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Synthesis of 2′(3′)-*O*-Glycyl Derivatives of Cytidylyl-(3′–5′)-inosine and 2′-Deoxycytidylyl-(3′–5′)-adenosine*

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ABSTRACT: The synthesis of the title compounds, potential substrates for ribosomal peptidyl transferase, is described. Orthoester **1**, prepared from inosine and ethyl *N*-benzyloxycarbonylaminoorthoacetate using methanesulfonic acid as catalyst, was condensed with *N*-acetyl-2′,5′-di-*O*-acetylcytidine 3′-phosphate (**2**) in the presence of dicyclohexylcarbodiimide in pyridine to give intermediate **3**. The latter was hydrolyzed in 80% formic acid at 0° to afford the 2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycyl derivative **4a**. Hydrogenolysis of compound **4a** in 80% acetic acid at 0° using palladium catalyst gave the title derivative **4b**. Similarly, the *N*-dimethyl aminomethylene orthoester of adenosine 5′-phosphate (**5**) was condensed with *N*-dimethylaminomethylene-5′-*O*-(4-methoxy)trityl-2′-deoxycytidine (**6**) in the presence of dicyclohexylcarbodiimide in pyridine to afford (after removal of *N*-dimethylaminomethylene groups) the protected derivative **7**. Compound **7** was detritylated and hydrolyzed in 80% acetic acid at room temperature to the 2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycyl derivative **8a** which was hydrogenolyzed (Pd) in 80% acetic acid at 0° to the title product **8b**.

Previous communications in this series (Rychlik *et al.*, 1967, 1969, 1970a) dealt with the problem of the substrate specificity of ribosomal peptidyl transferase, an enzyme which is believed to be responsible for the biosynthesis of all proteins in living organisms. We have found, using, as simple models, aminoacyl nucleosides and nucleotides instead of aminoacyl tRNA, that the substrate specificity of the enzyme depends on three major factors: (a) character of the nucleoside (base and sugar) moiety of a given aminoacyl derivative; (b) nature of the aminoacyl residue comprising a given aminoacyl nucleoside or nucleotide; (c) nature and chain length of oligonucleotide attached to the 5′ position of a given aminoacyl nucleoside.

Whereas 2′(3′)-*O*-glycyladenosine (A-Gly[1]) itself is inactive in the peptide chain transfer reaction catalyzed by peptidyl ribosomal transferase, it is a fairly good acceptor of the peptide chain when either a uridylyl-3′ or, still better, cytidylyl-3′ residue[2] is attached to the 5′-hydroxyl group of the parent compound. On the other hand, UpU-Gly, which does not contain any nucleoside units typical of the terminal trinucleotide of aminoacyl tRNA, exhibits no activity in peptide chain transfer. In order to pursue in greater detail the problem of acceptor activity of aminoacyl oligonucleotides in peptide chain transfer reaction catalyzed by peptidyl ribosomal transferase, we undertook the synthesis of two additional derivatives, CpI-Gly and dCpA-Gly. The former represents a relatively minor structural alteration in the molecule of CpA-Gly which was found to be a good substrate for peptidyl ribosomal transferase (Rychlik *et al.*, 1967). It is interesting to note that a similar structural change in the terminal unit of tRNA, *i.e.*, enzymic deamination of CpCpA to CpCpI, resulted in retention of amino acid acceptor activity of modified tRNA (Li and Su, 1967). Moreover, as shown previously (Rychlik *et al.*, 1969) I-Phe retains about 30% of transfer activity of A-Phe as determined in a ribosomal system containing poly(lysyl)- or *N*-acetylphenylalanyl-tRNA's as donors of peptide (or *N*-acylaminoacyl) residue.

