Journal of Chromatography, 198 (1980) 247–255
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,023

METAL CHELATE AFFINITY CHROMATOGRAPHY

I. INFLUENCE OF VARIOUS PARAMETERS ON THE RETENTION OF NUCLEOTIDES AND RELATED COMPOUNDS

PATRICK HUBERT* and JERKER PORATH*
Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala (Sweden)
(First received May 16th, 1980; revised manuscript received June 12th, 1980)

SUMMARY

The influence of various parameters, such as pH, ionic strength and temperature, on the retention of different nucleotides and related compounds on copper chelate gels has been investigated in order to understand the respective roles played by the different solute constituents (*i.e.*, heterocyclic bases, sugars and phosphate groups) in the interaction and to define optimal conditions for subsequent application to the fractionation of oligo- and polynucleotides.

INTRODUCTION

The investigation of the mode of action of biological macromolecules (proteins, nucleic acids, polysaccharides, etc.) has been hampered for a long time by the lack of convenient, rapid and high-yield techniques for the isolation of these substances in a pure form. Although affinity chromatography and related methods have afforded considerable improvements during the last decade, the search for purification techniques based on new principles still remains a very challenging motivation.

The observation that amino acids and nucleotides are able to form specific complexes with metallic cations and, more generally, with electron-acceptor ligands, prompted the development of two new fractionation procedures: electron donor–acceptor (or charge-transfer) chromatography and metal chelate affinity chromatography (ligand exchange chromatography).

The latter technique has been mainly studied for protein and amino acid fractionation purposes[1–6] and has so far received only limited attention in the nucleotide field[4,7,8]. However, during the last 25 years, much work has been devoted to the study of interactions between metallic cations, especially those belonging to the first transition period, and ribo- and deoxyribonucleic acids and their constituents (bases, nucleosides,

* Present address: Laboratoire de Chimie Physique Macromoleculaire, ENSIC, 1 Rue Grandville, 54000 Nancy, France.

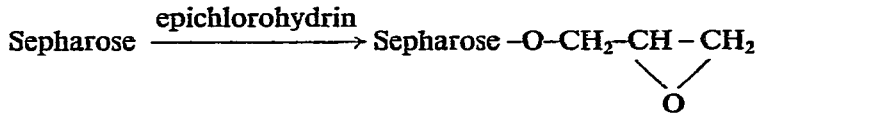
0021-9673/80/0000–0000/$02.25

nucleotides). It turns out that all of the physico-chemical techniques employed (spectrophotometry[9–12], nuclear magnetic resonance spectroscopy[13–16] and polarography[17]) provide evidence that these compounds interact strongly with metals to form complexes involving the phosphate group and/or the heterocyclic bases, depending on the experimental conditions. Further, these interactions are specific, as striking differences occur between the purines and the pyrimidines and even among different compounds of the same class (adenine and guanine derivatives, for instance).

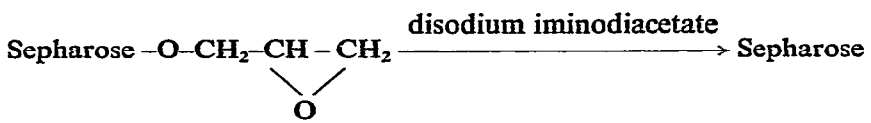
These observations led us to investigate whether these specific interactions might be used for nucleic acid fractionation on the basis of base composition by chromatography on metal chelate adsorbents, and to this end we have studied the influence of different parameters on the adsorption behaviour of nucleotides on copper chelate gels.

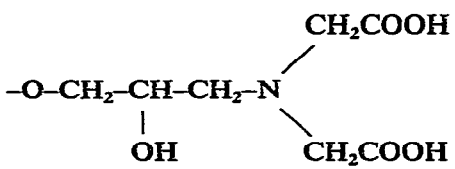
EXPERIMENTAL

The gel used for the metal chelate affinity chromatography experiments was prepared according to the following scheme:



(1)





(2)

The epoxy-activated Sepharose 6B (125 g), prepared from Sepharose 6B (Pharmacia, Uppsala, Sweden) according to the published procedure[18], was suspended in 100 ml of 2 *M* sodium carbonate solution and the mixture was shaken gently for 24 h in a water-bath (60–65°C) after the addition of 20 g of sodium iminodiacetate (Fluka, Buchs, Switzerland). After cooling the reaction vessel, the gel was carefully washed on a glass filter funnel successively with water, 0.1 *M* sodium carbonate solution, 0.01 *M* sodium acetate solution and finally water. The biscarboxymethylamino-Sepharose 6B thus obtained was kept in a cold room after addition of sodium azide (4 parts per 10,000) to prevent bacterial growth. The amount of iminodiacetic acid bound, as determined by nitrogen analysis, was 1017 μmole per gram of dry gel.

