Complexes of Oligonucleotides with Polynucleotides on Solid Supports†

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ABSTRACT: Complementary polynucleotide·oligonucleotide complexes on solid supports were investigated. Two anion exchangers were used as solid supports: a macroreticular polystyrene resin (Amberlite IRA-938) which is a strong anion exchanger; and an aminoethyl polyacrylamide gel (aminoethyl Bio-Gel P-30) which is a weak anion exchanger. The systems poly(C)·oligo(I) and 2poly(U)·oligo(A) were studied comparatively both on the supports and in aqueous salt solutions. The hydrogen-bonded complexes on the solid phase were studied under conditions in which the polynucleotides were bound ionically but the oligomers were not. Thermal melting profiles of the complexes were obtained by uv spectral measurement of the amount of oligonucleotide released through a stepwise heating procedure in a cuvet. The more highly ordered polymers and oligomers show a lower affinity for the supports than the less ordered ones. On the supports the H-bonded complexes usually had a T_m about 2° higher than in solution. This stabilization may result from the contribution of ion-exchange groups to the local ionic strength (particularly at low salt concentrations) and from an entrope advantage in complex formation, as a result of preimmobilization of the polynucleotide by the support. The topography of the support is clearly also important in this regard, since the polynucleotide is held in a conformation which may by either favorable or unfavorable for helix formation. The latter effect is more pronounced at a low ionic strength, when the polynucleotide is bound most firmly. The implication of this study to the biological systems and to the designing of a solid phase system for template-directed oligonucleotide condensations is discussed.

Attaching nucleic acids and synthetic polynucleotides to various solid column materials has become a common practice in recent years. Both covalent linkage (Gilham, 1968; Poonian *et al.*, 1971) and physical immobilization (Wada and Kishizaki, 1968; Rosenberg *et al.*, 1972) have been used to fix the polymers on a variety of supports, including cellulose, agarose, and acrylamide gel. Such polynucleotide supports have found wide application in affinity column chromatography. They are particularly well suited for hybridization studies in which a support carrying a single-stranded nucleic acid can be used to fractionate complementary oligonucleotides upon application of a temperature gradient (Gilham, 1964; Gilham and Robinson, 1964). Hydroxylapatite columns have also been used for thermal chromatography of oligonucleotide·polynucleotide complexes (Niyogi and Thomas, 1968). In this case, as in the present paper, conditions employed were such that the oligonucleotide bound to the column only if it complexed with the polynucleotide.

Although nucleic acid columns are well established as an analytical tool, no one has as yet reported any detailed physical–chemical study on the hydrogen-bonded associations of nucleic acids on solid supports *vis-à-vis* their behavior in solution. The effect of solid supports on such interactions should be of considerable interest when one considers the fact that polymerizations in biological systems occur in association with solid surfaces of membranes (such as the DNA polymerase system) or of organelles (such as the ribosome–polysome systems).

Is it possible that such solid-associated polymerization systems have evolved because these reactions occur more efficiently when the reactants are immobilized on a support? This idea is not completely without experimental evidence. It has been shown that ribosomes contribute immensely to the stability of the tRNA·mRNA complex (McLaughlin *et al.*, 1966). Although the tRNA, no doubt, binds to the ribosome at other sites in addition to the mRNA codon, McLaughlin *et al.* speculate that the ribosome may stabilize the complex "by providing spatial restrictions which strengthen the interaction between the trinucleotidyl residue of the sRNA anticodon and the complementary sequence of the messenger." The ribosome would thus stabilize the tRNA·mRNA complex by reducing some of the negative entropy requirement in the interaction which would otherwise be much larger in a "free" codon–anticodon interaction.

A similar entropy advantage is observed when small associating species become a part of larger macromolecules. Eisinger has demonstrated that a trinucleotide which shows no binding to its complementary trinucleotide in solution will associate appreciably with a tRNA molecule having a complementary anticodon (Eisinger, 1971). Furthermore, two tRNA molecules having anticodons complementary to each other associate very strongly (Eisinger, 1971). Eisinger first concludes that the anticodon regions of tRNA have similar and complementary structures. In addition, the experiments showed that the decreased mobility of trinucleotide segments when they are properly structured as parts of tRNA molecules increases the stability of their complex with this entropy advantage (Eisinger *et al.*, 1971).

A similar entropy effect is encountered when one compares the thermal stability of the poly(C)·oligo(I) complex *vs.* that of 2poly(U)·oligo(A) (Tazawa *et al.*, 1972). The former is markedly less stable than the latter whereas the corresponding polymer–polymer complexes have similar stabilities. The explanation is basically that oligomers lose more entropy upon

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helix formation with polymers than the polymers do. In poly(C)·oligo(I), a string of oligomers has to be immobilized for every polymer whereas in 2poly(U)·oligo(A), a string of oligomers is immobilized for only the first strand of poly(U). Adding the second strand of poly(U) is essentially a polymer·polymer association with no need for additional entropy loss of the oligomers.

We report in this paper studies on polynucleotide·oligonucleotide associations on two solid supports, both anion exchangers. The poly(C)·oligo(I) and 2poly(U)·oligo(A) complexes were deemed suitable for this study because their behavior in aqueous solution is already well known (Lipsett *et al.*, 1961; Michelson and Monny, 1967; Cantor and Chin, 1968; Tazawa *et al.*, 1972; Blake, 1972). Besides our interest in the physical chemistry problem already outlined, we were further attracted by the possibility of using such a solid-phase system for template-directed synthesis of nucleic acids—a "chemical ligase" reaction. Template-directed condensation of mononucleotides and oligonucleotides has already had limited success in aqueous solution (Naylor and Gilham, 1966; Sulston *et al.*, 1968, 1969; Weimann *et al.*, 1968; Schneider-Bernloehr *et al.*, 1970; Shabarova and Prokofiev, 1970; Renz *et al.*, 1971).

