CHARGE-TRANSFER AND WATER-MEDIATED CHROMATOGRAPHY

II. ADSORPTION OF NUCLEOTIDES AND RELATED COMPOUNDS ON ACRIFLAVIN-SEPHADEX

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SUMMARY

Low-molecular-weight compounds are linearly adsorbed on acriflavin-Sephadex, and desorption by variation of pH, salt or temperature is therefore not necessary. The V_E/V_T values of the tested substances under the experimental conditions varied in the range 1–15, thus allowing good separations on moderate-sized columns. The adsorption capacity is surprisingly high, indicating the great potentiality for large-scale fractionation of oligonucleotides. Secondary cooperative adsorption effects can be controlled by temperature, pH, ionic strength and/or polarity of the eluent (which should increase the versatility of charge-transfer adsorption in nucleotide chemistry).

INTRODUCTION

The role played by charge-transfer complexes in biology is not yet clear, even though this type of molecular complex was identified more than a century ago. Charge transfer is based on an interaction between molecules which can mutually release and receive energy by subsequent energy coupling and then pass into an energetically more favourable state. Only weak forces are involved in the formation of charge-transfer molecular complexes. Whereas the covalent bonds of organic compounds have lengths of less than 1.6 Å, and hydrogen bonds about 1.8 Å, the contact distance in charge-transfer complexes is in the range 3.0–3.4 Å. Therefore, this type of interaction is relatively weak, as reflected by its low enthalpy of dissociation (usually in the range 0.5–4.5 kcal/mole).

Different types of charge-transfer complexes may occur and were grouped into three different classes of donors by Slifkin[1]. The properties of aromatic and pseudo-aromatic donors and acceptors are determined by the type and number of aromatic rings and of electron-attracting or electron-releasing substituents. It seems that the

most important interactions in connection with chromatography of biomolecules should occur between two conjugated systems involving a π–π interaction. However, other types of change-transfer interaction may also occur (*e.g.*, n–π complexes formed between the lone-pair electrons of oxygen or sulphur and the aromatic or pseudo-aromatic ring systems).

Early attempts to develop charge-transfer chromatography or electron donor–acceptor chromatography (CT or EDA chromatography) for biochemical compounds have not been successful because (1) adsorption effects on polysaccharide gel derivatives have been very weak, (2) solubility requirements limit the selection of the ligand substances to relatively simple aromatic compounds (it should be noted that the results of extensive studies in organic media which have been discussed elsewhere[2] do not appear to be very promising) and (3) preparation methods for such adsorbents have only recently become available. After exploratory experiments and theoretical studies[3,4] our research was concentrated on some classes of substances known to be strong acceptors (*e.g.*, benzoquinone and phthalazine and nitrophenyl ethers). Acceptable results were not obtained until dinitrochlorobenzene was coupled to thiolated Sephadex[5] and acriflavin to epoxy-Sephadex[4].
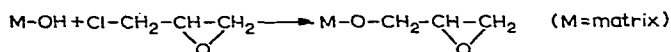
Most studies on biological charge transfer have been carried out on purines and pyrimidines[1], undoubtedly due to present interest in the role of nucleic acids in genetics and molecular biology. For example, extensive studies have been made on complexes formed between DNA and mutagenetic agents such as acridine, proflavin and related compounds[6,7]. The mutagens, which become intercalated between the base pair of the nucleic acids, have been shown to be charge donors[8]. It has been suggested that interactions between mutagens and purines are caused by electron charge transfer. Another case given in the literature is the action of an antibiotic actinomycin D, which inhibits the synthesis of ribosomal nucleic acid. This effect was attributed to interactions between the antibiotic and the guanosine and cytosine residues in the cistron and/or the ribosomal RNA[9,10]. During the interaction betceen operator and repressor in the operon for lactose a terminal part of the repressor containing 50 % of the aromatic amino acids interacted with the DNA fragment[11].

We consider these interactions to be so interesting and important that we have embarked upon a programme to explore ligands for nucleotide adsorption by electron donor–acceptor mechanisms. We hope that studies in this field may shed some light on ligand–oligonucleotide interactions and that the knowledge gained might be used to develop separation processes. For this purpose, we have performed a systematic study on the chromatographic behaviour of some purine and pyrimidine bases and on nucleosides, nucleotides and related compounds using π-electron-rich derivatives of Sephadex. The different parameters involved in charge-transfer chromatography, especially when using acriflavin as a ligand, and some preliminary results showing the importance of this method are presented. The drawbacks and some possible applications of charge-transfer chromatography are also discussed.
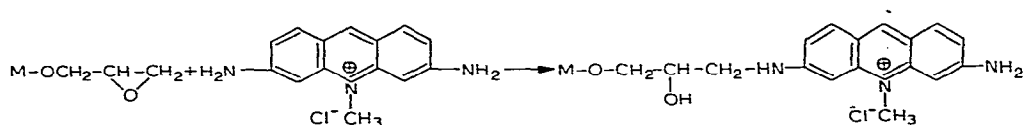
EXPERIMENTAL

