Journal of Chromatography, 371 (1986) 71–81
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1000

ENZYMATIC KINETIC ANALYSES THAT EMPLOY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

COMPETITION BETWEEN OROTATE- AND HYPOXANTHINE/GUANINE-PHOSPHORIBOSYLTRANSFERASES FOR A COMMON SUBSTRATE

SUNG H. CHUNG and DONALD L. SLOAN*
Department of Chemistry and Program in Biochemistry, City College of the City University of New York, New York, NY 10031 (U.S.A.)

SUMMARY

Enzymatic assay procedures that employ high-performance liquid chromatography (HPLC) have been 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 (≈ 15 μM) concentration of phosphoribosyl α-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 these 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 (a 1.7:1 ratio). These relative activities were then employed in an *in vitro* reaction competition analysis. The results were similar to the those obtained from experiments where equivalent OPRTase and HGPRTase activities were employed and reveal 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.

INTRODUCTION

High-performance liquid chromatography (HPLC) has proven to be an effective method for monitoring the course of many enzymatic reactions[1]. One of the

0021-9673/86/$03.50

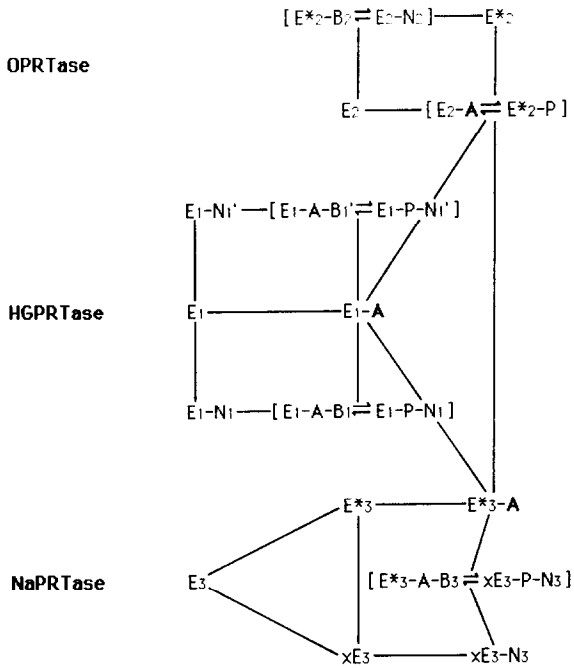

Fig. 1. The combined kinetic mechanisms of HGPRTase (E_1), OPRTase (E_2) and NaPRTase (E_3). Abbreviations: A = PRibPP; B_1 = hypoxanthine; B_1' = guanine; B_2 = orotate; B_3 = nicotinate; N_1 = inosine monophosphate; N_1' = guanosine monophosphate; N_2 = orotidine monophosphate; N_3 = nicotinate monophosphate; P = pyrophosphate. E_2^* and E_3^* represent covalent phosphoribosyl-ORPTase and phosphoryl-NaPRTase enzyme forms, respectively, whereas xE_3 represents the non-covalent phosphoryl-NaPRTase complex.

most interesting and valuable uses of this technique has been to measure the rates of two or more reactions occurring simultaneously[2–6]. Recently, we employed HPLC[2] to detect the effects of the utilization of phosphoribosyl α-1-pyrophosphate (PRibPP)* by hypoxanthine/guanine phosphoribosyltransferase (HGPRTase) on the utilization of this substrate by both nicotinate phosphoribosyltransferase (NaPRTase) and orotate phosphoribosyltransferase (OPRTase). The reactions catalyzed by OPRTase (eqn. 1), HGPRTase (eqns. 2 and 3) and NaPRTase (eqn. 4) are shown below. The most interesting result of this preliminary study[2] was that the irreversible enzymatic reaction (NaPRTase) eventually succeeded in utilizing all of the equivalents of PRibPP under the conditions we had designed for the assay solution. Although these phosphoribosyltransferases catalyze similar reactions, each reaction proceeds by way of a different kinetic mechanism[3,4,7], as illustrated in Fig. 1. These

