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Determination of ionization constants by paper electrophoresis

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Dimensionless apparent ionization constants of charged low-molecular-weight species may be obtained from paper-electrophoretic data at 20–25°C with buffers (*I* 0.1–0.5) of measured pH (1.5–12.5) containing oxalate ions. Relative mobilities rather than absolute mobilities were measured by using glycerol and *m*-nitrobenzenesulphonate respectively as standards of zero and unit mobility. Application of the procedure to ionizations of adenine, adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 3′:5′-cyclic AMP, ADP, ADP-glucose, agrocin 84 and ATP is described.

The use of known ionization constants for the optimization of pH conditions for the separation of charged species by electrophoresis in stabilized media has been recognized for many years (Consden *et al.*, 1946). Despite the obvious attraction of a method capable of multiple simultaneous determinations, the converse proposal, for the estimation of unknown ionization constants by studying electrophoretic mobilities of low-molecular-weight species as a function of pH or other ion concentration, has received scant attention (Kraus & Smith, 1950; Michl, 1952; MacWilliams, 1956).

The reasons have been succinctly discussed (Shaw, 1969), and it has been pointed out that the use of a stabilizing medium makes the measurement of reproducible absolute mobilities an almost impossible task, owing to the effect on ion mobility produced by capillary flow, electro-osmosis, adsorption, molecular sieving and temperature.

The purpose of the present paper is to demonstrate that, by the choice of conditions to minimize capillary flow, together with the selection of suitable reference compounds, voltage gradients and the potassium salts of anionic buffers containing oxalate ions, it is possible to obtain self-consistent estimates of ionization constants over the temperature range 20–25°C and *I* 0.1–0.5. The method involves a study of relative, rather than absolute, mobilities of charged species as a function of pH, over the range pH 1.5–12.5. It is not intended to replace the accurate determination of ionization constants by potentiometric and spectrometric procedures (Albert & Serjeant, 1971), but rather to supplement these methods when they are inapplicable.

Useful features to biochemists are that no knowledge of the absolute concentration of the compound of interest is required and that the minimum amount of material required is determined solely by the limits of detection that are used. It is particularly suitable for the characterization of labile, biologically active or radioactive compounds and has been repeatedly used in this laboratory for the characterization of the nucleotide bacteriocins known as agrocin 84 and 108 (Roberts *et al.*, 1977; Tate *et al.*, 1979). The monotony of repeated electrophoresis at different pH values is considerably alleviated by the fact that a large number of species can be examined simultaneously; indeed it is a particular virtue of the method that the component of interest need not be highly purified. The only requirement is that its location on the completed electrophoretogram be unambiguously known.

The determination of ionization constants over the range pH 1.5–12.5 for adenine, 2′-deoxyadenosine, 3′-deoxyadenosine, adenosine, 5′-AMP, 3′:5′-cyclic AMP, ADP, ADP-glucose, agrocin 84 and ATP is described.

Materials and methods

Materials

Reference compounds were purchased from the following suppliers: sodium *m*-nitrobenzenesulphonate, ICN Pharmaceuticals, Life Sciences Group, Plainview, NY, U.S.A.; sodium naphthalene-1,5-disulphonate, Hopkin and Williams, Chadwell Heath, Essex, U.K.; Orange G, Eastman Organic Chemicals, Eastman Kodak Co., Rochester, NY, U.S.A.; sodium bis(*p*-nitrophenylphosphate), adenine, adenine nucleosides and adenine nucleotides, Sigma, St. Louis, MO, U.S.A.; glycerol and all buffer salts, BDH Ltd., Poole, Dorset, U.K.

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Apparatus

The apparatus (Tate, 1968) was a modification (which prevents evaporation from the paper) of a simple solvent-cooled system (Markham & Smith, 1951); the original carbon tetrachloride coolant was replaced by tetrachloroethylene.

Buffers

To avoid discontinuities in the pH–mobility profiles, only anionic species have been utilized, and in each case the cation has been restricted to K^+. Incorporation of 0.05 M-oxalate in each buffer system prevented the retardation of anionic species by polyvalent cationic impurities that are usually present in the paper. Potassium salts or the free acid of oxalic acid and the second buffer component were dissolved in 0.75 times the final volume and the pH was adjusted with solid KOH to yield the desired pH (±0.1 pH unit). The solution temperature was then adjusted to 25°C, and finally the volume was adjusted to give a solution that was 0.05 M with respect to the sum total of oxalate series and likewise 0.05 M with respect to the sum total concentration of the second buffer component. The final pH (Bates, 1968) was then accurately measured (±0.02 pH unit) at 25°C with a Radiometer model-26 pH-meter with a combined glass/calomel (Hg_2Cl_2) electrode pair (GK2301B). The pH-meter was standardized at pH 1.0, 1.68, 4.00, 6.48, 9.18 and 13.0 with the following solutions: 0.134 M-HCl/0.05 M-KCl, 0.05 M-potassium tetraoxalate, 0.05 M-potassium hydrogen phthalate, Radiometer phosphate buffer, 0.01 M-sodium tetraborate, 0.132 M-NaOH/0.05 M-KCl.

