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City University of New York PH.D. 1987

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OROTATE PHOSPHORIBOSYLTRANSFERASE FROM BAKER'S YEAST: STUDIES OF THE INTERACTION WITH SUBSTRATES

BY

ROBERT WAYNE ASHTON

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.

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Dedicated to

Lorimer Astor Ashton,

Josephine Ena Ashton,

and Diane Marie Ashton.

Is there anything more wonderful on earth,

Our marvellous planet,

Than the miracle of man!

. . . . . .

He has mastered the mysteries of language:

And thought, which moves faster than the wind,

He has tamed, and made rational.

Political wisdom too, all the knowledge

Of people and States, all the practical

Arts of government he has studied and refined,

Built cities to shelter his head

against rain and danger and cold

And ordered all things in his mind.

—Sophocles

Acknowledgements

Some debts can never be repaid; they can only be passed on. Such a debt I owe to my mentor Dr. Donald L. Sloan who has guided me in my endeavor to become a scientist. His seemingly endless stores of patience and good humor have never ceased to amaze me, and will always inspire me.

I thank my committee, Dr. Sharon Cosloy, Dr. David Lavallee, Dr. Charlotte Russell, and Dr. Willaim Sweeny for their patience, interest, and advice. I would especially like to thank Dr. Russell for always having had the time to listen to my woes, chemical or otherwise, and for her guidance. My thanks, also, to Dr. Cosloy, in whose laboratory I was first introduced to *Salmonella typhimurium*; to Dr. Stanley Stein for the amino acid analysis; and to Dr. Charlie Grubemeyer for the use of his Hi-density fermentor. To Dr. Meyer Fishman, thanks for having the best MBRS program in the world, at City College.

To my former colleagues, Susan, Charlotte, Danyal, Aybar, Dean, Greg, Rosalyn, Cong, Sungyung, Raymond, Lewis, Lenny, and Linda, thanks for the memories! Thanks, Florence, for listening.

TABLE of CONTENTS

Schemes Page

INTRODUCTION

Our laboratory is concerned with the allocation of the 5-phosphoribosyl 1-pyrophosphate (PRibPP) pool among the ten enzymes (in yeast) called the phosphoribosyltransferases (Figure 1). The metabolic pathways utilizing these enzymes have a complex interrelationship due to their common substrate, PRibPP, and the interdependent relationship of the end products, the pyrimidine, purine, and pyridine nucleotides. Thus the study of these enzymes is of current interest, not only for the sake of basic knowledge, but also due to possible contributions to the chemotherapeutic treatment of cancer, parasitic diseases, and defects in purine or pyrimidine metabolism.

With regard to the metabolism of PRibPP, hyperuricemia, an excess of uric acid in the blood, has been shown to result from an increase in the PRibPP pool due to increased PRibPP synthetase (PRSase) activity which accelerates the *de novo* purine nucleotide synthesis pathway (Becker *et al.*, 1982). This pathway can also be accelerated by increased PRibPP aminotransferase activity resulting from a defect in allosteric control of the enzyme, an overactive enzyme, or by increased PRibPP levels (Kelly and Wyngaarden, 1974). Increased PRibPP levels can be caused by an increased rate of synthesis of PRibPP or by a defect in the salvage enzyme, hypoxanthine guanine phosphoribosyltransferase (HGPRTase), which is responsible for synthesis of quanine and inosine nucleotides (Lipstein *et al.*, 1978). Thus, one metabolic disease, hyperuricemia, which can lead to gout (Jones *et al.*, 1962), can result from any one of several enzymic defects in this complex system in which *de novo* purine, *de novo* pyrimidine, *de novo* pyridine and salvage pathways are interconnected.

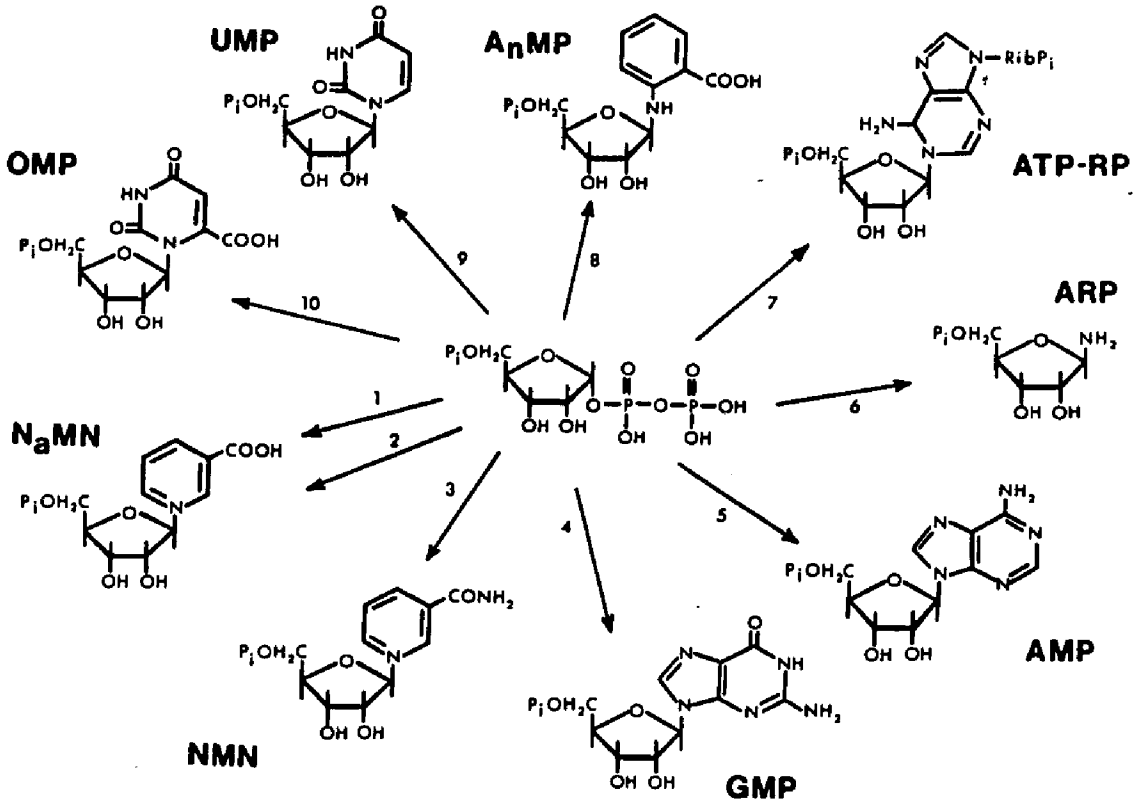
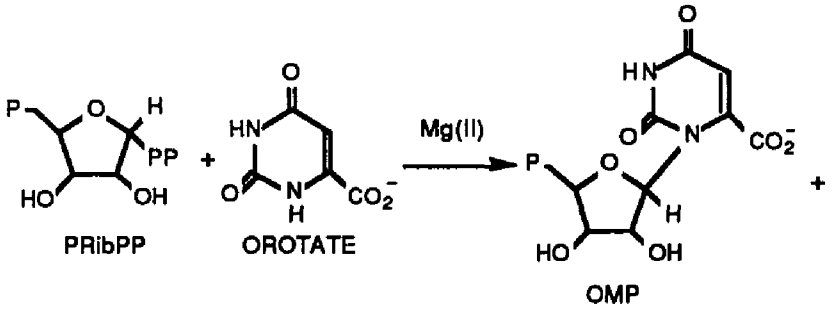


FIGURE 1. Phosphoribosyltransferase (PRTase)-catalyzed reactions and the metabolic fate of phosphoribosyl 1-pyrophosphate. The following enzymes are represented: 1) nicotinate PRTase, 2) quinolate PRTase, 3) nicotinamide PRTase, 4) hypoxanthine/guanine PRTase, 5) adenine PRTase, 6) glutamine-dependent aminotransferase, 7) ATP-PRTase, 8) anthranilate PRTase, 9) uracil PRTase and 10) orotate PRTase.

My role in this work has been the study of orotate phosphoribosyltransferase (EC 2.4.2.10) from *Saccharomyces cerevisiae* (Baker's yeast) and its interaction with its substrates, PRibPP and orotate. This enzyme links the PRibPP pool to the *de novo* pyrimidine nucleotide synthesis pathway by the following reaction:



(1)

This reaction was first observed in Baker's yeast by Lieberman *et al.*, (1955). The enzyme purifies as a dimer with a molecular weight of approximately 39,000 to 40,000 (Victor *et al.*, 1979a; Umezu *et al.*, 1971) and a sedimentation coefficient of 3.4 S (Yoshimoto *et al.*, 1978). The subunits have identical molecular weights of approximately 20,000 (Victor *et al.*, 1979a). Lieberman *et al.*, (1955) reported that yeast OPRTase (YOPRTase) has an absolute requirement for a metal ion activator, as has been found for this activity in all organisms studied since (Musick, 1981; Hammond and Gutteridge, 1983). Victor *et al.*, (1979b) have reported that YOPRTase is activated by Mg(II) or Mn(II). These investigators reported that Mn(II) binds to YOPRTase with a maximal stoichiometry of 4:1 (metal:enzyme), with cooperativity between the metal binding sites. Their kinetic analysis revealed that both Mg(II) and Mn(II) activated YOPRTase in a biphasic manner, suggesting that both a metal enzyme complex and metal free enzyme can catalyze the YOPRTase reaction. This is a Model III metal activation mechanism as described by London and Steck

(1969). Victor *et al.*, (1979b) performed proton relaxation rate enhancement studies that suggested an enzyme-substrate-metal bridge ternary complex was formed when PRibPP was incubated with enzyme and Mn(II). They also provided evidence that the PRibPP metal complex, which exists in a 1:1 ratio (Victor *et al.*, 1979b; Syed *et al.*, 1987) is the true substrate for YOPRTase.

Victor *et al.*, (1979a) determined that YOPRTase utilizes a Bi Bi Ping Pong kinetic mechanism, based on initial velocity studies, product inhibition studies, and half-reaction isotope exchange studies. They obtained parallel initial velocity reciprocal plots for both forward and reverse YOPRTase reactions and a pattern of competitive and mixed inhibition for the product inhibition studies. They also observed an exchange of [^{32}PP] into PRibPP, or [^{14}C]orotate into OMP when they examined the two separate half-reactions of YOPRTase. The partial exchanges required the presence of metal ions. These observations suggested a classic Bi Bi Ping Pong mechanism (Cleland, 1970), which would require the existence of an enzyme substrate intermediate (Douderoff *et al.*, 1947). Some of the other enzymes which display these characteristics are bacterial sucrose phosphorylase (Mieyal and Abeles,1972), acetate kinase (Anthony and Spector, 1972; Todhunter and Purich 1975), and nucleoside diphosphokinase (Morrison and Heyde, 1972). Also, the transaminases as a group catalyze their reactions *via* the ping-pong mechanism, utilizing the prosthetic group, pyridoxal 5-phosphate (PLP), to form a Schiff base intermediate in a binary enzyme-substrate complex. Glutamic oxaloacetate transaminase is a mechanistic prototype of this group of enzymes (Velick and Vavra, 1962).

Abeles and his colleagues' work with sucrose phosphorylase provides a case study of an enzyme utilizing the Ping-Pong mechanism with which to compare the

information that has been obtained from studies of YOPRTase. The reaction catalyzed by sucrose phosphorylase is as follows:

$$\text{SUCROSE+ Pi} \longleftrightarrow \text{FRUCTOSE + GLUCOSE-1-P} \qquad (2)$$

The sucrose phosphorylase reaction occurs with retention of configuration, with the α 1, 2 linkage of D-glucose to fructose being replaced by a α linkage to Pi. The OPRTase catalyzed reaction occurs with an inversion of configuration, with the C-1 carbon of PRibPP having an α configuration which becomes β in OMP. Whereas the double displacement mechanism expected for a Ping Pong mechanism nicely explains the action of sucrose phosphorylase, the inversion of configuration for the reaction catalyzed by YOPRTase can be explained by one of the following alternative mechanisms; an odd number of S_N2 displacements involving at least two enzyme residues and orotate; or an S_N1 dissociation of PPi to form a carbocation-like transition state or carbocation intermediate, followed by the front-side attack of an enzyme residue which is then displaced by the back-side attack of orotate.

Goitein *et al.*, (1978) have provided evidence for an S_N1 dissociation mechanism involving a carbocation-like transition state or a carbocation intermediate. They performed kinetic isotope experiments using PRibPP labeled with tritium at the C-1 carbon and obtained an α secondary effect of 1.17 for the YOPRTase reaction. This strongly suggests that a carbocation-like transition state or carbocation intermediate is involved in the rate limiting step of the reaction. Interestingly, they found that the HGPRTase and ATP-PRTase reaction mechanisms are also likely to proceed with a carbocation-like transition state or intermediate, although both of these enzymes are known to form ternary complexes with their substrates (Ali and Sloan, 1982; Morton and Parsons, 1976). Perhaps the observed order of binding and release of substrates

does not reflect the underlying chemistry of the catalytic mechanism utilized by the PRTases.

Abeles and his colleagues found it difficult to isolate a glucosyl-enzyme intermediate form of sucrose phosphorylase due to hydrolysis of the intermediate, which prevented it from accumulating. It proved necessary to denature the enzyme or chemically modify it in order to trap the intermediate (Voet and Abeles, 1970). These investigators mixed the enzyme with ^{14}C sucrose labeled in the glucose moiety and rapidly quenched the reaction by adjusting it to pH 3. The result was protein containing 1 to 1.9 moles of glucose per mole of enzyme (Detoma and Abeles, 1970). When the glucosyl enzyme was suspended in methanol only glucose was released, with no formation of methylglucoside. This result, along with the sensitivity of the intermediate to alkaline hydrolysis, lead to the conclusion that C-1 of the glucosyl moiety is bound to the oxygen atom of a carboxyl residue of the enzyme. In order to prove this a differential labelling experiment was performed with the acid denatured ^{14}C-glucosyl-enzyme complex (Detoma and Abeles, 1970). The complex was treated with carbodiimide and glycine ethylester in order to block the carboxyl groups of the enzyme. The glucosyl intermediate was then cleaved by a mild alkaline treatment and the enzyme was again exposed to carbodiimide, but now in the presence of labeled glycine ethyl ester. Detoma and Abeles observed the incorporation of the ^{14}C label. The control experiment was performed with enzyme that had not been exposed to substrate and no incorporation of label into the control enzyme occurred. These methods, while ingenious, did not demonstrate the existence of a catalytically competent intermediate. It was our plan to use ^{13}C-NMR to demonstrate the existence of such an intermediate at the active site of YOPRTase.

Chemical modification is a useful tool for studying an enzyme's catalytic mechanism. However, the results from this type of experiment must be cautiously

interpreted. At best it can provide a means to examine the nature of the residues located at the active site of an enzyme. Pyridoxal 5-phosphate (PLP) is a useful chemical modifier since it can be highly specific for active site lysines (Raetz and Auld, 1972). Lysine is often found at the active sites of enzymes that bind organophosphates (Means and Feeney, 1971). Because of this role it is not surprising that lysine may be located at the active site of several of the PRTases. Chemical modification experiments have indicated that lysine may be at the active site of hog liver quinolinate phosphoribosyltransferase (QPRTase), (Taguchi, H. and Iwai, K., 1976; Musick, 1981), rat liver hypoxanthine guanine phosphoribosyltransferase (HGPRTase), (Natsumeda *et al.*, 1977), and Ehrlich ascites adenine phosphoribosyltransferase (APRTase), (Gadd and Henderson, 1970). Grove and Levy (1979) have shown that though several lysines on the enzyme are modified, only one mole of PLP per mole of active site inactivates *Salmonella typhimurium* anthranilate phosphoribosyltransferase (Anth-PRTase), suggesting a single essential lysine at the active site. Histidine, cysteine and arginine have also been identified as playing roles at the active sites of the phosphoribosyltransferases. Sloan and Strauss (1984) found that diethylpyrocarbonate and *p*-bromophenacyl bromide each inactivated YOPRTase. They determined that PRibPP in the presence of Mg(II) protects against the inactivation caused by these modifiers, thus implicating histidine as an active site residue. Taguchi and Iwai (1976) have provided some evidence that histidine, cysteine, and arginine are at the active site of hog liver QPRTase. Rat liver HGPRTase is inactivated by sulfhydryl modifying reagents (Natsumeda *et al.*, 1977; Krenitsky, T.A. and Papaioannou, R., 1969) as is Ehrlich ascites HGPRTase (Krenitsky and Papaioannou, 1969). Ehrlich ascites APRTase has sulfhydryl groups that are susceptible to chemical modification and possibly involved in binding PRibPP (Gadd and Henderson, 1970). Uridine phosphoribosyltransferase (UPRTase), (found only in yeast and bacteria) is inactivated by

mercurials and protected against this inactivation by UMP (Natalini *et al.*, 1979). Umezu *et al.*, (1971) have reported that YOPRTase is unaffected by the sulfhydryl modifying reagents iodoacetate and *p*-chloromercuribenzoate.

Mouse Ehrlich ascites OPRTase has been shown to be associated with orotate decarboxylase (ODCase) to form the bifunctional protein UMP synthase (McClard *et al.*, 1980). Also, in the disease causing parasites *Trypanosoma cruzi* and *Crithidia luciliae* both OPRTase and ODCase are associated with glycosomes (Hammond and Gutteridge, 1983; Pragobpol *et al.*, 1984). Traut and Jones (1977; 1979) have reported that UMP synthase undergoes substrate and effector dependent association. They found that in the presence of OMP the 3.6 S monomer of UMP synthase associates to a 5.6 S dimer. However, in the presence of PRibPP, phosphate, or other anionic effectors the enzyme forms a 5.1 S dimer.

Jones and her colleagues have provided evidence for substrate channeling of the product of the OPRTase active site, OMP, to the ODCase active site, as a result of the fusion of the two activities (Traut and Jones, 1977; Traut and Jones, 1979). They have shown that the highly efficient coupling of the two activities accounts for the low steady state concentration of OMP found in mammalian tissues, 50 to 100 nM, as compared to a steady state concentration of 40 μM found in yeast (Hitchings, G. H., 1973). They have also shown that a conformational change in the complex occurs upon OMP formation. Traut (1982) has suggested that there were evolutionary pressures for the fusion of OPRTase and ODCase activities. He states that the reason for the efficient coupling of the two activities is the need to protect OMP from degradation by the high level of pyrimidine nucleotidase activity found in mammalian cells.

