excellent reductant for monitoring the mercury alkane thiolates. Although pure stannous chloride does not reduce methylmercury, cadmium chloride mixed with stannous chloride has been reported to be an efficaceous reductant under alkaline condition (4, 5). However, since we have found that this was not effective in our system, we employed sodium borohydride, a more potent reductant in the low pH ranges. Because of its extreme unstability, sodium borohydride was dissolved in 0.05 N NaOH solution to make it stable and mixed with acidic solution at the inlet for immediate use. In order to increase its efficacy of sodium borohydride, mercuric compounds from HPLC were previously mixed with oxidizing agent. The analytical sensitivity for methylmercury increased by a factor of about 10 and for inorganic mercury 1.5-fold. Reduction of mercuric compounds by sodium borohydride was not interfered with by the presence of cysteine.

Compared to the UV detector, resolution obtained by the CVAA detector is slightly inferior as shown in Figure 5. This slightly poor resolution of peaks is considered due to peak broadening during long transport, but it may be possible to improve by minimization of dead volume in reducing vessel and tubing.

At a low concentration such as 20 ng each of mercury thiolate, the peaks of thiolates are indistinguishable from the background signals on the chromatogram of UV detector. However, the CVAA detector offers a chromatogram with larger peaks of thiolates and with less background signals (Figure 5). This is because CVAA detector selectively picks up mercury compounds and has little matrix interference, resulting in much better sensitivity than UV detector.

The CVAA detector described in our study enables a selective and sensitive analysis of mercuric compounds continuously separated through HPLC. We believe that the combination of HPLC and mercuric specific CVAA detection will provide a powerful method for speciation of mercuric compounds in the environmental field.

ACKNOWLEDGMENT

The author wishes to thank A. Hata and K. Tsuchiya for their experimental assistances and G. Ohi for his valuable discussion in preparing the manuscript.

Registry No. Mercury methane thiolate, 21094-80-4; mercury ethane thiolate, 811-50-7; mercury propane thiolate, 4080-28-8; mercury butane thiolate, 23601-34-5; mercury, 7439-97-6.

LITERATURE CITED

(1) Westöö, G. *Acta Chem. Scand.* **1967**, *21*, 1790–1800.
(2) "Official Methods of Analysis of the Association of Official Analytical Chemists"; Association of Official Analytical Chemists: Washington, DC, 1980.
(3) Westöö, G. *Acta Chem. Scand.* **1966**, *20*, 2131–2137.
(4) Magos, L. *Analyst (London)* **1971**, *96*, 847–853.
(5) Magos, L., Clarkson, T. W. *J. Assoc. Offic. Anal. Chem.* **1972**, *55*, 966–971.
(6) Jones, D. R., IV; Manahan, S. E. *Anal. Chem.* **1976**, *48*, 502–505.
(7) Fraley, D. M., Yates, D. A., Manahan, S. E., Stalling, D., Petty, J. *Appl. Spectros.* **1981**, *35*, 525–531.
(8) Fraley, D. M.; Yates, D. A.; Manahan, S. E. *Anal. Chem.* **1979**, *51*, 2225–2229.
(9) Brinckman, F. E.; Blair, W. R.; Jewett, K. L.; Iverson, W. P. *J. Chromatogr. Sci.* **1977**, *15*, 493–503.
(10) Koizumi, H.; Hadeishi, T.; McLaughlin, R. *Anal. Chem.* **1978**, *50*, 1700–1701.
(11) McCrehan, W. A. *Anal. Chem.* **1981**, *53*, 74–77.
(12) McCrehan, W. A.; Durst, R. A. *Anal. Chem.* **1978**, *50*, 2108–2112.
(13) Inoue, S.; Hoshi, S.; Sasaki, M. *Bunseki Kagaku* **1982**, *31*, E243–E246.
(14) Häring, N.; Ballschmiter, K. *Talanta* **1980**, *27*, 873–879.
(15) Van Loon, J. C.; Radziuk, B.; Kohn, N.; Lichwa, J.; Fernandez, F. J.; Kerber, J. D. *At. Absorpt. Newsl.* **1977**, *16*, 79–83.
(16) Willeford, B. R.; Veening, H. *J. Chromatogr.* **1982**, *251*, 61–88.
(17) Oda, C. E., Ingle, J. D. *Anal. Chem.* **1981**, *53*, 2030–2033.
(18) Bushee, D.; Krull, I. S., Savage, R. N., Smith, S. B. *J. Liq. Chromatgr.* **1982**, *5*, 463–478.
(19) Koirtyohann, S. R.; Khalil, M. *Anal. Chem.* **1976**, *48*, 136–139.

RECEIVED for review October 27, 1982. Accepted November 19, 1982.

Mechanisms of Retention in Reversed-Phase Liquid Chromatographic Separation of Ribonucleotides and Ribonucleosides and Their Bases

Mona Zakaria[1] and Phyllis R. Brown*

Department of Chemistry, University of Rhode Island, Kingston, Rhode Island 02881

Eli Grushka

Department of Inorganic and Analytical Chemistry, Hebrew University, Jerusalem, Israel

The effects of the mobile phase variables, pH, organic modifier, and ionic strength on the retention behavior in the reversed-phase liquid chromatographic separation of the 5′-mono-, di-, and triphosphate nucleotides and their nucleosides and bases were investigated. The retention behavior observed suggests that for nucleotides at high pH, and their nucleosides and bases, the solvophobic model of reversed-phase chromatography is mainly operative; however for nucleotides at pH 2.95, the silanophilic model or another model in addition to or in place of the solvophobic model is more appropriate.

Purine and pyrimidine compounds are important biochemically, both in the free form as coenzymes and as components of nucleic acids. For years research on these compounds was hampered by the lack of methods and analytical tools which were sufficiently efficient and sensitive. In the past decade, much progress has been made on the separation of nucleotides and nucleosides and their bases by high-performance liquid chromatography (HPLC). Traditionally, these compounds were separated with the ion-exchange mode (*1–8*). However, since nucleosides and their bases do not generally contain ionic moieties, it was found that excellent resolution and rapid analyses of these compounds could be obtained by the reversed-phase mode (RPLC) with microparticulate octadecylsilica as the stationary phase (*9*). More recently, it has been reported that some nucleotides could also be separated by RPLC with or without ion pairing (*10, 11*).

