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Studies on the Interactions of Nucleotides, Polynucleotides, and Nucleic Acids with Dihydroxyboryl-Substituted Celluloses†

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ABSTRACT: Nucleotides and polynucleotides which contain a free 2′,3′-diol group are capable of forming specific complexes with the two cellulose derivatives: *N*-(*m*-dihydroxyborylphenyl)carbamylmethylcellulose and *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose. In chromatography on columns prepared from these celluloses the retention volume of a particular mono- or polynucleotide depends on (i) the pH of the elution solvent, (ii) the ionic strength and the nature of the cations in the elution solvent, (iii) the number of negative charges in the vicinity of the diol group, and (iv) the nature of the nucleotide bases in the vicinity of the diol group. In the case of those polynucleotides which do not possess a 2′,3′-diol group a method has also been devised for the binding of these molecules to the substituted celluloses. The method involves the derivatization of the terminal phosphate group of the polymer with sorbitol or *N*-methylglucamine. The incorporated polyhydroxy group then serves as the moiety which undergoes complex formation with the cellulose-bound dihydroxyboryl groups. These methods can be applied to the study of the primary structure of nucleic acids and to the development of procedures for the chemicoenzymatic synthesis of polynucleotides of defined sequence.

Ribonucleosides and certain sugars and other polyols have been shown to form specific complexes with two new cellulose derivatives which contain covalently bound dihydroxyboryl groups (Weith *et al.*, 1970). One of the derivatives, *N*-(*m*-dihydroxyborylphenyl)carbamylmethylcellulose was prepared by reaction of the azide of carboxymethylcellulose with *m*-aminobenzeneboronic acid while the other, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose was obtained by the condensation of *N*-(*m*-dihydroxyborylphenyl)succinamic acid with aminoethylcellulose in the presence of *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)-ethylcarbodiimide. The complexes formed with these cellulose derivatives are thought to consist of cyclic structures resulting from the reaction of a pair of hydroxyl groups on the polyol (the 2′,3′-diol groups in the case of ribonucleosides) with the dihydroxyboryl groups on the cellulose. Accordingly, these complexes are considered to be analogous to those structures known to be formed by certain polyols in aqueous borate solutions. In anticipation of the use of these new cellulose derivatives in chemical studies on nucleic acids the present work was directed to the investigation of the various factors which might affect the complex-forming capacity of nucleotides, polynucleotides, and nucleic acids, and a preliminary report of the results has been published (Rosenberg *et al.*, 1970).

Nucleotides. A number of nucleosides and nucleoside mono-, di-, and triphosphates have been chromatographed at pH 7.5 on columns of DBCM-cellulose[1] and their retention volumes measured. A comparison of these volumes in Table I shows that there are substantial differences in the degree of binding to the cellulose exhibited by the nucleotides and their parent nucleosides. For example, while there is a moderate amount of binding of the nucleoside, uridine, in the presence of the dilute buffer solution, there does not appear to be any complex formation between the cellulose and the various phosphate derivatives of this nucleoside since, under the same conditions, these derivatives can be eluted in volumes equal to the void volumes of the columns. However, the inclusion of 1 M sodium chloride in the elution solvent

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[1] Abbreviations used are: DBCM-cellulose, *N*-(*m*-dihydroxyborylphenyl)carbamylmethylcellulose; DBAE-cellulose, *N*-[*N*′-(*m*-dihydroxyborylphenyl)succinamyl]aminoethylcellulose.

