# Synthesis and Biological Activities of Cyclic AMP Analogs Modified in the I, 2, and 2'-Positions

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Analogs of adenosine-3',5'-cyclic phosphate (cAMP) modified in positions 2 (Cl, Br, SCH<sub>3</sub>) and 2' (2,4-dinitrophenyl) and doubly modified in positions 1 and 2 (N<sup>1</sup>O and Cl), 2 and 2' (Cl and 2,4-dinitrophenyl), have been synthesized by convenient methods. These derivatives have been examined as alternative activators of cAMP-dependent protein kinase isolated from bovine muscle and as alternative substrates for a cyclic phosphodiesterase from bovine heart. All analogs activated the kinase, most of them being more effective than cAMP. All were degraded by the diesterase, several at lower rates.

In recent years cAMP has been established as one of the most important small regulatory molecules in the whole field of life (1). Since cAMP is involved in the regulation of mammalian hormone systems (2), protein synthesis (3), cell division and transformation of cells (4, 5), it seems possible that mistakes in cAMP metabolism are responsible for some diseases (2). Several research groups have modified the cAMP molecule by chemical synthesis in different positions of the base, the ribose and the phosphate moiety (6), to get analogs which are more specific and have higher biological activity and cell penetration than cAMP itself, and may, therefore, be useful as chemotherapeutics. Besides these pharmacological aspects, biological investigations with cAMP analogs may provide insight into molecular interactions of the nucleotide and its different protein receptors.

In several laboratories the C-8 and N-6 position of the base moiety have been extensively modified (7-10). The C-2 position which might be responsible for specificity differences between cAMP and cGMP has been substituted by an amino group (10). The 2'-hydroxyl group has been acylated with different carboxylic acid anhydrides (11)to give relatively unstable esters. The most thoroughly investigated cAMP analog, 2',6-dibutyryl-cAMP (11), shows various interesting biological activities (12), but it frequently remains very difficult to decide which species is the active compound in the cell system, for the unstable 2'-ester bond can be hydrolyzed by the incubation medium as well as by enzymatic reaction (13, 14).

We recently reported in a short communication a chemically more stable substituent for the 2'-position, a 2,4-dinitrophenyl ether, and two new C-2 modified analogs, chloro- and bromo-derivatives (15). Here we describe their detailed synthesis, some other modified analogs based on these compounds, and their biological activities.

### Chemical Synthesis of the cAMP Analogs

For modification of the C-2' position we used cAMP (1) as starting material.



On treating (1) with 2,4-dinitrofluorobenzene in dimethylformamide in the presence of di-isopropylethylamine as base one obtains neither fluorination of phosphorous as with AMP (16), nor substitution of the 6-amino-group as with basic amino groups (17), but only the dinitrophenyl ether (2), which was completely stable under physiological conditions (pH 5-9). Subsequent treatment of 2 with *n*-butyric acid anhydride as described by Falbriand *et al.* (11) gives the N-6 acylated derivative (3). This compound may mimic the activities of dibutyryl-cAMP with the advantage of greater stability at the 2'-substituent.

The C-2-substituted derivatives (6) were prepared by phosphorylation of the 2-halonucleosides (4) (synthesized according to Montgomery and Hewson (18)) with POCl<sub>3</sub> in triethyl phosphate (19), and subsequent cyclization of the 5'-phosphate (5) with dicyclohexylcarbodiimide in anhydrous pyridine/DMSO, following the procedures of Symons (20), with a modified isolation technique for the products.

The chlorine atom in 6a is capable of undergoing nucleophilic attack (21) and was substituted by treatment with sodium methylmercaptide in anhydrous methanol to yield the 2-S-methylmercapto-derivative 7.

When 6a was treated with 2,4-dinitrofluorobenzene in the same way as cAMP, the 2,2'-modified analog 8 was obtained.

