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MECHANISM OF ZWITTERION-PAIR CHROMATOGRAPHY

I. NUCLEOTIDES

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SUMMARY

The basis of retention of zwitterionic solutes in reversed-phase liquid chromatography in the presence of zwitterionic pairing agents has been studied using long-chain amino acids (11-aminoundecanoic acid, C11AA and 12-aminododecanoic acid, C12AA), a tripeptide (L-leucyl–L-leucyl–L-Leucine, LLL), and a long-chain diamine (1,10-diaminodecane, C10DA). The adsorption has been determined for each pairing agent under the conditions used for chromatography to show up any correlation between retention and surface concentration of the pairing agents. Eluents were water–methanol (88:12) or water–dimethylformamide (90:10) buffered with acetic acid–acetate, or phosphoric acid–phosphate in the pH range 3–7. The most characteristic feature of zwitterion-pair chromatography is the existence of maxima in retention of zwitterionic solutes at pH where both solute and pairing agent exist as zwitterions. With C11AA and C12AA as pairing agents this occurs at pH of 4.4 and with LLL as pairing agent at pH of 3.5. The peak in retention is most noticeable when the ionic strength of the buffer is small (in the millimolar-range). The pH behaviour of retention in the presence of zwitterionic pairing agents is in striking contrast to that in the presence of C10DA which not only provides much reduced retention when present at the same surface concentration of amino groups, but shows no marked dependence on pH.

It is concluded that zwitterionic pairing agents provide a new mode of retention for zwitterionic solutes, namely the formation in the stationary phase of quadrupolar ion pairs between the zwitterionic forms of the solute and pairing agent when an appropriate pH is employed.

Illustrative separations of mono-, di- and triphosphate nucleotides, and of selected monophosphate nucleotides are shown.

INTRODUCTION

In a previous paper[1] we demonstrated that excellent separations of nucleotides could be achieved on a reversed-phase silica gel (ODS Hypersil^{R}) with predominantly aqueous eluents containing millimolar concentrations of zwitterionic pairing agents (11-aminoundecanoic acid, C11AA and 12-aminododecanoic acid, C12AA) when,

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without the added pairing agent, little or no retention could be observed. The separations achieved by this method showed excellent plate efficiency, selectivity and flexibility. The term "zwitterion-pair chromatography" was coined for this form of ion-pair chromatography in which both the solute and the pairing agent could exist as zwitterions, but it was not clear from the preliminary work whether or not retention depended upon the formation of quadrupolar ion-pairs or the more conventional dipolar ion-pairs. However, it was observed that while adsorption of C11AA varied somewhat with pH, the variation of column capacity ratio, k', was much more dramatic with the greatest retention occurring at about pH 4.2 where there was near maximal overlap of the zwitterionic forms of the pairing agent and the nucleotides. Somewhat similar results had been obtained in connection with the chromatography of tetracyclines in the presence of ethylene diamine tetraacetate (EDTA)[2] when the idea of zwitterion-pairing was first suggested.

Undoubtedly if genuine quadrupolar ion pairing occurred it should be associated with retention maxima at pH values around those where maximum overlap of the zwitterionic forms of the solutes and pairing agents exists. However, such maxima could also arise from interactions of oppositely charged forms of the solute and pairing agent if the range of overlap of the zwitterionic forms was small. It was the purpose of this work to see whether the expected maxima could be clearly observed and, if so, to distinguish between the two possible explanations of the enhanced retention brought about by addition of the zwitterionic pairing agents. Since it had previously been shown by several groups[3–5] that there exists a strong correlation between retention and concentration of adsorbed pairing agent we have determined the adsorption isotherms for the pairing agents used in this study over a wide range of conditions to be certain that major changes in retention were not simply due to changes in the concentration of adsorbed pairing agent. We have also measured the degree of retention over the pH range 3–7 under a variaty of conditions of ionic strength.

With dilute buffers, maxima in retention have been found at pH of 4–4.2 with C11AA and C12AA, and at pH of 3.5 with L-leucyl–L-leucyl–L-leucine (LLL) as zwitterionic pairing agents. The maxima are most marked with buffers of lowest ionic strength. When 1,10-diaminodecane (C10DA) is used as pairing agent no maxima are observed and the overall retention in the region of pH 4 is very much less than with C11AA added to give the same surface concentration of amino groups. The results provide strong evidence for the formation of quadrupolar ion pairs being the main cause of the enhanced retention produced by the amino acids as pairing agents.

EXPERIMENTAL

Adsorption isotherms were determined by the breakthrough method previously reported[1] using a refractive index monitor (Optilab Multiref Model 902, Vallingby, Sweden) coupled to the chromatographic column which was supplied with eluent by a membrane microdosing pump (Model 1515; Orlita, Giessen, G.F.R.). The whole equipment downstream of the pump was thermostatted by circulating water at 25°C, and was additionally mounted within an air-box likewise thermostatted at 25°C.

Liquid chromatography was carried out using a thermostatted photometer–

column-oven unit maintained at 25°C(Shandon Southern Products, Runcorn, Great Britain). The column was supplied with eluent by a single piston reciprocating pump (Model 110; Altex, Berkeley, CA, U.S.A.). Samples were introduced by a Rheodyne injection valve (Model 7120; Rheodyne, Berkeley, CA, U.S.A.). Columns were 5 mm bore and 100 or 125 mm long (Shandon Southern Products) and were packed with ODS Hypersil (Shandon Southern Products) using isopropanol as the slurry liquid and a pressure of 400 bar. The isopropanol-slurry was followed by 200 ml hexane before conditioning the column with methanol and finally eluent.