TABLE I: Retention Volumes of Nucleosides and Nucleotides on Columns of DBCM-Cellulose.[a]

Compound	Volume (ml)			
	Buffer	Buffer + 1 M NaCl	Buffer + 0.1 M $MgCl_2$	Buffer + 1 M NaCl–0.1 M $MgCl_2$
Cytidine	15.3	36.4	40.0	
Uridine	15.7	42.0	41.0	44.0
Adenosine	31.0	136.0	121.0	
Uridine 3′,5′-diphosphate	8.3	10.1	10.5	
Uridine 5′-phosphate	8.4	18.0	23.8	
Uridine 5′-diphosphate	8.3	15.2	28.1	
Uridine 5′-triphosphate	8.3	13.3	24.3	25.0
Adenosine 5′-phosphate		32.1	49.0	
Adenosine 5′-diphosphate		24.4	59.3	
Adenosine 5′-triphosphate		19.2	44.6	

[a] The columns had dimensions 40 × 0.6 cm, and the elutions were carried out at 20° and at a flow rate of about 3 ml/hr. Each elution solvent contained the buffer, 0.05 M *N*-methylmorpholinium chloride, and the final pH of each solvent was adjusted to 7.5 at 20°. The retention volumes were taken as the volumes of each solvent required to elute the sample and were measured from the point of addition of the sample at the top of the column to the point of its peak concentration eluting from the bottom.

causes a considerable increase in the retention volumes of all ribonucleosides and ribonucleotides. This effect has been observed before in the case of the nucleosides (Weith *et al.*, 1970) and the explanation given at that time was dependent on the assumption that the tetrahedral boronate anion, $-Ph[B(OH)_3]^-$ is the active species in complex formation. It was suggested that the p*K* for the process

$$-PhB(OH)_2 + H_2O \rightleftarrows -Ph[B(OH)_3]^- + H^+$$

would depend, to some extent, on the amount of salt in the elution solvent. The p*K* would be expected to increase in the presence of low salt concentrations due to the field effects of the negative charges of the residual carboxyl groups on the cellulose together with field effects arising from the negatively charged boronate anions themselves. On the addition of high-salt concentrations however, the p*K* of the ionization should tend to decrease due to the shielding of these charges on the surface of the cellulose. Thus, at a fixed pH, an increase in salt concentration should increase the concentration of boronate anions with a resulting increase in retention volumes. In addition, the different degree of binding displayed by the various nucleosides was attributed to the superimposition of other binding forces, such as H bonding, which have been observed before in the chromatography of nucleic acid components on other cellulose derivatives.

While it is reasonable to assume that these considerations should also apply to the phosphate derivatives of nucleosides it is clear from Table I that, in these cases, there is a further factor affecting the degree of complex formation. In the presence of 1 M sodium chloride the nucleotides are bound less strongly than their parent nucleosides, and, in addition, it appears that the greater the total negative charge on the nucleotide the smaller the retention volume. These effects can be understood in terms of the influence of charge on the stability of the complex itself assuming that, in complexing with diols, the benzeneboronate anions on the cellulose maintain their tetrahedral configuration and negative charge. The location of further negative charges on the diol moiety would then be expected to reduce the degree of complex formation through charge interaction. These effects then would manifest themselves in decreased retention volumes for the nucleotides compared with their parent nucleosides and the amount of this decrease should depend on the total negative charge on the nucleotide (triphosphate > diphosphate > monophosphate). This decreased stability due to charge interaction must also be partly counteracted by shielding derived from the high sodium chloride concentration since, in the absence of added salt, the uridine nucleotides experience no interaction with the cellulose.

The use of a divalent cation in the elution solvent distinguishes these two shielding effects even further. In the case of nucleosides, magnesium ions at 0.1 M are about as efficient as 1 M sodium ions in enhancing their binding to the cellulose whereas the divalent cation is substantially more efficient in enhancing the binding of nucleotides (Table I). This difference must arise from some extra shielding of the phosphate and polyphosphate groups due to specific interactions of the magnesium ions with these charged groups. This conclusion is supported, to some extent, by the anomalous behavior of the nucleoside diphosphates. In contrast to the order of binding of the nucleotides as judged by the order of elution in the presence of sodium ions, the diphosphates are the most strongly bound in the presence of magnesium ions. Apparently, greater shielding results from the particular interaction of magnesium ions with the nucleoside diphosphates than occurs with the corresponding monophosphates and triphosphates.