* Abbreviations used: ADP = adenosine diphosphate; ATP = adenosine triphosphate; HGPRTase = hypoxantine/guanine phosphoribosyltransferase from yeast; H = hypoxanthine; HPLC = high-performance liquid chromatography; IMP, Imp = inosine monophosphate; NaPRTase = nicotinate phosphoribosyltransferase; O = orotate; OMP, Omp = orotidine monophosphate; OPRTase = orotate phosphoribosyltransferase; Pi = monophosphate ion; PPi = pyrophosphate ion; PRibPP = phosphoribosyl α-1-pyrophosphate.

mechanisms must be interrelated because of the utilization of a common substrate, PRibPP.

$$\text{orotate} + \text{PRibPP} \rightleftharpoons \text{orotidine 5'-phosphate} + \text{PPi} \quad (1)$$

$$\text{hypoxanthine} + \text{PRibPP} \rightleftharpoons \text{inosine 5'-phosphate} + \text{PPi} \quad (2)$$

$$\text{guanine} + \text{PRibPP} \rightleftharpoons \text{guanosine 5'-phosphate} + \text{PPi} \quad (3)$$

$$\text{nicotinate} + \text{PRibPP} + \text{ATP} \rightarrow \text{ADP} + \text{Pi} + \text{nicotinate nucleotide} + \text{PPi} \quad (4)$$

In this paper, we describe a detailed kinetic analysis of the competition for PRibPP between two reversible enzymes in yeast, OPRTase and HGPRTase. We have examined this competition using equivalent mass concentrations of the two enzymes and using the physiological concentrations of HGPRTase and OPRTase that were determined to exist in the yeast cell cytosol. This latter experiment 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.

MATERIALS AND METHODS

Materials

Baker's yeast (Budweiser brand) was obtained from Valente Yeast (Flushing, NY, U.S.A.). PRibPP (sodium salt), hypoxanthine, inosine monophosphate (IMP), orotic acid, orotidine monophosphate (OMP), and triethanolamine were supplied by Sigma (St. Louis, MO, U.S.A.). All eluents for HPLC and water were doubly distilled, deionized, and filtered through membrane filters, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical grade.

Enzyme purification

HGPRTase and OPRTase were purified from baker's yeast to apparent electrophoretic homogeneity through the use of published procedures[3,4]. The units of these enzyme activities are defined as the μmoles IMP formed per min and the μmoles OMP formed per min, respectively.

High-performance liquid chromatography

A Waters (Milford, MA, U.S.A.) HPLC system, equipped with two solvent delivery pumps (Models 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 × 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 (Fig. 2a), involving the M-45 pump, was used for the studies presented in this paper. The column was equilibrated with a relatively high (50 m*M*) concentration of ammonium phosphate buffer (pH 6.0). Samples (10 μl) from solutions containing the two enzymes and substrates were injected with a Hamilton (Reno, NV, U.S.A.) 801 microliter syringe. Nucleotides and bases in the eluent were detected at 254 nm with a 0.02 absorbance setting. All of the solvents used in the chromatographic procedures were cleaned by vacuum filtration through a 0.45-μm HA Millipore filter.

Enzymatic assay procedures

As illustrated in Fig. 2b, measurements of the initial velocities of the HGPRTase- and OPRTase-catalyzed reactions, together and separately, by HPLC, were accomplished using modifications by Hanna and Sloan[8] and by Ali and Sloan[3] of the method described by Flaks[9]. The complete assay mixture consisted of 1.0 ml of 24 m*M* triethanolamine buffer (pH 8.0), containing 12 m*M* magnesium chloride, 0.1 ml of orotate, 0.1 ml of hypoxanthine, and 10 μl 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). Then the reaction was initiated by the addition of 10 μl of PRibPP. Aliquots of this solution were removed at appropriate time intervals, and the reaction occurring in each was terminated by heating the aliquots in a boiling water bath for 1 min. Each sample was filtered through a 0.45-μm HA Millipore filter prior to the HPLC injection.

