Synthesis of Nucleotides with Specific Radiolabels in Ribose

Adenosine 5'-phosphate was synthesized with 2H or 14C label specifically located as [1-2H]AMP, [1-14C]AMP, [5-2H]AMP, and [5-14C]AMP. The synthesis was accomplished from adenine and glucose or adenine and ribose using enzymes from the pentose pathway and/or from the purine salvage pathways. Structural analysis of the compounds confirmed the locations of the radiolabels. The methods provide a general scheme for the efficient synthesis of adenine nucleotides of high purity with 2H or 14C at any stable position on the ribose ring. Synthesis of [5-14C]AMP and [1-2H] dAMP from the corresponding ribonucleotides was accomplished with ribonucleotide reductase. Labeled inosine was prepared by enzymatic dephosphorylation and deamination of labeled AMP.

These compounds have been used to measure the secondary kinetic isotope effects on the acid-catalyzed hydrolysis of the N-glycosidic bond of AMP, dAMP, and inosine and the corresponding primary kinetic isotope effects with AMP. Acid hydrolysis in 0.1 or 0.2 N HCl at 50 °C gave 1H/2H secondary kinetic isotope effects of 1.23 ± 0.01, 1.26 ± 0.01, and 1.230 ± 0.003 for AMP, dAMP, and inosine, respectively. The primary kinetic isotope effect for 14C/12C was 1.049 ± 0.010 for AMP. The apparent rate constants for hydrolysis under these conditions were similar for inosine and AMP and were in the range 10-4 to 10-5 M-1 sec-1. Acid hydrolysis of dAMP is approximately 1000-fold faster than AMP but gives a similar 1H/2H kinetic isotope effect. The results of secondary isotope effects indicate that the transition states for the acid-catalyzed hydrolysis of the N-glycosidic bonds of inosine, AMP, and dAMP have similar bonding to 1-2H in the transition state and have considerable carbocation character. Results with [1-14C]AMP demonstrate that a significant primary isotope effect can be measured in the acid solvolysis of the N-glycosidic bond of AMP.

As a prelude to the study of heavy atom isotope effects on the enzyme-catalyzed hydrolysis of the N-glycosidic bond of AMP by several AMP nucleosidases (see accompanying paper (23)), isotope effects have been measured on the acid-catalyzed hydrolysis of AMP, dAMP, and inosine. Secondary deuterium isotope effects for adenine and inosine were reported originally by Romero et al. (1). The values of the secondary tritium isotope effects reported here for inosine serve as controls to establish the validity of the methods for measuring isotope effects. Secondary 2H isotope effects have not been previously reported for the acid-catalyzed hydrolysis of AMP, dAMP, or inosine. In addition, primary 14C isotope effects have not been reported for the acid-catalyzed hydrolysis of purine nucleosides or nucleotides. The values reported here are consistent with the previous proposals that acid-catalyzed hydrolysis of purine nucleosides (2-4) and nucleotides (5) proceed through transition states with considerable carbocation character. The results of these studies also provide values which can be compared to the kinetic isotope effects for AMP nucleosidases which catalyze the same hydrolytic reaction with AMP, but with a rate enhancement of approximately 103 (6).

In this paper, we present procedures for the enzymatic synthesis of adenine nucleotides of high purity and with specific labels of 2H and 14C in the ribose ring. Enzymatic synthesis of the desired compounds eliminates most of the by-products associated with chemical synthesis and allows the synthesis of small amounts of nucleotide with high specific radioactivity. Most of the enzymes and other reagents required for these synthetic procedures are commercially available or can be prepared using procedures which are described here or in the literature. The methods provided here can be applied to the specific labeling of a wide variety of both purine and pyrimidine nucleotides at any position of the ribose rings and should prove useful for kinetic isotope studies with other enzymes.

**EXPERIMENTAL PROCEDURES**

**Materials**

1-[14C]Ribose, [2-14C]Glucose, [6-14C]Glucose, [2-3H]Glucose, and [6-3H]Glucose were purchased from New England Nuclear or Amersham. Compounds labeled with 14C or 2H were supplied with specific radioactivities of 60 mCi/mmol and 12-30 Ci/mmol, respectively. NaBH4, (350 mCi/mmol) and Formula 950-A scintillation fluid were purchased from New England Nuclear. Sephadex G-10 and DEAE-Sephadex-A25 were from Pharmacia Fine Chemicals. HPLC reverse phase C18 Bondpak was purchased from Waters Associates. Norit A charcoal was from J. T. Baker Chemical Co. Ribonolactone and aminopterin were purchased from Sigma. Pyruvate kinase (2.7.1.40) and yeast hexokinase (2.7.1.1) grade 300 were from P-L Biochemicals. Myokinase (2.7.4.3) from rabbit muscle was from either Boehringer.