Finally, both sodium and magnesium ions appear to exert their effects at the same sites since the addition of 1 M sodium chloride to the elution solvent containing magnesium chloride does not appreciably alter the retention volumes. In these experiments as well as those reported below, uridine 3′,5′-diphosphate has been used as a control. This molecule does not have a *cis*-diol group and it is used to detect any binding to the cellulose derivatives resulting from interactions other than those involving the cyclic boronate structure. A similar pattern of the retention volumes shown in Table I has also been obtained using the DBAE-cellulose.

Dinucleoside Phosphates. It has been shown above as well as in the previous publication (Weith *et al.*, 1970) that, due to secondary binding forces, the nature of the base can exert a strong influence on the retention volumes of nucleosides and nucleotides, and that the resulting order of elution from columns of the derivatized celluloses is C, U, G, A. These effects are expected to be operative in the interactions of the celluloses with polynucleotides also and, in order to investigate the phenomenon further, 15 dinucleoside phosphates have been examined. With these results it is now possible to quantitatively evaluate the effects on retention volumes caused by a particular nucleotide base in either the 3′ position (the position containing the 2′,3′-diol group) or the 5′ position of a dinucleotide. The information gained in this study will be of value in predicting the retention volumes of polynucleotides containing known nucleotide sequences.

The results listed in Table II indicate that the type of nucleo-

TABLE II: Observed and Predicted Retention Volumes of Dinucleoside Phosphates on Columns of DBCM-Cellulose.[a]

Dinucleoside Phosphate CpY or XpC	V_R[b] (ml)	$\alpha_{CpY}V_S$[c]	$\alpha_{XpC}V_S$	$\frac{\alpha_{CpY}}{\alpha_{CpC}}$	$\frac{\alpha_{XpC}}{\alpha_{CpC}}$
CpC	25	15		1.0	
CpU	32	22		1.5	
CpG	64	54		3.6	
CpA	65	55		3.6	
CpC	25		15		1.0
UpC	26		16		1.0
GpC	46		36		2.4
ApC	52		42		2.8

Dinucleoside Phosphate XpY	$\frac{\alpha_{XpC}}{\alpha_{CpC}}$	$\frac{\alpha_{CpY}}{\alpha_{CpC}}$	$\frac{\alpha_{XpY}}{\alpha_{CpC}}$[d] (Calcd)	V_R[e] (Calcd) (ml)	V_R (Obsd) (ml)
UpU	1.0	1.5	1.5	33	33
GpU	2.4	1.5	3.6	64	65
ApU	2.8	1.5	4.2	73	77
UpG	1.0	3.6	3.6	64	66
ApG	2.8	3.6	10.0	160	154
UpA	1.0	3.6	3.6	64	65
GpA	2.4	3.6	8.6	139	132
ApA	2.8	3.6	10.0	160	156

[a] The columns had dimensions 40 × 0.6 cm, and each elution was carried out with 0.05 M *N*-methylmorpholinium chloride–1 M sodium chloride (pH 7.5) at 20° and at a flow rate of about 3 ml/hr. [b] The retention volume (V_R) of each compound was taken as the volume of buffer required to elute it and was measured from the point of addition to the column to the point of its peak concentration eluting from the bottom. [c] $\alpha_{CpY}V_S$ for a particular dinucleoside phosphate is obtained from the expression $V_R = V_C + \alpha_{CpY}V_S$, where V_C = void volume of the columns (= 10 ml); α_{CpY} = partition coefficient for the dinucleoside phosphate, CpY; V_S = volume of the stationary phase. [d] The ratio $\alpha_{XpY}/\alpha_{CpC}$ is obtained from $\alpha_{XpY}/\alpha_{CpC} = (\alpha_{XpC}/\alpha_{CpC})(\alpha_{CpY}/\alpha_{CpC})$. [e] Calculated from $V_R = V_C + \alpha_{XpY}V_S = V_C + \alpha_{XpY}(15/\alpha_{CpC}) = 10 + (\alpha_{XpY}/\alpha_{CpC})15$.