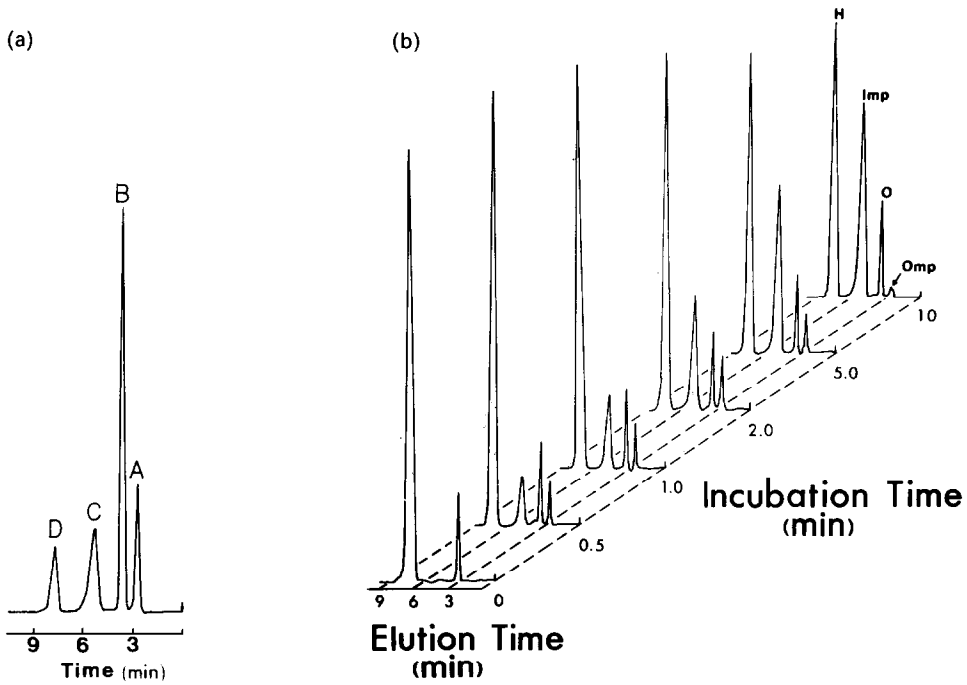

Fig. 2. (a) Separation of four components in a simultaneous two-enzymatic reaction system. Column, 25 cm × 3.9 mm Waters μBondapak C_{18}; mobile phase, 50 m*M* ammonium phosphate (pH 6); flow-rate, 1.2 ml/min; detection, at 254 nm with a 0.02 absorbance setting. Peaks: A = orotidine monophosphate; B = orotic acid; C = inosine monophosphate; D = hypoxanthine. (b) The elution profiles of the incubation mixture that illustrate the simultaneous detection of the OPRTase- and HGPRTase-catalyzed reactions over a 10-min time period. Conditions as in (a). The incubation solution contained: 24 m*M* triethanolamine (pH 8), 12 m*M* magnesium chloride, 20 μ*M* orotic acid, 80 μ*M* hypoxanthine, 40 μ*M* PRibPP, and appropriate amounts of OPRTase and HGPRTase in a final volume of 1.22 ml.

RESULTS

Competition 1

We employed equivalent mass concentrations (1 μg) of OPRTase and HGPRTase to examine the competition between these two enzymes for 100 μM PRibPP. We expected HGPRTase to dominate this competition study, 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 Fig. 3 and under this condition, the initial rate of OMP synthesis and the equilibrium reactant concentrations were greatly affected by the presence of HGPRTase activity. The concentration of OMP initially produced in this reaction decreases almost immediately after the incubation proceeds and continues to disappear until a new, highly reduced equilibrium concentration is reached. In contrast, the presence of the OPRTase activity had no discernable effect on the HGPRTase-catalyzed reaction under these conditions. We had not ruled out the possibility that certain components of the HGPRTase-catalyzed reaction might affect the OPRTase activity. Therefore, we determined 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 I, there is no significant difference in the OPRTase activities, when the four different sets of experiments are compared. Thereafter, we designed a detailed competition study more favorable to the OPRTase activity.

