from −0.3 to 0.4 e Å^{-3} about the bromide ion. The final conventional R factor is 2.4%.[21]

The structure of **11**·HBr was solved and refined in an analogous manner. Its final difference Fourier has no features greater than 0.2 e Å^{-3} in magnitude. The final R factor is 2.3%.[21]

The absolute configurations of **3**·HBr and **11**·HBr were established by comparison of the observed and calculated structure factors of the significant Bijvoet pairs of reflections[22] for each salt. For **3**·HBr there were 24 pairs of reflections for which $F_o(hkl)$ and $F_o(\overline{hkl})$ were both greater than 30 e and $|\Delta F_o| = ||F_o(hkl)| - |F_o(\overline{hkl})||$ was greater than 1.6 e. The corresponding differences in the calculated structure factors, ΔF_c, agreed in sign with their respective ΔF_o for all 24 pairs. For **11**·HBr, the signs of all but one of the ΔF_c agreed with the signs of the corresponding ΔF_o for the 48 pairs of reflections with F_o greater than 15 e and ΔF_o greater than 1.9 e.

(21) Listings of structure factors, coordinates, and thermal parameters for **3**·HBr and **11**·HBr will appear following these pages in the microfilm edition of this volume of the Journal. Single copies may be obtained from the Reprint Department, ACS Publications, 1155 Sixteenth St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for micofiche.

(22) A. F. Peerdeman, A. J. van Bommel, and J. M. Bijvoet, *Proc. Kon. Ned. Akad. Wetensch., Ser. B*, **54**, 16 (1951).

Acknowledgments. We wish to thank our Physical Chemistry Department, under the direction of Dr. R. P. W. Scott, for the analytical and spectral data. We are indebted to Dr. F. Scheidl for the microanalyses, to Dr. W. Benz for the mass spectral determination, to Dr. T. Williams for the nuclear magnetic resonance data, and especially to Dr. V. Toome for the ultraviolet, ORD, and CD determinations.

Thiophosphate Analogs of Nucleoside Di- and Triphosphates

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Abstract: The chemical synthesis of thiophosphate analogs of nucleoside di- and triphosphates bearing a sulfur at the terminal phosphorus atom by the use of *S*-2-carbamoylethyl thiophosphate is described. They can readily be oxidized to their disulfides and are only slowly degraded by alkaline phosphatase. Their reactivity with 5,5′-dithiobis(2-nitrobenzoic acid) was investigated and compared with that of other nucleoside thiophosphates.

Nucleotide analogs modified by replacement of an oxygen of the phosphate group by sulfur have recently been described, and have shown interesting behavior with enzymes involved with nucleic acid metabolism.[1] Analogs of nucleotide anhydrides bearing a sulfur at the α phosphorus atom have also been prepared, and the triphosphates have been used as substrates for DNA-dependent RNA polymerase to obtain polynucleotides containing a thiophosphate backbone.[2] Adenosine 5′-*O*-(1-thiotriphosphate) is also a substrate for C-C-A pyrophosphorylase, and using this enzyme adenosine 5′-*O*-phosphorothioate can be incorporated into $tRNA^{Phe}$.[3] The compound is, however, a competitive inhibitor for phenylalanyl-tRNA synthetase. These examples illustrate the usefulness of the 1-thiophosphate analogs, but a difficulty arises in their use in kinetic studies with enzymes, since the compounds exist as diastereoisomeric pairs due to the asymmetry at the α phosphorus atom. It is possible for the two isomers to have quite different behavior toward a particular enzyme, and this can present difficulties in interpretation of kinetic results. In the extreme, it is possible that one diastereoisomer is a substrate, while the other is an inhibitor. An analogy for this is provided by the *O*-(*p*-nitrophenyl) ester of thymidine 5′-*O*-thiophosphate, one diastereoisomer of which is a substrate for snake venom phosphodiesterase, while the other is an inhibitor.[4] When the isomers can be separated, as is the case with uridine 2′,3′-*O*,*O*-cyclophosphorothioate,[5] the isomerism can be useful to help define the stereochemistry of enzymatic processes.[6] However, for di- and triphosphates, which are difficult to crystallize, separation of the isomers is likely to be impossible. It was therefore thought of interest to attempt the preparation of di- and triphosphate analogs bearing sulfur on the terminal phosphorus atom, since these compounds would have no asymmetry at this site. The compounds were also of interest as potential affinity labels for enzymes containing thiol or disulfide groups in the region of their active site, because of the possibility of disulfide formation with the thiophosphate residue.

Preparation of nucleoside 5′-di- and -triphosphates is normally achieved by activation of a nucleoside 5′-phosphate followed by attack on the activated product using either orthophosphate or pyrophosphate ion. Because of the possibility of attack of thiophosphate ion on such a system *via* either sulfur or oxygen, two possible products can be envisaged from this type of reaction, one having the sulfur on the terminal phosphorus, and the other having a sulfur bridge between the two phosphorus atoms. The first activation method employed was that using *N*,*N*′-carbonyldiimidazole.[7] The reaction of adenosine 5′-phosphoroimidazolate with thiophosphate ion under the reported conditions gave no diphosphate-like product, even after allowing the reaction mixture to stand for a much longer time than is

(1) F. Eckstein, *J. Amer. Chem. Soc.* **92**, 4718 (1970), and references cited therein.

