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STEREOCHEMICAL COURSE OF THE BASE-CATALYZED HYDROLYSIS OF CYCLIC 2′-DEOXYADENOSINE 3′,5′-[^{17}O, ^{18}O]MONOPHOSPHATE

SHUJAATH MEHDI, JEFFREY A. CODERRE and JOHN A. GERLT[1]
Department of Chemistry, Yale University, New Haven, CT 06511, U.S.A.

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Abstract—At 100° and in 0.2 M $Ba(OH)_2$ the R_p diastereomer of cyclic 2′-deoxyadenosine 3′,5′-[^{17}O, ^{18}O]monophosphate is hydrolyzed to a 4:1 mixture of the S_p diastereomer of 3′-[^{16}O, ^{17}O, ^{18}O]dAMP and the R_p diastereomer of 5′-[^{16}O, ^{17}O, ^{18}O]dAMP, respectively, demonstrating that the hydrolysis reaction is accompanied by complete inversion of configuration at phosphorus. This finding establishes that pseudorotation of pentacoordinate reaction intermediates is unimportant in this chemical hydrolysis reaction.

Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) is well established as an important effector molecule which regulates a wide variety of biochemical processes. For example, in *Escherichia coli* the intracellular concentration of cyclic AMP responds to the presence or absence of glucose in the growth medium, and this variation in cyclic AMP levels is responsible for the expression of catabolite repression of the transcription of the structural genes for enzymes of nonessential catabolism. In higher organisms, the intracellular concentration of cyclic AMP is controlled by extracellular stimuli such as a number of peptide hormones, and the resulting variations in cyclic AMP levels allosterically activate or inhibit the catalytic activity of appropriate target enzymes. A major research goal of this laboratory is to provide fundamental information about the chemical and physical properties of the cyclic AMP molecule[2] as well as to establish detailed chemical mechanisms for the reactions catalyzed by the enzymes which are responsible for its synthesis and breakdown, adenylate cyclase and cyclic AMP phosphodiesterase, respectively.

Knowledge of the stereochemical course of the conversion of reactants to products provides valuable mechanistic information which can serve to eliminate mechanisms for interconverting reactants and products. This is true for enzymic reactions as well as nonenzymic reactions, and considerable advances have been made recently in the methodology for stereochemical studies of displacement reactions at tetrahedral P.[3] For enzymic systems, the results of stereochemical studies commonly are used to deduce whether the reaction catalyzed by the enzyme involves the necessary formation of a covalent adduct between the phosphoryl group being transferred and a nucleophile in the active site.[4] Such conclusions are thought reliable because each nucleophilic displacement reaction in the active site of an enzyme occurs by an in-line, direct displacement pathway, whose stereochemical course is inversion of configuration at P. Thus, an inversion of configuration, which implies a single displacement (or in general an odd number of displacements), is interpretted as a direct displacement of the donor nucleophile by the accepting nucleophile, whereas a retention of configuration, which implies two displacements (or in general an even number of displacements), is interpretted as a two step mechanism involving the necessary formation of a phosphorylated enzyme intermediate. The attractiveness of stereochemical studies lies in the fact that very important mechanistic information can be obtained for enzymes which are available only in small amounts or in impure form, as long as there is a sufficient amount of the desired catalytic activity to convert workable amounts of substrate to product. Since the availability and/or purity of enzymes are real considerations for both adenylate cyclases and cyclic nucleotide phosphodiesterases, we have chosen stereochemical studies for our initial investigations of the mechanisms of the reactions catalyzed by these enzymes.

We have reported that both cyclic[5] and acyclic[6] phosphodiesters can be prepared chiral by virtue of O isotope substitution of the phosphoryl oxygens and that the configurations of such materials can be established by utilizing the phenomenon of [^{18}O]-perturbations on the ^{31}P NMR resonances of phosphate esters which was described independently by the laboratories of Cohn,[7] Lutz[8] and Lowe.[9] Using cyclic [^{16}O, ^{18}O]dAMP as the substrate, we found that the pyrophosphorolysis reaction catalyzed by the adenylate cyclase purified from *Brevibacterium liquefaciens* occurs with inversion of configuration at P;[10] by microscopic reversibility, the nucleophilic displacement of pyrophosphate from the α-P atom of the nucleoside triphosphate substrate must occur with inversion of configuration at P. This stereochemical result is most easily explained by the direct interconversion of nucleoside triphosphate and cyclic nucleotide without the necessary formation of a nucleotidylated enzyme intermediate.

We have also prepared cyclic [^{17}O, ^{18}O]dAMP[11] so that the stereochemical course of its hydrolysis could be studied using water with the natural abundance of O isotopes as solvent. We have determined that the stereochemical course of the reaction catalyzed by a commercially available cyclic nucleotide phosphodiesterase is inversion of configuration at P;[12] this result is most easily explained by the direct attack of water on the cyclic nucleotide substrate to form the acyclic 5′-mononucleotide product. The same stereochemical result was obtained by Eckstein and Stec *et al.* when a diastereomer of the phosphorothioate analog of cyclic AMP, cyclic AMPS, was used as the substrate.[13] Thus result and our result obtained with the O chiral substrate provide one comparison (of several now available) which demonstrates that the stereochemical course, and presumably most other features of the catalytic mechanism, are unaffected by the substitution of a phosphoryl O atom with a S atom, even though cyclic AMPS is processed by the phosphodiesterase approx. 1/200 as rapidly as the natural substrate.

