Journal of Chromatography, 329 (1985) 321–330
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 744

PHYSICO-CHEMICAL CHARACTERIZATION OF CYCLIC NUCLEOTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. CATION BINDING IN THE MOBILE PHASE

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(First received February 22nd, 1985; revised manuscript received March 21st, 1985)

SUMMARY

The retention of nucleoside monophosphoric acids and cyclic nucleotide derivatives was measured on LiChrosorb RP-18 as a function of the cation concentration in the mobile phase. The retention increased upon cation binding due to the reduction in electronic charge on the solute molecule. The hyperbolic dependence of the capacity factor on the concentration of K^+ or Mg^{2+} in the eluent allowed the calculation of apparent stability constants for cyclic nucleotide–metal ion complexes. The results indicate large differences with respect to cation binding, depending both on the nature of the cation and the structure of the phosphate group. Base modifications, on the other hand, have only minor effects on the stability constants. Among the cations studied, Mg^{2+} shows the highest affinity for the cyclic phosphate group. It is concluded that Mg^{2+} may be complexed to cyclic nucleotides within living systems.

INTRODUCTION

Adenosine 3′,5′-monophosphate (cAMP) plays a central rôle in the regulation of growth and metabolism of many cell types[1,2]. In order to find substances that might mimic the action of cAMP or that might act as antagonists to this cyclic nucleotide, several hundred analogues have been synthesized and tested for their biological activities (see refs. 3,4 for reviews). Such studies with this pharmacologically promising class of compounds are hampered by the fact that there is little quantitative information about the underlying physico-chemical properties of cAMP derivatives.

The development of a sound theoretical basis of retention in reversed-phase high-performance liquid chromatography (RPLC) has revealed[5–9] that the solvent plays a dominant rôle in retention, so that measurement of the capacity factor under appropriate conditions can give quantitative information on certain physico-chemical phenomena taking place in solution[10–12]. Our long-term aim is to apply this non-

0021-9673/85/$03.30

analytical potential of RPLC to the characterization of cAMP and its systematically modified analogues[4].

It has been shown that the capacity factor is strongly related to the liquid–liquid distribution coefficient, log P, of neutral compounds[13–16] or weak acids and bases[17,18], provided that the capacity factor of the unionized state of the solute is known, thus enabling corrections to be made for ionization[19]. Consequently, RPLC retention parameters have been successfully employed either to calculate log P values or to describe directly the hydrophobic nature of bioactive compounds in studies on quantitative structure–activity relationships[14–21]. However, cyclic nucleotides in their unionized state cannot be analysed on alkyl-bonded stationary phases due to the instability of the matrix outside a mobile phase pH range of 1.5–7.5. The measurement of the hydrophobicity of charged solutes is difficult because complex formation with oppositely charged mobile phase components may be superimposed on the hydrophobic effect which controls the retention of neutral compounds[20,21]. Therefore, the effect of cations on the retention behaviour of negatively charged nucleotides must be ascertained. The present study is focused on alkali- and alkaline-earth-metal cations and their interactions with nucleotides, because these metal ions are not only common buffer constituents but also may be complexed to nucleotides within living systems.

Recently, Cohen and Grushka[22] reported on the dependence of the capacity factors of nucleotides, nucleosides and their bases on the concentration of Mg^{2+} and some transition-metal cations in the mobile phase. For nucleotide–Mg^{2+} complexes, the authors found a decrease in the capacity factor with an increase in the magnesium concentration in the mobile phase, and concluded that the nature of the base, and not the relative charges, controls the retention of nucleotides in RPLC. This finding is surprising because it is known that Mg^{2+} binds to the phosphate group[23], thereby reducing its net negative charge so that the nucleotide–Mg^{2+} complex should exhibit enhanced rather than reduced retention. In fact, Horváth *et al.*[12] showed this to be the case, theoretically as well as experimentally, and were furthermore able to calculate from retention data the stability constants of several nucleoside triphosphate–metal complexes which were in good agreement with constants obtained by other techniques.

