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Use of potentiometric titration for determination of α - and ω -peptide bonds in poly(aspartic acid) and poly(glutamic acid)

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sociation constant pK_{app} according to the Henderson-Hasselbalch equation:

$$pK_{app} = pH + \log [(1 - \alpha_d)/\alpha_d] \quad (1)$$

where α_d is the degree of dissociation,

$$\alpha_d = \alpha + ([H^+] - [OH^-])/C \quad (2)$$

Here, α , $[H^+]$, $[OH^-]$, and C , stand for the degree of neutralization, for the molar concentrations of H^+ , OH^- , and all the carboxyls, respectively. The pK_{app} is plotted versus α_d ; the curve obtained consists approximately of two straight lines with different slopes. These lines are extrapolated to $\alpha = 0$, and the values obtained are considered to be those of the intrinsic dissociation constants pK (3.25 and 4.35). The titration curve is graphically derived, and the value of α (0.29), where the derivation reaches its maximum, is taken as the mole fraction of the more acidic carboxyl. In their recent papers,^{3,10} the determination of this mole fraction is based on the knowledge of the evaluated pK values.

The object of the present paper is to show that the approach of Harada and coworkers is not correct and to evaluate what information about the nature of peptide bonds in poly(Asp) and poly(Glu) may be gained by means of potentiometric titration.

ANALYSIS OF THE TITRATION CURVES OF POLY(ASP) AND POLY(GLU)

Let us at first suppose that the carboxyls of a polymer behave as those of a low-molecular-weight compound and calculate Eqs. (1) and (2) in detail. K_1 and K_2 are the dissociation constants of the first and second carboxyls, respectively; k is the mole fraction of the first carboxyl; α_1 and α_2 their degrees of dissociation; C , $[COO^-]_1$, $[COO^-]_2$, $[COOH]_1$, $[COOH]_2$, $[OH^-]$, and $[H]$, respectively, are the concentrations of all carboxyls, of the first and second carboxyls in the dissociated form, of the first and second carboxyls in the undissociated form, of OH^- ions and of H^+ ions; α_d is the degree of dissociation of both carboxyls; α is the degree of neutralization; $[Na^+]$ is the concentration of added NaOH; and K_w is the ionic product of water.

In dilute solutions, where the activity coefficients are close to unity, K_1 and K_2 may be expressed by the following equations:

$$K_1 = \frac{[COO^-]_1[H]}{[COOH]_1}, \quad K_2 = \frac{[COO^-]_2[H]}{[COOH]_2} \quad (3)$$

The degree of neutralization is defined as

$$\alpha = [Na^+]/C \quad (4)$$

The conditions of electroneutrality is

$$[Na^+] + [H] = [OH^-] + [COO^-]_1 + [COO^-]_2 \quad (5)$$

and the mole fraction of the first carboxyl is

$$k = ([\text{COOH}]_1 + [\text{COO}^-]_1)/C \quad (6)$$

For the ionic product of water, we have

$$K_w = [\text{H}] [\text{OH}^-] \quad (7)$$

α may be expressed from the combination of these equations (the square brackets are henceforth omitted):

$$\alpha = \frac{K_1 k}{K_1 + \text{H}} + \frac{K_2(1 - k)}{K_2 + \text{H}} - \frac{\text{H}}{C} + \frac{K_w}{\text{H}C} \quad (8)$$

and α_d may be written according to Eq. (2):

$$\alpha_d = \frac{K_1 k}{K_1 + \text{H}} + \frac{K_2(1 - k)}{K_2 + \text{H}} = k\alpha_1 + (1 - k)\alpha_2 \quad (9)$$

At higher ionic strengths, activity coefficients must be introduced in Eq. (3). Equations (8) and (9) remain formally the same, only K_1 and K_2 in this case are not the true thermodynamic dissociation constants, but are concentration constants (i.e., multiplied by the activity coefficients of COO^- and H^+ and divided by the activity coefficient of COOH).

