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Esterification of Terminal Phosphate Groups in Nucleic Acids with Sorbitol and Its Application to the Isolation of Terminal Polynucleotide Fragments†

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ABSTRACT: The exposure of mono- and polynucleotides to 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and high concentrations of sorbitol results in the esterification of their monosubstituted phosphate groups. The presence of the sorbitol moiety permits these derivatives to bind strongly at pH 8.7 to columns of chromatographic supports containing the dihydroxyboryl group and to be subsequently released by elution with buffers at pH 5.5. The procedure constitutes a method for the isolation of polynucleotide fragments arising from the terminals of nucleic acids. A new method for the preparation of the chromatographic supports involves the synthesis of the 1,3-propanediol cyclic ester of *m*-[[3-(*N*-succinimidoxycarbonyl)propanoyl]amino]benzeneboronic acid and its condensation with aminoethylcellulose or aminoethylpolyacrylamide. The reagent is readily prepared by reaction of *N*-[*m*-(dihydroxyboryl)phenyl)]succinamic acid with 1,3-propanediol to protect the boronate moiety followed by esterification with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide.

In earlier reports from this laboratory we described the preparation of cellulose derivatives containing the dihydroxyboryl group and the use of these supports as novel chromatographic materials for the fractionation of sugars, nucleotides, polynucleotides, and nucleic acids (Weith et al., 1970; Rosenberg et al., 1972). Separations of molecules belonging to these classes arise from differential stabilities of the complexes that they form with the immobilized dihydroxyboryl groups; the complexes are presumed to consist of cyclic boronate structures formed between such groups and pairs of contiguous hydroxyl groups that possess the appropriate conformation. The chromatographic supports were constructed by condensing *N*-[*m*-(dihydroxyboryl)phenyl]succinamic acid with an aqueous suspension of aminoethylcellulose, aminoethylpolyacrylamide, or amino-substituted glass in the presence of a water-soluble carbodiimide (Weith et al., 1970; Duncan and Gilham, 1975). The cellulose derivative, DBAE-cellulose,[1] has also been exploited in procedures for the isolation of 3′-terminal fragments from RNA molecules and for the purification of tRNA isoacceptors (Rosenberg and Gilham, 1971; Duncan and Gilham, 1975; McCutchan et al., 1975). The material is now commercially available and is in widespread use for these as well as other special separation problems such as the isolation of 5′-terminal fragments from eukaryotic messenger and viral ribonucleic acids that contain terminal 5′-linked nucleoside polyphosphate groups. One difficulty with the use of DBAE-cellulose prepared by the above method arises out of the apparently incomplete substitution of the amino groups on the cellulose. This necessitates the use of chromatographic solvents containing relatively high salt concentrations in order to minimize the binding of polynucleotide fragments through the residual anion-exchange capacity of the aminoethylcellulose (Rosenberg et al., 1972). The present work describes the development of two new experimental advances in this separation technique: a new method for the efficient preparation of DBAE-cellulose and DBAE-polyacrylamide that minimizes the ion-exchange problem, and a method for the introduction of "handles" into nucleic acids that permits the isolation of terminal polynucleotide fragments from DNA and RNA on DBAE supports.

Synthesis of DBAE-cellulose and DBAE-polyacrylamide. The compound chosen as a reagent for the preparation of the chromatographic supports has the structure *m*-[[3-(*N*-succinimidoxycarbonyl)propanoyl]amino]benzeneboronic acid, and the method used for the synthesis of its 1,3 propanediol cyclic ester (IV) is shown in Scheme I. *N*-[*m*-(Dihydroxyboryl)phenyl]succinamic acid (II) prepared from succinic anhydride and *m*-aminobenzeneboronic acid (I) is converted with 1,3-propanediol to the cyclic ester III to protect the dihydroxyboryl group during the subsequent activation step. The ester, upon condensation with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide, gives IV in 78% yield. In aqueous solution at pH values above 8 this activated carboxylic acid reacts rapidly with suspensions of chromatographic supports containing primary amino groups. The extent of the reaction, in each case, can be estimated by observing the absorbance changes in the reaction solution that are appropriate for the loss of the reagent's chromophore and for the appearance of the chromophore corresponding to the released *N*-hydroxysuccinimide. Thus, aminoethylcellulose and aminoethylpolyacrylamide can be substituted with di-

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[1] Abbreviations used: DBAE, *N*-[*N*′-[*m*-(dihydroxyboryl)phenyl]-succinamyl]aminoethyl; EPC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride; DEAE, diethylaminoethyl.

