5-Iodoribose 1-Phosphate, an Analog of Ribose 1-Phosphate

ENZYMATIC SYNTHESIS AND KINETIC STUDIES WITH ENZYMES OF PURINE, PYRIMIDINE, AND SUGAR PHOSPHATE METABOLISM*

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The 5'-deoxy-5'-iodo-substituted analogs of adenosine and inosine are cytotoxic to tumor cells that have high activities of 5'-methylthioadenosine phosphorylase and purine nucleoside phosphorylase, respectively. (Savarese, T. M., Chu, S.-H., Chu, M. Y., and Parks, R. E., Jr. (1984) Biochem. Pharmacol. 34, 361–367). 5-Iodoribose 1-phosphate (5-IRib-1-P), the common intracellular metabolite of these 5'-iodonucleosides, has been synthesized enzymatically from 5'-deoxy-5'-iodoadenosine via adenosine deaminase from Aspergillus oryzae and human erythrocytic purine nucleoside phosphorylase. The purification and chemical properties of 5-IRib-1-P are described. The analog sugar phosphate inhibited purine nucleoside phosphorylase from human erythrocytes, phosphoglucomutase from rabbit muscle, and 5'-methylthioadenosine phosphorylase from Sarcoma 180 cells with Kₜ values of 26, 100, and 9 μM, respectively. Enzymes that react with 5'-phosphoribosyl 1-pyrophosphate (P-Rib-PP), P-Rib-PP amidotransferase, hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, and orotate phosphoribosyltransferase-orotidylate decarboxylase from extracts of Sarcoma 180 cells, were inhibited with Kₜ values of 49, 465, 307, and 275 μM, respectively. 5-IRib-1-P had no effect on P-Rib-PP synthetase. Since the Kₜ values of the analog sugar phosphate for 5'-methylthioadenosine phosphorylase and P-Rib-PP amidotransferase are much lower than the Kₜ values of the natural substrates, Pₐ or P-Rib-PP which are reported to be present at nonsaturating concentrations under physiological conditions, these enzymes could be significantly inhibited by 5-IRib-1-P in intact cells.

The metabolism of 5'-deoxy-5'-methylthioadenosine, the ubiquitous by-product of S-adenosylmethionine in the synthesis of the polyamines, spermidine, and spermine, has been studied as a target for the development of chemotherapeutic agents (1–4). Exogenous 5'-methylthioadenosine inhibits both isolated enzymes and intact cells (5–10). Intracellular 5'-methylthioadenosine concentrations in the submicromolar range (11) suggest that it is either rapidly metabolized or excreted. The known catabolic pathway for this nucleoside in mammalian cells involves phosphorylase by 5'-methylthioadenosine phosphorylase to form adenine and 5-methylthioribose 1-phosphate (12). These products are salvaged to adenine nucleotides (13) and methionine (14, 15), respectively. Although normal tissues examined to date contain this phosphorylase, several mammalian tumors, including freshly isolated human leukemia cells, are deficient in this enzyme and secrete 5'-methylthioadenosine into the culture medium (1, 16, 17). 5'-Methylthioadenosine is not a substrate for mammalian adenosine deaminase (3).

Structure-activity relationship studies of 5'-methylthioadenosine phosphorylase have identified several 5'-methylthioadenosine analogs that are inhibitors (2) or alternative substrates (18). Among the latter are analogs that release cytotoxic adenine analogs, pentose 1-phosphate analogs, or both. Adenines or inosines substituted by a halogen atom on C(5') serve as substrates for 5'-methylthioadenosine phosphorylase or purine nucleoside phosphorylase, respectively (19, 20). The iodinated derivatives, 5'-IAdo and 5'-IIno, were cytotoxic to several cell lines in vitro (21). 5'-IAdo, which yields adenine and 5-IRib-1-P upon phosphorolytic cleavage, was cytotoxic to the L5178Y mouse lymphoblastic leukemia line (ID₅₀ = 9.0 μM) which has high 5'-methylthioadenosine phosphorylase activity (1.1 nmol units/mg of protein). By contrast, this analog was nontoxic to the L1210 mouse leukemia cell line (ID₅₀ >100 μM) which is deficient in this phosphorylase. However, the purine nucleoside phosphorylase activity in L1210 cells is about 15 times higher than in L5178Y cells. Thus, growth of L1210 cells was inhibited by 5'-IIno (ID₅₀ = 7.5 μM), whereas L5178Y cells were relatively insensitive (ID₅₀ >100 μM). Adenine was only weakly cytotoxic (ID₅₀ >100 μM), and hypoxanthine was nontoxic to these cell lines. These results indicate that intracellular formation of 5-IRib-1-P, the common product, was responsible for the cytotoxicity of both 5'-iodinated nucleosides. Fig. 1 shows the generation of 5-IRib-1-P by the reactions of 5'-methylthioadenosine phosphorylase or purine nucleoside phosphorylase with 5'-IAdo or 5'-IIno, respectively. It should be noted that replacement of the hydroxyl group on C(5') of the ribose by an iodine atom prevents reaction of the nucleoside analogs

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with nucleoside kinases and blocks conversion of the iodinated pentose 1-phosphate to a pentose 5-phosphate by phosphoglucomutase or phosphoribomutase.

Since 5-IRib-1-P is an analog of both 5-methylthioribose 1-phosphate and Rib-1-P, it is possible that the mechanism of cytotoxicity involves the inhibition of enzymes of purine or pentose phosphate metabolism. To test this hypothesis, 5-IRib-1-P was synthesized enzymatically to permit kinetic analyses with relevant enzymes. Preliminary accounts of some of the results have been presented elsewhere (22, 23), and complete descriptions of these and other studies are shown in Ref. 24.

