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Synthesis and Biological Activity of Some Purine 5′-Thio-5′-deoxynucleoside 3′,5′-Cyclic Phosphorothioates†

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ABSTRACT: 5′-Deoxy-5′-thioadenosine 3′,5′-cyclic phosphorothioate (5′-thio-cAMP) and 5′-deoxy-5′-thioinosine 3′,5′-cyclic phosphorothioate (5′-thio-cIMP) were synthesized and their biological properties *in vitro* were compared to those of cAMP and cIMP. cIMP and 5′-thio-cAMP were hydrolyzed at approximately half the rate of cAMP by cAMP phosphodiesterase and stimulated cAMP-dependent protein kinase approximately half as well as cAMP. By comparison, the doubly modified cAMP derivative, 5′-thio-cIMP, exhibited a synergistic decrease in biological activity in which it was neither a substrate for phosphodiesterase nor an activator of protein kinase. A study of the inhibition of four different cAMP phosphodiesterases showed that 5′-thio-cIMP and cIMP exhibited a different spectrum of inhibition from that of 5′-thio-cAMP. A kinetic analysis of the inhibition of the cAMP phosphodiesterases from rabbit lung and beef heart revealed that 5′-thio-cAMP, 5′-thio-cIMP, and cIMP were all competitive inhibitors of both enzymes.

We have been interested in the effects of modification of the purine (Muneyama *et al.*, 1971; Bauer *et al.*, 1971; Meyer *et al.*, 1972), carbohydrate (Miller *et al.*, 1973), and phosphate moieties (Meyer *et al.*, 1973) of 3′,5′-cyclic nucleotides on biological activity in cyclic nucleotide dependent enzyme systems. Although several reports have appeared concerning the effect of heterocyclic modification of 3′,5′-cyclic nucleotides on the resulting biological activity (Muneyama *et al.*, 1971; Bauer *et al.*, 1971; Meyer *et al.*, 1972; Drummond and Severson, 1971; Free *et al.*, 1971; and Anderson *et al.*, 1972), few reports have dealt with alterations at the cyclic phosphate moiety. Compounds which involve structural modification of the cyclic phosphate moiety, such as substitution of an exocyclic oxygen by either nitrogen (Meyer *et al.*, 1973) or sulfur (Eckstein, 1970), or the substitution of an endocyclic oxygen by either methylene (Jones *et al.*, 1970) or nitrogen (Murayama *et al.*, 1971), have been shown to be either extremely weak or inactive in the activation of phosphorylase *b* kinases (Drummond and Powell, 1970) or cyclic nucleotide dependent protein kinases (Kuo and Greengard, 1970), or in the stimulation of synthesis of inducible enzyme messenger RNA (Anderson *et al.*, 1972).

The compounds studied in this investigation, 5′-deoxy-5′-thioadenosine 3′,5′-cyclic phosphorothioate (**5**, 5′-thio-cAMP[1]) and 5′-deoxy-5′-thioinosine 3′,5′-cyclic phosphorothioate (**7**, 5′-thio-cIMP), are novel cAMP (**1**) analogs where the endocyclic phosphate oxygen has been substituted by sulfur.

Experimental Section

Synthetic

Thin-layer chromatography was run on either (A) Baker-flex cellulose F (developed with acetonitrile–0.1 M ammonium chloride, 7:3) or (B) Merck Silica Gel-254F, 0.25 mm (developed with chloroform–ethyl acetate, 1:1). Evaporations were accomplished using a Buchler rotating evaporator under reduced pressure at <40°. Ultraviolet spectra were determined on a Cary 15 spectrometer. Analytical samples were dried over P_2O_5 for 12 hr at 0.01 mm (82°). Inosine 3′,5′-cyclic phosphate was prepared as previously described (Meyer *et al.*, 1972).

