uridine 3′,5′-O,O-cyclophosphate anion;[21] in the latter case the conformation *trans,gauche* is required by the six-membered phosphodiester ring.

(f) The Cation. In the initial stages of this structure analysis the triethylammonium cation appeared to be ordered and displayed almost C(3) symmetry. When a difference Fourier synthesis was computed at the end of the refinement procedure three more atoms showed up in chemically reasonable positions. These atoms correspond to disordered α-carbon atoms and render the symmetry of the cation nearly C_{3v}, Figure 6. Apart from the disorder this result is not too surprising since a triethylammonium ion has two ways to arrange its α-carbon atoms even if the locations of the nitrogen and β-carbon atoms remain fixed. This disorder of the triethylammonium cation has also been observed by Schwalbe and Lipscomb[22] but is not a general phenomenon; recently an ordered structure with this cation has been examined.[21]

Packing Arrangement. The packing of the molecules within the unit cell is indicated in two schematic projections down the shortest axis (on 010, Figure 7) and down the *c* axis (on 001, Figure 8). The molecules are arranged in fishbone manner. They are linked by hydrogen bonds which are indicated by short intermolecular contacts in the direction of the *b* axis but not in the *a*, *c* plane. The hydrogen bonds are formed between translation equivalent molecules along the *b* axis and between molecules which are symmetry related by the twofold screw axes parallel to *b* at $a = 0$, $c = +1/4$ and $a = 1/2$, $c = 3/4$. Along these symmetry elements extend zigzag bands of triethylammonium uridine 2′,3′-O,O-cyclophosphorothioate molecules (Figure 7). These zigzag bands are symmetry related to each other by the twofold screw axes at $a = 1/2$, $c = 1/4$, and $a = 0$, $c = 3/4$.

The hydrogen bonds between atoms O(5′)–O and O(4)–N(3) are indicated in Figure 8. It should be noted that the distance O(4)–C(5) is only 3.3 Å long. Similar short contacts between aromatic C–H groups and proton acceptors have been observed by several authors[10,21,23] and were correlated with hydrogen bonding forces.

Acknowledgment. The authors thank Professor F. Cramer for support, Dr. B. Krebs for stimulating discussions, Miss H. Schmidt for skillful technical assistance, and the Deutsche Forschungsgemeinschaft for financial assistance for parts of this work.

(21) C. L. Coulter, *Acta Crystallogr., B*, **25**, 2055 (1969).
(22) C. H. Schwalbe and W. N. Lipscomb, *J. Amer. Chem. Soc.*, **91**, 194 (1969).
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Nucleoside Phosphorothioates

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Abstract: The synthesis of some nucleoside 5′-phosphorothioates is described. They are resistant to alkaline phosphatase. On reaction with acetic anhydride the formation of only one mixed anhydride can be detected by ^{31}P nmr. It is hydrolyzed by aqueous pyridine to a mixture of nucleoside 5′-phosphate and nucleoside 5′-phosphorothioate. Adenosine 5′-phosphorothioate can be cyclized to adenosine 3′,5′-cyclic phosphorothioate. 3′,5′-Dithymidine phosphorothioate is resistant to snake venom and spleen phosphodiesterase and is a competitive inhibitor for these enzymes. Uridine 2′,3′-O,O-cyclic phosphorodithioate is obtained by reaction of 5′-O-acetyluridine and P_2S_5 and is a substrate for pancreatic ribonuclease.

Although there exist a great number of nucleotide analogs in which either the sugar or the base is modified, only a small number of analogs are known in which the phosphate group is altered. Among these are nucleoside phosphites,[2] nucleoside phosphonates,[3] homonucleoside phosphonates,[4] and dialky esters of nucleoside phosphorothioates.[5] We have recently[1] synthesized nucleoside phosphorothioates, analogs in which the phosphate is modified to a thiophosphate group. We hoped that such nucleotide analogs would be hydrolyzed by phosphatases and phosphodiesterases, respectively, with slower rates than the parent compounds and therefore be of interest particularly in biochemical investigations where an increased stability of the phosphate ester bond is desired.

A number of recent publications show that nucleoside phosphorothioates[6] as well as phosphorothioate analogs of polynucleotides[7] do indeed possess properties which make them suitable tools for studies of enzymes involved in nucleic acid metabolism.

The synthesis of nucleoside 5′-phosphorothioates was conveniently carried out by thiophosphorylation

