# The Effect of Urea, Formamide, and Glycols on the Secondary Binding Forces in the Ion-Exchange Chromatography of Polynucleotides on DEAE-Cellulose*

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*Received December 17, 1962*

The secondary binding forces affecting the column chromatography of polynucleotides on DEAE-cellulose can be eliminated, for practical purposes, by incorporating urea, formamide, or glycols into the eluting solvent. Thus, in the presence of urea the polynucleotides resulting from the action of pancreatic deoxyribonuclease on salmon testis DNA have been separated according to their degree of polymerization, and characterized. The separation is dependent on their net negative charge and almost independent of their base composition. The nature of the secondary binding forces between polynucleotides and cellulose has been studied by paper chromatography and hydrogen binding has been tentatively assigned the major role.

Progress in structural studies on nucleic acids has been limited by the techniques available for fractionating mixed polynucleotides. At present, only small polynucleotides can be successfully separated. The methods most commonly used to achieve the separations are ion-exchange and paper chromatography, and paper electrophoresis. But these methods are not useful for larger polymers containing a mixture of purine and pyrimidine bases. For example, the heterogeneous polynucleotides obtained by enzymic degradation of nucleic acids have been fractionated on columns of polystyrene anion-exchange resins (Volkin and Cohn, 1953; Sinsheimer, 1952), and more recently on columns of DEAE-cellulose (Staehelin, 1961; Staehelin *et al.*, 1959). In these studies, the selective properties of the adsorbents resulted in the separation of the individual components of the digest up to the tri- and into the tetranucleotide level. Some overlap and coincidence of peaks were noted even with these small polymers, and the separation of higher members proved to be impossible. In the case of polystyrene resins, in addition to the lack of resolution there was the added difficulty of eluting larger polymers.

On the other hand, it had been shown that homopolymers of deoxyribonucleotides could be readily separated even beyond the octanucleotide level by chromatography on DEAE-cellulose (Tener *et al.*, 1958). More recently, this technique has also been used to separate mixed pyrimidine (Spencer and Chargaff, 1961) and mixed purine (Habermann, 1962) deoxyribopolynucleotides resulting from the degradation of apurinic acid and apyrimidinic acid, respectively. Again, fair separation of the higher polymers was achieved.

The inability to separate the larger mixed polynucleotides containing both purine and pyrimidine bases was apparently the consequence of different secondary binding forces between the ion-exchange resin and the purine and pyrimidine bases in the polymers. For studies underway in this laboratory on the chemical structure of nucleic acids, it was considered necessary to develop a technique which would eliminate these secondary binding forces so that the separation of mixed polynucleotides would be independent of their base composition. This paper reports a method for doing this, that is, a method for separating polynucleotides on the basis of their net negative charge. In the accompanying article (Tomlinson and Tener, 1963), we describe an application of this technique for determining the end-groups of nucleic acids.

* This work has been supported by the United States Public Health Service, Grant C-5342. A preliminary report of this work has appeared (Tomlinson and Tener, 1962).

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## EXPERIMENTAL[1]

### *Materials*

Reagent-grade urea (Baker and Adamson) was used; 7 M solutions made from most lots showed negligible optical density (less than 0.02) at 260 mμ, but if a higher absorbance was encountered it could be removed by treating the solution with active charcoal. Strong urea solutions can extract ultraviolet-absorbing materials from rubber tubing and some plastic tubing. Rubber tubing can sometimes be freed of interfering substances by warming for several hours in a 7 M urea solution.

Reagent-grade formamide was used directly. It showed an optical density of over 1 at 260 mμ, but a negligible amount at 271 mμ. DEAE-cellulose was purchased from Brown Company, Berlin, N. H. AE-cellulose (aminoethyl cellulose) powder and DEAE-cellulose paper were manufactured by W. & R. Balston Ltd., England. DEAE-Sephadex was obtained from Pharmacia, Uppsala, Sweden. Tris hydrochloride buffer was prepared by titrating a solution of tris-(hydroxymethyl)aminomethane to the desired *p*H with hydrochloric acid. Optical density measurements were done on a Cary Model 11 spectrophotometer in 1-cm cells. An optical density unit is that amount of material per milliliter which gives a reading of 1 at the designated wavelength.

### *Enzymes*

Pancreatic deoxyribonuclease I (DNase I) and phosphomonoesterase from *Escherichia coli* were obtained from Worthington Biochemical Corp., Freehold, N. J. Phosphorus analyses were performed by the technique of Fiske and SubbaRow (1925).

