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5-Phosphorylribose 1-α-Methylenebisphosphonate: Properties of a Substrate Analog of 5-Phosphorylribose 1-α-Diphosphate[1]

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The enzymatic synthesis of 5-phosphorylribose 1-α-methylenebisphosphonate (PRPCP), an analog of 5-phosphorylribose 1-α-diphosphate (PRPP), has been achieved by incubating Mg^{2+}, β,γ-methylene ATP, and ribose 5-phosphate with pure *Salmonella typhimurium* PRPP synthetase (EC 2.7.6.1). The PRPCP was purified from the reaction mixture by ion-exchange chromatography, and was isolated as the ammonium salt. It was characterized by phosphate and ribose contents, and by ^{31}P NMR spectroscopy. A study of the rates of hydrolysis of PRPP and PRPCP at 37°C shows that the methylene analog is more stable to chemical hydrolysis at pH's 4, 7, and 10. The products of base hydrolysis of PRPCP are methylenebisphophonate and ribose 5-phosphate. PRPCP serves as a good alternate substrate for mammalian orotate phosphoribosyltransferase (EC 2.4.2.10), but is a very poor substrate for this enzyme derived from yeast. PRPCP should be a useful analog in kinetic studies of phosphoribosyl transferases because its chemical decomposition product, methylene bisphosphonate, is identical to the nonnucleotide product produced by these enzymes.

INTRODUCTION

The design of phosphonate analogs is a successful approach to the study of enzymes involved with transfer or hydrolysis of phosphate esters (*1*). The effects of such substrate analogs on their target enzymes are often useful in elucidating the mechanism involved. The only analog of phosphorylribose-pyrophosphate (PRPP)[3] that has been described (*2*) is 5-phosphoribosyl-1-methylene bisphosphonate (PRPCP), which differs from the natural substrate by having the pyrophosphate "bridge" oxygen replaced by a $-CH_2-$ group (see Scheme 1). PRPCP has not been fully characterized (*2*). As a prelude to the synthesis of other methylene analogs of PRPP, the synthesis of PRPCP was repeated and, for the first time, the rates of hydrolysis at 37°C of both PRPP and PRPCP at pH's 4, 7, and 10 have

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[3] Abbreviations used: PCP, methylenebisphosphonate; PRTase, phosphoribosyltransferase; PRPP, 5-phosphorylribose 1-α-diphosphate; PRPCP, 5-phosphorylribose 1-α-methylenebisphosphonate; OMP, orotidine 5′-phosphate.

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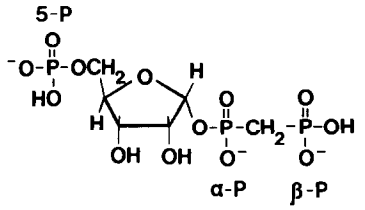

SCHEME 1.

been established. PRPCP was also found to be an alternate substrate of orotate phosphoribosyltransferase, orotate PRTase, derived from yeast and mammalian sources (*3*).

EXPERIMENTAL PROCEDURES

Reagents. Reagents were obtained from the usual commercial sources, and were of the highest purity available.

Enzymes sources. Orotate PRTase from yeast was obtained from Sigma Chemical Company as a mixture with OMP decarboxylase. Mouse and human orotate PRTase (uridylate synthase) were prepared in this laboratory. Mouse orotate PRTase was prepared by ammonium sulfate precipitation from an extract of Ehrlich ascites carcinoma (*4*). Human orotate PRTase derived from erythrocytes was extracted from hemolysates in a manner similar to published procedures (*5*, *6*). The yeast, mouse, and human enzymes generally had specific activities of 7.0, 14.5 and 0.05 nmol min^{-1} mg^{-1}, respectively.

Analog preparation. To a mixture of 5 mM AMPPCP (β,γ-methylene ATP), 10 mM ribose 5-phosphate, and 5 mM $MgSO_4$ in a mixed buffer containing 50 mM triethanolamine, 50 mM potassium phosphate, 0.37 mM EDTA, adjusted to pH 8.0, was added 9 units of *Salmonella typhimurium* PRPP synthetase (kindly supplied to us by Dr. R. Switzer and K. Gibson) in a total volume of 12 ml. The reaction mixture was incubated for 45 min at 37°C, and then placed on ice. The chilled mixture was treated for 10 min with an equal volume of chilled 20% w/v acid-washed Norit. The suspension was centrifuged, and the clear supernatant was applied to a DEAE-Sephadex column (*7*). Application of a linear gradient (0.2–0.8 M) of ammonium formate yielded a clear separation of PRPCP from ribose 5-phosphate. Fractions which contained PRPCP were precipitated with ice-cold methanol/acetone (0.5/5, v/v). The ammonium salt of PRPCP was washed with methanol/acetone and then acetone, and was finally dried at 0°C under vacuum over P_2O_5.

