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ISOTACHOPHORETIC DETERMINATION OF MOBILITY AND pK_a BY MEANS OF COMPUTER SIMULATION

III*. EVALUATION OF MOBILITY AND pK_a OF FIFTEEN NUCLEOTIDES AND SEVEN PHOSPHORUS OXOACIDS AND THEIR ISOTACHOPHORETIC SEPARATION

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SUMMARY

A computational method was applied for the evaluation of the absolute mobilities (m_0) and pK_a values of fifteen 5'-nucleotides and seven phosphorus oxoacids (POAs) from the observed qualitative indices, R_E, obtained by isotachophoresis. The nucleotides examined were the sodium salts of AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP and UTP and the POAs were the sodium salts of hypophosphorous, phosphorous, orthophosphoric, pyrophosphoric, triphosphoric, trimetaphosphoric and tetrametaphosphoric acids. Using the evaluated m_0 and pK_a values, the separability of seven POAs and fifteen nucleotides was assessed by computer simulation. With a leading electrolyte at pH 4.7 buffered by creatinine, the isotachophoretic separation of fifteen nucleotides and pyrophosphate, triphosphate and tetrametaphosphate ions was simulated. This was confirmed by experiments and the simulated calibration characteristics of some POAs were compared with the observed values.

INTRODUCTION

The isotachophoretic separation of nucleotides has been studied by several workers. Beckers and Everaerts[1] studied the separation behaviour of twelve nucleotides**, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, UMP, UDP and UTP, varying the pH of leading electrolytes (pH_L) in the range 3–8 by the use of a

* For Part II, see ref. 6.

** Abbreviations: AMP, ADP and ATP = adenosine-5'-mono-, di- and triphosphates; CMP, CDP and CTP = cytidine-5'-mono-, di- and triphosphates; GMP, GDP and GTP = guanosine-5'-mono-, di- and triphosphates; IMP, IDP and ITP = inosine-5'-mono-, di- and triphosphates; UMP, UDP and UTP = uridine-5'-mono-, di- and triphosphates.

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thermometric detector. As an example, the separation of eight nucleotides, UTP, UDP, GDP, ADP, UMP, GMP, AMP and CMP, at pH_L = 3.7 was carried out. They also reported the relative step heights of twenty nucleotides at pH_L = 3 and 4.5 using a conductivity detector[2]. In conclusion, they suggested a complicated separability for the nucleotides when the dependence of the pH on the effective mobilities is utilized[2]. Recently, Nukatsuka and Yoshida[3] improved the separability using an electrolyte system containing Mg^{2+} and 5% ethanol. Eleven nucleotides, *viz.*, AMP, ATP, CMP, CDP, CTP, GDP, GTP, IDP, ITP, UDP and UTP, were separated by complex formation and solvent effects.

As has been emphasized previously, isotachophoretic equilibria can be simulated and the technique can be utilized practically in an assessment of separability[4] and in quantitative[5] and qualitative analyses[6]. In this work, on the basis of the simulational procedure, the separation of fifteen 5'-nucleotides, *viz.* AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP and UTP, was attempted by taking advantage of a dependence of the pH_L on the effective mobilities. In the analytical study of the nucleotides, the coexistence of their hydrolysed phosphorus oxoacids (POA) should be considered, accordingly, seven POAs, including the hydrolysates, hypophosphorous (PO_2), phosphorous (PO_3), orthophosphoric (PO_4), pyrophosphoric (P_2O_7), triphosphoric (P_3O_{10}), trimetaphosphoric (P_3O_9) and tetrametaphosphoric acids (P_4O_{12}), were studied isotachophoretically. The separation of these POAs has already been reported by Yagi *et al.*[7].

To simulate the isotachophoretic equilibra, the absolute mobilities (m_0) and acid dissociation constants (pK_a) of the samples and the leading electrolyte constituents are necessary. The absolute mobilities of the nucleotides are not available in the literature, although the relative mobilities with respect to PO_2 have been reported by Kiso and Falk[8] for separability assessment in zone electrophoresis. The mobilities of POAs in the completely dissociated state have been reported, except for PO_3[9]. The mobilities of mono-, di- and trivalent ionic species of PO_4 have been reported[9], and these values were used here. To determine the mobilities and pK_a values, the values of the isotachophoretic index, R_E, were measured using different electrolyte systems in the pH_L range *ca.* 3–10.

EXPERIMENTAL

Stock sample solutions were prepared by dissolving the sodium salts of POAs and nucleotides (0.01 *M*) in distilled water. The sodium salts of four POAs, PO_2, PO_3, P_2O_7 and P_3O_{10}, were purchased from Tokyo Kasei Kogyo in the purest forms available. Synthesized and purified sodium salts of P_3O_9 and P_4O_{12} were provided by Dr. Ohashi of Kyusyu University. The fifteen sodium salts of the 5'-nucleotides, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP and UTP, were purchased from Sigma.

The isotachopherograms were obtained by the use of a Shimadzu IP-1B isotachophoretic analyser equipped with a potential gradient detector. The separating tube was 20–30 cm × 0.5 mm I.D. The driving currents applied were in the range 50–75 μA and all experiments were carried out at 25°C. For precise measurement of R_E values, acetic, propionic and butyric acids were used as internal standards to correct the asymmetric potential of the detector[9]. One of the three standards was

TABLE I

ABSOLUTE MOBILITIES AND pK_a VALUES OF LEADING ELECTROLYTE CONSTITUENTS AND INTERNAL STANDARDS USED IN SIMULATION (25°C)

m_0 = Absolute mobility ($cm^2\ V^{-1}\ sec^{-1}$) × 10^5; pK_a = thermodynamic acid dissociation constants, assumed values being used for Cl^-. BALA = β-alanine; EAC = ε-aminocaproic acid; CRE = creatinine; MP = γ-methylpyridine; IM = imidazole; MOR = morpholine; BA = benzylamine; CHA = cyclohexylamine; Ac = acetic acid; Prop = propionic acid; Buty = butyric acid.

Cation	m_0	pK_a	*Anion*	m_0	pK_a
$BALA^+$	37.5*	3.552	Cl^-	79.08	−3
EAC^+	29.8*	4.373	Ac^-	42.4	4.756
CRE^+	36.8*	4.848	$Prop^-$	37.1	4.874
MP^+	42.8*	6.08	$Buty^-$	33.8	4.82
IM^+	50.4*	7.15	$H_2PO_4^-$	34.2	2.161
MOR^+	42.5*	8.33	HPO_4^{2-}	59.1	7.207
BA^+	35.8*	9.33	PO_4^{3-}	71.5	12.325
CHA^+	33.6*	10.66			

* Evaluated by our isotachophoretic method.

selected to prevent the appearance of a mixed zone of the sample and the standard.