In addition to the constant 0.05 M-oxalate concentration, the following salts were employed in the stated pH ranges: pH 1.5–4.5, 0.05 M-potassium hydrogen sulphate; pH 4.5–6.6, 0.05 M-potassium dihydrogen citrate; pH 6.7–7.8, 0.05 M-potassium dihydrogen phosphate; pH 7.9–9.0, 0.05 M-tetrapotassium pyrophosphate; pH 9.4–11.0, 0.05 M-potassium hydrogen carbonate; pH 11.4–12.5, 0.05 M-dipotassium hydrogen phosphate.

Equilibration and minimization of capillary flow

Reproducible results cannot be obtained unless capillary flow is minimized. For this reason, uneven loss of moisture from the surface of the paper both during and after electrophoresis must be prevented. Evaporation from the surface of the paper is effectively eliminated with either solvent- or pressure-plate-cooled systems, but sufficient equilibration time must elapse before electrophoresis in order to minimize capillary flow from the buffer chambers towards the centre of the paper. To prevent siphoning through the paper it is necessary that the buffer levels are the same in each chamber.

The equilibration time for the electrophoresis unit was determined by the following procedure. A strip of Whatman 3MM filter paper (5 cm × 57 cm) was ruled crosswise at 5 cm intervals from the centre towards each end and dipped in buffer solution, lightly blotted and then spotted with 1 μl portions of 0.01 M-*m*-nitrobenzenesulphonic solution at each marked interval. The strip was then placed in the electrophoresis apparatus in contact with the wicks from the buffer chamber, for a fixed period of time (0, 0.25, 0.5, 1.0, 2 h) without application of the voltage gradient. The strip was then removed and pressed as dry as practicable between two sheets of Whatman 3MM paper lined with a disposable sheet of Whatman 1 paper and hung vertically to air-dry. Capillary flow during the drying procedure is virtually eliminated by this preliminary blotting procedure. An unsatisfactory drying technique is readily detected by displacement of the dyestuff markers on the zero-time-interval test strip. With increasing time of equilibration the rate at which the dyestuff markers near the ends of the strip are displaced towards the centre gradually diminishes and at 1 h the displacement distance is virtually the same as at 2 h. In the present work with a voltage gradient of 1563 $V \cdot m^{-1}$ a minimum equilibration time of 0.5 h has been found to give reproducible results for compounds with relative mobilities (m_N) of ±1.5 or less. A similar equilibration period was chosen by Frahn & Mills (1959) for a pressure-plate-cooled apparatus. Equilibration also minimizes pH changes diffusing from the electrode compartments. Elution of buffer from two segments at 15 cm from either side of the origin showed a pH difference of only 0.03 pH unit.

Electrophoresis and relative-mobility (m_N) measurements

Strips of Whatman 3MM filter paper (15 cm × 57 cm) ruled crosswise at the centre were spotted on this line at points 1.5 cm from each edge and also in the centre, with 1 μl portions of a standard reference solution, which was 0.01 M with respect to the following compounds: sodium *m*-nitrobenzenesulphonate, Orange G, sodium bis(*p*-nitrophenyl-phosphate), sodium naphthalene-1,5-disulphonate, 2′-deoxyadenosine, and 0.1 M with respect to glycerol. Samples (1 μl) to be studied were spotted in duplicate between the reference standards at 1 cm intervals. One end of the paper was immersed in the appropriate buffer to within 1 cm of the ruled origin and the buffer was permitted to rise by capillary action to the central origin line. Excess buffer was lightly blotted off, avoiding the central spotting zone, and the procedure was repeated with the remaining dry end. At this stage the dyestuff mixtures were still within 2 mm of their initial origin and the (wet weight)/(dry weight) ratio was approx. 2.5 : 1. The

