Biophysical Chemistry 13 (1981) 117–123
North-Holland Publishing Company

SPECTROPHOTOMETRIC DETERMINATION OF REACTION STOICHIOMETRY AND EQUILIBRIUM CONSTANTS OF METALLOCHROMIC INDICATORS. I. GENERAL CALCULATIONAL METHOD

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Received 15 July 1980

A calculational method is developed for spectrophotometric determination of stoichiometries and individual equilibrium constants in the complexing of metal ions with metallochromic indicators. Explicit expressions are developed for the calculation of all parameters necessary to describe mixtures of 1 : 1, 1 : 2 and 2 : 1 metal-indicator complexes. The analysis of titration curves entails a series of one-variable best-fit determinations based on mass action and conservation laws; this reduction in the number of degrees of freedom in the curve-fitting procedure yields greater resolution of the complexing parameters than is allowed by conventional methods. Since a common application of metallochromic indicators is to the determination of metal-binding properties of biological molecules, accurate description of metal-indicator complexing is vital for investigation of the regulatory roles of metal ions in biological events.

1. Introduction

Spectrophotometric titration is a frequently used method to determine reaction stoichiometries and equilibrium constants. The basic requirement of this technique is an analytical relationship between extent of reaction and optical signal, as, for instance, absorbance or fluorescence.

Spectrophotometric methods have been traditionally applied in the study of metal ion complex formation [1,2]. More generally, this approach is applicable to any ligand binding reaction which is associated with a change in an optical property, either directly or indirectly; the optical change can be via coupling, for instance, of a metal ion reaction with a reaction of the same metal ion to a metallochromic indicator.

Apart from the analytical aspects, it is well known that metal ions are an integral part of biological structures and functions. For instance, Ca^{2+} ions are essential and cannot be replaced by other divalent ions in a number of fundamental life phenomena such as nerve excitation, excitation-contraction coupling in muscle, neuro-stimulated hormone and transmitter secretion, etc. In order to identify the specific reactions and cellular components interacting with Ca^{2+} ions, various techniques have been developed to determine Ca^{2+} ions and changes in Ca^{2+} concentrations in biological systems. The applicability of each method is limited by its ability to select for Ca^{2+} over interference from H^+, Na^+, K^+ and Mg^{2+} ions, among others, by its sensitivity to the magnitude of Ca^{2+}-concentration changes and by its time resolution, which is important for kinetic measurements.

Significant progress was made when spectrophotometric methods using Ca^{2+} indicators such as murexide [3,4], arsenazo [5] and antipyrylazo [6] dyes were introduced into biophysical studies. Practical considerations for the selection of the appropriate indicator for a particular system have been outlined recently by Scarpa et al. [7]: in particular, arsenazo III and antipyrylazo III gain increasing attention. The absorbance spectra of these compositionally more complicated indicators do not show isosbestic wavelengths as calcium concentration is varied [8,9]. This observation indicates that Ca^{2+} binding stoichiometries for these two indicators are complicated, i.e., that more than one type of Ca^{2+} – indicator complex can be formed.

Any quantitative analysis of optical data in terms of metal ions bound to cell components requires knowledge of the stoichiometry, equilibrium constants and

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extinction coefficients. The present article describes a numerical method by which a particular ligand-binding matrix (e.g., metal indicator) can be optically and thermodynamically characterized over a wide range of concentrations. No sophisticated mathematical procedures are requires; rather, the method makes use of a self-evident property, namely, that the thermodynamic equilibrium constant characterizing any ligand-binding equilibrium is an intrinsic, and hence concentration-independent, quantity. However, this quantity is derivable from spectrophotometric or fluorometric data only if stoichiometries, equilibrium constants and extinction coefficients have been accurately chosen. The method is therefore essentially a trial-and-error approach requiring computerized fitting. However, by limiting the analysis at first to narrow concentration ranges, the parameter selection can be focused on only one type of complex in each titration, and each analytical procedure can be set up with only *one* variable.

