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Virial expansion of the free energy of a molecule with N inequivalent associating particles

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It is pointed out that, for a system of N particles which can associate reversibly with a molecule, to second order in perturbation the standard free energy is linear in each particle density plus terms in their pairwise products. This means that, because of symmetry, only $N(N+1)/2$ standard chemical potential parameters need be determined. The system is, therefore, highly overdetermined by available experimental studies. Furthermore, this form for the standard chemical potential is sufficiently orthogonalized so that evaluation of the individual elements of the chemical potential matrix can be made from data without noteworthy accuracy. Such a fitting process is carried out using data for hemoglobin, with oxygen, hydrogen ions, chloride ions, and 2,3 diphosphoglyserate as the associating particle systems. The analysis allows one to fit the Hill index, the Bohr effect, the equilibrium association constants, the shift of the oxygen gas pressure axis with changes in the pH, the chloride ion and diphosphoglyserate solute concentrations, and with temperature. The oxygenation data taken in a hyperbaric environment are interpreted to yield changes in the hemoglobin molecule's volume as the number of associating particles is varied.

INTRODUCTION

Gibbs's original work studying equilibrium in a multicomponent system developed the concept of a chemical potential μ_i to display the explicit dependence of the Gibbs free energy on the number N_i of particles of the ith component of the system.[1] Since the free energy is an extensive quantity, Euler's theorem on homogeneous functions may be used to determine the form of the Gibbs free energy to be

$$G=\sum_i N_i \mu_i , \tag{1}$$

with $\mu_i = \partial G/\partial N_i$. Euler's theorem states that a homogeneous function of degree h of the variables N_i must obey the equality[2]

$$hG(N_1,\ldots,N_j)=\sum_i N_i \frac{\partial G}{\partial N_i} . \tag{2}$$

Since an extensive parameter must be a homogeneous function of degree 1, Eq. (1) follows immediately.

The derivatives of the free energy are continuous, so one has the additional restriction on the chemical potentials that

$$\frac{\partial \mu_i}{\partial N_j}=\frac{\partial \mu_j}{\partial N_i} . \tag{3}$$

This is equivalent to saying that μ_i is a homogeneous function of degree 0. This follows from the corollary to Euler's theorem which states that the kth partial derivative with respect to any of its variables of a homogeneous function of degree h is a homogeneous function of degree $h-k$. In this case $h-k$ is 0.

The explicit calculations of the thermodynamic variables for liquids, imperfect gases, and strong electrolytes do not get much further in determining the general dependence of the chemical potential μ_i on the other particle numbers N_j, $j\neq i$, than is arrived at by Landau and Lifshitz, namely, that the free energy can have a term varying as $N_i N_j/V$, with V the volume of the system.[3-5] This result follows directly from Eq. (3) since if μ_i is to be a homogeneous function of degree 0, then it can only have a component which varies with N_j as N_j/V. Conversely, μ_j must have a component of equal magnitude which varies as N_i/V.

These results are possibly more familiar under the name of a virial expansion.[3] Any bulk variable, that is, any extensive parameter of a homogeneous physical system of indistinguishable particles, of which (pressure)$\times$(volume) is a specific example, can be expressed in terms of the number of particles as

$$pV=NkT[1+B(T)N/V+C(T)N^2/V^2+\cdots] . \tag{4}$$

The coefficients 1, B, C, etc., functions of T, are called the first, second, third, etc., virial coefficients. They are the ideal gas, the pair correction, the triplet correction, and so on.[6] The powers of V in the correction terms are sufficient to preserve the property that pV is a homogeneous function of degree 1. We have written the virial coefficients with positive sign, but since at temperatures sufficiently small with respect to the gas critical temperature $B(T)$ and $C(T)$ are generally observed to be negative, there is a lowering of the Gibbs free energy from that of an ideal gas at these temperatures when the particle density is increased. This must be the property of a system, for example, which shows a Joule-Thomson cooling with constant enthalpy expansion, $\partial T/\partial p|_h > 0$. Even

quantum systems such as ^{3}He and ^{4}He are observed to have negative B and C below their critical temperatures.[7]

The second virial coefficient arises from expanding the system potential energy in pair terms. For systems with no first-order pair terms, say, a Van der Waal's gas, these terms are from the second- and higher-order perturbation terms.[8,9] The third virial coefficient, the triplet term, comes in this case from the third- and higher-order perturbation terms from three-body potentials, plus third-order contributions from two-body potentials. This is the widely used result of the calculations of Axilrod and Teller which is used to discuss the nonpairwise additivity of the total potential which the triplet term implies.[10-12]

For a system with distinguishable particles, the N's in Eq. (4) are generalized by distinguishing them with subscripts. The virial coefficients also are indexed to each pair term, each triplet term, and so on. The terms are then summed over the virial coefficient indices.

The useful properties of the virial expansion for nonideal mixtures can be summarized as:
(i) The extensive parameters are homogeneous functions of the particle numbers, and vary, in successive approximations, as $\sum_i N_i\mu_i$, $\sum B_{ij}N_iN_j/V$, $\sum C_{ijk}N_iN_jN_k/V^2$, etc.[5]
(ii) The apparent grouping of the virial expansion terms as pair, triplet, N-plet, interactions is misleading, since though pair potential terms alone lead to the second virial coefficient, both two-body and three-body potentials contribute to the third virial coefficient, and pair through N-body potentials contribute to the Nth virial coefficient.
(iii) The contribution to the second virial coefficient coming from a second-order perturbation between equivalent classical particles will produce a relative lowering of the coefficient at low temperatures, irrespective of the nature of the particle pair potential.[9]
(iv) The temperature dependence of the virial coefficients will introduce an implicit temperature dependence into the extensive parameter.
(v) The chemical potential of a system is not the sum of the chemical potentials of its components because of the nonadditivity of potentials. It is the sum of the first virial expansion terms, plus half the sum of the second virial expansion terms, plus one-third the sum of the third virial expansion terms, and so on.

A framework which makes evident the properties of the free energy such as this one does would be very useful for understanding the equilibrium properties of a system of a molecule associating reversibly with several particles. More precisely, the physical systems which are of interest will include a large number of identical, mutually noninteracting molecules. Each one of these molecules associates reversibly with several particles. The observed average properties of this large number of molecules will be the same as an ensemble average over the available association states of a single molecule. Hence we can compare, without confusion, the statistical properties of the association of a single molecule with several particles and the observed mean state of association of many molecules. This is stated quantitatively by saying that, since the configuration integral of X noninteracting molecules is the Xth power of the configuration integral of one molecule divided by $X!$, then the free energy of X noninteracting molecules is X times that of a single molecule, plus an additive constant depending only on X and T. Since X will be constant in any reaction, X is a redundant coordinate. Hence we limit our discussion to the standard free-energy change in the association process, that is, the change in free energy of a single molecule when it associates reversibly with N particles. If this "virial expansion" framework has been developed it is in an idiom unfamiliar to experimentalists dealing with such situations.[13-15] There are several such systems of central physical and biological concern which are studied but for which such a framework of the virial expansion has not been used to simplify the analysis of the experimental observations. Some biological examples are:

(a) The ion-specific conductance systems in the nerve axon membrane. The properties of these systems of variable potassium- and sodium-ion conductance create the form of the electrical action potential transmitted along nerves. This system associates hydrogen, magnesium, and calcium ions. It also has a dependence of its free energy on the transmembrane potential.

