]}{1 - (A)(B)}$$

5. Scatchard Analysis

The binding constants for the protein-drug interaction were analyzed by means of the Scatchard equation (20,21,22,23,24,25). In applying the Scatchard equation to a drug-protein interaction, several theoretical considerations must be kept in mind.

A. The interaction must be reversible and the data must be obtained at equilibrium.

B. All groups on the protein molecule capable of interacting must have identical affinities for the drug molecules, and the affinity of any group must be unaffected by the binding of drug molecules with other groups. Sharp breaks in the various linear curves will be obvious only if the affinities of the several species of receptors differ widely.

C. Systematic deviation of the data in the direction of reduced binding may result from electrostatic repulsion by initially bound ions of subsequent ions of the same charge.

D. If the drug is not varied through a sufficient range the extrapolations will not even approximate the true maximum number of available reacting functional groups but will only show the number of groups readily available, i.e. those with highest affinity for the drug molecules.

With these assumptions, the Scatchard equation could be derived from the mass law (1). The derivation is as follows:

$$X + P_T \rightleftharpoons P_T X \qquad K_d = \frac{[X][P_T]}{[P_T X]}$$

where $[P_T]$ is the concentration of free receptors; $[X]$ is the concentration of unbound drug; $[P_T X]$ is the concentration of combined receptors; and K_d is the dissociation constant. If (N) represented the number of receptor groups carried by each protein molecule and $[P_o]$ the molar concentration of the total protein, $N[P_o]$ represented the total concentration of receptors, $[P_T] + [P_T X]$:

$$[P_T] = N[P_o] - [P_T X]$$

$$K_d = [X]\ N\ [P_o] - [P_T X] \Big/ [P_T X]$$

$$[X]\ N\ [P_o] - [X]\ [P_T X] = K_d\ [P_T X]$$

$$[X]\ N\ [P_o] = K_d + [X]\ [P_T X]$$

$$\frac{[P_T X]}{N\ [P_o]} = \frac{[X]}{K_d + [X]}$$

$$\frac{[P_T X]}{[P_o]} = \frac{N\ [X]}{K_d + [X]}$$

This expression was identical in form to the Langmuir adsorption isotherm.

$$y = \frac{x}{m} = \frac{bc}{\frac{1}{a} + c}$$

where:

x = amount of substance adsorbed
m = weight of adsorbant
c = concentration of unadsorbed material
a = constant
b = constant

The expression correctly expressed the fact that at high values of c the adsorbant becomes saturated and the whole

term approached a limiting value of b . The Langmuir isotherm was entirely equivalent to the mass law equilibrium.

$$\frac{[P_T X]\ K_d}{[P_o]} + \frac{[P_T X][X]}{[P_o]} = N\,[X]$$

$$\frac{[P_T X]\ K_d}{[P_o]} = N\,[X] - \frac{[P_T X][X]}{[P_o]}$$

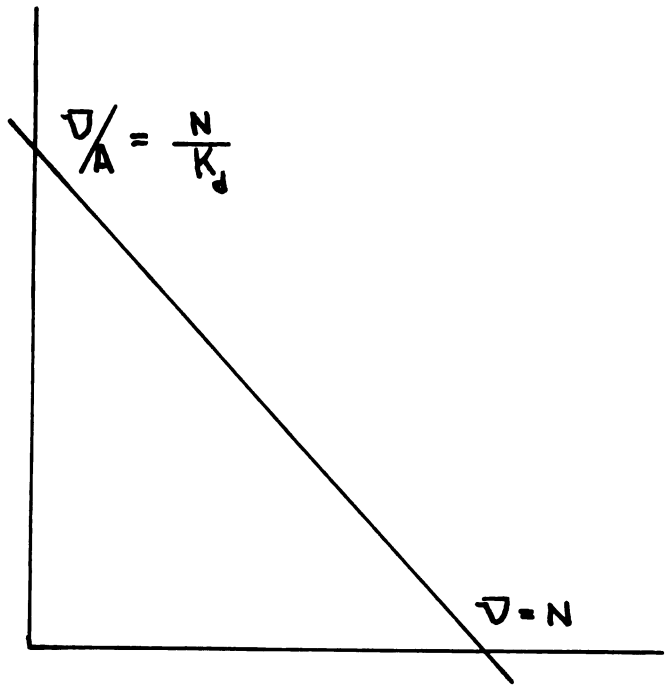
$$\frac{[P_T X]}{[P_o]} = \frac{N[X]}{K_d} - \frac{[P_T X]\,[X]}{[P_o]\ K_d}$$

$$\frac{\dfrac{[P_T X]}{[P_o]}}{[X]} = \frac{N}{K_d} - \frac{\dfrac{[P_T X]}{[P_o]}}{K_d}$$

