The Role of Hydrophobic Bonding in the Binding of Organic Compounds by Bovine Hemoglobin*

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ABSTRACT: A study of the binding of 17 organic compounds by bovine hemoglobin has been made using the technique of equilibrium dialysis. It is shown that the process closely resembles the adsorption of organic compounds by bovine serum albumin. The binding appears to depend almost entirely on hydrophobic bonding and is linearly related to the partitioning of the compounds between 1-octanol and water. Hydrogen bonds in particular appear to be unimportant. The steric requirements of the process are nonspecific, at least within the limits studied. Representative members of the molecules studied with hemoglobin were found not to adsorb to myoglobin (whale). This fact suggests that organic compounds are probably held in the interfaces among the four subunits of the hemoglobin molecule rather than within a particular subunit.

The interaction of organic compounds with proteins is an extremely important biochemical problem with many interesting facets. One of these is the extent to which drugs or metabolically necessary compounds will be restricted in their movements in the cell or intercellular fluids such as blood. Such restriction of movement will depend on how firmly they are bound by the protein with which they come into contact. It has been our thesis that drugs as well as other organic compounds find their sites of action by a random walk process (Hansch *et al.*, 1965c). The probability of a particular drug finding its site of action in a given interval of time will be greatly reduced if it is strongly bound by the proteins or lipids it meets. Since hydrophobic bonding appears to play a most important role in the binding process, it is with this action that we are at present concerned. While our knowledge of the binding of organic substances has been greatly increased through extensive studies of both a theoretical (Klotz *et al.*, 1958) and a practical nature (Scholtan, 1964), we are still not able to make good quantitative predictions of the binding of an untested compound onto a given protein. We have become interested in this problem through studies designed to rationalize the structure–activity relationship of biologically active congeneric groups of organic compounds (Hansch and Deutsch, 1966; Deutsch and Hansch, 1966; Hansch and Fujita, 1964).

One of the most important forces holding proteins and small organic molecules together appears to be that termed hydrophobic bonding (Kauzmann, 1959). While this is a complex force made up of dispersion forces as well as the "pressure" of the aqueous phase on the lipophilic portions of the molecules, we have found that the relative strength of such bindings can be estimated using the extrathermodynamic method (Leffler and Grunwald, 1963) of Hammett. We have discovered that the way biologically active congeners bind to enzymes (Hansch *et al.*, 1965a) or to BSA[1] (Hansch *et al.*, 1965b) rather closely parallels the way they partition between octanol and water. The success of this method depends on the fact that large changes in electronic binding by members of a series are not present and that steric hindrances other than those contained in the octanol–water model do not intervene. Even when important steric and electronic forces are involved, their effects can often be separated by means of regression analysis using free-energy-based substituent constants (Hansch *et al.*, 1965a).

The partition coefficient is of course an equilibrium constant and it may be used to derive a substituent constant analogous to the Hammett σ parameter

$$\pi = \log P_X - \log P_H \qquad (1)$$

In eq 1, P_X is the partition coefficient of derivative X in a congeneric series and P_H that of the parent compound. We have come to consider π to be a measure of the hydrophobic binding power of a function.

In a study of the binding of 19 phenols by BSA (Hansch *et al.*, 1965b), the following relationship was found to hold

$$\log \frac{1}{C} = 0.681\pi + 3.483 \qquad (2)$$

where $n = 19$, $r = 0.962$, and $s = 0.133$. In eq 2, C is the molar concentration of phenol necessary to produce a 1:1 complex of phenol and protein using the equilibrium dialysis technique of Klotz (Glick, 1956).

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[1] Abbreviation used: BSA, bovine serum albumin.

The correlation coefficient is represented by r, s is the standard deviation, and n is the number of points used to derive the constants. The binding constant is proportional to $1/C$. The very good linear free energy relationship between log $1/C$ and π is indicated by the high correlation coefficient. It was found that when a term in σ was added to eq 2, no improvement in correlation occurred. This indicates that the electronic effects of the substituents have no highly specific effect not contained[2] in the parameter π. The excellent correlation shown in eq 2 also indicates that no critical steric effects involved in the fit of the phenols to the binding site are apparent. The rational results obtained with albumin encouraged us to investigate the interaction of organic compounds with another protein. Bovine hemoglobin was chosen because rather pure material is relatively inexpensive, because of its biological importance, and because of the extensive knowledge of its structure.

