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CHROMATOGRAPHIC BEHAVIOUR OF TERNARY NUCLEOTIDE-METAL CATION–AMINO ACID COMPLEXES

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SUMMARY

The ternary complex between a nucleotide, a metal cation and an amino acid can be viewed as a simple model of the active centre of certain enzymatic systems. Attempts have been made to characterize this important complex chromatographically. When a purine nucleotide was injected into a mobile phase containing a buffer plus a metal cation and an amino acid, the retention of the nucleotide was vastly different from that in a mobile phase containing only one of these components. The synergistic effect of the metal cation and the amino acid is an indication of the formation of the ternary complex in the chromatographic column. Mobile phases containing leucine and one of the following cations were investigated: Cu^{2+}, Cd^{2+}, Zn^{2+} or Mn^{2+}. The order of retention increase, due to the presence of the metal cation, was Cu(II) > Cd(II) > Zn(II) > Mn(II). The effect was more pronounced with the adenosine nucleotides. Within a nucleotide family, the retention of the diphosphate changed the most. In certain cases, the chromatographic data enabled calculation of the formation constants of the ternary complexes, which were found to be in excellent agreement with the few literature values available.

INTRODUCTION

Many enzymes require divalent metal cations for activation. Among these are the enzymes which act on nucleotides, *e.g.*, the adenylate systems or the kinase systems. Considerable effort has been made to characterize the nature of the interactions between the metal cations and the nucleotide and/or the enzyme[1]. The current interest in nucleotide–metal cation–amino acid (NMA) ternary complexes is attributed to the fact that these complexes can be regarded as simple models for the naturally occurring active centres of some substrate–metal–enzyme systems.

While there is a substantial body of literature dealing with ternary complexes of nucleotides metal ions and a third component[2–11], very little information is available with regard to NMA ternary complexes. In view of their importance, the paucity of published work concerning the characterization of these complexes is surprising. The papers of Sigel and co-workers[2,5,8] are, to the best of our knowledge, the only ones to discuss such ternary complexes in aqueous solutions. Using techniques such as NMR, UV and circular dichroism (CD) spectroscopy, and potentiometric mea-

surements, these workers studied systems containing a nucleotide, an amino acid and a metal cation. The results of these investigations seem to indicate the existence of ternary complexes, in which the metal cation acts as a bridge between the amino acid and the phosphate part of the nucleotide. The metal ion is thought to mediate the interactions between the nucleotide base and the alkyl part of the amino acid. The whole complex, in fact, may be viewed as a metal ion-bridged adduct. The hydrophobic interactions thus induced may enhance the stability of the complex and, more importantly perhaps, may create specific environments that are of biological importance.

The effective charge of the ternary complex, if such exists, is expected to be different from that of the nucleotide alone. In addition, because of the alkyl moiety of the amino acid and the base part of the nucleotide, the whole complex should present a more hydrophobic surface to its surroundings. Therefore, the retention of the ternary complex should be different from that of the nucleotide alone. Thus, chromatography could be used to investigate the properties of the complex. The aim of our communication is to present preliminary results, indicating the feasibility of using chromatographic data to identify and characterize the ternary complex, NMA.

Before a study dealing with the ternary complex can be launched, the binary interactions between the various components of the complex should be understood. The chromatographic behaviour of amino acids in the presence of metal cations is well known[12]. Similarly, Cohen and Grushka[13] recently characterized the retention behaviour of nucleotides and its dependence on various metal cations. The effect of amino acids on the retention of nucleotides will be discussed in this work. Thus, the foundations have been laid for an examination of the chromatographic properties of nucleotides in the presence of both amino acids and metal cations. For that purpose, nucleotides were injected into four different mobile phases containing: (a) neat acetate buffer (pH 5.6), (b) buffer and metal cation, (c) buffer and amino acid and (d) buffer with metal cation and amino acid. The difference in the retention of the solutes was interpreted as an indication of interactions between the nucleotides and the mobile phase component(s). The results support the hypothesis of the formation of the ternary NMA complex in the chromatographic column. In addition, the formation constants of the ternary complexes were calculated, whenever possible, from the concentration dependence of the retention.