moist paper was then placed on the supporting frame, immersed in coolant and the ends pressed in in intimate contact with the saturated wicks from the buffer chambers. The electrodes and cooling coil were placed in position and the system was equilibrated for 0.5 h before applying 750 V to the electrodes. This corresponds to a voltage gradient of 1563 $V \cdot m^{-1}$, which was maintained for 1.5 h. The observed current depends upon the buffer composition, but was generally in the range 80–120 mA. Under these conditions with a cooling-water supply at 18–22°C and an initial coolant temperature of 20–23°C, the temperature rise of the coolant at the end of electrophoresis was less than 2°C. At completion of the electrophoresis the paper was removed, blotted dry, and air-dried as described under 'Equilibration and minimization of capillary flow'. Reference standards and components of interest were then detected by their u.v. (254 nm) absorption or other appropriate techniques (bioassay, radioactivity, specific reagents, etc.); glycerol may be detected with the acetone/$AgNO_3$-dip procedure (Trevelyan *et al.*, 1950), followed by a dip of ethanolic 0.5 M-NaOH; for phosphate and pyrophosphate buffers it is also necessary to steam the papers for a few minutes after the alkali treatment. Alternatively the glycerol may be detected by using a periodic acid reagent (Weiss & Smith, 1967). Displacement distances from glycerol are measured, to the nearest millimetre, between spot centres. Relative mobilities (m_N) are calculated from the displacement distance traversed by each component of interest divided by the mean displacement distance for the three *m*-nitrobenzenesulphonate spots (approx. 11 cm). Relative mobilities are denoted as positive when the component of interest moves in the same direction from glycerol as the chosen reference standard, and as negative when it moves in the opposite direction.

Ionization-constant calculations

If n is the maximum number of dissociable protons of a polyprotic acid $(H_nB)^m$ of charge m, where m may be zero or a positive or negative integer, then the stepwise Bronsted apparent ionization constant ${}^BK_a$, for the first ionization:

$$(H_nB)^m \rightleftarrows (H_{n-1}B)^{m-1} + H^+ \qquad (1)$$

may be expressed by denoting species concentrations in square brackets ([ ]) and H^+ activity with braces ({ }) as follows:

$${}^BK_a = \frac{[H_{n-1}B]\cdot\{H\}}{[H_nB]} \qquad (2)$$

where charges and species parentheses have been omitted for clarity.

If the mobility, m_N, relative to *m*-nitrobenzenesulphonate of the first ionization product $H_{n-1}B$ is m_1 and the relative mobility of the fully protonated acid H_nB is m_0, then, at pH values where the first ionization of H_nB is incomplete, the net relative electrophoretic mobility $\bar{m}$ of the equilibrium mixture of the acid and its ionization product, will have values that are intermediate between the mobility of the acid (m_0) and its ionization product (m_1). The numerical value of the net mobility ($\bar{m}$) is determined by the sum of the products of the respective mobilities and the partial molar fractions of the acid and its ionization product as shown in eqn. (3), for a single ionization step:

$$\bar{m} = m_0\cdot\frac{[H_nB]}{[H_nB]+[H_{n-1}B]} + m_1\cdot\frac{[H_{n-1}B]}{[H_nB][H_{n-1}B]} \qquad (3)$$

By substituting eqn. (2) in eqn. (3) and rearranging terms, the Bronsted apparent ionization constant. ${}^BK_a$, may be derived from any of the linear equations, eqns. (4), (5) or (6). For the purposes of calculation, the H^+ activity, {H}, is equated with the antilogarithm of the negative value of the measured pH, i.e. $\{H\} = 10^{-pH}$.

$$\frac{1}{\{H\}} = \frac{1}{{}^BK_a}\cdot\frac{(\bar{m}-m_0)}{(m_1-\bar{m})} \qquad (4)$$

$$\bar{m} = m_0 + {}^BK_a\cdot\frac{(m_1-\bar{m})}{\{H\}} \qquad (5)$$

$$\bar{m} = m_1 + \frac{1}{{}^BK_a}\cdot\{H\}\cdot(m_0-\bar{m}) \qquad (6)$$

From eqns. (5) and (6) is it apparent that, providing either the relative mobility of the acid (m_0) or its ionization product (m_1) is discernible as a plateau in the pH–relative-mobility profile, then the linear regression coefficients for the least-squares solution of eqns. (5) and (6) will provide the Bronsted apparent dissociation constant or its reciprocal, together with the remaining unknown relative mobility, i.e. m_0 or m_1. If both m_1 and m_0 are known, eqn. (4) may be used.

For two successive partially overlapping constants ($1.0 \leqslant pK_2 - pK_1 \leqslant 2.7$) estimates of the unknown intermediate mobility and the two overlapping constants may be made by using eqns. (5) and (6) to portions of the data where overlap is not significant, in a similar manner to the treatment (Albert & Serjeant, 1971) of overlapping ionization constants derived from spectrometric data.

Regardless of whether or not the constants overlap, the predicted mean mobility ($\bar{m}_p$) may and should be recalculated at each pH by eqn. (7) by using the derived pK_a values and relative mobilities.

$$\bar{m}_p = \frac{m_0 + m_1\cdot 10^{pH-pK_1} + m_2\cdot 10^{2pH-pK_1-pK_2} + \cdots}{1 + 10^{pH-pK_1} + 10^{2pH-pK_1-pK_2} + \cdots} \qquad (7)$$

In the case of a single ionization, only the first two terms of the multiplicand and the divisor are required. Similarly, for three or more ionizations, eqn. (7) can be expanded to include the appropriate additional terms.

