Journal of Chromatography, 201 (1980) 265–279
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,996

CHROMATOGRAPHY OF NUCLEOSIDE PHOSPHATES ON DEAE-SPHERON 1000 WITH ETHANOL–AMMONIUM FORMATE AS ELUENT

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SUMMARY

The chromatography of 5′-mono-, di- and triphosphates of ribonucleosides on DEAE-Spheron 1000 was studied using various ammonium formate–ethanol mixtures as eluent with pH from 3.5 to 5. The results of log k' measurements have been described by a second-degree polynomial with three variables, and the reduced height of a theoretical plate function with a three-dimensional linear equation. The influence of all variables on the chromatographic behaviour of this system is described, particularly using the first derivatives of log k' with respect to all variables. An example of a preparative separation is presented.

INTRODUCTION

In a recent paper on the separation of 5′-nucleoside monophosphates[1] the advantages of the weak anion exchanger DEAE-Spheron 1000 were outlined. This anion exchanger is prepared by bonding diethylaminoethyl groups to a glycol–methacrylate polymeric matrix in a bead form[2]. In contrast to separations on polystyrene-based anion exchangers[3] the adsorption of nucleotides to a hydrophilic matrix is much weaker and therefore may be conducted at room temperature. The exchange capacity of DEAE-Spheron is comparable to that of polystyrene ion exchangers; its mechanical rigidity is higher and it can therefore be used at higher pressure gradients. Silica gel-based ion exchangers are mechanically more stable[4,5] and can be used at even higher pressure gradients, but their chemical stability is inherently lower.

We have attempted to study separations on DEAE-Spheron 1000 under widely varied experimental conditions to see whether the selectivity may be changed not only by changing the concentration of the anion and the pH, but also by varying the concentration of ethanol in the eluent. With respect to preparative separations eluents with high contents of ethanol are of particular interest, because with increasing ethanol concentration the rate of on-column decomposition of di- and triphosphates decreases.

0021-9673/800000–0000/$02.25

EXPERIMENTAL

Chromatography

A home-built chromatograph[1] and 25 cm × 4 mm I.D. glass columns[6] were used for the measurement of capacity factors and the number of theoretical plates. The column temperature was maintained at 25°C. Chromatograms were obtained using a fixed-wavelength (254 nm) detector and an MTA Kutész (Budapest, Hungary) stripchart recorder. Preparative separations were performed in 10 mm I.D. glass columns. Conductivity detection was used to check the formate concentration in step gradient elution.

Buffers were prepared from weighed amounts of ammonium formate; distilled water and measured volumes of ethanol were added. After dissolution the pH was adjusted with concentrated formic acid and the final volume was made up with water.

All pH measurements were made with a GK 2351 C combined glass electrode and a PHM 63 digital pH meter (Radiometer, Copenhagen, Denmark), calibrated with buffers supplied by the manufacturer. No corrections to the recorded pH values have been made; it is believed that the difference between recorded and true pH value is less than 0.1 unit even at an ethanol concentration of 40%[7].

The nucleotides were supplied by Sigma (St. Louis, MO, U.S.A.) and all other chemicals by Lachema (Brno, Czechoslovakia). Stock solutions of the substances were prepared in distilled water and stored in a freezer (*ca.* −20°C). The amounts of the individual substances in the 10-μl samples injected into the chromatograph were about 1 μg.

The void volume (V_0) of the column was measured by a slight fluctuation of buffer solution concentration at the inlet into the chromatograph, as described previously[1]. The retention volumes (V_R) of the solutes were evaluated at the peak maxima as the peaks were almost symmetrical. The capacity factors (k') were calculated from the equation

$$k' = (V_R - V_0)/V_0 \tag{1}$$

The peak widths (W) were measured at the baseline by graphical extrapolation of inflection points. The number of theoretical plates (N) was calculated from

$$N = 16\,(V_R/W)^2 \tag{2}$$

and the reduced height of a theoretical plate[8] (h) from

$$h = l/Nd \tag{3}$$

where l is the column length and d the diameter of the sorbent particles.

Computations

All computations were made with a Varian 620L computer with a 24K memory, programmed in Basic (E Basic version). The least-square error approximation of experimental points for capacity factors was made by

$$\log k' = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_4x_1^2 + a_5x_2^2 + a_6x_3^2 + a_7x_1x_2 + a_8x_1x_3 + a_9x_2x_3 \tag{4}$$

where $x_1 = \log c$, $x_2 = \text{pH}$ and $x_3 = 0.01d$, c being the molar concentration of ammonium formate solution and d the volume percentage concentration of ethanol.

The reduced height of a theoretical plate was approximated by the linear equation

$$\log h = b_0 + b_1x_1 + b_2x_2 + b_3x_3 \tag{5}$$

The normal equations for least-squares fit were solved using Cholesky's method[9]. Weights of all experimental points were set equal to unity.

RESULTS AND DISCUSSION

The approximation of experimental points by eqn. 4 is illustrated in Figs. 1–3, where the logarithm of capacity factor is plotted against molar concentration of ammonium formate. Comparing Fig. 1 with Fig. 2, it can be seen that with increasing pH the capacity factors of adenosine-based phosphates increase and approach the values for guanosine-based phosphates. An increase in ethanol concentration (Fig. 3) results in an increased selectivity of guanosine–adenosine phosphate pairs.

