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INTERACTION OF DNA WITH HYDROXYAPATITE

STUDIES ON THE EFFECT OF THE PHOSPHATE CONCENTRATION OF THE COLUMN EQUILIBRATION AND WASHING BUFFER

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SUMMARY

The ability of hydroxyapatite to bind DNA effectively in phosphate solutions used for column equilibration, sample loading and column washing has been examined. It was demonstrated that substantial amounts of DNA (up to 40%) were eluted in the washing buffer when the phosphate concentration in the lysing solution or urea–phosphate used for column equilibration, sample loading and column washing was 0.24 *M*. A reduction in the phosphate concentration from 0.24 to 0.15 *M* in urea–phosphate solution led to almost 100% binding, whereas a similar reduction in the lysing solution did not. A modified method for loading and eluting DNA from hydroxyapatite columns is presented.

INTRODUCTION

Hydroxyapatite (HaP) is widely used for nucleic acid separation[1–4]. It has been used for separating RNA and DNA after *in vitro*[3] and *in vivo*[5] exposure to chemical carcinogens, in order to analyse each for covalent adducts. However, to avoid ambiguity in this type of analysis two factors require close attention: (1) the maximum useful capacity of HaP for DNA and (2) the critical relationship between the phosphate concentration and nucleic acid[1,6] as well as protein[7,8] adsorption.

Previously published[5,9] HaP chromatographic procedures tend to suggest that samples can be applied as solutions in which the phosphate concentration is as high as 0.24 *M*. Attempts to use these procedures for establishing the maximum useful capacity of hydroxyapatite for DNA revealed that almost 20% of the DNA loaded was not effectively bound by the column. Hence it was considered useful to examine the effect of the phosphate concentration of the column equilibration, loading and washing buffer on the DNA–HaP interaction.

MATERIALS AND METHODS

Calf thymus DNA and bovine serum albumin (BSA), fraction V, were obtained

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from Sigma Chemical Company (London, U.K.), whereas yeast RNA and hydroxyapatite were from BDH (Poole, U.K.).

Loading and elution of sample from HaP column

HaP columns (3.0 cm × 1.0 cm) were first equilibrated with either lysing solution (LS) or urea–phosphate (UP). Aliquots of DNA, RNA or BSA solution (1 mg/ml) in lysing solution were then loaded. The columns were washed as described by Shoyab[5] or Markov and Ivanov[9]. In some experiments the phosphate concentration in the washing buffer solutions was changed, as indicated in Results.

Quantitation of nucleic acids and protein

Fractions of 2 ml were collected and the presence of nucleic acid or protein was monitored spectrophotometrically. The absorbance of nucleic acids was measured at 260/320 nm while that of protein was measured at 280 nm. The amounts of nucleic acids or protein recovered from the column could then be estimated.

RESULTS

When purified calf thymus DNA was loaded and eluted from HaP as described by Shoyab[5], 30–40% was eluted in the washing buffer (data not shown). Consequently the phosphate concentration in the lysing solution was reduced from 0.24 to 0.15 *M*. Though this change did not lead to complete binding, it decreased the proportion of DNA that was prematurely eluted, from 30–40% to 9–19% (Table I,

TABLE I

EFFECT OF REDUCING THE PHOSPHATE CONCENTRATION OF THE LYSING SOLUTION AND UREA–PHOSPHATE ON THE DNA–HaP INTERACTION

Known amounts of DNA in lysing solution were loaded on 3.0 cm × 1.0 cm HaP columns pre-equilibrated with lysing solution containing 0.15 *M* (A) or 0.05 *M* (B) sodium phosphate. The columns were washed sequentially with LS (8 *M* urea, 1.0% sodium dodecyl sulphate, 0.01 *M* EDTA, 0.15 *M* sodium phosphate, pH 6.8), UP (8 *M* Urea, 0.15 *M* sodium phosphate, pH 6.8) and 0.1 *M* sodium phosphate, pH 6.8 to remove any unbound DNA (These three solutions are referred to as "washing buffer"; the concentration of phosphate in LS and UP was reduced to 0.05 *M* in condition B.) The bound DNA was eluted by 0.24 and 0.48 *M* sodium phosphate, pH 6.8 (referred to as eluting buffer). RNA and BSA were separately dissolved in lysing solution, loaded and eluted using condition B.

