taminated with some oxidation residues derived from 9-BBN.

This alcohol sample was treated[13a] with 20 mg (0.08 mmol, ~10 equiv) of (+)-α-methoxy-α-((trifluoromethyl)phenyl)acetyl chloride (95.7% ee), to give ~2 mg of the (*R*)-MTPA esters. The 250-MHz ^{1}H NMR spectra showed a characteristic doublet at δ 0.715 (J = 6.25 Hz) for the *R,R* diastereomer. Graphical curve resolution of the very small shoulder due to the upfield signal of the δ 0.706 doublet (which is due to the *R,S* diastereomer) was done in several different fashions at both 250 and 500 MHz to estimate a (93 ± 5):1 ratio for the *R,R* and *R,S* diastereomers (see Figure 1). Correction for the optical purity of the (+)-MTPA chloride implies an enantiomeric excess of 97 ± 5% for the (2*Z*,5*R*)-diene product **4**.

For confirmation of the correctness of the ^{1}H NMR assignments, this sample of the (*R*)-MTPA ester was spiked with an authentic sample of (*R*)-**8** (23% ee). The 250-MHz ^{1}H NMR spectrum of this mixture showed a major doublet at δ 0.716 and a minor doublet at δ 0.706.

B. Analysis of Cope Product 5. The amount of enriched (*E*)-diene **5** on hand proved insufficient for hydroboration and Mosher ester analysis. As a result, the absolute configuration and optical purity of **5** were determined by hydroboration and esterification of a mixture of **4** and **5**. A 9-mg (0.045 mmol) sample of the Cope products **4** and **5**, which had been somewhat enriched in **5** by preparative GC (**4**:**5** = 1.6:1), was hydroborated with 0.063 mmol of 9-BBN exactly as described in part A to give 4.7 mg (48%) of a 1.55:1 ratio of (*Z*)- and (*E*)-3,5-dimethyl-6-phenyl-5-hepten-1-ols, respectively.

This alcohol sample was treated[13a] with 20 mg of (+)-α-methoxy-α-((trifluoromethyl)phenyl)acetyl chloride (95.7% ee) to give 8.1 mg (85%) of the corresponding (*R*)-MTPA esters. The 250-MHz ^{1}H NMR spectrum showed a doublet at δ 0.715 for the *R,R* diastereomer of the *Z* isomer and a doublet at δ 0.947 (J = 6.26 Hz) for the *R,S* diastereomer of the *E* isomer. A small shoulder for the *R,R* diastereomer of the *E* isomer could be seen,for which graphical curve resolution at 250 MHz allowed a (92 ± 6):1 ratio of diastereomers to be estimated. Correction for the purity of the (+)-MTPA chloride used indicates an enantiomeric excess of 96 ± 6% for the diene product **5**.

For confirmation of the ^{1}H NMR assignments, this sample of (*R*)-MTPA esters was spiked with an authentic sample of the *E* ester prepared from (*R*)-**17** (23% ee). The 250-MHz ^{1}H NMR spectra showed retention of the doublet at δ 0.948 and the buildup of a doublet at δ 0.958 (J = 6.60 Hz).

Thermal Rearrangement of (*R*)-Diene 3. Preparation of (2*Z*,5*R*)-3,5-Dimethyl-2-phenyl-2,6-heptadiene (4) and (2*E*,5*S*)-3,5-Dimethyl-2-phenyl-2,6-heptadiene (5) and Determination of Absolute Configurations and Optical Purities. A 22-mg (0.11 mmol) sample of (*R*)-**3** (99% ee based on the enantiomeric purity of **1**) was sealed in an evacuated Pyrex ampule and heated at 240 °C for 16 h. The brown residue was purified by filtration through a short plug of silica gel (hexane) to give 11 mg (50%) of a 1:1 mixture of dienes **4** and **5**.

Hydroboration with 9-BBN and esterification[13a] with (+)α-methoxy-α-((trifluoromethyl)phenyl)acetyl chloride (95.7% ee) gave a mixture of diastereomeric (*R*)-MTPA esters. The 250-MHz ^{1}H NMR spectrum showed a doublet for the *R,R,Z* diastereomer **8** at δ 0.716 (J = 6.63 Hz) and a doublet for the corresponding *R,S,E* diastereomer at δ 0.948 (J = 6.60 Hz). Because of the small quantity of this sample available, accurate isomer ratios could not be determined. As with the product from the $PdCl_2$-catalyzed reaction, the *R,S,Z* diastereomer and the *R,R,E*-diastereomer were detectable only as small shoulders and are clearly minor (<15%) components.

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Registry No. 1, 83541-10-0; **1**·Et ester, 83478-46-0; (*R,R*)-**2**, 83478-45-9; (*R,S*)-**2**, 83478-32-4; **3**, 83541-11-1; **4**, 83478-33-5; **5**, 83478-34-6; **6**, 83478-35-7; **7**, 83478-36-8; (*R,R*)-**8**, 83478-37-9; (*R,S*)-**8**, 83478-38-0; **9**, 83478-54-0; **10**, 1117-61-9; **11**, 83541-12-2; **12**, 83541-13-3; **13**, 83478-39-1; **14**, 83478-40-4; **15**, 83478-41-5; **16**, 83478-42-6; **17**, 83478-43-7; **18**, 83478-53-9; (2*S*,4*E*)-2-methyl-2-phenyl-4-hexen-1-ol, 83478-44-8; (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride, 20445-33-4; (±)-(*E*)-2-methyl-2-phenyl-4-hexen-1-ol, 83541-14-4; ethyl iodide, 75-03-6; (3*S*,5*E*)-2,3-dimethyl-3-phenyl-5-hepten-2-ol, 83478-47-1; (1*R*,4*R*)-6-(benzyloxy)-4-methyl-1-phenylhexanol, 83478-48-2; (1*S*,4*R*)-l-(benzyloxy)-4-methyl-1-phenylhexanol, 83478-49-3; (2*R*,4*R*)-6-(benzyloxy)-2,4-dimethyl-1-phenyl-1-hexanone, 83478-50-6; (2*S*,4*R*)-6-(benzyloxy)-2,4-dimethyl-1-phenyl-1-hexanone, 83478-51-7; 7-(benzyloxy)-3,5-dimethyl-2-phenyl-2-heptanol, 83478-52-8; bis(acetonitrile)palladium dichloride, 14592-56-4; (±)-(*Z*)-3,5-dimethyl-6-phenyl-5-hepten-1-ol, 83541-15-5; (±)-(*E*)-3,5-dimethyl-6-phenyl-5-hepten-1-ol, 83541-16-6.

Hydrolysis of Adenosine 5′-Triphosphate: An Isotope-Labeling Study[1]

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Abstract: We have used a combination of ^{18}O-labeling experiments and kinetic studies to clarify the nonenzymatic hydrolytic pathways of adenosine 5′-triphosphate (ATP) at pH values ranging from 0 to 8.3. In 1 N and 0.1 N HCl, the data are consistent with the hypothesis that hydrolysis occurs by addition–elimination, with initial attack 93% γ and 7% β; both lead only to ADP + P_i. In the subsequent hydrolysis of the ADP to AMP + P_i, attack is 83% β and 17% α. At pH 8.3, the data are consistent with the hypothesis that hydrolysis occurs by elimination–addition. Over the entire pH range studied, we detected no oxygen exchange between water and ATP, ADP, or P_i. Nonenzymatic hydrolysis and isotopic analysis of the resultant P_i comprise a preferred means of assaying the isotopic enrichment of [γ-^{18}O]ATP to be used in studies of enzymatic processes.

A clear picture of the mosaic of events at the molecular level that together constitute the enzymatic hydrolysis of adenosine 5′-triphosphate (ATP),[3] as, for example, in muscle contraction, remains an unattained objective. Among the many modern experimental tools that today loom large in biochemical research, the use of stable isotope labeling as a mechanistic probe[4] has

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(3) Appreviations used: ATP, ADP, and AMP = adenosine 5′-tri-, -di-, and -monosphosphate; P_i and PP_i = inorganic phosphate and pyrophosphate. O with no superscript mass specification denotes unlabeled oxygen and, in particular, the ^{16}O isotope.

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appeared particularly promising, but in studies of ATP hydrolysis this promise has been only partially fulfilled. Unanswered questions center largely in three aspects of the work. (i) More than a single reaction path may be followed. (ii) Exchange of oxygen atoms with the molecular environment could complicate the task of translating isotopic analyses into chemical information. (iii) An optimal strategy for use of the ^{18}O-labeling approach, including both the synthesis of labeled compounds and the required isotopic analyses, has not been established.

