The origin of multiply sigmoid curves of pH-dependence

The partitioning of groups among titration pK values

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An acid, H_nA , with n ionizing groups is known to have the same titration curve as an equimolar mixture of n hypothetical monobasic acids, whose dissociation constants are known as the 'titration constants' of the real acid. We show that the pH-dependence of any property of H_nA is also represented by the sum of one-site titration curves, characterized by these same titration constants. Since one such property is the degree of dissociation of one of the dissociating groups, a fraction of each group shows each of the various titration pK values, so that the group partitions among them. The n groups therefore share the same n titration pK values but differ in the fractions belonging to each. The one H^+ ion per molecule that titrates with each pK is thus made up of the fractions, one from each group, that share this pK value. A group may possess a single pK value, in that it contributes virtually all of this pK and almost nothing to the others, only if either (1) it titrates in a different pH range from the other groups or (2) its affinity for H^+ is unaffected by their ionization state.

INTRODUCTION

Simms (1926) pointed out that the titration curve of a dibasic acid was identical with the sum of the titration curves of two monobasic acids. He symbolized the dissociation constants of these hypothetical acids, called titration constants, as G_1 and G_2 , and he gave the relation between them and the molecular constants K_1 and K_2 . The present treatment points out that other (e.g. spectroscopic) properties are usefully expressed in terms of these titration constants. In particular, each group of a multi-valent molecule titrates as if a fraction of it had each of the dissociation constants of the molecule.

TREATMENT

Background on dibasic acids

A dibasic acid, H_2A , is considered, which dissociates to HA^- , and then to A^{2-} (the negative charges being merely relative to whatever charge H_2A possesses). HA^- represents both of the two forms that differ according to which group loses a hydron [Bunnett & Jones (1988) explain this term for H^+]. The molecular constants K_1 and K_2 are defined by the equations:

$$K_1 = [H^+][HA^-]/[H_2A]$$

and

$$K_2 = [H^+][A^{2-}]/[HA^-]$$

If the two hypothetical acids, whose titrations add to give that of H_2A , are represented as HX and HY, then:

$$G_1 = [H^+][X^-]/[HX]$$

and

$$G_2 = [H^+][Y^-]/[HY]$$

Dixon (1975) pointed out that there were two hypothetical acids HX and HY such that (when the concentrations are relative, i.e. divided by the total concentration $[A]_t = [H_2A] + [HA^-] + [A^{2-}] = [X]_t = [HX] + [X^-]$ etc.):

$$\begin{aligned} [HA^-] &= [HX] \cdot [Y^-] + [X^-] \cdot [HY] \\ &= \text{a constant multiplied by } [HX] \cdot [Y^-] \\ &= \text{a different constant multiplied by } [X^-] \cdot [HY] \end{aligned}$$

and that:

$$\begin{aligned} [A^{2-}] &= [X^-] \cdot [Y^-] \\ [H_2A] &= [HX] \cdot [HY] \end{aligned}$$

and he gave one form of expressing their pK values as functions of the molecular values. These acids, i.e. those whose titration curves are factors of the Michaelis (1914) functions, are precisely the ones treated by Simms (1926), i.e. those whose titration curves summate to give that of the dibasic acid, although Dixon (1975) was unaware of this. He noted that the values of G_1 and G_2 were unreal if $K_1 < 4K_2$. The titration pK values are $pK^* \pm \log\{[m + (m^2 - 4)^{1/2}]/2\}$ when pK^* and m are defined by equating the molecular pK values with $pK^* \pm \log m$ (Dixon, 1988); they approach the molecular values when $pK_2 \gg pK_1$.

Derivation for dibasic acids

Simms (1926) was concerned with the acid–base titrations, but pK values are mainly used in studying properties other than hydron release. Suppose that some property changes as H_2A is titrated to A^{2-} , and suppose that the change is normalized to unity. Hence H_2A shows a value of zero, and A^{2-} one of unity. Then suppose that HA^- possesses it to degree a . The two forms of HA^- may possess it to different degrees, but the uncertainties in assigning a value to the ratio between them have been pointed out by Dixon (1976). The individual groups in H_2A will not, in general, follow one-site titration curves, because the pK of each will change as the other titrates.