Reversed-phase chromatography is an excellent alternative

[1] Present address: Hoffmann-La Roche, Inc., Nutley, NJ.

0003-2700/83/0355-0457$01.50/0

to ion-exchange chromatography because of its broad scope which can be used to separate compounds having a wide range of polarity and molecular weights. For nucleic acid components there are several advantages: (1) reproducibility of separations, (2) ease of operation, (3) rapidity of reequilibration after gradient elution, and (4) ability to use the same column for nucleotides as well as nucleosides and bases by varying the mobile phase parameters (*12–19*).

Although ionic compounds such as nucleotides are traditionally separated by ion-exchange chromatography, it has been found with many groups of ionic or ionizable compounds such as amino acids (*20*, *21*), peptides (*21*), and pteroyl-oligo-α-L-glutamates (*22*) that reversed-phase chromatography is also suitable and has great potential for these separations. In ion-exchange, the mechanism of retention is mainly interaction between the fixed charges on the stationary phase and the oppositely charged solute molecules. When purines, pyrimidines, and their nucleosides which are charged only under certain eluent conditions are separated by anion-exchange chromatography with an acidic eluent, they are minimally retained (*6*, *7*, *23*). The retention of nucleotides is dependent on the number of negative changes on the phosphate groups. Thus in anion-exchange chromatography using acidic eluents, the nucleosides and bases elute early followed by the monophosphate nucleotides, the diphosphates, and finally the triphosphates (*1–4*, *6*, *7*). Furthermore, it has been shown that increases in ionic strength decrease significantly the capacity factors of nucleotides (*4*) while increases in the percent organic modifier have minimal effects on retention behavior.

In reversed-phase chromatography in which the stationary phase is nonpolar, the order of retention is reversed (*8*). Nonpolar compounds are strongly retained. Ionic compounds have minimal retention since the hydrophobic effect which largely governs the magnitude of retention is decreased when the charge on the solute molecule increases (*22*). Thus according to the solvophobic theory of RPLC (*12*) it is predicted that in the RPLC separation of nucleic acid compounds, nucleotides should elute first, followed by their bases and nucleosides. The order of elution of nucleotides should be in order of charge: triphosphates followed by diphosphates and then monophosphates.

In a previous study done on the structure–retention relationship of a selected group of nucleic acid compounds, we found that some simple rules could be formulated which correlated structure to retention in reversed-phase chromatography (*17*). However, only a limited number of nucleosides, bases, and 5′-monophosphate nucleotides were studied. Moreover the rules were formulated mainly using an eluent system of pH ~5.6. Recently Van Haastert studied a group of adenine compounds and also formulated some rules for both RPLC and ion exchange retention behavior (*18*, *19*). However little attention has been paid to the RPLC retention behavior of the biologically important 5′-mono-, di-, and triphosphate ribonucleotides. Therefore in order to determine if the rules formulated apply more generally and if the RPLC behavior of nucleotides could be predicted, we systematically determined the effects of pH, organic modifier, and ionic strength on retention behavior of complete sets of compounds which are important in nucleic acid metabolism. The purines include adenine, guanine, hypoxanthine, and xanthine and the pyrimidines include cytosine, uracil, and thymine. The sets of compounds included the base, the corresponding ribonucleoside, and 5′-mono-, di-, and triphosphate ribonucleotides.

EXPERIMENTAL SECTION

Instrumentation. A Waters liquid chromatograph, ALC 204 (Waters Associates, Milford, MA), 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 an HP 3380 A integrator (Hewlett-Packard, Avondale, PA). The column was a Whatman Partisil PXS 10/25 ODS-3 (Whatman, Inc., Clifton, NJ). The column contained 10% carbon of which 9.5% was in the form of octadecylsilyl groups and 0.5% trimethylsilyl capping reagent.

Table I. k' Values and pK_{aa} and pK_{ab} Values[a] (*23*) of Purine and Pyrimidine Bases

	pK_{ab}	pK_{aa}	k' at pH 2.2	k' at pH 6.3
adenine	4.15	9.8	0.97	8.58
guanine	3.2	9.6	0.84	2.79
hypoxanthine	2.0	8.9	1.40	2.20
xanthine	0.8	7.5	2.46	2.68
cytosine	4.45	12.2	0.14	0.72
uracil	−3.4[b]	9.5	0.89	0.95
thymine	—[b]	9.9	2.75	2.83

[a] Values mentioned only for the first gain or loss of a proton. [b] Extremely low pH needed for the protonation of the species. Since both nitrogens in uracil and thymine are involved in amide tautomerism, very little basic strength remains.

Reagents and Solutions. Potassium chloride, KCl, and potassium dihydrogen phosphate, KH_2PO_4, HPLC grade, were purchased from Fischer Scientific Co. (Medford, MA) and diluted to the desired concentration with doubly distilled deionized water. The pH of the solutions was then adjusted with phosphoric acid or potassium hydroxide (Mallinckrodt Chemical Works, St. Louis, MO). Methanol and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI). Nucleoside, nucleotide, and base standards, from Sigma Chemical Co. (St. Louis, MO), were dissolved to 5×10^{-3} M in 0.02 F KH_2PO_4 buffer (pH 6.0).

Procedures. Prior to use, the column was allowed to equilibrate for 2 h at a flow rate of 1.5 mL/min under the mobile phase conditions used. It should be noted that for good reproducibility with this type of column exact equilibration time and conditions must be maintained especially if the mobile phase contains 70% or more of an aqueous solution. All separations were isocratic, using a 0.02 F KH_2PO_4 solution which was adjusted for pH, percent organic modifier, or ionic strength for individual experiments. A flow rate of 1.5 mL/min was used and all experiments were carried out at ambient temperature.

RESULTS AND DISCUSSION

Effects of pH. In general the retention behavior of the purine and pyrimidine bases is typical of the classical concept of reversed-phase chromatography in which charged species elute rapidly while neutral molecules are retained on the hydrophobic packing. For example, when the fractions, α_N, of the molecules present in the neutral form are plotted as a function of the pH, the graphs are similar to the graphs of k' vs. pH (Figure 1). Thus retention of the bases is related to the pK_a of the compound (Table I) (*24*) and increases as the neutral fraction present increases and the fraction present in the cationic form, α_+, decreases. In contrast to adenine, it was found that guanine has an inflection point in the k' curve at a lower pH, corresponding to its lower pK_a value (*24*); i.e., earlier attainment of partial neutrality.