In the hope of reducing these ambiguities, we undertook an ^{18}O-labeling study, using mass spectrometry for the isotopic analyses, to supplement previous studies on the nonenzymatic hydrolysis of ATP.[5-18] We drew added incentive for the present investigation from our discovery during a recent literature review[19] that organic chemists and mass spectrometrists, both working with phosphate esters and related species, had remained, in some important respects, oblivious to each other's efforts for a quarter of a century.

The nonenzymatic hydrolysis of ATP can in principle take place via four primary pathways:

attack on P_γ: $H_2O + ATP \rightleftharpoons ADP + P_i$ (1)

attack on P_β: $H_2O + ATP \rightleftharpoons P_i + ADP$ (2)

$H_2O + ATP \rightleftharpoons AMP + PP_i$ (3)

attack on P_α: $H_2O + ATP \rightleftharpoons PP_i + AMP$ (4)

The hydrolysis of ADP can occur via two primary pathways:

attack on P_β: $H_2O + ADP \rightleftharpoons AMP + P_i$ (5)

attack on P_α: $H_2O + ADP \rightleftharpoons P_i + AMP$ (6)

The hydrolysis of PP_i proceeds via one pathway, the result of which is the incorporation of the oxygen from water into one-half of the P_i generated by the route

$$H_2O + PP_i \rightleftharpoons 2P_i \quad (7)$$

A prior kinetic study in one of our laboratories provided pertinent data on the hydrolysis of ATP in 0.01 M aqueous solutions at 70 °C in the pH range 0–10.[20] In particular, it produced evidence that rules out PP_i formation via either P_β or P_α attack, i.e., paths 3 and 4, and consequently their contribution to P_i yield via path 7 is also negligible. Hydrolysis rates were determined with a liquid-chromatographic technique that also permitted examination of the products generated at various stages of the reaction. The findings indicated that (i) hydrolysis in 1 N ($t_{1/2}$ = 5 min; 10^4k = 1390 min^{-1}) and 0.1 N ($t_{1/2}$ = 35 min; 10^4k = 198.0 min^{-1}) HCl probably occurs by an addition–elimination mechanism in which intermediates with pentacovalent phosphorus, oxyphosphoranes, are generated from the protonated acid, the neutral acid, and the monoanion; (ii) hydrolysis at pH 8.3 ($t_{1/2}$ = 100.5 h; 10^4k = 1.15 min^{-1}) probably occurs by the elimination–addition mechanism, in which the monomeric metaphosphate anion, PO_3^-, is formed as an intermediate from the tetraanion, ATP^{4-}, and the trianion, $ATPH^{3-}$, in accord with an earlier suggestion.[21] The mechanism of hydrolysis of the dianion, which is one of the species present in the solution in the pH range 2–6, remains obscure. Over the entire pH range 0–10, ATP hydrolysis proceeds in stepwise fashion, leading first to ADP and then to AMP, with release of a molecule of P_i at each step. The second step is slower than the first by factors of 2.3 and 3.5, respectively, in 1 N and 0.1 N HCl and by a factor of 3 at pH 8.3.

A framework for our experimental approach is fairly well-defined from experience with ^{18}O-labeling in studies of enzymatic reactions of ATP.[22] Typically, ATP with the P_γ group enriched in ^{18}O is hydrolyzed and the ^{18}O content of the released P_i is taken as the basis for a chain of inference to the intervening chemistry.[23-34] A procedure commonly employed in the past to follow the tagged atoms consists of conversion of the recovered P_i to CO_2 for isotopic analysis by mass spectrometry.[23,24,27-29,35-38] This CO_2 strategy has come to be recognized as wasteful of information; such waste can be avoided by converting the P_i instead to a volatile phosphate ester, provided only such esterification can be accomplished with no oxygen exchange. Trimethyl[25,26,30-32,34,39-42] and tris(trimethylsilyl)[25,33,43,44] phosphates have been so employed. We elected to work with the trimethyl ester.

Such a labeling experiment requires knowledge of the isotopic content not only of the hydrolysis product but also of the reactant ATP—in particular, the P_γ group of the labeled ATP. One means used to obtain this information has been a control experiment, hydrolysis by myosin CaATPase, which has been considered to lead to P_i containing exclusively three oxygen atoms derived from the ATP P_γ group and one from water.[32,33] Thus, the isotopic composition of the P_i resulting from such a control hydrolysis of [γ-^{18}O]ATP has served an essential function in providing a starting point for evaluating that of the ATP P_γ group. Even though we

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have used this procedure, we have been concerned about how free such hydrolyses are of contributions from other possible reaction paths. One objective of the present study was to find an independent method of arriving at the isotopic composition of the ATP P_γ group and thereby to test the validity of the calcium-mediated enzymatic hydrolysis for this purpose. As is reported below, we have achieved this objective.

Experimental Section

We have examined via ^{18}O labeling the hydrolysis of ATP in 1 N and 0.1 N HCl and at pH 8.3. We started with a two-pronged approach, hydrolyzing labeled ATP with ordinary water and unlabeled ATP with ^{18}O-enriched water. We soon abandoned the latter, however, in view of the large uncertainties introduced by the low isotopic enrichments of the water employed (8–20%) and consequently of the P_i produced.

To test for possible oxygen exchange between P_i and medium, we have treated labeled KH_2PO_4 with ordinary water and unlabeled KH_2PO_4 with ^{18}O-enriched water, also in 1 N and 0.1 N HCl and at pH 8.3, using 0.01 M solutions at 70 ± 1 °C in all cases. The results of these tests are presented in Table I.[45] They reveal no exchange.

Pyridine was dried by refluxing with and distilling from calcium hydride; it was stored over 4-Å sieves. Nucleotides were analyzed by high-performance liquid chromatography (HPLC) using procedures previously described.[20] ^{18}O-Enriched water was obtained from Bio-Rad Laboratories.

Materials. ^{18}O-Enriched potassium dihydrogen phosphate was prepared by a modification of the procedure of Risley and Van Etten.[39] Phosphorus pentachloride (0.82 g, 3.9 mmol) was added in small portions over 10 min to ^{18}O-enriched water (0.50 g, 25 mmol, 50 mol % excess) at 20 °C. The reaction was run in a drybox under a nitrogen atmosphere. After the addition was complete the solution was sealed in a small flask and kept at 50 °C for 48 h. The excess water and hydrogen chloride were removed under vacuum (0.5 mm) at 20 °C, and the remaining syrup was dissolved in 2 mL of water. The pH of the solution was adjusted to 4.5 with 10% potassium hydroxide solution, and the potassium dihydrogen phosphate was precipitated by the addition of two volumes of 100% ethanol. The salt was washed with 100% ethanol and then with ether. After the mixture was dried for 45 min at 100 °C, 433 mg (83%) of the phosphate salt was isolated.

Bis(4'-morpholino-*N*,*N*'-dicyclohexylcarboxamidinium) adenosine 5'-diphosphomorpholidate was made as described by Wehrli et al.[46] Analysis by HPLC showed it to be free of nucleotide impurities.

[γ-^{18}O]Adenosine 5'-triphosphate was prepared by a slight modification of the procedure of Wehrli et al.[46] ^{18}O-Enriched potassium dihydrogen phosphate (326 mg, 2.25 mmol) dissolved in 3 mL of water was applied to a 10 × 1.25 cm column of Bio-Rad AG 50W-X8 resin (H^+ form). The column was eluted with 30 mL of water and the eluent evaporated under vacuum (0.5 mm) at 20 °C to a syrup. The syrup was dissolved in 15 mL of dry pyridine and tri-*n*-butylamine (417 mg, 2.25 mmol) was added. The pyridine was evaporated under vacuum (0.5 mm) at 20 °C. The evaporation was repeated 3× with 15 mL aliquots of pyridine to render the salt anhydrous. Residual pyridine was removed by repeating the evaporation 3× with dry benzene. The residue was kept under vacuum at 20 °C to remove the last traces of solvent. The adenosine 5'-diphosphomorpholidate salt (800 mg, 0.75 mmol) was rendered anhydrous by evaporating 4× with 15-mL aliquots of pyridine. Residual pyridine was removed by evaporating 3× with benzene. The salt was dried a further hour at 20 °C.

The dried phosphate salt was dissolved in 5 mL of anhydrous dimethyl sulfoxide (Me_2SO) and added to the morpholidate. The flask that contained the phosphate was rinsed twice with 4-mL aliquots of Me_2SO. The combined Me_2SO solutions were kept at 35 ± 1 °C for 30 h. The reaction was ~80% complete at this time. Attempts to allow the reaction to go further to completion resulted in lower yields.