The method has so far been applied to spectrophotometeric titrations of arsenazo I, arsenazo III and antipyrylazo III. Whereas arsenazo I exhibits a simple 1 : 1 stoichiometry with Ca^{2+} ions [10], both arsenazo III [8] and antipyrylazo III [9] form three complexes, 1 : 1, 1 : 2 and 2 : 1. Description of arsenazo III is given in Part II of this series.

Although the analytical method is outlined here in terms of metal ion-indicator interaction, the procedure may be applied to *any* ligand-binding reaction associated with an optical signal.

2. Optical signal and concentration of complexes

The relationship between the concentration of bound ligands in various types of complexes and an optical quantity is particularly simple when the optical signal, S, can be linearly related to the concentration of species X_i contributing to S; i.e., when a kind of general Lambert-Beer law is exhibited:

$$S = \sum_i a_i [X_i], \tag{1}$$

where a_i is the intrinsic optical coefficient of species X_i; square brackets denote concentration.

Using now the interaction of a metal ion, M, with an indicator, I, as an example, eq. (1) is expressed as

$$S = a_M [M] + a_I [I] + \sum_{p,q} a_{pq} [M_p I_q]; \tag{2}$$

a_{pq} is the intrinsic coefficient of the complex $M_p I_q$ with the stoichiometric numbers p of M and q of I molecules. For metallochromic indicators (which change in optical absorbance on complex formation with metal ions), eq. (2) takes the form

$$A_\lambda = \epsilon_{\lambda,I} [I] + \sum_{p,q} \epsilon_{\lambda,pq} [M_p I_q]. \tag{3}$$

In eq. (3), A_λ is the absorbance per cm at a given wavelength λ, and $\epsilon_{\lambda,i}$ is the extinction coefficient of species i at wavelength λ; in the visible wavelength range the absorbance of ions like Ca^{2+}, Mg^{2+} or alkali metal ions is negligibly small. The sum in eq. (3) is over all complexes.

For the data analysis, it is useful to define an absorbance difference according to

$$\Delta A = A - \epsilon_I [I_T], \tag{4}$$

where $[I_T]$ denotes the total indicator concentration and ϵ_I is the extinction coefficient of the unbound indicator; the subscript λ will be henceforth implicit. Mass conservation requires that

$$[I_T] = [I] + \sum_{p,q} q [M_p I_q]. \tag{5}$$

Inserting eqs. (3) and (5) into eq. (4), we obtain an expression relating a measurable quantity with the contributions of the complexes:

$$\Delta A = \sum_{p,q} q \Delta\epsilon_{pq} [M_p I_q], \tag{6}$$

where the difference in the extinction coefficients *per mole of indicator* is given by

$$\Delta\epsilon_{pq} = \epsilon_{pq}/q - \epsilon_I. \tag{7}$$

A spectrophotometric titration of the indicator with metal ions gives data sets (ΔA, $[M_T]$), where $[M_T]$ is the total concentration of metal ions, corresponding to a single value of $[I_T]$; these are the primary data for the analysis.

3. Determination of indicator extinction coefficient

Accurate determination of the extinction coefficient ϵ_I can be hampered by cations from both buffer and indicator salt. A simple dilution procedure may be used which allows precise determination of ϵ_I at a given wavelength. From eqs. (3)–(6) we derive

$$\frac{A}{[I_T]} = \epsilon_I + \frac{\Sigma_{p,q}\, q\Delta\epsilon_{pq}[M_pI_q]}{[I_T]} . \tag{8}$$

In a sequence of dilutions (with distilled water) the concentration of any of the possible metal ion complexes approaches zero faster than $[I_T]$. Therefore, the second term of the rhs of eq. (8) approaches zero in the limit of small $[I_T]$:

$$\epsilon_I = \lim_{[I_T]\to 0} A/[I_T] . \tag{9}$$

Eq. (9) indicates that (graphical) extrapolation from small, but finite, $[I_T]$ values to $[I_T] = 0$ should yield a reliable estimate of ϵ_I. Application of this method is shown in the following article.