(2) F. Eckstein and H. Gindl, *Eur. J. Biochem.*, **13**, 558 (1970).

(3) E. Schlimme, F. v.d. Haar, F. Eckstein, and F. Cramer, *ibid.*, **14**, 351 (1970).

(4) D. J. Shire and F. Eckstein, manuscript in preparation.

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

(6) D. A. Usher, D. J. Richardson, Jr., and F. Eckstein, *Nature (London)*, **228**, 664 (1970).

(7) D. E. Hoard and D. G. Ott, *J. Amer. Chem. Soc.*, 87, 1785 (1965).

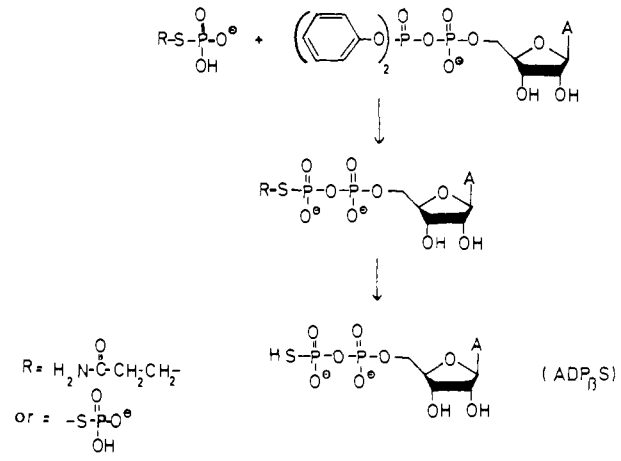
necessary with the orthophosphate ion. After 3 days, the only new product was P^1,P^2-diadenosine 5′-pyrophosphate, which was detected by electrophoresis and tlc. This is a recognized by-product in the preparation of nucleotide anhydrides.[7]

Since thiophosphate ion showed low reactivity toward the phosphoroimidazolate, another procedure was attempted using a more reactive leaving group for activation of the mononucleotide. The procedure used was that described by Michelson in which P^1-diphenyl P^2-adenosine 5′-pyrophosphate is allowed to react with orthophosphate.[8] With thiophosphate, no sulfur-containing product was detected after the standard reaction time of 3 hr. After 3 days, small amounts of two sulfur-containing compounds were present with electrophoretic mobilities at pH 7.5 similar to adenosine di- and triphosphates, respectively. The diphosphate-like material was rapidly degraded by alkaline phosphatase to adenosine and was not further studied. The product which behaved like adenosine 5′-triphosphate at pH 7.5 was found to have a phosphorus to adenine ratio of 2:1, and at pH 3.5 had the same electrophoretic mobility as adenosine 5′-diphosphate. In this respect, it was identical with adenosine 3′,5′-*O*,*O*-diphosphate. Degradation with alkaline phosphatase gave adenosine 3′-thiophosphate, this product then being stable to further degradation.[1] From this evidence, it seems likely that the compound is adenosine 3′-*O*-thiophosphate 5′-phosphate. An analogy for the formation of this compound is provided by the dismutation reactions of nucleoside 5′-polyphosphates in pyridine, where at least one compound having a 3′-phosphate group is formed, probably by an intramolecular phosphorylation.[9]

Due to the difficulties encountered above, another approach was attempted, which is an extension of the procedure used for the preparation of nucleoside 5′-*O*-thiophosphates.[1] Adenosine 5′-phosphate was protected by conversion to the N^6,2′-*O*,3′-*O*-triacetate,[10] and then allowed to react with triimidazole 1-phosphinesulfide. Successive treatments with acetic acid and ammonia to remove all protecting groups gave a small amount of diphosphate-like product. However, this was shown not to contain sulfur, and was in fact adenosine 5′-diphosphate. It seems likely that the reaction proceeded in the expected fashion, but that under the acidic conditions necessary to remove the second imidazole group, sulfur was also lost.

As noted above, the presence of the P=S grouping in the thiophosphate ion apparently has an adverse effect on the reactivity of the ion toward an activated adenosine 5′-phosphate derivative. It was therefore decided to protect the sulfur by esterification, in the hope that this might lessen its influence. A requirement for the ester grouping was that it should be removable under reasonably mild conditions. The group chosen for this purpose was that introduced by Cook,[11] the carbamoylethyl group (Scheme I). Reaction of *S*-2-carbamoylethyl thiophosphate with P^1-diphenyl P^2-adenosine 5′-pyrophosphate proceeded smoothly, and after removing the protecting group under alkaline

Scheme I



conditions, adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) was isolated in 35% yield by chromatography on DEAE-cellulose. The compound was identified by its electrophoretic mobility (identical with adenosine 5′-diphosphate at pH 3.5 and 7.5), its different chromatographic behavior compared to adenosine 5′-diphosphate, its nonidentity with adenosine 5′-*O*-(1-thiodiphosphate), and the development of a white color when a chromatogram was sprayed with a starch–iodine solution. The compound was only very slowly degraded by alkaline phosphatase, about 50% being degraded in 12 hr under conditions where adenosine 5′-diphosphate is degraded in less than 10 min. The compound behaves as a competitive inhibitor for this enzyme ($K_i = 6.6 \times 10^{-5}$ *M*). Evidence that the sulfur is situated on the terminal phosphorus and not between the two phosphorus atoms is provided by the reversible oxidation of the compound to its disulfide using potassium ferricyanide or hydrogen peroxide, and by its reaction with Ellman's reagent (see below). The disulfide was found to be stable to alkaline phosphatase. It could be readily separated from the free thiophosphate by tlc or paper electrophoresis.