Eluents were water–methanol (88:12) or water–dimethylformamide (90:10). The pH was adjusted within the range 3–7 by addition of phosphate buffer mixture (KH_2PO_4–Na_2PO_4–H_3PO_4), acetate buffer mixture ($NaCH_3COO$–CH_3COOH), pure acetic acid, or pure phosphoric acid. Methanol was HPLC grade (Rathburn Chemicals. Walkerburn, Great Britain). Dimethylformamide was AnalaR Reagent (BDH, Poole, Great Britain). C11AA, C12AA and C10DA were obtained from Aldrich (Gillingham, Great Britain). Nucleotides were obtained from BDH Biochemicals or from Sigma (Poole, Great Britain)

RESULTS AND DISCUSSION

Adsorption isotherms

Recent investigations of the mechanism of ion-pair chromatography using monopolar long-chain pairing agents[3–5] have established that the degree of retention of monopolar solutes can be closely correlated with the surface concentration of the adsorbed pairing agent. Thus, for a given pairing agent and eluent, the column capacity ratio, k', is almost exactly proportional to the surface concentration, C_{ads}, of

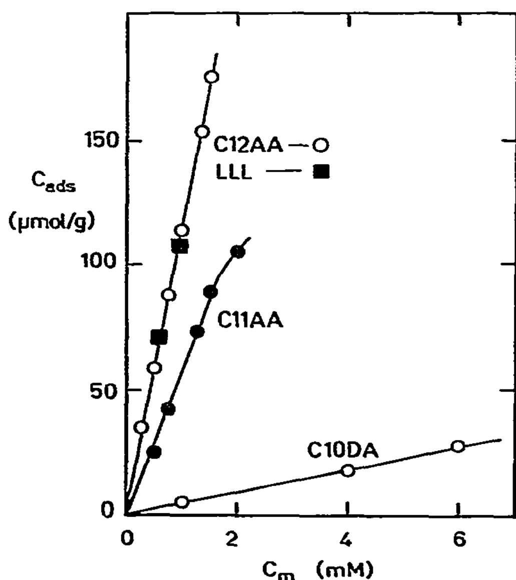


Fig. 1. Adsorption isotherms for C12AA, LLL, C11AA and C10DA. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 75 mM phosphate buffer at pH 5.8.

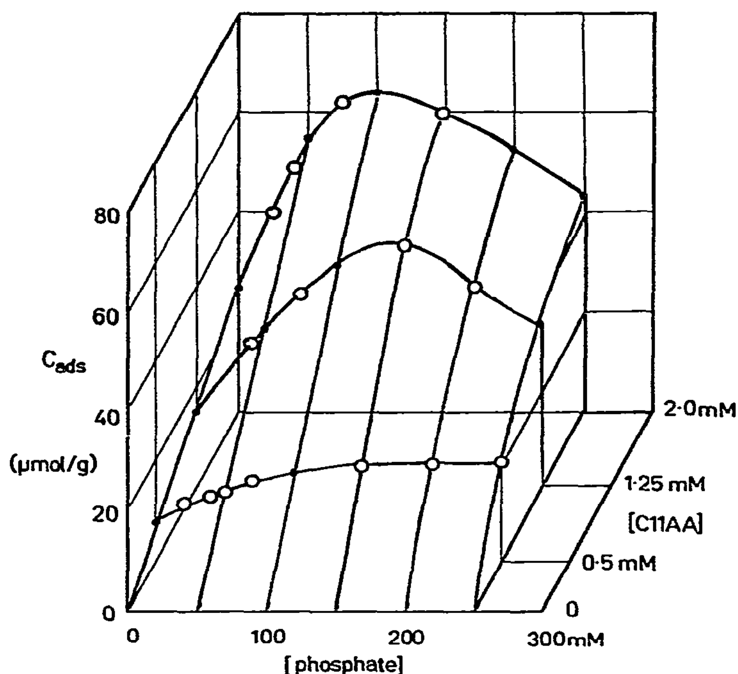


Fig. 2. Adsorption isotherms for C11AA at different phosphate buffer concentrations. Column packing, ODS Hypersil. Eluent, water–dimethylformamide (90:10) containing phosphte buffer at pH 5.35.

the pairing agent up to the point when micelle formation begins in the eluent. At this point or just before it multiple ion clusters form in the eluent and the retention may become constant or even begin to fall while C_{ads} continues to increase. In our previous study[1] somewhat similar results were reported for the retention of nucleotides in the presence of added C11AA. It has also been noted[4,5] that with a series of homologous pairing agents, while the adsorption coefficients differ greatly, the degree of retention for a given surface concentration is relatively little dependent upon the chain length of the pairing agent. With these results in mind we considered it essential to determine the adsorption isotherms of the various pairing agents used in this study over the range of eluent compositions used.