4. Analysis of spectrophotometric titrations

As discussed above, for the metallochromic indicators arsenazo III and antipyrylazo III, the absence of isosbestic (or isochromic) points in the absorbance spectra at different calcium concentrations indicates that the stoichiometric relationship of the metal-indicator interaction can not be simple. In such cases, conventional graphical methods can not be applied to evaluate equilibrium constants and extinction coefficients. Therefore, a new calculational method was developed. Although the method is numerical rather than graphical, computations involving stoichiometric numbers *less than 3* can be handled straightforwardly, without the need to numerically fit a cubic polynomial involving up to six variable parameters.

Metallochromic indicators are usually small molecules, and only low-order complexing with metal ions is to be expected. For example, using the method described below, the stable complexes of both arsenazo III and antipyrylazo III were found to be CaI, CaI_2 and Ca_2I; the distribution of Ca and I among the three complexes is strongly a function of $[Ca_T]$ and $[I_T]$.

Spectrophotometric and fluorometric titration of metallochromic indicators with metal ions involves observing the change in optical absorbance of fluorescence at a particular wavelength and for a fixed $[I_T]$ as $[M_T]$ is varied. The present approach requires several titrations, so as to cover a wide range of $[I_T]$ values.

4.1. Single type of complexation

Analysis of the titration data is begun with the purely operational assumption that only one type of reaction is involved,

$$pM + qI \rightleftharpoons M_pI_q , \tag{10}$$

characterized by an *overall* dissociation constant

$$K'_{pq} = [M]^p [I]^q / [M_pI_q] , \tag{11}$$

where [M] and [I] represent concentrations of free metal and free indicator, respectively. From eqs. (5) and (6), and the mass conservation law for metal,

$$[M_T] = [M] + p[M_pI_q] , \tag{12}$$

the expression for K'_{pq} can be transformed into

$$K'_{pq} = \frac{\{[M_T] - p\Delta A/(q\Delta\epsilon_{pq})\}^p \{[I_T] - \Delta A/\Delta\epsilon_{pq}\}^q}{\Delta A/(q\Delta\epsilon_{pq})} \tag{13}$$

It is recognized that if the interaction between M and I is totally covered by reaction model (10), then the variation of ΔA with varying $[M_T]$ and $[I_T]$ will always be such as to predict the same value for K'_{pq} via eq. (13). Therefore, in assuming model (10) to be correct, the analysis reduces to finding that combination of parameters $(p, q, \Delta\epsilon_{pq})$ which predict constancy for K'_{pq}. In practice, model (10) accurately covers the range of reactant concentrations where only 1 : 1 complexing occurs; in cases where higher-order complexation is important, contributions from other, lower-order, complexes are also to be expected and the model (10) would be inadequate.

Analysis of the metal-indicator data was therefore always begun with the (not necessarily true) assumption that model (10) holds, with $p = q = 1$, and all plausible $\Delta\epsilon_{11}$ values were scanned in search of a value which yielded the same value for K_{11} for each titration point (the prime has been dropped to indicate that K_{11} is an elementary equilibrium constant). The range

of $\Delta\epsilon_{1i}$ to be covered is suggested by the magnitude of ΔA relative to $[I_T]$.

Such a simple model is expected to be adequate when either reactant concentration is sufficiently low. In fact, subsequent steps in the method rely on the accurate determination of $\Delta\epsilon_{11}$ and K_{11}, using eq. (13).

Equilibrium constants must be adjusted for changes in ionic composition of the reaction medium as the concentration of ionic reactants is changed; these corrections are described in the Appendix.

The advantage of the present method is two-fold: a computerized calculation allows easily for adjustment of activity coefficients with changing ionic strength of the reaction medium, and the method can be readily extended to more complicated situations where simpler graphical methods are impractical, namely, to solutions containing more than one type of complex.

