Purine Nucleoside Phosphorylase: Kinetics, Mechanism, and Specificity

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SUMMARY

Ribosyl exchange reactions between purines and purine nucleosides catalyzed by crystalline purine nucleoside phosphorylase from calf spleen were markedly stimulated by inorganic phosphate. Initial velocity and product inhibition analyses were performed for the enzymic synthesis of inosine from ribose 1-phosphate and hypoxanthine. An ordered sequential mechanism involving the isomerization of the free enzyme and the initial combination of the purine base with one of the forms of the free enzyme is most consistent with these results.

The specificity of the enzyme toward the purine base in the synthesis of ribonucleosides was investigated. In order of decreasing effectiveness, ribosyl acceptors were guanine, hypoxanthine, xanthine, 6-mercaptopurine, and allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine]. Oxoallopurinol [4,6-dihydroxypyrazolo-(3,4-*d*)-pyrimidine] was a very poor substrate. Adenine, azathioprine [6-(1-methyl-4-nitro-5-imidazolyl)thiopurine], and uracil did not produce detectable amounts of ribonucleoside. Guanosine, deoxyguanosine, and inosine were shown to be effective pentosyl donors in exchange reactions.

INTRODUCTION

Mammalian purine nucleoside phosphorylase[1] has been shown to catalyze ribosyl-exchange between free bases and nucleosides (1–5). The mechanism of this transfer is uncertain. Enzymes catalyzing trans-deoxyribosylation reactions which are inactive toward deoxyribose 1-phosphate have been described in bacteria but not in mammalian tissues (6). Kritskii has reported that inorganic phosphate greatly stimulates the transribosylation activity of a purine nucleoside phosphorylase preparation repeatedly precipitated with ammonium sulfate (1). Abrams *et al.* have reported that a purified preparation of purine nucleoside phosphorylase from calf spleen catalyzes pentosyl transfer from deoxyguanosine or guanosine to guanine in the absence of added inorganic phosphate (3). As has been previously pointed out (7), exchange criteria for determining mechanisms must be interpreted with caution because their validity depends on the complete absence of acceptor molecules from the system. This report presents studies on ribosyl exchange reactions and the kinetics of nucleoside synthesis and a proposed enzyme mechanism consistent with these data. The specificity of the enzyme is also reported.

[1] Purine nucleoside:orthophosphate ribosyltransferase (EC 2.4.2.1).

MATERIALS AND METHODS

Materials. Inosine was purchased from the California Biochemical Corporation; guanosine and deoxyguanosine from Schwarz BioResearch, Inc.; β-D-ribose 1-phosphate, di(cyclohexylammonium) salt, from P-L Biochemicals, Inc.; guanine-8-

^{14}C, hypoxanthine-8-^{14}C, xanthine-8-^{14}C, and adenine-8-^{14}C from Volk Isotopes; uracil-2-^{14}C and 6-mercaptopurine-8-^{14}C from the New England Nuclear Corporation; D-ribose-1-^{14}C from Nuclear Chicago; crystalline purine nucleoside phosphorylase (calf spleen) from the Boehringer Mannheim Corp. Allopurinol-6-^{14}C [4-hydroxypyrazolo(3,4-*d*)pyrimidine], oxoallopurinol-6-^{14}C [4,6-dihydroxypyrazolo(3,4-*d*)pyrimidine], and azathioprine-8-^{14}C [6-(1-methyl-4-nitro-5-imidazolyl)thiopurine] were synthesized in this laboratory (8, 9) by G. B. Elion and H. N. Yeowell.

Assay of exchange and nucleoside synthesis. The assay solutions (0.25 ml) were incubated at 38° and pH 7.0. The buffer, incubation time, concentration of substrates, and amount of enzyme protein are specifically designated in the text. The reactions were stopped by the addition of 0.6 ml of absolute ethanol. Aliquots of the ethanolic solutions were spotted on Whatman 3 MM paper and developed in 5% disodium phosphate–isoamyl alcohol (2:1, v/v) (2 layer) in an ascending direction for 17 hr. R_F values in this solvent have been previously reported (5). Nucleoside synthesis was determined by scanning the chromatograms for radioactivity in an Atomic Associates 4 π Scanner (Model RSC 160).

The rate of synthesis of inosine from hypoxanthine and ribose 1-phosphate, both at 1 mM concentrations, was a linear function of the enzyme concentration. Under these conditions, linearity was maintained up to 50% conversion of hypoxanthine to inosine. This relatively wide range of linearity is consistent with the fact that the equilibrium of the reactions favors nucleoside synthesis (10, 11).