TABLE III: Retention Volumes of Polynucleotides on Columns of DBCM-Cellulose.[a]

Compound	0.05 M Morpholinium Chloride–0.1 M $MgCl_2$ (pH 8.5) (ml)
Uridine 3′,5′-diphosphate	10.1
Uridine 5′-phosphate	55.1
Uridylyl-(3′–5′)-uridine[b]	>65.0
$pU(pU)_4pU$	37.0
$pU(pU)_npU$ ($n \geq 7$)	9.5–34.0
$pA(pA)_4pA$[b]	>70.0
$N(pN)_npGp$[c]	9.6
$N(pN)_npG$[b,c]	>40

[a] The column had dimensions 40 × 0.6 cm and each elution was carried out at 20° and at a flow rate of 3 ml/hr. The retention volume was taken as the volume of buffer required to elute the compound and was measured from the point of addition at the top of the column to the point of its peak concentration eluting from the bottom. [b] The actual retention volumes at pH 8.5 of these samples were not determined. When the polymers did not appear in the eluates after the indicated volumes of pH 8.5 buffer had been passed through the column the polynucleotide was recovered by elution with about one void volume of 0.05 M sodium 2-(*N*-morpholino)-ethanesulfonate–0.1 M $MgCl_2$ (pH 6.0). [c] N = A, C, or U; $13 \leq n \leq 16$.

tide base occurring in both the 3′ and 5′ positions is important in determining the retention volume of each molecule. For a quantitative evaluation of these effects cytidylyl-(3′–5′)-cytidine (CpC) is chosen as the reference dinucleoside phosphate because, in the previous studies, it had been noted that, of the four nucleosides, cytidine has the smallest retention volume, a result which indicates that this nucleoside undergoes the least secondary interaction (if any) with the cellulose derivatives. By the comparison of the retention volumes of dinucleoside phosphates containing cytidine and another nucleoside with the retention volume of CpC it is possible to estimate the magnitude of secondary binding effects of any nucleoside in the 3′ or 5′ position relative to cytidine in the corresponding position. For example, in Table II, the ratios of the partition coefficients of CpA and ApC to that of CpC are used as measures of the binding of A relative to C in the 3′ position and the 5′ position, respectively. On the assumption that the binding effects resulting from the 3′ and 5′ positions of a dinucleoside phosphate are multiplicative a value for the ratio of the partition coefficient of ApA to that of CpC can be calculated. The calculated partition coefficient of ApA is then used to obtain a calculated retention volume, and this value is compared with the observed retention volume. The calculated retention volumes of all the other dinucleoside phosphates not containing cytidine are derived in a similar way, and the values obtained are in agreement with the observed volumes.

The results in Table II also indicate that the enhancement of the binding of U, G, and A relative to C in the 5′ position is less than the corresponding enhancement caused by these three bases relative to C in the 3′ position. This observation suggests that, in polynucleotides, the influence which a particular nucleotide base in the chain has on the retention volume may decrease as its distance from the 3′ terminus increases. However, it will be necessary to study the interactions of some specific tri- and tetranucleotides with the celluloses to establish such a rule.

Oligo- and Polynucleotides. The solvent conditions which promote the strong retention of oligo- and polynucleotides have also been investigated. It had already been shown in the earlier work that the pH of the elution solvent is an important determinant of the retention volumes of nucleosides because of its capacity to control the concentration of boronate anions on the cellulose. Charged compounds are also apparently subject to this effect as can be seen by the retention volumes of uridine 5′-phosphate at pH 7.5 and pH 8.5 (Tables I and III). In studies on polynucleotides also it can be shown that raising the solvent pH favors retention with the maximum effect being obtained at about pH 8.5. Further increases above this pH do not produce any increase in binding while a decrease in

TABLE IV: Retention of Transfer RNA on Columns of DBCM- and DBAE-Cellulose.[a]