Results and discussion

Fig. 1 shows the pH–relative-mobility (m_N) profiles, which establish the useful ranges for three secondary reference anions with *m*-nitrobenzenesulphonate as a primary standard ($m_N = 1.0$). The direction magnitude and pH-dependence of electro-osmotic buffer flow in this cellulose-stabilized medium can be seen from the apparent relative mobility of the pencilled starting line and is due to the actual electro-osmotic displacement towards the cathode of the zero-mobility marker, glycerol ($m_N = 0.0$). Mean relative mobilities for plateau regions in the stated pH ranges for some secondary standards are as follows: naphthalene-1,5-disulphonate, pH 1.6–12.4, $m_N = 1.44 \pm 0.04$ (56); Orange G, pH 1.8–9.0, $m_N = 0.72 \pm 0.04$ (32); picric acid, pH 2.9–12.4, $m_N = 0.69 \pm 0.03$ (38); bis(*p*-nitrophenylphosphate), pH 1.6–12.4, $m_N = 0.54 \pm 0.02$ (53). The mean absolute mobility of the *m*-nitrobenzenesulphonate ion over the range pH 1.6–12.4 is 1.4 $(\pm 0.1) \times 10^{-8} m^2 \cdot s^{-1} \cdot V^{-1}$ (56).

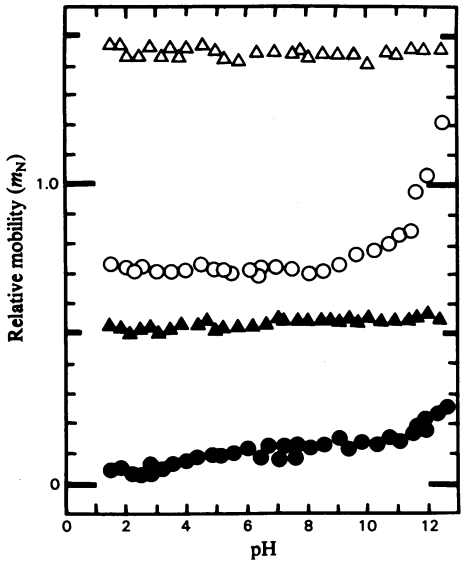

Fig. 1. *pH–relative mobility profiles for some secondary standards in paper electrophoresis and the electro-osmosis correction*

Mobilities (m_N) are expressed relative to *m*-nitrobenzenesulphonate and glycerol as primary standards of unit and zero mobility respectively. A series of anionic buffers with overlapping pK_a values and incorporating an 0.05 M-oxalate concentration as a chelation agent, were used as their potassium salts for the electrolytes. Symbols used: △, naphthalene-1,5-disulphonate; ○, Orange G; ▲, bis(*p*-nitrophenylphosphate); ●, the apparent relative mobility of the pencilled origin due to the actual electro-osmotic migration of the zero-mobility marker towards the cathode.

The choice of appropriate reference standards is arbitrary, but nevertheless certain aspects should be kept in mind. First there should be no change in the ionization state of the standard over the pH range of interest, hence the predominance of strong acids such as the sulphonic acids and di-substituted phosphoric acids as possible candidates for the range above pH 2.0. The case of picric acid deserves special comment; picric acid has a pK_a of −0.33 (Ives & Mosely, 1966), and the error introduced by using it as a primary standard at pH 1.7 (or in other words, 2 pH units above its pK_a, where it is 99% ionized) would seem to be acceptable. However, the remaining 1% protonated picric acid is continuously extracted from the surface of the paper by the solvent-cooled system, which means that it is usually below the level of detection at the end of the run in all buffers with a pH below 2.7.

Much of the preliminary work was done with the 1-phenylazo-2-naphthol-3,5-disulphonate dyestuff Orange G, which has been used in other work (Sanger *et al.*, 1965); however, its significant adsorption to cellulose (R_F 0.64) in aqueous oxalate buffers, together with the ionization of its phenolic hydrogen above pH 9 (Fig. 1), make it less satisfactory than the commercially available *m*-nitrobenzenesulphonate ion (R_F 0.85). The latter compound was chosen after consideration of an earlier suggestion (Frahn & Mills, 1959) to use the less readily available *p*-nitrobenzenesulphonate ion. Similarly much of the preliminary work used fructose as a conveniently detected non-migrating marker for the zero-mobility standard, but as the work was extended to higher pH values, the ionization of the anomeric hydroxy group became apparent and glycerol was substituted for fructose. The seminal work of Frahn & Mills (1959) demonstrated that glycerol is not significantly ionized in 0.1 M-NaOH.

In the course (2 h) of equilibration and electrophoresis, diffusion of most compounds results in a final spot diameter of 0.8 cm. This area (0.5 cm^2) carries approx. 13 μl of buffer solution and, assuming a uniform distribution of solute, the maximum final concentration of solute (other than buffer) is 0.8 mM.

