PHENYL BORONATE COLUMN FRACTIONATION OF HIGH MOLECULAR WEIGHT RNA

by

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ABSTRACT

The interactions of borate ion with 1,2-cis-diol groups are useful in fractionating mixtures of biological molecules. Separation of RNAs, up to the size of tRNAs, from other biomolecules using phenyl boronate agarose (PBA) column has been shown. In this work, we used phenyl-boronate agarose column to separate normal E. coli 16S ribosomal RNA from 16S ribosomal RNA with a blocked 3'-terminus. The blocking group was a 3',5'-cytidine (5'-32p)-bisphosphate. Using a 30 um boronate/ml of agarose (PBA-30) column we obtained reproducible separations at 22°C. The results show that this method is useful in preparing high molecular weight RNA of high specific activity for sequence analysis. It is also effective for separation of nuclease digestion products.

INTRODUCTION

The specific interactions of borate with cis-diols can be exploited for use in separating oligo and polyribonucleotides. Borate binds to a wide variety of ribonucleosides, ribonucleotides and ribonucleic acids because of the 1,2-cis-diol group at the 2' and 3' positions of the ribose (1). Borate gels are also used to separate RNA from DNA (2,3). Polynucleotides or nucleotides which are either 2' or 3' phosphorylated do not interact with boronate. This is attributable to blockage of one the hydroxyl groups. The interactions of boronate with the cis-diol group that form a tetrahedral complex can be disrupted by a competing cis-diol such as sorbitol. A shift in pH or monovalent ion concentration will also alter the stability of complex formation (4,5). Separation of RNAs of various sizes on phenyl boronate agarose columns depends upon the amount of the boron attached to the agarose. Sample concentration and ionic strength of buffers also affect the fractionation (1,4). Hydrophobic and hydrogen bonding are important because they also modulate the strength of bonding.

Several different boronate containing supports are commonly used to separate RNAs (4,6,7,8). Separation of RNAs with these supports is ascribed to the presence of free cis-diol groups. Using boronate columns we wanted to enrich 16S rRNA that had been 3' end blocked and labeled using T4 RNA ligase in the presence of 3',5'-cytidine(5'-32p)-bisphosphate (pCp) (9). Boronate columns should not bind labeled RNA because of the presence of a phosphate on one of the 3' end cis-diols. For sequecing large RNA molecules, such as 16S rRNA, enrichment of the labeled material is particulary useful because big RNAs often label poorly with T4 RNA ligase (9,10,11). To achieve this enrichment we initially attempted to employ the only published boronate based procedure for separation and nearly quantitative recovery of large rRNAs (4). We unexpectedly found, using the method described, that both blocked and nonblocked large rRNAs were retained by boronate agarose columns. This communication did not describe a control using blocked rRNA to confirm separation dependent upon the presence of cis-diols (4).

Using phenyl boronate agarose column, we have established conditions which permit separation of large rRNAs bases on the existence or lack of free 2',3'-cis-diols. At $22^{0}C$, a 30 um boronate/ml of agarose column renders the most effective partitioning between 3' end blocked and nonblocked rRNA. The column can also separate large RNase H digestion products. In sequence analysis RNase H is useful for generating specific restriction like fragments of RNA (12).

MATERIALS AND METHODS

Boronate Columns

Boronate derivatized agarose packed in columns was purchased from Amicon Corporation. Gel matrices with 10, 30, and 60 um of boron/ml of agarose respectively were packed into 2 ml bed volume (0.9 x 3.0). These are designated as PBA-10, PBA-30 and PBA-60 columns. The columns were used at room temperature but stored at $4^{o}C$ when not in use. Before use, the columns were allowed to equilibrate at room temperature for at least 1 hour. The columns were washed with 14 ml of application buffer prior to sample application: 50 ml Hepes, 10 mM $MgCl_2$, 0.2 M NaCl pH 8.5. Samples were applied in 0.5 ml (1 A_{260} RNA) to 1 ml (2 A_{260} RNA) of application buffer and allowed to flow into the column matrix. Then 0.1 ml of application buffer was allowed to flow into the column. At this point the flow was stopped and the sample was allowed to equilibrate in the column for at least 30 minutes. The columns were then washed with 14 ml of application buffer and fractions were collected at a flow rate of 1.5, ml/min (PBA-10), 0.5 ml/min (PBA-30) and 0.3 ml/min (PBA-60). Bound RNA was eluted by washing the column with the following buffer: 50 mM Hepes, 10 mM $MgCl_2$, 0.2 M NaCl, 0.1 M sorbitol pH 8.5. The columns can be reused up to 10 times. The columns were regenerated with 12 ml of 50 mM Acetate pH 5.0.