Synthesis of charge-transfer adsorbent

Selected cross-linked dextran (Sephadex G-25) was activated by epichlorohydrin[12] according to the following reaction:

$$M-OH + Cl-CH_2-CH-CH_2 \longrightarrow M-O-CH_2-CH-CH_2 \quad (M=matrix)$$
$$\underset{O}{\diagdown\diagup} \qquad\qquad \underset{O}{\diagdown\diagup}$$

A 250-ml volume of Sephadex G-25 (Pharmacia, Uppsala, Sweden) was thoroughly washed, after swelling, on a glass filter funnel with distilled water and was introduced with 125 ml of 2 M sodium hydroxide solution, 12.5 ml of epichlorohydrin (Kebo, Stockholm, Sweden) and 0.5 g of sodium borohydride (Merck, Darmstadt, G.F.R.) into a three-necked 2-l flask. An additional 125 ml of 2 M sodium hydroxide solution and 62.5 ml of epichlorohydrin were slowly and continuously introduced simultaneously over a period of 4–5 h with moderate stirring. Stirring was continued overnight at room temperature. The epichlorohydrin derivative of Sephadex G-25 (Sephadex G-25 ECD) was then collected on a glass filter funnel and thoroughly washed with distilled water and ethanol–0.02 M potassium hydroxide (1:1, v/v) for immediate use under the coupling conditions described below.

A 10-g amount of acriflavin (Fluka, Buchs, Switzerland) and the activated Sephadex G-25 were mixed with 200 ml of ethanol–0.02 M potassium hydroxide (1:1, v/v) with stirring at 40° overnight according to the following coupling reaction:

The gel was then washed successively on the glass filter funnel with water, ethanol (50%), acetic acid (0.05 M) and water, until a colourless effluent was obtained. The gel used in the chromatographic experiments presented here was analysed for its nitrogen content, and was found to contain 125 μmole of acriflavin per gram of dry gel.

Chromatographic procedure

Columns of acriflavin-Sephadex G-25 were equilibrated in a suitable buffer, depending on the experimental purposes. Samples were dissolved in 0.1 ml of eluent solution and run on the column at room temperature at a flow-rate of 10 ml/h if not otherwise stated. The charge transfer is virtually independent of time as demonstrated by the finding that variations in flow-rate and/or the size of the column do not effect the V_E/V_T values (reduced elution volumes), where V_E and V_T are the elution volume of the substance studied and the total bed volume of the column, respectively. Such values were calculated from the chromatograms obtained after measurement of the absorbance of the eluate at 254 nm with a Uvicord spectrophotometer. With our experimental conditions, the maximum retention capacity of the gel was about 0.1–1 mg of nucleotides per millilitre of swollen acriflavin-Sephadex G-25.

RESULTS

Chromatographic behaviour of adenosine and AMP on different Sephadex derivatives

Different derivatives coupled to Sephadex were studied for their abilities to interact with adenosine or AMP (Table I). Introduction of aromatic substituents into the Sephadex gel increased the affinity of the support for adenosine and AMP. It is known that Sephadex interacts with phenol, tryptophan, uric acid and π-electron-

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF ADENOSINE AND AMP ON DIFFERENT ADSORBENT GELS

Sample, 50 μl of adenosine or AMP at a concentration of 3 μg/μl in 0.1 M ethylmorpholine buffer (pH 7); column dimensions, 15 cm $\times$ 1 cm I.D.; flow-rate 10 ml/h; temperature, 24°. V_E/V_T = reduced elution volume of sample chromatographed.

Gel	V_E/V_T	
	Adenosine	AMP
G-25 (Fine)	1.55	1.18
G-25 ECD	1.50	1.13
G-25 S-chloranil	2.07	1.10
G-25 ECD chlorophenothiazine	1.91	1.01
G-25 S dinitrophenyl	2.45	1.20
G-25 S pentachlorophenyl	2.29	0.92
G-25 S dicyanochloroquinone	2.28	1.14
G-25 ECD acriflavin	2.40	4.55

containing solutes[13]. Sephadex-based charge-transfer adsorbents seem to act by two different kinds of "aromatic interactions", one involving the substituent, the other the hydroxyl group of the matrix or the matrix-bound water[3]. The spacer arm introduced in the gel for coupling the ligand does not seem to play an appreciable role in these interactions. The strength of the interaction between the solute and Sephadex derivatives is dependent on the nature of the ligand (e.g., type of ring system and its content of electron-attracting or -releasing substituents). As acriflavin-Sephadex yielded the largest differences in retention values for the test substances (Table I), it was selected for further studies.

