A New Model for the Binding of Flexible Ligands to Proteins*

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ABSTRACT: The interactions between substituted alkanes and proteins are analyzed by a new theoretical model, developed in the previous papers of this series. This model takes into account the ability of flexible molecules to bind in a large number of configurations (including many in which not all segments of the bound molecule are in contact with the surface) and therefore differs from the theory of multiple equilibria.

When such configurational flexibility is considered, the ligands will bind neither independently nor in identical configurations, even when all of the binding sites are chemically the same, so that flexible ligands cannot be expected to bind in accordance with the single-class model of the theory of multiple equilibria. The nonindependence of the ligands is responsible for a new type of negative cooperativity. It is found that biphasic isotherms, such as those observed in many experiments, are obtained only if the binding region includes a fairly large number of nonpolar sites (which interact with the hydrocarbon segments of the ligand) as well as ionic sites (which bind the ionic head group). Furthermore, with this new theory, it is possible to explain the experimental observation that the strength of binding increases with the chain length of the ligand, while the theory of multiple equilibria predicts the opposite chain-length dependence. And finally, the model is used to illustrate the limited nature of the conclusions which can be drawn from class analyses of experimental isotherms.

In this communication, a new theoretical model is presented for the analysis of the binding of small molecules to proteins. In contrast to the commonly used theory describing multiple-binding equilibria (Klotz, 1953; Steinhardt and Reynolds, 1969), this model takes into account the effects of ligand flexibility when chain-like molecules bind to a protein. The conformational entropy contributions arising from such flexibility may alter the equilibrium properties of protein–ligand systems considerably.

In the commonly used theory of multiple equilibria (Klotz, 1953; Steinhardt and Reynolds, 1969), it is assumed that all ligand molecules are bound to the protein with equal intrinsic binding constants (or can be grouped into distinct classes, within each of which the intrinsic binding constants are equal). Furthermore, the internal degrees of freedom of the bound ligands are neglected and therefore it must be assumed that all ligands in a given class bind in an identical manner. Given these assumptions, it can be shown (Klotz, 1953; Steinhardt and Reynolds, 1969) that if a maximum of M_i ligands is bound in the ith class, each with an intrinsic association constant, $k_{\text{int}}^{(i)}$, then the average number of ligands bound per protein molecule, $\bar{N}$, is related to the concentration of free ligand, c, by the equation

$$\bar{N} = \sum_{i=1}^{n} \frac{M_i k_{\text{int}}^{(i)} c}{1 + k_{\text{int}}^{(i)} c} \qquad (1)$$

where n is the number of classes.

The above assumptions of the theory of multiple equilibria may be reasonable for small ions and other rigid, monofunctional molecules (Figure 1a). However, many ligands encountered in protein binding studies (i) are composed of several functional groups, not all of which have to be in contact with the protein simultaneously, and (ii) possess internal flexibility due to the possibility of rotation about single bonds, even when bound (Figure 1b). Because of factor i, it is more realistic to consider that the binding of such ligands occurs through interaction with several sites, where each site is defined as an area on the protein surface which serves as a point of attachment for a single functional group, or *segment*, of the ligand in question. This definition also allows an investigation of binding to protein surfaces which contain functional groups differing chemically. As a result of factor ii, a ligand molecule can bind in a large number of configurations, including many in which not all segments of the ligand are in contact with the protein (*i.e.*, desorbed loops and free ends may occur).

The present model is based on an explicit consideration of both factors cited. The details of the theoretical derivations, as well as an analysis of the physicochemically significant results, are presented elsewhere (Laiken and Némethy, 1970a,b). The main features of the theory are summarized here, followed by a discussion of the conclusions of biochemical interest.