### *Deoxyribonucleic Acid Digests*

Powdered salmon testis DNA (500 mg) was allowed to hydrate in 70 ml 0.015 M magnesium acetate (*p*H 7.0) overnight at room temperature in the presence of a few drops of chloroform. Then, 0.5 mg of crystalline DNase I was added and the digestion allowed to proceed at room temperature and constant *p*H until complete (3 hours). The reaction was followed by observing the optical density changes of the solution at 271 mμ (a 52% increase) and the amount of sodium hydroxide (total, 1.43 ml 0.1 N) required to neutralize

[1] The following abbreviations have been used: DNase I, pancreatic deoxyribonuclease I; Tris, tris(hydroxymethyl)-aminomethane.

the secondary phosphate groups liberated during the hydrolysis. When no further change was observed, the reaction mixture was heated at 100° for 5 minutes, cooled to room temperature, and filtered. The filtrate was stored at −20° until needed.

*DEAE - Cellulose Columns.*—DEAE - cellulose was freed of fines by repeated settling and decantation from a large volume of water. The settled material was made into a thin slurry by adding 0.5–1.0 M ammonium carbonate (or 1 M sodium chloride) solution (about 20 bed volumes), and poured into a chromatographic column half filled with the same solution. Because the DEAE-cellulose was packed in the salt solution instead of water, the cellulose showed no tendency to mat and a uniform bed could be obtained very rapidly. Excess liquid was allowed to run out the bottom of the column, but sufficient liquid was retained over the bed to allow all air bubbles to escape while adding the slurry of DEAE-cellulose. Air pressure of about 5 lb psi was used to pack the column, with fresh additions of slurry being added until the desired bed depth was attained. The column was washed with 2 M ammonium carbonate (or 4 M sodium chloride) until the optical density of the eluate at 260 mμ was zero (about 10 bed volumes), and then with distilled water (about 5 bed volumes) until all the salt had been removed. If the column was to be used in the acetate form it was washed with 1 M sodium acetate until all the other anion was removed, and then with an additional 3 bed volumes of 1 M sodium acetate (*p*H 7.5 or *p*H 4.8), followed by distilled water until the effluent was salt free. Columns were extruded and repacked before each run. If they were used again directly their flow rates became very poor.

*Chromatographic Separation*

(a) EXPLORATORY COLUMNS.—Preferred procedure. (Some graphs show earlier variations of the method involving carbonate and acetate as eluting anions.) An aliquot of the DNase I digest containing 216 OD units (271 mμ) of polymer (*ca.* 24 μmoles of phosphorus) in 3 ml was added to a column (20 × 1.8 cm) of DEAE-cellulose (chloride) and washed in with a small amount of water. The elution of the column was then begun with a linear gradient (Parr, 1954) formed by 1 liter of 7 M urea to which 25 ml 0.1 M Tris hydrochloride *p*H 7.8 had been added (the urea affects buffers somewhat), and 1 liter of 0.3 M sodium chloride plus 7 M urea to which 25 ml of the buffer had also been added. When the column was to be run at an acid *p*H, 25 ml 0.1 N sodium acetate *p*H 4.7 was substituted for the Tris buffer.

The flow rate was 1 ml per minute, and aliquots were collected in a time-flow fraction collector and their optical density read at 271 mμ (where all four deoxyribonucleotides show molecular extinctions [average 9100] within 10% of each other).

(b) PREPARATIVE COLUMNS.—Improved separation of larger polymers was achieved by using a longer column and a more gradual gradient. For example, a digest of 0.5 g of DNA was run onto a column (3.5 × 100 cm) of DEAE-cellulose and eluted with a linear gradient formed by 8 liters 7 M urea containing 250 ml 0.1 M Tris hydrochloride, *p*H 7.8, and 8 liters 0.3 M sodium chloride, 7 M in urea, and also containing 250 ml of the same buffer.

*Isolation Procedure* (cf. Rushizky and Sober, 1962);

Fractions containing a peak were pooled, diluted with 3 to 5 volumes of water, and adjusted to about *p*H 8, if necessary. They were next run onto a column of DEAE-cellulose (carbonate) (20 × 2.4 cm for 0.2 mmoles of polymer), and the column was washed well with water and then with 0.02 M ammonium carbonate (*p*H 8.4) until chloride ion (silver nitrate test) was absent from the effluent. The optical density of the washings was checked to ensure that no nucleotide was eluted. The polymer was then eluted with 2 M ammonium carbonate and the effluent concentrated to dryness *in vacuo* with a bath temperature below 30°. Water was added to the residue and the solution again concentrated to dryness. The process was repeated until no ammonium carbonate remained; usually three evaporations were required.

