References

Akers, H. A., Llinás, M. and Neilands, J. B. (1972), *Biochemistry 11*, 2283.
Anke, T., and Diekmann, H. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 27*, 259.
Atkin, C. L. (1970), Ph.D. Dissertation, University of California, Berkeley, Calif.
Atkin, C. L., and Neilands, J. B. (1968), *Biochemistry 7*, 3734.
Black, D. St. C., Brown, R. F. C., and Wade, A. M. (1971), *Tetrahedron Lett. 47*, 4519.
Chinard, F. P. (1952), *J. Biol. Chem. 199*, 91.
Emery, T. (1966), *Biochemistry 5*, 3694.
Emery, T. F., and Neilands, J. B. (1962), *J. Org. Chem. 27*, 1075.
Garibaldi, J. A., and Neilands, J. B. (1955), *J. Amer. Chem. Soc. 77*, 2429.
Isowa, Y., Takashima, T., Ohmori, M., Kurita, H., Sato, M., and Mori, K. (1972), *Bull. Chem. Soc. Jap. 45*, 1464.
Jirgl, V. (1965), *Anal. Biochem. 13*, 381.
Klages, F., and Sitz, H. (1959), *Chem. Ber. 92*, 2606.
Llinás, M. (1971), Ph.D. Dissertation, University of California, Berkeley, Calif.
Luckey, M., Pollack, J. R., Wayne, R., Ames, B. N., and Neilands, J. B. (1972), *J. Bacteriol. 111*, 731.
Micetich, R. G., and MacDonald, J. C. (1965), *J. Biol. Chem. 240*, 1692.
Neilands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27*, 29.
Seifter, S., Gallop, P., Michaels, S., and Meilman, E. (1960), *J. Biol. Chem. 235*, 2613.
Snow, G. A. (1954), *J. Chem. Soc. A*, 2588.
Snow, G. A. (1970), *Bacteriol. Rev. 34*, 99.

Synthesis and Biological Activity of Some 2′ Derivatives of Adenosine 3′,5′-Cyclic Phosphate†

Jon P. Miller,* Dennis A. Shuman, Mieka B. Scholten, Mary K. Dimmitt, Charles M. Stewart, Tasneem A. Khwaja, Roland K. Robins, and Lionel N. Simon

ABSTRACT: A series of 2′ derivatives of cAMP were synthesized and their biological properties *in vitro* were investigated by studying their ability to serve as substrates for and inhibitors of cAMP phosphodiesterases, and to activate cAMP-dependent protein kinases. The unblocked 2′-OH in the ribo configuration was found to be necessary for the activation of protein kinase by cAMP. 2′-O-Substituted derivatives and 2′-deoxy-cAMP were relatively good substrates for and inhibitors of cAMP phosphodiesterases while 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate was both a relatively poor substrate and inhibitor. The product of phosphodiesterase action of the 2′-*O*-substituted derivatives was found to be the corresponding 2′-*O*-substituted 5′-nucleotide. N^6-Butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP were studied in the same way. The results indicate that N^6-butyryl-cAMP is the active form of N^6,2′-*O*-dibutyryl-cAMP.

Various derivatives of adenosine 3′,5′-cyclic phosphate, most notably N^6,2′-*O*-dibutyryl-cAMP[1] and several 8-alkylthio-cAMP analogs (Free *et al.*, 1972), have been shown to be more effective than cAMP itself in many whole cell systems (see reviews by Robison *et al.*, 1971, and Drummond and Severson, 1971). Reasons for the low activity of cAMP might include the low permeability of cAMP across cell membranes and its intracellular or extracellular hydrolysis by cAMP phosphodiesterase. It has been proposed that N^6,2′-*O*-dibutyryl-cAMP is both more rapidly transported across cell membranes and less rapidly hydrolyzed by the phosphodiesterase (Posternak *et al.*, 1962; Robison *et al.*, 1971). It has further been suggested that the acylated derivatives of cAMP were deacylated by soluble esterases before becoming active (Posternak *et al.*, 1962; Henion *et al.*, 1967). Exposure of HeLa S3 cells to N^6,2′-*O*-dibutyryl-cAMP led to intracellular levels of N^6-butyryl-cAMP that were 30 times that of basal cAMP levels (Kankel and Hilz, 1972; Kankel *et al.*, 1972). Consistent with these observations, it has been shown that N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP were not hydrolyzed by cAMP phosphodiesterase from beef heart (Moore *et al.*, 1968), liver (Levine and Washington, 1970; Menahan *et al.*, 1969), adipose tissue (Hepp *et al.*, 1969; Blecher *et al.*, 1971), or brain (Drummond and Powell, 1970). Thus, in some systems, at least, N^6-butyryl-cAMP is postulated to be the active metabolite of N^6,2′-*O*-dibutyryl-cAMP (Kankel *et al.*, 1972); however, a comparable understanding of the biologically active form of the 2′ derivatives of cAMP has awaited investigation. In this paper, we wish to report on the *in vitro* enzymatic properties of some representative 2′ derivatives of cAMP in comparison with N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP.

