CHROM. 4873

FRACTIONATION OF DNA ON A METAL ION EQUILIBRATED CATION EXCHANGER

I. CHROMATOGRAPHIC PROFILES OF DNA ON AN IR-120 Al^{3+} COLUMN

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SUMMARY

Amberlite IR-120, a polystyrene sulphonate type of cation exchanger, equilibrated with Al^{3+} has been used for the fractionation of DNA in conjunction with a discontinuous gradient elution procedure. This affords a quantitative and reproducible recovery of DNA in seven distinct fractions each differing in base composition.

Rechromatography can be successfully carried out on the major DNA fractions obtained by fractionation on the IR-120 Al^{3+} column.

INTRODUCTION

Although several procedures exist for the resolution of DNA[1] they are rather unsatisfactory due to experimental limitations like low capacity of the column, high UV positive shedding in the blank, irreversible adsorption, clogging up of the column, poor reproducibility, incomplete recovery, need for drastic eluting agents, slow flow-rates leading to inactivation of marker molecules, etc. In many cases fine resolution is also not possible. The need for the development of an alternate procedure devoid of these drawbacks, therefore, still exists.

Amberlite IR-120 resin equilibrated with Al^{3+} has been employed for the purification of several phosphate metabolising enzymes from agave juice[2]. As a logical extension of this work, studies were initiated on the fractionation of DNA using an IR-120 Al^{3+} column, an account of which follows.

EXPERIMENTAL AND RESULTS

Deoxyribonucleic acid

DNA was isolated from buffalo liver (Mammalia, Ruminantia), the chief milking animal in this country, by SEVAG's procedure[3]. It was a white, fibrous, 85 to 90% pure preparation containing 0.5 to 3.0% protein residue and was free from RNA contamination. Purity of DNA was examined by usual methods[4-7].

The fibrous nature suggested native DNA which was subsequently confirmed

by hyperchromicity data[8], *i.e.*, an increase in the UV absorbance by 40% at 260 mμ and by enzymatic studies[9].

The sodium salt of DNA was also isolated from *Bacillus subtilis* and phage T_2 using SEVAG's procedure[3] and KIRBY's phenol method[10], respectively. Both the preparations were tested for their purity and nativeness by the usual procedures[4–9].

IR-120 Al^{3+} column

10 g of dry regenerated Amberlite IR-120 Na^+ form (BDH, Great Britain), a polystyrene sulphonate, equilibrated with 0.2 *M* aluminium chloride solution was used for the IR-120 Al^{3+} column. Glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was percolated through the column till the pH of the inflow and effluent were the same. This column was then used for fractionation purposes.

Al^{3+} was specifically chosen as a counter-balancing cation as it is nontoxic, noncorrosive and inert in biological systems.

The following environmental factors, likely to influence the chromatographic behaviour of DNA, were studied.

Capacity of the IR-120 Na^+ form of the adsorbent for Al^{3+}

A known amount of 0.2 *M* aluminium chloride solution, judged as in excess from pilot experiments, was percolated through 10 g of the regenerated Na^+ form of the IR-120 resin at a rate of 10–15 ml per h. The column was washed with sufficient amount of glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) to remove any loosely retained Al ions and the amount of Al^{3+} in the effluent and buffer washings was determined by GENTRY AND SHERRINGTON's method[11]. The results are cited in Table I which shows that 11.08 mg of Al^{3+} can be maximally retained per g of IR-120 Na^+ form of the resin.

TABLE I

CAPACITY OF IR-120 Na^+ FORM OF THE ADSORBENT FOR Al^{3+}

Description	*Amount of Al^{3+} (mg)*
Loaded on 10 g Na^+ form of resin	563.50
Present in effluent and buffer washings	452.70
Retained on 10 g Na^+ form of resin	110.80
Capacity of 1.0 g resin for Al^{3+}	11.08

Capacity of the IR-120 Al^{3+} form of the adsorbent for DNA

135 ml of a homogeneous solution of DNA (0.5 mg/ml), in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*), was loaded on to a column containing 10 g IR-120 Al^{3+} resin and allowed to percolate at the rate of 10–15 ml per h, which ensured satisfactory equilibrium conditions. 5 ml fractions were collected till there was no DNA in the effluent. The column was then washed with three bed volumes of the above buffer to remove any loosely retained DNA. DNA adsorbed on IR-120 Al^{3+} column was desorbed by 1.0 *N* sodium hydroxide solution and estimated by BURTON's re-

