Activity of Polynucleotide Phosphorylase with Nucleoside Diphosphates Containing Sugar Ring Modifications†

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ABSTRACT: A number of nucleoside 5′-diphosphates containing modifications in their sugar rings have been synthesized, and the capacity of these nucleotides to act as substrates for polynucleotide phosphorylase has been examined. The 5′-diphosphates of 9-β-D-arabinofuranosyladenine (*ara*-A) and 3′-deoxyadenosine were prepared by phosphorylation of the nucleosides with phosphorus oxychloride followed by condensation of the resulting 5′-phosphates with inorganic phosphate using 1,1′-carbonyldiimidazole as the activating agent. The 5′-diphosphate of each ox-red nucleoside (a nucleoside in which the $C_{2'}$–$C_{3'}$ bond has been cleaved) was synthesized by oxidation of the 2′,3′-*cis*-diol groups in the 5′-diphosphates of adenosine, cytidine, guanosine, and uridine with sodium periodate followed by the reduction of the resulting dialdehydes with sodium borohydride. Similar conditions were also used to prepare the ox-red nucleosides as well as their 5′-phosphates and 5′-triphosphates. In a study of the capacity of modified nucleotides to add to a small oligoribonucleotide in the presence of polynucleotide phosphorylase, two classes of activity were exhibited: (i) the addition of a few residues of the nucleotide as in the case of the diphosphates of *ara*-A, 2′-deoxynucleosides, and (under certain conditions) 2′-*O*-(α-methoxyethyl)nucleosides; (ii) the addition of only one nucleotide residue as in the case of the diphosphates of the ox-red nucleosides and 3′-deoxyadenosine. The activity displayed by the latter class may be of value as a method for the radioactive labeling of the 3′ terminals of polyribonucleotides and RNA.

The enzyme, polynucleotide phosphorylase, has been incorporated into a number of methods for the synthesis of polyribonucleotides of defined sequence. The synthetic methods derive from the observation that, in the presence of 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates, the enzyme permits only one nucleotide addition to take place at the 3′ terminus of an oligoribonucleotide (Mackey & Gilham, 1971; Bennett et al., 1973; Sninsky et al., 1974). The limited addition reaction allows the synthesis of defined sequence polymers because the methoxyethyl group can be subsequently removed from the oligonucleotide product under mild acidic conditions, permitting a succession of single addition reactions to be carried out. Nucleoside diphosphates containing other $O^{2'}$-blocking groups have been employed in a similar way and these include the corresponding isovaleryl (Kaufmann et al., 1971), dihydrocinnamoyl (Kikuchi et al., 1975), and *o*-nitrobenzyl (Ikehara et al., 1976) derivatives. The present work concerns a study of some other modifications in the ribose rings of nucleoside 5′-diphosphates that result in limited enzymatic addition to oligoribonucleotides. A preliminary account of the work has been published (Sninsky et al., 1975).

Cordycepin and *ara*-adenosine[1] were phosphorylated at their 5′-hydroxyl positions with phosphorus oxychloride using a modification (Eckstein et al., 1975) of the method of Yoshikawa et al. (1967). The two 5′-phosphates, which were obtained in about 70% yield, were then converted to the corresponding 5′-diphosphates by activation with 1,1′-carbonyldiimidazole using a modification of the method of Cramer & Neunhoeffer (1962). The resulting imidazolidates, on reaction with inorganic phosphate, gave the 5′-diphosphates of *ara*-adenosine and cordycepin, which were subsequently purified by ion-exchange chromatography with yields of 47% and 65%, respectively.

The ox-red nucleoside 5′-diphosphates were prepared by subjecting each of the four nucleoside diphosphates to oxidation with sodium periodate followed by reduction with sodium borohydride. The conversion in each case was quantitative and the products were isolated by ion-exchange chromatography. The 5′-phosphates and 5′-triphosphates of ox-red nucleosides have also been prepared in the same way from the corresponding nucleotides. In the case of ox-red adenosine 5′-diphosphate, the structure was confirmed on the basis of its conversion by enzymatic dephosphorylation to ox-red adenosine. Ox-red adenosine, together with the other three ox-red nucleosides, were similarly prepared from the corresponding nucleosides by periodate oxidation and borohydride reduction, and small amounts of each product were purified by paper chromatography. In the case of ox-red adenosine and ox-red cytidine, however, larger quantitites were also purified by the use of ion-exchange resins to replace the contaminating inorganic ions with volatile salts. These two products were subsequently obtained in crystalline form and subjected to elemental analysis. The synthesis of ox-red nucleosides has been reported previously (Lerner, 1970), although, in this work, the methods used did not permit the crystallization of the products.

