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DIFFERENCES IN THE BEHAVIOR OF THE PURINE AND PYRIMIDINE BASES ON DEXTRAN COLUMNS AT DIFFERENT pH VALUES*

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SUMMARY

When the carboxyl groups of a highly cross-linked dextran gel are converted to their acid form, the gel can act as a cation exchanger towards purines and pyrimidines containing basic groups, and as a result the distribution coefficients of such compounds are markedly increased over those values obtained on neutral dextran. At alkaline pH's these same carboxyl groups interact in an opposite manner with the acidic groups of purines and pyrimidines to lower their distribution coefficients. Choosing a proper balance between the charge on the matrix of the gel, the charges of the compounds themselves, and the adsorptive effect of the compounds for the ether groupings of the dextran gel makes possible the analytical separation of purines and pyrimidines at acidic or alkaline pH's.

Desalting of the purine and pyrimidine bases can be accomplished on dextran columns at neutral pH, since many of the common salts such as chlorides, acetates, formates, phosphates, and iodides precede the bases as a sharp and distinct band.

INTRODUCTION

While examining highly cross-linked inert gels for desalting of nucleotides, UZIEL AND COHN[1] observed a partial separation of nucleosides and bases as well as a separation of salts from some of these compounds. Polyacrylamide gels and cross-linked dextrans have also been used by investigators[2–6] as chromatographic materials for the separation of purines and pyrimidines.

This present investigation is concerned with both the desalting and the analytical separations of purines and pyrimidines on a highly cross-linked dextran material (Sephadex G-10). In the latter application the small number of carboxyl groups contained on the Sephadex matrix[7] (see also Pharmacia literature) is used to advantage in making these separations. At alkaline pH's these negative charges account, in part, for the sharp separations of uracil (U), cytosine (C), guanine (G), and adenine (A). These same carboxyl groups act in a different manner if the dextran is pretreated with acid. Under this condition, large increases are noted for the distribution co-

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efficients (K_D) of C, A, and G compared to those determined on a column of neutral Sephadex. As a consequence of these changes in K_D, the separation of purines and pyrimidines can also be accomplished at acidic pH's. In this report these differences in the behavior of purines and pyrimidines at different pH's on columns of acidic, neutral, and alkaline Sephadex are discussed in terms of gel filtration, adsorption, and the charge effect or lack of charge of the compounds themselves as interacting with the anionic charge of the dextran matrix.

Desalting of purine and pyrimidine samples is accomplished on "neutral" Sephadex columns. Common salts such as acetates, formates, phosphates, iodides, and chlorides form sharp and distinct bands that precede the water elution of purines and pyrimidines from the G-10 columns.

EXPERIMENTAL

All the analytical separations and the majority of the desalting experiments were carried out on Sephadex G-10 (obtained from Pharmacia) contained in columns having bed dimensions of 1.5 × 86 cm. The maximum sample volume applied to this size column was 5 ml; in a few of the desalting experiments, a sample volume of 25 ml was applied, with the aid of a Sephadex flow adaptor, to G-10 columns having bed dimensions of 2.5 × 180 cm. The Sephadex was swollen in water for at least 1 h before the columns were packed. The gel slurry was added intermittently as excess water was drained from the bottom of a column. Over fifteen column volumes of water were percolated through the gel bed at a rate of about 1 ml/min before a column was used for an experiment.

Sephadex packed in this manner was considered a neutral dextran. Acidic dextran was prepared by passing about five column volumes of 0.01 *M* hydrochloric acid through the gel beds of these previously water-packed neutral columns; before use, excess acid was removed with a water rinse until the effluents had a neutral pH. Alkaline gel beds were prepared by equilibrating a neutral dextran column with alkaline buffer solutions.

The amount of each purine or pyrimidine added to these columns ranged from 2–5 μmoles. However, the concentration of guanine* in a sample introduced to a column was never greater than 0.4 μmoles/ml and upon subsequent elution its concentration was usually reduced by a factor of about ten. At these dilute concentrations and with the flow rates employed here, guanine was always recovered quantitatively, in contrast to the findings reported by BRAUN[2].

In the desalting experiments salts were present in samples at an initial concentration of approximately 1 molar. To avoid the fractionation of the conjugate acids and bases of buffers[1], the pH of these samples was adjusted to 7.0 prior to their desalting by a dextran column. The presence of chloride in column fractions was detected qualitatively by the silver nitrate test and quantitatively by titration with mercuric nitrate[8]. Purines, pyrimidines, Dextran Blue (Pharmacia), and hemoglobin were monitored qualitatively at 254 nm with an ISCO ultraviolet analyzer, Model UA, and measured quantitatively in a Model DB Beckman spectrophotometer at

* A 2-m*M* solution of guanine can be prepared at pH 12. Supersaturated solutions arise as the pH is lowered. From such solutions guanine precipitates erratically with time.