TABLE II

CAPACITY OF IR-120 Al^{3+} FORM OF THE ADSORBENT FOR DNA

Description	*DNA expressed as O.D. of DPA positive material*
Loaded on 10 g IR-120 Al^{3+} resin	182
Present in effluent and buffer washings	44
Retained on 10 g IR-120 Al^{3+} resin	138
Eluted with 1.0 *N* NaOH from IR-120 Al^{3+}	136
Capacity of 1.0 g IR-120 Al^{3+} resin for DNA	13.6 $\cong$ 5.0 mg

action[4]. The results are given in Table II. It shows that 13.6 O.D. units of DNA are maximally retainable per g of the Al^{3+} form of the IR-120 resin.

Effect of the amount of adsorbent on the profiles of DNA

Two columns containing respectively 2 g and 10 g of IR-120 Al^{3+} resin were employed. The procedure followed for the adsorption and elution is discussed in detail under *Typical chromatographic profiles of DNA*. The fractions collected were assayed for DNA content by BURTON's reaction[4].

It was found that the amount of adsorbent used did not have any effect on the nature of the DNA profiles. COHN[12] found that the amount of adsorbent used affects the profiles if the sample exceeds or approaches the capacity of the column. Similar observations have also been made by TOMPKINS[13]. However, the present studies using an IR-120 Al^{3+} column were carried out below the capacity of the column. Recently however, AYAD *et al.*[14] found that the resolution of the transforming principles of *B. subtilis* could be improved by increasing the amount of adsorbent layer in which fractionation occurs.

Effect of the amount of DNA on the profiles

20 O.D. and 40 O.D. units of DNA was loaded on two separate IR-120 Al^{3+} columns, each containing 10 g of the adsorbent. Adsorption and elution was carried out by a procedure described under typical chromatographic profiles. The fractions collected were assayed as before[4].

It was found that the amount of DNA did not have any effect on the nature of the profiles. The percentage distribution of DNA was practically the same in different fractions in spite of the different amounts of DNA loaded. Furthermore, there was no cumulative effect due to eluting agents as observed by DAVILA *et al.*[15].

Effect of the aging of DNA on the profiles

Freshly prepared DNA, and DNA stored for six months at 25° and 4° were chromatographed on an IR-120 Al^{3+} column under an identical set of experimental conditions keeping other parameters constant, *viz.* source of DNA, method of isolation and deproteinisation etc.

Aging or storage of DNA had no effect on its chromatographic behaviour. Theoretically, the chromatographic profiles need not change unless DNA is associated

