

Phosphorothioate Analogues of Nucleotides

FRITZ ECKSTEIN

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, Germany

Received November 29, 1978

Nucleotides are involved in a multitude of biological processes.¹ They are the building blocks for the genetic material, DNA, and its transcript, RNA. As nucleoside 5'-triphosphates, they are the precursors of these polymers to which they are transformed by the corresponding enzymes, the nucleotide polymerases. The most common nucleoside 5'-triphosphate is adenosine 5'-triphosphate (ATP), which was discovered about 50 years ago as a constituent of muscle,^{2,3} signalling its importance for the storage and transfer of energy. In its dual role as a phosphate as well as an adenylate donor, ATP interacts with a host of enzymes which often occupy positions strategic for the regulation of the flow of metabolites. As the substrate for adenylate cyclase it is also the precursor for the ubiquitous adenosine 3',5'-cyclic phosphate (cAMP), which is responsible for the relay of hormone messages into cells.

Because of the central role that nucleotides play in the functioning of the cell it is understandable that they arouse enormous interest among biochemists. For the majority of nucleotide-dependent enzymatic processes the overall reaction has been unravelled. However, a more detailed description of the way nucleotides—and in particular ATP—bind to a given enzyme as well as the detailed mechanistic steps involved in the transfer of phosphate or adenylate is only just now emerging.

To facilitate such studies a whole array of ATP analogues have been developed over the years. Those analogues which carry a modification in the triphosphate chain, the site of many enzymatic reactions, are of particular importance^{4,5} since they leave the nucleoside part of the molecule unaltered. The goal of most of these modifications has been to increase the stability of the molecule against enzymatic cleavage, thus facilitating the determination of the importance of bond cleavage for a particular ATP-mediated effect. Adenylylimidodiphosphate is the outstanding representative of this type of nonhydrolysable analogue. Such analogues are dead-end inhibitors which are not turned over by enzymes, and as a consequence they do not allow the study of mechanistic details of an enzyme reaction. For this purpose one requires analogues which are substrates. Furthermore, if one is interested in stereochemical studies of enzyme reactions involving phosphate or adenylate transfer, the analogue requires as a chiral center that phosphorus at which the reaction occurs. It is in this respect that nucleoside phosphorothioates differ most significantly from the hitherto available nucleotide analogues. In this class of analogues a nonbridging oxygen of the phosphate group is replaced by sulfur.⁶ A variety of phosphorothioate

analogues of ATP and ADP such as ADP α S, ADP β S, ATP α S, ATP β S, and ATP γ S⁷ can be synthesized (see Figure 1). Of course, this substitution is not restricted to adenine nucleotides but can be introduced into any ribonucleotide or deoxyribonucleotide. Considering the structures of ADP α S, ATP α S, and ATP β S, it is apparent that the phosphorus atom to which the sulfur is attached is now chiral, leading to the existence of diastereomeric pairs of such nucleotides. These compounds open the way to the investigation of stereochemical details of an enzymatic reaction in that it is possible—as will be shown in this Account—to determine whether a reaction proceeds with inversion or retention of configuration at a particular phosphorus. They also allow the study of stereoselectivity of an enzyme for a particular diastereomer. Both these approaches furnish information on the active site of an enzyme and are thus valuable for an understanding of its mechanism.

The application of this class of analogues is not restricted to this area of investigation. As has become apparent over the years, nucleoside phosphorothioates are sometimes very poor substrates for certain enzymes paralleling the characteristics of the dead-end inhibitors mentioned above. Because of their close structural similarity to nucleoside phosphates, they have thus become useful tools for the dissection of reaction sequences in more complex biological systems, as will be described later. The advantage over adenylyl imidodiphosphate^{4,5} is that sulfur can be introduced into any position of the tri- or diphosphate chain or even the monophosphate of a nucleotide molecule, whereas the imido analogue has so far been restricted to the analogue with the imino group between the β and γ phosphorus of a triphosphate. When the sulfur is introduced at the α position of ATP or dATP it can be transferred enzymatically into products such as RNA or DNA, where it can be used to advantage for the binding of metals or the study of restriction enzymes.

As this brief Account will show, nucleoside phosphorothioates have found wide application and have helped answer questions in various areas of biochem-

(1) Metzler, D. E. "Biochemistry"; Academic Press: New York, 1977.

(2) Lohmann, K. *Naturwissenschaften*. 1929, 17, 624.

(3) Fiske, C. H.; Subbarow, Y. *Science*. 1929, 70, 381.

(4) Yount, R. G. *Adv. Enzymol.* 1975, 43, 1.

(5) Yount, R. G. *Annu. Rev. Biochem.* In press.

(6) Eckstein, F. *Angew. Chem.* 1975, 87, 179; *Angew. Chem., Int. Ed. Engl.* 1975, 14, 160.

(7) The following abbreviations are used: ADP α S, adenosine 5'-O-(1-thiodiphosphate); ADP β S, adenosine 5'-O-(2-thiodiphosphate); ATP α S, adenosine 5'-O-(1-thiotriphosphate); ATP β S, adenosine 5'-O-(2-thiotriphosphate); ATP γ S, adenosine 5'-O-(3-thiotriphosphate); cAMPs, adenosine 3',5'-cyclic phosphorothioate. The abbreviations for phosphorothioate analogues other than adenosine phosphate derivatives are based on the same principle. U γ pS, uridine 2',3'-cyclic phosphorothioate; Up(S)A, uridylyl (3'-5')-adenosyl O,O'-phosphorothioate; Up(S)Me, uridine 3'-O-(methyl phosphorothioate); dbcAMP, N⁶,O^{2'}-dibutyryladenosine 3',5'-cyclic phosphate; poly[d(A-T)], alternating copolymer of deoxyadenylate and thymidylate; poly[r(U_n-A)], alternating copolymer derived from UTP and ATP α S.

