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Binding of Apolar Molecules by Serum Albumin

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The binding of 33 organic compounds to bovine serum albumin at pH 7.4 and 37° was studied using equilibrium dialysis. The affinity of the neutral molecules for the albumin is well correlated with their octanol-water partition coefficients. This is not true for molecules which are more than 50% ionized at pH 7.4. The constants in the linear free energy relationship derived for the 25 neutral molecules agree well with those obtained for other kinds of molecules. It is shown that the octanol-water and the isobutyl alcohol-water reference systems for defining hydrophobic character yield comparable results.

It has long been recognized that organic compounds are, in varying degrees, absorbed by serum protein.[1] However, it is only recently that successful attempts have been made to quantitatively correlate chemical structure with binding.[2-12] Such results are illustrated in the extrathermodynamic equations 1 and 2. In eq 1, C represents the molar concentration of organic compound necessary to produce a molar 1:1 complex with pure bovine serum albumin (2.5×10^{-5} M) in equilibrium dialysis. The octanol-water partition coefficient is represented by P, n represents the number of compounds tested, r is the correlation coefficient, and s is the standard deviation from regression. The numbers in parentheses are the 95% confidence intervals. The study was made at 4°. In eq 2, B represents the per cent penicillin bound and F the per cent free. This study was made at room temperature (about 22°).† The parameter π is defined[13] as $\pi_X = \log P_X - \log P_H$ where P_X is the partition coefficient of a derivative and P_H that of the parent molecule. In eq 1, all of the molecules are neutral un-ionized species while in eq 2, all of the compounds are acids which, at the experimental pH of 7.4, will be almost completely ionized. The carboxylate function of the penicillins is a constant feature of each of the 79 derivatives. Changes in the binding of each of the members is well correlated by differences in hydrophobic character of the various substituents defined by π.

1:1 Binding of Miscellaneous Compounds by Bovine Serum Albumin[7]

	n	r	s	
$\log \frac{1}{C} = 0.75(\pm 0.07) \log P + 2.30(\pm 0.15)$	42	0.960	0.159	(1)

Binding of Penicillins by Human Serum[5]

$\log (B/F) = 0.49\pi - 0.63$	79	0.924	0.134	(2)

The present report is concerned with a study of the binding of organic compounds reported some time ago.[14] It was shown then that the data in Table I could be roughly correlated with the water solubility of the organic compounds. Since this report it has been shown that partition coefficients provide a better reference system for the correlation of chemical structure with activity in biological processes.[15] In reconsidering this unpublished work we have used the data in Table I to derive eq 3 *via* the method of least squares. The conditions used in studying the binding of the molecules in Table I were similar to those used in obtaining the data for eq 1 except that in Table I the solution was buffered to pH 7.4 and a temperature of 37° was employed. Equation 3 is highly significant statistically; $F_{1,23} = 190.7$; $F_{1,3\,\alpha\,0.005} = 9.6$.

	n	r	s	
$\log \frac{1}{C} = 0.666(\pm 0.10) \log P + 2.600(\pm 0.22)$	25	0.945	0.242	(3)

Experimental Section

Binding Measurements. Equilibrium dialysis is a standard procedure.[16] The use of ultraviolet absorption to determine concentration permits observations on compounds with characteristic ultraviolet absorption in the region of protein absorption without the necessity of developing an assay procedure. This method requires care in manipulation; it is therefore described in detail.

Armour's bovine crystalline albumin (BSA) and a single lot of Armour's bovine albumin powder (Fraction V) were used. The crystalline material has very little dialyzable absorption, the powder a small amount in the lower ultraviolet. These absorptions are blanked. Binding on the powder was just perceptibly but not significantly higher than that on the crystalline albumin.

In a typical experiment, graded volumes of compound in pH 7.4 0.1 M phosphate buffer solution were pipetted into glass screw-top

†A. E. Bird, private communication.

