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# Synthesis and Biochemical Studies of Various 8-Substituted Derivatives of Guanosine 3′,5′-Cyclic Phosphate, Inosine 3′,5′-Cyclic Phosphate, and Xanthosine 3′,5′-Cyclic Phosphate†

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ABSTRACT: Several 8-substituted derivatives of guanosine 3′,5′-cyclic phosphate (cGMP), inosine 3′,5′-cyclic phosphate (cIMP), and xanthosine 3′,5′-cyclic phosphate (cXMP) were synthesized and their biochemical properties compared to corresponding 8-substituted adenosine 3′,5′-cyclic phosphate (cAMP) derivatives. cGMP was brominated to give 8-bromo-cGMP which was subsequently used to synthesize *via* nucleophilic reactions, 8-hydroxy-, 8-dimethylamino-, 8-methylamino-, 8-benzylamino-, 8-*p*-chlorophenylthio-, 8-benzylthio-, and 8-methylthio-cGMP. Deamination of cGMP and 8-bromo-cGMP gave cXMP and 8-bromo-cXMP, respectively. 8-Bromo-, 8-azido-, 8-hydroxy-, 8-methylthio-, 8-ethylthio-, 8-benzylthio-, and 8-*p*-chlorophenylthio-cIMP were prepared by deamination of the respective 8-substituted cAMP. Hydrogenation of 8-azido-cIMP gave 8-amino-cIMP. Thiation of 8-bromo-cIMP gave 8-thio-cIMP.

The substituted cGMP derivatives were specific for lobster muscle cGMP-dependent protein kinase whereas the 8-substituted cAMP derivatives were specific for bovine brain cAMP-dependent protein kinase. The relative potency of the 8-substituted cGMP and cAMP analogs was Br, OH, SR > cyclic nucleotide > $NR_1R_2$. cIMP and its 8-substituted derivatives exhibited specificity for the cAMP-dependent protein kinase. Only 8-hydroxy-cIMP and 8-*p*-chlorophenylthio-cIMP were more effective than c-IMP at activating either protein kinase. The 8-substituted derivatives were tested for their ability to inhibit the hydrolysis of cAMP and cGMP by phosphodiesterases from beef heart and rabbit lung. Some cGMP derivatives were better inhibitors of cAMP hydrolysis than of cGMP hydrolysis, while some cAMP derivatives were better inhibitors of cGMP hydrolysis than of cAMP hydrolysis. cGMP hydrolysis was inhibited preferentially by cAMP and cIMP derivatives with aromatic 8 substituents, while the inhibition of cAMP hydrolysis did not show this preference. cXMP was hydrolyzed at a rate only 3% that of cAMP by rabbit kidney phosphodiesterase. Of the 8-substituted derivatives, 8-methylthio-GMP and 8-amino-cIMP were substrates for this enzyme, and were hydrolyzed very slowly.

Guanosine 3′,5′-cyclic phosphate[1] (cGMP) has been implicated in the cellular mediation of hormone action (Hardman *et al.*, 1966). Subsequent studies have shown that administration of acetylcholine resulted in the accumulation of cGMP in heart (George *et al.*, 1970), brain (Ferrendelli *et al.*, 1970), and ductus deferens (Schultz *et al.*, 1972). 8-Bromo-cGMP was able to mimic the action of the cholinergic agents in human lung fragments (Kaliner *et al.*, 1972), and a variety of 8-substituted cGMP and cIMP derivatives were able to affect blood glucose and corticosterone levels as well as blood pressure and heart rate in rats (Paoletti *et al.*, 1973).

Results suggesting that the physiological actions of cGMP are inverse to those of cAMP have been reported in smooth muscle (Puglisi *et al.*, 1971; Ball *et al.*, 1970), renal cortex (Goodman *et al.*, 1972; Pagliara and Goodman, 1969, 1970), lysosomes (Ignarro and Colombo, 1973), antigen-sensitive lymphocytes (Strom *et al.*, 1972), psoriatic lesions (Voorhees *et al.*, 1973), cultured heart cells (Krause *et al.*, 1972), and bacterial cell free extracts (Zubay *et al.*, 1970; Emmer *et al.*, 1970). Clonal proliferation of lymphocytes resulted in a 10–50-fold increase in cGMP levels, while cAMP levels were essentially unaffected (Hadden *et al.*, 1972). Also, it is now well established that increased levels of cAMP are associated with reduced growth rate and induction of differentiation (Van Wijk *et al.*, 1972, and references therein). The growth inhibitory effects of cAMP, which may be mediated in part by the inhibition of precursor transport into cells (Kram *et al.*, 1973), is antagonized by cGMP (Kram and Tomkins, 1973). The available data suggest that cAMP limits growth, possibly by promoting differentiation (Kram *et al.*, 1973), while cGMP stimulates growth at the expense of differentiation.

Toward the goal of developing useful biochemical agents for investigating the functions and mechanisms of action of cGMP and developing potential pharmacological agents, we here report the detailed methods of synthesis of 8-substituted derivatives of cGMP, cIMP (**12**), and cXMP (**10**). Previously,

† From ICN Pharmaceuticals, Inc., Nucleic Acid Research Institute, Irvine, California 92664. *Received August 6, 1973.* Portions of these studies were reported at The First International Conference on the Physiology and Pharmacology of Cyclic AMP, Milan, July 1971, and at The Fifth International Congress on Pharmacology, San Francisco, Calif., July 1972.

[1] Abbreviations used are: cAMP (**22**), adenosine 3′,5′-cyclic phosphate; cGMP (**1**), guanosine 3′,5′-cyclic phosphate; cIMP (**12**), inosine 3′,5′-cyclic phosphate; cXMP (**10**), xanthosine 3′,5′-cyclic phosphate. dbcAMP and dbcGMP are $N^6$,2′-*O*-dibutyryladenosine 3′,5′-cyclic phosphate and $N^2$,2′-*O*-dibutyrylguanosine 3′,5′-cyclic phosphate, respectively.

methods for the direct introduction of various substituents into the 8 position of purine nucleosides and nucleotides have been developed (Holmes and Robins, 1964, 1965; Long *et al.*, 1967; Ikehara *et al.*, 1965a,b, 1969; Ikehara and Muneyama, 1966; Ikehara and Uesugi, 1969). Recently, the usefulness of these methods was further extended with the synthesis of various 8-substituted adenosine 3′,5′-cyclic phosphates (Ikehara and Uesugi, 1969; Muneyama *et al.*, 1971; Posternak *et al.*, 1971; Tesser *et al.*, 1972; Boehringer Mannheim G.m.b.H, 1973; Ikehara *et al.*, 1973). As a continuation of our study on the synthesis and biological activity of nucleoside 3′,5′-cyclic phosphates, we explored the syntheses of 8-substituted guanosine, inosine, and xanthosine 3′,5′-cyclic phosphates.

The present studies relate to structure–activity relationships with respect to the ability of these compounds (1) to activate partially purified cGMP- and cAMP-dependent protein kinases, (2) to serve as substrates for 3′,5′-cyclic nucleotide phosphodiesterase, and (3) to inhibit the hydrolysis of cAMP and cGMP. The biological activities of these derivatives will be compared with those of similarly substituted cAMP derivatives.

## Experimental Section

*Chemical Syntheses.* Tlc samples were dissolved in 0.1 N $NH_4OH$ and developed on Merck cellulose F plates with either solvent system A ($CH_3CN$–0.1 M $NH_4Cl$, 7:3), or B (*n*-$C_4H_9OH$–HOAc–$H_2O$, 5:2:3), where $R_A$ and $R_B = R_F$ compound/$R_F$ reference in the respective solvent system. Evaporations were performed under diminished pressure at <40°. Unless otherwise stated, analytical samples were dried at 80–100° (0.01 mm) for 12 hr. Compounds were desalted by absorption onto charcoal (Barnebey-Cheney, UU 50–200 mesh) and by elution with $H_2O$–EtOH–$NH_4OH$ (1:1:0.1). The 8-substituted cAMP derivatives described in the present paper have previously been reported by Muneyama *et al.* (1971). cIMP (**12**) was prepared as previously described by Meyer *et al.* (1972).

