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Ho, Hsu-Tso

NUCLEOSIDE PHOSPHOROTHIOATE WITH CHIRALLY SUBSTITUTED PHOSPHORUS

The Ohio State University

Ph.D. 1983

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NUCLEOSIDE PHOSPHOROTHIOATE WITH CHIRALLY
SUBSTITUTED PHOSPHORUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Hsu-Tso Ho

* * * * *

The Ohio State University
1983

Reading Committee:
Professor Perry A. Frey
Professor Michael H. Klapper
Professor David H. Ives
Professor Ming Daw Tsai

Approved By

[Signature]
Adviser
Department of Chemistry
To my dearest parents:

Their love, understanding and sacrifice made my dream come true, even if it was as far away as the other side of the world.
ACKNOWLEDGMENTS

I would like to express my sincerest appreciation to Professor Perry Frey for his guidance throughout the course of my graduate research and his generous help in preparation of this dissertation. I am very grateful to members of Dr. Frey's research group for providing constructive discussions and timely encouragement.

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"Synthesis of Nucleoside $^{18}$O Pyrophosphorothioates with
Chiral $^{18}$O Phosphorothioate Group of Known Configuration.
Stereochemical Orientations of Enzymatic Phosphorylations
of Chiral $^{18}$O Phosphorothioates." John P. Richard,
Hsu-Tso Ho, Perry A. Frey, J. Am. Chem. Soc. 100, 7756
(1978).

"Evidence Implicating Cyclo-Diphosphates as Intermediates
in Reactions of Nucleoside Phosphorothioates with Cyanogen
Bromide." R. Douglas Sammons, Hsu-Tso Ho, Perry A. Frey,

FIELDS OF STUDY

Major Field: Biochemistry

Studies in stereochemistry of enzyme-catalyzed phosphoryl group transfer, under the direction of Professor Perry A. Frey.
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CHAPTER 1
INTRODUCTION

1.1 Phosphoric Esters and Anhydrides: Stereochemistry and Mechanisms of Substitution

Enzyme catalyzed reactions involving the transfer of the phosphoryl or the nucleotidyl groups of nucleotides are ubiquitous in biological systems. Among the nucleotides, the most commonly employed is adenosine 5'-triphosphate (ATP). It is the precursor of one of the four major building blocks of RNA. It mediates energy transfer in many biological processes including the transition of chemical free energy into work. And it is also involved in regulating the flow of metabolites at strategic points in metabolism. It is converted by adenylate cyclase into 3',5'-cyclic adenosine monophosphate which relays hormonal messages in cells.

Because of the essential roles that ATP plays in the normal functioning of cells, many investigations have been undertaken to unravel the mechanisms of nucleotide-dependent enzymatic reactions. Although ATP was discovered more than 50 years ago (1,2), detailed analyses of the manner in which the nucleotide is bound
by a given enzyme and of the detailed mechanisms by which the phosphoryl and adenylyl groups are transferred have been pursued mainly over the past 20 years. A great deal has been learned through kinetic studies on a wide spectrum of enzymes catalyzing substitution at phosphorus. Techniques such as isotope tracing with $^{32}\text{P}$- or $^{18}\text{O}$-labeled phosphates (3,4) and isotope scrambling with $^{18}\text{O}$-labeled phosphates (5-7) have been applied to determine which bonds are cleaved, which phosphoryl groups of the substrates are transferred and whether a phosphorylated intermediate may be involved in the catalytic pathway. The recent development of high resolution magnetic resonance spectrometers has made NMR techniques increasingly powerful means of obtaining information about the interactions among enzymes, nucleotides and metal ions (8-16).

The phosphoric acid molecule as well as the phosphoryl groups in organophosphates or polyphosphates are tetrahedral and do not spontaneously exchange substituents at significant rates. Consequently, phosphates can be chiral molecules, analogous to compounds containing saturated carbon, when the substituents of the tetrahedral phosphorus differ from one another in the physical sense. Knowledge obtained
from stereochemical studies of the reactions of phosphates should provide information concerning the mechanisms by which the reactions proceed. Because of the rapid advances in the synthesis of chirally labeled phosphates and in the development of techniques for configuration analysis, such information is now well documented for more than thirty enzyme-catalyzed phosphoryl group transfer reactions.

Based on mechanistic and kinetic studies on the non-enzymatic reactions, associative and dissociative mechanisms of nucleophilic substitution at tetrahedral phosphorus in phosphates have been proposed (17-19).

The dissociative mechanism described by equation 1 is an $S_{N}1$ mechanism, in which the leaving group is expelled in the rate-limiting step and a planar electrophilic metaphosphate intermediate is produced. The planar intermediate is then captured by a nucleophilic acceptor, from either face if it is a truly free species. This mechanism must result in loss of stereochemical configuration at a chiral phosphorus center.

\[
\begin{align*}
\text{OR} & \quad \text{R}_1 \quad \text{R}_2 \\
\text{P} & \quad \text{OR}_1 \quad \rightarrow \quad \text{P} \\
\text{R}_2 \text{OH} & \quad \text{OR}_2 + \quad \text{R}_2 \text{O} \quad \text{P}
\end{align*}
\]
Three associative mechanisms have been proposed. One is a concerted $S_N^2$ mechanism (equation 2), in which a substituting nucleophile attacks from the side opposite to the leaving group and displaces the latter in a single step through a pentacovalent, most likely trigonal-bipyramidal transition state. The stereochemical result of this mechanism may well be inversion of configuration at a chiral phosphorus center. In the second associative mechanism, the incoming nucleophile approaches again from the opposite side of the leaving group (in-line attack), but in this case a trigonal-bipyramidal intermediate is formed, with both the nucleophile and the leaving group occupying the apical positions as shown in equation 3. With the departure of the leaving group, a product with inverted configuration at the chiral phosphorus center is generated. The third mechanism is illustrated in equation 4.
In the first intermediate the incoming nucleophile occupies an apical position and the leaving group is at an equatorial position. Before the leaving group can depart, a pseudorotation must take place to form a second intermediate in which the leaving group occupies an apical position from which it may depart. Reactions that proceed by this mechanism will result in retention of configuration at a chiral reaction center.
Reactions proceeding according to equations 2 and 3 have been termed "in-line" displacements, while those proceeding according to equation 4 have been called "adjacent" displacements.

Knowledge of the stereochemical course of substitution at phosphorus in such reactions can in principle be useful in distinguishing among those proposed mechanisms in non-enzymatic reactions. The pseudorotation mechanism has been observed in non-enzymatic reactions involving pentacovalent phosphorus compounds; and the rules for pseudorotation have been discussed by Westheimer (17,19). However, the adjacent mechanism has not been found among enzyme catalyzed phosphoryl group transfer reactions. Moreover, stereochemical analysis of enzymatic reactions should not be expected to provide evidence for the metaphosphate mechanism of equation 1 because of the probability that the interactions between such an intermediate and the active site of an enzyme, protecting it from being captured by water, would also control the stereochemistry and prevent racemization.

Stereochemical analysis of enzymatic phosphoryl and nucleotidyl group transfer has provided a different type of information about mechanisms. The findings
from over thirty stereochemical studies uniformly show that each displacement proceeds by an in-line mechanism as indicated by inversion of configuration at the chiral phosphorus center. In each study of an enzymatic phosphoryl or nucleotidyl group transfer for which there is conclusive nonstereochemical evidence implicating a covalent E-P intermediate, the stereochemistry has been found to proceed with overall retention of configuration. In reactions in which no nonstereochemical evidence of E-P has ever been obtained, the stereochemical analysis has demonstrated inversion of configuration at the chiral phosphorus center.

The stereochemical test is therefore a strong indicator of whether phosphoryl group transfer proceeds with a single displacement or double displacement pathway. Overall inversion is consistent with an odd number of displacements, while retention with an even number of displacements. Listed in Table 1 are the stereochemical courses of some enzymes that have been studied in this way.

1.2 Chiral Nucleoside Phosphorothioates

Methods for synthesizing chirally substituted nucleoside phosphorothioates as substrates for the enzymes of interest, and also procedures for the
TABLE 1
Stereochemical Course of Enzymic Substitution at Phosphates

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Stereochemistry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphorylases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease A, T₁, T₂</td>
<td>Inv-Inv</td>
<td>20-23</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Ret</td>
<td>24</td>
</tr>
<tr>
<td>E. aerogenes nonspecific phosphohydase</td>
<td>Inv</td>
<td>25</td>
</tr>
<tr>
<td>Snake venom phosphodiesterase</td>
<td>Ret</td>
<td>26,27,51</td>
</tr>
<tr>
<td>3',5'-cAMP phosphodiesterase</td>
<td>Inv</td>
<td>28</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>Inv</td>
<td>29</td>
</tr>
<tr>
<td>Myosine ATPase</td>
<td>Inv</td>
<td>30</td>
</tr>
<tr>
<td>Mitochondrial ATPase</td>
<td>Inv</td>
<td>31</td>
</tr>
<tr>
<td>Rabbit muscle-sacroplasmic reticulum ATPase</td>
<td>Ret</td>
<td>32</td>
</tr>
<tr>
<td>Exonuclease (bovine spleen)</td>
<td>Ret</td>
<td>33</td>
</tr>
<tr>
<td>Staphylococcal nuclease</td>
<td>Inv</td>
<td>34</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Ret</td>
<td>35</td>
</tr>
<tr>
<td><strong>Phosphotransferases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>Inv</td>
<td>36</td>
</tr>
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<td>Glycerokinase</td>
<td>Inv</td>
<td>37-39</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Stereochemistry</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Inv</td>
<td>37-39</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>Inv</td>
<td>37-39</td>
</tr>
<tr>
<td>Nucleoside diphosphate kinase</td>
<td>Ret</td>
<td>40</td>
</tr>
<tr>
<td>Nucleoside phosphotransferase</td>
<td>Ret</td>
<td>41</td>
</tr>
<tr>
<td>Adenosine kinase</td>
<td>Inv</td>
<td>42</td>
</tr>
<tr>
<td>Acetate kinase</td>
<td>Inv</td>
<td>43</td>
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<tr>
<td>Phosphoglycerate mutase</td>
<td>Ret</td>
<td>44</td>
</tr>
<tr>
<td>Polynucleotide kinase</td>
<td>Inv</td>
<td>45,52</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
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<td>46</td>
</tr>
<tr>
<td>Creatine kinase</td>
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<td>47</td>
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<tr>
<td>Glucokinase</td>
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<td>48</td>
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<tr>
<td>Phosphofructokinase</td>
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<td>49</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>Ret</td>
<td>50</td>
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<tr>
<td>PEP carboxykinase</td>
<td>Inv</td>
<td>53</td>
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</table>

**Nucleotidyltransferases**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Stereochemistry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPG pyrophosphorylase</td>
<td>Inv</td>
<td>54</td>
</tr>
<tr>
<td>Gal-1-P uridylyltransferase</td>
<td>Ret</td>
<td>40</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>Inv</td>
<td>55,56</td>
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<tr>
<td>DNA polymerase</td>
<td>Inv</td>
<td>57,58</td>
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</table>
Table 1 (continued)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>tRNA nucleotidyl transferase</td>
<td>Inv</td>
<td>59</td>
</tr>
<tr>
<td>Adenylyl cyclase</td>
<td>Inv</td>
<td>60, 61</td>
</tr>
<tr>
<td>Polynucleotide phosphorylase</td>
<td>Inv</td>
<td>62</td>
</tr>
<tr>
<td>T₄ RNA ligase</td>
<td>Inv</td>
<td>63</td>
</tr>
<tr>
<td>ATP sulfurylase</td>
<td>Inv</td>
<td>64</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>Inv</td>
<td>65</td>
</tr>
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</table>

**ATP-dependent Synthetases**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Stereochemistry</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPP synthetase</td>
<td>Inv</td>
<td>66</td>
</tr>
<tr>
<td>AcetylCoA synthetase</td>
<td>Inv</td>
<td>67</td>
</tr>
<tr>
<td>Aminoacyl transferase</td>
<td>Inv</td>
<td>11</td>
</tr>
</tbody>
</table>
analysis of phosphorus configurations, have been developed in the past few years. Since enzymatic reactions of ATP can involve substitution at $P_\alpha$, $P_\beta$ and $P_\gamma$ as illustrated in I, sulfur containing analogs of ATP, ADP and AMP with chiral phosphorus center have been of particular interest.

Nucleotidyl transferases, -cyclases and some ATP-dependent synthetases catalyze the substitution at $P_\alpha$. Substitution at $P_\gamma$ is catalyzed by ATPases, phosphorylases and some other ATP-dependent synthetases. Substitution at $P_\beta$ is catalyzed by pyrophosphotransferases. $P_\alpha$ and $P_\beta$ are prochiral centers and can be made chiral by stereospecific replacement of the nonbridging-oxygen with sulfur, $^{18}O$ or $^{17}O$. $P_\gamma$ is pro-prochiral and can be made chiral by stereospecifically replacing two of the three equivalent oxygens with one sulfur and one heavy oxygen isotope ($^{18}O$ or $^{17}O$), or by chirally enriching with one $^{18}O$ and one $^{17}O$ to give chiral $[^{16}O,^{17}O,^{18}O]$phosphate.
1.2.1 Synthesis

A variety of thiophosphate analogs of ATP, ADP and AMP have been synthesized. Adenosine-5'-monophosphorothioate (AMPS) is prepared by phosphorylating adenosine with thiophosphoryl chloride and working-up in H$_2$O or H$_2^{18}$O (57,68) (equation 5). AMPS can be coupled to phosphate or pyrophosphate by the general procedure introduced by Michelson (69) (equation 6) to synthesize ADPaS and ATPaS (23). Similar

$$\text{HO-}$$

$$\text{O}$$

$$\text{A}$$

$$\text{HO-}$$

$$\text{OH}$$

$$\text{1. SPCI}_3$$

$$\text{2. H}_2\text{O}$$

$$\text{S}$$

$$\text{O-P-O-}$$

$$\text{O}$$

$$\text{HO-}$$

$$\text{OH}$$

(5)

$$\text{R = H, PO}_3^-$$

procedures are applied to the synthesis of UMPS, UDPaS, UTPaS (70) and UDPaS-Glc (40). The (R$_p$) and (S$_p$)
isomers of these α-thionucleotides all can be separated by chromatographic methods (26,45,55,71-73). Based on the stereospecificities of certain enzymes, procedures of combination of chemical and enzymatic processes have also been developed to successfully prepare pure isomers of ADPaS, ATPaS (70) and UDPaS-Glc (40) as illustrated in equations 7 to 10.

$$\begin{align*}
(R_p+S_p)-ADPaS + P-Arg & \xrightarrow{\text{Arginine kinase}} (R_p)-ATPaS + (S_p)-ADPaS \\
(R_p+S_p)-ADPaS + PEP & \xrightarrow{\text{Pyruvate kinase}} (R_p)-ADPaS + (S_p)-ATPaS \\
(R_p+S_p)-UTPaS + Glc-1-P & \xrightarrow{\text{UDPG pyrophosphorylase}} (S_p)-UDPaS-Glc + (S_p)-UTPaS + PPi \\
(R_p+S_p)-UDPaS-Glc + PPi & \xrightarrow{\text{UDPG pyrophosphorylase}} (R_p)-UDPaS-Glc + (R_p)-UTPaS + Glc-1-P
\end{align*}$$

($R_p$)-ATPγS, $\gamma^{18}O(\beta,\gamma)^{18}O$ (36) was synthesized by first condensing $(S_p)-ADPaS[\alpha^{18}O,\alpha,\beta^{18}O]$ with 2',3'-methoxymethylene-AMP by Michelson's procedure and then removing the blocking adenosyl and methoxymethylene group.
As shown in Figure 1, adenosine 5'- (2-thiodiphosphate), ADPβS, has been synthesized by first coupling AMP with S-carbamoylethyl phosphorothioate (74) or coupling AMPS with 2',3'-methoxymethylene-AMP (75), using Michelson's condensing technique. The blocking groups would then be removed under relatively mild conditions to give ADPβS. The pure diastereomers of ATPβS can be prepared by stereoselectively phosphorylating ADPβS in the presence of Mg^{++} (i) by PEP and pyruvate kinase to give (S_p)-ATPβS or (ii) by acetyl-P, arginine-P, 1,3-diphosphoglycerate or creatine-P in the presence of the respective phosphokinase to give (R_p)-ATPβS. When AMPS,^{18}O_2 is condensed with 2',3'-methoxymethylene-AMP by Michelson's procedure, the product, the diastereomeric mixture of the half-blocked dinucleoside [^{18}O]-pyrophosphorothioates, can be separated by liquid chromatography. The pure diastereomers can be chemically degraded individually to (R_p) and (S_p) [β-^{18}O]ADPβS (111). The configurations at the chiral phosphorus centers have been assigned by the 31P NMR analysis of [α-^{18}O]ADPαS, which were derived by alternative chemical degradation of the precursor, half-blocked dinucleoside [^{18}O]-pyrophosphorothioate, without altering the chiral phosphorus center. Chiral [^{18}O]-monophosphorothioate
FIGURE 1. Synthesis of adenosine-5'-[2-thiodiphosphate].
Figure 1.
monoesters such as \((R_p)-\) and \((S_p)-[{}^{18}\text{O}]\text{AMPS}\) (29,76), have also been synthesized in the late 1970's.

\((R_p)-\) and \((S_p)-3',5'-\text{cyclic AMPS}\) (77) have been synthesized from protected 3',5'-cyclic AMP which was converted to the diastereomeric mixture of phosphoro-anilidate. The pure diastereomers obtained after thin layer chromatographic separation were converted to the corresponding phosphorothioates individually (equation 11). The replacement of the anilidate group with sulfur proceeds with retention of configuration at the chiral phosphorus center (78). Synthesis of 2'-deoxy, 3',5'-cyclic nucleoside thiophosphate has also been accomplished (79,80).

The phosphorothioates, with very few exceptions, have been found to be acceptable substrates for the enzymes of interest. They generally react more slowly than the natural substrates. Substitution of sulfur for oxygen, although it introduces perturbation in the
reaction process, is not expected to cause major changes in the reaction mechanism. Independent stereochemical studies carried out on seven enzymes, 3',5'-cyclic AMP phosphodiesterase (28,82), adenylyl cyclase (60,61), glycerokinase (37,38), snake venom phosphodiesterase (26,51), hexokinase, pyruvate kinase (37,83) and polynucleotide kinase (45,52), with both [18O] nucleoside phosphorothioates and [16O,17O,18O]-labeled chiral nucleotides, gave consistent conclusions and confirmed the validity of thionucleotides in studying stereochemistry.

