Journal of Chromatography, 255 (1983) 151–162
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,383

STATIONARY PHASE EFFECTS ON THE REVERSED-PHASE LIQUID CHROMATOGRAPHIC RETENTION BEHAVIOR OF PURINE AND PYRIMIDINE COMPOUNDS

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SUMMARY

The effects of stationary phase variables on the reversed-phase chromatographic behavior of nucleotides, nucleosides and bases were investigated. The parameters examined were the carbon chain length and bonded-phase polymericity as well as the per cent carbon coverage and loading, all known to affect the accessibility of the solute molecule to free silanols. As suggested by a previous study on mobile phase effects, solvophobic forces primarily govern the purine or pyrimidine nucleoside and base ring interaction with the hydrocarbonaceous packing. In addition, silanol–phosphate interactions seem to play a major role in the retention behavior of nucleotides at low eluent pH.

INTRODUCTION

Solvophobic forces are generally known to control the chromatographic behavior of molecules in reversed-phase systems[1]. However, under certain mobile phase conditions, several classes of compounds may defy the solvophobic elution rules: purine and pyrimidine nucleotides are such examples when a low pH buffer eluent is used[2]. Similar deviations, observed for crown ethers[3] and organic amines[4], have been attributed to silanophilic interactions between the solute and accessible silanols at the surface of the alkyl-silica bonded phase. Residual silanols which can contribute to the retention of ionic molecules have, in fact, been proven to exist[5].

In order to determine the effect of silanophilic interactions on the chromatographic retention of nucleotides in reversed-phase systems, stationary phase parameters which affect directly the accessibility of residual silanols were varied. Thus, at a low pH, nucleic acid constituents were chromatographed on columns with different carbon chain length and phase polymericity (Partisil ODS-3 *vs.* Partisil C_8), and per cent carbon coverage and loading (Partisil ODS-2 *vs.* Partisil ODS).

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0021-9673/83/0000-0000/$03.00

MATERIALS AND METHODS

A Waters liquid chromatograph, ALC 204 (Waters Assoc., Milford, MA, U.S.A.), was used in the course of this project. A Waters 440 UV dual-wavelength detector monitored the effluent at 254 and 280 nm. Retention times were recorded by means of the HP 3380A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Potassium dihydrogen phosphate, KH_2PO_4, high-performance liquid chromatography (HPLC) grade, was purchased from Fischer Scientific (Medford, MA, U.S.A.) and diluted to 0.02 *M* with doubly-distilled deionized water. The pH of the solution was then adjusted to 2.95 with phosphoric acid (Mallinckrodt, Saint Louis, MO, U.S.A.). Methanol was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Nucleoside, nucleotide and base reference compounds, from Sigma (St. Louis, MO, U.S.A.), were dissolved to $5 \cdot 10^{-3}$ *M* in 0.02 *M* KH_2PO_4 buffer (pH 6.0).

All columns were obtained from Whatman (Clifton, NJ, U.S.A.). These were: Partisil-10 ODS (5% carbon loading), Partisil-10 ODS-2 (15% carbon loading), Partisil-10 ODS-3 (10% carbon loading) and Partisil-10 C_8 (9% carbon loading). The first three are polymeric bonded phases since trifunctional octadecylsilane is used as the bonding agent onto either dried silica (ODS), "moisture-controlled" silica (ODS-2) or dried silica with trimethylchlorosilane capping (ODS-3)[6]. The C_8 is a truly monomeric phase since a monofunctional octylsilane is the bonding reagent onto dried silica with trimethylchlorosilane capping. The coverage of available silanols is 95% for both the ODS-3 and C_8 packings, whereas it is only approximately 75% and 50% for the ODS-2 and ODS stationary phases, respectively. Silanolic coverage was determined by the manufacturer[6] based on the retention of nitrobenzene with heptane for eluent[7].

Prior to use, the columns were allowed to equilibrate with the mobile phase for 2 h at a flow-rate of 1.5 ml/min. All data were obtained under the isocratic elution mode, using a 0.02 *M* KH_2PO_4 solution, initially adjusted to pH 2.95, and to which various percentages of organic modifier were added to make up the desired eluent. Flow-rate was 1.5 ml/min and temperature was ambient.