Fig. 1 shows the adsorption isotherms on ODS Hypersil for the four pairing agents, C12AA, LLL, C11AA and C10DA using a standard eluent of water–methanol (88:12) containing 75 m*M* phosphate buffer pH 5.8. The isotherms are linear over the range studied. Surface concentrations, C_{ads}, ranged up to 180 μmol/g for the most strongly adsorbed C12AA. C12AA and LLL are twice as strongly adsorbed as C11AA, and C11AA is about twelve times as strongly adsorbed as C10DA under the same conditions. Since the surface area of Hypersil before bonding is about 170 m^2/g (ref. 6), the surface concentrations based upon the original area ranged up to about 1 μmol/m^2. This compares with a concentration of bonded groups of about 2.5 μmol/m^2. Typical concentrations used in the chromatographic experiments were from 35–90 μmol/g or 0.2–0.5 μmol/m^2. In comparing k' values using C11AA and

C10DA the eluent concentrations of the pairing agents were chosen so as to provide about the same concentrations of amino groups, namely about 40 μmol/g.

Fig. 2 shows three dimensional isotherms for C11AA adsorbed from water–dimethylformamide (90:10) for different concentrations of phosphate buffer up to 250 mM with pH 5.3. At low buffer concentrations the isotherms are linear up to a concentration of pairing agent in eluent, C_m, of 2 mM, but when the phosphate concentration exceeds about 100 mM the isotherms become increasingly curved towards the C_m axis. At low eluent concentrations of C11AA, increase in the phosphate buffer concentration causes a gradual increase in C_{ads}, but at higher concentrations of C11AA, C_{ads} passes through a maximum. The maximum occurs at lower buffer concentrations the higher is C_m. In the chromatographic experiments described below the concentrations of C11AA and buffer were held below 2 mM and 100 mM respectively where the isotherms are linear and C_{ads} increases smoothly with buffer concentration.

Fig. 3 shows how pH affects the adsorption of C11AA from the standard eluent containing 75 mM phosphate buffer. For any pH between 3 and 7 the isotherms are more or less linear, but there is a flat maximum in the dependence of C_{ads} upon pH at pH 4–4.5. Data from Fig. 1 for pH 5.8 and from Fig. 4 for 0.75 mM C11AA are included in Fig. 3. For C10DA C_{ads} at an eluent concentration of 4 mM was found to be 19 μmol/g independent of pH in the range 5–7 (see Fig. 5).

The results on the adsorption of C11AA, C12AA and C10DA may be summarised as follows:

(1) Addition of CH_2 to the chain of C11AA increase its adsorption by a factor of about two.

(2) Replacement of CH_2NH_2 in C11AA by COOH to give C10DA reduces adsorption by a factor of twelve.

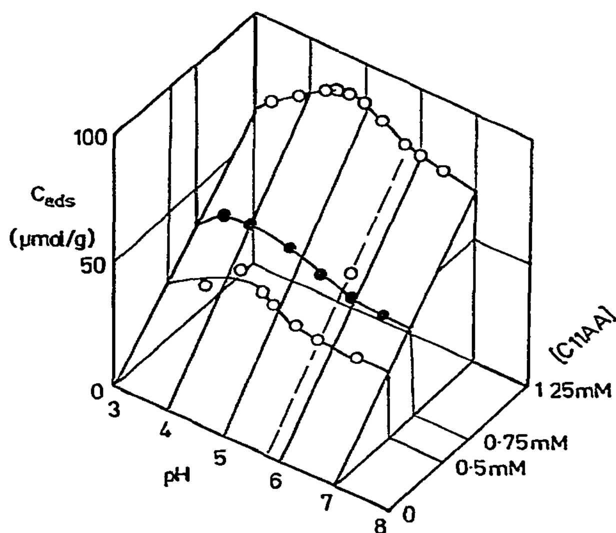


Fig. 3. Adsorption isotherms for C11AA at different pH. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 75 mM phosphate buffer.

(3) The adsorption isotherms of the three pairing agents are essentially linear in the chromatographically useful range.

(4) Addition of phosphate buffer increases C_{ads} slightly when the concentration of C11AA is low. At higher concentrations of C11AA in eluent a maximum in C_{ads} is found as phosphate concentration increases.

(5) pH has only a slight effect on C_{ads} for C11AA adsorbed from 75 m*M* phosphate buffer. A flat maximum is observed at pH 4–4.5.

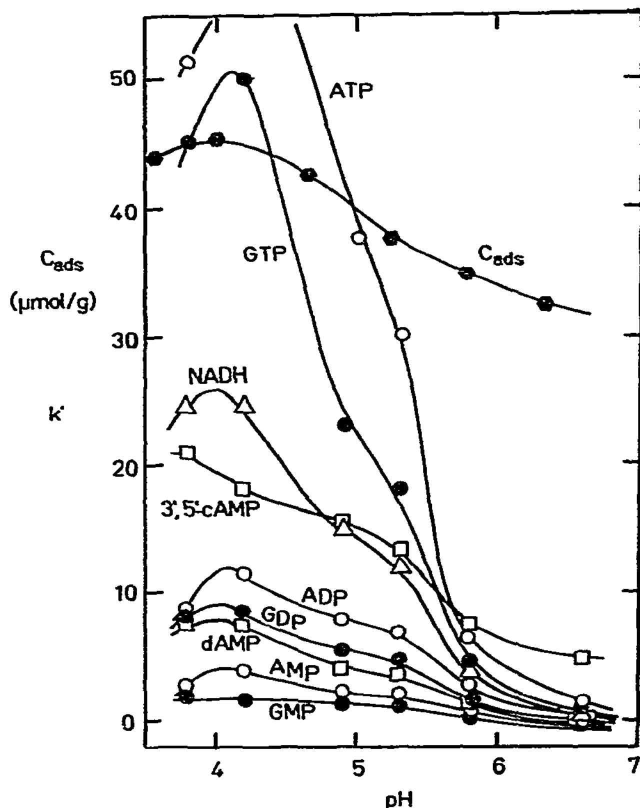


Fig. 4. Dependence of k' of nucleotides and of C_{ads} for C11AA upon pH. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 75 m*M* phosphate buffer and 0.75 m*M* C11AA as pairing agent.

