Mechanism of Mevalonate Pyrophosphate Decarboxylase: Evidence for a Carbocationic Transition State†,‡

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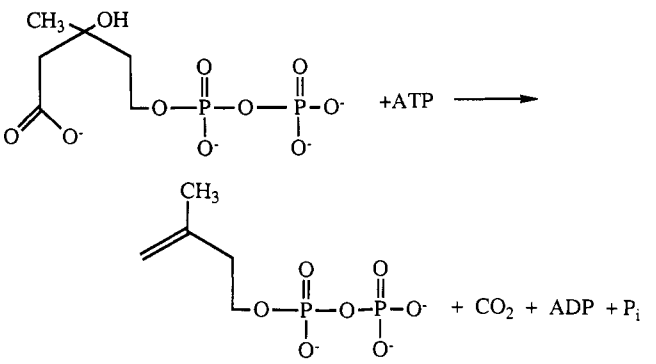
ABSTRACT: Mevalonate pyrophosphate decarboxylase catalyzes the decarboxylation of mevalonate pyrophosphate to isopentyl pyrophosphate. The mechanism of action of this enzyme was investigated to elucidate the mechanism of inhibition by 3-hydroxy-3-(fluoromethyl)-5-pyrophosphopentanoic acid (**II**). It was previously found that **II** is a competitive inhibitor ($K_i = 0.01$ μM) of the enzyme reaction [Reardon, J. E., & Abeles, R. H. (1987) *Biochemistry 26,* 4717−4722; Nave, J. F., d'Orchymont, H., Ducep, J. B., Piriou, F., & Jung, M. J. (1985) *Biochem. J. 227,* 247−254]. We have now observed that **II** is decarboxylated 2500-fold more slowly than mevalonate pyrophosphate (3-hydroxy-3-methyl-5-pyrophosphopentanoic acid, **I**). The enzyme was exposed to saturating concentrations of **II** and ATP and then passed through a Penefsky column to remove excess substrate. The enzyme was denatured immediately upon emerging from the Penefsky column. Nearly 1 equiv of both 3-phospho-3-(fluoromethyl)-5-pyrophosphopentanoic acid and ADP was bound to the enzyme. 3-Hydroxy-5-pyrophosphopentanoic acid (**III**) is phosphorylated at the secondary hydroxyl group and released from the enzyme without decarboxylation. This reaction is 30-fold slower than the rate of decarboxylation of **I**. The introduction of the 3-fluoromethyl group as well as the removal of the 3-methyl group results in low rates of decarboxylation. These substrate analogs have decreased electron density relative to the tertiary carbon of the substrate. Therefore, the transition state of the decarboxylation step has considerable carbocationic character. Further support for the carbocationic transition state is provided by the finding that *N*-methyl-*N*-carboxymethyl-2-pyrophosphoethanolamine (**IV**) inhibits the enzyme reaction with $K_i = 0.75$ μM. **IV** is probably a transition-state analog in which the positively charged nitrogen atom is analogous to the carbocation.

Mevalonate pyrophosphate decarboxylase is required for the biosynthesis of cholesterol and other terpenes and catalyzes the reaction shown in Scheme 1. It phosphorylates (Lindberg et al., 1962) and decarboxylatively anti-eliminates (Cornforth et al., 1966) the tertiary hydroxyl group of mevalonate pyrophosphate with overall inversion of configuration of the phosphate (Iyengar et al., 1986). The reaction is sequential and ordered (Jabalquinto & Cardemil, 1989). Two mechanisms for catalysis were proposed. A stepwise mechanism in which ATP phosphorylates the hydroxyl group of mevalonate pyrophosphate (3-hydroxy-3-methyl-5-pyrophosphopentanoic acid, **I**) (see Figure 1 for structures) followed by concomitant decarboxylation and expulsion of P_i (Reardon & Abeles, 1987; Nave et al., 1985; Lindberg et al., 1962) is shown in Scheme 2. A concerted mechanism was also considered (Reardon & Abeles, 1987; Lindberg et al., 1962).

The inhibition of mevalonate pyrophosphate decarboxylase is of interest since inhibition of this enzyme could decrease the rate of cholesterol formation. Inhibition of mevalonate pyrophosphate decarboxylase by 6-fluoromevalonate pyrophosphate (**II**) was reported ($K_i = 0.01$ μM) (Reardon &

Scheme 1



Abeles, 1987; Nave et al., 1985). The 6,6-difluoro- and 6,6,6-trifluoromevalonate pyrophosphate compounds are poorer inhibitors than **II** (Reardon & Abeles, 1987). It has been suggested that the inhibition by **II** is due to the formation of a ternary complex consisting of **II**, ATP, and enzyme. The fluoro substituent may prevent the phosphorylation of the tertiary alcohol group of **II** (Reardon & Abeles, 1987). We carried out experiments to further define the mechanism of action of the enzyme, as well as the mechanism of its inhibition by **II**.

MATERIALS AND METHODS

DL-Mevalonic acid lactone and 5,6-dihydro-2*H*-pyran-2-one were obtained from Aldrich. [^{3}H]$NaBH_4$ and [γ-^{32}P]-

