Interaction of poly(A) with different adsorbents for affinity chromatography of nucleic acids

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Chromatography on adsorbents for separation of mRNA containing poly(A) has given interesting results, even if the nature of the occurring interaction was not always well understood. In the present study we report the chromatographic behaviour of poly(A) homopolynucleotides on different substituted matrices: poly(U)-; poly(A)-; phenyl-, octyl-, ethanolamine-, acriflavin- and DNA-Sepharose; oligo-dT and MN-cellulose. Using different experimental conditions as ionic strength, neutral salt, pH, temperature, buffer composition it was possible to evaluate the participation of electrostatic, hydrophobic hydrogen-bonding, and/or charge-transfer interaction. Furthermore, it is shown that poly(A) interacts non-specifically with matrices like acriflavin or DNA-Sepharose, as well as with oligo-dT cellulose or poly(U)-Sepharose.

Chromatography on adsorbents containing immobilized polynucleotides has been applied in the purification of a number of proteins such as RNA-proteins, histones, DNA- or RNA-polymerases, etc. (for a review see ref. 1). In turn, the corresponding proteins can be immobilized and utilized for the isolation of nucleic acids, taking advantage of their mutual biospecific affinity[2]. Besides the possibility of studying the hybridization which occurs between complementary polynucleotides, nucleic acid affinity chromatography is excellent for separating these biomolecules according to their base composition, using matrix-bound polynucleotides and/or dyes of known structure and specificity[2,3]. Unfortunately, non-specific interactions between the proteins, and/or the polynucleotides, with the immobilized compound are often ignored. For example, the separation of poly(A)-containing nucleic acids on poly(U)-Sepharose is considered to occur by a simple 'base-pairing' mechanism[4]. However, the fact that relatively drastic conditions for elution of the mRNA-poly(A) from the column are needed suggests that interactions other than hydrogen bonding are of considerable importance. In order to study the nature of the interaction between polynucleotides in affinity systems, a series of experiments on the chromatographic behaviour of synthetic poly(A) on different supports has been performed. In the present paper it is shown that poly(A) adsorbs promptly to affinity adsorbents used for fractionation of nucleic acids by a mechanism that includes, among others, hydrogen bonding, electrostatic, hydrophobic and charge-transfer interactions according to the nature of the ligand, the matrix, and/or the coupling procedure employed during immobilization. The participation of either of these interactions in the adsorption of poly(A) to such adsorbents has been estimated under different conditions by varying pH, temperature, salt concentration and buffer composition.

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Experimental

Matrices and chemicals

^{3}H-poly(A) and ^{3}H-poly(U) (*MW* 100000, specific activity 2–10 Ci/mmol of AMP or UMP) were purchased from New England Nuclear Co. (Boston, Mass., USA). Unlabelled poly(A) and poly(U) (*MW* 100000) and MN-cellulose were from Sigma (St. Louis, Miss., USA). Phenyl-, octyl-, poly(A)-, poly(U)-Sepharose 4B, and Sepharoses 2B and 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Oligo-dT-cellulose (about 10 nucleotides chain length) came from Collaborative Res. Inc. (Waltham, Mass., USA). DNA-Sepharose 2B and ethanolamine-Sepharose 2B were prepared as described before[5]. Acriflavin (Fluka AG., Switzerland) was coupled to Sepharose 4B according to the method described in ref 6.

Samples

A stock solution (100 mg/ml) of poly(A) was prepared with 10 mM Tris- HCl buffer, pH 7.6, and mixed with 5 μl of ^{3}H-poly(A) (specific activity 20×10^7 c.p.m./ml). This mixture (100 μl; about 10×10^3 c.p.m.) was applied to each column according to the conditions described in the legends to the respective figures. The radioactivity was measured by liquid scintillation in an ABAC-SL40 Intertechnique scintillation counter using Instagel (Packard Instruments Co., Inc., Downers, Ill., USA). For the study of the pH effect, the stock solution was prepared in the following buffers: 50 mM glycine- HCl, pH 3; 50 mM sodium acetate- acetic, pH 4; 50 mM Tris- HCl, pH 5.6, 7.6, and 8.6. All experiments were run at room temperature unless otherwise stated.

