Synthesis and Enzymic Activity of 8-Acyl and 8-Alkyl Derivatives of Guanosine3′,5′-Cyclic Phosphate†

Leon F. Christensen,* Rich B. Meyer, Jr., Jon P. Miller, Lionel N. Simon, and Roland K. Robins

ABSTRACT: Several new 8-alkyl and 8-acyl derivatives of guanosine 3′,5′-cyclic phosphate (cGMP) and inosine 3′,5′-cyclic phosphate (cIMP) were prepared by direct alkylation or acylation of the parent cyclic nucleotide via free radicals generated in situ. These compounds have been examined for their ability to stimulate a cGMP-dependent protein kinase, and several of the cGMP derivatives were as active in this regard as cGMP. These compounds proved to be quite ineffective when tested for their ability to activate an adenosine 3′,5′-cyclic phosphate (cAMP) dependent protein kinase. In addition, these 8-substituted cGMP derivatives are not substrates for a phosphodiesterase preparation from rabbit kidney, but do show inhibition of the hydrolysis of cAMP by crude phosphodiesterase preparations from rabbit lung and beef heart.

The various biochemical functions of guanosine 3′,5′-cyclic phosphate (cGMP) are currently under elucidation in numerous laboratories. Cholinergic stimuli in vivo are associated with increased tissue levels of cGMP and exogenously added cGMP can produce cholinomimetic responses (George et al., 1970; Kuo et al., 1972; Ferrendelli et al., 1970; Puglisi et al., 1971; Kaliner et al., 1972a,b; Lee et al., 1972). cGMP is also implicated in the growth control of cells (Hadden et al., 1972; Kram and Tomkins, 1973; Seifert and Rudland, 1974). In general, cGMP stimulates cell growth while cAMP inhibits cell growth and stimulates cell differentiation. This apparent antagonism between the cellular events mediated by cGMP and those mediated by cAMP has been observed in a number of physiological systems (see Miller et al., 1973, and references cited therein). Most recently, evidence has been presented that is consistent with an involvement of cGMP in the regulation of prostaglandin synthesis and release (Stoner et al., 1973).

Our laboratory (Miller et al., 1973) and others (Paoletti et al., 1973) have recently reported the synthesis of a number of 8-alkylthio-, 8-arylthio-, and 8-alkylamino-cGMP derivatives, along with 8-hydroxy- and 8-bromo-cGMP, some of which have shown the ability to selectively stimulate a purified cGMP-dependent protein kinase from lobster tail but did not activate the cAMP-dependent kinase from bovine brain (Miller et al., 1973). Many of these compounds were, in addition, resistant to hydrolysis by cyclic nucleotide phosphodiesterase.

We now wish to report the synthesis of a novel class of cGMP analogs, e.g., 8-alkyl- and 8-acyl-cGMP derivatives, which act as activators of cGMP and cAMP dependent protein kinases and as substrates and inhibitors of phosphodiesterases. The homolytic alkylation and acylation procedures employed in the synthesis of compounds reported here may have implications in the understanding of possible chromosome damage by free radicals.

Experimental Section

Synthetic. Tlc samples were dissolved in 0.1 *N* NH_4OH and developed on Woelm silica gel F-254 plates with either solvent system A (CH_3CN–0.1 *N* NH_4Cl, 8:2), or B (*n*-BuOH–HOAc–H_2O, 5:2:3). Evaporations were performed under diminished pressure at <40°. Compounds were desalted by adsorption on charcoal (Barnebey-Cheney UU, 50–200 mesh), washing with water, and elution with H_2O–EtOH–NH_4OH (50:45:5). Chromatography on Dowex 50-X2 (100–200 mesh, H^+) resin was performed on a standard 4.5 × 75 cm column unless otherwise stated. Column eluates were monitored at 254 and 313 nm. The ultraviolet spectra reported in Table I were determined on a Cary 15 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. ^1H nuclear magnetic resonance (NMR) spectra in Me_2SO-d_6 or D_2O were recorded on a Hitachi-Perkin-Elmer R-60A with sodium 2,2-dimethyl-2-silapentanesulfonate as the internal reference and are reported in Table I.

8-Methylguanosine 3′,5′-Cyclic Phosphate (**2**). To a precooled (10°) solution of cGMP · Na · $4H_2O$ (**1**, 15 g, 34.1 mmol) in 3000 ml 0.3 *N* H_2SO_4 were added simultaneously and dropwise, solutions of $FeSO_4 \cdot 7H_2O$ (56.8 g, 204 mmol, in 340 ml of H_2O) and *tert*-butyl hydroperoxide (*t*-BuOOH) (10.9 ml, 136 mmol, in 340 ml of H_2O). Addition required 1.5 hr, after which the stirring was continued 0.5 hr. The mixture was then desalted using a column containing 400 ml of charcoal. The eluate was evaporated, then diluted to 100 ml with H_2O. A 5-ml aliquot was applied to a Dowex 50-X2 (100–200 mesh, H^+) column (4.5 × 75 cm) and eluted with water. Two major bands eluted from the column, the first corresponded to cGMP and the second to **2**. This second fraction was evaporated and the resulting solid suspended in Me_2CO, filtered, and air dried to give 480 mg (75%) of **2**. *Anal.* Calcd for $C_{11}H_{14}N_5O_7P \cdot 5H_2O$: C, 35.87; H, 4.10; N, 19.01. Found: C, 35.67; H, 4.24; N, 18.77.

8-Acetylguanosine 3′,5′-Cyclic Phosphate (**6**). cGMP · Na · $4H_2O$ (**1**, 2.0 g, 4.54 mmol) was dissolved in 100 ml of H_2O, then diluted with 100 ml of HOAc and 25 ml of 3 *N* H_2SO_4. The solution was cooled to 10° and 12 ml of acetaldehyde was added. Separate solutions of $FeSO_4 \cdot 7H_2O$ (10 g, 36 mmol in 60 ml of H_2O) and $(NH_4)_2S_2O_8$ (8.22 g, 36 mmol in 60 ml of H_2O) were added simultaneously over 1

† From the ICN Pharmaceuticals, Inc., Nucleic Acid Research Institute, Irvine, California 92664. *Received September 30, 1974.*

hr; stirring was continued an additional 0.5 hr. The solution was diluted with 300 ml of H_2O and desalted on charcoal (60 ml). After elution and evaporation, the residue in a small amount of H_2O was applied to a Dowex 50-X2 (100–200 mesh, H^+) column (4.5 × 75 cm) and eluted with water. Monitoring the eluate at 254 and 313 nm showed two close moving bands. The fractions corresponding to the first band, which absorbed at both wavelengths, were combined and evaporated. The residue was suspended in MeOH, filtered, and dried in vacuo at 80° to give 808 mg (43%). *Anal.* Calcd for $C_{12}H_{14}N_5O_8P \cdot 1.75H_2O$: C, 34.41; H, 4.21; N, 16.72. Found: C, 34.07; H, 3.94; N, 16.95. Recrystallization of the residue from the charcoal column eluate from H_2O gave the NH_3 salt of **6** suitable for further transformation.