It is assumed that the protein surface may be represented by a regular lattice of binding sites, each of which binds a single ligand segment, and that the number of configurations of the bound ligand molecules can be determined by counting the number of possible arrangements on the lattice. The configurations may be enumerated most simply if each bound molecule is represented in the partition function according to its state of adsorption (Roe, 1965), S, which describes the distribution of adsorbed and desorbed segments along the ligand.

$$S = \{d_0, s_1, d_1, s_2, d_2, \ldots . s_{l-1}, d_{l-1}, s_l, d_0'\} \qquad (2)$$

Here s_i and d_i are the number of segments in the ith adsorbed stretch and desorbed loop, respectively, d_0 and d_0' are the number of segments in the two free ends of the ligand, and l is the number of adsorbed stretches. The partition function for the system is

$$\Xi = \sum_{N=0}^{M} \frac{1}{N!} \left(\prod_{i=0}^{N} q(i) \right) e^{\mu N/kT} \qquad (3)$$

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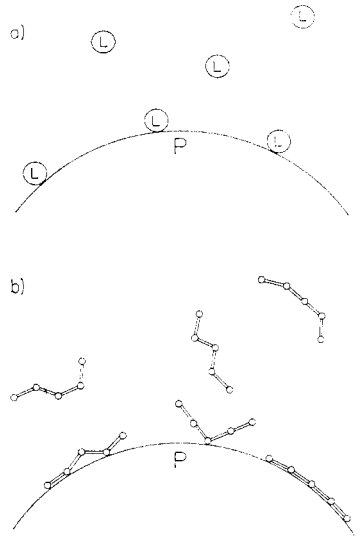

FIGURE 1: Schematic illustration of the binding of various ligands to a protein. (a) Small ions and other rigid ligands (L) bind to a protein (P) with no changes in configuration. (b) Flexible ligands may assume a large number of configurations in solution and in the bound state.

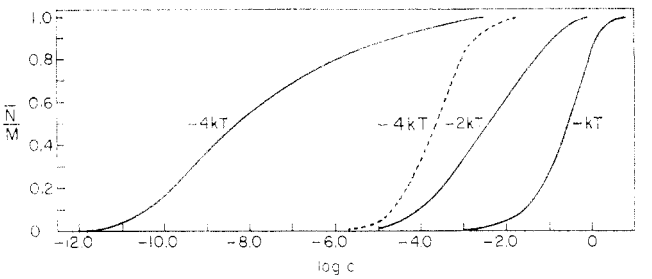

FIGURE 2: Binding isotherms for a homogeneous binding surface. The curves represent the binding of ligands with eight segments to a surface containing 162 binding sites, all of identical chemical composition. $\bar{N}/M$ is the fractional saturation of the surface (M = 20). The solid curves represent binding isotherms for the site–segment interaction energies ϵ indicated on the graph. If the ligands bind rigidly as in the theory of multiple equilibria, the dashed curve is obtained for the site–segment interaction energy $\epsilon = -4kT$.

where M is the maximum number of ligands which can bind to the protein (the value of M is determined by the size of the lattice and the number of segments in the ligand), μ is the chemical potential of the ligand, and $q(i)$ is the partition function for the ith bound ligand. The latter may be evaluated by assigning a statistical weight, $t(S)$, to each state of adsorption

$$q(i) = \sum_{\{S\}} t_i(S) \tag{4}$$

where the summation is over all possible states of adsorption for a given chain length. $t_i(S)$ is a count of all configurations available to the ith ligand with a given state of adsorption and also contains Boltzmann weighting factors corresponding to the number and kind of protein site–ligand segment contacts formed. From the partition function, it is possible to calculate the average number of ligands bound per protein molecule, $\bar{N}$ ($\bar{N} = kT\,[(\partial \ln \Xi)/\partial\mu]$), as a function of the concentration of free ligand, c (corresponding to an experimental binding isotherm) equilibrium constants for the binding of successive ligands, and parameters describing the average configuration of the bound ligands (such as the fraction of segments on the surface, number and length of loops, etc.; for details, see Laiken and Némethy, 1970a).