Columns

Pasteur pipettes were packed with 200 μl of gel. A fibreglass cap was attached to the bottom of each column

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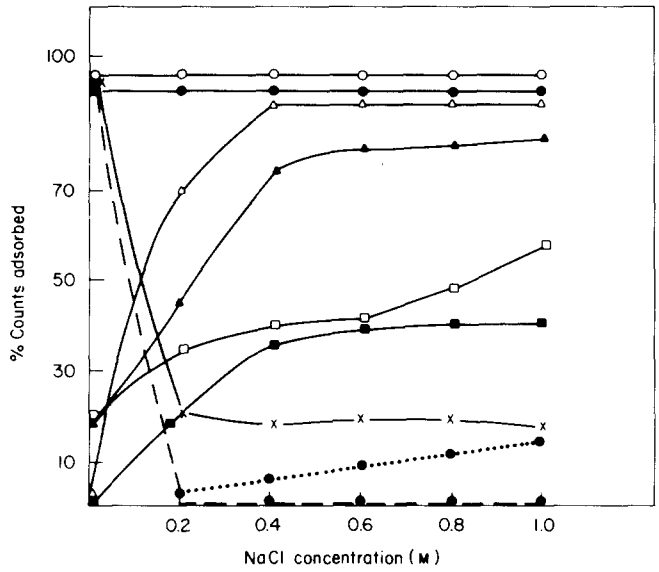

Figure 1. Salt effect. Adsorption of ^{3}H-poly(A) to different matrices as a function of salt concentration. The columns were equilibrated at 4 C with NaCl in 10 mM Tris HCl buffer, pH 7.6, at the concentrations indicated. The percent radioactivity adsorbed with respect to the total amount of material applied is plotted. ●, Acriflavin-Sepharose; ○, poly(U)-Sepharose; △, oligo-dT-cellulose; ▲, DNA-Sepharose; ■, MN-cellulose; □, octyl-Sepharose; ×, poly(A)-Sepharose; ●···●, phenyl-Sepharose; ●---●, ethanolamine-Sepharose

to assure the physical entrapment of the gel. The columns, run in triplicate, were equilibrated as indicated in the figure legends. The reported values correspond to the averages of at least three experiments, with a standard deviation of about 5%.

Results and discussion

Salt effect

The chemical nature of poly(A) suggests that the ionic strength of the medium may be important for the capacity of the molecule to interact with other compounds. At neutral pH and low ionic strength, the poly(A) molecule carries a considerable negative charge. Therefore, and since charges are efficiently neutralized by counterions, it is not surprising that the electrostatic attraction causing adsorption of poly(A) to the positively charged ethanolamine-Sepharose decreases with salt concentration (*Figure* 1). As has been shown, the activation of polysaccharide matrices using BrCN for the coupling of primary amines is considered to occur via formation of isourea linkages which are positively charged[7,31,32]. Such positively charged groups should also be present in poly(U)- and poly(A)-Sepharose, commercially prepared by the same procedure. This is noticed more clearly in the case of poly(A)-Sepharose (*Figure* 1). From *Figure* 1 it is clear that as the salt concentration is increased in the cases of poly(U)- and poly(A)-Sepharose other mechanisms of adsorption become relevant: poly(U)-Sepharose, for instance, might adsorb poly(A) by hydrogen bonding, whereas the adsorption of poly(A) to poly(A)-Sepharose can be accounted for in terms of 'stacking'[8]. In a control experiment it has been found that poly(A) shows no significant affinity for unsubstituted Sepharose under similar conditions.

DNA-Sepharose, on the other hand, does not possess important adsorption capacity towards poly(A) at low salt concentration, resembling neutral oligo-dT-cellulose in this respect (*Figure* 1). The low pH employed for the coupling of DNA to BrCN-activated Sepharose[5] is probably the reason why the number of positive charges introduced on the matrix, is lower[9].

As could be expected, oligo-dT-cellulose commercially prepared by irradiation, lacks affinity for poly(A) at low ionic strength (*Figure* 1). This is simply due to repulsion between the negatively charged polymers at such conditions. The same effect would occur between poly(A) and the polynucleotide matrices described above but, as pointed out, it cannot be observed, due to the presence of undesirable positive charges on those adsorbents. With increasing salt concentration charges are partially neutralized, and adsorption of poly(A) to its complementary matrices [poly(U)-, DNA-Sepharose and oligo-dT-cellulose] is enhanced. Considering a mechanism based on hydrogen bonding, the difference in adsorption capacity between these matrices is mainly attributed to their different degrees of substitution and/or chain-length.

