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## SEPARATION OF NUCLEOSIDES ON POLYACRYLAMIDE GEL COLUMNS

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### SUMMARY

A new method is described for the separation of nucleosides of both the ribo- and the deoxyribo-series. Polyacrylamide gel (Bio-Gel P-2, Bio Rad, Richmond, Calif.) columns are used. At alkaline pH the elution order is I, G+T, C, A; at neutral pH the elution order is C+T, I, A, G. The unresolved fraction may be separated at the other pH. The advantages of this new separation technique are the following: recoveries from the columns are quantitative; nucleosides are eluted in very small volumes; loads are close to those used in paper chromatography; eluted nucleosides show extremely clean spectra.

The method is also useful for the separation of purines and pyrimidines, and of nucleotides from nucleosides.

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### INTRODUCTION

We have developed a new method for determining the base composition of DNA which has none of the drawbacks of the classical procedure based on acid hydrolysis<sup>1,2</sup> and base separation by paper chromatography<sup>3</sup>, namely: (a) some amount of base destruction during the hydrolysis; (b) incomplete recovery of the bases from paper; (c) unsatisfactory spectral quality of the eluted bases.

This new procedure involves the use of polyacrylamide gel columns to separate the deoxyribonucleosides obtained by enzymatic digestion of DNA. The enzymatic digestion has been carried out in the present work with spleen enzymes, acid deoxyribonuclease, exonuclease and acid phosphomonoesterases<sup>4-6</sup>; this subject will be discussed in detail elsewhere<sup>8</sup>. The fractionation of nucleosides on polyacrylamide gel columns has been inspired by results reported by other authors<sup>7,9</sup>. Both ribo- and deoxyribo-nucleosides, and bases may be separated.

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## MATERIALS AND METHODS

Polyacrylamide gel (Bio-Gel P-2, 200-400 mesh) was purchased from Bio Rad, Richmond, Calif. The material was suspended in water and allowed to swell for 3 h or more. "Fines" were discarded by repeated decantation and the gel was then equilibrated with the buffer to be used in the chromatographic work. More recently, the gel was suspended in 0.1 M EDTA, pH 7.0, and then thoroughly washed with triple-distilled water, which was used throughout.

Columns were prepared by loading the Biogel suspension with stirring, using a funnel mounted on the top of the column. The gel was supported by a nylon net; the column outlet was made of capillary polyethylene tubing and the dead volume (from the column outlet to the recording cell) was 0.1-0.5 ml.

Chromatographic runs were performed at room temperature; flow rates of about 20 ml/h were obtained by using a Technicon (Chauncey, N. J.) peristaltic pump. The ultraviolet absorption of the column effluent was monitored at 253.7 m $\mu$  using a Uvicord (LKB, Stockholm, Sweden). Usual loads for a complete nucleoside analysis were 2-4  $A_{271 \text{ m}\mu}$  units in 0.05-0.2 ml. Reliable results were, however, also obtained at the level of 0.5  $A_{271 \text{ m}\mu}$  units.

Ultraviolet spectra and absorptions were measured using a Cary 15 recording spectrophotometer and a Zeiss PMQ II spectrophotometer, respectively. Buffers obtained from the chromatographic columns were used as reference solutions. Recoveries were checked by collecting the nucleoside fractions in 5- or 10-ml volumetric flasks, adjusting to the mark and determining the absorption at pH 2.0.

Nucleosides and nucleotides of the ribo- and deoxyribo- series, and bases were commercial products (Calbiochem., Los Angeles, Calif.). Degradation of DNA to nucleosides was done by successive digestions with spleen acid deoxyribonuclease<sup>4</sup>, exonuclease<sup>5</sup>, and acid phosphomonoesterases<sup>6</sup>. Dephosphorylated oligonucleotides obtained by successive acid deoxyribonuclease and acid phosphomonoesterases digestions, were degraded by spleen exonuclease<sup>6</sup> and the resulting mixture of nucleosides and nucleotides was used.