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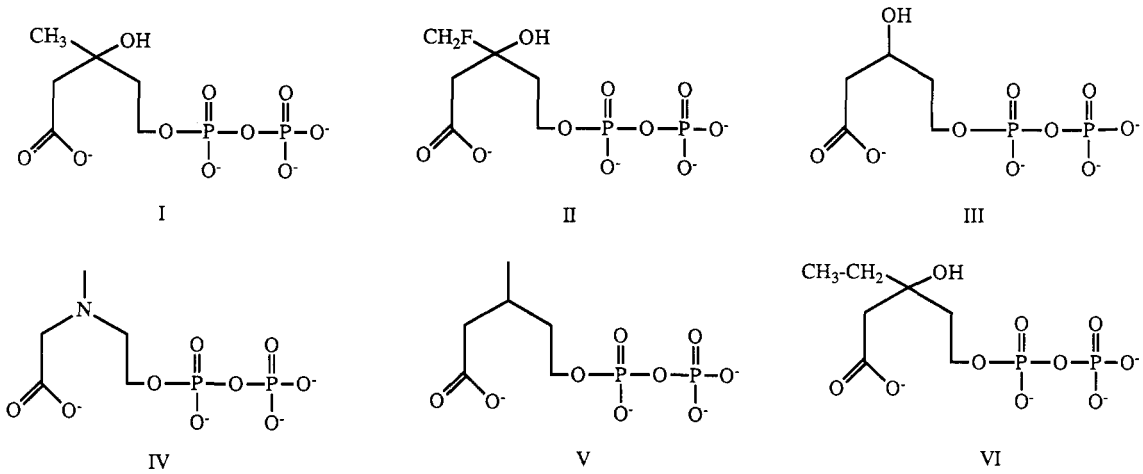
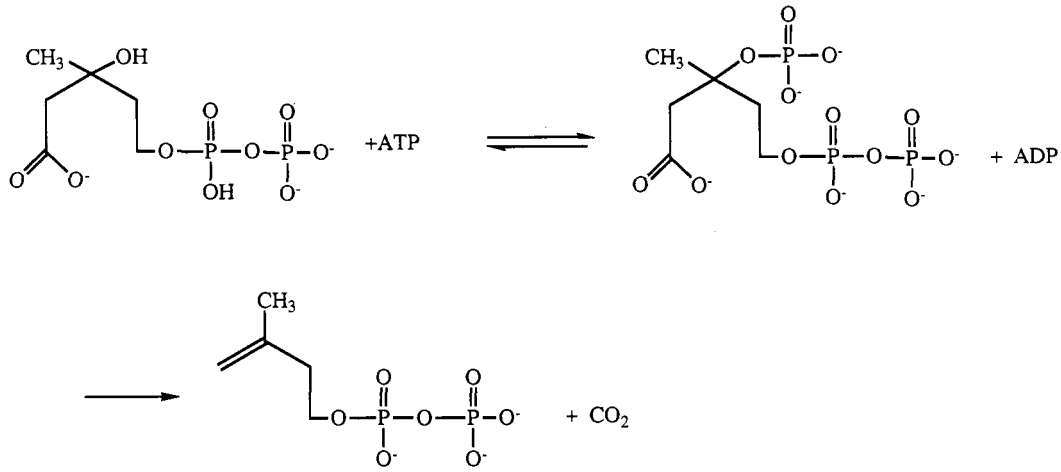


FIGURE 1: Structures of substrates and intermediates considered in this study.

Scheme 2



ATP were obtained from New England Nuclear. [8-^{3}H]ATP was obtained from American Radiolabelled Chemicals. Ethyl bromofluoroacetate was obtained from Specialty Chemicals.

Proton, phosphorus, and carbon NMR spectra were obtained at 300, 121, and 75 MHz, respectively, with a Varian XL-300 spectrometer. Proton reference peaks were TMS (0 ppm in $CDCl_3$) and HOD (4.76 ppm in D_2O). Carbon-13 reference peaks were $CHCl_3$ (77 ppm in $CDCl_3$) and methanol (49 ppm in D_2O). The phosphorus-31 reference peak was 85% phosphoric acid (external, 0 ppm in a sealed capillary tube surrounded by D_2O). Liquid scintillation counting was done using a Beckman LS-100C. Samples were dissolved in Amersham Biodegradable Counting Scintillant (BCS) and counted for either 1, 2, or 5 min with either carbon-14 or phosphorus-32 iso-sets.

Coupled Enzyme Assay. The change in absorbance at 340 nm of a 1-mL assay solution containing ATP (5 mM), $MgCl_2$ (6 mM), lactic dehydrogenase (Sigma, 15 units), pyruvate kinase (Sigma, 15 units), PEP (5 mM), Tris (0.1 M), KCl (0.1 M), NADH (0.6 mM), and enzyme was measured before and after addition of substrate. The reaction was carried out at 25°C and pH 7.

Kinetic Parameters. K_m and K_i values were determined from Lineweaver–Burk plots using initial velocities. Substrate concentrations used were 10–100 μM (**I**), 0.4–4.0 μM (**II**), 20–200 μM (**III**), and 15–150 μM (**VI**). Inhibitor concentrations used were 0.005–0.1 μM (**II**), 20–200 μM (**III**), and 0.3–3 μM (**IV**). Product formation was determined by the coupled enzyme assay.

Nucleotide-Exchange Experiments. A solution, composed of enzyme (0.14 unit; 1 unit is 1 μmol of product per minute), **II** (5 mM) or **I** (5 mM), ATP (5 mM), [β-^{32}P]ADP (5 mM, 650 cpm/nmol), $MgCl_2$ (20 mM), and Tris (0.1 M) in a total volume of 100 μL at pH 7, was incubated at 25 °C for 60 or 2 min, respectively. The enzyme was quenched by boiling for 3 min, and the precipitated enzyme was separated by centrifugation. The supernatant fluid was applied to an anion-exchange column (Dowex, 1 × 8–400, formate counterion, 0.5 × 5 cm, preequilibrated with water), and the column was washed with 20 mL of 5 M formic acid (elutes ADP). ATP was eluted with 10 mL of 5 M formic acid containing 0.8 M ammonium formate. Fractions (1 mL) were collected and counted directly.

Analysis of Reaction Products. A solution, composed of enzyme (0.01 unit), **III** (5 mM), [γ-^{32}P]ATP (5 mM, 8800 cpm/nmol), $MgCl_2$ (10 mM), and Tris (0.1 M) in a total volume of 100 μL at pH 7, was incubated at 37 °C for 24 h. In another experiment, the solution was composed of enzyme (0.10 unit) [3-^{3}H]**III** (5 mM, 10 cpm/nmol), ATP (5 mM), $MgCl_2$ (10 mM), and Tris (0.1 M) in a total volume of 100 μL at pH 7. The solutions were filtered (Amicon, Centricon-10, 5000*g*, 75 min) to remove the enzyme, and the filtrate was applied to an anion-exchange column (QAE-Sephadex, HCO_3 counterion, 1.5 × 7.0 cm, preequilibrated with water). The column was washed with water and the bound components were eluted with a linear gradient (100 mL of water and 100 mL of 1 M TEAB, 3.5 mL/fraction). Aliquots (100

μL) from each fraction of the ^{32}P experiment were dissolved in 4 mL of BCS and counted. Aliquots (1 mL) from each fraction of the ^{3}H experiment were dissolved in 19 mL of BCS and counted. For ^{31}P-NMR studies, a solution composed of enzyme (0.02 unit), **III** (20 mM), ATP (10 mM), $MgCl_2$ (20 mM), and Tris (0.1 M) in a total volume of 500 μL at pH 7 was incubated at 25 °C for 24 h and analyzed by ^{31}P-NMR.

