*Journal of Chromatography*, 95 (1974) 65–74


CHROM. 7492

# FRACTIONATION OF RNA ON A METAL ION EQUILIBRATED CATION EXCHANGER

## II. CHROMATOGRAPHIC BEHAVIOUR OF RNA, SUBJECTED TO DIFFERENT TREATMENTS, ON AMBERLITE IR-120 $Al^{3+}$ COLUMNS

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(Received April 5th, 1974)

SUMMARY

Purines, pyrimidines, nucleosides and nucleotides are not retained on Amberlite IR-120 $Al^{3+}$ columns and partial enzymic digest is retarded to a small extent. Hydroxylamine-treated RNA has a lower affinity and denaturation seems to have some influence on the retention of RNA. Temperature, pH and divalent cations affect the chromatographic behaviour. All of these results show that, apart from the effects of chain length and certain functional groups such as nitrogenous bases, secondary structure also plays a role in the chromatographic behaviour of RNA on Amberlite IR-120 $Al^{3+}$ columns.

## INTRODUCTION

In Part I[1], we described a procedure for the chromatographic fractionation of RNAs on Amberlite IR-120 $Al^{3+}$ columns. This paper deals with the chromatographic behaviour of RNAs subjected to different treatments.

In general, it has been found that the chromatographic behaviour is considerably influenced by chain-length and molecular weight, conformation and tertiary structure, base composition, nature of the adsorbent, etc. Some of these factors have been studied in detail in this work.

The physical state of the RNA molecule can have an effect on its chromatographic behaviour and, in order to explore this aspect, studies on the chromatographic behaviour on Amberlite IR-120 $Al^{3+}$ columns were carried out with denatured RNA, hydroxylamine-treated RNA and partial and complete enzymic digests of RNA, and the results obtained were compared with those for native RNA.

## EXPERIMENTAL

### *RNA materials*

*Native RNA*. Buffalo liver RNA, isolated and deproteinized by the chloroform–

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amyl alcohol method of Sevag *et al.*, and characterized for its purity and nativeness, was used in the chromatographic studies.

*Denatured RNA.* Denaturation of RNA involves the destruction of its hydrogen-bonded structure into a disordered state, with a consequent increase in UV absorption. Denaturation of RNA can be effected by heat treatment and other methods.

In this work, thermal denaturation was used because controlled heat treatment does not cause any side-reactions, such as hydrolysis and deamination, and it is very specific for denaturation, requires less time and results in no chemical interactions. RNA solution was heated at 96° for about 30 min and then rapidly cooled to room temperature in an ice-bath.

*Hydroxylamine-treated RNA.* Hydroxylamine reacts selectively with the pyrimidine bases of the polynucleotide chain and modifies them, without having any effect on purine moieties. The reaction is quantitative and pH dependent. Uracil residues are modified at pH 10.0 and cytosine residues at pH 6.0 (ref. 3).

In this work, an RNA solution was treated with 5.0 *M* hydroxylamine solution at pH 6.5 at 4° for about 96 h. After the reaction, the pH was readjusted to 4.0 and the hydroxylamine was removed by extensive dialysis against acetate buffer (pH 4.0, ionic strength ($\mu$) = 0.05). This solution was then used for the chromatographic studies on an Amberlite IR-120 $Al^{3+}$ column.

*Partial digest of RNA.* Degradation of RNA by specific cleavage of the phosphodiester bonds gives rise to a partial digest of RNA.

RNA was incubated with agave ribonuclease[4] at 37° for 2 h to give a partial digest of RNA. Agave (*Agave cantala*) is a xerophyte and the extracts of the fleshy leaves were used as the source of the enzyme. Preliminary experiments showed that complete hydrolysis of RNA requires 24 h by the same enzyme under an identical set of experimental conditions.

*RNA hydrolyzate.* RNA was depolymerized to mononucleotides by agave ribonuclease at 37° for 24 h.

*Chromatographic behaviour of RNAs*

The chromatographic behaviour of the above RNAs was studied as follows. A known amount of the specifically treated RNA was chromatographed on an Amberlite IR-120 $Al^{3+}$ column and the adsorbed RNA was eluted using 0.05 *M* ammonium acetate solution[1]. Fractions of 10-ml volume were collected and analyzed for their RNA content by the thymol iron(III) chloride–hydrochloric acid method[5].