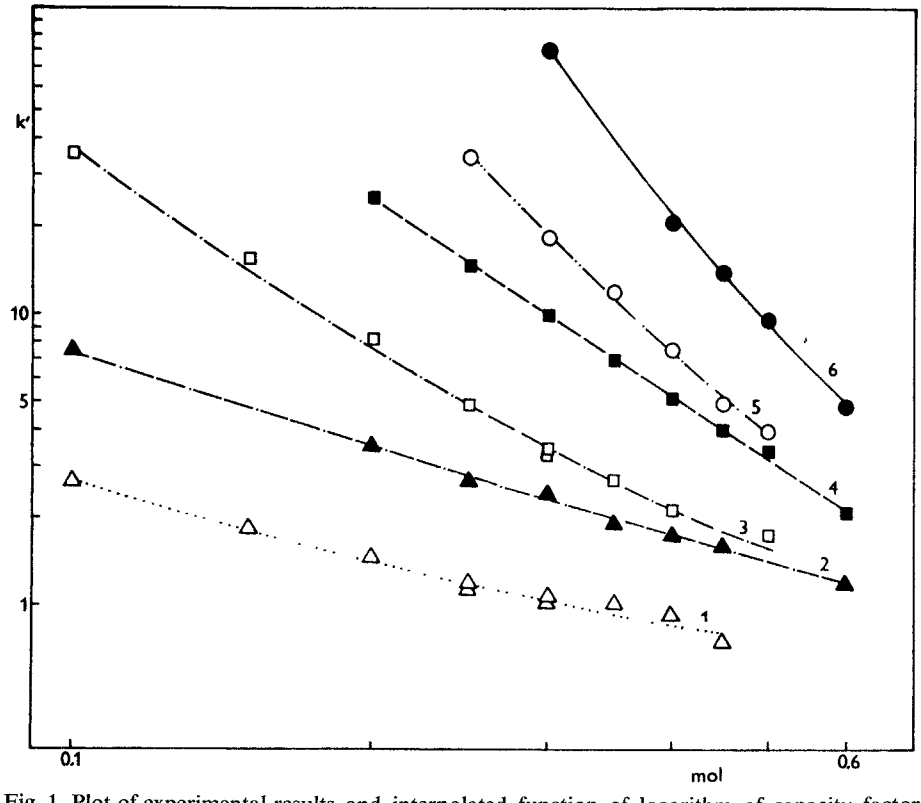

Fig. 1. Plot of experimental results and interpolated function of logarithm of capacity factor (k') *versus* molar concentration of ammonium formate (logarithmic scale) at pH 3.5; no ethanol in eluent. Curves: 1 = 5′-AMP; 2 = 5′-GMP; 3 = 5′-ADP; 4 = 5′-GDP; 5 = 5′-ATP; 6 = 5′-GTP.

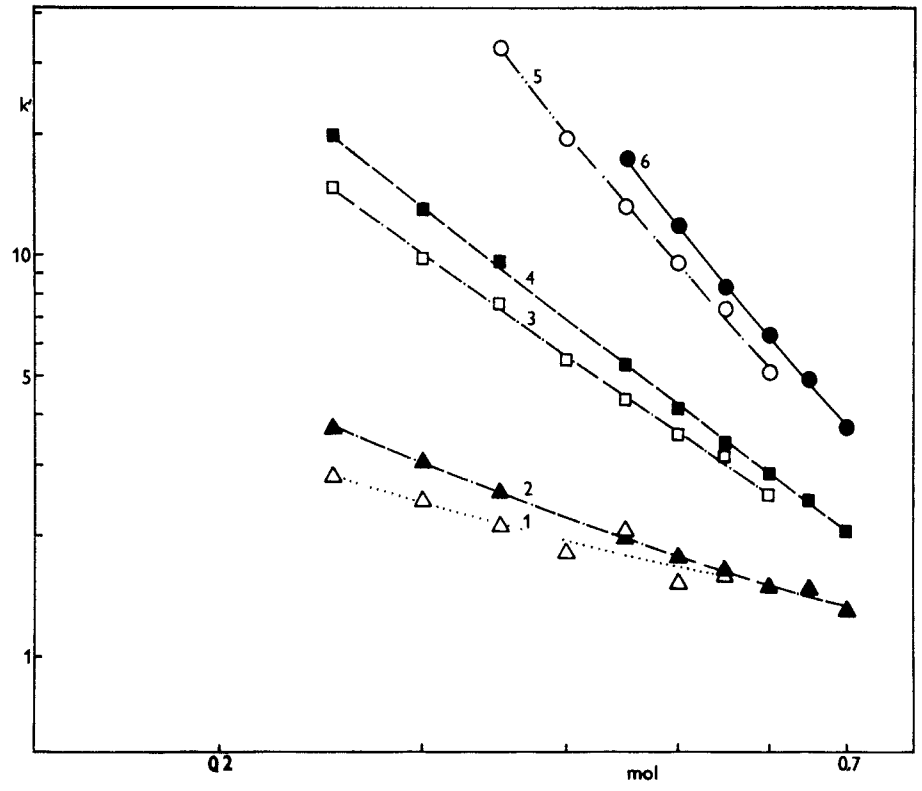

Fig. 2. As Fig. 1 at pH 5.

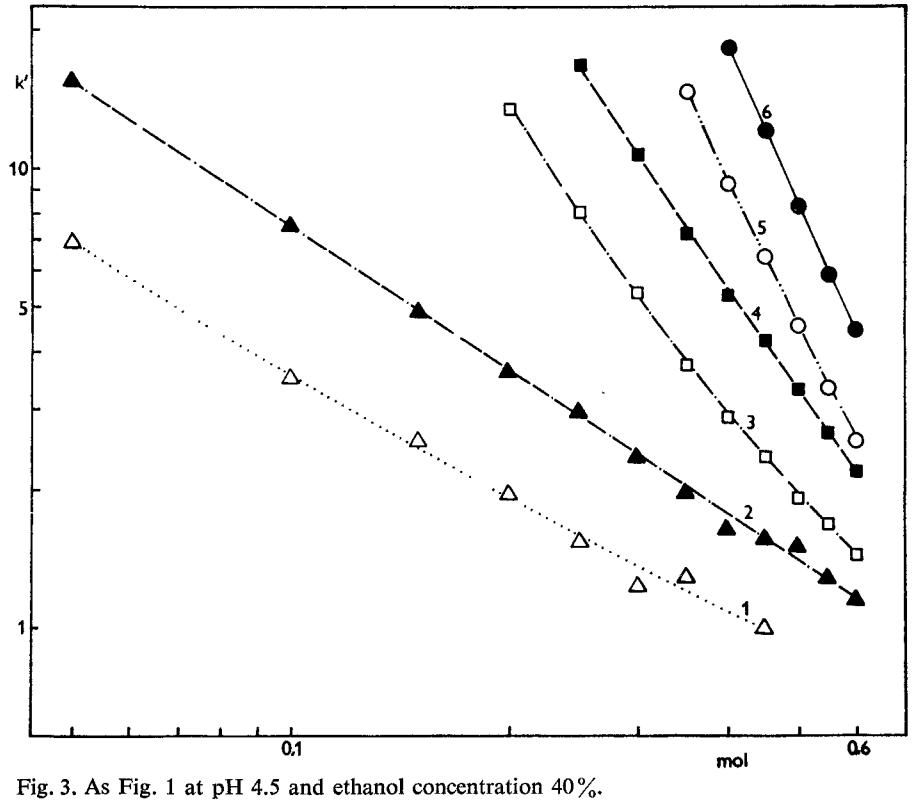

Fig. 3. As Fig. 1 at pH 4.5 and ethanol concentration 40%.