Influence of structure on the interaction

In order to elucidate the charge transfer of the various nucleotides present in RNA or DNA participation, we chromatographed different selected solutes on G-25 ECD acriflavin (Table II). A strong interaction between heterocyclic aromatic compounds (adenine, adenosine and AMP) and acriflavin was observed. Serine and glucose did not interact significantly while a slight adsorption occurred due to phosphorylation of these compounds. It is interesting that the amino group of serine did not play an important role in the interaction process. The influence of the phosphate groups present in the nucleotides is more complex. For example, in ethylmorpholine buffer (pH 7), a strong interaction occurred between acriflavin and AMP (V_E/V_T = 4.55) while adenosine had appreciably less affinity for the adsorbent in this buffer system. The opposite was observed with ethanolamine buffer.
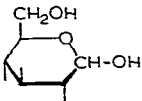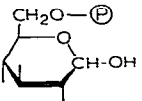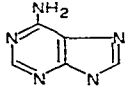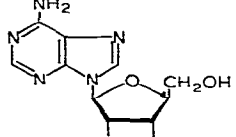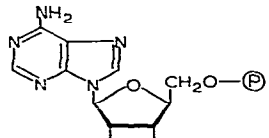
Dependence on ionic strength

The unchanged ligand may interact hydrophobically with the adsorbent at all ionic strengths[14]. It was therefore of interest to evaluate the influence of the ionic strength on charge-transfer interaction as a result of variations in either salt or amine concentration. The effect of a neutral salt on the interaction procedure is shown in Fig. 1 (Von Hippel and Scheich[18] have defined a neutral salt as a strong electrolyte which

TABLE II

INFLUENCE OF THE DIFFERENT COMPONENTS OF THE NUCLEOTIDES INTER-
ACTING WITH ACRIFLAVIN

The chromatographic experiments were performed under the same experimental conditions as on Sephadex G-25 ECD acriflavin described in Table I. Serine and phosphoserine were detected by the ninhydrin method and glucose and glucose 6-phosphate by the orcinol method.

Compound	Formula	V_E/V_T	
		Ethylmorpholine–acetic acid, 0.1 M (pH 7)	Ammonium acetate–acetic acid, 0.1 M, ethanolamine, 0.2 M (pH 6)
Serine	HO–CH$_2$–CH–COOH, NH$_2$	0.87	
Phosphoserine	P–O–CH$_2$–CH–COOH, NH$_2$	1.16	
Glucose	CH$_2$OH ... CH–OH	0.87	1.02
Glucose 6-phosphate	CH$_2$O–Ⓟ ... CH–OH	1.16	1.02
Adenine	NH$_2$ ...	3.25	3.26
Adenosine	NH$_2$ ... CH$_2$OH	2.45	2.50
Adenosine monophosphate (AMP)	NH$_2$ ... CH$_2$O–Ⓟ	4.48	1.88

dissolves significantly in water without bringing about a major change in solution pH). The graph shows that there is no effect of salt concentration on the acriflavin–adenosine interaction ($V_E/V_T = 2.45$). In contrast, the adsorption of AMP and CMP was considerably diminished on increasing the salt concentration. The phosphate group of the nucleotide apparently increases its interaction with acriflavin at low salt concentrations by affecting the electron distribution.

