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Determination of dissociation constants of cytokinins by capillary zone electrophoresis ☆

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Abstract

A method for the pK_a determination, based on mobility data measured by capillary zone electrophoresis, was applied to cytokinins and their analogs. The combination of charged mobility standards with an uncharged electroosmosis marker, injected in the uncoated capillary simultaneously with the measured substances, allows one to minimize the number of runs, reduce their duration and, in addition, to inform on the run-to-run stability of electroosmosis and on contingent side-effects. pK_a values of investigated cytokinins and their analogs ranged from 2.8 to 4.0 at 25°C in the phosphate and acetate buffers of ionic strength 0.015 *M*. Standard deviations of the constants, obtained by the non-linear fitting of equations for the pK_a calculation, were 3–5-times lower than standard deviations from the linear fitting or from the point-to-point calculation utilizing the Hendersson–Haselbalch equation. The equation of Boltzman sigmoid offers two checks on reliability of effective mobilities that serve as the raw data in the pK_a calculation. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Dissociation constants; Cytokinins

1. Introduction

Physiologically active substances are topical research subjects of modern biology, molecular biology, medicine, physiology, biochemistry and, consequently, of analytical chemistry. The phytohormones cytokinins support cell division, bud formation and herbaceous stem ramification. Some structural analogs of cytokinins exhibit cytostatic efficiency and are studied as potential drugs. The cytokinins and their analogs are either adenine or adenosine derivatives with the 6-amine group substituted by an aryl or alkyl (Table 1). The resulting secondary amine group gives weakly basic properties to the compounds.

The knowledge of pK_a values of cytokinins and their analogs is helpful both in their biochemical or pharmacological research and in the development of electrophoretic methods for the analysis of the compounds. The determination of pK_a values of cytokinins by established classical methods, tit-

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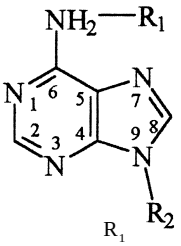
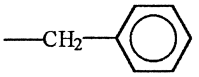
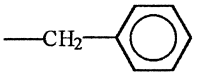
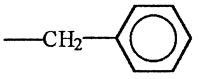
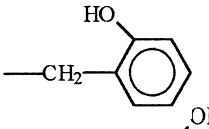
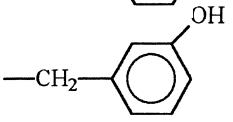
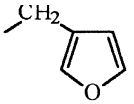
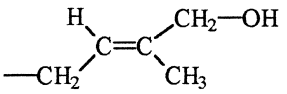
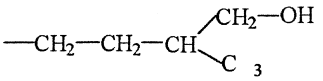
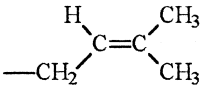
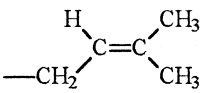
Table 1
Studied compounds



Abbreviation	Name	R_1	R_2
BAP	6-Benzylaminopurine		-H
BAPR	6-Benzylaminopurinriboside		*O*-Ribofuranosyl
BAPG	6-Benzylaminopuringlucoside		*O*-Glucopyranosyl
OTR	*o*-Topolinriboside		*o*-Ribofuranosyl
MTR	*o*-Topolinriboside		*o*-Ribofuranosyl
KR	Kinetinriboside		*o*-Ribofuranosyl
TZR	*trans*-Zeatinriboside		*o*-Ribofuranosyl
DHZR	Dihydrozeatinriboside		*o*-Ribofuranosyl
IPA	Isopentenyladenine		-H
IPR	Isopentenyladenosine		*o*-Ribofuranosyl

rimetry and photometry [1–3] meets difficulties: (1) the solubility of the compounds in water is seldom high enough to meet the needs of titrimetry and photometry. (2) Inexpressive changes in optical spectra of the compounds linked with their charging deteriorate both the accuracy and precision of the photometric pK_a determination. (3) Low purity is the general weakness of all laboratory preparations of cytokinins. (4) Commercially available cytokinins and their analogs are expensive.

Modern electrophoretic methods are potent enough to overcome all of these difficulties [4,5]. Mobility changes caused by the varying pH, ranging from zero to the ionic mobility of the compound, are large enough and may be measured with high precision. Purity of the used compound preparation is never the problem because of spontaneous purification of its zone during the electrophoretic transport. Capillary zone electrophoresis (CZE) is preferred generally in the measurement of input data because of its experimental simplicity and easier calculation of pK_a values, in comparison to isotachophoresis [5]. The investigated compounds are injected in submillimolar concentrations that are lower by a few orders of magnitude than the concentrations necessary for, e.g., titrimetric pK_a measurements.