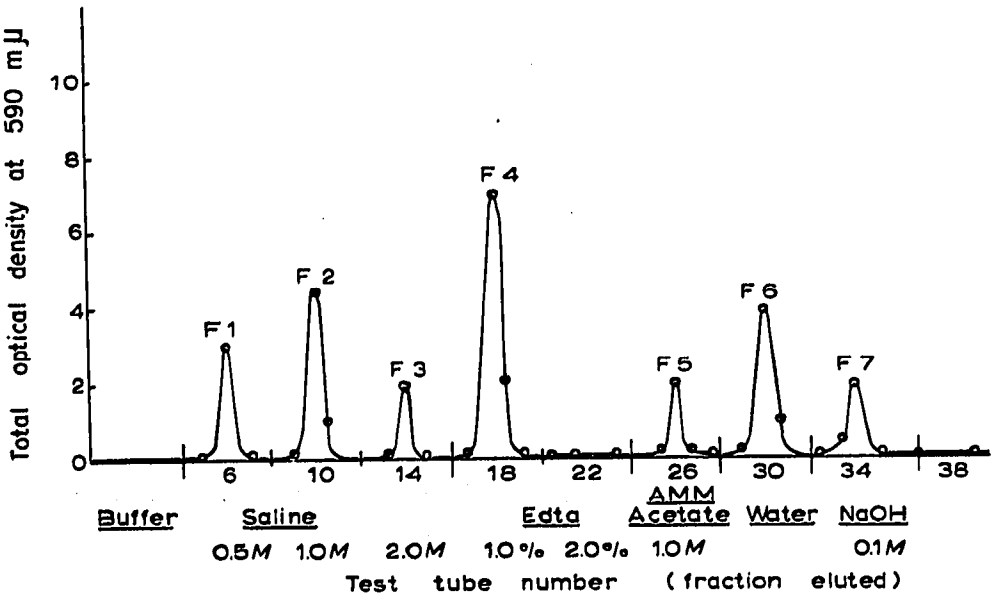

Fig. 1. Typical chromatographic elution profile of buffalo liver DNA (SEVAG's method[3]) on an IR-120 Al^{3+} column.

with deoxyribonuclease activity, which causes depolymerisation and subsequently results in deviation from the typical profiles.

Typical chromatographic profiles of DNA

A homogeneous solution of DNA, in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*), was loaded on to an IR-120 Al^{3+} column, and percolated at the rate of 10–15 ml per h. The effluent was collected, and the column was washed with at least three bed volumes of the above buffer to remove any loosely retained DNA. Adsorbed DNA was then desorbed by 100 ml of each of the following eluting agents in the given sequence: 0.5 *M*, 1.0 *M*, 2.0 *M* saline; 1.0%, 2.0% EDTA; 1.0 *M* ammonium acetate, glass distilled water and finally 0.1 *M* sodium hydroxide solution. The fractions, each of 25 ml, were collected and assayed for DNA content by BURTON's reaction[4]. The same procedure was followed in all the above studies.

The percentage of total eluted DNA by different eluting agents is given in Table III. It shows that DNA is 100% retainable and can be recovered completely by different eluting agents in seven well-defined fractions (F_1–F_7). Fig. 1 shows a

TABLE III

TYPICAL CHROMATOGRAPHIC PROFILES OF BUFFALO LIVER DNA ON AN IR-120 Al^{3+} COLUMN

Percent retention	*Percent elution by*								*Percent elution*
	Saline			*EDTA*		*Ammonium acetate 1.0 M*	*Distilled water*	*Sodium hydroxide 0.1 M*	
	0.5 M	*1.0 M*	*2.0 M*	*1.0%*	*2.0%*				
	F_1	F_2	F_3	F_4	F'_4	F_5	F_6	F_7	
100	10.0	15.0	7.0	33.0	Nil	8.0	17.0	10.0	100

TABLE IV

BASE COMPOSITION OF DNA FRACTIONS OBTAINED ON AN IR-120 Al^{3+} COLUMN

Fraction eluted by	1×10^{-2} *M moles of*				$\frac{A+T}{G+C}$	$\frac{Pu}{Py}$	*Nature of fraction*
	A	*G*	*C*	*T*			
Unfractionated DNA	5.51 ± 0.16	4.62 ± 0.12	4.81 ± 0.08	5.58 ± 0.08	1.17	0.96	A–T rich
0.5 *M* saline	5.25 ± 0.10	5.09 ± 0.12	4.88 ± 0.00	5.25 ± 0.15	1.06	1.02	A–T rich
1.0 *M* saline	4.84 ± 0.09	6.48 ± 0.09	6.82 ± 0.00	5.12 ± 0.12	0.76	0.93	G–C rich
2.0 *M* saline	3.99 ± 0.11	4.28 ± 0.03	4.40 ± 0.12	4.03 ± 0.06	0.93	0.98	G–C rich
1.0% EDTA	15.74 ± 0.10	15.46 ± 0.14	15.50 ± 0.25	15.18 ± 0.14	1.00	1.02	A = G
1.0 *M* ammonium acetate	2.01 ± 0.07	2.86 ± 0.21	2.48 ± 0.00	2.33 ± 0.05	0.85	0.99	G–C rich
Glass distilled water	2.67 ± 0.21	3.32 ± 0.00	3.47 ± 0.13	2.95 ± 0.00	0.85	0.94	G–C rich
0.1 *M* sodium hydroxide	4.17 ± 0.06	4.26 ± 0.00	3.94 ± 0.18	4.12 ± 0.01	1.00	1.02	Almost A = T = G = C

typical chromatographic profile after the total O.D. at 590 mμ has been plotted against the test tube number of the fraction eluted.

