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SOME ASPECTS OF FRACTIONATION OF DNA ON AN IR-120 Al^{3+} COLUMN

VII. EFFECT OF THE TISSUE AND SOURCE VARIATION ON THE CHROMATOGRAPHIC PROFILES OF DNA

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SUMMARY

The effect of variations in the tissue and source on the chromatographic profiles of DNA using an IR-120 Al^{3+} column was studied. For this purpose, DNA preparations were isolated from buffalo liver, kidney, brain and lung (tissue variation, same source) and from the livers of buffalo, humans, albino rat, pigeon and frog (source variation, same tissue). The pure and native preparation, in each case, were chromatographed and their profiles were studied. Buffalo liver DNA was completely retainable, while DNA preparations from the other tissues and sources were either partially retainable or non-retainable. They became completely retainable, however, after equilibration with 0.001 *M* Mg^{2+}, indicating a major influence of Mg^{2+}. The elution profiles of all Mg^{2+}-equilibrated DNAs were similar. Mg^{2+} possibly stabilized a specific conformation of DNA necessary for retention on the IR-120 Al^{3+} column. Mg^{2+} could be replaced by Mn^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+} and Hg^{2+} in conferring the retainability on buffalo kidney DNA which was originally retainable to an extent of only 7.0 %. Arginine also had an equivalent effect.

INTRODUCTION

Conflicting results exist in the literature for the effect of tissue and source variations on the chromatographic profiles of DNA. KIT[1,2] showed, with ECTEOLA-cellulose as an adsorbent, that the chromatographic profiles of DNA preparations from the spontaneous AKr lymphoma do not differ from those of the normal AKr mouse-spleen cells, nor do those of transplanted tetraploid lymphomas differ greatly from those of C_3H or DBA-2 mouse-spleen cells. It was further noted that the differences in the profiles of DNA from diploid and tetraploid lymphomas were also not significant, and the elution profiles of DNA of diploid and tetraploid carcinomas were shown to be similar to those of the two melanomas, the normal spleen cells and the lymphoma. It was further shown that the elution profiles of DNA of lung, thymus, kidney and liver of mouse, and brain, spleen and kidney of rat did not show any noticeable differences.

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However, BENDICH *et al.*[3] and POLLI AND SHOOTER[4] observed that the chromatographic profiles of DNA from different tissues and different sources differed significantly and emphasised that DNA preparations from vastly different tissues and different sources may have similar physico-chemical and biological properties. BENDICH *et al.*[5] showed that the chromatographic profiles of regenerating rat-liver DNA are different from those of normal adult rat-liver DNA. BENDICH *et al.*[6] also studied the chromatographic profiles of irradiated DNA, DNA containing 5-bromouracil, normal thymus DNA, DNA from the white cells of an untreated patient with chronic granulocytic leukemia and from a wide variety of other sources and concluded that there are differences in the profiles of DNA from tissue-to-tissue and source-to-source. CRAMPTON *et al.*[7] attempted the solvent fractionation of DNA from tissues of ox, pig, man, sea urchin and coliphage T_6 and showed that the resolution is based on the differences in base composition.

DUBUY *et al.*[8] distinguished DNAs from brain nuclei, mitochondria of mice and from nuclei of kinetoplasts of *Leishmania minriettii*. SUEOKA AND CHENG[9], SMITH AND QUAYLE[10] and SMITH[11] showed that DNA from different classes of the same group have different base compositions. A DNA preparation from a marine crab, *Cancer borialis*, could be fractionated into two different bands in a caesium chloride gradient. MINDICH AND HOTCHKISS[12] fractionated artificial mixtures of DNAs from different bacterial species and studied the different factors affecting the resolution. They found that DNA from *Micrococcus lysodeikticus* could be separated from that of the *Pneumococcal* and *Escherichia coli* DNA by chromatography on a methylated bovine serum albumin-coated kieselguhr (MSAK) column.

Thus, according to one school of thought, all tissues may have similar DNA molecules, the DNAs may not differ from each other in parameters such as chain length (molecular weight), conformation etc., and tissue specificity is still observed. Tissue specificity is shown to be due to "preferential supression" of some activity in one tissue, whilst "full freedom" exists for the same activity in other tissues.

