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REVERSED-PHASE RETENTION OF NUCLEIC ACID COMPONENTS

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I. INTRODUCTION

A. Background

The factors which affect the physiological balance of nucleotides, nucleosides and bases occupy many areas of biochemical, biomedical and genetic research. These components play central roles in the formation and function of the nucleic acids. In addition, many of the purines and pyrimidines serve as regulators and/or messengers in physiological processes. Thus, analogs of these components have been found useful as chemotherapeutic and antibiotic agents.

The purines and pyrimidines found within a physiological matrix can originate from de novo or salvage pathways, enzyme catalyzed degradation of tissues, nucleic acid catabolism and dietary sources. Depending on the matrix, these compounds can be found in micromolar to femtomolar concentrations. Often, the levels of the nucleosides, nucleotides and bases can be related to normal metabolic states or to abnormalities caused by disease. For the neoplastic diseases, the methylated nucleic acid components are of particular interest. The methylated bases occur mainly in tRNA and to a lesser extent in rRNA. Unlike the major nucleic acid components,

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there appears to be no mechanisms to recycle the methylated compounds. Thus, alterations in the excreted levels of the minor components are believed to reflect the extent of tRNA modification and metabolism in proliferative cells.

The importance of the purine and pyrimidine compounds made essential the development of reliable analytical techniques for rapid and sensitive determinations in complex matrices. While many different methods have been applied, the advent of high-performance liquid chromatography (HPLC) most greatly facilitated routine separation and measurement of nucleic acid components in biological materials.

Since the introduction of HPLC [1,2], the technique has had an explosive growth in its application to assays of ionic, polar, nonvolatile and thermally labile compounds. In addition, the wide variety of chromatographic modes made possible separations which previously required long analysis times, extensive purification and laborious derivatization procedures [3].

The liquid chromatographic determination of nucleic acid components can be traced to the research of Cohn [4]. Separations were obtained with polystyrene-divinylbenzene ion-exchange resins (100-μm) packed in open-columns. With these early methods, chromatographic capacities were high; however, long analysis times, poor efficiencies and solute degradation were some of the major drawbacks.

In an attempt to overcome the limitations associated with the open-column systems, pellicular packings were developed [1] and applied to purine and pyrimidine separation by Horvath and Lipsky [5], Kirkland [6], Brown [7] and others [8,9]. The pellicular packings were prepared by coating the resin around an inert glass or silica bead usually 40-70 μm in diameter. A notable advantage of the pellicular packings was an increase in efficiency. In addition, the rigidity of these solid core particles made possible the use of high flow-rates. However, these stationary phases were compromised by low capacity and instability since the outer shell could be striped from the support.

Following the suggestion by Stewart and Perry[10], the problem of column instability was resolved by chemically bonding the material to the silica support. Shortly thereafter, the use of bonded stationary phases in HPLC gained widespread acceptance mainly through the research of Halasz and Sebestian [11], Kirkland [12] and others.

The chronomatographic systems currently available were made possible by the development of microparticulate chemically bonded stationary phases [13]. The distinct feature of the modern day packings is the use of totally porous spherical or irregularly shaped silica particles 3-, 5- or 10-μm in diameter. In comparison with the pellicular packings, the silica microparticles have greater capacities due to higher surface area. In addition, efficiencies are vastly improved since the area in which the mobile phase could stagnate is reduced. While the use of such small particles would have also been desirable in the early days of HPLC, methods were not available to obtain the siliceous material within a uniform size distribution. Moreoever, neither the chromatographic instrumentation nor the technology in packing columns was sufficiently developed.

Initially, many researchers felt that only the ion-exchange HPLC methods would give adequate separation of nucleic acid components [14-16]. However, the reversed-phase mode of HPLC (RPLC) showed greater promise for purine and pyrimidine determinations [17,18]. In fact, since the advent of the microparticulate chemically bonded packings, RPLC has had a spectacular ascent and has emerged as the most widely accepted type of liquid chromatography [17-24]. Separations of nucleosides, nucleotides and bases are readily obtained using largely aqueous eluents [17-19] or with ion-pairing agents [20-22].

The popularity of RPLC is due to the many advantages offered over the other chromatographic modes. Among the advantages are:

1. Operational simplicity and ease of use;
2. High efficiency, reproducibility and sample turnover;
3. Column stability and long lifetimes;

4. Simultaneous separation of a broad scope of closely related and vastly different compounds, including compounds with a wide range of polarities, ionic states and molecular weights;
5. The variety of different types of reversed-phases commercially available, as well as those which can be prepared in-house;
6. The ability to manipulate the mobile phase to tailor separations for compounds with unique features;
7. The ease in which separations are optimized;
8. The ability to determine physicochemical properties such as hydrophobicity, dissociation constants and complex-formation constants.

The latter five advantages are perhaps the most significant aspects of RPLC. This article will review these factors in terms of their relationships to purine and pyrimidine separations. In addition, some applications will be presented. For more detailed coverage of specific applications and associated procedures involved in the analysis of complex matrices, the recent publications by Brown and co-workers[25-28] are suggested. The general reviews by Horvath[24,29], Karger[23] and Cooke[30] on RPLC are also suggested. For those interested in the evolution of liquid chromatography, Ettre[31] has given a historical overview. Moreover, the extensive coverage of recent literature pertaining to liquid chromatography by Majors, et al[32] is strongly recommended.

B. Purine and Pyrimidine Structures

The basic structures and numbering of the purine and pyrimidine rings are shown in Figure 1. These nitrogen heterocycles are referred to as the base or aglycone; the base is the primary structure from which the nucleoside or nucleotide is derived. The term nucleoside is used for the compounds which have a ribosyl substituent on the base structure (Figure 2). A nucleotide (Figure 3) is a nucleoside with one, two or three phosphate groups in place of the hydroxyl substituents on the ribosyl moiety. In addition, it is possible for many of these compounds to exist in several tautomeric forms. The environment in which the compound is present as well as

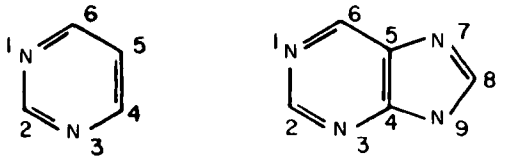

FIGURE 1

Structure and systematic numbering of the pyrimidine base (A) and the purine base (B).

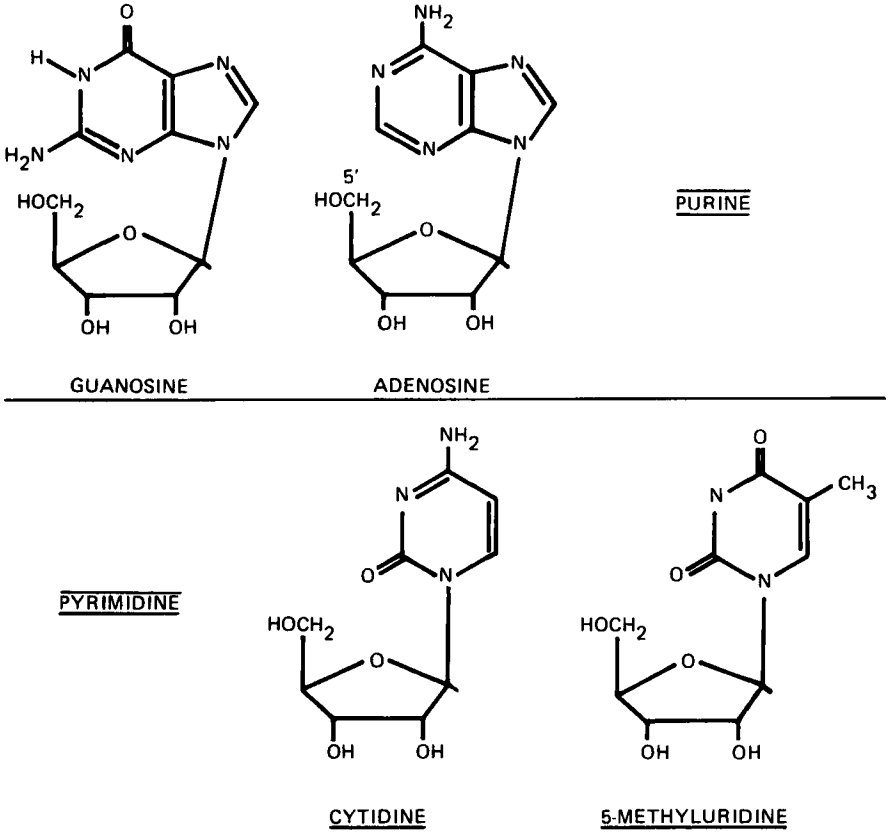

FIGURE 2

Some structures of purine and pyrimidine nucleosides.

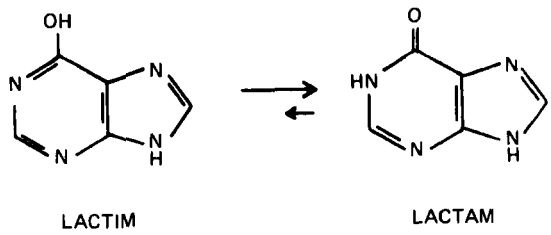

FIGURE 3

Adenosine 5'-monophosphate showing the three levels of structure.

neighboring substituents dictate which tautomeric form predominates; an example of the lactim-lactam structures of hypoxanthine are shown (Figure 4). In most cases, the lactam and amino form predominate in neutral aqueous solutions.