N^6-Formyl-5′-iodo-2′,3′-O-isopropylidene-5′-deoxyadenosine (**3**). A mixture of N^6-formyl-2′,3′-*O*-isopropylidene-5′-*O*-toluene-*p*-sulfonyladenosine (Jahn, 1965) (**2**) (55.4 g, 0.11 mol) and NaI (129 g, 0.86 mol) in 2.6 l. of acetone was refluxed for 12 hr with stirring. The reaction was treated as in the procedure for 5′-iodo-5′-deoxy-2′,3′-*O*-isopropyli-

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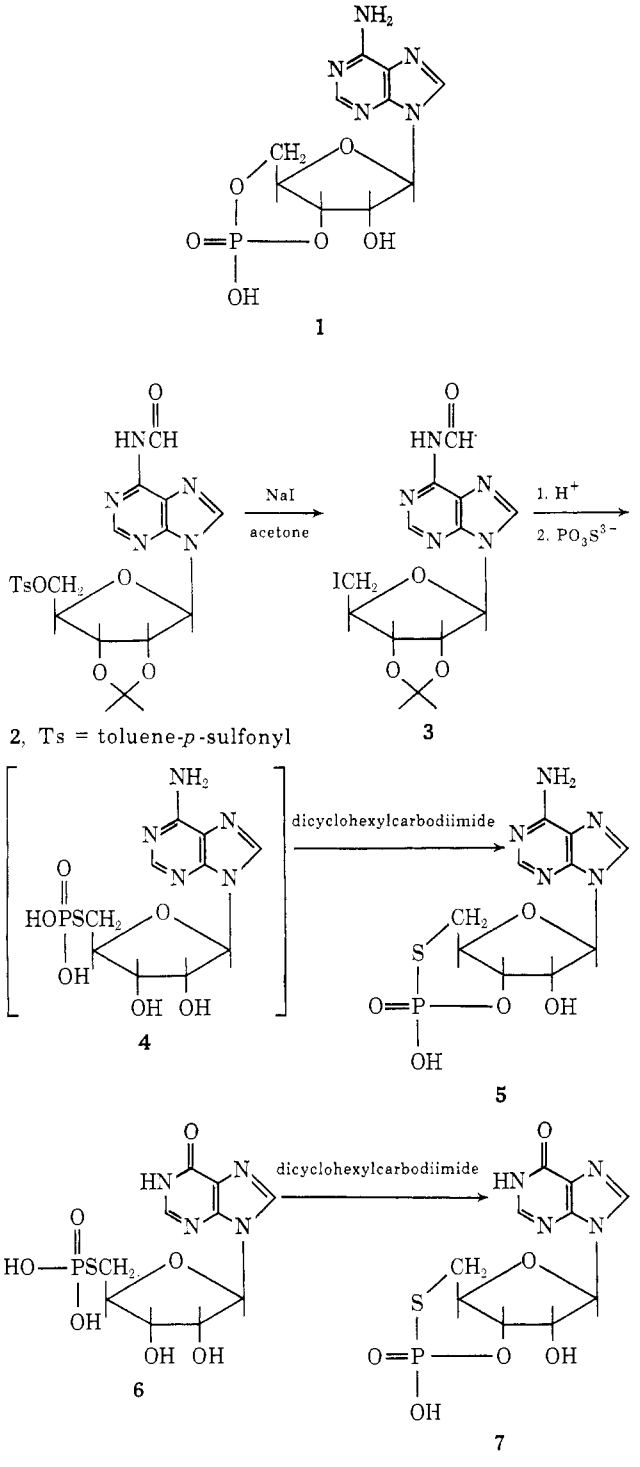
[1] Abbreviations used are: cAMP, adenosine 3′,5′-cyclic phosphate; cIMP, inosine 3′,5′-cyclic phosphate; 5′-thio-cAMP, 5′-deoxy-5′-thioadenosine 3′,5′-cyclic phosphorothioate; and 5′-thio-cIMP, 5′-deoxy-5′-thioinosine 3′,5′-cyclic phosphorothioate.

SCHEME I



deneinosine (Hampton *et al.*, 1968) to give, after concentration and cooling of the ethyl acetate extractant, 60 g of **3**, mp 153–155° (from two runs), of sufficient purity for subsequent reaction: $\lambda_{max}^{pH\ 1}$ 269, 258 (s), 278 (s) nm (ε 14,100, 12,800, 11,400); $\lambda_{max}^{pH\ 11}$ 258, 288 nm (ε 13,200, 7100).

Anal. Calcd for $C_{14}H_{16}IN_5O_4$: C, 37.76; H, 3.62; N, 15.73. Found: C, 37.63; H, 3.89; N, 15.52.

Proton magnetic resonance (pmr) spectra of the product in Me_2SO-d_6 was consistent with structure **3**. Thin-layer chromatography (tlc) of the product exhibited $R_{3/2}$ = 1.3 with system B, which also revealed a trace impurity of slower mobility.