To be able to follow rationally all changes in this complicated system, where several components of the eluent (formate and hydrogen ions and ethanol) may change the behaviour of single solutes, the description using eqns. 4 and 5 was chosen as the most appropriate. The linearized equation for capacity factors would not reflect accurately the shape of experimentally measured functions; on the other hand, the higher degree polynomials would obviously be superfluous and would follow deviations mainly caused by experimental errors. It may not be excluded that deviations from linearity in the log k' equation are not caused by changes in the void volume with changes in the buffer concentration or by other influences, but even then the computed function reflects correctly the behaviour of the system if we are interested in chromatographic separations. A further advantage of compression of the results in this form is the possibility of making subsequent optimizations[10].

In Table I are summarized the coefficients of eqn. 4 for points measured within the ranges of ammonium formate concentration 0.02–0.7 M, pH 3.5–5 and volume concentration of ethanol 0–40% for most compounds. The concentration range of ammonium formate for di- and triphosphates was smaller; no measurements were made at concentrations where the capacity factors would be too high.

TABLE I

COEFFICIENTS OF LOG k' EQUATION

Compound	a_0	a_1	a_2	a_3	a_4	a_5	a_6	a_7	a_8	a_9
5′-AMP	−2.92	−0.730	1.07	−0.009	0.110	−0.095	0.267	0.018	−0.232	−0.149
5′-ADP	−3.77	−1.31	1.48	0.949	0.404	−0.144	−0.497	−0.053	−0.544	−0.330
5′-ATP	−5.86	−4.88	2.30	1.15	−0.937	−0.223	−1.14	0.220	−0.707	−0.378
5′-GMP	−1.72	−1.37	0.677	−1.06	0.038	−0.067	0.520	0.103	−0.218	0.113
5′-GDP	−2.12	−2.08	0.910	−0.786	0.438	−0.096	0.141	−0.057	−0.346	0.075
5′-GTP	−3.61	−4.20	1.60	−0.684	0.949	−0.171	−0.093	0.246	0.260	0.063
5′-CMP	−1.33	0.479	0.330	−0.760	0.130	−0.026	0.341	−0.213	−0.423	0.127
5′-CDP	−2.42	−0.366	0.730	0.362	0.253	−0.064	−0.411	−0.240	−0.798	−0.063
5′-CTP	−3.48	−0.666	1.31	0.366	0.993	−0.134	−0.864	−0.317	−0.974	−0.053
5′-UMP	−0.255	−1.19	−0.122	−0.607	0.013	−0.024	0.023	0.084	−0.303	−1.19
5′-UDP	−0.738	−1.85	0.153	−0.158	0.333	−0.009	−0.268	0.049	−0.609	0.049
5′-UTP	−3.13	−4.12	1.24	0.479	0.219	−0.134	−0.702	0.205	−0.151	−0.055

The coefficients a_0 decrease with increasing number of phosphate groups attached to the nucleoside; the same applies to a_1 coefficients. The coefficients a_2 increase with increasing number of phosphate groups, whereas a_3 has the lowest value for monophosphates and attains roughly the same value for di- and triphosphates. Similar regularities could also be found for the coefficients a_4, a_5 and a_6, reflecting the concave or convex bending of parabolic functions. The value of a_4 for 5′-ATP is probably influenced by experimental error; one erroneous point with too high a k' value causes bending of the interpolated function in an other direction. Most values of coefficients a_7–a_9 are small, fluctuate around zero and probably cannot be meaningfully interpreted.

In Table II are summarized the coefficients for the log h equation. With increasing concentration of ammonium formate the height of a theoretical plate in-

TABLE II

COEFFICIENTS OF LOG h EQUATION

Compound	b_0	b_1	b_2	b_3
5'-AMP	1.676	0.077	−0.011	0.544
5'-ADP	1.367	0.258	0.090	0.508
5'-ATP	2.141	0.781	−0.056	0.549
5'-GMP	1.754	0.174	−0.014	0.427
5'-GDP	1.704	0.363	0.015	0.453
5'-GTP	1.962	0.736	−0.024	0.523
5'-CMP	1.553	0.116	0.042	0.376
5'-CDP	1.974	0.377	−0.034	0.356
5'-CTP	1.905	0.573	−0.015	0.559
5'-UMP	1.571	0.272	0.059	0.414
5'-UDP	1.581	0.343	0.038	0.439
5'-UTP	1.897	0.920	0.011	0.403

creases; this is more pronounced for compounds with more phosphates groups attached (coefficient b_1). The influence of pH is not significant (b_2); on the other hand, the decrease in efficiency with increasing ethanol concentration manifests itself by a consistently high value of b_3.

The statistical characteristics (Table III) demonstrate that the fit is reasonably good. Under the heading *Error k'* are summarized the standard deviations of computed k' values from experimental points. The least-squares fitting was computed for log k' values; therefore the relative deviations are more or less constant for all values of k', but the absolute value of the standard deviation increases with increasing k'. One erroneous point for 5'-ATP manifests itself by a high value of the k' error.

TABLE III

STATISTICS OF APPROXIMATIONS

Compound	*No. of points*	*Error log k'*	*Error k'*	*Error log h*
5'-AMP	82	0.013	0.189	0.086
5'-ADP	82	0.031	0.494	0.104
5'-ATP	56	0.032	2.30	0.050
5'-GMP	83	0.009	0.126	0.066
5'-GDP	72	0.008	0.343	0.064
5'-GTP	45	0.005	0.637	0.042
5'-CMP	67	0.020	0.093	0.100
5'-CDP	76	0.021	0.545	0.073
5'-CTP	60	0.013	0.403	0.071
5'-UMP	76	0.019	0.168	0.081
5'-UDP	75	0.012	0.553	0.078
5'-UTP	48	0.008	0.424	0.052

Figs. 4 and 5 illustrate the influence of ammonium formate concentration on the capacity factors of purine and pyrimidine nucleotides, respectively. Figs. 6 and 7 show the influence of pH and Figs. 8 and 9 the concentration of ethanol on capacity factors. All of these graphs were computed from approximated functions and clearly demonstrate the influence of single eluent parameters on separation.