Fig. 2 shows the pH–relative-mobility profiles for adenine, adenosine, AMP, ADP and ATP. The stepwise nature of the ionizations can readily be seen, including the increase in mobility of adenosine

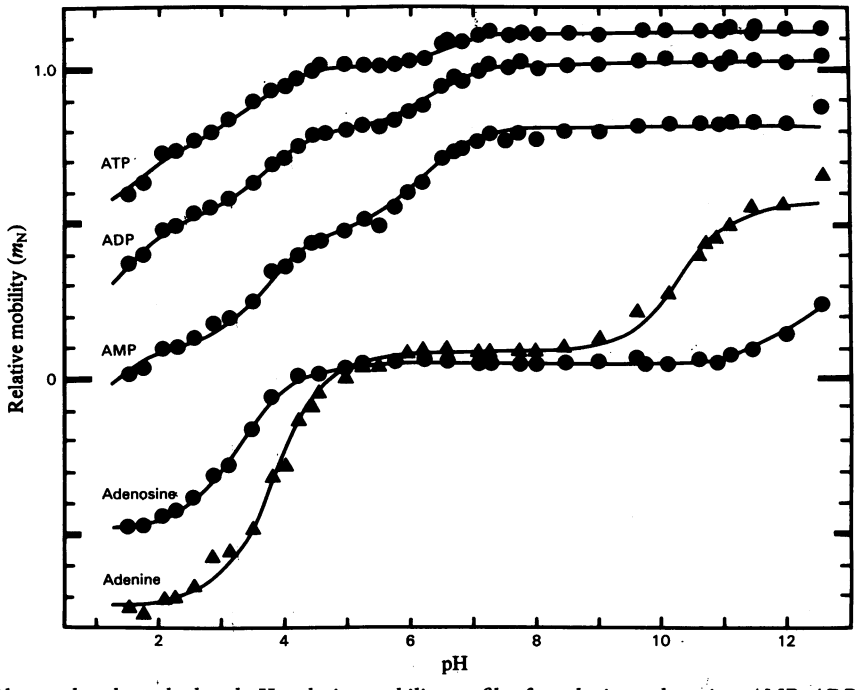

Fig. 2. *Observed and recalculated pH–relative-mobility profiles for adenine, adenosine, AMP, ADP and ATP*
Mobilities (m_N) are expressed relative to *m*-nitrobenzenesulphonate and glycerol as primary standards of unit and zero mobility respectively. A series of anionic buffers with overlapping pK_a values (incorporating 0.05 M-oxalate as a chelation agent) were used as their potassium salts for the electrolytes. Symbols used: ▲, adenine; ●, adenosine, AMP, ADP and ATP. Note that the uncharged species of adenine and adenosine in the range pH 5–9 are not coincident with zero. The curves represent the relative mobilities, recalculated by using eqn. (7) from the Materials and methods section. Single-species relative mobilities and the dimensionless pK_a values derived from the data are listed in Table 2.

above pH 11, which can probably be ascribed to ionization of hydroxy groups in the ribose moiety. It is also clear from the data at pH 12.5 in Table 1 that those nucleosides bearing a 2′-hydroxy group are more readily ionized than the 2′-deoxynucleoside, a point that was also noted in spectrometric studies by Fox & Shugar (1952).

It is noteworthy that, for most species bearing aromatic-ring systems, including the adenine derivatives in Fig. 2, the plateau for the various uncharged species does not coincide with the zero-mobility marker, i.e. glycerol. This is a direct consequence (Frahn & Mills, 1959) of the electro-osmotic flow of buffer towards the cathode and the differing degrees of adsorption of the neutral species to the cellulose. Glycerol (R_F 0.95) in oxalate buffer systems provides a reasonable approximation to a non-adsorbed, non-migrating zero-mobility marker.

Successful control of adsorption of such strongly chelating anions as the inositol phosphates was first achieved by Seiffert & Agranoff (1965) with an oxalate buffer system. It is this feature, coupled with the use of relative, rather than absolute, mobilities, as well as the minimization of capillary flow both during and after electrophoresis, that have combined to provide the extremely reproducible relative mobilities shown in Fig. 2. A previous study (Kitaoka, 1979) of the same compounds demonstrates the difficulties of interpretation as well as the discontinuous nature of pH–absolute-migration-distance profiles.