Table I shows the m_0 and pK_a values of the electrolyte consdtituents used in the calculations. Their values were mainly taken from the literature[9,10] but several of the mobilities used were determined by our isotachophoretic method.

The leading electrolyte systems used are summarized in Table II, together with the calculated concentrations and effective mobilities of the leading electrolyte constituents. Electrolyte systems 1–13 were used for the R_E measurements of POAs. The leading electrolytes were 10 m*M* HCl solutions and the pH_L was adjusted in the range 3.1–10.2 by adding β-alanine (BALA; pH_L = 3.1), ε-aminocaproic acid (EAC; 4.2), γ-methylpyridine (MP; 5.5 and 6.0), imidazole (IM; 6.6 and 7.2), morpholine (Mor; 8.2), benzylamine (BA; 8.8 and 9.2) and cyclohexylamine (CHA; 9.7 and 10.2). Electrolyte systems, 14–24 were used for the nucleotides. The leading electrolytes were 10 m*M* HCl solutions and the pH_L was adjusted in the range 3.2–7.2 by adding BALA (pH_L = 3.2 and 3.6), creatinine (CRE; 4.62), MP (5.1 and 6.0) and IM (6.5 and 7.2). The leading electrolytes contained an additive such as 0.2% Triton X-100, 0.02% poly(vinyl alcohol) (PVA; degree of polymerization = 50) or 0.2% hydroxypropylmethylcellulose (HPMC; viscosity of 2% aqueous solution = 4000 cP). For the R_E measurements, Triton X-100 and PVA were used, as 0.2% HPMC solution is very viscous and the effect of viscosity on R_E values has not been studied in detail.

The terminating electrolytes used were 10 m*M* caproic acid, 10 m*M* pelargonic acid and 10 m*M* 2-(N-morpholino)ethanesulphonic acid. The pH of the terminating electrolyte was adjusted appropriately by adding the buffer used for the preparation of leading electrolytes. The pH measurement was carried out by the use of a Horiba F-7ss expanded-scale pH meter. Electrolyte systems 25 and 26 were those used in ref. 2.

The observed relative step heights[2] (chlorate as standard) were converted into R_E values as described previously[6] and the converted R_E values were used for m_0 and pK_a evaluation, together with the observed R_E values. R_E is the ratio of the potential

TABLE II

ELECTROLYTE CONDITIONS, EFFECTIVE MOBILITIES AND CONCENTRATIONS OF LEADING ZONE CONSTITUENTS (25°C)

Leading ion, Cl^-; terminating ions, *n*-caproate (Nos. 1, 2, 9–24), peralgoate (Nos. 3 and 4) and 2-(N-morpholino)ethanesulphonate (Nos. 5–8). pH_L = pH of leading electrolyte; C_L^* = total concentration of leading ion (mM); $\bar{m}_L$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of leading ion × 10^5; $C_{B,L}^*$ = total concentration (m*M*) of buffer; $\bar{m}_{B,L}$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of buffer ion × 10^5; Std. (R_E) = internal standard and the simulated R_E value (E_S/E_L); ClO_3 chlorate ion.

No.	pH_L	C_L^*	$\bar{m}_L$	*Buffer*	$IC_{B,L}^*$	$\bar{m}_{B,L}$	*Std.* (R_E)
1	3.12	9.53	74.79	BALA	11.70	−25.51	Ac (8.155)
2	4.20	9.57	74.78	EAC	15.28	−16.48	Ac (3.560)
3	5.51	9.53	74.79	MP	11.84	−31.56	Ac (2.072), Prop (2.416)
4	6.00	10.37	74.63	MP	18.13	−22.36	Ac (1.975), Prop (2.288)
5	6.60	9.57	74.78	IM	12.01	−37.20	Prop (2.212)
6	6.68	9.57	74.78	IM	12.08	−32.45	Ac (1.996), Prop (2.324)
7	7.20	9.57	74.78	IM	19.27	−23.18	Prop (2.194)
8	7.20	10.37	74.63	IM	20.84	−23.15	Ac (1.910), Prop (2.196)
9	8.20	10.26	74.65	MOR	17.11	−23.28	Ac (1.907), Prop (2.193)
10	8.76	10.26	74.65	BA	12.75	−25.96	Prop (2.196)
11	9.19	10.26	74.64	BA	16.98	−19.52	Ac (1.909)
12	9.72	10.26	74.64	CHA	11.38	−27.27	Ac (1.909), Prop (2.195)
13	10.22	10.26	74.61	CHA	13.83	−22.67	Ac (1.906)
14	3.23	10.02	74.69	BALA	13.48	−23.76	Ac (7.541)
15	3.65	10.02	74.69	BALA	20.86	−15.95	Ac (5.799), Prop (7.242)
16	4.64	10.02	74.69	CRE	15.58	−21.35	Ac (2.748). Prop (3.314) Buty (3.529)
17	5.11	10.02	74.69	MP	10.98	−35.70	Buty (2.761)
18	5.17	10.02	74.69	MP	11.12	−35.24	Prop (2.521), Buty (2.744)
19	6.00	10.02	74.69	MP	17.53	−22.37	Buty (2.505)
20	6.03	10.27	74.64	MP	18.51	−21.70	Buty (2.501)
21	6.50	10.02	74.69	IM	12.04	−38.77	Buty (2.440)
22	6.58	10.02	74.69	IM	12.45	−37.50	Prop (2.214), Buty (2.436)
23	7.15	10.02	74.69	IM	19.05	−24.50	Prop (2.196)
24	7.22	9.99	74.70	IM	20.54	−22.63	Prop (2.195)
25*	3.00	10.00	74.70	BALA	11.28	−27.11	ClO_3 (1.186)
26*	4.50	10.00	74.70	EAC	22.01	−11.97	ClO_3 (1.186)

* Systems 25 and 26 are the electrolyte systems used in ref. 2.

gradient (E, V cm^{-1}) of a sample zone (E_S) to that of the leading zone (E_L), $R_E = E_S/E_L$, which corresponds to the ratio of the effective mobility of the leading ion in its zone to that of the sample ion in its zone ($R_E = \bar{m}_L/\bar{m}_S$) from the equality of the velocities of migrating zones.