Use of 1.0 *M* ammonium acetate first and then of 1.0% EDTA did not alter typical chromatographic behaviour of DNA.

Base composition of different fractions of DNA

The base composition of the DNA fractions was studied to see if DNA fractionated according to the differences in base composition.

Each fraction was concentrated on a rotating evaporator and then precipitated by chilled ethanol. The precipitate was hydrolysed by MARSHAK AND VOGEL'S procedure[16]. Aliquots of each fraction were chromatographed on Whatman No. 1 paper using isopropanol–hydrochloric acid–water, 65:16.6:18.4 as the solvent. Simultaneously, a standard solution of each base was chromatographed. After a 17 h development, the chromatogram was removed from the chamber and dried in air at room temperature. The resolved bases were detected under a Chromatolite lamp, and eluted in 5 ml of 0.1 *N* hydrochloric acid and the O.D. was recorded between 230 to 290 mμ. Blank strips opposite each of these bases were cut from the paper and the O.D. of each of these blanks was subtracted from the O.D. of the corresponding base. The molar extinction coefficients of standard solutions of adenine, guanine, cytosine and thymine agreed closely with the reported values serving as a check on the procedure. The base ratios calculated are presented in Table IV which gives the base composition data of the unfractionated DNA and its seven distinct fractions. The recovery of the bases is greater than 90%. Variations in base composition are considered to be experimentally significant and are also confirmed by OTH'S procedure which was modified by ROGER *et al.*[17]. The base composition data obtained by two different methods, based upon two different approaches, are in good agreement as shown in Table V.

From Tables IV and V it is clear that unfractionated DNA as well as 0.5 *M* saline elutable fraction are A–T rich, while the subsequent two fractions elutable by 1.0 *M* and 2.0 *M* saline are G–C rich. With a change in the nature of eluting agent, *i.e.* with 1.0% EDTA, the fraction coming out has all the bases in almost equal pro-

TABLE V

BASE RATIOS OF DNA FRACTIONS

Fraction eluted by	*A/G ratio by*	
	Paper chromatography	*Modified Oth's procedure*
Unfractionated DNA	1.17	1.15
0.5 *M* saline	1.06	1.10
1.0 *M* saline	0.76	0.90
2.0 *M* saline	0.93	0.98
1.0% EDTA	1.00	1.04
1.0 *M* ammonium acetate	0.85	0.90
Glass distilled water	0.85	0.88
0.1 *M* sodium hydroxide	1.00	—

TABLE VI

RECHROMATOGRAPHY OF DNA FRACTIONS ON AN IR-120 Al^{3+} COLUMN

Fraction loaded	*Percent retention*	*Eluting agent*	*Percent elution*	*Total elution*	*Inference*
0.5 *M* Saline (F_1)	100	0.5 *M* saline alone	100	100	Homogeneous fraction
1.0% EDTA (F_4)	100	Glycine–sodium hydroxide (F 4.1)	52	100	Heterogeneous fraction originally or heterogeneity due to dissociation in absence of Mg^{2+}
		0.5 *M* Saline (F 4.2)	15		
		1.0% EDTA (F 4.3)	34		
Glycine–sodium hydroxide (F 4.1)	100	Glycine–sodium hydroxide alone	100	100	Possibly homogeneous fraction
0.5 *M* Saline (F 4.2)	100	0.5 *M* saline alone	100	100	Possibly homogeneous fraction
1.0% EDTA (F 4.3)	100	Glycine–sodium hydroxide (F 4.3a)	43	100	Heterogeneous originally or as a result of disaggregation in absence of Mg^{2+}
		0.5 *M* saline (F 4.3b)	58		
1.0% EDTA (F_1) + 0.005 *M* Mg^{2+} 4°, 24 h	100	1.0% EDTA alone	100	100	Homogeneous aggregate as a result of Mg^{2+} binding

portions. The fractions eluted by 1.0 *M* ammonium acetate and glass distilled water are again G–C rich. The last fraction eluted by 0.1 *M* sodium hydroxide is slightly A–T rich.