*8-Bromoguanosine 3′,5′-Cyclic Phosphate* (**2**). To a suspension of cGMPNa·$4H_2O$ (**1**, 70.4 g, 0.16 mol) in 300 ml of $H_2O$ was added, over a 3-min period, a solution of bromine (9.3 ml) in 900 ml of $H_2O$. The solution was stirred for a further 15 min and then purged vigorously for 3 hr with $N_2$. The resulting solution was then passed through a Dowex 50-X8 (pyridinium, 100–200 mesh, 7 × 21 cm) column and the nucleotide was eluted with $H_2O$. The aqueous eluent was concentrated to *ca.* 100 ml and 200 ml of EtOH was added to cause crystallization. The crystals were filtered and washed with 70% aqueous EtOH to give 60 g (72% of the pyridinium salt of **2**. *Anal.* Calcd for $C_{10}H_{11}BrN_5O_7P \cdot C_5H_5N \cdot H_2O$: C, 34.56; H, 3.48; N, 16.12; Br, 15.33. Found: C, 34.64; H, 3.39; N, 15.95; Br, 15.40. The sodium salt of **2** was obtained by passing an aqueous solution of the pyridinium salt of **2** through a Dowex 50-X8 ($Na^+$, 100–200 mesh) column. The aqueous eluent was evaporated to a small volume and 2 volumes of EtOH was added to precipitate **2** ($Na^+$). *Anal.* Calcd for $C_{10}H_{10}BrN_5O_7PNa \cdot 0.75H_2O$: C, 26.13; H, 2.52; N, 15.23; Br, 17.38. Found: C, 26.46; H, 2.90; N, 15.01; Br, 17.30. The free acid **2** was obtained by passing an aqueous solution of the $Na^+$ or pyridinium salt of **2** through a Dowex 50-X8 ($H^+$) column: $\lambda_{max}^{pH\ 1}$ 259 nm, 256 sh (ε 21,200, 19,400), $\lambda_{max}^{pH\ 11}$ 260 nm (ε 19,200); $R_A$ 1.10, $R_B$ 0.78.

*8-Dimethylaminoguanosine 3′,5′-Cyclic Phosphate* (**3**). 8-Bromo-cGMP (**2**, 1.0 g, 2.36 mmol) and anhydrous dimethylamine (12 ml) in 30 ml of MeOH was stirred in a bomb at 120° for 17 hr. The solution was passed through a Dowex 50-X8 ($H^+$, 100–200 mesh, 3 × 16 cm) column. Coevaporation of the aqueous eluent with EtOH gave 0.69 g (71%) of crystalline **3**: $\lambda_{max}^{pH\ 1}$ 262 nm, 290 sh (ε 18,200, 11,900), $\lambda_{max}^{pH\ 11}$ 265 nm (ε 16,200); $R_A$ 1.30, $R_B$ 0.91.

*Anal.* Calcd for $C_{12}H_{17}N_6O_7P \cdot 1.25H_2O$: C, 35.08; H 4.78; N, 20.45. Found: C, 35.21; H, 5.09; N, 20.06.

*8-Methylaminoguanosine 3′,5′-Cyclic Phosphate* (**4**). 8-Bromo-cGMP (**2**, 1.0 g, 2.36 mmol) was treated with anhydrous methylamine as in the procedure for **3** and chromatographed on Dowex 50-X8 ($H^+$, 100–200 mesh, 3 × 16 cm) column. The column was eluted with EtOH–0.1 N HCl (1:1), and the eluent was codistilled with EtOH to a white solid. The solid was triturated with EtOH and filtered to give 0.43 g (43%) of **4**: $\lambda_{max}^{pH\ 1}$ 253 nm, 290 (ε 16,100, 9000), $\lambda_{max}^{pH\ 7}$ 258 nm, 297 (ε 15,300, 7700), $\lambda_{max}^{pH\ 11}$ 260 nm (ε 14,700); $R_A$ 0.76, $R_B$ 0.79.

*Anal.* Calcd for $C_{11}H_{15}N_6O_7P \cdot 3H_2O$: C, 30.84; H, 4.94; N, 19.62; P, 7.23. Found: C, 30.97; H, 4.67; N, 19.42; P. 6.98.

*8-Benzylaminoguanosine 3′,5′-Cyclic Phosphate* (**5**). 8-Bromo-cGMP (**2**, 1.5 g, 3.5 mmol), benzylamine (12 ml), and MeOH (90 ml) were stirred in a bomb at 105° for 14 days. The solution was evaporated and the residue partitioned between $CHCl_3$ (400 ml) and $H_2O$ (100 ml). The aqueous layer was applied to a Dowex 50-X8 ($H^+$, 100–200 mesh, 5 × 12 cm) column. The column was eluted with $H_2O$ to remove **5** (783 mg, 45%, after evaporation of appropriate fractions): $\lambda_{max}^{pH\ 1}$ 255 nm, 288 (ε 20,100, 10,000); $\lambda_{max}^{pH\ 11}$ 263 nm (ε 17,400); $R_A$ 2.49, $R_B$ 1.73.

*Anal.* Calcd for $C_{17}H_{19}N_6O_7P \cdot 3.5H_2O$: C, 39.77; H, 5.10; N, 16.36. Found: C, 39.66; H, 5.15; N, 16.43.

*8-p-Chlorophenylthioguanosine 3,′5′-Cyclic Phosphate, Sodium Salt* (**6**). An $H_2O$ (25 ml)–MeOH (500 ml) suspension of 8-bromo-cGMP (**2**, 10 g, 23.6 mmol), *p*-chlorobenzenethiol (3.7 g, 25.7 mmol), and NaOAc (4 g) was refluxed for 24 hr. The opaque solution was filtered and evaporated to a solid. The solid was triturated 3 × 100 ml with hot EtOH and then dissolved in a minimum amount of hot $H_2O$. The aqueous solution was cooled and filtered to yield 8.3 g (66%) of **6**: $\lambda_{max}^{pH\ 1}$ 275 nm (ε 21,500), $\lambda_{max}^{pH\ 11}$ 296 nm (ε 21,500); $R_A$ 2.85, $R_B$ 1.91.

*Anal.* Calcd for $C_{16}H_{14}ClN_5O_7PSNa \cdot 1.25H_2O$: C, 36.10; H, 3.12; N, 13.15; S, 6.02. Found: C, 36.18; H, 2.94; N, 13.32; S, 6.30.

*8-Hydroxyguanosine 3′,5′-Cyclic Phosphate, Ammonium Salt* (**7**). A solution of 8-bromo-cGMP (**2**, 10 g, 23.6 mmol), NaOAc (10 g, 18.5 mmol), glacial HOAc (250 ml), and $Ac_2O$ (50 ml) was refluxed for 12 hr. The solution was diluted to 2 l. and desalted by a charcoal column (5 × 21 cm). The aqueous EtOH–$NH_3$ eluent was kept at room temperature for 5 days and then at 60° for 35 hr. The solution was concentrated to 100 ml and added to a Dowex 50-X8 ($H^+$, 100–200 mesh, 6 × 48 cm) column. The column was washed with $H_2O$ and the first uv-absorbing fraction was discarded. The second fraction was concentrated, the pH adjusted to 7–8, and the solution added to Dowex 1-X2 (formate, 100–200 mesh, 4.5 × 10 cm). The column was washed with 500 ml of $H_2O$ and then the product was eluted with a gradient of 1 l. of $H_2O$ *vs.* 1 l. of 2.5 N $HCO_2H$–1.5 M $NH_4HCO_2$. The appropriate fractions were coevaporated with EtOH and passed through a Dowex 50 ($H^+$) column. The aqueous eluent was codistilled with EtOH to a solid. The solid was dissolved in dilute $NH_4OH$ and coevaporated with EtOH to give 2.4 g (26%) of **7**: $\lambda_{max}^{pH\ 1}$ 246 nm,

294 (ε 11,400, 9500); $\lambda_{max}^{pH\ 11}$ 248 nm, 278 (ε 10,000, 8700); $R_A$ 0.67, $R_B$ 0.46.

*Anal.* Calcd for $C_{10}H_{12}N_5O_8PNH_3 \cdot 1.5H_2O$: C, 29.63; H, 4.47; N, 20.73. Found: C, 29.54; H, 4.25; N, 20.48.

*8-Methylthioguanosine 3′,5′-Cyclic Phosphate* (**8**). A suspension of 8-bromo-cGMP (**2**, 0.88 g, 2.1 mmol) and thiourea (0.5 g, 6.6 mmol) in MeOH was refluxed for 26 hr. The solution was cooled in an ice bath, NaOMe (0.32 g, 5.9 mmol) was added, and then a MeOH (5 ml) solution of MeI (0.4 ml) was added dropwise. The solution was stirred overnight and codistilled with EtOH to a solid. The solid was precipitated from an aqueous solution with EtOH. The precipitate was dissolved in $H_2O$ and the pH of the solution was adjusted to 2 with concentrated HCl. EtOH was added to the solution which was then cooled to cause crystallization of 0.29 g (33%) of **8**: $\lambda_{max}^{pH\ 1}$ 273 nm (ε 17,700), $\lambda_{max}^{pH\ 11}$ 283 nm (ε 14,900); $R_A$ 1.09, $R_B$ 0.80.

*Anal.* Calcd for $C_{11}H_{14}N_5O_7PS \cdot 2H_2O$: C, 30.91; H, 4.24; N, 16.38; S, 7.50. Found: C, 30.84; H, 4.07; N, 16.19; S, 7.72.