Equations (8) and (9) allow us to calculate theoretically all the usual plots of titration data: pH vs α (titration curve) according Eq. (8); $\text{p}K_{\text{app}}$ vs α according to Eqs. (1) and (9); and $d\text{pH}/d\alpha$ vs α (differential titration curve) by deriving the function given by Eq. (8):

$$\frac{d\text{pH}}{d\alpha} = \frac{1}{\text{H} \ln 10} \left(\frac{kK_1}{(K_1 + \text{H})^2} + \frac{(1 - k)K_2}{(K_2 + \text{H})^2} + \frac{1}{C} + \frac{K_w}{\text{H}^2 C} \right)^{-1} \quad (10)$$

The condition for an extreme in the differential titration curve may be calculated from the second derivation of Eq. (8):

$$\frac{kK_1(\text{H} - K_1)}{(\text{H} + K_1)^3} + \frac{(1 - k)(\text{H} - K_2)}{(\text{H} + K_2)^3} - \frac{1}{C} + \frac{K_w}{\text{H}^2 C} = 0 \quad (11)$$

The limit of the function (1) for $\alpha_d \rightarrow 0$, i.e., $\text{H} \rightarrow \infty$, according to Eqs. (9) and (1) is

$$\lim_{\alpha_d \rightarrow 0} K_{\text{app}} = kK_1 + (1 - k)K_2 \quad (12)$$

One of the most characteristic features of the dissociation equilibrium of polyacids is a continuous increase in $\text{p}K_{\text{app}}$ with increasing α_d , which is caused by the increasing difficulty of removal protons from polyions with increasing charge.¹¹ Several theoretical models have been developed (e.g., Refs. 12, 13), but none of them describes the titration of a polyanion better than semiquantitatively. $\text{p}K_{\text{app}}$ may be expressed by an empirical polynomial expansion¹⁴:

$$\text{p}K_{\text{app}} = \text{p}K_0 + \phi_1\alpha + \phi_2\alpha^2 + \dots \quad (13)$$

where $\text{p}K_0$, ϕ_1 , $\phi_2 \dots$ are empirical constants whose physicochemical meaning may be discussed. Equation (13) is consistent with the so-called extended Henderson-Hasselbalch equation¹⁴:

$$\text{pH} = \text{p}K + n \log [(1 - \alpha)/\alpha] \quad (14)$$

where n and $\text{p}K$ are empirical constants, and n approaches 2. $\text{p}K_0 = \lim_{\alpha_d \rightarrow 0} \text{p}K_{\text{app}}$ is the so-called intrinsic dissociation constant, i.e., the dissociation constant at the very start of titration, when the dissociation is not influenced by any charge on the polymer.

Let us try to evaluate, at least qualitatively, the manifestation of the polymer effect in the dissociation behavior of poly(Asp). [The case of poly(Glu) is analogous.] As there is no pronounced steric difference between the two types of carboxyls, the acidity is, at the beginning of the titration, primarily due to the induction effect. The carboxyl of I is separated from the amide nitrogen, and from the carbonyl as well, by two carbon atoms. The carboxyl of II is separated from the nitrogen by one carbon atom and from the carbonyl by two carbons. Consequently, the electronegative nitrogen induces greater acidity to the latter. On the other hand, the polymer acquires, as the titration proceeds, a negative charge, which causes the increase of both $\text{p}K$'s. The carboxyl of I is farther from the polymer chain and is therefore less affected. Thus, the difference in the acidity of the two types of carboxyls becomes lower during the course of the titration.

DISCUSSION

The theoretical titration curve with the constants evaluated by Harada and coworkers⁸ (Fig. 1) really contains an inflection point caused by considering two dissociation constants in Eq. (8). Figure 2 shows several differential titration curves calculated for different values of k . These