The interpretation of stereochemical results is based in part on the assumption that pseudorotatory processes involving trigonal bipyramidal intermediates[14] are unimportant; this assumption is supported by experimental evidence which shows that those enzymic reactions for which independent chemical data exist conclusively demonstrating the participation of a covalent intermediate invariably proceed with retention of configuration and those reactions for which direct transfer of the phosphoryl group is accepted invariably proceed with inversion of configuration. However, in their study of the cyclic nucleotide phosphodiesterase,[13]Eckstein and Stec postulated an alternative mechanism also consistent with the stereochemical outcome in which a nucleotidylated enzyme intermediate is formed with retention of configuration, i.e. with pseudorotation, and breaks down to product with inversion of configuration; such a mechanism does not violate the principle of microscopic reversibility. This mechanism was proposed since no stereochemical data were available for any ring-opening reaction involving a 6-membered ring monoanionic phosphodiester; as a result, the relative energies of potential trigonal bipyramidal intermediates in the enzyme catalyzed reaction could not be evaluated, and this suggested that the in-line mechanism deduced from the stereochemical result should be viewed as tentative.

In the research described in this paper, we have examined the stereochemical course of the base-catalyzed hydrolysis of one of the diastereomers of cyclic [^{17}O, ^{18}O]dAMP,[11] thereby providing the necessary experimental data to allow evaluation of the alternative mechanism proposed by Eckstein and Stec.[13] Our results demonstrate that the hydrolysis of the 6-membered cyclic phosphate ester ring in the 3′,5′-cyclic nucleotide proceeds with complete inversion of configuration at P to form either of the observed hydrolysis products, 3′- and 5′-dAMP; this finding renders unlikely the alternative mechanism proposed by Eckstein and Stec. In addition, this study and our previously reported work on the cyclic nucleotide phosphodiesterase reaction[12] provide the first comparison of the stereochemical consequences of the enzymic and nonenzymic hydrolysis of a phosphate ester.

RESULTS

Despite the pronounced thermodynamic instability of 3′,5′-cyclic nucleotides,[2] these 6-membered ring cyclic phosphodiesters are kinetically very stable. Using the reaction conditions (0.2 M $Ba(OH)_2$ and 100°) described by Khorana *et al.*,[15] cyclic dAMP is converted in 90 min to a mixture of 3′- and 5′-dAMP in approx. 50% yield. Integration of a ^{31}P NMR spectrum of the mixture showed that the 3′- and 5′-mononucleotides are formed in a 4:1 ratio, with the remainder of the P being present as inorganic phosphate. Under these conditions, the mononucleotides are observed to hydrolyze slowly to inorganic phosphate, with no evidence of isomerization being apparent.

Since our experimental plan was to determine the stereochemical course of the hydrolysis of cyclic [^{17}O, ^{18}O]dAMP in water having the natural abundance of O isotopes, it was first necessary to determine unambiguously whether the hydrolysis reaction proceeds with C–O or P–O bond cleavage since this information could remain cryptic in the stereochemical study. In addition, hydrolysis via C–O bond cleavage would be of little interest to enzymic reactions involving phosphate esters which proceed with P–O bond cleavage. Unlabeled cyclic dAMP was hydrolyzed according to Khorana's conditions using water which was enriched to the extent of 45.5% with ^{18}O.

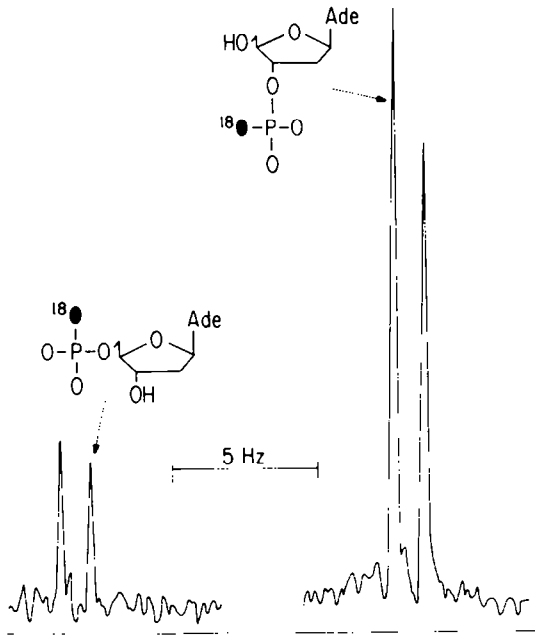


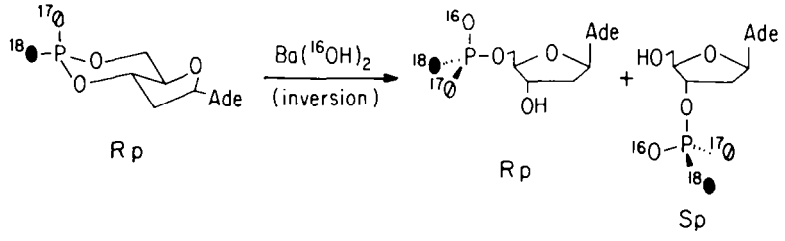
Fig. 1. Proton-decoupled ^{31}P NMR spectrum at 81 MHz of 3′- and 5′-dAMP derived from the hydrolysis of unlabeled cyclic dAMP in water enriched 45.5% with ^{18}O. The spectrum was obtained with a 200 Hz sweep width and a 3 sec acquisition time; 434 transients were collected prior to multiplication of the total free induction decay (FID) initially by the exponential linebroadening factor of −0.64 Hz and then an apodization factor of 1.1 sec and finally Fourier transformation. The approximate chemical shift of the 5′-dAMP is 3.85 ppm and that of 3′-dAMP is 3.33 ppm.

After lyophilization of the solvent, neutralization with triethylammonium bicarbonate, and centrifugation to remove the precipitated barium carbonate, the ^{31}P NMR spectrum shown in Fig. 1 was obtained. Two resonances are observed for both the 3′- and 5′-dAMP products, with the downfield resonance in each set being that of unlabeled phosphate ester and the upfield being that of phosphate ester labeled with a single ^{18}O.[7-9] The isotopic compositions calculated from the ratio of the intensities of the resonances for each mononucleotide (46.2% ^{18}O for the 3′-dAMP and 44.0% ^{18}O for the 5′-dAMP) are in good agreement with the isotopic composition of the solvent (45.5% ^{18}O); no resonances attributable to multiply labeled species can be detected. Therefore, the data derived from this spectrum demonstrate that each mononucleotide is formed with P–O bond cleavage and that no exchange of solvent oxygen with phosphate ester oxygen occurs under the reaction conditions.