*8-Benzylthioguanosine 3′,5′-Cyclic Phosphate* (**9**). A suspension of 8-bromo-cGMP (**2**, 3.7 g, 8.7 mmol) and thiourea (2.0 g, 26 mmol) in MeOH was refluxed for 12 hr. The solution was cooled in an ice bath and NaOMe (1.5 g, 28 mmol) and then 3.1 ml of α-bromotoluene were added. After 30 min, the solution was evaporated and the residue suspended in EtOH and filtered. The solid was dissolved in *ca.* 35 ml of warm $H_2O$ and filtered. The filtrate was cooled to room temperature and the pH adjusted to 2 with concentrated HCl to cause crystallization of 2.2 g (52%) of **9**: $\lambda_{max}^{pH\ 1}$ 273 nm, 290 sh (ε 13,800, 11,400); $\lambda_{max}^{pH\ 7}$ 277 nm (ε 15,700); $\lambda_{max}^{pH\ 11}$ 293 nm, 275 sh (ε 13,600, 9700); $R_A$ 2.11, $R_B$ 1.66.

*Anal.* Calcd for $C_{17}H_{18}N_5O_7PS \cdot H_2O$: C, 42.06; H, 4.15; N, 14.42. Found: C, 42.29; H, 4.19; N, 14.53.

*Xanthosine 3′,5′-Cyclic Phosphate* (**10**). To a solution of $cGMPNa \cdot 4H_2O$ (**1**, 2.0 g, 4.5 mmol) in 100 ml of 50% aqueous HOAc was added dropwise over a period of 30 min a solution (20 ml) of $NaNO_2$ (2.0 g, 29 mmol). After a further 90 min, ammonium sulfamate (2.5 g) was added, and the resulting solution was stirred overnight. The solution was desalted on a charcoal column and the eluate was evaporated to a solid. The solid was dissolved in 40 ml of $H_2O$ and the solution was added to a Dowex 50-X2 ($H^+$, 100–200 mesh, 4.5 × 75 cm) column. The column was eluted with $H_2O$ to remove three uv-absorbing compounds. The major peak was lyophilized to yield 800 mg (48%) of white **10**: $\lambda_{max}^{pH\ 1}$ 234 nm, 260 (ε 8500, 9900), $\lambda_{max}^{pH\ 11}$ 247 nm, 276 (ε 10,900, 9700); $R_A$ 0.98, $R_B$ 0.43.

*Anal.* Calcd for $C_{10}H_{11}N_5O_8P \cdot H_2O$: C, 32.98; H, 3.60; N, 15.38. Found: C, 33.05; H, 3.37; N, 15.63.

*8-Bromoxanthosine 3′,5′-Cyclic Phosphate* (**11**). To a suspension of 8-bromo-cGMP ($Na^-$) (**2**, 1.0 g, 4.5 mmol) in 30 ml of glacial HOAc was added $NaNO_2$ (1.0 g, 15 mmol) in 10 ml of $H_2O$. The resulting solution was stirred overnight at room temperature. The solvent was evaporated and the residue in 10 ml of $H_2O$ was passed through a Dowex 50-X2 ($H^+$, 100–200 mesh, 4.5 × 75 cm) column. The appropriate fractions were taken to dryness and codistilled with EtOH until dry. The final residue was dissolved in EtOH and 4 volumes of EtOAc was added to precipitate 320 mg (33%) of **11**: $\lambda_{max}^{pH\ 1}$ 250 nm, 263 (ε 12,300, 12,500), $\lambda_{max}^{pH\ 11}$ 262 nm, 280 sh (ε 13,500, 12,500); $R_A$ 0.82, $R_B$ 0.63

*Anal.* Calcd for $C_{10}H_{10}BrN_4O_8P$: C, 28.25; H, 2.37; N, 13.18. Found: C, 28.02; H, 2.56; N, 12.97.

*8-p-Chlorophenylthioadenosine 3′,5′*-Cyclic Phosphate (**34**). 8-Bromoadenosine 3′,5′-cyclic phosphate (**23**, 20.0 g, 50 mmol), *p*-chlorobenzenethiol 20 g, 0.14 mol), and NaOCH (13.1 g) were refluxed in 400 ml of absolute MeOH overnight and then allowed to cool to room temperature. The resulting solid was filtered and washed with 100 ml of MeOH followed by 400 ml of $Et_2O$. The solid was dissolved in hot (80–90°) $H_2O$ and acidified to pH 2 with 1 N HCl. After cooling, the precipitate was filtered, washed with $H_2O$, EtOH, and $Et_2O$, and then recrystallized from methoxyethanol to yield 18.7 g (81%) of **34**: $\lambda_{max}^{pH\ 1}$ 281 nm (ε 15,900); $\lambda_{max}^{pH\ 11}$ 283 nm (ε 13,750); $R_A$ 2.51, $R_B$ 1.68.

*Anal.* Calcd for $C_{16}H_{15}ClN_5O_7PS \cdot 0.5H_2O$: C, 39.96; H, 3.35; N, 14.56. Found: C, 40.07; H, 3.15; N, 14.34.

*8-Bromoinosine 3′,5′-Cyclic Phosphate* (**13**). NaOH (1 N) was added dropwise to a stirring suspension of 8-bromo-cAMP (**23**, 15.0 g, 36.7 mmol) in 50 ml of $H_2O$ until solution occurred. $NaNO_2$ (19.0 g, 0.28 mol) was added, followed by the dropwise addition of 30 ml of glacial HOAc. After the mixture was stirred overnight at room temperature, the solvent was evaporated and the residue codistilled twice with 20 ml of glacial HOAc. The final residue was taken up in 30 ml of $H_2O$ and passed through Dowex 50-X8 ($H^+$, 100–200 mesh, 700 ml) column. The column was eluted with $H_2O$, the uv-absorbing fractions were pooled, and the solvent was evaporated. The residue was codistilled twice with EtOH. The final solid was suspended in EtOH and filtered to give 10.5 g (70%) of **13**: $\lambda_{max}^{pH\ 1}$ 253 nm (ε 13,900); $\lambda_{max}^{pH\ 11}$ 258 nm (ε 12,600); $R_A$ 1.30, $R_B$ 0.84.

*Anal.* Calcd for $C_{10}H_{10}BrN_4O_7P$: C, 29.36; H, 2.46; N, 13.69. Found: C, 29.11; H, 2.45; N, 13.59.

*8-Azidoinosine 3′,5′-Cyclic Phosphate* (**14**). **14** was prepared from 8-azido-cAMP (**33**, 10 g, 27 mmol) and $NaNO_2$ (10 g, 0.14 mol) in 200 ml of $H_2O$ and 50 ml of glacial HOAc and passed through a Dowex 50-X8 ($H^+$, 100–200 mesh, 700 ml) column as in the procedure for compound **13**. The residue was codistilled with absolute EtOH until dry, then recrystallized from EtOH and dried at 50° to yield 6.65 g (56%) of **14**: $\lambda_{max}^{pH\ 1}$ 273 nm (ε 15,700); $\lambda_{max}^{pH\ 11}$ 280 nm (ε 13,000); $R_A$ 1.49, $R_B$ 0.93.

*Anal.* Calcd for $C_{10}H_{10}O_7N_8P \cdot 2H_2O$: C, 29.49; H, 2.97; N, 24.07. Found: C, 29.33; H, 3.19; N, 24.06.

*8-Hydroxyinosine 3′,5′-Cyclic Phosphate* (**15**). Glacial HOAc (50 ml) was added dropwise to a solution of 8-hydroxy-cAMP (**24**, 4 g, 1.2 mmol) and $NaNO_2$ (4g, 58 mmol) in 20 ml of $H_2O$. After the mixture was stirred for 2 days at room temperature, the solvent was evaporated and the residue codistilled once with $H_2O$ and dissolved in 80 ml of $H_2O$. This solution was loaded onto a column (2 cm i.d.) of 80 ml of Bio-Rex 5 (Bio-Rad) (formate, 100–200 mesh). The column was washed with $H_2O$ and then a 2-l. gradient of 0–4 N formic acid followed by 4 N formic acid. The appropriate fractions were pooled and evaporated to dryness <30°. The residue was codistilled four times with EtOH <30°. The final residue was dissolved in EtOH, 20 volumes of ether was added, and the precipitated solid was filtered and dried under high vacuum at room temperature to yield 2.3 g (52%) of **15**: $\lambda_{max}^{pH\ 1}$ 253 nm, 283 sh (ε 11,000, 5400); $\lambda_{max}^{pH\ 11}$ 263 nm (ε 11,700); $R_A$ 0.89, $R_B$ 0.55.

*Anal.* Calcd for $C_{10}H_{11}N_4O_8P \cdot 0.33H_2O \cdot 0.33C_2H_5OH$: C, 34.85; H, 3.74; N, 15.24. Found: C, 34.85; H, 3.69; N, 15.00.