Fritz Eckstein was born in Darmstadt, Germany. He studied chemistry at the University of Bonn, where he received the Ph.D. in 1960. Following three years of postdoctoral work with E. Baer at the University of Toronto and with R. B. Woodward at Harvard University, he has been with the Max-Planck-Institut für experimentelle Medizin in Göttingen. He is affiliated also with the University of Göttingen as an "ausserplanmässiger" Professor.

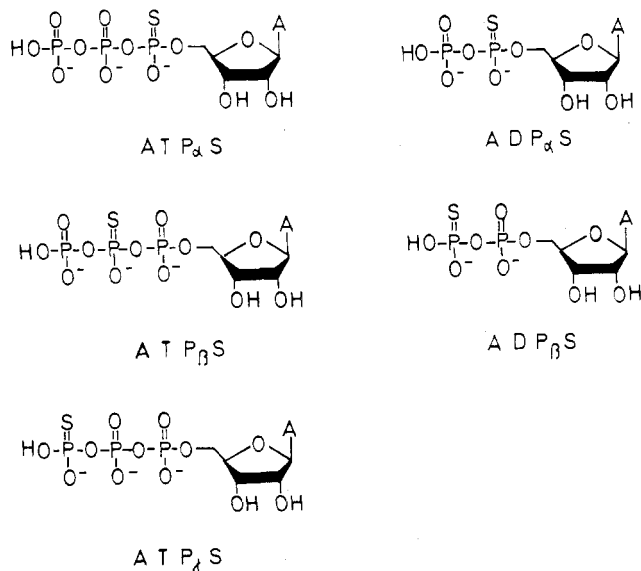


Figure 1. Formulas of various phosphorothioate analogues of adenosine nucleotides.

istry. Since the last review of this subject appeared in 1975,⁶ sufficient progress has been made to warrant another synopsis.

Synthesis and Configurational Assignment of the Diastereomers of ATP_αS and ATP_βS

The mixture of the two diastereomers—arbitrarily named A and B—of ATP_αS can be synthesized chemically.⁸ They can be distinguished by high-resolution ³¹P NMR^{9,10} or high-pressure liquid chromatography (LC).¹¹ Their separation can be achieved by the use of enzymes which act specifically or preferentially on one of the isomers of ATP_αS. Thus, nucleoside diphosphate kinase⁸ and hexokinase¹² degrade specifically the A-isomer of ATP_αS. Other kinases can be employed to phosphorylate one isomer of ADP_αS or to produce only one isomer from the (prochiral) AMPS. Thus, pyruvate kinase and myokinase are specific for the synthesis of ATP_αS A-isomer from a mixture of ADP_αS isomers.⁸ A combination of myokinase and pyruvate kinase can be employed to produce ATP_αS A-isomer directly from AMPS.^{9,10} Alternatively, one can phosphorylate a mixture of isomers of ADP_αS with creatine kinase to give predominantly the B-isomer of ATP_αS; incubation with hexokinase specifically degrades the contaminating A-isomer.¹³ In our laboratory ATP_αS B-isomer is normally prepared by creatine kinase catalyzed phosphorylation of ADP_αS, B-isomer remaining after reaction of the mixture of diastereomers with pyruvate kinase as described above. Unfortunately, no enzyme has yet been found to phosphorylate specifically AMPS to ADP_αS (or ATP_αS) B-isomer.

(8) Eckstein, F.; Goody, R. S. *Biochemistry*. 1976, 15, 1685. The absolute configurations given for the structures of the diastereomers of ADP_αS (Figure 5) and ADP_βS (Figure 6) in ref 8 are incorrect. They should be reversed.

(9) Sheu, K. F. R.; Frey, P. A. *J. Biol. Chem.* 1977, 252, 4445.

(10) Jaffe, E. K.; Cohn, M. *Biochemistry* 1978, 17, 652.

(11) Burgers, P. M. J.; Eckstein, F. *Proc. Natl. Acad. Sci. U.S.A.* 1978, 75, 4798; see ref 8 for footnote.

(12) Stahl, K. W.; Schlimme, E.; Eckstein, F. *FEBS Lett.* 1974, 40, 241.

(13) In chloroplasts, ADP_αS A-isomer acts as phosphate acceptor in phosphorylation and ATP_αS A-isomer as substrate for the light-triggered ATPase (Strotmann, H.; Bichel-Sandkötter, S.; Edelmann, K.; Eckstein, F.; Schlimme, E.; Boos, K. S.; Lustorff, J. *Biochim. Biophys. Acta* 1979, 545, 122.

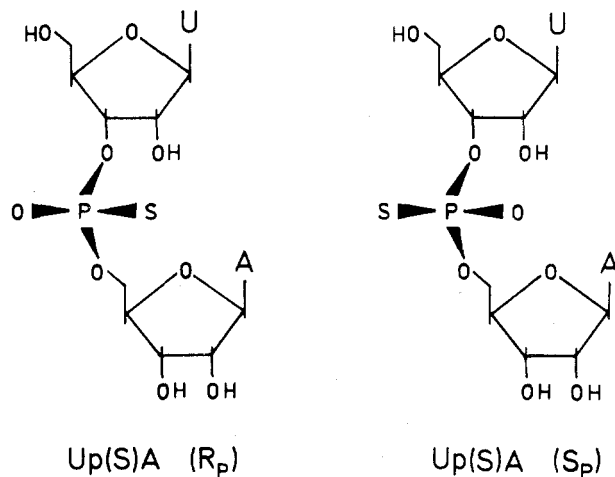


Figure 2. S and R configuration of Up(S)A.