Another approach to the preparation of this analog involved protection of the thiophosphate by oxidation to its disulfide with iodine in hydrochloric acid,[12] and use of this ion as the attacking species in a reaction with P^1-diphenyl P^2-adenosine 5′-pyrophosphate. After removal of the protecting thiophosphate group by reduction with 2-mercaptoethanol, adenosine 5′-*O*-(2-thiodiphosphate) was obtained in 25% yield.

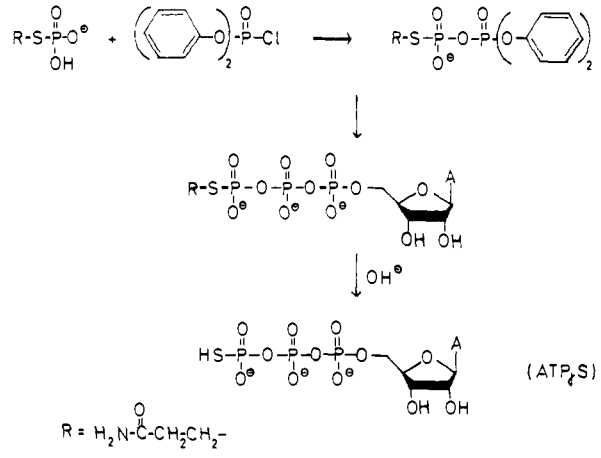
For the preparation of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) an extension of the first method described above for the 2-thiodiphosphate was attempted. Adenosine 5′-diphosphate was activated by reaction with diphenyl phosphorodichloridate, and the product (not isolated) was treated with *S*-2-carbamoylethyl thiophosphate. After removal of the protecting group with 0.2 *N* sodium hydroxide at 100°, and chromatography on DEAE-cellulose and DEAE-Sephadex, adenosine 5′-*O*-(3-thiotriphosphate) was obtained in *ca.* 4% yield. The compound was slightly more stable than the 2-thiodiphosphate to alkaline phosphatase, about 48 hr being needed for 50% hydrolysis under the standard conditions used. Electrophoretically, the compound was identical with adenosine 5′-triphosphate at pH 3.5 and 7.5. It could be distinguished from the

(8) A. M. Michelson, *Biochim. Biophys. Acta*, **91**, 1157 (1964).
(9) J. R. Reiss and J. G. Moffatt, *J. Org. Chem.*, **30**, 3381 (1965).
(10) R. K. Ralph and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 2926 (1961).
(11) A. F. Cook, *ibid.*, **92**, 190 (1970).
(12) E. Thilo and E. Schöne, *Z. Anorg. Chem.*, **259**, 226 (1949).

latter by tlc on cellulose impregnated with polyethylenimine (PEI cellulose). Like the 2-thiodiphosphate, the compound could be oxidized with ferricyanide or hydrogen peroxide to give a disulfide which could be reduced back to the starting material with dithiothreitol or 2-mercaptoethanol.

Since this method resulted in a low yield of the desired product, an alternative procedure was investigated (Scheme II). *S*-2-Carbamoylethyl thiophosphate was

Scheme II



activated by reaction with diphenyl phosphorochloridate, and the product, which was not characterized, was allowed to react with adenosine 5′-diphosphate. Following the same hydrolysis and work-up procedure as before, the 3-thiotriphosphate was isolated in 25% yield after DEAE–cellulose chromatography slightly contaminated with inorganic thiophosphate and adenosine 5′-triphosphate, or in slightly lower yield following DEAE–Sephadex chromatography. The absence of adenosine 5′-triphosphate in this product was best shown by tlc on PEI–cellulose. The 5′-*O*-(3-thiotriphosphates) of uridine and guanosine were prepared in the same way.

In an attempt to estimate the amounts of sulfur in the two compounds whose preparations have been described, their reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent[13]) was investigated. The extent of reaction with the reagent was estimated from the light absorbance at 412 mμ of the released 2-nitro-5-thiobenzoic acid ion. It was found that with both compounds the extent of reaction was very small, unless a large excess of Ellman's reagent was used. By stepwise addition of the reagent, the theoretical limit of reaction could be approached, but never reached. Clearly, the reaction is reversible, and the position of equilibrium very much favors the existence of the thiophosphates in the reduced state. Table I shows equilibrium constants calculated from the spectrophotometric data for the two analogs, along with those of several other analogs. In their calculation, it was assumed that only mixed disulfide formation between a thiophosphate and 2-nitro-5-thiobenzoic acid was of importance, and that symmetrical disulfide formation was negligible, due to the large excess of Ellman's reagent used. Relative initial rates of reaction under identical conditions are also given (Table I).

(13) G. A. Ellman, *Arch. Biochim. Biophys.*, 82, 70 (1959).