*Enzymic Digestion*

(a) PHOSPHOMONOESTERASE. — An excess of *E. coli* phosphomonoesterase was added to the samples in 0.01 M Tris buffer, *p*H 8.0, 0.01 M in magnesium chloride. Digestions were carried out for 12 hours at 37°. A drop of chloroform was added routinely to all enzymic incubation mixtures, with both phosphomono- and phosphodiesterase, to eliminate the hazard of bacterial contamination. Inactivation of the phosphomonoesterase prior to phosphodiesterase treatment of the samples was accomplished by passing the incubation mixtures through a Dowex 50 (ammonium) resin column, concentrating them *in vacuo*, and then heating them for 1 hour on a boiling water bath.

(b) SNAKE VENOM PHOSPHODIESTERASE.—This enzyme was prepared by the method of Koerner and Sinsheimer (1957) from commercial lyophilized snake venom (*Crotalus adamateus*). The purified product showed no detectable phosphomonoesterase activity under the conditions used to degrade the polynucleotides in the peaks. The degradation was carried out by dissolving a sample of each polymer from (a) in 0.2 M ammonium bicarbonate buffer, *p*H 7.6, 0.01 M in magnesium chloride. Then an aliquot of the snake venom phosphodiesterase was added to each and the mixtures were incubated for 24 hours at 37°. No polynucleotides remained.

(c) SPLEEN PHOSPHODIESTERASE.—Worthington's spleen phosphodiesterase was further purified by the method of Razzell and Khorana (1961). No detectable phosphomonoesterase activity was present in the purified product under the conditions described for its use. The remaining samples from (a) were each dissolved in 0.2 M ammonium acetate, *p*H 5.4, 0.01 M in ethylenediaminetetraacetic acid and 0.01 M in Tween 80. An excess of spleen phosphodiesterase was added to each and the mixtures were incubated for 24 hours at 37°. These conditions were sufficient to completely degrade the polynucleotides.

*Ratio Determinations*

The incubation mixtures from (b) and (c) were resolved into nucleoside fractions and nucleotide fractions by column chromatography on DEAE-cellulose in the carbonate form.

*Paper Chromatography*

Whatman 1 or Whatman 40 paper was used in a descending technique. DEAE-cellulose paper was washed in several changes of Tris buffer *p*H 7.5 until the *p*H remained constant, and then with water until salt free. The paper was dried before use.

## RESULTS

Initial chromatographic separations were conducted with DEAE-cellulose (carbonate) columns, and ammonium carbonate in the eluting system. Results of these studies are shown in Figure 1. The elution pattern of a digest of DNA in 0, 4 M, and 8 M urea are shown. The marked effect of the urea on the elution pattern can be noted.

The successive peaks (Fig. 1c) contain, respectively, mononucleotides (tubes 31–40), dinucleotides

TABLE I
ANALYSES OF POLYNUCLEOTIDE FRACTIONS

| Peak | Fraction Nos. | $\epsilon$/P[a] | Total Phosphorus Solidus Monoesterase Phosphorus | Nucleotide/ 5'-Nucleoside | Nucleotide/ 3'-Nucleoside |
|---|---|---|---|---|---|
| Mono | 32–37 | 10,000 | 1.2 | — | — |
| Di | 44–53 | 8,130 | 2.1 | 1.2 | 1.5 |
| Tri | 55–68 | 7,880 | 3.3 | 1.9 | 2.1 |
| Tetra | 72–85 | 7,210 | 4.0 | 3.2 | 2.8 |
| Penta | 87–101 | 6,970 | 5.1 | 4.2 | 4.5 |
| Hexa | 103–117 | 6,730 | 6.3 | 5.2 | 5.0 |
| Hepta | 119–131 | 6,770 | 7.4 | 6.5 | 5.7 |
| Higher | 132–150 | 6,610 | 8.1 | 7.1 | 7.0 |

[a] $\epsilon$/P: optical density at 271 m$\mu$ per mole of phosphorus.

(tubes 43–54), trinucleotides (tubes 55–69), and so forth, that is, each peak differs from its neighbors by one negative charge. The characterization of each peak was achieved through the following observations: (a) the order of elution from the column, (b) the ratio of phosphomonoesterase-sensitive phosphorus to total phosphorus, (c) the ratio of nucleoside from the 5'-hydroxyl end or the 3'-hydroxyl end to nucleotide obtained by treating a peak with phosphomonoesterase followed by snake venom or spleen phosphodiesterase, and (d) the decreasing $\epsilon$/P (optical density per phosphorus) value with increasing size. The results are summarized in Table I.