According to the other school of thought, however, every tissue is assigned a special function controlled by a specific kind of DNA. The special function may possibly be due to the specific enzymes, which in turn may be an expression of the specific DNA present in the tissue. For example, DNA present in liver, the centre of many metabolic activities in the living organism and having a function different to those of other tissues, may be different from that in other tissues. In other words, different tissues may have different kinds of DNA molecules.

In view of these contradictions in the literature, it was of interest to study the chromatographic behaviour of DNA from different tissues and different sources, using an IR-120 Al^{3+} column[13].

EXPERIMENTAL AND RESULTS

The IR-120 Al^{3+} column

Ten grams of dry regenerated Amberlite IR-120 (Na^+ form), polystyrene sulphonate, were equilibrated with a 0.2 *M* aluminium chloride solution to give the IR-120 Al^{3+} column[14]. Glycine–sodium hydroxide buffer (pH 8.6; 0.054 *M*) was percolated through the column until the pH of the influent and effluent were the same. This column was then used for studying the chromatographic behaviour of DNA

samples from different tissues and different sources, the method of isolation being identical in all cases.

Deoxyribonucleic acids

The sodium ·· DNA used in these experiments was isolated by the method of SEVAG *et al.*[15] us. following tissues and sources.

(1) *Tissue variation.* Liver, kidney, lung and brain of buffalo (*Mammalia, Ruminantia*).

(2) *Source variation.* Livers from buffalo (*Mammalia, Ruminantia*), humans, albino rat, pigeon (*Columbia livia*) and frog (*Rana tigrina*).

DNA samples isolated from these different tissues and sources were white, fibrous, native and fairly pure. They were associated with various amounts (1.0–5.0 %) of residual protein and were devoid of RNA contamination. The nativity and purity were examined as discussed earlier[14,16].

Procedure

DNAs, thus isolated, were dissolved in the glycine–sodium hydroxide buffer to give homogeneous solutions. The solutions were loaded on different IR-120 Al^{3+} columns, the columns were washed with three bed volumes of the buffer to strip off any loosely retained DNA, and wherever adsorption occurred, DNAs from the columns were eluted with different eluting agents in the usual sequence[14]. Fractions (each 25 ml) were collected and the DNA contents were determined by BURTON's reaction[17] (the details of the experimental procedure have been discussed earlier[14]).

It was observed that buffalo-liver DNA alone was retained completely, whilst the other DNA preparations were either not retained or only partially retained.

DNA samples that were partially retainable or non-retainable were equilibrated with magnesium chloride at 4° for 24 h, the effective concentration of Mg^{2+} being 0.001 *M*. Mg^{2+} equilibration was tried as the close interrelationship between Mg^{2+} and both the structural and the functional integrity of polyribosomes is well known[26,29,33,46]. Also, a critical concentration-dependent retention and elution of DNA from an Amberlite IRC-50 Mg^{2+} column was known[18]. Mg^{2+}-equilibrated DNAs were again chromatographed on the IR-120 Al^{3+} column. They were found to be retainable on the column to a very large extent and were eluted by the usual elution procedure[14].

TABLE I

EFFECT OF TISSUE VARIATION ON THE CHROMATOGRAPHIC PROFILES OF DNA FROM BUFFALO (SAME SOURCE, IDENTICAL METHODOLOGY) ON IR-120 Al^{3+} COLUMN

Tissue	*Retention (%)*		*Elution (%)*	*Profiles after Mg^{2+} equilibration*
	Before Mg^{2+} equilibration	*After Mg^{2+} equilibration*		
Liver	100	100	100	Typical seven fractions (F_1–F_7)
Kidney	7.0	95.0	98.0	,,
Brain	0	85.0	85.0	,,
Lung	0	90.0	102.0	,,