Formally, the model bears some similarity to the statistical-mechanical theories of polymer adsorption at a surface (Silberberg, 1962; Roe, 1965). However, it differs from the latter through the inclusion of several features to make the lattice a suitable representation of a compactly folded protein molecule. A two-dimensional lattice with uniform coordination number at each site (*e.g.*, $z = 6$) would correspond to an infinite planar surface. By introducing a small number of sites with a lower coordination number (*e.g.*, $z = 5$), it is possible to modify the lattice so that it can be folded over the surface of a polyhedron (Caspar and Klug, 1962). If twelve pentacoordinated lattice points are introduced on a hexacoordinated lattice at regular intervals, an icosahedron is obtained (Caspar and Klug, 1962), which is assumed to represent the surface of an approximately spherical protein. The total number of hexacoordinated sites can be varied by changing the extent of subdivision of each face of the icosahedron. With this lattice, the mathematical treatment readily permits the use of sites which differ chemically (Laiken and Némethy, 1970a). Thus, the lattice sites may have ionic, polar, or nonpolar character, providing a reasonable representation of a protein surface. Similarly, the ligand may contain segments of a variable chemical nature (*e.g.*, long-chain carboxylic acids and alkyl sulfates consist of one ionic and several nonpolar segments). If the entire surface of the protein is not available for binding, the partition function easily can be modified (Laiken and Némethy, 1970a) to describe a small patch of binding sites which then could be placed on a surface of any shape. The model in its present form treats the surface of the protein molecule as invariant, *i.e.*, it is assumed that no changes in protein conformation occur upon ligand binding. If such changes can occur, a different surface lattice would have to be used for each conformation. The theory could be modified to include conformational changes, but only with an increase in the number of parameters which would render verification uncertain. Multilayer binding of ligands, although not included in the calculations described here, also could be considered by a modification of the theory.

The flexibility of the ligands and the fact that the lattice can accommodate several ligand molecules result in phenomena which differ from those expected if the commonly used theory of multiple equilibria (Klotz, 1953; Steinhardt and Reynolds, 1969) were applicable to such systems.

Nonindependence of Bound Ligand Molecules. The availability of vacant binding sites on the protein is reduced more strongly with increasing saturation than the space available to desorbed segments. As a result, configurations of the ligand with a larger fraction of desorbed segments are preferred for large $\bar{N}$. Thus, the binding of successive ligands does not occur independently and the average configuration of the bound ligand changes as additional ligands are bound, even though the site–segment interaction energy remains the same. This is the case for homogeneous binding surfaces on which all sites are chemically equivalent as well as for heterogeneous binding surfaces.

When homogeneous binding surfaces are treated in the commonly used theory of multiple equilibria, it is assumed that all ligands bind independently and have an identical, rigid configuration when bound, so that the equilibrium constants for the binding of successive ligands differ only by a statistical factor which accounts for the different ways of arranging the ligands on the protein. As a result, the binding

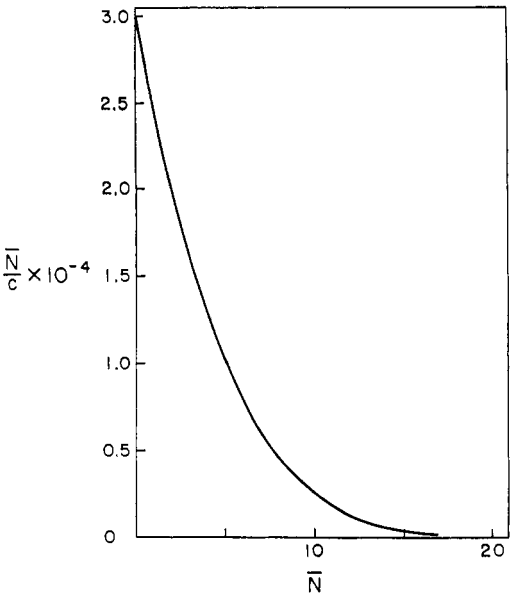

FIGURE 3: Scatchard plot of binding data calculated with the present model. The curve shown here corresponds to the binding isotherm for $\epsilon = -2kT$ in Figure 2. The plot is not linear although the binding surface is homogeneous.