The percentage retention of the treated RNAs and the percentage elutions of the total adsorbed RNA and the fractions obtained are given in Table I. The elution profiles are given in Fig. 1.

*Effect of temperature on the chromatographic profiles*

The temperature during the adsorption and elution cycle was kept at 4°, 27° or 50°, and its influence on the chromatographic behaviour of buffalo liver RNA was studied, using three separate Amberlite IR-120 $Al^{3+}$ columns. For a temperature of 4°, the column was operated in an Allwyn refrigerator. For a temperature of 27°, the jacketted column was operated with water circulating at 27 ± 1° and the temperature was regulated by a thermoregulator. Stirring was carried out so as to avoid variations in temperature. The third column was maintained at 50° by means of a jacketted col-

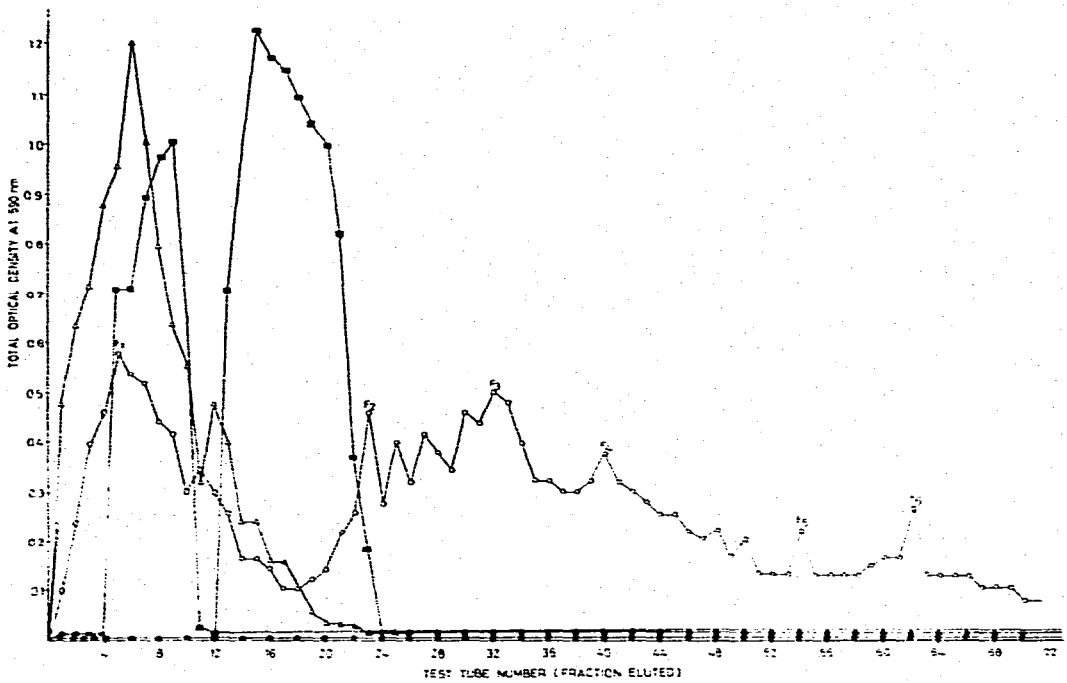


Fig. 1. Chromatographic elution profiles of buffalo liver RNA, subjected to different treatments, on Amberlite IR-120 $Al^{3+}$ columns. ○---○, Native; ○—○, partial digest; ●—●, complete digest; ■—■, hydroxylamine-treated.

TABLE I

EFFECT OF PRE-TREATMENT OF RNA ON CHROMATOGRAPHIC BEHAVIOUR

| *RNA* | *Retention (%)* | *Elution (%)* | *Number of fractions elutable by 0.05 M ammonium acetate* |
|---|---|---|---|
| Native RNA | 100 | 100 | 6 ($F_1$–$F_6$) |
| Denatured RNA | 100 | 100 | Not elutable by 0.05 *M* ammonium acetate; elutable only by 1.0 *M* ammonium acetate in one fraction |
| Hydroxylamine-treated RNA | 40 | 100 | 2 |
| Partial digest | 25 | 100 | 1 |
| Complete digest | 0 | – | |

umn through which water at 50 ± 1° was circulated by a circulating pump connected to a thermostat, which was adjusted to 50 ± 1° with the aid of a contact thermoregulator. A uniform temperature was maintained by constant stirring.