The value, y , of the property at any pH is given by $y = a \cdot [HA^-] + [A^{2-}]$, or, using the Michaelis (1914) functions:

$$y = \frac{a + K_2/[H^+]}{[H^+]/K_1 + 1 + K_2/[H^+]} \quad (1)$$

We can suppose that this is in general equal to the sum of two one-site saturation curves. Thus, if we attribute a value of zero to the property possessed by HX and HY, b to the value possessed by X^- and $(1-b)$ to the value possessed by Y^- , then:

$$y = \frac{b}{1 + [H^+]/G_1} + \frac{1-b}{1 + [H^+]/G_2} \quad (2)$$

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or

$$y = \frac{b \cdot G_1}{G_1 + [H^+]} + \frac{(1-b) \cdot G_2}{G_2 + [H^+]} \quad (3)$$

Eqns. (1) and (2) can be represented respectively as:

$$y = \frac{a \cdot K_1 \cdot [H^+] + K_1 \cdot K_2}{[H^+]^2 + K_1 \cdot [H^+] + K_1 \cdot K_2} \quad (4)$$

and

$$y = \frac{[b \cdot G_1 + (1-b) \cdot G_2] \cdot [H^+] + G_1 \cdot G_2}{[H^+]^2 + (G_1 + G_2) \cdot [H^+] + G_1 \cdot G_2} \quad (5)$$

These two expressions are identical if the Simms (1926) relationships:

$$G_1 \cdot G_2 = K_1 \cdot K_2 \quad (6)$$

and

$$G_1 + G_2 = K_1 \quad (7)$$

hold, provided that the following equation also holds:

$$b = \frac{a \cdot (G_1 + G_2) - G_2}{G_1 - G_2} \quad (8)$$

This can be represented as:

$$b = \frac{a \cdot (R + 1) - 1}{R - 1} \quad (9)$$

where $R = G_1/G_2$.

The identity of eqns. (4) and (5) shows that the change of any property can be treated as the sum of changes in the hypothetical acids HX and HY, i.e. that all properties of the dibasic acid follow the sum of one-site curves (Fig. 1).

Features of the representation

No complexity is met in acid-base titrations, because if hydron release is the only property studied, $a = 0.5$, since production of HA^- from H_2A releases half as much as production of A^{2-} does. By eqn. (9), if $a = 0.5$, then $b = 0.5$.

When the titration pK values are separated, so that $G_1 \gg G_2$, i.e. $R \gg 1$, again $b = a$. When the titration pK values are identical, b becomes infinite or indeterminate; this corresponds with the impossibility of assigning fractions between two identical acids. Alternatively, if the titration values are close, and $a \neq 0.5$, then $b \neq a$. Indeed b can go outside the limits 0 to 1 even when a is within these limits; more precisely, it does so when a is outside the limits $1/(R+1)$ and $R/(R+1)$. An example is given in Fig. 1(c).

Titration constants can prove useful in handling problems of pH-dependence. Dixon *et al.* (1987), for example, were able to use them to generalize a treatment of independent groups to cover interacting groups in demonstrating that for enzyme-catalysed reactions in which H^+ is taken up in one direction and released in the other, and the dependence of rate (strictly specificity constant) on pH follows a theoretical bell-shaped curve, then the pK characterizing the alkaline side of the bell for the direction of hydron uptake is identical with that characterizing the acid side of the bell for the reverse direction. It may be important to recognize the possibility of the paradoxical situation shown in Fig. 1(c), that, although a property changes in a single direction as hydrons are removed, it may have to be considered as changing in the reverse direction in one of the hypothetical one-site acids used to model the phenomenon when titration constants are used.

Application to the titration of groups

Dixon & Tipton (1973) pointed out that the titration curve of

Fig. 1. pH-dependence a property of a dibasic acid, and its analysis by using titration constants

The bold curves show the dependence of the property, y , on pH. It is normalized so that H_2A does not possess it, HA^- possesses it to degree a and A^{2-} possesses it to unit degree. It can be represented as the sum $a \cdot [HA^-] + [A^{2-}]$. The dependence of y on pH may be expressed as the sum of two one-site curves, as shown: $b \cdot [X^-] + (1-b) \cdot [Y^-]$, where HX and HY are the two hypothetical one-site acids with the titration pK values. In each case the fraction a has been set at 0.3. (a) Typically doubly sigmoid dependence of the property is shown when the titration pK values are well separated, here by 3 units, giving $b = 0.2996$ by eqn. (9). From the equations given by Dixon (1988), the molecular pK values are 3.001 apart [i.e. $2 \cdot \log(p+1/p)$, where the difference in titration values is $2 \cdot \log p$], and the width at half-height of the bell-shaped curve for $[HA^-]$ is 3.104. (b) With the titration pK values 1 unit apart, the change of property on dehydronation of H_2A to A^{2-} appears more like a single sigmoid curve, but is still the sum of two sigmoid curves. Here the molecular pK values differ by 1.08, and the width at half-height of the bell-shaped curve for $[HA^-]$ is 1.73, and $b = 0.256$. (c) With close titration pK values, 0.2 apart, the overall curve is again not obviously doubly sigmoid. Here the paradoxical situation arises that b and $(1-b)$ go outside the limits 0 to 1, so the overall curve is the sum of positive and negative single-site curves. Here $b = -0.38$, the molecular pK values are 0.62 apart, and the width at half-height of the curve for $[HA^-]$ is 1.54.

a single group of a dibasic acid, where a is the fraction of it that is dissociated in the monoanion HA^- , can be regarded as if a fraction a of it titrated with pK_1 , and a fraction $(1-a)$ with pK_2 . They noted that this was only an approximation, and failed unless $pK_2 \gg pK_1$. The use of titration constants allows analogous but precise statements to be made.