This equation was in the form of the linear equation $y = mx + b$. In the Scatchard analysis $\dfrac{[P_T X]}{[P_o]}$ equals ∇ and was the abcissa while $\dfrac{\dfrac{[P_T X]}{[P_o]}}{[X]}$ represented ∇/A and was the ordinate. The graphical representation of the equation was as follows:

$$\frac{\frac{[P_T X]}{[P_o]}}{[X]} = \frac{N}{K_d} - \frac{\frac{[P_T X]}{[P_o]}}{K_d}$$

$$\frac{\frac{[P_T X]}{[P_o]}}{[X]} = V/A$$



$$\frac{[P_T X]}{[P_o]} = V$$

As $\frac{\frac{[P_T X]}{[P_o]}}{[X]}$ tends to zero $\frac{\frac{[P_T X]}{[P_o]}}{K_d} \rightarrow \frac{N}{K_d}$ and $\frac{[P_T X]}{[P_o]} \rightarrow N$

As $\frac{\frac{[P_T X]}{[P_o]}}{K_d}$ tends to zero $\frac{\frac{[P_T X]}{[P_o]}}{[X]} \rightarrow \frac{N}{K_d}$

TABLE I

DUPLICATE FLUOROMETRIC DETERMINATIONS FOR HUMAN SERUM ALBUMIN WITH MADDS

Initial Protein Concentration (gm%)	Initial Drug Concentration (mcg/ml)	First Duplicate Inside Bag[a] (mcg/ml)	First Duplicate Outside Bag[a] (mcg/ml)	Second Duplicate Inside Bag[a] (mcg/ml)	Second Duplicate Outside Bag[a] (mcg/ml)
0.100	1.00	1.61 1.69	0.49 0.49	1.61 1.54	0.49 0.49
0.100	2.00	3.03 2.96	1.19 1.08	3.01 2.82	1.10 1.05
0.100	5.00	6.04 5.83	3.21 3.15	6.04 6.10	3.28 3.34
0.100	7.00	8.31 8.19	5.27 5.24	8.30 8.42	5.29 5.37
0.100	10.00	10.42 10.64	7.40 7.42	10.54 -	7.40 -
0.100	15.00	15.35 15.55	12.10 12.14	15.55 -	12.21 -
0.600	1.00	2.96 3.09	0.16 0.16	3.02 2.95	0.14 0.17
0.600	2.00	5.94 5.94	0.32 0.34	5.76 5.80	0.38 0.38
0.600	5.00	14.00 13.82	1.41 1.37	13.19 13.40	1.49 1.34

[a]Measurements made after equilibrium dialysis.

TABLE II

DIALYSIS OF HUMAN SERUM ALBUMIN WITH MADDS

Initial Protein Concentration (mcmoles/l)	Initial Drug Concentration (mcmoles/l)	Drug Concentration Inside Bag After Dialysis (mcmoles/l)	Drug Concentration Outside Bag After Dialysis (mcmoles/l)	ν	ν/A
87.0	3.45 (1.0mcg/ml)	10.2 (2.95mcg/ml)	0.55 (0.16mcg/ml)	0.112	2.03×10^5
87.0 (0.60 gm%)	6.87 (2.0mcg/ml)	20.2 (5.85mcg/ml)	1.21 (0.35mcg/ml)	0.218	1.79×10^5
87.0	17.2 (5.0mcg/ml)	46.9 (13.60mcg/ml)	4.83 (1.40mcg/ml)	0.484	1.00×10^5
14.5 (0.10 gm%)	3.45 (1.0mcg/ml)	5.59 (1.62mcg/ml)	1.69 (0.49mcg/ml)	0.269	1.59×10^5
14.5	6.87 (2.0mcg/ml)	10.2 (2.95mcg/ml)	3.79 (1.10mcg/ml)	0.441	1.16×10^5
14.5	17.2 (5.0mcg/ml)	20.7 (6.00mcg/ml)	11.2 (3.25mcg/ml)	0.654	5.84×10^4
14.5	24.1 (7.0mcg/ml)	28.6 (8.30mcg/ml)	18.3 (5.30mcg/ml)	0.71	3.90×10^4
14.5	34.4 (10.0mcg/ml)	36.3 (10.54mcg/ml)	25.5 (7.40mcg/ml)	0.745	2.92×10^4
14.5	51.7 (15.0mcg/ml)	52.9 (15.35mcg/ml)	41.9 (12.15mcg/ml)	0.800	1.90×10^4

TABLE III

DUPLICATE FLUOROMETRIC DETERMINATIONS FOR MOUSE SERUM ALBUMIN WITH DDS

Initial Protein Concentration (gm%)	Initial Drug Concentration (mcg/ml)	First Duplicate Inside Bag[a] (mcg/ml)	First Duplicate Outside Bag[a] (mcg/ml)	Second Duplicate Inside Bag[a] (mcg/ml)	Second Duplicate Outside Bag[a] (mcg/ml)
0.100	1.00	0.80 0.79	0.72 0.69	0.81 0.80	0.72 0.74
0.100	2.00	1.80 1.74	1.61 1.56	1.67 1.66	1.49 1.53
0.100	5.00	4.84 4.79	4.42 4.31	4.86 -	4.31 -
0.100	10.00	9.59 9.70	8.88 9.17	9.56 -	8.86 -
0.500	0.500	0.50 0.52	0.31 0.28	0.51 0.47	0.32 0.33
0.500	2.00	1.87 1.89	1.16 1.24	1.95 1.98	1.18 1.32

[a] Measurement made after equilibrium dialysis.