Additional evidence which shows that components of the nuclease digest are separated on the basis of their net charge is found in the accompanying article (Tomlinson and Tener, 1963). When a peak is treated with phosphomonoesterase and rechromatographed, it emerges from the column at about the same place as polymers containing two nucleotide residues less.

In Figure 1c, the *p*H of the eluting solution was initially about 8.4, but increased to about 9.0 as the elution proceeded, because of loss of carbon dioxide. This *p*H change of ammonium as well as potassium bicarbonate solutions is particularly noticeable in the

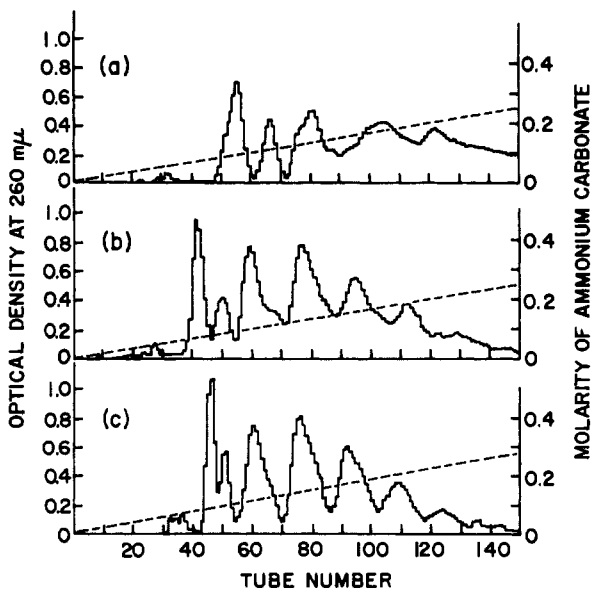


FIG. 1.—The separation of components of a DNase I digest (550 OD units at 260 m$\mu$) of salmon testis DNA on a DEAE-cellulose (carbonate) column (35 × 2.4 cm); eluting solution, a linear gradient of ammonium carbonate (*p*H 8.4) containing (a) no urea, (b) 4 M urea, and (c) 8 M urea. Fraction size, 18 ml.

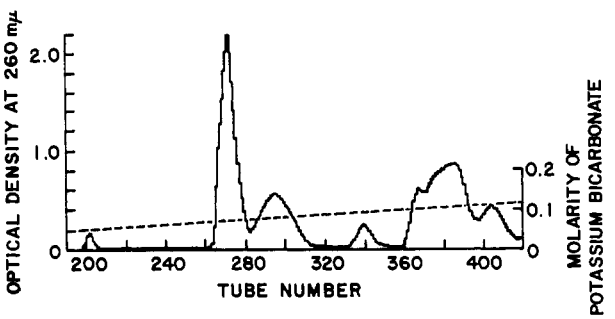


FIG. 2.—Separation of mono-, di-, and trinucleotides from 4000 OD units (260 m$\mu$) of the DNase I digest on a DEAE-cellulose (carbonate) column (69 × 3.4 cm) with a linear gradient 7 M in urea with potassium bicarbonate as shown. Fraction size, 22 ml. See text for discussion.

presence of urea, even in a closed system under air pressure. As a consequence of this loss the exact salt concentration in the eluting solutions was not known and, in addition, the higher *p*H was sufficient to ionize the ring hydroxyl group (*pK* 9.38) of guanylic acid residues. The small peak (tube 51) which came between the di- and trinucleotide peaks was found to be dinucleotides containing at least one guanylic acid residue. Figure 2 shows a partial elution curve at the dinucleotide level from a large column using 8 M urea and an increasing potassium bicarbonate (*p*H 8.9) gradient. The small peak (tube 340) in front of the trinucleotide is deoxyguanylyldeoxyguanylic acid, and the intermediate sized peak (tube 295) contained dinucleotides with one deoxyguanylic acid residue.

