and water (14:1:3, v/v/v) for 24 hr at room temperature. The unprotected hexadecanucleotide showed a trace of faster traveling impurity in solvent E (R_f values are shown in Table I). The spectral properties of d-T(pTpApCpT)$_3$pTpApC were λ_{max} 263 mμ, λ_{min} 234 mμ, and $\epsilon_{280}/\epsilon_{260} = 0.60$ in water.

Removal of Monomethoxytrityl Group from d-MMTr-TpTpApC Using Acetic Acid–Pyridine Buffer. Ammonium d-MMTr-TpTpApC (70 OD_{260} units) was dissolved in 0.5 ml of a mixture of acetic acid, pyridine, and water (14:1:3). Aliquots (0.05 ml) were taken at different time intervals and evaporated with pyridine. The residue was analyzed by paper chromatography in solvent A. Half-life of the compound was found to be 5 hr at room temperature After 24 hr d-TpTpApC was practically the only nucleotidic compound detected in solvent A. The spot was eluted, treated with concentrated ammonia at 37° for 12 hr, and chromatographed in solvent E. Again a single spot was observed. When the above acetic acid–pyridine treatment was prolonged to 5 days, again only a single product corresponding to d-TpTpApC (solvents A and E) was observed, and no evidence of depurination was obtained. After 10 days at room temperature, the formation of new minor ultraviolet-absorbing products corresponding, presumably, to the removal of adenine was observed (solvents A and E).

Allosteric Linkage

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Abstract: For any system there exists a binding potential, $\mathcal{J}$, which is a function of the chemical potentials, μ, of all the components present save one, the reference component. This has the property that $\partial\mathcal{J}/\partial\mu_i = n_i$, where n_i is the amount of component i per unit of reference component. In the case of a macromolecule which exists in several allosteric conformations all in equilibrium with one another, $\mathcal{J}$ assumes the special form given by eq 1.5 or 1.6, becoming what we call the allosteric binding potential. This considerably simplifies the discussion of the macromolecule and predicts various key features of its behavior in its reactions with ligands. It leads at once to the concept of allosteric linkage, a type of linkage which arises exclusively from the prevalence of equilibrium between the various conformations, independently of whether these, by themselves, show any linkage effects at all. Allosteric linkage, when heterotropic, may be either positive or negative; when homotropic, it is always positive (or cooperative). In the case where the macromolecule contains only a single site for a ligand X, equilibrium between the various conformations has the result that the ligand equilibrium curve for X necessarily assumes the form of a simple titration curve. When the number of sites is greater than one, the situation is of course more complex. In such cases, however, the median ligand activity of the macromolecule as a whole, which gives the total work of saturating it with ligand, may be expressed very simply in terms of the median ligand activities of the various forms by eq 4.3 or 4.4 The introduction of the allosteric binding potential clarifies the whole concept of homotropic linkage in an allosteric macromolecule and leads to a sharper distinction between the true and apparent interaction free energy. It likewise clarifies the concept of heterotropic linkage and the regulation to which it can give rise. In particular, it shows that the potential fineness of allosteric control of an enzyme by its activators and inhibitors increases with the number of sites for them in the macromolecule (eq 6.4; see also section 10c). Moreover it brings out the fact that whenever the interactions are allosteric in origin the shape of the ligand equilibrium curve for a given ligand cannot be invariant for changes of the ligand activity of the control ligands, nor, in general, will the curve be symmetrical. (An exception is of course the one- or two-site case.) Finally, the introduction of the binding potential leads to an expression for the heat of combination of the macromolecule with a ligand, which shows how this quantity depends on the heats of the various allosteric transitions (eq 9.3). An analysis of the ligand equilibria of hemoglobin in the light of these principles shows that they are not inconsistent with the idea that the various interactions displayed, both the heterotropic and homotropic ones, are predominantly allosteric in origin. This leads, almost perforce, to the conclusion that the major part of the interactions arises within the $\alpha\beta$ subunits. The interactions between these subunits, whether in the same or different molecules, though of decisive importance in producing the observed values of the Hill interaction parameter, n, are much smaller.

Previous discussions of linkage[1-3] have been pitched on the most general note, without heed for mechanism. Now, in view of mounting interest in allosteric transitions as a possible source of regulation in enzymes, and indeed in working proteins generally, the time would seem ripe for a more detailed analysis, directed specifically at those linkage effects, commonly known as allosteric effects, which arise from the prevalence of equilibrium between different conformational forms in a macromolecule. The task is greatly lightened by the introduction of an expression for the binding potential of the macromolecule, which we shall call the *allosteric binding potential.* It is with this that we begin; we end, as usual, with a discussion of hemoglobin, which provides an incomparable test body for all such ideas.

1. The Allosteric Binding Potential

In order to introduce the concept of the allosteric binding potential a few words about the binding potential in general are called for by way of orientation. The binding potential is a thermodynamic concept which applies to any system but is particularly relevant to a macromolecule containing a number of interacting sites for several different ligands.[3] It is closely related to the grand canonical partition function of statistical

(1) J. Wyman, *Advan. Protein Chem.*, 4, 407 (1948).
(2) J. Wyman, *ibid.*, **19**, 223 (1964).
(3) J. Wyman, *J. Mol. Biol.*, **11**, 631 (1965).

mechanics, although, being a wholly macroscopic concept, it is more general. Let P be any one of the various thermodynamic potentials and let its first partial derivatives with respect to the composition variables, for constant values of the physical variables, be denoted by μ. Then it can be shown that there exists a corresponding potential, Π, which is a function of all the μ except one, namely that corresponding to whichever component is chosen as reference component, and which has the property that

$$n_i = \frac{\partial \Pi}{\partial \mu_i}$$

where n_i is the amount of component i in the system per unit of reference component. This potential, Π, we call the binding potential, designating it by the Russian character corresponding to Roman L. Each n_i given in this way may be regarded as the amount of component i "bound" by the reference component. We enclose the word "bound" by quotation marks, because of the very general sense in which it is used, for it is not restricted to material which enters into chemical combination with the reference component but includes as well all other forms which may be present. However, when the amount of nonchemically bound material may be neglected, or allowed for, as is often the case when the reference component is a macromolecule possessing many ligand binding sites, then the word may with good approximation be taken in its more familiar and literal sense. It is under these conditions that the relationship between the binding potential and the grand partition function is clearest. If, in particular, P is identified with the Gibbs free energy, then of course the μ_i's become the same as the ordinary chemical potentials, and each μ_i, except for a constant depending on the choice of the standard state, may be replaced by $RT \ln x$, where x is the corresponding activity. By resorting to second derivatives we arrive at once at a variety of useful linkage relations which describe the interrelations of function in the macromolecule, and the introduction of the binding potential leads to the concept of functional maps which clarify the notions of linkage groups, linked functions, and linked sites. With this brief introduction we now proceed to the formulation of an expression for the binding potential of an allosteric macromolecule, or, as we may say, for the allosteric binding potential.

Suppose that a macromolecule M exists in a number of discrete conformational forms 1, 2, ..., r, all in equilibrium with one another. The problem is to derive an expression for the total binding potential, Π, of the macromolecule in terms of the individual binding potentials, Π_i, of the various forms. Let us arbitrarily choose one form, say 1, as a reference form and denote by L_i' the ratio of any other form i to this form. This means that L_i' is the equilibrium constant for the transition from form 1 to form i and of course implies that $L_1' = 1$. Further, let us denote by $\bar{X}$ the total amount of any ligand X bound per mole of macromolecule, and by μ_x and x the chemical potential and activity of X, respectively. Finally, let ν_i' be the fraction of the molecules present in form i, *i.e.*

$$\nu_i' = L_i'/\Sigma L_i' \qquad (1.1)$$

Then[3]

$$\bar{X} = \frac{\partial \Pi}{\partial \mu_x} = \frac{\partial \Pi}{RT \partial \ln x} = \sum_{i=1}^{r} \nu_i' \bar{X}_i = \sum_{i=1}^{r} (L_i' \bar{X}_i / \sum_{i=1}^{r} L_i') \qquad (1.2)$$

But

$$\bar{X}_i = \frac{\partial \Pi_i}{RT \partial \ln x} \qquad (1.3)$$

Moreover

$$L_i' = L_i e^{(\Pi_i - \Pi_1)/RT} \qquad (1.4)$$

where L_i is a constant which gives the value of L_i' when $(\Pi_i - \Pi_1) = 0$. It follows that

$$\Pi = RT \ln \sum_{i=1}^{r} L_i e^{\Pi_i/RT} \qquad (1.5)$$

as may be verified by differentiation.

By writing the expression in this way, we avoid giving arbitrary priority to any one of the forms. It is to be noted, however, that one of the L's must always be equal to 1, namely that corresponding to whichever form is chosen as the reference form.

Since addition of a constant to Π makes no difference, the summation in eq 1.5 may be divided by ΣL_i, with the result

$$\Pi = RT \ln \sum_{i=1}^{r} \nu_i e^{\Pi_i/RT} \qquad (1.6)$$

Here, it should be especially emphasized that the ν's are constants which give the values of the ν''s when each $L_i' = L_i$; they may be interpreted as mole fractions and as such are subject to the condition $\Sigma \nu_i = 1$.[4]

Equations 1.5 and 1.6 may be compared with the corresponding expressions for the binding potential when there is no equilibrium between the forms; these are simply

$$\Pi = \sum_{i=1}^{r} \left(\frac{L_i}{\Sigma L_i} \Pi_i \right) \qquad (1.7)$$

and

$$\Pi = \sum_{i=1}^{r} \nu_i \Pi_i \qquad (1.8)$$

2. Allosteric Linkage

Let us fix our attention on two ligands X and Y. Suppose that each Π_i is the sum of two terms, Π_{ix} and Π_{iy}, one a function of x and the other a function of y. This means that there is no direct linkage between X and Y in any one form or in the whole system in the absence of equilibrium between the forms. When, however, there *is* equilibrium between the forms, then unless either Π_{ix} or Π_{iy} is the same for all the forms, it will be impossible to separate Π in eq 1.5 or 1.6 into

(4) It should be noted that the possibility of expressing the allosteric binding potential in this simple way results from the fact that the allosteric transitions involve no change of molecular weight, *i.e.*, no association or dissociation. In a dissociating system the situation is much more complex, owing to the fact that the ratios of the forms are no longer directly identifiable with equilibrium constants. For example, if any one of the forms is a t-mer of form 1, then it will not be possible in general to write an *explicit* analytical expression for the binding potential, since this would involve the solution of a tth degree equation.

the sum of two potentials, one a function of x and the other a function of y, and there will be a linkage of the two ligands mediated by the conformational equilibria. Such linkage we call *allosteric linkage*.

The situation may perhaps be clearer if we express each Π_i in terms of a polynomial N_{xy} in x and y, as is always possible with better or poorer approximation.

$$\Pi_i = RT \ln N_{xy} \qquad (2.1)$$

In general, if the number of X binding sites is t and the number of Y binding sites s, N_{xy} will be of the form

$$\sum_{i=0}^{t} \sum_{j=0}^{s} K_{ij} x^i y^j$$

where the K's are constants, K_{00} being equal to 1. When there is no direct linkage between X and Y, then N_{xy} will be factorable into the product of two polynomials, one in x and one in y.

$$N_{xy} = N_x N_y = (1 + K_1 x + \ldots + K_t x^t)(1 + M_1 y + \ldots + M_s y^s) \qquad (2.2)$$

Equation 1.5 then becomes

$$\Pi = RT \ln \Sigma L_i N_{ix} N_{iy} \qquad (2.3)$$

and eq 1.6 becomes

$$\Pi = RT \ln \Sigma \nu_i N_{ix} N_{iy} \qquad (2.4)$$

If and only if either N_x or N_y is the same for all values of i will Π degenerate into the sum of two terms, one a function of x and one a function of y. This is illustrated by the case where there are only two forms and where the polynomials are both of first degree. Then

$$\Pi = RT \ln [\nu_1(1 + K_1 x)(1 + M_1 y) + \nu_2(1 + K_2 x)(1 + M_2 y)] = RT \ln [1 + (\nu_1 K_1 + \nu_2 K_2)x + (\nu_1 M_1 + \nu_2 M_2)y + (\nu_1 K_1 M_1 + \nu_2 K_2 M_2)xy]$$

The condition of factorability is

$$(\nu_1 K_1 + \nu_2 K_2)(\nu_1 M_1 + \nu_2 M_2) = \nu_1 K_1 M_1 + \nu_2 K_2 M_2$$

which, introducing $\nu_1 + \nu_2 = 1$, yields

$$M_1(K_2 - K_1) = M_2(K_2 - K_1)$$

It is evident that factorability requires either

$$M_1 = M_2 \text{ or } K_1 = K_2$$

This condition is both necessary and sufficient, and, except in this degenerate case, the allosteric equilibrium must give rise to heterotropic interactions. These may be either positive or negative, depending on whether K_2/K_1 and M_2/M_1 are both either greater or less than 1 or whether one is greater than 1 and the other less.[5]

Apart from degenerate cases, allosteric equilibrium also leads inevitably to homotropic interactions. Suppose the activity of ligand Y is held constant and that in each of the r conformations the t X binding sites are all independent. Then eq 2.4 becomes

$$\Pi = RT \ln \sum_{i=1}^{r} \nu_i (1 + k_{i1}x)(1 + k_{i2}x) \ldots (1 + k_{it}x) \qquad (2.5)$$

(5) As in previous discussions the term heterotropic is used here for interactions between sites which bind different ligands; homotropic is used for interactions between sites which bind the same ligand.