Nomenclature problems abound in the description of the purines and pyrimidines since many are known by systematic, biochemical and trivial names; thus complicating a general presentation of the original reference material. Furthermore, there exists a variety of symbols and abbreviations which can be used to represent a single compound; this is especially true for those compounds with many substituents. Therefore, in an attempt to facilitate the presentation, a single format was adopted and used throughout. The format is based on the IUPAC-IUB guidelines[33]; examples of names and symbols for the nucleic acid components are given in Table I.

C. Physical Properties

For the chromatographic analysis of the nucleic acid fragments, ionization constants and spectral properties are useful (Table II). As a guide in the development of chromatographic conditions, the extent of dissociation expected in any particular eluent can be used to predict retention and/or elution order. In addition, identifications of the nucleosides, nucleotides and bases are facilitated by their absorption characteristics at several wavelengths or pH values.

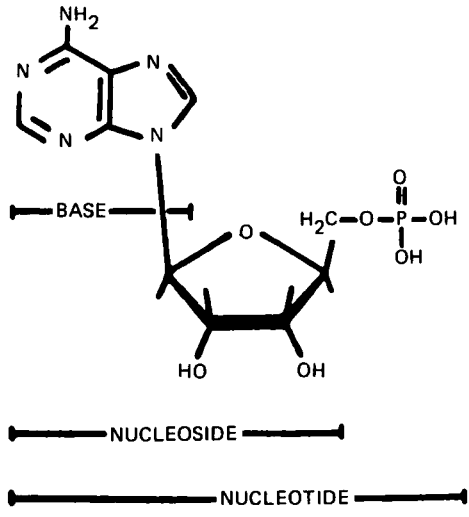

FIGURE 4

Tautomeric structures of hypoxanthine.

II. REVERSED-PHASE LIQUID CHROMATOGRAPHY

A. Separation Mechanism

The mechanism of separation in RPLC is not well understood and is the subject of extensive research. Models based on partition[34], absorption[35], dispersive interaction[36], compulsory absorption[37], solubility[38,39], solvophobic interaction[40,41] and mixed solvophobic-silanophilic interaction[42,43] have been proposed. While certain features tend to overlap in these models, a unified theory of retention in reversed-phase systems has yet to be developed[44].

From the studies of reversed-phase retention, it is apparent that many complex factors are involved. It is generally acknowledged that the interactions between the bulk mobile phase and the solute are predominant in the mechanism of retention with reversed-phase systems. However, the role of the stationary phase is less certain. While solute-stationary phase interactions are largely seen to be secondary to the solute-mobile phase interactions, in-

TABLE I

NAMES AND ABBREVIATIONS FOR NUCLEIC ACID COMPONENTS

Bases

Uracil (Ura)
Thymine (Thy)
Adenine (Ade)
Guanine (Gua)

Nucleosides[a]

Uridine (Urd, U)
Thymidine (Thd, T)
Adenosine (Ado, A)
Guanosine (Guo, G)

Nucleotides[b]

Uridine 5'-monophosphate (UMP, pU)
Uridine 5'-diphosphate (UDP, ppU)
Uridine 5'-triphosphate (UTP, pppU)

Examples of 2'-Deoxyribonucleosides[a]

Deoxyuridine (dUrd, dU)
Deoxythymidine (dThd, dT)

Example of 2'-Deoxyribonucleotide[b]

Deoxycytidine 5'-monophosphate (dCMP)

[a] The three letter abbreviations are associated with topics of chemical change; the capital letter symbols imply the source was a nucleic acid.

[b] The position of the phosphate group can be indicated with the prefix 2', 3', or 5' for simple nucleotides and by 2', 3'- and 3', 5'- for cyclic nucleotides; the lower case p to the left indicates a 5'-phosphate and to the right a 3'-phosphate for the oligonucleotides.

stances where the stationary phase contributes significantly to retention have been noted. In addition, the role of solute structure is not well defined; frequently models of the retention process derived from the observed behavior of one class of compounds fail to describe the retention of another class of compounds. In particular,

TABLE II

ULTRAVIOLET AND IONIZATION DATA FOR SOME NUCLEIC ACID COMPONENTS

Compound	pK_1	pK_2	λ Max (nm)		
			Acidic	Neutral	Basic
Cyt	4.5	12.2	276	267	282
Cyd	4.2	12.5	280	270	273
Thy	---	9.9	265	265	291
Thd	---	9.7	267	267	267
Ade	4.2	9.8	263	261	269
Ado	3.5	12.5	257	260	259
Gua	3.2	9.6	248, 276	246, 276	274
Guo	1.6	9.2	256	254	256,266
Hyp	2.0	8.-	248	250	263
Ino	1.2	8.8	248	249	253
AMP	3.7	6.6	257	260	259
ADP	3.9	7.2	257	260	259
ATP	4.0	7.7	257	260	259

this can be seen when the retention of purines is compared to non-polar or moderately polar hydrocarbons[45].

1. Solvophobic Theory in RPLC

The solute and mobile phase interactions which are seen primarily to govern reversed-phase retention are ascribed to hydrophobic or solvophobic effects[40,41]. The hydrophobic effect is an entropically driven phenomenon which accounts for the clustering of non-polar solutes in water; the salient feature is that the organizing force is based on repulsion by the solvent rather than an attraction by the solutes. The solvophobic theory[46-48] is a generalized variant of hydrophobicity that is applicable to the eluents commonly used in RPLC and in principle employs accessible physicochemical data.

Horvath and co-workers[40,49] have adapted the solvophobic theory to describe the mobile phase generated selectivity observed in reversed-phase systems. Within the framework of this model, the driving force for retention is mainly due to an increase in the entropy of the mobile phase accompanying the transfer of the solute from the liquid phase to the stationary phase. It is important to note that the stationary phase is assumed to play only a passive role in the retention mechanism; this assumption is valid only when the nature of solute binding to the stationary phase is weak and nonspecific.

The treatment of physicochemical phenomena underlying both ionized and nonionized solute retention in RPLC with the solvophobic theory has been given in detail[40,49]. However, the theory on the influence of the solvent on the associations of nucleic acid components has not been fully developed. Investigations in this direction have been made by Sinanoglu[46-48]; similar calculations, although differing in details, were also given by Pullman[50].

The reversible association of the solute with the reversed-phase is based on the thermodynamic properties associated with the tendency to minimize the site of cavities formed by the solute molecules in the hydro-organic mobile phase; the interactions of the solute and the alkyl-chains of the stationary phase are be-

lieved to be purely hydrophobic in nature and not due to electrostatic or hydrogen bonding effects. Retention is expressed in terms of the overall standard free energy change (ΔG_T) associated with the transfer of the solute from the mobile phase to the stationary phase by

$$\text{In } k' = \ln \phi - \Delta G_T/RT \qquad (1)$$

where k' is the capacity factor of the solute, ϕ is the phase-ratio of the column, R is the gas constant and T is the temperature. The ΔG_T term represents the sum of two free energy changes. The first is involved with the association of the solute and the alkyl-chain in the gas phase, while the second corresponds with the transfer of the solute, alkyl-chain and solute-stationary phase complex individually into the mobile phase. The second step accounts for the free energy changes arising from the formation of the cavity, as well as the extent of interactions with solvent molecules.

For hydro-organic mobile phases, the solvophobic expression for retention of unionized solutes is given by

$$\ln k'_o = g + \frac{N\Delta A + 4.836N^{1/3}(K^e-1)V^{2/3}}{RT}\gamma \qquad (2)$$

where k'_o is the capacity of the un-ionized solute, g is a constant which largely accounts for the van der Waals' contribution to the free energy of interaction between solvent and solute, N is Avogadro's number, ΔA is the contact surface area of the solute with the stationary phase, K^e is the microscopic cavity factor, V is the mole volume of the eluent, and γ is the eluent surface tension. This relationship reveals that $\ln k'_o$ is linearly related to surface tension, as well as to the hydrocarbonaceous surface area. In addition, the surface tension can be expressed as a function of ionic strength such that a plot of $\ln k'_o$ versus ionic strength also yields a straight line.

For ionized solutes, the relationship becomes more complex but can be given in a simplified form by

$$\ln k'_z = a' + b' * f(w) + c' \Delta A \qquad (3)$$

where k'_z is the capacity factor of the charged solute, f(w) represents the effect of solute charge on the interactions and a', b' and c' are column and solvent dependent parameters. Under isocratic conditions, this relationship predicts that a plot of ln k'_z (corrected for electrostatic effects) versus hydrophobic surface area yields a straight line. Moreover, the slope of the line remains constant when eluents of different pH but otherwise identical composition are used.

2. Mixed-Mode Interaction

Recently, Horvath and co-workers[42,43] introduced a dual-binding concept to account for solutes which deviate from the solvophobic theory. In addition to solvophobic forces, interactions between the solute and free silanols of the reversed-phase are possible. To denote this behavior, the authors used the term 'silanophilic' interaction. Apparently, the silanophilic interactions are more likely to occur with the short alkyl-chain reversed-phases than with the long alkyl-chain phases[45].