The various nucleoside diphosphates were tested for their ability to permit nucleotide additions to the trinucleotide, pA-A-A, in the presence of polynucleotide phosphorylase. The results are listed in Table I. The chromatographic elution pattern obtained for the products of the reaction involving *ara*-ADP (Figure 1) shows that this diphosphate behaves in a manner similar to that observed for the 2′-deoxynucleoside 5′-diphosphates in an earlier study (Batey & Gilham, 1974). The enzyme allows a small number of nucleotide additions to the trinucleotide acceptor with both of these substrates and, under conditions that favor the diaddition oligonucleotide, pA-A_2-dA-dA, as the major product with dADP as substrate, the monoaddition product, pA-A_2-araA, is the major species

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[1] Abbreviations used: 3′dA (cordycepin), 3′-deoxyadenosine; *ara*-A (*ara*-adenosine), 9-β-D-arabinosyladenine; A(MeOEt), 2′-*O*-(α-methoxyethyl)adenosine; ox-redA (ox-red adenosine), 9-[1′,5′-dihydroxy-4′(*S*)-hydroxymethyl-3′-oxapent-2′(*R*)-yl]adenine.

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TABLE I: Additions of Nucleotides to pA-A-A with Polynucleotide Phosphorylase.

5′-Diphosphate	pA-A-A (mM)	Nucleoside diphosphate (mM)	Enzyme (units/mL)	Time (h)	Yields of products (%)	
					Monoaddition	Diaddition
ara-Adenosine	0.7	3.0	18	5.5	52	32
Cordycepin	0.8	3.0	13	7.0	>95	0
Deoxyadenosine	1.0	3.0	10	5.5	9	83[a]
Ox-red adenosine	0.5	2.4	15	2.0	>95	0
Ox-red cytidine	0.5	2.4	15	2.0	>95	0
Ox-red guanosine	0.5	5.0	30	10.0	>95	0
Ox-red uridine	0.5	5.0	30	10.0	>95	0

[a] The reaction mixture also contained an 8% yield of the triaddition product, pA-A_2-dA_2-dA.

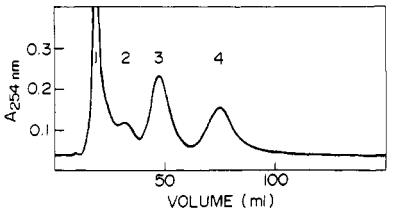

FIGURE 1: Elution pattern from the analysis of the reaction of pA-A-A (93 nmol) with *ara*-ADP and polynucleotide phosphorylase using ion-exchange chromatography with solvent system C (Experimental Section). Peak identification: (1) *ara*-ADP; (2) pA-A-A; (3) pA-A_2-*ara*-A; (4) pA-A_2-*ara*-A-*ara*-A.

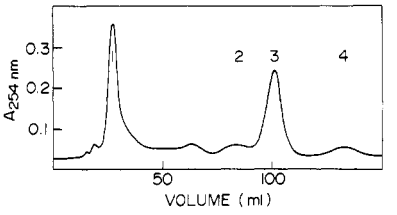

FIGURE 2: Elution pattern from the analysis of the reaction of pA-A-A with 3′dADP and polynucleotide phosphorylase using ion-exchange chromatography with solvent system B (Experimental Section). Peak identification: (1) 3′dADP; (2) pA-A-A; (3) pA-A_2-3′dA; (4) pA-A_3-3′dA.

in the *ara*-ADP reaction.