Pentose determinations. Pentose determinations were performed essentially as described by Albaum and Umbreit (*8*). Samples (1.0 ml) were treated with 1.1 ml orcinol/$FeCl_3$ reagent (10 vol 0.1% $FeCl_3 \cdot 6H_2O$ in 12 M HCl plus 1 vol 10% orcinol in 95% ethanol) and heated at 100°C for 30 min. The absorbance was then measured at 670 nm. Ribose 5-phosphate · 3/2 H_2O was used as a standard.

Phosphorus determinations. Total phosphorus was determined as described by Ames (*9*).

^{31}P NMR spectroscopy. Proton spin-decoupled ^{31}P NMR spectra were obtained using a Bruker WM 250 FT NMR operating at 101.3 MHz. An inverse gated–proton-decoupled experiment was conducted for three consecutive timepoints for the base-catalyzed hydrolysis of PRPCP (Fig. 3). This method provided more accurate integrals by minimizing nuclear Overhauser enhancement. Chemical shifts are reported relative to 85% H_3PO_4; downfield shifts are assigned positive values. Solvents and other parameters were as indicated in the figure legends.

Enzyme assays. Orotate PRTase activity was measured at 37°C in the presence of an excess of orotidylate decarboxylase to quantitatively convert OMP formed to UMP and CO_2. The rate of reaction was measured either radiochemically by following the incorporation of label from [7-^{14}C]orotate into $^{14}CO_2$ (method A) or spectrophotometrically by following the decrease in absorbance at 290 nm due to conversion of orotate to UMP (method B, yeast enzyme only). Method A: Reaction mixtures contained 50 mM Tris–HCl (pH 7.7), 5 mM $MgCl_2$, 0.1 mM [7-^{14}C]orotate (600 cpm/nmol), and 2 mM dithiothreitol. Varying concentrations of PRPCP and/or PRPP were added as substrate to the assay mixtures, and the reactions were initiated by the addition of orotate PRTase (mouse and human enzymes). The amount of $^{14}CO_2$ which was released was then measured (*9*). Mouse Ehrlich ascites orotidylate decarboxylase is not inhibited by 100 μM PRPP or PRPCP, the maximum amount used in kinetic studies. Method B: Reaction mixtures (1.0 ml) contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer (pH 7.5), 5 mM $MgSO_4$, 5 mM mercaptoethanol, various concentrations of PRPP and/or PRPCP, and 0.27 mg of the yeast enzyme. The difference in the absorbance of orotate and UMP at 290 nm was $\Delta\Sigma_{290} = 5.1\ mM^{-1}$. Reactions were initiated by the addition of orotate (final concentration, 0.1 mM).

Hydrolysis of PRPP or PRPCP. Reaction mixtures contained 1 mM PRPP or PRPCP and 0.2% sodium azide (which did not alter the pH of the buffer) in either 50 mM potassium formate buffer (pH 4.0), 3-4-morpholinepropanesulfonate buffer (pH 7.0), or glycine buffer (pH 10.0). Vessels, incubated at 37°C, were capped to protect against evaporation, and a low concentration of uridine was added so that its absorbance could be measured to check whether evaporation had occurred. Samples were taken from the hydrolysis mixtures at indicated times, and [PRPP] or [PRPCP] was measured immediately.

The concentrations of PRPP and PRPCP were determined by measuring the stoichiometric conversion of [7-^{14}C]orotate to $^{14}CO_2$ in the presence of an excess of mouse orotate PRTase and OMP decarboxylase. A two- to fivefold greater amount of enzyme was used for determination of PRPCP.