8-Butyrylguanosine 3′,5′-cyclic phosphate (**7**) was synthesized as per the preparation of **6** using 10 g (22.7 mmol) of **1** and 50 ml of butyraldehyde. After desalting on charcoal 4.04 g (36%) of slightly contaminated ammonium salt of **7** was obtained by crystallization from H_2O. Passage of the filtrate through Dowex 50-X2 column gave, after evaporation and suspension of the residue in acetone, 3.42 g (34%) of pure **7.** For analysis a small amount of the ammonium salt was passed through Dowex 50-X2, and the residue after evaporation was suspended in acetone, filtered, and dried in vacuo at 80° for 18 hr. *Anal.* Calcd for $C_{14}H_{18}N_5O_8P \cdot 1.5H_2O$: C, 38.02; H, 4.79; N, 15.83. Found: C, 38.13; H, 4.64; N, 15.77.

8-Isobutyrylguanosine 3′,5′-Cyclic Phosphate Ammonium Salt (**8**) and *8-Isopropylguanosine 3′,5′-Cyclic Phosphate* (**3**). Compound **8** was prepared as per **6,** using 10 ml of 2-methyl-1-propanal and 2 g (4.55 mmol) of **1.** After elution from the Dowex 50-X2 column the fractions corresponding to the first major band were evaporated with an excess NH_4OH to give 0.98 g (47%) of **8.** *Anal.* Calcd for $C_{14}H_{18}N_5O_8P \cdot NH_3 \cdot 1.5H_2O$: C, 36.61; H, 5.27; N, 18.30. Found: C, 36.84; H, 5.29; N, 17.92.

Unreacted **1** eluted from the Dowex 50-X2 column immediately after **8.** Continued elution with water produced another major band having uv absorption at 254 nm but not at 313. The fractions corresponding to this band were evaporated and the residue was suspended in methanol to give **3.** In a reaction using 10 g (22.7 mmol) of **1** the yield of **3** was 2.11 g (22%) after drying in vacuo at room temperature. *Anal.* Calcd for $C_{13}H_{18}N_5O_7P \cdot 2H_2O$: C, 36.89; H, 5.24; N, 16.54. Found: C, 36.64; H, 5.58; N, 16.42.

8-Benzoylguanosine 3′,5′-cyclic phosphate (**9**) was prepared as per **6** using benzaldehyde (100 ml) and 10 g (22.7 mmol) of **1.** The reaction mixture was evaporated; the residue was triturated with Et_2O, then H_2O. The residue was stirred in H_2O (800 ml) while the pH was adjusted to 8 using 2 *N* NaOH. The filtered solution was adjusted to pH 1.5 with concentrated HCl, diluted to 2 l. with H_2O, and applied to an Amberlite XAD-4 column (4 × 70 cm, 850 ml). The column was washed with several liters of H_2O and then with a 12-l. gradient from 0 to 75% MeOH in H_2O. The fractions corresponding to the major band eluting from the column were evaporated; the residue was dissolved in 100 ml of MeOH and dropped into 1000 ml of Et_2O. The resulting solid was filtered and dried in vacuo at room temperature to give 1.5 g (14%) of **9.** *Anal.* Calcd for $C_{17}H_{16}N_5O_8P$: C, 45.44; H, 3.58; N, 15.58. Found: C, 45.16; H, 3.70; N, 15.44.

8-Carbamoylguanosine 3′,5′-Cyclic Phosphate (**10**). To a stirred solution of **1** (10 g, 22.7 mmol) in 500 ml of H_2O was added 500 ml of HOAc, 50 ml of 3 *N* H_2SO_4, and 100 ml of formamide. After cooling to 10° 100 g of $FeSO_4 \cdot 7H_2O$ was added. A solution of 82.2 g of $(NH_4)_2S_2O_8$ in 300 ml of H_2O was then added dropwise over 0.75 hr followed by stirring an additional 0.5 hr. After work-up as for **6,** the product was crystallized from H_2O at 0°. Filtration and drying in vacuo at room temperature gave 6.1 g (63%) of **10.** *Anal.* Calcd for $C_{11}H_{13}N_6O_8P \cdot 2H_2O$: C, 31.14; H, 4.04; N, 19.80. Found: C, 31.02; H, 3.85, N, 18.20.

8-(1-Hydroxyethyl)guanosine 3′,5′-Cyclic Phosphate (**11**). Compound **6,** ammonium salt (5.0 g, 10.5 mmol), was stirred in H_2O (75 ml) with $NaBH_4$ (400 mg, 10.5 mmol) for 0.5 hr. The pH was adjusted to 2 with concentrated HCl and the solution was then desalted using a charcoal column (100 ml). The eluate was evaporated, the residue was dissolved in 125 ml of MeOH, and an equal volume of Et_2O was added. The resulting solid was filtered and dried in vacuo at room temperature to give 3.10 g (69%) of the ammonium salt of **11.** For analysis, a portion was passed through a Dowex 50-X8 column in water. The fractions containing product were evaporated, and the residue was triturated with MeOH and dried in vacuo at room temperature. *Anal.* Calcd for $C_{12}H_{16}N_5O_8P \cdot 2H_2O$: C, 33.89; H, 4.74; N, 16.47. Found: C, 33.99; H, 4.86; N, 16.08.

