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THE CYBERNETICS OF BIOLOGICAL MACROMOLECULES *

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This paper develops the concept of linkage as it applies to the binding of ligands by a polyfunctional macromolecule, or system of macromolecules, under equilibrium, steady-state, and transient conditions.

1. Introduction

It used to be said, in accordance with Newtonian philosophy, that everything in this world of ours is to some degree related to everything else. That was before the days of Heisenberg and the uncertainty principle. But I suppose that at a macroscopic level the same may still be maintained. Nowhere is this interdependence of macroscopic events more apparent than in living organisms, whose very name stems from recognition of their high degree of organization; and nowhere in the organism is this more evident than in the polyfunctional macromolecules of which it is composed. Here the interrelated events to a large extent involve and depend upon the specific binding of smaller molecules which may be regarded as ligands. These may be such simple inorganic substances as proton, oxygen, CO_2, phosphate or other ions, which react with specific sites in the macromolecule, as in the respiratory proteins; or they may be more complex substances such as hormones and neurotransmitters, or even other macromolecules. The interactions may involve the successive binding of molecules of a given ligand by a set of sites specific for that ligand, or the binding of different ligands by their many sites. On the one hand, we speak of the interactions as homotropic; on the other, as heterotropic. Both types are associated with phenomena of cooperativity and anticooperativity. Not only do these linkage effects, as they are called, involve the interdependence of the binding of small ligands by a given macromolecule, but they also involve the control of reactions *between* macromolecules. Thus, whenever a given reaction involving macromolecules leads to an uptake or release of a given ligand, then owing to a difference between the binding of that ligand by the different macromolecular species involved, the equilibrium constant will be a function of the activity of the ligands. This applies equally to polymerization or simple conformational change, and indeed, in polyphase systems, to the distribution of a macromolecule between the many phases. It is basic to the phenomena of macromolecular assembly.

In this paper I give a brief account, in terms of simple macroscopic concepts, of these cybernetic phenomena (for that is what they really are) as they show up under equilibrium, steady-state, and transient conditions. The simplest case is that of a one-phase system which exists in a state of thermodynamic equilibrium, and we begin with that.

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2. A one-phase system at equilibrium

In this case we know that the total energy E is uniquely determined by values of the total volume V, total entropy S, and total amount X of each of r components: $E=E(S, V, X_1, \ldots X_r)$. From this the temperature T, pressure p, and the chemical potential μ of each component are obtained as the corresponding first partial derivatives:

$$T=\partial E/\partial S, \quad p=-\partial E/\partial V, \quad \mu_i=\partial E/\partial X_i. \tag{1}$$

It is to be noticed that E has one particularly important property: if each of the variables $(S, V, X_1, \ldots X_r)$ is multiplied by a factor α, E is multiplied by the same factor. This means that E is first-order homogeneous in those variables. It follows that each of the above derivatives is zero-order homogeneous in the same variables—its value is independent of the size of the system. These derivatives are said to be intensive (or internal), as distinct from S, V and X_i, which are extensive (or external).

From eq. (1) we can, by equating cross derivatives, obtain a variety of linkage relations such as

$$\partial\mu_i/\partial X_j=\partial\mu_j/\partial X_i, \quad \partial\mu_i/\partial S=\partial T/\partial X_i. \tag{2a}$$

Such relations serve to reduce the number of experimental quantities required to describe the system, and often make it possible to substitute an easier measurement for another more difficult one.

The list of linkage relations such as eq. (2a) may be greatly increased by application of a set of Legendre transformations to the energy function to obtain a corresponding set of new functions, or potentials, in which one or more of the extensive variables is replaced by a corresponding intensive one. For example, one such transformation would be to $\phi=E+pV-TS-\mu_1X_1=\phi(p, T, \mu_1, X_2, \ldots)$, which has the property that $d\phi=V\,dp-S\,dT-X_1\,d\mu_1+\mu_2\,dX_2+\ldots$ and yields a variety of new linkage relations such as

$$(\partial X_1/\partial X_2)_{p,T,\mu_1,\ldots}=-(\partial\mu_2/\partial\mu_1)_{p,T,X_2,\ldots} \tag{2b}$$

It will be seen that these potentials occur in pairs of opposites, with one exception. Owing to the fact that the energy is first-order homogeneous, its opposite, in which all the extensive variables are replaced by corresponding intensive ones, vanishes identically. That this should be so is obvious from physical considerations, for it is clear that from the values of p, T, and the chemical potentials μ of the components of a system nothing can be inferred as to its absolute size.