The Me_2SO solution was poured into 80 mL of cold water and applied to a 33 × 2.8 cm column of DEAE-cellulose (HCO_3^- form). The column was washed with 150 mL of water and then eluted with a linear gradient of triethylammonium bicarbonate (0.005–0.35 M) at a flow rate of 1.5 mL/min. Fractions of 18–20 mL were collected and ~3 L of buffer was required.

The fractions containing ATP were pooled and evaporated to dryness under vacuum at 20 °C. The residue was evaporated 4× with 50 mL of methanol to remove any residual buffer. The tetrakis(triethylammonium) salt that was isolated was converted to the tetrasodium salt with an excess of sodium iodide in acetone. The final yield was 280 mg (63% based on the morpholidate). The purity of the product was determined by high-performance liquid chromatography using a Whatman Partisil PXS 10/25 SAX ion-exchange column (25 cm × 4.6 mm i.d.). Elution was with 0.50 M potassium phosphate (pH 4.5). Detection was by UV at 260 nm. The ATP was generally 99.5% pure, the only detectable impurity being 0.5% ADP.

Hydrolysis of Labeled and Unlabeled ATP in Aqueous Buffers. In all cases the hydrolyses were carried out in 0.01 M solutions at 70 ± 1 °C. The buffer solutions employed were 1 N HCl, 0.1 N HCl, and pH 8.3 (*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid). The ^{18}O-enriched buffers were analyzed by treating 5–10 mg of phosphorus pentachloride with excess buffer solution, followed by heating at 50 °C for 24–48 h. The phosphate produced was isolated as magnesium ammonium phosphate as described below. The hydrolyses in 1 N and 0.1 N HCl were allowed to proceed to complete reaction to AMP and $2P_i$. The hydrolysis at pH 8.3 was stopped after 25% of the ATP had reacted; at this point the product contained ~96% ADP and 4% AMP.

The inorganic phosphate produced in the hydrolyses was isolated by a method similar to that of Boyer[47] as follows. The solution was adjusted to pH 2 and the nucleotides removed by treatment with acid-washed charcoal (Darco G-60). After adjustment to pH 9 with 3 M ammonium hydroxide the solution was concentrated under vacuum (0.5 mm) at 20 °C to 0.5 mL. An equal volume of magnesia mixture was added and the solution left at 5 °C for several hours to allow complete precipitation of the magnesium ammonium phosphate. The phosphate was isolated by centrifugation and washed with small volumes of cold 1:3 ammonia–water, ethanol, and finally with ether. The salt was dried with a stream of nitrogen and stored at −20 °C. No loss of label was observed over periods up to 1 month under these conditions.

Conversion of Magnesium Ammonium Phosphate to Trimethyl Phosphate. The magnesium ammonium phosphate was suspended in 1 mL of water and 1 N hydrochloric acid added dropwise until it just dissolved. The solution was applied to a 6 × 0.8 cm column of Bio-Rad AG 50W-X8 resin (H^+ form) and eluted with several bed volumes of water. The eluent was evaporated to dryness under vacuum (0.5 mm) at 20 °C and the residue dissolved in 0.25 mL of methanol. A solution of diazomethane in ether was added dropwise until the yellow color of diazomethane persisted. Excess diazomethane, ether, and some of the methanol were removed under reduced pressure (30 mm) at ~10 °C to a final volume of 100–150 μL. The trimethyl phosphate solution was analyzed for interfering impurities by gas chromatography (6 ft × $^1/_8$ in. 10% Carbowax 20 M column at 150 °C).

Isotopic Analyses: Manipulations. The constraints of the sample-preparation procedure dictated that the trimethyl phosphates for analysis be introduced into the mass spectrometer in the form of ~1% solutions in 90:10 CH_3OH:H_2O. We set as a goal isotopic analyses good to 1% absolute. This level of uncertainty thus corresponds to 100 ppm of the total solution. For such an analysis, prior separation of the phosphate from at least most of the solvent and possible interfering species is essential. As an extra precaution against unforeseen sources of interference, all too common in mass spectrometry of phosphate esters,[48] we employed two different experimental procedures. In one, which seems to have been adopted as standard by other workers in this area, the solution passes through a gas-chromatographic column that is directly coupled to the mass spectrometer (GC/MS); the chromatographically separated trimethyl phosphate peak is then analyzed on the fly as it emerges from the column. The second, which is described briefly elsewhere,[32] comprises probe microdistillation within the ionization chamber in combination with high-resolution mass measurement (6000–10 000) to separate the desired ion beams from isobaric ones that might also be present (probe/MS). A well-controlled temperature-programmable probe, coupled with the use of 5-Å molecular sieve as sample support, enhances the separation of trimethyl phosphate from solvent and smooths the distillation curve of the phosphate into the ion source, helping to maintain ion currents at a useful level long enough to allow several repetitive scans of the spectrum.

The GC/MS system consists of a Hewlett-Packard 5710A gas chromatograph directly coupled to a Hewlett-Packard 5980A mass spectrometer with a Hewlett-Packard 5933A data system. The separation was accomplished with a 6 ft × $^1/_8$ in. 3% OV-17 column using an oven temperature of 90 °C (isothermal).

The probe/MS measurements were made on a CEC Model 21-110B mass spectrometer. For facilitation of data acquisition and manipulation, the analogue output was fed to a Data General Nova 2/10 computer, using Kratos DS50S software and associated interface. High accuracy in mass measurement was a prime objective in design of the 21-110

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instrument, but accuracy in measuring peak intensities, which constitute the primary data for isotopic analysis as for all quantitative analysis, was a distinctly secondary consideration. Moreover, rapid scanning and nonconstant sample vapor pressure, both inherent in probe injection as in GC/MS, tend to further degrade the quality of intensity measurements.[49] Under such circumstances, averaging over multiple scans is an essential ingredient of data improvement, but the need for multiple scans increases the importance of close control of the rate of sample evolution from the probe. Sample-introduction techniques based on probe injection but incorporating instrumental and procedural modifications to meet these requirements have been referred to as micromolecular distillation or microdistillation.[50] For optimum temperature control, we employed a quartz temperature-programmable probe[51] that can be cooled/heated from −100 to +400 °C. To enhance the separation of trimethyl phosphate from solvent, we packed the sample tube with 5-Å molecular sieve[52] as a support.[53,54] With this combination of techniques, as shown in Figure 1,[45] the ester volatilizes smoothly and its maximum rate of evolution coincides with minima in the corresponding curves for both methanol and water. Other supports that we tried proved less satisfactory.

In both the GC/MS and probe/MS procedures, we measure electron-impact (electron energy 70 eV) spectra, scan a limited mass range (135–155 and 125–180, respectively), and average the isotopic distribution calculated from the molecular ion peak intensities in usually 7–10 consecutive scans. Standard deviations of single measurements for individual isotopic species from the average are usually <1% absolute by GC/MS and <2% absolute by probe/MS.

Isotopic Analyses: Calculations and Results. The 70-eV mass spectrum of trimethyl phosphate[55] shows a molecular ion peak, m/z 140, of high intensity, with but little interference from other peaks in the immediate vicinity: a fragment ion peak at m/z 139 and naturally occurring heavy isotopic peaks at 141 and 142 of respective intensities 3.5%,[55b] 3.5%,[56] and 0.8%[56] that at 140. An incremental contribution at 141, apparently varying with sample pressure from zero to ~5% of the intensity at 140, is attributed to the protonated molecule, arising via an ion molecule reaction. For optimal isotopic analyses with, say, deuterium labeling, these interferences would call for modification of the analytical procedure,[57] but the possible errors they might introduce become vanishingly small for present purposes in consequence of the 2-u mass difference between ^{16}O and ^{18}O. Hence the isotopic analysis boils down simply to measuring peak intensities over the range of m/z 139–149, removing the contributions of naturally occurring heavy isotopic species, discarding the residues of odd m/z values, and normalizing those of m/z 140, 142, 144, 146, and 148—molecular ion peaks containing respectively 0, 1, 2, 3, and 4 ^{18}O atoms—to a total of 100%.

Table II[45] shows four isotopic analyses so arrived at over a 5-month period on a product from reaction of PCl_5 with $H_2{}^{18}O$ and two analyses made a year apart on a second, similar product of lower isotopic purity. Each number listed is itself an average derived from 7–10 consecutive scans, as described above. The examples shown were chosen randomly, and we take the good agreement as evidence of both satisfactory repeatability and long-term stability of the samples.

Table III[45] shows comparative analyses obtained with sample introduction by directly coupled gas chromatography and by probe microdistillation. The results are practically indistinguishable. Thus they render highly unlikely any serious interference that might have persisted in either experimental technique.

Table IV[45] shows the use of the binomial coefficients[58] to relate atom % ^{18}O enrichment to the isotopic composition of phosphate randomly labeled in all four positions. For high enrichment, atom % ^{18}O is calculated from the ratio of triply labeled to quadruply labeled phosphate.