In contrast to this type of activity, the diphosphates of cordycepin and the four ox-red nucleosides permit only single additions to the trinucleotide acceptor (Table I). The elution pattern corresponding to the cordycepin diphosphate reaction is shown in Figure 2. However, in addition to the major product, pA-A_2-3′dA, the presence of a small amount of another product with a chain length of five nucleotides is apparent. It has been shown that the amount of this side product does not increase with reaction time and it has been assigned the structure, pA-A_3-3′dA. This pentanucleotide could have been produced by an addition of cordycepin phosphate to pA-A_2-A, a tetranucleotide that can arise in the reaction mixture by (i) phosphorolysis of pA-A-A with inorganic phosphate to form ADP and (ii) subsequent addition of ADP to pA-A-A. This type of behavior has been noted before in reactions involving additions to oligonucleotide acceptors with 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates as substrates (Sninsky et al., 1974). Such sequence rearrangements were shown to be avoidable by providing, in situ, a separate enzyme system for the removal of inorganic phosphate either present or formed in the reaction mixture. In the case of the addition with the ox-red nucleoside diphosphates no sequence rearrangements were detectable. The elution pattern corresponding to the reaction of pA-A-A with ox-red CDP is shown in Figure 3. Similar patterns were obtained from the analyses of reactions containing each of the other three ox-red nucleoside diphosphates as substrate.

In an earlier report on the study of single enzymatic addition of blocked nucleotides to oligoribonucleotide acceptors using 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates as donors (Sninsky et al., 1974), it was stated that the substitution of Mn^{2+} for Mg^{2+} as the divalent cation in the reaction mixture permits a small amount of double addition. This effect has now been clearly demonstrated by exposing the dinucleotide, pA-A, Mn^{2+} ion, and 2′-*O*-(α-methoxyethyl)adenosine 5′-diphosphate to a relatively large amount of polynucleotide phosphorylase for an extended time. Under these conditions, pA-A-A(MeOEt) and pA-A-A(MeOEt)-A(MeOEt) are formed in yields of 70% and 30%, respectively.

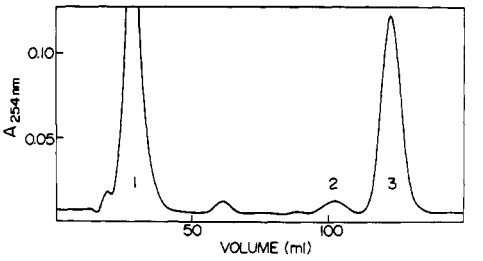

FIGURE 3: Elution pattern from the analysis of the reaction of pA-A-A (48 nmol) with ox-red CDP and polynucleotide phosphorylase using ion-exchange chromatography with solvent system B (Experimental Section). Peak identification: (1) ox-red CDP; (2) pA-A-A; (3) pA-A_2-ox-redC.

The various oligonucleotides prepared in this study have been characterized by the measurement of their chromatographic retention volumes and, in some cases, by analyses of their nucleotide components (Table II). The analyses of nucleotide compositions were carried out by alkaline hydrolysis followed by chromatographic separation of the products (Ho & Gilham, 1973; Sninsky et al., 1974). As expected, the terminal internucleotide bonds in pA-A_2-*ara*-A-*ara*-A and pA-A-A(MeOEt)-A(MeOEt) are not cleaved in this type of degradation. The penultimate nucleoside in each case does not contain a 2′-hydroxyl group with the cis configuration relative to the phosphodiester group and so, on alkaline treatment, the terminal two nucleosides are obtained in the form of a dinucleoside phosphate.

With these results it is possible to distinguish two classes of modified nucleoside 5′-diphosphates that exhibit limited additions to oligoribonucleotides in the presence of polynucleotide phosphorylase. The diphosphates of one class possess a 3′-hydroxyl group together with a cis 2′-hydrogen atom or a cis 2′-hydroxyl group containing a small blocking group. In the presence of Mn^{2+} ions, these substrates permit limited multiple

TABLE II: Characterization of Oligonucleotides.

Oligonucleotide	$\epsilon_{254nm} \times 10^{-3}$	Chromatographic retention (volume mL) in solvent system A	B	C	Products from alkaline hydrolysis
pA-A	25.8	57			
pA-A-A	36.3	82	105	36	
pA-A_2-dA	50.4	110			
pA-A_2-dA-dA	64.5	138			
pA-A_2-3′dA	50.4	102			pAp:Ap:3′dA, 1.1:1.9:1
pA-A-A(MeOEt)	36.3	67			
pA-A-A(MeOEt)-A(MeOEt)	50.4	86			pAp:Ap:A(MeOEt)-A(MeOEt), 1.0:0.9:1.0
pA-A_2-*ara*-A	50.4	121		48	
pA-A_2-*ara*-A-*ara*-A	64.5	168		74	pAp:Ap:*ara*-A-*ara*-A, 1.1:1.9:1.0
pA-A_2-ox-redA	50.4		128		pAp:Ap:ox-redA, 1.0:2.1:1.1
pA-A_2-ox-redC	42.8		124		pAp:Ap:ox-redC, 1.0:2.0:1.0
pA-A_2-ox-redG	48.3		159		
pA-A_2-ox-redU	46.3		133		