The retention behavior of the nucleosides also relate to their pK_a values (Table II). Inosine, uridine, and thymidine, which are largely neutral in the pH range covered, do not undergo a significant change in retention with increasing pH. However, for adenosine and cytidine which are protonated at pH 2.2, the capacity factors are smaller than at pH 6.3 where the compounds are neutral. The k' of cytidine increases only moderately when the pH of the eluent goes from 2.2 to 6.3 while that of adenosine increases dramatically, an increase that cannot be explained solely on the basis of pK_a.

As would be expected with reversed-phase chromatography, all 5′-monophosphate nucleotides have lower k' values than

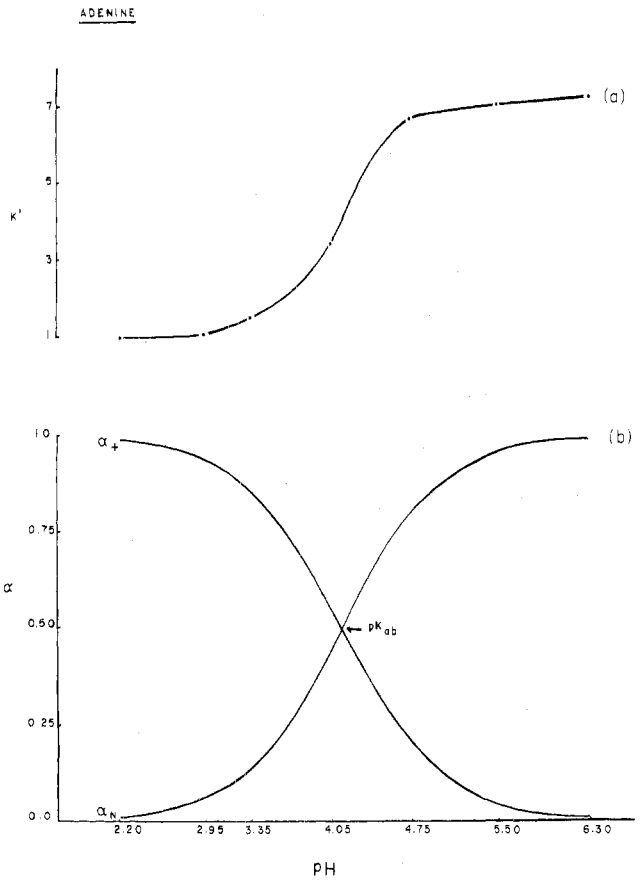

Figure 1. (Upper graph) k' vs. pH of adenine. k' values obtained by using a 0.02 F KH_2PO_4 buffer eluent at different pH values. For chromatographic conditions, see Methods. (Lower graph) Positive fraction (α_+) and neutral fractions (α_N) vs. pH of adenine.

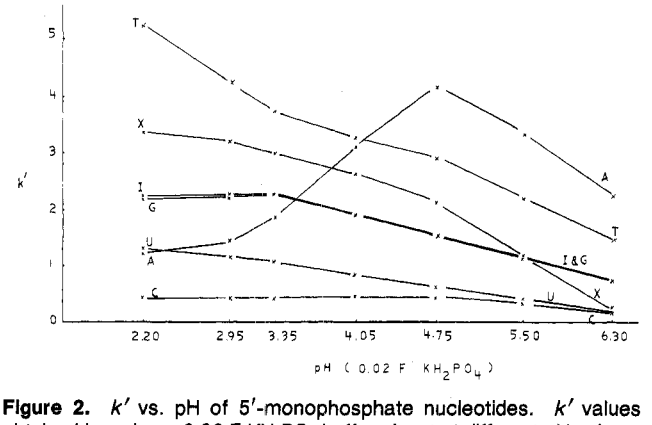

Figure 2. k' vs. pH of 5′-monophosphate nucleotides. k' values obtained by using a 0.02 F KH_2PO_4 buffer eluent at different pH values.

Table II. pK_{ab} and pK_{aa} Values (*23*) and k' Values for the Nucleosides at pH 2.20 and pH 6.30

		pK_{ab}	pK_{aa}[a]	k' at pH 2.20	k' (pH 6.30)
purines	Ado	3.5	12.5	2.64	22.5
	Guo	1.5	9.2	4.27	6.94
	Ino	1.2	8.8	5.31	5.66
	Xao	<2.5	5.7	9.21	3.07
pyrimidines	Cyt	4.15	12.5	0.650	1.45
	Urd		9.2	1.96	2.09
	Thd		9.8	8.95	9.12

[a] Nucleosides have an additional $pK_{aa} \simeq 12$ which corresponds to the ionization of the sugar hydroxyl.

their corresponding nucleosides (Table III). However, the retention order of these nucleotides in relation to their corresponding bases depends on the pH of the eluent. At pH 6.3 the nucleotides elute before their corresponding bases, behavior consistent with that predicted in reversed-phase systems. However, at pH 2.2, the bases elute before their respective 5′-monophosphate nucleotides, behavior typical of anion exchange systems.