8-(1-Hydroxybutyl)guanosine 3′,5′-Cyclic Phosphate (**12**). One gram (2.26 mmol) of **7** was treated with $NaBH_4$ as for **11.** After acidification the solution was passed through a Dowex 50-X2 (100–200 mesh, H^+) column (4.5 × 70 cm). The fractions corresponding to product were evaporated, dissolved in a small amount of MeOH, and dropped into Et_2O (200 ml). The moist solid was collected on a filter and dried in vacuo at room temperature to give 846 mg of **12.** An analytical sample was recrystallized from MeOH–EtOH. *Anal.* Calcd for $C_{14}H_{20}N_5O_8P \cdot 2H_2O$: C, 37.09; H, 5.34; N, 15.45. Found: C, 36.81; H, 5.16; N, 15.38.

8-(1-Hydroxy-2-methylpropyl)guanosine 3′,5′-Cyclic Phosphate (**13**). Compound **8** (500 mg, 1.03 mmol, ammonium salt) was reduced as in synthesis of **11.** After desalting on charcoal the residue was passed through a Dowex 50-X2 column and eluted with H_2O. The appropriate fractions were evaporated to dryness, and the residue was suspended in Me_2CO, filtered, and dried in vacuo at room temperature to give 158 mg (33%) of **13.** *Anal.* Calcd for $C_{14}H_{20}N_5O_8P \cdot 2.5H_2O$: C, 36.37; H, 5.45; N, 15.15. Found: C, 35.88; H, 5.38; N, 15.43.

8-(α-Hydroxybenzyl)guanosine 3′,5′-Cyclic Phosphate (**14**). Compound **9** (100 mg, 0.22 mmol) was treated with $NaBH_4$ (60 mg) in 2 ml of H_2O for 0.5 hr. The pH was adjusted to 1 with 1 *N* HCl. The resulting crystals were filtered and air dried to give 65 mg (59%). For analysis a portion was passed through a short silica gel column eluting with 30% MeOH in $CHCl_3$. After evaporation the residue was suspended in 1 *N* HCl, filtered, washed with water, and dried in vacuo. *Anal.* Calcd for $C_{17}H_{18}N_5O_8P \cdot 3H_2O$: C, 40.40; H, 4.79; N, 13.86. Found: C, 40.50; H, 4.43; N, 13.73.

8-Acetylguanosine 3′,5′-Cyclic Phosphae Thiosemicarbazone (**15**). Compound **6** (840 mg, 2 mmol) and thiosemicarbazide (180 mg, 2 mmol) in water (10 ml) were heated on steam bath for 1 hr. After cooling the solid was filtered and dried in vacuo at 80° to give 690 mg (59%) of **15.** *Anal.* Calcd for $C_{13}H_{17}N_8O_7PS \cdot 2.5H_2O$: C, 30.89; H, 4.39; N, 22.17. Found: C, 30.79; H, 4.49; N, 21.79. Compounds **16, 17,** and **18** were prepared from **6** and the appropriate hy-

drazine derivative in the same fashion:

8-Acetylguanosine 3',5'-cyclic phosphate semicarbazone (**16**): yield 81%. *Anal.* Calcd for $C_{13}H_{17}N_8O_8P \cdot 1.5H_2O$: C, 33.12; H, 4.27; N, 23.77. Found: C, 32.94; H, 4.01; N, 23.54.

8-Acetylguanosine 3',5'-cyclic phosphate 4-phenylsemicarbazone (**17**): yield 84%. *Anal.* Calcd for $C_{19}H_{21}N_8O_8P \cdot 3H_2O$: C, 39.73; H, 4.74; N, 19.51. Found: C, 39.97; H, 4.71; N, 19.79.

8-Acetylguanosine 3',5'-cyclic phosphate phenylhydrazone (**18**): yield 95%. *Anal.* Calcd for $C_{18}H_{20}N_7O_7P \cdot 2.5H_2O$: C, 41.38; H, 4.82; N, 18.76. Found: C, 41.11; H, 5.13; N, 18.46.

8-Benzylguanosine 3',5'-Cyclic Phosphate (**4**). Compound **4** was synthesized as per the preparation of **6** using 4.0 g (9.1 mmol) of **1** and 20 ml of toluene. The reaction mixture was evaporated to dryness and the residue taken up in H_2O for desalting. The eluate from charcoal was evaporated, and the residue, in 100 ml of H_2O, was passed in two portions through a Dowex 50-X2 column and eluted with water. Two major bands emerged from the column, the first corresponding to **1** and the second to **4.** The appropriate fractions were condensed to a small volume. The crystalline product was filtered and dried in vacuo at room temperature to give 250 mg (6%) of **4.** *Anal.* Calcd for $C_{17}H_{18}N_5O_7P_1 \cdot 2H_2O$: C, 43.32; H, 4.70; N, 14.86. Found: C, 43.14; H, 4.58; N, 14.75.

8-Neopentylguanosine 3',5'-Cyclic Phosphate (**5**). To a solution of **1** (2.0 g, 4.54 mmol) and $FeSO_4 \cdot 7H_2O$ (6.3 g, 22.7 mmol) in H_2O (100 ml) was added HOAc (100 ml) and 3 *N* H_2SO_4 (25 ml). The mixture was cooled to 5–10° with some solid precipitate resulting. 2,4,4-Trimethyl-2-hydroperoxypentane (3.32 g, 22.7 mmol) (Hoffman, 1960) was added dropwise with vigorous stirring over 0.5 hr after which the stirring continued 0.5 hr. The solution was evaporated, then redissolved in water (1000 ml) and desalted on charcoal (50 ml). After evaporation of the appropriate fractions, the residue was dissolved in a small amount of water and applied to Dowex 50-X2 column and eluted with water. Fractions containing cGMP eluted first. These were evaporated and suspended in acetone to give cGMP (890 mg, 2.5 mmol). The second uv absorbing band eluting from the column contained **5.** These fractions were evaporated, dissolved in 15 ml of 1 *N* NH_4OH, filtered, and adjusted to pH 1 with concentrated HCl. After several hours, the crystals were filtered, washed with water, and dried in vacuo at room temperature to give 345 mg (16%, or 35% based on unrecovered **1**) of **5.** *Anal.* Calcd for $C_{15}H_{22}N_5O_7P \cdot 3.5H_2O$: C, 37.66; H, 6.11; N, 14.64. Found: C, 37.63; H, 5.90; N, 14.51.

