ELSEVIER

Biophysical Chemistry 100 (2003) 123–129

Biophysical Chemistry

www.elsevier.com/locate/bpc

Hemoglobin–oxygen equilibria: retrospective and phenomenological perspective☆

Irving M. Klotz*

Northwestern University, Evanston, IL 60208-3113, USA

Received 17 January 2002; accepted 14 April 2002

Abstract

The origin of the concept of a molecular complex between oxygen and hemoglobin can be traced to Stokes, a century and a half ago. Subsequently, physicochemical concepts of equilibria provided a path to quantitative formulations of these ligand–receptor interactions. Then, it took a quarter of a century before a proper format was prepared in terms of four stoichiometric equilibria and their associated binding constants. Since then, experimental measurements of these stoichiometric binding constants have consistently disclosed that successive values of K_1 to K_4 are accentuated above those expected if every subunit of hemoglobin maintained the same, intrinsic, unchanging affinity for oxygen. An alternative analysis of the observed cooperative interactions has been obtained by extracting the roots of the polynomial of the stoichiometric binding equation and deriving an alternative binding equation containing constants that for O_2–Hb are complex numbers. These constants have the dimensions and properties of equilibrium constants. They provide some novel phenomenological insights into ligand–receptor equilibria.

Keywords: Ligand–receptor interactions; Stoichiometric binding constants; Complex number constants

1. Introduction

Hemoglobin has occupied a pre-eminent position in fundamental research in physiology and biophysics for a century and a half. It has also provided, among a wide range of biological insights, a primary molecular system for the elucidation of ligand–receptor interactions in general.

In the basic life sciences, it is a fully accepted concept that a physiological or pharmacological effector must be bound by a receptor to initiate a biological response. However, this view was not at all obvious or intuitive 150 years ago.

It is often difficult to pinpoint precisely the first appearance of a new insight. In hindsight, it seems apparent that it was observations by a physicist of perturbations in hemoglobin spectra in the presence of oxygen that provided the first glimpse of a molecular interaction between these two substances. Stokes, in the middle of the 19th century,

☆ This was an area of interest to Professor John T. Edsall throughout his scientific career. For a very detailed review of the history of research on hemoglobin, see J.T. Edsall, 'Blood and hemoglobin: the evolution of knowledge of functional adaptation in a biochemical system', J. Hist. Biol. 5(2) (1972) 205–257.

*Corresponding author. Tel: +1-847-491-3546; fax: +1-847-491-7713.

E-mail address: i-klotz@northwestern.edu (I.M. Klotz).

0301-4622/03/$ - see front matter
PII: S0301-4622(02)00273-9

was Lucasian Professor at Cambridge University, one in the long line of towering theoretical physicists from Isaac Newton to Stephen Hawking to hold that appointment. In addition to being a great theorist, he was also a talented experimentalist, particularly in developing spectroscopic methods for examining chemical substances and reactions and for studying blood. It was Stokes who first recognized, from changes in spectra when oxygen (O_2) was removed from and subsequently added to blood, that oxygen combined with hemoglobin[1], i.e. that a complex was formed [1].

During the last decades of the 19th century, this insight was generalized, particularly by Ehrlich and Langley, to a wide range of interactions of natural and synthetic substances that act upon tissues and organs. It was Ehrlich [2] who coined the famous aphorism *Corpora non agunt nisi fixata* which translated becomes, ''A substance cannot be (biologically) active unless it is 'fixed' (bound to a receptor).''

2. The equilibrium constant

The idea of an O_2–hemoglobin complex proposed by Stokes provided a concrete molecular image, but was only qualitative in nature. Before connections to thermodynamics could be established, the conception of an equilibrium constant had to emerge. The germ of this concept appeared first in papers of Guldberg and Waage (in the 1860s) who formulated the Law of Mass Action [3]. Subsequently, Gibbs and von Helmholtz introduced the free energy function and van't Hoff built a bridge from theoretical thermodynamics to equilibrium chemical phenomena. Among other relationships, the equation

$$\Delta G^\circ = -RT\ln K \tag{1}$$

where K is the equilibrium constant and ΔG° the corresponding standard free energy was derived, and it provided the foundation for connecting O_2–hemoglobin equilibria to thermodynamic quantities. Thus, it became apparent that the route to quantitative assessments of hemoglobin–ligand interactions lies through the respective equilibrium constants.