Table VII. Hydrolysis of [γ-^{18}O]ATP at pH 8.3

	A	B	C	D	E	F	G	H
		calcd for x = 96.3%						
			path 1 hydrolysis		paths 5, 6			discrepancies,
mol wt	product analysis[a,b]	ATP P_γ	Σ = 100%	Σ = 96%	Σ = 4%	totals, col D, E	obsd (col A)	col F less col G
148	0.2	86.00	0.00	0.00	0.00	0.00	0.2	−0.2
146	84.6	13.22	89.31	85.74	0.00	85.74	84.6	1.1
144	9.8	0.78	10.31	9.90	0.00	9.90	9.8	0.1
142	2.3	0.04	0.42	0.40	3.85	4.25	2.3	2.0
140	3.1	0.00	0.01	0.01	0.15	0.16	3.1	−2.9

[a] $R = 9.8/84.6 = 0.11584$, [b] $x = 300/(3 + R) = 96.3\%$. [b] $R \equiv (\%\ [^{18}O_2]P_i)/(\%\ [^{18}O_3]P_i)$; $x \equiv$ atom % ^{18}O in P_γ. See Table VI for deviation of the relationship between R and x.

For low enrichment, where the abundances of triply and quadruply labeled material approach zero, atom % ^{18}O is calculated from the ratio of singly labeled to unlabeled phosphate.

Table V[45] shows the calculation of atom % ^{18}O from the isotopic analyses of two samples from reaction of PCl_5 with ^{18}O-enriched water. In one, the enrichment so found is 99.1%; in the other, 8.8%. In both, the complete isotopic distributions calculated in turn from these values for atom % enrichment are virtually identical with the analyses, as they should be.

Assume now that the P_γ group of a labeled ATP, such as we have been considering, is converted by hydrolysis to P_i that retains three of the four P_γ oxygen atoms and acquires one from water. Table VI[45] shows the isotopic distribution for this product, calculated on the continuing assumption of random statistics. The atom % ^{18}O content of the P_γ group is readily calculated from the ratio of doubly to triply labeled phosphate.

We now have on hand the information needed to allow translation of the isotopic composition of P_i resulting from hydrolysis of [γ-^{18}O]ATP into the desired chemical information—the isotopic composition of the enriched ATP and the contributions of various reaction pathways to the hydrolysis: (i) the statistical distribution of combinations of four oxygens from labeled positions and the converse relationship by which we can calculate x, the ^{18}O content of the ATP P_γ group, from such a distribution; among the hydrolysis products, this distribution is associated with the primary product of P_β attack by water, path 2; (ii) the statistical distribution of combinations of oxygens from three out of four labeled positions, as expected in P_i arising by path 1 from [γ-^{18}O]ATP and, again, the converse relationship; (iii) a 96:4 split between P_γ and P_β contributions to the P_i when the hydrolysis is carried out at pH 8.3; and (iv) a 50:50 split between P_γ and P_β contributions to the P_i released in 1 N and 0.1 N HCl.

Table VII shows the isotopic analysis of the product of a pH 8.3 hydrolysis and the attendant calculations. The analysis listed in column A yields the value 96.3 atom % for x. This value in turn allows calculation of the isotopic compositions of the ATP P_γ group, column B, and of the hydrolysis product expected from path 1, column C. The small differences between columns A and C signify that the bulk of the hydrolysis product can be accounted for by path 1. The composition of the 96% of product derived from P_γ is hence approximated by the distribution in column D, which is obtained from that in column C by applying a scaling factor of 0.96. The 4% of product derived from P_β is assumed to result from paths 5 and 6, hence to contain only oxygen atom from a labeled position, the $\beta\gamma$ bridge, and isotopic purity in this position is 96.3%; the resultant composition of the P_β contribution appears in column E. The total calculated product composition, obtained by summing the individual isotopic species in columns D and E, is shown in Column F. The observed composition, in column A, is reproduced in column G to facilitate comparison. Discrepancies between columns F and G are listed in column H. The largest of these discrepancies are associated with the unlabeled and singly labeled species: −2.9% and 2.0%, respectively. Their origins are uncertain. The small discrepancy for the quadruply labeled species, comprising the entire amount found in the isotopic analysis, lies within the uncertainty of the measurements; we have taken it as effectively zero.

Table VIII shows the corresponding data and calculations for hydrolysis in 1 N HCl of the same [γ-^{18}O]ATP preparation from which the data in Table VII were obtained. On the preliminary assumption that the values for doubly and triply labeled species in the isotopic analysis, column A, represent part of a single statistical distribution, free of contributions from other sources, we used these values to arrive at a first approximation for x, atom % ^{18}O in the ATP P_γ group, of 96.2%. The

(49) McLafferty, F. W. "Interpretation of Mass Spectra", 2nd ed.; W. A. Benjamin: Reading, MA, 1973; p 8.

(50) (a) Grigsby, R. D.; Hansen, C. O.; Mannering, D. G.; Fox, W. G.; Cole, R. H. *Anal. Chem.* **1971**, *43*, 1135. (b) Schronk, L. R.; Grigsby, R. D.; Scheppele, S. E. *Anal. Chem.* **1982**, *54*, 748. (c) Grigsby, R. D., private communications.

(51) Purchased from Masspec, Inc., College Station, TX.

(52) For an excellent review of the properties of molecular sieves, see: Breck, D. W. *J. Chem. Educ.* **1964**, *41*, 678.

(53) Schumacher, E.; Taubenest, R. *Helv. Chim. Acta* **1966**, *49*, 1439.

(54) Brown, P.; Djerassi, C. *Tetrahedron* **1968**, *24*, 2949.

(55) (a) Bafus, D. A.; Gallegos, E. J.; Kiser, R. W. *J. Phys. Chem.* **1966**, *70*, 2614. (b) Our spectrum is in good accord with that reported by Bafus et al. except that their m/z 139:m/z 140 intensity ratio is higher than ours by a factor of 10×, presumably the result of a slipped decimal point.

(56) Beynon, J. H. "Mass Spectrometry and its Applications to Organic Chemistry"; Elsevier: New York, 1960; Appendix 1.

(57) Cf. Albert, D. K.; Meyerson, S. *Anal. Chem.* **1967**, *39*, 1904.

(58) Beynon, J. H. "Mass Spectrometry and its Applications to Organic Chemistry"; Elsevier: New York, 1960; pp 299–300.

Table VIII. Hydrolysis of [γ-^{18}O]ATP in 1 N HCl

	A	B	C	D	E	F	G	H	I
		statistical distn		residue,	path 1 hydrolysis		$(P_\gamma)_\gamma$, path 1, col F	$(P_\beta)_\gamma$(b), path 5, col F	$(P_\beta)_\gamma$(a), path 5,
mol wt	product analysis[a,b]	Σ = 100%	scaled to 3.1 at 148	col A less col C[b,c]	Σ = 100%	scaled to 43.2 at 146	scaled to Σ = 46.4%	scaled to Σ = 2.2%	Σ = 44.2%, 96.2% $^{18}O_1$
148	3.1	85.57	3.1	0.0	0.00	0.0	0.0	0.0	0.0
146	43.7	13.59	0.5	43.2	88.97	43.2	41.2	2.0	0.0
144	5.2	0.83	0.0	5.2	10.61	5.2	5.0	0.2	0.0
142	42.2	0.04	0.0	42.2	0.45	0.2	0.2	0.0	42.5
140	5.9	0.00	0.0	5.9	0.01	0.0	0.0	0.0	1.7
			3.6			48.6	46.4	2.2	44.2

	J	K	L	M	N	O	P	Q	R
mol wt	$(P_\beta)_\beta$(a), path 5, Σ = 3.4%, unlabeled	$(P_\beta)_\beta$(b), path 5, Σ = 0.2%, 2 oxygens 96.2% labeled	$(P_\gamma)_\gamma$, path 1, (col G)	$(P_\beta)_\gamma$, path 5, (col H, I)	$(P_\gamma)_\beta$, path 2, (col C)	$(P_\beta)_\beta$, path 5, (col J, K)	total (col L, M, N, O)	obsd (col A)	discrepancies col P less col Q
148	0.0	0.0	0.0	0.0	3.1	0.0	3.1	3.1	0.0
146	0.0	0.0	41.2	2.0	0.5	0.0	43.7	43.7	0.0
144	0.0	0.2	5.0	0.2	0.0	0.2	5.4	5.2	0.2
142	0.0	0.0	0.2	42.5	0.0	0.0	42.7	42.2	0.5
140	3.4	0.0	0.0	1.7	0.0	3.4	5.1	5.9	−0.8
	3.4	0.2	46.4	46.4	3.6	3.6	100.0	100.1	