(b) The system controlling the chemical transmitters in the synaptic cleft, the electrically insulating gap between the nerve ending and the muscle fiber membrane. This system has a dependence of its free energy on the transmembrane potential, and it also associates reversibly with hydrogen, magnesium, and calcium ions, and the chemical transmitter, acetylcholine.

(c) The heme protein molecule, and in particular, in hemoglobin form, reversibly associates oxygen molecules, hydrogen, and other ions. The experimental data for this system are available in abundance, so we have chosen it as a system with which to illustrate the possible advantages of using the virial expansion framework to define the particle-dependent and temperature-dependent properties of the free energy. The experimental data are then used only to derive the magnitudes of a small

number, $N(N+1)/2$, of second virial coefficient magnitudes which are needed to define the free energy of a molecule with N associating particles.

It is not a simple matter to translate with great rigor the arguments used to derive the virial coefficients for a fluid to arrive at the form of the free energy of a single particle which can have several particles associating reversibly with it.[16] Instead, we make a heuristic derivation of the form that the change in free energy of a single molecule, the so-called "standard free energy," must have when any one of N particles is associated with it. The result which we find is that the standard free-energy change will be expressible in a virial expansion framework identical with the one expressed above but with the volume V set equal to 1. We find that it has the linear Gibbs term, a term bilinear in the particle association numbers N_i, which is equivalent to a pair interaction $\mu_{ij} N_i N_j$, a term trilinear in the particle association numbers which is equivalent to a three-body interaction, and so on.

These terms give the change of chemical potentials of the individual particles associated with the molecule the symmetry defined by Eq. (3), as if it were a homogeneous function of zero degree. This symmetry is useful to exploit to understand how the change in the number of one particle associating with the molecule can cause a change in the number of a different particle that is associating with the molecule.

As an example of the usefulness of explicitly indicating the dependence of the particle chemical potential on the density of the other particles in association with the molecule, and the usefulness of the symmetry of the interactions, we use this form to describe the association of oxygen with heme protein, and particularly hemoglobin. The other associating particles in this case are protons, chlorine ions, and phosphoglyserates.

In the following sections we give a brief introduction to the oxygen-heme protein system. In an effort to be as explicit as possible the basic equations of association equilibrium are then presented with reference to this particular system. The form of the perturbed standard free energy is then explored through conventional thermodynamic perturbation theory.[17] The parameters that enter into the chemical potential are then evaluated using existing experimental data determined with the oxygen-hemoglobin system.

OXYGEN-HEME PROTEIN

The oxygen molecule binds reversibly with a class of molecules which have the generic name of heme protein. These are iron-porphyrin compounds in which the iron is always in reduced form, Fe^{++}. This binding has proven to be interesting not only because it is a process of fundamental importance for animal life, but also because the details of the process are fascinating. The binding of oxygen to these heme proteins proves to vary rapidly in a particular range of pressure of the oxygen gas of the heme protein environment; see Fig. 1. This property is important, for example, in the use of hemoglobin, a particular heme protein, to transport oxygen in mammalian blood. It allows the hemoglobin to become saturated with oxygen in the lungs, and it allows the burden of oxygen to be almost totally discharged in body matter with low oxygen content. This signoidal property of the oxygenation curve of heme proteins has been studied widely, so that it is well documented as to the way the oxygenation curve changes shape and translates along the oxygen gas pressure axis as elements in the heme protein environment, the acidity, the NaCl concentration and the concentration of organic phosphates, in particular, 2,3 diphosphoglycerate, are varied.

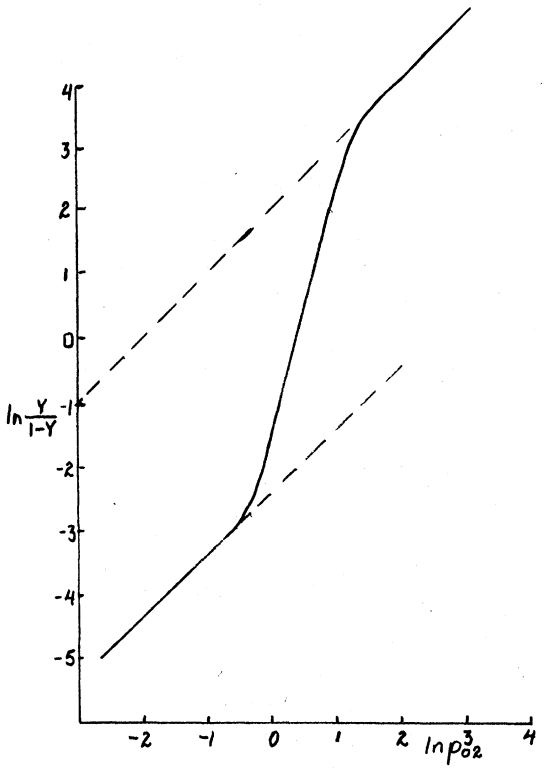

FIG. 1. Typical heme protein oxygenation curve: Y is the fractional saturation of the oxygenation of the heme protein, p_{02} is the pressure of the oxygen gas with which the heme protein has been equilibrated.

The chemistry of the heme proteins is well in hand. The amino acid sequences in their structure are known. The stereo chemistry is understood. The structure of myoglobin and hemoglobin have

been determined. The conformational changes with oxygenation have been determined. The thermodynamics of the oxygen association process has been presented. Finally, many models and theories of oxygen binding have been produced.[18]

In the past these models have been explored in terms of the ideal chemical equilibrium constants, the first virial terms, for the successive addition of oxygens to the heme protein. Pairing models do modify these equilibrium constants to include *ad hoc* pair interactions between oxygens. However, the effects of other particles which associate with the heme protein on these equilibrium constants, so that they depart from ideal behavior, are then included by observing the manner in which these equilibrium constants vary with the ambient density of the other associating particles.

The method used here is therefore quite different, since we predict the form of the variation of the chemical potentials with all the probabilities of association of all the particles, and merely use the data to establish the constants of proportionality. The form of the free energy shows why the experiments must be analyzed in this way. With such a dependence of the free energy on the particle association probabilities it is impossible to make an investigation of the oxygen association while varying a single other associating particle number. Increasing the ambient oxygen gas pressure does increase the probability of oxygen association with the heme protein, but it also varies the numbers of the other particles already associating with the molecule. The chlorine ions, phosphoglyserates, and residue ionization all vary at the same time. The conclusion is that the experimental data for oxygenation of hemoglobin appears to be more complicated than necessary because the experiments do not view a normal coordinate of the system, but a combination of a number of variations of a number sufficient to alias the more simple behavior one might naively expect to see.

STRATEGY

The measurement of the fractional saturation of the oxygenation of heme proteins as a function of the ambient oxygen gas partial pressure is a common procedure. The typical form of the data is shown in Fig. 1. In essence, this is a measurement of the chemical potential of the partially oxygenated heme protein as a function of the fractional saturation of the ability of the heme protein to bind oxygen. The measurement of the heme protein chemical potential is made in terms of the precisely calculable chemical potential of molecular oxygen gas. The analysis that follows burdens itself with the study of the empirically determined chemical potential when the type and concentration of particles other than oxygen in the environment of the heme protein are varied.