Desalting purine nucleoside phosphorylase. Boehringer Mannheim crystalline purine nucleoside phosphorylase is shipped and stored as an ammonium sulfate suspension. This preparation was desalted by passing it through a Sephadex G-100 column of dimensions 3×30 cm equilibrated at 23° with 0.02 M Tris-hydrochloride-buffer, pH 7.0, containing 2 mM mercaptoethanol. The enzyme activity was eluted in a single peak with the protein in an effluent volume of 72 ml. The void volume of the column, as determined with blue Dextran 2000, was 52 ml. The desalted enzyme solution was stored at —28°.

At a ribose 1-phosphate and guanine concentration of 1 mM and pH 7.0, the crystalline purine nucleoside phosphorylase preparation, before Sephadex treatment, catalyzed the synthesis of 61 μmoles of ribonucleoside per minute per milligram of protein. With hypoxanthine as the substrate, this specific activity value was 32. Sephadex treatment usually resulted in the reduction of the specific activity by a factor of ten. The Sephadex-treated preparation was used in all exchange and kinetic studies. The untreated preparation was employed only for the determination of the specificity of ribonucleoside synthesis.

Protein concentration was determined by the method of Lowry *et al.* (12).

RESULTS

It was observed that relatively crude preparations of purine and pyrimidine nucleoside phosphorylases from guinea pig small intestine (5) lost their ability to catalyze ribosyl exchange between free bases and ribonucleosides after gel filtration on a Sephadex G-100 column equilibrated with 0.02 M Tris buffer, pH 7.0. This activity was restored if inorganic phosphate was added to the assay mixtures. These preliminary observations suggested that the mechanism of the exchange reaction was via a ribose 1-phosphate intermediate. Experiments designed to test this hypothesis were performed with crystalline calf spleen purine nucleoside phosphorylase.

In agreement with the preliminary findings with crude preparations, the ribosyl exchange between ^{14}C-hypoxanthine and inosine catalyzed by crystalline purine nucleoside phosphorylase was greatly stimulated by inorganic phosphate (Table 1). Arsenate did not substitute for phosphate. Both phosphate and arsenate inhibited the synthesis of inosine from hypoxanthine and ribose 1-phosphate. Exchange between ribose and inosine or ribose 1-phosphate was not detectable.

TABLE 1

Phosphate and arsenate effects on exchange reactions

The final reaction mixtures (0.25 ml) had a concentration of 1 mM ^{14}C-hypoxanthine or ^{14}C-ribose, 1 mM inosine or ribose 1-phosphate, 0.08 M buffer, and 18.7 μg of crystalline purine nucleoside phosphorylase protein per milliliter. Controls lacked inosine and ribose 1-phosphate. Incubations were carried out for 20 min. The chromatograms were developed in *n*-butanol–water (84:16, v/v) in an ascending direction. R_F values for hypoxanthine, inosine, ribose, and ribose 1-phosphate were 0.27, 0.12, 0.21, and 0, respectively. In all cases, any radioactive product detected was inosine.

Substrates		Percent conversion to other ^{14}C-labeled compounds		
^{14}C-labeled	Nonradioactive	Tris buffer	Phosphate buffer	Arsenate buffer
Hypoxanthine	Inosine	<0.5[a]	25	<0.5
Ribose	Inosine	<0.5	<0.5	<0.5
Hypoxanthine	Ribose 1-phosphate	70	33	14
Ribose	Ribose 1-phosphate	<0.5	<0.5	<0.5

[a] Below limits of detectability of the method.

Kinetics

The rate of the exchange reaction between ^{14}C-hypoxanthine and inosine was found to be dependent on the concentration of inorganic phosphate (Fig. 1). The reciprocal of the phosphate concentration was a linear function of the reciprocal of the initial velocity. Optimal activity was attained when the phosphate concentration approached a stoichiometric level.

Double reciprocal plots of initial velocity *vs* hypoxanthine concentration (variable substrate) with ribose 1-phosphate as the nonvariable substrate are shown in Fig. 2. At different fixed concentrations of ribose 1-phosphate, the relationship between $1/v$ and the reciprocal of the hypoxanthine concentration yielded a family of lines which differed both in slope and ordinate intercept. The same pattern was seen where ribose 1-phosphate was the variable substrate and hypoxanthine the nonvariable substrate (Fig. 3). In both cases, the reciprocal of the apparent maximal velocity (ordinate intercept) was a linear function of the reciprocal of the nonvariable sub-

