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COMPARISONS OF THE OROTATE PHOSPHORIBOSYLTRANSFERASE AND HYPOXANTHINE/GUANINE PHOSPHORIBOSYLTRANSFERASE ACTIVITIES IN YEAST

BY

SUNG H. CHUNG

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1987

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

7/17/87
Date

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July 17, 1987
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The City University of New York

Abstract

COMPARISONS OF THE OPRTASE AND HGPRTASE ACTIVITIES IN YEAST

BY

SUNG H. CHUNG

Adviser: Professor Donald L. Sloan

Enzymatic assay procedures that employ high-performance liquid chromatography (HPLC) have proven to be sensitive and versatile methods for accomplishing kinetic analyses of enzyme-catalyzed reactions, with nucleotides as substrates or products. Both orotate phosphoribosyltransferase (OPRTase) and hypoxanthine/guanine phosphoribosyltransferase (HGPRTase) have been purified from Baker's yeast and analyzed kinetically using a modification of published HPLC procedures. Because these two enzymes exist in the cytosol of yeast and might compete for the limiting (1-13 μM) concentration of phosphoribosyl 1-pyrophosphate (PRibPP), we elected to examine both equilibrium and steady-state effects of one enzymatic reaction on the other with HPLC. First, under the condition of equivalent mass concentrations of OPRTase and HGPRTase, the initial rate of orotidine monophosphate synthesis and the equilibrium state were greatly affected by the presence of HGPRTase activity. In contrast, the presence of the OPRTase activity had no effect on the HGPRTase-catalyzed reaction under the same conditions. Second, to examine a competition by these enzymes for PRibPP in vivo, we have established that the total activities (units/ml) of OPRTase and HGPRTase in yeast cell extracts were 740

units/ml and 450 units/ml, respectively. These relative activities were then employed in an in vitro reaction competition analysis. The results were similar to those obtained from experiments where equivalent OPRTase and HGPRTase activities were employed and revealed profound initial velocity and equilibrium effects of one reaction on the other. Thus a real competition between these enzymes for PRibPP may occur in the yeast cell cytosol, as determined by this unique HPLC competition assay procedure.

Several pyrimidine base analogs and xanthine derivatives were examined, to determine whether they are alternative substrates or inhibitors of yeast OPRTase and HGPRTase, respectively. These studies showed that both enzymes have a fairly selective specificities for their base substrates. Most of these compounds studied were relatively poor inhibitors or had no inhibitory or substrate capabilities.

Non-reacting molecular sieve HPLC studies suggested the occurrence of a concentration-dependent equilibrium between monomeric and dimeric HGPRTase forms. However, the monomeric form of HGPRTase was observed to dominate in solution, in the presence of Mg-PRibPP in the eluting buffer. According to previously described cross linking studies and this HPLC study, the catalytically active form of yeast HGPRTase may be the monomeric form of the enzyme.

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First of all, I would like to thank my thesis advisor Dr. Donald L. Sloan for helping me go through years I have spent here. He has always encouraged and cheered me up when I faced difficulties in my personal life as well as my research. I have always believed that my Lord leads and fills my life with what I would need to be successful and Dr. Sloan was one of those people who I really needed to meet.

I also thank my committee members, Dr. H. Schulz, Dr. T. Haines, Dr. S. Cosloy, and Dr. T. Krulwich for their encourgement, patience and kindness.

From the bottom of my heart, I really appreciate my parents. They have been praying for me, especially my scientific career and spiritual life, since I came to this country. I like to share this glory with my lovely wife, Kyunghie.

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INTRODUCTION

Phosphoribosyl 1-pyrophosphate (PRibPP) is utilized by a group of enzymes known as the phosphoribosyltransferases (PRTases), each of which is specific for a divalent metal ion and a nitrogenous base. The acceptors of the ribose-5-phosphate group are pyridine (Preiss et al., 1957), pyrimidine (Hatfield et al., 1964), or purine bases (Kornberg et al., 1955), the amide of glutamine (Goldthwaith, 1956), ATP and anthranilic acid utilized in first enzymatic steps of His and Trp synthesis, respectively (Crowley, 1964; Smith et al., 1960), or ammonia (Hartman, 1963). PRTase-catalyzed reactions and the metabolic fate of PRibPP have been presented previously (Musick, 1981; Sloan et al., 1984).

Since all PRTases need PRibPP as a common substrate, PRibPP may play an important role as a regulator of these nucleotide biosynthetic pathways. The intracellular concentration of PRibPP in normal human erythrocytes ranges from 1 to 5 μM whereas its concentrations in cultured fibroblasts may be as high as 13 μM (Kelley et al., 1970). These values are substantially lower than the Km value for the PRibPP amidotransferase in bacteria (Nierlich et al., 1965), avian liver (Wyngaarden et al., 1959), and mammalian adenocarcinoma cells (Hill et al., 1969) (230 to 470 μM). It is noteworthy that this range of intracellular PRibPP concentration is also lower than the Km for most other PRTase enzymes. Thus, the concentration of PRibPP within the cell may also be rate-limiting for enzymes other than PRibPP amidotransferase. A regulation of intracellular PRibPP concentration can be carried out via different pathways. The metabolism of PRibPP synthesis is summarized in Figure 1.

As Musick (1981) has noted, the kinetic and the structural properties of the

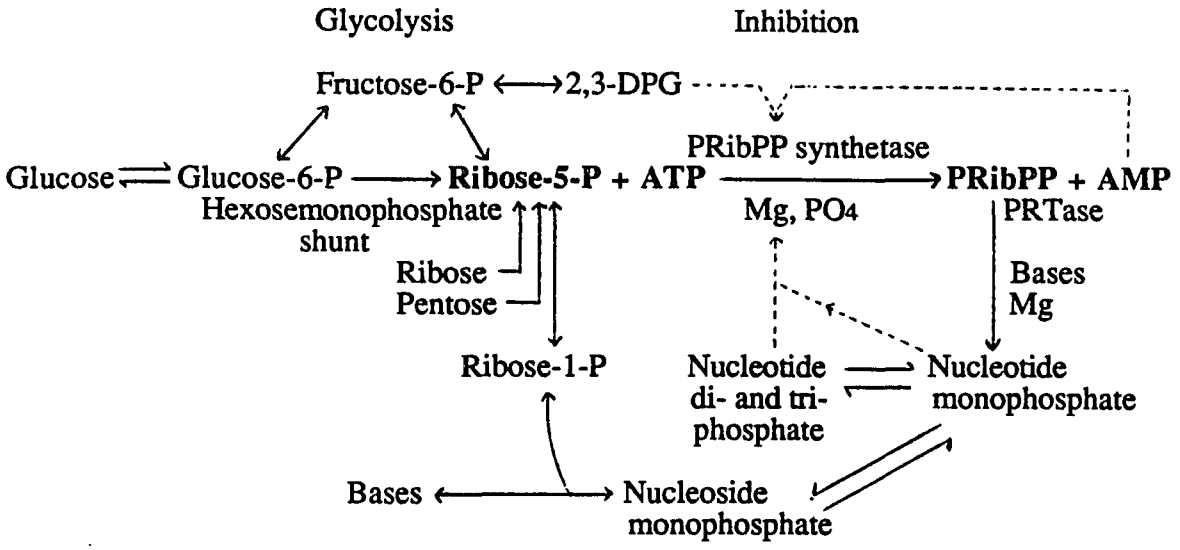


Figure 1. Regulation of intracellular phosphoribosyl 1-pyrophosphate (PRibPP) concentration. PRibPP formation is regulated by the availability of ribose-5-phosphate, which can be produced by the glycolytic pathway, the hexosemonophosphate shunt, or the degradation of ribonucleotides. PRibPP synthetase is inhibited by 2,3-diphosphoglycerate (DPG), adenosine 5-monophosphate (AMP), as well as a number of purine and pyrimidine ribonucleotides as indicated by the dotted lines. ATP = adenosine triphosphate, PRTase = phosphoribosyltransferase

PRTases are quite variable, even when a single tissue source such as yeast (Saccharomyces cerevisiae) is examined (Victor et al., 1979a; Ali and Sloan, 1982; Schmidt et al., 1979; Nagy and Ribet, 1977; Natalini et al., 1979; Hanna et al., 1983;Natalini et al., 1986).

In our laboratory, Baker's yeast has been chosen as a source of the PRTases, because these enzymes can be isolated from this tissue in relatively large quantities. We started out to survey the PRTase activities in yeast using reversed-phase high-performance liquid chromatography (HPLC). Initially, we asked how the relatively small cellular concentration of PRibPP (1-13μM) is distributed among these nucleotide synthetic pathways, because the mechanisms of control of PRibPP allocation for this simple eukaryotic organism may provide a model for control mechanisms in mammalian cells, as well as in parasitic organisms. A Water's μBondapak C_{18} reverse-phase HPLC column was employed to monitor several enzymatic reaction components simultaneously. Gradient elution procedures were perfected that allowed the separation of the following PRTase assay components: adenosine monophosphate, nicotinate mononucleotide, orotate, adenosine triphosphate, nicotinate, adenosine diphosphate and nicotinamide. In preliminary experiments (Sloan et al., 1984), the above gradient system, a 20 min linear gradient ranging from 0% to 100% 25 mM ammonium phosphate buffer (pH 6), was employed to examine the PRibPP utilization by orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10), hypoxanthine-guanine phosphoribosyltransferase (HGPRTase, EC 2.4.2.8), and nicotinate phosphoribosyltransferase (NaPRTase, EC 2.4.2.11) catalyzed reactions. The reactions catalyzed by OPRTase (eqn.1), HGPRTase (eqn.2 and 3) and NaPRTase (eqn.4) are shown below.

orotate + PRibPP $\rightleftharpoons$ orotidine 5'-phosphate + PPi (1)

hypoxanthine + PRibPP $\rightleftharpoons$ inosine 5'-phosphate + PPi (2)

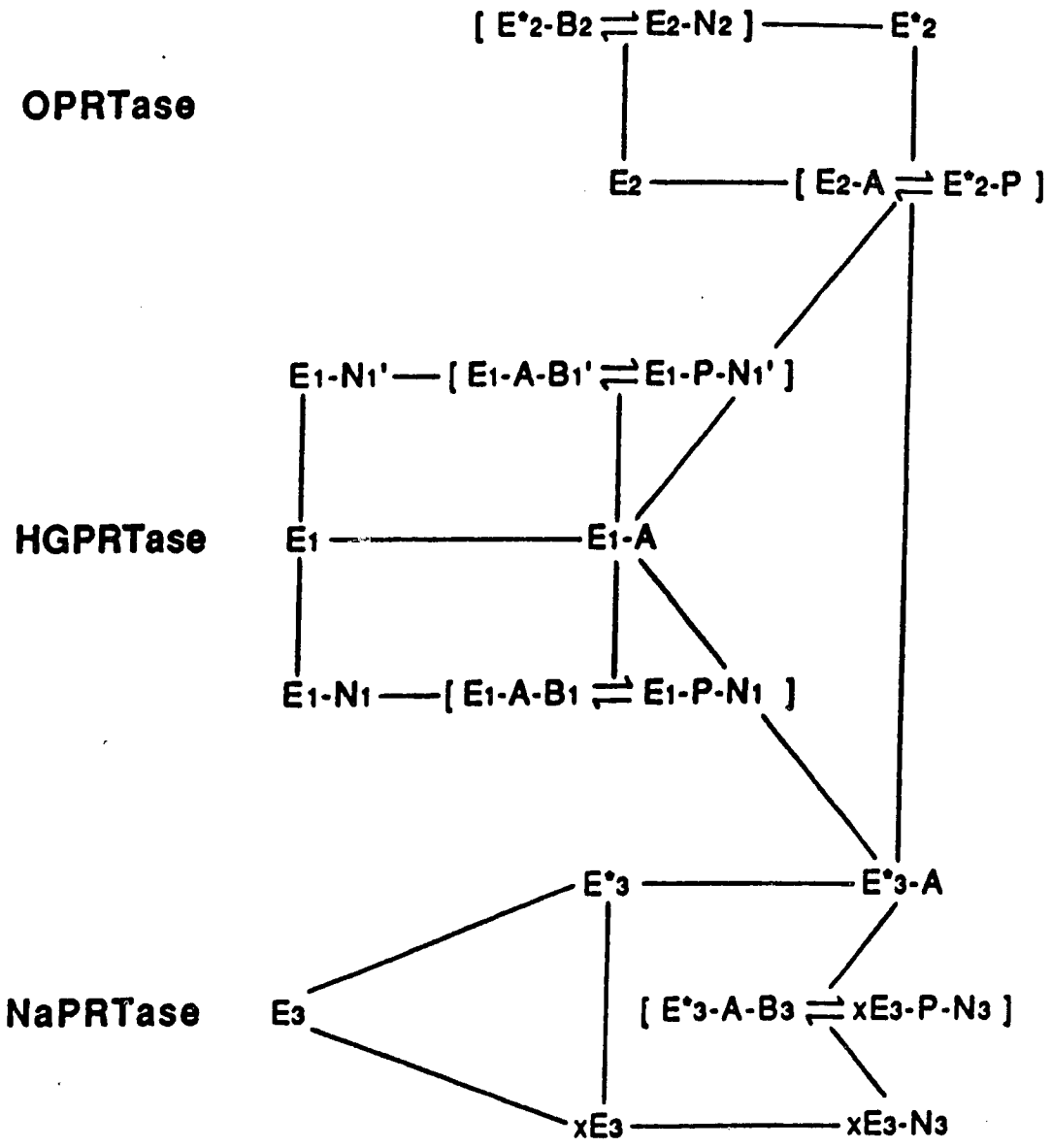
guanine + PRibPP $\rightleftharpoons$ guanosine 5'-phosphate + PPi (3)

nicotinate + PRibPP + ATP $\longrightarrow$ ADP + Pi + nicotinate nucleotide + PPi (4)

The most interesting result of this preliminary study was that the irreversible enzymatic reaction (of NaPRTase) eventually succeeded in utilizing all of the equivalents of PRibPP, under the conditions used in this assay. Although these phosphoribosyltransferases catalyze similar reactions, each reaction proceeds by way of a different kinetic mechanism (Ali and Sloan, 1982; Hanna et al., 1983; Victor et al., 1979), as illustrated in Figure 2. These mechanisms must be interrelated because of the utilization of a commom substrate, PRibPP. My role in this project was to carry out a detailed kinetic analysis of the competition for PRibPP between two reversible enzymes in yeast, OPRTase and HGPRTase. I have examined this competition under three different conditions (see TABLE 1). The experiment, using the physiological concentrations of OPRTase and HGPRTase, marks the first time that these two reactions have been analyzed simultaneously at levels corresponding to an in vivo situation, and demonstrates further the usefulness of this type of HPLC assay procedure.

In all organisms thus far examined, OPRTase exhibits an absolute requirement for Mg(II) or Mn(II) (Kasbekar et al., 1964; Ashihara, 1978; Yoshimoto et al., 1978; Belser and Wild, 1978; Silva and Hatfield, 1978) with optimal activity achieved at ~ 5 mM Mg(II) ion, the probable physiological activator. Studies with substrate analogs have demonstrated that the enzyme from human and beef erythrocytes requires a 2,4-

Figure 2. The combined kinetic mechanisms of HGPRTase (E_1), OPRTase (E_2) and NaPRTase (E_3). Abbreviations: A = PRibPP; B_1 = hypoxanthine; B'_1 = guanine; B_2 = orotate; B_3 = nicotinate; N_1 = inosine monophosphate; N'_1 = guanosine monophosphate; N_2 = orotidine monophosphate; N_3 = nicotinate monophosphate; P = pyrophosphate. E^*_2 and E^*_3 represent covalent phosphoribosyl-OPRTase and phosphoryl-NaPRTase enzyme forms, respectively, whereas xE_3 represents the non-covalent phosphoryl-NaPRTase complex.


OPRTase
[E*2-B2 ⇌ E2-N2]
E*2
E2
[E2-A ⇌ E*2-P]
E1-N1'
[E1-A-B1' ⇌ E1-P-N1']
HGPRTase
E1
E1-A
E1-N1
[E1-A-B1 ⇌ E1-P-N1]
E*3
E*3-A
NaPRTase
E3
[E*3-A-B3 ⇌ xE3-P-N3]
xE3
xE3-N3

diketo-substituted pyrimidine nucleus (Silva and Hatfield, 1978). Kinetic analyses of pyrimidine analogs as ligands of yeast OPRTase were carried out in this laboratory and by others (Victor et al., 1979; Dahl et al., 1959). It has been demonstrated that the inhibitor and substrate specificities of mammalian, bacterial, protozoal, and yeast OPRTase are quite different (O'Donovan and Neuhard, 1970; Henderson and Paterson, 1973). According to the literature survey by Niedzwicki and his colleagues (1984), the mammalian OPRTase has a quite broad selectivity for base substrates. For instance, the OPRTase of bovine erythrocytes can utilizes somewhat unusual uric acid and xanthine as base substrates (Hatfield and Wyngaarden, 1964). In contrast, yeast OPRTase appears to be highly specific for its base substrate. So far, only 5-fluoroorotate and 5-azaorotate (oxonic acid) have been idetified as alternative substrates of yeast OPRTase (Victor et al., 1979; Ashton and Sloan, unpublished data). I have examined several pyrimidine bases, listed in TABLE 5, as substrates or inhibitors of purified yeast OPRTase. None of them serve as substrates except 5-fluoroorotate and oxonic acid. (5-Bromo- and 5-Iodoorotate were initially thought to be poor substrates of yeast OPRTase, but subsequent NMR experiment demonstrated that those compounds are contaminated with 8 to 10% of orotate.) These experiments have revealed that the placement of bulky substituents at the 5-position of orotate disallowed the use of the structure as substrate by this enzyme. We reasoned that the orotate analog oxonic acid would not be prevented sterically from binding to the yeast OPRTase active site and might serve as a substrate for this enzyme just as it had been demonstrated for several mammalian enzymes (Cihák and Sorm, 1972). We reasoned further that an aldehyde group at the 6-position would allow binding of the orotate ring to the enzyme active site, and that this analogue (orotaldehyde) might also serve as a substrate. Interestingly, newly synthesized orotaldehyde, by a direct oxidation method,

is not a substrate, but inactivates yeast OPRTase over a period of time instead. Similar to pyridoxal 5-phosphate (PLP), the aldehyde group of orotaldehyde has been shown to react with the side chain amino group of the lysine residue, which might be present at the active site of the enzyme. For a couple of decades, the chemical modification of enzymes has been a useful experimental tool for enzymologists to understand how enzymes work, although critical interpretations of those experimental results are always necessary. The developments in molecular biology over the past few years allow investigators to change any amino acid residue in a protein by another, via site-directed mutagenesis of the gene (Hutchison et al., 1978; Smith, 1985). Using today's sophisticated new approaches, the precise and often delicate future changes in amino acids that in and near the active sites of several yeast PRTases, will be able to illuminate the interdependent roles of catalytic groups, whenever either the complete amino acid sequence or tertiary structural information is available.

As shown in equations 2 and 3, HGPRTase catalyzes the Mg(II) dependent transfer of a phosphoribosyl group from PRibPP to the purine bases hypoxanthine or guanine (Krenitsky et al., 1969; Arnold and Kelley, 1971; Olsen and Milman, 1974). Unlikely the human and yeast HGPRTase, *Leishmania donovani* promastigotes and *Escherichia coli* seemed to contain distinctive xanthine PRTase and guanine-xanthine PRTase, respectively (Tuttle and Krenitsky, 1980; Liu and Milman, 1983). In addition to guanine-xanthine PRTase, E. coli utilizes adenine (Hochstadt-Ozer and Stadtman, 1971) and hypoxanthine-guanine PRTases (Martin and Yang, 1972). Although Nussbaum and Caskey (1981) reported that neither adenine nor xanthine acted as substrate for the yeast HGPRTase, controversy still exists as to the substrate specificity of yeast HGPRTase. Several xanthine-derivatives have been examined as possible substrates or inhibitors of yeast HGPRTase in this work. These studies were

undertaken because a preliminary studies had indicated that xanthine is a poor substrate for the yeast HGPRTase catalyzed reaction, and that 3-methylxanthine and 1,3,7-trimethylxanthine (caffeine) might inhibit this activity. We planned to explore these reactions in detail to determine the kinetic mechanism for XMP formation and to determine the relative values of the inhibition constants for the five xanthine derivatives, listed later.

Finally, during our examination of the electrophoretic properties of the purified OPRTase (Victor et al., 1979), HGPRTase (Ali and Sloan, 1982; Sloan et al., 1984), and NaPRTase (Hanna et al., 1983) we noted some similarities (and differences) in the sizes and subunit structures of these yeast enzymes. OPRTase and HGPRTase are dimeric proteins (with 23,000 MW and 26,000 MW subunits respectively), whereas NaPRTase is a 40,000 MW monomer, as determined by SDS-gel electrophoresis. However, these results did not reveal whether monomer/dimer association equilibria occurred in solutions containing OPRTase or HGPRTase, or what the apparent molecular weight of the catalytically active forms of these enzymes would be. Bifunctional crosslinking reagents, glutaraldehyde and dimethyl suberimidate, were first employed to examine the subunit association-dissociation equilibria of both enzymes. The SDS gel electrophoresis patterns of the enzymes, after incubation with these crosslinkers, in the absence and presence of Mg(II) and PRibPP, suggested that the PRibPP-OPRTase complex is predominantly dimeric, whereas PRibPP-HGPRTase occurs primarily as an active monomeric structure. In this paper, molecular sieve TSK-HPLC was used to confirm the previous electrophoretic results (Furman and Neet, 1983).