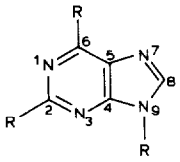
RESULTS

Retention characteristics

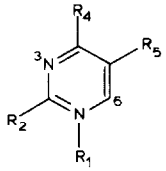
The retention behavior of bases, nucleosides and nucleotides (Fig. 1), solutes with a wide range of polar or ionic character, was studied on four different stationary phases. Data obtained using the ODS-3 and C_8 columns were compared. In view of the similar carbon loading and coverage of those stationary phases, differences in solute retention could be attributed to bonded chain length and polymericity effects. Similarly, the ODS-2 and ODS data were compared, and resulting variations in retention related to differences in carbon loading and coverage.

Chain length and polymericity: ODS-3 vs. C_8. Nucleosides and bases were retained longer on the ODS-3 than on the C_8 column, when a phosphate buffer at a pH value of 2.95 was used as eluent (Table I). Retention decreased on both columns upon addition of methanol to the mobile phase (Fig. 2).

Conversely, diphosphonucleotides were retained longer on the C_8 than on the ODS-3 stationary phase with the aqueous buffer eluent (Table I). On both columns,



Purine structures



Pyrimidine structures

Purine ring substituents			*Solute*	*Pyrimidine ring substituent*				*Solute*
R_2	R_6	R_9		R_1	R_2	R_4	R_5	
H	NH_2	H	Adenine	H	OH[a]	NH_2	H	Cytosine
NH_2	OH[a]	H	Guanine	H	OH[a]	OH[a]	H	Uracil
H	OH[a]	H	Hypoxanthine	H	OH[a]	OH[a]	CH_3	Thymine
OH[a]	OH[a]	H	Xanthine					
		Ribose	Corresponding nucleoside	Ribose				Corresponding nucleoside
		Ribose, 1 PO_4^{3-}[b]	Nucleoside monophosphate	Ribose, 1 PO_4^{3-}[b]				Nucleoside monophosphate

Fig. 1. Structure of the purine and pyrimidine compounds. [a] Keto–enol tautomerism exists. In the text, keto substitution mentioned. [b] If 2 PO_4^{3-}, nucleoside diphosphate; 3 PO_4^{3-}, nucleoside triphosphate.

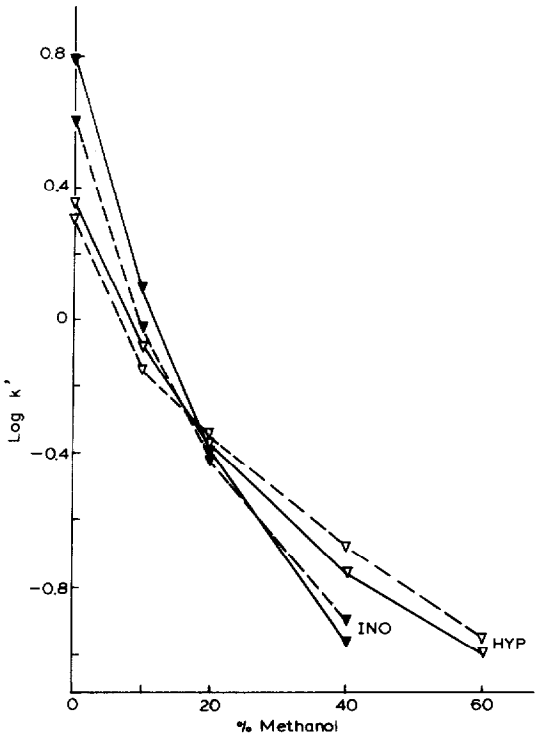


Fig. 2. Chromatographic behavior of a nucleoside (Ino) and its base (Hyp) in 0.02 *M* KH_2PO_4 buffer (pH 2.95) for both ODS-3 (——) and C_8 (– – –) columns.