**EXPERIMENTAL PROCEDURES**

**Enzymatic Synthesis of 8-Amino-5'-iodoguanosine**

To determine whether 8-amino-5'-iodoguanosine could be synthesized from 5-IRib-1-P and 8-aminoguanosine, the reaction with purine nucleoside phosphorylase was monitored by means of a Perkin-Elmer Model 402 scanning UV spectrophotometer and by reversed-phase HPLC. In the spectrophotometric assays, the incubation mixtures contained 250 µM 8-aminoguanosine, 200 µM 5-IRib-1-P, and 3 units of purine nucleoside phosphorylase in 1 ml of 25 mM Tris-C1 buffer, pH 7.5, and were incubated at 37 °C for 20 h. For HPLC assays, the reaction mixture contained 250 µM 8-aminoguanosine, 290 µM 5-IRib-1-P, and 3 units of purine nucleoside phosphorylase in 1 ml of 50 mM Tris-C1 buffer, pH 7.5, and were incubated at 37 °C for 20 h. The fractions containing 8-amino-5'-iodoguanosine with purine nucleoside phosphorylase was monitored by means of a Perkin-Elmer spectrophotometer at 258 nm (19).

**Enzyme Preparations and Determination of Kinetic Parameters**

The Vmax and K values were calculated from double-reciprocal plots (1/v versus 1/S) and Dixon plots (1/v versus I), respectively, by linear regression analyses. Inhibition of P-Rib-PP amidotransferase, adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, and orotate phosphoribosyltransferase-OMP decarboxylase was assayed by a competitive spectrophotometric method modified from Ray and Roscelli (34). The reaction mixture contained, in a final volume of 1 ml, 25 mM Tris-C1, pH 7.4, 2.5 mM MgCl2, 50 mM imidazole HCl, pH 7.3, 5 mM NADP, 3 µM glucose 1,6-bisphosphate, 0.08 unit of glucose-6-phosphate dehydrogenase, 0.13 unit of rabbit muscle phosphoglucomutase, and various concentrations of 5'-IAdo and 5'-IIno, respectively.

**Multiple Enzyme Inhibitions by 5-Iodorebose 1-Phosphate**

The reactions of 5'-methylthioadenosine phosphoribosyltransferase (MTAPase) and purine nucleoside phosphorylase (PNP) with 5'-IAdo and 5'-IIno, respectively, were followed by reversed phase HPLC. In the spectrophotometric assays, the incubation mixtures were started by the addition of P-Rib-PP and stopped by adsorbing 20 µl of the reaction mixture onto a Millipore filter support and placing in a scintillation vial with 1 ml of 1 N KOH. The assay mixtures in 500 µl contained 20 mM Tris-C1, 2.5 mM MgCl2, 50 mM imidazole HCl, pH 7.3, 5 mM NADP, 30 µM glucose 1,6-bisphosphate, 0.42 unit of glucose-6-phosphate dehydrogenase, 21.6 units of rabbit muscle phosphoglucomutase, and various concentrations of Rib-1-P.

P-Rib-PP Amidotransferase, Hypoxanthine-guanine Phosphoribosyltransferase, Adenine Phosphoribosyltransferase, and Orotate Phosphoribosyltransferase-OMP Decarboxylase from Sarcoma 180 Cells— Cells were obtained from female CD1 mice 6–7 days after intraperitoneal inoculation with about 2.5 × 10⁶ cells. The cells were washed twice with saline, and extracts were prepared as described elsewhere (36). P-Rib-PP amidotransferase activity was 0.0675 µmol/min/ml packed cells and was assayed by measuring the production of radiolabeled glutamate from [14C]glutamine (37). The assay mixture contained, in a final volume of 200 µl, 50 mM Tris-C1, pH 7.5, 5 mM MgCl2, 0.06 µCi [14C]glutamine, various concentrations of P-Rib-PP, and an appropriate volume of cell extract. After a 20-min preincubation with the cell extract at 37 °C, the reaction was started by the addition of P-Rib-PP and stopped by adsorbing 20 µl of the reaction mixture to a 25-mm DE-81 disc. All discs from a single experiment were immersed in 2 liters of distilled water and washed three times with 2 liters of water. Each dried disc was placed in a scintillation vial in 7 ml of scintillant for determination of the radioactivity.

Adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase were assayed by a modification of the method of Green and Martin (38) by measuring the production of radiolabeled AMP and IMP from [14C]adenine and [14C]hypoxanthine, respectively. The assay mixtures in 200 µl contained 50 mM Tris-C1, pH 7.6, 5 mM MgCl2, 0.1 mM adenosine or hypoxanthine, 0.25 µCi/ml [14C]adenine or [14C]hypoxanthine, various concentrations of P-Rib-PP, and the cell extract. The reaction was started by the addition of an aliquot of the cell extract to the assay mixture at 37 °C and was stopped by applying a 20-µl aliquot of the reaction mixture to a 25-mm DE-81 disc. Each disc was washed with about 20 ml of 1 M ammonium formate under suction on a Millipore filter support and then in a scintillation vial with 1 ml of 1 N HCl and 7 ml of scintillant fluid and counted.

Orotate phosphoribosyltransferase-OMP decarboxylase was assayed by the method of Jones et al. (39) which measured the production of [14C]CO2 from [14C]orotic acid. The [14C]CO2 was trapped in a center well which contained a Whatman 3MM filter disc soaked with 50 µl of 5 N KOH. The assay mixtures in 500 µl contained 20 mM Tris-C1, pH 7.5, 0.42 unit of glucose-6-phosphate dehydrogenase, 21.6 units of rabbit muscle phosphoglucomutase, and various concentrations of Rib-1-P.

The reactions of 5'-methylthioadenosine phosphoribosyltransferase (MTAPase) and purine nucleoside phosphorylase (PNP) with 5'-IAdo and 5'-IIno, respectively. Both analogs are used to determine the Vmax values obtained from the double-reciprocal plots were used to determine the K values from the Dixon plots. Similar values were determined from the intersection of two lines obtained at different fixed P-Rib-PP concentrations and from the equation, K = Ks/(1 + [S]/Ks), which is applicable to competitive inhibitors.

**Purine Nucleoside Phosphorylase from Human Erythrocytes—** Purine nucleoside phosphorylase was partially purified as described above (26) and chromatographed on calcium phosphate gel-cellulose (27). A fraction with a specific activity of 5 units/mg protein was dialyzed against 1 mM Tris-C1, pH 7.5, containing 1 mM dithiothreitol. The standard assay for purine nucleoside phosphorylase coupled with xanthine oxidase was employed (27).