(1) Habilitationsschrift, Mathematisch-Naturwissenschaftliche Fakultät Braunschweig, 1968; a preliminary report of a portion of this work has been published: F. Eckstein, *J. Amer. Chem. Soc.*, **88**, 4292 (1966); part of this work was supported by the Deutsche Forschungsgemeinschaft.
(2) A. Holy and F. Sorm, *Collect. Czech. Chem. Commun.*, **31**, 1562 (1966).
(3) D. H. Rammler, L. Yengoyan, A. V. Paul, and P. C. Bax, *Biochemistry*, **6**, 1828 (1967); A. Holy, *Tetrahedron Lett.*, 881 (1967).
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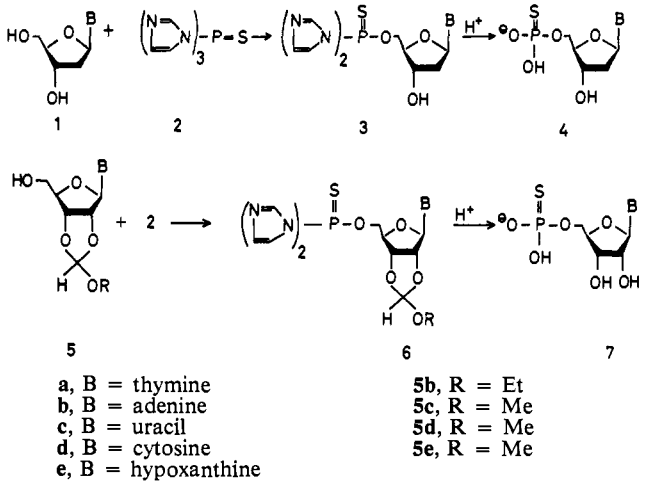
Table I. Synthesis of Nucleoside 5′-Phosphorothioates

Nucleoside 5′-phosphorothioate	Starting material	Solvent	Yield, %	Anal—Calcd, % C	H	N	P	S	Found, % C	H	N	P	S	Nucleoside P
4a	3′-Acetylthymidine	Pyridine	30	31.32	3.42	7.33	8.10	8.38	31.70	3.93	6.98	8.52	7.78	
4a	Thymidine (**1a**)	Pyridine	35											
4d	N-Dimethylaminomethylenecytidine[a]	Pyridine	25	29.50	2.76	11.51	8.49	8.74	29.60	3.25	11.04		9.53	0.95
7b	2′,3′-Ethoxymethylideneadenosine (**5b**)[b]	DMF	20	27.09	3.63	15.81	6.99	7.22	27.55	3.90	15.53		7.57	0.97
7c	2′,3′-Methoxymethylideneuridine (**5c**)[c]	Pyridine	31	26.78	3.22	6.95	7.68	7.95	27.17	3.11	6.53	7.50	7.56	
7e	2′,3′-Methoxymethylideneinosine (**5e**)[c]	Pyridine	35	28.02	3.53	13.08	7.24	7.47	29.02	3.09	12.92		7.96	0.87

[a] A. Holy and J. Zemlicka, *Collect. Czech. Chem. Commun.*, **32**, 3159 (1967). [b] F. Eckstein and F. Cramer, *Chem. Ber.*, **98**, 995 (1965). [c] B. E. Griffin, M. Jarman, C. B. Reese, and J. E. Sulston, *Tetrahedron*, **23**, 2301 (1967).

of the unprotected 2′-deoxyribonucleosides (**1**) or the protected ribonucleosides (**5**) with triimidazolyl-1-phosphinesulfide (**2**) in pyridine or dimethylformamide–tetrahydrofuran. Treatment of the reaction solution with 80% aqueous acetic acid at 100° for 15 min to remove the imidazole groups from **3** or **6** (Scheme I)

Scheme I



a, B = thymine
b, B = adenine
c, B = uracil
d, B = cytosine
e, B = hypoxanthine

5b, R = Et
5c, R = Me
5d, R = Me
5e, R = Me

did not lead to extensive hydrolysis although hydrolysis was observed on treatment of isolated nucleoside 5′-phosphorothioates with acetic acid.[1] The reaction solution was sufficiently buffered by imidazole to suppress this hydrolysis. Completion of the reactions **3** → **4** and **6** → **7** could easily be checked by electrophoresis. The synthesis of nucleoside 5′-phosphorothioates was accompanied by the formation of varying amounts of a compound bearing one positive charge as indicated by electrophoresis. The nature of this material was not further investigated. 5′-O-Tritylthymidine could only be thiophosphorylated to a negligible extent.[1] Thiophosphorylation of unprotected thymidine (**1a**) led to formation of thymidine 5′-phosphorothioate (**4a**) which was contaminated by approximately 2.6% of thymidine 3′-phosphorothioate. Differentiation between the 5′- and the 3′-phosphorothioate was achieved by desulfurization of the isolated mixture of compounds with DMSO,[1] hydrolysis of the nucleoside phosphates with crude snake venom which is specific for nucleoside 5′-phosphates, and separation of thymidine and thymidine 3′-phosphate by paper chromatography in system A.[8] In cases where a small contamination of the 2′-deoxynucleoside 5′-phosphorothioate by the 3′ isomer can be tolerated, protection of the 3′ OH group can therefore be omitted. Furthermore, the NH_2 group of adenosine did not react with triimidazolyl-1-phosphinesulfide (**2**). Recently the selective thiophosphorylation of adenosine at the 5′ position with $PSCl_3$ was reported.[6a]

Nucleoside 5′-phosphorothioates and nucleoside 5′-phosphates could be separated on DEAE-Sephadex A 25 columns. The nucleoside phosphate, because of its slightly higher pK_a value,[9] was eluted ahead of the nucleoside phosphorothioate. They also showed large differences in R_F values on paper chromatography in system B (Table III).