Based on the solvophobic-silanophilic model for RPLC, Zakaria, et al[51] explained the increase in the retention of nucleotides with increasing methanol concentration to the predominance of silanophilic interactions. However, under most chromatographic conditions, the RPLC retention of nucleosides, nucleotides and bases can be attributed mainly to solvophobic forces.

B. Structure-Retention Relationships

With the solvophobic theory, it is possible to predict retention based on solute structure and physicochemical characteristics[52-54]. However, relationships between the structure of the nucleic acid components and their reversed-phase chromatographic behavior have not been extensively studied. Recently, Brown and Grushka[55] noted the relationships between purine and pyrimidine substituents to reversed-phase retention. These relationships are as follows:

1. Any substituent that causes charge formation decreases k'.

2. Any substituent that causes a tautomeric shift will affect k'.

3. Both group and position of substituents affect k' in the order OH<H<NH_2<NHR. Methyl groups approximately double the k' value of the parent compound.

4. In nucleosides, the addition of the ribosyl group increases k' of the comparable base structure.

5. In the deoxyribonucleosides, the loss of the OH group in the 2'-position increases the k' over that of the ribonucleoside.

6. In nucleotides, linear phosphate groups decrease k', but the cyclic phosphate group increases k' compared to that of the corresponding ribonucleosides.

7. Based on the energy of vertical base stacking in aqueous solutions, pyrimidines elute before similarily substituted purines.

The postulations given by Brown and Grushka[55] were further evaluated by Assenza and Brown[56]. In this work, the functional group contributions to retention of a large variety of purine compounds were investigated. The quantitative determination of the functional group effects was based on extra-thermodynamic linear free energy relationships and is equivalent to other substituent parameters[52-54]. Table III shows the quantified group increment terms calculated from the observed retention of the purines with several reversed-phases. As can be seen from Table III, the group increment terms determined with columns A and B (both octadecylsilica) follow the trends noted by Brown and Grushka[55]. However, column D (trimethylsilica) is seen to have different interactions with the substituents.

The lack of correlation in selectivity between the reversed-phases was used by Assenza and Brown[56] to demonstrate a method in predicting the structure of unknown purine compounds. Capacity factors obtained on two different reversed-phases were compared with the tabulated group terms in such a way to give a list of possible substituents whose sum equaled the observed values.

C. Effect of Chromatographic Parameters

During the past decade many attempts have been made to describe and predict solute retention of RPLC. In general, the app-

TABLE III

CALCULATED T_j VALUES

Position	Substituent	Column A	B	C	D	Compounds Used
R_1	Methyl	0.890	2.34	1.23	0.892	m_1-Hyp
R_2	Oxo	0.220	-0.294	0.0520	-0.663	Xan
	Amino	0.110	-0.477	0.0160	0.440	Gua
	Methyl	0.820	0.724	1.10	0.690	m_2-Ade
	Methylamino	1.18	1.32	1.03	0.933	m_2-G
	Dimethylamino	2.45	3.42	2.09	1.97	m_2^2-G
R_3	Methyl	1.70	2.17	1.39	0.875	m_3-Xan
R_6	Oxo	-0.750	-1.91	-0.954	-0.795	Hyp
	Amino	0.320	-0.127	0.264	0.257	Ade
	Methyl	1.14	1.26	1.18	0.777	m_6-Pur
	Methylamino	2.20	3.02	1.23	1.42	m_6-Ade
	Dimethylamino	3.41	4.68	2.19	2.62	m_6^2-Ade
	Thio[1]	1.28	1.48	-0.538	3.20	SH_6-Pur
	Imino[2]	-2.03	-4.04	-1.33	-0.595	m_1-Ade
R_7	Methyl (base)	0.790	0.656	1.44	1.07	m_7-Gua
	Methyl (N'side)[4]	-0.670	-1.78	-0.460	0.460	m_7-G
R_8	Oxo	-0.802	-1.99	-1.10	-1.08	UA
R_9[3]	Ribo	1.19	1.02	0.870	0.109	l
	d-Ribo	1.31	1.45	1.31	0.561	dl
	c-Ribo	1.56	0.940	0.680	-0.584	cl
	mp-Ribo	-0.740	-1.89	-1.47	-1.92	AMP
	dp-Ribo	-1.24	-2.72	-3.51	-3.10	ADP
	tp-Ribo	-2.74	-5.22	-3.73	-3.36	ATP
	Purine	1.92	2.54	2.15	0.933	Purine

1 Substituent is in the thione form (Lactam).

2 Represents protonated form.

3 Ribo-B-ribofuranosyl, 2'-deoxribofuranosyl, 3', 5'-cyclicmonophosphate, 5'-monophosphate, 5'-diphosphate, and 5'-triphosphate, respectively.

4 See text for explanation

roach has either been empirical or thermodynamically based. In the empirical approach, retention data for a set of solutes are obtained over a variety of chromatographic conditions and are used to derive valid relationships. With the thermodynamic approach, a theoretical description is developed based on pertinent fundamental thermodynamic or physicochemical parameters; an example of this is the solvophobic theory. A major problem in the empirical description is the inability to directly apply the derived constants to other phase systems or solutes; in addition, these relationships may be valid only over a small range of conditions. The major drawback to the theoretical description is that no simple functions with rigorous thermodynamic basis exist between the solute and retention. However, both methods have made possible the evaluation, optimization and comparison of different chromatographic systems; as well as to formulate new directions to unravel the chromatographic process.

1. Stationary Phase Effects

The packingsused in RPLC are prepared by chemically bonding hydrocarbon chains of various lengths and configurations to the microparticulate silica support[13,37,57,58]. In addition, the reversed-phases can be categorized as either a 'brush' or 'bulk' packing (Figure 5). The brush-type phases result from the reaction of monofunctional silanes (e.g., trimethylchlorosilaine) with the silica microparticle; since each molecule of the silane reagent reacts with only one silanol group, a monomeric reversed-phase is produced. The bulk-type material is prepared from silica using a difunctional or trifunctional silanizing reagent; the resulting product is a cross-linked polymeric reversed-phase. Other reagents can be used to produce bonded phases which differ in the site of the linkage[27]; however, most commercial reversed-phases are prepared as shown in Figure 5.

Due to the differences between the surface structure of the reversed-phases, retention characteristics can vary widely. A major difficulty in establishing the role of the stationary phase in the chromatographic process is due to the lack of detailed

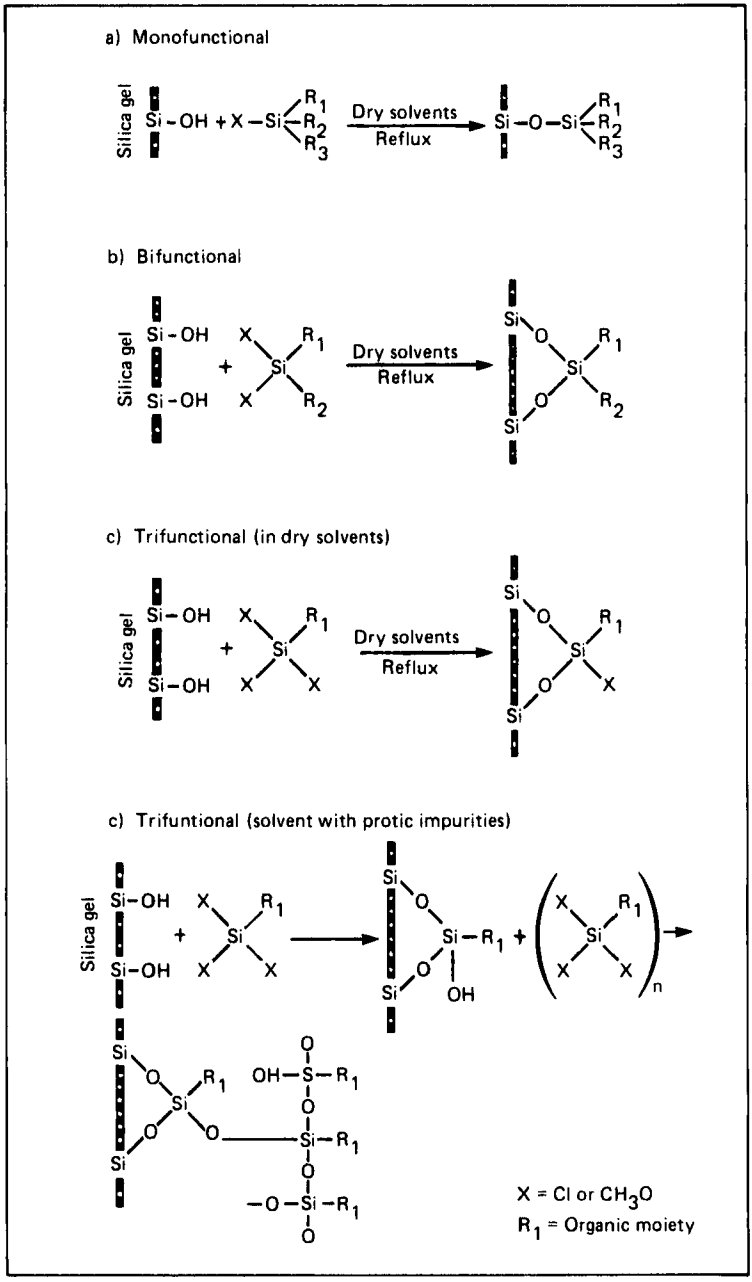

FIGURE 5

Schematic representation for the preparation of bonded reversed-phases. Reproduced from E. Grushka and E.J. Kikta, Anal. Chem., 49, 1004A (1977).

specifications. The selectivity differences observed in practice can be attributed to many factors pertaining to the stationary phase. These factors include:

1. Particle size and shape.
2. Porosity, specific surface area and pore size distributions of the unbound silica.
3. Chemical nature of the bonded hydrocarbon.
4. The configuration of the hydrocarbons.
5. The surface concentration of accessible silanol and siloxane groups.
6. Surface concentration of bonded hydrocarbon.