Substrate channeling may also be a consequence of the unfavorable equilibrium of the reaction catalyzed by OPRTase. OPRTase and ATP-PRTase are the only PRTases known to favor the pyrophosphorolysis reaction (Musick, 1981; Ismande and Handler,

1961). The equilibrium constants for the forward OPRTase reaction have been reported to be 0.12 (Lieberman *et al.*, 1955), 0.07 at pH 7.4 and 37°C (Traut and Jones, 1977) and 0.49 at pH 8 and 25°C (Victor *et al.*, 1979). The utilization of the ping pong mechanism by the yeast enzyme may be a development that more effectively couples the hydrolysis of pyrophosphate to the synthesis of OMP. The free energy ($\Delta G'$) for the overall YOPRTase forward reaction is positive with a upper limit of 1.6 kcal/mole, based on the K_{eq} value of 0.07 at pH 7.4 and 37°C. The $\Delta G'$ for the hydrolysis of pyrophosphate is –4.2 kcal/mole at pH 7 and 25°C (Segel, 1976). Therefore, the overall reaction leading to the formation of 1 mole of OMP and two moles of inorganic phosphate from 1 mole of PRibPP and 1 mole of orotate will be favored with a $\Delta G'$ (lower limit) of –2.6 kcal/mole. Though the synthesis of OMP is thermodynamically favored when coupled to the the hydrolysis of pyrophosphate, the question arises as to whether or not it is kinetically favorable under the *in vivo* steady state equilibrium conditions of the yeast cell. The OPRTase enzyme faces stiff competition for PRibPP, which must be allocated among all the PRTases. Although the PRibPP steady state concentration in yeast is not known, it is unlikely that it is significantly higher than the the yeast PRTases' Km values for this metabolite. These values range from approximately 20 to 50 μM (Musick, 1981). Were YOPRTase to utilize a mechanism requiring a ternary complex of substrates and products (i.e. an ordered bi bi, or random bi bi mechanism) it seems likely that the products OMP and PPi (with respective Km values of 8 and 96 μM, and Ki values of 10 and 131 μM, Victor *et al.*, 1979a), could be rapidly cycled back to PRibPP and orotate (with respective Km values of 38 and 35 μM, and Ki values of 42 and 63 μM, Victor *et al.*, 1979a) before their release from the active site to the medium. The amount of OMP synthesized by YOPRTase would be highly sensitive to the competition for PRibPP. The ping pong mechanism allows the coupling of the first partial reaction of

YOPRTase, the cleavage of PRibPP, to the hydrolysis of pyrophosphate. Switzer (1969) has reported a value of –7.0 kcal/mole for the hydrolysis of PRibPP at pH 7.5 and 25°C. The enzymatic cleavage should therefore be highly favored. We can use the average of the Km and Ki values given above to calculate estimated binding and dissociation ΔG' values for PRibPP and PPi of –6 and +5.4 kcal/mole respectively, and assume that the ΔG' of PRibPP hydrolysis is stored in the enzyme-PRib intermediate. Upon combining the estimated ΔG' values for PRibPP binding and PPi release with the ΔG' of hydrolysis of PPi we get an estimated ΔG' value of –5.6 kcal/mole for the first partial reaction of YOPRTase coupled to the hydrolysis of PPi. Therefore PPi is released from the active site and hydrolyzed by inorganic pyrophosphatase, in a highly favored reaction, prior to, or concurrently with, the binding of orotate and the formation of OMP. This renders the reversal of the reaction unlikely, due to the low levels of free PPi available for binding to OPRTase while OMP is still bound. This may be a variation on the common intermediate theme discussed by Lehninger (1971). In this case the common intermediate would be an enzyme-substrate intermediate in which part of the free energy of the hydrolysis of PRibPP is stored. Also, the formation of this intermediate is rendered irreversible by the hydrolysis of PPi.

The kinetic mechanism of the OPRTase activity of UMP synthetase is not known, however, McClard *et al.*, (1984) have suggested that it may differ from the YOPRTase mechanism. They base this suggestion on the differing ability of the enzymes to utilize the PRibPP analog 5–phosphorylribose 1-α-methylenebisphosphonate (PRPCP) as a substrate. These investigators have reported PRPCP to be a fair substrate for OPRTase from mouse carcinoma and human erythrocytes. The mouse enzyme had a Km value of 4.9 μM and a Vmax 11.4 fold less than the Vmax for PRibPP, when PRCPP was used as a substrate. The human enzyme had a Km value of 8 μM and a Vmax 8.2 fold less than the Vmax for PRibPP. They found PRCPP to be an extremely poor substrate

for YOPRTase. It had a Km value of 550 μM and a Vmax 940 fold less than the Vmax for PRibPP.

The only other PRTase known to favor the pyrophosphorolysis reaction, ATP-PRTase, utilizes an ordered sequential mechanism (Morton and Parsons, 1976; Kleeman and Parsons, 1976). Also, evidence has been provided for the use of a Bi Bi Ping-Pong kinetic mechanism by two other PRTases, UPRTase and APRTase (Natalini *et al.*, 1979; Thomas *et al.*, 1973; Hochstadt, 1978). Neither of these enzymes is readily reversible (Natalini *et al.*, 1979; Hori and Henderson, 1966). Thus any attempt to rationalize YOPRTase's use of a ping-pong mechanism remains highly speculative.

YOPRTase exhibits a high degree of specificity for its substrates. As discussed above a single PRibPP analog, PRPCP, has been reported to be a fair substrate for mammalian OPRTase, but an extremely poor one for YOPRTase (McClard *et al.*, 1984). The mammalian OPRTase enzyme is known to utilize the following pyrimidines or analogs as substrates, uracil, thymine, 6-azathymine, 6-azauracil, 5-fluorouracil, 5-iodouracil, uric acid, xanthine, 2, 4-dihydroxypteridine (Silva and Hatfield, 1978), 5-fluoroorotate (Dahl *et al.*, 1959), 5-azaorotate (also called oxonic acid) (Cihak and Sorm, 1972), and orotic hydrazide (Krauss *et al.*, 1983). The YOPRTase enzyme is more discriminating in its pyrimidine base requirements and most of the compounds listed above are not substrates for YOPRTase. 5-Fluoroorotate has been demonstrated to be an alternate substrate for purified YOPRTase (Victor *et al.*, 1979a), and no studies are known that examine 5-azaorotate or orotic hydrazide as substrates for YOPRTase. Figure 2 illustrates a number of orotate analogs that have been examined as possible substrates for OPRTase. Of the derivatives halogenated at position 5, only 5-fluororotate is a substrate, with a Km of 27 μM (Victor *et al.*, 1979a), the chloro, bromo, and iodo derivatives having no activity (Dahl *et al.*, 1959).

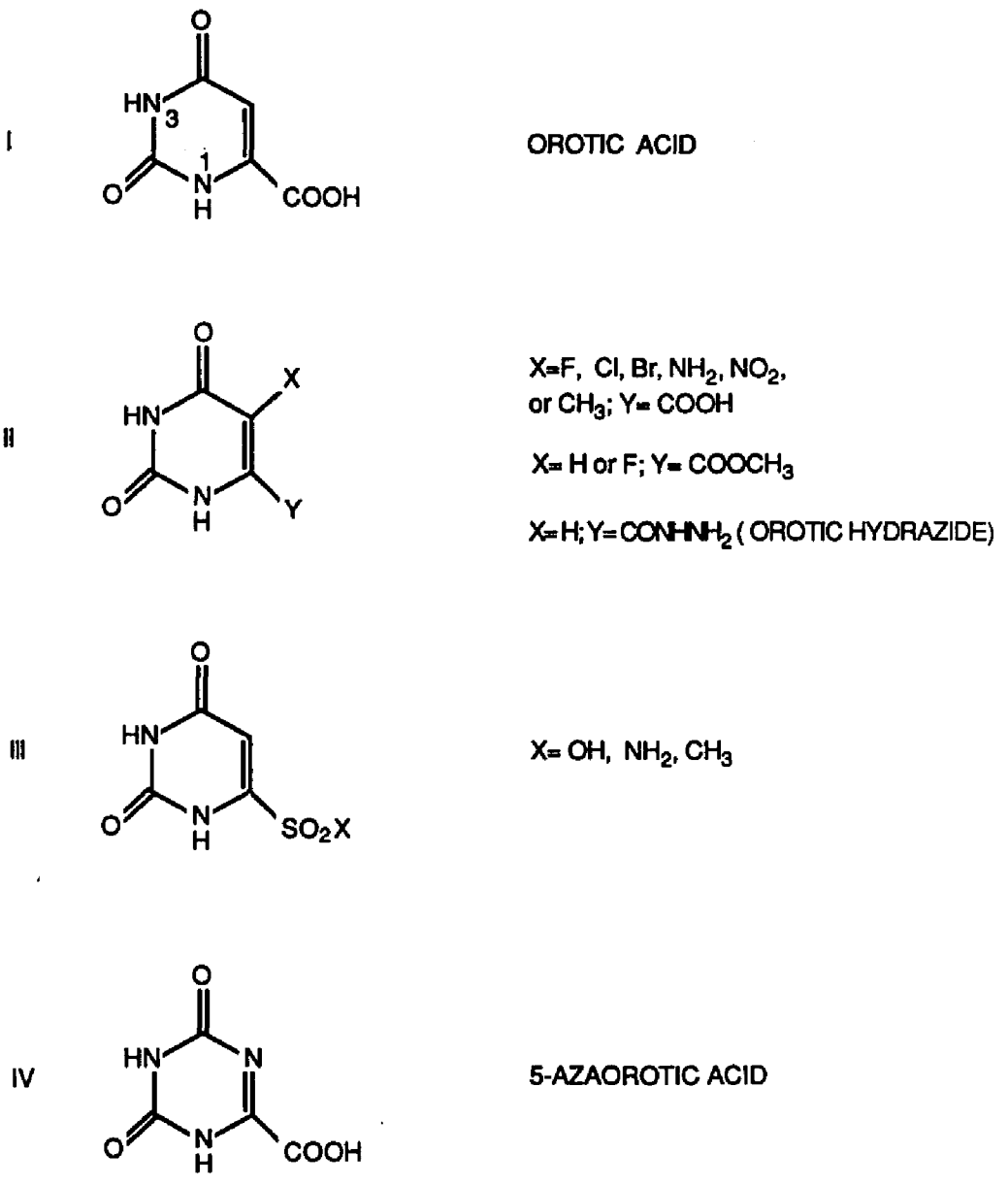


FIGURE 2. Orotic acid and substrate analogs that have been examined as substrates for OPRTase.

Dahl showed that when groups bulkier than hydrogen or fluorine, such as amine, nitro, or methyl groups, are substituted at position 5 (Figure 2, compound II) substrate activity is lost. The sulfonated derivatives shown in Figure 2 (compound III) uracil 6-sulphonic acid, uracil 6-sulphamide, and uracil 6–methylsulfone, are not substrates for, but do inhibit, OPRTase (Holmes, 1956). Cooper (1958) found that uracil 6-sulphonic acid and it's derivatives inhibit by reacting irreversibly with cysteines of OPRTase. Niedzwicki *et al.*, (1984) have reported a set of structure-activity relationships for orotate analogs that interact with mouse liver OPRTase. Their general findings are as follows; substitution at N1 diminishes binding; the oxo group at position 2 is required; binding is enhanced when N3 is replaced by an endocylic oxygen or a methylene group and abolished when substituted with a methyl group; the oxo group at position 4 is required; electron-withdrawing functional groups at C5 enhance binding as does replacing C5 with an endocyclic imino group; and substitution of the C6 carboxyl group with the methylester of a carboxyl group enhances binding, as does substitution with a hydroxyl or a methylsulfone group.

The regulation of OPRTase has been extensively studied in *Escherichia coli* and *Salmonella typhimurium*. Based on their studies of *Salmonella typhimurium* mutants that required uracil and arginine or cittruline for growth, Back and Woods (1953) proposed that uracil represses the formation of an enzyme (or enzymes) involved in pyrimidine synthesis. Yates and Pardee (1957) suggested that uracil, or an end product derived from it, controlled the levels of the enzymes involved in *de novo* pyrimidine nucleotide synthesis in *E. coli*. They studied *E. coli* mutants lacking in dihydroorotase (DHOase) or dihydroorotic acid dehydrogenase (DHOdeh). When these mutants were grown on a medium lacking uracil, Yates and Pardee found high levels of the enzymes specific for orotic acid synthesis, aspartate transcarbamylase (ATCase), DHOase, DHOdehase, OPRTase, and ODCase. Upon adding uracil to the

medium these enzymes' levels were repressed to normal "constitutive" levels. Beckwith *et al.*, (1962) believed that their studies with *Salmonella typhimurium* UMP kinase "leaky" mutants indicated that DHOase, DHOdehase, OPRTase and ODCase were linked and coordinately expressed, while ATCase was separately controlled. They attributed differential changes in the various enzyme activities to differing degrees of enzyme inhibition. Dennis and Herman (1970) provided evidence that the synthesis of DHOdehase and OPRTase were not coordinately controlled. Speculation that these enzymes were under the control of a single operon was ended by the findings that the genes for these enzymes were unlinked, and scattered, on both the *E. coli* and *Salmonella typhimurium* chromosomes, (Beck and Ingraham, 1971; Taylor and Trotter, 1972; Sanderson,1972). From their studies with *Salmonella typhimurium* mutants, Schwartz and Neuhard (1975) proposed that ATCase, OPRTase, and ODCase expression were controlled by a uridine containing nucleotide in a noncoordinate manner, and that a cytidine nucleotide controlled the expression of DHOase and DHOdehase in a coordinate manner. They suggested that uridine and cytidine di- or tri--phosphates were the regulatory agents. Kelln *et al.*, (1975) examined mutants deficient in uridine monophosphate kinase, cytidine deaminase and cytidine triphosphate synthetase. These mutants allowed them to examine the effects of inadequate cytidine and uridine nucleotide pools separately, and without generalized pyrimidine starvation. They found that DHOase and DHOdehase were subject to repression by cytosine (or a derivative), OPRTase and ODCase were subject to repression by uracil (or a derivative), and ATCase was subject to derepression by cytosine (or a derivative) and repression by uracil (or a derivative). They were not able to correllate these activity levels with nucleotide pool size and suggested that UTP and CTP were not the regulatory agents. Jensen (1979) found that *Salmonella typhimurium* mutants deficient in IMP dehydrogenase, and therefore GTP deficient,

had elevated UTP levels and lowered CTP levels. The levels of ATCase, OPRTase, and ODCase were elevated while DHOase and DHOdehase levels were lowered. Upon adding guanine to the growth medium normal pyrimidine metabolism and enzyme levels were established. Based on these findings, Jensen proposed that a normal purine nucleotide pool size was essential for repressive pyrimidine effects to be observed. He pointed out that the failure of Kelln *et al.*, (1975) to observe a correllation between CTP and UTP levels and pyrimidine enzyme levels may have been due to the high ATP and GTP levels that were present in the mutants studied by Kelln and his coworkers. Despite repeated attempts, none of these investigators had been able to isolate a mutant in which the regulation of the pyrimidine nucleotide synthesis enzyme levels could be shown to be irreversibly decoupled from the pyrimidine nucleotide pool. Then Jensen *et al.*, (1982) reported the isolation of a *Salmonella typhimurium* mutant which simultaneously had high levels of UTP and CTP and high levels of ATCase, OPRTase, and ODCase, while DHOase and DHOdehase were unaffected. Jensen and his coworkers determined the cause to be a mutation in one of the subunits of RNA polymerase and suggested that the expression of these enzymes was controlled by an attenuation mechanism.

Poulsen *et al.*, (1983) have isolated and sequenced the *E. coli* OPRTase gene. They found that the leader sequence of this gene contains what may be a *rho*-independent transcriptional terminator sequence (a G-C rich region capable of forming a stem-loop structure), preceded by a thymidylate rich region. This is convincing evidence that the expression of *E. coli* OPRTase is under transcriptional control and regulated by an attenuation mechanism. They also found evidence that OPRTase is expressed coordinately with another protein of unknown function. Poulsen *et al.*, (1984) have asserted that the reading frame containing OPRTase and the unknown protein constitute an operon, with the unknown protein transcribed first. Initiation of

transcription of this operon was found to be independent of pyrimidine pool levels. However, pyrimidine pool levels were directly related to the rate of termination of transcription in the intercistronic space between the end of the unknown protein's gene and the start of the OPRTase gene.

The following thesis reports several studies of substrate and substrate analog interactions with YOPRTase. These studies were initiated in order to further characterize the underlying chemistry of the reaction catalyzed by YOPRTase. I have examined the kinetics of the YOPRTase catalyzed reaction with 5-azaorotic acid as substrate; enzymatically synthesized [1-^{14}C]PRibPP and [1-^{13}C]PRibPP, and studied their interaction with YOPRTase by means of binding studies and ^{13}C-NMR. The specific goals of the ^{13}C-NMR studies were to characterize an enzyme-substrate intermediate at the active site of YOPRTase and to learn how such an intermediate is stabilized. Chemical modification studies were performed with uracil 6–aldehyde, an analog of orotic acid and a novel chemical modifier for lysine residues. Pyridoxal 5-phosphate, a classical chemical modifier of lysine residues, was also used to examine the role of lysine at the active site of YOPRTase. HPLC gel filtration studies were performed to examine the quaternary structure of YOPRTase under varying conditions of protein concentration and substrate availability.

MATERIALS AND METHODS

1) PURIFICATION OF OROTATE PHOSPHORIBOSYLTRANSFERASE FROM YEAST

Materials. Adipic dihydrazide, ammonium sulfate, bovine serum albumin (BSA), orotic acid, orotidine 5'-monophosphate (OMP), PRibPP, sodium periodate, and Tris-HCl were purchased from Sigma Chemical Co.; magnesium chloride, manganese chloride, n-octanol, potassium phosphate, and toluene from Fisher Scientific Co.; DEAE cellulose and hydroxylapatite from Bio-Rad Laboratories; Blue Sepharose, CNBr-activated Sepharose 4B and Sephacryl S-200 from Pharmacia; potassium acetate from Baker and Adamson products.

a) Autolysis. Ten lbs. of Baker's yeast (*Saccharomyces cerevisiae*, Budwieser brand from Valente Yeast Co., Flushing N. Y.) was dispersed into 3 l of 0.3 M potassium phosphate buffer, pH 8, at 30°C. The yeast suspension was gently mixed with a motorized stirrer and 500 ml of toluene was added to begin the autolysis. The pH was monitored and maintained at a value of 8 with 5 N KOH. When the pH remained constant (about 3 hrs after adding toluene) the autolysate was placed in a cold room at 4°C and left there overnight, without stirring. The next day the autolysate was spun using stainless steel cups in a Sorvall RC-5 centrifuge with a GSA rotor (unless otherwise stated the GSA rotor is used throughout the purification), at 9,000 RPM for 20 min. at 4°C. Unless otherwise stated all the following procedures, except the centrifugations, took place in a cold room at 4°C.

b) Ammonium Sulfate Precipitation. To prevent foaming, 5 ml (approximately 0.1% of the preparation volume) of n-octanol was added to the 9.2 l of autolysate, which was adjusted to pH 5 with 8 N acetic acid. After the adjustment the autolysate

volume was 9.63 l.