Fig. 1 shows the dependence on pH_L of the observed R_E values of seven POAs. The black circles show the observed R_E values and the curves were plotted using the best-fitted m_0 and pK_a values. Fig. 1 also shows the R_E–pH_L curve of orthophosphoric acid (PO_4), which was obtained by the simulation using m_0 and pK_a values from the literature[9]. The discontinuities in the curves are due to the different buffers used. An increase in the R_E values at low pH_L results from a decrease in the effective mobilities of the samples. The plateau of the curves in Fig. 1 indicates that an ionic species with the same charge exists mainly in the corresponding pH_L range. From the R_E values

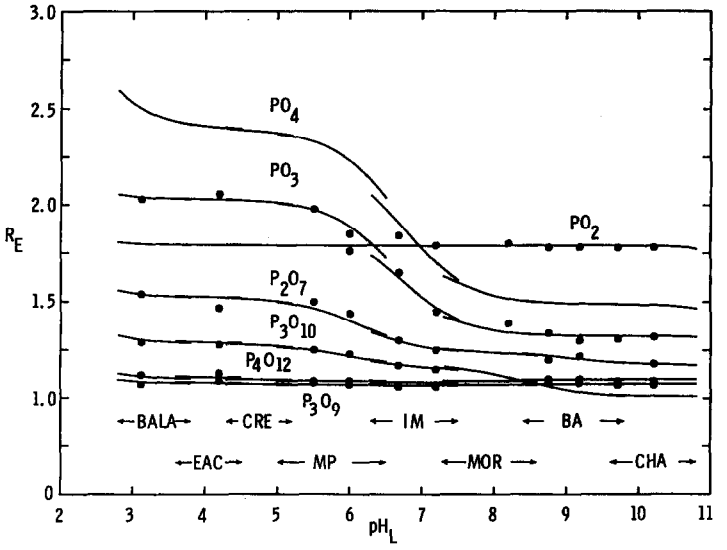

Fig. 1. Observed R_E values (●) of hypophosphorous (PO_2), phosphorous (PO_3), pyrophosphoric (P_2O_7), triphosphoric (P_3O_{10}), trimetaphosphoric (P_3O_9) and tetrametaphosphoric acid (P_4O_{12}). The curves show the dependence on pH_L of simulated R_E values using best-fitted mobility and pK_a. The dependence on pH_L of simulated R_E values of orthophosphoric acid (PO_4) is also shown. For abbreviations of buffers, see Table I.

at the plateau, m_0 can be determined. The pH_L values at points of inflection of curves correspond approximately to the pK_a values. In anionic analysis, the pH of sample zone is always higher than the pH_L. Therefore, the pK_a value (7.2 for PO_4) is higher than the pH_L value at the point of inflection (*ca.* 6.7 for PO_4). From the measurement of the R_E values near the point of inflection, pK_a can be evaluated. In practice, the m_0 and pK_a values were obtained simultaneously from the observed R_E values by the least-squares method used to draw the exact R_E *vs.* pH_L curves.

Fig. 2A and B show the dependence of the pH_L on the observed R_E values of the fifteen nucleotides and the best-fitted R_E–pH_L curves. It can be seen that in the pH_L range 5–8, each of the R_E–pH_L curves of mono-, di- and triphosphates of five nucleosides merge into one of three bands, suggesting that even the separation of the five different nucleotides with the same kind of phosphates is impossible at $pH_L > 5$. However, mono-, di- and triphosphates of a nucleoside may be easily separated in the pH_L range *ca.* 3–8. The details concerning the separability will be discussed later.

RESULTS AND DISCUSSION

The computational procedures for the evaluation of m_0 and pK_a values have been reported previously[6,11]. The evaluation of the m_0 and pK_a values of nucleotides and POAs and the data processing were carried out on an SORD M223 microcomputer and an NEC MS-120 minicomputer. A Watanabe WX4671 *X–Y* plotter was also used.

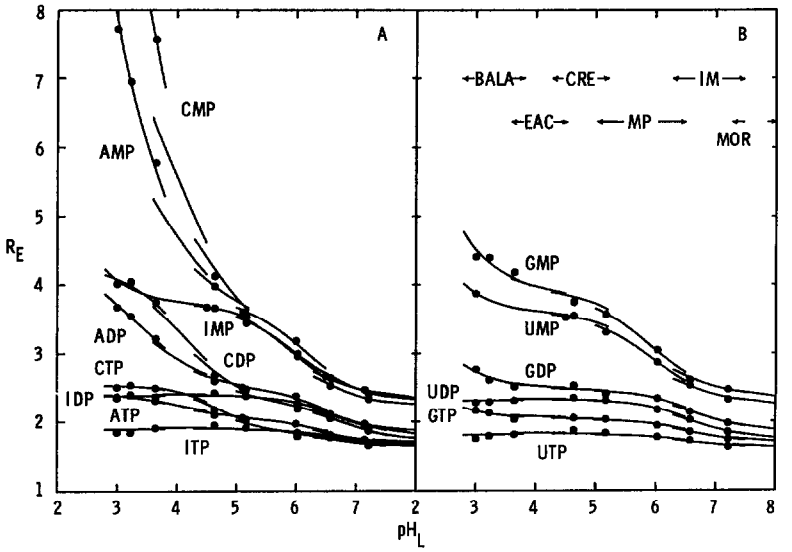

Fig. 2. Observed R_E values (●) of AMP, ADP, ATP, CMP, CDP, CTP, IMP, IDP, ITP, GMP, GDP, GTP, UMP, UDP and UTP. The curves show the dependence on pH_L of simulated R_E values using best-fitted mobility and pK_a. For abbreviations of buffers, see Table I.

Mobility and pK_a evaluations

The least-squares method was applied to the observed R_E values and the m_0 and pK_a values of POAs and nucleotides were determined. Table III shows the observed and the best-fitted R_E values of PO_2, PO_3, P_2O_7, P_3O_{10}, AMP ADP, ATP, GMP, GDP and GTP and their effective mobilities and the concentrations of the sample zone constituents. Good agreement was obtained between the observed and best-fitted R_E values. The agreement was also good for the R_E values converted from the relative step heights at $pH_L = 3$ (ref. 2), confirming the utility of the R_E index for identification. Table IV summarizes the determined m_0 and pK_a values together with the previously reported values[9,10,12]. Several m_0 and pK_a values are not given in Table IV, and the corresponding ionic species may have very small pK_a values, so the precise mobilities could not be obtained by isotachophoresis in principle. Several m_0 and pK_a values were fixed in the least-squares method. The fixed values were mainly taken from the literature but some were fixed appropriately because of the limitation of the pH_L range for R_E measurements in the present experiments. The mobilities of 34 ionic species of nucleotides and seven ionic species of POA were newly determined. The pK_1 value of IMP obtained was very different from that given in the literature[12]. It should be noted that the R_E measurements were carried out in the pH_L range of 3.2–7.2, so some uncertainty is inevitable for the determined pK_a values for $pK_a < ca.\ 3$ and $pK_a > ca.\ 7$.