TABLE I

CAPACITY FACTORS (k') OF SOME BASES, NUCLEOSIDES AND NUCLEOTIDES ON ODS-3 AND C_8 USING A 0.02 M KH_2PO_4 ELUENT (pH 2.95)

	$k'_{ODS\text{-}3}$	k'_{C_8}
Bases		
Hyp	2.23	2.10
Xan	2.99	2.19
Nucleosides		
Urd	2.10	1.73
Ino	6.14	4.05
Guo	6.75	4.12
Nucleoside monophosphates		
CMP	0.500	0.323
UMP	1.26	1.12
TMP	4.52	3.64
AMP	1.67	1.09
IMP	2.76	2.21
GMP	2.74	1.97
XMP	3.67	2.38
Nucleoside diphosphates		
CDP	1.05	1.09
UDP	12.0	17.6
TDP	26.1	45.1
ADP	4.29	5.86
IDP	22.3	33.0
GDP	17.7	24.8
XDP	28.4	–

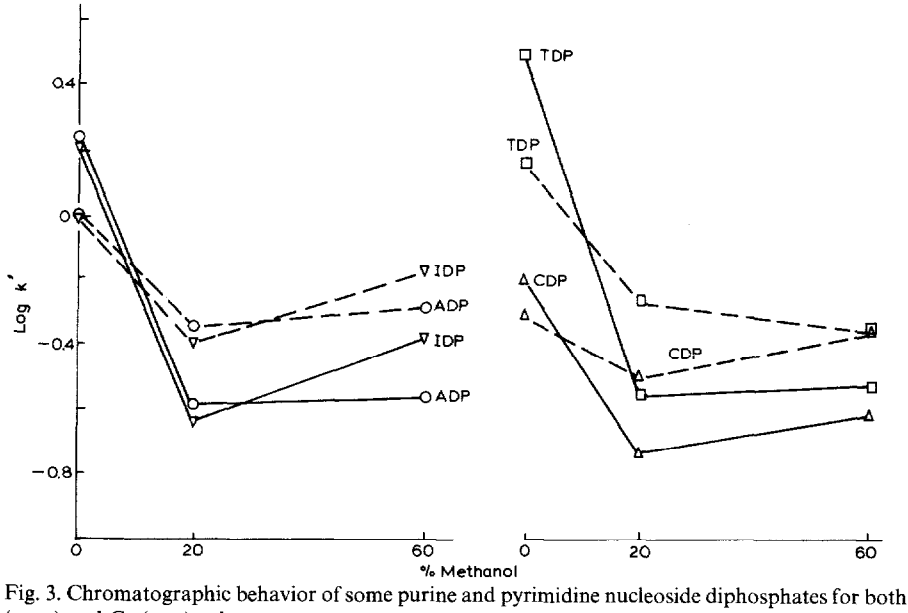


Fig. 3. Chromatographic behavior of some purine and pyrimidine nucleoside diphosphates for both ODS-3 (——) and C_8 (– – –) columns.

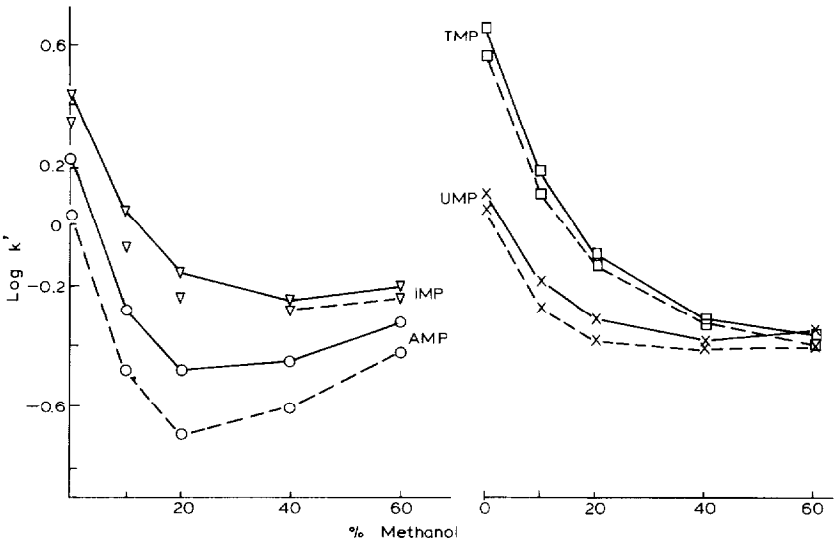


Fig. 4. Chromatographic behavior of some purine and pyrimidine nucleoside monophosphates for the ODS-3 (——) and C_8 (– – –) columns.

elution was enhanced by the addition of small amounts of methanol to the mobile phase and retarded by the addition of more substantial amounts. At a 60% eluent composition of methanol, the retention of the diphosphonucleotides on the ODS-3 packing was similar to, if not greater than, that on the C_8 (Fig. 3).