1.2.2 Configurational Analysis of Chiral Phosphorothioates

The assignments of absolute configurations of the chiral thiophosphate molecules are based ultimately upon correlations with the crystal structures of a few phosphorothioates whose structures have been determined. The method was introduced by Eckstein's laboratory in the study of pancreatic nuclease A (55). The first assignment of the absolute configuration to a chiral nucleotide thiophosphate, the endo-2',3' cyclic UMPS, which has the \( (R_p) \) configuration, was done by x-ray crystallographic analysis (81). One of the chemically synthesized Up(S)A diastereomers, which can be converted to endo-2',3'-cyclic UMPS by RNase with inversion of configuration,
was also a far better substrate for snake venom phosphodiesterase. This epimer of Up(S)A was assigned the (R_p) configuration. One ATPαS epimer was cleaved by snake venom phosphodiesterase much faster than its counterpart and was, therefore, also assigned the (R_p) configuration. This assignment has been confirmed independently by other methods (26,84).

Relative configurational assignments are made by analysis of the stereoselectivities of enzymes acting on the Mg^{2+}-thionucleotides complexes and also by \(^{31}\)P-NMR spectroscopy. The stereospecificities of enzymes for metal-thionucleotides are summarized in Table 2. Because the enzymes do not always exhibit absolute stereospecificity for one isomer or the other, \(^{31}\)P-NMR is the more positive way to assign the configurations of the nucleotides, provided at least 2 \(\mu\)moles of sample are available.

The diastereoisomers of many nucleotide phosphoro-thioates can be effectively distinguished by \(^{31}\)P-NMR spectrometry as demonstrated by Frey (89) and Cohn (92). The reported P_\(\alpha\) chemical shifts for \(\alpha\)-thionucleotide epimers differ by 0.2-0.5 ppm [(R_p) upfield]. The difference in P_\(\beta\) chemical shifts of the epimers of ATPβS was reported to be about 0.1 ppm [(S_p) upfield]. The
## TABLE 2

Stereoselectivities for Some Enzymes for α- and β-Thionucleotides

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;-thionucleotide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;β&lt;/sub&gt;S</td>
<td>8,70</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>(R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;β&lt;/sub&gt;S</td>
<td>85</td>
</tr>
<tr>
<td>Arginine kinase</td>
<td>(R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;β&lt;/sub&gt;S</td>
<td>70,86</td>
</tr>
<tr>
<td>Acetate kinase</td>
<td>(R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;β&lt;/sub&gt;S</td>
<td>70,87</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;β&lt;/sub&gt;S</td>
<td>11,88</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (ATP site) (S&lt;sub&gt;p&lt;/sub&gt;) ADP&lt;sub&gt;a&lt;/sub&gt;S (AMP site)</td>
<td>70 (89)</td>
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<tr>
<td>Snake venom phosphodiesterase</td>
<td>(R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S (R&lt;sub&gt;p&lt;/sub&gt;) Up(S)&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>26,55</td>
</tr>
<tr>
<td>AcetylCoA synthetase</td>
<td>(R&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>90</td>
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<td>Polynucleotide phosphorylase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ADP&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>62,91</td>
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<tr>
<td>tRNA nucleotidyl transferase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>59</td>
</tr>
<tr>
<td>E. coli RNA polymerase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) ATP&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>55,56</td>
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<tr>
<td>E. coli DNA polymerase I</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) dATP&lt;sub&gt;a&lt;/sub&gt;S</td>
<td>58</td>
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<tr>
<td>Reverse transcriptase</td>
<td>(S&lt;sub&gt;p&lt;/sub&gt;) dTTP&lt;sub&gt;a&lt;/sub&gt;S</td>
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Table 2 (continued)

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<th>Enzyme</th>
<th>Mg(^{2+})-thionucleotide</th>
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<tr>
<td>UDP-glC pyrophosphorylase</td>
<td>((R_p)) UTPaS</td>
<td>40,56</td>
</tr>
<tr>
<td></td>
<td>((S_p)) UDPaS-Glc</td>
<td>40</td>
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<tr>
<td>Gal-1-P uridylyl-transferase</td>
<td>((R_p)) UDPaS-Glc</td>
<td>40</td>
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<tr>
<td>3-phosphoglycerate kinase</td>
<td>((S_p)) ATPaS ((S_p)) ATP(_{\beta})S</td>
<td>10</td>
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31P-NMR chemical shifts of the thiophosphoryl-phosphorus in adenosine-5'-phosphorothioates are shown in Table 3.

For nucleoside [18O] phosphorothioates, the bridging and nonbridging 18O can also be distinguished by 31P-NMR spectroscopy (93). The phosphorus bonded directly to an 18O will exhibit about 0.020 ppm upfield shift per 18O atom for single bonded 18O and about 0.04 ppm per 18O for double bonded 18O. Since bridging 18O is single bonded and nonbridging 18O is at least partially double bonded, they are distinguishable.

1.3 Chiral Phosphates

1.3.1 Synthesis

Unfortunately not all enzyme systems will tolerate the sulfur substituent in a phosphate. It has been found that alkaline phosphatase and phosphoglycerate mutase do not accept phosphothioates as substrates. To overcome this obstacle, chiral [16O,17O,18O]phosphomonoesters and (R_p)- and (S_p)-3',5'-cyclic AMP 18O have been synthesized. Two procedures for the elegant synthesis of chiral [16O,17O,18O]phosphomonoesters have been developed independently by Knowles' research group (24,38,94) and by Lowe's research group (95,96). Knowles' procedure started by reacting P17OCl3 with
TABLE 3

$^{31}$P-NMR Chemical Shifts$^a$ of Thiophosphoryl-Phosphorus in Adenosine Phosphorothioates

<table>
<thead>
<tr>
<th></th>
<th>$\delta_\text{P}$ (ppm)</th>
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<tbody>
<tr>
<td>$(S_p)$ ATP$\alpha$S</td>
<td>42.97</td>
</tr>
<tr>
<td>$(R_p)$ ATP$\alpha$S</td>
<td>42.74</td>
</tr>
<tr>
<td>$(R_p)$ ATP$\beta$S</td>
<td>30.33</td>
</tr>
<tr>
<td>$(S_p)$ ATP$\beta$S</td>
<td>30.24</td>
</tr>
<tr>
<td>ATP$\gamma$S</td>
<td>33.08</td>
</tr>
<tr>
<td>$(R_p)$ ADP$\alpha$S</td>
<td>40.96</td>
</tr>
<tr>
<td>$(S_p)$ ADP$\alpha$S</td>
<td>40.68</td>
</tr>
<tr>
<td>ADP$\beta$S</td>
<td>33.05</td>
</tr>
<tr>
<td>AMP$\alpha$S</td>
<td>43.20</td>
</tr>
</tbody>
</table>

$^a$Values of chemical shifts are expressed relative to $H_3PO_4$ at pH 9.
(-)-ephedrine to give a separable mixture of 2-chloro-1,3,2-oxazaphospholidin-2-ones II (Figure 2a) and followed by treating the major isomer with two successive displacements to introduce RO- and H\(^{18}\)O- to the phosphoryl groups (III and IV). The procedure was adapted to the synthesis of chiral \([\gamma-^{16}\)O,\(^{17}\)O,\(^{18}\)O]ATP (43). In Lowe's procedure, \(^{18}\)O was introduced to first generate (1R,2S)-[1-\(^{18}\)O]-1,2-dihydroxydiphenylethane V (Figure 2b) which was treated with (i) P\(^{17}\)OCl\(_3\) and (ii) ROH to form the five-member cyclic triester. Hydrogenolysis of the \([^{16}\)O,\(^{17}\)O,\(^{18}\)O]-labeled species (III, IV and VI) gave corresponding chiral phosphates.

Synthesis of \((R_p)\)- and \((S_p)\)-3',5'-cyclic AMP \(^{18}\)O was accomplished by a procedure similar to that of the synthesis of 3',5'-cyclic AMPS (equation 11), substituting \([^{18}\)O] benzaldehyde (52) or C\(^{18}\)O\(_2\) (35) for C\(^{18}\)O\(_2\).

### 1.3.2 Configurational Analysis of Chiral Phosphates

Knowles and associates have devised the methods for determining the absolute configuration of a labeled phosphoric monoester. The key molecule in the analysis is \((S)-1,2\)-propanediol-(R\(_p\))-1-[\(^{16}\)O,\(^{17}\)O,\(^{18}\)O]phosphate, which can be obtained by \(E\). \textit{coli} alkaline phosphatase catalyzed transfer of the phosphoryl group from a chiral \([^{16}\)O,\(^{17}\)O,-\(^{18}\)O]phosphomonoester to \((S)-\text{propan-1,2-diol with overall}

FIGURE 2. Synthesis of chiral $[^{16}O,^{17}O,^{18}O]$phosphomoesters.
Figure 2.
configurational retention (24). The (S)-1,2-propanediol-(R_p)-1-[^16O,^17O,^18O]phosphate VII is cyclized chemically with random loss of one peripheral phosphorus oxygen (Figure 3). Alkylation of one of the epimeric oxygens of the cyclic chiral phosphates generates chromatographically separable syn- and anti- (to the methyl group) isomeric cyclic triesters. Four out of the six methylation products contain ^17O and therefore are silent in ^31P-NMR (41). The two species containing only ^16O and ^18O are shown in Figure 3. The syn-isomer VIII contains double bonded ^18O while the anti-isomer IX contains single bonded ^18O. In the ^31P-NMR spectrum the chemical shifts of syn- and anti-isomers are separated by about 0.1 ppm, while the isotope induced upfield chemical shifts for P=^18O and P-^18O- are about 0.04 ppm and 0.02 ppm, respectively. Therefore the position of ^18O can be identified and the configurational assignment at the phosphorus of chiral phosphomonoester VII can be made (97). The two ^18O-labeled species that are derived from the "in-line" ring closure and methylation of (R_p)- and (S_p)-(S)-1,2-propanediol-1-[^16O,^17O,^18O]phosphate are shown in equations 12 and 13. Although the ^17O enrichment is not 100%, the percentage of ^16O,^18O present in the
FIGURE 3. Analysis of chiral $[^{16}O,^{17}O,^{18}O]$phospho-
monoesters.
Figure 3.
(R_p)-phosphomonoester \rightarrow \text{syn} + \text{anti} \quad (12)

(S_p)-phosphomonoester \rightarrow \text{syn} + \text{anti} \quad (13)

$^{17}O$-source can be quantitatively corrected and the analysis procedure is secure. Configurational assignment to cyclic [$^{18}O$]phosphodiesters is made by the same principle (37,79).

1.4 Enzyme-Metal-Nucleotide Complexes

Most of the enzyme catalyzed phosphoryl group transfer reactions require metal ions as cofactors. Mg(II) is the most commonly present divalent ion; it forms numerous diastereomeric metal-nucleotide complexes that are in rapid equilibrium in aqueous solution. To study the metal-nucleotide complexes as enzyme substrates, several different approaches have been employed.

Cohn and Jaffe (8,11) demonstrated a reversal or relaxation of the stereospecificity of hexokinase toward the diastereomers of ATPδS and ATPαS with hard ions such as Mg(II) and soft ions such as Cd(II). Mg(II) prefers
oxygen ligands while Cd(II) prefers sulfur ligands. The reversal of hexokinase specificity for ($R_p$) ATP$_p$S to $S_p$ epimer upon replacing Mg(II) with Cd(II) suggested that the active metal-ATP complex was coordinated through the $\beta$-phosphorus and that Cd(II) coordinated with S while Mg(II) coordinated with O. This coordination switch would enable the AMP moieties of the two epimers to occupy the same binding locus in the active site. This is illustrated by structures X and XI. The other epimers with Mg(II) coordinated only to O at $P_\beta$ and Cd(II) coordinated to S would result in improper placement of the AMP moiety or distortion of the chelate ring in the active site.

![Diagram of structures X and XI](image)

The retention of stereospecificity for ATP$_p$S independent of which metal ion is involved, Mg(II) or Cd(II), suggested that either the $\alpha$-phosphorus is not coordinated with the metal ion or the specific binding conformation at the active site of hexokinase permits only oxygen to be coordinated with a metal ion. Similar studies have been carried out for pyruvate kinase (II), arginine kinase.
(98), creatine kinase (86), and phosphoglycerate kinase (10).

Cleland and coworkers (14,15) introduced the use of the exchange inert CrATP and Co(NH$_3$)$_4$ATP complexes for stereochemical analysis. The absolute configuration of (β,γ)-bidentate Co(NH$_4$)$_3$ATP complexes have been determined (99). Studies done on the substrate activity of (R$_p$) Co(NH$_3$)$_4$ATP with hexokinase strongly suggest that the absolute configuration of the active isomer of Mg(II)·ATPβS is also (R$_p$). This is known as the screw-sense isomer, structures X and XII. The Λ and Δ screw-sense isomeric configurations were defined by Cleland.

Both the metal-thionucleotide and exchange-inert Cr(III)· and Co(NH$_3$)$_4$·nucleotide complexes introduce chemical perturbation to the metal·nucleotide complexes which conceivably could lead to ambiguities in interpretation. The availability of regio-specific and stereospecific $^{17}$O-adenosine nucleotides (60,100,101) in combination with Mn(II) ESR spectroscopy provides a
method for studying the stereochemical configuration of enzyme-bound species with minimal perturbation by substituting Mn(II) for Mg(II). In many cases this substitution is relatively innocuous with respect to the velocity of enzymatic reaction. The superhyperfine coupling between the nuclear spin of $^{17}O$ ($I = 5/2$) and the unpaired electron spin of a paramagnetic ion such as Mn(II) can often be detected in the ESR spectrum for Mn(II) when the $^{17}O$ is directly ligated in Mn(II) (102-104). The quadrupolar effect of $^{17}O$ is detected as an inhomogeneous broadening and an associated lowering of the amplitude of the ESR signals. ESR spectra for solutions of creatine kinase·Mn(II)ADP·creatine·nitrate complex (104) with ADP, $(R_p) [\alpha-^{17}O]ADP$ and $(S_p) [\alpha-^{17}O]ADP$ gave the following results: the signals for the sample with $(S_p) [\alpha-^{17}O]ADP$ were inhomogeneously broadened whereas spectra for the corresponding samples with ADP and $(R_p) [\alpha-^{17}O]ADP$ were indistinguishable. These data showed that Mn(II) was preferentially coordinated to the pro-S oxygen at the $\alpha$-phosphorus of ADP and that the preferred stereochemical configuration of the $\alpha,\beta$ chelate ring has the $\Delta$ screw-sense. Earlier studies by Reed and Leyh (102) had demonstrated that ADP is an $\alpha,\beta$ bidentate ligand for Mn(II) in the complex.
Other approaches such as Mg(II)-introduced $^{17}$O-NMR signal broadening (105) and Mn(II)-introduced $^{31}$P-NMR relaxation (106,107) have also been employed.

1.5 Research Goal

Developments in the synthesis of thionucleotides have reached the point where sulfur can be introduced stereospecifically to replace any of the peripheral oxygens on the polyphosphate moieties of nucleotides with $^{18}$O or $^{17}$O or both placed at any other positions that might be desired. Yet progress in preparing heavy oxygen isotope labeled chiral phosphates has been mostly limited to the synthesis of $[^{16}$O,$^{17}$O,$^{18}$O]phosphomonoesters. The fact that not all enzymes tolerate thionucleotides makes it worthwhile to develop a general procedure to prepare chiral nucleotides such as $[^{\alpha-17}$O]- (or $[^{\alpha-18}$O]-)ADP, $[^{\beta-17}$O]- (or $[^{\beta-18}$O])-ATP, $[^{\alpha-16}$O,$^{17}$O,$^{18}$O]ADP and $[^{\beta-16}$O,$^{17}$O,$^{18}$O]ATP. In addition to stereochemical studies, these chiral nucleotides can also be very useful for studying the stereoconfiguration of enzyme-metal-nucleotide complexes at the active site, as demonstrated in the case of creatine kinase.

Chirally labeled $[^{\alpha-18}$O]dATP and $[^{\alpha-18}$O]dADP have been prepared by adenylyl cyclase-catalyzed pyrophosphorolysis of chiral $[^{18}$O]-3',5'-cyclic dAMP (60). This required the rather difficult synthesis of $(R_p)$- and $(S_p)$ $[^{18}$O]cyclic
dAMP and the purification of adenylyl cyclase. The purpose of this research was to develop a procedure by which the stereospecific replacement of the sulfur atom from a chiral thionucleotide with a heavy oxygen isotope could be accomplished.

The $^{31}$P[$^{18}$O]- and $^{31}$P[$^{17}$O]-NMR spectroscopy provides a nondestructive analysis technique. To employ this method one needs more than two micromoles of material and a high resolution multinuclei NMR spectrometer. For some purposes this can be an inconvenient amount of material. To get around this, a GC-mass spectral analysis procedure was developed. With the latter analysis technique one could get by with much less material and still obtain accurate data.
2.1 Chemicals, Coenzymes, and Enzymes

The following chemicals were purchased from Aldrich Chemical Company: acenaphthene, barium oxide, calcium hydride, citric acid, cobalt(II) nitrate, 2-cyanoethyl phosphate (barium salt), cyanogen bromide, N,N'-dicyclohexylcarbodiimide, diphenyl phosphorochloridate, formic acid, hexamethylphosphoramidite, diethylene glycol, lithium chloride, lithium hydroxide, 2,6-lutidine, 2-mercaptoethanol, molecular sieves (4A), N-[N'-methyl-N''-nitroso(aminooethyl)]benzamide, phosphorus oxychloride, sodium periodate, tetrabutylammonium hydroxide, thiophosphoryl chloride, tributylamine, triethyl phosphate, trimethyl orthoformate, trimethyl phosphate, tri-n-octylamine, uridine and uridine-5-monophosphate.

HEPES, TRIZMA hydrochloride, PIPES, dithioerythritol, dithiothreitol, glucose, glucose-6-phosphate, sodium pyruvate, adenylate kinase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, hexokinase, lactate-dehydrogenase, pyruvate kinase, acetyl phosphate,
adenosine 5'-diphosphate, adenosine 5'-monophosphate, adenosine 5'-triphosphates, NADH, NADP, phosphoenolpyruvate, L-cysteine, alumina Type WB-5 DEAE-Sephadex A-25 ion exchanger, SP-Sephadex C-25, ion exchanger, lactic acid, 5,5'-dithio bis-(2-nitrobenzoic acid), and ethylene-diaminetetraacetic acid were purchased from Sigma Chemical Company.

Acetate kinase and adenosine were purchased from Boehringer Mannheim.

HPLC grade reagents: monobasic potassium phosphate, methanol, acetonitrile, methylene chloride and n-heptane, and reagent grade chemicals: triethylamine, benzene, barium oxide, ammonium molybdate, DMF, ferric chloride, potassium citrate, phosphorus pentoxide and 85% phosphoric acid were purchased from Fisher Chemical Company. Dowex AG-50 and deuterium oxide (88.7%) were purchased from Bio-Rad Laboratories. \[^{18}\text{O}]\text{H}_2\text{O}\) was purchased from Monsanto Research Corporation, Mound Laboratory.

