and this ancillary activity was eluted just before the acetyltransferase peak in the Sephacryl profile.

The stabilities of this and other aminoglycoside-modifying enzymes (R. G. Coombe and A. M. George, unpublished results) to gel filtration on Sephadex columns in which there is substantial loss of enzyme activity have been reported (Williams & Northrop, 1976; Umezawa et al., 1973).

In these experiments, the criteria for homogeneity of the enzyme were (a) elution of the purified enzyme from Sephacryl and DEAE-Sephacel columns in superimposed activity–protein peaks and (b) a single protein band coincident with acetyltransferase activity in acrylamide gel electrophoresis.

Stability studies and definition of buffer and ion requirements established limits for the successful purification of AAC(3)-V with maximal retention of activity, and for its subsequent employment in kinetic, pH, and substrate studies.

References

Alpers, D. H., Appel, S. H., & Tomkins, G. M. (1965) *J. Biol. Chem. 240*, 10.

Benveniste, R., & Davies, J. (1971a) *FEBS Lett. 14*, 293–296.

Benveniste, R., & Davies, J. (1971b) *Biochemistry 10*, 1787–1796.

Benveniste, R., & Davies, J. (1973) *Annu. Rev. Biochem. 42*, 471–505.

Biddlecome, S., Haas, M., Davies, J., Miller, G. H., Rane, D. F., & Daniels, P. J. L. (1976) *Antimicrob. Agents Chemother. 9*, 951–955.

Coombe, R. G., & George, A. M. (1981) *Aust. J. Chem. 34*, 547–554.

Davies, J., & O'Connor, S. (1978) *Antimicrob. Agents Chemother. 14*, 69–72.

Davies, J., & Smith, D. I. (1978) *Annu. Rev. Microbiol. 32*, 469–518.

Devaud, M., Kayser, F. H., & Huber, U. (1977) *J. Antibiot. 30*, 655–664.

George, A. M. (1980) Ph.D. Thesis, Sydney University.

Haas, M. J., & Dowding, J. E. (1975) *Methods Enzymol. 43*, 611–628.

Holmes, R. K., Minshew, B. H., Gould, K., & Sanford, J. P. (1974) *Antimicrob. Agents Chemother. 6*, 253–262.

Inouye, S., & Ogawa, H. (1964) *J. Chromatogr. 13*, 536–541.

Kawabe, H., Kondo, S., Umezawa, H., & Mitsuhashi, S. (1975) *Antimicrob. Agents Chemother. 7*, 494–499.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Maehr, H., & Schaffner, C. P. (1964) *Anal. Chem. 36*, 104.

Majumdar, M. K., & Majumdar, S. K. (1969) *Appl. Microbiol. 17*, 163.

Mason, D. J., Dietz, A., & Smith, R. M. (1961) *Antibiot. Chemother. (Washington, D.C.) 11*, 118.

Matsuhashi, Y., Yagisawa, M., Kondo, S., Takeuchi, T., & Umezawa, H. (1975) *J. Antibiot. 28*, 442–447.

Umezawa, H., Yamamoto, H., Yagisawa, H., Kondo, S., Takeuchi, T., & Chabbert, Y. A. (1973) *J. Antibiot. 26*, 407–417.

Wagman, G. H., Marquez, J. A., & Weinstein, M. J. (1968) *J. Chromatogr. 34*, 210–215.

Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384.

Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.

Williams, J. W., & Northrop, D. B. (1976) *Biochemistry 15*, 125–131.

Yagisawa, M., Kondo, S., Takeuchi, T., & Umezawa, H. (1975) *J. Antibiot. 28*, 486–489.