260 nm after 15-ml fractions had been collected; acetates, formates, and the iodate ions were assayed by the latter method but at a wavelength of 230 nm.

Distribution coefficients were calculated from the following equation[7,9]:

$$K_D = \frac{V_e - V_0}{V_1}$$

where V_e is the elution volume of a solute, V_0 is the void volume of the dextran column, and V_1 is the internal volume of the gel beads. In these experiments it is assumed that sodium chloride has a K_D of 1; hence, the V_e for sodium chloride gives the value for $V_0 + V_1$. Both Blue Dextran and hemoglobin[9] (about 10 mg/ml in 0.1 *M* NaCl), used interchangeably, gave the same value in determinations of V_0. The difference between the V_e for sodium chloride and V_0 yielded the value for V_1.

RESULTS AND DISCUSSION

Desalting and distribution coefficients for neutral dextran columns

Fig. 1 shows the elution curves for sodium chloride and hemoglobin, from which the column constants were determined. Also given in this figure are the elution volumes and the K_D values for U, C, G, and A as determined on neutral Sephadex. The fact that the four bases have K_D values greater than 1 indicates that in addition to the sieve effect of gel filtration an additional mechanism is also acting during the elution of these compounds from the dextran gel. This additional adsorptive effect

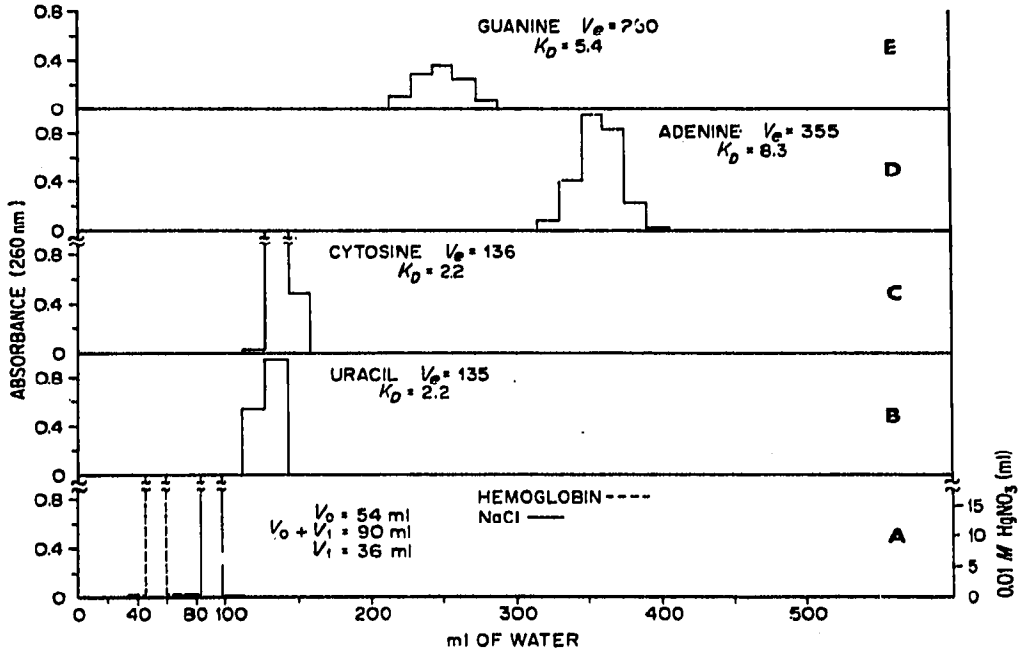

Fig. 1. Elution data from a neutral dextran column (1.5 × 86 cm) from which the distribution coefficient of purines and pyrimidines is calculated. Curve A: Determination of column constants, as shown, using hemoglobin and NaCl from a 5-ml sample volume (Cl^- determined by titration with mercuric nitrate as indicated on the right-hand ordinate). Curves B–E: The four bases sorbed separately from 5-ml sample volumes containing water or salts at pH 7; all elutions were made with water at a flow rate of about 1 ml/min.

had been noticed earlier by GELOTTE[7] for a number of heterocyclic and aromatic compounds including several purines and pyrimidines. Adsorption onto the gel matrix has been effectively used by SWEETMANN AND NYHAN[6] to separate nucleosides and various purines and pyrimidines as well as their methylated isomers on Dextran G-10 gels. DETERMANN AND WALTER[10] propose that in Sephadex gels it is ether groupings that interact with aromatic and heterocyclic compounds, causing them to be retained longer than expected on a dextran matrix. As will be shown in later discussions in addition to the sieve action of gel filtration and this adsorptive effect, the charge of the dextran matrix and the charge of ionized compounds play important roles in the elution behavior of the purines and pyrimidines at pH's other than in the neutral range.