An analogous phenomenon was observed when we increased the ionic strength of ammonium acetate buffer at pH 6 by addition of ethanolamine (Fig. 2). In this instance, the interaction between adenosine and acriflavin remained constant ($V_E/V_T =$
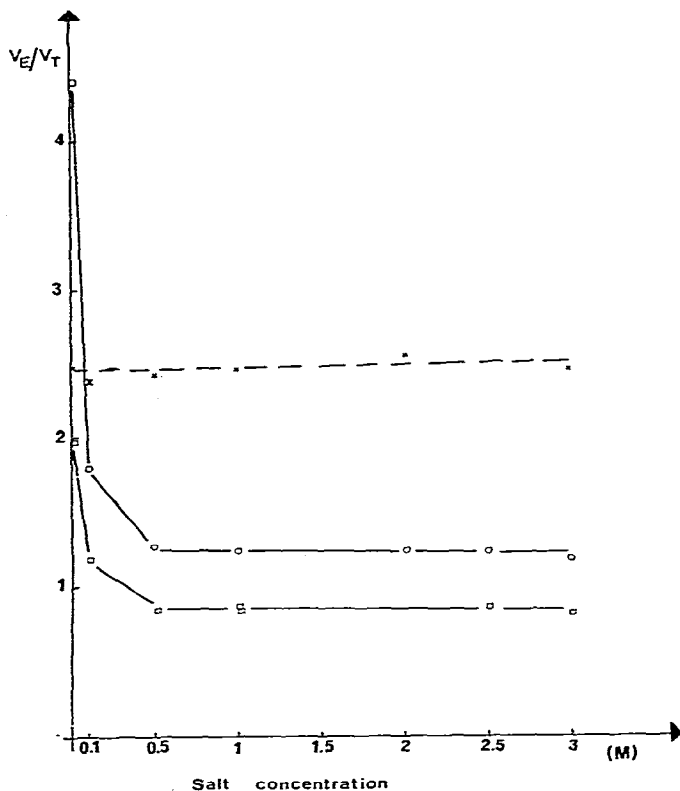
Fig. 1. Dependence of interactions on salt concentration. A 50-μl volume of adenosine ($\times$), adenosine monophosphate (AMP) (O) and cytosine monophosphate (CMP) ($\square$) were applied on Sephadex G-25 ECD acriflavin in 0.1 M ethylmorpholine buffer (pH 7) at different NaCl concentrations at room temperature.
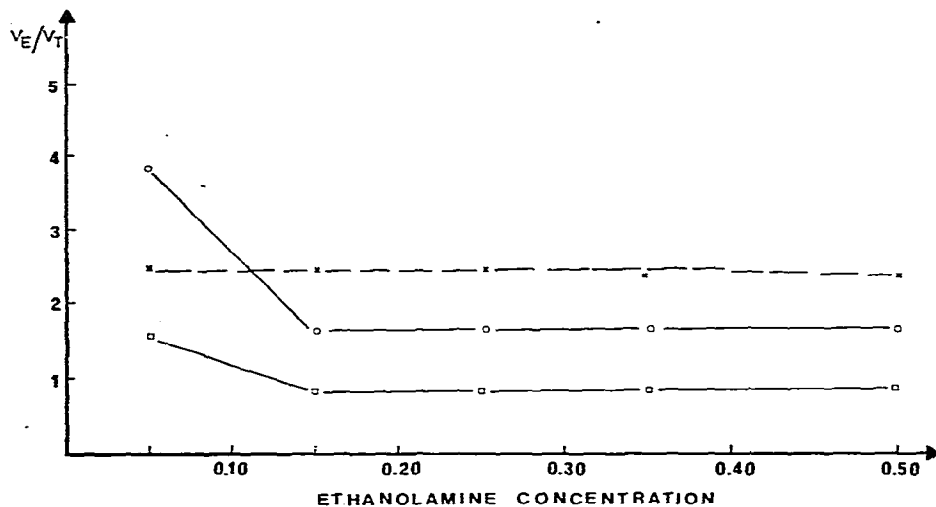

Fig. 2. Dependence of interactions on ionic strength of the buffer. A 50-μl volume of adenosine ($\times$), adenosine monophosphate (AMP) (O) and cytosine monophosphate (CMP) ($\square$) were applied on Sephadex G-25 ECD acriflavin in 0.1 M ammonium acetate buffer (pH 6) at different ethanolamine concentrations (M) at room temperature.

TABLE III

INFLUENCE OF THE IONIC STRENGTH AND THE BUFFER COMPOSITION ON ACRIFLAVIN–AMP INTERACTION

Buffer ammonium acetate–acetic acid, 0.1 M (pH 6)	V_E/V_T	Conductivity $(10^{-2}$ S/cm)
Ethanolamine, 0.2 M	1.75	14
Ethanolamine, 0.5 M	1.75	23
Ethanolamine, 2.0 M	1.70	41
NaCl, 0.1 M	1.79	11
NaCl, 0.5 M	1.28	46
NaCl, 2.0 M	1.24	90