Table III. k' Values for Sets of Purine and Pyrimidine Compounds at pH 2.2 and pH 6.30

	at pH 2.2	at pH 6.30
Ade	0.97	8.58
Ado	2.64	22.5
AMP	1.31	2.46
Gua	0.88	2.79
Guo	4.27	6.94
GMP	2.16	0.70
Hyp	1.40	2.20
Ino	5.31	5.66
IMP	2.52	0.69
Xan	2.46	2.68
Xao	9.21	3.07
XMP	3.32	0.23
Cyd	0.14	0.72
Cyt	0.65	1.45
CMP	0.43	0.14
Ura	0.89	0.95
Urd	1.96	2.09
UMP	1.27	0.21
Thy	2.75	2.83
Thd	8.95	9.12
TMP	5.15	1.43

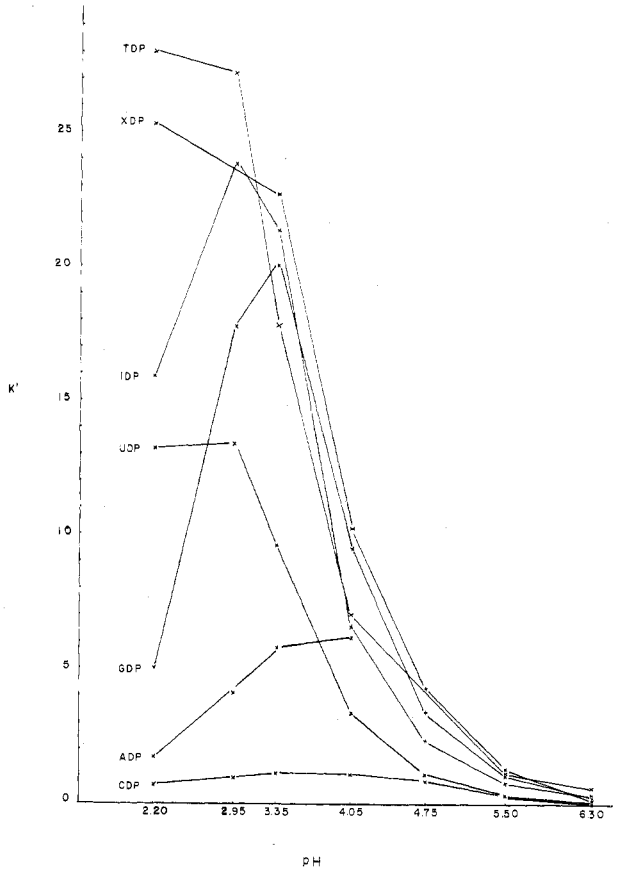

Figure 3. k' vs. pH of 5′-diphosphate nucleotides. k' values obtained by using a 0.02 F KH_2PO_4 buffer eluent at different pH values. For chromatographic conditions, see Methods.

The effect of pH on the 5′-monophosphate nucleotides is shown in Figure 2. With CMP there is relatively little change

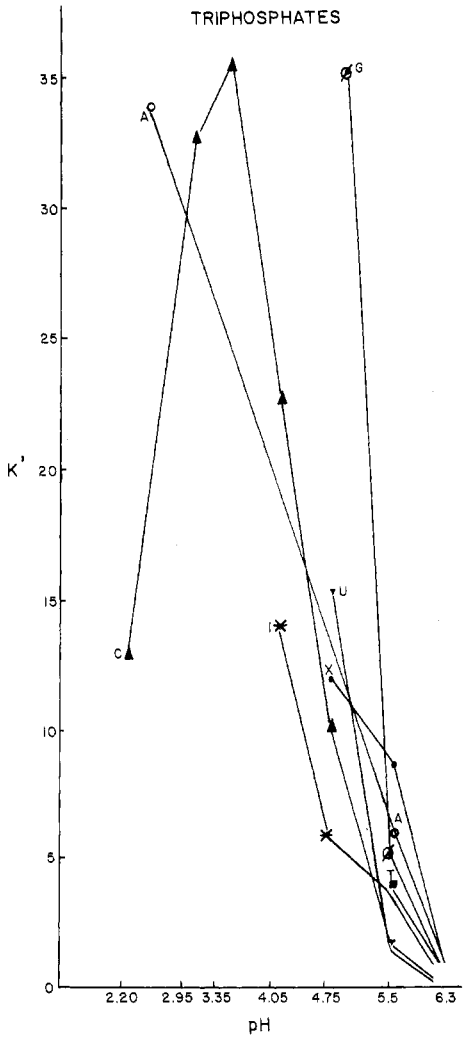

Figure 4. k' vs. pH of 5′-triphosphate nucleotides. k' values obtained by using a 0.02 F KH_2PO_4 buffer eluent at different pH values. For chromatographic conditions, see Methods.

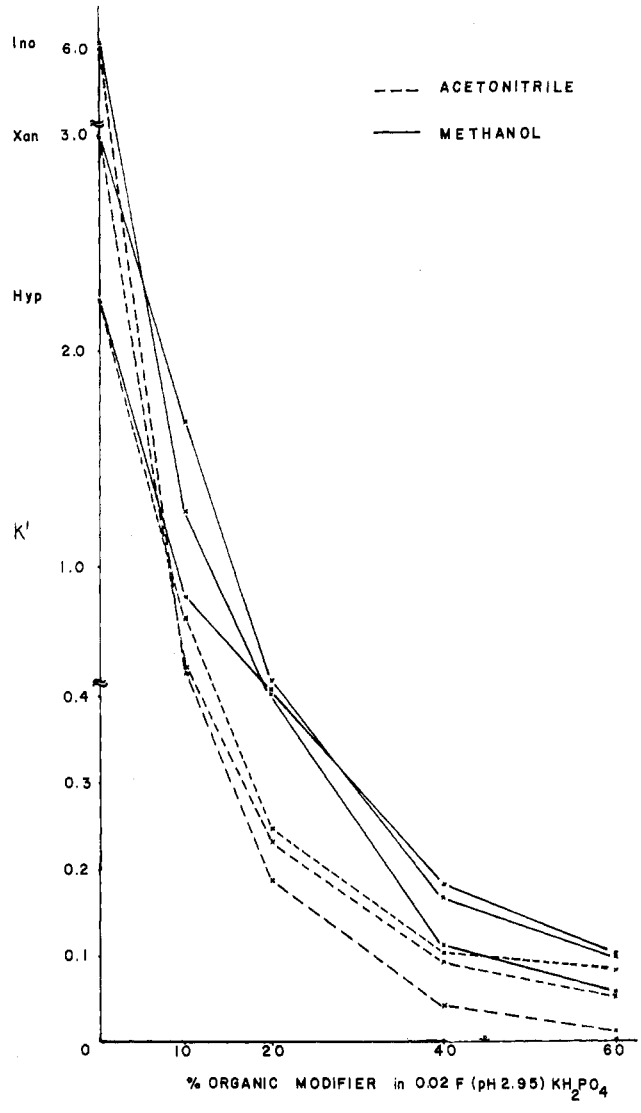

Figure 5. k' values of the nucleosides and bases vs. percent methanol and acetonitrile in 0.02 F KH_2PO_4 (pH 2.95). For chromatographic conditions, see Methods.

in k' with increasing pH, while the k' values of all the other nucleotides except AMP decrease with increasing pH. The diphosphates are well separated from each other at low pH as are the triphosphates (Figure 3 and 4). However, at higher pH (~6.30) when the phosphate groups have undergone further ionization and acquire an additional charge, the compounds elute closely near the void volume; thus the behavior at the higher pH is that expected according to the solvophobic mechanism in the reversed-phase mode whereas the retention behavior at the lower pH indicates a different mechanism (or mechanisms) is operative.