[a] $R = 5.2/43.7 = 0.11899$,[b] $x = 300/(3 + R) = 96.2\%$.[b] [b] See footnote *a* to Table VII. [c] $R = 5.2/43.2 = 0.11965$; $x = 300/(3 + R) = 96.2\%$. Assign column C to $(P_\gamma)_\beta$, path 2.

statistical distribution of species containing four oxygen atoms from such a pool, column B, applies not only to the ATP P_Y group but also to product derived from P_γ but via hydrolytic attack on P_β, i.e., reaction path 2. This product, which we designate $(P_\gamma)_\beta$,[59] is taken to be the source of the 3.1% of product containing four tagged atoms. Its contribution, column C, is obtained by scaling the distribution in column B to a value of 3.1 for the quadruply labeled species. Removing this contribution from the total product leaves a residue, column D, from which we derive a second approximation for x. In view of its identity with the first approximation, we accept the value so obtained, 96.2%; and the distribution in column C can now be assigned with confidence to $(P_\gamma)_\beta$. The corresponding isotopic distribution for path 1 hydrolysis, column E, is scaled in column F to a value for the $^{18}O_3$ species of 43.2, to accord with column D. We note, however, that the sum of the contributions in columns C and F exceeds by 2.2% the 50% required by the experimental protocol to be derived from P_γ. This 2.2% we assign, as its most probable source, to $(P_\beta)_\gamma$, i.e., path 5, from ATP in which labeled phosphoryl was inadvertently incorporated in the β position during the synthesis. Such a model must contain oxygens from the 96.2 atom % ^{18}O pool in the two nonbridging P_β positions as well as in the $\beta\gamma$ bridge to account for the data. Our data do not reveal whether a fourth such oxygen occupies the $\alpha\beta$-bridging position. Products derived from this heavily labeled P_β will be indicated by (b) in the remainder of this discussion; products from ordinary P_β, by (a).

The contents of column F are prorated accordingly between $(P_\gamma)_\gamma$, from path 1, in column G, and $(P_\beta)_\gamma$(b), from path 5, in column H. Total $(P_b)_\gamma$ = total $(P_\gamma)_\gamma$ = 46.4% of total P_i; $(P_\beta)_\gamma$(a) then = 46.4 − $(P_\beta)_\gamma$(b) = 44.2; this contribution appears in column I. Total $(P_\beta)_\beta$ is set equal to total $(P_\gamma)_\beta$ (column C), 3.6, and prorated between $(P_\beta)_\beta$(a) and $(P_\beta)_\beta$(b) in the same proportions as $(P_\beta)_\gamma$(a) and $(P_\beta)_\gamma$(b), i.e., (a):(b) = 44.2:2.2 = 3.4:0.2; these contributions appear in columns J and K, respectively. The indicated contributions are collected in columns L, M, N, and O, and summed in column P for comparison with the observed composition, in column Q. Discrepancies between columns P and Q are listed in column R. Again, these discrepancies are of about the same magnitude as the uncertainty of the measurements. In summary, the data and treatment displayed in Table VIII lead to the following conclusions: (1) ^{18}O enrichment of the ATP P_γ group is 96.2 atom %; (2) 4.7% of the P_β groups are similarly enriched, presumably through some inadvertent reaction during synthesis of the labeled ATP; (3) 93% of initial attack by water occurs at P_γ and 7% at P_β.

(59) In this notation, throughout the discussion below, the subscript within the parentheses denotes the phosphoryl group released as P_i and the subscript outside the parentheses denotes the position of primary hydrolytic attack on the ATP.

Table IX. Hydrolysis Products from a Common [γ-^{18}O]ATP

	pH 8.3		1 N HCl		myosin CaATPase[a]	
M_r	obsd	acctd for	obsd	acctd for	obsd	acctd for
148	0.2	0.0	3.1	3.1	0.1	0.0
146	84.6	84.6	43.7	43.7	80.8	80.8
144	9.8	9.8	5.2	5.4	15.5	15.5
142	2.3	3.9	42.2	42.7	1.8	1.0
140	3.1	0.2	5.9	5.1	1.9	0.0
	100.0	98.5	100.1	100.0	100.1	97.3
derived, x = atom %	96.3		96.2		94.0	

[a] See ref 60.

Table X. Hydrolysis Products from a Second Common [γ-^{18}O]ATP

	0.1 N HCl		1 N HCl		pH 8.3	
M_r	obsd	acctd for	obsd	acctd for	obsd	acctd for
148	3.5	3.5	3.4	3.4	0.7	0.0
146	42.6	42.6	43.4	43.4	81.8	81.8
144	6.1	6.2	5.5	5.7	9.2	9.2
142	41.6	41.7	41.9	41.9	5.5	4.0
140	6.2	6.0	5.9	5.6	2.7	0.1
	100.0	100.0	100.1	100.0	99.9	95.1
derived, x = atom %	95.4		95.9		96.4	

In Table IX we have collected the observed isotopic distributions, derived values of x, and appropriately calculated isotopic distributions for products of hydrolysis of the [γ-^{18}O]ATP preparation at pH 8.3 (Table VII), in 1 N HCl (Table VIII), and by myosin CaATPase.[60] The [γ-^{18}O]ATP preparation employed in this set of experiments was not subjected to hydrolysis in 0.1 N HCl. Table X shows the observed

(60) We are indebted to Professor H. M. Levy, State University of New York at Stony Brook, for the myosin and for carrying out the enzymatic hydrolysis.

isotopic distributions, and similarly derived values of x, for products from hydrolysis of a second [γ-^{18}O]ATP preparation in 0.1 N and 1 N HCl and at pH 8.3. Hydrolysis in 0.1 N HCl gave results in close accord with those obtained in 1 N HCl. To facilitate comparison of corresponding "observed" and "accounted for" isotopic distributions, we have scaled the latter in each case to the observed value of the triply labeled species. The values of ATP P_γ ^{18}O content derived from the nonenzymatic hydrolysis at all pHs employed are essentially indistinguishable. That from the calcium control enzymatic hydrolysis in Table IX is less than the nonenzymatic by 2%.

The results shown in Tables IX and X are typical. Several independent [γ-^{18}O]ATP preparations and hydrolyses have checked each other closely. We have typically obtained isotopic enrichments of 99 atom % ^{18}O in [^{18}O]P_i preparations from $H_2{}^{18}O$ of 99% isotopic purity, and 96 atom % ^{18}O in the γ-phosphoryl group of the [γ-^{18}O]ATP synthesized from such [^{18}O]P_i. Isotopic analysis of the P_i obtained from the complete hydrolysis of this typical [γ-^{18}O]ATP to AMP + 2P_i in 1 N and 0.1 N HCl consistently indicates the inadvertent incorporation of ~4% of enriched phosphoryl groups in the β position. In some of our earlier experiments, this value reached 9%; in a few instances, it dropped as low as 2%.

Path 6 does not appear in the calculations above because the data in Tables VII, VIII, and IX do not distinguish between the two possible paths, (5) and (6), for hydrolysis of the ADP formed from ATP in the initial hydrolyses. To fill this gap, we supplemented the [γ-^{18}O]ATP hydrolyses by similar experiments on [β-^{18}O]ADP. Again, hydrolyses in acid and in base gave nearly identical values for the enrichment in the terminal phosphoryl group in the nucleotide. Likewise, hydrolytic attack here, too, took place predominantly at the terminal phosphoryl group; the relative probabilities we obtained are 83% β and 17% α.

In the pH 8.3 hydrolysis, Table VII, the isotopic distribution of the 4% product derived from intermediate ADP should in principle be corrected for the few percent of highly enriched P_β. But the correction is less than the uncertainties of the data and so has been omitted. Similarly, in the 1 N HCl hydrolysis, Table VIII, the isotopic distribution of that portion of the product derived from P_β via P_α attack, i.e., path 6, on intermediate ADP containing highly enriched P_β is in error if the $\alpha\beta$-bridging oxygen is also labeled. Here also, the possible error incurred is obscured by the uncertainties of the data, and we have not attempted any correction.

Discussion

The labeling and kinetic studies complement each other, and the combination constitutes the basis for a comprehensive picture of the nonenzymatic hydrolysis of ATP over the pH range of 0–10. These studies do not, of course, establish conclusively the mechanisms by which hydrolysis occurs at the various pH values. However, as a useful conceptual framework to simplify the discussion, we have followed the mechanistic hypothesis utilized[20] to accommodate experiments in which reactions of ATP with water and alcohols were carried out in aprotic solvents.

