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Short Communication

A novel method for the determination of protonation constant

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Abstract

It is for the first time that the use of paper electrophoresis is described for the assessment of protonation constants The method is based on migration of a spot of the peptide with variation of pH of background electrolyte containing 0.1 M perchloric acid. The graph between pH *vs* mobility gives information about nature of dissociation and helps in calculating them. The calculated values are fairly in agreement with literature values.

Key words: Paper electrophoresis, protonation constants, mobility.

1. Introduction

Protonation of peptides in general has been studied mainly with electrometric technique using glass electrode which gives reliable results. The ionophoretic technique has been recently introduced for investigating formation of complexes and determination of stability constants of mononuclear complexes [1,2]. Usual procedure is to study the mobility of metal ion spot on paper strip soaked with background electrolyte buffered at a fixed pH containing progressively increasing concentration of the ligand. In these laboratories the procedure has been drastically modified [3-7]. Here the concentration of liganding sample is kept constant but hydrogen ion concentration of background electrolyte is progressively decreased by addition of an alkali solution. Thus the previous technique failed to elucidate the effect of change of relative concentration of the different ionic species of a liganding sample. The technique modified by us has also been used for the study of mixed complexes for the first time [8-13].

The electrophoretic technique usually suffers from a number of defects. Temperature during electrophoresis, capillary flow on the paper strip, electroosmosis, adsorption and molecular 'sieving' affect the mobility of charged moieties. The technique described here is almost free from these 'vitiating' factors. The technique is very handy and simple. It gives results fairly in agreement with those of the literature values.

2. Experimental

Instrument: Electrophoresis equipment (Systronics model 604, India) has been used. The apparatus consists of a PVC moulded double tank vessel. In order to avoid error due to heat

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generation during electrophoresis, two hollow rectangular plates each of one kg weight covered with thin plastic paper have been used through which thermostated water is circulated. The tank is closed with transparent PVC moulded lid to prevent moisture changes that may upset the equilibrium in paper strip. Each electrolyte tank contains a separate pt-wire electrode. Voltage variation is eliminated with an electrical stabilizer.

pH measurements were made with Elico model L_{1-10} pH meter using glass electrode.

Chemicals: A.R. grade perchloric acid sodium hydroxide and all peptides (B.D.H.) are used.

0.28% (w/v) solution of ninhydrin in acetone was used for detection of peptide spots. A saturated solution of silver nitrate (A.R.) in acetone (pure) was sprayed on paper and subsequently fumed with ammonia to detect glucose spot.

Procedure: The level of hollow base plate in the instrument was made horizontal with a spirit level. 150 ml of 0.1 M perchloric acid (A.R.B.D.H.) was taken in each tank of the electrophoretic apparatus. The two tank solutions were levelled by siphoning them through a bent tube. These precautions were taken to check any gravitational and hydrodynamic flow. Paper strips (Whatman No. 1) of 30×1 cm^2 size were soaked in the background electrolyte and the excess electrolyte solution was blotted by pressing them gently within the folds of dry filter paper sheets. The strips in duplicate were then spotted with aqueous solutions (0.01 M) in the centre with a micropipette and were subsequently placed on the base plate and sandwiched under the upper hollow metallic plate with end of strip and were allowed to remain as such for 15 minutes. A 200 volt potential difference was applied between the tank solutions to initiate electrophoresis.

The electrophoresis was carried out for 60 minutes. The hollow plates were well circulated with thermostated water (35° C) throughout the experimental period. Afterwards strips were taken out with the help of glass and dried at horizontal plate form and the spots detected. This observation was repeated at different pHs of the background electrolyte (variation in pH was made by the addition of caustic soda solution). The distance recorded in duplicate differed within ±5% and the average distance of the duplicates was noted for calculations. The distance travelled towards anode was assumed to be negative and those towards cathode to be positive. The actual distance of sample spot takes into account the distance travelled by the reference glucose spot. The potential gradient through the strip was found to be 7.5 V/cm.