Apparent pK_a values and relative mobilities (m_N) for the species involved in the ionization may readily be obtained by the application of analogous equations to those used for the determination of ionization constants by spectrophotometry (Albert & Serjeant, 1971). Table 1 shows the application of eqn. (5) to the data for adenosine, 2′-deoxyadenosine and 3′-deoxyadenosine in the pH range 1.5–10.6. First, the mean value of the plateau region (pH 5.5–10.6) corresponding to the uncharged species provides the value of m_1 in eqn. (5); secondly, this value is used to calculate the function $(m_1-\bar{m})/10^{-pH}$ for each pair of pH and net-

Table 1. *Calculation of pK_a values and single species relative mobilities for adenosine, 2'-deoxyadenosine and 3'-deoxyadenosine*

Data from the plateau region (pH 5.5–10.6) provides the uncharged species means (m_1) for adenosine [0.047 ± 0.01 (16)], 2'-deoxyadenosine [0.043 ± 0.01 (16)] and 3'-deoxyadenosine [0.035 ± 0.01 (16)]; these values are then used for a least-squares solution of the linear eqn. (5) from the text and by the use of the net mobilities (m_N) as ordinate values and the calculated function $10^{pH}(m_1 - m_N)$ as abscissa values for each pH over the range pH 1.51–4.0. Data for pH 5.4 are excluded because the difference ($m_1 - m_N$) is less than three times the S.D. in the plateau region. The correlation coefficients, the relative mobility of the protonated species (m_0) and the pK_a values were as follows: adenosine $r^2 = 0.98$, $m_0 = -0.46 \pm 0.01$ (10), $pK_a = 3.40 \pm 0.02$ (10); 2'deoxyadenosine, $r^2 = 0.98$, $m_0 = -0.48 \pm 0.01$ (10), $pK_a = 3.52 \pm 0.02$ (10); 3'-deoxyadenosine, $r^2 = 0.95$, $m_0 = -0.47 \pm 0.02$ (10), $pK_a = 3.59 \pm 0.04$ (10). These values have been inserted in the simplest form of eqn. (7) to recalculate the predicted mobilities (m_p) shown. The restricted data above pH 10.6 could be similarly treated with eqn. (6), but the limited ionization of the deoxynucleosides to yield anions would introduce large errors. The predicted data (pH 12.0–12.5) for adenosine were obtained by using data from Table 2.

	Adenosine		2'-Deoxyadenosine		3'-Deoxyadenosine	
pH	m_N	m_p	m_N	m_p	m_N	m_p
1.51	−0.47	−0.46	−0.49	−0.47	−0.51	−0.47
1.51	−0.49	−0.46	−0.50	−0.47	−0.50	−0.47
2.04	−0.44	−0.44	−0.47	−0.46	−0.47	−0.46
2.04	−0.45	−0.44	−0.46	−0.46	−0.46	−0.46
3.08	−0.28	−0.30	−0.32	−0.34	−0.33	−0.35
3.08	−0.29	−0.30	−0.33	−0.34	−0.32	−0.35
3.50	−0.16	−0.18	−0.23	−0.22	−0.22	−0.24
3.50	−0.17	−0.18	−0.21	−0.22	−0.24	−0.24
4.00	−0.06	−0.06	−0.09	−0.09	−0.12	−0.10
4.00	−0.06	−0.06	−0.09	−0.09	−0.11	−0.10
4.54	0.00	0.01	0.0	0.0	−0.01	−0.01
4.54	0.01	0.01	0.01	0.0	−0.01	−0.01
5.48	0.03	0.04	0.04	0.04	0.02	0.03
5.48	0.04	0.04	0.03	0.04	0.02	0.03
5.95	0.06	0.05	0.07	0.04	0.06	0.04
5.95	0.06	0.05	0.06	0.04	0.06	0.04
6.50	0.03	0.05	0.03	0.04	0.02	0.04
6.50	0.03	0.05	0.03	0.04	0.02	0.04
7.11	0.05	0.05	0.04	0.04	0.04	0.04
7.11	0.05	0.05	0.04	0.04	0.04	0.04
7.55	0.03	0.05	0.04	0.04	0.03	0.04
7.55	0.03	0.05	0.04	0.04	0.03	0.04
8.46	0.04	0.05	0.04	0.04	0.03	0.04
8.46	0.05	0.05	0.04	0.04	0.03	0.04
9.63	0.06	0.05	0.06	0.04	0.05	0.04
9.63	0.07	0.05	0.06	0.04	0.05	0.04
10.63	0.06	0.05	0.03	0.04	0.03	0.04
10.63	0.06	0.05	0.03	0.04	0.03	0.04
12.00	0.14	0.16	0.07	—	0.07	—
12.00	0.14	0.16	0.07	—	0.07	—
12.54	0.23	0.22	0.09	—	0.12	—
12.54	0.24	0.22	0.09	—	0.12	—

relative-mobility ($m_N = \bar{m}$) values in the range pH 1.5–4.5. Finally, a plot of m_N versus the function $(m_1 - m_N)/10^{-pH}$ will provide the apparent ^BK_a and the relative mobility (m_0) of the fully protonated species as the slope and intercept of the linear plot. Preferably a least-squares solution of the data will, in addition, provide a correlation coefficient and an error for m_0. A programmable calculator simplifies the calculations and provides a very simple means for checking the goodness-of-fit to the data by recalculating the predicted mobilities (m_p) with the appropriate form of eqn. (7). Note that $pK_a = -\log {}^BK_a$.