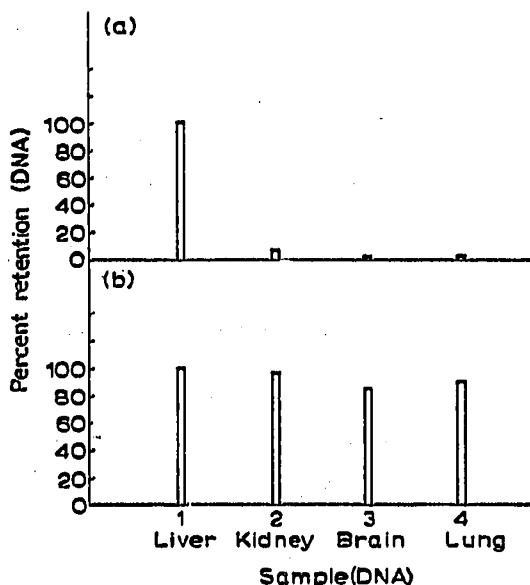

Fig. 1. Percentage retention of DNA from various buffalo tissues on IR-120 Al^{3+} column, (a) before Mg^{2+} equilibration and (b) after Mg^{2+} equilibration.

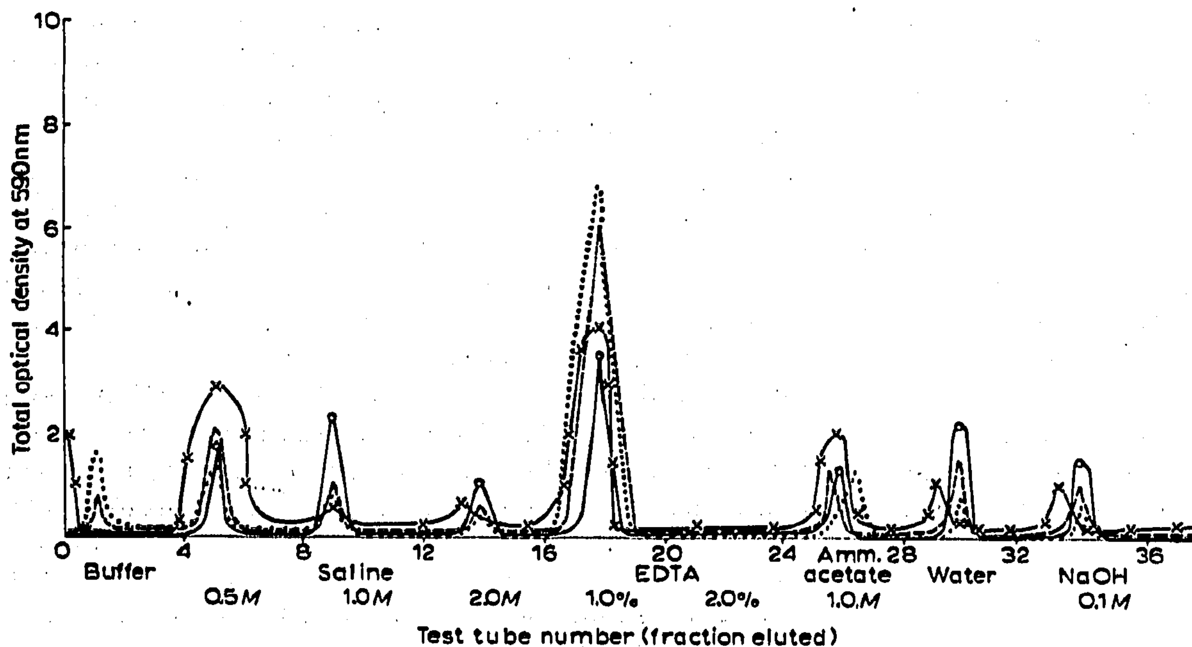

Fig. 2. Chromatographic elution profiles of DNA from different buffalo tissues (isolated by SEVAG's method[15]) on IR-120 Al^{3+} column. ○—○, liver; ----, kidney; ...., lung; ×—×, brain.

The DNA content of the fractions, in each case, was determined by BURTON's reaction[17].

