COLUMN CHROMATOGRAPHY OF NUCLEOTIDES OVER THYMIDYLATE-CELLULOSE*

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INTRODUCTION

Chromatographic techniques which utilize biochemically specific absorbents to purify materials are beginning to be of considerable importance. Naturally occurring nucleic acids can be linked to insoluble supports for the isolation of complementary strands of nucleic acids[1]. Synthetic polynucleotides containing only one kind of purine or pyrimidine base also have been coupled through phosphate[2] and pyrophosphate[3] linkage to cellulose and by ultraviolet irradiation[4] to polyvinyl chloride. Such derivatives have been used to isolate soluble ribonucleic acids which accept amino acid adenylates[5].

The present investigation was undertaken to exemplify more completely the general utility of the technique whereby an easily prepared nucleotide-cellulose compound effects chromatographic separation of materials which contain a base that selectively complexes through complementary hydrogen bonding.

EXPERIMENTAL

Thymidylate-cellulose

The acid form of thymidine-5′-monophosphate was obtained by dissolving 1 g of the diammonium salt (Sigma Chemical Co.) in water, adding a slight equivalent excess of formic acid, and evaporating to dryness under reduced pressure at 50°. To remove water of hydration, the white residue was dissolved in anhydrous pyridine and again evaporated to dryness. The pyridinium thymidine-5′-phosphate was dissolved in 100 ml of anhydrous pyridine and stirred into a suspension which contained 5 g of dicyclohexylcarbodiimide (Eastman Organic Chemicals) dissolved in 50 ml of anhydrous pyridine and 50 g of cellulose powder (Whatman CF 11), previously dried overnight at 50° over P_2O_5 *in vacuo*. The contents were shaken in a stoppered flask for 4 days at 30°. The thymidylate-cellulose was filtered off and washed thoroughly by resuspending and filtering from pyridine, ethanol, water, again ethanol, and finally diethyl ether. The colorless product was dried in air and then at 40° *in vacuo*.

The yield for esterification of thymidylate residues to cellulose was determined

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by the release of material with maximum absorbance at 267 mμ upon hydrolysis of 100 mg portions of the cellulose derivative with 5 ml of 1 *N* HCl at 100°. After cooling, cellulose was centrifuged off, and the supernatant solutions were read against an acid blank in the Beckman DU spectrophotometer.

Column chromatography

Approximately 2.5 mg amounts of mononucleosides and 5'-mononucleotides (Sigma Chemical Co.) were dissolved in 5 ml of 1 *M* sodium chloride containing 0.01 *M* sodium phosphate buffer at pH 7. The solution was chilled and poured over 10 g of thymidylate-cellulose in a 1.5 × 21 cm column which was previously equilibrated with the sodium chloride–phosphate buffer at 5°. The compounds were eluted with 100 ml of the cold salt-buffer and 10 ml fractions collected. The absorbance at 260 mμ was measured against the salt-buffer blank in the spectrophotometer.

Similar chromatography was effected with 1 mg samples of polyadenylic acid, obtained from Dr. SEVERO OCHOA of New York University, before and after hydrolysis in 0.1 *N* HCl for 5 min at 100°. The nucleotides with lower molecular weight were eluted with 250 ml of the salt-buffer at 5°, whereas those with higher molecular weight were eluted with an additional 250 ml of the salt buffer at 25°.

Yeast soluble nucleic acid (General Biochemicals Inc.) was chromatographed in the same manner as with polyadenylate, except 2.5 mg in 5 ml of salt buffer was poured on the column at 25°, followed by lowering the temperature to 4° before elution was started.

RESULTS

The rate of release of thymidylate residues upon acid hydrolysis of thymidylate-cellulose is shown in Fig. 1. Essentially all of the thymidylate is liberated in a *quasi* first-order manner within 5 h to give a maximum absorbance of 7 at 267 mμ. The molar extinction coefficient at this wavelength for thymidylate in acid is $9.6 \cdot 10^3$, so that its molarity in the 5 ml of supernatant solution obtained from a 100 mg portion of the cellulose derivative was estimated to be $7.3 \cdot 10^{-4}$. Thus, approximately 140 mg of thymidylic acid was esterified per 10 g of cellulose.