Cytokinins and their analogs are compounds of very low basicity and their pK_a values have never been measured before. The aims of this study are to extend the CZE method for the pK_a determination [4–9] to these compounds and to other singly charged very weak bases of very low solubility, to make the method more precise and less laborious, and to compare pK_a data from different calculation procedures.

2. Theoretical

The equilibrium:

$$BH^+ \leftrightarrow B + H^+ \tag{1}$$

describes the dissociation of cytokinins and other monovalent bases. The stoichiometric dissociation constant, K_a, relevant to it, may be defined by equilibrium concentrations of the species BH^+, B and H^+, $[BH^+]$, [B] and $[H^+]$, respectively, that are expressed usually in *M*.

$$K_a = \frac{[H^+][B]}{[BH^+]} \tag{2}$$

In buffered systems that are standard in electrophoresis, $[H^+]$ is identical to the total concentration of protons in the background electrolyte, c_H. However, the experimentally measurable pH value is the negative decadic logarithm of the (relative) activity of the hydrogen ion in the solution, a_H, which, for concentrations expressed in the molarity scale, is [10]:

$$pH = -\log \frac{c_H y_H}{c_H^0} = -\log \frac{[H^+] y_H}{c_H^0} \tag{3}$$

where y_H is the single ion activity of the hydrogen ion, c_H^0 is an arbitrary constant representing the standard state condition and equal to 1 *M* [11]. In order to keep the consistency of quantities in Eq. (2), the corresponding activities have to be substituted for [B] and $[BH^+]$, too. By analogy with the hydrogen ion activity, expressions for the equilibrium activities of deprotonated and protonated forms of the base, a_B, a_{BH}, respectively, may be written:

$$a_B = \frac{[B] y_B}{c_B^0} \tag{4a}$$

$$a_{BH} = \frac{[BH^+] y_{BH}}{c_{BH}^0} \tag{4b}$$

Using these activity expressions, the equation correlating the equilibrium concentrations of interacting species H^+, BH^+ and B, $[H^+]$, $[BH^+]$ and [B], respectively, with the thermodynamic dissociation constant, K_a^{th}, is obtained [12]. Thus, with respect to the meanings of c_H^0 [11], c_B^0 and c_{BH}^0, which are equal to 1 *M*, the equation for the dimensionless K_a^{th} has the form:

$$K_a^{th} = \frac{[H^+][B]}{[BH^+]} \cdot \frac{c_{BH}^0}{c_H^0 c_B^0} \cdot \frac{y_H y_B}{y_{BH}} = K_a \cdot \frac{c_{BH}^0}{c_H^0 c_B^0} \cdot \frac{y_H y_B}{y_{BH}} \tag{5}$$

The activity of uncharged B, y_B, is generally considered to be 1 [4,5]. Consequently, pK_a^{th} is:

$$pK_a^{th} = pH - \log \frac{[B]}{[BH^+]} + \log y_{BH} \quad (6)$$

because $c_{BH}^0/c_H^0 = 1$.

The activity of a single ion of valency, z, and of the effective hydrated diameter, a, given in nanometers, in the solution of low ionic strength, I, given in mol/l, is [4,5]:

$$-\log y = \frac{0.5085 z^2 \sqrt{I}}{1 + 3.28 a \sqrt{I}} \quad (7)$$

Hydrated diameters of ions are seldom known. The approximate value of $a = 0.5$ nm is therefore recommended for the calculation of y as the generally applicable approximation [4,5].

According to the Tiselius′ concept, the effective mobility of a species present in solution in i coexisting forms, bound each to the other by fast equilibria, u_{eff}, is [13,14]:

$$u_{eff} = \sum_i u_i x_i \quad (8)$$

u_i is the ionic mobility of the ith form of the species and x_i is its molar fraction. Using this concept, the effective mobility of the monovalent base, B, is:

$$u_{eff} = xu = \frac{[BH^+]}{[B] + [BH^+]} u \quad (9)$$

Here, u is the ionic mobility of the protonated base and x is its equilibrium molar fraction in solution at the used pH. Eq. (9), rearranged to the form:

$$\frac{[B]}{[BH^+]} = \frac{u_{eff}}{u - u_{eff}} \quad (10)$$

and substituted to Eq. (6), gives the Hendersson–Haselbalch equation (Eq. (11)) which correlates pK_a^{th} with the experimentally accessible data, pH, u and u_{eff}:

$$pK_a^{th} = pH - \log \frac{u_{eff}}{u - u_{eff}} + \log y_{BH} \quad (11)$$

The pH dependence of the effective mobility of a weak base has the form of its titration curve (Fig. 1) [14]. The form of the titration curve is identical to that of the Boltzman sigmoid as may be evidenced easily by inserting Eq. (5) into Eq. (9). The resulting equation:

$$u_{eff} = \frac{u}{1 + \frac{K_a^{th}}{[H^+]}} = \frac{u}{1 + 10^{(pH - pK_a)}} \quad (12)$$

is a particular case of the Boltzman sigmoid having the most general mathematical form:

$$y = \frac{a}{b + \exp\left(\frac{x + c}{d}\right)} + e \quad (13)$$

Here, a, b, c, d and e are constants. By applying Eq. (13) to a mobility shift, caused by the pH controlled splitting off of one proton from a protonated base, the Boltzman sigmoid equation has the form:

$$u_{eff} = \frac{(u - u_0)}{1 + \exp \frac{pH - pK_a^{th}}{d pH}} + u_0 \quad (14)$$

where u, u_0 are the mobilities of the highly and less protonated forms of the base, respectively. Evidently, $u_0 = 0$ for the completely deprotonized base.

The Boltzman sigmoid is therefore an alternative of the Hendersson–Haselbalch equation for the computer fitting of experimental data and Eq. (14) is its most suitable form. In addition to the pK_a^{th}, the limiting mobilities of the highly and less protonated forms, u and u_0, respectively, result from the fitting of Eq. (14). Thus, a check on the reliability of the experimental raw data is possible if u is accessible experimentally, too. The slope of the tangent in the inflect point of the Boltzman sigmoid is:

$$k = -\frac{u - u_0}{4 d pH} \quad (15)$$

The comparison of this value of the slope with the theoretical slope:

$$k = -\frac{u}{4 \log e} \approx -\frac{u}{1.7372} \quad (16)$$

offers another possibility to check the suitability of raw data. The necessity to measure four experimental points in the pH-mobility dependence for the fitting with the Eq. (14), supposing $u_0 \neq 0$ is not sufficient

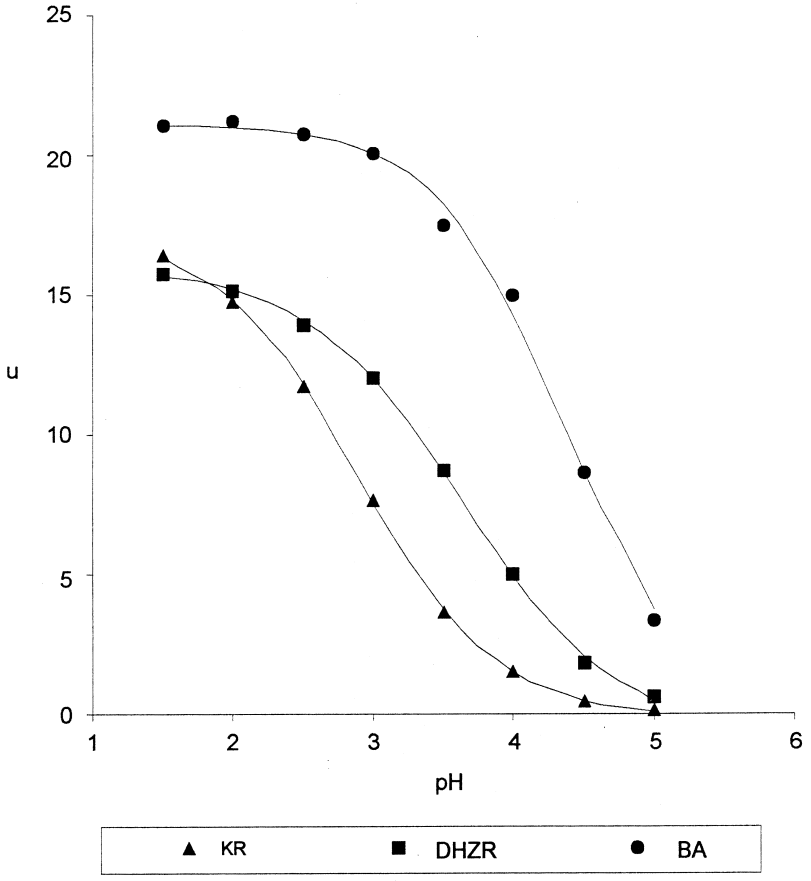


Fig. 1. pH dependencies of effective mobilities measured with kinetinriboside (KR), dihydrozeatinriboside (DHZR) and isopentenyladenine (IPA).

from the statistical point of view. At least six experimental points are recommended.