Both adsorption and elution were carried out as previously described[1] but at the temperature stated. The effects of temperature on the adsorption and elution are shown in Table II. Fig. 2 shows the elution profiles.

*Effect of pH variation on the adsorption of RNA*

The size, shape and conformation of the RNA molecule are dependent on a

TABLE II

EFFECT OF TEMPERATURE VARIATION ON THE CHROMATOGRAPHIC PROFILES OF RNA

| *Temperature* (°C) | *Retention* (%) | *Elution* (%) | *Profiles* |
|---|---|---|---|
| 4 | 100 | 102 | 7 fractions ($F_1$–$F_7$) |
| 27 | 100 | 100 | 6 fractions ($F_1$–$F_6$) |
| 50 | 100 | 95 | 7 fractions ($F_1$–$F_7$) |

balance of different forces such as hydrogen bonding and Van der Waal's forces, and vary with changes in the environment. Thus a change in pH and/or temperature gives rise to changes in the overall structure of the molecule. It was therefore suggested by Peacocke[6] that the use of acid or alkali should be avoided, not only during the isolation of RNA but also in subsequent physico-chemical studies. Doty and Rice[7] showed that a decrease in pH causes structural alterations as a result of cleavage of hydrogen bonds. Hence, in addition to the study of the effect of temperature, the effect of pH, which affects the physical state of the molecule, was also studied.

RNA is unstable under alkaline conditions and even mild alkalinity is sufficient to bring about degradation to nucleotides. However, RNA is relatively stable under acidic conditions and, in order to study the effect of pH on the retention of RNA on Amberlite IR-120 $Al^{3+}$ columns, the adsorption studies were carried out at pH 4, 5 and 6.

Homogeneous solutions of RNA prepared in acetate buffer (0.05 $\mu$) at different pH values (4, 5 and 6) were loaded on three Amberlite IR-120 $Al^{3+}$ columns, previously equilibrated with acetate buffer of pH 4, 5 and 6, respectively. All other

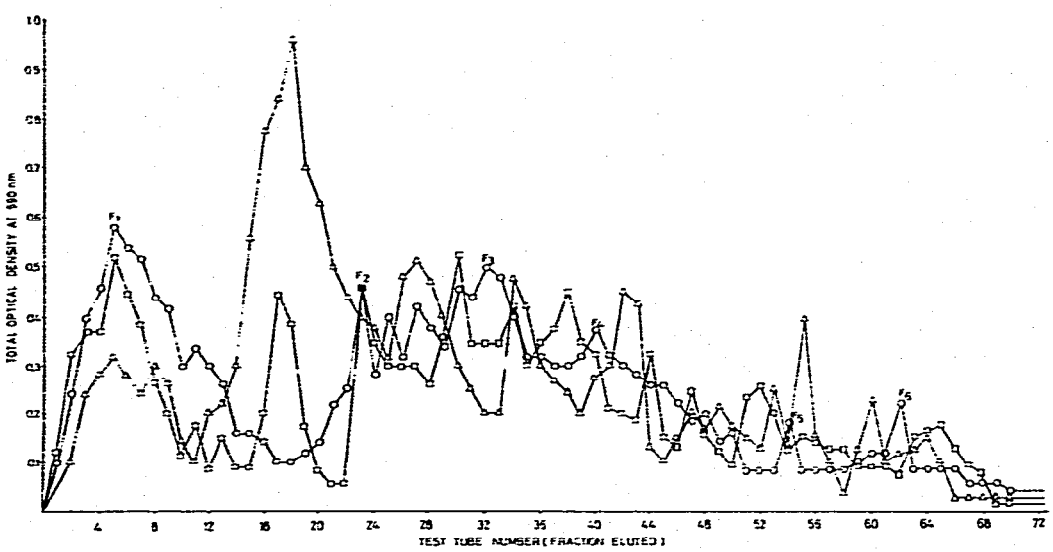


Fig. 2. Chromatographic elution profiles of buffalo liver RNA on Amberlite IR-120 $Al^{3+}$ columns at different temperatures: △—△, 4°; ○—○, 27°; □—□, 50°.

factors that are likely to affect the retention were kept constant. The column operation was carried out as described earlier[1].