5'-Methylthioadenosine Phosphorylase from Sarcoma 180 Cells— The cytosolic enzyme was partially purified from Sarcoma 180 cells as described previously (3) and applied to a chromatofocusing column (20). 5'-Methylthioadenosine phosphorylase emerged in two peaks from the chromatofocusing column; a large peak at pH 5.1 and a minor peak at 4.6. The specific activity of the large peak was 55.9 nmol/min/mg protein, indicating 21.5-fold purification.

5'-Methylthioadenosine phosphorylase activity was determined by monitoring the phosphorylisis of 5'-methylthioadenosine in the coupled spectrophotometric assay (3).
pH 7.6, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM orotic acid, 0.04 μCi/ml [¹⁴C]orotic acid, various concentrations of P-Rib-PP, and cell extracts. The reaction at 37 °C was started by the addition of an aliquot of the cell extract and was stopped by injection of 100 μl of 70% perchloric acid through the rubber cap with a syringe. The trapping of CO₂ from the acidified reaction mixture was allowed to proceed for 40 min. The center wells were removed from the tubes and placed in scintillation vials with 8 ml of scintillant fluid for counting.

P-Rib-PP Synthetase from Sarcoma 180 Cells—Cell extracts were prepared as above. After centrifugation at 105,000 × g for 1 h, the pellet was resuspended in 12 ml of 0.125 M sucrose containing 0.05 M KCl, 0.07 M Tris-Cl, pH 7.6, 1 mM dithiothreitol, and 2% Triton X-100. The suspension was dispersed by 20 half-s pulses from a Model W-2225R sonicator with the output setting at 5, before centrifugation at 10,000 × g for 1 h. The supernatant was used for assays by a modification of the method of Danis and Scholer (40) which measured the production of [¹⁴C]orotic acid. Each 200-μl reaction mixture contained 50 mM potassium phosphate buffer, pH 7.35, 120 μM ribose 5-phosphate; 1 mM ATP, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 mM dithiothreitol, and cell extract. The reaction at 37 °C was started by the addition of the cell extract, and, after 30 min in a shaking water bath, was stopped by heating each tube for 30 min at 115 °C. The P-Rib-PP in the reaction mixture was quantitated by the following method to compensate for the inhibition of the coupled enzymes, orotate phosphoribosyltransferase-OMP decarboxylase, by 5-IRib-1-P. After the reaction mixture was cooled, 50 mM potassium phosphate buffer, pH 7.35, 0.5 mg of orotate phosphoribosyltransferase-OMP decarboxylase (3 units/mg protein), 0.1 mM orotic acid, and 0.04 μCi/ml [¹⁴C]orotic acid were added to give a final volume of 500 μl. The reaction at 37 °C was started by addition of the enzymes and was stopped after 90 min by injection of 100 μl of 70% perchloric acid. Further procedures were followed as described above.

RESULTS

Substrate Activity with Nucleoside Phosphorylases and Synthesis of 8-Amino-5'-iodoguanosine—The purified 5-IRib-1-P served as a substrate for 5'-methylthioadenosine phosphorylase and purine nucleoside phosphorylase in the synthesis of 5'-1Ado, 5'-1Ino, and 5'-1Guo. The formation of the novel Guo analog was confirmed by the normal UV spectral shift for the formation 5'-IAdo, 5'-IIno, and 5'-IGuo. The formation of the novel Guo analog was confirmed by the normal UV spectral shift observed for the formation of 5'-IAdo and 5'-IIno was verified by their known elution pattern in analytical reversed-phase HPLC (see the Miniprint Section for retention times). The synthesis of 8-amino-5'-iodoguanosine was similarly confirmed by the UV spectral shift observed for the formation of 8- aminoquinoguanosine (19) and a distinct retention time in HPLC profiles. The synthesis of 5'-1Ado and 5'-1Ino was verified by their characteristic retention pattern in analytical reversed-phase HPLC (see the Miniprint Section for retention times). The synthesis of 8-amino-5'-iodoguanosine was similarly confirmed by the UV spectral shift observed for the formation of 8-aminoquinoguanosine (19) and a distinct retention time on analytical reversed-phase HPLC. As reported earlier, an amino group at C(8) of the purine base increases the affinity more than 10-fold; an iodine atom at C(5') of inosine increases affinity 3-fold, while decreasing the maximal velocity with purine nucleoside phosphorylase by 1000-fold (19). Consequently, the combination of these two modifications could result in a potent purine nucleoside phosphorylase inhibitor. Table III shows the affinities for purine nucleoside phosphorylase of bases and nucleosides substituted at C(5') or C(8). The new analog, 8-amino-5'-iodoguanosine, has a Kᵢ value of ~3.3 μM, which is intermediate between those for 8-aminoquinine and 8-aminoguanine and about 10-fold lower than the Kᵢ of guanosine.

Effects of 5-IRib-1-P on Specific Enzymes of Purine, Pyrimidine, and Sugar Phosphate Metabolism—Purified 5-IRib-1-P was tested as an inhibitor of purine nucleoside phosphorylase from human erythrocytes, phosphoglucomutase from rabbit muscle and 5'-methylthioadenosine phosphorylase, P-Rib-PP amidotransferase, hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, orotate phosphoribosyltransferase-OMP decarboxylase, and P-Rib-PP synthetase from Sarcoma 180 cells. The Kᵢ values are presented in Tables IV and V in comparison with the kinetic constants of inorganic phosphate and the natural sugar phosphates.