*The abbreviations used are: HPLC, high pressure liquid chromatography; P-Rib-PP, 5-phosphoribosyl-1-pyrophosphate.

As a prelude to the study of heavy atom isotope effects on...
reaction mixture was applied to a column of DEAE-Sephadex A-25 dissolved in the reaction mixture was stirred. After addition of 25 µl of the 0.5 M ribonolactone to NaB₃H₄ yielded sufficient 
kiferin (7). Ribonolactone and a crystal of bromothymol blue were


dissolved in 20% NaOH to give a 0.5 M solution and stored at -70 °C.


The mixture was incubated for 50 min at 37 °C. The supernatant was added to a column of DEAE-Sephadex A-25 (acetate form, 0.6 x 18 cm) and eluted with 20 mM acetic acid. Fractions containing the peak of 'H were pooled, lyophilized to dryness. Water was added and the mixture again lyophilized. This step was repeated and the sample was dissolved in 1 ml of 20 mM acetic acid and applied to a column (1 x 100 cm) of G-10 Sephadex in 20 mM acetic acid. The peak fractions were pooled, lyophilized, dissolved in 20 mM acetic acid, applied to another column of DEAE-Sephadex A-25 (acetate form, 0.7 x 18 cm) and eluted with 20 mM acetic acid. Fractions containing the peak of 'H were pooled, lyophilized, and dissolved in 95% ethanol. This fraction was used as the source of [1-'H]ribose for the synthesis of [1-'H]AMP.

The yields of [1-'H]ribose produced by [H³]hydroxyde reduction of ribonolactone were approximately 10% of theoretical. Gotoin et al. (8) reported the tendency of ribonolactone to be over-reduced to ribitol by NaBH₄. However, the use of a large molar excess of ribonolactone to NaBH₄ yielded sufficient [1-'H]ribose for enzymatic synthesis of AMP.

Preparation of Adenine Phosphoribosyltransferase—Production, assay, and purification of adenine phosphoribosyltransferase were based on the method of Hochstadt (9). A freshly growing culture of E. coli K12 in synthetic media (5 ml) was added to 50 ml of Korn-Weissbach (10) medium containing 0.5 µM amipron, pH 7.1 at 37 °C and used to inoculate 12 liters of Korn-Weissbach medium containing 0.3 µM amipron. The 20-liter container was agitated and aerated at 37 °C for about 18 h until the reading was 200 on a Klett colorimeter. The cells were harvested by centrifugation and washed with 0.01 M Tris-HCl, pH 7.1, containing 30 mM NaCl. Approximately 20 g of cells were obtained and suspended in 800 ml of 33 mM Tris-HCl, pH 7.1, followed by 800 ml of 40% sucrose in 33 mM Tris-HCl, pH 7.1, and 1.7 ml of 0.1 M Na-EDTA, pH 8.0. After the solution was stirred for 10 min at room temperature the cells were collected by centrifugation and the supernatant was drained carefully. The total cell pellets were rapidly suspended in 1600 ml of cold 20 mM MgSO₄ followed by centrifugation at 13,000 x g for 1 h. The supernatant was concentrated to 100 ml using an Amicon hollow fiber apparatus followed by dialysis against 50 mM Tris-HCl, pH 8.0. Solid (NH₄)₂SO₄ was added and the sample treated to yield the fraction precipitating between 0.45 and 0.56 saturation. The pellet was suspended in the minimum volume of 75 mM potassium phosphate buffer, pH 7.5, and was applied to a column (2.5 x 30 cm) of Sephacyr G-150, equilibrated with 75 mM potassium phosphate buffer, pH 7.5, and eluted with the same buffer. The fractions containing the maximum adenine phosphoribosyltransferase activity were pooled and concentrated by dialysis against solid ammonium sulfate or by collodion bag apparatus (25,000 M₉, retention). The specific activity of the enzyme was 0.7 and total yield was 91 units. The fractions were frozen in dry ice/ethanol and stored at -80 °C for several months without significant loss in activity.