Figure 3 shows the chromatographic separation of the DNase I digest in the absence and presence of 7 M urea with sodium acetate (*p*H 7.5) as the eluting

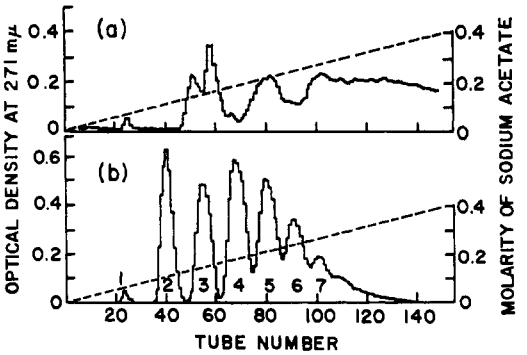


FIG. 3.—Separation of 200 OD units (271 m$\mu$) of the DNase I digest on a DEAE-cellulose (acetate) column (20 × 1 cm) with a linear gradient of sodium acetate (*p*H 7.5) with (a) no urea, (b) 7 M urea. Fraction size, 9.6 ml.

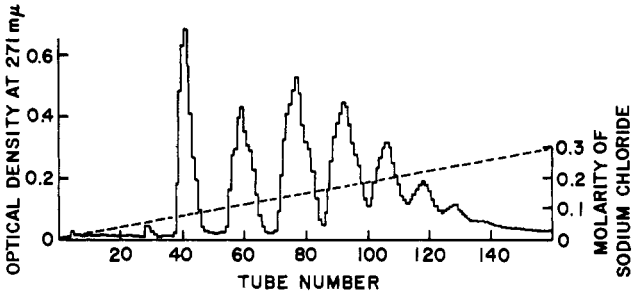


FIG. 4.—Separation of 216 OD units (271 mμ) of DNase I digest on a DEAE-cellulose (chloride) column (30 × 1.8 cm) with a linear gradient formed by mixing 1 liter of 7 M urea, 0.3 M in sodium chloride and containing 25 ml 0.1 M sodium acetate buffer *p*H 4.7, and 1 liter of 7 M urea containing 25 ml of the same buffer. Fraction size, 12 ml.

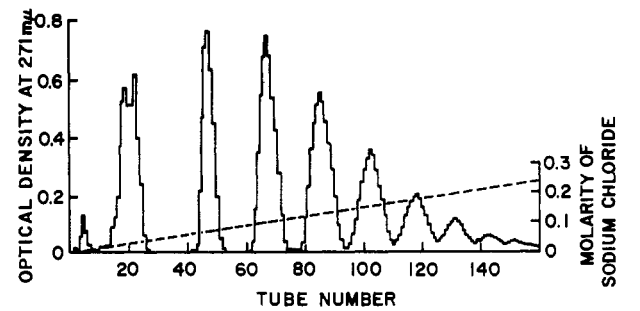


FIG. 5.—Separation of 216 OD units (271 mμ) of DNase I digest that had been dephosphorylated with phosphomonoesterase, DEAE-cellulose (chloride) column (30 × 1.8 cm). Eluting solution, same as in Fig. 4 except *p*H 5.0 buffer was used. Fraction size, 11 ml.

solution. The use of 7 M instead of 8 M urea, as in Figures 1 and 2, gave equally good separation of peaks while minimizing crystallization of urea in the eluent. It is now used routinely. Acetate proved to be more satisfactory than carbonate for separating the various polynucleotides because the *p*H of solutions could be controlled at lower values. However, more recently we have used buffered sodium chloride solutions for the elutions, since chloride not only gives a satisfactory elution pattern but also its removal can be readily checked during the isolation procedure. Figures 4 and 5 are chromatographic elution patterns with sodium chloride as the eluting salt.

In addition to the ionization of guanylic acid ring hydroxyl groups, other effects of *p*H are noted. Figure 4 shows an elution curve at *p*H 4.7, at which value the secondary phosphate dissociation in the molecules is largely repressed. The peaks emerge at a lower salt concentration than at *p*H 7.5—at the concentrations expected for polymers carrying approximately one less charge. The spectra of individual tubes within the trinucleotide peak showed that some separation of components was being effected. For example, in the trinucleotide peak, tube 56 had a $\lambda_{max}$ at 268 mμ, and tube 65 at 258 mμ. This partial separation within a peak was also noticed with columns run at *p*H 7.5.

Phosphomonoesterase was used to remove the terminal phosphate residues from the polymers in the DNase I digest. When these dephosphorylated polynucleotides were chromatographed in the urea systems on DEAE-cellulose, the elution pattern shown in Figure 5 was obtained. Peaks were eluted at much lower salt concentration than the corresponding phosphorylated derivatives, and excellent separation of higher members was achieved. Dinucleoside phosphates are not completely held by the DEAE-cellulose column under the conditions routinely used for desalting. Therefore, adsorption onto active charcoal was used to recover this fraction.