FUNDAMENTALS OF EQUILIBRIUM

Heme proteins can associate reversibly with many particles, oxygen molecules O_2, ionized hydrogen H^+, the chloride ion of NaCl, Cl, diphosphoglycerates, DPG, and other organic phosphates. Thus the equations of interest describe the heme protein Hb associated with the molecule a, $\mathrm{Hb}M_a$, which combines with molecule i, M_i, to give $\mathrm{Hb}M_aM_i$,

$$M_i + \mathrm{Hb}M_a \rightarrow \mathrm{Hb}M_aM_i . \tag{5}$$

Since heme protein associates more than two particles this equation must be repeated r times for each of the possible r states of the heme protein association for which an additional particle can be accepted. For this reason the notation M_a representing a molecule in Eq. (5) will be generalized so that M_a represents the ath possible arrangement of associating particles. For N distinct particles there will be $(N-1)!\sum_{n=2}^{N}[(N-n)!]^{-1}$ arrangements. This varies from $2.5(N-1)!$ at $N=4$ to $2.72(N-1)!$ for large N. In hemoglobin, for example, if one considers the 4 oxygens and the 24 protons equivalent, then with chlorine and DPG $N=4$, and there are 15 arrangements, which we will henceforth call N states. Considering the associated oxygens distinguishable gives 1956 N states.

Equilibrium requires equality of the chemical potentials μ_k of the separate systems:

$$\mu_{M_i} = \mu_{\mathrm{Hb}M_aM_i} - \mu_{\mathrm{Hb}M_a} . \tag{6}$$

The free energies of the particle systems are written in terms of the respective particle partition sums as

$$F_{M_i} = -kT \ln\left[\left(\sum_s \exp[-(E_i)_s/kT]\right)^{N_i} \Big/ N_i!\right], \tag{7}$$

where $F_{M_i} = G - pV$ is the Helmholtz free energy, k is Boltzmann's constant, and T is the solution temperature; $(E_i)_s$ is the energy of the sth state of the molecule M_i, and N_i is the number of indistinguishable molecules M_i in the solution. Stirling's formula for $N!$ and partial differentiation give the chemical potential as

$$\mu_{M_i} = -kT \ln\left[\left(\sum_s \exp[-(E_i)_s/kT]\right) \Big/ N_i\right]. \tag{8}$$

The partition sum is proportional to the volume of the solution, so μ is a homogeneous function of degree zero. This result, inserted into Eq. (6), leads to the law of mass action which relates the

numbers of the particles by

$$\frac{N_{ia}}{N_i N_a} = \frac{\sum_s e^{-(E_{ia})_s/kT}}{(\sum_s e^{-(E_i)_s/kT})(\sum_s e^{-(E_a)_s/kT})} . \quad (9)$$

N_i is the number of the molecule M_i, N_a is the number of heme proteins with the ath N state of associating particles, and N_{ia} is the number of heme proteins associating with the ath N state of associating particles plus the molecule M_i. E_i, E_a, and E_{ia} are the eigenenergies of these systems.

We are interested in the fractional saturation parameter, S_{ia}, of the molecule M_i for its association with the heme protein when it is associating with the ath N state of particles,

$$N_{ia}/(N_{ia}+N_a) = S_{ia} . \quad (10)$$

In Eq. (9) the factor $N_i/\sum_s e^{-(E_i)_s/kT}$ is, by Eq. (8), $\exp(\mu_{M_i}/kT)$. The other two sums can be defined in terms of F_{ia0} and F_{a0}, the standard free energy, the free energy, Eq. (7), with one particle:

$$e^{-F_{ia0}/kT} = \sum_s e^{-(E_{ia})_s/kT} . \quad (11)$$

These substitutions allow Eq. (10) to be written in the form

$$\frac{S_{ia}}{1-S_{ia}} = \exp(\mu_{M_i}/kT)\exp[-(F_{ia\,0}-F_{a0})/kT]$$
$$= \exp(\mu_{M_i}/kT)K_{ia}(T) . \quad (12)$$

K_{ia} is the reaction equilibrium constant, a function of the temperatures. $F_{ia\,0}-F_{a0}$ is the standard free energy of the reaction.

For the associating particle systems of particular interest, the chemical potentials can be taken to be in their simple ideal form. The particle concentrations are conventionally corrected to represent an activity parameter. We dispense with this nicety since these corrections are not the central issue in our discussion. For oxygen gas the partition sum can be carried out explicitly in terms of the molecular mass, m, Planck's constant h, Boltzmann's constant k, the absolute temperature T, and the gas partial pressure to give

$$e^{\mu_{O2}/kT} = (kT)^{-5/2}(2\pi m/h^2)^{-3/2}p_{02} \quad (13)$$
$$= e^{-22.367}p_{02}, \text{ at } T = 293\,^\circ\text{K}, \quad (14)$$

with p_{02} in Torr. Analogously, the other particles have chemical potentials which are the natural logarithms of the particle concentrations, plus a constant varying only with the temperature:

$$\mu_{H^+} = -kT\ln 10[\text{pH} - \text{pH}_0(T)],$$
$$\mu_{Cl} = kT(\ln c_{Cl} - \ln c_{Cl_0}), \quad (15)$$
$$\mu_{DPG} = kT(\ln c_{DPG} - \ln c_{DPG_0}) .$$

PERTURBED STANDARD FREE ENERGY

We can use conventional perturbation theory to derive an expression for the variation of the free energy of a stripped heme protein with the addition of associating particles.[17] The combined Hamiltonian of the heme protein plus particles is written as

$$\mathcal{H} = \mathcal{H}_0 + \sum_i V_i + \sum_{ij} V_{ij} + \sum_{ijk} V_{ijk} + \cdots , \quad (16)$$

where $\mathcal{H}_0$ is the Hamiltonian of the bare heme protein and $\sum_i V_i$ is the change in $\mathcal{H}_0$ with the addition of particles to the heme protein. This includes the particle kinetic energy plus the two-body potential interaction with the heme protein. The terms V_{ij} are corrections to the two-body heme protein-particle interaction due to the presence of a third body, plus the two-body interactions between the particles themselves. The V_{ijk} are the next-order correction terms. We neglect the terms beyond $\mathcal{H}_0$ and V_i since the relatively great distances between particles makes them probably small. Furthermore, these two terms allow one to establish the form of the virial expansion corrections, which is all that is the intention here.