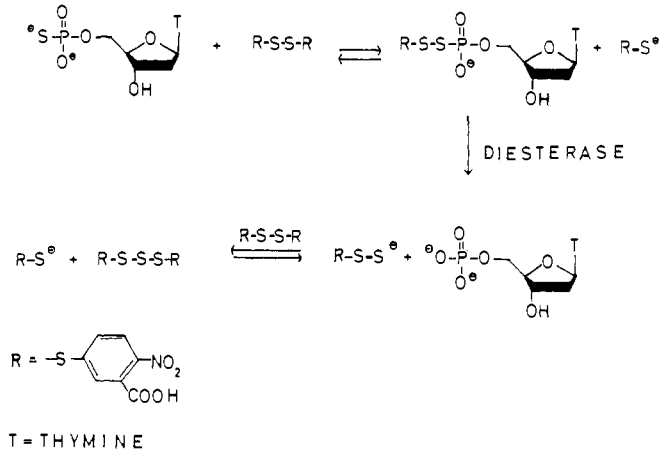
Table I. Reaction of Thiophosphate Analogs with Ellman's Reagent

Compd	Rel initial reaction rate	K^a
AMPS[b]	1.0	3.22×10^{-2}
TMPS[b]	0.88	3.45×10^{-2}
ADPβS	0.33	4.15×10^{-2}
ATPγS	0.091	2.32×10^{-2}
ATPαS[2]	0.081	5.35×10^{-3}
3′(2′)UMPS[1]	0.41	

[a] K = equilibrium constant for the reaction RPSH + R′SSR′ ⇄ RPSSR′ + R′SH, where R = a nucleoside or nucleotide and R′ = 2-nitro-5-thiobenzoic acid. [b] Reference 1.

It is of interest to note that addition of snake venom phosphodiesterase to the mixture of Ellman's reagent and thymidine 5′-*O*-thiophosphate or adenosine 5′-*O*-thiophosphate resulted in the release of 2 equiv of colored anion. The most likely reaction path to explain this result is the following (Scheme III).

Scheme III



Presumably the last stage is driven to completion, or nearly so, by the large excess of Ellman's reagent used. This explains the formation of 2 equiv of anion.

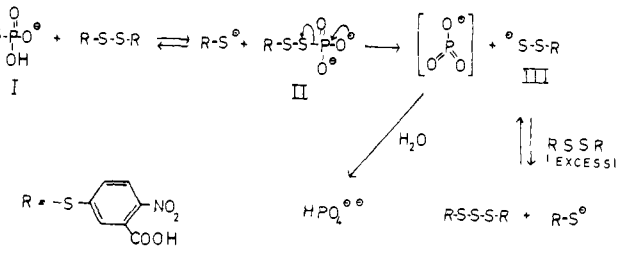
The intermediate disulfide seems a likely substrate for the enzyme, because of its resemblance to a diester of orthophosphoric acid. An alternative reaction scheme, in which the analog is desulfurized by the phosphodiesterase and the resulting hydrogen sulfide reacts with Ellman's reagent liberating 2 equiv of anion, could be ruled out in the case of adenosine 5′-thiophosphate, since reaction of the phosphodiesterase with the analog in the absence of Ellman's reagent resulted in much slower desulfurization.

Addition of snake venom phosphodiesterase to the mixtures of thiodi- or triphosphates with Ellman's reagent caused no further release of color, indicating that in this case the intermediate disulfides are not recognized as substrates.

In the reaction of uridine 3′(2′)-*O*-thiophosphate with Ellman's reagent, the production of 2-nitro-5-thiobenzoic acid did not stop until 2 equiv of the anion were released. Chromatography of the products showed that complete conversion to uridine 2′,3′-*O*,*O*-cyclic phosphate had occurred. Presumably this compound was formed by intramolecular attack of the 2′(3′)-hydroxyl group on the mixed disulfide of uridine 3′(2′)-*O*-thiophosphate and 2-nitro-5-thiobenzoic acid.

Since it is known that thiophosphate ion can reduce disulfide bonds in certain enzymes,[14] an attempt was made to compare the reactivities of simple thiophosphate ion and the nucleotide analogs toward Ellman's reagent. Reaction of thiophosphate with the reagent resulted in the rapid release of twice as much 2-nitro-5-thiobenzoate as expected, even using a comparatively small excess of the disulfide. Furthermore, from the kinetics of the reaction, it was clear that two separate steps are involved, the first being faster than the second. It seems likely that the following sequence of reactions occurs (Scheme IV).

Scheme IV



This scheme results in the liberation of 2 equiv of anion/equiv of thiophosphate. The suggested mechanism of breakdown is based on the high reactivity of the disulfide III as a leaving group, as already indicated in the formation of uridine 2′,3′-*O*,*O*-cyclic phosphate from uridine 3′(2′)-*O*-thiophosphate as described above.

Due to the more complex reaction of thiophosphate ion with Ellman's reagent, it was not possible to compare its reactivity with that of the analogs. However, a comparison with cysteine was obtained by mixing cysteine and Ellman's reagent in a 1:1 molar ratio. This resulted in liberation of the maximum theoretical amount of colored anion, indicating a high equilibrium constant for this reaction. This would suggest that cleavage of cysteine disulfide bonds in proteins by the analogs is unlikely, unless it results in some radical change in structure leading to a more stable configuration of the polypeptide chain. However, since the analogs apparently have low reducing power, it follows conversely that their disulfides should have high oxidizing power, *i.e.*, that they would react readily with reactive thiol groups such as that in cysteine.