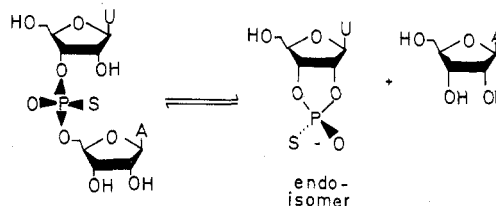


Figure 3. Transesterification of Up(S)A isomer R by RNase A.

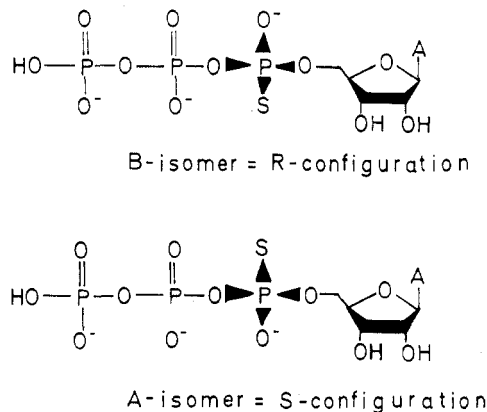


Figure 4. Absolute configuration of diastereomers of ATP_αS.

The mixture of the two diastereomers (A and B) of ATP_βS can also be obtained by chemical synthesis.⁸ They can be distinguished by ³¹P NMR,¹⁰ but so far not by LC. Enzymatically they can be synthesized from ADP_βS. Pyruvate kinase produces predominantly (85%) the A-isomer of ATP_βS,¹⁴ whereas acetate kinase yields the B-isomer.⁸ ATP_βS A-isomer can be purified of contaminating B-isomer by incubation with hexokinase.¹⁴ Conversely, myosin may be used to degrade specifically the A-isomer of ATP_βS.⁸ As will be shown below, the choice of cation is critical in directing the stereoselectivity of these kinase reactions.

The determination of the absolute configuration of these diastereomers is also based on enzymatic analyses¹¹ (see Figure 2). It was found that ATP_αS B-isomer is a substrate for snake venom phosphodiesterase, being degraded about 500 times faster than the A-isomer. This stereoselectivity also applies to the hydrolysis of the two diastereomers of Up(S)A, which were synthesized chemically. They can be separated by column chromatography and distinguished by

(14) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* 1978, 253, 4823.

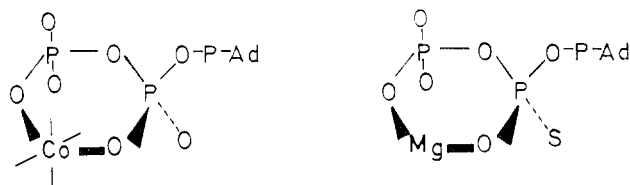


Figure 5. Absolute configuration of diastereomers of ATP β S.

LC.^{11,15} The isomer with the shorter retention time is degraded by snake venom phosphodiesterase about 1200 times faster than the other. Transesterification of this isomer with RNase A produces U>pS endoisomer (see Figure 3). On the basis of earlier work,^{16,17} this allows the assignment of the *R* configuration according to the Cahn-Ingold-Prelog rules¹⁸ to this isomer of Up(S)A. Since this *R*-isomer is also the substrate for snake venom phosphodiesterase, these results establish the requirement of this configuration for the active site of this enzyme (see Figure 4). Accordingly, ATP α S B-isomer must also possess the *R* configuration and, conversely, the A-isomer must have the *S* configuration. The stereoselectivity of snake venom phosphodiesterase for the *R*-isomer has recently been confirmed by the X-ray structural analysis of that isomer of the *p*-nitrophenyl ester of adenosine 5'-phosphorothioate, which is resistant to this enzyme. It has, as predicted, the *S* configuration.¹⁹

The establishment of the absolute configuration of the diastereomers of ATP β S¹⁴ links the phosphorothioate analogues to the Co³⁺ complexes of ATP²⁰ (see Figure 5). Of the two β,γ -bidentate Co(NH₃)₄³⁺ complexes of ATP, only one is a substrate for hexokinase; its absolute configuration was established by X-ray structural analysis.²¹ Jaffee and Cohn found that in the presence of Mg²⁺ only the B-isomer of ATP β S is a substrate for hexokinase. This, then, must have the same stereochemical arrangement as the active Co(NH₃)₄³⁺ complex in order to fit into the active site and to react with glucose. This correlation establishes the *R* configuration for the B-isomer and the *S* configuration for the A-isomer of ATP β S. With the absolute configuration of the various diastereomers known, one is in a position to describe the substrate specificity of an enzyme for a particular diastereomer in the α or β position of the di- or triphosphate chain of a nucleotide.

Metal Complexes of ATP, ATP α S, and ATP β S

It is very often postulated that a particular salt of ATP is the substrate for a given enzyme. What is often not appreciated is that the formation of such a salt results in the formation of a new center of chirality at the α and/or β phosphorus. Such salts exist as pairs of diastereomers, and some examples are given in Figure 6 for the Mg²⁺ salt. When we try to understand the binding of ATP to the active site of an enzyme, it is of

