An Improved Method for Determination of Acid Dissociation Constants of Peptides

Xiaojun Fang,[1] Quintus Fernando,[1] Sydney O. Ugwu,[2] and James Blanchard[2,3]

Received February 20, 1995; accepted May 22, 1995

Purpose. The method described here enables the proton dissociation constants of several amino acid residues of a peptide to be determined simultaneously in aqueous solution without prior knowledge of the exact concentration of the peptide.

Methods. The method used here employs a non-linear fitting program, the BEST program, or a linear least-squares method in combination with the BEST program. These methods are discussed in detail with an emphasis on the quality of the potentiometric titration data that are obtained. Two representative peptides, one with two proton dissociation constants (K_{a1}, K_{a2}) and the other with four proton dissociation constants (K_{a1}–K_{a4}) were used to illustrate the advantages and the limitations of these two complementary methods.

Results. The pK_a values of TVL, a schizophrenia-related tripeptide, were found to be 3.62 (±0.02) and 7.17 (±0.05); the pK_a values of ELTLQE, a hexapeptide, were found to be 2.32, 3.77, 4.58 and 7.74.

Conclusions. The methods reported here are generally applicable to a variety of peptides. The possibility of integrating these procedures into a preparative chromatographic system for the "on-line" assessment of the pKa values of peptides during the purification stage is an attractive and novel feature of this method.

KEY WORDS: dissociation constants; peptide; improved method.

INTRODUCTION

Recent advances in recombinant DNA and hybridoma techniques have resulted in an increasing number of peptide and protein drugs (*1,2*). At present most of these agents must be injected since their large size, instability, and high polarity limit their administration by other routes. Thus, dosage formulation is critical if their clinical utility is to be realized. Knowledge of the physical properties of these agents becomes especially important in guiding the formulation of delivery systems.

One important physical property of peptides and proteins is the degree of dissociation of their constituent amino acids. In order to assess this parameter it is necessary to know their acid dissociation constants, i.e., pK_a values. One of the most widely used methods for pKa determination is potentiometry (*3–6*). Other methods, such as spectrophotometry, are generally less accurate, more time consuming, or difficult to use. These methods are employed when potentiometric titrations are inappropriate, e.g., determination of pK_a's < 2 or >12, or for a compound with low aqueous solubility (*7,8*). In addition to these methodological problems, peptides present additional challenges due to their expense, hygroscopicity, limited stability, and the relatively low solubility of their non-salt forms.

Despite the many advantages of the potentiometric method for pK_a determinations, there are some problems commonly encountered in the pK_a determination of commercially available peptides. It is extremely difficult to obtain a peptide of high purity free of low levels of acid or base contaminants for several reasons. First, acetate salts of peptides often contain a non-stoichiometric amount of acetic acid, which originates from the conversion of the peptide to its acetate salt and which depends on the nature of the peptide and the vacuum employed in the course of the removal of the acetic acid. Second, most chloride or acetate salts of peptides are hygroscopic, and determination of the amount of peptide by weighing is subject to error. This error, introduced by contamination with water, is significant when working with a small quantity of the peptide. Third, if a peptide is supplied in the form of a chloride or trifluoroacetate salt, it will probably contain a non-stoichiometric amount of HCl or TFA.

Although the methodology for performing potentiometric titrations is fairly well standardized (*3–6*), the sophistication of the data analysis can influence greatly the quality and reliability of the results. Advances in computing technology over the past few years coupled with publication of the BEST program in 1988 have enabled us to improve greatly the analysis of potentiometric titration data.

The presence of weak acid or strong acid contaminants in a peptide introduces enormous uncertainties in the potentiometric determination of the pK_a values because these contaminants consume the strong base added during the potentiometric titration. In the past it was necessary to ensure that samples to be titrated were free of such contaminants and to perform several replicate titrations in order to have confidence in the data, especially for polyprotic compounds (*3*). In this report we have proposed a general method for determining the pKa values of polyprotic peptides which we have validated for two peptides: TVL with two dissociable protons, and ELTLQE with four. This method possesses a number of advantages over other methods. First, it is generally applicable to the determination of the pK_a values of peptides in the range of 3 to 11. Second, the titration requires only about 0.02 millimoles of peptide. Third, the method requires no prior knowledge of the amount of peptide used in the potentiometric titration and thus the peptide need not be weighed accurately, thereby obviating the need for an expensive microbalance to accurately weigh small quantities of peptides. In addition, the method allows a determination of the purity of the peptide.

MATERIALS AND METHODS

Materials

A 50% (w/w) NaOH solution was purchased from Fisher Scientific (Tustin, California); potassium acid phthalate (99.95–100.05%) from Aldrich Chemical Co., Inc. (Milwau-

[1] Department of Chemistry, University of Arizona, Tucson, Arizona 85721

[2] Department of Pharmaceutical Sciences, College of Pharmacy University of Arizona, Tucson, Arizona 85721.

[3] To whom correspondence should be addressed.