Although the individual polynomials are completely factorable, their summation is not, and therefore the system must display *stabilizing* homotropic interactions which arise from the conformational equilibria;[3] however, the degree of the polynomial remains equal to t, and therefore the maximum value of the Hill parameter, n, cannot exceed t, as of course we know from more general considerations.[6]

As an example to show that the summation cannot be completely factored, consider the simple case recently discussed by Monod, Wyman, and Changeux[7] where there are only two forms and where all the k's for each form are the same. Then the summation in eq 2.5 becomes

$$\nu_1(1 + k_1 x)^t + \nu_2(1 + k_2 x)^t$$

which gives

$$1 + t(\nu_1 k_1 + \nu_2 k_2)x + \frac{t(t-1)}{2}(\nu_1 k_1^2 + \nu_2 k_2^2)x^2 + \ldots$$

In order that the polynomial be completely factorable, it is necessary that the ratio of *each* coefficient to the preceding one be equal to or less than its statistical value,[8] *e.g.*, $(\nu_1 k_1 + \nu_2 k_2)^2 > \nu_1 k_1^2 + \nu_2 k_2^2$. But,

(6) This parameter is by definition the slope at any point of a Hill plot in which $\ln (\bar{x}/(1 - \bar{x}))$ is represented as a function of $\ln x$ (or μ_x), $\bar{x}$ being the fractional saturation of the macromolecule with ligand X. It is useful as an index of the interaction energy which is realized in saturating the macromolecule with ligand (see section 5).

(7) J. Monod, J. Wyman, and J. P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).

(8) This principle, being so nearly self-evident, was given without accompanying proof (ref 3, footnote to p 640). It is so fundamental, however, to the whole discussion of linkage that we take this occasion to present one. Consider the expression $(1 + kx)^t$. When expanded this gives rise to a polynomial in which, by definition, the coefficients K have their statistical values ($K_i = k^i t!/i!(t - i)!$). Now modify one of the factors $1 + kx$ to $1 + ukx$, where u may be either greater or less than 1. The resulting polynomial may be written in the form

$$1 + (1 + \eta)tkx + \ldots + \frac{t!}{i!(t-i)!}(1 + i\eta)k^i x^i + \frac{t!}{(i+1)!(t-i-1)!}[1 + (i+1)\eta]k^{i+1}x^{i+1} + \ldots$$

where $\eta = (u - 1)/t$. Now, when the coefficients have their statistical values

$$\frac{K_{i+1}}{K_i(K_1/t)}\frac{t-1}{i+1} = 1$$

In the present case this ratio is $[1 + (i + 1)\eta]/[(1 + \eta)(1 + i\eta)]$. The difference between the denominator and numerator will be found to be $i\eta^2$, which is greater than 0. Consequently the ratio in question is less than 1. With the aid of the principle of mathematical induction the argument may be extended to the case where *all* the factors are made different.

We also take the occasion to make clear that by stabilizing (or positive, or cooperative) interactions we mean interactions which have the effect of decreasing the work required to saturate the macromolecule with ligand. If the sites are independent of one another, this work has a certain value, and, as we have just proved, the coefficients of the polynomial for the binding potential satisfy a certain inequality. Destabilizing (or negative, or anticooperative) interactions, since they act wholly to increase this work, must strengthen this inequality for every value of i. Consequently, if the inequality is violated for *any* value of i, at least some of the sites must be subject to stabilizing interactions. On the other hand, some stabilizing interactions, provided they are small enough in relation to other effects, can be present without showing up in this way. Thus violation of the inequality is a sufficient, but not a necessary, condition to establish the existence of stabilizing interactions. Commonly stabilizing interactions show up in values of the Hill parameter, n, which are greater than 1; nevertheless, though stabilizing interactions always lead to an increase of n above the value which it would otherwise have, they do not necessarily raise it above unity. All this will become clearer as the discussion proceeds.

There is a further point. As we have seen, the allosteric binding potential is always expressible in terms of a polynomial of the same degree as the polynomials for the various conformational forms. This

taking account of the fact that $\nu_1 + \nu_2 = 1$, it is easy to show that this requires $(k_1 - k_2)^2 \leqq 0$, a condition which will only be fulfilled in the degenerate case where $k_1 = k_2$, *i.e.*, where the two conformations are indistinguishable in respect to the binding of X.

It should be realized that when the r polynomials N_{xy} have certain factors in common, these may be removed and then appear as an additional term (or terms) in the expression for the binding potential in accordance with eq 1.5 or 1.6. Moreover, it is superfluous to assume that the individual binding potentials are themselves factorable. If they are not, then of course the system is subject to linkage effects in addition to those arising from the allosteric equilibria.[9]

3. A Unique Case

As a special case consider a system which contains only one X binding site in each of r conformations (or allosteric forms). Then

$$\Pi = RT \ln \sum_{i=1}^{r} \nu_i (1 + K_i x) N_{iy} = RT \ln \sum_{i=1}^{r} (\nu_i N_{iy} + \nu_i K_i N_{iy} x) \quad (3.1)$$

For any value of y the polynomials N_{iy} are constants. Consequently, if we are considering only the binding of X, we may write eq 3.1 as

$$\Pi = RT \ln \left(1 + \frac{\Sigma \nu_i N_{iy} K_i x}{\Sigma \nu_i N_{iy}}\right) \quad (3.2)$$

This is identical with the binding potential of a single site molecule which exists in only one conformation, except that the single constant K is replaced by

$$\bar{K} \equiv \frac{\Sigma \nu_i N_{iy} K_i}{\Sigma \nu_i N_{iy}} \quad (3.3)$$

$\bar{K}$ may be regarded as a weight average of the K_i's, the weighting factors being $\nu_i N_{iy}$. In this particularly simple case the Hill parameter, n, acquires the value of exactly 1 as a result of the allosteric equilibria.

It is interesting to compare the ligand equilibrium curves for the system in the presence and in the absence of the allosteric equilibria. Figure 1 represents the case where there are only two allosteric forms, of rather widely different affinities for X. Curve a (equilibrium present) corresponds to eq 3.2, curve b (equilibrium absent) to eq 1.7 or 1.8. The introduction of equilibrium gives rise to a complete changeover from a two-step curve to a single-step curve with the Hill parameter, $n = 1$. As will be seen below, this simple "all or nothing" behavior stands in contrast to the more complex behavior of a system containing several X binding sites.

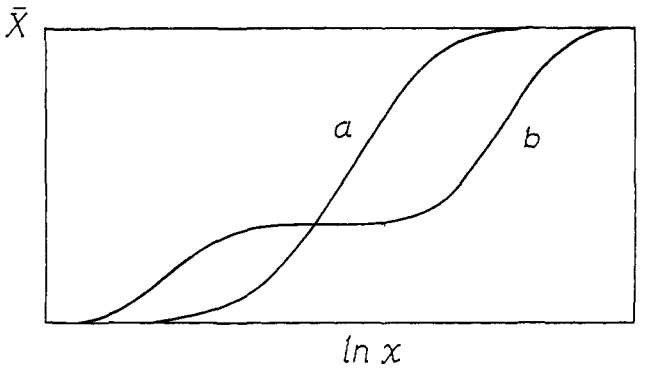

Figure 1. Ligand equilibrium curve of a molecule which contains a single site for a ligand X and which exists in two conformational forms: curve a, with conformational equilibrium; curve b, without conformational equilibrium (see text).

Equation 3.3 shows the way in which $\bar{K}$ varies with y. Since when $y = 0$ each $N_{iy} = 1$, $\bar{K}_0$ (the value of $\bar{K}$ when $y = 0$) is given by

$$\bar{K}_0 = \Sigma \nu_i K_i$$

Consequently eq 3.3 may also be written as

$$\bar{K} = \bar{K}_0 \frac{\Sigma \nu_i N_{iy} K_i}{(\Sigma \nu_i N_{iy})(\Sigma \nu_i K_i)} \quad (3.4)$$

This may be compared with the expression for K in the simple one-site, one-conformation case when there is direct interaction between X and Y. Here the binding potential is

$$\Pi = RT \ln (N_y' + N_y K_0 x)$$

which gives

$$K = K_0 N_y / N_y' \quad (3.5)$$

When the various Y binding sites are independent, this becomes

$$K = K_0 \frac{(1 + m_1 y)(1 + m_2 y) \ldots}{(1 + m_1' y)(1 + m_2' y) \ldots} \quad (3.6)$$

In contrast, the right-hand member of eq 3.4 will not in general be factorable in this way.

4. The Median Ligand Activity

In dealing with ligand binding by a macromolecule, it is often convenient to introduce the concept of the median ligand activity.[2] This is defined as that value x_m of the ligand activity x such that

$$\int_{x=0}^{x_m} \bar{X} \, d \ln x = \int_{x_m}^{\infty} (t - \bar{X}) \, d \ln x \quad (4.1)$$

where t is the number of X binding sites. It is a direct measure of the *total* work, ΔF, done in saturating the macromolecule with ligand.

$$\Delta F = tRT \ln x_m \quad (4.2)$$

The median ligand activity can be given a very simple formulation with the aid of the binding poten-

results from the prevalence of the allosteric equilibria. It is worth noting that, when the equilibria are suppressed, it is no longer possible to formulate the binding potential in this way. This follows at once from eq 1.8, which leads to the result

$$\Pi = RT \ln \prod_i (1 + K_{i1} x + \ldots + K_{it} x^t)^{\nu_i}$$

The ν_i's are of course, as mole fractions, all less than unity. Similarly it is clear that, when a macromolecule dissociates, the binding potential cannot be expressed in terms of a polynomial of any degree, since the ν_i's then involve the solution of quadratic or higher degree equations. See footnote 4.

(9) Throughout this discussion we have assumed a discrete number of conformations, corresponding to a finite value of r. By increasing r indefinitely it would seem that it should be possible, formally at least, to extend the principle of allosteric linkage to embrace the case where there is a continuous transformation of the conformation of the macromolecule as the ligand activity increases.

tial. In terms of the binding potential eq 4.1 becomes

$$\frac{1}{RT}\int_{x=0}^{x_m}\frac{\partial \mathcal{J}}{\partial \ln x}d\ln x = t\ln x\Big|_{x_m}^{\infty} - \frac{1}{RT}\int_{x_m}^{\infty}\frac{\partial \mathcal{J}}{\partial \ln x}\,d\ln x$$

whence

$$t\ln x\Big|_{x_m}^{\infty} = \frac{1}{RT}\mathcal{J}\Big|_{x=0}^{\infty}$$

If we express $\mathcal{J}$ in terms of a polynomial[10]

$$\mathcal{J} = RT\ln(1 + K_1x + \ldots + K_tx^t)$$

this gives

$$t\ln x\Big|_{x_m}^{\infty} = \ln(1 + K_1x + \ldots + K_tx^t)\Big|_{x=0}^{\infty}$$

which may be written as

$$-\ln x_m{}^t = 0 + \lim_{x\to\infty}\ln\left(\frac{1 + K_1x + \ldots + K_tx^t}{x^t}\right) = \ln K_t \quad (4.3)$$

Consequently

$$\left(\frac{1}{x_m}\right)^t = K_t \quad (4.4)$$

from which it follows that at the median point of the equilibrium curve the concentrations of the completely liganded and completely unliganded forms of a macromolecule are the same.

In the case of an allosteric system, where

$$\mathcal{J} = RT\ln(\nu_1N_1 + \nu_2N_2 + \ldots + \nu_rN_r) \quad (4.5)$$

eq 4.4 becomes

$$\left(\frac{1}{x_m}\right)^t = \nu_1K_{1t} + \nu_2K_{2t} + \ldots + \nu_rK_{rt} = \nu_1\left(\frac{1}{x_{m1}}\right)^t + \nu_2\left(\frac{1}{x_{m2}}\right)^t + \ldots + \nu_r\left(\frac{1}{x_{mr}}\right)^t \quad (4.6)$$

In words, the reciprocal of $x_m{}^t$ is the average of the reciprocals of the several $x_{mi}{}^t$ values.

In the event that one of the forms contains a smaller number of X binding sites than the others, the corresponding value of K_t is zero (the corresponding $x_m = \infty$) and that form contributes nothing to the value of x_m for the system as a whole except through the values of the ν for the other forms.

