Adenosine 3′,5′-Cyclic Phosphorothioate: Synthesis and Biological Properties†

Fritz Eckstein,* Lauritz P. Simonson, and Hans-Peter Bär

ABSTRACT: Adenosine 3′,5′-cyclic phosphorothioate (cAMPS) was synthesized. Its interaction with cyclic nucleotide phosphodiesterase from beef heart and rabbit brain as well as with cAMP-dependent protein kinase from beef heart was studied and compared with that of cAMP. Both diastereomers of cAMPS are slowly hydrolyzed by the diesterases with rates about $1/50$ to $1/150$ that of cAMP. The hydrolysis of cAMP (K_m = 2.3×10^{-5} M) by the enzyme from heart is inhibited by cAMPS with $K_i = 2.3 \times 10^{-5}$ M. Protein kinase is activated by cAMPS almost as effectively as by cAMP. The binding of [^{3}H]cAMP to the protein kinase is inhibited by cAMPS, the dissociation constant for cAMPS being at least ten times lower than that of cAMP.

In an effort to modify the biological properties of adenosine 3′,5′-cyclic phosphate, a large number of analogs has been synthesized with alterations in the base, the sugar, and the phosphate part of the molecule (Simon *et al.*, 1973). In an attempt to synthesize cAMPS, one of us has reacted adenosine 5′-phosphorothioate with *N,N*-dimethylformamide dimethyl acetal and triisopropylbenzenesulfonyl chloride (Eckstein, 1970). Although certain aspects of the reaction could not be explained, such as the absence of any cAMP formation as well as the inability to detect the two expected diastereomers, the product was assumed to be cAMPS based on its electrophoretic mobility, its elementary analysis, and its chemical shift in the P nmr.

This compound was, unexpectedly, neither substrate nor inhibitor for 3′,5′-cyclic nucleotide phosphodiesterases from various sources (Eckstein and Bär, 1969). Because of these discrepancies it was desirable to develop an independent synthesis for cAMPS.

In this publication evidence is presented that the compound synthesized previously (Eckstein, 1970) was adenosine 5′-*S*-methylphosphorothioate and that cAMPS can be obtained by reacting adenosine 5′-*O,O*-bis(*p*-nitrophenyl)phosphorothioate with potassium *tert*-butoxide. The interactions of cAMPS with 3′,5′-cyclic nucleotide phosphodiesterases as well as with cAMP-dependent protein kinase from beef heart are described.

Experimental Section

General Procedures and Materials

Electrophoresis was performed using Schleicher and Schüll 2043 b (washed) paper in 0.1 M triethylammonium bicarbonate (pH 7.5) or borate buffer (pH 10) at 2000 V. The same paper was used for paper chromatography. Ultraviolet absorption measurements were carried out using a Zeiss PMQ II which was equipped with a Servogor recorder for kinetic studies. The ^{1}H and ^{31}P nmr spectra were recorded with a Bruker-Physic HFX 60 spectrometer equipped with a Fourier transform unit Bruker-Data-System B-NC12. Chemical shifts are given in δ units (parts per million) downfield from external Me_4Si for ^{1}H spectra and relative to 30% aqueous phosphoric acid as external standard for ^{31}P spectra. Adenosine deaminase (calf intestine, *ca.* 200 U/mg), snake venom phosphodiesterase (*Crotalus terr. terr. ca.* 1.5 U/mg), alkaline phosphatase (calf intestine, *ca.* 35 U/mg), and cAMP phosphodiesterase (beef heart, *ca.* 35 U/mg) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). *Crotalus atrox* and *Crotalus adamanteus* snake venoms were obtained from Sigma Chemical Co., St. Louis, Mo. 2′,3′-Diacetyladenosine was obtained from Papierwerke Waldhof-Aschaffenburg (Mannheim, Germany) and Sigma Chemical Co.

Cyclic nucleotide phosphodiesterase from rabbit brain was prepared as described by Drummond and Perrott-Yee (1961). Partially purified cAMP-dependent protein kinase from beef heart was prepared according to the procedure described by Rubin *et al.* (1972) through the DEAE-cellulose chromatography step. This preparation was utilized for both protein kinase and cAMP binding studies.