Very recently, matrix-bound dyes have been employed for affinity chromatography of nucleic acids taking advantage of their base specificity[3,6,40]. Therefore, it was interesting to look at the chromatographic behaviour of poly(A) on this type of adsorbent. For this purpose, acriflavin-Sepharose was prepared by using a coupling procedure that avoids the introduction of charges on the matrix[6]. The data plotted in *Figure* 1 suggest that the adsorption of poly(A) to acriflavin-Sepharose may be practically salt-independent as a result of mixed effects of electrostatic, hydrophobic, and charge-transfer interactions. Evidence in favour of a charge-transfer association between poly(A) and acriflavin-Sepharose, at moderate salt concentration, has been collected. For instance, free poly(U) is able to displace poly(A) from acriflavin-Sepharose columns provided that conditions for poly(A) poly(U) complex formation are adequate (in our case, 10 mM Tris HCl, pH 7.6, with 0.4 M NaCl are optimal for elution). Competition of poly(U) with poly(A) for adsorption on acriflavin-Sepharose is excluded because poly(U) has no affinity for acriflavin-Sepharose under these conditions (results not shown). Interestingly, similar effects have been observed when cellulose[10-12] and/or millipore filters[13] have been used in the purification of poly(A)-containing nucleic acids. Although no explanation has been put forward, due to lack of information about the structure of cellulose, some authors have proposed the participation of aromatic interactions between the lignin aromatic groups of cellulose and the bases of polynucleotides[11]. The fact that only purine polynucleotides possess a significant affinity for these adsorbents supports further the idea that aromatic interactions of the type of charge-transfer are not unusual with poly(A).

For the binding of poly(A) to acriflavin-Sepharose, the model for the interaction between this kind of dye and nucleic acids, as proposed by Lerman[15], seems very suitable: that is, both external (binding of the dye directed to the phosphate groups) and intercalated (binding of the dye between the bases) associations between poly(A) and acriflavin-Sepharose are feasible, with accessibility under a given set of conditions being the sole restriction.

The ability of nucleic acids to enhance the solubilization of organic compounds in aqueous systems has been known for several years[16]. Nevertheless, the contribution

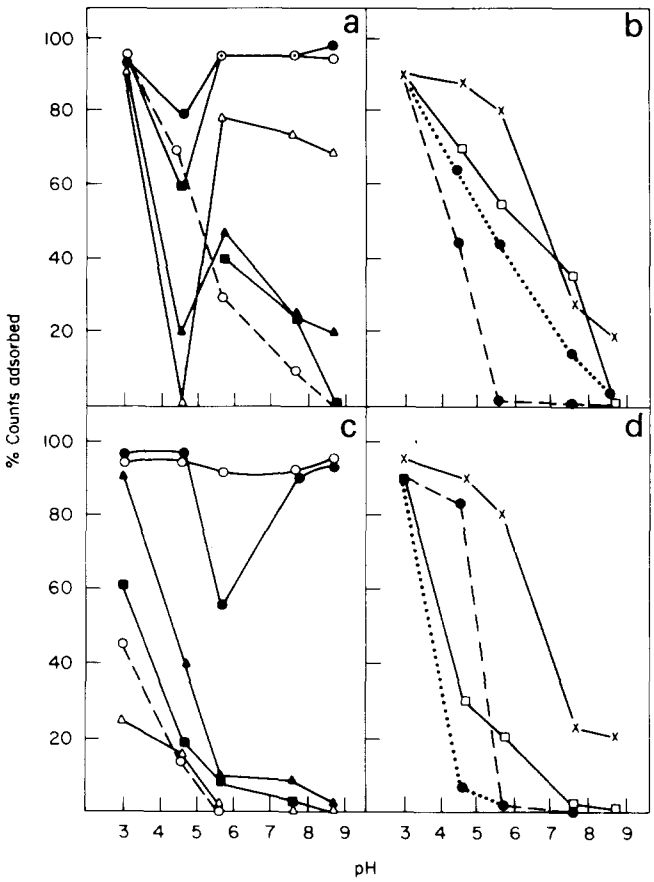

Figure 2. pH effect. Adsorption of ^{3}H-poly(A) to different matrices as a function of the pH. The percent radioactivity adsorbed with respect to the total amount of material applied is plotted: (a) and (b) give results of experiments performed with buffer containing 0.4 M NaCl; (c) and (d) results with the same buffers but in the absence of salt. All the experiments were run at 20 C. ○ - - - ○, Glass-wool; other symbols as in *Figure* 1