0724-8741/95/1000-1423$07.50/0

kee, Wisconsin); concentrated HNO_3 from EM Science (Gibbstown, New Jersey); boric acid from Sigma Chemical Co. (St. Louis, Missouri); siliconizing fluid from Princeton Applied Research (Princeton, New Jersey); a tripeptide, the schizophrenia related peptide (TVL) from Peptides International (Louisville, Kentucky) was used as received. The structure of fully protonated TVL is shown in Fig. 1 (a). A hexapeptide (ELTLQE) was obtained from Merck Sharpe & Dohme Research Laboratories (Rahway, New Jersey), and used as received. The structure of fully protonated ELTLQE is shown in Fig. 1 (b). All other chemicals used were from Mallinckrodt, Inc., Paris, Kentucky, and were of analytical grade.

Potentiometry

Potentiometric measurements of hydrogen–ion concentration were performed in a 150 mL glass jacketed vessel provided with a magnetic stirrer and a tightly fitting rubber stopper. The latter was equipped with inlet and outlet tubes for nitrogen, a buret for delivery of NaOH, a glass pH electrode, a salt-bridge, made of 2% agarose gel containing saturated KCl, which was connected to a saturated calomel reference electrode, and a thermometer. During the titration the tip of the buret was immersed in the solution. The temperature in the titration vessel was controlled by circulation of thermostated water through the jacket. To minimize the effect of nitrogen flow on the evaporation of the titration solution, the nitrogen was saturated with water vapor by passing it through a 0.1 M $NaNO_3$ solution. A siliconized titration vessel was used for titration of the peptide, ELTLQE.

Standard NaOH solutions were prepared from 50% (w/w) NaOH solution and CO_2-free deionized H_2O and the prepared NaOH solutions were stored in KIMAX precision bore burets with three-way stopcocks. The NaOH solutions were maintained under a nitrogen atmosphere to prevent CO_2 absorption. The carbonate content of the standard NaOH solutions, determined as described earlier (*9*), was found to be $\leq 0.9\%$. The molarities of the NaOH solutions were determined with potassium acid phthalate using phenolphthalein as indicator. A stock HNO_3 solution, prepared with CO_2-free deionized H_2O, was standardized with a standard NaOH solution. Stock $NaNO_3$ solution (1.000 M) was prepared by dissolving 42.50 g $NaNO_3$ in 500.0 mL CO_2-free deionized H_2O.

(A) Conversion of Measured pH Values to $[H^+]$ Moles/L

A BECKMAN Φ-72 pH Meter was calibrated with NIST (National Institute of Standards and Technology) buffers (pH = 4.00 and 7.00). Titration of 50.00 mL of 2.060 mM HNO_3 solution in the presence of 0.100 M $NaNO_3$ gave an experimental curve for converting measured pH to $-\log[H^+]$. The calculated ion-product of water using the Debye-Hückel equation (*10*) was $10^{-13.80}$ at an ionic strength of 0.10 at 25.0°C.

(B) Determination of Acid Dissociation Constants of Boric Acid

A 40.00 mM boric acid solution was prepared by dissolving the desired amount of previously dried H_3BO_3 in CO_2-free deionized water. 5.000 mL of the boric acid solution was transferred to a 50 mL volumetric flask followed by addition of 1.000 mL (0.1028 mmol) of nitric acid and 5.000 mL of 1.000 M sodium nitrate. The resulting solution was diluted to 50.00 mL with CO_2-free deionized water. 40.00 mL of the diluted solution was transferred to the titration vessel, and potentiometric titration was performed at 25.0°C.

(C) Determination of Acid Dissociation Constants of the Peptides

A weighed amount (7–20 mg) of peptide was placed in the titration vessel followed by addition of a desired volume of stock HNO_3, a desired amount of $NaNO_3$ and a desired volume of CO_2-free deionized H_2O to make the initial volume in the titration vessel between 30 and 40 mL. The potentiometric titration was performed at 25.0°C under nitrogen, and the final concentration of $NaNO_3$ in the titration vessel was 0.100 M.

RESULTS AND DISCUSSION

(A) Conversion of Measured pH to $[H^+]$ moles/L

The best fit parameters for a representative linear regression line obtained in our laboratory for the conversion of experimentally measured pH to $-\log[H^+]$ is listed in Eq. 1.

$$-\log[H^+] = 1.000(4) \times pH_{measured} - 0.038(2) \quad (1)$$

($R^2 = 0.9999$, where R is the correlation coefficient). The values in parentheses are the uncertainties in the last digits of the slope and Y-intercept values of the straight line. The linear conversion plot was prepared solely with titration data points obtained in the acidic region to avoid the effect of possible random fluctuation of the glass electrode potential in the high pH region. The effect of fluctuations on pH measurements in the high pH region was observed as a deviation of the titration data points from the calculated straight line. The uncertainty in the values of $pH_{measured}$ with the BECKMAN Φ-72 is 0.001. Based on the calculated propagation of uncertainties (*11*), the uncertainty in the values of $-\log[H^+]$ is about 0.015 at $pH_{measured} < 3.5$ and about 0.04 at $pH_{measured}$ near 11. Theoretically, the slope of the straight line (Eq 1) should be 1 with a Y-intercept of −0.084 calculated from the Debye-Hückel equation (*11*) under the experimental conditions employed. However, the Y-intercept value is rarely the same as the value calculated from the Debye-Hückel equation because of the uncertainties introduced by variations in the experimental conditions under which the parameters used in the Debye-Hückel equation are determined, which include (1) variation in the selection of background electrolyte; (2) variation in the manner in which the junction potentials at various interfaces are maintained in the potentiometric titration; and (3) uncertainties in the determination of these parameters used in the Debye-Hückel equation, which are not reported by Meites (*12*). Therefore, we prefer to obtain values of the hydrogen-ion concentration from an experimental calibration curve rather than calculating the hydrogen-ion concentration from activity coefficients of the hydrogen ion obtained from the Debye-