Earlier studies of human erythrocytic purine nucleoside phosphorylase indicated that its reaction follows an ordered Bi Bi sequence with the nucleoside the first substrate to add, followed by P₀ and with the pentose 1-phosphate the first product to leave, followed by the purine base (42). Therefore, 5-IRib-1-P was examined as a product inhibitor in comparison with Rib-1-P, with P₀ as the variable substrate. 5-IRib-1-P showed greater affinity than Rib-1-P. Two Kᵢ values were determined for both sugar phosphates because purine nucleoside phosphorylase showed nonlinear double-reciprocal plots with P₀ or Rib-1-P as the variable substrate for inosine phosphorylase or guanosine synthesis, respectively. Fig. 2 illustrates the biphasic nature of a double-reciprocal plot that yielded Kᵢ values of 60 and 417 μM for P₀. Double-reciprocal plots with Rib-1-P as the variable substrate for guanosine synthesis were also biphasic and yielded Kᵢ values of 131 and 326 μM. When the pentose 1-phosphates were used as inhibitors at concentrations of P₀ >100 μM, Rib-1-P and 5-IRib-1-P appeared to act as competitive inhibitors since the y-coordinate of the intersect of the lines coincided with the reciprocal Vₘₐₓ; Kᵢ values of 110 and 26 μM, respectively, were determined from the Dixon plots of Fig. 3, a and b. At lower P₀ concentrations, a noncompetitive inhibition was observed and an apparent Kᵢ value is given. The reaction rates observed at high concentrations of the inhibitor, Rib-1-P or 5-IRib-1-P, are faster than those predicted from the extrapolation in the Dixon plot of the lines obtained at low concentrations of inhibitor. This suggests that, although they function as inhibitors, the pentose 1-phosphates may also induce negative cooperativity.

With 5'-methylthioadenosine phosphorylase, a Kᵢ value for P₀ of 440 μM was determined with freshly prepared enzyme. However, after storage at −20 °C, the values was twice as high. Savarese et al. (3) determined a Kᵢ value for P₀ of 3.5 mM with 5'-methylthioadenosine phosphorylase from Sarcoma 180 cells. Kᵢ values of 7.5, 21, and 0.2 mM have been reported for P₀ for the enzyme from human peripheral lymphocytes (43), human erythrocytes (44), and rat liver (45), respectively. 5-IRib-1-P was examined as a product inhibitor with P₀ as the variable substrate. Inhibition of 5'-methylthioadenosine phosphorylase was especially pronounced. The Kᵢ value of 5-IRib-1-P was 50-100 times lower than the Kᵢ value of P₀. The natural product, 5'-methylthioribose 1-phosphate, was not available for comparison. The affinity of 5-IRib-1-P was found to be ~200-fold greater than that of Rib-1-P.

The primary substrate for phosphoglucomutase is Glc-1-P, but Rib-1-P also reacts with low affinity and ~25-fold lower activity (46). Markedly different kinetic parameters for Rib-

### Table III

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ (μM)</th>
<th>Kᵢ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-IIno</td>
<td>12°</td>
<td></td>
</tr>
<tr>
<td>8-Adenosine</td>
<td>0.2-1.2</td>
<td>4°</td>
</tr>
<tr>
<td>8-Aminoguanosine</td>
<td>17°</td>
<td></td>
</tr>
<tr>
<td>8-Amino-5'-iodoguanosine</td>
<td>3.3</td>
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</table>

*Data were obtained from Ref. 19.
*μM Value was obtained from Ref. 41.
Multiple Enzyme Inhibitions by 5-Iodoribose 1-Phosphate

TABLE IV
Kinetic constants of 5-IRib-1-P with phosphorylases and phosphoglucomutase

Purine nucleoside phosphorylase, 5'-methylthioadenosine phosphorylase, and phosphoglucomutase were measured by coupled spectrophotometric assays with inosine, 5'-methylthioadenosine, and glucose 1,6-bisphosphate, respectively, as the saturating substrates. All the $K_i$ values were determined from Dixon plots. The $K_m$ and $K_i$ values are the mean ± S.D. of 2-3 determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Kinetic parameter</th>
<th>μM</th>
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<tr>
<td>Purine nucleoside phosphorylase</td>
<td>$P_i$</td>
<td>$K_m$ (low)*</td>
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<tr>
<td></td>
<td></td>
<td>$K_m$ (high)*</td>
<td>417</td>
<td></td>
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<tr>
<td></td>
<td>$P_i$</td>
<td>$K_i$</td>
<td>26 ± 1</td>
<td></td>
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<tr>
<td></td>
<td>5-IRib-1-P</td>
<td>$K_i$</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At 150, 200 μM</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>$P_i$</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>At 50 μM</td>
<td>$K_i$</td>
<td>110</td>
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<td>Rib-1-P</td>
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<td>270</td>
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<td>At 100, 200 μM</td>
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<td></td>
<td>$P_i$</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>At 50 μM</td>
<td>$K_i$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-Methylthioadenosine phosphorylase</td>
<td>$P_i$</td>
<td>$K_m$</td>
<td>440*</td>
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<tr>
<td></td>
<td>5-IRib-1-P</td>
<td>$K_i$</td>
<td>860 ± 23</td>
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<tr>
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<td>At 0.5, 1 mM</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rib-1-P</td>
<td>$K_i$</td>
<td>9 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At 1 mM</td>
<td>$K_i$ (high)*</td>
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<tr>
<td>Phosphoglucomutase</td>
<td>Glc-1-P</td>
<td>$K_m$</td>
<td>21 ± 0</td>
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<tr>
<td></td>
<td></td>
<td>$K_i$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-IRib-1-P</td>
<td>$K_i$</td>
<td>100 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>At 10, 20 μM</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Rib-1-P</td>
<td>$K_m$</td>
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<td></td>
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<tr>
<td></td>
<td>At 10, 20 μM</td>
<td>$K_i$</td>
<td>1,000 ± 64</td>
<td></td>
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<tr>
<td></td>
<td>Rib-1-P</td>
<td>$K_i$ (high)*</td>
<td>1,148 ± 52</td>
<td></td>
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</tbody>
</table>

* Different $K_m$ values of $P_i$ were determined at low (<500 μM) and high (>500 μM) concentration ranges.

** The $K_m$ value of $P_i$ in a fresh enzyme preparation before storage at -20°C.

1-P have been reported for the enzyme from different sources. The $K_i$ and $K_m$ values for Rib-1-P were reported as 25 and 11 mM, respectively, for human erythrocytic phosphoglucomutase (47), whereas the $K_m$ of Rib-1-P was 0.9 mM for rabbit muscle phosphoglucomutase (48). The present results with Rib-1-P and 5-IRib-1-P may reflect the use of two different assay procedures for phosphoglucomutase and phosphoribomutase activity. The $K_i$ value of 5-IRib-1-P for phosphoglucomutase is high and similar to the $K_m$ value of Rib-1-P, when Rib-1-P is the variable substrate. When Glc-1-P was used as substrate, the $K_i$ value of 5-IRib-1-P decreased by about 10-fold to 100 μM.