Competition 2

In an attempt to recreate more closely the *in vivo* conditions under which these reactions occur, we have established that the total activities (units/ml) of OPRTase and HGPRTase in a yeast cell extract were 740 units/ml and 450 units/ml, respectively. These relative activities were then employed in an *in vitro* reaction competition analysis. As shown in Fig. 4a, the initial velocities of OMP synthesis and the equilibrium states were apparently affected by the presence of HGPRTase activity, es-

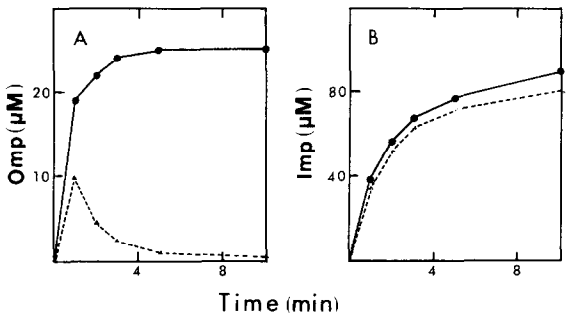

Fig. 3. Appearance of the nucleotides OMP and IMP using equivalent mass concentrations (1 μg for each enzyme) of OPRTase and HGPRTase. The reaction mixture contained: 24 mM triethanolamine (pH 8), 12 mM magnesium chloride, 80 μM orotic acid and/or 80 μM hypoxanthine, 100 μM PRibPP, and appropriate amounts of OPRTase and/or HGPRTase in a final volume of 1.22 ml. (A) OMP formation in the presence (dotted line) and absence (solid line) of the complete HGPRTase assay mixture. (B) IMP formation in the presence (dotted line) and absence (solid line) of the complete OPRTase assay mixture.

TABLE I

EFFECTS OF THE PRESENCE OF EITHER HGPRTase, HYPOXANTHINE, OR IMP ON THE 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	19.5	27.5	41	54.5
80 μM orotate 80 μM hypoxanthine + OPRTase	13.5	19	27	41	50
80 μM orotate 83.3 μM IMP + OPRTase	14	19	32	45	58

pecially at the lowest (20 μM) concentration of PRibPP. Meanwhile, the HGPRTase activity shown in Fig. 4b was only slightly affected by the presence of the highest (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. Double reciprocal plots were constructed during this study because the HGPRTase and OPRTase kinetic mechanisms can be distinguished by this graphical analysis, and because competitive and non-competitive inhibitions are readily distinguished. As shown in Fig. 5, the parallel lines that are observed for the OPRTase-catalyzed $1/v$ *versus* 1/PRibPP plot, during which we made use of several fixed concentrations of orotate in the absence of the HGPRTase activity, were no longer observed when the HGPRTase activity was present. HGPRTase activity was present, a series of intersecting lines were characterized, with the value of the x-intercept decreasing with increasing hypoxanthine concentration. In contrast, the $1/v$ *versus* 1/PRibPP plot for the HGPRTase-catalyzed reaction for a series of fixed concentrations of hypoxanthine, is composed of a series of intersecting lines, and the point of intersection appears to change in the presence of the OPRTase activity. 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[7] (eqn. 1) is less than 1 (favoring the reverse pyrophosphorolysis of OMP), the concentration of OMP is reduced most dramatically. These results are analogous to those obtained under conditions where equivalent OPRTase and HGPRTase activities were employed (data not shown).