Repeated chromatographic fractionation

Rechromatographic study of two major fractions, *viz.* the 0.5 *M* saline and 1.0% EDTA elutable fractions was carried out. These fractions were concentrated on a rotatory evaporator and precipitated by two volumes of distilled ethanol. The precipitate dissolved easily in the glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) in case of 0.5 *M* saline elutable fraction, but not in case of the 1.0% EDTA elutable fraction. The latter was dissolved in 2.0% sodium carbonate solution and the pH was adjusted back to 8.6.

Rechromatography of the 0.5 M saline elutable fraction. A homogeneous solution of the 0.5 *M* saline elutable fraction (F_1) was loaded on to the IR-120 Al^{3+} column and was found to be 100% retainable. It was also quantitatively eluted with 0.5 *M* saline only. The results are given in Table VI. It should be noted that the 0.5 *M* saline elutable fraction is re-elutable by the same eluting agent indicating its chromatographic homogeneity.

Rechromatography of the 1.0% EDTA elutable fraction. A homogeneous solution of the 1.0% EDTA elutable fraction (F_4) was loaded on to the IR-120 Al^{3+} column and was found to be 100% retainable. When it was eluted by the usual sequence of eluting agents it was found that the adsorbed material is separated into three fractions, a glycine–sodium hydroxide elutable (F 4.1), a 0.5 *M* saline elutable (F 4.2) and a 1.0% EDTA elutable (F 4.3) fraction. No elution was possible with intermediate eluting agents like 1.0 *M* and 2.0 *M* saline.

Further chromatographic procedure. F 4.1 and F 4.2 were adsorbed on a fresh IR-120 Al^{3+} column and eluted at the same position on elution. F 4.3 was found to be 100% retainable on a fresh column and could be resolved into two fractions, a glycine–sodium hydroxide elutable (F 4.3a) and a 0.5 *M* saline elutable (F 4.3b) fraction.

Rechromatography of the 1.0% EDTA elutable fraction (F_4) after Mg^{2+} equilibration. After concentration and precipitation, the precipitate was dissolved and equilibrated with 0.005 *M* Mg^{2+} at 4° for 24 h (0.005 *M* Mg^{2+} ion concentration was expected to be sufficient to nullify the chelating effect of any traces of EDTA remaining). This fraction was then chromatographed on a fresh IR-120 Al^{3+} column and was found to be 100% retainable and could only be eluted at its original location and height by 1.0% EDTA.

The results are given in Table VI and Fig. 2. The latter depicts the chromatographic profiles of the 1.0% EDTA elutable fraction and subfractions, before and after Mg^{2+} equilibration. It is worth noting that the F_4 fraction subfractionated in the absence of Mg^{2+} appears as a single entity after Mg^{2+} equilibration.

Chromatographic behaviour of B. subtilis DNA and phage T_2 DNA

A homogeneous solution of *B. subtilis* DNA and phage T_2 DNA in glycine–sodium hydroxide buffer (pH 8.6, 0.054 *M*) was separately loaded on to an IR-120 Al^{3+} column and its behaviour was studied under experimental conditions identical to those used for the buffalo liver DNA fractionation.