3. Results and discussion

The plot of overall electrophoretic mobility of peptide spot against pH gives a curve with a number of plateaus as shown in figs.1 and 2. A plateau is obviously indicative of a pH range where mobility is practically constant due to the predominance of a particular ionic species. Thus every plateau indicates the formation of a specific ionic species resulting from peptides.

The first plateau occurring in the region of low pH values with positive mobility must be due to the protonation of amino group; with increase of pH, the speed of the spot decreases due to deprotonation. The decrease continues till the second plateau is reached. The ionic species in the case of all peptides are dipolar ions. With overall neutral charge which is confirmed since the speed is zero in the region of second plateau.

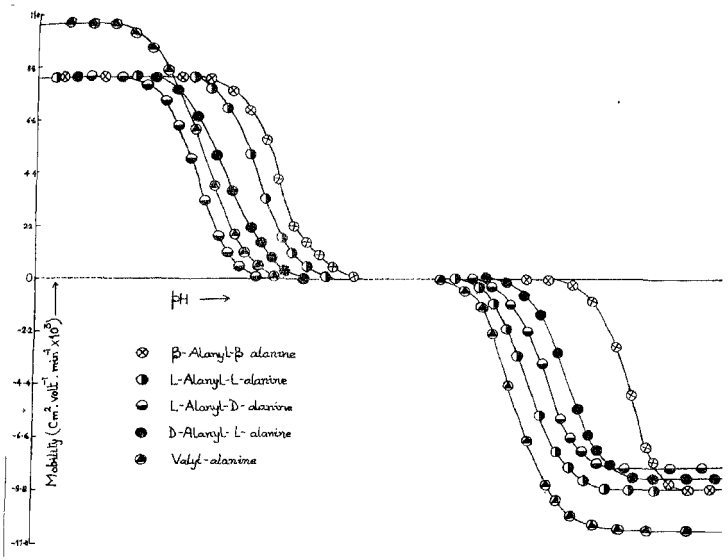

FIG. 1. Mobility curves (Temp. 35 °C, ionic strength 0.1).

On further increase of pH, mobility further decreases and becomes constant in the range of third plateau. The last plateau which lies in the negative region of the curve is due to the formation of the anionic species of peptides. The mobility remains unchanged on further increase of pH.

Considering the protonated variety of all peptides as H_2L, dipolar ion as HL and anionic variety as L (charges on the ions are being ignored). The dissociation can be expressed as:

$$H_2L \underset{}{\overset{k_1}{\rightleftharpoons}} HL + H$$

$$HL \underset{}{\overset{k_2}{\rightleftharpoons}} L + H$$

The concentration of a dipolar ion, and anionic species can be expressed as:

$$[HL] = \frac{k_1 [H_2L]}{[H]}$$

$$[L] = \frac{k_1 k_2 [H_2L]}{[H]^2}$$

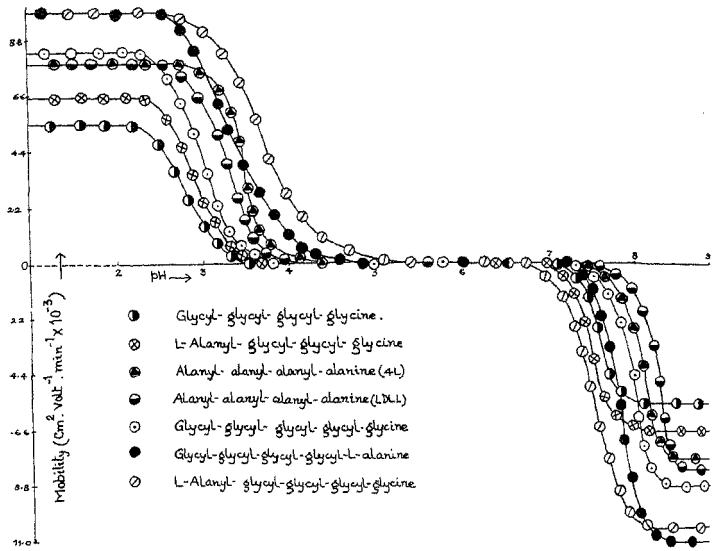

FIG. 2. Mobility curves (Temp. 35°C, ionic strength 0.1).

