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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PYRIMIDINE BASES AND NUCLEOSIDES

APPLICATION OF SOLVOPHOBIC THEORY

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SUMMARY

The chromatographic behaviour of some natural and modified pyrimidine bases and nucleosides on an octadecyl stationary phase was studied. The retention and selectivity parameters of the separation of the compounds studied were derived on the basis of solvophobic theory. The mechanism of base and nucleoside interactions with the surface of the hydrocarbonaceous stationary phase is discussed. The best separation is observed at pH 3.5 for the bases and at pH 4.8–5.2 for the nucleosides. An increase in the solute surface tension results in an increased selectivity of separation. When the surface tension and the ionic strength of the mobile phase are not kept constant, there are considerable deviations in retention from that predicted by solvophobic theory.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is widely used to determine the components of nucleic acids in different materials[1–7]. The need to find optimal separation conditions makes it important to determine the interrelationship between the retention parametes, the solute state and the structure, in order to be able to predict the chromatographic behaviour of the compounds studied and to search for the optimal conditions of analysis.

The HPLC of pyrimidine bases and nucleosides has been studied by many workers and the results obtained have been generalized and reviewed[1–4]. Earlier publications[5–8] were largely concerned with solving the practical problems of analysing different components of nucleic acids and their analogues and with investigating the effects of the stationary phase properties on retention[9–13]. Far fewer papers have been devoted to studying the interrelationship between the structure of pyrimidine bases

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and nucleosides and their retention[3,14–16]. The study of the chromatographic behaviour of different compounds in order to derive quantitative characteristics within the framework of the currently popular solvophobic theory of retention[15,17,18] has not yet been attempted.

Attempts to explain the chromatographic behaviour of pyrimidine derivatives sometimes run into difficulties. It is observed, for example, that it is very difficult to elucidate the specific features of the retention of pyrimidine bases, because acid and base groups and different substituents are simultaneously present in the molecule. The conclusion has been drawn that the effect of pH on capacity factors is intricate and the relationship between the retention and ionization constants for these compounds is non-existent[19]. The more than double increase in the retention time of 5-methylated bases was interpreted as resulting from the compounds being converted into the lactim form and from the "stacking" process present[19]. The concept of vertical "stacking" to explain the increase in the chromatographic retention of the bases under RP-HPLC conditions has been employed[16,19]. The chromatographic behaviour is observed to be little changed for 6-aza pyrimidine bases[19], whereas their acid–base properties they are very different from those of the unmodified compounds.

The absence of a relationship between the retention and acid–base constants and the ionized state of pyrimidine derivatives in solution is at variance with the basic propositions in solvophobic theory for the retention of ionogenic substances on hydrophobic sorbents[15,18] and also with the results of studies of the effect of the mobile phase composition on the retention of bases and nucleosides[4,16].

The aims of this work were to study the chromatographic behaviour and the relationship between the thermodynamic characteristics of the retention of pyrimidine compounds and their structure and state in solution and to determine the thermodynamic retention and selectivity parameters for natural and modified analogues of bases and nucleosides, to elucidate the reason for the deviations, reported by Štulik and Pacáková[19], of the chromatographic behaviour of pyrimidine derivatives from that predicted by solvophobic theory and to determine the optimal conditions for the chromatography of these compounds on a hydrophobic sorbent.

EXPERIMENTAL

Chromatographic conditions

The experiments were performed on an HG-1305 liquid chromatograph (Nauch-Pribor Assoc., Orel, U.S.S.R.) equipped with a variable-wavelength detector set 265 nm (sensitivity 0.005 a.u.f.s.) and on an LKB (Bromma, Sweden) liquid chromatographic system consisting of a Model 2151 variable-wavelength monitor, a Model 2150 HPLC pump, a Model 2152LC controller, a Model 2154 injector and a Model 2220 recording integrator. The columns used were a Silasorb C_{18} (15 μm) glass column (15.0 × 0.1 cm I.D.) (Lachema, Brno, Czechoslovakia) connected to the HG-1305 chromatograph and a Separon SIX C_{18} (5 μm) column (15.0 × 3.3 mm I.D.) (Laboratorni pristroje, Prague, Czechoslovakia) or Bondapak C_{18} (10 μm) column (30.0 × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) connected to the LKB liquid chromatographic system. The mobile phase consisted of 0.1 *M* phosphate buffer in 0.1–2.5 *M* ammonium sulphate solution at a flow-rate of 0.03 ml/min on the HG-1305 chromatograph and 0.4 ml/min on the LKB system.