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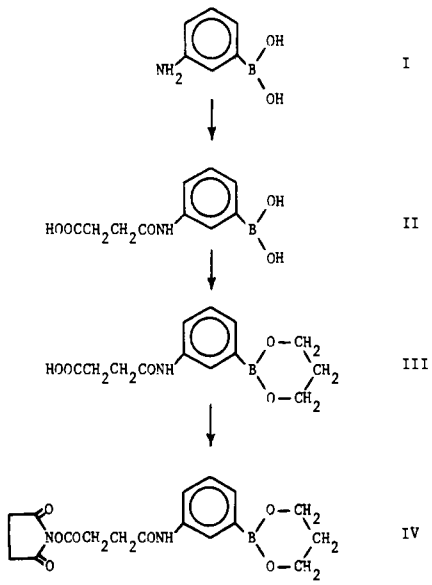
Scheme I



hydroxyboryl groups with yields of 67% and 91%, respectively (based on the amino group content of the polymers as specified by the manufacturers). The derivatized supports are subsequently treated with *N*-succinimidyl acetate in order to eliminate any residual primary amino groups.

The two substituted polymers were tested for their ability to bind ribonucleosides and ribonucleotides and were found to exhibit the same behavior as that observed for similar supports prepared by the water-soluble carbodiimide method. In addition, the materials were tested for the presence of residual ion-exchange groups that would manifest themselves in the ionic binding of tRNA under conditions where binding through complex formation with the dihydroxyboryl groups would be minimized. In the presence of 0.05 M sodium acetate buffer at pH 4.5 a column of DBAE-cellulose bound tRNA to the extent of 98%. The bound RNA could be released, however, by incorporation of 1 M sodium chloride into the elution buffer. In contrast to this behavior, DBAE-polyacrylamide bound less than 10% of the tRNA under the low salt conditions. In the presence of solutions of higher pH, both supports act as complexing agents for the terminal *cis*-diol groups of the tRNA molecules. Thus, with solutions consisting of 1 M sodium chloride—0.1 M $MgCl_2$, buffered at pH 8.7, only 2% and 4% of the tRNA sample were eluted from columns of the cellulose and polyacrylamide supports, respectively.

With the results of these experiments, it is apparent that the new method of introducing the dihydroxyboryl group into solid supports is a more efficient and economical procedure and that it yields products that are somewhat superior to those prepared by the carbodiimide-mediated condensation. DBAE-polyacrylamide, synthesized by the new approach, appears to be essentially free of unsubstituted amino groups, although it is clear that the corresponding cellulose derivative still retains some ion-exchange capacity. The difference in behavior of these products may be simply a result of the different methods used in the manufacture of the ion exchangers. Aminoethylcellulose is prepared by aminoalkylation, and it is likely that it contains some tertiary amino groups which would not be subject to acylation by the reagent IV.

Esterification of Nucleotides and Polynucleotides with Sorbitol. Separations involving DBAE-substituted supports may also be applied to polynucleotides and nucleic acids that do not possess terminal 2′,3′-diol groups. The procedure involves the incorporation of an appropriate polyhydroxyl "handle" into one or the other of the terminal positions of the polynucleotide. For this work, the sugar alcohol sorbitol was chosen because it exhibits a strong binding capacity with dihydroxyboryl-substituted cellulose (Weith et al., 1970). In preliminary experiments (Rosenberg *et al.*, 1972), it was shown that the sorbitol moiety could be readily attached to the phosphate group of a nucleotide by using a method developed earlier for the conversion, in aqueous solution, of phosphomonoesters to phosphodiesters (Naylor & Gilham, 1966).