Identification of 3-(Fluoromethyl)-3-buten-1-yl Pyrophosphate. A solution, composed of enzyme (0.10 unit), [5-^{3}H]-**II** (0.5 mM, 106 cpm/nmol), ATP (5 mM), $MgCl_2$ (6 mM), and Tris (20 mM) in a total volume of 500 μL at pH 7, was incubated at 25 °C for 4 h, filtered through a membrane as above, and analyzed by anion-exchange chromatography (Pharmacia FPLC, Mono-Q HR5/5). In a control experiment the enzyme was omitted. The filtrate (100 μL) was applied to the column, the column was washed with 5 mL of water, and the bound components were eluted using a linear gradient (10 mL of water and 10 of mL 20 mM Tris (pH 7) containing 0.5 M KCl, 0.5 mL/fraction, 0.5 mL/min). Each fraction was completely dissolved in 4 mL of BCS and counted. The above filtrate (100 μL) was mixed with alkaline phosphatase (Sigma, 200 units) and Tris (200 mM) in a total volume of 150 μL at pH 9 and was incubated at 37 °C for 2 h before it was filtered again. The filtrate was analyzed by ion-exclusion HPLC (Bio-Rad Aminex HPX-87H, 300 × 7.8 mm). The material was eluted isocratically (5 mM H_2SO_4, 0.5 mL/min, 0.5 mL/fraction). The fractions were entirely dissolved in 4 mL of BCS and counted. Authentic 3-(fluoromethyl)-3-buten-1-ol was chromatographed under the same conditions.

Dialysis Experiments. An apparatus with two 150-μL chambers, separated by a dialysis membrane (MW_{co}, 10 000), was employed. The apparatus was maintained at 4 °C on a wrist-action shaker for 16 h. One chamber contained enzyme (0.12 unit) in a volume of 100 μL. The other solution contained [β-^{32}P]**II** (100 nM, 100 cpm/nmol), ATP or AMP-PNP[1] (5 mM), $MgCl_2$ (6 mM), and Tris (20 mM) in a total volume of 100 μL at pH 7. After completion of the reaction, solutions were removed and analyzed by scintillation counting.

Penefsky Experiments. Enzyme containing bound components was separated quickly from nonbound components according to a published procedure (Penefsky, 1979). Solutions, composed of enzyme (0.13 unit), **II** (0.01 mM), [γ-^{32}P]-ATP (2 mM, 3 × 10^6 cpm/nmol) or [8-^{3}H]ATP (2 mM, 1 × 10^5 cpm/nmol), $MgCl_2$ (2 mM), and Tris (50 mM) in a total volume of 50 μL at pH 7, were incubated at 4 °C for 15 min, applied to a Penefsky column, and centrifuged into a receiving test tube containing 33 μL of concentrated perchloric acid. In other experiments, solutions were composed of enzyme (0.13 unit), [β-^{32}P]**II** (0.01 mM, 6.3 × 10^6 cpm/nmol), ATP (2 mM), $MgCl_2$ (2 mM), and Tris (50 mM) in a total volume of 50 μL at pH 7. The filtrate was neutralized with 2 M KOH, and the precipitate was centrifuged in an Eppendorf tube. The supernatant was analyzed by anion-exchange chromatography. The Penefsky column was a precentrifuged 1-mL plastic syringe plugged with glass wool and filled with Biogel P30M (Bio-Rad), which was previously equilibrated with 50 mM Tris (pH 7). All Penefsky spins were done in a swing-bucket, tabletop, clinical centrifuge (IEC) for 2 min at maximum speed. Chromatography was performed on a Beckman HPLC equipped with a Whatman (Partisil PXS 10/25 SAX) column. The material was applied to the column, the column was washed with water, and the bound components were eluted using a linear gradient (0−100% B in 60 min at 0.5 mL/min, 0.5 mL/fraction; A was water, and B was 1 M KCl and 0.5 M KH_2PO_4 at pH 4.5). For ^{32}P material, each fraction was counted without scintillant. For ^{3}H material, 100 μL from each fraction was dissolved in 4 mL of BCS and counted.

Synthetic Procedures. Pyrophosphorylation of lactones was performed by a published procedure (Reardon & Abeles, 1987). The lactone precursors of compounds **I**, **II**, and **V** were prepared according to published procedures (Reardon & Abeles, 1987; Quistad & Cerf, 1981; Quistad et al., 1982). [5-^{3}H]**II** was prepared as above, with the exception that $NaBH_4$ was replaced with [^{3}H]$NaBH_4$. 3-(Fluoromethyl)-3-buten-1-ol was kindly provided by Dr. Dale Poulter. Tris-(tetrabutylammonium)pyrophosphate was prepared according to a published procedure (Woodside et al., 1988). Jones reagent is composed of 8 N chromium trioxide and 8 N sulfuric acid in water. Zinc was activated by sequential washing in 5% HCl, water, ethanol, and ether. Ozonolysis was performed on a Welsbach Ozonator (set at 120 V). Flash chromatography was performed according to a published procedure (Still et al., 1978).

3-Hydroxy-5-pyrophosphopentanoate, Lithium Salt (**III**). A solution of 5,6-dihydro-2*H*-pyran-2-one (0.86 mL, 10 mmol), mercuric acetate (6.38 g, 20 mmol), perchloric acid (5 μL of 70%), water (10 mL), and THF (10 mL) was stirred at 23 °C, in the dark, for 36 h. Potassium phosphate (5 mL of 1 M, pH 8) and THF (40 mL) were added to the solution. The solution was cooled on ice, and $NaBH_4$ (ca. 0.6 g, 16 mmol) was added slowly. The mixture was saturated with NaCl and extracted with 3 × 20 mL of THF. The organic pool was dried with K_2CO_3. The solvent was removed under reduced pressure. The crude product was submitted to flash chromatography with ethyl acetate (R_f = 0.3) to give tetrahydro-4-hydroxy-2*H*-pyran-2-one (0.27 g, 2.3 mmol, 23%), an oil. ^{1}H-NMR ($CDCl_3$): 4.3 (m, C*H*(OH)), 4.4 (m, CH_2O), 2.7 (m, $C(O)CH_2$), 2.0 (m, CH_2CH_2O).

Tetrahydro-4-hydroxy-2*H*-pyran-2-one (0.12 g, 1.0 mmol) was pyrophosphorylated to give 3-hydroxy-5-pyrophosphopentanoate, lithium salt (63 mg, 0.2 mmol, 20%). ^{1}H-NMR (D_2O): 4.1 (m, CH(OH)), 4.0 (m, CH_2O), 2.3 (m, $C(O)CH_2$), 1.7 (m, CH_2CH_2O). ^{13}C-NMR (D_2O): 183, 69, 48, 40, 20 ppm. ^{31}P-NMR (D_2O): −4.6 (d, J = 20 Hz, β), −8.4 (d, J = 20 Hz (heteronuclear decoupled); td, J = 20, 5 Hz (homonuclear decoupled), α).