Oxidation of 6a with a large excess of *m*-chloro-perbenzoic acid or monoperphthalic acid gave the  $N^1$ -oxide 9 in only very poor yield even after 1 week, while cAMP could be quantitatively oxidized under these conditions.

While the compounds 2, 6a, and 6b, which were to be used as starting materials for further modifications, were prepared in such amounts that pmr spectra could be measured to confirm their structures, the analogs 3, 7, 8, and 9 were synthesized only in small amounts sufficient for determination of their biological activities. Their structures were unambiguously established by uv-spectra and chromatographic behavior. Thus, 7 showed the pH-dependent uv-spectrum typical of 2-S-alkyl-substituted adenosine (21), 8 the characteristic dinitrophenyl shoulder at 300 nm together with the spectrum of 2-chloro-derivatives, and 9 a bathochromically shifted adenosine-3',5'-cyclic phosphate- $N^1$ -oxide spectrum (22) (see Table 1). We tried, in addition, to alkylate the oxygen of the cAMP- $N^1$ -oxide 10 (11) with benzyl bromide according to the methods described by Montgomery *et al.* and Fuji *et al.* (23). Although nearly all the starting material disappeared, it was not possible to



isolate the pure  $N^1$ -benzyloxy derivative 11 either by different chromatographic methods or by precipitation from various solvents. We could only determine the chromatographic behavior and the uv-spectrum of the compound, which showed a change

from the typical N<sup>1</sup>-oxide spectrum ( $\lambda_{max}$  232 nm) to that of a N<sup>1</sup>-benzyloxy compound ( $\lambda_{max}$  260 nm).



Scheme 3

# **Biochemical Studies**

To obtain initial information about the biological activities of the synthesized analogs, we studied their behavior as substrates for two enzymes, cyclic phosphodiesterase and protein kinase, which, together with adenyl cyclase, play the most important role in cAMP metabolism (24). Both enzymes were obtained from commercial sources and purified to the same degree as used by other authors who have tested cAMP analogs (7-9).

For comparison with compound 9, cAMP- $N^1$ -oxide, which was first synthesized by Posternak (11, 22), has also been introduced into the biochemical studies, since it has previously been tested only in cell extracts (22) and not in isolated enzyme systems.

### Phosphodiesterase Studies

All compounds were substrates for the cyclic phosphodiesterase from bovine heart and were hydrolyzed to the corresponding 5'-monophosphates. Comparing their relative extents of hydrolysis under identical conditions, we found differences depending on the type of modification (see Table 1).

The  $N^1$ -oxide 10 and the S-methyl derivative 7 were cleaved at about the same rate as cAMP. Chloro- and bromo-substitution in position C-2 (compounds **6a** and **6b**) caused only a slight decrease in the rate of hydrolysis, but this was further attenuated, being only 35-40% of the rate of cAMP if the 2'-hydroxyl group was substituted (compounds 2 and 8). The 2',6-modified analog 3 was a very poor substrate, and was not hydrolyzed during the test period. Hydrolysis was achieved only with a much larger amount of enzyme and after a longer incubation period.

The results show that modifications of the base moiety in position N-1 and C-2 do not confer any remarkable stability against hydrolysis by the phosphodiesterase; the enzyme-substrate interaction is much more influenced by substitution of the 2'-hydroxyl group. The high stability of the 2',6-disubstituted compound 3 is consistent with the result Michal *et al.* have obtained with 2',6-dibutyryl cAMP, using the same enzyme (9).