Materials

Compound names are abbreviated as in Table I. 6-AzaCyd was obtained from Institute of Molecular Biology and Genetics of the Ukrainian S.S.R. Academy of Sciences and 5-AzaCyd and Ara-C from Chemical Dynamic Corp. (South Plainfield, NJ, U.S.A.). Other bases and nucleosides were supplied by (Reachim, Moscow, U.S.S.R.). Orthophosphoric acid and ammonium sulphate were obtained commercially (analytical-reagent grade) and were used without further purification. Water was doubly distilled and filtered for HPLC use.

RESULTS AND DISCUSSION

All the compounds studied are ampholytes, but within the pH range used in RP-HPLC (1–8) they exhibit either basic or acidic properties. The values of the constants that characterize the acid–base properties are given in Table I. The pK_a and pK_b values of 6-AzaCyd, 6-AzaCyt, 6-AzaUrd and Ara-C were determined spectrophotometrically. The pK_b value of 5-AzaCyd was determined by the RP-HPLC method, which requires a much smaller amount of material[20]. The pK values were determined at an ionic strength of 0.3.

As can be seen from the results in Table I, the replacement of carbon atoms in the pyrimidine heterocycle at position 6 by a more electronegative nitrogen atom appreciably increases the acidic and decreases the basic properties of the compounds. The effect of replacement of a carbon atom at position 5 is much less pronounced.

The changes in the acid–base properties of pyrimidine bases and nucleosides significantly affect their chromatographic behaviour. Fig. 1 shows the dependences of the capacity factors (k') of the compounds studied on the eluent pH at constant ionic strength. The sharp decrease in the capacity factors with transition of the compounds from the molecular into the ionized form is observed for 5- and 6-aza deriv-

TABLE I

pK_{ab} AND pK_{aa} VALUES OF BASES AND NUCLEOSIDES

Compound	pK_{aa}	pK_{ab}
Bases		
Uracil (Ura)	9.5*	
Cytosine (Cyt)		4.5*
Thymine (Thy)	9.9*	
6-AzaCytosine (6-AzaCyt)		1.57
6-AzaUracil (6-AzaUra)	7.00	
6-AzaThymine (6-AzaThy)	7.16	
Nucleosides		
Cytidine (Cyd)		4.2*
Uridine (Urd)	9.2*	
Thymidine (Thd)	9.8*	
6-AzaCytidine (6-AzaCyd)		1.38
6-AzaUridine (6-AzaUrd)	6.7	
Arabinosinecytosine (Ara-C)		4.35
5-AzaCytidine (5-AzaCyd)		4.00

* From ref. 2.