The interactions of the analogs with several enzyme systems, among them DNA-dependent RNA polymerase and polynucleotide phosphorylase, are under investigation in our laboratory.

Experimental Section

General Procedures. Paper chromatography was performed by the descending method, using Schleicher and Schüll 2043b (washed) paper in system A (ethanol–1 *M* ammonium acetate, 7:3, v/v) and Whatman 3 MM paper in system B (isobutyric acid–2 *N* NH_4OH–0.2 *N* EDTA, 120:12:2, v/v). Thin-layer chromatography (tlc) using Merck silica gel F_{254} plates was carried out using system A. Ion exchange tlc was performed with MN-Polygram CEL 300 PEI/UV plates (Machery and Nagel, Düren, Germany) eluting with 0.75 *M* KH_2PO_4 adjusted to pH 3.4 with concentrated HCl. Schleicher and Schüll 2043b (washed) paper was used for high-voltage electrophoresis in 0.1 *M* triethylammonium bicarbonate (pH 7.5) or 0.05 *M* ammonium formate buffer (pH 3.5) at 2200 V for 1.5 hr. Sulfur-containing spots were detected on paper or tlc plates using a spray containing sodium azide (1 g), soluble starch (1 g), and iodine (1 g).[15] Using this spray, compounds containing readily oxidizable sulfur appeared as white spots on a blue background. Ultraviolet absorption measurements were made using a Cary Model 14, Zeiss PMQII, or Unicam SP-1800 instrument. ^{31}P nmr spectra were recorded with a Perkin-Elmer R-10 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 (parts per million) from standard. Whatman DE-23 or DE-52 cellulose and DEAE-Sephadex A-25 were used for ion exchange chromatography using a linear gradient of triethylammonium bicarbonate.

Degradations with alkaline phosphatase (5–10 μl) were carried out using 0.3–1 μmol of nucleotide in 0.05–0.15 ml of 0.1 *M* Tris-HCl buffer (pH 8.0) at 37° for the length of time stated. The products were analyzed by tlc in system A and electrophoresis at pH 3.5 or 7.5. Non-sulfur-containing nucleotides present as impurities in the preparations were estimated as follows. The uv absorbance of a solution containing the analog (*ca.* 2 A_{260} units in 2.5 ml of 0.1 *M* Tris-HCl buffer, pH 8.0) and adenosine deaminase (2 μl of solution) was measured at 265 mμ. Alkaline phosphatase (5 μl) was added, and the decrease in the absorbance at 265 mμ was measured. For complete degradation, $\epsilon_M = -8200$. Alkaline phosphatase (purity grade I, calf intestine) and adenosine deaminase were purchased from Boehringer and Söhne (Mannheim, Germany), as was snake venom phosphodiesterase.

Phosphate determinations were carried out by the methods of Hurst and Becking[16] or of Chen, *et al.*[17]

Table II. Thin-Layer Chromatography of Nucleotide Anhydrides and Their Thiophosphate Analogs

Compd	R_f values: Silica gel	R_f values: PEI–cellulose
ADP	0.06	0.49
ADPβS	0.12	0.27
ADPαS[a]	0.08	0.33
ADPβS disulfide	0.49	
ATP	0.03	0.25
ATPγS	0.06	0.10
ATPαS[b,c]	0.03	0.12
ATPγS disulfide	0.37	0.08
GTP	0.0	0.13
GTPγS	0.0	0.05
UTP	0.07	0.50
UTPγS	0.08	0.18
UTPγS disulfide	0.50	

[a] Adenosine 5′-*O*-(1-thiodiphosphate). [b] Adenosine 5′-*O*-(1-thiotriphosphate). [c] Reference 2.

Adenosine 5′-*O*-(2-Thiodiphosphate) (ADPβS). (1) Adenosine 5′-phosphate (347 mg, free acid, 1 mmol) was added to dry methanol (5 ml) and tri-*n*-octylamine (0.43 ml, 1 mmol), and the mixture was warmed gently until solution was obtained. The solvent was removed under reduced pressure, and the residue was dried by repeated evaporation of 5-ml aliquots of dry DMF. Dry dioxane (7 ml) was added to the residue, followed by dry DMF (1 ml) if solution did not occur easily. Diphenyl phosphorochloridate (0.3 ml) was added to the solution, followed by tri-*n*-butylamine (0.3 ml). The white precipitate obtained at this stage redissolved slowly on stirring. After standing for 3 hr at room temperature, the solvent was removed by evaporation, and dry ether (50 ml) was added to the residue with shaking. The mixture was allowed to stand at 4° for 0.5 hr, and the ether was then removed by decantation. Dry dioxane (5 ml) was added to the residue, and the resulting suspension was evaporated to dryness. A solution of *S*-2-carbamoylethyl thiophosphate (tri-*n*-butylammonium salt, 2 mmol)[18]

(14) H. Neumann, R. F. Goldberger, and M. Sela, *J. Biol. Chem.*, **239**, 1536 (1964).

(15) D. J. Shire, Göttingen, unpublished results.

(16) R. O. Hurst and G. C. Becking, *Can. J. Biochem. Physiol.* **41**, 469 (1963).