MATERIALS

Baker's yeast (Budweiser Brand) was obtained from Valente Yeast, Inc. (Flushing, NY). Sephacryl S-200 and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ), and a Cellex D (DEAE cellulose) and Biogel HTP (hydroxyapatite) were obtained from Bio-Rad Laboratories (Richmond, CA). The following chemicals were supplied by Sigma Chemical Company: orotic acid, OMP, hypoxanthine, IMP, activated charcoal, celite (diatomaceous earth), ammonium phosphate (monobasic), triethanolamine, POPSO (piperazine-N,N'-bis[2-hydroxypropanesulfonic acid]), selenium dioxide, sodium bisulfite, xanthine, 3-methylxanthine, 1,3-dimethylxanthine, 1,7-dimethylxanthine, 3,7-dimethylxanthine, 1,3,7-trimethylxanthine (caffeine). 6-Methyluracil was purchased from Aldrich Chemical Company, Inc. All other chemicals were reagent grade.

METHODS

PURIFICATION OF OPRTASE

All purification steps were performed at 4°C unless otherwise stated. Yeast OPRTase was purified using procedures published previously (Umezu et al., 1971; Victor et al., 1979), with a few modifications.

(1) Autolysis: 25 lbs. of Baker's yeast (Budweiser Brand obtained from Valente Yeast Co., Flushing, N.Y.) was suspended in 7.5 liter of 0.3 M potassium phosphate buffer, pH 8. The suspension was gently mixed by a motor-driven stirrer in a water bath, which was kept at 30°C. A volume of 1250 ml of toluene was then added to initiate the autolysis procedure. The pH was monitored occasionally and adjusted to pH 8 with 5N KOH (about 650 ml of 5N KOH was used). The pH of the suspension remained at a value of 8 after 2 hours of addition of toluene. Then the autolysate was placed in a cold room (4°C) and allowed to stand overnight. Next day the autolysate was spun in a Sorvall RC-5 superspeed refrigerated centrifuge for 30 min at 9,000 rpm using a GSA rotor. Yeast cell debris (precipitate from the centrifugation) was discarded and the supernatant from centrifugation was collected and filtered through cheesecloth to remove fluffy lipid materials.

(2) Ammonium Sulfate Fractionation: The filtrate from the autolysis was adjusted to pH 5 with 8N-acetic acid after addition of 20 ml of n-octanol in order to prevent foaming. To obtain a 50% ammonium sulfate fraction, 3,374 g of solid ammonium sulfate was added fairly slowly to the 10.8 liter of acidified filtrate with gentle stirring (313 g of ammonium sulfate / liter of filtrate). It took several hours to complete the addition, and the solution was then placed in the cold room overnight to allow yeast proteins to precipitate completely. The precipitate, which contained the

OPRTase activity, was obtained by centrifugation at 9,000 rpm for 25 min, and then redissolved in a minimal amount of 25 mM Tris-HCl buffer, pH 8. This turbid suspension was then adjusted to pH 8 with 1N KOH prior to dialysis. The dialysis of the solution was conducted overnight against 20 liters of 10 mM Tris-HCl, pH 8.

(3) Manganese Treatment: The dialyzed solution was spun at 9,000 rpm for 20 min to remove undissolved materials. To get rid of nucleic acids present in this enzyme preparation, 165 mL of 1 mM $MnCl_2$ was added to 3,150 ml of the supernatant (final concentration of $MnCl_2$ was 50 mM), and the solution was stirred gently for 30 min and then centrifuged. The supernatant from centrifugation was dialyzed against 10 liters of 10 mM Tris-HCl, pH 8.

(4) Ethanol Fractionation: The total volume of the dialyzed solution was 3,000 ml and it was divided into 4 fractions for ethanol treatment. To each batch of 750 ml of the above protein solution was added 100 ml of 2 M potassium acetate buffer, pH 6, containing 10 mM orotate. The solution was cooled to below 0°C in a salt-ice water bath and chilled 95% ethanol was added to the solution, with swirling, to a 15% ethanol concentration (180 ml ethanol per 1,000 ml solution). The temperature of the solution was checked after each addition of ethanol and it was maintained close to 5°C. The precipitate obtained from the 15% ethanol treatment was discarded by centrifugation at 9,000 rpm for 15 min. The supernatant was then treated with additional ethanol to bring its concentration to 50% (820 ml ethanol per 1,000 ml original solution). The 50% precipitate, recovered from centrifugation, was then dissolved in a minimal volume of 25 mM Tris-HCl, pH 8. After stirring for a time to allow maximal solubilization, the suspension was centrifuged and the precipitate was resuspended and extracted with the same buffer. The supernatants were pooled together and then dialyzed overnight against 10 liters of 25 mM Tris-HCl, pH 8. The

next day, appropriate amounts of $MgCl_2$ and orotate (final concentrations were 2 mM and 1 mM , respectively) were added to the above dialyzed preparation with stirring (final volume was 980 ml) in order to stabilize OPRTase.

(5) Heat Treatment: The dialyzed active ethanol fraction was poured into four stainless steel centrifuge cups and placed in a water bath at 53°C for 5 min. They were then transferred immediately to an ice bath for 10 min. The denatured proteins were removed by centrifugation at 9,000 rpm for 20 min. The supernatant was then filtered through Whatman No.1 filter paper.

(6) Gel Filtration Chromatography: The clarified supernatant was concentrated with an Amicon ultrafiltration apparatus and a PM-10 membrane. A pre-swollen suspension of Sephacryl S-200 was used in this step instead of any Sephadex G-series because of two merits of this material. a) The unique chemical structure of Sephacryl makes it exceptionally rigid. Therefore excellent flow rates can be obtained in both large and small columns. b) All Sephacryl types are specially treated to reduce the number of active centers where adsorption can occur ensuring good recoveries. After the Sephacryl S-200 column (1.5 x 105 cm) had been equilibrated with 10 mM Tris-HCl buffer (pH 8, containing 40 mM NaCl and 1 mM orotate), 8 ml of Blue Dextran 2000, 1 mg/ml, was loaded on the column to determine a void volume of the column (Vo = 220 ml). Thereafter, 50 ml of previous concentrated enzyme preparation was applied to the column for each run. A huge and single elution peak of protein came out after about 250 ml of elution volume had been collected, and those fractions containing over 20% of the activity found in the most active portion were pooled.

(7) Cellex D (DEAE Cellulose) Chromatography: The pooled Sephacryl S-200 fraction was concentrated as described above and dialyzed overnight against 20 liters of 10 mM potassium phosphate buffer, pH 8, containing 1 mM orotate. The dialysate

was then applied to the DEAE column, which was eqilibrated with the same buffer. The protein was eluted with a linear gradient made up of 500 ml each of 10 mM and 200 mM potassium phosphate buffer, pH 8. The elution profile of the DEAE column was composed of one minor peak followed by one major peak. The active portions of the second major peaks from three runs of DEAE were pooled and concentrated to 60 ml. The concentrated enzyme preparation was then dialyzed overnight against the same buffer.

(8) Bio-Gel HTP (Hydroxyapatite) Chromatography: The concentrated enzyme solution from DEAE chromatography was loaded onto an hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer, pH 8, containing 1 mM orotate. A linear gradient (300 ml each of 10 mM and 200 mM potassium phosphate buffer at pH 8) was applied to the column to elute the enzyme. A single and sharp elution profile of protein was obtained, and those fractions having activities over 20% of the most active fraction were collected and dialyzed overnight against 10 liters of 100 mM of potassium phosphate buffer, pH 7.2.

(9) OMP-Sepharose Affinity Chromatography: OMP-Sepharose affinity column was prepared by following procedures. a) CNBr-activated Sepharose 4B, purchased from Pharmacia Fine Chemicals, was coupled to adipic acid dihydrazide (Lamed et al., 1973). b) Oxidized OMP was then added to the centrifuged hydrazide-Sepharose and this suspension was allowed to stand at room temperature, with occasional swirling, for 4 hours (Dodin, 1981). Excess (unbound) oxidized OMP was washed away with 3 M NaCl until no absorbance at 280 nm was detected and the column was equilibrated with 100 mM potassium phosphate buffer at pH 7.2. The protein solution collected from hydroxyapatite was loaded slowly onto the affinity column and washed with 50 ml of 100 mM potassium phosphate buffer, pH 7.2. This wash was followed by a

wash with 10 mM potassium phosphate buffer, pH 8 until the absorbance at 280 nm disappeared. The enzyme was detached from the column by addition of 10 ml of 1 mM OMP solution. Those fractions containing enzyme were pooled and concentrated to 8 ml with an Amicon ultrafiltration unit, and enzyme-bound OMP was removed completely from the enzyme solution by dialyzing for 6 hours against 4 liters of 10 mM potassium phosphate buffer, pH 8, containing 1 mM orotate.

SPECTROSCOPIC ASSAY OF OPRTASE

Measurements of the initial velocities of the phosphoribosyl transfer reaction were carried out spectrophotometrically at 25°C using a Cary-15 double beam recording spectrophotometer, according to the method of Umezu et. al. (1971), using an extinction coefficient of 3950 $M^{-1}cm^{-1}$ determined previously. The final concentrations of the assay components in 1 ml of assay solution were 50 mM Tris-HCl buffer, pH 8, 1 mM $MgCl_2$, 100 μM PRibPP, 300 μM orotate, and an appropriate amount of enzyme was added to this assay mixture to initiate the enzymatic reaction.

PURIFICATION OF HGPRTASE

HGPRTase was purified from yeast using a published procedure in this lab (Ali and Sloan, 1982). Baker's yeast 11 lbs was suspended in 4.5 liters of 0.3 M potassium phosphate buffer at pH 8 and 500 ml of toluene was added to initiate an autolysis. This autolysis step was carried out at 30°C water bath for 4 hours with occasional swirling. The pH of the suspension was checked periodically and adjusted to 8 with 5N KOH. The suspension was then placed in the cold room and allowed to stand overnight. The aqueous layer was collected by centrifugation at 9,000 rpm for 30 min and clarified through cheesecloth, and then ammonium sulfate (to 60% saturation)

was added to precipitate the enzyme. The precipitate was removed by centrifugation and dissolved in 1 mM potassium phosphate buffer,pH 8, containing 15% glycerol and 1 mM DTT, and then dialyzed against the same buffer. Thereafter 100 ml each of the dialyzed enzyme solution was placed in stainless steel centrifuge cups and heat treatment was accomplished by incubating the samples at 70°C for 5 min and then quickly cooling them to 4°C. Denatured proteins were removed by centrifugation and 10% glycerol and 1 mM DTT were added to the supernatant to stabilize the HGPRTase. For the remainder of the procedure, all buffers contained 15% glycerol and 1 mM DTT if not specified otherwise.

Heat-treated enzyme solution was dialyzed against 1 mM potassium phosphate, pH 7.5, for 4 hours, and applied to the hydroxyapatite column, which had been equilibrated with the same buffer. The enzyme was then eluted with a linear gradient made up of 500 ml each of 1 mM and 100 mM potassium phosphate buffer, pH 7.5. Those fractions having HGPRTase activity were pooled and concentrated by Amicon pressure ultrafiltration. (Three attempts at hydroxyapatite chromatography were made, due to a failure to bind the enzyme to the column material, and as far as my experience is concerned, this hydroxyapatite chromatography step might be a necessary step prior to a GMP-Sepharose affinity chromatography to obtain the pure HGPRTase from yeast).

The enzyme solution obtained from hydroxyapatite was dialyzed and applied to a GMP-Sepharose column, equilibrated with 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 15% glycerol and 1 mM DTT. The GMP-Sepharose column was prepared according to published procedures (Huges et al., 1975; Schmidt et al., 1979). The column was washed with the equilibrating buffer containing 1.2 M KCl until no detectable protein eluted. The enzymatic activity was then released by adding 5 mM

GMP to the buffer, a procedure first described by Gutensohn et al. (1976). The fractions with HGPRTase activity were pooled and concentrated by Amicon pressure ultrafiltration.

The HGPRTase solution from the GMP-Sepharose was concentrated further by centrifuging in a Centricon 10 unit at 1,500 rpm for the required length of time to obtain a minimal amount of enzyme sample (Prior to use, each Centricon 10 unit was rinsed by spinning it with 2 ml of deionized water to remove trace amounts of glycerin and sodium azide). To remove enzyme-bound GMP and contaminants, 100 µl each aliquot of enzyme solution was applied to a HPLC gel filtration column (Bio-Sil TSK 250 purchased from Bio-Rad Co.), as a final step of HGPRTase purification procedure, which was equilibrated with a 0.2 M sodium sulfate and 0.02 M sodium phosphate buffer, pH 6.8. The elution profile was composed of two major peaks, and fractions containing HGPRTase activity were collected and concentrated by ultrafiltration.

ASSAY OF HGPRTASE ACTIVITY

Activities of HGPRTase-catalyzed reaction were measured according to the method, originally described by Flaks (1963). The assay mixture consisted of 0.1 ml of 1 mM hypoxanthine (91 µM final concentration), 0.2 ml of 2 mM PRibPP (360 µM final concentration), 0.1 ml of 100 mM $MgCl_2$, 0.1 ml of 0.25 M potassium phosphate buffer (pH 7.4), and water to a final volume of 1.1 ml. The mixture was placed in a 38°C water bath and the reaction, initiated by adding 20 µl of enzyme solution, was allowed to proceed for 15 min. The reaction was terminated by heating the sample in a boiling water bath for 2 min. Denatured protein was removed by centrifugation and the supernatant was filtered through a 0.45-mm HA Millipore filter prior to HPLC injection.

COMPETITION BETWEEN OPRTASE AND HGPRTASE FOR A COMMON SUBSTRATE

OPRTase (0.54 mg/ml) and HGPRTase (0.32 mg/ml) purified from Baker's yeast to apparent electrophoretic homogeneity were used in this study. When necessary, each enzyme was diluted with the following buffers; a) OPRTase with 50 mM Tris-HCl, pH 8, containing 0.2 mM orotate and b) HGPRTase with 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 15% glycerol and 1 mM DTT.

(1) High-Performance Liquid Chromatography: A Waters Associates (Milford, MA, U.S.A.) HPLC system, equipped with two solvent delivery pumps (Models M-6000A and M-45), a Model 660 solvent programmer, Model U6K sample injector, Model 440 absorbance detector, and a Houston Instruments (Austin, TX, U.S.A.) Omniscribe chart recorder was used in the assay procedure. A single 25 cm x 3.9 mm Waters μBondapak C_{18} column was placed on-line with the solvent delivery system at a flow rate of 1.2 ml/min. An isocratic elution system (Figure 3), involving the M-45 pump, was employed in this competition study. The column was equilibrated with a 50 mM ammonium phosphate buffer, pH 6. Samples (10 μl) from solutions containing the two enzymatic assay components were injected with a Hamilton (Reno, NV, U.S.A.) 801 microliter syringe. Nucleotides and bases in the eluant were detected at 254 nm with a 0.02 absorbance setting. All of the solvents used in the chromatographic procedures were clarified by vacuum filtration through a 0.45 μm HA Millipore filter.

(2) Enzymatic assay procedures: The initial velocities of the HGPRTase- and OPRTase-catalyzed reactions, measured together and separately, with HPLC, were quantitated from interpolation of linear plots of the reactant peak height vs concentration

Figure 3. Separation of four components in a simultaneous two enzymatic reaction system of HGPRTase and OPRTase. Peaks: A = orotidine monophosphate; B = orotic acid; C = inosine monophosphate; D = hypoxanthine.

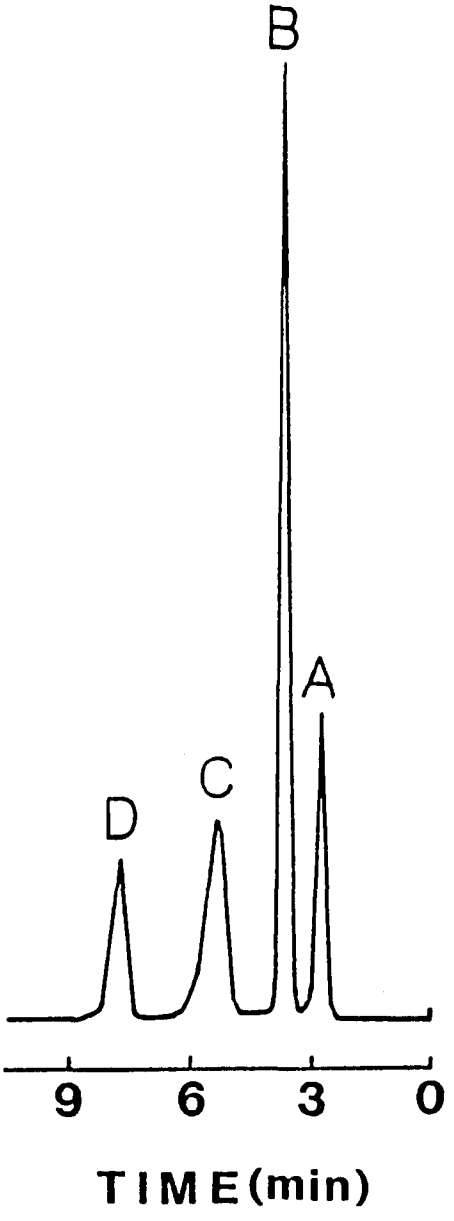
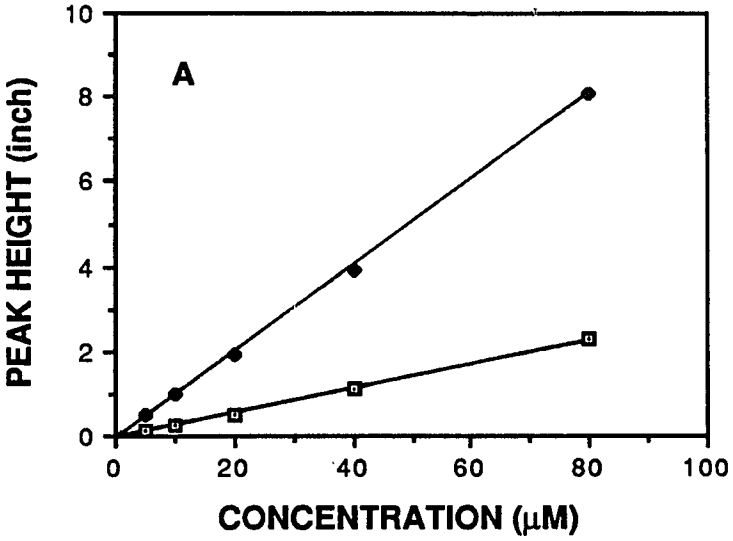
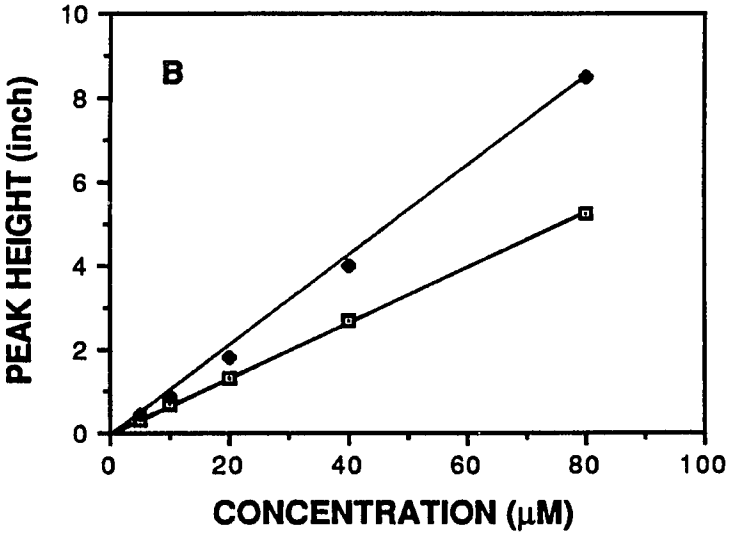

B
A
C
D
9
6
3
0
TIME(min)

Figure 4. Plots of peak height (inch) vs concentrations (μM) of the four components in this enzymatic reaction. Panel A depicts OMP (closed squares) and orotic acid (open squares). Panel B depicts IMP (closed squares) and hypoxanthine (open squares).


