Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5239–5242, September 1980
Biophysics

Ligand-linked phase changes in a biological system: Applications to sickle cell hemoglobin

(biophysics/functional linkage/polyphasic system)

JEFFRIES WYMAN* AND STANLEY J. GILL†

*Istituto di Chimica Biologica, Citta Universitaria, 00185, Rome, Italy; and †Department of Chemistry, University of Colorado, Boulder, Colorado 80309

Contributed by Jeffries Wyman, May 9, 1980

ABSTRACT **Polyphasic linkage is a close analog of the allosteric and polysteric linkages shown by many biological macromolecules. Like them, it gives rise to both homotropic and heterotropic effects. It is governed by a group of linkage potentials applicable to each separate phase and also, subject to certain conditions, by a group of lower order applicable to the whole system, globally. A good example of polyphasic linkage is provided by sickle cell hemoglobin which, under suitable conditions and subject to control by oxygen, precipitates out of solution to form what appear to be microtubules. This is but one instance of the way in which macromolecular assembly and the formation of subcellular structures generally can be regulated by various small molecules acting as ligands.**

The concept of linkage, both homotropic and heterotropic as it applies to the binding of ligands by a macromolecule in a one-phase system, has been developed and applied in detail to allosteric and polysteric (aggregating) systems in earlier papers (1–4). In the present paper we show how this macroscopic concept finds expression in the more general case of a polyphase system containing any number of phases and components.

Polyphasic linkage

Consider a system consisting of three components X, Y, and Z held at constant temperature and pressure. This may exist as a single phase (2 degrees of freedom) or break up into a number of different phases, of which, however, only three at most can coexist in equilibrium. In that case there is a triple point (0 degrees of freedom). To obtain a clearer picture of the situation, choose Z as the reference component. Then, in each phase the binding potential Π (2) will be a function of the chemical potentials μ_x and μ_y of each of the two other components in accordance with the differential equation

$$d\Pi = \bar{X}d\mu_x + \bar{Y}d\mu_y \qquad [1]$$

where $\bar{X} = \partial\Pi/\partial\mu_x$ and $\bar{Y} = \partial\Pi/\partial\mu_y$ are, respectively, the amounts of X and Y bound (in the most general sense of that term) per unit of Z (in that phase). These barred quantities are thus normalized extensive variables in distinction from the intensive quantities T, p, and the μs. Eq. 1 corresponds, apart from sign, to the Gibbs–Duhem equation, Π being minus the chemical potential of the reference component Z.

When there is only one phase the system will be defined by a two-dimensional surface in three-dimensional Cartesian space where the x and y coordinates give the values of μ_x and μ_y, respectively, and the z coordinate gives the value of Π. When there are two phases, α and β, each will be represented in the same way and the equilibrium of the two will be defined by the line of intersection of the two surfaces. Because this line represents equilibrium, it gives rise to the relationship

$$d\Pi^\alpha - d\Pi^\beta = (\bar{X}^\alpha - \bar{X}^\beta)d\mu_x + (\bar{Y}^\alpha - \bar{Y}^\beta)d\mu_y = 0$$

or

$$\frac{d\mu_y}{d\mu_x} = -\frac{\bar{X}^\alpha - \bar{X}^\beta}{\bar{Y}^\alpha - \bar{Y}^\beta}. \qquad [2]$$

In general, each $\bar{X}$ and $\bar{Y}$ will be a function of both μ_x and μ_y, and integration of this equation gives μ_y as a function μ_x, subject to an undetermined constant which depends on the choice of standard states. Eq. 2, or its integral, defines the projection on the xy plane of the line of intersection of the two surfaces in space and thus provides for a familiar type of phase diagram. On one side of the line one surface lies above the other; on the other side, it lies below it. Depending on the choice of the reference component, the character of the diagram will be different.

It will be noticed that Eq. 2 is the exact counterpart of the Clausius–Clapeyron equation which describes the variation of vapor pressure with temperature for a single substance. Its integration is simplified where there is no XY linkage in either phase, so that the variables are separable. But whether or not this is the case, and, indeed, whether or not analytical expressions for $\bar{X}$ and $\bar{Y}$ are available, we know that the integral must exist. Clearly, depending on the form of the functions $\bar{X}(\mu_x, \mu_y)$ and $\bar{Y}(\mu_x, \mu_y)$, the phase diagram may show all sorts of patterns. We may be sure, however, that for each phase the binding potential Π, by which $\bar{X}$ and $\bar{Y}$ in that phase are determined, will be a single-valued function of μ_x and μ_y.