Experimental Section

Materials. The resin used in these studies is a macroreticular anion-exchange resin known as Amberlite IRA-938. It is a strong anion exchanger carrying quaternary ammonium exchange groups. The pore diameter ranges from 25,000 to 230,000 Å. It has an exchange capacity for inorganic ions of 3.7 mequiv/g. The Amberlite IRA-938 used in this study was a gift from Rohm and Haas Co., and the resin is now commercially available through Mallinckrodt distributors.

The other solid support used in this study was aminoethyl Bio-Gel P-30. It was a gift from Dr. H. M. Dintzis who prepared it by heating Bio-Gel P-30 (100–200 mesh) at 100° for 8 hr with ethylenediamine (Inman and Dintzis, 1969). It is thus a weak anion exchanger consisting of a polyacrylamide gel bearing primary amines. The exchange groups have a pK_a of about 9.2. A similar product aminoethyl Bio-Gel P-60 having an inorganic exchange capacity of 2 mequiv/g is now commercially available from Bio-Rad Laboratories.

All unlabeled polynucleotides used were commercial preparations from various sources. Tritiated poly(U) was from Miles Chemical Co.

Oligomers of inosinic acid (I_6 and I_8) were a gift from Dr. and Mrs. Ichiro Tazawa (Tazawa *et al.*, 1972). A_4 was obtained from Dr. Lou-Sing Kan and also from Miles Chemical Co. Oligoadenylates A_4p, A_5p, and A_6p were prepared by Dr. Seiichi Uesugi. The preparation involved limited digestion of poly(A) with Micrococcal nuclease. The oligomers were purified by paper chromatography on Whatman 1 developed with 1-propanol–concentrated NH_3–H_2O (55:10:35). A_2 was a commercial preparation from Miles Co. The neutral deoxynucleoside ethyl phosphotriester dAp(Et)dA was a gift from Dr. Paul S. Miller (Miller *et al.*, 1971).

Methods. Loading Polynucleotides on Supports.[1] The gel was swollen in an excess volume of 0.05 M Tris (pH 7.2) for 2 days before use. The resin was washed extensively with buffer to remove any free uv-absorbing material. Polynucleotides were loaded on both supports by a batch procedure. In a typical resin loading procedure, 1 ml of wet resin was stirred with 0.02 mmol[2] of polynucleotide in 10 ml of 10^{-3} M neutral phosphate buffer for 4 days at room temperature. The resin was found to have the following capacities for polynucleotides: poly(U), 0.14 mmol/g of dry resin; poly(A), 0.12 mmol/g; poly(C), 0.06 mmol/g. A wet volume of 1 ml of resin corresponds approximately to 0.123 g dry weight. The kinetics of loading the gel were considerably faster. Again 1 ml of gel was stirred at room temperature with 0.02 mmol of polynucleotides in 10 ml of 0.05 M Tris (pH 7.2). In this case, however, binding was complete in about 3 hr; 1 ml of gel could easily bind 0.02 mmol of polynucleotides, and loadings as high as 0.05 mmol/ml wet gel were sometimes achieved.

Melting Curves. All polynucleotide·oligonucleotide melting curves were done in a Cary 15 spectrophotometer with a temperature-controlled cuvet holder. At low temperatures, the sample compartment was flushed with dry nitrogen to prevent condensation. Melting in solution was monitored by uv absorbance at the wavelength showing maximum hypochromicity. Such melting curves were found to be accurately reversible if the temperature was increased or lowered at 1°/min.

Melting the complexes on solid supports is a novel technique and deserves some detailed description. Approximately 0.1 ml of resin or gel carrying a known amount of polynucleotide was put in a 3-ml cuvet and 3 ml of the complementary oligonucleotide at the appropriate concentration was added. As will be explained under Results, the salt concentration was always such that the polynucleotide was ionically bound to the support but the oligomer was not. The solution was stirred with a fine stream of water-saturated nitrogen bubbles so that the support particles remained suspended. The cuvet was cooled down to the starting temperature, and 1 hr was allowed for the formation of the hydrogen-bonded complex to reach an equilibrium. Then the bubbling was stopped, the solid allowed to settle, and the uv spectrum of the supernatant recorded. Next, the temperature was raised a few degrees with the resumption of the nitrogen bubbling. After a suitable equilibration period, bubbling was interrupted, and another spectrum was recorded. This procedure was continued until the complex had melted completely.

Preliminary kinetic studies indicated that equilibration at each temperature required at least 20 min with the gel and at least 35 min with the resin. The absorbance at the peak position of the spectra was plotted as a function of temperature to yield a melting curve. It should be noted that in this procedure, the spectrophotometer measures only the chromophoric material in the supernatant, *i.e.*, the oligonucleotide that has been melted off the polynucleotide-resin. The absorbance readings plotted are therefore at the wavelength at which the oligomer has its maximum absorption. Parallel lines were drawn through the low and high temperature plateau regions in the melting curve, and T_m is defined as the temperature located midway between these two lines. Our experience indicated that the accuracy of the T_m determination was about ±0.2°.

Results

The formation of hydrogen-bonded oligonucleotide·polynucleotide complexes on ion exchangers can be shown only under conditions in which the polynucleotide is bound

[1] Amberlite IRA-938 will hereafter be referred to simply as resin and aminoethyl Bio-Gel P-30 as gel.

[2] Moles of polynucleotides and oligonucleotides refer to base residues throughout the paper.

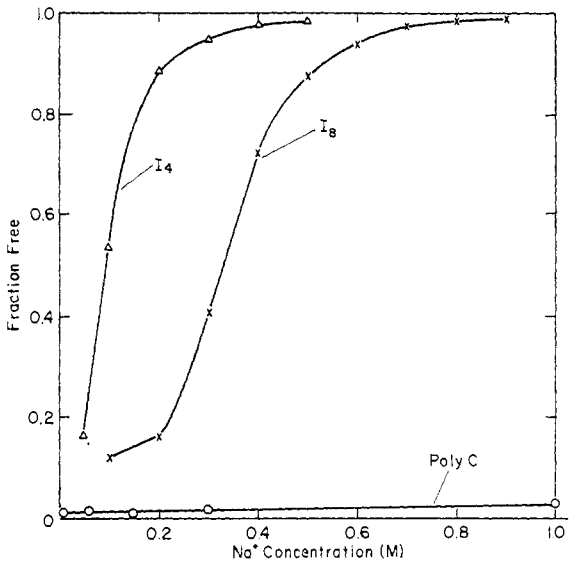

FIGURE 1: Binding of I_4, I_8, and poly(C) to aminoethyl Bio-Gel as a function of Na^+ concentration, room temperature, pH 7.2.