Effect of Organic Modifier. The effects of organic modifier were examined in 0.02 F KH_2PO_4 eluent systems at two different pH values: pH 2.95 and pH 5.50. At the lower pH the nucleosides and bases exhibited a decrease in k' with increasing amounts of methanol, behavior expected in a reversed-phase system (Figure 5). However, the curvature in the plot indicates the mechanism is more complicated than the solvophobic model alone. Thus, there may be ion pair formation with phosphate ion or adsorption on the unreacted silanols. For all the nucleotides except the thymine nucleotides, with increasing methanol concentration there was an initial decrease in log k' values, followed by an increase in k' values. For the monophosphate nucleotides, the log k' values increased when there was between 20 and 40% methanol in the mobile phase (Figure 6). For the diphosphate nucleotides, the increase in k' values started at approximately 10% methanol (Figure 7). This increase in k' with increasing organic modifier is contrary to behavior predicted by the solvophobic theory. While the retention behavior of nucleosides and their bases (*16*) as well as the nucleotides at pH 5.5 is in accordance with the solvophobic theory of reversed-phase chromatography, the nucleotides exhibit anomalous behavior in an eluent of pH 2.95.

In all experiments where acetonitrile was used instead of methanol as the organic modifier, in general the effects were similar to those of methanol except for faster elution. This effect is expected in reversed-phase systems since acetonitrile is less polar than methanol.

Therefore, in eluents of pH 5.5, the retention behavior of nucleotides is typical of and can be attributed mainly to a reversed-phase solvophobic phenomena; however, in a mobile phase of pH 2–3, other mechanisms or dual mechanisms of retention appear to be operative.

Effect of Ionic Strength. The ionic strength of the aqueous eluent was increased first by using higher phosphate buffer concentrations (pH 2.95). The increased buffer concentrations did not change significantly the retention behavior of the nucleosides, bases, and 5′-monophosphate nucleotides other than improving the symmetry of the inosine and guanosine peaks. However, with the di- and triphosphate nucleotides, increasing the concentration of buffers decreased the k' values as is illustrated in graphs of k' vs. ionic strength

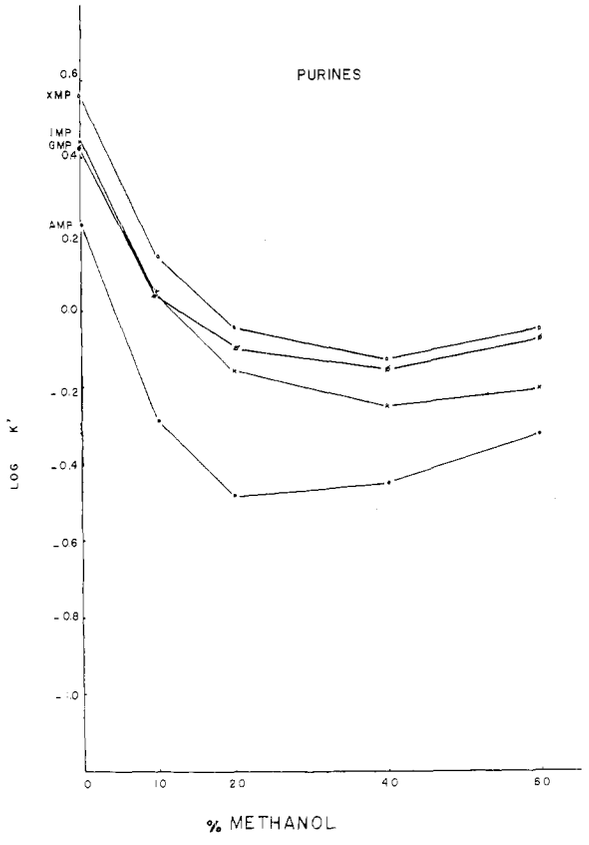

Figure 6. log k' of purine 5'-monophosphate nucleotides vs. percent methanol in 0.02 F KH_2PO_4 (pH 2.95). For chromatographic conditions, see Methods.

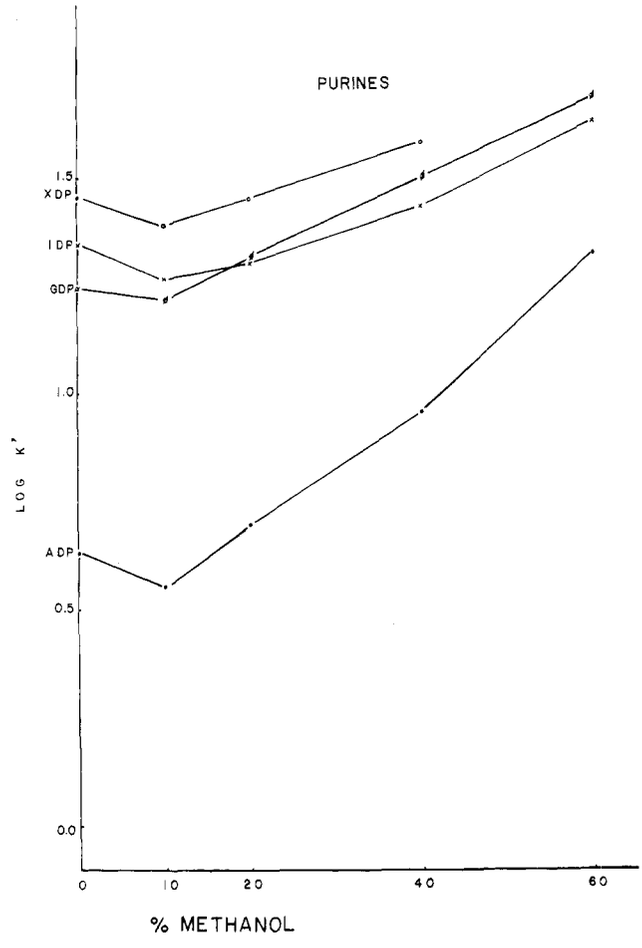

Figure 7. log k' of purine 5'-diphosphate nucleotides vs. percent organic modifier in 0.02 F KH_2PO_4 (pH 2.95). For chromatographic conditions, see Methods.

of the cytosine and adenine families (Figure 8). In order to determine whether the change in retention was due to the presence of phosphate ions per se in the medium or the increase in ionic strength, we kept the concentration of KH_2PO_4 buffer at 0.02 F and gradually increased the ionic strength, μ, by the addition of KCl. Similar but slightly more pronounced decreases in k' were obtained when increased amounts of KCl were present.