Separability assessment

Fig. 3 shows the graph of effective mobilities *vs.* pH_S (pH of sample zone) for seven POAs, PO_2, PO_3, PO_4, P_2O_7, P_3O_{10}, P_3O_9 and P_4O_{12}. The graphs were plotted using the evaluated constants on the assumption that the ionic strength is zero, *i.e.*,

TABLE III

OBSERVED AND BEST-FITTED R_E VALUES OF PHOSPHORUS OXOACIDS AND NUCLEOTIDES, EFFECTIVE MOBILITIES AND CONCENTRATIONS OF ZONE CONSTITUENTS (25°C)

No. = number of electrolyte system shown in Table II; R_E = ratio of potential gradients, E_S/E_L; $\bar{m}_S$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) × 10^5 of sample ion; pH_S = pH of sample zone; C_S^t = total concentration (mM) of sample ion; C_B^t = total concentration (mM) of buffer; $\bar{m}_B$ = effective mobility ($cm^2\ V^{-1}\ sec^{-1}$) of buffer ion × 10^5; I = ionic strength of sample zone × 10^3.

Compound	*No.*	R_E			$\bar{m}_S$	pH_S	C_S^t	$C_{B,S}^t$	$\bar{m}_{B,S}$	I
		Observed	*Best-fitted*	*Deviation (%)*						
PO_2	4	1.76	1.79	−1.60	41.7	6.10	8.12	15.9	−20.2	8.12
	6	1.84	1.79	2.97	41.9	6.79	7.32	10.2	−33.6	7.32
	8	1.79	1.79	0.19	41.8	7.31	7.92	18.3	−20.2	7.92
	9	1.80	1.79	0.67	41.7	8.30	8.04	14.9	−21.2	8.04
	10	1.78	1.79	−0.54	41.7	8.85	8.24	10.7	−25.0	8.24
	11	1.78	1.79	−0.54	41.7	9.28	8.22	15.0	−17.9	8.24
	12	1.78	1.79	−0.53	41.7	9.89	8.17	9.52	−26.4	8.25
	13	1.78	1.79	−0.42	41.7	10.4	7.81	12.2	−20.2	8.06
	Mean error			0.93						
PO_3	1	2.03	2.04	−0.53	36.6	3.34	6.77	9.78	−22.1	6.72
	2	2.06	2.03	1.42	36.8	4.32	7.39	13.3	−14.9	7.42
	3	1.98	1.97	0.29	37.9	5.67	6.69	9.50	−29.2	7.33
	4	1.85	1.89	−2.11	39.5	6.15	6.79	15.9	−19.1	8.60
	6	1.65	1.62	1.79	46.2	6.87	5.12	11.0	−31.7	9.75
	8	1.45	1.47	−1.35	50.8	7.30	5.05	19.5	−20.4	12.1
	9	1.39	1.34	3.51	55.7	8.26	4.70	16.1	−21.9	13.7
	10	1.34	1.33	0.79	56.2	8.81	4.71	11.9	−25.0	14.0
	11	1.30	1.33	−1.97	56.3	9.24	4.70	16.1	−18.5	14.1
	12	1.31	1.32	−1.14	56.3	9.79	4.69	10.6	−26.4	14.1
	13	1.32	1.32	−0.28	56.4	10.3	4.63	13.1	−21.4	14.1
	Mean error			1.38						
P_2O_7	1	1.54	1.54	0.27	48.7	3.25	4.01	10.6	−23.3	10.6
	2	1.47	1.52	−3.53	49.1	4.27	4.19	14.2	−15.3	12.7
	3	1.50	1.46	2.53	51.2	5.61	3.80	10.6	−29.8	13.2
	4	1.44	1.40	2.48	53.1	6.08	3.83	17.0	−20.4	15.6
	5	1.30	1.30	−0.02	57.5	6.69	3.17	11.2	−35.2	16.2
	8	1.25	1.26	−0.70	59.3	7.26	3.26	20.0	−21.6	18.7
	10	1.20	1.22	−1.93	61.0	8.81	2.93	12.3	−24.6	21.2
	11	1.22	1.20	1.25	62.0	9.24	2.75	16.5	−18.3	22.5
	13	1.18	1.18	0.01	63.2	10.3	2.49	13.5	−21.1	24.4
	Mean error			1.41						
P_3O_{10}	1	1.29	1.30	−0.59	57.6	3.20	2.93	11.1	−23.8	17.1
	2	1.28	1.28	−0.32	58.2	4.25	2.99	14.7	−15.4	18.0
	3	1.25	1.24	0.66	60.2	5.57	2.74	11.2	−30.0	19.0
	4	1.23	1.22	0.69	61.1	6.05	2.77	17.6	−20.9	22.2
	6	1.17	1.17	0.35	64.1	6.73	2.37	12.1	−34.1	22.1
	8	1.15	1.16	−0.81	64.4	7.24	2.48	20.4	−21.8	24.7
	Mean error			0.57						

(*Continued on p. 202*)

TABLE III (*continued*)

Compound	*No.*	R_E			$\bar{m}_S$	pH_S	C_S^t	$C_{B,S}^t$	$\bar{m}_{B,S}$	I
		Observed	*Best-fitted*	*Deviation (%)*						
AMP	14	6.95	6.92	0.50	10.8	3.99	5.17	9.26	− 9.93	2.69
	15	5.78	5.67	1.92	13.2	4.20	5.14	13.2	− 6.84	3.29
	16	3.98	3.98	−0.08	18.8	4.98	5.26	11.1	−15.1	5.01
	17	3.61	3.64	−0.90	20.5	5.52	4.87	6.35	−31.9	5.36
	19	3.19	3.15	1.29	23.7	6.28	4.30	13.5	−16.2	6.74
	21	2.67	2.71	−1.62	27.5	6.82	3.69	8.31	−33.1	8.00
	23	2.47	2.45	0.97	30.5	7.36	3.46	15.6	−19.0	9.22
	25	7.72	7.86	−1.86	9.50	3.88	5.28	6.84	−11.9	2.41
	Mean error			1.14						
ADP	14	3.55	3.53	0.46	21.1	3.64	3.78	10.0	−16.3	6.11
	15	3.22	3.20	0.69	23.4	3.95	3.66	17.5	−10.5	7.19
	16	2.60	2.63	−1.04	28.4	4.84	3.46	12.4	−17.6	9.66
	17	2.51	2.51	−0.07	29.7	5.35	3.28	7.61	−33.7	9.88
	19	2.38	2.35	1.41	31.8	6.18	3.10	14.5	−18.3	11.0
	21	2.12	2.15	−1.19	34.8	6.72	2.81	9.14	−34.9	12.2
	23	1.97	1.96	0.49	38.1	7.30	2.64	16.5	−20.4	13.9
	25	3.68	3.71	−0.87	20.1	3.47	3.85	7.76	−19.7	5.62
	Mean error			0.78						
ATP	14	2.40	2.36	1.59	31.6	3.48	3.05	11.0	−19.2	10.6
	15	2.31	2.30	0.35	32.5	3.84	2.95	18.3	−12.3	11.7
	16	2.10	2.12	−0.90	35.3	4.79	2.63	13.1	−18.5	14.4
	17	2.06	2.06	0.22	36.3	5.28	2.48	8.40	−33.9	14.7
	19	1.97	1.96	0.63	38.2	6.14	2.35	15.2	−19.2	16.1
	21	1.83	1.84	−0.48	40.6	6.66	2.17	9.73	−35.7	17.3
	23	1.74	1.74	0.16	43.0	7.27	2.06	17.0	−21.2	19.0
	25	2.35	2.39	−1.65	31.3	3.29	3.07	8.90	−22.8	10.2
	Mean error			0.72						
GMP	14	4.39	4.31	1.91	17.3	3.71	4.81	9.52	−15.0	4.28
	15	4.18	4.07	2.52	18.3	4.01	4.92	16.6	−9.56	4.66
	16	3.75	3.81	−1.72	19.6	4.93	5.07	11.0	−16.1	5.35
	18	3.57	3.58	−0.19	20.9	5.57	4.56	6.57	−31.2	5.70
	20	3.05	3.02	0.85	24.7	6.30	4.01	14.6	−15.8	7.60
	22	2.61	2.64	−1.02	28.3	6.85	3.44	8.74	−32.3	8.59
	24	2.47	2.45	0.62	30.4	7.42	3.28	17.1	−17.4	9.45
	25	4.40	4.50	−2.36	16.6	3.55	4.79	7.34	−18.1	4.07
	Mean error			1.40						
GDP	14	2.61	2.64	−1.30	28.2	3.53	3.33	10.7	−18.5	8.87
	15	2.51	2.55	−1.49	29.3	3.86	3.36	18.0	−11.9	9.51
	16	2.53	2.47	2.44	30.3	4.81	3.43	12.5	−18.2	10.3
	18	2.40	2.43	−1.44	30.7	5.37	3.30	7.84	−33.4	10.2
	20	2.34	2.33	0.40	32.0	6.21	3.21	15.4	−17.7	11.3
	22	2.14	2.14	−0.05	34.9	6.79	2.81	9.54	−33.5	12.2
	24	1.97	1.97	−0.11	37.9	7.37	2.60	18.0	−18.6	13.9
	25	2.77	2.73	1.51	27.4	3.35	3.34	8.55	−21.8	8.45
	Mean error			1.09						