In the case of dCpA-Gly the structure of the penultimate nucleoside unit of CpA-Gly is modified by replacing the 2′-hydroxyl group with hydrogen atom. As noted earlier (Rychlik *et al.*, 1969) a similar structural change in the terminal residue (A-Phe) led to almost complete loss of transfer activity in dA-Phe. The comparison of the effect of

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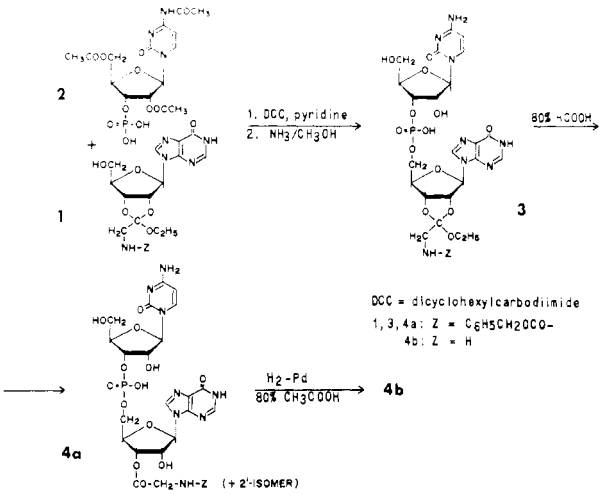
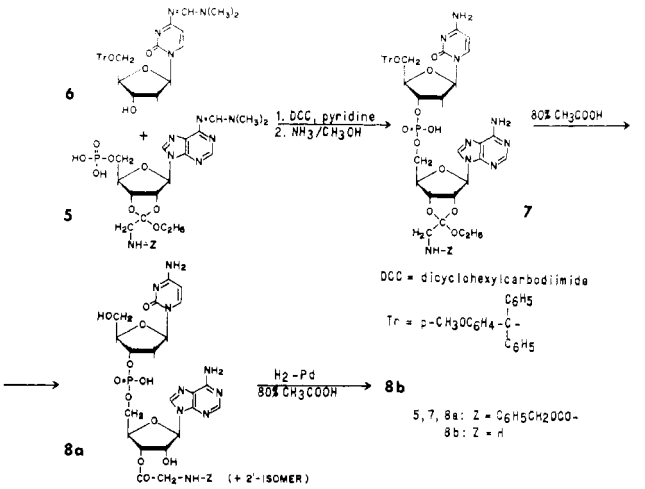
[1] Abbreviations used are: A-Gly, 2′(3′)-*O*-glycyladenosine; CpI-Gly, cytidylyl-(3′–5′)-(2′)3′-*O*-glycylinosine; dCpA-Gly, 2′-deoxycytidylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpCpA, cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-adenosine; CpCpI, cytidylyl-(3′–5′)-cytidylyl-(3′–5′)-inosine; A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; dA-Phe, 2′-deoxy-3′-*O*-L-phenylalanyladenosine; CpA, cytidylyl-(3′–5′)-adenosine; UpU-Gly, uridylyl-(3′–5′)-2′(3′)-*O*-glycyluridine; UpA-Gly, uridylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpA-Gly, cytidylyl-(3′–5′)-2′(3′)-*O*-glycyladenosine; CpI, cytidylyl-(3′–5′)-inosine; dCpA, 2′-deoxycytidylyl-(3′–5′)-adenosine; dC, 2′-deoxycytidine; A, adenosine; Cp, cytidine 3′-phosphate; Gly, glycine; I, inosine; pA(OEt)(CH_2NHZ), 2′,3′-*O*-(*N*-benzyloxycarbonyl)aminomethylethoxymethyleneadenosine 5′-phosphate; I(OEt)(CH_2NHZ), 2′,3′-*O*-(*N*-benzyloxycarbonyl)aminomethylethoxymethyleneinosine; MeOTrdCpA(OEt)(CH_2NHZ), 5′-*O*-(4-methoxy)trityl-2′-deoxycytidylyl-(3′–5′)-2′,3′-*O*-(*N*-benzyloxycarbonyl)-aminomethylethoxymethyleneadenosine; CpI-(ZGly), cytidylyl-(3′–5′)-2′(3′)-*O*-(*N*-benzyloxycarbonyl)glycylinosine; dCpA(ZGly), 2′-deoxycytidylyl-(3′–5′)-2′(3′)-*O*-(*N*-benzyloxycarbonyl)adenosine.