8-Acetylinosine 3',5'-Cyclic Phosphate (**20**). To a solution of 3.0 g (8.6 mmol) of inosine 3',5'-cyclic phosphate (**19,** Meyer et al., 1972) and 25 ml of acetaldehyde in 300 ml of 0.3 *N* H_2SO_4 at 10° were added simultaneously and dropwise, 30 g of $FeSO_4 \cdot 7H_2O$ in 180 ml of H_2O and 24.7 g of $(NH_4)_2S_2O_8$ in 180 ml of H_2O. After 0.5 hr of additional stirring, the solution was desalted. To the residue from evaporation of the charcoal eluate was added aqueous alcohol and 6 g of silica gel. The solvent was evaporated and the dried residue was added to the top of a silica gel column (60 g), packed in and eluted with 30% MeOH in $CHCl_3$. Product appeared first from the column, followed by **19.** Appropriate product-containing fractions were pooled and evaporated, and the residue was dissolved in H_2O and passed through Dowex 50 (H^+). Evaporation of the eluate and trituration of the residue with Me_2CO gave 430 mg (11%). *Anal.* Calcd for $C_{12}H_{13}N_4O_8P \cdot 3H_2O$: C, 33.81; H, 4.49; N, 13.14. Found: C, 34.04; H, 4.52; N, 13.30.

8-Benzoylinosine 3',5'-Cyclic Phosphate (**21**). Compound **21** was synthesized in a manner similar to **6** using 10.0 g (28.6 mmol) of **19** and benzaldehyde. After desalting the residue was dissolved in H_2O and applied to an Amberlite XAD-4 column (4 × 70 ml, 800 ml). The column was washed with H_2O then eluted with a 6-l. gradient from 0 to 75% MeOH in H_2O. The fractions corresponding to the major band eluting near the end of the gradient were evaporated. The residue was dissolved in H_2O and passed through a Dowex 50 (H^+) column. Evaporation of the eluate gave 205 mg (1.5%) of **21** after drying in vacuo at room temperature. *Anal.* Calcd for $C_{17}H_{15}N_4O_8P \cdot 1.5H_2O$: C, 44.26; H, 3.92; N, 12.14. Found: C, 44.35; H, 3.83; N, 12.20.

Biological Methods. The methods for evaluation of the cyclic nucleotides as activators of bovine brain cAMP dependent and lobster tail cGMP dependent protein kinases, and for determination of the ability of the compounds to serve as substrates and inhibitors of phosphodiesterase, were as previously described (Miller et al., 1973).

Results and Discussion

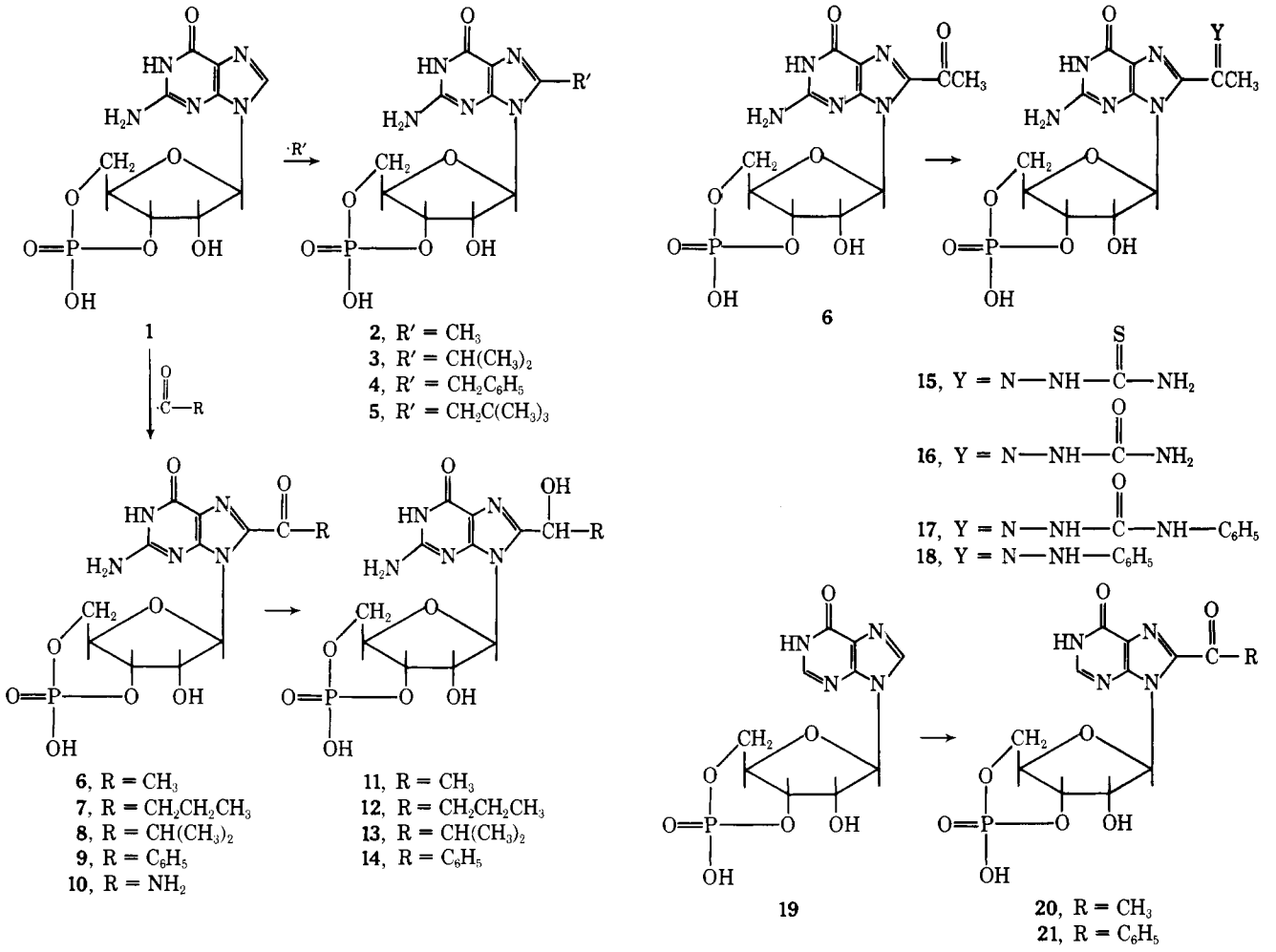
Synthetic. Historically, 8-alkylpurines have been synthesized by Traube cyclization of a 4,5-diaminopyrimidine with the appropriate carboxylic acid fragment (Robins, 1967). The only 8-acylpurine derivatives known prior to the present work are 8-acetylcaffeine, 8-propionylcaffeine, and 8-acetyltheophylline (Ehrhart and Hennig, 1956), prepared from 8-cyanocaffeine and 8-(1-hydroxyethyl)theophylline, respectively. For the purposes of the present study, it was desirable to develop methods of introduction of alkyl and acyl groups directly into the performed guanosine 3',5'-cyclic phosphate molecule.