*Expt.**	*% Distribution of recovered macromolecule*			
	A		*B*	
	Washing buffer	*Eluting buffer*	*Washing buffer*	*Eluting buffer*
1	19.0	81.0	–	–
2	9.0	90.0	–	–
3	–	–	3.0	97.0
4	–	–	0.0	100.0
5	–	–	92.0	8.0
6	–	–	63.0	36.0

* Mean load of DNA in expts. 1–4: 106 μg. Load of RNA in expt. 5: 500 μg; of BSA in expt. 6, 1600 μg.

expts. 1 and 2, column A). However, total binding was subsequently achieved when the phosphate concentration in both the lysing solution and urea–phosphate was reduced to 0.05 *M* (Table I, expts. 3 and 4, column B). Evidently, a considerable reduction in phosphate concentration is required for satisfactory DNA binding when using Shoyab's procedure.

Apart from satisfactory binding, another parameter that deserves attention is the purity of the DNA eluted from HaP columns, especially when it is being isolated from tissue lysate. One of the ways to achieve this is by ensuring that the phosphate concentration of the washing buffer solution is just sufficient to elute RNA and protein without desorbing the DNA. This condition is not satisfied by 0.05 *M* sodium phosphate which enhanced the DNA–HaP interaction but did not effect the complete elution of RNA and protein from the column (Table I, expts. 5 and 6, column B). As a result of this, Shoyab's procedure was abandoned.

Markov and Ivanov[9] used a method slightly different from Shoyab's to load and elute DNA from HaP columns. In their procedure, lysing solution was omitted and the columns were equilibrated with urea–phosphate solution containing 0.2 *M* sodium phosphate. When DNA was loaded and eluted using this procedure, a significant amount was also eluted in the washing buffer, UP (8 *M* urea, 0.24 *M* sodium phosphate, pH 6.8) and 0.15 *M* sodium phosphate, pH 6.8 (data not shown). Hence the concentration of phosphate in UP was reduced to 0.15 *M*. As a result, 100% binding was achieved (Table II, expts. 1 and 2, column A). Under the same conditions, BSA was loaded and completely eluted in the washing buffer (Table II, expts. 3 and 4, column A). However, when RNA was applied about 75% of it was eluted

TABLE II

EFFECT OF REDUCING THE PHOSPHATE CONCENTRATION OF UREA PHOSPHATE SOLUTION ON THE ADSORPTION OF DNA, RNA AND BSA TO HaP

DNA, BSA and RNA were dissolved separately in lysing solution and 0.5 ml of each was loaded on 3.0 cm × 1.0 cm HaP columns pre-equilibrated with urea–phosphate (UP). After loading, the columns were washed with UP (8 *M* urea, 0.15 M sodium phosphate, pH 6.8), 0.15 *M* sodium phosphate (A) and additionally with 0.20 *M* sodium phosphate (B) and then tightly bound DNA, BSA or RNA was eluted with 0.48 *M* sodium phosphate, pH 6.8.

*Expt.**	*% Distribution of recovered macromolecule*						
	A			*B*			
	UP	*0.15 M*	*0.48 M*	*UP*	*0.15 M*	*0.20 M*	*0.48 M*
1	0.0	0.0	100.0	–	–	–	–
2	0.0	0.0	100.0	–	–	–	–
3	2.1	97.9	0.0	–	–	–	–
4	2.5	97.7	0.0	–	–	–	–
5	55.6	19.3	25.6	–	–	–	–
6	57.5	18.3	24.3	–	–	–	–
7	–	–	–	54.6	11.9	33.5	0.0
8	–	–	–	0.0	0.0	50.0	50.0

* Mean loads: 230 μg DNA in expts. 1, 2 and 8; 1640 μg BSA in expts. 3 and 4; 160 μg RNA in expts. 5–7.

in the washing buffer while the remaining 25% was subsequently eluted in 0.48 *M* sodium phosphate, the eluting buffer (Table II, expts. 5 and 6, column A).

Consequently, 0.2 *M* sodium phosphate was introduced as an additional washing solution after 0.15 *M*. This solution caused RNA to be eluted completely in the washing buffer (Table II, expt. 7, column B), but when DNA was chromatographed under identical conditions the 0.20 *M* sodium phosphate desorbed 50% of the DNA (Table II, expt. 8, column B).

The effect of 0.20 *M* sodium phosphate on the adsorption of DNA and RNA to hydroxyapatite, Table II, suggests that the elution of both substances overlaps at this concentration. To resolve this, a gradient elution of DNA and RNA from HaP was performed with the sodium phosphate concentration between 0.15 and 0.20 *M* (see Fig. 1). It was deduced that 0.18 *M* phosphate can desorb RNA completely without any effect on the stability of the DNA–HaP interaction.