*[3-^{3}H]3-Hydroxy-5-pyrophosphopentanoate, Lithium Salt ([3-^{3}H]***III**). Jones reagent was added dropwise to a solution of tetrahydro-4-hydroxy-2*H*-pyran-2-one (0.3 g, 2.6 mmol) and acetone (30 mL) until an orange color persisted. 2-Propanol was added to the mixture until the orange color disappeared. The mixture was vacuum filtered through a pad of celite with the aid of some acetone. The solvent was removed under reduced pressure. The crude product was submitted to flash chromatography with ethyl acetate (R_f = 0.23) to give β-oxo-δ-valerolactone (0.2 g, 1.7 mmol, 67%), an oil. ^{1}H-NMR ($CDCl_3$): 4.6 (t, J = 6 Hz, CH_2O), 3.6 (s, $C(O)CH_2$), 2.7 (t, J = 6 Hz, CH_2CH_2O).

[^{3}H]$NaBH_4$ (2.5 M in 10 mM NaOH, 2220 cpm/nmol) was slowly added to an ice-cold, stirring solution of β-oxo-

[1] Abbreviations: AMP-PNP, adenylyl imidodiphosphate; TEAB, triethylammonium bicarbonate; FPLC, fast protein liquid chromatography.

δ-valerolactone (0.11 g, 1.0 mmol), ethanol (1 mL), and water (1 mL) until the starting material was consumed. The pH of the solution was maintained at 4.5 using 2 M HCl. The crude product was submitted to flash chromatography with ethyl acetate (R_f = 0.3) to give [4-^{3}H]tetrahydro-4-hydroxy-2*H*-pyran-2-one (0.12 g, 1.0 mmol, 100%), an oil. The NMR data was identical to that for the nonradioactive compound.

[4-^{3}H]Tetrahydro-4-hydroxy-2*H*-pyran-2-one (0.12 g, 1.0 mmol) was pyrophosphorylated to give [3-^{3}H]-3-hydroxy-5-pyrophosphopentanoate, lithium salt (63 mg, 0.2 mmol, 20%), a solid. The NMR data was identical to that for the nonradioactive compound.

*[β-^{32}P]-3-Hydroxy-3-(fluoromethyl)-5-pyrophosphopentanoate, Triethylammonium Salt ([β-^{32}P]*II*).* A solution of 3-hydroxy-3-(fluoromethyl)-5-pyrophosphopentanoate, lithium salt (10 mg, 29 μmol), and HCl (300 μL of 1 M) was boiled for 15 min. LiOH (4 M) was added until pH 7, and the resulting solution was lyophilized to give 3-hydroxy-3-(fluoromethyl)-5-phosphopentanoate, lithium salt. ^{1}H-NMR (D_2O): 4.4 (d, J = 45 Hz, CH_2F), 3.9 (m, CH_2O), 2.4 (s, $C(O)CH_2$), 1.9 (m, CH_2CH_2O). ^{31}P-NMR (D_2O): 3.8 (t, J = 5 Hz (homonuclear decoupled); s (heteronuclear decoupled)).

A solution of 3-hydroxy-3-(fluoromethyl)-5-phosphopentanoate, lithium salt (17 mM), [γ-^{32}P]ATP (0.83 μM, 1.3 × 10^{10} cpm/nmol), $MgCl_2$ (17 mM), mevalonate phosphate kinase (0.03 unit), Tris (20 mM), and KCl (20 mM) in a total volume of 300 μL at pH 7 was incubated at 25 °C for 30 min. ATP (1 μmol) was added, and the solution was incubated at 25 °C for another 120 min. The solution was boiled for 3 min and centrifuged. The supernatant fluid was submitted to QAE-Sephadex chromatography to give [β-^{32}P]-3-hydroxy-3-(fluoromethyl)-5-pyrophosphopentanoate, triethyl ammonium salt (3.0 mM, 6.3 × 10^6 cpm/nmol).

*3-Hydroxy-3-ethyl-5-pyrophosphopentanoate, Lithium Salt (***VI***).* A solution of ethyl propionate (6.9 mL, 60 mmol), allyl bromide (15.6 mL, 180 mmol), diethyl ether (50 mL), and THF (50 mL) was added, dropwise, to a mixture of magnesium turnings (5.8 g, 240 mmol), diethyl ether (10 mL), and THF (10 mL). The mixture was stirred at 25 °C for 8 h. Seventy grams of ice was stirred in. Concentrated HCL (ca. 50 mL) was added to the mixture until pH 7. The aqueous layer was extracted with diethyl ether (3 × 75 mL). The organic pool was extracted with sodium bicarbonate (100 mL) and dried with magnesium sulfate. The solvent was removed under reduced pressure to give 4-hydroxy-4-ethyl-1,6-heptadiene (7.3 g, 52 mmol, 87%), an oil. ^{1}H-NMR ($CDCl_3$): 5.9 (m, CH_2CHCH_2C), 5.2 (m, CH_2CHCH_2C), 2.2 (d, J = 8 Hz, CH_2CHCH_2C), 1.5 (q, J = 8 Hz, CH_2CH_3), 0.9 (t, J = 8 Hz, CH_3CH_2).

Ozone was bubbled through a solution of 4-hydroxy-4-ethyl-1,6-heptadiene (7.3 g, 52 mmol) and methylene chloride (100 mL) at −78 °C until a blue color persisted. Oxygen was bubbled through the solution for 20 min. A solution of $NaBH_4$ (10 g, 263 mmol), water (8.5 mL), and ethanol (8.5 mL) was slowly added to the solution, and stirring was continued for 8 h at 4 °C. Concentrated HCl was added to the mixture until pH 5 was obtained. The mixture was extracted with ethyl acetate (5 × 40 mL). The organic pool was washed with potassium bicarbonate-saturated water, and the solvent was removed under reduced pressure. The residue was dissolved in 150 mL of acetone, and Jones reagent was added until an orange color persisted. 2-Propanol was added to the mixture until the orange color disappeared. The mixture was vacuum filtered through a pad of celite with the aid of some acetone, and the solvent was removed under reduced pressure. The crude product was submitted to flash chromatography with diethyl ether to give 5-hydroxy-5-ethylpyran-2-one (1.7 g, 12 mmol, 20%), an oil. ^{1}H-NMR ($CDCl_3$): 4.5 (m, CH_2O), 2.6 (m, $CH_2C(O)$), 1.9 (m, CH_2CH_2O), 1.6 (q, J = 8 Hz, CH_2CH_3), 1.0 (t, J = 8 Hz, CH_3CH_2).