RNA	Per Cent Bound[b]				
	DBCM-Cellulose		DBAE-Cellulose		
	Buffer + 0.1 M $MgCl_2$	Buffer + 0.1 M $MgCl_2$ + 20% Dimethyl Sulfoxide	Buffer + 1 M NaCl	Buffer + 1 M NaCl–0.1 M $MgCl_2$	Buffer + 1 M NaCl–0.1 M $MgCl_2$ + 20% Dimethyl Sulfoxide
Unfractionated tRNA	0	27	0	15	80
Purified fMet-tRNA				0	>95

[a] The columns had the dimensions 40 × 0.6 cm, and each elution was carried out at 20° and at a flow rate of about 3 ml/hr. Each elution solvent contained the buffer, 0.05 M morpholinium chloride, and, after addition of the appropriate salt, the pH was adjusted to 8.7 in each case. [b] The per cent of RNA which is not eluted from the columns after several void volumes of the particular elution solvent has passed through. The bound RNA is then recovered with one void volume of 0.05 M sodium 2-(*N*-morpholino)ethanesulfonate–1 M NaCl (pH 5.5).

the pH from this value gradually diminishes the retention volume until, at about pH 6.0, there is no interaction between any compound and the cellulose derivatives. Thus, the most suitable solvent for the retention of polynucleotides on DBCM-cellulose consists of a pH 8.5 buffer together with 0.1 M magnesium chloride. In the case of the DBAE-cellulose it is also necessary to add 1.0 M sodium chloride to the solvent in order to minimize the binding of polynucleotides through the residual anionic-exchange capacity of the cellulose (resulting from those aminoethyl groups which are not derivatized during its preparation).

Table III lists some examples of the retention of polynucleotides. In the case of the homopolymers it is again noted that the presence of purines enhance the retentions to a significant extent. The study of the effect of chain length on retention volume was carried out using uridine homopolymers in which this extra bonding effect due to the base is at a minimum. The observed result that an increase in chain length causes a decrease in retention volume could be due to an increase in the steric restriction in the approach of the larger molecules to the active sites on the cellulose. Alternatively, the reduced retentions might result from the increase in charge on the molecules and its resultant influence on the degree of complex formation as described above. It appears that the latter effect may be the more important consideration in the binding of smaller polynucleotides. For example, uridylyl-(3′–5′)-uridine which is almost twice the size of uridine 5′-phosphate contains one less charge and is bound much more strongly.

Polynucleotides of chain length 15 and containing mixed bases are also readily bound to the cellulose if they possess 2′,3′-diol groups at their terminals (Table III). The larger polynucleotides obtained directly from the ribonuclease T_1 digestion of RNA are not retained by DBCM-cellulose, because they contain 3′-terminal phosphate groups. However, after treatment with phosphatase to remove these terminal phosphate groups the polymers are strongly bound and an elution solvent of pH 6 is required for their subsequent recovery.

tRNA. Steric effects as well as charge interactions may become important considerations in the binding of larger polynucleotides. For example, under the conditions used for the DBCM-cellulose chromatography of the molecules listed in Table III, tRNA is not bound, and on columns of DBAE-cellulose, with solvents containing sodium and magnesium chlorides, only small fractions of the tRNA mixture can be retained. Since the tRNA preparation is known to consist of molecules which contain 2′,3′-glycol groups at their 3′ terminals it seemed probable that the lack of binding was due, at least in part, to the inability of their terminals to reach the active sites on the cellulose. This effect would then constitute steric hindrance to complex formation by virtue of the compact shape of the molecules and would be consistent with the relatively rigid secondary structure that is known to exist in these RNA species.

On the basis of these considerations experiments were performed using solvents which might be expected to partly loosen the RNA structure. Thus, it was found that the addition of 20% dimethyl sulfoxide to the sodium chloride–magnesium chloride solvent results in the substantial binding of unfractionated tRNA to columns of DBAE-cellulose (Table IV). The effect of the addition of dimethyl sulfoxide is even more dramatic in the case of a purified tRNA species. $tRNA^{fMet}$, which does not interact with the DBAE-cellulose in the sodium–magnesium chloride solvent, is completely bound on the addition of 20% dimethyl sulfoxide. More recent experiments have shown that essentially all of the unfractionated tRNA mixture can be bound to DBAE-cellulose if the absorption is carried out in the presence of the sodium–magnesium–dimethyl sulfoxide solvent at 0° (Duncan and Gilham,[2] 1972).