In view of this it is convenient for many purposes to choose one of the components as a reference component, and express the energy and all the extensive variables of which it is a function as amounts per unit of reference component [2,3]. This is possible because of the first-order homogeneous property of the energy. It reduces the number of independent variables by one and gives us a function, the "normalized" energy, whose opposite *does* exist, and to which all possible Legendre transformations are applicable. These tranformations form an abelian group, of order 2^{r+2-1} and having the symmetry of an $(r+2-1)$-dimensional rectangle, which is isomorphic with the group of potentials which it generates. From this group all possible linkage relations applicable to the normalized system may at once be obtained. The opposite of the energy normalized with respect to the amount of any one of the chemical components is, except for sign, the same as what has been called the binding potential, or linkage, and denoted by Π. It has the property that

$$\partial\Pi/\partial\mu_i=\bar{X}_i=\partial\Pi/RT\,\partial\ln x_i \tag{3a}$$

where x_i is the activity of component i and $\bar{X}_i$ is its amount per unit of reference component (macromolecule). Similarly, $\partial\Pi/\partial T=\bar{S}$ and $\partial\Pi/\partial p=-\bar{V}$. From this, by equating cross derivatives, we obtain such linkage relations as

$$\partial\bar{X}_i/\partial\mu_j=\partial\bar{X}_j/\partial\mu_i, \quad \partial\bar{S}/\partial\mu_i=\partial\bar{X}/\partial T.$$

By introducing another member of the group, e.g. $\Pi'=\Pi-\bar{X}_2\mu_2=\Pi'(T, p, \mu_1, \bar{X}_2, \mu_3, \ldots)$ for which

$d\Pi' = \bar{S}\,dT - \bar{V}\,dp + \bar{X}_1\,d\mu_1 - \mu_2\,d\bar{X}_2 + \ldots$ and again taking account of the equality of cross derivatives we obtain

$$(\partial\bar{S}/\partial\bar{X}_2)_{T,p,\mu_1,\ldots} = -(\partial\mu_1/\partial T)_{p,\mu,X_2,\ldots}, \tag{3b}$$

as the analogue of eq. (2b) applicable to the normalized system.

An example of the power of this approach is provided by one of the components of trout hemoglobin (Hb trout I) [4]. A Hill plot of CO binding by this hemoglobin as measured at two temperatures and fixed pH is shown in fig. 1. If we multiply both sides of eq. (3b) by T and introduce the relation $RT\,d\ln p_{CO} = d\mu_{CO}$ we obtain $(\partial\bar{H}/\partial\bar{CO})_{T,pH} = -RT^2(\partial \ln p_{CO}/\partial T)_{\bar{CO},pH}$ where $\bar{H}$ denotes heat content per heme equivalent. When applied to the results shown in fig. 1 this tells us at once that there is a change of sign in the heat of CO binding by this four-site molecule from a negative to a positive value as the reaction proceeds. This surely reflects some profound change in the macromolecule accompanying ligation. The subject of ligand-induced changes in the state of a macromolecule as a basis of linkage will be discussed in section 3. In order to prepare the way for it we pause to consider the effect of the ligands on the equilibrium constant for a reaction involving several macromolecules.

It is easily shown, by comparison with the Gibbs-Duhem equation, that Π is equal to minus the chemical potential of the reference component expressed as a function of the intensive variables p, T, and the chemical potentials μ, of each of the ligands. This provides the basis for a general formulation of the equilibrium constant for a reaction involving a set of macromolecules. Take the simple case of the reaction A → B. For this reaction we can write

$$\Pi_B - \Pi_A = -\Delta G_0 = RT \ln K,$$

K being the equilibrium constant. Consequently

$$K = K_0 \exp[(\Pi_B - \Pi_A)/RT], \tag{4}$$

where K_0 is a constant depending on the choice of standard states. Since the two Πs are functions of p, T, and the chemical potentials of all the ligands, eq. (4) embodies the dependence of the equilibrium constant