The influence of these factors has been reviewed by Halasz[58]. In all of the proposed retention mechanisms, solute retention has been related in some way to the carbon content of the stationary phase. The most controversial aspect relates to the effect of alkyl-chain length. With longer chains, the amount of organic content per unit column volume increases, generally resulting in increased retention. However, chain length alone has no specific role[37,45,57]; the different selectivities observed with the various linear alkyl-chain phases are due to hydrocarbonaceous surface area and the accessibility of residual silanols[45]. Referring to Table III, it can be seen that the octylsilica column (C) more strongly retained purine than one of the octadecylsilica columns (column A) due to higher hydrocarbonaceous surface area. In addition, certain selectivity differences can be seen with the trimethylsilica column (D) which is probably due to interactions with residual silanols. According to the recent work by Horvath[45], the good correlation between the selectivities of columns A and B is due to homoenergetic behavior. Columns A and C are said to be homeoenergetically related. The differences between A and D are due to heteroenergetic behavior.

Zakaria and Brown[59] recently studied the effects of the stationary phase on purine and pyrimidine retention; the results of this investigation corroborates other research in this area[39-43, 51, 56-58]. As was expected from the solvophobic

theory, nucleoside and base retention was greater on packings with higher hydrocarbonaceous surface area. Selectivity differences were also observed with the octadecylsilica packings having different surface coverages. Moreover, retention outside of the solvophobic theory was found for nucleotides chromatographed on the phases with lower hydrocarbonaceous surface area; methanol enhanced the silanophilic interactions which in turn altered the selectivities for compounds with electron-rich substituents.

2. Mobile Phase Effects

a. pH

The effects of mobile phase pH on purine and pyrimidine separations have been studied in detail[17,49,51,60,61]. Figure 6 illustrates the change in retention for nucleosides and bases with pH. In addition, the effect of pH on some nucleotides is shown (Figure 7). As can be seen, the compounds which can ionize over the pH range of 2-7 display dramatic changes in their capacity factors when the pH is near the pK_a. Horvath[49] found that this behavior can be adequately described by a simple equation. For a monoprotic acid the relationship is given by

$$k' = \frac{k'_o + k'_2 \frac{K_a}{[H^+]}}{1 + \frac{K_a}{[H^+]}} \qquad (4)$$

and for a base by

$$k' = \frac{k'_o + k'_z \frac{[H^+]}{K_a}}{1 + \frac{[H^+]}{K_a}} \qquad (5)$$

where k'_z in equation 4 is the capacity factor of the negatively charged solute and in equation 5 for the positively charged solute; all other terms have their usual meaning.

According to equations 4 and 5, the capacity factor changes with pH in a sigmoidal fashion. The midpoint of the transition occurs at a pH equal to the apparent pK_a of the compound. Figure 8 shows the basic behavior of Cyt; also shown is the change in

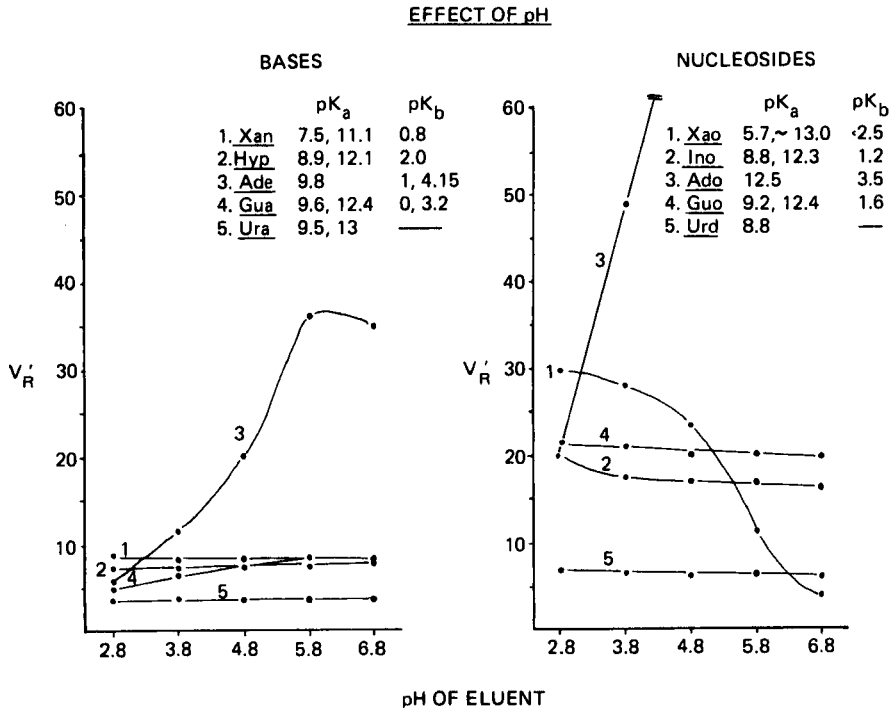

FIGURE 6

Effect of pH of the mobile phase on the retention of nucleosides and bases on a reversed-phase column. The ionic strength was held constant by the addition of KCl to the 0.01 M phospate buffer. Column: μBondapack C_{18}. Flow: 1.5 ml/min. Temperature: ambient. From reference #17.

solute charge over the pH range. As a rule of thumb, the ratio of the capacity factor in the neutral state (k'_o) to the ionic state (k'_z) is approximately 3-4 for the purines and pyrimidines. In addition, a plot of ln k' versus pH is found to be linear over the pH range of $pK_a \pm 1.5$.

From these relationships, it is readily possible to optimize separations from reported values of pK_a (corrected for temperature or ionic strength); Gehrke[60] has also shown that methanol

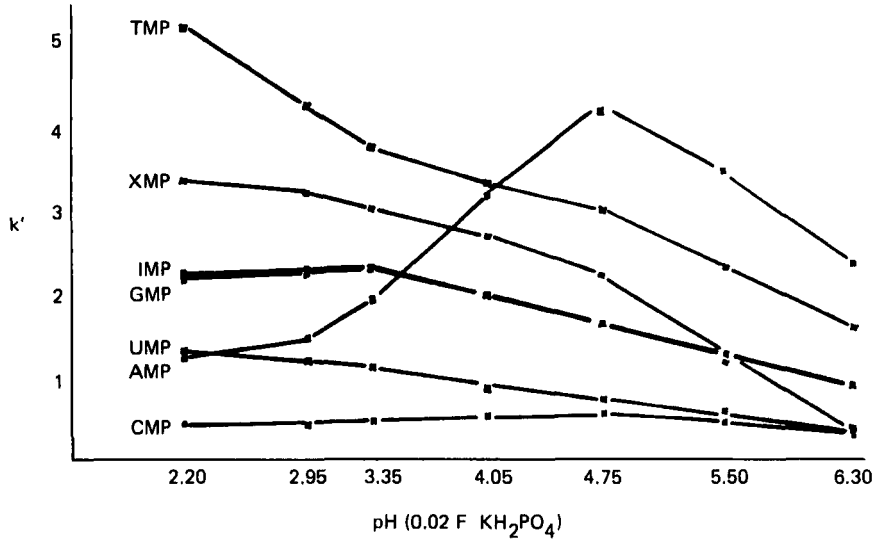

FIGURE 7

Effect of pH on the retention of nucleotide monophosphates. Conditions are as in Fig. 6, except the column was a Partisil-ODS. From reference #51.

does not appreciably change the pH relationship. In addition, solute pK_a can be determined from chromatographic behavior[49,62].

b. Organic Modifier

The retention behavior of nucleic acid components in the presence of increasing concentrations of organic modifier is typical of that expected from the solvophobic theory; the capacity factor decreases with increasing organic content of the mobile phase. Both the surface tension and dielectric constant of the hydro-organic mobile phase dictate the retention of purines and pyrimidines in solvophobic chromatography.