The elution pattern from chromatography of mononucleosides on thymidylate-cellulose is illustrated in the top half of Fig. 2. Both thymidine and cytidine are eluted immediately following displacement of the volume of liquid held up by the cellulose derivative. Some slight retardation of guanosine is effected, but adenosine is retained best. The elution of mononucleotides, illustrated in the bottom half of this figure, is somewhat more rapid. The same order of appearance of compounds in the effluent is found with 5'-adenylate retained best.

The elution pattern from chromatography of a preparation of polyadenylate is illustrated in the top half of Fig. 3. Most of the material is not eluted at 5°, but is readily stripped off the column at 25°. The opposite is true after acid hydrolysis, as shown in the bottom half of this figure.

The elution pattern from chromatography of a soluble preparation of ribonucleic acid from yeast on thymidylate-cellulose is illustrated in the bottom half of Fig. 4. Some resolution of material is apparent, since a small fraction of polynucleotide, probably rich in adenine, was eluted at 25°.

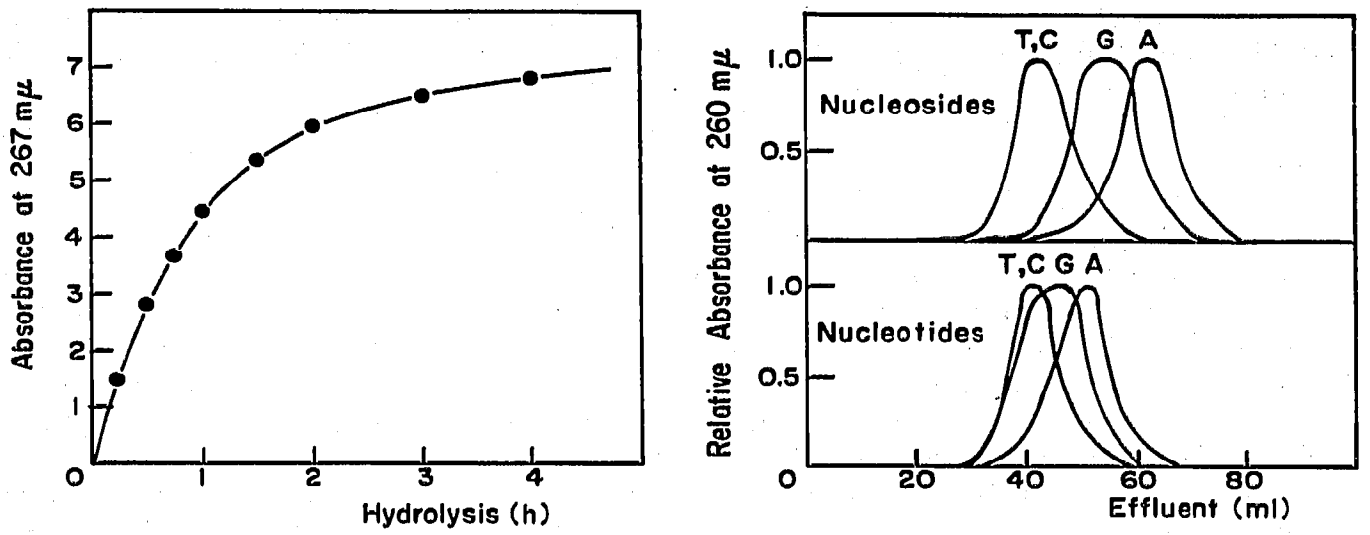

Fig. 1. Rate of acid hydrolysis of thymidylate-cellulose.

Fig. 2. Chromatography of mononucleosides (top) and mononucleotides (bottom) on thymidylate-cellulose. The letters indicate the base component: T = thymine; C = cytosine; G = guanine; A = adenine.