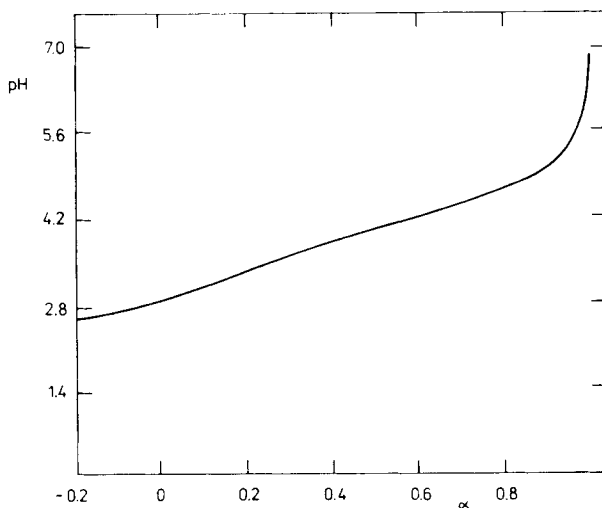


Fig. 1. The theoretical titration curve calculated according to Eq. (8) with the values of the constants those evaluated by Harada and coworkers (Ref. 8): $\text{p}K_1 = 3.25$, $\text{p}K_2 = 4.35$, $k = 0.29$, $C = 10^{-2}M$, and $\text{p}K_w = 14$.

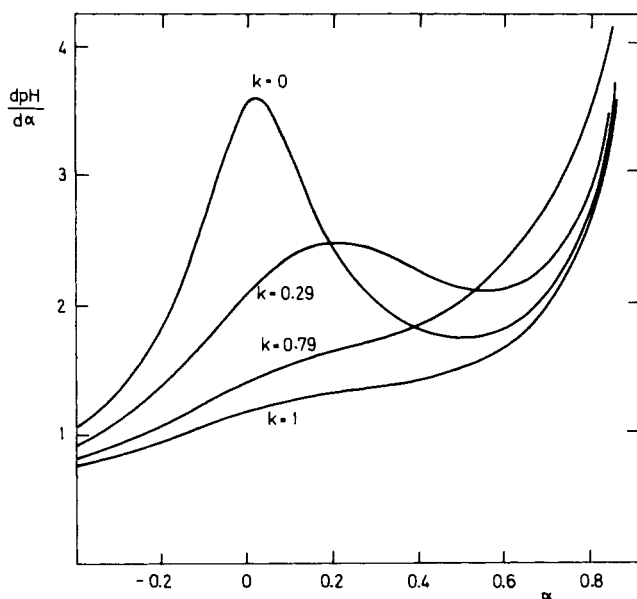


Fig. 2. The theoretical differential titration curve calculated according to Eq. (10) for several values of k and the values of the other constants as in Fig. 1.

curves do not always display extremes for $\text{pH} < 7$; the condition for an extreme (inflection in Fig. 1) is given by Eq. (11). This equation does not always have a real root for $\text{pH} < 7$, i.e., there are certain k , K_1 , K_2 , and C for which the titration curve does not contain any inflection. The increase of pH in these cases is mostly due to the approach of the main equivalence point and not to the titration of the carboxyl, i.e., the third term in Eqs. (8) or (11) overlaps the first two. Consequently, the absence of the inflection point by no means indicates that the two types of carboxyls are not present. On the other hand, there may be an inflection point at the beginning of the curve even for $k = 0$.

The degree of neutralization at which the titration curve reaches the first inflection point (α_i) may be calculated according to Eqs. (11) and (8). It depends, in general, on all constants in these equations. Figure 3 illustrates the dependence of α_i on k and C . It is clearly seen that k is not equal to α_i ; e.g., for $k = 0.29$, as supposed by Harada and coworkers,⁸ $\alpha_i = 0.204$ (the difference is about 42% of α_i); or for the evaluated $\alpha_i = 0.29$, the corresponding k is 0.355. The last α_i for which the titration curve displays an inflection is about 0.48. Concentration is also a substantial factor in Eq. (11), the titration curve would not show an inflection, e.g., for $C < 10^{-3}M$ and $k = 0.29$, or $C < 10^{-2}M$ and $k = 0.5$, $\text{p}K_1 = 3.25$ and $\text{p}K_2 = 4.35$.