TABLE IV

DIALYSIS OF MOUSE SERUM ALBUMIN WITH DDS

Initial Protein Concentration (mcmoles/l)	Initial Drug Concentration (mcmoles/l)	Drug concentration Inside Bag After Dialysis (mcmoles/l)	Drug Concentration Outside Bag After Dialysis (mcmoles/l)	$\bar{\nu}$	$\bar{\nu}/A$
72.5 (0.50 gm%)	2.01 (0.50mcg/ml)	2.01 (0.50mcg/ml)	1.25 (0.31mcg/ml)	0.010	8.3×10^3
72.5	8.06 (2.0mcg/ml)	4.96 (1.92mcg/ml)	0.039 (1.23mcg/ml)		7.8×10^3
14.5 (0.10 gm%)	4.03 (1.0mcg/ml)	3.23 (0.80mcg/ml)	2.90 (0.72mcg/ml)	0.023	7.6×10^3
14.5	8.06 (2.0mcg/ml)	6.94 (1.72mcg/ml)	6.25 (1.55mcg/ml)	0.047	7.5×10^3
14.5	20.1 (5.0mcg/ml)	19.5 (4.83mcg/ml)	17.8 (4.42mcg/ml)	0.113	6.3×10^3
14.5	40.3 (10.0mcg/ml)	38.8 (9.62mcg/ml)	36.2 (8.97mcg/ml)	0.180	4.96×10^3

TABLE V

DUPLICATE FLUOROMETRIC DETERMINATIONS FOR HUMAN SERUM ALBUMIN WITH DDS

Initial Protein Concentration (gm%)	Initial Drug Concentration (mcg/ml)	First [a] Duplicate Inside Bag (mcg/ml)	First [a] Duplicate Outside Bag (mcg/ml)	Second [a] Duplicate Inside Bag (mcg/ml)	Second [a] Duplicate Outside Bag (mcg/ml)
0.100	2.00	1.77 1.86	1.52 1.60	1.79 1.67	1.54 1.50
0.100	5.00	4.60 4.78	4.12 4.25	4.71 ----	4.22 ----
0.100	10.00	9.07 9.04	8.42 8.32	8.75 8.86	8.06 8.20
0.100	15.00	14.01 14.23	13.22 12.82	13.89 14.11	13.25 -----
0.100	20.00	21.00 21.21	20.02 20.05	20.86 20.69	19.80 19.58
0.100	30.00	30.15 30.30	29.29 28.09	29.55 -----	29.11 -----
0.500	0.500	0.52 0.56	0.27 0.37	0.52 0.61	0.32 0.26
0.500	2.00	2.32 2.41	1.39 1.43	2.21 2.25	1.28 1.31
0.500	5.00	6.55 6.32	3.99 4.07	6.35 6.54	4.12 4.03

[a] Measurements made after equilibrium dialysis.

TABLE VI

DIALYSIS OF HUMAN SERUM ALBUMIN WITH DDS

Initial Protein Concentration (mcmoles/l)	Initial Drug Concentration (mcmoles/l)	Drug Concentration Inside Bag After Dialysis (mcmoles/l)	Drug Concentration Outside Bag After Dialysis (mcmoles/l)	$\bar{\nu}$	$\bar{\nu}/A$
72.5 (0.50gm%)	2.01 (0.50mcg/ml)	2.21 (0.55mcg/ml)	1.20 (0.30mcg/ml)	0.014	1.16×10^4
72.5	8.06 (2.0mcg/ml)	9.27 (2.30mcg/ml)	5.44 (1.35mcg/ml)	0.053	9.74×10^3
72.5	20.1 (5.0mcg/ml)	26.0 (6.45mcg/ml)	16.3 (4.05mcg/ml)	0.133	8.22×10^3
14.5 (0.10gm%)	8.06 (2.0mcg/ml)	7.14 (1.77mcg/ml)	6.21 (1.54mcg/ml)	0.064	1.02×10^4
14.5	20.1 (5.0mcg/ml)	18.9 (4.70mcg/ml)	16.9 (4.20mcg/ml)	0.138	8.17×10^3
14.5	40.3 (10.0mcg/ml)	36.0 (8.93mcg/ml)	33.1 (8.21mcg/ml)	0.200	6.01×10^3
14.5	60.4 (15.0mcg/ml)	56.7 (14.05mcg/ml)	52.8 (13.10mcg/ml)	0.260	4.90×10^3
14.5	80.6 (20.0mcg/ml)	84.5 (20.95mcg/ml)	80.0 (19.85mcg/ml)	0.305	3.80×10^3
14.5	121.0 (30.0mcg/ml)	121.0 (30.00mcg/ml)	116.0 (28.82mcg/ml)	0.330	2.90×10^3