TABLE III (*continued*)

Compound	No.	R_E			$\bar{m}_S$	pH_S	C_S	$C_{B,S}$	$\bar{m}_{B,S}$	I
		Observed	*Best-fitted*	*Deviation (%)*						
GTP	14	2.13	2.11	0.75	35.3	3.45	2.50	11.4	−19.8	13.7
	15	2.03	2.07	−1.94	36.1	3.81	2.51	18.7	−12.9	14.4
	16	2.05	2.04	0.64	36.7	4.77	2.54	13.2	−18.8	15.3
	18	2.03	2.01	0.89	37.1	5.32	2.46	8.61	−33.5	15.1
	20	1.94	1.96	−0.83	38.2	6.17	2.42	16.1	−18.5	16.5
	22	1.85	1.84	0.50	40.6	6.74	2.17	10.1	−34.3	17.3
	24	1.74	1.74	−0.16	42.9	7.34	2.04	18.7	−19.3	19.1
	25	2.16	2.15	0.28	34.7	3.26	2.51	9.21	−23.2	13.2
	Mean error			0.75						

the systems are not in the isotachophoretic steady state. However, the dependence of pH_S on these graphs can be used for the separability assessment, to a first approximation. From Fig. 3, it seems that the seven POAs can be separated in the pH_L range *ca.* 3–5.5, but from Fig. 1 (isotachophoretic steady state) the separation of P_3O_9 and P_4O_{12} is difficult at any pH_L. At pH_L above *ca.* 3, P_3O_9 is trivalent and P_4O_{12} is tetravalent and the absolute mobility of $P_4P_{12}{}^{4-}$ is considerably larger than that of $P_3O_9{}^{3-}$ (Table IV and Fig. 3). However, the effective mobility of P_3O_9 is slightly larger than that of P_4O_{12} in the isotachophoretic steady state, because the effective mobilities of polyvalent ions are strongly affected by ionic strength as estimated from Onsager's equation. When P_3O_9 or P_4O_{12} is not present in a given sample, the separation of seven POAs may be fairly easy in the pH_L range *ca.* 3–5.5. Fig. 4 shows the simulated and observed isotachopherograms of PO_2, PO_3, PO_4, P_2O_7, P_3O_9 and P_3O_{10} under the separation conditions of pH_L = 3.3 buffered by adding BALA. The separation was complete, as expected.

The separation of these POAs at pH_L = 5.5 buffered by histidine was reported by Yagi *et al.*[7]. The sample zones were detected in the order P_3O_9, P_4O_{12}, P_3O_{10}, P_2O_7, PO_2, PO_3 and PO_4. Although the order is the same as that in this study (pH_L = 6, buffer = MP), the separability of P_3O_9 and P_4O_{12} was considerably improved by the use of histidine. The difference in the separability suggests that an ion-pair interaction may occur between histidine and P_3O_9, P_4O_{12}, etc., decreasing the effective mobilities. The interaction may be fairly strong. The details of the ion-pair formation will be described elsewhere. For precise determinations of m_0 and pK_a values, of course, the use of a negligibly interacting buffer such as MP is desirable.

Fig. 5 shows the dependence of the pH on the effective mobilities of the fifteen nucleotides, which were plotted using the evaluated m_0 and pK_a values at an ionic strength of zero. The separation of these nucleotides is apparently very difficult compared with the POAs. The separation of eleven nucleotides, AMP, ATP, CMP, CDP, CTP, GDP, GTP, IDP, ITP, UDP and UTP, has been reported by Nukatsuka and Yoshida[3] using as the leading electrolyte 10 m*M* HNO_3 solution containing 2.9 m*M* Mg^{2+} and 5% ethanol (pH = 3) buffered by adenosine. Under these conditions, sufficient differences in the effective mobilities of nucleotides for separation were obtained by utilizing the complex-forming effect between Mg^{2+} and nucleotides and

TABLE IV

DETERMINED ABSOLUTE MOBILITIES AND pK_a VALUES OF SIX PHOSPHORUS OXOACIDS AND FIFTEEN NUCLEOTIDES (25°C)

m_1–m_5 = Absolute mobilities ($cm^2\ V^{-1}\ sec^{-1}$) × 10^5 of mono- – pentavalent ions; pK_1–pK_5 = thermodynamic acid dissociation constants. Figures in parentheses are literature values. For pK_a negative values were used. For mobilities appropriate values were used.