## RESULTS

Fig. 1A and 1B are composite patterns showing the chromatographic behaviour of deoxyribonucleosides, as run separately from each other on alkaline or neutral columns; the solvents used were 0.01 M sodium carbonate-0.001 M EDTA, (pH 10.6) and 0.01 M ammonium acetate-0.001 M EDTA (pH 7.4), respectively.

Fig. 2 shows plots of  $K_{av}$  (available) against  $(V_e - V_0)$ , as obtained in the two cases just mentioned.  $K_{av}$  is equal to  $\frac{1}{V_t - V_0} (V_e - V_0)$ ;  $V_t$  is the total column volume,  $V_0$  the void volume (as determined by using Dextran Blue, Pharmacia, Uppsala, Sweden), and  $V_e$  the elution volume, as measured at the maxima of the peaks.  $V_e$  and the band width of the chromatographic component are independent of the nucleotide load in the range (0.1-1.0  $\mu$ moles) used.  $K_{av}$  is independent of changes in the packed volume of the columns.

Recoveries were calculated for two kinds of samples, namely isolated deoxyri-

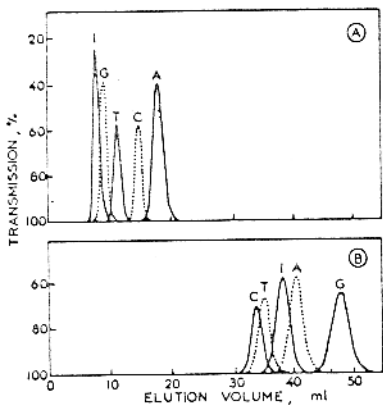


Fig. 1. Composite pattern showing the chromatographic behaviour of deoxyribonucleosides. A. 30  $\mu$ l of 0.02 M solutions of deoxyribonucleosides were loaded on a 0.75 cm  $\times$  33.2 cm Bio Gel P 2 column ( $V_t = 14.68$  ml). Elution was carried out with 0.01 M sodium carbonate - 0.001 M EDTA, pH 10.6, at a flow rate of about 8 ml/h.  $V_0$  was equal to 6.08 ml. B. 30  $\mu$ l of 0.02 M solutions of deoxyribonucleosides were loaded on a 0.91 cm  $\times$  51.6 cm Bio-Gel P-2 column ( $V_t = 33.8$  ml). Elution was carried out with 0.01 M ammonium acetate - 0.001 M EDTA, pH 7.4, at a flow rate of 18.3 ml/h.  $V_0$  was equal to 13.2 ml. In both cases columns were thermostated at 20°.

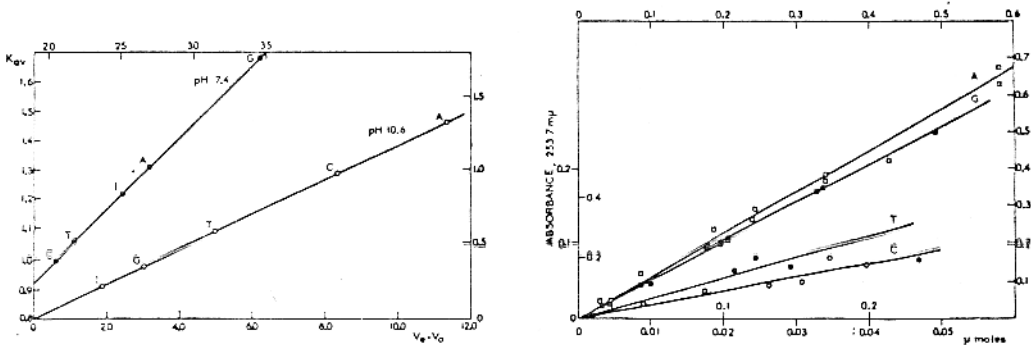


Fig. 2. Plots of  $K_{av}$  versus  $V_e - V_0$  for deoxyribonucleosides run on Bio Gel P2 columns under the experimental conditions given in the legend to Fig. 1. Left-hand ordinate and upper abscissa refer to the set of data obtained at pH 7.4; right-hand ordinate and lower abscissa refer to the data obtained at pH 10.6.  $V_e - V_0$  is given in ml.