TABLE VII

Dialysis of Human Serum Albumin with DDS
pH-9.8

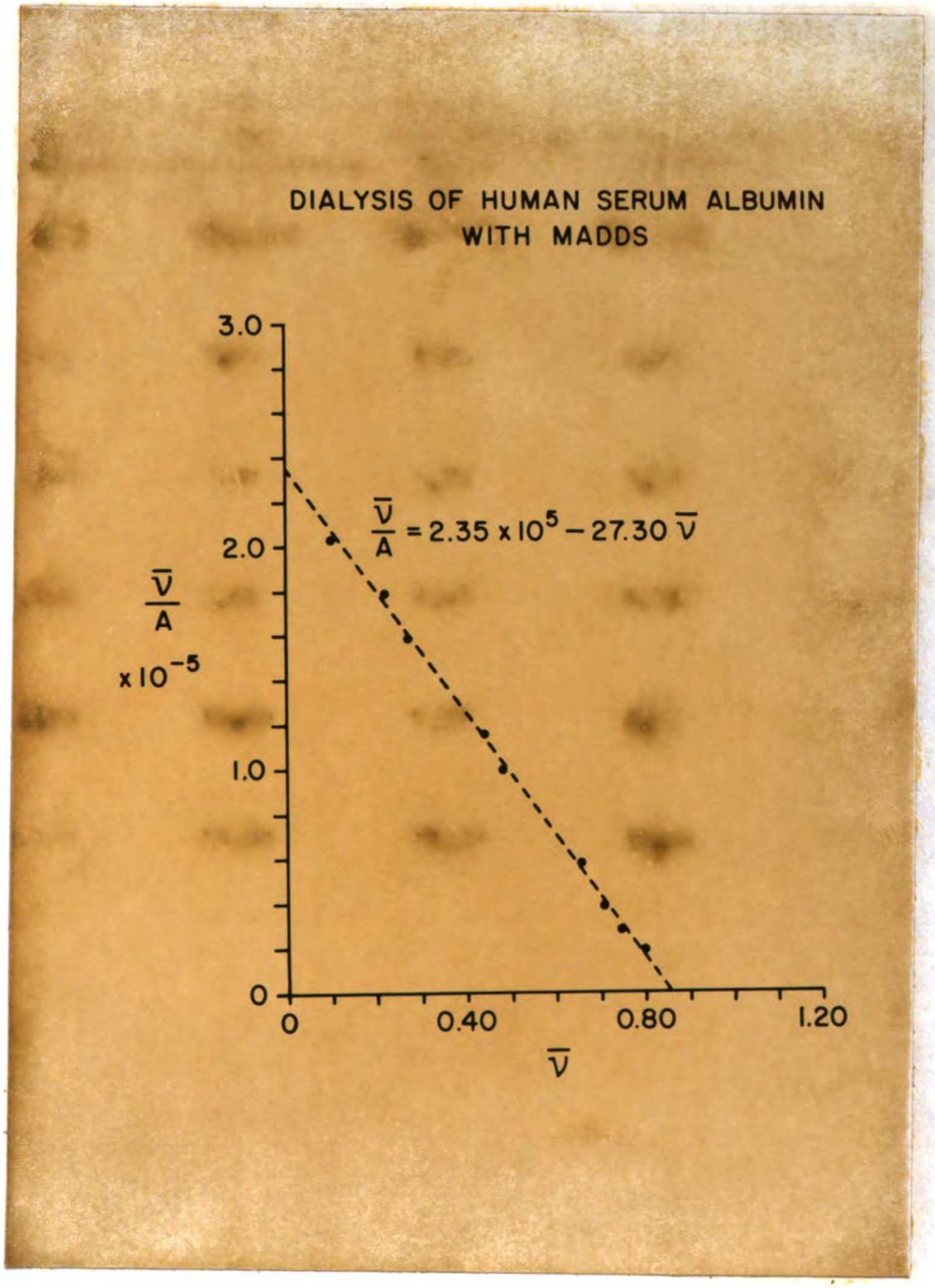
Initial Protein Concentration (gm%)	Initial Drug Concentration (mcg/ml)	Average Concentration Inside Bag (mcg/ml)	Average Concentration Outside Bag (mcg/ml)	∇
0.100	1.00	0.78	0.81	0
0.100	2.00	1.50	1.55	0
0.100	5.00	4.58	4.57	0
0.100	10.00	9.06	9.25	0
0.100	15.00	14.14	14.28	0