Equation 4.6 enables us to explore the effect of the interactions resulting from the allosteric equilibria on the work required to saturate the macromolecule with ligand X. Since, as we have seen, these interactions are always of a stabilizing kind, it is clear, in a general way, that they must act to decrease this work, but it is worthwhile to analyze the situation in detail. Consider first the case where there are only two allosteric forms, 1 and 2, each of which contains t X binding sites. Then

$$\frac{1}{x_m{}^t} = \frac{\nu_1}{x_{m1}{}^t} + \frac{\nu_2}{x_{m2}{}^t}$$

The total work of saturating the macromolecule is therefore

$$\Delta F = RT\ln x_m{}^t = -RT\ln\left(\frac{\nu_1}{x_{m1}{}^t} + \frac{\nu_2}{x_{m2}{}^t}\right) \quad (4.7)$$

In the absence of the allosteric transition the work would be, by eq 1.7 and 1.8

$$\Delta F^* = RT\nu_1\ln x_{m1}{}^t + RT\nu_2\ln x_{m2}{}^t \quad (4.8)$$

The difference between the two may be written as

$$\Delta F^* - \Delta F = RT\ln\left[x_{m1}{}^{\nu_1 t}x_{m2}{}^{\nu_2 t}\left(\frac{\nu_1}{x_{m1}{}^t} + \frac{\nu_2}{x_{m2}{}^t}\right)\right] \quad (4.9)$$

If we make use of the relation

$$\nu_1 + \nu_2 = 1$$

the expression in brackets becomes

$$\left(\frac{x_{m2}{}^t}{x_{m1}{}^t}\right)^{\nu_2}\left[1 + \nu_2\left(\frac{x_{m1}{}^t}{x_{m2}{}^t} - 1\right)\right]$$

Consequently, introducing

$$z \equiv x_{m1}/x_{m2}$$

$$\Delta F^* - \Delta F = RT\ln\left[\frac{1 + \nu_2(z^t - 1)}{z^{\nu_2 t}}\right] \equiv RT\ln\lambda \quad (4.10)$$

In the two limiting cases where $\nu_2 = 0$ or $\nu_2 = 1$, $\lambda = 1$ for all values of z; also, in the special case where $z = 1$, $\lambda = 1$ for all values of ν_2. In all these events $\Delta F^* - \Delta F = 0$. Otherwise λ is always greater than 1 and therefore $\Delta F^* - \Delta F > 0$. This is apparent from a graph of the numerator and denominator of the expression for λ plotted against z^t. Clearly we need consider only values of $z \geqq 1$, since the case of $z < 1$ becomes the same as that of $z > 1$ when the roles of the two conformations are interchanged. Figure 2 shows such a graph. Curve a, a straight line of slope ν_2, is for the numerator, curve b, uniformly concave downward and of slope ν_2 at $z^t = 1$, is for the denominator. Except in the limiting cases, $\nu_2 = 0$ and $\nu_2 = 1$, curve a always lies above curve b.

We emphasize that $\Delta F^* - \Delta F = RT\ln\lambda$ gives the decrease in the work required to saturate the macromolecule with ligand which results from the allosteric transition between the two conformations. Since λ is always greater than 1, this is always positive.

(10) Although there are good grounds, on the basis of the mass law, for doing this, the procedure might seem to limit the generality of the results which follow. Actually all that is assumed in the argument given here is that when x (and μ) $\to \infty$, the amount of X bound by the macromolecule approaches t, the number of sites, and that when $x \to 0$, the amount bound goes to zero. Then, for large values of x

$$\frac{1}{RT}\frac{\partial \mathcal{J}}{\partial \ln x} \to t \qquad (x \to \infty)$$

whence

$$\mathcal{J} = RT\ln x^t + \text{constant} = RT\ln K_tx^t$$

On the other hand, for small values of x

$$\frac{1}{RT}\frac{\partial \mathcal{J}}{\partial \ln x} = 0$$

whence

$$\mathcal{J} = \text{constant}$$

Since the value of the constant makes no difference, it may be set equal to zero, which corresponds to a value of 1 for the constant term of the polynominal. This procedure produces 4.3.

Although the proof just given is based on a value of $r = 2$, the principle may readily be extended to any value of r. To do so we have only to treat $(r - 1)$ of the conformations formally as a single conformation characterized by a median ligand activity given by

$$\frac{1}{x_m{}^t} = \left(\frac{\nu_1}{x_{m1}{}^t} + \ldots + \frac{\nu_{r-1}}{x_{m(r-1)}{}^t}\right) \Big/ (\nu_1 + \ldots + \nu_{r-1})$$

and repeat the reasoning just employed, making use of the principle of mathematical induction.

When there is another ligand Y for which there are s sites, the polynomial in the expression for the binding potential, whether for the macromolecule as a whole or for each conformation, is of the form

$$\sum_{i=0}^{t} \sum_{j=0}^{s} K_{ij} x^i y^j$$

where $K_{00} = 1$. In this case, in order to obtain the median ligand activity x_m or y_m, we resort to a procedure which we may call normalizing the polynomial. This consists in reducing the leading term to unity when either x or y is treated as the sole variable; it reduces the polynomial to the standard form for determining the corresponding median activity. Thus if x is taken as the variable we divide the polynomial by the sum of all the terms which are of zero degree in x, thereby normalizing it in x; if y is taken as the variable, we divide by the sum of all the terms which are of zero degree in y, thereby normalizing it in y. Normalization in x yields

$$\frac{1}{x_m{}^t} = \frac{K_{t0} + K_{t1}y + \ldots + K_{ts}y^s}{K_{00} + K_{01}y + \ldots + K_{0s}y^s} \qquad (4.11.1)$$

Normalization in y yields

$$\frac{1}{y_m{}^s} = \frac{K_{0s} + K_{1s}x + \ldots + K_{ts}x^t}{K_{00} + K_{10}x + \ldots + K_{t0}x^t} \qquad (4.11.2)$$

These two equations show how the work of saturating the macromolecule with either ligand varies with the activity of the other. When they are written in logarithmic form and differentiated, they yield the two familiar results

$$\frac{\mathrm{d} \ln x_m}{\mathrm{d} \ln y} = \frac{\bar{Y}_{x\to\infty} - \bar{Y}_{x\to 0}}{s} \qquad (4.12.1)$$

$$\frac{\mathrm{d} \ln y_m}{\mathrm{d} \ln x} = \frac{\bar{X}_{y\to\infty} - \bar{X}_{y\to 0}}{t} \qquad (4.12.2)$$

It is of interest to consider the two limiting values of x_m corresponding to $y = 0$ and $y \to \infty$; likewise those of y_m corresponding to $x = 0$ and $x \to \infty$. Since $K_{00} = 1$ it follows at once that these are

$$\left(\frac{1}{x_m{}^t}\right)_{y=0} = K_{t0} \qquad \left(\frac{1}{x_m{}^t}\right)_{y\to\infty} = \frac{K_{ts}}{K_{0s}} \qquad (4.13.1)$$

$$\left(\frac{1}{y_m{}^s}\right)_{x=0} = K_{0s} \qquad \left(\frac{1}{y_m{}^s}\right)_{x\to\infty} = \frac{K_{ts}}{K_{t0}} \qquad (4.13.2)$$

We verify that

$$\left(\frac{1}{x_m{}^t}\right)_{y=0}\left(\frac{1}{y_m{}^s}\right)_{x\to\infty} = \left(\frac{1}{y_m{}^s}\right)_{x=0}\left(\frac{1}{x_m{}^t}\right)_{y\to\infty} \qquad (4.14)$$

which means that it makes no difference, in saturating the macromolecules, in which order we introduce the ligands, as of course we know from general principles. The total work of doing so is

$$\Delta F = RTt \ln (x_m)_{y=0} + RTs \ln (y_m)_{x\to\infty} = RTs \ln (y_m)_{x=0} + RTt \ln (x_m)_{y\to\infty} \qquad (4.15)$$

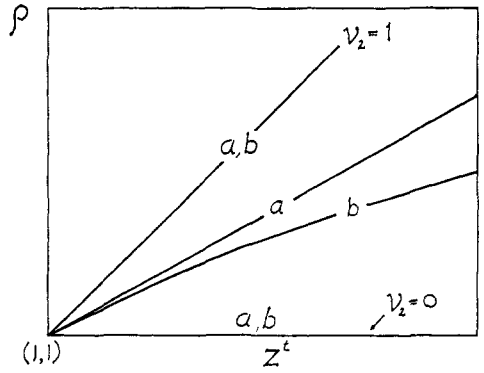

Figure 2. Graph of numerator (a) and denominator (b) of the ratio λ in eq 4.10.

It should be realized that eq 4.11, 4.13, 4.14, and 4.15 apply equally well either to any one of the allosteric forms or to the system as a whole and are independent of whether or not the interactions are all of allosteric origin. It is of interest, however, to consider specifically the behavior of the system as a whole in the special case where the polynomial for each of the forms is factorable into a polynomial in x and a polynomial in y, the heterotropic interaction energies being exclusively allosteric. Then

$$N_i = N_{ix}N_{iy}$$

where

$$N_{ix} = 1 + K_{i1}x + \ldots + K_{it}x$$

and

$$N_{iy} = 1 + M_{i1}y + \ldots + M_{is}y^s$$

In this case eq 4.11.1 becomes

$$\frac{1}{x_m{}^t} = \frac{\sum_{i=1}^{r} \nu_i N_{iy} K_{it}}{\sum_{i=1}^{r} \nu_i N_{iy}} \qquad (4.16)$$

When $y = 0$ this yields

$$\left(\frac{1}{x_m{}^t}\right)_{y=0} = \sum_{i=1}^{r} \nu_i \left(\frac{1}{x_{mi}{}^t}\right)_{y=0} \qquad (4.17.1)$$

and, when $y \to \infty$

$$\left(\frac{1}{x_m{}^t}\right)_{y\to\infty} = \frac{\sum_{i=1}^{r} \nu_i \left(\frac{1}{y_{mi}{}^s}\right)_{y=0}\left(\frac{1}{x_{mi}{}^t}\right)_{y=0}}{\sum_{i=1}^{r} \nu_i \left(\frac{1}{y_{mi}{}^s}\right)_{x=0}} \qquad (4.17.2)$$

In both cases $(1/x_m{}^t)$ is an average, but in one the weighting factors are simply the ν_i; in the other, $\nu_i(1/y_{mi}{}^s)_{x=0}$.

Corresponding expressions hold for $1/y_m{}^s$.

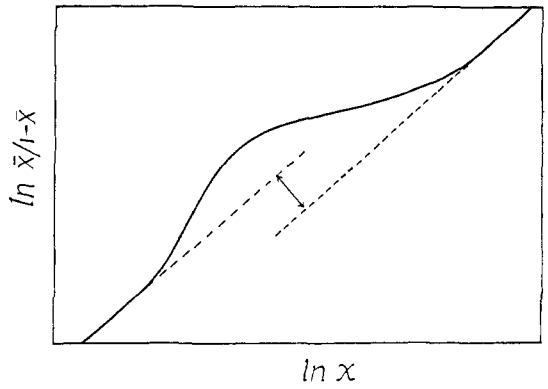

Figure 3. A hypothetical Hill plot.

Equations 4.17.1 and 4.17.2 lead to the result

$$\left(\frac{1}{x_m{}^t}\right)_{y\to\infty}\left(\frac{1}{y_m{}^s}\right)_{x=0} = \left(\frac{1}{x_m{}^t}\right)_{y=0}\left(\frac{1}{y_m{}^s}\right)_{x\to\infty} = \sum_{i=1}^{r}\nu_i\left(\frac{1}{x_{mi}{}^t}\right)_{y=0}\left(\frac{1}{y_{mi}{}^s}\right)_{x=0} \quad (4.18)$$

Here the left-hand and middle members give, each equally well, the total work, ΔF, of saturating the macromolecule with *both* ligands, as formulated in eq 4.15; the right-hand member gives the average value of

$$\left(\frac{1}{x_{mi}{}^t}\right)_{y=0}\left(\frac{1}{y_{mi}{}^s}\right)_{x=0}$$

the weights being $\nu_1, \nu_2, \cdots, \nu_r$.

If there were no allosteric transitions, *i.e.*, if the system were frozen in its unliganded state, the mole fractions of the various forms maintaining their initial values, then the work of saturating the macromolecule with both ligands would be

$$\Delta F^* = RT\sum_{i=1}^{r}\nu_i \ln (x_{mi}{}^t)_{y=0} + RT\sum_{i=1}^{r}\nu_i \ln (y_{mi}{}^s)_{x=0} \quad (4.19)$$

Let us suppose for the moment that there are only two conformations ($r = 2$). Then

$$\Delta F^* - \Delta F = RT \ln \nu_1 x_{m1}{}^t y_{m1}{}^s + RT \ln \nu_2 x_{m2}{}^t y_{m2}{}^s + RT \ln \left[\frac{\nu_1}{x_{m1}{}^t y_{m1}{}^s} + \frac{\nu_2}{x_{m2}{}^t y_{m2}{}^s}\right] = RT \ln (x_{m1}{}^t y_{m1}{}^s)^{\nu_1}(x_{m2}{}^t y_{m2}{}^s)^{\nu_2}\left(\frac{\nu_1}{x_{m1}{}^t y_{m1}{}^s} + \frac{\nu_2}{x_{m2}{}^t y_{m2}{}^s}\right) \quad (4.20)$$

In this equation each x_m refers to the value corresponding to $y = 0$ and each y_m to the value corresponding to $x = 0$. If we replace $x_{m1}{}^t y_{m1}{}^s$ by $x_{m1}{}^t$ and $x_{m2}{}^t y_{m2}{}^s$ by $x_{m2}{}^t$, then this equation becomes the same as (4.9), and we may repeat the same argument used for the one-ligand case to show that in the two-ligand case also $\Delta F^* - \Delta F \geq 0$; as before, we may extend the principle to any value of r.