The chromatographic behavior of the monophosphonucleotides resembled that of the nucleosides and bases in that they exhibited greater retention on the ODS-3 than C_8 column with the buffer eluent (Table I). This behavior was not altered upon the addition of organic modifier to the mobile phase (Fig. 4).

Carbon loading and coverage: ODS-2 vs. ODS. Except for CTP, all bases, nucleosides and other nucleotides were retained longer on the column with higher carbon loading and coverage (ODS-2) at pH 2.95, and in the absence of an organic modifier (Table II). The decrease in retention with decreasing coverage seems more substantial for the nucleosides, bases and nucleoside monophosphates than their more ionized di- and triphosphate derivatives, as indicated by the $k'_{ODS\text{-}2}/k'_{ODS}$ values obtained for all solutes (Table II).

The log k' *vs.* % methanol plots show a continuous decrease in retention with increasing amounts of methanol for the nucleosides, bases (Fig. 5) and nucleoside monophosphates (Fig. 6). The same behavior is not observed for the di- (Fig. 7) or triphosphates (Fig. 8) which are retained even longer on either ODS-2 or ODS columns as the composition of methanol in the mobile phase is increased from 20 to 60%. Thus, the stepwise addition of a phosphate group to purine and pyrimidine nucleosides can be seen to affect markedly their chromatographic behavior.

At 60% methanol in the buffer eluent, all solutes are retained longer on the column with lower carbon coverage and loading (Figs. 5–8). In the nucleotide series, $k'_{ODS}/k'_{ODS\text{-}2}$ is greatest for the monophosphates and smallest for the triphosphates.

TABLE II

k' VALUES OF SOME BASES, NUCLEOSIDES AND NUCLEOTIDES ON ODS-2 AND ODS USING A 0.02 M KH_2PO_4 ELUENT (pH 2.95), AND $k'_{ODS\text{-}2}/k'_{ODS}$ VALUES

	$k'_{ODS\text{-}2}$	k'_{ODS}	$k'_{ODS\text{-}2}/k'_{ODS}$
Bases			
Hyp	11.1	4.37	2.54
Xan	10.3	4.01	2.57
Nucleosides			
Urd	6.90	1.60	4.31
Ino	21.6	4.38	4.93
Guo	10.6	4.78	2.23
Nucleoside monophosphates			
CMP	1.38	0.657	2.09
UMP	1.48	0.646	2.28
TMP	7.06	1.97	3.58
AMP	4.12	1.45	2.84
IMP	3.66	1.29	2.84
GMP	4.53	1.50	3.02
XMP	4.98	1.46	3.41
Nucleoside diphosphates			
CDP	0.623	0.492	1.26
UDP	0.778	0.514	1.51
TDP	3.04	1.42	3.91
ADP	1.72	1.01	1.70
IDP	1.62	0.989	1.64
GDP	2.35	1.18	1.99
XDP	1.67	0.900	1.85
Nucleoside triphosphates			
CTP	0.728	0.873	0.834
UTP	1.52	1.34	1.14
TTP	3.33	2.54	1.31
ATP	2.38	2.15	1.11
ITP	3.41	1.76	1.94
GTP	3.85	2.70	1.43
XTP	2.58	1.98	1.30

Selectivity

Selectivity was studied for various solute pairs and can be represented by $\alpha = k'_{S\text{-}G}/k'_S$ where S is a solute and G a group substituent on that solute[8]. Data obtained for the different stationary phases were compared in order to elucidate the contribution of different groups to solute retention.

Chain length and polymericity: ODS-3 vs. C_8. Under aqueous conditions, α was observed to be greater for the ODS-3 than the C_8 column when the substituent is a ribose ($k'_{nucleoside}/k'_{base}$), and smaller when the substituent group G is a phosphate ($k'_{monophosphate}/k'_{nucleoside}$, $k'_{diphosphate}/k'_{monophosphate}$) (Table III). The latter trend is more pronounced for the second rather than first phosphate substitution.

