Cation-Exclusion Chromatography on Anion Exchangers: Application to Nucleic Acid Components and Comparison with Anion-Exchange Chromatography†

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ABSTRACT: Ion-exclusion chromatography, previously demonstrated for the separation of anionic substances on cation-exchange material (Singhal, R. P. (1972), *Arch. Biochem. Biophys. 152*, 800), has been extended to the separation of cationic substances on anion-exchange material. A model mixture of nucleosides, derived from ribonucleic acid and including cations as well as uncharged species, can be resolved rapidly and efficiently at an acid pH on columns of an anion exchanger if the latter is comprised of small and uniformly sized beads (Aminex A-25, 17-μm diameter). The plate heights calculated from the results ($\simeq$0.2 mm) are superior to those seen in ion exchange on the same columns and are comparable to those reported earlier (Singhal, 1972) for anion exclusion on cation-exchange columns of similar physical characteristics. The charged (cationic) substances, if not retarded by partition effects, appear within elution volumes less than the liquid content of the column, thus justifying the term "exclusion," and respond to pH changes and to the Donnan effect in a manner predictable from their pK values and the ionic strength of the medium. An uncharged substance, in the absence of partition effects (*e.g.*, ^3H_2O), will appear at one liquid column volume. Partition effects are shown by retardation beyond one liquid column volume and by further retardation with increased ionic strength ("salting-out"), and these effects can be manipulated, together with pH and the addition of organic solvents, to maximize resolutions and reduce the time for a given separation. A direct comparison between cation-exclusion and anion-exchange separations on identical columns confirms the superiority of the former in certain practical aspects (*e.g.*, smaller plate heights leading to better resolution, shorter analysis times, and use of dilute, volatile eluents). Applications of cation-exclusion chromatography to mixtures of various modified nucleosides, deoxynucleosides, and bases, as well as to the compositional analysis of microgram quantities of tRNA, are illustrated.

The characteristics and advantages of ion-exclusion chromatography were discovered in work with nucleosides and bases on sulfonated polystyrene cation-exchanger Aminex A-6 (Singhal, 1972a; Singhal and Cohn, 1972a). In studying and developing the new phenomenon, Singhal (1972b) noted that although the commonly used anion exchanger, Dowex 1 (a quaternary ammonium polystyrene), responded to ion-exclusion principles in a predictable manner, it yielded separations that were less than satisfactory from a practical standpoint. It was surmised that this situation arose from the fact that the particles of Dowex 1, even in the dimensions of 300–400 mesh, were both larger and less uniform in size than Aminex A-6. Since both uniformity and smallness of size could reasonably be expected to play a decisive role in reducing dispersion of the peaks as they move down the column, hence reducing overlap and increasing resolution, we sought to reexamine the phenomenon with an anion-exchange material of the same physical nature as Aminex A-6. This we found in Aminex A-25, and the results reported here indicate that, on comparable material, cation-exclusion chromatography on an anion exchanger is as effective as anion-exclusion chromatography on a cation exchanger in separating substances of controllable ionic charge from each other and in handling the uncharged nucleosides and bases simultaneously encountered in nucleate hydrolysates.

Materials

The anion exchangers, Aminex A-25 (17.5 $\pm$ 2 μm) and 300–400 mesh Dowex 1-X8 (56–38 μm) (see Sober, 1970), were obtained from Bio-Rad Laboratories, Richmond, Calif., and from Dr. Norman G. Anderson, respectively. Samples of 2-thiouridine, 5-(methylaminomethyl)-2-thiouridine, 6-isopentenyladenosine, 6-isopentenyl-2-(methylthio)adenosine, and uridin-5-oxyacetic acid and an uncharacterized modified guanosine ("Q" in Nishimura, 1972a) were gifts from B. C. Pal, J. A. Carbon, R. H. Hall, N. G. Leonard, and S. Nishimura, respectively. Purified arginine transfer RNA (*Escherichia coli*) was supplied by A. D. Kelmers. Other nucleic acid components and enzymes were obtained from a variety of commercial sources.

Methods

Column Preparation and Operation. The exchangers were prepared for use by washing successively with several bed volumes of alkali (1 M NaOH), water, acid (1 M HCl), and water. Ultraviolet-absorbing contaminants were removed by successive treatments with about ten bed volumes of 50% acetone, 50% ethanol, and water. The formate and acetate forms were prepared by treating the (chloride-form) exchangers with 3 M sodium formate and 3 M sodium acetate, respectively. Finally, the exchangers were washed with water and equilibrated with the appropriate eluent solution.