A
PEAK HEIGHT (inch)
CONCENTRATION (μM)
0
2
4
6
8
10
0
20
40
60
80
100


B
PEAK HEIGHT (inch)
CONCENTRATION (μM)
0
2
4
6
8
10
0
20
40
60
80
100

(Figure 4). The complete assay mixture consisted of 1.0 ml of 24 mM triethanolamine buffer (pH 8), containing 12 mM $MgCl_2$, 0.1 ml each of the appropriate concentrations of orotate and hypoxanthine, and of 10 µl each of OPRTase and HGPRTase in a final volume of 1.22 ml. The mixture was placed in a 37°C water bath, and a 0.2 ml aliquot was taken from the mixture at time zero (control). The reaction was then initiated by the addition of 10 µl of PRibPP. Aliquots of this solution were removed at appropriate time intervals and heated in a boiling water bath for 1 min to terminate the reaction. Each sample was filtered through a 0.45 µm HA Millipore filter prior to the HPLC injection. The HPLC elution profiles were utilized to determine the time course of the reaction as described previously (Ali et al., 1982; Hanna et al., 1980). Variations on this basic assay mixture were also employed during, which appropriate bases and enzymes were either excluded or included. These changes are described in the appropriate figure legends.

(3) Determination of total activities of OPRTase and HGPRTase in yeast cell extract: 1 lb Baker's yeast was suspended in 450 ml of 0.3 M potassium phosphate buffer, pH 8, and 50 ml of toluene was added to this suspension. Periodically the pH adjustment to 8.0 was carried out with 5N KOH and this autolysis procedure has been continued at 30°C water bath until no change of pH was observed. The yeast cell debris was removed by centrifugation at 10,000 rpm for 30 min, and a clarified supernatant was used for enzyme assays (there was no significant difference in a total activity of individual enzyme when undialyzed and dialyzed supernatants were compared). Saturating concentrations of PRibPP (1 mM final) and bases (300 µM final), and 10 µl of aliquot of supernatant were employed in the enzyme assays.

Experimental schemes for this competition study were summarized in a Table 1.

TABLE 1

Experimental scheme for the competition study between OPRTase and HGPRTase activities

Competition	[OPRTase]/[HGPRTase]	[PRibPP]	[Bases]
1	equivalent mass concentration (1 μg)	100 μM	80 μM
2	equivalent activity*	10 - 500 μM	300 μM
3	in-vivo activity ratio (OPRT/HGPRT = 1.6)**	20 - 100 μM	20 - 160 μM

* The unit of these enzyme activities are defined as the μmoles OMP formed per min and the μmoles IMP formed per min, respectively.

** The total activities (units/ml) of OPRTase and HGPRTase in yeast cell extracts were 740 and 450 units/ml, respectively (1.6 to 1 ratio)

OROTALDEHYDE STUDY

(1) Synthesis of Orotaldehyde (6-formyluracil): It has been observed previously that orotaldehyde can be easily prepared by a direct oxidation of 6-methyl uracil with selenium dioxide in refluxing acetic acid in good yield (Donleavy et al., 1943). A second procedure was employed in our laboratory (Johnson et al., 1931), which is more tedious than the direct oxidation procedure outlined below.

Initially, 31.5 g of 6-methyluracil (Aldrich chemical, Lot # 05915LJ) and 33.3 g of selenium dioxide (Aldrich chemical, Lot # 15F3443) were dissolved in 800 ml of glacial acetic acid, and refluxed for 6 hours at 55°C with mechanical stirring. During this time, the white suspension of selenium dioxide was gradually replaced by the dark green selenium. The hot reaction mixture was filtered through Whatman No. 1 filter paper and the selenium cake was extracted with 250 ml of boiling acetic acid. The combined yellow filtrate and extract were evaporated to dryness using a vacuum evaporator, giving 31 g of a yellow solid.

The above yellow solid gave positive 2,4-dinitrophenylhydrazone (DNP) test, which can be used for aldehyde/ketone identification purposes. 2,4-dinitrophenylhydrazine reagent was prepared, and one drop of sample (dissolved in a minimum amount of ethanol) was added to this reagent, following a procedure described in Organic Structure Determination by J. Pasto and C.R. Johnson (1969). When one drop of material was added, a precipitate formed on top of the DNP solution, and (once it was shaken) a yellow to orange-red precipitate formed, which is considered to be a positive test.

The crude orotaldehyde, which still contained some selenium and excess selenium dioxide, was purified as follows. The yellow solid material was dissolved in

350 ml of warm water and undissolved solid was removed by filtration. To the clarified solution (150 ml) 5 ml of a saturated solution of sodium bisulfate (30 g of sodium bisulfate in 60 ml of water) was added, and boiled with 10 g of active charcoal and celite for 10 min. While this suspension was hot, it was filtered and the filtrate was acidified with concentrated hydrochloric acid to pH 1. The above charchol/celite cake was extracted with a minimum amount of hot water, and the pH of the filtrate was adjusted to 1. Upon cooling at room temperature, partially pure orotaldehyde was collected. An analytical sample, which was to be used in the kinetic studies, was prepared by recrystallization from water (100 mg of sample was dissolved in 4 ml of warm water and cooled in refrigerator). The resulting off-white solid was dried completely in P_2O_5-vacuum desiccator.

(2) Determination of Purity of synthesized orotaldehyde: A Waters μBondapak C_{18} reverse-phase HPLC was employed to compare purities between unrecrystallized and recrystallized samples under following chromatographic conditions; 5% MeOH as mobile phase, 0.6 ml/min flow rate, and 0.5 AUFS. Thereafter, recrystallized sample (10 mg) was dissolved in 1 ml of warm water and freeze-dried with a lyophilizer, and the dehydrated sample was then reconstituted in 1 ml of D_2O for the NMR experiment. The melting point of sample was also examined.

(3) Inactivation Studies with Orotaldehyde: To 15 μl of OPRTase was added 285 μl of 50 mM POPSO (piperazine-N,N'-bis[2-hydroxypropanesulfonic acid]) buffer, pH 8, in the presence and absence of orotaldehyde. The incubations were allowed to proceed at 25 °C and 25 μl aliquots were removed at 1, 3, 6, 10, 20, 30, 45, 60, and 90 min. The enzyme activities were measured spectrophotometrically in an assay mixture consisting of 150 μM PRibPP, 100 μM orotate, 1 mM $MgCl_2$, and 50

mM potassium phosphate buffer at pH 8 in a final volume of 1 ml. In this study 1, 2, 3, 5, and 8 mM final concentrations of orotaldehyde were employed.

(4) Flow Dialysis Experiments: To 25 µl of enzyme were added 475 µl of 50 mM POPSO buffer, pH 8, containing 4 mM final concentration of orotaldehyde. The incubations were allowed to proceed for 45 min (during this time, enzyme activities were measured at 1, 5, 15, 30, 45 min). Thereafter, a 200 µl volume of the enzyme solution containing orotaldehyde was placed in the upper chamber of the flow dialysis unit and 50 mM POPSO buffer, pH 8, was passed through the lower chamber which was separated from the upper chamber by a dialysis membrane. Aliquots of 25 µl were taken and assayed at 6, 12, 18, 55, and 75 min.

(5) Protection Studies: The effects of $MgCl_2$, orotate, PRibPP, Mg-orotate, or Mg-PRibPP on OPRTase inactivation by orotaldehyde (4 mM final concentration) was assayed. To 5 µl of enzyme was added 95 µl of 50 mM POPSO buffer, pH 8, in the presence and absence of orotaldehyde and the above stated ligands. The reaction was allowed to proceed for 60 min. In all cases, after a 45 min of incubation, equilibrium had been reached. The residual activities of OPRTase was measured spectroscopically after 60 min, according to the assay method described in an earlier section.

(6) pH-dependent Studies: The inactivation of OPRTase (0.5 mg/ml) by orotaldehyde was studied in potassium phosphate buffers (0.05 ionic strength) at several constant pH values (6.1, 7.0, 7.5, 7.9, 8.5, and 9.0). A 10 mM orotaldehyde solution was mixed with an equal volume of 100 mM potassium phosphate buffer (at the desired pH value) containing the enzyme, to give an orotaldehyde concentration of 5 mM and final concentration of the 50 mM for phosphate buffer. Aliquots of 25 µl were removed at 1, 4, 7, 10, 20, 30, 45, and 60 min and assayed for residual activity. If it is assumed that the reactive side chain amino group of the lysine residues of the

enzyme (E) react with orotaldehyde (A) to yield the corresponding Schiff base (EA) with no significant concentration of the intermediate carbinolamine present, the equilibrium may be expressed as:

$$E + A \underset{k\text{-}o}{\overset{ko}{\rightleftharpoons}} EA \qquad (5)$$

At any pH, then, K_{pH} = [EA]/[E][A]. If the inactivation of enzyme is studied at a concentration of orotaldehyde in great excess over enzyme, the concentrations of E and EA may be calculated from a knowledge of total enzyme concentration (E_{total}), the enzyme activity at the inception of the inactivation reaction (Act_o), and the residual activity after completion of reaction (Act_∞). Thus, K_{pH} may be calculated as:

$$K_{pH} = \frac{[E_{total}] - [E_{total}]Act_\infty/Act_o}{[E_{total}]Act_\infty/Act_o[A]} \qquad (6)$$

(7) Inhibitor Studies: In the first experiment, OPRTase, PRibPP (25 - 200 μM), and orotaldehyde were incubated in the dark for 15 min, and the reaction was initiated by addition of 100 μl of 3 mM orotate (300 μM final concentration). Next, with a series of orotate concentrations (25 - 300 μM), the reaction was started by addition of 50 μl of 4 mM PRibPP (200 μM final concentration). The assay mixture contained final concentrations of 25 mM POPSO buffer, pH 8, 2 mM $MgCl_2$, and appropriate concentrations of PRibPP and orotate in a 1 ml final volume.

In the second experiment, initial velocities of the OPRTase-catalyzed reaction ,using fixed concentrations of orotate or PRibPP under two different sets of conditions, were measured in the presence of orotaldehyde. The assay mixture contained same

constituents as above and the reaction was initiated by adding 10 µl of diluted OPRTase.

PYRIMIDINE BASE ANALOGS STUDY

(1) HPLC Assay: The OPRTase activity was estimated by determining the elution peak height of OMP over time, that corresponds to an OMP concentration in the standard curve (shown in Figure 4). OMP synthesis was measured through the use of reverse-phase HPLC column with cytidine monophosphate as an internal standard, and mobile phase of ammonium phosphate (50 mM, pH 6) at a flow rate of 1 ml/min. The assay mixture in the inhibition study consisted of 0.1 ml of 0.24 M Tris-HCl buffer, pH 8, containing 12 mM $MgCl_2$, 0.1 ml of orotate, 1.0 ml of orotate analogs, and 5 µl of enzyme. 0.2 ml of aliquot was taken at zero time for control and the reaction was started by adding 25 µl of 20 mM PRibPP. Each 200 µl aliquot was removed at appropriate time intervals (8, 15, 60, and 120 seconds) and the enzymatic reaction was terminated with boiling water. Prior to inject samples, 10 µl of 0.2 mM cytidine monophosphate was added to each sample as the internal standard.

(2) Spectroscopic assay: Inhibition studies with several orotate analog compounds have been initiated at room temperature using a Cary 15 spectrophotometer. The assay mixture contained 830 µl of 30 mM POPSO buffer, pH 8, containing 2.4 mM $MgCl_2$ in the absence (control) and presence of each orotate analog, 20 µl of a series of fixed concentration of orotate (25, 33, 50, 66, 100, 133, 150 µM), 50 µl of 10 mM PRibPP. A volume of 20 µl of forty times diluted stock enzyme was added to start the reaction.

PURINE BASE ANALOGS STUDY

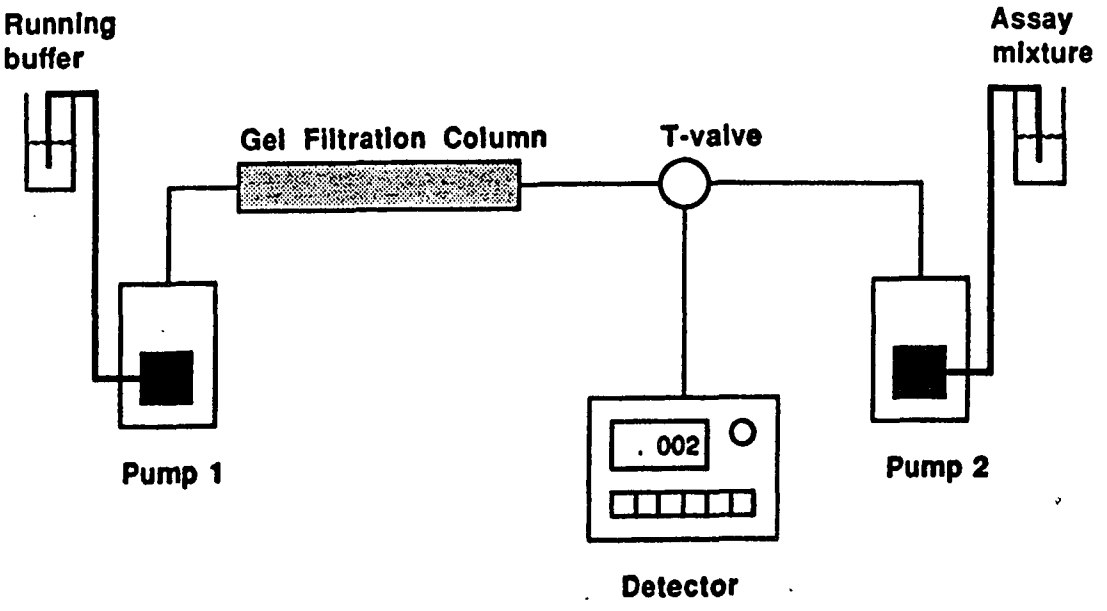
Xanthine and 5 xanthine-derivative compounds were employed in this study to determine whether any of these compounds was an alternate substrate or inhibitor of yeast HGPRTase. 400 µl of total assay volume contained 130 µl of 24 mM Tris-HCl buffer, pH 7.4, containing 6 mM $MgCl_2$, 50 µl of hypoxanthine or guanine, and 20 µl of 10 mM PRibPP (500 µM final concentration). At time zero, a 100 µl aliquot was removed and the reaction was initiated by the addition of 10 µl of ten times diluted HGPRTase.

RELATIONSHIP BETWEEN HGPRTASE SUBUNIT AND ACTIVITY

A Waters HPLC instrument equipped with the same accessories described previously was employed in this study. All samples were dissolved in buffers made up with high-conductivity water, which had been processed with a Gelman "Water-1" apparatus. Samples were injected onto the HPLC column with a Hamilton 801 microliter syringe, subsequent to their filtration through a 0.45 mm HA Millipore filter.

A schematic representation of chromatographic system is shown in Figure 5. For studies involving non-reacting gel filtration, 50 µl aliquots of the appropriate diluted HGPRTase were placed onto a TSK-250 molecular sieve column, flowing at a rate of 1 ml/min at room temperature. The elution buffers for these experiments were 100 mM TEA, pH 6.8, alone and TEA containing either 1 mM Mg(II) or 100 µM PRibPP and 1 mM Mg(II), for appropriate experiments. A T-connector valve was employed to mix the eluant from the column with HGPRTase assay mixture (500 µM PRibPP, 200 µM hypoxanthine, 5 mM Mg(II), 50 mM potassium phosphate, pH 7.4), which was placed on-line with the elution material at a flow rate of 0.1 ml/min with a second pump. The resulting mixed solution was fed through a reaction line consisting

Figure 5. Schematic representation of high-performance liquid chromatographic system. The flow rates of Pump 1 and 2 were set at 1.0 and 0.1 ml/min, respectively in this study.


Running buffer
Assay mixture
Gel Filtration Column
T-valve
. 002
Pump 1
Pump 2
Detector

of 15 feet of 1/16 inch tubing, leading to the 440 detector set at 254 nm. Since the assay procedure is essentially that described by Hill (1970), the appearance of IMP (maximal absorbance at 257 nm) can easily be monitored, and with this detection the active form of HGPRTase can be located and characterized. Apparent molecular weights were determined from an interpolation of linear plots of natural log molecular weight vs elution time for the marker proteins: bovine serum albumin, egg albumin, beta-lactalbumin, trypsinogen, and ribonuclease A.

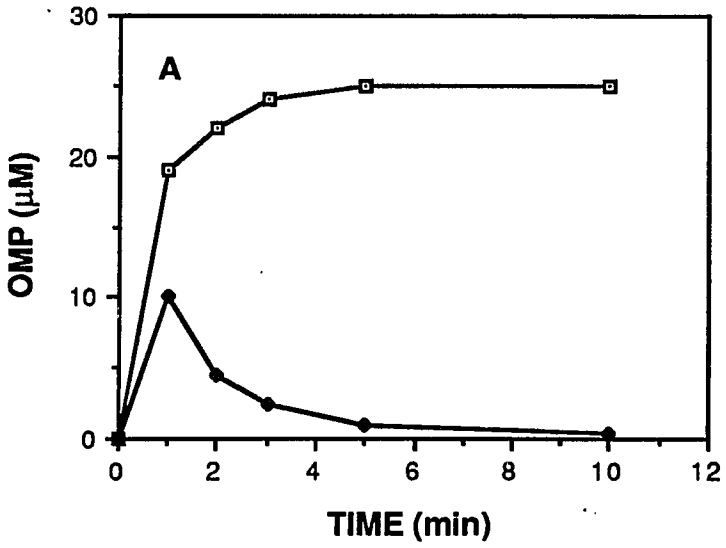
RESULTS

COMPETITION BETWEEN OPRTASE AND HGPRTASE FOR A COMMON SUBSTRATE

(1) Competition #1: First of all, we employed equivalent mass concentrations (1 μg) of OPRTase and HGPRTase to examine the competition between these two enzymes for 100 μM PRibPP. The HGPRTase-catalyzed reaction was expected to dominate this competition, since the specific activity of HGPRTase is approximately 10 times higher than that of OPRTase (1000 units/mg and 120 units/mg for HGPRTase and OPRTase, respectively). As shown in Figure 6A and under this condition, the initial rate of OMP synthesis and the equilibrium reactant concentrations were greatly affected by the presence of the HGPRTase activity. The concentration of OMP initially produced in this reaction decreases almost immediately after the reaction was initiated and continues to disappear until a new, highly reduced equilibrium concentration is reached. In contrast, the presence of the OPRTase activity had no discernible effect on the HGPRTase-catalyzed reaction under the same conditions (Figure 6B). However, we had not ruled out the possibility that certain components of the HGPRTase-catalyzed reaction might affect the OPRTase activity. Therefore, we examined the effects of the presence of either HGPRTase itself, hypoxanthine, or IMP on the OPRTase-catalyzed reaction profile over a 10 min incubation period. As shown in TABLE 2, there is no significance difference in the OPRTase activities, when the four different sets of experiments are compared. Thereafter, we designed detailed competition studies more favourable to the OPRTase activity.

(2) Competition #2: In this experiment, equivalent activities of both enzymes were employed. While each base concentration (orotate and hypoxanthine) was fixed to a saturating concentration (300 μM), the concentration of a common substrate,

Figure 6. Appearance of the nucleotides OMP and IMP using equivalent mass concentrations (1 μg for each enzyme) of OPRTase and HGPRTase. The reaction mixture contained: 24 mM triethanolamine (pH 8), 12 mM magnesium chloride, 80 μM orotic acid and/or 80 μM hypoxanthine, 100 μM PRibPP, and appropriate amounts of OPRTase and/or HGPRTase in a final volume of 1.22 ml. (A) OMP formation in the presence (closed square) and absence (open square) of the complete HGPRTase assay mixture. (B) IMP formation in the presence (closed square) and absence (open square) of the complete OPRTase assay mixture.


A
OMP (μM)
30
20
10
0
0
2
4
6
8
10
12
TIME (min)

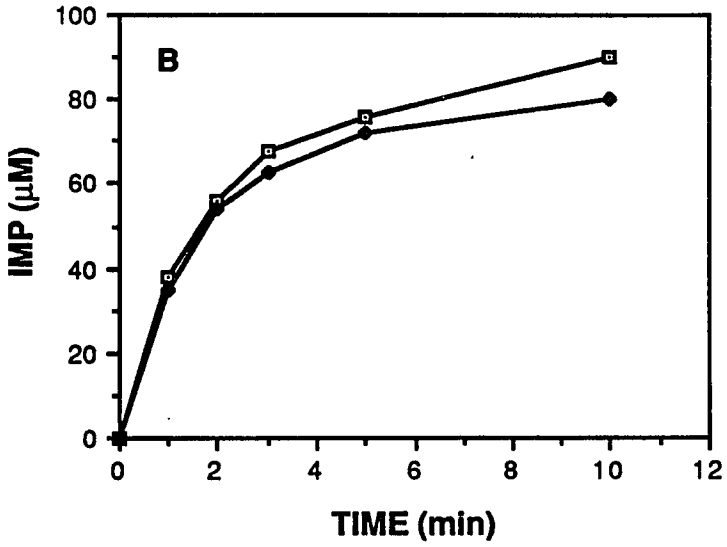

B
IMP (μM)
100
80
60
40
20
0
0
2
4
6
8
10
12
TIME (min)

TABLE 2

Effects of the presence of either HGPRTase, hypoxanthine, or IMP on OPRTase-catalyzed reaction profile over a 10 min incubation period

The PRibPP concentration in all experiments is 100 μM.