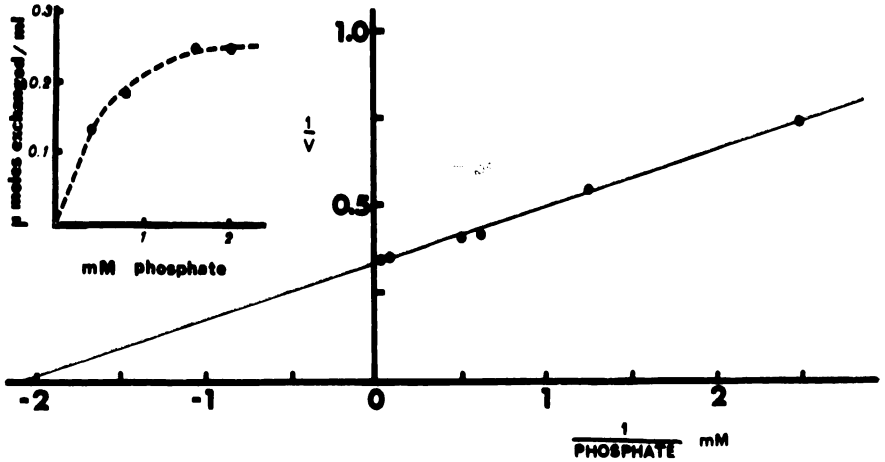

FIG. 1. *Phosphate dependence of the ribosyl exchange reaction between ^{14}C-hypoxanthine and inosine*

Reaction mixtures contained 1 mM ^{14}C-hypoxanthine, 1 mM inosine, 0.01 M Tris-hydrochloride buffer, and 37.4 μg of enzyme protein per milliliter. The reactions were stopped after 30 min incubations. v is expressed as micromoles × 10 of ^{14}C-hypoxanthine exchanged per milliliter per 30 min.

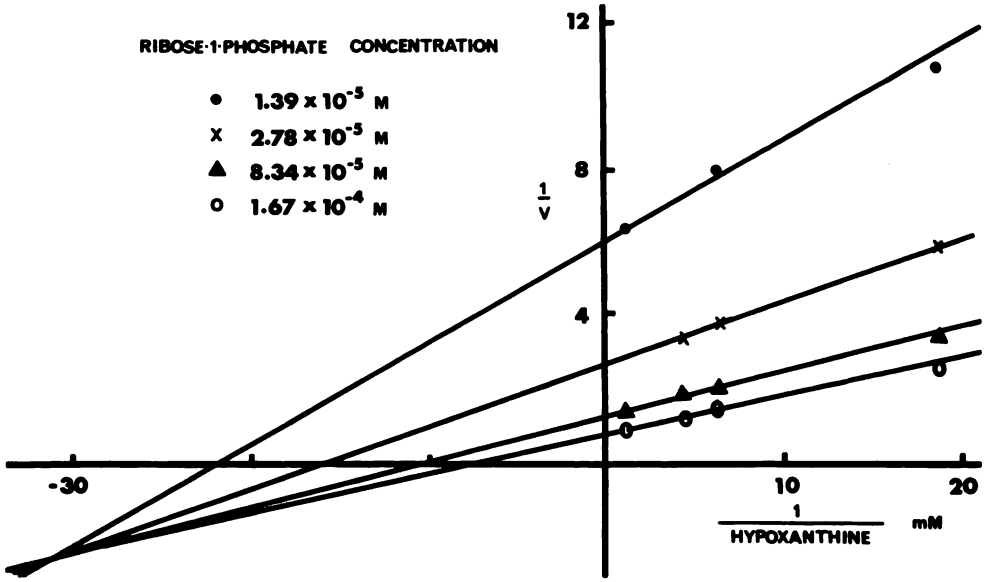

FIG. 2. *Double reciprocal plots of initial velocity against hypoxanthine concentration*

Reaction mixture concentrations of Tris-hydrochloride buffer and enzyme protein were 0.08 M and 37.5 μg/ml, respectively. Incubations were carried out for 10 min. v is expressed as micromoles × 10 of inosine synthesized per milliliter per minute.