The ribonokinase assay solution contained 1 mM ATP, 2 mM MgCl₂, 1 mM D-ribose, 10 mM KCl, 0.5 mM P-enolpyruvate, 100 mM Tris-HCl, pH 8.0, 0.1 mM NADH, 2 units of pyruvate kinase, and 2 units of lactate dehydrogenase in a volume of 1 ml. Enzyme was added to the reaction mixture which lacked ribose to establish the base-line rate of NADH oxidation at 340 nm. Ribose was added and the difference in A₅₆₀ was used to estimate enzymatic activity.

Synthesis of [5-'3H]AMP and [5-'3H]AMP from Ribose—Labeled AMP was synthesized from ribose in reaction mixtures which consisted of 50 mM potassium phosphate, pH 7.5, 10 µM P-ribosyl-1,5 cyclic phosphate (50 mCi/mmol) or [6-3H]Glucose labeled to specific radioactivity of 50-100 mCi/mmol) or [6-3H]Glucose labeled to specific radioactivity of 50-100 mCi/mmol. The culture was incubated at 37 °C for at least 6 months without significant loss in activity.

The reaction mixture was placed in boiling H₂O for 3 min and cooled on ice. The labeled ATP was converted to AMP by the addition of glucose to a final concentration of 0.25 mM, 25 units/ml of hexokinase, and 200 units/ml of myokinase followed by incubation for 30 min at 37 °C. The reaction mixture was mixed with boiling H₂O for 3 min.

Synthesis of [5-'3H]AMP and [5-'3H]AMP from Glucose—Labeled AMP was synthesized from glucose in reaction mixtures which consisted of 50 mM potassium phosphate, pH 7.8, 50 mM glycylglycine, 0.1 mM MgCl₂, 1 mM ATP, 50 mM NaDP, 20 mM P-enolpyruvate, 2 units/ml of hexokinase, 2 units/ml of myokinase, 7 units/ml of glucose-6-P dehydrogenase, 1 unit/ml of 6-P-gluconate dehydrogenase, 5 units/ml of phosphoribosimomerase, 25 units/ml of pyruvate kinase, 1.3 units/ml of P-Rib-PP synthetase, 0.5 units/ml of adenine phosphoribosyltransferase, 1.5 units/ml of ribokinase, 5 units/ml of myokinase, and 20 units/ml of pyruvate kinase, and 1 mM labeled glucose. Carrier-free ['3H]glucose ([6-3H]Glucose labeled to specific radioactivity of 250 mCi/mmol) was dissolved under vacuum. The above reagents were added in the order listed except ribokinase was added to start the reaction. The solution was incubated for 30-60 min at 37 °C. The reaction mixture was placed in boiling H₂O for 3 min and the ATP was converted to AMP by the addition of glucose to a final concentration of 0.25 mM, 25 units/ml of hexokinase, and 200 units/ml of myokinase followed by incubation for 30 min at 37 °C. The reaction mixture was mixed with boiling H₂O for 3 min.

The luciferase assay was prepared by addition of 5 ml of H₂O, stored at -60 °C, and used repeatedly for at least 6 months without significant loss in activity.
2.5 ml of 40 mM MgCl₂ (pH 7.4), and 2.5 ml of 10 mM Na arsenate (pH 7.4) to one vial of luciferase. A standard curve was obtained with ATP used as the luciferase preparation. The total volume of assay was 0.5 ml, consisting of 0.45 ml of the luciferase mixture and 0.05 ml of the sample containing the ATP. The reaction was initiated by addition of the ATP solution into the luciferase solution using a 50-μl Hamilton syringe.

**Purification of Labeled AMP**—The reaction mixtures were applied to a column (1 × 100 cm) of Sephadex G-10 and eluted with 20 mM acetic acid at room temperature. Radioactive fractions which eluted at the position of AMP were further analyzed by HPLC using reverse phase C₁₈ bondedpak (4 mm × 30 cm) in 0.1 M potassium phosphate, pH 5.0. Fractions from G-10 Sephadex which contained the AMP were pooled and applied to a column (0.6 × 15 cm) of DEAE-Sephadex A-25 acetate form. The column was washed with 20 ml of 20 mM acetic acid and the AMP was eluted with a 100-ml linear gradient of 0.5-1.0 M acetic acid. The fractions were analyzed for radioactivity and for AMP using HPLC. Fractions with >99% purity of AMP as determined by HPLC were pooled and lyophilized to dryness. The dried material was dissolved in H₂O and stored at −20 °C. The same column was used for analysis of AMP phase HPLC as described above except 0.1 M ammonium acetate, pH 5.0, was used as eluant and up to 0.6 ml of sample was applied. The AMP fractions were pooled, lyophilized, dissolved in H₂O, and stored at −20 °C.