Table 2 summarizes the pK_a and relative-mobility data for single ionic species that have been obtained by this method. It is worthwhile to consider the units applicable to such pK_a values. By definition, the relative-mobility data are clearly dimensionless, and likewise the operational definition of the pH scale (Bates, 1968) is also dimensionless, hence any value

Table 2. *Summary of pK_a values and single-species relative mobilities obtained by paper electrophoresis*
Errors and correlation coefficients (r) are derived from least-squares fits to eqns. (4), (5) or (6). Questionable data for AMP without error values was obtained by an iterative fit of eqn. (7) to minimize the residual error. For comparison, the apparent ionization constants obtained potentiometrically at 25°C and I0.15 by Alberty *et al.* (1951) are listed (Lit. pK_a). Dimensionless pK_a values obtained by electrophoresis in this Table were from data measured at 20–25°C. I was 0.1–0.5, and the concentration of the measured components was less than 0.8 mM.

Compound	Species net charge	m_N	pK_a	r	Lit. pK_a
Adenine	+1	−0.73 ± 0.01 (9)	3.82 ± 0.04 (9)	0.96	4.12
	0	0.09 ± 0.02 (20)	10.37 ± 0.04 (7)	0.96	9.75
	−1	0.57 ± 0.01 (7)			
Adenosine	+1	−0.47 ± 0.01 (10)	3.40 ± 0.02 (9)	0.98	3.63
	0	0.05 ± 0.01 (16)	12.0 ± 0.1 (4)	0.92	
	−1	0.27 ± 0.03 (4)			
2′-Deoxyadenosine	+1	−0.48 ± 0.01 (10)	3.52 ± 0.02 (10)	0.98	
	0	0.04 ± 0.01 (16)			
3′-Deoxyadenosine	+1	−0.47 ± 0.02 (10)	3.59 ± 0.04 (10)	0.95	
	0	0.04 ± 0.01 (16)			
5′-AMP	+1	−0.4 ± ? (3)	1.7 ± ? (3)		
	0	0.09 ± 0.01 (20)	3.68 ± 0.02 (10)	0.99	3.74
	−1	0.50 ± 0.02 (20)	6.17 ± 0.20 (8)	0.92	6.05
	−2	0.82 (8)			
3′:5′-Cyclic AMP	0	0.04 ± 0.01 (10)	3.62 ± 0.02 (10)	0.99	
	−1	0.47 ± 0.01 (14)			
ADP-glucose	−1	0.36 ± 0.01 (10)	3.93 ± 0.04 (10)	0.94	
	−2	0.65 ± 0.02 (10)			
Agrocin 84	−1	0.36 ± 0.02 (6)	3.26 ± 0.06 (6)	0.94	
	−2	0.60 ± 0.02 (15)			
5′-ADP	0	0.18 ± 0.08 (4)	1.5 ± 0.2 (4)	0.8	
	−1	0.53 ± 0.01 (16)	3.87 ± 0.06 (9)	0.9	3.95
	−2	0.82 ± 0.01 (16)	6.28 ± 0.04 (7)	0.9	6.26
	−3	1.02 ± 0.02 (40)			
5′-ATP	−1	0.51 ± 0.06 (4)	1.80 ± 0.4 (4)	0.6	
	−2	0.77 ± 0.01 (7)	3.52 ± 0.04 (7)	0.96	4.0
	−3	1.01 ± 0.01 (8)	6.5 ± 0.2 (10)	0.5	6.48
	−4	1.12 ± 0.01 (41)			

derived from pH and relative-mobility values alone is also dimensionless. Numerically the values obtained are in reasonable proximity to those obtained in the similar ranges of temperature (20–25°C) and ionic strength (I 0.1–0.5) by more conventional procedures, as shown in Table 2.

Limitations

The method is undoubtedly time-consuming and in part this stems from the relatively low voltage gradients that are used to minimize the temperature rise in the cooling solvent. On the other hand, the 0.5 h capillary-equilibration time at the beginning of each electrophoresis cannot be decreased further without seriously jeopardizing the reproducibility of the relative mobilities. There is a continuous change in ionic strength from approx. 0.1 at the beginning to 0.5 at the end of the pH scale, but as the shape of the curves in Fig. 2 demonstrates, this does not result in any serious changes in adsorption to the cellulose support, although there is almost certainly an effect on the individual pK_a values. It is possible that the ionic-strength changes could be estimated, and compensated for, by the addition of a background electrolyte such as KNO_3, but the improvement in accuracy is unlikely to be worth the additional effort, in view of the fact that there still remains a degree of uncertainty as to the actual temperature of the buffer on the paper, a factor that will also affect significantly the pK_a values.