4.2. *Two types of complexation*

If it is found that at higher $[M_T]$ and/or $[I_T]$ values, the assumption of only one type of complex contributing to ΔA is incorrect, an enhanced formalism must be invoked which allows for *either* 1 : 2 *or* 2 : 1 complexation in addition to the 1 : 1 species. The appearance of the higher-order species is apparent through a progressively increasing deviation of the K_{11}-values calculated from eq. (13) from the (constant) value obtained at low concentrations.

Experiments can be designed so as to resolve the individual thermodynamic and optical properties of the complexation reactions

$$MI + I \rightleftharpoons MI_2 \tag{14}$$

$$MI + M \rightleftharpoons M_2I. \tag{15}$$

Determination of $\Delta\epsilon_{12}$ and K_{12}, corresponding to reaction (14), can be based on the establishment of constancy for K_{12}, by writing an expression similar to eq. (13) and employing $\Delta\epsilon_{12}$ as the single variable. Experimentally, a mixture of MI and MI_2 complexes is expected for low $[M_T]$ and moderate-to-high $[I_T]$ values (relative to the already determined value of K_{11}). For reaction (14), mass conservation laws take the form

$$[M_T] = [M] + [MI] + [MI_2], \tag{16a}$$

$$[I_T] = [I] + [MI] + 2[MI_2], \tag{16b}$$

and the absorbance change, relative to the $[M_T] \approx 0$ value, is expressible as (cf. eq. (6))

$$\Delta A = \Delta\epsilon_{11}[MI] + 2\Delta\epsilon_{12}[MI_2]. \tag{17}$$

The correctness of assuming an MI and MI_2 mixture as being responsible for ΔA can be established from the definition of K_{12}:

$$K_{12} = \frac{[MI]\,[I]}{[MI_2]} = \frac{2\Delta\epsilon_{12}[M]\,[I]^2}{\Delta A K_{11} - [M]\,[I]\,\Delta\epsilon_{11}}, \tag{18}$$

where we have substituted for $[MI]_2$ from eq. (17), and have included the definition

$$[MI] = [M]\,[I]/K_{11}. \tag{19}$$

Eq. (18) for K_{12} contains three "unknowns", $\Delta\epsilon_{12}$, [M] and [I], but the expression can be reduced to one independent variable due to the mass conservation laws (16a) and (16b). [M] can be expressed in terms of $\Delta\epsilon_{12}$ and [I], e.g., by substituting in eq. (16a) for $[MI_2]$ from eq. (17), using eq. (19) for [MI]:

$$[M] = \frac{K_{11}(2[M_T]\Delta\epsilon_{12} - \Delta A)}{2\Delta\epsilon_{12}K_{11} + [I](2\Delta\epsilon_{12} - \Delta\epsilon_{11})}. \tag{20}$$

Making the same substitutions for $[MI_2]$ and [MI] in eq. (16b), and using eq. (20) for [M], gives [I] as a root of a quadratic polynomial, dependent only on $\Delta\epsilon_{12}$:

$$[I] = -B/2 + \{(B^2 - 4C)^{1/2}\}/2, \tag{21}$$

where

$$B = \{K_{11} + (1 - \Delta\epsilon_{11}/\Delta\epsilon_{12})([M_T] - \Delta A/(2\Delta\epsilon_{12})\}$$

$$\times \{1 - \Delta\epsilon_{11}/(2\Delta\epsilon_{12})\}^{-1} - ([I_T] - \Delta A/\Delta\epsilon_{12}),$$

$$C = -K_{11}([I_T] - \Delta A/\Delta\epsilon_{12})\{1 - \Delta\epsilon_{11}/(2\Delta\epsilon_{12})\}^{-1}.$$

A selected value for $\Delta\epsilon_{12}$ fixes the value of [I] via eq. (21), which in turn fixes the value of [M] by eq. (20); eq. (18) can then be used to calculate the value of K_{12} corresponding to that choice of $\Delta\epsilon_{12}$. The case $\Delta\epsilon_{11} = 2\Delta\epsilon_{12}$ is a singular point, but can be treated with these expressions as a limit case.