The fraction a of a group under study that is dissociated in HA^- is simply one example of the normalized property considered above, since all of this group is dissociated in A^{2-} . Fig. 1(a) can therefore represent the dissociation of a single group, and it is a

Fig. 2. Titration of one group of a dibasic acid

The Figure demonstrates that part of the group $-AH$ in the molecule $HA-BH$ titrates with one pK and part with another. When $pH = pK_A$, $[HA-BH] = [-A-BH]$; when $pH = pK_B$, $[HA-BH] = [HA-B^-]$. When $pH = pK_1$ (where $K_1 = K_A + K_B$), $[HA-BH] = [-A-BH] + [HA-B^-]$. The concentration of $-A^-$, i.e. $[-A-BH] + [-A-B^-]$, clearly follows a doubly sigmoid curve. The text shows that a fraction titrates with a pK of pG_1 and a fraction with pG_2 (which will be very close to pK_1 and pK_2 for values as far apart as shown). To show such a separation of titration pK values (here 3.0), with an appreciable fraction of each group titrating with each (here fractions of 0.6 and 0.4), the group pK values of $-AH$ and $-BH$ in $HA-BH$ must be comparable (here differing by 0.18), and the pK of group $-AH$ must be markedly lower in $HA-BH$ than in $-A-B^-$, and hence that of $-BH$ markedly lower in $HA-BH$ than in $-A-BH$ (here by 2.38).

precise statement that a fraction b of this group dissociates with a pK of pG_1 and a fraction $(1 - b)$ with a pK of pG_2 . And indeed, if $pK_2 \gg pK_1$, then this reduces to the statement made by Dixon & Tipton (1973). The titration of a group partly with one pK and partly with another is illustrated in Fig. 2. The **bold** curve shows the fraction of the group $-AH$ that is dissociated, i.e. the sum of the fractional concentrations of $-A-BH$ and $-A-B^-$.

This may be applied to cysteine, an example carefully analysed by Benesch & Benesch (1955). Throughout the pH range of interest, the carboxy group is overwhelmingly dissociated, so the treatment and symbols will refer only to the two-site system of the aminothiols. Thus ' H_2A ' represents $-O_2C-CH(-NH_3^+)-CH_2-SH$, K_1 is the molecular dissociation constant for the loss of a hydron from this form, and so yields HA^- , a mixture of $-O_2C-CH(-NH_3^+)-CH_2-S^-$ and $-O_2C-CH(-NH_2)-CH_2SH$, which in turn loses a hydron with a molecular constant K_2 to yield A^{2-} , i.e. $-O_2C-CH(-NH_2)-CH_2-S^-$.

It is taken that the form of cysteine with one net negative charge is 68% $-O_2C-CH(-NH_3^+)-CH_2-S^-$ and 32% $-O_2C-CH(-NH_2)-CH_2-SH$. Benesch & Benesch (1955) reasonably argued that these were the best values that could be inferred, although Dixon (1976) has pointed out that direct measurement is impossible and all such assignments contain uncertainties [ingenious ways of minimizing these uncertainties have been developed (e.g. Metzler *et al.*, 1973)]. Dixon & Tipton (1973) suggested that the thiol group could be described by saying that 68% of its thiol group had a pK of 8.36 (pK_1) and 32% one of 10.53 (pK_2). Applying the above treatment, the degree of loss of H^+ from the thiol group is $0.68 \cdot [HA^-] + [A^{2-}]$. From the expressions given by Dixon (1988), the titration constants are negligibly different from the molecular constants (because these are so far apart), and, by eqn. (8), b differs negligibly from a , so the statement that 68% of the thiol group has a pK of 8.36 and 32% one of 10.53 (and, conversely, that the ammonio group $-NH_3^+$ has the same two pK values, 32% of it possessing that of

Table 1. Assignment of groups to titration pK values for cysteine

The fractions of each group that titrate with the three pK values are shown. The entries also represent the fractions of each pK due to the three groups. The lowest pK is due to the carboxy group entirely, not because it is independent of the other groups, but because it is overwhelmingly converted into the $-CO_2^-$ as the pH is raised before appreciable dissociation of other groups. The data are those of Benesch & Benesch (1955), who attributed group pK values of 8.53 and 10.03 to the mercapto group in the forms $-O_2C-CH(-NH_3^+)-CH_2-SH$ and $-O_2C-CH(-NH_2)-CH_2-SH$ respectively, and 8.86 and 10.36 to the ammonio group in the forms $-O_2C-CH(-NH_3^+)-CH_2-SH$ and $-O_2C-CH(-NH_3^+)-CH_2-S^-$ respectively. The titration pK values differ negligibly from the molecular values, since these differ so greatly from each other.