Sample Preparation

Mid-log phase E. coli D-10 cells were the source of 16S rRNA and 23S rRNA. Ribosomal RNAs were prepared from phenol extracts of cell lysates. Ribosomal 16S and 23S RNA was isolated by zone ultracentrifugation (13). The 3'-termini of 16S rRNA were radio labeled with (5'-32p)pCp using T4 RNA Ligase (14). Uniformly labeled 23S rRNA was prepared from cells grown in the presence of (32p)-orthophosphate.

RESULT AND DISCUSSION

Table 1 list the buffers used in this work. PBA-60, PBA-30, and PBA-10 columns were tested. We have found that matrix gel PBA-30 columns are best for separating large RNAs like 16S ribosomal RNA (1542 nucleotides) based on the presence of 3'-terminus cis-diols. To demonstrate this separation a mixture of normal and 32p-3' end labeled 16S rRNA was applied in the various sample application buffers (A,B,C,D) shown in Table 1. The 3'-phosphorylation on the labeled RNA should interfere with binding to the columns.

Buffers A and C have been reported to be good application buffers when RNA separations are required (1,4). However,we have found that only buffer D is suitable as an application buffer when large RNAs are applied to the columns. In contrast with previous investigators (3,4), we found that buffers A and B were not able to remove 16S RNA from the column, whether or not the 3'-end was blocked. Binding to the column occurred at either $4^{o}C$ or $22^{o}C$. These application buffers did work as previosly reported when low molecular weight RNAs, such as tRNA, were passed through the columns. Buffer D with 0.2 M NaCl reduced nonspecific binding of 3' end blocked 16S rRNA which occurred when buffer B was used. Buffer E and F are elution buffers, but optimal recovery of cis-diol containing 16S rRNA with buffer E requires sorbitol. The sorbitol provides competing cis-diols. Low pH alone was sufficient to elute small RNAs.

Figure 1 shows a typical elution profile PBA-30, where 70% of the normal 16S rRNA which has free 3' cis-diols is retained and approximately 80% of the 3' end blocked RNA passes through the column. The material which was not retained on the column was reapplied to the column to determine if the sample would distribute again in the same manner. Again, approximately 20% of the blocked material was bound to the column suggesting that the retention observed in the first passage through the column was most likely because of nonspecific interactions. Furthermore, when the retained material was eluted, dialyzer into application buffer and reapplied approximately 80% of the previously retained blocked RNA eluted. We found 23S rRNA to have comparable elution and retention characteristics as 16S rRNA. PBA-10 columns (Figure 2) do not retain either free 3' cis-diol 16S rRNA or blocked 16S rRNA, while PBA-60 (Figure 3) retains both type of 16S rRNA independent of the application buffer used.

To show the utility of the columns in separating RNase H digestion products we prepared a synthetic DNA which was complementary to the 3'-end of 16S rRNA. The DNA (pTAAGGAGGTG) was hybridized with (5'-32p)pCp 3'-end blocked 16S rRNA and then incubated with RNase H. The RNase H will only attack the DNA/RNA hybrid. The digestion product for the large rRNA fragment has a free 3'-end and was bound to the column. This RNA was eluted, relabeled with (5'-32p)pCp and it migrates on polyacrylamide gels in a zone expected for a "16S"

like rRNA molecule (Figure 4). Recoveries for this rRNA fragment and one that we have generated that is 1400 nucleotides in length are typically in the 70% to 80% range. We have also found that the columns can only be used four times before reproducability is lost.

Table 1

Composition of Application and Elution Buffers

Buffers	Composition
A	50 mM Hepes, 10mM $MgCl_2$, 20% v/v 95% EtoH, pH 8.5 with NaoH
B	50 mM Hepes, 10 mM $MgCl_2$, pH 8.5 with NaoH
C	50 mM Hepes, 10 mM $MgCl_2$, 20% v/v 95% EtoH, 0.2 M NaCl, pH 8.5 with NaoH
D	50 mM Hepes, 10 mM $MgCl_2$, 0.2 NaCl pH 8.5 with NaoH
E	100 mM Tris pH 8.5 with HCL
F	50 mM Hepes, 10 mM $MgCl_2$, 0.2 M NaCl, 0.1 M Sorbitol pH 8.5 with NaoH
G	50 mM Acetate pH 5.0