Hückel equation (*10*). The value of the slope in Equation 1 may deviate slightly from 1 mainly due to errors introduced during calibration of the pH meter with the NIST buffers. The uncertainty in the experimentally determined slope is the major source of the uncertainty in the determination of $-\log[H^+]$, especially in the high pH region.

(B) Verification of the Method with Boric Acid

Boric acid was used as a standard to validate the proposed method since it is difficult to obtain a high-purity peptide with known pK_a values and known stoichiometry. Boric acid is a good standard acid because it is available in a highly pure form, it is not hygroscopic and thus the amount of boric acid used can be weighed accurately. The dissociation equilibria of boric acid are complicated, thus it is not feasible to obtain the total number of millimoles of H^+, $(N_H+)_{tot}$, in the very high pH region. Therefore, during the titration of boric acid, the addition of NaOH was stopped after the first equivalence point was reached. The pK_a, the $(N_H+)_{tot}$ and the amount of boric acid used in the titration were determined by the BEST program. Acetic acid was initially assumed to be present in the titration solution and its amount was determined by the BEST program. The pK_a of acetic acid at 25.0°C and 0.10 ionic strength is 4.564, calculated using the Debye-Hückel equation (*10,12*). The simulated titration curve is shown in Fig. 2 and the results obtained by the BEST program are listed in Table I. The pK_a of boric acid determined here is in excellent agreement with the value previously reported (*12*). The BEST program indicated that a small amount of a weak acid component, which is insignificant in comparison to the amount of boric acid, was present.

(C) Determination of Acid Dissociation Constants of the Peptides

Peptides generally consist of polyprotic weak acids and their successive acid dissociation constants are determined with the aid of the BEST program (*9*), which is a non-linear weighted least-squares fitting program for all the points on the titration curve. The BEST program calculates the $-\log[H^+]$ directly on the basis of the input parameters and minimizes the weighted sum of squares of $-\log[H^+]$ residuals, while refining the pK_a's to their "best" values. The BEST program also has the capability of refining the initial number of millimoles of all ionizable species in the system.[4] A fully protonated peptide with m protonation sites, after acidification with excess strong acid, e.g., HCl, can generally be represented as HA_l^{n+}, where n^+ represents the number of positive charges on the fully protonated peptide. It is necessary to acidify the peptide solution before performing the potentiometric titration in order to measure the first pK_a of the peptide. The number of millimoles of strong acid added is $(N_{SA})_{add}$. After acidification, a solution of the peptide generally also contains acetic acid, as HAc, and Cl^- as a counter ion. The total number of millimoles of titratable protons, which includes the free hydrogen ions, and the hy-

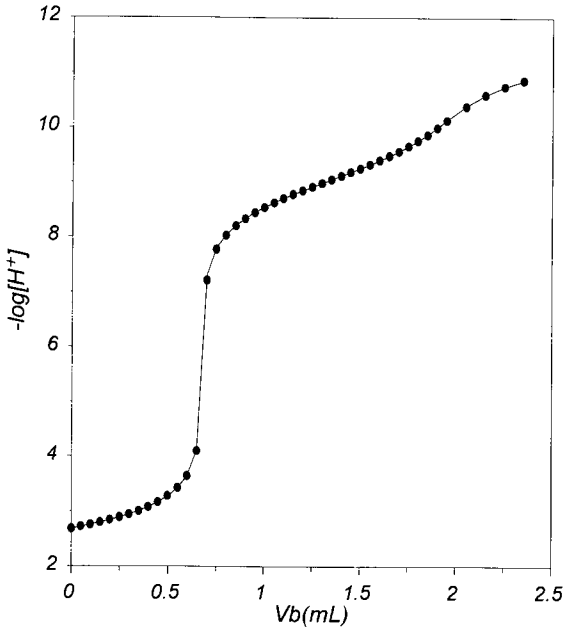


Fig. 2. Potentiometric titration curve of a mixture of 4.001 mM boric acid and 2.056 mM nitric acid. ●: the experimental points; solid line: the titration curve simulated by the BEST program with $\sigma_{fit} = 0.004$.

[4] Motekaitis, R. J. (private communication).