3. Experimental

A Spellman CZE 1000R high-voltage power supply (Plainview, NY, USA) and a Jasco 875 UV–Vis spectrophotometer (Tokyo, Japan) were the main parts of the laboratory set-up used for electrophoretic experiments. The detector was equipped with a laboratory-made capillary holder allowing temperature control of the capillary by water, supplied by the water bath U 15^{C} thermostat (Prüfgeräte-Werk Medingen, Germany). The water temperature, set at 24.5°C, fluctuated within ±0.1°C. The driving voltage was adjusted according to the background electrolyte conductivity. Joule heat production inside the capillary of approximately 0.075 W, was kept approximately constant except for the phosphate buffer, pH 1.5 (Table 2). An uncoated thin-wall fused-silica capillary (Capillary Columns, Bratislava, Slovak Republic) of 50 cm (40 cm separation distance)×50 μm I.D.×100 μm O.D. was used. Quartz lenses, sandwiching the capillary in the holder, increased the intensity of the measuring light beam, passing through the 0.3-mm detection window, up to 50% intensity of the reference light beam. The detector output was processed by the CI 105 integrator and the TZ 4200 strip chart recorder (Laboratory Instruments, Prague, Czech Republic).

2-[*N*-Morpholino]ethanesulfonic acid (MES), 6-

Table 2
Used buffers[a]

pH	Buffering acid	*C* (mmol/l)	*U* (kV)	*P*[b] (W)
1.5	Phosphoric acid	200	6	0.15
2.0	Phosphoric acid	37	7.5	0.07
2.5	Phosphoric acid	20	12	0.08
3.0	Phosphoric acid	17	12	0.07
3.5	Acetic acid	288	15	0.08
4.0	Acetic acid	100	15	0.07
4.5	Acetic acid	42	15	0.07
5.0	Acetic acid	24	15	0.08
5.5	Acetic acid	18	15	0.08
5.5	MES	75	15	0.07
6.0	MES	34	15	0.06

[a] Common cation of the buffers: Tris. Ionic strength $I=0.015$ *M*, except for the phosphate buffer, pH 1.5 having $I=0.035$ *M*).
[b] Total power input to the capillary.

benzylaminopurine (BAP), 6-benzylaminopurin-riboside (BAPR), 6-benzylaminopuringlucoside (BAPG), kinetinriboside (KR), *trans*-zeatinriboside (TZR), dihydrozeatin riboside (DHZR), isopentenyladenine (IPA), and isopentenyladenosine (IPR) (Table 1) were from Sigma–Aldrich; new cytokinin derivatives, *o*-topolinriboside (OTR) and *m*-topolinriboside (MTR) were synthesized [15] in the Institute of Experimental Botany, the Academy of Sciences of the Czech Republic (Olomouc, Czech Republic). Compounds serving as charged mobility standards (benzylamine, brucine) and the buffer constituents (Table 2) except for MES were purchased from Lachema (Brno, Czech Republic). Mesityl oxide was from Merck.

Buffers (Table 2) were prepared from weak acids dissolved in freshly boiled distilled water. The pH of each buffer was adjusted by Tris using the CORNING 240 pH meter (Corning Glass Works, Corning, NY, USA) and the Radelkis 0808P electrode. The ionic strength (I) of the buffers was 0.015 *M*; except for the phosphate buffer which had $I=0.035$ *M*.

A fresh separation capillary was activated by 0.1 *M* NaOH overnight, flushed by distilled water for 1 min and by the buffer for 1 h at least (usually overnight) up to the rough leveling off of the electroosmosis. Then, it was conditioned for 30 min using a voltage of 5–10 kV, lower than that used in the respective runs with the buffer, and flushed with the buffer again for 3 min. Between runs, the capillary was washed with the buffer for 3 min. Before any change of the buffer, the capillary was cleaned and reactivated with 0.1 *M* NaOH for 10 min. Then, electroosmosis was stabilized using the described flushing procedure. The stabilized capillary was stored overnight in the buffer; for a longer storing period, distilled water was used.