[1] Hemoglobin from blood had been crystallized in 1840.

For hemoglobin, the earliest such studies came from Hüfner [4,5]. Assuming a molecular weight of 16 000 for hemoglobin, corresponding to 1 mol of its content of linked iron, he presented the oxygenation equilibrium in terms of a 1:1 stoichiometric complex. Viewed as a combination of species (Hüfner focused on the dissociation direction), one writes

$$\mathrm{Hb} + \mathrm{O_2} \rightleftharpoons \mathrm{HbO_2} \tag{2}$$

Adopting the format of his contemporary physical chemists (Nernst, Ostwald, Arrhenius), Hüfner formulated the appropriate ratio of equilibrium concentrations, which for Eq. (2) is

$$\frac{(\mathrm{HbO_2})}{(\mathrm{Hb})(\mathrm{O_2})} = K \tag{3}$$

where the parentheses represent concentration. The moles of bound O_2, $[HbO_2]$, per mole of total protein, $[Hb] + [HbO_2]$, may be represented by B, which is also the fractional saturation, Y, of the hemoglobin in a one-to-one complex. With some simple algebraic manipulations (see [6]), one can show, using Eq. (3), that

$$B = \frac{K(\mathrm{O_2})}{1 + K(\mathrm{O_2})} \tag{4}$$

In graphical form, this equation corresponds to a rectangular hyperbola (Fig. 1). Hüfner found that his experimental observations fitted a curve of the shape shown in Fig. 1. Because at half-saturation $Y = B = 0.5$, one finds from Eq. (4) that

$$K = \frac{1}{(\mathrm{O_2})_{\mathrm{at}\ Y=0.5}} \tag{5}$$

That is how Hüfner evaluated K for his hemoglobin–oxygen interaction.

This procedure, however, uses only a single point, the concentration of free O_2 at $B = 0.5$. Obviously, it would be preferable to include all of the experimental measurements. In modern times with sophisticated algebraic fitting procedures and vast computer resources, it would be a straightforward matter to find the best value of K from Eq. (4) using all the experimental data.

Even before modern computational procedures were available, several graphical techniques were

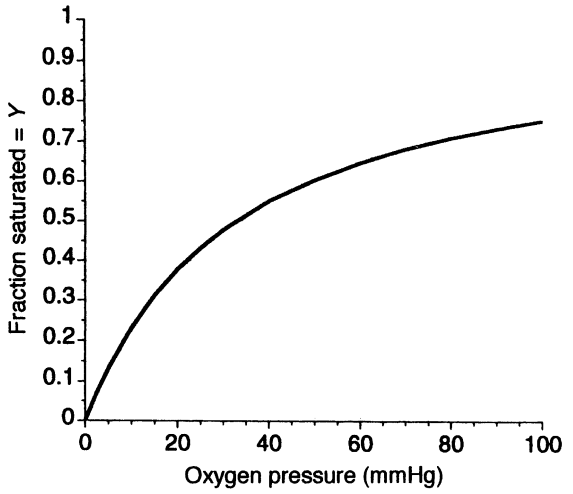

Fig. 1. Oxygen uptake curve for a hemoglobin that can bind only one made of O_2 at saturation, and is half-saturated at an oxygen pressure near 30 mmHg.

developed for the conversion of Eq. (4) to a linear transform (see [6]). These have acquired different proper names, but were all originally formulated by Wolff in the 1920s (see [7,8]).

For a univalent stoichiometry between ligand and receptor, as in Hüfner's treatment of O_2–Hb, these graphical procedures have little to offer in the presence of modern algebraic computational treatments.

By the beginning of the 20th century, it had become apparent that Hüfner's formulation of the Hb/O_2 equilibrium in terms of a one-to-one complex could not be correct. More careful and extensive measurements by Bohr [9,10] showed unequivocally (Fig. 2), that the oxygen uptake curve was sigmoidal in shape. An equilibrium constant of the form shown in Eq. (3) cannot lead to a sigmoidal oxygen uptake curve. Evidently, Eq. (2) is not an appropriate representation of the Hb/O_2 equilibrium.