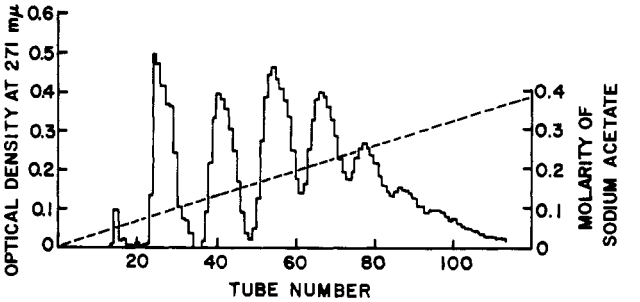


FIG. 6.—Separation of 200 OD units (271 mμ) of the DNase I digest on DEAE-cellulose (acetate) column (20 × 1 cm); eluting solution, a linear gradient of sodium acetate, *p*H 7.5 and 8 M in formamide as shown. Fraction size, 13 ml.

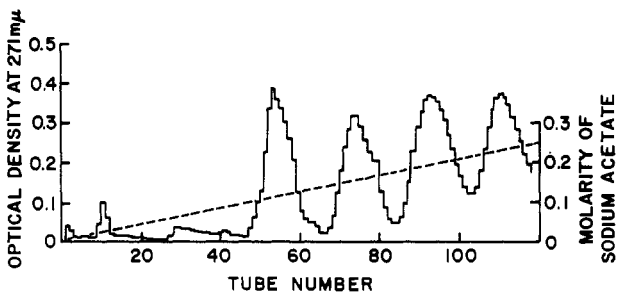


FIG. 7.—Partial separation of 200 OD units (271 mμ) of the DNase I digest on a DEAE-cellulose (acetate) column (20 × 1 cm); eluting solution, a linear gradient of sodium acetate, *p*H 7.5 and 8 M in ethylene glycol. Fraction size. 10.5 ml.

Other reagents have been used in place of urea to overcome secondary binding forces between DEAE-cellulose and polynucleotides. Figure 6 shows the elution curve obtained from the DNA digest when the eluting solution contains 8 M formamide. The picture is similar to that with 8 M urea. Glycols can also be used in the eluting systems. Figure 7 is an elution curve of the DNA digest with 8 M ethylene glycol in the eluting solvent. A somewhat similar pattern is obtained with 8 M propylene glycol. The individual peaks are not as well separated, and they are eluted at higher salt concentrations. One disadvantage of the glycol-containing systems is the greater viscosity of the solutions and thus slower flow rates through the columns. Propylene glycol is particularly poor on this account. But glycols and formamide do offer an advantage over urea in those cases where low temperatures are required. Urea will crystallize from 7 M solutions at 0°, whereas the glycol and formamide solutions remain homogeneous.

The $R_F$ values of deoxyribonucleosides on Whatman 1 paper with various solvents are shown in Table II. With water as the developing solvent, the nucleosides did not move at the front but were considerably retarded. When the temperature of the chromatographic system was lowered to 4° the purine deoxyribonucleosides moved more slowly. Seven molar urea and 4 M sodium trichloroacetate pushed the nucleosides near the solvent front. Four molar sodium chloride had little effect on their migration, 75% methanol did not affect their $R_F$ values, and pure methanol retarded their movement somewhat.

When DEAE-celulose paper was used (Table III) somewhat similar results were noted.

TABLE II

$R_F$ VALUES OF DEOXYRIBONUCLEOSIDES ON WHATMAN 1 PAPER

| Nucleoside[a] \ Solvent | Water, 22° | Water, 4° | 7 M Urea | 4 M Sodium Trichloroacetate | 4 M Sodium Chloride | 75% Methanol | Pure Methanol |
|---|---|---|---|---|---|---|---|
| TdR | 0.79 | 0.76 | 0.94 | 0.97 | 0.82 | 0.74 | 0.63 |
| CdR | 0.77 | 0.76 | 0.92 | 0.97 | 0.75 | 0.72 | 0.55 |
| AdR | 0.53 | 0.45 | 0.89 | 0.96 | 0.41 | 0.55 | 0.35 |
| GdR | 0.60 | 0.46 | 0.89 | 0.96 | 0.53 | 0.53 | 0.36 |

[a] The abbreviations TdR, CdR, AdR, and GdR are used for the deoxyribosides of thymine, cytosine, adenine, and guanine, respectively

TABLE III

$R_F$ VALUES OF DEOXYRIBONUCLEOSIDES ON DEAE-CELLULOSE PAPER

| Nucleoside[a] \ Solvent | Water, 22° | 7 M Urea | 4 M Sodium Trichloroacetate |
|---|---|---|---|
| TdR | 0.82 | 0.95 | 0.86 |
| AdR | 0.68 | 0.93 | 0.80 |
| CdR | 0.81 | 0.97 | 0.83 |
| GdR | 0.63 | 0.96 | 0.80 |

[a] The abbreviations TdR, CdR, AdR, and GdR are used for the deoxyribosides of thymine, cytosine, adenine, and guanine, respectively.