Synthetic Procedures

O,O-Bis(p-nitrophenyl) Phosphorochloridothioate. A solution of *p*-nitrophenol (27.8 g) in anhydrous ether (100 ml) was added dropwise over 30 min under ice-cooling and stirring to a solution of $PSCl_3$ (21 ml) in anhydrous pyridine (15.5 ml) and dichloromethane (5 ml). Stirring was continued for 6 hr or overnight at room temperature. The precipitate was filtered off and the filtrate evaporated. Methylcyclohexane (70 ml) was added to the residue and evaporated again. The residue was dissolved in $CHCl_3$ and chromatographed on a SiO_2 column (4 × 50 cm) with $CHCl_3$–petroleum ether (7:3, v/v).

With the first liter of eluent mostly *p*-nitrophenyl phosphorodichloridothioate was eluted, with the second the bis(*p*-nitrophenyl) phosphorochloridothioate. The eluents are evaporated; the residue was dissolved in benzene (20 ml) and petroleum ether (bp 40–60°) added slowly. Yield of the dichloride 15 g, mp 52° (lit. 53–54°; Tolkmith, 1958); yield of the monochloride 5.5 g, mp 82°. *Anal.* Calcd for $C_{12}H_8N_2O_6PSCl$: C, 38.46; H, 2.14; Cl, 9.46; N, 7.47; P, 8.26; S, 8.55. Found: C, 38.60; H, 2.26; Cl, 9.22; N, 7.41; P, 8.64; S, 8.22.

2′,3′-Diacetyladenosine 5′-O,O-Bis(p-nitrophenyl)phospho-

†From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Göttingen, Germany (F. E.), and the Department of Pharmacology, University of Alberta, Edmonton, Canada (L. P. S. and H.-P. B.). *Received March 26, 1974.* This work was supported in part by the Deutsche Forschungsgemeinschaft (F. E.) as well as by the Medical Research Council of Canada and the Alberta Heart Foundation (H.-P. B.).

[1]Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; cAMPS, adenosine 3′,5′-cyclic phosphorothioate; EGTA, ethylene glycol bis(β-aminoethyl ether)tetraacetic acid.

rothioate. To 2′,3′-diacetyladenosine (3.50 g, 10 mmol) dissolved in anhydrous pyridine (2 ml) was added bis(*p*-nitrophenyl) phosphorochloridothioate (4.0 g). After 3.5 hr at room temperature inspection by thin-layer chromatography (tlc) (SiO_2, $CHCl_3$/MeOH, 95:5) revealed completion of the reaction. The reaction solution was evaporated; the residue was taken up in $CHCl_3$ and washed with 10% acetic acid (two times), H_2O, saturated $NaHCO_3$ solution (two times), and H_2O to neutrality. The organic phase was dried over Na_2SO_4 and evaporated and the residue recrystallized from benzene (25 ml). After the solution is cooled, the crystals are collected, dissolved in $CHCl_3$, and precipitated with petroleum ether. Yield 4.2 g, mp 152–154°, λ_{max}(MeOH) 266 nm (ϵ 15,000 l. mol^{-1} cm^{-1}). *Anal.* Calcd for $C_{26}H_{24}O_{12}N_2PS$: C, 45.28; H, 3.50; N, 14.21; P, 4.49; S, 4.65. Found: C, 44.35; H, 3.76; N, 12.72; P, 4.72; S, 4.23.

Adenosine 5′-O,O-Bis(p-nitrophenyl)phosphorothioate. The diacetyl compound (3.5 g) was suspended in methanol saturated with NH_3 at 0° (200 ml) and stirred at room temperature until everything had gone into solution (30 min). The solution was evaporated, the residue was taken up in little MeOH, and the material was crystallized by addition of $CHCl_3$. The crystals were recrystallized from 60 ml of ethanol. Yield 1.0 g, mp 164–165°; λ_{max}(MeOH) 265 nm (ϵ 15,000 l. mol^{-1} cm^{-1}). *Anal.* Calcd for $C_{22}H_{20}O_{10}N_7PS$: C, 43.63; H, 3.32; N, 16.19; P, 5.11; S, 5.29. Found: C, 43.43; H, 3.88; N, 16.17; P, 5.02; S, 5.24.