Table II. Hydrolysis of Uridine Cyclic Phosphorothioates by Pancreatic Ribonuclease

Uridine 2′,3′ derivative	$K_m \times 10^3$, M	k_{+2}, sec^{-1}
Cyclic phosphate	6.2	2.5
Cyclic phosphorothioate (*endo*)	6.2	0.5
Cyclic phosphorothioate (*exo*)	50	0.5
Cyclic phosphorodithioate	40	0.5

To check whether nucleoside 5′-phosphorothioates were degradable by alkaline phosphatase, adenosine 5′-phosphorothioate (**2b**) was tested in a coupled assay with adenosine deaminase. The formation of inosine was followed at 265 mμ. After an immediate burst of inosine formation which corresponded to a contamination by 5% adenosine 5′-phosphate, there was no further change at 265 mμ. Addition of 5′-AMP yielded the calculated decrease at 265 mμ, showing that the enzymes were still active. Incubation of the other nucleoside 5′-phosphorothioates here reported also led to formation of about 5% nucleoside. The reisolated nucleoside 5′-phosphorothioates were completely stable to *E. coli* alkaline phosphatase from Worthington, although they showed about 3% nucleoside formation with calf intestinal enzyme, possibly due to some diesterase activity as mentioned below. The only nucleoside phosphorothioates tested for inhibition of phosphatases, thymidine 5′-phosphoro-

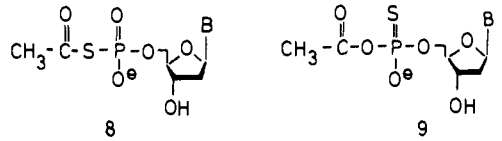
(8) This analysis was kindly carried out by Dr. J. Skoda, Prague.

thioate (**4a**) and uridine 5′-phosphorothioate (**7c**), proved to be competitive inhibitors.[9] Uridine 3′-phosphorothioate, on the other hand, obtained by ring opening of the crystalline isomer of uridine 2′,3′-O,O-cyclic phosphorothioate[10] with pancreatic ribonuclease, was hydrolyzed by alkaline phosphatase to uridine as shown by electrophoresis and paper chromatography. In which way the 2′ OH group plays a decisive role in this reaction is not as yet clear. Breslow, *et al.*,[11] have recently shown that O-*p*-nitrophenyl phosphorothioate also is a substrate for alkaline phosphatase, whereas Neumann[12] found a number of O-alkyl phosphorothioates to be resistant. From the data available so far the only conclusion one can draw is that the nature of the ester group in phosphorothioate esters affects their stability toward alkaline phosphatase, in contrast with phosphate esters.[13] This influence might reflect the stringent requirement for proper alignment of susceptible ester linkage and functional groups of the active site for catalysis, which might be disturbed by substitution of oxygen by sulfur.

Nucleoside 5′-phosphorothioates are not resistant, however, to snake venom phosphodiesterase, but are converted to nucleoside 5′-phosphates.[14]

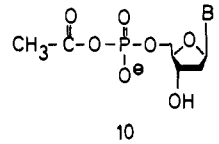
Solutions of nucleoside 5′-phosphorothioates (10 m*M*) in Tris–HCl buffer (0.1 *M*, pH 8.0) were stable for at least 3 days at 37°, the lyophilized Na^+ salts for at least 7 months at 4°.

Reaction of thymidine 5′-phosphorothioate (**4a**) with acetic anhydride in pyridine followed by hydrolysis of the reaction products with 50% aqueous pyridine led to a mixture of products containing 70% 3′-O-acetylthymidine 5′-phosphorothioate and 20% of 3′-O-acetylthymidine 5′-phosphate as judged by degradation with alkaline phosphatase.[15] We have applied ^{31}P nmr in an attempt to clarify the mechanism of this P–S bond cleavage. In principle this acetylation could lead to the two mixed anhydrides **8** and **9**.



8

9



10

B = thymine

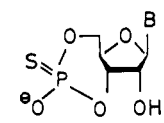
Following the addition of acetic anhydride (2 ml) to a solution of **4a** (pyridinium salt, 1 mmol) in pyridine (1 ml), we observed disappearance of the signal for **4a** (δ = −51.0 ppm) in its ^{31}P nmr spectrum and appearance of one new singlet (δ = −45.5 ppm). Addition of water (0.5 ml) to this solution gave rise to two new signals, one for thymidine 5′-phosphorothioate (**4a**) (δ = −46.7 ppm) and one for thymidine 5′-phosphate (δ = 0 ppm). In this solvent mixture, the chemical shifts of **4a** and 5′-dTMP were slightly different from those in pyridine and the signals of H_3PO_4 and 5′-dTMP coincided. This finding suggests that only one mixed anhydride (**8**) (δ = −45.5 ppm) was formed, although at the present time it cannot be excluded with certainty that the chemical shifts of **8** and **9** coincide. Hyrolysis of **8** by nucleophilic attack of H_2O or pyridine on the carbonyl carbon atom would lead to C–S bond fission and formation of thymidine 5′-phosphorothioate (**4a**), whereas attack on the P atom would cleave the P–S bond. Further studies are needed to clarify the mechanism of this hydrolysis.