In this paper, we examine the effect of cations such as K^+ and Mg^{2+} upon the retention of nucleoside monophosphoric acids and some cyclic nucleotides in RPLC. It will be shown that the cations bind to the phosphate group leading to enhanced retention of the complex. The results indicate large differences in the affinity of the cations for complexation, depending both on the nature of the cation and the structure of the phosphate group. At certain metal concentrations in the eluent, all solute molecules are in the form of complexes so that a well defined reference state of the solute is at hand which enabled us to measure the hydrophobic nature of a large variety of cAMP derivatives. We will report on these results in a subsequent paper.

MATERIALS AND METHODS

The chromatographic equipment consisted of a Constametric III pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), a Model UV-III UV detector (LDC), a Model 7125 injection valve equipped with a 20-μl sample loop (Rheodyne, Berkeley,

CA, U.S.A.) and a Servogor Model S pen recorder (Metrawatt, Nürnberg, F.R.G.). The stainless-steel column (25 cm × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.) was packed by the slurry technique as previously described[14]. The stationary phase was 10-μm LiChrosorb RP-18 (Merck, batch No. VV 1106) known to possess a very high surface coverage of octadecyl ligands[24]. The column was used without further treatment in all experiments. The mobile phase consisted of methanol (Baker HPLC reagent) and different volume fractions of either potassium phosphate buffer (pH 6.6) or the chlorides of alkali- and alkaline-earth-metal cations. The pH of the latter was adjusted to 6.6 by small amounts of Tris [tris(hydroxymethyl)aminomethane]. After use of the chlorides, great care was taken to wash the column and apparatus extensively with distilled water in order to avoid damage to the chromatograph.

A 5-μl volume of a 0.1 m*M* sample solution was injected and the retention times were measured with a stop-watch. The reproducibility of the retention time measurements was better than ± 1% so that in all experiments two independent runs were carried out. The flow-rate was 2.0 ml/min at room temperature and the void volume of the column was determined as previously described[14]. The standard error of log k' determinations was less than ± 0.005.

Fig. 1 shows the structures of the cyclic nucleotides which were analysed in this study. Adenosine 3',5'-monophosphate (cAMP), 8-bromoadenosine 3',5'-monophosphate (8-Br-cAMP), 8-aminoadenosine 3',5'-monophosphate (8-NH_2-cAMP), guanosine 3',5'-monophosphate (cGMP), adenosine 2',3'-monophosphate (2',3'-cAMP), adenosine 5'-monophosphate (5'-AMP), adenosine 3'-monophosphate (3'-AMP), adenosine 2'-monophosphate (2'-AMP) and adenosine were purchased from Boehringer (Mannheim, F.R.G.). Adenosine 3',5'-monophosphorothioate, Rp-isomer (Rp-cAMPS) and adenosine 3',5'-monophosphorothioate, Sp-isomer (Sp-cAMPS) were synthesized in our laboratory according to Baraniak *et al.*[25].

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms illustrating the drastic effect of cations on the elution of nucleotides. With 10% methanol in water as the eluent, the charged 5'-AMP and cAMP are eluted close to the void volume of the column, whereas adenosine shows considerable retention (chromatogram A in Fig. 2). In this case, the effects of the two and one negative charges, respectively, located at the phosphate moiety predominate over any hydrophobic interactions of the purine base with the mobile and stationary phases which, in turn, determine the retention of the neutral adenosine. This finding is not in accord with the data of Cohen and Grushka[22] who found that, in the absence of Mg^{2+} in the mobile phase, cAMP was retained much more strongly than adenine. These authors, however, used as eluent an aqueous acetate buffer of pH 5.6 and 0.04 *M* ionic strength, the composition of which was, unfortunately, not given. As will be shown below, under such mobile phase conditions, cAMP is already present as a nucleotide–cation complex so that the apparent "hydrophobicity" of cAMP can be traced back to an effective charge neutralization upon complex formation.