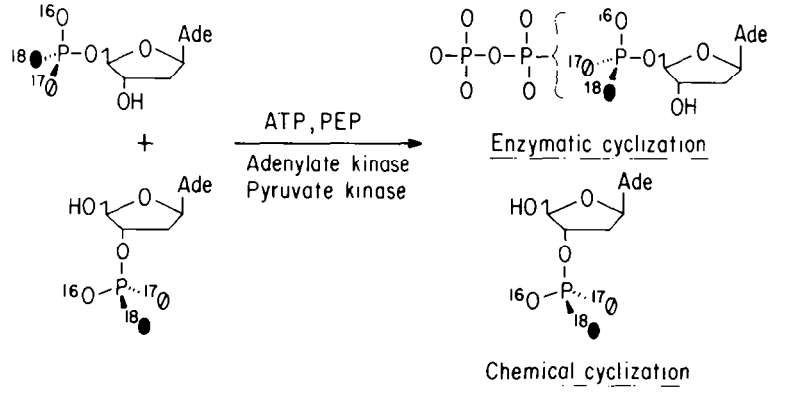
Five hundred μmoles of the R_p diastereomer of cyclic [^{17}O, ^{18}O]dAMP[11] were hydrolzed in 90 min using Khorana's conditions (Scheme 1). The structures for the products shown in Scheme 1 are those expected if the hydrolysis reaction yields each mononucleotide with inversion of configuration at P. Following purification by ion-exchange chromatography on DEAE-Sephadex A-25, 250 μmoles of a 4:1 mixture of [^{16}O, ^{17}O, ^{18}O]-chiral 3′- and 5′-dAMP were isolated. Although it is possible to easily separate a mixture of 3′- and 5′-AMP by ion-exchange chromatography, such is not the case for a mixture of 3′- and 5′-dAMP; however, our method of configurational analysis does not require separation of the mixture at this stage.

The 5′-dAMP present in the mixture of mononucleotides was pyrophosphorylated by the coupled action of adenylate kinase and pyruvate kinase in the presence of a trace of ATP and excess phosphoenolpyruvate (PEP) (Scheme 2).[12,16] The resulting mixture of three types of triply labeled dATP (formed by virtue of the inability of adenylate kinase to discriminate among the three stable isotopes of O present in the phosphoryl group of the chiral 5′-dAMP) was separated from unreacted 3′-dAMP by ion-exchange chromatography. Fifty μmoles of labeled dATP and 200 μmoles of oxygen chiral 3′-dAMP were recovered.

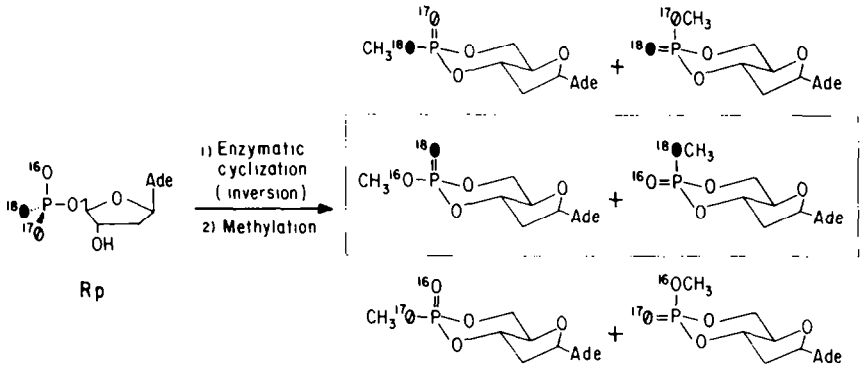
The configurational analysis of the 5′-dAMP product was accomplished by first using the adenylate cyclase from *B. liquefaciens* to convert the mixture of three types of triply labeled dATP to a mixture of three types of doubly labeled and chiral cyclic dAMPs; this enzymic reaction has been established to proceed with inversion of configuration at P.[12] Following the procedure described by Lowe *et al.*,[17] the K-salt of the isolated cyclic dAMP was methylated with methyl iodide in dimethyl sulfoxide solution and in the presence of 18-crown-6 to afford a nearly quantitative yield of the equatorial and axial methyl esters of cyclic dAMP. The structures of the methyl esters obtained from these enzymic and chemical transformations (assuming that the hydrolysis reaction occurred with inversion of configuration) are shown in Scheme 3. The ^{31}P NMR spectrum of the methyl esters is shown in Fig. 2; the downfield set of four resonances is associated with the equatorial ester and the upfield set of four resonances is associated with the axial ester. Each set of resonances is composed of four signals due to the perturbations caused by zero, one, two and three bonds between ^{18}O and ^{31}P, with an increasing number of such bonds leading to progressively upfield changes in chemical shift; resonances associated with species



Scheme 1. Barium catalyzed hydrolysis of the R_p diastereomer of cyclic [^{17}O, ^{18}O]dAMP, assuming inversion of configuration at phosphorous.



Scheme 2. Pyrophosphorylation of 5′-dAMP in the mixture of 3′- and 5′-dAMP.



Scheme 3. The methyl esters obtained from the 5′-dAMP.

