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Chromatography of Nucleic Acids on Cross-Linked Cyclodextrin Gels Having Inclusion-Forming Capacity

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ABSTRACT

The characterization by UV spectral methods of the interaction between cyclodextrins and nucleic acid components yields the following information. Adenine nucleotides are found to interact most strongly with cycloheptaamylose, presumably by inclusion of the base within the cyclodextrin cavity. Factors influencing the interaction include pH, the position of phosphorylation of nucleotides, and the degree of polymerization of oligonucleotides. When epichlorohydrin cross-linked cycloheptaamylose gel is used as a stationary phase for nucleic acid chromatography, adenine-containing compounds are retained most strongly. The factors affecting complex formation with free cycloheptaamylose have the same effects on gel chromatography of nucleic acids. It appears that cyclodextrin stationary phases are useful for fractionation of nucleic acids and of other classes of compounds with functional groups which can interact with cyclodextrin cavities.

INTRODUCTION

As potential components of polymers or resins with specific complexing ability, the cyclodextrins are interesting candidates. Cyclodextrins are cyclic α-1,4-oligoglucopyranosides having six or more anhydroglucose residues per molecule. These compounds are natural products resulting from the digestion of starch by an amylase from Bacillus macerans. Their unique cyclic structure endows the cyclodextrins with an equally unique physical property, i.e., the ability to form inclusion complexes with a variety of molecules with functional groups fitting into the cyclodextrin cavity. This property of cyclodextrins has been the object of a large number of studies, from which Thoma and Stewart [1] have compiled an extensive review.

The binding of a "substrate" by the cyclodextrin may bring susceptible bonds, such as ester bonds, into proximity with the hydroxyl groups of the cyclodextrin. This may lead to catalysis of the hydrolysis of such bonds, with the kinetics of the process being analogous to those of enzyme-catalyzed reactions. A number of such enzyme analog studies have been reviewed by Cramer and Hettler [2], and many of these illustrate the high degree of specificity involved in inclusion-complex formation.

The present investigation on cyclodextrin-nucleic acid interactions is the result of a search for compounds which would perturb the UV spectrum of specific transfer ribonucleic acid (tRNA) molecules in a manner indicative of their secondary structure. Since nucleic acid bases are about the same size as a number of other compounds which form complexes with cyclodextrins, interaction was anticipated.

The basic technique used to characterize the interaction was UV difference spectroscopy. The following results describe the parameters which affect the interaction of nucleic acids with free cyclodextrins in solution [3]. It will later be seen that these same parameters hold for the binding of nucleic acids by cyclodextrins which are immobilized as cross-linked gels.

EXPERIMENTAL SECTION

Difference Spectra

UV difference spectra were measured in a Cary 15 spectrophotometer using the dual compartment cuvettes designed by Yankeelov [4]. Cyclodextrin solutions were pipetted into one side of the reference and the sample cuvettes, while nucleic acid solutions were placed in the remaining sides. In this configuration a baseline was determined by scanning from 320 to 220 nm. The contents of the sample cuvette

were then mixed, and the scan was repeated to determine the difference spectrum.

Gel Preparation

The procedure of Solms and Egli [5] was modified in order to reduce the amount of cross-linking to the minimum required for mechanically stable gels. Fifty grams of cycloheptaamylose was added to 20 ml water and worked into a smooth paste to which 60 ml of 50% (w/v) NaOH containing 50 mg $NaBH_4$ was added. This procedure was necessary to prevent lumps from forming. This mixture was then blended vigorously with 34 ml epichlorohydrin at 50°. A Virtis homogenizer, having a water-jacketed flask connected to a circulating thermostat, was found to be convenient for this purpose. Temperature control was essential since considerable heat was evolved during gel formation. Formation of the gel occurred within 30-45 min of epichlorohydrin addition. At this time cooling water was circulated through the jacket of the flask. The gel was washed at least three times with acetone, followed by water, until the pH of the wash was 8-9. The particle size of gels was reduced to a size suitable for column chromatography by treatment in a Potter-Elvejehm type tissue homogenizer with a loose-fitting glass pestle. Fines were removed by settling and decantation.