The use of elution solvents containing dimethyl sulfoxide in the present work was prompted by the earlier observations that this substance is effective in destroying secondary structure of nucleic acids (Helmkamp and Ts'o, 1961) and that aqueous solutions containing different concentrations of the denaturant are capable of affecting the secondary structure of a bacteriophage RNA to different extents (Strauss *et al.*, 1968). In the present case it is clear that the effect by the dimethyl sulfoxide on the binding of tRNA is due to an effect on the tRNA molecules themselves since the retention volumes exhibited by the smaller polynucleotides described above are not affected by the inclusion of the denaturant in the various elution solvents used. It is concluded that the function of the denaturant in these binding studies involves the

[2] Unpublished data.

loosening of the structures of the tRNA molecules near the 3′ terminals, thereby relieving some of the steric restriction to the approach of their 2′,3′-diol groups to the dihydroxyboryl groups on the cellulose.

Nucleotides and Polynucleotides Derivatized with Sorbitol. The use of the above methods is restricted to the study of RNA molecules, polyribonucleotides, and ribonucleotides which contain unsubstituted 2′,3′-diol groups at their terminals. However, it was considered that it might be possible to devise methods which would involve a more general application of the binding of polynucleotides to dihydroxyboryl-substituted cellulose. Such methods would then be available for the study of monomers and polymers in the DNA series, as well as those RNA species which do not contain unsubstituted 2′,3′-diol groups. One such method consists of the derivatization of the terminal phosphate groups of a polynucleotide or nucleic acid with a polyhydroxy compound which would be expected to interact strongly with cellulose-bound dihydroxyboryl groups.

In the initial studies sorbitol was chosen as the polyhydroxy compound to be incorporated. The choice was made on the basis of two considerations: (i) it exhibits a very large retention volume on DBCM-cellulose (Weith *et al.*, 1970) and (ii) the polyol displays a large solubility in water, a property that would permit its condensation with terminal phosphate groups of polynucleotides under relatively mild conditions. The derivatization is accomplished by use of a method previously developed for the synthesis, in aqueous solution, of phosphodiesters from monoesters (Naylor and Gilham, 1966). Aqueous solutions of nucleotides and polynucleotides can be converted to the appropriate diesters through activation of the terminal phosphate groups with *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodiimide in the presence of relatively high concentrations of an alcohol. In the present application it can be shown that, under these conditions, thymidine 5′-phosphate is converted to the sorbitol ester in essentially quantitative yield. It is expected that the product of this reaction consists of a number of isomers depending on the relative reactivity of the various hydroxyl groups in sorbitol. However, this possibility does not appear to present a problem since it is observed that all of the product binds strongly to DBCM-cellulose whereas the parent nucleoside, thymidine shows no interaction (Table V).

Polynucleotides can also be derivatized with sorbitol and, in each case, the resultant product shows substantial binding to the cellulose. In the case of the uridine homopolymers which consist of a mixture of large oligonucleotides with 5′-terminal phosphate groups, the sorbitol-derivatized product could not be eluted from the cellulose at pH 8.5. tRNA which also contains a 5′-terminal phosphate group can be derivatized in a similar way. This RNA is not normally bound to DBCM-cellulose in the absence of dimethyl sulfoxide. After reaction with sorbitol, however the RNA displays strong binding at pH 8.5.

More recently, studies have begun on the use of a polyhydroxy compound which appears to have some advantages over sorbitol. *N*-Methylglucamine reacts with terminal phosphate groups under similar conditions to those used for the incorporation of sorbitol—the predominant product, however, is the corresponding phosphoramidate. Polynucleotides which have been derivatized with this polyol are also strongly bound to the cellulose and furthermore, after chromatographic separation, the methylglucamine group can be easily removed from the polymers by mild acid treatment. The details of these experiments together with a description of the derivatization of polynucleotides containing terminal mono-, di-, and triphosphate groups will be the subject of a forthcoming publication.