Fig. 3. Plots of the absorptions at 253.7  $m\mu$  as read at the maxima of the peaks on the logarithmic paper of the LKB-recorder, against the amounts of deoxyribonucleosides loaded on Biogel P2 columns. A and G, upper abscissa, right-hand ordinate. Lower abscissa, left-hand ordinate: T, inner values; C, outer values.

bonucleosides and deoxyribonucleosides in artificial mixtures. In the first case, recoveries were 98-100 %, in the second they were better than 96 %. By plotting the absorptions, as read at the maxima of the peaks on the logarithmic paper of the LKB recorder against the amounts of nucleosides loaded, straight lines were obtained for the purine derivatives (up to 0.6  $\mu$ moles), whereas rather scattered results were obtained with the pyrimidine derivatives, in a lower range of absorptions (fig. 3). This seems to be due to the fact that purine nucleosides have absorption maxima close to the wavelength of the Uvicord source.

Owing to the lack of resolution of C and T at pH 7.4 and of G and T at pH 10.6, analyses of deoxyribonucleoside mixtures were performed by concentrating the un-

fractionated material in a rotary evaporator and re-running it at the other pH. The mechanical losses encountered in the concentration process were determined by measuring again the  $A_{271}$  of the samples before the second chromatography: they were found to be less than 10% and were, obviously, taken into account. Alternatively the relative proportions of nucleosides in the first unresolved fraction was determined from its ultraviolet spectrum at acid pH.

A typical analysis of an *Escherichia coli* DNA digest is shown in Fig. 4; in this case the first column was run at pH 10.4 and the second one at 7.4. Fig. 5 shows the

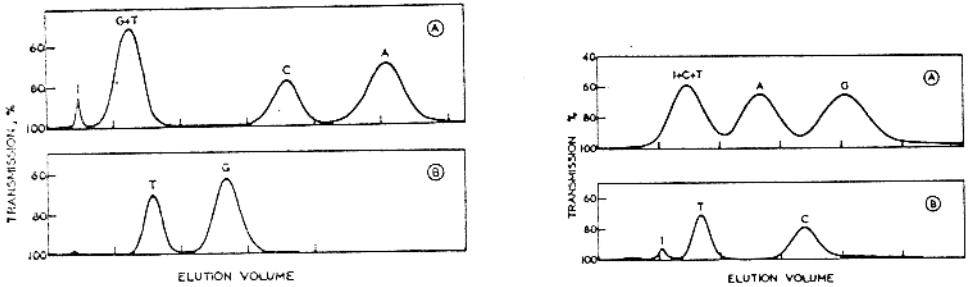


Fig. 4. Chromatography on Bio-Gel P-2 columns of the deoxyribonucleosides obtained by enzymatic digestion from an *E. coli* DNA sample. A.  $4.212 A_{271} m\mu$  units in 0.2 ml of  $2.10^{-3}$  M ammonium carbonate (pH 10.2) were chromatographed on a  $0.9 \text{ cm} \times 80 \text{ cm}$  column equilibrated with the same buffer. The first small peak is deoxyinosine (formed by a trace contamination of adenosine deaminase in the particular exonuclease preparation used); the second is a mixture of deoxyguanosine and thymidine; the third is deoxycytidine and the fourth is deoxyadenosine. B. The G+T fraction was evaporated *in vacuo*, dissolved in 0.2 ml of  $2.10^{-4}$  M sodium phosphate (pH 7.2) and chromatographed on a  $0.6 \text{ cm} \times 50 \text{ cm}$  column equilibrated with the same buffer. The amount loaded was  $2.641 A_{271} m\mu$  units. The first peak is thymidine, the second peak deoxyguanosine.