Column Chromatography

Columns were poured under flow and equilibrated with the appropriate buffer. Gels prepared as above occupied about 5 ml bed volume/g dry gel. Chromatographic runs were made at room temperature unless specified otherwise. Column effluents were monitored at 254 nm with a UV flow monitor, since all of the compounds used have significant absorbances at this wavelength.

RESULTS AND DISCUSSION

As with other classes of compounds, the cyclodextrins showed a great deal of specificity on interaction with nucleic acids. When individual nucleotides were used as potential guest molecules with cyclohexaamylose or cycloheptaamylose, no interaction was found with the former compound. Examination of accurate space-filling models (Corey-Pauling-Koltun) indicated that the cyclohexaamylose cavity was too small to allow entry of any bases of these nucleotides.

With cycloheptaamylose, not only the nature of the base but the

position of the phosphate group in the nucleotide were found to influence the interaction. This is illustrated in Fig. 1, which shows the difference spectra resulting from inclusion complex formation between cycloheptaamylose and a number of adenine nucleotides. Nucleotides

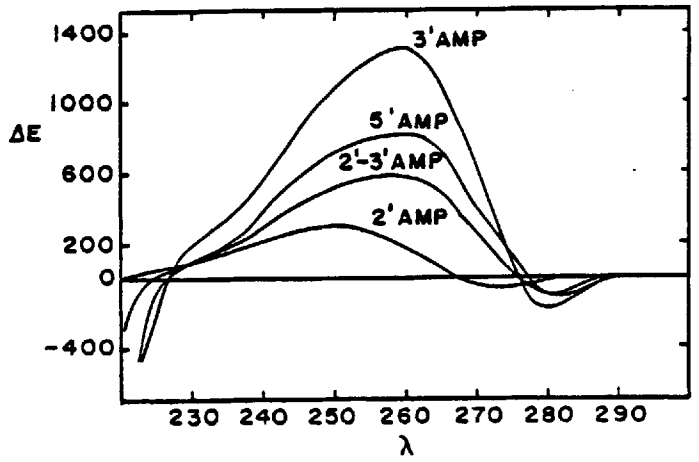

FIG. 1. The interaction of cycloheptaamylose with various adenosine monophosphates. TSC buffer (0.01 M Tris and 0.1 M NaCl, pH 7.6), 0.01 M cycloheptaamylose. All difference spectra are presented as the spectrum of the free compound minus that of the complex. (Note: 2'-3'AMP is adenosine 2',3'-cyclic monophosphate.)

containing bases other than adenine gave no significant spectral changes on mixing with cycloheptaamylose. Pyrimidine bases are apparently too small to completely fill the cavity, while guanine, having one more group attached to the purine ring than adenine, is too large. The position of the phosphate group in adenine nucleotides has a strong effect on the interaction. Cycloheptaamylose-adenine nucleotide complexes are most stable when the ionic phosphate group remains in the aqueous environment outside the cyclodextrin cavity. This is reflected in the greater spectral shifts seen with adenosine 3'-monophosphate (3'-AMP) and adenosine 5'-monophosphate (5'-AMP) as compared with the 2'-nucleotides having the base and phosphate on adjoining ribose carbon atoms.

The pH has a considerable influence on inclusion complex formation. Focusing on the titration curve for 3'AMP in Fig. 2, in the pH range from 7.5 to 5.0, the phosphate group undergoes protonation (pK_a = 5.9)