K_{11} and $\Delta\epsilon_{11}$ are here treated as known quantities and $\Delta\epsilon_{12}$ values are scanned for that value which results in the same value calculated for K_{12} for all data points ($[M_T]$, $[I_T]$, ΔA). Such a $\Delta\epsilon_{12}$ value can be found, provided that a significant contribution to ΔA arises

from MI_2 complexing. (As with eq. (13), the expression for K_{12} must be multiplied by the appropriate ionic strength correction factor described in the Appendix.)

Investigation of M_2I-complexing, reaction (15) can be most easily done at low $[I_T]$ and moderate-to-high $[M_T]$ levels. The relevant mass conservation equations are

$$[M_T] = [M] + [MI] + 2[M_2I], \tag{22a}$$

$$[I_T] = [I] + [MI] + [M_2I], \tag{22b}$$

and the experimental signal is assumed to be

$$\Delta A = \Delta\epsilon_{11}[MI] + \Delta\epsilon_{21}[M_2I]. \tag{23}$$

Proceeding as above, one obtains an expression for K_{21} in terms of [M] and [I]:

$$K_{21} = \frac{[MI][M]}{[M_2I]} = \frac{\Delta\epsilon_{21}[M]^2[I]}{\Delta A K_{11} - \Delta\epsilon_{11}[M][I]}. \tag{24}$$

Eqs. (22a) and (23) give

$$[M] = \frac{K_{11}([M_T]\Delta\epsilon_{21} - 2\Delta A)}{\Delta\epsilon_{21}K_{11} + [I](\Delta\epsilon_{21} - 2\Delta\epsilon_{11})}. \tag{25}$$

Inserting this result in eq. (22b) yields the expression for [I] in terms of $\Delta\epsilon_{21}$:

$$[I] = -D/2 + \{(D^2 - 4E)^{1/2}\}/2, \tag{26}$$

where

$$D = \{K_{11} + (1 - \Delta\epsilon_{11}/\Delta\epsilon_{21})([M_T] - 2\Delta A/\Delta\epsilon_{21})\} \times \{1 - 2\Delta\epsilon_{11}/\Delta\epsilon_{21}\}^{-1} - ([I_T] - \Delta A/\Delta\epsilon_{21}),$$

$$E = -K_{11}([I_T] - \Delta A/\Delta\epsilon_{21})(1 - 2\Delta\epsilon_{11}/\Delta\epsilon_{21})^{-1}.$$

As in the previous case, all plausible values of $\Delta\epsilon_{21}$ are scanned for the value which predicts the same value for K_{21} for each titration point.

4.3. Three types of complexation

In many cases the distribution of complexes may be adjusted so as to focus either on $MI - MI_2$ or $MI - M_2I$ mixtures, simply by suitably selecting $[M_T]$ and $[I_T]$. If such a separation of MI_2 and M_2I can not be achieved, then the formalism must be extended to include all three species, MI, MI_2 and M_2I in the optical measurement. If one follows the above calculational format, solving for either K_{12} or K_{21}, then one is faced with solving a cubic polynomial for [I]: on the other hand, this may be circumvented by a numerical technique, as described below.

It will be assumed here that $[M_T]$ and $[I_T]$ can be selected so as to achieve *either* the $MI - MI_2$ *or* the $MI-M_2I$ mixture, but not both. We assume, for example, that the species M_2I can be isolated from MI_2; the converse case can be handled in an identical manner. Therefore, K_{11}, K_{12}, $\Delta\epsilon_{11}$ and $\Delta\epsilon_{12}$ are here treated as determined quantities and we seek that value of $\Delta\epsilon_{21}$ which predicts constancy for K_{21}.