Adenosine 3′,5′-Cyclic Phosphorothioate. Adenosine 5′-bis(*p*-nitrophenyl)phosphorothioate (1 g) was dissolved in 750 ml of anhydrous dimethylformamide and 25 ml of 1 M potassium *tert*-butoxide added. After the mixture was stirred at room temperature for 20 min, acetic acid (ca. 6 ml) was added for neutralization. The solution was evaporated, the residue was dissolved in water and passed over an ion-exchange column (Merck I, H^+-form, 3 × 30 cm), the eluate was evaporated, and the residue was dissolved in water and sufficient triethylammonium bicarbonate (1 M) to obtain complete solution. The solution was evaporated to dryness, and the residue was dissolved in water and chromatographed on a SiO_2 column (3 × 30 cm) with acetone–benzene–water (8:2:0.75, v/v). Fractions of about 150 ml were collected. The first three fractions contain mainly *p*-nitrophenol, and the following four mainly cAMPS as judged by electrophoresis in borate buffer, pH 10. The mobility of this compound was almost identical with that of cAMP. The fractions containing the cAMPS were pooled, evaporated, dissolved in water, and chromatographed on a DEAE-Sephadex column (1.5 × 30 cm) with a linear gradient of 500 ml each of water and 0.2 M triethylammonium bicarbonate. The cyclic phosphorothioate was eluted at 0.18–0.2 M buffer. The pooled fractions were evaporated, the buffer was removed by repeated (three times) evaporations with methanol, and the residue was recrystallized from ethanol (5 ml). Yield 45 mg, mp 195–205°.

The mother liquor was evaporated and chromatographed on paper (2 sheets 55 × 55 cm) in isopropyl alcohol–water–ammonia (7:1:2, v/v). The main band was excised, eluted with 50% aqueous methanol, filtered, evaporated, transformed into the triethylammonium salt by passage over a Merck I-ion exchange column (triethylammonium form), and recrystallized from ethanol. Yield 52 mg, mp 198–204°; total yield 14%; ^{31}P nmr (H_2O) −53.22 and −54.27 ppm; λ_{max}(H_2O) 269 nm (ϵ 14,900 l. mol^{-1} cm^{-1}). Electrophoretic mobility pH 7.5: R_{cAMPS} 1.0, R_{cAMP} 1.10, $R_{5'\text{-AMP}}$ 2.0; borate pH 10.0: R_{cAMPS} 1.0, R_{cAMP} 1.10, $R_{5'\text{-AMP}}$ 2.0. Development of blue color with periodate and benzidine (Cifronelli and Smith, 1954). *Anal.* Calcd for $C_{16}H_{27}N_6O_5PS$: C, 43.08; H, 6.09; N, 18.82; P, 6.93; S, 7.18. Found: C, 43.20; H, 5.90; N, 18.76; P, 6.93; S, 7.11.

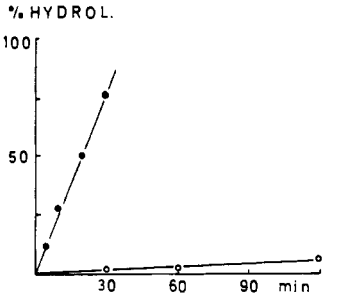


FIGURE 1: Hydrolysis of cAMP and cAMPS by cyclic phosphodiesterase from beef heart. The rates of hydrolysis at 37° were determined as described in the Experimental Section. The reaction solution contained 0.1 M Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 15 mM cAMP, and 0.12 U of phosphodiesterase in 1 ml. Total volume 360 μl. Aliquots (30 μl) were separated by electrophoresis. cAMP (●); cAMPS (○).