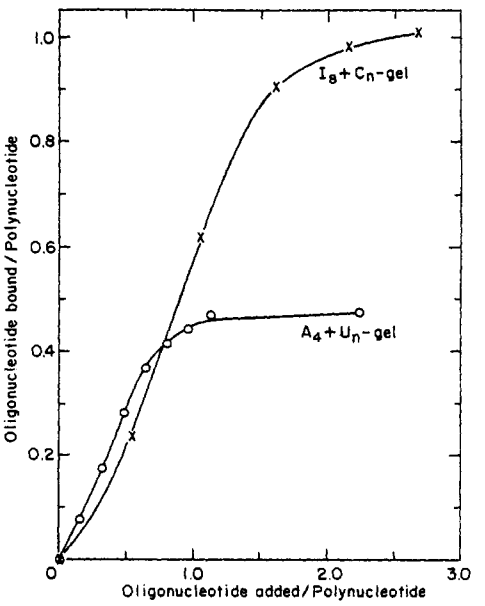

FIGURE 2: Absorption isotherms for binding oligonucleotides to their complementary polynucleotide-gels in 0.7 M NaCl and 0.05 M Tris (pH 7.2) at 4°.

ionically while the oligomer is not. Figure 1 shows the elution of I_4 and I_8 (about 3 ODU (248 nm) each) from the gel (0.1 ml of wet gel) as a function of NaCl concentration at room temperature. The gel was stirred in 3 ml of 0.05 M Tris (pH 7.2). After each addition of concentrated NaCl solution, 20 min was allowed for equilibration; 95% of I_4 is eluted at 0.3 M NaCl, and 95% of I_8 is eluted at 0.63 M (Figure 1). In 1.0 M NaCl, C_n is still completely bound by the gel; it can be eluted nearly totally in 1.5 M salt. Thus, a sodium chloride concentration of 0.75 M was chosen as a suitable condition of ionic strength for studying oligonucleotide·polynucleotide association on the gel.

With the resin, considerably higher ionic strength was required to prevent the ionic binding of oligonucleotides. Polynucleotides were eluted only at very high salt concentrations (over 2 M). A_n, in fact, could not be eluted with NaCl from the resin because it precipitates at a salt concentration lower than that required for elution. For the purpose of quantitative evaluation of the amount of polynucleotides bound to the resin, A_n could be hydrolyzed inside the resin with alkali. Similarly, U_n and C_n could be removed in digested form by either alkaline or pancreatic RNase hydrolysis. The mononucleotides are eluted at relatively low salt concentration. I_4, I_6, and I_8 are all eluted from the resin by 1.5 M NaCl, which was chosen as the standard condition for oligomer–polymer associated studies on the resin.

To demonstrate specific association of oligo(I) with poly(C) on the resin, 3 ml of I_6 or I_8 solution having an OD (248 nm) of 1.0 was stirred in the cold room (3°) with 0.1 ml of resin previously loaded with poly(C) (C_n-resin) in 1.5 M NaCl and 0.01 M phosphate buffer (pH 7.3). The absorbance of the supernatant dropped nearly to zero as the oligo(I) was bound to the poly(C) on the resin. The association was shown to be base specific; poly(U) resin (U_n-resin) did not bind oligo(I). Binding of A_4 to gel loaded with poly(U) (U_n-gel) in 0.7 M NaCl–0.05 M Tris (pH 7.2) was demonstrated in a similar experiment.

There was some problem with unspecific binding of the oligonucleotides to resin, presumably by stacking of the base rings on the aromatic groups of the Amberlite. This binding was not ionic, since the nucleosides inosine and adenosine as well as the neutral dimer dAp(Et)dA were bound to the unloaded resin to a certain extent. Adenosine was bound slightly more than inosine, probably because of the greater stacking tendency of the former. This kind of binding was not encountered with the gel. In the resin experiments with longer oligomers, unspecific binding turned out to be less of a problem. Intramolecular stacking of the bases in the oligomer appeared to hinder stacking on the resin. Binding of I_6 to the resin is more extensive at room temperature than at 0°. This difference undoubtedly reflects the greater rigidity of I_6 at 0° due to self-stacking. Under the conditions used for studying oligomer·polymer associations on the resin, the unspecific binding amounted to less than 10% of specific binding.

Experiments to determine the stoichiometry of the oligonucleotide·polynucleotide associations are illustrated in Figure 2. The C_n-gel carried 0.3 μmol of C_n in 0.1 ml of gel, and the U_n-gel contained 0.5 μmol of U_n in 0.1 ml of gel. The gel was stirred in a 3-ml solution containing 0.7 M NaCl and 0.05 M Tris (pH 7.2). The oligonucleotide was added stepwise in concentrated solution. After each increase in oligomer concentration, the cuvet was chilled in ice for 5 min and stirred in the cold room for 30 min. Results in Figure 2 demonstrate that I_8 forms a 1:1 complex with C_n-gel and A_4 forms a 1:2 complex with U_n-gel. With the resin, similar stoichiometry experiments revealed additional complexity. Only about 40% of the residues in C_n on C_n-resin appeared to be available for binding I_8. Perhaps the rest of the C_n was bound in regions of the resin where there is insufficient room for the $I_8 \cdot C_n$ complex to form.