Since the ionic strength affected only minimally the retention behavior of nucleosides and bases, classical reversed-phase behavior is indicated. However, the pronounced effect of the ionic strength on the chromatographic behavior or the di- and triphosphate nucleotides suggests that charge interaction, perhaps via silanophilic interaction, may play a significant role in the RPLC retention of the nucleotides (*25*).

Therefore, for the nucleosides and bases which we examined, we agree with the findings of Van Haastert (*18*) that in RPLC the pH of the mobile phase influences both capacity factors and selectivity and that the effects can be directly correlated to the pK_a of the solutes. However, the very high k' of adenosine at pH 6.3 compared to the capacity factors of other nucleosides or to that of adenosine itself at pH 2.2 is hard to explain in terms of pK_a. For the nucleotides, the effect of pH is not straightforward. At pH 6.3 where there is higher electron density on the phosphate moiety than at pH 2.2, the retention order is that expected in a reversed-phase system. Thus, solutes with the largest charge have weaker hydrophobic interactions with the stationary phase and hence decreased retention. The anomalous behavior of the nucleotides at pH 2.2 may have several possible explanations. The configuration of the nucleotides may be such that the tail of the nucleotide containing the phosphate group is tucked under the base moiety and the charges are hidden within the molecule itself. Thus, the effects of solute size and solubility which would predominate could explain the retention behavior. Another possible explanation is that molecules in which the base is positively charged and the phosphate group negatively charged would tend to behave like a neutral molecule. A third explanation is that the nucleotides which are zwitterionic at this pH could be pairing with themselves, thus acting as an ion pair or effectively as a neutral molecule (*26*, *27*).

Of all the compounds examined, the adenine compounds exhibit retention behavior which is most difficult to understand, e.g., the extremely high k' of adenosine at higher pHs (Table II), the increase and then decrease of k' values of AMP with increasing pH (Figure 2), the relatively low k' of ADP and very high k' of ATP at pH 2.2 (Figure 3 and 4). The other group of compounds which is of interest is the cytosine series which have shown minimal retention with most ion-exchange and reversed-phase separations (*4–8*, *16*). It is interesting to note, however, that although pH has minimal effect on cytosine and its nucleoside and 5'- monophosphate nucleotide (k' mainly <1), pH did affect the triphosphate nucleotide and high ionic strength greatly decreased the retention. In addition, k' values of CDP were greatly increased in eluents containing a high percent of organic modifier. For CMP, the retention was higher both in aqueous buffer and in buffer containing 60% organic modifier. Thus although Van Haasterts rules for RPLC chromatography of nucleobases, nucleosides, and nucleotides (*18*) apply to the adenine compounds which he studied, they are not general rules which can apply to families of naturally occurring purine and pyrimidine compounds.

The majority of the rules we had formulated for predicting retention behavior in reversed phase (*17*) generally apply to