The injected samples, mixed just before measurements, consisted of 10 μl of a stock solution of cytokinins (solution I) and of 15 μl of a stock solution of mobility standards (solution II). Solution I was prepared by dissolution of two or three cytokinins, selected from the set of investigated compounds and having sufficiently different mobilities, in water at concentrations of approximately $1\cdot10^{-3}$ *M* each. No decomposition of both stock solutions of cytokinins and injected samples was observed during the measurement of raw mobility data. Solution II, prepared for each of the buffers separately, contained $5\cdot10^{-4}$ *M* benzylamine, $5\cdot10^{-4}$ *M* brucine and $5\cdot10^{-2}$ *M* mesityl oxide in the buffer–water (1:1) mixture. The samples were introduced into the capillary by siphoning for 15 s; the capillary inlet–outlet difference was 14.4 cm. Mobility data, corrected for electroosmosis, are expressed in 10^{-9} m^2 V^{-1} s^{-1} units.

4. Results and discussion

Electrophoretic determination of dissociation constants of weak electrolytes [4–8] is, in fact, the particular case of determination of equilibrium constants in solution [12]. The knowledge from the investigation of electrophoretic determination of stability constants, e.g., Ref. [16], called alternatively association [17] or binding constants [18–21], are thus relevant to the determination of dissociation constants, too. The fact that the input experimental data are decisive for the accuracy of the calculated constant if a correct theoretical model and a proper calculation procedure are used [12], is of primary importance. pK_a values of cytokinins have never been measured before. However, based on pK_a data of related compounds, they are expected to drop below 3 for some of them. Thus, the pH of the buffers used in the measurements of effective mo-

Table 3
The comparison of effective mobilities of kinetinriboside calculated from Eqs. (17–19)

pH	Eq. (17)[a]		Eq. (18)[b]		Eq. (19)[c]
	u	SD[d]	u	SD[d]	u
2.0	14.77	0.247	14.41	0.007	14.42
2.5	11.76	0.035	11.67	0.028	11.71
3.0	7.64	0.017	7.30	0.170	7.54
3.5	3.60	0.021	3.52	0.078	3.63
4.0	1.52	0.007	1.17	0.240	1.51
4.5	0.43	0.000	0.17	0.184	0.43
5.0	0.15	0.007	−0.15	0.205	0.14

[a] Mobility standard: mesityl oxide.
[b] Mobility standards: benzylamine, brucine.
[c] Mobility standards: brucine, mesityl oxide.
[d] With respect to data calculated from Eq. (19).

bilities of cytokinins has to range from 2 to 5 at least. Buffers of constant ionic strength (Table 3) were used in order to eliminate the influence of different ionic strengths on raw effective mobilities [14]. The buffers of ionic strength 0.015 *M* were selected as a reasonable compromise between the necessity to minimize the Joule heat production inside the capillary and the requirement for sufficient buffering capacity [7]. In order to minimize electric conductivity of the buffers, weak acids producing univalent anions were used and the Tris cation was preferred to the sodium cation. Phosphate buffer, pH 1.5 was included in the buffer set in order to make sure that an unexpected additional protonation of the investigated bases was absent in strongly acidic media. The ionic strength of this buffer exceeded 0.015 *M* (Table 2). Thus, mobilities measured in phosphate buffer, pH 1.5 were not included in the raw data for the calculation of dissociation constants.

In order to keep the temperature inside the capillary constant, the driving voltage was adjusted according to electric conductivities of the buffers. The temperature increase in the capillary, estimated from the mean Joule heat input 0.075 W and from the capillary parameters [22], was approximately 0.5°C. The standard temperature 25°C inside the capillary was approximated by setting the temperature of the thermostatic water to 24.5°C.

4.1. Determination of raw mobilities

The necessity to measure effective mobilities approaching zero (Fig. 1) and to utilize the highly acidic background electrolytes preclude to use the coated capillary. The common correction for electroosmosis, based on an uncharged electroosmosis marker, was completed with the correction method utilizing two mobility standards [23]. Mesityl oxide served as the uncharged electroosmosis marker, benzylamine ($pK_a = 9.33$) and brucine ($pK_a = 8.28$) [24] were selected as the charged high- and low-mobility standards (Fig. 2), respectively. Their ionic mobilities, measured at 25°C in the phosphate, acetate and MES buffers of $I = 0.015$ *M* using mesityl oxide as the electroosmosis marker, were independent of compositions of these buffers. The mean values of mobilities of benzylamine and brucine were 30.40 ± 0.03 and $17.01 \pm 0.02 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}, respectively.