Melting curves on solid supports were done as described under Methods. Figure 3 shows melting curves obtained with I_8 and C_n in solution and on the resin. Note that the absorbance in (b) starts nearly at zero; below 5°, nearly all the I_8 is complexed with the C_n-resin. The T_m of the oligo(I)·C_n complex on the resin was found to be 2° higher than that in solution. This was about the average amount of stabilization observed with the supports in a number of experiments summarized in Table I. With the series A_np (n = 4–6) and U_n-gel, there is a slight increase in stabilization with increasing oligonucleotide chain length.

The T_m of $I_8 \cdot C_n$-resin was investigated as a function of oligonucleotide concentration. The results showed considera-

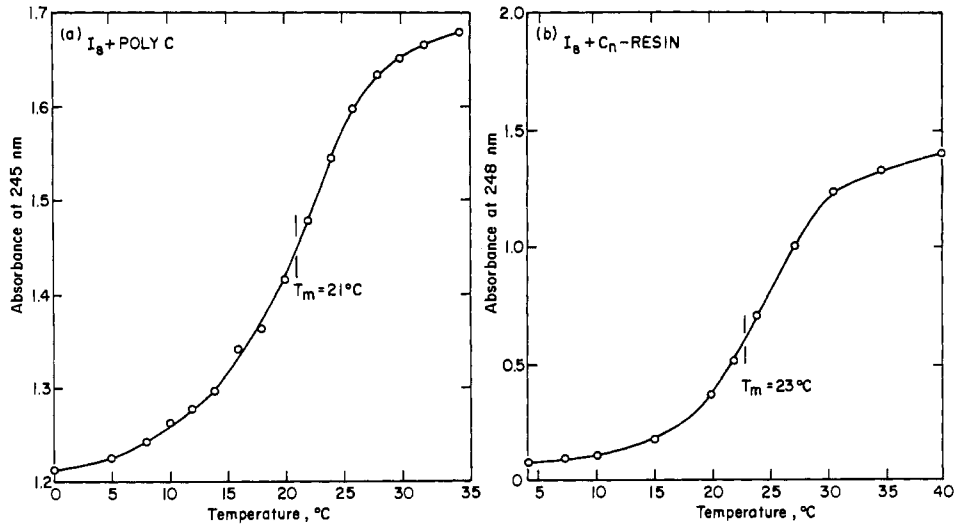

FIGURE 3: Comparison of $C_n \cdot I_8$ melting in solution and on the resin. Solutions contained 1.5 M NaCl and 0.01 M K_2PO_4 (pH 7.3): (a) melting in solution; (b) melting on resin.

ble scattering because of the difficulty in acquiring precise measurement of the melting curves on the resin. A plot of $1/T_m$ *vs.* the natural logarithm of the free oligomer strand concentration at T_m could be fitted by a straight line with the same slope as that obtained previously in solution containing 0.01 M Mg^{2+} (Tazawa *et al.*, 1972). Since the slope of this line is determined by the enthalpy of association, ΔH of the oligo(I)·poly(C) complex formation on the resin must be close to that in solution determined in our laboratory, *i.e.*, −6.2 kcal/mol per base pair (Tazawa *et al.*, 1972). This finding suggests that the observed stabilization could be due to an entropy advantage (the $T\Delta S$ term) acquired on the surface of the resin. However, in view of the small difference in stability (2°), no definite conclusion can be made.

In 0.75 M salt, U_n is bound to the gel, but the binding is probably loose and thus allows the polymer considerable freedom of motion. It seemed reasonable that the entropy advantage conferred by the solid support in the immobilization of the polynucleotide could be increased by reducing the salt concentration. Lower ionic strength, however, requires using shorter oligonucleotides which would not be bound to the gel ionically at this salt concentration. With A_4, the ionic strength of the binding experiment can be reduced to 0.3 M NaCl. As seen in Table I, the stability of the $2U_n \cdot A_4$ complex in 0.75 M NaCl is almost the same in the gel (0.3° higher) as that in solution. Reduction of the salt concentration from 0.75 to 0.3 M decreased the T_m as expected but also produced a larger difference in T_m (1.4°, Table I) between the complex in the gel *vs.* that in the solution. This larger difference in T_m at lower ionic strength may be partially due to the charge neutralization of the positively charged gel, however. For instance, the difference in T_m for $A_4p \cdot 2U_n$ in gel *vs.* that in solution in 0.75 M NaCl is 1.8° instead of the 0.3° observed for $A_4 \cdot 2U_n$ in gel *vs.* solution (Table I). The positively charged gel may exert a larger influence on the stability of the oligomer·polymer complex which has a higher ratio of negative charge/mass.

Ionic strength not only influences the binding of the oligonucleotide·polynucleotide complexes to the solid phase but

TABLE I: Comparison of Melting Temperatures of Oligonucleotide·Polynucleotide Complexes in Solution and on a Solid Support.[a]

System	Ionic Strength	T_m (°C) Support	T_m (°C) Solution	ΔT_m (°C)
$I_8 + C_n$-resin	1.5	23	21	2
$I_8 + C_n$-gel	0.75	22	20	2
$A_4p + 2U_n$-gel	0.75	25.4	23.6	1.8
$A_5p + 2U_n$-gel	0.75	35.0	32.8	2.2
$A_6p + 2U_n$-gel	0.75	41.8	39.1	2.7
$A_4 + 2U_n$-gel	0.75	30.6	30.3	0.3
$A_4 + 2U_n$-gel	0.30	24.7	23.3	1.4

[a] Oligonucleotide concentrations were always the same in corresponding support and solution melting experiments. Except for the experiment with C_n-resin, polynucleotide to oligonucleotide ratios were always 1:1 for poly(C) and oligo(I) and 2:1 for poly(U) and oligo(A). In the resin experiment, a threefold excess of C_n-resin was required to bind all the I_8. Residue concentrations of oligo(I) and poly(C) were 1.2×10^{-4} M. In the series A_4p, A_5p, and A_6p, a constant oligomer strand concentration of 10^{-5} M was used. A_4 had a residue concentration of 6×10^{-5} M.