Fundamental equations for the $MI - MI_2 - M_2I$ mixture are

$$[I_T] = [I] + [MI] + 2[MI_2] + [M_2I], \tag{27a}$$

$$[M_T] = [M] + [MI] + [MI_2] + 2[M_2I], \tag{27b}$$

and

$$\Delta A = \Delta\epsilon_{11}[MI] + 2\Delta\epsilon_{12}[MI_2] + \Delta\epsilon_{21}[M_2I]. \tag{28}$$

Eliminating $[M_2I]$ between eqs. (27a) and (27b) gives an expression for [M] in terms of only one unknown, [I]:

$$[M] = \frac{\{2([I_T] - [I]) - [M_T]\}K_{11}K_{12}}{3[I]^2 + K_{12}[I] - K_{11}K_{12}}. \tag{29}$$

Eq. (28) can be written in terms of all three unknowns [M], [I] and $\Delta\epsilon_{21}$ using eq. (27b),

$$\Delta A = (\Delta\epsilon_{11} - \Delta\epsilon_{21}/2)[M][I]/K_{11} + (2\Delta\epsilon_{12} - \Delta\epsilon_{21}/2)[M][I]^2/(K_{11}K_{12}) + (\Delta\epsilon_{21}/2)([M_T] - [M]), \tag{30}$$

but can be reduced to a function in only [I] and $\Delta\epsilon_{21}$ by eq. (29). The numerical method involves finding that value of [I], between $[I] = [I_T]$ and $[I] = 0$, which satisfies eqs. (29) and (30) for a selected value of $\Delta\epsilon_{21}$: the resulting set of values ([M], [I]) predict a value for K_{21}, where

$$K_{21} = \frac{[MI][M]}{[M_2I]} = \frac{K_{12}[M]^2[I]}{([I_T] - [I])K_{11}K_{12} - [M][I]K_{12} - 2[M][I]^2}. \tag{31}$$

Eq. (31) is obtained by substituting for $[M_2I]$ from eq. (27a). $\Delta\epsilon_{21}$ values can therefore by scanned to find that value which leads to the sets of values ($[M]$, $[I]$) which yield the *same* value of K_{21} for each titration point.

The equilibrium constant for the overall disproportionation reaction

$$MI_2 + M \rightleftharpoons 2MI \tag{32}$$

can be immediately calculated as

$$K = [MI_2][M]/[MI]^2 = K_{11}/K_{12}. \tag{33}$$

The entire set of calculations may be checked for consistency by incorporating the calculated values of K_{11}, K_{12} and K_{21} into eqs. (27a) and (27b) via eqs. (18), (19) and (31), and recalculating ΔA from eq. (28) using the determined values of $\Delta\epsilon_{11}$, $\Delta\epsilon_{12}$ and $\Delta\epsilon_{21}$ for each experimental point.

5. Determination of metal binding to biological systems

In general, a metallochromic indicator can be used to determine metal-binding properties of a biological system if the total concentration of each reactant and the equilibrium constants of the various metal-indicator complexes are known. Hence, the analytical problem is essentially the converse of the one discussed to this point: optical and binding properties of the indicator are assumed known and one must find the *effective* value of $[M_T]$ corresponding to the measured value of ΔA.

The effective total metal concentration sensed by the indicator is the actual $[M_T]$ *minus* the "concentration" of metal ions which have been effectively removed due to binding by biological components in the mixture, $[M_b]$:

$$[M_T] - [M_b] = \sum_{p,q} p[M_pI_q] + [M]. \tag{34}$$

$[M_b]$ may be determined by scanning the entire range $0 < [M_b] < [M_T]$, solving for the corresponding values of $[M]$ and each $[M_pI_q]$ (e.g. by the computer program given in ref. [11]) and comparing the calculated values of ΔA to the measured value. In this way, each experimental data set (ΔA, $[M_T]$, $[I_T]$) yields a corresponding set of calculated values ($[M_b]$, $[M]$), and from a sufficiently large number of different $[M_T]$ and $[I_T]$ values the metal-binding properties of the biological material can be determined. An example of the method is afforded by the application of antipyrylazo III to the determination of Ca^{2+}-binding by acetylcholine receptor protein [9].