Adenosine 5′-S-Methylphosphorothioate. Adenosine 5′-phosphorothioate (Murray and Atkinson, 1968) (Na^+ salt, 0.3 mmol) was stirred in methanol (30 ml) and methyl iodide (75 μl) added. After stirring for 1 hr at room temperature, electrophoresis at pH 7.5 indicated that the reaction was about 90% complete. The solution was evaporated, and the residue taken up in water and chromatographed on a DEAE-cellulose column (carbonate form, 2 × 25 cm) with a linear gradient of 800 ml each of water and 0.2 M triethylammonium bicarbonate. The compound was eluted at about 0.1 M. The fractions were combined, evaporated, and reevaporated with methanol to remove the buffer. Yield 3200 A_{260} units (0.21 mmol). The material was chromatographed on paper with isopropyl alcohol–ammonia–water (7:1:2, v/v), and the main band was eluted with 50% aqueous methanol and turned into the triethylammonium salt by passage over a Merck I-ion exchanger (triethylammonium form) and evaporation of the eluate. The material did not crystallize but was precipitated from an ethanolic solution with ether. Electrophoretic mobility (pH 7.5): R_{AMPSMe} 1.0, R_{cAMP} 0.90, $R_{5'\text{-AMP}}$ 1.85; borate pH 10.0: R_{AMPSMe} 1.0, R_{cAMP} 0.66, $R_{5'\text{-AMP}}$ 1.22; ^{31}P nmr (H_2O) −21.6 ppm; ^1H nmr 2.19 ppm (J = 13 Hz; d, 3 H). No development of a blue colour with periodate and benzidine (Cifronelli and Smith, 1954). *Anal.* Calcd for $C_{17}H_{31}N_6O_6PS$: C, 42.66; H, 6.52; N, 17.56; P, 6.47; S, 6.70. Found: C, 42.91; H, 6.59; N, 17.64; P, 6.33; S, 6.57.

Enzymatic Procedures

Cyclic Nucleotide Phosphodiesterase. The hydrolysis of cAMPS by beef heart enzyme was followed by separation of the reaction products by electrophoresis at pH 7.5, elution of the uv active spots with 0.05 M Tris-HCl, pH 7.6, and spectrophotometric determination of the nucleotide concentration. For a direct comparison of the rates of cleavage of cAMPS and cAMP (Figure 1), the rate of cAMP hydrolysis was also determined by this method. Inhibition experiments with beef heart enzyme were performed by an optical procedure, monitoring the rate of inosine formation at 265 nm ($\Delta\epsilon_{265}$ 8400 l. mol^{-1} cm^{-1}) in the presence of excess adenosine deaminase (0.01 mg/ml) and alkaline phosphatase (0.01 mg/ml), at a temperature of 23°.

Protein Kinase and [^{3}H]cAMP Binding. Both protein kinase and binding assays were carried out in the same buffer system containing 50 mM sodium acetate (pH 6.2), 10 mM $MgCl_2$, 0.3 mM EGTA, 0.2 mM EDTA, 1 mg/ml of histone II, 33 μg/ml

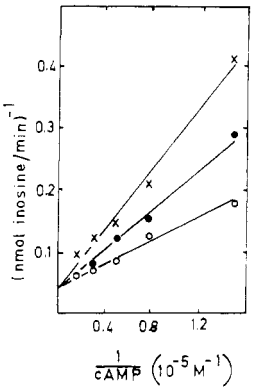


FIGURE 2: Inhibition of cAMP hydrolysis by cyclic phosphodiesterase by cAMPS. The rates of hydrolysis at 23° were determined as described in the Experimental Section. The reaction solution contained 10 mM Tris-HCl (pH 7.6), 1.5 mM $MgCl_2$, and in 1 ml of 0.6 U of alkaline phosphatase, 0.6 U of adenosine deaminase, and 1.7 mU of phosphodiesterase; no inhibitor (○); 11.5 μM cAMPS (●); 46 μM cAMPS (x).

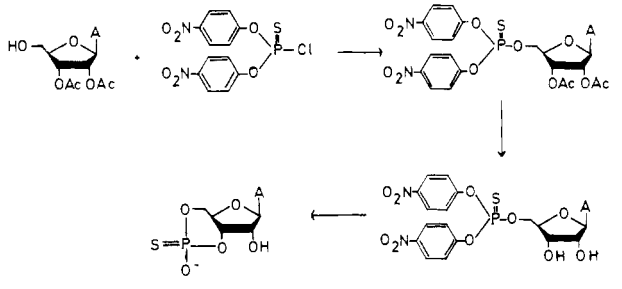
of protein kinase, and varying concentrations of cAMP or cAMPS. Kinase assays were initiated by the addition of 10 μl of 2 mM [γ-^{32}P]ATP (>10^6 cpm) to 90 μl of the medium described above. Incubations were carried out for 11 min at 30°. The reactions were terminated and processed by the filter paper precipitation technique described by Reimann *et al.* (1971).