5-Hydroxy-5-ethylpyran-2-one (1.3 g, 9 mmol) was pyrophosphorylated to give 3-hydroxy-3-ethyl-5-pyrophosphopentanoate, lithium salt (0.2 g, 0.58 mmol, 6%), a solid. ^{1}H-NMR (D_2O): 4.0 (m, CH_2), 2.4(s,$CH_2C(O)$), 1.9 (m, CH_2CH_2O), 1.6 (q, J = 8 Hz, CH_2CH_3), 0.9 (t, J = 8 Hz, CH_3CH_2). ^{13}C-NMR (D_2O): 181, 73, 62, 45, 38, 32, 8 ppm. ^{31}P-NMR (D_2O): −4.6 (d, J = 20 Hz, β), −8.5 (d, J = 20 Hz (heteronuclear decoupled); td, J = 20, 6 Hz (homonuclear decoupled), α).

*N-(Carboxymethyl)-N-methylpyrophosphoethanolamine(***IV***).* To an ice-cold, stirred solution of *N*-methylethanolamine (3.8 g, 50 mmol) and pyridine (12 g, 150 mmol) was added, slowly, benzyl chloroformate (4.3 g, 25 mmol). Stirring was continued for an additional hour at room temperature. The solution was diluted with 150 mL of diethyl ether, washed twice with 100 mL of 3% H_2SO_4, washed once with 100 mL of water, and dried with $MgSO_4$. The solvent was removed under reduced pressure to give *N*-(carbobenzoxy)-*N*-methylethanolamine (2.5 g, 12 mmol), an oil. ^{1}H-NMR ($CDCl_3$): 7.4 (m, phenyl), 5.1 (s, $CH_2OC(O)$), 3.8 (t, CH_2OH), 3.5 (t, NCH_2), 3.0 (s, CH_3).

To an ice-cold, stirred solution of *N*-(carbobenzoxy)-*N*-methylethanolamine (0.21 g, 1.0 mmol) and triethanolamine (0.15 g, 1.5 mmol) in 3 mL of methylene chloride was added, slowly, a solution of methanesulfonyl chloride (0.13 g, 1.1 mmol) in 0.5 mL of methylene chloride. Stirring was continued for an additional hour at room temperature. The crude product was submitted to flash chromatography with 50% ethyl acetate/hexane (R_f = 0.35) to give *N*-(carbobenzoxy)-*N*-methylmethanesulfonylethanolamine (0.18 g, 0.64 mmol), an oil. ^{1}H-NMR ($CDCl_3$): 7.4 (m, phenyl), 5.1 (s, $CH_2OC(O)$), 4.4 (m, CH_2OS), 3.6 (t, NCH_2), 3.0 (s, SCH_3), 2.9 (m, NCH_3).

A solution of *N*-(carbobenzoxy)-*N*-methylmethanesulfonylethanolamine (0.18 g, 0.64 mmol), tris(tetrabutylammonium) pyrophosphate (0.67 g, 0.7 mmol), tetrabutylammonium iodide (0.03 g, 0.07 mmol), and 1.5 mL of acetonitrile was stirred at room temperature for 8 h. The solvent was removed under reduced pressure, and the residue was diluted in 5 mLof water. The resulting solution was passed, with the aid of 50 mL of water, through a column of Dowex-50 (lithium counterion, 1.5 × 5 cm). The effluent was lyophilized, dissolved in 10 mL of water, and hydrogenated with hydrogen gas in the presence of about 50 mg of 10% palladium on carbon. The catalyst was removed by centrifugation, and the solution was adjusted to pH 11. One milliliter of 1 M potassium bromoacetate (pH 11) and 1 mL of 1 M KP_i (pH 11) was added to the solution, and the latter was stirred at room temperature for 8 h. The solution was submitted to anion-exchange chromatography (QAE-Sephadex-A25; 1.5 × 8 cm, bicarbonate counterion, preequilibrated with water). The column was washed with 50 mL of water, and the bound components were eluted using a linear gradient (50 mL of water and 50 mL of TEAB (pH 7.5); 4 mL/fraction). The fractions containing the desired product

were pooled, concentrated under reduced pressure, and passed, with the help of 50 mL of water, through a Dowex-50 column (lithium counterion, 1.5 × 5 cm). The effluent was lyophilized to give *N*-(carboxymethyl)-*N*-methylpyrophosphoethanolamine (**IV**, 0.1 g, 0.4 mmol), a solid. ^{1}H-NMR (D_2O): 4.2 (m, CH_2O), 3.7(s, $C(O)CH_2$), 3.5 (m, NCH_2CH_2), 2.9(s, CH_3). ^{13}C-NMR (D_2O): 160, 63, 62, 59, 44 ppm. ^{31}P-NMR (D_2O): −4.7 (d, J = 20 Hz, β), −9.2 (d, J = 20 Hz (heteronuclear decoupled); td, J = 20, 5 Hz (homonuclear decoupled), α).

[β-^{32}P]Adenosine Diphosphate ([β-^{32}P]ADP). A solution composed of myokinase (Sigma, 92 unit), [γ-^{32}P]ATP (10 mM, 33 × 10^3 cpm/nmol), AMP (10 mM), $MgCl_2$ (20 mM), and Tris (0.1 M) in a total volume of 500 μL at pH 7 was incubated at 37 °C for 2 h. The solution was boiled for 3 min and centrifuged. The supernatant fluid was applied to an ion-exchange column (Dowex, 1 × 8-400, formate counterion, 0.5 × 5 cm, preequilibrated with water), the column was washed with water, and the bound components were eluted using a linear gradient (50 mL of water and 50 mL of 5 M formic acid, 3 mL/fraction). Only one peak of radioactivity was eluted. These fractions were pooled and lyophilized. The resulting residue was dissolved in water to give a solution of [β-^{32}P]ADP (13 mM, pH 6, 1660 cpm/nmol). The ADP concentration was determined by the coupled enzyme assay.