addition to the oligonucleotide acceptor. Some possible reasons why these diphosphates do not undergo extensive polymerization have been discussed in an earlier report (Batey & Gilham, 1974). The second class would consist of diphosphates of those nucleosides (cordycepin and the ox-red nucleosides) that do not have a sugar ring with a 3′-hydroxyl group. These substrates are clearly unable to support a second nucleotide addition to the acceptor. This phenomenon may simply be due to the inability of the single-addition product to locate a hydroxyl group in the correct spatial configuration on the enzyme surface to permit the required nucleophilic attack on the α-phosphate moiety of the adjacent nucleoside diphosphate.

These conclusions may be useful in explaining some earlier observations on the activity of certain modified nucleotides with polymerizing enzymes. For example, it has been shown that, while *ara*-ADP does not, itself, polymerize in the presence of polynucleotide phosphorylase and is not incorporated into poly(A) during the polymerization of ADP, it causes a lag period in the onset of ADP polymerization (Lucas-Lenard & Cohen, 1966). Similar experiments with *ara*-uridine 5′-diphosphate have indicated that, while this diphosphate does not appear to act as a polymerization substrate, it possesses the ability to inhibit the polymerization of UDP (Michelson et al., 1962). Ox-red adenosine 5′-diphosphate has recently been shown to inhibit the polymerization of ADP with polynucleotide phosphorylase (Smrt et al., 1975), a result that can now be understood in terms of the single addition of the ox-red nucleotide, yielding chains that are unable to undergo further elongation. In addition, the results obtained with cordycepin 5′-diphosphate and polynucleotide phosphorylase are consistent with some related studies of Shigeura & Boxer (1964) who concluded that the inhibition of the activity of RNA polymerase from *Micrococcus luteus* by cordycepin 5′-triphosphate arises out of the incorporation of single cordycepin moieties into the terminals of polynucleotide chains and the inability of these chains to permit the addition of further nucleotides.

Finally, two immediate applications of the present work are indicated. One of these concerns the specific single addition reactions displayed by the diphosphates of cordycepin and the ox-red nucleosides as a possible method for the 3′-terminal labeling of polyribonucleotide chains in RNA structural studies. In this application, the labeling of terminals with ox-red nucleosides may be of particular interest, since radioactively labeled nucleoside 5′-diphosphates are currently available in high specific activities from a number of commercial sources, and these can be converted to the appropriate single addition substrates within a few hours. The second application arises out of the observation that separate sets of conditions exist that will permit either one or two additions of a 2′-*O*-(α-methoxyethyl)nucleotide to an oligoribonucleotide. This flexibility should be of some value in the use of polynucleotide phosphorylase in methods for the construction of polynucleotides of defined sequence.

Experimental Section

Materials. Cordycepin, *ara*-adenosine, 1,1′-carbonyldiimidazole, and Sephadex G-10-120 cross-linked dextran were obtained from Sigma Chemical Co., St. Louis, Mo. The chromatographic materials Dowex 1-X2, Dowex 1-X4, and beaded polyacrylamide were purchased as AG 1-X2 (−400 mesh), AG 1-X4 (−400 mesh), and Bio-Gel P-2 (200–400 mesh), respectively, from Bio-Rad Laboratories, Richmond, Calif. *Micrococcus luteus* polynucleotide phosphorylase was the product of Boehringer Mannheim Corp., New York, N.Y., and a unit of its activity is defined as the amount required to polymerize 1 μmol of UDP in 15 min at 37 °C. Alkaline phosphatase (Grade I) from calf intestine was obtained from the same source, and a unit of its activity is defined as the amount required to release 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min at 25 °C. 2′-*O*-(α-Methoxyethyl)adenosine 5′-diphosphate was prepared as previously described (Bennett & Gilham, 1975). The oligonucleotides, pA-A and pA-A-A, were obtained by the partial degradation of poly(A) with pig liver nuclei ribonuclease as described by Bennett et al. (1973).