Ammonium sulfate (3,014 g) was slowly added to the 9.63 l of autolysate, with gentle stirring. When all the ammonium sulfate had dissolved the solution was left to sit overnight, without stirring. The next day the solution was spun at 9,000 RPM for 20 min. The supernatant had no detectable YOPRTase activity. The 0-50% (at 4°C) pellets were recovered and reconstituted with 2.5 l of 25 mM Tris-HCl, pH 8. The suspension was adjusted to pH 8 with 1N KOH, then dialyzed overnight, against 18 l of 10 mM Tris-HCl, pH 8, and again, against 20 l of the same buffer. The dialysate was spun at 9,000 RPM for 20 min. The supernatant volume was 3.1 l.

c) Ethanol Fractionation. To remove nucleic acids the solution was made 50 mM in $MnCl_2$ by the addition of 163 ml of 1 M $MnCl_2$ with stirring. Thereafter, the solution was spun for 1 hr at 9,000 RPM for 20 min. The volume of the preparation was now 3.3 l.

The following procedure was performed outside of the coldroom. A 750 ml volume of the preparation was removed and mixed with 100 ml of 2 M potassium acetate, pH 6, and 10 mM orotate. The solution was cooled to 1°C in a salt ice-water bath and 180 ml of 95% ethanol (EtOH) (all EtOH used in this procedure was cooled to -32°C prior to use) was slowly added with stirring. After the addition of the first 50 ml of EtOH the solution temperature was allowed to drop to -2°C. After all the EtOH was added the temperature was brought to -5°C or slightly below and the 15% EtOH solution was spun at 9,000 RPM for 10 min. at -10°C. The supernatant was placed in an acetone dry-ice bath and maintained at -10°C as 820 ml of 95% EtOH was slowly added. The 50% EtOH solution was then spun at 9,000 RPM for 10 min. at -10°C or below. These steps were repeated for the remaining enzyme preparation with solution volumes proportionally adjusted depending on the amount of remaining enzyme preparation. The recovered 50% EtOH pellets were reconstituted in 200 ml of 25 mM

Tris-HCl, pH 8. The suspension was adjusted to pH 8 with 1 N KOH, then stirred for 1 hr. It was dialyzed overnight against 18 l of 25 mM Tris-HCl, pH 8. The final volume was 980 ml, which was made 2 mM in $MgCl_2$ and 1 mM in orotate; each was added directly and the solution was stirred for 3 hrs.

d) Heat Treatment. The heat treatment was performed by placing, at most, 200 ml of the enzyme preparation in a stainless steel centrifuge cup then bringing it to 53°C in a hot water bath for 5 min. Precipitate was removed by centrifugation at 9,000 for 20 min. and discarded. The supernatant from the heat treatment was separated into 50 ml aliquots and frozen for storage.

e) Molecular Sieve Chromatography. The heat treated YOPRTase preparation was applied, 50 ml at a time, to a Sephacryl S-200 column equilibrated with buffer containing 10 mM Tris-HCl, pH 8, and 1 mM orotate. NaCl, which is a buffer component in the gel chromatography procedure described by Victor *et al.*, (1979a) and Umezu et al (1971), was not used, eliminating the need for a dialysis prior to DEAE cellulose chromatography. YOPRTase activity eluting from the column was pooled and frozen for storage.

f) DEAE Cellulose Chromatography. Approximately 250 ml of the YOPRTase preparation was applied to a DEAE column (25 x 2.5 cm) equilibrated with buffer containing 10 mM potassium phosphate, pH 8, and 1 mM orotate. The column was developed with 200 ml of the equilibration buffer and a 900 ml linear gradient of 10 to 200 mM potassium phosphate, pH 8, with 1 mM orotate, was applied. YOPRTase eluting from the DEAE column was pooled and concentrated with an Amicon ultrafiltration unit fitted with an Amicon PM10 membrane. The concentrated solution was dialyzed overnight against 10 l of 10 mM potassium phosphate, pH 8, with 1 mM orotate, then frozen for storage.

g) Hydroxylapatite Chromatography. A 100 ml volume of YOPRTase preparation was placed on a hydroxylapatite column (25 x 2.5 cm) previously equilibrated with 10 mM potassium phosphate, pH 8, and 1 mM orotate. The column was developed with 100 ml of the same buffer then a 500 ml linear gradient of 10 to 200 mM potassium phosphate, pH 8, with 1 mM orotate, was applied. Eluting fractions containing YOPRTase were pooled and concentrated. The concentrated enzyme solution was frozen for storage.

h) Blue Sepharose Chromatography. The YOPRTase from the previous step (36 ml) was dialyzed against 4 l of 10 mM Tris-HCl, pH 8, at 4°C for 4 hr. This was followed by an overnight dialysis against a fresh change of the same buffer. A 10 ml aliquot of YOPRTase was applied to a Blue Sepharose column with a gel bed of 58 ml, and equilibrated with 10 mM Tris-HCl, pH 8. The column was washed with 200 ml of 10 mM Tris-HCl, pH 8, and YOPRTase was eluted with a 6 ml aliquot containing 10 mM Tris-HCl, pH 8, 10 mM PRibPP, and 10 mM $MgCl_2$. A total of three columns were run. Eluting fractions containing YOPRTase activity were pooled and concentrated to 9.5 ml.

i) OMP Sepharose Chromatography. The YOPRTase from the Blue Sepharose step was combined with several YOPRTase samples from previous purifications. This mixture was dialyzed against 100 mM potassium phosphate, pH 7.2, prior to its application to an OMP sepharose affinity column. OMP sepharose was prepared from OMP, adipic dihydrazide, CNBr-activated Sepharose 4B and sodium periodate according to the method of Dodin (1981). After the sample application, the column was washed with 30 ml of 100 mM potassium phosphate, pH 8, followed by a wash with 10 mM potassium phosphate, pH 8, until the absorbance at 280 nm dropped to baseline. The YOPRTase was eluted from the column with a 10 ml aliquot of 17 mM PRibPP and 17 mM $MgCl_2$.

j) BIO-SIL TSK HPLC Gel Filtration. YOPRTase, from the OMP Sepharose chromatography step, that was to be used in the experiments examining YOPRTase subunit association, was applied to a Bio-Gel TSK-250 HPLC gel filtration column purchased from Bio-Rad. The running buffer was 100 mM TEA, pH 6.8, and the flow rate was 1 ml/min. Fractions were collected with a LKB Redirac fraction collector.

k) Spectroscopic Assay. YOPRTase was assayed by following the decrease in absorbance at 295 nm, as orotic acid and PRibPP are utilized to form OMP, with a Cary 15 recording spectrophotometer. A typical assay mixture contained 50 mM Tris-HCl, pH 8, 100 μM PRibPP, 300 μM orotic acid, and 1 mM $MgCl_2$ in a final volume of 1 ml. For specific activity determinations the PRibPP concentration was raised to 1 mM and YOPRTase was diluted in 50 mM Tris-HCl, pH 8, containing 1 mg/ml BSA.

l) Bio-Rad Protein Assay. All protein assays were performed with the Bio-Rad protein determination kit using the Bio-Rad Protein Standard II (bovine serum albumin) as the calibration standard.

2) PRibPP SYNTHETASE PURIFICATION

Materials. Ammonium phosphate, EDTA, glucose, ribose 5-phosphate (R5P), streptomycin sulfate, and triethanolamine were purchased from Sigma Chemical Co.; acetic acid from Fisher Scientific Co.; sodium fluoride from J. T. Baker Chemical Co.

PRibPP synthetase was purified according to the method of Switzer and Gibson (1978). Salmonella LT-2 was cultured in the 100 l fermentor at Hunter College, with the kind permission and advice of Dr. W. Sweeney. The incubation was performed at 37°C on the E medium of Vogel and Bonner (1956) containing 0.5% glucose. The cell

yield was 617 g, of which 215 were used for the isolation of PRibPP synthetase. The remainder was frozen and stored.

a) Sonication. The cells were suspended in 40 ml of 50 mM potassium phosphate, pH 7.5 (extract buffer), and sonicated, with a Sonifier Cell Disrupter (Model W185 from Heat Systems-Ultrasonics Inc., 150 watts maximum), 40 ml at a time for 4 x 20 sec, with cooling on ice. The solution was diluted further, with 600 ml of the extract buffer, to 1 l, prior to being spun at 10,000 RPM (GSA rotor) for 60 min. The supernatant volume was 1 l.

b) Heat Treatment. A 100 ml volume of 10% (W/V) streptomycin sulfate in distilled water was added dropwise with stirring to the supernatant from the previous step. The solution was then heat treated as follows: 200 ml of the solution was placed in a stainless steel centrifuge can and rapidly brought to 54°C by heating it in a boiling water bath. The solution was then maintained at 55°C in a warm water bath, for 5 min. At the end of 5 min the can was placed in an ice-water bath in order to rapidly cool the solution. The heat treated solution was spun at 10,000 RPM, yielding a supernatant of 1,030 ml.

c) Ammonium Sulfate Precipitation. To the supernatant from the previous step, a 215.3 g quantity of solid ammonium sulfate was slowly added with stirring. After the salt dissolved the solution was allowed to sit overnight in a cold room, on ice, without stirring. The solution was spun at 10,000 RPM and the pellet was dissolved in 55 ml of 50 mM potassium phosphate, pH 7.5, containing 25% ammonium sulfate. This solution was quick-frozen in a dry ice-acetone bath and stored at -20°C.

d) First Acid Precipitation. The solution from the previous step was thawed. It was then adjusted to pH 4.6 by the dropwise addition of 1 N acetic acid, while being held at 0°C in an ice-water bath. The solution was then spun at 17,000 g at 4°C for 10 min in the SS-34 rotor (this rotor was used for all the remaining centrifugations in this

procedure). The supernatant was discarded and the precipitate was resuspended in 45 ml of 50 mM potassium phosphate, pH 7.5. This solution was quick-frozen as above.

e) Second Ammonium Sulfate Precipitation. The solution from the second step was thawed and spun at 17,000 g for 10 min to remove any precipitate. One-half volume of 50 mM potassium phosphate, pH 7.5, saturated with ammonium sulfate, was added to the solution and the mixture was allowed to stand on ice for 10 min. It was then spun for 15 min at 17,000 g at 4°C. The supernatant was discarded and the precipitate suspended in 50 mM potassium phosphate, pH 7.5, at 18% saturation with ammonium sulfate (100 g/l). The solution was triturated for 15 min with a glass rod then spun at 17,000 g for 20 min at 4°C. The supernatant was discarded and the pellet was suspended in 40 ml of 50 mM potassium phosphate, pH, 7.5. The solution was quick-frozen as above.

f) Second Acid Precipitation. The solution from the previous step was thawed and spun for 10 min at 17,000 g at 4°C. The precipitate was discarded and the supernatant was treated as in the first acid precipitation. The pellet was suspended in 25 ml of 50 mM potassium phosphate, pH 7.5, and quick-frozen as before.

g) Blue Sepharose. The solution from the previous step was thawed and applied to a Blue Sepharose column equilibrated with buffer containing 50 mM triethanolamine, 50 mM potassium phosphate, pH 7.5, and 2 mM $MgCl_2$. The column was washed with two column volumes of the above buffer, then with 5 column volumes of 50 mM potassium phosphate, pH 7.5. Enzyme was eluted with 100 ml of 50 mM potassium phosphate, pH 7.5, containing 25 mM each of $MgCl_2$ and 25 mM ATP. The fractions containing protein were pooled and brought to 75% saturation with ammonium sulfate to precipitate the protein. The solution was allowed to sit overnight at 4°C and the pellet was collected the following day by centrifugation. The pellet was suspended in

10 ml of 50 mM potassium phosphate, pH 7.5, separated into 1 ml fractions, and quick-frozen.

h) HPLC Assay. PRibPP synthetase was assayed by using HPLC to follow the decrease in ATP and the increase in AMP as ATP and ribose 5-phosphate (R5P) are utilized to form PRibPP. A Waters HPLC instrument equipped with a Model 600A solvent delivery system, Model U6K sample injector, Model 440 absorbance detector, and a Houston Omniscribe chart recorder were used for this study. A Sepralyte SAX 5 μM quaternary amine column, purchased from Analytichem International, was used with an isocratic elution buffer of 0.1 M ammonium phosphate, pH 2.7, at a flow rate of 1 ml/min. The assay mixture contained 50 mM triethanolamine, 50 mM potassium phosphate, pH 8, 2.0 mM ATP, 5.0 mM R5P, 10.0 mM $MgCl_2$, 0.37 mM EDTA, and 25.0 mM NaF in a final volume of 1 ml. Enzyme samples (2 to 10 μl) were added to 1.0 ml of the assay mixture and the assays were incubated for 30 min with aliquots removed at 2.5, 5, 15, and 30 min. The reaction was quenched by heating the aliquots in a boiling water bath for 2 min. Twenty μl aliquots were injected into the HPLC, at a flow rate of 1 ml/min.

3) RIBOKINASE PURIFICATION

Materials. Antifoaming A Emulsion, ATP, deoxyribose, dithiothreitol (DTT), lactate dehydrogenase, magnesium sulfate, NADH, phosphoenol pyruvate, protamine sulfate, pyruvate, pyruvate kinase, and ribose were purchased from Sigma Chemical Co.; bacto-tryptone, tryptone, and yeast extract from Difco Laboratories; ethyleneglycol, ferrous ammonium sulfate, glycerol, and n-butanol from Fisher Scientific Co.; Dowex-AG1-X8 from Bio-Rad Laboratories; Phenyl Sepharose from

Pharmacia; [1-^{14}C]ribose from Amersham Corp.; and Hydrofluor from National Diagnostics.

a) Incubation. Salmonella-LT-2 for the ribokinase purification was grown and harvested as follows. An overnight incubation was prepared by inoculating, after autoclaving, a 50 ml volume of medium containing 1g casamino acids, 1.4 g K_2HPO_4 and 0.6 g KH_2PO_4. The Salmonella-LT-2 strain used was kindly provided by Dr. S. Cosloy (City College), who also advised me in its handling. L-Broth was prepared with 10 g tryptone, 5 g yeast extract, 5 g NaCl, and was made up to 1 liter with water. It was inoculated with the overnight culture and separated into 4 parts for a second overnight incubation. Hi-density L-Broth was prepared by mixing 80 g of bacto-tryptone, 40 g yeast extract, 20 g NaCl, 20 ml 0.1 M $CaCl_2$, 20 ml 1 M $MgSO_4$, and 4 ml 10 mM $Fe(NH_4)(SO_4)_2$ with water to give a total volume of 4 l. The medium was placed in a Lab-Line Hi-Density Fermentor. The incubation vessel was set to rotate at 370 RPM and the temperature was maintained at 35°C. The medium was then inoculated with the 1 l overnight culture. Aliquots were removed at intervals to check the Klett and pH values. The Klett reading taken immediately following the inoculation was 66 and the pH was 6.95. Fifteen minutes after the inoculation, 50 ml of 50% glycerol and 50 ml of 1 M dibasic sodium phosphate were added. After three hr, when the Klett had reached 550, a 4.2 g quantity of deoxyribose in 30 ml of water (this solution was previously autoclaved), was added to the incubation. After 30 min the Klett was 620 and the pH 6.99. One hundred ml of a 1M dibasic sodium phosphate solution was added. After another 30 min the Klett was 670. One hr after the addition of the sodium phosphate the Klett was 660. At this point the incubation was ended and the cells harvested. A few drops of Antifoaming A Emulsion were added and the culture was poured into a large Erlenmeyer flask which was placed in an ice bath. The cells were collected by centrifugation at 7,500 RPM for 10 min at 4°C in a Sorvall RC-5B centrifuge using a GSA rotor. The final cell yield was 178 g, which was frozen

before proceeding with the isolation of ribokinase. Dr. C. Grubemeyer (New York University) graciously allowed me the use of his Lab-Line Hi-Density Fermentor and advised me in its use.

b) Sonication. Forty-eight grams of cells were suspended in 100 ml of extract buffer containing 50 mM triethanolamine-HCl, pH 8.1, 1 mM EDTA and 1 mM dithiothreitol. Forty ml aliquots were sonicated (4 x 15 sec), using a Heat Systems-Ultrasonics Sonifier Cell Disrupter, with cooling on ice between sonications. The resulting suspension was spun for 90 min at 13,200 g and the pellet was discarded. The supernatant was made up to 290 ml with 140 ml of extract buffer. A Bio-Rad protein determination assay gave a value of 14.8 mg/ml for the cell extract, which was diluted to 10 mg/ml by the addition of 175 ml of extract buffer.

c) Protamine Sulfate Precipitation. A 0.5% protamine sulfate solution in extract buffer was prepared. One ml aliquots of the diluted cell extract were used to determine the amount of 0.5% protamine sulfate solution needed to precipitate the RKase activity. Up to 0.45 ml/ml of the protamine solution could be added before RKase pelleted upon centrifugation. Therefore 159 ml of the 0.5% protamine sulfate solution was added, dropwise with rapid stirring, to the 353 ml of cell extract. The mixture was spun at 9,000 RPM for 90 min at 4°C using the GSA rotor. The supernatant was decanted and adjusted to pH 7.0 with 1 N HCl to yield 510 ml of solution. Another 0.1 ml of 0.5% protamine sulfate per ml of cell extract (51 ml) was added to precipitate ribokinase activity. This solution was immersed in an ice-water bath and allowed to sit for 15 min without stirring. The solution was then spun at 13,000 RPM for 5 min in a SS-34 rotor. The pellet was suspended in 10 ml of buffer containing, 0.6 M ammonium sulfate, 50 mM potassium phosphate, pH 6.1, 1 mM EDTA, and 1 mM dithiothreitol (DTT). The protamine sulfate II was frozen for storage. This preparation, when thawed, was dialyzed against 10 mM potassium phosphate, pH 6.8, 25% ammonium

sulfate with 1 mM EDTA and 1 mM DTT. After dialyzing for 5 hr the protamine sulfate II solution was spun at 12,000 RPM in the SS-34 rotor for 90 min at 4°C.

d) Phenyl Sepharose Chromatography. A Phenyl Sepharose column (1.5 x 28 cm) was prepared according to Pharmacia's instructions. It was washed with one bed volume of distilled water followed by one bed volume of EtOH; then washed with two bed volumes of n-butanol; then one bed volume of EtOH followed by one bed volume of distilled water. The column was equilibrated with buffer containing 25% ammonium sulfate, 50 mM triethanolamine, pH 7.5, 1 mM EDTA and 1 mM DTT. The 25% ammonium sulfate protein solution was applied to the column, which was developed with 100 ml of the equilibration buffer. A 300 ml linear gradient was applied. It consisted of 25% ammonium sulfate, to 50% ethylene glycol in 10 mM potassium phosphate, pH 6.8, both containing 1 mM EDTA and 1 mM DTT. Ten ml fractions were collected. The major peak of RKase activity eluted in tubes 40 through 43. Tubes 41 and 42 were pooled and concentrated to 2 ml in a 10 ml Amicon unit. The RKase sample was frozen and stored at -76°C.

e) Ribokinase Assays. The two assays used during the ribokinase purification, were published by Horecker and Hoffee (1974). The first, an assay utilizing [1–^{14}C]ribose was used in the early stages and for the specific activity determinations. The assay mixture contained 12.5 mM ribose, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and 50 mM Tris, pH 7.5. A 31 µl volume of ^{14}C-ribose was added to 10 ml of this solution. An 80 µl aliquot of this solution contained 30,940 CPM. The reaction was initiated by adding 10 µl of enzyme preparation to 80 µl of assay mixture and 10 µl of 50 mM ATP in 50 mM Tris, pH 7.5. The reaction was quenched by boiling for 2 min and the assay mixture was applied to a 0.5 ml Dowex AG1-X8 column (200-400 mesh; Cl^- form). The column was washed with 3 x 2 ml of water and bound ^{14}C-R5P was eluted with 3 x 1 ml 0.1 N HCl. The column was regenerated with 2 column

volumes of 2 N HCl followed by 3 column volumes of water. Eluted ^{14}C samples were mixed with 4 ml of Hydrofluor and counted. The second assay was spectrophotometric, and was found to be useful in the later stages of the purification. The assay mixture contained 0.1 mM NADH, 10 mM $MgCl_2$, 10 mM KCl, 10 mM ATP, 10 mM ribose, 3.3 mM PEP, 50 mM TEA, pH 7.5, 3 units of lactate dehydrogenase, and 5 units of pyruvate kinase, in a final volume of 1 ml. The addition of ribokinase activity to this assay mixture resulted in an absorbance decrease at 340 nm.