Tissue variation. The percentage retention of DNA with and without Mg^{2+} equilibration is given in Table I and Fig. 1. Table I also gives the percentage elution of the total retained DNA. The elution profiles are given in Fig. 2 where total optical density at 590 nm is plotted against the test-tube number or the fraction eluted.

Source variation. The percentage retention of DNA with and without Mg^{2+} equilibration is given in Table II and Fig. 3. Table II also gives the percentage elution

TABLE II

EFFECT OF SOURCE VARIATION ON THE CHROMATOGRAPHIC PROFILES OF DNA FROM LIVERS (SAME TISSUE, IDENTICAL METHODOLOGY) ON IR-120 Al^{3+} COLUMN

Source	*Retention (%)*		*Elution (%)*	*Profiles after Mg^{2+} equilibration*
	Before Mg^{2+} equilibration	*After Mg^{2+} equilibration*		
Buffalo	100	100	102	Typical seven fractions (F_1–F_7)
Human	25.0	100	100	,,
Albino rat	45.0	100	103	,,
Pigeon	40.0	75.0	100	,,
Frog	5.0	100	98.0	,,

of the total adsorbed DNA and the elution profiles obtained are given in Fig. 4, where total optical density at 590 nm is plotted against the test-tube number or the fraction eluted.

DISCUSSION

From Tables I and II, it is clear that DNA from buffalo liver is 100 % retainable on the IR-120 Al^{3+} column, even in the absence of Mg^{2+}, whilst DNAs from other tissues and sources are either partially retainable or non-retainable under comparable experimental conditions. It is important to emphasize that if the method of isolation was different, then the differences in retainability could be explained as it was earlier[19]. As the methods of isolation and deproteinisation are the same, however, one would expect the retainabilities to be similar. Equilibration of DNA with 0.001 *M* Mg^{2+}

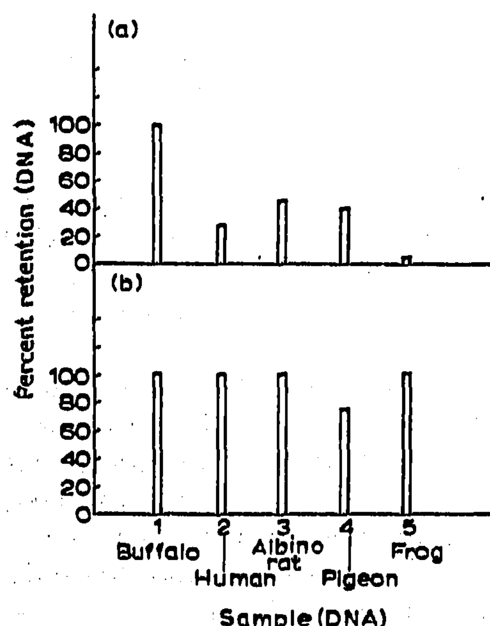

Fig. 3. Percentage retention of liver DNA from various sources on IR-120 Al^{3+} column, (a) before Mg^{2+} equilibration and (b) after Mg^{2+} equilibration.

at 4° for 24 h, however, removed the differences in retainability in each case, making non-retainable DNA retainable to a great extent, irrespective of tissue and source variation. Once DNA the had been adsorbed, all DNAs were completely elutable and gave characteristic profiles yielding seven well defined fractions[14].

KIT[1,2] showed that the chromatographic profiles of DNA from various tissues from the same source and of the same tissue from various sources are not distinguishable when different variables such as the method of deproteinisation and isolation, control over deoxyribonuclease activity, or the physical state of the molecule, are kept constant. The present author's earlier findings[19,20] support the same view. This view has been substantiated by other workers[21-25].