## DISCUSSION

Preliminary evidence indicates that the major secondary binding force between polynucleotides and DEAE-cellulose is hydrogen bonding. Hydrophobic interaction, which plays such an important role in chromatography of polynucleotides on polystyrene ion-exchange resin (Cohn, 1961; Tener *et al.*, 1958), does not seem to be an important factor in their separation on DEAE-cellulose.

The secondary binding force between cellulose and purine and pyrimidine derivatives can be readily demonstrated by noting the $R_F$ values of nucleosides on paper chromatograms where electrostatic binding forces are absent (Table II). If no interaction occurred between the cellulose and the nucleosides, the nucleosides would have moved with the solvent front. Actually, their $R_F$ values in water on Whatman 1 paper are considerably less than one at 22° and even smaller at 4°. This effect is particularly noticeable with purine deoxyribosides. Further, some solutions which disrupt the forces maintaining the secondary structure of various macromolecules (Kauzmann, 1959) also have a profound effect on the $R_F$ values of nucleosides. For example, 7 M urea (Rice and Doty, 1957) and 4 M sodium trichloroacetate (cf. Hamaguchi and Geiduscheck, 1962) move the nucleosides very near the front—actually at a urea and a salt front, respectively. On the other hand, neither 75% nor pure methanol increased the $R_F$ values of the nucleosides. In fact, with pure methanol, the values decreased with respect to water. This effect and the decrease in $R_F$ values with decreasing temperature indicate that hydrogen bonding is much more important than hydrophobic bonding in this interaction between nucleosides and cellulose.

The $R_F$ values for nucleosides on DEAE-cellulose paper differ only slightly from those obtained on Whatman 1 paper (Table III). The somewhat hydrophobic diethylaminoethyl ether groups, therefore, have little effect on the binding forces between nucleosides and cellulose. Here again, 7 M urea and 4 M sodium trichloroacetate moved the nucleosides to the front. Also, in column chromatography of polynucleotides on DEAE-cellulose, neither 75% methanol nor 7 M ethanol overcame secondary binding forces, but 7 M urea, 8 M formamide, and 8 M ethylene and propylene glycol did.

In studies on the effect of urea on hydrogen bonding, it was concluded that urea is not a good hydrogen bond breaker in aqueous solutions (see Kauzmann, 1959; Levy and Magoulas, 1962), and its role in disrupting secondary structure of proteins is primarily one of disrupting hydrophobic bonds. Scheraga *et al.* (1962) have more recently pointed out that thermodynamic data do not support the idea that hydrophobic bonds are the sole force contributing to the thermal stability of protein conformations, and also that urea may produce a variety of effects on molecules in solutions (Steinberg and Scheraga, 1962). However, since the secondary forces which bind polynucleotides to DEAE-cellulose were strongly affected by urea (and formamide), but not by alcohol, it would appear likely that the major secondary binding force is hydrogen bonding. Further studies are needed to confirm this proposal.

A factor which will enhance hydrogen bonding (and other secondary forces) is the electrostatic interaction between polynucleotides and DEAE-cellulose. Since it was apparent from the paper chromatographic studies that purine and pyrimidine bases are primarily responsible for the interaction between nucleosides and cellulose, the amount of the cellulose-nucleoside complex will depend on the type and concentration of base residue present. In the chromatography of polynucleotides on DEAE-cellulose, the electrostatic forces increase the effective concentration of the bases in the vicinity of the cellulose, and thus would be expected to increase the secondary binding forces. An additional consequence of electrostatic interaction is that these secondary binding forces would be expected to differ for various isomers of a nucleotide because each would be bound with a different orientation for its base residue. Thus it is possible to explain the separation of adenosine-2′- and 3′-phosphates (Staehelin, 1961) at an alkaline *p*H where the phosphate groups are completely ionized.

Another binding force which could be involved in these separations is the interaction of the Watson and Crick type between polymers adsorbed onto the DEAE-cellulose by electrostatic forces and those moving down the column. No evidence is available to suggest the importance of this type of binding, but urea solutions would be expected to eliminate it. Bautz and Hall (1962) and Gilham (1962) have recently reported chromatographic systems making use of this binding force for the separation of nucleic acids and polynucleotides.