TABLE VIII

Dialysis of Human Serum Albumin with DDS
pH-5.6

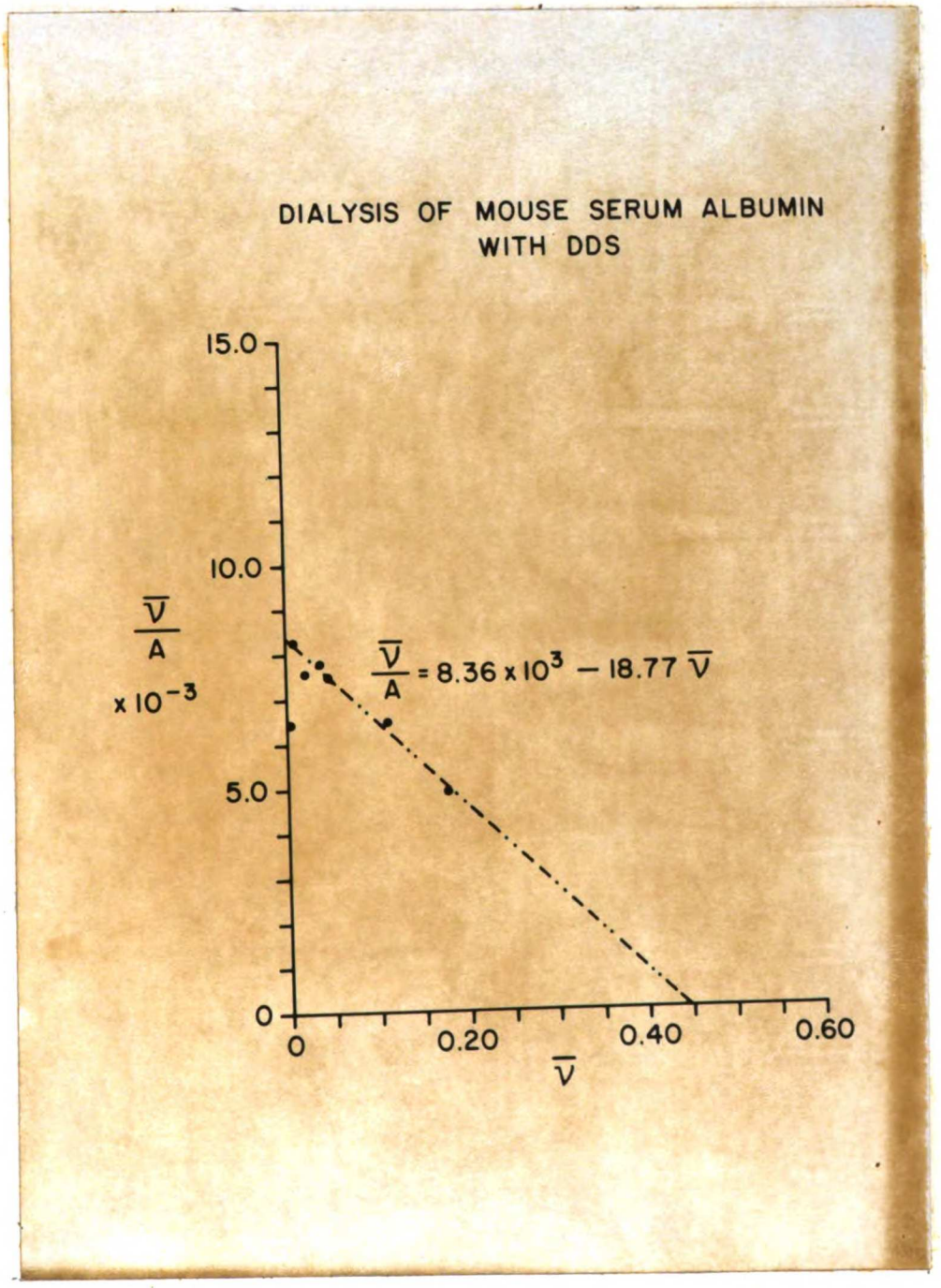
Initial Protein Concentration (gm/%)	Initial Drug Concentration (mcg/ml)	Average Concentration Inside Bag (mcg/ml)	Average Concentration Outside Bag (mcg/ml)	ν
0.100	1.00	0.76	0.76	0
0.100	2.00	1.76	1.64	0.033
0.100	5.00	4.05	3.93	0.033
0.100	10.00	9.13	8.77	0.128
0.100	15.00	12.55	12.00	0.156