[2] The latter resembles most closely the terminal unit of aminoacyl tRNA.

SCHEME I



SCHEME II



the same type of structural change in the terminal and penultimate nucleoside unit of CpA sequence is therefore of obvious interest.

Results and Discussion

CpI-Gly was prepared according to a general procedure described earlier for the synthesis of UpU-Gly, UpA-Gly, and CpA-Gly (Chladek and Zemlicka, 1967). One of the key components in the preparation, derivative **1**, was obtained by orthoester exchange of inosine with ethyl *N*-benzyloxycarbonylaminoorthoacetate (Zemlicka and Chladek, 1966) in the presence of methanesulfonic acid in dimethylformamide (yield 89%). Compound **1** was condensed in the usual manner (Chladek and Zemlicka, 1967) with *N*-acetyl-2′,5′-di-*O*-acetylcytidine 3′-phosphate (**2**) (Lohrmann and Khorana, 1964) using dicyclohexylcarbodiimide in pyridine. The crude reaction product was deacetylated with methanolic ammonia and purified by DEAE-cellulose column chromatography and preparative paper chromatography to give **3** in 20% yield. The ultraviolet spectrum of the latter corresponds to that of CpI (A. Holy, unpublished results). Pancreatic ribonuclease degradation of **3** led to **1** and Cp leaving intact 5% of the (2′–5′) isomer. Hydrolysis of **3** in 80% formic acid (Chladek and Zemlicka, 1967) for 30 min at 0° gave a quantitative yield of **4a**. Removal of *N*-benzyloxycarbonyl group by hydrogenolysis in 80% acetic acid at 0° over palladium oxide on barium sulfate yielded CpI-Gly (**4b**, 77%), which was characterized by usual criteria (Chladek and Zemlicka, 1967). Pancreatic ribonuclease degradation of **4b** showed no increase in the amount of (2′–5′) isomer relative to **3**.

For the preparation of dCpA-Gly an approach more common in the synthesis of 2′-deoxyribooligonucleotides, *i.e.*, condensation of protected 5′-nucleotide with a blocked nucleoside component carrying a free 3′-hydroxyl group, was employed. The amino group of the cyclic orthoester of adenosine 5′-phosphate (Zemlicka and Chladek, 1968) was blocked by reaction with dimethylformamide dimethyl acetal (Zemlicka and Holy, 1967) in dioxane at room temperature. The *N*-dimethylaminomethylene derivative **5** was then used in the form of a pyridinium salt in condensation with *N*-dimethylaminomethylene-5′-*O*-(4-methoxy)trityl-2′-deoxycytidine (**6**) (Zemlicka and Holy, 1967) using dicyclohexylcarbodiimide in pyridine. After removal of both *N*-dimethylaminomethylene groups with ammonia in methanol, the crude reaction product was chromatographed on a DEAE-cellulose column to give **7** (55.5% yield) whose ultraviolet spectrum resembled that of CpA (Calbiochem, 1964). Hydrolysis of **7** was effected in 80% acetic acid for 36 hr at room temperature to give the 2′(3′)-*O*-(*N*-benzyloxycarbonyl)-glycyl derivative **8a** in 67% yield. Paper chromatography of **8a** in the solvent 2-propanol–ammonium hydroxide–water (7:1:2) resulted in hydrolysis of the ester linkage (Chladek and Zemlicka, 1967) with the formation of dCpA. The latter was stable toward deoxyribonuclease; however, it suffered ready degradation by *Vipera russelli* venom to give dC and A as the only products. Removal of *N*-benzyloxycarbonyl group of **8a** by hydrogenolysis in 80% acetic acid at 0° over palladium oxide on barium sulfate gave dCpA-Gly (**8b**) in 59% yield, which was characterized in terms of the usual criteria (Chladek and Zemlicka, 1967).