2.45). This value was different from that obtained for AMP in the presence of a neutral salt ($V_E/V_T = 1.25$). In order to clarify this unexpected difference, we carried out some experiments in which the conductivity of the buffer was varied (Table III). For the same ionic strength (e.g., interpolated for a conductivity of $41 \cdot 10^{-2}$ S/cm) the V_E/V_T value differs; in 2 M ethanolamine $V_E/V_T = 1.70$, and in 0.1 M ammonium acetate–0.5 M sodium chloride (pH 7) buffer $V_E/V_T = 1.25$. In both buffer systems a decrease in the ionic interaction between the phosphate group of AMP and acriflavin was observed, although different limiting V_E/V_T values seemed to be approached. Therefore, it appeared that an amine and a neutral salt in the buffer acted differently in the interaction of ligand and solute. These findings led us to focus part of our study on the effects of different amines on the V_E/V_T values of AMP (Fig. 3). The amines were chosen to reflect different electron donor properties. As shown in Fig. 3, the
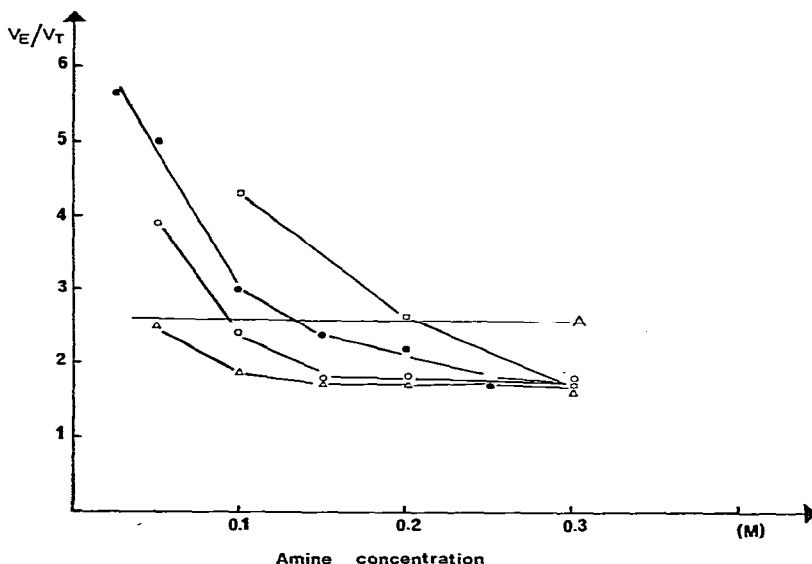

Fig. 3. Effect of different amines on acriflavin–AMP interactions. The samples were applied on Sephadex G-25 ECD acriflavin using different types of amines included in 0.1 M ammonium acetate buffer (pH 6). ●, Triethylamine; △, diethylenetriamine; ○, ethanolamine; □, ethylmorpholine; ine A, adenosine.

strength of interaction depends firstly on the nature of the amine, and secondly on its concentration. No influence on complex formation between adenosine and acriflavin was observed, the V_E/V_T value remaining constant at about 2.45. The presence of amines in the buffer system helped to decrease the AMP–acriflavin interaction by a mixed mechanism, where the different alkyl substituent on the amino group plays a role as well as the ionic strength of the buffer. Diethylenetriamine (0.1 M) seemed to abolish the electrostatic interaction between acriflavin and AMP, thereby allowing an excellent separation of adenosine and AMP. As the amine buffer systems are volatile the subsequent isolation of the solutes is facilitated.

Effect of neutral salts

The influence of salt on the adsorption of aromatic compounds to hydrophobic or charge-transfer gels is probably due to a number of factors exerted on the bio-molecule as well as on the matrix. The effectiveness of salt in promoting interactions between acriflavin and nucleoside or nucleotide varied according to the chemical nature of the ions present in the medium. Ions of neutral salts have been arranged according to their salting-in and salting-out properties (Hofmeister series). When the effect of different salts (or ions) in promoting hydrophobic or charge-transfer inter-action are considered, a problem arises of how to compare the effects of monovalent and polyvalent ions. This comparison can be made, for example, at constant salt concentration or at constant ionic strength. Salt concentration was kept constant in a series of experiments (Table IV). For monovalent cations, no appreciable change appeared in the adenosine–acriflavin or in the AMP–acriflavin interaction, but for the anions the salt effect corresponded to the Hofmeister series, *i.e.*, increasing chaotropic effects reduced the interaction. Polyvalent ions decreased the interaction even more.

TABLE IV

EFFECT OF IONS ON ACRIFLAVIN–AMP OR –ADENOSINE INTERACTION

50 μl of adenosine or AMP at a concentration of 3 μg/μl in 0.1 M ammonium acetate buffer (pH 6) containing 2 M of a neutral salt in the same kind of column as described Table I.