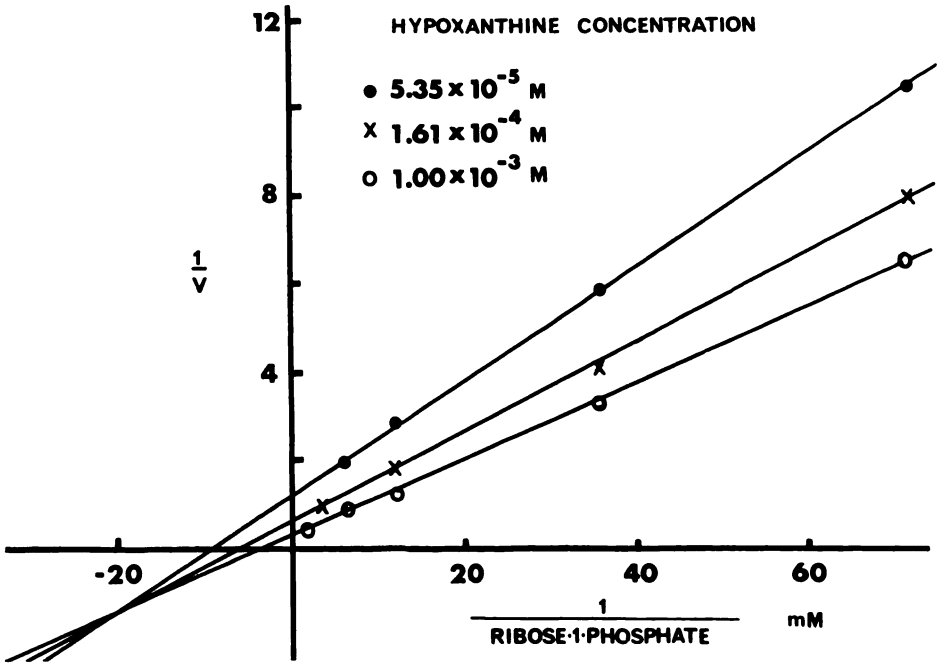

FIG. 3. *Double reciprocal plots of initial velocity against ribose 1-phosphate concentration*

Reaction mixture concentrations of Tris-hydrochloride buffer and enzyme protein were 0.08 M and 37.4 μg/ml, respectively. Incubations were carried out for 10 min. v is expressed as micromoles × 10 of inosine synthesized per milliliter per minute.

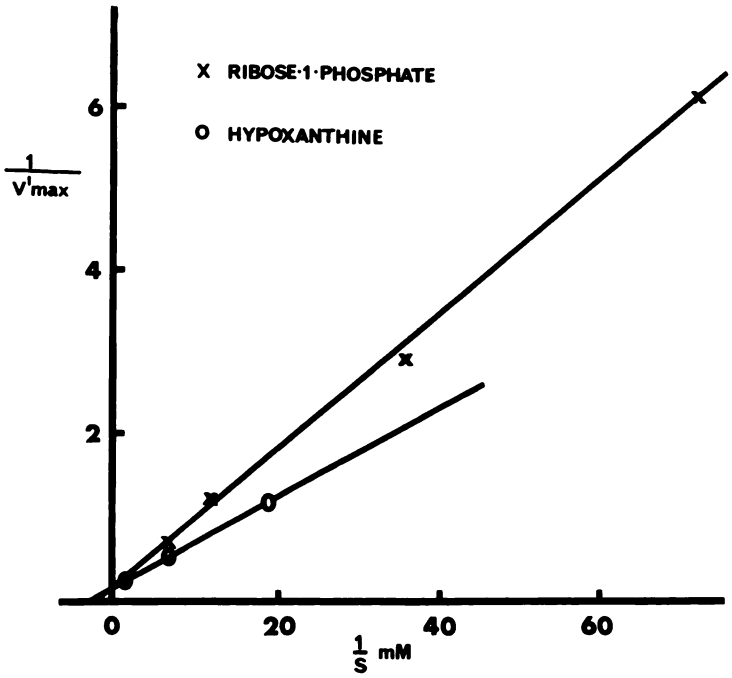

FIG. 4. *Secondary plots of intercepts* ($1/V'_{max}$) *of the lines in the double reciprocal plots in Figs. 2 and 3 against the reciprocals of the millimolar concentrations of the nonvariable substrate*

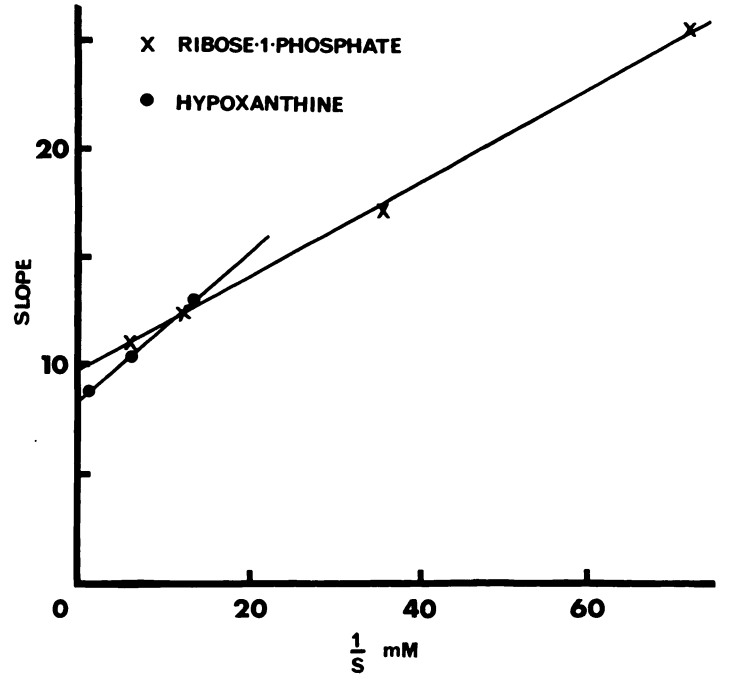

FIG. 5. *Secondary plots of the slopes of the lines in the double reciprocal plots in Figs. 2 and 3 against the reciprocals of the millimolar concentrations of the nonvariable substrate*