**Conversion of AMP to dAMP**—The synthesis of dAMP was based on the procedure described by Brinkley et al. (12). The reaction mixture for conversion of AMP to ATP consisted of 0.1 M sodium acetate, pH 7.3, 50 mM potassium phosphate, pH 7.7, 10 mM phosphoenolpyruvate, 2 mM MgCl₂, 0.5 mM ATP, 1.5 mM labeled AMP, 100 units/ml of pyruvate kinase, and 25 units/ml of myokinase in a final volume of 222 μl. The mixture was incubated for 15 min at 37 °C and the reaction was terminated by placing the tube in boiling H₂O for 3 min. The reaction mixture for conversion of AMP to dATP consisted of the above mixture which was made to 25 mM dithiothreitol, 10 mM EDTA, 1 mM dNTP, and 0.5 mg/ml of enzyme β₂ (freshly prepared), and 1 unit of nucleoside triphosphate reductase (in 0.1 M sodium acetate, pH 7.3). The mixture (472 μl) was flushed with N₂, sealed, and incubated for 60 min at 37 °C. The conversion of AMP to dATP was conducted in the above reaction mixture which was made to 11 mM glucose, and to which was added 125 units of myokinase and 40 units of hexokinase for a final volume of 557 μl. The reaction mixture was incubated for 60 min at 37 °C.

The production of dAMP was followed by using C₁₈ bondedpak reverse phase HPLC with 0.1 M ammonium phosphate adjusted to pH 5.0 with HCl in 5% methanol. The flow rate was 2 ml/min. The labeled dAMP compounds were purified using Sephadex G-10 as described in a previous section and the HPLC method described above except 0.1 M ammonium acetate, pH 5.0, with 5% methanol was used as the eluant. The HPLC fractions which contained the labeled dAMP were pooled, lyophilized to dryness, dissolved in −200 μl of H₂O, and used as the source of labeled dAMP for kinetic isotope effect experiments.

**Analysis of Labeled AMP**—Labeled AMP was converted to ribose 5-P₄O₃ and adenosine in a reaction mixture containing 100 mM triethanolamine-HCl, pH 8.0, 0.5 mM ATP, 1 mM MgCl₂, and 0.1 unit/ml of AMP nucleosidase. Samples were incubated at 30 °C for sufficient time to convert >98% of the AMP to ribose 5-P. The solution was adjusted to pH 9 using 1 M KOH, 1 unit/ml of alkaline phosphatase was added, and the mixture was incubated at 30 °C for 30 min to convert ribose 5-P₄O₃ to ribose. The solution was applied to a column (0.5 × 3.5 cm) of DEAE-Sephadex A-25 (acetate form) and eluted with 20 mM acetic acid. The ribose fractions were lyophilized to dryness and used as the source of ribose for structural analysis.

The analysis of the C-1 position of ribose followed the procedure of Jones and Stoodley (13) shown in Scheme 1. Approximately 1⁰⁶ cpm of labeled ribose was combined with 50 mg of ribose, 170 mg of I₂, and 3.2 ml of methanol. The mixture was heated to 40 °C and 3.5 ml of 10% KOH in methanol (w/v) was added dropwise. The reaction was incubated for 20 min, then cooled to room temperature. The precipitate was sedimented and washed twice with 2 ml of methanol and twice with 2 ml of ether. The methanol/ether washes were combined and analysed for volatile (H₂O) and nonvolatile (ribose) radioactivity. The potassium ribonate precipitate was dried and dissolved in H₂O or 0.5 mM potassium phosphate, pH 5.8. Comparison of the radioactivity in the potassium ribonate and H₂O released into the H₂O demonstrates the fraction of H present in the 1 position. The conversion of ribose to potassium ribonate gave approximately a 50% yield as determined by the ribose content of the methanol wash.
into glass scintillation vials. To each 0.50-g sample, 0.50 ml of H$_2$O was added together with 9.0 ml of scintillation fluid. The mixture was shaken and analyzed for radioactivity on a scintillation counter. Fractions containing a minimum of 95% of the total radioactivity in the ribose 5-PO$_4$ were counted. This was essential since the separation of [H]ribose 5-PO$_4$ and [C]ribose 5-PO$_4$ showed a small isotope effect on the charcoal column. Isotopic effects on charcoal chromatography have also been reported for labeled methanes (15).