Figure 4 illustrates the dependence of α_i on $\text{p}K_1$ and $\text{p}K_2$. It is seen that the necessary condition for the existence of an inflection is the requirement of a certain minimal difference between $\text{p}K_2$ and $\text{p}K_1$ and that α_i approaches k when this difference increases. The assumption that $\alpha_i = k$ could be applied when $\text{p}K_2 - \text{p}K_1$ is large, e.g., for poly(α , ϵ - lysine).¹⁵

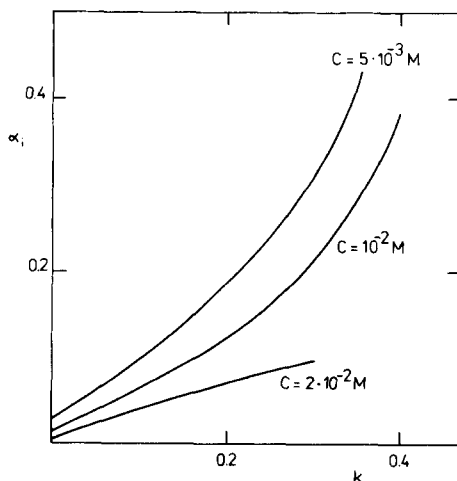


Fig. 3. Plot of degree of neutralization where the titration curve reaches the first inflection point (α_i) vs k for three different concentrations C and the values of the other constants as in Fig. 1. Calculated according to Eqs. (8) and (11).

The evaluation of the inflection point suffers from great experimental error. The calculated $d\text{pH}/d\alpha$ of the first maximum (corresponding to the equivalence of the first carboxyl) is very flat compared with the second (the main equivalence) and is 540 times smaller.

The plot of $\text{p}K_{\text{app}}$ vs α_d (Fig. 5) shows that the extrapolation of Harada and coworkers⁸ cannot be applied. The value of $\text{p}K_{\text{app}}$ for $\alpha_d \rightarrow 0$ is given by Eq. (12). An extrapolation of some breaks in the curve has no founda-

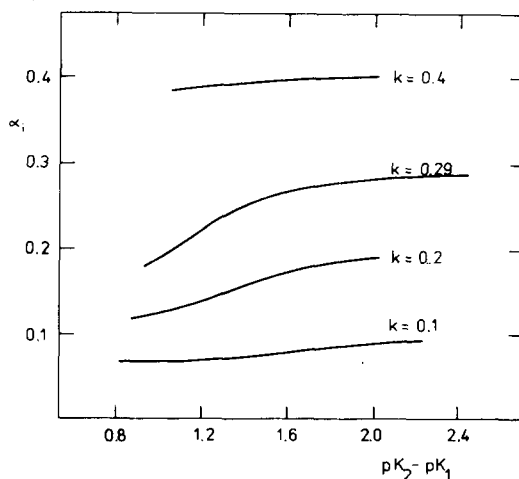


Fig. 4. The dependence of α_i on the difference between $\text{p}K_1$ and $\text{p}K_2$ for several values of k . The variable is $\text{p}K_2$. The values of the other constants are the same as in Fig. 1. Calculated according to Eq. (8) and (11). The curves start at the smallest $\text{p}K_2 - \text{p}K_1$ for which Eq. (11) has a real root at $\text{pH} < 7$.

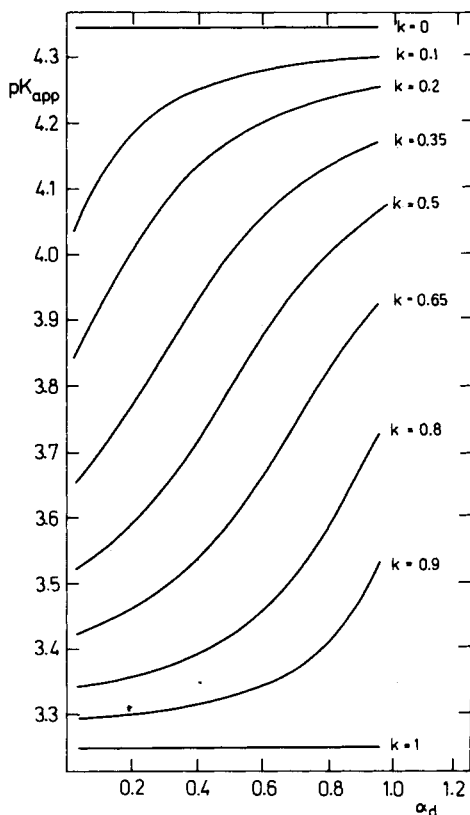


Fig. 5. The plot of pK_{app} vs α_d for several values of k and the values of the other constants as in Fig. 1. Calculated according to Eqs. (1) and (9).