*8-Methylthioinosine 3′,5′-Cyclic Phosphate* (**16**). **16** was prepared from 8-methylthio-cAMP (**26**, 4.0 g, 10.7 mmol) and $NaNO_2$ (5.0 g, 72 mmol) in 25 ml of $H_2O$ and 15 ml of glacial HOAc and then passed through a Dowex 50-X8 ($H^+$, 100–200 mesh, 400 ml) column, as in the procedure for compound **13**. The residue was codistilled twice with EtOH and refluxed in EtOH for 15 min and the resulting suspension cooled and

filtered to yield 3.25 g (81%) of **16**: $\lambda_{max}^{pH\ 1}$ 268 nm ($\epsilon$ 12,250); $\lambda_{max}^{pH\ 11}$ 275 nm ($\epsilon$ 12,050); $R_A$ 1.26, $R_B$ 0.91.

*Anal.* Calcd for $C_{11}H_{13}N_4O_7PS$: C, 35.11; H, 3.48; N, 14.89. Found: C, 34.92; H, 3.68; N, 14.77.

*8-Ethylthioinosine 3',5'-Cyclic Phosphate* (**17**). **17** was prepared from 8-ethylthio-cAMP (**27**, 2 g, 5.1 mmol) and $NaNO_2$ (2.5 g, 36 mmol) in 10 ml of $H_2O$ and 7 ml of glacial HOAc, and then passed through a Dowex 50-X8 ($H^+$, 100–200 mesh, 200 ml) column, as in the procedure for compound **13**. The residue was codistilled twice with EtOH, slurried in EtOH, and filtered to yield 0.68 g (34%) of **17**: $\lambda_{max}^{pH\ 1}$ 270 nm ($\epsilon$ 15,400); $\lambda_{max}^{pH\ 11}$ 277 nm ($\epsilon$ 15,400); $R_A$ 1.85, $R_B$ 1.32.

*Anal.* Calcd for $C_{12}H_{15}N_4O_7PS$: C, 36.92; H, 3.87; N, 14.35. Found: C, 37.03; H, 4.09; N, 14.14.

*8-Benzylthioinosine 3',5'-Cyclic Phosphate* (**18**). Glacial HOAc (50 ml) was added dropwise to a stirring solution of sodium 8-benzylthio-cAMP (**28**, 15 g, 33 mmol) and $NaNO_2$ (15 g, 0.22 mol) in 100 ml of $H_2O$. After the mixture was stirred overnight at room temperature, the solvent was evaporated and the residue codistilled twice with $H_2O$. The final residue was taken up into 20 ml of $H_2O$ by dropwise addition of 2 N NaOH. The solution was adjusted to pH 2.5 with 1 N HCl, and the solid which precipitated was filtered and washed with 20 ml of ice-cold $H_2O$ and then 20 ml of EtOH. The filtrate and washings were combined, evaporated to half-volume, and seeded. The subsequent crop of crystals was filtered and washed as before. The two crops of crystals were combined to yield 9.7 g (65%) of **18**: $\lambda_{max}^{pH\ 1}$ 276 nm ($\epsilon$ 13,100); $\lambda_{max}^{pH\ 11}$ 283 nm ($\epsilon$ 13,550); $R_A$ 2.13, $R_B$ 1.76.

*Anal.* Calcd for $C_{17}H_{17}N_4O_7PS$: C, 45.13; H, 3.78; N, 12.38; S, 7.08. Found: C, 45.00; H, 3.79; N, 12.49; S, 7.21.

*8-p-Chlorophenylthioinosine 3',5'-Cyclic Phosphate Ammonium Salt* (**19**). 8-*p*-Chlorophenylthio-cAMP (**34**, 15.0 g, 31.2 mmol) was dissolved in 300 ml of $H_2O$ by the addition of 2 N NaOH to pH 10 and warming on a steam bath. The solution was evaporated to dryness and 500 ml of glacial HOAc added. The suspension was stirred while $NaNO_2$ (15.0 g, 0.22 mol) in 30 ml of warm $H_2O$ was slowly added. After the mixture was stirred for 30 min, a second portion of $NaNO_2$ (5.0 g, 0.07 mol) was added. The resulting solution was stirred overnight and the solvent was evaporated. The residue was dissolved in 200 ml of 1:1 MeOH–$H_2O$ and passed through a Dowex 50-X8 ($H^+$, 100–200 mesh, 5 × 25 cm, prewashed with 1:1 MeOH–$H_2O$) column. The column was eluted with 1:1 MeOH–$H_2O$ and the uv-absorbing fractions were pooled and evaporated. The residue was codistilled with ethanol until it solidified. The solid was recrystallized from EtOH to give 9.6 g of the free acid of **19**. The ammonium salt was prepared by recrystallization of the free acid (1.0 g) from 0.1 N $NH_4OH$ (8 ml) to yield 0.6 g (40% overall) of **19**: $\lambda_{max}^{pH\ 1}$ 256 nm, 275 sh ($\epsilon$ 15,600, 15,000); $\lambda_{max}^{pH\ 11}$ 244 nm, 282 ($\epsilon$ 12,900, 15,800); $R_A$ 2.55, $R_B$ 1.54.

*Anal.* Calcd for $C_{16}H_{14}ClN_4O_7PSNH_3$: C, 39.23; H, 3.49; N, 14.29. Found: C, 39.25; H, 3.39; N, 14.07.

*8-Aminoinosine 3',5'-Cyclic Phosphate* (**20**). 8-Azido-cIMP (**14**, 1.0 g, 2.7 mmol) was dissolved in 50 ml of $H_2O$ by the addition of concentrated $NH_4OH$, then 250 mg of 10% Pd on charcoal was added. The suspension was shaken under 40 psi $H_2$ at room temperatue overnight. The catalyst was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in absolute MeOH, and a small amount of decolorizing charcoal was added. The suspension was filtered through diatomaceous earth. The filtrate was evaporated to dryness and the residue dissolved in $H_2O$ and the pH adjusted to 2 with 1 N HCl. The crystals which formed were filtered off and washed with $H_2O$ to yield 590 mg (69%) of **20**: $\lambda_{max}^{pH\ 1}$ 253 nm, 283 sh ($\epsilon$ 13,450, 4250); $\lambda_{max}^{pH\ 11}$ 261 nm ($\epsilon$ 14,300); $R_A$ 0.57, $R_A$ 0.58.

*Anal.* Calcd for $C_{10}H_{12}N_5O_7P$: C, 34.79; H, 3.50; N, 20.28. Found: C, 34.61; H, 3.64; N, 20.10.

*8-Thioinosine 3',5'-Cyclic Phosphate* (**21**). 8-Bromo-cIMP (**13**, 1.0 g, 2.5 mmol) and thiourea (0.38 g, 6.3 mmol) were refluxed overnight in 25 ml of absolute EtOH. The solvent was evaporated and the residue was dissolved in 20 ml of $H_2O$ and filtered and the filtrate evaporated to 5 ml. The solution was loaded onto a Dowex 50-X8 ($H^+$, 100–200 mesh, 200 ml) column. The column was eluted with $H_2O$ and the appropriate fractions were pooled and evaporated to dryness. The residue was stirred in MeOH until a solid formed. The solid was filtered and washed with MeOH to yield 300 mg (31%) of **21**: $\lambda_{max}^{pH\ 1}$ 291 nm ($\epsilon$ 13,450); $\lambda_{max}^{pH\ 11}$ 288 nm ($\epsilon$ 17,500); $R_A$ 1.16, $R_B$ 0.61.

*Anal.* Calcd for $C_{10}H_{11}N_4O_7PS \cdot 1.5H_2O$: C, 30.85; H, 3.62; N, 14.39. Found: C, 30.81; H, 3.67; N, 14.14.

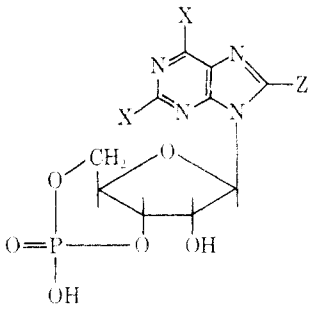
*Biological Methods. Enzyme Preparations.* The cAMP phosphodiesterases were purified from a 30,000*g* supernatant of beef heart, rabbit lung, or rabbit kidney. The procedure, which was used for all three tissues, involved $(NH_4)_2SO_4$ fractionation (the 0–50% of saturation fraction was used), and DEAE-cellulose chromatography (the protein eluting from the column between 0.08 and 0.4 M KCl was used). The details of the purifications have been reported previously (Miller *et al.*, 1973). Bovine brain cAMP-dependent protein kinase was purified through the DEAE-cellulose step as previously described (Kuo and Greengard, 1969a,b). Lobster tail muscle cGMP-dependent protein kinase was purified through the DEAE-cellulose step as described by Kuo and Greengard (1970). We have succeeded in obtaining this kinase only from lobster greater than 2 kg in weight.