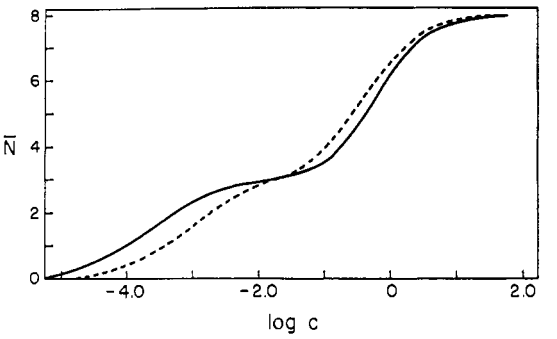

FIGURE 4: Isotherms for the binding of a five-segment carboxylic acid to a region on a protein molecule containing 42 lattice sites of the following composition: 3 ionic and 39 nonpolar sites (——); 3 ionic, 12 polar, and 27 nonpolar sites (---), with polar and ionic sites located near each other.

isotherms (expressed as $\bar{N}$ *vs.* log c) for such systems are symmetrical about their midpoints, and the Scatchard and double-reciprocal plots are linear (Klotz, 1953; Steinhardt and Reynolds, 1969). On the other hand, in the present model the equilibrium constants for the binding of successive ligands vary in a manner which reflects the changing configuration of the bound ligands as well as simple statistical considerations. Therefore, the binding isotherms for homogeneous surfaces calculated using the present model are not symmetrical (Figure 2), but are broader at high levels of saturation, as discussed in the next section. As a result of this asymmetry, the Scatchard plots fail to be linear (Figure 3). In other words, flexible ligands usually cannot be expected to conform to the simple, single-class model of the theory of multiple equilibria (*cf.*) discussion below.

Negative Cooperativity. The assymmetry of the calculated binding isotherms (Figure 2) results from the above-mentioned fact that the binding of successive ligands does not occur independently. Thus, the decrease in the number of vacant surface sites as additional ligands are bound reduces the number of configurations available to subsequently bound ligands and makes their binding more difficult, broadening the isotherm as saturation is approached. Since the lattice contains a sufficient number of sites to bind M ligands, simple steric hindrance is not involved for $\bar{N} < M$. This flattening of the calculated binding curves, due to the first ligand molecules decreasing the affinity of the vacant sites for subsequent ligands, can be referred to as *negative cooperativity* because of its formal similarity to the broadening of enzyme–substrate binding curves (Levitzki and Koshland, 1969). However, the negative cooperativity of the present model arises from the interdependence of the configurational entropies of the bound ligands and therefore differs from the more familiar forms of negative cooperativity attributed either to conformational changes in multisubunit proteins (allosteric effects; see Koshland *et al.* (1966) and Levitzki and Koshland (1969)) or to direct energetic interaction between the ligands (such as the electrostatic effects in multiple ionization; see Klotz, 1953).

In contrast to the other types of negative cooperativity, the negative cooperativity of the present model becomes more pronounced when the interaction energy between the lattice sites and the ligand segments becomes stronger (Figure 2). With a more favorable energy of interaction, the first ligands bind preferentially in configurations with a larger fraction of their segments adsorbed, causing a more rapid depletion of the vacant surface sites.

Binding to Chemically Heterogeneous Surfaces. If the present model is used to represent the interaction of a protein with a given flexible ligand, the lattice must include sites which differ chemically, representing the functional groups commonly found on proteins. As an illustrative example, one may consider the binding of fatty acids to a protein surface which contains a small number of ionic sites (*e.g.*, ϵ-amino groups), which bind only the carboxyl segment of the ligand (with an energy $\epsilon = -5.0$ kcal/mole), and a fairly large number of nonpolar sites, which bind only the hydrocarbon segments (with a free energy of -0.65 kcal/mole of CH_2 groups).[1] A biphasic binding isotherm is obtained (Figure 4), similar to those observed experimentally for the substituted alkanes (Goodman, 1958). In the first phase of the isotherm, the ligands bind with the carboxyl segment as well as some hydrocarbon segments attached. The intermediate plateau occurs at a value of $\bar{N}$ equal to the number of ionic sites (*i.e.*, saturation of the latter) but only partial saturation of the nonpolar sites. At higher ligand concentrations, saturation of the remaining nonpolar sites is possible, with the ligands binding in configurations with a free carboxyl end.