DISCUSSION

The use of HPLC assay procedures to monitor enzyme-catalyzed reactions has been well documented[1], and we contend that the greatest use of this technique will be in areas where several enzymatic reactions can be analyzed simultaneously under

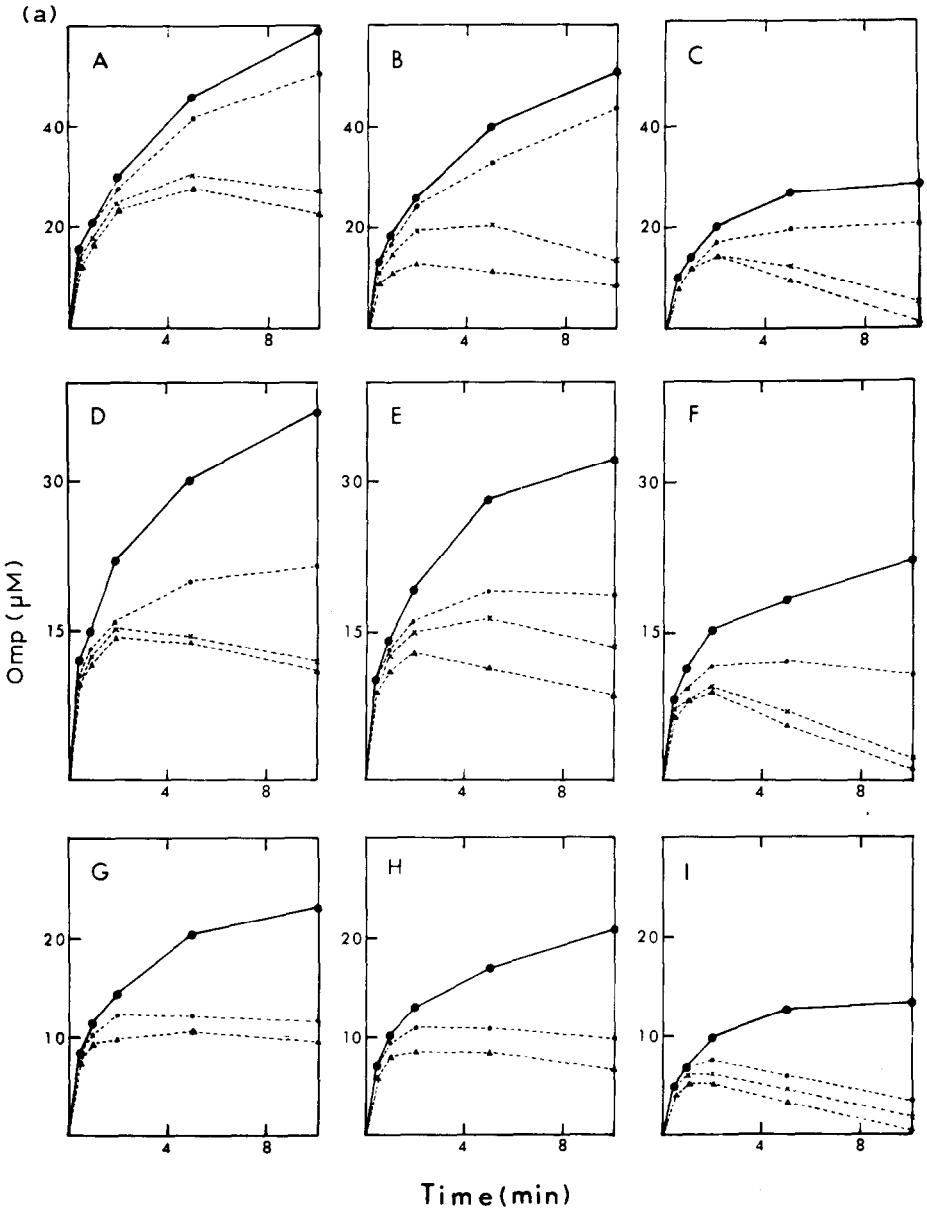

Fig. 4.