Acidic Hydrolysis. The addition–elimination sequence proposed for the hydrolysis of ATP in 1 N and 0.1 N HCl most likely proceeds via an oxyphosphorane intermediate.[20,21,61-65] Oxyphosphoranes have been observed in solution in equilibrium with phosphate esters.[66] Formulas **1** and **4** in Figure 2 depict, respectively, the oxyphosphoranes to be expected from addition of water to the P_γ and P_α atoms of [γ-^{18}O]ATP, and they suggest a plausible rationale for the formation of only one of these intermediates, **1**, in the rate-limiting step of the hydrolytic pathway. The main difference between formulas **1** and **4** seems to be the extent of steric hindrance provided by the adenosyl-5′ group (Ad) relative to the proton when the respective ligands (OAd vs. OH) are situated in the equatorial positions of trigonal-bipyramidal

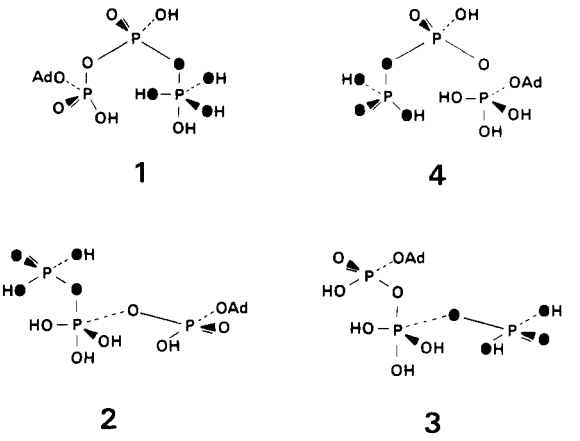

Figure 2. Oxyphosphorane intermediates corresponding to possible primary reaction pathways 1–4 in hydrolysis of [γ-^{18}O]ATP in 1 N and 0.1 N HCl by the addition–elimination mechanism. **1** denotes attack by water on P_γ: **2** and **3**, on P_β; **4**, on P_α. (●) denotes the heavy isotope, ^{18}O. (Reprinted with permission from ref 1b.)

5-coordinate phosphorus. The configuration of the oxyphosphoranes with ADP or PP_i as ligands in an apical position is based on the relatively high apicophilicity[62-64] of the pyrophosphate group. On purely electronic grounds, one would expect similar apicofugicity for the ADP and PP_i ligands. Hence we attribute the observed preference for the addition of water to the P_γ atom vs. the P_α atom in the acidic hydrolysis of ATP to the difference in steric features shown in formulas **1** and **4**.

The same argument can rationalize the observed preference for attack by water at the P_β atom vs. the P_α atom of ADP in 1 N and 0.1 N HCl. Here the two formulas that correspond to **1** and **4** would have AMP or P_i, respectively, as ligands in the apical position of the oxyphosphorane. Again, we suggest that water adds to the P_β atom to give an oxyphosphorane with equatorial OH, in preference to the P_α atom to give an oxyphosphorane with equatorial OAd, mainly for steric reasons. The difference in rate between the hydrolyses of ADP and ATP to AMP + P_i and ADP + P_i, respectively, may reflect a lower electrophilicity of the P_β atom in ADP, which carries an AMP substituent, than of the P_γ atom in ATP, which carries an ADP substituent.

A possible reason for the preference of attack by water at the P_γ atom vs. the P_β atom of ATP in strongly acidic media is suggested by a comparison between formulas **1** and **4**, on the one hand, and **2** and **3**, on the other. Formulas **1** and **4** contain one strongly apicophilic ligand in the apical position of the phosphorane. Formulas **2** and **3** contain one relatively weaker ligand in the apical position, and a second apicophilic ligand in an equatorial position. This type of ligand distribution on pentacovalent phosphorus[62-64] appears to favor oxyphosphoranes of type **1**/**4** over those of type **2**/**3**.

The water attack that does occur at the P_β atom of ATP results in the loss of P_i (formula **2**) and not of AMP (formula **3**). Preference for a smaller P_i ligand over a larger one in the apical position may be, at least partly, the reason for this discrimination between the two possible pathways. No enzyme is known that catalyzes the addition of water to the P_β atom of ATP; i.e., there is no known "P_β-ATPase". However, three enzymes have been reported[67] that catalyze attack by hydroxylic functions at P_β of ATP. In all cases, the products are AMP and the product of pyrophosphoryl transfer to the hydroxyl group. Thus, the directions of cleavage in nonenzymatic and enzymatic nucleophilic attacks at P_β of ATP are different. The P_β group of ATP is of the type $XX'PO_2H$, rather than of the type XPO_3H_2 exemplified by the P_γ group. The available evidence supports the view that monoionizable phosphates as such,[68] or as their monoanions,

(61) For a compilation of basic papers on the oxyphosphorane intermediate hypothesis, see: (a) McEwen, W. E.; Berlin, K. D., Eds. "Organophosphorus Stereochemistry"; Dowden, Hutchinson and Ross: Stroudsbrug, PA, 1975; Vol I, II. (b) Ramirez, F.; Hansen, B.; Desai, B. *J. Am. Chem. Soc.* **1962**, *84*, 4588. (c) Ramirez, F.; Madan, O. P.; Desai, N. B.; Meyerson, S.; Banas, E. M. *J. Am. Chem. Soc.* **1963**, *85*, 2681.

(62) Gillespie, P.; Hoffman, P.; Klusacek, H.; Marquarding, D.; Pfohl, S.; Ramirez, F.; Tsolis, E. A.; Ugi, I. *Angew. Chem., Int. Ed. Engl.* **1971**, *10*, 687.

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(68) Ramirez, F.; Marecek, J. F.; Tsuboi, H.; Chaw, Y. F. *Phosphorus Sulfur* **1978**, *4*, 325.

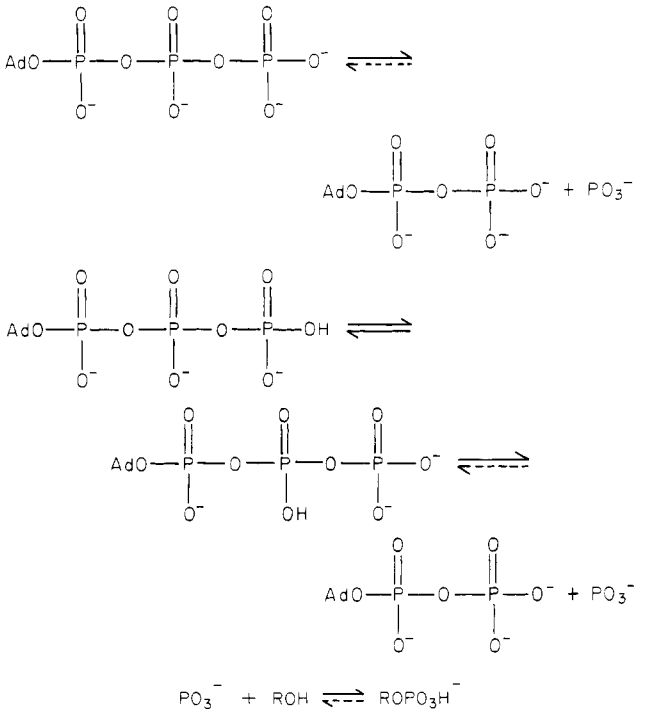
$XX'PO_2^-$, undergo nucleophilic substitutions by the addition–elimination mechanism exclusively. The few enzymes that direct ATP hydrolysis to give AMP and a pyrophosphorylated hydroxyl function may orient the tightly bound MgATP and the nucleophile in such a way as to produce an oxyphosphorane with the conformation type **3** rather than **2**, i.e., with apical AMP rather than apical P_i.

The addition–elimination mechanism here postulated and the formation of an oxyphosphorane as an intermediate are in line with previous work[69,70] in which actual, stable oxyphosphoranes were synthesized containing phosphate and pyrophosphate ligands in an apical position of trigonal-bipyramidal pentavalent phosphorus. Those oxyphosphorane models for ATP hydrolysis are stable because they lack ionizable OH ligands on the pentavalent phosphorus. Nonetheless, their formation and behavior in solution support the postulated formation of analogues in the ATP hydrolyses.

