

2' Derivatives of Guanosine and Inosine Cyclic 3',5'-Phosphates. Synthesis, Enzymic Activity, and the Effect of 8-Substituents[†]

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ABSTRACT: A series of representative derivatives of guanosine cyclic 3',5'-phosphate (cGMP) and inosine cyclic 3',5'-phosphate (cIMP) which contained modifications in either the 2' position or the 8 and 2' positions were synthesized. Three types of derivatives were investigated: (1) derivatives in which the 2' position has been altered to produce a 2'-deoxynucleoside cyclic 3',5'-phosphate or a 9- β -D-arabinofuranosylpurine cyclic 3',5'-phosphate; (2) 2'-*O*-acyl derivatives; and (3) doubly modified derivatives containing a 2' modification [as in (1) and (2)] and an 8-substitution. 2'-Deoxyinosine cyclic 3',5'-phosphate and 9- β -D-arabinofuranosylhypoxanthine cyclic 3',5'-phosphate were obtained by HNO_2 deamination of 2'-deoxyadenosine cyclic 3',5'-phosphate and 9- β -D-arabinofuranosyladenine cyclic 3',5'-phosphate (ara-cAMP), respectively. Treatment of 8-bromo-2'-*O*-(*p*-toluenesulfonyl)adenosine cyclic 3',5'-phosphate with NaSH yielded the intermediate 8,2'-anhydro-9- β -D-arabinofuranosyl-8-mercaptopoadenine cyclic 3',5'-phosphate, which was converted directly to 2'-deoxyadenosine cyclic 3',5'-phosphate (dcAMP) by treatment with Raney nickel. 8-Bromo-2'-*O*-(*p*-toluenesulfonyl)guanosine cyclic 3',5'-phosphate was converted to 8,2'-anhydro-9- β -D-arabinofuranosyl-8-mercaptoguanine cyclic 3',5'-phosphate, and the latter was desulfurized with Raney nickel to give 2'-deoxyguanosine cyclic 3',5'-phosphate. Ara-cAMP, 9- β -D-arabinofuranosylguanine cyclic 3',5'-phosphate, and 9- β -D-arabinofuranosyl-8-mercaptoguanine cyclic 3',5'-phosphate have been previously reported (Mian et al., 1974, *J. Med. Chem.* 17, 259). 8-Bromo-2'-*O*-acetylinosine cyclic 3',5'-phosphate and 8-[*(p*-chlorophenyl)thio]-2'-

O-acetylinosine cyclic 3',5'-phosphate were produced by acylation of 8-bromoinosine cyclic 3',5'-phosphate and 8-[*(p*-chlorophenyl)thio]inosine cyclic 3',5'-phosphate, respectively; while 8-bromo-2'-*O*-butyrylguanosine cyclic 3',5'-phosphate was synthesized by bromination of 2'-*O*-butyrylguanosine cyclic 3',5'-phosphate. The enzymic activities of these derivatives were investigated (1) as activators of cAMP-dependent (bovine brain) and cGMP-dependent (lobster tail muscle) protein kinases, (2) as substrates for a cyclic nucleotide phosphodiesterase (rabbit kidney), and (3) as inhibitors of the hydrolysis of both cAMP and cGMP by two cyclic nucleotide phosphodiesterase preparations (rabbit lung and beef heart). Both the 2' derivatives and 8-substituted 2' derivatives of both cIMP and cGMP were less than one-hundredth as active as cAMP or cGMP at activating the cAMP-dependent protein kinase or the cGMP-dependent protein kinase, respectively. All of the 2' derivatives of cIMP and cGMP were hydrolyzed at significant rates by phosphodiesterase, while the 8-substituted 2' derivatives were completely or substantially resistant to enzymic hydrolysis. The results of the studies of the inhibition of cAMP and cGMP hydrolysis by these derivatives showed that (1) 2' derivatives were, in general, better inhibitors than the corresponding 8-substituted 2' derivatives; (2) O^2 -acyl derivatives and 2'-deoxy derivatives were similar in inhibitory properties and in turn were better inhibitors than the ara derivatives; and (3) all the derivatives inhibited cGMP hydrolysis to an equal or greater extent than they inhibited cAMP hydrolysis.

Analogs of adenosine cyclic 3',5'-phosphate (cAMP) and guanosine cyclic 3',5'-phosphate (cGMP) containing chemical modifications in various portions of these molecules have provided insight into the manner in which the parent cyclic nucleotides interact with the enzymes of their metabolism (for reviews, see Simon et al., 1973; Meyer and Miller, 1974). Modifications of the carbohydrate moiety of cAMP have revealed that an unsubstituted 2'-hydroxyl group in the *ribose* configuration is required for the activation of cAMP-dependent protein kinase but not for the uti-

lization of the derivatives by cyclic nucleotide phosphodiesterase (Miller et al., 1973a; Boswell et al., 1973). Studies on the activation of protein kinase by various 2',8-disubstituted derivatives of cAMP have shown that the general inactivity of 2' derivatives of cAMP as activators of the kinase was not overcome by addition of an 8-substituent (Khwaja et al., 1975), even though many 8-substituted derivatives of cAMP activate the kinase more efficiently than cAMP itself (Muneyama et al., 1971, 1975; Bauer et al., 1971; DuPlooy et al., 1971; Miller et al., 1973b).

8-Substituted derivatives of cAMP specifically activate only cAMP-dependent protein kinase while derivatives of cGMP with corresponding 8-substituents were specific for cGMP-dependent protein kinase (Miller et al., 1973b). 8-Substituted derivatives of inosine cyclic 3',5'-phosphate (cIMP), by comparison, exhibited significant activity with both kinases but demonstrated partial specificity for the cAMP-dependent protein kinase. At the same time corresponding 8-substituents produced similar effects on the

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Table I: Ultraviolet Spectra of the Nucleotides.

No.	λ_{max} , nm ($\epsilon \times 10^{-3}$)		
	pH 1	pH 7	pH 11
6	261 (11.4) 281 (sh, 7.6)		263 (11.1)
9	252 (14.7)	252 (14.7)	257 (13.4)
11	247 (11.5)	247 (11.5)	251 (12.1)
13	256 (16.2) 275 (sh, 14.2)	256 (16.4) 275 (sh, 14.4)	244 (13.0) 282 (15.6)
14	256 (14.2)	252 (16.0)	257 (14.4)
	278 (sh, 9.9)	274 (sh, 10.6)	
15	257 (11.8)	253 (13.3)	260 (11.8)
	282 (sh, 8.3)	277 (sh, 8.7)	
16	260 (15.5) 275 (sh, 12.8)		270 (13.6)

ability of the resulting cAMP or cGMP derivatives to activate their respective kinases.