Group	Titration pK value...			Sum for group
	1.9	8.36	10.53	
Carboxy, $-CO_2H$	1.00	0	0	1
Mercapto, $-SH$	0	0.68	0.32	1
Ammonio, $-NH_3^+$	0	0.32	0.68	1
Sum for pK	1	1	1	

8.36 and 68% that of 10.53) is justified, and is no longer an approximation. The approximation Dixon & Tipton (1973) made was to use molecular constants instead of titration constants. If an acid H_2A had closer pK values, a and b would differ, as would the titration values from the molecular values.

Just as each group partitions among the different pK values, so the pK values partition among the groups. Each titration pK is made up of contributions from the different groups (Table 1).

Reconciliation of approaches when groups are independent

In general $K_1 = K_A + K_B$, where K_A and K_B are the dissociation constants of the two groups in the molecule when the other group is hydronated. Similarly, $1/K_2 = 1/K_A' + 1/K_B'$, where K_A' and K_B' are the dissociation constants of these two groups when the other is dehydronated. When the groups are independent, i.e. the hydronation state of one does not affect the other's dissociation constant, then $K_A = K_A'$ and $K_B = K_B'$, so $K_1 = K_A + K_B$ and $1/K_2 = 1/K_A + 1/K_B$. Comparison with eqns. (6) and (7) identifies K_A and K_B , the group constants, with G_1 and G_2 , the titration constants. This is fairly obvious, since if the groups are independent they represent the acids HX and HY that were defined as independent in the treatment.

The approach of the previous section can be reconciled with the fact that each group has its own pK under these circumstances. The fraction of HA^- that has the first group dissociated is $K_A/(K_A + K_B)$, so this is a of the above treatment. Putting this into eqn. (8), when $G_1 = K_A$ and $G_2 = K_B$, gives $b = 1$, i.e. all of this group titrates with the pK of $pG_1 = pK_A$.

Generalization to n groups

Appendix 1 gives a generalization from the above treatment of two groups to n groups. It does so in four parts. In the first, it establishes that the sum of concentrations of all the species with the same number of bound hydrons, i.e. $[H_{n-r}A^r]$ where r can have any value from 0 to n , is the product of $n-r$ terms of the type $[HX_r]$ and r terms $[X_r^-]$, one term for each of the hypothetical monobasic acids HX_r , whose dissociation constants are called $G_1, G_2, \dots, G_n$. In other words, the Michaelis (1914) functions have factors that represent titration curves of monobasic acids. The second part establishes that these constants are the titration constants of Simms (1926), since the titration curve of the multi-valent acid is identical with that of the sum of all n acids HX_r .

The third part shows that every property of the molecule, possessed in different degree by each of the ionic forms, follows the sum of one-site curves, in that the pH-dependence of this property is duplicated by assigning a fraction of its change with pH to the dissociation of each of the hypothetical acids HX_j . In the final part of the Appendix we consider the conditions for the titration constants to be real. We show that the condition that each molecular constant should be 4 times that for the next hydron to dissociate is sufficient to ensure that all titration constants are real. If this condition holds, then:

$$pK_1 < pG_1 < (pK_1 + pK_2)/2 < pG_2 < (pK_2 + pK_3)/2 < \dots < pG_{n-1} < (pK_{n-1} + pK_n)/2 < pG_n < pK_n$$

so that the successive titration pK values are close to the successive molecular pK values. We also point out that the whole analysis does not collapse if a pair of constants is not real; we merely replace two of the one-site curves by factors representing $[H_2X]$, $[HX^-]$ and $[X^{2-}]$, where H_2X is a dibasic acid with positively co-operative dehydronation.

Appendix 2 shows why fewer assignments of dissociation constant describe the titration of the groups than are required for specifying all the group constants.

DISCUSSION

The first practical consequence of this analysis is that the variation of properties with pH can be presented as the sum of one-site curves. There is no need to interpret data in more complex ways, such as fitting them to the bell-shaped pH-dependences of the concentrations of intermediate forms.

Another implication resides in the fact that each group in a molecule titrates as the sum of one-site curves. Hence, if some property, such as absorbance or fluorescence, shows a doubly or triply sigmoid dependence on pH, there is no need to postulate that dissociation of more than one group directly affects this property. Provided that hydronation of other groups affects the dissociation constant of the one group, so that it partitions among different titration pK values, then the multiply sigmoid pH-dependence of the property may merely reflect the degree of hydronation of this single group.