Effects of Inositol Hexasulfate on the Oxygen Affinity of Hemoglobin: Verification of the Integral Function Theory of Thermodynamic Linkage†

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ABSTRACT: A detailed series of experimental measurements have been carried out to investigate the effects of inositol hexasulfate (IHS) on the oxygen binding curves of human hemoglobin. The data provide a critical test of the integral function theory for the mutual interaction of two ligands binding to a nondissociating macromolecule [Ackers, G. K. (1979) *Biochemistry 18*, 3372–3380]. This theory, which is required for cases where the fractions of bound and unbound ligands are of comparable magnitude, was found to predict quantitatively the observed effects. The experimentally determined variation of the median oxygen concentration with IHS concentration was analyzed by least-squares methods to determine the IHS binding constants for unliganded and fully oxygenated hemoglobin. The derived constants are in good agreement with independent estimates of their values, providing further verification of the theoretical treatment. General aspects of the integral function approach to thermodynamic linkage are briefly outlined. The importance of this approach for treating physiological situations is discussed.

In many physiological circumstances, as well as in vitro experimental situations, there is a need to understand the mutual influence of several small molecules in their binding reactions to a macromolecule. A frequently encountered case is that where the binding curve of one ligand, X, to a macromolecule, M, is influenced by the presence of a second ligand molecule, D, which also binds to M. Heterotropic effects in the regulation of allosteric proteins (Monod et al., 1965; Benesch & Benesch, 1967) are common examples of these phenomena.

Until recently there has been no theoretical treatment of this problem applicable to conditions where the concentrations of the "regulatory" molecule D in its bound and unbound forms are of comparable magnitude. This condition seems likely to be common under physiological circumstances and is certainly the case for in vitro experiments with regulatory

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0006-2960/82/0421-0875$01.25/0

molecules of high affinity. Recently an exact thermodynamic theory has been developed to account for the effects of a "regulatory" ligand (e.g., organic phosphate binding to hemoglobin) upon the binding curve of a second ligand (e.g., oxygen) in terms of total concentration of the "regulatory" ligand (Ackers, 1979). This treatment follows a general approach, termed the integral function theory of thermodynamic linkage (cf. Ackers & Halvorson, 1974; Johnson et al., 1976; E. G. Clawsey, B. W. Turner, and G. K. Ackers, unpublished results), which extends the previous approaches to the theory of linked functions (e.g., Wyman, 1948, 1964; Weber, 1972, 1975). A brief discussion of the principal differences of approach is presented under Discussion of this paper. For the case of the two ligands X and D, this theory opens up the possibility of interpreting experiments in previously inaccessible ranges of the experimental variables where concentrations of M–D complexes and unbound D are of comparable magnitude. In this paper we present the results of an experimental study aimed at testing this new theory.

The experimental system studied is that of human hemoglobin binding both oxygen and the organic phosphate inositol hexasulfate (IHS).[1] Interest in the influence of organic phosphate interactions on hemoglobin oxygen binding has a 2-fold motivation: (a) certain organic phosphates, notably DPG, IPP, ATP, and GTP, act as potent regulators of hemoglobin function under physiological conditions; (b) these compounds are very effective tools for probing and manipulating the quaternary structural transitions which accompany the cooperative interactions in hemoglobins [for a general review, see Benesch & Benesch (1974)]. For purposes of the present study, IHS was chosen because its binding affinities to deoxyhemoglobin and oxyhemoglobin are sufficiently high to exhibit the special features predicted by the new linkage treatment. A previous study of IHS effects on oxygen binding by human hemoglobin has been presented by Benesch et al. (1977). An especially desirable feature of the IHS molecule is that all six sulfate residues are completely ionized in the region of neutral pH, permitting convenient independent determination of the binding constants by the proton absorption method (Bucci, 1974).

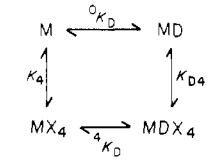
Theoretical Considerations

The detailed theoretical treatment related to the present experimental study has been presented elsewhere (Ackers, 1979). Here we summarize briefly the thermodynamic relationships used in the analysis of our data.