In accordance with what has just been said regarding equilibrium between two phases, a triple point, representing equilibrium among three phases, will be seen to represent the intersection of three nonparallel surfaces, one for each phase, in three-dimensional space. Any such point corresponds to a unique pair of values of μ_x and μ_y and thus to a particular point on the phase diagram—i.e., in the $\mu_x\mu_y$ plane. Suppose we construct a plane in our three-dimensional space which lies indefinitely close to the triple point and cuts each of the three phase separation lines. The result will be an indefinitely small tetrahedron of which the three faces that intersect at the triple point will become more and more nearly planar as the size of the tetrahedron diminishes—i.e., as the constructed plane moves toward the triple point. The triple point may be seen in terms of the projection of this tetrahedron on the $\mu_x\mu_y$ plane.

So far we have limited ourselves to a system of three components. Suppose now we introduce additional components Q,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: Hb S, sickle cell hemoglobin.

W, ... Then we have a greater number of degrees of freedom and the possibility of a greater number of phases existing together. In each phase, Eq. 1 now becomes

$$d\Pi = \overline{X}d\mu_x + \overline{Y}d\mu_y + \overline{Q}d\mu_q + \overline{W}d\mu_w. \qquad [3]$$

For constant values of μ_q and μ_w this of course reduces to Eq. 2 and everything is the same as before *except* that the constant of integration and, in general, $\overline{X}$ and $\overline{Y}$ will be functions of the new variables. Thus, the character of the phase diagram will depend on these new variables. In this enlarged case the binding potential in each phase will be represented by an $n - 1$ dimensional surface in an n-dimensional space, where n is the total number of components (including the reference component). Thus, that which in the three-component case was a line in a two-dimensional phase diagram becomes an $(n - 2)$-dimensional surface in the n-component case. Similarly, that which was originally a triple point now becomes an $(n - 3)$-dimensional surface. And so on, until we reach a true point in our n-dimensional space, a "multiple point," where the number of degrees of freedom is reduced to zero. Any geometrical or topological considerations applicable to the three-component case can of course, formally, be generalized to the n-component case although it is impossible to visualize n-dimensional space.

From these considerations it is not difficult to see how phase equilibria provide the basis for both homotropic (cooperative) and heterotropic (control) linkage phenomena in a multicomponent system. If in particular the reference component is a macromolecule, we may regard the other components as its ligands, and the phase equilibria play the same role as the allosteric and polysteric equilibria analyzed in previous discussions of linkage. In fact we have a sequence or hierarchy of control mechanisms conceptually all the same, resting as they all do on the same principle of ligand-linked conformational change (in the broadest sense of that term): allosteric (5), polysteric (4), polyphasic.

Phase transitions illustrated by hemoglobin

A glimpse of the way in which these principles find expression in a biological system is provided by sickle cell hemoglobin, Hb S, an abnormal hemoglobin which is the cause of sickle cell anemia in man (6). Although Hb S differs from normal human hemoglobin (Hb A) by only one amino acid substitution in each of the two β chains, its behavior is very different. At concentrations less than about 20%, it is indistinguishable from Hb A, at least in respect to its oxygen-binding properties. At higher concentrations, however, it undergoes an oxygen-linked phase transition (7, 8) which generates a large amount of cooperativity, measured values of the Hill parameter n rising well above the value ≈ 3 characteristic of the solution (9). The system has recently been reinvestigated with the aid of a new method for measuring oxygen binding and analyzed in terms of the foregoing concepts (10, 11).

Briefly, the resulting picture may be described as follows. When a solution of Hb S of sufficiently high concentration is progressively deoxygenated, then at a certain critical oxygen pressure, which depends on the protein concentration, deoxyhemoglobin begins to precipitate, forming a solid phase or gel (Fig. 1). This consists of elongated aggregates, which have been compared with microtubules. If the total amount of water in the system is held constant (that is, if the system is closed with respect to water but open with respect to oxygen), the liquid phase becomes progressively more dilute as deoxygenation proceeds and there will be a corresponding increase in the activity (vapor pressure) of the solvent, water, as a result of the water–hemoglobin interaction which, to a high degree of approximation, is independent of oxygenation. (This means that in the liquid phase there is no oxygen-water linkage mediated by hemoglobin.) By plotting the measured values of μ_{H_2O} against μ_{O_2} we obtain a phase diagram of the type discussed above.