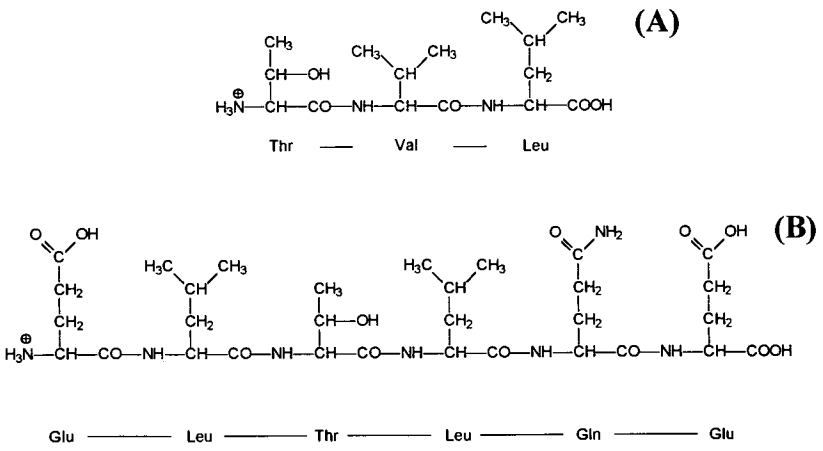


Fig. 1. (A) Structure of fully protonated peptide, TVL; (B) Structure of fully protonated peptide, ELTLQE.

Table I. Final Refined Values of the Variables and the Acid Dissociation Constants in the Potentiometric Titration of Boric Acid at 25.0°C

$(N_{Ac})_{tot}$		$(N_{A1})_{tot}$		pK_a	
0.0021[a]	0[b]	0.1549[a]	0.1604[b]	9.04[c]	9.06 (ref. 13)

[a] Value refined by the BEST program, the σ_{fit} = 0.004. The uncertainty in the number of millimoles is not obtainable from the BEST program.
[b] Value calculated from the concentration of the standard solution. A1 represents the conjugate base of boric acid.
[c] Value refined by the BEST program; the uncertainty in the pK_a value is less than 0.01.

drogen ions in the peptide as well as in the acetic acid, is represented as $(N_H+)_{tot}$ after acidification of the solution. The total number of millimoles of peptide and acetic acid are represented by $(N_{A1})_{tot}$ and $(N_{Ac})_{tot}$, respectively.

The pK_a values, the number of millimoles of the peptide $(N_{A1})_{tot}$, the number of millimoles of acetate ions $(N_{Ac})_{tot}$, and the total millimoles of titratable hydrogen ions $(N_H+)_{tot}$, present are refined simultaneously by the BEST program. The total number of millimoles of titratable protons, which include free hydrogen ions and hydrogen ions in both the peptide and acetic acid, is represented as $(N_H+)_{init}$ before acidification of the solution with strong acid, and is calculated as follows:

$$(N_H+)_{init} = (N_H+)_{tot} - (N_{SA})_{add} \quad (2)$$

The number of millimoles of strong acid contaminants, designated as $(N_{SA})_{cont}$, such as hydrochloric acid or trifluoroacetic acid, in the initial peptide sample is determined as follows:

$$(N_{SA})_{cont} = (N_H+)_{init} - (m(N_{A1})_{tot} + (N_{Ac})_{tot}) \quad (3)$$

A comparison of the values of $(N_H+)_{init}$, $(N_{A1})_{tot}$ and $(N_{Ac})_{tot}$ provides information on the quantity and purity of the peptide present.

The fully protonated TVL peptide is a diprotic acid which has two pK_a values. The experimental titration data (filled-circles) as well as the titration curves (solid lines) simulated by the BEST program are shown in Figs. 3 (a)–(c). The TVL peptide used in the potentiometric titrations was obtained as an acetate salt, and therefore, the number of millimoles of acetate ion is included in the refinement by the BEST program. The refined results listed in Table II, however, indicate that $(N_{Ac})_{tot}$ is zero within experimental error in both experimental trials with TVL. Table II also shows that the $(N_H+)_{init}$ determined from Eq 2 is slightly less than the number of millimoles of the peptide, $(N_{TVL})_{tot}$ in both trials. Therefore, we believe that we were provided with the peptide mainly as an ampholyte, rather than its acetate salt. The conclusion that the peptide is in its ampholyte form is also supported by the observation that, before the TVL peptide solution was acidified with nitric acid, the $pH_{measured}$ value of the solution was 5.438 which is very close to the average, ca. 5.39, of the two pK_a values of the TVL. If the peptide was supplied in the form of its acetate salt, the expected initial calculated pH value would have been around 4.2. The $(N_{SA})_{cont}$ values calculated from Eq 3 are less than zero in both trials, indicating that the TVL peptide was not contaminated with any strong acids.

In any non-linear refinement procedure, especially when many parameters have to be refined, small differences in the values of the parameters being refined can cause similar effects on the minimization process, resulting in difficulties with correlation of these parameters in the refinement. Therefore, the number of parameters to be refined simultaneously in a non-linear refinement procedure should be kept at a minimum. This can be achieved using an independent linear least-squares method to predetermine the total number of millimoles of titratable protons in the solution.