Table III shows the amount of RNA adsorbed at the different pH values. Fig. 3 shows the percentage retention of RNA as a function of the variation in pH.

TABLE III

EFFECT OF pH VARIATION ON THE ADSORPTION OF RNA

| *pH* | *Retention (%)* |
|---|---|
| 4.0 | 100 |
| 5.0 | 28 |
| 6.0 | 15 |

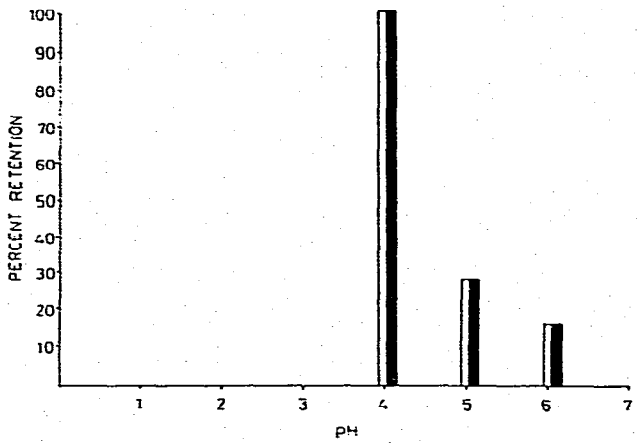


Fig. 3. Effect of variation in pH on the percentage retention of RNA on Amberlite IR-120 $Al^{3+}$ columns: □, in absence of $Mg^{2+}$; ■, in presence of $Mg^{2+}$.

*$Mg^{2+}$ equilibration and RNA retention*

Several reports exist regarding the role of divalent cations such as $Mg^{2+}$ and $Mn^{2+}$ and polyamines such as spermine, spermidine and putrescine in determining the conformation of RNA[8–13]. In view of the multiple role of $Mg^{2+}$ in affecting the structure of RNA and background information on the functioning of Amberlite IR-120 $Al^{3+}$ columns, it was thought worthwhile to study the effect of this ion on the chromatographic profiles of RNA on Amberlite IR-120 $Al^{3+}$ columns.

TABLE IV

EFFECT OF $Mg^{2+}$ IONS ON THE CHROMATOGRAPHIC PROFILES OF RNA

| *Effective concentration of $Mg^{2+}$ ions (M)* | *Retention (%)* | *Elution (%)* | *Profile* |
|---|---|---|---|
| 0 | 100 | 100 | 6 fractions ($F_1$–$F_6$) |
| 0.005 | 100 | 95 | 6 fractions ($F_1$–$F_6$) |

Buffalo liver RNA, isolated by the method of Sevag *et al.*[2], was dissolved in acetate buffer (pH 4.0, 0.05 $\mu$) and equilibrated with $Mg^{2+}$ ions (the effective concentration being 0.005 *M*) for 24 h at 4°. It was then chromatographed on an Amberlite IR-120 $Al^{3+}$ column as described earlier[1].

Table IV shows the percentage of RNA adsorbed and the amount of total RNA eluted. Fig. 4 shows the elution profiles of RNA in the presence of $Mg^{2+}$ ions.