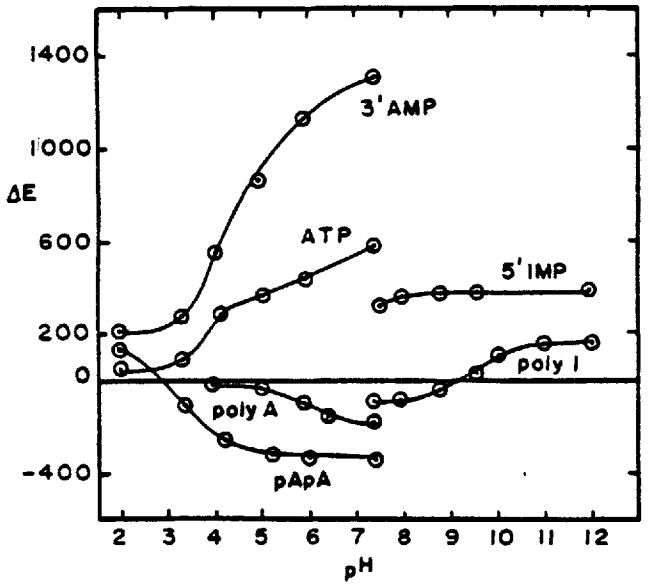

FIG. 2. The effect of pH on cycloheptaamylose interaction. 0.01 M cycloheptaamylose was used. All solutions contained 0.1 M NaCl and 0.01 M appropriate buffers.

while below 5.0 the amino group of the adenine base is titrated (pK_a = 3.7). Both of these protonations affect the magnitude of the spectral change. Our interpretation of this was that neutralization of the secondary phosphate lessened its solvation and weakened the complex, while placing a positive charge on the amino group of the adenine ring greatly reduced its affinity for the cycloheptaamylose cavity.

Once adenine residues are polymerized, the nature of the interaction of the bases with cycloheptaamylose is somewhat modified. In a polynucleotide such as poly(adenylic acid) (poly A), the bases are involved in base-stacking interactions which reduce their exposure to cycloheptaamylose and also cause a reversal of the sign of the spectral change. This is illustrated in Fig. 3, which shows a comparison of the cycloheptaamylose-induced difference spectrum of 5'-AMP with those for a series of polyadenylates from a chain length of two up to hundreds. The effect of chain length is seen to level off after the heptanucleotide is reached.

With the above type of information as background, we attempted to analyze the cycloheptaamylose-induced difference spectra of several

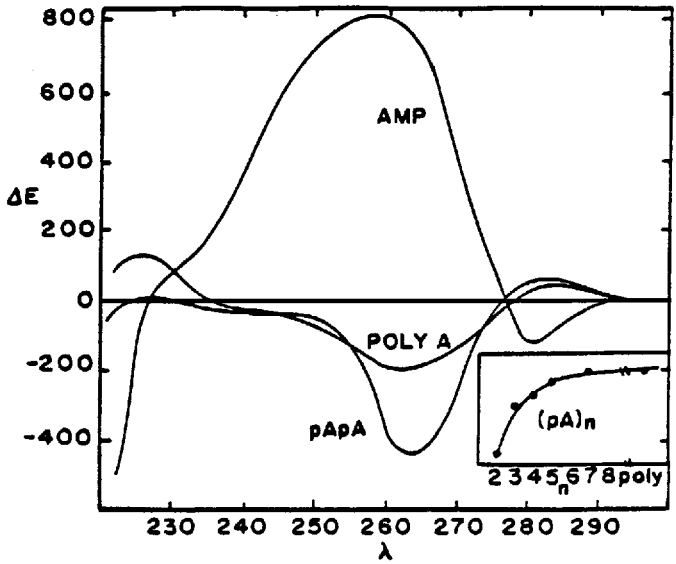

FIG. 3. The effect of polymerization of adenylic residues on cycloheptaamylose interaction. TSC buffer, 0.01 M cyclodextrin; inset is chain length n vs ΔE_{263} for oligoadenylic acids. The ordinate of the inset is the same as for the overall figure.

purified transfer ribonucleic acids. Figure 4 shows the difference spectra obtained with three different tRNA's under three different ionic conditions. It is obvious that the adenine bases in these tRNA's are not readily accessible to cycloheptaamylose, especially in the presence of magnesium ions, a requirement for the biological activity of tRNA. When these tRNA's are hydrolyzed to their constituent nucleotides however, the typical cycloheptaamylose-AMP difference spectrum is again seen (Fig. 5).