In this paper, we wish to report on the in vitro enzymic properties of some representative derivatives of cGMP and cIMP modified in either the 2' or in both the 2' and 8 positions so that a comparison can be made with the corresponding 2' and 8,2' derivatives of cAMP.

Experimental Section

Ultraviolet spectra were recorded on a Cary 15 and, where not reported, were consistent with the reported structure. The purity of all compounds was verified by thin-layer chromatography on either Merck Silica Gel 254-F or Baker-flex Cellulose-F plates developed in either acetonitrile-0.1 N NH₄Cl, 7:3 and 4:1, or 2-propanol-H₂O-concentrated NH₄OH, 7:2:1. The ¹H nuclear magnetic resonance (NMR) spectra were determined on a Hitachi Perkin-Elmer R-20A and elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., or Het. Chem. Co., Harrisonville, Mo. Pertinent uv spectra are recorded in Table I. All compounds were homogeneous by thin-layer chromatography (TLC) and were verified to be phosphodiesters by electrophoresis at pH 7.2. Additionally, the integrity of the cyclic nucleotide moiety of the nucleotides containing ribose was verified by the presence of an apparent singlet ($J_{H_1'-H_2'} < 1$) in the ¹H NMR for the anomeric proton (Jardetzky, 1962).

2'-Deoxyadenosine Cyclic 3',5'-Phosphate (3, dcAMP). A stirred suspension of 8-bromo-2'-O-(*p*-toluenesulfonyl)-adenosine cyclic 3',5'-phosphate (1, 8Br-O^{2'}-Ts-cAMP, 11.72 g, 20 mmol, Mian et al., 1974), dimethylformamide (120 ml), and 60 ml of 1 N NaSH was kept at room temperature overnight, then evaporated under reduced pressure from EtOH repeatedly. The residual solid was dissolved in MeOH (250 ml) with 25 ml of 2 N NH₄OH. To this solution Raney nickel (ICN-K&K Labs) (70 g) was added, and the stirred mixture was refluxed for 16 hr. The reaction mixture was filtered through a Celite pad and washed with MeOH (200 ml) containing 2 N NH₄OH (25 ml). The filtrate and washings were combined and evaporated; the residue was dissolved in MeOH (30 ml) and precipitated with Et₂O to obtain several crops of 3; recrystallization from aqueous EtOH gave 4.98 g (71.1%) of 3. Anal. Calcd for C₁₀H₁₁N₅NaO₅P·H₂O: C, 32.53; H, 3.55; N, 18.96. Found: C, 32.46; H, 3.61; N, 18.90.

8,2'-Anhydro-9- β -D-arabinofuranosyl-8-mercaptopguanine Cyclic 3',5'-Phosphate (5, 8HS-8,2'-anhydro-ara-cGMP). The Na salt of 8-bromo-2'-O-(*p*-toluenesulfonyl)-

guanosine cyclic 3',5'-phosphate (4, 8Br-O^{2'}-Ts-cGMP, 2.7 g, 5 mmol) was converted to its ammonium salt and the dried material was suspended in 1-butanol (110 ml) and refluxed with thiourea (0.57 g, 7.5 mmol) for 4 hr. The reaction mixture was kept at ambient temperature overnight and filtered. The precipitate was washed with 1-butanol and dried to give 2.07 g of 5 in almost quantitative yield containing traces of *p*-toluenesulfonic acid but suitable for further transformations. A sample was purified by chromatography on a DEAE-cellulose (DE 52, HCO₃⁻ form) column with a gradient (0.05–0.3 M) of triethylammonium bicarbonate (pH 7.3). The fractions containing product were pooled and evaporated to obtain pure 5 as the Et₃N salt. Anal. Calcd for C₁₀H₁₁N₅O₆PS·C₆H₁₅N·H₂O: C, 36.36; H, 5.49; N, 15.90. Found: C, 36.12; H, 5.79; N, 15.72.

2'-Deoxyguanosine Cyclic 3',5'-Phosphate (6, dcGMP). A mixture of 5 (0.38 g, 1 mmol), 25 ml of MeOH, 5 ml of 2 N NH₄OH, and 6 g of Raney nickel was stirred and refluxed for 18 hr. The reaction mixture was filtered through a Celite pad and washed with MeOH (20 ml) containing 2 N NH₄OH (2.5 ml). The filtrate and washings were combined and evaporated to yield 0.1 g of 6 as a white solid, homogeneous on TLC. On paper electrophoresis (pH 4 and 7) it exhibited a mobility similar to cGMP.

2'-Deoxyinosine cyclic 3',5'-phosphate (7, dcIMP) was prepared from 2'-deoxyadenosine cyclic 3',5'-phosphate (3) in 45% yield by the procedure and work-up used in the preparation of cIMP from cAMP (Meyer et al., 1972).

2'-O-Acetyl-8-bromoinosine Cyclic 3',5'-Phosphate Triethylammonium Salt (9, 8Br-O^{2'}-Ac-cIMP). A solution of 8-bromoinosine cyclic 3',5'-phosphate (8, 29 g, 71 mmol) in 10 ml of Et₃N, 200 ml of pyridine, and 150 ml of Ac₂O was stirred overnight at room temperature. The solvent was removed in vacuo and the gum which remained crystallized upon scratching. The crude solid was filtered, washed with EtOAc, and dissolved in a small volume of MeOH. EtOAc was added until crystallization began. The solid was filtered and dried to yield 27 g (69%) of 9. Anal. Calcd for C₁₂H₁₂BrN₄O₈P·C₆H₁₅N: C, 39.14; H, 4.92; N, 12.68; Br, 14.46. Found: C, 38.85; H, 4.65; N, 12.55; Br, 14.66.