The number of millimoles of free H^+ and the volume of the peptide solution after acidification are represented as $(N_{SA})_{free}$ and V_a mL, respectively. During the titration of the above acidified peptide solution a volume, V_b mL, of a strong base titrant, e.g., NaOH, of concentration C_b moles/L is added to the solution. In the high pH region of the titration curve, where all titratable hydrogen ions are neutralized by the NaOH added and the pH value is approximately two units larger than the highest pK_a estimated for the peptide, the charge balance equation for the system is approximated as

$$[Na^+] + (n-m)[A_1^{(n-m)+}] = [OH^-] + [Cl^-] + [Ac^-] \quad (4)$$

The mass balance equation for Cl^- and Na^+ at high pH is:

Table II. Final Refined Values of the Variables and the Acid Dissociation Constants in the Potentiometric Titration of the Peptide, TVL, at 25.0°C

$(N_{H^+})_{tot}$ mmol	$(N_{SA})_{add}$ mmol	$(N_{H^+})_{init}$ mmol	$(N_{SA})_{cont}$ mmol	$(N_{Ac})_{tot}$ mmol	$(N_{A1})_{tot}$ mmol	pK_1	pK_2
0.1297[a]	0.1028	0.0269	−0.0398	0.00003[a]	0.03323[a]	3.63[a]	7.12[a]
0.1295[b]	0.1028	0.0267	−0.0391	0.00003[b]	0.03288[b]	3.64[b]	7.11[b]
0.0698[c]	0.0315	0.0383	−0.0103	0.00006[c]	0.04001[c]	3.60[c]	7.21[c]

[a] Values refined by the BEST program with the titration data of trial 1, the σ_{fit} = 0.016 (9). The number in parenthesis is the uncertainty in the last digit of σ_{fit}. The concentration of NaOH used was 0.01546 M.
[b] Values determined with the titration data of trial 1 by the linear least-squares method in combination with the BEST program. The concentration of NaOH used was 0.01546 M.
[c] Values determined from trial 2 by the BEST program, the σ_{fit} = 0.004. The concentration of NaOH used was 0.1207 M.