An alternative to Eq. (2) that can produce a sigmoidal shape in the oxygen uptake curve for hemoglobin is [11]

$$\mathrm{Hb} + n\mathrm{O_2} = \mathrm{Hb(O_2)}_n \tag{6}$$

This equation presumes that Hb has a capacity to hold n moles of O_2 and that these moles of O_2 are bound simultaneously, not in a stepwise fashion. Under these circumstances, fractional saturation as a function of O_2 pressure is given by the equation

$$Y = \frac{K(\mathrm{O_2})^n}{1 + K(\mathrm{O_2})^n} \tag{7}$$

From this, one can also obtain the relation

$$\log\frac{Y}{1-Y} = \log K + n\log(\mathrm{O_2}) \tag{8}$$

which is known as the Hill equation. The corresponding graph, the Hill plot, should be a straight line with a slope of n, the number of sites for O_2 (or in the general case for the ligand) on the receptor molecule of hemoglobin. Corresponding graphs are often published for other ligand–receptor combinations.

It is not widely recognized that the Hill equation is essentially an empirical one, despite the conjunction of Eq. (6) with Eq. (7). Even with hemoglobin (a particularly favorable system for its application), experimental measurements (in the range that fit a linear Hill plot) yield a non-integer value for n of 2.5; yet it is now known that hemoglobin has four binding sites for O_2 and not a fractional number.

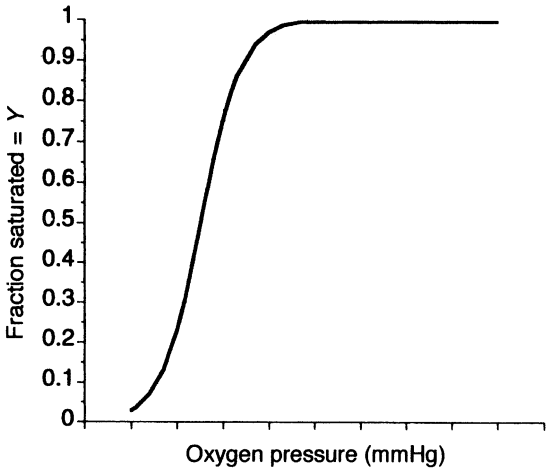

Fig. 2. Sigmoidal shape of oxygen uptake curve for hemoglobin representative of those described by Bohr et al. [9,10] with dog blood.

Furthermore, to utilize Eq. (8) to correlate binding results, one must be able to reach saturation of the receptor. This goal is generally not feasible experimentally.

For a multiple-site receptor (i.e. $n>1$), Eq. (6) is a highly oversimplified representation. First of all, the binding of ligand in most cases occurs in successive steps, each of which would have an equilibrium constant. Secondly, it is necessary to appreciate that for a multi-site receptor ($n>1$) one must distinguish between two different types of equilibrium constant (12, 6), which we shall designate 'stoichiometric' and 'site', respectively.[2]

For hemoglobins constituted of four subunits, oxygen binding can be presented in terms of four stoichiometric binding constants. On the other hand, a full description in terms of individual sites requires 32 site-binding constants, although this number can be reduced substantially if one assumes specific molecular models.

In the present exposition, we shall focus on the phenomenological stoichiometric binding formulation.

3. Stoichiometric equilibrium constants for oxygen–hemoglobin equilibria

From a stoichiometric perspective, the stepwise binding of O_2 to a tetrameric Hb is represented by the equilibria

$$\mathrm{Hb}+\mathrm{O_2}=\mathrm{Hb}\{\mathrm{O_2}\} \quad K_1=\frac{(\mathrm{Hb}\{\mathrm{O_2}\})}{(\mathrm{Hb})(\mathrm{O_2})} \tag{9}$$

$$\mathrm{Hb}\{\mathrm{O_2}\}+\mathrm{O_2}=\mathrm{Hb}\{\mathrm{O_2}\}_2 \qquad K_2=\frac{(\mathrm{Hb}\{\mathrm{O_2}\}_2)}{(\mathrm{Hb}\{\mathrm{O_2}\})(\mathrm{O_2})} \tag{10}$$

$$\mathrm{Hb}\{\mathrm{O_2}\}_2+\mathrm{O_2}=\mathrm{Hb}\{\mathrm{O_2}\}_3 \qquad K_3=\frac{(\mathrm{Hb}\{\mathrm{O_2}\}_3)}{(\mathrm{Hb}\{\mathrm{O_2}\}_2)(\mathrm{O_2})} \tag{11}$$

$$\mathrm{Hb}\{\mathrm{O_2}\}_3+\mathrm{O_2}=\mathrm{Hb}\{\mathrm{O_2}\}_4 \qquad K_4=\frac{(\mathrm{Hb}\{\mathrm{O_2}\}_4)}{(\mathrm{Hb}\{\mathrm{O_2}\}_3)(\mathrm{O_2})} \tag{12}$$

