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The separation of nucleotides by isotachophoresis

Many papers in the literature have dealt with methods for the separation of nucleotides[1-6], some of which are based on the principle of electrophoresis. SILVER *et al.*[2] reported on the separation of nucleotides by high-voltage paper electrophoresis, for instance. A complete separation of thirteen nucleotides was obtained in an electrolyte system containing a citrate buffer and EDTA at pH 4.05. The time required for analysis in these experiments was *ca.* 70 min. ZAK *et al.*[3] carried out the analysis of nucleotides at pH 3.1 and 9.6, also by paper electrophoresis. STRANSKY[4] described the separation of the adenosine nucleotides by paper electrophoresis, for which the optimum pH was found to be 4–5.

In most of these methods, which show the possibility of separating nucleotides by electrophoretic methods, the principle of zone electrophoresis is used, in which a swamping background electrolyte is needed. Because of this requirement, no general means of detection, *e.g.*, a conductometric or thermometric method, can be used. Colour reactions, UV absorption and other specific methods are obviously the most suitable methods of detection.

Isotachophoresis is an electrophoretic method in which all ionic species of the sample move in successive zones with a speed equal to that of the leading zone, during the steady state. Each zone contains only one ionic species of the sample, and its concentration is adjusted to that of the leading electrolyte according to the modified Ohm's law.

In previous papers[7-10] it has been shown that isotachophoresis is an analytical method that can be used for separating charged particles, both qualitatively and quantitatively. In this paper the qualitative separation of some nucleotides by isotachophoresis is described.

Apparatus and chemicals

Basically, the electrophoretic equipment consists of a capillary tube (I.D. 0.45 mm; O.D. 0.75 mm), connected to two electrode reservoirs. The introduction of the sample is carried out by means of a four-way tap. The capillary tube is mounted in a modified Liebig cooler and is surrounded by a water jacket. The detection is thermometric by using thermocouples (copper-constantan, 0.015 mm), which are mounted around the capillary tube. A further explanation was given earlier[11].

All the nucleotides (5'-substituted) used were produced by Sigma Chemical Company, St. Louis, U.S.A. The other chemicals were of p.a. quality, produced by Merck. Double-distilled water was used as the solvent.

Experimental and results

In order to obtain an optimum separation, maximum differences in the mobilities must be present in the electrolyte system chosen. The effective mobility is defined as:

$$m_{eff} = \Sigma \gamma_i \cdot \alpha_i \cdot m_i$$

where α_i is the dissociation constant, m_i is the absolute mobility and γ_i is a cor-

rection factor. The pH of the electrolyte system, which regulates the degree of dissociation of the nucleotides, is an important factor as it affects the effective mobilities.

Although it is possible to calculate the effective mobilities for the different systems[7,8] from data such as pK values and absolute mobilities in order to choose an optimum electrolyte system, experiments had to be carried out to find this optimum system as exact data on the nucleotides are not available.

The step-heights* were measured at pH 3.4–7, for the mono-, di- and tri-

TABLE I

CONDITIONS FOR THE DIFFERENT ELECTROLYTE SYSTEMS

System	*pH*	*Leading electrolyte*	*Current* (μA)	*Terminator*
WAdCl	3.4	0.01 N HCl + adenosine	70	Caproic acid
WαNCl	3.7	0.01 N HCl + α-naphthylamine	70	Caproic acid
WAnCl(1)	4.2	0.01 N HCl + aniline	70	Pivalic acid
WAnCl(2)	4.6	0.01 N HCl + aniline	70	Pivalic acid
WPyrCl	5.0	0.01 N HCl + pyridine	70	Cacodylic acid
WHisCl	6.0	0.01 N HCl + histidine	70	Cacodylic acid
WImCl	7.0	0.01 N HCl + imidazole	70	Benzyl-*dl*-asparigine

TABLE II

STEP-HEIGHTS OF THE NUCLEOTIDES FOR THE DIFFERENT SYSTEMS

The step-heights are given in millimetres from the top of the step-heights of the leading electrolyte zones.