The simultaneous injection of two, constantly charged mobility standards and of an uncharged electroosmosis marker with cytokinins allowed one to compare three methods for electroosmosis correction. (i) Correction based on the migration time of the electroosmosis marker only, (ii) correction utilizing the electroosmosis marker and one charged mobility standard and (iii) correction based on two charged mobility standards.

The equation:

$$u_x = \left(\frac{1}{t_x} - \frac{1}{t_0}\right) \cdot \frac{Ll}{V} \tag{17}$$

supplies mobilities corrected for electroosmosis. The total length of the capillary, L, its migration distance, l, and the driving voltage, V, have to be known precisely in addition to the migration times of the compound, t_x, and of the electroosmosis marker, t_0. Random changes in experimental conditions as well as small systematic deviations from the expected ones are not corrected for. Thus, even consecutive injections cannot secure raw data of the highest precision if they are calculated from markedly different t_0 and t_x values (Table 3).

The analyte mobility, u_x, may be obtained from its migration time, t_x, using migration times t_A, t_B, and mobilities u_A, u_B of mobility standards A, B, respectively [23] disregarding the presence or the absence of electroosmosis during the run (method iii):

$$u_x = u_A + (u_B - u_A) \cdot \frac{t_B}{t_A - t_B} \cdot \frac{t_A - t_x}{t_x} \tag{18}$$

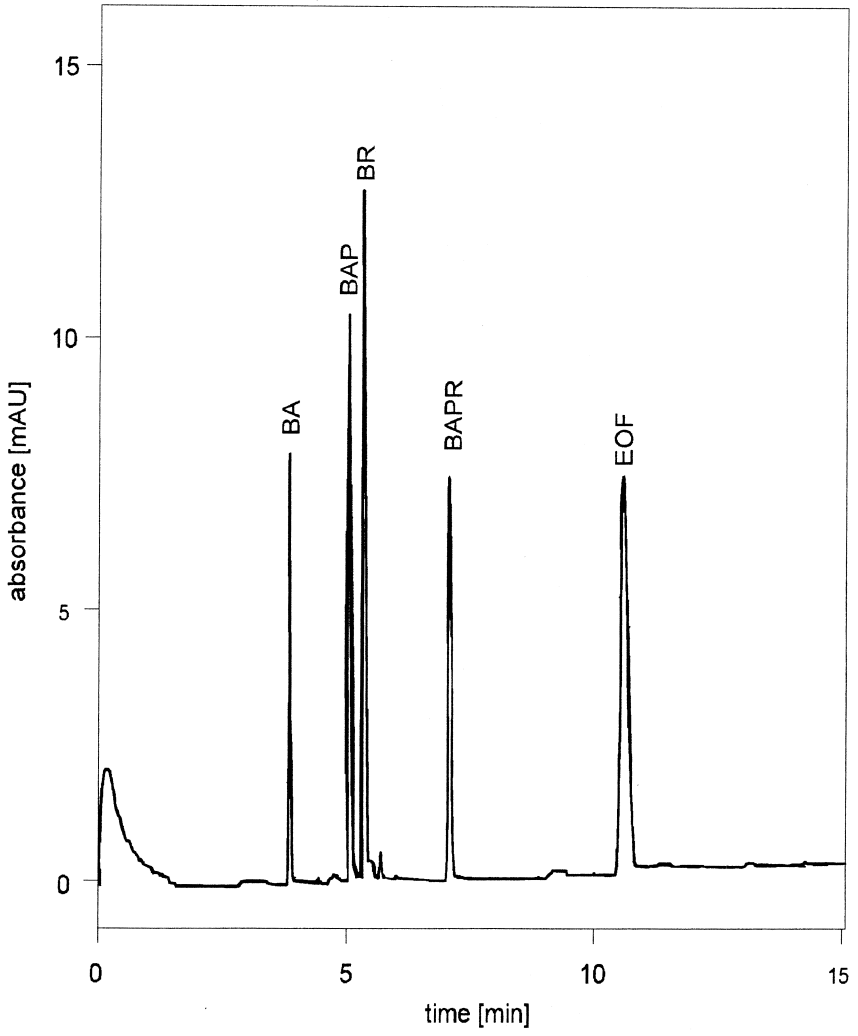


Fig. 2. Typical record of the measurement of effective mobilities of cytokinins. Buffer: 0.017 *M* Tris–phosphate, pH 3.0 (I=0.015 *M*). Cytokinins: BAP=6-benzylaminopurine; BAPR=6-benzylaminopurineriboside. Mobility standards: BA=benzylamine; BR=brucine; EOF=electroosmosis marker (mesityl oxide).