All pH measurements were made at about 25°, after the addition of any organic solvent if such was included. Three different dimensions of columns were employed: 10.8 cm $\times$ 6.35 mm (Chromatronix, Catalog No. LC-6M-43), 24 cm $\times$

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5 mm, and 50 cm × 5 mm (Uziel *et al.*, 1968). The columns were jacketed, and constant temperature (50°) was maintained by a Haake water bath. Narrow Teflon tubing (0.3 mm, i.d.) was employed for all connections. The production of undesirable air bubbles was effectively prevented by collecting the effluent through 15–20 ft of narrow tubing that terminated 2 m above the spectrophotometer cell, and by constantly stirring the eluent at room temperature and pressure.

RNA Hydrolysis. Hydrolysates of glutamate tRNA and arginine tRNA (*E. coli*) also served as sources of common and modified nucleosides. Rapid and complete hydrolysis of tRNA samples to nucleosides using a volatile buffer (Singhal, R. P., unpublished) was achieved in the following way. Transfer RNA equivalent to about 50–65 μg was dissolved in 10 μl of 0.2 M ammonium carbonate containing 1 mM magnesium acetate (pH ≃8.7) to which was added 5 μl of a mixture of three enzymes: pancreatic ribonuclease (12.5 μg or 37.5 units), snake venom phosphodiesterase (12.5 μg or ≃10 potency units), and alkaline phosphatase (7.1 μg or 0.25 unit). The sample was digested in polyethylene microcentrifuge tubes (capacity 400 μl) at 50° (±2°) for 3 hr. As little as 0.10 A_{260} unit of tRNA (5–6 μg) has routinely been hydrolyzed and assayed by the procedures described here with satisfactory results (1.0 A_{260} unit is the amount of substance in 1 ml of a solution giving an absorbance of 1.0 when measured at 260 nm with a pathlength of 1.0 cm).

Sample Application. An off-column, septum-type sample injector (Chromatronix Model 164A-11) was used to introduce solutions by means of a Hamilton syringe (catalog no. 705) without interrupting the flow of eluents. Generally a sample containing a mixture of nucleic acid components (nucleosides, deoxynucleosides, and bases) of about 0.4 A_{260} unit (≃40 nmol) in 10–15 μl of eluent solution was applied to the column. Samples prepared in eluent buffer were preferred to prevent pH alteration, although direct application of about 10 μl of RNA hydrolysate in 0.2 M ammonium carbonate (pH 8.7) did not alter the peak positions. However, large sample volumes (more than 50 μl) and much salt (see Singhal, 1972b, for influence of salt) in the sample yielded increased peak widths and losses in peak resolution, respectively.

Continuous Monitor and Recorder. The ultraviolet-monitoring unit introduced by Anderson (1962) and modified by Uziel *et al.* (1968) was used for all measurements. Essentially, it contains identical small volume (50 μl), quartz-windowed flow cells (10-mm pathlength) housed in a double-beam (Beckman) spectrophotometer. The effluent was monitored at two different wavelengths simultaneously (260 and 280 nm, or 260 and 320 nm in the case of 4-thiouridine) on a logarithmic chart as a series of points that can be treated as histograms or as Gaussian distribution curves (Singhal, 1972b) for quantitation.

Millimolar extinction coefficients of nucleosides at pH 3.75 and at 260 nm were calculated from those determined at pH values well away from their pK values (Beaven *et al.*, 1955; Sober, 1970) and adding the appropriate absorbances due to ionized and nonionized species (see Albert and Sergeant, 1971).

Data Presentation (Singhal and Cohn, 1972b). Inasmuch as the most important results are peak positions and widths relative to volume (or time), these are presented as bars of length 2W (W = width of a peak at half its maximum height and is thus independent of the observed peak height; see peak G in Figure 3) placed so that the midpoints are at the positions of the peaks ($\bar{V}_x$). Each bar thus extends a distance (volume or time) of $+W$ and $-W$ from the peak position.