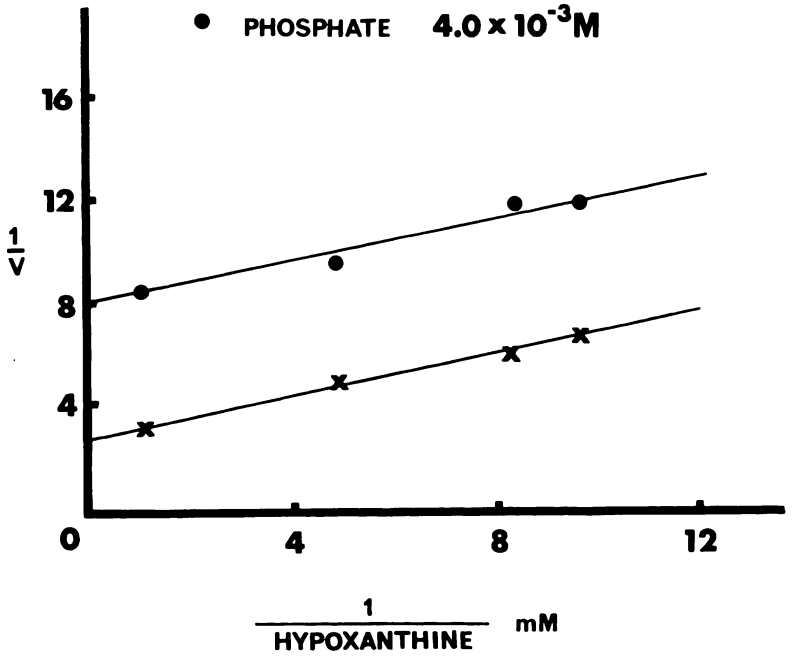

FIG. 6. *Product inhibition by inorganic phosphate*

Double reciprocal plot of initial velocity against hypoxanthine concentration. Reaction mixture concentrations of ribose 1-phosphate, Tris-hydrochloride buffer, and enzyme protein were 0.33 mM, 0.08 M, and 18.5 μg/ml, respectively. Incubations were carried out for 10 min. v is expressed as micromoles × 10^2 of inosine synthesized per milliliter per minute.

strate concentration (Fig. 4). Likewise, the slope was a linear function of the reciprocal of the nonvariable substrate concentration (Fig. 5). From the secondary plots in Fig. 4 the concentration-independent Michaelis constants were determined to be 4×10^{-4} M for hypoxanthine and 5×10^{-4} M for ribose 1-phosphate.

The double reciprocal plots in Figs. 6–8 show the effects of the products, phosphate and inosine, upon the initial rate of nucleoside synthesis. With hypoxanthine as the variable substrate, inhibition by phosphate (Fig. 6) was clearly not of a competitive nature.[2] A definite distinction between uncompetitive and noncompetitive inhibition cannot be made from the data, although the slopes of the two lines appear similar. With hypoxanthine as the variable substrate, inhibition by inosine (Fig. 7) was noncompetitive. At this concentration of ribose 1-phosphate (2 mM), apparent product activation was obtained at high hypoxanthine concentrations and the double reciprocal plots departed from linearity. The intercept and slope of the linear range of each double reciprocal plot was a linear function of the inosine concentration (Fig. 7). With ribose 1-phosphate as the variable substrate, inhibition by inosine (Fig. 8) was not of a competitive nature since the apparent maximal velocity was lowered. The apparent activation at low ribose 1-phosphate concentrations is most probably a result of the ambiguity inherent in the method employed to determine nucleoside synthesis. Ribosyl exchange and net nucleoside synthesis are not distinguishable by this method. With ribose 1-phosphate as the variable substrate, inhibition by inorganic phosphate (Fig. 8) appeared competitive. However, a rigorous distinc-

[2] Inhibition will be called competitive, uncompetitive, or noncompetitive, respectively, when the slope, ordinate intercept, or both, of lines resulting from double reciprocal plots are a function of the inhibitor concentration.