TABLE 1

| Compound                        | No. | α    |
|---------------------------------|-----|------|
| cAMP                            | 1   | 1.00 |
| 2′-NO <sub>2</sub> phe-cAMP     | 2   | 0.35 |
| 6-Bu-2'NO <sub>2</sub> phe-cAMP | 3   | 0.00 |
| 2Cl-cAMP                        | ба  | 0.80 |
| 2Br-cAMP                        | 6b  | 0.75 |
| 2CH₃S-cAMP                      | 7   | 1.00 |
| 2Cl-2'NO2phe-cAMP               | 8   | 0.40 |
| 2Cl-N <sup>1</sup> O-cAMP       | 9   | 0.50 |
| N <sup>1</sup> O-cAMP           | 10  | 1.00 |
|                                 |     |      |

EFFECT OF CAMP PHOSPHODIESTERASE FROM BOVINE HEART ON CAMP AND ITS ANALOGS<sup>a</sup>

<sup>*a*</sup>  $\alpha$  = rates of hydrolysis of test compounds/rates of hydrolysis of cAMP.

# Protein Kinase Studies

The cyclic AMP-dependent protein kinase from beef heart was activated by all the analogs described. To elicit information about their activation behavior compared with that of cyclic AMP, we measured the dependence of enzyme activity against the concentration of the nucleotide for each derivative and obtained the activation profiles shown in Figs. 1 and 2. Some analogs (Fig. 1) began to show activation at a concentration approximately 10 times higher than that of cyclic AMP, while others started at the



FIGS. 1 AND 2. Activity of the cyclic AMP-dependent protein kinase from bovine muscle in the presence of various concentrations of cyclic AMP and cyclic AMP-analogs. Incubation conditions are described in the Experimental section.  $\blacktriangle$  cAMP (1).  $\blacksquare$  2'NO<sub>2</sub>phe-cAMP (2).  $\bigcirc$  N<sup>1</sup>O-cAMP (10).  $\textcircled{\bullet}$  2Cl-2'-NO2phe-cAMP (8). × 2Cl-N<sup>1</sup>O-cAMP (9). □ 6-Bu-2'NO2phe-cAMP (3). © 2Br-cAMP (6b). ◊ 2ClcAMP (6a). ♦ 2CH<sub>3</sub>S-cAMP (7).

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same concentration (Fig. 2). Nearly all analogs caused maximal activity of the enzyme at higher concentrations than cyclic AMP, but most of them effected a higher degree of activation. The 2-mercaptomethyl- (7) and the 2-bromo derivative (**6b**) reached the highest degree, which was approximately 60% higher than that of cyclic AMP. A 25-30% better activation was caused by 2'-dinitrophenyl- (2)<sup>1</sup>, 2-chloro- (**6a**), and 2-chloro-2'-dinitrophenyl-cAMP (**8**). The 6-butyryl-2'-dinitrophenyl analog **3** was only a slightly better activator, while those compounds oxidized in position N-1 (**9** and **10**) reached the same degree as cyclic AMP.

All compounds modified in position N-1 or C-2 caused only slight, or, for the most part, no inhibition of the enzyme activity at high concentrations (>10<sup>-4</sup> M). This phenomenon, typical of cAMP and other analogs (7, 8, 24, 25), is only shown by 2'-dinitrophenyl- and 6-butyryl-2'-dinitrophenyl-cAMP (2 and 3).

### Discussion of the Biological Results

Our modifications of the cAMP molecule in positions N-1, C-2, or O-2' influence the shape of the activation profile of protein kinase and the rate of cleavage by the diesterase, but do not prevent interaction between the analogs and the proteins. This is in agreement with the results obtained by other authors on modifying positions N-6, N-7, or C-8 (7, 8, 26). From all the results available, one may assume that cAMP can be extensively modified in the base moiety without losing its ability to activate protein kinase or to serve as a substrate for the cyclic phosphodiesterase.

There is no answer at present to the question of whether cAMP analogs might be useful as chemotherapeutics. But if higher ability to activate protein kinases and better stability against cyclic phosphodiesterases are essential for such pharmacological agents as is supposed by many authors (7, 8, 26), some of the analogs described in this paper might be of interest for investigation of their behavior in cell extracts or in the complete organism.