Hydrochloric acid, nitric acid, sulfuric acid and ammonium hydroxide were obtained from Hi-Pure Chemical, Inc. Ligroine and triethylphosphate were from Eastman Kodak Company. Dioxane, MgCl\(_2\), MnCl\(_2\), and potassium phosphate (monobasic and dibasic) were from MC/B Manufacturing Chemists.
Most chemicals and enzymes were used as received, with the exception of solvents, which were dried and repurified as described below, and cyanogen bromide, which was stored desiccated in the dark.

2.2 Purification of Solvents

Triethyl phosphate was mixed with barium oxide and allowed to stand for 24 hours before distilling in vacuo. Redistilled triethyl phosphate was stored over molecular sieves (4A) in the dark. Thiophosphoryl chloride and phosphorus oxychloride were redistilled and desiccated under N₂. Pyridine was mixed with calcium hydride and allowed to stand overnight, redistilled and stored over potassium hydroxide in the dark. N,N-dimethylformamide was dried by mixing with powdered barium oxide and allowing the mixture to stand overnight. It was then distilled from alumina. Redistilled DMF was stored over molecular sieves (4A) in the dark.

Tri-n-butylamine was stirred with calcium hydride for 12 hours and then distilled in vacuo. Redistilled tri-n-butylamine was desiccated under N₂. Tri-n-ethylamine was redistilled before use. Hexamethylphosphoramide was distilled in vacuo prior to being used. Ethyl ether, if not from a freshly opened can, was passed through an alumina (Basic Type WB-5) column
just prior to being used. Dioxane was stored overnight with molecular sieve 4A and distilled. Redistilled dioxane stored over BaO in the dark was percolated through an alumina column prior to use. Small aliquots of alumina treated dioxane were tested for the presence of peroxides. They were mixed with equal volumes of water and a few crystals of KI were added. A yellow color from oxidation of $I^-$ to $I_2$ signaled the presence of peroxide. The alumina treatment was repeated until a colorless clear solution was obtained in the KI-test.

2.3 Ion Exchange Columns

DEAE-Sephadex A-25 ion exchanger was prepared by permitting the exchanger to swell in 1.0 M TEAB for several hours. After being packed in a column having the desired dimensions, the column of ion exchanger was washed first with four volumes of 1.0 M KHCO$_3$ and then with four volumes of 0.1 M TEAB buffer at pH 7.8. Regeneration of the column was done by a similar procedure.

SP-Sephadex C-25 ion exchanger was prepared by permitting the resin to swell in 1.0 M aqueous salt solution. Depending on the cation desired to be exchanged, the following salts were used: pyridinium chloride for pyridinium ion, sodium chloride for sodium
ion, potassium chloride for potassium ion, and lithium chloride for lithium ion. The swelled exchanger was poured as a slurry into a column having the desired dimension. The column was washed first with four volumes of the same 1.0 M salt solution and then with five volumes of water. The final water effluent was tested with AgNO₃ to verify the absence of chloride ions. Regeneration of the column material was by a similar procedure.

Chelex 100 was prepared by swelling up in water for 24 hours and stored at 4°C.

Dowex 50W-X2 cation exchanger was prepared by permitting it to swell in water for a few hours and then packing a column having the desired dimensions. The packed column was washed with four volumes of base and acid in the following order: 1.0 N NaOH, water, 1.0 N HCl, water. The column was ready for exchanging protons at this stage. If another cation was required, four volumes of a one molar aqueous solution of the chloride salt of the desired cation were passed through the column and water was then passed through until the effluent was salt free (negative of silver nitrate test).
2.4 Chromatographic Methods

Thin layer chromatography was performed using Eastman silica gel plates containing a fluorescent indicator with solvent system A (water/conc. ammonium hydroxide/n-propanol, 1:3:6) and on Baker cellulose poly(ethylenimine) plates with system B (0.75 M potassium phosphate, pH 3.5) or system C (1.0 M lithium chloride). Nucleotides were visualized under a UV lamp. Nucleotide phosphorothioate could be distinguished by the absorbance of UV light and the temporary staining with iodine vapor. Inorganic phosphate and thiophosphate were visualized by spraying with the Hanes-Isherwood reagent (108).

Nucleotides synthesized in this research were routinely purified by ion exchange column chromatography through DEAE-Sephadex A-25, using TEAB in the elution gradients. TEAB was prepared by bubbling CO$_2$ gas generated from dry ice through a glass filter and into a 1.0 M aqueous solution of triethylamine with stirring until the pH of the solution reached 7.6. The stock solution was stored at room temperature in a tightly capped bottle. The pH of the solution was checked prior to use, and when necessary CO$_2$ gas was bubbled into the stock solution to bring the pH down to 7.6.
Sample nucleotides in solution at pH 7.6 with ionic strength at 0.1 M or less were absorbed to columns of DEAE-Sephadex A-25 that had been equilibrated with 0.1 M TEAB at pH 7.6 (see above). Nucleotides and thio-nucleotides were eluted from the columns using linear gradients of TEAB at pH 7.8. The gradients were prepared by adding equal volumes of two different concentrations of TEAB buffer to two identical flasks connected by a bridge filled with the buffer of lower concentration. The latter buffer was stirred continuously while the gradient was drawn from this flask to feed the column. Fractions were collected, and those containing nucleotides were identified by measuring their absorbance at 260 nm. Peak fractions were pooled and TEAB buffer was removed by flash evaporation using a rotary evaporator connected to a vacuum pump that was protected with a dry ice-acetone cooled trap. The bath temperature was kept below 30°C at all times. When TEAB was being removed, the initial residue was twice taken up in 95% ethanol and evaporated to insure that all the buffer salts were removed. The final residue was dissolved in a minimum amount of water and stored at -15°C after the pH of the solution had been adjusted to 10 with triethylamine.
In preparation for further use in synthesis, the triethylammonium salts of nucleotides and nucleoside phosphorothioates were converted to tri-n-octylammonium salts to render them soluble in organic solvents. The triethylammonium salt of a nucleotide was passed through a column of Dowex-50 (pyridinium form) which had at least a 20-fold excess of exchange capacity. The flow-through contained the pyridinium salt of the nucleotide, which was concentrated to dryness by rotary evaporation in vacuo. The residue was dissolved in a minimal volume of methanol and tri-n-octylamine was added to the solution (one equivalent for all nucleoside monophosphates or monophosphorothioates, two equivalents for nucleoside diphosphates or thiodiphosphates). The mixture was stirred until it clarified and methanol was removed in vacuo. The residue was desiccated with P$_2$O$_5$ under vacuum for 20 hours to insure absolute dryness.

High performance liquid chromatography (HPLC) was carried out using a Waters Associates liquid chromatograph equipped with ultraviolet absorbance and refractive index detectors and a Sargent-Welch dual pen recorder. Waters Associates reverse phase C$_{18}$-Bondapak columns were used to separate nucleotides, with potassium phosphate buffers as the mobile phase. Water and all
aqueous solutions used for HPLC were cleaned up by filtering through a Waters type HA 0.45 μm Millipore filter each day. All the organic solvents used for HPLC were filtered through a Waters FH Millipore filter and stored.

Phosphate buffer for HPLC was prepared as described. A 1.0 M stock solution of potassium phosphate, pH 6.3, was filtered through a Waters FH Millipore filter and stored at room temperature. Eluting buffer was prepared by diluting the stock solution to the desired concentration with filtered water and degassing prior to use. When a methanol-water solvent was needed, the calculated amount of filtered HPLC grade methanol was added to the desired volume of stock solution and filtered water was then added to adjust the total volume. All solvents used for HPLC were degassed.

2.5 Spectrophotometric and Colorimetric Analysis

Ultraviolet absorbance measurements were made by using either a Cary 118C spectrophotometer or a Unicam SP 1800 spectrophotometer equipped with a Unicam AR-25 linear recorder. The concentrations of adenosine nucleotides and nucleotide phosphorothioates were calculated using measured $A_{259}$ values and the extinction coefficient $\epsilon = 1.5 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.
and NADPH were similarly calculated from $A_{340}$ and the extinction coefficient $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Thiocyanate was detected by reaction with Fe(III) under acidic conditions to produce the red ferric monothiocyanate ion which has a maximal absorbance at 455 nm (equation 14). The test solution contained

$$\text{Fe}^{+3} + \text{SCN}^- + H_2O^+ \rightarrow [\text{Fe(SCN)}]^+^2 \quad \text{(red)}$$

The test solution contained 0.1 ml 5% ferric chloride in 0.1 N HCl and 10 µl of unknown sample diluted to a total volume of 1 ml. Excess Fe(III) is necessary to avoid the formation of Fe(SCN)$_3$.

Inorganic phosphate was determined qualitatively by adding an aliquot of sample solution to 0.7 ml of testing reagent with the final volume adjusted to 1 ml. The reagent was prepared by mixing one part of freshly prepared 10% ascorbic acid solution with six parts of 0.389% ammonium molybdate·4H$_2$O in 1 N H$_2$SO$_4$ solution. The testing solution was kept at 45°C for 20 minutes and optical densities at 660 nm were checked. Solutions containing inorganic phosphate gave a blue color.
Nucleotide phosphorothioates with the thiophosphoryl group at the terminal position were determined by mixing an aliquot of the solution to be tested with 0.1 ml of 11.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) dissolved in 0.1 M Tris-HCl at pH 8.0, the final volume being adjusted to 1 ml by addition of H₂O. Absorbance at 412 nm was measured and the extinction coefficient \( \varepsilon = 11.2 \times 10^3 \text{ M}^{-1}\text{cm}^{-1} \) was used to calculate the concentration of thiophosphoryl groups.

2.6 Enzymatic Assays

The concentration of AMP was determined enzymatically by measuring the molar equivalent of NADH consumed in its phosphorylation to ATP by a coupled assay system containing adenylate kinase, pyruvate kinase and lactate dehydrogenase. See equations 15 to 18 for the stoichiometry. The assay mixture contained the following: 40 mM Tris-HCl at pH 8.0, 1 mM DTT, 100 mM KCl, 10 mM MgCl₂, 0.1 mM NADH, 1 mM PEP, 0.1 mM ATP and excess lactate dehydrogenase, pyruvate kinase and adenylate kinase. After AMP was added, the decrease in \( A_{340} \) was recorded and the AMP concentration was calculated with an extinction coefficient of 2.0 x 6200.
AMP$^{2-}$ + ATP$^{4-}$ $\xrightarrow{\text{adenylate kinase}}$ 2 ADP$^{3-}$ (15)

2H$^+$ + 2 ADP$^{3-}$ + 2 PEP$^{3-}$ $\xrightarrow{\text{pyruvate kinase}}$ 2 ATP$^{4-}$ + 2 CH$_3$COCO$_2^-$ (16)

2 CH$_3$COCO$_2^-$ + 2 NADH + 2 H$^+$$\xrightarrow{\text{lactate dehydrogenase}}$ 2 CH$_3$CHOHCO$_2^-$ + 2 NAD$^+$ (17)

Sum: AMP$^{2-}$ + 2 PEP$^{3-}$ + 2 NADH + 4 H$^+$$\xrightarrow{\text{pyruvate kinase}}$ ATP$^{4-}$ + 2 CH$_3$CHOHCO$_2^-$ + 2 NAD$^+$ (18)

The concentration of ADP was assayed by determining the molar equivalent of NADH oxidized in the pyruvate kinase, lactate dehydrogenase coupled assay system (equations 16 and 17). The system contained 40 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM PEP, 2 mM MgCl$_2$, 0.5 mM DTT, 0.1 mM NADH and an excess of pyruvate kinase and lactate dehydrogenase. After the addition of ADP the decrease of absorbance at 340 nm was recorded and the concentration of added ADP calculated using the extinction coefficient $\varepsilon = 6.2 \times 10^3$ M$^{-1}$·cm$^{-1}$ for NADH.

The concentration of glucose-6-phosphate was measured by determining the NADPH generated upon glucose-6-phosphate dehydrogenase catalyzed oxidation of
glucose-6-phosphate to 6-phosphogluconolactone according to equation 19. Glucose-6-phosphate was added to an assay mixture containing 25 mM Tris-HCl, pH 8.0, 0.1 mM 

\[
glucose-6-P + NADP^+ \rightarrow 6\text{-phosphogluconolactone} + NADPH + H^+ \tag{19}
\]

NADP\(^+\) and an excess of glucose-6-phosphate dehydrogenase. The amount of glucose-6-phosphate was calculated from the increase of the absorbance at 340 nm due to the NADPH formed, using the extinction coefficient 6.2 \(\times\) 10\(^3\) M\(^{-1}\) cm\(^{-1}\) for NADPH.

To determine the concentration of ATP in a solution, an aliquot was added to an assay mixture which contained the following ingredients: 25 mM Tris-HCl, pH 8.0, 5 mM MgCl\(_2\), 5 mM glucose, 0.1 mM NADP\(^+\), and an excess of hexokinase and glucose-6-phosphate dehydrogenase. In this assay hexokinase catalyzed the phosphorylation of glucose to glucose-6-P by the ATP in the sample. Glucose-6-P dehydrogenase then catalyzed the oxidation of glucose-6-P by NADP\(^+\) according to equation 19; and the NADPH produced, which was equivalent to the ATP in the sample, was calculated from the increase in \(A_{340}\) and the extinction coefficient for NADPH at 340 nm (6.2 \(\times\) 10\(^3\) M\(^{-1}\) cm\(^{-1}\)).
Gas Chromatographic and Mass Spectroscopic Analysis

Oxygen-18 enriched phosphate and thiophosphate were converted to the trimethyl esters in preparation for gas chromatographic-mass spectroscopic analysis. Diazomethane (CH$_2$N$_2$) used to methylate thiophosphate was prepared as described by Sekiya and colleagues (109). N-[N'-methyl-N'-nitroso(aminomethyl)]benzamide (2 g) was added to a suspension consisting of 1.7 g of crushed KOH in 5 ml of diethylene glycol and 10 ml of diethyl ether in a flask. The reaction mixture was stirred at ambient temperature for one hour. Diazomethane appeared in the ether layer, giving it a bright yellow color. The flask was connected by a 1/16 in ID Tygon tube to a second container which was cooled in an ice-water bath. The CH$_2$N$_2$-ether solution was distilled into the second flask by gently heating the reaction flask to 40°C while stirring the mixture.

Phosphate or thiophosphate samples were dried by rotary evaporation and taken up in 0.5 to 1 ml methanol. The solution was acidified with HCl and the pH verified by adding a very small aliquot of the sample to a piece of damp pH paper. Immediately after the pH had dropped to <2, diazomethane-ether was added to the solution in successive 0.5 ml aliquots until the solution remained
permanently yellow. The solution was kept at ambient temperature for 20 minutes before the ether-methanol solvent was carefully blown away using a gentle stream of nitrogen gas. To avoid large losses of permethylated phosphates, it is important not to remove the last trace of solvent.

Triethyl phosphate and triethyl phosphorothioate were prepared by alkylating the phosphate samples with diazoethane in a similar procedure. Diazoethane was obtained by addition of N-ethyl, N-nitrosourea (0.5 g) to a small flask containing 5 ml 40% KOH and 20 ml of ethyl ether. The suspension was stirred with a Teflon stir bar at ambient temperature until all the solid material disappeared and the diazoethane appeared in the ether layer as a strong orangeyellow color. C₂H₅N₂-ether was distilled as described above for diazomethane.

All operations involving the preparation of diazalkanes and the permethylation of phosphates were carried out in a fume hood. Rubber gloves, safety glasses and protective clothing were worn as further protection against these toxic gases.

The peralkylated phosphate and thiophosphate samples were subjected to gas chromatographic-mass spectroscopic analysis using a Finnigan 4021 GC-mass spectrometer.
system. A 10% SE-30 glass column was used for the gas chromatography.

2.8 Enzymatic Degradation of ATP

The $P_{\gamma}$, $P_{\beta}$, and $P_{\alpha}$ phosphoryl groups of ATP were isolated as phosphate for analysis of $^{18}$O content by the systematic enzymatic degradation outlined in Figure 4. $[^{18}O]$ ATP from the desulfurization of ATP$\beta$S with cyanogen bromide in $H_2^{18}$O (phosphate buffer) was first converted to ADP and glucose-6-phosphate. The 10 ml reaction mixture contained 25 mM Tris-HCl buffer at pH 8.0, 1 mM $^{18}$O-ATP, 10 mM glucose and 5 mM MgCl$_2$ as well as hexokinase (5 units). After incubation at ambient temperature for one hour, the amount of glucose-6-phosphate generated was determined by withdrawing an aliquot of the reaction mixture and assaying enzymatically for glucose-6-phosphate (section 2.6). After verifying that the reaction was complete, the products were purified by DEAE-Sephadex A-25 chromatography through a 0.7 x 18 cm column using a 0.25 liter TEAB linear gradient increasing from 0.05 M to 0.3 M. Fractions containing ADP were detected by their absorbance at 260 nm. Those containing glucose-6-phosphate were detected by enzymatic analysis (section 2.6). The glucose-6-phosphate obtained here contained the $\gamma$-phosphoryl group of ATP.
FIGURE 4. Degradation scheme for the isolation of $P_\alpha$, $P_\beta$, and $P_\gamma$ of ATP as phosphate.
Figure 4.
The ADP isolated above was desalted as described in section 2.4 and its β-phosphoryl group transferred to glucose by the coupled actions of adenylate kinase and hexokinase, in which the β-phosphoryl group of ADP was first transferred to a second ADP, forming ATP which then donated this group to glucose to produce glucose-6-phosphate. The reaction mixture contained the following components: 25 mM Tris-HCl buffer at pH 8.0, 10 mM MgCl₂, 2 mM ADP, 20 mM glucose, 1 mM DTT, adenylate kinase (5 units), and hexokinase (5 units) in a total volume of 5 ml. The glucose-6-phosphate dehydrogenase assay was again used to follow the formation of glucose-6-phosphate. The phosphoryl group transferred to glucose was exclusively from the β-phosphoryl group of ¹⁸O-ATP.

The reaction products were purified by chromatography through a DEAE-Sephadex A-25 column (0.7 x 18 cm) using a 0.2 liter linear TEAB gradient, increasing in concentration from 0.08 M to 0.2 M at pH 8.0. AMP and glucose-6-phosphate-containing fractions were identified by their A₂₆₀ values and enzymatic analysis for glucose-6-phosphate. Fractions containing AMP and glucose-6-phosphate from this column (representing Pα and Pβ of ATP) and those containing glucose-6-phosphate (representing Pγ) from the preceding column were separately
pooled and desalted by the procedure described in section 2.4 for desalting nucleotides.