*Enzyme Assays.* The assay for inhibition of cAMP hydrolysis by the rabbit lung and beef heart enzymes contained in 0.5 ml: 25 $\mu$mol of Tris-HCl (pH 7.5), 5 $\mu$mol of $MgCl_2$, 20–200 $\mu$g of phosphodiesterase protein, 80 nmol of 8-[$^3$H]cAMP (350,000 cpm), and varying concentrations of the 3',5'-cyclic nucleotide being tested as an inhibitor. The same conditions were used with both enzymes for the assay of the inhibition of cGMP hydrolysis, except that 160 nmol of 8-[$^3$H]cGMP (1,100,000 cpm) replaced the 8-[$^3$H]cAMP. The incubation times were determined from pilot assays to give kinetically valid data. The mixture was heat-inactivated to terminate the reaction, and treated with 5'-nucleotidase (crude *Crotalus atrox* venom) to convert the 5'-nucleotide to a nucleoside. The unreacted 3',5'-cyclic nucleotide was absorbed onto Dowex 1-X2 and the radioactivity of the nucleoside fraction determined.

When testing cAMP derivatives as substrates for rabbit kidney phosphodiesterase, the standard reaction mixture contasned in 0.60 ml: 3.0 $\mu$mol of cyclic nucleotide, 30 $\mu$mol of Tris-HCl (pH 7.5), 6 $\mu$mol of $MgCl_2$, 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. The reaction mixtures were also analyzed by tlc as previously described (Miller *et al.*, 1973). The details of the phosphodiesterase substrate and inhibition assays have been previously described (Miller *et al.*, 1973).

The assay for the stimulation of both the cAMP-dependent and the cGMP-dependent protein kinases contained in 0.1 ml: 5 $\mu$mol of NaOAc (pH 6.0), 1 $\mu$mol of $MgCl_2$, 20 $\mu$g of histone

TABLE I: Chemical Structures of 8-Substituted 3′,5′-Cyclic Nucleotide Analogs.



| Compd | X | Y | Z | Compd | X | Y | Z |
|---|---|---|---|---|---|---|---|
| **1** | OH | $NH_2$ | H | **18** | OH | H | $SCH_2C_6H_5$ |
| **2** | OH | $NH_2$ | Br | **19** | OH | H | S-*p*-$C_6H_4Cl$ |
| **3** | OH | $NH_2$ | $N(CH_3)_2$ | **20** | OH | H | $NH_2$ |
| **4** | OH | $NH_2$ | $NHCH_3$ | **21** | OH | H | SH |
| **5** | OH | $NH_2$ | $NHCH_2C_6H_5$ | **22** | $NH_2$ | H | H |
| **6** | OH | $NH_2$ | S-*p*-$C_6H_4Cl$ | **23** | $NH_2$ | H | Br |
| **7** | OH | $NH_2$ | OH | **24** | $NH_2$ | H | OH |
| **8** | OH | $NH_2$ | $SCH_3$ | **25** | $NH_2$ | H | SH |
| **9** | OH | $NH_2$ | $SCH_2C_6H_5$ | **26** | $NH_2$ | H | $SCH_3$ |
| **10** | OH | OH | H | **27** | $NH_2$ | H | $SC_2H_5$ |
| **11** | OH | OH | Br | **28** | $NH_2$ | H | $SCH_2C_6H_5$ |
| **12** | OH | H | H | **29** | $NH_2$ | H | $NH_2$ |
| **13** | OH | H | Br | **30** | $NH_2$ | H | $NHCH_3$ |
| **14** | OH | H | $N_3$ | **31** | $NH_2$ | H | $N(CH_3)_2$ |
| **15** | OH | H | OH | **32** | $NH_2$ | H | $NHCH_2C_6H_5$ |
| **16** | OH | H | $SCH_3$ | **33** | $NH_2$ | H | $N_3$ |
| **17** | OH | H | $SC_2H_5$ | **34** | NH | H | S-*p*-$C_6H_4Cl$ |

(Worthington HLY), 0.5 nmol of ATP-[$\gamma$-$^{32}$P], protein kinase enzyme (20–200 $\mu$g), 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), 0.05 ml of each reaction mixture was absorbed onto 2.3 cm diameter Whatman 3MM paper disks, and the disks were dried in a stream of cool air. The disks were then plunged into 10% $Cl_3CCO_2H$ (~2 ml/disk) and soaked for 10 min with occasional swirling. They were then washed in a similar manner sequentially in 5% $Cl_3CCO_2H$ (three times), EtOH, and $Et_2O$. After the disks were thoroughly dried, the radioactivity in each disk was determined in a liquid scintillation spectrometer, using 3 ml of a toluene solution of 0.5% 2,5-diphenyloxazole and 0.04% 2,2′-*p*-phenylenebis(5-phenyloxazole).

## Results

*Chemical.* Bromination of cGMP (**1**) (see Table I for structures) gave 8-bromo-cGMP (**2**) which was analyzed both as the $Na^+$ and as the crystalline pyridinium salts. Metathesis of the bromo group of 8-bromo-cGMP (**2**) occurred in good yield and with minimum side reactions when the free acid of 8-bromo-cGMP (**2**) was treated with sulfur, nitrogen, and oxygen containing nucleophiles at elevated temperatures. In this manner, treatment of a methanolic solution of 8-bromo-cGMP (**2**) with dimethylamine, methylamine, benzylamine, and *p*-chlorobenzenethiol gave the respective 8-substituted cGMP derivatives **3**, **4**, **5**, and **6**. 8-Hydroxy-cGMP (**7**) was prepared by the method of Ikehara *et al.* (1969) by the reaction of 8-bromo-cGMP (**2**) in refluxing NaOAc–glacial HOAc. Thiourea and cGMP gave as an intermediate 8-thio-cGMP which was then alkylated with methyl iodide and $\alpha$-bromotoluene to give 8-methylthio-cGMP (**8**) and 8-benzylthio-cGMP (**9**), respectively. cGMP and 8-bromo-cGMP (**2**) were deaminated by the conventional method of $NaNO_2$ in acetic acid to give xanthosine 3′,5′-cyclic phosphate (cXMP, **10**) and 8-bromo-cXMP (**11**). It should be noted that the uv spectra of 8-bromo-cXMP (**11**) was found to differ significantly from that of 8-bromoxanthosine reported by Holmes and Robins (1964)[2] and Long *et al.* (1967). These two literature procedures for 8-bromoxanthosine were repeated and were found to yield identical products (tlc, systems A and B) whose uv spectra were similar to our 8-bromo-cXMP (**11**).

As a further extension of 8-substituted cGMP analogs, 8-substituted cIMP compounds, which might be considered as 2-desamino-cGMP analogs, were prepared, where feasible, by deamination of previously reported (Muneyama *et al.*, 1971) and herein reported (*i.e.*, *p*-chlorophenylthio-cAMP, **34**) 8-substituted cAMP derivatives. By this method, 8-bromo-cIMP (**13**), 8-azido-cIMP (**14**), 8-hydroxy-cIMP (**15**), 8-methylthio-cIMP (**15**), 8-ethylthio-cIMP (**17**), 8-benzylthio-cIMP (**18**), and 8-*p*-chlorophenylthio-cIMP (**19**) were synthesized. 8-Amino-cIMP (**20**) and 8-thio-cIMP (**21**) were more readily accessible by hydrogenation of 8-azido-cIMP (**14**) to 8-

TABLE II: Activation of cGMP-Dependent and cAMP-Dependent Protein Kinases by 8-Substituted Derivatives of cGMP.

| Compd | Lobster Muscle cGMP-Dependent Protein Kinase[a] $K_a$ for cGMP/$K_a$ for Derivative | Bovine Brain cAMP-Dependent Protein Kinase[a] $K_a$ for cAMP/$K_a$ for Derivative |
|---|---|---|
| cGMP (**1**) | 1.0 | 0.023 |
| 8-Bromo-cGMP (**2**) | 4.0 | 0.037 |
| 8-Hydroxy-cGMP (**7**) | 2.5 | 0.030 |
| 8-Methylthio-cGMP (**8**) | 4.9 | 0.025 |
| 8-Benzylthio-cGMP (**9**) | 4.5 | 0.030 |
| 8-*p*-Chlorophenylthio-cGMP (**6**) | 5.2 | 0.058 |
| 8-Methylamino-cGMP (**4**) | 0.32 | 0.0012 |
| 8-Dimethylamino-cGMP (**3**) | 0.020 | 0.0036 |
| 8-Benzylamino-cGMP (**5**) | 0.005 | 0.0001 |

[a] $K_a$ is the activation constant determined using a Lineweaver–Burk plot. The $K_a$ for the activation of the cGMP-dependent protein kinase by cGMP was $1.7 \times 10^{-7}$ M, and the $K_a$ for the activation of the cAMP-dependent protein kinase by cAMP was $2.0 \times 10^{-7}$ M. The assays were performed as described in Biological Methods.