On acetylation of thymidine 5′-phosphate (δ = +1.0 ppm) in the same way, the mixed anhydride **10** (δ = +8.6 ppm) was formed. This type of mixed anhydride, as already demonstrated for acetyl phenyl phosphate,[16] is hydrolyzed with C–O bond fission.

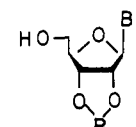
Attention should be drawn to the sensitivity of the chemical shifts to solvent and cation. Thus, in water the Na^+ salt of thymidine 5′-phosphate had δ = −3.94 ppm and that of thymidine 5′-phosphorothioate δ = −45.5 ppm.[17]

The synthesis of adenosine 3,5′-cyclic phosphorothioate (**11**), an analog of adenosine 3′,5′-cyclic phosphate, a common intracellular mediator of the action of several hormones, was undertaken. Nucleoside 3′,5′-cyclic phosphates are generally synthesized by ring closure of nucleoside 5′-phosphates with dicyclohexylcarbodiimide.[18] Since activation of nucleoside 5′-phosphorothioate with dicyclohexylcarbodiimide leads to nucleoside 5′-phosphate rather than nucleoside 5′-phosphorothioate derivatives, we tried ring closure of adenosine 5′-phosphorothioate with triisopropylbenzenesulfonyl chloride. This activating agent yields the phosphate as well as the phosphorothioate derivative.[15] To avoid undesired side reactions the amino group of adenosine 5′-phosphorothioate was protected with N,N-dimethylformamide dimethyl acetal. After chromatography on DEAE-cellulose we obtained adenosine 3′,5′-O,O-cyclic phosphorothioate (**11**) in 50% yield. There was no adenosine 3′,5′-cyclic phosphate formed in this reaction although a mixture of the phosphate and the phosphorothioate was expected. A more detailed study on the mechanism of activation by triisopropylbenzenesulfonyl chloride is needed to explain this result.

As observed with dinucleoside phosphate–dinucleoside phosphorothioate[15] as well as nucleoside 2′,3′-



11, B = adenine



12, B = uracil

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(12) H. Neumann, *J. Biol. Chem.*, 243, 4671 (1968).

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(14) F. Eckstein and H. P. Baer, *Biochim. Biophys. Acta*, 191, 316 (1969).

(15) F. Eckstein, *Tetrahedron Lett.*, 1157 (1967).

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(17) The spectra of the Na^+ salts were kindly taken by Dr. A. Schmidpeter and Dr. H. Brecht, München.

(18) M. Smith, G. J. Drummond, and H. G. Khorana, *J. Amer. Chem. Soc.*, 83, 698 (1961).

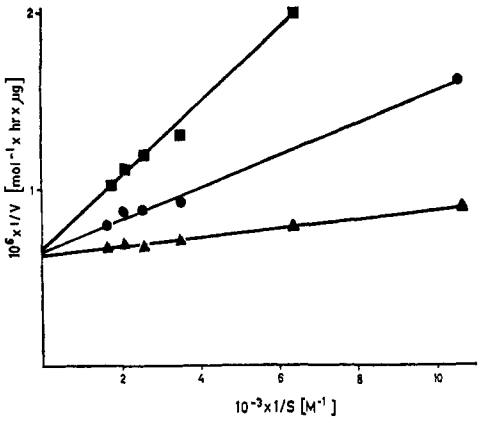


Figure 1. Lineweaver-Burk plot of hydrolysis of 5′-thymidine *p*-nitrophenyl phosphate by snake venom phosphodiesterase with 3′,5′-dithymidine phosphorothioate (**13**) as inhibitor: ▲, no inhibitor; ■, 210 μM inhibitor; ●, 86 μM inhibitor; $K_m = 4.3 \times 10^{-5}$ M; $K_i = 3.5 \times 10^{-5}$ M.

cyclic phosphate[19]–nucleoside 2′,3′-cyclic phosphorothioate pairs, the phosphorothioate had a higher R_f value in system A.

In adenosine 3′,5′-cyclic phosphorothioate (**11**) the P atom is asymmetric and since it is attached to an asymmetric substituent, D-ribose, it should, like uridine 2′,3′-cyclic phosphorothioate,[10] exist as two diastereomers. ^{31}P nmr, however, revealed only one singlet. This is in contrast to nucleoside 2′,3′-cyclic phosphorothioate where the chemical shifts of the two isomers differ by 1 ppm. Since the cyclization will in all probability not have proceeded stereoselectively, we have to assume that the difference in chemical shift of the two diastereomers of adenosine 3′,5′-cyclic phosphorothioate is so small that we could not detect it. Work is under way to try to separate the two isomers.