The two glucose-6-phosphate solutions and the AMP solution were all subjected to alkaline phosphatase digestion to generate inorganic phosphate. The reaction mixtures contained the following components: 0.1 M Tris-HCl buffer at pH 8.0, 0.1 M NaCl, 10 mM glucose-6-phosphate (or AMP) and alkaline phosphatase (5 units) in a total volume of 1.0 ml. The solution was incubated at 37°C for 2 hours and applied onto a 0.7 x 18 cm DEAE-Sephadex A-25 column to purify the inorganic phosphate. A linear gradient of TEAB buffer at pH 8.0 increasing in concentration from 0.05 to 0.14 M and having a total volume of 0.15 liter was passed through the column to elute the products. Phosphate-containing fractions were detected by the colorimetric test (section 2.5), pooled, and desalted as described for nucleotides (section 2.4). The purified phosphate samples were subjected to $^{31}$P-NMR analysis to determine their $^{18}$O-enrichment.

2.9 Enzymatic Synthesis of Nucleoside [${}^{18}$O]Phosphorothioates

Adenylate kinase and [γ-${}^{18}$O]ATPγS (supplied by Sheu and Richard) was used to thiophosphorylate AMP, producing [β-${}^{18}$O]ADPβS and ADP (36). The reaction
mixture contained the following: 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 2 mM AMP, 1 mM dithioerythritol, 0.2 mM [γ⁻¹⁸O]ATPγS and adenylate kinase (4.5 units·ml⁻¹) and was incubated at ambient temperature for 100 minutes. Sixty A₂₆₀ units of [γ⁻¹⁸O]ATPγS were used and the [β⁻¹⁸O]ADPβS was purified by chromatography through a DEAE-Sephadex A-25 column (1.0 x 20 cm). Elution was performed with a one liter linear gradient of TEAB, increasing from 0.1 M to 0.4 M. Fractions containing [β⁻¹⁸O]ADPβS were pooled, desalted (section 2.4) and stored at pH 10, -15°C for further use. The yield of [β⁻¹⁸O]ADPβS was 89.5%.

(Rp) [¹⁸O]ATPβS was prepared by phosphorylation of [β⁻¹⁸O]ADPβS with acetyl phosphate catalyzed by acetate kinase (70). The reaction mixture contained: 75 mM Tris-HCl, pH 7.8, 7.5 ml MgCl₂, 0.6 mM dithiothreitol, 30 mM acetyl phosphate, 3 mM [β⁻¹⁸O]ADPβS and acetate kinase (120 units·ml⁻¹) and was incubated at 28°C for 8 hours. Eighteen A₂₆₀ units of [β⁻¹⁸O]ADPβS obtained from above were used and [¹⁸O]ATPβS was purified by DEAE-Sephadex A-25 column (0.7 x 15 cm) chromatography. A 0.5 liter linear gradient of TEAB, increasing from 0.25 M to 0.45 M, was used to elute the products. Fractions containing [¹⁸O]ATPβS were pooled, desalted and stored at pH 10, -15°C. The yield was 70%.
(S_p) [\textsuperscript{18}O]ATP\textsubscript{8}S was prepared by phosphorylating [\textbeta-\textsuperscript{18}O]ADP\textsubscript{8}S with pyruvate kinase and PEP, using the procedure described in section 5.3. [\textbeta-\textsuperscript{18}O]ADP\textsubscript{8}S was obtained from the adenylate kinase reaction described earlier in this section.

2.10 \textsuperscript{31}P-NMR Analysis

Samples of 2 to 2.5 ml for NMR analysis contained the following: 1-5 mM nucleotide, 30-50 mM EDTA and 40% D\textsubscript{2}O with pH adjusted to 9-10. The spectrometer was field frequency locked to the deuterium resonance of D\textsubscript{2}O in the solvent. The temperature of the samples was kept at 25°C. Shifts upfield from an 85% orthophosphoric acid standard were assigned negative values. Samples were contained in 10 mm Wilmad precision NMR tubes. During the course of this research, three different NMR spectrometers have been used: a Bruker 200 mHz, a Nicolet 200 mHz, and a Bruker 360 mHz.
CHAPTER 3
REACTIONS OF NUCLEOSIDE PHOSPHOROTHIOATES
WITH CYANOGEN BROMIDE

3.1 Chiral Phosphorothioates as Precursors of Chiral Phosphates

The development of chemical syntheses of nucleoside-phosphorothioates and nucleoside [18O] phosphorothioates by several research groups in the 1970's (36, 70, 74, 75, 80) greatly accelerated the investigations of the stereochemistry of enzyme catalyzed phosphoryl group and nucleotidyl group transfer reactions.

The phosphorothioate analogs of nucleotides and other biological phosphates are usually acceptable substrates for enzymes. There is little reason to expect the sulfur substituent to alter the stereochemical course of a single displacement at phosphorus. In a few cases in which the stereochemical courses of the reactions have been studied with both chiral thiophosphate and chiral phosphate substrates, the results have been the same (Chapter 1.2). However, not all phosphoryl group-transferring enzymes will accept the sulfur-containing nucleotides as substrates. Alkaline phosphatase from
E. coli is one example. One of the alternatives is to synthesize heavy oxygen isotope-labeled chiral phosphates. Chiral $[^{16}O,^{17}O,^{18}O]$phosphate monoesters can be synthesized by two general procedures devised separately in Knowles' laboratory (94) and in Lowe's laboratory (96). Compounds such as 5'-[γ-(S)-$^{16}O,^{17}O,^{18}O$]ATP (38,43), 2-[(R)-$^{16}O,^{17}O,^{18}O$]phospho-D-glycerate (38,44), α-D-glucose 1-[(S)-$^{16}O,^{17}O,^{18}O$]phosphate (110) have been prepared and utilized in the study of several phosphokinase systems in the two laboratories.

The synthesis of phosphoanhydrides stereospecifically labeled with heavy oxygen isotope cannot be accomplished by similar procedures. Gerlt and Coderre enzymatically synthesized (S$_p$) [α-$^{18}O$]dADP with $^{18}O$-oxygen at a nonbridging position from cyclic $[^{18}O]$dAMP (79). Yet the procedure cannot be used for [β-$^{18}O$]ATP synthesis; and adenylate cyclase required to convert (R$_p$) or (S$_p$) 3',5'-cyclic $[^{18}O]$AMP to chiral α-$^{18}O$ nucleotides is not readily available. Moreover the synthesis of chiral $[^{18}O]$cdAMP is difficult.

The availability of chirally substituted phosphorothioates makes them attractive and logical precursor molecules for the synthesis of chirally substituted phosphates if stereospecific displacement of sulfur
from the chiral centers with \(^{18}\text{O}\) or \(^{17}\text{O}\) could be achieved. Pure epimers of \([\alpha-{^{18}\text{O}}]\text{ADP}\alpha\text{S}, [\alpha-{^{18}\text{O}}]\text{ATP}\alpha\text{S}, [\beta-{^{18}\text{O}}]-\text{ADP}\beta\text{S}, [\beta-{^{18}\text{O}}]\text{ATP}\beta\text{S}\) and \(\text{AMPS}^{18}\text{O}\) all can be synthesized in good yields (111,112), so that a reagent or reagents that could promote the stereospecific displacement of sulfur by \(^{17}\text{O}\) from \(\text{H}_2^{17}\text{O}\) would make the synthesis of \([\alpha-{^{17}\text{O}},{^{18}\text{O}}]\text{ADP}, [\alpha-{^{17}\text{O}},{^{18}\text{O}}]\text{ATP}, [\beta-{^{17}\text{O}},{^{18}\text{O}}]\text{ATP}\) and \([\alpha-{^{16}\text{O}},{^{17}\text{O}},{^{18}\text{O}}]\text{AMP}\) by one general procedure possible.

In this work cyanogen bromide was chosen to activate the sulfur atom in the \(\text{ATP}\beta\text{S}\) molecule in the expectation that the resulting thiocyanate group would be displaced stereospecifically by a water molecule or a hydroxyl ion. Cyanogen bromide is an electrophilic compound which is known to react preferentially with sulfur, e.g., in the thioether group of methionine. It is therefore reasonable to expect the strongly electron-withdrawing cyanide group in \(\text{BrCN}\) to react with the sulfur atom of a nucleoside phosphorothioate XIII and increase its electrophilic reactivity at the chiral phosphorus center, thereby promoting nucleophilic attack and displacement of \(\text{SCN}^-\) by water or hydroxyl ion. The \(pK_a\) value of thiocyanic acid is estimated to be between 0.85 and -1.8, making the thiocyanate group the most stable leaving group available at the reaction
center. The pK\textsubscript{a}'s of the other two leaving groups in this case would be near 7 for O\textsubscript{3}PO-Ado and near 12 for inorganic phosphate.

![Chemical Structure]

3.2 Reaction of ATP\&S with Cyanogen Bromide

The reaction sequence originally envisaged for the synthesis of e.g., (S\textsubscript{p}) [\beta\textsuperscript{-18}O]ATP is outlined in Figure 5. (R\textsubscript{p}) [\beta\textsuperscript{-18}O]ADP\&S would be synthesized by the procedure of Richard and Frey (111) and enzymatically phosphorylated stereoselectively to (S\textsubscript{p}) [\beta\textsuperscript{-18}O]ATP\&S (30,70). This would then be permitted to react with BrCN in H\textsubscript{2}O (or H\textsubscript{2}\textsuperscript{17}O) to produce, presumably with inversion of configuration, (S\textsubscript{p}) [\beta\textsuperscript{-18}O]ATP (or (S\textsubscript{p}) [\beta\textsuperscript{-17}O,\textsuperscript{18}O]ATP).

In preliminary experiments, ATP\&S was converted to ATP in 60% yield upon reaction in water with a 10-fold excess of BrCN, verifying the potential value
FIGURE 5. Hypothetical conversion of $\left(R_p\right) [\beta^{18}\text{O}]$-ADP$\beta$S to $\left(S_p\right) [\beta^{18}\text{O}]$ATP

Shown is the route originally envisaged but not realized because of a complicating side reaction involving the formation of a cyclo-diphosphate as an unexpected intermediate. The actual course of the reaction of ATP$\beta$S with BrCN is outlined in Figure 12.
Figure 5.
of BrCN as a reagent for promoting desulfurization.

The reaction mixture consisted of the triethylammonium salt of ATPβS (10 μmoles) dissolved in 1 ml of H₂O and combined with 100 μmoles of BrCN. The reaction mixture was kept at ambient temperature for 4 hours. A blank solution consisted of BrCN and water and a control reaction mixture contained 10 μmoles of the sodium salt of ATP and 100 μmoles of BrCN. All solutions were maintained at ambient temperature for four hours. The pH's of the solutions were checked hourly and 10 μl aliquots of the ATPβS and ATP-containing solutions were withdrawn to test for the thiocyanate ion using the FeCl₃ test (Chapter 2). The results in Table 4 were obtained, showing declining pH's in all solutions due to the hydrolysis of BrCN but the appearance of thiocyanate only in the reaction mixture containing ATPβS and BrCN.

One microliter aliquots of the reaction mixture were withdrawn at 0.5 and 2.5 hours and analyzed by thin layer chromatography on a silica gel coated plate (Chapter 2). Two major UV absorbing spots were detected comigrating with standard ATP and ADP. A third spot was stained by I₂, indicating the presence of sulfur. Similar samples of the control solution containing ATP
TABLE 4

Thiocyanate Formation in the Reaction of ATPβS with BrCN

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Reaction solution (ATPβS + BrCN)</th>
<th>Control solution (ATP + BrCN)</th>
<th>Blank (BrCN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>$A_{455}$</td>
<td>pH</td>
</tr>
<tr>
<td>0.5</td>
<td>5.5</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>0.09</td>
<td>-</td>
</tr>
<tr>
<td>1.5</td>
<td>4.6</td>
<td>-</td>
<td>5.2</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>0.17</td>
<td>-</td>
</tr>
<tr>
<td>2.5</td>
<td>4.6</td>
<td>-</td>
<td>5.1</td>
</tr>
</tbody>
</table>
and cyanogen bromide showed only one UV absorbing spot which comigrated with standard ATP.

The nucleotides were purified from the reaction mixture initially containing ATPβS and BrCN, with the results presented in Figure 6. Two major UV absorbing peaks were eluted from the column. The first compound obtained in 31% yield was identified as ADP by thin layer chromatography and by its activity as a substrate for pyruvate kinase. The second compound obtained in 60% yield was identified as ATP (i) by thin layer chromatography, (ii) as a substrate for hexokinase, and (iii) by its $^{31}$P-NMR spectrum (proton decoupled), which consisted of a doublet for $P_\gamma$ at 5.5 ppm upfield from $H_3PO_4$, $J_{\beta,\gamma} = 19.86$ Hz, a doublet for $P_\alpha$ at 10.8 ppm, $J_{\alpha,\beta} = 19.49$ Hz, and a triplet for $P_\beta$ at 21.4 ppm, $J = 19.85$.

The above experiment showed that BrCN might be a suitable reagent for removing sulfur from a thio-nucleotide and producing the corresponding nucleotide in good yield. However, efforts to repeat this experiment met with limited success. The reaction products, though still consisting of some ATP, were often largely ADPβS and ADP. Based on experience with ATPβS under other circumstances, uncontrolled pH changes were thought to be a factor contributing to the low yield of
FIGURE 6. Chromatography of products from the reaction of ATPßS with BrCN.

After 4 hours the reaction mixture consisting of 10 μmoles of ATPßS and 100 μmoles of BrCN in 1 ml of H₂O was chromatographed through a 1.0 x 12 cm column of DEAE-Sephadex A-25 eluted with a 500 ml linear gradient of TEAB increasing from 0.3 M to 0.5 M. Fractions were collected and A₂₆₀ of selected fractions were measured. Fractions under the two peaks were pooled and desalted by rotary evaporation (Chapter 2). Pooled fractions 9-13 (Peak A) contained 3.1 μmoles of ADP and pooled fractions 16-27 (Peak B) contained 6.0 μmoles of ATP.

Symbols: •——•, A₂₆₀
Figure 6.
ATP. Cyanogen bromide, which is known to react with tertiary amines according to equation 20, might also have been diverted to side reactions by samples of ATPβS that might not have been completely free of the TEAB used in its chromatographic purification.

\[ \text{BrCN} + R_3N \rightarrow R_3N^+CN + Br^- + R_2NCN + RBr \]  \hspace{1cm} (20)

To evaluate the compatibility of BrCN with buffers, various buffer solutions were tested by measuring the pH change upon adding BrCN. The pH of a mixture containing 33 mM buffer at the desired pH, and 90 mM BrCN was monitored for 30 min. Among the buffers tested were sodium acetate at pH 4.1 and 5.0, sodium citrate at pH 6.2, HEPES buffer at pH 7.0 and sodium phosphate at pH 7.0. In these tests HEPES was found to be incompatible with BrCN. It was the only one that underwent a large pH change, from 7.0 down to 3.9 in 30 min, presumably due to a reaction occurring between BrCN and HEPES. The pH values of the other solutions remained within 0.2 of the starting pH.

Trial experiments were carried out in a search for suitable conditions of pH, buffer and ratio of ATPβS to BrCN. In the first series a solution containing 10 mM ATPβS and a buffer was treated with BrCN at the concentration shown in Table 5. The reaction mixtures were
### TABLE 5

**ATP Yields in Reactions of ATPβS with BrCN**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>ATPβS (mM)</th>
<th>BrCN (mM)</th>
<th>ATP-yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>7.4</td>
<td>10</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Na acetate</td>
<td>4.1</td>
<td>10</td>
<td>100</td>
<td>18</td>
</tr>
<tr>
<td>Na acetate</td>
<td>5.0</td>
<td>10</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Na citrate</td>
<td>6.0</td>
<td>10</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>Na citrate</td>
<td>6.0</td>
<td>10</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>Na phosphate</td>
<td>7.0</td>
<td>10</td>
<td>100</td>
<td>64</td>
</tr>
<tr>
<td>Na phosphate</td>
<td>7.0</td>
<td>10</td>
<td>50</td>
<td>64</td>
</tr>
<tr>
<td>Na phosphate</td>
<td>7.0</td>
<td>10</td>
<td>20</td>
<td>64</td>
</tr>
</tbody>
</table>
kept at ambient temperature for 2 hours and cysteine was added to consume excess BrCN. The amount of ATP generated was determined by enzymatic analysis as described in Chapter 2. The ATP yields ranged from 18 to 64% under various conditions (see Table 5). Satisfactory conditions appeared to be 0.1 M sodium phosphate at pH 7 with 10 mM ATPβS and 20 mM BrCN.

3.3 Reaction of ATPβS with BrCN in \((^{18}O)H_2O\)

To determine the origin of the oxygen that displaced sulfur from ATPβS in the BrCN-mediated desulfurization, the reaction was carried out in \((^{18}O)H_2O\) as solvent, since water was considered to be the most probable source of oxygen. Phosphate buffer was also a possible source, however, so it was important to verify the origin of this oxygen. The incorporation of \(^{18}O\) into ATP could be detected by the effect of incorporated \(^{18}O\) on the \(^{31}P\)-NMR chemical shifts of \(P_\alpha\), \(P_\beta\), and \(P_\gamma\) in the ATP produced (93,113).

To conserve \(H_2^{18}O\) the reaction was carried out on a small scale with three repetitions, \(H_2^{18}O\) being recovered after each reaction and reused in the next. The triethylammonium salt of ATPβS (10 μmoles) was dried by rotary evaporation under vacuum as described in Chapter 2. To the residue in a 10 ml pear-shaped flask was added 0.5 ml
of 0.1 M sodium phosphate buffer at pH 7.0. The solution was frozen as a thin film on the wall of the flask and lyophilized. \( ^{18} \text{O}\)H\(_2\)O (0.5 ml, 95.2\% \( ^{18} \text{O} \)) and BrCN (20 \( \mu \text{moles} \)) were added to the dried residue. BrCN was added from a freshly prepared stock solution in DMF. After 30 min at ambient temperature, 12 \( \mu \text{moles} \) of cysteine crystals were added to the solution to consume unreacted BrCN. \( ^{18} \text{O}\)H\(_2\)O was recovered by connecting the flask through a three-way connecting tube to a water aspirator and a second flask that was cooled in liquid nitrogen. The temperature of the reaction flask was maintained with a lukewarm bath, and the \( ^{18} \text{O}\)H\(_2\)O was transferred and trapped in the liquid \( N_2 \)-cooled flask (0.4 ml). ATP\(_{\beta S}\) (8 \( \mu \text{moles} \)) lyophilized with sodium phosphate as described above was dissolved in the 0.4 ml \( ^{18} \text{O}\)H\(_2\)O recovered from the first reaction. BrCN (16 \( \mu \text{moles} \)) was added as above and the same reaction procedure repeated. The second recovery of \( ^{18} \text{O}\)H\(_2\)O (0.37 ml) was again used in the desulfurization of 7 \( \mu \text{moles} \) of ATP\(_{\beta S}\) with 14 \( \mu \text{moles} \) of BrCN. The final recovery of \( ^{18} \text{O}\)H\(_2\)O was 0.35 ml and \( ^{18} \text{O} \)-enrichment in the \( ^{18} \text{O}\)H\(_2\)O recovered was measured by mass spectroscopy.