In accord with the solvophobic model, an empirical relationship which greatly simplifies the prediction of the effect of solvent composition on retention is given by[63,64]

$$k' = k'_w e^{mC} \qquad (6)$$

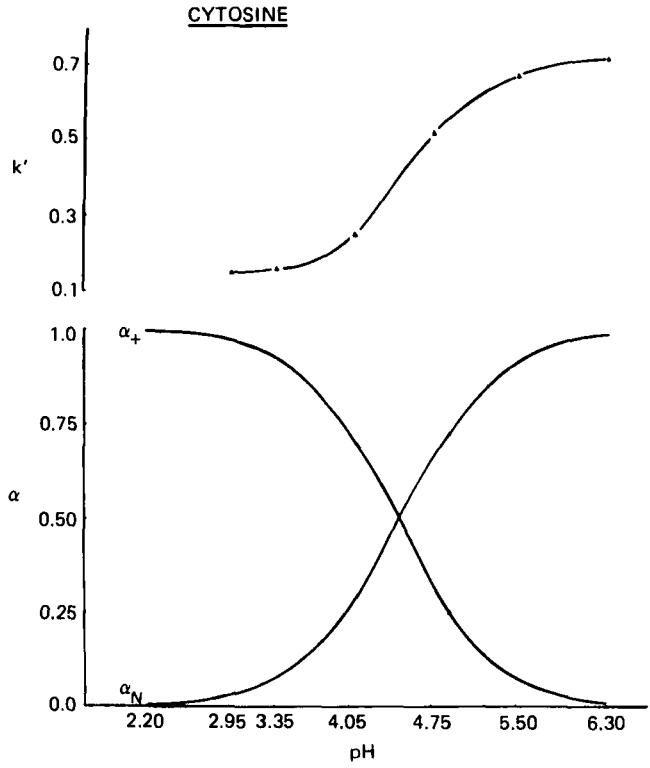

FIGURE 8

Effect of pH on Cyt (upper). Also shown is the relative change in the charge of the molecule. Conditions are as in Fig. 7. In lower graph α_+ is the ratio of the cytosine molecules in the positively charged state to the total number of cytosine molecules, and α_N is the number of cytosine molecules in the neutral state to the total number of cytosine molecules. From Reference #51.

where k'_w is the capacity factor in the totally aqueous eluent and m is the slope of the ln k' versus the concentration (C) of organic modifier in the mobile phase. Figure 9 shows this relationship for purines and pyrimidines. Hartwick and co-workers[63,64] used equation 6 to predict purine and pyrimidine retention in both isocratic and gradient elution. Generally, the magnitude of the slope can

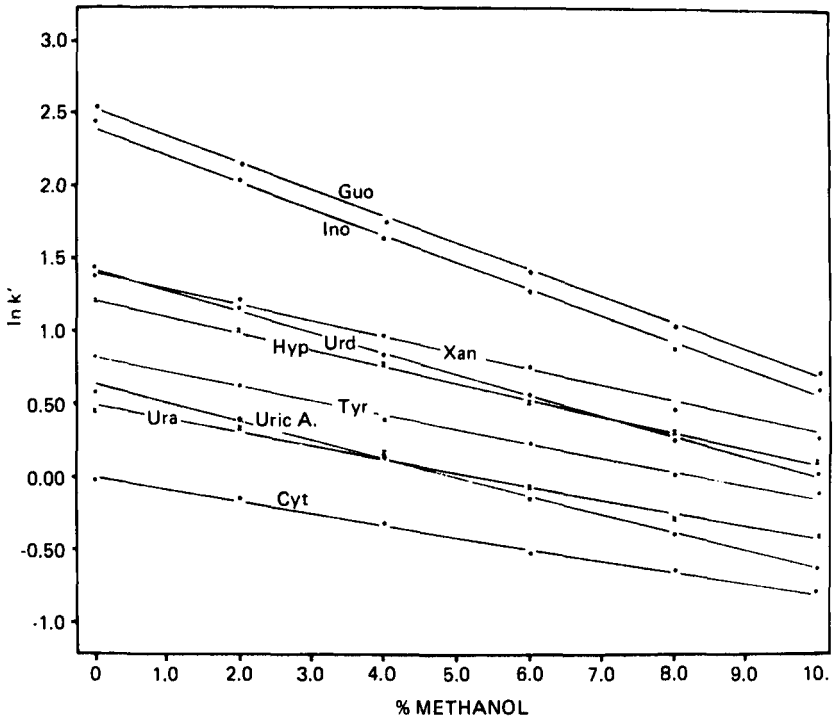

FIGURE 9

The effect of methanol concentration in the mobile phase on retention. Tyr: tyrosine. Uric A.: uric acid. Column: Partisil-ODS. Flow: 1.0 ml/min. Temperature: ambient. From reference #63.

be estimated from partition coefficients. In addition, there is a linear relationship between ln k'_w and m; solutes which are more strongly retained with the neat aqueous eluents are affected to a greater extent by changes in methanol concentration leading to steeper slopes[60,63,64]. Moreover, it is possible to determine the additive effects of the substituents on m[53,54]; Gehrke[60] has indirectly noted these substituent effects.

Anomalous retention is noted for nucleotides[51]. At acidic pH, nucleotide retention increased with increasing organic contents

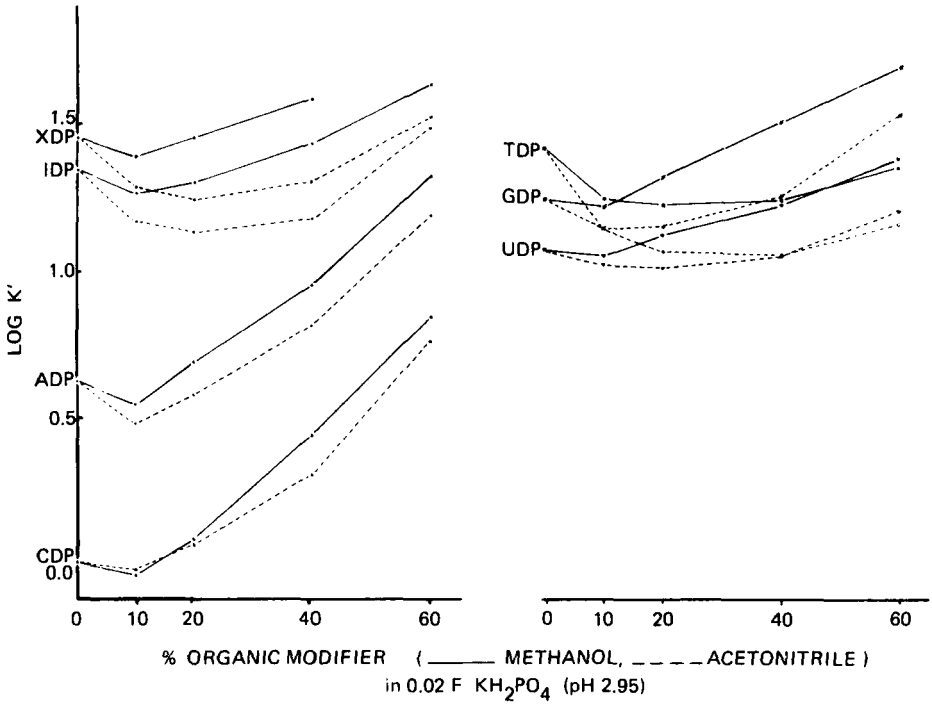

FIGURE 10

Anomalous behavior of nucleotides with increasing organic modifier. Conditions as in Fig. 7. From reference #51.

in the mobile phase (Figure 10). This contradiction to the solvophobic theory suggests silanophilic interactions[42]. However, at pH values close to neutrality the nucleotides are seen to behave like the nucleosides and bases (Figure 11).

c. Ionic Strength

Changing the ionic strength of the mobile phase has little effect on the retention of nucleosides and bases[51,61]. However, the ionic strength effect is more pronounced in the case of ribonucleotides at pH where the compound is ionized. Figure 12 shows the observed effect of ionic strength on nucleotide retention. With increasing ionic strength the ribonucleotide triphosphates, which have the largest negative charge, are subject to the greatest

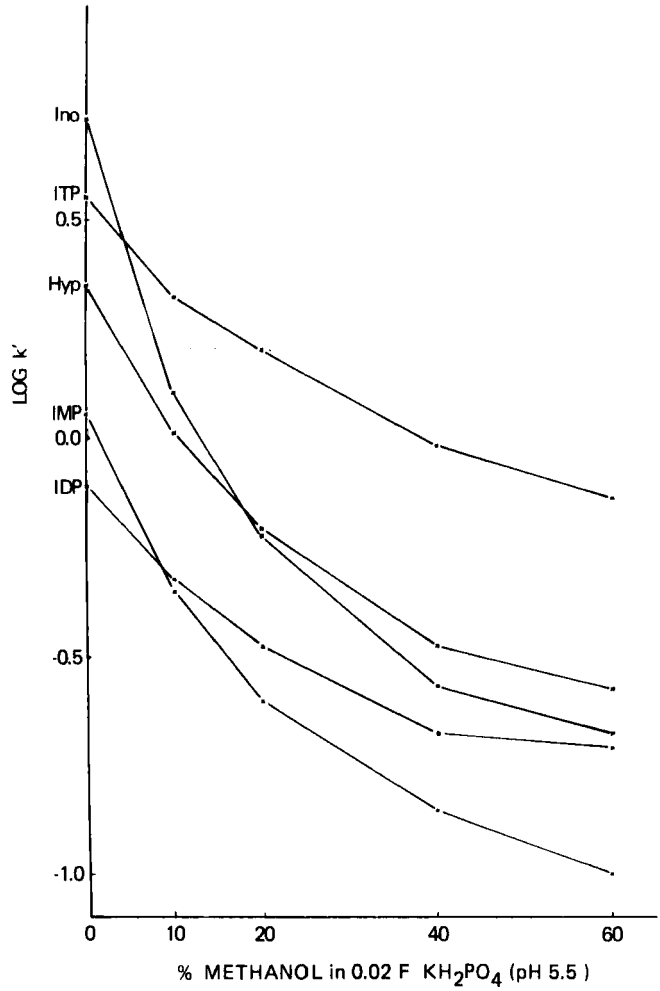

FIGURE 11

Solvophobic behavior of nucleotides (and corresponding nucleoside and base) with increasing methanol concentration in near neutral eluent. Conditions as in Fig. 7. From reference #51.