Ions in ammonium acetate-acetic acid, 0.1 M (pH 6)	V_E/V_T	
	Adenosine	AMP
Rb$^+$	2.26	1.41
K$^+$	2.40	1.41
Na$^+$	2.45	1.27
Cs$^+$	2.26	1.41
Li$^+$	2.26	1.27
Mg^{2+}	1.84	1.47
Al^{3+}	1.15	0.95 ▼
Cl$^-$	2.45	1.27
Br$^-$	2.15	1.21
I$^-$	1.95	1.08
SCN$^-$	1.60	1.08 ▼

(cations: Increasing chaotropic effect; anions: Increasing salting-out effect)

Effect of temperature

Complexes can be characterized using thermodynamic parameters. The enthalpy of dissociation ($\Delta H°$) is the most commonly used parameter, and is obtained

from the variation of the dissociation constant (K_c) with absolute temperature from the Van 't Hoff equation. The effect of temperature on V_E/V_T is shown in Fig. 4. The adsorption of AMP and adenosine on acriflavin gels decreased when the temperature was increased. In aqueous buffer systems (*e.g.*, ethylmorpholine–acetic acid, 0.1 M, pH 7) solute–ligand interactions decreased more slowly with adenosine to a value of 1.70 at 50°. The effect of higher temperature on the buffer composition is considered not to be appreciable.
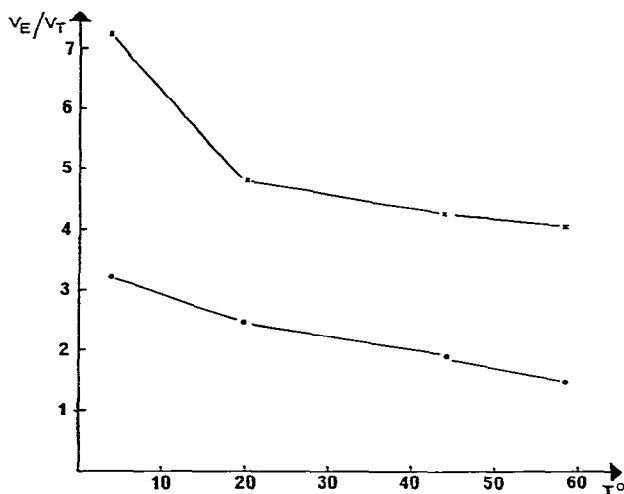

Fig. 4. Effect of temperature on acriflavin–adenosine or –AMP interactions in 0.1 M ethylmorpholine buffer (pH 7). ●, Adenosine; ×, AMP.

Effect of pH

Naturally, pH can play an important role in the adsorption of solute on a charge-transfer gel carrying ionized groups such as quaternary nitrogen on acriflavin. Variation in the pH may modify the charge of either the biomolecule or the adsorbent. By changing the pH, a net charge may be introduced in the ligand which can be either of the same sign as or of opposite sign to the charge on the biomolecule. In the former instance, the repulsive effect will affect negatively the adsorption phenomena (Table V). In the low acidity range, the tendency towards decreased interaction reflects protonation of the adenosine amino group which has a pK_a value of 3.3[15] and as a consequence complexing is impeded. In the presence of neutral salt and at high salt concentration, such as with 2 M sodium chloride solution, the ionic effect due to the phosphate group can be suppressed for AMP–acriflavin interaction (Table V). The pH range 5–7 appears optimal for complex formation, in accord with the results of Tsibris *et al.*[16].

Effect of solvent

As shown in Table VI, addition of ethylene glycol to a concentration of 50% decreases the gel–solute interaction. Urea in high concentration also reduces the adenosine– and AMP–acriflavin interactions, presumably by preventing hydrogen bond

TABLE V

EFFECT OF pH ON ACRIFLAVIN–ADENOSINE OR –AMP INTERACTION

Buffer composition			pH	V_E/V_T	
				Adenosine	AMP
Ethylmorpholine,	0.1	M	7	2.44	4.55
Ethylmorpholine,	0.1	M,			
NaCl,	2	M	7	2.40	1.30
Ammonium acetate,	0.1	M,			
ethanolamine,	0.15	M	6	2.45	1.75
Ammonium acetate,	0.1	M,			
ethanolamine,	0.15	M	5	2.45	1.75
HCOOH/NaOH,	0.1	M	3.2	1.32	2.37
HCOOH/NaOH,	0.1	M,			
NaCl,	2	M	3.2	1.28	1.70

TABLE VI

EFFECT OF SOLVENT COMPOSITION ON ACRIFLAVIN–ADENOSINE OR –AMP INTERACTION

Buffer composition			V_E/V_T			
			4°		20°	
			Adenosine	AMP	Adenosine	AMP
Ethylmorpholine,	0.1	M (pH 7)	3.24	7.22	2.40	4.80
Ammonium acetate,	0.1	M				
ethanolamine,	0.15	M (pH 6)	3.14	2.58	2.50	1.70
Ethylmorpholine,	0.1	M,				
NaCl,	2	M (pH 7)	1.83	1.54	2.45	1.25
Ethylmorpholine,	0.1	M,				
urea,	3	M (pH 7)	1.98	3.47	1.75	2.23
Ethylmorpholine,	0.1	M,				
ethylene glycol,	50%	(pH 7)	1.56	4.07	1.71	2.78

formation between solute and adsorbent. Upon increasing the urea concentration, the adenosine–acriflavin interaction decreases to a limiting V_E/V_T value of ca. 1.50 at about 6 M at room temperature (unpublished results).