5′-Deoxy-5′-thioadenosine 3′,5′-Cyclic Phosphorothioate (**5**). A suspension of **3** (4 g, 9 mmol) was stirred in 120 ml of methanol–1 N HCl (1:2) at 75° for 1 hr. The solution was cooled to +4° and neutralized with solid LiOH to pH 9. Trilithium thiophosphate (Akerfeldt, 1962) (8 g) was added to the solution which was then stirred for 5 hr at 45°. Ethanol (125 ml) was added and after 0.5 hr the precipitate was removed by filtration. The filtrate was diluted to 4 l. with methanol–water (1:3) and added to a DEAE column (7 × 20 cm, HCO_3^-). The column was washed with methanol–water (1:1) and then eluted with a gradient of 3 l. of 30% aqueous methanol containing 50 ml of 1 M triethylammonium bicarbonate *vs.* 3 l. of 30% aqueous methanol containing 750 ml of 1 M triethylammonium bicarbonate, to elute 5′-deoxy-5′-thioadenosine 5′-phosphorothioate (**4**). To the appropriate fractions (TOD_{260} = 7.5 × 10⁴) was added 1.6 g (5.5 mmol) of 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine and the mixture was coevaporated several times with methanol and then with pyridine to a foam. The foam was dissolved in 2.5 l. of pyridine, 10 g (48 mmol) of dicyclohexylcarbodiimide was added, and the solution was then refluxed for 2 hr. The reaction was treated in the usual manner (Smith *et al.*, 1961) to remove dicyclohexylcarbodiimide and dicyclohexylurea. The resulting aqueous extract was concentrated to 30 ml and added to a Dowex 5-X8 column (HCO_3^-, 100–200 mesh, 3 cm × 11 cm). The product was eluted with a gradient of 1 l. of water *vs.* 1 l. of 2 N formic acid to give 130 mg (4%) of 5′-deoxy-5′-thioadenosine 3′,5′-cyclic phosphorothioate (**5**) which was isolated after lyophilization of the appropriate fractions: $\lambda_{max}^{pH\ 1}$ 257 nm (ε 14,200); $\lambda_{max}^{pH\ 11}$ 258 nm (ε 14,500); $\lambda_{max}^{pH\ 7}$ 259 nm (ε 14,400); $R_{5/1}$ = 1.1 (solvent A).

Anal. Calcd for $C_{10}H_{12}N_5O_5PS \cdot H_2O$: C, 33.06; H, 3.88; N, 19.27; S, 8.82. Found: C, 33.10; H, 3.85; N, 18.95; S, 8.91.

5′-Deoxy-5′-thioinosine 3′,5′-Cyclic Phosphorothioate Ammonium Salt (**7**). A water–methanol solution of 5′-deoxy-5′-thioinosine 5′-phosphorothioate (Hampton *et al.*, 1969; Haga *et al.*, 1971), triethylammonium salt **6** (2.4 g, 4.3 mmol), and 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine (1.25 g, 4.42 mmol) was coevaporated with *N*,*N*-dimethylformamide and then pyridine to a dry foam. The foam was dissolved in 2 l. of pyridine containing dicyclohexylcarbodiimide (9 g, 44 mmol) and the solution was refluxed for 4 hr and cooled, and 200 ml of water was added. The solution was evaporated, *in vacuo*, and dicyclohexylcarbodiimide and dicyclohexylurea were removed as described in the previous procedure for **5**. The aqueous extractant was added to a DEAE column (7 × × 20 cm, HCO_3^-) and the column was washed with 2 l. of 0.02 M triethylammonium bicarbonate. Elution of the column with a gradient of 3 l. of 0.025 M triethylammonium bicarbonate *vs.* 3 l. of 0.3 M triethylammonium bicarbonate removed **7**. The appropriate fractions were coevaporated with methanol, *in vacuo*, and the product was converted to the ammonium salt with Dowex 50 (NH_4^+) to give 224 mg (13%) of **7**: $\lambda_{max}^{pH\ 1}$ 249 nm (ε 9700); $\lambda_{max}^{pH\ 11}$ 253 nm (ε 10,800); $\lambda_{max}^{pH\ 7}$ 248 nm (ε 10,000); $R_{7/6}$ = 1.1 (solvent A).

Anal. Calcd for $C_{10}H_{14}N_5O_5PS \cdot NH_3 \cdot 2H_2O$: C, 30.07; N, 4.53; H, 17.53. Found: C, 29.69; N, 4.72; H, 17.20.