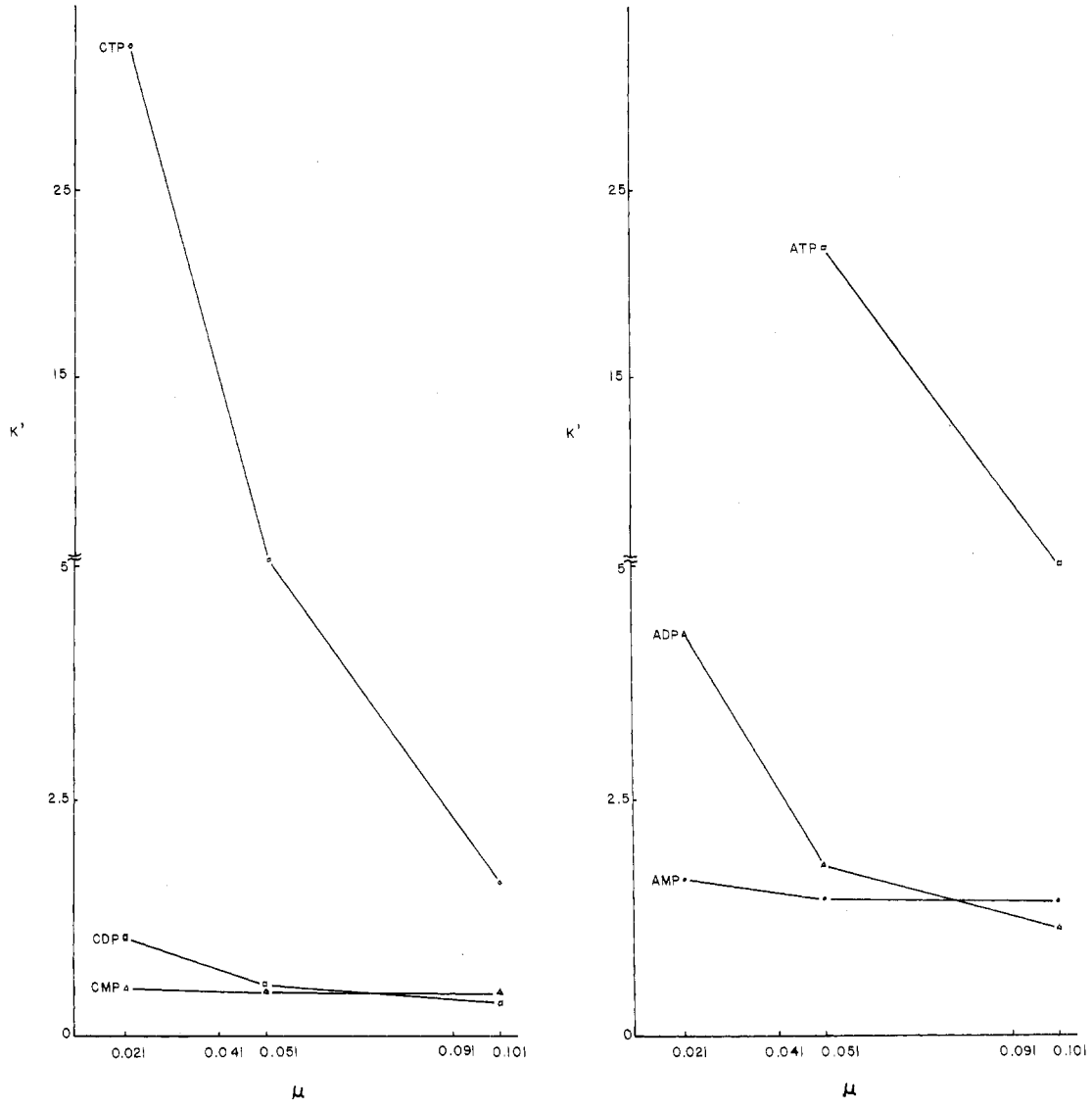

Figure 8. k' values of the mono-, di-, and triphosphate nucleotides of cytosine and adenine vs. μ, the ionic strength. The ionic strength was increased by increasing the concentration of the phosphate buffer eluent. For chromatographic conditions, see Methods.

most of the naturally occurring purine and pyrimidine compounds except for nucleotides at a low pH (2.2). Therefore, compounds which exist mainly as the aromatic tautomer or in which the molecule is charged, elute earlier. In addition, the substitution of a ribosyl or methyl group increases the k' of the comparable base (Table III). However, in general the rules do not apply in the case of amine substituents. In the purine series at pH 6.3, the substitution by an amine for a hydroxyl increased k'; however, in an eluent of pH 2.2, the k' decreased. With the substitution of an amine for the hydroxyl in the pyrimidines, as in the case of cytosine and uracil compounds, the cytosine compounds eluted before the respective uracils (Table III); hence the rule of OH < H < NH_2 did not apply universally.

It was also observed in initial experiments that the chromatographic behavior of the mono-, di-, and triphosphate nucleotides was affected by stationary phase characteristics such as the length of the carbon chain, the percent carbon loading, and the extent of silanol capping. These data support the postulation that the silanophilic mechanism (25) plays a prominent role in separation of nucleotides. However, the retention behavior of nucleosides and bases was not influenced by these stationary phase parameters; thus the limited stationary phase data we have obtained so far supports the solvophobic mechanism for the separation of the nonionic compounds. Systematic studies on the effects of stationary phase parameters on the retention behavior of purine and pyrimidine compounds are now in progress.

In general, the RPLC retention behavior of the nucleosides and bases can be explained mainly by the solvophobic theory of reversed-phase chromatography. However for the data reported here on the nucleotides it is indicated that a dual mechanism or another mechanism in place of the solvophobic mechanism is operative in the reversed-phase separations of the 5′-mono-, di-, and triphosphate nucleotides, especially in a low pH eluent.

ACKNOWLEDGMENT

The authors thank R. Ken Forcé, Raymond P. Panzica (University of Rhode Island, Kingston, RI), John O. Edwards (Brown University, Providence, RI), Imre Molnár (Knauer, Germany), Csaba Horváth (Yale University, New Haven, CT), Richard A. Hartwick (Rutgers University, Piscataway, NY), and Anté M. Krstulović (Manhattanville, NY) for their helpful discussions and Fred Rabel (Whatman, Inc., Clifton, NJ) and Klaus Lohse (Kratos, SID, Westwood, NJ) for their technical assistance.

Registry No. Adenine, 73-24-5; guanine, 73-40-5; hypoxanthine, 68-94-0; xanthine, 69-89-6; cytosine, 71-30-7; uracil, 66-22-8; thymine, 65-71-4; Ado, 58-61-7; Guo, 118-00-3; Ino, 58-63-9; Xao, 146-80-5; Cyt, 65-46-3; Urd, 58-96-8; Thd, 50-89-5; AMP, 61-19-8; GMP, 85-32-5; IMP, 131-99-7; XMP, 523-98-8; CMP,

63-37-6; UMP, 58-97-9; TMP, 365-07-1; TDP, 491-97-4; XDP, 29042-61-3; IDP, 86-04-4; UDP, 58-98-0; GDP, 146-91-8; ADP, 58-64-0; CDP, 63-38-7; ATP, 56-65-5; CTP, 65-47-4; ITP, 132-06-9; XTP, 6253-56-1; UTP, 63-39-8; GTP, 86-01-1; TTP, 365-08-2.

