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SEPARATION OF SINGLE-STRANDED FROM DOUBLE-STRANDED NUCLEIC ACIDS USING ACRIFLAVIN–AGAROSE CHROMATOGRAPHY

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SUMMARY

A simple and rapid method is described for the separation of single-stranded from double-standed nucleic acids. Samples are applied in a solution containing 0.5 *M* potassium chloride on to an acriflavin–agarose gel (degree of substitution 5 μmole/ml of gel). The double-stranded structures pass through whereas the single-stranded nucleic acids are adsorbed on the column.

INTRODUCTION

The most commonly used method for the separation of single-stranded from double-stranded nucleic acids is chromatography on hydroxyapatite[1,2]. This procedure, based on the interaction between the negative phosphate groups of the nucleic acids and the positive calcium ions on the surface of hydroxyapatite crystals[3], permits the preparation of relatively pure double-stranded nucleic acids which bind more tightly than single-stranded ones[4]. Hydroxyapatite chromatography does not involve direct interaction with the purine and pyrimidine bases of the nucleic acid.

More recently, new chromatographic methods for the separation of nucleic acids and related compounds have been developed[5–7], based on the ability of some dyes to interact with nucleic acids[5–8]. For example, acridines can interact either with DNA through its intercalating properties[9,10] or with RNA through a weaker mechanism[11]; both interactions are essentially based on charge-transfer or π–π interactions[8]. Preliminary results show that these differences in the interacting properties of acriflavin, either with DNA or with RNA, could be exploited for separation purposes[23].

In this paper we propose a new technique for the rapid separation or removal of single-standed from double-stranded nucleic acids.

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EXPERIMENTAL

Synthesis of acriflavin adsorbent

Acriflavin–agarose gels were synthesized as described earlier[7] using 4% agarose (Ultrogel A4) as a support. The agarose gels were activated by epichlorohydrin and then the acriflavin was immobilized at 40°C in ethanol–potassium hydroxide solution (1:1) for several hours. The determination of the degree of substitution of acriflavin ligand was effected by nitrogen microanalysis. Acriflavin–Ultrogel Type II is now commercially available from Reactifs I.B.F. (Villeneuve la Garenne, France).

Preparation of nucleic acid samples

[^{3}H]Thymidine-labelled DNA from mice plasmacytoma cells (pl. DNA) was prepared as described previously[12], solubilized in 10 m*M* Tris–HCl (pH 7.6) at 3 mg/ml, and sonicated for 10 sec at 0°C (MSE ultrasonic power unit, 20 kHz). Under these conditions, the pl. DNA fragment size varies between 300 and 1000 base pairs. pBR322 was prepared by caesium chloride banding and DNA was digested by the restriction endonuclease BamHI [the reaction was performed at 37°C for 2 h in 6 m*M* Tris–HCl (pH 7.9), 6 m*M* magnesium chloride, 450 m*M* sodium chloride and 15 mg/ml of bovin serum albumin]. When stated, DNA was end-labelled at the 5′-terminus in an exchange reaction that contained [γ-^{32}P]ATP and polynucleotide kinase[13]. In some experiments, plasmacytoma DNA was chromatographed on a hydroxyapatite column (volume 1.5 ml, flow-rate 30 ml/h at 20°C) in 30 m*M* Tris–phosphate buffer (pH 7.8) containing 7 m*M* 2-mercaptoethanol and 4 m*M* magnesium chloride[1]. The DNA that was eluted from hydroxyapatite with a phosphate concentration of 500 m*M* was considered to be double stranded and called HAP-DNA. Some plasmacytoma cell DNA was treated with S1 nuclease (S1-DNA) as described below. The oviduct messenger RNA (mRNA) was purified using the lithium chloride–urea method[14]; the size of ovalbumin mRNA is 1870 nucleotides[15]. The synthesis of chicken oviduct complementary DNA (cDNA) was performed using [α-^{32}P]ATP as described earlier[16].

