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SEPARATION AND ANALYSIS OF NUCLEIC ACIDS AND THEIR CONSTITUENTS BY ION-EXCLUSION AND ION-EXCHANGE COLUMN CHROMATOGRAPHY

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I. INTRODUCTION

The recent commercial development of high pressure liquid chromatographic instruments, and the availability of small and uniformly sized resin beads has enhanced the role of column chromatography for the rapid assay of nucleic acid components. A major advancement in the purification and analysis has been due to the recent demonstration that the ion exclusion is more or equally effective than the ion exchange as a separation principle for the resolution of purine and pyrimidine bases, nucleosides, deoxynucleosides and nucleotides[1-3]. This article deals with the recent applications of separation principles that have been employed for the separation of nucleic acid constituents. The reader is referred to two review articles of Cohn for the earlier works on this subject[4,5]. Separations by chromatography and electrophoresis on paper and thin layers are not described here; several monographs and review articles are available in the literature[6-12]. The reader is also referred to a recent methodological study for the base analysis of ribopolynucleotides by chemical tritium labeling which utilizes two dimensional chromatography on thin layers of cellulose as a separation means[13,14].

The separation of nucleic acid constituents on columns of polystyrene resins, which bear either cationic (sulfite) or anionic (quaternary amine) functional groups, is based upon two main opposing principles: ion exchange and ion exclusion. (a) the anion exchange occurs between an anion and a resin with anionic functional groups. Similarly, the cation exchange occurs between a cation and a resin with cationic functional groups. (b) the ion exclusion

is a manifestation of Donnan effect. It separates ionized from nonionized substances by excluding the charged species due to repulsion as opposed to attraction in the ion-exchange. However, ion exclusion utilizes the same resin matrix as does ion-exchange but at desorbing, as opposed to absorbing conditions. Repulsion of ions rather than attraction is deliberately sought. Thus, one uses "cation exchanger" for anion-exclusion chromatography[1,3,15] and an "anion exchanger" for cation-exclusion chromatography[2].

Though ion exclusion and ion exchange are two opposing principles, both may occur at the same time in the separation of a mixture of nucleic acid constituents. Besides these two ionic principles, nonionic interactions are also important. Hydrophobic interactions and partition chromatography play an important and independent role in determining the distribution coefficient of organic compounds on polystyrene matrices. For example, purine compounds are retarded on polystyrene exchangers more than are equally charged pyrimidine compounds; this has been rationalized by noting the more "organic" nature of the purines (compared to pyrimidines of similar structure) and the benzenoid nature of the polystyrene matrix. If an attraction of an organic molecule to an organic solvent (the matrix) exists, it should be possible to demonstrate a "salting out" effect. Such has been observed in ion-exclusion studies; the higher the ionic strength, the higher the distribution coefficient of a nonionized molecule undergoing partition chromatography, hence the later it appears in the elution sequence. This accounts for the relative positions of like-charged purines and pyrimidines and for the effect of ionic strength on the absolute position of uncharged purines and thus underscores the important role of partition chromatography in ion exclusion chromatography[2].

The nonionic interactions are generally manipulated by adding an organic solvent to the eluant. They are also influenced by the amount of salt in the eluant. On the other hand, the ionic interactions are mainly effected by the ionic state of the solute. Hence, the extent of ionic interactions between resin matrix and the solutes is controlled by the pH of the environment.

Kelmers et al.[16] developed "reversed-phase" chromatography as a means of achieving separations of individual transfer RNA species. These separations utilize columns of small beads of an inert solid material coated with basic quaternary aliphatic ammonium derivatives and involve both ionic and hydrophobic interactions between the solutes and the coating material. Although originally developed for separations at the macromolecular level, reversed-phase chromatography has been successfully applied to separations of small molecules.

Reversed-phase chromatography, involving hydrophobic organic substances immobilized on inert supports [such as aluminum oxide, kieselguhr, polytrifluorochloroethylene (Kel F), and polyfluoroethylene (Teflon)] effects a simple partition of solutes between an immobilized organic phase and a mobile aqueous phase. This is the opposite of partition chromatography on paper, in which the organic phase is the mobile one. The "reversed-phase" chromatography developed for the separation of tRNAs[16] actually involves both ion exchange and differential solubility, as pointed out in 1965 by Kelmers et al.[17] The results from a recent work[18] indicate that the smaller nucleic acid components are resolved on these columns in a manner essentially identical to that observed in "anion-exchange" chromatography on polystyrene materials. Hence the only difference between what has been called reverse-phase anion-exchange chromatography and the more conventional anion-exchange chromatography on polystyrene resins lies in the difference between the alkyl and aryl substances holding the ion-exchange groups, which are quaternary ammonium derivatives in both cases. The differentiation sought by calling one "reversed phase" and the other "anion exchange" is thus misleading; both are both, and both are a mixture of ion exchange and partition.

II. THEORETICAL CONSIDERATIONS

Data Presentation in Quantitative Terms

Peak Dimensions. The most important features of a chromatographic separation are peak positions and peak widths relative to volume or time[3,19]. The results are best expressed as bars of length $2W$ and plotted so that the midpoints are at the peak positions, $\overline{V}_x$. (W is the peak width at 50% of the maximum height of the peak, and $\overline{V}_x$ is ml or min from application of the sample to the apex of the peak.) Thus, each bar covers a distance (in volume or time) representative of $+W$ and $-W$ from the peak position. Both W and $\overline{V}$ are expressed in column bed volumes [(cross-section of column) X (column length)] or minutes, or both. $\overline{V}_x$ in bed volumes is numerically equal to the volume distribution coefficient of a substance under experimental conditions[1-3,19].

Separation of Peaks. Two neighboring bars that just touch indicate a resolution factor (R_w) of 1.0 [$R_w = (\overline{V}_b - \overline{V}_a)/(W_b + W_a)$]. A value of 1.0 for R_w is adequate for easy quantitation even in proportions of 100 to 1 of the two neighboring substances[19].

Efficiency of a Chromatographic System. The "height-equivalent of a theoretical plate" (HETP) offers a quantitative measure and comparison of column efficiency for each component (peak) under each separation condition and each resin. The plate height is determined from the equation: $HETP = 0.18\ (W/\overline{V}_x)^2 L$ (see Gleuckauf[20,21]), where $\overline{V}_x$ is the total volume to the peak (less any volume between the end of the column and the detector) from the start of elution, and L is the height of the resin bed in millimeters. The HETP of each substance, in millimeters, is indicated under its $2W$ bar on the figures.

The usefulness of these parameters in expressing the results was demonstrated by Singhal and Cohn[19]. The authors explain that

many factors affect the efficacy, which may be expressed quantitatively in terms of resolution (R) of two substances and the plate height (HETP) shown by a single substance. Both involve two experimentally determinable variables, the distribution coefficient (D) and the dispersion or variation (σ). The former can be determined by either equilibration or column experiments and is subject to a simple mass law approach based on affinity for the exchanger. The latter is determinable only by column experiments and is a measure of the overall efficiency of the experimental conditions, mechanical as well as chemical. When σ is combined with the distribution coefficient and the length of the column to give plate height (HETP), we have a quantitative measure of the efficiency of a given exchanger with respect to the given substance under the total experimental conditions employed, i.e., a quantitative measure of the qualitative and often misleading term, "sharpness," of a peak. In contrast to resolution, it is a measure of the behavior (number of exchanges) of a single substance.

III. PRACTICAL CONSIDERATIONS

1. Column Preparation and Operation

The exchangers are cleaned by washing with several volumes of alkali (1M NaOH), acid (1M HCl or HCOOH), 50% ethanol, 50% acetone and water. The treated resin is centrifuged at each step to avoid losses in decantation. This treatment removes most ultraviolet-absorbing free material in most ion-exchange resins. The ammonium (cation exchanger) and acetate (anion exchanger) forms are prepared by treating the resins with 3M ammonium formate and 3M sodium acetate, respectively.

Reversed-phase column material (commonly known as RPC-5), consisting of beads of polychlorotrifluoroethylene coated with a quaternary ammonium derivative [methyltrialkyl(C_8-C_{10}) ammonium chloride], is prepared by thoroughly mixing 2.5 ml of 1% quaternary amine derivative in chloroform for each gram of beads. The dry powder obtained, after removing chloroform at 20°C, is suspended

in 1.0M acetate or chloride, as desired, and stirred at about 20°C for 2 h to remove air bubbles. Finally, the suspension is poured into an extension tube fitted on top of a jacketed column [the bead support and column top are made of identical adjustable pistons and porous (10-μm diameter pores) discs]. Uniform packing of the beads is achieved by passing the suspension through a column at about 0.5 ml/min flow rate for about 2 hr. The bed height obtained in this manner does not shrink more than 2% upon repeated use of the column[18].

Constant temperature is maintained by using a circulatory water bath. The production of undesirable air bubbles, especially at high temperatures and with organic solvents in the eluant, is effectively prevented by passing the effluent through a very narrow bore tubing (0.3 mm i.d.) and collecting it at a level two meters higher than the spectrophotometer cell.

2. Hydrolysis of Nucleic Acids

The nucleic acid analysis can be carried out at the free base, the nucleoside or the nucleotide level. The nucleoside level has emerged as a favorite because of its freedom from isomeric pairs (as in alkaline hydrolyzate of RNA) and from strong chemicals (as in freeing the bases of pyrimidine nucleosides). Nucleosides can be obtained by mild enzymic means from both RNA and DNA.

(a) DNA.

Deoxynucleosides. DNase I and venom phosphodiesterase, each 10 mg per ml, and alkaline phosphatase, 3 mg per ml are prepared in 0.2 M ammonium carbonate. The three enzymes are mixed in 1:1:2 proportions, respectively. To each salt-free A_{260} unit of DNA sample in 10 μl of 0.2 M ammonium carbonate, 5 μl portion of the three enzyme mixture containing 35 units of DNase, 10 units of venom diesterase, and 0.25 units of alkaline phosphatase, is added in a microtube. The hydrolysis is complete in about 90 minutes at 50°C.

Bases. The RNA hydrolysis to bases is generally incomplete and unsatisfactory. The purine bases are removed from DNA under mild acid conditions (pH 1.6 at 37°C for 25 hr or pH 2.8 at 100°C for 1 hr), however drastic conditions are required to free the pyrimidine bases. Concentrated, 90-100% formic acid[23] at 175°C for 2 hr, 6 M hydrochloric acid[24] at 120°C for 2 hr or 12 M perchloric acid[25] at 100°C for 1 hr have been employed for a quantitative recovery of purine and pyrimidine bases from DNAs. Since the hydrolysis has to be carried out in sealed tubes and some loss of bases due to high acidity occurs, the method is cumbersome and thus DNA estimation at nucleoside level is preferred.

(b) RNA.

Nucleosides. The conditions for enzymic hydrolysis of tRNA to nucleosides in an easily volatilizable buffer were investigated recently[22]. Approximately 1.0 A_{260} unit (50 µg) of tRNA in 10 µl of 0.2 M ammonium carbonate (pH 8.7) containing 1 mM magnesium acetate is mixed with 5 µl of a mixture of three enzymes: pancreatic RNase (37 units), venom phosphodiesterase (10 units), and alkaline phosphatase (0.25 unit). The last two enzymes should be free of a contaminant deaminase, which transforms adenosine into inosine during the hydrolysis. Though tRNAs are hydrolyzed at a slower rate initially when Mg^{2+} is included in the digest, a complete RNA hydrolysis is achieved in 2 hr under these conditions with or without Mg^{2+}. The modified nucleosides are released slowly, such as pseudouridine in 3 hr. Hence, the hydrolysis is routinely carried out at 50°C for 4 hr or at 37°C for 15 hr.

2'-3'-nucleotides. One A_{260} unit of tRNA in 0.3 M KOH (10-15 µl) is hydrolyzed at 37°C for 18 hr. Excess alkali is removed by adding an equal quantity of $HClO_4$ and sedimenting the insoluble $KClO_4$ in the cold. The crystalline pellet is washed twice with cold 10 mM $HClO_4$ to recover any adsorbed nucleotides. Some deamination of cytidylic acids may occur during such alkaline hydrolysis[18].

3'-nucleotides. One A_{260} unit of oligonucleotides in 10 µl of 50 mM ammonium acetate, pH 4.5 is mixed with 3 µl of three

enzymes (RNase T_2, 2 units; RNase T_1, 25 units; pancreatic RNase, 1.5 units). The hydrolysis is complete, as no oligonucleotides are detectable at 37°C after 4 hr (see the note in ref. 18, p. 14).

5'-nucleotides. One A_{260} unit of oligonucleotides in 10 µl of 0.2 M ammonium carbonate and 1 mM magnesium acetate (pH 8.7) containing 10 units of phosphodiesterase I (freed of 5'-nucleotidase activity[26]) is incubated at 50°C for 2-3 hr to produce a complete hydrolysis.

Oligonucleotides. (a) RNase T_1. Fifty A_{260} units of tRNA, dissolved in 200 µl of 20 mM Tris-HCl, pH 7.4 are incubated at 50°C for 15 min. Thereafter, 5 µl of RNase T_1 (free of RNase T_2 activity, Calbiochem) containing 400 units is added, and the mixture is digested at 37°C for 30 min. The reaction is stopped by adding 300 µl of 8 M urea. The digest is acidified, when desired, to convert cyclic phosphates into 3'-phosphates.

(b) Pancreatic RNase. Ten A_{260} units of tRNA and 100 µg (300 units) of pancreatic ribonuclease, in 30 µl of 20 mM Tris-acetate with 2 mM EDTA, pH 7.4, are incubated at 50°C for 2 hr. To convert cyclic phosphates into 3'-phosphates, the mixture is made acidic (pH 3 to 4) with 1 M HCl and incubated at 20°C for another 2 hr. The digest is mixed with an equal volume of 8 M urea and is applied directly to the column. No adverse effects due to the presence of enzyme in the samples are noted[18].

The addition of urea immediately after the hydrolysis, besides avoiding nonspecific-bond splitting, prevents aggregation of oligonucleotides and thus helps in the total recovery of large oligonucleotides from the column[22].

Acid-soluble pool. Rat liver obtained after decapitation is directly homogenized with a Potter-Elvehjem homogenizer in 0.75 M $HClO_4$ (ref. 27). A 5% liver homogenate in cold $HClO_4$ is separated by centrifugation. The pellet is washed twice with small volumes of cold 10 mM $HClO_4$. The washings and the supernatant are combined, and the mixture is directly applied to the reversed-phase column.