A review reflecting increased recent interest in homolytic alkylation and acylation of heterocyclic systems has recently appeared (Minisci and Porta, 1974). These authors found that these homolytic addition reactions attain unusual regiospecificity and are applicable to a wide variety of substrates when applied to protonated heteroaromatic bases. This reaction, however, has been virtually uninvestigated in the field of biologically significant heterocycles. Only recently Kawazoe et al. (1972) have examined homolytic methylation of guanine, guanosine, and 5'-guanylic acid and found that 8-methylguanine was produced from the action of guanine, *tert*-butyl hydroperoxide, and $FeSO_4$, in the presence of dilute sulfuric acid.

In the presence of $FeSO_4$, *tert*-butyl hydroperoxide decomposes to yield acetone and the methyl radical (Minisci et al., 1970). When cGMP (**1**) was treated with these reagents in 0.3 *N* H_2SO_4 solution, 8-methylguanosine 3',5'-cyclic phosphate (**2**) was isolated in 75% yield (Scheme I). Compound **2** was characterized by the uv spectra, which was similar to cGMP, and the absence of the C-8 proton in the ^1H NMR. No chromatographic evidence was found for the cleavage of the cyclic phosphate or ribosyl moieties under these conditions.

It has been shown that 2,4,4-trimethyl-2-hydroperoxypentane (Hoffman, 1960) undergoes β scission in the presence of Fe^{2+} to give the neopentyl radical and acetone (Minisci et al., 1970). Treatment of cGMP with trimethylhydroperoxypentane under the conditions used for the synthesis of **2** gave 8-neopentylguanosine 3',5'-cyclic phosphate (**5**) in 16% yield. The benzyl radical has been gener-

Scheme I



ated from toluene with Fe^{2+} and a radical initiator (Hutton and Walters, 1965); we examined the reaction of this radical with protonated cGMP. Thus, cGMP and toluene in aqueous acetic acid with $FeSO_4$ and ammonium persulfate gave 8-benzylguanosine 3′,5′-cyclic phosphate (**4**) in 6% yield.

Hydrogen abstraction from the carbonyl carbon of aldehydes by a radical source has been shown (Caronna et al., 1969; Gardini and Minisci, 1970; Minisci and Porta, 1974) to yield the acyl radical, a highly reactive acylating agent. We investigated these species as reagents for the synthesis of 8-acyl-cGMP derivatives. Acetaldehyde and cGMP, in the presence of *t*-BuOOH, $FeSO_4$, and 0.3 *N* H_2SO_4, yielded 8-acetylguanosine 3′,5′-cyclic phosphate (**6**); however, in initial experiments a significant amount of 8-methyl-cGMP (**2**) was also formed. To avoid the possibility of methylation competing with hydrogen abstraction from the aldehyde, ammonium persulfate was used as a radical source subsequently. In this manner, **6** was readily obtained in 43% yield. Using the appropriate aldehyde under these conditions, 8-*n*-butyryl- (**7**), 8-isobutyryl- (**8**), and 8-benzoylguanosine 3′,5′-cyclic phosphate (**9**) were prepared from cGMP. Formamide and cGMP under these conditions gave 8-carbamoylguanosine 3′,5′-cyclic phosphate (**10**).

Side reactions were minimal in the homolytic acylation reaction with one notable exception. Isobutyraldehyde and cGMP gave **8** in 47% yield, but tlc revealed another component in addition to unreacted cGMP. This product was isolated in 22% yield and identified as 8-isopropylguanosine 3′,5′-cyclic phosphate (**3**), obtained presumably by partial decarbonylation of the isobutyryl radical.

The novel 8-acyl-cGMP derivatives were shown to undergo the usual reactions characteristic of ketones. Treatment of **6–9** with $NaBH_4$ in water readily gave 8-(1-hydroxyethyl)- (**11**), 8-(1-hydroxybutyl)- (**12**), 8-(1-hydroxy-2-methylpropyl)- (**13**), and 8-(α-hydroxybenzyl)guanosine 3′,5′-cyclic phosphate (**14**), respectively. Compound **6** also yielded a thiosemicarbazone (**15**), semicarbazone (**16**), 4-phenylsemicarbazone (**17**), and phenylhydrazone (**18**) upon treatment with the appropriate reagent in water. Additional reactions of this versatile class of compounds are under investigation.

The structures of the 8-acyl-cGMP derivatives were assigned by the ^1H NMR spectra, which showed the expected new peaks of the substituent in addition to the loss of the C-8 proton. In all cases the anomeric proton appeared as an apparent singlet, verifying the integrity of the cyclic phosphate ring (Jardetzky, 1962). The uv spectra maxima and tlc data are given in Table I.

To investigate the scope of homolytic alkylation and acylation with regard to substrate, adenosine 3′,5′-cyclic phosphate was treated with *t*-BuOOH and $FeSO_4$ in dilute acid. No evidence, however, of 8-methyl-cAMP was found. Treatment of cAMP with acetaldehyde, $FeSO_4$, and persulfate in aqueous acid also failed to yield any detectable 8-acetyl-cAMP.

Table I: Physical Properties of 8-Substituted cGMP Derivatives.