EXPERIMENTAL

Reagents

All nucleotides and amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals, including metal sulphates, were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). These were all of analytical regent AR grade. The water used for the mobile phase was purified in our laboratory using a Seral purifying system, resulting in water having a resistivity > 18 MΩ.

Apparatus

The experiments were performed with a SP8000 liquid chromatograph (Spectra-Physics, Mountain View, CA, U.S.A.) equipped with a thermostatted oven, a data system and a variable-wavelength UV detector. The column (250×4 mm I.D.) was a LiChrosorb RP-18 cartridge (Merck, Darmstadt, F.R.G.).

Procedure

Chromatography was carried out with 0.1 M acetate buffer (pH 5.6), containing the metal cations and/or the amino acids. Prior to use, the acetate buffers and all the samples were filtered through a 0.3-μm Millipore Type PH membrane filter. The mobile phase flow-rate was kept constant at 1 ml/min the column temperature was maintained at 37°C and the detector was set at 260 nm. The solutes were prepared in micro test-tubes and were kept frozen at −23°C to prevent decomposition.

RESULTS AND DISCUSSION

Before describing the results, the choice of metal cations and amino acid used in this study should be explained. To demonstrate the existence of the ternary complex, leucine (leu) was chosen as the amino acid additive. It was felt that leu, being a moderately hydrophobic amino acid, should enhance the stability of the ternary complex, if it exists. The cations examined [Cu^{2+}, Zn^{2+}, Cd^{2+} and Mn^{2+}] were chosen for their different coordinating abilities with nucleotides[5,13]. Only the purine nucleotides were investigated, since their ternary complexes are believed to be more stable than those of the pyrimidine nucleotides.

The capacity factors of the adenosine and guanosine nucleotides, in the four mobile phases, are given in Tables I–III. In Table I, k_0' indicates the values of the capacity factors in a neat acetate buffer mobile phase. In addition, Table I lists the capacity factors of the nucleotides in the presence of various concentrations of leu. The retention of the nucleotides is practically unaffected by the presence of the amino acid, indicating little or no interactions between the two species. Mitchell and Sigel[4] reached the same conclusions, based on their NMR investigation. Metal cations, on the other hand, can have a large influence on the retention of nucleotides[13]. Table III shows the effect of Cu^{2+} at various concentrations. In all cases, increasing the copper ion concentration increases the retention of the solutes. The effect is most pronounced with the diphosphate nucleotides (NDP), and least pronounced with the monophosphate nucleotides (NMP). This is in agreement with the results obtained previously in our laboratory[13].

TABLE I

CAPACITY FACTORS OF NUCLEOTIDES, INJECTED INTO A 0.1 M ACETATE BUFFER (pH 5.6) CONTAINING VARIOUS CONCENTRATIONS OF LEUCINE.

k_0' indicates the capacity factors in acetate buffer only.

Solute	k_0'	Conc. of leu × 10^5 M				
		4	8	16	50	100
AMP	2.10	2.11	2.15	2.14	2.14	2.09
ADP	0.64	0.67	0.67	0.68	0.68	0.69
ATP	0.40	0.49	0.49	0.52	0.54	0.58
GMP	0.60	0.62	0.63	0.64	0.64	0.63
GDP	0.19	0.18	0.20	0.20	0.20	0.18
GTP	0.07	0.07	0.08	0.09	0.09	0.10

TABLE II

CAPACITY FACTORS OF NUCLEOTIDES INJECTED INTO A 0.1 M ACETATE BUFFER (pH 5.6) CONTAINING VARIOUS CONCENTRATIONS OF Cu^{2+}