Chromatographic procedures

The biscarboxymethylamino-Sepharose 6B was packed into columns (13–15 cm × 1 cm I.D., $V_T \approx$ 10–12 ml) and washed with water prior to loading with 20 m*M* copper(II) sulphate solution (Merck, Darmstadt, G.F.R.). After saturation had been reached, excess of copper was removed by washing with 2 bed volumes of water. At this stage, the amount of Cu^{2+} bound was 899 μmole per gram of dry gel. The copper chelate adsorbent was then washed with the desired buffer until all loosely bound Cu^{2+} had been eluted.

The nucleotides and their derivatives (Sigma, St. Louis, MO, U.S.A.) were dissolved in water to a concentration of 2.5 m*M*, except for the poorly soluble adenine and guanine, which were dissolved to saturation by careful heating and were injected in 400-μl samples.

The retention of solutes is expressed in terms of reduced elution volumes (V_E/V_T), V_E being the elution volume of the maximum of the peak obtained either by recording (Altex UV minotor, RDK recorder) at 280 nm (cell 0.25 cm) or by measuring the absorbance at 260 nm (Beckman 25 spectrophotometer) of the effluent fractions collected.

In all chromatographic experiments the flow-rate was maintained at 11.7 ml/h by a peristaltic pump (Pharmacia).

RESULTS

As a preliminary control, the retention of different nucleotide derivatives was tested on Sepharose 6B and biscarboxymethylamino-Sepharose 6B in Tris–hydrochloric acid and citrate–phosphate buffers (0.05 *M*, pH 7.0, 1 *M* sodium chloride). In all instances the reduced elution volumes obtained were in the range 1.2–1.4. Hydrophobic interactions with the gel, which are likely to occur at these high salt concentrations, may account for the slight retardation observed.

Influence of buffer composition

The retention of all of the solutes tested was strongly affected by the composition of the eluting buffers, as shown in Table I.

With buffers containing substituted amino groups, the adsorption of solutes increased as the p*K* of the base involved in the buffer was decreased. This may be interpreted in terms of competition between the nucleotides and the buffer components. In fact, the metal chelate gel interacts with electron-donor solutes and therefore tends to become increasingly "saturated" during equilibration as the nucleophilic character of the buffer constituents is increased: NH_4Cl, p*K* 9.25 > Tris, p*K* 8.08 > N-ethylmorpholine, p*K* 7.65. The interaction of copper with the nucleotides is therefore less favoured as the Cu^{2+} ion becomes increasingly engaged with the electron-donor components of the buffer.

According to this interpretation, one would expect greater retardation when the elution is performed with low p*K* buffers. However, with the chosen citrate–phosphate buffer (citric acid, higher p$K = 6.39$), the opposite result was obtained, probably because citrate is a well known chelating agent for metallic cations, and is therefore able to prevent the metal from any further interaction with the solutes tested.

TABLE I

INFLUENCE OF BUFFER COMPOSITION (pH 7.0)

Compound	*Citrate–phosphate, 0.05 M/NaCl, 1 M*	*Tris–HCl, 0.05 M/NaCl, 1 M*	*Tris–HCl, 0.05 M/NH_4Cl, 1 M*	*Ethylmorpholine–acetic acid, 0.1 M/NaCl, 1 M*
Adenine	>20	>20	—	—
Adenosine	2.30	6.10	2.50	8.55
AMP	1.70	5.75	1.95	9.65
Guanine	—	>20	—	—
Guanosine	1.85	3.95	2.15	4.50
GMP	1.50	4.10	1.95	5.75
Thymine	1.35	1.35	1.35	1.40
Uracil	—	1.10	—	—
Cytosine	—	1.40	—	—

Dependence of the retention on the ionic strength and the nature of electrolytes added to the eluting buffer

The retention of nucleotides and derivatives on copper chelate gels is not based on simple electrostatic interactions, as the V_E/V_T values are actually enhanced by higher salt concentrations and the presence of chaotropic ions, as shown in Tables II–IV.