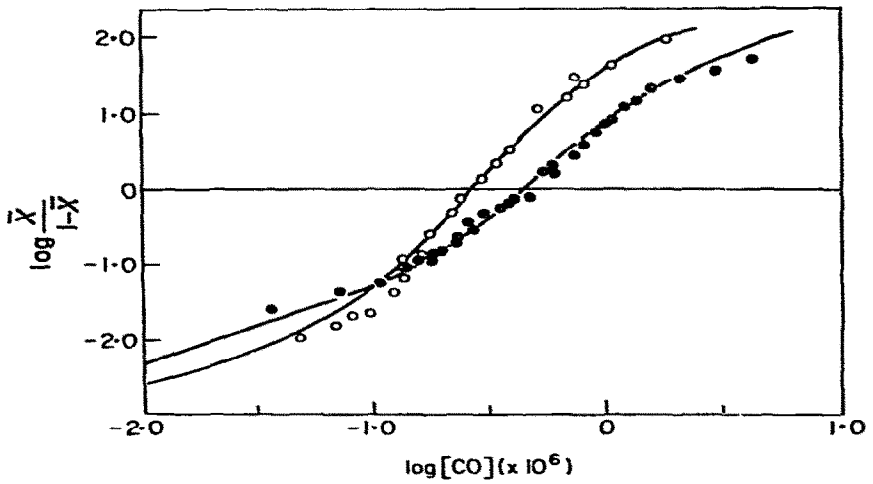


Fig. 1. Hill plots for the binding of CO by deoxy Hb trout I at 20°C (●) and at 4°C (○), in 0.1 M phosphate buffer at pH 6.8.

for the macromolecular reaction on all these quantities. Written in differential form

$$RT\,\mathrm{d}\ln K=\mathrm{d}\Pi_B-\mathrm{d}\Pi_A=(\bar{S}_B-\bar{S}_A)\,\mathrm{d}T-(\bar{V}_B-\bar{V}_A)\,\mathrm{d}p+\Sigma(\bar{X}_{iB}-\bar{X}_{iA})\,\mathrm{d}\mu_i. \tag{5}$$

it becomes an immediate source of the van 't Hoff equation for the reaction as it occurs at constant p and constant values of the chemical potentials of the ligands; alternatively it shows how K varies with the activity of a given ligand in accordance with the difference between the binding of that ligand by two macromolecules at a given temperature. Furthermore, introducing the equality of the cross derivatives, it shows how the heat of the reaction varies with the chemical potentials of the ligands and provides other similar relations. It is, in particular, applicable to the case where a macromolecule exists in several different forms (or conformations) which differ in their binding properties for their ligands. Likewise, if we treat entropy (and heat) as ligands, it governs the effect of temperature on thermal transitions of a macromolecule such as the melting of DNA [5] or the reversible denaturation of a protein. More generally it applies to polymerization, or to such reactions as the reversible combination of an antigen with its antibody, or, finally, to macromolecular assembly. A good example of the latter is provided by certain of the hemocyanins which consist of disparate subunits whose association is sensitive to various ions and to oxygen [6].

3. Linkage mechanisms

Up to now our discussion has been purely phenomenological. Like all thermodynamic analyses it has had nothing to say about mechanism, that is, about the molecular basis for the various linkage effects. Moreover, due to its complete generality, it has not distinguished true binding, governed by mass law relations, from binding represented by activity effects. Indeed, in borderline cases it is hard to do so. Sometimes, to be sure, there are specific and obvious spectral changes associated with the true binding of a ligand, as in the case of oxygen binding by the respiratory proteins; also there may be NMR signals associated with the binding of specific ions. But this is by no means always the case, and more often than not we lack, as it were, the necessary "spectacles" to distinguish sharply between the two.

Why is it, in the case of true binding, that the presence of a molecule of ligand at one site in a large molecule should affect the probability of the binding of another molecule of ligand, the same or different, at another site, perhaps very far away? Sometimes of course this may be due to gross electrostatic effects, as in the case of proton binding by a large polybasic acid such as a protein. But this cannot be an explanation of the positive homotropic cooperativity observed in many respiratory proteins and enzymes. Nor can such cooperativity be explained on the basis of the instability of free radicals as in the case of the *nearly* two-step oxidation of many small organic molecules such as the quinones.

It has now been fairly well established that the explanation of a great variety of linkage effects involving often widely separated sites in large molecules is to be found in ligand-induced changes in the physical state of the macromolecule. Such changes may be purely conformational, without change of molecular weight, or they may involve aggregation or dissociation. In the former case, which is the simpler one to deal with, they are called allosteric; in the latter, polysteric. Conceptually the picture is extremely simple, based on the general principle that whenever to a macromolecular system at equilibrium a given ligand is added, the equilibrium will shift in such a way as to favor the forms which have the higher affinity for the ligand so that the total work of liganding is diminished. This immediately provides a qualitative explanation of why homotropic interactions so often show positive cooperativity whereas the heterotropic ones can be either positively or negatively cooperative [7].

The concept may be made more precise by introduction of the relations given above for the effect of a ligand on an equilibrium involving macromolecules [eqs. (4) and (5)]. This is particularly simple in the case of an allosteric system where the macromolecule exists in a number of different conformations 1, 2, ... t.