(6) C11AA is about twice as strongly adsorbed from water–methanol (88:12) as from water–dimethylformamide (90:10).

(7) Although no quantitative experiments were carried out on the effect of temperature, it was observed that the amount of pairing agent adsorbed decreased with increase of temperature. Accordingly thermostatting of the system was essential for reproducible results. In an independent study on the adsorption of cetylpyridinium by ODS Hypersil[7], an enthalpy of adsorption of −32 kJ/mol was observed corresponding to a temperature coefficient of about 5% per degree.

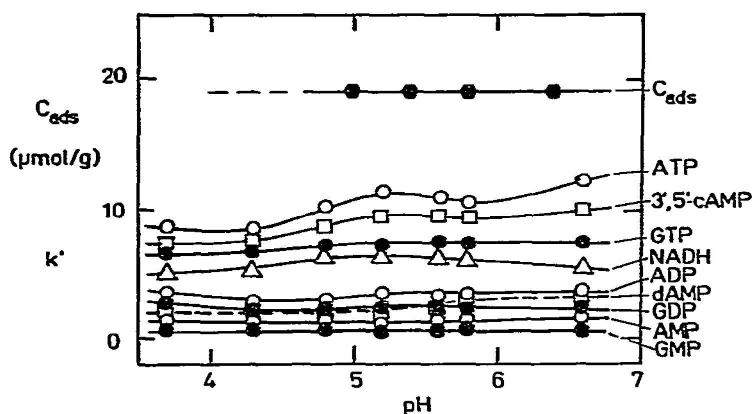


Fig. 5. As for Fig. 4 but with 4 mM C10DA as pairing agent.

Dependence of retention upon pH, C11AA and C10DA

Figs. 4 and 5 compare the effects of pH upon retention of nucleotides when using C11AA and C10DA as pairing agents and standard eluent containing 75 mM phosphate. The concentrations of pairing agents, 0.75 mM for C11AA and 4 mM for C10DA were chosen to give approximately the same surface concentrations of $-NH_3^+$ groups. The effect of pH upon k' for the two pairing agents may be seen to be completely different. With C10DA (Fig. 5) a fall in pH from 7 to 3 causes a slight overall decline in retention while the surface concentration of C10DA remains constant at 19 μmol/g (giving 38 μmol/g of amino groups). By contrast, with C11AA as pairing agent (Fig. 4), there is a massive increase in k' as the pH is reduced from 7 to

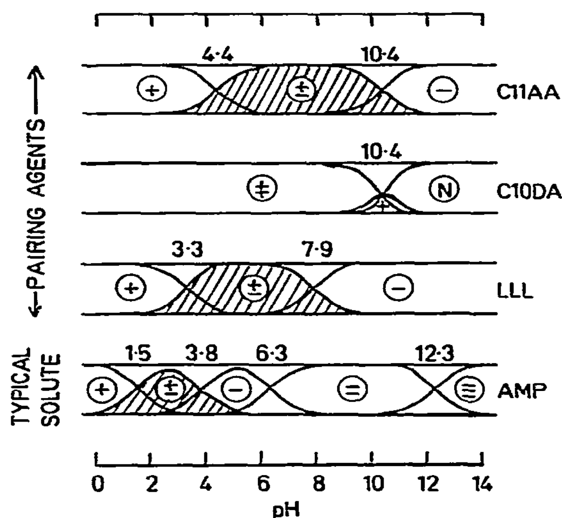
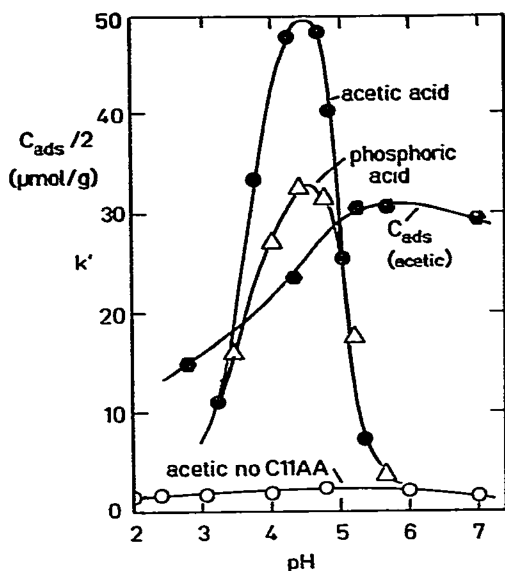


Fig. 6. Ionisation diagrams showing the fractions of different ionic forms as a function of pH for pairing agents, C11AA, C12AA, C10DA and LLL, and for a typical solute, AMP. Note that higher phosphates have additional primary phosphate ionisations at pH around 1.5–2.