Table V presents the effects of 5-IRib-1-P as an inhibitor for the four phosphoribosyltransferases with P-Rib-PP as the variable substrate. P-Rib-PP amidotransferase bound 5-IRib-1-P ~100 times more tightly than Rib-1-P. The $K_i$ value was about 2-fold lower than the $K_m$ value for P-Rib-PP. Hypoxanthine-guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, and orotate phosphoribosyltransferase-OMP decarboxylase from Sarcoma 180 cell extracts were only **
weakly inhibited by 5-IRib-1-P. The analog sugar phosphate had no effect on Sarcoma 180 P-Rib-PP synthetase.

**DISCUSSION**

5-Iodoribose 1-phosphate, the toxic metabolite common to the phosphorylisis of either 5'-IAdo or 5'-IIno, has been found to inhibit a variety of reactions that use 5-methylthioribose-1-P, Rib-1-P, Glc-1-P, or P-Rib-1-PP as substrates. Inhibition studies with 5'-methylthioadenosine phosphorylase and purine nucleoside phosphorylase, enzymes that generate 5-IRib-1-P intracellularly, showed that the analog has $K_i$ values for these enzymes that are lower than the $K_m$ values of the natural substrate, P$_i$. The affinity of 5-IRib-1-P is 3-4-fold higher than that of Rib-1-P for purine nucleoside phosphorylase. This is similar to the difference in the $K_m$ values of 5'-IIno (12 $\mu$M) and Ino (33 $\mu$M) for purine nucleoside phosphorylase (19). In intact cells, phosphorolytic reactions are favored for both purine nucleoside phosphorylase and 5'-methylthioadenosine phosphorylase due to the efficient removal of the purine base by reaction with a phosphoribosyltransferase. Since intracellular P$_i$ concentrations range from 0.5 to 2 mM (49), neither enzyme is saturated with P$_i$. Thus, 5-IRib-1-P may act as a product inhibitor of these enzymes. The $K_i$ value of 5-IRib-1-P is 16 times lower than the $K_m$ value of P$_i$ for purine nucleoside phosphorylase at the physiological P$_i$ concentration range, whereas the $K_i$ value of 5-IRib-1-P is 50-100 times lower than the $K_m$ value of P$_i$ for 5'-methylthioadenosine phosphorylase. These results suggest that the analog sugar phosphate could be much more inhibitory to 5'-methylthioadenosine phosphorylase than to purine nucleoside phosphorylase in intact cells. However, final judgment on the relative affinities of P$_i$ and 5-IRib-1-P for both enzymes must be reserved since the $K_m$ values may represent the upper limits of the substrate dissociation constants. In addition, widely different $K_m$ values for P$_i$ have been reported for 5'-methylthioadenosine phosphorylase. Savarese et al. (3) gave a $K_m$ value of 3.5 mM for the enzyme in the cytosol of Sarcoma 180 cells. In the present study, 21-fold purified enzyme obtained by chromatofocusing showed a $K_m$ value of 440 $\mu$M with freshly prepared enzyme and 860 $\mu$M after storage at -20 °C. Although Rib-1-P is not a natural substrate for 5'-methylthioadenosine phosphorylase, its affinity was compared with that of 5-IRib-1-P because 5-methylthioribose-1-P was not available. The $K_i$ value of 5-IRib-1-P is ~200-fold lower than that of Rib-1-P, whereas the $K_m$ value of 5'-IAdo is ~140-fold lower than that of adenosine. Thus, as with purine nucleoside phosphorylase, substitution on C(5') of the nucleoside by an iodine atom results in a similar increase in the affinities of both the nucleoside and the resulting pentose 1-phosphate.

In studies with purine nucleoside phosphorylase, two different $K_m$ values were determined for both P$_i$ and Rib-1-P at high and low substrate concentration ranges. This indicates negative cooperativity induced at high substrate concentrations. Similar phenomena were observed previously with a nucleoside or base (19, 50) as the substrate, as well as with P$_i$ (51-53). The apparent $K_m$ values of the bovine liver enzyme for P$_i$ were reported to be 130 $\mu$M at low substrate concentrations and 360 $\mu$M at high substrate concentrations (51). With the bovine thyroid enzyme, values of 350 and 1490 $\mu$M were obtained (52). In contrast, earlier results from our laboratory with human erythrocytic purine nucleoside phosphorylase indicated substrate activation by P$_i$ only at pH 6.0 and below (53). The erythrocytic enzyme is subject to post-translational modifications that markedly affect the degree of cooperativity (54). This phenomenon is most pronounced in the most acidic electrophoretic variants and may differ from one enzyme preparation to another. Fig. 3, a and b, indicates that the apparent activations induced by the inhibitory sugar phosphates were additive to that caused by the substrate, P$_i$.  

**Fig. 2.** Double-reciprocal plot of the reaction of human erythrocytic purine nucleoside phosphorylase when P$_i$ is used as the variable substrate. Purine nucleoside phosphorylase was measured by the coupled spectrophotometric assay at 293 nm with inosine as the saturating substrate. Velocity is expressed as the change in absorbance/min × 10$^4$. The reaction mixture contained purine nucleoside phosphorylase, 50 mM Hepes, pH 7.3, 0.5 mM inosine, 0.02 units of xanthine oxidase, and variable concentrations of P$_i$ in 1 ml.