As reported elsewhere, adenosine 3′,5′-cyclic phosphorothioate is neither substrate nor inhibitor to cyclic 3′,5′-nucleotide phosphorodiesterase[14,20] and does not activate phosphorylase b kinase.[20] It attracts, however, myxamoebae of *Dictyostalium discoideum*.[21]

In an attempt to find an inhibitor for pancreatic ribonuclease on the nucleoside cyclophosphate level, we had synthesized earlier uridine 2′,3′-O,O-cyclic phosphorothioate. The two diastereomers obtained, however, turned out to be substrates with different K_m but identical k_{+2} values.[10] Expecting introduction of another sulfur atom to produce a cyclic phosphorothioate which was resistant to ribonuclease, we permitted 5′-O-acetyluridine to react with P_2S_5 at room temperature in pyridine. After treatment with NH_3 we obtained 2′,3′-cyclic phosphorothioate (**12**), which also was a substrate for pancreatic ribonuclease. It was hydrolyzed to, presumably, uridine 3′-phosphorodithioate without loss of sulfur. That the product was the 3′ and not the 2′ isomer was assumed from the known specificity of the enzyme for cyclic phosphates, but was not proved. The K_m and k_{+2} values obtained from a Lineweaver–Burk plot are summarized in Table II together with the respective values for uridine cyclic phosphate and cyclic phosphorothioate. It is apparent that uridine cyclic phosphorodithioate has almost an

(19) F. Eckstein and H. Gindl, *Chem. Ber.*, **101**, 1670 (1968).
(20) G. J. Drummond and C. A. Powell, *Mol. Pharmacol.*, in press.
(21) T. M. Konijn, Utrecht, private communications.

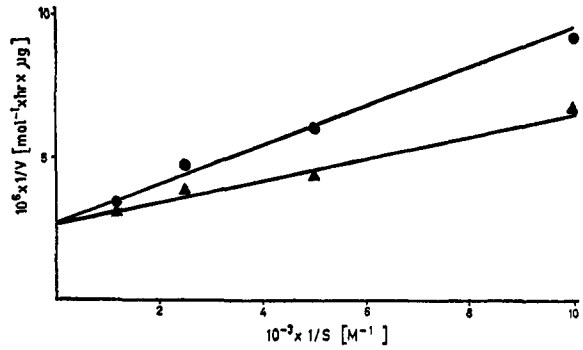
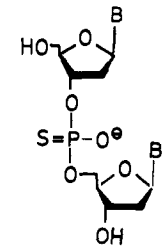


Figure 2. Lineweaver–Burk plot of hydrolysis of 3′-thymidine *p*-nitrophenyl phosphate by spleen phosphodiesterase with 3′,5′-dithymidine phosphorothioate (**13**) as inhibitor: ▲, no inhibitor; ●, 3.3 mM inhibitor; $K_m = 1.4 \times 10^{-3}$ M; $K_i = 4.2 \times 10^{-3}$ M.

identical K_m value with that of the noncrystalline isomer of uridine 2′,3′-cyclic phosphorothioate in which ribose and sulfur are in the *exo* configuration.[22] The crystalline isomer, where ribose and sulfur are in the *endo* configuration, has a lower K_m value. This result would, therefore, indicate that only sulfur in the *exo* configuration interferes with the binding to the enzyme.

We reported earlier that 3′,5′-dithymidine phosphorothioate (**13**) is resistant to snake venom and spleen phosphodiesterase.[15] No hydrolysis of 3′,5′-dithymidine phosphorothioate was detected even after 10 hr at 37°, using 25 times the amount of enzyme necessary to hydrolyze TpT. Inhibition experiments with 5′-thymidine *p*-nitrophenyl phosphate and 3′-thymidine *p*-nitrophenyl phosphate, respectively, showed that 3′,5′-dithymidine phosphorothioate is a competitive inhibitor for these two enzymes (Figures 1 and 2). The resistance of 3′,5′-dithymidine phosphorothioate cannot be attributed to a lack of binding. It is bound by these enzymes just as well as TpT, as indicated by K_i, if one assumes K_m to represent the dissociation constant of the enzyme–substrate complex, although this assumption is not necessarily valid. This consideration does not take into account the existence of diastereomers of the inhibitor, which might have different K_i values. Although a slower rate of hydrolysis for the phosphorothioate might be expected, the complete resistance cannot be explained on a purely chemical basis. Until one has a better understanding of the mechanism of action of these enzymes, one explanation might be a slight change in geometry of the inhibitor as compared to the substrate which is not so much reflected in K_i but which prevents the proper interaction of 3′,5′-dithymidine phosphorothioate with the functional groups of the active site. This change of geometry



13, B = thymine

(22) W. Saenger and F. Eckstein, *Angew. Chem.*, **81**, 566 (1969); *Angew. Chem. Int. Ed. Engl.*, 8, 595 (1969).