tion. The figure also shows that the shape of a curve such as, for example, for $k = 0.9$ does not give any evidence for one type of carboxyl.⁹

The evaluation of the experimental data would be even more complicated if the polymer effect were not neglected. Harada and coworkers⁸ pretend to resolve this problem by applying Eq. (14), in which the effect of the electrostatic interactions is approximated by considering interactions between neighboring elements of the chain.¹³ They put $n = 1$ and $\alpha_1 = \alpha_d/k$, $\alpha_2 = (\alpha_d - k)/(1 - k)$, which is not correct—see Eq. (9); α_1 is not independent of α_2 . A more correct solution would be to introduce expansion (13) into Eq. (8), but this equation would contain too many empirical constants to be of any use.

The polymer effect may be compensated a great deal for $\alpha < 0.7$ – 0.8 by adding a neutral salt¹¹; k should therefore be determined by titration in its presence. For instance, the titration curve of poly(α -Glu) obtained in the presence of $2M$ NaCl or $0.01M$ $MgCl_2$ and plotted according to Eq. (1) is almost a straight line, parallel with the x -axis, for $0.1 < \alpha < 0.8$.¹⁶ The experimental data may be evaluated according to Eq. (8) by the least squares method. However, the presence of a salt lowers the values not only

of the constants ϕ_1, ϕ_2, \dots in Eq. (13), but also of pK_0 , which was theoretically reasoned¹⁴ and experimentally evaluated for poly(Glu)¹⁷ and poly(Asp).¹⁶ Therefore, pK_1 and pK_2 cannot be determined in salt-free water according to Eq. (8). An approximate value of pK_1 may be obtained by extrapolation to $\alpha = 0$ and recalculation according to Eq. (12) ($K_1 > K_2$, and thus K_2 may be neglected). The value of pK_2 cannot be obtained by extrapolation for $\alpha_d \rightarrow 1$ because of the polymer effect.

These quantitative evaluations may be applied only on polymers not exhibiting a conformational transition. However, the above discussions may also be useful for conformational studies. Potentiometric titration of poly(Asp) and poly(Glu) has been applied mainly in the investigation of their secondary structure.^{7,16-20} Although conformational transition induced by changes of pH is undoubted in poly(Glu)¹⁷ and rare, if at all, in poly(Asp),^{5,16,18-20} irregularities have been detected in the potentiometric behavior of poly(Asp) not displayed by poly(Glu); e.g., the titration curves of the polymers measured by different laboratories under similar conditions differ substantially from each other—being sometimes anomalously curved or even containing a hump that, in the presence of a neutral salt, becomes more definite¹⁶; the increase in pK_{app} with α_d cannot be reduced by the presence of a neutral salt for $\alpha_d < 0.7$;^{16,18} and some other irregularities. These uninterpreted facts can be easily explained in the light of the recent finding that this polymer probably contains two types of carboxyl groups of different acidity.²

CONCLUSIONS

Potentiometric titration is certainly not the most suitable method for the problem discussed. Nmr spectroscopy provides much more accurate information. On the other hand, titration may exceed the accuracy of chemical-modification methods, assuming that no intramolecular transpeptidation takes place during the procedure and that the incomplete conversion in subsequent modification steps is the same for I and II. Potentiometric titration is available in every laboratory and offers rapid qualitative or, if conformational transitions do not interfere, even acceptable quantitative results. The inflection point, when not at the beginning of the titration curve (at $\alpha < 1$), indicates the presence of both types of peptide bonds. Their molar ratio may be determined in the presence of a neutral salt according to Eq. (8), and pK_1 may be obtained by extrapolation and calculation according to Eq. (12).

All calculations in this presentation were performed on a Wang 600 calculator.

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