From an oxygenation curve of hemoglobin, measured under any particular set of conditions (temperature, pH, ionic strength, presence of various ligands, etc.), a rigorous determination of the hemoglobin's affinity for oxygen is provided by the median oxygen concentration, $\bar{X}_m$ (Wyman, 1964):

$$\bar{X}_m = \int_0^1 \ln (X)\, d\bar{Y} \qquad (1)$$

where (X) is the concentration of unbound oxygen, X, in equilibrium with tetrameric hemoglobin, M, and $\bar{Y}$ is the fractional saturation with oxygen. The median $\bar{X}_m$ measures the total free energy ΔG required to saturate the oxygen binding sites under the specified conditions: $\Delta G = 4RT \ln \bar{X}_m$, where R is the gas constant and T the absolute temperature. Thus $\bar{X}_m$ is an equilibrium constant for binding four oxygen molecules onto the hemoglobin tetramer. In the case where a second ligand D (e.g., organic phosphate) also binds to the macromolecule M, the value of $\bar{X}_m$, corresponding to any specified concentration of D, is related in an exact way to the thermodynamic constants of the linkage scheme



(2)

where K_4 and K_{D4} are equilibrium constants for binding four oxygens by tetrameric hemoglobin in the absence of D and in the presence of saturating amounts of D, respectively. These constants are directly calculable from the median concentrations of the oxygen binding curves in the absence of D and with saturating amounts of D by the relationship $(\bar{X}_m)^{-4} = K_4$ or K_{D4}, respectively. ${}^0K_D$ and ${}^4K_D$ are the respective equilibrium constants for binding 1 mol of D to the tetramers in the unliganded and fully oxygenated forms [cf. Ackers (1979) for more detailed definitions and derivations]. The fundamental relationships between these constants and the experimentally determined median ligand concentration $\bar{X}_m$ are given in eq 3 and 4 for a solution of hemoglobin tetramers (concentration of M_t in molar heme) in the presence of organic phosphate at total concentration, (D_t):

$$\log P_m = \log P_m{}^0 + \frac{1}{4}\log\frac{{}^0K_D}{{}^4K_D} + \frac{1}{2(M_t)}\left[\frac{1}{{}^4K_D} - \frac{1}{{}^0K_D}\right] + \frac{1}{2(M_t)}\phi \ln\frac{{}^0K_D}{{}^4K_D} + \frac{A_1}{2(M_t){}^0K_D} - \frac{A_2}{2(M_t){}^4K_D} + \left[\frac{1}{4} + \frac{\phi}{2(M_t)}\right]\ln\frac{[(A_1+1)/{}^0K_D] + [[2\phi + (M_t)]/2]}{[(A_2+1)/{}^4K_D] + [[2\phi + (M_t)]/2]} + \frac{\phi}{2(M_t)}\ln\frac{A_2 + \phi{}^4K_D + 1 + [(M_t)/(2\phi)]}{A_1 + \phi{}^0K_D + 1 + [(M_t)/(2\phi)]} \qquad (3)$$

$${}^0K_D K_{D4} = K_4{}^4K_D \qquad (4)$$

where $A_1 = [(1 + \phi{}^0K_D)^2 + (M_t){}^0K_D]^{1/2}$, $A_2 = [(1 + \phi{}^4K_D)^2 + (M_t){}^4K_D]^{1/2}$, $\phi = (D_t) - (M_t)/4$, P_m is the median partial pressure, related to $\bar{X}_m$ through Henry's law constant, $K_H = \bar{X}_m/P_m$, and $P_m{}^0$ is the median pressure in the absence of phosphate. Equation 4 is merely a statement of conservation of energy around the linkage scheme. Equation 3 is the principal result from the new theory. A special case of this theory expresses the variables in terms of the concentration of unbound organic phosphate:

$$\log P_m = \log P_m{}^0 + \frac{1}{4}\log\frac{1 + {}^0K_D(\bar{D})}{1 + {}^4K_D(\bar{D})} \qquad (5)$$