3. Data Presentation

Though the separations are best illustrated by the elution profile, the method is impractical in presenting several experiments in a limited space. It also lacks quantitation of sharpness and resolution. Peak positions and widths relative to volume or elution time are the two most important results. They can be illustrated as bars of length $2\underline{W}$ ($\underline{W}$ = width of a peak at half its maximum height) placed so that the midpoints are at the positions of peaks ($\overline{V}_x$). Thus, each bar will represent a distance in volume or time of $+\underline{W}$ and $-\underline{W}$ from the peak position[1-3].

Two neighboring bars that just touch indicate a resolution factor ($\underline{R}_w$) of 1.00 [$\underline{R}_w = (\overline{V}_b - \overline{V}_a)/(\underline{W}_b + \underline{W}_a)$], which is sufficient to allow quantitation even up to ratios of 100:1 of the two neighboring species[19]. The height equivalent of a theoretical plate (HETP) can be calculated for each peak (see section II). It allows a quantitative comparison of column efficiency for each substance under each separation condition.

IV. INFLUENCE OF SEPARATION PARAMETERS

1. pH

The nucleic acid components are amphoteric in nature. They contain amino groups that form cations at acid pH's, such as in cytidine, adenosine and guanosine. The hydroxyls of uridine and guanosine form anions at basic pH's. The phosphate group of a nucleotide bears an anionic charge commencing at pH<1, and another anion commencing at pH 4 (see fig. 1 in ref. 10). The hydroxyls of ribose have a pK around 13. They are used most effectively in the formation of a complex with borate, which introduces an additional anion and helps in the separation of 2'-, 3'-nucleotides from 5'-nucleotides[28,29]. The nucleic acid constituents bear a net cationic or anionic charge depending upon the pH of the medium, the eluant. Hence, the pH is the most important factor. The eluant pH's when selected judiciously, can separate these compounds by either anion exclusion or cation exchange on the same column.

Figure 1 illustrates the effect of pH on the four most common nucleosides separated on a column of cation-exchange resin[1]. Adenosine and cytidine (pK_b 3.5 and 4.1, respectively) become cations in the region of pH 4, hence they appear last in cation-exchange analysis at this pH (Fig. 1, top row). Uziel et al.[30] separated the four nucleosides (and other) under these conditions, where uridine and guanosine are resolved due to non-ionic forces, but found unsatisfactory resolution of the three uridines. However, these uridines and the other major nucleosides separate satisfactorily on a similar cation-exchange column at

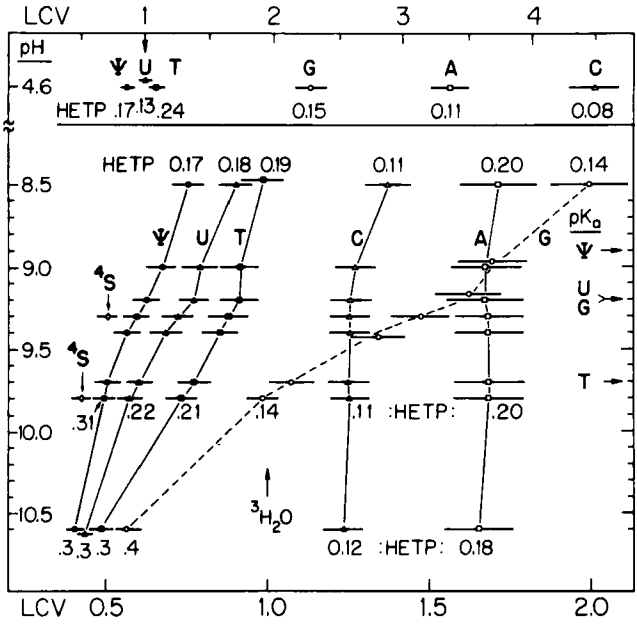

FIGURE 1

Effect of pH upon elution volume and plate height (HETP) in anion-exclusion[1] and cation-exchange[30] (pH 4.6) chromatography of ribonucleosides. Column: Aminex A-6, 50 cm x 5 mm; liquid column volume (LCV), 8 ml. Eluants: 0.4 M $HCOONH_4$ at pH 4.65; 50 mM NH_4OH adjusted with AcOH for pHs 8.5-9.8; and 10 mM NH_4OH, pH 10.6. Flow rate: 0.2 ml/min; 50°C. [Reproduced from R. P. Singhal, Arch. Biochem. Biophys. 152, 800 (1972), by permission of the Academic Press, Inc.]

alkaline pH values (Fig. 1), where no nucleoside has a positive (attractive) charge, and hence the nucleosides are incapable of binding to the neagtively charged resin. With the proper choice of exchange materials and pH conditions, partially ionized components (in this case, five uridines, inosine, and guanosine) thus can be resolved in less than one liquid column volume of eluant[1,15].

The five uridines (2-thiouridine, 4-thiouridine, pseutouridine, uridine, and ribothymidine) appear at pH 9.8 in the order of increasing pK_a (8.1, 8.2, 8.9, 9.2, and 9.7, respectively), which is the order of decreasing anionic charge at this pH. The relation between elution position and pK_a values of these anions on the cation exchanger clearly indicates that they are variably repelled or excluded from the negatively charged matrix. The pH of the eluting buffer determines their net anionic charge, hence the extent of their partial exclusion. The separation of adenosine and cytidine on an anion exchanger at acid pH may be similarly explained[9].

Figure 2 illustrates the effect of pH on peak positions (volumn distribution coefficients in bed volumes) and HETP of the common 5'-ribonucleotides resolved by anion-exclusion chromatography on a column of Aminex A-6 (ref. 3). The four major nucleotides appear as one broad band at neutral pH or pH 5. However, as the eluant is made acidic, the front band is resolved into two bands or into individual peaks. From pH 5 to 2.5, the positions of the three uridylate analogs remain unchanged, but guanylate is slightly retarded, thus completely resolving the major nucleotides. The guanylate retardation can possibly be explained by the loss of net negative charge, which is due to the ionization of the basic amino group, and due to the purinic, hydrophobic nature (more affinity for the stationary organic phase of the resin matrix than for the aqueous eluant) of the molecule.

This dual retardation effect is better demonstrated in the separation of cytidylate from adenylate, where the two are variably retained on the column, as the cationization (pK_b's 4.5 and 3.8,

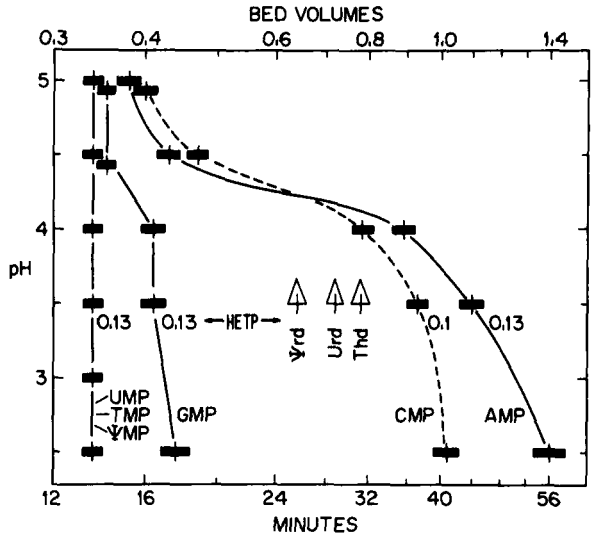

FIGURE 2

Effect of pH upon elution volume and plate height (HETP) in anion-exclusion chromatography[1] of 5'-ribonucleotides and uridines. Column: Aminex A-6, 50 cm x 5 mm; bed volume, 9.8 ml. Eluants: 5 mM HCOOH at pH 2.5; 20 mM NH_4COOH + HCOOH for pH 3-5. Flow rate: 0.25 ml/min; 50°C. [Reproduced from R. P. Singhal, Eur. J. Biochem. 43, 245 (1974), by permission of the Fed. Eur. Biochem. Soc.]

respectively) counteracts the anionic character of the primary phosphate group. The relative positions of the last two nucleotides at pH 4.5 and 5, however, cannot be explained by differences between pyrimidines and purines. It can be calculated on the basis of the Donnan effect that the actual pH inside the ion-exchange bed is somewhat different from that outside, in the solution, due to the local environment (anions of the resin matrix in dilute-salt solutions) and temperature of the column. Therefore, the peak positions of solutes cannot precisely be predicted on the basis of their ionization constants. Presumably the actual pH in the column environment is appreciably lower than the apparent pH 3.5, which causes a total cationization of the basic group in cytidylate and in adenylate. Hence, the cytidylate and adenylate may behave as neutral molecules under these conditions.

Figure 3 illustrates the influence of pH on the anion-exchange chromatography of nucleosides. Cytidine and adenosine that appear last in cation exchange (cations at pH 4.6, see fig. 1) behave as neutral molecules; hence their elution positions are not influenced. However, uridine and guanosine develop an anionic charge in the region of pH 9. The two nucleosides are sorbed to the resin more and more strongly as their anionic character is enhanced progressively by raising the pH. Adenosine and guanosine are retarded more than cytidine and uridine, respectively, because the two purine nucleosides bear enhanced hydrophobic interactions, hence they are strongly sorbed to the organic resin[19].

The effects of varying the rate of pH change and the ionic concentration (pH and salt gradient) on the elution position of 2', 3'-nucleotides have been described[31]. The relative distribu-

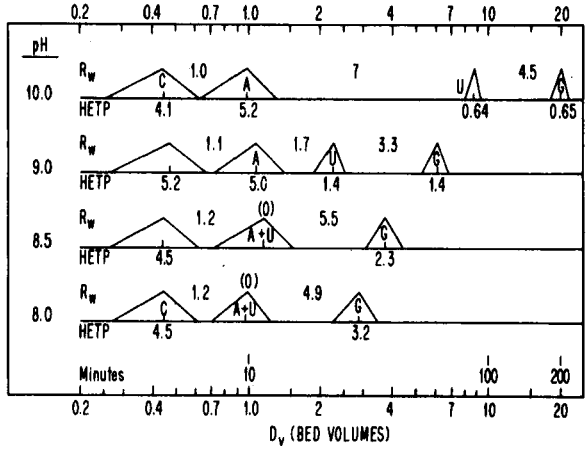

FIGURE 3

Effect of pH upon elution volume (volume distribution coefficient, D_V), resolution factor (R_W) and plate heights (HETP) in anion-exchange chromatography of ribonucleosides[19]. Column: Dowex 1-X8, 50 cm x 2.5 mm; bed volume, 2.45 ml. Eluant: 0.2 M NH_4OAc containing 5% 2-propanol; flow rate, 5.1 cm/min; 50°C. [Reproduced from R. P. Singhal and W. E. Cohn, Anal. Biochem. 45, 585 (1972), by permission of the Academic Press, Inc.]

tion of the nucleotides depends upon the ratio (R) of the rate of change to the ionic strength [R = (GV)/S, where G is the rate of pH change in pH units per liter of the eluant, V is the wet resin volume and S is the ionic strength]. A large number of nucleotides have been separated by this principle[31].

2. Ionic Strength

(a) Ion Exchange.

The ionic strength of the eluting ion (examples: Cl^- and Na^+ in anion- and cation-exchange chromatography, respectively) influences the peak positions of substances that truly undergo ion exchange. However, the substances that appear near the front of the chromatogram, hence excluded, are not influenced by changes in the eluting salt [except when a low ionic strength causes a partial exclusion of the excluded species; see section (b) below]. Figure 4 illustrates such effects. As the anion exchanger is eluted with increasing molarities of the acetate ion, the retention of two anions (uridine and guanosine) is decreased, but those of uncharged species (adenosine and cytidine) is constant. The analysis time can be shortened by using the higher molarities, but it may result in the loss of resolution of some species, for example, uridines in Fig. 4(a).

(b) Ion Exclusion.

The concentration of the eluting ion is not expected, a priori, to influence the peak positions, as the uncharged substances obviously do not exchange in ion-exclusion conditions. Singhal[1], while studying the effect of ionic strength on nucleiside separation by anion exclusion found that higher ionic strength decreases the retention (increases the exclusion) of the ionized species, but results in a constant or increased elution volume for the nonionized species (see Fig 4 in ref. 1). This observation is explained[1] on the basis of the Donnan effect.

The higher the salt concentration, the higher the pH inside the cation-exchange beads, leading to a greater degree of ionization of the partially penetrating solutes and thus to increased re-

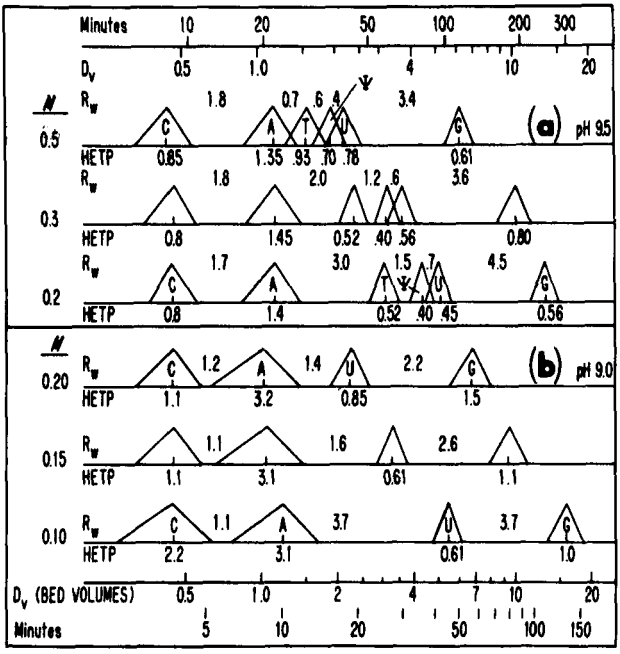

FIGURE 4

Effect of ionic strength of eluant upon elution volume (distribution coefficient, D_V), resolution factor (R_W) and plate height (HETP) in anion-exchange chromatography of ribonucleosides[19]. Column: Dowex 1-X8, 24 cm x 5 mm, bed volume, 4.7 ml; 50°C. Eluants: NH_4OAc (5% 2-propanol) at concentrations shown, at (a) pH 9.5, 1.3 cm/min, and (b) pH 9.0, 2.9 cm/min. [Reproduced from same paper as Figure 3.]

pulsion and earlier elution. On the other hand, uncharged substances are "salted out"[32,33] and relatively retarded by increased ionic strength. Sargent and Rieman[34] made use of this "salting-out" property to separate a mixture of 11 uncharged amines on a cation-exchange resin by eluting the column successively with 1.75, 1.32, and 0.22 M K_3PO_4. Thus, a complex mixture of nonionized solutes (such as the more strongly bound methylated nucleosides present in tRNA) can be resolved in ion-exclusion chromatography by eluting the column with a decreasing salt gradient, the opposite of that used in ion-exchange work.