Polyadenylic acid ([2,8-^{3}H]adenylate) was purchased from New England Nuclear (Boston, MA, U.S.A.); poly(U) from Miles Labs. (Slough, Great Britain).

Nuclease S1 treatment

Nuclease S1 specifically splits the phosphodiester bonds of single-stranded nucleic acids[17]. The treatment was effected by mixing 30 μl of 0.1 *M* acetate buffer (pH 4.5) containing 10 m*M* zinc chloride, 0.3 *M* potassium chloride, 30 μl of a buffered solution of nucleic acid sample and 0.5 μg of nuclease S1, and adjusting the volume to 0.1 ml. After incubation for 2 h at 50°C the reaction was stopped by addition of 0.5 ml of 10 m*M* Tris–HCl (pH 7.6) containing 0.5% SDS and precipitated with 2 volumes of ethanol. An aliquot was absorbed in Whatman paper for radioactivity measurement[18].

Chromatography

Pasteur pipettes containing a fibre-glass cap at the bottom were packed with 200–300 μl of acriflavin gel; unless otherwise stated, the degree of substitution of this gel was 5 μmole of ligand per millilitre of gel. The columns run in triplicate were

equilibrated in 10 m*M* Tris–HCl (pH 7.6) containing 0.5 *M* potassium chloride. Samples were applied on to the column in the equilibration buffer (20–50 μl per assay); the column was washed with 2.5 ml of the same buffer, then with 5 ml of 10 m*M* Tris–HCl buffer (pH 7.6) and finally with 5 ml of Tris–HCl buffer (pH 7.6) containing 0.5% of SDS. The radioactivity in each fraction was measured after absorption of an aliquot of each fraction in Whatman paper (1 × 2 cm)[18]. The reported values correspond to the average of at least three experiments with a relative standard deviation of 5%.

The adsorption capacity of acriflavin–agarose is dependent on the nature of the particular nucleic acid. Its adsorption capacity for a synthetic poly(A), molecular weight 100,000, is approximately 1 mg/ml of gel in 10 m*M* Tris–HCl buffer (pH 7.6) containing 0.5 *M* potassium chloride. For ovalbumin mRNA, molecular weight 550,000, its adsorption capacity is *ca.* 0.4 mg/ml of gel. In fact, the adsorption capacity on acriflavin gel depends on the size of the single-stranded nucleic acid; this is explained by a multi-point attachment phenomenon.

RESULTS

Design of the gel

As stated previously[7,9,10], acriflavin can interact with nucleic acid purines or pyrimidines through two binding mechanisms: the primary one occurs by intercalation between DNA base pairs whereas the second involves mainly electrostatic interaction between the positive charge of the dye and the DNA phosphate.

In order to decrease the strength of interaction between acriflavin and nucleic acid bases, we have shown[7] that an increase in ionic strength will prevent the electrostatic effect between the dye and the nucleotide phosphate without affecting the charge-transfer or π–π interactions. On the other hand, the strength of this interaction depends on the number of adsorption centres: either the size of the nucleic acid or the degree of substitution of the dye on the gel. As shown Table I, a high salt concentration does not affect significantly the adsorption of single-stranded nucleic acids on

TABLE I
INFLUENCE OF THE DEGREE OF ACRIFLAVIN SUBSTITUTION OF THE GEL

20 μl of radiolabelled poly(A) (5 μg) or DNA (10 μg) (specific activity 10^4 cpm/μl) were applied on three different substituted gels as described under Experimental.

Degree of substitution (μmole/ml of gel)	*Nucleic acid*	*Adsorption (%) (10 mM Tris–HCl, pH 7.6, + 500 mM KCl)*	*Elution of adsorbed material (%) (10 mM Tris–HCl, pH 7.6, + 0.1% SDS)*
5.02	Poly(A)	80	56
	DNA	51	42
15.50	Poly(A)	88	9
	DNA	92	11
19.70	Poly(A)	90	–
	DNA	94	–

various acriflavin-substituted gels. Such a phenomenon is not observed with DNA molecules. DNA adsorption increases when the degree of substitution of acriflavin on the gel increases. Furthermore, a high ligand density favours multi-point attachment and makes desorption of DNA difficult or impossible, whereas a low ligand density (5 μmole of acriflavin per millilitre of gel) leads to a good recovery of the adsorbed fraction.

