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COUPLING OF NUCLEIC ACIDS TO AGAROSE: A BIOSPECIFIC SUPPORT FOR THE PURIFICATION AND/OR STUDY OF THE INTERACTIONS OF RELATED COMPOUNDS

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SUMMARY

DNA and RNA bound to an insoluble matrix provide an excellent biospecific support in affinity chromatography which can be applied to many problems in molecular biology such as the purification of specific proteins or nucleic acids and the nature of the interactions between nucleic acids and proteins. A study has been made of the different parameters which affect the binding mechanism between nucleic acids and cyanogen bromide-activated epichlorohydrin-desulphated agarose, a hypothetical binding mechanism is proposed.

INTRODUCTION

Affinity chromatography, which employs ligands fixed to an insoluble matrix, is now extensively used, but there are few examples of the use of insolubilized derivatives of nucleic acids. During the last few years several methods have been introduced for studying the interactions between nucleic acids: Hall and Spiegelman[1] studied interactions between DNA and RNA in solution, and Bautz and Hall[2] studied interactions between phage RNA and phage DNA.

Some of the non-covalent interactions between nucleic acids and proteins are highly specific, *e.g.*, those of repressor–DNA[3], histone–DNA[4], ribosomal protein–rRNA[5], etc. While studying the problems of the specificity of proteins associated with mRNA[6], we looked for methods which would allow us to characterize the parameters of interaction between these two types of macromolecules. The coupling of RNA or DNA to an insoluble matrix of the polysaccharide type provides an excellent tool for studying these interactions according to the principles of affinity chromatography.

Coupling of homooligonucleotides through a phosphate group to hydroxyl groups of cellulose was performed by Gilham[7]. This oligonucleotide–cellulose, for instance oligo-dT-cellulose, is still used to separate poly A-containing nucleic acids

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and also to study the mechanism of action of some enzymes such as the DNA polymerase of Kornberg[8]. Recently, Poonian *et al.*[9] described a technique for the coupling of nucleic acids, using the cyanogen bromide (CNBr) activation of agarose described by Axén *et al.*[10]. They observed that RNA and single chains of DNA were bound to cyanogen bromide (CNBr)-activated agarose at pH 8. This binding can be compared to that between amino groups of proteins and CNBr-activated agarose. Wagner *et al.*[11] showed that, for the synthetic polynucleotides (poly rI), coupling to agarose was possible through the terminal phosphate of the polynucleotide if the pH of the reaction medium was slightly acidic (pH = 6); in this last case, the possibility of binding through an amino group was excluded because inosine has no amino groups.

In this study, we analyze the mode of coupling and the physicochemical properties of ribonucleic acid–epichlorohydrin-desulphated agarose (ECD)-agarose as a biospecific support.

MATERIALS AND METHODS

Reagents

Sepharose 2B and CNBr-activated Sepharose 4B are beaded agarose products of Pharmacia (Uppsala, Sweden). CNBr and formamide were purchased from Merck (Darmstadt, G.F.R.), ^{3}H- or ^{14}C-labelled cyclohexanol, glucose, glucose-6-phosphate, adenosine, AMP, ethanolamine and methylamine were from NEN (Dreieichenhain, G.F.R.) and ^{125}I from DEA (Saclay, France).

Extraction and purification of ribosomal RNA

Plasma cell tumours RPC_5, grown on Balb/c mice, were used. Labelled nucleic acids were obtained by injecting tumour-bearing mice intraperitoneally with 0.2 mCi of [^{3}H]uridine and killing the mice 24 h later. Ribosomal RNA (rRNA) from lyophilized tumours was extracted with phenol at pH 5 in the cold (0–4°). The aqueous phase was deproteinized with chloroform–isoamyl alcohol and the RNA precipitated with ethanol. After dissolution in Tris–HCl buffer (0.1 *M*, pH 7.5), the RNA was treated with pre-incubated pronase (50 μg/ml) for 14 h at 4°. The RNA was precipitated with 1.5 *M* NaCl and the supernatant containing the tRNA was discarded. After dissolution in water and a further precipitation with ethanol, rRNA was finally dissolved in water to give a concentration of 10–20 mg/ml and checked for degradation by polyacrylamide gel electrophoresis[12].