RESULTS AND DISCUSSION

PRPCP recovered from ion-exchange chromatography was shown to have the correct composition by two chemical criteria. The compound was determined to have 3.04 ± 0.09 (SD) mol total phosphorus per mol pentose and, in addition, the

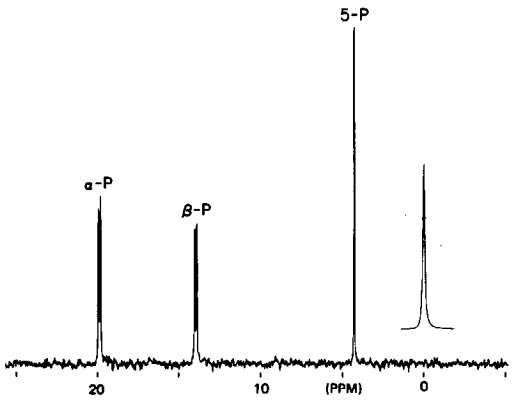

FIG. 1. ^{1}H-decoupled ^{31}P NMR spectrum of PRPCP in 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate buffer (pH 8.0), 5 mM Na_2EDTA, 20% D_2O (Inset: 85% H_3PO_4).

product had the predicted ^{31}P NMR spectrum (Fig. 1). Peak assignments are +4.27 (5-P), +13.94 (β-P), and +19.81 (α-P) (see Scheme 1); the assignments of α-P and β-P were made according to an unambiguous proton-coupled spectrum (not shown). The two mutually coupled downfield ^{31}P nuclei ($J \sim 12$ Hz) have chemical shifts that are consistent with phosphonate resonances (*11*). These spectra provide an indication of the purity of the synthesized analog.

At low pH or elevated temperatures PRPP decomposition is rapid (*12*). The introduction of a methylene group, which has reduced electronegativity (*14*), into the anhydride linkage results in an increased resistance to hydrolytic cleavage. Indeed, PRPCP is significantly more stable than PRPP across a wide pH range (Fig. 2). First-order rate constants (h^{-1}), for hydrolysis were (pH 4) 0.107 for PRPP and 0.049 for PRPCP; (pH 7) 0.022 for PRPP and 0.006 for PRPCP; and (pH 10) 0.023 for PRPP and 0.007 for PRPCP. The ates of hydrolysis at pH 10 and pH 7 are both slower than that at pH 4. Kornberg *et al.* (*12*) showed that there was a similar relation between the rates of hydrolysis of PRPP at pH values between 3.1 and 9.0 at 65°C. The only product of mild acid hydrolysis of PRPCP was presumably PCP, which resulted from scission at the anomeric carbon. PCP was also the only product of base-catalyzed (pH 10) hydrolysis, as demonstrated by monitoring the reaction by ^{31}P NMR; the α-P and β-P resonances were progressively replaced by the PCP signal throughout the course of hydrolysis (Fig. 3 and Table 1). At the first timepoint the PCP signal was not present; by 312 h the PCP signal had grown to 23.0% of the total integrated area (Table 1). This result was not unexpected, since a 1,2-cyclic intermediate, as is found in the case of base-catalyzed hydrolysis of PRPP (*13*), is not a reasonable intermediate for PRPCP. Anchimeric assistance by the adjacent 2-OH, as is observed for PRPP (*13*), is probably not a reasonable contributor to base-catalyzed hydrolysis of PRPCP, since such a mechanism would not allow any reasonable leaving group to attain an axial position in the pentavalent intermediate (Fig. 4A). A pseudorotation about the -CH^2 group (ψ_{CH_2}) would be required to situate the 1-oxygen axial to another oxygen