(Continued on p. 78

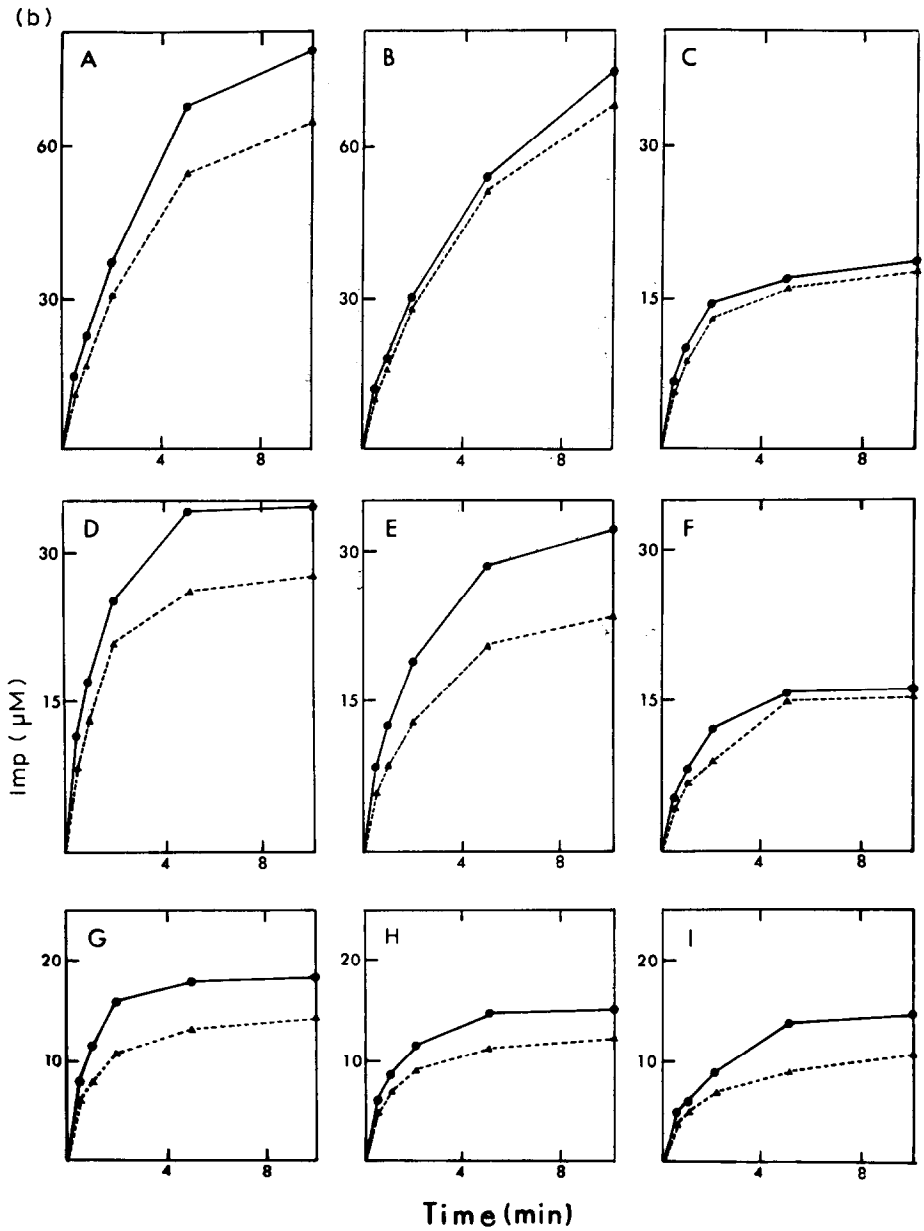

Fig. 4. (a) OMP synthesis in the presence (dotted line) and absence (solid line) of the complete HGPRTase assay mixture. The assay conditions and incubation mixture are as described in under Materials and methods and in Table II, respectively, except that, among the three dotted lines, the bottom line (▲.....▲) represents OMP appearance in the presence of 160 μM hypoxanthine, the middle line (×.....×) represents this appearance in the presence of 80 μM hypoxanthine, and the top line (●.....●) represents this appearance in the presence of 20 μM hypoxanthine. (b) 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 Materials and methods and in Table II, respectively, except that the dotted line represents IMP appearance in the presence of 160 μM orotate.