of hydrophobic bonding between polynucleotides in affinity systems is ignored in most cases. Quite recently it has been possible to purify nucleic acids by a salting-out mechanism on unsubstituted Sepharoses[17,36,37]. The separation is ascribed mainly to discrimination between polynucleotides of different hydrophobicity, which are more or less retained in the columns[17]. Thus, polynucleotides should be adsorbed on hydrophobic adsorbents according to the ionic strength of the media, as in the case of proteins[38]. This is shown in *Figure* 1 for poly(A) on octyl- and phenyl-Sepharoses, though in the latter case aromatic interactions may also be involved. In the same way, MN-cellulose shows an important salt-dependent adsorption capacity towards poly(A)[10-12]. Still, as discussed above, the adsorption capacity of MN-cellulose towards purine-polynucleotides is most probably related to aromatic interactions of the type of charge-transfer. This will be further demonstrated by the effect of temperature on the adsorption of poly(A) to this matrix as described below.

pH Effect

Poly(A) undergoes conformational changes as a result of variations in the pH of the medium[18-19]. Such structural modifications affect its ability to interact with other compounds. As shown in *Figure* 2 the pH effect is also influenced by the presence or absence of salt (*Figures* 2*a*, 2*b*, 2*c* and 2*d*, respectively). In general, it is considered that these parameters affect directly the overall charge of the molecule in question. For example, at low pH the poly(A) molecule is less charged and its tendency to adsorb hydrophobically to matrices like octyl-Sepharose, and probably phenyl-Sepharose and MN-cellulose, might be expected to increase (*Figures* 2*b* and 2*d*).

When pH is lowered, the repulsion between negatively charged polynucleotides is decreased. Yet no tendency of poly(A) forming hydrogen bonds with its complementary immobilized polynucleotides (oligo-dT-cellulose, poly(U)- and DNA-Sepharose) is observed (*Figure* 2*c*). This might be due to protonation of the adenine amino groups. Only if the pH is above the pK of the basic amino-group (pK = 3.45), and a certain amount of salt is present, is the hydrogen-bonding effect observed (*Figure* 2*a*). As pH, and consequently the overall negative charge of the molecules, increases the repulsion between the similarly charged polymers may account for the decreased capacity of adsorption at higher pH.

Unfortunately, the chromatographic behaviour of poly(A) on the adsorbents under study at pH lower than 5.6 is dubious. From a control experiment it has been found that glass wool possesses a strong adsorption capacity at acidic pH. This effect is particularly pronounced when salt is present (*Figure* 2*a*). The nature of the adsorption of poly(A) to glass wool under such conditions remains unclear.

Temperature effect

The molecular structure of poly(A) is also affected by variations in temperature[17-21]. For example, at neutral pH and moderate salt concentration the poly(A) molecule possesses a single-stranded helical configuration at 0 C[20]. This helical structure disappears in a non-cooperative way as temperature increases. For this reason, one may expect that the interacting abilities of free poly(A) with other compounds are temperature dependent.

A typical temperature effect plot for the association of complementary polynucleotides is shown in *Figure* 3. The observed T_m values under these conditions are in good agreement with those previously reported for the association of this type of polynucleotides[22,39]. The exceptional stability of the poly(A) poly(U)-Sepharose com-

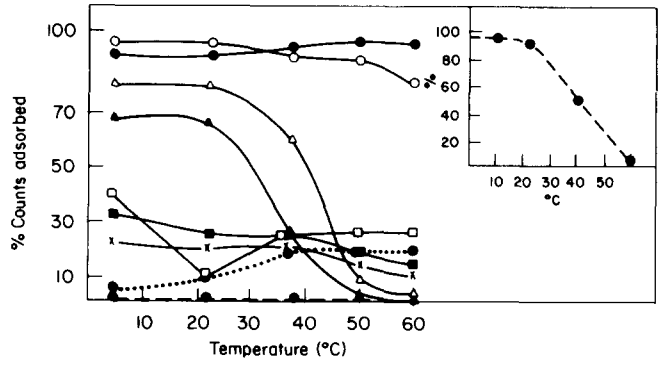

Figure 3. Temperature effect. Adsorption of ^{3}H-poly(A) to different matrices as a function of temperature. The columns were calibrated with 0.4 M NaCl in 10 mM Tris HCl buffer, pH 7.6, in a temperature chamber. The percent radioactivity adsorbed with respect to the total amount of material is plotted. Symbols as in *Figure* 1