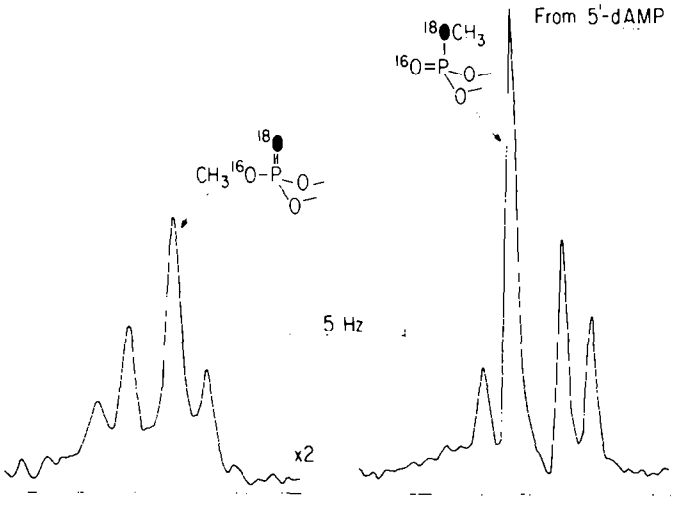
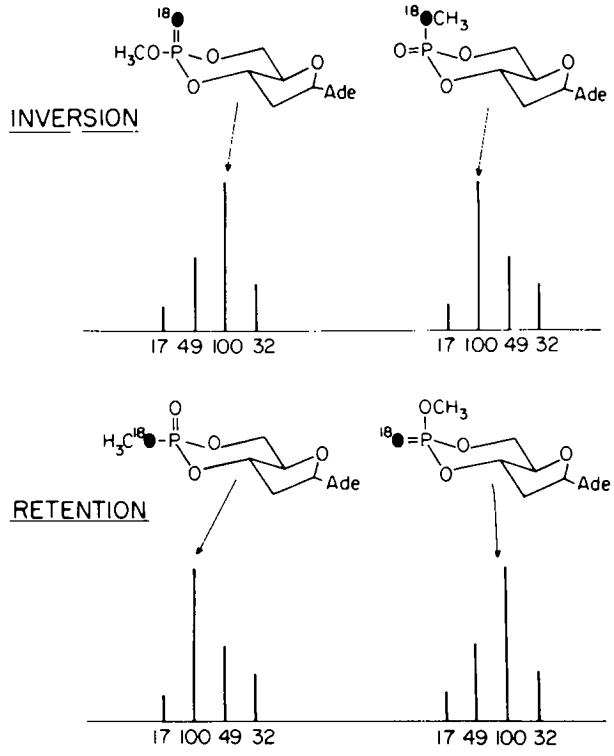


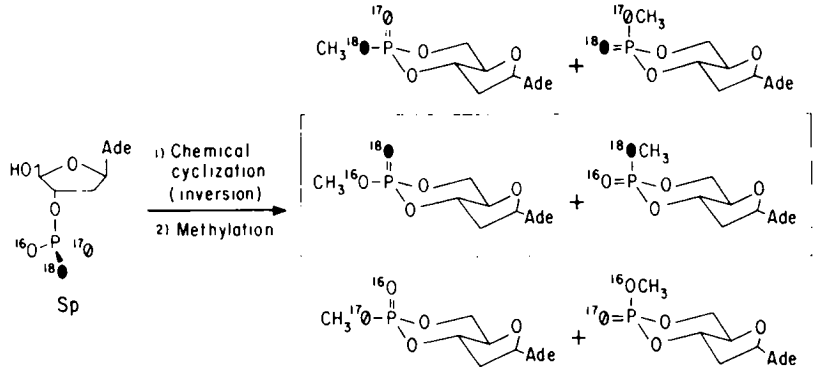
Fig. 2. Proton-decoupled ^{31}P NMR spectrum at 81 MHz of the methyl esters of labeled cyclic dAMP derived from the 5′-dAMP obtained by the hydrolysis of cyclic [^{17}O, ^{18}O]dAMP. The spectrum was obtained with a 200 Hz sweep width and a 3 sec acquisition time; 434 transients were collected prior to multiplication and Fourier transformation of the total FID as described in the legend in Fig. 1. The approximate chemical shift of the equatorial methyl ester is −1.93 ppm and that of the axial methyl ester is −3.30 ppm.

containing ^{17}O are not observable in this spectrum due to the extensive line broadening resulting from the quadrupolar relaxation mechanism provided by the ^{17}O nucleus.[18] Given the ^{17}O and ^{18}O enrichments of the starting cyclic [^{17}O, ^{18}O]dAMP, it is possible to calculate the expected intensities of the four resonances for each ester assuming either complete inversion or complete retention of configuration in the hydrolysis reaction, and these are shown in Scheme 4. Comparison of these idealized spectra with that shown in Fig. 2 easily allows the conclusion that the hydrolysis reaction to form 5′-dAMP proceeded with inversion of configuration at P, i.e. the predominant [^{16}O, ^{18}O]-labeled triesters are those shown in the box in Scheme 3. Within the experimental error of this method of configurational analysis, the magnitudes of the ratios of the configurationally pertinent resonances (those with one and two bonds between ^{18}O and ^{31}P) are in accord with *complete* inversion of configuration at the chiral phosphodiester P atom.

The configurational analysis of the isolated 3′-dAMP product was accomplished following a chemical activation and cyclization sequence of reactions. The 3′-dAMP was reacted with diphenylphosphorochloridate under anhydrous conditions, and the anhydride formed between 3′-dAMP and diphenyl phosphate was exposed to potassium t-butoxide, resulting in the formation of a mixture of the three types of doubly labeled chiral cyclic dAMP; Lowe has estimated the stereospecificity of this reaction to be in excess of 94%.[17] Following the procedure used for the configurational analysis of the 5′-dAMP, the labeled cyclic dAMPs were methylated with methyl iodide, and the structures of the methyl esters obtained from the chemical transformations (assuming that the hydrolysis reaction occurred with inversion of configuration) are shown in Scheme 5. The ^{31}P NMR spectrum of the methyl esters is shown in Fig. 3. The intensities of the four resonances to be expected for each ester assuming either complete



Scheme 4. The ^{31}P NMR spectra of the methyl esters of labeled cyclic dAMP assuming either inversion of retention of configuration in the hydrolysis reaction.