While CpI-Gly released a nascent polypeptide chain from poly(lysyl)-tRNA in a cell-free *Escherichia coli* ribosomal system, dCpA-Gly was shown to be only very slightly active (Rychlik *et al.*, 1970b). This fact could well indicate the role of the penultimate nucleotide unit of the acceptor sequence of aminoacyl-tRNA in interactions with ribosomal peptidyl transferase.

Materials and Methods

The general methods employed in the present study as well as the degradation with pancreatic ribonuclease have been described in an earlier work (Chladek and Zemlicka, 1967, 1968).

Chromatography. A descending technique on Whatman No. 1 paper was used with solvents S_1, 2-propanol–concentrated ammonium hydroxide–water (7:1:2), and S_2, 1-butanol–acetic acid–water (5:2:3). Thin-layer chromatography was performed on silica gel coated aluminum foils in the solvents S_3, dichloromethane–methanol (9:1), S_4, dichloromethane–methanol (4:1), and S_5, dichloromethane–methanol (1:1). The solvents S_3 and S_4 were also used for preparative thin-layer chromatography which was performed on 3-mm thick nonadhering (loose) layers of silica gel 30–60 μ containing fluorescent indicator (Chladek and Zemlicka, 1968) and (including the solvent S_5) 0.2% of triethylamine. R_F values are listed in Table I.

Paper Electrophoresis. An apparatus essentially the same as that described by Markham and Smith (1952) was used with the following buffers on Whatman No. 1 paper: 0.05 M sodium hydrogen phosphate (pH 7.5), 0.05 M sodium hydrogen citrate (pH 3.4), and 1 M acetic acid (pH 2.4).

Experimental Section[3]

2′,3′-O-(N-benzyloxycarbonyl)aminomethylethoxymethyleneinosine (**1**). A mixture of inosine (0.54 g, 2 mmoles), ethyl *N*-benzyloxycarbonylaminoorthoacetate (Zemlicka and Chladek, 1966; 1.22 g, 4 mmoles), dimethylformamide (10 ml), and methanesulfonic acid (0.2 ml) was shaken until it became homogeneous and then allowed to stand for 19 hr at room temperature. Sodium hydrogen carbonate (0.84 g, 0.01 mole) was added with shaking, and the mixture was filtered. The filter cake was washed with dimethylformamide and the clear filtrate was evaporated (0.1 mm Hg) at 40° (bath temperature). The residue was dissolved in dichloromethane (25 ml) and precipitated by the addition of ether (100 ml) followed by petroleum ether (bp 40–60°, 100 ml). The product, **1** (0.87 g, 89%), was washed with petroleum ether (50 ml) and dried at room temperature at 0.1 mm Hg. This material which contained a trace of inosine (thin-layer chromatography, S_3) was used for the preparation of intermediate **3**.

A sample of **1** (0.38 g) for analysis was purified *via* preparative thin-layer chromatography (15 × 40 cm, S_3) and the zone was eluted with S_4 (25 ml). The product was precipitated from dichloromethane (3 ml) with ether (25 ml) and petroleum ether (25 ml) to give 0.19 g (50%) of **1** which was dried in the manner described above. The infrared spectrum (chloroform) of **1** showed the following bands: 3380 cm^{-1} (NH, hypoxanthine), 3200–3300 cm^{-1} (OH-bonded), 3451 cm^{-1} (NH, urethane), 1707 and 1692 cm^{-1} (CO). *Anal.* Calcd for $C_{22}H_{25}N_5O_8 \cdot H_2O$: C, 52.27; H, 5.38; N, 13.86. Found: C, 52.59; H, 5.49; H, 13.37.