Double channel liquid scintillation counting to determine H and C was performed on a Searle Delta 300 instrument with channel A recording <1% of H counts and channel B recording >99% of H counts. The fraction of C radioactivity appearing in each channel was determined for each set of experiments by placing approximately 10$^6$ cpm of [1-14C]ribose in 0.5 g of 10 mM ribose 5-PO$_4$. The [1-14C] ribose sample was prepared for counting as described above. All vials were counted 10-20 min each in a cyclic repetitive fashion five to ten times until greater than 5 x 10$^6$ counts in channel A had been accumulated. No systematic variation was observed in counting rates due to the time required for counting. The counts from channel A of all the vials containing ribose 5-PO$_4$ from each column were summed. The same procedure was used for channel B. The total cpm from channel A and total cpm from channel B were used in subsequent calculations.

Isotope Effect Measurements—Labeled AMP with H and C in the desired positions was combined with unlabeled AMP and HCl to final concentrations of 1.25 mM and 0.1 M, respectively. The total radioactivity of AMP in reaction mixtures was >10$^6$ cpm in the C channel. To obtain the most accurate determination of the ratio of C/H, the amount of H radioactivity which appeared in the H channel was at least three times the amount of C which appeared in the C channel. Except where indicated, kinetic isotope effect measurements were determined with at least triplicate determinations. The total volume of reaction mixtures was either 100 or 300 µl. The samples which contained AMP and inosine were incubated at 50°C until 10-20% of the substrate was converted to products. Hydrolysis of dAMP was much more rapid than AMP and incubations were at 30°C. The reaction was terminated by neutralizing the HCl with NaOH and the addition of triethanolamine-HCl (pH 8.0) to a final concentration of 50 mM. Aliquots of 100 µl from the reaction mixtures were applied to charcoal columns for separation of adenine and AMP from ribose 5-PO$_4$ or deoxyribose 5-PO$_4$. A control for determination of the kinetic isotope effect was the analysis of reaction mixtures using the procedure above where no acid was included in the incubation. The total H cpm in the ribose 5-PO$_4$ sample was determined by Equation 1.

Total H cpm = (channel B cpm) - (C channel ratio x channel A cpm) (1)

The C/C primary isotope effect was measured with a mixture of [1-14C]AMP and [5'-3H]AMP as substrates, and Equation 2 was used to calculate the observed isotope effect.

Observed C/C isotope effect

$\frac{\text{H cpm (total)}}{\text{C cpm (channel A)}}$ at 15% hydrolysis

$= \frac{\text{H cpm (total)}}{\text{C cpm (channel A)}}$ at either 0 or 100% hydrolysis

(2)

The H/H secondary isotope effect was measured with a mixture of [1-5H]AMP and [5'-14C]AMP as substrates and Equation 3 was used to calculate the observed isotope effect.

Observed H/H isotope effect

$\frac{\text{C cpm (channel A)}}{\text{H cpm (total)}}$ at 15% hydrolysis

$= \frac{\text{C cpm (channel A)}}{\text{H cpm (total)}}$ at either 0 or 100% hydrolysis

(3)

To correct the measured kinetic isotope effects to 0% AMP hydrolysis, Equation 4 was used

$IE = \ln \left(1 - \frac{1}{f \text{R}}\right) / \ln (1 - f)$

(4)

where $IE$ is the actual isotope effect, $IE_{max}$ is the observed isotope effect, and $f$ is the fraction of the AMP converted to product as determined by the fraction of C (channel A) counts converted to ribose 5-PO$_4$. All kinetic isotope effect values have been adjusted to 0% of AMP hydrolysis. The standard error values for isotope effects were determined by the method described in Ref. 16 which takes into account the error of the counting and the standard deviation of multiple determinations.