Fig. 5. Chromatography on Bio Gel P 2 columns of the deoxyribonucleosides obtained by enzymatic digestion from an *E. coli* DNA sample. A.  $5.240 A_{271} m\mu$  units in 0.2 ml of  $2.10^{-4}$  M sodium phosphate (pH 7.2) were chromatographed on a  $0.9 \text{ cm} \times 80 \text{ cm}$  column equilibrated with the same buffer. The first peak is a mixture of deoxyinosine, deoxycytidine and thymidine, the second peak is deoxyadenosine and the third peak is deoxyguanosine. B. The unresolved fraction was evaporated *in vacuo*, dissolved in 0.2 ml of  $2.10^{-3}$  M  $(\text{NH}_4)_2\text{CO}_3$  (pH 10.2) and chromatographed on a  $0.9 \text{ cm} \times 50 \text{ cm}$  column equilibrated with the same buffer. The amount loaded was  $3.200 A_{271} m\mu$  units. The first peak is deoxyinosine, the second thymidine and the third deoxycytidine. One division on the abscissa of Figs. 4 and 5 is equal to 4.7 ml.

analysis of the same digest run first at pH 7.4 and then at pH 10.4; it is clear that the resolution of the peaks is not as good as in the first case; furthermore the small amount of deoxyinosine (since a spleen exonuclease preparation containing a trace of adenosine deaminase was purposely used in this experiment) present in the digest is not separated in the first chromatography but only in the second one. Table I shows an analysis of an *E. coli* DNA.

While we have been mostly concerned with the analysis of deoxyribonucleosides, the present method can also be used for the nucleosides of the ribo-series (which show the same behaviour as the deoxy compounds) and for the bases; the behaviour of purines and pyrimidines will be described elsewhere.

Another useful application of P 2 Biogel columns is the separation of mixtures of nucleosides and nucleotides at neutral pH: under these conditions nucleotides are eluted before nucleosides and these are partially separated among themselves (Fig. 6). The mononucleotide fraction never shows up as a symmetrical peak; in

TABLE I

NUCLEOSIDE ANALYSIS OF *E. coli* DNA

The first column was run at pH 10.4; the second column was run at pH 7.4. Spectra were determined at pH 2.0. Values reported are absorptions at the specified wavelengths, multiplied by the volumes of the nucleoside fractions (11 ml). The values in parentheses are theoretical values.

	$\lambda(m\mu)$							250	280	$\mu\text{moles}^*$	$\frac{A+T}{G+C}$	$\frac{A+G}{C+T}$
	250	255	258	260	267	271	280					
G	1.562	1.705	1.672	1.617	1.276	1.300	1.133	0.96(1.02)	0.70(0.70)	0.147		
T	0.759		1.100	1.166	1.265	1.200	0.836	0.65(0.65)	0.71(0.72)	0.139		
											0.99	1.04
A	1.770		2.130	2.130		1.350	0.490	0.83(0.83)	0.23(0.24)	0.132		
C	0.350		0.760	0.910		1.314	1.960	0.38(0.43)	2.15(2.16)	0.148		

\* Calculated using  $A_{\text{max}}$  values as given by Calbiochem.

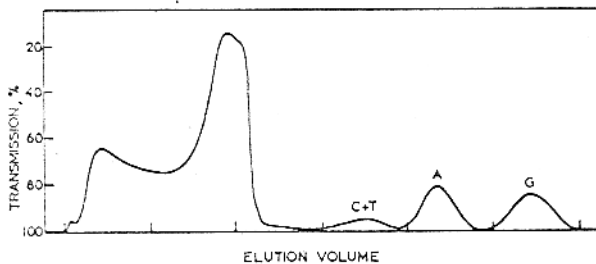


Fig. 6. Chromatography of a dephosphorylated acid deoxyribonuclease digest of a calf thymus DNA after exonuclease treatment on a 0.75 cm  $\times$  47 cm Bio-Gel P-2 column equilibrated with sodium phosphate buffer, pH 7.2. Nucleotides are eluted first, in two peaks; deoxyribonucleosides (from the 3'-phosphate ends of the oligonucleotides) are eluted in the order deoxycytidine + thymidine, deoxyadenosine, deoxyguanosine.

most instances it is split into two poorly resolved peaks. Oligonucleotides, if present, precede the mononucleotide peak(s). This property of the column makes it ideally suited for the study of terminal nucleosides in oligonucleotides<sup>9</sup>.