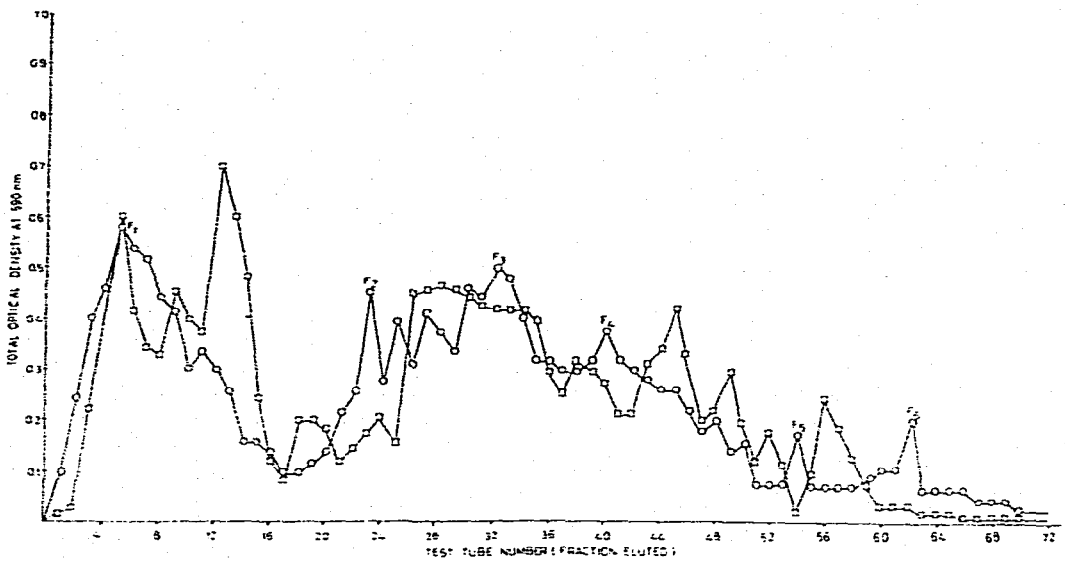


Fig. 4. Chromatographic elution profile of buffalo liver RNA, equilibrated with $Mg^{2+}$ ions, on Amberlite IR-120 $Al^{3+}$ columns: ——, without equilibration; – – –, $Mg^{2+}$ equilibration.

## RESULTS AND DISCUSSION

### *Effect of thermal denaturation*

From Table I and Fig. 1, it is clear that native RNA is completely retained and eluted quantitatively into six fractions ($F_1$–$F_6$).

Denatured RNA obtained by heating for 30 min was also found to be completely retained. It could not be eluted by 0.05, 0.1 and 0.5 *M* ammonium acetate solution and could be eluted only by 1.0 *M* ammonium acetate solution as a single fraction. The appearance of denatured RNA as a single fraction possibly indicates that different species of RNA after denaturation (heating for 30 min at 96 and sudden cooling, with no opportunity for annealing to occur) have lost their fine differences, if any. Denatured RNA is held more strongly and the slight differences that exist among different RNAs in their affinity for $Al^{3+}$ are masked by the overwhelming contribution of the denatured structure which makes the retention stronger, resulting in one peak, which is eluted only at an increased molarity of ammonium acetate.

Goldthwait and Kerr[14] studied the effect of temperature on the chromatographic behaviour of tRNA and rRNA using an ECTEOLA-cellulose column and observed that at elevated temperatures tRNA is more strongly adsorbed and can be eluted only by 1.0 *M* sodium chloride solution. The effect of high temperature appears to involve the destruction of the secondary structure, resulting in stronger adsorption.

Strong retention may be due to multiple linkages, the dissociation of which requires a high salt concentration. The present results on the Amberlite IR-120 $Al^{3+}$ column point in this direction. Popa *et al.*[15] also found that formaldehyde-treated rat liver RNA behaved differently than the untreated RNA on a methylated albumin on Celite (MAC) column. This altered behaviour was thought to be due to the changed secondary structure.

*Partial and complete digests*

A partial digest of RNA with RNase showed less adsorption (Table I) after passage through an Amberlite IR-120 $Al^{3+}$ column and could be eluted completely in two fractions, a minor and a major one (Fig. 1). The decreased adsorption and ease of elution of the partial digest of RNA indicate that the Amberlite IR-120 $Al^{3+}$ column has a lower affinity towards oligonucleotides and no affinity for mononucleotides (Table I, Fig. 1), as revealed by the non-retention of the complete digest of RNA and also of an artificial mixture of mononucleotides on this column. Earlier workers[16,17] showed that neither mononucleotides nor amino acids are retained on this column, while polymers have a higher affinity. If molecular sieving has any role in retention on Amberlite IR-120 $Al^{3+}$ columns, it is likely to be in the reverse order, *i.e.*, it will exclude relatively low-molecular-weight material while retaining high-molecular-weight material.