Table 1. Effect of buffer composition[a]

Gel	% Radioactivity adsorbed					
	0.4 M KCl	0.4 M NaCl	0.4 M CsCl	0.4 M LiCl	4 mM $MgCl_2$	4 mM $CaCl_2$
Poly(U)-Sepharose 4B	93	98	94	94	88	92
Acriflavin-Sepharose 4B	95	98	95	95	92	96
Oligo-dT-cellulose	90	90	90	86	79	87
Mn-cellulose	50	27	73	52	85	35
DNA-Sepharose 2B	20	77	14	7	84	86
Octyl-Sepharose 4B	39	5	55	54	62	55
Poly(A)-Sepharose 4B	5	25	21	36	67	34
Phenyl-Sepharose 4B	10	10	21	35	30	36
Ethanolamine-Sepharose 2B	0	0	24	38	85	74
Sepharose 4B	0	0	0	12	10	32

[a] The columns were equilibrated at 20 C with the corresponding saline in 10 mM Tris HCl buffer, pH 7.6. Figures show the percent radioactivity adsorbed with respect to the total amount of ^{3}H-poly(A) applied in each case. Further details in Experimental section. The salts are arranged according to their increasing chaotropicity: K > Na > Cs > Li > Mg > Ca (refs 30 and 35)

plex is again evidenced by an almost complete absence of a temperature effect in the range 4 60 C, though it decreases at higher temperatures. In other experiments, however, it has been noticed that free poly(U) when it complexes with poly(A)-Sepharose does show a melting point within this range (results not shown here). This fact suggests that the temperature effect for polynucleotide association is related to the nature of the immobilized ligand. Therefore it is conceivable that some restrictions on the mobility of the ligand may occur when it is coupled to a matrix, thus limiting the conformational changes that would be observed with the ligand in solution[20].

Hydrophobic interactions are favoured by increasing temperature[24]. Therefore, the temperature effect is also good evidence against hydrophobic bonding as the main reason for adsorption of free poly(A) to MN-cellulose and poly(A)-Sepharose (*Figure* 3). In such cases, there is a tendency for adsorption to decrease as a function of temperature (*Figure* 3). In contrast, hydrophobic adsorbents such as octyl-Sepharose become more efficient with increasing temperature (*Figure* 3).

The inset of *Figure* 3 shows the effect of temperature on the adsorption of free poly(A) to ethanolamine-Sepharose in the absence of salt, supporting the previously proposed electrostatic mechanism of adsorption of free poly(A) to this gel.

Although charge-transfer and electrostatic interactions should be reduced by increasing temperature[25], acriflavin-Sepharose is still a strong adsorbent for free poly(A) over the full range of 4 60 C (*Figure* 3). This observation suggests that the increasingly favoured hydrophobic interaction between free poly(A) and acriflavin-Sepharose following a rise in temperature dominates.

Buffer composition effect

A large number of reports have put in evidence the effect of the buffer composition on molecular interactions of nucleic acids[26–30]. The role of certain ions regarded as chaotropes[30], for example, has been related to denaturation, dissociation and extension of these polymers[30]. From *Table* 1 it appears that a generalization about a cationic effect on the chromatographic behaviour of free poly(A) is not possible. While with some adsorbent binding is favoured as chaotropicity increases, with others