Solute	Concn. of Cu^{2+} × 10^5 M			
	4	8	16	32
AMP	2.12	2.13	2.13	2.20
ADP	1.09	1.37	1.69	1.89
ATP	0.50	0.54	0.59	0.79
GMP	0.67	0.69	0.73	0.81
GDP	0.25	0.38	0.56	0.67
GTP	0.07	0.10	0.11	0.12

TABLE III

CAPACITY FACTORS OF NUCLEOTIDES, INJECTED INTO A 0.1 M ACETATE BUFFER (pH 5.6) CONTAINING VARIOUS CONCENTRATIONS OF Cu^{2+} PLUS LEUCINE IN EQUIMOLAR QUANTITIES

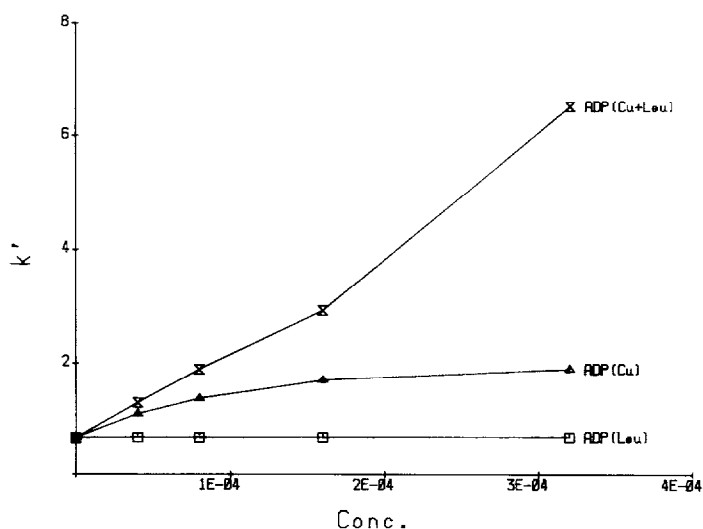| Solute	Concn. of Cu^{2+} + leu × 10^5 M			
	4	8	16	32
AMP	2.19	2.19	2.20	2.43
ADP	1.30	1.90	2.92	6.51
ATP	0.61	0.80	1.13	1.93
GMP	0.72	0.81	0.90	1.45
GDP	0.53	0.85	1.21	3.49
GTP	0.08	0.16	0.26	0.48

Fig. 1. the dependence of the capacity factor of ADP on the concentration (M) of leu or Cu^{2+} or leu + Cu^{2+} in a 0.1 M acetate buffer (pH 5.6) as mobile phase. In the last case, the concentration of leu was always equal to that of Cu^{2+}.

Table II shows the k' values as a function of the concentration of both Cu^{2+} and leu. The large effect is obvious. In fact, the increase in the retention, as compared to that in the neat buffer mobile phase, is greater than the sum of the changes in retentions in Tables I and II; *i.e.*, the effect of the two additives is synergistic. Fig. 1 shows this vividly in the case of ADP. The great increase in the capacity factors of the nucleotides in the presence of both Cu^{2+} and leu indicates strong interactions between the three components. The presence of each of the three species is required if large retention changes are to occur. This requirement is taken as an indication of the formation of a ternary complex in the chromatographic column.