Table III. Paper Chromatography of Nucleoside Phosphates and Nucleoside Phosphorothioates

Compd	System A	System B	Compd	System A
5′-dTMP	0.29	0.43	Uridine	0.73
4a	0.24	0.25	Uridine 2′,3′-cyclic phosphate	0.58
5′-dCMP	0.20	0.49		
5′-CMP		0.34	Uridine 2′,3′-cyclic phosphorothioate	0.66
4d	0.20	0.31		
5′-AMP	0.22	0.51	Uridine 2′,3′-cyclic phosphorodithioate	0.73
7b	0.19	0.28		
5′-UMP	0.21	0.25	Adenosine 3′,5′-cyclic phosphate	0.54
2′,(3′)-UMP		0.34		
7c	0.18	0.13	Adenosine 3′,5′-cyclic phosphorothioate	0.70
Uridine 3′-phosphorothioate		0.11		
5′-IMP	0.12	0.20		
7e	0.12	0.14		

could be caused by the larger van der Waals radius or the higher polarizability of sulfur. Not all enzymes are equally sensitive to such changes. For example, DNA-dependent RNA polymerase accepts nucleoside 5′-O-(1-thiotriphosphates) as substrates.[23] In this connection, the mirror image situation where oxygen is replaced by sulfur in the enzyme and not in the substrate is of interest. When serine was modified to cysteine in the active site of thiol-subtilisin the enzyme was found to be inactive toward normal substrates although all other properties were unchanged.[24]

Summarizing, it can be said that in some cases nucleoside phosphorothioates are substrates, in some competitive inhibitors, and in others, *e.g.*, adenosine 3′,5′-cyclic phosphorothioate, there seems to be no interaction with the respective enzyme at all. At the present stage of our knowledge the type of interaction between a given nucleoside phosphorothioate and a certain enzyme seems to be impossible to predict.

Experimental Section

General Procedures. Paper chromatography was performed by the descending technique using Schleicher and Schüll 2043 b (washed) paper for system A (ethanol–1 *M* ammonium acetate, 7:3, v/v) and Whatman 3 MM paper for system B (isobutyric acid–2 *N* NH_4OH–0.2 *N* EDTA, 120:72:2, v/v). Paper electrophoresis was performed with Schleicher and Schüll 2043 b (washed) paper at 1500 V using 0.1 *M* triethylammonium bicarbonate buffer (pH 7.5) or 0.05 *M* ammonium formate (pH 3.5). In all thiophosphorylation reactions dried solvents were employed. Ultraviolet absorption measurements were carried out using a Zeiss PMQ II or a Cary Model 14 instrument. ^1H nmr spectra were recorded with a Perkin-Elmer R 10 spectrometer. Chemical shifts are given in δ units (parts per million) downfield from internal TMS. ^{31}P nmr spectra were recorded with the same spectrometer in connection with a Northern Scientific HS 544 Digital Memory oscilloscope with 30% aqueous phosphoric acid as standard. Chemical shifts are given in δ units (ppm) from standard. Ion exchange chromatography was carried out using Whatman DE 23 cellulose or DEAE-Sephadex A 25 in the bicarbonate form. A linear gradient of triethylammonium bicarbonate was used. Adenosine deaminase, snake venom phosphodiesterase, calf intestinal alkaline phosphatase, and pancreatic ribonuclease were purchased from Boehringer and Söhne (Mannheim, Germany), and *E. coli* alkaline phosphatase from Worthington. Spleen phosphodiesterase was a kind gift of Dr. H. Sternbach, Göttingen.

Unless stated otherwise, degradation with alkaline phosphatase was carried out by incubating 1 μmol of nucleotide in 0.1 ml of 0.1 *M* tris–acetate buffer (pH 8.0) for 12 hr at 37° with 10 μg of the calf intestinal or 100 μg of the *E. coli* enzyme. Degradation with snake venom phosphodiesterase (10 μg) was carried out in the same way. Radioactivity was measured in a liquid scintillation counter (Packard Tricarb Model 4312). To determine the radioactivity of compounds after paper chromatography, the uv-active spots were eluted with 5 ml of water–MeOH (1:1, v/v), the optical density was measured, and 0.1 ml applied on a Whatman 3 MM filter paper disk, dried, and counted. Phosphate determinations for the ratio of nucleoside/P were performed according to King.[25]

Triimidazolyl-1-phosphine Sulfide (2). A solution of $PSCl_3$ (4.85 g, 2.98 ml, 29 mmol) in dry tetrahydrofuran (40 ml) was added to a solution of recrystallized and dried imidazole (11.5 g, 170 mmol) in dry tetrahydrofuran under stirring. After 1.5 hr the precipitate was removed by filtration under exclusion of moisture and washed with tetrahydrofuran, and the combined solutions were evaporated to dryness. The residue was taken up in dry tetrahydrofuran (100 ml) and filtered, and the filtrate evaporated to dryness and dried in a desiccator: yield 7.1 g (90 %) of a white solid; mp 145–150°; ^1H nmr ($CDCl_3$) 2.27, 2.69, and 2.89 (1:1:1); ^{31}P nmr (pyridine) −26.0. *Anal.* Calcd for $C_9H_9N_6PS$: N, 31.71; P, 11.68; S, 12.10. Found: N, 31.40; P, 11.35; S, 12.01.