Experimental Section

Synthetic. N^6,2′-*O*-Dibutyryl-cAMP, N^6-butyryl-cAMP, and 2′-*O*-butyryl-cAMP were purchased from Sigma Chemical

† Contribution from ICN Nucleic Acid Research Institute, Irvine, California. *Received December 6, 1972.*

[1] Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; 2′-*O*-acetyl-cAMP, 2′-*O*-acetyladenosine 3′,5′-cyclic phosphate; 2′-*O*-methyl-cAMP, 2′-*O*-methyladenosine 3′,5′-cyclic phosphate; 2′-*O*-Dnp-cAMP, 2′-*O*-(2,4-dinitrophenyl)adenosine 3′,5′-cyclic phosphate; 2′-*O*-butyryl-cAMP, 2′-*O*-butyryladenosine 3′,5′-cyclic phosphate; N^6-butyryl-cAMP, N^6-butyryladenosine 3′,5′-cyclic phosphate; N^6-2′-*O*-dibutyryl-cAMP, N^6-2′-*O*-dibutyryladenosine 3′,5′-cyclic phosphate; ara-cAMP, 9-β-D-arabinofuranosyladenine 3′,5′-cyclic phosphate; and 2′-deoxy-cAMP, 2′-deoxyadenosine 3′,5′-cyclic phosphate.

Co. All three compounds werd chromatographed on thin layer to ascertain purity. The N^6,2′-*O*-dibutyryl-cAMP contained ~2% of 2′-*O*-butyryl-cAMP and no detectable cAMP. The N^6-butyryl-cAMP contained ~3% of 2′-*O*-butyryl-cAMP and ~6% cAMP. The 2′-*O*-butyryl-cAMP contained ~3% of N^6,2′-*O*-dibutyryl-cAMP and ~3% cAMP. These levels of impurities were judged to be insignificant and the compounds were used as received without further purification. 2′-*O*-Acetyl-cAMP was synthesized by the procedure of Falbriard *et al.* (1967). Ara-cAMP (Khwaja *et al.*, 1972) and 2′-deoxy-cAMP (Khwaja and Robins, unpublished results) were synthesized from cAMP. Aquascint II and 8-[^{3}H]cAMP were obtained from ICN Chemical and Radioisotope Division.

Thin-layer chromatography was performed on 0.1-mm Brinkman EM cellulose glass plates prewashed with distilled water. The solvent systems used were A (acetonitrile–0.1 M ammonium chloride, 7:3), B (acetonitrile–0.1 M ammonium chloride, 4:1), and C (isopropyl alcohol–concentrated ammonium hydroxide–water, 7:1:2). Evaporations were performed under diminished pressure at less than 40°. Ultraviolet spectra were determined on a Cary 15 spectrometer. High-voltage paper electrophoresis was run at 12.5 V/cm for 90 min in 25 mM potassium phosphate buffer (pH 7.0) on Whatman No. 3MM paper.

2′-O-Methyladenosine 3′,5′-Cyclic Phosphate.[2] 2′-*O*-Methyladenosine (1.0 g, 3.56 mmol) (Broom and Robins, 1964) was stirred in 9 ml of trimethyl phosphate at ice-bath temperature and 0.70 ml of $POCl_3$ was added. After 90 min Et_3NHCO_3 (0.5 M) was added to the reaction mixture until the pH reached 7.5. The solution was concentrated and any crystals which had formed were filtered. The filtrate was evaporated *in vacuo* and the resulting solid residue was dissolved in 15 ml of H_2O and applied to a Whatman DE-52 cellulose column (HCO_3^- form, 28 × 6.3 cm). Elution with a gradient of H_2O–0.05 M Et_3NHCO_3 (3:3, v/v) yielded 1.36 g of 2′-*O*-methyladenosine 5′-phosphate (Rottman and Heinlein, 1968) triethylammonium salt after evaporation of the appropriate fractions. Electrophoresis of the product in phosphate buffer (pH 7.2) showed the 2′-*O*-methyladenosine 5′-phosphate to be homogeneous with a mobility identical with that of adenosine 5′-phosphate. 2′-*O*-Methyladenosine 5′-phosphate triethylammonium salt (1.36 g, 2.41 mmol) and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine (0.87 g, 3.1 mmol) were dissolved in 75 ml of pyridine and 15 ml of H_2O. The solution was evaporated and azeotroped with pyridine. The resulting glass was dissolved in 300 ml of pyridine and was added dropwise to a refluxing solution of 1.25 g (6.0 mmol) of dicylohexylcarbodiimide in 300 ml of pyridine over 1 hr. The solution was refluxed an additional 2 hr and then 10 ml of H_2O was added and the solution evaporated to dryness. The residue was partially dissolved in H_2O and filtered, and the filtrate was evaporated. The residue was dissolved in 0.5 M Et_3NHCO_3, concentrated, and added to a Whatman DE-52 cellulose column (HCO_3^- form, 30 × 6.3 cm). Elution with a gradient of H_2O–0.5 M Et_3NHCO_3 (3:3, v/v) yielded 0.72 g of the triethylammonium salt of 2′-*O*-methyladenosine 3′,5′-cyclic phosphate after evaporation of the proper fractions. The cyclic nucleotide was dissolved in 20 ml of H_2O and the pH was adjusted to 2 with concentrated HCl; addition of EtOH gave crystalline 2′-*O*-methyladenosine 3′,5′-cyclic phosphate: $\lambda_{max}^{pH\ 1}$ 256 nm (ε 14,780), $\lambda_{max}^{pH\ 11}$ 258 nm (ε 15,170).

Anal. Calcd for $C_{11}H_{14}N_5O_6P \cdot 2.5H_2O$: C, 34.02; H, 4.93; N, 18.03; P, 7.97. Found: C, 34.40; H, 5.30; N, 18.16; P, 7.93.