APPLICATIONS AND DISCUSSION

　　The chromatographic behaviour of nucleotides, nucleosides, their corresponding bases and other related compounds has been studied under various conditions (Figs. 1–4, Tables I–VI). It was found that the nucleosides, e.g., adenosine, are less ·retarded than their corresponding bases (Fig. 5 and Table II). The ribose residue affects the adsorption by its hydrophilic character which increases the polarity of the complex and/or by steric hindrance. It was also observed that the reduced·elution volumes fall into two categories, the purine compounds being more retarded than the pyrimidines (Figs. 1 and Fig. 5). This finding and earlier results[4] are in agreement with some k values obtained from approximate solutions of the Schrödinger equation[17]
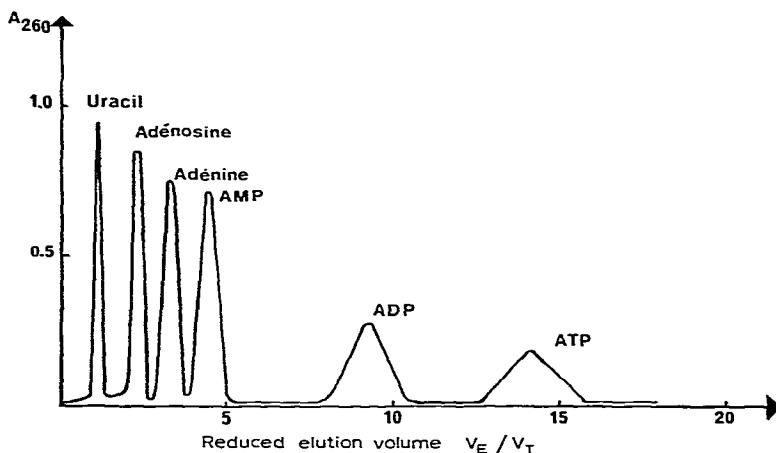
Fig. 5. Chromatographic resolution of mononucleotides, nucleosides and bases on Sephadex G-25 ECD acriflavine in 0.1 M ethylmorpholine buffer (pH 7) at room temperature.

and with some dissociation constants (K_c) given for the complexes of riboflavin with the respective purine and pyrimidine compounds[16]. These results also agree with the findings that adenosine and guanosine are better electron donors than thymine, uracil and other pyrimidine analogues. Hence adsorption seems to parallel the electron donor properties of the solute, as we have shown for aromatic amino acids[4].

The influence of the phosphate group of the nucleotide on adsorption on acriflavin-Sephadex is surprisingly complex. As mentioned above, some ionic interaction, due to the positive charge on acriflavin, can be favoured by using buffers of low ionic strength (Figs. 1 and 2). Thus, chromatography permits a satisfactory separation of homologous series of nucleotides and their corresponding nucleosides. The separation depends upon attraction between the positive charge on the nitrogen atom in acriflavin and the ionized phosphate as well as on the number and kind of interacting groups of the oligonucleotides (Fig. 6).

The effect of the phosphate group is strong at low ionic strength but can be decreased or abolished by increasing the salt concentration to a point at which the charge-transfer interaction remains. However, as shown in results with phosphoserine and phosphoglucose (Table II), the phosphate group *per se* may not be the site of interaction. Rather, the phosphate group of the AMP may exert its influence indirectly by changing the configuration of the nucleotide molecule, and/or by affecting the electron distribution over the heterocyclic ring system. As a provisional hypothesis we propose that an intramolecular complex is formed at high salt concentration. This results in an intramolecular arrangement that inhibits or decreases the π–π interaction between the two conjugated systems involved in charge-transfer complex formation; two explanations for this seem possible:

(i) the alignment for orbital overlapping with the acriflavin ligand is sterically hindered, and/or