RESULTS

Synthesis and Analysis of Labeled AMP—The enzymatic synthesis of [1-3H]AMP, [1-14C]AMP, [5'-3H]AMP, and [5'-14C]AMP was accomplished using the coupled enzymatic steps shown in Scheme 3. The conversion of the sugar to ATP was monitored by analyzing samples of the reaction mixture with luciferase. An example of the conversion is shown in Fig. 1. The reaction was driven to ATP by excess P-enolpyruvate, pyruvate kinase, and myokinase. After production of ATP approached a maximum, the reaction was terminated by heating, and the ATP converted to AMP by the addition of glucose, hexokinase, and myokinase (indicated as the final conversion in Scheme 3). Incorporation of labeled glucose or ribose into ATP and conversion back to AMP gave 60-93% overall yields (Table I). The purity of the labeled AMP was established with the experiments summarized in Tables II and III. The enzymatic degradation of labeled AMP by AMP nucleosidase demonstrated that the radioactivity originally present in AMP eluted as ribose 5-PO$_4$ after treatment with enzyme (Table II). Labeled AMP samples were analyzed for the presence of ribose 5-PO$_4$ or other material which would coelute with ribose 5-PO$_4$ and thereby interfere with subsequent measurement of isotope effect. Table III indicates that <0.02% of the labeled material elutes in the position for ribose 5-PO$_4$. Following treatment with AMP nucleosidase, the labeled ribose 5-PO$_4$ eluted in fractions 3-6 from the charcoal column. Structural analysis of ribose isolated from labeled AMP indicated that the H and C labels were present in the desired position in the ribose ring (Tables IV and V).

Isotope Effect on Acid Hydrolysis of AMP and Inosine—Primary kinetic isotope effects were determined with a mixture of [1-14C]AMP and [5'-3H]AMP as substrates. Secondary isotope effects were measured with a mixture of [1-14C]AMP and [5'-14C]AMP as substrates. As a control for the methods and the structures of the labeled substrates, the secondary isotope effect on the acid-catalyzed hydrolysis of inosine was measured. Inosine was produced from labeled AMP by the combined action of alkaline phosphatase and adenosine deaminase as described under "Experimental Procedures". The acid-catalyzed hydrolysis of inosine is known to exhibit a significant secondary kinetic isotope effect (1). The acid-catalyzed hydrolyses of AMP and inosine showed secondary isotope effects of 1.23 ± 0.01 and 1.230 ± 0.003, respectively (Table VI). The secondary isotope effect when dAMP was hydrolyzed in HCl was 1.259 ± 0.006. Similar studies to determine the primary kinetic isotope effect on the acid-catalyzed hydrolysis of AMP gave a C/C isotope effect of 1.049 ± 0.008 (Table VI). Analysis of the products of the acid hydrolysis of AMP by HPLC gave adenine and ribose 5-PO$_4$ as the sole products during the incubation times used for glycosidic bond hydrolysis. Similar experiments were attempted to establish the isotope effects associated with base-catalyzed hydrolysis of adenosine and AMP. In both cases, the reaction rates were very slow and HPLC analysis of the products indicated significant degradation to products other than the purine base and ribose or ribose 5-PO$_4$. Similar results have been reported for chemical studies of base-catalyzed solvolysis of purine nucleosides (17).
Isotope Effects on N-Glycosidic Bond Hydrolysis

SCHEME 3

FIG. 1. Enzymatic production of ATP from glucose. The concentration of ATP in the incubation mixture was determined by the luciferase assay. The reaction mixture for ATP synthesis initially contained approximately 1 mM ATP, 1 mM radioactive glucose, and the remainder of the ingredients shown in Scheme 3 in a volume of 0.5 ml. The reaction was initiated by the addition of hexokinase. Additional details of the procedure are given under "Experimental Procedures."

TABLE I
Efficiency of AMP synthesis

The distribution of radioactivity was determined by analyzing an aliquot of the reaction mixtures on HPLC as described under "Experimental Procedures." Counts which coeluted with authentic ATP and ADP were assumed to be these nucleotides but were not further analyzed. Unincorporated label eluted primarily at the solvent front which contained sugars and sugar phosphates.

<table>
<thead>
<tr>
<th>Intended product</th>
<th>Substrate</th>
<th>AMP</th>
<th>ATP-ADP</th>
<th>Unincorporated label</th>
<th>Yield as %</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1-3H]AMP</td>
<td>[1-3H]Ribose</td>
<td>38,400</td>
<td>400</td>
<td>1,590</td>
<td>93</td>
</tr>
<tr>
<td>[1-3C]AMP</td>
<td>[1-3C]Ribose</td>
<td>28,400</td>
<td>400</td>
<td>1,590</td>
<td>93</td>
</tr>
<tr>
<td>[5-3H]AMP</td>
<td>[5-3H]Glucose</td>
<td>8,160</td>
<td>289</td>
<td>3,090</td>
<td>71</td>
</tr>
<tr>
<td>[5-3C]AMP</td>
<td>[5-3C]Glucose</td>
<td>30,100</td>
<td>1,240</td>
<td>18,500</td>
<td>60</td>
</tr>
</tbody>
</table>

An additional control for the observed kinetic isotope effects is the incorporation of heavy isotopes at positions which are insensitive to the chemical reaction. In the case of the acid solvolysis of AMP, a mixture of [5-3H]AMP and [5-3C]AMP provides a control for artifacts which could arise during hydrolysis and/or sample preparation. As indicated in Table VI, the observed isotope effect was near zero for this reaction and was different from either the primary or secondary isotope effects obtained with the heavy atoms in the isotopically sensitive position.