Nucleoside 5′-Phosphorothioates (Table I). A solution of triimidazolyl-1-phosphine sulfide (**2**) (5.3 g, 20 mmol) in 100 ml of the solvent indicated in Table I was added to a solution of the nucleoside (10 mmol) in 100 ml of the same solvent. Where dimethylformamide was used as a solvent for the nucleoside, **2** was dissolved in 100 ml of tetrahydrofuran. After 12 hr at room temperature, the solvent was evaporated and the residue taken up in 100 ml of 80% acetic acid, kept at 100° for 15–20 min, and evaporated to dryness. Where alkali labile protective groups had to be removed this residue was left for 2 hr with 100 ml of concentrated ammonia and was then evaporated. The reaction mixture was purified on a DEAE-cellulose column. The nucleoside 5′-phosphorothioate (4 mmol) was dissolved in water (10 ml, brought to pH 7.0 with ammonia) and inorganic phosphate precipitated by addition of a solution of barium acetate (18.9 g) in water (pH 7.0) (35 ml). After 3 hr at 4°, the precipitate was removed by centrifugation and washed several times with water (pH 7.0), and the combined aqueous solutions were concentrated by evaporation to a small volume (5 ml). The solution was analyzed for phosphate and converted into the free acid by passing over Merck I (H^+) ion exchanger. The effluent was evaporated to dryness and passed over Merck I (Na^+) ion exchanger and the effluent concentrated to a small volume (10 ml), and lyophilyzed.

Uv spectra and mobilities in electrophoresis at pH 3.5 and 7.5 of all nucleoside 5′-phosphorothioates were identical with the ones of the corresponding nucleoside 5′-phosphates. The content of nucleoside 5′-phosphates was determined by degradation with alkaline phosphatase and separation of the reaction products by paper chromatography in system A. This contamination amounted to 3–5% for all nucleoside 5′-phosphorothioates described.

Degradation of Adenosine 5′-Phosphorothioate by Alkaline Phosphatase. Adenosine 5′-phosphorothioate (1.6 μmol) was dissolved in Tris-HCl buffer (0.1 *M*, pH 7.4, 3 ml), calf intestinal alkaline phosphatase (10 μg, 10 μl) added, and the formation of inosine upon addition of adenosine deaminase (20 μg, 10 μl) followed at 265 mμ in a Cary spectrophotometer. After 15 min more phosphatase (50 μg, 50 μl) was added and after another 60 min 5′-AMP (1.6 μmol) was added.

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[^{35}S]Uridine 3′-Phosphorothioate. [^{35}S]Uridine 2′,3′-cyclic phosphorothioate, crystalline isomer (750 mμmol, 7 mμmol = 7951 cpm), was dissolved in 0.3 ml of Tris–acetate buffer (0.05 *M*, pH 7.4) and pancreatic ribonuclease (159 μg in 10 μl of buffer) added. After 12 hr at room temperature, the solution was chromatographed in system A and the radioactivity of the uv-active spots counted as described under General Procedures. A total of 60 mμmol of [^{35}S]uridine 2′,3′-O,O-cyclic phosphorothioate (1.2 mμmol = 1226 cpm) as well as 600 mμmol of [^{35}S]uridine 3′-phosphorothioate (12 mμmol = 12,807 cpm) was isolated. The latter traveled with uridine 2′(3′)-phosphate in electrophoresis at pH 7.5.

Hydrolysis of [^{35}S]Uridine 3′-Phosphorothioate by Alkaline Phosphatase. [^{35}S]Uridine 3′-phosphorothioate (0.3 μmol) was dissolved in 0.1 ml of 0.1 *M* Tris–acetate (pM 8.0) and 200 μg of alkaline phosphatase was added. After 12 hr at 37°, the reaction solution was analyzed by electrophoresis. Only uridine (0.28 μmol) could be recovered. A control experiment without enzyme indicated no change in the starting material.

Adenosine 3,′5′-Cyclic Phosphorothioate (11). Adenosine 5′-phosphorothioate (Na^+ salt, 2.4 g, 6 mmol) was dissolved in dry dimethylformamide (100 ml), dimethylformamide dimethyl acetal (10 ml) added, and the reaction mixture stirred overnight. Approximately 0.6 mmol had not gone into solution after this period. The solution was decanted, dried by codistillation with pyridine, and evaporated to dryness, and the residue taken up in dimethylformamide (800 ml). This solution was added dropwise over 5 hr under stirring to a solution of triisopropylbenzenesulfonyl chloride (8 g) in pyridine (600 ml). After another 5 hr water (10 ml) was added and the solution evaporated to dryness after 30 min. The residue was treated for 2 hr with concentrated ammonia (100 ml) and again evaporated to dryness. The reaction mixture was purified by chromatography on DEAE-cellulose. The resulting triethylammonium salt was converted to the K^+ salt by passage over a column of Merck I ion exchanger (K^+ form) and lyophilized: yield 1.2 g (50 %) of a white powder; $\lambda_{max}^{H_2O}$ 259 mμ (ε 15,000); ^{31}P nmr (H_2O) −18.7. In electrophoresis at pH 7.5 the material had a relative mobility of 0.83 to adenosine 3′,5′-cyclophosphate.

Anal. Calcd for $C_{10}H_{17}N_5O_5PSK \cdot 4H_2O$: N, 15.30; P, 6.80; S, 7.01. Found: N, 15.56; P, 5.90; S, 6.57.