It is noteworthy, although paradoxical, that all the n dissociating groups share the same n pK values, namely the titration pK values of the molecule. These groups are likely to differ greatly in how they partition among the titration pK values. Not only do different fractions of each group titrate with the different titration pK values (rows in Table 1), but the whole of each pK is made up by the contributions of the different groups that titrate with it (columns in Table 1).

A fully independent group contributes all of one pK and nothing to any of the others. A group may contribute all of one

pK even if it is not independent, provided that it differs greatly in acid strength from any groups whose ionization affects its own affinity for H^+ . With cysteine (Table 1), for example, the carboxy group contributes to only one pK , not because its dissociation constant is independent of the hydronation state of the other groups, but because they are fully hydronated over the pH range in which it titrates.

It is not only the titrations of the n ionizing groups that are constrained to partition among the n titration pK values; it is

also the pH-dependence of any property of the molecule. This emphasizes the principle that assigning a change of property to a particular pK cannot, by itself, tell the location of a hydron (Jencks, 1969); it does not imply which group needs to be hydronated or dehydronated for the property to be expressed, and more than one group may contribute to this pK .

In enzymes such as ribonucleases and aspartic peptidases, where two ionizing groups participate in catalysis, one hydronated (often acting as a general acid) and one dehydronated (often acting as either a general base or a nucleophile), our analysis shows that it would only be for an extreme difference that the query 'which carboxyl group has the lower pK_a value' (Sielecki *et al.*, 1990) would be appropriate; the realistic question is how the two identified groups partition between the two pK values.

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REFERENCES

- Benesch, R. E. & Benesch, R. J. (1955) *J. Am. Chem. Soc.* **77**, 5877–5881
- Bunnett, J. F. & Jones, R. A. Y. (1988) *Pure Appl. Chem.* **60**, 1115–1116
- Dixon, H. B. F. (1975) *Biochem. J.* **151**, 271–274
- Dixon, H. B. F. (1976) *Biochem. J.* **153**, 627–629
- Dixon, H. B. F. (1988) *Biochem. J.* **253**, 911–913
- Dixon, H. B. F. & Tipton, K. F. (1973) *Biochem. J.* **133**, 837–842
- Dixon, H. B. F., Brocklehurst, K. & Tipton, K. F. (1987) *Biochem. J.* **248**, 573–578
- Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 182–189, McGraw-Hill, New York
- Metzler, D. E., Harris, C. M., Johnson, R. J., Siano, D. B. & Thomson, J. A. (1973) *Biochemistry* **12**, 5377–5392
- Michaelis, L. (1914) *Die Wasserstoffionenkonzentration*, pp. 30–39, Springer, Berlin [English translation (1926), pp. 56–69, Ballière, Tyndall and Cox, London]
- Sielecki, A. R., Fedorov, A. A., Boodhoo, A., Andreeva, N. S. & James, M. N. G. (1990) *J. Mol. Biol.* **214**, 143–170
- Simms, H. S. (1926) *J. Am. Chem. Soc.* **48**, 1239–1250

APPENDIX 1

Details of generalization to n groups

Factorization of the Michaelis functions

If there are n groups, there will be $n+1$ forms ranging from H_nA to A^{n-} . The concentration of H_nA is given by:

$$[H_nA] = 1/(1 + K_1/[H^+] + K_1 \cdot K_2/[H^+]^2 + \dots)$$

or

$$[H_nA] = [H^+]^n / ([H^+]^n + K_1 \cdot [H^+]^{n-1} + K_1 \cdot K_2 \cdot [H^+]^{n-2} + \dots)$$

where the constants K are the molecular dissociation constants, such that:

$$K_r = [H^+] \cdot [H_{n-r}A^{r-}] / [H_{n-r+1}A^{(r-1)-}]$$

Loss of the first hydron gives $H_{n-1}A^-$, and $[H_{n-1}A^-]$ is obtained from $[H_nA]$ by multiplying it by $K_1/[H^+]$. We multiply the expression for $[H_{n-r+1}A^{(r-1)-}]$ by $K_r/[H^+]$ to obtain that for $[H_{n-r}A^{r-}]$, so:

$$[H_{n-r}A^{r-}] = \frac{K_1 \cdot K_2 \dots K_r \cdot [H^+]^{n-r}}{[H^+]^n + K_1 \cdot [H^+]^{n-1} + K_1 \cdot K_2 \cdot [H^+]^{n-2} + \dots} \quad (10)$$

Let us give the name $f([H^+])$ to the denominator, so that:

$$f([H^+]) = [H^+]^n + K_1 \cdot [H^+]^{n-1} + K_1 \cdot K_2 \cdot [H^+]^{n-2} + \dots \quad (11)$$

Such a polynomial in general factorizes to give:

$$([H^+] + G_1) \cdot ([H^+] + G_2) \dots ([H^+] + G_n)$$

for some constants G . We have used the symbol G for these constants, because we show below that they are the titration constants. The relations between the molecular constants K and the titration constants G are found by equating the coefficients of each power of $[H^+]$. Thus (cf. Simms, 1926):

$$\begin{aligned} K_1 &= G_1 + \dots + G_n \\ K_1 \cdot K_2 &= G_1 \cdot G_2 + G_1 \cdot G_3 + \dots \end{aligned}$$

so

$$K_2 = (G_1 \cdot G_2 + \dots) / (G_1 + \dots + G_n)$$

i.e. (the sum of products of all pairs of distinct titration constants) divided by (the sum of all distinct titration constants).