Ionic species	*System*						
	WAdCl	*WαNCl*	*WAnCl(1)*	*WAnCl(2)*	*WPyrCl*	*WHisCl*	*WImCl*
AMP	536	476	400	310	304	290	162
ADP	318	268	224	170	164	186	108
ATP	204	184	150	118	100	146	82
GMP	388	350	352	290	290	300	162
GDP	230	210	176	152	140	192	112
GTP	172	160	120	104	100	160	88
CMP	740	624	472	346	300	250	—
CDP	356	312	276	192	—	184	100
CTP	176	188	168	124	108	142	68
UMP	328	318	324	264	—	270	—
UDP	172	164	168	136	—	178	100
UTP	—	120	104	98	—	130	78

* The step-height is a qualitative factor in isotachophoresis and is a measure of the effective mobility.

phosphates of adenosine, uridine, guanosine and cytidine. The conditions of the electrolyte systems used are listed in Table I and in Table II the step-heights measured for the nucleotides are given for the different systems. All the step-heights were measured with the same thermocouple. Small differences in step-heights, shown in the figures and listed in Table II, can be present when different thermocouples, mounted at different positions on the capillary tube (in order to give the possibility of using several lengths for the separation), are used.

Fig. 1 shows the graphical representation of the step-heights in the different systems. At higher pHs (5–7), the differences in step-heights are rather small and