Chromatography of various nucleic acids

The above results indicate a difference in adsorption at a low degree of substitution on acriflavin–agarose between DNA and single-stranded homopolynucleotides. This fact can be exploited to separate single-stranded from double-stranded nucleic acids. For this reason we have chromatographed different nucleic acid samples: single-stranded [poly(A); cDNA]; double-stranded [poly(A)–poly(U) hybrid; cDNA–mRNA hybrid; DNA]; nuclease S1-treated hybrid or DNA; DNA obtained from hydroxyapatite chromatography. Fig. 1A shows that single-stranded nucleic acids are strongly adsorbed on immobilized acriflavin. With double-stranded nucleic acid (Fig. 1B), one can see that a larger amount of the material is in the flow-through fraction than in the adsorbed fraction. Nevertheless, some nucleic acids are adsorbed on the gel through their single-stranded structure: DNA may possess a single-stranded tail due to the extraction procedure itself; some poly(A)–poly(U) and cDNA–mRNA hybrids have single-stranded tails: the two partners do not have the same sizes [the sizes of poly(A) and poly(U) are not homogeneous, and ovalbumin mRNA and its cDNA do not have the same number of nucleotides[15]]. Another possibility could be that the strands of the hybrid are not well associated; if the hybridization is performed with an excess of mRNA (10-fold) for 36 h, only 15–18% of the labelled nucleic acids is in the adsorbed fraction. Indeed, we observed (Fig. 1C) that the cDNA–mRNA hybrid or

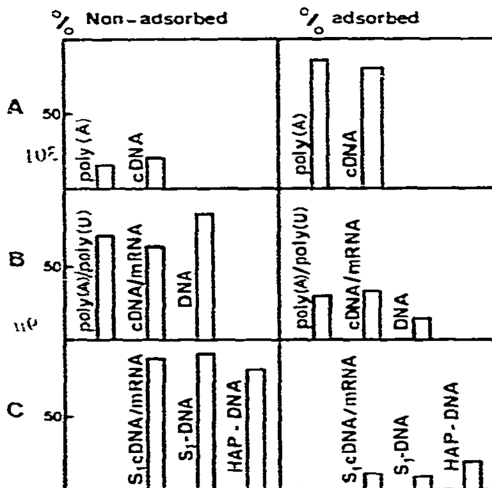

Fig. 1. Nucleic acids distribution. Eight different nucleic acids samples from various origins were applied separately on acriflavin–agarose. The amount of labelled nucleic acids varied between 5 and 10 μg per sample. (A) Single-stranded nucleic acids: polyA and ovalbumin complementary DNA (cDNA). (B) Double stranded structures: poly(A)–poly(U) hybrid; ovalbumin complementary DNA oviduct messenger RNA hybrid (cDNA-mRNA); plasmacytoma DNA. (C) S1-treated cDNA–mRNA; S1-treated plasmacytoma DNA; plasmacytoma DNA purified by hydroxyapatite. The chromatography was performed as described under Experimental. Hybridization was performed by adding a 4-fold excess of poly(U) or oviduct mRNA with [^{3}H]poly(A) or [^{32}P]cDNA, respectively, to 10 mM Tris–HCl buffer containing 0.3 M NaCl and 1 mM EDTA. The reaction mixture was incubated for 3 h at 50°C.

DNA that has been pre-treated with nuclease S1 (which digests the single-stranded moiety) is not adsorbed on acriflavin gel: only small amounts are retained on the column. On the other hand, with HAP-DNA obtained from hydroxyapatite chromatography, a larger amount of DNA is retained on the acriflavin column; this suggests that the HAP-DNA contained some single-stranded structures, which may be discriminated by acriflavin chromatography. As a control we have shown that 30% of the adsorbed HAP-DNA is sensitive to the action of nuclease S1.