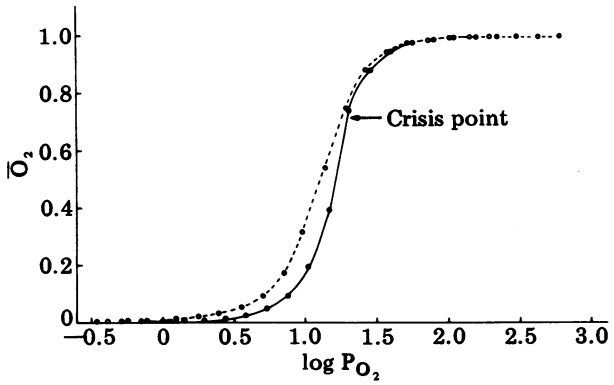

FIG. 1. Oxygen-binding curve (●) of Hb S under gelling conditions (Hb S, 0.30 g/liter). Dotted curve is for Hb S in solution throughout (0.15 g/liter). Arrow marks crisis point where gel phase appears.

Because the total amount of oxygen present in the system is the sum of the amounts present in each phase, from this it is possible to calculate the overall binding curve of the system. We know (or assume) a fixed water content and characteristic binding curve for the solid phase. In the liquid phase the amount of oxygen is given by the binding curve measured at lower concentrations, at which there is no solid phase. (This is known to be independent of concentration, another aspect of the absence of any significant oxygen-water linkage in the liquid phase.) The only additional information required is the activity coefficient of hemoglobin in solution, which can be obtained from osmotic pressure or sedimentation measurements (12). (Note that the activity coefficient is simply an expression of the homotropic interaction operative in water "binding.") Conversely, we could work backwards from determinations of the overall oxygen-binding curve and data on the activity coefficient to obtain the chemical potentials of water and hence construct the phase diagram. To the molecular biologist the case of Hb S is of interest on the one hand as an example of how a ligand-linked phase change can give rise to cooperativity and on the other as an example of how structural changes (e.g., the formation of a new phase) may be introduced and controlled by a set of small ligands.

Phase changes involving $\Delta\overline{H}$ and $\Delta\overline{V}$

We have assumed throughout this discussion that the temperature T and pressure P are held constant. Now S and V may be treated formally as ligands of chemical potential T and $-P$ respectively. Consequently, by substituting T or $-P$ for one of the μs we can obtain what might be called a hybrid phase diagram. Any such diagram will provide us with values of the entropy or volume change associated with the transfer of a particular component from one phase to another. For example, if T is substituted for μ_x and $\overline{S}$ for $\overline{X}$ in Eq. 2, we obtain

$$\frac{d\mu_y}{dT} = -\frac{\overline{S}^\alpha - \overline{S}^\beta}{\overline{Y}^\alpha - \overline{Y}^\beta}. \qquad [4]$$

This equation may also be written in a form more closely related to calorimetry as

$$RT^2\frac{d\ln y}{dT} = -\frac{\overline{H}^\alpha - \overline{H}^\beta}{\overline{Y}^\alpha - \overline{Y}^\beta}$$

where y denotes activity and $\overline{H}$ is heat content (per mol of macromolecule). In applying it, it must be remembered that the chemical potentials of other ligands, such as X, are to be held constant.

A more general formulation

The case presented by Hb S is particularly simple because there is only one liquid phase, and in the liquid phase there is no water-oxygen linkage. But the principles involved in its analysis are the same as in the general case. Let us spell them out in greater detail.