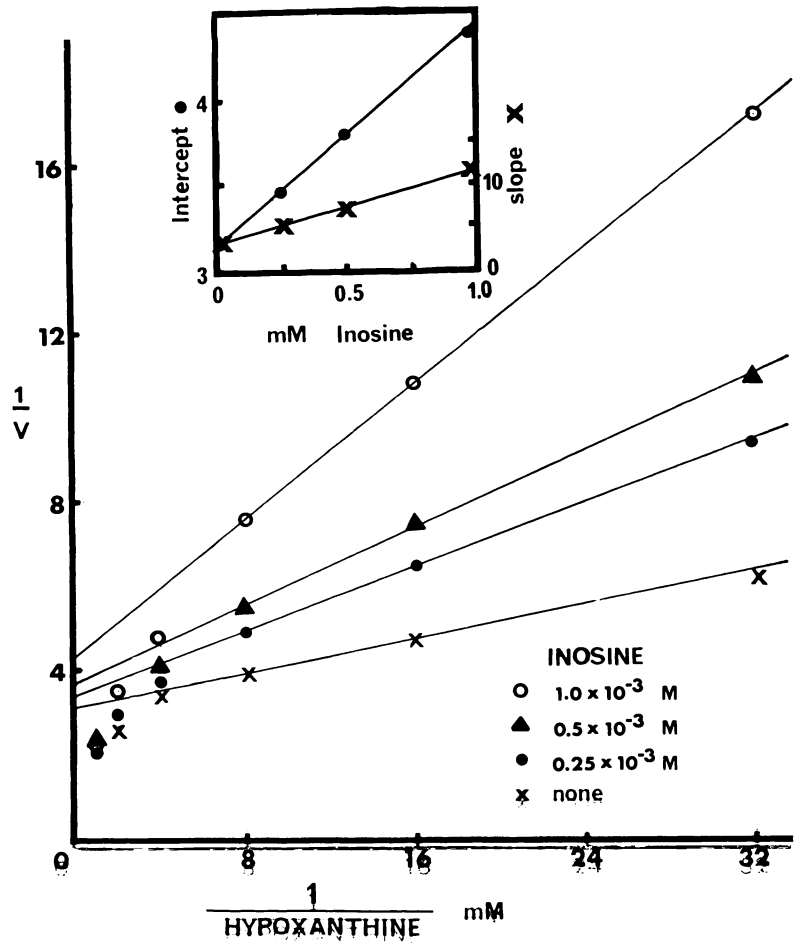

FIG. 7. *Product inhibition by inosine*

Double reciprocal plot of initial velocity against hypoxanthine concentration. Reaction mixture concentrations of ribose 1-phosphate, Tris-hydrochloride buffer, and enzyme protein were 2.0 mM, 0.08 M, and 18.5 μg/ml, respectively. Incubations were carried out for 15 min. v is expressed as micromoles × 10^2 of inosine synthesized per milliliter per minute.

tion between competitive and noncompetitive inhibition cannot be made from the data. Fitting by the method of least-squares yielded lines which had close but not identical ordinate intercepts.

Substrate Specificity

Table 2 presents the rates of ribonucleoside synthesis catalyzed by crystalline purine nucleoside phosphorylase from ribose 1-phosphate and ^{14}C-base, both at 1 mM concentration. Guanine and hypoxanthine were the best substrates. Under these conditions, allopurinol was converted to the ribonucleoside at approximately 1/10th the rate of its analog, hypoxanthine; and oxoallopurinol at approximately 1/3000th the rate of its analog, xanthine. 6-Mercaptopurine was converted to the ribonucleoside at 1/4th the rate of hypoxanthine. No detectable products were formed with azathioprine, adenine, or uracil.

Inosine, guanosine, and deoxyguanosine were effective pentosyl donors with both hypoxanthine and guanine in the presence of added inorganic phosphate (Table 3). No exchange could be detected with any combination of these donors and acceptors