Chemical Hydrolysis

A 23 mM solution of compound was incubated for 6 hr at 50° in the following buffers which contained 43% dimethyl sulfoxide: 0.05 M HCl–KCl, pH 2; 0.05 M NaOAc–HOAc, pH 3; 0.05 M K_3PO_4, pH 7; and 0.05 M glycine–NaOH, pH 9. Hydrolysis products were examined by tlc with system A and by electrophoresis at pH 7.2.

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Enzyme Preparations. The cAMP phosphodiesterases were purified from a 30,000*g* supernatant of beef heart, beef brain, rabbit lung, or rabbit kidney. The procedure, which was used for all four tissues, involved ammonium sulfate fractionation (the 0–50% saturation fraction was used) and DEAE-cellulose chromatography (the protein eluting from the column between 0.08 and 0.4 M KCl was used). The details of the purifications have been previously reported (Miller *et al.*, 1973). Bovine brain cAMP-dependent protein kinase was purified through the DEAE-cellulose step as previously described (Kuo and Greengard, 1969).

Enzyme Assays. The assay for inhibition of cAMP phosphodiesterase contained, in 1.0 ml, 50 μmol of Tris-HCl (pH 7.5), 10 μmol of $MgCl_2$, 10–200 μg of phosphodiesterase protein, 100 nmol of 8-[^{3}H]cAMP (350,000 cpm), and varying concentrations of the nucleoside cyclic phosphate being tested as an inhibitor. The incubation times (4–10 min) were determined from pilot assays to give kinetically valid data. The mixture was heat inactivated to terminate the reaction and treated with 5′-nucleotidase (crude *Crotalus atrox* venom) to convert the 5′-phosphate product to a nucleoside. The untreated nucleoside cyclic phosphate was absorbed onto Dowex 1 and the radioactivity of the nucleoside fraction determined.

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, 30 μmol of Tris-HCl (pH 7.5), 6 μmol of $MgCl_2$, and 0.1–0.3 mg of phosphodiesterase protein. After an appropriate incubation period the reaction was terminated by heating and treated with bacterial alkaline phosphatase, and the phosphate released assayed colorimetrically. The details of the phosphodiesterase substrate and inhibition assays have been previously described (Miller *et al.*, 1973). The assay for stimulation of protein kinase by cAMP analogs and the method for the determination of K_a value were performed as previously described (Muneyama *et al.*, 1971).

Results

Perhaps one of the most critical structural features within the purine 3′,5′-cyclic nucleotide is the 3′,5′-cyclic phosphate ring. As a means of probing the importance of the cyclic phosphate ring the synthesis and *in vitro* biological activity of 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) were investigated. 2′,5′-Dideoxy-5′-thio-9-β-D-ribofuranosylnucleoside 3′,5′-cyclic phosphorothioates have been synthesized by selective thiophosphorylation of the 3′-hydroxyl group of 2′-deoxyribonucleosides with subsequent cyclization to the 2′,5′-dideoxy-5′-thioribonucleoside 3′,5′-cyclic phosphorothioate by intramolecular nucleophilic displacement (Chladek and Nagyvary, 1972). These 2′-deoxy-5′-thiocyclic nucleotides lacked, however, a 2′-hydroxyl group, which is known (Miller *et al.*, 1973) to greatly influence the biological properties of a 3′,5′-cyclic nucleotide. Our synthesis of 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) was undertaken to more clearly study the importance of the cyclic phosphate ring in the presence of an intact 2′-hydroxyl group in the β-D-ribo configuration for the biological activity of cAMP.

The synthesis of the 5′-deoxy-5′-thio-9-β-D-ribofuranosylnucleoside 3′,5′-cyclic phosphorothioates **5** and **7** was investigated by selective 5′-thiophosphorylation followed by dicyclohexylcarbodiimide-mediated intramolecular cyclization.

Treatment of N^6-formyl-2′,3′-*O*-isopropylidene-5′-*O*-toluene-*p*-sulfonyladenosine (**2**) (Jahn, 1965) with sodium iodide in refluxing acetone gave in good yield 5′-deoxy-N^6-formyl-5′-iodo-2′,3′-*O*-isopropylideneadenosine (**3**) (Scheme I). Acid hydrolysis of **3** gave as the major chromatographic product 5′-deoxy-5′-iodoadenosine which was treated directly with trilithium thiophosphate to give 5′-deoxy-5′-thioadenosine 5′-phosphorothioate (**4**). 5′-Deoxy-5′-thioadenosine 5′-phosphorothioate (**4**) exhibited an electrophoretic mobility at pH 7.2 very similar to adenosine 5′-phosphate and was acid labile, as expected (Hampton *et al.*, 1969; Haga *et al.*, 1971). Intramolecular cyclization of **4** by dicyclohexylcarbodiimide in refluxing pyridine occurred in low yield to give 5′-thio-cAMP (**5**). 5′-Thio-cIMP (**7**) was similarly prepared by dicyclohexylcarbodiimide cyclization of 5′-deoxy-5′-thioinosine 5′-phosphorothioate (**6**) (Hampton *et al.*, 1969; Haga *et al.*, 1971).