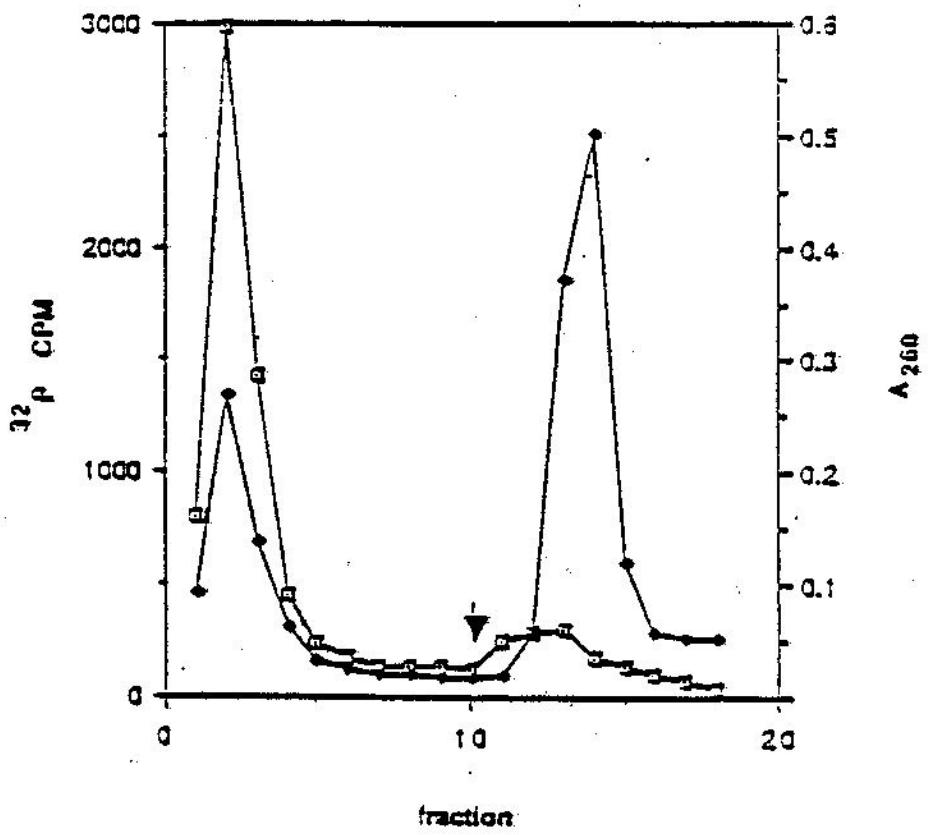

Figure 1: Column chromatographic separation of 16S rRNA on PBA-30. Two-milliliter bed volume PBA-30 was loaded with 2 A_{260} unlabel 16S rRNA (●——●) and 8 x 10^3 cpm (5'-32p)pCp 3'-end blocked 16S rRNA (■——■). The samples were applied in buffer D and eluted with buffer F. The change to elution buffer is shown by the arrow. Fraction volumes are 1.5 ml and flow rate was 0.5 ml/min.