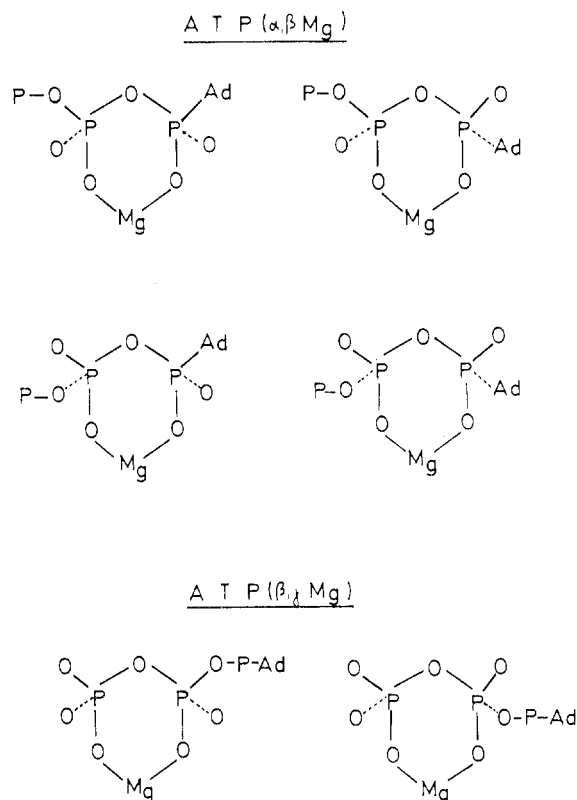


Figure 6. Examples of diastereomers of ATP-Mg²⁺ complexes.

considerable importance to know which of the diastereomers is bound to produce an active enzyme-substrate complex. This is normally not possible since these diastereomers exchange ligands rapidly when not bound to enzyme. By the use of metal ions such as Cr³⁺ or Co(NH₃)₄³⁺, where ligand exchange is slow, Cornelius and Cleland²⁰ succeeded in separating such diastereomers. In the case of nucleoside 5'-O-(1-thiotriphosphates) and -(2-thiotriphosphates) such diastereomers are inherent in the structure and are independent of the presence of a cation. They are stable; i.e., once isolated they do not interconvert (racemize) even though the cation might exchange rapidly.

The discrimination by an enzyme for a particular diastereomer can have its basis in two phenomena: either it may be due to the enzyme's selection of a particular isomer of the substrate-metal complex, with the metal ion remaining coordinated to the substrate in the active site, or it can be due to the enzyme's replacing the metal ion by a positively charge group in the active site, with only one of the diastereomers fulfilling the spatial requirements for such an arrangement. Studies with only one metal salt of the phosphorothioates will not distinguish between the two alternatives. However, a recent discovery by Jaffee and Cohn¹⁴ seems to offer a method for deciding between the two alternatives. They showed that Cd²⁺ coordinates to sulfur rather than oxygen and that Co²⁺ coordinates to both. This contrasts with the case for Mg²⁺, which coordinates—as also determined by X-ray structural analysis for diethyl *O,O*-phosphorothioate²²—only to oxygen in a phosphorothioate.

Working with the diastereomers of ATP β S and hexokinase, they found that, in the presence of Mg²⁺,

(15) Burgers, P. M. J.; Eckstein, F. *Biochemistry* 1979, 18, 592.

(16) Saenger, W.; Suck, D.; Eckstein, F. *Eur. J. Biochem.* 1974, 46, 559.

(17) Usher, D. A.; Erenrich, E. S.; Eckstein, F. *Proc. Natl. Acad. Sci. U.S.A.* 1972, 69, 115.

(18) Cahn, R. S.; Ingold, C.; Prelog, V. *Angew. Chem., Int. Ed. Engl.* 1966, 5, 385. It should be emphasized that the designation of absolute configuration of the phosphorothioates is made without consideration of the cation and is, thus, independent of it.

(19) Burgers, P. M. J.; Eckstein, F.; Sathyanarayana, R.; Saenger, W. Manuscript in preparation.

(20) Cornelius, R. D.; Cleland, W. W. *Biochemistry* 1978, 17, 3279.

(21) Merritt, E. A.; Sundaralingam, M.; Cornelius, R. D.; Cleland, W. W. *Biochemistry* 1978, 17, 3274.

(22) Schwalbe, C. H.; Goody, R. S.; Saenger, W. *Acta Crystallogr., Sect. A.* 1973, 29, 2264.

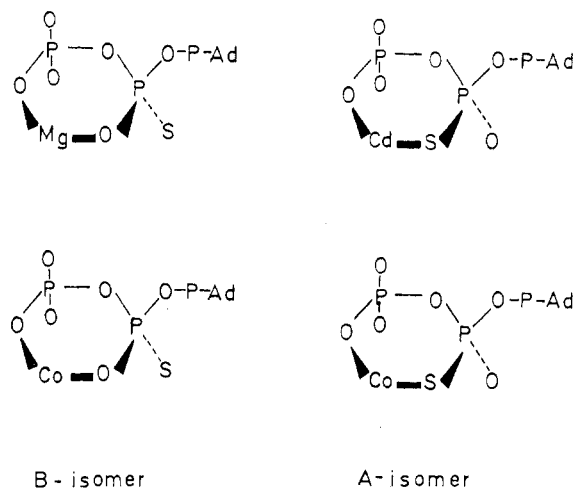


Figure 7. Configurations of diastereomers of ATP β S-metal complexes required of substrates for hexokinase.

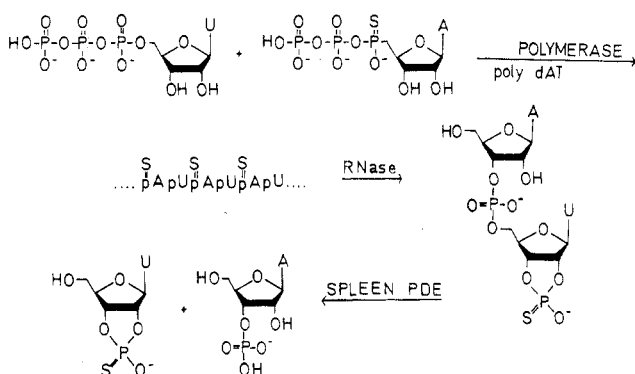


Figure 8. Scheme for the determination of absolute configuration of the phosphorothioate internucleotide linkage in poly[r(U₅A)].

the B-isomer (*R* configuration) is a substrate, while with Cd²⁺ the A-isomer and with Co²⁺ both isomers are substrates. As can be seen in Figure 7 the spatial arrangement of the various groups is identical for all these cases. Stereospecificity is, therefore, expected to be reversed in an enzymatic reaction when one changes from Mg²⁺ to Cd²⁺ and lost when one changes to Co²⁺, as long as these cations stay coordinated to the substrate in the enzyme-substrate complex. If they do not, then the stereospecificity should be unaltered by this change of cation. Thus, the work with the phosphorothioate analogues can provide not only information on the structure of the metal-substrate chelate being selected by an enzyme but also information as to whether this metal ion stays bound to the substrate once fixed in the active site.