6. Discussion

Basic to any computation of metal-indicator binding is the accurate determination of metal and indicator concentrations. Commerically available metallochromic indicators are often impure and the exact concentration of indicator needs first to be determined; a purification procedure applicable to azo-dyes has been outlined by Kendrick [12]. Estimates of total metal concentration may also be in error due to metal contamination from the indicator, buffer and added salts. Other ions which compete for the indicator may also be present and have an interfering effect.

With these precautions, solutions of various indicator concentrations can be titrated with metal ions and the present analytical treatment may be applied. The main advantage of the present method over conventional computerized and graphical approaches is the higher sensitivity afforded by having only one variable parameter in each curve fitting procedure. This resolved, for instance, controversies on the stoichiometry of Ca^{2+} binding to arsenazo III and antipyrylazo III, verifying the existence of stable 1 : 2 and 2 : 1 Ca^{2+}–indicator complexes for both indicators (Murexide is also known to form 1 : 2 complexes in methanol, i.e., two murexide ions chelate one Ca^{2+} ion [13]).

The above procedure may be bypassed if one is interested only in measurement of metal uptake or release, that is, only in *changes* in $[M_b]$ rather than its total value. In such cases, the optical signal can be calibrated by observing changes in ΔA after addition of known amounts of metal ion to the reaction mixture. On the other hand, the present technique is useful for characterizing metal-binding isotherms which cover the wide range of metal-binding sites typical of large biological molecules [9].

Appendix

Activity Coefficient Corrections

Since the reaction partners M and I are ions and [M] during titrations was increased up to the 10^{-1}M range, ionic strength dependencies of reaction equilibria have to be considered. As is well known, the true thermodynamic equilibrium constant, K_{th}, is expressed as the product of the apparent equilibrium constant, K, and the thermodynamic activity coefficients, $f_i^{\nu_i}$, of the reacting species i, with stoichiometric number ν_i and charge number z_i (both with sign):

$$K_{th} = K \prod_i f_i^{\nu_i}. \tag{A1}$$

The ionic strength adjustment, $\Pi_i f_i^{\nu_i}$, must be calculated for each titration point ($[M_T]$, $[I_T]$) and the expressions for the apparent dissociation equilibrium constants given here must in each case be multiplied by $\Pi_i f_i^{\nu_i}$ to obtain K_{th}: it is actually the constancy of K_{th}, independent of ionic strength, which is sought in each case.

Usually $\Pi_i f_i^{\nu_i}$ may be approximated by a semiempirical expression using the Debye-Hückel approximation [14], in the form

$$\log \prod_i f_i^{\nu_i} = -\sum_i \nu_i z_i^2 \frac{A(I_c)^{1/2}}{1 + B\bar{a}(I_c)^{1/2}} + C I_c \sum_i \nu_i \tag{A2}$$

In eq. (A2), A and B are constants depending on temperature T and the dielectric constant D; at $T = 293$ K and $D = 80$,

$A = 0.507\ \mathrm{M}^{-1/2}$,

$B = 0.328 \times 10^8\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1/2}$,

$\bar{a}$ is the distance of closest approach between the reaction partners, and the ionic strength, I_c, is defined as

$$I_c = (0.5) \sum_i z_i^2 c_i, \tag{A3}$$

where c_i is the concentration of ionic species i. The last term on the rhs of eqs. (A2) contains the adjustable parameter C characteristic for the salt determining the values if I_c.

Specification of charge numbers z_i is usually obvious for metal ions, but the *effective* charge number may be less than the net charge for larger structures, because not all the charged groups contribute to the charge of the reaction site with the metal ion.

Acknowledgement

The financial support of the Deutsche Forschungsgemeinschaft, grant Ne 227, and the Stiftung Volkswagenwerk is gratefully acknowledged.

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