If the charged mobility standard B is replaced by an uncharged electroosmosis marker, (method ii) Eq. (18) has the form:

$$u_x = u_A - u_A \cdot \frac{t_0}{t_A - t_0} \cdot \frac{t_A - t_x}{t_x} \quad (19)$$

Eqs. (18) and (19) avoid the necessity to know the used voltage and the capillary dimensions. In addition, they suppress the influences of random changes, instabilities and small deviations in experimental conditions on calculated mobilities [23]. It allows cutting down the number of consecutive sample injections to two injections only as a rule supposing that electroosmosis is constant during the runs. However, the necessity to inject two mobility standards whose mobilities are pH-independent in the used pH range, sufficiently different each to other and known precisely is the issue. Eqs. (18) and (19) supply the best data if the unknown mobility u_x is within the range given by mobilities of the standards A and B, u_A, u_B, and close to one of them. Thus, effective mobilities approaching zero, calculated

from Eq. (18), suffer from increasing errors and may be even senseless (Table 3). Eq. (19) is therefore more suitable for effective mobilities approaching zero. Consequently, injection of at least one charged mobility standard and an electroosmosis marker is recommended from the viewpoints of precision of measured effective mobilities and experimental demands. Total analysis time, given by the migration time of electroosmosis, ranged from 10 to 20 min in our experiments (Fig. 2).

There are two additional benefits associated with the use of at least one charged mobility standard and an uncharged electroosmosis marker. Constant electroosmosis, evidenced by the absence of the systematic shift of t_0, is necessary for correct mobility data disregarding the method used for the electroosmosis correction. Thus, it is highly recommendable to monitor the electroosmosis during the runs. The run-to-run magnitude of t_0 allows this. The anomalous changes in effective mobilities of compounds whose pK_a is measured are indicated by unexpected changes in migration times of charged mobility standards in the used buffer. Anomalous drops in mobilities of both mobility standards and investigated cytokinins observed in the tartarate buffer (Fig. 3), for which the ion pairing between the tartarate anion and organic cations is responsible probably, may serve as relevant example.

4.2. Calculation procedures

The highly popular Hendersson–Haselbalch equa-

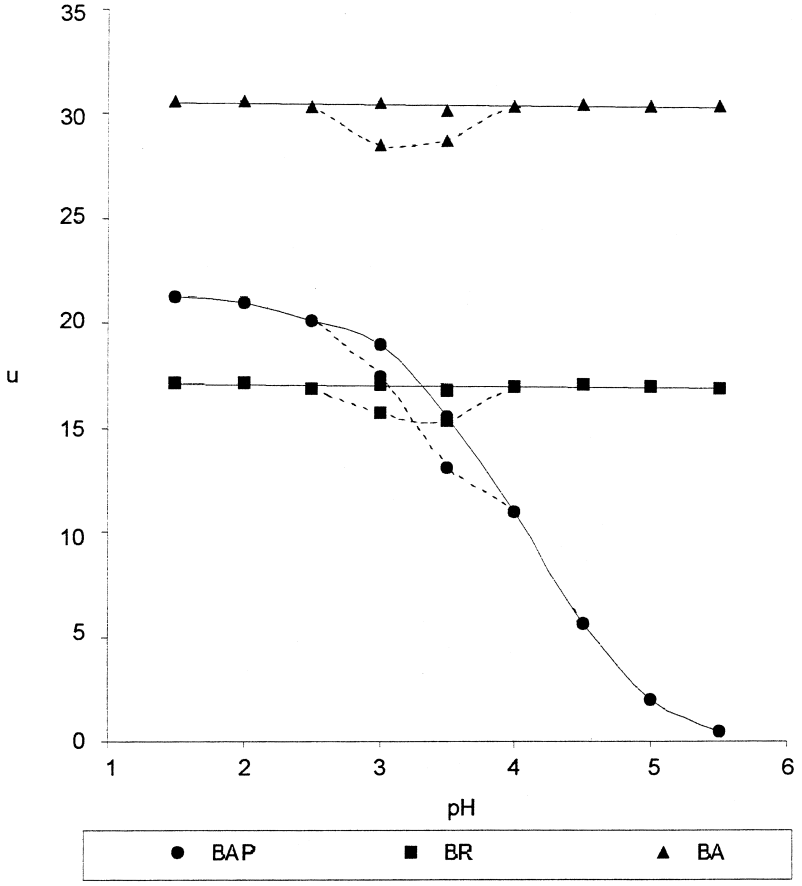


Fig. 3. Anomalous shapes of pH dependencies of mobilities of 6-benzylaminopurine (BAP), brucine (BR) and benzylamine (BA) measured in tartarate buffers (dashed lines) and regular shapes of these dependencies measured in phosphate and acetate buffers (solid lines).