In this case the amount of ligand X bound per macromolecule is given by

$$\bar{X}=(M_1\bar{X}_1+M_2\bar{X}_2+\dots M_t\bar{X}_t)/(M_1+M_2+\dots M_t),$$

where each M denotes the concentration of macromolecule in a particular conformation. From this, by assuming the activity of each form of the macromolecule to be proportional to M and writing the equilibrium constant for the transformation from conformation 1 to conformation i as $K_{1i}=(K_{1i})_0 \exp[(\Pi_i-\Pi_1)/RT]$ and $\bar{X}_i$ as $\partial\Pi_i/\partial\mu_X$ we obtain

$$\bar{X}=\frac{\exp(\Pi_1/RT)\partial\Pi_1/\partial\mu_X+(K_{12})_0\exp(\Pi_2/RT)\partial\Pi_2/\partial\mu_X+\dots(K_{1t})_0\exp(\Pi_t/RT)\partial\Pi_t/\partial\mu_X}{\exp(\Pi_1/RT)+(K_{12})_0\exp(\Pi_2/RT)+\dots(K_{1t})_0\exp(\Pi_t/RT)}$$

$$=(\partial\ln/\partial\mu_X)\left[\exp(\Pi_1/RT)+(K_{12})_0\exp(\Pi_2/RT)+\dots(K_{1t})_0\exp(\Pi_t/RT)\right]$$

$$=(\partial\ln/RT\partial\ln x)\left[\nu_{10}\exp(\Pi_1/RT)+\nu_{20}\exp(\Pi_2/RT)+\dots\nu_{t0}\exp(\Pi_t/RT)\right],$$

where the ν_0 s denote the mole fractions of the macromolecule in the various conformations in the absence of ligand (or where the ligand is in some specified standard state). From this, taking account of the relation $\partial\Pi/\partial\mu_X=\bar{X}$ applicable to the *whole system*, we obtain as binding potential for the whole macromolecule the expression

$$\Pi=RT\ln\sum_i\nu_{i0}\exp(\Pi_i/RT). \tag{6}$$

Here Π is a function of p, T, and the chemical potentials μ of all the ligands; when differentiated partially with respect to any one of these it gives the value of the corresponding extensive variable expressed per unit of reference component (macromolecule). (It should be noted that when we differentiate with respect to temperature the factor RT is to be treated as a constant. This is due to the way in which activities are defined in terms of chemical potentials.)

Except for the approximation involved in taking the activity of each form of the macromolecule as proportional to its concentration, eq. (6) is quite general and applies equally well to true binding or to binding associated with activity effects or to a combination of the two. But when we limit ourselves to true binding, say of ligand X at constant p and T, the equation may be expressed in a more readily applicable form. If we assume that in each conformation there are q independent sites and that the binding to each is described by a simple mass law equation, then

$$\bar{X}_i=k_{i1}x/(1+k_{i1}x)+\dots k_{it}x/(1+k_{it}x)=\partial\Pi_i/\partial\mu_X=\partial\Pi_i/RT\partial\ln x$$

and integration gives

$$\Pi_i=RT\ln(1+k_{i1}x)(1+k_{i2}x)\dots(1+k_{iq}x).$$

As a result eq. (6) becomes

$$\Pi=RT\ln\sum_i\nu_{i0}(1+k_{i1}x)(1+k_{i2}x)\dots(1+k_{iq}x). \tag{7}$$

We have only to differentiate this with respect to $\mu_X=RT\ln X$ to get the total binding of X per macromolecule. If we further assume, as in the so-called MWC model, that there are only two (quarternary) conformations and that the sites in each are identical as well as independent, then eq. (7) becomes

$$\Pi=RT\ln\left[\nu_{10}(1+k_{11}x)^q+\nu_{20}(1+k_{12}x)^q\right]=RT\ln\left[(1+k_{11}x)^q+L_0(1+k_{12}x)^q\right], \tag{8}$$

where $L_0=\nu_{20}/\nu_{10}$. In this special case it follows from eq. (8) that the binding curve ($\bar{X}$ versus $\ln x$) is at every point steeper than a simple titration curve. More generally, quite apart from the shape of the curve, it is clear that the total free energy of binding is always reduced as a result of the allosteric transitions, which we might describe as a progressive yielding of the macromolecule to the ligand.