One has, from perturbation theory, the energy of the heme protein with N associated particles, in terms of the representation for the stripped molecule states,

$$E(N)_r = E_r^{(0)} + \sum_i (V_i)_{rr} + \sum_s \frac{|\sum_i (V_i)_{rs}|^2}{E_r^{(0)} - E_s^{(0)}} + \cdots . \quad (17)$$

Since, by definition, the standard free energy is

$$F(N)_0 = -kT\ln\left(\sum_r e^{-E_r/kT}\right), \quad (18)$$

by expanding the logarithm one finds,

$$F(N)_0 = F(0)_0 + \sum_i \bar{V}_i - \frac{1}{2}\sum_r \sum_s \frac{|\sum_i (V_i)_{rs}|^2 (w_{s0} - w_{r0})}{E_r^{(0)} - E_s^{(0)}}$$
$$- \frac{1}{2kT}\left\langle \left[\sum_i (V_i)_{rr} - \sum_i \bar{V}_i\right]^2 \right\rangle . \quad (19)$$

The w_{s0} are the canonical Gibbs probabilities for the states s. If the states in the second-order sum have energy differences small compared to kT, the double sum reduces by closure, $\sum_s V_{rs}V_{sr} = V_{rr}^2$, to be equal to the last term. In general one has a lowering of the standard free energy in second order for any interaction. Part of this second-order contribution will be temperature dependent, and part will be temperature independent.

The terms in Eq. (19) that depend upon a single particle, such as $\bar{V}_i$ and $|(V_i)_{rs}|^2$, will be weighted by that particles occupation probability N_i. The terms in $|\sum_i (V_i)_{rs}|^2$ depending upon two particles will be weighted by the product of their respective

expectation, $N_i N_j$. For example, if the ith particle has matrix elements between the r and s states of the heme protein so that $(V_i)_{rs} \neq 0$, but the jth particle does not so that $(V_j)_{rs} = 0$, then the free energy will increase linearly with N_i, and the chemical potential of the ith particle is a constant. If, on the other hand, two particles perturb, more or less, the same states of the heme protein so that $(V_i)_{rs} \neq 0 \neq (V_j)_{rs}$, the free energy will increase linearly with $N_i N_j$. In other words, if the particles perturb only the local states of the molecule, such as the local vibration states at each heme site, the free energy will vary as N_i and the chemical potential will be constant. If they perturb states which must be described in terms of the molecule as a whole, the free energy will vary bilinearly as $N_i N_j$ and the chemical potential of the ith particle will have a component varying with N_j.

This bilinearity in the second-order perturbation has the effect of making the particles appear to have pair interactions between them. The pair interaction is not direct, since we omitted that term in Eq. (16), but it is the result of the type of interaction each particle has with the total heme protein molecule. The third-order terms will give an apparent triplet interaction, and so on. The bilinear variation of the free energy with the pairlike terms in $N_i N_j$, can be the result of the second-order perturbation of the energy, but it can also arise from the linear first-order terms, V_{rr}, and the nonlinearity of the distribution function, the last term in Eq. (19).

The virial expansion of the free energy of a molecule which associates with several particles will thus be of the form described in the Introduction. The first virial coefficient will be composed of the terms linear in the probability of the particle to associate. The second virial coefficient will be represented in the particle bilinear probabilities. The third virial coefficient in the trilinear triplet probabilities, and so on.

In the following sections we briefly sketch the method in which this virial expansion framework is useful in analyzing a number of experimental observations on a particular heme protein, hemoglobin.

SECOND-ORDER STANDARD CHEMICAL POTENTIALS

The heme protein can associate with several systems of particles, oxygen, protons, chlorine ions, and diphosphoglyserate, in particular. If we limit the particles to these particles which reversibly associate with the heme protein, then to second order in perturbation and to first order in particle number, the standard free energy of a single heme protein would have the form,

$$F(N_1, \ldots, N_k)_0 - F(0, \ldots, 0)_0 = \sum_i \mu_i N_i + \sum_i \sum_{j \neq i} \mu_{ij} N_i N_j , \quad (20)$$

where N_i is 0 or 1, depending upon whether the ith particle is associated with the heme protein.

The change of the standard free energy, $F_{ia0} - F_{a0}$, in Eq. (12) is the change in the standard free energy when the ith particle is certainly added to a heme protein which is certainly associated with the ath N state of associating particles. This ath N state would be represented by the set of numbers, $(N_{1a}, N_{2a}, \ldots, N_{ia} = 0, \ldots, N_{ka})$, with each N_{ja} taking on the values 1 or 0 for a given N state, and with N_i being certainly 0 for the N state with which the ith particle can be associated. Hence

$$F_{ia0} - F_{a0} = \mu_{\mathrm{Hb}i} + \sum_{j \neq i} \mu_{ij} N_{ja} . \quad (21)$$

For N distinguishable associating particles, because of symmetry, there will be only $N(N+1)/2$ parameters of the free energy to be determined to represent it to second order in perturbation.

In an experimental situation, with many heme proteins in solution, the statistical probability of association is observed. This is the saturation parameter S_{ia}, defined in Eq. (10). In case the saturation parameters of the different N states are not distinguishable, the average of S_{ia} over all the N states will be determined. From Eq. (12) one finds that the variation of S_{ia} with the free-energy parameters μ_{ij} and the N state numbers N_{ja} is

$$\begin{aligned} dS_{ia} &= S_{ia}(1 - S_{ia})\, d[\mu_i/kT + K_{ia}] \\ &= S_{ia}(1 - S_{ia})\, d\Big[(\ln c_i - \ln c_{i0}) \\ &\qquad - \Big(\mu_{\mathrm{Hb}i} + \sum_{j \neq i} \mu_{ij} N_{ja}\Big)\Big/kT\Big] . \end{aligned} \quad (22)$$

The basic drama of the whole system is now revealed. To average S_{ia} over the N states, write it in terms of S_{i0}, its value with all $N_j = 0$, plus the correction given by Eq. (22), when the N_{ja} are not zero.

$$\begin{aligned} S_i &= \frac{1}{n} \sum_{a=1}^{n} S_{ia} \\ &= S_{i0} - \frac{1}{n} \sum_{a=1}^{n} S_{ia}(1 - S_{ia}) \sum_{j \neq i} \mu_{ij} N_{ja}/kT . \end{aligned} \quad (23)$$

The factor $S_{ia}(1 - S_{ia})$ has a maximum of $\frac{1}{4}$ at $S_{ia} = \frac{1}{2}$. Near this point, for a change in chemical potential of $3kT$, S_{ia} will vary from 0.18 to 0.82, and the factor $S_{ia}(1 - S_{ia})$ will vary by $\pm 20\%$. Out-

side this range, the effect of the coefficient $S_{ia}(1 - S_{ia})$ is to reduce the difference in S_{ia} for different N states. The first-order value for S_i then will be given by Eq. (23) with $S_{ia}(1 - S_{ia})$ taken equal to the constant $S_i(1 - S_i)$. The average is then over N_{ja}, which is by definition S_j. Iteration of this solution in Eq. (23) by substituting for $S_{ia}(1 - S_{ia})$ will develop small corrections in $S_i S_j$. Since we neglected these terms at the outset, we are logically correct to neglect them now.

This means that to first order in the particle associated N-state numbers, N_{ja}, the N states can be averaged in Eqs. (12), (21), and (22) by replacing S_{ia} by S_i, and N_{ja} by S_j.