# EXPERIMENTAL SECTION

### General Procedures

Ultraviolet spectra were recorded using a Cary 16 or a Unicam SP 1800 spectrometer; nmr spectra were measured with a Bruker Multikern spectrometer HX 60/4-12. Chemical shifts are reported in  $\delta$ -units relative to tetramethylsilane (TMS). Thin-layer chromatography was performed on Merck silica gel F 254 or PF 254 plates using the solvent systems A (*n*-propanol-concd ammonia-water, 7:1:2, v/v) and B (acetonebenzene-water, 8:2:1, v/v).

Paper chromatography was performed by the descending technique on Schleicher & Schüll 2043b paper. The solvent systems were A or C (ethanol-1 M ammoniumacetate, 7:3, v/v). Paper electrophoresis was also carried out on Schleicher & Schüll 2043b paper using 0.1 M triethylammonium bicarbonate buffer pH 7.4. Chromatographic data and the uv spectra of all compounds synthesized are given in Table 2.

<sup>1</sup> In a recently published work (J. P. Miller *et al.*, *Biochemistry* **12** (1973) 1010) 2'-dinitrophenylcAMP has been shown to stimulate protein kinases from bovine heart and bovine brain very poorly. The differences between activation of these enzymes and our protein kinase from bovine muscle is highly interesting.

|                              |     | t]<br>solv | c<br>ents | Paper chror<br>solv | natography<br>ents | Electrophoresis<br>solvent | uv-Spectra<br>methanol        |
|------------------------------|-----|------------|-----------|---------------------|--------------------|----------------------------|-------------------------------|
| Compound                     | No. | в          | A         | ¥                   | C                  | D                          | $\lambda_{\max}$ , (nm)       |
| cAMP                         | 1   | 0          | 1.0       | 1.00                | 1.00               | 1.00                       | 259                           |
| 2'-NO2phe-cAMP               | 7   | 0.27       | 1.20      | 1.85                | 1.46               | 0.65                       | 259, 300 (shoulder)           |
| 6-Bu-NO2phe-cAMP             | e   | 0.35       | 1.30      | 2.29                | 1.85               | 0.81                       | 274; 255, 284, 300 (shoulder) |
| 2CI-cAMP                     | 68  | 0.13       | 1.06      | 1.24                | 1.42               | 0.81                       | 265                           |
| 2Br-cAMP                     | 6b  | 0.11       | 1.10      | 1.42                | 1.45               | 0.87                       | 264                           |
| 2CH <sub>3</sub> S-cAMP      | 7   | 0.18       | 1.11      | 1.27                | 1.19               | 0.56                       | 268, (pH 1); 234, 271 (pH 7)  |
| 2Cl-2'NO2phe-cAMP            | œ   | 0.08       | 1.13      | 2.05                | 1.54               | 0.49                       | 264, 294 (shoulder)           |
| 2CI-N <sup>1</sup> O-cAMP    | 6   | I          | I         | 0.81                | 0.84               | 1.60                       | 236, 265, 296 (shoulder)      |
| N <sup>1</sup> O-cAMP        | 10  | [          | 0.92      | 0.64                | 0.63               | 1.24                       | 232, 262, 292 (shoulder)      |
| N <sup>1</sup> O-benzyl-cAMP | 11  | 0.08       | 1.12      | 1.76                | 1.57               | 0.68                       | 259                           |
|                              |     |            |           |                     |                    |                            |                               |

Chromatographic Data and uv-Spectra of the Compounds Prepared<sup>4</sup>

**TABLE 2** 

" For solvent systems see Experimental section, general procedures.

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# MATERIALS

DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, DEAE 52 from Whatman.

Cyclic AMP (1) was a product from Boehringer, Mannheim. 2-Chloroadenosine (4a) and 2-bromoadenosine (4b) were prepared in general following literature procedures (17) but purification of the reaction products was performed by preparative thinlayer chromatography on Merck PSC-Fertigplatten Silicagel 60 F 254 using chloroform/ methanol (8:2) as solvent system.