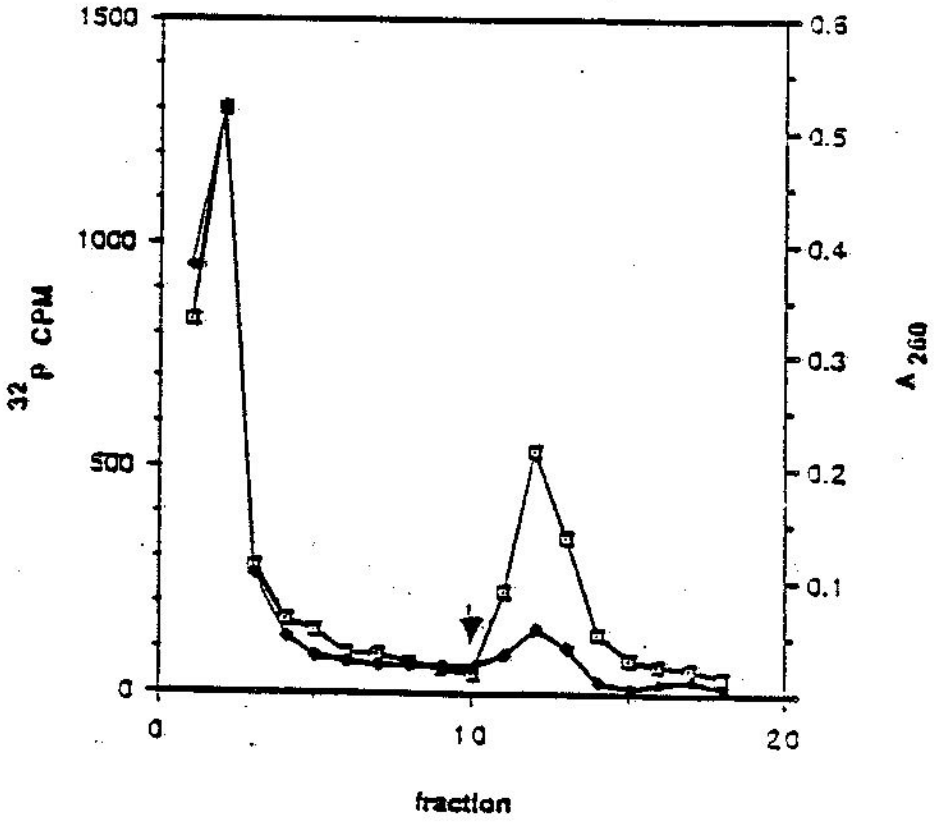

Figure 2: Column chromatographic separation of 16S rRNA on PBA-10. Column was loaded with 1.5 A_{260} unlabeled 16S rRNA (⟶) and 5 x 10^3 cpm labeled 16S rRNA (⟶). The column conditions and fraction size were the same as Figure 1. The flow rate was 1.5 ml/min.

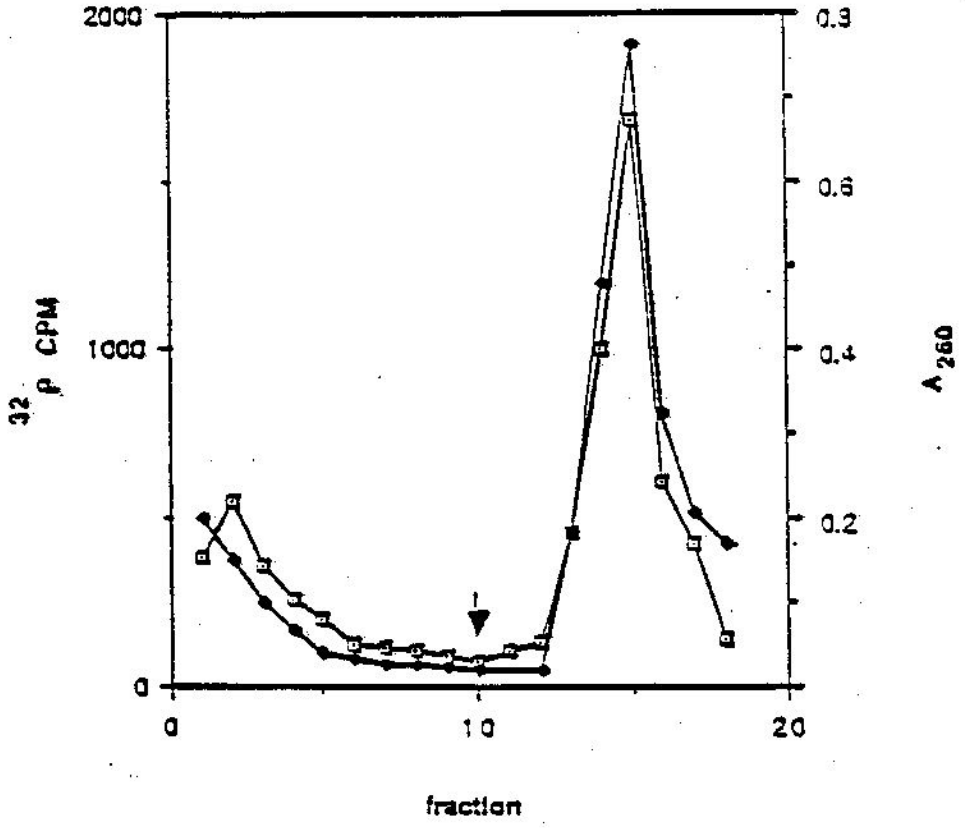

Figure 3: Column chromatographic separation of 16S rRNA on PBA-60. The column was loaded with 2.5 A_{260} unlabel 16S rRNA (⟵⟶). The apparently conditions were the same as in figure 1. The flow rate was 0.3 ml/min.