Consequently we verify that the total work of saturating the macromolecule with *both* ligands is always diminished as a result of the allosteric equilibria. But the values of x_m or y_m may either increase or decrease with increasing activity of the other ligand; *i.e.*, the heterotropic interactions may be either positive or negative. Thus the work of saturating the macromolecule with either ligand alone, the activity of the other one being held constant, may be either increased or diminished as a result of the heterotropic interactions.

5. The Hill Plot and the Apparent Interaction Energy

The Hill plot gives useful information about the homotropic reactions, of whatever origin, in *any* system.[2] Here we consider specifically the behavior of the Hill plot in an *allosteric* system.

The Hill plot is one in which

$$\ln \frac{\bar{X}}{t - \bar{X}} = \ln \frac{\bar{x}}{1 - \bar{x}}$$

$\bar{x} = \bar{X}/t$ being the fractional saturation of the macromolecule with ligand, is shown as ordinate *vs.*

$$\ln x = (\mu_x - \mu_{x0})/RT$$

as abscissa. In the simple case where the macromolecule exists in only one conformation and contains only one site, such a plot of course gives a straight line of unit slope, the value of $\ln x$ for which $\ln \bar{x}/(1 - \bar{x}) = 0$, *i.e.*, $\bar{x} = 1/2$, being $-\ln K$, where K is the equilibrium constant for the reaction. The total work of saturating the macromolecule is therefore

$$\Delta F = -RT \ln K = RT \ln x_m = RT \ln x_{1/2}$$

and there is no question of any interaction energy between sites, of whatever origin.

If the macromolecule contains several sites, then, quite apart from any question of conformation, unless the sites are all alike and independent, the Hill plot will no longer be straight but will show one or more right-hand or left-hand deviations or kinks. However, unless the interactions between certain of the sites are infinite, it will approach an asymptote of unit slope at each end (see Figure 3). In the case where all the sites are identical, the perpendicular distance between the final and initial asymptotes, multiplied by $RT\sqrt{2}$, gives the average value of the free energy of interaction of the sites, due to whatever cause, realized per site in saturating the macromolecule with ligand. When the final asymptote lies above the initial one this interaction energy is, as a matter of convention, taken to be positive; in the opposite case, as negative.

In case the sites are *not* all identical but are independent, the Hill plot will show only right-hand displacements, and the final asymptote will lie below the initial one, exactly as in the case of identical sites which interact negatively. Thus the difference between the final and initial asymptotes, taken as positive when the final asymptote lies above the initial one, gives only a minimum value for the total interaction energy between the sites realized in saturating the macromolecule with ligand. We shall call this the *apparent interaction energy*.

Not only does the Hill plot, through its asymptotes, provide information about the minimum value of the *total* free energy of interaction realized in completely saturating the macromolecule with ligand, it also gives information about the point value of the free energy of interaction realized, per site, at any degree of saturation $\bar{x}$ in the system as a whole. If we denote the slope of the Hill plot at saturation $\bar{x}$ by n, then the minimum value of this quantity is given by

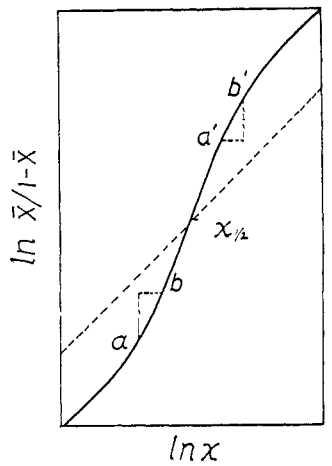

Figure 4. A hypothetical Hill plot to show symmetry requirements; see ref 13.

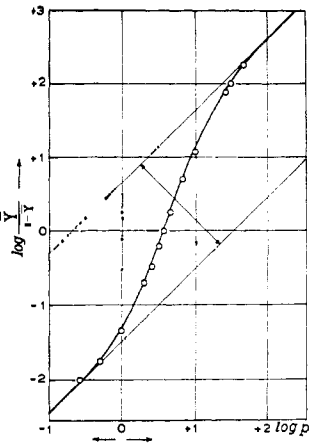

Figure 5. A Hill plot of the oxygen equilibrium of sheep hemoglobin in 0.2 *M* phosphate buffer of pH 9.1 at 19°; based on data of F. J. W. Roughton, A. L. Otis, and R. L. J. Lyster, *Proc. Roy. Soc.* (London), **B144**, 29 (1955).

$$\Delta F_I = \frac{RT}{\bar{x}(1-\bar{x})}\left(1 - \frac{1}{n}\right) \qquad (5.1)$$

Moreover, as follows from this relation, the minimum free energy of interaction, per site, for the whole system realized in passing from saturation $\bar{x}_1$ to saturation $\bar{x}_2$ is simply $RT\sqrt{2}$ times the projection of the line joining points 1 and 2 on an axis normal to the asymptotes, *i.e.*, one making an angle of 135° with the abscissa axis. It will be seen that unless the value of $n > 1$, *i.e.*, unless the Hill plot bends to the left, then the minimum value of the free energy of interaction, *i.e.*, the apparent interaction free energy, is negative.

Figure 5 shows a Hill plot for the oxygen equilibrium of sheep hemoglobin. This curve, which is characteristic of the ligand equilibrium of many proteins, shows only a single left-hand kink, and the distance between the estimated asymptotes corresponds to an apparent free energy of interaction of about 3000 cal per site, although it will be seen that the exact position of the asymptotes is subject to a good deal of uncertainty. It should be emphasized, however, that this value is only an apparent one, and if hemoglobin is an allosteric protein, the true value must be greater than this, for, if the allosteric transitions were suppressed, the distribution of conformations remaining the same as in the absence of ligand, the Hill plot would show one or more downward kinks and the final asymptote would lie below the initial one. It should never be forgotten that the apparent free energy of interaction determined from a Hill plot can only be equated to the true free energy of interaction if we assume that, in the absence of interactions, the plot is a straight line of unit slope.

Let us consider a little more closely what may be expected for the Hill plot of a macromolecule containing many sites and existing in several conformations. Of course, if the sites are all the same in all the conformations, then the curve will be a straight line irrespective of whether or not the conformations are in equilibrium. On the other hand, as we have pointed out, if there is no equilibrium, the conformations remaining in fixed proportions, and if the sites are not all alike, the Hill plot will show one or more right-hand kinks, and the *apparent* interaction free energy will be negative, although actually of course there is no interaction.

The interesting cases are those in which there is allosteric equilibrium between the conformations. A unique one is that in which there is only one site in each conformation. Then, as we have seen (section 3), the ligand equilibrium is always that of a simple one-site molecule, and the Hill plot is a straight line of unit slope. The apparent interaction free energy will therefore be zero; the true free energy of interactions, however, is certainly greater.

In order to deal with more complex cases we derive a general expression for the apparent interaction energy as determined from the asymptotes of a Hill plot. Let the binding potential be

$$\Pi = RT \ln (1 + K_1 x + \ldots + K_t x^t)$$

Then it follows from eq 1.1 that in the limiting case where $x \to 0$, and $\bar{x} \to 0$

$$\frac{\bar{x}}{1-\bar{x}} = \frac{K_1 x}{t} \qquad (5.2.1)$$

which corresponds to a median ligand activity

$$x_m = \frac{t}{K_1} \qquad (5.2.2)$$

On the other hand, when $x \to \infty$ and $\bar{x} \to 1$

$$\frac{\bar{x}}{1-\bar{x}} = \frac{tK_t x}{K_{t-1}} \qquad (5.3.1)$$

which corresponds to a median ligand activity

$$x_m = \frac{K_{t-1}}{tK_t} \qquad (5.3.2)$$

These two limiting equations fix the positions of the asymptotes of the Hill plot and give for the apparent interaction energy per site the expression

$$\Delta F_I = RT \ln (x_m)_{\bar{x}\to 0} - RT \ln (x_m)_{\bar{x}\to 1} = RT \ln \left(\frac{t^2 K_t}{K_1 K_{t-1}}\right) \equiv RT \ln \rho \qquad (5.4)$$

If the ratio ρ is greater than 1, then the final asymptote of the Hill plot lies above the initial one and the apparent interaction energy is positive.

Now the statistical values of K_t and K_{t-1} are[8]

$$K_t^* = \left(\frac{K_1}{t}\right)^t \text{ and } K_{t-1}^* = t\left(\frac{K_1}{t}\right)^{t-1} \qquad (5.5)$$

(By statistical values we mean here the values corresponding to a polynomial resulting from t independent and identical sites, *i.e.*, those given by the expansion of $(1 + kx)^t$.) Consequently, if the ratio ρ is equal to 1, which means that the two asymptotes of the Hill plot coincide, then the ratio (K_1/K_{t-1}) has its statistical value, and conversely.

Now consider the special case where there are r conformations, each of which contains t identical sites, characterized by a constant k_i in conformation i. Then

$$K_1 = t\sum_{i=1}^{r}\nu_i k_i = t(\bar{k})$$

$$K_{t-1} = t\sum_{i=1}^{r}\nu_i k_i^{t-1} = t(\overline{k^{t-1}}) \qquad (5.6)$$

$$K_t = \sum_{i=1}^{r}\nu_i k_i^t = (\bar{k^t})$$

Here the bar stands for an average taken over all the conformations, each weighted according to its ν.

In terms of these average values

$$\rho = \frac{(\bar{k^t})}{(\bar{k})(\overline{k^{t-1}})} \qquad (5.7)$$

but it can be readily proved as a general statistical principle that this ratio is always greater than 1.[11] Therefore in this significant case the Hill plot *always* gives a positive apparent energy of interaction, irrespective of the value of the various k_i.

It is interesting to compare the apparent free energy of interaction read from a Hill plot with the true value obtained by taking into account the final asymptote which *would be* approached if no conformation changes were permitted. This final asymptote is determined by the limiting value of the ratio $(\bar{x}/1 - \bar{x})$ approached as $x \to \infty$. This is easily shown to be

$$\frac{\bar{x}}{1-\bar{x}} = \frac{x}{\dfrac{\nu_1}{k_1} + \dfrac{\nu_2}{k_2} + \ldots} = \frac{x}{(\overline{k^{-1}})}$$

where $(\overline{k^{-1}})$ is the mean value of the reciprocals of the k's. It corresponds to a median ligand activity

$$(x_m)_{\bar{x}\to 1} = (\overline{k^{-1}})$$

Under these same conditions the *initial* asymptote and its corresponding median ligand activity are the same as before.

$$(x_m)_{\bar{x}\to 0} = \frac{1}{(\bar{k})}$$

The apparent value of ΔF_I in the absence of any conformational change is therefore

$$\Delta F_I = RT\ln\frac{1}{(\bar{k})} - RT\ln(\overline{k^{-1}}) = -RT\ln(\bar{k})(\overline{k^{-1}}) \qquad (5.8)$$

Since $(\bar{k})(\overline{k^{-1}})$ is necessarily greater than unity, this is always negative. The true value of the free energy of interaction when the conformational changes are permitted exceeds the apparent value read from a Hill plot by this amount.

The considerations just presented apply to the case where the t sites in each of the r conformations are identical. When they are not, the situation becomes much more complex, and the apparent free energy of interaction no longer need to be positive.