2′-O-(2,4-Dinitrophenyl)adenosine 3′,5′-Cyclic Phosphate.[3] To a stirred suspension of cAMP (3.29 g, 10 mmol) in *N*,*N*-dimethylformamide (20 ml) was added Et_3N (7.4 g, 40 mmol) and 2,4-dinitrofluorobenzene (3.72 g, 20 mmol). The solution was maintained under anhydrous conditions at room temperature overnight. MeOH (20 ml) was added and the solution evaporated *in vacuo*. The residual gum was dissolved in EtOH (30 ml) and the product precipitated with excess Et_2O (200 ml). The gummy precipitate was repeatedly triturated with H_2O to obtain a pale powder (5.63 g). The powder was dissolved in MeOH by addition of 2 N NH_4OH and reprecipitated by acidification. The precipitate was dissolved in H_2O by addition of 2 N NH_4OH and the solution was acidified with 2 N HCl to cause precipitation of 2′-*O*-(2,4-dinitrophenyl)adenosine 3′,5′-cyclic phosphate (4.7 g, 97%) $\lambda_{max}^{pH\ 1}$ 256, 294 sh nm (ε 23, 900), $\lambda_{max}^{pH\ 11}$ 258, 291 sh nm (ε 24,000).

Anal. Calcd for $C_{16}H_{14}N_7O_{10}P_1 \cdot H_2O$: C, 37.42; H, 3.11; N, 19.10. Found: C, 37.52; H, 3.21; N, 18.99.

Methods

The cAMP phosphodiesterases were purified from rabbit lung and kidney and beef cerebral cortex and heart. The method of preparation described below was used for all four tissues. All operations were performed at 4°. Fresh tissue was homogenized in 3 volumes of buffer I (0.33 M sucrose–10 mM imidazole-HCl (pH 7.5–10 mM magnesium acetate) for 1 min in a Waring blender at full speed. The homogenate was centrifuged at 30,000*g* for 1 hr and the supernatant was filtered through glass wool previously washed with buffer I. Solid $(NH_4)_2SO_4$ was added slowly to 50% of saturation, the suspension was stirred for 20 min and the precipitate was collected at 20,000*g* for 20 min. The precipitate was dissolved in buffer I (10 ml/100 ml of original homogenate) and dialyzed against a 100-fold excess of buffer II (buffer I without sucrose) for 18 hr. The precipitate that formed during dialysis was removed by centrifugation and the clarified protein solution was applied to a column of DEAE-cellulose (S&S Type 70, 20 ml of packed column volume/100 g of tissue) previously equilibrated with buffer II. The column was eluted at 1.0 ml/min with buffer II until the $A_{280\ nm}$ of the effluent was ≤0.05. The column was then eluted with buffer II containing 0.4 M KCl. All fractions (10 ml) with an $A_{280\ nm}$ of 0.2 or greater that had a A_{280}:A_{260} ratio of 1.0 or greater were pooled and dialyzed for 18 hr against a 100-fold excess of buffer II. The dialyzed enzyme was clarified by centrifugation and frozen at −20°. The protein concentration varied between 1 and 2 mg per ml.

cAMP-dependent protein kinase was purified through the DEAE-cellulose step as previously described (Miyamato *et al.*, 1969; Kuo and Greengard, 1969). The assay for the stimulation of this enzyme by cAMP and the determination of K_a values were performed as previously described (Muneyama *et al.*, 1971).

[2] Direct alkylation of cAMP has also been reported to give 2′-*O*-methyl-cAMP (Tazawa *et al.*, 1972).

[3] 2′-*O*-Dnp-cAMP was obtained in an attempt to prepare adenosine 3′,5′-cyclic phosphorofluoridate. The synthesis of 2′-*O*-Dnp-cAMP was recently reported by Jastorff and Freist (1972) after this work had been completed.

TABLE I: Stimulation of cAMP-Dependent Protein Kinases by 2′ Derivatives of cAMP.[a]

Compound	K_a' Value	
	Bovine Brain	Bovine Heart
cAMP	1.0	1.0
2′-*O*-Acetyl-cAMP	0.0057	0.0040
2′-*O*-Methyl-cAMP	0.0023	0.0017
2′-*O*-Dnp-cAMP	0.0008	0.0012
2′-*O*-Butyryl-cAMP	0.0030	0.0017
N^6-Butyryl-cAMP	0.84	0.92
N^6-2′-*O*-Dibutyryl-cAMP	0.0010	0.0033
Ara-cAMP	0.0013	0.0029
2′-Deoxy-cAMP	0.0038	0.0048

[a] Values reported are K_a' values; where $K_a' = K_a$ for cAMP/K_a for test compound. K_a is the activation constant. The K_a values for cAMP were 4×10^{-8} and 7×10^{-8} M for the enzymes from brain and heart, respectively. The assay was performed as described under Methods.

TABLE II: 2′ Derivatives of cAMP as Substrates for High K_m cAMP Phosphodiesterases.