No.	λ_{max}(nm)($\epsilon \times 10^{-3}$) pH 1	pH 7	pH 11	R_{cGMP}[a] A	B	^{1}H NMR Solvent[c]	H-1′	δ, ppm[d] (8 Substituent)
2	260 (13.3)	252 (13.8)	257 (13.2)	1.0	1.1	I	5.83	2.56 (s, 3)
3	261 (15.1)	253 (15.6)	258 (14.7)	1.8	1.9	I	5.96	1.37 (d, 6), 3.30 (m, 1)
4	262 (16.2)	256 (18.0)	262 (16.2)	2.1	2.4	I	5.77	2.51 (s, 2), 7.34 (s, 5)
5	262 (15.7)	256 (17.4)	259 (15.7)	1.9	1.7	II	5.81	1.04 (s, 9), 2.76 (s, 2)
6	330, 276 (16.2, 8.1)	330, 276 (16.2, 7.9)	349 (17.6)	2.2	1.8	II	6.69	2.63 (s, 3)
7	331, 277 (15.8, 7.6)	332, 277 (16.1, 7.6)	350 (16.8)	2.9	2.4	I	6.77	0.99 (t, 3), 1.7 (m, 2)
8	330, 275 (16.2, 7.8)	331, 275 (16.3, 7.8)	348 (17.0)	3.0	2.6	I	6.79	1.24 (d, 6)
9	351, 271 (14.9, 13.4)	352, 271 (14.7, 13.1)	375, 253 (16.1, 16.1)	3.1	2.6	II	6.56	7.6 (m, 3), 8.1 (m, 2)
10	300, 275 (14.2, 14.8)	300, 275 (14.1, 14.3)	312 (15.1)	1.3	1.3	II	6.91	6.2 (bs, 2, exchangeable with D_2O)
11	261 (14.8)	257 (16.8)	262 (14.3)	1.2	1.4	I	6.19	1.69 (d, 3)
12	261 (15.8)	257 (17.1)	262 (15.3)	1.8	2.1	II	6.22	
13	262 (15.4)	258 (17.6)	262 (15.3)	1.9	2.1			
14	262 (21.0)	258 (23.1)	267 (20.3)	2.2	2.2			
15	335, 282 (32.2, 12.5)	334, 283 (32.6, 13.0)	347, 275 (29.2, 9.5)	1.9	2.1			
16	317 (23.0)	314 (24.9)	324 (22.7)	1.0	1.8			
17	318 (26.9)	317 (28.4)	327 (25.4)	2.1	2.6			
18	373, 287 (26.4, 8.9)	347, 292 (30.2, 13.3)	350 (30.2)	3.1	1.7			
20	297 (7.7)	297 (7.6)	321 (7.0)	1.6[b]	1.2[b]	II	6.82	2.72 (s, 3), 8.29 (s, 1, H-2)
21	310, 269, 227 (12.2, 11.5, 11.1)	310, 269, 227 (12.2, 11.5, 11.0)	341, 265 (9.5, 11.5)	3.9[b]	2.4[b]	II	6.50	7.7 (b, 3), 8.15 (m, 2), 8.30 (s, 1, H-2)

[a] R_{cGMP} = mobility of compound/mobility of cGMP. [b] R_{cIMP}. [c] Solvent I, D_2O; II, Me_2SO-d_6. [d] Downfield from internal DSS. H-1′ is an apparent singlet in all cases. J = 7 Hz for the split CH_3 groups.

Inosine 3′,5′-cyclic phosphate (**19**), however, proved to be a substrate for acylation, as noted by the formation of 8-acetyl- (**20**) and 8-benzoylinosine 3′,5′-cyclic phosphate (**21**) from cIMP and acetaldehyde and benzaldehyde, respectively, in dilute acid with $(NH_4)_2S_2O_8$ and $FeSO_4$. In each case, however, the yield (11 and 1.5%, respectively) was considerably lower than with the corresponding cGMP derivative.

Compounds **20** and **21** were confirmed as 8-substituted inosines by a deuterium exchange experiment. The 8-proton of cIMP exchanges with deuterium when heated at 80° for 1 hr in D_2O containing NaOD (Bullock and Jardetzky, 1964). The ^{1}H NMR spectra of **20** and **21** showed signals corresponding to one purine C–H proton. Treatment of **20** and **21** with D_2O under conditions which gave complete exchange of the 8-proton of cIMP had no effect on these ^{1}H NMR signals. They therefore correspond to H-2 and the acyl substituent is at the 8 position of **20** and **21.**

Biochemical. The new 8-substituted purine cyclic nucleotides were examined for their ability to stimulate both a cAMP-dependent protein kinase isolated from beef brain and a cGMP-dependent protein kinase isolated from lobster tail (see Table II). The intracellular action of both cAMP and cGMP has been postulated (Kuo and Greengard, 1969; Kuo et al., 1972) to be mediated by its activation of a protein kinase.

The ability of the cyclic nucleotides to activate the protein kinases is expressed as K_a' as defined in footnote *a*, Table II. The two kinases are highly selective for their respective cyclic nucleotide, although not absolutely so: cAMP has a K_a' of 0.015 with the lobster muscle protein kinase while cGMP has a K_a' of 0.023 with the bovine brain protein kinase.

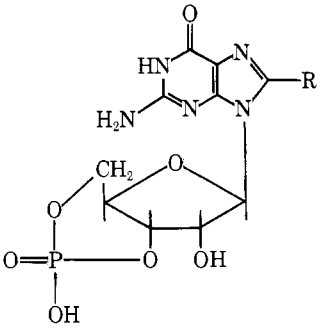
The K_a of cAMP for the cAMP-dependent protein kinase is almost the same (see footnote *a*, Table II) as the K_a of cGMP for the cGMP-dependent protein kinase. Comparison of the K_a' values is, therefore, an accurate guide to the type of kinase stimulation seen at a given cyclic nucleotide concentration.

The 8-acyl- and 8-alkyl-cGMP derivatives reported here showed a remarkable specificity for the cGMP-dependent enzyme, as shown in Table II. Only 8-butyryl-cGMP showed even modest ability to stimulate the cAMP dependent kinase, having a K_a' of 0.064 for this enzyme. None of the other compounds derived from cGMP demonstrated a K_a' better than 0.01 with the cAMP dependent protein kinase.

When assayed as activators of the cGMP-dependent protein kinase, most of the 8-substituted cGMP's proved to be fair to excellent. The 8-carbamoyl- (**10**), 8-(1-hydroxyethyl)- (**11**), and 8-(α-hydroxybenzyl)-cGMP (**14**) proved to be superior to cGMP in their ability to activate this kinase.