[2] The uv spectra and elemental analysis data for the product prepared by the method of Holmes and Robins (1964) were: $\lambda_{max}^{pH\ 1}$ 245 nm, 266 ($\epsilon$ 12,050, 11,900), $\lambda_{max}^{pH\ 11}$ 257, 280 (sh) ($\epsilon$ 9250, 11,550). *Anal.* Calcd for $C_{10}H_{11}BrN_4O_6 \cdot H_2O$: C, 31.51; H, 3.41; N, 14.70; Br, 20.96. Found: C, 31.85; H, 3.37; N, 14.75; Br, 21.11.

TABLE III: Activation of cGMP-Dependent and cAMP-Dependent Protein Kinases by 8-Substituted Derivatives of cAMP.[a]

| Compd | Bovine Brain cAMP-Dependent Protein Kinase $K_a$ for cAMP/$K_a$ for Derivative | Lobster Muscle cGMP-Dependent Protein Kinase $K_a$ for cGMP/$K_a$ for Derivative |
|---|---|---|
| cAMP (**22**) | 1.0 | 0.015 |
| 8-Bromo-cAMP (**23**) | 2.9 | 0.027 |
| 8-Hydroxy-cAMP (**24**) | 2.8 | 0.020 |
| 8-Thio-cAMP (**25**) | 3.8 | 0.017 |
| 8-Methylthio-cAMP (**26**) | 2.4 | 0.043 |
| 8-Ethylthio-cAMP (**27**) | 2.0 | 0.020 |
| 8-Benzylthio-cAMP (**28**) | 2.1 | 0.061 |
| 8-*p*-Chlorophenylthio-cAMP (**34**) | 18. | 0.098 |
| 8-Amino-cAMP (**29**) | 1.5 | 0.0003 |
| 8-Methylamino-cAMP (**30**) | 0.38 | 0.0003 |
| 8-Dimethylamino-cAMP (**31**) | 0.59 | 0.0011 |
| 8-Benzylamino-cAMP (**32**) | 0.023 | 0.0001 |
| 8-Azido-cAMP (**33**) | 0.68 | 0.0002 |

[a] All procedures and conditions were the same as in Table II. $K_a$ values for **24**, **25**, and **29** were reported by Muneyama *et al.* (1971).

TABLE IV: Activation of cGMP-Dependent and cAMP-Dependent Protein Kinases by 8-Substituted Derivatives of cIMP and cXMP.[a]

| Compd | Lobster Muscle cGMP-Dependent Protein Kinase $K_a$ for cGMP/$K_a$ for Derivative | Bovine Brain cAMP-Dependent Protein Kinase $K_a$ for cAMP/$K_a$ for Derivative |
|---|---|---|
| cIMP (**12**) | 0.085 | 0.59 |
| 8-Bromo-cIMP (**13**) | 0.020 | 0.14 |
| 8-Hydroxy-cIMP (**15**) | 0.71 | 0.85 |
| 8-Thio-cIMP (**21**) | 0.025 | 0.062 |
| 8-Methylthio-cIMP (**16**) | 0.11 | 0.16 |
| 8-Ethylthio-cIMP (**17**) | 0.090 | 0.32 |
| 8-Benzylthio-cIMP (**18**) | 0.22 | 0.17 |
| 8-*p*-Chlorophenylthio-cIMP (**19**) | 0.84 | 1.0 |
| 8-Amino-cIMP (**20**) | 0.0095 | 0.02 |
| 8-Azido-cIMP (**14**) | 0.030 | 0.067 |
| cXMP (**10**) | 0.0044 | 0.016 |
| 8-Bromo-cXMP (**11**) | 0.0045 | 0.0017 |

[a] All procedures and conditions were the same as in Table II, except that the $K_a$ for the activation of the cGMP-dependent protein kinase by cGMP was $1.4 \times 10^{-7}$ M, and the $K_a$ for the activation of the cAMP-dependent protein kinase by cAMP was $2.9 \times 10^{-7}$ M.

amino-cIMP (**20**), and by thiation of 8-bromo-cIMP (**13**) with thiourea to 8-thio-cIMP (**21**), respectively.

The structures and purities of the compounds reported were confirmed by elemental analysis, tlc in two systems, and by pmr and uv spectra.

*Biological.* The lobster muscle cGMP-dependent protein kinase and the bovine brain cAMP-dependent protein kinase are specific for their respective cyclic nucleotides (Kuo and Greengard, 1969a,b, 1970). The results in Table II show that the various 8-substituted derivatives of cGMP retained this specificity for the cGMP-dependent protein kinase. In addition, the 8-bromo (**2**), 8-hydroxy (**7**), 8-methylthio (**8**), 8-benzylthio (**9**), and 8-*p*-chlorophenylthio (**6**) analogs were better than cGMP (**1**) at activating the cGMP-dependent protein kinase. The 8-methylamino (**4**), 8-dimethylamino (**3**), and 8-benzylamino (**5**) analogs were less active than cGMP. For comparison, derivatives of cAMP with the same chemical substituents in the 8 position of the purine ring were analyzed for their ability to activate both the cAMP-dependent and the cGMP-dependent protein kinases. The results (Table III) show that the 8-substituted cAMP derivatives retain their specificity for the cAMP-dependent protein kinase.

The ability of 8-substituted derivatives of cIMP to stimulate the two protein kinases is summarized in Table IV. cIMP (**12**) had a $K_a$ only about twice that of cAMP with the cAMP-dependent protein kinase, but had a $K_a$ more than 10 times that of cGMP with the cGMP-dependent protein kinase. 8-Bromo-cIMP (**13**), like cIMP, was seven times more effective with the cAMP-dependent protein kinase than with the cGMP-dependent protein kinase. In addition, 8-ethylthio-cIMP (**17**) was approximately four times more effective with the cAMP-dependent protein kinase, and 8-thio-cIMP (**21**), 8-amino-cIMP (**20**), and 8-azido-cIMP (**14**) were twice as effective.

This slight specificity for the cAMP-dependent protein kinase was lost with 8-hydroxy-cIMP (**15**), 8-methylthio-cIMP (**16**), 8-benzylthio-cIMP (**18**), and 8-*p*-chlorophenylthio-cIMP (**19**), which were essentially equal in relative activity with the two protein kinases. In comparing the various cIMP analogs as activators of the cAMP-dependent protein kinase, only 8-hydroxy-cIMP (**15**) and 8-*p*-chlorophenylthio-cIMP (**19**) were more effective than cIMP. While all the other derivatives were less active than cIMP, the 8-bromo-cIMP (**13**), 8-alkylthio (**16**, **17**), and 8-arylthio (**18**, **19**) derivatives were more active than 8-amino-cIMP (**20**) and 8-azido-cIMP (**14**). When comparing these same analogs as activators of the cGMP-dependent protein kinase, essentially the same pattern of relative activity was seen.

Also shown in Table IV are the results with cXMP (**10**) and 8-bromo-cXMP (**11**). cXMP (**10**) was 40 times less active than cIMP with the cAMP-dependent protein kinase. 8-Bromo-cXMP (**11**) was an additional tenfold less active. In addition, cXMP (**10**) showed significant preference for the cAMP-dependent protein kinase, while 8-bromo-cXMP (**11**) was more active with the cGMP-dependent protein kinase.

The relative rates of hydrolysis of the cyclic nucleotide derivatives which were substrates for the rabbit kidney 3′,5′-cyclic nucleotide phosphodiesterase are shown in Table V. cGMP and cIMP were hydrolyzed at significant rates which were about half that of cAMP. cXMP (**10**) was hydrolyzed only very slowly by the rabbit kidney enzyme. We have also

TABLE V: Relative Rates of Hydrolysis of Various Cyclic Nucleotides by Rabbit Kidney Cyclic Nucleotide Phosphodiesterase.

| Compd | Rel Rate of Hydrolysis[a] |
|---|---|
| cAMP (**22**) | 1.0 |
| cGMP (**1**) | 0.53 |
| 8-Methylamino-cGMP (**4**) | 0.07 |
| cIMP (**12**) | 0.46 |
| 8-Amino-cIMP (**20**) | 0.08 |
| cXMP (**10**) | 0.03 |

[a] The rates of hydrolysis are expressed relative to that of cAMP which was 16 nmol of 5′-AMP formed per min per 230 μg of protein under the assay conditions described in Biological Methods.

found that 8-methylamino-cGMP (**4**) and 8-amino-cIMP (**20**) were hydrolyzed at a very slow but measurable rate by the rabbit kidney enzyme. No detectable hydrolysis products were seen after 60 min with any of the other derivatives. Increasing the enzyme two-, five-, and tenfold did not result in the formation of any measurable hydrolysis products. Examination of the reaction mixtures by tlc showed that none contained any detectable 5′ nucleotide.