FIGURE I



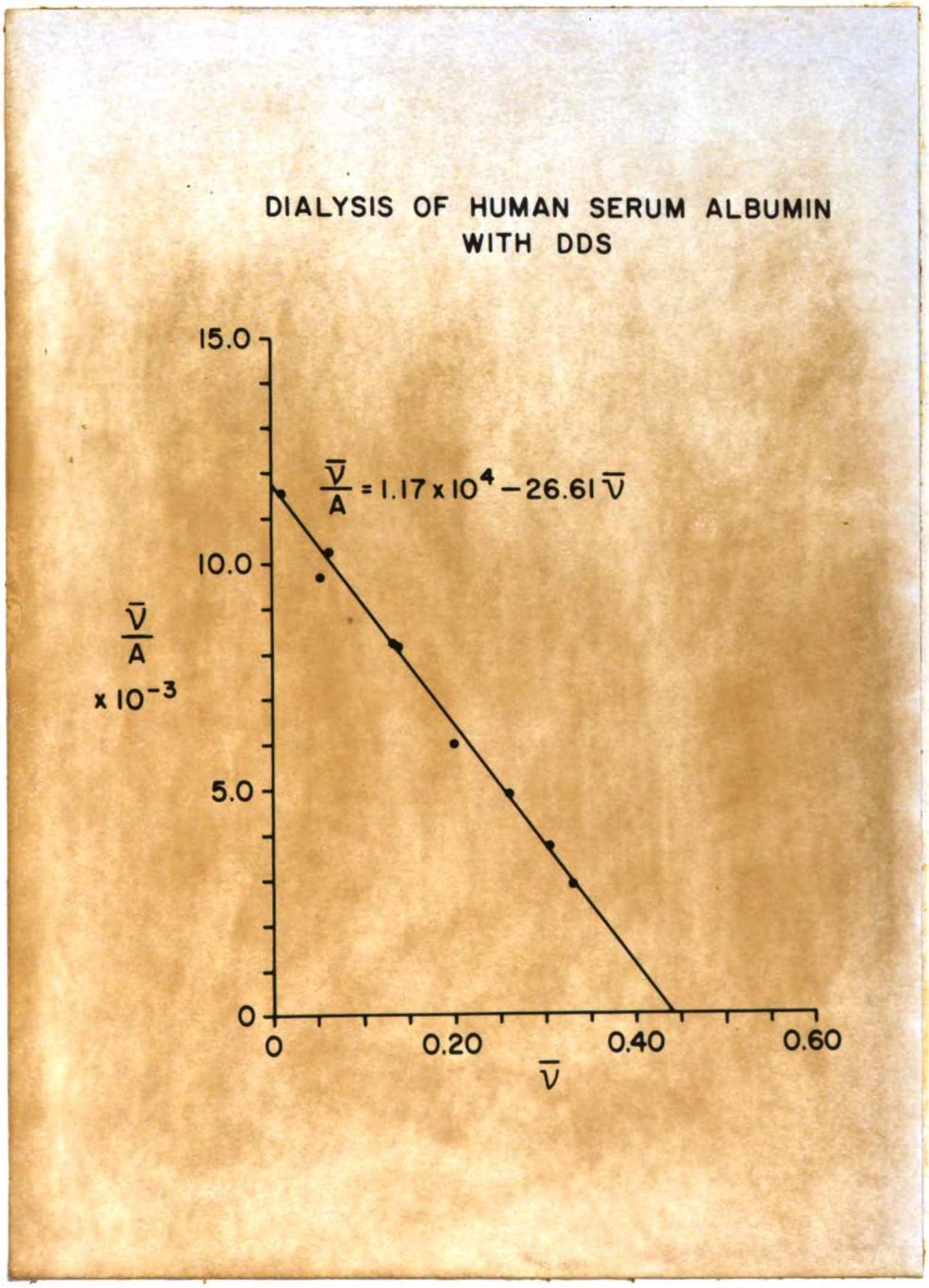
Scatchard plot of dialysis of MADDS with HSA. The data from Table II have been ploted.

FIGURE II



Scatchard plot of dialysis of DDS with MSA. The data from Table IV have been ploted.

FIGURE III



Scatchard plot of dialysis of DDS with HSA. The data from Table VI have been plotted.

RESULTS

1. Binding of MADDS by HSA

The results of the study of the binding of MADDS by HSA are tabulated in Tables I and II and the Scatchard plot is shown in Figure I. The points appear all to lie on the same straight line, suggesting that only one species of binding site is present (1,27,28). A straight line was fit to the points on the Scatchard plot by the method of least squares (30). The equation of the regression line is:

$$\nu/A = 2.35 \times 10^5 - 27.30\,\nu.$$

The ν intercept is 0.86; because ν should be a small whole number, the intercept is interpreted to indicate only one binding site per albumin molecule. The ν/A intercept is 2.35×10^5; because at $\nu = 0$, $\nu/A = N K_a$ and $N = 1$, then $K_a = 2.35 \times 10^5$.