Similarly:

$$K_r = \frac{(\text{sum of all products of } r \text{ distinct titration constants})}{(\text{sum of all products of } r-1 \text{ distinct titration constants})}$$

and similarly:

$$K_1 \cdot K_2 \dots K_r = \text{sum of all products of } r \text{ distinct titration constants} \quad (12)$$

Hence from eqn. (10):

$$[H_{n-r}A^{r-1}] = \frac{[H^+]^{n-r} \cdot (\text{sum of all products of } r \text{ distinct constants})}{([H^+] + G_1) \cdot ([H^+] + G_2) \dots ([H^+] + G_n)} \quad (13)$$

There are n acids HX_j for $j = 1, 2, \dots, n$. Since $[HX_j] = [H^+]/([H^+] + G_j)$ and $[X_j^-] = G_j/([H^+] + G_j)$, $[H_{n-r}A^{r-1}]$ = sum of all products of $n-r$ terms $[HX_j]$ and r terms $[X_j^-]$. Since $[HX][Y^-]$ = a constant multiplied by $[HY][X^-]$, $[H_{n-r}A^{r-1}]$ = a constant multiplied by any $n-r$ terms $[HX_j]$ and r terms $[X_j^-]$.

Hence each Michaelis (1914) function may be represented as the product of one-site titration curves. The concentration of the species that has lost r hydrons is obtained by multiplying the expressions for $n-r$ hydronated forms of HX_j with those for r dehydronated forms X_j^- , as previously shown for $n = 2$ (Dixon, 1975).

Titration constants

If the constants G are titration constants, then the total alkali taken up in titrating the n hypothetical acids HX will be equal to that taken up on titrating H_nA , and it is the sum of n terms of the type $G_j/([H^+] + G_j)$. The denominator of this will be $f([H^+])$ (see eqn. 11). The numerator will be the sum of n terms G_j multiplied by the product of $([H^+] + G_j)$ as j takes the $n-1$ values different from j . The coefficient of $[H^+]^{n-r}$ in this expression is then the sum of terms G_j times $G_{j_1} \cdot G_{j_2} \dots G_{j_{r-1}}$ with $j_1, j_2, \dots, j_{r-1}$ distinct and different from j . A product of the form $G_{i_1} \cdot G_{i_2} \dots G_{i_r}$ will therefore arise in r different ways since we can take j to be i_1 or i_2 or ... or i_r . Using eqn. (12), we see that the coefficient of $[H^+]^{n-r}$ in the numerator is $r \cdot K_1 \cdot K_2 \dots K_r$. The total alkali taken up is thus the sum of $r \cdot K_1 \cdot K_2 \dots K_r [H^+]^{n-r} / f([H^+])$ for $r = 1$ to n .

Since each form $H_{n-r}A^{r-1}$ has the concentration $K_1 \cdot K_2 \dots K_r [H^+]^{n-r} / f([H^+])$, and requires r hydrons to be removed for its formation, this is exactly the quantity of alkali required. Hence the titration curve of the real acid H_nA is identical with that of the sum of the n hypothetical acids HX , and the values of G are their dissociation constants, namely the titration constants of H_nA .

Properties follow single-site titration curves

Any property of the acid can be normalized so that H_nA possesses it to zero extent, A^{n-} to unit extent and each of the other ionic forms $H_{n-r}A^{r-}$ to extent a_r . We find constants b such that, if each X_j^- is ascribed the property to extent b_j , the pH-dependence of the property is described. Hence, just as with hydron release of the previous section, any other property is expressed as changes of independent acids that possess the titration constants.

The value, P , of the property at a given pH is the sum of n terms $a_r \cdot [H_{n-r}A^{r-}]$, i.e. the sum of n terms $a_r \cdot K_1 \cdot K_2 \dots K_r \cdot [H^+]^{n-r} / f([H^+])$ for $r = 1$ to n . We plan to ascribe this to the n dissociated forms, so that the hypothetical ion, X_j^- , possesses the property to the extent b_j . The mixture of these acids possesses it to the extent of the sum of all terms $b_j \cdot G_j / ([H^+] + G_j)$.