Table I. Binding of Organic Compounds by Bovine Serum Albumin at pH 7.4 and 37°

Compound	Log P^a	Log $1/C$ Obsd	Log $1/C$ Calcd[b]	X/M at c = 1 mg %[c]	B_4 1 mg %, %[d]
Sulfanilamide[e]	−0.78*	2.44	2.07	0.0047	16
4-Methoxyaniline[e]	0.78	2.94	3.11	0.14	36
4-Aminoacetophenone[e]	0.82	3.02	3.14	0.23	48
Sulfapyridine	0.00*	3.03	2.59	0.17	40
Phenol[e]	1.46*	3.22	3.56	0.3	56
4-Methylaniline	1.39*	3.29	3.52	0.40	61
4-Aminopropiophenone	1.32	3.39	3.47	0.39	61
4-Nitroaniline	1.39*	3.40	3.52	0.45	64
4-Chloroaniline	1.83	3.63	3.81	0.77	76
4-Aminobutyrophenone	1.82	3.65	3.80	0.77	76
4-Methylphenol[e]	1.94*	3.65	3.88	0.8	76
4-Bromoaniline	2.03	3.94	3.94	1.55	86
2-Naphthylamine	2.25	3.95	4.09	1.43	85
4-Aminovalerophenone	2.32	4.04	4.13	1.78	88
4-Chlorophenol	2.39*	4.04	4.18	1.7	87
8-Hydroxyquinoline	2.02*	4.06	3.93	1.85	88
6-Methyl-8-hydroxyquinoline	2.52	4.09	4.27	2.1	89
4-Iodoaniline	2.35	4.23	4.15	2.5	91
4-Aminobiphenyl	3.03	4.38	4.61	3.2	93
4-Aminocaprophenone	2.82	4.50	4.47	4.3	94
5,7-Dimethyl-8-hydroxyquinoline	3.02	4.53	4.60	3.9	94
4-Bromophenol	2.59*	4.67	4.31	3.8	94
5-Chloro-8-hydroxyquinoline	2.95	5.01	4.55	4.8	95
2-Hydroxybenzofuran	3.45	5.15	4.89	11.7	98
4-Hydroxybenzofuran	3.45	5.22	4.89	11.6	98
Salicylic acid	2.26*	8.14[f]	4.09	2.0	89
Sulfadiazine	−0.08*	4.02[f]	2.54	0.23	48
Sulfathiazole	0.05*	4.01[f]	2.62	0.93	79
Benzoic acid[e]	1.81*	6.61[f]	3.80	1.1	81
Sulfamethazine	0.27*	3.91[f]	2.77	1.5	86
Sulfamerazine	0.14*	3.61[f]	2.68	0.80	76
4-Hydroxybenzoic acid	1.51*	6.06[f]	3.59	0.45	64
4-Nitrophenol	1.96*	4.30[f]	3.89	1.6	86

[a]Values marked by an asterisk were experimentally determined; others are calculated; see Experimental Section. [b]Calculated using eq 3. [c]Milligrams adsorbed per gram of protein at c = 1 mg %. [d]Per cent of compound bound in 4% albumin solution at a free drug concentration of 1 mg %. [e]Log $1/C$ for these compounds is slightly less accurate. [f]These compounds are more than 50% ionized under experimental conditions; log $1/C$ refers to the neutral form of the molecule.

bottles. Additional buffer was added to make a standard volume. Nine-inch lengths of 29/32 "Nojax" visking cellulose tubing were cleared of extraneous ultraviolet absorption by heating to simmer in distilled water, rinsed, and knotted at one end. Albumin solution (10 ml) was introduced with a pipette. After knotting above the solution, 2 in. of tubing was cut from each end, leaving 5 in. of tubing. The end into which the protein was added was rinsed to remove a residue of albumin. Such a "sack" is placed in each bottle containing compound solution, and to one other containing the standard volume of buffer only, handling with forceps. This is a "blank" for the "protein series," and serves to measure the slight dialyzable absorption of the albumin. Before the tying sequence, the hands are washed with soap; knots are made without handling the body of the sack to prevent contamination with absorptive material present in human sweat.[17]

A similar series of bottles is prepared covering a concentration range of the compound somewhat lower than that of the protein series. Buffer only (10 ml) is knotted into 5-in. lengths of tubing, one of these is placed in each bottle, and in one with buffer only for a "blank." This "control series" serves to determine the amount of compound which adsorbs on the sack material. Both series are equilibrated overnight at 37° with mild agitation.