Biphasic isotherms also are obtained following the introduction of a small number of sites representing polar groups (such as those of the peptide backbone), which can bind both types of ligand segments (with $\epsilon = -2.7$ to -1.5 kcal per mole for charged segments and -0.08 to -0.23 kcal per mole for CH_2 groups). If the number of such sites is small, there is little change in the general features of binding, although the separation between the two phases of the isotherm is reduced (Figure 4). However, if a sufficiently large number of polar sites is introduced (for example, if less than one-half of the lattice sites are nonpolar sites), the intermediate plateau disappears almost entirely. Therefore, that biphasic isotherms have been observed experimentally for several substituted

[1] Details of the arrangements of the sites on the lattice and a derivation of the energy values used is presented elsewhere (Laiken and Némethy, 1970b).

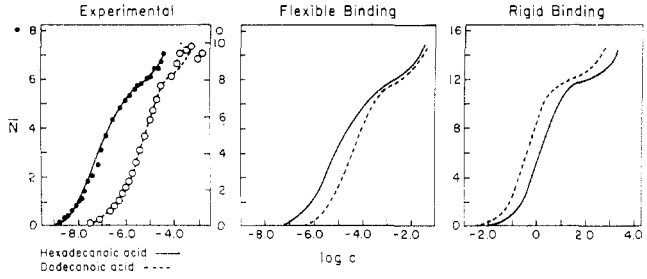

FIGURE 5: Comparison of experimental isotherms (left) for the binding of hexadecanoic (palmitic) and dodecanoic (lauric) acids to serum albumin (Goodman, 1958) with the isotherms calculated using the present model for flexible binding (center) and the theory of multiple equilibria, in which the ligands bind rigidly (right). To facilitate the comparison of the graphs, the ordinates have been chosen so that all intermediate plateaus occur at the same vertical level.

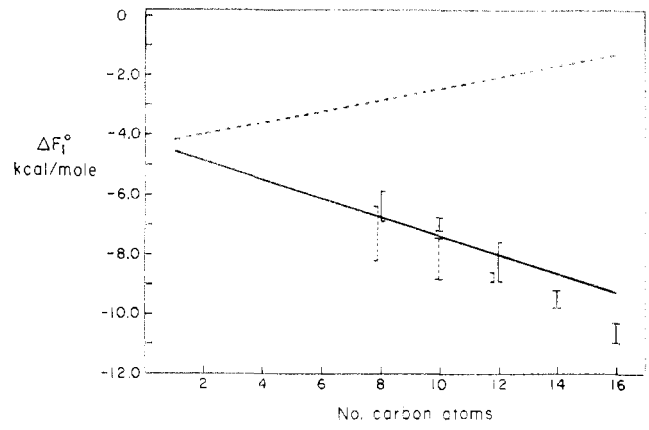

FIGURE 6: The variation in ΔF_1°, the change in free energy for the binding of the first ligand, with the number of carbon atoms in the ligand. In the present model for flexible binding, ΔF_1° decreases with increasing chain length (——), as observed experimentally for both the carboxylic acids (**I**) and the alkyl sulfates (**I**) binding to serum albumin. The bars indicate the ranges of the experimental values reported in the literature (references are listed in the text). In contrast, in the theory of multiple equilibria ΔF_1° increases as the chain length of the ligand increases (---). The results with the present model and the theory of multiple equilibria were calculated with a lattice containing 12 ionic sites and 150 nonpolar sites.

alkanes (Goodman, 1958) is a significant result if binding data are to be used to provide clues about the chemical nature of the binding region, since a biphasic isotherm will not be obtained unless the binding region includes a fairly large number of nonpolar sites as well as ionic sites.

Effect of Ligand Chain Length. Only the present model can explain the experimental observation (Karush and Sonenberg, 1949; Teresi and Luck, 1952; Goodman, 1958; Ray *et al.*, 1966; Reynolds *et al.*, 1967, 1968; Spector *et al.*, 1969) that the strength of binding increases with the chain length of the ligand (Figure 5). In contrast, the commonly used theory of multiple equilibria (Klotz, 1953; Steinhardt and Reynolds, 1969), which implicitly assumes rigid binding of the ligands, predicts a decrease in the strength of binding with increasing ligand chain length. In the latter, the neglect of the internal degrees of freedom of the bound ligand implies that all configurational entropy is lost upon binding, resulting in a large configurational entropy loss per segment which dominates the free-energy change per segment. Furthermore, the isotherms obtained with the assumption of rigid binding occur at concentrations several orders of magnitude above those observed experimentally, whereas the positions of the isotherms of the present model are much closer to those actually observed.