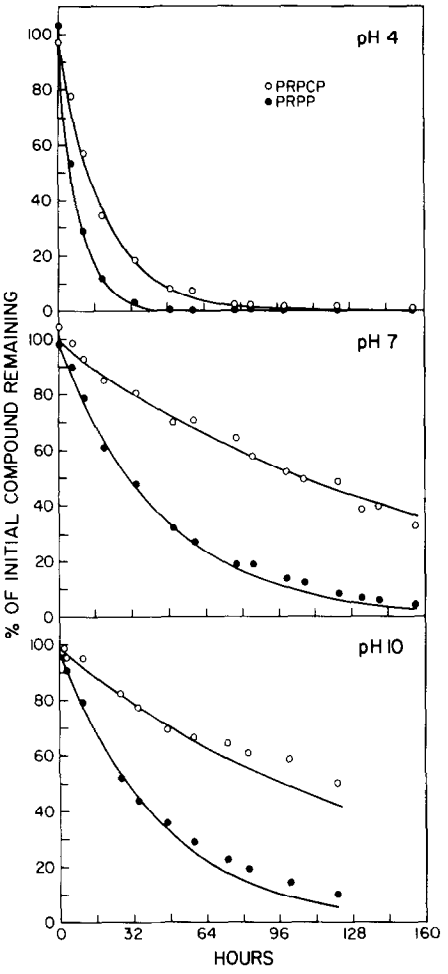

FIG. 2. Hydrolysis of PRPP (●) or PRPCP (○) at 37°C. Reaction mixtures contained PRPP or PRPCP (initially 1 mM) and 0.2% sodium azide in either 50 mM potassium formate buffer (pH 4.0), 4-morpholinepropanesulfonate buffer (pH 7.0), or glycine buffer (pH 10.0) and were held at 37°C. Reaction conditions were as described under Experimental Procedures. The zero time points were not used as 100%; rather, this value was determined by a linear extrapolation from samples, taken at the initial intervals plotted as log PRPP or PRPCP remaining vs time. The points plotted here are an average of the values determined for triplicate or duplicate samples, while the curve was theoretically determined from the rate constants. Rate constants were varied using the observed points until a best fit was achieved.

atom prior to formation of a 2-methylene bisphosphonate product (Fig. 4A). No such species was, in fact, detected by ^{31}P NMR (Fig. 3), indicating that the energy required for pseudorotation must negate any advantage offered by assistance from the neighboring -OH. The decreased rate of hydrolysis may therefore reflect the fact that hydrolysis at the anomeric carbon, to produce methylene bisphosphonate, is the only route available (Fig. 4B) at high pH.

Murray *et al.* (*2*) demonstrated that PRPCP could serve as a substrate analog

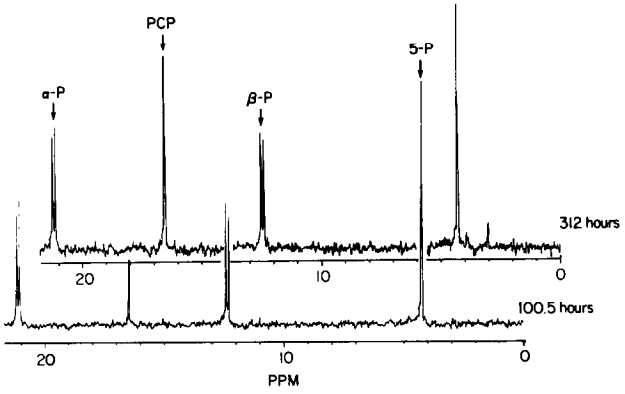

FIG. 3. Course of hydrolysis of PRPCP at pH 10 monitored by ^{31}P NMR. The reactions were performed as described under Experimental Procedures, and the sample contained 0.2% NaN_3 plus 20% D_2O. Resonances are labeled by arrows. A control (similar to Fig. 1) was run at the initial timepoint (0 h). The spectra were obtained from 178, 179, and 122 transients for the 0- (not shown), 100.5-, and 312-h timepoints, respectively.

for nicotinamide PRTase from rat (EC 2.4.2.12), and PRPP amidotransferase (glutamate-amidating, EC 2.4.2.14) and hypoxanthine PRTase (EC 2.4.2.8) derived from Ehrlich ascites carcinoma. PRPCP was also found to be a competitive inhibitor with respect to PRPP of adenine PRTase derived from Ehrlich ascites carcinoma (EC 2.4.2.7); however, PRPCP did not act as a substrate or an inhibitor of yeast orotate PRTase under the conditions used (*2*).

Our studies indicate that PRPCP is indeed an alternate substrate of orotate PRTase derived from yeast and mammalian sources, although the yeast enzyme turns over PRPCP at only 0.1% of the rate observed for PRPP as inferred from kinetic experiments that employed [7-^{14}C]orotate (assay method A, above). It was

TABLE 1

THE RELATIONSHIP BETWEEN ^{31}P NMR PEAK SIGNALS AND THE PERCENTAGE OF TOTAL INTEGRATED AREA IN THE BASE-CATALYZED HYDROLYSIS OF PRPCP

	Percentage of total integrated area[a]		
Peaks	0 h	100.5 h	312 h
5-P	31.9	31.0	27.7
α-P and β-P	68.1	58.8	49.3
PCP	0.0	10.2	23.0

[a] 100% is equivalent to the total integrated area under the four peaks for each time interval.