[2] In the literature, these are also widely called 'macroscopic' and 'microscopic' equilibrium constants, respectively. Such designations seem inappropriate because numerical values for both are expressed in a macroscopic unit molarity.

Table 1
Some characterizing binding constants[a] for oxygen–hemoglobin complexes

	Stoichiometric Binding Constants	$(K_i/K_1)_{\mathrm{ideal}}$	Virtual binding constants
Sheep hemoglobin [18]	$K_1=0.1124$	[1]	$K_\alpha=0.30e^{0.24\pi i}$
	$K_2=0.1974$	0.375	$K_\beta=0.30e^{-0.24\pi i}$
	$K_3=0.1475$	0.1667	$K_\gamma=0.27e^{0.30\pi i}$
	$K_4=1.996$	0.0625	$K_\delta=0.27e^{-0.30\pi i}$
Human hemoglobin [19]	$K_1=0.0188$	[1]	$K_\alpha=0.21e^{0.26\pi i}$
	$K_2=0.0566$	0.375	$K_\beta=0.21e^{-0.26\pi i}$
	$K_3=0.407$	0.1667	$K_\gamma=0.21e^{0.24\pi i}$
	$K_4=4.28$	0.0625	$K_\delta=0.21e^{-0.24\pi i}$
Human hemoglobin cross-linked [20]	$K_1=0.3497$	[1]	$K_\alpha=0.49e^{0.20\pi i}$
	$K_2=0.1409$	0.375	$K_\beta=0.49e^{-0.20\pi i}$
	$K_3=0.2686$	0.1667	$K_\gamma=0.39e^{0.31\pi i}$
	$K_4=2.802$	0.0625	$K_\delta=0.39e^{-0.31\pi i}$

[a] In mm^{-1}.

where $K_1...K_4$ are the successive stoichiometric equilibrium constants. The fractional saturation of tetrameric hemoglobin, Y, and the moles of bound O_2 per mole protein, B, are then expressed by the equation

$$B=4Y=\frac{K_1(\mathrm{O_2})+2K_1K_2(\mathrm{O_2})^2+3K_1K_2K_3(\mathrm{O_2})^3+4K_1K_2K_3K_4(\mathrm{O_2})^4}{1+K_1(\mathrm{O_2})+K_1K_2(\mathrm{O_2})^2+K_1K_2K_3(\mathrm{O_2})^3+K_1K_2K_3K_4(\mathrm{O_2})^4} \tag{13}$$

For hemoglobin, this formulation[3] was published by Adair [13], shortly after his osmotic pressure experiments established that hemoglobin is a tetramer.

Some classical and some more recent experimental values for K_1, K_2, K_3 and K_4 are listed in Table 1. The stoichiometric equilibrium constants are always real positive numbers.

Also listed in Table 1 are the relative values that the successive stoichiometric constants would have if all four subunits maintained exactly the same affinity for O_2 as the fractional saturation Y progressed from 0 to 1. These ratios are designated $(K_i/K_1)_{\mathrm{ideal}}$. It is obvious in Table 1 that for O_2–Hb the successive stoichiometric affinities are

[3] Several others, before and after Adair, derived completely general equations for the binding of n moles of a ligand by a multivalent receptor [6,12,14–17].