The N<sup>1</sup>-oxide (10) was synthesized following the general procedure described by Falbriand *et al.* (11) using *m*-chloroperbenzoic acid instead of monoperphthalic acid.

### Preparation of cAMP Analogs

2'-O-[2,4-dinitrophenyl-]adenosine-3',5'-cyclic phosphate(2'NO<sub>2</sub>phe-cAMP) (2). Adenosine-3',5'-cyclic phosphate(1)(329 mg, 1 mmole) was suspended in absolute dimethylformamide (40 ml). After addition of diisopropylethylamine (Hünig base) (560  $\mu$ l) and 2,4-dinitrofluorobenzene (500  $\mu$ l, 4 mmole), the reaction mixture was kept at room temperature. After 2 days the solvent was evaporated nearly to dryness and the residue chromatographed on four preparative silica gel plates (20 × 40 cm) using chloroform in the first run and then chloroform/methanol (6:4) in a second. 2 was identified by its typical uv-spectrum and isolated by elution from silica gel with methanol. Yield 320 mg (66%).

pmr (d<sub>6</sub> DMSO): sugar: H-4' and H-5' unidentified, H-3': 4.98–5.37 (1H, m) H-2': 5.98 (1H, J=5 Hz), H-1': 6.3 (1H, s); base: HN<sub>2</sub>-group: 7.35 (2H, s), H-2: 8.18 (1H, s) H-8: 8.36 (1H, s) dinitrophenyl group: 7.65 (1H, d, J=9 Hz), 8.50 (1H, dd,  $J_2=9$  Hz,  $J_2=2.5$  Hz), 8.75 (1H, d, J=2.5 Hz).

2'-O-[2,4-dinitrophenyl-]-N<sup>6</sup>-butyryl-adenosine-3',5',cyclic phosphate (6Bu-2'NO<sub>2</sub>phecAMP) (3). 2'-O-[2,4-dinitrophenyl-]adenosine-3',5'-cyclic phosphate (2) (15 mg, 30  $\mu$ moles) was dried by evaporation with absolute pyridine (1 ml), dissolved in a mixture of pyridine/n-butyric acid anhydride (5:1.5, v/v, 1 ml), and kept at room temperature. The reaction was followed by thin-layer chromatography (system B). After 5 days the formation of **3** was practically quantitative. The reaction mixture was evaporated to dryness under the reduced pressure of an oil diffusion pump (<10<sup>-3</sup> Torr), redissolved in 250  $\mu$ l methanol, and chromatographed on four analytical thin-layer plates (20 × 20 cm) using solvent system B. **3** was isolated in more than 90% yield (spectrophotometrically determined) by elution with methanol and subsequent evaporation of the solvent.

2-Chloro-adenosine-(3',5')-cyclic phosphate (2 Cl-cAMP) (**6a**). 2-Chloroadenosine (**4a**) (115 mg, 6000 OD<sub>264</sub> units, 0.4 mmole (17)) was dissolved in triethyl phosphate (1.5 ml) and POCl<sub>3</sub> (0.15 ml) added. The solution was kept for 16 hr at 4°C. The excess of POCl<sub>3</sub> was decomposed by addition of water (3 ml) and the acidic reaction mixture adjusted to pH 4 with triethylamine. The 2-chloroadenosine-5'-phosphate (**5a**) was isolated as the triethylammonium salt from a column of DEAE-Sephadex A-25 (110 ml) with a gradient of 0.05–0.3 *M* triethylammonium bicarbonate buffer (1.5 liters each). Yield: 4030 OD<sub>264</sub> units (0.265 mmole (17) 66%). **5a** (3040 OD<sub>264</sub> units, 0.2 mmole (17)) and dicyclohexylcarbodiimide (DCCD) (510 mg) were heated in a mixture of absolute pyridine/absolute dimethylsulfoxide (850 ml/100 ml) under reflux for 90 min. After cooling, water (15 ml) was added, and the mixture filtered to remove dicyclohexyl urea, and evaporated to dryness at 30°C, under reduced pressure ( $<10^{-3}$  Torr) of an oil-diffusion pump. (If such a pump is not available, dimethylsulfoxide can be removed by the following procedure: Methylene chloride (250 ml) was added to the solution and the mixture extracted with 1 N NaOH (25 ml). The NaOH-phase was extracted five more times with methylene chloride (100 ml) to remove the last traces of DMSO, then neutralized with an ion exchanger (Dowex 50, H<sup>+</sup>-form)). The analog **6a** was separated from starting material **5a** and by-products by column chromatography on DEAE-cellulose (HCO<sub>3</sub>-form) (120 ml) using a gradient of water-0.1 *M* triethylammoniumbicarbonate (1.5 liters each). Yield: 1820 OD<sub>264</sub> units (0.12 mmole, 54%).