Extraction and purification of DNA

Isolated nuclei from tumours were dissolved in 0.5% sodium dodecyl sulphate (SDS) and 1 *M* sodium perchlorate, deproteinized with phenol–chloroform–isoamyl alcohol, precipitated with ethanol and collected on a glass rod. After dissolution in 0.1 *M* Tris–HCl buffer (pH 7.5), the remaining proteins were digested by pronase for 14 h at 4°. The DNA was again precipitated with ethanol, washed and redissolved in 0.15 *M* sodium chloride–0.015 *M* sodium citrate (SSC)[13].

In vitro labelling of nucleic acids with ^{125}I

RNA and heat-denatured DNA were iodinated according to the procedure of Commerford[14], as modified by Getz *et al.*[15]. Iodinated RNA was filtered through Sephadex G-25 with sterilized water. Iodinated DNA was treated with 0.5 *M* KOH for

1 h at 37°, then filtered through Sephadex G-25 equilibrated with 0.01 *M* KOH. The DNA solution was neutralized and concentrated ten times by addition of one ninth of its volume of formamide and evaporation of the water in a lyophilization apparatus. The DNA was adjusted in the coupling buffer (buffer A: 2-morpholinoethanesulphonic acid (MES) 40 m*M*, adjusted to pH 6.5 with NaOH–80% formamide). Specific activities of $5 \cdot 10^4$–$2 \cdot 10^5$ cpm/μg of nucleic acid were thus obtained and were adequate for our needs.

Preparation and activation of ECD–agarose

The method of Porath *et al.*[16] was used for the preparation of ECD-agarose with Sepharose 2B (Pharmacia). Inactive ECD-agarose was activated by CNBr according to the method of Axén *et al.*[10]. The ECD-agarose gel (10 ml) was suspended in one volume of sterilized water (4°) containing 2 g of CNBr. The pH was immediately adjusted to 11 and maintained at this value by titration with 6 *M* NaOH. The temperature was maintained at 4° throughout the titration. When the reaction was complete, the gel was rapidly washed with 10 volumes of buffer A.

Preparation of immobilized molecules

CNBr-activated ECD-agarose was suspended in one volume of buffer A, containing a known concentration of the nucleic acid, and incubated overnight at 20° in slowly rotating polystyrene test-tubes. The gel was then washed on a ground-glass funnel with the following reagents: 10 vol. of a 1 m*M* phosphate buffer (pH 7) containing 90% formamide, 10 m*M* ethylenediaminetetraacetate (EDTA) and 0.2% N-lauroylsarcosine (NLS); 10 vol. of 10 m*M* Tris–HCl buffer (pH 7), containing 25% formamide, 10 m*M* EDTA and 700 m*M* NaCl; 100 ml of water. It was stored in the reaction buffer A. The free reactive amino group was deactivated in 0.2 *M* glycine–NaOH buffer (pH 8) overnight. A precise volume of the gel was then suspended in 2 ml of distilled water in a Packard vial. In order to determine the concentration of bound nucleic acid of known specific activity, the sample was mixed with 5 ml of Instagel (Packard) with vigorous shaking and the radioactivity was measured by liquid scintillation spectrometry (Intertechnique ABAC/SL 40).

RESULTS AND DISCUSSION

Choice of the matrix and incubation buffer

In order to select the optimum conditions, four different types of matrices were examined for their ability to bind RNA. The results are shown in Table I. The most efficient binding was obtained with CNBr-activated ECD-Sepharose 2B. The advantage of ECD-agarose is its ability to resist high temperatures and high concentrations of solvents and chaotropic ions, to remain active over a wide pH range and to show a high capacity to react with the nucleic acid as ligand. This matrix was then used in all of the experiments. The study was made with RNA or DNA bound to agarose, but for simplicity, we describe only the case of RNA–agarose.

The optimal binding was obtained with a mixture of 40 m*M* MES buffer (pH 6.5) containing 80% formamide (Table II), which could modify the secondary structure of the nucleic acid to give a denatured random coil conformation more favourable for coupling.