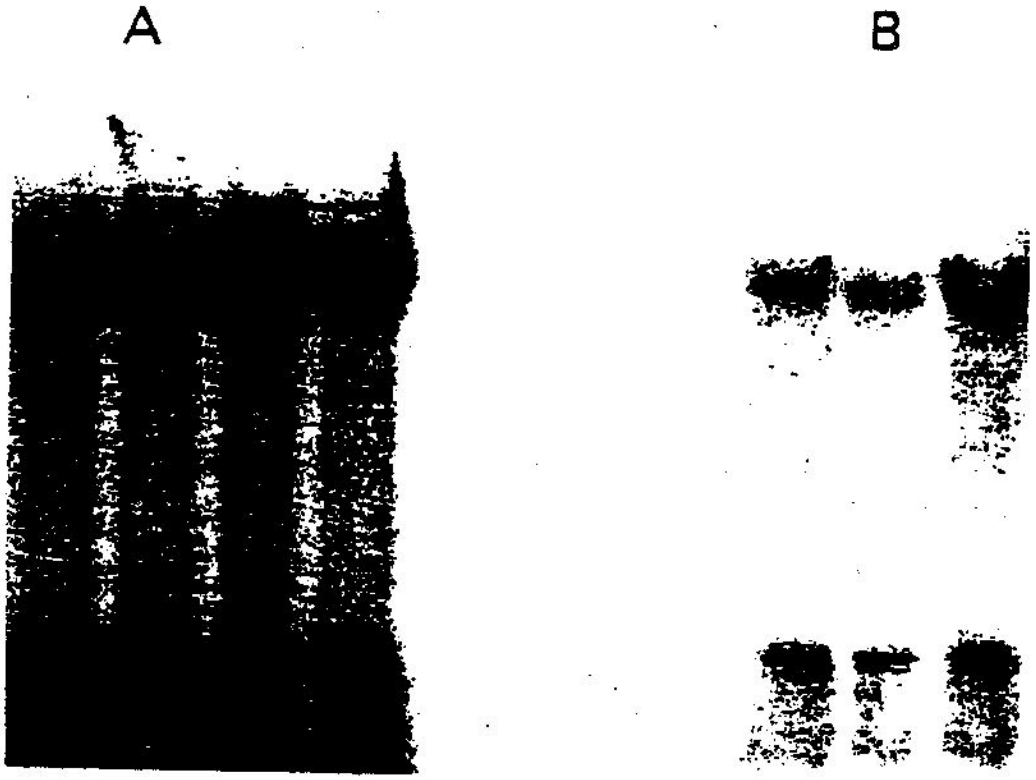

Figure 4: Autoradiograph of a polyacrylamide showing 3'-end labeled 16S rRNA. A. 3'-end [32p]pCp labeled normal 16S rRNA purified by zone ultracentrifugation. B. 3'-end [32p]pCp labeled RNase H digested 16S rRNA purified on a PBA-30 column. The polyacylamide gel was a split 2.8% - 10% denaturing gel (15x10 cm, 0.75 mm thick) and the running buffer was 50 mM Tris-base, 1 mM EDTA pH 8.3. Running time was 2 hours at 5 mA.

REFERENCES

1. In "Boronate Ligands in Biochemical Separations". Amicon Corporation (1981).

2. Ackerman, S., Cool, B., and Furt, J.J. (1979) Anal. Biochem. 100, 174-178.

3. Scott, H., Rudloff, E., and Schmidt, P. (1973) Biochem. 12, 932-938.

4. Pace, B., and Pace, R. (1980) Anal. Biochem. 107, 128-135.

5. Rosenberg, M. (1974) Nucleic Acids Res. 1, 653-671.

6. Igloi, G.L., and Kossel, H. (1987) in Methods in Enzymology (Ray Wu ed), Vol. 155, pp. 433-448, Academic Press Inc.

7. McCutchan, T.F., and Soll, D. (1975) Nucleic Acids Res. 2, 853-864.

8. Duncan, R.E., and Gilham, P.T. (1975) Anal. Biochem. 66, 532-539.

9. Bruce, A.G., and Uhlenbeck, O.C. (1978) Nucleic Acids Res. 5, 3665-3677.

10. Uhlenbeck, O.C., and Gumport, R.I. (1982) in the Enzyems (P.D. Boyer ed.) New York: Academic Press, pp.31-58.

11. Uhlenbeck, O.C. (1983) TIBS, March 94-96.

12. Donis-Keller, H. (1979) Nucleic Acids Res. 7, 179-192.

13. Schaup, H.W., Green, M.,and Kurland, C.G. (1971) Molec. Gen. Genetics 112, 1-8.

14. Meyhack, B., Pace, B., Uhlenbeck, O.C., and Pace, N.R. (1978) Proc. Natl. Acad. Sci. USA 75, 3045-3049.