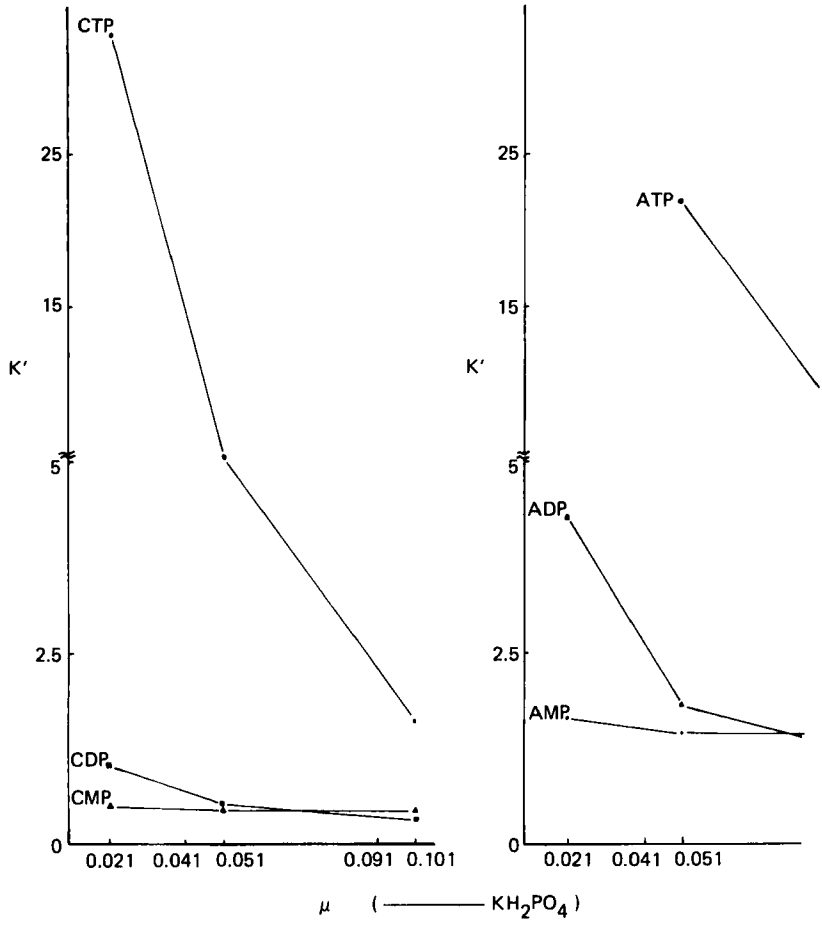

FIGURE 12

Effect of ionic strength on nucleotide retention. Condition as in Fig. 7. From reference #51.

decrease in retention. The data in Figure 12, however,are not in total agreement with that observed by Taylor, et al[61]; it is believed that the differences arise from the use of different reversed-phases.

Taylor[61], Christman[65] and Whitehouse and Greenstock[66] have taken advantage of the ionic strength effect to optimize

nucleotide separations. However, it should be noted that applications which call for the use of high concentrations of phosphate buffer can damage the column; care should therefore be taken not to leave the stationary phase exposed to the salts for prolonged periods.

3. Temperature Effects

Column temperature is a parameter which has received little attention. The vast majority of RPLC separations are conducted at ambient temperatures. Horvath, et al[1] studied the effect of temperature on the separation of nucleotides in ion-exchange HPLC; Gehrke, et al[60] briefly examined the effect of temperature on RPLC separations of nucleosides. However, temperature is an important parameter in terms of reproducibility[67], increased efficiency[68] and optimization[68,69].

The most important aspect of temperature in purine and pyrimidine separation is its effect on selectivity and resolution. From equation 1, the following expression for retention can be derived

$$\ln k' = \Delta H/RT + \Delta S/R + \ln \phi \qquad (7)$$

where ΔH and ΔS are the enthalpy and entropy changes associated with the retention process. Equation 7 predicts a linear relationship between ln k' and 1/T; the slopes of the van't Hoff plots give the enthalpy term while the intercept is the sum of the entropy and phase-ratio terms. In addition, the relationship can also be given by

$$k' = k'_s e^{nT} \qquad (8)$$

Where k'_s is the capacity factor at some standard state and n is the slope of the ln k' versus T plot.

Horvath and co-workers[69] demonstrated the concomitant dependence of solute retention on column temperature and solvent composition. The enthalpy change experimentally deduced was found to be compensated by an entropy change indicative of a constant mechanism over a relatively wide range of temperatures and organic modifier concentrations. The following equation,

$$\ln k' = -\frac{\Delta H}{R}\left(\frac{1}{T} - \frac{1}{p}\right) - \frac{\Delta G}{Rp} + \ln \phi \qquad (9)$$

where p is the compensation temperature and ΔG is the change in the Gibbs free energy, shows that the value of the compensation temperature is constant; usually 600-700 ^{o}K for reversed-phase systems which are described by the solvophobic model. The dependence of k' on temperature and solvent composition is given in equation 10:

$$\ln k' = A_1 C\left(1 - \frac{T}{p}\right) + A_2/T + A_3 \qquad (10)$$

where C is the concentration of the organic modifier. The terms A_1, A_2, and A_3 represent empirical constants:

$$A_1 = m\Delta H_c(0)/Rp$$
$$A_2 = -\Delta H_c(0)/R \quad \text{or} \quad -[\Delta H_c(0) + \Delta H_n(0)]/R$$
$$A_3 = \Delta S(0) + \ln \phi$$

where $\Delta H_c(0)$ and $\Delta S(0)$ are the enthalpy and entropy change for retention in neat aqueous eluents and the constant m again refers to the slope of ln k' versus C plots. In addition, where the enthalpy is not fully compensated, a correction factor $\Delta H_n(0)$ is used and has been found to be constant for the solutes and chromatographic system investigated[69].

The significance of these relationships represent a major step forward in establishing a rigorous treatment of reversed-phase retention. A finding that the A_1, A_2, and A_3 terms vary linearly with solute structure[69] indicated that they were subject to linear free energy relationships; this has been subsequently verified[53,54]. The importance of the linear free energy relationships is that a library of pertinent substituent constants can be established and used to predict reversed-phase retention based on solute structure a priori. Thus, the substituent effects noted in Table III can be expanded to account for temperature and methanol effects; research along these lines have recently been conducted in our laboratory. Moreover, inclusion of pH and buffer effects in terms of the substituent constants[70] should also be possible for the purines and pyrimidines.

Another important aspect of the temperature relationship was demonstrated by Diasio and Wilburn[71]. The authors used sub-ambient column temperature to improve the separation of fluorouracil metabolites.

4. Ion-Pairing

The use of ion-pairing in RPLC has extended the utility of the method to include simultaneous separation of ionized and non-ionized compounds[20-22]. Although the exact mechanism of reversed-phase ion-pairing is subject to controversy, three distinct views have been proposed. The first model suggests the formation of ion-pairs in the mobile phase[72,73] prior to interaction with the stationary phase. The second view stipulates an ion-exchange mechanism[20] where the ion-pairing agents in the mobile phase transform the stationary phase into an ion-exchanger. The third approach suggests that the retention mechanism is due to ion-interaction[74]. With this model, ion-pairs are not formed in the mobile phase; rather it is assumed that the lipophilic ions are in a dynamic equilibrium. This results in the formation of an electrical double layer on the hydrocarbonaceous stationary phase. Thus, retention is based on an electrostatic attraction due to the surface charge density of the ion-pairing ions and from a sorption effect with the stationary phase.

Hoffman and Liao[20] first used an ion-pairing agent for the separation of major ribonucleotides with an octadecylsilica column. Although complete resolution of all compounds was not possible (Fig. 13), the potential and utility of the method was demonstrated. Subsequent work by Darwish and Prichard[22] with more efficient columns greatly improved these separations. An interesting application of ion-pairing was given by Erhlich and Erhlich[75] for the separation of DNA bases (Figure 14). In addition, a new ion-pairing technique, zwitterion-pair[21], was shown to be very flexible and has great potential for further development.

Also classified as an ion-pairing type of technique is the use of metal ions to enhance separations and alter elution orders.