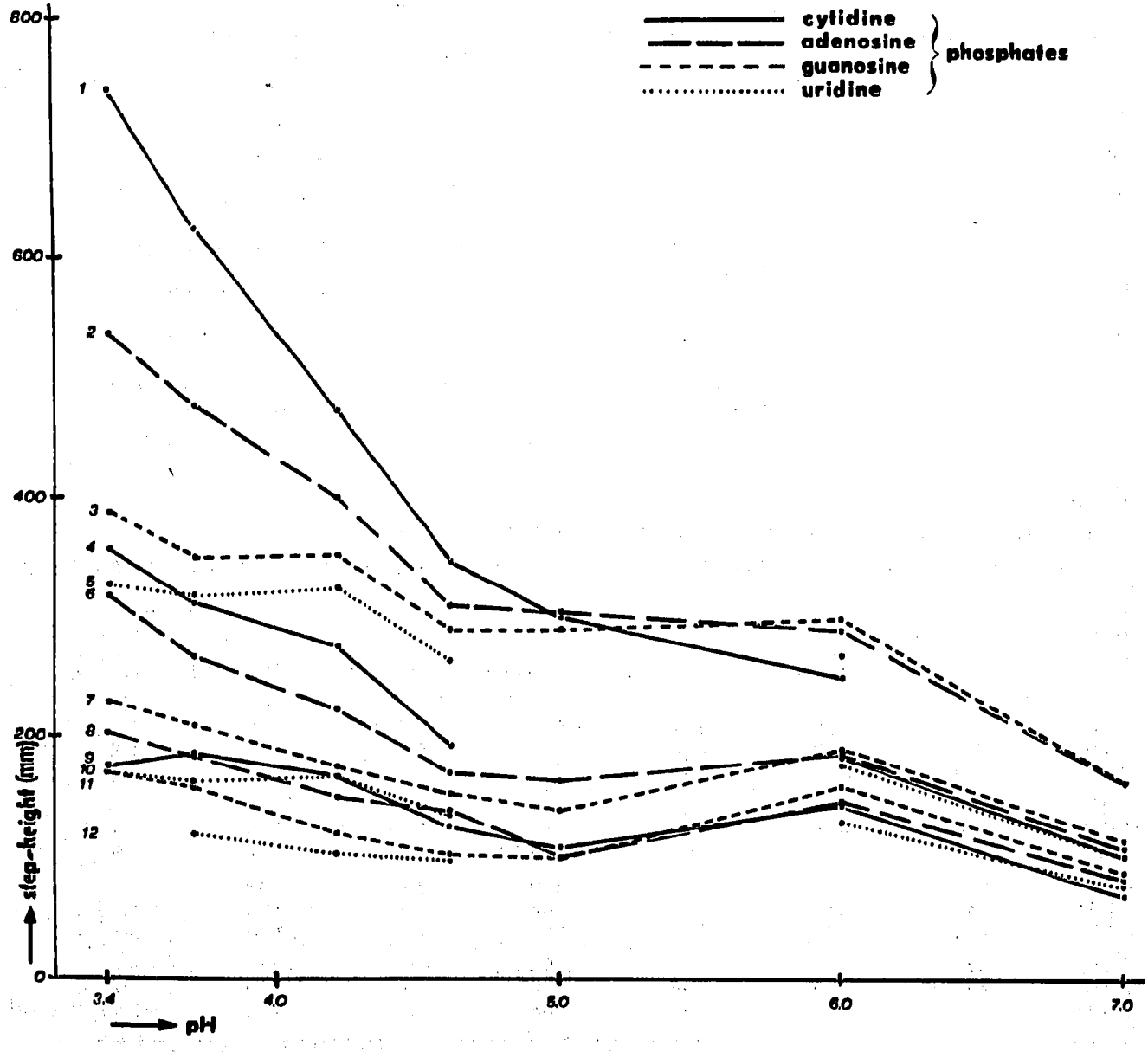

Fig. 1. Graphical representation of the step-heights in the different systems. 1 = CMP; 2 = AMP; 3 = GMP; 4 = CDP; 5 = UMP; 6 = ADP; 7 = GDP; 8 = ATP; 9 = CTP; 10 = UDP; 11 = GTP; 12 = UTP.

these systems are not suitable for the separation of complex mixtures. However, the separation of the mono-, di- and triphosphates of each nucleotide is possible. Figs. 2a and b show the electropherograms for the separations of adenosine and uridine phosphates, respectively, at pH 6.0. In the sample used to obtain Fig. 2a, pyrophosphate was also present. The time required for the separation was about 20 min.