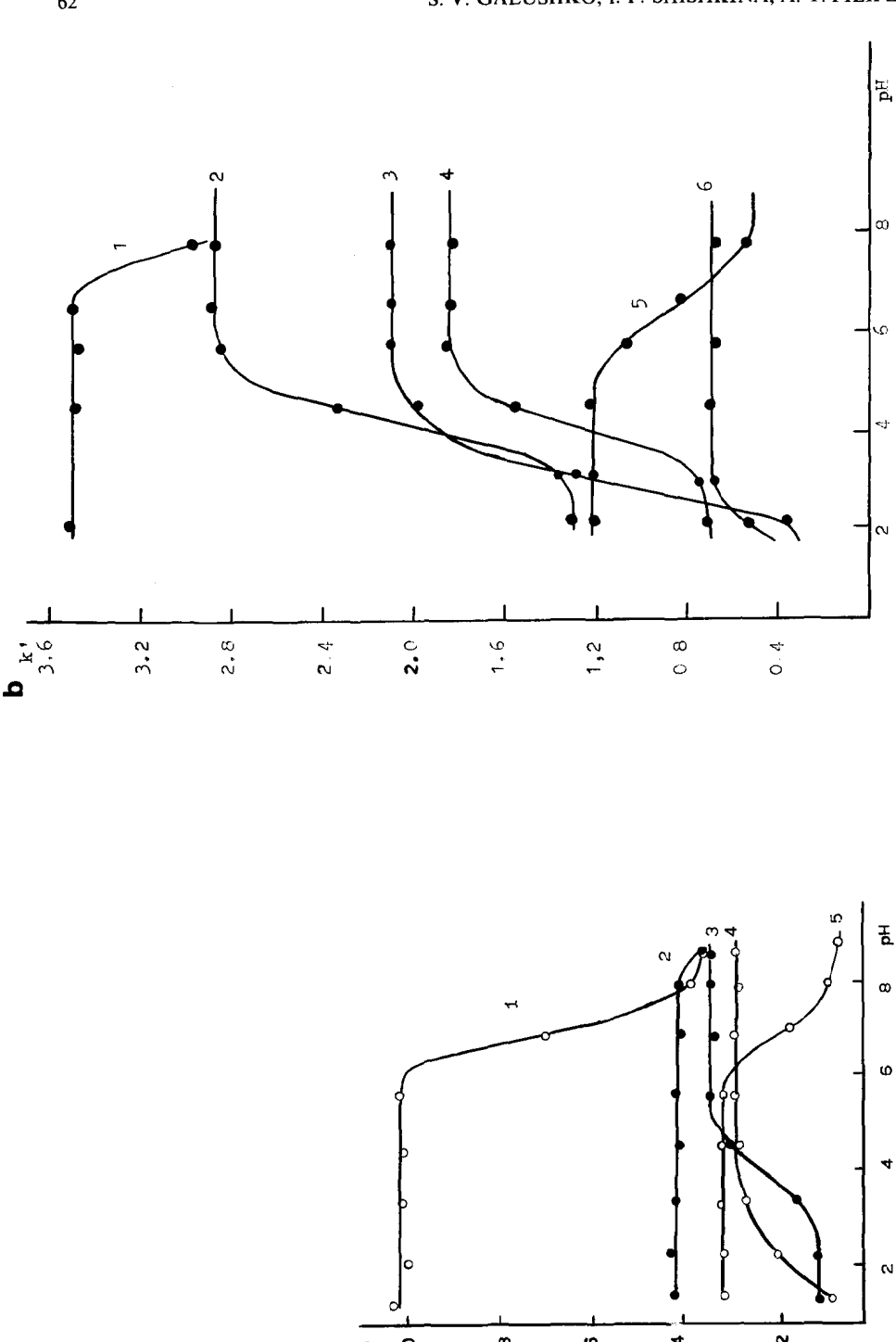

Fig. 1. Effect of pH on the retention of pyrimidine bases and nucleosides. Stationary phase: Silasorb C_{18} (15 μm). Mobile phase: 1.0 *M* ammonia sulphate in 0.1 *M* phosphate buffer. (a) 1 = 6-AzaThy; 2 = Ura; 3 = Cyt; 4 = 6-AzaCyt; 5 = AzaUra. (b) 1 = Urd; 2 = Ara-C; 3 = 5-AzaCyd; 4 = Cyd; 5 = 6-AzaUrd; 6 = 6-AzaCyd. Curves for Cyd and Ara-C correspond to those theoretically calculated by using eqn. 1.

atives at much lower pH than that for unmodified bases and nucleosides. As was shown theoretically and confirmed experimentally, the retention of weak acids and bases is defined as follows[15,18,20]:

$$k' = \frac{k'_0 + ak'_i}{1 + a} \quad (1)$$

where k'_0 and k'_i are the capacity factors of the molecular and ionized forms, respectively, $a = K_a/[H^+]$ for weak acids and $[H^+]/K_a$ for weak bases and K_a is the acid dissociation constant. At constant ionic strength, we obtained the values of k'_0 and k'_i for the compounds which, within the range of the mobile phase pH values (1–8), can be completely in molecular and ionized forms, and using eqn. 1 we calculated the dependences $k' = f(pH)$.