2'-O-Butyrylinosine Cyclic 3',5'-Phosphate (11, O^{2'}-Bt-cIMP). Inosine cyclic 3',5'-phosphate sodium salt (10, 3 g, 8.5 mmol) and 4-dimethylaminopyridine (0.15 g, 1.2 mmol) were stirred in 50 ml of dimethylformamide and 25 ml of butyric anhydride for 2 hr at room temperature. The solvent was evaporated and the residue was codistilled twice with EtOH. The final residue was dissolved in EtOH and approximately 3 volumes of EtOAc was added. The precipitate was filtered and washed with EtOAc. A second precipitation from EtOH with EtOAc yielded an analytical sample, 324 mg (9%) of 11. Anal. Calcd for C₁₄H₁₆N₄NaO₈P: C, 39.81; H, 3.81; N, 13.26. Found: C, 39.59; H, 4.07; N, 12.95.

2'-O-Acetyl-8-[(*p*-chlorophenyl)thio]inosine Cyclic 3',5'-Phosphate (13, 8pClPhS-O^{2'}-Ac-cIMP). 8-[(*p*-chlorophenyl)thio]inosine cyclic 3',5'-phosphate (12, 6.0 g, 12.2 mmol, Miller et al., 1973b) was dissolved in MeOH containing 3 ml of triethylamine. The solution was evaporated to dryness and the residue was dissolved in a mixture of dimethylformamide (100 ml) and acetic anhydride (50 ml) containing 4-dimethylaminopyridine (305 mg, 2.5 mmol). The solution was stirred for 2 hr at room temperature, the solvent was evaporated, and the residue dissolved in a minimum volume of 1:1 MeOH-H₂O. This was passed through a Dowex 50 (H⁺, 100–200 mesh, 4.5 × 15 cm, prewashed

with 1:1 MeOH-H₂O column. The eluate was evaporated to a small volume and the solid was filtered and washed with H₂O. Recrystallization from H₂O yielded 5.43 g (86%) in two crops. Anal. Calcd for C₁₈H₁₆ClN₄O₈PS: C, 41.99; H, 3.13; N, 10.88. Found: C, 41.79; H, 3.36; N, 10.75.

2'-O-Acetylguanosine Cyclic 3',5'-Phosphate (14, O²-Ac-cGMP). A dimethylformamide (40 ml) suspension of the Na salt of cGMP·4H₂O (1.0 g, 2.7 mmol), 4-dimethylaminopyridine (0.1 g, 0.82 mmol), and 20 ml of acetic anhydride was stirred 2 hr. The resulting solution was evaporated to dryness; the residue was dissolved in 1:1 MeOH-H₂O and passed through a Dowex 50-X8 (H⁺, 100-200 mesh, 2 × 8 cm, prewashed with 1:1 MeOH-H₂O) column. The eluate was evaporated to dryness and the residue suspended in EtOH, filtered, and dried to yield 600 mg (57%) of **14**. Anal. Calcd for C₁₂H₁₄N₅O₈P·H₂O: C, 35.56; H, 3.97; N, 17.28. Found: C, 35.69; H, 3.98; N, 16.81.

2'-O-Butyrylguanosine Cyclic 3',5'-Phosphate Sodium Salt (15, O²-Bt-cGMP). The Na salt of cGMP·4H₂O (2.0 g, 5.4 mmol) and 4-dimethylaminopyridine (0.15 g, 1.23 mmol) were dissolved in 50 ml of dimethylformamide and 5 ml of butyric anhydride. After stirring at room temperature for 3 hr the reaction mixture was filtered and the filtrate evaporated to dryness. EtOH was added to the residue and the resulting solid was filtered, washed with EtOH, and dried to yield 870 mg (37%) of **15**. Anal. Calcd for C₁₄H₁₄N₅NaO₈P: C, 38.45; H, 3.91; N, 16.01. Found: C, 38.35; H, 4.07; N, 15.93.

8-Bromo-2'-O-butyrylguanosine Cyclic 3',5'-Phosphate Triethylammonium Salt (16, 8Br-O²-Bt-cGMP). Bromine water (saturated at room temperature) (150 ml) was added dropwise over a 2-hr period to a solution of the Na salt of O²-Bt-cGMP (**15**, 11.5 g, 26 mmol) in 500 ml of H₂O. The solution was stirred for an extra 0.5 hr, then N₂ was bubbled through until the color changed from orange to light yellow. The solution was evaporated to ~300 ml and diluted with 300 ml of MeOH. This was passed through a Dowex 50-X8 (H⁺, 100-200 mesh, 5 × 24 cm, prewashed with 1:1 MeOH-H₂O) column. The eluate was evaporated to dryness and the residue was codistilled with EtOH until dry. Trituration of the foam with acetone gave 10.5 g of the crude free acid of **16**. This was dissolved in MeOH containing 5 ml of triethylamine and evaporated to dryness, and the residue was dissolved in CHCl₃ and placed onto a 30-g silica gel column (packed in CHCl₃). The column was washed with CHCl₃ and then the product was eluted with 1:9 MeOH-CHCl₃. The appropriate fractions were pooled and evaporated to dryness. CHCl₃ was added to the residue and the resulting solid was filtered and dried to yield 5 g (32%) of **16**. Anal. Calcd for C₁₄H₁₆BrN₅O₈P·C₆H₁₅N: C, 40.34; H, 5.41; N, 14.11; Br, 13.42. Found: C, 40.18; H, 5.40; N, 14.06; Br, 13.45.

The syntheses of 9-β-D-arabinofuranosylguanine cyclic 3',5'-phosphate (ara-cGMP) and of 9-β-D-arabinofuranosyl-8-mercaptopurine cyclic 3',5'-phosphate (8HS-ara-cGMP) have been previously described by Mian et al. (1974); and the synthesis of 9-β-D-arabinofuranosylhypoxanthine cyclic 3',5'-phosphate (ara-cIMP) has been previously described by Revankar et al. (1975), who used the method of Meyer et al. (1972) to deaminate 9-β-D-arabinofuranosyladenine cyclic 3',5'-phosphate.¹ N²,O²-Dibutyryl-

guanosine cyclic 3',5'-phosphate (N²,O²-Bt₂cGMP) and N²-butyrylguanosine cyclic 3',5'-phosphate (N²-Bt-cGMP) were purchased from Sigma. The purity of these compounds, determined by thin-layer chromatography as previously described (Miller et al., 1973a), was as follows: N²,O²-Bt₂cGMP contained ~2% O²-Bt-cGMP and <1% cGMP; and N²-Bt-cGMP contained ~2% N²,O²-Bt₂cGMP, ~2% O²-Bt-cGMP, and <1% cGMP. They were used as received without further purification.