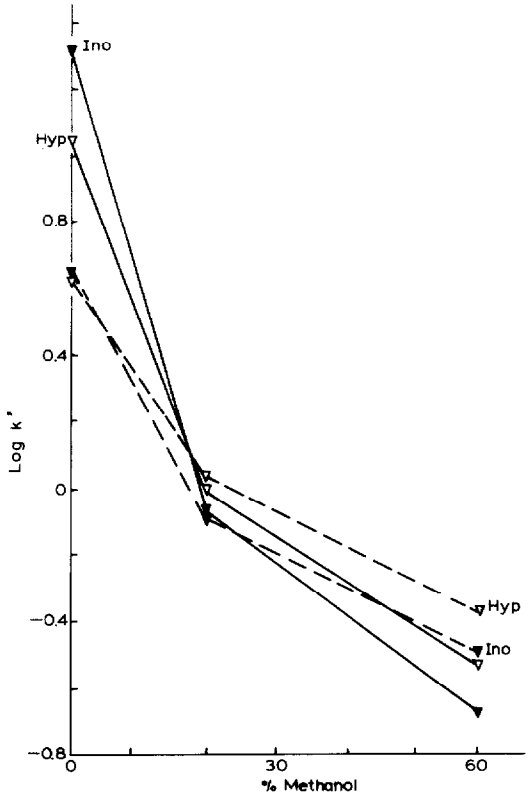


Fig. 5. Chromatographic behavior of a nucleoside (Ino) and its base (Hyp) on ODS-2 (——) and ODS (– – –) stationary phases in 0.02 *M* KH_2PO_4 (pH 2.95).

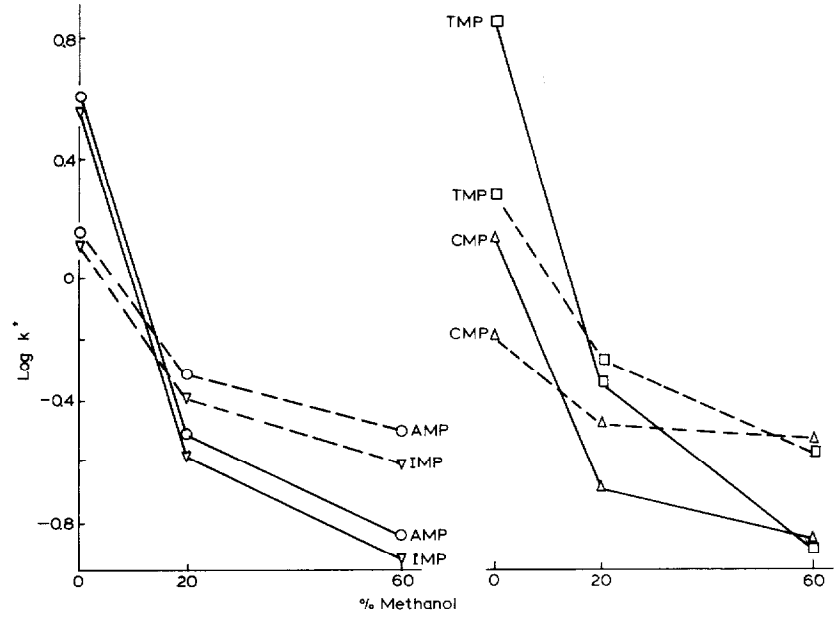


Fig. 6. Chromatographic behavior of sume purine and pyrimidine nucleoside monophosphates on ODS-2 (——) and ODS (– – –) stationary phases.