When the 8-substituted cGMP derivatives were grouped into classes according to substituent, it was found that the K_a' values of compounds within each class were similar. Group 1, the 8-alkyl analogs **2–5,** had generally poor K_a' values, with 8-isopropyl-cGMP (**3**) showing the best stimulation (K_a' = 0.70). Group 2, the 8-acyl-cGMP's **6–9,** were slightly more active as a class. Group 3, the 8-(1-hydroxyalkyl) derivatives **11–14,** were noticeably better in their ability to stimulate the kinase. In fact, when each member of group 3 was compared with its respective member of group 2 (i.e., **11** with **6,** etc), a constant three- to fivefold increase in K_a' was realized upon conversion of the keto function to a hydroxy function. The fourth group, the hydrazine

Table II: Activation of Protein Kinase and Inhibition of Phosphodiesterase Activity by the 8-Substituted Derivatives of cGMP and cIMP.



No.	R	Protein Kinase,[a] K_a' Bovine	Protein Kinase,[a] K_a' Lobster	Phosphodiesterase Inhibition,[b] I_{50} (μM) Lung	Phosphodiesterase Inhibition,[b] I_{50} (μM) Heart
1	H	0.023	1.0		
2	CH_3	0.015	0.066	400	200
3	$CH(CH_3)_2$	<0.001	0.70	330	100
4	$CH_2C_6H_5$	<0.001	0.10	130	140
5	$CH_2C(CH_3)_3$	<0.001	0.16	70	100
6	$COCH_3$	<0.001	0.56	70	170
7	COC_3H_7	0.064	0.20	70	90
8	$COCH(CH_3)_2$	<0.01	0.13	50	11
9	COC_6H_5	<0.005	0.68	50	50
10	$CONH_2$	<0.001	1.5	400	100
11	$CHOHCH_3$	<0.01	1.8	400	170
12	$CHOHC_3H_7$	<0.001	0.62	133	29
13	$CHOHCH(CH_3)_2$	<0.001	0.72	180	38
14	$CHOHC_6H_5$	<0.005	2.3	140	>300
15	$C(NNHCSNH_2)CH_3$	<0.005	0.052	26	50
16	$C(NNHCONH_2)CH_3$	<0.005	0.031	200	125
17	$C(NNHCONHC_6H_5)CH_3$	<0.01	0.11	150	40
18	$C(NNHC_6H_5)CH_3$	<0.005	0.44	2000	100
19	H	0.59	0.085	100	3.9
20	$COCH_3$	0.022	0.0041	2000	1000
21	COC_6H_5	0.070	0.011	400	70

[a] K_a' is the ratio of K_a of the dependent cyclic nucleotide (cAMP for bovine brain and cGMP for lobster tail) to the K_a of the test compound. The K_a of cAMP for the bovine brain kinase is 2.0×10^{-7} M and the K_a of cGMP for the lobster tail kinase is 1.7×10^{-7} M. [b] I_{50} is the concentration of test compound necessary for 50% inhibition of the cleavage of cAMP (concentration of cAMP = 1.6×10^{-7} M). The I_{50} of theophylline is 200 μM against rabbit lung and 100 μM against beef heart.

derivatives of 8-acetyl-cGMP, were less active than all of the above, except for **18**, 8-acetylguanosine 3′,5′-cyclic phosphate phenylhydrazone, with a K_a' of 0.44. It was shown in a previous paper (Miller et al., 1973) that 8-bromo-cGMP possessed a K_a' of 4 for this cGMP dependent protein kinase. It is surprising to note that 8-methyl-cGMP (**2**) had a K_a' some 60 times less active than the otherwise sterically similar 8-bromo compound.

The two 8-acyl-cIMP analogs reported here were very poor activators of both protein kinases, showing slightly greater specificity for the cAMP dependent enzyme. This is in agreement with the greater activation of the cAMP dependent kinase by cIMP itself, and with the preference for the cAMP dependent enzyme by the other 8-substituted cIMP derivatives which have been reported (Miller et al., 1973).

All compounds were examined for their susceptibility to hydrolysis by cyclic nucleotide phosphodiesterase. Both cGMP and cIMP were hydrolyzed at approximately one-half the rate of cAMP by the rabbit kidney enzyme preparation used for this experiment. The new 8-substituted derivatives were not substrates (rate of hydrolysis <0.05 that of the rate of cAMP hydrolysis) within the limits of detection of our assay, with the sole exception of 8-(1-hydroxybutyl)-cGMP (**12**), which was hydrolyzed at a rate 0.18 times the rate of hydrolysis of cAMP. This finding is in agreement with the general conclusion that 8-substitution of a purine 3′-5′-cyclic ribonucleotide greatly reduces the ability of the resulting derivative to serve as a substrate for cyclic nucleotide phosphodiesterase (Muneyama et al., 1971; Miller et al., 1973; Meyer and Miller, 1974).

All of the compounds in this report were also examined for their ability to inhibit the hydrolysis of cAMP by both rabbit lung and beef heart cyclic nucleotide phosphodiesterase, as is shown in Table II. Some of the compounds inhibited the rate of cAMP hydrolysis by 50% at concentrations in the 10^{-5}–10^{-4} range, within the same order of magnitude as was observed for the 8-bromo, 8-thio, and 8-amino derivatives of cGMP previously reported from these laboratories (Miller et al., 1973). As was the case with the latter compounds (Miller et al., 1973), the 8-alkyl- and 8-acyl-cGMP derivatives reported here were competitive inhibitors of the enzyme.

Russell et al. (1973) have shown that in rat liver at least three phosphodiesterase activities are present: a high affinity cGMP-hydrolyzing enzyme, a low affinity enzyme which hydrolyzes both cAMP and cGMP, and a high affinity negatively cooperative cAMP phosphodiesterase inhibited by cGMP. Although the 8-substituted derivatives of cGMP did show inhibition of the hydrolysis of cAMP in the crude rabbit lung and beef heart preparations of phosphodiesterase we have used, it is conceivable that they have a stronger affinity for the cGMP binding sites in these preparations. They may, therefore, be better inhibitors of the hydrolysis of cGMP in such a crude preparation. This possibility was not examined but will be the subject of future work.

The remarkable specificity shown by the 8-alkyl- and 8-acyl-cGMP derivatives for the cGMP-dependent protein kinase, coupled with their apparent lack of hydrolysis by phosphodiesterase, indicates that this class of compound may prove very useful in the study of biological responses involving cGMP.