3′,5′-Cyclic nucleotides are known to be more stable to acidic hydrolysis than their respective 5′-nucleotides (Smith *et al.*, 1961; Drummond *et al.*, 1964). The same relative stability was observed with 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) compared to their respective 5′-deoxy-5′-thionucleotides **6** and **4**. No hydrolysis of the cyclic thionucleotides **5** or **7** was detected by tlc or electrophoresis after 6 hr at 50° in pH 2, 3, 7, or 9 buffers, whereas the respective 5′-deoxy-5′-thionucleotides **4** and **6** were completely hydrolyzed after a few minutes at 60° in pH 3 buffer (Haga *et al.*, 1971).

Pmr spectra of the 5′-deoxy-5′-thionucleoside 3′,5′-cyclic phosphorothioates **5** and **7** were consistent with their structures. Of particular interest was the upfield shift of the 5′-methylene (~1 ppm) and 4′-H hydrogens[2] (relative to the oxygen isostere) due to the less electronegative sulfur atom.

Dreiding molecular models show that the cyclic phosphorothioate ring of **5** and **7** is slightly larger and less strained than the oxygen isostere; however, the similarity in the coupling constants of 1′-H (J = *ca.* 1 Hz) of 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) to cIMP and cAMP (J = 1 Hz, Jardetzky, 1962) suggests that there is little difference in carbohydrate conformation between the 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) with their oxygen isosteres.[3]

The biological properties of 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**) were compared to their parent cyclic nucleotides, cAMP and cIMP, respectively. Table I compares the ability of these four compounds to stimulate bovine brain cAMP-dependent protein kinase. 5′-Thio-cAMP (**5**) and cIMP were approximately one-half as potent as cAMP at activating the protein kinase, whereas 5′-thio-cIMP (**7**) was only one-thousandth as active as cAMP.[4]

The relative rates of hydrolysis by rabbit kidney cAMP

[2] The 5′-H's and the tentatively assigned 4′-H occurred as a broad multiplet similar to that reported by Chladek and Nagyvary (1972) for the 5′-H's of 5′-deoxy-5′-thiothymidine 3′,5′-cyclic phosphorothioate. Unfortunately, the integral of the latter broad multiplet was not reported and the possibility that the 4′-H occurred within this multiplet instead of at ~4.5 ppm, where the 3′-H of thymidine 3′,5′-cyclic phosphate resonates (Smith *et al.*, 1972; Lapper *et al.*, 1972), is unknown.

[3] The same conclusion is now also evident by comparison of the 1′-H of 5′-deoxy-5′-thiothymidine 3′,5′-cyclic phosphate (Chladek and Nagyvary, 1972) with the 1′-H of thymidine 3′,5′-cyclic phosphate (Lapper *et al.*, 1972; Smith *et al.*, 1972).

[4] A K_a' of 0.001 may well represent no real activation. This would be a K_a for 5′-thio-cIMP of 4×10^{-5} M. Equal concentrations of ATP, ADP, or AMP can produce a measurable disassociation of the regulatory from the catalytic subunit of protein kinase (J. P. Miller and L. N. Simon, unpublished results).

TABLE I: Stimulation of Bovine Brain cAMP-Dependent Protein Kinase by 5′-Deoxy-5′-thionucleoside 3′,5′-Cyclic Phosphorothioates.[a]

Compd	K_a' Value
cAMP	1.0
5′-Thio-cAMP (**5**)	0.51
cIMP	0.59
5′-Thio-cIMP (**7**)	0.001

[a] Values reported as K_a' values, where $K_a' = K_a$ for cAMP/K_a for test compound and K_a is the activation constant. The K_a value for cAMP was 4×10^{-8} M. The assay was performed as described in the Experimental Section.

phosphodiesterase of these same four compounds are compared in Table II. Both 5′-thio-cAMP (**5**) and cIMP were substrates for the enzyme (29 and 46% of the rate of cAMP, respectively) whereas 5′-thio-cIMP (**7**) was not a substrate. Even when this latter compound was incubated six times longer than cAMP, no hydrolysis was detected. Chromatographic analysis of the reaction mixture showed that the nucleoside cyclic phosphate was still intact.