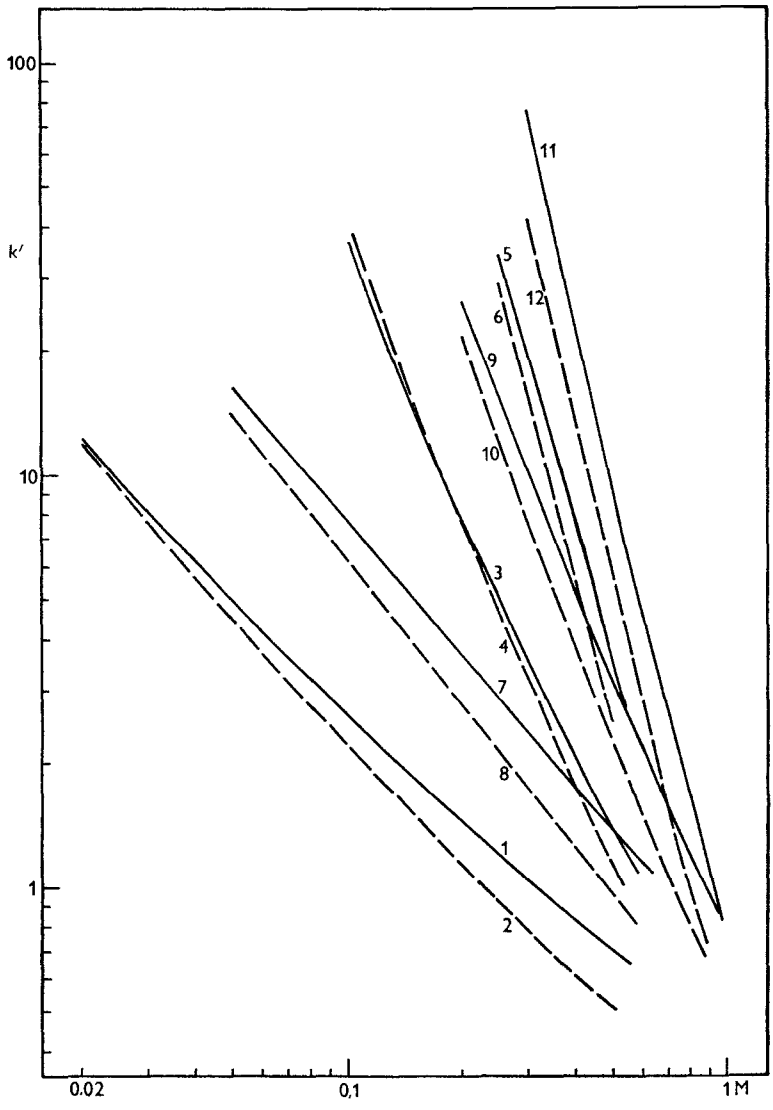

Fig. 4. Function of log k' *versus* ammonium formate concentration (logarithmic scale) for purine nucleotides. Ethanol concentration: full curves (odd numbers), 0%; broken curves (even numbers), 40%. Eluent pH: 3.5. Curves: 1, 2 = 5'-AMP; 3, 4 = 5'-ADP; 5, 6 = 5'-ATP; 7, 8 = 5'-GMP; 9, 10 = 5'-GDP; 11, 12 = 5'-GTP.

For the separation of mono-, di- and triphosphates the most important factor is the concentration of ammonium formate. As can be seen from Figs. 4 and 5, by a proper choice of gradient the separation can be performed successfully; however, isocratic separation of all mono-, di- and triphosphates is clearly impossible. Intersection of the curves for mono- and diphosphates and di- and triphosphates occurs at a concentration of ammonium formate of about 0.5 *M* (5'-GMP and 5'-ADP; 5'-GDP and 5'-ATP in Fig. 4) for purine nucleotides, and this concentration does not

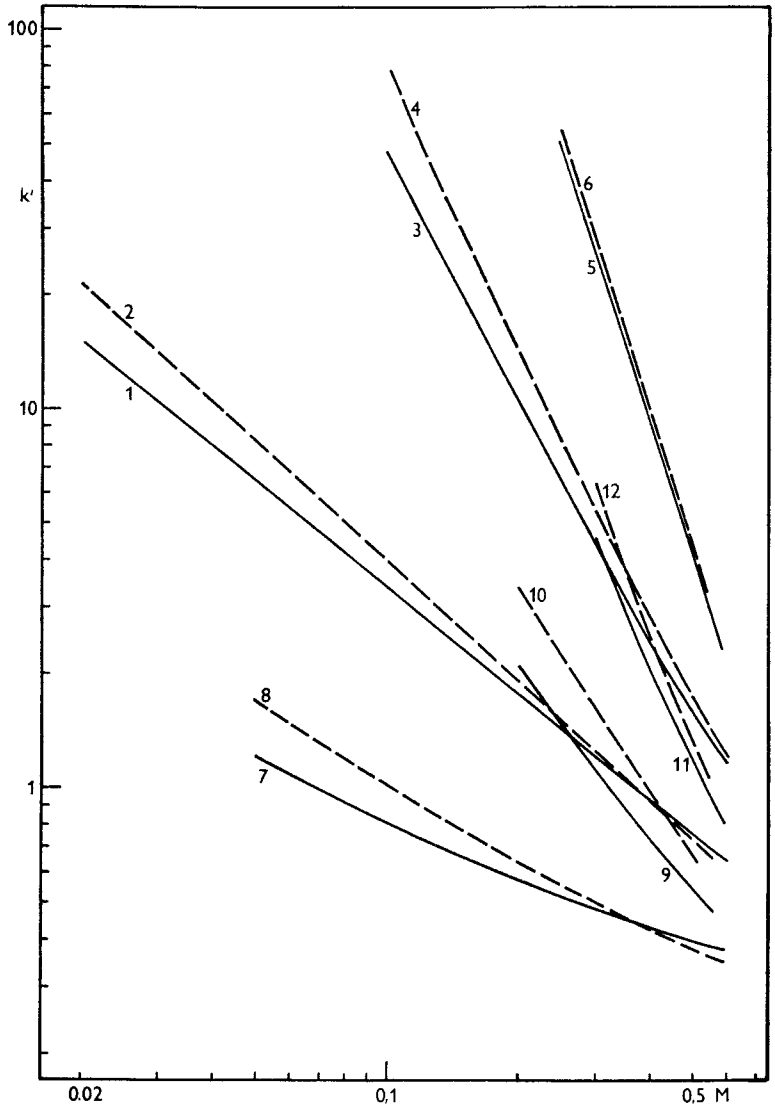

Fig. 5. As Fig. 4, for pyrimidine nucleotides. Curves: 1, 2 = 5′-UMP; 3, 4 = 5′-UDP; 5, 6 = 5′-UTP; 7, 8 = 5′-CMP; 9, 10 = 5′-CDP; 11, 12 = 5′-CTP.