TABLE II

INFLUENCE OF THE IONIC STRENGTH

Compound	*Tris–HCl, 0.01 M, pH 7.0*	*Tris–HCl, 0.05 M, pH 7.0 + × mole/l NaCl*				
		0 M	*0.1 M*	*0.2 M*	*0.5 M*	*1 M*
AMP	2.35	3.0	4.90	5.65	5.5	5.75
Adenosine	7.80	5.15	6.55	6.20	5.75	6.10
GMP	2.05	2.75	4.10	4.20	3.5	4.1
Guanosine	4.6	3.65	4.20	4.0	3.6	3.9
Thymidine	—	1.3	1.2	1.15	1.15	1.2

TABLE III

INFLUENCE OF THE NATURE OF CATIONS (1 *M*) INCLUDED IN THE BUFFER (TRIS–HCl 0.05 *M*, pH 7.0)

Compound	*LiCl*	*NaCl*	*KCl*
AMP	4.4	5.75	8.5
Adenosine	5.1	6.1	6.6
GMP	3.5	4.1	5.7
Guanosine	3.9	3.9	4.1
Thymidine	1.4	1.4	1.4

The adsorption increases with the ionic strength up to a plateau at 0.1–0.2 *M* sodium chloride. Further, the retention seems to be directly related to the size of the ions involved in the eluting buffer (Li < Na < K and F < Cl < Br). KSCN, the

TABLE IV

INFLUENCE OF THE NATURE OF ANIONS (1 *M*) INCLUDED IN THE BUFFER (TRIS–HCl 0.05 *M*, pH 7.0)

Compound	*KF*	*KCl*	*KBr*	*KSCN*
AMP	4.7	8.15	8.7	1.4
Adenosine	4.15	6.1	6.6	1.8
GMP	4.95	5.25	5.3	1.25
Guanosine	3.75	3.7	3.85	1.6
Thymidine	1.3	1.3	1.3	1.3

most bulky, plays a different role, owing to its ability to react with the Cu^{2+} ions in solution.

The effect of electrolytes on the retention can be interpreted as a modification of the affinity of Cu^{2+} for its solvation water molecules; the weakening of the forces between Cu^{2+} and water induced by salt consequently facilitates the adsorption of nucleotides.

The results obtained in Tris–hydrochloric acid of low molarity (0.01 instead of 0.05 *M*) deserve special attention (Table II). From the effect of ionic strength discussed above, one would expect a slight decrease in the retention in this buffer. On the other hand, when the molarity of the buffer is lowered, the capacity of the electron-donor groups of the Tris to saturate the copper is decreased (*cf.*, Table I). More binding sites are available for the solutes, which can thus be retained more efficiently. Further, with molecules bearing a phosphate group (AMP, GMP), the low ionic strength may allow the phosphate to participate in the interaction, as pointed out by different workers[10,13–17,19–21].

The fact that nucleosides are effectively more retained supports the hypothesis according to which the lesser saturation of Cu^{2+} by the buffer components plays a major role in the retention. On the other hand, the decreased retention of nucleotides suggests that the repulsive electrostatic interactions with the matrix have, in this instance, an even more important effect.

Contribution of hydrophobic interactions in the retention process

Hydrophobic interactions with the matrix and the spacer arm are involved in the adsorption phenomenon. This is demonstrated by the fact that additives that weaken hydrophobic and other water structure-dependent interactions (*e.g.*, ethylene glycol) cause non-neglectable decreases in the V_E/V_T values (Table V).

TABLE V

INFLUENCE OF ETHYLENE GLYCOL ON RETENTION

Buffer: Tris–HCl, 0.05 *M*, pH 7.0.

Compound	*Ethylene glycol concentration (%)*	
	0	*50*
AMP	3.0	2.40
Adenosine	5.15	3.35
GMP	2.75	2.40
Guanosine	3.65	2.80

However, even in 50% ethylene glycol the retention still remains very high. This point, and the fact that the adsorption does not increase with increasing temperature (Table VI), lead to the conclusion that water structure-dependent interactions are not the main factor in the overall retention process.

TABLE VI

INFLUENCE OF TEMPERATURE ON THE RETENTION

Buffer: Tris–HCl, 0.05 *M*, pH 7.0/NaCl, 1 *M*.

Compound	*Temperature (°C)*	
	4	22
AMP	6.10	5.75
Adenosine	8.15	6.10
GMP	4.40	4.10
Guanosine	5.40	3.95

pH dependence

The pH obviously plays a major and very complex role, as it affects not only the nucleophilic character of the buffer components and the electron-donor properties of the nucleotides, but also the metal chelate structure and stability.