Materials and Methods

The hemoglobin used was doubly crystallized, electrophoretically pure material obtained from Calbiochem, Los Angeles, Calif. On dissolving in distilled water, a small amount of material ~3% (apparently denatured protein) did not go into solution and was removed by filtration. Preliminary experiments indicated that better precision in results was obtained when the hemoglobin was dialyzed against distilled water. The dialysis was allowed to run for 1 week at 0° although a shorter time might have sufficed. It was found that hemoglobin freshly made up before dialysis and hemoglobin which had been dialyzed and allowed to stand protected from the air at 0° for several weeks had the same adsorption spectra. Essentially all of the hemoglobin must have been in the unoxidized form, although for adsorption work this is probably not an important point since Schoenborn (1965) has recently shown that reduced and oxyhemoglobin (horse) as well as the methemoglobin all adsorb xenon in essentially the same way. It was found that consistent results could be obtained on different lots of hemoglobin by different individuals.

The myoglobin was also obtained from Calbiochem. It was whale skeletal muscle myoglobin of grade A quality. Before using, it was filtered and dialyzed in the same fashion used for the hemoglobin.

The procedure for determining the binding affinity was exactly the same as that employed to obtain the constants used in our albumin work (Hansch *et al.*, 1965b) except that a concentration of hemoglobin or myoglobin of 600 mg/250 ml of H_2O was used. The experiments with both hemoglobin and myoglobin were conducted in doubly distilled water without the presence of any salts or buffers. Our previous work with albumin showed the same result with buffered and distilled water solutions. Assuming a molecular weight of 64,500 (Andrews, 1964), the solution was 3.7×10^{-5} M. Because of the limited solubility of some of the compounds and the somewhat lower binding power of hemoglobin, we have used the point where the ratio of moles of bound compound/mole of hemoglobin is 0.5 for comparison rather than the point where the ratio is 1 used in our albumin work. Using 0.5 for comparison, it was possible in most examples to obtain points on either side of this value and thus avoid extrapolations. The ratio of bound compound to protein appeared to be a linear function of C in the range of ratios investigated (~0.2–1.0). The concentrations necessary to produce the 0.5 complex were found by determining the amount of compound

TABLE I: Concentrations of Organic Compounds Necessary to Produce 0.5 Ratio of Protein Complex.

Substance	Log P[a]	Obsd Log $1/C$	Calcd Log $1/C$	Δ Log $1/C$
4-Fluorophenol	1.77	3.15	3.14	0.01
4-Chlorophenol	2.39	3.70	3.55	0.15
4-Bromophenol	2.59	4.00	3.68	0.32
4-Iodophenol	2.91	3.94	3.90	0.04
4-Phenylphenol*	3.58	4.52	4.34	0.18
3-Chlorophenol	2.50	3.39	3.62	0.23
3-Trifluoromethylphenol	2.95	3.94	3.92	0.02
1-Naphthol*	2.70	3.75	3.76	0.01
4-Chloroaniline*	1.83	3.22	3.18	0.04
4-Bromoaniline	2.03	3.24	3.31	0.07
4-Nitroaniline	1.37	2.98	2.87	0.11
3-Nitroaniline	1.39	2.78	2.89	0.11
1-Naphthylamine*	2.23	3.62	3.45	0.17
4-Aminoazobenzene*	3.36	4.08	4.20	0.12
4-Nitrochlorobenzene	2.39	3.33	3.55	0.22
Naphthalene	3.37	4.19	4.20	0.01
2-Acetylnaphthalene*	2.82	3.58	3.84	0.26

[a] The values of P were determined experimentally (Fujita *et al.*, 1964), except for those marked by an asterisk. These were calculated taking advantage of the additive nature of log P (Iwasa *et al.*, 1965). For example, it was assumed that π for Cl on aniline would be the same as on phenol. Hence, log P 4-chloroaniline = log P aniline + πchlorophenol. 1-Naphthol was calculated by adding πOH (−0.67), obtained from log P phenol − log P benzene, to log P for naphthalene (3.37). The additive nature of log P can be illustrated in the calculation of log P for aminoazobenzene. One could subtract 0.5 for each methyl group in 4-dimethylaminoazobenzene (log P = 4.60, Hansch *et al.*, 1965c) to azobenzene to get 3.60. Alternatively, one could add πNH_2 (−0.46) from the nitrobenzene system to azobenzene (3.82: Fujita *et al.*, 1964) to obtain 3.36. The authors used the latter value since the closer the value of P is to 1, other factors being equal, the more accurate its value is likely to be.