change with addition of ethanol. On the other hand, the position of the intersections for pyrimidine nucleotides (Fig. 5) occurs at a lower concentration, but is shifted to higher eluent concentration on addition of ethanol.

Another important parameter is the acidity of the eluent (Figs. 6 and 7). This parameter may strongly influence the separation of nucleotides with easily protonatable NH_2 groups (adenosine and cytidine nucleotides) from uridine and guanosine nucleotides. The curves for eluents with and without ethanol run mostly parallel for pyrimidine nucleotides (Fig. 7); only the curves for monophosphates show a slight

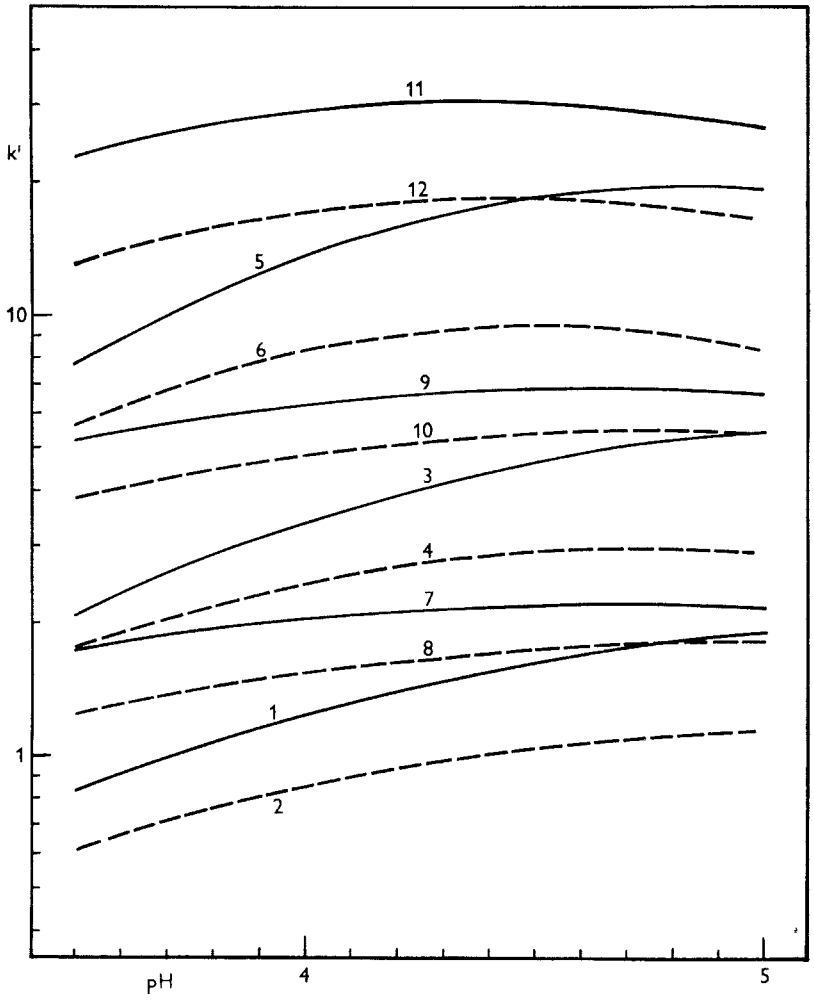

Fig. 6. Function of log k' *versus* pH of eluent for purine nucleotides. Ammonium formate concentration: 0.4 *M*. Ethanol concentration: full curves (odd numbers), 0%; broken curves (even numbers), 40%. Curves as in Fig. 4.

divergence. This is most evident for all adenosine-based nucleotides (Fig. 6). The difference between purine and pyrimidine nucleotides is most pronounced if the influence of ethanol concentration on the capacity factors is followed (Figs. 8 and 9). For most purine nucleotides the capacity factors decrease with increasing ethanol concentration (Fig. 8), but they remain essentially constant for pyrimidine nucleotides (Fig. 9). This behaviour is not strongly influenced by changes in pH.

When we tried to correlate the values of the coefficients of the capacity factor equations with the structures of the nucleotides (Table I), poor results were obtained. This is due to the limited range of variables for which experiments could be carried out, inherent experimental errors and the fact that the influence of every variable is divided into three terms in the log k' equation.