**Fig. 3.** Inhibition of purine nucleoside phosphorylase by Rib-1-P and 5-IRib-1-P. Purine nucleoside phosphorylase was measured by the coupled spectrophotometric assay with inosine as the saturating substrate. The enzyme was preincubated with 0.02 units of xanthine oxidase, 0.5 mM inosine, 50 mM Hepes, pH 7.5, and inhibitor in 1 ml until a low level of background activity due to P$_i$ contamination was eliminated. The reaction at 30 °C was started with addition of P$_i$. Velocity is expressed as the change in absorbance/min at 293 nm with xanthine oxidase, 0.5 mM inosine, 50 mM Hepes, pH 7.5, and inhibitor in 1 ml until a low level of background activity due to P$_i$ contamination was eliminated. The reaction at 30 °C was started with addition of P$_i$.
Multiple Enzyme Inhibitions by 5-Iodoribose 1-Phosphate

Apparent activation by purine nucleoside phosphorylase inhibitors has also been seen with 8-aminoguanine (19) and 1-beta-rutinosyl-1,2,4-triazole-3-carboxamide (55).

Since the C(5') atom of the ribose is blocked by the iodine substitution, 5-IRib-1-P is incapable of serving as a substrate for phosphoglucomutase or phosphoribomutase. However, it acts as an inhibitor of phosphoglucomutase with substantially greater affinity than Rib-1-P when Glic-1-P is the substrate, i.e. about 100-fold. Although 5-I Rib-1-P was more inhibitory than Rib-1-P when Glic-1-P was the substrate, phosphoglucomutase is not likely to be a significant target for 5-I Rib-1-P because the natural substrate has a much higher affinity. For reasons that have not yet been examined, the affinity of 5-I Rib-1-P is about 10-fold lower when the secondary substrate, Rib-1-P, is studied. The phosphoglucomutase reaction is complex and employs glucose 1,6-bisphosphate as an intermediate metabolite. From steric considerations, it seems possible that 5-I Rib-1-P behaves as an analog of glucose 1,6-bisphosphate or of ribose 1,5-bisphosphate in these reactions. These interesting findings are worthy of further detailed study. Ideally, one should examine phosphoribomutase rather than phosphoglucomutase. Unfortunately, phosphoribomutase is not available commercially and, although it has been purified from rat liver and studied in detail (56), it is highly unstable.

Since P-Rib-1-PP amidotransferase, adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, and orotate phosphoribosyltransferase-OMP decarboxylase are key enzymes for purine and pyrimidine synthesis de novo and salvage, kinetic studies were performed to determine the effects of 5-I Rib-1-P. With P-Rib-1-PP amidotransferase, 5-I Rib-1-P (K_i = 49 g M) has 100-fold greater affinity than Rib-1-P (K_i = 4700 g M). The 5-I Rib-1-P value of 5-I Rib-1-P is half of the K_m value of P-Rib-1-PP. P-Rib-1-P may be a rate-limiting substrate of Sarcoma 180 P-Rib-1-PP amidotransferase for purine biosynthesis de novo. The K_m value for P-Rib-1-PP is 104 g M, whereas its intracellular concentration is 5-30 g M under normal resting conditions (57, 58). Since the K_m value of 5-I Rib-1-P is 49 g M, it may cause significant inhibition of P-Rib-1-PP amidotransferase in these cells under physiological conditions. However, since intracellular P-Rib-1-PP levels can be modified by factors such as elevated glucose or P_i concentration, decreased di- and triphosphate nucleotide levels, or blockade of purine or pyrimidine biosynthesis de novo (59-62), it is possible that these cells could overcome the inhibition. When adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, and orotate phosphoribosyltransferase. OMP decarboxylase, the low affinities for the analog sugar phosphate and the high affinities for the natural substrate, P-Rib-1-PP, indicate that potent inhibitions of these enzymes by 5-I Rib-1-P would not be expected in intact cells. P-Rib-1-PP synthetase was not inhibited by 5-I Rib-1-P.

While 5-I Rib-1-P inhibits several enzymes of pyrimidine, and sugar phosphate metabolism, its affinity for these enzymes differs considerably, and the importance of the inhibitions must be evaluated in relation to the K_m and intracellular concentrations of the relevant substrates. Under physiological conditions, P_i and P-Rib-1-PP may be nonsaturating substrates for 5'-methylthioadenosine phosphorylase and P-Rib-1-PP amidotransferase, respectively. Since these enzymes are most strongly inhibited by 5-I Rib-1-P, they may be important targets for the analog sugar phosphate in intact cells. Studies performed to date (24) with intact L5178Y cells showed that both purine synthesis de novo and P-Rib-1-PP accumulation were inhibited by 5'-1Ado, while purine salvage was not affected significantly. When the component of inhibition that could be attributed to the adenine released from the nucleoside was subtracted from the total effect, only purine synthesis de novo appeared to be inhibited by 5-I Rib-1-P itself. This finding agrees with the present results with the individual enzymes. In contrast, purine synthesis de novo was not inhibited by 5'-1Ado in intact HL-60 cells. These results indicate tissue or species differences in the purine metabolic pathways of L5178Y and HL-60 cells. These limited experiments with the two cell lines suggest that the cytotoxicity of 5'-iodonucleosides is not due to the inhibition of purine metabolism, since inhibition of purine synthesis de novo would be counteracted by the concomitant release and salvage of the adenine or hypoxanthine moiety. Recent preliminary experiments have shown that incubation of human erythrocytes and HL-60 cells with 5'-Iino and 5'-1Ado, respectively, can result in intracellular accumulation of 5-I Rib-1-P to high levels. Further studies are necessary to elucidate the mechanisms of toxicity of the analog sugar phosphate with different cell lines.