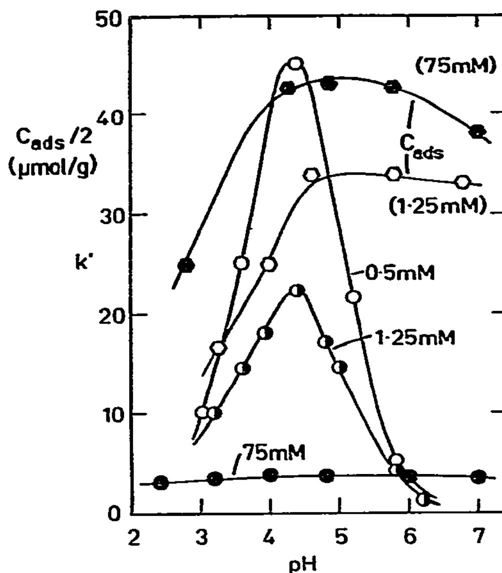


Fig. 7. Dependence of k' for AMP upon pH, with pH adjusted by pure acetic acid or pure phosphoric acid, and dependence of C_{ads} for C11AA upon pH. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 1.25 mM C11AA.

Fig. 8. Dependence of k' for AMP upon pH, with pH adjusted by acetate buffers of different strengths (marked on lines), and dependence of C_{ads} for C11AA upon pH for low and high buffer strengths. Other conditions as for Fig. 7.

about 4.2, and there is evidence for a maximum in k' of several of the nucleotides at about pH 4.2. Over the pH range 3–7 the surface concentration of C11AA changes by only about 40%, while k' values change by up to 50-fold. Evidently the small change in C_{ads} does not in any way account for the massive changes in k' values. Again comparing Figs. 4 and 5 it is noted that at pH around 7 retention with C11AA is only a fraction of that with C10DA, but is many times greater at pH 4.2. Finally a weak maximum (Fig. 5) or bulge (Fig. 4) in the k'-dependence is observed at pH 5.3 for both pairing agents which is superimposed upon the general trends.

Over the entire pH range, as shown for the ionisation diagrams given in Fig. 6, the amino groups of C11AA and C10DA are fully ionised (pK_a 10.4). Likewise the primary phosphate groups of the nucleotides are fully ionised at any pH over 3 (pK_a < 2). Thus the strength of the interactions of the amino groups with the phosphate groups must remain constant over the pH range 3–7. This is in agreement with the absence of any strong variation of k' with pH when using C10DA as pairing agent. The slight fall in k' as pH is reduced may be due to the onset of a repulsive interaction between the positive N atoms of the nucleotides (pK_a in range 3–4) and the amino groups of C10DA as pH is reduced.

The strong increase in k' when using C11AA as pH is reduced from 7 to 4.2 can only be due to an increasing interaction between the partially ionised carboxyl group of C11AA (pK_a 4.4) and the partially ionised nitrogen of the nucleotides (pK_a 3–4). These interactions which will appear relatively weak when averaged over all mole-

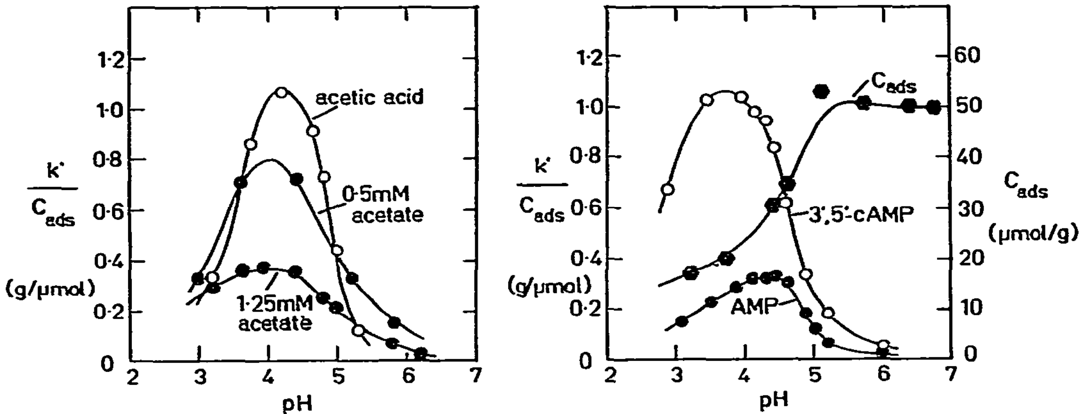


Fig. 9. Dependence of k'/C_{ads} upon pH for AMP. Data and conditions as for Figs. 7 and 8.

Fig. 10. Dependence of k'/C_{ads} upon pH for AMP and 3'5'-cylic AMP with C12AA as pairing agent, and dependence of C_{ads} for C12AA upon pH. Column packing, ODS Hypersil. Eluent, water–dimethylformamide (90:10) containing 1.0 mM C12AA, pH adjusted by acetic acid.

cules will nevertheless reinforce the strong interactions of the phosphate groups and the ionised amino groups of C11AA which remain unchanged over the pH range. The weak interactions will, however, vary over the pH range due to the variation in the degrees of ionisation of the two species and should be a maximum at a pH which is midway between the pK_a values of the two weakly interacting groups, that is at a pH of about 4.1 (taking pK_a for the nucleotide as 3.8, the value for adenosine phosphates). This predicted value for maximal retention agrees gratifyingly with the observed value of 4.2. When the pH is reduced below 4.2 the decreasing degree of ionisation of the carboxyl group of C11AA more than compensates for the increasing ionisation of the nitrogen of the nucleotides, and k' begins to fall.