In view of the results described above, as well as many others, which show that cyclodextrins possess considerable selectivity for guest molecules, it was apparent that stationary phases containing cyclodextrins could be a valuable addition to present liquid chromatographic practice.

Since cyclodextrins contain large numbers of hydroxyl groups, they can be cross-linked in alkaline solution using such bifunctional reagents as epichlorohydrin. Solms and Egli [5] first reported the application of this method for the preparation of cyclodextrin gels with selective retention properties. Wiedenhof et al. [6] have developed an

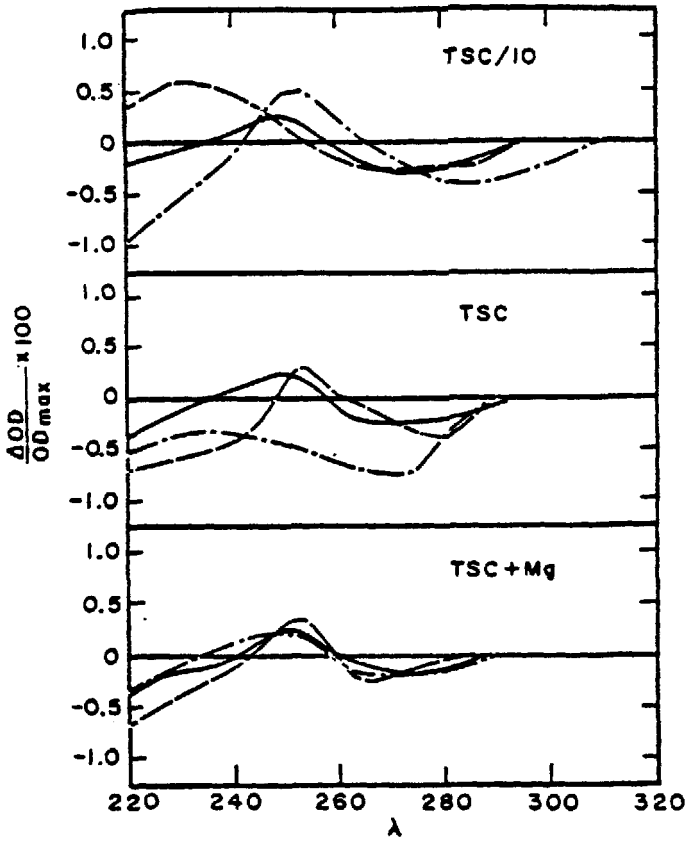

FIG. 4. Comparison of the cycloheptaamylose difference spectra of three tRNA's under three ionic conditions. TSC + Mg contained 20 mM $MgCl_2$. (—) Yeast valine tRNA; (— —) Escherichia coli phenylalanine tRNA; (— -) yeast arginine tRNA.

emulsion polymerization process for the above reaction which yields bead-form cyclodextrin resins with attendant improvements in flow properties during column chromatography. Such epichlorohydrin-cycloheptaamylose gels were therefore used in attempts to fractionate nucleic acids and their constituents [7]. This investigation was undertaken for two reasons. The first was a practical one; that was to provide another basis for fractionation to those interested in nucleic acid chemistry. The second reason was to examine whether

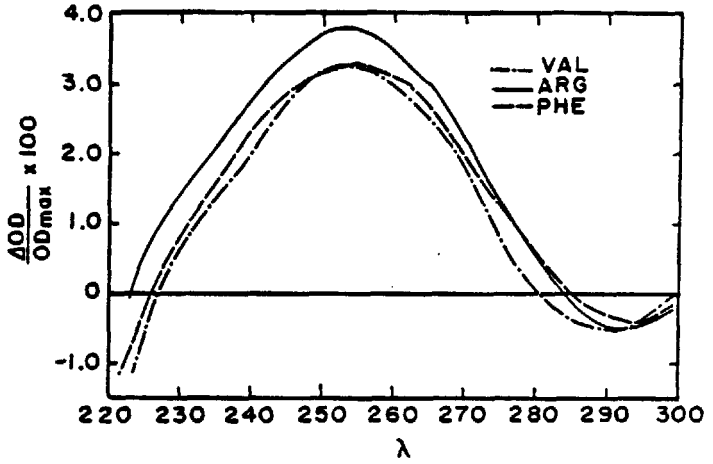

FIG. 5. Cycloheptaamylose difference spectra of NaOH digested tRNA's. The three tRNA samples were hydrolyzed in 0.3 M NaOH at 37° for 18 hr, neutralized, and diluted into TSC for spectral measurements.

the conditions for complex formation by cyclodextrin solutions were the same as those for cyclodextrin gels.