Sample	m_1	m_2	m_3	m_4	m_5	pK_1	pK_2	pK_3	pK_4	pK_5
PO_2	45.1 (44.3)					1.1*				
PO_3	40.0	65.9				1.3*	7.086 (6.70)			
P_2O_7	29**	57.9	76.4	89.4 (99.4)		1*	1.9*	6.6*	9.6*	
P_3O_{10}	–***	48**	74.7	89.3	113.0*	–	1.1*	2.3*	6.5*	9.24*
P_3O_9	–	–	83.7 (86.6)			–	–	2.05*		
P_4O_{12}	–	–	75.6	94.7 (97.1)		–	–	–	2.74*	
AMP	22.6	39.5				3.981 (3.74)	6.791 (6.1–6.4)			
ADP	19.2	36.7	53.7			–	4.101 (3.95)	7.056 (6.1–6.7)		
ATP	–	37.5	49.2	64.7		–	–	4.418 (4.0)	7.064 (6.0–6.95)	
CMP	23.7	40.6				4.468 (4.44–4.5)	6.705 (6.3–6.6)			
CDP	19.5	40.0	57.3			–	4.782 (4.36–4.6)	7.349 (6.4–6.6)		
CTP	–	36.0	54.4	66.8		–	–	5.255 (4.8)	7.35** (6.6)	
GMP	21.7	38.0	54.3**			2.845 (2.3–2.4)	6.512 (5.92–6.1)	9.5** (9.38–9.4)		
GDP	18.7**	37.3	52.9	68.5**		–	2.958 (2.9)	7.116 (6.3)	9.5** (9.6)	
GTP	–	34**	49.8	64.1	78.4**	–	–	3.044 (3.3)	7.158 (6.5)	9.5** (9.3)
IMP	22.6	38.2	53.9**			2.575 (1.54)	6.545 (6.04)	9.5**		
IDP	–	38.3	54.2	70.2**		–	–	7.169	9.5**	
ITP	–	–	52.8	65.0	77.3**	–	–	–	7.2	9.5**
UMP	23.4	39.7	56**			2.499	6.529 (6.3–6.4)	9.5**		
UDP	–	39.4	56.0	72.6**		–	–	7.088 (6.5)	9.5** (9.4)	
UTP	–	–	54.9	66.8	78.8**	–	–	–	7.1 (6.6–7.1)	9.5** (9.5–9.7)

* Fixed in the least-squares method. The values were taken from the literature.

** Fixed in the least-squares method. The values were assumed appropriately taking into account preliminary calculations.

*** –, These values could not be determined isotachophoretically in principle.

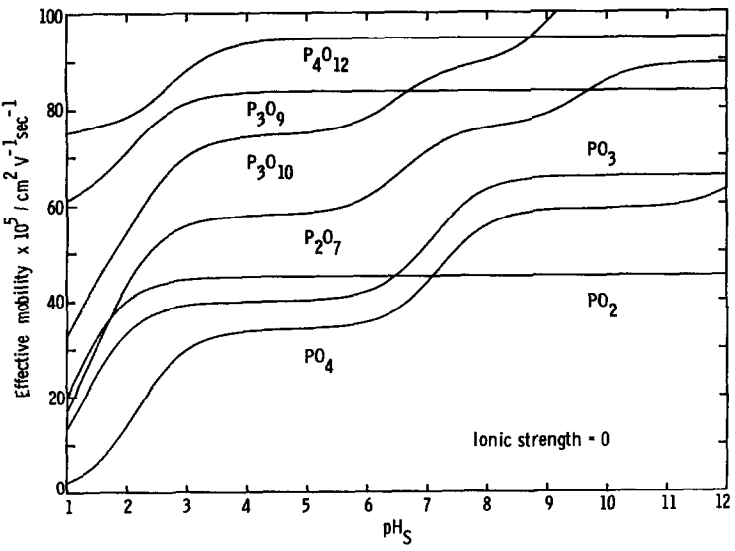

Fig. 3. Dependence on pH_L of effective mobilities of seven phosphorus oxoacids at ionic strength zero. The curves are not for the isotachophoretic steady state. pH_S = pH of samples.

the solvent effect on the mobilities and pK_a values. The simulation is complicated with the electrolyte system used; in particular, the lack of stability constants of Mg–nucleotide complexes in 5% ethanol solution makes the simulation impossible at present. As the most fundamental aspect of isotachophoretic separations, the dependence of the pH on the effective mobilities was utilized in this work. The separation behaviour can be simulated using the determined m_0 and pK_a values. It should be noted that the effective mobilities of samples are very sensitive to the pH_L when the pK_a values of the samples are greater than pH_L and the proper choice of the pH_L

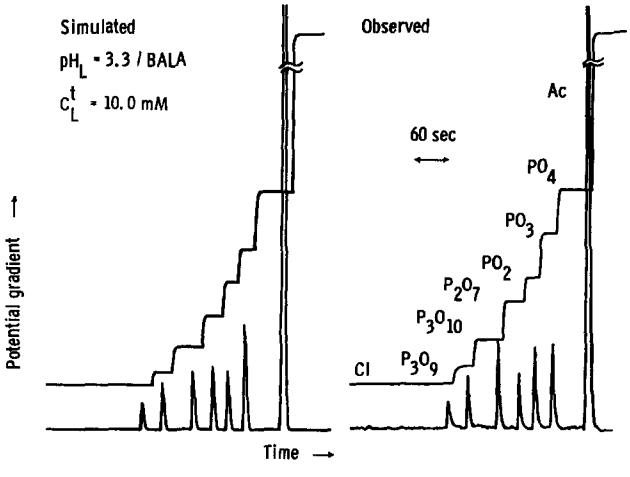

Fig. 4. Simulated and observed isotachopherograms of six phosphorus oxoacids at pH_L = 3.3 (β-alanine buffer). The terminator is acetic acid (Ac). Current = 50 μA.

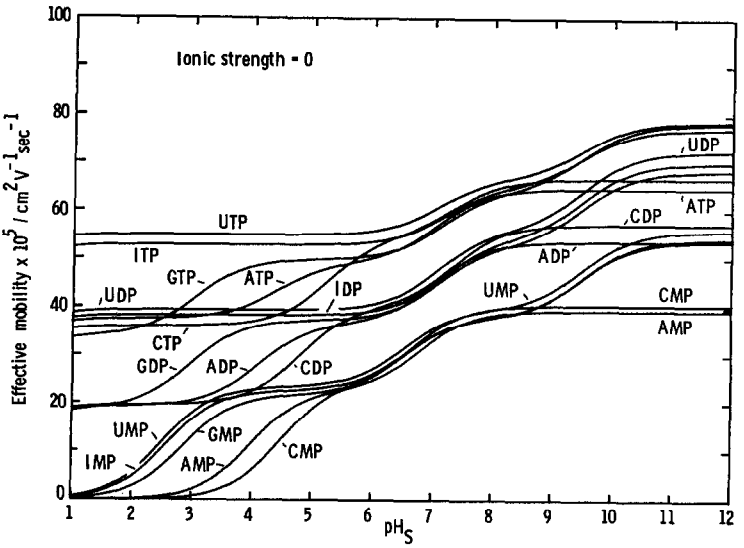

Fig. 5. Dependence on pH_L of effective mobilities of fifteen nucleotides at ionic strength zero. The curves are not for the isotachophoretic steady state. pH_S = pH of samples.

is of decisive importance for effective separations. From Fig. 5, it is apparent that for a good separation of these nucleotides, pH_L should be in the range 4.5–5. As shown in Fig. 5, in the optimal pH range the curves of ten or more nucleotides are almost flat.