tion (Eq. (11)) is used for the point-by-point calculation of pK_a. The equation defines the effective mobility of a substance, u_{eff}, in terms of mobility of its protonated form, u, solution pH and of the equilibrium constant pK_a. Thus, u is necessary for the calculation in which identical statistical weight is ascribed to each of the experimental points as in the linear regression. Random errors of raw effective mobilities remain unconsidered just as in the linear regression; it affects adversely the pK_a mean and, mainly, its standard deviation (Table 4).

The whole set of the experimental data is utilized in one computing step by programmes for non-linear fitting. Such an approach makes it possible to suppress random errors and, in this way, to decrease the standard deviation of the calculated pK_a. Both the pK_a and the mobility of the charged form of the base, u, are treated as adjustable parameters and their values that give the best fit are obtained simultaneously (Table 5). The comparison of regression procedures for the calculation of pK_a from the Hendersson–Haselbalch equation revealed that much higher number of input effective mobilities is necessary for the weighted linear regression compared to the non-linear regression. Perfect agreement of dissociation constants from the weighted linear regression and from the non-linear regression reported in Ref. [8] was obtained only if the sufficient number of raw mobilities was used for the weighted linear regression.

The perfect mutual agreement of pK_a data calculated from Eqs. (11) and (14) (Table 4) evidences the equivalency of the equations as far as the pK_a calculation only is concerned. The 3–5-times lower standard deviations of pK_a from the non-linear computer fitting, applied to Eq. (14), advocates this computing procedure.

The direct comparison of pK_a of some of the cytokinins, derived from electrophoretic measurements, with their dissociation constants obtained by another method is not possible because of the absence of such data. However, pK_a of 6-benzylaminopurine, determined by UV spectrometry and by differential pulse voltammetry, equal to 4.09 and 4.19, respectively [25] agree nicely with the pK_a data calculated from electrophoretic mobilities (Table 4). It supports the credibility of all pK_a data summarized in Table 4.

Table 4
Dissociation constants of cytokinins obtained by the point-to-point calculation from the Hendersson–Haselbalch equation (Eq. (11)), and by the non-linear fitting with the Boltzman sigmoid (Eq. (14)), and their standard deviations (SDs)

	Eq. (11)		Eq. (14)	
	pK_a	SD	pK_a	SD
BAP	3.88	0.10	4.00	0.02
BAPR	3.06	0.13	2.94	0.03
BAPG	3.02	0.10	2.90	0.03
OTR	3.08	0.09	3.04	0.04
MTR	3.00	0.07	2.92	0.05
KR	2.90	0.03	2.82	0.02
TZR	3.10	0.07	3.05	0.03
DHZR	3.55	0.05	3.59	0.03
IPA	4.24	0.06	4.32	0.06
IPR	3.43	0.10	3.46	0.03

Table 5
Ionic mobilities of charged cytokinins obtained by the non-linear fitting of the Boltzman sigmoid (Eq. (14)) and measured in the 200 mmol/l phosphate buffer, pH 1.5, corrected for the ionic strengths $I=0.015$ and 0.035 M, respectively

	Eq. (14)		Measured[a]	
	u	SD	u	SD
BAP	25.87	0.17	25.98	0.12
BAPR	21.50	0.45	20.50	0.06
BAPG	20.21	0.37	19.23	0.07
OTR	20.72	0.50	20.13	0.08
MTR	20.62	0.43	19.81	0.06
KR	21.86	0.23	20.81	0.07
TZR	20.62	0.38	20.09	0.08
DHZR	20.42	0.27	20.07	0.07
IPA	25.67	0.38	26.05	0.02
IPR	21.24	0.27	20.81	0.03

[a] Ionic mobility supposing the protonation of the compound is complete at pH 1.5 and the interaction of the respective cation with the monophosphate anion is absent.

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