## DISCUSSION

The main advantages of the present procedure over already existing ones are the following: (a) quantitative recoveries; (b) small fraction volumes; (c) high sensitivity; (d) clean spectral properties of the eluted compounds.

It may be interesting to consider the nature of this type of separation. Fig. 7 shows the variation of the elution volume of isolated nucleosides at different pH values of the eluent. Two different phenomena may be noticed: (a) the elution volumes of purines is higher than that of pyrimidines when nucleosides are almost undissociated (neutral pH) or totally dissociated (very high and very low pH values); (b) the elution volume and the band width of nucleosides is minimal when they are completely ionized; this may be due to a decrease of the diffusion constant of nucleosides upon ionization; (c) at intermediate ionization values the relative importance of the two

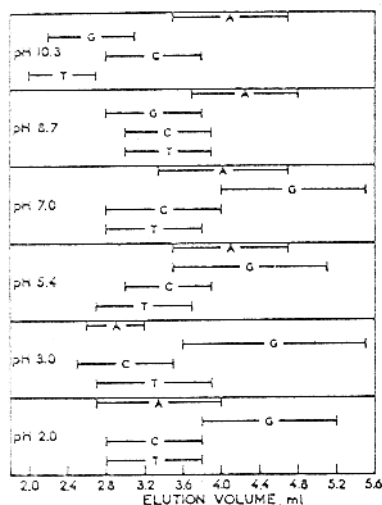


Fig. 7. Elution volumes of deoxyribonucleosides chromatographed at different pH values on a 0.49 cm  $\times$  17.5 cm column of Bio Gel P 2. Bars indicate the spread of every peak.

factors determining the chromatographic behaviour of nucleosides, namely their affinity for the polyacrylamide matrix and their ionization, may be judged from the elution volumes; the ionization seems to be a more important factor than the interaction with the gel, particularly for the purine nucleosides, as shown, for instance, by the fact that at pH 10.3 T (pK 9.9) has a smaller elution volume than G (pK 9.2).

Results which are, in some respects, similar to those reported here, have been obtained by other authors<sup>10-13</sup> working with Sephadex columns. These have the disadvantage, compared with the polyacrylamide gel columns, that nucleosides are eluted in much larger volumes and that the chromatographic separations takes a much longer time.

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#### REFERENCES

- 1 E. VISCHER AND E. CHARGAFF, *J. Biol. Chem.*, 176 (1948) 715.
- 2 A. MARSHAK AND H. J. VOGEL, *J. Biol. Chem.*, 189 (1951) 597.
- 3 G. R. WIATT, in E. CHARGAFF, AND J. N. DAVIDSON, *The Nucleic Acids*, Vol. I, Academic Press, New York, 1955, p. 243.
- 4 G. BERNARDI, A. BERNARDI AND A. CHERSI, *Biochim. Biophys. Acta*, 129 (1966) 1.
- 5 A. BERNARDI AND G. BERNARDI, *Biochim. Biophys. Acta*, 155 (1968) in the press.

- 6 A. CHERSI, A. BERNARDI AND G. BERNARDI, *Biochim. Biophys. Acta*, 129 (1966) 12.
- 7 M. UZIEL AND W. E. COHN, *Biochim. Biophys. Acta*, 103 (1965) 539.
- 8 A. N. SCHWARTZ, A. W. YEE AND B. A. ZABIN, *J. Chromatogr.* 20 (1965) 154.
- 9 M. CARRARA AND G. BERNARDI, *Biochemistry*, submitted for publication.
- 10 T. HOHN AND W. POLLMAN, *Z. Naturforsch.*, 18b (1963) 919.
- 11 T. HOHN AND H. SCHALLER, *Biochim. Biophys. Acta*, 138 (1967) 466.
- 12 R. BRAUN, *Biochim. Biophys. Acta*, 142 (1967) 267.
- 13 G. DIRHEIMER AND J. P. EBEL, *Bull. Soc. Chem. Biol.*, 49 (1967) 447.

*Biochim. Biophys. Acta*, 155 (1968) 1-7