The prime interaction which brings about the strong retention on addition of C11AA when the pH is about 4.2 must therefore be due to a quadrupolar interaction of the zwitterionic forms of the nucleotide and the pairing amino acid.

The low retention of the nucleotides in the presence of C11AA at high pH when compared to the retention in the presence of C10DA is most likely due to the increased repulsive interaction between the carboxyl group of the amino acid and the phosphate groups of the nucleotides. This is especially notable for the nucleotides containing secondary phosphate groups with their pK_a of around 6.3. It is notable that 3',5'-cyclic AMP which has no secondary phosphate group is much less affected than the other nucleotides.

Dependence of retention upon pH and ionic strength: C11AA, C12AA and LLL

It is well established in ion-exchange and in ion-pair chromatography that a reduction in the ionic strength of the eluent increases retention by reducing the competition for charged sites in the stationary phase[8]. In order to provide maximum scope for zwitterion pairing and so for the exhibition of a maximum in retention as pH is varied, the retention of selected nucleotides in eluents of very low ionic strength was investigated. With the present group and with standard eluents containing low buffer concentrations it proved impossible to elute di- and triphosphate nucleotides

with reasonable k' values, and therefore only the monophosphates AMP and 3′,5′-cyclic AMP were used for the experiments now described.

Fig. 7 shows the dependence of k' for AMP upon pH in the presence of C11AA with pH adjustment by (i) pure acetic acid, and (ii) pure phosphoric acid, and without addition of C11AA with pH adjustment by pure acetic acid. The isotherm for C11AA in the presence of acetic acid is shown. The k' values for AMP in the presence of C11AA show strong maxima at pH of around 4.4. The adsorption of C11AA, however, falls quite steeply as the pH is reduced below about 5.2. This decrease is proportionately less marked with higher concentrations of buffer (see Fig. 8) and then occurs at slightly lower pH. The fall in C_{ads} with decrease of pH is presumably due to the decreasing ionisation of the carboxyl group and the development of a net positive charge by C11AA. There is only slight retention of AMP in the absence of C11AA.

Fig. 8 shows corresponding curves when the pH is adjusted by acetate buffers of various strengths from 0.5 to 75 mM. With the weak buffers strong maxima in k' as a function of pH are observed but even with buffer concentrations as low as 1.25 mM the maximum retention is reduced by a factor of two compared to that with no buffer present. Increase of buffer concentration to 75 mM increases the adsorption of C11AA significantly but greatly reduces the retention of AMP at pH 4.5. Evidently the zwitterion-pairing effect is extremely sensitive to the presence of other ions in the eluent.

At pH below 5 the adsorption of C11AA decreases significantly especially with buffers of low ionic strength. In order to allow for this change in C_{ads}, the data from Figs. 7 and 8 where acetic acid and acetate were used have been replotted in Fig. 9 after dividing the k' values by the concentration of adsorbed C11AA. The normalised curves so obtained confirm that the maxima are not associated with changes in C_{ads}. The maxima in the normalised curves now occur at pH in the range 4.0–4.2 with the maximum being sharpest when pure acetic acid is used to adjust pH. The pH for the maximum is now almost exactly that predicted, that is midway between the pK_a of the nucleotides (3.8) and of the carboxylic acid group (4.4), and corresponds to maximal overlap of the zwitterionic forms of AMP and C11AA.

Fig. 10 shows similar data for C12AA as pairing agent and water–dimethylformamide (90:10) as eluent, again normalised to unit surface concentration of pairing agent. Retention is significantly lower as is expected when dimethylformamide is used as organic modifier, but the maximum in retention is still relatively sharp and occurs at pH 4.2 with AMP and at pH 3.7 for 3′,5′-cyclic AMP. Again 3′,5′-cyclic AMP behaves somewhat differently from the other nucleotides.

Fig. 11 shows results obtained with the tripeptide LLL. With AMP and LLL the range of zwitterion overlap is greater than with C11AA and AMP (see Fig. 6) since the first ionisation of the peptide occurs at pH 3.3 instead of 4.4. The position of the peak retention now occurs at pH 3.5, again midway between the relevant pK_a values of the nucleotide (3.8) and the pairing agent (3.3). Retention of AMP in the presence of 1.0 mM LLL is much less than in the presence of a similar concentration of C11AA in spite of the stronger adsorption of LLL. This may be due to the unfavourable stereochemistry of the interaction of LLL and the nucleotide.

The following conclusions may be drawn from the results of the last two sections.

(1) Retention of nucleotides in the presence of C10DA is little dependent upon

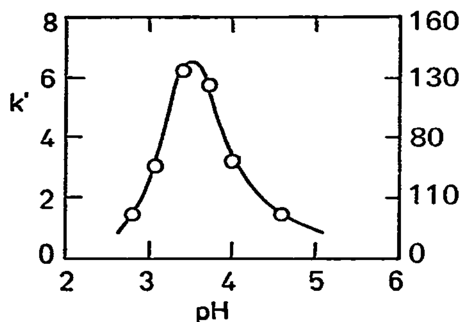


Fig. 11. Dependence of k' for AMP upon pH with LLL as pairing agent, and dependence of C_{ads} for LLL upon pH. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 0.5 mM phosphate buffer and 1.0 mM LLL.

pH. Retention in presence of C11AA changes greatly with pH showing maximum retention at pH 4.2.