Chromatography. Ion-exchange chromatography was performed according to the methods of Asteriadis et al. (1976). Columns of Dowex 1-X2 (−400 mesh) had dimensions, 0.4 × 100 cm, and elution was carried out, under pressure, at 12–24 mL/h with the solvent system: 200 mL of 40% ethanol containing a linear gradient of (A) 0.2–0.4 M NH_4Cl (pH 8); or (B) 0.2–0.3 M NH_4Cl (pH 8); or (C) 0.3–0.5 M NH_4Cl (pH 8); or (D) 0.1–0.4 M NH_4Cl (pH 8). Paper chromatography was carried out in the descending mode on Whatman 3MM chromatographic paper with the solvent systems: (E) isopropyl alcohol–concentrated NH_4OH–water (7:1:2, v/v); (F) *n*-propyl alcohol–concentrated NH_4OH–water (55:10:35, v/v); (G) ethanol–1 M NH_4OAc, pH 7.0 (7:3, v/v); (H) *n*-butyl alcohol–acetic acid–water (5:2:3, v/v).

5′-Phosphates of ara-Adenosine and Cordycepin. The dried nucleoside (1.0 mmol) was dissolved in warm triethyl phosphate (2.5 mL) and the solution was cooled to 4 °C. Phosphorus oxychloride (110 μL) was added and the mixture kept at 4 °C for 2.5 h. Aqueous barium acetate (10%, 10 mL) was then added, followed by triethylamine (1 mL). Ethanol (30 mL) was added and the mixture was allowed to stand at 4 °C for 10 min and then centrifuged. The resulting pellet was washed with 70% aqueous ethanol (3 × 20 mL) and was then extracted with water (6 × 30 mL) until no further UV-absorbing material could be dissolved. The combined aqueous washes were concentrated in vacuo to dryness. Analysis of the product in each case by paper chromatography with solvent system E showed that the 5′-phosphates of *ara*-adenosine and cordycepin were obtained in 69% and 76% yields, respectively.

TABLE III: Chromatographic Data for Nucleotides.

Nucleotide	Retention volume (mL) in solvent system D	R_f[a] in solvent system F	G	H
ADP	83	1.00	1.00	1.00
ATP	106	0.96	0.41	0.82
dAMP	48	1.19	2.58	2.54
dADP	74	1.10	1.34	1.28
ara-AMP	54	1.09	1.83	1.96
ara-ADP	85	0.93	0.79	1.09
3'dAMP	48	1.22	2.50	2.50
3'dADP	73	1.01	1.21	1.25
ox-red AMP	37	1.22	2.80	2.32
ox-red ADP	65	1.07	1.16	1.34
ox-red ATP	77	1.02	0.80	1.01
ox-red CMP	42	1.18	3.41	2.05
ox-red CDP	65	1.06	1.59	1.26
ox-red GMP	52	1.04	2.59	2.11
ox-red GDP	75	0.94	1.25	1.17
ox-red UMP	41	1.19	4.44	2.31
ox-red UDP	69	1.10	2.20	1.51

[a] Values listed are relative to the R_f value of ADP.

5'-Diphosphates of ara-Adenosine and Cordycepin. The 5'-phosphates of *ara*-adenosine and cordycepin were converted to the corresponding mono[tri(*n*-butyl)ammonium] salts, and phosphorylated with a mixture of 1,1'-carbonyldiimidazole and mono[tri(*n*-butyl)ammonium] phosphate using the procedure described by Bennett & Gilham (1975) for the phosphorylation of 2',3'-di-*O*-(α-methoxyethyl)guanosine 5'-phosphate. The products in each case were subjected to ion-exchange chromatography on a column (50 × 3 cm) of DEAE-cellulose (Whatman DE 23) using 6 L of 5% BuOH containing a linear gradient of 0–0.3 M $Et_3NH^+HCO_3^-$ (pH 7.5) as the eluting solvent. The 5'-diphosphates of *ara*-adenosine and cordycepin were each obtained between elution volumes of 2–2.5 L with yields of 47% and 65%, respectively. The products were desalted by repeated evaporation in vacuo of their aqueous solutions. Chromatographic data are listed in Table III.