Scheme 5. The methyl esters obtained from the 3′-dAMP.

inversion or complete retention of configuration in the hydrolysis reaction are also those presented in Scheme 4. Thus, the spectrum in Fig. 3 allows the conclusion that the hydrolysis reaction to form 3′-dAMP proceeded with inversion of configuration at phosphorus, i.e. the predominant [^{16}O, ^{18}O]-labeled triesters are those shown in the box in Scheme 5. The relative intensities of the signals associated with species with zero and three bonds between ^{18}O and ^{31}P demonstrate a small amount of washout of O isotope during the chemical activation reaction; this was approximated as 17%. Based on this figure and the error inherent in this method of configurational analysis, the magnitudes of the ratios of the configurationally pertinent resonances are in accord with *complete* inversion of configuration at the chiral phosphodiester P atom.

DISCUSSION

In principle four trigonal bipyramidal species can be formed by the attack of hydroxide ion on the tetrahedral P atom of cyclic dAMP; these are shown in Scheme 6. Structures **1** and **2** result from the attack of the hydroxide ion opposite the 3′- and 5′-ester O atoms, respectively; structures **3** and **4** result from the attack of hydroxide ion adjacent to the ester O atoms. These structures are formulated on the basis of one of the accepted rules for nucleophilic displacement reactions at P,[14] i.e. the attacking nucleophile occupies an apical position.

These rules also specify that leaving groups can depart only from apical positions;[14] therefore, **1** and **2** can breakdown directly to 5′- and 3′-dAMP, respectively, whereas **3** and **4** must first undergo a pseudorotation to place either ester O atom in an apical

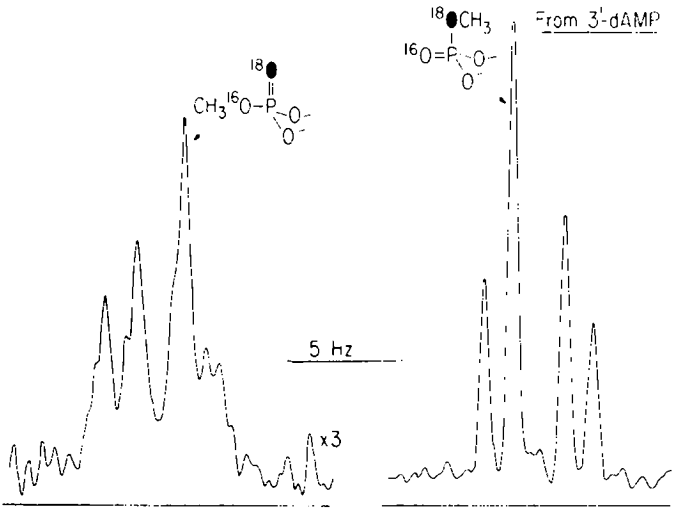
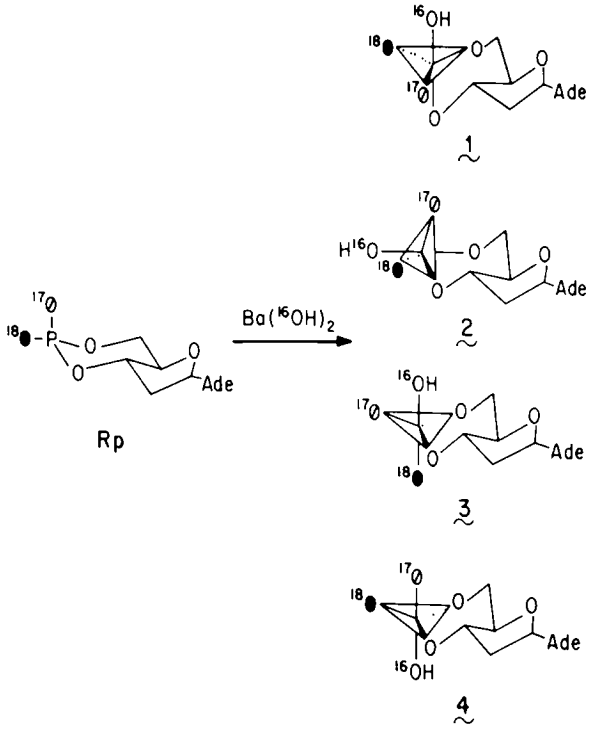


Fig. 3. Proton-decoupled ^{31}P NMR spectrum at 81 MHz of the methyl esters of labeled cyclic dAMP derived from the 3′-dAMP obtained by the hydrolysis of cyclic [^{17}O, ^{18}O]dAMP. The spectrum was obtained with a 400 Hz sweep width and a 3 sec acquisition time; 2693 transients were collected prior to multiplication and Fourier transformation of the total FID as described in the legend to Fig. 1. The approximate chemical shift of the equatorial methyl ester is −1.93 ppm and that of the axial methyl ester is −3.30 ppm.

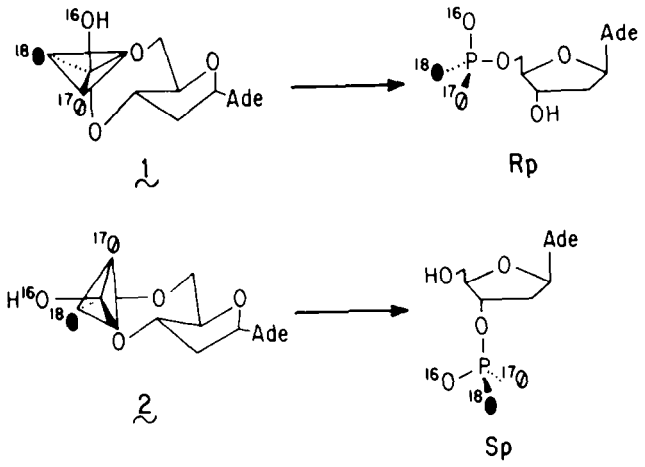


Scheme 6. The trigonal bipyramidal species which can be formed by cyclic [^{17}O, ^{18}O]dAMP.