4) STUDIES WITH 5-AZAOROTIC ACID

a) Initial Velocity Study. The enzyme used in the study was purified to a specific activity of 54. The protein concentration was 0.2 mg/ml as determined using the Bio-Rad protein assay with protein standard II. The study was performed over a concentration range of 20 to 200 μM for PRibPP and 33.3 to 300 μM for 5-azaorotic acid (also known as oxonic acid) in an assay mixture consisting of 1 mM $MgCl_2$ with 50 mM Tris-HCl, pH 8. Volumes of 3 μl of YOPRTase were used for each assay. PRibPP was added to begin the reaction, which was followed by observing the decrease in absorbance at 270 nm. The initial velocity was calculated using a molar extinction coefficient of 3,789 (the molar extinction coefficient at 254 where absorbance is maximal is 6,404). The data were analyzed using Cleland's algorithms for the statistical analysis of ping pong and sequential mechanisms (Cleland, 1979). Revelant parts of his FORTRAN code were translated to BASIC for use on a NEC 8201 personal computer.

b) HPLC Assay of 5-Azaorotic Acid Reaction. A Waters Associates HPLC

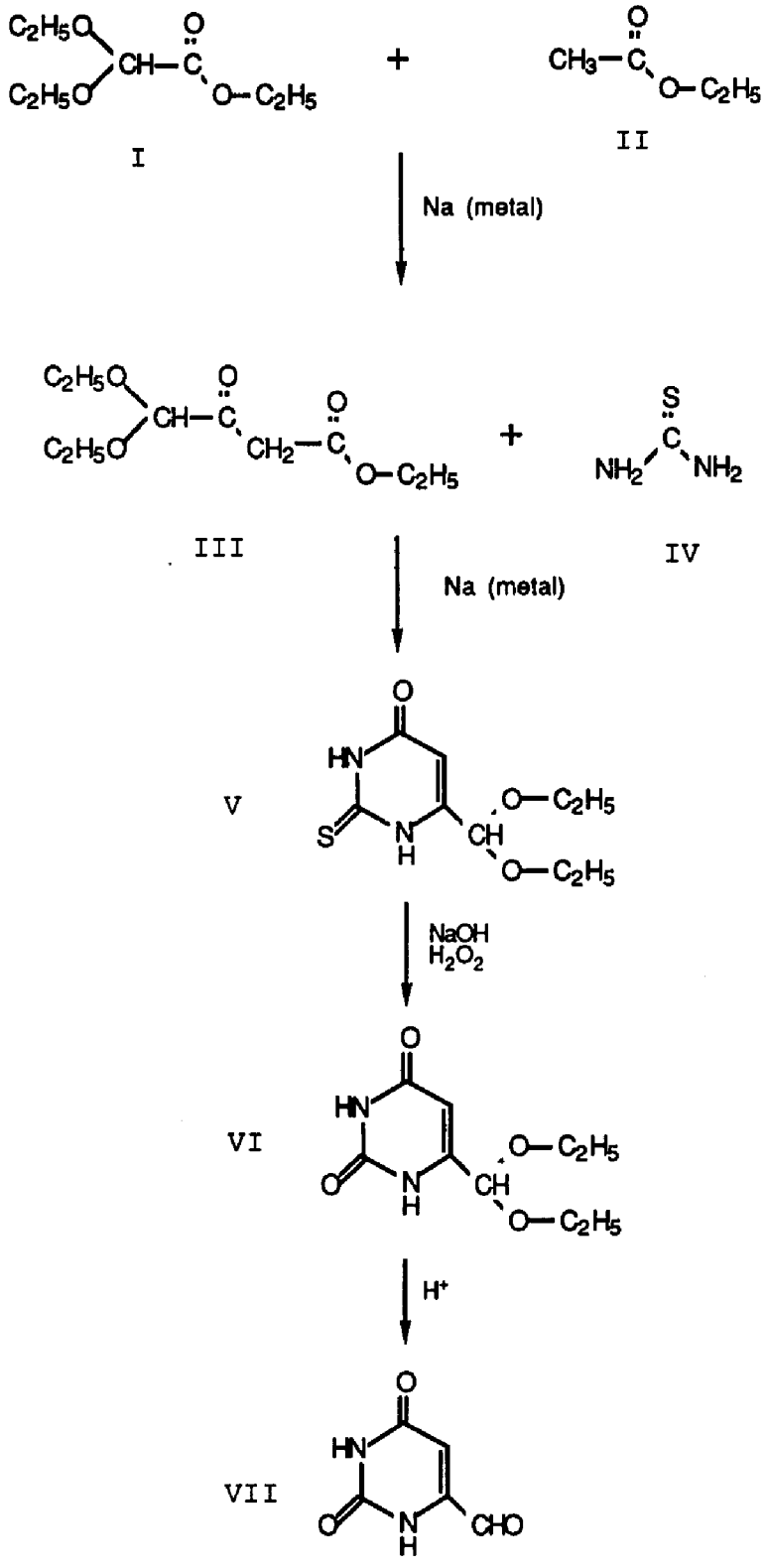
instrument made up of a Model 6000A pump, a U6K injector, a Model 440 UV detector (254 nm) and a μBondapak C_{18} column, along with a Houston Omniscribe chart recorder were the HPLC equipment used in this experiment. Aliquots were removed from an incubation mixture containing, YOPRTase, 100 μM 5-azaorotate, 1 mM $MgCl_2$, 100 μM PRibPP, and 50 mM Tris-HCl, pH 8, injected onto the column and eluted with an isocratic elution buffer consisting of 0.15 M ammonium phosphate, pH 2.7.

c) Metal Activation Studies With 5-Azaorotate. An assay mixture containing 300 μM 5-azaorotate, 200 μM PRibPP, and 50 mM TEA, pH 8, was passed through a Chelex-100 column as was the YOPRTase preparation used in this study. The assay mixture was then made up to one of the following concentrations in Mg^{2+}; 10, 30, 50, 70, 90, 100 μM, 0.25, 0.50, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 50, or 90 mM. The reaction was initiated by the addition of a 20 μl aliquot of YOPRTase and followed spectrophotometrically at 270 nm.

5) STUDIES WITH URACIL 6-ALDEHYDE

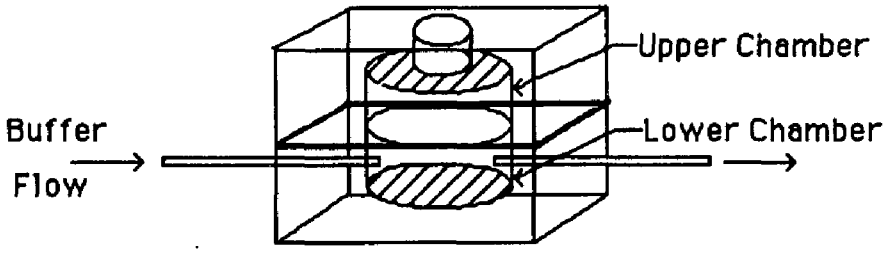
a) YOPRTase Inactivation. The uracil 6-aldehyde used in this experiment was synthesized by Mr. Dean Cuebas according to the procedure of Johnson and Schroeder (1931). An outline of the procedure is shown in scheme 1. To 70 μl of enzyme was added 70, 35, 17.5 or 8.75 μl of 20 mM uracil 6-aldehyde in 50 mM potassium phosphate buffer, pH 8. The incubation mixture was then made up to 140 μl with 50 mM potassium phosphate buffer, pH 8, to give uracil 6-aldehyde concentrations of 10, 5, 2.5 and 1.25 mM for each incubation. The control was 70 μl of enzyme mixed with 70 μl of buffer. The incubations were made at 25°C and 15 μl aliquots were taken at 1,

SCHEME 1. Outline of Uracil 6-aldehyde synthesis. (I) Ethyl diethoxy-acetate, (II) Ethyl acetate, (III) Ethyl γ, γ-diethoxyacetate, (IV) Thiourea, (V) Diethylacetal of 2-thiouracil 6-aldehyde, (IV) Diethylacetal of uracil 6–aldehyde, (VII) uracil 6-aldehyde.


I
II
Na (metal)
III
IV
Na (metal)
V
NaOH
H_2O_2
VI
H^+
VII

5, 10, 20, 30, 40, 50, and 60 mins. YOPRTase activity was measured spectrophotometrically in an assay mixture consisting of 150 μM orotate, 100 μM PRibPP, 1 mM $MgCl_2$ and 50 mM potassium phosphate buffer, pH 8, in a final volume of 1 ml.

b) Flow Dialysis: YOPRTase Reactivation. A 150 μl volume of YOPRTase with a specific activity of 27 and a protein concentration of 0.14 mg/ml was incubated with 150 μl of 5 mM uracil 6-aldehyde for 3 hrs. A rectangular piece of membrane cut from Spectra/Por dialysis membrane tubing (MW cutoff, 12,000-14,000) was clamped in place between the upper chamber and lower chambers of a flow dialysis unit. A schematic of the flow dialysis unit is shown below in Scheme 2:



SCHEME 2

The enzyme mixture was placed in the upper chamber and buffer containing 300 μM orotate and 1 mM $MgCl_2$ in 100 mM potassium phosphate buffer, pH 8, was passed through the lower chamber. Aliquots of 20 μl were removed and assayed at 0, 5, 10, 20, 30, 40, and 60 minutes. This experiment was repeated with the incubation mixture being made approximately 3 mM in sodium borohydride by the addition of 10 μl of a 90 mM solution prior to beginning the flow dialysis.

c) Protection Studies. Solutions of 10 mM PRibPP, 10 mM OMP, 10 mM PPi, 3 mM orotate, 20 mM uracil 6-aldehyde, and 10 mM $MgCl_2$ were made up in 50 mM

potassium phosphate, pH 8. YOPRTase was added to incubation mixtures containing (in the absence or presence of 1 mM $MgCl_2$) final concentrations of 5 mM uracil 6–aldehyde, 1 mM substrate, 50 mM potassium phosphate, pH 8, in a total volume of 20 µl. The incubations were carried out in a 25°C water bath, in the dark, for 30 min. A 10 µl aliquot from each incubation was then assayed for remaining YOPRTase activity. The samples were assayed using the reverse pyrophosphorolysis YOPRTase reaction. The assay mixture contained 100 µM OMP, 500 µM PPi and 1 mM $MgCl_2$ in 50 mM potassium phosphate buffer, pH 8, in a final volume of 1 ml.

6) CHEMICAL MODIFICATION OF YOPRTase LYSINE RESIDUES WITH PLP

Materials. Pyridoxal 5-phosphate (PLP), orotic acid, OMP, and PRibPP were purchased from Sigma Chemical company; potassium phosphate and magnesium chloride from Fisher Scientific Company; Spectralab dialysis tubing from Thomas Scientific company. Centricon-10 ultrafiltration units were purchased from Amicon. The flow dialysis unit was purchased from Thomas Scientific Co. and modified by sealing the screw hole in the side of the upper chamber and drilling a hole through the top of the unit, this allowed samples to be easily added or removed. The spectrophotometric assay was used for the enzyme assays.

a) Time Course Of PLP Inactivation Of YOPRTase. YOPRTase (specific activity, 182 µmoles/min /mg; protein concentration, 0.52 mg/ml) was diluted 1:5 with 1 mg/ml BSA in 25 mM POPSO (purchased from Sigma Chemical Co.), pH 8. A 100 µl volume of enzyme was mixed with 100 µl of 200 µM PLP, 25 mM POPSO, pH 8, and incubated at 30°C in the dark. Aliquots of 20 µl were removed at timed intervals of 0.5, 1, 2.5, 5, 10, 15, 30, and 60 min, mixed with 20 µl of 1 mM sodium borohydride

in 25 mM POPSO, pH 8, then placed on ice. Aliquots of 5 µl were assayed in an assay mixture containing 50 mM Tris, pH 8, 1 mM $MgCl_2$, 300 µM orotate, and 100 µM PRibPP. The experiment was repeated with 100 µl volumes of enzyme being added to 100 µl of 20 µM PLP, 25 mM POPSO, pH 8. A control experiment was performed by mixing enzyme with an equal volume of 25 mM POPSO, pH 8, incubating the sample at 30°C in the dark, and removing aliquots for assay at 0 and 60 min.

b) Concentration Dependence Of PLP Inactivation Of YOPRTase. YOPRTase was incubated with the following concentrations of PLP, 0, 10, 20, 50, 100, 250, 500, 1,000, and 2,000 µM, with 50 mM POPSO, pH 8. These samples were incubated at 25°C for 20 min. After the incubation $NaHB_4$ was added to each of the samples which were placed on ice for 10 min.

c) Reactivation Of PLP Inactivated YOPRTase. In an early experiment, YOPRTase was incubated at 25°C for 30 min alone or in the presence of 25 mM PLP in 50 mM potassium phosphate, pH 8. Two runs were performed for each type of incubation with one of each duplicate set being treated by the addition of 30 mM sodium borohydride. The incubations were then subjected to a 90 min dialysis against 50 mM potassium phosphate, pH 8, in a flow dialysis unit. The YOPRTase activity was then determined spectrophotometrically. A later set of experiments were performed as follows. Two incubation mixtures were prepared. For each, 30 µl of enzyme was mixed with 30 µl of 2 mM PLP, and 25 mM POPSO, pH 8. One sample was incubated for 10 min at 30°C in the dark and the other was exposed to the room lights. A control was prepared by adding 30 µl of enzyme to 30 µl of 25 mM POPSO, pH 8. It was incubated for 10 min at 30°C in the dark. After the incubations, 20 µl aliquots were removed from each sample and added to test tubes containing 5 µl of 25 mM sodium borohydride in 25 mM POPSO, pH 8. Another set of 20 µl aliquots were removed from each sample and added to test tubes containing 5 µl of 25 mM POPSO, pH 8. The samples were placed on ice for ten min., then each was placed in a

separate Centricon-10 unit. The test tubes were rinsed with 200 µl of 50 mM Tris-HCl, pH 8, and the rinse was added to the corresponding sample. The samples were subjected to ultrafiltration by spinning them at 5,000 g for 60 min then adding 200 µl of 50 mM Tris-HCl, pH 8. This was repeated three times, after which the samples were recovered, lyophilized, and reconstituted with 50 µl of a solution containing 25 mM POPSO, pH 8, and 1 mg/ml BSA. Activity measurements were performed using the spectroscopic assay.

d) PLP Protection Studies. An experiment was performed in which YOPRTase was incubated with 1 mM PLP and the following substrates; 1 mM PRibPP, 1 mM PRibPP with 1 mM Mg^{2+}, 1 mM OMP, 1 mM OMP with 1 mM $MgCl_2$, 1 mM PPi, 1 mM PPi with 1 mM $MgCl_2$, and 1 mM orotate. Velocity measurements were made using the spectrophotometric assay.

e) Competition Studies With PLP. The YOPRTase used in these studies had a specific activity of 182 and a protein concentration of 0.52 mg/ml. The enzyme was diluted 1:2 with 25 mM POPSO, pH 8, for use in the initial velocity studies, by examining the competition between PLP and the substrates PRibPP and orotate. YOPRTase was added to an assay mixture containing PLP and the substrate whose concentration was to be varied, either PRibPP or orotate. For the studies with PRibPP the final 1 ml assay mixture contained 25 mM POPSO, pH 8, 2 mM $MgCl_2$, 300 µM orotate, PRibPP at 25, 33, 50, 100, or 200 µM, and PLP at 0 µM, 125 µM, or 250 µM. PRibPP concentration was varied through the listed values as the PLP concentration was held constant at one of the concentrations given. A 2 µl aliquot of YOPRTase was added to the assay mixture containing both PLP and PRibPP and the mixture was allowed to incubate at 30°C, in the dark, for 5 min prior to the addition of orotate to initiate the reaction. Upon the addition of orotate the reaction was followed spectrophotometrically at 295 nm. The studies with various orotate concentrations

differed as follows. YOPRTase was incubated in assay mixtures containing PLP at 0, 125, or 250 μM and orotate at 60, 75, 100, 150, or 300 μM. The reaction was initiated with PRibPP at a final concentration of 100 μM.

f) Amino Acid Analysis of PLP Treated YOPRTase. Six YOPRTase samples were prepared for amino acid analysis. The YOPRTase used in this study had a concentration of 0.52 mg/ml and a specific activity of 182. Enzyme and all other solutions used in this experiment were buffered with 50 mM POPSO, pH 8. Six samples were prepared as follows. For sample 1, a 20 μl volume of YOPRTase was mixed with 20 μl of 50 mM POPSO, pH 8. For sample 2, a 10 μl volume of 50 mM POPSO, pH 8, was added to 20 μl of enzyme. For samples 3, and 4, 10 μl volumes of 4 mM PLP were added to 20 μl of enzyme. Sample five was prepared by mixing 20 μl of YOPRTase with 10 μl of 4 mM PLP, 10 μl of 40 mM PRibPP, and 1 μl of 50 mM $MgCl_2$. Sample 6 was prepared by mixing 20 μl of enzyme with 10 μl of 10 mM PRibPP and 10 μl of 4 mM PLP. All samples were incubated at 30°C for 15 min in the dark. Ten μl aliquots of 50 mM sodium borohydride were added to samples 2, 4, 5, and 6. Ten μl aliquots of 50 mM POPSO, pH 8, were added to samples 1 and 3. The samples were each placed in a Centricon-10 unit and subjected to ultrafiltration with three changes of 1 ml of 50 mM POPSO, pH 8. The samples were recovered and taken to the laboratory of Dr. S. Stein (CABM, Rutgers University), who graciously performed the amino acid analysis. The amino acid analysis techniques included gas phase protein hydrolysis, postcolumn reaction with o-pthaldialdehyde/2-mercaptoethanol reagent (OPA) for amino acid detection, and postcolumn reaction with sodium hypochlorite for imino acid (proline) detection.

g) PLP Incorporation into YOPRTase. YOPRTase remaining from the [1–^{13}C]NMR experiments was used for this study. The specific activity of this enzyme was 104. Twenty-five millimeter glass filters (#31) and a vacuum suction

apparatus were purchased from Schleicher & Schuell. Protosol and Econofluor were purchased from New England Nuclear. [^{3}H]sodium borohydride, with a specific activity of 9.6 mCi/mmol, was purchased from Amersham Corporation. All manipulations with [^{3}H]sodium borohydride, up to and including the addition of 10% trichloroacetic acid (TCA) to samples, were performed under a fume hood. The [^{3}H]sodium borohydride was dissolved in 100 µl of 50 mM POPSO, pH 9, resulting in a [^{3}H]sodium borohydride concentration of 104 mM. A 10 µl aliquot of this solution was added to 2.8 ml of buffer containing 50 mM sodium borohydride, and 50 mM POPSO, pH 8, resulting in a solution with a specific activity of 1.57 x 10^5 DPM/nmol. YOPRTase, in 20 µg quantities, was incubated with the following concentrations of PLP, 0, 10, 20, 50, 100, 250, 500, 1,000, and 2,000 µM, with 50 mM POPSO, pH 8, in 40 µl volumes. These samples were incubated at 25°C for 20 min. After the incubation 10 µl volumes of the diluted [^{3}H]$NaHB_4$ were added to the samples which were then placed on ice for 10 min. Aliquots of 50 µl of 1 mg/ml BSA in 50 mM POPSO, pH 8, were added to each sample. One ml aliquots of ice-cold 10% TCA were added to the samples which were allowed to sit on ice for 30 min. Each sample was applied to a glass filter held in a vacuum suction apparatus. The filters were washed ten times with 10 ml volumes of ice-cold 10% TCA followed by a wash with 10 ml of 95% ethanol. The filters were allowed to dry prior to placing them in liquid scintillation vials with 0.5 ml of Protosol. Vial caps were lined with Teflon cap liners and tightly fitted to the vials which were left overnight in a 37°C water bath. The next day 5 ml volumes of Econofluor and 50 µl volumes of glacial acetic acid (to quench chemiluminescence) were added to each vial. The samples were placed in a Tracor Analytic 6895 liquid scintillation counter for counting.