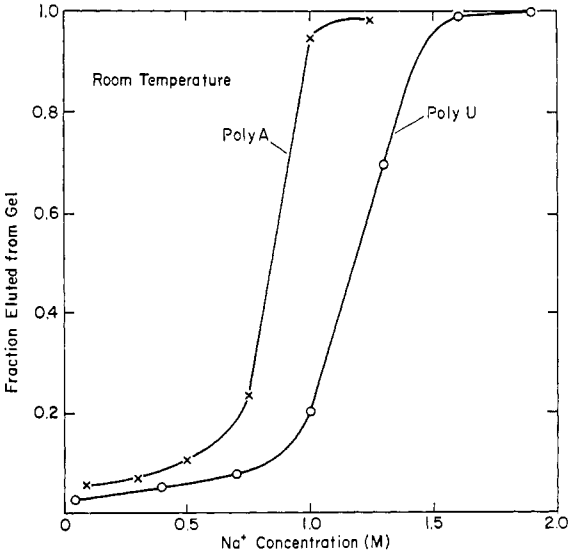

FIGURE 4: Elution of A_n and U_n from the gel by NaCl, room temperature, pH 7.2.

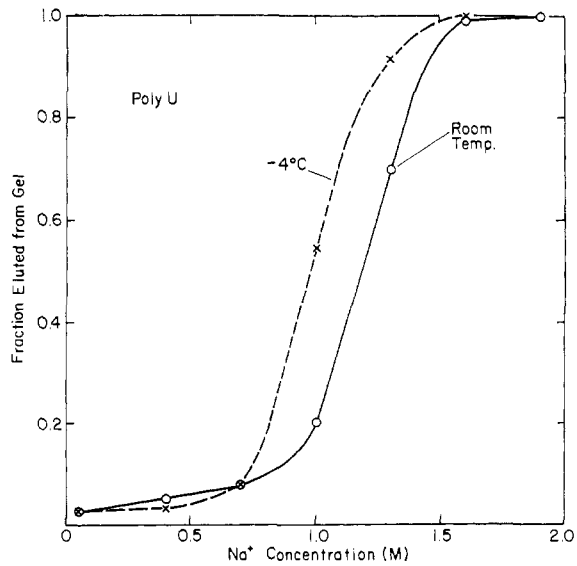

FIGURE 5: Elution of poly(U) from gel by NaCl at −4° and at room temperature.

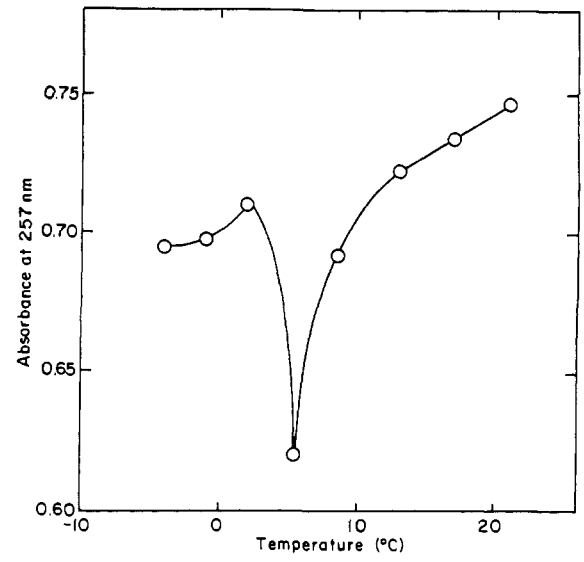

FIGURE 6: Binding of A_2 to U_n-gel as a function of temperature in 0.7 M NaCl–0.05 M Tris (pH 7.2).

also affects the conformation of the polynucleotides. It is important to evaluate the effect of polynucleotide conformation on its interaction with the solid phase at a given ionic strength. In Figure 4, the elution of A_n and U_n from the gel at room temperature by varying concentration of NaCl is shown. The experiment was done essentially the same way as those shown in Figure 1. A_n was eluted from the gel at a lower concentration of NaCl (near completion at 1.0 M) than the poly(U) (near completion at 1.5 M). Since the A_n is known to be much more highly stacked in a strong salt solution than poly(U) (Leng and Felsenfeld, 1966; Thrierr *et al.*, 1971) this experiment suggests that the polynucleotides which have a more stacked, rigid structure would be likely to have a lower affinity toward the gel. This effect may be similar to the aforementioned observation on the unspecific binding of I_6 to the resin which is lower at 0° than at room temperature. To test this reasoning further, the elution of U_n was compared between −4° and room temperature (Figure 5). The data clearly indicate that U_n is eluted from the gel at −4° by a NaCl concentration 0.2 M lower than that at room temperature. U_n is known to form a hairpin helix (Thrierr *et al.*, 1971) in solutions of high ionic strength at low temperature. Study on the binding of $A_n \cdot U_n$ to the gel turned out not to be fruitful due to a very slow kinetic situation and to the precipitation of the helical complex in the gel at high salt concentration. These experiments do indicate that the affinity of the polynucleotides for the gel may be substantially diminished when the polynucleotide assumes a greater degree of secondary structure and a more rigid conformation. This conclusion is amply supported in the following experiments on the binding of the $A_2 \cdot U_n$ complex to the gel.