For practical purposes it is apparent that the use of urea does allow mixtures of ribo- (Tomlinson and Tener, 1962) or deoxyribopolynucleotides to be separated according to the number of net negative charges on each polymer, and independently of their base composition. The suppression of secondary binding

forces is not absolute, however, since the leading and tailing edges of each peak have different spectra, and on columns of high resolving power some separation within a peak becomes evident (cf. Fig. 5). It can be noted, too, that at *p*H values where a polymer has partially ionized groups it will separate as if it had a fractional charge (cf. Fig. 2), and will be eluted between peaks of unit charge.

Preliminary studies have been done on the use of urea solutions in the separation of polynucleotides on other anion-exchangers. AE-cellulose (carbonate) columns did not give a good separation of polymers of the DNase I digest of DNA when the elution was carried out with ammonium carbonate. The incorporation of 8 M urea into the eluant did not improve the separation. On the other hand, the use of urea did improve somewhat the separation of mixed polymers on DEAE-Sephadex A-25 (carbonate), but higher salt concentrations were required to elute the peaks than were required with DEAE-cellulose. However, it should be pointed out that chromatography of polynucleotides on DEAE-Sephadex is not a simple ion-exchange process. For example, we have found that mixed thymidylic acid polymers prepared by the dicyclohexylcarbodiimide method (Tener *et al.*, 1958) can be readily separated on DEAE-Sephadex A-25 into discrete peaks by an increasing gradient of ammonium carbonate. When an individual peak from this column was isolated and rechromatographed on DEAE-cellulose (carbonate), again with an ammonium carbonate gradient, two or more peaks resulted and the major peaks were found to be the linear and corresponding cyclic nucleotide. This procedure has proven to be very useful for purifying these polymers, and it does show that their separation on DEAE-Sephadex is not necessarily a function of their ionic charge but is dependent on their size.

No evidence has been obtained for a reaction between polynucleotides and ammonium cyanate which forms slowly by breakdown of urea (Stark *et al.*, 1960).

## References


Bautz, E. F. K., and Hall, B. D. (1962), *Proc. Nat. Acad. Acad. Sci. U. S. 48*, 400.

Cohn, W. E. (1961) *in* Chromatography, Heftman, E., ed., New York, Reinhold, p. 554.

Fiske, C. H., and SubbaRow, Y. (1925), *J. Biol. Chem. 66*, 375.

Gilham, P. T. (1962), *J. Am. Chem. Soc. 84*, 1311.

Habermann, V. (1962), *Biochim. Biophys. Acta 55*, 999.

Hamaguchi, K., and Geiduscheck, E. P. (1962), *J. Am. Chem. Soc. 84*, 1329.

Kauzmann, W. (1959), *in* Advances in Protein Chemistry, vol. 14, New York, Academic, p. 1.

Koerner, J. F., and Sinsheimer, R. L. (1957), *J. Biol. Chem. 228*, 1049.

Levy, M., and Magoulas, J. P. (1962), *J. Am. Chem. Soc. 84*, 1345.

Parr, C. W. (1954), *Biochem. J. 56*, xxvii.

Razzell, W. E., and Khorana, H. G. (1961), *J. Biol. Chem. 236*, 1144.

Rice, S. A., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 3937.

Rushizky, G. W., and Sober, H. A. (1962), *Biochim. Biophys. Acta 55*, 217.

Scheraga, H. A., Nemethy G., and Steinberg, I. Z. (1962), *J. Biol. Chem. 327*, 2506.

Sinsheimer, R. L. (1952), *J. Biol. Chem. 208*, 445.

Spencer, J. H., and Chargaff, E. (1961), *Biochim. Biophys. Acta 51*, 209.

Staehelin, M. (1961), *Biochim. Biophys. Acta 49*, 11.

Staehelin, M., Sober, H. A., and Peterson, E. A. (1959), *Arch. Biochem. Biophys. 85*, 289.

Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.

Steinberg, I. Z., and Scheraga, H. A. (1962), *J. Am. Chem. Soc. 84*, 2890.

Tener, G. M., Khorana, H. G., Markham, R., and Pol, E. H. (1958), *J. Am. Chem. Soc. 80*, 6223.

Tomlinson, R. V., and Tener, G. M. (1962), *J. Am. Chem. Soc. 84*, 2644.

Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 703(this issue).

Volkin, E., and Cohn, W. E. (1953), *J. Biol. Chem. 205*, 765.