6. The Regulatory Action of Allosteric Transitions

There is another way of looking at the situation. Consider an allosteric system in which there are only two conformations. Then by eq 1.1

$$\bar{X} = \frac{1}{1+L'}\bar{X}_1 + \frac{L'}{1+L'}\bar{X}_2 \qquad (6.1)$$

Here L' is of course a function of the activities of all the components, including X, in accordance with eq 1.4. Let us fix our attention on ligand Y, which we may regard as a regulator for the combination of X with the macromolecule—it may act either as an activator or an inhibitor. If the interactions stem exclusively from allosteric equilibria, then the saturations $\bar{X}_1$ and $\bar{X}_2$ are independent of y. Consequently

$$\left(\frac{\partial\bar{X}}{\partial\ln y}\right)_x = \left(-\frac{L'}{(1+L')^2}\bar{X}_1 - \frac{L'^2}{(1+L')^2}\bar{X}_2 + \frac{L'}{1+L'}\bar{X}_2\right)\frac{\partial\ln L'}{\partial\ln y} = \frac{L'}{(1+L')^2}(\bar{X}_2 - \bar{X}_1)\frac{\partial\ln L'}{\partial\ln y}$$

but by eq 1.4

$$\frac{\partial\ln L'}{\partial\ln y} = (\bar{Y}_2 - \bar{Y}_1)$$

Consequently

$$\left(\frac{\partial\bar{X}}{\partial\ln y}\right)_x = \frac{L'}{(1+L')^2}(\bar{X}_2 - \bar{X}_1)(\bar{Y}_2 - \bar{Y}_1) \qquad (6.2)$$

(11) A proof is as follows. Consider the three averages

$$(\bar{k}) = \frac{k_1 + k_2 + \ldots + k_n}{n}$$

$$(\overline{k^{t-1}}) = \frac{k_1^{t-1} + k_2^{t-1} + \ldots + k_n^{t-1}}{n}$$

$$(\bar{k^t}) = \frac{k_1^t + k_2^t + \ldots + k_n^t}{n}$$

In order to demonstrate that $(\bar{k^t}) > (\bar{k})(\overline{k^{t-1}})$, we have only to show that

$$(\bar{k^t}) - (\bar{k})(\overline{k^{t-1}}) = \frac{(k_1^t + k_2^t + \ldots)}{n} - \frac{(k_1 + k_2 + \ldots)(k_1^{t-1} + k_2^{t-1} + \ldots)}{n^2} > 0$$

The middle member, after multiplication by n^2, gives

$$k_1(k_1^{t-1} - k_2^{t-1}) + k_1(k_1^{t-1} - k_3^{t-1}) + \ldots + k_n(k_n^{t-1} - k_1^{t-1}) + k_n(k_n^{t-1} - k_2^{t-1}) + \ldots = (k_1 - k_2)^2(k_1^{t-2} + k_1^{t-3}k_2 + \ldots + k_2^{t-2}) + (k_1 - k_3)^2(k_1^{t-2} + k_1^{t-3}k_3 + \ldots + k_3^{t-2}) + \ldots$$

but this is certainly greater than zero.

A familiar special case results from setting $t = 2$; the mean-square value of a quantity is always greater than the square of its mean value. The proof may readily be extended to show that

$$(\overline{k^{i+j+\cdots}}) > (\bar{k^i})(\bar{k^j})\ldots$$

It follows from this that in the system under consideration (r conformations in equilibrium, each containing t identical sites) the ratio of *each K* to the preceding one in the polynomial in the expression for the binding potential is greater than its statistical value.

In terms of the *fractional* saturations $\bar{x}$ and $\bar{y}$, *i.e.*, saturations per X or Y binding site, this becomes

$$\left(\frac{\partial \bar{x}}{\partial \ln y}\right)_x = \frac{L'}{(1 + L')^2}(\bar{x}_2 - \bar{x}_1)(\bar{y}_2 - \bar{y}_1)s \qquad (6.3)$$

In order to avoid any invidious distinction between the two forms, eq 6.3 may be written in terms of this mole fractions ν_1' and ν_2' as

$$\left(\frac{\partial \bar{x}}{\partial \ln y}\right)_x = \nu_1'\nu_2'(\bar{x}_2 - \bar{x}_1)(\bar{y}_2 - \bar{y}_1)s \qquad (6.4)$$

We emphasize that in these equations, L', in distinction from L used in earlier sections, refers to the *actual* value of the equilibrium constant between the two forms *at the prevailing values of x and y;* likewise the ν''s, in distinction from the ν's employed earlier, refer to the *actual* values of the mole fractions represented by the two forms at the prevailing values of x and y.

Equation 6.4 may be generalized to any number of forms with the result

$$\left(\frac{\partial \bar{x}}{\partial \ln y}\right)_x = s\sum_i\sum\nu_i'\nu_j'(\bar{x}_i - \bar{x}_j)(\bar{y}_i - \bar{y}_j) \qquad (6.5)$$

where the summation is to be extended over all pairs of values of i and j regardless of order.

It will be seen that the left-hand side of eq 6.5 is a simple measure of the work required to be done through the regulator Y in order to produce a given change in the amount of ligand X (substrate) bound per site and thus, in the case of an enzyme system, to produce a given change in the activity of the enzyme. This work is given by $RT(\partial \ln y/\partial \bar{x})_x$. It is significant that it is inversely proportional to the number s of Y binding sites per macromolecule (enzyme), and, if this is an oligomer made up of protomers each containing one site, to the number of protomers in the oligomer (to use the Monod terminology). A large molecule (large value of s) is thus more susceptible to regulation than a small one (for an example see section 10c).

We may use the same procedure to explore the homotropic interactions (self-regulation) of ligand X. Even if there were no direct dependence of $\bar{X}_i$ on x (as if, for example, we were considering values of x for which $\bar{X}_1$ was essentially 0 and $\bar{X}_2$ essentially t), then there would still be a dependence of $\bar{X}$ on x due to the allosteric transition (see Figure 1). In the case where there are only two conformations, the contribution to $(\partial \bar{X}/\partial \ln x)y$ arising from the allosteric transition alone is given by

$$\frac{\partial \bar{X}}{\partial \ln x} = \nu_1'\nu_2'(\bar{X}_2 - \bar{X}_1)^2 \qquad (6.6)$$

whence

$$\frac{\partial \bar{x}}{\partial \ln x} = t\nu_1'\nu_2'(\bar{x}_2 - \bar{x}_1)^2$$

It will be seen that this is always positive, *i.e.*, *homotropic* allosteric interactions are always of a stabilizing type, in distinction from the heterotropic ones which may be either positive ($\bar{X}_2 - \bar{X}_1$ and $\bar{Y}_2 - \bar{Y}_1$ of same sign) or negative ($\bar{X}_2 - \bar{X}_1$ and $\bar{Y}_2 - \bar{Y}_1$ of opposite sign). The argument may be readily generalized to the case where there are any number of conformations. Thus homotropic interactions always act to increase the value of the parameter n of the Hill plot and so to increase the apparent interaction free energy, whether this be positive or negative.[12]

7. Variance and Invariance

It is a consequence of eq 1.5 or 1.6 that, except for special cases, allosteric linkage between several ligands makes the shape of the equilibrium curve for each ligand dependent on the chemical potentials of all the other ligands. This becomes apparent when we recall that the curve is a plot of $\partial\Pi/\partial\mu_x$ *vs.* μ_x. It is illustrated simply by the case where there are only two ligands X and Y. Write the binding potential as

$$\Pi = RT \ln \Sigma\nu_i e^{\Pi_{ix} + \Pi_{iy}}$$

Then clearly if and only if Π_{iy} is the same for all forms, *i.e.*, for all values of i, will $\partial\Pi/\partial\mu_x$ be independent of μ_y. This condition means of course that there is no linkage between X and Y. But if Π_{iy} is *not* the same for all values of i, then not only will there be heterotropic interactions between X and Y but also homotropic interactions involving Y as well as X. These, as we have seen, are sure to be of a stabilizing type, *i.e.*, cooperative. So, in any system in which there are heterotropic interactions between two ligands which are of allosteric origin, there must also be homotropic allosteric interactions for each ligand, which are necessarily cooperative, and in general the equilibrium curves for each ligand will be dependent in shape on the chemical potential of the other ligand. The principle may be extended to any number of ligands.

The only exception to this principle regarding the variance of shape of the ligand equilibrium curve is the special case where there is only a single site for one or more of the ligands. In this special case the equilibrium curve for that ligand will, as we have seen, always be the same as that for a single-site molecule existing in only one conformation, *i.e.*, the Hill parameter n will be equal to 1; thus the curve will always be invariant.

8. Symmetry

A striking feature of certain ligand equilibrium curves (*e.g.*, the oxygen equilibrium curve of hemoglobin) is their symmetry, or at least near-symmetry. The formal conditions of symmetry were developed elsewhere some years ago for any system in which the binding potential (*i.e.*, the ligand equilibrium) can be expressed in terms of a polynomial.[1] If we write

$$\Pi = RT \ln (1 + K_1x + \ldots + K_tX^t)$$

(12) Consider the special case of a macromolecule which contains t sites and exists in two conformations, 1 and 2, the affinities of the sites in conformation 2 for ligand X, though not necessarily the same, being all much greater than those of the sites in conformation 1. By eq 6.6

$$n = \frac{\partial \ln [\bar{x}/(1 - \bar{x})]}{\partial \ln x} = \frac{1}{\bar{x}(1 - \bar{x})}\frac{\partial \bar{x}}{\partial \ln x} = \frac{t\nu_1'\nu_2'}{\bar{x}(1 - \bar{x})}(\bar{x}_2 - \bar{x}_1)^2$$

Also

$$\bar{x} = \nu_1'\bar{x}_1 + \nu_2'\bar{x}_2$$

Owing to the large difference between the sites in the two conformations, there will be a wide range of values of x within which $\bar{x}_2 \cong 1$ when $\bar{x}_1 \cong 0$, and therefore $\bar{x} \cong \nu_2'$, $(1 - \bar{x}) \cong (1 - \nu_2') = \nu_1'$. As a result, anywhere within the range, $n \to t$, which is its upper limit. If ν_2 (the value of ν_2' for $x = 0$) is sufficiently small, this value of $n \cong t$ will hold over substantially the whole of the equilibrium curve; however, at the two ends of the curve, where $x \to 0$ and $x \to \infty$, $n \to 1$.

then these conditions are

$$x_{1/2} = \frac{1}{K_t^{1/t}}, \cdots, \frac{K_i}{K_t^{i/t}} = \frac{K_{t-i}}{K_t^{(t-i/t)}}, \cdots \quad (8.1)$$

where $x_{1/2}$ is the value of x required to half-saturate the macromolecule. It should be emphasized that although they are applicable in particular to an allosteric system, they are of much wider scope and are wholly independent of the nature of the binding or the mechanism which gives rise to the interactions, being subject only to the limitation regarding the form of the binding potential. As we have shown earlier in this paper, the median ligand activity x_m is given by $x_m = 1/K_t^{1/t}$. Consequently, symmetry demands that $x_{1/2} = x_m$, which is obvious. Moreover, as follows very simply from eq 8.1, at the midpoint of the equilibrium curve ($x = x_{1/2}$, $\bar{x} = 1/2$) the concentrations of "conjugate" intermediates, *i.e.*, MX_i and MX_{t-i}, are always equal to one another, which is also more or less obvious. Further, it is easy to show that if we express the ligand activity as a ratio of x to x_m, *i.e.*, introduce $w = x/x_m$, the conditions of symmetry require that the expression for the binding potential itself become symmetrical in its coefficients.[13]

$$\mathcal{J} = RT \ln \left[1 + \frac{K_1 w}{K_t^{1/t}} + \frac{K_2 w^2}{K_t^{2/t}} + \cdots + \frac{K_2 w^{t-2}}{K t^{2/t}} + \frac{K_1 w^{t-1}}{K t^{1/t}} + w^t \right] \quad (8.2)$$

In an allosteric system the conditions for symmetry will not in general be realized. Consider, for example, the case where the ligand equilibrium curve for each conformation is symmetrical, as when the corresponding polynomial is simply $(1 + kx)^t$. Suppose there are only two conformations. Then the polynomial in the expression for the total binding potential is

$$1 + (\nu_1 k_1 + \nu_2 k_2)tx + \ldots + (\nu_1 k_1{}^{t-1} + \nu_2 k_2{}^{t-1})tx^{t-1} + (\nu_1 k_1{}^t + \nu_2 k_2{}^t)x^t$$

Consequently the condition of symmetry given by the second of the equations becomes

$$\frac{\nu_1 k_1 + \nu_2 k_2}{(\nu_1 k_1{}^t + \nu_2 k_2{}^t)^{1/t}} = \frac{\nu_1 k_1{}^{t-1} + \nu_2 k_2{}^{t-1}}{(\nu_1 k_1{}^t + \nu_2 k_2{}^t)^{(t-1)/t}} \quad (8.3)$$

This will not in general be satisfied, although it is always satisfied in the special cases when $t = 1$ or $t = 2$. We have already seen that when $t = 1$ the ligand equilibrium curve is the same as a simple titration curve ($n = 1$).

It should be made clear that the functional symmetry discussed here is not to be confused with the geometrical symmetry which has been proposed elsewhere as a basis for allosteric transitions.[7] Although attempts have been made to relate functional and structural symmetry,[14] it is apparent from this discussion that there need be no connection between them. The system of constraints leading to the conservation of symmetry in allosteric transitions, proposed by Monod and his collaborators,[7] represents as it were a mechanism within a mechanism. Actually, from a thermodynamic point of view, the mere existence of differences of ligand affinity between different conformations is enough to generate the complex conformational equilibria that result in allosteric linkage.

9. The Heats of Liganding

Consider first the case where there is only one ligand. Then

$$\mathcal{J} = RT \ln \sum \nu_i N_{ix} \quad (9.1)$$

where N_{ix} is a polynomial in x of degree t. Consequently

$$x_m{}^t = \frac{1}{\Sigma \nu_i K_{it}}$$

but

$$\Delta H = -tRT^2 \frac{\partial \ln x_m}{\partial T} = RT^2 \frac{\partial \ln \Sigma \nu_i K_{it}}{\partial T} = \left[RT^2 \Sigma \nu_i K_{it} \frac{\partial \ln K_{it}}{\partial T} + RT^2 \Sigma K_{it} \frac{\partial \nu_i}{\partial T} \right] \Big/ \Sigma \nu_i K_{it} \quad (9.2)$$

where ΔH is the increase in total heat accompanying the saturation of the macromolecule (all t sites).