Binding assays in the presence and absence of cAMPS contained 0.2 mM unlabeled ATP and various concentrations of [^{3}H]cAMP (5×10^{-9}–4×10^{-7} M) with a specific activity of approximately 20 Ci/mmol. Incubations were kept at 30° for 90–120 min and were then processed as described by Gilman (1970), using ice-cold 20 mM phosphate buffer (pH 6) for washing of membrane filters.

Results

Synthesis. The reaction scheme for the synthesis of adenosine 3′,5′-cyclic phosphorothioate is shown in Scheme I. Bis(*p*-nitrophenyl) phosphorochloridothioate was synthesized by adaption of a procedure given for the synthesis of *p*-nitrophenyl phosphorochloridothioate (Tolkmith, 1958). Both compounds are obtained by this method and can easily be separated. The cyclization of adenosine 5′-*O*,*O*-bis(*p*-nitrophenyl)phosphorothioate was achieved by reaction with potassium *tert*-butoxide in dimethylformamide (Borden and Smith, 1966). The compound is characterized by its large negative chemical shift in the ^{31}P nmr and the presence of two signals separated by approximately 1 ppm due to the two diastereomers as also observed for nucleoside 2′,3′-cyclic phosphorothioates (Eckstein *et al.*, 1972). The electrophoretic mobility in borate buffer as well as the reaction with periodate and benzidine (Cifronelli and Smith, 1954) indicate the absence of the 2′- and 3′-hydroxyl groups.

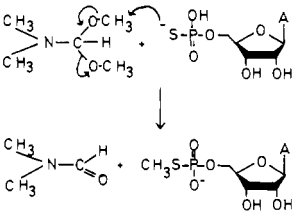
SCHEME I



The compound reported previously (Eckstein, 1970) shows the presence of the *cis*-hydroxyl groups by these two criteria. Because of this and its migration in electrophoresis at pH 7.5 like a phosphodiester it is now assumed that this compound was adenosine 5′-*S*-methylphosphorothioate. Synthesis of this compound by an unambiguous route developed by Cook (1970) by reaction of adenosine 5′-phosphorothioate (Murray and Atkinson, 1968) with methyl iodide showed that the two compounds are identical by all the criteria mentioned above as well as by their susceptibility to snake venom phosphodiesterase and the presence of a methyl group in the ^{1}H nmr spectrum.

Reaction of adenosine 5′-phosphorothioate with *N*,*N*-dimethylformamide dimethyl acetal under the conditions described (Eckstein, 1970) leads to adenosine 5′-*S*-methylphosphorothioate presumably by the following reaction (Scheme II).

SCHEME II



Interaction with Enzymes. When cAMPS was incubated with cyclic nucleotide phosphodiesterase from beef heart, the compound was hydrolyzed very slowly (Figure 1). The hydrolysis of cAMPS had reached 25% after 6 hr and 48% after 12 hr. The concentrations of cAMP and the analog in this experiment were 10^{-2} M and thus about 1000-fold higher than the K_m value of cAMP and K_i value of cAMPS (see below). The rates measured were therefore practically the V_{max} values. They differ approximately by a factor of 50. Similar results were obtained with phosphodiesterase from rabbit brain, cAMPS being hydrolyzed approximately at $^1/_{150}$ the rate of cAMP. Because of this large difference in rate it was possible to measure the inhibition of cAMP hydrolysis by cAMPS (Figure 2). With the heart enzyme, the K_i value for cAMPS obtained from the Lineweaver–Burk plot is 2.5×10^{-5} M and thus very similar to the K_m value of cAMP ($= 2.3 \times 10^{-5}$ M).