It will be seen that the expansion of the sum in eq. (7) always gives rise to a polynomial. This may or may not be factorable into subpolynomials with real positive coefficients (no others are physically acceptable). If it *is* completely factorable into first-degree factors the allosteric macromolecule will be indistinguishable in terms of functional binding from a simple molecule with q independent sites. In this case the binding curve (normalized to a single site) can nowhere be steeper than a simple titration curve: conversely, if at any point the curve *is* steeper than a simple titration curve, we know at once that the polynomial is *not* completely factorable and that there are site interactions. A "prime" factor (with real positive coefficients) may be interpreted in terms of a set of interacting sites. The question of the factorability of the binding polynomial has been the subject of much discussion. It takes one back to the nineteenth century algebra of Cayley and Sylvester [7].

When we take account of the presence of other chemical ligands the polynomial will include other activities, one for each ligand. Here again the concept of factorability comes in. If this mixed polynomial is factorable into separate polynomials, one for each ligand, it means of course that the ligands are independent. Otherwise they will be either positively or negatively linked.

The behavior of various hemoglobins and some other macromolecules has been exhaustively studied, both kinetically and under conditions of equilibrium, in accordance with these concepts. In the case of human hemoglobin (HbA) the oxygen binding curve at fixed pH can be surprisingly well accounted for on the basis of the simplest allosteric model—the MWC model with only two conformations in each of which the four oxygen combining sites are the same and independent, and the existence of two such quarternary conformations has been confirmed by X-ray crystallography. In HbA, however, studies of the heterotropic influence of other ligands, particularly protons, when analyzed in terms of the allosteric model, suggest that there must be additional allosteric effects local to each of the four subunits (the 2α and 2β chains). It is an easy matter to expand the formal relations developed above to take account of such a situation and indeed to generalize eq. (6) to include a hierarchy of nested conformational changes, one inside the other. To do this we have only to write eq. (6) as $\Pi/RT = \ln \Sigma \nu_{10} \exp(\Pi_1/RT)$ and express each Π_1/RT in the same way. Upon continuing the process we have an expression which is reminiscent of Mandelbrot's [8] geometrical concept of fractals ("scaled fractals"); patterns which look the same seen under any degree of magnification.

From a teleological point of view heterotropic interactions, as a basis of regulation and control at a molecular level, are of the greatest importance, particularly in enzymes, and the allosteric mechanism which makes them possible must be regarded as a major development in macromolecular evolution. Homotropic interactions too, represented in the steepening of the binding curve for a given ligand, say oxygen in the case of the respiratory proteins, can be of great value in transport phenomena. The steepness of a binding curve is limited by the number of sites in the macromolecule, or in its subunits. In the case of some of the larger hemocyanins and some of the giant invertebrate hemoglobins, the number of sites may exceed 100, and the steepness of the binding curve may be very great. Closer consideration, however, suggests that these super-large molecules do not change conformation as a unit, but exist in constellations of sites forming more or less independent allosterically functioning subunits [10]. A possible reason for this might be a kinetic one; the relaxation time associated with a conformational change would be expected to increase with the size of the unit (think of the effect of size and shape on the rotary diffusion of a macromolecule) and if it were too great it might impair the efficiency of the circulating vehicle of transport. There are indications that in the kinetics of the oxygenation of the hemocyanins the conformational change is rate limiting, just the opposite of what is found in hemoglobin [11].

4. Polysteric and polyphasic linkage

When we come to polysteric linkage, involving the ligand-induced association or dissociation of a macromolecule, the underlying principles remain the same, but the formalism is more complex, and it is no

longer possible to formulate the binding potential in terms of a single polynomial like that given above [12,13]. A typical case is provided by human hemoglobin. Under non-physiological conditions, namely at very high dilution, this hemoglobin undergoes an oxygen-induced dissociation into dimers ($\alpha\beta$ subunits) and the tetramer-dimer equilibrium gives rise to much the same cooperativity of oxygen binding as the normal allosteric change [14]. In sickle cell hemoglobin (HbS), which is the cause of the genetic disease known as sickle cell anemia in man, a similar polysteric linkage is greatly magnified and moved up into the physiological range. When HbS above a certain critical concentration is deoxygenated it polymerizes to form elongated aggregates which have been likened to microtubules, and which distort the cells in such a way as to impede their free passage through the capillaries. This extreme instance of a polysteric phenomenon, which really amounts to a ligand-controlled phase change, generates a large amount of cooperativity of oxygen binding, in itself not detrimental, indeed rather the reverse. In the present connection, however, it is of interest rather as an example of the way in which a ligand can serve as a mechanism of control for the formation of a subcellular structure as a new phase—at almost every level a living cell is to be thought of as a polyphasic system. Thus, to the phenomena of allosteric and polysteric linkage we may add that of polyphasic linkage.