Each of the four common deoxyribonucleoside 5′-phosphates can be quantitatively converted into the corresponding sorbitol derivative by treating the nucleotide at pH 5.5 for 2 h at room temperature with 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide in the presence of a large excess of the sugar alcohol. This is clearly demonstrated by the elution patterns obtained from the chromatographic analysis of the condensation reactions on ion-exchange resins. The four sorbitol nucleotides were readily isolated by adsorption to DEAE-cellulose. The elution patterns obtained from the chromatography of the products on a column of DBAE-cellulose show that, in contrast to the parent nucleotides, each sorbitol derivative binds strongly at pH 8.7 and is quantitatively released by subsequent elution at pH 5.5. The assignment of the phosphodiester structure to the derivative rests on the observation that they are resistant to the action of alkaline phosphatase and on the fact that they can be converted back to the parent nucleotides by exposure to snake venom phosphodiesterase. In addition, it was shown that the sorbitol derivatives are resistant to the action of sodium hydroxide (1 M NaOH, 2 h at 37 °C).

Under the conditions used for the preparation of sorbitol nucleotides, a mixture of oligonucleotides (chain lengths 2–25; obtained from deoxyribonuclease I digestion of calf thymus DNA) can be derivatized to the extent of about 70% as judged by the proportion of the product that can be bound to DBAE-cellulose. However, the yields of incorporation of sorbitol tend to decrease with increasing chain length of polynucleotide. For example, two samples of polydeoxyribonucleotides with average chain lengths of about 10 and 100 gave derivatization yields of 85% and 60%, respectively. This decrease in yield may be simply a response to steric effects arising from polynucleotide secondary structure, an idea that is supported by experiments on the derivatization of unfractionated tRNA. The 5′-terminal phosphate groups of these molecules are converted to their sorbitol diesters in less than 25% yield under the above conditions, and even at 60 °C, the incorporation yield does not exceed 30%. However, in the presence of denaturing conditions (7 M urea), the yields of derivatization of tRNA and long polynucleotides at room temperature are increased to 75%. The derivatized tRNA binds strongly to DBAE-cellulose, an effect that appears to be a response to cyclic boronate structures formed with the 5′-sorbitol moiety rather than with the 2′,3′-diol group at the 3′ terminus. Unmodified tRNA has been shown to bind poorly to the cellulose in the absence of magnesium ions (Rosenberg et al., 1972), whereas sorbitol-tRNA exhibits strong binding under these conditions.

Experiments on the specificity of the reaction indicate that the incorporation of sorbitol is confined to the terminal phosphate groups of polynucleotides. Derivatization of 5′-^{32}P-labeled calf thymus DNA followed by deoxyribonuclease I digestion yields ^{32}P-labeled fragments, 75% of which can be retained by DBAE-cellulose chromatography at pH 8.7. Subsequent elution with the low pH buffer yields the bulk of

the radioactivity, unaccompanied by any detectable ultraviolet-absorbing material. This results is consistent with the expectation that sorbitol incorporation into internal regions of the polynucleotide chain occurs at very low level. This view is supported also by the observation that polynucleotides whose terminal phosphate groups have been removed by treatment with alkaline phosphatase before exposure to the sorbitol reaction conditions do not bind to DBAE-cellulose. The results of this experiment also indicates that phosphodiester cleavage is not a significant side reaction under the sorbitol derivatization conditions.

The results of these experiments indicate that the method should be useful in the isolation of polynucleotide fragments deriving from the phosphorylated 5′ or 3′ terminals of DNA. With RNA molecules, the procedure would be restricted to the isolation of 5′-terminal polynucleotides because any phosphate group located at the 3′ terminals in such species would be expected to cyclize with the adjacent hydroxyl group rather than undergo the sorbitol esterification reaction. However, in these cases, 3′-terminal polyribonucleotides could be isolated by first removing the 3′-phosphate group enzymatically and then exploiting the binding capacity of the resulting 2′,3′-diol group. An extension of the potential to isolate terminal DNA fragments would involve the ability to select out series of restriction endonuclease fragments that possess different chain lengths and share a common terminus. This isolation would constitute a simplified procedure for the mapping of restriction endonuclease sites. Another possible application concerns the use of the esterification reaction to render particular terminal phosphate groups in DNA or DNA fragments insensitive to alkaline phosphatase. This would allow the specific labeling of subsequent restriction endonuclease cleavage sites with ^{32}P via the phosphatase–polynucleotide kinase technique and permit sequence analysis near these sites without the necessity of prior fractionation of the endonuclease fragments.