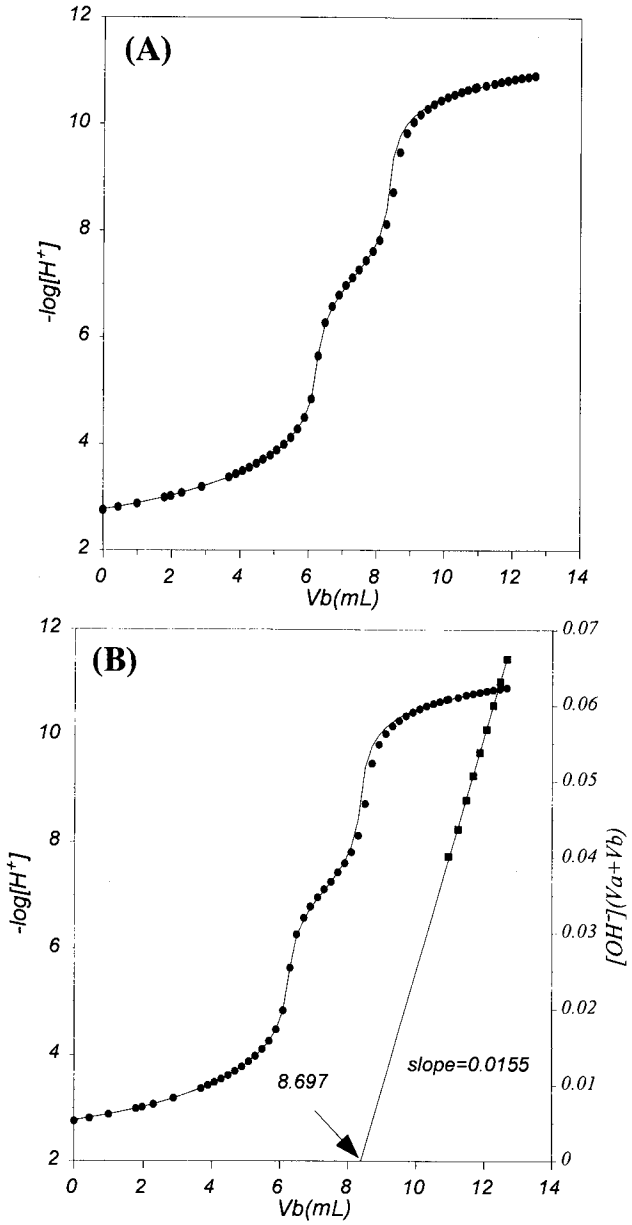


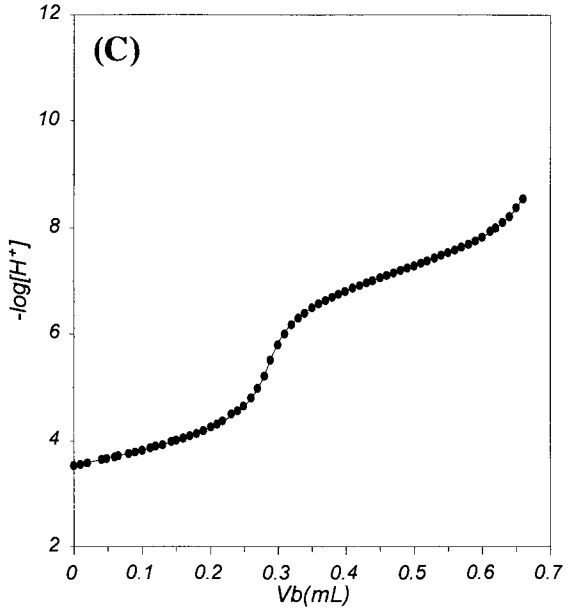
Fig. 3. (A) Potentiometric titration curves of 14.1 mg/40.00 mL of TVL peptide acidified with 0.1028 mmol of nitric acid; ●: the experimental points; solid line: the simulated titration curve by the BEST program with σ_{fit} = 0.016. (B) Potentiometric titration curves of 14.1 mg/40.00 mL of TVL peptide acidified with 0.1028 mmol of nitric acid; ● and ■: the experimental points; curved solid line: the simulated titration curve by the linear method in combination with the BEST program; straight solid line: $[OH^-](V_a+V_b)$ vs V_b. (C) Potentiometric titration curves of 7.6 mg/29.31 mL of TVL peptide acidified with 0.0315 mmol of nitric acid; ●: the experimental points; solid line: the simulated titration curve by the BEST program with σ_{fit} = 0.004.

$$[Cl^-] = \{(N_{SA})_{free} + n[A_1^{(n-m)+}](V_a + V_b)\}/(V_a + V_b) \quad (5)$$

$$[Na^+] = C_bV_b/(V_a + V_b) \quad (6)$$

and the mass balance equation for Ac^- is given by

$$[Ac^-] = (N_{Ac})_{tot}/(V_a + V_b) \quad (7)$$



By substituting Eqs 5, 6 and 7 into Eq 4, multiplying the resulting equation by $(V_a + V_b)$ and rearranging, Eq 8 is obtained.

$$[OH^-](V_a + V_b) = C_bV_b - \{(N_{SA})_{free} + (m[A_1^{(n-m)+}] + [Ac^-])(V_a + V_b)\} \quad (8)$$

The mass balance equation for the total number of millimoles of H^+, (which includes the free hydrogen ions as well as the hydrogen ions bound in the peptide and acetic acid), after acidification with strong acid can be expressed as:

$$(N_H+)_{tot} = (N_{SA})_{free} + (m[A_1^{(n-m)+}] + [Ac^-])(V_a + V_b) \quad (9)$$

at pH values ≥2 units higher than the highest pK_a of the peptide. Substitution of Eq 9 into Eq 8 gives:

$$[OH^-](V_a + V_b) = C_bV_b - (N_H+)_{tot} \quad (10)$$

A plot of $[OH^-](V_a + V_b)$ versus V_b yields a straight line. The slope of this line is C_b and the Y-intercept gives $(N_H+)_{tot}$. After the $(N_H+)_{tot}$ is determined, the BEST program is used to determine the number of millimoles of the peptide and acetate and all the p*K*a values of the peptide by refining these values simultaneously. The titration data for the TVL peptide were also analyzed by this linear least-squares method in combination with the BEST program to demonstrate the usefulness of this method. The results shown in Fig. 3 (b) and listed in Table II obtained by the linear least-squares method in combination with the BEST program are essentially the same, within the margin of error, as those obtained by the BEST program alone. The average of the two pK_a's of the TVL peptide are 3.62(±0.02) and 7.17(±0.05). The titration data of trial 2 were not analyzed by the linear least-squares method because the potential of the glass electrode did not reach an equilibrium value for values of $pH_{measured}$ approaching 9.0, and therefore, the titration was stopped before a high pH was reached.

The peptide ELTLQE, which is a tetraprotic acid when fully protonated, was used in an attempt to demonstrate the

advantage of the linear method in combination with the BEST program in the treatment of potentiometric titration data. It did not serve this purpose, however, because the pH values measured in the high pH region were subject to large errors originating from the slow response of the glass electrode in this region. The experimental titration data (empty-circles) of peptide ELTLQE are shown in Fig. 4. A straight line of slope 0.077, represented by Eq 10, was generated and is shown in Fig. 4. The slope, which was significantly smaller than the concentration of NaOH (0.1024 M) used in the titration, indicated the invalidity of the titration data in the high pH region. The titration was not repeated because only a limited amount of the peptide was available. The ELTLQE peptide used in our potentiometric titrations does not contain acetate ion because, according to the supplier, it was freeze-dried from an HPLC eluent containing 0.1% TFA and the initial pH of the ELTLQE peptide solution before acidification was found to be 3.386. Therefore, only the four pK_a's, the number of millimoles of peptide and the total number of millimoles of titratable protons were refined by the BEST program. The acetate ion was excluded from the refinement. The refined results are listed in Table III and the simulated titration curve (solid line) is shown in Fig. 4. In order to increase the weight of the titration data points associated with the protonation of the peptide, data points in the very low and high pH regions were ignored in the refinement. The four pK_a's of the ELTLQE peptide are 2.32, 3.77, 4.58 and 7.74. The disadvantage of using the BEST program alone for a peptide with several pK_a values is demonstrated in Figure 4, and Table III, when we assumed no prior knowledge of the presence of acetate ions in the sample and allowed the BEST program to determine the number of millimoles of acetate ion. The results obtained by including acetate ion in the refinement are completely different from those obtained by a refinement without the acetate ion, as shown in Table III. However, the goodness of fit, σ_{fit} (*9*), for both simulated titration curves are very similar, as shown by the superimposition of the dashed-line on the solid-line shown in Fig. 4. We, therefore, recommend that the number of fitted parameters be minimized using our linear least-squares method before employing the BEST program, especially when several pK_a values have to be determined simultaneously.