Acknowledgments

We are indebted to Ms. Mieka Scholten for technical assistance, to Mr. O. P. Crews, Jr. and his staff for large-scale preparation of intermediates, and to Dr. D. A. Shuman for helpful discussions.

References

Bullock, F. J., and Jardetzky, O. (1964), *J. Org. Chem. 29,* 1988.

Caronna, T., Gardini, G. P., and Minisci, F. (1969), *J. Chem. Soc., Chem. Commun.,* 201.

Ehrhart, G., and Hennig, I. (1956), *Arch. Pharm. (Weinheim, Ger.) 289,* 453.

Ferrendelli, J. A., Steiner, A. L., McDougal, D. B., Jr., and Kipnis, D. M. (1970), *Biochem. Biophys. Res. Commun. 41,* 1061.

Gardini, G. P., and Minisci, F. (1970), *J. Chem. Soc. C,* 929.

George, W. J., Polson, J. B., O'Toole, A. G., and Goldberg, N. D. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 398.
Hadden, J. W., Hadden, E. M., Haddox, M. K., and Goldberg, N. D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3024.
Hoffman, J. (1960), *Org. Syn. 40,* 76.
Hutton, J. and Waters, W. A. (1965), *J. Chem. Soc.,* 4254.
Illiano, G., Tell, G. P. E., Siegel, M. I., and Cuatrecasas (1973), *Proc. Natl. Acad Sci. U.S.A. 70,* 2443.
Jardetzky, C. D. (1962), *J. Amer. Chem. Soc. 84,* 62.
Kaliner, M., Orange, R. P., and Austen, K. F. (1972a), *J. Exp. Med. 136,* 556.
Kaliner, M. A., Orange, R. P., Laraia, P. J., and Austen, K. F. (1972b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 748.
Kawazoe, Y., Maeda, M., and Nushi, K. (1972), *Chem. Pharm. Bull. 20,* 1341.
Kram, R. and Tomkins, G. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1659.
Kuo, J. F. and Greengard, P. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 1349.
Kuo, J. F. Lee, T.-P., Reyes, P. L., Walton, K. G., Donnelly, T. E., Jr., and Greengard, P. (1972), *J. Biol. Chem. 347* 16.
Lee, T.-P., Kuo, J. F., and Greengard, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3287.
Meyer, R. B., Jr., and Miller, J. P. (1974), *Life Sci. 14,* 1019.
Meyer, R. B., Shuman, D. A., Robins, R. K., Bauer, R. J., Dimmitt, M. K., and Simon, L. N. (1972), *Biochemistry 11,* 2704.
Miller, J. P., Boswell, K. H., Muneyama, K., Simon, L. N., Robins, R. K., and Shuman, D. A. (1973), *Biochemistry 12,* 5310.
Minisci, F., Bernardi, R., Bertini, F., Galli, R., and Perchinumno, M. (1971), *Tetrahedron 27,* 3575.
Minisci, F., Galli, R., Malatesta, V., and Caronna, T. (1970), *Tetrahedron 26,* 4083.
Minisci, F., and Porta, O. (1974). *Adv. Heterocycl. Chem. 16,* 123.
Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10,* 2390.
Paoletti, R.,Berti, F., Spano, P. F., Michal, G., Weimann, G., and Nelboeck, M. (1973), *Pharmacol. Res. Commun. 5,* 87.
Puglisi, L., Berti, F., and Paoletti, R. (1971), *Experientia 27,* 1187.
Robins, R. K. (1967), *Heterocycl. Compd. 8,* 162.
Russell, T. R., Terasaki, W. L., and Appleman, M. M. (1973), *J. Biol. Chem. 248,* 1334.
Seifert, W. E., and Rudland, P. S. (1974), *Nature (London) 248,* 138.
Stoner, J., Manganiello, V. C., and Vaughan, M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3830.

Kinetics of Interactions between Antibodies and Haptens†

Thomas W. Smith* and Keith M. Skubitz

ABSTRACT: Association and dissociation kinetics of antibody–hapten interactions of high affinity and specificity have been determined by newly developed techniques using dextran-coated charcoal for rapid separation of free and antibody-bound hapten. Interactions of 12 combinations of four antibody populations (rabbit digoxin-specific antibody, sheep digoxin-specific antibody, rabbit ouabain-specific antibody, and rabbit digitoxin-specific antibody) with three haptens ([^{3}H]digoxin, [^{3}H]ouabain, and [^{3}H]digitoxin) have been studied in terms of both association and dissociation kinetics, and compared in selected instances with association constants determined under equilibrium conditions. Association rate constants determined under both second-order and pseudo-first-order conditions were found to be similar for all antibody–hapten pairs studied (range 0.87–1.7 $\times 10^7$ M^{-1} sec^{-1}), and were comparable to values previously estimated for antibodies to dye haptens of markedly lower affinity. In contrast, dissociation rate constants varied markedly from 1.9 $\times 10^{-4}$ to 1.7 $\times 10^{-2}$ sec^{-1}. Ratios of association to dissociation rate constants measured by these methods were in satisfactory agreement with average intrinsic association constants measured under equilibrium conditions. These studies support the concept that the major kinetic variable governing antibody–hapten interactions is the rate of dissociation of the complex, and that the strength of antibody–hapten association is determined principally by the activation energy for dissociation.

Despite the existence of an extensive literature on antibody–hapten interactions (Sehon et al., 1971) and a plethora of recent studies using such interactions as the basis for radioimmunoassay of minute concentrations of biologically active substances (Yalow, 1973; Smith and Haber, 1973), there are relatively few studies bearing on the kinetic determinants of antibody–hapten interactions. This is due in large part to the extremely rapid nature of antibody–hapten association reactions, which has necessitated the use of cathode-ray polarography (Schneider and Sehon, 1962), temperature jump relaxation (Froese et al., 1962), and spectrophotometric or fluorimetric measurements together with stopped-flow instrumentation (Day et al., 1963). Studies by Sehon and his colleagues (1963) and by Day et al.

† From the Cardiovascular Division, Peter Bent Brigham Hospital, the Cardiac Unit, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115. *Received September 16, 1974.* Supported by U.S. Public Health Service Grant HL-18003. Dr. Smith is an Established Investigator of the American Heart Association.