Purification of Mevalonate Pyrophosphate Decarboxylase. Fifty grams of bakers' yeast, suspended in 100 mL of 10 mM KP_i (pH 7) (buffer A), was homogenized with a Bead Beater (Biospec Products) at 4 °C for 6 min (3 homogenizations with 4-min rests between runs), using 120 mL of 0.5-mm glass beads. The resulting suspension was centrifuged (1 h at 27000*g*). The supernatant fluid was heated to 60 °C and centrifuged (30 min at 12000*g*). The supernatant fluid was acidified with 1 M acetic acid to pH 5.0 and centrifuged (15 min at 12000*g*). The supernatant fluid was neutralized using concentrated KOH, and 1 vol of buffer A saturated with $(NH_4)_2SO_4$ was slowly added. The resulting mixture was centrifuged (30 min at 12000*g*). The supernatant fluid was saturated with $(NH_4)_2SO_4$, stirred for 30 min, and centrifuged (30 min at 12000*g*). The pellet was dissolved in and dialyzed (Viskase seamless cellulose tubing, MW_{co} 10K) against buffer A before it was applied to an anion-exchange column (DE-23 Whatman; 6 × 30 cm, preequilibrated with buffer A). The column was washed with 2 L of the same buffer, and the bound components were eluted using a linear gradient (1 L of buffer A and 1 L of 500 mM KP_i (pH 7); 22 mL/fraction). The protein concentration (absorbance at 280 nm) and the mevalonate pyrophosphate-dependent ATPase activity (coupled enzyme assay) were measured; active fractions were pooled, saturated with $(NH_4)_2SO_4$, and centrifuged (30 min at 12000*g*). The pellet was dissolved in and dialyzed against 20 mM Tris (pH 7) before it was applied to an ion-exchange column (Pharmacia FPLC Mono-Q HR10/10, preequilibrated with 20 mM Tris (pH 7)). The column was washed with 20 mL of 20 mM Tris (pH 7), and the bound components were eluted using a linear gradient (30 mL of 20 mM Tris (pH 7) and 30 mL of 20 mM Tris (pH 7) containing 1 M KCl, 2.0 mL/min, 2 mL/fraction). The protein concentration and the mevalonate pyrophosphate-dependent ATPase activity were measured for each fraction, and the active fractions were pooled. The pool was concentrated (Amicon, Centriprep-10), diluted with 1 mM KP_i (pH 7), and applied to an adsorption column (hydroxyapatite−agarose, HA-Ultrogel, 1.4 × 6.5 cm, preequilibrated with 1 mM KP_i (pH 7). The column was washed with 200 mL of 1 mM KP_i (pH 7), and the bound components were eluted using a linear gradient (500 mL of 1 mM KP_i (pH 7) and 500 mL of 100 mM KP_i (pH 7), 20 mL/fraction). Active fractions were pooled and concentrated. $(NH_4)_2SO_4$ was added to the pool until a concentration of 1.7 M was reached. This solution was applied to a hydrophobic interaction column (Pharmacia Phenyl-Superose HR5/5, preequilibrated with 50 mM KP_i (pH 7) containing 1.7 M $(NH_4)_2SO_4$). The column was washed with 5 mL of the same buffer, and the bound components were eluted using a linear gradient (5 mL of 50 mM KP_i (pH 7) containing 1.7 M $(NH_4)_2SO_4$ and 5 mL of 50 mM KP_i (pH 7), 1 mL/fraction, 0.5 mL/min). Active fractions were pooled, concentrated (Amicon, Centricon-10), diluted in 1 mM KP_i (pH 7), and applied to an affinity column (Matrexgel Blue A, 1.5 × 7.5 cm, preequilibrated in 1 mM KP_i (pH 7)). The column was washed with 10 mL of the same buffer, and the bound components were eluted with a linear gradient (50 mL of 1 mM KP_i (pH 7) and 50 mL 1 mM KP_i (pH 7) containing 1 M KCl). Active fractions were pooled and concentrated (Amicon, Centricon-10) to give a 1.0 mg/mL protein solution with specific activity of 504 min^{-1}. Native PAGE (Blattler, 1972) revealed a single band at about 80 000 g/mol. Denaturing SDS−PAGE (Laemmli, 1970) revealed a single band at 43 000 g/mol. A summary of the purification is described in Table 1.

Table 1: Purification of Mevalonate Pyrophosphate Decarboxylase

	protein (mg)	total act. (μmol/min)	sp. act. (μmol/min/mg)
$(NH_4)_2SO_4$ (50−100%)	3600	37	0.01
DE-23	360	48	0.13
Mono-Q	32	30	0.94
hydroxyapatite	15	25	1.7
phenyl-Superose	6	20	3.3
Matrex-gel Blue	2	12	6.0

Purification of Mevalonate Phosphate Kinase. Fifty grams of bakers' yeast, suspended in 100 mL of buffer A, was homogenized as before. $(NH_4)_2SO_4$ was added to the supernatant fluid to 60%, and the resulting mixture was centrifuged (30 min, 12000g). $(NH_4)_2SO_4$ was added to the supernatant fluid to 80%, and the resulting mixture was centrifuged (30 min, 12000*g*). The pellet was dissolved in and dialyzed against buffer A before it was applied to an ion-exchange column (DE-32 Whatman, 6 × 30 cm, preequilibrated in buffer A). The column was washed with 2 L of buffer A, and the bound components were eluted using a linear gradient (0.6 L of buffer A and 0.6 L of buffer A containing 1 M KCl, 20 mL/fraction). Active fractions were pooled, concentrated (ultrafiltration, Amicon YM10), and taken up in buffer A containing 1.7 M $(NH_4)_2SO_4$ (buffer B). The resulting solution was applied to a hydrophobic interaction column (Pharmacia FPLC, Phenyl-Superose HR5/5, preequilibrated with buffer B). The column was washed with 5 mL of buffer B, and the bound components were eluted using a linear gradient (5 mL of buffer B and 5 mL of buffer A, 1 mL/fraction, 0.5 mL/min). Active fractions were concentrated (Amicon, Centricon-10) to give a 30 mg/mL protein solution with a specific activity of 5.6 min^{-1}. This enzyme preparation did not catalyze ATP hydrolysis in the absence of its substrate, 3-hydroxy-3-methyl-5-phosphopentanoic acid.

Table 2: Kinetic Constants[a]

compd	K_{cat} (min^{-1})	K_m (μM)	K_i (μM)
I	500 ± 60	15 ± 5	
II	0.20 ± 0.04	0.8 ± 0.2	0.010 ± 0.005
III	14 ± 3	40 ± 10	50 ± 20
IV			0.8 ± 0.03
V			>1000
VI	60 ± 10	30 ± 10	

[a] Kinetic constants were calculated with PSI-Plot (Poly Software International, Ltd., Salt Lake City, UT).