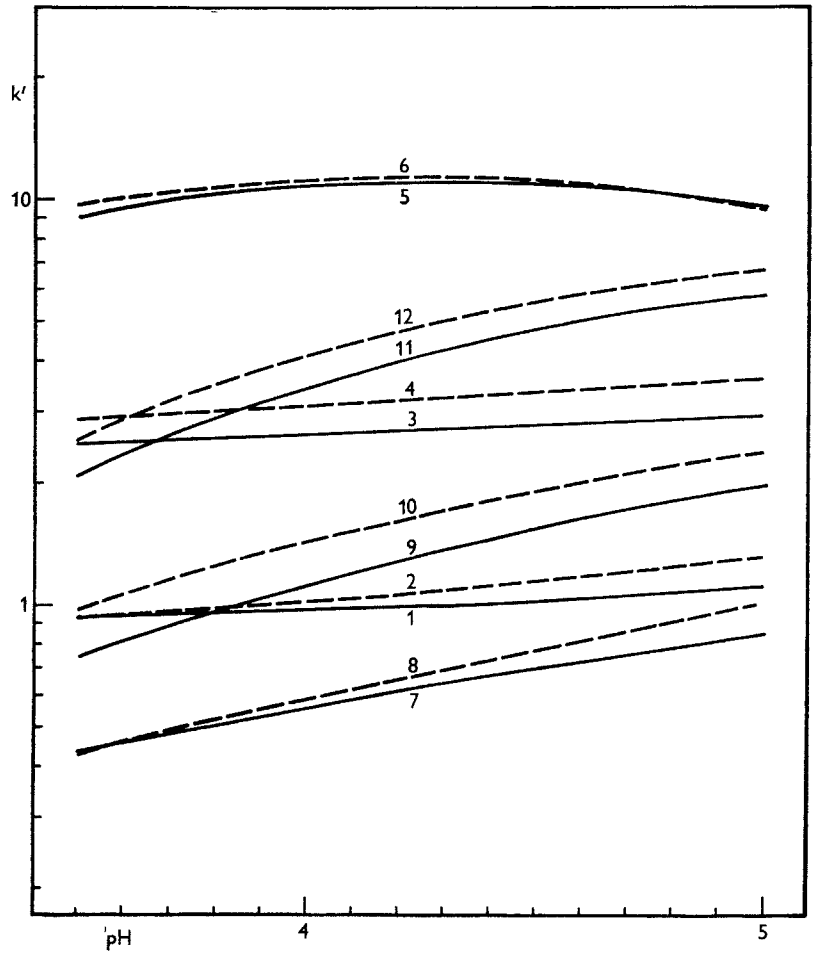

Fig. 7. As Fig. 6, for pyrimidine nucleotides. Curves as in Fig. 5.

If values of log k' and first derivatives of log k' with respect to the logarithm of the ammonium formate concentration, pH and volume concentration of ethanol at various points of the experimentally studied range of variables are compared (Tables IV–VII), some relationships seem to be evident.

The increments of log k' with increasing number of phosphate groups are only weakly influenced by the structure of the nucleotide base; they seem be slightly lower only for cytidine nucleotides. The increment between log k' values for 5'-CDP–5'-CMP in Table VII is probably too low owing to experimental errors connected with the measurement of very low k' values. The increments are mostly influenced by anion concentration; the influence of ethanol and pH is smaller. The first derivatives of log k' with respect to the logarithm of anion concentration (d log k'/d log c) increase regularly with increasing number of phosphate groups. They are slightly lower at higher anion concentrations, and the influence of ethanol concentration and pH is negligible. Why its value is always smaller for 5'-CTP than for the other triphosphates is not clear.

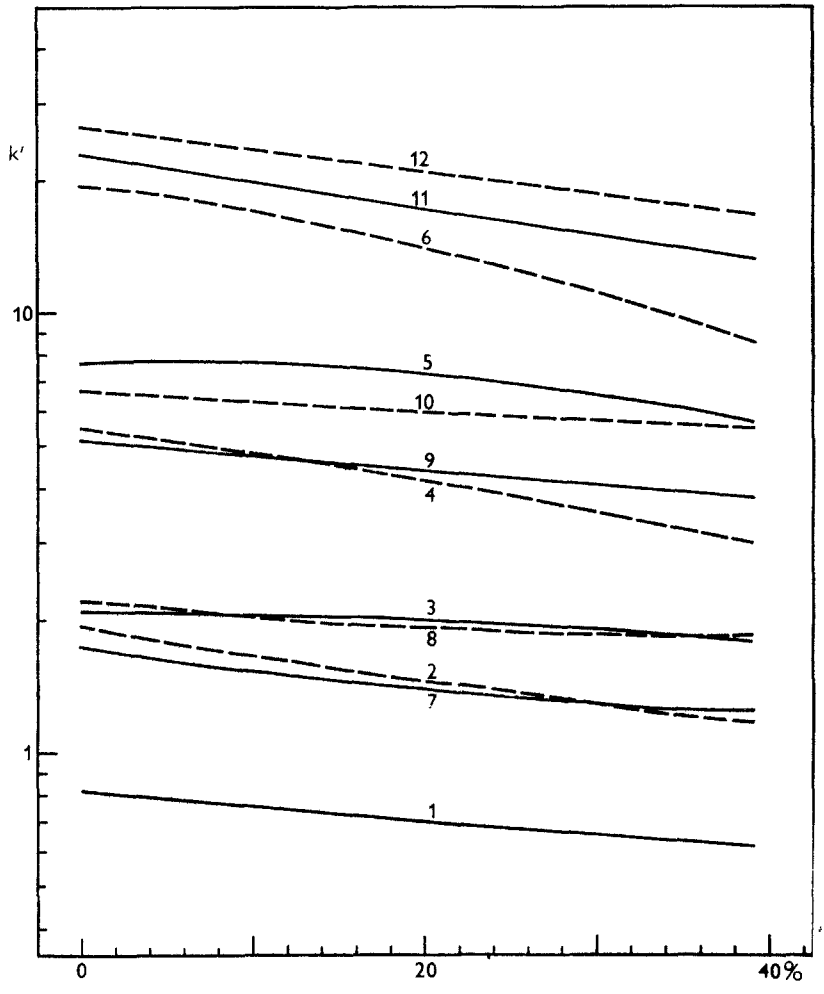

Fig. 8. Function of log k' *versus* ethanol concentration (vol.-%) for purine nucleotides. Ammonium formate concentration: 0.4 *M*. Full curves (odd numbers), pH 3.5; broken curves (even numbers), pH 5. Curves as in Fig. 4.