An example of the influence of ionic strength on anion exclusion of nucleotides is illustrated in Figure 5. As the ionic strength of the eluant is increased from 10 to 100 mm, neutral molecules (nucleosides) remain uneffected in this range. However, disregarding the slight shift in positions of excluded uridylate and guanylate, the diminised retentions of cytidylate and adenylate is perhaps due to the decreased partition effect, which is perhaps caused by changes in their conformational properties in very dilute salt solutions.

The increased retention of the two nucleotides at lower ionic strength is explained[3] by increase in the Donnan effect, which alters the pH of the local environment, hence the ionization of the nucleotides. As the salt concentration is decreased, the differ-

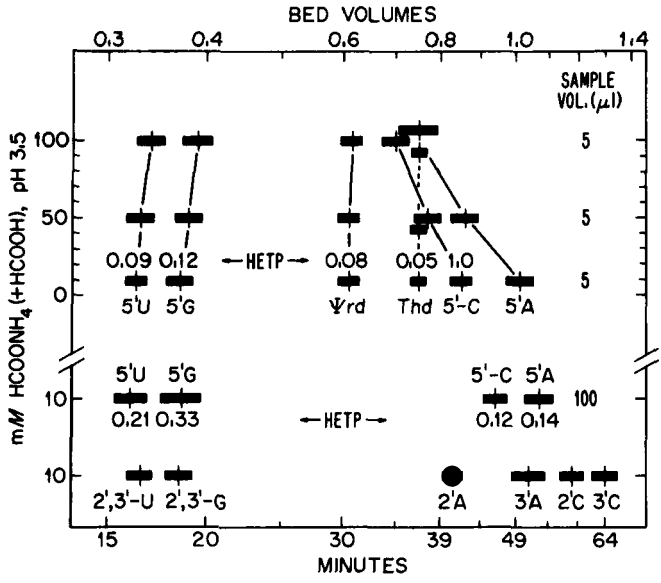

FIGURE 5

Effect of ionic strength of eluant and sample volume on elution volume, resolution and plate height (HETP) in anion-exclusion chromatography of ribonucleosides[1]. Column: Aminex A-6, 50 cm x 5 mm; bed volume, 9.8 ml. [Reproduced from R. P. Singhal, Eur. J. Biochem. 1974, 43, 245, by permission of the Fed. Eur. Biochem. Soc.]

ence between the environment of the resin solution inside the resin bead (more acidic) and that outside (less acidic) tends to increase due to establishment of the Donnan equilibrium. Thus, when solutes enter into the resin matrix from a low-salt solution (e.g., 10 mM), they encounter an environment of lower pH than when they enter from a high-salt solution (e.g., 100 mM). The net negative charge on the nucleotide therefore diminishes or disappears as the lower pH inside the beads causes enhanced cationization of the base moiety. Thus, in dilute solutions the anionic repulsion which causes an early elution of nucleotides is lost, and nonionic forces like partition effect increase their adsorption. Both forces increase retention in dilute salt solutions.

3. Temperature

In ion exchange, all substances, as examined for nucleosides[19,30], elute early on increasing temperature with parallel increases in plate heights but with parallel decreases in resolutions. The temperature effect is not uniform in ion exclusion[1]. As illustrated in Figure 6, when temperature is lowered, pseudouridine and uridine appear near the front in less eluant volumes. As the temperature is raised, ribothymidine (except at 30°C), uanosine, cytidine, and adenosine move closer to the front, peak widths become smaller, and the efficiency improves (small plates). The displacement of adenosine with temperature is greater than that of any other nucleoside.

The practical benefits of shortened analysis time (a factor of more than 2.5 between 30 and 80°C), lower plate heights, and lower column pressure (a factor of 2 between 30 and 80°C) must be weighed against the disadvantages of the loss in resolution (the guanosine-cytidine-adenosine triplet in Fig. 6), as well as the stability of the compounds and the analytical system (plastic fittings, resin, eluant, etc.). A temperature of about 50°C has been found satisfactory[1-3,15,18,19,30]. Recently, optimum resolutions of several modified nucleosides have been reported[35] by cation-exchange chromatography[30] by using different combinations of ionic strength and

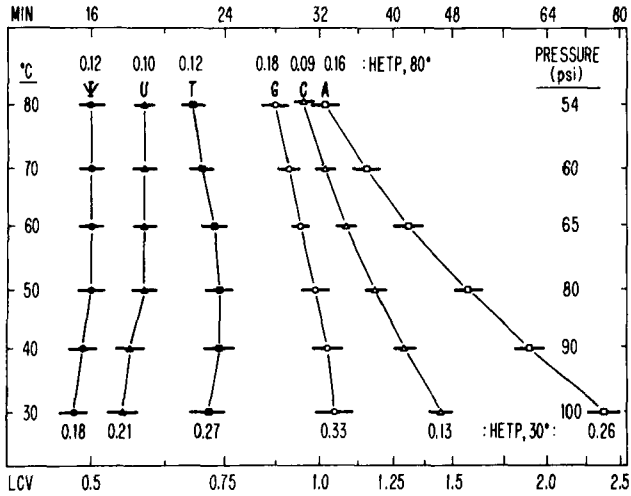

FIGURE 6

Effect of temperature upon elution volume and plate height (HETP) in anion-exclusion chromatography of ribonucleosides[1]. Column: Aminex A-6, 50 cm x 5 mm; LCV, 8 ml. Eluant: 50 mM NH_4OH neutralized to pH 9.8 with AcOH; flow rate: 1.25 cm/min. [Reproduced from R. P. Singhal, Arch. Biochem. Biophys. 152, 800 (1972), by permission of the Academic Press, Inc.]

column temperatures ranging from 0.1 M at 40°C to 0.4 M ammonium formate at 75°C.

4. Organic Solvent

Highly hydrophobic species are not readily desorbed by aqueous eluants. For example, 2-methylthio-6-isopentyladenosine is sorbed 20 times stronger than most common nucleosides in cation-exchange chromatography[30]. Such substances appear as broad bands, hence remain practically undetectable. Organic solvents, when added to the aqueous eluant, result in increased solubilities of compounds especially containing one or more methyl groups. The enhanced solubility in the moving phase (eluant) causes a loss in the hydrophobic interaction between the solute and the benzoid resin matrix. Consequently, these substances appear early when eluated with organic solvents.

The addition of various solvents to the eluting buffer has been explored in ion-exchange chromatography[19]. The addition of ethanol has a marked effect on purines than on pyrimidines. Adenosine is influenced more than guanosine. Isopropanol has the same effect as ethanol. Of all the other solvents tested[19], 1-butanol (7%) and 1-propanol (10%) were found satisfactory for nucleoside separation by anion-exchange chromatography. Similarly ethanol has been reported[36] to improve and hasten the resolution of minor, hydrophobic nucleosides in cation-exchange chromatography[30].

The influence of organic solvents on both anion and cation-exclusion chromatography has been studied[1,2]. Ethanol has a marked influence on more lipophilic and nonionized compounds. Figure 7 illustrates the addition of ethanol to the eluant in anion exclusion of nucleosides. Ionically repelled compounds are influenced insignificantly. However, the substances that are retained

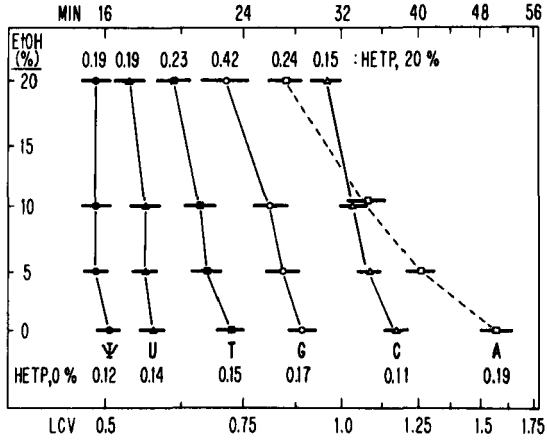

FIGURE 7

Effect of ethanol upon elution volume and plate height (HETP) in anion-exclusion chromatography of ribonucleosides[1]. Column: Aminex A-G, 50 cm x 5 mm; LCV, 8 ml. Eluant: 20 mM $(NH_4)_2CO_3$, pH 9.8 plus ethanol as shown; flow rate, 1.25 cm/min; 50°C. [Reproduced from same paper as figure 6.]

strongly due to partition and hydrophobic properties are effected differently (example: adenosine is influenced more than cytidine). Such additives have significant advantages in speeding up the analysis and sometimes in eliminating disadvantageous overlaps (such as 10% ethanol in Fig. 7). The decrease in efficiency with the addition of ethanol (compare plate heights at 0 and 20% ethanol) and increase in the operating pressure are insignificant disadvantages.

The effects of solvents on the adsorption mechanisms of aromatic compounds on ion-exchange resins have been reported in a series of publications[37,40]. The adsorption is assigned to hydrogen bond or π-bond interactions between an aromatic compound and a counter ion of the anion exchanger. Thus, the most important factor in the adsorption is the ability of the solvent to form a hydrogen bond. The Van der Waals' forces have insignificant influence on distribution coefficient values. The effect of six organic solvents on elution positions (K_d's) of a number of aromatic compounds, containing different functional groups, on several ion exchangers has been described by those workers[40].

5. Flow Rate

The time required to complete an analysis is regulated by controlling the flow rate of the eluant[41]. Equilibrium operating conditions are gradually lost and the elution curve is skewed (+W >-W) as flow rate is increased[19]. Significant losses in resolution and chromatographic efficiency (plate height) occur with only small changes in flow rate (see Fig. 9 in Ref. 19; Table I in Refs 1-3; Fig. 6 in Ref. 30). A compromise must be made between a reduction in analysis time and a loss in resolution, especially when two peaks are separable by only a resolution factor approaching one.

6. Resin Particle Size

A resin of high cross-linkage excludes ionic solutes effectively as the fixed ionic concentration inside the resin is sufficiently high. On the other hand, a resin of low-cross-linkage causes enhanced sorptions of nonionic substances[33]. A rapid

"equilibrium" (exclusion or exchange) is achieved by low cross-linked and small particle-size resins.

The influence of resin particle size on cation-exclusion and on anion-exchange chromatography was studied recently[2]. Figure 8 illustrates the effect of resin bead size and uniformity. The exchanger of larger and heterogeneous particle size (Dowex 1: 47 ±10 μm) resolves the nucleosides less effectively than a similar ion exchanger that has one-third the particle diameter and a narrow particle size range (Aminex A-25: 17.2 ±2 μm). The plate heights (HETP) for each component and the total analysis times are appreciably reduced with the small and homogenous resin beads. Thus, small and uniform beads of ion exchanger improve resolutions in both ion-exclusion and ion-exchange chromatography.

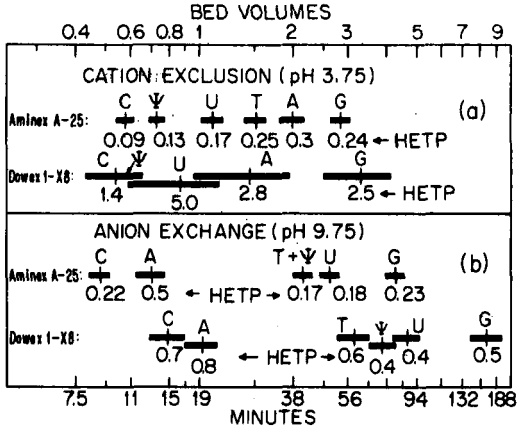

FIGURE 8

Effect of resin bead size and uniformnity upon separation and plate height (HETP) of ribonucleosides, and comparison of cation-exclusion[2] and anion-exchange[1] chromatography. Columns: Aminex A-25 (17.5 ±2 μm) and Dowex 1-X8 (47 ±10 μm) in 24 cm x 5 mm. Eluants: (a) 20 mM $HCOONH_4$ + 15 mM HCOOH, pH 3.75, at 1.5 cm/min; (b) 0.3 M NH_4OAc + 7% 1-butanol, pH 9.75, at 1.4 cm/min; 50°C. [Reproduced from R. P. Singhal and W. E. Cohn, Biochemistry 12, 1532 (1973), by permission of the American Chemical Society.]

7. Sample Size

A fairly large volume of the sample, when applied to a column at an ionic strength lower than the eluant, does not cause any adverse effects in ion-exchange chromatography. However, the volume of the sample solutions in ion-exclusion chromatography is theoretically limited by the volume of solution adsorbed by the resin and in practice is found to be considerably less than the excluded volume[42].

Two experiments with 10 mM ammonium formate as eluant (see Fig. 5), where the same amount of 5'-nucleotide mixture is applied in 5 and 100 µl, respectively, show that while positions of uridylate and guanylate remain unchanged, cytidylate and adenylate are appreciably retarded in anion exclusion. The sample volume appears to influence the partition of weakly ionized substances more than strongly charged species. The plate heights of only early excluded peaks change (increase) by increases in the sample volume.

V. SEPARATIONS

The objective of the experiments described here is not to recommend a single, immutable procedure. The examples chosen are those that appear best to illustrate various principles and modification potentialities. They are recent and perhaps meet most requirements of a modern biochemist. However, no single procedure is best for all purposes. Practical modifications can be introduced in these (and other) procedures by judiciously varying one or more separation factors for any given situation.