Cytidylyl-(3′–5′)-2′,3′-O-(N-benzyloxycarbonyl)aminomethylethoxymethyleneinosine (**3**). Compound **3** was prepared according to a previously described method (Chladek and Zemlicka, 1967) from **1** (0.49 g, 1 mmole) and *N*-acetyl-2′,5′-di-*O*-acetylcytidine 3′-phosphate (Lohrmann and Khorana, 1964) allowing a reaction time of 4 days. The crude product, after deacetylation with ammonia in methanol (20 hr at room temperature), was chromatographed on a DEAE-cellulose column and then subjected to preparative paper chromatography on Whatman No. 3 MM paper (2 sheets) to give **3** (1575 OD_{260} units, 19.5%) which was homogeneous on thin-layer chromatography in S_5, paper chromatography in S_1 and S_2, and electrophoresis in phosphate buffer. An ultraviolet spectrum of **3** in 0.01 N HCl showed E_{250}/E_{260} 1.00, E_{290}/E_{260} 0.67, a spectrum essentially similar to that of CpI (A. Holy, unpublished results). Degradation with pancreatic ribonuclease gave Cp and I in the ratio of 0.84:1 (both products were identified by paper chromatography in the solvent S_1 and ultraviolet spectrum in 0.01 N HCl after elution of the corresponding spots) in addition to 5% of pancreatic ribonuclease-resistant (2′–5′) isomer. The half-life of **3** in 80% acetic acid at room temperature was 2.25 hr (determination, *cf.* Chladek and Zemlicka, 1967; Zemlicka and Chladek, 1966).

[3] All evaporations were carried out *in vacuo* at bath temperatures below 30° unless stated otherwise.

TABLE I: Paper and Thin-Layer Chromatography.

Compound	S_1	S_2	S_3	S_4
dC	0.54			
A	0.59			
Cp	0.15	0.12		
dCpA	0.27	0.11		
CpI	0.18	0.06		
Gly	0.39			
I	0.46	0.36		
pA(OEt)(CH_2NHZ)	0.53	0.89	0.04	0.12
I(OEt)(CH_2NHZ) (**1**)	0.78		0.65	
CpI(OEt)(CH_2NHZ) (**3**)	0.50			
MeOTrdCpA(OEt)(CH_2NHZ) (**7**)	0.85	0.71	0.19	0.48
CpI(ZGly) (**4a**)	0.15[a]			
dCpA(ZGly) (**8a**)	0.27[a]	0.5		
CpI(Gly)[b] (**4b**)	0.19[a]	0.04		
dCpA(Gly)[b] (**8b**)	*a*	0.1		

[a] Hydrolysis to parent dinucleoside phosphate and Gly (*cf.* Chladek and Zemlicka, 1967). [b] Yellow color with ninhydrin.

Cytidylyl-(3′–5′)-2′(3′)-O-(N-benzyloxycarbonyl)glycylinosine (**4a**). A solution of **3** (480 OD_{260} units, 34.6 μmoles) in 80% formic acid (6 ml) was allowed to stand for 30 min at 0° (bath temperature), then diluted with water and lyophilized. A quantitative yield of **4a**, homogeneous in S_2 and hydrolyzed by S_1 to CpI (Chladek and Zemlicka, 1967), was obtained.