DISCUSSION

Synthesis of specific radiolabels in the ribose of AMP has made use of the enzymes of the pentose shunt and the purine salvage pathways. These methods gave good yields with spe-
Isotope Effects on N-Glycosidic Bond Hydrolysis

### Table IV
**Structural analysis of ribose from [5'-³H]AMP and [5'-¹⁴C]AMP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific radioactivity</th>
<th>cpm/mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>[5'-³H]Ribose</td>
<td>0.65 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>H dimedone complex of formaldehyde from C-5</td>
<td>0.68 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>[5'-¹⁴C]Ribose</td>
<td>0.43 × 10⁶</td>
<td></td>
</tr>
<tr>
<td>C dimedone complex of formaldehyde from C-5</td>
<td>0.42 × 10⁶</td>
<td></td>
</tr>
</tbody>
</table>

### Table V
**Structural analysis of ribose from [1'-³H]AMP and [1'-¹⁴C]AMP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent</th>
<th>K ribonate</th>
<th>Unreacted</th>
<th>BeCO₂</th>
<th>Minimum label in 1-ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1'-³H]Ribose</td>
<td>11,400</td>
<td>610</td>
<td>4,600</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>[1'-¹⁴C]Ribose</td>
<td>821,000</td>
<td>485,000</td>
<td>330,000</td>
<td>477,000</td>
<td>96</td>
</tr>
</tbody>
</table>

### Table VI
**Kinetic isotope effect on the acid-catalyzed hydrolysis of AMP, inosine, and dAMP**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Kinetic isotope effect</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>[5'-³H]AMP + [1'-¹⁴C]AMP</td>
<td>1.049 ± 0.009 (5)</td>
<td>50 °C, 0.1 N HCl</td>
</tr>
<tr>
<td>[5'-¹⁴C]AMP + [1'-³H]AMP</td>
<td>1.236 ± 0.010 (6)</td>
<td>50 °C, 0.1 N HCl</td>
</tr>
<tr>
<td>[5'-¹⁴C]Inosine +</td>
<td>1.230 ± 0.003 (5)</td>
<td>50 °C, 0.2 N HCl</td>
</tr>
<tr>
<td>[5'-¹⁴C]dAMP +</td>
<td>1.259 ± 0.006 (3)</td>
<td>30 °C, 0.1 N HCl</td>
</tr>
<tr>
<td>[5'-³H]dAMP + [5'-¹⁴C]AMP</td>
<td>0.984 ± 0.002 (3)</td>
<td>50 °C, 0.1 N HCl</td>
</tr>
</tbody>
</table>

The reliability of the method for the determination of the kinetic isotope effect was confirmed by comparing values obtained in this paper with published values. The acid hydrolysis of inosine has been reported to give a ³H secondary kinetic isotope effect of 1.175 ± 0.009 at a rate of ~3 × 10^-⁶ s⁻¹ (1). The acid hydrolysis of inosine reported here gave a ³H secondary kinetic isotope effect of 1.230 ± 0.003 at a rate of ~5 × 10^-⁶ s⁻¹. Using the value of 1.44 for the Swain relationship, the measured ³H isotope effect is 1.16 when adjusted to a ³H kinetic isotope effect. These values agree quite closely, substantiating the method of determining the kinetic isotope effects described here. The kinetic isotope effect determination of the acid hydrolysis of inosine also helps to establish the position of the isotopic labels in AMP, since the isotope effect would be lower than the observed effect if the ³H and ¹⁴C labels were not in the appropriate positions. The data in Tables IV and V showed that the labels are in the expected position.