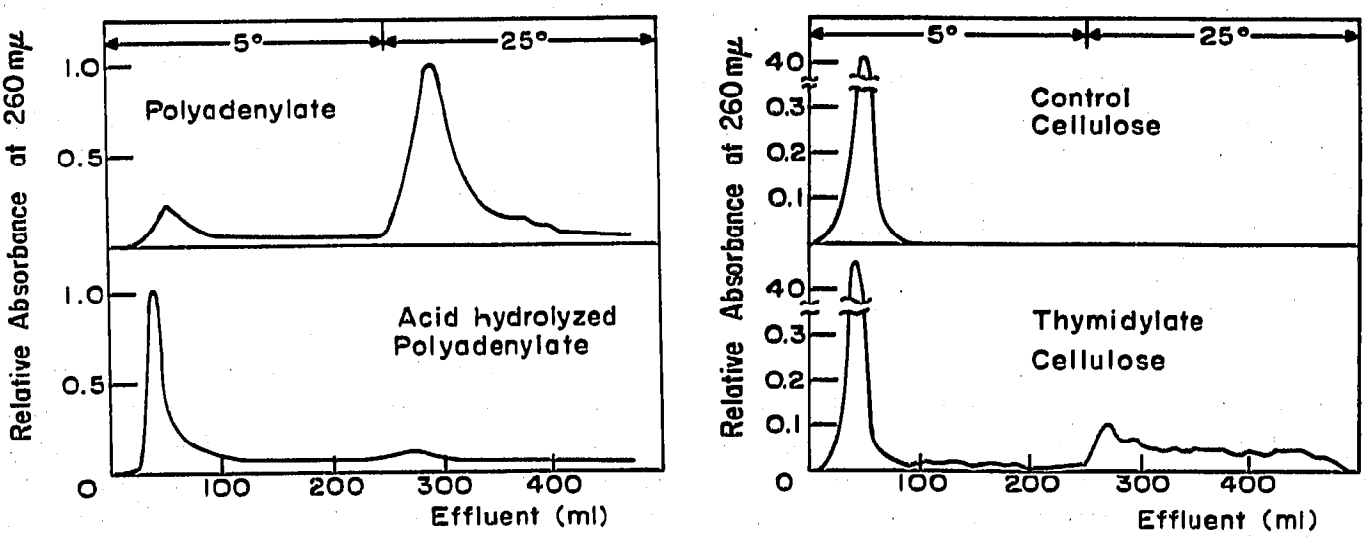

Fig. 3. Chromatography of polyadenylate (top) and acid-hydrolyzed polyadenylate (bottom) on thymidylate-cellulose.

Fig. 4. Chromatography of soluble ribonucleic acid on cellulose (top) and on thymidylate-cellulose (bottom).

None of the materials tested showed any retention on control columns of plain cellulose. The top half of Fig. 4 shows the behavior of yeast soluble ribonucleic acid on such a control column.

DISCUSSION

Coupling of a nucleotide to an inert solid by functional groups which do not preclude some selective bonding to a different nucleotide offers an advantage during purification of the latter. Though secondary forces can affect the absolute retentions of materials, the relative contribution of hydrogen bonding between base pairs, *i.e.* adenine *versus* thymine or uracil and guanine *versus* cytosine, is predominant.

As expected, adenine-containing compounds are best retained by the thymidylate moieties of the cellulose derivative. The generally better retention of nucleosides than nucleotides is probably due to electrostatic repulsion in the latter between their anionic phosphate groupings and those in the thymidylate residues on the cellulose. The slight retardation of guanosine and 5'-guanylate during chromatography may reflect the greater basicity of these compounds.

The greater the number of adenylate units within a polymer, the more effectively is the material retained on the thymidylate-cellulose. Acid hydrolysis of polyadenylate led mainly to formation of the adenylate monomer which was readily eluted in the cold. Only a small portion of the soluble ribonucleic acid was retained on the thymidylate-cellulose; however, the fact that an increase in temperature was required for complete elution would indicate that at least a small portion of the adenine moieties were not internally hydrogen bonded and thus were retained on the column.

The general principal of attachment of specific reagents or natural substances to cellulose or cellulose derivatives has recently been applied for selective removal of such proteins as antibodies[6], enzymes[7,8], and avidin[9]. Sufficient progress of a similar nature has now been made in the area of nucleic acids to warrant further development of nucleotide-cellulose chromatography.

SUMMARY

The synthesis of thymidylate-cellulose was accomplished through condensation of 5'-thymidylate and cellulose using dicyclohexylcarbodiimide in pyridine. The thymidylate-cellulose was shown to selectively retain adenosine, 5'-adenylate, and polyadenylate compounds under conditions of high salt concentration and low temperature which favor hydrogen bonding of the complementary base. Increased temperature was effective during elution of polyadenylate and yeast soluble nucleic acid. Acid hydrolysis was used to cause extensive cleavage of polynucleotides to form mononucleotides, thus indicating the differences in retention on thymidylate-cellulose of polymers as opposed to monomers.

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