A comprehensive study on the formation of the $A_2 \cdot U_n$ complex on the gel has provided much information about the influence of conformation on binding to a solid support. A melting experiment of A_2 and U_n-gel in 0.75 M salt is shown in Figure 6. At this ionic strength U_n is bound to the gel (Figure 5) while A_2 is not unless it is complexed with poly(U). The maximum absorbance attainable in this experiment was 1.8, with a contribution of 1.1 from U_n and 0.7 from A_2 under noninteracting conditions. The absorbance (257 nm) value of nearly 0.7 measured at −4° (Figure 6) indicates that the U_n at this temperature was bound to the gel without formation of complexes with A_2 which was left in solution. This result was unexpected since the complex $2U_nA_2$ was found to have a T_m of 7.6° in solution under the same conditions. The unusual "melting curve" in Figure 6 shows first a small increase with increasing temperature followed by a decrease to a minimum representing about 15% binding of A_2. After reaching a minimum in absorbance, the curve rises again. This portion of the curve resembles the later stage of a normal melting curve. In 0.75 M Na^+ solution, the T_m of a self-complexed U_n helix is 3.8°, while the T_m of $A_2 \cdot 2U_n$ is 7.6°. The results in Figure 6, however, suggest that at a temperature (−4°) below both T_m values, U_n is bound to the gel in such a form that it is unavailable for binding A_2. From the present data, it is not known whether or not U_n on the gel surface is in the form of a double-stranded, hairpin helix which is the helical conformation of U_n in solution. As the temperature is increased, this covered structure of U_n becomes partially melted, but the U_n remains in the gel at this ionic strength (Figure 5), and thus becomes available for binding A_2. This is probably the explanation for the dip in absorbance at 5.5° observed in Figure 6. The slight increase of absorbance from 11 to 20° is most likely due to the decrease of hypochromicity of A_2 in solution upon unstacking at higher temperature.

This interesting phenomenon was further investigated with the neutral deoxyadenosine dinucleoside monophosphate ethyl phosphotriester (dAp(Et)dA). This neutral dimer forms a 1A–2U complex with U_n readily in salt solution with a T_m significantly higher than that of the $dA_2 \cdot 2U_n$ complex (Miller *et al.*, 1971). This neutral dimer, however, does not bind ionically to the gel even at very low salt concentration as anticipated. The T_m values of the dAp(Et)dA·2(U)$_n$ complex in various salt solutions are shown in Table II; these values are useful for the comparative study of this complex on the gel. It is interesting to note the high T_m value of this complex (19.7° in 0.2 M $MgCl_2$) and the fact that the T_m in 0.01 M $MgCl_2$ is actually higher than that in 0.01 M $MgCl_2$ + 0.75 M NaCl. This effect which has been observed previously (Lipsett *et al.*, 1961) indicates that the replacement of the Mg^{2+} ion bound to the complex by Na^+ reduces the stability of the complex.

Table III shows the results of several attempts to form a complex between dAp(Et)dA and U_n-gel under various conditions. At 4°, there was no evidence of any binding of dAp(Et)dA to U_n-gel either in 0.01 M $MgCl_2$ or 0.75 M NaCl even

TABLE II: Melting Temperatures of $2U_n \cdot dAp(Et)dA$ in Solution.[a]

Salt	T_m (°C)
1.0 M NaCl	9.5
0.01 M $MgCl_2$	12.0[b]
0.75 M NaCl + 0.01 M $MgCl_2$	9.5
0.2 M $MgCl_2$	19.7

[a] dAp(Et)dA was used at a residue concentration of 5×10^{-5} M, and the poly(U) was 10^{-3} M. Solutions contained 0.05 M Tris (pH 7.2). [b] This result is from Miller *et al.* (1971). In this experiment, total nucleotide concentration (both components) was 5×10^{-5} M.

though this temperature is considerably below the T_m (12 and 9.5°, Table II) of the helical complex in solution in these salt solutions. At a lower temperature (−4°) and higher salt concentration (1 M NaCl, with and without 0.02 M $MgCl_2$), the absorbance of the supernatant actually became higher than the input absorbance of dAp(Et)dA, indicating that elution of poly(U) from the gel began to take place. This is perhaps expected in view of the elution profile shown in Figure 5, in which about 50% of U_n can be eluted from the gel at −4° in 1.0 M NaCl. Only when more salts were added at 4° was a partial binding of dAp(Et)dA to U_n-gel observed as indicated by the decrease of the original input absorbance. It should be noted that the binding of dAp(Et)dA to U_n-gel does not correlate with the T_m values of the $dAp(Et)dA \cdot 2U_n$ complex in the corresponding salt solutions as shown in Tables II and III. The T_m of the solution complex in the medium where the dAp(Et)dA *did not bind* to the U_n-gel (0.01 M $MgCl_2$, T_m = 12°; 0.75 M NaCl, $T_m \geq 9.5°$) can be larger, equal to, or smaller than the T_m of the solution complex in the medium where dAp(Et)dA *partially binds* to the U_n-gel (0.75 M NaCl + 0.01 M $MgCl_2$, T_m = 9.5°; 0.15 M $MgCl_2$, $T_m \leq 19.7°$). It seems clear that the covered form of U_n on the gel surface at low temperature and moderate ionic strength can be made available for binding A_2 (or dAp(Et)dA) by increasing either the temperature (Figure 6) or the salt concentration (Table III). It is interesting to note that Mg^{2+} is nearly 100-fold more effective than Na^+ in raising the T_m of the complex in solution but only about fourfold as effective in eluting polynucleotides from the gel. The stabilization must involve a specific interaction with Mg^{2+}, whereas the increased eluting power is probably simply due to the greater ionic strength resulting from divalent ions. This difference allowed us to increase the stability of the complex with Mg^{2+} without elution of the polymer. This is the reason for the choice of salt concentrations in Tables II and III.

In order to investigate further the effect of salt concentration on the binding of dAp(Et)dA to the U_n on the gel surface, an experiment with $[^3H]U_n$-gel was done at −4° (Figure 7). This experiment is also useful for providing more details confirming one of the experiments (1.0 M NaCl, −4°) in Table III. This experiment can be started from a very low salt concentration (0.05 M), since the neutral dAp(Et)dA does not bind to the gel without complex formation with U_n in the gel. The salt concentration was increased by sequential addition of an appropriate volume of 5 M NaCl followed by an equilibration period of 45 min with stirring by bubbling nitrogen. Then the gel particles were allowed to settle and the absorbance of the supernatant and the radioactivity in a 50-μl