(17) P. S. Chen, T. Y. Tosibara, and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

(18) Prepared by passing a solution of the lithium salt (2 mmol)[11] over a column of Merck I ion exchanger (pyridinium form) followed by evaporation and re-solution of the residue in methanol containing tri-*n*-butylamine (2 mmol). After removal of the solvent, the product was dried by repeated evaporation of 5-ml aliquots of dry pyridine.

in dry pyridine (6 ml) was added, and the solution was allowed to stand for 3 hr at room temperature, after which time a precipitate had formed. The pyridine was removed by evaporation under reduced pressure, 0.2 *N* NaOH (80 ml) was added, and the resulting cloudy solution was heated for 10 min at 100°. After cooling, the mixture was neutralized with Merck I ion exchanger (pyridinium form), treated with 2-mercaptoethanol (0.5 ml), filtered, and chromatographed on DEAE-cellulose, using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) from 0.05 to 0.3 *M*. The product was eluted at about 0.22 *M* (5400 A_{260} units, 35% yield from adenosine 5′-phosphate). The compound was electrophoretically identical with adenosine 5′-diphosphate, but gave a white color with the sulfur spray; adenosine:phosphorus = 1:2.07; $\lambda_{max}^{H_2O}$ 259 mμ (ε 15,000). The adenosine 5′-diphosphate content was not more than 1%, as judged by degradation with alkaline phosphatase.

(2) Dipotassium dihydrogen dithiophosphate[19] (302 mg, 1 mmol) was converted to the pyridinium salt by passage of a solution in 50% aqueous methanol down a column of Merck I ion exchanger in the pyridine form. Tri-*n*-butylamine (2 mmol) was added to the effluent, which was then evaporated under reduced pressure. The residue was dried by repeated evaporation of dry pyridine. A solution of this product in dry pyridine (4 ml) was then substituted for *S*-2-carbamoylethyl thiophosphate in the procedure described above, using 0.5 mmol of adenosine 5′-phosphate. After standing for 16 hr at room temperature, the solvent was removed under reduced pressure, and the residue was dissolved in water (20 ml), treated with 2-mercaptoethanol (2 ml), and chromatographed as before on DEAE-cellulose (2000 A_{260} units, 26% yield from adenosine 5′-phosphate). This product was identical by electrophoresis, tlc, and paper chromatography in system B (R_{ADP} = 0.75) with that obtained using *S*-2-carbamoylethyl thiophosphate. Its chemical and enzymatic properties were also identical.

Adenosine 5′-*O*-(3-Thiotriphosphate) (ATPγS). *S*-2-Carbamoylethyl thiophosphate (Li^+ salt, 0.5 mmol) was converted to the pyridinium salt by passage over Merck I ion exchanger, and then converted to the mono(tri-*n*-octylammonium) salt by addition of tri-*n*-octylamine (0.22 ml, 0.5 mmol) to a solution in methanol. The solvent was removed under reduced pressure, and the residue was dried by repeated evaporation of dry DMF. The product was then dissolved in dry dioxane (3.5 ml), and diphenyl phosphorochloridate (0.15 ml) followed by tri-*n*-butylamine (0.23 ml) were added. The solution was allowed to stand at room temperature for 2 hr, and then evaporated under reduced pressure. Ether (10 ml) was added to the residue, and after shaking for 1–2 min, petroleum ether (40–60°, 20 ml) was added and the mixture was allowed to stand at 4° for 0.5 hr. The supernatant was decanted, the residue was dissolved in dry dioxane (3 ml), and the solution was concentrated under reduced pressure. A solution of adenosine 5′-diphosphate [mono(tri-*n*-octylammonium), mono(tri-*n*-butylammonium) salt, 0.25 mmol][20] in dry pyridine (3 ml) was added to the remaining syrup, and the solution was allowed to stand for 2 hr at room temperature. A precipitate formed slowly during this time. The solvent was removed under reduced pressure, 0.2 *N* NaOH (20 ml) was added, and the mixture was heated at 100° for 10 min. After cooling, the cloudy solution was neutralized with Merck I ion exchange resin (pyridinium form), treated with 2-mercaptoethanol (0.5 ml), and chromatographed on DEAE-cellulose using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5, 0.15–0.4 *M*). The product was eluted at about 0.28 *M* (800 A_{260} units, slightly contaminated with adenosine 5′-diphosphate and thiophosphoric acid). The compound could be obtained pure by chromatography on DEAE-Sephadex using a linear gradient of triethylammonium bicarbonate buffer (pH 7.5, 0.35–0.6 *M*). The product was eluted at about 0.5 *M*. It was electrophoretically identical with adenosine 5′-triphosphate. Nonsulfur-containing contaminants were not detectable by tlc or by degradation with alkaline phosphatase; adenosine:phosphorus = 1:2.94; $\lambda_{max}^{H_2O}$ 259 mμ (ε 15,000).

Uridine 5′-*O*-(3-Thiotriphosphate) (UTPγS) and Guanosine 5′-*O*-(3-Thiotriphosphate) (GTPγS). These compounds were synthesized in the same way as adenosine 5′-*O*-(3-thiotriphosphate). They were identical on electrophoresis with the corresponding nucleoside 5′-triphosphates but could be readily distinguished from these by PEI-cellulose tlc. They could be oxidized to the disulfides by ferricyanide or H_2O_2.