As can be seen from Fig. 1, the calculated curves correspond exactly to the experimental values, and the position of points $0.5(k' + k'_i)$ correspond to the pK_{ab} values for Cyd, Ara-C and Cyt on the pH axis. Other workers obtained the dependences $k' = f(pH)$ for different pyrimidine bases that were basically different from those obtained in this work (Fig. 1). They used a 0.1 *M* citrate–phosphate buffer, the pH of which was varied from 2 to 6 by adding phosphoric acid or sodium hydroxide. When 0.1 *M* citric acid is neutralized sodium citrate solution is finally obtained, *i.e.*, a weakly acidic solution with low ionic strength is transformed into a solution of a totally dissociating salt of the same molar concentration, but with a much higher ionic strength.

It should be noted that citric and acetic acids, in addition to other organic acids, are surface-active substances that lower appreciably the surface tension of a solution[21]. The salts of these acids are far less capable of lowering the surface tension of solutes[21]. Therefore, when the solution of an organic acid goes into an acid salt solution of the same concentration, surface tension of the mobile phase may rise appreciably[21], and this is known to lead to a change in capacity factor[17,18]. If such buffers are used, the capacity factor of an ionogenic substance changes not only with a change in the ionic state of such a compound, but also with changes in the ionic strength and mobile phase surface tension[18].

Fig. 2 shows the dependences of the capacity factors of some pyrimidine compounds in 0.1 *M* acetate buffers. These dependences are similar to those given in ref. 19 for citrate buffers and very different from those in phosphate buffers where the ionic strength and surface tension remain almost constant. The results obtained indicate that the behaviour of the pyrimidine compounds studied corresponds to that predicted by solvophobic theory for ionogenic compounds and is dependent on the acid–base properties of the compounds. The deviations in buffer solutions of weak organic acids observed here and previously[19] may be attributed to the fact that the ionic strength and surface tension of mobile phase solutes are not kept constant.

According to "solvophobic" theory[17,18], the energy for retaining substances on hydrophobic sorbents is defined by the sum of the energies due to Van der Waals (ΔF_{VdW}) and electrostatic (ΔF_{es}) interactions in a solution and the energy needed to produce a cavity in a solvent, ΔF_{cav}:

$$-RT \ln k' = \Delta F_{as.gas} + \Delta F_{cav} + \Delta F_{VdW} + \Delta F_{es} + \varphi \quad (2)$$

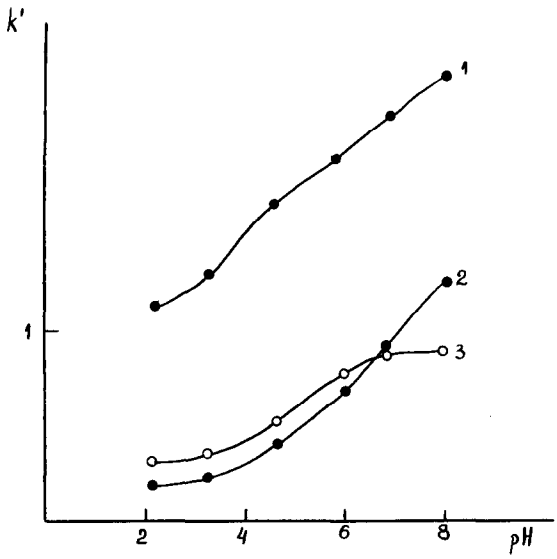

Fig. 2. Effect of pH on the retention of nucleosides. Stationary phase: Silasorb C_{18} (15 μm). Mobile phase: 0.1 *M* acetate buffer. 1 = Cyd; 2 = 6-AzaCyd; 3 = 6-AzaUrd.