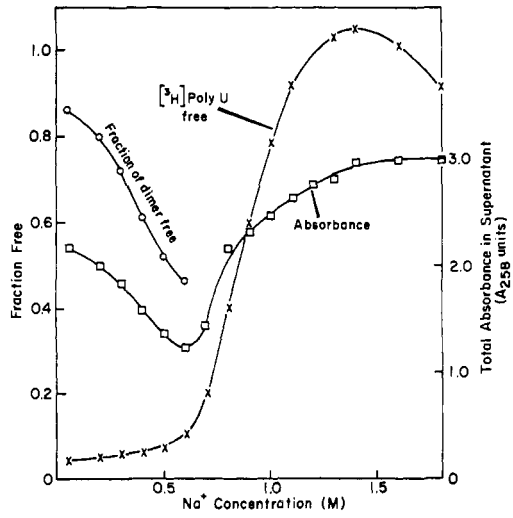

FIGURE 7: Dependence of $2U_n$-gel·dAp(Et)dA formation on Na^+ concentration in 0.05 M Tris (pH 7.2) at −4°.

sample were measured. This procedure was repeated after each salt addition throughout the experiment. After an appropriate volume and concentration adjustment, the data are presented in Figure 7.

In the region of 0.05–0.6 M salt, the absorbance in the supernatant decreases as the salt concentration increases (Figure 7). All the $[^3H]U_n$ was found to remain in the gel. This decrease in absorbance in the supernatant reflects the binding of dAp(Et)dA to the U_n in the gel. From the curve denoted by "Fraction of dimer free" in Figure 7, about half of the dAp(Et)dA was bound to the U_n-gel at −4°, 0.6 M NaCl. As the salt concentration is increased to 0.7 M and higher, the absorbance begins to increase and the elution of $[^3H]U_n$ from the gel is clearly indicated by the radioactivity measurement. Under this condition, it is no longer possible to calculate accurately the portion of dAp(Et)dA remaining free outside the gel because of various considerations, such as the hypochromicity of the $dAp(Et)dA \cdot 2U_n$ complex, etc. (Thus, the curve denoted by "Fraction of dimer free" is terminated at 0.6 M salt in Figure 7.) At 0.85 M NaCl, about half of the $[^3H]$-U_n is eluted from the gel, confirming the observation listed in Table III about the experiment conducted at −4°, 1.0 M NaCl. The anomalous decrease in the free $[^3H]U_n$ curve above 1.4 M Na^+ is due to interference with the liquid scintillation

TABLE III: Experiments on Forming a Complex between dAp(Et)dA and U_n-Gel.[a]

Temp (°C)	Salt	Fraction of Dimer Bound
4	0.01 M $MgCl_2$	0
4	0.75 M NaCl	0
−4	1.0 M NaCl	Increase in absorbance in solution indicating elution of U_n
−4	1.0 M NaCl + 0.02 M $MgCl_2$	
4	0.75 M NaCl + 0.01 M $MgCl_2$	0.23
4	0.15 M $MgCl_2$	0.18

[a] In these experiments, 0.1 ml of gel bearing 0.3 μmol of U_n was stirred with 2.9 ml of dAp(Et)dA solution having a residue concentration of 5×10^{-5} M.

counting by the large amount of salt in the samples. It should be noted that the data in Figure 5 indicate that about 1.0 M NaCl is required to elute 50% of the U_n from the gel at −4° in the *absence* of dAp(Et)dA. This observation suggests that complex formation of dAp(Et)dA with poly(U) facilitates the elution of U_n from the gel. One is tempted to interpret the data in Figure 7 to mean the T_m of dAp(Et)dA·U_n-gel at 0.6 M NaCl is −4°, which is much below the estimated T_m (~5°) of the dAp(Et)dA·$2U_n$ complex in 0.6 M NaCl solution. However, this is not a correct interpretation, since the data in Figure 6 concerning the rA_2·U_n-gel complex would indicate a T_m of 5–6° at 0.7 M NaCl by the same reasoning. It is known that the dAp(Et)dA·$2U_n$ complex has a slightly higher T_m than rA_2·$2U_n$ complex in solution (Miller *et al.*, 1971; Tazawa *et al.*, 1970). This inconsistency clearly indicates that the binding of dAp(Et)dA to U_n-gel is strongly influenced by the interaction between the U_n and the gel, and this U_n-gel interaction is both temperature and salt dependent. Thus, the binding data of dAp(Et)dA to U_n-gel cannot be interpreted simply from the viewpoint of the stability of the dAp(Et)dA·U_n complex.

Discussion

The above study on the formation of oligonucleotide·polynucleotide complexes on the charged surface of solid supports reveals two basic factors for consideration. The first factor is the contribution of the ion-exchange groups of the support to the local ionic strength, resulting in a higher stability of the complex. This factor probably is not important when the salt concentration of the solution is sufficiently high (0.75–1.5 M), though the stabilization of the complex by the support as shown in Table I may still arise partially from this contribution. Niyogi and Thomas (1968) found that complexes of oligo(A) and poly(U) on hydroxylapatite were stabilized about 2–3° higher relative to solution in 0.3 M NaCl–0.02 M sodium phosphate buffer (pH 6.8). They attributed this added stability to an increase in the local ionic strength of the buffer near the hydroxylapatite surface. In addition, they suggested that perhaps the hydroxylapatite crystal surface provides a pattern of interacting groups that stabilized the polynucleotide duplex.

The second factor concerns the structural aspects of the charged groups on the solid support *vis-à-vis* the structure of the complex and of the individual components in the complex. On the one hand, this structural factor can enhance the stability of the complex by a partial immobilization of the components in the complex, thereby reducing the loss of entropy upon complex formation. The enhancement of the T_m of the complex on solid support *vs.* that in free solution reported in Table I and Figure 3 may reflect this contribution. Besides the supportive evidence about this stabilizing effect cited in the introduction, Martinson (1973) has observed marked stabilization of native DNA by hydroxylapatite under conditions favoring binding of the DNA to the support. He believes that the hydroxylapatite interferes with the unwinding process required by denaturation. On the other hand, the charged groups on the surface of the support can also interfere with the formation of the complex by forcing the polynucleotide to assume a conformation unfavorable for binding the complementary oligonucleotides. Our study also provides clear evidence for such a phenomenon. When the oligo(I) and polynucleotides assume a more rigid and stacked conformation, they have a lower affinity toward the support. Similarly, U_n in the dAp(Et)dA·$2U_n$ complex also has a lower affinity for the gel than does free U_n. At a low temperature and moderate to low ionic strength, the U_n is bound to the gel in such a "covered" form that it becomes unavailable for binding A_2 or dAp(Et)dA. Such a "covered" form of U_n on the gel surface can be melted by increasing the temperature or the salt concentration, conditions which lead to less immobilization of the polynucleotides on the charged surface.