1. Bases.

(a) Cation-Exchange Resin

(i) Anion-exclusion chromatography. Figure 9 indicates the separation[1] of both major purine, pyrimidine bases, and rare methylated species at pH 10. Adenine and cytosine are not resolved at either pH 9.5 or at pH 9.8, but they are at pH 10. The minor component, 2-methyladenine, moves farther away from 5-methylcytosine as the eluant pH is raised from 9.5 to 10. The early elution of

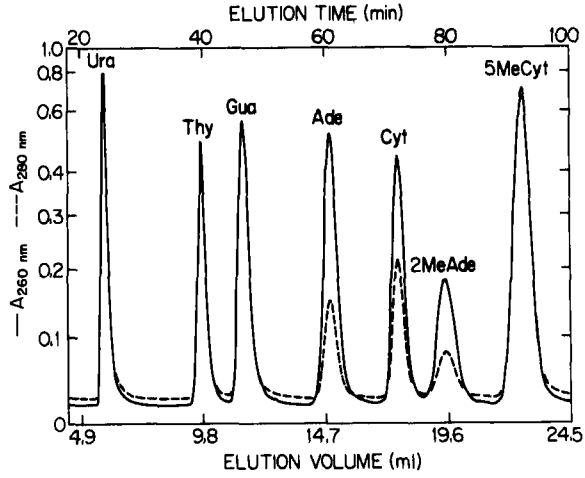

FIGURE 9

Separation of purine and pyrimidine bases by anion-exclusion chromatography[1]. Column: Aminex A-6, 50 cm x 5 mm. Elution: 20 mM $(NH_4)_2CO_3$, pH 10.0; 0.25 ml/min; 50°C. [R. P. Singhal, unpublished work.]

adenine compounds with an increase in pH is ascribed to an increase in the anionization of adenine (pK_a, 9.8) in this pH range. The six common bases can be resolved satisfactorily (R_w > 1) in about 60 min by eluting at a fast flow rate. The elution positions of major nucleosides and deoxynucleosides in relation to bases have been described (see Fig. 8 in ref. 1). [See also anion-exclusion chromatography on molecular-sieve materials, subsection 2(a).]

(ii) Cation-exchange chromatography. Uziel et al.[43] studied the separation of cytosine, adenine, guanine and uracil on an Aminex A-6 column. When pH is raised from 4.75 to 6.75, maintaining ionic strength (0.4 M formate) and temperature (50°C) constant, cytosine and adenine elute early, but the other two species appear at the same positions. A routine analysis of the four bases can be carried out at pH 5.2 in about 25 min at a moderate column pressure, 200 psi.

(b) Anion-Exchange Resin

(i) Cation-exclusion chromatography. Weak hydrochloric acid[4,5], and recently, a dilute buffer solution[2] have been used to separate the major bases on an anion-exchange column. Figure 10 illustrates such separations. Adenosine is ionized less than cytosine at pH 3.75 (pK_b's 4.1 and 4.5, respectively), hence the former appears late. This is also due to the hydrophobic nature of the purine base. The present method is adequate when uracil is not present in the sample (Fig. 10). Both uracil and adenosine can be resolved by selecting an appropriate pH or by adding ethanol to

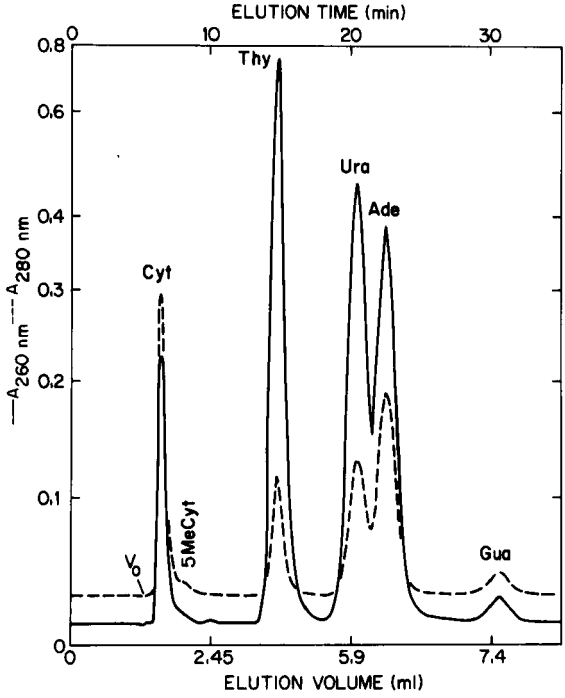

FIGURE 10

Separation of the common purine and pyrimidine bases by cation-exclusion chromatography[2]. Column: Aminex A-25, 10.8 cm x 6.35 mm. Elution: 2 mM CH_3COOH ($+NH_4OH$), pH 3.75; 0.25 ml/min; 50°C. [R. P. Singhal, unpublished work.]

the eluant. The buffer pH should not be selected very acidic since it causes deamination of cytosine at such high temperatures.

(ii) Anion-exchange chromatography. All bases of the nucleic acids, except cytosine derivatives, are anionized at alkaline pH's. Hence, a pH value can be determined where the difference in their anionic character is maximum. However, other properties, such as hydrophobic groups, should also be considered in predicting separations on the basis of the ionization. Figure 11 illustrates a separation of the common bases of the nucleic acids on a Dowex-1 x-8 column. Since the peaks are well resolved ($R_w \geq 1$), a faster flow rate can be used to reduce the time of analysis.

2. Nucleosides and Minor Components

(a) Cation-Exchange Materials

(i) Anion-exclusion chromatography. At alkaline pH's, uridines and guanosines behave as anions, and cytidine and adenosine as uncharged molecules [see section IV(1)]. On a cation exchanger, the anions are resolved by anion exclusion by making use of the differences in their ionizations at a given pH. The uncharged species are resolved by nonionic principles, such as parti-

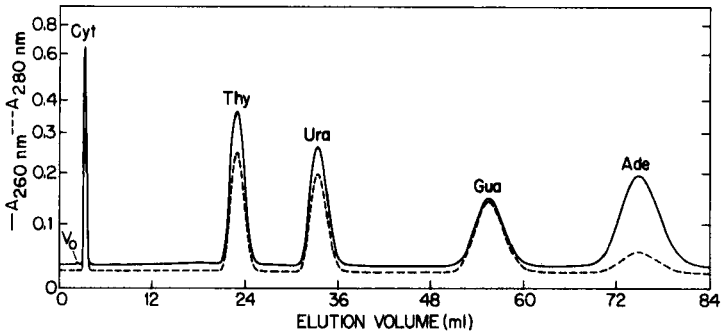

FIGURE 11

Separation of the common purine and pyrimidine bases by anion-exchange chromatography[19]. Column: Dowex 1-X8, 25 cm x 5 mm. Elution: 0.3 M $(NH_4)_2CO_3$ plus 7% 1-butanol, pH 9.7, 50°C; 0.2 ml/min. [R. P. Singhal, unpublished work.]

tion, hydrophobic nature, size, etc. The elution position of certain compounds may depend equally on both ionic and nonionic properties (for example, guanosine in Fig. 1). The common nucleosides of tRNA's can be separated at either pH 9.3 or 9.8 in about 35 min by this principle[15]. Figure 12 illustrates an analysis of 0.52 A_{260} units of glutamate tRNA (E. coli). All components show remarkable resolutions under these conditions in about 13 ml and 1 hr (Fig. 12C). The method should be used with caution where the quantitation of alkali-unstable minor components, such as 7-methyl-

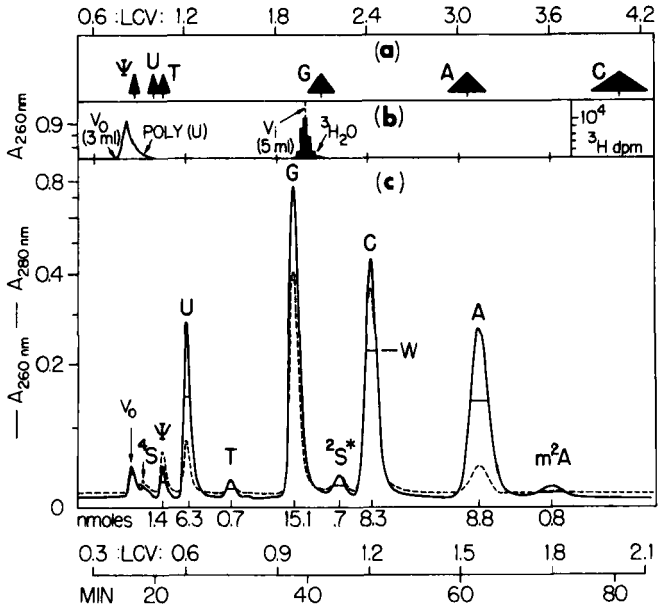

FIGURE 12

Comparison of cation-exchange[30] and anion-exclusion[1] chromatography of ribonucleosides (panels a and c, respectively). Analysis of nucleosides from glutamate tRNA (quantities in nanomoles are indicated under each peak). Column: Aminex A-6, 50 cm x 5 mm. Eluants: 20 mM $(NH_4)_2CO_3$ at pH 9.8, 50°C; 1 cm/min (panels b and c); 0.4 M HCO_2NH_4 at pH 4.65, 50°C. Panel b shows the elution positions of polyuridylate and ^3H_2O under these conditions; the two indicate the excluded volume and total liquid volume (LCV, 8 ml), respectively for this column. [R. P. Singhal, Arch. Biochem. Biophys. 152, 800 (1972), by permission of the Academic Press, Inc.]

guanosine is important. However, the degradation product of such compounds can be assayed with confidence. Figure 13 describes elution positions of several minor ribonucleosides deoxynucleosides and bases under separation conditions of Fig. 12. Several minor nucleosides do not separate satisfactorily from the other nucleosides under these conditions[1]. However, these components can be separated by judiciously selecting the separation parameters.

The effluent in this and other ion-exclusion methods is practically free of salt and can be volatilized easily. This property is important when isolation and characterization of minor components or a base composition of the base-labeled nucleic acid is sought. This method was applied for determining the degree of modification of glutamate tRNA with cyanogen bromide. The reaction, which transforms a minor component, 5-methylaminomethyl-2-thiouridine [5(MeNHMe)2Srd] into a uridine derivative, was determined by following the disappearance of the minor component that separated

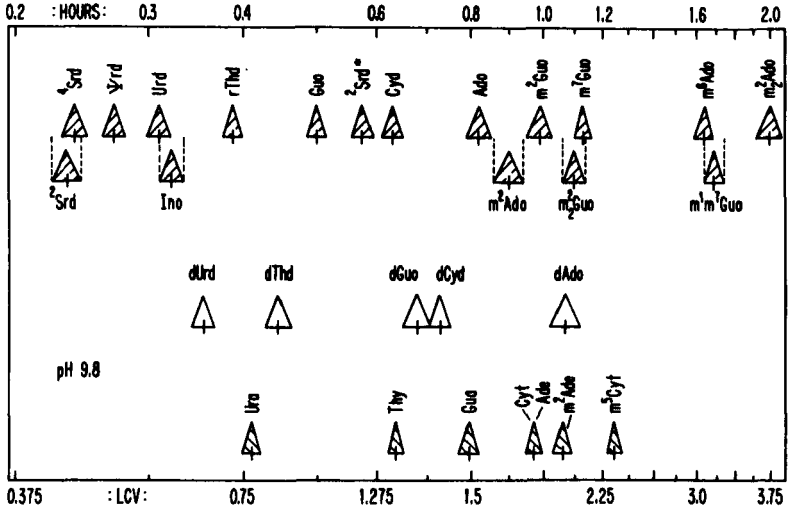

FIGURE 13

Elution positions of the common bases, deoxynucleosides and of the major and some minor ribonucleosides in anion-exclusion chromatography[1] at pH 9.8 on a cation-exchange column. See legend to Fig. 12(c) for details. [Reproduced from same paper as Fig. 12.]

as a distinct peak[22] (Fig. 14). The anion exclusion was used recently in determining the in vivo-modification of precursor molecules of tRNAs (E. coli), and thus, a complete modification of precursor tRNAs was not found to be a prerequisite for its maturation[44,45].

To answer the question of whether or not the composition of a highly purified tRNA preparation agrees with the primary structure of its major component and if so, to what extent, Singhal and Best[46] recently examined five purified tRNAs by ion-exclusion column chromatography of nucleosides and by thin-layer separation of in vitro-labeled nucleoside derivatives. The authors concluded that neither of the two analytical systems can be used alone to detect and quantitate every one of the nucleoside species present in tRNAs. However, the column methods have practical advantages of speed, scale and range of chromatographic conditions (pH, temperature) without loss of versatility. They describe positions of several modified nucleosides: 2-methyladenosine, 5-methylaminomethyl-2-thiouridine, uridin-5-oxyacetate, photoproduct, pyrimidinone (4-5) cytidine, and discuss disadvantages in the analysis by ^{3}H-labeling and separation by bidimensional chromatography[13,14].

(ii) Anion-exclusion chromatography on molecular-sieve materials. Separation of ribonucleosides, deoxynucleosides, and purine and pyrimidine bases have been reported on so-called molecular-sieve materials [polyacrylamide gels (47-50) or Sephadex columns (51-63, 65)]. In fact, these separations are also due to ion exclusion, since the molecular sieve materials do have some ion-exchange properties arising from the very weakly ionizable carboxyl, hydroxyl, and amide groups. The separations have been performed at pH values that induce charges of the same sign on both matrix and solutes. Further support for this interpretation comes from the fact that many of the solutes employed were eluted in less than one liquid column volume, indicating exclusion from the matrix. The strong retention of nonionized species on these matrices is certainly due to nonionic forces since both kinds of gels have a