In a second set of experiments we chromatographed a mixture of single- and double-stranded nucleic acids which were radiolabelled differently. We first observed (Table II) that up to 86% of the DNA is located in the flow-through fraction whereas most of the single-stranded nucleic acid poly(A) or cDNA is in the adsorbed fractions, as confirmed by their almost complete hydrolysis by the S1 treatment. As shown above, some DNA is adsorbed on the acriflavin, as they possess a single-stranded moiety; if the DNA possess a pure double-stranded structure (pBR322 DNA), its rate of adsorption decreases to 4%. The cDNA–mRNA hybrid which is in the flow-through fraction (Fig. 1) is not hydrolysed by S1 nuclease (6%), whereas the adsorbed fraction is sensitive to S1 (71% of the material is hydrolysed). Moreover, the single-stranded mRNA which is in excess is essentially located in the adsorbed fraction.

DISCUSSION

A rapid and direct method for the separation or removal of single-stranded from double-stranded nucleic acids has been developed. Using low-substituted acriflavin agarose gel, at a high salt concentration (0.5 *M* potassium chloride) the double-stranded nucleic acids pass through the gel whereas the single-stranded nucleic acids are adsorbed; if a double-stranded nucleic acid has a single-stranded region, it will be retained on the acriflavin gel (see Fig. 1 and Table II). This can be applied to the hybrids.

We suggest the following adsorption mechanism. In either instance, a high salt

TABLE II

SEPARATION OF NUCLEIC ACIDS

Four different samples containing 5–10 μg of nucleic acids, (i) plasmacytoma DNA, (ii) ovalbumin cDNA + plasmacytoma DNA, (iii) poly(A) + plasmid pBr322 DNA, (iv) plasmid pBr322 DNA, were applied on to an acriflavin–agarose column (5 μmole/ml of acriflavin). The non-adsorbed and adsorbed fractions were pooled separately, and precipitated with 2.5 ml of absolute ethanol at −70°C for 30 min. Then the pellets were resuspended in buffer and aliquots were tested for radioactivity and nuclease S1 sensitivity. The recovery of the column was up to 98% for double-stranded DNA and up to 80% for single-stranded nucleic acids.

Sample	*Species*	*Non-adsorbed*		*Adsorbed*	
		Radioactivity (%)	*S1 Nuclease sensitivity (%)*	*Radioactivity (%)*	*S1 Nuclease sensitivity (%)*
(i)	[^{3}H]pl. DNA	86	5	14	20
(ii)	[^{32}P]ov cDNA +	6	84	94	97
	[^{3}H]pl. DNA	87	2	13	71
(iii)	[^{3}H]poly(A) +	8	50	92	85
	[^{32}P]pBr322 DNA	96	0	4	1
(iv)	[^{32}P]pBr322 DNA	96	0	4	0

concentration prevents electrostatic interactions between the positive charge of acriflavin and the nucleic acid phosphates. Nevertheless, under such conditions single-stranded nucleic acids are adsorbed on the gel through a π–π or charge-transfer interaction[5,7], which was not much affected by the presence of salt, and secondly through a hydrophobic effect which may even be increased by the salt[19]. Moreover, the adsorption of single-stranded nucleic acids is reinforced by a multi-point attachment on the acriflavin gel[6,20,21]. In contrast, an increase in the salt concentration decreases the affinity constant of the drug for double-stranded nucleic acids, as was observed in free solution with ethidium bromide[22]. This has been explained by a contraction of the double helix which prevents or decreases the inside penetration of the drug. Moreover, the intercalation of the acriflavin drug between double-stranded nucleic acid base pairs may be restricted, as the acriflavin is covalently bound to polysaccharide gel through a three-carbon spacer.

In conclusion, this method for the separation of single-stranded from double-stranded nucleic acids is easy to use, versatile, works at room temperature with good recovery, and permits the purification of large double-stranded fragments. In addition, it may complement hydroxyapatite chromatography, which separates single-stranded nucleic acids not retained on the column from the double-stranded structures[1,2].

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