Total peptides distribute themselves in the form of different ionic species. The following expression holds for total concentration

$$L_T = \{ 1 + \frac{k_1}{[H]} + \frac{k_1 k_2}{[H]^2} \} [H_2 L]$$

The relative abundance of a particular anionic species of the peptide depends upon the pH of the background electrolyte in which it is present. Under the influence of electric field imposed on the strip, the peptide spot which is essentially a conglomeration of a number of species in equilibrium will move as single entity whose mobility in an electric field can be given by the equation:

$$U = u_n f_n$$

where f_n and u_n are molefraction and mobility of a particular species of the peptide under investigation. Using this equation in the present case the overall mobility is given by the expression:

$$U = u_{H_2L} \cdot f_{H_2L} + u_{HL} \cdot f_{HL} + u_L f_L$$

where u_{H_2L}, u_{HL}, and u_L are mobilities of H_2L, HL and L respectively and f_{H_2L}, f_{HL} and f_L are their molefractions. Substituting the values of molefraction f_{H_2L} by $(1+K_1/[H]+k_1 k_2/[H]^2)^{-1}$; f_{HL} by $K/[H](1+K_1/[H]+k_1 k_2/[H]^2)^{-2}$; f_L by $\frac{K_1 K_2}{[H]^2}$ in the above equation and simplifying we obtain:

$$U = \frac{u_{H_2L} + u_{HL} \cdot K_1/[H] + u_L \cdot k_1 k_2/[H]^2}{1 + K_1/[H] + K_1 K_2/[H]^2}$$

This expression is a general expression correlating overall mobility 'U' with mobility of individual species present in the spot as well as with their relative abundance.

For calculating the first dissociation constant k_1, first and second plateaus have been considered. The average mobility corresponds to a point at which $k_1/[H] = 1$. The values of k_1 can thus be calculated. Similarly this principle of average mobility is used again, for calculation of K_2 between the second and third plateau and for K_3, the third and fourth plateau are to be kept in mind. Values of K_s are reported in Table I. The calculated values are fairly agreeable with those determined by others. The variation is partly due to variation in temperature and ionic strength.

Table I
Protonation constants of some peptides
(Temp. 35°C, ionic strength 0.1)

Peptides	Ionophoretic value		Literature value		Ref No.
	log K_1	log K_2	log K_1	log K_2	
Gly-gly-gly-gly	7.58	2.80	7.87	3.18	14
L-ala-gly-gly-gly	7.49	2.89	7.85 7.97	3.18 3.30	14
Ala-ala-ala-ala	8.12	3.46	8.02	3.36	14
Ala-ala-ala-ala (LDDLL)	8.32	3.30	8.35	3.38	14
Gly-gly-gly-gly-gly	8.00	3.00	7.86	3.39	14
Gly-gly-gly-gly-L-ala	7.58	3.31	7.86	3.37	15
L-Ala-gly-gly-gly-gly	7.75	3.62	7.86 7.78	3.38 3.49	14 15
L-Ala-ala	9.27	3.90	9.30 9.37	3.95 4.02	14 15
L-Ala-L-ala	8.00	3.65	8 05 8.17 8.15	3.20 3.30 3.31	14 17 17
L-Ala-D-ala	8.29	2.98	—	—	—
D-Ala-L-ala	8.30	2.87	8.30	2.35	—
Val-ala	7.83	3.32	—	—	—

The precision of the method is limited to that of paper electrophoresis. The order of uncertainty is $\pm 5\%$. Obviously this cannot replace the more reliable methods but definitely provides a new method worth developing and perhaps with further refinements may prove valuable.

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