RESULTS AND DISCUSSION

Decarboxylation of **I**. The reaction catalyzed by mevalonate pyrophosphate decarboxylase (Scheme 1) was assayed by measuring the consumption of NADH in a spectrophotometric assay involving the coupling enzymes pyruvate kinase and lactate dehydrogenase. This is referred to as the coupled enzyme assay. The rates of P_i production and ADP formation were compared under the following experimental conditions: **I** (0.5 mM) (kinetic constants are given in Table 2), ATP (5 mM), and $MgCl_2$ (6 mM) at 25 °C and pH 7. The rate of P_i production (k_{cat} = 504 min^{-1}), assayed by a published procedure (Lanzetta et al., 1979), was identical to the rate of ADP formation, which was determined by the coupled enzyme assay method.

Evidence for a two-step process (Scheme 2) might be obtained from nucleotide-exchange experiments. A solution containing enzyme (0.14 unit), **I** (5 mM), ATP (5 mM), [β-^{32}P]ADP (5 mM, 650 cpm/nmol), and $MgCl_2$ (20 mM) was allowed to react for 2 min at 25 °C (about 20% of total reaction). The solution was boiled to quench the reaction and was centrifuged to separate the denatured enzyme. The supernatant fluid was placed on a Dowex-1 column to separate ADP and ATP. There was no detectable increase in radioactive ATP. The exchange rate was less than 15% of the catalytic rate. The failure to detect exchange does not necessarily imply a concerted reaction. A stepwise reaction could occur where ADP is not released before decarboxylation, or where ADP is released before fast decarboxylation.

Role of the 3-Methyl Group. The contribution of the methyl group of the substrate to the catalytic reaction was examined. We synthesized 3-hydroxy-5-pyrophosphopentanoic acid (**III**), and it was found to be a substrate for the enzyme. For ADP formation k_{cat} = 14 min^{-1}, 3% of the rate obtained with **I**, and K_m = 44 μM. The rate of P_i formation was undetectable. The lack of P_i formation suggested that a phosphorylated compound had accumulated. To determine the structure of the product, a solution containing enzyme (0.01 unit), **III** (5 mM), [γ-^{32}P]ATP (5 mM, 8800 cpm/nmol), and $MgCl_2$ (10 mM) was incubated at 37 °C for 24 h. The reaction mixture was filtered to remove the enzyme, and the filtrate was analyzed on a QAE-Sephadex column. In addition to ATP, a new peak of radioactivity was observed at 0.55 M triethylammonium bicarbonate (TEAB). This radiolabeled compound results from the action of the enzyme on **III**. Possibly, the compound is phosphorylated **III**. Such a compound is expected to elute at a TEAB concentration greater than 0.48 M, the TEAB elution concentration of ATP. The above experiment was repeated with [3-^{3}H]**III** and non-isotopic ATP. Analysis of the product mixture by QAE-Sephadex revealed the presence of unreacted **III** (0.46 M TEAB) as well as a peak eluting at 0.55 M TEAB, which cochromatographed with the compound derived from [γ-^{32}P]ATP. These results demonstrate that the enzyme transfers the γ-phosphate of ATP to **III**. Phosphate could be transferred to the carboxyl, hydroxyl, or pyrophosphate moiety of **III**. Experiments were carried out to distinguish among these possibilities. The ^{32}P-labeled fractions eluting at 0.55 M TEAB were brought to pH 9.1 and maintained at this pH for 24 h at 25 °C. Rechromatography on QAE-Sephadex revealed no change in R_f and no new peak; i.e., the product is stable at pH 9. This result eliminates the possibility that the carboxyl group of **III** was phosphorylated (Di Sabato & Jencks, 1961). ^{31}P-NMR of the reaction product was determined. Enzyme (0.02 unit), **III** (20 mM), ATP (10 mM) and $MgCl_2$ (20 mM), were incubated for 24 h at 25 °C. A doublet with a chemical shift of 2.3 ppm and a coupling constant of 6 Hz was observed, indicating that the phosphate of the product is attached to a secondary alkyl carbon (Mavel, 1973). Other peaks in the spectrum were also present and were found to be signals from the phosphate atoms of ATP, ADP, and **III**. Taken together, these results demonstrate that the enzyme catalyzes the phosphorylation of the secondary hydroxyl group of **III** to give 3-phospho-5-pyrophosphopentanoic acid.

The decarboxylated product of [3-^{3}H]-3-hydroxy-5-pyrophosphopentanoic acid, [3-^{3}H]-3-buten-1-yl pyrophosphate, was undetectable; therefore, the rate of decarboxylation of phosphorylated **III** is decreased at least 300-fold compared to **I**. The methyl group is electron donating and increases the rate of reactions that are thought to occur by a carbocationic transition state. For example, *tert*-butyl bromide undergoes β-elimination at a rate 10 000-fold faster than that of *sec*-butyl bromide in acetonitrile at 25 °C (Martin et al., 1965). *tert*-Butyl chloride hydrolysis is 40-fold faster than that of isopropyl chloride in 80% ethanol/water at 25 °C (Taft, 1956). The rate decrease in decarboxylation of **I** brought about by the removal of the methyl group indicates that the transition state for the decarboxylation of **I** has carbocationic character and is stabilized by the methyl group.

Action of Enzyme on **II**. It was of interest to determine whether or not **II** is chemically modified by the decarboxylase. We reexamined the inhibition by **II** and found it to be a competitive inhibitor with K_i = 0.01 μM, as reported previously (Reardon & Abeles, 1987; Nave et al., 1985). Slow binding was not observed. The possibility had been considered that ATP is unable to phosphorylate **II** and that at steady state an ATP−**II**−enzyme complex predominates (Reardon & Abeles, 1987). We determined, by equilibrium dialyses, that **II** does not bind to the enzyme in the presence of AMP-PNP, an ATP analog with a nonhydrolyzable $\beta-\gamma$ phosphate bond (Yount, 1975). AMP-PNP was found to be a competitive inhibitor with ATP, K_i = 3 ± 1mM (S. Dhe-Paganon, unpublished). One dialysis chamber contained enzyme, and the other chamber contained 0.04 equiv of [β-^{32}P]**II** at a concentration of 100 nM. After 16 h, the radioactive inhibitor was equally distributed between the two chambers. This was found when either AMP-PNP or ATP was used. Possibly, **II** must be phosphorylated to achieve inhibition. Nucleotide-exchange experiments were performed to further explore this suggestion. A solution containing enzyme (0.14 unit), ATP (5 mM), $MgCl_2$ (20 mM), [β-^{32}P]ADP (5 mM, 650 cpm/nmol), and **II** (5 mM) was allowed to react for 1 h at 25 °C. The reaction mixture was boiled to quench the reaction and centrifuged to remove the denatured protein. The supernatant fluid was placed on

a Dowex-1 column to separate ADP and ATP. There was no detectable increase in radioactive ATP. The exchange rate is less than 15% of the catalytic rate. The failure to detect exchange may be due to the lack of ADP release from the inhibited complex.