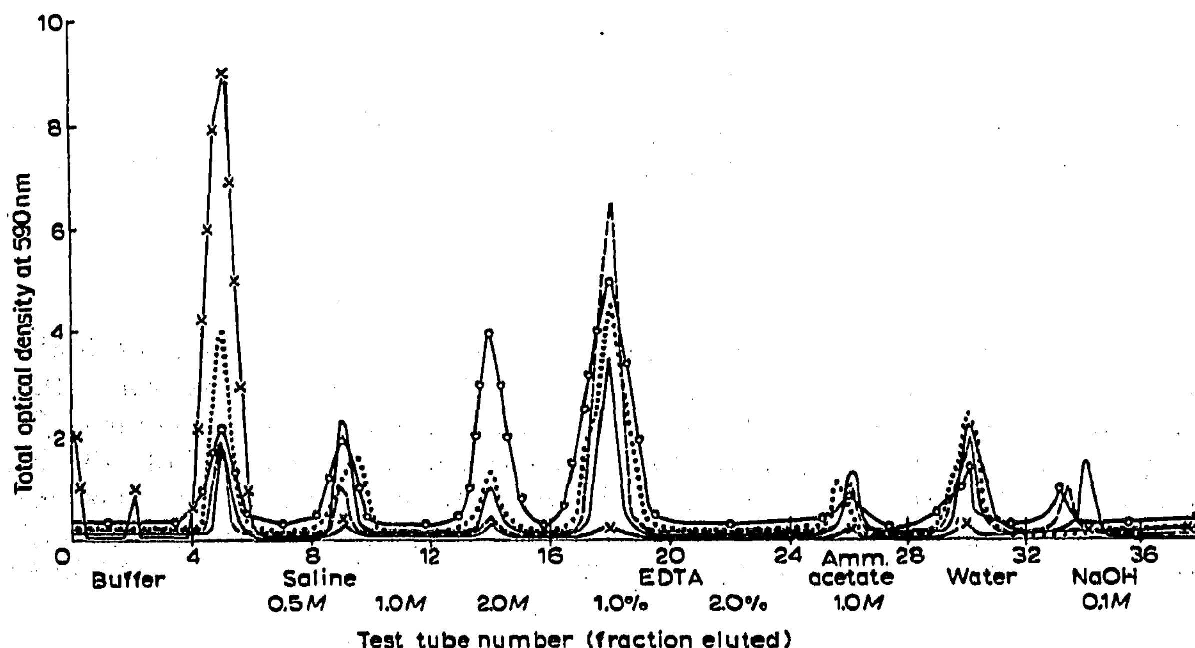

Fig. 4. Chromatographic elution profiles of liver DNA from different sources (isolated by SEVAG's method[15]) on IR-120 Al^{3+} column. o—o, buffalo; ---, human; ......, albino rat; ×—×, pigeon; o—o frog.

KIT[21], SMITH and coworkers[22,23] and KONDO AND OSAWA[24] showed that DNAs from different tissues or different sources are not distinguishable. LANGRIDGE *et al.*[25] also showed that DNAs from vastly different sources with different physico-chemical and biological properties give almost indistinguishable X-ray patterns. In other words, their profiles could be indistinguishable.

The present observations on the IR-120 Al^{3+} column, under comparable experimental conditions, are similar to those above. The similarity in the chromatographic profiles may be due to the fact that (i) there may not be genuine differences in the overall structure of DNAs from different tissues and sources; (ii) the existing chromatographic methods fail to distinguish between differences in DNAs; (iii) the differences may be so small that no physico-chemical method can enlarge and identify them; or (iv) the differences may be exhibited only at a biological level.

It is fairly certain, however, that the heterogeneity at chain-length level, conformational level, compositional level (*i.e.*, A-T or G-C rich level) or at the nucleo-

tide sequence level does exist. More experimental evidence and theoretical evaluation is necessary to obtain further insight into this matter.

In the light of the results in Tables I and II and their graphical representation in Figs. 2 and 4, it is difficult to decide whether a definite distinction in DNAs from different tissues or different sources exists; nevertheless, the necessity for Mg^{2+} for the satisfactory retention of DNAs from certain tissues and sources is striking.

Mg^{2+} equilibration and DNA retention

Mg^{2+} is likely to have a finite role in the structural integrity of DNA molecules in several ways.