Adler and Rich (1962) studied polynucleotide–polynucleotide association on cellulose columns in which one of the polynucleotides was covalently linked to the support. They found that the complex of U_n with an A_n column had a T_m 14° lower than the complex in solution and also displayed a broader thermal transition. They explained this difference by the fact that the A_n was firmly attached to the cellulose at several places so that only shorter segments of A_n were left to form a complex with U_n.

This work and reports in the literature clearly indicate that the interaction of a solid support with polynucleotides can *stabilize* or *destabilize* complexes formed with oligomers or with other polymers depending on the conditions (salt concentration, temperature, etc.) and the structural features of the surface of the support relative to the conformation of the polynucleotides and the complex. More knowledge about this interaction may allow us to construct experimental systems for such particular advantages. One such possibility is to build a solid support system for an efficient polynucleotide-directed condensation of oligonucleotides. Such studies are currently in progress in our laboratory.

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Synthesis and Properties of Adenosyl- and Methylepicobalamin†

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ABSTRACT: Adenosyl-13-epicobalamin and methyl-13-epicobalamin have been prepared by reacting 13-epicob(I)alamin with 5′-*p*-toluenesulfonyladenosine and methyl iodide, respectively. The isomerization of the cobalamins at carbon-13 in trifluoroacetic acid is affected by the nature of the upper axial ligand. Treatment of methylcobalamin in trifluoroacetic acid does not yield the expected methyl-13-epicobalamin and reaction of 13-epicob(I)inamide with methyl iodide does not give an equimolar mixture of methylaquo-13-epicobinamide and aquomethyl-13-epicobinamide. The electronic spectra, circular dichroism, and optical rotatory dispersion of adenosyl-13-epicobalamin and methyl-13-epicobalamin are distinct from those of the cobalamins. Like the corresponding cobalamins, the two epicobalamins are converted to their "base off" forms in acid; however, the pK_a values for the 5,6-dimethylbenzimidazole moiety are lower (2.8 and 2.2) than those of the cobalamins (3.5 and 2.7). These lower pK_a values suggest that the inversion of the propionamide side chain at carbon-13 affects the electronic character of the cobalt atom. Adenosyl-13-epicobalamin does not function as a coenzyme in the ribonucleotide reductase system of *Lactobacillus leichmannii*, and acts as an inhibitor when incubated with adenosylcobalamin. Adenosyl-13-epicobalamin, methyl-13-epicobalamin, cyano-13-epicobalamin, and aquo-13-epicobalamin do not form active holoenzyme when incubated with N^5-methyltetrahydrofolate–homocysteine cobalamin methyltransferase apoenzyme from *Escherichia coli* B. However, these four epicobalamins were found to inhibit active holoenzyme formation with methylcobalamin.

When cyanocobalamin is treated with trifluoroacetic acid, or other highly acidic reagents, a mixture of cyanocobalamin, cyanocobinamide, and two darker colored corrinoids is formed (Bonnett *et al.*, 1971). Similar corrinoids are formed when corrinoid carboxylic acids such as cobyric acid are dissolved in strong acid. These new corrinoids are virtually indistinguishable from the corrinoids on the basis of electrophoretic behavior or ir spectra; while the electronic spectra show small but significant differences. On the other hand, their chiroptical and chromatographic properties are distinctly different.

The structure of these new corrinoids was elucidated by X-ray analysis. The X-ray data established that the propionamide side chain e attached to C-13 is projected up instead of down relative to the plane of the corrin ring. This inversion of configuration at C-13 causes a change in the conformation of ring C, while the rest of the molecule is not significantly altered (Stoeckli-Evans *et al.*, 1972). Scott *et al.* (1973) observed a downfield shift of one of the methyl groups at C-12 in the ^{13}C nuclear magnetic resonance spectrum (nmr) of dicyano-13-epicobinamide confirming the anticlinal relationship of this methyl group to the propionamide side chain at C-13. Hodgkin (Stoeckli-Evans *et al.*, 1972) has pointed out that in adenosylcobalamin the 5′-deoxyadenosyl moiety lies directly above C-13, a position which is occupied by the propionamide side chain in the 13-epicobalamins and thus the adenosyl moiety in adenosyl-13-epicobalamin must be positioned differently. On the other hand, the methyl moiety of methyl-13-epicobalamin would not be expected to be sterically hindered.

Because microbiological assays with *Escherichia coli* showed that cyano-13-epicobalamin is about 10% as active as cyanocobalamin (Bonnnett *et al.*, 1971) we decided to synthesize the two coenzyme forms of 13-epicobalamin and to determine their biological activity. This paper describes the synthesis of adenosyl-13-epicobalamin and methyl-13-epicobalamin. The physical and chemical properties as well as their coenzymatic activities are also reported.

Materials and Methods

Materials. Cyanocobalamin and DL-N^5-methyltetrahydrofolate were obtained from Sigma Chemical Co., DL-N^5-methyl-[^{14}C]tetrahydrofolate was from Amersham/Searle, *S*-adenosyl-L-methionine chloride was from P-L Biochemicals, 5′-*O*-(*p*-tolylsulfonyl)adenosine was from Zellstofffabrik Waldhof, and [^{13}C]methyl iodide, 61.8% enriched, was from Prochem. Ribonucleotide reductase from *Lactobacillus leichmannii* was kindly supplied by Dr. R. L. Blakley and N^5-

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