5. Linkage under steady-state conditions

Thus far we have limited the discussion to linkage under conditions of equilibrium where the principles of classical thermodynamics are applicable. True equilibrium however is rare in biological systems. More commonly such systems exist in a steady state or a transient state. This is true of all functioning enzymes, and even the circulating blood when looked at as a whole, is to be regarded as representing a steady state [15]. Seen from the outside, superficially, a steady state may be hard to distinguish from an equilibrium state, but in terms of the governing equations there is all the difference in the world between them. In the case of a steady state there are no potentials to rely on, and the resulting powerful reciprocal linkage relations described above are lacking. As a result we are left alone with the kinetic equations. In a large number of cases of biological interest these will be first order, and in what follows we limit ourselves to such.

Consider a simple allosteric system in which a macromolecule exists in two conformations T and R in each of which it can combine with a ligand X at a single site. Then there will be four possible states of the molecule which can be represented by a square and which we designate by 1, 2, 3, 4.

$$\begin{array}{ccc} (1)\ \mathrm{T} & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & \mathrm{TX}\ (2) \\ k_1 \uparrow\downarrow k_{-1} & & k_{-3} \uparrow\downarrow k_3 \\ (4)\ \mathrm{R} & \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} & \mathrm{RX}\ (3) \end{array}$$

If we limit ourselves to one-step transitions, the system will be described by 8 kinetic constants $k_1, \ldots k_{-4}$, as shown, and if we denote the activity (concentration) of the ligand by X we have the four kinetic equations

$$\begin{aligned} \dot{T} &= -(k_2x+k_{-1})T + k_{-2}TX + 0RX + k_1R, \\ \dot{TX} &= k_2xT - (k_{-2}+k_3)TX + k_{-3}RX + 0R, \\ \dot{RX} &= 0T + k_3TX - (k_4+k_{-3})RX + k_{-4}xR, \\ \dot{R} &= k_{-1}T + 0TX + k_4RX - (k_1+k_{-4}x)R, \end{aligned} \tag{9}$$

where dots denote time derivatives. It will be seen that these equations provide for the conservation of the macromolecule. Moreover, if x is held constant they are linear, and it can be shown that their solution involves three relaxation times given by the roots λ of the secular equation

$$\begin{vmatrix} [-(k_2x+k_{-1})-\lambda] & k_{-2} & 0 & k_1 \\ k_2 & [-(k_{-2}+k_3)-\lambda] & k_{-3} & 0 \\ 0 & k_3 & [-(k_4+k_{-3})-\lambda] & k_{-4}x \\ k_{-1} & 0 & k_4 & [-(k_1+k_{-4}x)-\lambda] \end{vmatrix} = 0, \qquad (10)$$

one of the roots being 0. In this simple case a full solution is possible and it can be shown that there is a unique critical point (in the mathematician's sense of the term) asymptotically stable in the large. That is, there is a steady state which will always be approached regardless of the starting point. Provided the velocity constants satisfy the condition of the microscopic balance, namely that the product of those pointing clockwise round the square be equal to that of those pointing counterclockwise, this steady state will be a true equilibrium. But when this condition is not fulfilled certain unexpected things can happen and in the steady state there will be a circulation of the macromolecule round the square in one direction or the other depending on the values of the constants. The amounts of the various forms of the macromolecule are proportional to the cofactors of the elements of the top row of the matrix obtained from eq. (9) and the binding curve of even a one-site macromolecule may show cooperativity or anticooperativity [16].

When other conformations and other ligands (for each of which there may be more than one site) are present, the number of governing equations is increased by one for each new form of the macromolecule, and the square will be replaced by a multidimensional cube. But the principles illustrated by the simpler case remain the same. There will always be a steady state, unique and asymptotically stable in the large. The number of relaxation times involved in the approach to this steady state will be one less than the number of different forms of the macromolecule. The amounts of the various forms in the steady state will be proportional to the cofactors of the elements of the first row of the now enlarged matrix obtained from the governing equations, and thus there will be a linkage between the binding of the various ligands and at the same time the conformation of the macromolecule will be controlled by the various ligand activities. Microscopic balance now depends on equality of the clockwise and counterclockwise products of the constants round each face of the cube; and in the absence of such balance there will be a circulation of the macromolecule round the edges of the cube as in the simpler two-dimensional case. Under these conditions the macromolecule can act as a free energy transducer [16,17].