A quantitative demonstration of the chain-length dependence is presented in Figure 6, in which ΔF_1°, the change in free energy for the binding of the first ligand, is plotted as a function of the number of carbon atoms in the ligand. In the present theory, in which the flexibility of the bound ligands is included, ΔF_1° becomes more negative with increasing chain length, as observed experimentally for the binding of carboxylic acids and alkyl sulfates to serum albumin (Karush and Sonenberg, 1949; Teresi and Luck, 1952; Goodman, 1958; Ray *et al.*, 1966; Reynolds *et al.*, 1967, 1968; Spector *et al.*, 1969). In contrast, if it is assumed that the ligands bind rigidly, as in the theory of multiple equilibria, ΔF_1° increases with increasing chain length. The latter result is obtained by assuming that all ligand segments contact the surface of the protein but that the ligands lose all configurational entropy upon binding (Laiken and Némethy, 1970a). Thus, the existence of multiple contacts between the ligand and the protein is *not* sufficient in itself to produce an increase in the strength of binding with increasing chain length, as suggested by Steinhardt and Reynolds (1969).

The slope of the experimental ΔF_1° *vs.* chain-length curve is somewhat steeper than that of the curves calculated using the present theory. The most plausible explanation for this discrepancy (Laiken, 1970) is that all configurations of the unbound ligand have been equally weighted in the theory. Actually, some preference for compactly folded configurations is expected, which would minimize unfavorable water–hydrocarbon contacts. Such folding would reduce the number of configurations of the free ligand in solution. Therefore, less entropy would be lost upon binding, causing ΔF_1° to become more negative. This effect would be larger for the longer ligands, resulting in a steeper slope for the calculated curves. A considerably more negative value for the strength of the CH_2 segment–nonpolar site interaction also would cause the slope to become steeper, but this cannot be justified on physical grounds (Laiken and Némethy, 1970b).

Limitations of Class Analyses of Binding Data. The isotherms of the present model may be used to demonstrate potential errors that may arise from an uncritical use of class analyses in the application of the theory of multiple equilibria. In the upper graph of Figure 7, the theoretical curve represents an isotherm calculated with the present model when octanoic acid binds to a lattice on which 12 ligands bind in the first phase of the isotherm and 20 bind in the second phase. Using a nonlinear least-squares algorithm (Laiken and Printz, 1970), the best two-class fit of this isotherm according to the theory of multiple equilibria (eq 1) was obtained with classes containing 11 and 23 members, which correspond approximately to the number of ligands binding in each phase of the isotherm. Although not illustrated, a somewhat better fit is possible with three classes, simply because of the additional statistical degrees of freedom. In this case, the 11 members of the high-energy class are divided into two new classes. Similarly, when experimental data have been analyzed using a three-class fit (Goodman, 1958), the sum of the first two classes was found to correspond approximately to the position of the intermediate plateau. The latter most likely represents the number of ionic sites in the binding region, and the division of the high-energy class into two classes appears to have no physical significance.

However, the above analysis of a calculated isotherm was performed on a complete isotherm in which the plateau of the second phase of binding actually was observed. Experimental data for binding in the absence of protein conformational changes (to which the present model is applicable) usually are available only for that short portion of the second phase immediately following the intermediate plateau region (see Figure 5). Therefore, it is of interest to consider the analysis of an incomplete isotherm. The latter can be formed from the calculated curves of the present model by cutting off a large portion of the second phase, as in the bottom graph of Figure 7. A two-class fit of this truncated isotherm gives classes containing 12 and 66 members. In other words, although the size of the first class still corresponds to the number of ligands bound in the first phase of the isotherm, the size of the second class bears *no relationship whatsoever* to the number of ligands actually bound in the second phase. A class which consists of a large number of members, representing the binding of ligands in the second phase, has been postulated by several workers from analyses of such incomplete isotherms (Teresi and Luck, 1952; Goodman, 1958; Spector *et al.*, 1969) and is probably a very misleading estimate of the actual number of ligands which could bind in the second phase. Therefore, the only physically significant information which can be obtained from a class analysis of incomplete biphasic isotherms is the number of ionic sites, and this quantity could, in many cases, be obtained simply from the position of the intermediate plateau.