The elution position of these four bases on neutral Sephadex does not change significantly, regardless of the presence or absence of salts in the 5-ml sample volumes. When buffering substances are present, it is necessary to adjust the pH of the sample to 7 before it is applied to the column. Otherwise, large variations (due to the fractionation of conjugate acids and bases)[1] in pH occur in the region of the salt breakthrough. In the case of acetate and formate buffers, this phenomenon can cause erratic elution profiles for cytosine when such buffers contained in samples are at a pH of between 3 and 6; at these pH's double peaks have been observed for cytosine.

Neutral acetates and formates have the same V_e ($\sim$90 ml) that was found for chloride, whereas sodium iodate tends to be eluted slightly ahead of these salts. Although not studied here, HOHN AND POLLMANN[3] have found that the lithium, iodide, and phosphate ions are not retarded on Sephadex columns and consequently were also assigned a K_D equal to unity.

Chromatography and distribution coefficients determined on acidic dextran columns

Fig. 2 shows that uracil elutes in the same position on acidic Sephadex as on a neutral G-10 column (see Fig. 1). However, the K_D of G changes from 5.4 to 8.9, and even greater changes are noted for C and A; the K_D values of these compounds change, respectively, from 2.2 and 8.3 to K_D values of > 15 for C and a value > 23 for A. The mechanism involved in changing these K_D values is probably that of ion exchange. Sephadex has some carboxyl groups ($\sim$0.01 mequiv./g)[7] throughout its three-dimensional dextran matrix. At low pH, these carboxyl groups would be in their acid form and would stay that way even if a column were water washed. Such a column would then act like a cation exchanger and hence retain A, C, and G, whose basis groups attract a proton from the carboxyl group and become positively charged and then become ionically bound to the now negatively charged carboxyl groups. U, on the other hand, does not have any basic group and consequently elutes in the same position on either neutral or acidic dextran columns. This difference in retention of the four bases suggests that analytical separations would be possible on acidic dextran columns. However, this can be accomplished only if a delicate balance is made between the adsorptive effects of the bases and their attraction to ion exchange. The bottom curve of Fig. 2 shows such a separation in which A is eluted ahead of G; the elution position of these two purines is reversed on neutral Sephadex columns. If 0.001 *M* acetic acid at pH 4 is used throughout the entire elution, U and C separate by a wide margin, but A tends to regain the position it holds on neutral Sephadex; and the result is that A and G are eluted together. If 0.01 *M* acetic acid is