The results of an investigation of the inhibition of four different cAMP phosphodiesterases by the compounds under study are summarized in Table III. cIMP and theophylline were also investigated as inhibitors of the enzymes in a parallel study. The I_{50} values for theophylline were similar for the four enzymes under study. In contrast, 5′-thio-cAMP (**5**) was a much better inhibitor than theophylline against the heart and lung enzymes, but about equal in potency to theophylline against the brain and kidney enzymes. By comparison, 5′-thio-cIMP (**7**) was a much better inhibitor than theophylline against all the enzymes except the lung enzyme where 5′-thio-cIMP (**7**) was approximately equal in inhibitory capability to theophylline. cIMP demonstrated approximately the same spectrum of inhibitory capability as that of 5′-thio-cIMP (**7**). The heart enzyme was quite sensitive to inhibition by both 5′-thio-cAMP (**5**) and 5′-thio-cIMP (**7**), while the other three enzymes were sensitive to inhibition by one, but not both, of the 5′-thiocyclic nucleotide analogs.

The kinetics of inhibition of the rabbit lung and beef heart phosphodiesterases were studied and the results are summarized in Figure 1. It was found that 5′-thio-cAMP (**5**), 5′-thio-cIMP (**7**), and cIMP were all competitive inhibitors of both enzymes. The K_i values for these three compounds as inhibitors of the two enzymes were determined by the graphical method of Dixon (1953) and are summarized in Table IV. As was seen in the comparison of the I_{50} values (Table III), 5′-thio-cAMP (**5**) was a better inhibitor of the lung phosphodiesterase while cIMP and 5′-thio-cIMP (**7**) were both better inhibitors of the heart phosphodiesterase.

TABLE II: 5′-Deoxy-5′-thionucleoside 3′,5′-Cyclic Phosphorothioates as Substrates for Rabbit Kidney cAMP Phosphodiesterase.[a]

Compd	Rel Rate of Hydrolysis
cAMP	100
5′-Thio-cAMP (**5**)	29
cIMP	46
5′-Thio-cIMP (**7**)	0

[a] The rates of hydrolysis by each enzyme are expressed relative to that of cAMP. The actual rate of cAMP hydrolysis was 30 nmol of 5′-nucleotide formed/min. The rates were determined by colorimetric analysis of the phosphate produced by the action of bacterial alkaline phosphatase on the 5′-nucleotide product of phosphodiesterase hydrolysis.

TABLE III: Inhibition of cAMP Phosphodiesterases by 5′-Deoxy-5′-thionucleoside 3′,5′-Cyclic Phosphorothioates.[a]

	I_{50} (μM)			
Compd	Beef Heart	Beef Brain	Rabbit Lung	Rabbit Kidney
5′-Thio-cAMP (**5**)	12	250	2.4	100
5′-Thio-cIMP (**7**)	2.2	11	200	3.4
cIMP	3.8	24	80	22
Theophylline	130	230	250	160

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 in the Experimental Section.

Discussion

Previous studies have shown that modification of the cyclic phosphate ring of cAMP by substitution of an exocyclic oxygen by sulfur or nitrogen (Meyer *et al.*, 1973) or endocyclic oxygen by 5′-methylene reduced by several orders of magnitude the ability of the analog to activate protein kinase (Drummond and Powell, 1970; Kuo and Greengard, 1970). The present studies confirm the importance of the cyclic phosphate ring of cAMP for optimal activation of protein kinase. 5′-Thio-cAMP was found to be approximately one-half as potent as cAMP for the activation of protein kinase, demonstrating that, while the 5′-oxygen was necessary for the optimal activation of the kinase, substitution of the endocyclic oxygen with sulfur was better tolerated than substitution with a methylene ($-CH_2-$) group, *e.g.*, in adenosine 3′,6′-cyclic phosphonate (Drummond and Powell, 1970; Kuo and Greengard, 1970).