*Hydroxylamine-treated RNA*

Hydroxylamine-treated RNA shows less adsorption (Table I) and is eluted in two major fractions (Fig. 1), the location of which on the chromatogram indicates that they too have little affinity for Amberlite IR-120 $Al^{3+}$ columns. This lower affinity may be due to the destruction of pyrimidine bases by hydroxylamine.

The heat treatment results only in a change in conformation, unlike the action of RNase, where a partial or complete degradation takes place. The behaviour of hydroxylamine-treated RNA as well as of partial and complete digests of RNA indicates that a polymeric structure is essential for retention on Amberlite IR-120 $Al^{3+}$ columns. As long as a polymer maintains a specific type of conformation, its retention on the column under an identical set of experimental conditions is reproducible. Once the requisite conformation has been modified, however, the adsorption and elution patterns change, and the profiles of modified RNA in the present work confirmed this result. Goehler and Doi[18] observed large differences in the chromatographic profiles of *Bacillus subtilis* RNA modified by iodine oxidation and thiosulphate reduction. A change in the RNA profiles on an MAK column, due to substitution of 5-fluorouracil for uracil, was noted by Lowrie and Bergquist[19].

*Effect of temperature variation*

It is clear from Table II and Fig. 2 that buffalo liver RNA is fractionated into six fractions ($F_1$–$F_6$) at 27° and into seven fractions ($F_1$–$F_7$) at 4° and 50°. Adsorption of these RNAs seems to be independent of temperature, *i.e.*, the adsorption is complete irrespective of the temperature. As the retention proper of RNA on Amberlite IR-120 $Al^{3+}$ columns is independent of temperature, the mechanism involved in retention is unlikely to be that associated with the classical adsorption phenomenon, which is influenced by temperature.

At 4 °, there seems to be a gradual shift in the elution profiles towards the left, indicating dissociation or partitioning of an RNA–$Al^{3+}$ complex, favouring easy desorption or elution from the column. Similarly, at 50 ° there also seems to be an equilibrium in favour of easy elution from the column. There is not only a shift in the locations of the fractions but also a significant change in their distribution. The appearance of an additional fraction in both instances seems to be reproducible.

The present results seem to be comparable with those obtained by Koza[20] on the separation of enterovirus RNA on calcium phosphate, where it was noted that the elution patterns were similar irrespective of the temperature, but there was a change in the distributions in different fractions. Retardation of elution at higher temperatures due to the melting of RNA on DEAE-cellulose and DEAE-Sephadex was observed by Baguley *et al.*[21]. This effect did not seem to occur in the present work, where temperature had only a marginal effect on the chromatographic patterns, unlike in the MAC column, where higher temperatures had to be resorted to in order to obtain complete elution of rRNA from a mammalian source[22–24]. It was noted that even by raising the temperature to 90 ° and using 1.5 *M* sodium chloride solution, not more than 90% elution could be achieved. Kubinski *et al.*[25] observed that the chromatographic elution profiles of RNA from poliovirus-infected amnion cells on a MAC column were greatly influenced by temperature.

Variation in temperature is known to bring about conformational changes in nucleic acids[26,27]. May[28] observed that temperature influences both dissociation and aggregation. Although elution at high temperature may be more rapid than at lower temperatures, chromatography at a lower temperature is desirable as it reduces the risk of cleavage of labile bonds and enzymic degradation. The present results on the change in distribution indicate that marginal changes in conformation may be the reason for the type of behaviour observed.

*pH variation*

Table III shows that there is complete retention at pH 4.0 and a marked decrease in retention at pH 5.0 and 6.0. Changes in pH are known to bring about structural transitions in RNA[6,7,26,27]. The pH changes in the present work may bring about such transitions in the RNA molecule, resulting in decreased retention of RNA on the Amberlite IR-120 $Al^{3+}$ column.

The interaction between $Al^{3+}$ and RNA seems to be optimal at pH 4 rather than at pH 6. Had the retention involved the anionic species of RNA, one would expect a better retention at pH 6. Apparently the least anionic and possibly the least charged species seem to be interacting with $Al^{3+}$ ions.