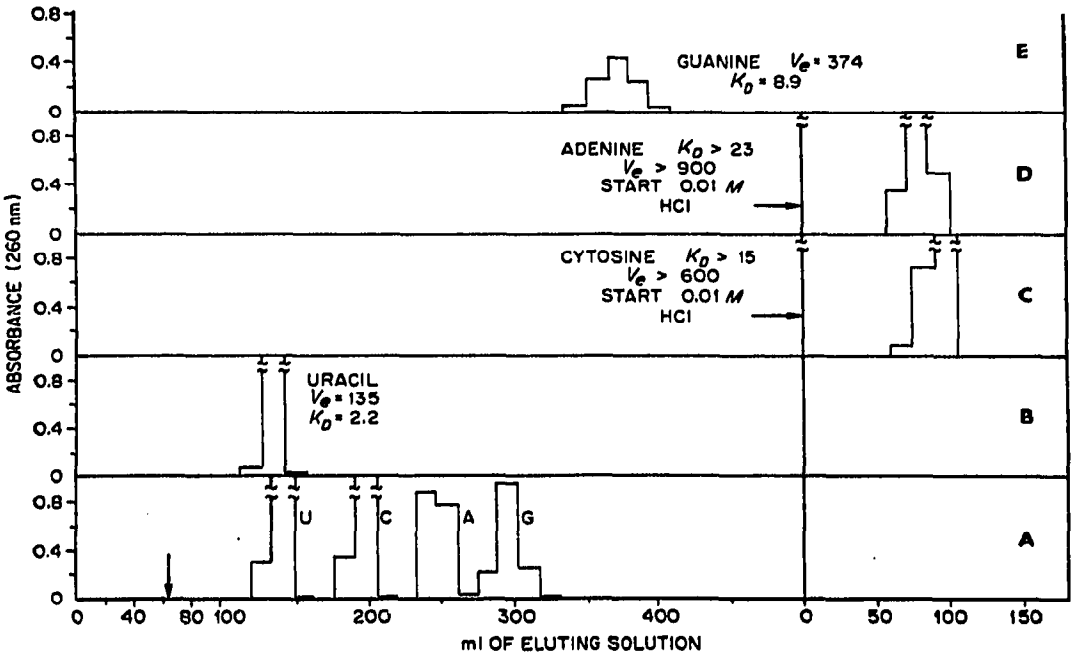

Fig. 2. Behavior of the purine and pyrimidine bases on an acidic dextran column (1.5 × 86 cm). Top curves (B–E): Individual purines and pyrimidines sorbed to columns from 5 ml of water or salt solution at pH 7; U and G were eluted with water, A and C were eluted with 0.01 *M* HCl, as indicated, after (respectively) 900 ml (curve D) and 600 ml (curve C) of water had passed through these two columns. Bottom curve: A mixture of U, C, A, and G sorbed to the column from 5 ml of water or salt solution at pH 7; even though the sample is applied at pH 7, the column is equilibrated with 0.01 *M* acetic acid before use. After a water rinse of 65 ml had passed through the column (indicated by the arrow), the bases were eluted with 0.01 *M* acetic acid; all elutions were made at a rate of about 1 ml/min.

used, the situation is reversed: A and G separate but U and C are eluted together. By using about 65 ml of water to carry out the initial part of the elution before switching to 0.01 *M* acetic acid, all four bases can be separated as shown in the bottom curve of Fig. 2. The amount of water used for the initial part of the elution is fairly critical. For example, if only 50 ml of water is used, A and G separate well but U and C overlap, and if about 90 ml of water is used, U and C separate cleanly but A and G elute essentially as one peak. Salts initially present in a sample do not interfere with these analyses.

Acidic dextran can be converted to its neutral form by equilibrating an acidic G-10 column with a neutral acetate solution (~0.1 *M*). Following a water rinse to remove the acetate solution, this column now gives elution patterns for the purines and pyrimidines that are indistinguishable from those demonstrated in Fig. 1.

Chromatography on alkaline dextran columns

In the pH range 9–10, U, G, and A can exist as anions by virtue of their acidic groupings, whereas C remains uncharged and ionizes only at a much higher pH. As noted by SWEETMAN AND NYHAN[6], these charges decrease the adsorption of the anionic purines and pyrimidines. However, over a narrow range of pH close to the acidic pK values of U, G, and A, separations can be effected in which the negative charge of the compounds and the negative charge on the dextran matrix as well as

adsorptive effects are advantageously used to make analytical separations. This is demonstrated in Fig. 3, which shows the separation of U, C, G, and A that was accomplished with an ammonium chloride–ammonium hydroxide solution at pH 9.7.

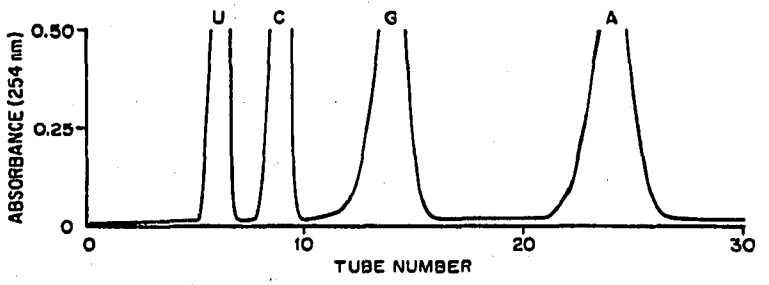

Fig. 3. ISCO tracings showing the separation of purines and pyrimidines on a dextran column (1.5 × 86 cm) at alkaline pH. After equilibration with 0.01 *M* NH_4OH–0.00$_5$ *M* NH_4Cl at pH 9.7, a mixture of U, C, G, and A was sorbed to the column from 5 ml of this same buffer. Elution of the bases was also carried out with the ammoniacal buffer at a flow rate of about 1 ml/min. Chart speed of ISCO was 1 in./h and tube volume was 15 ml.

As the pH is varied, uncharged cytosine retains the same position but the position of the other bases varies considerably. For instance, at pH 9.3, U and C are eluted together while G and A separate cleanly; and at pH 10, U is eluted first from the dextran column followed by a peak that contains C + G, which precedes a single peak containing A. These changes in elution position with changes in pH appear to be involved in a delicate balance between the ionization of U ($pK_a = 9.5$), cytosine (none between pH 9 and 10), guanine ($pK_a = 9.6$) and adenine ($pK_a = 9.8$), the negative charge of the dextran matrix due to carboxyl groups, and the adsorptive attraction of dextran for heterocyclic compounds that has been discussed earlier.

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