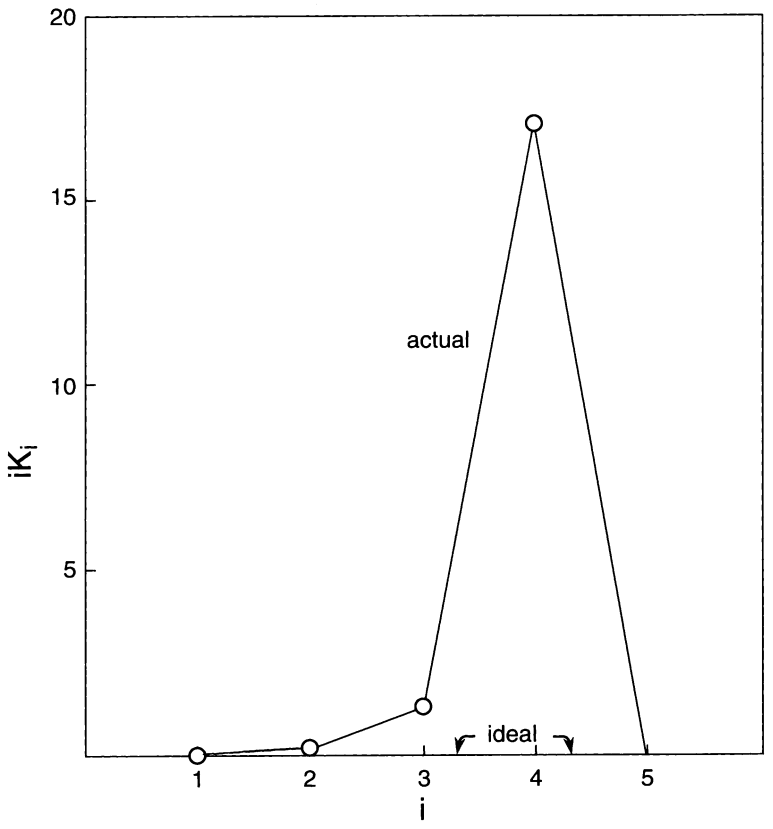

Fig. 3. Affinity profile for uptake of O_2 by hemoglobin. On the scale displayed, the ideal line is so close to the abscissa axis that the two cannot be seen separately.

accentuated with increasing uptake of O_2 by hemoglobin. In anthropomorphic terminology such behavior is ascribed to 'cooperative interactions'.

The trends in accentuations above the ideal can be presented in a more quantitative fashion in a graphical affinity profile [6]. For ideal receptors,

$$i(K_i)_{\text{ideal}} = (n+1)\ K - Ki \tag{14}$$

where K is the intrinsic affinity constant of a non-interacting site. This normalized equation tells us that for an ideal receptor–ligand complex, a graph of $i(K_i)_{\text{ideal}}$ vs. i will present a straight line, a convenient reference form. An example of the actual behavior of O_2–Hb is illustrated in Fig. 3. For this human hemoglobin [19], the accentuations in affinity at each successive step are so strong that the comparison line for ideal behavior is essentially invisible because it lies so close to the abscissa axis.

When the successive stoichiometric constants K_i are accentuated in magnitude above the ideal value, then the roots of the partition function (the denominator) in the equation for B, Eq. (13) for O_2–Hb, are complex numbers [6]. These roots also appear in an alternative algebraic expression for B. For O_2–Hb, that equation is

$$B = \frac{K_\alpha(O_2)}{1+K_\alpha(O_2)} + \frac{K_\beta(O_2)}{1+K_\beta(O_2)} + \frac{K_\gamma(O_2)}{1+K_\gamma(O_2)} + \frac{K_\delta(O_2)}{1+K_\delta(O_2)} \tag{15}$$

The coefficients $K_\alpha ... K_\omega$ have the dimensions of equilibrium constants and are algebraically linked

to the stoichiometric constants $K_1...K_i$. However, they do not correspond to any particular step in the successive equilibria of Eqs. (9)–(12), or to the binding by any specific subunit of hemoglobin. Since each term in Eq. (15) has the form of that for binding of a ligand by a single isolated unit, the K_ωs may be designated as 'virtual' equilibrium constants. In Eq. (15) these virtual constants faithfully reproduce the experimentally observed dependence of B on (O_2) as does the classical Eq. (13) with its stoichiometric binding constants.

When the binding affinity is accentuated in successive stoichiometric steps, then the values of $K_\alpha...K_\omega$ are imaginary numbers. Specifically for oxygen–hemoglobin equilibria, the virtual binding constants are of the form

$$K_\alpha = A_1 e^{i\theta_1}; \quad K_\beta = A_1 e^{-i\theta_1}$$
$$K_\gamma = A_2 e^{i\theta_2}; \quad K_\delta = A_2 e^{-i\theta_2} \tag{16}$$

two pairs of conjugate numbers. Numerical values of these constants for specific preparations of hemoglobin are presented in Table 1. With these values inserted into Eq. (15), one obtains a much simpler algebraic expression than that provided by Eq. (13) to present oxygen saturation of hemoglobin quantitatively and analytically.