All the standard free-energy differences, Eq. (21) for the particles depend more or less on the association probabilities of all the other particles. Hence, if the association probability for an oxygen at a particular heme site, S_{02i}, is changed, then the standard free-energy differences of all the other particles change. But this leads to a change in the association probabilities of these other systems by Eq. (22), for all particles which are not saturated, $S_j = 1$, or stripped, $S_j = 0$. Hence the standard free-energy difference with respect to the subject oxygen must change, and it can never be constant except under the special condition that all the other particles are stripped or saturated. A change in temperature induces a similarly involved set of variations. This is so although the chemical potentials of the free particles, the first term on the right in Eq. (22), with which the heme proteins are in equilibrium are held constant. Holding the pH of the electrolyte constant, or the concentration of NaCl constant does not insure that the number of protons associated with the heme protein residues, or the chlorine ions associated with it are constant. It is, in fact, these second-order adjustments of association probabilities that account for the rather bizarre behavior of the oxygenation curve as the properties of the surrounding electrolyte are changed. In the following sections we discuss the determination of the free-energy parameters of a particular heme protein, hemoglobin, from experimental data. The basic tactic used in understanding the experimental data is one suggested by Eq. (22). The change in the chemical potential of particle i, say the surrounding oxygen gas, when the association probability of that particle is held constant, $dS_i = 0$, and the association probability of a second particle is changed from stripped to saturated, $dS_j = 0 \rightarrow 1$, is just μ_{ij} except for a correction for the second-order changes in the S_k of the remaining particles. The second observation is that the major effect of the change in the association probability of a particle on the behavior of the other associating systems occurs when that particle's association probability is $\frac{1}{2}$.

OXYGEN-OXYGEN TERMS

A heme protein can have several sites for binding oxygen molecules. Call the saturation parameters for these sites S_{02i}, where the index i distinguishes the different heme binding sites. According to Eq. (22) we have, for the oxygens,

$$dS_{02i} = S_{02i}(1 - S_{02i})\left[d(\ln p_{02}) - \sum_{j \neq 02i} \mu_{02ij}\, dS_j / kT\right], \tag{24}$$

with similar equations for the non-oxygen particles.

The experimental data do not distinguish the separate oxygen particles, so we must average S_{02i} over the i sites. The averaged saturation parameter is quite universally called Y.

$$Y = \frac{1}{N_0} \sum_{i=1}^{N_0} S_{02i}, \tag{25}$$

where N_0 is the number of heme sites that can bind oxygen. If one observes the average oxygen saturation parameter Y, it is impossible to determine more than the mean of μ_{02i02j}, since to first order in the particle saturation parameters only its mean is observed. Represent the mean as μ_{0202}. The set of equations, Eq. (24), averaged as in Eq. (25), then have the solution with all solute concentrations constant and only $\ln p_{02}$ varying,

$$dY = \frac{Y(1 - Y)\, d\ln p_{02}}{1 + Y(1 - Y)[(N_0 - 1)\mu_{0202}/kT - N_0 \sum_{i \neq 02} (\mu_{02i}/kT)^2 S_i(1 - S_i)]}. \tag{26}$$

One of the earliest attempts to describe this system was made by A. V. Hill in 1910. He wrote a relationship between the oxygen saturation parameter Y and the oxygen gas pressure p_{02} in the form that it would have for an nth order association:

$$Y = Kp_{02}^n / (1 + Kp_{02}^n). \tag{27}$$

To represent the observed sigmoidal oxygenation curve, the Hill index n must vary with Y. For hemoglobin, for example, near $Y = \frac{1}{2}$, $n \sim 3$; while for $Y \rightarrow 0$, or 1, $n \rightarrow 1$. According to Eq. (27) one has

$$dY = nY(1 - Y)\, d\ln p_{02}. \tag{28}$$

We conclude therefore that n is defined by Eq. (26) to be the reciprocal of the denominator on the right-hand side. This proves to be a remarkably accurate prediction of the Y dependence of n in terms of a constant chemical potential parameter, μ_{0202}. Tyuma *et al.* give a plot of n as a function of $\log p_{02}$ in their Fig. 7.[19] These data were taken for hemoglobin stripped of DPG in low NaCl concentration and at fixed pH. As we can show from the magnitudes of the parameters determined later, the terms in the sum in the denominator of Eq. (26) contribute 5% to this term, so they can be ignored. The DPG concentration is zero, so S_{DPG} is zero. Apparently the NaCl concentration is low enough so that S_{Cl} is nearly zero. The proton density S_{zi} is near $\frac{1}{2}$, so it is the major correction. In this case μ_{0202} is determined by the peak value of n, 2.5, to be $-0.8kT_0$. The width of the n curve at $Y=\frac{1}{4},\frac{3}{4}$, is slightly greater than predicted. This extra width can be explained by allowing the individual oxygen-oxygen terms to vary from $-0.8kT_0$ by $\pm 0.1kT_0$. Thus these experimental data appear to be in excellent agreement with Eq. (26). Here and in the remaining text these energies will be given in units of kT_0, the thermal energy at room temperature.

Tyuma *et al.* also graph n for hemoglobin in 2mM DPG. Using the values for the oxygen-DPG chemical potential derived below, the correction of the DPG-dependent term is 0.09. For this case the peak value of n is 3 and μ_{0202} is determined to be $-0.80kT_0$, in good agreement with the stripped case. The width of the n-dependent curve suggests a slower variation of n with Y than would be predicted by a constant coefficient of the $Y(1-Y)$ factor in the denominator of Eq. (26). This is to be expected since the associated DPG density also varies with Y. For these data S_{DPG} is near one and it decreases with Y. Hence the correction increases as Y approaches 1. The net effect is to reduce the variation by a factor of about 2 as Y goes to 1. As Y goes to zero the correction disappears, so the variation of n on deoxygenation is predicted to vary as it does in stripped hemoglobin. This is the behavior reported by Tyuma *et al.*

Integration of Eq. (26) yields the true chemical potential, that is, the particle-dependent chemical potential as

$$\ln p_{02} - \ln p_{02}|_{Y=1/2} = \ln\frac{Y}{1-Y} + \frac{3\mu_{0202}}{kT}\left(Y-\tfrac{1}{2}\right) - 4\sum_{i\neq 02}\int_{1/2}^{Y}\frac{\mu_{02i}}{kT}S_i(1-S_i)\,dY\,. \tag{29}$$

The valence number N_0 has been taken that of hemoglobin, 4. The last two terms in this equation represent the off-set between the $Y=0$ and $Y=1$ asymptotes of the oxygenation curve in $\ln[Y/(1-Y)]$. For hemoglobin the oxygen-oxygen term contributes about 2.4 to this off-set, the protonation, 0.12 at pH 7.4, and the NaCl and DPG have contributions that could account for amounts equal to that of the oxygen-oxygen term. The data of Tyuma *et al.* give this factor for stripped hemoglobin as 3.6 at pH 7.4 and 3.2 at pH 9.1. The unknown NaCl concentration could account for the difference.