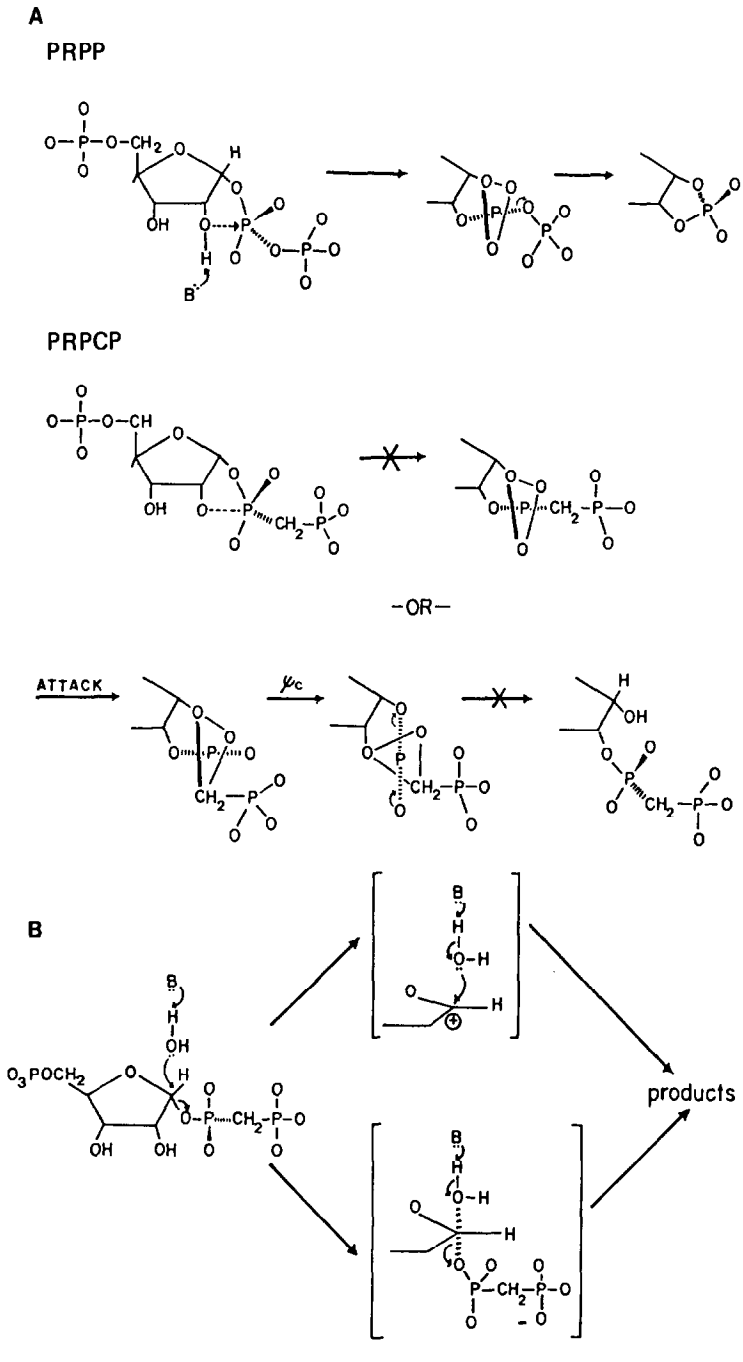

FIG. 4. (A) Anchimeric assistance by the 2-OH in hydrolysis of PRPP as described by Khorana *et al.* (*13*), and the similar but unlikely mechanism for PRPCP. The less electronegative -CH_2- (*14*) would not be expected to accept a pair of electrons nor would be expected to assume an axial position in the pentavelent intermediate. A pseudorotation (Ψ_{CH_2}) would be required to yield the 2-methylene bisphosphonate hydrolysis product (not observed). (B) Likely single mechanism available for base-catalyzed hydrolysis of PRPCP. The S_N1 or S_N2 attack by solvent water is the only suitable route to products PCP and ribose 5-P.

TABLE 2

COMPARISON OF THE KINETIC CONSTANTS OF PRPCP AND PRPP AS SUBSTRATES FOR MOUSE EHRLICH ASCITES CARCINOMA, HUMAN ERYTHROCYTE, AND YEAST OROTATE PRTASE ENZYMES

OPRTase from	K_m(μM) PRPP	K_m(μM) PRPCP	$\frac{K_m(\text{PRPCP})}{K_m(\text{PRPP})}$	$\frac{V_{max}(\text{PRPP})^a}{V_{max}(\text{PRPCP})^{a,b}}$	Substrate selectivity[c]
Mouse carcinoma	4.9	3.7	0.75	11.4	8.6
Human erythrocyte	8	3	0.38	8.2	3.1
Yeast	19	550	28	940	26,300

[a] Each set of values was measured at the same concentration of enzyme (E_t).