Table III and Fig. 1 show some interesting trends. While all nucleotides are affected by the presence of Cu^{2+} (or any other cation studied) and leu, the extent of the effect is not equal for all solutes. In general, the relative change in retention, as measured by the ratio k'/k_0', is greater in the case of the guanosine nucleotides. Within a family, the NDPs show the greatest change in retention. The smallest change occurred in the case of the NMPs. This is similar to the trend observed when only Cu^{2+} was present in the mobile phase (*c.f.*, Table II). The large effect on the retention of the NDPs can be attributed to two causes. Under the conditions of the present study, the NTPs have a -3 charge, the NDPs a -2 charge and the NMPs a -1 charge. Thus, the divalent metal ion neutralizes the charge more effectively in the case of the NDPs. Moreover, in the case of the ternary complex, the alkyl side chain of the amino acid can interact with the basic part of the nucleotide. For steric reasons, this interaction is favoured when the nucleotide is a diphosphate. Fig. 2 illustrates this with space-filling models. The alkyl side chain is closer to the base for NDP than for NTP. As a consequence, the size of the cavity in the centre of the complex is much greater for NTP, a fact which makes for better solvation by the aqueous mobile phase. The NDP possesses, proportionately, a larger hydrophobic surface, a therefore, its retention time is longer. As a result, the retention order is reversed, as shown in Fig. 3. In a neat buffer mobile phase, the retention order is NTP < NDP < NMP, while with Cu^{2+} and leu in the mobile phase the order is NTP < NMP < NDP.

When the Cu^{2+} is replaced by other cations, similar retention patterns for the nucleotides are observed. For example, Table IV shows the capacity factors of the nucleotides as a function of the concentrations of Cd^{2+} and leu. The retention, similar to that in the Cu^{2+} + leu case, increases as the concentration of the additive is increased. However, the rate of increase is much smaller with Cd^{2+} in the mobile phase. A note should be made here with regard to the concentration scale of the two cations. The maximum concentrations in the case of Cu^{2+} + leu was $3.2 \cdot 10^{-4}$ M. At that concentration, the increase in the k' value of, say, GTP was six-fold. When Cd^{2+} is present instead of Cu^{2+}, much greater concentrations must be used in order to see appreciable changes in the retention, and even then, the effect of the cation is much less pronounced. At the highest concentration used, $1.5 \cdot 10^{-3}$ M, the change in the retention of GDP was less by a factor of 3.