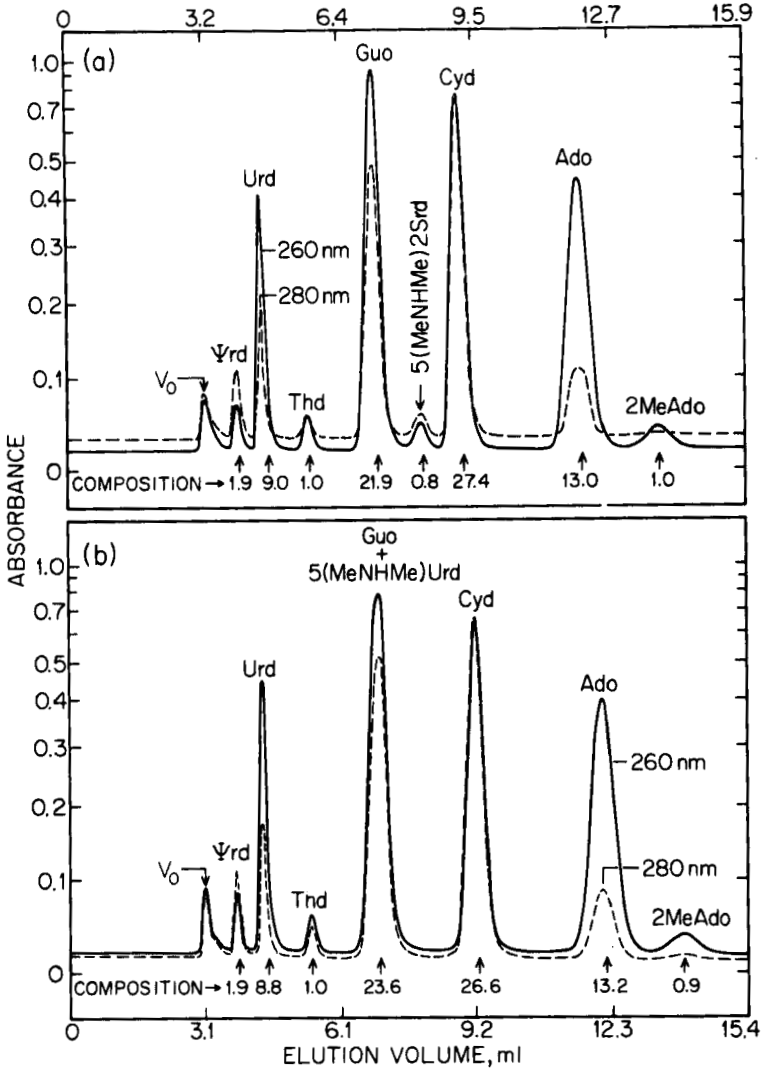

FIGURE 14

Comparison of nucleosides derived from (a) untreated and (b) CNBr-treated glutamate $tRNA_2$ by anion-exclusion chromatography[1]. The quantities in nanomoles are indicated under each peak. [Reproduced from R. P. Singhal, Biochemistry 13, July 2, 1974 issue, by permission of the American Chemical Society.]

hydrophobic backbone structure. The original observation[64], K_D values of aromatic and heterocyclic compounds on the gel columns are greater than one, is in parallel to this argument. Very recently, Prasada Rao and Cherayil[65] reported the separation of thionucleosides and their derivatives on both polyacrylamide (Bio-Gel P-2) and Sephadex (G-10) columns by eluting with dilute salt solutions at about pH 10.5. Since the anionically charged thionucleosides were excluded in less than one liquid column volume and the thionucleosides containing strong hydrophobic groups were retarded strongly, the separations on these[65] and on phosphocellulose[66] columns are due to anion-exclusion chromatography. Group separations of bases, nucleosides and nucleotides on gel columns, using this principle, were reported recently[67] (see section below).

(iii) Cation-exchange chromatography. Adenosine and cytidine are cations at an acid pH, hence they exchange with a cation exchanger. Though both uridine and guanosine are uncharged and excluded at about pH 4.6, guanosine appears after uridine compounds due to its more organic nature. A separation of several nucleosides by this principle is illustrated in Fig. 15. Here, the uridine group of nucleosides are poorly resolved because they lack charge differences and possess very little nonionic differences [compare cation exchange (Fig. 12a) and anion exclusion (Fig. 12c) by using the same column]. The plate heights observed under the conditions of both anion exclusion and cation exchange range between 0.2 to 0.3 mm. However, the anion exclusion has the advantage of speed, dilute volatile eluant and superior resolutions (uridines, minor components).

To enhance the desorption of the strongly bound minor nucleosides and bases, three modifications have been introduced in this cation-exchange method[30]: increase in the ionic strength[35,36], pH[36] and temperature[35], and addition of ethanol to the eluant[36] [see sections IV(3) and (4)]. Figure 16 illustrates the effect of increasing the pH, ionic strength and the addition of ethanol (compare conditions of Figs. 15 and 16). While several minor components

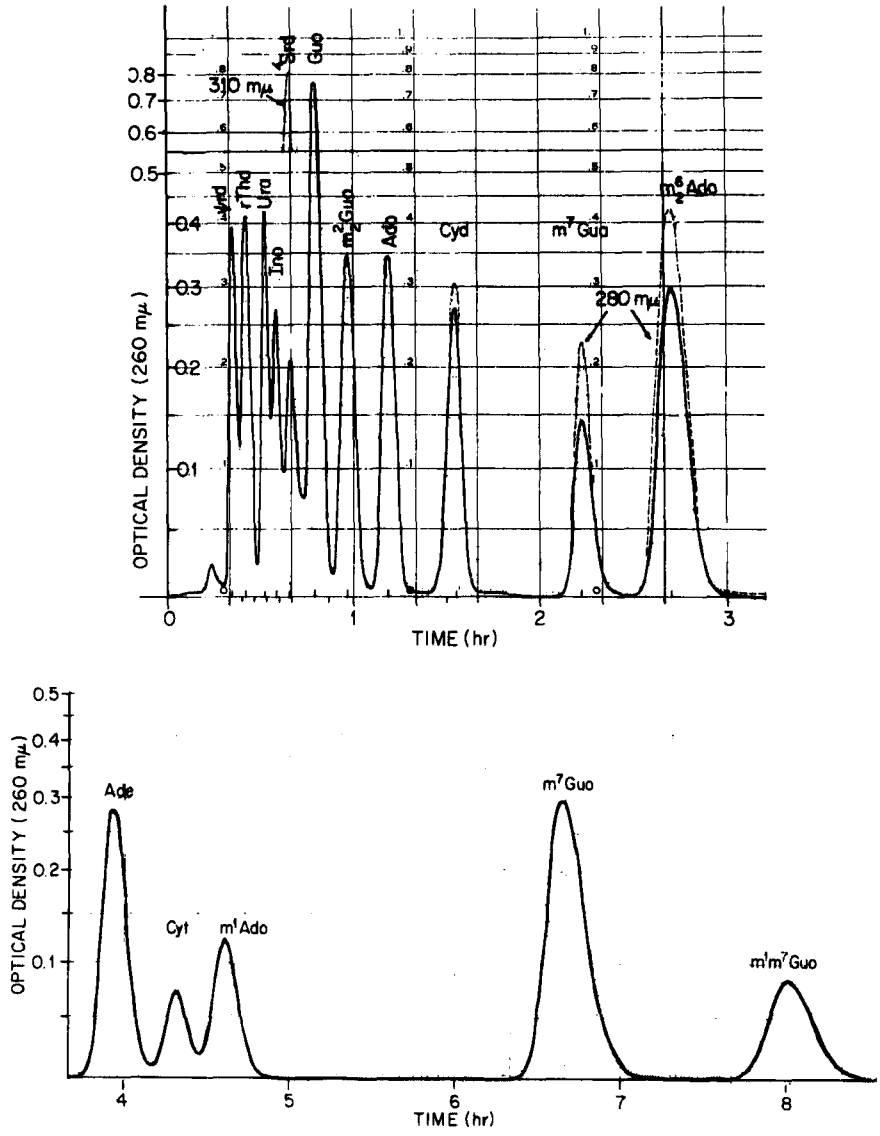

FIGURE 15

Elution positions of several bases and ribonucleosides in cation-exchange chromatography[30]. Column: Aminex A-6, 23 cm x 6 mm. Elution: 0.4 M HCO_2NH_4, pH 4.65, 48°C, 0.26 ml/min, 24 psi. [The original figure, modified for clarity, is reproduced from Uziel et al. 25, 77 (1968) by permission of the authors and the Academic Press, Inc.]

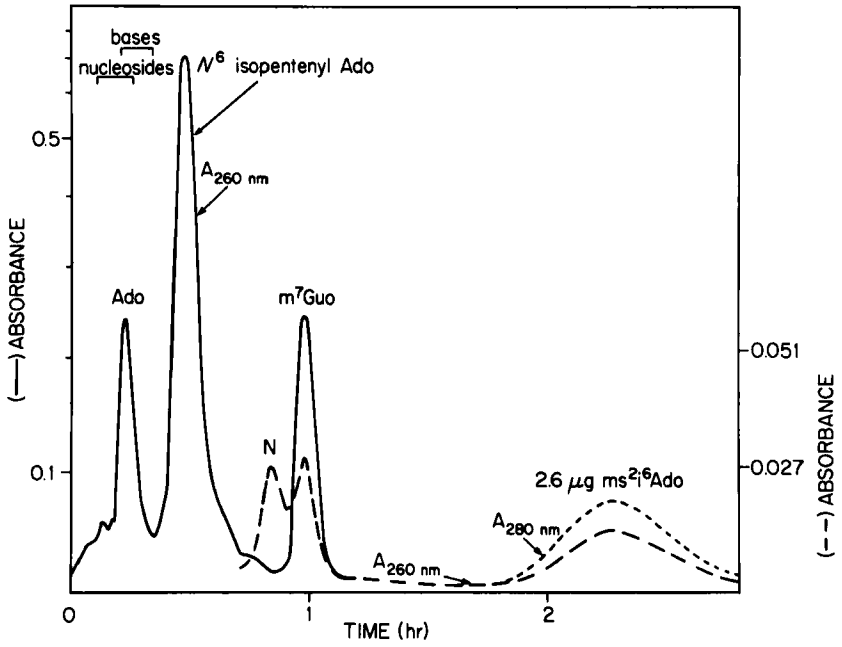

FIGURE 16

Elution positions of bases and ribonucleosides in a modified[36] cation-exchange[30] chromatography. Column: Aminex A-6, 13 cm x 5 mm. Elution: 0.85 M CH_3COONH_4, pH 5.7 and 15% ethanol, 49°C, 0.25 ml/min. N represents an unknown component present in tyrosine $tRNA_1$ hydrolyzate (broken curves). [The original figure, modified for clarity, is reproduced from M. Uziel and C. Koh, J. Chromatogr. 59, 188 (1971) by permission of the authors and the Elsevier Publishing Co.]

are satisfactorily resolved for quantitation, all common nucleosides and bases appear as a single band near the front (Fig. 16).

(b) Anion-Exchange Resin

(i) Cation-exclusion chromatography. At acid pH values, as discussed above, cytidine and adenosine group of nucleosides are cations while uridine and guanosine are uncharged species. On an anion exchanger, the former two species are excluded[1,2]. The degree of their exclusion depends upon their pK_b values. This is a situation opposite to cation exchange where the cations are exchanged. But, nonionic species elute unadsorbed in both cases.

Figure 17 illustrates an analysis of arginine tRNA (E. coli) at a picomole level. Though uridines are uncharged and not excluded at acid pHs, they appear between two excluded cations, cytidine and adenosine. Their particular position indicates little nonionic interactions, but they are enough to resolve the three uridine species. Better resolutions of 7-methylguanosine, cytidine and pseudouridine peaks were achieved recently by substituting Aminex A-28 (8 μm beads) for Aminex A-25 (17 μm beads). The minor components, such as 4-thiouridine and methylated species that appear late in the chromatogram, can be desorbed in less volume and time by introducing a buffer of slightly high ionic strength after the appearance of the guanosine peak.

In this procedure, 4-thiouridine is resolved from all other uridines. And, the use of an acid eluant avoids any degradation of alkali-unstable compounds. Figure 18 illustrates the elution positions of the major and some minor ribonucleosides, common deoxy-

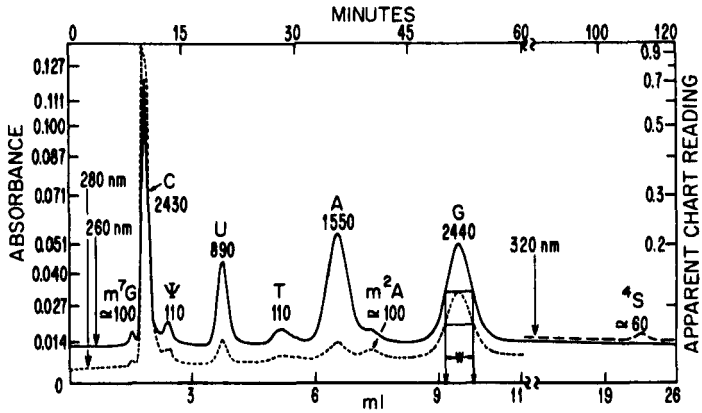

FIGURE 17

Analysis of about 4 μg of nucleoside mixture of arginine tRNA by cation-exclusion chromatography[2]. Column: Aminex A-25, 10.8 cm x 6.35 mm. Elution: 20 mM $HCOONH_4$ +15 mM HCOOH, pH 3.75, 50°C, 0.185 ml/min. The quantities in picomoles are indicated under each peak. [Reproduced from R. P. Singhal and W. E. Cohn, Biochemistry 12, 1532 (1973), by permission of the American Chemical Society.]

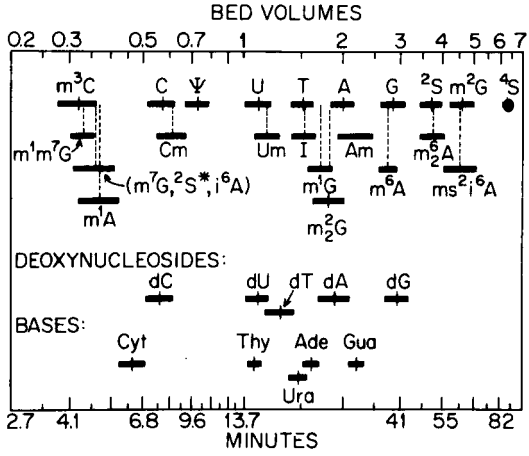

FIGURE 18

Cation-exclusion chromatography[2] of purine and pyrimidine bases, deoxynucleosides and ribonucleosides (top four rows). See legend to Fig. 17 for separation conditions. [Reproduced from same paper as Fig. 17.]

nucleosides and bases. Twenty one out of 24 nucleosides show some degree of separation, which can be improved by chosing appropriate parameters. The method has been used in the characterization of oligonucleotides. Thus, Fig. 19 illustrates an analysis of the decanucleotide[22]. The fragment was derived from the anticodon loop of glutamate tRNA after a modification with bisulfite, which causes a cytidine to uridine to uridine transition[22,68].