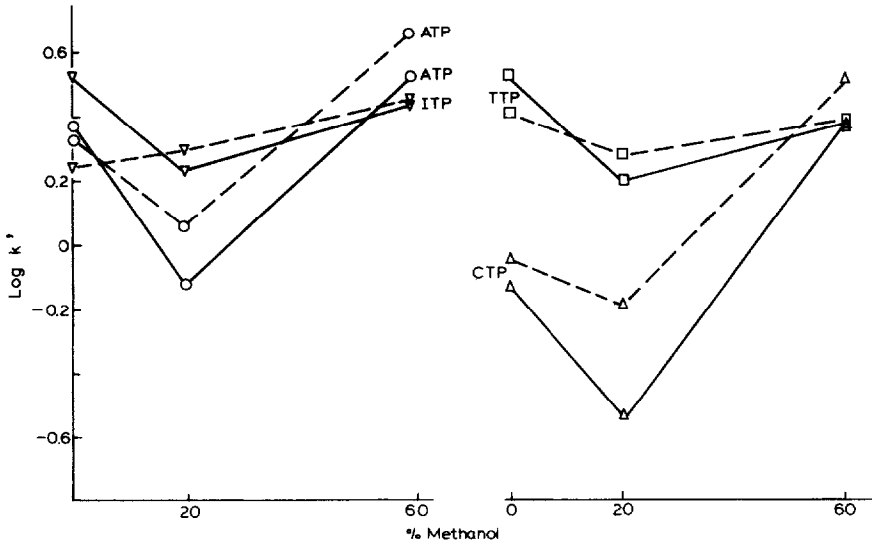


Fig. 7. Chromatographic behavior of some purine and pyrimidine nucleoside diphosphates on ODS-2 (——) and ODS (– – –) stationary phases.

Within each solute category (base, nucleoside, monophosphate, diphosphate), the introduction of a keto group (k'_{XMP}/k'_{IMP}, k'_{Xan}/k'_{Hyp}) or an amino group (k'_{GMP}/k'_{IMP}, k'_{GDP}/k'_{IDP}) at position 2 of the purine ring resulted in a greater selectivity for the ODS-3 in comparison to the shorter chain, monomeric C_8 column. Conversely, this selectivity was decreased when an amino was replaced by a keto group on position 6

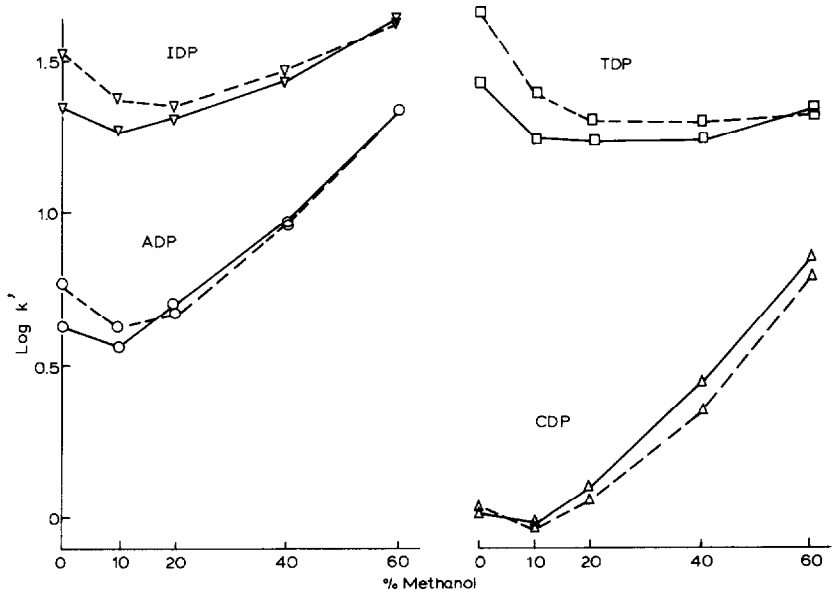


Fig. 8. Chromatographic behavior of some purine and pyrimidine nucleoside triphosphates on ODS-2 (——) and ODS (– – –) stationary phases.

of the purine ring (k'_{IMP}/k'_{AMP}, k'_{GMP}/k'_{AMP}, k'_{GDP}/k'_{ADP}). Finally, whereas α was larger on the ODS-3 column with a methyl G substituent on position 5 of uridine monophosphate (k'_{TMP}/k'_{UMP}), it was smaller than on the C_8 column when uridine diphosphate (k'_{TDP}/k'_{UDP}) was the substituted solute (Table IIIa).

Upon addition of methanol to the mobile phase (60% methanol in buffer), selectivity decreased for both the ODS-3 and C_8 columns, when the substituent of the solute pairs was a ribose moiety, a methyl group at the 5-position of pyrimidine or a keto group at the 2-position of purine. However, α increased when the substituent was a phosphate or an amino group at the 2-position of purine (Table IIIb).

It is worth noticing that, in presence of the organic modifier, the α values obtained for the ODS-3 column were smaller than those for the C_8 only in the case of second phosphate group substitution ($k'_{diphosphate}/k'_{monophosphate}$). The results for the 5-methyl pyrimidine substituent are exactly the reverse of those under aqueous conditions (Table III).