The most serious source of error becomes apparent when attempts (Table 2) are made to obtain pK_a values for two species whose relative mobilities approach to within 0.1 relative-mobility unit. The final deprotonation step of ATP, with a pK_a near 6.5 and a correlation coefficient of only 0.5, provides a relevant example. In cases such as this, iterative adjustment of pK_a values in the relative-mobility predictor eqn. (7) can be used to minimize the residual error and thereby improve the goodness-of-fit, but unless the mobility differences used in eqns. (4), (5) and (6) are greater than three times the S.D. in the plateau regions, the derived pK_a values are of poor precision.

Advantages

A signal advantage of the method lies in its ability to yield reproducible apparent pK_a values of biologically active materials by using minute quantities of crude extracts from natural products. These pK_a values may then be used to devise effective isolation procedures, such as for the nucleotide bacteriocin agrocin 84 (Roberts *et al.*, 1977). The utility of these dimensionless pK_a values lies not in their approximate relationship to published pK_a values, but rather by virtue of their simultaneous determination, to the ease with which they may be used to rank the acid strength of labile compounds such as the deoxynucleosides and nucleoside diphosphate sugars. The pH–mobility profiles also provide a sensitive and useful criterion for identity in the comparison of natural and synthetic samples.

Significant differences in the relative mobility of closely related single ion species of the same charge type can be useful indicators of substantial size or shape differences between the ions. Thus ADP-glucose and agrocin 84 have mobilities of 0.65 ± 0.02 and 0.60 ± 0.02 respectively for the species of charge -2. Both compounds contain adenine, glucose and phosphorus in the ratio 1:1:2, but the lower mobility of the agrocin 84 dianion indicated the likely presence of another substituent, which was later identified (Tate *et al.*, 1979) as a 2,4-dihydroxy-4-methylpentanamide.

In conclusion, it should be noted that with a suitable choice of reference compounds, buffers and conditions, the procedure should also be applicable to the determination of stability constants for other equilibria such as the borate complexes (Michl, 1952; Frahn & Mills, 1959) and calcium complexes (Mills, 1961; Angyal & Mills, 1979) of carbohydrates.

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References

Albert, A. & Serjeant, E. P. (1971) *The Determination of Ionization Constants*, 2nd edn., pp. 44–59, Chapman and Hall, London

Alberty, R. A., Smith, R. M. & Bock, R. M. (1951) *J. Biol. Chem.* **193**, 425–434

Angyal, S. J. & Mills, J. A. (1979) *Aust. J. Chem.* **32**, 1993–2001

Bates, R. G. (1968) in *C.R.C. Handbook of Biochemistry: Selected Data for Molecular Biology* (Sober, H. A., ed.), pp. J190–J194, The Chemical Rubber Co., Cleveland, OH

Consden, R., Gordon, A. H. & Martin, A. J. P. (1946) *Biochem. J.* **40**, 33–41

Fox, J. J. & Shugar, D. (1952) *Biochim. Biophys. Acta* **9**, 369–384

Frahn, J. L. & Mills, J. A. (1959) *Aust. J. Chem.* **12**, 65–89

Ives, D. J. D. & Mosely, P. G. N. (1966) *J. Chem. Soc. B*, 757–761

Kitaoka, Y. (1979) *J. Chromatogr.* **168**, 241–245

Kraus, K. A. & Smith, G. W. (1950) *J. Am. Chem. Soc.* **72**, 4329–4330

MacWilliams, E. (1956) in *Techniques of Organic Chemistry*, 2nd edn. (Weissberger, A., ed.), vol. 3, part 1, p. 127, Interscience Publishers, New York and London

Markham, R. & Smith, J. D. (1951) *Nature (London)* **168**, 406–408

Michl, H. (1952) *Monatsh. Chem.* **83**, 737–747

Mills, J. A. (1961) *Biochem. Biophys. Res. Commun.* **6**, 418–421

Roberts, W. P., Tate, M. E. & Kerr, A. (1977) *Nature (London)* **265**, 379–381

Sanger, F., Brownlee, C. G. & Barrell, B. G. (1965) *J. Mol. Biol.* **13**, 373–398

Seiffert, U. B. & Agranoff, B. W. (1965) *Biochim. Biophys. Acta* **98**, 574–581

Shaw, D. J. (1969) *Electrophoresis*, pp. 99–103, Academic Press, London and New York

Tate, M. E. (1968) *Anal. Biochem.* **23**, 141–149

Tate, M. E., Murphy, P. J., Roberts, W. P. & Kerr, A. (1979) *Nature (London)* **280**, 697–699

Trevelyan, W. E., Proctor, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445

Weiss, J. B. & Smith, I. (1967) *Nature (London)* **215**, 638