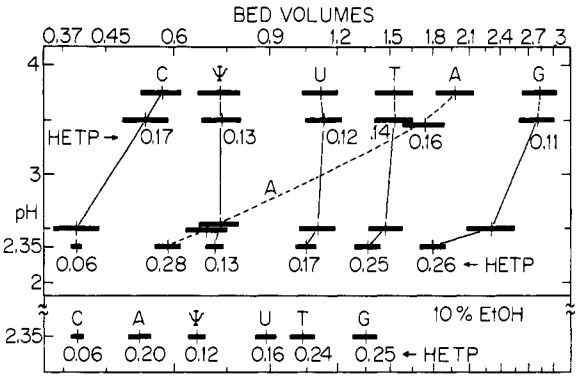

FIGURE 1: Effect of pH upon resolution, elution volume, and plate height (HETP) in cation-exclusion chromatography. Bio-Rad Aminex A-25 columns: (1) 10.8 cm × 6.35 mm and (2) 50 cm × 5 mm (only for the analysis at pH 2.35). Eluents: 0.01 M $HCOONH_4$ + HCOOH for pH values 3.75 and 3.50; 0.05 M HCOOH for pH 2.50; and 0.10 M HCOOH for pH 2.35. The bottom analysis eluent contained 10% ethanol. Flow rates: 0.57 and 1.0 cm/min for columns 1 and 2, respectively; temperature, 50°. Each bar covers twice the observed width (W) at half-maximum peak height; the center of each bar indicates the peak location. The independence of W from actual peak height is shown by the two W values entered on the G peak in Figure 3. HETP is in millimeters per theoretical plate. A, C, G, T, U, and ψ are adenosine, cytidine, guanosine, ribothymidine, uridine, and pseudouridine, respectively (IUPAC–IUB, 1970).

Two neighboring bars that just touch indicate a resolution factor (R_w) of 1.00 [$R_w = (V_b - V_a)/(W_b + W_a)$], which is sufficient to allow easy quantitation even up to ratios of 100:1 of the two neighboring components (Singhal and Cohn, 1972b). The "height-equivalent of a theoretical plate" (HETP) is derived from the formula HETP = $0.18(W/\bar{V}_x)^2L$ (Glueckauf, 1955a,b), where $\bar{V}_x$ is the total volume to the peak (after subtracting the volume between the end of the column and the detector) from the beginning of the elution, and L is the length of the column in millimeters. HETP, in millimeters, is placed on the figures. It allows a quantitative comparison of column efficiency for each substance under each separation condition and for each resin. Both W and $\bar{V}$ are expressed in bed volumes (cross-section of column × length of column) or minutes, or both. As noted earlier, $\bar{V}_x$ in bed volumes is numerically equal to the volume distribution coefficient for substance x under the conditions of the experiment (Mayer and Tompkins, 1947).

Results

Separations of Major Nucleosides by Cation Exclusion

A mixture of the common nucleosides (adenosine, cytidine, guanosine, and uridine) and uridine analogs (pseudouridine and ribothymidine) served as the model on which to study the resolutions obtained by cation-exclusion chromatography on anion-exchange columns.

Influence of Separation Parameters. pH. Figure 1 illustrates the infuence of pH on peak positions (volume distribution coefficient), R_w, and HETP of the common nucleosides resolved by cation exclusion on columns of the anion-exchanger Aminex A-25. Pyrimidines appear before purines, and cations emerge before noncations at pH 4. As the pH is lowered, the two cations, cytidine and adenosine (pK_b 4.1 and 3.4, respectively), become more positively charged; hence they appear nearer the front (are more excluded). Adenosine is about 30% ionized at pH 3.75, but it is appreciably retarded

TABLE I: Effect of Flow Rate on Resolution and Plate Heights of Nucleosides in Cation-Exclusion Chromatography.[a]

Flow Rate (cm/min)	Resolution (R_w)					Plate Heights (HETP)					
	C-ψ	ψ-U	U-T	T-A	A-G	C	ψ	U	T	A	G
1.06	2.1	3.4	2.2	1.8	2.2	0.09	0.13	0.17	0.25	0.30	0.24
1.41	1.3	3.1	1.9	1.7	1.8	0.20	0.21	0.24	0.35	0.39	0.32
1.76	1.2	2.6	1.8	1.5	1.7	0.23	0.36	0.25	0.35	0.45	0.39

[a] Aminex A-25, 24 cm × 5 mm column; eluent 50 mM $HCOONH_4$, pH 3.8, 50°.

due to its purine nature (Cohn, 1955, 1967). The earlier appearance of guanosine as the pH is lowered may be rationalized in similar fashion ($pK_b \simeq 1.6$).