If we put this sum over the common denominator $f([H^+])$, we obtain some polynomial:

$$c_0 + c_1[H^+] + \dots c_{n-1}[H^+]^{n-1} \text{ over } f([H^+])$$

The mathematical theory of 'partial fraction decompositions' shows that conversely any expression $(c_0 + c_1[H^+] + \dots c_{n-1}[H^+]^{n-1}) / f([H^+])$ can be written as the sum of terms $b_j \cdot G_j / ([H^+] + G_j)$, provided that the G_j values are distinct.

Hence every property follows the same titration constants. We can check the normalization requirements by putting $[H^+]$ equal to zero and infinity. When $[H^+]$ is infinite, all the values of $[X_j^-]$ are zero, so P is zero. When $[H^+]$ is zero, the property is unity, and also the sum of all terms b_j . Hence the b values represent the degree to which each titration constant is ascribed the change of property on passing from H_nA to A^{n-} .

The condition for the factors to be real

Throughout the above arguments we have assumed that the polynomial:

$$f([H^+]) = [H^+]^n + K_1 \cdot [H^+]^{n-1} + K_1 \cdot K_2 \cdot [H^+]^{n-2} + \dots + K_1 \cdot K_2 \dots K_n$$

factorizes as $([H^+] + G_1) \cdot ([H^+] + G_2) \dots ([H^+] + G_n)$ for n distinct values $G_1, G_2, \dots, G_n$. This is just the condition that f has n real roots $-G_1, -G_2, \dots, -G_n$, i.e. is equal to zero when $[H^+] = -G_1, -G_2$ etc. When $n = 2$ the condition for there to be two real roots is just that $K_1 > 4K_2$. For higher values of n the corresponding conditions are much more complicated and involve the discriminant of the polynomial. For example, when $n = 3$ the condition is that:

$$K_1^2 \cdot K_2 - 4K_1 \cdot K_2^2 - 4K_1^2 \cdot K_3 - 27K_2 \cdot K_3^2 + 18K_1 \cdot K_2 \cdot K_3 > 0$$

A more manageable condition that certainly ensures that there are n real roots is that the dissociation constants decrease sufficiently rapidly. We show below that, if:

$$K_1 > 4 \cdot K_2 > 4^2 \cdot K_3 > \dots > 4^{n-1} \cdot K_n \quad (14)$$

then the polynomial f will have n real roots. (It may still have n real roots without this condition holding.)

Suppose that the inequalities (14) hold and consider the value of f at $[H^+] = -\sqrt{K_r \cdot K_{r+1}}$. We show that the dominant term in the polynomial f at this value of $[H^+]$ is the $(r+1)$ st, namely:

$$K_1 \cdot K_2 \dots K_r \cdot [H^+]^{n-r}$$

Call this term T_r . The $(r+2)$ nd is:

$$\frac{K_{r+1} \cdot T_r}{[H^+]} = -\sqrt{\left(\frac{K_{r+1}}{K_r}\right)} \cdot T_r$$

and $\sqrt{K_{r+1}/K_r}$ is less than $\frac{1}{2}$ because of (14). Similarly the $(r+3)$ rd term is $(K_{r+2}/K_r) \cdot T_r$ with K_{r+2}/K_r at most $\frac{1}{16}$. The successive terms alternate in sign and decrease in magnitude so that their sum, from the $(r+2)$ nd to the last term, lies between 0

and $-T_r/2$. The terms before T_r alternate in sign and decrease in magnitude so their sum also lies between 0 and $-T_r/2$. Adding all of the terms together we see that $f(-\sqrt{K_r \cdot K_{r+1}})$ lies between T_r and $(T_r - T_r/2 - T_r/2) = 0$. So $f(-\sqrt{K_r \cdot K_{r+1}})$ has the same sign as T_r , that is positive when $n-r$ is even and negative when $n-r$ is odd. Since f takes opposite signs at $[H^+] = -\sqrt{K_{r-1} \cdot K_r}$ and at $[H^+] = -\sqrt{K_r \cdot K_{r+1}}$, it must have a root $-G_r$ between them. When we apply this argument for each different value of r (in the extreme cases letting $[H^+]$ equal $-K_1$ and $-K_n$) we obtain n real roots $-G_1, -G_2, \dots, -G_n$ satisfying

$$K_1 > G_1 > \sqrt{K_1 \cdot K_2} > G_2 > \sqrt{K_2 \cdot K_3} > \dots > G_{n-1} > \sqrt{K_{n-1} \cdot K_n} > G_n > K_n$$

i.e.

$$pK_1 < pG_1 < (pK_1 + pK_2)/2 < pG_2 < (pK_2 + pK_3)/2 < \dots < pG_{n-1} < (pK_{n-1} + pK_n)/2 < pG_n < pK_n.$$

This demonstrates our contention, and also shows, incidentally, that the titration constants G will be of the same order of magnitude as the successive dissociation constants K . The term $(pK_r + pK_{r+1})/2$ is the pH at which $[H_{n-r}A^-]$ is maximal if, as is likely, this pH is much greater than pK_{r-1} and much less than pK_{r+2} . If each $K_r \gg K_{r+1}$, then each $G_r \approx K_r$.