7) ENZYMATIC COUPLED ASSAY PROCEDURE EMPLOYING HPLC

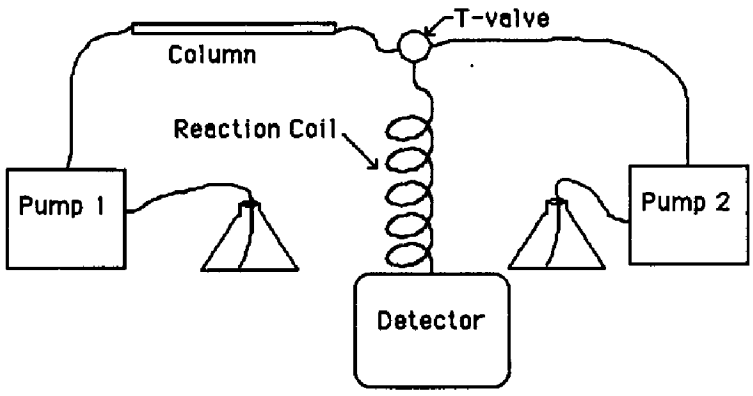
Waters Associates Model 6000A pump, U6K injector, and Model 440 UV detector (254 nm) were placed on-line with an Analytichem quaternary amine column. An isocratic elution buffer, containing 0.15 M ammonium phosphate (pH 2.7), was used to characterize the elution times of ATP, ADP, AMP, OMP, and orotate. The assay for PRSase consisted of 100 μM ATP, 1 mM ribose 5-phosphate, 1 mM $MgCl_2$, 50 mM triethanolamine, and 50 mM potassium phosphate, pH 8, in 2 ml. The reaction was initiated by the addition of 10 to 25 μl of PRSase and 0.3 ml volumes were removed at measured time intervals over the period of an hour. The samples were filtered through a 0.45 μM Millipore filter and 20 μl aliquots were injected onto the the HPLC column. Control assays were also performed in the absence of R5P in order to account for any non-R5P dependent activities. The assay mixture for ribokinase consisted of 100 μM ATP, 1 mM $MgCl_2$, 25 mM NaF (to inhibit myokinase), and 50 mM triethanolamine, pH 7. The incubation was performed at 37°C and was initiated by the addition of a 10 μl volume of ribokinase. Aliquots of 0.2 ml were removed at measured intervals and prepared for injection as described above. The assay solution for the four step synthesis of OMP from ribose consisted of 1 mM ATP, 10 mM ribose, 10 mM $MgCl_2$, 25 mM NaF, 500 μM orotate, 50 mM triethanolamine and 50 mM potassium phosphate, pH 7. The reaction was initiated by the addition of 5 μl of RKase, 10 μl of PRSase, and 5 μl of pyrophosphatase. Aliquots were removed at timed intervals over a period of 90 min and prepared for injection as described above.

8) QUATERNARY STRUCTURE DETERMINATION

Materials. Triethanolamine, PRibPP, and orotate were purchased from Sigma Chemical Co. $MgCl_2$ was from Fisher Scientific Co. A Waters HPLC instrument equipped with a Model 600A solvent delivery system, Model M-45 solvent delivery system, Model U6K sample injector, Model 440 absorbance detector, and a Houston Omniscribe chart recorder were used for this study. The HPLC gel filtration column used was a BIO-SIL TSK-250 analytical column (300 x 7.5 mm). The YOPRTase used in this experiment had a specific activity of 182 μmoles/min./mg.

a) Non-reacting Gel Experiments. YOPRTase was diluted to final concentrations of 0.1, 0.25, 0.5, 1.0 and 5.0 μg/ml in 100 mM TEA, pH 6.8. For the studies involving non-reacting gel filtration, aliquots of 50 μl of diluted enzyme were loaded onto the column which was run at a flow rate of 1 ml/min. The pump pressure was 250 psi. The experiments were performed at an ambient room temperature of 23°C. The running buffers used contained 100 mM TEA, pH 6.8, or 1 mM $MgCl_2$ and 100 mM TEA, pH 6.8, or 100 μM PRibPP, 1 mM $MgCl_2$, and 100 mM TEA, pH 6.8, depending on the experiment. A T-valve was used to mix the eluent from the column with YOPRTase assay mixture containing 500 μM PRibPP, 300 μM orotate, 2 mM $MgCl_2$, and 50 mM Tris, pH 8. The assay mixture was pumped at a flow rate of 0.3 ml/min using the second high pressure pump. The solution from the T-valve was fed into a reaction line consisting of 15 feet of 1/16 inch ID tubing. This led to the absorbance detector, set to a wavelength of 254 nm. At this wavelength an absorbance increase corresponds to an orotate and PRibPP dependent formation of orotidine 5'–monophosphate. The position of the eluting YOPRTase was determined by observing the position of eluting OMP, which was recorded as an absorbance peak.

A schematic of the experimental apparatus is shown below in Scheme 3:



SCHEME 3

Apparent molecular weights were determined by linear regression analysis using a calibration plot of the natural log of molecular weight *versus* the elution times for the molecular weight marker proteins, bovine serum albumin, egg albumin, β-lactalbumin, and ribonuclease A.

b) Reacting Gel Experiments. For the reacting gel studies the assay mixture was replaced with 100 mM TEA, pH 6.8. The elution buffer contained 100 μM PRibPP, 100 μM orotate, 1 mM $MgCl_2$, and 100 mM TEA, pH 6.8.

c) Experiments Using Higher YOPRTase Concentration. YOPRTase was injected at concentrations that allowed the direct observation of eluting protein by its UV absorbance at 254 nm. YOPRTase with a protein concentration of 0.54 mg/ml was injected onto the column in 10 μl aliquots and the column eluent was fed directly into the detector.

In another experiment the column was equilibrated with 100 mM TEA, pH 6.8, then with 100 mM TEA, pH 6.8, 10 μM OMP and 1 mM $MgCl_2$. Aliquots of

YOPRTase (200 µl) were injected onto the HPLC gel filtration column at concentrations of 60 µg/ml. Eluting YOPRTase was observed by its absorbance at 254 nm.

9) [1-^{14}C]PRibPP SYNTHESIS

a) Synthesis and Purification. This procedure is from Goitein *et al.*, (1978). [1–^{14}C]Ribose (50 mCi/mmole) was purchased from New England Nuclear. Activated charcoal and celite (diatomaceous earth) were purchased from Sigma. A 10 ml incubation mixture was prepared containing 50 mM potassium phosphate, 50 mM triethanolamine, pH 7.5, 4 mM ATP, 10 mM $MgCl_2$, 150 µl of [1-^{14}C]ribose (10 µCi), 100 µl of PRibPP synthetase (30 units) and 25 µl of ribokinase (5 units). The incubation was performed at room temperature. After 3 hr the reaction was quenched by the addition of 1 g neutralized, activated charcoal and 1 g of celite. The mixture was stirred and allowed to sit on ice for 5 min. It was then filtered through a Whatman No. 1 filter using a Hirsch funnel and an aspirator flask. The filtrate was placed on a DEAE-cellulose column (25 x 1.5 cm) that had been equilibrated with distilled water. The column was developed with 10 ml of distilled water, a 100 ml linear gradient of 0 to 0.4 KCl was applied, and 2 ml fractions were collected. Aliquots of 25 µl were removed from every other fraction, diluted with 3 ml of Hydrofluor and counted. Fractions 37 through 45 (the second peak of radioactivity, which is PRibPP) were pooled, lyophilized, and then reconstituted to 5 ml with distilled water. A 2.5 ml aliquot of the [1-^{14}C]PRibPP was applied to a Sephadex G-10 column (15 x 1.5 cm) previously equilibrated with distilled water. Fractions of two ml were collected and fractions 4 and 5, which had the highest CPM, were pooled, lyophilized, then stored at

-76°C. The [1-^{14}C]PRibPP sample was reconstituted with 0.5 ml of 50 mM Tris-HCl, pH 8, prior to its use in the flow dialysis experiment.

b) HPLC Assay of Labeled PRibPP. A 10 µl aliquot of [1-^{14}C]PRibPP was added to an assay mixture containing 1 µl of YOPRTase, 10 µl of ODCase (1 mg/ml), 300 µM orotate, 1 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8. A second 10 µl aliquot of [1-^{14}C]PRibPP was added to another assay mixture sample, prepared as above, but with 11 µl of buffer added instead of enzyme. The assay mixtures were incubated at room temperature for 30 min (earliest studies) to 1.5 hr (most recent assay), then heated in boiling water for 2 min to quench the reaction. The samples were filtered with Millipore 0.45 µM HA filters and 20 µl aliquots were injected onto a µBondapak C_{18} reverse phase HPLC column and eluted with an isocratic elution buffer of 15 mM ammonium phosphate, pH 5.5. The column was calibrated with OMP, UMP and orotate standards.

10) GEL FILTRATION OF THE YOPRTase-[1-^{14}C]PRibPP COMPLEX

A [1-^{14}C]PRibPP sample was lyophilized and reconstituted with water to 1 ml. It was applied to a Sephadex G-10 column (12 x 1 cm) previously equilibrated with 50 mM Tris-HCl, pH 8, and 1 ml fractions were collected. A 200 µl aliquot of the eluting fraction with the highest CPM, containing 1 x 10^5 DPM was incubated with 3 µl of 100 mM $MgCl_2$, 3 µl of 10 mM pyrophosphate and 100 µl of YOPRTase at a concentration of 0.49 mg/ml, and having a specific activity of 110 µmoles/min/mg, for 20 min. A 2 µl aliquot of pyrophosphatase (1 mg/ml) was added and the incubation was continued for another 20 min. The incubation mixture was applied to a Sephadex Superfine G-25 column equilibrated with Tris-HCl, pH 8. Fractions of 10 drops each

were collected with the LKB Redi-Rac fraction collector. For each fraction, 10 µl aliquots were assayed for enzyme activity and 25 µl aliquots were mixed with 1.5 ml of hydrofluor for liquid scintillation counting.

11) [1-^{13}C]PRibPP SYNTHESIS

a) Synthesis. The incubation mixture for the synthesis of [1-^{13}C]PRibPP contained the following: 50 mM potassium phosphate, 50 mM TEA (pH 7.5), 12 mM $MgCl_2$, and 10 mM ATP. The [^{13}C]ribose (99.8% enriched at the C1 position), was kindly provided by Dr. A. Serriani of Omnicrom Inc. (Ithaca, N. Y.). A 29.8 mg quantity of [1-^{13}C]ribose was added to 50 ml of the incubation mixture, resulting in a [1-^{13}C]ribose concentration of 4 mM. To this solution was added 100 µl of ribokinase (20 units) and 0.5 ml of PRibPP synthetase (148 units). The progress of the incubation was followed by removing 20 µl aliquots at timed intervals and observing the formation of UMP at 295 nm using an assay mixture containing orotate, $MgCl_2$, YOPRTase and orotidine 5'-monophosphate decarboxylase (ODCase). When the reaction ceased the incubation mixture was placed on ice. Then 5 g of neutralized activated charcoal and 5 g of celite were stirred into the solution which was left to sit on ice for 5 min. The suspension was filtered and the filtrate collected in an aspirator flask, using Whatman No. 1 filter disks and a Buchner funnel with suction. The charcoal-celite bed was washed with 50 ml of ice-cold water. The wash was collected and added to the filtrate. The charcoal-celite treatment and wash was repeated wih this solution. The second filtrate plus wash solution was adjusted to pH 7 with 1 N KOH.

b) DEAE Cellulose Chromatography. The following steps were performed in a cold room at 4°C. The crude [1-^{13}C]PRibPP solution was applied to a DEAE cellulose column (29.5 x 1.5 cm) previously equilibrated with water. The column was

developed with 40 ml of water, then a 100 ml linear gradient of 0 to 0.4 M KCl was applied and 2 ml fractions were collected. The fractions were assayed for ribose with the orcinol reagent assay. Fractions from the last peak to elute (fractions 36 through 40) were pooled, lyophilized, and reconstituted with 2 ml of water. This sample was then applied to a Sephadex G-10 column (14.5 x 1.2 cm) previously equilibrated with water. The eluting fractions containing [1-^{13}C]PRibPP (as determined enzymatically with the OPRTase spectrophotometric assay) were pooled and lyophilized, then reconstituted with 0.4 ml of 20 mM potassium phosphate, pH 7.5. The amount of [1-^{13}C]PRibPP obtained was determined with the OPRTase-ODCase spectrophotometric assay.

12) FLOW DIALYSIS: PRibPP BINDING STUDY

POPSO buffer, and Tris-HCl were purchased from Sigma Chemical Co. Spectra/Por dialysis membranes were from Thomas Scientific. A Technilab flow dialysis unit was modified by sealing the screw hole on the side of the upper chamber, then drilling a hole through its top for the addition of samples. The flow dialysis unit was prepared for the experiment by attaching the lower chamber to a LKB 2120 Varioperpex II pump and filling it with buffer. A stirring flea was placed in the lower chamber and a piece of dialysis membrane (MW cutoff, 12,000-14,000) that had been boiled once in a 1 mM EDTA solution and twice in distilled water was sandwiched between the two halves of the flow dialysis unit, which were then screwed together. The unit was placed atop a stirring plate. The tubing leading from the lower chamber to the fraction collector was 1/16th of an inch OD and kept to a minimum length. Buffer containing 1 mM Tris, pH 8, and 1 mM $MgCl_2$, was pumped through the lower

chamber at a flow rate of 6.5 ml per minute as 2 ml fractions were collected by a LKB 2112 Redirac fraction collector. A calibration experiment was performed first. A 200 µl aliquot of [1-^{14}C]PRibPP (1.65 x 10^6 DPM) was added to 0.25 ml solution of 1 mM Tris, pH 8, and 1 mM $MgCl_2$ in the upper chamber with mixing by a stirring flea. Immediately after the addition, the fraction collector was started. When the 16th fraction began collecting a 5 µl aliquot containing 0.45 M PRibPP, 1 M $MgCl_2$ and 50 mM Tris, pH 8, was added. A total of 48 fractions were collected. From every other fraction a 0.6 ml aliquot was removed and diluted in 5 ml of Bray's solution for liquid scintillation counting. The experiment with enzyme was then performed. A fresh membrane was placed in the flow dialysis unit and 250 µl of YOPRTase (4 mg/ml) was added to the upper chamber. Upon the addition of 200 µl of [1-^{14}C]PRibPP the fraction collector was started. As every eighth fraction began collecting, a 5 µl aliquot containing 1.8 mM PRibPP and 50 mM Tris, pH 8, was added to the upper chamber. When the 48th fraction began collecting a 5 µl aliquot containing 0.45 M PRibPP, 1 M $MgCl_2$ and 50 mM Tris, pH 8, was added instead. Again, aliquots of 0.6 ml were removed from every other fraction and counted in 5 ml of Bray's solution. I later learned that microprecipitation of phosphorylated compounds will occur in Bray's solution. Therefore the liquid scintillation cocktails were shaken prior to counting. The samples used to produce the Scatchard plot (fractions 6, 14, 22, 30, 38, 46, and 56) were counted four times for ten min each and were shaken prior to each count. The average of the four counts was used for the calculations.

13) [1-^{13}C]NMR OF YOPRTase-[1-^{13}C]PRibPP MIXTURE

[1-^{13}C]PRibPP was synthesized as discussed above. Experiments were

performed on the JEOL-400 and IBM-200WP NMR instruments at Hunter and CCNY, respectively. The 4 mm coaxial tubes, 5 mm and 10 mm NMR tubes were purchased from Wilmad Glass Company as were Tetramethylsilane (TMS), 99.5% D_2O and adapters to fit 5 mm NMR tubes within 10 mm NMR tubes. All spectra of ribose, [1–^{13}C]ribose, and their phosphorus derivatives were obtained at the ambient temperature of the probe or at 19°C, using the IBM-200WP at 50 MHz, with a spectral width of 11 kHz, an acquisition time of 0.74 s, a pulse width of 5 μs and broadband proton decoupling. The proton-decoupled spectra of the YOPRTase-[1-^{13}C]PRibPP mixture was obtained on the JEOL-400 at 19°C at 100 MHz with a 0.55 s acquisition time and a pulse width of 8 μs with a total accumulation time of approximately 11 hr.