The first term in the numerator on the right yields $\Sigma \nu_i K_{it} (\Delta H_i)_x$, where $(\Delta H_i)_x$ is the increase in total heat accompanying the saturation of form i with ligand X.

If we introduce $\nu_i = L_i / \Sigma L_i$, the second term may be dealt with by the methods employed in section 6 and yields

$$\Sigma\Sigma \nu_i \nu_j (K_{it} - K_{jt})(\Delta H_{ij})_c$$

where $(\Delta H_{ij})_c$ is the increase of total heat accompanying the transition from conformation i to conformation j in the absence of ligand (compare this with eq 6.5). As in the earlier case, the summation is to be extended over all pairs of values of i and j regardless of order.

The total result is therefore

$$\Delta H = \frac{\Sigma \nu_i K_{it} (\Delta H_i)_x}{\Sigma \nu_i K_{it}} + \frac{\Sigma \nu_i \nu_j (K_{it} - K_{jt})(\Delta H_{ij})_c}{\Sigma \nu_i K_{it}} \quad (9.3)$$

Here ΔH gives the *total* heat of saturating the macromolecule with ligand X, *i.e.*, the heat of saturating all t sites. It is to be emphasized that the ν's are the mole fractions of the various forms in the absence of ligand and are constant. Equation 9.3 shows how the total ΔH involves the heats of transition between the various allosteric forms as well as the heats of liganding of the forms separately. Each of the two terms on the right represents an average.

When another ligand, Y, enters into the picture, the situation becomes more complicated, as will be seen from eq 4.16, for we now have also to take account of the temperature derivatives of the N_{iy}, *i.e.*, the heats of combination with ligand Y of each of the conformations. We shall not attempt to write down the general equation. Nevertheless it may perhaps be worthwhile to consider in passing the simplest possible case, that where there are only two allosteric forms and where there is

(13) Another way of looking at symmetry is in terms of the Hill plot (Figure 4). Symmetry of the ligand equilibrium curve demands symmetry of the corresponding Hill plot. Since the interaction energy involved in passing from saturation a to saturation b is proportional to the difference of ordinate between a and b, clearly the interaction energy realized in symmetrically located steps must be the same, *i.e.*, that involved in passing from 10 to 20% saturation, and must be the same as that involved in passing from 80 to 90% saturation.

(14) D. W. Allen, K. F. Guthe, and J. Wyman, *J. Biol. Chem.*, **187**, 393 (1950).

only one site for each of the two ligands (see section 3). The equilibrium constant for X is then simply

$$\bar{K} = \frac{\nu_1 K_1(1 + M_1 y) + \nu_2 K_2(1 + M_2 y)}{\nu_1(1 + M_1 y) + \nu_2(1 + M_2 y)}$$

where the M's are the equilibrium constants for the Y binding site in each of the two forms. In the absence of Y, the right-hand member degenerates into a form which may be dealt with by the treatment just given; in the presence of a large amount of ligand it passes over into

$$\bar{K} = \frac{\nu_1 M_1 K_1 + \nu_2 M_2 K_2}{\nu_1 M_1 + \nu_2 M_2}$$

from which ΔH is obtained by differentiation with respect to T.

10. Hemoglobin as an Allosteric Protein

Hemoglobin is generally regarded as the type case of an allosteric protein; indeed it was the study of mammalian hemoglobin that first suggested the concept of conformation change as a source of interaction in a macromolecule containing several sites for several different ligands.[15] It is challenging therefore to see how far the behavior of hemoglobin conforms to the principles just developed.

a. **The Oxygen Equilibrium of Mammalian Hemoglobin.** Under a wide variety of conditions the mammalian hemoglobins exist as tetramers containing two α chains and two β chains. As such they contain four sites, the four hemes, one in each chain, each of which combines with a single molecule of oxygen (or any one of various other ligands). There are also certain other sites, about whose identity and position there has been much speculation, which bind proton and which are linked with the oxygen-combining sites. This heterotropic linkage, known as the Bohr effect, is positive below pH $\sim$6 and negative above it. In addition to the heterotropic interactions between proton and oxygen, there are also homotropic interactions between the oxygen combining sites, the apparent homotropic free energy of interaction per site as determined from the asymptotes of a Hill plot being in the neighborhood of 3000 cal (see Figure 5).

The most striking features of the oxygen equilibrium of mammalian hemoglobin are the following. (1) The equilibrium curves, $\bar{Y}$ or $\bar{x}$ *vs.* ln x, are very nearly invariant in shape for changes of pH. (2) They are also nearly symmetrical. (3) The interactions represented by the Bohr effect can be explained, phenomenologically at least, by assuming that there are two proton-binding sites per chain, the same for each chain, which are oppositely affected by oxygenation but are independent of one another. (4) The equilibrium curves are, if anything, even more invariant in shape for changes of temperature than for changes of pH,[16] and the effect of temperature can be fairly well accounted for by assigning to each oxygen-binding site an intrinsic heat of oxygenation which is independent of the degree of protonation of the molecule, and to each of the two oxygen-linked proton-binding sites a heat of ionization which is independent of the degree of oxygenation. (5) The required value of the heat of oxygenation is, within the errors, the same as that observed in myoglobin, a single-chain molecule containing only one heme, which shows no appreciable homotropic interactions and scarcely any heterotropic ones. Of the required heats of ionization of the oxygen-linked acid groups, one lies in the range characteristic of a carboxyl group and the other in that characteristic of an imidazole group. (6) The characteristic homotropic interactions, and in particular the value of $n \cong 3$, are not greatly changed at high ionic strength where the tetramers are very largely dissociated into $\alpha\beta$ subunits; on the other hand, the Bohr effect is much reduced.

It would seem, in view of the analysis just given, that items 1, 2, and 3 would rule out any interpretation of the behavior of hemoglobin in terms of allosteric linkage. Moreover, the simple behavior represented by items 4 and 5 would appear difficult to reconcile with such an interpretation. Item 6 ($n = 3$ in a two-site molecule) is hard to explain on any basis. On the other hand, strong evidence from a variety of sources indicates that the combination of hemoglobin with ligand is accompanied by profound conformational changes and suggests therefore that the homotropic and heteotropic interactions are both basically allosteric. If different conformations have different ligand affinities and the system is in equilibrium, allosteric effects must come into play.

Faced with these opposite and equally compelling indications, we offer the following suggestion, which is in fact the amplification of an idea proposed many years ago on the basis of much more limited evidence.[1] Let us assume that the sites within the $\alpha\beta$ pairs interact very strongly and that there are much weaker interactions between pairs. This idea is consistent with the fact that hemoglobin H (β_4) and other systems containing only one kind of chain fail consistently to show any significant interactions, heterotropic or homotropic. If the subunits were completely stabilized, then n would be equal to 2 and the homotropic interaction would be infinite for each such pair. A further relatively small secondary interaction between the pairs might then be expected to raise n somewhat above 2, say to the observed value of 2.7–3. The Hill plot would have the form shown in Figure 6 with an asymptote of 2 at each end. The ligand equilibrium curve, as in the case of a two-site macromolecule, would be symmetrical, and at the same time the value of n would be relatively insensitive to the secondary effects arising from the relatively weak interaction between the $\alpha\beta$ pairs. Thus whether at high ionic strength, where the oxygenated molecules are partly dissociated into $\alpha\beta$ pairs, these interactions between the pairs were due to an association–dissociation equilibrium (for which there is some evidence) or to a simpler type of interaction between separate molecules, concentration changes would have relatively little effect on the over-all picture; indeed the transition from intramolecular to intermolecular interaction consequent on dissociation might scarcely be noticed. Since the interaction energy within the $\alpha\beta$ subunits is infinite, any allosteric transformation to which it was due would necessarily be complete (100%) as a result of oxygenation. Thus if the Bohr

(15) J. Wyman and D. W. Allen, *J. Polymer Sci.*, 7, 499 (1951).

(16) In the case of tuna fish hemoglobin, the curves change grossly with pH but are invariant for changes of temperature; see A. Rossi Fanelli and E. Antonini, *Nature*, **186**, 895 (1960).

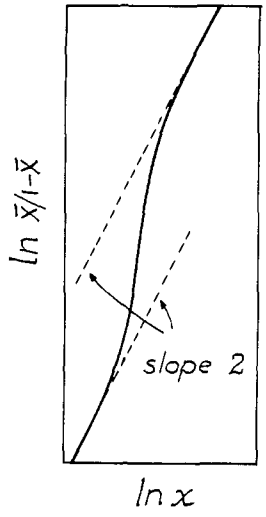

Figure 6. Hypothetical Hill plot for extreme case of two completely stabilized $\alpha\beta$ subunits subject to secondary interaction.

effect arose from heterotropic interactions within the $\alpha\beta$ pairs, it would be expressible in terms of a simple difference in the pK values of the oxygen-linked acid groups in the two conformations (assuming only two to be involved), just as it is found to be. If the free-energy change accompanying the allosteric transition within the $\alpha\beta$ subunits, as well as the much smaller free-energy change representing the interaction between them, both represented entropy effects, then we should also have an explanation of the observed temperature invariance of the equilibrium curves and the unexpectedly simple behavior of the heats. The hypothesis that ΔH for the transition is essentially zero is in good agreement with the fact that the inherent heat of oxygenation of native hemoglobin is the same as that of myoglobin and various modified hemoglobins in which the heme–heme interactions and Bohr effect have been eliminated.

Clearly this extreme interpretation of the behavior of hemoglobin is inconsistent with the facts. The slope of the asymptotes in the Hill plot is unity, not 2, and the apparent interaction energy is not infinite but only about 3000 cal. Let us see therefore how much of the picture, in its main outlines so plausible, remains when we reduce the interaction energy *within* the $\alpha\beta$ subunits to a finite value, keeping it, however, much greater than that *between* these units.[17]

Consider first the $\alpha\beta$ pairs. Assume that these exist in two conformations, 1 and 2, which in the absence of ligand occur at the mole fractions ν_1 and ν_2 and are characterized by the equilibrium constants k_1 and k_2, the two sites having the same oxygen affinity in each conformation. We wish to explore the values of $n = n_{1/2}$ at half-saturation and the apparent and real free energies of interaction within the pairs in relation to various values of the constants.

The binding potential of a pair is given by

$$\Pi = RT \ln [\nu_1 (1 + k_1x)^2 + \nu_2(1 + k_2x)^2] \quad (10.1)$$

By setting

$$K_1 = 2(\nu_1k_1 + \nu_2k_2)$$

and

$$K_2 = \nu_1k_1^2 + \nu_2k_2^2$$

in eq 5.4, we obtain for the apparent free energy of interaction, *per site*

$$\Delta F_I = RT \ln \frac{\nu_1k_1^2 + \nu_2k_2^2}{(\nu_1k_1 + \nu_2k_2)^2} = RT \ln \rho \quad (10.2)$$

If we introduce $z = k_2/k_1$, the expression for ρ becomes

$$\rho = \frac{1 + \nu_2(z^2 - 1)}{(1 + \nu_2(z - 1))^2} \quad (10.3)$$

The expression for $n_{1/2}$ may be obtained directly from the general equation given earlier,[18] namely

$$n_{1/2} = \frac{\sum_{i=0}^{t}(4i^2 - t^2)K_i x_{1/2}^i}{t\sum_{i=0}^{t}K_i X_{1/2}^i} \quad (10.4)$$

By setting $t = 2$ and identifying $x_{1/2}$ with $x_m = 1/\sqrt{K_2}$ on the grounds that the ligand equilibrium curve of *any* two site molecules is necessarily symmetrical (see section 8), we then obtain

$$n_{1/2} = \frac{2}{1 + \frac{K_1}{2\sqrt{K_2}}} = \frac{2}{1 + \frac{\nu_1k_1 + \nu_2k_2}{\sqrt{\nu_1k_1^2 + \nu_2k_2^2}}} = \frac{2}{1 + \frac{1}{\sqrt{\rho}}} \quad (10.5)$$

We see that in this simple two-site case n depends only on the apparent free energy of interaction, $\Delta F_I = RT \ln \rho$.

We know that large values of ΔF_I and $n_{1/2}$ (approaching 2) will be realized when k_1 and k_2 are widely separated and the form with the lower k is predominant in the absence of ligand; this means of course that combination with ligand involves a large conformational transition. Figure 7 shows values of ΔF_I and $n_{1/2}$ calculated as a function of ν_2 for several different large values of z from eq 10.2 and 10.5. It will be seen from the figure that we need consider only values of z greater than 100 or 200, and that a value between about 500 and 1000 combined with a value of $-\log \nu_2$ between about 2 and 4 (*i.e.*, $10^{-4} < \nu_2 < 10^{-2}$) will give an interaction energy per heme of 2500–3000 cal and a value of $n_{1/2}$ in the range 1.7–1.9, which is about what we are looking for.