(ii) Anion-exchange chromatography. As mentioned earlier (see anion-exclusion), uridine and guanosine compounds are anionized at alkaline pH values; hence, they can exchange on an anion-exchange column or can undergo anion exclusion on a cation-exchange column. The members of the uridine group (ribothymidine, pseudouridine, and uridine) are resolved in these two cases by virtue of their slightly different pK_a values, all of which lie in the region of 9. Figure 20 illustrates separations of several nucleosides. The early appearance of 6-isopentenyladenosine is of particular interest (compare its position in cation exchange, Fig. 16). The

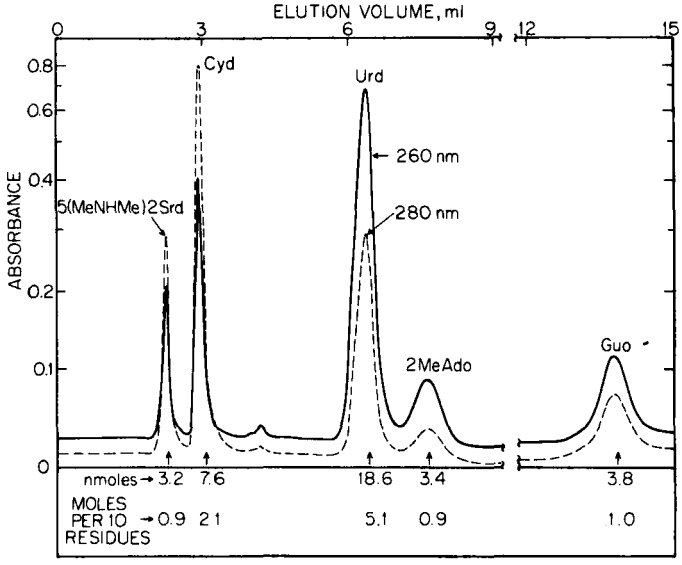

FIGURE 19

Analysis by cation-exclusion chromatography[2] of ribonucleosides of a decanucleotide (peak 3 in Fig. 30b) derived from bisulfite-modified glutamate $tRNA_2$. Sample: enzymatic hydrolysate of the decanucleotide, 0.36 A_{260} unit. Column: Aminex A-28, 18 cm x 6.3 mm. Elution: 1 mM NH_4OAc, pH 3.8, at 50°C and 0.2 ml/min. The quantities in nanomoles are indicated under each peak. [Reproduced from R. P. Singhal, Biochemistry 13, July 2, 1974 issue, by permission of the American Chemical Society.]

separation of minor components (Fig. 21, pH 9.7) can be improved by varying the separation parameters. The effect of pH on separation (R_w) of uridines is illustrated in this case. The plate heights in anion-exchange chromatography on Dowex 1 columns varies between 0.4 to 0.8 mm. However, this can be reduced to 0.2 mm, thus making it compatible to other analytical systems by using anion exchangers of smaller and more uniform bead size, such as Aminex A-25 or A-28 (see Fig. 8)[19].

3. Deoxynucleosides.

The deoxynucleosides can be separated similar to ribonucleosides on both anion-and cation-exchange resins by either ion-ex-

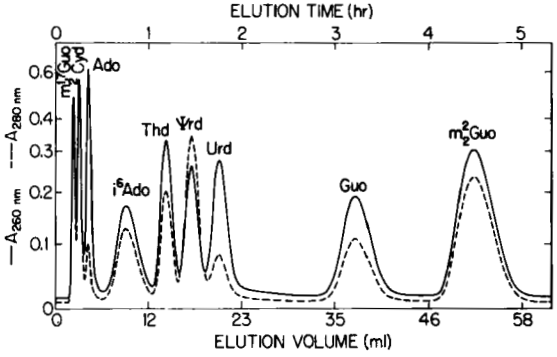

FIGURE 20

Anion-exchange chromatography[19] of ribonucleosides. Column: Dowex 1-X8, 25 x 0.5 cm. Elution: 0.3 M NH_4OAc + 7% 1-butanol, pH 9.7, 50°C, 1 cm/min. [R. P. Singhal, unpublished work.]

change or ion-exclusion procedures. Since deoxynucleosides differ from ribonucleosides very little (pK values), their separations generally require minor modifications of the ribonucleoside separa-

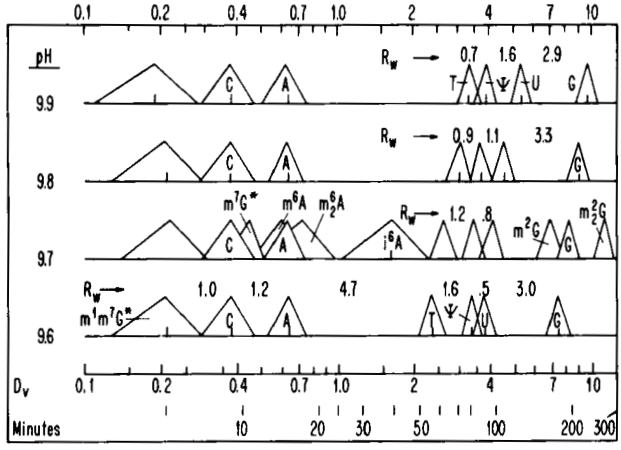

FIGURE 21

Anion-exchange chromatography[19] of ribonucleosides. Separation at pH 9.7 indicates elution positions of several minor components. For column and elution conditions, see legend to Fig. 20. [Reproduced from R. P. Singhal and W. E. Cohn, Anal. Biochem. 45, 585 (1972), by permission of the Academic Press, Inc.]

tion conditions. Thus the common deoxynucleosides are resolved by anion-exclusion at pH 9.5 (Fig. 22), but the ribonucleosides require a slightly higher pH. The elution positions of deoxynucleosides with respect to ribonucleosides and bases have been investigated[1,2] (see Fig. 13 for anion exclusion and Fig. 18 for cation exclusion).

4. Group Separation and Complex Formation.

To investigate each species of nucleotides, nucleosides, and bases, a prior fractionation of the sample into three broad categories is often helpful. An early attempt[47] to resolve these three groups on a polyacrylamide gel column was unsatisfactory (mixed peaks). Recently, pH conditions and other parameters were explored[69] for this anion-exclusion chromatography[1]. A satisfactory separation of the three groups in the presence of borate is shown in Fig. 23.

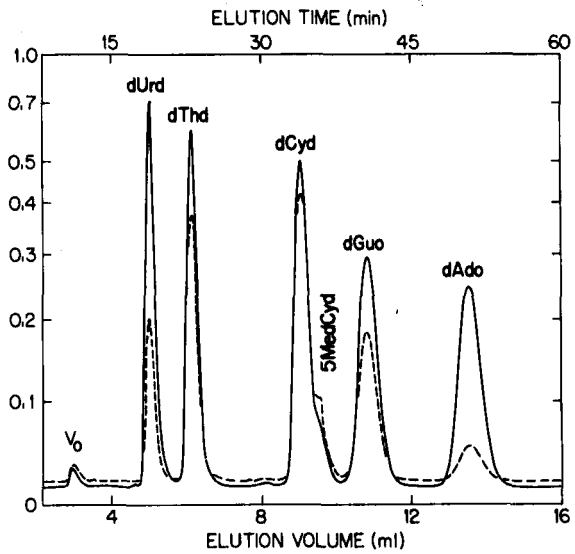

FIGURE 22

Anion-exclusion chromatography[1] of deoxynucleosides. Column: Aminex A-6, 28 cm x 6.35 mm. Elution: 20 mM $(NH_4)_2CO_3$, pH 9.5, 50°C, 0.28 ml/min, 75 psi. [R. P. Singhal, unpublished work.]

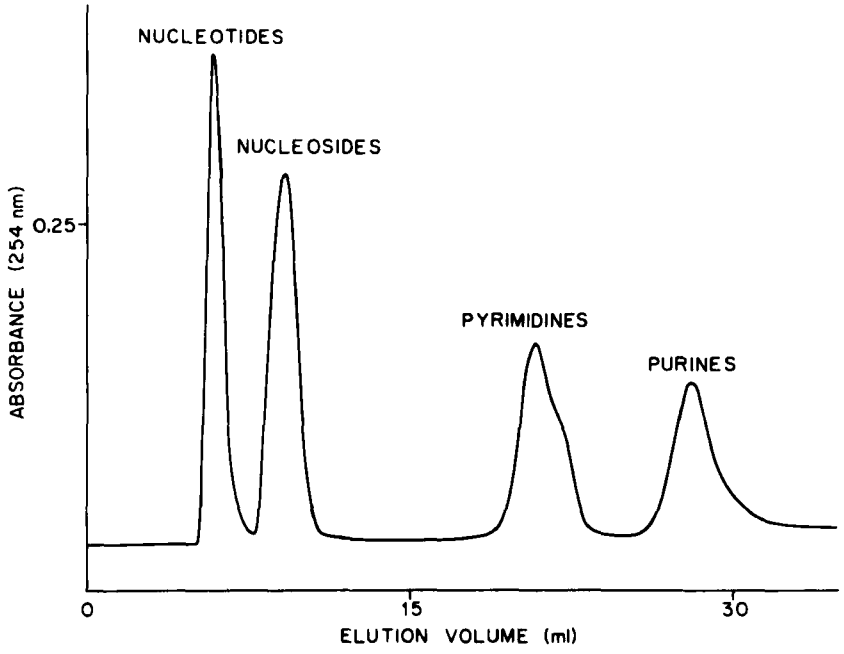

FIGURE 23

Group separation of ribonucleotides, ribonucleosides and purine and pyrimidine bases by anion-exclusion chromatography[1] in the presence of borate ions. Column: Bio-Gel P-2, 200-400 mesh, 70 cm x 6.2 mm, (Void volume, 5.8 ml). Elution: 20 mM potassium tetraborate (+HCOOH), pH 8.9, 0.35 ml/min, 140 psi. [Reproduced from J. X. Khym, Anal. Biochem. 58, 638 (1974), by permission of the author and the Academic Press, Inc.]

A borate salt complexes with cis glycols at alkaline pH's. The complex introduces an anionic charge[4,29,70]. Thus, in the presence of borate at alkaline pH's, all of four groups of ribonucleosides and 5'-ribonucleotides become anions. Since guanine and uridine bases have pK_a values in the region of pH 9, guanosine and uridine compounds become anionic in contrast to cytidine and adenosine compounds under these conditions. These differences in anionic charges and nonionic properties, observed in the presence of borate, can be exploited advantageously for their chromatographic separations.

Thus, borate complexing has been used in other specific separations, such as sugars[29], sugar phosphates[4,29], ribonucleosides from deoxynucleosides[71-73], 5'-ribonucleotides from 2',3'-ribonucleotides[4,5] and free tRNA's from aminoaylated tRNA's[74,75], which lack cis glycols.

The ribose moiety of ribonucleosides and 5'-ribonucleotides can be oxidized by periodate to cleave the 2', 3'-bond. The product, a ribose containing aldehyde groups at both 2'- and 3'-positions, chromatographs like the original compound in the absence of borate. The dialdehyde can be reduced by $NaHB_4$ to a dialcohol derivative, which possesses a reduced anionic character. Or, it can be reacted with primary amines to obtain products of a Schiff-base nature. These products may be reduced to yield amino groups at the original aldehyde sites. The chemical modification of the ribose moiety introduces changes in the anionic and perhaps also in the nonionic characters of the entire molecule. Separations on the basis of these modifications have been achieved by column chromatography[76]. By using NaB^3H_4 for the reduction of dialdehydes, ribose derivatives can be labeled in vitro. The method has been exploited for a sensitive quantitation and separation of RNA hydrolysates by partition chromatography[13,14].

5. Nucleotides.

A. Simple nucleotides.

The simple nucleotides are the purine and pyrimidine nucleosides that are esterified to a phosphoric acid residue [see section IV(1) for ionization properties]. Thus 2'-, 3'- and 5'-nucleotides of both ribose and deoxyribose series, 2':3'-cyclic and 3':5'-cyclic nucleotides are known. The discovery and characterization of these compounds is credited to the pioneer work of Cohn[77,78] and others[79-81].

(i) Cation-exchange resin. The anionic character of nucleotides at an acid pH is due to differences in the cationic charge of the bases and the anionic charge of the phosphate group linked to the ribose moiety; therefore, differences in the net

negative charges of nucleotides can be expected (see refs. 10 and 18 for net charges at a given pH). The charge differences among nucleotides, in conjunction with their variable partitions between mobile (aqueous eluant) and stationary (organic resin matrix) phases, are ascribed for chromatographic differences. The nucleotide separation on a cation exchanger that was originally ascribed to cation-exchange[82], are in fact due to anion exclusion[3]. Anion exclusion of nucleotides (Fig. 24, also see Fig. 2) is superior to anion-exchange chromatography in several aspects (smaller plate

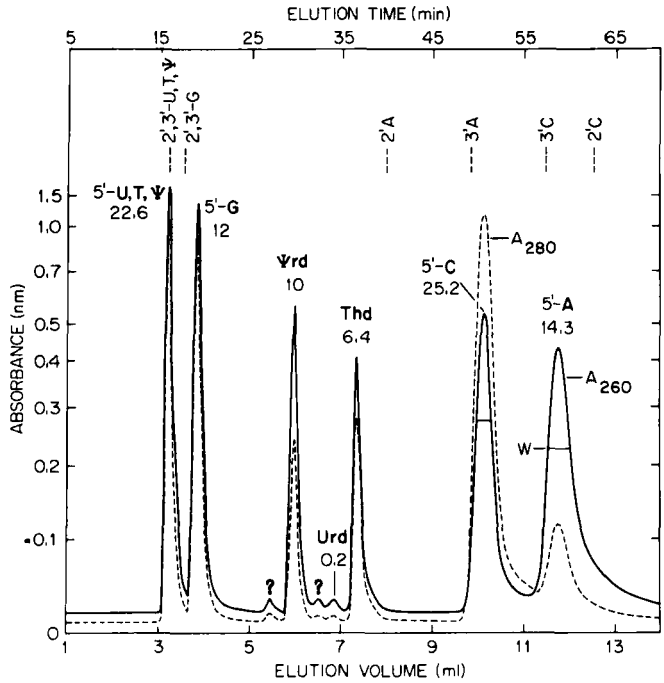

FIGURE 24

Separation of a mixture of 5'-ribonucleotides and three common uridines by anion-exclusion chromatography[3]. The positions of the isomeric 2'- and 3'-nucleotides are indicated by dashed lines at the top. Column: Aminex A-6, 50 cm x 5 mm. Elution: 10 mM $HCOONH_4$, pH 3.50, 50°C, 0.2 ml/min. [Reproduced from R. P. Singhal, Eur. J. Biochem. 43, 245 (1974), by permission of the Fed. Eur. Biochem. Soc.]

heights, better resolutions, salt-free eluants, speed). The two methods were compared recently[3]. The nucleosides (such as derived from the 3' end of tRNAs) can be resolved by cation exchange[30] after analysing the sample for mononucleotides by anion-exclusion chromatography[3].