Experiment	OMP (μM) formed				
	Incubation time (min)				
	0.5	1.0	2.0	5.0	10
80 μM orotate OPRTase	14	19	30	45	55
80 μM orotate OPRTase HGPRTase	15	20	28	41	55
80 μM orotate OPRTase 80 μM hypoxanthine	14	19	27	41	50
80 μM orotate OPRTase 84 μM IMP	14	19	32	45	58

PRibPP, was varied from 10 to 500 μM. As shown in Figure 7, during the 10 min incubation period, the equilibria of OPRTase-catalyzed reaction were significantly affected by the presence of HGPRTase assay components. Initial velocities were also greatly reduced when the PRibPP concentration was placed below 50 μM (Figure 7D-F). HGPRTase-catalyzed reaction profiles in this competition study were also determined and are shown in Figure 8, in which initial velocities of HGPRTase-catalyzed reaction, in the presence of OPRTase assay components, appeared to be slightly reduced at below 25 μM of PRibPP concentration (Figure 8E and F). Depending upon these enzymatic reactions data (Figure 7 and 8), double-reciprocal plots of initial velocities vs PRibPP were constructed (Figure 9). As shown in Figure 9A, the plot of OPRTase-catalyzed reaction in the presence of HGPRTase activity appeared to be biphasic, whereas the plot of HGPRTase-catalyzed reaction in the absence and presence of OPRTase activity showed a competitive pattern (Figure 9B).

(3) Competition #3: In an attempt to recreate more closely the in vivo conditions under which these reactions might occur, we determined the total activities (units/ml) of OPRTase and HGPRTase in a yeast cell extract and found them to be 740 units/ml and 450 units/ml, respectively. These relative activities were then employed in an in vitro reaction competition analysis. In Figure 10 is illustrated one of the elution profiles of simultaneous enzymatic reactions in this experiment. The elution profile was defined initially by two nitrogenous bases and we were then able to monitor concentration changes in four reaction components simultaneously during the 10 min incubation period. As shown in Figure 11, the initial velocities of OMP synthesis and the equilibrium states were apparently affected by the presence of HGPRTase activity, especially at the lowest (20 μM) concentration of PRibPP. Meanwhile, the HGPRTase activity shown in Figure 12 was only slightly affected by the presence of the highest

Figure 7. OMP synthesis in the presence (dotted line) and absence (solid line) of the HGPRTase assay mixture. Equivalent enzymatic activities for both enzymes were employed. Panel A through F were differentiated by six employed concentrations of PRibPP in this experiment: 500, 200, 100, 50, 25 and 10 μM respectively. The assay conditions and incubation mixture are as described in under Methods.

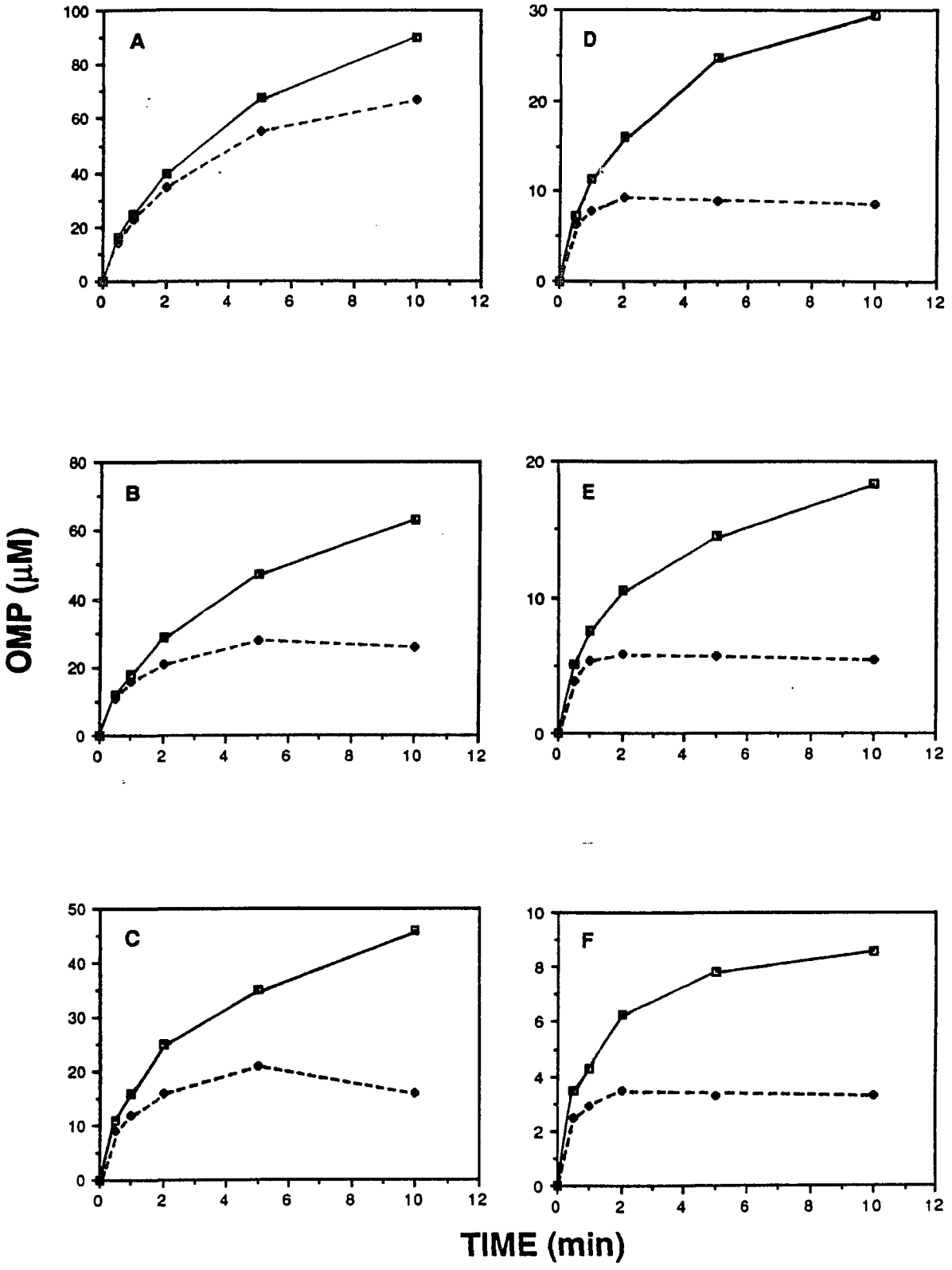

A
B
C
D
E
F
OMP (μM)
TIME (min)

Figure 8. IMP synthesis in the presence (dotted line) and absence (solid line) of the OPRTase assay mixture. Other experimental conditions are the same as defined in Figure 7.

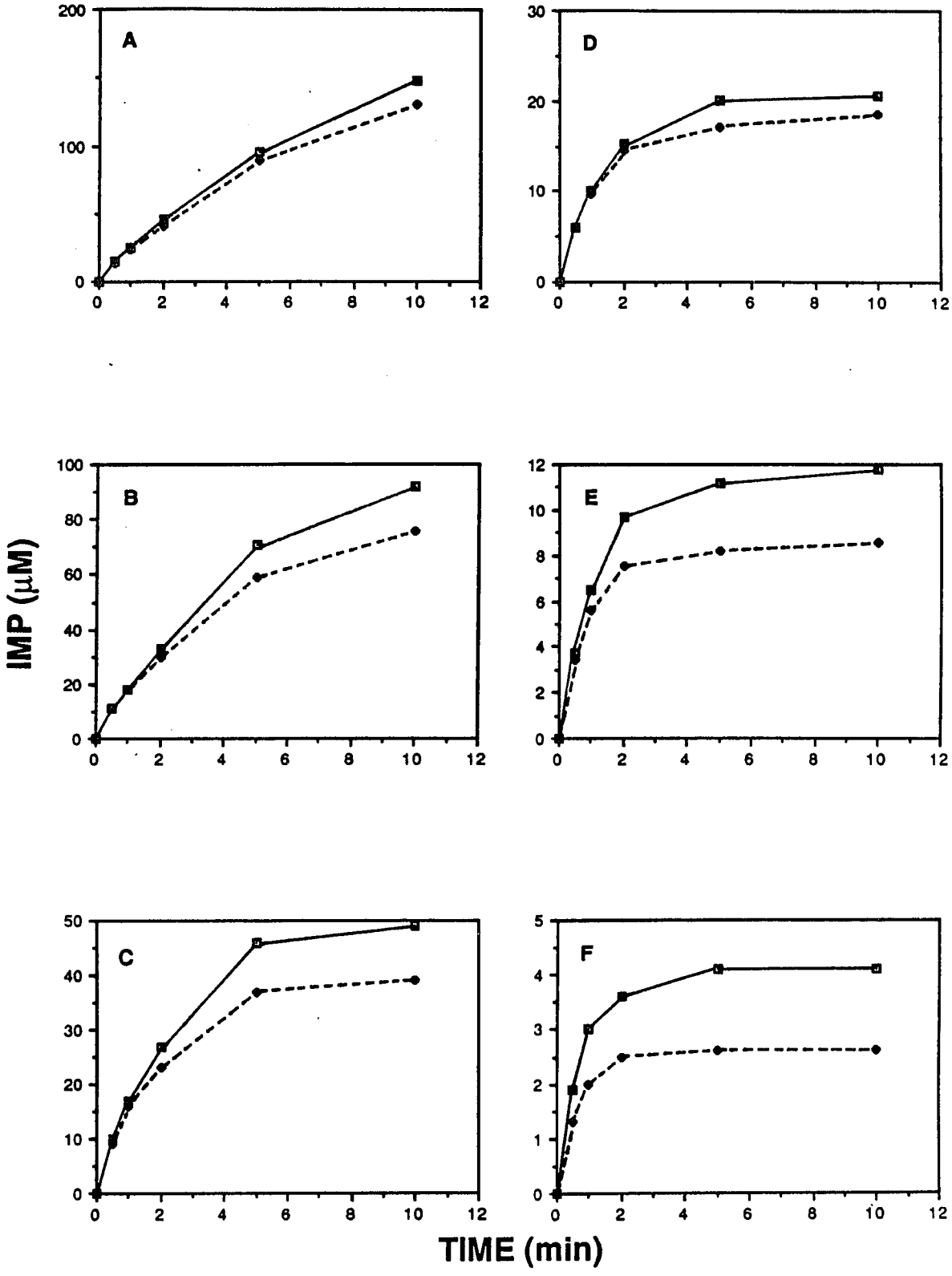
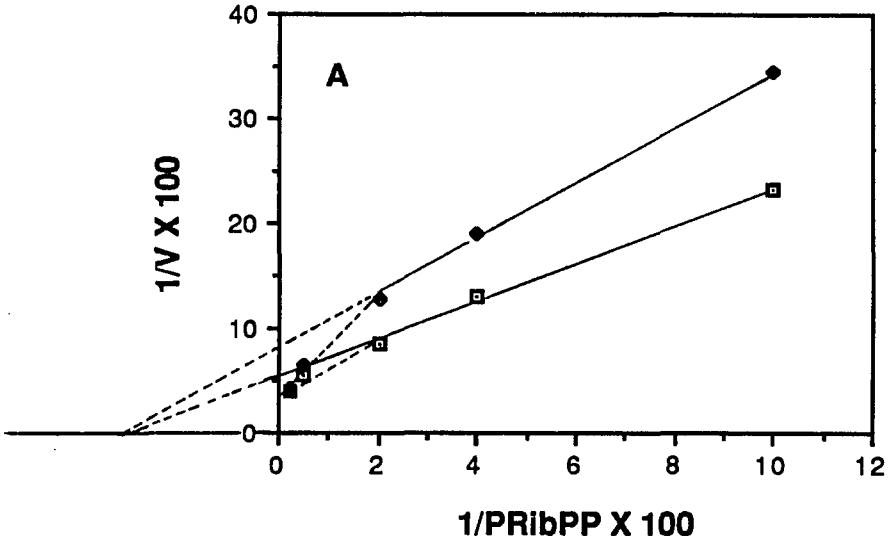

A
B
C
D
E
F
IMP (μM)
TIME (min)

Figure 9. Double reciprocal plots from the competition #2 experiment. Panel A represents the 1/OMP formation vs 1/PRibPP plot in the absence (open squares) and presence (closed squares) of the HGPRTase activity. Panel B represents the 1/IMP formation vs 1/PRibPP plot in the absence (open squares) and presence (closed squares) of the OPRTase activity.


A
1/V X 100
1/PRibPP X 100
0
10
20
30
40
0
2
4
6
8
10
12

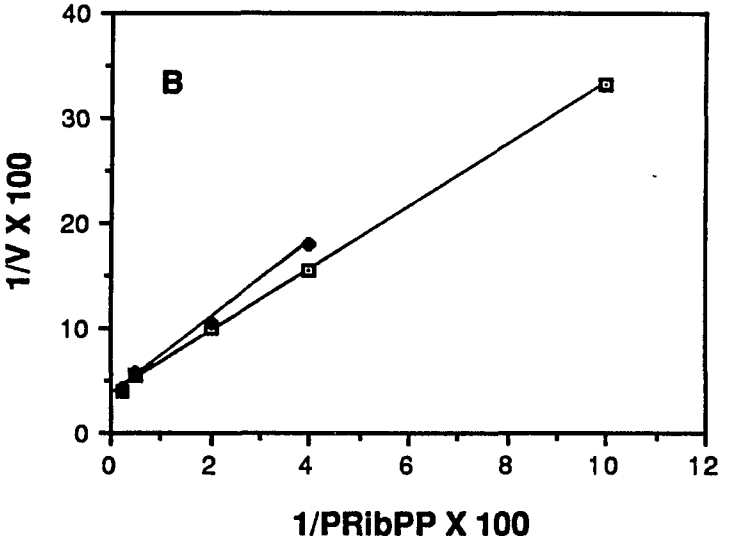

B
1/V X 100
1/PRibPP X 100
0
10
20
30
40
0
2
4
6
8
10
12

Figure 10. A typical HPLC elution profile of the enzyme incubation mixture that illustrates the simultaneous detection of the OPRTase- and HGPRTase-catalyzed reactions over a 10 min period. The incubation solution contained: 24 mM triethanolamine (pH 8), 12 mM $MgCl_2$, 20 μM orotic acid, 80 μM hypoxanthine, 40 μM PRibPP, and appropriate amounts of OPRTase and HGPRTase in a final volume of 1.22 ml.

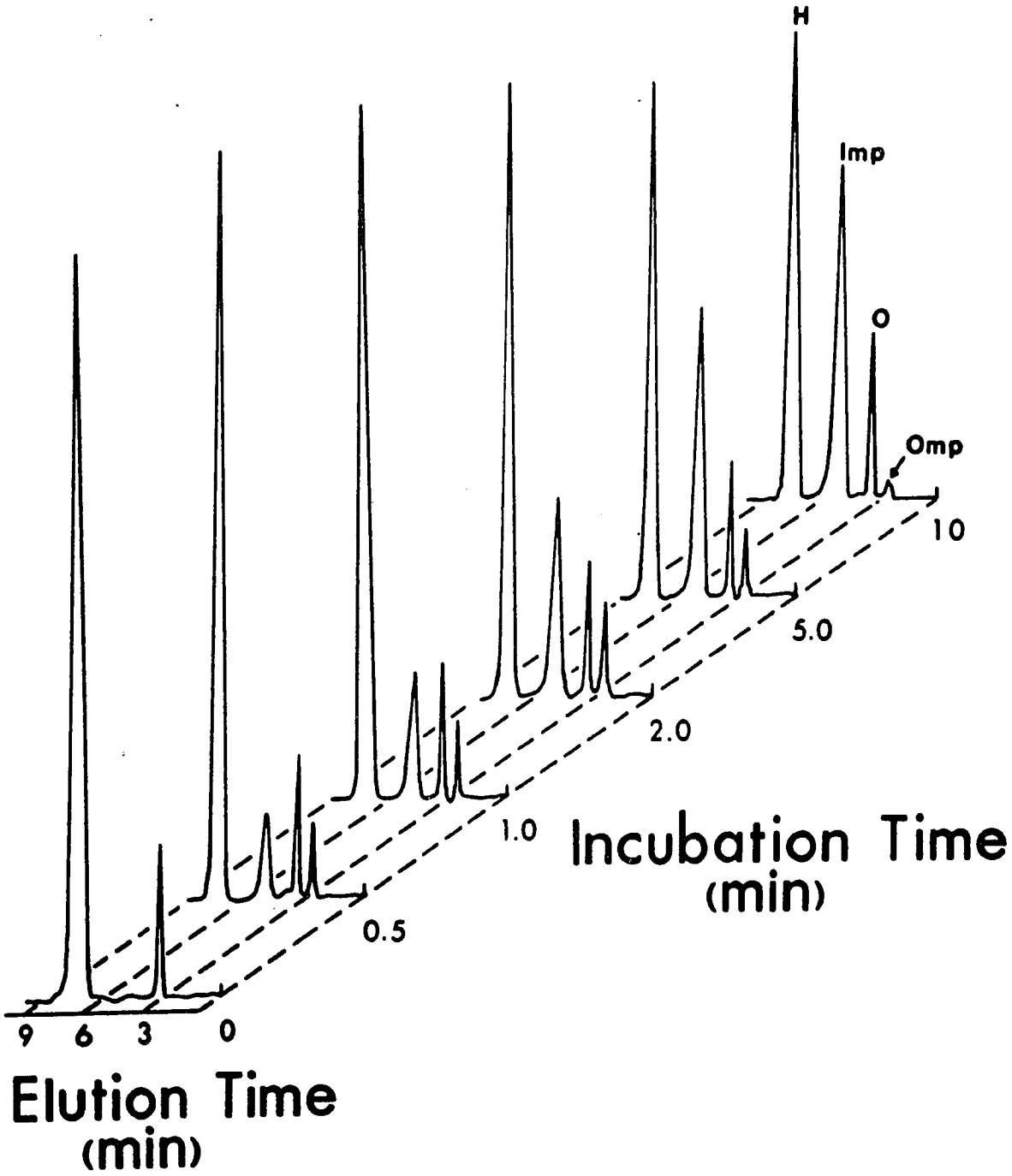

H
Imp
O
Omp
10
5.0
2.0
1.0
0.5
0
Incubation Time
(min)
9
6
3
0
Elution Time
(min)

Figure 11. OMP synthesis in the presence (dotted line) and absence (solid line) of the complete HGPRTase assay mixture. The assay conditions and incubation mixture are as described in under Methods and in TABLE 3, respectively, except that, among the three dotted lines, the bottom line (▲---▲) represents OMP appearance in the presence of 160 μM hypoxanthine, the middle line (×---×) represents its appearance in the presence of 80 μM hypoxanthine, and the top line (●---●) represents its appearance in the presence of 20 μM hypoxanthine. Panel A through I were differentiated by three employed concentrations of PRibPP (100, 40, and 20 μM) and orotate (160, 80, and 20 μM) as following: Panel A through C for three PRibPP concentrations with 160 μM orotate, Panel D through F with 80 μM orotate, and Panel G through I with 20 μM orotate.