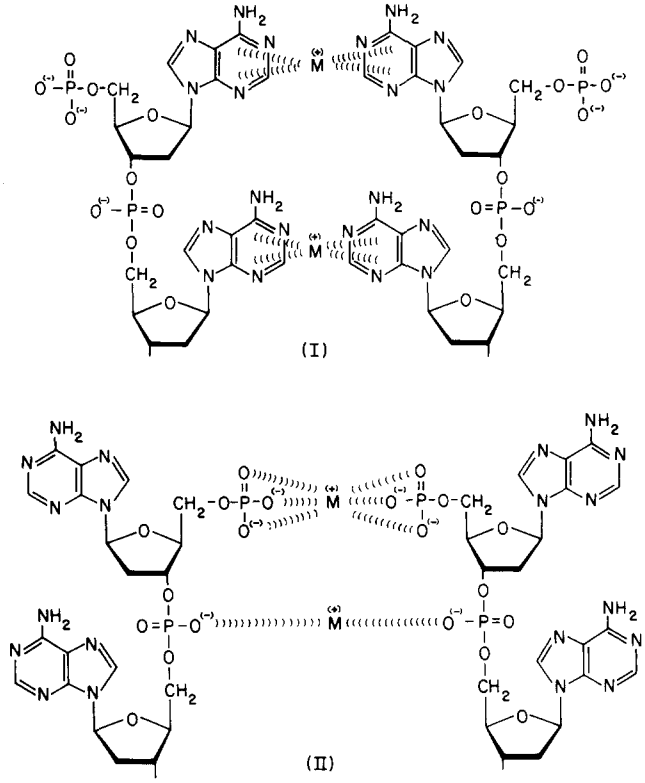

Figure 4. Models for the interaction between polynucleotides at low ionic strength in the presence of Mg^{2+} and Ca^{2+}. (I), 'sandwich-complex'; (II), 'bridge-complex'

the result is the opposite. This might be due to several factors acting on the molecules in question: changes in conformation, the water structure, neutralization of charges, chelate binding and possibly 'sandwich' complexes between aromatic structures and metals (*Figure* 4).

An interesting observation that arises from *Table* 1 concerns the effect of Ca^{2+} and Mg^{2+} on the tendency of poly(A) to bind to those adsorbents. For instance, a hydrogen bond mechanism for the adsorption of poly(A) to oligo-dT-cellulose, and or DNA-Sepharose, appears very unlikely in view of the low ionic strength of the buffer. Neither can the large adsorption capacity of MN-cellulose towards poly(A) in this case be ascribed to

Table 2. Effect of buffer composition[a]

Gel	% Radioactivity adsorbed								
	NaCl concentration (M)			1.0 M NaSCN	6.0 M Urea		90% Formamide		
	0	0.4	1.0		+0 M NaCl	+0.4 M NaCl	+0 M NaCl	+0.4 M Nacl	1% SDS
Poly(U)-Sepharose 4B	96	96	98	96	76	80	90	13	18
Acriflavin-Sepharose 4B	96	96	98	20	90	0	90	32	6
Poly(A)-Sepharose 4B	95	20	20	10	70	0	94	57	7
Oligo-dT-cellulose	0	90	90	85	0	0	0	0	0
Mn-cellulose	0	35	40	15	10	10	10	25	0
DNA-Sepharose 2B	0	70	84	22	0	0	0	15	0
Octyl-Sepharose 4B	20	40	45	20	20	30	5	33	0
Phenyl-Sepharose 4B	0	8	20	10	0	0	0	13	0
Ethanolamine-Sepharose 4B	90	0	0	0	90	0	50	10	0
Sepharose 4B	0	0	0	0	0	0	0	10	0

[a] The columns were equilibrated at 20 C with 10 mM Tris Hcl buffer, pH 7.6 containing the compound or salt as indicated. The percentages of radioactivity adsorbed with respect to the total amount of ^{3}H-poly(A) applied are shown.

salting-out effects. Consequently, the models described in *Figures* 4*a*, 4*b* are proposed. First, in the case of polynucleotide matrices, the negatively charged phosphate groups of both polymers, poly(A) and the ligand, should assume opposed directions in order to minimize electrostatic repulsion. Thereafter, the bases may approach each other closely enough to complex with the metal in a 'sandwich' fashion (*Figure* 4*a*). It is clear that in the case of MN-cellulose, the first step may be omitted. Another alternative would be to consider the metals as 'bridges' between phosphate groups (*Figure* 4*b*). However, this latter model implies that the hydrophobic bases would be exposed to the media, making the possibility less probable.

There are many examples of metal-π-complexes in biological systems[28] and their participation in maintaining biological structures[33]. Moreover, the fact that Mg^{2+} and Ca^{2+} are essential for the complex reactions involving polynucleotides, e.g. protein synthesis, suggests that they are especially important in biomolecular interactions between aromatic structures[33].

Both acceptor capacity[34] ($Mg^{2+} > Li^+ > Ca^{2+} > Na^+ > K^+ > Cs^+$) and chaotropicity[30,35] ($Ca^{2+} > Mg^{2+} > Li^+ > Cs^+ > Na^+ > K^+$) of cations certainly contribute to the effect expected from the ionic strength of their solutions alone. Hence, it is very difficult to draw any conclusion about their action, particularly with molecules (proteins and polynucleotides) whose structural conformations depend on these parameters.