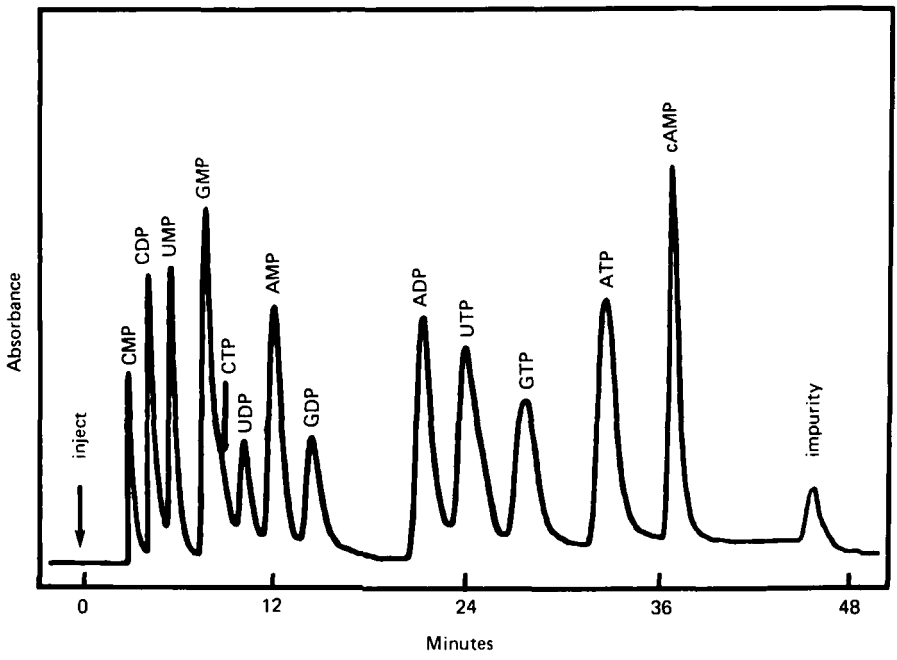

FIGURE 13

Ion-pairing separation of nucleotides. Mobile phase gradient: (A) 0.025 M tetrabutylammonium hydrogen sulfate and 0.050 M KH_2PO_4 plus 0.080 M NH_4Cl at pH 3.9; (B) 0.025 M tetrabutyl ammonium hydrogen sulfate and 0.10 M KH_2PO_4 plus 0.2 M NH_4Cl at pH 3.4 in 30% methanol. *Operating conditions:* 40-min gradient program (concave-8) at 1.0 ml/min. From reference #20.

Chow and Grushka[76] used Mg^{2+} in the mobile phase to dramatically improve the separation of nucleotides, nucleosides and bases. Later, Horvath, et al[77] reported the use of RPLC to measure the association constants of the metal binding to nucleotides.

5. Gradient Elution

A detailed treatment of the use of gradient elution in the separation of nucleosides and bases has been given[63,64]. Using the relationship shown in equation 6, Hartwick, et al[63] determined that nucleoside and base separations could be optimized sys-

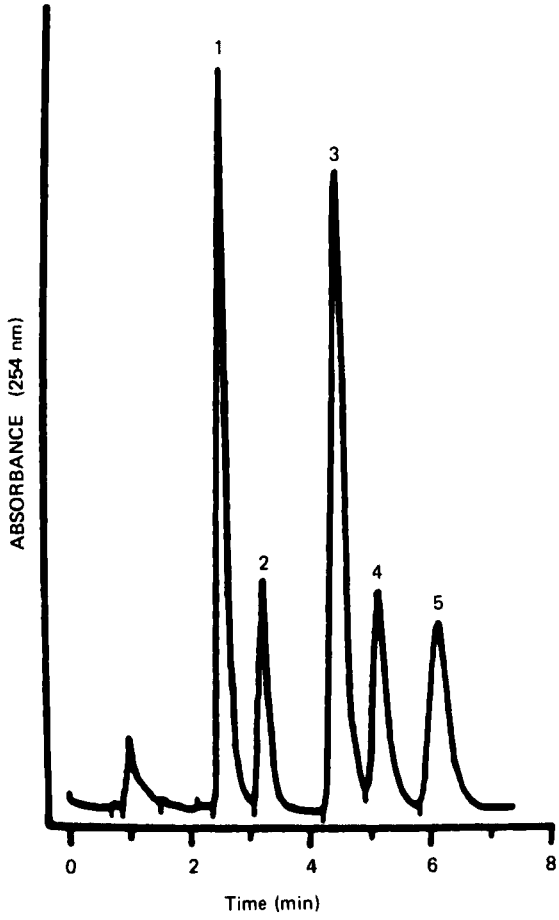

FIGURE 14

Separation of nucleic acid bases. Peak Identities: 1. Ura, 2. 5-M-Cyt, 3. Gua, 4. Cyt, and 5. Thy. All about 200-pmol. The separation was obtained with a C_{18} column and an eluent containing 5-mM heptane sulfonate and 2.5-mM potassium phosphate (pH 5.6). Flow 2 ml/min. From reference #75.

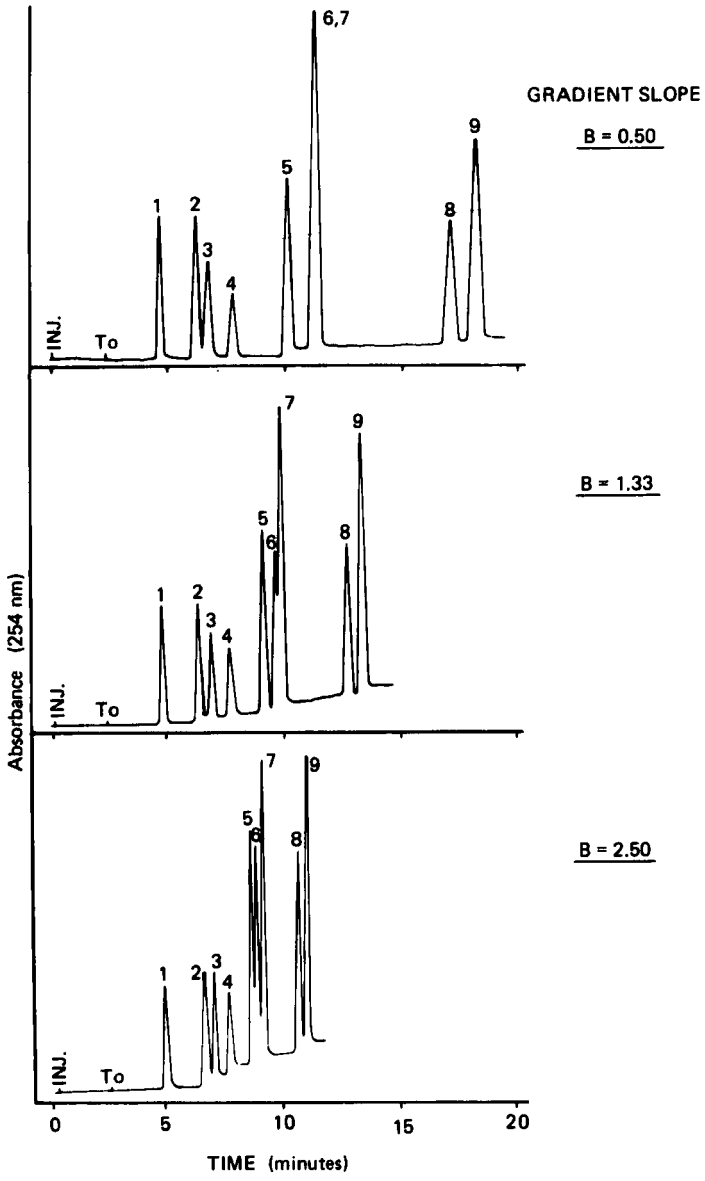

FIGURE 15

Chromatograms of the compounds in Fig. 9 showing the effect of the gradient slope (% methanol per minute). Conditions as in Fig. 9. From reference #63.

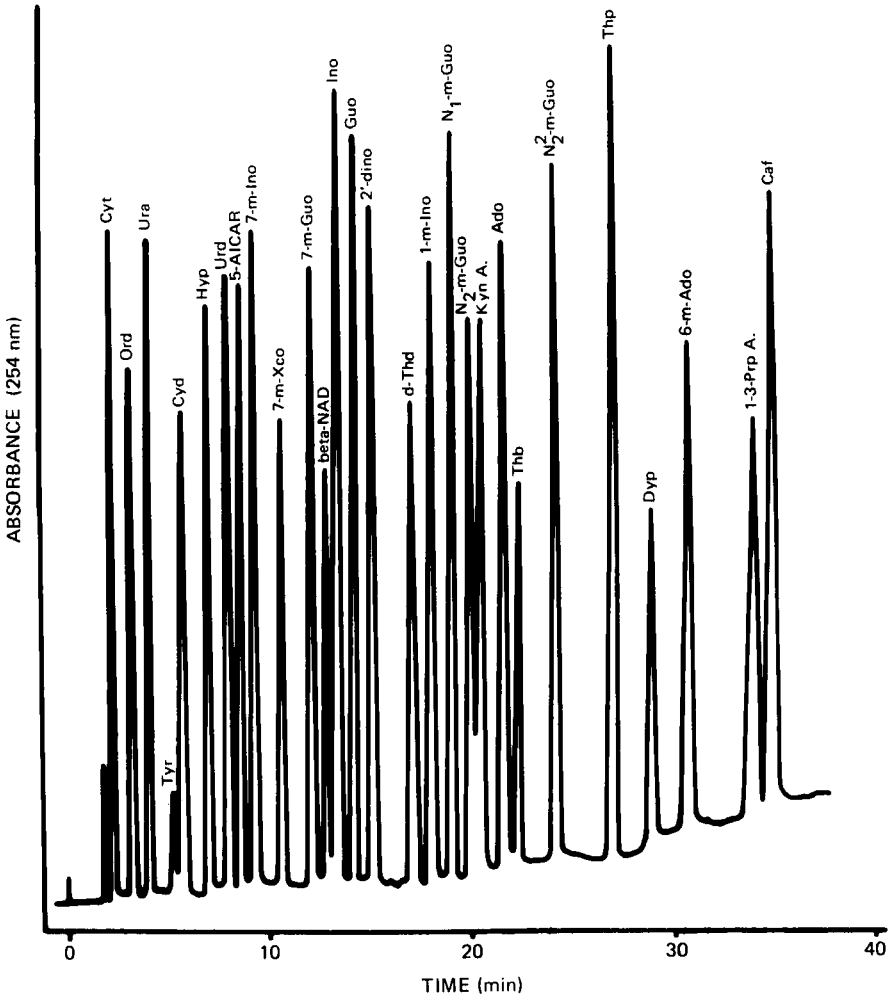

FIGURE 16

Maximized gradient separation of 29 nucleic acid components and other biologically important compounds. Column: uBondapak C_{18}. Chromatographic conditions: Primary eluent- 0.01 M KH_2PO_4 (pH 5.6). Secondary eluent- 60% methanol (v/v). Flow- 1.5 ml/min. Gradient- linear 0.69% methanol/min. From reference #64.