Ox-red Nucleosides. Each nucleoside (4.0 mmol) was dissolved in 10 mL of water (50 mL in the case of guanosine), and the solution was cooled to 0 °C and treated with sodium periodate (4.1 mmol). After 1 h at 0 °C the solution was adjusted to pH 10 by the addition of dilute NaOH, and sodium borohydride (20 mmol) was added. The mixture was kept at 0 °C for a further 5 h and then treated with excess acetone and allowed to stand at room temperature for 1 h, after which time the solution was concentrated in vacuo. Small quantities of the ox-red nucleosides were purified by paper chromatography with solvent system E, while the bulk of the products from adenosine and cytidine were purified for analysis by ion-exchange chromatography. The aqueous solution of the ox-red product was passed through a column containing 30 g of Dowex 1-X4 (HCO_3^- form) and then through a column of 30 g of Dowex 50W-X8 (pyridinium form). The solution was evaporated to dryness in vacuo and the residue was recrystallized from ethanol. Anal. (ox-red adenosine, mp 139–140 °C) Calcd for $C_{10}H_{15}N_5O_4$: C, 44.61; H, 5.61; N, 26.01. Found: C, 44.54; H, 5.52; N, 25.80. Anal. (ox-red cytidine, mp 165 °C) Calcd for $C_9H_{15}N_3O_5$: C, 44.08; H, 6.17; N, 17.14. Found: C, 44.25; H, 6.10; N, 17.13. R_f values obtained on Whatman 3MM paper with solvent system E were: ox-red adenosine, 0.61; ox-red cytidine, 0.61; ox-red guanosine, 0.41; ox-red uridine, 0.55; adenosine, 0.55; deoxyadenosine, 0.63; *ara*-adenosine, 0.50; cordycepin, 0.64.

Ox-red Nucleoside 5'-Diphosphates. The sodium salt of the nucleoside 5'-diphosphate (0.04 mmol) dissolved in 2 mL of water at 0 °C was treated with sodium periodate (0.06 mmol), and, after the periodate had dissolved, the solution was kept at 0 °C for 1 h. The reaction mixture was then treated with $Na_2B_2O_4 \cdot 10H_2O$ (20 mg) and then with sodium borohydride (0.3 mmol). After the solution had been allowed to stand at 0 °C for 5 h, it was diluted to 5 mL with water and applied to a column (100 × 0.7 cm) of Dowex 1-X2 ion-exchange resin. Separation was effected at 35 mL/h with 400 mL of 40% ethanol containing a linear gradient of 0.1–0.4 M NH_4Cl that had been adjusted to pH 8 with ammonia. The conversion was essentially quantitative and the fractions containing the single UV-absorbing peak in each case were combined and desalted by concentrating the solution to a small volume and passing it through a column (60 × 1 cm) of Sephadex G-10 using 20% ethanol as the eluting solvent. The ox-red nucleoside 5'-phosphates and 5'-triphosphates may be prepared under the same reaction conditions.

The structure of the ox-red adenosine 5'-diphosphate was confirmed by dephosphorylation. The diphosphate (4 A_{260nm} units) in 25 μL of 0.4 M sodium tris(hydroxymethyl)methylaminopropanesulfonate (pH 9.0 at 25 °C) was incubated at 37 °C with 1 unit of alkaline phosphatase for 3 h. The product was chromatographically indistinguishable from ox-red adenosine prepared as described above.

Addition of Modified Nucleotides to Adenosine Trinucleotide. Each reaction mixture (total volume 60–100 μL) contained 0.5–1.0 mM pA-A-A, 2.4–5.0 mM nucleoside 5'-diphosphate, 0.1 M sodium tris(hydroxymethyl)methylaminopropanesulfonate (pH 9.0 at 25 °C), 0.01 M $MnCl_2$, and polynucleotide phosphorylase (10–30 units/mL). After incubation at 37 °C for a specified time, the products of the reaction were separated by ion-exchange chromatography. Representative chromatographic elution patterns are shown in Figures 1–3. The chromatographic data for each case are listed in Table II, and the details of the reaction conditions and the yields of the various products are shown in Table I. The yields of products were calculated using ϵ_{254nm} values (Table II) that were estimated by the method of Sninsky et al. (1974).

Reaction of pA-A with 2'-O-(α-Methoxyethyl)adenosine 5'-Diphosphate. A mixture of pA-A (0.75 μmol) in 0.1 mL of 1.0 M sodium tris(hydroxymethyl)methylaminopropanesulfonate)pH 9.0 at 25 °C), 0.1 M $MnCl_2$ (0.1 mL), 20.0 mM 2'-*O*-(α-methoxyethyl)adenosine 5'-diphosphate (0.1 mL), and 0.7 mL of polynucleotide phosphorylase (30 units/mL) was incubated at 37 °C for 24 h. The products were separated by ion-exchange chromatography with solvent system A and the yields obtained for pA-A-A(MeOEt) and pA-A-A-(MeOEt)-A(MeOEt) were about 70% and 30%, respectively.