We determined that slow turnover of **II** occurred. The enzyme catalyzes **II**-dependent ATP hydrolysis at a rate of 0.19 min^{-1}, 2500-fold less than the rate obtained with **I**. The product of this reaction was identified as 3-(fluoromethyl)-3-buten-1-yl pyrophosphate by chromatographic analysis. Enzyme (0.10 unit), [5-^{3}H]**II** (0.5 mM, 106 cpm/nmol), ATP (5 mM), and $MgCl_2$ (6 mM) were incubated at 25 °C for 4 h. The reaction mixture was filtered to remove the protein component and analyzed on a Mono-Q anion-exchange column using a linear salt gradient. A new peak was detected at 0.25 M KCl as well as a minor peak of unreacted **II**. The elution position of the new peak is consistent with decarboxylated **II**, ie., 3-(fluoromethyl)-3-buten-1-yl pyrophosphate. The new peak was further characterized by exposing it to alkaline phosphatase. The resulting solution was analyzed by chromatography on an ion-exclusion column. Two prominent peaks co-eluted with an authentic sample of 3-(fluoromethyl)-3-buten-1-ol (24 and 38 min), and the two other peaks (minor) co-eluted with fluoromevalonate (18 and 21 min (closed and open forms)). This is evidence that 3-(fluoromethyl)-3-buten-1-yl pyrophosphate is generated from **II** by the action of mevalonate pyrophosphate decarboxylase. In the dialysis experiment described above, II was converted to product in the presence of ATP and therefore no ATP−**II**−enzyme complex could be detected. As pointed out above, the decarboxylation of **II** proceeded 2500-fold more slowly than the decarboxylation of **I**.

The fluorine group is electron withdrawing and decreases the rate of reactions with a carbocationic transition state. For example, when one of the hydrogen atoms of *tert*-butyl chloride is replaced by a fluorine atom, the rate of solvolysis in 80% ethanol/water at 25 °C decreases by about 4000-fold (Taft, 1956). The slow rate of enzymatic decarboxylation of **II** provides further evidence that the transition state for decarboxylation has carbocationic character.

*Composition of Enzyme−**II** Complex at Steady State.* The composition of the enzyme−inhibitor complex at steady state was examined. A mixture consisting of enzyme (0.13 unit), **II** (0.01 mM), [γ-^{32}P]ATP (2 mM, 3×10^6 cpm/nmol), and $MgCl_2$ (2 mM) was allowed to react at 4 °C for 15 min and then passed through a Penefsky column to separate enzyme-bound and free small molecules. The effluent from the Penefsky column was immediately quenched with perchloric acid, neutralized with base, and analyzed by ion-exchange HPLC. The total radioactivity corresponded to 0.4 mol of radioactive phosphate per mole of enzyme in the effluent. The chromatographic analysis displayed two radioactive peaks of equal intensity (31 and 39 min). [γ-^{32}P]ATP elutes at 31 min. The compound eluting at 39 min is possibly phosphorylated **II**. In order to confirm this identification, the experiment was repeated using [β-^{32}P]**II** and non-isotopic ATP. Two peaks of radioactivity were detected: one at 24 min, which cochromatographed with [β-^{32}P]**II**, and another at 39 min. 3-Phospho-5-pyrophosphopentanoic acid, obtained from the previous experiments, eluted at 39 min. These data indicate that at steady state the inhibited complex contains 3-phospho-3-(fluoromethyl)-5-pyrophosphopentanoic acid. No radioactivity remained bound to the enzyme when **II** was replaced with the natural substrate, **I**. Another experiment analogous to the one described above was done using [8-^{3}H]ATP and non-isotopic **II**. The HPLC analysis revealed the existence of 0.2 equiv of ADP in the column filtrate. These results show that the enzyme−inhibitor complex also contains ADP; this explains the lack of observable nucleotide exchange. We conclude that the fluorine atom does not hinder phosphorylation of the substrate but does hinder decarboxylation of the intermediate. The rate of decarboxylation of the phosphorylated compound is decreased at least 2500-fold compared to that of **I**. The slow decarboxylation of **II** is probably due to the destabilization of the transition state by the inductive effect of fluorine.

Effect of a Transition-State Analog. If the enzyme active site is designed to stabilize a carbocationic transition state, it should bind strongly to a substrate analog containing a positively charged nitrogen atom. A number of examples are known where a positively charged nitrogen atom forms a strong complex with an enzyme that catalyzes a reaction involving a transition state with carbocationic character (Reardon & Abeles, 1986; Ta et al., 1992). The compound *N*-(carboxymethyl)-*N*-methylpyrophosphoethanolamine (**IV**) was synthesized and found to be a competitive inhibitor against **I** with $K_i = 0.75$ μM. The carbon analog of this compound, 3-methyl-5-pyrophosphopentanoic acid (**V**), bound poorly to the enzyme ($K_i > 1$ mM). Taken together, these results further support the involvement of a carbocationic transition state.

Steric Requirements of the Active Site of the Enzyme. Unfavorable steric interactions could be responsible for the poorer inhibition observed with polyfluorinated compounds compared to the monofluorinated compound. To explore the steric requirements of the binding site, an analog of **I**, 3-hydroxy-3-ethyl-5-pyrophosphopentanoic acid (**VI**), was synthesized and found to be a substrate for the enzyme. It was found by using the coupled enzyme assay that the enzyme catalyzed the production of ADP from **VI** with a k_{cat} of 160 min^{-1}, 32% of the rate obtained with **I**, and a K_m of 33 μM. These results indicate considerable steric tolerance. Steric hindrance is probably not responsible for the higher K_i of the di- and trifluoro compounds. Since the carbon−fluorine bond has a large dipole moment, it is likely that unfavorable dipole interactions with the protein are responsible for the poor inhibition by the di- and trifluoro compounds. Unfavorable dipolar interactions were previously invoked to explain anomalous behavior of polyfluorinated inhibitors (Allen & Abeles, 1989).

Conclusion. Mevalonate pyrophosphate decarboxylase catalyzes the phosphorylation of **II** and **III**. This finding strongly supports a two-step mechanism: phosphorylation followed by decarboxylation. The slow decarboxylation of phosphorylated **II** and **III**, as well as the inhibition by **IV**, a possible transition-state analog, indicates a transition state with carbocationic character.

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