If instead of Cu^{2+} or Cd^{2+}, one uses Zn^{2+} or Mn^{2+} the effect on the retention of the nucleotides diminishes still further. This is clearly seen in Fig. 4, where k' values for GDP are plotted as a function of the concentration of leu and Cu^{2+}, Cd^{2+}, Zn^{2+} or Mn^{2+}. In all cases, the retention increases with the concentration of the additives. The extent of increase is in the order $Cu^{2+} > Cd^{2+} > Zn^{2+} > Mn^{2+}$, in agreement with the extent of complex formation as reported by Sigel *et al.*[8]. The
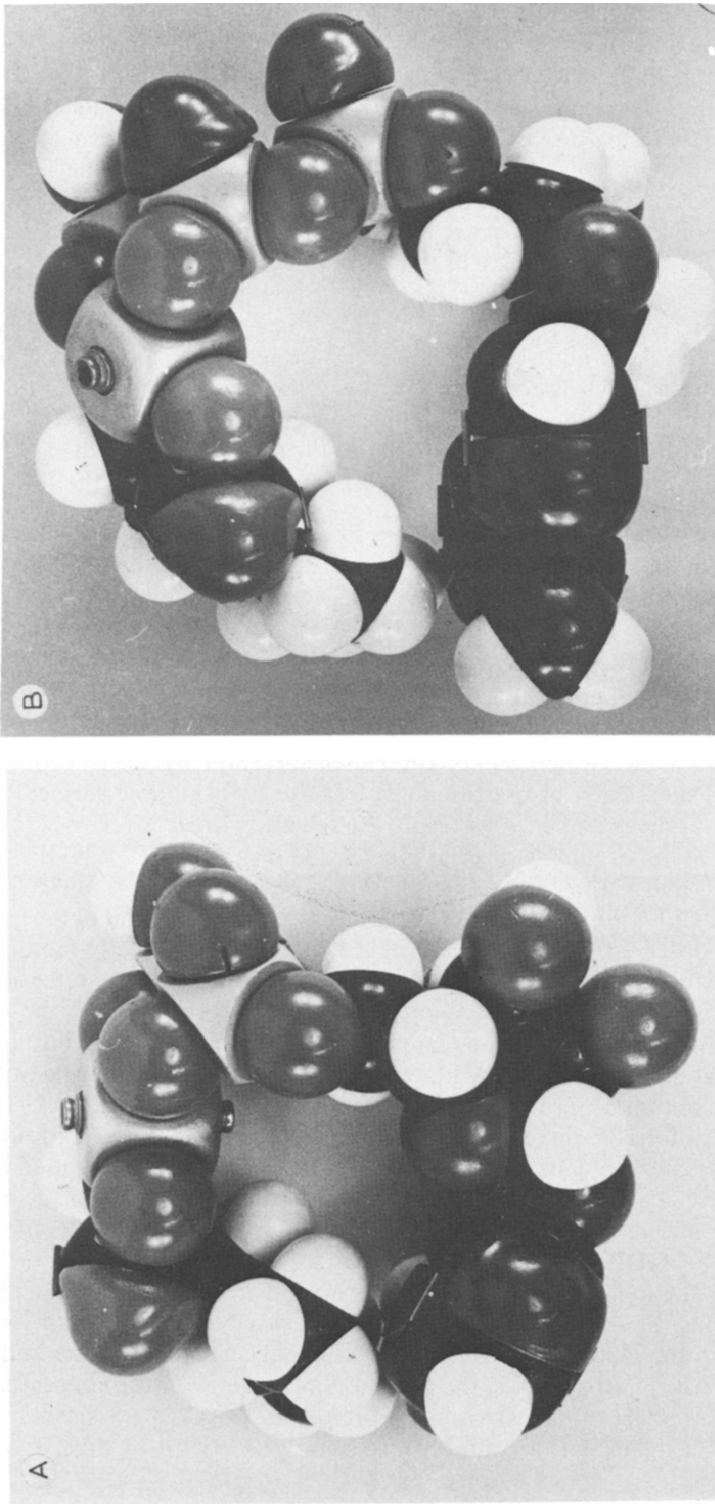
Fig. 2. Space-filling models showing the ternary complex of ADP–Cu^{2+}–leu (A) and of ATP–Cu^{2+}–leu (B). The photographs were taken so as to accentuate the central cavity and the hydrophobic surface of each complex.
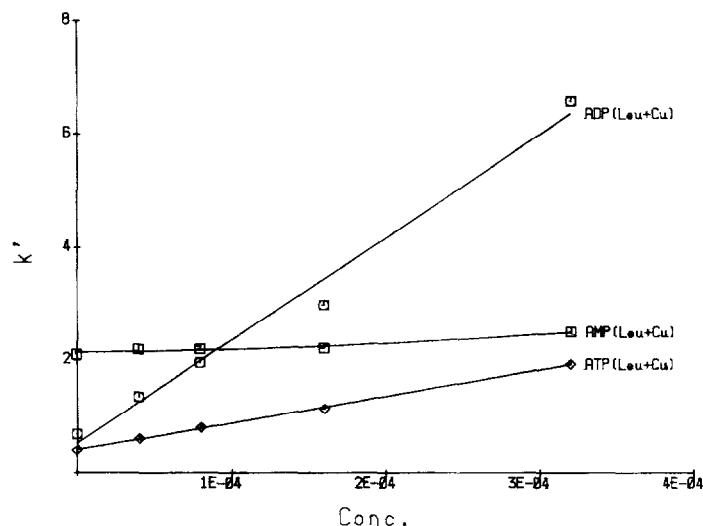
Fig. 3. The dependence of the capacity factors of the three adenosine nucleotides on the concentration (*M*) of leu + Cu^{2+} in a 0.1 *M* acetate buffer (pH 5.6) as mobile phase. In each case, the concentration of the amino acid was equal to that of the metal cation, corresponding to the values on the concentration axis [at different concentrations (*M*)].

trend is perhaps not surprising; it follows, in general, the stability of the metal–nucleotide and metal–amino acid binary complexes.