Carbon loading and coverage: ODS-2 vs. ODS. When a phosphate buffer eluent was used, selectivity decreased with decreasing coverage except for those solute pairs where the substituent G is a phosphate group. Upon addition of methanol at a 60% composition in the mobile phase, α increased in both ODS-2 and ODS columns when

TABLE III

SELECTIVITY DATA, $\alpha_G = k'_{S\text{-}G}/k'_S$, FOR THE ODS-3 AND C_8 COLUMNS UNDER AQUEOUS (a) AND HYDROORGANIC (b) MOBILE PHASES

(a) 0.02 *M* KH_2PO_4, pH 2.95; (b) 60% methanol in (a).

Group G	*(a)*		*(b)*	
	C_8	*ODS-3*	C_8	*ODS-3*
Phosphate				
IDP/IMP	14.9	8.09	70.4	68.7
ADP/AMP	5.38	2.57	55.0	44.3
IMP/Ino	0.546	0.450	9.85	10.8
GMP/Guo	0.478	0.406	9.00	13.4
UMP/Urd	0.647	0.600	10.7	12.4
Nucleoside				
Ino/Hyp	1.93	2.75	0.520	0.565
Keto				
Xan/Hyp	1.04	1.34	0.960	0.956
XMP/IMP	1.08	1.33	1.32	1.41
IMP/AMP	2.03	1.65	1.56	1.32
GMP/AMP	1.81	1.64	1.98	1.76
GDP/ADP	4.23	4.12	–	2.35
Amino				
GMP/IMP	0.891	0.993	1.26	1.34
GDP/IDP	0.752	0.794	–	1.14
Methyl				
TMP/UMP	3.25	3.59	1.02	0.991
TDP/UDP	2.56	2.18	0.889	0.924

G was a phosphate, and decreased with a ribose, 2-purine keto, 2-purine amino or 5-pyrimidine methyl group.

DISCUSSION

The retention and selectivity data obtained with stationary phases of different chain length, carbon coverage and loading were studied both under aqueous and hydro-organic mobile phases. The eluent composition is known to affect the configuration of the octadecyl and octyl chains which, in turn, affects solute chromatographic behavior.

Retention characteristics

In aqueous mobile phases, bonded hydrocarbonaceous chains collapse hydrophobically[9] and form a network which, when sufficiently dense, tends to impede the solute access to residual silanols. Under such condition, the retention of the solutes studied was found greater on the stationary phase with a higher carbon loading (ODS-2) in comparison to that with a lower carbon loading (ODS). Indeed, the extent of solute retention is known to increase with the surface concentration of bonded chains in reversed stationary phases[10].

With columns of a higher coverage (ODS-3 and C_8) and the same buffer eluent, negatively charged diphosphonucleotides, unlike nucleosides and bases, were retained longer on the C_8 than on the ODS-3 phase (Table I). This may be attributed to silanophilic interactions, more allowed by the monomeric C_8 bristles than by the polymeric ODS-3 network.

The addition of methanol to the mobile phase solvates the polymeric chains and initiates changes in their configuration which lead to the unfolded chain state[9,11]. This is hypothesized to increase the surface area of the bonded phase that is available for solute interactions, as well as to facilitate interactions with residual silanols. Thus, mixed mechanisms may be responsible for the greater retention of nucleosides and bases on the C_8 compared to the ODS-3 packing, at 60% methanol in the buffer eluent. The solvophobic behavior of these solutes is nevertheless well demonstrated by the decrease in their log k' values with increasing amounts of methanol in the mobile phase. The importance of the "purine or pyrimidine ring–hydrocarbon chain" interactions in governing primarily the retention of nucleosides and bases has already been discussed[2]. Conversely, with all stationary phases examined, the log k' *vs.* % methanol plots for the nucleotides denote the occurrence of phenomena dictated by other than solvophobic forces.

The faster elution of the nucleotides with the low-covered, low-carbon loaded ODS column (Table II), as compared to columns with higher coverage (Table I), indicates that the chromatographic behavior of these solutes is partly due to their interaction with the hydrocarbonaceous packing, probably through their hydrophobic purine or pyrimidine base ring. However, the increase in their log k' values with higher methanol percentages, which is more pronounced as the number of negatively charged phosphate groups increases, indicates the occurrence of phosphate–silanol interactions, possibly through hydrogen-bond formation. For columns with less coverage and loading, especially those which have not been capped, these forces are important enough to affect chromatographic behavior. Thus, there is greater reten-

tion of all nucleic acid components on the ODS, as compared to the ODS-2 stationary phase, in presence of 60% methanol in the buffer eluent.