RESULTS

1) OPRTase PURIFICATION

This purification is based on the procedure of Victor *et al.*, (1979), which was adapted from Umezu *et al.*, (1971). The result of a typical purification is shown in Table I. The purification resulted in an OPRTase preparation with a specific activity of 400. Sephacryl S-200 was used instead of the Sephadex G-100 used in the previous procedures. This significantly accelerated the gel filtration stage, since Sephacryl S-200 can be subjected to faster flow rates without the gel bed packing, causing the flow to become intolerably slow. Not adding NaCl to the buffer in the Sephacryl S-200 chromatography step allowed the elimination of a dialysis step prior to DEAE cellulose chromatography. Usually two peaks of OPRTase activity (a minor and a major peak) are eluted from the DEAE column. However, little or no minor peak was found when this dialysis step was eliminated. The introduction of the OMP affinity column allowed for the consistent purification of OPRTase that ran as a single band on electrophoresis gels or on the HPLC TSK-250 analytical gel filtration column. The OPRTase specific activity of 400 μmol/min./mg reported herein is six times the value of 65 μmol/min./mg that Victor *et al.*, (1979) reported for their preparation.

2) PRibPP SYNTHETASE PURIFICATION

The result of the PRSase purification is shown in Table II. This purification resulted in an enzyme preparation with a specific activity of 168, based on the HPLC

Table I. OPRTase Purification From Baker's Yeast

Stage	Volume (ml)	Protein (mg/ml)	Total Activity	Specific † Activity	% Recovery
Autolysis	9200	46.3	7667	0.018	100
Ammonium Sulfate	3100	30.3	6951	0.074	90.6
Ethanol	980	57.3	4212	0.075	54.9††
Heat Treatment	825	50	4950	0.120	64.6
Sephacryl-200	1200	20	3504	0.146	45.7
DEAE	200	6.5	1482	1.14	19.3
Hydroxylapatite	33	13.3	1251	2.85	16.3
Blue Sepharose	9.5	1.24	1225	104	16
OMP Sepharose	6	0.89	2136	400	---†††

†Specific activity is defined as µmoles OMP/min./mg enzyme.

††The sample used for this activity determination was stored in the presence of EtOH and may have been partially inactivated.

†††Several OPRTase samples from different preparations were combined for this step.

Table II. PRibPP Synthetase Purification From S. Typhimurium

STAGE	Volume (ml)	Protein (mg/ml)	Total Activity	Specific† Activity	% Recovery
Sonication	1000	11.9	2178	0.183	100
Streptomycin Sulfate	1030	2.76	2246	0.790	103
$(NH_4)_2SO_4$ Fractionation	50	2.26	2373	21	109
Acid Precipitation	----	---	----	-----	---
$(NH_4)_2SO_4$ Precipitation	40	0.82	2427	74	111
Acid Precipitation	----	---	-----	-----	---
Blue Sepharose	10	1.76	2957	168	136

†Specific activity is defined as μmoles PRibPP/min./mg enzyme.

assay, and a total protein yield of 17.6 mg. The unusual increase in % activity recovered for each step is due to activation of the enzyme by the quick-freezing step, as was reported by Switzer and Gibson (1978). The HPLC assay was found to be useful at all stages of the purification, requiring only small corrections for endogenous ATPase activity in the earliest stages.

3) RIBOKINASE PURIFICATION

The ribokinase purification procedure, described herein, was developed by me in this laboratory, although the protamine sulfate precipitation stage was from a procedure published by Schimmel *et al.*, (1974). The results are shown in Table III. The purification yielded a RKase preparation with a specific activity of 3.59, approximately one fourth of the value of 15 (in the absence of activating cations) reported in the original procedure. However, the preparation from this laboratory did not undergo aggregation to form an insoluble protein precipitate upon freezing and thawing, as did the preparation of the earlier workers.

4) 5-AZAOROTATE KINETICS

a) Initial Velocity Study. The initial velocity data were statistically fitted to both Ordered Bi Bi and Bi Bi Ping Pong models with the latter yielding the lowest standard errors for the fit. The initial velocity patterns (Figures 3 & 4) were plotted from the calculated values determined by fitting the data to a Bi Bi Ping Pong kinetic model using Cleland's algorithms (Cleland, 1979). Listings of the BASIC programs used are found in Appendix I. The calculated kinetic parameters and their standard errors for

Table III. Ribokinase Purification From *S. typhimurium*

STAGE	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL ACTIVITY	SPECIFIC† ACTIVITY	% RECOVERY
Sonication	290	14.8	928	0.22	100
Protamine Sulfate	----	-----	----	----	----
25% $(NH4)_2SO_4$	10	56	230	0.41	25
Phenyl Sepharose Frac. 40 & 43	7	7.14	77	1.54	8
Phenyl Sepharose Frac. 41 & 42	2	19.5	140	3.59	15

†Specific activity is defined as µmoles R5P/min./mg enzyme.

FIGURE 3. Kinetic analysis of the alternate substrate azaorotate/oxonate. Double reciprocal plot of the initial velocity of nucleotide formation *versus* PRibPP concentration over a series of fixed concentrations of the alternate substrate (30-300 mM). The spectroscopic assay procedure used in this study was described in "Materials and Methods"

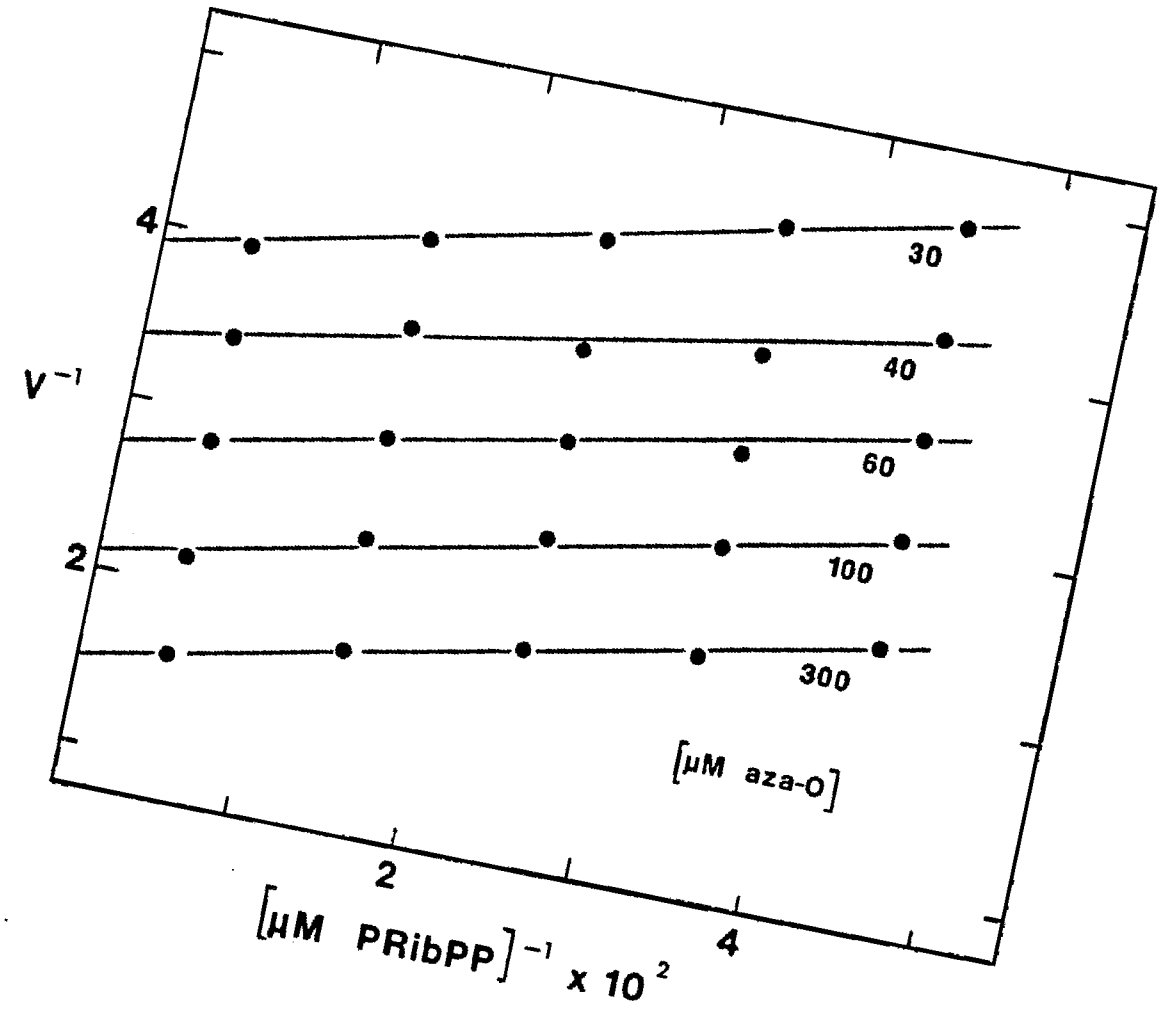
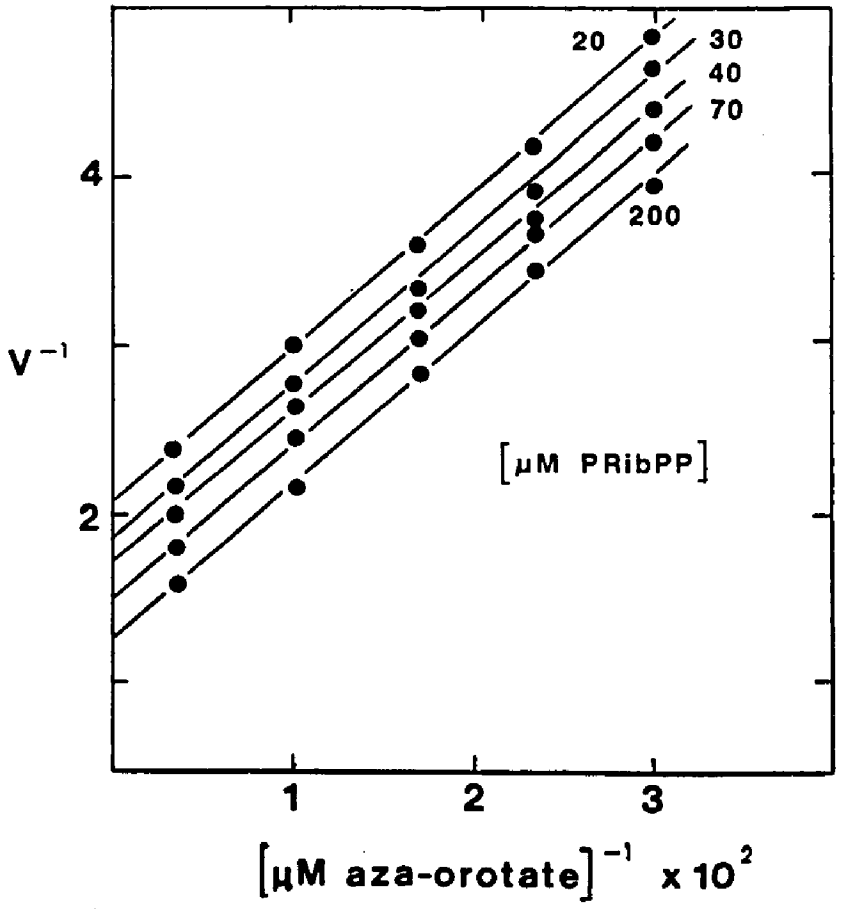

4
2
V^{-1}
30
40
60
100
300
[μM aza-O]
2
4
[μM PRibPP]$^{-1}$ x 10^2

FIGURE 4. Kinetic analysis of the alternate substrate azaorotate/oxonate. Double reciprocal plot of the initial velocity of nucleotide formation *versus* alternate substrate concentration over a series of fixed concentrations of PRibPP (20-200 mM). The spectroscopic assay procedure used in this study was described in "Materials and Methods"


20
30
40
70
200
4
2
V^{-1}
[μM PRibPP]
1
2
3
[μM aza-orotate]$^{-1}$ x 10^2

PRibPP and 5-azaorotate are 16.0 ± 0.02 μM, and 75.5 ± 0.07 μm, respectively. The V_{max} value was 8.29 ± 0.004 nmol/min (13.8 μmoles/min./mg), and sigma, the standard error for the overall fit to the ping pong model, was 2.3×10^{-3}. The structures of orotic acid and 5-azaorotate are shown in Figure 5 for comparison. Table IV compares the YOPRTase kinetic parameters for 5-azaorotate with those determined for orotate and 5-fluororotate by Victor *et al.*, (1979). Based on the Kcat/Km values of 5.2×10^5, 2.5×10^5, and 6.1×10^4 for 5-fluoroorotate, orotate, and 5-azaorotate, respectively, 5-fluoroorotate has the greatest specificity for YOPRTase, having 2.1 times the Kcat/Km of orotate which in turn has 4.1 times more specificity than 5-azaorotate.

b) HPLC Assay of 5-Azaorotate Reaction. There was the appearance of a peak corresponding to the possible formation of nucleotide. It was transient, attaining only a small peak height and then spreading and disappearing during the time course of the reaction.

c) Metal Activation Studies With 5-azaorotate. This experiment has shown that metal is required for the OPRTase catalyzed reaction with 5-azaorotate, however the data has not been fitted to a model mechanism for metal activation.

5) STUDIES WITH URACIL 6-ALDEHYDE

a) OPRTase Inactivation. Spectroscopic studies indicated that uracil 6-aldehyde was not a substrate for YOPRTase. However, it was found to inhibit the normal YOPRTase reaction with orotate and PRibPP when present in the assay mixture. Further studies showed that at least part of the inhibition was due to the inactivation of YOPRTase. Figure 6 shows the time- and concentration-dependent inactivation of

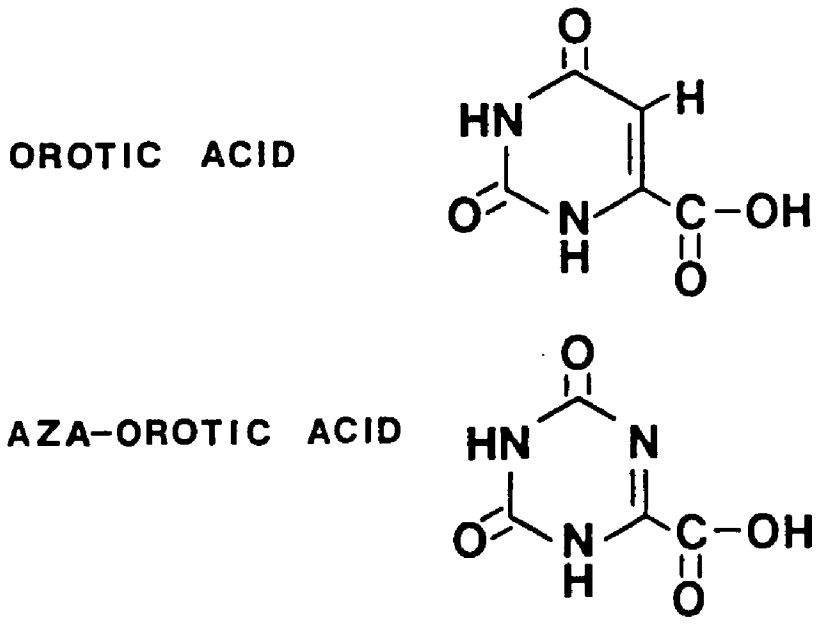


FIGURE 5. Comparison of the structures of orotic acid and azaorotic Acid. The other name for azaorotic acid is oxonic acid.

Table IV
Kinetic Parameters Of YOPRTase Substrates

	Km (mM)	Vmax (μmoles/min./mg)	Kcat (s^{-1})	Kcat/Km (s^{-1} M^{-1})
Orotate	38.0	28.5	9.5	2.5×10^5
5-Fluororotate	27.0	42.0	14.0	5.2×10^5
5-Azaorotate	75.5	13.8	4.6	6.1×10^4

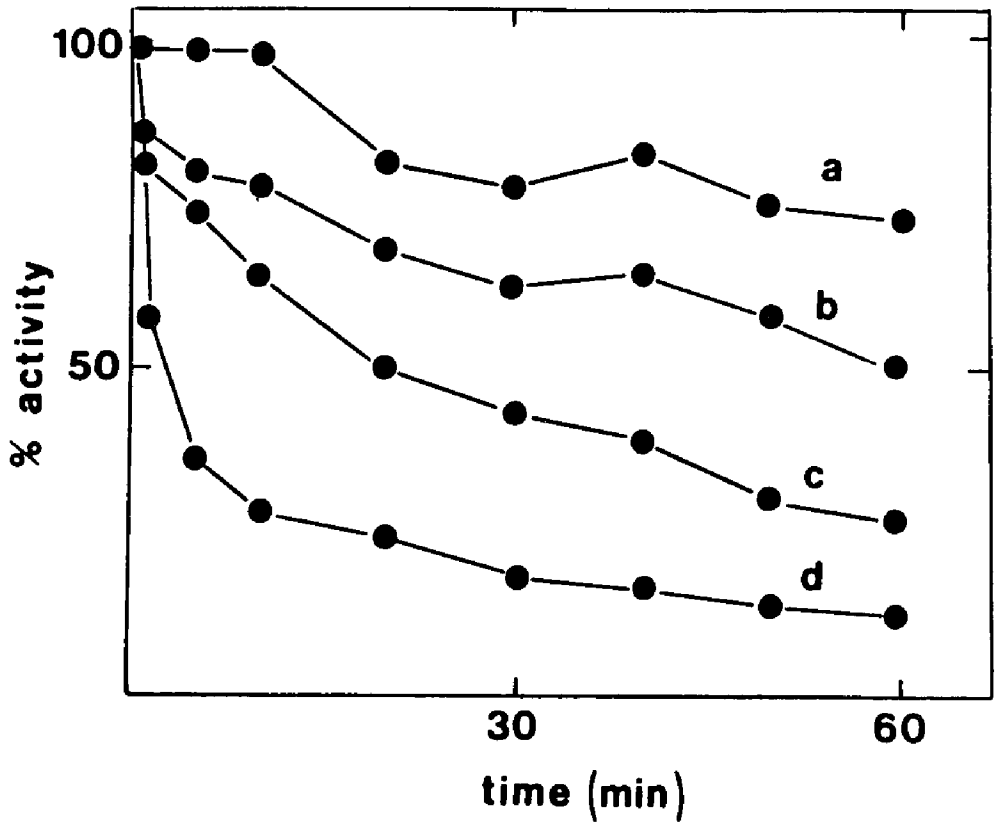


FIGURE 6. The effect of the addition of uracil 6-aldehyde (orotaldehyde) to an incubation mixture containing YOPRTase. A time-dependent inactivation was characterized over a range of aldehyde concentrations: a) 1.3 mM, b) 2.5 mM, c) 5mM, and d) 10 mM. The spectroscopic assay procedure for YOPRTase was employed in these studies and is described in "Material and Methods".