Uridine 2′,3′-Cyclic Phosphorodithioate (12). 5′-Acetyluridine (960 mg, 3.3 mmol) and P_2S_5 (448 mg, 4 mmol) were dissolved in pyridine (8 ml) and left for 1 hr at room temperature and for a further hour at 60°. The yellow solution was evaporated and the residue treated with concentrated ammonia (50 ml) for 2 hr. After evaporation of the solvent, the residue was chromatographed on DEAE-cellulose. The reaction products obtained were uridine 2′,3′-cyclic phosphorothioate (2000 OD_{260} units, 0.2 mmol), identical by electrophoresis and paper chromatography with an authentic sample, a compound with the same mobility in electrophoresis as uridine 2′(3′)-phosphate, presumably uridine 2′(3′)-phosphorodithioate (2000 OD_{260} units), and uridine 2′,3′-cyclic phosphorodithioate (10,000 OD_{260} units) as a thick oil which crystallized on standing. Recrystallization from EtOH yielded 0.40 mg (28%) of white needles: mp 164–166°; $\lambda_{max}^{H_2O}$ 260 mμ (ε 10,000); ^{31}P nmr (H_2O) −126; in electrophoresis at pH 7.5 the material had a relative mobility of 0.90 to uridine 2′,3′-cyclic phosphate.

Anal. Calcd for $C_{15}H_{25}N_3O_6PS_2$ (as a monotriethylammonium salt): N, 9.59; P, 7.08; S, 14.64. Found: N, 9.36; P, 6.97; S, 13.94.

[^{35}S]Uridine 2′,3′-cyclic phosphorodithioate was synthesized in the same way using [^{35}S]P_2S_5.

[^{35}S]Uridine 3′-Phosphorodithioate. [^{35}S]Uridine 2′,3′-cyclic phosphorodithioate (**12**) (1 μmol, 12 mμmol = 4884 cpm) was dissolved in 0.3 ml of Tris–acetate buffer (0.05 *M*, pH 7.4) and pancreatic ribonuclease (450 μg in 30 μl of buffer) added. After 18 hr at room temperature the solution was chromatographed in system A and the radioactivity counted as described under General Procedures. [^{35}S]Uridine 2′,3′-cyclic phosphorodithioate (120 mμmol, 3.4 mμmol = 1156 cpm) and 350 mμmol of [^{35}S]uridine 3′-phosphorodithioate (7 mμmol = 2821 cpm), which was identical in electrophoresis at pH 7.5 with uridine 2′(3′)-phosphate, were isolated.

Hydrolysis of Uridine Cyclic Phosphates by Pancreatic Ribonuclease (Table II). To a 1-ml solution of dimethylglutaric acid buffer (pH 7.0, 0.1 *M*) and NaCl (0.1 *M*) in a thermostated cuvette, 2–15 μl of substrate solution was added. The reaction was started by addition of 10–30 μl of enzyme solution. The concentration of enzyme was determined by measuring the extinction at 277 mμ assuming ε = 9800. The reaction was followed at 283 mμ at 25° in a Cary 15 spectrophotometer. Initial velocities were determined by a first-order rate plot, K_m and k_{+2} values by a Lineweaver–Burk plot.

Inhibition of Diesterases. Inhibition experiments with snake venom phosphodiesterase were performed at 25° in a Gilford Model 2000 recorder connected with a Beckman Model DUR spectrophotometer. The liberation of *p*-nitrophenol was followed at 400 mμ assuming ε = 17,200. The reaction solution (3 ml) contained Tris-HCl buffer (pH 8.7, 0.3 mmol), protein (1 μg), and substrate and inhibitor as indicated in Figure 1.

In experiments with spleen phosphodiesterase, the reaction solution (300 μl) contained succinate buffer (pH 5.6, 50 μmol), 1% Tween 80 (10 μl), protein (0.68 μg), and substrate and inhibitor as indicated in Figure 2. The liberation of *p*-nitrophenol after 4 min of incubation at 25° was determined by transfer of 50 μl of the incubation solution to 1.0 ml of 0.1 *N* NaOH and measurement of the optical density of the resulting solution at 400 mμ.

Communications to the Editor

Detection of a "Michaelis" Complex by Spin Labeling in a Model Enzyme System

Sir:

The detection and characterization of enzyme–substrate intermediate complexes is critical to the elucidation of enzymatic reaction mechanisms. Although numerous studies on proteolytic enzymes have been concerned with the direct detection of covalent acyl enzyme intermediates, the techniques employed in these investigations have not been generally suitable for the direct observation of noncovalent "Michaelis" enzyme–substrate complexes.[1] Frequently, evidence for the postulation of the formation of intermediate Michaelis complexes has come from the kinetic dependence of the enzymatic reactions on either the substrate or enzyme concentration (*i.e.*, saturation effects). We wish to report the use of the spin-labeling technique[2] to detect directly a noncovalent "Michaelis" complex as well as a covalent complex in a model enzyme catalyzed hydrolysis of an ester. The model enzyme employed is cycloheptaamylose, one of the cyclodextrins, which are toroidal polysaccharides known to catalyze the hydrolysis of phosphate[3] and carboxylic[4]

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