(*i*) *Mg^{2+} as ionic crosslink.* Mg^{2+} may act as "ionic crosslink", giving a particular conformation to the DNA molecules, that is fortuitously acceptable to the IR-120 Al^{3+} column. FELSENFELD *et al.*[26] showed that a triple-stranded structure can be formed from polyadenylic acid and polyuridylic acid (2:1) in the presence of Mg^{2+}. They considered the triple helix formation as a model for a combined DNA double helix with a complementary single strand of RNA. This observation suggests the important role of Mg^{2+} in attributing a particular conformation to the DNA molecule. Requirements for divalent cations such as Mg^{2+} and in some cases Zn^{2+} (ref. 27) in DNA and RNA polymerase reactions have been shown by many workers. It is felt that Mg^{2+} may have multiple functions[27].

Various concentrations of Mg^{2+} in the eluting agent were initially used by FRANKEL AND CRAMPTON[18] and later developed by MINDICH AND HOTCHKISS[12] for the fractionation of DNA on an Amberlite IRC-50 Mg^{2+} column, where perhaps Mg^{2+} served as "ionic crosslink" in the conformation of the molecule. An effort to disturb or deform the particular conformation resulted in desorption and subsequent elution. A good example is that of ribosome–ribonucleoprotein particle formation. According to KEIICHIRO *et al.*[28], transition metals determine the conformation of RNAs. REVEL AND HIATT[29] have shown Mg^{2+} requirement for the formation of the *m*RNA–*r*RNA–*t*RNA complex. SILMAN *et al.*[30] presented evidence for the requirement of Mg^{2+} and spermine in the aggregation of bacterial and mammalian ribosomes. When *E. coli* cells were cultured in Mg^{2+}-deficient media, most of the ribosomes disappeared without loss of viability[31, 32]. When Mg^{2+} was added to the media, such ribosome-free cells began to rebuild their ribosomes. This further showed the significance of Mg^{2+} in the biological system. Mg^{2+} salts and ethanol were used in the precipitation of polynucleotides by RAZZELL[33]. He stated that metal ions directly affected the net charge, aggregation and solubility, although the conformation may be affected indirectly.

(*ii*) *Mg^{2+} as a stabilizer of subunits.* Mg^{2+} may be reversibly bound so as to stabilize an association of subunits[34] of the macromolecule or to confer a particular conformation, helical or folded, which may be acceptable to the IR-120 Al^{3+} column, hence effecting DNA retention. The present authors' earlier data[10] also support this view.

(*iii*) *Mg^{2+} as counter-ions.* Mg^{2+} may function as counter-ion to the negatively charged DNA anion under the conditions used. It is difficult, however, to explain why Na^{+} (ref. 35) do not have the same effect.

The possibility that Mg^{2+} acts as counter-ion was supported by AMES AND DUBIN[36], who also noted that polyamines have a role similar to that of Mg^{2+} in the

maintenance of a particular conformation of the DNA molecule. At phsysiological pH, DNA carries a high density of negative charge. For this reason, *in situ* DNA is found associated with various types of cations, *viz.* histones and oligoamines, such as spermine, spermidine, putrescine and cadaverine. It is claimed that these molecules play a role in the neutralisation of DNA and hence in conferring, preserving or stabilizing certain conformations.

(*iv*) *Mg^{2+} as an integral part of DNA.* Mg^{2+} may form an integral part of the whole DNA molecule, which may somehow be "missing" in the DNA preparations from tissues and sources other than buffalo liver.

The possibility that Mg^{2+} may form an integral part of the DNA molecule has been speculated by CAVALIERI *et al.*[37] By using equilibrium dialysis and spectrophotometric titration, they showed that 1 mole of Mg^{2+} is bound to 5 moles of P_i of calf-thymus DNA. The presence of serine and threonine in the DNA molecule was reported by BORENFREUND *et al.*[38]. BENDICH AND ROSENKRANZ[39] suggested the presence of one serine moiety for every 500–1000 nucleotide residues in the DNA molecule in the chain. They further suggested[39] that part of the serine may occur as *o*-phosphoserine which is known to form a Mg^{2+} complex[40] of sufficient stability. Thus, Mg^{2+} constituting an integral part of the DNA molecule may give a possible explanation of the facts.