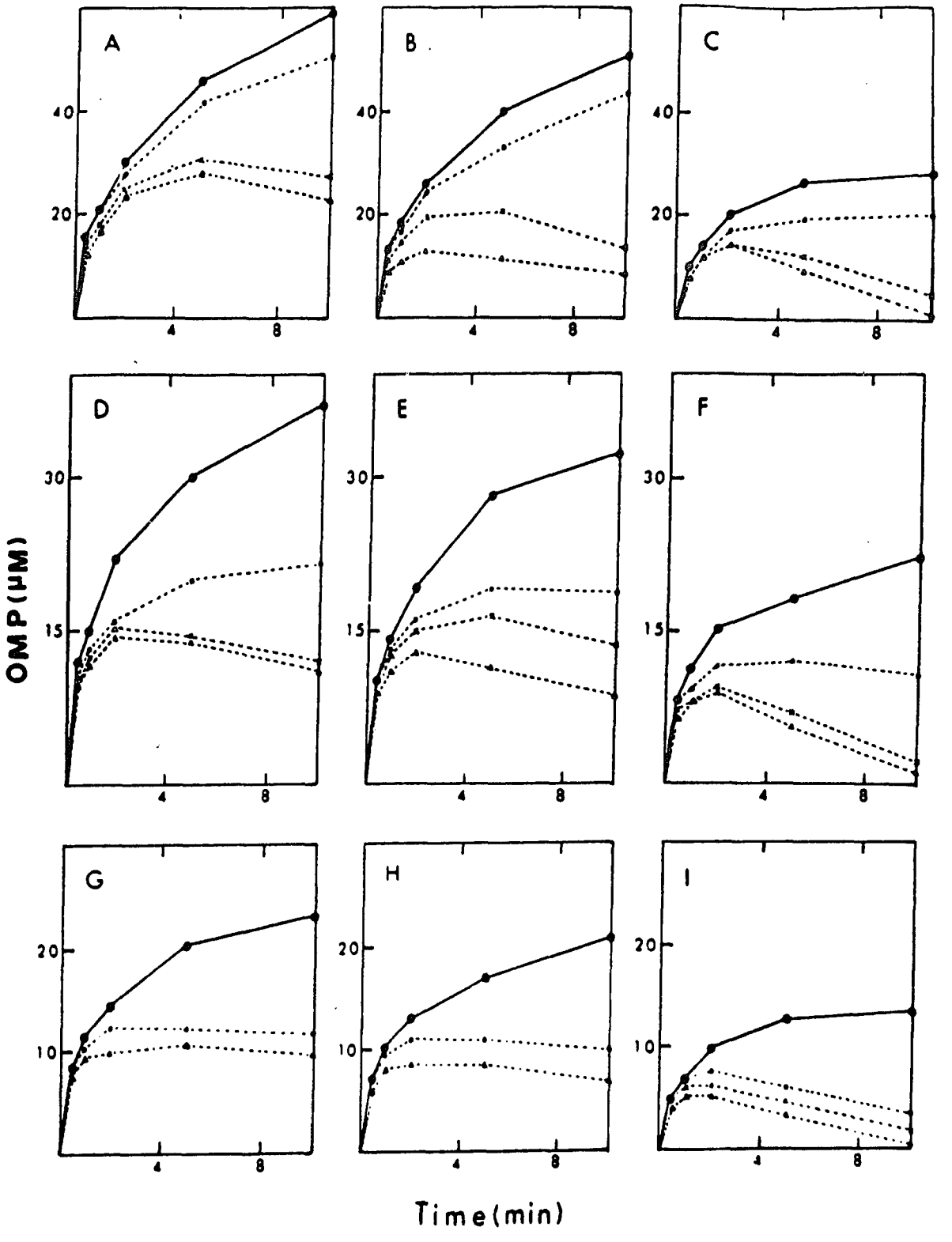
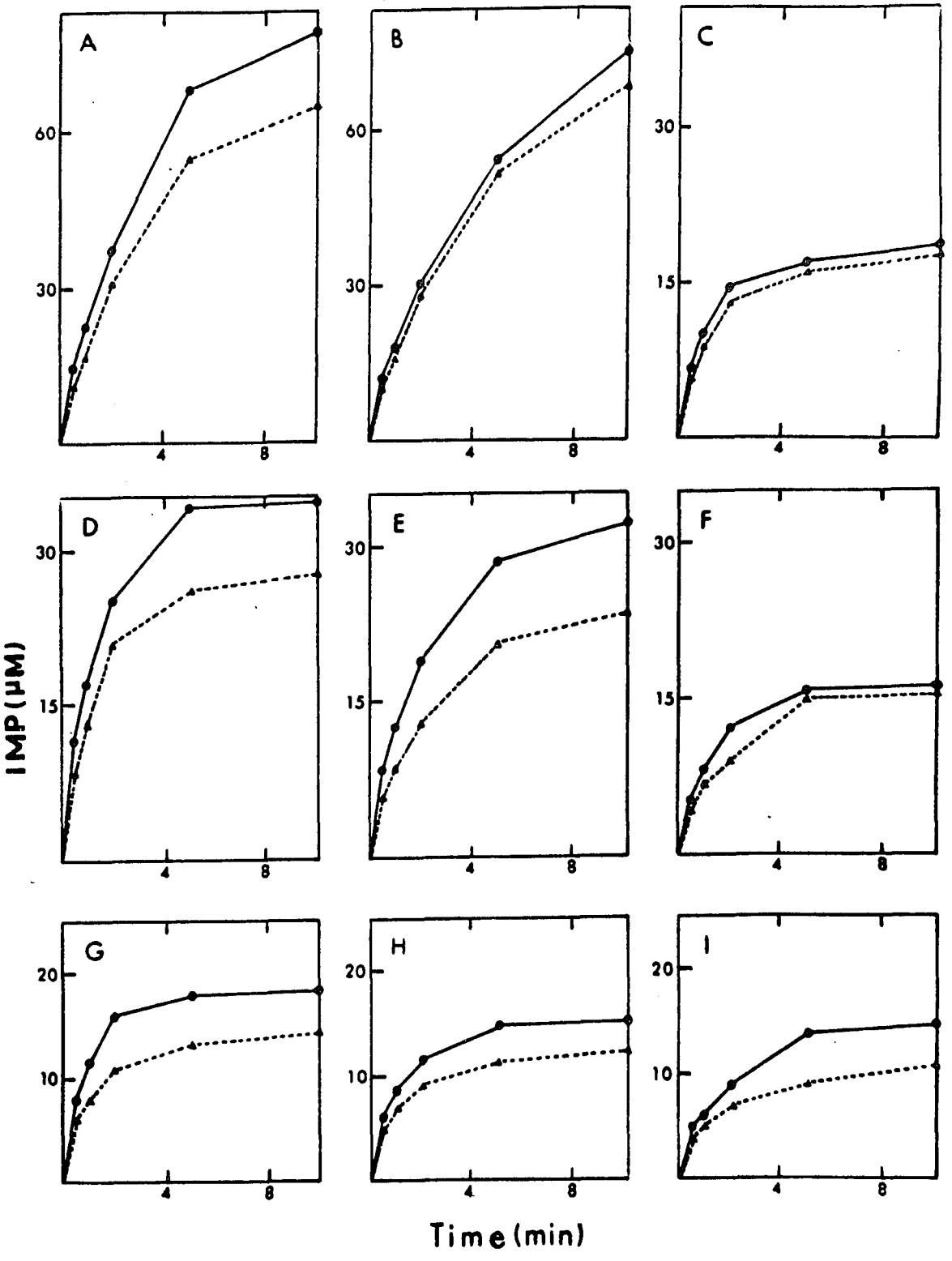

A
B
C
D
E
F
G
H
I
40
20
30
15
10
4
8
OMP(μM)
Time(min)

Figure 12. IMP synthesis in the presence (dotted line) and absence (solid line) of the complete OPRTase assay mixture. The assay conditions and incubation mixture are as described in under Methods and in TABLE 3, respectively, except that the dotted line represents IMP appearance in the presence of 160 μM orotate. Other experimental conditions are the same as defined in Figure 11.


A
B
C
D
E
F
G
H
I
60
30
15
20
10
4
8
IMP (μM)
Time (min)

(160 μM) concentrations of orotate and OPRTase. Next, we measured the initial velocities (v) of each enzymatic reaction to clarify further the effects of one enzyme reaction on the other. Lineweaver-Burk plots were constructed based on the data of this study because the HGPRTase and OPRTase kinetic mechanism can be distinguished by this graphical analysis, and because inhibition patterns such as a competitive, noncompetitive, uncompetitive, or mixed type are readily distinguished. The values of initial velocities of each enzyme-catalyzed reaction and in the presence of the other enzyme activity are shown in TABLE 3, and double reciprocal plots (Figure 13) were drawn by using experimental data presented in TABLE 3. As shown in Figure 13, the parallel lines that were observed for the OPRTase-catalyzed 1/v vs 1/PRibPP plot (Figure 13A), at several fixed concentrations of orotate in the absence of the HGPRTase activity, were no longer observed when the HGPRTase activity was present. In the presence of HGPRTase activity, a series of intersecting lines were characterized (Figure 13B), with the value of the x-intercept decreasing with increasing hypoxanthine concentration. In contrast, the 1/v vs 1/PRibPP plot for the HGPRTase-catalyzed reaction for a series of fixed concentrations of hypoxanthine, is composed of a series of intersecting lines (Figure 13C), and the point of intersection appears to change in the presence of the OPRTase activity (Figure 13D). As expected, the equilibrium concentrations of all the reactants were altered by the presence of both enzymatic activities, but because the equilibrium constant for the formation of OMP (eqn.1) is less than 1 (favouring the reverse pyrophosphorolysis of OMP), the concentration of OMP is reduced most dramatically.

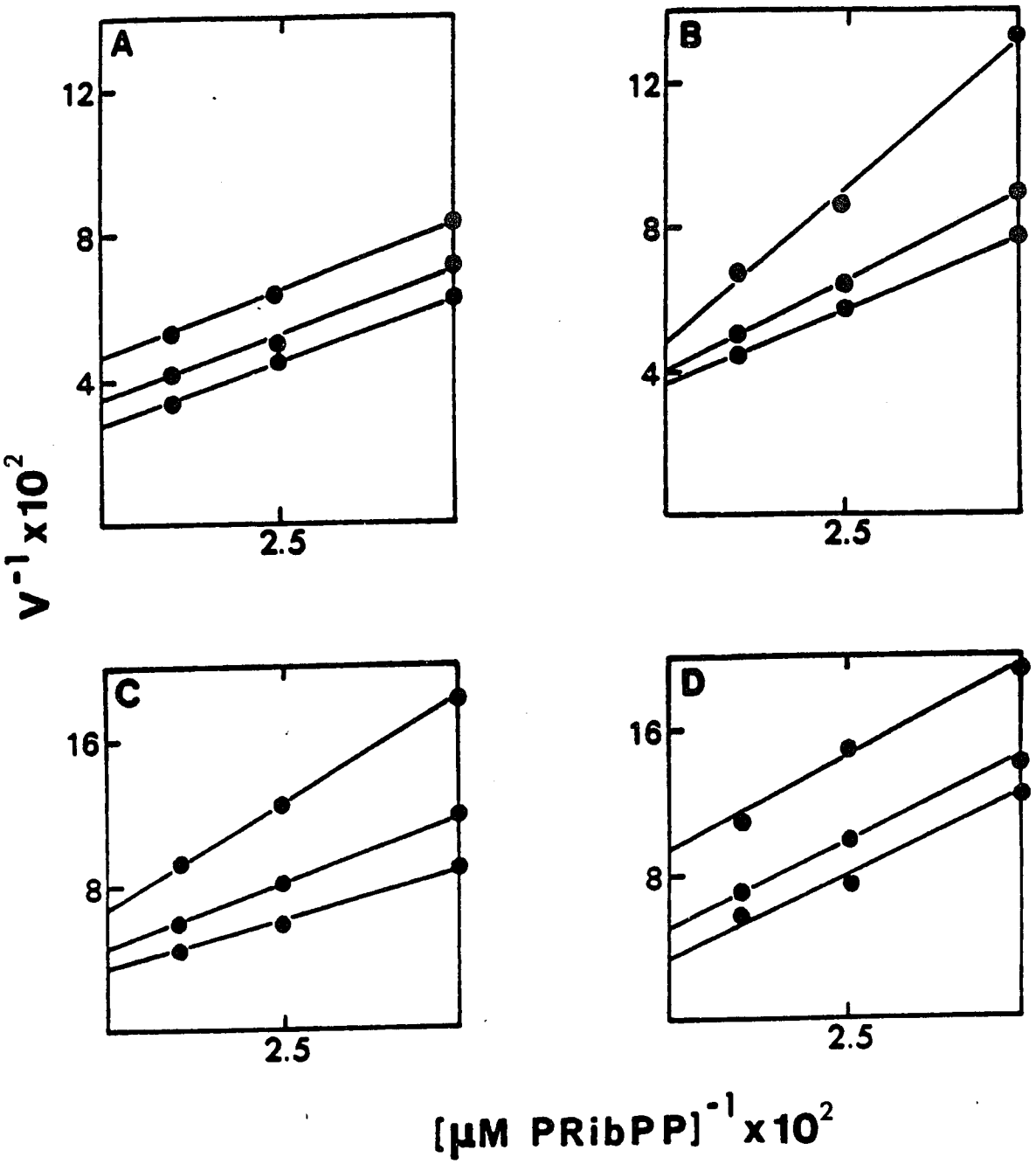
TABLE 3

Effects of the presence of HGPRTase and OPRTase assay components on OPRTase and HGPRTase activities, respectively

Experiment	PRibPP concentration (μM)					
	100	40	20	100	40	20
	OPRTase initial velocity (μmoles OMP/min)			HGPRTase initial velocity (μmoles IMP/min)		
OPRTase						
(1) 160 μM O*	29	22	16			
(2) 80 μM O	24	20	14			
(3) 20 μM O	19	16	12			
HGPRTase						
(4) 160 μM H**				23	17	12
(5) 80 μM H				18	13	9
(6) 20 μM H				11	8	6
OPRTase/HGPRTase						
(7) 160 μM O + 160 μM H	23	17	13	17	14	8
(8) 80 μM O + 160 μM H	20	16	11	18	14	9
(9) 20 μM O + 160 μM H	15	12	8	19	13	9
OPRTase/HGPRTase						
(10)160 μM O + 160 μM H	23	17	13	17	14	8
(11)160 μM O + 80 μM H	24	17	14	16	10	7
(12)160 μM O + 20 μM H	28	19	15	9	7	5

* orotate
** hypoxanthine
Experiments 7-12 represent competition studies and they are presented as the bottom lines in Figure 11 and 12.

Figure 13. Double reciprocal plots from experimental data presented in TABLE 3. Panels A and B both represent the 1/OMP formation vs 1/PRibPP plots: (A) for experiments 1-3 in TABLE 3 and (B) for experiments 7-9. Panels C and D represent the 1/IMP formation vs 1/PRibPP plots: (C) for experiments 4-6 and (D) for experiments 10-12.


A
B
C
D
12
8
4
16
8
2.5
$V^{-1} \times 10^2$
$[\mu M\ PRibPP]^{-1} \times 10^2$

OROTALDEHYDE STUDY

Preliminary experiments revealed that newly-synthesized orotaldehyde is not a substrate of OPRTase, but modifies this enzyme instead. Orotaldehyde was synthesized by a direct oxidation method (Zee-cheng and Cheng, 1967), and was recrystallized from water. Reverse-phase HPLC column was employed to monitor any nitrogen containing contaminants which might be present in this chemical preparation. As shown in Figure 14, the elution profile was composed of three peaks. The relatively small peaks (1st and 3rd peaks) were identified as orotate and 6-methlyuracil, respectively, by comparison to standard materials on HPLC under the previously described chromatographic condition. A peak corresponding to orotaldehyde is predicted to emerge after orotate and before 6-methyluracil on reverse-phase HPLC. After recrystallization (Figure 14B), I was able to obtain a 97% pure preparation of orotaldehyde. Thereafter, more than 100 injections unto HPLC column were made and the portion of a second huge peak was collected to get a more completely purified orotaldehyde. The pooled solutions containing orotaldehyde were freeze-dried and the solid sample was then redissolved in 0.5 ml of D_2O for an NMR experiment. There are two nonexchangable protons (with deutrium) within the orotaldehyde structure; one at the 5-position and another at the carbonyl group. As shown in Figure 15, we had two distinct peaks which had approximately the same intensity, the peak at 8.9477 ppm represents the aldehyde substituent, whereas the peak at 5.8387 ppm represents the 5-ring proton. Kinetic studies has been initiated using this synthesized and purified orotaldehyde.

Incubation of yeast OPRTase with orotaldehyde resulted in a decrease of the catalytic activity to an equilibrium value, when the orotaldehyde concentration is varied from 1 to 8 mM (Figure 16). In each incubation the catalytic activity decreased to an

Figure 14. HPLC elution profiles of newly-synthesized orotaldehyde under the chromatographic conditions described in Methods. The profile A and B depict orotaldehyde before and after a recrystallization from water, respectively.

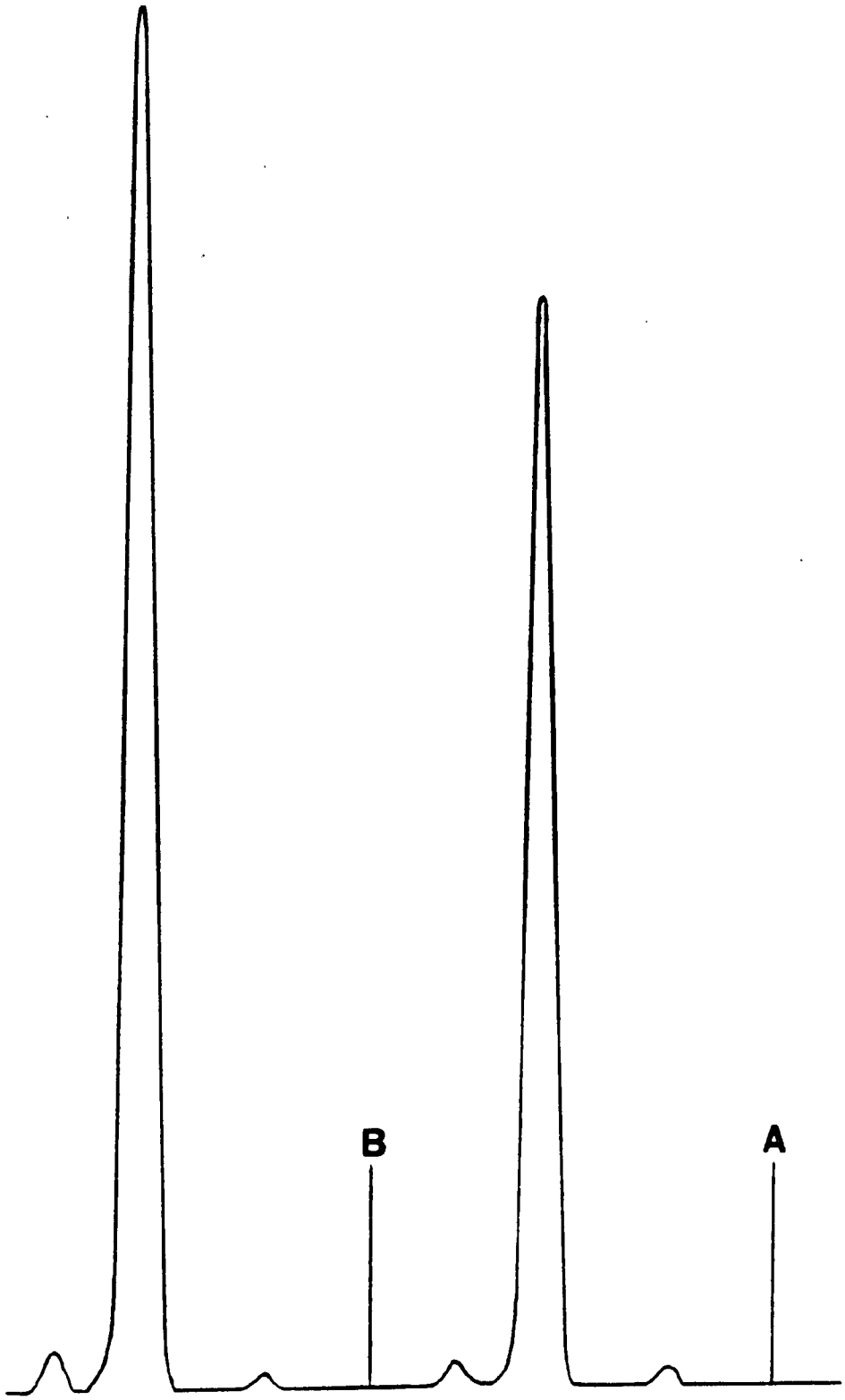
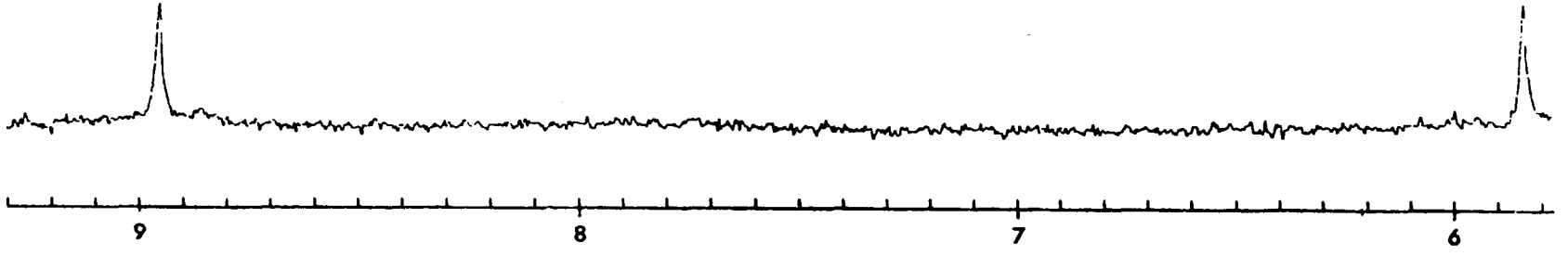

B
A

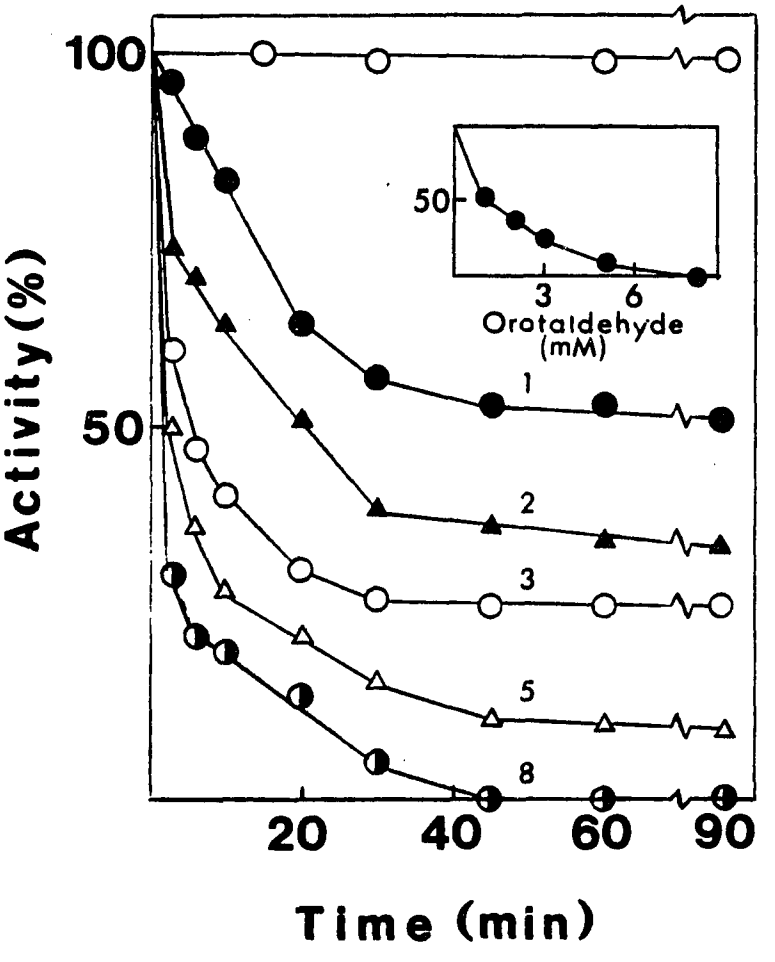
Figure 15. 300-MHZ ^1H NMR spectra of purified orotaldehyde in D_2O.