TABLE V: Retention Volumes of Nucleotides and Polynucleotides Derivatized with Sorbitol on Columns of DBCM-Cellulose.[a]

Compound	Volume (ml) pH 7.5	pH 8.5
Thymidine	10.8	
Sorbitol-pT	60.0	
$pU(pU)_npU$ ($n \geq 7$)		9.5–34
Sorbitol-$pU(pU)_npU$ ($n \geq 7$)[b]		>120
tRNA		9.4
Sorbitol-tRNA[b]		>65

[a] The columns had dimensions 40 × 0.6 cm, and each elution was carried out at 20° and at a flow rate of 3 ml/hr. The retention volumes were taken as the volumes of buffer required to elute the compounds and were measured from the point of addition at the top of the column to the point of their peak concentration eluting from the bottom. The two buffer solutions used were 0.05 M *N*-methylmorpholinium chloride–0.1 M $MgCl_2$ (pH 7.5) and 0.05 M morpholinium chloride–0.1 M $MgCl_2$ (pH 8.5). [b] These substances were not eluted from the columns at pH 8.5, and, after the indicated volumes of the pH 8.5 buffer had passed through, the polymers were subsequently recovered by changing the elution solvent to 0.05 M sodium 2-(*N*-morpholino)ethanesulfonate–0.1 M $MgCl_2$ (pH 6.0).

Conclusion

On the basis of the results of the present study two new methods have already been developed for the study of nucleic acid structure and synthesis. One method employs the substituted celluloses to selectively bind and subsequently isolate the 3′-terminal polynucleotides (containing the *cis*-glycol group) which are formed by specific enzymic cleavage of large ribonucleic acid molecules. The internal polynucleotide fragments which lack the *cis*-glycol group do not bind, and the terminal polynucleotide can be subsequently recovered from the cellulose in a relatively pure condition. The method has been used to isolate and compare the 3′-terminal polynucleotides derived from the ribonucleic acids of three bacteriophages (Rosenberg *et al.*, 1971; Rosenberg and Gilham, 1971). The cellulose derivatives have also been used in a new procedure for the synthesis of polynucleotides of defined sequence. Nucleoside 5′-diphosphates containing certain blocking groups at their 2′ positions can be used to add nucleotides, in a stepwise fashion, to a growing polynucleotide chain (Mackey and Gilham, 1971). The addition is catalyzed by the enzyme, polynucleotide phosphorylase and, at the end of each synthetic step, the polynucleotide product contains a 2′-blocking group at its terminus. By chromatography on the dihydroxyboryl-substituted cellulose this product can then be readily separated from the starting polynucleotide since the latter contains a free 2′,3′-diol group at its terminus.

The present work may also find some application in se-

quence analysis studies of both DNA and RNA. For example, the derivatization of the terminal phosphate group of a nucleic acid fragment with sorbitol or *N*-methylglucamine followed by partial digestion with an endonuclease would yield a complex mixture of oligo- and polynucleotides. Chromatography of this mixture on DBCM-cellulose would allow the isolation of only those fragments which contain the original terminal phosphate group and, after fractionation of these fragments, the comparison of each member with the next larger and next smaller fragments should permit the nucleotide sequence of substantial sections of the original molecule to be determined.