Interaction of ATP α S and ATP β S with Polymerases

It was found rather early that ATP α S as well as UTP α S are substrates for *E. coli* DNA-dependent RNA polymerase. With the separation of the diastereomers it became possible to demonstrate that only one of these—the A-isomer—was accepted as substrate and that the phosphorothioate linkage produced has the *R* configuration.²³ This was performed as follows: ATP α S A-isomer and UTP were polymerized to poly[r(U₅A)] using poly[d(A-T)] as template (see

(23) Eckstein, F.; Armstrong, V. W.; Sternbach, H. *Proc. Natl. Acad. Sci. U.S.A.* 1976, 73, 2987.

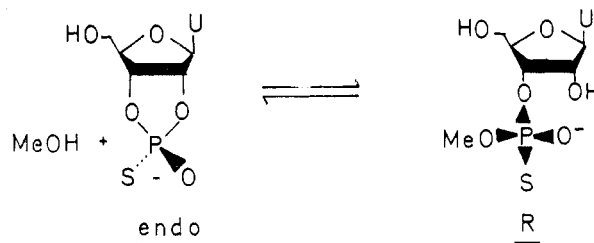


Figure 9. Transesterification of U>pS with methanol by RNase A.

Figure 8). Limited digestion with RNase A resulted in the formation of A-U>pS, which was cleaved by spleen phosphodiesterase to Ap and U>pS.

The latter was found to have the endo configuration. As mentioned above, earlier work on the mechanism of RNase has furnished the information that this enzyme transesterified the endo-isomer of U>pS with methanol to Up(S)Me with the *R* configuration, following an in-line mechanism¹⁶ (see Figure 9). This was also shown for the transesterification with cytidine.¹⁷ Because of microscopic reversibility it follows—and could be shown experimentally with Up(S)Me—that the phosphorothioate internucleotide linkage with the *R*-configuration is transesterified to U>p(S) endo isomer. The isolation of this isomer from the RNase digest of the polymer leads to the conclusion that its internucleotide linkage had the *R* configuration. Since the *S* configuration has been established for ATP α S A-isomer, it follows that the polymerization had proceeded with inversion of configuration. The simplest explanation for this result is an S_N2-like reaction where the incoming 3'-OH group of the growing RNA chain and the leaving pyrophosphate group of ATP α S are positioned for an in-line mechanism in the apical positions of a trigonal bipyramid.²⁴ This inversion of configuration is found not only for the elongation but also for the initiation step of this enzyme,²⁵ where, as analyzed by LC, the *R*-isomer of pppAp(S)U is synthesized from ATP and UTP α S A-isomer in the presence of T7 DNA.²⁶ By a similar analysis it could be shown that tRNA nucleotidyltransferase²⁷ and polynucleotide phosphorylase²⁸ follow the same stereochemical course. The more recent discovery that snake venom phosphodiesterase hydrolyses essentially only the *R*-diastereomer of the internucleotide phosphorothioate linkage has facilitated the analysis of phosphorothioate polymers. This approach was employed in the analysis of the product of polymerization of dATP α S A-isomer on polydT-oligodA as template-primer with *E. coli* DNA-dependent DNA polymerase I.²⁹ This enzyme also produces the *R*-diastereomer in the internucleotide linkage.

For the polymerases a unified picture emerges. All accept only the substrate with the *S* configuration

(24) Westheimer, F. H.; *Acc. Chem. Res.* 1968, 1, 70. For a most recent review covering the topological implications of displacement at phosphorus in nonenzymatic as well as enzymatic reactions including nucleoside phosphorothioates, see: Westheimer, F. H. In "Molecular Rearrangements", de Mayo, P., Ed.; Interscience: New York and London, in press.

(25) Johnston, D. E.; McClure, W. R. In "RNA Polymerase", Losick, R., Chamberlin, M., Ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1976.

(26) Armstrong, V. W.; Yee, D.; Eckstein, F. Manuscript in preparation.

(27) Eckstein, F.; Sternbach, H.; von der Haar, F. *Biochemistry* 1977, 16, 3429.

(28) Burgers, P. M. J.; Eckstein, F. *Biochemistry* 1979, 18, 450.

(29) Burgers, P. M. J.; Eckstein, F. *J. Biol. Chem.* in press.

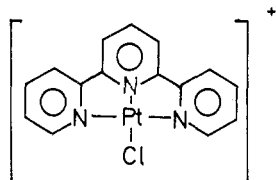


Figure 10. Chloro(terpyridine)platinum(II).

around the α -phosphorus, in the presence not only of Mg^{2+} but also of Co^{2+} (and even of Cd^{2+} in the case of RNA polymerase²⁶), and all polymerize with inversion of configuration.³⁰

Differences seem to exist, however, in the stereoselectivity at the β phosphorus. *E. coli* DNA-dependent RNA polymerase does not distinguish between the two isomers of $ATP\beta S$. Both are about equally good substrates in the presence of Mg^{2+} or Co^{2+} , but in the presence of Cd^{2+} the B-isomer is the preferred substrate. In contrast, *E. coli* DNA-dependent DNA polymerase in the presence of Mg^{2+} accepts only $dATP\beta S$ A-isomer as substrate. When the cation is changed from Mg^{2+} to Co^{2+} or Mn^{2+} , which coordinate to oxygen as well as sulfur, both isomers are about equally good substrates.²⁹ A simple interpretation of these results at the present time is that, in the active site of DNA polymerase, the metal cation is coordinated with the β but not the α phosphorus. In the case of RNA polymerase the same situation seems to exist with respect to the α phosphorus but, because of the lack of stereospecificity for the β phosphorus, the presence of the coordination of metal or the lack of it is difficult to determine. It is presently the subject of further investigation. Of course, this approach cannot analyze for ligands on the γ phosphorus.