Compound	Rel Rate of Hydrolysis[a]			
	Beef Heart	Beef Brain	Rabbit Lung	Rabbit Kidney
cAMP	100	100	100	100
2′-*O*-Acetyl-cAMP	58	55	43	50
2′-*O*-Methyl-cAMP	49	39	20	30
2′-*O*-Dnp-cAMP	17	6	12	11
2′-*O*-Butyryl-cAMP	81	71	22	37
N^6-Butyryl-cAMP	8	7	16	3
N^6,2′-*O*-Dibutyryl-cAMP	7	3	1	3
Ara-cAMP	18	15	25	17
2′-Deoxy-cAMP	92	59	38	67

[a] The rates of hydrolysis by each enzyme are expressed relative to that of cAMP. The actual rates of cAMP hydrolysis (nmol of 5′-nucleotide formed per min) were 12, 22, 12, and 30 by the heart, brain, lung, and kidney enzymes, respectively. The rates were determined by colorometric determination of the phosphate produced by the action of bacterial alkaline phosphatase on the 5′-nucleotide product of phosphodiesterase hydrolysis.

When testing cAMP derivatives as substrates for cAMP phosphodiesterase, the standard reaction mixture contained in 0.60 ml: 3.0 μmol of cAMP or cAMP derivative, 6 μmol of $MgCl_2$, and 0.1–0.3 mg of phosphodiesterase protein. The incubation times were determined from pilot assays to give kinetically valid data. Incubation was from 10 to 45 min at 30°. The reaction was terminated by heating to 90° for 2.5 min. Control tubes contained enzyme inactivated in the same way before the incubation. The mixtures were centrifuged to remove denatured protein and a 50-μl portion of the supernatant was chromatographed on cellulose thin layer. The plates were developed in a suitable solvent and then visualized under ultraviolet light. Each spot was scraped from the plate; the material was eluted from the cellulose with 0.1 M $(NH_4)_2CO_3$ or distilled H_2O and the amount was quantitated optically. Control experiments with 8-[^{3}H]cAMP showed that >96% of the material applied to the plate was recovered.

A second method was also used to determine the amount of 5′-phosphate product formed from phosphodiesterase action on the cAMP analogs. An identical reaction mixture was incubated and the reaction was terminated by heating. A control reaction contained heat-inactivated enzyme. Then 100 μg (10 μl) of bacterial alkaline phosphatase (Worthington BAPSF or BAPC) was added and incubation was continued for 1 hr at 30°. Control experiments with 5′-AMP and 2′-*O*-methyl-5′-AMP showed that under these conditions, there was stoichiometric conversion of these compounds to adenosine and 2′-*O*-methyladenosine, respectively. The reaction with alkaline phosphatase was terminated by addition of 100 μl of 55% trichloroacetic acid. The precipitate was removed by centrifugation and the supernatants were analyzed for inorganic phosphate by a modification of the method of Lowry and López (1946).

In experiments where the analogs were tested as inhibitors of phosphodiesterase, the reaction contained in 1.0 ml: 30 μmol of Tris-HCl (pH 7.5), 10 μmol of $MgCl_2$, 10–200 μg of phosphodiesterase protein, and 160 pmol or 100 nmol of 8-[^{3}H]cAMP, for the assay of the low K_m and high K_m phosphodiesterases, respectively. Approximately 350,000 cpm/assay was present at both cAMP concentrations. The concentration of the cAMP analogs was varied over an appropriate range. The reaction mixture was incubated at 30° for 10 min, and then heated to 90° for 2 min to terminate the reaction. An identical reaction mixture was heat inactivated immediately for the determination of the background radioactivity of the assay. After the reaction was cooled to room temperature, 0.1 ml of a 1-mg/ml solution of snake venom (*Crotalus atrox*) was added to each reaction mixture which was then incubated for 10 min at 30°. Next, 1 ml of a 1:3 suspension of neutral, base-washed Dowex 1-X8 was added to each tube, which was then mixed well and centrifuged for 10 min at 1000 rpm; 0.1 ml of the supernatant from each tube was added to 3 ml of Aquascint II and the radioactivity of each assay was determined in a liquid scintillation spectrometer.

Results

In order to ascertain the relative importance of the 2′ position of cAMP in binding to enzymes which utilize cAMP, each of the derivatives was studied as an activator of the cAMP dependent protein kinase and as a substrate and an inhibitor of the cyclic nucleotide phosphodiesterase. These properties were compared with those reported for N^6,2′-*O*-dibutyryl-cAMP, N^6-butyryl-cAMP, and 2′-*O*-butyryl-cAMP (Posternak *et al.*, 1962; Henion *et al.*, 1967).

Activation of cAMP-Dependent Protein Kinases by 2′-O-Substituted cAMP Analogs. The ability of the 2′-O-substituted cAMP analogs to stimulate protein kinase was tested, using enzymes from bovine brain and heart. The three butyryl analogs were also tested for comparison. As can be seen in Table I, all derivatives with a 2′-O substitution were less than $^1/_{100}$th as active as cAMP. N^6-Butyryl-cAMP, by comparison, was almost as active as cAMP itself.

2′ Derivatives of cAMP as Substrates for cAMP Phospho-

diesterase. Table II gives the results of a study comparing the susceptibility of the 2′ derivatives of cAMP to cAMP phosphodiesterases purified from rabbit lung, rabbit kidney, beef heart, and beef brain. These hydrolysis rates were determined by measuring the phosphate removed from the 5′-nucleotide product with bacterial alkaline phosphatase. Essentially the same results were obtained when the hydrolysis rates were determined by chromatographic separation and quantitation of the resultant 5′-nucleotide.