The data in Table VII give an example of the tremendous change in retention provoked by a variation of only one pH unit. Also, in citrate–phosphate buffer, 0.05 *M*, 1 *M* sodium chloride, pH 6.0 (results not shown), all of the solutes are readily eluted, including guanine and adenine ($V_E/V_T > 20$ in citrate–phosphate buffer, pH 7.0; *cf.*, Table I).

TABLE VII

DEPENDENCE OF THE INTERACTION ON pH

Buffer: Tris–HCl, 0.05 *M*/NaCl, 1 *M*.

Compound	*pH*	
	7.0	*8.0*
AMP	5.75	1.35
Adenosine	6.10	1.85
GMP	4.10	1.45
Guanosine	3.95	1.80

"Cooperativity" effect

The enhanced adsorption observed on passing from the mono- to the dinucleotides (Table VIII) must be due to multi-point attachment.

At pH 8.0, under conditions where guanine and adenine nucleotides are almost unretarded (*cf.*, Table VII), the presence of two purine rings in the same molecule allows differentiation between adenine and guanine derivatives, although not enough to permit a clear separation on a column of reasonable dimensions.

At pH 7.0, there is a tremendous effect of cooperativity on purine dinucleotides. On the other hand, the presence of a pyrimidine ring in the dinucleotide molecule

TABLE VIII

"COOPERATIVE" EFFECT ON RETENTION

Buffer: Tris–HCl, 0.05 *M*/NaCl, 1 *M*.

pH 7.0				*pH 8.0*			
GMP	4.10	GpG	16	GMP	1.45	GpG	3.9
AMP	5.75	ApA	35	AMP	1.35	ApA	3.6
		GpU	3.55				

does not significantly affect the retention, which remains similar to that of the corresponding purine mononucleotide.

The practical application of these observations was demonstrated in a fractionation experiment in which an artificial mixture consisting of GpU, GpG and ApA was applied on a short copper chelate gel column (length 2.7 cm, V_T 2.1 ml). The elution profile obtained reflects the complete resolution of the three compounds (Fig. 1).

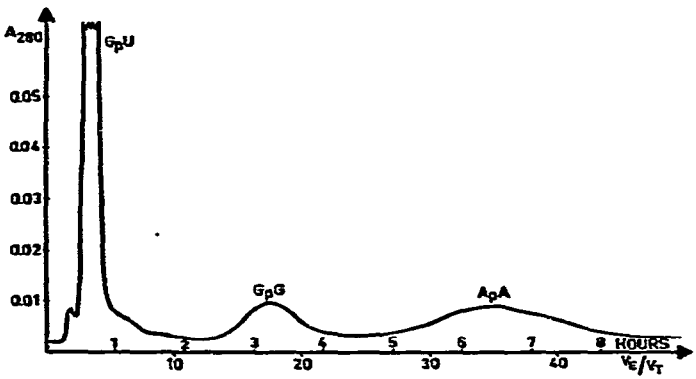


Fig. 1. Elution profile of a mixture consisting of GpU (*ca.* 0.15 mg), GpG (*ca.* 0.1 mg) and ApA (*ca.* 0.2 mg) chromatographed on copper chelate biscarboxymethylamino-Sepharose 6B. Column: 2.7 × 1 cm I.D., V_T 2.1 ml. Eluting buffer: Tris–HCl, 0.05 *M*, pH 7.0/NaCl, 1 *M*. Flow-rate: 11.7 ml/h. Profile obtained on recording at 280 nm (cell 0.25 cm).

DISCUSSION

The retention of nucleotides and derivatives on copper chelate gels appears to be complex. Electron donor–acceptor, hydrophobic and presumably also electrostatic interactions are all involved in the overall adsorption process. According to the data obtained, the retentions increase in the order purines ≫ pyrimidines, with adenine > guanine and cytosine ≈ thymidine ≈ uracil, and generally bases ≫ nucleosides ≥ nucleotides.

These observations are in good agreement with results reported in the literature for species free in solution, except that guanine nucleotides and nucleosides are generally claimed to bind copper more efficiently than adenine derivatives[22]. Although both phosphate groups and heterocyclic bases have been proved to be involved in

the binding to copper when free in solution[9,13,19,22–24], our data with immobilized Cu^{2+} suggest that the adsorption proceeds mainly via interactions with the heterocyclic bases. Under our conditions a major role of phosphate group in the interaction with the metal ion seems unlikely. In fact, it is either ruled out by high salt conditions or cancelled owing to the repulsion by the negatively charged groups borne by the gel.