The structure and purity of the 5-I Rib-1-P used in these studies have been confirmed by several criteria: 1) stoichiometry of ribose and phosphorus is in agreement with the proposed structure; and 2) it reacted with 5'-methylthioadenosine phosphorylase and adenine and with purine nucleoside phosphorylase and hypoxanthine to produce the expected 5'-iodonucleosides. The availability of this iodinated sugar phosphate has also enabled the enzymatic synthesis of a proposed (19) purine nucleoside phosphorylase inhibitor, 8- amino-5'-iodoguanosine. This new analog has an affinity intermediate between those of 5'-Iino and 8-aminoguanine. Intracellular phosphorylation of 8-amino-5'-iodoguanosine would liberate both 8-aminoguanine, a potent inhibitor of purine nucleoside phosphorylase (K_i = 0.2-1.2 g M), and the cytotoxic sugar phosphate, 5-I Rib-1-P. The synthesis of this analog illustrates the potential usefulness of enzymatic procedures for the preparation of other base-modified 5'-halogenated nucleosides. Such analogs might release nonsalvageable or cytotoxic purine bases when they are cleaved intracel- lularly by the action of purine nucleoside phosphorylase or 5'-methylthioadenosine phosphorylase. If a purine that is unreactive or poorly reactive with hypoxanthine-guanine phosphoribosyltransferase or adenine phosphoribosyltrans- ferase were released, e.g. 8-aminoguanine (19) or 8-aza-adenine, the effect of 5-I Rib-1-P on purine synthesis de novo would not be counteracted. Moreover, if a cytotoxic base such as 6-thioguanine were released, its activation by hypoxan- thine-guanine phosphoribosyltransferase would be potent- iated by the inhibition of biosynthesis de novo which depletes natural purine nucleotides and provides more P-Rib-1-PP for the salvage reaction. Thus, the base and sugar moieties might act synergistically to inhibit purine nucleotide metabolism and cell division.

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Preparation of Enzymes - Non-specific adenine deaminase from \textit{Aspergillus flavus} was extracted from the crude amylease preparation with 50% ethanol and further by a modification of a published procedure in order to eliminate contaminating nucleosidase activity (26). The deaminase activity was eluted from a Sephadex A-50 column (2.5 x 2 cm) with a convex gradient of 0.5 L of 10 mM potassium phosphate buffer (pH 7.0) and 1.5 L of 10 mM potassium phosphate buffer, pH 7.0. Fractions 23 to 30, containing most of the deaminase activity (73% units), were free of detectable nucleosidase activity and were pooled. High ionic strength (0.5 M NaCl) and increased phosphate (0.5 M Na2HPO4) gave the highest activities. The adenine deaminase activity was determined by monitoring the deamination of adenine at 265 nm. Nucleosidase activity was determined by reversed phase HPLC as described below.

Human erythrocruorin nucleoside phosphorylase was partially purified at the New England Enzyme Center, Boston, MA, by a large-scale method (26). A fraction with a specific activity of 0.5 unit/mg protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM diethanolamine. Enzymic activity was determined at 25°C by the standard assay for inosine phosphorylase coupled with the xanthine oxidase reaction (27).

Enzymic Synthesis and Purification of 5-Iodo-1-Phosphate

5'-Iodo-1-phosphate was synthesized enzymically from 1-Phospho-5'-ribose 1-Phosphate, a gift of MCB Uanufacturing, Chemists, Inc., Uedford, MA; the analytical and semipreparative reversed-phase HPLC columns (7.8 x 300 mm) were purchased from Waters Associates, Milford, MA. The purine nucleoside phosphorylase (8000 units/mg) and xanthine oxidase (1000 units/mg) were purified as described. The final reaction was adjusted to pH 6.5, frozen, thawed and passed through a 0.22 mm filter to remove insoluble reaction products, especially uric acid. Compounds with molecular weights 320, 1250, 1500 and 3700, were removed by gel filtration on a Sephadex G-25 column (50 mm x 1.5 cm) and dialyzed against 50 mM Tris-HCl, pH 7.0. The fractions that eluted between 90 min and 240 min were collected and lyophilized. The product was dissolved in 10 ml of water and chromatographed again on the descending column to decrease further the amount of contaminating cytosine. The final product was lyophilized for storage at -20°C.

Purification of 5-IBP-1-Phosphate - The reaction mixture was adjusted to pH 6.5, frozen, thawed and passed through a 0.22 mm filter to remove insoluble reaction products, especially uric acid. The compound was purified by ion-pair chromatography on a semipreparative reversed phase HPLC column (7.5 x 30 mm) which was equilibrated and eluted with 100 mM sodium phosphate buffer, pH 6.5. The effluent was monitored by absorbance at 254 nm and collected at 2.5 ml fractions which were assayed for ribose, and inorganic and total orthophosphate by the methods of Perry and Logan (29). Phosphate, and total phosphate by the Fiske and SubbaRow tests (30), respectively. The fractions that eluted between 90 min and 140 min were collected and lyophilized. The resulting powder was dissolved in 4 ml of water, filtered through a Sephadex G-25 column (50 mm x 1.5 cm) which was equilibrated and eluted with water. The nucleoside fractions were collected and lyophilized. The product was dissolved in 10 ml of water and chromatographed again on the descending column to decrease further the amount of contaminating cytosine. The final product was lyophilized for storage at -20°C.

After reversed phase ion pair HPLC the product showed weak UV absorbance at 254 nm. The purity of this fraction was tested by rechromatography on an analytical reversed phase HPLC. Approximately 450 nm (by the orcinol test) 5-IBP-1-Phosphate consistently showed a sharply split peak detected by absorption at 254 nm with a retention time of 3 min. This fraction was orcinol positive and yielded 5'-111m when incubated with hypoxanthine and pyrophosphate. Three different solvent systems a) 0 to 100% methanol gradient in 10 mM phosphate buffer, pH 6.5 b) 20 mM methanol isocratically, and c) 20% methanol isocratically were used for the analytical reversed phase HPLC. Based on their reactivity in the nucleoside synthesis assay (described below) with components of the split peak as active substrates.

Cumulative recoveries during synthesis and purification of 5-IBP-1-Phosphate, based solely on determinations of the ribose content by the ortcinol procedure, indicated 100% recovery after deamination, 84% after phosphorilation and 78% after RNase digestion. The reaction steps were monitored by analytical reversed phase HPLC, which indicated that degradation of 5-IBP was 3% and phosphorylation of 5'-IAdo was 100%. Even though degradation was not complete, the reaction was stopped to avoid bacterial growth. Theorically, the ribose content should have decreased by 10% by deamination recovery was indicated by the orcinol assay. After ion-pair reversed phase HPLC, 5-IBP-1-Phosphate was separated from other ribose-containing compounds and recovery was found to be only 2%. The reason for low yield has not been determined, but it could have resulted from a low level of hydrolysis remaining in the fungal adenine deaminase preparation or the instability of the iodide or phosphate substituent.