APPENDIX 2

Number of assignments

There are $n \cdot 2^{n-1}$ group constants for the acid H_nA , since each of the n groups has 2^{n-1} constants, as it has a different constant according to whether each of the other groups is hydronated or dehydronated. Not all these can be independently varied, since the equilibrium constant for going to any form from any other must be independent of path. Hence the number of separately variable constants is 1 less than the total number of different forms, since each isomer of each $H_{n-r}A^-$ can have any equilibrium constant for formation from any chosen form, say H_nA . Since the number of isomers of H_nA , $H_{n-1}A^-$, $H_{n-2}A^{2-}$ etc. are given by the terms of the binomial expansion of $(1+1)^n$, the number of forms is 2^n , and the number of separately assignable group constants is $2^n - 1$.

We now calculate the number of assignments of values that can be made to titration values and the allotment of groups to them. Each of the n titration pK values can be assigned a value. Each of the n groups is assigned to these. For each of the first $n-1$ groups, $n-1$ values are assigned for the fraction of the group allotted to the first $n-1$ pK values (the last pK takes the rest of the group, so no assignment is made for it). The n th group takes the remainder of each of the pK values. Hence there are $n + (n-1)^2$ separate assignments to be made, i.e. $n^2 - n + 1$. This is markedly smaller than the number of assignable group constants when n is large (see Table A1).

If a pair of factors is unreal, then instead of being able to represent properties as following the sums of one-site curves, i.e. sums to which the concentration $[HX]$ or $[X^-]$ contributes for each hypothetical acid H_2X , two such terms may be replaced by one of the expressions for $[H_2X]$, $[HX^-]$ and $[X^{2-}]$, where H_2X is a hypothetical dibasic acid with positively co-operative hydronation, i.e. for which $K_1 < 4K_2$.

Note added in proof (received 6 June 1991)

We thank Rev. Dr. A. R. Peacocke for telling us of the work of von Muralt (1930), who also generalized the treatment of Simms (1926) to n groups, as we do in the second section of Appendix 1. We also thank Dr. F. J. C. Rossotti for pointing out that our statement in the 'Treatment' section, that concentrations $[H_nR]$ etc. are relative to the sum of the concentrations of all forms, needs repetition for Appendix 1.

REFERENCES

- Dixon, H. B. F. (1975) *Biochem. J.* **151**, 271-274
 Michaelis, L. (1914) *Die Wasserstoffionenkonzentration*, pp. 30-39, Springer, Berlin [English translation (1926), pp. 56-69, Baillière, Tyndall and Cox, London]
 Simms, H. S. (1926) *J. Am. Chem. Soc.* **48**, 1239-1250
 von Muralt, A. L. (1930) *J. Am. Chem. Soc.* **52**, 3518-3523

At first it seems paradoxical that, although $2^n - 1$ separate assignments of group constants can be made, fewer assignments are needed to specify the titrations of all the groups in the molecule. To illustrate why, we take the assignment of values for an acid H_4Q . If we assign the fraction of each of the groups dehydronated in the form H_3Q^- , we specify the fractions of each of the four forms that differ according to which of four groups, A, B, C or D, is dehydronated. But when we specify the four fractions that represent how much of each group is dehydronated in the mixture of tautomers that comprise H_2Q^{2-} , we fail to specify how much of each of its six forms (differing according to which pair of groups, AB, AC, AD, BC, BD or CD is dehydronated) there is. Hence the fact that there are two more assignable group pK values in Table A1 for $n = 4$ than there are assignments in the full description of the titrations of all four groups. Similarly with H_5Q , stating the fraction of each group hydronated defines the distribution of tautomers of H_4Q^- and HQ^{4-} . But for each of H_3Q^{2-} and H_2Q^{3-} , only five specifications are needed to say how much of each group is hydronated in each, whereas ten specifications are needed of the ten pairs AB, AC, AD, AE, BC, BD, BE, CD, CE and DE, dehydronated or hydronated respectively, in each. Hence there are ten more specifications of group constants possible than those needed to describe the titration of the groups, again shown in Table A1.

Table A1. Numbers of group dissociation constants and assignments of groups among titration constants

Groups	n	1	2	3	4	5	6	7	8	9	10
Group pK values	$n \cdot 2^{n-1}$	1	4	12	32	80	192	448	1024	2304	5120
Independently assignable group pK values	$2^n - 1$	1	3	7	15	31	63	127	255	511	1023
Allotments of titration pK values	$n^2 - n + 1$	1	3	7	13	21	31	43	57	73	91