In eq 5 $(\bar{D})$ is the concentration of unbound phosphate in equilibrium with the hemoglobin at the median point P_m. Equation 5 and equations of similar form can be used to derive values of the constants ${}^0K_D$, ${}^4K_D$, and $P_m{}^0$ from experimental measurements of P_m as a function of organic phosphate concentration provided the binding of D is sufficiently weak that the approximation $(\bar{D}) \simeq (D_t)$ may be used (Benesch et al., 1971, 1976, 1977; Bare et al., 1974; Edalji et al., 1976; Baldwin, 1975; Szabo & Karplus, 1976). A major prediction of the theoretical treatment that leads to both eq 3 and 5 is that for a regulatory ligand of high affinity, most of the observable variation of P_m with (D_t) will occur in a region of the

[1] Abbreviations: DPG, 2,3-diphosphoglycerate; IHP, *myo*-inositol hexaphosphate; IHS, *myo*-inositol hexasulfate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; ATP, adenosine triphosphate; GTP, guanosine triphosphate; IPP, *myo*-inositol pentaphosphate.

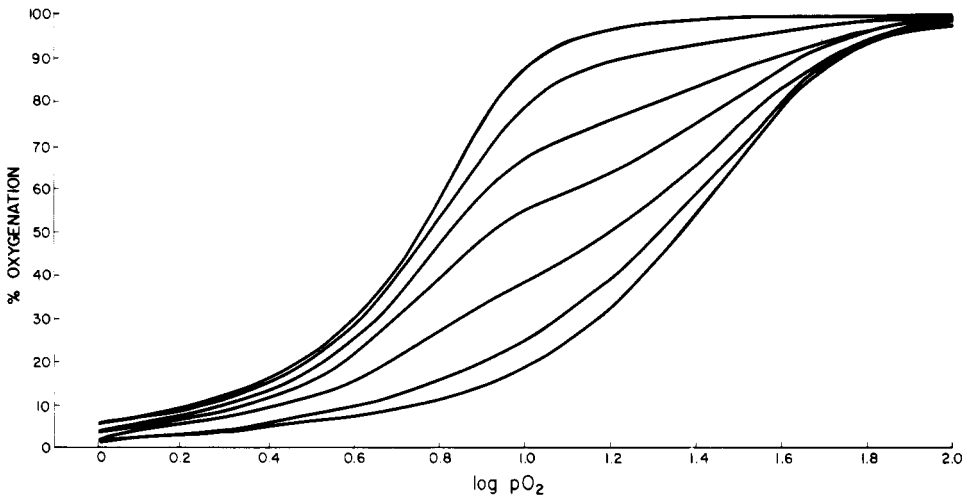


FIGURE 1: Oxygenation curves of hemoglobin in the presence of inositol hexasulfate (IHS). Hemoglobin concentration 1 mM (as tetramer); total IHS concentration from left to right: 0, 0.1, 0.3, 0.5, 0.7, 1.0, and 1.8 mM; all measurements at 25 °C in 0.05 M Bis-Tris buffer, pH 7.3; total chloride concentration 0.1 M.

experimental variables not describable by eq 5 when it is used with the approximation $(\bar{D}) \simeq (D_t)$. Prior to the present study, no series of experimental values [P_m vs. (D_t)] has existed which would permit critical testing of this prediction or of the quantitative validity of eq 3. In order to evaluate eq 3, it was necessary to obtain oxygenation curves which were densely spaced throughout the region where P_m is most sensitive to (D_t). From results of the previous experimental study (Benesch et al., 1977) and theoretical calculations (Ackers, 1979), we were able to design such a series of experiments.

Methods

Hemoglobin was prepared as described previously (Benesch et al., 1972) from adult human blood. *myo*-Inositol hexasulfate (IHS) was prepared by Terra Marine Bioresearch, La Jolla, CA, by the method of Fatiadi (1970). The concentrations of solutions of IHS were determined by direct weighing on the basis of a molecular weight of 924. All measurements were made at 25 °C in 0.05 M Bis-Tris buffer, pH 7.3, with a total chloride concentration of 0.1 M.