where $\Delta F_{as.gas}$ is the change in free energy resulting from a substance interacting with a surface in the phase and φ is the phase ratio in the column. The decrease in retention when a substance goes from the molecular to the ionized form is determined almost solely by changes in the electrostatic interaction of the substance with the solvent[18]. By varying the hydrogen ion concentration and keeping the ionic strength and surface tension constant, we can determine the thermodynamic difference between the two forms of the substance, caused by a change in electrostatic interaction in the mobile phase:

$$-\Delta(\Delta F_{es}) = RT \ln \frac{k'_0}{k'_i}$$

The values of k'_0 and k'_i were derived from the results of studying the dependences $k' = f(pH)$ (Fig. 1). When total ionization could not be achieved in the chromatographic experiment, the values of k'_i were calculated using eqn. 1. The results are given in Table II. The values show that 5- and 6-aza derivatives are characterized by large $\Delta(\Delta F_{es})$ values and this may indicate both the smaller size of the molecules and the lower dipole moments compared with other compounds[18].

It has been shown that for uncharged forms, only the following term changes with changing surface tension and ionic strength of a solution[18]:

$$-\Delta F_{cav} = [N\Delta\Phi + 4.836N^{1/3}(k^e - 1)V^{2/3}]\gamma \quad (3)$$

where N is Avogadro's number, V the molar volume of the eluent, $\Delta\Phi$ the area of hydrophobic contact with the hydrocarbon sorbent surface, k^e a constant dependent

TABLE II

RETENTION PARAMETERS CHARACTERIZING THE TRANSFORMATION OF PYRIMIDINE BASES AND NUCLEOSIDES FROM THE MOLECULAR TO THE IONIZED FORM

Base	$-\Delta(\Delta F_{ex})$ *[1.0 M $(NH_4)_2SO_4$] (kJ/mol)*	*Nucleoside*	$-\Delta(\Delta F_{ex})$ *[1.0 M $(NH_4)_2SO_4$] (kJ/mol)*
Cyt	2.9	Cyd	2.4
Ura	2.2	Urd	2.1
Met-Ura	2.2	5-AzaCyd	4.8
6-AzaCyt	4.7	6-AzaCyd	4.2
6-AzaUra	3.8	6-AzaUrd	2.3
6-AzaThy	2.9	Ara-C	2.0

on the solvent properties[17,22] and γ the surface tension. By finding the dependence $\ln k' = f(\gamma)$, we obtained the values of $\Delta\Phi$ and ΔF_{cav} for the compounds studied (Table III).

The results obtained make it possible to draw some conclusions concerning the interaction of molecules with a hydrocarbonaceous stationary phase. For instance, Table III shows that the transition from a pyrimidine to a triazine ring decreases the area of molecular contact with the hydrophobic sorbent surface.

With 6-azathymine, substitution of a nitrogen atom for a CH group in the 6-position in the heterocyclic ring has an insignificant effect on the hydrophobic contact area. In unmethylated derivatives, the same substitution results in a significant decrease in this parameter (Table III). This fact indicated that the orientation of the 6-AzaThy relative to the sorbent surface is different to that for 6-AzaCyt and 6-AzaUra. The results in Tables III and IV show that a considerable contribution to the interaction of thymine and 6-azathymine with the surface comes from methyl groups. The Van der Waals radius of methyl and methylene groups is about 2.0 Å[23], hence the Van der Waals area of these groups is 50.3 Å^2. According to our results, the introduction of a methyl group into the base molecule leads to an increase in the hydrophobic contact area of 50–60 Å (Table IV). We can therefore conclude that a 6-AzaThy and Thy methyl group penetrates completely into the surface hydrocar-