Table 2 shows the action of detergents, chaotropic anions, polarity-reducing agents and hydrogen bond-breaking agents on the adsorption of poly(A) to the matrices under study. In the cases where hydrogen bond formation is the main reason for binding, urea and formamide are very efficient in preventing adsorption of poly(A) (oligo-dT-cellulose and DNA-Sepharose) (*Table* 2). In contrast, the failure of urea to suppress the adsorption of poly(A)- to poly(U)-Sepharose emphasizes again the participation of types of interactions other than hydrogen bonding. Since SDS is an excellent eluant (*Table* 2), the association between poly(A)- and poly(U)-Sepharose is thought to occur with a significant contribution from hydrophobic bonding. This explains the larger efficiency of formamide (90%, v/v) as compared to urea in preventing the formation of a poly(A)–poly(U) complex (*Table* 2). In other words, formamide (90%, v/v) owes its effect both to disruption of hydrogen bonds and to polarity-reducing properties that weaken hydrophobic interaction[38]. As shown in the case of octyl-Sepharose, urea only partially decreases the tendency of poly(A) to adsorb to this matrix hydrophobically (*Table* 2), while formamide (90%, v/v) is again more efficient than urea as a hydrophobicity-suppressing agent in this example. The chaotropic anion SCN^- affects mainly the adsorption of poly(A) to hydrophobic matrices (octyl-Sepharose, phenyl-Sepharose), charge-transfer adsorbents (acriflavin-Sepharose, MN-cellulose) and the complementary DNA-Sepharose, but shows no effect in the cases of poly(U)-Sepharose and oligo-dT-cellulose. This seems to indicate that the hydrogen bonding between these last two matrices and poly(A) is strong enough to resist the action of SCN^-. In the case of DNA-Sepharose, it is probable that the thymidine-rich[5] sequences on DNA cannot always be accessible for complexing through hydrogen bonding with poly(A), and that the binding of poly(A) to DNA-Sepharose is to a large extent non-specific.

The remarkabe ability of urea to prevent the adsorption of poly(A) on acriflavin-Sepharose, indicated the electron donor nature of urea. The action of urea consists of providing the necessary electronic cloud for the electron-deficient acriflavin ring system, thus preventing the adsorption of poly(A) by this mechanism. The presence of salt is only required to eliminate electrostatic effects between poly(A) and acriflavin-Sepharose. On the other hand, the efficiency of SDS in disrupting the acriflavin poly(A) complex could be interpreted as evidence for participation of a hydrophobic bonding mechanism in the adsorption of poly(A) to acriflavin-Sepharose. Even if hydrophobic contributions are not fully eliminated, it appears difficult to ascribe the ability of free poly(U) to displace poly(A) from acriflavin-Sepharose columns to a detergent-like action. The SDS effect might be explained simply in terms of the formation of a micelle structure around the hydrophobic acriflavin molecule causing a strong repulsion towards poly-(A).

Conclusions

The chemical structure of poly(A) enables the molecule to interact with different compounds electrostatically, hy-

drophobically, by hydrogen bonding, and/or charge-transfer mechanisms. Of interest for polynucleotide associations, these possibilities have been estimated with synthetic poly(A) and different adsorbents under various conditions of pH, temperature, salt concentration and buffer composition. For practical purposes, several recommendations are suggested for chromatography of poly(A)-containing nucleic acids on affinity adsorbents containing polynucleotide- or dye-bound matrices: apart from reduction of salt concentration, poly(A)-nucleic acids can be eluted from oligo-dT-cellulose columns by increasing the temperature[41]. Urea, formamide and detergents can be useful when strong binding is present as a result of the participation of other less specific interactions than hydrogen bonding (in cases like poly(U)-Sepharose, acriflavin-Sepharose, DNA-Sepharose, etc.). Chaotropic salts should be avoided, since their use does not always lead to an efficient desorption and there is a large risk of causing irreversible modifications of the structures involved[30,35]. Divalent ions like Ca^{2+} and Mg^{2+} in the buffer may cause non-specific adsorption by enhancing aromatic interactions, therefore their presence should be restricted to a minimum.

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