The importance of the conformational structure of cAMP, particularly that of the intact cyclic phosphate ring, is exemplified by the fact that neither adenosine 5′-phosphate (Kuo

TABLE IV: K_i Values for 5′-Deoxy-5′-thionucleoside 3′,5′-Cyclic Phosphorothioates as Inhibitors of cAMP Phosphodiesterases.[a]

	K_i (μM)	
Compd	Rabbit Lung	Beef Heart
5′-Thio-cAMP (**5**)	3.5	15
5′-Thio-cIMP (**7**)	120	2.6
cIMP	60	1.8

[a] The K_i values were determined from Dixon plots of the data in Figure 1.

and Greengard, 1969) nor adenosine-5′-methylphosphonate activate protein kinase (Muneyama *et al.*, 1971). Sundaralingam and Abola (1972) have reported, however, that adenosine 3′,6′-cyclic phosphonate "can assume the conformational characteristics of natural cAMP." The above results combined with the fact that oxygen and sulfur are congeners, *i.e.*, exhibiting similar chemical (electronic) properties, suggests that not only the conformational structure of cAMP but also the electronic properties of the endocyclic 5′ substituent of cAMP are important for the most efficient activation of protein kinases.

Both 5′-thio-cAMP (**5**) and cIMP, each containing one modification of the parent molecule, were approximately one-half as potent as cAMP at activating protein kinase. By comparison, 5′-thio-cIMP (**7**), which contains the modifications present in both the latter two compounds, was essentially ineffective at activating protein kinase. The study of these same compounds as substrates for cAMP phosphodiesterase demonstrated the same profile of activity, namely that 5′-thio-cAMP (**5**) and cIMP were hydrolyzed at one-third and one-half the rate of cAMP, respectively, while 5′-thio-cIMP (**7**) was not hydrolyzed at all. These results indicate that the site on the regulatory subunit of protein kinase and the active site of phosphodiesterase that bind cAMP can tolerate a single cAMP modification of either N^6 deamination (cIMP) or endocyclic sulfur substitution at the 5′ position (5′-thio-cAMP (**5**)). However, when these two modifications were present in the same molecule, there was a synergistic effect which resulted in the complete inability of the resulting compound (5′-thio-cIMP (**7**)) to either activate protein kinase or serve as substrate for phosphodiesterase.

The results indicate that not only are both the 6-amino group and the 5′-oxygen important for the optimal interaction between cAMP and these enzymes, but they also suggest that there is an interaction between the two portions of the active sites[5] of these enzymes that bind the 6-amino and 5′-oxygen moiety of the cAMP molecule, respectively. The two modifications appear to be (from model building considerations) far enough removed from one another that they probably do not together affect some third separate area of the molecule to abolish the biological activity of the disubstituted compound. In addition, if the two modifications were acting independently to affect their own particular binding points within the active site, then one would expect to observe a simple additive effect of having both modifications in the same molecule. One possible explanation for the synergism actually observed can be found in the induced fit theory of substrate binding to an enzyme active site proposed by Koshland (1958, 1959, 1963). Utilization of this theory to rationalize these results would indicate that there is some type of interaction between the two postulated sites, *e.g.*, the portion of the active site that interacts with the 6-amino group and a second portion of the active site that interacts with the 5′-oxygen. It can be envisioned that, for example, interaction of the 6-amino group with its respective part of the active site produces an allosteric change of conformation in the second part of the active site that interacts with the 5′-oxygen. The reverse order of this mechanism as well as a mutual effect of each portion of the active site upon the other portion is equally plausible.

Even though 5′-thio-cIMP (**7**) does not interact with cAMP phosphodiesterase in a way that allows it to be hydrolyzed, the observation that it is a competitive inhibitor of the phosphodiesterase shows that it does indeed reversibly bind to the active site of the enzyme in a way very similar to the binding of cAMP. Since both 5′-thio-cAMP (**5**) and cIMP are also competitive inhibitors of phosphodiesterase but at the same time are hydrolyzed, these data suggest that both the N^6-amino and 5′-oxygen of cAMP are necessary for not only the binding to but also the actual catalysis by the phosphodiesterase.