A volume of the original compound solution is placed in a bottle and shaken with the experiment. The ultraviolet absorption of this aliquot is compared with that of the fresh solution to give evidence against chemical breakdown. After dialysis, the dialysate solutions are read for compound concentrations at an appropriate wavelength.

To calculate the binding, the absorbance of the blank is subtracted from that of the bottle dialysates in each series, and the free compound concentration determined. The concentration times the total volume in the bottle (dialysate plus sack) gives the amount of free compound in milligrams. This figure is subtracted from the known milligrams of compound added initially to each bottle to give the milligrams adsorbed. In the case of the control series, the compound is adsorbed only on the cellulose tubing. An isotherm for this series showing the X/M *vs.* equilibrium free concentration in mg % is constructed. From this isotherm, the milligrams of compound adsorbed on the tubing at the concentration of each bottle in the protein series is read off. Subtracting this value from the number of milligrams adsorbed on both protein and sack gives the milligrams adsorbed on the protein, and the milligrams adsorbed per gram of albumin (X/M) directly. A graph of these values *vs.* the free concentration in mg % gives the isotherm for the compound on albumin, and from this the X/M value at 1 mg % and elsewhere in the concentration range. Plot of data in isotherm form provides a valuable measure of the internal consistency of the experiment.

At dialysis equilibrium, the free compound concentration is assumed to be equal on both sides of the semipermeable membrane. The increased concentration inside the sack is due to that bound on the albumin. The per cent binding is then

$$B = \frac{S - D}{S} \times 100 \tag{4}$$

where S = concentration in presence of albumin and D is free concentration in mg %. This is suitable for a specific system.

A more general equation providing for variation in albumin concentration is obtained as follows: substitute for S its equivalent $c + PY$, where c is free concentration in mg % (D), P the g % albumin, and Y the mg of compound adsorbed per gram. Then

$$B = \frac{100}{1 + \frac{c}{PY}} \tag{5}$$

This gives the per cent binding at any point in the concentration range of the isotherm for a specific concentration of protein.

The log P in Table I marked by an asterisk are experimental values. The other values were calculated using additivity principles.[18] Since π values for substituents on aniline and phenol are very similar,[13] π values from the phenol system were used to calculate the aniline derivatives. For example: $\log P_{\text{4-bromoaniline}} = \log P_{\text{aniline}} + \pi_{\text{4-Br}} = 0.90 + 1.13 = 2.03$. For the aminoacylphenones, the value of

π_{CH_3CO} was calculated from the regression equation relating aniline and benzene. From this equation the π value for the CH_3CO group is found to be 0.47 log unit greater when the substituent is on aniline than when it is on benzene ($\pi = -0.55$). Hence, log $P_{4\text{-aminoacetophenone}}$ is calculated as: $-0.55 + 0.47 + 0.90 = 0.82$. For the higher members of the series, 0.5 was added for each CH_2 unit. Log $P_{2\text{-naphthylamine}}$ was calculated by adding $\pi_{(CH)_4}$ of 1.35 to log P_{aniline}. $\pi_{(CH)_4}$ is an average from nine systems.[9] To the measured value of 2.02 for 8-hydroxyquinoline, π values were added to obtain log P for the derivatives. Log $P_{4\text{-aminobiphenyl}}$ = log P_{aniline} + log P_{benzene} = 0.90 + 2.13 = 3.03. Log P values for hydroxydibenzofurans were calculated by adding π_{OH} (−0.67) to log $P_{\text{dibenzofuran}}$ (4.12).[18]

The eight molecules at the bottom of Table I marked by *f* were not used in formulating eq 3. These compounds are more than 50% ionized at pH 7.4. The values given for log $1/C$ for these molecules are based on the amount of *neutral* compound present. Log P for these compounds is also for the neutral form of the molecule. The molecules marked by *e* have binding constants of somewhat lower accuracy than the others of Table I.