Suppose there are two phases, α and β, and three components. Choose one of these as the reference component; the other two, X and Y, may be regarded as its ligands. Then the system, as it moves along the phase line, is subject to the following equations, in which brackets stand for a functional relationship:

$$\begin{aligned} \mu_y &= [\mu_x], \\ \overline{X}^\alpha &= [\mu_x], \\ \overline{X}^\beta &= [\mu_x], \\ \overline{Y}^\alpha &= [\mu_x], \\ \overline{Y}^\beta &= [\mu_x] \\ \overline{X} &= f\overline{X}^\alpha + (1-f)\overline{X}^\beta \\ \overline{Y} &= f\overline{Y}^\alpha + (1-f)\overline{Y}^\beta. \end{aligned} \quad [5]$$

Here, $\overline{X}$ and $\overline{Y}$ are the total amounts of X and Y in the whole system per unit of reference component, and f represents the fraction of the reference component (macromolecule) present in phase α. The first five equations result from the equilibrium between the two phases and from the binding potential for each phase; the two other equations are stoichiometric. If the system is open with regard to both X and Y, as it is when X and Y are free to pass in and out of the system as μ_x is varied, then of course, although μ_y, $\overline{X}^\alpha$, $\overline{X}^\beta$, $\overline{Y}^\alpha$, and $\overline{Y}^\beta$ are all uniquely determined by μ_x, we can say nothing as to f and, consequently, the total amounts of the two ligands bound per macromolecule—namely, $\overline{X}$ and $\overline{Y}$.

This situation is realized when we start from a point P_1 in the phase diagram (Fig. 2) and add increasing amounts of X while the system is exposed to an infinite reservoir of Y of chemical potential μ_y. As $\overline{X}$ increases, so does μ_x until it reaches the value μ_x^* on the phase line. At this point, phase β makes its appearance and the reference component begins to enter that phase. Because in general $\overline{X}^\alpha \neq \overline{X}^\beta$ and $\overline{Y}^\alpha \neq \overline{Y}^\beta$, the transition will be accompanied by a change of $\overline{X}$ and $\overline{Y}$ at constant μ_x and μ_y. The process, which we might describe as crossing the phase line, will continue until phase α is exhausted. During its course, the two-phase system will show infinite cooperativity of ligand binding like a simple allosteric system containing an infinite number of binding sites (3). Because S and V may be regarded as ligands, the process will be the exact counterpart of the melting of ice or the vaporization of water. We know that the relative amounts of the two phases—e.g., ice and water—are completely undetermined at equilibrium. In order to specify them it is necessary to fix the total volume (or entropy) of the system. In the same way, in the case of ligands, it is necessary to specify either $\overline{X}$ or $\overline{Y}$ in order to fix f and consequently to determine the amount of the other. When this is done, then instead of crossing the phase line, the system will move along it as in the experiments on Hb S described above.

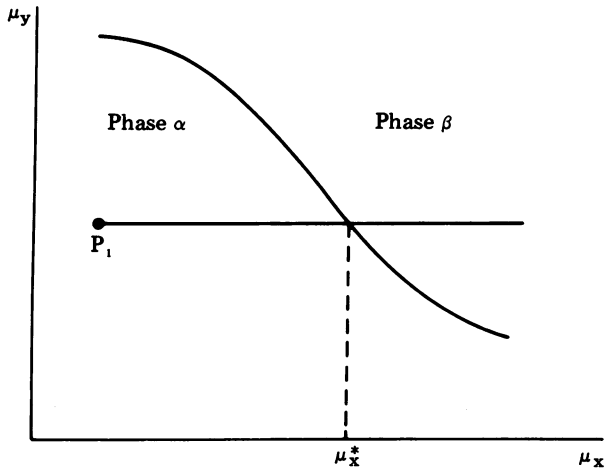

FIG. 2. Phase diagram. See text.

What happens when we have a fourth component W (as a third ligand)? Then, our equations become expanded to

$$\begin{aligned} &\mu_y = [\mu_x, \mu_w], \overline{X}^\alpha = [\mu_x, \mu_w], \ldots, \overline{W}^\beta = [\mu_x, \mu_w] \\ &\overline{X} = f\overline{X}^\alpha + (1-f)\overline{X}^\beta \\ &\overline{Y} = f\overline{Y}^\alpha + (1-f)\overline{Y}^\beta \\ &\overline{W} = f\overline{W}^\alpha + (1-f)\overline{W}^\beta. \end{aligned} \quad [6]$$

Everything is just the same except that we now have one more degree of freedom and the binding curve for Y depends not only on μ_x but also on μ_w (or $\overline{W}$). Clearly, the result can be generalized to any number of the components and phases. For each additional phase it is necessary to know another f in order to specify the relative amounts of the phases and hence define the state of the system. This can be achieved by specification of the total amount of any additional normalized extensive property ($\overline{Y}$, $\overline{S}$, $\overline{V}$, etc.)