It was found that neither of the DNA preparations were retained in absence or

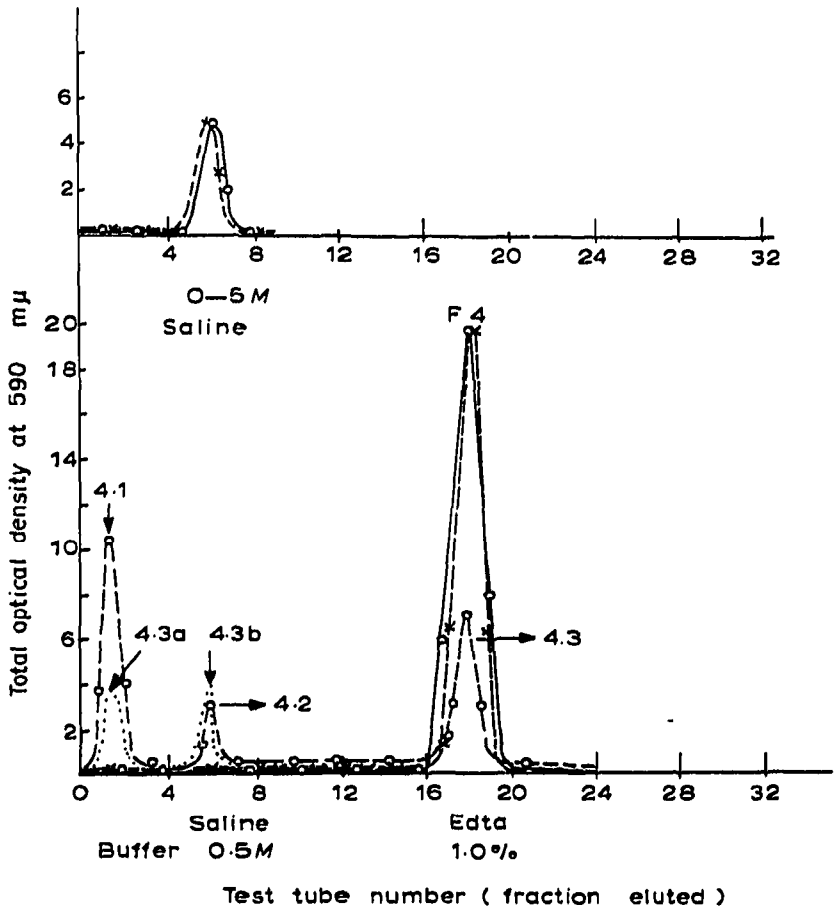

Fig. 2. Rechromatography of DNA fractions on an IR-120 Al^{3+} column. (a) O—O, chromatography of F_1; ×— — —×, rechromatography of F_1. (b) O—O, chromatography of F_4; O— —O, rechromatography of F_4 to give 4.1, 4.2 and 4.3; ×— — —×, rechromatography of F_4 + Mg^{2+}; · · · · ·, rechromatography of $F_{4.3}$ (4.3a and 4.3b).

presence of Mg^{2+}. Mg^{2+} equilibration was tried to see whether it could modify the behaviour. It is difficult to indicate precisely the reason for the failure in retention of these preparations. It could be that rapidly dividing DNAs are not acceptable by the column as could be judged from chromatographic behaviour of human hepatoma DNA[9]. It looks as if the IR-120 Al^{3+} column resolves DNA from rapidly dividing cells and that of somatic tissue.

DISCUSSION

The present fractionation procedure is not conventional ion-exchange chromatography, as in addition to the resin, Al ions are also involved in the adsorption as revealed from equilibrium dialysis data[9]. It could be looked upon as complex-ion-exchange chromatography or ligand-exchange chromatography.

Although a discontinuous gradient elution procedure has certain limitations, for routine explorative purposes it is quick and convenient, particularly for manual

operations. Fractionation can easily be carried out on any scale by adjusting the size of the column; furthermore, the setting up of the column is extremely simple and inexpensive. Discontinuous gradient elution was therefore employed in the present studies and was found to be reasonably satisfactory, when the basic technique had been established, for the general study of the heterogeneity of DNA.

It is known that fractions with high G–C content are eluted with a decreasing salt concentration. The odd elution behaviour of A–T rich fractions with a low salt concentration in the present studies (0.5 *M* saline) has also been noted by CHENG AND SUEOKA[18] in the case of mouse testes DNA profiles on a methylated serum albumin kieselguhr column. This was explained by possible contamination due to an unusual base like methylcytosine. In the present studies however no unusual base was detected and it is unlikely that DNA from a mammalian source will contain any odd base.

In the light of these observations, it was concluded that the IR-120 Al^{3+} column effects the fractionation of DNA extensively and reproducibly, the basis being differences in base composition.

The possibility that the three fractions obtained on rechromatography of the F_4 fraction are genuinely independent fractions, which were eluted together as a single peak by the somewhat drastic and indiscriminate elutability of EDTA in the first chromatographic separation of the DNA is unlikely as rechromatography of the F 4.3 fraction yields two more fractions (F 4.3a, F 4.3b) yet again. Another possibility that 1.0% EDTA elutable fraction (F_4) has undergone structural changes due to its conversion to DNA–Al complex as a result of previous passage through the adsorbent and therefore may have diminished its affinity towards the adsorbent, is not supported by existing circumstantial data[9]. Depolymerisation or denaturation as a result of the passage of the DNA through the adsorbent does not explain the rechromatographic behaviour. It has already been proved that denaturation does not significantly alter the chromatographic behaviour of DNA[19].