Discussion

The correlation of eq 3 is a good one, embracing almost a 1000-fold difference in affinity of the small molecules for BSA. This is about a fourfold greater range than was used in eq 1. Equation 3 accounts for about 90% of the variance ($r^2 = 0.91$) in log $1/C$. The other 10% must be split between experimental error and shortcomings of the octanol-water model. Within the limits of our model, binding is controlled by log P. No special allowance is made for the steric or electronic characteristics of the various functional groups other than that contained in log P. Binding of neutral molecules to BSA is a very nonspecific process which is not sensitive to the geometry of the substrate, differences in hydrogen-bonding ability, or the ability to form charge-transfer complexes. Only aromatic compounds were used in the formulation of eq 3; however, for eq 1 aliphatic compounds were found to fit in the same equation as aromatic ones.

Compounds **26-33**, which are marked by an asterisk in Table I, are 50% or more ionized at the experimental pH of 7.4. In order to compare these with the others of Table I, log $1/C$ is given in terms of the neutral form. Hence the rather high values for log $1/C$. In all of these instances the compounds are more poorly bound than one would expect from the log P value for the neutral form and *much* more poorly bound than one would expect from log P for the ionic form. Log P for the un-ionized form of salicylic acid is 2.26, while log P for the sodium salt is −0.85. The sodium salt has 1000 times less affinity for the octanol phase. This indicates that since these ionized compounds bind to BSA, the binding must be heavily dependent on the affinity of anions for cationic centers in BSA. Since the negative charges on the ionic forms of compounds **26-33** are delocalized in a variety of ways, it is not possible to correct for the ionic contribution to binding for such a mixed set of molecules.

The results contained in eq 1 and 3 can be compared with those of Scholtan[6] who showed that the binding of a variety of drugs to human albumin could be correlated with log P values from isobutyl alcohol. He obtained the general result

$$\log K = 0.9 \log P_{\text{isobutyl alcohol}} + \log K_p \quad (6)$$

In eq 6, K is a binding constant comparable to that of eq 1. The intercept, K_p, was found to vary with different series of drugs, being especially dependent on charge. Equation 7,

$$\log K = 0.9 \log P_{\text{isobutyl alcohol}} - 0.05 \quad (7)$$

for example, was found to hold for a set of cardenolides.

We have established[19] the relationship between log P_{octanol} and log $P_{\text{primary butyl alcohols}}$ shown in eq 8. There

	n	r	s	
$\log P_{\text{butyl alcohol}} = 0.70 \log P_{\text{octanol}} + 0.38$	57	0.993	0.123	(8)

is not a significant difference for log P values in *n*-butyl alcohol or isobutyl alcohol; hence the two types have been used to formulate eq 8. Substituting eq 8 into eq 7 gives eq 9. The slope of eq 9 is quite close to that of eq 3. One

$$\log K = 0.65 \log P + 0.3 \quad (9)$$

would expect it to be somewhat lower than eq 1 but comparable to eq 3 since the binding was studied at 37°. The slopes of linear free energy relationships decrease with an increase in temperature.[20]

The results embodied in eq 3 extend the relationship found in eq 1 to new molecules. Equation 3 also establishes the fact that the slope of the relationship between binding constants and log P decreases with increasing temperature. By means of eq 8 and 9 it appears possible to relate linear free energy relationships between various kinds of partitioning systems involving macromolecules as well as pure organic solvents.

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