Experimental Section

Materials. *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)-ethylcarbodiimide-*p*-toluenesulfonate, sorbitol, and *N*-methylglucamine were purchased from Aldrich Chemical Co., Milwaukee, Wis., and the nucleotides, dinucleoside phosphates, polyadenylic acid, and polyuridylic acid were products of P-L Biochemicals Inc., Milwaukee, Wis. Yeast tRNA and 2-(*N*-morpholino)ethanesulfonic acid were obtained from Calbiochem, La Jolla, Calif. Morpholine and *N*-methylmorpholine were Eastman products and were redistilled before use. The DBCM-cellulose and DBAE-cellulose were prepared as previously described (Weith *et al.*, 1970). The G-terminal oligonucleotides were prepared and purified by the methods described by Robinson *et al.* (1969). The sample of fmet tRNA was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

Preparation of Polynucleotides. Pig liver ribonuclease was purified and assayed by the procedure described by Heppel (1966) and was used to degrade polyuridylic acid to a mixture of oligonucleotides of the type: $pU(pU)_npU$ ($12 \geq n \geq 0$). The enzyme (10 units) and polyuridylic acid (15 mg) were dissolved in 0.07 M potassium phosphate buffer (pH 7)–0.13 M magnesium chloride, and the mixture was kept at 37° for 35 min. The digestion was then stopped by rapidly cooling the mixture to 0° and then lowering the pH to 3 using 1 N hydrochloric acid. The mixture was then neutralized with ammonia and fractionated by descending paper chromatography on Whatman No. 3MM paper using the solvent system: *n*-propyl alcohol (55 ml)–concentrated ammonia (10 ml)–water (35 ml). The resulting well-defined bands were cut out and eluted, and their chain lengths were determined by alkaline hydrolysis. A small quantity of each band (about 5 ODU_{260}) was dissolved in 0.25 N NaOH (0.2 ml) and kept at 37° for 20 hr. The products were examined using a new chromatographic procedure for the separation of all the nucleosides and nucleotides that can be formed in alkaline hydrolyses (Asteriadis and Gilham,[2] 1970). For this method a column (100 × 0.2 cm) of Dowex 1-X4 ion-exchange resin (400 mesh, chloride form) was packed in the presence of 0.5 M ammonium chloride–20% ethanol solution. The column was washed with 20% ethanol and then 0.4 ml of 1 N sodium hydroxide followed by 1 ml of 20% ethanol. The alkaline digest is diluted to 1 ml and applied to the column. Fractionation is effected by a linear gradient of chloride ion formed from 100 ml of 20% ethanol and 100 ml of 0.5 M ammonium chloride–20% ethanol, pH 10, at a flow rate of 8 ml/hr. The chain lengths are then determined by the molar ratios, pUp:Up:U. A similar procedure was used to prepare and characterize the series of adenosine oligonucleotides.

Thymidine 5'-Sorbitol Phosphate. Sorbitol (3 g) and thymidine 5'-phosphate (sodium salt, 0.025 mmole) were dissolved at 60° in 0.5 M sodium 2-(*N*-morpholino)ethanesulfonate (pH 5.5, 0.5 ml). *N*-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethylcarbodiimide-*p*-toluenesulfonate (300 mg) was added, and the mixture was kept at 50° for 6 hr with occasional stirring. The product was diluted with water and applied to a column (40 × 1.5 cm) of DEAE-cellulose (Whatman DE-23, HCO_3^- form), and chromatography was effected with 0.02 M ammonium bicarbonate at a flow rate of 60 ml/hr. The order of elution was thymidine 5'-sorbitol phosphate (yield > 90%), *p*-toluenesulfonate, and thymidine 5'-phosphate. Paper chromatography on Whatman No. 3MM paper gave the R_F of the product relative to that of thymidine 5'-phosphate as 1.22 in the solvent system: *n*-propyl alcohol (55 ml)–concentrated ammonia (10 ml)–water (35 ml), and 1.23 in solvent system: ethanol (70 ml)–1 M ammonium acetate (pH 7) (30 ml).

The sorbitol derivatives of other nucleotides and oligonucleotides were prepared in a similar way. In the case of the derivatization of tRNA, the product can be isolated from the reaction mixture by precipitation with three volumes of ethanol.

Retention Volumes. The chromatographic experiments with the various compounds on the columns of the two cellulose derivatives were carried out on quantities of 2–5 ODU_{260} and their elution positions were determined spectrophotometrically.

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