As an illustration of these ideas we have, in the last section, chosen Hb S, where the phase behavior is controlled by the true chemical binding of a ligand (oxygen). Another quite different example is provided by a mixture of two largely immiscible ligands such as benzene and water with added solutes, say alcohol and acetone. Here there is no chemical binding, and in this special case the two liquid phases are sufficiently dilute to be describable by Henry's and Raoult's laws. This facilitates formulation of the binding potential for each individual phase. Both examples bring out the complexities involved in any rigorous definition of solubility.

On the existence of a binding potential in polyphasic systems

In the case of a one-phase system, we know of course that there is always a binding potential Π, which is minus the chemical potential of the reference component and is but one member of a group of linkage potentials applicable to the system. The question arises as to whether anything like this holds in a polyphasic system.

By specifying the total volume V, total entropy S, and total amount of each of the n components of a thermodynamic system of any given number of phases, we completely determine its state. (In case this is not clear it will become so from a consideration of Eq. **5** or **6**.) This means that the total energy and the total amount of each phase are determined by these $n + 2$ extensive variables. From the energy function so defined we obtain values of the corresponding $n + 2$ intensive quantities T, P, and the chemical potentials μ of all the components as the appropriate first partial derivatives. (Remember that formally we may treat entropy and volume as components of "chemical potential" T and P, respectively.)

We can always apply a set of Legendre transformations to the energy function as defined above to obtain a set of new functions in which some of the extensive variables are replaced by corresponding intensive ones. These functions are a source of useful linkage relations. But in doing this we are limited to such transformations as lead to variables in terms of which the state of the system—in particular, the sizes of the phases and

the value of the energy—are defined. In the case of a one-phase system this prevents our passing from the energy function defined in terms of the $n + 2$ extensive variables to its opposite defined wholly in terms of the intensive ones. It is an expression of the fact that the energy is first-order homogeneous in the extensive variables, with the result that its opposite does not exist. (This means that the Jacobian of its first partial derivatives—in other words, the Hessian—vanishes; and it is this which is the source of the Gibbs–Duhem equation.)

In the case of the one-phase system this difficulty can be overcome by fixing the value of one of the extensive variables, which we identify as the reference component (usually a macromolecule). The result is that the energy, now normalized as the energy per unit reference component, becomes a function of $n + 2 - 1$ normalized extensive variables. This function cannot be first-order homogeneous and is therefore susceptible to all possible Legendre transformations. These transformations form a group of order 2^{n+2-1} which is isomorphic with the group of potentials which it generates and has the symmetry of an $n + 2 - 1$ dimensional rectangle in hyperspace. Of this group, the binding potential Π is a particularly useful member.

In the case of a polyphasic system (p phases) it is necessary to fix the value of one additional extensive variable for each added phase in order to determine the relative sizes of the phases, and consequently the number of extensive variables susceptible to transformation is reduced from $n + 2 - 1$ to $n + 2 - p$, with the result that the order of the group of permitted Legendre transformations and the corresponding group of linkage potentials is reduced to 2^{n+2-p}. Again the group will have the symmetry of a multidimensional rectangle, in this case $n + 2 - p$. It will be seen that $n + 2 - p$ is the number of degrees of freedom of the system as given by the phase rule in its familiar form.

A p-phase system in which the amounts of p components are fixed, so that the system is characterized by a 2^{n+2-p} order group of linkage potentials, may be described as "well defined." (We might also describe it as being normalized with respect to p extensive variables). Now it will be seen that, by fixing the total amounts of p components, we are in fact combining them into a single multiple component (recall the definition of a component). Thus, the binding potential Π of a well-defined system may be seen as minus the chemical potential of a multiple component whose properties are a weighted mean of those of its constituents. Indeed, looked at entirely from the outside there is really no difference between a well-defined one-phase system of two components and a well-defined two-phase system of three components, or, more generally, between a well-defined p-phase system of n components and a well-defined $(p + 1)$-phase system of $n + 1$ components.