YOPRTase incubated with uracil 6-aldehyde. This inactivation could be due to a reaction involving bimolecular collisions between enzyme and uracil 6-aldehyde, or a reaction occurring within a binary complex of enzyme and uracil 6-aldehyde. If the inactivation occurs within a binary complex the inactivation should proceed with first-order kinetics (Walsh, 1979). Figure 7 shows the result of an attempt to fit the data to a first-order kinetic model. At low concentrations this model appears to fit the data. However, at high concentrations the kinetics of the inactivation are more complex. This does not rule out the formation of a binary complex. The kinetics could be complicated by interactions at two or more sites on the enzyme. Also, there is a carry over of uracil 6-aldehyde from the incubation mixture to the assay mixture. The possibility that uracil 6-aldehyde could also be a competitive inhibitor of YOPRTase means that the observed activity could be the result of inactivation and kinetic inhibition. The finding that uracil 6-aldehyde inactivates YOPRTase led to speculation that Schiff base formation, that is the formation of a covalent imine linkage between amine and aldehyde functional groups, was occurring between the aldehyde group of uracil 6-aldehyde and lysine on YOPRTase. Experiments were designed to test this possibility.

b) Flow Dialysis: YOPRTase Reactivation. A diagnostic feature of enzyme inactivation due to Schiff base formation is its reversibility (Anderson *et al.*, 1966). YOPRTase attains levels of zero activity when incubated with uracil 6–aldehyde for periods of two to three hours, without treatment with sodium borohydride. When YOPRTase was inactivated by an incubation with uracil 6-aldehyde, then subjected to flow dialysis, 50% of the activity was recovered (Figure 8). When, prior to flow dialysis, the inactivated YOPRTase was treated with sodium borohydride to render the inactivation irreversible by reduction of postulated lysine-uracil 6-aldehyde linkages, only 12% of the original activity was recovered (Figure 8). It should be noted that

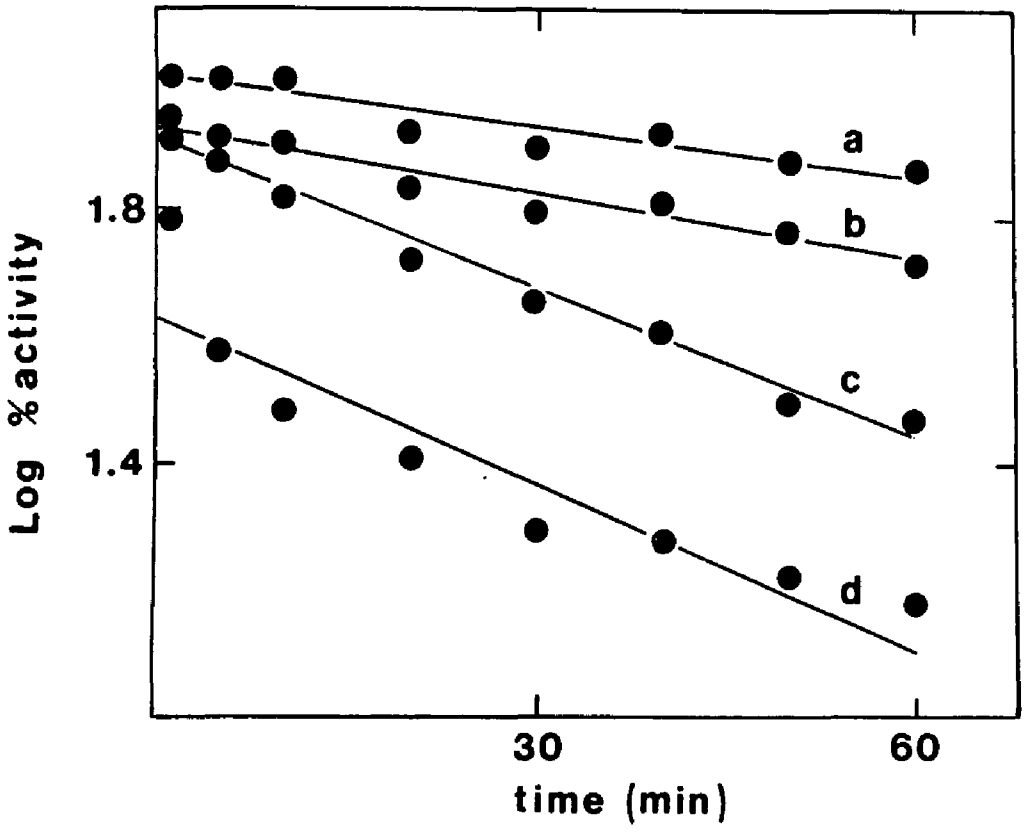


FIGURE 7. The effect of the addition of Uracil 6-aldehyde (orotaldehyde) to an incubation mixture containing YOPRTase. These data are the same as Figure 6 except that the results have been placed in semilog form and then fitted to a pseudo first-order kinetic model.

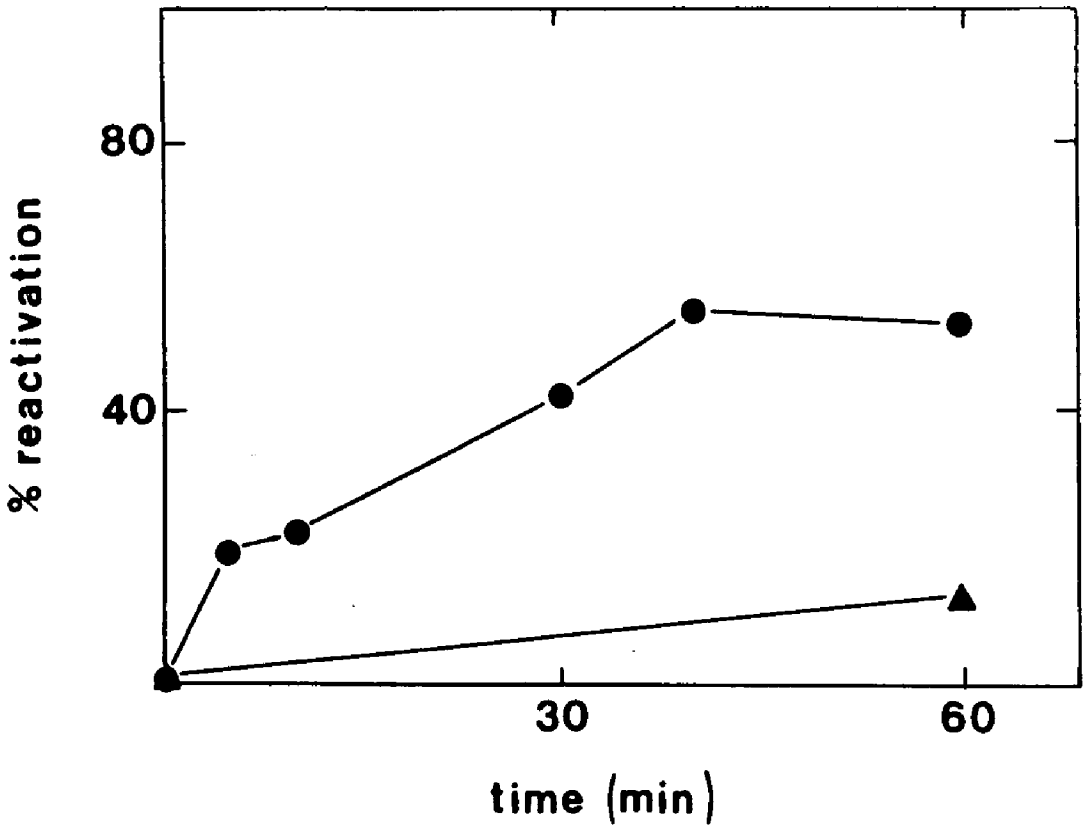


FIGURE 8. The effect of the addition of uracil 6-aldehyde (orotaldehyde) to an incubation mixture containing YOPRTase. The results of a time-dependent flow dialysis experiment subsequent to the addition of the aldehyde to YOPRTase. The closed circles represent enzyme activity after an incubation with uracil 6-aldehyde alone, whereas the closed triangles represent the enzyme after an incubation with the aldehyde, followed by the addition of sodium borohydride.

sodium borohydride reduces not only the Schiff base imine bond to a carbinolamine bond, but also reduces free aldehydes to their alcohol derivatives, eliminating their ability to form Schiff base linkages. The recovery of partial activity upon dialysis suggests that modification of lysine is only part of the reason for the inactivation.

c) Protection Studies. Substrate protection against inactivation of an enzyme by a reagent is evidence that the reagent is active site directed (Walsh, 1979). Table V lists the results of substrate protection studies performed with uracil 6-aldehyde. All substrates, except 1 mM PPi and 1 mM OMP in the absence of $MgCl_2$, provided a significant degree of protection for YOPRTase against inactivation during an incubation with 5 mM uracil 6-aldehyde. Upon adding 1 mM $MgCl_2$ to the incubation with those substrates they also showed significant protection. PRibPP at 1 mM, and in the presence of 1 mM $MgCl_2$, provided the most protection, with YOPRTase retaining 80% of its original activity.

6) CHEMICAL MODIFICATION OF YOPRTase LYSINE RESIDUES WITH PLP

a) Time Course Of Inactivation. It was decided to examine the inactivation of YOPRTase using pyridoxal 5-phosphate (PLP), a classical modifier of lysine residues (Raetz and Auld, 1972). Upon incubating YOPRTase with PLP at 30°C, it was instantaneously inactivated, within the time resolution of the experiment, to a level of activity dependent on the concentration of PLP, as shown in Figure 9. This experiment demonstrates that an equilibrium level of inactivity is attained that is dependent upon the PLP concentration. Chen and Engel (1975) showed, for glutamate dehydrogenase, that the reason for this equilibrium level of inactivation was the active site directed binding of PLP in a noncovalent and covalent manner. They proposed that the initial binding

Table V

Substrate Protection Against Inactivation of Orotate Phosphoribosyltransferase by Uracil 6-Aldehyde.

Incubation	% Activity Remaining
Control	100
5 mM Uracil 6-Aldehyde	23
5 mM Uracil 6-Aldehyde + 1 mM Mg^{2+}	24
5 mM Uracil 6-Aldehyde + 1 mM Orotate	59
5 mM Uracil 6-Aldehyde + 1 mM Orotate + 1 mM Mg^{2+}	37
5 mM Uracil 6-Aldehyde + 1 mM OMP	28
5 mM Uracil 6-Aldehyde + 1 mM OMP + 1 mM Mg^{2+}	56
5 mM Uracil 6-Aldehyde + 1 mM PPi	18
5 mM Uracil 6-Aldehyde + 1 mM PPi + 1 mM Mg^{2+}	50
5 mM Uracil 6-Aldehyde + 1 mM PRibPP	54
5 mM Uracil 6-Aldehyde + 1 mM PRibPP + 1 mM Mg^{2+}	80

Activity was measured spectrophotometrically using the reverse pyrophosphorolysis assay in an assay mixture containing 100 μm OMP, 500 μM PPi, 1 mM $MgCl_2$, and 50 mM potassium phosphate, pH 8, in a final volume of 1 ml.

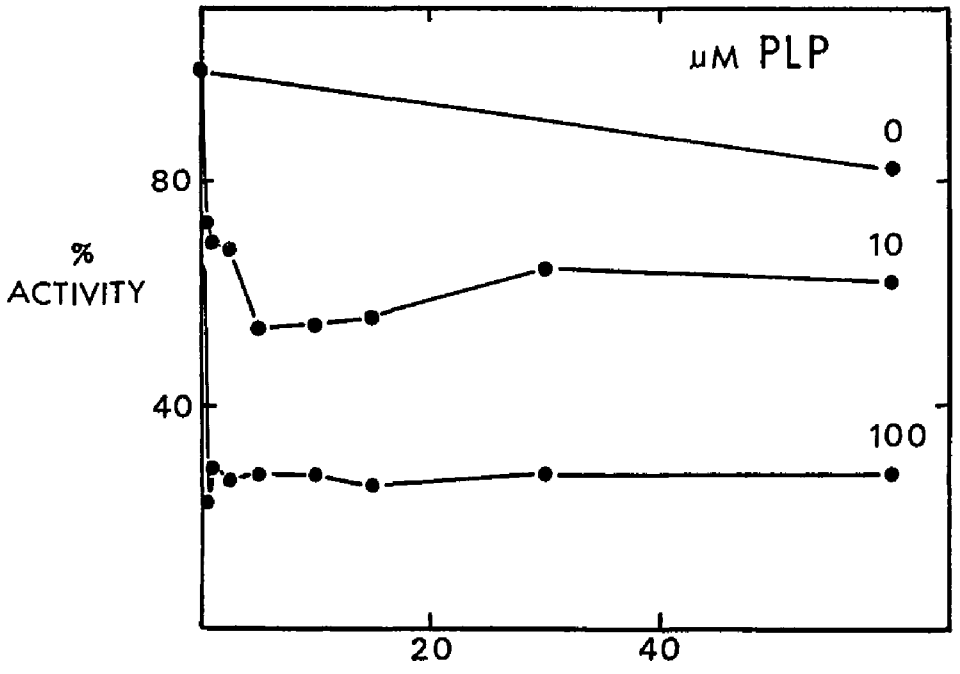


FIGURE 9. The effect of an addition of pyridoxal phosphate (PLP) to an incubation mixture containing YOPRTase. This time-dependent inhibition of the enzyme activity by 10 μM and 100 μM reagent was rendered irreversible by the addition of sodium borohydride and examined using the spectroscopic assay procedure described in "Materials and Methods".

interaction between PLP and enzyme is noncovalent and reversible. The initial step is followed by a covalent bond formation step which is also reversible. Thus an equilibrium is established between free PLP, noncovalently bound PLP, and covalently bound PLP. The addition of sodium borohydride freezes this equilibrium distribution of nonbonded and covalently bonded PLP with the noncovalent complex forming active enzyme and pyridoxol 5'-phosphate. As the PLP concentration is increased the level of inactivation is increased but never goes to completion since there is always some amount of noncovalent complex remaining (Figure 9). This equilibrium level of inactivation is rendered irreversible by the addition of sodium borohydride which reduces the covalently linked PLP-lysine groups and the free PLP.

b) Concentration Dependence of PLP Inactivation Of YOPRTase. The concentration dependence of PLP inactivation of YOPRTase is shown in Figure 10, which shows the YOPRTase activity remaining after 20 min incubations at 25°C with increasing concentrations of PLP, followed by the addition of sodium borohydride.

c) Reactivation Of PLP Inactivated YOPRTase. Table VI lists the results of early reactivation studies in which 25 mM concentrations of PLP were utilized. The experiment is presented because of the striking increase in YOPRTase activity. Natsumeda *et al.*, (1977) reported an activation of HGPRTase when incubated with 2, 4, 6–trinitrobenzenesulfonic acid, a reagent which is known to react with amine groups and thiols (Fields, 1971). They had no explanation for the increased activity. When I used lower concentrations of PLP this activation was not observed. It will require further study if the observed increase in YOPRTase activity is to be understood. The results of the most recent reactivation experiment, which utilized YOPRTase of greater purity and PLP concentrations of 1 mM, are given in Table VII. Only 50% of the activity is recovered when YOPRTase is incubated with 1 mM PLP then subjected to ultrafiltration in the presence of Tris-HCl. Tris, which contains an amine group, can

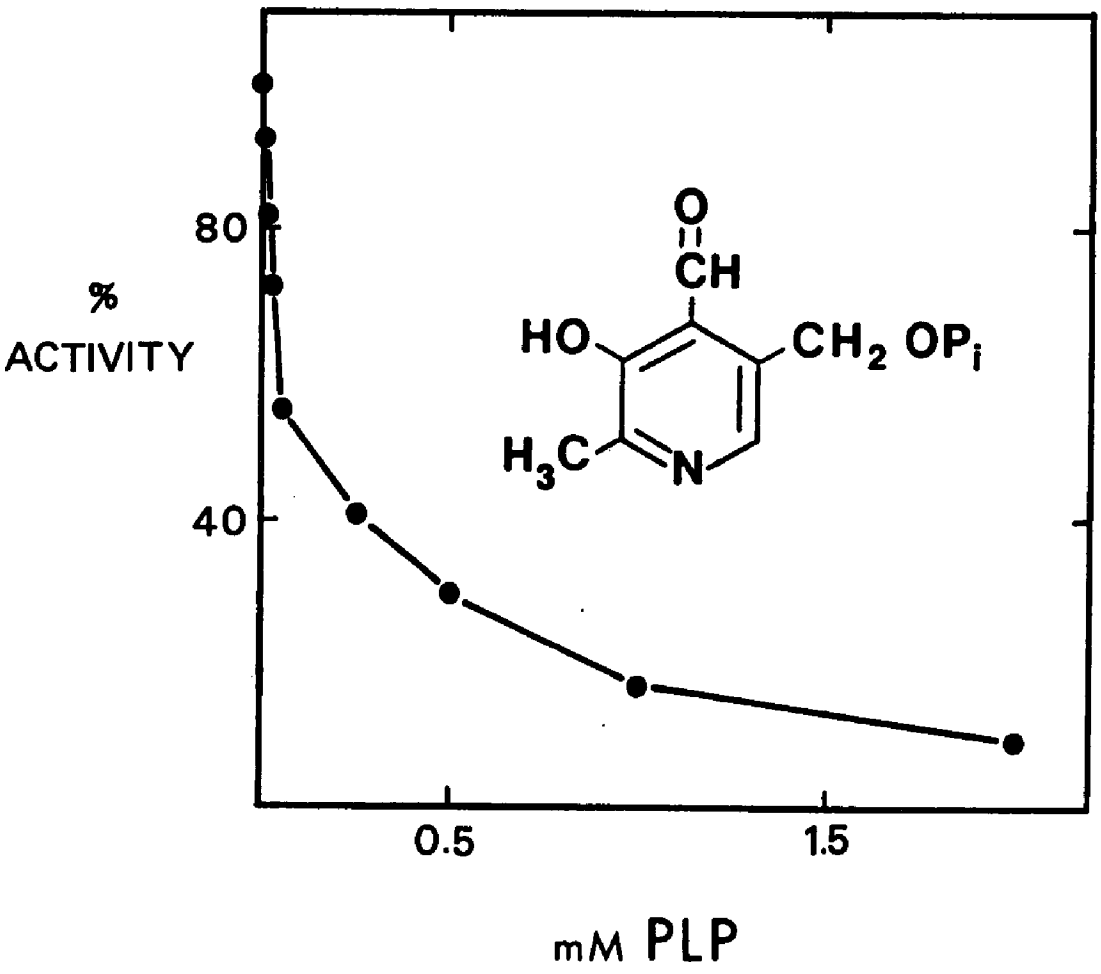


FIGURE 10. The effect of an addition of pyridoxal 5-phosphate (PLP) to an incubation mixture containing YOPRTase. This PLP concentration-dependent inhibition of the enzyme activity, after an incubation period of 15 min., was examined using the spectroscopic assay procedure described in "Materials and Methods". The structure of pyridoxal 5-phosphate has been included for comparison with orotate (Figure 4).