Smaller peak widths at pH 2.35 than those at pH values 3.75, 3.5, and 2.5 (Figure 1) may be ascribed to differences in column dimensions (50 cm × 5 mm *vs.* 10.8 cm × 6.35 mm, respectively) or to other physical factors involved in the construction of the columns. The HETP values are altered only insignificantly with changes in eluent pH.

Since adenosine, besides other nucleosides, is well separated from two neighboring peaks, and no evidence of depurination of nucleosides or deoxynucleosides was noted in this dilute eluent (≃0.02 M $HCOONH_4$ + 0.03 M HCOOH) at pH 3.75, this eluent was chosen for further investigations.

SOLVENT. The addition of an organic solvent (ethanol, to 10%) to the eluent (0.10 M formic acid, pH 2.35) has a marked influence (compare the two bottom rows in Figure 1) on nucleosides that are more lipophilic in nature (guanosine, adenosine, and 5-methyluridine). All six compounds are eluted in 13 ml by an eluent containing ethanol as compared with 18 ml for an eluent having no solvent. The efficiency after the addition of ethanol (compare HETP at 0 and 10% ethanol concentrations, pH 2.35) remains unaltered. The earlier studies (Singhal and Cohn, 1972b) indicate that different kinds of alcohols influence a chromatographic separation in about the same way. Hence, it is concluded that organic solvents like ethanol offer significant advantages in shortening the time of analysis. This is in accord with our earlier observations with anion-exchange (Singhal and Cohn, 1972b) and anion-exclusion (Singhal, 1972b) chromatography.

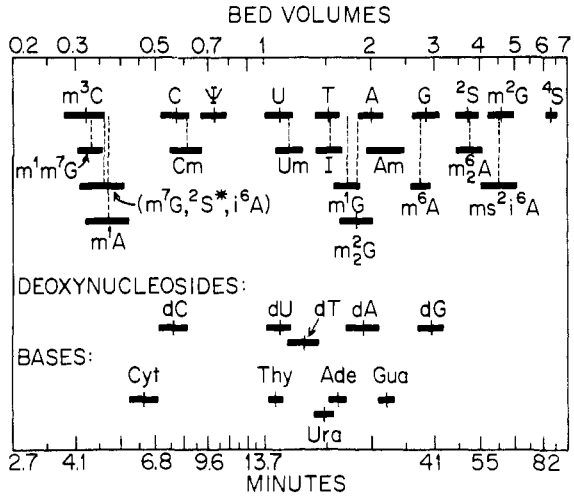

FIGURE 2: Cation-exclusion chromatography of (1) the major and some minor ribonucleosides, (2) common deoxyribonucleosides, and (3) bases, at pH 3.75 on an anion-exchange column; a composite of 25 experiments. Aminex A-25, 10.8 cm × 6.35 mm; eluent, 0.01 M $HCOONH_4$ + ≃0.02 M HCOOH, pH 3.75; flow rate 0.77 cm/min; temperature 50°. For mode of plotting and symbols of major components, see legend to Figure 1. Minor nucleosides (IUPAC–IUB, 1970): 1-methyladenosine, m^1A; 6-methyladenosine, m^6A; 6-dimethyladenosine, m_2^6A; isopentenyladenosine, i^6A; 6-isopentenyl-2-methylthioadenosine, ms^2i^6A; 2′-*O*-methyladenosine, Am; 2′-*O*-methylcytidine, Cm; 3-methylcytidine, m^3C; 1-methylguanosine, m^1G; 2-methylguanosine, m^2G; 2-dimethylguanosine, m_2^2G; 7-methylguanosine, m^7G; 1,7-dimethylguanosine, m^1m^7G; inosine, I; 2-thiouridine, ^2S; 5-(methylaminomethyl)-2-thiouridine, $^2S^*$; 4-thiouridine, ^4S; pseudouridine, ψ; ribothymidine, T; 2′-*O*-methyluridine, Um. 5-Methyldeoxycytidine appears almost exactly with deoxycytidine (elution volumes of 0.56 and 0.57, respectively).

FLOW RATE. As the flow rate is increased, efficiency and resolution decline (HETP increases and R_w decreases). Table I illustrates analyses at three different flow rates. Significant losses in resolution and efficiency occur with only small changes in flow rate. From a practical standpoint, a compromise must be made between a reduction in analysis time (40% less time is required by increasing the flow rate from 1 to 1.8 cm/min) and a loss in resolution, particularly when two components are separable by only a resolution factor approaching 1.0.