NMR-spectrum (d<sub>4</sub> methanol): H-8: 8.29 (1H, s), H-1': 6.05 (1H, s) H-2', H-3', H-4', H-5': 4.20–4.80 (5H, m), triethylammonium ion: 1.34 (9H, t, J = 7 Hz), 3.20 (6H, q, J = 7 Hz).

2-Bromo-adenosine-3',5'-cyclic phosphate (2 Br-cAMP) (6b). 2-Bromoadenosine (4b) (144 mg, 6420  $OD_{265}$  units, 0.43 mmole (17)) was phosphorylated with POCl<sub>3</sub> (0.22 ml) in triethyl phosphate (1.1 ml), and 2-bromo-adenosine-5'-phosphate (5b) isolated as described for the corresponding chloro-compound. Yield: 3870  $OD_{265}$  units (0.26 mmole (17), 61 %). The 0.26 mmole 5b were subsequently converted to the cyclic phosphate 6b as described above.

Yield: 2160 OD<sub>265</sub> units (0.145 mmole (17), 56%).

NMR-spectrum (d<sub>4</sub> methanol): H-8: 8.15 (1H, s), H-1': 6.03 (1H, s), H-2', H-3', H-4', H-5': 4.15-4.70 (5H, m) triethylammonium ion: 1.35 (9H, t, J = 7 Hz), 3.20 (6H, q, J = 7 Hz).

2-S-Methylmercapto-adenosine-3',5'-cyclic phosphate (2  $CH_3S$ -cAMP) (7). To a solution of 2-chloroadenosine-3',5'-cyclic phosphate (**6a**) (150  $OD_{264}$  units, 10  $\mu$ mole (17)) in methanol (700  $\mu$ l), was added 1 N sodium methyl mercaptide (20) (30  $\mu$ l). The mixture was kept in a sealed tube at 100°C for 2 hr and 7 then isolated by chromatography on a small DEAE-cellulose column (HCO<sub>3</sub>-form) (11 ml) using a gradient of water  $\rightarrow 0.05 M$  triethylammonium bicarbonate buffer (300 ml each). Vield: 48 OD-4 units (3.6  $\mu$ mole (20) 36 %)

Yield: 48 OD<sub>234</sub> units (3.6  $\mu$ mole, (20), 36%).

2-Chloro-2'-O-[2,4-dinitrophenyl]-adenosine-3',5'-cyclic phosphate (2 Cl-2'NO<sub>2</sub>phecAMP) (8). 2-Chloroadenosine-3',5'-cyclic phosphate (6a) (150 OD<sub>264</sub> units, 10  $\mu$ mole) was dissolved in dimethylformamide (100  $\mu$ l), diisopropyl-ethylamine (Hünig base) (5  $\mu$ l), and 2,4-dinitrophenyl fluorobenzene (5  $\mu$ l) was added and the mixture kept for 2 days at room temperature. After evaporation of the solvents, 8 was separated by chromatography on two analytical thin-layer plates (20 × 20 cm) using first chloroform, subsequently solvent system (B), and isolated by elution with methanol.