Interaction of Phosphorothioate-Containing Polymers with Heavy Metals

The introduction of phosphorothioate groups into polynucleotides by polymerases as described above is not only of mechanistic interest. Depending on which of the nucleoside triphosphates carries the sulfur, this group can be incorporated base specifically into the polymer, with the great advantage that the bases themselves remain unmodified. Sulfur represents a new functional group in such a polymer; it differs from oxygen, for example, in its capacity to bind heavy metals. This has been exploited by Strothkamp and Lippard who could show that a (terpyridine)platinum complex can specifically and stoichiometrically be bound to the phosphorothioate groups in a mixed polymer of the type $poly[r(-A-U)]^{31,32}$ (see Figure 10). Of course this complex can also bind by intercalation to unmodified polynucleotides, but the concentration necessary for this interaction is considerably greater than that for the binding to phosphorothioate and, thus, does not interfere. Similarly, this interaction has been shown for tRNA, where the two last residues had first been removed and were then enzymatically replaced by CTP α S and ATP α S, thus introducing two phosphorothioate groups at the 3' terminus.³³ These experi-

(30) Inversion of configuration has also been determined for UDP-glucose pyrophosphorylase for which UTP α S B-isomer is the substrate [Sheu, K. F. R.; Frey, P. A. *J. Biol. Chem.* 1978, 253, 3378].

(31) Strothkamp, K. G.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* 1976, 73, 2536.

(32) Lippard, S. J. *Acc. Chem. Res.* 1978, 11, 211.

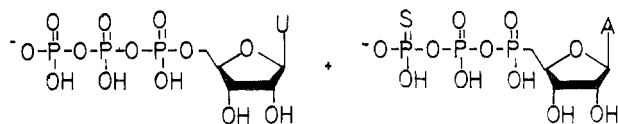


Figure 11. Transcription of poly[d(A-T)] with UTP and ATP γ S.

ments demonstrate that two such platinum complexes can be accommodated at two neighboring phosphorothioates.

We have demonstrated earlier that phosphorothioate groups can also be base specifically incorporated into natural DNA.³⁴ Using the plus strand of either $\phi X174$ or fd phage as template, one can synthesize with the help of DNA polymerase and ligase the double-stranded closed circular DNA where the minus strand carries the phosphorothioate groups. This replication proceeds with high fidelity, as judged by the radioactivity incorporated and, probably more importantly, from the digestion with restriction enzymes.³⁵ This digestion, although proceeding rather slowly if the phosphate group to be cleaved is replaced by a phosphorothioate, yields the same fragments as the parent DNA. The modified DNA can also bind the platinum complex specifically.³⁶ These findings lend themselves to the suggestion that one should investigate such polynucleotide-Pt complexes by electron microscopy to see whether this approach might be useful for the detection of newly synthesized DNA or similar problems. Such investigations are under way.³²

Not only Pt complexes can bind to phosphorothioate groups. In an elegant study Huang and collaborators utilized the interaction of Hg^{2+} with phosphorothioates.^{37,38} Their work is of particular importance for the isolation of newly synthesized RNA. In a preliminary study it had been observed³⁹ that ATP γ S is a substrate for DNA-dependent RNA polymerase. With this compound as substrate the internucleotide linkage is the same as that with ATP since thiopyrophosphate is displaced. The polymers from two such reactions are, however, different. If the newly synthesized RNA has ATP at its 5' end, then this will be an ATP γ S in the reaction with the modified nucleotide (see Figure 11). This has been used to advantage for the separation of RNA by binding such ATP γ S containing RNA on Hg-Sepharose, from which it can be displaced by elution with mercaptoethanol.^{37,38} This, then, is a method for separating newly synthesized RNA from endogenous RNA. There are essentially two types of promoters on native DNA, one resulting in mRNA with ATP at the 5' end, the other with GTP. When we

(33) Szalda, P. J.; Strothkamp, K. G.; Eckstein, F.; Sternbach, H.; Lippard, S. J. Manuscript in preparation.

(34) Vosberg, H. P.; Eckstein, F. *Biochemistry* 1977, 16, 3633.

(35) Vosberg, H. P.; Eckstein, F. Manuscript in preparation.

(36) Strothkamp, K. G.; Vosberg, H. P.; Eckstein, F.; Lippard, S. J. Unpublished results.

(37) Reeve, A. E.; Smith, M. M.; Pigiet, V.; Huang, R. C. C. *Biochemistry* 1977, 16, 4464.

(38) Smith, M. M.; Reeve, A. E.; Huang, R. C. C. *Biochemistry* 1978, 17, 493.

(39) Goody, R. S.; Eckstein, F.; Schirmer, R. H. *Biochim. Biophys. Acta.* 1972, 276, 155.