When 50 m*M* potassium phosphate buffer (pH 6.6) is used as eluent instead of water (chromatogram B in Fig. 2), the retention of the uncharged adenosine is little affected whereas the capacity factors of the nucleotides greatly increase. This

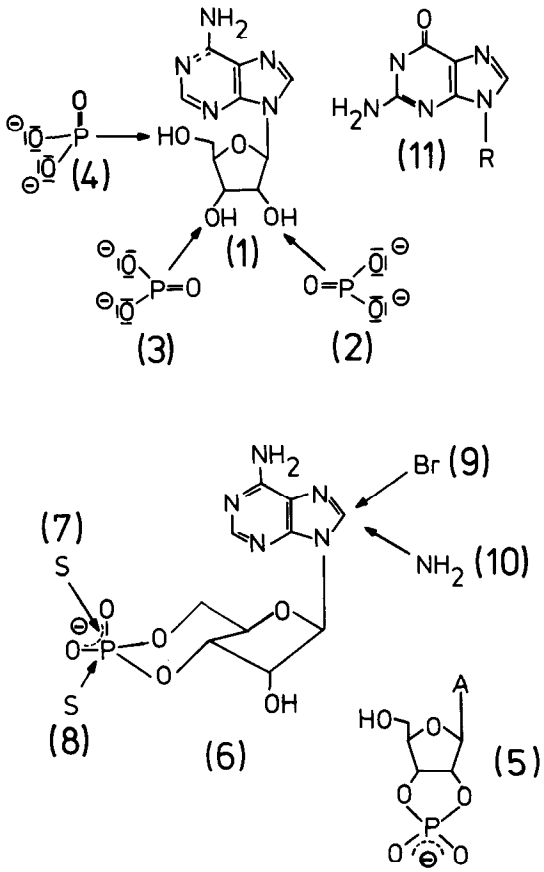

Fig. 1. Structures of nucleosides and nucleotides. 1 = Adenosine; 2 = adenosine 2'-monophosphate (2'-AMP); 3 = adenosine 3'-monophosphate (3'-AMP); 4 = adenosine 5'-monophosphate (5'-AMP); 5 = adenosine 2',3'-monophosphate (2',3'-cAMP); 6 = adenosine 3',5'-monophosphate (cAMP); 7 = adenosine 3',5'-monophosphorothioate; Sp-isomer (Sp-cAMPS); 8 = adenosine 3',5'-monophosphorothioate, Rp-isomer (Rp-cAMPS); 9 = 8-bromoadenosine 3',5'-monophosphate (8-Br-cAMP); 10 = 8-aminoadenosine 3',5'-monophosphate (8-NH_2-cAMP); 11 = guanosine 3',5'-monophosphate (cGMP), with R as in 6. A = Adenine.

effect is explained by complex formation between the metal ion and the phosphate group of the nucleotide, thereby reducing the net negative charge of the solutes and thus enhancing retention of the complex[26]. Charge neutralization is less effective for the doubly charged 5'-AMP as for cAMP, as is seen from the differences in retention times.

The specific binding of K^+ to the phosphate group of nucleotides is confirmed in Fig. 3, which shows the variation of the capacity factors of the isoelectronic nucleoside monophosphoric acids 2'-AMP, 3'-AMP and 5'-AMP, with the concentration of K^+ in the mobile phase. This cation shows a comparable affinity for the different nucleotides (see below), but the capacity factors of the resulting complexes at cation saturation concentrations are very different, thus substantiating the influence of the structure of the phosphate group upon the polarity of the nucleotide–K^+