We have found that bovine heart and rabbit lung contain 3′,5′-cyclic nucleotide phosphodiesterase activities with $K_m$ values for both cAMP and cGMP of 0.2–0.8 μM (J. P. Miller and L. N. Simon, in preparation). Table VI compares the ability of these 8-substituted analogs of cAMP, cGMP, cIMP, and cXMP to inhibit the hydrolysis of cGMP by these enzyme preparations. The concentration of the [$^3$H]cGMP substrate was 0.32 μM. The $I_{50}$ of cAMP was 200 times this concentration. With the lung enzyme, the $I_{50}$ for cAMP was approximately 300 times this concentration. cIMP compared to many of its 8-substituted analogs was one of the better inhibitors of cGMP hydrolysis by the two enzyme preparations, while cXMP (**10**) was a significantly less effective inhibitor. The results in Table VI also indicate that each of the 8-substituted derivatives of cAMP, cGMP, and cXMP were essentially equal in potency as inhibitors of cGMP hydrolysis. The few exceptions to this generalization require mention. 8-*p*-Chlorophenylthio-cAMP (**34**) was the best inhibitor of all the compounds tested with the lung enzyme. 8-*p*-Chlorophenylthio-cGMP (**6**), by comparison, was not a remarkable inhibitor of this enzyme. This same difference was seen to a lesser extent when comparing 8-benzylthio-cAMP (**28**) and 8-benzylamino-cAMP (**32**) with 8-benzylthio-cGMP (**9**) and 8-benzylamino-cGMP (**5**), respectively. In addition, 8-benzylthio-cIMP (**18**) was a better inhibitor than 8-benzylthio-cGMP (**9**), but not as effective as 8-benzylthio-cAMP (**28**). In general, the results indicated that regardless of the chemical structure of the parent cyclic nucleotide, the hydrolysis of cGMP was preferentially inhibited by compounds containing an aromatic substituent in the 8 position.

Table VII compares the ability of these 8-substituted analogs of cAMP, cGMP, cIMP, and cXMP to inhibit the hydrolysis of cAMP by these two enzyme preparations. The concentration of [$^3$H]cAMP substrate was 0.16 μM. The $I_{50}$ of cGMP or cIMP was about 20 times this concentration. With the lung enzyme, the $I_{50}$ for cGMP or cIMP was approximately 500 times this concentration. cXMP (**10**), which was a very poor substrate compared to cGMP and cIMP, had $I_{50}$ values 10 and 150 times those for either cGMP or cIMP with the lung and heart enzymes, respectively. The heart enzyme was more sensitive to inhibition by cGMP, cIMP, and cXMP (**10**) than was the lung enzyme. The large difference (25–30-fold) in $I_{50}$ values between the two enzymes for cGMP and cIMP was not seen with any of the 8-substituted derivatives of any of the four parent cyclic nucleotides.

Comparison of the data in Tables VI and VII in terms of $I_{50}$/[S] values revealed that half the compounds were essentially equal in their ability to inhibit cGMP hydrolysis and cAMP hydrolysis. Of the remaining compounds, some were significantly better inhibitors of cAMP hydrolysis than of cGMP hydrolysis when compared in terms of $I_{50}$/[S] ratios: 8-bromo-cAMP (**23**) and 8-*p*-chlorophenylthio-cGMP (**6**) with both lung and heart, 8-amino-cAMP (**29**) in lung only, and 8-amino-cIMP (**20**) in heart only (compare Tables VI and VII). At the same time, other compounds were significantly better inhibitors of cGMP hydrolysis than of cAMP hydrolysis when compared in terms of $I_{50}$/[S] ratios: 8-benzylamino-cAMP (**32**), 8-dimethylamino-cGMP (**3**), and cXMP (**10**) in lung and heart; 8-*p*-chlorophenylthio-cAMP (**34**), cIMP, and 8-bromo-cXMP (**11**) in lung only; and 8-ethylthio-cAMP (**27**) in heart only.

## Discussion

The various physiological functions of cGMP (**1**) and the mechanisms by which it elicits these functions are only beginning to be discovered and understood. Chemical analogs of cGMP which could enter cells, which would be resistant to enzymatic degradation, and which could specifically mimic the action of cGMP will undoubtedly prove to be powerful tools in investigations of the functions and mechanisms of action of cGMP. The significant activity of some of the cGMP derivatives in the histamine-induced bronchospasm and the passive cutaneous anaphylaxis systems suggests that the analogs are entering cells (M. Van Winkle and L. N. Simon, unpublished results). The activity of several 8-substituted derivatives of cAMP in isolated cells (Free *et al.*, 1971) and in tissue preparations (Rubin *et al.*, 1971) suggests that cyclic nucleotides do penetrate cells. With few exceptions, the 8-substituted derivatives of cGMP (**1**), cIMP (**12**), cXMP (**10**), and cAMP (**22**) were resistant to enzymatic hydrolysis by a rabbit kidney 3′,5′-cyclic nucleotide phosphodiesterase preparation. A number of the cGMP analogs were more effective than cGMP at activating the lobster muscle cGMP-dependent protein kinase and all of the cGMP analogs retained their specificity for this kinase. It is not known if the physiological actions of cGMP are mediated by a cGMP-dependent protein kinase, but the lobster muscle cGMP-dependent protein kinase was used because it presented an uncomplicated cell-free system that was specific for cGMP for the evaluation of those analogs.

Experiences in our laboratory with 8-substituted derivatives of cAMP as inducers of hepatic tyrosine aminotransferase (J. P. Miller and L. N. Simon, in preparation), as well as work from other laboratories with these same analogs on the effects on growth rate and enzyme induction (Van Wijk *et al.*, 1972), indicate that the 8-substituted derivatives of cAMP behave very similarly to cAMP, but produce more pronounced effects of longer duration due to their stability toward hydrolysis. On the basis of their ability to activate cGMP-dependent protein kinase, we might expect the 8-substituted analogs of cGMP to be more potent agonists of cGMP functions than cGMP.

All of the 8-substituted cGMP derivatives specifically

TABLE VI: Inhibition of cGMP Hydrolysis by 8-Substituted Derivatives of cAMP, cGMP, cIMP, and cXMP.[a]

| | 8-Substituted Cyclic Nucleotide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | cAMP | | cGMP | | cIMP | | cXMP | |
| 8 Substituent | Rabbit Lung $I_{50}$ ($\mu$M) | Beef Heart $I_{50}$ ($\mu$M) | Rabbit Lung $I_{50}$ ($\mu$M) | Beef Heart $I_{50}$ ($\mu$M) | Rabbit Lung $I_{50}$ ($\mu$M) | Beef Heart $I_{50}$ ($\mu$M) | Rabbit Lung $I_{50}$ ($\mu$M) | Beef Heart $I_{50}$ ($\mu$M) |
| -H | 140 | 68 | | | 18 | 12 | 520 | 180 |
| -Br | 870 | 150 | 610 | 95 | 1200 | 550 | 170 | 1400 |
| -OH | 620 | 1500 | 870 | 910 | 1900 | 1600 | | |
| -SH | 590 | 200 | | | 2300 | 820 | | |
| $-SCH_3$ | 450 | 88 | 470 | 440 | 1300 | 430 | | |
| $-SC_2H_5$ | 110 | 42 | | | 600 | 120 | | |
| $-SCH_2C_6H_5$ | 16 | 24 | 300 | 180 | 36 | 95 | | |
| -S-*p*-$C_6H_4Cl$ | 6.0 | 61 | 280 | 350 | 100 | 130 | | |
| $-NH_2$ | 1200 | 570 | | | 1300 | 3500 | | |
| $-NHCH_3$ | 380 | 200 | 350 | 390 | | | | |
| $-N(CH_3)_2$ | 910 | 390 | 170 | 60 | | | | |
| $-NHCH_2C_6H_5$ | 19 | 25 | 600 | 950 | | | | |
| $-N_3$ | 530 | 280 | | | 870 | 500 | | |

[a] The inhibition of cGMP hydrolysis was performed as described in Biological Methods. The rates for the uninhibited reactions were 3.3 pmol per 7 min per 120 $\mu$g of protein and 2.0 pmol per 7 min per 24 $\mu$g 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.

activated the lobster tail muscle cGMP-dependent protein kinase, while the 8-substituted cAMP derivatives specifically activated the bovine brain cAMP-dependent protein kinase. The relative potency of the 8-substituted cGMP analogs as activators of either the cGMP-dependent or cAMP-dependent protein kinase followed essentially the same trend: Br, OH, SR > cGMP > $NR_1R_2$. The 8-substituted cAMP analogs also demonstrated a similar order of potency with both protein kinases: Br, OH, SH, SR > cAMP > $NR_1R_2$, $N_3$.