Biochemical Methods

Enzyme Preparations. The cyclic nucleotide phosphodiesterases were prepared as reported previously (Miller et al., 1973a). Bovine brain cAMP-dependent protein kinase and lobster tail muscle cGMP-dependent protein kinase were purified through the DEAE-cellulose step as previously described (Kuo, 1972).

Enzyme Assays. The assay for inhibition of cAMP or cGMP hydrolysis by the rabbit lung and beef heart enzymes contained in 0.5 ml: 25 μmol of Tris-HCl (pH 7.5); 5 μmol of MgCl₂; 20–200 μg of phosphodiesterase protein; 80 pmol of [8-³H]cAMP or [8-³H]cGMP (350,000 cpm); and varying concentrations of the 3',5'-cyclic nucleotide being tested as an inhibitor. The incubation times were determined from pilot assays to give kinetically valid data. The reaction mixtures were processed as previously described (Miller et al., 1973a).

When testing cAMP derivatives as substrates for rabbit kidney phosphodiesterase, the standard reaction mixture contained in 0.60 ml: 3.0 μmol of cyclic nucleotide; 30 μmol of Tris-HCl (pH 7.5); 6 μmol of MgCl₂; and 0.1–0.3 mg of phosphodiesterase protein. After an appropriate incubation period (usually 10–60 min), the reaction was terminated by heating and treated with bacterial alkaline phosphatase, and the phosphate released was assayed colorimetrically (Lowry and López, 1946). The details of the phosphodiesterase substrate and inhibition assays have been previously described (Miller et al., 1973a).

The assay for the stimulation of both the cAMP-dependent and the cGMP-dependent protein kinases contained in 0.1 ml: 5 μmol of NaOAc (pH 6.0); 1 μmol of MgCl₂; 20 μg of histone (Worthington HLY); 0.5 nmol of [γ -³²P]ATP; protein kinase enzyme (20–200 μg); and various concentrations of the 3',5'-cyclic nucleotide being tested as an activator (10⁻⁹–10⁻³ M). After a suitable incubation time to give kinetically valid data (4–20 min), the reaction mixtures were processed as previously described (Miller et al., 1973b).

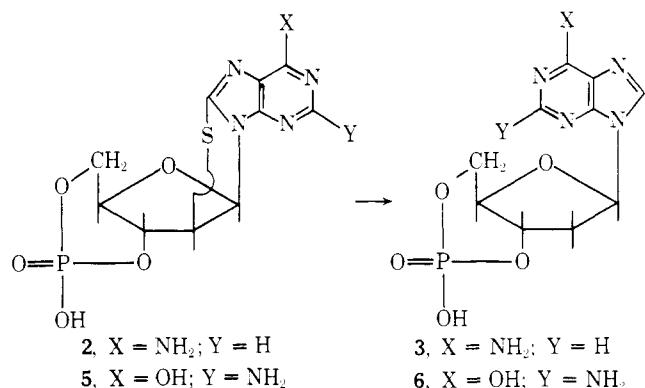
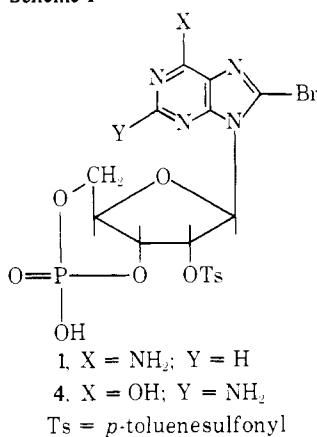
Chemistry. The synthesis of the cyclic 3',5'-phosphates of deoxyadenosine and deoxyguanosine by N,N'-dicyclohexylcarbodiimide-mediated cyclization of the corresponding 5'-phosphates was first reported by Drummond et al. (1964). We report here a new synthesis of these compounds from the parent cAMP and cGMP via desulfurization of the corresponding 8,2'-anhydro-9-β-D-arabinofuranosyl-8-mercaptopurine cyclic 3',5'-phosphates, as shown in Scheme I. A similar route has previously been used in the synthesis of 5'-dAMP and 5'-AMP by Ikehara and Uesugi (1970).

Treatment of the previously reported (Mian et al., 1974) 8Br-O²-Ts-cAMP (**1**) with NaSH in dimethylformamide gave the intermediate 8HS-8,2'-anhydro-ara-cAMP (**2**), which was not isolated but was converted directly to dcAMP (**3**) by treatment with Raney nickel.

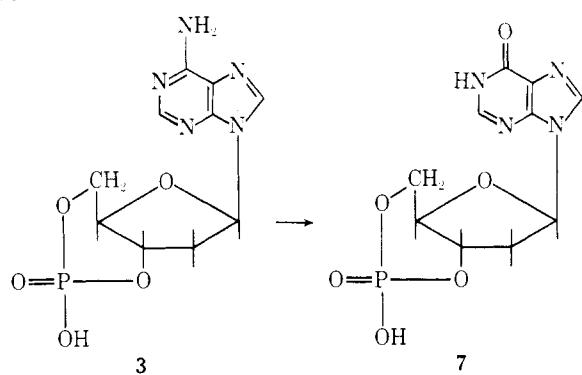
In a similar fashion, 8Br-O²-Ts-cGMP (**4**) was converted into the thioanhydronucleotide (**5**) with thiourea, then

¹ Ara-cIMP was first synthesized by A. M. Mian by this same method (G. R. Revankar, personal communication).