TABLE III

CHARACTERISTICS OF THE COMPOUND–SORBENT–SURFACE CONTACT

Base	$\Delta\Phi$ (Å^2)	$-\Delta F_{cav}$ *(H_2O) (kJ/mol)*	*Nucleoside*	$\Delta\Phi$ (Å^2)	$-\Delta F_{cav}$ *(H_2O) (kJ/mol)*
Cyt	23	15.6	Cyd	77	38.8
Ura	33	19.9	Urd	86	43.2
6-AzaCyt	11	10.4	6-AzaCyd	61	31.8
Thy	85.8	42.7	5-AzaCyd	69	35.6
6-AzaThy	80.1	40.3	Ara-C	82	40.9
6-AzaUra	19.9	14.3	6-AzaUrd	63	32.8

TABLE IV

CONTRIBUTIONS TO THE CHANGE IN FREE ENERGY OF ADSORPTION

Compounds	$\Delta(\Delta\Phi)$ (Å^2)	$\Delta(\Delta F_{int})$ [H_2O] (kJ/mol)	$\Delta(\Delta F_{int})$ [2.5 M $(NH_4)_2SO_4$] (kJ/mol)	$\Delta(\Delta F_{cav})$ [H_2O] (kJ/mol)	$[\Delta(\Delta F_{vdW}) + \Delta(\Delta F_{es}) + \Delta(\Delta F_{as.gas})]$ (kJ/mol)
Ura–6-AzaUra	13	0.1	0.55	5.6	−5.5
Cyt–6-AzaCyt	12	0.01	0.45	5.2	−5.19
Thy–6-AzaThy	6	1.1	1.3	2.6	−1.5
Cyd–6-AzaCyd	16	2.1	2.7	6.9	−4.8
Urd–6-AzaUrd	23	2.2	3.1	9.9	−7.7
Cyd–5-AzaCyd	8	0.7	1.0	2.8	−2.1
Cyd–Ara-C	5	0.9	0.0	2.2	−1.3
Cyd–Cyt	54	2.3	4.4	23.3	−21.0
Urd–Ura	53	2.3	4.4	22.8	−20.5
6-AzaCyd–6-AzaCyt	50	0.1	2.06	21.6	−21.5
6-AzaUrd–6-AzaUra	43	0.1	1.7	18.6	−18.5
Ara-C–Cyt	59	3.6	5.98	25.4	−21.8
Thy–Ura	53	2.0	4.2	22.8	−20.8
6-AzaThy–6-AzaUra	60	1.2	3.6	25.9	−24.7

bonaceous layer. The insignificant contact area of unmethylated pyrimidine bases indicates that the hydrophilic heterocycle is largely in a water layer and contacts poorly with surface alkyl radicals. It is clear that with 6-AzaThy, the introduction of a nitrogen atom into a heterocycle that is almost entirely outside the hydrocarbonaceous layer may affect in a very insignificant manner the hydrophobic contact area.

One of the basic points in the chromatographic process is the selectivity of separation and the possibility of controlling this parameter. Using eqns. 2 and 3, we can obtain an expression for the separation selectivity of a substance:

$$\ln \alpha = \frac{N(\Delta\Phi_1 - \Delta\Phi_2)\gamma}{RT} + \frac{\Delta(\Delta F_{es}) + \Delta(\Delta F_{as.gas})}{RT} + \frac{\Delta(\Delta F_{VdW})}{RT} = \frac{\Delta(\Delta F_{int})}{RT}$$

Tabel IV gives the values obtained from the contributions to selectivity that characterize the difference in intermolecular interactions in a substance–surface–solution system.

The change in free energy of adsorption on introduction of a methyl group is 2–4 kJ/mol, depending on the mobile phase surface tension (Tabel IV). This value is in agreement with the data reported in ref. 19, which can be used to calculate that on introduction of a methyl group into the pyrimidine base molecule, the change in free energy of adsorption is about 3 kJ/mol. This value is a fairly typical increment ΔF_{int} for methyl and methylene groups in different compounds[24] under RP-HPLC conditions. Hence there is no reason to refer to any special behaviour of the methylated derivatives of pyrimidine bases or to try to explain this by shifts in tautomeric equilibrium and in the stacking process, as attempted elsewhere[19]. Also, it should to be noted that stacking is unlikely under HPLC conditions when the concentration of the compound concerned is much less than 10^{-2} M, a concentration at which the process is still observed[25].