When comparing ($K_a$ for cGMP)/($K_a$ for cIMP) with ($K_a$ for cAMP)/($K_a$ for cIMP) for the cGMP- and cAMP-dependent protein kinases, respectively, cIMP exhibited specificity for the cAMP-dependent protein kinase. A similar comparison showed that cXMP, although a much poorer activator of both kinases, was also more specific for the cAMP kinase. Addition of an 8 substituent to cIMP had a greater enhancing effect on the ability of the resulting derivatives to activate the cGMP-dependent protein kinase than it did on their ability to activate the cAMP-dependent protein kinase. A comparison of the effect of adding substituents at C-8 of cGMP on the ability of the resulting compounds to activate the cGMP-dependent protein kinase (Table II), with the effect of adding these same substituents to C-8 of cAMP, on the ability of the resulting compounds to activate the cAMP-dependent protein kinase (Table III) showed that any one particular substituent had about the same relative effect on the ability of the resulting cGMP and cAMP analog to activate its respective protein kinase. Together, these results indicate that 8-substituted derivatives of cIMP have a greater than expected activity with the cGMP-dependent protein kinase, and a lesser than expected activity with the cAMP-dependent protein kinase.

The acute specificity of the cAMP-dependent and the cGMP-dependent protein kinases for their respective cyclic nucleotide lies in the regulatory subunits of the two protein kinases (Miyamoto *et al.*, 1973). The similarity of both the 8-substituted cGMP and cAMP analogs in their relative abilities to activate either the cAMP-dependent or the cGMP-dependent protein kinase suggests that the binding sites on the regulatory subunits of these two kinases are very similar with respect to the effect of 8 substitution of the cyclic nucleotide. The relative potency of the 8-substituted derivatives of cIMP with the two protein kinases was different from that observed with the 8-substituted derivatives of cGMP and cAMP. The mechanism by which a particular 8 substituent on a purine 3′,5′-cyclic nucleotide affects the activation of a protein kinase is difficult to ascertain from these studies. The few examples studied suggest, however, that the effect is complex and involves more than a simple purine–carbohydrate conformational change imposed by the 8 substituent. Nevertheless, these studies do show that an 8 substituent on cGMP, cIMP, and cAMP can have a dramatic effect on protein kinase activation. This was best examplified by the 8-*p*-chlorophenylthio substituent which resulted in a one order of magnitude improvement in $K_a$ values.

The studies on the susceptibility of these analogs to hydrolysis by the rabbit kidney phosphodiesterase indicate that, while modifications in the 6 position (cIMP) did not drastically alter the susceptibility to hydrolysis, modification of both the 6 and the 2 position (cXMP) drastically decreased the ability of the cyclic nucleotide to serve as a substrate. Michal *et al.* (1970) have reported that cXMP was hydrolyzed to 20% the extent of cAMP under their experimental conditions by a bovine heart enzyme preparation. We have found that a preparation of bovine heart cyclic nucleotide phosphodiesterase prepared as previously described (Miller *et al.*, 1973) was able to hydrolyze cXMP at 9% the rate of cAMP under the same experimental conditions described in Biological Methods for the rabbit kidney enzyme. The fact that 8-methylamino-cGMP and 8-amino-cIMP were the only compounds of all the 8-substituted derivatives under study that served as substrates for the phosphodiesterase is interesting since 8-amino-cAMP was the only one of a number of 8-substituted derivatives of cAMP that was a substrate for the rabbit kidney enzyme (Muneyama *et al.*, 1971).

In examining the data presented on the inhibition of cGMP and cAMP hydrolysis by the analogs, it should be kept in mind that the $K_m$ values for both cAMP and cGMP with the

TABLE VII: Inhibition of cAMP Hydrolysis by 8-Substituted Derivatives of cAMP, cGMP, cIMP and cXMP.[a]

| | 8-Substituted Cyclic Nucleotide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | cAMP | | cGMP | | cIMP | | cXMP | |
| 8-Substituent | Rabbit Lung | Beef Heart | Rabbit Lung | Beef Heart | Rabbit Lung | Beef Heart | Rabbit Lung | Beef Heart |
| | $I_{50}$ ($\mu$M) | | $I_{50}$ ($\mu$M) | | $I_{50}$ ($\mu$M) | | $I_{50}$ ($\mu$M) | |
| –H | | | 87 | 3.0 | 100 | 3.9 | 1200 | 520 |
| –Br | 40 | 7.7 | 150 | 48 | 400 | 100 | 2300 | 3300 |
| –OH | 150 | 500 | 260 | 330 | 670 | 170 | | |
| –SH | 20 | 48 | | | 2000 | 250 | | |
| $-SCH_3$ | 69 | 24 | 80 | 67 | 1300 | 220 | | |
| $-SC_2H_5$ | 33 | 77 | | | 130 | 100 | | |
| $-SCH_2C_6H_5$ | 53 | 17 | 170 | 140 | 67 | 13 | | |
| $-S$-$p$-$C_6H_4Cl$ | 200 | 22 | 29 | 17 | 130 | 46 | | |
| $-NH_2$ | 83 | 33 | | | 100 | 84 | | |
| $-NHCH_3$ | 180 | 33 | 72 | 50 | | | | |
| $-N(CH_3)_2$ | 130 | 140 | 220 | 140 | | | | |
| $-NHCH_2C_6H_5$ | 150 | 110 | 59 | 170 | | | | |
| $-N_3$ | 80 | 43 | | | 400 | 200 | | |

[a] The inhibition of cAMP hydrolysis was performed as described in Biological Methods. The rates for the uninhibited reactions were 1.7 pmol per 10 min per 140 $\mu$g of protein and 1.9 pmol per 10 min per 38 $\mu$g of protein for the lung and heart enzymes, respectively. The $I_{50}$ is defined in Table VI.

enzyme preparations from bovine heart and rabbit lung were 0.2–0.8 $\mu$M (J. P. Miller and L. N. Simon, in preparation). cGMP was a more effective inhibitor of cAMP hydrolysis than was cAMP as an inhibitor of cGMP hydrolysis. The inhibition of cAMP hydrolysis by cGMP has been observed by others with enzyme preparations from bovine heart (Beavo *et al.*, 1970; Goren and Rosen, 1972). cAMP was a better inhibitor of cGMP hydrolysis by the heart than by the lung enzyme, while cIMP was an excellent inhibitor of both enzymes. Harris *et al.* (1973) reported that cGMP and cIMP were more potent inhibitors of the hydrolysis of cAMP by a cat heart preparation than were a group of 8-substituted derivatives of cAMP. In addition, they demonstrated that cGMP, cIMP, and 8-bromo-cAMP were all competitive inhibitors of the cat heart enzyme. At least part of the reason why cIMP was a good inhibitor of cGMP hydrolysis may be because cIMP could serve as an alternate substrate for the enzyme. With the exception of 8-amino-cAMP (Muneyama *et al.*, 1971), all the 8-substituted derivatives of cAMP, cGMP, cIMP, and cXMP were essentially not significant substrates, indicating that they could not be inhibiting cGMP hydrolysis by acting as alternate substrates for the enzymes. In fact, 8-amino-cAMP was one of the poorer inhibitors of cGMP hydrolysis among the 8-substituted cAMP derivatives. The results on the inhibition of cGMP and cAMP hydrolysis by the analogs showed that some of the cGMP derivatives were better inhibitors of cAMP hydrolysis than of cGMP hydrolysis, while some of the cAMP derivatives were better inhibitors of cGMP hydrolysis than of cAMP hydrolysis. It is important to note that in the experiments on the inhibition of cAMP hydrolysis, the concentration of [$^3$H]-cAMP substrate was only half the concentration of [$^3$H]cGMP used as substrate for the measurement of cGMP hydrolysis. In this respect, it is interesting to note that 50% of the $I_{50}$ values for cAMP hydrolysis were approximately half the $I_{50}$ values for the same compound as an inhibitor of cGMP hydrolysis, indicating no preference for inhibiting the hydrolysis of either cyclic nucleotide. The better inhibitors of cGMP hydrolysis were cAMP and cIMP derivatives with aromatic 8 substituents. Of all the compounds described here, 8-*p*-chlorophenylthio-cAMP (**34**) is the most intriguing, being approximately 67 times more active as an inhibitor of cGMP hydrolysis when compared in terms of $I_{50}$/[S] ratios. While the protein kinases showed strong preference for an 8-arylthio over an 8-arylamino substitution, this specificity was not seen with the phosphodiesterases.

The results on the inhibition of cAMP hydrolysis by these analogs are remarkable only in that there was so little variation in $I_{50}$ values between compounds and between enzymes for these 8-substituted analogs. This lack of variation is significant when compared with the striking differences in $I_{50}$ values for inhibition of cGMP hydrolyxis between similarly substituted compounds of different parent cyclic nucleotides, and differences between the lung and heart enzymes for particular compounds. In addition, the preferential inhibition of cGMP hydrolysis by compounds containing an aromatic 8 substitution was not seen with cAMP hydrolysis. These comparisons suggest there are significant differences between the cAMP and cGMP phosphodiesterase activities of both bovine heart and rabbit lung.

## Acknowledgments

We thank Ms. Mieka Scholten, Ms. Mary Dimmitt, and Mr. Randy Bauer for their valuable technical assistance, Dr. James Drummond for his help in obtaining New England lobster that would yield the cGMP-dependent protein kinase, and Dr. Rich Meyer for helpful discussions during the preparation of this report.

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