Secondary deuterium kinetic isotope effects on the acid-catalyzed hydrolysis of the N-glycosidic bond of inosine has given an isotope effect (³H/²H) of 1.175 ± 0.009 (1). This value has been interpreted in terms of a transition state with the C—N bond cleavage well developed and considerable oxycarbonium character in the ribose. Calculations of limiting deuterium isotope effects for N-glycosidic bonds of purine nucleosides were originally reported to be 1.21–1.25 based on interconversion of nucleoside and oxycarbonium ion (20). More recently, deuterium secondary isotope effects in excess of 1.30 have been observed for the equilibrium interconversion of a carbon sp³ center and a noncarboxylation sp² center (21). A tritium secondary kinetic isotope effect of 1.41 has been reported for the ³H hydrolysis of F-riboisopropylphosphate (8). The results of isotope effect studies are consistent with kinetic studies on the acid-catalyzed hydrolysis of adenine nucleosides with substituents on both the adenine and ribose rings. Garrett and Mehta (2) provided evidence that the reactive species of adenosine during acid hydrolysis is the protonated dication with N-1 being protonated first followed by addition of the second proton at N-7. This species was proposed to undergo an A-1 solvolysis. The combined evidence from chemical and secondary isotope effect data thus provide support for the dissociative cleavage of the N-glycosidic bond of purine nucleosides. The observed ³H/²H isotope effect on the acid-catalyzed hydrolysis of AMP was found to be the same as that for inosine (Table VI). Thus, the presence of the 5'-PO₄ does not alter the nature of the transition state which has been proposed to result from the protonation of the purine and its subsequent loss from the ribose 5-PO₄.

Measurement of the secondary ³H/²H kinetic isotope effect on dAMP solvolysis gave a value which is only slightly greater than for AMP or inosine. Since dAMP lacks the 2'-hydroxyl group which can delocalize electrons around C-1' in AMP and inosine, the electrons are more readily lost to the purine ring, causing increased sensitivity to acid solvolysis. Even though the rate of hydrolysis of dAMP is ~1000 times greater than AMP, the nature of the transition states for both reactions appears to be similar based on the magnitude of the secondary isotope effects. The transition state thus remains oxycarbonium-like, and the rate enhancement must therefore be a result of the destabilized electrons near C-1'. These conclusions provide independent confirmation of the proposal that the major effect of the 2'-hydroxyl is inductive (2).

The ¹⁴C primary isotope effect has not been previously reported for the acid-catalyzed hydrolysis of purine nucleosides or nucleotides. From chemical models, studies, it is ex-
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Expected that an $S_n\text{1}$ reaction such as that proposed for acid-catalyzed hydrolysis of the $N$-glycosidic bond will result in a primary $^{13}\text{C}/^{14}\text{C}$ kinetic isotope effect of nearer unity than the upper limit of approximately 1.15 (22). An example is provided by the hydrolysis of the pyrophosphoryl group from C-1 of P-Rib-PP, which is known to proceed by an $S_n\text{1}$ mechanism. This reaction gives kinetic isotope effects of 1.02 for the primary $^{13}\text{C}/^{14}\text{C}$ effect at C-1, and 1.41 for the secondary $^1\text{H}/^2\text{H}$ effect at C-1 (8). The measured primary kinetic isotope effect for $^{12}\text{C}/^{14}\text{C}$ at C-1' of AMP is 1.049 ± 0.009 (Table VI) is well within the range of 1.00 to 1.08 expected for a dissociative mechanism and establishes that bonding to C-1' of AMP in the transition state is considerably looser than in the substrate or product.

In summary, the results presented here demonstrate a facile method for the enzymatic synthesis of adenine nucleotides with C-1' and C-5' $^3\text{H}$ and $^{14}\text{C}$ radiolabels in the ribose. The methods are easily adaptable, simply by substitution of starting materials, to the synthesis of nucleosides with label in other positions of the ribose or the purine moiety. Hydrolytic studies of $^3\text{H}$- and $^{14}\text{C}$-labeled nucleosides, nucleotides, and deoxynucleotides in acid establish the magnitude of the primary and secondary kinetic isotope effects in C-N glycosidic bond cleavages. Finally, reliable and rapid methods for isolating the radiolabeled riboses have been used to establish the validity of the kinetic isotope effect technique. These techniques are used in the subsequent paper to establish the kinetic isotope effects for the enzymatic hydrolysis of the N-glycosidic bond of AMP and dAMP (23).

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Synthesis of nucleotides with specific radiolabels in ribose. Primary 14C and secondary 3H kinetic isotope effects on acid-catalyzed glycosidic bond hydrolysis of AMP, dAMP, and inosine.

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