The renown mathematician Jacques Hadamard is credited [21] with the observation

> ''The shortest path between two truths in the real domain passes through the complex domain.''

To what extent do the virtual binding constants lead in this direction?

Since K_ω has the dimensions and characteristics of a binding constant, it seems appropriate to insert it into Eq. (1) to obtain a corresponding free energy change. Starting with the Cartesian form of a complex number we write

$$K_\omega = a + ib = \sqrt{a^2+b^2}\, e^{i\ \arctan(b/a)} \tag{17}$$

and thereby obtain the exponential form. Then we recognize that (b/a) is the tangent not only of the angle arctan (b/a), but also of the angles, arctan $[(b/a)+2\pi]$, arctan $[(b/a)+2\pi\cdot 2]$, ... arctan $[(b/a)+2\pi\cdot n]$, where $n=0, +1, +2....$. Consequently, we can change Eq. (17) into

$$K_\omega = a + ib = \sqrt{a^2+b^2}\, e^{i\ [\arctan(b/a)+2\pi n]} \tag{18}$$

Proceeding to insert this result into Eq. (1), we find

$$\begin{aligned}\Delta G^\circ &= -RT\ln(a+ib)\\ &= -1/2\ RT.\ln(a^2+b^2) - iRT[\arctan\ (b/a)+2\pi n]\\ n &= 0, \pm 1, \pm 2\end{aligned} \tag{19}$$

Thus, in the Argand plane, ΔG° is a many-valued function corresponding to many equivalent states in the complex domain.

Turning to a different area, we can return from the complex domain of $K_\alpha...K_\omega$ to the real domain of $K_1...K_2$ by finding equations for the latter in terms of the former [6]. For tetrameric hemoglobin these are,

$$\begin{aligned}K_1 &= K_\alpha + K_\beta + K_\gamma + K_\delta\\ K_1K_2 &= K_\alpha K_\beta + K_\alpha K_\gamma + K_\alpha K_\delta + K_\beta K_\gamma\\ &\quad + K_\beta K_\delta + K_\gamma K_\delta\\ K_1K_2K_3 &= K_\alpha K_\beta K_\gamma + K_\alpha K_\beta K_\delta\\ &\quad + K_\alpha K_\gamma K_\delta + K_\beta K_\gamma K_\delta\\ K_1K_2K_3K_4 &= K_\alpha K_\beta K_\gamma K_\delta\end{aligned} \tag{20}$$

Now, if we insert the individual forms of Eq. (16) for each of its virtual constants, we obtain a format that permits us to explore the variation of each stoichiometric binding constant, as we vary θ_1 and θ_2, respectively, in the exponential complex numbers.

For each θ, we can explore the range $0 \le \theta \le \pi/2$, which correlates with the relative contribution of the imaginary to the real component in the complex number. The effects of such trends on the values of $K_1...K_4$ can then be revealed. Thereafter, one can also delineate the corresponding algebraic equation for the fractional saturation of hemoglobin as a function of O_2 concentration.

Having entered the field of complex variables, one gains access to new functions for analyses of ligand–receptor binding data.

The binding constant K_α (Eq. (15)) being a complex number can be represented as a vector, of length A, in the complex (Argand) plane. In orthogonal notation, $A=a+ib$, and $a^2+b^2=A^2$, which we can designate as the Pythagorean area, A_{Py}. But in the complex (Argand) plane one can also define a function $a^2+(ib)^2=A_{Ar}$. In contrast to A_{Py}, A_{Ar} may take on positive, zero or negative values, yet dimensionally it is an area. Bernoulli converted the elementary problem of obtaining the area (of a quadrant) of a circle into a formulation using complex variables [21], and thereby proved that $i^i=e^{-\pi/2}$. What insights are hidden in A_{Ar}?