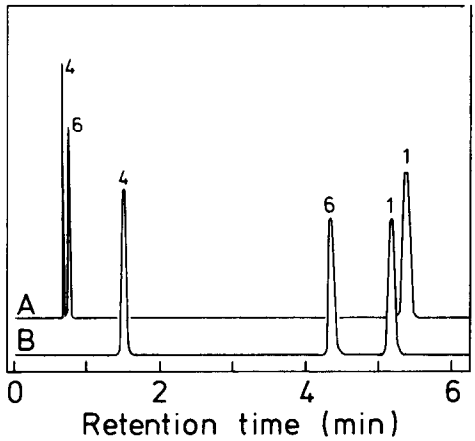

Fig. 2. Separation of 5'-AMP (4), cAMP (6) and adenosine (1) on a LiChrosorb RP-18 column. Mobile phases: A, 10% methanol (v/v) in water; B, 10% methanol (v/v) in 50 m*M* potassium phosphate buffer (pH 6.6). Flow-rate 2.0 ml/min.

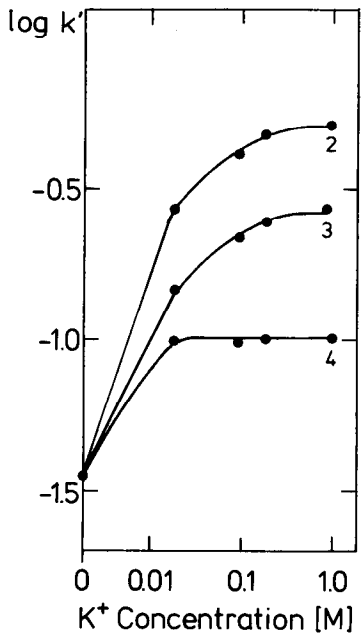

Fig. 3. Effect of potassium concentration (in *M*) in the mobile phase on the retention of 2'-AMP (2), 3'-AMP (3) and 5'-AMP (4). Chromatographic conditions: LiChrosorb RP-18; 20% methanol (v/v) in potassium phosphate buffer (pH 6.6); flow-rate 2.0 ml/min.

complex. These structural differences are manifested also by slightly different acid dissociation constants[27] and may be related to a steric effect involving differences in proximity to the hydroxyl groups of the sugar moiety and the nitrogens of the purine ring (Fig. 1). Obviously, RPLC under appropriate mobile phase conditions is a sensitive tool with which to detect minor structural variations within charged groups.

Horváth *et al.*[12] have presented a theoretical framework for the treatment of complex formation in RPLC which can be used to determine stability constants, *e.g.*, for metal binding to nucleotides[12,28]. Accordingly, the dependence of the capacity factor of the solute, k', on the cation concentration in the mobile phase, [C], can be expressed by

$$k' = \frac{k_0 + k_c[C]}{1 + K[C]} \quad (1)$$

where k_0 and k_c represent the capacity factors of the free solute and the complex, respectively. Provided that (i) [C] is much larger than the concentration of the solute, (ii) complex formation takes place in the mobile phase and (iii) association of the complex to the stationary phase is not limited by the number of accessible ligands, the stability constant of the complex, K, can be evaluated. Under our experimental conditions, the three premises are fulfilled since we use a very small solute concentration (< 1 nmol) and since the complexing metal ion is not supposed to bind to the non-polar stationary phase so that complex formation must occur within the mobile phase.

Implicit to the model of Horváth *et al.*[12], which is formally similar to the Michaelis–Menten equation, is the assumption that no other equilibria determine retention. This is, however, unlikely for metal binding by nucleotides because metal ions can form different complex species besides a 1:1 complex[29] and, furthermore, nucleotides may form dimers and other aggregates by base stacking in aqueous solution[30]. Therefore, the K values determined by RPLC represent the overall stability constant for all complex species[12,28], and do not necessarily reflect the physico-chemical meaning of a stability constant in solution. Nevertheless, Horváth's approach is very useful to determine apparent stability constants for the metal–nucleotide complexes which can be used to compare the affinity of different nucleotides for the same cation, or *vice versa*, to assess the efficiency of the binding of different metals to the same nucleotide[22,28]. From Fig. 3, we have determined the stability constants according to a graphical procedure[12] and found log K values (in M^{-1}) of 2.2, 2.1 and 2.0 for 5'-AMP, 2'-AMP and 3'-AMP. These results are in accord with the sequence of affinity reported by Khan and Martell[27] for binding of Mg^{2+} to the same nucleotides.