TABLE I

ASSAY OF RNA BINDING ON DIFFERENT TYPES OF CNBr-ACTIVATED AND NON-ACTIVATED AGAROSE

0.75 mg of [^{3}H]RNA (85 cpm/μg) or [^{125}I]RNA were incubated with 0.25 ml of the different activated gels, bringing the total volume to 1 ml with buffer A. After 12 h, the gel was washed and its radioactivity was measured as described previously (see Materials and methods).

Matrix	*[^{3}H]RNA*		*[^{125}I]RNA*	
	cpm/ml of gel	*RNA fixed (%)*	*cpm/ml of gel*	*RNA fixed (%)*
1 CNBr-activated Sepharose 4B (Pharmacia)	70,000	28	575,000	30
2 CNBr-activated Sepharose 2B	113,000	45	735,000	49
3 CNBr-activated ECD-Sepharose 2B	163,000	65	1100,000	64
4 Non-activated ECD-Sepharose 2B	3,500	1.5	20,000	1.8

TABLE II

SELECTION OF THE INCUBATION BUFFER

In each case, the activated ECD-agarose (1 ml) was incubated with 250 μg of [^{125}I]RNA.

Buffer	*RNA bound (μg)*
MES (20 m*M*, pH 6.5)	28
MES (40 m*M*, pH 6.5)	30
MES (400 m*M*, pH 6.5)	18
MES (200 m*M*, pH 6.5)	18
MES (40 m*M*, pH 6.5) + 25% formamide	39.5
MES (40 m*M*, pH 6.5) + 50% formamide	94
MES (40 m*M*, pH 6.5) + 80% formamide	112

Dependence on the nucleic acid concentration

The amount of RNA coupled increases with increasing RNA concentration up to a limiting value (Fig. 1A). This may be due to steric hindrance of access to the active sites. Sometimes it was necessary to use nucleic acids labelled with different tracers when higher specific activities[17] were needed. There is no appreciable difference in the coupling of differently labelled RNAs to the support (Fig. 1B). This shows that the iodination did not alter the binding mechanism.

Time dependence

At room temperature, coupling was usually almost complete in 5 h. After a short linear increase, the process of coupling tended towards a limiting value which was reached after nearly 4 h (Fig. 2). All of the binding samples were incubated overnight.

The kinetics of reaction of the RNA molecules probably differ according to the extent of steric hindrance. The portion which reacts in the early stage of the assay might be bound in more "open" regions of the gel and will therefore be more accessible for protein contact and complex formation. A shorter reaction time may thus be preferable even at the expense of a lower yield.

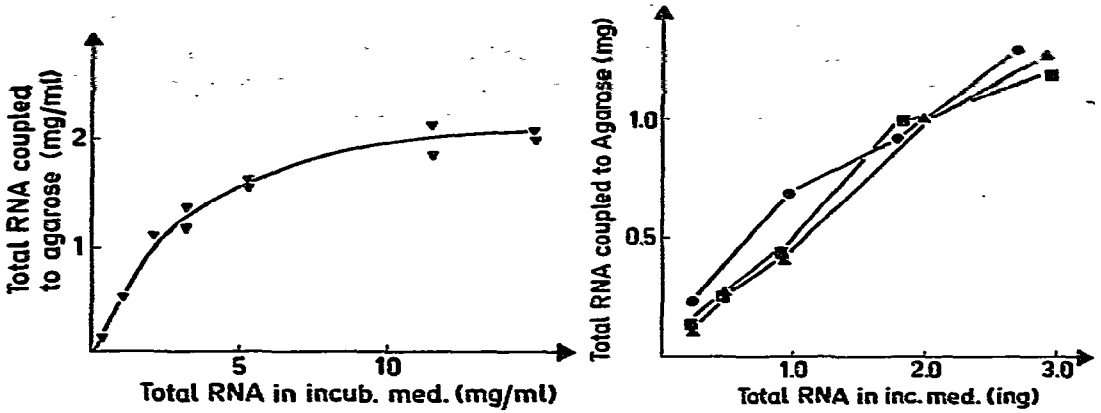


Fig. 1. (A) Effect of RNA concentration on the amount of RNA coupled. RNA was coupled to 1 ml of activated ECD-agarose in buffer A overnight, and the sample was washed and its radioactivity measured (see Materials and methods). (B) Effect of differently labelled RNA on the amount of RNA coupled. (●—●), *in vitro* [^{125}I]RNA; ■—■, *in vivo* [^{3}H]RNA; ▲—▲, *in vivo* [^{3}H]RNA subsequently labelled *in vitro* with (cold) ^{128}I.