TABLE II

EFFECTS OF THE PRESENCE OF HGPRTase AND OPRTase ASSAY COMPONENTS ON OPRTase AND HGPRTase ACTIVITIES, RESPECTIVELY

Experiments 1–3 represent OPRTase activities only: Experiment 1 is shown in graphs A, D and G of Fig. 4a; experiment 2 is shown in graphs B, E and H; experiment 3 is shown in graphs C, F and I. Experiments 4–6 represent HGRPTase activities only and they are depicted in Fig. 4b using same pattern as Fig. 4a. Experiments 7–12 represent competition studies and they are presented as the bottom lines (▲.....▲) in both figures.

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	19.5	14			
(3) 20 μM O	19	16	12			
+ HGPRTase						
(4) 160 μM H				22.5	17	11.5
(5) 80 μM H				18	12.5	8.5
(6) 20 μM H				11	8.0	5.5
+ OPRTase + HGPRTase and fixed H (160 μM)						
(7) 160 μM O	23	17	13	17	14.3	8.0
(8) 80 μM O	20	15.5	11	18	13.3	9.0
(9) 20 μM O	15	11.5	7.5	19	13.3	9.0
+ OPRTase + HGPRTase and fixed O (160 μM)						
(10) 160 μM H	23	17	13	17	14.3	8.0
(11) 80 μM H	24	17	13.5	16	10	7.0
(12) 20 μM H	27.5	18.5	14.5	9.0	6.7	5.2

conditions that approach, as closely as possible, those found in a living cell. As shown in Fig. 1, the allocation of PRibPP among three phosphoribosyltransferases offers a series of interrelated reactions that 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. Moreover, the new elution conditions described in this paper have proven to be superior to those described previously[2] for the detection of OPRTase activity. To demonstrate the effectiveness of this methodology, we have presented, in this article, our preliminary evaluation of the initial velocities of the HGPRTase- and OPRTase-catalyzed reactions under a variety of enzyme concentration ratios, and we 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 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

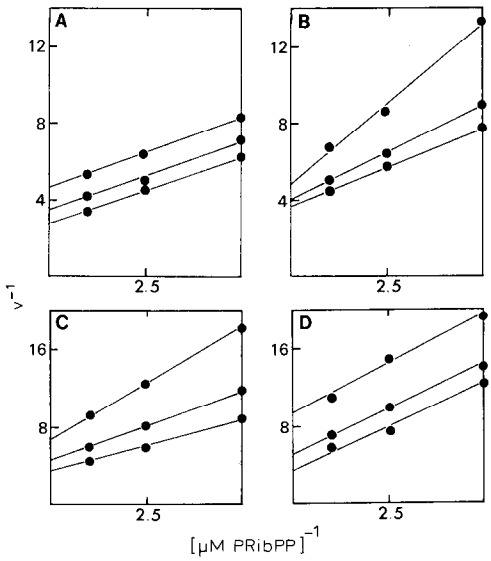

Fig. 5. Double reciprocal plots from experimental data presented in Table II. Graphs A and B both represent 1/OMP *versus* 1/PRibPP plots: (A) for experiments 1–3 in Table II and (B) for experiments 7–9. Graphs C and D both represent 1/IMP *versus* 1/PRibPP plots: (C) for experiments 4–6 and (D) for experiments 10–12.

purified HGPRTase and by the reversibility of the OPRTase-catalyzed reaction, and 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, equivalent effects of one assay system on the initial velocity of the other nucleotide synthesis will occur. (3) After the ratio of the HGPRTase and OPRTase activities (1:1.7) 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 II) being similar to velocities observed for the equivalent activity competition studies. Apparent respective V_{max} 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 (Fig. 4a). Under these conditions in the yeast cytosol, the two activities would indeed compete for the micromolar concentration of PRibPP[10] present in these cells.