These considerations apply to any system of p phases. It should be realized, however, that any point at which a new phase makes its appearance will be a singular point—although the energy and its first derivative are continuous, the second and higher order derivatives are not. Such a point was observed by Gill *et al.* (10) in experiments on the oxygen binding of Hb S and was called a "crisis point" (see Fig. 1). It was the point at which the gel first made its appearance.

If the system is well defined only up to a certain number of phases, then when additional phases are introduced it becomes indeterminate and this will be reflected in its geometrical representation in hyperspace. Thus, the famous ruled surface in 3-space described by Gibbs will have its counterpart in a corresponding surface in hyperspace. All this points to the way in which linkage and cooperativity can be seen in terms of the geometry and topology of a multidimensional plot. For an essay on the relationship between thermodynamics and geometry see the article by Weinhold (13).

Comparison of polyphasic and allosteric systems

The preceding considerations bring out the close analogy between an allosteric system and a polyphasic one. Consider a two-phase system. The parameter f (see Eqs. **5** and **6**), which specifies the distribution of the reference component between the two phases, corresponds exactly to the single ν (given by L) which represents the conformational equilibrium in the case of a two-state allosteric system (5). Subject to activity coefficients, which are uniquely determined by the binding potentials in each phase, it is an equilibrium constant, and, just as in the allosteric case, its value is determined by the chemical potentials of the various ligands (or their amounts per unit of reference component) in each of the phases.

In the case of a p-phase system there are $p - 1$ fs, corresponding to $p - 1$ equilibrium constants, to be determined; analogously, in the case of an allosteric system, where there are r conformations, there are $r - 1$ Ls (or ν ratios) to be determined. Thus, in either case we are concerned with the effect of a ligand, or set of ligands, on an equilibrium constant. The only difference between the two cases is a practical one involving activity coefficients. In the allosteric case, where the macromolecule is present in a single phase, we may safely forget them, because, to a high degree of approximation, they cancel out. In the polyphasic case this is not so. The most familiar biological example of a polyphase system is whole blood which, with good approximation, may be treated as a two-phase system consisting of erythrocytes and plasma. It is interesting that in the case of sickle cell anemia there is appearance and disappearance of a new phase within the erythrocytes during the course of circulation. This might be taken as an example of a conformational change within a conformational change such as has sometimes been postulated in the case of allosteric systems.

All this brings out two things. In the first place, from a formal and operational point of view, it establishes the broad generality of the concept of a group of linkage potentials, which apply equally to any well-defined system, whether of one, two, or more phases. In the second place, from a physical point of view, it reveals the underlying sameness of three mechanisms of regulation and control in biological systems—allosteric, polysteric, and polyphasic—all of which rest on the principle of ligand-linked "conformational" change.

The authors would like to record their indebtedness to W. G. Bardsley and Richard Woolfson for valuable discussions during their visit to Rome. This work was supported by National Science Foundation Grant PCM 772062 to J.W. and National Institutes of Health Grant HL 22325 to S.J.G.

1. Wyman, J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1464–1468.
2. Wyman, J. (1965) *J. Mol. Biol.* **11**, 631–644.
3. Colosimo, A., Brunori, M. & Wyman, J. (1974) *Biophys. Chem.* **2**, 338–344.
4. Colosimo, A., Brunori, M. & Wyman, J. (1976) *J. Mol. Biol.* **100**, 47–57.
5. Monod, J., Wyman, J. & Changeux, J. P. (1976) *J. Mol. Biol.* **12**, 88–118.
6. May, A. & Huehns, E. R. (1976) *Br. Med. Bull.* **32**, 223–233.
7. Minton, A. P. (1976) *J. Mol. Biol.* **100**, 519–542.
8. Hofrichter, J., Ross, P. D. & Eaton, W. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3035–3039.
9. Gill, S. J., Sköld, R., Fall, L., Shaeffer, T., Spokane, R. & Wyman, J. (1978) *Science* **201**, 362–364.
10. Gill, S. J., Benedict, R. C., Fall, L., Spokane, R. & Wyman, J. (1979) *J. Mol. Biol.* **130**, 175–189.
11. Gill, S. H., Spokane, R., Benedict, R. C., Fall, L. & Wyman, J. (1980) *J. Mol. Biol.* **140**, 299–312.
12. Ross, P. D. & Minton, A. (1977) *J. Mol. Biol.* **112**, 437–452.
13. Weinhold, F. (1976) *Phys. Today* **29**(3), 23–30.