LITERATURE CITED

(1) Cohn, W. E. *J. Am. Chem. Soc.* **1980**, *72*, 1471.
(2) Anderson, N. G. *Anal. Biochem.* **1962**, *4*, 269.
(3) Burtis, C. A. *J. Chromatogr.* **1970**, *51*, 183.
(4) Horváth, C. G.; Preiss, B.; Lipsky, S. R. *Anal. Chem.* **1967**, *39*, 1422.
(5) Horváth, C. G.; Lipsky, S. R. *Anal. Chem.* **1969**, *41*, 1227.
(6) Brown, P. R. *J. Chromatogr.* **1970**, *52*, 257.
(7) Hartwick, R. A.; Brown, P. R. *J. Chromatogr.* **1975**, *112*, 651.
(8) Hartwick, R. A.; Brown, P. R. *J. Chromatogr.* **1976**, *126*, 679.
(9) Gehrke, C. W.; Kuo, K. C.; Zumwalt, R. W. *J. Chromatogr.* **1980**, *147*, 129.
(10) Anderson, F. S.; Murphy, R. C. *J. Chromatogr.* **1976**, *121*, 251.
(11) Anderson, N. E.; Liao, T. C. *Anal. Chem.* **1977**, *49*, 2231.
(12) Horváth, C. G.; Melander, W. *Am. Lab. (Fairfield, Conn.)* **1978**, 17.
(13) Karger, B. L.; Giese, R. W. *Anal. Chem.* **1978**, *50*, 1048A.
(14) Brown, P. R.; Krstulović, A. M. *Anal. Biochem.* **1979**, *99*, 1.
(15) Brown, P. R.; Hartwick, R. A.; Krstulović, A. M. "Advances in Chromatography"; Giddings, J. C., et al., Eds.; Marcel Dekker: New York, 1980; Vol. 18, p 101.
(16) Hartwick, R. A.; Assenza, S. P.; Brown, P. R. *J. Chromatogr.* **1979**, *186*, 647.
(17) Brown, P. R.; Grushka, E. *Anal. Chem.* **1980**, *52*, 1210.
(18) van Haastert, P. J. M. *J. Chromatogr.* **1981**, *210*, 229.
(19) van Haastert, P. J. M. *J. Chromatogr.* **1981**, *210*, 241.
(20) Hill, P. W.; Walters, F. H.; Wilson, T. D.; Stuart, J. D. *Anal. Chem.* **1979**, *51*, 1338.
(21) Molnár, I.; Horváth, C. G. *J. Chromatogr.* **1977**, *142*, 623.
(22) Bush, B. T.; Frenz, J. H.; Melander, W. R.; Horváth, C. G.; Cashmore, A. R.; Dryer, R. N.; Knipe, J. D.; Coward, J. K.; Bertino, J. R. *J. Chromatogr.* **1979**, *168*, 343-353.
(23) Brown, P. R.; Bobick, S.; Hanley, F. L. *J. Chromatogr.* **1974**, *99*, 587.
(24) Sober, H. A., Ed. "Handbook of Biochemistry—Selected Data for Molecular Biology", 2nd ed.; (H. A. Sober, ed.). The Chemical Rubber Co.: Cleveland, OH, 1970.
(25) Nahum, A.; Horváth, C. *J. Chromatogr.* **1981**, *203*, 53.
(26) Knox, J. H.; Jurand, J. *J. Chromatogr.* **1981**, *203*, 85.
(27) Knox, J. H.; Jurand, J. *J. Chromatogr.* **1981**, *218*, 341.

RECEIVED for review March 9, 1982. Resubmitted September 24, 1982. Accepted November 15, 1982. This research has been supported by Grant No. 5 R01 CA17603-06 and the American Hoechst Fellowship.

Liquid Chromatography of Alkali and Alkaline Earth Metal Salts on Poly(benzo-15-crown-5)- and Bis(benzo-15-crown-5)-Modified Silicas

Masaki Nakajima, Keiichi Kimura,* and Toshiyuki Shono

Department of Applied Chemistry, Faculty of Engineering, Yamada-Oka, Suita, Osaka 565, Japan

The chromatographic behavior of alkali and alkaline earth metal halides on crown ether modified silicas was investigated with water or water/methanol as the mobile phase. Alkali metal halides, LiX, NaX, KX, RbX, and CsX (X = Cl, Br, or I), can be successfully separated on poly(benzo-15-crown-5)- or bis(benzo-15-crown-5)-modified silica by elution with water or water/methanol mixtures. The separation of alkali metal halides having a common cation is also possible on the modified silicas. The chromatographic behavior of the modified silicas was found to reflect the excellent cation selectivity of poly- and bis(benzo-15-crown-5)s significantly. These silicas were also tested for their usefulness in separation of alkaline earth metal chlorides.

Macrocyclic polyethers, which are commonly called "crown ethers", have the ability to form stable complexes with various metal cations (*1*). They have various cation selectivities which are related mainly to the relative size of cation and crown ether ring. Recently, it has been reported that the crown compounds can be applied in the field of analytical chemistry, such as solvent extraction (*2–5*), ion-selective electrode (*6–8*), and liquid chromatography (*9–17*).

One of their most important applications in analytical chemistry may be in liquid chromatography, where they may be used as components of either the mobile or the stationary phase. With respect to the former approach, a few studies have been reported on the separation of sulfonic acids on silica gel (*9*) and of amino compounds on a hydrophobic stationary phase (*10, 11*) using crown ether containing mobile phase. There are extensive investigations (*13–17*) for the latter approach. Some crown compounds are immobilized on insoluble organic supports such as cross-linked polystyrene and condensation polymers and coated on silica gel. Separations of some metal cations, anions, and organic compounds are attained by using the stationary phases.

Our previous works (*2–5*) on solvent extraction of alkali picrates have made it clear that poly- and bis(crown ether)s form sandwich-type 2:1 crown ether unit to cation complexes intramolecularly with particular metal cations. The poly(crown ether)s possessing neighboring crown ether moieties have excellent selectivities and great cation-binding abilities compared with the corresponding monomeric analogues. Taking advantage of the attractive cation-binding properties of the poly(crown ether)s, we have modified silica gel with poly- and bis(benzo-15-crown-5) (*12*), aiming at excellent stationary phases for high-performance liquid chromatography (HPLC) of metal ions. This paper describes in detail the syntheses of poly(benzo-15-crown-5)- and bis(benzo-15-crown-5)-modified silicas, and their chromatographic behavior on separation of alkali and alkaline earth metal halides.

EXPERIMENTAL SECTION

Syntheses of Stationary Phases. The schemes of the syntheses and the expected structures of poly(benzo-15-crown-5)- and bis(benzo-15-crown-5)-modified silicas employed here are illustrated in Figure 1.

Poly(benzo-15-crown-5)-Modified Silica. Silica gel (Wako-gel LC-10H, 10 μm, irregular) was activated by refluxing in concentrated HCl for 4 h, followed by washing with water repeatedly, and dried overnight under vacuum at 80 °C. To this activated silica gel (13.39 g), a toluene solution (70 mL) of (3-aminopropyl)triethoxysilane (0.045 mmol, 10.0 g) was added and then stirred for 30 min. The mixture was kept at 80 °C, the solvent being evaporated gradually. After the reaction, the NH_2-modified silica gel (I) was suspended in dry methylene chloride, and then methacrylic anhydride (0.05 mol, 6.61 g) was added dropwise in

0003-2700/83/0355-0463$01.50/0