Separation of Other Nucleic Acid Components

The conditions for the best separation of the six common ribonucleosides were applied to determine the behaviors of minor ribonucleosides, deoxyribonucleosides, and purine and pyrimidine bases. The parameters for the best resolution of each substance were not independently investigated.

Rare Nucleosides. The results presented in Figure 2 indicate that 21 ribonucleosides out of 24 studied show some degree of separation under the conditions chosen. Only three nucleosides (7-methylguanosine, 5-(methylaminomethyl)-2-thiouridine, and isopentenyladenosine) appear in the same position. An appropriate choice of column size and separation parameters (pH, temperature, flow rate) should resolve, if not all of the 21 compounds, at least the nucleosides derived from a single tRNA (see below for an example).

Sulfur-containing nucleosides (2- and 4-thiouridines, 6-isopentenyl-2-(methylthio)adenosine) are strongly retained on this anion exchanger. The positive charge on 5-(methylaminomethyl)-2-thiouridine ($^2S^*$) may be responsible for its almost total exclusion. This method thus provides an excellent tool for isolation and quantitation of 4-thiouridine (uncontaminated with other uridines), present in almost all tRNAs from *E. coli* [an exception is the *E. coli* glutamate tRNA (Singhal, 1971)].

Interestingly, the 2′-*O*-methyl derivatives of cytidine and

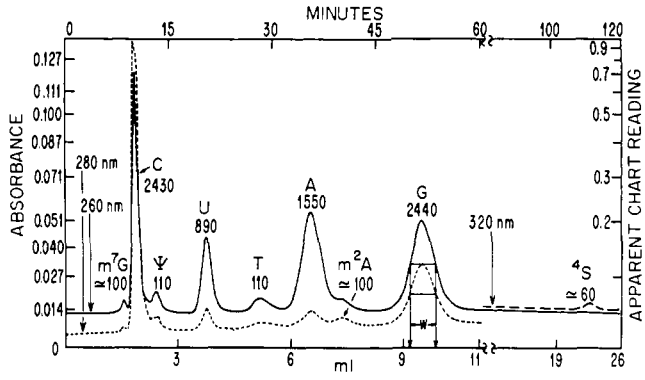

FIGURE 3: Separation of about 4 μg of nucleoside mixture of a purified arginine tRNA (*E. coli*) on Aminex A-25; column, 10.8 cm $\times$ 6.35 mm. A total of 8 nmol (0.08 A_{260} unit at pH 3.75) of nucleosides obtained by enzymatic hydrolysis (pancreatic ribonuclease, snake venom phosphodiesterase, alkaline phosphatase mixture; for details, see Methods) of arginine tRNA at pH 8.7, in 1 μl of solution, without removing the salts or adjusting the pH, was applied to the column and eluted with 0.02 M $HCOONH_4$ + 0.015 M HCOOH, pH 3.75, at 0.57 cm/min or 0.185 ml/min (40 psi pressure), and 50°. For mode of plotting, and symbols used, see legends to Figures 1 and 2. The quantities indicated (in picomoles) for each peak were calculated from their millimolar extinction coefficients at pH 3.75: 14.7 (Ado), 6.75 (Cyd), 11.7 (Guo), 9.95 (Urd), 9.0 (Thd), 7.77 (ψrd), 10.7 (Me^7Guo), and 14.7 (Me^2Ado) at 260 nm; and 17.3 (^{4}Srd) (see Hall, 1971) at 320 nm. The peak labeled m^7G is actually the alkali degradation product of 7-methylguanosine, an isocytosine derivative.

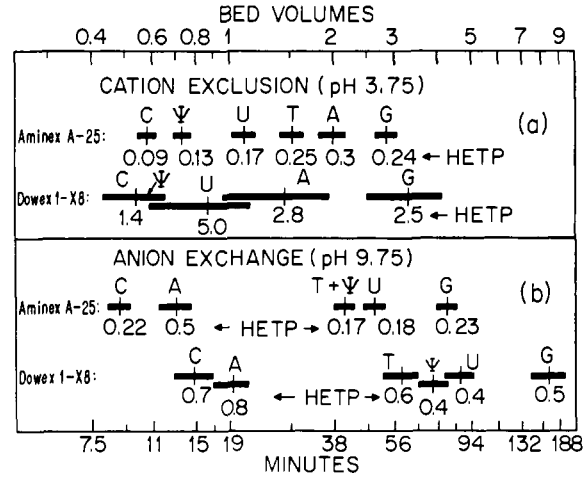

FIGURE 4: Comparison of cation-exclusion and anion-exchange chromatography, and influence of resin bead size and uniformity; Aminex A-25 (17.5 $\pm$ 2 μm) and Dowex 1-X8 (47 $\pm$ 10 μm) in 24 cm $\times$ 5 mm columns; temperature, 50°. Eluents: (a) 0.02 M $HCOONH_4$ + 0.015 M HCOOH, pH 3.75, at 1.5 cm/min; and (b) 0.30 M NH_4OAc + 7% 1-butanol, pH 9.75, at 1.4 cm/min. For mode of plotting and symbols, see legends to Figures 1 and 2.