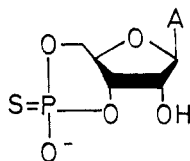


Figure 12. Adenosine 3',5'-cyclic phosphorothioate.

use either ATP γ S or GTP γ S this method allows the separation of the mRNA originating from these two types of promoters.³⁸ Obviously this technique rests on the intactness of the 5-terminus of the RNA, which often might be modified or lost by editing.

cAMPS, a Slowly Hydrolyzable Analogue of cAMP

The chemical synthesis of the mixture of the two diastereomers of adenosine 3',5'-cyclic phosphorothioate (cAMPS) has been described⁴⁰ (see Figure 12). Various attempts to separate these isomers, which would be of considerable interest for mechanistic studies, have not been successful, although this has been achieved in the case of cTMPS.⁴¹ cAMPS is only slowly hydrolyzed by beef heart phosphodiesterase but can stimulate protein kinase.⁴⁰ These early observations stimulated research into areas where cAMPS could be used as an alternative cAMP analogue to the commonly used and also slowly hydrolyzed dbcAMP. It was thought that cAMPS might be useful since it does not generate butyric acid on hydrolysis, which at times complicates the interpretation of results obtained with dbcAMP. The rat parotid gland was the first biological system tested to see whether cAMPS could penetrate cell membranes.⁴² In this system it is known that β -adrenergic agonists stimulate the secretion of amylase. Since the level of cAMP increases at the same time, it is believed that this compound mediates the signal given by the hormone. If this is so, amylase secretion should also be observed by the administration of cAMP alone. This experiment, however, gives negative results because cAMP is hydrolyzed rather rapidly so that it never reaches an appreciable concentration in the cell. With dbcAMP, however, this secretion could be induced, indicating that this compound is able to penetrate into the cell where it is then probably hydrolyzed to cAMP. The experiments conducted with cAMPS showed that it was just as efficient as dbcAMP in this stimulation, presenting indirect evidence that it also could penetrate the cell membrane.

Since then cAMPS has been applied to other systems. The antigen-stimulated histamine release of mast cells could be shown to be inhibited by cAMPS at similar concentrations and to a similar extent as dbcAMP.⁴³ Similar results were obtained on the lipolysis of fat cells,⁴⁴ the production of androgen binding protein, and the conversion of testosterone to 17 β -estradiol as late responses of cultured Sertoli cells to follicle stimulating hormone.⁴⁵ Although cAMPS was not superior to dbcAMP in any of these systems, these experiments establish cAMPS as a complementary analogue which

can establish beyond doubt that the effects seen with the latter are not due to release of butyric acid or the triggering of some other response unrelated to cAMP. However, in studying chemotaxis and cell development of the slime mould, *Dictyostelium discoideum*, cAMPS is definitely superior to dbcAMP, which does not elicit any responses in this system. Again, because of its slow rate of enzymatic hydrolysis, cAMPS becomes a useful analogue.⁴⁶ It helped to clarify that chemotaxis, as well as the induction of cAMP pulses, does not demand hydrolysis of cAMP but rather that binding to receptors on the cell surface is sufficient to evoke these effects.⁴⁷ According to results obtained with cAMPS, a similar mechanism seems to be operative in the induction of stalk cell differentiation.⁴⁷

The Adenylate Cyclase System and Its Interaction with Nucleoside Phosphorothioates

One approach to the synthesis of the isomers of cAMPS is to try to use the diastereomers of ATP α S for enzymatic cyclization. At least with a crude enzyme preparation from Ehrlich ascites cells which still contained high ATPase activity, this has failed.⁴⁸ Both isomers of ATP α S were bound by the cyclase, but no formation of product could be detected. It is hoped that experiments with purer enzyme preparations will be more rewarding. It could be shown, however, that ATP γ S is a substrate for the cyclase. This has been confirmed recently with calf thymocyte plasma membranes.⁴⁹

The hormone-stimulated adenylate cyclase from pigeon or turkey erythrocytes interacts not only with ATP but also with GTP.⁵⁰ The hormone apparently induces the binding of GTP, which is essential for the cyclase activation. The binding protein also possesses a GTPase activity which cleaves GTP to GDP. This hydrolysis terminates the activation. Analogues of GTP which cannot be hydrolyzed by the GTPase activity have, therefore, been used in this system to obtain increased or prolonged activation. It turned out that GTP γ S is the most potent activator.⁵⁰ It is not hydrolyzed by the GTPase activity⁵¹ and can retain activation for a much longer time than any other non-hydrolyzable analogue, presumably because, in addition to its resistance to hydrolysis, its rate of dissociation is very low. The introduction of GTP can be stopped efficiently by GDP β S, which allows one to measure the turn-off rate of the cyclase.⁵² GDP is not efficient since it is hydrolyzed rapidly.

Studies of Phosphate Transfer with ATP γ S

Nucleoside 5'-O-(3-thiotriphosphates) such as ATP γ S are substrates for nucleoside diphosphate kinase from beef liver and can thus be used for the transfer of phosphorothioate to nucleoside diphosphates.³⁹ However, for the enzyme from *E. coli* which is contained in small amounts in ribonucleotide reductase

(46) Mato, J. M.; Konijn, T. M. *FEBS Lett.* 1977, 75, 173.

(47) Rossier, C.; Gerisch, G.; Malchow, D.; and Eckstein, F. *J. Cell. Sci.* 1978, 35, 321.

(48) Bär, H. P.; Simonson, L. P.; Eckstein, F. *FEBS Lett.* 1974, 41, 199.

(49) Monneron, A.; d'Alayer, J. *FEBS Lett.* 1978, 90, 157.

(50) Pfeuffer, Th.; Helmreich, E. J. M. *J. Biol. Chem.* 1975, 250, 867.

(51) Cassel, D.; Selinger, Z. *Biochem. Biophys. Res. Commun.* 1977, 77, 868.

(52) Cassel, D.; Eckstein, F.; Lowe, M.; Selinger, Z. *J. Biol. Chem.* Submitted for publication.