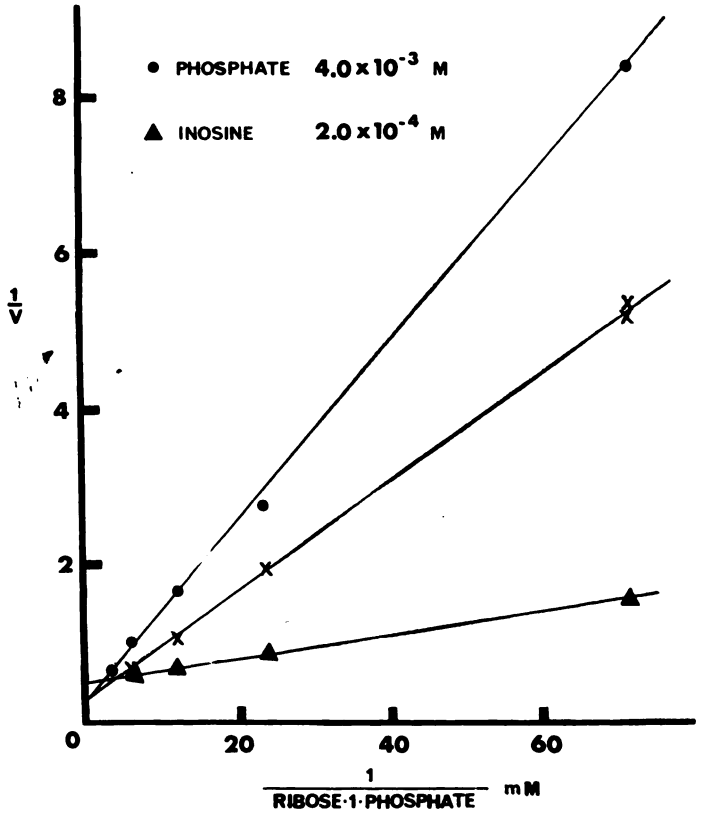

FIG. 8. *Product inhibition by inorganic phosphate and inosine*

Double reciprocal plot of initial velocity against ribose 1-phosphate concentration. Reaction mixture concentrations of ^{14}C-hypoxanthine, Tris-hydrochloride buffer, and enzyme protein were 0.206 mM, 0.08 M, and 18.5 μg/ml, respectively. Incubations were carried out for 10 min. v is expressed as micromoles $\times 10^3$ of inosine synthesized per milliliter per minute.

in the absence of added inorganic phosphate.

The crystalline purine nucleoside phosphorylase preparation contained no pyrophosphorylase or xanthine oxidase activity.

Inhibitors

Diisopropyl phosphorofluoridate (1 mM reaction mixture concentration) did not inhibit the catalysis by purine nucleoside phosphorylase of exchange between hypoxanthine and inosine or the synthesis of inosine from ribose 1-phosphate and hypoxanthine.

DISCUSSION

The nomenclature of Cleland (13) will be employed throughout this discussion. The nucleoside synthesis and phosphorolysis catalyzed by purine nucleoside phosphorylase involves two substrates and two products. The reaction will therefore be referred to as "Bi Bi."

The kinetic patterns in Figs. 2 and 3 fit the rate equation of the form:

$$v = \frac{V_{max}}{1 + K_A/(A) + K_B/(B) + K_{AB}/(A)\,(B)}$$

(A) and (B) represent the concentrations of the two substrates; K_A and K_B represent the corresponding Michaelis constants; while K_{AB} equals $K_A \cdot K_B$.

It is apparent from this rate equation that both the slopes and intercepts of the lines resulting from double reciprocal plots are functions of the concentration of either substrate when it is treated as the non-

TABLE 2
Specificity of purine nucleoside phosphorylase for ribonucleoside synthesis

Reaction mixtures had a final concentration of 1 mM ribose 1-phosphate, 1 mM ^{14}C-ribosyl acceptor, and 0.02 M Tris-hydrochloride buffer. The reactions were stopped after incubation for 20 min. With guanine, the reaction mixture concentration was 0.67 mM because of its low solubility.

^{14}C-ribosyl acceptor	Reaction mixture protein concentration (μg/ml)	Ribonucleoside formed (μmoles/ml/protein/min)
Guanine	0.30	61
Hypoxanthine	0.14, 0.30, 0.60	32
Xanthine	1.4	15
6-Mercaptopurine	1.4	8.5
Allopurinol	1.4, 6.0	3.1
Oxoallopurinol	300	0.005
Adenine	6.0	<0.04
Azathioprine	300	<0.0008
Uracil	300	<0.0008

TABLE 3
Specificity of ribosyl exchange reactions catalyzed by purine nucleoside phosphorylase

The final reaction mixtures (0.25 ml) had a concentration of 1 mM ^{14}C-hypoxanthine or 0.67 mM ^{14}C-guanine, 1 mM ribosyl donor, 0.08 M buffer, and 18.7 μg of crystalline purine nucleoside phosphorylase protein per milliliter. Controls lacked a ribosyl donor. Incubations were carried out for 20 min.

^{14}C-ribosyl acceptor	Ribosyl donor	^{14}C-Nucleoside formed %	
		Tris buffer	Phosphate buffer
Hypoxanthine	Inosine	<0.5[a]	9
Hypoxanthine	Guanosine	<0.5	6
Hypoxanthine	Deoxyguanosine	<0.5	4
Guanine	Inosine	<0.5	14
Guanine	Guanosine	<0.5	16
Guanine	Deoxyguanosine	<0.5	16

[a] Below limits of detectability of the method.

variable substrate. If a "ping pong" or shuttle mechanism were operative, the term

$$\frac{K_{AB}}{(A)\,(B)}$$

would be absent from the rate equation, and the kinetic patterns would be those in which the intercepts, but not the slopes of the lines, would be a function of the concentrations of the nonvariable substrate. The reaction mechanism must therefore be of a sequential type.

Product inhibition patterns of sequential Bi Bi mechanisms can distinguish among all but two mechanisms (13). The present data (Figs. 6–8) eliminate mechanisms involving more than one substrate-product pair which show competitive inhibition. This leaves two classes of sequential mechanisms for consideration: Ordered Bi Bi and Iso Ordered Bi Bi.[3] Each Ordered Bi Bi mechanism has two possible addition sequences, therefore four alternatives must be considered.