[b] Value estimated using V measured at [S] = K_m, and the formula $V_{max} = v$ ([S] + K_m)/[S].

[c] Substrate selectivity = catalytic efficiency of PRPP/catalytic efficiency of PRPCP = [V_{max} (PRPP)/V_{max} (PRPCP)] × [K_m (PRPCP)/K_m (PRPP)].

therefore necessary to obtain the kinetic parameters, V_{max} and K_m, indirectly. We assumed that the binding of PRPCP as a substrate would be the equivalent of PRPCP binding as a competitive inhibitor, with respect to PRPP, to the orotate PRTase. As expected, we were able to measure the apparent K_I for PRPCP from such an experiment (Fig. 5). On the above assumption, the value of K_m is 550 μM for PRPCP[4] as compared to 19 μM for PRPP (Table 2).

We have determined that PRPCP does have a slight ability to act as a donor of the phosphoribosyl group using yeast orotate PRTase, and is readily used by the mouse Ehrlich ascites or human erythrocyte orotate PRTase. The values of V_{max}/K_m for each substrate indicate the relative preference of the enzyme for PRPP over PRPCP. The yeast enzyme displays a high selectivity for PRPP over PRPCP (Table 2). This effect is due to both a large decrease in affinity (about 28-fold) and a very large decrease in turnover rate (about 940-fold). This high preference for PRPP is in sharp contrast to what is observed for this enzyme from two mammalian sources. Both mammalian orotate PRTase enzymes show slightly higher apparent affinities for the methylene analog than for PRPP. The mouse and human enzymes readily recognize PRPCP, but apparently have some difficulty in removing the PCP moiety on the way to formation of the phosphoribosyl–enzyme intermediate (*16*). Therefore, this analog may be serving as a probe of some substantial difference in mechanism between the mammalian and yeast enzymes.

Since PRPP decomposes spontaneously to 1,2-cyclic phosphorylribose 5-phosphate, this compound is always present when one studies the kinetics of any of the several phosphoribosyl transferases. No one has established whether this by-product binds to these enzymes and has influenced the various kinetic analysis of these enzymes. PRPCP may therefore be valuable as an alternate substrate for use in kinetics studies of phosphoribosyl transferase because PCP, whether formed

[4] Strictly speaking, this value represents the true dissociation constant of the Michaelis–Menton ES complex, and should be regarded as a minimum estimate of the kinetic K_m. The calculated value of V_{max}/K_m is, therefore, an upper limit, and the resulting value of selectivity of PRPP/PRPCP (Table 2) is a lower limit.

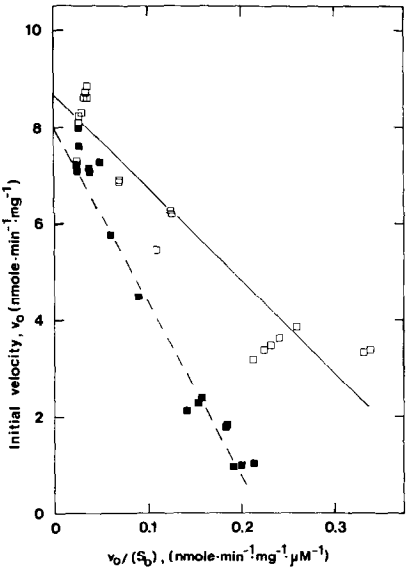

FIG. 5. Eadie–Hofstee plot of kinetic data obtained for yeast orotate PRTase using PRPP as substrate either alone (□) or in the presence of 0.5 mM PRPCP (■) as an inhibitor. Under the latter conditions, PRPCP is not a significant substrate.

chemically or enzymatically, would be the only product formed other than the nucleotide.

That the mammalian enzymes actually bind PRPCP more tightly and that turnover is substantially decreased (Table 2) suggests that other methylene analogs of PRPP could serve as antimetabolites. Isosteric analogs, which contained a methylene group in place of oxygen at the anomeric carbon, would be of primary interest since such compounds would be resistant to removal of the pyrophosphate (or rather its phosphinyl or phosphonyl isostere) group after binding. The synthesis of a required intermediate of such compounds has been accomplished by McClard (*17*), and efforts to synthesize anomeric methylene analogs of PRPP are under way.

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