(ii) weakening of the electron-donor properties of AMP. This hypothesis is reinforced by the fact that if we choose pentachlorophenyl as a ligand coupled to Sephadex, the AMP–pentachlorophenyl interaction is expressed by a low V_E/V_T value
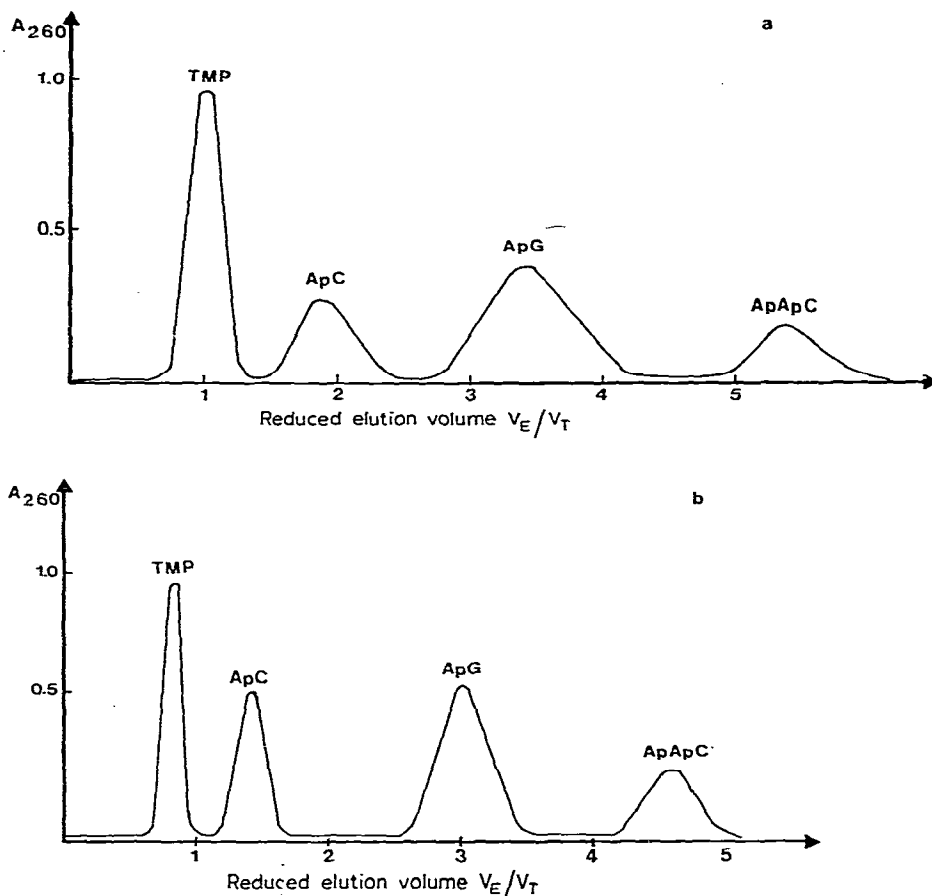
Fig. 6. Chromatographic resolution of oligonucleotides: thymidine monophosphate (TMP) adenylyl-cytidine (ApC), adenylylguanosine (ApG) and adenylyladenylylcytidine (ApApC). (a) 0.1 M ammonium acetate buffer (pH 6), 0.2 M ethanolamine at 20°; (b) 0.1 M ethylmorpholine buffer (pH 7), 2 M NaCl at 20°.

(Table I) and is independent of the ionic strength of the eluate buffer (unpublished results).

AMP–acriflavin is an adsorption complex of the charge-transfer type, as the gel turns darker[4] at very high concentration of adenosine monophosphate and in the presence of sodium chloride. The influence of the phosphate group in the oligonucleotides is suppressed.

When several adsorption centres are introduced into the solute, cooperative charge-transfer will increase the strength of the adsorption considerably (see Fig. 6). Mono-, di- and trinucleotides can thus easily be separated on the basis of the number and character of nucleotide units. Purines are more strongly adsorbed than pyrimidines with the same number of base units. The temperature dependence has been demonstrated in a previous publication[4].

Even when ionic interaction is suppressed, there is still considerable retention

of the oligonucleotides (Fig. 6). However, chaotropic ions inhibit or prevent charge-transfer complex formation.

The ligands or solutes used are more or less hydrophobic and hydrophobic adsorption can therefore be expected. The opposite influence of temperature on adsorption can serve as a guide for the estimation of the relative importance of hydrophobic interaction *versus* charge transfer. Increases in temperature should decrease the V_E/V_T values for charge-transfer adsorption. Consequently, the temperature effect (Fig. 4) supports the hypothesis that electron charge transfer is more important for the systems in the present study than is hydrophobic interaction. It is difficult to avoid other kinds of interactions, such as hydrogen bond formation.

ACKNOWLEDGEMENTS

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