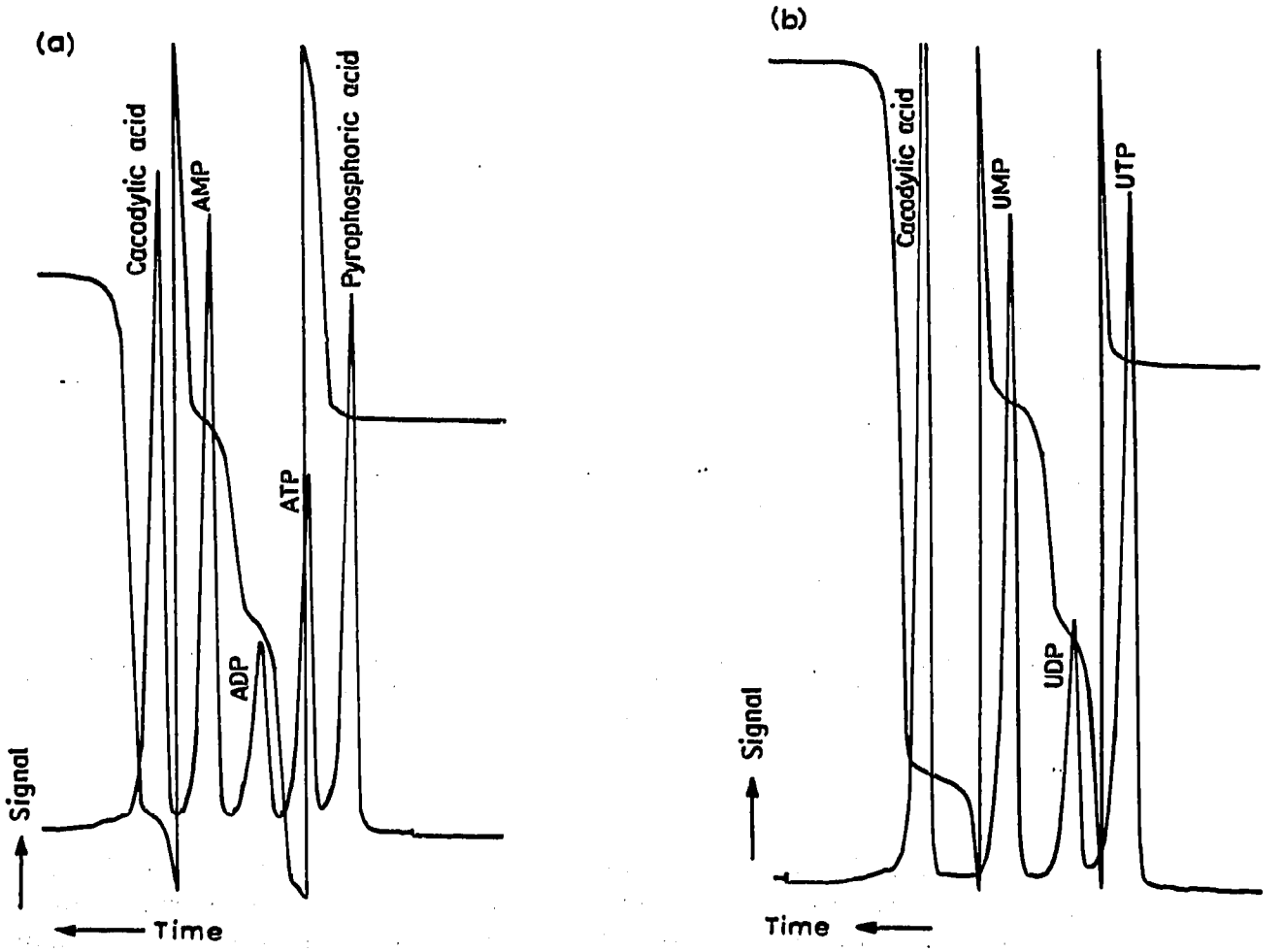

Fig. 2. (a) Separation of pyrophosphate, ATP, ADP and AMP in the system WHisCl at pH 6.0. The terminator is cacodylic acid. (b) Separation of UTP, UDP and UMP in the system WHisCl at pH 6.0. The terminator is cacodylic acid.

At lower pHs, the step-heights diverge and greater differences are obtained. Such systems are more suitable for the separation of complex mixtures. As an example, the electropherogram for the separation of the nucleotides UTP, UDP, GDP, ADP, UMP, GMP, AMP and CMP is shown in Fig. 3. A complete separation could easily be obtained in 30 min at pH 3.7.

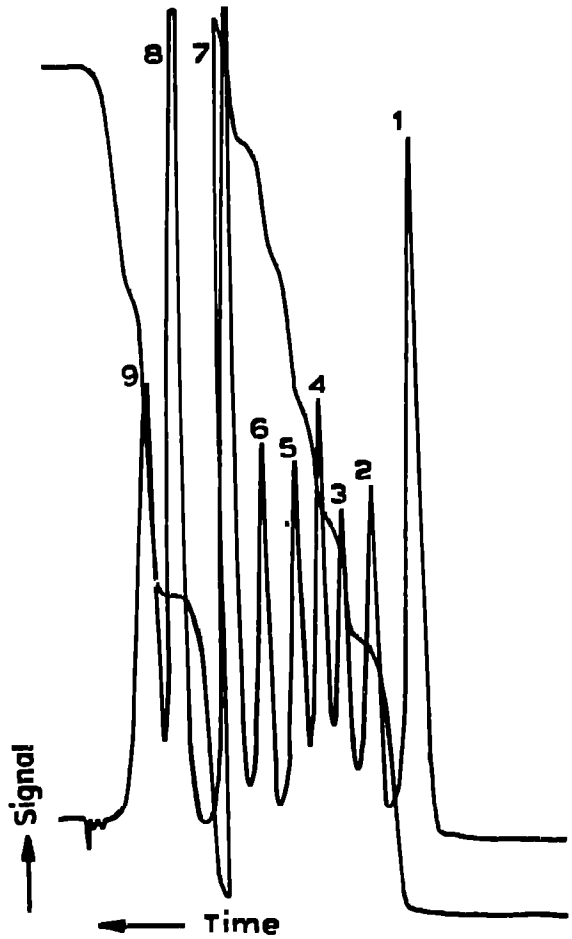

Fig. 3 Separation of a mixture of nucleotides at pH 3.7, in the system WαNCl. The terminator is caproic acid. The sample contained 1 = UTP; 2 = UDP; 3 = GDP; 4 = ADP, 5 = UMP; 6 = GMP; 7 = AMP; 8 = CMP, 9 = caproic acid.

Lower pHs can rarely be used, however, because the low effective mobilities at these pHs require a greater potential than is available. Not all nucleotides could be separated at these pHs, but the use of a UV detector, at more wavelenghts, can solve this problem[6].

Discussion

Although the principle of isotachophoresis differs totally in comparison with zone electrophoresis, the results obtained in this work correspond remarkably with those obtained by other workers. The sequence of the nucleotides at pH 4.05 (ref. 2) is similar and the optimal pHs correspond with those obtained eleswhere[2,4]. The results of zone electrophoresis and isotachophoresis can be compared.

For the separation of complex mixtures of nucleotides by isotachophoresis, the use of a counterflow of electrolyte and/or a combination of more systems may be necessary. In particular, a counterflow of electrolyte can be applied if the differ-

ences in concentrations between the ionic species of the sample are large and the differences in effective mobilities are small.

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