Oxidation of Analogs. Adenosine 5′-*O*-(2-Thiodiphosphate) Disulfide. Oxidation of adenosine 5′-*O*-(2-thiodiphosphate) was carried out with either potassium ferricyanide or hydrogen peroxide.

(1) Potassium ferricyanide solution (0.1 *M*, 100 μl) was added to the diphosphate analog (1 μmol) dissolved in water (10 μl), and after 5 min at room temperature, the whole solution was applied to chromatography paper, and the mixture was separated by electrophoresis at pH 7.5 and 2200 V. At this pH the disulfide has a mobility equal to that of adenosine 5′-phosphate. After elution in methanol-water (1:1, v/v), the product was obtained in *ca.* 60% yield. It was readily distinguished from the starting material by tlc (Table I). Addition of 2-mercaptoethanol to the disulfide resulted in rapid reduction to the original thiodiphosphate.

(2) For preparation of the disulfide on a larger scale, oxidation with hydrogen peroxide was more convenient. Adenosine 5′-*O*-(2-thiodiphosphate) (6 μmol in 0.8 ml of water) was treated with 3% hydrogen peroxide (0.1 ml in a 100-ml round-bottomed flask),[21] and the solution was then immediately evaporated to dryness. Water (1 ml) was added to the residue and again immediately removed by evaporation. This process was repeated three times to remove hydrogen peroxide. As long as the starting material was free of thiophosphoric acid, this procedure resulted in quantitative conversion to the disulfide. Longer contact time with hydrogen peroxide must be avoided to prevent further oxidation of the product.

Adenosine 5′-*O*-(3-Thiotriphosphate) Disulfide. Oxidation of adenosine 5′-*O*-(3-thiotriphosphate) could be achieved in the same way as described for the 2-thiodiphosphate. With potassium ferricyanide under the conditions described above, a 50% yield of disulfide was obtained. For larger scale oxidations, hydrogen peroxide was used. On paper electrophoresis at pH 7.5, the compound had a mobility relative to adenosine 5′-triphosphate of 0.82, and was readily distinguished from the latter by tlc (Table II). Reduction with 2-mercaptoethanol gave the starting material.

Stability of the Analogs against Alkaline Phosphatase. Five A_{260} units of each of the respective analogs in Tris–HCl buffer (0.1 *M*, pH 8.0, 50 μl) were treated with alkaline phosphatase (5 μl) at 37°. The mixture was examined by tlc in system A after 2, 30, and 48 hr. Adenosine 5′-*O*-(2-thiodiphosphate) was degraded completely after 48 hr, whereas adenosine 5′-*O*-(3-thiotriphosphate) was less than 50% degraded. Detectable degradation of the 2-thiodiphosphate occurred after 2 hr. Under the same conditions, adenosine 5′-diphosphate was degraded completely after 10 min.

The stability of adenosine 5′-*O*-(2-thiodiphosphate) was high enough to measure inhibition kinetics in the degradation of adenosine 5′-phosphate. Using the assay system described before, the rate of cleavage could be followed by the rate of deamination of adenosine by adenosine deaminase. The inhibitor had a K_i value of 6.6×10^{-5} *M*, compared with a K_M value for adenosine 5′-*O*-phosphate of 5.95×10^{-5} *M*.

The disulfides of the two analogs were stable against alkaline phosphatase under the conditions described above.

Reactions of Thiophosphate Analogs with Ellman's Reagent. About 2 A_{260} units of the analog (in the case of thymidine 5′-*O*-thiophosphate about 0.7 A_{267} unit) were dissolved in 2.5 ml of 0.1 *M* Tris–HCl buffer (pH 7.6) in a quartz glass cell (path length 1 cm) and the exact absorbance at 260 or 267 mμ, respectively, was measured. Ellman's reagent (usually 50 μl of a solution in 0.1 *M* Tris–HCl buffer (pH 7.6) saturated at 4°; concentration of the reagent = 11.8 m*M*) was added, and the increase in absorbance at 412 mμ was continuously recorded. When the absorbance had reached a constant level (usually within 5 min), more Ellman's reagent (50 or 100 μl) was added, and the increase again recorded. From these data, the relative initial rate for each analog was calculated (allowing for slight variations in the amount of analog

(19) Prepared by the method of Thilo and Schöne,[12] with the modification that 2 mol of thiophosphate/mol of iodine was used instead of the reported ratio of 1:1. The latter results in a twofold excess of iodine. The identity of the product was confirmed by its elemental analysis (Calcd for $K_2H_2P_2O_6S_2$: H, 0.7; P, 20.5; S, 21.2. Found: H, 0.84; P, 20.35; S, 21.21) and ^{31}P nmr spectrum (H_2O, −1.5). Addition of dithiothreitol caused a shift in the signal to the position of thiophosphoric acid at the appropriate pH (−44).

(20) Adenosine 5′-diphosphate (Na^+ salt, 0.25 mmol) was converted to the pyridinium salt by passage of a solution in 50% aqueous methanol over a column of Merck I (pyridinium form). After removal of solvent, methanol (10 ml), tri-*n*-octylamine (0.25 mmol), and tri-*n*-butylamine (0.25 mmol) were added to the residue. After stirring for 0.5 hr, solution had occurred. The solvent was removed by evaporation, and the residue was dried by repeated evaporation of 5-ml aliquots of dry pyridine.