The rate of ATP hydrolysis decreases sharply in the pH range 1–4 as the pH of the medium increases; this decrease is most simply accounted for if species with relatively large rate constants are being replaced by a species with a relatively smaller rate constant. The pK_a for the equilibrium $ATPH^{3-} \rightleftharpoons ATP^{4-} + H^+$ has the value 7.3 and represents protonation of an oxygen atom in ATP. The pK_a for the equilibrium $ATPH_2^{2-} \rightleftharpoons ATPH^{3-} + H^+$ has the value 4.3 and corresponds to protonation of the 6-amino group. The pK_as for the subsequent equilibria $ATPH_3^- \rightleftharpoons ATPH_2^{2-} + H^+$ and $ATPH_4 \rightleftharpoons ATPH_3^- + H^+$ are close to or below 1.5. The present work furnishes no information on whether the dianion, $ATPH_2^{2-}$, undergoes hydrolysis by addition–elimination or elimination–addition or whether the two mechanisms are competitive. ATP and ADP undergo hydrolysis at very similar rates at pH 4.

Alkaline Hydrolysis. The rate of hydrolysis of ATP levels off in the pH range 4–7 and then decreases rapidly in alkaline medium, leveling off again only at about pH 9.3. Thus the rates of the fast hydrolysis in 1 N HCl and the slow hydrolysis at high basicity differ by a factor of about 4200. The present isotope-labeling study shows that the hydrolysis of ATP at pH 8.3 proceeds exclusively by cleavage of the $P_\beta O\text{-}P_\gamma$ bond. At this low acidity, the species to be considered are the tetraanion, ATP^{4-}, and the trianion. The low reaction rate of hydrolysis of ATP in basic medium appears to be associated with the elimination–addition mechanism.

Formation of the monomeric metaphosphate ion, PO_3^-, as an intermediate was first suggested 25 years ago[71,72] and has received support from more recent research.[73–77] Although PO_3^- has not been observed in solution, its presence in the gas phase at moderate temperatures (ca. 200 °C) has been verified.[78] The newer evidence for PO_3^- formation in the hydrolysis of ATP in basic solution[20] is based on the formation of *tert*-butyl phosphate, isopropyl phosphate, and methyl phosphate at similar rates when solutions of the tetra-*n*-butylammonium (M^+) salts of the trianion in acetonitrile are allowed to react with limited amounts of the corresponding alcohols at 30 °C: $ATPH^{3-}M^+_3 + ROH \rightleftharpoons ROPO_3H^-M^+ + ADPH^{2-}M^+_2$. Analogous results are obtained with the tetraanion salt, $ATP^{4-}M^+_4$. A rapid nucleophilic addition of *tert*-butyl alcohol to the 4-coordinate phosphorus atom and, in particular, the insensitivity of this addition to the structure of the alcohol appear implausible. However, addition of the alcohol to the electrophilic 3-coordinate phosphorus accommodates the results nicely.



The proton shift[79,80] shown prior to the formation of metaphosphate from the trianion allows loss of the metaphosphate ion rather than of the less stable metaphosphoric acid, $HOPO_2$.

Isotopic Assay of [γ-^{18}O]ATP. The present work provides a convenient and trustworthy method to assay the isotopic enrichment of [γ-^{18}O]ATP for use in investigations of enzymatic hydrolysis of ATP. An element of circularity is inherent in the past practice of making such assays by means of another enzymatic reaction. For example, in researches on the mechanism of reaction of MgATP under catalysis by actomyosin MgATPase during muscle contraction,[32,81–83] the isotopic enrichment of [γ-^{18}O]ATP was established by means of myosin CaATPase. Myosin catalyzes the hydrolysis of CaATP as it does the hydrolysis of MgATP. However, no muscle contraction results from the action of myosin CaATPase. There is, in fact, question as to the ability of actin to activate the hydrolysis of CaATP by myosin, in contrast with that of MgATP. *With our present method of assay for* [γ-^{18}*O*]*ATP we have verified that under optimum experimental conditions one can, indeed, obtain a fairly accurate picture* (*within some 2%*) *of the isotopic enrichment of* [γ-^{18}*O*]ATP *by the myosin-CaATPase assay.* However, under less nearly optimum conditions, the enzymatic assay by myosin CaATPase is subject to greater uncertainties and its usefulness for establishing the isotopic enrichment of [γ-^{18}O]ATP becomes problematic.

Summary. We have utilized ^{18}O isotopic labeling with two different mass spectral analytical techniques to define the positions of attack by water on the tripolyphosphate chain of ATP and on the diphosphate chain of ADP in 1 N and 0.1 N HCl and at pH 8.3. We have demonstrated that the nonenzymatic hydrolysis of [γ-^{18}O]ATP, at any of the pHs studied although perhaps most simply at pH 8.3, provides a more reliable assay of the isotopic content of ATP than does the CaATPase reaction commonly used. However, by carrying out the latter reaction with scrupulous

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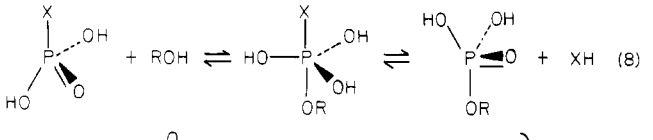
attention to experimental conditions, we have obtained agreement within 2% by the two methods. A careful check on the possibility of oxygen exchange between water and ATP, ADP, and P_i yielded no evidence for such exchange over the pH range studied. Finally, the labeling results from ATP hydrolysis in either 1 N or 0.1 N HCl yielded an extra dividend in revealing the inadvertent incorporation during the synthesis of [γ-^{18}O]ATP of a few percent of highly enriched phosphoryl in the β position.

Postscript. A report by Banks et al.[84] of the use of kinetic and tracer techniques to study some of the same questions that we have addressed appeared after completion of the present manuscript. A referee expressed concern over the "significantly different" results found in the two studies. In the hydrolyses of ATP and ADP in ^{18}O-enriched 3 N perchloric acid at 25 °C, Banks et al. report that the relative contributions of pathways 1:2 = (63 ± 4):(32 ± 4), and those of pathways 5:6 = 67:33, with the added observation, however, that *the latter ratio increases with decreasing acidity*. In addition, they report that some 5% of the P_i evolved in ATP hydrolysis under these conditions forms via PP_i intermediate from either path 3 or 4. They are in accord with us in finding no evidence for oxygen exchange between ATP, ADP, or P_i and medium. In view of differences in both the experimental conditions employed for the hydrolyses and the design of the labeling experiments, we are encouraged that the two sets of findings agree as well as they do. The bulk of Banks' data comes from hydrolyses of 3 N perchloric acid, outside the pH range that we explored. Banks converted the recovered P_i to CO_2 for isotopic analysis, telescoping into one value the five data points obtainable via conversion to trimethyl phosphate. Moreover, Banks' labeling data are derived entirely from hydrolyses of unlabeled nucleotide in ^{18}O-enriched water. This approach in our hands proved unreliable even at enrichments far greater than the 1.29% employed by her and her associates; however, the direct measurement of ion abundance ratios, as performed by isotope ratio mass spectrometers such as that used in their work, makes possible a marked increase in accuracy and precision.[4,85] Some discrepancies exist between the conclusions drawn from the two studies, but we are much more impressed by the extent of agreement than by that of disagreement.

Relevance of the Present Study to the Mechanism of Enzymatic ATP Reactions. Studies of nucleophilic substitutions of aryl phosphate have led to the conclusion[74-77] that, in general, a diprotonated phosphoryl group, XPO_3H_2, undergoes substitutions by the addition–elimination mechanism, while the unprotonated species, XPO_3^{2-}, reacts by elimination–addition. This work indicated also[74-77] that, in light of all presently available evidence, the addition–elimination mechanism involves an oxyphosphorane as intermediate (reaction 8),[61-66] while the elimination–addition



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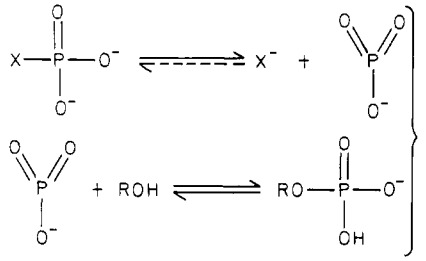

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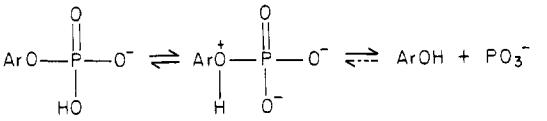
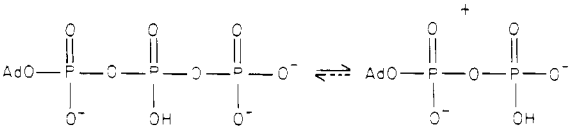
mechanism involves the monomeric metaphosphate ion as intermediate (reaction 9).[71-73]

In contrast to the diprotonated and the unprotonated species, which react by a given mechanism regardless of the structure of the phosphate, the monoprotonated species, XPO_3H^-, is capable of reacting by one or the other of the two mechanisms described above, depending on the structure of the phosphate. Thus the 2,4-dinitrophenyl phosphate monoanion reacts by addition–elimination while the 4-nitrophenyl phosphate monoanion reacts by elimination–addition. These differences seem to depend on the electrophilicity of the phosphorus (higher in the dinitrophenyl ester) and on the basicity of the ester oxygen atom (higher in the mononitrophenyl ester). The observations were rationalized by assuming, in accord with a prior suggestion,[79,80] that formation of the metaphosphate ion from a phosphomonoester monoanion requires a proton shift of some sort, e.g.:



In the case of 4-nitrophenyl phosphate, where elimination–addition occurs in both $ArOPO_3^{2-}$ and $ArOPO_3H^-$, formation of PO_3^- is faster from the dianion than from the monoanion in acetonitrile and in alcohol solution, but in aqueous solution the order is reversed. This is one of several significant medium effects noted in the study[74-76] and was rationalized in terms of greater solvation of the more polar ground state, i.e., the phosphates, than of the less polar transition states, i.e., the transition state leading to the oxyphosphorane intermediate or to the metaphosphate intermediate, depending on which mechanism is operative. The dianion is assumed to be solvated by water to a greater extent than the monoanion.