The purification of ATP produced, 50.4\% overall yield, in these three reactions is described in the legend to
Figure 7, which shows the chromatographic elution profile. The $^{31}$P-NMR signals corresponding to the $P_β$ region in this sample of $[^{18}O]$ATP is shown in Figure 8. The substitution of sulfur by the hydroxyl group from $[^{18}O]H_2O$ would result in the incorporation of $^{18}O$ in the $β$-phosphorus of ATP. As shown by Cohn and Hu (93,113), the introduction of $^{18}O$-oxygen into a phosphoryl group causes upfield shift of the phosphorus NMR signal. The isotope induced shift is approximately 0.04 ppm in the case of double bonded oxygen-18 and 0.02 ppm in the case of single bonded oxygen-18. Since the reaction was carried out in 95.2% enriched $[^{18}O]H_2O$, the $^{31}$P-NMR signals for $P_β$ of the ATP isolated would have consisted exclusively of the isotope-shifted signals if sulfur had been replaced by 95% $^{18}O$. The unshifted signal would have appeared upon addition of unlabeled ATP. Surprisingly, the $^{18}O$-isotope shifted signals and the unshifted signals were observed at about equal intensities for $P_β$ without added unlabeled ATP (Figure 8, Part A). Moreover, as shown by Part B of Figure 8, the doublet signals for $P_γ$ were broadened relative to the doublet of $P_α$, suggesting that it actually consisted of two unresolved doublets, corresponding to $^{18}O$-isotope shifted and unshifted signals. This could have resulted from $^{18}O$
FIGURE 7. Chromatographic purification of ATP from reaction of ATPβS with BrCN in $^{18}O\text{H}_2\text{O}$.

The products of the three reactions described in the text were dissolved with distilled water and combined. Purification of ATP generated was by DEAE-Sephadex A-25 anion exchanger chromatography using a 1 x 20 cm column and a one liter linear gradient of TEAB buffer increasing from 0.2 M to 2.45 M according to the procedure described in section 2.4. Fractions of 7.5 ml volume were collected at 10 min intervals. The fractions 49-67 containing 12.7 μmoles of ATP were pooled and desalted as described in Chapter 2. The residue was dissolved in 2 ml 35% D$_2$O and adjusted to pH 8.0 by addition of NaOH in preparation for $^{31}$P-NMR analysis.

Symbols: •—•, $A_{260}$; o—o, $A_{412}$.
Figure 7.
FIGURE 8. $^{31}$P-NMR spectrum of $[^{18}$O$]_{\text{ATP}}$.

Shown in Part A is the $\beta$-region of the $^{31}$P-NMR spectrum obtained for $[^{18}$O$]_{\text{ATP}}$ produced by reaction of ATP$_{3}$S with BrCN and purified as described in Figure 7. The two triplet signals corresponding to ATP and $[^{18}$O$]_{\text{ATP}}$ are centered at -21.60 ppm and -21.63 ppm, respectively, the latter corresponding to the "isotope shifted" signal of $[^{18}$O$]_{\text{ATP}}$. Shown in Part B is the entire $^{31}$P-NMR spectrum showing doublet signals for $P_{\alpha}$ at -11.03 ppm and $P_{\gamma}$ at -5.97 ppm. Note that the lines in the $P_{\gamma}$ doublet are significantly broader than those in the $P_{\alpha}$ doublet, suggesting that the $P_{\gamma}$ doublet may consist of two unresolved doublets.
Figure 8.
being distributed almost evenly between the β- and γ-phosphoryl groups. To ensure that the dilution of isotope at $P_\beta$ could not have resulted from dilution of $[^{18}O]H_2O$ used as solvent, the recovered $[^{18}O]H_2O$ was subjected to mass spectroscopic analysis. The $^{18}O$-enrichment of the $[^{18}O]H_2O$ was found to be higher than 83%. Therefore the low enrichment at either the β- or γ-phosphoryl group could not be attributed to dilution of isotope in $[^{18}O]H_2O$ in the course of its repeated recovery in the experimental procedure.

To clarify the pattern of $^{18}O$ incorporation, the $[^{18}O]ATP$ was systematically degraded in such a way that each of the three phosphoryl groups was separately isolated as inorganic phosphate; and these samples were subjected to $^{31}P$-NMR analysis to determine their $^{18}O$ contents. The spectra shown in Figure 9 were obtained, showing that $P_\beta$ and $P_\gamma$ contained approximately equivalent $^{18}O$-enrichments. The enrichments in both were somewhat less than that indicated for $P_\beta$ in Figure 8, Part A. This could have resulted from dilution by traces of inorganic phosphate in the reagents used for the degradation or, alternatively, by alkaline phosphatase-catalyzed exchange of $^{16}O$ for $^{18}O$ in phosphate during the degradation. The significant fact is that both
FIGURE 9. \([\beta^{18O}, \gamma^{18O}]\)ATP from the reaction of ATP\(_8\)S with BrCN in \([^{18O}]H_2O\).

The sample of \([^{18O}]\)ATP prepared for the \(^{31}\)P-NMR spectral analysis in Figure 8 was enzymatically degraded as described in section 2.8 in such a way that \(P_\alpha\), \(P_\beta\), and \(P_\gamma\) were separately isolated as inorganic phosphate. These samples were then individually analyzed for the presence of \(^{18}O\) as revealed by the isotope-shifting effect of \(^{18}O\) on the \(^{31}\)P-NMR chemical shift of inorganic phosphate (113,114).

The smaller slightly upfield signals in the spectra for the phosphate samples derived from \(P_\beta\) and \(P_\gamma\) of \([^{18O}]\)ATP correspond to \([^{18O}]HPO_4\)\(^-\), while the more intense signals correspond to \(HPO_4\)\(^-\). No such \(^{18}O\)-isotope shifted signal is associated with the \(^{31}\)P-NMR spectrum of phosphate derived from \(P_\alpha\). This means that in the reaction of ATP\(_8\)S with BrCN, sulfur is displaced by oxygen derived partly from \([^{18O}]H_2O\) and partly from some other source.
P_β and P_γ contained equivalent $^{18}$O-enrichment, while P_α was not enriched with $^{18}$O.

3.4 Reactions of $(S_p)[\beta,\gamma$-bridging-$^{18}$O]ATPβS and $(S_p)\,[\beta$-$^{18}$O]ATPβS with BrCN

The fact that $^{18}$O was incorporated into P_γ in an amount equivalent with that at P_β when desulfurization was carried out in $[^{18}$O]$H_2O$ suggested that the second source of oxygen for displacing sulfur at P_β might be oxygen bonded to P_γ or P_α; that is, part of the thio-cyanate in cyanated ATPβS might have been displaced by an oxygen in the P_γ or P_α-phosphoryl groups.

To further investigate this possibility, a sample of $^{18}$O-labeled ATPβS with $^{18}$O placed selectively in the $\beta,\gamma$-bridging position of the triphosphate moiety, $(S_p)[\beta,\gamma$-bridging-$^{18}$O]ATPβS, was synthesized and converted to ATP by reaction with BrCN in $H_2O$. The compound was prepared by enzymatically phosphorylating a sample of $(S_p)[\beta$-$^{18}$O]ADPβS according to equation 21, using PEP and rabbit muscle pyruvate kinase as the

\[
\text{Ado-O-P-O-P} + \text{PEP} \rightarrow \text{Ado-O-P-O-P} + \text{Pyruvate} (21)
\]
phosphorylating system. A second sample with nonbridging $^{18}\text{O}$ was prepared by phosphorylation of (R$_p$) $[^{18}\text{O}]\text{ADP}\beta S$ according to equation 22, using the same phosphorylating system. This sample of (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$ was also desulfurized by reaction with BrCN in H$_2$O.

\[
\begin{array}{c}
\text{Ado-O-P-O-P} \\
\text{O} \\
\text{S} \\
\text{O} \\
\text{O}
\end{array}
\begin{array}{c}
+ \\
\text{PEP}
\end{array}
\Rightarrow
\begin{array}{c}
\text{Ado-O-P-O-P} \\
\text{O} \\
\text{S} \\
\text{O} \\
\text{O}
\end{array}
\begin{array}{c}
+ \\
\text{Pyruvate}
\end{array}
\]

The $^{31}\text{P}$-NMR spectra of (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$ and (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$ shown in Figure 10 verify the positions of $^{18}\text{O}$-enrichment in these samples. Thus Part A shows the effect of $^{18}\text{O}$ on the chemical shifts of both P$_\beta$ and P$_\gamma$ in (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$, whereas Part B shows the effect only on P$_\beta$ in (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$.

Figure 11 shows the $^{31}\text{P}$-NMR spectra of $[^{18}\text{O}]\text{ATP}$ obtained from the reactions of these samples of $^{18}\text{O}$-enriched ATP$\beta S$ with BrCN in H$_2$O. In Part A (Figure 11) the P$_\gamma$ and P$_\beta$ regions for $[^{18}\text{O}]\text{ATP}$ obtained from (S$_p$) $[^{18}\text{O}]\text{ATP}\beta S$ show that the bridging-$^{18}\text{O}$ in the substrate has been partially randomized to both the
FIGURE 10. $^{31}$P-NMR spectra of ($S_p$) [$\beta,\gamma$-bridging-$^{18}$O$]ATP\beta S$ and ($S_p$) [$\beta$-$^{18}$O$]ATP\beta S$.

The enzymatic phosphorylation of ($S_p$) [$\beta$-$^{18}$O$]ATP\beta S$ and ($R_p$) [$\beta$-$^{18}$O$]ADP\beta S$ to the title compounds is illustrated in equations 21 and 22 and described in Chapter 5. The spectra were obtained with proton spin decoupling except the lower spectra in Part A.

Part A: The upper spectrum is the $P_\beta$ and $P_\gamma$ region of ($S_p$) [$\beta,\gamma$-bridging-$^{18}$O$]ATP\beta S$ and the lower spectrum is the same region after adding an approximately equimolar ATP\beta S.

Part B: The upper spectrum is the $P_\beta$ and $P_\gamma$ region of ($S_p$) [$\beta$-$^{18}$O$]ATP\beta S$ and the lower is the same region after adding equimolar ATP\beta S.
Figure 10
FIGURE 11. $^{31}$P-NMR spectra of $^{18}$OATP produced by reaction of $(S_p) [\beta,\gamma$-bridging-$^{18}$O]ATP$\beta$S and $(S_p) [\beta-^{18}$O]ATP$\beta$S with BrCN.

The reaction conditions were as described in section 3.3 (10 mM $^{18}$O-enriched ATP$\beta$S, 20 mM BrCN, 50 mM KPi) except that the title compounds were substituted for ATP$\beta$S and the reactions were carried out in H$_2$O. The $^{18}$OATP produced was purified and prepared for $^{31}$P-NMR analysis as described in Figure 7 (yield 50%). The $^{31}$P-NMR spectra were obtained without proton spin decoupling. Part A: The spectrum of $^{18}$OATP obtained from $(S_p) [\beta,\gamma$-bridging-$^{18}$O]ATP$\beta$S. Part B: The P$_\beta$ region is shown in the upper spectrum for $^{18}$OATP obtained from $(S_p) [\beta-^{18}$O]ATP$\beta$S. The lower spectrum is the same region after adding an equivalent amount of ATP. The P$_\gamma$ and P$_\alpha$ doublets (not shown) were enhanced but otherwise unchanged by added ATP.
Figure 11
Figure 11
FIGURE 12. Hypothetical pathway for the production of ATP in the reaction of ATP8S with BrCN.
β,γ-bridging and β-nonbridging positions of ATP. The ratio of \(^{18}O\)-isotope shifted to unshifted signals for \(P_γ\) is about 3:2 in \([^{18}O]ATP\) but at least 10:1 in \((S_p)\) \([γ,β\)-bridging-\(^{18}O\)]ATP\(\beta\)S. Similarly, the ratio of bridging to nonbridging \(^{18}O\) bonded to \(P_β\) in ATP is about 3:2, whereas in the substrate it was at least 10:1. Thus the reaction of \((S_p)\) \([β,γ\)-bridging-\(^{18}O\)]ATP\(\beta\)S with BrCN leads to ATP by a mechanism that involves partial transfer of bridging-\(^{18}O\) in the substrate to a β-nonbridging position in the product. Part B of Figure 11 shows the \(P_β\) region of \([^{18}O]ATP\) produced from \((S_p)\) \([β-^{18}O]\)]ATP\(\beta\)S, verifying that nonbridging \(^{18}O\) at \(P_β\) in the substrate remains nonbridging in the product.

3.5 Conversion of ATP\(\beta\)S to ATP by Reaction with BrCN in Water. The Probable Mechanism

The results of experiments described in the foregoing sections suggest that the conversion of ATP\(\beta\)S to ATP by reaction with BrCN in phosphate buffer proceeds by the pathways outlined in Figure 12. ATP\(\beta\)S most likely reacts with BrCN as indicated to form the hypothetical S-cyanated intermediate XIII which, though not directly detected in these experiments, is expected to be produced by the nucleophilic displacement of \(Br^-\) from BrCN by the sulfur in ATP\(\beta\)S. BrCN is known to be quite reactive with sulfur,
even with sulfur in thioethers such as methionine. This reactivity is the basis of the first chemical step in the specific cleavage of methionyl peptide bonds in proteins by reaction with BrCN (115). The cleavage generates specific "BrCN peptides" that are often used in the amino acid sequence analysis of proteins.

Intermediate XIII apparently reacts further to produce ATP by two different routes, one of which involves the direct reaction of H$_2$O with the $\beta$-phosphorus to displace thiocyanate. This leads to the incorporation of $^{18}$O from [18O]H$_2$O into the $\beta$-phosphorus of ATP, and is the route that was originally expected for the conversion of ATP$\beta$S to ATP. The second route is the internal displacement of thiocyanate by a $\gamma$-phosphoryl oxygen, forming a second intermediate XIV, a cyclo-diphosphate. This intermediate, whose formation was not anticipated, is also quite reactive with water, reacting at the $\gamma$-position with cleavage of either bridging P-O bond to $P_\gamma$ and producing ATP. Intermediate XIV leads to the incorporation of $^{18}$O into $P_\gamma$ of ATP when the reaction is carried out in [18O]H$_2$O; and it also leads to the randomization of bridging $^{18}$O in the reaction of (S$_\beta$) $[^{18}$O-bridging-$P_\gamma$]ATP$\beta$S to both bridging and nonbridging $P_\beta$ positions of ATP. Again, intermediate XIV is hypothetical since it is not directly observed.
The two salient facts leading to the proposal that part of the reaction involves the cyclo-diphosphate XIV as an intermediate are the incorporation of solvent oxygen into $P_\gamma$ of ATP and the partial randomization of $\beta,\gamma$-bridging oxygen in ATP$\beta$S to bridging and nonbridging positions at $P_\beta$ of ATP. This mechanism involves intramolecular transfer of $P_\gamma$-oxygen in ATP$\beta$S to $P_\beta$ of ATP. Both of the above facts could be explained equally well by a mechanism involving intermolecular transfer of $P_\gamma$-oxygen through a dimeric intermediate such as XV shown below. This would arise from the mutual displacement of thiocyanate from $P_\beta$ of intermediate XIII in Figure 12 by a $P_\gamma$-oxygen of a second molecule. Hydrolysis of XV would occur in such a way as to lead to the observed results. The formation of XV does not appear to be very probable. However, since it would explain the results, and since XIV has not been identified definitively, it must be given serious consideration.
Two other experimental facts are not consistent with the involvement of XV in the randomization of bridging oxygen or the incorporation of solvent oxygen into $P_Y$. In addition to undergoing hydrolysis to ATP, XV should also produce substantial amounts of AMP, whereas only small amounts of AMP were produced. More significantly, the reaction of a $P_Y$-phosphoryl oxygen of ATPβS with $P_B$ of intermediate XIII (Figure 12) would be chemically similar to the reaction of inorganic phosphate with the same center. Such a reaction would produce the hypothetical intermediate XVI shown below. This might undergo hydrolysis to ATP, but it

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{O} \\
\text{O} & \quad \text{P} & \quad \text{O} & \quad \text{P} & \quad \text{O} & \quad \text{Ado} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} \\
\text{O} & \quad \text{P} & \quad \text{O} & \quad \text{Ado} \\
\end{align*}
\]

would do so in $[^{18}\text{O}]_2\text{H}_2\text{O}$ without incorporating $^{18}\text{O}$ into ATP. Since the reaction was carried out in the presence of phosphate buffer at 10 times the concentration of ATPβS, intermediate XVI should predominate over XV if the displacement of thiocyanate by phosphoryl group is intermolecular. Available evidence is not consistent with the involvement of XVI, suggesting that XV also is not involved.

Hydrolytic opening of the cyclo-diphosphate XIV by reaction of water at $P_Y$ and not $P_B$ is consistent with the
well-known properties of cyclo-triphosphate esters. Alkyl and dialkyl cyclo-triphosphates are known to undergo facile hydrolysis in water, with the orientation of the addition of water and cleavage of the rings being such as to result in the expulsion of the best leaving group from the center of hydrolysis (112,116). If water were to react at \( P_\beta \) of XIV, the \( pK_a \) of the conjugate acid of the leaving group (\( P_\gamma \)) would be between 6 and 7; whereas, when water adds to \( P_\gamma \) of XIV, the \( pK_a \) of the conjugate acid of the leaving group (\( P_\beta \)) is much lower, perhaps between 2 and 3. So the addition of water is at \( P_\gamma \).

A similar mechanism prevails in the reaction of ADP\( \alpha \)S with BrCN, which was studied by R. D. Sammons (112,118).