Cytidylyl-(3′–5′)-2′(3′)-O-glycylinosine (**4b**). A solution of **4a** (432 OD_{260} units, 31 μmoles) in 80% acetic acid (13.5 ml) was hydrogenated over palladium oxide–barium sulfate catalyst (52.5 mg, Kuhn and Haas, 1955) at 0° (bath temperature) in an open vessel for 3.75 hr. The mixture was filtered through a Celite bed and the filtrate was lyophilized to give **4b** (332 OD_{260} units, 77% yield) which was judged to be homogeneous in S_2 and which was hydrolyzed by S_1 to CpI, Gly, and a small amount of glycinamide. Likewise, **4b** appeared to be a single component in electrophoresis in acetic acid with a mobility of 5.6 relative to CpI. However, in citrate buffer traces of both Gly and an unknown, ninhydrin-positive component (0.7 the mobility of CpI) were detected and **4b** showed a mobility of 4.4 that of CpI. The ultraviolet spectrum (0.01 N HCl) corresponded to that of CpI (A. Holy, unpublished results): λ_{max} 251, 274 nm; λ_{min} 231, 260–262 nm; E_{250}/E_{260} 0.62. Degradation with pancreatic ribonuclease (Chladek and Zemlicka, 1967) showed, in addition to Gly (formed by hydrolysis), the presence of 5% of resistant (2′–5′) isomer, Cp and I in the ratio of 0.84:1 (identified by paper chromatography in S_1 and the ultraviolet spectra of the corresponding spots in 0.01 N HCl). Hydrolysis of compound **4b** (1 μmole) in 0.2 N NaOH (0.2 ml) for 30 min at room temperature gave Gly and CpI as the sole products which were identified by paper chromatography in S_1 and S_2 as well as by electrophoresis in 1 M acetic acid and citrate buffer.

N-Dimethylaminomethylene-2′,3′-O-(N-benzyloxycarbonyl)aminomethylethoxymethyleneadenosine 5′-Phosphate (**5**). A solution of 2′,3′-*O*-(*N*-benzyloxycarbonyl)aminomethylethoxymethyleneadenosine 5′-phosphate (Zemlicka and

Chladek, 1968; 16,575 OD_{257} units, 1.1 mmoles) and dimethylformamide dimethyl acetal (2 ml, *ca.* 20 mmoles) in dioxane (20 ml) was held overnight at room temperature. Lyophilization of the mixture led to a quantitative yield of **5** whose ultraviolet spectrum (ethanol) corresponded to that of *N*-dimethylaminomethyleneadenosine (Zemlicka and Holy, 1967): λ_{max} 315 nm; λ_{min} 235 nm.

5'-O-(4-Methoxy)trityl-2'-deoxycytidylyl-(3'–5')-2',3'-O-(N-benzyloxycarbonyl)aminomethylethoxymethyleneadenosine (**7**). A solution of **5** (1.1 mmoles) in 50% pyridine was passed over a column of Dowex 50 (pyridinium form, 2 × 8 cm) and the column was washed with 50% pyridine (*ca.* 200 ml). The combined eluates were evaporated to dryness at 0.1 mm, *N*-dimethylaminomethylene-5'-*O*-(4-methoxy)trityl-2'-deoxycytidine (Zemlicka and Holy, 1967; 1.25 g, 2.22 mmoles) was added, and the mixture was then rendered anhydrous by repeated evaporation with pyridine. The residue was dissolved in pyridine (10 ml), dicyclohexylcarbodiimide (4.12 g, 0.02 mole) was added, and the reaction mixture was held at room temperature for 6 days. Water (2 ml) was added, after which the mixture was allowed to stand for 5 hr at room temperature and was then extracted with petroleum ether (bp 40–60°, 3 × 20 ml). The aqueous layer was evaporated *in vacuo* and the residue was dissolved in saturated methanolic ammonia (50 ml) and allowed to stand overnight at room temperature. The solution was then evaporated *in vacuo* and the residue was chromatographed on a DEAE-cellulose column (bicarbonate form, 87 × 3.5 cm) using a linear gradient of triethylammonium bicarbonate (2 l. of 50% methanol in the mixing vessel, 2 l. of 0.2 M triethylammonium bicarbonate in 50% methanol in the reservoir, flow rate 2 ml/min, 17-ml fractions were taken). Fractions 91–180 containing **7** were combined and evaporated *in vacuo* and the residue was coevaporated twice with methanol to give 12,975 OD_{260} units (55.5%, calculated for ϵ_{260} 20,800) of chromatographically homogeneous (S_1) **7**; the ultraviolet spectrum (0.01 N HCl, E_{250}/E_{260} 0.80, E_{280}/E_{260} 0.75, E_{290}/E_{260} 0.48) corresponded to that of CpA (Calbiochem, 1964). Fluorescent, nonnucleotidic impurities were removed by preparative thin-layer chromatography (17 × 7 cm, S_4) of **7** (6370 OD_{260} units, 0.303 mmole); the zone containing **7** was eluted with methanol–2% triethylamine (50 ml), and eluate was evaporated *in vacuo* to give 5587.5 OD_{260} units (87.5%) of 7.[4]