(ii) Anion-exchange materials. The first successful separations of nucleotides were obtained by anion-exchange chromatography[77]. Since then, the method has been in wide application[79,80,83,84] and has been modified[85] to accommodate separations of bases and nucleosides in the same analysis. Thus, the anion-exchange chromatography on small and uniform beads was recently reinvestigated[3] to develop systems that can be used to separate the major nucleotides in single eluants (Fig. 25) and to separate most nucleosides, nucleotides and other hydrolysis products in one analysis (see Fig. 7 in ref. 3).

The separations in so-called reversed-phase chromatography, as mentioned earlier (section I), are also due to nonionic interactions and to the anion-exchange properties of the immobilized quaternary ammonium derivatives[18]. The separation of major nucleotides (Fig. 26) and several minor components on "reversed-phase" columns has been achieved[3,86]. A rapid analysis is achieved by using a shorter column (see Fig. 3 in ref. 18; Figs. 9 and 10 in ref. 86) and complex mixtures are resolved by eluting with a linear salt gradient[18]. These separations require the use of dilute salt solutions as eluants. If ammonium acetate is employed at a low ionic strength, the adsorbed quaternary ammonium derivative (anion-exchanger) is mobilized from the inert matrix. However, this practical problem can be overcome by substituting sodium chloride for ammonium acetate[18]. Recently, Holton et al.[87] substituted silylated silica for polychlorotrifluoroethylene, the inert support for RPC-5. These workers report insignificant bleeding of the alkyl ammonium salt while eluting with dilute ammonium acetate solutions, and observe satisfactory resolutions of several simple nucleotides and oligonucleotides.

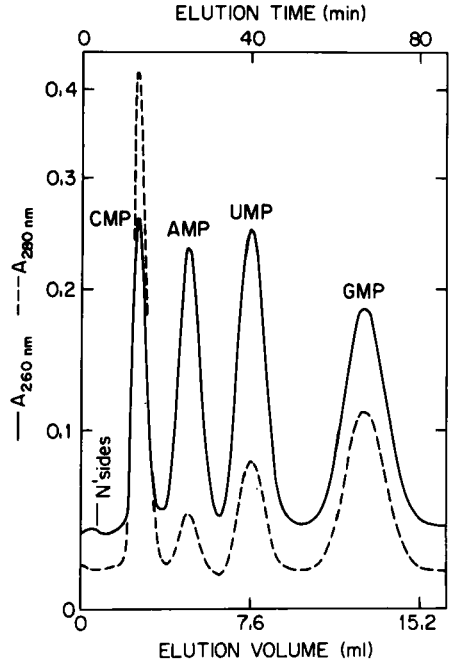

FIGURE 25

Anion-exchange chromatography[3] of the common 5'-ribonucleotides. Column: Dowex 1-X8, 400 mesh, 25 cm x 2 mm. Elution: 0.25 M NH_4OAc containing 20% 2-propanol, pH 3.3, 50°C, 0.2 ml/min. [R. P. Singhal, unpublished work.]

B. Nucleoside Polyphosphates.

After the introduction of ion-exchange chromatography for the separation of nucleic acid components[77,82], numerous investigators have since variously modified[31,85,88] the basic method to develop a practical procedure for mapping the substances of interest present in biological fluids (such as, citric acid cycle intermediates[89], biosynthesis of nucleic acid[90] and acid-soluble pool components[79,91]). These substances are bases, nucleosides, simple nucleotides and pyrophosphorylated forms of common nucleosides [such as, adenine, adenosine, adenosine 3'-phosphate, 5'-monophosphate (AMP), 5'-diphosphate (ADP) and 5'-triphosphate (ATP), and

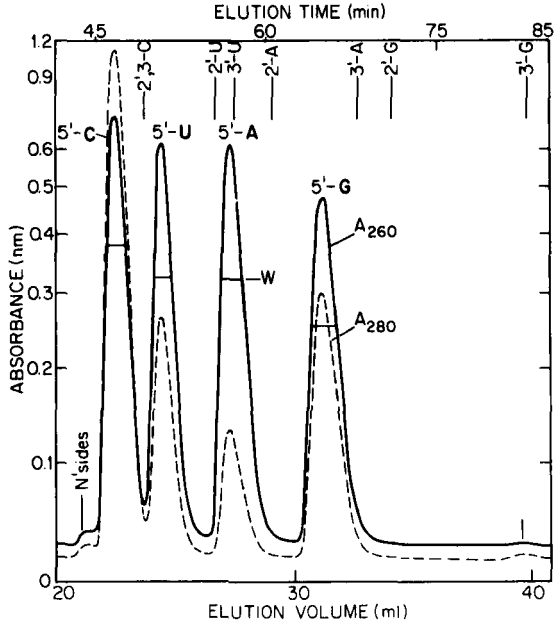

FIGURE 26

Anion-exchange ("reversed-phase") chromatography of ribonucleotides[18]. Column: RPC-5, 100 cm x 6.35 mm. Elution: 0.15 M NH_4OAc - 0.28 M AcOH, pH 4.4, 50°C, 0.5 ml/min, 160 psi. The positions of isomeric 2'- and 3'-nucleotides are indicated by vertical lines at the top. [Reproduced from R. P. Singhal, Biochim. Biophys. Acta 319, 11 (1973), by permission of the Elsevier Publishing Co.]

adenosine 3':5'-cyclic phosphate]. Earlier separations of these compounds were achieved on two anion-exchange columns under very acidic conditions, where the substances are unstable. Figure 27 illustrates a mapping of the acid soluble components of rat liver cytoplasm. The order of elution of the monophosphates is repeated in the di- and triphosphates on this "reversed-phase" column.

The separation of a complex mixture of nucleotides can be improved[4] by using a linear[92], discontinuous[93,94], or other[95,96] mode of salt gradient; a constant[4,80] or falling[31] pH; volatile[72-97] or other kinds[98-101] of eluants; a cellulose anion-exchanger[102-105],

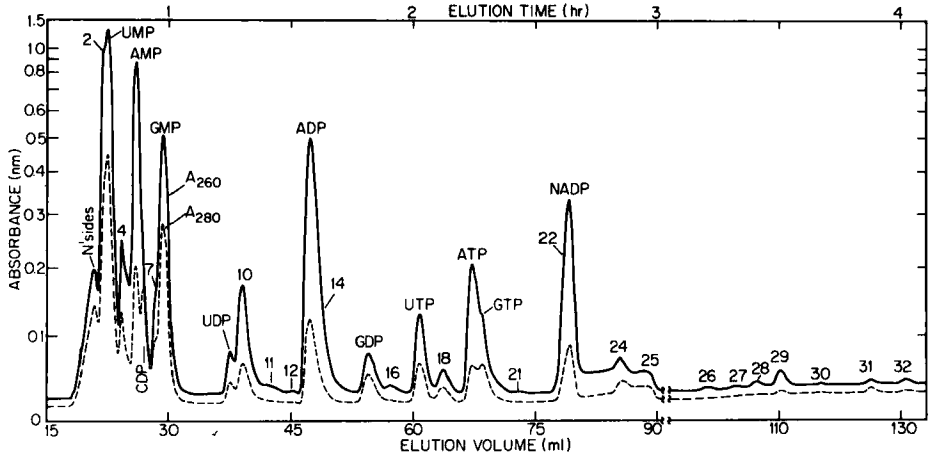

FIGURE 27

Anion-exchange ("reversed-phase") chromatography of ultraviolet-absorbing components of the "acid-soluble" fraction of rat liver[18]. Column: RPC-5, 98 cm x 6.3 mm. Elution: linear gradient from 0.15 M to 0.93 M NH_4OAc, pH 4.4; 100 ml of each component; 50°C, 0.5 ml/min, 160 psi. [Reproduced from same paper as Figure 26.]

or by using a hydroxylapatite ion-exchange material[106]. Recently, the separation of ribonucleoside 5'-mono, -di, and -triphosphates on an anion-exchange column of small and uniform beads was reported[107] (Fig. 28). These separations are achieved with a dilute solution of sodium citrate at an alkaline pH. The bases and nucleosides that may interfere in these separations can be removed by a prior chromatography (see Fig. 23).

C. Oligonucleotides.

The oligonucleotides are fragments of nucleic acids, where nucleosides are linked together by phosphodiester bonds. (5'-carbon of ribose is linked through a phosphoric group to 3'-carbon of another ribose). The hydrolysis of RNAs and DNAs with specific enzymes yield fragments of varying sizes. Basically three kinds of ion-exchange materials have been employed for the separation of oligonucleotides. They are, anion-exchange resin[108] (Dowex-1 X2),

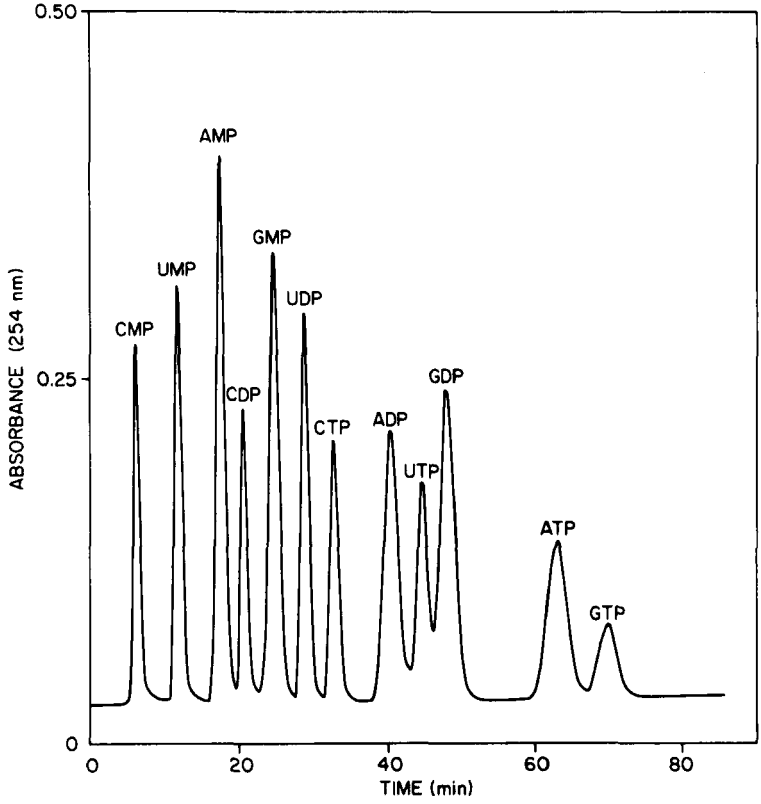

FIGURE 28

Anion-exchange separation of 5'-mono-, di-, and triphosphates. Column: Aminex A-27, 10 cm x 6.2 mm. Elution: concave gradient, a closed constant volume mixing vessel containing 25 mM sodium citrate and an open reservoir containing 0.5 M citrate, both at pH 8.3; 70°C, 0.6 ml/min, 40 psi. [Reproduced from J. X. Khym, J. Chromatogr. (1974) in press, by permission of the author and the Elsevier Publishing Co.]

modified cellulose[109] (DEAE-cellulose, DEAE-Sephadex), and the so-called "reverse-phase"[18,86,110].

Figure 29 illustrates a separation of the oligonucleotides, derived from a pancreatic RNase digest of glutamate tRNA, on a reversed-phase-5 column with a linear gradient of ammonium acetate

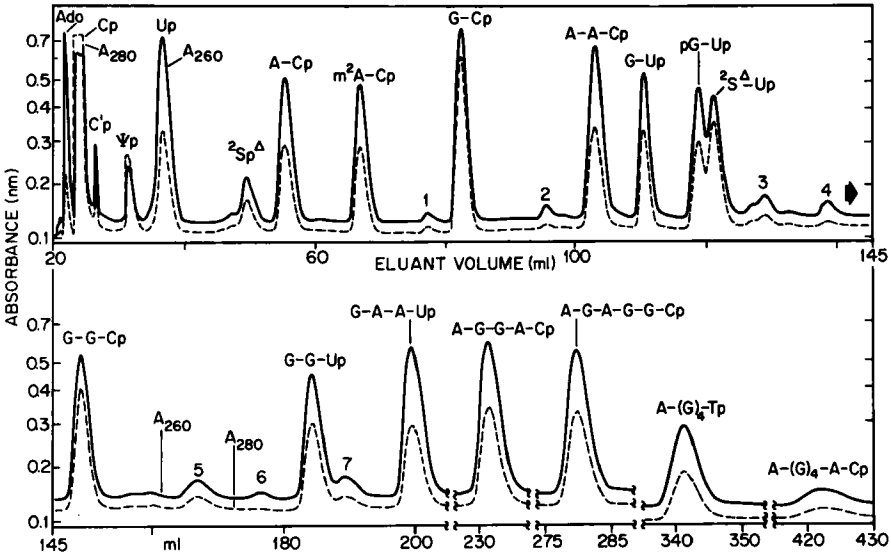

FIGURE 29

Anion-exchange ("reversed-phase") chromatography[18] of a pancreatic ribonuclease digest of glutamate tRNA. Column: RPC-5, 100 cm x 6.35 mm. Elution: first with 20 ml of the starting solution (0.4 M) followed by a linear acetate gradient, 0.4 to 4.0 M NH_4OAc ($+NH_4OH$), pH 9.8 (250 ml each), 50°C, 0.5 ml/min, 180 psi. [Reproduced from same paper as Figure 26.]

at pH 9.8. The method provides homogenous peaks of most components, from pyrimidine mononucleotides to the long-chained oligonucleotides that are difficult to resolve on DEAE-cellulose or DEAE-Sephadex columns[105] (cf. Fig. 2 in ref. 68). The resolutions at an alkaline pH are superior due to enhanced charge differences[18] (see below). This separation method[18] was recently applied for determining the bisulfite modification sites in glutamate tRNA. The two chromatograms, RNase T_1 digests from control and modified tRNA's (Fig. 30), are similar except for the presence of three extra peaks in that of the modified tRNA[22]. [tRNA fragments, (1) C-C-A_{OH} and (2) C_3-U-^{2}S-U-C-m^2A-C-Gp (Fig. 30a) are transformed into (1) C-U-A_{OH}, and (2) C_2-U_2-^{2}S-U_2-m^2A-C-Gp and C_2-U_2-^{2}S-U_2-m^2A-U-Gp (peaks 1-3, respectively in Fig. 30b) by this modification].