In order to get an idea of the completeness of the transition accompanying combination with ligand when the constants lie in this range, let us arbitrarily set $\nu_2 = 10^{-3}$ ($\nu_1 \cong 1$) and $z = 500$. Since the equilibrium constant for the transition is given by

$$\frac{\nu_2'}{\nu_1'} = L_2' = \frac{\nu_2(1 + k_2x)^2}{\nu_1(1 + k_1x)^2}$$

it follows that saturation with ligand, which corresponds to $x \rightarrow \infty$, must cause a change in ν_2 from 10^{-3} to $10^{-3}z^2 = 250$, in other words, a virtually complete

(17) In the original suggestion the sites were supposed to be arranged as at the corners of a rectangle with an interaction constant of 400 for interactions along the short sides of the rectangle and a constant of 4 for interactions along the long sides, there being no interaction corresponding to diagonals. This naive model gives a total interaction energy, per heme, of about 2200 cal as compared with the value of 2500–3000 cal of more exact data more recently obtained from Hill plots. Of this 2200 cal, about 1800 come from the strongly interacting pairs and about 400 from the weakly interacting pairs.

(18) See eq 4 in ref 1.

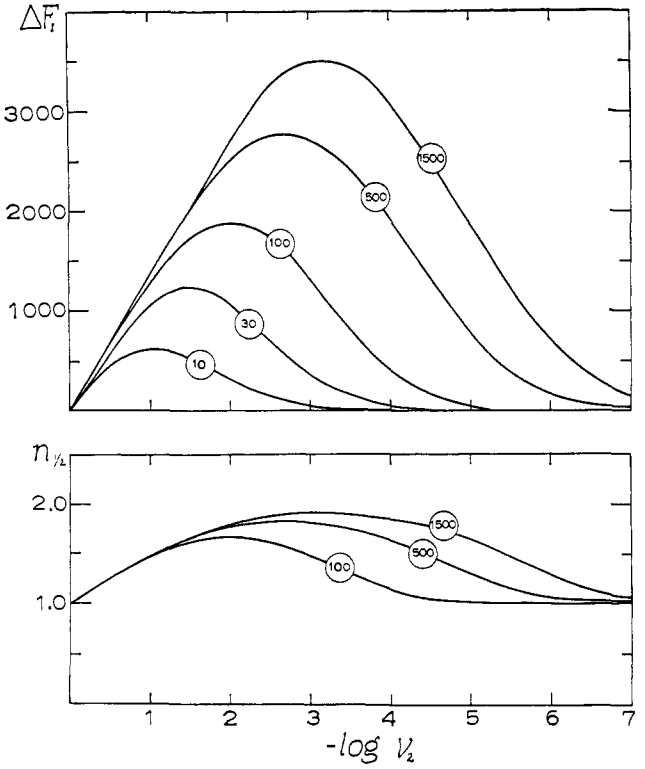

Figure 7. Values of apparent interaction energy ΔF_I and $n_{1/2}$ for the $\alpha\beta$ subunits as a function of log ν_2 for various values of z (from calculations in text).

transition. Moreover, in view of the high degree of stabilization, *i.e.*, the very high interaction energy within the pairs, the value of ν_2 will vary nearly linearly with the degree of oxygenation.

Under these conditions the value of $n_{1/2}$ is 1.8 and the value of the apparent interaction energy of the $\alpha\beta$ pair is 2780 cal. This apparent free energy of interaction represents of course a minimum value, as we have insisted throughout. The true value exceeds this by an amount determined by the final asymptote of the hypothetical Hill plot in the absence of any conformational change. In accordance with eq 5.8 this excess is given by $RT \ln \overline{k^{-1}}\bar{k}$, which in the present case becomes

$$RT \ln \left(\frac{\nu_1}{k_1} + \frac{\nu_2}{k_2}\right)(\nu_1 k_1 + \nu_2 k_2) =$$

$$RT \ln \left(\nu_1 + \frac{\nu_2}{z}\right)(\nu_1 + \nu_2 z) \cong RT \ln [1 + (10^{-3}/500)][1 + (10^{-3} \times 500)] = RT \ln 1.5 = 240 \text{ cal} \quad (10.6)$$

This almost negligibly small figure results from the fact that ν_2 remains so small (10^{-3}) throughout.[19]

(19) It should be emphasized that the interaction free energy is a measure of the extent to which the introduction of ligand at any degree of saturation is diminished as a result of the previous introduction of ligand. Thus the total free energy of interaction refers to the difference between the free energy (per site) of introducing the last increment of ligand under actual conditions and the value which it would have if there were no intreactions. It is not to be confused with the diminution in the actual work of saturating the macromolecule with ligand which results from the interactions, as formulated in section 4, and represents an integral effect of the interactions. In the present case the latter quantity, per site, is, by eq 4.10

$$\frac{RT}{2} \ln \left[\frac{1 + \nu_2(z^t - 1)}{z^{t/\nu_2}}\right] = \frac{RT}{2} \ln \left[\frac{1 + 10^{-3}(25 \times 10^4 - 1)}{(25 \times 10^4)^{1/1000}}\right] = 1645 \text{ cal}$$

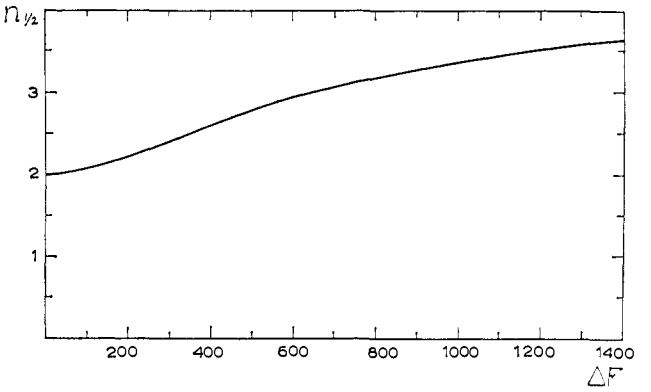

Figure 8. Values of $n_{1/2}$ *vs.* the secondary interaction energy ΔF_I between the $\alpha\beta$ subunits (from calculations in text).

So far our considerations have been directed to the $\alpha\beta$ subunits, which, alone, have an interaction energy of 2700 cal per site and a value of $n_{1/2} = 1.8$. The question next arises as to how much additional interaction energy between these subunits would be required to raise $n_{1/2}$ from this value to the observed value of 2.8–3.0. For this purpose, in order to make the calculations as simple as possible, we treat the $\alpha\beta$ subunits as if they were completely stabilized, with $n = 2$. Then the binding potential degenerates into the simple form

$$\text{J}\!\!\text{I} = RT \ln [\nu_1(1 + k_1^2x^2)^2 + \nu_2(1 + k_2^2x^2)^2] \quad (10.7)$$

Our assumption is equivalent to supposing that the tetramer exists in two conformations in each of which the completely stabilized $\alpha\beta$ dimers have a different oxygen affinity. This is not unreasonable if we suppose that the conformation of the dimers (as determined by oxygenation) affects the equilibrium between the two forms of the tetramer. Differentiation of (10.7) with respect to $RT \ln x^2$ gives the number of oxygenated $\alpha\beta$ units per tetramer. The free energy of interaction, per $\alpha\beta$ unit, is determined by the values of $\nu_1 = 1 - \nu_2$ and k_2/k_1. However, for our present purpose we need not go into this. Equation 10.7 may be used as it stands to obtain the free energy of interaction per double site, *i.e.*, per $\alpha\beta$ subunit, as a function of ρ treated as a parameter. Since in this case of two identical $\alpha\beta$ subunits, each completely stabilized, the ligand equilibrium curve is symmetrical, $x_{1/2} = x_m$, and we obtain the value of $n_{1/2}$ on the basis of eq 10.4 in terms of the same parameter ρ.

$$n_{1/2} = \frac{4}{1 + (1/\sqrt{\rho})} \quad (10.8)$$

This is the same as (10.5) with the factor 2 replaced by 4; this replacement results from the fact that in eq 10.7 J$\!\!$I is expressed as a function of x^2 instead of x. Figure 8 shows values of $n_{1/2}$ plotted against ΔF_I per site as obtained in this way. It is striking what small values of ΔF_I, *e.g.*, 600 cal per site, suffice to raise $n_{1/2}$ from 2 to ~3. The actual case, where the subunits are not completely stabilized ($n_{1/2}$, say, = 1.8 instead of 2) should not be greatly different.[20] This calculation rounds out the discussion given above of the anomalously high values of n observed under conditions where the full hemoglobin molecules are largely dissociated into dimers.

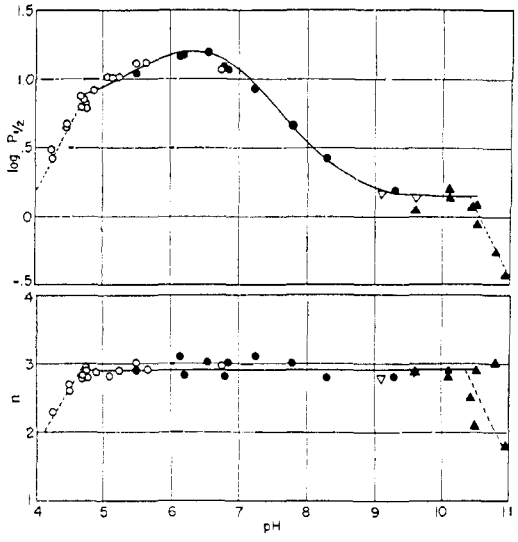

Figure 9. Bohr effect and values of n for human hemoglobin in various buffers in absence of added salt (20°): ●, in 0.2 M phosphate; ○, in 0.4 M acetate; ▽, in 0.05 M borate; ▲, in 0.4 M glycine; from E. Antonini, J. Wyman, A. Rossi Fanelli, and A. Caputo, *J. Biol. Chem.*, **237**, 2773 (1962).

We are now in a position to consider the pH invariance of the ligand equilibrium curves. The total observed Bohr effect for the mammalian hemoglobins, *i.e.*, the change in log $x_{1/2}$ = log x_m (identifying pO_2 with x) between pH 9 and 6.2, is slightly over 1. This means that x_m at pH 6.2 is a little more than ten times what it is at pH 9. If we suppose that this is all due to interactions within the subunits and, as before, set $k_2/k_1 = 500$, we obtain from eq 4.6

$$\frac{(x_m)^2_{pH6}}{(x_m)^2_{pH9}} = \frac{(1 + \nu_2 500^2)_{pH9}}{(1 + \nu_2 500^2)_{pH6}} \cong 100$$

Provided the ν's are not much less than 10^{-4}, this gives

$$\frac{(\nu_2)_{pH9}}{(\nu_2)_{pH6}} \cong 100$$

Reference to Figure 7 shows that a shift of $-\log \nu_2$ by 2 units centered about a mean value of near 3 does not lead to any very significant change of either $n_{1/2}$ or the value of the apparent free energy of interaction. There will not therefore be any very significant change in ΔF_I or $n_{1/2}$ for the whole molecule (*i.e.*, the tetramer) over the whole range of the Bohr effect, and the oxygen equilibrium curve will be essentially pH invariant. Moreover, both at pH 6 and 9 the conformational change of the $\alpha\beta$ subunits accompanying oxygenation will be nearly 100%. On the other hand, at pH <4.7 or >10, where we know the $\alpha\beta$ subunits begin to dissociate into monomers, there should be a sharp drop in n, as well as in x_m, since it is known that the isolated chains have a very high oxygen affinity. Both these expectations are in fact fulfilled in a striking way (see Figure 9).

Since there is a virtually 100% transition from one conformation to the other accompanying oxygenation, it now becomes intelligible why the Bohr effect can be explained so well by the original simple-minded interpretation in terms of two independent oxygen-linked groups per site which have different pK values in the oxygenated and deoxygenated states of the system. Moreover, since in either the oxy or deoxy forms the conformation is virtually fixed ($\nu_2 \cong 1$ or 0), it also becomes intelligible why there are no homotropic interactions between the protons in either form.

It is a striking fact that hemoglobin H (β_4) and various modified hemoglobins in which the interactions are lacking all have a much higher oxygen affinity than native hemoglobin. Indeed almost anything that one does to native hemoglobin tends to increase its oxygen affinity. Moreover, in most of these forms both the homotropic and heterotropic reactions are greatly reduced or lacking. Now there is evidence from the reaction of native hemoglobin with dye that it is the *deoxy* form which is exceptional. It seems likely therefore that in hemoglobin H and the various modified hemoglobins this form is suppressed, or at least greatly reduced, ν_2 approaching 1 instead of being very small as in native hemoglobin. If this were the case we should get just the kind of change in log $x_{1/2}$ which is observed.