The fact that the bases are far more strongly adsorbed than the corresponding nucleosides and nucleotides emphasizes the negative role played by the sugar and phosphate moieties. As the formation of the glycosidic linkage to ribose or esterification at the 5'-ribose position seems to cause no major charge redistribution in the adenine ring[25], it appears therefore that steric hindrance and modification of the organization of water molecules around the solutes must be the main explanations for the effect observed.

Recently, Chow and Grushka[26] have shown that nucleosides and nucleotides can be separated by high-performance liquid chromatography on columns of silica with cobalt-complexed ligands. Together with our findings, their study indicates the potential applications of metal chelate adsorbents for nucleotide and oligonucleotide fractionation. As the main interaction involved in the adsorption process concerns the heterocyclic bases of these compounds, one can reasonably expect an extension of this system to other small heterocyclic molecules such as vitamins, hormones and drugs.

From the very high retention observed with dinucleotides one must expect considerable adsorption of solutes capable of multi-point attachment to the metal chelate gels. In connection with protein and nucleic acid purification, this may present difficulties during pH gradient elution, owing to the simultaneous leakage of metal ions[2,3,6]. The study of pre-washing conditions, allowing a subsequent enhanced stability of the chelate over a wide pH range, which is at present in progress, will complement the present work and provide the basic information required for the purification of nucleic acids by metal chelate affinity chromatography.

ACKNOWLEDGEMENTS

P.H. thanks the NFR and the CNRS for their financial support during his stay in Uppsala. We acknowledge a grant from The Swedish Work and Environmental Fund (Arbetarskyddsfonden). We are also grateful to Dr. David Eaker for linguistic revision of the manuscript, and to Mrs. Inga Johansson for secreterial help.

REFERENCES

1 V. A. Davankov and A. V. Semechkin, *J. Chromatogr.*, 141 (1977) 313.
2 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
3 B. Lönnerdal, J. Carlsson and J. Porath, *FEBS Lett.*, 75 (1977) 89.
4 A. Goździcka-Józefiak and J. Augustyniak, *J. Chromatogr.*, 131 (1977) 91.
5 J. P. Lebreton, *FEBS Lett.*, 80 (1977) 351.
6 V. G. Edy, A. Billiau and P. de Somer, *J. Biol. Chem.*, 252 (1977) 5934.
7 R. M. Kothari, *Chromatogr. Rev.*, 12 (1970) 127.
8 D. W. Gruenwedel and J. C. C. Fu, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 2002.
9 M. Fiskin and A. Beer, *Biochemistry*, 4 (1965) 1289.
10 J. P. Schreiber and M. Daune, *Biopolymers*, 8 (1969) 139.
11 Y. A. Shin, *Biopolymers*, 12 (1973) 2459.

12 J. M. Rifkind, Y. A. Shin, J. M. Heim and G. L. Eichorn, *Biopolymers*, 15 (1976) 1879.
13 M. Cohn and T. R. Hughes, *J. Biol. Chem.*, 237 (1962) 176.
14 G. L. Eichorn, P. Clark and E. D. Becker, *Biochemistry*, 5 (1966) 245.
15 N. A. Berger and G. L. Eichorn, *Biochemistry*, 10 (1971) 1847.
16 N. A. Berger and G. L. Eichorn, *Biochemistry*, 10 (1971) 1857.
17 D. Bach and I. R. Miller, *Biopolymers*, 5 (1967) 161.
18 J. Porath and L. Sundberg, *Protides Biol. Fluids*, 18 (1970) 401.
19 G. L. Eichorn and P. Clark, *Proc. Nat. Acad. Sci. U.S.*, 53 (1965) 586.
20 H. Britzinger, *Biochim. Biophys. Acta*, 77 (1963) 343.
21 H. Moll, P. W. Schneider and H. Britzinger, *Helv. Chim. Acta*, 47 (1964) 1837.
22 E. Frieden and J. Alles, *J. Biol. Chem.*, 230 (1957) 797.
23 J. Eisinger, R. G. Shulman and B. M. Szymanski, *J. Chem. Phys.*, 36 (1962) 1721.
24 G. L. Eichorn, in G. L. Eichorn (Editor), *Inorganic Biochemistry*, Vol. II, Elsevier, Amsterdam, 1973, p. 1191.
25 D. B. Boyd, *Theor. Chim. Acta*, 14 (1969) 402.
26 F. K. Chow and E. Grushka, *J. Chromatogr.*, 185 (1979) 361.