In view of the importance of the ternary complexes, it would be of interest to measure their formation constants. Little information can be found in the literature regarding these constants. To the best of our knowledge, only Sigel *et al.*[8] have obtained and reported the formation constants of such ternary complexes. Horváth *et al.*[14] and Grushka and Chow[15] have shown that liquid chromatography can be used to measure formation constants. A modification of this approach will be used here in an attempt to utilize some of the data collected for the calculation of these potentially important constants.

TABLE IV

CAPACITY FACTORS OF NUCLEOTIDES, INJECTED INTO A 0.1*M* ACETATE BUFFER (pH 5.6) CONTAINING VARIOUS CONCENTRATIONS OF Cd^{2+} PLUS LEUCINE IN EQUIMOLAR QUANTITIES

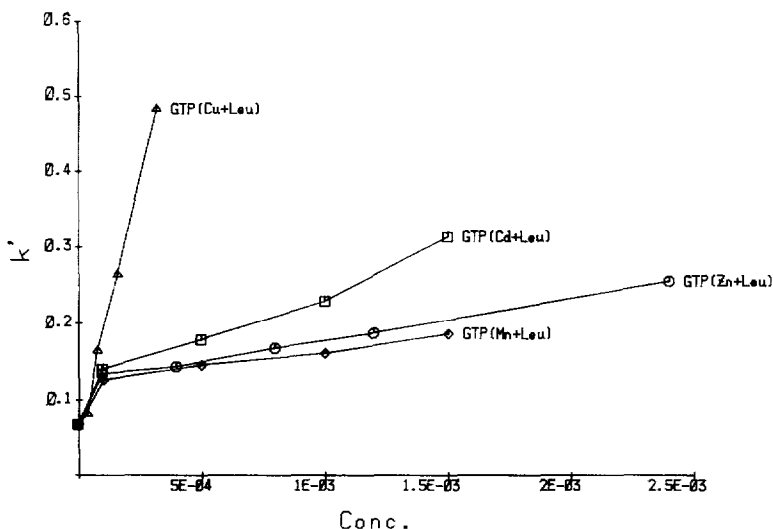| Solute	Concn. of Cd^{2+} + leu × 10^4 M			
	1	5	10	15
AMP	2.20	2.40	2.47	2.65
ADP	1.05	1.18	1.41	1.50
ATP	0.63	0.71	0.90	0.96
GMP	0.73	0.91	1.06	1.15
GDP	0.28	0.55	0.59	0.68
GTP	0.14	0.18	0.28	0.31

Fig. 4. The effect of the nature of the metal ion on the retention of GTP. In addition to the metal ions, the acetate buffer mobile phase contained leu in concentrations equal to those of the cations. The lines serve to connect the experimental points; they are not the result of any fitting procedure.

For the purpose of the present treatment, it will be assumed that leucine and the metal cation form a binary complex in the mobile phase, and that this binary complex is the predominant species in that phase. The injected nucleotides interact with the binary complex, in the mobile phase, to yield a ternary complex. Using these assumptions, the capacity factor of the nucleotides can be expressed as

$$k' = \frac{[Nu]_s + [NuMA]_s}{[Nu]_m + [NuMA]_m} \tag{1}$$

where Nu represents the nucleotides, MA the metal cation–amino acid complex and the subscripts s and m indicate concentrations in the stationary and mobile phase, respectively. From the definition of the capacity factor of the nucleotides in the neat buffer

$$k'_0 = \frac{[Nu]_s}{[Nu]_m} \tag{2}$$

of the complexed nucleotides

$$k'_c = \frac{[NuMA]_s}{[NuMA]_m} \tag{3}$$

and of the formation constant

$$K_f = \frac{[NuMA]}{[Nu][MA]} \tag{4}$$

eqn. 1 can be rewritten as:

$$k' = \frac{k'_0 k'_c K_f[MA]_m}{1 + K_f[MA]_m} \tag{5}$$

The derivation of eqn. 5 assumed, in addition to the qualifications above, that the buffer does not interact with the nucleotides, amino acids or the metal cations, and that the ternary complex is formed only in the mobile phase. Under these conditions, the concentration dependence of the capacity factors of the nucleotides allow the calculation of the formation constants of the ternary complexes. However, to be physically meaningful, the experimental data must meet three main requirements:

(a) Horváth et al.[14] have shown that the concentration of the injected solute should be roughly of the same order of magnitude as the reciprocal of the formation constant, or else the results are unreliable;

(b) the slopes of the concentration dependences of the k' values should be fairly steep, otherwise the linearization techniques necessary to extract the formation constants are subject to undesirable errors;

(c) the number of experimental points should be sufficient to minimize errors in the regression procedures. These conditions limit the usefulness of the data collected here to the calculation of the complex formation for the NDPs and NTPs with Cd^{2+} + leu and with Zn^{2+} + leu, and for NDP, NTP and NMP with Cu^{2+} + leu. Table V shows the formation constants for the above systems. Also given are the very few existing literature values. With the exception of the Cu^{2+} + leu case, the agreement is excellent. Considering the assumptions and approximations made in the derivation and calculation, the agreement is most gratifying, signifying again the value of the chromatographic technique as a method for obtaining physico-chemical parameters. The discrepancy in the Cu^{2+} + leu case might be attributed to the fact that the concentrations of the injected nucleotides were about two orders of magnitude greater than the expected value of the formation constants (see above discussion). However, it must be kept in mind that the literature value given in Table V is the only one available, and it too may be in error.

The effect of the cation on the order of the formation constants is as expected:

TABLE V

LOGARITHM OF FORMATION CONSTANTS OF THE TERNARY COMPLEXES WITH THREE DIFFERENT METAL IONS

The values were calculated from eqn. 5. Values in parentheses are taken from ref. 8.

Solute	Log K_f		
	Zn	Cd	Cu
AMP	—	3.09	4.14
ADP	2.75	3.26	4.40
ATP	3.19 (3.23)	3.40 (3.51)	4.94 (6.9)
GMP	—	2.77	4.40
GDP	2.91	2.89	4.68
GTP	3.17	3.36	4.80

$K_f(Cu) > K_f(Cd) > K_f(Zn)$, in agreement with the retention order (*cf.*, Fig. 4). This order is also similar to the order of the formation constants of the nucleotide–metal cation binary complexes[7]. Within a nucleotide family, the K_f values seem to follow the order NTP > NDP > (NMP), the parentheses signifying uncertainties due to possible difficulties with the Cu^{2+} + leu data. Most interesting is the fact that K_f values for the NDPs are usually less than the corresponding values for the NTPs, even though the retention of the former is affected most by the presence of the cation and amino acid. Evidently, the factors controlling the retention do not necessarily play a major rôle in determining the stabilities of the complexes.

Table V indicates that the formation constants of the adenosine nucleotide complexes are larger than those of the quanosine nucleotide complexes. The exact meaning of this trend is not immediately clear.

CONCLUSIONS

The chromatographic behaviour of nucleotides in the presence of metal cations and amino acids seems to point to the formation of ternary nucleotide–metal cation–amino acid complexes in the column during chromatography. This is a very exciting observation since it could offer additional possibilities for the investigation of these important compounds in physiological concentration ranges. Studies are now being pursued in our laboratory to examine the effect of different amino acids and pH values on the stability of the complex. The results of characterizing the ternary complexes can shed light on such paramount topics as metal-mediated activation and/or passivation of enzymes. Related information on stereoselective interactions between amino acids and nucleotides, induced by the metal cations, could also be obtained from the chromatographic results. An extension of this stereoselective interaction to enzyme–nucleic acid complex is an intriguing possibility.

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