(2) Buffer strength greatly affects retention: k' decreases greatly as ionic strength increases up to 75 mM and the effect is already noticeable with an ionic strength of 1.25 mM.

(3) In eluents of low ionic strength the maximum in k' as a function of pH is most pronounced.

(4) The maximum retention as a function of pH occurs at a pH which is midway between the pK_a values for formation of zwitterionic forms of the pairing agent and the nucleotide. This occurs at pH 4.2 for C11AA–AMP and C12AA–AMP, and at pH 3.5 for LLL–AMP. The predicted pH-values are 4.1, 4.1 and 3.5 respectively.

(5) The occurrence of maxima in k' as a function of pH is independent of buffer concentration, nature of buffering system, nature of pairing agent and nature of solute within the limits of this study.

Examples of separations

Separations of nucleotides in the past have normally been carried out by ion-exchange chromatography (for references see ref. 1). The results are generally poor in terms of plate efficiency, and gradient elution is required to separate mono- from di- from triphosphates. As shown previously[1] addition of C11AA allows reversed-phase separations of the three groups to be carried out in an isocratic separation with high plate efficiency. Fig. 12 shows such a separation using optimal conditions. At pH 5.4 there is good resolution of the three groups from one another but fairly small retention of the monophosphates. C10DA instead of C11AA at the same surface concentration gives much poorer separation of these compounds as seen from Fig. 13. Selectivity and peak shape are both poor. In this respect C10DA differs from cetyltrimethylammonium used successfully by Gilbert[9] for nucleotide separations. The reason for the poor peak shape with C10DA, particularly for ATP is not clear. If the asymmetry arose from a non-linear partition isotherm for ATP, the position of the peak maximum would be expected to depend upon sample size. The comparison of the broken with the full line in Fig. 13 shows that this is not the case. Conventional chromatographic theory would then argue that the broadness of the peak must arise

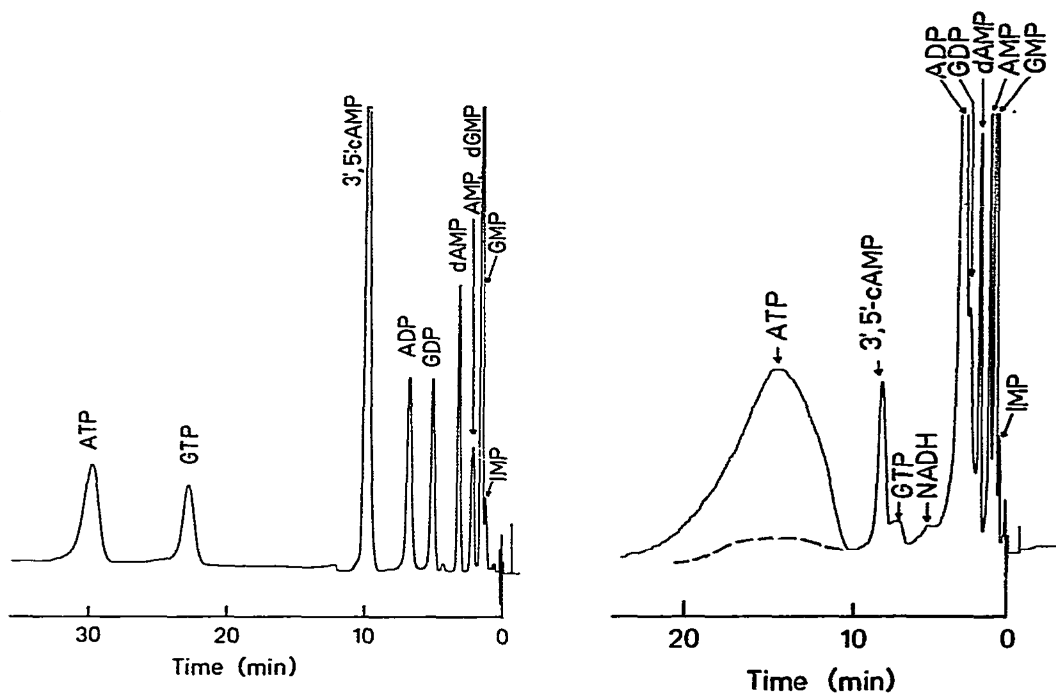


Fig. 12. Separation of mono-, di-, and triphosphate nucleotides using C11AA as pairing agent. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 75 m*M* phosphate buffer (pH 5.45) and 1.0 m*M* C11AA. Column 100 × 5 mm I.D. Flow-rate, 1.2 ml/min. Detector, UV at 254 nm.

Fig. 13. Separation of nucleotides using C10DA as pairing agent. Packing, column and eluent as Fig. 12 except pH 4.25 and 4.5 m*M* C10DA used as pairing agent.

from slow equilibration of ATP between the mobile and stationary phases, but there seems no obvious reason why this should be the case with C10DA and not with the other pairing agents which have been used.