The procedure of Markov and Ivanov[9] was, therefore, modified as follows. The column was equilibrated with UP containing 0.15 instead of 0.24 *M* sodium phosphate. After loading, the columns were washed sequentially with UP, 0.15 *M* and additionally with 0.18 *M* sodium phosphate before eluting the DNA with 0.48 *M* sodium phosphate, pH 6.8. This procedure was successfully used to establish the maximum useful capacity of HaP columns and also for separating RNA and DNA in nucleic acid extracts.

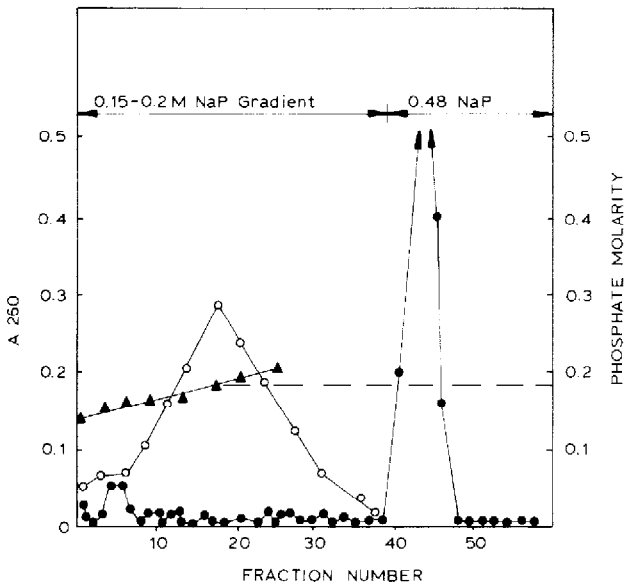

Fig. 1. Continuous gradient elution of 1.0 mg RNA (○—○) and 0.30 mg DNA (●—●) from a 3.0 cm × 1.0 cm HaP column. The column was equilibrated and loaded as described in Table II. After washing with UP, gradient elution between 0.15 *M* (40 ml only) and 0.20 *M* sodium phosphate (40 ml only) was commenced. Two-ml fractions were collected and the absorbance at 260 nm and conductivity of each were measured. Conductivities were converted into phosphate molarities (▲—▲) using an appropriate calibration curve.

DISCUSSION

The chromatographic behaviour of DNA loaded on HaP columns pre-equilibrated and subsequently washed with lysing solution or urea–phosphate containing different amounts of phosphate described in this paper highlights a problem inherent in a number of published procedures for HaP column chromatography. These reports[5,9] suggest that DNA can be loaded on HaP columns pre-equilibrated and later washed with solutions whose phosphate concentration is 0.24 *M*. On the basis of the present observations, this concentration appears to be rather high for these purposes and may be responsible for the premature elution of DNA in the washing buffer, as experienced in this work and by others[10]. This view is confirmed by the observation (see Table II) that DNA can even be eluted by 0.20 *M* phosphate. Furthermore, this single factor, more than any other, explains why all attempts to bind DNA in lysing solution or urea–phosphate containing a higher phosphate concentration (0.24 *M*) were completely unsuccessful. Still, a recent report[11], like the earlier ones[5,9] recommends 0.24 *M* phosphate for column equilibration and DNA loading. In view of the present results, it is probable that some proportion of DNA is eluted prematurely from the column when 0.24 *M* sodium phosphate is used for column equilibration and DNA loading.

Since the concentration of phosphate is a potent factor that determines the strength of nucleic acid–HaP interaction[1,6], the actual concentration of phosphate in the lysing solution used for sample loading, column equilibration and washing should be evaluated and stated. The reason is that (as in this work) when solid urea is dissolved in a solution of 0.24 *M* sodium phosphate, the volume changes lead to a new phosphate concentration of *ca.* 0.15 *M*, that shown to allow complete DNA binding. Indeed, this is the way Johnson and Illan[11] prepared their 8 *M*/0.24 *M* urea–phosphate solution. The concentration (0.24 *M*) stated does not reflect the actual concentration of phosphate in this solution, and may therefore be misleading.

HaP has been successfully used for different nucleic acid separation problems. One of these is the separation of DNA and RNA present in cellular or nuclear lysates after *in vivo*[5] and *in vitro*[3] modification by radiolabelled chemical carcinogen. This enables investigators to establish which of the two is more susceptible to chemical modification by the carcinogen. The use of HaP in this kind of investigation and the fact that DNA can be desorbed from HaP columns prematurely under certain conditions as noted in this paper indicate the importance of accurate monitoring of the purity of RNA or DNA eluted from HaP columns, especially when the sample chromatographed contains both substances and HaP is the sole separation and purification technique used. In such situations, the problem of cross-contamination can be minimized if the appropriate concentration of phosphate for column equilibration, sample loading and column washing is not exceeded.

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