#	CURSOR	FREQ	PPM	INTEGRAL	INTENSITY
1	1031	1790.721	8.9477	.423	4.559
2	2045	1168.525	5.8387	.418	4.642



PPM FROM TMS

Figure 16. Inactivation of OPRTase (0.5 mg/ml) with various concentrations of orotaldehyde as a function of time. Represented with each line are the mM concentrations of orotaldehyde employed. The enzymatic reaction was monitored spectroscopically at 25°C in 50 mM potassium phosphate buffer (pH 8) containing 100 μM orotate, 150 μM PRibPP and 1 mM $MgCl_2$.


100
50
Activity(%)
20
40
60
90
Time (min)
1
2
3
5
8
50
3
6
Orotaldehyde
(mM)

equilibrium state within 45 - 60 min, and the plot of residual activities (being determined after reaching to equilibrium) vs orotaldehyde concentration is also shown as an inset of Figure 16. A complete inactivation was obtained for highest concentration (8 mM) of orotaldehyde, suggesting that an essential lysine residue(s) at the active site of OPRTase was(were) completely coupled to this aldehydic chemical modifier by formation of a reversible Schiff base. The rate of OPRTase inactivation with orotaldehyde was slower than that with pyridoxal phosphate (PLP), which was demonstrated previously in our lab by R.W. Ashton, and requires higher concentrations of orotaldehyde for a comparable degree of inactivation, suggesting that the phosphate group of PLP might facilitate the precise positioning of PLP with respect to the active groove of yeast OPRTase.

The observation of 60% inactivation, after 45 min incubation of OPRTase with 4 mM orotaldehyde, which was then partially reversed to 35% inactivation by flow dialysis (Figure 17), suggests that orotaldehyde is fairly tightly held at the OPRTase active site, even if no Schiff base is formed. In this regard, an attempt was made to fit the inactivation data, assuming that the inactivation follows pseudo-first order kinetics. As shown in Figure 18, at low concentrations of orotaldehyde this kinetic model appears to fit the data. However, at higher concentrations it is apparent that the kinetics are more complex. At these concentrations there may be a sufficient concentration of orotaldehyde present in the assay mixture for a competition between it and orotate to become significant. In order to understand this complicated kinetics pattern, inhibition studies of orotaldehyde against orotate or PRibPP were initiated (shown later).

Protection, by substrates and $MgCl_2$, against the inactivation of OPRTase by orotaldehyde was also studied (TABLE 4). Orotate and PRibPP provided some protection in the absence of $MgCl_2$, and Mg(II) itself had no effect on the extent of the

Figure 17. Reactivation of OPRTase-catalyzed reaction, which was initially inactivated with 4 mM orotaldehyde, by a flow dialysis procedure. An arrow indicates the time (45 min) at which the flow dialysis was initiated.

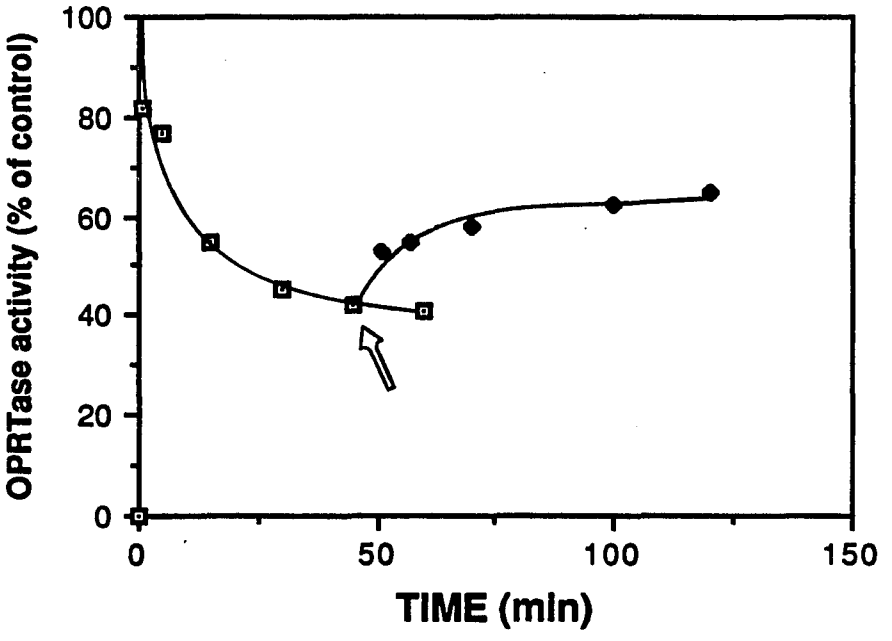

OPRTase activity (% of control)
100
80
60
40
20
0
0
50
100
150
TIME (min)

Figure 18. Pseudo-first order plots of inactivation of OPRTase with various orotaldehyde concentrations. The experimental points were obtained from time-dependent inactivation study depicted in Figure 16.

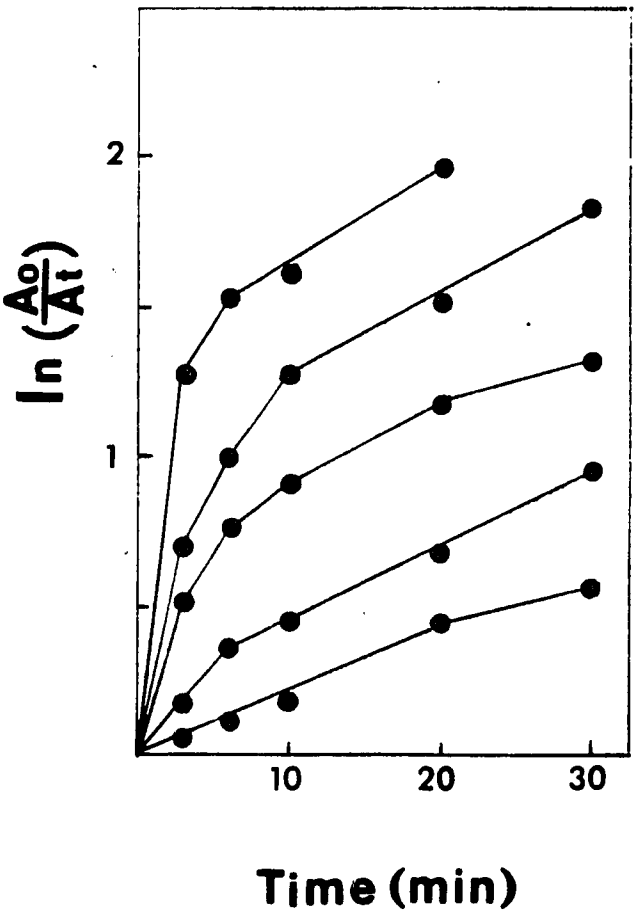

2
1
$\ln\left(\frac{A_o}{A_t}\right)$
10
20
30
Time (min)

TABLE 4

Protection by substrates and Mg(II) against the inactivation of OPRTase by orotaldehyde

Addition	Activity at equilibrium (% of control)
None	20
$MgCl_2$ (10 mM)	20
Orotate (1.25 mM)	26
Orotate (1.25 mM) + $MgCl_2$ (10 mM)	30
PRibPP (1.25 mM)	43
PRibPP (1.25 mM) + $MgCl_2$ (10 mM)	59
PRibPP (5 mM)	70

* Inactivation of OPRTase by 4 mM orotaldehyde was performed at 25°C in the incubation mixture containing enzyme (0.5 mg/ml), 50 mM POPSO buffer (pH 8), and the stated ligand concentration. In all cases, after a 45 min of incubation, equilibrium had been reached. Activity was measured spectrosopically after 60 min, according to the assay method described in Methods section.

inactivation. Upon incubation along with $MgCl_2$, all substrates protected the enzyme against inactivation by orotaldehyde. In the presence of $MgCl_2$, orotate demonstrated only a slightly increased protective ability against inactivation, contrasting a previous proposal (Dodin et al., 1982) that the Mg(II)-orotate is the true substrate for all OPRTase activities. The large increase in protection afforded by PRibPP in the presence of Mg(II) again supports that Mg(II)-PRibPP is the true substrate for OPRTase activities and also suggests that orotaldehyde may reside at the PRibPP binding site.

Time-dependent inactivation of OPRTase by 5 mM orotaldehyde at several pH values is shown in Figure 19. When the pH of an incubation medium is alkaline, the rate of inactivation was increased. The values of log K_{pH} determined from the degree of enzyme inactivation have been plotted as a function of pH in Figure 20A. As shown in Figure 20A, the equilibrium constants for the formation of the orotaldehyde-inactivated enzyme at pH values greater than 8 appear to decrease. Rates of inactivation of enzyme by orotaldehyde were also plotted as a function of pH (Figure 20B). The observed curve may be interpreted as a dependence of the rate of inactivation on the mole fraction of the conjugate base form of a functional group on the enzyme, as illustrated below,

$$\begin{array}{l} E + A \longrightarrow EA \\ \downarrow\uparrow \\ EH^+ \end{array} \qquad (7)$$

where E represents yeast OPRTase and A represents orotaldehyde. These results suggest the formation of a Schiff base between orotaldehyde and a deprotonated lysine residue.

Figure 19. Time-dependent inactivation of OPRTase by 5 mM orotaldehyde at several pH values. The numbers on these curves indicate the assay pH values employed in this study.

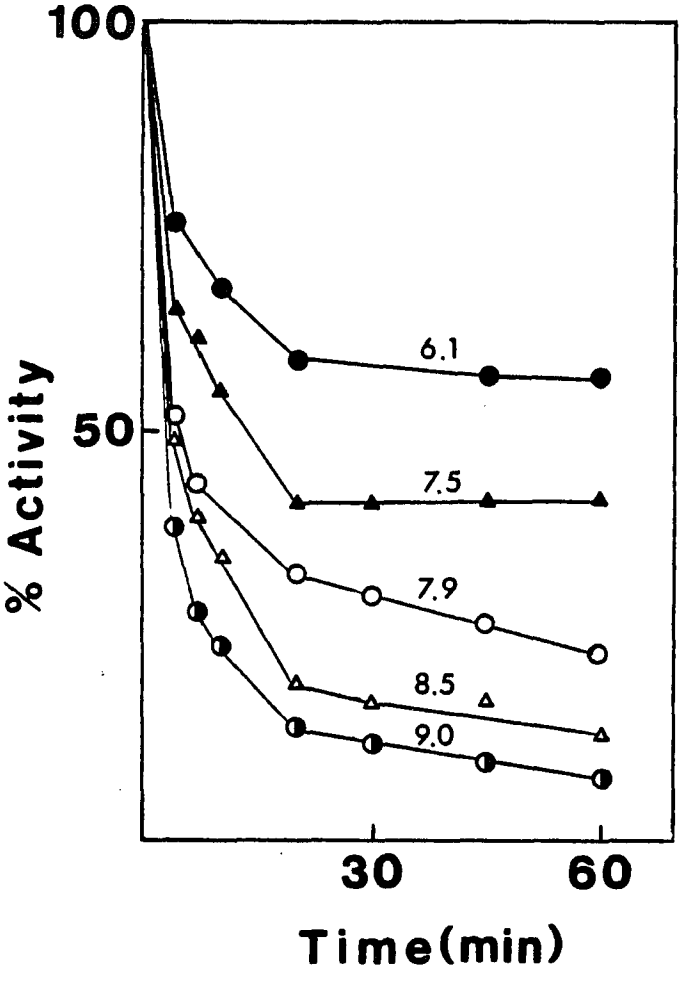
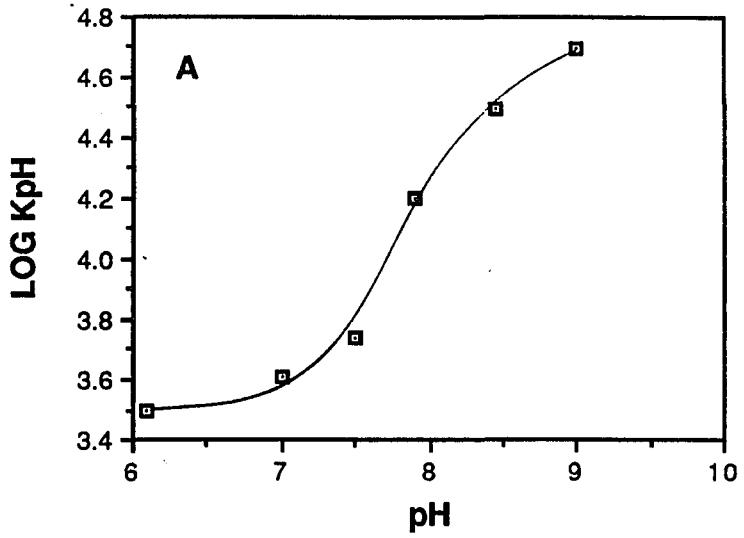
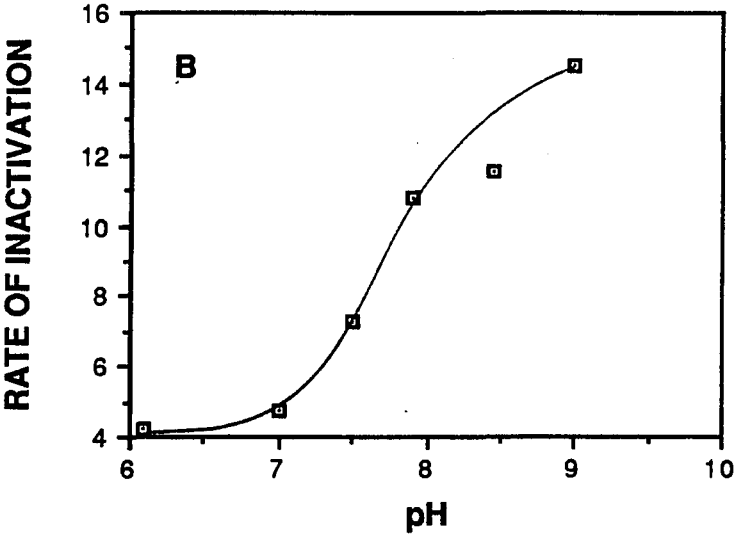

100
50
% Activity
6.1
7.5
7.9
8.5
9.0
30
60
Time(min)

Figure 20. (A) A plot of the equilibrium constants for the formation of orotaldehyde-inactivated OPRTase vs pH, (B) A plot of the initial rate of inactivation of OPRTase vs pH. In both cases, 5 mM orotaldehyde was employed.


A
LOG KpH
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
6
7
8
9
10
pH


B
RATE OF INACTIVATION
16
14
12
10
8
6
4
6
7
8
9
10
pH

Time-independent initial velocities of the OPRTase-catalyzed reaction in the presence of orotaldehyde were measured spectrophotometrically at 25°C. First, the concentration of PRibPP was varied from 25 to 200 μM, while the orotate concentration was fixed to 300 μM. In this case, two concentrations (0.5 and 1 mM) of orotaldehyde were employed. As shown in Figure 21A, reciprocal plots of 1/v vs 1/PRibPP suggest a non-competitive inhibition mode. In order to calculate an inhibition constant (Ki) for non-competitive inhibition, Dixon's graphical method was employed, along with the following equation.

$$1/v = 1/V\,(1+K_m/S)(1+i/K_i) \qquad (8)$$

In Figure 21B, the intersection of the pattern of lines allows the calculation of the Ki value (which lies on the base-line). This calculated Ki value for orotaldehyde was 1.25 mM. Next, the concentration of orotate was varied from 25 to 300 μM while the PRibPP concentration was fixed at 200 μM. In this experiment, three concentrations (0.25, 0.5, and 1 mM) of orotaldehyde were employed. A graphical analysis of 1/v vs 1/orotate plots also demonstrated a non-competitive inhibition mode (Figure 22A). A Dixon plot (Figure 22B) allowed the calculation of the inhibition constant for orotaldehyde equal to 0.15 mM.

PYRIMIDINE BASE ANALOGS STUDY

Kinetic analyses with pyrimidine base analogs are presented in TABLE 5. Among the orotate analogs tested, oxonic acid and 5-fluoroorotate have been shown by others to be alternate substrates of yeast OPRTase. The other compounds listed in TABLE 5 have been examined as inhibitors of the enzyme. A previously described experiment demonstrated that 6-formyluracil (orotaldehyde) appeared to be a non-

Figure 21. (A) Double reciprocal plot of 1/V vs 1/PRibPP in the absence (bottom line) and presence of orotaldehyde (middle and top lines), (B) Dixon plot of 1/V vs [inhibitor] over a series of concentrations of PRibPP.