Scheme I



Scheme II

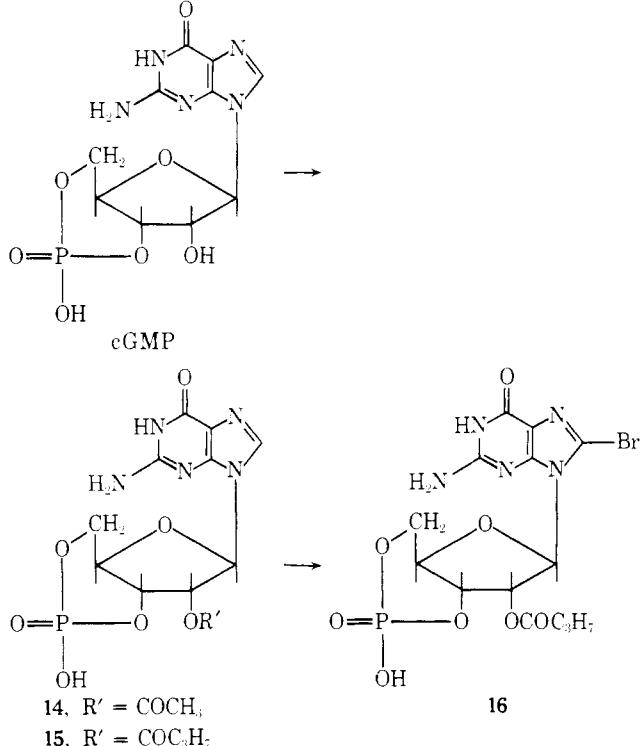
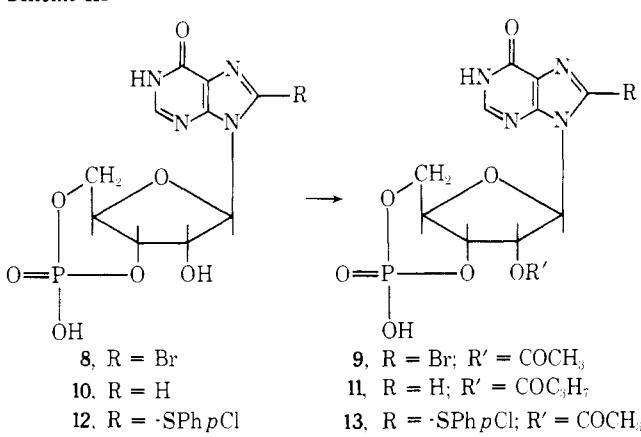


desulfurized with Raney nickel to give dcGMP (6).

Deamination of dcAMP (3) with NaNO₂ in aqueous acetic acid (Meyer et al., 1972) gave the corresponding derivative of hypoxanthine, dcIMP² (7) (Scheme II).

The customary procedure of acylation using acetic anhydride in pyridine with a trialkylammonium salt of the cyclic nucleotide converted 8Br-cIMP (8) to its *O*^{2'}-acetyl derivative (9). As a general method of acylation, however, the use of 4-dimethylaminopyridine as a catalyst in conjunction with the carboxylic anhydrides was found to be superior to the former methods because work-up was facilitated. This reagent has been used for rapid acylation of sterically hindered tertiary alcohols (Höfle and Steglich, 1972). Thus the sodium salt of cIMP (10) was converted to its *O*^{2'}-butyryl derivative (11) in dimethylformamide-butyric anhydride with a catalytic amount of 4-dimethylaminopyridine. Like-

Scheme III



wise 12 was acetylated as its triethylammonium salt with Ac₂O-dimethylformamide containing a catalytic amount of 4-dimethylaminopyridine to give 13.

The synthesis of the 8-bromo-*O*^{2'}-acyl derivatives of cGMP was accomplished from cGMP. The sodium salt of cGMP (as a crystalline tetrahydrate) was readily acetylated or butyrylated with the corresponding acyl anhydride and a catalytic amount of 4-dimethylaminopyridine in dimethylformamide to give *O*^{2'}-Ac-cGMP (14) and *O*^{2'}-Bt-cGMP (15), respectively. Bromination of 15 with Br₂ in H₂O gave 8Br-*O*^{2'}-Bt-cGMP (16). These reactions are illustrated in Scheme III.

Results and Discussion

Activation of cGMP- and cAMP-Dependent Protein Kinases. Each of the 2' and 8,2' derivatives were examined for their ability to activate the cGMP-dependent protein kinase from lobster tail muscle and the cAMP-dependent protein kinase from bovine brain. These results are compared in Table II. All of the analogues containing a 2' or 8,2' modification were less than 1/100 as active as either cAMP and cGMP as activators of their respective kinase. These results

² 7 was first synthesized by Drummond et al. (1964) by the cyclization of dIMP.

Table II: Activation of cAMP- and cGMP-Dependent Protein Kinases by 2' and 8,2' Derivatives of cGMP and cIMP.^a

Compound	Lobster Muscle cGMP-Dependent Protein Kinase	Bovine Brain cAMP-Dependent Protein Kinase
	K_a for cGMP/ K_a for Derivative	K_a for cAMP/ K_a for Derivative
cAMP	0.015	1.0
cGMP	1.0	0.023
dcGMP (6)	0.005	0.002
ara-cGMP	0.009	0.001
$O^{2'}\text{-Ac-cGMP}$ (14)	0.002	0.001
$O^{2'}\text{-Bt-cGMP}$ (15)	0.006	0.003
$N^2, O^{2'}\text{-Bt}_2\text{cGMP}$	0.005	0.0009
$N^2\text{-Bt-cGMP}$	0.029	0.001
8HS-8,2'-anhydro-ara-cGMP (5)	0.001	0.0007
8HS-ara-cGMP	0.001	0.0008
8Br- $O^{2'}\text{-Bt-cGMP}$ (16)	0.006	0.001
8Br- $O^{2'}\text{-Ts-cGMP}$ (4)	0.003	0.002
cIMP	0.085	0.59
dcIMP (7)	0.001	0.002
ara-cIMP	0.0007	0.0005
$O^{2'}\text{-Bt-cIMP}$ (11)	0.0003	0.0008
8Br- $O^{2'}\text{-Ac-cIMP}$ (9)	0.003	0.001
8pClPhS- $O^{2'}\text{-Ac-cIMP}$ (13)	0.001	0.0005

^a K_a is the apparent activation constant determined using a Line-weaver-Burk plot. The K_a for the activation of cGMP-dependent protein kinase by cGMP was $2.0 \times 10^{-7} M$, and the K_a for the activation of the cAMP-dependent protein kinase by cAMP was $1.3 \times 10^{-7} M$. The assays were performed as described in Biochemical Methods.

extend our previously reported results on the inactivity of 2' derivatives of cAMP as activators of the bovine brain cAMP-dependent protein kinase (Miller et al., 1973a) which demonstrated that an unblocked 2'-OH in the ribo configuration was necessary for the activation of this kinase by cAMP and its derivatives.