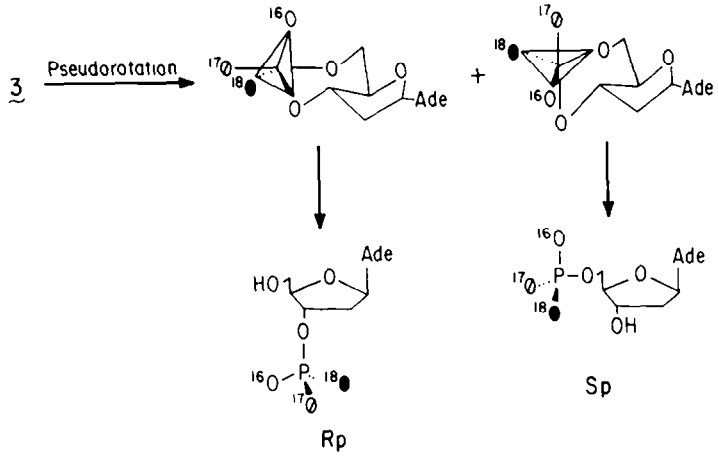
position. Reaction pathways involving **1** and **2** will yield products with inversion of configuration at P, as shown in Scheme 7. Reaction pathways involving **3** and **4** must involve a pseudorotation if these are to yield products, and the analyses shown in Schemes 8 and 9 reveal that such reactions will be accompanied by retention of configuration at P. If the hydrolysis were to involve equal contributions by all four trigonal bipyramidal species, the acyclic products could be formed with racemization at the chiral P atom.

The results we have presented establish that the hydrolysis of cyclic [^{17}O, ^{18}O]dAMP proceeds with inversion of configuration to form both 3′- and 5′-dAMP (Scheme 1), thereby implying that the products are formed only from trigonal bipyramidal species **1** and **2** without the intervention of either **3** or **4**, i.e. *pseudorotatory processes are unimportant in the chemical hydrolysis of the cyclic phosphodiester.*

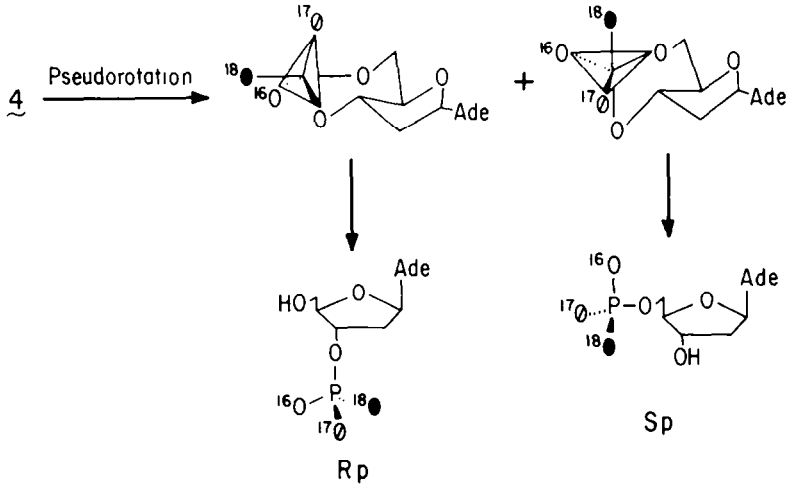
The study of phosphoranes and the reactions of P(III) and P(IV) compounds which involve the inter-



Scheme 7. Reaction pathways for trigonal bipyramidal species **1** and **2**.



Scheme 8. Reaction pathway for trigonal bipyramidal species **3**.



Scheme 9. Reaction pathway for trigonal bipyramidal species **4**.

mediacy of P(V) species has led to generalizations concerning the relative apicophilicities of substituents in trigonal bipyramidal species, with electron withdrawing atoms or groups preferring apical positions and electron donating atoms or groups preferring equatorial positions.[19] Whereas these preferences are clearly apparent in studies of displacement reactions of acyclic systems, they can be altered by the presence of a ring. It is accepted that 5-membered rings prefer an apical–equatorial rather than a diequatorial arrangement, since the former disposition is considered to be of lower conformational energy by virtue of less bond angle distortion.[20] No experimental data exist which allow prediction of the preferred arrangement of 6-membered rings in trigonal bipyramidal species, although the results of a quantum mechanical study of the trigonal bipyramidal species derived from cyclic AMP suggest that the apical–equatorial geometry is of lower energy.[21] Accordingly, the conclusions derived from our stereochemical study regarding the contributions by structures **1**, **2**, **3** and **4** in Scheme VIII cannot be definitively explained. However, it is pertinent to point out that under the strongly basic reaction conditions the trigonal bipyramidal species are present as their dianions,[22] therby permitting the presence of two electron donating anionic O atoms in the equatorial plane; this situation would increase the stability of **1** and **2** relative to **3** and **4**.

In view of our finding that the chemical hydrolysis of cyclic dAMP proceeds without the intervention of pseudorotation, the alternate mechanism proposed by Eckstein and Stec for the cyclic nucleotide phosphodiesterase reaction involving the formation of a nucleotidylated enzyme intermediate with retention of configuration of ^{31}P is very unlikely. Admittedly, the results of chemical experiments need not be directly relevant to the mechanisms of enzymic reactions, but it is likely that the geometry of an enzyme active site would be optimized to stabilize a single transition state or intermediate intervening between substrate and product; stablization of two trigonal bipyramidal structures would require differing geometries of cationic residues or metal ions to stabilize the anionic charges of such species. Moreover, consideration of molecular models reveals that a pseudorotation involving a molecule such as cyclic AMP could require extensive movement of the atoms far removed from the P center, and such motion is not easily rationalized in terms of the specificity of the enzyme.