Most strikingly, N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP were found to be very poor substrates for each of the four enzymes. In comparison, 2′-*O*-butyryl-cAMP, 2′-*O*-acetyl-cAMP, and 2′-*O*-methyl-cAMP, which do not contain an N^6 substitution, were quite good substrates for cAMP phosphodiesterase, as was 2′-deoxy-cAMP. 2′-*O*-Dnp-cAMP, a 2′-*O*-ether containing a relatively large steric and electron-withdrawing substituent at the 2′ position, was a poor substrate. Ara-cAMP was also hydrolyzed at a relatively slow rate compared to cAMP. There were significant variations in tissue specificity; for example, while 2′-*O*-acetyl-cAMP and ara-cAMP were each hydrolyzed at essentially the same rate by all four enzymes (50 and 25%, respectively), 2′-*O*-butyryl-cAMP was split four times faster by the heart enzyme than by the lung enzyme (81% *vs.* 22%). Of the four cAMP phosphodiesterase preparations investigated, the beef heart phosphodiesterase appeared to be the least sensitive to modifications of cAMP in the 2′ position. With the exception of ara-cAMP, the heart enzyme preparation was able to hydrolyze each of the 2′ derivatives of cAMP at a faster rate than any of the three other enzyme preparations. By comparison, the lung enzyme preparation was the most sensitive to changes in the cAMP molecule at the 2′ position, and hydrolyzed most of the derivatives at a slower rate than did the other three enzyme preparations.

For those derivatives that were substrates, the nature of the products of phosphodiesterase action was also investigated. The product in all cases migrated like a nucleoside monophosphate upon electrophoresis in phosphate buffer at pH 7.0. The product of the action of phosphodiesterase on 2′-*O*-methyl-cAMP was shown to be exclusively 2′-*O*-methyladenosine 5′-phosphate by chromatographic comparison in the solvent systems A and B (see Experimental) with an authentic sample of the corresponding 5′-nucleotide. With 2′-*O*-acetyl-cAMP as substrate, the product was 2′-*O*-acetyladenosine 5′-phosphate. This material was chromatographically distinct from 2′-*O*-acetyl-cAMP, cAMP, and 5′-AMP, in systems A and B and migrated as a diionic species upon electrophoresis at pH 7.0. When treated with 5 N NH_4OH for 1 hr at 50° (to remove the 2′-*O*-acetyl group), the product was chromatographically identical with 5′-AMP. By the same criteria, 2′-*O*-butyryl-cAMP was shown to be hydrolyzed by phosphodiesterase to 2′-*O*-butyryl-5′-AMP. Under the conditions used in the phosphodiesterase incubation, there was no detectable deacylation of the 2′-*O*-acyl-cAMP derivatives to cAMP by any of the four enzyme preparations. The ether linkage of the 2′-*O*-methyl and 2′-*O*-(2,4-dinitrophenyl) substituents were also stable under the incubation conditions used.

2′ Derivatives of cAMP as Inhibitors of cAMP Phosphodiesterase. All tissues so far examined contain at least two cAMP phosphodiesterase activities; one with a K_m for cAMP of approximately 10^{-7} M (low K_m) and a second with a K_m of approximately 10^{-4} M (high K_m).

Table III summarizes results of a study of the inhibition of the low K_m cAMP phosphodiesterases from the four tissues.

TABLE III: Inhibition of Low K_m cAMP Phosphodiesterases by 2′ Derivatives of cAMP.

Compound	I_{50} (μM)[a]			
	Beef Heart	Beef Brain	Rabbit Lung	Rabbit Kidney
2′-*O*-Acetyl-cAMP	10	150	5.0	110
2′-*O*-Methyl-cAMP	8.7	230	4.8	41
2′-*O*-Dnp-cAMP	26	57	25	89
2′-*O*-Butyryl-cAMP	5.6	23	5.6	36
N^6-Butyryl-cAMP	37	230	46	180
N^6,2′-*O*-Dibutyryl-cAMP	37	770	230	320
Ara-cAMP	24	1200	190	800
2′-Deoxy-cAMP	21	210	11	180
Theophylline	130	230	250	160

[a] I_{50} equals the concentration of compound that produces 50% of maximal inhibition. The concentration of cAMP used was 1.7×10^{-7} M and the assay was performed as described under Methods.

The results are expressed as I_{50} values[4] and the cAMP concentration in all cases was 1.6×10^{-7} M. The I_{50} values for theophylline were found to be very similar for each of the four enzyme preparations. The various cAMP derivatives, on the other hand, demonstrated large differences in potency as well as large differences in tissue specificity with respect to their inhibitory ability. A comparison of the four 2′-O-substituted derivatives; *i.e.*, acetyl-, methyl-, butyryl-, and Dnp-cAMP shows that the lung and heart enzymes were more sensitive than the brain and kidney enzymes to inhibition by compounds containing relatively small substituents in the 2′-O position. With the larger 2,4-dinitrophenyl substituent, the lung and heart enzymes were somewhat less sensitive to inhibition. The brain enzyme appeared to be more sensitive to inhibition when the derivative contained the bulkier 2,4-dinitrophenyl group and 2′-*O*-butyryl group compared to the inhibition produced by the smaller 2′-*O*-methyl and 2′-*O*-acetyl group.

A comparison of the three butyryl derivatives of cAMP against all four enzymes revealed that 2′-*O*-butyryl-cAMP was a better inhibitor than N^6-butyryl-cAMP which in turn was a better inhibitor than N^6,2′-*O*-dibutyryl-cAMP. The only exception to this order was with the heart enzyme where the latter two compounds were equally potent. N^6-Butyryl-cAMP, like the 2′-O derivatives compared above, was a better inhibitor of the heart and lung enzymes than the brain and kidney enzymes.