[2] We have found (Fujita *et al.*, 1964) that although π is approximately constant from system to system, electronic or steric interaction of substituents may affect its value.

bound by 10 ml of aqueous hemoglobin solution at 4 to 5 concentrations. For each point it was necessary to find the amount of compound bound by the dialysis bag in order to be sure of the amount bound by the protein. It was noted that binding by the bag was roughly inversely proportional to log P, while binding by the protein was directly proportional to log P. The concentrations were then plotted against the molar ratio of compound:protein and C for the ratio of 0.5 was determined graphically. The results are given in Table I. We have made our correlations of binding *vs.* hydrophobicity using log P instead of π since we wished to study a variety of compounds rather than a single series.

Discussion

In extending our study of the interaction of organic compounds from BSA to hemoglobin, we first examined a series of eight phenols (Table I) for direct comparison with the binding of phenols by albumin (Hansch *et al.*, 1965b). The linear relationship between log $1/C$ and log P was again found to prevail. We then studied six aromatic amines and, finally, three neutral compounds. The good linear relationship between the binding constant and log P for the diverse group of 17 compounds is quantitatively shown in eq 3 which was derived by the method of least squares.

$$\log \frac{1}{C} = 0.666 \log P + 1.960 \qquad (3)$$
$$(0.11) \qquad (0.28)$$

where $n = 17$, $r = 0.941$, and $s = 0.163$. The values in parenthesis are the 90% confidence intervals. In order for better comparison of eq 2 and 3, eq 2 can be placed on a log P basis by substituting $\pi = \log P_X - 1.46$ into eq 2. The value of 1.46 is log P for phenol.

$$\log \frac{1}{C} = 0.681 \log P + 2.489 \qquad (4)$$

Comparison of eq 3 for the adsorption of 17 miscellaneous compounds by bovine hemoglobin with eq 4 for the adsorption of 19 phenols by BSA shows that the two processes are extremely close in character. The slopes for the two curves are, for all practical purposes, identical. There is a difference of 0.529 between the intercepts, indicating that BSA has a greater affinity for organic compounds than hemoglobin. The close correspondence between the slopes for the two equations indicates that the atmosphere of the protein material surrounding the bound molecules in the hemoglobin and BSA is quite similar.

While eq 3 was derived for the condition where 0.5 mole of compound was bound/mole of hemoglobin, the results for the 1:1 complex obtained by extrapolation are quite similar as shown in eq 5.

$$\log \frac{1}{C} = 0.713 \log P + 1.512 \qquad (5)$$
$$(0.11) \qquad (0.27)$$

where $n = 17$, $r = 0.950$, and $s = 0.160$.

The fact that amines, phenols, and neutral compounds are equally well accommodated by eq 3 reveals that hydrophobic bonding, as *defined* by our octanol–water model, is the most important factor in the binding process. Of special note is the good fit of the naphthalene molecule which does not have hydrogen bonding ability. Work in progress indicates that eq 4 for BSA also fits many molecules other than phenol equally well. Here, too, naphthalene and molecules with little or no hydrogen bonding character fit well. Thus, the type of binding we are considering is nonspecific for more or less neutral, nonionic organic compounds.

It is interesting that even though various kinds of amido and carbonyl groups must be in or near the binding sites, the many different functions attached to the phenols and amines are well predicted. Groups as diverse as NH_2, OH, NO_2, CN, CF_3, and $COCH_3$ all appear to be equally well fit by eq 3 and 4.

Although the binding by the two proteins parallels partitioning between octanol and water, the fact that the slopes in eq 3 and 4 are about 0.7 rather than 1 is significant. The main driving force for the formation of the hydrophobic bond is the large increase in entropy (Kauzmann, 1959) which accrues when the water envelope surrounding the apolar part of the organic compound is destroyed in its transfer to the nonpolar phase. The slopes of less than one in eq 3 and 4 indicate that the free-energy change in the transfer of a molecule from water to protein is not as great as the free-energy change from water to octanol. If we assume this to be related to ΔS rather than ΔH, according to Kauzmann, we can picture the molecules bound in the protein as being placed under more constraint than those in octanol.

Also of interest are the implications for the stereochemistry of the adsorption by the two proteins. At least within the bounds set by phenol, 4-phenylphenol, naphthol, and aminoazobenzene, no special steric hindrance to adsorption is evident. Up to this point the compounds we have studied have all been aromatic with various attached functions. These essentially planar molecules might show a different adsorption pattern than a bulky compound such as a quinuclidine derivative. Experiments to explore this aspect of protein adsorption are in progress.