Oxygenation curves were determined by using a modified Hemoscan oxygen analyzer (Aminco). Each oxygenation curve was automatically recorded and subsequently digitized manually. The data points from each curve were analyzed for the median oxygen concentration according to eq 1. This and all other calculations were performed on a Hewlett-Packard 1000 system. The median values were converted into partial pressures P_m by using a value of 1.69×10^{-6} M mm^{-1} for Henry's law constant (Wilhelm et al., 1977). The values of P_m of each IHS concentration were subjected to nonlinear least-squares analysis by using a fitting program described elsewhere (Johnson et al., 1976; Ackers & Johnson, 1981). This program determines confidence limits for the estimated parameters as well as their values.

Independent determinations of the binding constants for IHS to unliganded and oxyhemoglobins were carried out by the proton absorption method (Bucci, 1974; Benesch et al., 1976; Edalji et al., 1976).

For all oxygen equilibrium experiments, the hemoglobin concentration was 4.0×10^{-3} M (in units of molar heme). At this concentration the fraction of dimers was negligible (Mills et al., 1976).

Results and Discussion

Oxygenation Curves. A series of 22 oxygenation curves were obtained in the presence of various concentrations (D_t) of IHS, ranging from 1×10^{-4} to 2.0×10^{-3} M, i.e., 0.1–2.0 mol/Hb tetramer. In addition, 11 oxygenation curves were measured in the absence of IHS to obtain an accurate estimate of P_m^0. The value of P_m^0 was found to be 4.8 ± 0.2 mmHg. A subset of the oxygenation curves is shown in Figure 1, illustrating the principal features observed. It can be seen that IHS has a dramatic effect on both the positions and shapes of the oxygenation curves. The curves are highly asymmetric in general. At intermediate ratios of IHS to Hb, they exhibit a pronounced bimodality. Thus the approximation, commonly employed, of substituting in thermodynamic equations partial pressures at half-saturation, P_{50}, rather than the median values, P_m, is clearly to be avoided. In these data the P_{50} values differ from corresponding P_m values by as much as 10% (2 mmHg), with the largest deviations being at IHS/Hb ratios greater than 0.8.

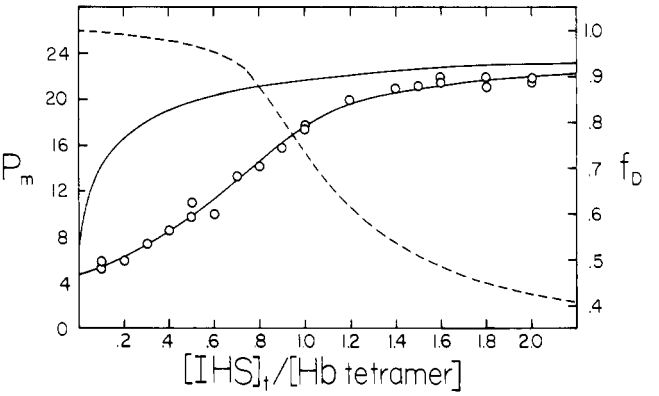


FIGURE 2: Effect of IHS on affinity for oxygen. Circles are median values, P_m, of partial pressure. Each median value was determined from the oxygenation curve measured at the total concentration of IHS, $[IHS]_t$, against which the median is plotted on this graph. The solid curve through the median points represents the best fit to eq 3. The solid curve to the left shows the predicted variation of P_m with IHS concentration according to eq 5 (see text). The dashed curve shows the fraction of $[IHS]_t$ which is bound to the hemoglobin at an O_2 pressure equal to P_m. The upper limit value for P_m is 25.2 mmHg (see text).