Derivatives with respect to pH at lower anion concentrations (Table IV) are influenced only by the structure of the base and by the substutition of the base by amino groups (Table IV). At higher anion concentrations (Table VII) these values remain constant for mono- and diphosphates, but for triphosphates a more or less pronounced increase is observed. Both ethanol concentration and pH influence the value of this derivative, as would be expected. The derivative of log k' with respect to ethanol concentration is negative at low buffer concentration for purine nucleotides and positive for all pyrimidine nucleotides. This is clear evidence of different anion exchanger–solute interactions for both types of compounds. This interaction is influenced both by ethanol concentration and pH (Tables V–VII) at higher anion con-

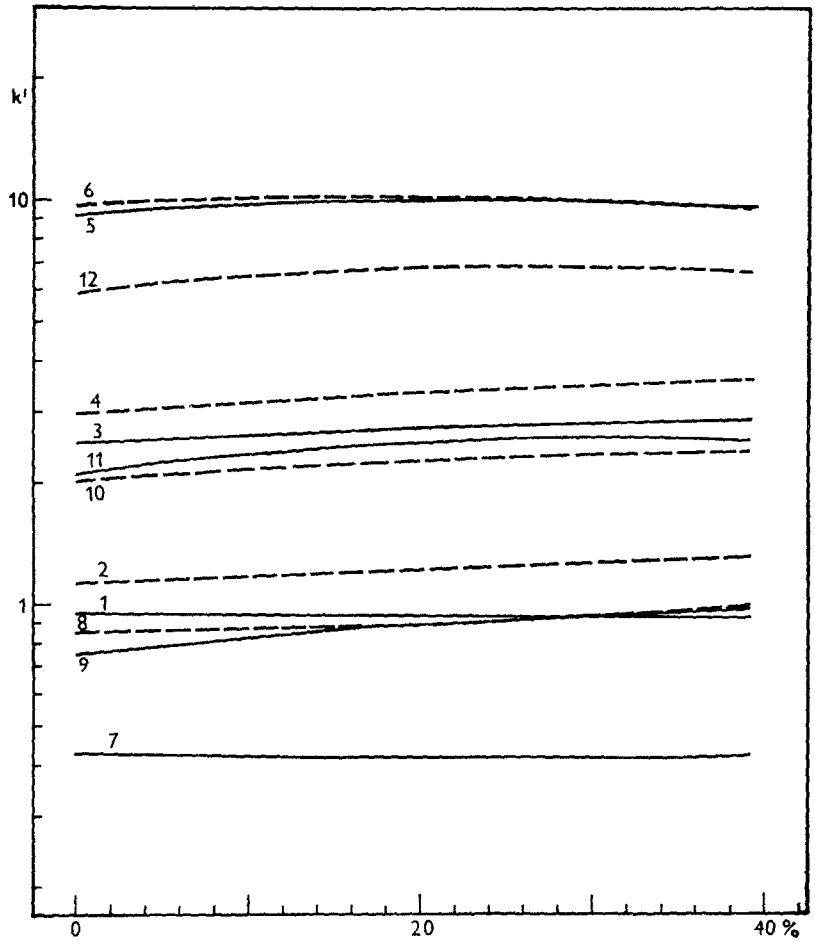

Fig. 9. As Fig. 8, for pyrimidine nucleotides. Curves as in Fig. 5.

TABLE IV

VALUES OF LOG k' AND FIRST DERIVATIVES OF LOG k' FUNCTION WITH RESPECT TO x_1, x_2 AND x_3 VARIABLES

Ammonium formate concentration, 0.3 M; pH, 4; ethanol concentration, 20%.

Compound	*Log k'*	*Δ log k'*	*∂ log k'/∂x₁*	*∂ log k'/∂x₂*	*∂ log k'/∂x₃*
5'-AMP	0.098	–	−0.821	0.260	−0.378
5'-ADP	0.730	0.632	−2.059	0.292	−0.283
5'-ATP	1.483	0.753	−3.169	0.279	−0.449
5'-GMP	0.355	–	−1.046	0.112	−0.281
5'-GDP	1.023	0.668	−2.384	0.129	−0.248
5'-GTP	1.875	0.652	−4.157	0.110	−0.607
5'-CMP	−0.184	–	−0.597	0.260	0.106
5'-CDP	0.331	0.515	−1.753	0.333	0.364
5'-CTP	0.990	0.659	−3.169	0.393	0.317
5'-UMP	0.111	–	−0.934	0.053	0.090
5'-UDP	0.730	0.619	−2.127	0.057	0.249
5'-UTP	1.512	0.782	−3.563	0.047	0.059

TABLE V

VALUES OF LOG k' AND FIRST DERIVATIVES OF LOG k' FUNCTION WITH RESPECT TO x_1, x_2 AND x_3 VARIABLES

Ammonium formate concentration, 0.6 M; pH, 3.5; no ethanol in eluent.

Compound	*Log k'*	*Δ log k'*	*∂ log k'/∂x₁*	*∂ log k'/∂x₂*	*∂ log k'/∂x₃*
5'-AMP	−0.210	—	−0.717	0.391	−0.481
5'-ADP	0.009	0.219	−1.680	0.486	−0.083
5'-ATP	0.263	0.254	−3.701	0.651	−0.016
5'-GMP	0.056	—	−1.031	0.188	−0.611
5'-GDP	0.332	0.276	−2.079	0.227	−0.447
5'-GTP	0.665	0.333	−3.761	0.343	−0.522
5'-CMP	−0.426	—	−0.327	0.197	−0.220
5'-CDP	−0.368	0.058	−1.321	0.338	0.320
5'-CTP	−0.100	0.268	−2.218	0.442	0.397
5'-UMP	−0.188	—	−0.907	0.028	−0.076
5'-UDP	0.062	0.250	−1.829	0.072	0.149
5'-UTP	0.335	0.273	−3.503	0.254	0.322

TABLE VI

VALUES OF LOG k' AND FIRST DERIVATIVES OF LOG k' FUNCTION WITH RESPECT TO x_1, x_2 AND x_3 VARIABLES

Ammonium formate concentration, 0.6 M; pH, 5; ethanol concentration, 20%.

Compound	*Log k'*	*Δ log k'*	*∂ log k'/∂x₁*	*∂ log k'/∂x₂*	*∂ log k'/∂x₃*
5'-AMP	0.031	—	−0.736	0.074	−0.598
5'-ADP	0.278	0.247	−1.869	−0.013	−0.776
5'-ATP	0.560	0.282	−3.512	−0.113	−1.039
5'-GMP	0.121	—	−0.919	0.011	−0.234
5'-GDP	0.397	0.276	−2.062	−0.045	−0.278
5'-GTP	0.704	0.307	−3.338	−0.159	−0.466
5'-CMP	−0.182	—	−0.732	0.144	0.107
5'-CDP	0.024	0.206	−1.841	0.133	0.061
5'-CTP	0.291	0.267	−2.886	0.030	−0.029
5'-UMP	−0.065	—	−0.842	0.127	0.131
5'-UDP	0.182	0.247	−1.877	0.052	0.115
5'-UTP	0.433	0.251	−3.225	−0.160	−0.041

TABLE VII

VALUES OF LOG k' AND FIRST DERIVATIVES OF LOG k' FUNCTION WITH RESPECT TO x_1, x_2 AND x_3 VARIABLES

Ammonium formate concentration, 0.6 M; pH, 4; ethanol concentration, 20%.