Selectivity

The general increase of selectivity with increasing hydrocarbonaceous chain length has been attributed to entropy factors and preferential orientation of solute moieties within the bonded phase layer[12]. Selectivity has also been reported to increase with increasing water content of the mobile phase[11]. However, deviations from these trends have been shown to exist, especially when the contributions of certain groups to selectivity, $\alpha_G = k'_{S-G}/k'_S$, are strongly dependent on free, accessible silanols. Both phosphate and 6-amino purine substituents are examples of such groups, since their α_G value is larger on the C_8 than on the ODS-3 column wherein free silanols are less accessible (Table III). Moreover, upon use of the organic modifier and subsequent solvation of the hydrocarbonaceous chains, the 2-amino purine substituent also interacts with residual silanols, as indicated by an increase in the relative retention of GMP/IMP in both C_8 and ODS-3 columns (Table III).

The phosphate group–silanophilic interaction is also apparent from selectivity data obtained with columns of different coverage. Indeed, α_G has been shown to increase with decreasing coverage as an effect of polar group contribution and greater silanophilic interactions, and to decrease with decreasing coverage when resulting from a hydrophobic group contribution[8]. Polar groups, or groups which contain electron-rich atoms (phosphate, amino) could in fact hydrogen-bond with the silanols which are not ionized at the acidic pH used.

The configuration of a solute within the high-coverage, bonded phase layer is undoubtedly important in affecting these forces since the 2-amino purine substituent seems to interact with the free silanols only upon addition of the organic modifier, *i.e.*, chain solvation and greater accessibility of the residual groups. In columns with a lower coverage, methanol may compete with the solutes for the silanophilic interactions[8]. This may explain why, unlike for the high-coverage ODS-3 and C_8, only the phosphate substituent resulted in larger α_G values on the ODS-2 and ODS stationary phases when the methanol composition was increased to 60%.

From the above discussion, one can see the dependence of chromatographic behavior on the stationary phase, both in the configuration of its bonded chains and accessibility of its free silanols, as well as on the solute molecule proper, with its polar, electron-rich silanophilic groups and non-polar, hydrophobic moieties. The examination of retention data on different packings helped elucidate the types of interactions, whether resulting from silanophilic or solvophobic phenomena, that are possible between the stationary phase and solute classes of different ionic character. Furthermore, the analysis of selectivity data revealed which solute groups were responsible for the various interactions.

CONCLUSIONS

The effects of mobile phase parameters such as pH, ionic strength, % organic modifier, on the retention of the bases, nucleosides and nucleotides have already been discussed[2]. The present study of their chromatographic behavior on different stationary phases supports the conclusions previously reached concerning solute–

stationary phase interactions. In summary, although retention mechanisms can concur, solvophobic forces primarily govern the purine or pyrimidine ring interaction with the hydrocarbonaceous packing in nucleosides and bases, whereas silanophilic forces are mostly responsible for the retention of polar groups such as the phosphate moiety in nucleotides. Electron-rich substituents such as phosphate or amino can indeed hydrogen-bond with residual silanols on the stationary phase. Entropy effects are found to influence, in particular, amino–silanol interactions.

The addition of methanol to the mobile phase enhances the occurrence of mixed mechanisms since it increases the chain surface area available for hydrophobic ring interactions, while it allows easier access to residual silanols by polar solute groups. Methanol may also compete with the solutes for silanophilic interactions, especially in columns with low coverage. In addition, the presence of methanol may change the solvated state of the solutes, causing differences in retention behavior.

Clearly, all stationary phase–mobile phase, mobile phase–solute, solute–stationary phase interactions affect solute retention. Interactions with the stationary phase may involve the hydrocarbonaceous chains and/or the accessible residual silanols.

ACKNOWLEDGEMENTS

We would like to thank Dr. Henri Colin (École Polytechnique, Laboratoire de Chimie Analytique Physique, Palaiseau, France) and Professor Eli Grushka (Hebrew University, Department of Inorganic and Analytical Chemistry, Jerusalem, Israel) for their helpful comments, and Dr. Frederic Rabel (Whatman Inc., Clifton, NJ, U.S.A.) for his extensive help and cooperation in the project.

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