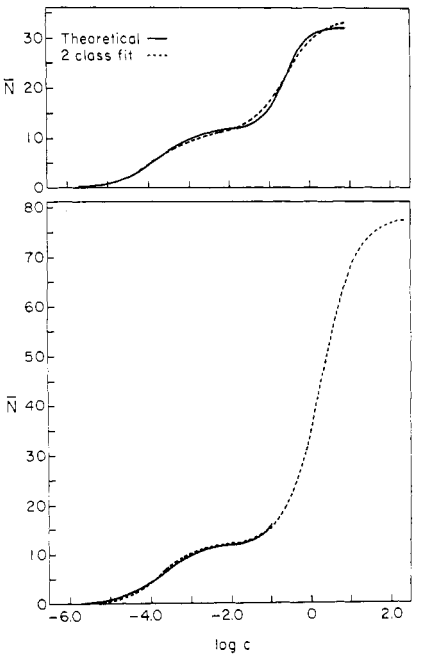

FIGURE 7: Class analysis of an isotherm calculated using the present model, as discussed in the text.

Discussion

The most important application of the model presented here is the calculation of binding isotherms for protein–ligand interactions. It should be noted that the calculation of a single isotherm by the theory which matches a particular experimental isotherm is neither an adequate test of the model nor an indication that the assumed size of the surface lattice and the arrangement of the various types of lattice sites resemble the actual protein surface. In general, a given experimental isotherm can be reproduced by various assumptions about the surface lattice in the present model and by other theoretical models which consider ligand flexibility (Laiken, 1970). This is because the manner in which the surface is represented, as opposed to the important feature of ligand flexibility, has relatively little effect on the most significant results and conclusions (Laiken and Némethy, 1970b). However, the presence of certain features in binding data (such as biphasic isotherms) may provide some general information about the nature of the protein surface (such as the *relative number* of nonpolar and ionic sites; see Laiken and Némethy, 1970b).

The extent of occupancy of the various classes of chemically different sites may be calculated as a function of the saturation of the surface (Laiken and Némethy, 1970a). Since occupancy of a site may alter its reactivity, the study of changes in the titration behavior of ionizable groups or reactivity of other functional groups as a result of ligand binding would be an additional application of the model discussed here. Differences between the relative changes in reactivity of such groups and the fractional saturation of the surface, both as functions of ligand concentration, could be used to verify the prediction that the average configuration of the bound ligands changes with saturation.

Applicability of the Present Theory and the Theory of Multiple Equilibria. The commonly used theory of multiple equilibria (Klotz, 1953; Steinhardt and Reynolds, 1969), in which the ligands bind in a rigid configuration, can be regarded as a special case of the model discussed here (Laiken and Némethy, 1970a). In the binding of flexible ligands, the theory of multiple equilibria might be applicable in certain special circumstances. (i) If only a small patch of sites is available for the binding of the ligand segments, only one configuration of the fully adsorbed ligand may exist. A linearly arranged set of nonpolar sites of limited length, surrounded by polar sites, is a possible example. However, the ligand still could form loops, which would have to be treated by the present, more general model. (ii) If an array of binding sites not only is limited in size, but is situated in a "groove," loop formation might be negligible since the site–segment interaction energies would be larger when the interacting groups are partially shielded from the solvent. (iii) Each ligand may be isolated from the others when bound. For example, the protein surface might contain several well-separated patches of nonpolar sites. This would enable the ligands to bind independently, in the sense of this paper, so that the theory of multiple equilibria might be used. However, the number of configurations available to the flexible alkyl chain of the bound ligand probably would be different from that for the free ligand.

A good test to determine which theory is applicable in a given experimental system is provided by the comparison of the binding of several ligands, especially those belonging to a homologous series. In this case, the predictions of the present theory and those of the theory of multiple equilibria differ markedly, as discussed above (Figures 5 and 6) and in detail elsewhere (Laiken and Némethy, 1970b). For the binding of substituted alkanes to serum albumin, only the present model is able to predict the correct dependence of the strength of binding on the chain length of the ligand.