Confidence limits were determined for the two intercept values (30). For $\nu = 0$, $\nu/A = 2.35 \times 10^5$, the 95% confidence limits are $(2.35 \pm .335) \times 10^5$. For $\nu/A = 0$, $\nu = 0.86$, the 95% confidence limits are 0.86 ± 0.16.

2. Binding of DDS by MSA

The results of the study of the binding of DDS by MSA are tabulated in Tables III and IV and the Scatchard plot is shown in Figure II. A straight line is suggested by the points indicating only one species of binding site on the albumin molecule. A straight line was fit to the points on the Scatchard plot by the method of least squares. The regression line is:

$$\nu/A = 8.36 \times 10^3 - 18.77\,\nu.$$

The ν intercept is 0.445, and is interpreted to indicate one-half

binding site per albumin molecule. The $\bar{\nu}/A$ intercept is 8.36×10^3; because at $\bar{\nu} = 0$, $\bar{\nu}/A = nK_a$ and $n = 0.445$, then $K_a = 1.67 \times 10^4$.

Confidence limits were determined for the two intercept values (30). For $\bar{\nu} = 0$, $\bar{\nu}/A = 8.36 \times 10^3$, the 95% confidence limits are $(8.36 \pm 0.295) \times 10^3$. For $\bar{\nu} = 0.445$, $\bar{\nu}/A = 0$, the confidence limits are 0.445 ± 0.02.

3. Binding of DDS by HSA

The results of the study of the binding of DDS by HSA are tabulated in Tables IV and VI and the Scatchard plot is shown in Figure III. The points appear all to lie on the same line, suggesting that only one species of binding site is present. A straight line was fit to the points of the Scatchard plot by the method of least squares. The equation for the regression is:

$$\bar{\nu}/A = 1.17 \times 10^4 - 26.61\bar{\nu}.$$

The $\bar{\nu}$ intercept is 0.45; the intercept is interpreted to indicate one-half binding site per albumin molecule. The $\bar{\nu}/A$ intercept is 1.17×10^4, because at $\bar{\nu} = 0$, $\bar{\nu}/A = nK_a$ and $n = 0.45$, then $K_a = 2.34 \times 10^4$.

Confidence limits were determined for the two intercept values. For $\bar{\nu} = 0$, $\bar{\nu}/A = 1.17 \times 10^4$, the 95% confidence limits are $(1.17 \pm 0.29) \times 10^4$. For $\bar{\nu} = 0.45$ $\bar{\nu}/A = 0$, the 95% confidence limits are 0.45 ± 0.08.

The binding studies of DDS by human serum albumin at pH 9.6 and pH 5.8 are tabulated in Table VII and Table VIII, respectively. The studies at pH9.8 indicate that binding declined to zero, as evidenced by the fact that equal concentrations of drug are found both inside and outside the bag. At pH 5.8 the binding was reduced by approxi-

mately 50%.

In previous work by Biggs and Levy (8), the percent binding of undiluted human plasma with DDS and MADDS approached 80% and 99%, respectively. The present work shows that the binding of MADDS by the albumin molecule is significantly greater than that of DDS. MADDS has ten times the affinity for the albumin molecule that DDS has.

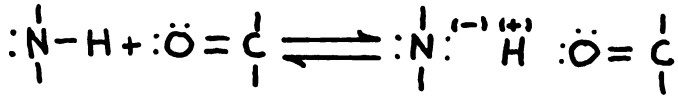
DISCUSSION

1. Binding Characteristics of HSA

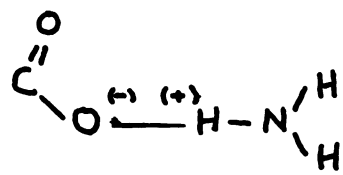
It has been shown that DDS is moderately bound to the HSA molecule. Attention was focused on the mechanism of binding between DDS and the albumin molecule.

The mechanism of binding might be ionic. The albumin molecule exists in a net negative state at physiologic pH (7.4), attributable to the beta carboxyl group of aspartic acid and the gamma carboxyl group of glutamic acid, and DDS can exist in an ionized, protonated state. The Ka of DDS has been determined to be 1.0×10^{1} ($pK_a = 1$). With a pK_a of 1, DDS exists totally in an uncharged state at pH 7.4. In addition, other experiments have shown that the surface of the albumin molecule contains positively, but not negatively, charged groups (31, 32). It must be assumed that the binding mechanism is something other than ionic.

Albumin is a globular protein (45% alpha-helix), consisting of a single polypeptide chain with an N-terminal aspartic acid residue (33). Its tertiary structure and stability are dependent upon hydrogen bonds between NH and CO groups of adjacent turns of the peptide spiral which forms the helix, and salt bridges which are formed by the mutual attraction of the positively and negatively charged groups of the peptide chain.