The interaction of cyclic nucleotides with cations has scarcely been analysed. Since analogues of cAMP are frequently used in studies on the mode of action of cAMP, it is of special interest whether base modifications affect the complexation behaviour of the cyclic phosphate. We have also selected two stereochemically different adenosine 3',5'-monophosphorothioates, Sp-cAMPS and Rp-cAMPS (Fig. 1), because they are modified in the charged region, and because they exhibit strikingly different biological actions in cAMP-dependent systems[31–34].

Fig. 4 reveals that in general cyclic nucleotides are much more sensitive to binding by K^+ than are the monophosphoric acids and, furthermore, that the stability constants are roughly equal for all compounds (Table I). Neither the different location of the cyclic phosphate as in 2',3'-cAMP nor the replacement of one oxygen by a sulphur atom seems to change the affinity for the cation and thus the electronic structure of the phosphate group. This is an interesting finding because Rp-cAMPS

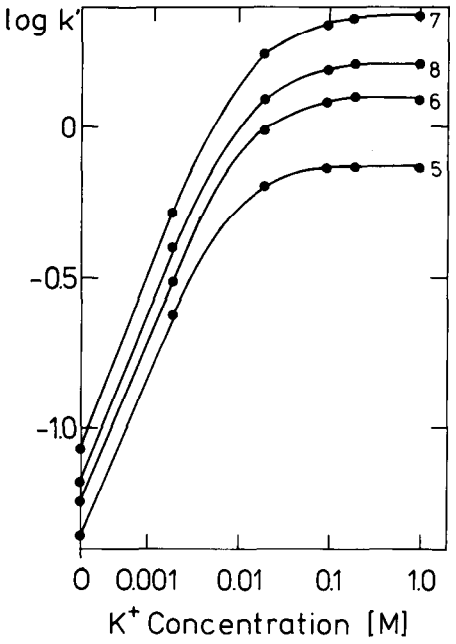

Fig. 4. Effect of potassium concentration in the mobile phase on the retention of 2',3'-cAMP (5), cAMP (6), Rp-cAMPS (8) and Sp-cAMPS (7). Chromatographic conditions as in Fig. 3.

specifically antagonizes the effect of cAMP[32] which may now be related to steric, but not to electronic effects of the substituent. The influence of K^+ on the retention behaviour of base-modified cyclic nucleotides is shown in Fig. 5A. Although the substituents cover a wide range with respect to their polarity, the stability constants of the nucleotide–K^+ complexes are similar (Table I), indicating that the structure of the purine ring is of minor importance for complex formation.

A second variable determining the stability of the nucleotide complexes is the nature of the cation. In addition to K^+, we have analysed the effects of Li^+, Na^+,

TABLE I

STABILITY CONSTANTS, log K (M^{-1}), OF METAL–NUCLEOTIDE COMPLEXES

log K was determined graphically from retention data as in ref. 12. Abbreviations of compounds as in Fig. 1. n.d. = Not determined.

Compound	*Cation*	
	K^+	Mg^{2+}
2',3'-cAMP	2.8	4.1
cAMP	2.9	4.2
8-Br-cAMP	3.0	4.2
8-NH_2-cAMP	2.8	4.2
cGMP	2.8	4.1
Sp-cAMPS	2.7	n.d.
Rp-cAMPS	2.8	n.d.