These ideas offer a possible clue to the question of whether a given phosphate may react by one type of mechanism in the absence of an enzyme and by another in its presence. The observed reactivities of ATP and of phenyl, 4-nitrophenyl, and 2,4-dinitrophenyl phosphates suggest that, among these aryl phosphates, the 4-nitrophenyl represents the closest model for ATP. As stated above, both the dianion and the monoanion of 4-nitrophenyl phosphate react by elimination–addition. In the absence of an enzyme, the tetraanion, ATP^{4-}, and the trianion $ATPH^{3-}$, also react by elimination–addition. If one assumes that one of the functions of the enzyme is to direct the protonation of ATP^{4-} at one of the two oxyanions of P_γ, i.e., if the enzyme prevents the proton shift that is thought to be essential for the formation of PO_3^- from $ATPH^{3-}$ (see reaction 10), then the trianion would react



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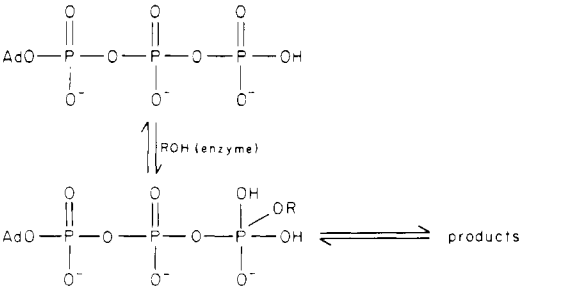

(11)

by the addition–elimination mechanism at the active site of the enzyme (reaction 11) and by elimination addition if no enzyme is present (reaction 10).

Acknowledgment. This work was supported by a grant from the National Science Foundation (CHE 79-04985 to F.R.). We thank D. K. Albert, Standard Oil Co., for orientation and guidance

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in selection and use of the sample support, P. J. Ihrig, G. G. Jones, Eric Jagel, and Carl Myerholtz, Standard Oil Co., for varied contributions to the development of optimal mass spectral and related computing procedures, H. M. Levy, State University of New York, for an enzymatic hydrolysis, as noted in footnote 60, and R. D. Grigsby, Texas A&M University, and R. D. Minard, The Pennsylvania State University, for allowing us access to unpublished material.

Registry No. Adenosine 5′-triphosphate, 56-65-5; [γ-^{18}O]adenosine 5′-triphosphate, 73116-39-9.

Supplementary Material Available: Tables giving data on treatment of P_i with water to test for oxygen exchange (Table I), isotopic analyses over the indicated time spans of two products from reactions of PCl_5 with $H_2{}^{18}O$ (Table II), isotopic analyses via GC/MS and probe/MS (Table III), isotopic analysis of P_i from reaction of PCl_5 with $H_2{}^{18}O$ (Table IV), isotopic analysis of P_i from reaction of PCl_5 with $H_2{}^{18}O$ (Table V), and calculation of ATP P_γ isotopic composition from analysis of P_i from hydrolysis in which the resultant P_i retains three of the four P_γ oxygen atoms and acquires one from water (Table VI), and figure showing evolution of methanol, water, and trimethyl phosphate from probe packed with 5-Å molecular sieve (Figure 1) (8 pages). Ordering information is given on ay current masthead page.

Conformational Studies of *S*-Adenosyl-L-homocysteine, a Potential Inhibitor of *S*-Adenosyl-L-methionine-Dependent Methyltransferases[1]

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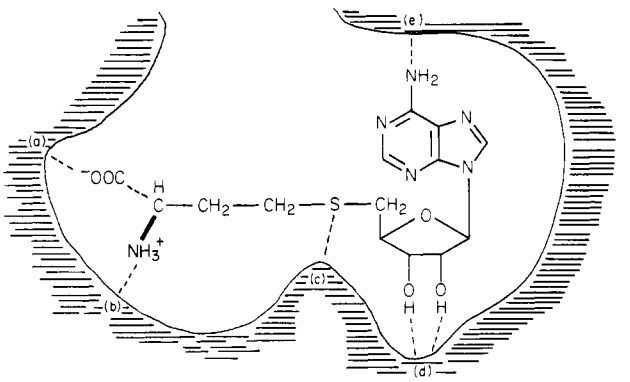
Abstract: The spatially favored conformation of *S*-adenosyl-L-homocysteine (SAH), a potent inhibitor of *S*-adenosyl-L-methionine (SAM) dependent methyltransferases, was studied by X-ray diffraction, 200-MHz ^{1}H nuclear magnetic resonance (NMR), and theoretical methods. The crystal used for X-ray analysis was monoclinic, of space group *C*2, with a = 45.942 (16) Å, b = 5.687 (1) Å, c = 15.627 (6) Å, β = 100.28 (5)°, and Z = 8. The final R index was 0.12 for 3243 independent reflections measured by Cu Kα radiation. The conformation of two crystallographically independent molecules was anti for the glycosyl bond, C(3′)-endo– or C(1′)-exo–C(2′)-endo sugar puckering, gauche/gauche for the orientation about the C(4′)–C(5′) bond, trans or gauche$^+$ about the C(α)–C(β) bond, trans about the C(β)–C(γ) bond, and gauche$^-$ or gauche$^+$ about the C(γ)–S bond. On the other hand, analysis of ^{1}H NMR spectra in ^2H_2O and $(C^2H_3)_2SO$ solutions showed a lack of conformational preference and approximately equal populations of rotational isomers. For elucidation of the energetically stable conformation of the SAH molecule, classical potential energy calculations were carried out, using the minimization technique. Each of the rotatable bonds varied for C(3′)-endo or C(2′)-endo ribose puckering. The torsion angles about the glycosyl, C(4′)–C(5′) and C(5′)–S bonds are highly important for energetically favored conformations. The SAH molecule favors two types of conformers for C(2′)-endo and three types for C(3′)-endo ribose puckering; the latter conformers are energetically less favorable by about 2–3 kcal/mol.

Biological transmethylation reactions are usually achieved by methyltransferases, utilizing *S*-adenosyl-L-methionine (SAM) as a methyl donor. *S*-Adenosyl-L-homocysteine (SAH), a demethylated metabolite of SAM, can bind to these methyltransferases and potentially inhibit their catalytic reaction. These enzymes are restimulated by adenosylhomocysteinase, which degrades SAH.[2] In tissue, the SAH and SAM level is equivalent;[3,4] therefore, SAH may be a key molecule in the regulatory mechanism of biological transmethylation.[5-7]

For elucidation of the functional groups of SAH necessary for inhibition and for development of more potent inhibitors, inhibitory experiments of SAH analogues have been performed for many methyltransferases such as catechol *O*-methyltransferase, hydroxyindole *O*-methyltransferase, and transfer RNA methyltransferase.[8-10] These enzymes generally show strict specificity for the structural features of SAH; the functional groups of primary importance for binding to the enzymes are terminal carboxyl (a) and amino (b) groups, sulfur atom (c), hydroxyl groups (d), and the amino group of the adenine base (e).[11]

Knowledge about the spatial arrangement of these functional groups is necessary to elucidate the substrate specificity of methyltransferases.



We now report the crystal structure of the SAH molecule as determined by the X-ray diffraction method and discuss the

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(1) Preliminary results for X-ray analysis were reported in *J. Chem. Soc., Chem. Commun.* 671 (1981).
(2) For reviews, see: F. Salvatore et al., "The Biochemistry of *S*-Adenosylmethionine", Columbia University Press, New York, 1977; S. K. Shapiro, F. Schlenk, Eds., "Transmethylation and Methionine Biosynthesis", University of Chicago Press, Chicago, 1965.
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