The inactivity of the 8,2' derivatives (4, 5, 9, 13, 16, and 8HS-ara-cGMP) with the kinases is most interesting in light of the observation that 8Br-cGMP is four times more potent than cGMP as an activator of the cGMP-dependent kinase and that 8pClPhS-cIMP was almost as active as cGMP at activating the cGMP-dependent kinase and equal to cAMP at activating the cAMP-dependent kinase (Miller et al., 1973b). These results therefore show that the improvement in the ability to activate protein kinase caused by an 8-substitution on cGMP or cIMP (Miller et al., 1973b) is negated by the modification of the 2' position. The same conclusion with respect to cAMP was drawn from studies on 8,2' derivatives of cAMP (Khwaja et al., 1975).

The current interest in the physiological functions and mechanism of action of cGMP (for review see Goldberg et al., 1973) and the increasing use of $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$ in experiments to test the effect of exogenous cGMP (Puglisi et al., 1971; Krause et al., 1972; Goldberg et al., 1973; Silverman and Epstein, 1975) have led us to compare the cGMP-dependent protein kinase activities of $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$, $N^2\text{-Bt-cGMP}$, and $O^{2'}\text{-Bt-cGMP}$ (15) (Table II). $N^2\text{-Bt-cGMP}$ was more than an order of magnitude less active than cGMP with the cGMP-dependent kinase. This is in contrast to the essentially similar activities of cAMP and N^6 -butyryladenosine cyclic 3',5'-phosphate ($N^6\text{-Bt-cAMP}$) with a cAMP-dependent protein kinase from bovine brain (Miller et al., 1973a). This observation as well as other data

Table III: Hydrolysis of 2' and 8,2' Derivatives of cGMP and cIMP by Rabbit Kidney Phosphodiesterase.^a

Compound	Relative Rate of Hydrolysis	
	Relative to cAMP	Relative to Parent Cyclic Nucleotide
cAMP	1.0	
cGMP	0.53	1.0
dcGMP (6)	0.21	0.40
ara-cGMP	0.31	0.58
$O^{2'}\text{-Ac-cGMP}$ (14)	0.25	0.47
$O^{2'}\text{-Bt-cGMP}$ (15)	0.19	0.36
$N^2, O^{2'}\text{-Bt}_2\text{cGMP}$	0.08	0.15
$N^2\text{-Bt-cGMP}$	0.08	0.15
8HS-8,2'-anhydro-ara-cGMP (5)	0.06	0.11
8HS-ara-cGMP	0.07	0.13
8Br- $O^{2'}\text{-Bt-cGMP}$ (16)	0.08	0.15
8Br- $O^{2'}\text{-Ts-cGMP}$ (4)	<0.05	<0.1
cIMP	0.46	1.0
dcIMP (7)	1.21	2.6
ara-cIMP	0.53	1.2
$O^{2'}\text{-Bt-cIMP}$ (11)	0.55	1.2
8Br- $O^{2'}\text{-Ac-cIMP}$ (9)	<0.05	<0.1
8pClPhS- $O^{2'}\text{-Ac-cIMP}$ (13)	<0.05	<0.1

^a The rates of hydrolysis are expressed relative to that of cAMP (rate for cAMP/rate for derivative) which was 46 nmol of 5'-AMP formed per min per 390 μg of protein under the assay conditions described in Biochemical Methods.

(Drummond and Powell, 1970; Kaukel et al., 1972; Miller et al., 1973b) have lead to the conclusion that $N^6\text{-Bt-cAMP}$ is the active metabolite of $N^6, O^{2'}\text{-dibutyryladenosine cyclic 3',5'-phosphate}$ ($N^6, O^{2'}\text{-Bt}_2\text{cAMP}$). The data presented here indicate that, unless a concentration of $N^2\text{-Bt-cGMP}$ greater than approximately $10\mu\text{M}$ was produced in a cell after exposure to $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$, it would not be possible for $N^2\text{-Bt-cGMP}$ to be the active metabolite of $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$. If $10\mu\text{M}$ $N^2\text{-Bt-cGMP}$ could not be produced in a cell, then $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$ would elicit its physiological effect only after it has been converted to cGMP. These conclusions are only valid, of course, if the actions of cGMP are mediated by a protein kinase such as the one studied here, as has been suggested (Kuo et al., 1971). If indeed cGMP is the active metabolite of $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$, then any system in which the latter is used must be capable of deacylating it. These data show that $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$ should not be viewed as being congeneric with $N^6, O^{2'}\text{-Bt}_2\text{cAMP}$ and caution should be exercised when using $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$ to test the effects of exogenous cGMP in any experimental system. The lack of a response may simply indicate the lack of a deacylating activity or the presence of a high phosphodiesterase activity, which would degrade the cGMP as rapidly as it is generated from the $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$.

Hydrolysis by Cyclic Nucleotide Phosphodiesterase. Table III summarizes the results of a study on the hydrolysis of these derivatives by rabbit kidney phosphodiesterase. In general, the 2' derivatives of cGMP and cIMP are all hydrolyzed at significant rates by the phosphodiesterase. The rate of hydrolysis of 2'-deoxy- (6), $O^{2'}\text{-Ac}$ (14), and $O^{2'}\text{-Bt-cGMP}$ (15) relative to cGMP are very close to the respective rates of hydrolysis of 2'-deoxy-, $O^{2'}\text{-Ac}$, and $O^{2'}\text{-Bt-cAMP}$ relative to cAMP (Miller et al., 1973a).

The results with the 2' derivatives of cIMP are intriguing. Ara-cIMP and $O^{2'}\text{-Bt-cIMP}$ (11) are hydrolyzed at a slightly greater rate than cIMP. DcIMP (7) was hydrolyzed

Table IV: Inhibition of cAMP and cGMP Hydrolysis by 2' and 8,2' Derivatives of cGMP and cIMP.^a