Although C11AA provides excellent separations of the mono-, di-, and triphosphates, it has proved difficult to resolve a large range of monophosphates, even when the k' values are increased by change in pH and buffer concentration. For this group LLL gives very good selectivity as shown in Fig. 14. IMP and GMP now elute after AMP whereas with C11AA they elute before. The relative retention of the deoxy- to unreduced monophosphates remains roughly the same at about 1.3–1.5. Unfortunately with LLL, IMP and GMP are no longer resolved. To obtain complete resolution of the chosen group of nucleotides it is necessary to use a mixture of the two pairing agents, which breakthrough experiments show are independently adsorbed. Optimal results with such a mixture are shown in Fig. 15. The separations in Figs. 12, 14 and 15 show excellent plate efficiencies of between 3000 and 5000 for 125-mm columns.

Evidently variation of the nature and concentration of the pairing agent, and of the pH and ionic strength of the buffer, provide great flexibility in the control of retention and selectivity so that the method should be readily adaptable to any specific problem.

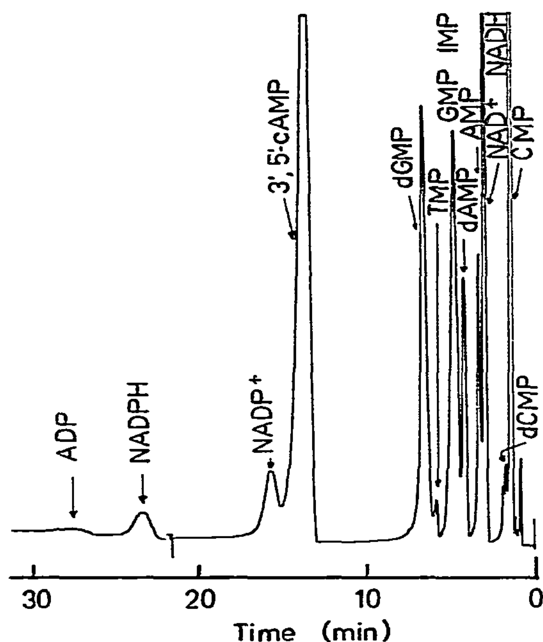


Fig. 14. Separation of mainly monophosphate nucleotides using LLL as pairing agent. Column packing, ODS Hypersil. Eluent, water–methanol (88:12) containing 2.3 mM phosphate buffer (pH 3.3) and 1.0 mM LLL. Column 125 × 5 mm I.D. Other conditions as in Fig. 12.

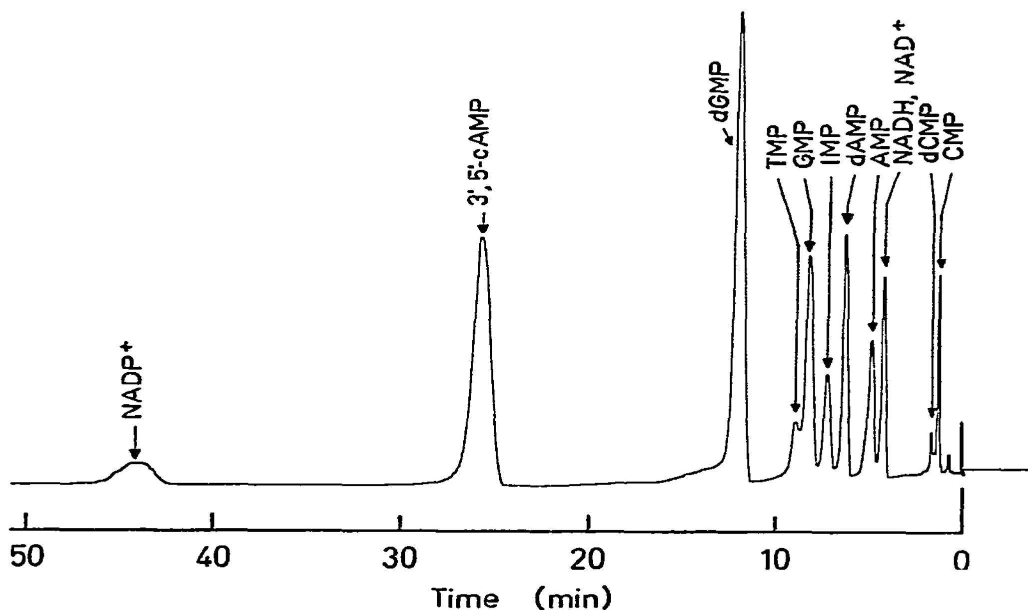


Fig. 15. As for Fig. 14 except 1.0 mM C11AA and 1.0 mM LLL used as mixed pairing agent.

CONCLUSIONS

The experiments described above, we believe, provide compelling evidence that under the correct conditions of pH zwitterionic solutes can interact strongly with zwitterionic pairing agents by the formation of quadrupolar ion pairs in reversed-phase HPLC systems. This new form of ion-pair chromatography offers great flexibility in the control of retention and selectivity by adjustment of the nature and concentration of the pairing agent, by control of pH and of buffer concentration. Thus it is possible to tune the conditions to separate nucleotides according to the number of phosphate groups, or to separate individual nucleotides with the same number of phosphate groups. Because formation of quadrupolar ion pairs involves the formation of two strong interactions between the solute and the pairing agent, it should be possible to exploit zwitterion-pair methods in the resolution of enantiomers by using an optically active zwitterion-pairing agent.

ACKNOWLEDGEMENT

The authors wish to record their thanks to the Science Research Council of Great Britain for their generous support of this work.

NOTE ADDED IN PROOF

Separation of enantiomers by this method has now been achieved and will be reported shortly (*J. Chromatogr.*, in press).

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