The reliability of the p*K*a values and the estimation of the total number of the millimoles of titratable protons and acetate ions determined potentiometrically, rests exclusively on the reliability of the potentiometric determination of hydrogen–ion concentration. The validity of the experimental curve for the conversion of measured pH to $-\log[H^+]$ can be checked using a Gran plot (*13*) for a strong acid-strong base titration:

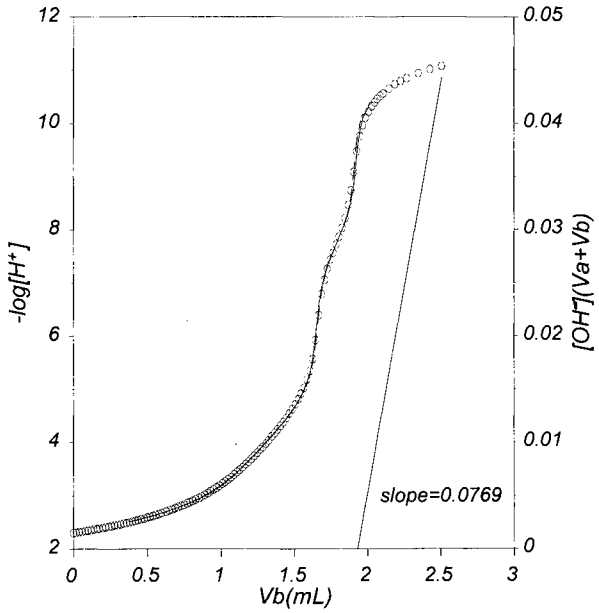


Fig. 4. Potentiometric titration curves of 20.4 mg/21.00 mL of ELTLQE peptide acidified with 0.1092 mmol of nitric acid; ○: the experimental points; curved solid line: the simulated titration curve by the BEST program with σ_{fit} = 0.012; straight solid line: $[OH^-](V_a+V_b)$ vs V_b; curved broken line: the simulated titration curve by the BEST program by assuming the presence of acetate in the sample. The broken line is superimposed on the solid line.

$$[H^+](Va + Vb) = -CbVb + CaVa \quad (11)$$

$$[OH^-](Va + Vb) = CbVb - CaVa \quad (12)$$

where, *C*a represents the initial concentration of nitric acid. An experimental conversion curve was obtained with the strong acid, HNO_3 and the hydrogen-ion concentration at each titration point was calculated using the experimental conversion curve. The titration data obtained in the acidic region were plotted and a straight line (represented by Eq 11) was obtained with a slope of $-Cb$ and a Y-intercept of *CaVa*. The titration data obtained in the basic region were plotted and a straight line (represented by Eq 12) was obtained with a slope of *C*b and a Y-intercept of $-CaVa$. If the conversion curve is valid, the two Y-intercepts and two

Table III. Final Refined Values of the Variables and the Acid Dissociation Constants in the Potentiometric Titration of the Peptide, ELTLQE, at 25.0°C

$(N_{H^+})_{tot}$ mmol	$(N_{SA})_{add}$ mmol	$(N_{H^+})_{init}$ mmol	$(N_{SA})_{cont}$ mmol	$(N_{Ac})_{tot}$ mmol	$(N_{A1})_{tot}$ mmol	pK_1	pK_2	pK_3	pK_4
0.1973[a]	0.1092	0.0881	−0.0235	0	0.02789[a]	2.32[a]	3.77[a]	4.58[a]	7.74[a]
0.1962[b]	0.1092	0.0870	−0.0171	0.01749[b]	0.02603[b]	−1.79[b]	3.20[b]	4.26[b]	7.72[b]

[a] Values determined by the BEST program, assuming the $(N_{Ac})_{tot}$ to be zero in the refinement, the σ_{fit} = 0.012. The concentration of NaOH used was 0.1024 M.

[b] Values determined by the BEST program, without assuming the $(N_{Ac})_{tot}$ to be zero in the refinement. The negative value of pK_1 is irrational for this peptide and immediately indicates an erroneous output of the refinement program regardless of the small σ_{fit} (0.008) that was achieved by the refinement.

slopes obtained for the straight lines represented by Eqs 11 and 12 should be identical within experimental error. Due to contamination of the NaOH solution with CO_2, the slope calculated from Eq 12 should be smaller than the slope calculated from Eq 11 and should reflect the extent of contamination of NaOH by CO_2. The Gran plots and the titration curve of HNO_3 with NaOH are shown in Fig. 5; the slopes calculated from Eqs. 11 and 12 are 0.1208(±0.0006) and 0.120(±0.001), respectively. The Y-intercepts calculated from Eqs. 11 and 12 are 0.1028(±0.0003) and −0.1031(±0.0002), respectively. These results demonstrate the reliability of the potentiometric determination of hydrogen–ion concentration.