2'-Deoxycytidylyl-(3'–5')-2'(3')-O-(N-benzyloxycarbonyl)-glycyladenosine (**8a**). A solution of **7** (3020 OD_{260} units, 0.145 mmole) in 80% acetic acid (7 ml) was maintained at room temperature for 36 hr and then lyophilized. The residue was treated with chloroform (5 ml), ether (25 ml), and petroleum ether (bp 40–60°, 25 ml) and the solid **8a** was removed by filtration and dried *in vacuo* (2035 OD_{260} units, 67%). This material contained traces of unidentified products of R_F 0.09 and 0.63, respectively, as determined by paper chromatography (S_1). Impure **8a** (1754 OD_{260} units, 84 μmoles) was applied (in 80% acetic acid) on one sheet of Whatman No. 3 MM paper which was developed in S_2, the zone of **8a** was eluted with 80% acetic acid, and the eluate was freeze-dried to give 1075 OD_{260} units (61%) of compound **8a**, chromatographically (S_1 and S_2) uniform; in S_1 it was hydrolyzed to dCpA. The latter reaction was used for the preparation of dCpA which was then isolated by preparative paper chromatography in the same solvent and found stable toward deoxyribonuclease.

Degradation of dCpA with Vipera ruselli venom. A sample of dCpA (37.5 OD_{260} units, 2.1 μmoles) in water (0.05 ml) was incubated with 100 μg of enzyme in Tris buffer (pH 10, 0.1 ml) for 4 hr at 37°. Paper chromatography in S_1 showed the presence of dC and A. No starting material was detected.

2'-Deoxycytidylyl-(3'–5')-2'(3')-O-glycyladenosine (**8b**). Compound **8a** (1250 OD_{260} units, 60 μmoles) was hydrogenated in 80% acetic acid (5 ml) over palladium/charcoal catalyst (10%, 0.1 g, Merck, Darmstadt, Germany) 2 hr at 0° (bath temperature) in an open vessel. The filtered catalyst was washed with cold 80% acetic acid and the filtrate was lyophilized to give 733 OD_{260} units (59%) of chromatographically (S_2, in S_1 decomposition to dCpA and Gly was observed) homogeneous **8b**. On paper electrophoresis (citrate buffer), the product moved as a double spot[5] (4.9 of mobility of dCpA) and gave a yellow color with ninhydrin. The ultraviolet spectrum (0.01 N HCl) corresponded to that of CpA (Calbiochem, 1964); E_{250}/E_{260} 0.77, E_{280}/E_{260} 0.77; E_{290}/E_{260} 0.54; λ_{max} 264 nm; λ_{min} 235 nm.

Acknowledgments

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[4] The yields were determined after dissolving the product in methanol and heating the corresponding aliquot in 0.01 N HCl for 2 hr at 100° (bath temperature).

[5] This observation is ascribed to a partial separation of 2'- and 3'-*O*-glycyl derivatives. Similar partial separation of some 2'- and 3'-*O*-aminoacyl adenosines by paper chromatography was observed earlier (McLaughlin and Ingram, 1964).

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Analysis of Transcription *in Vitro* Using Purine Nucleotide Analogs*

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ABSTRACT: The process of DNA transcription by purified *Escherichia coli* RNA polymerase has been studied using the 5′-triphosphates of 8-azaguanosine and formycin. While the rate of polymerization of these analogs is influenced by the choice of template and by the ionic environment, it is at all times lower than that of the normal counterparts. Analysis of the different steps in transcription reveals that, compared with the natural purine nucleotides, (1) the initiation of chains by 8-azaguanosine triphosphate and formycin triphosphate is low; (2) the analogs support a rate of chain elongation indistinguishable from normal; (3) the expected spontaneous release of newly synthesized RNA chains from the ternary transcription complex does not occur with either analog. The differences in the extent of chain initiation and release account quantitatively for the decreased rate of transcription in the presence of the analogs.