For a more precise assessment of the separation conditions, the isotachophoretic equilibria of seven POAs and fifteen nucleotides were simulated. Fig. 6 shows the simulated dependence of pH_L on the effective mobilities of PO_2, PO_3, PO_4, P_2O_7, P_3O_{10}, P_3O_9, P_4O_{12}, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, UTP and propionic acid as an internal standard. The curves overlap with each other, suggesting that the separation of all the samples at once is impossible. However, as estimated from Fig. 5 in the pH_L range *ca.* 4.5–5, especially at pH_L = *ca.* 4.7 (creatinine buffer), the fifteen nucleotides, three POAs, P_3O_9 or P_4O_{12}, P_3O_{10}, and P_2O_7 may be separated experimentally.

Fig. 7 shows the simulated R_E *vs.* pH_L curves. The R_E values at pH_L = 4.7 were 1.10 (P_4O_{12}), 1.28 (P_3O_{10}), 1.51 (P_2O_7), 1.82 (UTP), 1.90 (ITP), 2.04 (GTP), 2.11 (ATP), 2.19 (CTP), 2.31 (UDP), 2.39 (IDP), 2.47 (GDP), 2.61 (ADP), 2.70 (CDP), 3.51 (UMP), 3.65 (IMP), 3.81 (GMP), 3.94 (AMP) and 4.26 (CMP). The differences in the R_E values are small, but may be sufficient for separation. The effective mobilities under these conditions can be calculated from $(74.7/R_E) \cdot 10^{-5}$.

The optimum pH_L range may be limited, as CTP may form a mixed zone with UDP at lower pH_L and with ATP at higher pH_L. Fig. 8 shows the simulated and observed isotachopherograms at pH_L = 4.7. The terminator is pelargonic acid. As the simulated isotachopherogram shows, fifteen nucleotides, P_2O_7, P_3O_9, P_3O_{10} and the internal standard, propionic acid, could be separated. Satisfactory agreement was obtained between the simulated and the observed isotachopherograms, but the simulated R_E value of pelargonic acid (4.72) is smaller than the observed value. The

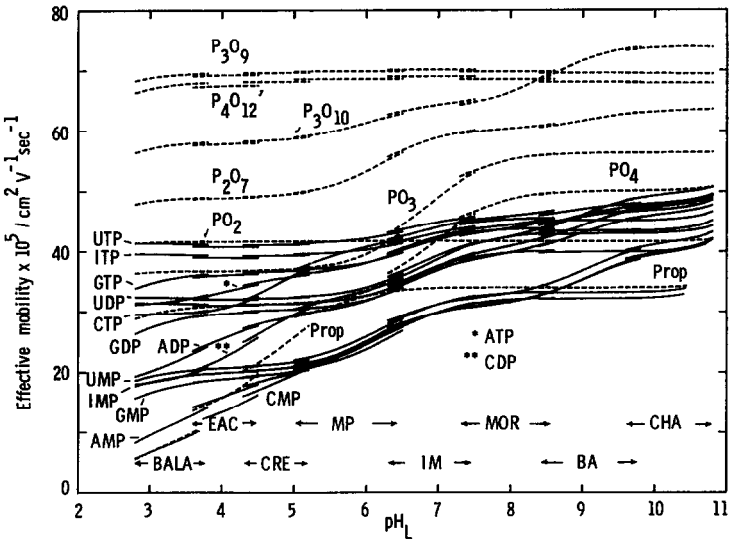

Fig. 6. Dependence on pH_L of the simulated effective mobilities of seven phosphorus oxoacids (broken curves), fifteen nucleotides (solid curves) and propionic acid (Prop; broken curve) at the isotachophoretic steady state. For abbreviations of buffers, see Table I.

m_0 and/or pK_a used[6] may be uncertain. Sharp zone boundaries of IMP and GMP could not be observed when the concentration of additive (PVA or HPMC) was low (*e.g.*, 0.05%). The observed isotachopherogram in Fig. 8 was obtained using a leading electrolyte containing 0.2% HPMC.

Thus, it has become apparent that the fifteen nucleotides can be separated by

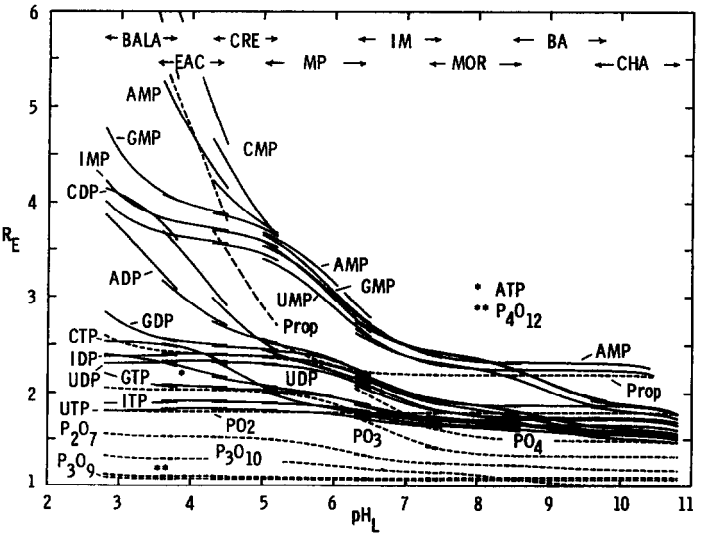

Fig. 7. Dependence on pH_L of the simulated R_E values of seven phosphorus oxoacids (broken curves), fifteen nucleotides (solid curves) and propionic acid (Prop; broken curve) at the isotachophoretic steady state. For abbreviations of buffers, see Table I.