Experimental Procedures

Materials. Aminoethylcellulose (0.33 mequiv/g) and Sephadex G-50-80 were purchased from Sigma Chemical Co., St. Louis, MO, and aminoethylpolyacrylamide (aminoethyl Bio-Gel P-150, 1.1 mequiv/g) and Dowex 1-X4 ion-exchange resin (AG 1-X4, −400 mesh) were purchased from Bio-Rad Laboratories, Richmond, CA. Deoxyribonucleoside 5′-phosphates and polynucleotide kinase were obtained from P-L Biochemicals, Milwaukee, WI. Snake venom phosphodiesterase, alkaline phosphatase, deoxyribonuclease I and calf thymus DNA were the products of Worthington Biochemical Corp., Freehold, NJ. Yeast tRNA and salmon sperm DNA were obtained from Calbiochem, LaJolla, CA. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EPC) was the product of Pierce Chemical Co., Rockford, IL.

m-[[3-(N-Succinimidoxycarbonyl)propanoyl]amino]-benzeneboronic Acid 1,3-Propanediol Cyclic Ester (IV). *N*-[*m*-(Dihydroxyboryl)phenyl]succinamic acid (II, 4.74 g), prepared by the method of Weith et al. (1970), and 1,3-propanediol (1.52 g) were dissolved in dry dioxane (40 mL) and the solution was heated on a boiling water bath for 10 min. The solvent was removed in vacuo and the resulting cyclic boronate (III) was dried by twice dissolving it in dry dioxane (40 mL) and evaporating the solution to dryness. The product was finally dissolved in dry dioxane (100 mL). *N*-Hydroxysuccinimide (2.3 g) and then dicyclohexylcarbodiimide (4.13 g) were dissolved in the solution. The mixture was allowed to stand overnight, and the precipitated dicyclohexylurea was then removed by filtration. The precipitate was washed with dioxane, and the filtrate and combined washings were evaporated to dryness. The residue was recrystallized from dioxane–ethyl acetate to yield the product IV (5.84 g, 78%), mp 174–176 °C. Anal. Calcd for $C_{17}H_{19}BN_2O_7$: C, 54.57; H, 5.12; B, 2.89; N, 7.49. Found: C, 54.33; H, 5.08; B, 2.91; N, 7.68.

N-Succinimidyl Acetate. *N*-Hydroxysuccinimide (4.8 g) was dissolved in dry pyridine (10 mL), and acetic anhydride (6 mL) was added dropwise to the solution. The mixture was allowed to stand overnight and was then evaporated to dryness in vacuo. The residue was treated with water and again dried in vacuo. Recrystallization from water gave 3.5 g of the product (mp 130 °C). The ester with mp 130 °C had been previously prepared by treating *N*-hydroxysuccinimide with acetic acid and dicyclohexylcarbodiimide (De Groot et al., 1966).

[[N-[N′-[m-(Dihydroxyboryl)phenyl]succinamyl]amino]-ethyl]cellulose. Aminoethylcellulose (1 g, 0.33 mequiv/g) was washed twice by centrifugation with 20-mL portions of 1 M sodium hydroxide and then washed a number of times with water until the alkali had been removed. The cellulose was then stirred as a suspension in water (20 mL) while a solution of *m*-[[3-(*N*-succinimidoxycarbonyl)propanoyl]amino]-benzeneboronic acid 1,3-propanediol cyclic ester (IV, 150 mg, 0.4 mmol) in warm dioxane (2 mL) was added dropwise. The pH of the suspension was set at 9 by the addition of dilute NaOH, and the cellulose was stirred for 2 h and then washed repeatedly with water. The DBAE-cellulose was shown to contain at least 0.22 mmol of boronic acid group per g by spectrophotometric analysis of a portion of the combined washings that had been treated with 1 M NH_4OH (using ϵ_{250} 19 000 for the amide of *N*-[*m*-(dihydroxyboryl)phenyl]-succinamic acid and ϵ_{250} 7600 for *N*-hydroxysuccinimide in 1 M NH_4OH). Any unreacted amino groups in the product were acetylated by treating the DBAE-cellulose with 200 mg of *N*-succinimidyl acetate under the same conditions.

A column (10 × 1 cm) of the cellulose was tested for its pH-dependent ability to bind two samples (30 A_{260} units each) of tRNA. In the presence of 0.05 M NH_4OAc (pH 4.5) as eluting buffer the column bound 98% of the tRNA, and release of this material could be effected by subsequent elution with 1 M NaCl–0.05 M NH_4OAc (pH 4.5). The RNA was also bound to 98% when applied to the column in 1 M NaCl–0.1 M $MgCl_2$–0.05 M morpholine hydrochloride–20% dimethyl sulfoxide (pH 8.7), and release was effected with 1 M NaCl–0.05 M NH_4OAc–20% dimethyl sulfoxide (pH 4.5).