In order to initiate a quantitation of these results, we have selected a chemical mechanism that would be the simplest representation of the competition for PRibPP by two reactions. This reaction is described as a reversible consecutive two-stage process with a single, reversible first stage and a single, initial substance, and is illustrated by eqn. 5, where the parameters [O] and [I] represent the concentrations of OMP and IMP respectively, and where PRibPP is the initial substance ([S]).

$$[O] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [S] \overset{k_2}{\rightarrow} [I] \tag{5}$$

As described by Rodigin and Rodiguina[11], the concentrations of each of the reactants, at a time (t) during the reaction, can be defined by eqns. 6–9, where $[S]^0$ represents the initial concentration of PRibPP and where X_1 and X_2 are the two roots of the following quadratic equation (eqn. 9), taken with the reverse signs. Our initial computations suggest that the shape of the curves shown in Figs. 3 and 4 can be generated with these equations, and we are currently designing a computer program to provide the best fits for the experimental results from a catalogue of possible k_1, k_2, and k_{-1} values.

$$[O] = k_{-1}\,[S]^0\,[(1/X_2 - X_1)\,e^{-X_1 t} + (1/X_1 - X_2)\,e^{-X_2 t}] \tag{6}$$

$$[S] = [S]^0\,[(k_1 - X_1/X_2 - X_1)\,e^{-X_1 t} + (k_1 - X_2/X_1 - X_2)\,e^{-X_2 t}] \tag{7}$$

$$[I] = [S]^0\,\{1 - [k_2(k_1 - X_1)/(X_2 - X_1)]\,e^{-X_1 t} - [k_2(k_1 - X_2)/X_2(X_1 - X_2)]\,e^{-X_2 t}\} \tag{8}$$

$$X^2 + X(k_{-1} + k_1 + k_2) + k_1 k_2 = 0 \tag{9}$$

Once the best fit for the kinetic constants has been obtained, we will take the next progressive step in these calculations by introducing the saturation condition (enzyme substrate complex formation) required for enzymatic reactions. These new studies will again demonstrate the value of enzymatic assay procedures that employ HPLC.

ACKNOWLEDGEMENTS

This research was supported by NIH grant No. AM-20183 and by a grant from the City University of New York Research Foundation. We wish to acknowledge the contributions of Ted Dashman who participated in the HPLC experiments and in several valuable discussions.

REFERENCES

1 D. L. Sloan, *Adv. Chromatogr.*, 23 (1984) 97.
2 D. L. Sloan, L. Z. Ali, D. Aybar-Batista, C. Yan and S. L. Hess, *J. Chromatogr.*, 316 (1984) 43.
3 L. Z. Ali and D. L. Sloan, *J. Biol. Chem.*, 257 (1982) 1149.
4 L. S. Hanna, S. L. Hess and D. L. Sloan, *J. Biol. Chem.*, 258 (1983) 9745.
5 R. W. Ashton and D. L. Sloan, *J. Chromatogr.*, 317 (1984) 245.
6 A. P. Halfpenny and P. R. Brown, *J. Chromatogr.*, 349 (1985) 275.
7 J. Victor, L. B. Greenberg and D. L. Sloan, *J. Biol. Chem.*, 254 (1979) 2647.
8 L. S. Hanna and D. L. Sloan, *Anal. Biochem.*, 103 (1980) 230.
9 J. B. Flaks, *Methods Enzymol.*, 6 (1963) 144.
10 I. H. Fox and W. N. Kelley, *Ann. Int. Med.*, 74 (1971) 424.
11 N. M. Rodigin and E. N. Rodiguina, *Consecutive Chemical Reactions —Mathematical Analysis and Development*, Nostrand, Princeton, NJ, 1964, p. 24.