Compound	*Log k'*	*Δ log k'*	*∂ log k'/∂x₁*	*∂ log k'/∂x₂*	*∂ log k'/∂x₃*
5'-AMP	−0.139	—	−0.754	0.266	−0.448
5'-ADP	0.147	0.286	−1.816	0.276	−0.446
5'-ATP	0.445	0.298	−3.733	0.346	−0.661
5'-GMP	0.044	—	−1.022	0.144	−0.347
5'-GDP	0.346	0.302	−2.119	0.146	−0.352
5'-GTP	0.692	0.346	−3.585	0.184	−0.529
5'-CMP	−0.352	—	−0.518	0.196	−0.021
5'-CDP	−0.174	0.178	−1.601	0.265	0.124
5'-CTP	0.126	0.273	−2.571	0.298	0.024
5'-UMP	−0.168	—	−0.926	0.079	−0.001
5'-UDP	0.120	0.288	−1.926	0.072	0.066
5'-UTP	0.459	0.339	−3.431	0.109	0.013

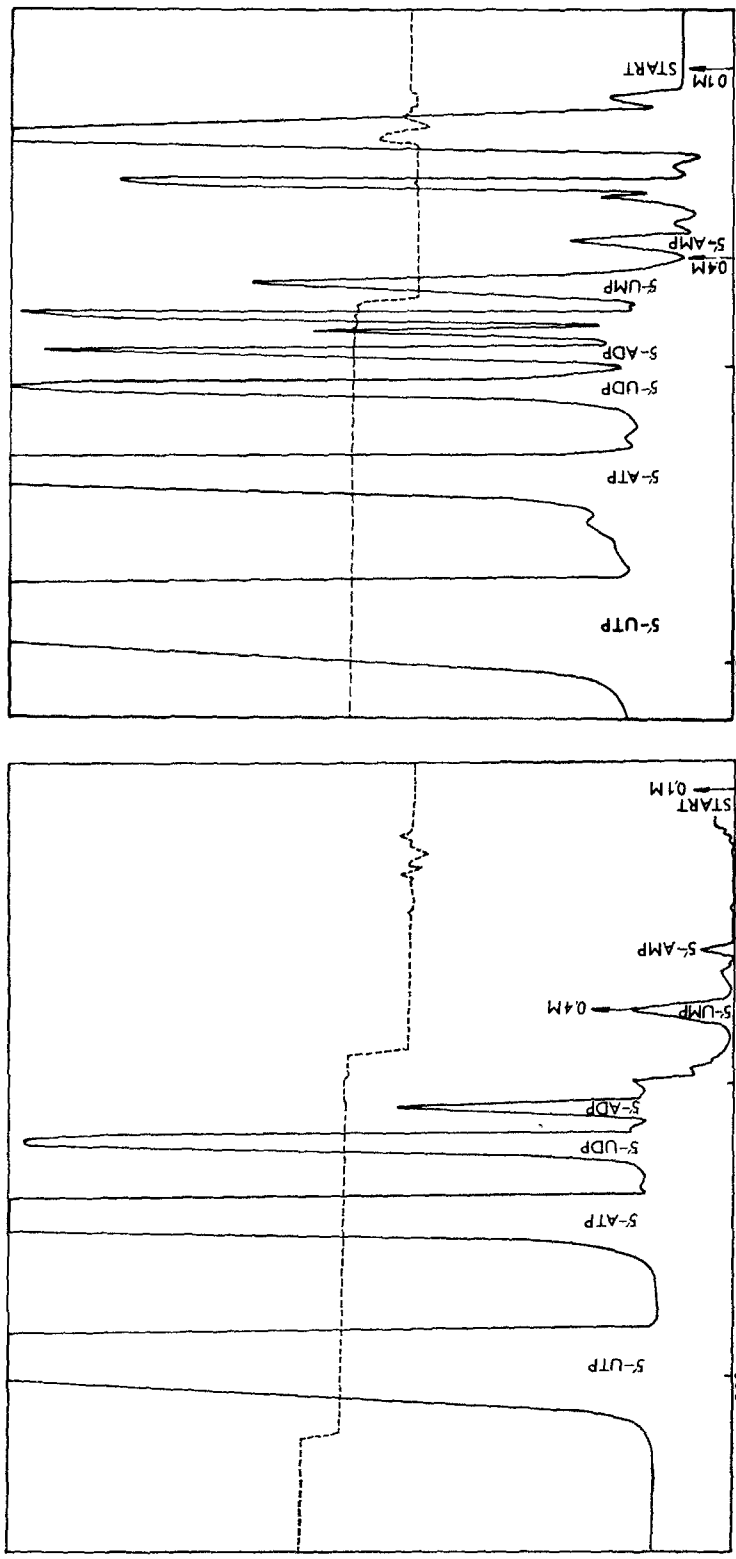

Fig. 10. Separation of a synthetic sample of 5'-AMP, 5'-ADP, 5'-ATP, 5'-UMP, 5'-UDP and 5'-UTP. Vertical axis; full line, absorbance (linear scale, arbitrary units); broken line, eluent conductivity (logarithmic scale, arbitrary units). Column, 250 × 10 mm I.D.; eluent, ammonium formate (0.1 and 0.4 *M*) (pH 3.5) —40% ethanol; temperature, 25°C; total amount of sample, 20 mg.

Fig. 11. As Fig. 10, using a real sample from a biological experiment.

centration as the log k' values of di- and triphosphates are more strongly dependent on ethanol concentration than the log k' values of monophosphates.

The practical application of the system is illustrated in Figs. 10 and 11 for the preparative chromatography of nucleotides. The detailed knowledge of the system enabled us to choose the conditions under which a complicated biological system could be effectively separated.

The results of this study will enable one to make a rational choice of an optimal chromatographic system for nucleotide separations and, by comparison with results for nucleotide separations on other sorbent, will enable one to draw conclusions about the mechanism of the interactions between the solute, eluent and anion exchanger.

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