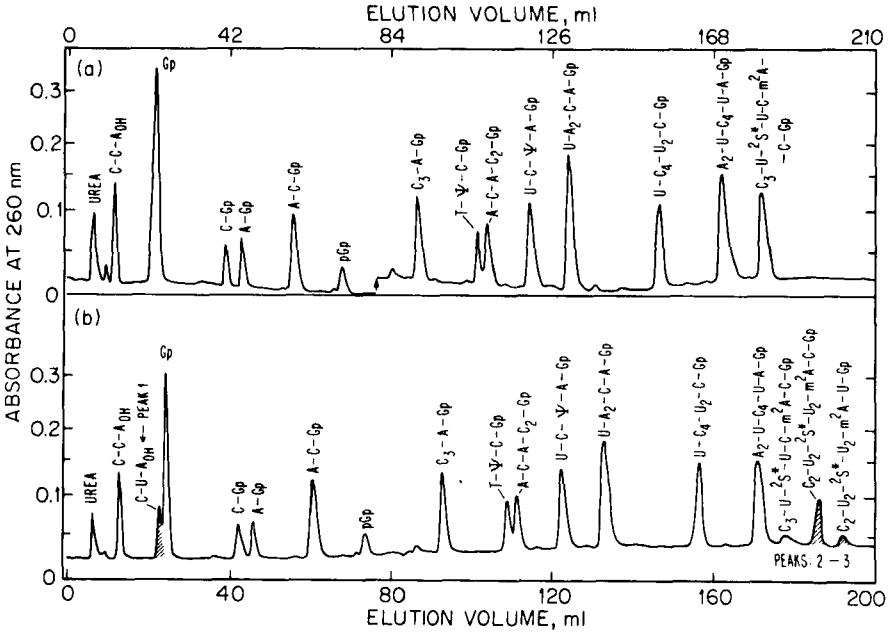

FIGURE 30

Comparison of oligonucleotides from ribonuclease T_1 digests of (a) normal and (b) bisulfite-treated glutamate $tRNA_2$. Column: RPC-5, 25 cm x 6.3 mm. Elution: linear gradient of 0.1 to 0.75 M NaCl, pH 9.8 (20 mM Na_2CO_3-$NaHCO_3$), 100 ml each, at 50°C, 0.5 ml/min, 100 psi. Sample: 4 A_{260} units of tRNA hydrolysed with RNase T_1. [Reproduced from R. P. Singhal, Biochemistry 13, July 2, 1974 issue, by permission of the American Chemical Society.]

Figure 31 illustrates a separation of pyrimidine oligonucleotides of DNA, obtained by acid hydrolysis. These first separations were achieved on Dowex 1-Cl^- columns with a linear gradient of hydrochloric acid[108], the method has been modified by other workers (for example, see ref. 112). The examples of the use of the DEAE-cellulose for the separation of oligonucleotides are shown in Figs. 32 and 33 below.

The appearance of the sequence of oligonucleotides in the three kinds of anion exchangers depends upon the net anionic charges. At an acid pH, cytosine, adenine and guanine (pK_b's: 4.5,

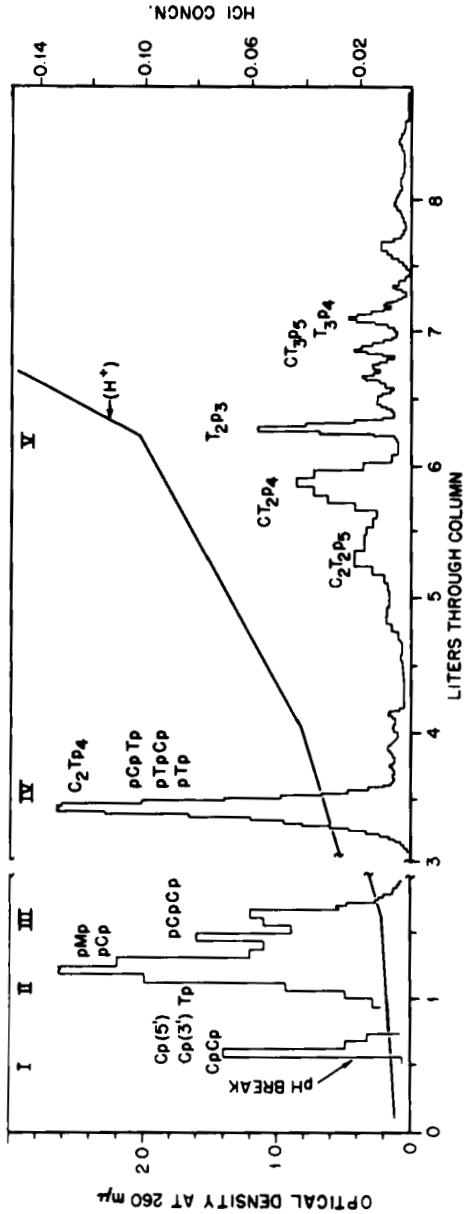

FIGURE 31

Anion-exchange chromatography of pyrimidine deoxynucleotides, obtained by acid digestion of DNA. Column: Dowex 1-Cl^-, 200-400 mesh, 10 cm x 1 cm^2. Elution: linear gradients of Cl^- as shown. [Reproduced from W. E. Cohn and E. Volkin, Biochim. Biophys. Acta, 24, 359 (1957), by permission of the authors and the Elsevier Publishing Co.]

4.1 and 3.2, respectively) are cationized; hence, the net anionic charge of the oligonucleotide, containing these residues, is reduced. On the other hand, at an alkaline pH, thymine, guanine, uracil and the secondary phosphate of the terminal residue (pK_a's: 9.9, 9.6, 9.5 and 6, respectively) are anionized, thus enhancing the net anionic charge and exchange capacity of the oligonucleotide. (pH 9.8 permits a separation of uridine isomers[1,19].) Besides these ionic effects, nonionic differences are equally important in predicting the elution sequence of oligonucleotides. (Oligonucleotides containing purine bases are retained by the ion exchanger more than those containing pyrimidine bases). These nonionic interactions are often problematic (low recovery, poor resolutions). They can be suppressed by operating at an elevated temperature[113,114] [see section IV(3)], and by adding an organic solvent or 7 M urea to the eluant. Figure 32 illustrates the effect of urea[115-118] on the separation of oligonucleotides of DNA by DEAE-cellulose chromatography[115]. Figure 33 shows a separation of the

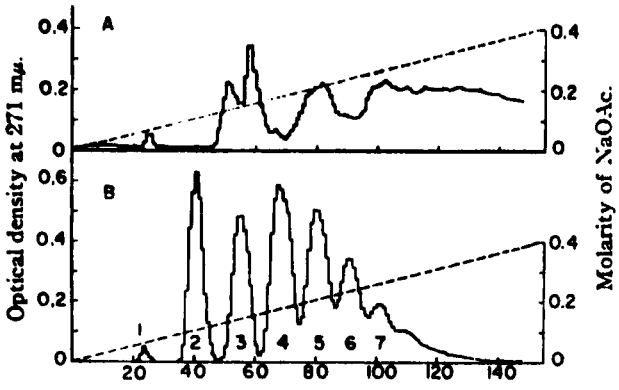

FIGURE 32

Effect of urea on the anion-exchange chromatography of deoxyoligonucleotides. Sample: DNase digest of salmon testes DNA. Column: DEAE-cellulose, 20 cm x 1 cm. Elution: linear gradient of NaOAc (pH 7.5) as shown, total volume 1.5 l. (A) Without urea. (B) Same, with 7 M urea. [Reproduced from R. V. Tomlinson and G. M. Tener, J. Amer. Chem. Soc. 84, 2644 (1962), by permission of the authors and the American Chemical Society.]

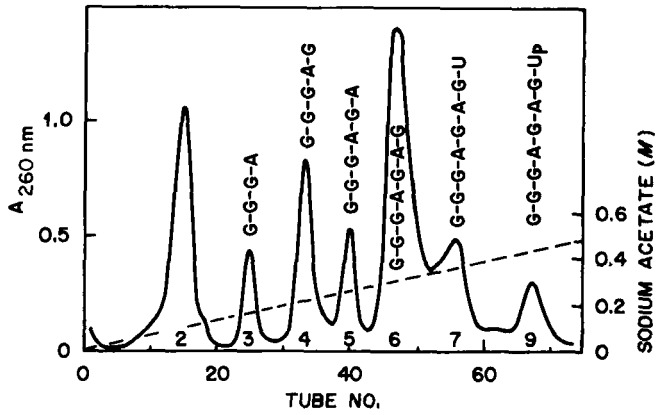

FIGURE 33

Separation of the products of a sequentially degraded octanucleotide. Sample: the octanucleotide was first dephosphooxylated (peak 7), then partially degraded by an exonuclease. Column: DEAE-cellulose, 30 cm x 3.5 mm. Elution: linear gradient of acetate, as shown, containing 7 M urea; 1.2 ml per tube. [Reproduced from Holley et al., Biochem. Biophys. Res. Commun. 17, 389 (1964), by permission of the authors[119] and the Academic Press, Inc.]

products of a sequentially degraded octanucleotide of tRNA. These satisfactory separations[119] were achieved on a DEAE-cellulose column with a linear gradient of sodium acetate containing 7 M urea. A low recovery of some guanylate-rich oligonucleotides from DEAE-Sephadex and "reversed-phase-5" columns have been reported[18,119A]. The guanylate oligomers (6 or more residues) aggregate on DEAE-Sephadex columns and remain strongly bound to the ion exchanger even in the presence of high concentration of sodium chloride and urea. Elution with guanidinium acetate, as they suggest[119A], can improve the guanylate-rich oligonucleotides; however, the eluants then must be desalted for further manipulations.

Desalting by ion-exchange. The volatilizable eluants[109,120] are removed by repetitive freeze-drying. The process is time consuming and unworthy for labile compounds. Nucleotides and oligonucleotides have been recovered from nonvolatile eluants by gel

filteration[22], simple adsorption chromatography on activated charcoal[121], or by anion-exchange chromatography on small DEAE-cellulose columns[122]. The last two methods finally require either a dilute ammonium hydroxide or ammonium bicarbonate solution for the recovery of adsorbed or exchanged substances. Loesche et al.[123] suggested a new approach, a salt of a volatile acid for the elution. The salt is removed by a cation exchanger (H^+ form), which exchanges the salt cations for protons. If $NaHCO_3$ is an eluant, the reaction can be expressed:

$$NaHCO_3 + \text{cation exchanger } (H^+) \rightarrow \text{cation exchanger } (Na^+) + CO_2 + H_2O$$

However, the method may be unsuitable for acid-labile compounds as free acid is evolved and the cation exchanger in H^+ form is very acidic.

6. Nucleic Acids.

At the present state of art, DNAs and RNAs are generally separated during isolation, such as fractionation by phenol or enzymatic destruction of one of the two nucleic acids. RNAs that are functionally different, in general, also vary in size. The property is often used for their fractionation by gel-filtration chromatography. Figure 34 illustrates a fractionation of DNase-treated nucleic acids on a Sephadex column[45]. This procedure provides groups of rRNA, precursor molecules of tRNAs (α and β species[45]), 5S RNA and tRNAs.

The fractionation of one group of nucleic acid has been achieved on a large variety of adsorbants and ion-exchange materials, for example: Keiselgurh coated with methylated albumin[124-131], polylysine[132], silica gel[132A], modified cellulose (DEAE-cellulose, phosphocellulose) and agarose (DEAE-Sephadex, Sepharose)[133-137], "reversed-phase" matrices[16,17,45,86,138,138A] (see section I), hydroxyapatite[139-144], modified polystyrene[145-147], and borate substituted cellulose[74] or polymethachrylate[75]. The reversed-phase-5 column and an anion-exchange column of small and uniform-size beads

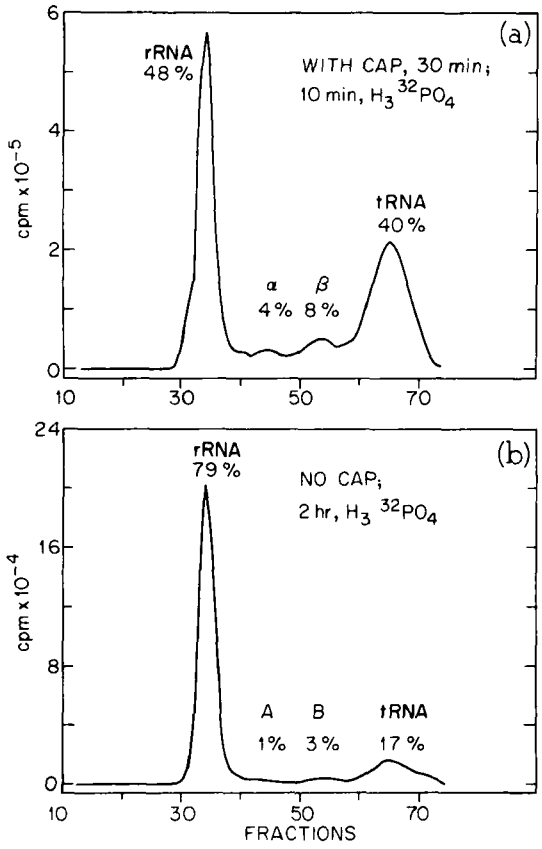

FIGURE 34

Separation of ^{32}P-RNAs (E. coli) by gel filteration. Column: Sephadex G-100, 90 cm x 1.6 cm; bed vol., 180 ml. Elution: 0.75 M NaCl with 10 mM NaOAc, pH 5.1, 4°C, 9 ml/h. (a) Pulse-labeled RNA, cells treated with chloramphinicol (CAP) in a low-phosphate medium. (b) Stable RNA. [Reproduced from J. Dijk and R. P. Singhal, J. Biol. Chem. 249, 645 (1974), by permission of the Am. Soc. Biol. Chem.]

were recently compared for the fractionation of bulk tRNAs under similar conditions. Improved resolutions and a possibility of ethanol addition to the eluant were noticed on the anion-exchange column[3] (Singhal and coworkers, unpublished results).

The polynucleotides are highly anionic in nature. Therefore, no fractionation by anion exclusion can be expected on a column of molecular-sieve material that is anionically charged. However, bulk tRNAs can be separated into two groups on Sephadex matrices of fine bead size (rich in carboxyl residues)[148,149]. They are apparently fractionated due to anion exclusion. It will be interesting to examine the separation of medium size nucleic acids (3-5 S) on columns of Sepharose 4B, which was recently found satisfactory for protein separation by anion exclusion[150].

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