This study and our previous examination of the stereochemical course of the enzyme catalyzed hydrolysis[12] of cyclic [^{17}O, ^{18}O]dAMP provide the first comparison of the stereochemical consequences of the enzymic and chemical *hydrolysis* of a phosphate ester.[23] The simplest explanation for the identical stereochemical results is that the reactions share a similar mechanism, i.e. the direct, in-line attack of water on the P to displace the ester O leaving group. The large rate acceleration exhibited by the enzyme catalyzed reaction, perhaps as large as 10^{15}, does suggest, however, that the enzyme optimizes catalysis through factors which cannot be provided by a divalent metal ion, e.g. more effective deprotonation of water to provide the hydroxide ion nucleophile, geometrically optimized charge neutralization of the phosphate ester anion, approximation of the hydroxide ion and phosphate ester, and a decrease in the polarity of the medium within the active site.

A caveat must be expressed about the mechanism proposed for the enzyme catalyzed reaction.[24] Nucleophilic catalysis by carboxylate groups in the enzyme catalyzed hydrolysis of phosphate esters would lead to the formation of acyl phosphate or acyl phosphate ester intermediates, which could hydrolyze with C–O rather than P–O bond cleavage. The stereochemical consequence of such a mechanism involving the participation of a covalent intermediate would be inversion of configuration; thus, an inversion of configuration should not be viewed as sufficient evidence to rule out covalent catalysis.

EXPERIMENTAL

Materials and methods. Adenylate kinase, pyruvate kinase, lactate dehydrogenase, phosphenolpyruvate, and ATP were purchased from Sigma. NADH was the product of P–L Biochemicals. All other chemicals were the finest grades commercially available.

The R_p diasteromer of cyclic [^{17}O, ^{18}O]dAMP was prepared as previously outlined.[11] The oxygen isotopes in the positions designated "^{17}O" in the Schemes are derived from $H_2{}^{17}O$, and mass spectral analysis showed that the isotopic composition of this position is 17% ^{16}O, 51% ^{17}O, and 32% ^{18}O; the isotopic content of the position designated "^{18}O" is approx. 99% ^{18}O. Unlabeled cyclic dAMP was prepared by a literature procedure.[15]

Adenylate cyclase from *B. liquefaciens* was isolated according to the published procedure.[25]

The ^{31}P NMR spectra shown in the Figures were obtained at 81 MHz using a Varian XL-200 spectrometer; the spectra were obtained with broadband proton decoupling. Other spectra were obtained at 32 MHz using a Varian CFT-20 spectrometer equipped with a phosphorus probe. Chemical shifts are measured relative to external 85% H_3PO_4, with positive values being downfield of this reference.

Hydrolysis of unlabeled cyclic dAMP. Two mL of a 20 mM solution of cyclic dAMP in 0.2 M $Ba(OH)_2$ was heated for 90 min in a polyethylene tube immersed in a boiling water bath. After the brown soln had cooled to room temp, excess 2 M triethylammonium bicarbonate was added, and the precipitated $BaCO_3$ was removed by centrifugation. The residue was repeatedly washed with water and centrifuged, and the combined superatant solns were evaporated to dryness by rotary evaporation. After removal of excess triethylammonium bicarbonate by repeated evaporation from EtOH the residue was dissolved in 1.2 mL of 0.1 M EGTA, pH 9.0, containing 20% D_2O. A ^{31}P NMR spectrum revealed the presence of 5′-dAMP ($\delta_p = 3.85$ ppm, triplet, $J_{HP} = 5$ Hz), 3′-dAMP ($\delta_p = 3.33$ ppm, doublet, $J_{HP} = 7.8$ Hz), and inorganic phosphate ($\delta_p = 2.63$ ppm) in an approximate ratio of 1:4:3, uncorrected for the NOE; a trace of cyclic dAMP was detected.

When 5′-dAMP was heated in $Ba(OH)_2$ soln under similar conditions, the only species detected by ^{31}P NMR were 5′-dAMP and inorganic phosphate.

In a similar experiment, the residue obtained following removal of $BaCO_3$ was dissolved in water and applied to a 2 × 30 cm column of DEAE-Sephadex A-25 (HCO_3^-), and the products of the hydrolysis were eluted with a 1.8 L linear gradient of 0–0.4 M triethylammonium bicarbonate, pH 7.5. The acyclic mononucleotides eluted together and were obtained in 50% yield. This mixture was dissolved in EGTA soln as previously described, and a ^{31}P NMR spectrum obtained under conditions to suppress the NOE and with a long pulse delay showed that 3′- and 5′-dAMP were present in a ratio of 4:1.

Demonstration of P–O *cleavage.* A hydrolysis was carried out as described in the previous section except that water enriched to the extent of about 50% with ^{18}O was used as solvent. After the hydrolysis, the water was recovered by

bulb-to-bulb distillation. A small amount of PCl_5 was hydrolyzed with 20 μL of the recovered water, and the resulting phosphoric acid was methylated with diazomethane; analysis by gas chromatography/mass spectrometry showed that the ^{18}O enrichment was 45.5%.

Following removal of the Ba, the mixture of mononucleotides was purified by ion-exchange chromatography. The mixture was dissolved in 0.5 mL D_2O and percolated through a small column of Chelex-100 (Na^+) contained in a Pasteur pipette plugged with a Whatman 934-AH glass fiber filter directly into an acid-washed 10 mm NMR tube; the column was washed with 0.5 mL H_2O, and 0.4 mL of a solution of 0.1 M EGTA, pH 9.0, was filtered into the NMR tube. This procedure was followed to ensure that narrow linewidths would be obtained when the 81 MHz ^{31}P NMR spectrum shown in Figure 1 was obtained.