uridine are slightly separated from their nonmethylated parents by cation exclusion; this is in contrast to no resolution at all of these species by anion exclusion (Aminex A-6, 28 cm $\times$ 6 mm column, unpublished observations). Minor components, like uridin-5-oxyacetic acid and an uncharacterized modified guanosine [which were termed "V" and "Q," respectively, by Nishimura (1972a)], were not eluted even after 5–7 column volumes.

Deoxynucleosides. The four common deoxynucleosides of DNA are well resolved under the conditions chosen for ribonucleosides (Figure 2). However, deoxyuridine is only partially separated ($R_w \leq 1$) from deoxythymidine. A slightly longer column should give better resolution of these two compounds. All five deoxynucleosides were stable under these conditions (pH 3.75, 50°).

Bases. The bottom row in Figure 2 illustrates the complete separation of the four common bases of DNA in 35 min. However, uracil (a major component of RNA) is not well resolved from adenine under these conditions. The separation efficiency of the purine and pyrimidine bases is superior (average HETP, 0.07 mm) to that of ribonucleosides and deoxyribonucleosides (average HETP values, 0.13 and 0.16 mm, respectively) in this system.

Application to Compositional Analysis of a tRNA. The separation of nucleosides obtained from an enzymic hydrolysate of arginine tRNA (*E. coli*) by cation exclusion on an anion exchanger is shown in Figure 3. This particular analysis utilized only 0.08 A_{260} unit (about 4–5 μg) of tRNA. Hence, minute amounts of nucleic acid samples can be isolated and analyzed by employing this analytical system and any sufficiently sensitive monitoring device. It should be noted that more distinct peaks of the minor components may be obtained by simply increasing the input of tRNA components or by increasing the sensitivity of the monitor.

Ion Exclusion and Ion Exchange: A Comparison

Influence of Resin Particle Size. Ion-exclusion chromatography on Aminex A-25 and Dowex 1-X8 (average particle diameters, about 17 and 47 μm, respectively) under identical conditions are presented in Figure 4a. The ion exchanger of larger and more varying particle size (Dowex 1) does not resolve the common nucleosides effectively by cation exclusion (Singhal, 1972b). However, the substances are very well separated ($R_w \gg 1$) on a similar ion exchanger that has one-third the particle diameter and a narrow particle size range (Aminex A-25).

The six common nucleosides were also separated on the two anion exchangers by using the preferred separation conditions for anion-exchange chromatography (Singhal and Cohn, 1972b) as shown in Figure 4b. Again, since cytidine and adenosine are resolved by nonionic phenomena, their separation is markedly improved on smaller particle resins. Similarly, the plate heights (HETP values) for each component and the total analysis times are appreciably reduced with the Aminex material. However, ribothymidine and pseudouridine, satisfactorily resolved (R_w = 0.96) on the larger particle resin, do not separate on Aminex A-25 columns under identical ion-exchange conditions.

Cation Exclusion vs. Anion Exchange. A direct comparison between ion-exclusion and ion-exchange separations (at pH values 3.75 and 9.75, respectively) of the common nucleosides (Figure 2) indicates that ion-exclusion chromatography on columns containing small beads of uniform size is superior for the following reasons: (1) all the six major components are satisfactorily resolved ($R_w \gg 1$ in each case); (2) plate heights of the early appearing, ionically excluded cytidine and other pyrimidines are appreciably smaller than those of similar early peaks in ion exchange; (3) analysis is completed in less eluent volume (3 *vs.* 5 column volumes) and less time (1 hr *vs.* 1.5 hr); (4) the eluent for ion exclusion is volatile, simple, almost free of salt, and requires no organic solvent; (5) the alkali-sensitive minor nucleosides (7-methylguanosine, 1,7-dimethylguanosine, and 1-methyladenosine) are stable in this analytical system (pH 3.75).