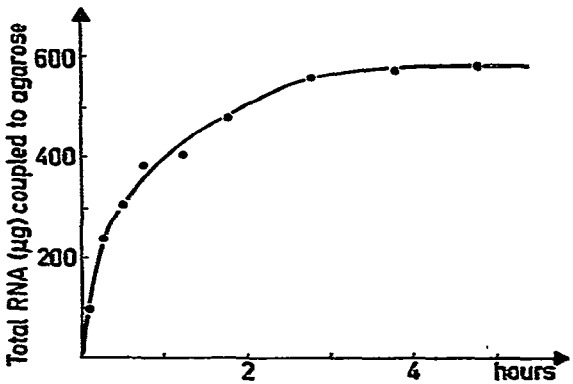


Fig. 2. Time course of the coupling reaction. 1.5 mg of labelled RNA were incubated with 0.15 ml of activated ECD-agarose for different incubation times and then washed and measured (see Materials and methods).

Dependence of binding on ion concentration

The binding of RNA was studied in the presence of several monovalent cations (Li^+, Na^+, K^+, Rb^+ and Cs^+) at various concentrations (Table III). In each case, it was found that the binding was reduced by *ca.* 70–90% as the concentration of the cation increased to 200 m*M*. However, some residual binding remained even when the concentration of the cation was 1 *M* (Fig. 3), and appeared to be insensitive to changes in the ionic environment. This can be explained by either an interaction between cations and binding sites on the agarose, or, more likely, by changes in the secondary structure of RNA brought about by the increased ionic strength. The same inhibition phenomenon is observed in the presence of chaotropic ions such as ClO_4^- or CCl_3COO^-, and can be explained by a competition between these ions and the RNA for the bindingsites on the insoluble matrix, or by a partial precipitation of the RNA.

TABLE III

EFFECT OF DIFFERENT CATIONS ON THE BINDING OF RNA ON AGAROSE

All of the experiments were carried out with 200 m*M* of each ion. 0.25 ml of CNBr-activated ECD-agarose were used, with 1 mg of [^{125}I]RNA, and incubated overnight.

Ion	*Fixed RNA (%)*
Standard	100
Li^+	12
Na^+	19
K^+	17
Rb^+	19
Cs^+	20
ClO_4^-	24
CCl_3COO^-	26

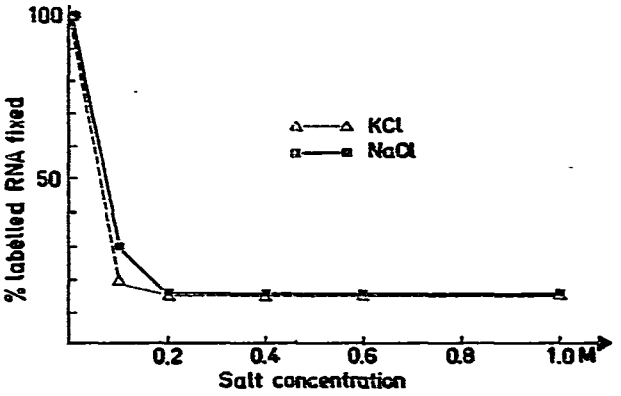


Fig. 3. Dependence of the binding on ion concentration. 1 mg of labelled RNA was incubated overnight with 0.25 ml of activated ECD-agarose bringing the total volume to 1 ml with buffer A containing different salt concentrations and then washed and measured.

Washing of the gel

After the coupling reaction between activated ECD-agarose and nucleic acids, the excess of RNA was washed away with various media (Table IV) in order to remove trace amounts of unbound adsorbed material which might otherwise disturb the subsequent experiments. The most efficient washing was obtained with 90% formamide.