The temperature dependence of the translation of the $\ln p_{02}$ axis of the oxygenation curve is given by Eq. (22). If the oxygen saturation parameter Y is held constant, then the temperature variation of the oxygen–heme protein standard chemical potential must be compensated for by a change in the chemical potential of the oxygen gas. Hence,

$$\frac{d\ln p_{02}}{d(1/T)} = -\tfrac{5}{2}T + \frac{\mu_{H02}}{k} + \frac{1}{kT}\frac{d\mu_{H02}}{d(1/T)}\,. \tag{30}$$

Benesch *et al.* find the derivative to be $-5500\,^\circ$K for stripped hemoglobin with NaCl 0.1 M at pH 7.3, and $Y=\frac{1}{2}$.[20] The first two terms of Eq. (30) yield a factor of $-7000\,^\circ$K, part of the difference must come from the last term. The rest of the difference must come from the variation of the chemical potential of the heme protein with thermal contraction.

The effect of hydrostatic pressure on the chemical potential is determined by the molecular volume,

$$\frac{\partial\mu}{\partial p} = v\,. \tag{31}$$

According to Eq. (22), observation of the shift of the hemoglobin oxygenation curve will determine the change of the difference of the standard chemical potentials of hemoglobin and hemoglobin with one additional oxygen molecule. This experiment has been done.[21] The result is that $kT\partial\ln p_{02}/\partial p = -300$ Å^3 at $Y=\frac{1}{2}$, pH = 7.1, DPG solute concentration 4.8 mM and the Cl ion solute concentration unspecified. This result says that, under these conditions, the volume of the hemoglobin molecule with oxygen is smaller than the hemoglobin molecule without oxygen by 300 Å^3. By analogy we argue, therefore, that thermal contraction, which increases with $1/T$, should also lower μ_{H02} and thus increase the magnitude of the $-7000\,^\circ$K term. At this point we wish only to make the point that this standard free energy difference must have a temperature dependence large enough to make the last term in Eq. (30) equal, at least, to $5T\,^\circ$K. For positive terms in μ_{H02} this would confirm the prediction of the $1/T$ dependence of the terms in Eq. (19), and in fact we find all the terms except μ_{0202} to be positive. The temperature dependence

obviously will vary with oxygen saturation and the concentration of the other saturating particles in the solute. We will return to a consideration of this aspect of Eq. (30) later. The pressure variation of the standard chemical potential will also be discussed at length below.

OXYGEN-DIPHOSPHOGLYCERATE TERMS

It is observed experimentally that one of the systems with the strongest interaction with the oxygen-hemoglobin system is 2, 3 diphosphoglyserate, DPG. There are several published experimental investigations of this system. The measured shift of $\ln p_{02}$, and in particular, the change in oxygen gas pressure at $Y=\frac{1}{2}$, with change in the DPG solute concentration, allows one to use Eqs. (12) and (21) to derive both the equilibrium association constant for DPG at $Y=\frac{1}{2}$, and the linear contribution of DPG to the oxygen-hemoglobin standard chemical potential. Explicitly,

$$kT_0\Delta \ln p_{02}|_{Y=1/2} = \int_0^{S_{DPG}} \left(\mu_{02DPG} - \sum_i S_i(1-S_i)\mu_{02i}\frac{\mu_{DPGi}}{kT_0}\right) dS_{DPG}. \tag{32}$$

The shift in $\ln p_{02}$ at $Y=\frac{1}{2}$, chlorine ion concentration 0.1 M, pH 7.3, as the DPG solute concentration is varied from zero to an amount sufficient to saturate the DPG association, is observed to be $1.4kT_0$.[19,20] The terms in the sum in Eq. (32) are calculated from the parameters of the other associating particles, as determined later, to be $0.5kT_0$; hence μ_{02DPG} is $1.9kT_0$.

Since the chemical potential of the DPG solute is proportional to the natural logarithm of the DPG solute concentration, Eq. (12) may be written in terms of the solute concentration c and the equilibrium association constant c_0 as

$$S_{DPG} = \frac{c/c_0}{1+c/c_0}. \tag{33}$$

The variation of the oxygen-hemoglobin standard chemical potential, measured by $\ln p_{02}$ at $Y=\frac{1}{2}$, with variation of the solute concentration c then determines the equilibrium association concentration c_0 to be 0.1 mM at $Y=\frac{1}{2}$, pH 7.3, and chloride concentration 0.1 M.

Equation (21) says that, symmetrically, the DPG standard chemical potential must vary with the oxygen association saturation parameter Y as

$$d\mu_{HDPG} = \left(\mu_{02DPG}N_0 + \sum_i \mu_{DPGi}\frac{dS_i}{dY}\right)dY, \tag{34}$$

where N_0 is the oxygen valence of the heme protein.

The effect of this Y-dependent term would appear, for example, in Eq. (12). The association constant will appear to vary with Y in this manner:

$$K_{HDPG}(Y,S_i) = K_{HDPG}(0,S_i)\exp\left[-\int_0^Y \left(\mu_{02DPG} - \frac{1}{kT}\sum_i S_i(1-S_i)\mu_{DPGi}\mu_{02DPG}\right)N_0\,dY/kT\right]. \tag{35}$$

This equation allows one to determine the association constant of DPG in deoxygenated hemoglobin. As Y drops from $\frac{1}{2}$, the associated chlorine increases. Using values of parameters determined later, this equation predicts the association constant for deoxygenated hemoglobin to be 3×10^{-5} M in terms of concentration, for chlorine ion concentration of 0.1 M. The experimental result is 1.5×10^{-5} M.[20] The difference is not significant.

Equations (34) and (22) also show that S_{DPG}, the association saturation of DPG will also vary with Y. Hence the oxygen–heme protein standard chemical potential will vary with Y and the association saturation of DPG. The contribution of this term to the magnitude of the Hill index n has been given above in Eq. (26).

The temperature dependence of $\ln p_{02}$ will depend on DPG concentration through the same standard chemical potential parameters. The μ_{ij} will vary to some extent as $1/T$. Take t to be the fraction that varies in this manner. Hence S_{DPG} and the other saturation parameters will vary with temperature also. And the S_i will vary with S_{DPG}. Including the explicit $1/T$ dependence one finds the DPG dependence of $\ln p_{02}$ to be

$$\Delta\frac{d\ln p_{02}}{d(1/T)} = \frac{1}{k}(1+t)N_0S_{DPG} \times\left(\mu_{02DPG} - \frac{1}{kT}\sum_i \mu_{02i}\mu_{DPGi}S_i(1-S_i)\right) \times\left(1+\frac{1}{2T}\frac{d\ln S_{DPG}}{d(1/T)}\right). \tag{36}$$

This factor has been observed experimentally to be 1700 °K for DPG solute concentrations of 0.25 and 1 mM, pH 7.3 and chloride ion concentration 0.1 M.[20] If one takes t equal to 1, the first factors multiplying the last one equal $2400S_{DPG}$ °K. The variation of $\ln S_{DPG}$ with $1/T$ will be negative, both because of thermal contraction and because of its standard chemical potential. At this state of our knowledge of these terms the agreement is satisfactory.

OXYGEN, CHLORIDE ION, AND DPG

In the manner described above one can evaluate the oxygen-chloride ion perturbation. For DPG at zero concentration, stripped hemoglobin, available data indicate that μ_{02Cl} is $2.5kT_0$, and that the equilibrium concentration of Cl at $Y=\frac{1}{2}$, $S_{DPG}=0$, and pH 7.3, is approximately 0.06 M.[20] For a DPG concentration of 0.25 mM, one must include the variation of S_{DPG} with associated chloride and the variation of associated chloride with DPG.