*Divalent cations*

From Table IV, it is clear that buffalo liver RNA isolated by the method of Sevag *et al.*[2] is completely retained on Amberlite IR-120 $Al^{3+}$ columns, irrespective of $Mg^{2+}$ equilibration. It is also quantitatively eluted into six fractions. There is, however, not only a change in the elution pattern but also in the distribution after $Mg^{2+}$ equilibration. These quantitative differences in elution profiles are possibly due to changed conformation because of $Mg^{2+}$ equilibration. Roberts *et al.*[29] found a large effect on the elution profiles of RNA on a DEAE column due to $Mg^{2+}$. The presence of $Mg^{2+}$ ions in the eluting agent in reversed-phase chromatography is known to

increase the tertiary structure and to facilitate easy elution[30]. Kelmers *et al.*[30] further noted that the presence of $Mg^{2+}$ ions alters the elution profiles. A large change in the elution pattern of RNA in the presence of $Mg^{2+}$ ions was observed by Otaka *et al.*[31] on DEAE-cellulose columns. Otaka *et al.*[31] further noted that the two peaks present in the absence of $Mg^{2+}$ ions become a single peak after $Mg^{2+}$ equilibration. Gillam *et al.*[32] also concluded that the strength of binding of tRNAs to BD-cellulose is dependent on the concentration of divalent cations in addition to other factors such as temperature and pH. Singh and Keller[33] noted that the distribution of rRNA in different peaks on a MAC chromatogram is dependent on the $Mg^{2+}$ concentration. They also found[33] that at higher $Mg^{2+}$ concentrations the 28S peak is prominent, while at low $Mg^{2+}$ concentrations the middle peak is prominent. It was further observed[33] that $Mg^{2+}$ ions do not change the basic location of the peaks but change the distribution. These workers further reported large differences in percentage recoveries on MAC columns in the absence and presence of $Mg^{2+}$ ions. However, in the present investigation, the percentage recovery was not a function of $Mg^{2+}$ concentration.

## CONCLUSION

The failure of Amberlite IR-120 $Al^{3+}$ to retain starch and its ability to retain histone[34] and other proteins and enzymes[17] indicates that, apart from macromolecular structure and chain length, certain functional groups such as nitrogenous bases are required for retention.

Further, the failure of Amberlite IR-120 $Al^{3+}$ to retain bases, nucleosides and nucleotides, the poor affinity of partial digests and hydroxylamine-treated RNA, the strong retention of heat-denatured RNA and the effects of pH, temperature and $Mg^{2+}$ ions on the elution profiles suggest that the secondary structure of RNA has some influence on its behaviour on Amberlite IR-120 $Al^{3+}$ columns. This behaviour is very similar to that of RNA on hydroxyapatite, methylated albumin silicic acid (MASA), BD-cellulose and reversed-phase chromatographic columns[30,32,35,36] and on modified cellulose such as DEAE-cellulose and ECTEOLA-cellulose[14,21,37]. Earlier workers[17,38] have shown that the secondary structures of proteins play an important role in their chromatographic behaviour on Amberlite IR-120 $Al^{3+}$ columns.

## ACKNOWLEDGEMENTS

The authors are grateful to Professor H. J. Arnikar, Senior Professor and Head of the Department of Chemistry, University of Poona, for providing the laboratory facilities and for his interest in this work. One of the authors (V.S.) thanks the University Grants Commission and the University of Poona for financial assistance.

## REFERENCES

1 V. Shankar and P. N. Joshi, *J. Chromatogr.*, 90 (1974) 99.
2 M. G. Sevag, B. D. Lackmann and J. Smolens, *J. Biol. Chem.*, 124 (1938) 425.
3 N. K. Kochetkov and E. J. Budwsky, *Progr. Nucl. Acid Res. Mol. Biol.*, 10 (1969) 417.
4 S. D. Sontakke, *Ph.D. Thesis*, University of Poona, Poona, 1973.
5 N. B. Patil, S. V. Bhide and N. R. Kale, *Carbohyd. Res.*, 29 (1973) 513.