Stability of coupled RNA

In the study of the interaction of nucleic acids with related compounds, the temperature is an important parameter. We analyzed the thermal stability of the RNA–ECD-agarose (Fig. 4). RNA–ECD-agarose, which had been stored for 3 weeks at 4° in 50% formamide, released some RNA fragments when it was heated to $> 70°$. This degradation could be due to thermal hydrolysis of the RNA[18]. In the case of RNA–ECD-agarose in Tris–HCl (10 m*M*, pH 7.6) stored under the same conditions, some RNA fragments began to detach at $> 35°$, possibly owing to

TABLE IV

EFFECT OF DIFFERENT WASHING SOLUTIONS AFTER THE FIXATION OF RNA ON THE ACTIVATED AGAROSE

0.75 mg of [^{125}I]RNA were incubated with 0.25 ml of CNBr-activated ECD-agarose under the experimental conditions in Table I. After incubation overnight, each sample was washed in a funnel with different solutions and its radioactivity was measured. Formamide washing is considered to elute all of the unbound RNA.

Washing solution	*Eluted RNA (%)*
90% Formamide	100
0.04 *M* MES	20–30
0.2 *M* KCl	50–60
3 *M* KCl	70–90
3 *M* Thiocyanate	60–80
6 *M* Urea	60–80

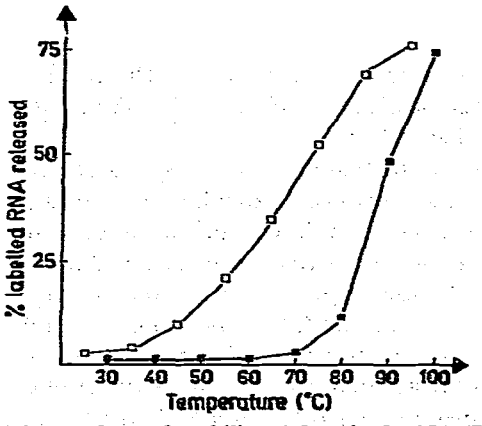


Fig. 4. Thermal stability. 0.25 ml of RNA–ECD-agarose in different buffers are heated at different temperatures for 12 h, washed and the percentage of labelled RNA released was measured. □, RNA–ECD-agarose stored in 10 m*M* Tris–HCl buffer (pH 7.6) for 3 weeks at 4°; ■, RNA–ECD-agarose stored in 50% formamide under sterile conditions.

RNAse degradation. This indicates that formamide provides an efficient preserving system against some contaminating nuclease activities.

Conservation of ligand properties

We tested the behaviour of RNA–agarose as a substrate for hydrolyzing enzymes (Table V). It can be seen that DNA or RNA was not completely hydrolyzed by deoxyribonuclease or ribonuclease, respectively. The incomplete hydrolysis may be explained by steric hindrance between the fragment of RNA bound to the matrix and the enzyme. Potassium hydroxide only removed part of the DNA, perhaps by denaturation of reassociated strands, but KOH is able to hydrolyze a few phosphodiester bonds in DNA and 0.5 *M* KOH may split some bonds between the matrix and ligand.

TABLE V

HYDROLYSIS OF DIFFERENT NUCLEIC ACIDS BOUND TO ECD-AGAROSE

1 ml of DNA–ECD-agarose (80 μg/ml) or RNA–ECD-agarose (50 μg/ml) was incubated at 37° for 30 min in 10 m*M* Tris–HCl buffer (pH 7.6) for RNAse (Worthington, Freehold, N.J., U.S.A.), and in 10 m*M* Tris–HCl buffer containing 3 m*M* magnesium acetate for DNAse (Sigma, St. Louis, Mo., U.S.A.). With 0.5 *M* KOH, the gel was incubated overnight at 37°.

Nucleic acid	*Hydrolysis reagent*	*Nucleic acid remaining (%)*
DNA		100
	DNAse (50 μg)	15
	KOH (0.5 *M*)	58
RNA		100
	DNAse (50 μg)	85
	KOH (0.5 *M*)	0.5
	RNAse (50 μg)	10

The mechanism of fixation

It is of great interest to know how the nucleic acid is bound to the matrix. In order to elucidate this mechanism, we tested the effect of several compounds containing different reactive functional groups (Table VI).