A set of self-consistent parameters is obtained by taking $\mu_{DPGCl}=0.7kT_0$. This drops the associated chloride by about a factor of 3, and it also drops the associated DPG by a third so that the variation of $\ln p_{02}$ is equivalent to an oxygen-chloride interaction of $0.28kT_0$, as it is observed to be.[20]

OXYGEN, pH, AND DPG

If one includes only associating protons, H^+, whose change in probability of association is noted by a change in the ionization of a heme residue, $dH^+=dz_i$, and DPG, one finds from Eq. (22), for $Y=\frac{1}{2}$,

$$\left.\frac{d\ln p_{02}}{d(p\mathrm{H})}\right|_{Y=1/2}=\frac{1}{kT}\left(\mu_{02\mathrm{DPG}}\frac{dS_{\mathrm{DPG}}}{dz_i}+\mu_{02zi}\right)\frac{dz_i}{d(p\mathrm{H})}\,. \tag{37}$$

One should include a sum over i, where i indexes the different residues which can be protonated. For a given pH range, only one residue with a pK in that range will be changing its protonation number z_i. Hence we dispense with the explicit summation. Equation (37) may be related to a result due to Wyman which has been used in the past to discuss the Bohr effect.[21] This relationship is derived in the accompanying Appendix.

The factor $dz_i/d(p\mathrm{H})$, the differential titration of hemoglobin, is a well known function. The factor dS_{DPG}/dz_i is given by Eq. (22), and it is proportional to $S_{DPG}(1-S_{DPG})$. Thus the left-hand side of Eq. (37), which is called the Bohr effect should be the same at vanishing DPG concentration, $S_{DPG}=0$, and at large DPG concentration c greater than 2 mM. The experimental data display such a variation.[20] This data allows one to determine $\mu_{02\,z_i}$ near pH 7.5 to be $0.07kT_0$ and μ_{DPGz_i} to be $0.3kT_0$.

These parameters now determine the variation of the DPG association constant with pH:

$$\frac{d\ln K_{DPG}}{d(p\mathrm{H})}=\frac{\mu_{DPGz_i}}{kT}\,\frac{dz_i}{d(p\mathrm{H})}\,. \tag{38}$$

The data of Benesch *et al.* give this factor as 2.41 for pH from 7.0 to 7.3, and 3.31 for pH from 7.3 to 7.8.[20] With μ_{DPGz_i} equal to $0.3kT_0$, the respective $dz_i/d(p\mathrm{H})$'s are 8.1 and 11.1, values which are in agreement with the differential titration values.[18]

OXYGEN AND HYPERBARIC EFFECTS

The variation of the standard chemical potential with pressure is formally written in terms of the true oxygen gas pressure at a given value of Y by means of Eq. (22):

$$kT\frac{d\ln p_{02}}{dp}=\frac{d\mu_{02}}{dp}+\frac{d\mu_{0202}}{dp}Y+\sum_i\left(\frac{d\mu_{02i}}{dp}S_i+\mu_{02i}\frac{dS_i}{dp}\right). \tag{39}$$

The available data give the variation of $\ln p_{02}$ with Y and DPH at pH 7.1 and unspecified chloride ion concentration.[22] With DPG saturated, it is -300 Å^3 at $Y=0.5$, -450 Å^3 at $Y=0.2$, and -240 Å^3 at $Y=0.8$. With DPG at a concentration less than 0.5 mM in the solute, these numbers are, respectively, -140, -290, and -80 Å^3. These data were taken with nitrogen as the pressurizing gas. With helium as the pressurizing gas, but with unspecified environment, these numbers are, respectively, -115, -205, and -15 Å^3. For the first two sets of data the term in the sum which depends on the DPG saturation parameter yields the result that,

$$-160\ \text{Å}^3=(1-S_{\mathrm{DPG}})\left(1+\frac{\mu_{02\,\mathrm{DPG}}}{kT}S_{\mathrm{DPG}}\right)\frac{d\mu_{02\,\mathrm{DPG}}}{dp}\,. \tag{40}$$

This means that $d\mu_{02DPG}/dp$ has a magnitude much greater than 160 Å^3; that is, the DPG, on associating, contributes to the decrease in the hemoglobin molecule's volume.

The derivative of Eq. (39) with respect to Y will have a constant term plus a term varying with Y. A variation of Y, as we have repeatedly stated before, brings about a complicated variation of the saturation parameters of the other associating particles. One must, in order to do a proper analysis, carry out an eigenvalue calculation to obtain the relative shifts. However, the approximate solution which we have used above is quite accurate and we continue to use it here. This means that in the derivative of Eq. (39) with respect to Y, the factor dS_i/dY is taken from Eq. (22) as

$$\frac{dS_i}{dY}=-S_i(1-S_i)\mu_{02i}\,. \tag{41}$$

The factor $S_i(1-S_i)$ will as usual vary with Y. At this point it is sufficient to replace it by its Taylor series expansion about its value at $Y=\frac{1}{2}$, where it has a value represented by S_i^0. This yields the result

$$kT\frac{d^2\ln p_{02}}{dY\,dp}=\left[\frac{d\mu_{0202}}{dp}+\sum_i\frac{d\mu_{02i}}{dp}\times\left(1-S_i^0(1-S_i^0)\frac{\mu_{02i}}{kT}\right)\right]+\left(\sum_i\frac{d\mu_{02i}}{dp}S_i^0(1-S_i^0)(2S_i^0-1)\right)(Y-\tfrac{1}{2}). \tag{42}$$

The first term in Eq. (42) is evaluated from the data as 350 Å^3, independent of DPG or pressurizing gas.

The coefficient of the second term is determined to be zero for the data taken using helium gas as the pressurizing gas, and it is −150 Å^3 for the data taken using nitrogen as the compressing gas, and variable DPG.

The conclusion to be reached is that DPG alone does not determine these coefficients in Eq. (42), but another particle, say the chloride ion, is also of importance. The difference in the data taken with nitrogen and helium gas thus could be explained by different chloride ion concentrations, the sign of the terms in the Y-dependent term being determined by whether the association saturation S_i^0 is greater or less than $\frac{1}{2}$.

The data also indicate that $d\mu_{0202}/dp$ could be positive. In this case the oxygen-oxygen coupling term μ_{0202} would decrease in magnitude with pressure. This term would indicate that the oxygen-oxygen coupling increases the volume of the oxygenated hemoglobin with respect to deoxygenated hemoglobin. This would decrease the volume decrease contributed by the single-particle term which was discussed in a preceding section.

We leave the discussion of the relationship of these parameters, as well as the standard chemical potentials, to the structure of hemoglobin as it is presently understood to a second paper.[23]

SUMMARY

For a mass of data we have shown that one can determine the oxygen–heme protein chemical potentials to second order in perturbation, which is a linear approximation in the associating particle densities, so that they form an internally consistent set of parameters. The magnitude of the parameters appears to depend little upon time or source. The parameter μ_{02z_i}, upon which the Bohr effect depends, is invariant over the years of data at $0.07kT_0$. The effect of DPG on oxygenation as reported by different laboratories is approximately $1.9kT_0$.