6 A. R. Peacocke, *Biochim. Biophys. Acta*, 14 (1954) 157.
7 P. Doty and S. A. Rice, *Biochim. Biophys. Acta*, 16 (1955) 446.
8 K. Fuwa, W. E. C. Wacker, R. Druyan, A. F. Bartholomay and B. L. Vallee, *Proc. Nat. Acad. Sci. U.S.*, 46 (1960) 1298.
9 M. Revel and H. H. Hiatt, *J. Mol. Biol.*, 11 (1965) 467.
10 N. Silman, M. Artman and H. Engelberg, *Biochim. Biophys. Acta*, 103 (1965) 231.
11 H. R. Mahler and E. Cordes, *Biological Chemistry*, Harper and Row, New York, Evanston, San Francisco, London, 1967, p. 124.
12 G. Felsenfeld, D. R. Davis and A. Rich, *J. Amer. Chem. Soc.*, 79 (1957) 2023.
13 M. Fujioka and I. Lieberman, *J. Biol. Chem.*, 239 (1964) 1164.
14 D. A. Goldthwait and D. S. Kerr, *Biochim. Biophys. Acta*, 61 (1962) 930.
15 L. Popa, V. Lăcătus, M. Popescu and R. Portocată, *Biochim. Biophys. Acta*, 149 (1967) 396.
16 R. M. Kothari, *Ph.D. Thesis*, University of Poona, Poona 1968.
17 M. V. Hegde, *Ph.D. Thesis*, University of Poona, Poona, 1970.
18 B. Goehler and R. H. Doi, *Proc. Nat. Acad. Sci. U.S.*, 56 (1966) 1047.
19 R. J. Lowrie and P. L. Bergquist, *Biochemistry*, 7 (1968) 1767.
20 J. Koza, *J. Chromatogr.*, 25 (1966) 325.
21 B. C. Baguley, P. L. Bergquist and R. K. Ralph, *Biochim. Biophys. Acta*, 108 (1965) 139.
22 A. V. Lichtenstein, E. G. Piker and V. S. Shapot, *Biochim. Biophys. Acta*, 138 (1967) 441.
23 K. A. O. Ellem and S. L. Rhode, *Biochim. Biophys. Acta*, 174 (1969) 117.
24 M. Stachelin, *Progr. Nucl. Acid Res. Mol. Biol.*, 2 (1963) 186.
25 H. Kubinski, G. Koch and O. Drees, *Biochim. Biophys. Acta*, 61 (1962) 332.
26 V. Luzzati, A. Mathis, F. Masson and J. Witz, *J. Mol. Biol.*, 10 (1964) 28.
27 T. I. Tikchonenko, G. A. Perevertaylo and E. Dobrov, *Biochim. Biophys. Acta*, 68 (1963) 500.
28 P. May, *J. Mol. Biol.*, 9 (1964) 263.
29 R. B. Roberts, R. J. Britten and E. J. Bolton, *Microsomal Particles Protein Syn., Pap. Symp., 1st*, 1958, p. 84.
30 A. D. Kelmers, H. O. Weeren, J. F. Weiss, R. L. Pearson, M. P. Stulberg and G. D. Novelli, *Methods Enzymol.*, 21C (1971) 9.
31 E. Otaka, S. Osowa, Y. Oota, A. Ishihama and H. Mitsui, *Biochim. Biophys. Acta*, 55 (1962) 310.
32 I. Gillam, S. Millward, D. Blew, M. von Tigerstrom, E. Wimmer and G. M. Tener, *Biochemistry*, 6 (1967) 3043.
33 H. Singh and D. Keller, *Biochim. Biophys. Acta*, 169 (1968) 150.
34 A. M. Diwan and P. N. Joshi, unpublished results.
35 G. Bernardi, *Methods Enzymol.*, 21D (1971) 95.
36 R. Stern and U. Z. Littauer, *Methods Enzymol.*, 21C (1971) 86.
37 B. C. Baguley, P. L. Bergquist and R. K. Ralph, *Biochim. Biophys. Acta*, 95 (1965) 570.
38 P. G. Batliwala, *Ph.D. Thesis*, University of Poona, Poona, 1972.