[[N-[N′-[m-(Dihydroxyboryl)phenyl]succinamyl]amino]-ethyl]polyacrylamide. Aminoethylpolyacrylamide (1 g, 1.1 meq/g) was suspended in 35 mL of water in a plastic centrifuge tube and allowed to stand overnight. The swollen polyacrylamide was washed with water by centrifugation and decantation several times and finally suspended in 20 mL of water. *m*-[[3-(*N*-Succinimidoxycarbonyl)propanoyl]amino]-benzeneboronic acid 1,3-propanediol cyclic ester (IV, 420 mg, 1.12 mmol), dissolved in 8 mL of dioxane, was added dropwise to the stirred suspension of the polymer while its pH was being maintained at 9.0 by the addition of dilute NaOH. After the addition was complete, the suspension was stirred for a further 4 h and then washed extensively with water. Spectrophotometric analysis of the washings indicated that the polyacrylamide contained 1.0 mequiv of dihydroxyboryl group per g. The product was suspended in 20 mL of water and then treated with a dioxane solution (2 mL) of *N*-succinimidyl acetate (500 mg) in the same way. The product was finally washed extensively with water.

A column (10 × 1 cm) of the substituted polyacrylamide was tested for its pH-dependent ability to bind two samples (90 A_{260} units) of tRNA. In the presence of 0.05 M NH_4OAc (pH 4.5) as eluting buffer, less than 10% of the tRNA was retained by the support. Application of the RNA to the column in 1 M NaCl–0.1 M $MgCl_2$–0.05 M morpholine hydrochloride–20% dimethyl sulfoxide (pH 8.7) resulted in 96% binding, and release was effected by elution with 1 M NaCl–0.05 NH_4OAc–20% dimethyl sulfoxide (pH 4.5).

Sorbitol Derivatization of Mononucleotides. Each deoxyribonucleoside 5′-phosphate (disodium salt, 100 A_{260} units) was dissolved in 0.5 mL of water, and the solution was adjusted to pH 5.5 with dilute HCl and added to an equal volume of 0.5 M sodium 2-(*N*-morpholino)ethanesulfonate (pH 5.5). Sorbitol (3 g) and EPC (300 mg) were added, the mixture was warmed briefly to dissolve all of the sorbitol, and the resulting syrup was allowed to stand at 20 °C for 2 h. The mixture was then diluted with water (15 mL) and a small portion was subjected to analysis by the method of Asteriadis et al. (1976). The analyses were carried out on a column (100 × 0.2 cm) of Dowex 1-X4 ion-exchange resin by elution at 10 mL/h with 200 mL of 10% EtOH containing a linear gradient of 0–0.5 M NH_4Cl that had been brought to pH 9.0 by addition of ammonia. In this system each sorbitol derivative has an elution volume of about one-half that of the parent nucleotide (e.g., sorbitol-pdA = 39 mL, pdA = 71 mL). The bulk of the reaction mixture in each case was diluted with water (200 mL) and applied to a column (25 × 4 cm) of DEAE-cellulose (Whatman DE-23) to remove the sorbitol. The column was washed extensively with water, and the product was recovered by elution with 1 M $Et_3NH^+HCO_3^-$ and subsequent removal of the volatile salt by evaporation in vacuo. Chromatography on Whatman No. 3MM paper with EtOH–1 M NH_4OAc, pH 7 (7:3, v/v), yielded R_f values (relative to pdA) of 1.0, 1.0, 0.8, and 1.3 for the four nucleotides, pdA, pdC, pdG, and pdT, respectively, and 1.4, 1.5, 1.3, and 1.7 for the corresponding sorbitol derivatives. R_f values (relative to pdA) in isopropyl alcohol–concentrated NH_4OH–water (7:1:2, v/v) were 1.0, 0.8, 0.4, and 1.2 for pdA, pdC, pdG, and pdT, respectively, and 2.2, 1.9, 1.4, and 2.6 for the corresponding sorbitol derivatives. Each purified derivative was shown to have a UV spectrum identical with that of the parent nucleotide. The derivatives display strong binding to columns of DBAE-cellulose in that they are totally retained in the presence of the pH 8.7 buffer and undergo release on exposure to the pH 5.5 buffer. The column had dimensions 40 × 0.4 cm and elution was carried out at 20 mL/h with 2 M NaCl–0.05 M morpholine hydrochloride–20% dimethyl sulfoxide (pH 8.7) which was subsequently changed to 2 M NaCl–0.2 M NaOAc–10% dimethyl sulfoxide (pH 5.5).