References

Caspar, D. L. D., and Klug, A. (1962), *Cold Spring Harbor Symp. Quant. Biol. 27*, 1.

Goodman, D. S. (1958), *J. Amer. Chem. Soc. 80*, 3892.

Karush, F., and Sonenberg, M. (1949), *J. Amer. Chem. Soc. 71*, 1369.
Klotz, I. M. (1953), *Proteins 1*, 727.
Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Laiken, N. (1970), Ph.D. Thesis, The Rockefeller University.
Laiken, N., and Némethy, G. (1970a), *J. Phys. Chem. 74*, 4421.
Laiken, N., and Némethy, G. (1970b), *J. Phys. Chem. 74*, 4431.
Laiken, S., and Printz, M. (1970), *Biochemistry 9*, 1547.
Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.
Ray, A., Reynolds, J. A., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2607.
Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6*, 937.
Reynolds, J. A., Herbert, S., and Steinhardt, J. (1968), *Biochemistry 7*, 1357.
Roe, R.-J. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 50.
Silberberg, A. (1962), *J. Phys. Chem. 66*, 1872, 1884.
Spector, A. A., John, K., and Fletcher, J. E. (1969), *J. Lipid Res. 10*, 56.
Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic Press.
Teresi, J. D., and Luck, J. M. (1952), *J. Biol. Chem. 194*, 823.

Microviscosity and Order in the Hydrocarbon Region of Micelles and Membranes Determined with Fluorescent Probes. I. Synthetic Micelles*

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ABSTRACT: The viscosity in micelle interiors, termed here as microviscosity, was derived from an adequate comparison of the degree of fluorescence depolarization of perylene or 2-methylanthracene when dissolved in the tested micelles and in American white oil U. S. P. 35. The latter was used as a reference system of known viscosities. In the series studied, lauryltrimethylammonium bromide, myristyltrimethylammonium bromide, cetyltrimethylammonium bromide (CTABr), and stearyldimethylbenzylammonium bromide, the determined microviscosities at 27° are all in the range of 17–50 cP. The change in microviscosity with temperature in this series was found to follow a simple exponential form with an activation energy in the range of 6.1–9.6 kcal mole^{-1}. Added salts affected only slightly the microviscosity values. Mixed micelles of perylene-labeled CTABr with cetyl alcohol or cholesterol and with sodium 1-hexadecanesulfonate, were used to test the effect of charge isolation and charge neutralization on the fluidity of the micelle interior. The microviscosity of these mixed micelles was found to increase rapidly with concentration of the admixed component, and at a molar ratio close to 1:1 microviscosities of several poises were obtained. The changes in apparent rotational diffusion with wavelength of excitation indicate that the depolarizing rotations are strongly anisotropic. In-plane rotations in perylene are ten times faster than out-of-plane rotations, independently of the medium (micelles, propylene glycol at −14°, propylene glycol–glycerol at 4°). This indicates that the resistance to the motion in the micelles must be close to isotropic. A summary of the findings presented leads to the conclusion that micelle interiors are similar in nature to aliphatic hydrocarbon solvents.

In general, the fluorescence emitted from molecules which are dispersed in a viscous medium is partially polarized. This is customarily expressed in terms of molecular anisotropy, r, or degree of polarization, p, which are measured at right angle to a polarized excitation beam and are defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

When $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed through a polarizer oriented parallel and perpendicular to the plane of polarization of the excitation beam. For a rotating fluorescent sphere the observed r or p values obey the well known Perrin (1926) equation in which r_0 and p_0 are the values

$$\frac{r_0}{r} = \frac{\frac{1}{p} - \frac{1}{3}}{\frac{1}{p_0} - \frac{1}{3}} = 1 + \frac{6R_s}{\lambda} \tag{2}$$

of r and p when the emitting molecules maintain their orientation excitation and emission (*e.g.*, in a very viscous solvent), R_s is the rate of rotation of the sphere and λ is the rate of fluorescence emission. The term r_0/r is defined here as the degree of depolarization.

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