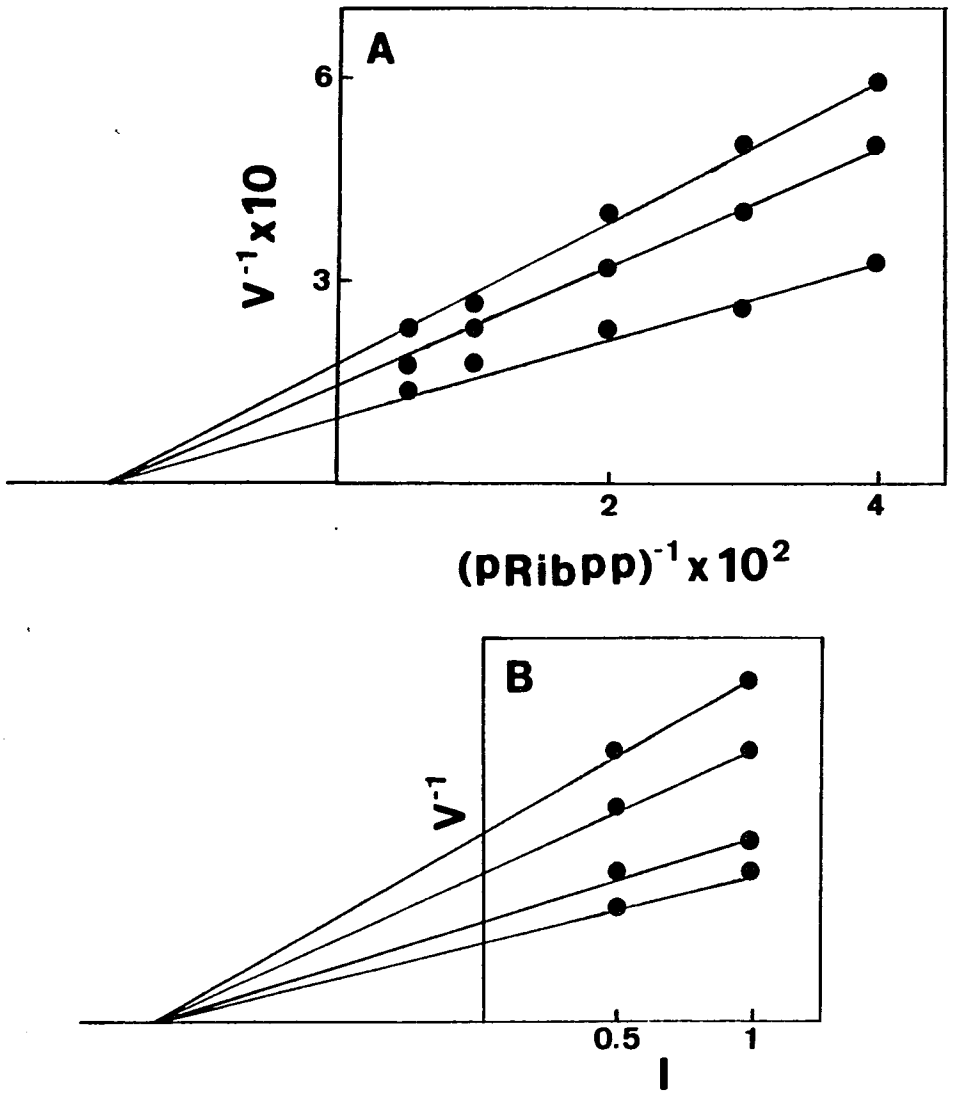
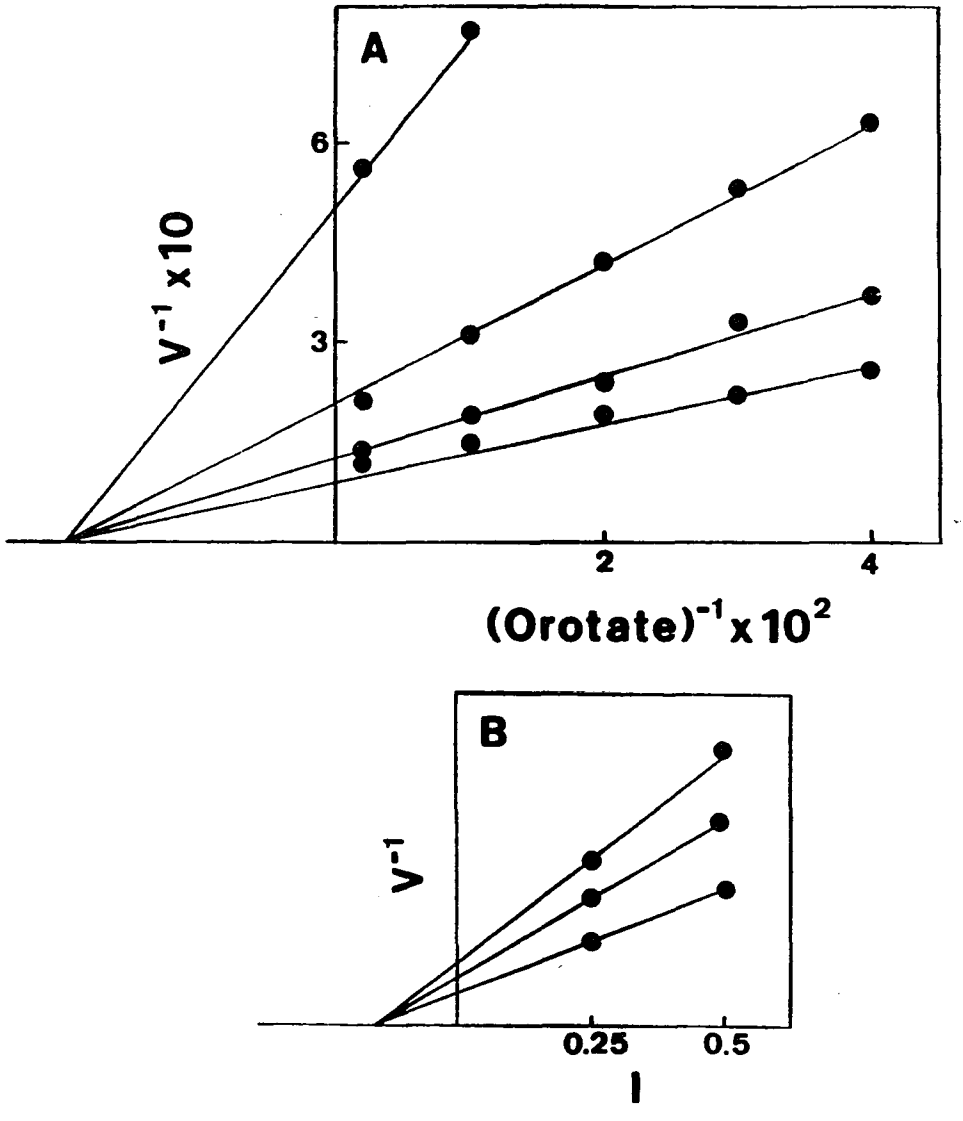

A
6
3
V^{-1}x10
2
4
$(pRibPP)^{-1}$ x 10^2
B
V^{-1}
0.5
1
I

Figure 22. (A) Double reciprocal plot of 1/V vs 1/orotate in the absence (bottom line) and presence of orotaldehyde (three lines except bottom one), (B) Dixon plot of 1/V vs [inhibitor] over a series of concentrations of orotate.


A
6
3
$V^{-1} \times 10$
2
4
$(Orotate)^{-1} \times 10^2$
B
V^{-1}
0.25
0.5
I

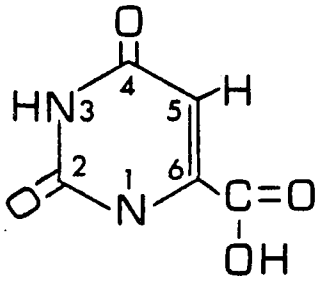


TABLE 5

Pyrimidine base analogs under study

Name/atom#	2	3	5	6	Source*
2-Thioorotate	SH				A
2-Aminoorotate	NH_2				H
3-Benzylorotate		C_6H_5			H
5-Aminoorotate			NH_2		A
5-Methylorotate			CH_3		H
5-Nitroorotate			NO_2		A
5-N-oxonate			(C→N)		A
5-Fluoroorotate			F		R
5-Bromoorotate			Br		R
5-Iodoorotate			I		R
5,6-Dihydrouracil			H_2	H_2	A
6-Methyluracil				CH_3	A
Uracil-6-acetic acid				CH_2COOH	H
6-Formyluracil				CHO	S

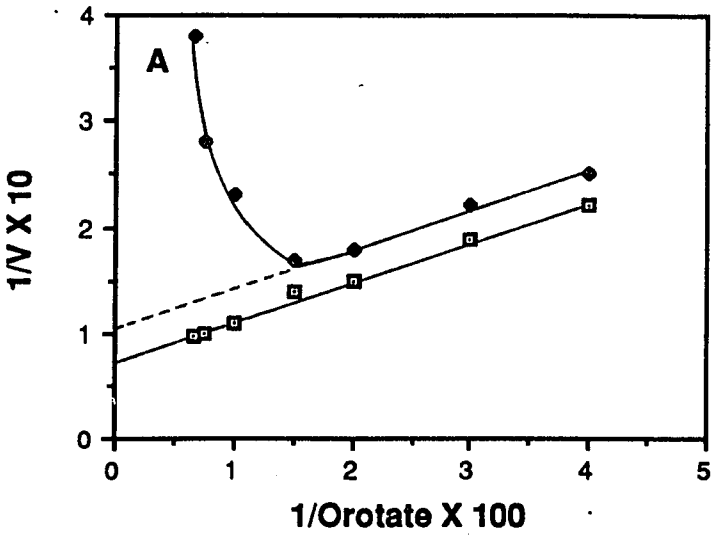
* A = Aldrich Chemical Company, Inc.
H = Hofmann-LaRoche
S = synthesized in our lab

competitive inhibitor of both substrates of OPRTase, PRibPP and orotate (see Figure 21 and 22). Interestingly, 5-aminoorotate showed an unique inhibition pattern at 0.8 mM (Figure 23A). When the concentration of orotate is increased, the degree of inhibition by 5-aminoorotate increased significantly. Meanwhile, 5-methylorotate (Figure 23B) and 5-nitroorotate appeared to be simple uncompetitive inhibitors. As for 2-thioorotate, two concentrations, 0.5 and 0.3 mM, were employed. As shown in Figure 24A, 2-thioorotate appeared to be a non-competitive inhibitor of yeast OPRTase. A Ki value of 0.68 mM was obtained from a Dixon plot (Figure 24B). For 2-aminoorotate, two concentrations, 0.7 and 0.4 mM, were utilized. As shown in Figure 25A, 2-aminoorotate also appeared to be a non-competitive inhibitor of yeast OPRTase ,with a Ki value of 0.94 mM (Figure 25B). A comparison of micromolar kinetic constants of alternate substrates and inhibitors of OPRTase at 25 and 37°C are shown in TABLE 6.

PURINE BASE ANALOGS STUDY

Initially, xanthine was examined as an alternative substrate of yeast HGPRTase, using the HPLC assay procedure. Several concentrations of xanthine, from 16.7 to 167 μM, and a saturating concentration of PRibPP (500 μM final) were utilized in this study, and they were incubated with enzyme for 3 min. A series of xanthine concentrations failed to produce a corresponding nucleotide. Thereafter, xanthine and five xanthine derivatives were examined as inhibitors of the enzyme activities. Two different substrates, hypoxanthine and guanine, were employed. In the HPLC profile of the enzyme-catalyzed reaction, we monitored depletion of substrate as well as formation of nucleotide. As shown in Figure 26, none of the compounds studied demonstrated any inhibitory effect on the HGPRTase-catalyzed reaction profiles,

Figure 23. (A) Double reciprocal plot of 1/V vs 1/orotate in the absence (open squares) and presence of 5-aminoorotate (closed squares), (B) Double reciprocal plot of 1/V vs 1/orotate in the absence (open squares) and presence of 5-methylorotate (closed squares). In both cases, PRibPP was fixed to a saturating concentration of 500 μM. The assay conditions are described in "Methods".


A
1/V X 10
1/Orotate X 100
0
1
2
3
4
5

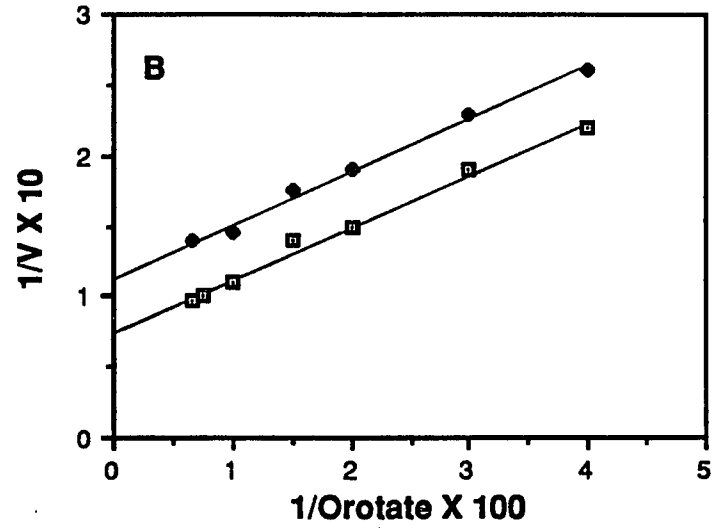

B
1/V X 10
1/Orotate X 100
0
1
2
3
4
5

Figure 24. (A) Double reciprocal plot of 1/V vs 1/orotate in the absence (bottom line) and presence of 2-thioorotate (middle and top lines), (B) Dixon plot of 1/V vs 1/[inhibitor] over various concentrations of orotate.

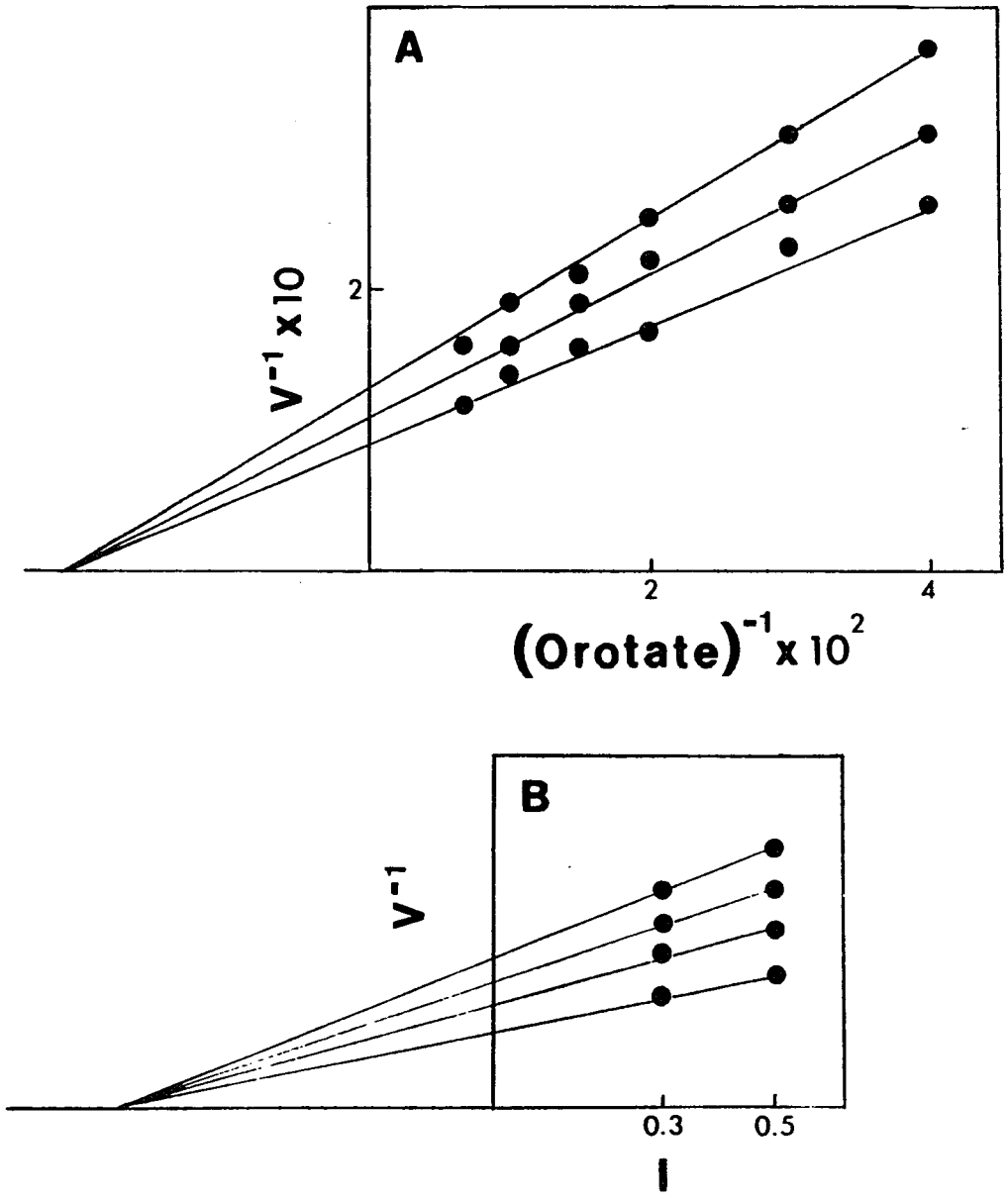
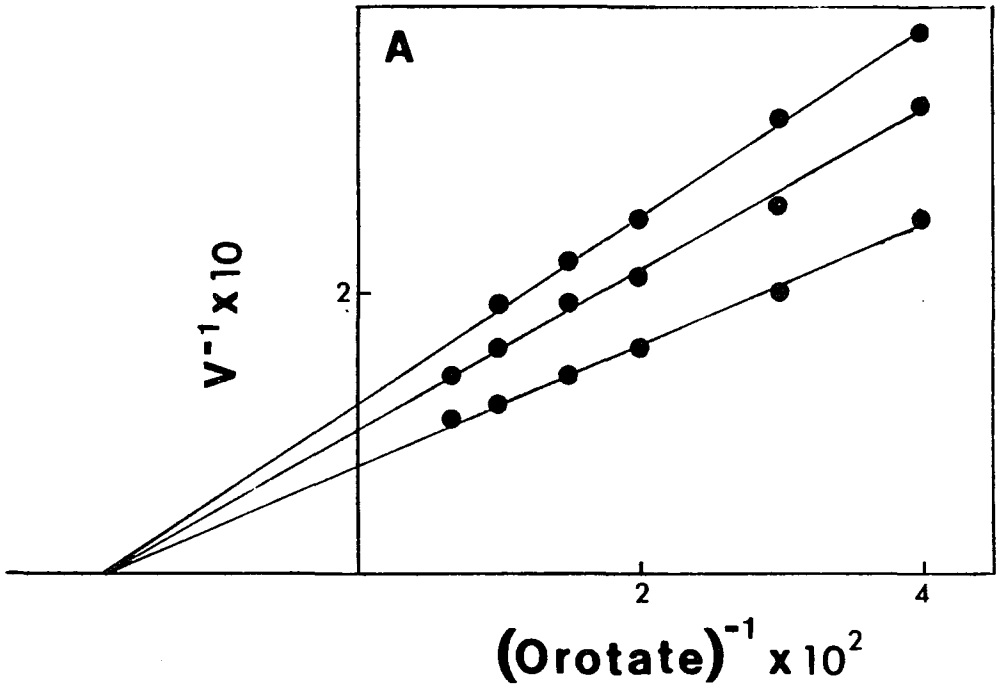

A
$V^{-1} \times 10$
2
2
4
$(Orotate)^{-1} \times 10^2$
B
V^{-1}
0.3
0.5
I

Figure 25. (A) Double reciprocal plot of 1/V vs 1/orotate in the absence (bottom line) and presence of 2-aminoorotate (middle and top lines), (B) Dixon plot of 1/V vs 1/[inhibitor] over various concenrations of orotate.


A
$V^{-1} \times 10$
2
2
4
$(Orotate)^{-1} \times 10^2$

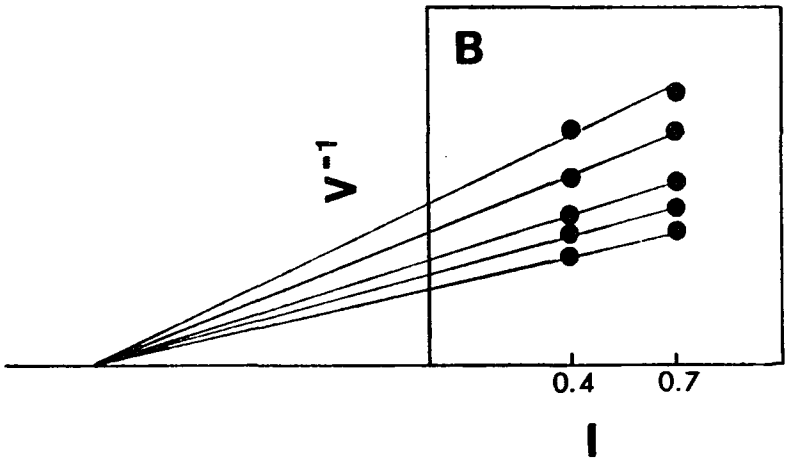

B
V^{-1}
0.4
0.7
I

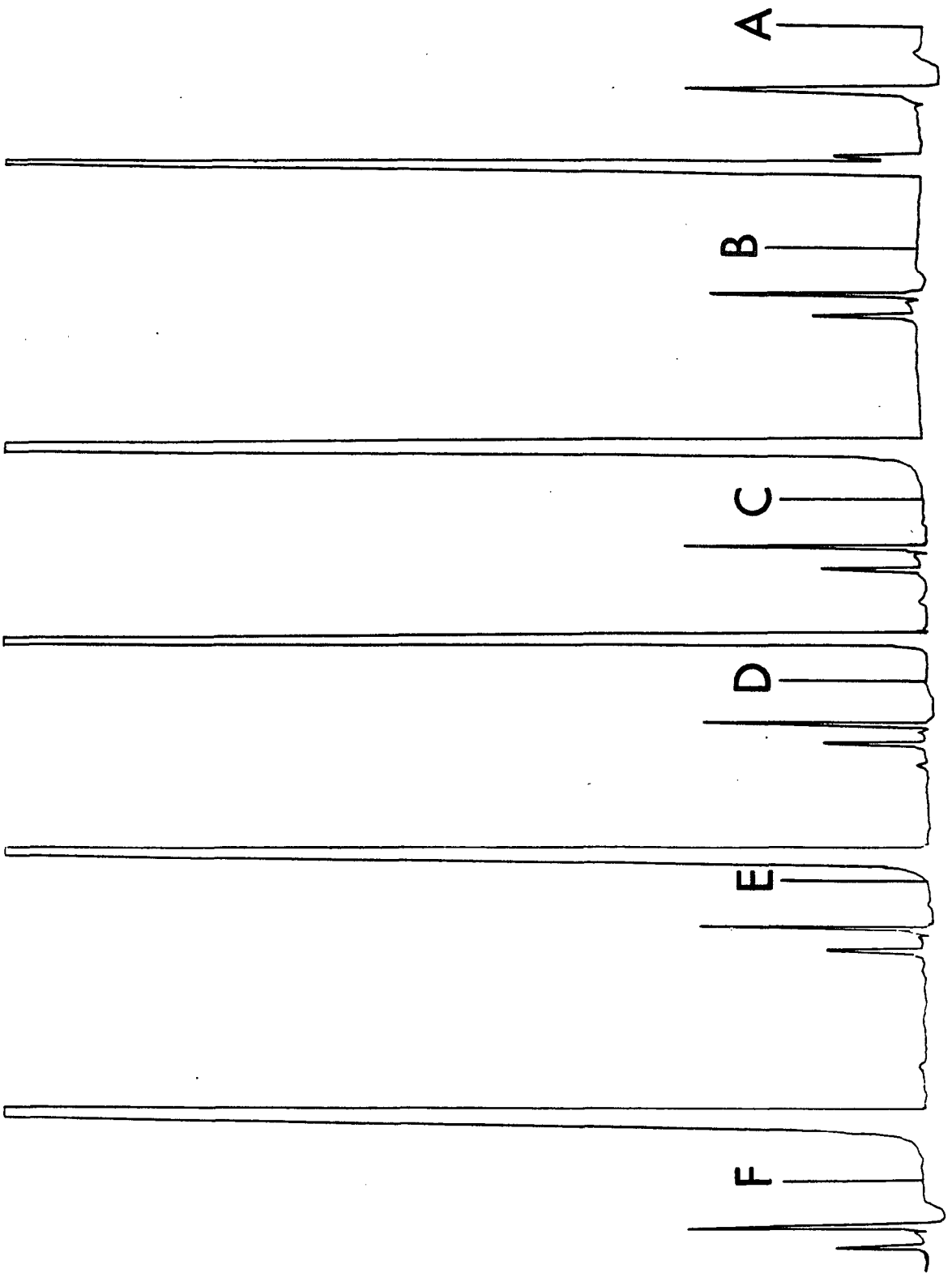
TABLE 6

Comparison of micromolar kinetic constants of alternate substrates and inhibitors of OPRTase at 25°C and 37°C

Analogues	25°				37°			
	Substrate		Inhibitor		Substrate		Inhibitor	
	Km	Vmax	type	Ki	Km	Vmax	type	Ki
Orotate	29	28			30	83		
5-Fluoroorotate	30	34			24	49		
Oxonate[1]	76	8			-	-		
2-Thioorotate			NC[2]	680			NA[3]	
2-Aminoorotate			NC	940			NA	
5-Aminoorotate			UC[4]	2400			NA	
5-Methylorotate			UC	1700			NA	
6-Formyluracil			NC	150			NA	

1 result obtained from R.W. Ashton
2 noncompetitive
3 not available
4 uncompetitive

Figure 26. Elution profiles of the HGPRTase-catalyzed IMP formation over a 2 min incubation period in the presence of xanthine (A), 1,3-dimethylxanthine (B), 3,7-dimethylxanthine (C), 1,7-dimethylxanthine (D), caffeine (E), and none (F).