First, adenosine, which does not possess a phosphate group, was bound very efficiently (291 nmoles) whereas 5'-AMP, which has a terminal phosphate group, was bound much less readily (117 nmoles). Under our experimental conditions, adenosine may be bound by its amino group, which becomes more positively charged and hence less reactive in AMP due to the influence of the phosphate group. This interpretation may be supported by the extent of fixation of ethanolamine (289 nmoles) or methylamine (241 nmoles). For coupling of amino groups the optimum pH is greater than 8. If necessary, the pH may be decreased but this will result in a decrease in the amount of the ligand coupled[19]. The coupling of RNA is more extensive at pH 8 than at pH 6 (ref. 17).

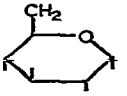
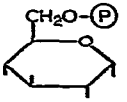
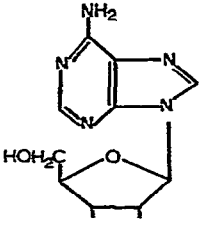
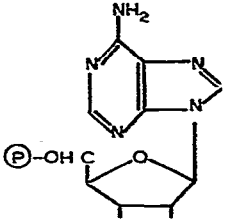
Secondly, the intervention of the phosphate group in the binding mechanism is supported by the finding of a difference in binding between glucose-6-phosphate (46 nmoles) and glucose (4 nmoles) or cyclohexanol (1 nmole): in the last two cases, binding by the hydroxy group seems very unlikely.

Consequently, if imido-carbonate is the reactive group after activation of agarose by CNBr, as was suggested elsewhere[10], it is difficult to understand how it can react with phosphate esters. One explanation may be that an ionic complex is formed between $=C=NH_2^+$ and phosphate and that, in a second step, the imido-carbonate may react with an adjacent hydroxyl group. In other words, phosphate having a p*K* of *ca.* 6 should facilitate the reaction by causing a local increase in substrate concentration. This hypothesis is in agreement with the fact that removal of a terminal phosphate from RNA with alkaline phosphatase results in less efficient binding to activated agarose (15–20%) than untreated RNA (100% fixation, reference point)[17]. Interestingly, some synthetic polynucleotides such as poly U (unpublished results) or poly rI (ref. 11), which do not possess amino groups, were coupled at pH 6. Another fact in favour of the binding via the intervention of the terminal phosphate is the total hydrolysis of RNA by KOH (Table V).

TABLE VI

BINDING OF VARIOUS SUBSTRATES ON ACTIVATED ECD-AGAROSE

0.5 ml of each labelled substrate (20 m*M*) in a buffer A were incubated overnight with 0.5 ml of CNBr-activated agarose. After incubation the sample was washed and its radioactivity measured.

Substrate	*Formula*	*Amount of substrate bound* (nmoles/ml agarose)*
Cyclohexanol		1
Glucose		4
Glucose-6-phosphate		46
Adenosine		291
Adenosine monophosphate (5'-AMP)		117
Ethanolamine	$HOCH_2CH_2NH_2$	289
Methylamine	CH_3NH_2	241

* Average from three experiments.

Approximate yields of the reaction can be calculated. In Table VI, the greatest binding is obtained for ethanolamine (300 nmole/ml); this corresponds to $20 \cdot 300 = 6$ μmole/g (1 g of Sepharose 2B swells to 20 ml) dry gel which is *ca.* 1% of the maximum amount of introduced active groups; usually, substitution of glycine at pH 8 corresponds to 500–600 μmole/g dry gel[19]. The hypothetical structure of agarose after activation with CNBr shows that one imido-carbonate group requires approximately two galactose residues, or 3.3 "mmole" active groups/g (ref. 19): one group per each of $3300/6 = 550$ galactose residues may contain a connecting link to ethanolamine. Consequently, RNA or DNA is probably attached by a single bond or at most by very few bonds.

CONCLUSION

Once fixed to agarose as a biospecific support, the nucleic acid preserves its ability to interact with other substances. The system is suitable for many different purposes, such as the purification, and the study of the interaction, of some proteins having a high affinity for messenger RNA[6], for the preparative isolation and purification of some genes of the genome of eukaryotic cells[20] and for the detection of very low ribonuclease activities[21].

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