In Figure 2 the values of P_m are plotted as a function of the molar ratio of IHS to hemoglobin tetramers. These data were fit to eq 3 and 5 by nonlinear least-squares analysis, yielding the estimated parameters shown in Table I. Equation 3 was found to provide an excellent fit to the data, as judged by the variance of the fit, the randomness in the distribution of residuals to the best fit, and the agreement between values of

Table I: Derived Values of Interaction Constants for Oxygen and IHS Binding to Hemoglobin

	fitted values[a] (confidence limits)	independent estimates
${}^0K_D$	1.12×10^6 (6.49×10^5, 1.92×10^6)	1.25×10^6 [b]
${}^4K_D$	1.27×10^3 (6.73×10^2, 2.39×10^3)	2.5×10^3 [b]
$P_m{}^0$	4.60 (4.36, 4.85)	4.88 ± 0.20
	variance of fit: 9.4×10^{-13}	

[a] Median values, P_m, derived from 22 oxygenation curves corresponding to various concentrations of IHS were analyzed by least-squares analysis according to eq 3. [b] Values taken from Benesch et al. (1977) were measured under identical buffer conditions to experiments of this study but pertain to a temperature of 20 °C and hemoglobin concentrations of 0.01 (${}^0K_D$) and 0.04 mM (${}^4K_D$).

all estimated parameters and their independently determined values [cf. Johnson et al. (1976) for a discussion of these criteria]. Table I shows the best-fit values obtained by allowing $P_m{}^0$, ${}^0K_D$, and ${}^4K_D$ to "float" simultaneously in the fitting procedure. The confidence limits corresponding to 65% probability are also given for the estimated parameter values. It was found that, within these confidence limits, the constants $P_m{}^0$ and ${}^0K_D$ are identical with the independently estimated values, while the estimated values of ${}^4K_D$ differ by a factor of 2. The least-squares program employed has been discussed extensively in previous publications (Johnson et al., 1976; Ackers & Johnson, 1981; Turner et al., 1981). The values shown in Table I represent unique fits to the data and are independent of all initial guesses within a factor of at least 10^2 of the value shown.

The statistical precision with which any of these parameters can be estimated is of course increased when one or more of them can be solidly anchored at independently estimated values, which are then held fixed in the fitting procedure. The present data set was found capable of defining any one of the three parameters to within a few percent when the other two were held fixed. Additionally, we carried out tests of the sensitivity of eq 3 and these data to variations in the hemoglobin concentration, (M_t). Values of $P_m{}^0$, ${}^0K_D$, and ${}^4K_D$ were held fixed at their best-fitted values (Table I), and the data were fit by eq 3 to the best value of (M_t). The program converged on 4.04×10^{-3} M with confidence limits of $\pm 0.25 \times 10^{-3}$ M and an excellent variance of 8.35×10^{-13}. By contrast, when (M_t) was fixed at 1×10^{-3} M and the data simultaneously fitted for $P_m{}^0$, ${}^0K_D$, and ${}^4K_D$, the variance increased to 1.3×10^{-8}, the distribution of residuals was highly skewed, and the estimated parameters were far outside the ranges of the independent estimates (i.e., ${}^0K_D$ was 4×10^{22}, ${}^4K_D$ was 6×10^{36}, and $P_m{}^0$ was 10^5 mmHg). These results indicate a high degree of sensitivity to hemoglobin concentration in the analysis of data according to eq 3.

The solid curve of Figure 2 through the median points was calculated from eq 3 by using the estimated constants given in Table I. It is seen that the major shift in P_m effected by IHS occurs in the range of IHS/Hb ratios below 1.0, and the transition is 84% complete at a ratio of 1.5. This nearly equimolar region is where bound IHS and unbound IHS exist in comparable proportion, as shown by the dashed curve of Figure 2. This fraction f_D of bound IHS was calculated by combination of eq 3 and 5; i.e., once the correct values of all parameters except ($\bar{D}$) are known from analysis of the data in terms of eq 3, then eq 5 which is rigorously correct can be used to determine ($\bar{D}$). The fraction, f_D, of bound IHS is then calculated as $1 - (\bar{D})/(D_t)$. An interesting feature of the solid curve of Figure 2 is the slow asymptotic approach to the upper limit value of 25.1 mmHg, corresponding to K_{D4} of eq 2. Such a slow approach of the full-saturation value could lead to errors in attempts to evaluate K_{D4} from experimental values of P_m at high concentrations of IHS. Even at an IHS concentration of 3 mM, P_m is only 23.2 mmHg.