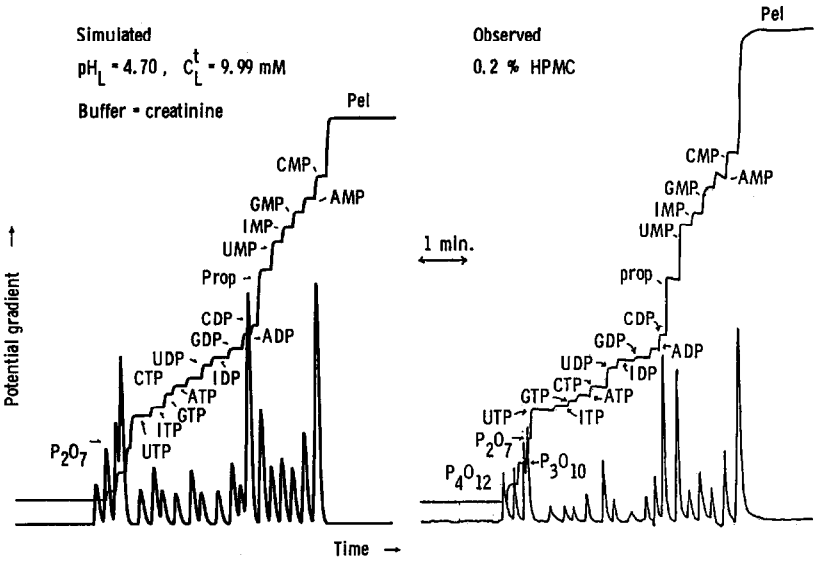

Fig. 8. Simulated and observed isotachopherograms of nineteen anions, AMP, ADP, ATP, CMP, CDP, CTP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, UTP, P_2O_7, P_3O_{10}, P_4O_{12} and propionic acid (Prop) at pH_L = 4.7 (creatinine buffer). The terminator was pelargonic acid. Current = 75 μA. Sample amounts *ca.* 1.2–3 nmol.

utilizing merely the pH dependence of the effective mobilities. A defect of the present electrolyte conditions is that the hydrolysis products, PO_4 (R_E = 2.39 at pH_L = 4.7) and PO_3 (2.02), cannot be distinguished from IDP (2.39) and GTP (2.04), respectively, by the use of a potential gradient detector. For this discrimination, a UV detector must be used, as PO_3 and PO_4 do not respond to UV light, but the nucleotides do. For their separation, a pH above 7.5 might be used, or complex-forming equilibria might be useful, as reported by Nukatsuka and Yoshida[3]. However, it should be noted that several mixed zones may be formed when UMP, IMP, ADP and GMP are added to the eleven nucleotides separated under the electrolyte conditions according to the reported PR ($=1/R_E$) values.

Calibration characteristics

Except for PO_4, the salts of commercial POAs contain appreciable amounts of impurities formed by hydrolysis and/or oxidation of the reagents used. Further, the stoichiometric forms and water contents are sometimes ambiguous. Therefore, the experimental determination of the calibration characteristics of the POAs is generally troublesome. Mikkers *et al.*[13] studied the calibration characteristics of PO_4, P_2O_7 and P_3O_{10} by a combination of photometric and isotachophoretic determinations. Fortunately, the calibration characteristics can easily be established by utilizing a simulation technique when the m_0 and pK_a values of the samples and the electrolyte constituents used are known[5]. For PO_4, the coefficient of the calibration line, a = n(nmol)/t(sec), could be calculated directly from the observed time-based zone length (t) and amount of sample injected (n) from a figure in ref. 13. Using the coefficient for PO_4, those for P_2O_7 and P_3O_{10} were evaluated by use of the observed values.

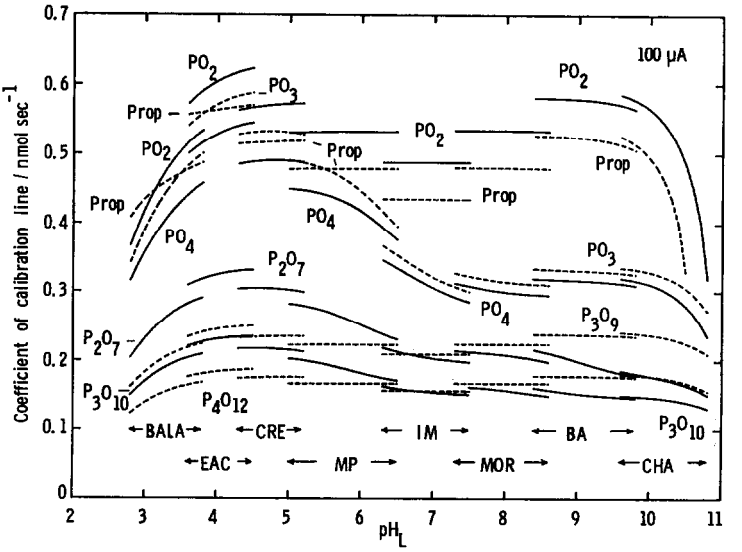

Fig. 9. Dependence on pH_L and buffers of the simulated coefficient of calibration line, a = n(nmol)/t(sec), of seven phosphorus oxoacids at the isotachophoretic steady state. The leading ion is 10 mM chloride. Current = 100 μA. For abbreviations of buffers, see Table I.

The coefficients obtained were 0.386, 0.227 and 0.178 for PO_4, P_2O_7 and P_3O_{10} and the simulated values were 0.391, 0.244 and 0.175, respectively, at 80 μA. The agreement between the observed and the simulated calibration characteristics is satisfactory, confirming the utility of the simulation.

Figs. 9 and 10 show the dependence of pH_L on the simulated coefficients of the calibration line in the pH_L range 2.8–10.8 using eight buffers at 100 μA. The

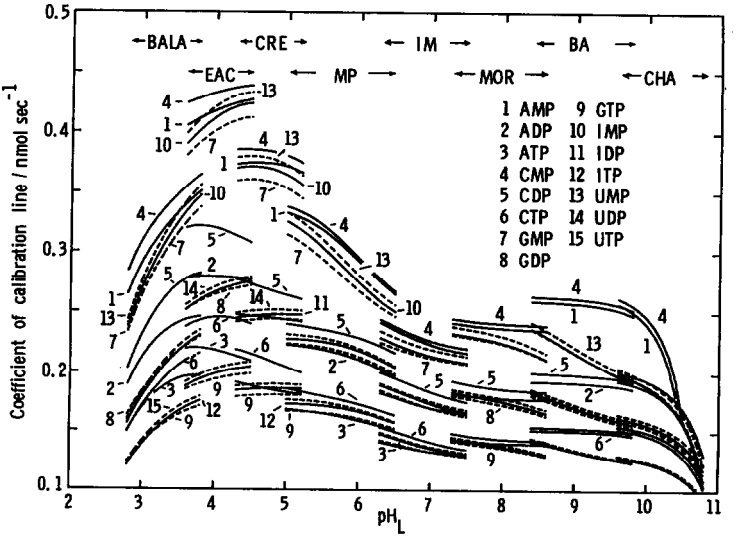

Fig. 10. Dependence on pH_L and buffers of the simulated coefficient of calibration line, a = n(nmol)/t(sec), of fifteen nucleotides. The leading ion is 10 mM chloride. Current = 100 μA.

leading ion is 10 m*M* Cl^-. These coefficients are convenient for a rough quantification of the POAs and nucleotides. The values of the coefficient under different current conditions (*i*) can be easily obtained as 100 *a*/*i*.

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