6. Free energy transduction

Suppose the macromolecule is an enzyme for which there are two substrates X and Y which are released by the enzyme in the forms X′ and Y′ as indicated in the following scheme:

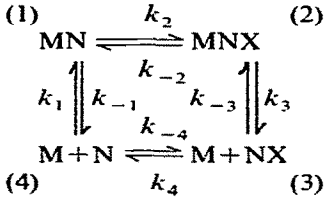
$$\begin{array}{ccccc} & & (1) & & (2) \\ \mathrm{M}+\mathrm{Y}' & \overset{k_y}{\underset{k_{-y}}{\rightleftharpoons}} & \mathrm{MY} & \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} & \mathrm{MXY} \\ & & k_1 \Big\| k_{-1} & & k_{-3} \Big\| k_3 \\ & & \mathrm{M} & \overset{k_{-4}}{\underset{k_4}{\rightleftharpoons}} & \mathrm{MX} \overset{k_x}{\underset{k_{-x}}{\rightleftharpoons}} \mathrm{M}+\mathrm{X}' \\ & & (4) & & (3) \end{array}$$

(In this simple example there is no need to consider a conformational change. The presence of a second ligand plays the same role.) Provided the activities of the substrates X and Y and their products X′ and Y′

are maintained at constant non-equilibrium values the kinetic equations will be linear and there will be a steady state in which there is a circulation of the enzyme round the square, clockwise or counterclockwise in accordance with whichever of the reactions $MX \rightarrow M + X'$ and $MY \rightarrow M + Y'$ is dominent, i.e., whichever involves the greater free energy change. Thus, the free energy derived from one reaction is used to drive the other. All this may be formulated more precisely if desired by writing down the equations in detail. It will then be seen how the addition of the constants for the side reaction destroys the condition of microscopic balance, assuming it to be obeyed in their absence [16].

7. The existence of a steady state when the equations are non-linear

Often, and in fact generally, the first-order kinetic equations will not be strictly linear. This will be so in polysteric systems and whenever the activities of the ligands are not maintained at strictly constant values. Under these conditions the problem of establishing whether or not there is a steady state (or critical point) unique and asymptotically stable as in the linear case, becomes vastly more complicated. To be sure, if all the constants satisfy the conditions of microscopic balance the physical principles of thermodynamics tell us that the system will surely come to thermodynamic equilibrium; but otherwise the question remains open. In the latter case there is no secular equation and the possibility of some kind of limit cycle cannot be ruled out. However, the problem has been rigorously dealt with, and the existence of a steady state, with all the implications which it carries, has been established in the case of a simple but not unrepresentative model system [15]. This system was suggested by consideration of the visual system involving rhodopsin which undergoes a series of photochemical reactions in the presence of light. It may be represented by the following scheme:



in which MN represents an intact macromolecule composed of a protein moiety N which has one site for a ligand X of activity (concentration) x, and a prosthetic group M, which does not combine with X.

This scheme represents the simplest case in which non-linearity results on the one hand from dissociation of the macromolecule and on the other from the changing activity of the ligand, which is present in fixed *total amount* as the reaction proceeds.

$$\begin{aligned}
\dot{MN} &= -(k_2x + k_{-1})\cdot MN + k_{-2}\cdot MNX + 0\cdot M\cdot NX + k_1\cdot M\cdot N,\\
\dot{MNX} &= k_2x\cdot MN - (k_{-2} + k_3)\cdot MNX + k_{-3}\cdot M\cdot NX + 0\cdot M\cdot N,\\
\dot{NX} &= 0\cdot MN + k_3\cdot MNX - (k_{-3}M + k_4)\cdot NX + k_{-4}x\cdot N,\\
\dot{N} &= k_{-1}\cdot MN + 0\cdot MNX + k_4\cdot NX - (k_1\cdot M + k_{-4}x)\cdot N.
\end{aligned} \tag{11}$$

It will be seen that these equations satisfy the condition of conservation of the macromolecule. At the same time they are subject to the constraints

$$M+MN+MNX+C_m, \quad MN+MNX+N+NX+C_n, \quad x=C_X-MNX-NX, \tag{12}$$

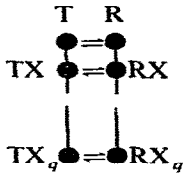
where C_m is half the total number of subunits and C_x is the total amount of ligand. From the first and second eqs. (12) it follows that $M=N$ which shows that it would be redundant to include an equation for M.