Thus, overlapping of the fractions due to the purging action of the eluting agent, structural alterations, depolymerisation, or denaturation as a result of passage through the adsorbent, do not explain the rechromatographic behaviour of the 1.0% EDTA elutable fraction (F_4). The subsequent emergence of two more fractions—F 4.3a and F 4.3b— suggests a labile character for the EDTA elutable fraction and its dissociation into subunits. The quantitative emergence of F_4 after Mg^{2+} equilibration as a single peak due to the coalescence of the F 4.1, F 4.2 and F 4.3 fractions adds to this view. The original EDTA fraction may be dissociated or disaggregated into three subunits by the demineralising action of EDTA giving three fractions and Mg^{2+} addition may cement them back together. Mg^{2+} is known to be a structural ingredient of polyribosomes, a critical concentration of which maintains its integrity as well as functional state[20,21]. OTAKA *et al.*[22] noticed that the chromatographic behaviour of RNA, on DEAE-cellulose, was very much dependent on the molarity of Mg^{2+}; two peaks found at low Mg^{2+} concentrations merged into a single peak at a high Mg^{2+} concentration. It is possible that Mg ions exhibit a unique role in the structural integrity of DNA as well in the present experiments. Mg ions have already been known to bring about conformational changes in nucleic acid molecules[23,24].

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REFERENCES

1 R. M. KOTHARI, *Chromatog. Rev.*, 12 (1970) 127.
2 A. S. SANE, *Ph. D. Thesis*, Poona University, India, 1968.
3 M. G. SEVAG, D. B. LACKMAN AND J. SMOLENS, *J. Biol. Chem.*, 124 (1938) 425.
4 K. BURTON, *Biochem. J.*, 62 (1956) 315.
5 B. L. HORECKER, P. A. SMYRNIOTIS AND H. KLENOW, *J. Biol. Chem.*, 205 (1953) 661.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
8 R. D. HOTCHKISS, *Methods in Enzymology*, 3 (1957) 710.
9 R. M. KOTHARI, *Ph. D. Thesis*, Poona University, India, 1968.
10 C. KIDSON AND K. S. KIRBY, *Biochim. Biophys. Acta*, 91 (1964) 627.
11 C. H. R. GENTRY AND L. S. SHERRINGTON, *Analyst*, 71 (1946) 432.
12 W. E. COHN, *Science*, 109 (1949) 377.
13 E. R. TOMPKINS, *Anal. Chem.*, 22 (1950) 1352.
14 S. R. AYAD, G. R. BARKER AND A. E. JACOB, *Biochem. J.*, 98 (1966) 3-P.
15 C. DAVILA, P. CHARLES AND L. LEDOUX, *J. Chromatog.*, 19 (1965) 396.
16 A. MARSHAK AND H. J. VOGEL, *Biochem. J.*, 46 (1951) 509.
17 M. ROGER, C. O. BECKMANN AND R. D. HOTCHKISS, *J. Mol. Biol.*, 18 (1966) 156.
18 T. Y. CHENG AND N. SUEOKA, *Science*, 141 (1963) 1194.
19 R. M. KOTHARI AND P. N. JOSHI, *Seminar in Cell Biology, Poona University, Poona, India, Oct. 1966.*
20 N. SILMAN, M. ARTMAN AND H. ENGELBERG, *Biochim. Biophys. Acta*, 103 (1965) 231.
21 J. GORDON AND F. LIPMANN, *J. Mol. Biol.*, 23 (1967) 23.
22 E. OTAKA, S. OSAWA, Y. OOTA, A. ISHIHAMA AND H. MITSUI, *Biochim. Biophys. Acta*, 55 (1962) 310.
23 B. D. MEHROTRA AND H. R. MAHLER, *Biochim. Biophys. Acta*, 91 (1964) 78.
24 L. STEVENS, *Biochem. J.*, 99 (1966) 11-P.