The substitution of a more electronegative nitrogen atom for a CH group in the 6-position of the nitrogenous heterocycle of bases and nucleosides decreases the area of molecular contact with a sorbent to a greater extent for the nucleosides. The introduction of a nitrogen atom in position 6 affects variously the free energy of adsorption. The change in the free energy of adsorption for the nucleosides is 2 kJ/mol greater than that for the bases. The introduction of a nitrogen atom into the heterocycle in nucleosides therefore leads to a greater change in the molecular interaction with the surface than for the bases. As a result, we can assume that the interactions of both the nitrogenous hetrocycle and the sugar moiety of the molecule with the surface are altered. This seems to account for the fact that the introduction of a sugar component into the molecule results in a greater increase in the hydrophobic contact area for unmodified heterocyclic compounds. With transition from a base to a nucleoside, the introduction of a nitrogen atom into the heterocycle markedly affects the free energy of adsorption (4.4 kJ/mol for the natural compounds and 1.7–2.1 kJ/mol for 6-aza derivatives in 2.5 M ammonium sulphate solution).

Table IV shows that the values of $\Delta(\Delta\Phi)$ and $\Delta(\Delta F_{cav})$ are large in these instances, the selectivity in base–nucleoside separation sharply increasing with increase in the surface tension of the mobile phase [$\Delta(\Delta F_{int}) \approx 0.0$ kJ/mol in water and 1.7–2.1 kJ/mol in 2.5 M ammonium sulphate solution]. The chromatographic parameters of 6-aza derivatives of nucleosides are very different to those for 5-AzaCyd. The

substitution of a nitrogen atom for a carbon atom in position 5 results in much smaller changes in both the hydrophobic contact area and all the other components of adsorption energy. This makes it possible to conclude that the contribution of a C-6 atom to the hydrophobic sorbent surface–nucleoside interaction is greater than that of a C-5 atom.