The reliability of the results calculated by the linear least-squares method in combination with the BEST program and the BEST program alone depend upon the number of data points obtained in the buffer region of the titration curve of the peptide because the BEST program minimizes the residual $-\log[H^+]$ for all data points on the titration curve. One may neglect some data points in the high and low pH regions, as we did with the ELTLQE peptide, in order to increase the weight of the data points obtained in the buffer region during the refinement by the BEST program. In general, the uncertainty of the refined parameters increases if fewer data points are used in a non-linear refinement program; we prefer, therefore, to obtain a large number of data points in the buffer region of the peptide. Therefore, the smallest possible increment in NaOH volume should be used in the potentiometric titration, as demonstrated in Fig. 3 (c). This is one of the fundamental limits to the accuracy of the method since the amount of peptide available for a potentiometric titration is usually very small. An alternative solution to this problem may be the use of a more dilute NaOH solution; in this case, however, maintenance of the ionic strength during the titration will be difficult because of the significant dilution effect resulting from the addition of the NaOH solution.

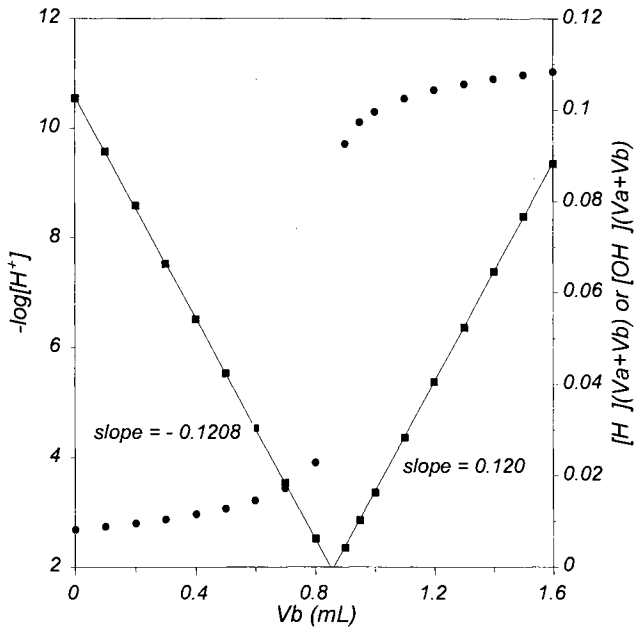


Fig. 5. ●: titration curve of 2.056 mM of nitric acid; ■: Gran plots of $[H^+](V_a+V_b)$ vs V_b and $[OH^-](V_a+V_b)$ vs V_b.

ACKNOWLEDGMENTS

The generous gift of the hexapeptide (ELTLQE) by Merck Sharpe & Dohme Research Laboratories is gratefully acknowledged.

REFERENCES

1. A. K. Banga, and Y. W. Chien. Systemic delivery of therapeutic peptides and proteins. *Int. J. Pharm.* 48:5–50 (1988).
2. C. G. Pitt. The controlled delivery of polypeptides and proteins. *Int. J. Pharm.* 59:173–196 (1990).
3. B. R. Glick and D. J. Leggett. A method for the determination of the pKa of α-N-benzoyl-L-arginine in the presence of an impurity. Analyt. Biochem. 70:563–571 (1976).
4. W. J. Lambert and R. J. Dalga. Potentiometric determination of thermodynamic and apparent dissociation constants by nonlinear least squares fitting. Drug Develop. Ind. Pharm. 16:719–737 (1990).
5. M. Meloun, and M. Bartos. Computer estimation of dissociation constants. Part IV. Precision and accuracy of potentiometric titrations. Mikrochim. Acta 108:227–239 (1992).
6. A. G. Gonzalez, and A. G. Asuero. Computation method for unbiased evaluation of equivalence volumes and ionization constants from potentiometric acid-base titrations. Analyt. Chim. Acta 257:29–33 (1992).
7. L. Z. Benet and J. E. Goyan. Potentiometric determination of dissociation constants. *J. Pharm. Sci.* 56:665–680 (1967).
8. G. A. Lewis. Determination of dissociation constants of sparingly soluble compounds. *Int. J. Pharm.* 18:207–212 (1984).
9. A. E. Martell, and R. J. Motekaitis. *Determination and Use of Stability Constants*. VCH Publishers, Inc., New York, NY, 1988.
10. M. D. Seymour, and Q. Fernando. Effect of ionic strength on equilibrium constants. *J. Chem. Educ.* 54:225–227 (1977).
11. H. A. Strobel, and W. R. Heineman. *Chemical Instrumentation: A Systematic Approach*. 3rd Ed., Chapter 10, John Wiley & Sons, Inc., New York, NY, 1989.
12. L. Meites, ed. *Handbook of Analytical Chemistry*, Section 1, 6–7, McGraw-Hill Book Company, Inc., New York, NY, 1963.
13. G. Gran. Determination of the equivalence point in potentiometric titrations. Part II. *Analyst* 77:661–671 (1952).