In view of the recent work on the binding of xenon by whale myoglobin (Schoenborn *et al.*, 1965), we investigated the adsorption of 4-bromophenol, 1-naphthol, naphthalene, 1-naphthylamine, 4-phenylphenol, 4-aminoazobenzene, and 4-bromoaniline on whale myoglobin. These molecules were among the most strongly bound by hemoglobin. The procedure used was the same as that employed for hemoglobin except that in the case of myoglobin, the molar concentration was about four times as great because of its

lower molecular weight. Even under these conditions myoglobin did not adsorb a measurable amount of any of the above seven molecules. The test concentrations were equivalent to or, in some cases, higher than those used with hemoglobin. This result implies that the small organic molecules are held between the subunits of the hemoglobin in a hydrophobic milieu. This region or regions (the binding appears to be linear up to at least 1 mole/mole of hemoglobin) must be rather large in relation to a benzene ring. While the work of Schoenborn shows that xenon can penetrate into the myoglobin molecule and is thus firmly bound, the molecules we have investigated are apparently too large to enter easily.

The above picture which emerges for the binding of organic compounds by hemoglobin is quite similar to that suggested for serum albumin (Weber and Young, 1964). Weber and Young have presented evidence that bovine serum albumin is made up of a number of globular subunits. On peptic hydrolysis, BSA is broken into the subunits which are shown to have greatly reduced binding power. Weber and Young have concluded that binding by the intact BSA probably occurs on the interfaces of the subunits.

The fact that hemoglobin binds organic compounds in proportion to their lipophilic character has a most important bearing on how such compounds are stored and distributed in the animal body. This is particularly important in the case of drugs. While a large amount of work has been carried out studying the hemolysis of red cells by organic compounds, only a few isolated unsystematic studies of the binding by hemoglobin have been made (Schanker *et al.*, 1964). It has been assumed that hemolysis indicates entry of compounds into the cell. Jacobs (1931), in an extensive survey of the subject, pointed out that there is no authenticated case where a lipoid-soluble substance has been shown to be unable to enter the erythrocyte. In a more recent study it was shown (Hutchinson, 1952) using ^{14}C-labeled alcohols that methanol and butanol enter the red cell at a rate too fast to measure. Equilibrium appeared to be reached in less than 60 sec. Since all of the evidence available indicates that organic compounds enter the red cell readily, and since we have shown them to be bound to hemoglobin in much the same fashion as to albumin, hemoglobin must be considered as a most important agent along with albumin in the transport and storage of lipophilic molecules. The large amount of hemoglobin present makes it particularly important. In fact, in the human, hemoglobin is present in more than four times the amount of serum albumin (Fruton and Simmons, 1958).

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References

Andrews, P. (1964), *Biochem. J. 91*, 222.

Deutsch, E. W., and Hansch, C. (1966), *Nature* (in press).

Fruton, J. S., and Simmons, S. (1958), *General Biochemistry*, 2nd ed, New York, N. Y., Wiley, p 163.

Fujita, T., Iwasa, J., and Hansch, C. (1964), *J. Am. Chem. Soc. 86*, 5175.

Glick, D. (1956), *Methods Biochem. Analy. 3*, 265.

Hansch, C., and Deutsch, E. W. (1966), *Biochim. Biophys. Acta* (in press).

Hansch, C., Deutsch, E. W., and Smith, R. N. (1965a), *J. Am. Chem. Soc. 87*, 2738.

Hansch, C., and Fujita, T. (1964), *J. Am. Chem. Soc.* 86, 1616.

Hansch, C., Kiehs, K., and Lawrence, G., (1965b), *J. Am. Chem. Soc. 87*, 5770.

Hansch, C., Steward, A. R., Iwasa, J., and Deutsch, E. W. (1965c), *Mol. Pharmacol. 1*, 207.

Hutchinson, C. (1952), *Arch. Biochem. Biophys. 38*, 35.

Iwasa, J., Fujita, T., and Hansch, C. (1965), *J. Med. Chem. 8*, 150.

Jacobs, M. H. (1931), *Ergeb. Biol. 7*, 1.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 37.

Klotz, I. W., Ayers, J., Ho, J. Y. C., Horowitz, M. G., and Heiney, R. E. (1958), *J. Am. Chem. Soc. 80*, 2132.

Leffler, J. E., and Grunwald, E. (1963), Rates and Equilibria of Organic Reactions, New York, N. Y., Wiley.

Schanker, L. S., Johnson, J. M., and Jeffrey, J. J. (1964), *Am. J. Physiol. 207*, 503.

Schoenborn, B. P. (1965), *Nature 208*, 760.

Schoenborn, B. P., Watson, H. C., and Kendrew, J. C. (1965), *Nature 207*, 28.

Scholtan, W. (1964), *Arzneimittel-Forsch. 14*, 1234.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 1415*, 1424.