The behavior of the nucleotide analogs suggests that the initiation sequences in calf thymus DNA and in T_4 phage DNA are different and heterogeneous.

Stereochemical specificity in transcription appears to be much more exacting for chain initiation and termination than for chain elongation.

Formycin and 8-azaguanosine, structural analogs of adenosine and guanosine, respectively (Roblin *et al.* (1945); Koyama *et al.* (1966)), inhibit nucleic acid synthesis in bacterial and mammalian cells (Shapiro *et al.* (1950); Chantrenne (1964); Hori *et al.* (1964); Ward *et al.* (1969)). The analogs resemble their normal counterparts in H-bonding potential and in the formation of base pairs with complementary template residues, and they are also effectively incorporated into RNA *in vivo* and *in vitro* (Shapiro *et al.* (1950); Smith and Matthews (1957); Matthews (1958); Brockman *et al.* (1959); Caldwell *et al.* (1966); Ward *et al.* (1969)).

Previous observations have indicated that formycin and 8-azaguanosine residues in polynucleotides are likely to produce structural anomalies because both of these nucleosides tend to exist in the *syn* conformation (Ward *et al.* (1969)) under conditions in which the natural nucleosides maintain the *anti* conformation. The analogs could therefore be expected to interfere with some of the steps in RNA synthesis, and the rate of RNA synthesis is indeed depressed significantly when FTP[1] or 8-azaGTP is substituted for ATP or GTP, respectively (Kahan and Hurwitz (1962); Ikehara *et al.* (1968)). However, the mechanism by which the analogs depress RNA synthesis has not been defined, and the relationship between *in vitro* RNA synthesis and cytotoxicity remains unclear. We have therefore reexamined the utilization of the analogs by RNA polymerase. The findings presented below demonstrate that the analogs behave differently in the initiation, elongation, and release of RNA chains; in comparison with the natural substrates, the analogs (1) function poorly in initiation, (2) function normally in chain propagation, and (3) the normal pattern of chain termination and the release of long polynucleotides from ternary complexes is altered when the newly synthesized product contains either base analog.

Materials and Methods

Escherichia coli RNA polymerase was prepared and assayed as previously described (Darlix *et al.*, 1969). The conditions of incubation for individual experiments are given in the figure legends. The ionic conditions were one of the following: (1) Tris-HCl, pH 8.5 $\times 10^{-2}$ M; $MgCl_2$, 5 $\times 10^{-3}$ M; $MnCl_2$, 10^{-3} M; β-mercaptoethanol, 8 $\times 10^{-3}$ M; (2) Tris-HCl, pH 8.5 8 $\times 10^{-2}$ M; $MgCl_2$, 10^{-2} M; KCl, 5 $\times 10^{-2}$ M; β-mercaptoethanol, 5 $\times 10^{-3}$ M; (3) as in 2, but KCl, 0.16 M.

Unless otherwise noted, nucleoside triphosphates were present at 10^{-4} M.

The termination factor, ρ, was extracted from *E. coli* and purified by a modification of the procedure of Roberts (1969).

RNA synthesis was monitored by following the incorporation of the appropriate radioactive nucleotide into material precipitable by 5% trichloroacetic acid using a Millipore filter method. The method of direct filtration through Millipore filters (Sentenac *et al.*, 1968) was also used, particularly for estimating the initiation and release of RNA chains. Measure-

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[1] Abbreviations used are: FTP, formycin triphosphate; 8-azaGTP, 8-azaguanosine triphosphate; MAK, methylated serum albumin kieselguhr.