Characterization of Oligonucleotide Products. Ion-exchange chromatographic fractions corresponding to each product were combined, concentrated, and desalted on a column (60 × 1 cm) of Bio-Gel P-2 beaded polyacrylamide with an eluting solvent of 20% ethanol as described previously (Sninsky et al., 1974). In the case of products containing the acid-labile methoxyethyl group, a few drops of ammonia were added to the combined fractions and to the eluting solvent to keep the pH above 7 during the desalting procedure. The composition of each oligonucleotide product was determined by alkaline hydrolysis and subsequent ion-exchange chromatography of the hydrolysis products on a column of Dowex 1-X4 using 200 mL of 20% ethanol containing a linear gradient of 0–0.5 M NH_4Cl that had been adjusted to pH 10 with ammonia (Ho & Gilham, 1973; Sninsky et al., 1974). In this

system the retention volumes (mL) of the various products were: ox-redC, 11; ox-redA, 13; A(MeOEt), 16; cordycepin, 19; A, 32; A(MeOEt)A(MeOEt), 45; A2′p, 69; A3′p, 74; pAp, 92. In the case of the analyses of oligonucleotides containing *ara*-adenosine moieties a similar analytical chromatographic system was used except that the elution solvent contained 40% ethanol. The corresponding retention volumes (mL) in this system were: *ara*-A, 33; *ara*-A-*ara*-A, 41; Ap, 61; pAp, 84.

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Characterization of Complexes of Superhelical and Relaxed Closed Circular DNA with H1 and Phosphorylated H1 Histones†

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ABSTRACT: Complexes of H1 histone and closed circular SV40 DNA have been analyzed on sedimentation velocity gradients. At 100 mM NaCl, the binding of H1 histone to relaxed DNA is cooperative, resulting only in rapidly sedimenting complexes. These complexes consist of an average of 40–45 molecules of H1 histone per molecule of relaxed DNA. Under the same conditions, the interaction of H1 histone with superhelical DNA yields two distinct types of complexes: a slowly sedimenting complex and a rapidly sedimenting complex. The slowly sedimenting complex contains an average of 10–12 H1 histone molecules per molecule of superhelical DNA. The rapidly sedimenting complex arises as a result of the cooperative binding of H1 histone to superhelical DNA; the number of H1 histone molecules per superhelical DNA molecule in this complex ranges from 45 to 80. The cooperative binding of H1 histone to DNA is salt sensitive. It is further demonstrated that, of four different species of phosphorylated H1 histone tested, all bind DNA and retain the ability to discriminate between superhelical and relaxed DNA with the same efficiency as the nonphosphorylated H1 histone. Sedimentation velocity gradient analysis of the complexes of one species of phosphorylated H1 histone and circular DNA indicates that, although the nature of the binding is qualitatively similar to that of nonphosphorylated H1 histone, there are quantitative differences.

The chromatin of eukaryotes is organized in a linear array of structural subunits called nucleosomes (Oudet et al., 1975; Olins & Olins, 1974). Nucleosomes consist of a core particle containing two each of the four histone classes H2A, H2B, H3, and H4 closely associated with 140 base pairs of DNA and less tightly associated with approximately 60 additional base pairs of DNA (Kornberg, 1974; Van Holde et al., 1974; Hewish & Burgoyne, 1973; Sollner-Webb & Felsenfeld, 1975; Noll, 1974; Simpson & Whitlock, 1976). Physical studies on nucleosome structure demonstrate that the DNA of the nucleosome is wound around the outside of the protein core (Baldwin et al., 1975) in a manner which is topologically equivalent to a supercoiling of the DNA (Germond et al., 1975).

The structural relationship of the fifth major class of histones, the very lysine-rich H1 histones, to this fundamental unit of chromatin is not known. H1 histone has been reported to be associated with the 60 base pair segment of nucleosomal DNA (Varshavsky et al., 1976) and with regions of internucleosomal DNA (Noll & Kornberg, 1977). H1 histone may induce higher order packaging of nucleosomes.

H1 histone is distinct from the other four histones in a number of ways. Calf thymus H1 histone, a protein of mol wt

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