(21) It is important to use a flask of this size or larger so that removal of hydrogen peroxide by evaporation is as rapid as possible.

used), as were the equilibrium constants. The latter could be calculated from single measurements, or graphically, after obtaining several points by stepwise addition of Ellman's reagent. The good straight line obtained in the graphical method indicated the validity of the assumption that symmetrical disulfide formation was negligible. Addition of snake venom phosphodiesterase (5 μl) to the mixtures of thymidine 5′-*O*-thiophosphate or adenosine 5′-*O*-thiophosphate and the reagent resulted in the production of 2 equiv of 2-nitro-5-thiobenzoic acid. When thiophosphoric acid was substituted for an analog in the above procedure, 2 equiv of colored anion were produced in a reaction which involved two steps, as shown by the kinetic data, the first step being significantly faster than the second. Extrapolation of the line for the second step back to zero time indicated that in each step 1 equiv of anion was released.

In the case of uridine 3′(2′)-*O*-thiophosphate, the characteristic pattern of reaching equilibrium after a few minutes was not observed. Instead, the absorbance at 412 mμ increased steadily until 2 equiv of the anion had been liberated. To investigate the products from this reaction, Ellman's reagent (150 μl, 10 mg/2 ml) was added to uridine 3′(2′)-*O*-thiophosphate (4.7 A_{260} units) in 300 μl of 0.1 *M* Tris–HCl buffer (pH 7.4) and the mixture was left overnight. Paper chromatography in system A then showed complete conversion to uridine 2′,3′-*O*,*O*-cyclic phosphate.

Reaction of Adenosine 5′-*O*-Thiophosphate with Snake Venom Phosphodiesterase. (1) Adenosine 5′-*O*-thiophosphate (2 A_{260} units) was dissolved in 0.1 *M* Tris–HCl (pH 7.6), and the light absorbance at 260 mμ was measured. Adenosine deaminase (3 μl), alkaline phosphatase (10 μl), and snake venom phosphodiesterase (5 μl) were added. The absorbance at 265 mμ was unchanged after 30 min, indicating that reaction was very slow under these conditions. Addition of Ellman's reagent (25 μl of a 11.8 m*M* solution) resulted in liberation of 2 equiv of 2-nitro-5-thiobenzoic acid/equiv of analog within 10 min.

(2) Adenosine 5′-*O*-thiophosphate (10 A_{260} units) was incubated overnight at 37° with snake venom phosphodiesterase (10 μl) in 100 μl of 0.1 *M* Tris–HCl buffer (pH 7.4). Paper chromatography in system A indicated that no adenosine had been formed. Alkaline phosphatase (10 μl) was then added, and the mixture was incubated for a further 2 hr at 37°. Paper chromatography in system A showed the presence of adenosine and adenosine 5′-*O*-thiophosphate in the ratio 6:4, indicating 60% desulfurization by the phosphodiesterase.

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Calculation of the Rotational Strengths of Mononucleosides[1]

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Abstract: The rotational strengths of the two longer wavelength transitions, B_{2v} and B_{1v}, of four mononucleosides (adenosine, guanosine, uridine, and cytidine) as a function of the glycosidic rotational angles have been investigated theoretically. The transition in each base is characterized by transition monopoles; the sugar is treated as a sum of bond polarizabilities. The interaction among these polarizabilities is also considered. Rotational strengths were calculated using three different sets of transition monopoles and many combinations of bond polarizabilities. We conclude that adenosine, uridine, and cytidine may have primarily one conformation, but that in guanosine the base is not definitely fixed with respect to the ribose. Calculation on different anomeric nucleosides of adenosine and uridine shows that the configuration at the anomeric carbon C-1′ determines the sign of the optical rotation. The configuration at C-2′ influences the glycosidic angular dependence of rotational strength more profoundly than that at C-3′ and C-5′. These results are in good agreement with experiments. The signs and magnitude of the calculated rotational strengths are in good agreement with experiment for the anti conformation of all the isomers of adenosine. As the conformation of the nucleosides in B-form DNA is quite different from the anti form, we calculate that the rotational strengths of the nucleosides in the polynucleotide are very different from those in solution.

Many workers have measured the circular dichroism (CD) and optical rotatory dispersion (ORD) of polynucleotides, and have shown that optical activity is an important tool for conformational assignments. Theories have been developed to facilitate the interpretation of the spectra of polynucleotides.[3,4] However, in these theories, the CD and ORD of the monomer units themselves have been ignored. Recently, experimental and theoretical studies of the optical activity and conformation of nucleosides have appeared.[5–7] In particular, an extended series of articles by Miles *et al.*,[8] have investigated this problem in detail.

In the present paper we have used an improved version of Kirkwood polarizability theory to include the presence of a classical polarizability near a quantum system. The rotational strengths of mononucleosides are calculated using transition monopoles on the bases interacting with polarizable bonds of the sugars. We try to examine the calculations critically. Three different sets of transition monopoles have been employed with various degrees of success. The effect of different values of bond polarizability and variation of the positions of furanosyl OH groups have also been examined. The calculated rotational strengths as a function of the glycosidic angle are in qualitative agreement

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