Incubation of cAMPS (5 mM) with snake venoms (0.5 mg/ml) of *Crotalus atrox* or *Crotalus adamanteus* for several hours did not lead to any degradation of cAMPS, as analyzed by paper chromatography of aliquots using isopropyl alcohol–ammonia–water (7:1:2) as solvent.

In our studies on protein kinase it was found that cAMPS was almost as effective as cAMP in stimulating the activity. Dose response studies showed that concentrations for half-maximal stimulation of kinase activity were about 4×10^{-8} and 8×10^{-8} M for cAMP and 10^{-7} M for cAMPS in two separate determinations (compare Figure 3).

The binding of [^{3}H]cAMP to the protein kinase could be effectively reduced by increasing concentrations of both cold cAMP and cAMPS, the latter appearing slightly more potent (Figure 4). To determine the dissociation constants of cAMPS, binding curves for [^{3}H]cAMP were determined in the presence and absence of two fixed concentrations of cAMPS, and the data were analyzed by three procedures. These included double reciprocal plots, the mathematical procedure of Klotz (1948) and an extended Scatchard analysis, involving replots of intercepts (or slopes) from Scatchard plots obtained in the absence and presence of cAMPS, and an iterative procedure useful in the case of tightly binding ligand analogs (to be published).

TABLE I: Dissociation Constants for cAMP and cAMPS with Protein Kinase.

Compd	Expt No.	Dissociation constant (10^8 M)[a] Method 1	Method 2	Method 3
cAMP	1	27.0 ± 2.5		24.6
	2	29.5 ± 1.6		28.7
cAMPS	1	3.5 ± 0.3	2.1 ± 0.4	1.23
	2	3.1 ± 0.2	1.9 ± 0.4	1.48

[a] Method 1: Double reciprocal plots, analogous to competitive enzyme inhibition analyses, computed with the use of the Fortran program COMP by Cleland (1963). Method 2: Stepwise calculations proposed by Klotz *et al.* (1948); values are means of ten calculations at different cAMPS and cAMP concentrations. The dissociation constant for cAMP was taken to be 22.2 × 10^{-8} M and the maximal binding 22 pmol of cAMP/mg of kinase as obtained by Fortran program HYPER (Cleland, 1963). Method 3: From replots of slopes of Scatchard plots in the presence and absence of two concentrations of cAMPS, and iterative refinement of results (procedure to be published).

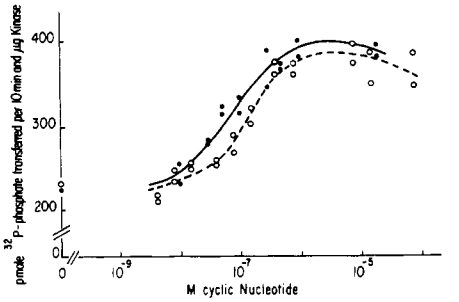


FIGURE 3: Stimulation of protein kinase from beef heart by cAMP (●) and cAMPS (○).

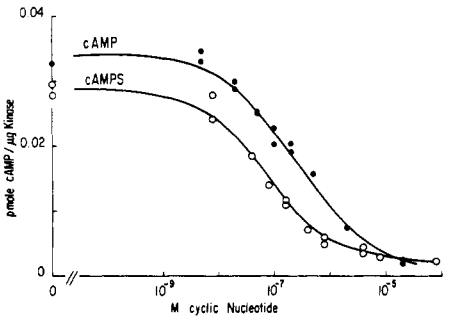


FIGURE 4: Inhibition of [^{3}H]cAMP (4 × 10^{-8} M) binding to protein kinase from beef heart in the presence of unlabeled cAMP (●) and cAMPS (○).

Results are summarized in Table I. All three procedures yielded similar values of dissociation constants in the range of 1–3 × 10^{-8} M indicating that cAMPS binds tightly to the protein kinase, *i.e.*, presumably to its regulatory subunit, the dissociation constant being 10–20 times lower than that for cAMP.

Discussion

Analogs of cAMP have been synthesized in the hope of discovering organ- or tissue-selective pharmacological agonists resistant to enzymatic hydrolysis. Further, derivatives and analogs of cAMP may be potentially useful in studying parameters of the mode of action of cAMP in stimulating protein kinase systems.