The last two derivatives under consideration, 2′-deoxy- and ara-cAMP, were found also to preferentially inhibit the enzymes from lung and heart over those from brain and kidney. The only exception to this is ara-cAMP which was 50 times more effective against the heart than the brain enzyme. In general, 2′-deoxy-cAMP was approximately equal in potency to those derivatives with a small substitution in the 2′ position while ara-cAMP was much less potent than the other compounds tested. The heart enzyme was an exception and did not demonstrate this differential sensitivity while the 2′-O-

[4] I_{50} = concentration of inhibiting compound that produces 50% inhibition of the reaction.

TABLE IV: Inhibition of High K_m cAMP Phosphodiesterases by 2′ Derivatives of cAMP.

Compound	I_{50} (μM)[a]			
	Beef Heart	Beef Brain	Rabbit Lung	Rabbit Kidney
2′-*O*-Acetyl-cAMP	190	160	260	150
2′-*O*-Methyl-cAMP	190	160	260	250
2′-*O*-Dnp-cAMP	190	75	450	75
2′-*O*-Butyryl-cAMP	580	170	180	210
N^6-Butyryl-cAMP	290	660	820	250
N^6,2′-*O*-Dibutyryl-cAMP	190	250	230	300
Ara-cAMP	1900	3300	1800	500
2′-Deoxy-cAMP	970	1100	450	500
Theophylline	580	330	900	150

[a] I_{50} equals the concentration of compound that produces 50% of maximal inhibition. The concentration of cAMP used was 1×10^{-4} M and the assay was performed as described under Methods.

substituted, ara, and 2′-deoxy derivatives demonstrated almost equal potency.

Table IV shows the results of the determination of the potency of inhibition of the high K_m cAMP phosphodiesterase activities of the four enzyme preparations by the 2′ derivatives of cAMP. Here the I_{50} values for theophylline vary appreciably among the four enzymes, while being relatively constant for 2′-*O*-acetyl- and methyl-cAMP. 2′-*O*-Dnp-cAMP was a better inhibitor of the brain and kidney enzymes than of the heart and lung enzymes. 2′-*O*-Butyryl-cAMP inhibited all four enzymes equally, with the single exception of the heart enzyme against which was a poorer inhibitor. In addition, this latter derivative was a poorer inhibitor of the heart enzyme than any of the other three derivatives of cAMP containing a substitution on the 2′ position.

As in the case of the low K_m enzymes, in general, 2′-deoxy-cAMP was a better inhibitor than ara-cAMP; but, unlike the low K_m activities, 2′-deoxy-cAMP was a poorer inhibitor than the 2′-O-substituted cAMP derivatives.

Discussion

In an effort to gain additional knowledge about the relationship of the carbohydrate moiety to the biological activity of cAMP, a series of 2′-modified derivatives of cAMP were synthesized and investigated. Falbriard *et al.* (1967) had previously reported on the synthesis of acyl derivatives of cAMP; however, the question of biological and chemical stability of the acyl derivatives created some doubt as to their true biological mechanism of action. The significance of the 2′ position of cAMP for interaction with protein kinase and phosphodiesterase was therefore studied by a comparison of 2′-*O*-acyl derivatives of cAMP with the more chemically stable 2′-*O*-methyl-, 2′-*O*-(2,4-dinitrophenyl)-, 2′-deoxy; and ara-cAMP derivatives.

All the compounds under study, which contained substituents or modifications at the 2′ position were very poor activators of protein kinase suggesting that the unblocked 2′-OH in a ribo configuration is necessary for interaction with the regulatory subunit of the protein kinase. The effect of 2′-O modification was also evident in stimulation of protein kinase by N^6-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP. Whereas N^6-butyryl-cAMP was almost as active as cAMP, N^6,2′-*O*-dibutyryl-cAMP was only $^1/_{1000}$th as active.

The procedure used for the preparation of all four phosphodiesterase activities produced a final preparation containing most (80–90%) of the activity present in the crude homogenate. In contrast to the results with protein kinase, all the 2′ derivatives of cAMP were substrates for the phosphodiesterases. Each of the four preparations contained both the low K_m and the high K_m phosphodiesterase activities. The K_m for the former activity was approximately 10^{-7} M and that for the latter in the order of 10^{-4} M for all four preparations. The 2′ derivatives of cAMP were tested as substrates for the high K_m phosphodiesterase at a substrate concentration of 5×10^{-3} M, which was 50 times the K_m of this enzyme. The higher concentration was necessary in order to obtain measurable rates with the method of assay employed. The product of phosphodiesterase hydrolysis of the 2′-substituted cAMP derivatives was found to be the corresponding 2′-substituted 5′-nucleotide. With respect to their susceptibility to enzymatic hydrolysis, all of the 2′ derivatives of cAMP investigated were substrates for the four different enzyme preparations (beef heart, beef brain, rabbit lung, and rabbit kidney), although to greatly varying extents. The necessity for an intact 2′-OH group for interaction of cAMP with the phosphodiesterase enzyme substitute appeared to be tissue specific as demonstrated (Table II) by the varying rates of hydrolysis of 2′-deoxy-cAMP (between 92 and 38%) by the four enzymes, where the beef heart enzyme was the most sensitive and the rabbit kidney the least sensitive to the absence of a 2′-OH group. That 2′-deoxy-cAMP is a substrate for phosphodiesterase to varying degrees is further verified by the results of Drummond and Powell (1970) with beef brain phosphodiesterase, by Michal *et al.* (1970) with rat adipose tissue and by the results of Nair (1966) with dog heart phosphodiesterase preparation.