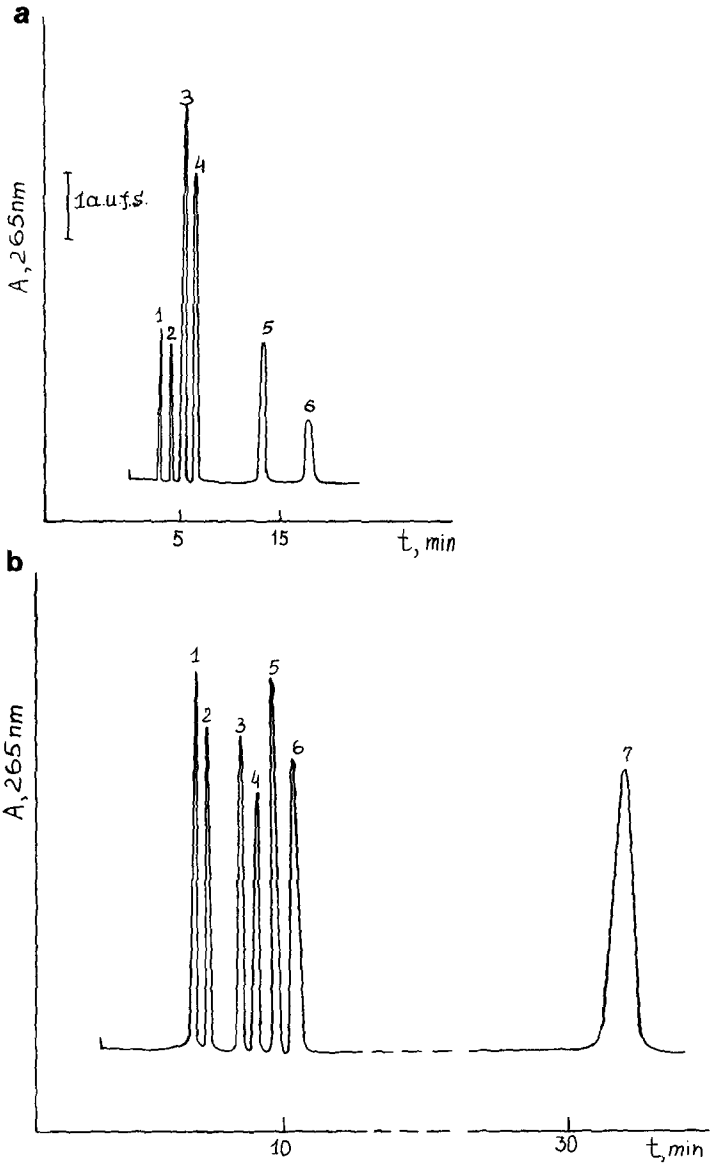

Fig. 3. Chromatograms of separation of nucleic acid components and their derivatives. (a) Stationary phase: Separon C_{18} (5 μm). Mobile phase: 1 *M* ammonium sulphate in 0.1 *M* phosphate buffer (pH 3.45). 1 = Cyt; 2 = 6-AzaCyt; 3 = 6-AzaUra; 4 = Ura; 5 = 6-AzaThy; 6 = Thy. (b) Stationary phase: Bondapak C_{18} (10 μm). Mobile phase: 0.125 *M* ammonium sulphate in 0.01 *M* phosphate buffer (pH 5.5). 1 = 6-AzaCyd; 2 = 6-AzaUrd; 3 = Cyd; 4 = 5-AzaCyd; 5 = Ara-C; 6 Urd; 7 = Thd.

The change in the position of a hydroxyl group relative to a ribose ring (Ara-C-Cyd) results in an increase in the free energy of adsorption of 10 kJ/mol. It is probable that the longer retention of Ara-C- compared with Cyd is due to the formation of an intramolecular hydrogen bond between the O-2 oxygen atom of the nitrogenous heterocycle and the hydrogen atom of an arabinose hydroxy group, resulting in a decreased interaction with the aqueous mobile phase. As the decreased molecular interaction with water leads to an insignificant increase in the area of hydrophobic contact by Ara-C compared with that by Cyd (5 Å), it is possible to conclude that only a small proportion of the atoms forming an intramolecular hydrogen bond make contact with the sorbent surface.

The data obtained show (Table IV and Fig. 2) that by increasing the ionic strength of the eluent, it is possible to enhance considerably the selectivity of separation of a compound when the difference in the sorbent surface contact area is more than 5–6 Å. The results obtained enable us to vary and calculate the selectivity of separating compound mixtures studied at different pH and ionic strength. The dependences $k' = f(pH)$ make it possible to conclude that the optimal pH range for the separation of nucleoside mixtures is 5.3–5.5. As long as Cyd and Ara-C are partially protonated, it is possible, under these conditions, to separate to a maximum extent the neighbouring peaks belonging to Urd-Ara-C-5AzaCyd-Cyd. The optimal pH range for the bases is 3.4–3.6.

At low ionic strength, the values of α that correspond to neighbouring peaks of the nucleosides are within the range 1.2–1.3 and those of the bases are within the range 1.1–1.2. To separate such mixtures completely, it is therefore necessary to apply columns with 2000–3000 theoretical plates, which is a realistic task. When low-performance columns are used, it is necessary to increase the selectivity and capacity factors by applying an eluent characterized by a higher surface tension, using up to 2.5 *M* ammonium sulphate solution (Table IV). Fig. 3a shows the chromatogram for the bases and Fig. 3b that for the nucleosides studied on a high-performance column when component separation is optimal under isocratic chromatographic conditions and with an aqueous eluent, which is known to be the best arrangement for routine analysis.

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