Mg^{2+} and Ca^{2+} on the retention of cAMP derivatives. Fig. 5B shows the plot of the capacity factor *versus* the concentration of Mg^{2+} in the eluent. The other cations behave similarly to K^+ (Li^+, Na^+) and to Mg^{2+} (Ca^{2+}) (data not shown). This is consistent with the finding that stability constants of nucleotide–cation complexes also differ little within the groups of alkali- and alkaline-earth-metal cations, respectively[23,35]. Fig. 5 clearly indicates that alkaline-earth-metal cations possess a greater affinity for the cyclic phosphate than do the alkali-metal cations. This difference, however, is not great enough to exclude strong competition of, *e.g.*, K^+ and Mg^{2+} for the same binding site. If a buffer of moderate to high ionic strength is used as in the work of Cohen and Grushka[22] and Mg^{2+} is added at much lower concentrations, the effect of Mg^{2+} on the retention may be obscured by the abundance of, *e.g.*, alkali-metal cations. This is of course not the case for the transition-metal ions which have high affinity for the purine base[22]. Interestingly, the capacity factors of the complexes at cation saturation concentration are independent of the nature of the cation (Fig. 5).

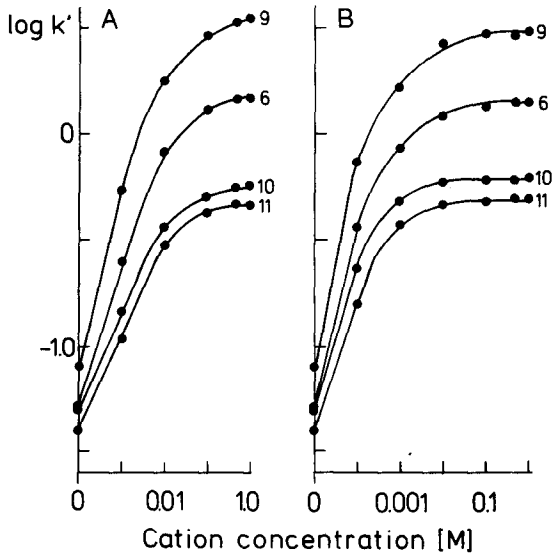

Fig. 5. Effect of cation concentration (in *M*) in the mobile phase on the retention of cGMP (11), 8-NH_2-cAMPS (10), 8-Br-cAMP (9) and cAMP (6). Chromatographic conditions: LiChrosorb RP-18 and 20% methanol (v/v) in water containing different concentrations of alkali- and alkaline-earth-metal chlorides: A, KCl; B: $MgCl_2$.

From the retention data we have determined the apparent stability constants (Table I). Taking into account the uncertainties in the log *K* values, the following conclusions can safely be drawn. Cyclic nucleotides are much better substrates for complex formation with metal cations than are the corresponding monophosphoric acids, due to the electronic structure of the phosphate group. The resulting cyclic nucleotide–metal complex may achieve partial electrical neutrality, as is seen from the similar retention times of complexed cAMP and adenosine (Fig. 2). Effective shielding of the charge is also possible with the divalent alkaline-earth-metal cations,

yielding complexes of the same polarity (Fig. 5). However, the affinity for Mg^{2+} is about a factor of 10 greater than for monovalent K^+, and about a factor of 100 greater than for K^+ in the complexation of adenosine monophosphoric acids. In view of the fact that both Mg^{2+} and the alkali-metal ions are present within cells in concentrations well above 100 m*M*, we may conclude that cAMP and cGMP are complexed also *in vivo*, preferentially to Mg^{2+}. Moreover, if the stability constants in Table I are related to the known values, *e.g.*, from binding of Mg^{2+} to ATP[12,23], the cyclic nucleotides may very well show a similar affinity for divalent cations. Since it is known that cations play an important rôle in the regulation of cAMP-dependent processes[1], it is tempting to speculate that cation binding by cyclic nucleotides may have important implications for the explanation of their mode of action.

We started to study the effects of cations on retention because we wished to determine the hydrophobicity of cAMP analogues. From the results above it is clear that two different well defined molecular species of the solute can be characterized experimentally, *i.e.*, the charged compound in the absence of cations in the mobile phase, and the complexed nucleotide at cation saturation concentration. The determination of the hydrophobicity of cAMP analogues will form the subject of the subsequent paper.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ja 246/3-4) and the Fonds der Chemischen Industrie. We are grateful to Sabine Schockert for her careful preparation of the manuscript.

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