The preliminary set of parameters which we suggest as useful is the following one: $\mu_{0202}=-0.8kT_0$, $\mu_{02\mathrm{DPG}}=1.9kT_0$, $\mu_{02z_i}=0.07kT_0$ (for pK's near 7.5), $\mu_{02\mathrm{Cl}}=2.5kT_0$, $\mu_{\mathrm{DPG}z_i}=0.3kT_0$, $\mu_{\mathrm{DPGCl}}=0.7kT_0$. The zero-order standard chemical potentials for each particle system, μ_{02}, μ_{DPG}, μ_{Cl}, μ_{z_i}, are determined by the equilibrium association constants, p_{02} at $Y=\frac{1}{2}$, K_{DPG}, K_{Cl}, and pK_i. Missing is the element $\mu_{\mathrm{Cl}z_i}$, which could be determined from the variation of the Bohr effect with chloride ion concentration. All of these standard chemical potential parameters should be expected to vary with temperature, in part, as $1/T$.

The special point which is made in this approach is that experiments with heme proteins, and with hemoglobin in particular, explore planes through an N-dimensional function space in which the oxygen–heme protein association function is mapped. The N dimensions are the saturation parameter axes of the N systems of particles which can possibly associate with the heme protein. Although the solute chemical potentials can be held constant, the experiments explore planes on which most of the individual saturation parameters vary. This means that what is being varied in a particular experiment is not only the oxygen saturation parameter and the ambient oxygen gas partial pressure, for example, but also being varied is the number of residue protons, of associated DPG, and chloride ions as dependent variables. The net effect is to warp the oxygenation curve from an analytically simple behavior to a much more complex one.

This more complex projection is usually discussed as if it were simply a cut in the $Y-p_{02}$ plane with the saturation parameters of the other associating systems held constant. Hence the common conclusion that oxyhemoglobin is not 100% characterizable.

This is an essential difficulty of these oxygenation studies. The resolution seems to be to evaluate, by successive steps, the elements of the standard chemical potential matrix, the μ_{ij}, and to gain insight from these more simple constants. The associating systems are sufficiently orthogonal in this representation so that the elements can be evaluated from data without noteworthy accuracy. They also describe something sufficiently physical and sufficiently definite so that there is hope that they will be of use to supplement the fund of structural knowledge already available.

One interesting result of this approach is its contradictions, at times, of the tenets in this field which have been established in the past using the conventional view. For example, the fact that the Hill index n is not 1 is usually taken to indicate an attractive, or cooperative, potential between oxygen molecules. From our results, Eq. (26), it is evident that n will also be greater than 1 because

of any interaction, of any sign, of the oxygen with any other particle.

APPENDIX

The Bohr effect calculation can also be made more abstractly by using the formal properties of differentials. Since the free energy of a single heme protein is a thermodynamic potential which is determined by the state of the system and not by the path by which it reached the state, dF is an exact differential. The Maxwell relations can therefore be used to derive the relationships between several standard chemical potentials. This type of calculation, which Wyman calls a linked function analysis, proceeds as follows.[21] Differentiate the standard free energy, Eq. (20), with respect to two particular particle association numbers, z_i and Y:

$$\frac{\partial^2 F}{\partial z_i \partial Y} = N_0 \left(\frac{\partial \mu'_{02}}{\partial z_i}\right)_Y = \frac{\partial^2 F}{\partial Y \partial z_i} = \left(\frac{\partial \mu'_{zi}}{\partial Y}\right)_{z_i}, \qquad \text{(A1)}$$

with $\partial F/\partial Y = \mu'_{02}$ and $\partial F/\partial z_i = \mu'_{zi}$. To keep the notation simple, we will not note explicitly that all the other N_i are constant. With the chain relation for partial differentiation one can express the relationship between the first and third terms in Eq. (A1) in the form:

$$\left(\frac{\partial z_i}{\partial Y}\right)_{\mu'_{zi}} = -N_0 \left(\frac{\partial \mu'_{02}}{\partial z_i}\right)_Y \left(\frac{\partial z_i}{\partial \mu'_{zi}}\right)_Y = -N_0 \left(\frac{\partial \mu'_{02}}{\partial \mu'_{zi}}\right)_Y . \qquad \text{(A2)}$$

In equilibrium, by Eq. (6), μ'_{zi} is equal to the chemical potential of the electrolyte, and μ'_{02} is equal to the chemical potential of the oxygen gas. Hence Eq. (A2) can be written in the form:

$$\sum_i \left(\frac{\partial z_i}{\partial Y}\right)_{\mathrm{pH}} = \frac{N_0}{2.3} \left(\frac{\partial \ln p_{02}}{\partial\, \mathrm{pH}}\right)_Y . \qquad \text{(A3)}$$

A sum has been made of the left side of the equation, since residues with different pK's will each contribute to this term.

Wyman gives this equation in slightly different notation. The factor N_0, the number of hemes per molecule, is sometimes erroneously omitted. Equation (A3) is awkward to use to confront experimental data. To use it Wyman was forced to make some approximations which are drastic analytically, but not drastic practically. The left-hand side of Eq. (A3) was replaced by a finite difference of the number of charges per heme on a fully oxygenated and a fully deoxygenated molecule. The oxygen gas pressure p_{02} varies in this range from infinity to zero. The right-hand side of Eq. (A3), which depends upon the $\ln p_{02}$, has to be evaluated from the mean value of $\ln p_{02}$ which is the logarithm of the geometric mean of these limit pressures. Wyman calls this mean the median pressure, and he approximated it by p_{02} for $Y = \frac{1}{2}$.

Equation (A3) may be transformed into the more convenient form which we have obtained above, Eq. (37), by setting the free-energy derivatives in Eq. (A1) equal to the constant, $\mu_{02z_i} N_0$, which the derivative represents. The chain rule for differentiation then yields the relation

$$\left(\frac{\partial z_i}{\partial Y}\right)_{\mu'_{zi}} = -\sum_i \mu_{02zi} N_0 \left(\frac{\partial z_i}{\partial \mu'_{zi}}\right)_Y . \qquad \text{(A4)}$$

Equation (A4) allows one to express Eq. (A3) in the form of Eq. (37) which is given in the main text. This trivial modification of Wyman's result, Eq. (A3), increases its usefulness immensely. The form we give is more useful for comparison with experimental data since it directly relates two conveniently available sets of data, the Bohr effect and the differential titration curves. Our Eq. (37) also clearly distinguishes the effect of the ionization of different residues on the chemical potential for oxygenation of the heme protein. The derivation given in the main text is certainly as simple as that given in this Appendix, which paraphrases Wyman. Furthermore, a formal derivation such as his does not allow one to derive the explicit temperature dependence of the coupling term, as we have been able to do by using the perturbation result, Eq. (19), to write down the phenomenological form of the free energy in Eq. (20). We thus add the information that the μ_{02zi} will have a component which varies as the reciprocal of the absolute temperature, and present the Bohr effect equation in a form which allows one to use conveniently the results of differential titration measurements. Finally, from our general result, Eq. (37), the effect of DPG, or other associating particles, on the Bohr effect is explicit.

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