Since all of the adenine nucleotides have the same molar extinction, the magnitude of the spectral shifts in Fig. 1 should be proportional to the degree of interaction with cycloheptaamylose. Nucleotides with large spectral changes should thus be retained strongly by the cycloheptaamylose gel. Comparison of Figs. 6 and 1 shows that this is so. If the liquid volume is taken as the elution volume of Cl^-, NH_4^+, or H_3O^+, it is evident that 5'-CMP is retained only slightly, then 2'-, 5'-, and 3'-AMP as predicted from Fig. 1.

The elution pattern in Fig. 6 was obtained at pH 8.1. If the pH of the eluting buffer was lowered to 3.0, no resolution was obtained. This is consistent with the spectral data in Fig. 2 which indicates little cycloheptaamylose-nucleotide interaction at pH 3.0.

Temperature also has an effect on the elution of nucleotides as demonstrated in Fig. 7. Panel a shows the elution pattern for the four major RNA-derived 3'-nucleotides at 25° C, while Panel b shows the higher retention evident at 0° C. A more detailed study has established 10° C as a sufficiently low temperature to separate RNA hydrolysates for nucleotide ratio analysis.

The next two figures illustrate the effects of variations in the sugar portion of nucleotides or nucleosides on binding to cycloheptaamylose gels. Figure 8 shows the elution pattern of a mixture of

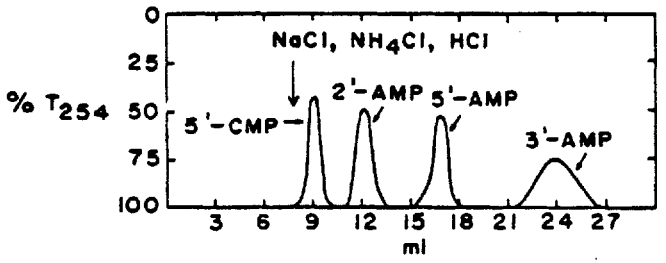

FIG. 6. Chromatography of three adenosine monophosphates and cytidine 5'-monophosphate on cycloheptaamylose gel. Sample contained 0.1 mg of each nucleotide in 0.1 ml; 0.6 × 45 cm bed; 2.8 ml/hr 0.01 M Tris-HCl, pH 8.1, at room temperature (25°C).

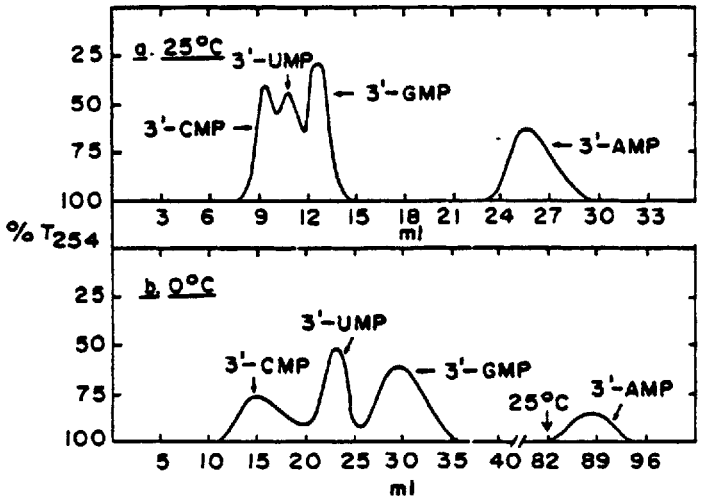

FIG. 7. Chromatography of the four major RNA-derived 3'-nucleotides on cycloheptaamylose gel. Samples contained 0.1 mg of each nucleotide in 0.1 ml; 0.6 × 45 cm bed; (a) 4.0 ml/hr 0.01 M Tris-HCl, pH 8.1, at room temperature; (b) 3.7 ml/hr same buffer at 0°C to 82 ml, then room temperature (25°C).

adenosine 5'-monophosphate and 2'-deoxyadenosine 5' monophosphate. The less polar deoxynucleotide binds more strongly to the gel.