(40) Eckstein, F.; Simonson, L. P.; Bär, H. P. *Biochemistry* 1974, 13, 3806.

(41) Zielinski, W. S.; Stec, W. *J. Am. Chem. Soc.* 1977, 99, 8365.

(42) Eckstein, F.; Eimerl, S.; Schramm, M. *FEBS Lett.* 1976, 64, 92.

(43) Eckstein, F.; Foreman, J. C. *FEBS Lett.* 1978, 91, 182.

(44) Kuntze, H.; Eckstein, F. Unpublished results.

(45) Dorrington, G. H.; Luis, B. G.; Fritz, L. B. Private communication.

preparations at least dGTP γ S is not a substrate.⁵³

In the earlier review on nucleoside phosphorothioates⁶ ATP γ S was described as a substrate for protein kinase as well as phosphorylase kinase. The latter enzyme phosphorylates the inactive glycogen phosphorylase *b* to the active phosphorylase *a*. Reaction with ATP γ S led to an active thiophosphorylase *a* which was resistant to the action of phosphatase, thus freezing the phosphorylase in an active state.⁵⁴ In a ³¹P NMR study on the role of pyridoxal phosphate⁵⁵ this thiophosphorylation has been used to differentiate between the various phosphate resonances from the phosphorylserine, the pyridoxal phosphate, and AMP. Phosphorothioate groups have a large chemical shift downfield from phosphoric acid, on the order of 40–50 ppm. Thus the thiophosphorylserine residue resonance obtained on thiophosphorylation of phosphorylase, as well as the adenosine 5'-phosphorothioate residue, are far removed from the pyridoxal phosphate resonance whose chemical shift as a function of various parameters can easily be followed without interference from other phosphate residues.

How far this enzymatic thiophosphorylation of proteins, or, even more interesting, the resistance of the thiophosphorylated protein against phosphatases is a general phenomenon is not clear at present. In at least one other instance, the thiophosphorylation of myosin light chains, this has been verified.⁵⁶ It could also be demonstrated that rat liver phosphofructokinase can be activated by the use of ATP γ S instead of ATP.⁵⁷ For the synthesis of the much needed [³⁵S]ATP γ S the exchange reaction employing phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase⁵⁸ was adapted, showing that these two enzymes can also handle the phosphorothioate group.

In an elegant piece of work Knowles and co-workers⁵⁹ synthesized chiral [¹⁸O,¹⁶O]ATP γ S starting with D-glyceric acid. This was converted chemically to the 3- and 2-[¹⁸O]phosphorothioates of D-glycerate, of which the latter was used as substrate for enolase and pyruvate kinase to produce chiral [¹⁸O]ATP γ S. Space does not permit a discussion of their work in detail. It

(53) v. Döbeln, U.; Reichard, P. *J. Biol. Chem.* **1976**, *251*, 3616.

(54) Gratecos, D.; Fischer, E. H. *Biochem. Biophys. Res. Commun.* **1974**, *58*, 960.

(55) Feldmann, K.; Hull, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 856.

(56) Morgan, M.; Perry, S. V.; Ottawa, J. *Biochem. J.* **1976**, *157*, 687.

(57) Brand, I.; Söling, D.; Eckstein, F. Unpublished results.

(58) Eckstein, F. *Biochim. Biophys. Acta* **1977**, *483*, 1.

(59) Orr, G. A.; Simon, J.; Jones, S. R.; Chin, G. J.; Knowles, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 2230.

must suffice here to mention their finding that all three enzymes they employ for phosphorothioate transfer (pyruvate kinase, hexokinase, glycerol kinase) follow the same stereochemical course, either all working with inversion or all with retention. In the light of what is known today, the former appears to be the most likely.

Conclusion

Nucleoside phosphorothioates have proved to be interesting compounds for a variety of reasons. On the one hand, they have been found useful in the hands of the more mechanistically inclined in studying the stereoselectivity of an enzyme for a particular isomer of the nucleoside di- or triphosphate as well as in determining whether the metal is still associated with the substrate on the enzyme active site. In connection with this, they can be used for determining the stereochemical course of a reaction involving either phosphate or nucleoside monophosphate transfer. Some of these aspects they have in common with other compounds such as the Cr³⁺ or Co(NH₃)₄³⁺ complexes and the chiral [^{16,17,18}O]phosphate esters.⁶⁰

On the other hand, in many complex biological systems, where nucleotides play a role, nucleoside phosphorothioates have proved to be of advantage, because of their slow rate of degradation, in dissecting individual steps in a sequence of reactions. It might well be that in the future this will be the area where they can be employed to the greatest advantage.

The most vexing part of working with nucleoside phosphorothioates is that it seems to be extremely hazardous to predict their behavior for a particular enzyme. This is a reflection of our still poor understanding of enzymatic reactions—a state which nucleoside phosphorothioates might help to improve.

I thank all collaborators who made contributions to this project and whose names are mentioned in the references for their enthusiasm, their involvement, and their hard work, which made nucleoside phosphorothioates so successful as an investigative tool. V. W. Armstrong, P. M. Burgers, and D. Yee kindly helped me in the preparation of the manuscript. The work carried out in my laboratory was in part financially supported by the Deutsche Forschungsgemeinschaft.

(60) The development of chiral [^{16,17,18}O]phosphate esters [Abbott, S. A.; Jones, S. R.; Weinmann, S. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1978**, *100*, 2558. Cullis, P. M.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1978**, 512] has opened the way for the determination of the stereochemical course of enzymatic phosphate transfer [Jones, S. R.; Kindman, V. A.; Knowles, J. R. *Nature (London)* **1978**, *275*, 564] without having to resort to the phosphorothioates. Although no direct comparison of the results obtained by the two approaches with the same enzyme has been documented so far, it will be interesting to see it being done.