Specificity of Mg^{2+} equilibration

To study whether retainability is specifically associated only with Mg^{2+}, the influence of the divalent cations Mn^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+} and Hg^{2+} was studied. The effect of arginine was also studied.

Buffalo-kidney DNA, which was retainable to an extent of only 7.0 %, was equilibrated with different divalent cations, the effective concentration being 0.001 *M* in each case, at 4° for 24 h and was then chromatographed on the IR-120 Al^{3+} column. The retainability of buffalo-kidney DNA with each individual cation was studied. In the case of arginine, the experimental conditions were the same as for the cations, except that 0.005 *M* arginine was used for equilibration.

The retainability of buffalo-kidney DNA in the presence of different divalent cations is given in Table III.

TABLE III

RETAINABILITY OF BUFFALO KIDNEY DNA ON IR-120 Al^{3+} COLUMN IN THE PRESENCE OF DIFFERENT DIVALENT CATIONS

Cation (0.001 M)	*Retention (%)*
No cation added	7
Mg^{2+}	95
Mn^{2+}	100
Zn^{2+}	100
Ni^{2+}	100
Ca^{2+}	100
Hg^{2+}	100
Arginine (0.005 *M*)	100

It is clear from Table III that 7.0 % retainable DNA after equilibration with various cations becomes 100 % retainable on the IR-120 Al^{3+} column. A similar improvement in retainability was also produced by 0.005 *M* arginine.

Polysaccharides, proteins, RNAs and DNAs are known to form intimate associations with metal ions in biological systems[41]. Proteins also form complexes or conjugates with DNA, either as such or with the aid of a cation. It is possible, therefore, that metal ions have an important role in the structure and hence in the expression of proteins or nucleic acids. Their presence, therefore, is unlikely to be a casual impurity or a stray association with these macromolecules.

The reason why these other divalent cations, Mn^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+} and Hg^{2+}, show similar behaviour to that of Mg^{2+} can be explained by taking into consideration their ionic radii and ionisation potentials, which may play a significant role in the conferring of particular conformations.

Metal ions induce slow intramolecular changes. Mg^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+} have almost identical ionic radii and ionisation potentials. They can, therefore, be expected to produce similar effects. It is difficult, however, to explain the effects of Ca^{2+} and Hg^{2+} on the same basis, as their ionic radii are greater than the others.

It is important to note that 0.005 *M* arginine showed a behaviour similar to Mg^{2+} by improving the retention of buffalo kidney DNA. Similar results have been found by other workers[42–45].

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REFERENCES

1 S. KIT, *Arch. Biochem. Biophys.*, 87 (1960) 324.
2 S. KIT, *Arch. Biochem. Biophys.*, 87 (1960) 334.
3 A. BENDICH, H. B. PAHL AND S. M. BEISER, *Cold Spring Harbor Symp. Quant. Biol.*, 21 (1956) 31.
4 E. E. POLLI AND K. V. SHOOTER, *Biochem. J.*, 69 (1958) 398.
5 A. BENDICH, P. J. RUSSELL, JR., AND G. B. BROWN, *J. Biol. Chem.*, 203 (1953) 305.
6 A. BENDICH, H. B. PAHL, G. C. KORNGOLD, H. S. ROSENKRANZ AND J. R. FRESCO, *J. Amer. Chem. Soc.*, 80 (1958) 3949.
7 C. F. CRAMPTON, R. LIPSHITZ AND E. CHARGRAFF, *J. Biol. Chem.*, 211 (1954) 125.
8 H. G. DUBUY, C. F. T. MATTERN AND F. L. RILEY, *Biochim. Biophys. Acta*, 123 (1966) 298.
9 N. SUEOKA AND TS'AL-YING CHENG, *J. Mol. Biol.*, 4 (1962) 161.
10 M. SMITH AND D. B. QUAYLE, *Nature*, 200 (1963) 676.
11 M. SMITH, *J. Mol. Biol.*, 9 (1964) 17.
12 L. MINDICH AND R. D. HOTCHKISS, *Biochim. Biophys. Acta*, 80 (1964) 73, 93.
13 R. M. KOTHARI, *Chromatogr. Rev.*, 12 (1970) 127.
14 R. M. KOTHARI, *J. Chromatogr.*, 52 (1970) 119.
15 M. G. SEVAG, D. B. LACKMAN AND J. SMOLENS, *J. Biol. Chem.*, 124 (1938) 425.
16 R. M. KOTHARI, *Ph. D. Thesis*, Poona University, 1968.
17 K. BURTON, *Biochem. J.*, 62 (1956) 315.
18 F. R. FRANKEL AND C. F. CRAMPTON, *J. Biol. Chem.*, 237 (1962) 3200.
19 R. M. KOTHARI, *J. Chromatogr.*, 54 (1971) 239.
20 R. M. KOTHARI, *J. Chromatogr.*, 53 (1970) 580.
21 S. KIT, *J. Biol. Chem.*, 235 (1960) 1756.
22 K. C. SMITH AND H. S. KAPLAN, *Fed. Proc.*, 18 (1959) 507.
23 K. C. SMITH, S. REBHUN AND H. S. KAPLAN, *Anal. Biochem.*, 1 (1960) 202.