Compound	I_{50} (μM)			
	cAMP Hydrolysis		cGMP Hydrolysis	
	Rabbit Lung	Beef Heart	Rabbit Lung	Beef Heart
cAMP			92	44
cGMP	86	3.0		
dcGMP (6)	57	4.0	10	4.4
ara-cGMP	49	39	74	120
$O^{2'}\text{-Ac-cGMP}$ (14)	160	3.0	9.4	4.7
$O^{2'}\text{-Bt-cGMP}$ (15)	200	4.4	6.9	3.8
$N^2, O^{2'}\text{-Bt}_2\text{cGMP}$	110	150	80	53
$N^2\text{-Bt-cGMP}$	180	67	22	29
8HS-8,2'-anhydro- ara-cGMP (5)	>1000	>1000	>1000	>1000
8HS-ara-cGMP	50	45	83	160
8Br- $O^{2'}\text{-Bt-cGMP}$ (16)	170	67	10	3.8
8Br- $O^{2'}\text{-Ts-cGMP}$ (4)	220	170	60	85
cIMP	100	3.8	18	12
dcIMP (7)	150	7.5	4.8	29
ara-cIMP	330	33	110	80
$O^{2'}\text{-Bt-cIMP}$ (11)	20	4.0	11	11
8Br- $O^{2'}\text{-Ac-cIMP}$ (9)	250	330	390	44
8pCIPhS- $O^{2'}\text{-Ac-cIMP}$ (13)	130	67	130	120

^aThe inhibition of cGMP and cAMP hydrolysis was performed as described in Biochemical Methods. The rates of cGMP hydrolysis for the uninhibited reactions were 3.7 pmol per 6 min per 140 μg of protein and 1.8 pmol per 8 min per 20 μg of protein for the lung and heart enzymes, respectively. The rates of cAMP hydrolysis for the uninhibited reactions were 2.0 pmol per 8 min per 200 μg of protein and 1.6 pmol per 8 min per 30 μg of protein for the lung and heart enzymes, respectively. The I_{50} is the concentration of compound that causes a 50% inhibition in the rate of the reaction.

at 2.5 times the rate of cIMP. A kinetic analysis of dcIMP (7) indicated that the K_m for this analogue with the phosphodiesterase was approximately equal to the K_m for cIMP (~ 5 mM) while the V_{max} for dcIMP (7) was two to three times the V_{max} for cIMP.

Even though the 2' derivatives of cGMP and cIMP are substrates for the phosphodiesterase, addition of 8-substituents (to yield 4, 5, 9, 13, 16, and 8HS-ara-cGMP) completely (4, 9, and 13) or substantially (5, 16, and 8HS-ara-cGMP) prevents the resulting 8,2' derivatives from serving as substrates for the enzyme. 8,2' derivatives of cAMP have also been shown to be resistant to enzymic hydrolysis (Khwaja et al., 1975). In these cases the allowable 2' modification was overruled by the unallowable 8-substitution.

The data on the hydrolysis rates for the butyrylated derivatives of cGMP ($N^2, O^{2'}\text{-Bt}_2\text{cGMP}$, $N^2\text{-Bt-cGMP}$, and $O^{2'}\text{-Bt-cGMP}$ [15]) show that both $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$ and $N^2\text{-Bt-cGMP}$ are only slowly hydrolyzed, while $O^{2'}\text{-Bt-cGMP}$ is hydrolyzed at a substantial rate.

Inhibition of cAMP and cGMP Hydrolysis. All of the derivatives were examined for their ability to inhibit the hydrolysis of both cAMP and cGMP by phosphodiesterases from rabbit lung and beef heart. The results are given in Table IV. Several general conclusions can be drawn from these data. (1) 2' derivatives were better inhibitors than the corresponding 8,2' derivatives (or $N^2, 2'$ derivatives, in the case of $N^2, O^{2'}\text{-Bt}_2\text{cGMP}$). In fact, 8HS-8,2'anhydro-ara-cGMP (5) was such a poor inhibitor that an I_{50} could not be determined. (2) $O^{2'}\text{-Acyl}$ derivatives and 2'-deoxy derivatives of cGMP and cIMP were similar in their inhibitory properties and in turn were better inhibitors than ara-cGMP and ara-cIMP, respectively. (3) In those cases where

there was a significant difference the 2' and 8,2' derivatives of cGMP and cIMP were better inhibitors of cGMP hydrolysis than of cAMP hydrolysis. Conclusions (1) and (2) are consistent with those drawn from a study on 8,2' derivatives of cAMP (Khwaja et al., 1975).

From the data presented here and from our previously reported studies (Miller et al., 1973a; Khwaja et al., 1975) two separate, but related, concepts are brought into focus. For the 2' and 8 positions, the structural requirements for the interaction of cAMP with the bovine brain cAMP-dependent protein kinase are generally the same as the structural requirements for the interaction of cGMP with the lobster tail muscle cGMP-dependent protein kinase. In addition, with both kinases, 8-substituents, in general, improve the protein kinase stimulating activity of the parent cyclic nucleotide, while 2'-modifications substantially eliminate this activity, and 8,2' derivatives are no more active than the corresponding 2' derivatives.

The cAMP-dependent kinase is specific for cAMP and 8-substituted cAMP analogues, and, in turn, the cGMP-dependent kinase is specific for cGMP and 8-substituted cGMP analogues (Miller et al., 1973b). In addition, studies on a series of 6-substituted and 2,6-disubstituted 9- β -D-ribofuranosylpurine cyclic 3',5'-phosphates have shown, as might be expected, that the cGMP-dependent kinase was highly specific for an amino group in the 2 position (Meyer et al., 1972, 1975). These observations show that these two kinases are essentially similar in those portions of their respective cyclic nucleotide binding sites that interact with the 8 and 2' positions. In addition, they are similar in their interaction with 8,2'-disubstituted cyclic nucleotide analogues. On the other hand, the portions of the binding sites on the cAMP-dependent and cGMP-dependent protein kinases that help to determine their cyclic nucleotide specificity would be expected to be different. The available data then suggest that it is those portions of the binding sites that interact with the 2 and 6 positions of the purine ring that determine at least in part the nucleotide specificity of the two protein kinases.