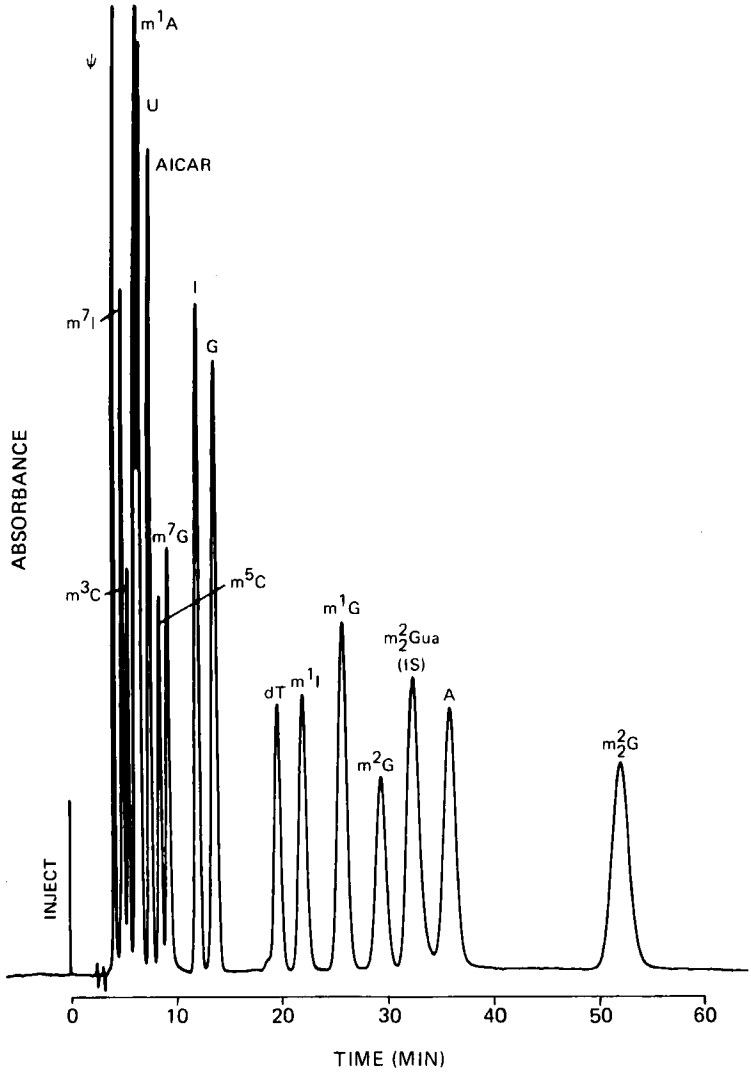

FIGURE 17

Reversed-phase isocratic separation of a mixture of 16 nucleosides. Column: uBondapak C_{18}. Eluent: 0.01 M $NH_4H_2PO_4$ (pH 5.1) in 60% methanol (v/v). Flow: 1.0 ml/min. From reference #78.

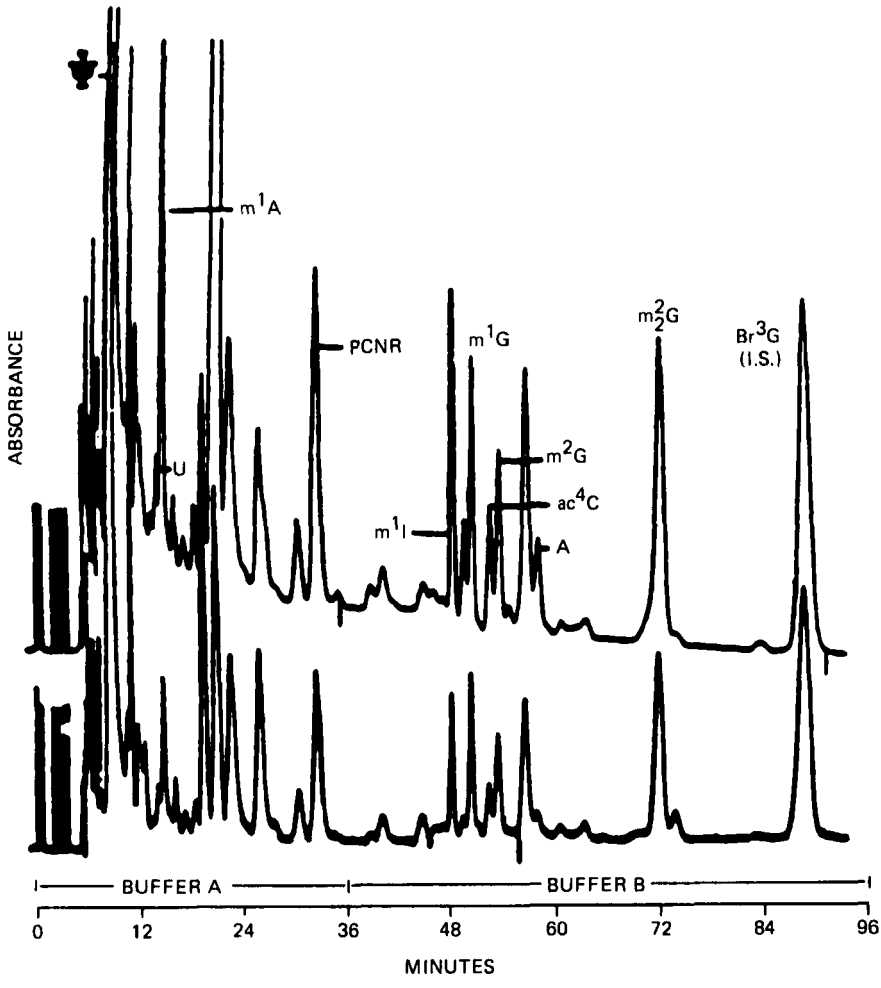

FIGURE 18

Step-gradient separation of nucleosides in urine. Column: μBondapak C_{18} (two in series). Eluents: A- 0.01 M $NH_4H_2PO_4$ (pH 5.3) in 2.5% methanol. B- 0.01 M $NH_4H_2PO_4$ (pH 5.1) in 8.0% methanol. Flow: 1.5 ml/min. Temperature: 35°C. Upper trace is taken at 254-nm, lower trace is 280-nm. From reference #60.

tematically through the selection of an appropriate gradient slope (Figure 15). In addition, this method was later used[64] to optimize the separation of nucleic acid components and other biologically important compounds (Figure 16).

While gradient elution extends the range of the number of compounds which can be analyzed in a given period of time, the method is not as sensitive as isocratic elution. Initially, Gehrke and co-workers[78] used isocratic elution to separate nucleosides (Figure 17); however, they later found[60] that the system could be improved without compromising sensitivity with the use of a step-gradient (Figure 18).

Through the use of multiple organic modifiers in the mobile phase[79], gradient elution methods appear to be needed less frequently. For example, a rapid method using an aqueous eluent containing methanol, acetonitrile and tetrahydrofuran was developed for the separation of caffeine and its metabolites in biological samples[80].

III. CONCLUSION

Reversed-phase HPLC is a powerful analytical technique for the separation and measurement of biologically important compounds. Within the last 5-6 years, the application of RPLC in the analysis of nucleic acid components has grown tremendously. Recent applications have included the analysis of tRNA hydrolysates[81], DNA hydrolsates[82], cyclic nucleotides[83], oligonucleotides[84,85], and enzymes[86]. Moreover, RPLC has been shown to have great potential in the field of clinical chemistry, especially in drug monitoring and early detection of disease states[25,28].

Recent research has shown the need for new models of the retention process which accounts for the active participation of the stationary phase. Despite the lack of a clear understanding of the retention mechanism, empirical relationships have been employed with excellent results. In addition, new approaches in the simultaneous optimization of several chromalographic parameters will greatly reduce the time required to develop separations[87].

The fact that the retention process is based on linear free energy relationships will result in further applications in the

determination of physicochemical data. The use of RPLC to evaluate purine octanol-water partition coefficients has been reported[88]. The biomedical significance in this area alone is vast.

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