Yield: 91 OD<sub>264</sub> units (50–60% determined by the amount of reisolated starting material **6a** (50 OD<sub>264</sub> units)).

2-Chloro-adenosine-3',5'-cyclic phosphate N<sup>1</sup>-oxide (2 Cl-N<sup>1</sup>O-cAMP) (9). 2-Chloroadenosine-3',5'-cyclic phosphate (6a) (150 OD<sub>264</sub> units, 10  $\mu$ mole) in methanol (750  $\mu$ l) was treated with *m*-chloroperbenzoic acid (17 mg, 100  $\mu$ mole) for 7 days in darkness at room temperature. The reaction was followed chromatographically and stopped when no additional formation of 9 was realized even after addition of more perbenzoic acid (10 mg) and heating for 2 hr at  $60^{\circ}$ C.

9 was separated from 6a by paper chromatography in solvent system (A) and isolated by elution with water/methanol (1:1) yield 28 OD<sub>236</sub> units (10-20%, determined by the amount of reisolated starting material 6a).

# **BIOCHEMICAL SECTION**

# Materials and Methods

Protein kinase from bovine muscle was a munificent gift from Dr. Wunderwald, Fa. Boehringer, Mannheim. Cyclophosphodiesterase from beef heart was bought from Boehringer, Mannheim.  $[\gamma^{-32}P]$ ATP was obtained from the Radiochemical Centre, Amersham, England. Protamine sulfate was kindly made available to us free of charge by Fa. Boehringer, Mannheim.

Quantitative spectrophotometric measurements were performed with a Zeiss uvspectrophotometer PMQ II. Paper-chromatographic separations were carried out on paper 2043b mgl (Schleicher & Schüll) in solvent system (A).

# Protein Kinase Experiments

Protein kinase activity was determined by measuring the incorporation of <sup>32</sup>P from  $[\gamma^{-32}P]ATP$  into protamine after a modified method of Rosen *et al.* (27). The incubation mixture (0.2 ml) contained 0.05 *M* potassium phosphate buffer, pH 7.0, 0.01 *M* MgSO<sub>4</sub>, 10 *M*  $[\gamma^{-32}P]ATP$  (sp act 2239 mCi/mmole), 0.01 *M* dithiothreitol, 0.3 mg of protamine sulfate, 0.5 mg of bovine serum albumin, 70 µg protein kinase, and cAMP or the analogs as indicated. Incubations were performed at 30°C for 2 min unless otherwise noted. After incubation according to the methods of Bollum (28), aliquots (30 µl) were transferred to discs of Whatman No. 3 MM filter paper and washed twice with 10% trichloroacetic acid each for 30 min at room temperature, twice with 5% trichloroacetic acid each for 30 min, with ethanol for 10 min, and finally with ether. The IR-dried discs were transferred to liquid scintillation vials, 10 ml of toluene-based scintillation solution were added, and the radioactivity was determined. One unit of protein kinase activity was defined as that amount of enzyme which will transfer 1 pmole of <sup>32</sup>P from  $[\gamma^{-32}P]$ -ATP to protamine in 2 min at 30°C. The enzyme activity was determined for cAMP and its derivatives over the concentration range of  $10^{-8}$ - $10^{-3} M$  cyclonucleotide.

# **Phosphodiesterase Studies**

For investigation of the rate of hydrolysis of the cyclic AMP analogs by beef heart cyclic phosphodiesterase, each incubation contained 0.3  $\mu$ mole cyclonucleotide, 50  $\mu$ l 0.05 *M* Tris-HCl buffer, pH 7.5/0.04 m*M* magnesium sulfate, and 100  $\mu$ g phosphodiesterase (sp act 0.1 *M*), and was incubated for 90 min, a time in which cyclic AMP was completely hydrolyzed. After paper chromatographic separation of the entire mixture, the proportion of the hydrolyzed product was determined spectrophotometrically after elution with water.

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