Characterization of 5-IBP-1-Phosphate

This Layer Chromatography (TLC) on silica gel plates was used to detect 5-IBP-1-Phosphate for small scale purification. Nucleosides and bases were identified by UV absorbance at 254 nm. Orcheforol chloride reagent was sprayed on the developed plates which were then heated at 50°C for 15 min. Blue spots indicated the presence of sugar-containing compounds. Elution with methylcellulose: methylethylketone: 36 FL:60:4 (7:1:4) gave separations with the Rf values of 0.85, 0.67, 0.52 and 0.58 for hypoxanthine, 5'-IAdo, uric acid and 5'-IBP-1-Phosphate, respectively.

Analytical Reversed Phase HPLC - A phenylalum 4.0 cm column (5.0 x 30 cm) was eluted with 10 mM cytochrome-cysteic acid, pH 6.5, at a flow rate of 1.2 ml/min. The retention times of sugar phosphates and other relevant compounds were 5'-IAdo (4 min), ribose (4 min), 5'-IBP-1-Phosphate (5 min), hypoxanthine (6 min), uric acid (7 min), 5'-IAdo (8 min) and 5'-IBP-1-Phosphate (13 min).

Chemical Characterization - In a typical preparation, 687 nmol of 5'-IAdo yielded 174 nmol of the purified 5-IBP-1-Phosphate after gel filtration, i.e. a yield of about 24%. The 5-IBP-1-Phosphate represented only 24% of the product by weight; the remainder was accounted for by cytosine or its nucleoside salt which were produced by the proton magnetic resonance spectra. Analysis showed an approximate 1:1 stoichiometry between the pentose sugar and acid-labile phosphate. Furthermore, the product was detectable on reversed phase HPLC by weak absorbance at 254 nm. The Kiyota iodide bond is reported to absorb maximally between 255 and 260 nm (31). To verify that the UV absorbance at 254 nm was due to the C-1 bond, 5-iododeoxyribosil was obtained by acid hydrolysis
of 5'-IAdo and chromatographed on reversed phase HPLC. The fraction detected by the orcinol and iodine tests (32) also showed a peak at 254 nm. The UV spectrum in water of the final preparation after gel filtration of the cyclohexylamine salt of 5'-IAdo-1-P had a peak at 240 nm with a molar absorption coefficient of 320 cm⁻¹ M⁻¹, which is somewhat lower than the reported value of 500 cm⁻¹ M⁻¹ for this type of bond in aliphatic iodides (31).

Proton Magnetic Resonance Spectroscopy—The spectra were obtained on a Brucker AM-250 spectrometer. About 1 mg of sample was lyophilized four times from 99.8% D₂O and then dissolved in 1 ml of D₂O. Chemical shifts were reported relative to 3-(trimethylsilyl)-1-propanesulfonic acid as the external reference. Chemical shifts and coupling constants are given in Table I. The proton magnetic resonance spectrum of 5'-IAdo-1-P showed a similar profile to that of Rib-1-P. However, the peak corresponding to the proton at C-5 is shifted upfield from 3.7 to 3.4 ppm, there are differences in coupling constants, J₅₋₆ and J₄₋₅ of 4-β and there is an inversion in the relative positions of 2-β and 4-β.

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\begin{array}{lll}
\text{Chemical shifts and coupling constants for 5'-IAdo-1-P and Rib-1-P} \\
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\]

- 5'-IAdo-1-P (in D₂O): 5.6 (q, 3b, J₅₋₆ 4.0 Hz), 5.0 (d, 3b, 8.5 Hz)
- 5'-Rib-1-P (in D₂O): 5.6 (q, 3b, J₅₋₆ 3.9 Hz), 5.0 (d, 3b, 8.5 Hz)

10 mM KH₂PO₄ buffer, pH 7.5, was used as the starting buffer. A linear increase of the second buffer from 0 to 100% at 9%/min was used for the gradient profile. The flow rate was 1.2 ml/min.

Enzymatic Synthesis of 5'-Iodonucleosides from 5'-IAdo-1-P—To confirm the synthesis of 5'-IAdo-1-P, fractions from the semipreparative reversed phase HPLC were tested for substrate activity with purine nucleoside phosphorylase and 5'-methylthioadenosine phosphorylase. The incubation mixture for 5'-methylthioadenosine phosphorylase contained 100 μM hypoxanthine or adenine, 1 unit of purine nucleoside phosphorylase, and 1 unit of 5'-iodo- or 5'-iidosadenosine. The reaction was carried out at 37°C for 10 min in 1 ml of 50 mM Tris·Cl buffer, pH 7.5, at 37°C on a shaking water bath. The reaction mixture was monitored by reversed phase HPLC and TLC. 5'-Iodonucleosides were readily separated from the respective bases and nucleosides by analytical reversed phase HPLC on a phenylpentyl (4) C₁₈ column. Separations were achieved with a programmed gradient, 10 mM potassium phosphate buffer (pH 5.5) as the starting eluent and 10 mM potassium phosphate buffer (pH 5.5) containing 50% methanol as the second eluent. The gradient profile had a linear increase of the second buffer from 0 to 100% at a rate of 9%/min, a 10 min isocratic elution at 100% and a linear decrease (-9%/min) until 0% was reached. The flow rate was 1.2 ml/min. Guanine, guanosine and 5'-jodosguanosine emerged with retention times of 5 min, 11 min, and 13 min; hypoxanthine, inosine and 5'-jodoinosine, 4 min, 8 min, and 12 min; adenosine, adenosine and 5'-jodoado, 8 min, 9 min and 15 min. Cellulose plates developed with 5% MgEDTA gave R₅ values of 0.46, 0.61, 0.67, 0.70, and 0.38 for hypoxanthine, 5'-jodoinosine, inosine, adenosine and 5'-jodoado, respectively. Elution with water gave a better separation of inosine and 5'-jodoinosine with R₅ values of 0.49, 0.60 and 0.72 for hypoxanthine, 5'-jodoinosine and inosine, respectively.
5-Iodoribose 1-phosphate, an analog of ribose 1-phosphate. Enzymatic synthesis and kinetic studies with enzymes of purine, pyrimidine, and sugar phosphate metabolism.
H S Choi, J D Stoeckler and R E Parks, Jr