Discussion

Ion-exclusion chromatography differs from ion-exchange chromatography on a charged matrix in that no exchange of ions takes place. Whereas conditions for adsorption of the solutes are employed for ion exchange, conditions that repel the solutes from the charged sites of the matrix are used for ion exclusion. The principal condition, pH, is in the range usually used for clearing or stripping a column of absorbed (exchanged) material. Repulsion of ions rather than attraction is deliberately sought. Thus, one uses a "cation exchanger" for anion-exclusion chromatography (Singhal, 1972b; Singhal and Cohn, 1972b) and, in the present instance, an "anion exchanger" for cation-exclusion chromatography.

The fact that a degree of exclusion from the interior of the matrix takes place is shown by the facts that certain substances appear at eluent volumes that are less than the total volume of liquid contained in the column (that is, they appear before [^{3}H]water in the anion-exclusion experiments of Singhal (1972b)) and that the degree of such exclusion is regulated in a predictable manner by regulating the net charge on the ion by pH adjustment. Further evidence is provided by the influence of ionic strength. At first sight, ionic strength may be thought to have no influence on a distribution coefficient determined solely by charge repulsion. However, the Donnan equilibrium that exists between the high concentration of fixed charge within the matrix ($\simeq$1 M) and the low concentration of similarly charged ions in the eluent ($\simeq$0.01 M) indicates that the pH within the matrix may be as much as 2 pH units away from that of the eluent. An ion that penetrates the matrix comes into an environment (liquid medium) differing in pH from that in the external medium by an amount determined by the difference in these two concentrations. Thus, the distribution coefficient of a given ion can be lowered by raising the ionic strength (Singhal, 1972b) but by a mechanism quite different from the mass action effect encountered in ion exchange. In ion exchange, the Donnan effect influences the distribution coefficient oppositely to the mass action effect; in ion exclusion, the latter is not involved.

Another effect of ionic strength may be ascribed to the fact that partition chromatography plays an important and independent role in determining the distribution coefficient of organic compounds on polystyrene matrices. It has long been noted that 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 ionic 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. If partition chromatography were not involved, no substance could be retarded beyond one liquid column volume under ion-exclusion conditions, which is obviously not the case.

In their separations of various saccharides on polystyrene exchangers by elution with 92% ethanol, Larsson and Samuelson (1965) and Jonsson and Samuelson (1967) noted some rather unexpected inversions in the order of elution volumes (distribution coefficients) between anion and cation exchange columns. If only partition chromatography, expected to be the sole governing element, were involved, the relative orders of elution should have remained unchanged. Their conclusion was that "the sorption mechanism is complex and with the data available at present no rigorous theoretical treatment can be given." However, we note that the distribution coefficients on their anion-exchange column increased with the number of hydroxyl groups on the sugars and decreased for the deoxy analogs of particular sugars. Since sugar hydroxyls are acids (albeit weak ones), it is possible that some anion exchange was taking place that modified the partition. The reversals in elution order on their cation-exchange column may then have arisen from exclusion of the same weak anions, again superimposed on simple partition.

Two variables, the size and uniformity of the resin particle, seem to play important roles in both ion-exclusion and ion-exchange chromatography. Small and homogeneous (size, shape) resin beads (*e.g.*, Aminex A-25) are superior to particles of large and varying diameter (*e.g.*, Dowex 1) in resolving complex mixtures of solutes (such as a mixture of the three uridines). Uniformity of size as a critically important factor in ion-exclusion chromatography is apparent in the work of Ehrlich *et al.* (1971), who noted the improvement in nucleoside separations on fine beads of a gel material (Sephadex G-10, which contains free carboxyl groups) when a sharply defined particle-size range replaced the usual mixture of particle sizes. Although not so interpreted by these workers, it seems apparent to us that this was a manifestation of ion-exclusion chromatography as well as of the practical importance of particle size and particle-size range in its application. The degree of cross-linkage and the ionic form of the exchanger are equally important in ion-exclusion separations (Singhal, 1972b).

The plate heights (HETP, a quantitative measure of column efficiency) observed under the conditions of cation-exclusion and anion-exchange chromatography with small and uniform resin beads (Aminex A-25) average about 0.2 mm for the nucleosides. These plate-height values are similar to those found in anion-exclusion (Singhal, 1972b) and superior to those observed in cation-exchange (Uziel *et al.*, 1968) and anion-exchange (Singhal and Cohn, 1972a) separations. The direct comparison between cation-exclusion and anion-exchange chromatography of the model compounds on the same columns demonstrates that ion exclusion on small and uniform exchanger particles is superior and has certain practical advantages.