Efforts to analyze the median values solely according to eq 5 under the approximation (D) = (D_t) were highly unsuccessful. ${}^0K_D$ and $P_m{}^0$ were found to be essentially infinitely correlated with each other in this data set, and the pair (${}^0K_D$ and ${}^4K_D$) was almost as unresolvable. All fits gave high variances and extreme skewing of residuals (Johnson et al., 1976). The most reasonable result was obtained when $P_m{}^0$ was fixed at 4.6 mm and ${}^0K_D$ at 1.1×10^6, and the data were analyzed for ${}^4K_D$ alone. A value of 6.33×10^3 was then obtained. When the correct values of $P_m{}^0$, ${}^0K_D$, and ${}^4K_D$ (Table I) were used in eq 5, the resulting predicted variation of P_m with (IHS) is shown in Figure 2 as the solid line to the left of the data points. It is strikingly clear that eq 5 does not provide an adequate description of the data when used with correct values of the constants. These findings confirm the predictions made earlier regarding the conditions where eq 3 is required (Ackers, 1979) and provide strong verification of the thermodynamic treatment which has been deduced to account for these effects.

Under normal physiological conditions within the red cell, the concentrations of hemoglobin tetramers and DPG molecules are nearly equal, i.e., about 5 mM (Rapoport & Guest, 1941), so that total DPG cannot be equated to free DPG and eq 5 is not applicable. Therefore eq 3 should make it possible for the first time to predict the effect of changes in DPG level on oxygen delivery under normal and pathological conditions. This would apply to situations where the red cell DPG level is elevated, such as anemias (Oelshlegel et al., 1972) or sojourn at high altitudes (Lenfant et al., 1968), and especially where the opposite is the case, as in transfusion of stored blood (Valeri, 1974) and certain glycolytic enzyme deficiencies (Metcalfe & Dhindsa, 1972; Rosa et al., 1978).

Finally, we wish to draw attention to certain aspects of the theory of linked functions which are illustrated by this study. The thermodynamic treatment of eq 3 is based on an approach we have called the integral function theory. This approach extends substantially the Wyman theory of linked functions, which has formed a useful basis for much understanding of linked reactions in macromolecular systems. The Wyman theory is based upon derivative relationships between the various experimental parameters. In the case of two linked binding functions, the Wyman theory provides a means of predicting, from derivative relationships, the directions of influence by the several ligands upon each other's binding curves. Other linked processes, such as subunit assembly and conformation change, are similarly treated. The derivative relationships of the Wyman approach have the virtue of providing a quantitative understanding of the rates of mutual variation among the linked processes. They do not, however, provide a framework for describing the *actual* variations among the experimental quantities. In order to do this, it is necessary to have relationships between the experimental parameters rather than their derivatives. One way of solving this problem is to attempt a "brute force" integration of the derivative relationships for the conditions of a particular experimental situation. An alternative method, which we have termed the integral function approach (E. G. Clawsey, B. W. Turner, and G. K. Ackers, unpublished results), is based upon the use of generalized functions which have the form of reaction constants, e.g., for subunit assembly (${}^xK_2$), for ligand

binding (X_{med}, ${}^{x}K_D$) or for conformation change (${}^{x}K_I$). Such a generalized constant is formulated in terms of its variation with (a) concentrations of the various molecular species comprising the system of interest, e.g., the variation of ${}^{x}K_2$ with X etc., and (b) the microscopic reaction constants of the system. When these functions are used, relationships are then established which permit interpretation of the various overall reactions (e.g., binding isotherms, assembly reactions corresponding to experimental constraints, ligands, etc.). An extensive discussion of this approach and its various uses will be presented elsewhere (E. G. Clawsey, B. W. Turner, and G. K. Ackers, unpublished results). Its application to experimental data on the ligand-linked subunit assembly of hemoglobin has been described previously (Mills et al., 1976). The present study demonstrates the successful application of the integral function approach to the mutual interactions of two ligands binding to a nondissociating macromolecule.