3.6 Dinucleotide-5'-[2-thiotriphosphates] as Precursors of (\( R_p \)) and (\( S_p \)) \( [\beta^{-18}\text{O}]\text{ATP} \)

The unexpected and novel rearrangement of ATP\( \beta \)S described in the foregoing sections makes its conversion to ATP by reaction with BrCN a poor method for preparing (\( R_p \)) and (\( S_p \)) \( [\beta^{-18}\text{O}]\text{ATP} \) from (\( S_p \)) and (\( R_p \)) \( [\beta^{-18}\text{O}]\)-ATP\( \beta \)S. The best that could be expected would be mixtures of (\( R_p \)) and (\( S_p \)) isomers enriched in one epimer. Thus if (\( R_p \)) \( [\beta^{-18}\text{O}]\text{ATP} \) reacted according to the pathway in Figure 12, with half of it being partitioned through intermediate XIV and with equal partitioning of ring oxygens in XIV upon hydrolysis, the ATP produced would
be a mixture consisting of 75% \((S_p) [\beta^{-18}O]ATP\) and 25% \((R_p) [\beta^{-18}O]ATP\). The procedure would be even less useful for synthesizing \((S_p) [\beta^{-17}O,^{18}O]ATP\) by reaction of \((R_p) [\beta^{-18}O]ATP\)S in \([^{17}O]H_2O\).

R. D. Sammons (100) and T. Meade (117) found that \(P^1,P^2\)-dialkyl pyrophosphorothioates react with BrCN in water to produce \(P^1,P^2\)-dialkyl phosphates in excellent yields with no sign of rearrangements. Sulfur was replaced by \(^{18}O\) from \([^{18}O]H_2O\) without dilution, and the reactions of \((R_p)\) and \((S_p)\) \(\beta\)-cyanoethyl-ADPaS were found to proceed with inversion of configuration (100). Reaction of \((R_p)\) \(\beta\)-cyanoethyl-ADPaS with BrCN in \([^{18}O]H_2O\) produced exclusively \((S_p)\) \(\beta\)-cyanoethyl \([\alpha^{-18}O]ADP\) as shown by R. D. Sammons. The structures of \((R_p)\) \(\beta\)-cyanoethyl-ADPaS and \((S_p)\) \(\beta\)-cyanoethyl \([\alpha^{-18}O]ADP\) are shown in equation 23.

\[
\text{\begin{align*}
\text{SII} & \quad -O-\text{Ado} \quad \text{BrCN/H}_2\text{O} \\
\text{---} \quad \rightarrow \\
\text{---} & \quad \text{O} \quad \text{P-O-Ado} \\
\text{---} & \quad \text{O} \quad \text{P-O-Ado} \\
\end{align*}}
\]

\((S_p)\) \(\beta\)-cyanoethyl \([\alpha^{-18}O]ADP\) was deblocked to \([\alpha^{-18}O]ADP\) by treatment with base. The NMR spectra of
a mixture of this [\(\alpha-^{18}O\)]ADP and authentic ADP showed that the \(^{18}O\) was located specifically at a \(P^\alpha\)-nonbridging position with no sign of scrambling in this case. Configurational analysis of the cobalt(III)tetrammine [\(\alpha-^{18}O\)]ADP complexes by \(^{31}P\)-NMR showed that substitution of \(^{18}O\) for sulfur proceeded stereospecifically with inversion of configuration at \(P^\alpha\).

In these experiments and those of T. Meade, the presence of an alkyl substituent on a terminal phosphoryl group prevented the internal displacement of thiocyanate; and this was in effect confirmed in the foregoing work with ATP\(\beta\)S, where no evidence of participation by the \(P^\alpha\)-oxygens was detected. These results implied that if the \(\gamma\)-phosphoryl group of ATP\(\beta\)S could be blocked by alkylation to an ester, using an easily removable alkyl group, one should be able to avoid oxygen scrambling and obtain stereochemically pure [\(\beta-^{18}O\)]ATP in a similar way.

In 1976 Eckstein and Goody (70) synthesized \(\gamma\)-cyanoethyl-ATP\(\beta\)S and deblocked it by treatment with base to generate ATP\(\beta\)S. The coupling procedure was based on the general phosphoanhydride synthesis procedure published by Michelson (69). The synthesis involved coupling ADP\(\beta\)S to diphenyl phosphorochloridate-activated cyanoethyl phosphate. In this laboratory \(\gamma\)-(p-nitrophenyl)-ATP\(\alpha\)S was similarly synthesized by coupling ADP\(\alpha\)S to diphenyl
phosphorochloridate-activated p-nitrophenyl phosphate (112). John P. Richard from this laboratory also synthesized P$^1$-adenosine-P$^3$-2',3'-methoxymethylene adenosine-5'-[1-thiotriphosphate] (36) by activating 2',3'-methoxymethylene-AMP with diphenyl phosphorochloridate and coupling with ADPβS. All these precedents suggested that the synthesis of a γ-alkyl-ATPβS could be accomplished by a well-established procedure, with several choices of easily removable alkyl blocking groups. If the P$_β$ epimers could be separated, they could then be separately desulfurized by reaction with BrCN in [18O]H$_2$O and the resulting products dealkylated at P$_γ$ to produce the P$_β$-epimers of [β-$^{18}$O]ATP.

3.7 Synthesis of (R$_p$ + S$_p$) P$^1$-Adenosine-P$^3$-2',3'-Methoxyethylene Adenosine-5'-[2-thiotriphosphate]

The reported yield of ATPβS prepared by Michelson's coupling of cyanoethyl phosphate with ADPβS was 12% (70), based on the ADPβS used. Coupling of diphenyl phosphorochloridate-activated AMPS to 2',3'-methoxymethylene-AMP has been a standard procedure in this laboratory to obtain P$^1$-adenosine-P$^2$-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] (111), which is the precursor for ADPβS. The yield in this synthesis ranged between 40% and 60%. Based on familiarity with this procedure and the reasonable coupling yield, 2',3'-methoxymethylene-AMP
was chosen to couple to ADPβS with the aim of obtaining \( P^1\)-adenosine-\( P^3\)-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] as a mixture of \( P_\beta \) epimers. The projected synthetic route is outlined in Figure 13. The same procedure used to obtain ADPβS from \( P^1\)-adenosine-\( P^2\)-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] could be applied here to deblock the desulfurized product and generate ATP.

This procedure produced very low yields of what appeared to be the desired product, based on its chromatographic properties and its chemical conversion to authentic ATPβS by periodate-cleavage and alkaline elimination of the unblocked adenosyl moiety and deblocking of the 2',3'-methoxymethylene adenosyl moiety. The major product appeared to be AMP, although this was not definitely identified. The yields were comparable to those reported by Eckstein in his synthesis of ATPβS, which were thought perhaps not to be optimal or to be attributable to instability of diphenyl phosphoro-chloridate-activated cyanoethyl phosphate in pyridine.

Whatever the reasons for Eckstein's low yield, failure of this procedure in the present effort was traced to extreme liability of ADPβS in pyridine solution, the optimal solvent for the Michelson phosphoanhydride
FIGURE 13. Projected synthetic route of \((R^p + S^p)\) 
\(p^1\)-adenosine-\(P^3\)-2',3'-methoxymethylene 
adenosine-5'-[2-thiotriphosphate].

Shown is the synthetic route that was originally proposed and attempted. The selection of this route was motivated by precedents set forth in the text. This route produced only nominal yields of the desired product, however, principally because ADPβS was unstable under the reaction conditions and decomposed faster than the rate of the coupling reaction.
Figure 13
synthesis. ADPβS was found to decompose quickly in pyridine to a compound that migrated with AMP on thin layer chromatograms. Therefore, by the time ADPβS had been dried by twice dissolving a desiccated sample in anhydrous pyridine followed by rotary evaporation of the pyridine in vacuo at low temperature to remove the last traces of water, it had largely decomposed; and further decomposition occurred once it had been redissolved in pyridine and combined with diphenyl phosphorochloridate-activated 2',3'-methoxymethylene-AMP.

Pyridine is the best solvent for the Michelson coupling, and in any case ADPβS turned out to be quite unstable in all other nonaqueous solvents tested in which its tri-n-alkyl-ammonium salt could be dissolved. Therefore, it must be concluded that the route outlined in Figure 13 cannot be useful for the desired purpose. It would be especially inappropriate for coupling (R₉) or (S₉) [β⁻¹⁸O]ADPβS, since these compounds are valuable reagents which should not be wasted in a synthesis that produces an intolerably low yield of the desired product.
Another method for synthesizing dinucleoside \([2\text{-thio} \text{triposphates}]\) was required. To be useful for the present purposes, such a method had to be applicable to the coupling of ADPβS to a phosphoric ester in high yield. This meant that the activated phosphoric ester had to be sufficiently reactive to couple with ADPβS faster than ADPβS decomposes under the reaction conditions. The coupling reaction should proceed in a nonaqueous solvent in which the tri-\(n\)-alkylammonium salts of ADPβS could be dissolved, preferably a solvent other than pyridine. The literature on nucleotide synthesis provided some leads that resulted in the eventual development of an efficient synthetic procedure.

In the late 1940's and early 50's, Todd and coworkers developed methods for linking together dissimilar molecules, nucleosides in particular, through phosphate or pyrophosphate residues in order to synthesize polynucleotides and nucleotide coenzymes. In the process they developed a procedure to synthesize 5'-O-nucleoside-benzyl phosphorochloridate \((119,120)\), which could then react with a second nucleoside or nucleoside monophosphate, forming an ester or phosphoanhydride by displacement of chloride ion (equation 24). ROH could be an alcohol, phosphate, nucleoside, or a nucleoside monophosphate. Debenzylation of the coupled products by catalytic
hydrogenation gave the desired phospho-compounds. Among the compounds they synthesized were FAD, UDP and UDPG.

Based on this approach in which a pyrophospho-anhydride linkage was formed by nucleophilic displacement of chloride from a phosphorochloridate by a second phosphoryl group, which proceeds in solvents other than pyridine, a new synthetic procedure outlined in Figure 14 was devised to prepare the desired compound. 2',3'-O-Methoxymethylene adenosine-5'-phosphorodichloridate was first prepared by reaction of 2',3'-O-methoxymethylene adenosine (121) with phosphorus oxychloride in triethyl phosphate. After removing unreacted phosphorus oxychloride and triethyl phosphate by vacuum distillation at 35°C, the residue was combined with the tri-n-butylammonium salt of ADP&$S$ in hexamethylphosphoramide (HMPA) for 30 min. After precipitating the nucleotides by adding diethyl ether and decanting the liquid layer, the residue was dissolved with TEAB buffer and purified by chromatography.
FIGURE 14. Synthesis of \((R_p + S_p) \, P^1\text{-adenosine-}P^3\text{-}2',3'\text{-methoxymethylene adenosine-}5'\text{-}[2\text{-thiotriphosphate}]\).

This new synthetic route to \(P_1^1, P_3^3\text{-}\)dinucleoside triphosphates was successfully developed to produce a high yield (60-65%) of the title compound. The product of coupling could be converted to ATP8 in 85-90% yield.
Figure 14
Because the thiophosphoryl group is in the center of the molecule, the synthesis can be carried out in a similar way starting with adenosine 5'-phosphorodichloridate and 2',3'-O-methoxymethylene adenosine-5'-[2-thiodiphosphate] as shown in Figure 15. With these two synthetic procedures, one has the potential capability to introduce $^{18}O$ specifically at either the $\alpha,\beta$-bridging or $\beta,\gamma$-bridging position of ATP$\beta$S, which is the product after removing the adenosyl group from one end and the methoxymethylene group from the other end of the molecule. If one starts with (R$_p$) $[\beta-^{18}O]ADP\beta S$, $^{18}O$-oxygen will be located on $\beta$-phosphorus of ATP$\beta$S at either nonbridging or $\alpha,\beta$-bridging position. Starting with (R$_p$) $[\beta-^{18}O]2',3'$-methoxymethylene-ADP$\beta$S, $^{18}O$ oxygen will end up on $\beta$-nonbridging or $\beta,\gamma$-bridging position as shown in Figure 15. If the $R_p$ and $S_p$ isomers of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] could be separated, the $^{18}O$ bridging and nonbridging molecules would thereby be separated. By the same token, the desulfurization of the $^{18}O$-enriched $P^1,P^3$-dinucleoside [2-thiotriphosphates] described above in water or $[^{17}O]H_2O$ followed by removal of the blocking groups in a similar way one should have access to (R$_p$) and (S$_p$) $[\beta-^{18}O]ATP$ or $[\beta-^{17}O,^{18}O]ATP$, or to ATP with $\alpha,\beta$-bridging-$^{18}O$ or $\beta,\gamma$-bridging-$^{18}O$. 
FIGURE 15. Projected syntheses of specifically $^{18}_O$-enriched nucleotides.
Figure 15
The detailed procedure for synthesizing \((R^+_p + S^-_p)\)
\(p^1\)-adenosine-\(p^3\)-2',3'-methoxymethylene adenosine-5'-
[2-thiotriphosphate] by the route outlined in Figures 14 and 15 is given in Chapter 5.

An interesting question arose in connection with the synthetic procedure in Figure 14. The immediate coupling product would be structure XVII shown below, in which one Cl would still be bonded to the P of the

2',3'-methoxymethylene-AMP moiety. It was assumed that this Cl would be hydrolytically removed during aqueous workup. The question that arose concerned the mechanism by which the hydrolysis would proceed. It might involve direct hydrolytic displacement, in which case workup in \([^{18}O]H_2O\) would result in the substitution of \(^{18}O\) for

![Chemical structure XIVII](image-url)
this Cl in the final product. Another mechanism was considered to be more probable, however. The relative positions of the P-Cl bond and the adenosyl phosphoryl oxygens suggested that chloride might be displaced by internal cyclization to the species XVIII, shown below.

\[
\begin{align*}
\text{XVIII}
\end{align*}
\]

In accord with well-known precedents cited above in section 3.5, XVIII could be expected to undergo hydrolysis during aqueous workup by addition of water to either fully esterified phosphorus. This would be accompanied by cleavage of the indicated bonds to eliminate the most
stable leaving group. This mechanism would result in the incorporation of $^{18}\text{O}$ into both the $P^1$ and $P^3$ groups of the product during workup in $[^{18}\text{O}]\text{H}_2\text{O}$.

The probability that this would be the predominant hydrolytic mechanism made the synthetic routes in Figures 14 and 15 very attractive, because the cyclization, an intermolecular coupling, would proceed rapidly subsequent to the coupling step and before aqueous workup, no doubt much faster than intermolecular coupling between two or more molecules of XVII, which could also occur prior to aqueous workup and would lead to oligomeric side products. The intramolecular coupling would probably supercede most or all other side reactions of XVII and ultimately lead through the predictable hydrolysis of XVIII to the desired product.

To determine whether the hydrolytic mechanism involves cyclization, the synthesis was carried out as usual except for the substitution of $[^{18}\text{O}]\text{H}_2\text{O}$ in the aqueous workup. Since the $^{31}\text{P-NMR}$ signals for $P^1$ and $P^3$ of the coupling product could not be readily distinguished, it was converted to $[^{18}\text{O}]\text{ATP}\delta\text{S}$ in which the $P$-resonances are well separated, with chemical shift values of -11.5 ppm for $P_\gamma$, 29.9 ppm for $P_\beta$, and -6.5 ppm for $P_\alpha$, all relative to $\text{H}_3\text{PO}_4$. The presence
of $^{18}O$ at any of these positions could be detected by the upfield shift it induces on the P-signal.

Figure 16 illustrates the chemical transformations involved as well as the consequences of $[^{18}O]H_2O$ workup in this experiment. The incorporation of $^{18}O$ into both P$^1$ and P$^3$ of the product is established by the $^{31}P$-NMR spectrum of $[^{18}O]$ATP$\delta$S shown in Figure 17. Note that the signals for both P$\alpha$ and P$\gamma$ are accompanied by $^{18}O$-isotope shifted signals of approximately equal intensity, showing that $^{18}O$ was incorporated with approximately equal frequency into P$^1$ and P$^3$, consistent with the chemical similarity of P$^1$ and P$^3$ in the hypothetical cyclic intermediate XVIII. These results strongly support the proposal that XVIII is the true coupling product between 2',3'-methoxymethylene-ADP$\delta$S and adenosine-5'-phosphorodichloridate.

3.8 Diastereomers of P$^1$-Adenosine-P$^3$-2',3'-Methoxymethylene-Adenosine-5'-[2-thiotriphosphate]

Efforts were made to separate the diastereomers of P$^1$-adenosine-P$^3$-2',3'-methoxymethylene-adenosine-5'-[2-thiotriphosphate] by HPLC. Typical elution profiles are shown in Figures 18 and 19.

Since only about 90 A$_{260}$ units of nucleotides were injected each time (Figure 19) and the long retention
FIGURE 16. Consequences of $[^{18}O]$H$_2$O workup in the coupling of adenosine-5'-phosphorodichloridate with 2',3'-methoxymethylene-ADP$_5$S.

$[^{18}O]$ATP$_5$S was synthesized, as described in Chapter 5, by carrying out the aqueous workup of the coupling reaction product in $[^{18}O]$H$_2$O and removing the adenosyl group in one end and the methoxymethylene in the other end. $^{31}$P-NMR spectrum of $[^{18}O]$ATP$_5$S, as shown in Figure 17, identified that $^{18}$O was incorporated with relatively equal frequency into either $P^1$ or $P^3$ position of the $P^1,P^3$-dinucleoside [2-thiotriphosphate].
Ado—O—P—Cl

\[ \text{Ado—O—P—Cl} \]

1. HMPA
2. \((^{18}\text{O})\text{H}_2\text{O}\)

\[ \text{Ado—O—P—O—P—O—P—O—P—O} \]

\[ \text{OCH}_3 \]

1. NaIO₄
2. HSCH₂CH₂OH
4. pH 10.5, 50°C, 20 mins

\[ \text{O—P—O—P—O—P—O—Ado} \]

\[ \text{O—P—O—P—O—P—O—Ado} \]
FIGURE 17. $^{31}$P-NMR spectrum of $[^{18}\text{O}]\text{ATP}\beta S$ prepared by the procedure in Figure 16.

$[^{18}\text{O}]\text{ATP}\beta S$ (10 μmoles) obtained from the procedure shown in Figure 16 was subjected to $^{31}$P-NMR analysis (proton spin decoupled). Part A: The spectra of the $P_\gamma$ region with chemical shifts centered at -4.91 ppm and -4.93 ppm for $^{16}\text{O}$-bonded and $^{18}\text{O}$-bonded species, respectively, and with a ratio of the intensities of 3.1:2, $J_{\beta,\gamma} = 28.29$ Hz. Part B: The spectra of the $P_\alpha$ region with chemical shifts centered at -10.23 ppm and -10.26 ppm for $^{16}\text{O}$-bonded and $^{18}\text{O}$-bonded species, respectively, and with a ratio of the intensities of 3.6:2, $J_{\alpha,\beta} = 26.87$ Hz. Part C: The $P_\delta$ region of the spectra showed two sets of triplets corresponding to $R_p$ and $S_p$ epimers, $\delta_p = 30.09$ ppm and 30.02 ppm, respectively, with no sign of $^{18}\text{O}$-induced chemical shifts.
Figure 17
FIGURE 18. Separation of $^\text{P}^1$-adenosine-$^\text{P}^3$-2',3'-methoxymethylene-adenosine-5'-[2'-thiotriphosphate] by HPLC.