A
B
C
D
E
F

during this 3 min incubation period. This finding did not agree with our preliminary studies that made use of a spectroscopic assay procedure. TABLE 7 lists the chemical structures of these compounds and the results of this study.

RELATIONSHIP BETWEEN HGPRTASE SUBUNITS AND ACTIVITY

The association-dissociation of yeast HGPRTase has been re-examined by using molecular-sieve HPLC (Furman and Neet, 1983). As illustrated in Figure 27, non-reacting gel HPLC was employed to examine the active form(s) of HGPRTase. Both active monomeric and dimeric forms were observed from elutions of a range (0.4 - 4 μg/50 μl) of HGPRTase concentrations onto the TSK column (Figure 5A - 5D). As a concentration of eluting enzyme increased, the peak corresponding to the dimeric form became bigger and sharper, suggesting that non-reacting HGPRTase exists in a concentration dependent, self association-dissociation equilibrium of monomers and dimers. The equilibrium was not rapid enough to see a single peak, as evidenced by the presence of overlapped peaks with apparent molecular weights of 25,200 ± 4,200 and 54,300 ± 4,000. Interestingly, when Mg (II) is included in the equilibration buffer (Figure 5E), the calculated monomer-to-dimer ratio increases to 8, and when both PRibPP and Mg (II) are included, this ratio increases even further so that the monomer is essentially the only detectable active species (Figure 5F). This suggests ,but does not prove, that Mg-PRibPP displays a strong "dissociative" behaviour and appears to alter the hydrodynamic properties of the HGPRTase in solution. Under the conditions of a standard assay of the HGPRTase, therefore, the monomeric species might be the predominant form.

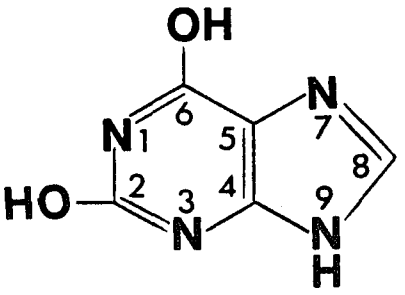


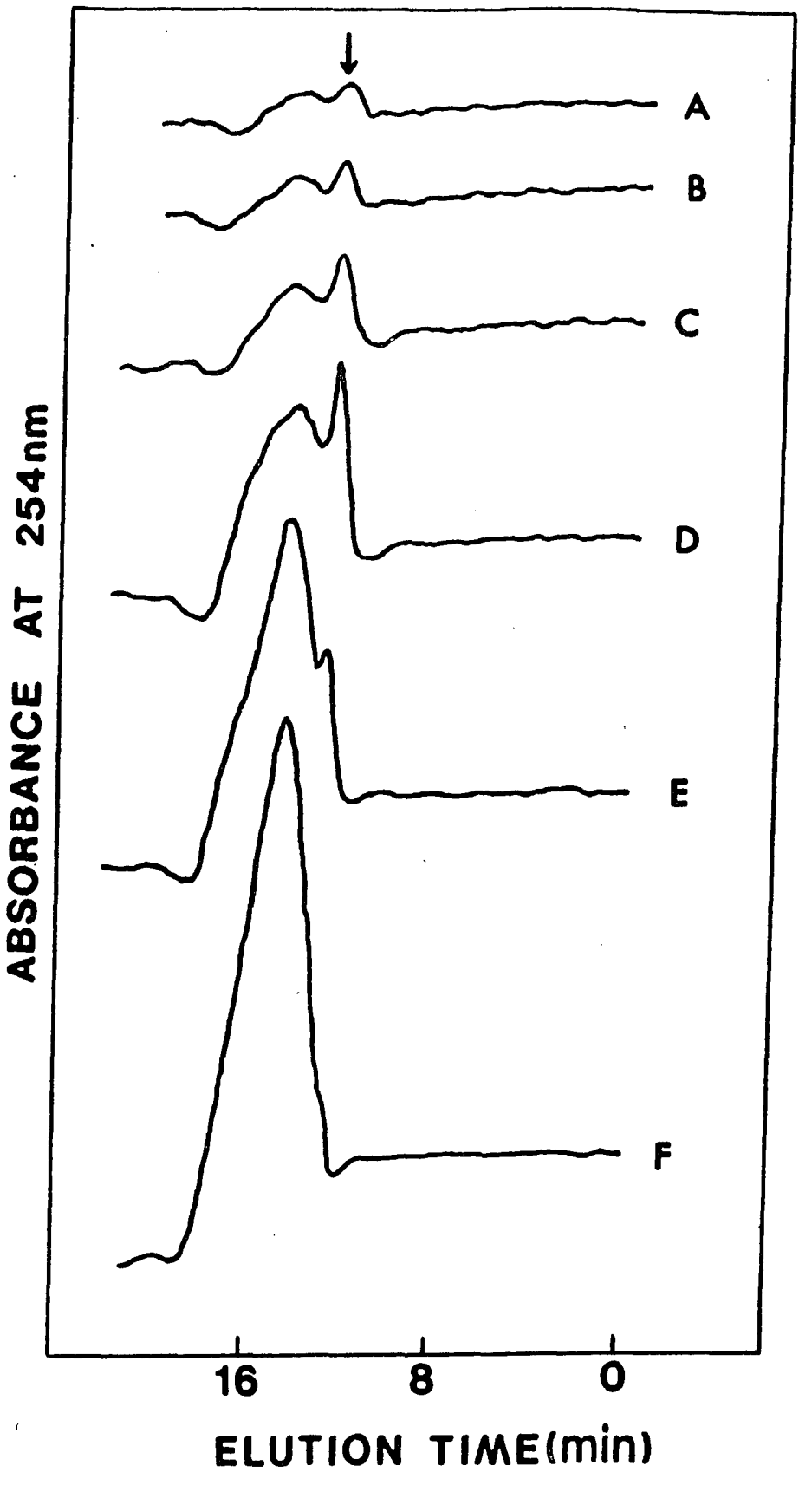
TABLE 7

Purine base analogs under study and the results

Name/atom#	1	3	7	Concentration	Sustrates	Inhibition
xanthine	-	-	-	0.5 mM	H*	-
3-methylxanthine		CH_3		2.5 mM	G**	-
1,3-dimethylxanthine (theophylline)	CH_3	CH_3		2.5 mM	H/G	-
3,7-dimethylxanthine (theobromine)		CH_3	CH_3	2.5 mM	H/G	-
1,7-dimethylxanthine	CH_3		CH_3	2.5 mM	H/G	-
1,3,7-trimethylxanthine (caffeine)	CH_3	CH_3	CH_3	2.5 mM	H/G	-

*H = hypoxanthine (5, 10, 20 and 40 μM were employed)
**G = guanine (10 amd 20 μM were employed)

Figure 27. Non-reacting molecular sieve HPLC elutions of HGPRTase: (A) 0.4 μg/ml enzyme eluted with 100 mM TEA (pH 6.8), (B) 0.8 μg/ml enzyme eluted with the same buffer, (C) 1.3 μg/ml enzyme eluted with the same buffer, (D) 4.0 μg/ml enzyme eluted with the same buffer, (E) 4 μg/ml enzyme eluted with 100 mM TEA (pH 6.8) containing 1 mM Mg(II), (F) 4 μg/ml enzyme eluted with 100 mM TEA (pH 6.8) containing 1 mM Mg(II) and 100 μM PRibPP. The production of IMP by the eluting enzyme was used as a detection device, as described in "Methods".


A
B
C
D
E
F
ABSORBANCE AT 254nm
16
8
0
ELUTION TIME(min)

DISCUSSION

The rate of metabolic processes depends on both the amount and the catalytic efficiency of the enzymes concerned. Control of enzyme formation by induction and repression, and control of enzyme degradation, are unlikely to provide an adequate mechanism for rapid adjustments in reaction rates. These forms of control are therefore often supplemented by methods that bring about the regulation of the activity of pre-existing enzyme molecules. The biological regulation of the activity of pre-existing enzyme can be accomplished by many different mechanisms, one of which would be explained by a following clinical case. Beardmore and his colleagues (1973) demonstrated that the activities of OPRTase and orotidylate decarboxylase are increased in erythrocytes from patients deficient in HGPRTase. The mechanism responsible for the increased activity of both enzymes has not been established. However, one possible cause of the increased activities of those enzymes is activation by phosphoribosyl 1-pyrophosphate (PRibPP), a compound known to be present in increased amounts in HGPRTase-deficient patients (Fox and Kelley, 1971). In contrast, patients with a deficiency of either adenine phosphoribosyltransferase or OPRTase have normal PRibPP levels in circulating erythrocytes. The fact, that the enzyme activities responsible for the de-novo pathway of pyrimidine nucleotide synthesis are regulated by the enzyme activity responsible for the salvage pathway of purine nucleotide synthesis (not vice versa), has been challenging us to examine the competition between yeast OPRTase and HGPRTase for a common substrate PRibPP. Hence the pyrimidine and purine nucleotide biosynthetic pathways are indeed interrelated and affected by variations in the intracellular PRibPP concentration. Although we do not know the exact physiological steady-state concentration of PRibPP

in yeast cytosol, a fluctuation in the natural levels of PRibPP may play an important part in biological control.

The use of HPLC assay procedures to monitor enzyme-catalyzed reactions has been well documented (Sloan, 1984), and I contend that the greatest use of this technique will be in areas where several enzymatic reactions can be analyzed simultaneously under conditions that approach, as closely as possible, those found in a living cell. As shown in Figure 2, the simultaneous utilization of PRibPP by three phosphoribosyltransferases is challenging to characterize kinetically. To our knowledge, HPLC provides the only effective means by which each reactant concentration can be monitored simultaneously over time.

Here, I have presented the preliminary evaluation of the initial velocities of the HGPRTase- and OPRTase-catalyzed reactions under at various enzyme concentration ratios, and I have determined the time course of these two reactions prior to the establishment of the overall reactant equilibria. These results can be summarized as follows. (1) Under conditions where OPRTase and HGPRTase are present in equivalent mass concentrations, the effect of the presence of the HGPRTase assay components on the OPRTase-catalyzed reaction is more profound than the effect of the presence of the OPRTase assay components on the HGPRTase-catalyzed reaction. This difference is caused by the higher specific activity of the purified HGPRTase and by the reversibility of the OPRTase-catalyzed reaction. (Victor et al., 1979, determined the equilibrium constant for the forward OPRTase-catalyzed reaction as 0.49 at pH 8 and 25°C. A Pyrophosphorolysis is therefore a more favorable reaction than a phosphoribosyltransfer reaction.) If these concentrations existed within the yeast cell, then the OPRTase activity would be at a distinct disadvantage in the yeast cell cytosol. (2) Under conditions where equivalent activities of the two enzymes are present in the

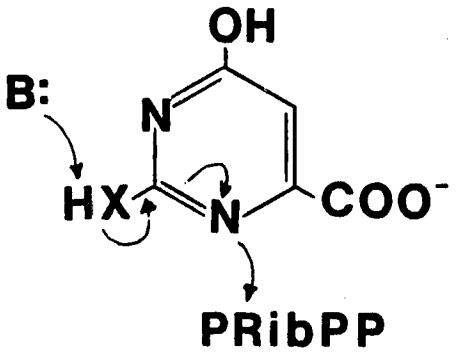
assay solution, initial velocities and equilibrium states of the OPRTase-catalyzed reaction are disturbed more than those of HGPRTase-catalyzed reaction. It is interesting to note that a double reciprocal plot of OPRTase-catalyzed reaction in the absence and presence of the HGPRTase assay components showed a non-linear inhibition pattern with respect to PRibPP, noncompetitive and then competitive at low and high concentrations of PRibPP, respectively (Figure 9A). In contrast, a double reciprocal plot of the HGPRTase-catalyzed reaction in the absence and presence of the OPRTase assay components showed a competitive inhibition pattern with respect to PRibPP (Figure 9B). (3) After the ratio of the HGPRTase and OPRTase activities (1:1.6) had been determined in yeast cell extracts, this ratio of activities was employed in a competition assay, with the result of the initial velocity investigation (TABLE 3). Apparent respective Vmax values, for this ratio of OPRTase and HGPRTase activities in competition, were determined to be 27.8 and 28.0 units/ml, whereas these values for the separate activities were determined to be 37.0 and 30.3 units/ml. In addition, after an extended period of OMP formation, the OPRTase catalysis is reversed to produce PRibPP for IMP synthesis. Under these conditions in the yeast cytosol, the two activities would indeed compete for the micromolar concentration of PRibPP present in these cell.

Most conventional techniques for the analysis of kinetic data in enzyme reactions consider only the " initial velocity," i.e., the rate of the reaction extrapolated to zero time. Recently, Barshop and his colleagues (1983) developed a computer system for the full time course of chemical kinetic reactions. The present computer system (they named it KINSYM) is an outgrowth of the program developed by Bates and Frieden (1973), which was written in the assembly language of a small computer, and now is largely machine independent and greatly increased in power and computational

efficiency. I hope that our full time course of competition reactions can be analyzed theoretically by using the KINSYM program, which was generously given us by Dr. Carl Frieden.

Niedzwick and his colleagues (1984) evaluated eighty pyrimidine base analogs as inhibitors of mouse liver OPRTase. Based on their finding and an extensive literature review, a structure-activity relationship has been formulated for the binding of pyrimidine base analogs to mammalian OPRTase. However, it must be emphasized that the inhibitor and substrate specificities of bacterial, protozoal, yeast, and mammalian (even different tissues) OPRTases are quite different. OPRTase from all organisms studied thus far (except yeast) is found in vivo as a bifunctional enzyme complex with the next enzyme of the sequence, orotidylate decarboxylase (ODCase) (Kasbekar et al., 1964; Ashihara, 1978; Rattke and Krauss, 1977; Jones et al., 1978; Brown and O'Sullivan, 1977; Traut and Jones, 1977; Reyes and Intress, 1978; Traut and Jones, 1979). As a consequence of this association, OMP is channeled from OPRTase to ODCase without an appreciable accumulation of OMP (Traut and Jones, 1977). Based on our kinetic results of compounds listed in TABLE 5, we suggested a preliminary structure-activity relationship for the binding of pyrimidine base analogs to yeast OPRTase. These results can be summarized as following: 1) Sulfur at the 2-position binds more tightly than an amino group at the same position. If one of the tautomeric forms (-SH in this case) is dominated at moderate acidic pH, then 2-thioorotate could be an alternative substrate of yeast OPRTase (Strauss and Sloan's proposal illustrated in Figure 28). However, this analog is not a substrate but an inhibitor. Thus the atom placed at this position must be oxygen for the compound to be a substrate. More experiments are needed to reveal why this is so. 2) Substitution with a benzyl group at 3-position abolishes binding. 3) Substitution with halogen atoms at 5-

Figure 28. Nucleophilic attack by base (B:) initiates an electron flow to the 1-ring nitrogen atom, and then the electron-rich nitrogen atom attacks a phosphoribosyl group of PRibPP with displacement of pyrophosphate.


OH
B:
N
HX
N
COO⁻
PRibPP

position generally diminishes binding. The bigger the atomic size is, the less tighter the compound binds to the enzyme. 4) Substitution with methyl or acetic acid at 6-position abolishes binding. Initial velocity studies showed that 6-formyluracil (orotaldehyde) inhibits OPRTase non-competitively with respect to both substrates. Moreover, the value of the inhibition constant of orotaldehyde against orotate is much smaller than that against PRibPP, suggesting that orotate and orotaldehyde may occupy the same binding site on yeast OPRTase. However, this compound may also competes with PRibPP, suggesting that perhaps the location of bound orotaldehyde is equidistance between the substrate binding sites. All of these spatial consideration provide a qualitative sizing of the OPRTase active site, which is illustrated in Figure 29.

The nature of the subunit association of HGPRTase from other tissue sources have been studied previously using crosslinking reagents. Paulus, Bieber and their colleagues (1980) have denoted that HGPRTase from beef brain is a trimeric protein composed of eqivalent 26,000 MW subunits and that PRibPP does not affect the relative concentration of the three enzyme forms. Holden and Kelley (1978) observed that the human HGPRTase is a tetrameric protein and that PRibPP has no effect on the relative distribution of the four molecular weight forms. These results contrast with our results for the yeast HGPRTase for which the presence of PRibPP alters significantly the monomer/dimer equilibria. In addition, our results with xanthine and its derivatives demonstrated that none of them are substrates or inhibitors of the yeast HGPRTase. Any substitution at three different positions of xanthine (1, 3 and 7) abolished the binding. The size of the base binding site on HGPRTase is illustrated in Figure 30.

In conclusion, we suggest that different effects of substrates and activators (or lack of such effects) on the activities and the monomer/dimer equilibria of PRTases that we have examined, may partially reveal a control mechanism for the allocation of

PRibPP in yeast into the important salvage purine nucleotide and de-novo pyrimidine nucleotide biosynthetic pathways.

Figure 29. A qualitative illustration of the orotate binding site of the yeast OPRTase. X and R represent substituents as depicted in TABLE 5.

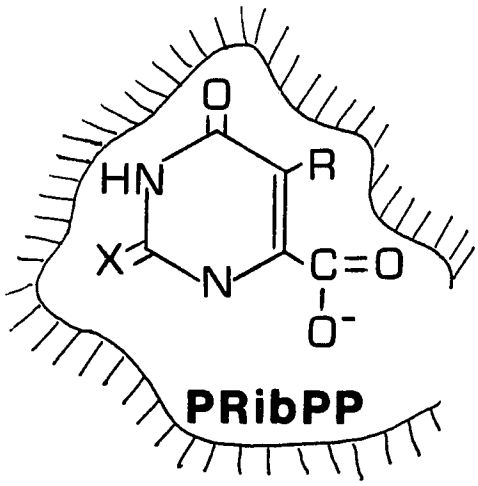
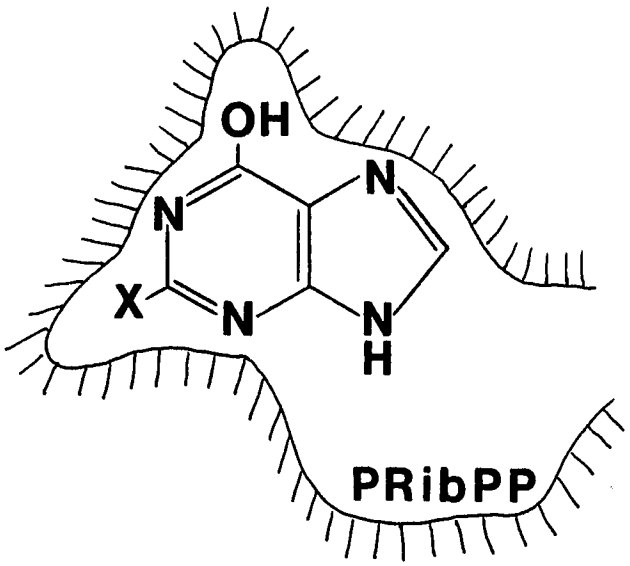

O
HN
R
X
N
C=O
O⁻
PRibPP

Figure 30. A qualitative illustration of the purine binding site of the yeast HGPRTase. The 1, 3, and 7-ring positions were substituted by methyl group(s) respectively during one of the kinetic studies, as depicted in TABLE 7. When X represents hydrogen the compound is hypoxanthine, hydroxyl for xanthine, and amino for guanine.


OH
N
N
X
N
N
H
PRibPP

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