Treatment of each of the sorbitol esters with snake venom phosphodiesterase under previously described conditions (Ho and Gilham, 1973) resulted in its conversion back to the corresponding nucleoside 5′-phosphate.

Sorbitol Derivatization of Oligonucleotides. Heat-denatured calf thymus DNA (40 A_{260nm} units) was dissolved in 4 mL of 0.05 M Tris-HCl–5 mM $MnCl_2$ (pH 7.6) and treated with 5 units of deoxyribonuclease I for 1 h at 37 °C. The enzyme was destroyed by adding 1 mL of 2 M NaOH and incubating the solution at 37 °C for 2 h. The mixture was adjusted to pH 7 with acetic acid, diluted with 10 volumes of water, and applied to a column (25 × 1 cm) of DEAE-cellulose. Chloride and acetate ions were removed from the column by washing with 0.05 M $Et_3NH^+HCO_3^-$, and the oligonucleotides were recovered by elution with 1 M $Et_3NH^+HCO_3^-$ and subsequent removal of the volatile salt by evaporation. A portion (10 A_{260} units) of the oligonucleotide product was dissolved in water (100 μL) and derivatized as described above by using 100 μL of the pH 5.5 buffer, 0.6 g of sorbitol, and 60 mg of EPC. Excess sorbitol was removed by the use of DEAE-cellulose, and the recovered oligonucleotide fraction was tested for its ability to bind to DBAE-cellulose as described above for the sorbitol nucleotides.

In a control experiment the remainder of the oligonucleotide fraction was treated with alkaline phosphatase (0.5 unit) in 1 mL of 0.05 M Tris-HCl (pH 8) for 30 min at 37 °C. The product was isolated by adsorption and elution from DEAE-cellulose as described above and then subjected to the same conditions for reaction with sorbitol and water-soluble carbodiimide. The isolation and the testing of the product for binding to DBAE-cellulose were carried out as described above.

Sorbitol Derivatization of tRNA. Transfer RNA (0.5 mg) in 0.2 mL of 0.25 M sodium 2-(*N*-morpholino)ethanesulfonate (pH 5.5) was treated with 120 mg of urea, 1.2 g of sorbitol, and 120 mg of EPC. The resulting syrup was allowed to stand at 20° for 2 h and then passed through a column (50 × 1 cm) of Sephadex G-50 to remove the smaller molecular species from the product. The binding of sorbitol-tRNA to DBAE-cellulose was carried out as described above for the sorbitol nucleotides.

Derivatization of DNA. Calf thymus DNA (10 A_{260nm} units) dissolved in 1 mL of 0.05 M Tris-HCl (pH 8) was treated with alkaline phosphatase (0.07 unit) for 30 min at 37 °C. The phosphatase was destroyed with alkali by using the method of Ho and Gilham (1973). The alkali was neutralized with acetic acid and the DNA was precipitated by the addition of three volumes of ethanol. The product was dissolved in 1 mL of 0.02 M $MgCl_2$–0.01 M mercaptoethanol–0.1 M Tris-HCl (pH 7.6) and exposed to polynucleotide kinase (20 units) and 1.8 nmol of [γ^{32}P]ATP (14.4 Ci/mmol) for 30 min at 37 °C. The excess of ATP was removed by passing the solution through a column (50 × 1 cm) of Sephadex G-50, and the labeled DNA was derivatized with sorbitol as described above for tRNA. A portion of the product, dissolved in 0.2 mL of 0.05 M Tris-HCl–5 mM $MnCl_2$ (pH 7.6), was treated with deoxyribonuclease I (10 units) for 30 min at 37 °C. The enzyme was inactivated by boiling the solution for 3 min in the presence of 5 mM EDTA and the product was tested for its ability to bind to DBAE-cellulose as described above for the sorbitol nucleotides.

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