Let us consider the value of ln $x_{1/2}$ = ln x_m for an $\alpha\beta$ subunit. This is given by

$$\frac{1}{x_m^2} = k_1^2(\nu_1 + \nu_2 z^2)$$

where as usual $z \equiv k_2/k_1$. Setting $\nu_2 = 10^{-3}$ and $z = 500$

$$\frac{1}{x_m^2} = k_1^2(1 + 500^2 \times 10^{-3})$$

On the other hand, setting $\nu_2 = 1$ and $z = 500$

$$\frac{1}{x_m^2} = k_1^2(500)^2$$

The ratio of the two values of x_m^2 is 1000, and thus we should expect an increase in log x_m of approximately 1.5 as a result of suppressing the deoxy conformation. This is not far from what is actually observed.[21]

(20) The extent of the error introduced by this revealing over-simplification becomes apparent when we write down the full expression for the binding potential of the system. This is

$$\Pi = RT \ln \{\nu_1'[\nu_1(1 + k_1x)^2 + \nu_2(1 + k_2x^2)]^2 + \nu_2'[\nu_1(1 + k_1z'x)^2 + \nu_2(1 + k_2z'x)^2]^2\}$$

where the ν''s refer to the mole fractions of the two conformations of the tetramer in the absence of ligand, and z' gives the factor by which k_1 and k_2 are increased in the tetrameric conformation of higher ligand affinity; this is the basis of the secondary interaction. If we set $\nu_2'/\nu_1' = 1/25$ and $z' = 5$, we then obtain for this secondary interaction a value of 780 cal, and n, for the median point of the equilibrium curve, rises from its value of 1.8 in the isolated pairs to a value of 2.7 in the tetramer, instead of from an ideal value of 2 to about 3, as estimated above. Since the value of $_m$ remains equal to $^1/_2$, the equilibrium curve remains symmetrical. We could of course juggle the figures in various ways, but it is clear that there is not much room if we are to reconcile a value of n approaching 3 with a total interaction energy of only about 3000 cal. The figure 3000, which is obtained from the position of the asymptotes in the Hill plots, is, however, subject to considerable uncertainty and the true value might well be somewhat higher. Indeed a recent reexamination of the Roughton data on sheep hemoglobins gave a value of ΔF_I = 3300 cal; the data of Allen, *et al.*,[14] on human hemoglobin gave a value of ΔF_I = 3600 cal.

(21) There may of course be other direct effects produced by the modifications. A special case is human hemoglobin digested by carboxypeptidase B. In this case the homotropic heme–heme interactions remain unchanged, but the Bohr effect is reduced to about one-third its normal value. Since the conformational change responsible for the homotropic interactions remains, we might explain this by supposing that elimination of the C terminal arginine residue of the α chains by the carboxypeptidase alters the environment of the oxygen-linked acid groups in a critical way, so that they are no longer affected by the change.

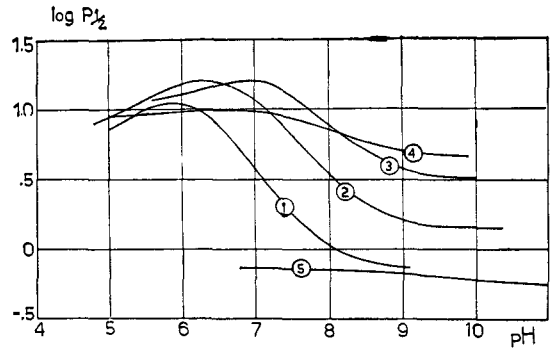

Figure 10. Bohr effect in human hemoglobin as affected by ionic strength: (1) in 2.5×10^{-3} *M* phosphate; (2) at moderate ionic strength (from Figure 9); (3) in buffered 2 *M* sodium chloride; (4) in buffered 5 *M* sodium chloride; (5) given for comparison, for horse or human myoglobin in 0.2 *M* phosphate or 0.1 *M* Tris HCl (from Antonini, *et al.*, in caption to Figure 9).

We conclude this subsection with a consideration of the influence of ionic strength. The effect of ionic strength is primarily on the Bohr effect, the value of n remaining essentially constant except at *very* low ionic strength where it drops nearly to 2. Figure 10 shows the way in which the Bohr effect decreases progressively as the ionic strength is raised until at last it nearly disappears. In a way this is not unexpected if our interpretation is correct. According to assumption the oxygen equilibrium is dominanted by interactions within the $\alpha\beta$ subunits, and the Bohr effect arises wholly within them. We know that at pH 6, where log x_m is a maximum, there can be no difference of proton bound, and therefore no charge difference, between the oxygenated and deoxygenated molecules. Consequently the conformational change within the $\alpha\beta$ subunits accompanying oxygenation (this is the important one) should be unaffected by salt concentration at this pH. However, if the differences in the strength of the oxygen-linked acid groups in the different conformations are due, as seems likely, to electrostatic interactions with neighboring charged groups, we should expect that increasing ionic strength would diminish them and so reduce the Bohr effect in the fashion observed.

A problem remains, however, regarding the secondary interactions between the $\alpha\beta$ subunits, which, although small in terms of interaction energy, are nevertheless decisive in raising the value of n from 2 to nearly 3. The fact that n maintains its high value in strong salt solutions where the oxygenated molecules are largely dissociated means that the intramolecular interactions between the $\alpha\beta$ subunits in the tetramer are largely taken over by intermolecular interactions between these same subunits when they are dissociated. These interactions might either represent the more ordinary type of intermolecular interaction in a solution or a true oxygen-linked association–dissociation equilibrium. The latter possibility, which has much to recommend it, has been analyzed in considerable detail.[2] It would require that a very substantial fraction of the *deoxygenated* molecules be associated as tetramers as long as the value of n remains high. Experiments bearing on this are at present inconclusive, although there is every evidence that the dissociation of hemoglobin in various ways is always a ligand-linked equilibrium.

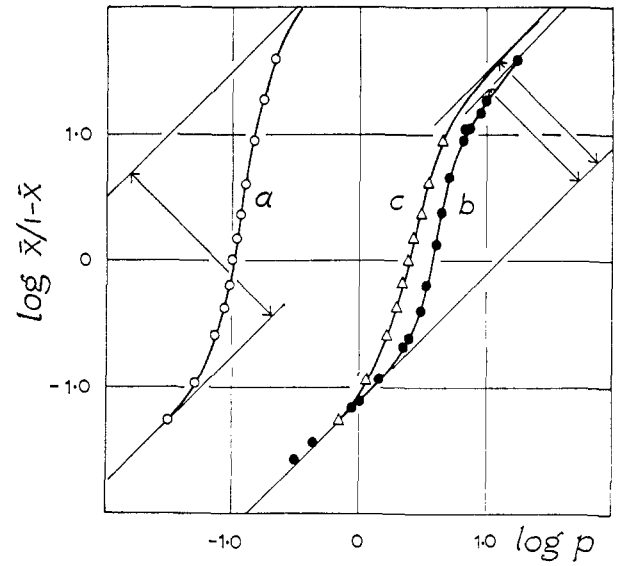

Figure 11. Hill plots of the oxygen equilibrium of three invertebrate pigments. Curve a: Erythrocruorin of *Arenicola cristata* (mol wt $\sim 3 \times 10^6$, number of sites >100) from results of D. W. Allen and J. Wyman, *J. Cell Comp. Physiol.*, **39**, 383 (1952). Curve b: Chlorocruorin as present in the blood of *Spirographis* (mol wt 2.75×10^6, number of sites ~80) from results of E. Antonini, A. Rossi Fanelli, and A. Caputo, *Arch. Biochem. Biophys.*, **97**, 336 (1962). Curve c: Hemocyanin of lobster (*Homerus americanus*) (mol wt ~825,000, number of sites 24) from results of S. M. Pickett, A. F. Riggs, and J. W. Larimer, *Science*, **151**, 1005 (1966). All measurements are at 20°; those on Erythrocruorin at pH 7.8 in 0.67 *M* phosphate buffer; those on Chlorocruorin at pH 7.6 in 0.1 *M* phosphate buffer; those on Hemocyanin at pH 7.7 in 0.05 *M* Tris buffer 0.025 *M* in calcium ion. Curve a, $n = 6$, $\Delta F \cong 3000$ cal; curve b, $n \cong 5$, $\Delta F = 1900$ cal; curve c, $n \cong 4$, $\Delta F = 2000$ cal.

b. The Oxidation–Reduction Equilibrium of Mammalian Hemoglobin. In an over-all way the oxidation–reduction behavior of hemoglobin parallels that with oxygen.[22] Both oxygenation and oxidation are accompanied by conformational changes, and the conformation of oxidized hemoglobin, at least in the crystal, appears to be the same as that of oxyhemoglobin. Both the equilibria show homotropic interactions between the hemes and are subject to a Bohr effect. However, the oxidation Bohr effect is dominated by the ionization of a water molecule which occupies the sixth coordination position of the iron atoms in ferrihemoglobin but is lacking in hemoglobin and oxyhemoglobin; the pK for this ionization is close to 8. Instead of being invariant in shape, the oxidation–reduction curves of hemoglobin become flatter at acid pH, n dropping from about 2.5 at pH 9 to nearly 1 at pH 6. As a related phenomenon, the directly measured apparent interaction free energy falls from close to 1300 cal at pH 8.6 to nearly zero (300 cal) at pH 6. Finally, the oxidation–reduction curves are for the most part *not* symmetrical. On the whole the picture is far easier to reconcile with the allosteric interpretation than is the oxygen equilibrium and indeed represents just about what we should expect for a system subject to allosteric transitions. The very large change in ΔF_I and n between pH 6 and 9 is readily intelligible on the basis of the large effect of the dissociation of the water molecule in ferrihemoglobin, which is not balanced at all by anything in ferrohemoglobin. The

(22) E. Antonini, J. Wyman, M. Brunori, J. F. Taylor, A. Rossi Fanelli, and A. Caputo, *J. Biol. Chem.*, **239**, 907 (1964).

only somewhat puzzling thing is why the homotropic interactions are not somewhat larger; those observed would hardly account for the nearly 100% conformational transition accompanying oxidation, which is indicated by X-ray studies on the crystal.

c. Invertebrate Pigments. Figure 11 contains Hill plots of the oxygen equilibria of several giant invertebrate respiratory proteins, which contain a very large number of sites. In all cases the value of n at half-saturation is high, although the total apparent free energy of interaction is low. Thus for spirographis hemoglobin $n_{1/2}$ is over 5 (as compared with about 3 for mammalian hemoglobin), but ΔF_I is only about 1800. These proteins would seem to offer a beautiful example of the principle brought out in section 6 about the way in which the regulatory sensitivity of an allosteric protein increases with the number of sites. Their behavior has a close bearing on the problem of what Francis Crick temptingly calls "the design of an enzyme."

11. The Occurrence of Allosteric Linkage

It will be seen from the analysis just given that the behavior of hemoglobin, as a test body, is indeed not irreconcilable with the requirements of allosteric linkage. A great number of other cases are also now on record of enzymes which show the characteristic behavior of allosteric proteins.[7] Nevertheless the question persists as to how comprehensive *is* the phenomenon of allosteric control in macromolecules generally? Certainly there must be other kinds of linkage which come into play as well. The binding, and at appropriate pH ionization, of a water molecule as a result of oxidation, which accounts for the very large oxidation Bohr effect in hemoglobin, is a case in point; yet even here the way in which this direct linkage becomes effective in the larger system of interactions in the molecule as a whole, the tetramer, would appear to involve allosteric transitions. Recently another type of mechanism involving long-range forces arising from the polarizability of the hemes has been suggested to explain specifically the interactions exhibited in the oxygen equilibrium of hemoglobin;[23] however, the phenomenon at stake is so general, being in no way limited either to heme proteins or to their oxygen equilibrium, that this explanation, apart from any objections which may be raised against it on grounds of the underlying quantum chemical calculations, is unconvincing. One can hardly refrain from looking for a common mechanism for a common body of phenomena, and one is led, almost by the principle of exclusion, to the conformational interpretation of the many similar cases of interaction and regulation observed in biological macromolecules. Certainly there can be little doubt that allosteric linkage is a phenomenon of widespread occurrence, offering a striking example of the adaptation of structure to function at a molecular level.[24]

Acknowledgments. I express my indebtedness to Professor Jacques Monod, whose introduction of the allosteric concept into the study of enzymes led me to write this paper; also to Dr. Francis Crick for a number of provocative discussions; likewise to Professors John Edsall and Rufus Lumry for various criticisms, and, of course, to my colleagues in Rome. The work was supported by a grant from the National Science Foundation.

(23) Professor Willard Libby, personal communication.

(24) Nevertheless in applying the principle to enzyme systems generally, a certain modicum of caution is necessary. No functioning enzyme can ever, by the very nature of the case, be in true equilibrium, and at best the study of an enzyme system is that of a system in a steady state. The analysis given in this paper is, strictly speaking, limited to systems in true equilibrium, and one should not be too brash in extending it. In any given case the basic question is how far the values of the various velocity constants involved are such that the enzyme substrate complex may be treated as being in equilibrium with the free substrate and free enzyme. When a system is not in equilibrium but in a steady state in which there is a constant degradation of energy, certain unexpected and paradoxical effects become possible.