The proof for the existence of a steady state even in this simple case is difficult and involved, and the reader is referred to the original paper [15]. It is noteworthy, however, that in the special case where the constants satisfy the condition of microscopic balance, the existence of a unique steady state asymptotically stable in the large follows immediately from the laws of thermodynamics, which may themselves be regarded as existence theorems based on experience. Thus, physical principles here provide the solution of what would seem to be a purely mathematical problem. Clearly, it would be of interest to see how far the above model system could be expanded without destroying the proof. In view of what has just been said, it might be conjectured that in any system like that above where, in the special case that the kinetic constants satisfy microscopic balance, there is thermodynamic equilibrium, there will, even in the absence of microscopic balance, be a unique and globally stable critical point.

8. Transients

The foregoing analysis has been limited to macromolecular systems either in thermodynamic equilibrium or in a steady state. When we attempt to extend it to transient states, involving an approach either to equilibrium or to a steady state, we are confronted by a far more difficult problem. Indeed when the kinetic equations are non-linear and we cannot even be sure that they admit of a solution in the large, and where in any case there is no secular equation, it would seem to be wholly beyond us. Nevertheless under limited conditions, especially when the equations are linear, much can be deduced from them, and much has indeed already been learned from experimental studies of the transient behavior of respiratory proteins and enzymes as they relax either to equilibrium or to a steady state. Most of these experiments have been carried out either by the temperature-jump or the stopped-flow method.

There has recently been given a rigorous analysis, within the framework of the preceding discussion, of relaxation in three particularly simple model systems in which the equations are treated as linear and allow of exact solution with the aid of the generating-function technique [19]. Each of these models represents a variant of the original two-state concerted model of Monod, Wyman and Changeux, which can be represented by the ladder



Here the arms of the ladder correspond to the two conformational states T and R of the macromolecule and the rungs to the allosteric transitions between the different liganded forms. Here we give only the briefest outline of what was done.

In accordance with the original MWC model it was assumed throughout that in each conformation the

sites were identical and independent, having the same velocity and equilibrium constants. In the first model one conformation was eliminated, so that the kinetic ladder was reduced to one side. In the second model the "structural" velocity constants, corresponding to the rungs of the ladder, were assumed to be all the same, independent of the degree of ligation. This corresponds to the most extreme departure from microscopic balance. It gives a set of eigenvalues of fairly simple form although the probability of site occupancy as the system approaches the steady state (as distinct from equilibrium) becomes a rather complicated function of time. In the third model the velocity of either the "on" reactions or the "off" reactions were supposed to be negligible, so that either the combination or dissociation of ligand was irreversible. This corresponds to many of the experiments made with the stopped-flow technique, and the model, quite apart from its inherent interest, is useful in providing a starting point for dealing with the general case by successive approximations in which we progressively eliminate irreversibility, starting from either end. Altogether, in spite of the highly limited nature of the models involved, this analysis is useful in the light it throws on the concept of transients.

9. Conclusions

In this brief account I have endeavored to develop in macroscopic terms the concept of linkage, both homotropic and heterotropic, which is so fundamental at every level of organization in living systems. In doing this I have focused attention on a macromolecule, or a set of interacting macromolecules, in the presence of a set of ligands as representing the middle ground from which one may proceed either upwards or downwards in scale. Three cases have been considered, namely when the system is in thermodynamic equilibrium, in a steady state, or in a transient state; and the analysis has been developed to cover three possible model systems, allosteric, polysteric, polyphasic. Metaphorically, one can think of a macromolecule as a community of interacting ligands, of which the solvent is an important member, linked through the macromolecule and interacting with it, and of larger systems as communities of interacting macromolecules. Thus, such diverse cybernetic phenomena as the Bohr effect or phosphate effect in respiratory proteins, the regulation of enzymes by activators and inhibitors, the interaction of antigen and antibody, macromolecular assembly, neurotransmission, are brought under one roof. In this picture everything hangs on the existence of a steady state, defined by the ligands, towards which, as towards a goal, the system is relaxing, without perhaps ever reaching it before it changes, or about which it is fluctuating whenever, as is often the case, the energy of transition from one state to another is of the order of kT. The times involved in the relaxations will vary enormously, from picoseconds to seconds, depending on the size of the elements involved, whether assemblies of macromolecules, or the smallest parts of a single macromolecule. One is reminded once again of the concept of scaled fractals and white noise, and if there were correlations between the fluctuations, as has been suggested [20], the white noise might be converted into brownian noise, and further, and this might provide a clue to the riddle of the enzymes—why it is that such large and complex molecules are required to perform what is often such a simple task, for example, the hydration of carbon dioxide by carbonic anhydrase.

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