Cation exclusion, though primarily applied here to nucleic acid components, can similarly be applied for the analysis of constituents of other macromolecules of biochemical importance. The common ribonucleosides in the present study may be regarded as model solutes, being a mixture of several charged and uncharged species. As illustrated in Figure 2, substances other than the major nucleosides—for example, rare nucleosides, deoxynucleosides, and purine and pyrimidine bases—are similarly resolved by this principle. In fact, even various ribonucleotides (2′-, 3′-, or 5′-nucleoside phosphates) that have strong negative charges can be resolved by anion-exclusion chromatography (Singhal, R. P., and Cohn, W. E., manuscript in preparation). Similarly, one may anticipate fractionation of tRNA species and oligonucleotides on a weakly negatively charged matrix, such as carboxymethylcellulose.

Since peak widths are narrow (small plate heights and

sharp peaks) in ion exclusion, sensitivity is enhanced. This is apparent from the example given for the analysis of arginine tRNA (*E. coli*) where about 8 nmol (0.08 A_{260} unit of tRNA) of nucleosides are separated and identified. The nucleoside composition found corresponds to about 22 cytidines, 22 guanosines, 14 adenosines, 8 uridines, 1 residue each of pseudouridine, ribothymidine, 7-methylguanosine, and 2-methyladenosine, and 0.6 mol of 4-thiouridine, for an arginine tRNA molecule reported to contain 75 residues. [7-Methylguanosine is degraded by the alkaline and temperature conditions of the enzymic digestion to an isocytosine derivative (Hall, 1971), with consequent loss of the quaternary ammonium charge that placed it so far ahead of cytidine in Figure 2. The degradation product, which still contains all the original nitrogen atoms, still precedes cytidine (Figure 3), but its formation may be prevented by choosing other hydrolysis conditions (*e.g.*, RNase T_2 and acid phosphatase).] Except for the loss of one cytidine residue and two dihydrouridines, which were not looked for, the composition is almost identical with what would be expected from the sequence recently suggested by Nishimura (1972b) and Murao *et al.* (1972) for this tRNA. The loss of cytidine and 4-thiouridine in some tRNA species appears to be caused by the formation of "cytidine–4-thiouridine adduct" [a pyrimidinone–cytidine symbolized as Pdo(4–5)Cyd (see Bergstrom and Leonard, 1972)] from exposure of the tRNA to light during the course of its isolation (Singhal, R. P., unpublished). A peak having an absorbance at 330 nm that is characteristic of Pdo(4–5)Cyd appears before the 4-thiouridine peak in anion exclusion at pH 9.8 (Singhal, 1972b).

This analysis illustrates the sensitivity of the method. The sensitivity of ion-exclusion chromatography in our hands is comparable to the thin-layer procedure (Randerath and Randerath, 1971) for the analysis of tritium derivatives of ribonucleosides.

In conclusion, ion-exclusion chromatography, which is based upon (1) repulsive forces between a resin surface and similarly charged solutes (ion exclusion) and (2) partition between nonionized solutes and the resin matrix (partition chromatography), can be employed for the analytical or preparative separation of substances of biochemical importance, such as the nucleic acid components employed here, by balancing charges (pH adjustment) and selecting other separation parameters. The resolution and efficiency of the separations achieved by this method appear superior to those obtained by ion-exchange and thin-layer procedures.

Added in Proof

Subsequent to the work reported in this paper, we became aware of two earlier papers in which ion exclusion played a part in effecting the chromatographic separations reported. Seki (1960) effected separations of a variety of phenols on cation exchangers at acid and at alkaline pH values and, although none was eluted earlier than one liquid column volume because of the large partition effects, noted that the order of elution was in general the order of decreasing anionic charge and hence of decreasing repulsion or exclusion. Lange and Hempel (1971) performed similar experiments on a number of aromatic acids, aldehydes, and alcohols but, although their results clearly show that the exclusion principle was operating, concluded that "dissociation cannot be of great importance for this kind of separation." None of these investigators, although noting the relative dissociation constants of their test substances, attempted to capitalize upon these by setting the pH (and other, less important, parameters) at values that would maximize the differences in net charge of the solutes. In most cases, they were operating at pH values far removed from the pK's of the substances in question, so that differential exclusion could not be brought into play.

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