Figure 9 shows that a rather subtle variation in the stereochemistry

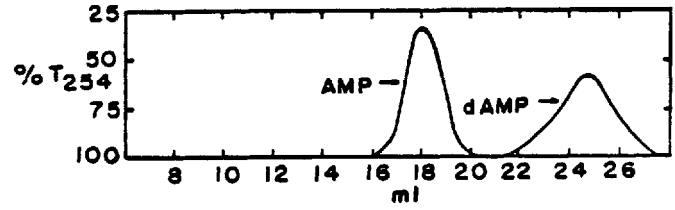

FIG. 8. Separation of adenosine 5'-monophosphate from deoxyadenosine 5'-monophosphate on cycloheptaamylose gel. Sample contained 0.1 mg of each nuclotide in 0.1 ml; 0.6 × 45 cm bed; 4.5 ml/hr 0.01 M Tris-HCl, pH 8.1, 20°C.

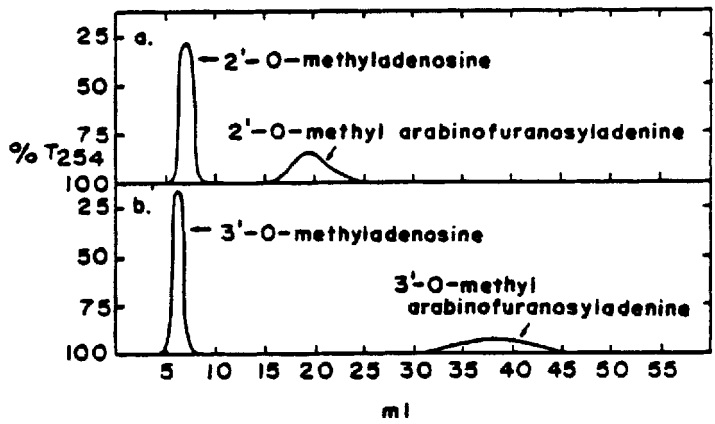

FIG. 9. Chromatography of O-methyladenosines and their arabinosyl analogs. 0.7 × 4.5 cm bed; 5.4 ml/hr 0.01 M Tris, pH 8.1, at room temperature (25°C). (a) 0.1 mg 2'-O-methyladenosine plus 0.05 mg 2'-O-methylarabinofuranosyladenine in 0.1 ml. (b) 0.1 mg 3'-O-methyladenosine plus 0.04 mg 3'-O-methylarabinofuranosyladenine in 0.1 ml.

of the sugar can lead to dramatic differences in elution from the gel. The epimeric compounds, 2'-O-methyladenosine and 2'-O-methyl arabinofuranosyladenine, or the 3'-O-methyl nucleosides have quite different elution volumes. In both cases the arabinose compounds bind much more strongly than the ribose.

Oligonucleotides containing adenine do not bind tightly to cycloheptaamylose gels, although some fractionation can be obtained. Most recently cycloheptaamylose gels containing anion exchange (diethylaminoethyl) groups

been prepared. These gels are quite useful in the separation of oligonucleotides and have also been used to fractionate tRNA's. This combination of anion exchange and inclusion-complexing ability expands the fractionation procedures available for fragments of RNA produced during base sequence analysis.

In summary it appears that cyclodextrin stationary phases can be of general use for the fractionation of compounds containing functional groups with varying affinities for the cyclodextrin cavity. Where these functional groups have readily measurable spectral properties, it may be possible to predict their chromatographic behavior on cyclodextrin gels by studying the difference spectra obtained by mixing with cyclodextrin solutions.

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