24 N. KONDO AND S. OSAWA, *Nature*, 183 (1959) 1602.
25 R. LANGRIDGE, W. E. SEEDS, H. R. WILSON, C. W. HOOPER, M. H. F. WILKINS AND L. D. HAMILTON, *J. Biophys. Biochem. Cytol.*, 3 (1957) 767.
26 G. FELSENFELD, D. R. DAVIES AND A. RICH, *J. Amer. Chem. Soc.*, 79 (1957) 2023
27 M. FUJIOKA AND I. LIEBERMAN, *J. Biol. Chem.*, 239 (1964) 1164.
28 F. KEIICHIRO, W. E. C. WACKER, R. DRUGAN, A. F. BARTHOLOMAY AND B. L. VALLEE, *Proc. Nat. Acad. Sci.*, 46 (1960) 1298.
29 M. REVEL AND H. H. HIATT, *J. Mol. Biol.*, 11 (1965) 467.
30 N. SILMAN, M. ARTMAN AND H. ENGELBERG, *Biochim. Biophys. Acta*, 103 (1965) 231.
31 B. J. MCCARTHY, *Biochim. Biophys. Acta*, 55 (1962) 880.
32 H. SUZUKI AND Y. HAYASHI, *Biochim. Biophys. Acta*, 87 (1964) 610.
33 W. E. RAZZELL, *J. Biol. Chem.*, 238 (1963) 3053.
34 R. ROLFE, *J. Mol. Biol.*, 4 (1962) 22.
35 R. M. KOTHARI, Unpublished results.
36 B. N. AMES AND D. T. DUBIN, *J. Biol. Chem.*, 235 (1960) 769.
37 L. F. CAVALIERI, A. ANGELOS AND M. E. BALIS, *J. Amer. Chem. Soc.*, 73 (1951) 4902.
38 E. BORENFREUND, E. FITT AND A. BENDICH, *Nature*, 191 (1961) 1375.
39 A. BENDICH AND H. S. ROSENKRANZ, *Prog. Nucleic Acid Res.*, 1 (1963) 223.
40 R. ÖSTERBERG, *Nature*, 179 (1957) 476.
41 B. G. MALMSTROM AND A. ROSENBERG, *Adv. Enzymol.*, 21 (1959) 130.
42 B. D. MEHROTRA AND H. R. MAHLER, *Biochim. Biophys. Acta*, 91 (1964) 78.
43 P. Y. CHENG, *Biochim. Biophys. Acta*, 102 (1965) 314.
44 R. BARBER AND M. NOBLE, *Biochim. Biophys. Acta*, 123 (1966) 205.
45 J. E. LEVESON AND A. R. PEACOCKE, *Biochim. Biophys. Acta*, 123 (1966) 329.
46 E. OTAKA, S. OSAKIA, Y. OOTA, A. ISHIHAMA AND H. MITSUI, *Biochim. Biophys. Acta*, 55 (1962) 310.