A 7.8 mm x 30 cm reverse phase $\mu$Bondapak C$_{18}$ column from Waters Associates was set up to resolve the diastereomers of the title compound. Ten microliters of sample containing 0.9 $A_{260}$ units of the diastereomers was injected each time in search of suitable conditions. Elution by 50 mM potassium phosphate buffer, pH 6.5, at 1.5 ml per minute is shown; the peaks appeared at about 50 minutes after injection. Several different conditions were tried and separation was not improved in any case.

Symbol: $A_{254}$, ___.
FIGURE 19. Separation of \(^{\text{P}1}\)-adenosine-\(^{\text{P}3}\)-2',3'-methoxymethylene-adenosine-5'-[2-thiotriphosphate].

Separation was done on a 8.40 x 250 mm Chromasil 5 \(\text{C}_{18}\) column purchased from Whatman Chemical Separation, Inc. A sample of 0.5 ml containing 90 A\(_{260}\) units of the title nucleotide was injected and eluted with 0.3 M potassium phosphate buffer, pH 6.8, with 10% methanol. The flow rate was 2 ml per minute and the first major peak with absorbance at 254 nm appeared between 225 minutes to 250 minutes. To determine the degree of overlapping, 4 ml fractions were collected and pooled into the three pools indicated in the figure. The first and last pools, designated as (I) and (II), were desalted for analysis as described in the text.
Figure 19 Fraction No.
time in the column caused the material to be eluted in a very broad peak, and since the concentration of phosphates was 0.3 M in the separations, desalting of pools from the HPLC column presented a problem. Desalting by gel filtration or DEAE-Sephadex chromatography using the volatile TEAB to elute the nucleotide would have been impractical. Ultrafiltration also proved to be impractical. A desalting procedure was developed based on salting out and solvent partitioning. The fraction pool was first concentrated to one-tenth of its original volume by rotary evaporation at 30°C. At this point some crystals appeared coming out of the solution, and the mixture was placed in an ice-water bath and stored for 2 hours before filtering the crystals. A minimum volume of H₂O/MeOH = 1:2 (v/v) was used to wash the crystals. The combined filtrate and wash was again concentrated on a rotary evaporator until a small amount of precipitate appeared. About two volumes of methanol were added to the suspension. Two liquid layers separated after the suspension was thoroughly shaken and set in an ice-water bath for 20 minutes. The absorbance at 260 nm of the upper layer was checked to verify that it contained more than 95% of the A₂60. If so, the upper layer was decanted by pipetting into a separate flask. If not, a small
volume of H₂O was added, the suspension was remixed, and after standing in an ice-water bath for 20 minutes the A₂₆₀ was again checked. This was repeated until >95% of the A₂₆₀ absorbing material appeared in the upper layer. The upper layer was further desalted by concentrating to a smaller volume using a rotary evaporator and again adding two volumes of methanol.

The concentration and methanol-water extraction process was repeated until the final total phosphate present was low enough to be removed by a small 1.5 x 15 cm DEAE-Sephadex A-25 column. Based on the capacity of the resin, the total phosphate should be less than 5 mmoles. The elution profiles of P¹-adenosine-P³-2',3'-methoxymethylene-adenosine-5'-[2-thiotriphosphate] from pools (I) and (II) after removing more than 90% of the phosphate are shown in Figure 20. The recovery of A₂₆₀ absorbing material was between 80 and 90%.

To determine the (R_p) and (S_p) diastereomer compositions of P¹-adenosine-P³-2',3'-methoxymethylene-adenosine-5'-[2-thiotriphosphate] obtained in pools (I) and (II), small samples were chemically degraded to ATP₈S as described above. The samples of ATP₈S obtained were analyzed for (R_p) ATP₈S using the hexokinase-glucose-6-P dehydrogenase assay procedure, which is known to be
FIGURE 20. Chromatographic desalting of pools (I) and (II) from Figure 19.

The two pools from five repetitions of the HPLC separation in Figure 19 were largely freed of potassium phosphate buffer as described in the text. The solutions, containing less than 5 mmoles of potassium phosphate, were chromatographed through 1.5 x 15 cm columns of DEAE-Sephadex A-25. Elution was with a 0.8 liter TEAB gradient, increasing from 0.1 M to 0.5 M at a flow rate of 1 ml·min⁻¹; fractions of 7 ml were collected. Fractions with absorbance at 260 nm were pooled and TEAB was removed by evaporation as described in Chapter 2. The elution profile for pool (I) is Part A and that for pool (II) is Part B. From pool (I) 3.5 µmoles and from pool (II) 6 µmoles of nucleotide were obtained.

Symbols: •—•, A₂₆₀; o---o, A₆₆₀ (phosphate).
Figure 20
highly selective for the (Rₚ) epimer. These assays showed that about 60% of ATP₈S from pool (II) was the (Rₚ) epimer, while about 10% from pool (I) was the (Rₚ) epimer. This assay indicated that pool (I) consisted of about 90% of (Sₚ) diastereomers and 10% of (Rₚ) diastereomers, while pool (II) was hardly enriched in either.

This approach to prepare pure diastereomers of dinucleoside 2-thiotriphosphates was abandoned as impractical. While the synthesis of the diastereomeric mixtures was efficient, the problem of separation proved to be intractable. Diastereomers with the (Sₚ) configuration at P₈ could be obtained with difficulty, but in only small amounts and still contaminated with 10% of the (Rₚ) diastereomers.

The separation problem is compounded by the fact that the methoxymethylene group in 2',3'-methoxymethylene-nucleosides and nucleotides has an optical center and both configurations are present. This leads to an additional two diastereomers in P¹-adenosine-P³-2',3'-methoxymethylene-adenosine-5'-[2-thiotriphosphate], giving a total of four diastereomers. Separation techniques that are powerful enough to resolve the (Rₚ) and (Sₚ) isomers also begin to resolve diastereomers that differ in methoxymethylene configuration, resulting
in cross contamination. This is clearly revealed in Figure 18, which shows two major peaks, both asymmetric due to the presence of other diastereomers. Unless all four diastereomers can be completely separated, the best that could be achieved would be that small amounts of one of the four, the first eluted from a column, could be isolated.
4.1 Configuration of Nucleoside $^{18}$O Phosphorothioates

Chiral nucleoside $^{18}$O phosphorothioates such as $R_p$- (or $S_p$) $[^{18}$O]$\text{AMP}$ and $R_p$- (or $S_p$) $[\beta^{18}$O]$\text{ADP}$ S can be stereoselectively phosphorylated by enzymatic action at the $R$ or $S$ oxygen of the thiophosphoryl group. As a result of this process, the $^{18}$O will be located in either a bridging or nonbridging position in the phosphorylated product as shown below (reaction 25).

$$R - O - P\quad +\quad ATP\quad \rightarrow\quad ADP\quad (25)$$

The outcome in a given case will depend on the $[^{18}$O]-thiophosphoryl configuration and stereoselectivity of the phosphorylating enzyme. Methods that can distinguish
the position of the $^{18}\text{O}$ as bridging or nonbridging provide the means for determining the configurations of chiral $[^{18}\text{O}]$thiophosphate centers. One such method is mass spectral analysis of the volatile derivatives of chemical degradation products.

Such a procedure has been devised in this laboratory (122). The method involved the chemical degradation outlined in Figure 21, in which the 2-thiotriphosphate moiety was cleaved from the nucleoside, methylated with diazamethane, hydrolyzed, and again methylated. Trimethyl phosphate and trimethyl phosphorothioate were analyzed for $^{18}\text{O}$ content in the parent molecular ions. Hydrolysis in $[^{18}\text{O}]\text{H}_2\text{O}$ verified the partitioning of bridging oxygen between trimethyl phosphate and trimethyl phosphorothioate, whereas nonbridging $^{18}\text{O}$ at the $\beta$-phosphoryl position of ATP$\beta$S could appear only in trimethyl phosphorothioate.

The fragmentation patterns of the trialkyl esters of phosphate have been assigned by Bafus et al. (123) and Santoro (124). Oxygen-18 enrichment in trimethyl phosphate and trimethyl phosphorothioate can be calculated from the relative intensities of the parent molecular ions, m and m+2 species, and the position of $^{18}\text{O}$ in the original nucleoside $[^{18}\text{O}]$phosphorothioate can then be deduced.
FIGURE 21. Analysis of $^{18}$O position, bridging or nonbridging, in nucleoside $[^{18}$O]phosphoro-thioates.
1. NaIO₄
2. 2- Mercaptoethanol
3. pH 10.5, 50°C, 30 min
4. DEAE- Sephadex Chromatography

1. CH₃OH, HCl
2. CH₂N₂

1. H₂O, 100°C, 60 min
2. CH₃OH, HCl, CH₂N₂

Figure 21
4.2 Stereochemical Course of the PEP Carboxykinase Reaction

To establish the stereochemical course of the PEP carboxykinase catalyzed phosphoryl group transfer reaction, $(R_p) \gamma^{18}O \text{ATP} \gamma S$ (125) was used as the thiophosphoryl group donor as shown in reaction 26.

\[ \text{Oxaloacetate} \xrightarrow{\text{Retention}} \text{CH}_2C=O-P\gamma^S + CO_2 + GDP \]

The $[^{18}O]$thiophosphoenolpyruvate generated was used as the substrate for pyruvate kinase acting on ADP to produce $[\gamma^{18}O]\text{ATP} \gamma S$, a process known to proceed with inversion of configuration at phosphorus (37-39). $[\gamma^{18}O]\text{ATP} \gamma S$ in turn served as the thiophosphoryl group donor in the adenylate kinase catalyzed phosphorylation of AMP to $[\beta^{18}O]\text{ADP} \beta S$, also with inversion of configuration (36). Since both pyruvate kinase and
adenylate kinase catalyzed the transfer with inversion of configuration at the chiral phosphorus center, the $[\beta^{18}O]$-ADPβS must have had the same configuration at $\beta$-phosphorus as that in $^{18}O$thiophosphoenolpyruvate. The configuration in $[\beta^{18}O]$ADPβS was determined by stereoselective phosphorylation using (i) pyruvate kinase and PEP, and (ii) acetate kinase and acetyl phosphate, which selectively phosphorylated the pro-$S$ and pro-$R$ $\beta$-oxygens in ADPβS, respectively (70,87). The $[^{18}O]$ATPβS was subjected to analysis to determine whether $^{18}O$ was bridging or nonbridging. The results of this analysis showing inversion of configuration in the PEP carboxykinase reaction are described in this chapter.

Preparation of $[^{18}O]$thiophosphoenolpyruvate from the PEP carboxykinase catalyzed reaction and pyruvate kinase catalyzed transfer of its $[^{18}O]$thiophosphoryl group to ADP forming $[^{\gamma^{18}}O]ATP\gamma S$ were carried out by John Richard and Rex Sheu in this laboratory. As described in section 2.9, adenylate kinase was used to catalyze the transfer of the $\gamma$-[$^{18}O]$thiophosphoryl group from $[^{\gamma^{18}}O]ATP\gamma S$ to AMP, generating $[\beta^{18}O]$-ADPβS which was divided into two parts and phosphorylated individually to form (R_p)- and (S_p) $[^{18}O]$ATPβS.
Samples of (R<sub>p</sub>) and (S<sub>p</sub>) [<sup>18</sup>O]ATP<sub>8S</sub> (0.9 μmoles each) obtained as described in section 2.9 were individually treated with 1.3 equivalents of NaIO<sub>4</sub> at neutral pH and ambient temperature for 15 minutes. Excess 2-mercaptoethanol was added to consume periodate and the solutions were heated at 50°C for 30 minutes at pH 10.5. The samples of [<sup>18</sup>O]2-thiotriphosphate were purified by chromatography through DEAE-Sephadex A-25 columns (0.7 x 12 cm). The cleaved adenosine moiety, mercaptoethanol and iodide were washed off the column with 30 ml of 0.3 M TEAB; and the samples of [<sup>18</sup>O]-thiotriphosphate were eluted with 30 ml of 0.7 M TEAB. After removing TEAB (described in section 2.4), the [<sup>18</sup>O]2-thiotriphosphates dissolved in 0.5 ml ethanol were alkylated with diazoethane (section 2.7) and then subjected to hydrolysis at 100°C for 1 hour in capped Eppendorf micro test tubes. The hydrolyzed products were again ethylated and analyzed by gas chromatography-mass spectroscopy (section 2.7).

Because of the anticipated instability of [<sup>18</sup>O]-thiophosphoenoxytriphosphate and the complication caused by the contamination of ATPase activity in the commercially obtained acetate kinase, the two [<sup>18</sup>O]ATP<sub>8S</sub> samples obtained amounted to only 0.9 μmoles each. To minimize
the loss of trialkyl esters of phosphate and thiophosphate while removing the solvent with a stream of N₂, diazomethane was used as the alkylation agent in place of diazomethane. Large losses of trimethyl esters had been observed at this step in earlier experiments.

The \(^{18}\)O-enrichments in the triethyl esters are given in Table 6. \(^{18}\)O was shown to be present in the pro-S position of \([^{18}\text{O}]\text{ADPβS}\), demonstrating the S-configuration at the \(\beta\)-phosphorus in contrast to the R-configuration of \((\text{R}^\text{p})\ [^{18}\text{O}]\text{GTPγS}\).

### 4.3 Mechanism of PEP Carboxykinase

The foregoing result demonstrating that PEP carboxykinase catalyzes thiophosphoryl group transfer with inversion of configuration suggests that the mechanism of the reaction does not involve a double displacement at phosphorus and a covalent thiophosphoryl-enzyme as an intermediate. As discussed in Chapter 1, such mechanisms involve overall retention of configuration in chiral thiophosphoryl groups.

In the case of PEP carboxykinase, it appears to be most likely that the phosphoryl donor, \((\text{R}^\text{p})\ [^{18}\text{O}]\text{-GTPγS}\) in this case, and oxaloacetate are simultaneously bound at adjacent sites in the ternary complex of
<table>
<thead>
<tr>
<th>Phosphorylating system</th>
<th>Sample</th>
<th>Triethyl phosphate</th>
<th>Triethyl thiophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate kinase</td>
<td>(R_p) [β-^{18}O]ATPβS</td>
<td>1.4 ± 1.2</td>
<td>84.3 ± 3.2^b</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>(S_p) [β-^{18}O]ATPβS</td>
<td>16.8 ± 1.0</td>
<td>53.4 ± 4.9</td>
</tr>
</tbody>
</table>

^aThe GC column temperature was programmed as follows: 90°C for the initial 6 minutes followed by 6°C per minute increasing up to 240°C. Triethyl phosphate has a retention time of 9 min and triethyl thiophosphate 13 minutes. Multiple scans were made at m/e 169-173 for triethyl thiophosphate and at m/e 98-102 for triethyl phosphate. The ions of interest are the m/e 99 (m) and 101 (m+2) for triethyl phosphate and m/e 170 (m) and m/e 172 (m+2) for triethyl thiophosphate.

^bThe ^18O-enrichment in (R_p) [γ-^{18}O]ATPγS used in this study was 87.0%.
E·MgGTP·oxaloacetate. This is consistent with the steady state kinetics, which requires such a ternary complex. The decarboxylation of oxaloacetate proceeds as described in equation 27 to produce the enolate anion of pyruvate.

\[
\begin{align*}
\text{CH}_2=\text{C}-\text{C}-\text{O}^- & \quad \rightarrow \quad \text{CO}_2 + \text{CH}_2=\text{C}-\text{C}-\text{O}^- \quad \text{(27)} \\
\end{align*}
\]

\[
\begin{align*}
\text{CH}_2=\text{C}-\text{C}-\text{O}^- & \quad \rightarrow \quad \text{CH}_2=\text{C}-\text{C}-\text{O}^- + \text{Mg-GDP} \quad \text{(28)} \\
\end{align*}
\]

The enolate displaces the \(\text{[^{18}O]}\)thiophosphoryl group of \((\text{R_p})\) \(\text{[\gamma-^{18}O]GTP}\gi\), producing \((\text{S_p})\) \(\text{[^{18}O]}\)thiophosphenol-pyruvate and GDP according to equation 28.

The mechanism outlined in equations 27 and 28 is a reasonable one that is consistent with all of the facts. Available evidence does not, however, definitively exclude the possibility that the thiophosphoryl transfer might be concerted with the decarboxylation step. Nor is
it possible to exclude the admittedly unattractive possibility of a triple or pentuple displacement mechanism on the basis of kinetic and stereochemical evidence alone. Evidence supporting such mechanisms is presently not available. There is, in contrast, precedent for equations 27 and 28 in both enzymatic and nonenzymatic reactions. The decarboxylation of oxaloacetate is catalyzed by metal ions as well as by enzymes (126); and the phosphorylation of enolpyruvate is catalyzed by pyruvate kinase (127). Inversion of configuration at phosphorus is also observed in the pyruvate kinase reaction (39), so equation 28 may be a common step in the pyruvate kinase and PEP carboxykinase reactions.

It is also interesting that pyruvate kinase itself catalyzes the decarboxylation of oxaloacetate (equation 27). It appears that these two enzymes have much in common.
CHAPTER 5
SYNTHESIS OF NUCLEOTIDES

A major part of the laboratory research described in the foregoing sections of this dissertation depended upon the availability of nucleotides containing sulfur or sulfur and \(^{18}O\) in place of \(^{16}O\) at known positions. These compounds had to be synthesized. In some cases the synthetic procedures had been published in detail and were reproduced as described, specifically the syntheses of (R) and (S) \([\beta-^{18}O]ADP\) (111), the originally described procedures for synthesizing ADP\(\beta\) (111) and ATP\(\beta\) (70), the synthesis of AMPS (68) and \([\alpha-^{18}O_2]AMPS\) (57,111). Some improvements were made in the synthesis of \([\alpha-^{18}O_2]AMPS\). 2',3'-Methoxymethylene adenosine and 2',3'-methoxymethylene-AMP were also synthesized by published procedures (126,127).

In the course of the research new, improved methods were developed for synthesizing ADP\(\beta\) and ATP\(\beta\). The new methods produce these nucleotides more conveniently and in much better yields than those originally reported by Eckstein and coworkers. An intermediate in the new synthesis of ATP\(\beta\) is \(P^1\)-adenosine-P\(^3\)-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate], a compound
that has heretofore not been described. The procedure for synthesizing this compound (Figure 14) produces a very good yield (>60%) and is a new method for synthesizing polyphosphates. It should be generally applicable to the synthesis of triphosphates.