HYROGEN BOND



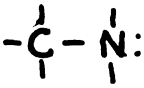
SALT BRIDGE

The disulfide bonds (-S-S-) between cysteine residues in the peptide chain are of secondary importance. Although the S-S bonds are of importance in maintaining conformation, they do not prevent flexibility of the peptide chain. Conformation does not change when numerous disulfide bonds are cleaved (34).

In examining the binding between HSA and DDS, it was found that binding (ν) declined to zero at pH 9.6. This change in condition towards a more alkaline pH causes rupture of the salt bridges by the conversion of NH_3^+ to NH_2 (34). Irreversible deamination and sulfhydryl-disulfide interchange also occur, disrupting the -S-S- bonds (35). It has been concluded based on polarized fluorescence studies that molecular dissociation occurs above pH 9 (36).

At pH 5.6 the binding of DDS by HSA was reduced by approximately one-half. Investigators have found by examining optical rotation and intrinsic viscosity that at lower pH values the albumin molecule undergoes molecular expansion. As the pH approaches 4.5, the albumin molecule undergoes isomerization and a change from the N form to a new F form results (35). Intermediate forms in various states of molecular expansion have been shown to exist between the N form and the F form.

The expansion is largely electrostatic in origin and takes place only after formation of an "expandable" intermediate in which intramolecular stabilizing forces have been lost. At lower pH values, protonation of COO^- to COOH occurs causing rupture of the salt bridges and also disruption of the hydrogen bond, thus eliminating intramolecular stabilizing forces.

It is evident that the binding of MADDS and DDS by human serum albumin is related to the tertiary structure of the albumin molecule with a possible coordinate covalent bond occuring between the carbon-bound amino group  of DDS and MADDS and the albumin molecule.

II. Binding Characteristics of DDS and MADDS

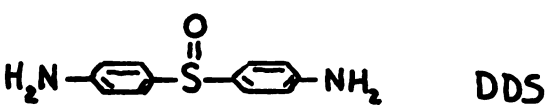
The ∇ intercepts for DDS and MADDS were as follows:

1. MADDS by HSA - 0.86
2. DDS by HSA - 0.45
3. DDS by HSA - 0.445

In the Scatchard equation, ∇ represents the number of binding sites of drug per albumin molecule and should approach a small whole number (1,27,28). MADDS was interpreted as having one binding site per molecule of HSA.

The number of binding sites for DDS on the HSA and MSA molecules was also interpreted as one. By definition, ∇ equals the number of moles of drug bound per mole of albumin. As a result, a ∇ value of 0.45 means that 0.45 mole of DDS is bound per mole of albumin; and a ∇ value of 0.86 indicates that 0.86 mole of MADDS is bound per mole of albumin. It appears that 2 molecules of albumin bind 2 molecules of MADDS for each molecule of DDS bound.

The structures of DDS and MADDS are as follows:



DDS

MADDS .

If it is assumed that the active site on the drug molecules is the terminal amino group, then DDS has two active sites for binding to the albumin molecule and can be considered a "bivalent" molecule. It is assumed that each molecule of DDS with two active binding sites could bind with two molecules of albumin.

III. Binding of DDS by HSA and MSA

Levy et al (8) have demonstrated that DDS behaves differently in diluted mouse plasma than in diluted human plasma. When the plasma of both species is diluted (1.0 gm%), the percent binding for mouse plasma drops significantly (70% to 5%).

It was first thought that the difference in binding between diluted human and mouse plasma was a result of a difference in the binding constants; it was thought that the binding constants for human plasma would be significantly greater. Knowing that serum albumin is quantatively the most important component in binding, the percent binding and association constants were determined for both albumin solutions. It was found that the association constants for HSA and MSA were approximately the same, 2.3×10^4 and 1.67×10^4, respectively; and the percent binding for diluted solutions of MSA (0.50 gm% - 35%-38% bound) was similar to that for human serum albumin (0.50 gm% - 37%-45%).

The differences in binding that occured between human and mouse plasma were not explained by the study of serum albumin.

SUMMARY

1. DDS is moderately bound to HSA and MSA with a K_a of 2.34×10^4 and 1.67×10^4, respectively. MADDS has approximately ten times the affinity for the HSA molecule than does DDS, with a K_a of 2.35×10^5.

It is speculated that the active site on the drug is the terminal amino group. Because DDS is an N,N'-diamino molecule, two active sites are present. It appears that two molecules of albumin bind two molecules of MADDS for each molecule of DDS bound.

2. The binding of DDS to the human albumin molecule is not of the ionic type. It is possible that DDS binding is related to the tertiary structure of albumin, and it is hypothesized that a coordinate covalent bond may occur between the carbon-bound amino group of DDS and the albumin molecule.

3. The differences in binding that occur between DDS and diluted mouse plasma and human plasma are not explained by the studies of serum albumin.

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