With such interests in mind, cAMPS was synthesized. The first claim to successful chemical synthesis of this compound has now been recognized as being incorrect, and it is likely that, in fact, adenosine 5′-*S*-methylphosphorothioate was obtained instead (Eckstein, 1970). Since such nucleoside 5′-*S*-alkylphosphorothioates are hydrolyzed by snake venom phosphodiesterase (Cook, 1969), our earlier results (Eckstein and Baer, 1969) are explained.

The route of synthesis of cAMPS described in this publication is similar to that of 5′-deoxy-5′-aminoadenosine 3′,5′-cyclic phosphorothioate (Jastorff and Krebs, 1972). Like nucleoside 2′,3′-cyclic phosphorothioates (Eckstein *et al.*, 1972), nucleoside 3′,5′-cyclic phosphorothioates can exist in the form of two diastereomers whose chemical shift in the ^{31}P nmr differs by approximately 1 ppm. Inspection of models of cAMPS reveals that one can distinguish between an endo (*S* configuration) and an exo isomer (*R* configuration) with respect to ribose and sulfur just as in the case of uridine 2′,5′-cyclic phosphorothioate. In the latter, the endo isomer has the smaller chemical shift (Saenger and Eckstein, 1970; Eckstein *et al.*, 1972) and it is not unlikely that the same is also true for cAMPS. The final answer, however, will have to be provided by X-ray structural studies. Attempts to separate the two isomers of cAMPS by crystallization are under way.

The cAMPS is very slowly hydrolyzed by cyclic nucleotide phosphodiesterase from both beef heart and rabbit brain cortex. In the case of the beef heart enzyme, it seems to be bound as well as cAMP, assuming that the K_m value for cAMP is closely related to the dissociation constant of the enzyme–substrate complex. The interpretation is complicated by the fact that two diastereomers are present. It is conceivable that one isomer is bound more tightly than the other. The rate by which they are hydrolyzed, however, does not seem to differ much, if at all, since no discontinuity in the course of complete hydrolysis was seen. Further, when the hydrolysis was stopped after 20 and 70% completion, respectively, the ratio of the diastereomers was determined by ^{31}P nmr and found to be unchanged in comparison to the starting material. It was hoped that preferential hydrolysis of one isomer would enable us to isolate the other in pure form. Phosphodiesterases from other sources than beef heart will be investigated to this end. For the diastereomers of 5′-deoxy-5′-aminoadenosine 3′,5′-cyclic phosphorothioate it has been reported that one is hydrolyzed by cyclic nucleotide phosphodiesterase whereas the other is not (Jastorff and Bär, 1973). It is interesting to compare cAMPS with the recently synthesized isomeric 5′-thio-5′-deoxyadenosine 3′,5′-cyclic phosphorothioate where a sulfur atom replaces the 5′-oxygen (Shuman *et al.*, 1973). A comparison of the data indicates that both compounds have very similar K_i values when tested with the phosphodiesterase from beef heart.

The presence of a sulfur atom in the phosphate group of cAMP appears to have little influence on the ability of the nucleotide to stimulate protein kinase activity. Our studies showed that cAMPS is approximately one-half as active as cAMP, and a very similar relationship has been reported for the 5′-thio analog referred to above (Shuman *et al.*, 1973). In the case of 5′-amino-5′-deoxyadenosine 3′,5′-cyclic phosphate, the introduction of a sulfur atom also reduced the activity to stimulate protein kinase, particularly in case of one of the diastereomers (Jastorff and Bär, 1973). Presently we do not know whether the diastereomers of cAMPS will show different ef-

fects in this system, but on the basis of the above study one would predict that this should be the case.