With the exception of 2′-*O*-Dnp-cAMP, the derivatives with a substitution on the 2′-OH group (acetyl, methyl, butyryl) were hydrolyzed at approximately the same rate as was 2′-deoxy-cAMP. The effect of decreased substrate capability of the 2′-O-substituted derivatives cannot, therefore, be explained solely by a steric effect. Additionally, 2′-*O*-butyryl-cAMP had a moderate rate of hydrolysis which was generally greater than the rate of hydrolysis of both the 2′-*O*-acetyl and 2′-*O*-methyl derivatives. These data, therefore, suggest that while the 2′-OH group is necessary for optimal interaction with the enzyme, the presence of the oxygen is of less importance than the presence of the hydroxyl hydrogen. A second possibility is that the effects on the ability of these two types of cAMP derivatives (2′-O substitution *vs.* 2′-OH removal) to interact with the enzyme are different, and that it is only coincidental that the rates of hydrolysis are similar.

The importance of the ribo configuration of the 2′-OH is illustrated by the relatively low rates of hydrolysis of ara-cAMP (Table II). This effect cannot be entirely explained by the lack of a hydroxyl in the ribo configuration because hydrolysis of ara-cAMP by all four enzymes was less than that of 2′-deoxy-cAMP. This effect could be additionally explained by either (a) an unfavorable interaction between the arabino-OH and the enzyme, and/or (b) by the presence of a potential rotational barrier (Schweizer and Robins, 1972) between the arabino OH and the adenine ring which could hinder the formation of a favorable conformation of ara-cAMP with the enzyme.

The comparative N^6-substituted derivatives, N^6-butyryl- and N^6,2′-*O*-butyryl-cAMP, were very poor substrates for the phosphodiesterases. This finding is consistent with the results of others (Moore *et al.*, 1968; Drummond and Powell, 1970; Michal *et al.*, 1970; Meyer *et al.*, 1972) who showed that N^6 substitution, in general, resulted in resistance of the compound to phosphodiesterase hydrolysis. The structure–activity relationship of the N^6- and 6-substituted 3′,5′-cyclic nucleotides deserves further investigation.

The manner in which derivatives of cAMP mimic the action of cAMP may, in part, be by inhibiting cAMP phosphodiesterase and thereby increasing endogenous cAMP levels. We have, therefore, examined the ability of the 2′ derivatives of cAMP to inhibit both the low and high K_m cAMP phosphodiesterases. Each of the phosphodiesterase inhibition studies were done at or near the K_m for the activity being investigated. The four phosphodiesterases used here all had low K_m values for cAMP of about 10^{-7} M, and the cAMP concentration used was 1.7×10^{-7} M. Whereas the I_{50} values for theophylline for all four enzymes were similar, the I_{50} values for the 2′ derivatives of cAMP showed distinct differences in tissue specificity and potency which were equal to or better than the I_{50} values of theophylline. The beef heart and rabbit lung were found to be the most sensitive to inhibition by all the derivatives. In general, the 2′-O-substituted and 2′-deoxy-cAMP derivatives were approximately equal in inhibitory activity and the more potent inhibitors of the low K_m phosphodiesterase enzymes. The N^6-butyryl derivatives were less active and ara-cAMP was the least active as inhibitors of these enzymes. As in the substrate studies, the conformation and presence of the 2′-OH is important for interaction of the cyclic nucleotide with the low K_m phosphodiesterase enzymes.

In contrast to the I_{50} values for the low K_m phosphodiesterases, the high K_m enzyme I_{50} values for theophylline varied more between enzymes while the I_{50} values for the 2′-O-substituted derivatives varied less. Similar to the low K_m results, N^6-butyryl-cAMP and 2′-deoxy-cAMP were equal in activity but somewhat less active than the above compounds, and ara-cAMP was the least active compound tested. It is interesting to note that 2′-*O*-butyryl-cAMP and N^6,2′-*O*-dibutyryl-cAMP exhibited similar I_{50} values against the high K_m enzyme while N^6-butyryl-cAMP was significantly less active. This is in contrast to the I_{50} values for the same three compounds against the low K_m enzyme where 2′-*O*-butyryl-cAMP was more active than the N^6-mono- and N^6,2′-*O*-dibutyryl-cAMP derivatives. This suggests that the mechanism of inhibition of the butyryl derivatives is different for the two enzymes of differing K_m values.

In comparing the ability of the derivatives to act as substrates and as inhibitors of the high K_m phosphodiesterase enzymes, the experimental procedures dictate that the inhibition studies take into account any competition for interaction with the enzyme between the cAMP derivative and cAMP itself, while the studies of the analogs as substrates were performed in the absence of cAMP and, therefore, measured no competition with cAMP. If the only means by which these analogs were acting as inhibitors of the phosphodiesterases was by competing with cAMP to be utilized as a substrate, then it would be expected that those derivatives which were the better substrates would also be the better inhibitors. Because the 2′-O-substituted derivatives were all (with the exception of N^6,2′-*O*-dibutyryl-cAMP) both good substrates and good inhibitors, the values for these compounds were used as a standard against which to compare the values for the other compounds. N^6-Butyryl-cAMP and ara-cAMP were both poor substrates and poor inhibitors. In contrast, N^6,2′-*O*-dibutyryl-cAMP, 2′-*O*-Dnp-cAMP, and 2′-deoxy-cAMP did not fit the pattern; N^6,2′-*O*-dibutyryl-cAMP and 2′-*O*-Dnp-cAMP were poor substrates but very good inhibitors, while 2′-deoxy-cAMP was a very good substrate but a poor inhibitor of the high K_m phosphodiesterases. This comparison suggests that for these latter compounds the mechanism of inhibition is quite different from one of simply mimicking the substrate. These findings are quite interesting in themselves, as well as in light of the report of Russell *et al.* (1972), which suggests that phosphodiesterase contains two binding sites for cAMP and demonstrates negative cooperativity. Further kinetic studies are needed to delineate the mechanism of phosphodiesterase inhibition by these 2′ derivatives of cAMP.