The new synthetic procedures are detailed in this chapter.

5.1 Synthesis of ADPβS

The synthesis of ADPβS was based on that described by Richard and Frey for (R_p) and (S_p) [β-¹⁸O]ADPβS (111). Several modifications were made to reduce the number of chromatographic steps, thereby enhancing the overall yield.

An aqueous solution containing 1 mmole of the triethyl-ammonium salt of 2',3'-methoxymethylene-AMP was dried by flash evaporation in a rotary evaporator. The residue was dissolved with 20 ml of methanol, and 1 mmol of tri-n-octylamine (0.45 ml) was added to the solution. The mixture was stirred until it became clear. After removing methanol by rotary evaporation, the residue was further dried by twice dissolving it in anhydrous dimethyl formamide (DMF) and removing the solvent by rotary evaporation. The DMF-dried 2',3'-methoxymethylene-AMP was desiccated in vacuo over P₂O₅ for 24 hours in
preparation for coupling to activated AMPS prepared as follows. The triethylammonium salt of AMPS (2 mmoles) was dried first by rotary evaporation to remove water. Methanol (40 ml) and tri-n-octylamine (2 mmoles, 0.9 ml) were added to the nucleotide. The mixture was stirred until a clear solution was obtained and then dried by rotary evaporation. The residue was further dried by twice dissolving it in anhydrous DMF and removing the solvent by rotary evaporation. The AMPS was then dried in vacuo over P$_2$O$_5$ for 24 hours.

The mono-tri-n-octylammonium salt of AMPS was dissolved in 8 ml of triethyl phosphate added directly to the round bottom flask in which AMPS had been desiccated. To the solution were added diphenyl phosphorochloridate (3 mmoles, 0.64 ml) and tri-n-butylamine (4 mmoles, 1 ml). The flask was stoppered and the mixture stirred at ambient temperature for 3 hours. A mixture consisting of 150 ml of petroleum ether (b.p. 60-80°C) and 50 ml of diethyl ether (new can) was added to the solution with stirring at the end of the 3 hour period. This mixture was kept at 0°C for 30 minutes. The ether layer was decanted and 4 ml of dioxane was added to the residue. The solution was evaporated to dryness using a rotary evaporator. The
dried mono-tri-n-octylammonium salt of 2',3'-methoxy-methylene-AMP was dissolved in 2 ml of pyridine and transferred to the flask containing \textsuperscript{1}P-diphenylphosphoryl-AMPS. The reaction mixture was stirred at ambient temperature for 16 hours. After removing pyridine by rotary evaporation, diethyl ether (20 ml) was added to the residue and water was used to extract the nucleotides until $A_{260}$ of the aqueous extracts were less than 0.5. The pH of the aqueous extract was immediately adjusted to 8 with NaOH to avoid losing the methoxymethylene group. Three mmoles of NaIO$_4$ were added to the aqueous extract and the solution was kept at ambient temperature for 30 minutes, at which time 30.0 mmoles of 2-mercapto-ethanol were added to quench the periodate degradation and reduce IO$_4^-$ to I$^-$. The pH of the solution was adjusted to 2 by adding HCl, and after 100 minutes at 25$^\circ$C the pH was adjusted to 10.5 by addition of NaOH. This solution was heated at 50$^\circ$C for 30 minutes. The solution was diluted to 2 liters and ADPβS was purified from this solution by chromatography as described in the legend to Figure 22.

The $^{31}$P-NMR spectrum (proton spin decoupled) of ADPβS obtained from above exhibited a $P_\alpha$ doublet at -11.79 ppm and a $P_\beta$ doublet at 32.82 ppm with $J_{\alpha,\beta} = \ldots$
Figure 22. Chromatographic purification of ADPβS.

The crude product solution contained in 2 liters was divided into two 1-liter aliquots, each of which was passed through a 4 x 50 cm column of DEAE-Sephadex A-25 in the HCO₃⁻ form (Chapter 2) to adsorb the nucleotides. Nucleotides were eluted from each column using a TEAB gradient having a total volume of 7 liters (3.5 liters of each component, see Chapter 2) and increasing from 0.2 to 0.45 M. Fractions of 23 ml were collected at 16 minute intervals, and selected fractions were analyzed for A₂₆₀ and DTNB reactivity. Shown is one of the elution profiles in which ADPβS was eluted in fractions 241-279. The yield of ADPβS was 575 µmoles from the two columns, or 57.5% based on 2',3'-methoxymethylene-AMP.

Symbols: ••••, A₂₆₀; o—o, DTNB reactivity.
31.74 Hz, in agreement with the values reported for \([\beta^{-18}O]ADP\deltaS\). Thin layer chromatography (as described in section 2.4) also showed that the ADP\deltaS prepared in this manner comigrate with authentic \([\beta^{-18}O]ADP\deltaS\) prepared by the procedure of Richard and Frey. ADP\deltaS was also converted to ATP\deltaS by two different enzymatic phosphorylating systems: (i) pyruvate kinase and PEP, and (ii) acetate kinase and acetyl phosphate as described in Chapter 2.

5.2 Synthesis of 2',3'-Methoxymethylene-ADP\deltaS

\(P^1\)-adenosine-\(P^2\)-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] was synthesized as described by Richard and Frey, except that the \((R_p)\) and \((S_p)\) epimers were not separated. The mixture was converted to 2',3'-methoxymethylene-ADP\deltaS by the following procedure. \((R_p)\) \(P^1\)-Adenosine-\(P^2\)-2',3'-methoxymethylene-5'-[1-thio-1-\(^{18}\)O-diphosphate] was also converted to \((S_p)\) 2',3'-methoxymethylene-[\(\beta^{-18}\)O]ADP\deltaS by the same procedure.

\(P^1\)-adenosine-\(P^2\)-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] \((300 \mu\text{moles})\) was dissolved in 30 ml water and the pH was then adjusted to 8.0. To the solution 450 \(\mu\text{moles}\) of NaIO\(_4\) was added. After stirring at ambient temperature for 30 minutes, 5 \(\mu\text{moles}\) of 2-mercaptoethanol was added and the pH of the solution
was adjusted to 10.5 with NaOH. The reaction mixture was kept at 50°C for 30 minutes and then diluted with water to a final volume of 200 ml for DEAE-Sephadex A-25 column chromatography. The elution profile is shown in Figure 23.

The 2',3'-methoxymethylene-ADPβS obtained gave a positive reaction with DTNB (procedure as described in Chapter 2), an identical UV absorption spectrum as that of 2',3'-methoxymethylene-AMP, and was not degraded by NaIO₄. The ³¹P-NMR spectrum of 2',3'-methoxymethylene-ADPβS gave two doublets with chemical shifts and a coupling constant essentially identical to that of ADPβS:  

δᵣᵣ = 11.07 ppm and  δᵣᵦ = 33.05 ppm with  Jᵣᵦ = 31.70 Hz.

5.3 Syntheses of (Sₚ) [β⁻¹⁸O]ATPβS and (Sₚ) [β,γ-bridging⁻¹⁸O]ATPβS

(Rₚ) [β⁻¹⁸O]ADPβS (20 μmoles) and (Sₚ) [β⁻¹⁸O]ADPβS (25 μmoles) were each converted to ATPβS by phosphorylation with PEP in the presence of pyruvate kinase, according to equations 29 and 30. In ATPβS generated from (Sₚ) [β⁻¹⁸O]ADPβS, the ¹⁸O ended up in the β,γ bridging position; whereas in ATPβS from (Rₚ) [β⁻¹⁸O]-ADPβS, the ¹⁸O remained in the nonbridging position (equation 30). The phosphorylation conditions were as described in the legend to Figure 24. The positions
FIGURE 23. Purification of 2',3'-methoxymethylene-adenosine-5'-[2-thiodiphosphate].

Reaction products were purified by chromatography on a 2.5 x 40 cm DEAE-Sephadex A-25 column. A 3.5 liter linear gradient of TEAB increasing from 0.1 M to 0.5 M was used to elute the compounds (see section 2.4 for procedure). Fractions of 23 ml were collected at a rate of 1.5 ml per minute. Fractions containing $A_{260}$ absorbing materials and thiophosphates were identified as described in Chapter 2. Fractions 119 to 142 were pooled and desalted as described in section 2.4. The pooled fractions contained 223.3 μmoles methoxymethylene-ADPβS, an overall yield of 74.4%, based on the extinction coefficient of 24,000 M$^{-1}$·cm$^{-1}$ for P$^1$-adenosine-P$^2$-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] and 15,000 M$^{-1}$·cm$^{-1}$ for the former.

Symbols: ●-●, $A_{260}$; ○-○, $A_{412}$.
FIGURE 24. Purification of \((S_p) [\beta-^{18}O]ATP\beta S\) and 
\((S_p) [\beta,\gamma\text{-bridging}-^{18}O]ATP\beta S\).

The phosphorylation system contained the following:
40 mM Tris-HCl, pH 8.0, 4 mM MgCl\(_2\), 380 mM KCl, 0.86 mM 
dithiothreitol, 2.5 mM PEP, 2 mM [\beta-^{18}O]ADP\beta S and
400 units of pyruvate kinase. \((R_p) [\beta-^{18}O]ADP\beta S\)
(20 \(\mu\)moles) and \((S_p) [\beta-^{18}O]ADP\beta S\) (25 \(\mu\)moles) were
prepared as described by Richard and Frey (111). The
reaction mixture was incubated at ambient temperature
for 6 hours and the reaction was monitored by thin
layer chromatography as described in section 2.4.
Reaction products were purified by DEAE-Sephadex A-25
column (1.5 x 25 cm) chromatography. A 0.8 liter
linear gradient of TEAB, increasing from 0.2 M to
0.55 M, was used to elute the products. Fractions of
7.5 ml were collected at the rate of 0.5 ml min\(^{-1}\) and
those under the major \(A_{260}\) absorbing peak (\(\bullet\)---\(\bullet\)) were
pooled and desalted as described in Chapter 2 in
preparation for BrCN treatment (section 3.4). The
yield of ATP\beta S was 85%. Shown in Figure 24 is the
elution profile of the \([^{18}O]ATP\beta S\) from \((R_p) [\beta-^{18}O]-
ADP\beta S\); an identical profile obtained for \([^{18}O]ATP\beta S\)
from \((S_p) [\beta-^{18}O]ADP\beta S\) is not shown here.
of $^{18}$O-enrichment were verified by $^{31}$P-NMR spectroscopy (Figure 10).

5.4 Synthesis of $(R_p + S_p) P^1$-Adenosine-$P^3$-2',3'-Methoxymethylene Adenosine-5'-[2-thiotriphosphate]

The syntheses were carried out by one procedure from two different sets of starting materials (Figure 15). The coupling of 2',3'-methoxymethylene adenosine-5'-phosphorodichloridate to ADP$\beta$S followed by working up in water and the coupling of adenosine-5'-phosphorodichloridate to 2',3'-methoxymethylene-ADP$\beta$S followed by
aqueous workup, both gave \((R_p + S_p) P^1\text{-adenosine-P}^3\text{-}
2',3'-\text{methoxymethylene adenosine-5'}-\text{[2-thiotriphosphate]}\)
as the major product.

The triethylammonium salt of 2',3'-methoxymethylene-ADP\(\beta\)S (or of ADP\(\beta\)S) (100 \(\mu\)moles) was converted to its
di-tri-n-butylammonium salt by the following procedure. The aqueous solution of the triethylammonium salt was
concentrated by rotary evaporation and the resulting
residue dissolved in 2 ml of methanol. Two equivalents
(200 \(\mu\)moles) of tri-n-butylamine was added to this
solution. Methanol was then removed by rotary evaporation in vacuo. The residue was dissolved in 2 ml of
water and the solution frozen as a thin film on the wall of the flask. The frozen sample was lyophilized until
dry (12 hours). Separately, adenosine (or 2',3'-
methoxymethylene adenosine) (250 \(\mu\)moles), which had been
desiccated over \(P_2O_5\) in vacuo at 110°C overnight, was
dissolved in 0.7 ml of triethyl phosphate by cautiously
swirling the suspension over an open flame. The clear
solution was immediately cooled in an ice-water bath.
Phosphorus oxychloride (325 \(\mu\)moles) was added to the
solution and the reaction mixture was stirred magnetically
at room temperature for 30 minutes. Triethyl phosphate,
unreacted phosphorus oxychloride, and hydrochloric acid
generated in the reaction were removed by vacuum distillation at 35-40°C into a receiving flask that was cooled in an ice-water bath. The lyophilized tributylammonium salt of 2',3'-methoxymethylene-ADPβS (or of ADPβS) was dissolved in 1.0 ml of hexamethylphosphoramide and transferred to the flask containing the crude adenosine-5'-phosphorodichloridate (or 2',3'-methoxymethylene-5'-phosphorodichloridate). The flask that originally contained the 2',3'-methoxymethylene-ADPβS (or ADPβS) was washed with an additional 0.5 ml of hexamethylphosphoramide; and the rinse was combined with the reaction mixture, which was sealed and stirred at room temperature for 2 hours. Diethyl ether (20 ml) was added to the reaction solution and the resulting suspension centrifuged. After decanting the supernatant fluid, the precipitate was dissolved with 10 ml 1 M TEAB, and stirred at ambient temperature for 1 hour. Reaction products were purified by ion exchange chromatography as described in Figure 25. The yield of P^1-adenosine-P^3-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] from this new synthetic procedure was about 60 to 65%, based on the thionucleotide used. The major product was judged to be P^1-adenosine-P^3-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] based on the following
FIGURE 25. Purification of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thio-phosphate].

The 10 ml 1 M TEAB solution containing products from reactions described in section 5.4 was diluted to 150 ml with water and purified by DEAE-Sephadex A-25 (2.5 x 40 cm) chromatography. Elution was done with a 2.4 liter TEAB linear gradient, increasing from 0.15 M to 0.5 M and fractions of 12 ml were collected at a rate of 1 ml per minute. Fractions 149 to 179 were pooled and desalted as described in Chapter 2 and 1575 $A_{260}$ units of the dinucleoside thiophosphate were recovered.

Symbols: $\bullet--\bullet$, $A_{260}$. 
findings. The major product collected from fractions 149 to 179 (Figure 25) has identical $r_f$ value as that of the corresponding product synthesized in low yield by coupling ADP$_8$S to 2',3'-methoxymethylene-AMP through diphenyl phosphorochloridate activation. The $^{31}$P-NMR spectrum of this compound in Figure 26 showed two groups of peaks present with chemical shifts in the region characteristic of the $\alpha$-phosphoryl and $\beta$-thiophosphoryl group of ATP$_8$S (92). Treating an aliquot of the solution containing this compound in the order (1) NaIO$_4$, (2) pH 2, ambient temperature, 100 minutes, (3) pH 10.5, 50°C, 30 minutes gave ATP$_8$S, as shown in a later section.

To obtain the maximum yield of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate], it is important to use freshly distilled phosphorus oxychloride and hexamethylphosphoramide; all glassware must be carefully dried prior to use.

This procedure has been used with (R$^p$) 2',3'-methoxymethylene [$\beta$-$^{18}$O]ADP$_8$S to prepare [$^{18}$O]-enriched products with similar results. It has also been performed with [$^{18}$O]H$_2$O workup for the coupling product to investigate aspects of the coupling and hydrolytic workup mechanisms as described in Chapter 3.
FIGURE 26. $^{31}$P-NMR spectrum of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thio-triphosphate].

Shown is the proton spin decoupled $^{31}$P-NMR spectrum obtained as described in Chapter 2 using the Nicolet 200 MHz instrument.

The $P^2$ signal appears downfield in the spectrum and has a chemical shift of 30.65 ppm and coupling constant of 24.86 Hz. The $P^1+P^3$ signals have a chemical shift centered at -11.71 ppm and coupling constant of 24.91 Hz. The integration ratio of the two sets of signals is approximately $(P^1+P^3):P^2 = 2:1$. 

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Figure 26
5.5 Preparation of ATPβS from $P^1$-Adenosine-$P^3$-2',3'-Methoxymethylene Adenosine-5'-[2-thiotriphosphate]

The method developed in this laboratory for preparing ADPβS from $P^1$-adenosine-$P^2$-2',3'-methoxymethylene adenosine-5'-[1-thiodiphosphate] can be applied with slight modification to the degradation of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] to ATPβS in excellent yield. The triethylammonium salt of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] (240 $A_{260}$ units) in 2 ml of aqueous solution was adjusted to pH 8.4. Sodium periodate (15 µmoles) was added to the solution with stirring. After incubation at ambient temperature for 20 minutes, 150 µmoles of 2-mercaptoethanol were added and the pH of the solution was adjusted to 2.0 with 1 N HCl. The solution was kept at ambient temperature for 120 minutes to allow for hydrolytic removal of the methoxymethylene group, and then the pH was readjusted to 10.5 with 1 N NaOH. The solution was kept in a 50°C water bath for 20 minutes during which the periodate-cleaved adenosyl group was removed by alkaline β-elimination, and it was then diluted to 20 ml with water before being subjected to ion exchange chromatography to purify ATPβS as described in Figure 27. One single major peak with
FIGURE 27. Purification of ATPβS from P\textsuperscript{1}-adenosine-
P\textsuperscript{3}-2',3'-methoxymethylene adenosine-5'-
[2-thiotriphosphate].

ATPβS was purified by ion exchange chromatography
on a DEAE-Sephadex A-25 column (1.5 x 15 cm). A
0.7 liter linear gradient of TEAB, increasing from
0.2 M to 0.6 M, was used to elute the product.
Fractions of 4.5 ml were collected at 10 minute
intervals. ATPβS was eluted in fractions 28 through
36, which were pooled and desalted as described in
Chapter 2.
absorbance at 260 nm was eluted between fractions 28 and 36. The pooled fractions contained 121 A$^{260}_2$ units of material. The product was identified as ATPgS by thin layer chromatography, $^{31}$P-NMR analysis and as a substrate for hexokinase. As expected, only half of the ATPgS added into the assay mixture was consumed since two diastereomers were expected in the synthesis. The $^{31}$P-NMR spectrum of ATPgS also indicated the presence of the two diastereomers. The yield of ATPgS from degradation of $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate] was 85.7% as calculated on the basis of the extinction coefficient 25,500 cm$^{-1}$.M$^{-1}$ for $P^1$-adenosine-$P^3$-2',3'-methoxymethylene adenosine-5'-[2-thiotriphosphate].

The extinction coefficient was obtained by treating the commercially available $P^1,P^3$-di(adenosine-5'-)triphosphate with snake venom phosphodiesterase and calculating the A$^{260}_2$ change before and after the enzyme treatment.
LIST OF REFERENCES


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125. (Rp) [γ-18O]GTPyS was prepared in this laboratory by the procedure of Richard and Frey.