The fact that the apparent binding affinity of cAMPS to the kinase system is about 10–20 times higher than that of cAMP is interesting and begs an explanation. Similar observations have been made with a number of 8-substituted cAMP derivatives (Simon *et al.*, 1973). One would have expected that binding and kinase stimulation would be more closely related, particularly since in the present study efforts have been made to perform both kinase and binding assays under identical conditions with respect to the composition of the assay media, except for the times of incubation (or preincubation). However, the latter difference seems to be of no consequence since we have shown (unpublished) that the rate of kinase stimulation by cAMPS is at least as rapid as that by cAMP, *i.e.*, it is virtually immediate upon addition of the nucleotide. A further possibility may be that binding of protein kinase and subsequent dissociation into regulatory (cyclic nucleotide binding) and catalytic subunits (Rubin *et al.*, 1972) are not necessarily coupled or simultaneous steps. Simon *et al.* (1973) discuss this effect, in terms of multiple binding sites for the nucleotides. It would be of interest to know details about the number and the type of nucleotide binding sites and the stoichiometry of binding and subunit dissociation.

Since cAMPS is essentially as effective as cAMP in stimulating protein kinase, but is hydrolyzed only slowly by phosphodiesterases, it is of interest to test this compound in biological systems where a long-term effect should predictably result. Presumably, the permeability characteristics of cAMPS will not differ much from those of cAMP, thus leaving the degree and rate of cell membrane permeation still as a major problem to its pharmacological use. However, introduction of other chemical groups, in analogy to acylated derivatives of cAMP such as dibutyryl cAMP, may lead to the development of analogs with good cell permeation characteristics and desirable intracellular functions in terms of the specificity and time course of cAMP-like action. In addition, the occurrence of cAMPS in form of a pair of diastereomers with phosphorus as the additional center of chirality offers the possibility to obtain information on the stereochemistry of the action of cyclic phosphodiesterases in analogy to the studies carried out with uridine 2′,3′-cyclic phosphorothioate and pancreatic RNase A (Usher *et al.*, 1970).

Attempts to synthesize cAMPS enzymatically using adenosine 5′-*O*-(1-thiotriphosphate) as substrate for adenylate cyclase from Ehrlich ascites cells were not successful (Bär *et al.*, 1974).

Acknowledgment

The authors thank Miss M. Goumet for excellent technical assistance and Mr. B. Seeger for measuring the nmr spectra.

References

Bär, H. P., Simonson, L. P., and Eckstein, F. (1974), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 41,* 199–202.

Borden, R. K., and Smith, M. (1966), *J. Org. Chem. 31,* 3247–3253.

Cifronelli, J. A., and Smith, F. (1954), *Anal. Chem. 26,* 1132–1134.

Cleland, W. W. (1963), *Nature (London) 198,* 463–465.

Cook, A. F. (1970), *J. Amer. Chem. Soc. 92,* 190–195.

Drummond, G. I., and Perrott-Yee, S. (1961), *J. Biol. Chem. 236,* 1126–1129.

Eckstein, F. (1970), *J. Amer. Chem. Soc. 92,* 4718–4723.

Eckstein, F., and Bär, H. P. (1969), *Biochim. Biophys. Acta 191,* 316–321.

Eckstein, F., Schulz, H. H., Rüterjans, H., Haar, W., and Maurer, W. (1972), *Biochemistry 11,* 3507–3512.

Gilman, A. G. (1970), *Proc. Nat. Acad. Sci. U. S. 67,* 305–312.

Jastorff, B., and Bär, H. P. (1973), *Eur. J. Biochem. 37,* 497–504.

Jastorff, B., and Krebs, T. (1972), *Chem. Ber. 105,* 3129–3202.

Klotz, I. M., Triwush, H., and Walker, F. M. (1948), *J. Amer. Chem. Soc. 70,* 2935–2941.

Murray, A. W., and Atkinson, M. R. (1968), *Biochemistry 7,* 4023–4029.

Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1986–1995.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972), *J. Biol. Chem. 247,* 36–44.

Saenger, W., and Eckstein, F. (1970), *J. Amer. Chem. Soc. 92,* 4712–4718.

Shuman, D., Miller, J. P., Scholten, M. B., Simon, L. N., and Robins, R. K. (1973), *Biochemistry 12,* 2781–2786.

Simon, L. N., Shuman, D. A., and Robins, R. K. (1973), *Advan. Cyclic Nucleotide Res. 3,* 225–353.

Tolkmith, M. C. (1958), *J. Org. Chem. 23,* 1685–1690.

Usher, D. A., Richardson, D. I., and Eckstein, F. (1970), *Nature (London) 228,* 663–665.