References

- Bauer, R. J., Swiatek, K. R., Robins, R. K., and Simon, L. N. (1971), *Biochem. Biophys. Res. Commun.* 45, 526.
- Boswell, K. H., Miller, J. P., Shuman, D. A., Sidwell, R. W., Simon, L. N., and Robins, R. K. (1973), *J. Med. Chem.* 16, 1075.
- Drummond, G. I., Gilgan, M. W., Reiner, E. J., and Smith, M. (1964), *J. Am. Chem. Soc.* 86, 1626.
- Drummond, G. I., and Powell, C. A. (1970), *Mol. Pharmacol.* 6, 24.
- DuPlooy, M., Michal, G., Weimann, G., Nelboeck, M., and Paoletti, R. (1971), *Biochim. Biophys. Acta* 230, 30.
- Goldberg, N. D., O'Dea, R. F., and Haddox, M. K. (1973), *Adv. Cyclic Nucleotide Res.* 3, 155.
- Höfle, G., and Steglich, W. (1972), *Synthesis*, 619.
- Ikehara, M., and Uesugi, S. (1970), *Tetrahedron Lett.*, 713.
- Jardetzky, C. D. (1962), *J. Am. Chem. Soc.* 84, 62.
- Kaukel, E., Mundhenk, K., and Hilz, H. (1972), *Eur. J. Biochem.* 27, 197.
- Khwaja, T. A., Boswell, K. H., Robins, R. K., and Miller, J. P. (1975), *Biochemistry*, 14, 4238.
- Khwaja, T. A., Harris, R., and Robins, R. K. (1972), *Tetrahedron Lett.*, 4681.
- Krause, E.-G., Halle, W., and Wollenberger, A. (1972),

Adv. Cyclic Nucleotide Res. 1, 301.

Kuo, J.-F. (1972), *Methods Md. Biol.* 3, 199.

Kuo, J.-F., Wyatt, G. R., and Greengard, P. (1971), *J. Biol. Chem.* 246, 7159.

Lowry, O. H., and López, J. A. (1946), *J. Biol. Chem.* 162, 421.

Meyer, R. B., Jr., and Miller, J. P. (1974), *Life Sci.* 14, 1019.

Meyer, R. B., Jr., Shuman, D. A., Robins, R. K., Bauer, R. J., Dimmitt, M. K., and Simon, L. N. (1972), *Biochemistry* 11, 2704.

Meyer, R. B., Jr., Uno, H., Shuman, D. A., Robins, R. K., Simon, L. N., and Miller, J. P. (1975), *J. Cyclic Nucleotide Res.* 1, 159.

Mian, A. M., Harris, R., Sidwell, R. W., Robins, R. K., and Khwaja, T. A. (1974), *J. Med. Chem.* 17, 259.

Miller, J. P., Beck, A. H., Simon, L. N., and Meyer, R. B., Jr. (1975), *J. Biol. Chem.* 250, 426.

Miller, J. P., Boswell, K. H., Muneyama, K., Simon, L. N., Robins, R. K., and Shuman, D. A. (1973b), *Biochemistry* 12, 5310.

Miller, J. P., Shuman, D. A., Scholten, M. B., Dimmitt, M. K., Stewart, C. M., Khwaja, T. A., Robins, R. K., and Simon, L. N. (1973a), *Biochemistry* 12, 1010.

Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry* 10, 2390.

Muneyama, K., Shuman, D. A., Boswell, K. H., Robins, R. K., Simon, L. N., and Miller, J. P. (1975), *J. Carbohydr. Nucleosides, Nucleotides* (in press).

Puglisi, L., Berti, R., and Paoletti, R. (1971), *Experientia* 27, 1187.

Revankar, G. R., Huffman, J. H., Allen, L. B., Sidwell, R. W., Robins, R. K., and Tolman, R. L. (1975), *J. Med. Chem.* 18, 721.

Silverman, P. M., and Epstein, P. M. (1975), *Proc. Natl. Acad. Sci. U.S.A.* 72, 442.

Simon, L. N., Shuman, D. A., and Robins, R. K. (1973), *Adv. Cyclic Nucleotide Res.* 3, 225.

The Synthesis and Properties of the Complete Complementary DNA Transcript of Ovalbumin mRNA[†]

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ABSTRACT: The synthesis of a complementary DNA copy (cDNA) of hen ovalbumin mRNA using AMV RNA-directed DNA polymerase was studied under different conditions of salt, deoxyribonucleotide concentrations, temperature, and time. It was observed that in the absence of monovalent cation at 46°C a complete transcript of ovalbumin mRNA could be effected by the enzyme. The minimum deoxyribonucleotide requirement for complete synthesis was 35 μM for dATP, dGTP, and dCTP and 200 μM dTTP. By a number of different experimental criteria which included sedimentation on alkaline sucrose gradients and electrophoresis in polyacrylamide gels containing 98%

formamide, direct electron microscope visualization, and protection of ovalbumin [125 I]mRNA from nuclease digestion it could be demonstrated that a considerable fraction of a complete mRNA transcript was indeed synthesized. The cDNA/ovalbumin mRNA hybrid had a T_m on hydroxylapatite of 92°C, indicating the synthesis of a RNA transcript with a high fidelity. When such a complete ovalbumin [3 H]cDNA was synthesized with a specific activity of 10^8 cpm/ μ g and hybridized to an excess of chick DNA, the kinetics of hybridization indicated that the cDNA was comprised of a nonrepetitive sequence.

RNA-directed DNA polymerases (reverse transcriptase) isolated from avian myeloblastosis virus (AMV) and Rous sarcoma virus (RSV) have been used to synthesize cDNAs¹ that are complementary to eucaryotic cell messenger RNAs

(Verma et al., 1972, 1974; Ross et al., 1972; Honjo et al., 1974; Thrall et al., 1974; Diggelman et al., 1973; Harris et al., 1973; Bishop et al., 1974). Such cDNAs have become widely employed as sensitive and specific hybridization probes in a variety of experiments designed to study gene frequency, the transcription of chromatin in vitro and HnRNA and mRNA metabolism (see review of Lewin, 1975; Rosen and O'Malley, 1975). It is generally observed that the average size of the cDNA product is smaller than that of the RNA template. For example with rabbit globin mRNA as template (10 S), cDNA products 5 S to 8 S have been described (Ross et al., 1972). Complementary DNA from MOPC 41 myeloma immunoglobulin light chain mRNA (14 S) had a major component of about 5 S (Honjo et al., 1974; Diggelman et al., 1973). With ovalbumin mRNA, cDNA has been described which was heterogeneous in size with an average sedimentation coefficient of 5 S

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¹ Abbreviations used are: cDNA_{ov}, ovalbumin complementary DNA; AMV, avian myeloblastosis virus; NT, nucleotides; dNTP, deoxyribonucleoside triphosphate; EDTA, disodium ethylenediaminetetraacetic acid; Hepes, N-2-hydroxypiperazine-N'-2-ethanesulfonic acid; Cot, the product of the total RNA or DNA concentration in moles of nucleotides per liter and the time in seconds.