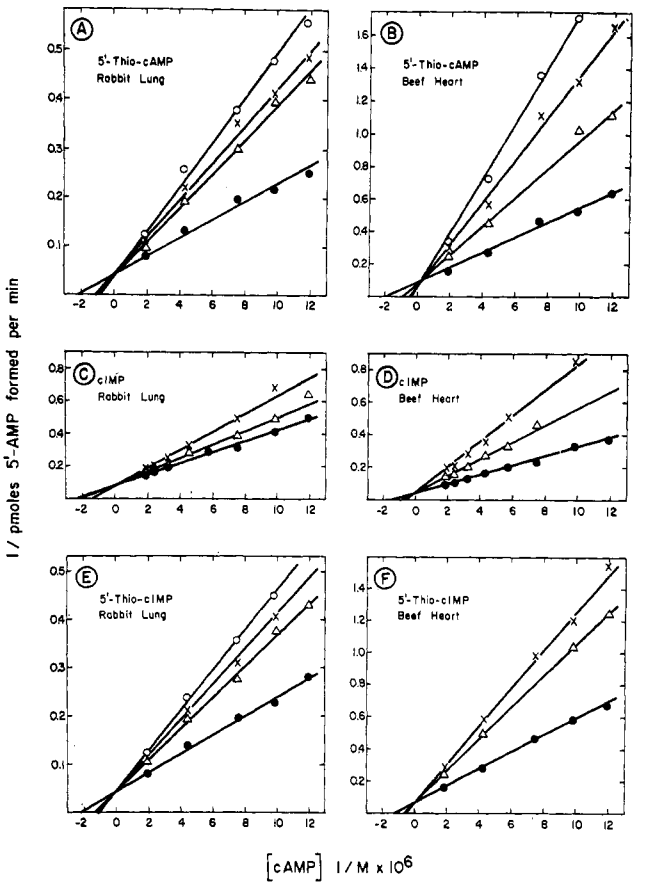

FIGURE 1: Kinetics of inhibition of rabbit lung and beef heart cAMP phosphodiesterase by 5′-thio-cAMP, cIMP, and 5′-thio-cIMP. The rates of cAMP hydrolysis were determined as described in the Experimental Section in the absence and the presence of various concentrations of the nucleoside cyclic phosphate being tested as an inhibitor: (A) no inhibitor (●), 1.5×10^{-6} M 5′-thio-cAMP (Δ), 2.4×10^{-6} M 5′-thio-cAMP (×), 3.6×10^{-6} M 5′-thio-cAMP (○); (B) no inhibitor (●), 7.5×10^{-6} M 5′-thio-cAMP (Δ), 1.5×10^{-5} M 5′-thio-cAMP (×), 3.5×10^{-5} M 5′-thio-cAMP (○); (C) no inhibitor (●), 2.8×10^{-5} M cIMP (Δ), 1.4×10^{-4} M cIMP (×); (D) no inhibitor (●), 2.0×10^{-6} M cIMP (Δ), 7.0×10^{-6} M cIMP (×); (E) no inhibitor (●), 1.0×10^{-5} M 5′-thio-cIMP (Δ), 1.0×10^{-4} M 5′-thio-cIMP (×), 1.0×10^{-3} M 5′-thio-cIMP (○); (F) no inhibitor (●), 1.3×10^{-6} M 5′-thio-cIMP (Δ), 2.5×10^{-6} M 5′-thio-cIMP (×).

Examination of the data (Tables III and IV) summarizing the inhibition of cAMP phosphodiesterases by the compounds under study indicates that cIMP and 5′-thio-cIMP (**7**) both demonstrate a different spectrum of inhibitory activity than does 5′-thio-cAMP (**5**). cIMP and 5′-thio-cIMP (**7**) had preferential activity against the heart, brain, and kidney enzymes while 5′-thio-cAMP (**5**) was active against the heart and lung enzymes. By comparison, the I_{50} for theophylline was approximately the same for all four enzymes. Unlike the results with these compounds as activators of protein kinase and as substrates for phosphodiesterase,

[5] We define the active site as all the portions of the protein (in this case either the regulatory subunit of the protein kinase or the phosphodiesterase) that interact with cAMP.

where either modification of the cAMP decreased the activity of the resulting compound to interact with these two enzymes, the data on the inhibition of phosphodiesterases from heart, brain, and kidney show that the doubly modified 5′-thio-cIMP (**7**) was the best inhibitor of these enzymes. The only exceptions to this generalization were the results with the lung enzyme where 5′-thio-cAMP (**5**) was the best inhibitor.

Since all the derivatives are competitive inhibitors of the phosphodiesterases, the differences in potency between enzymes are probably due to differences between the active sites of the enzymes themselves. The delineation of differences between the phosphodiesterases of various tissues, the multiplicity of phosphodiesterases within any one tissue, as well as studies on structure–activity relationships, can be approached by studies using cAMP analogs such as those described here.

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