Acknowledgments

We thank Benjamin W. Turner for help with the computational aspects of this work.

References

Ackers, G. K. (1979) *Biochemistry 18*, 3372–3380.

Ackers, G. K., & Halvorson, H. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4312–4316.

Ackers, G. K., & Johnson, M. L. (1981) *J. Mol. Biol. 147*, 559–582.

Baldwin, J. (1975) *Prog. Biophys. Mol. Biol. 29*, 225–320.

Bare, G. H., Alben, J. O., Bromberg, P. A., Jones, R. T., Brimhall, H., & Padilla, F. (1974) *J. Biol. Chem. 249*, 773–779.

Benesch, R., & Benesch, R. E. (1967) *Biochem. Biophys. Res. Commun. 26*, 162–164.

Benesch, R., Edalji, R., & Benesch, R. E. (1976) *Biochemistry 15*, 3396–3398.

Benesch, R. E., & Benesch, R. (1974) *Adv. Protein Chem. 28*, 211–237.

Benesch, R. E., Benesch, R., Renthal, R., & Gratzer, W. B. (1971) *Nature (London), New Biol. 234*, 174–176.

Benesch, R. E., Benesch, R., Renthal, R., & Maeda, N. (1972) *Biochemistry 11*, 3576–3579.

Benesch, R. E., Edalji, R., & Benesch, R. (1977) *Biochemistry 16*, 2594–2597.

Bucci, E. (1974) *Biochemistry 13*, 814–818.

Edalji, R., Benesch, R. E., & Benesch, R. (1976) *J. Biol. Chem. 251*, 7720–7721.

Fatiadi, A. C. (1970) *Carbohydr. Res. 12*, 293.

Johnson, M. L., Halvorson, H. R., & Ackers, G. K. (1976) *Biochemistry 15*, 5363–5371.

Lenfant, C., Torrance, J., English, E., Finch, C. A., Reynafarje, C., Ramos, J., & Faura, J. (1968) *J. Clin. Invest. 47*, 2652–2656.

Metcalfe, J., & Dhindsa, D. S. (1972) in *Oxygen Affinity of Hemoglobin and Red Cell Acid Base Status* (Rorth, M., & Astrup, P., Eds.) pp 613–628, Academic Press, New York.

Mills, F. C., Johnson, M. O., & Ackers, G. D. (1976) *Biochemistry 15*, 5350–5362.

Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.

Oelshlegel, F. J., Brewer, G. J., Penner, J. A., & Schoomaker, E. B. (1972) in *Hemoglobin and Red Cell Structure and Function* (Brewer, G. J., Ed.) pp 377–396, Plenum Press, New York.

Rapoport, S., & Guest, G. M. (1941) *J. Biol. Chem. 138*, 269.

Rosa, R., Prehu, M. O., Beuzard, Y., & Rosa, J. (1978) *J. Clin. Invest. 62*, 907–915.

Szabo, A., & Karplus, M. (1976) *Biochemistry 15*, 2869–2877.

Turner, B. W., Pettigrew, D. W., & Ackers, G. K. (1981) *Methods Enzymol. 76*, 596–628.

Valeri, C. R. (1974) in *The Red Blood Cell* (Surgenor, D. M., Ed.) pp 511–574, Academic Press, New York.

Weber, G. (1972) *Biochemistry 11*, 864–878.

Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.

Wilhelm, E., Battino, R., & Wilcock, R. J. (1977) *Chem. Rev. 77*, 219–241.

Wyman, J. (1948) *Adv. Protein Chem. 4*, 407–443.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 22–286.