Acknowledgment

We express our sincere appreciation to Dr. Don N. Harris of the Department of Biochemical Pharmacology, Squibb Institute for Medical Research, for his collaboration in the preliminary phases of this investigation and his helpful comments.

References

Blecher, M., Ro'Ane, J. T., and Flynn, P. D. (1971), *Arch. Biochem. Biophys. 142*, 351.

Broom, A. D., and Robins, R. K. (1964), *J. Amer. Chem. Soc. 87*, 1145.

Drummond, G. I., and Powell, C. A. (1970), *Mol. Pharmacol. 6*, 24.

Drummond, G. I., and Severson, D. L. (1970), *Annu. Rev. Med. Chem. 6*, 215.

Falbriard, J. G., Posternak, T., and Sutherland, E. W. (1967), *Biochim. Biophys. Acta 148*, 99.

Free, C. A., Chasin, M., Paik, V., and Hess, S. M. (1971), *Biochemistry 10*, 3785.

Henion, W. F., Sutherland, E. W., and Posternak, T. (1967), *Biochim. Biophys. Acta 148*, 106.

Hepp, K. D., Menahan, L. A., Wieland, O., and Williams, R. H. (1969), *Biochim. Biophys. Acta 184*, 554.

Jastorff, B., and Freist, W. (1972), *Angew. Chem., Intern. Ed. 11*, 713.

Kankel, E., and Hilz, H. (1972), *Biochem. Biophys. Res. Commun. 46*, 1011.

Kankel, E., Mundhenk, K., and Hilz, H. (1972), *Eur. J. Biochem. 27*, 197.

Khwaja, T. A., Harris, R., and Robins, R. K. (1972), *Tetrahedron Lett.*, 4681.

Kuo, J. F., and Greengard, P. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 1349.

Levine, R. A., and Washington, A. (1970), *Endocrinology 87*, 377.

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

Menahan, L. A., Hepp, K. D., and Wieland, O. (1969), *Eur. J. Biochem. 8*, 435.

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

Michal, G., Nelböck, M., and Wiemann, G. (1970), *Fresenius, Z. Anal. Chem. 252*, 189.

Miyamato, E., Kuo, J. F., and Greengard, P. (1969), *J. Biol. Chem. 244*, 6395.

Moore, P. F., Iorio, I. C., and McManus, J. M. (1968), *J. Pharm. Pharmacol. 20*, 368 (1968).
Muneyama, K., Bauer, R. J., Shuman, D. A., Robins, R. K., and Simon, L. N. (1971), *Biochemistry 10*, 2390.
Nair, K. G. (1966), *Biochemistry 5*, 150.
Posternak, T., Sutherland, E. W., and Henion, W. F. (1962), *Biochim. Biophys. Acta 65*, 558.
Robinson, G. A., Butcher, R. W., and Sutherland, E. W. (1971), Cyclic-AMP, New York, N. Y., Academic Press.
Rottman, F., and Heinlein, K. (1968), *Biochemistry 7*, 2634.
Russell, T. R., Thompson, W. J., Schneider, F. W., and Appleman, M. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1791.
Schweizer, M. P., and Robins, R. K. (1972), *Proc. 5th Intern. Jerusalem Symp., April.*
Tazawa, I., Tazawa, S., Alderfer, J. L., and Ts'o, P. O. (1972), *Biochemistry 11*, 4931.

CORRECTIONS

"The Nature of the Reverse Type I (Modified Type II) Spectral Change in Liver Microsomes," by John B. Schenkman,* Dominick L. Cinti, Sten Orrenius, Peter Moldeus, and Robert Kaschnitz, Volume 11, Number 23, November 7, 1972, page 4243.

Dr. Kaschnitz's name was erroneously spelled Kraschnitz, and his fellowship acknowledgment should have read, "Recipient of a short term fellowship from the European Molecular Biology Organization (Brussels)."

"Mechanism of Activation of Bovine Procarboxypeptidase A S_5. Alterations in Primary and Quaternary Structure," by Jack R. Uren and Hans Neurath, Volume 11, Number 24, November 21, 1972, page 4483.

The illustrations but not the legends of Figures 7 and 9 should be interchanged, *i.e.*, the legend of Figure 9 goes with Figure 7 and *vice versa*.

"A Calorimetric Investigation of the Copper–Bovine Plasma Albumin Interaction," by Fred H. Reynolds, Jr., R. K. Burkhard, and Delbert D. Mueller, Volume 12, Number 2, January 16, 1973, page 359.

On page 360, the first part of the second sentence under Results should read: "When $\bar{v}/A$ was plotted *vs.* $\bar{v}$. . ." On page 361, the units for the entropy changes in Table I should read cal mol^{-1} deg^{-1}.