An Ordered Bi Bi mechanism where hypoxanthine initially combines with the free enzyme, is not consistent with the noncompetitive inhibition between inosine and hypoxanthine (Fig. 7). If this mechanism were operative, competitive inhibition should have been observed.

Ordered Bi Bi and Iso Ordered Bi Bi mechanisms involving the initial addition of ribose 1-phosphate are not consistent with the following considerations.

1. Inorganic phosphate is required in stoichiometric amounts for optimal ribosyl exchange (Fig. 1). In addition, arsenate cannot substitute for phosphate in this reaction (Table 1). These observations strongly suggest that a free ribose 1-phosphate intermediate is involved in ribosyl exchange reactions catalyzed by purine nucleoside phosphorylase.

2. Kalckar (14) observed that a purine nucleoside phosphorylase preparation, treated with activated charcoal to free it of any clinging base or nucleoside, catalyzed a slow exchange of radioactive phosphate with ribose 1-phosphate. This obser-

[3] An Iso Theorell-Chance mechanism is a limiting case of an Iso Ordered Bi Bi mechanism where the concentration of the ternary complex is nil. The data (Fig. 8) cannot rigorously distinguish between competitive and noncompetitive inhibition; and therefore between Iso Ordered and Iso Theorell-Chance mechanisms.

vation is difficult to explain by an exchange mechanism which involves free purine base and nucleoside intermediates. This observation, however, is consistent with a mechanism involving binary enzyme-base and enzyme-nucleoside intermediates.

3. Lampen observed (15) that arsenolysis of ribose 1-phosphate by purified nucleosidases requires a small amount of added base; that this arsenolysis was considerably more rapid than the arsenolysis of nucleosides; and that cleavage of the nucleosides was inhibited by concentrations of the free base which did not prevent arsenolysis of the ester. These observations are not consistent with mechanisms where ribose 1-phosphate combines with the free enzyme.

The remaining alternative, an Iso Ordered Bi Bi mechanism (Fig. 9) involv-

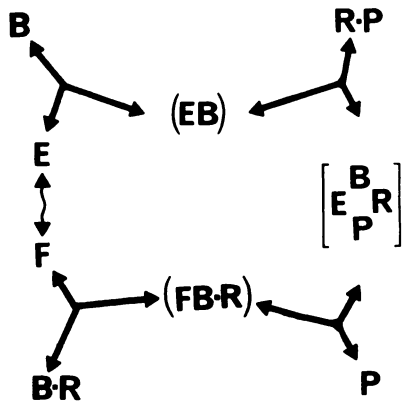

FIG. 9. *Schematic representation of an Iso Ordered Bi Bi mechanism for purine nucleoside phosphorylase*

.E and F represent isomeric forms of the free enzyme; P, phosphate; B, the purine base; and R, the pentosyl moiety.

ing an isomerization of the free enzyme and the initial combination of the purine base with one of the forms of the enzyme, is most consistent with the data available at the present time. This mechanism is strongly supported by the observations of Pinto and Touster (4) that a highly purified preparation of purine nucleoside phosphorylase exists in separable and interconvertible phosphorylase and transferase forms, and that hypoxanthine induces the transformation to the transferase form. The enzyme forms designated E and F in Fig. 9 would correspond to these transferase and phosphorylase forms, respectively. It would be of interest to investigate the possibility that the apparent product activation observed at high concentration of ribose 1-phosphate and hypoxanthine (Fig. 7) might be due to substrate effects on the isomerization between the phosphorylase and transferase forms of the enzyme.

Fiers and de Bersaques (16) have studied the kinetics of the arsenolysis of inosine catalyzed by a purine nucleoside phosphorylase preparation from human skin. They interpret their data as favoring a mechanism which involves the formation of a single ternary complex by ordered addition with the initial addition of phosphate.

Kim *et al.* (17) have recently proposed an ordered mechanism for purine nucleoside phosphorylase from human erythrocytes, in which the nucleoside is the first substrate to bind and the purine base is the last product to dissociate.

The specificity of purine nucleoside phosphorylase for ribonucleoside synthesis (Table 2) agrees with our previous finding (5) that allopurinol is a much better substrate for this enzyme than is oxoallopurinol. The inability of the S-substituted derivative of 6-mercaptopurine, azathioprine, to act as a substrate for this enzyme is consistent with the observations that the ribonucleoside of 6-methylthiopurine is not cleaved by cells which contain purine nucleoside phosphorylase (18, 19). It is also of interest that adenine is not effectively converted to a ribonucleoside by this enzyme. The observation that crystalline purine nucleoside phosphorylase can catalyze pentosyl transfer (Table 3) from both guanosine and deoxyguanosine is consistent with the conclusions of other workers that the activity toward deoxyribonucleosides and ribonucleosides are properties of the same enzyme (10, 11, 20, 21).

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