Hydrolysis of cyclic [^{17}O, ^{18}O]*dAMP*. A soln of 500 μmoles of the R_p diastereomer of cyclic [^{17}O, ^{18}O]dAMP in 25 mL of 0.2 M $Ba(OH)_2$ was heated for 90 min in a Teflon bottle immersed in a boiling water bath. After cooling and removal of the Ba, the acyclic mononucleotides were purified by chromatography on DEAE-Sephadex A-25. The fractions containing hydrolysis products were combined and evaporated to afford 260 μmoles of a mixture of 3′- and 5′-[^{16}O, ^{17}O, ^{18}O]dAMP.

Configurational analysis of 5′-[^{16}O, ^{17}O, ^{18}O]*dAMP*. A mixture (48 mL) with the following composition was prepared:[12,16] 5.80 mM of the mixture of the labeled 3′- and 5′-dAMP (1.16 mM 5′-dAMP and 4.64 mM 3′-dAMP), 1.15 nM ATP, 2.86 mM phosphoenolpyruvate, 2.9 mM NADH, 2.86 mM $MgCl_2$, 60 mM KCl, 32 mM Tris-HCL, pH 8.0, 25 U/mL adenylate kinase, 50 U/mL lactate dehydrogenase, and 10 U/mL pyruvate kinase. The progress of the reaction was monitored by measuring the absorbance at 340 nm, which decreased from 18.2 to a constant value of 3.2 in 95 min. A soln of 50 mM sodium pyruvate (5 ml) was added to convert the remaining NADH and NAD (since NADH coelutes with 3′-dAMP from DEAE-Sephadex). The mixture was applied to a 2 × 40 cm column of DEAE-Sephadex A-25 at 4° and eluted with a 2 L linear gradient of 0–0.7 M triethylammonium bicarbonate, pH 7.5. Appropriate fractions were evaporated to yield 50 μmoles of labeled dATP and 200 μmoles of chiral 3′-dAMP.

The isolated dATP was converted to cyclic dAMP in a mixture[12] (25 mL) which contained 2.0 mM labeled dATP, 10 mM pyruvate, 1 mM dithiothreitol, 20 mM $MgSO_4$, 0.1 M Tris-HCl, pH 9.0, 0.1 mg/mL BSA, and 0.023 U/mL adenylate cyclase from *B. liquefaciens*. After 4 hr, the mixture was applied to a 2 × 40 cm column of DEAE-Sephadex A-25 and eluted with a 1.8 L linear gradient of 0–0.4 M triethylammonium bicarbonate, pH 7.5. Appropriate fractions were evaporated to yield 50 μmoles of labeled cyclic dAMP.

Labeled cyclic dAMP (50 μmoles) was converted to the K-salt by percolation through a 2 mL column of Dowex-50 (K^+). After evaporation, 40 mg of 18-crown-6 was added to the residue,[17] and the mixture was dissolved and evaporated several times from water. The residue was dried by repeated evaporation from dry DMF and dissolved in 0.8 mL dry DMSO. MeI (140 μL) was added, and the mixture was stirred in a stoppered flask for 22 hr. The brown soln was filtered through a glass fiber filter into a 10 mm NMR tube, and 1.7 mL of d_6-DMSO was added. The 81 MHz ^{31}P NMR spectrum obtained is shown in Fig. 2.

Configurational analysis of 3′-[^{16}O, ^{17}O, ^{18}O]*dAMP*. The unreacted labeled 3′-dAMP isolated following the pyrophosphorylation of the labeled 5′-dAMP was contaminated with sulfate, which was introduced by using ammonium sulfate suspensions of adenylate kinase, and lactate dehydrogenase. The sulfate was removed by precipitation with barium acetate, and the excess Ba was then precipitated by the addition of excess triethylammonium bicarbonate. The solids were removed by centrifugation and washed with water, and the supernatant solns were combined and repurified by ion-exchange chromatography on DEAE-Sephadex A-25. Sulfate-contaminated 3′-dAMP (100 μmoles) was processed to yield 70 μmoles of pure mononucleotide.

The labeled 3′-dAMP was percolated through a column of Dowex-50 (pyridinium), and the eluant and washings were evaporated. The residue was suspended in MeOH and 1.1 equivs of tri-n-octylamine was added; the suspension was warmed at 60° until a clear soln was obtained.[17] Following removal of the MeOH, the residue was dried by repeated evaporation of dry DMF. The residue was dissolved in 0.9 mL dry DMF and 0.9 mL dry dioxane, and this soln was dried by stirring for 3 hr following addition of several 4 Å molecular sieves. Freshly distilled diphenylphosphorochloridate (0.9 equiv) and tri-n-butylamine (1 equiv) were added. After stirring for 30 min, a soln of 0.2 g of BuOK in 15 mL DMF was added. The reaction was quenched after 10 min by pouring it into 20 mL wet Amberlite IR-120 (pyrdinium) resin. The resin was removed by filtration and thoroughly washed, and the combined filtrates were evaporated to dryness. The residue was dissolved in 50 mL water, washed three times with ether, and the water was removed by rotary evaporation. An aqueous soln of the residue was applied to a 2 × 40 cm column of DEAE-Sephadex, and the labeled cyclic dAMP was eluted with a 1.8 L linear gradient of triethylammonium bicarbonate, pH 7.5. Appropriate fractions were evaporated to afford 40 μmoles of labeled cyclic dAMP.

This sample was methylated and prepared for ^{31}P NMR spectroscopy as described in the previous section. The 81 MHz ^{31}P NMR spectrum obtained is shown in Fig. 3.

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