The vectors for K_α and K_γ [Eq. (16)] can be added to give the sum vector $K_\Sigma=A_{\Sigma e}i\theta_\Sigma$. From its coordinates in the complex plane, one can calculate the corresponding Argand area A_Σ. How is A_Σ related to A_α and A_β, areas of the constituent vectors A_α and A_β? The Argand areas provide a return bridge from the complex domain into the real domain, so novel real relations between sum and constituent vectors should become available.

The closed loop formed by the vectors K_α, K_γ and K_Σ suggests the examination of other integrals, particularly Cauchy's contour integrals in the complex domain. Many powerful physical relationships have derived therefrom [21].

The binding constants K_α, K_β are calculated from the experimental data, B as a function of ligand concentration (Eq. (15)). Since that analytic expression contains terms that are complex, one may find that the chord length distance C, and the arc length distance, S, of segments of the curve do not become equal as $\Delta x\rightarrow 0$ [21]. In particular, the $\lim_{\Delta x\rightarrow 0}(S/C)<1$, i.e. the arc length is shorter than that of the straight-line chord. It is tantalizing to see if this astonishing behavior is present in binding curves that lie above that of an ideal one.

These and similar inquiries may open portals to new phenomenological insights into ligand–receptor interactions such as those in hemoglobin–oxygen equilibria.

References

[1] G.G. Stokes, On the reduction and oxidation of the colouring matter of blood, Proc. R. Soc. Lond. 13 (1864) 355–364.

[2] P. Ehrlich, Scientific principles, methods and results, Lancet 2 (1913) 445–451.

[3] J.R. Partington, A History of Chemistry, 4,, Macmillan and Co, London, 1964.

[4] G. Hüfner, On the law of dissociation of oxyhemoglobin and some related biological questions, Arch Anat. Physiol. (Physiol. Abteilung) (1890) 1–27

[5] G. Hüfner, Recent research on the dissociation of oxyhemoglobin, Arch. Anat. Physiol. (Anat. Abteilung) (1901) 187–217

[6] I.M. Klotz, Ligand–Receptor Energetics, John Wiley and Sons, New York, 1997.

[7] J.B.S. Haldane, Enzymes, Longmans, Green and Co, London, 1930.

[8] K.G. Stern, Allgemeine Chemie der Enzyme, Steinkopff, Dresden, 1932.

[9] C. Bohr, Theoretical treatment of oxygen uptake of hemoglobin, Zeit. Physiol. 17 (1904) 682–688.

[10] C. Bohr, K.A. Hasselbalch, A. Krogh, Biological connection of the influence of carbon dioxide pressure in blood on the binding of oxygen, Skand. Arch. Physiol. 16 (1904) 401–412.

[11] A.V. Hill, The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curve, J. Physiol. 40 (1910) iv–vii.

[12] E.Q. Adams, Relations between the constants of dibasic acids and of amphoteric electrolytes, J. Am. Chem. Soc. 38 (1916) 1503–1510.

[13] G.S. Adair, The oxygen dissociation curve of hemoglobin, J. Biol. Chem. 63 (1925) 529–545.

[14] K. Linderstrøn-Lang, The ionization of proteins, C. R. Trav. Lab. Carlsberg 7 (15) (1924) 29.

[15] H.S. Simms, Dissociation of polyvalent substances, J. Am. Chem. Soc. 48 (1926) 1239–1250.

[16] I.M. Klotz, F.M. Walker, R.B. Pivan, The binding of organic ions by proteins, J. Am. Chem. Soc. 68 (1946) 1486–1490.

[17] J. Wyman, Heme proteins, Adv. Protein Chem. 4 (1948) 407–531.

[18] F.J.W. Roughton, A.B. Otis, R.J. Lyster, The determination of the individual equilibrium constants of the four intermediate reactions between oxygen and sheep hemoglobin, Proc. R. Soc. Lond. B144 (1955) 29–54.

[19] K. Imai, Adair fitting to oxygen equilibrium curves of hemoglobin, Methods Enzymol. 232 (1994) 559–576.

[20] S. Miura, T.M. Ikeda-Saito, T. Yonetani, C. Ho, Oxygen equilibrium studies of cross-linked asymmetrical cyanomet valency hybrid hemoglobins: models for partially oxygenated species, Biochemistry 26 (1987) 2149–2155.

[21] P.J. Nahin, The Story of $\sqrt{-1}$, Princeton University Press, Princeton, NJ, 1998, p. 70, 105, 157 and 195.