Table VI

Activity† of YOPRTase after an Incubation for 30 min. with Pyridoxal 5-Phosphate in the Presence and Absence of Sodium Borohydride, and followed by a 90 min. Flow Dialysis Experiment.

Incubation	%Activity
Control	87
30 mM $NaBH_4$	68
25 mM PLP	320
24 mM PLP + 30 mM $NaBH_4$	40

†Activity was measured spectrophoto-metrically in an assay mixture containing 300 μM orotate, 100 mM PRibPP, 1mM $MgCl_2$, and 50 mM potassium phosphate, pH 8, in a final volume of 1 ml.

Table VII

Reactivation of YOPRTase, After Treatment with 1 mM Pyridoxal 5-Phosphate in the Presence and Absence of Sodium Borohydride, and After Ultrafiltration†.

Additions		
1 mM PLP	5 mM $NaBH_4$	% Activity Recovered
-	-	100
-	+	76
+	-	52
+	+	0

†YOPRTase was incubated for 10 min. in the presence and absence of 1 mM PLP in 25 mM POPSO buffer, pH 8. The enzyme was then treated or not treated with 5 mM $NaBH_4$ as indicated above. The incubation mixtures were subjected to ultrafiltration using Amicon Centricon-10 units with three additions of 200 µl volumes of 50 mM TRIS-HCl buffer, pH 8. The samples were lyophilized and reconstituted to equal volumes, then assayed with the standard standard spectroscopic assay.

form a Schiff base with PLP and reverse the formation of enzyme–PLP complexes. This result is similar to the result of the reactivation experiment with uracil 6-aldehyde.

When YOPRTase is treated with sodium borohydride alone, approximately 25% of its activity is lost (Table VI, Table VII). While this could be due to denaturation due to the bubbles formed by sodium borohydride in solution, another possibility comes to mind. If an enzyme–phosphoribosyl intermediate does exist, it could be an ester of a glutamic or aspartic acid at the active site. Such an intermediate may be subject to reduction by sodium borohydride, as are phosphoryl-enzyme intermediates (Dahl and Hokin, 1974; Degani and Boyer, 1973). Sloan and Strauss (1984) have determined that NH_2OH inactivates YOPRTase rapidly. This result, in light of studies with sucrose phosphorylase, lends support to the existence of a phosphoribosyl intermediate at the active site of YOPRTase. Alkaline hydroxylamine has been found to rapidly release glucose from sucrose phosphorylase by a displacement reaction in which NH_2OH is incorporated into the enzyme. A pH study with NH_2OH could possibly provide evidence for such an intermediate, and a provide a probe for its nature, allowing one to determine whether it is a thioester, which is labile to acidic NH_2OH, or a carboxy ester, which would be labile to alkaline NH_2OH (Walsh, 1979). Sodium borohydride can also cause a small amount of reductive cleavage of peptide bonds (Walsh 1979). The failure to observe a residual level of activity (the equilibrium level) after treatment with PLP and sodium borohydride (Table VII) is probably due to the failure of this residual activity to survive the lyophilization.

d) PLP Protection Studies. It was found that YOPRTase was partially or completely protected from inactivation when incubated with 1 mM substrate (PRibPP, orotate, OMP, or PPi) and 1 mM PLP at 30°C for 15 min. The results are presented in Table VIII. The degree of protection was dependent upon the nature of the substrate and the presence or absence of $MgCl_2$.

Table VIII

Substrate Protection Against Inactivation of YOPRTase, After Treatment with 1 mM Pyridoxal 5-Phosphate, and Followed by the Addition of 10 mM Sodium Borohydride†.

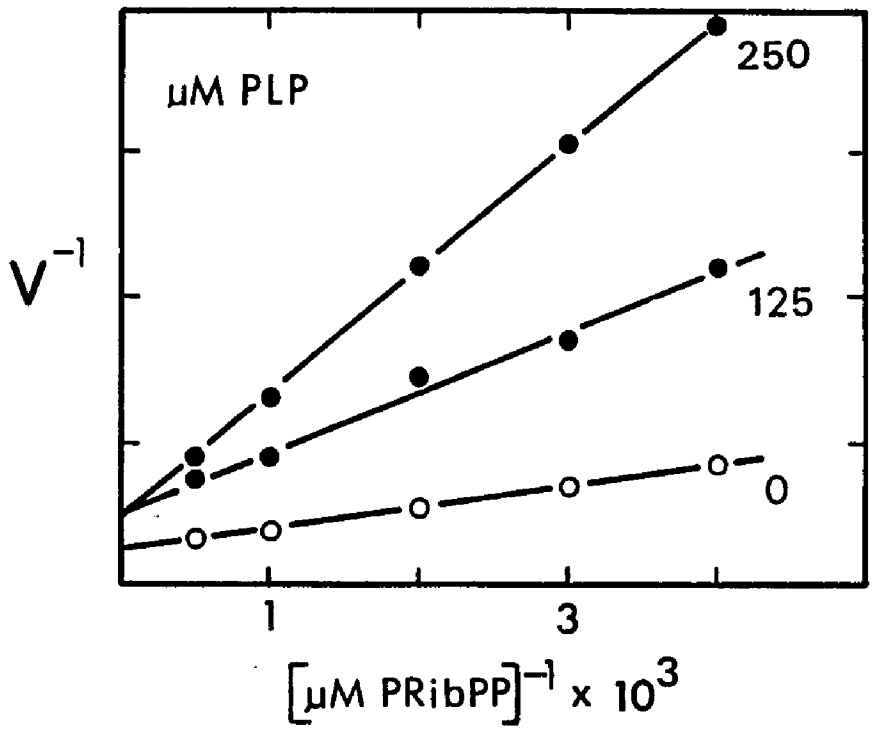
Additions	% Remaining Activity
Control (No PLP)	100
1 mM PLP	17 ± 1
1 mM PLP + 1 mM Orotate	18 ± 1
1 mM PLP + 1 mM PPi	15 ± 2
1 mM PLP + 1 mM PPi +1 mM $MgCl_2$	24 ± 3
1 mM PLP + 1 mM OMP	48 ± 2
1 mM PLP + 1 mM OMP + 1 mM $MgCl_2$	29 ± 1
1 mM PLP + 1 mM PRibPP	49 ± 2
1 mM PLP + 1 mM PRibPP + 1 mM $MgCl_2$ . .	111 ± 7

†YOPRTase was incubated with 1 mM PLP for 15 min. $NaBH_4$ was added to give a final concentraton of 10 mM. Enzyme activity was determined with the standard spectrophotometric assay.

e) Competition Studies With PLP. This experiment was performed to gather information on the extent to which the inactivation by PLP is active site directed, also, to examine the specificity within the active site, for the modification by PLP. Initial velocity determinations of YOPRTase activity were made in the presence of 0, 125, or 250 μM PLP at varied concentrations of PRibPP, while orotate was held constant. The experiment was repeated with orotate being varied while PRibPP was held constant. Upon plotting the data from these competition experiments two distinct patterns emerged which are shown in Figure 11. The reciprocal plot of the data from the experiment with PLP and varied PRibPP shows lines intersecting at the 1/V axis, indicating competitive inhibition. Therefore PRibPP and PLP compete for a binding site on YOPRTase. Also, YOPRTase in the presence of PLP, had a Vmax 50% of the value for YOPRTase in the absence of PLP. This suggests that PLP also interacts with YOPRTase at a site other than the PRibPP binding site. The reciprocal plot for the experiment with PLP and varied orotate resulted in a pattern with an intersection far to the left of the 1/V axis, indicating a mixed inhibition mode for the interaction of PLP and orotate with YOPRTase.. This, in combination with the evidence that PLP and PRibPP are competitive, is evidence that PLP and orotate interact with different sites within the active site of YOPRTase.

f) Amino Acid Analysis of PLP Inactivated YOPRTase. Amino acid analyses were performed to get direct chemical evidence for the modification of YOPRTase lysines. Table IX presents the results of the amino acid determinations of the hydrolysates of modified and unmodified YOPRTase. The values are the average of three determinations for each hydrolysate sample except experiment four, the experiment with YOPRTase treated with PLP and sodium borohydride, for which only two of the determinations were valid, due to technical problems. The error for the overall analysis is between 10% and 15%. Unfortunately, experiments to determine

FIGURE 11. Inhibition of YOPRTase activity by the addition of pyridoxal 5-phosphate (PLP) to the assay solution. The upper figure is a double-reciprocal plot of the initial velocity *versus* PRibPP concentration, in the presence of 0, 125 μM and 250 μM PLP. The lower figure is a double reciprocal plot of the initial velocity *versus* orotate concentration in the presence of these same PLP concentrations. The assay procedure used in this kinetic analysis is described in "Materials and Methods".


μM PLP
250
125
0
V^{-1}
1
3
$[\mu M\ PRibPP]^{-1} \times 10^3$

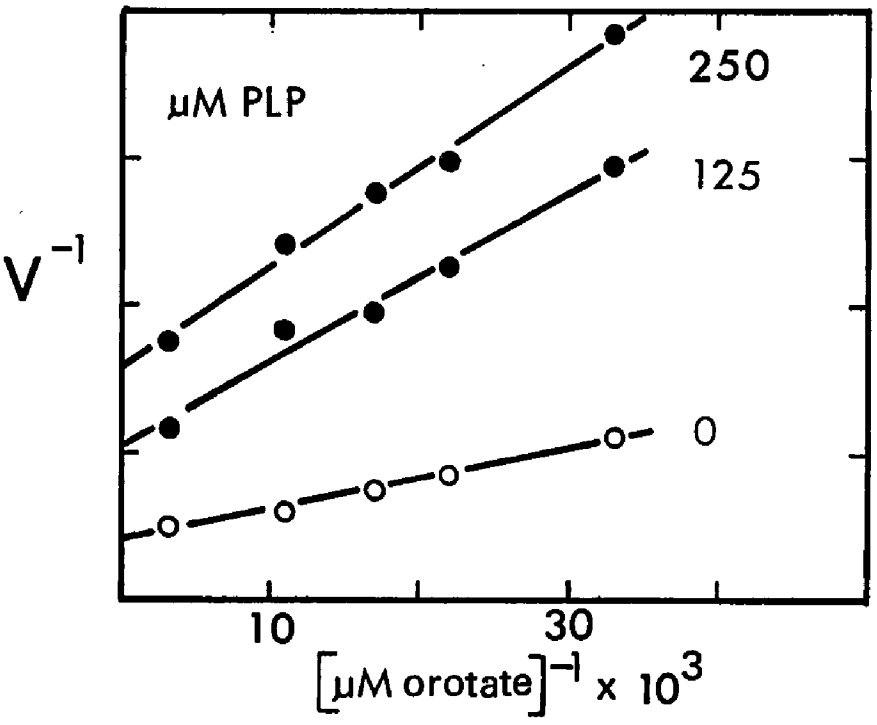

μM PLP
250
125
0
V^{-1}
10
30
$[\mu M\ orotate]^{-1} \times 10^3$

Table IX. Content of the hydrolysates: 1) YOPRTase alone, 2) YOPRTase incubated with 10 mM sodium borohydride, 3) YOPRTase incubated with 1.33 mM PLP, 4) YOPRTase incubated with 1.33 mM PLP, and 12.5 mM magnesium chloride, 5) YOPRTase incubated with 1O mM PRibPP, 12.5 mM magnesium chloride, 1 mM PLP, and 10 mM $NaBH_4$, 6) YOPRTase incubated with 1 mM PLP, 10 mM sodium borohydride and 2.5 mM PRibPP, 7) Average of means for each amino acid determination (excludes value for lysine in experiment # 4).

Table IX

AMINO ACID ANALYSIS OF OPRTase TREATED WITH PLP

	1	2	3	4	5	6	7
ASP†	22.6 ± 2.1	27.3 ± 1.4	27.6 ± 2.0	31.2 ± 0.1	29.2 ± 0.8	27.9 ± 0.5	27.6 ± 2.9
SER	19.0 ± 1.2	20.1 ± 1.6	17.8 ± 1.5	20.7 ± 0.2	18.5 ± 2.8	17.5 ± 0.8	18.9 ± 1.3
GLY	26.0 ± 1.6	33.0 ± 2.1	36.5 ± 0.6	35.7 ± 1.4	36.3 ± 6.1	29.8 ± 0.2	32.9 ± 4.2
GLU††	37.9 ± 0.9	38.8 ± 1.6	41.0 ± 2.1	44.1 ± 0.9	42.1 ± 3.6	39.5 ± 0.8	40.6 ± 2.3
THR	12.5 ± 0.7	13.8 ± 0.9	12.1 ± 1.4	15.0 ± 0.1	15.0 ± 2.6	13.9 ± 1.2	13.7 ± 1.2
PRO	9.7 ± 0.1	7.6 ± 1.3	11.7 ± 1.3	7.5 ± 0.6	————	15.1 ± 1.6	10.3 ± 3.2
ALA	34.6 ± 0.6	38.1 ± 3.7	38.2 ± 0.9	40.0 ± 1.6	38.7 ± 5.8	36.1 ± 1.3	37.6 ± 1.9
VAL	14.9 ± 0.5	15.1 ± 0.4	15.5 ± 1.3	19.7 ± 0.3	17.1 ± 1.1	15.5 ± 0.7	16.2 ± 1.9
MET	2.45 ± 0.3	1.9 ± 0.1	2.5 ± 0.1	2.7 ± 0.0	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.3
TYR	13.8 ± 0.7	13.4 ± 1.0	14.0 ± 0.7	14.9 ± 0.0	13.1 ± 1.3	13.8 ± 0.3	13.8 ± 0.6
ILE	29.8 ± 0.9	30.4 ± 1.0	28.3 ± 3.9	28.9 ± 1.4	32.8 ± 2.2	31.6 ± 0.7	30.3 ± 1.7
LEU	32.2 ± 2.1	33.0 ± 1.5	30.5 ± 2.5	31.9 ± 0.7	32.2 ± 2.7	33.3 ± 1.0	32.2 ± 1.0
PHE	17.1 ± 0.7	17.2 ± 0.9	16.2 ± 1.4	14.8 ± 0.9	17.4 ± 1.9	17.4 ± 1.0	16.7 ± 1.0
HIS	3.50 ± 0.1	3.7 ± 0.2	3.7 ± 0.3	2.8 ± 0.0	3.2 ± 0.3	2.84 ± 0.1	3.3 ± 0.4
LYS	26.1 ± 1.5	26.8 ± 1.6	29.3 ± 4.1	5.9 ± 0.3	27.8 ± 1.6	26.3 ± 0.7	27.3 ± 1.3
ARG	17.0 ± 2.1	24.4 ± 3.0	13.9 ± 1.9	9.6 ± 0.1	13.5 ± 3.6	9.8 ± 1.2	14.6 ± 5.4

†Value includes asparagine residues.

††Value includes glutamine residues.

cysteine were not performed. The values shown are based on estimated MW's of 40,000 and 120, for YOPRTase and an amino acid residue, respectively. In experiment 4, where YOPRTase has been incubated with 1.33 mM PLP for 15 min at 30°C, prior to the addition of sodium borohydride to a final concentration of 12.5 mM, a dramatic difference is seen in the number of lysines. This treatment has apparently resulted in the loss of approximately 21 lysine residues out of a total of 27 residues for the YOPRTase dimer, demonstrating that YOPRTase lysine residues are modifed by PLP. An unexpected result is the lack of evidence of any lysine modification when YOPRTase is incubated with both PRibPP and PLP prior to reduction with sodium borohydride. Perhaps the positively charged lysine residues are protected from modification by the highly anionic PRibPP. Also, when YOPRTase is treated with sodium borohydride alone no significant changes occur, except the large increase in arginine. However, the large variations seen in arginine are possibly due to contaminants eluting with arginine, which is the last residue to elute from the chromatographic column. The variations in glycine may be due to contamination, while those for proline were due to technical problems with the assay for imino acids. Either tryptophan was destroyed in the hydrolysis despite the addition of 1% thioglycollic acid, or tryptophan is not present in YOPRTase, contrary to Sloan and Strauss (1984). The values in column 7, the averages of the all the amino acid determinations, excluding lysine for experiment 4, were used to calculate the normalized amino acid content for a YOPRTase subunit of MW 18,550. This value is based on a MW determination that will be discussed later. The normalized amino acid content is shown in Table X. The values for YOPRTase cysteine and tryptophan are from Sloan and Strauss (1984).

TABLE X

YOPRTase AND *E. COLI* OPRTase AMINO ACID COMPOSITION

	YOPRTase	*E. coli*
ASP†	12	11
ASN	---	6
SER	9	9
GLY	15	19
GLU††	18	19
GLN	---	7
THR	6	9
PRO	5	6
ALA	16	23
VAL	7	12
MET	1	3
TYR	6	8
ILE	13	14
LEU	14	23
PHE	8	12
HIS	2	4
LYS	12	12
ARG	7	12
CYS	2	2
TRY	2	0

†Includes asparagine residues for YOPRTase.
††Includes glutamine residues for YOPRTase.

Also shown in Table X, for comparison, is the amino acid content of the *E. coli* OPRTase subunit, based on its DNA nucleotide sequence (Poulsen *et al.*, 1983).

g) PLP Incorporation Into YOPRTase. The purpose of this experiment was to determine both the total number of lysine residues modified for a given PLP concentration, and the number of lysines essential for activity, by correlating the amount of PLP incorporated with the degree of inactivation. However, the interpretation of this experiment is complicated by several factors. First, the blank runs (tritium containing samples with no protein were applied to filters) have CPM's that are very high relative to the number of counts incorporated into the protein. This could have been circumvented by using higher protein concentrations, but this was not an option open to me, due to the limited quantity of YOPRTase available. Another option, making and using [^{3}H]PLP, which should be much cleaner and also more readily quantified, was ruled out due to time constraints. Another consideration is that PLP may be causing the inactivation of YOPRTase by interactions other than the modification of lysine. Also, as discussed above it is possible that sodium borohydride could reduce an enzyme substrate intermediate and thus cause incorporation of ^{3}H in the absence of PLP. Indeed, a number of the control runs (YOPRTase treated with $NaBH_4$ in the absence of PLP) had high CPM's, and the one with the lowest was used to make the calculations.

When YOPRTase was incubated with increasing concentrations of pyridoxal 5-phosphate, followed by reduction with [^{3}H]sodium borohydride, then collected on glass filters and washed with 10% TCA, it was found that ^{3}H was incorprated into YOPRTase, thus demonstrating that PLP is incorporated into YOPRTase. Figure 12 shows the number of moles of PLP incorporated per mole of YOPRTase active sites as a function of the inactivation of YOPRTase. From the data it appears that there are three lysines modified per subunit as YOPRTase approaches full inactivation.

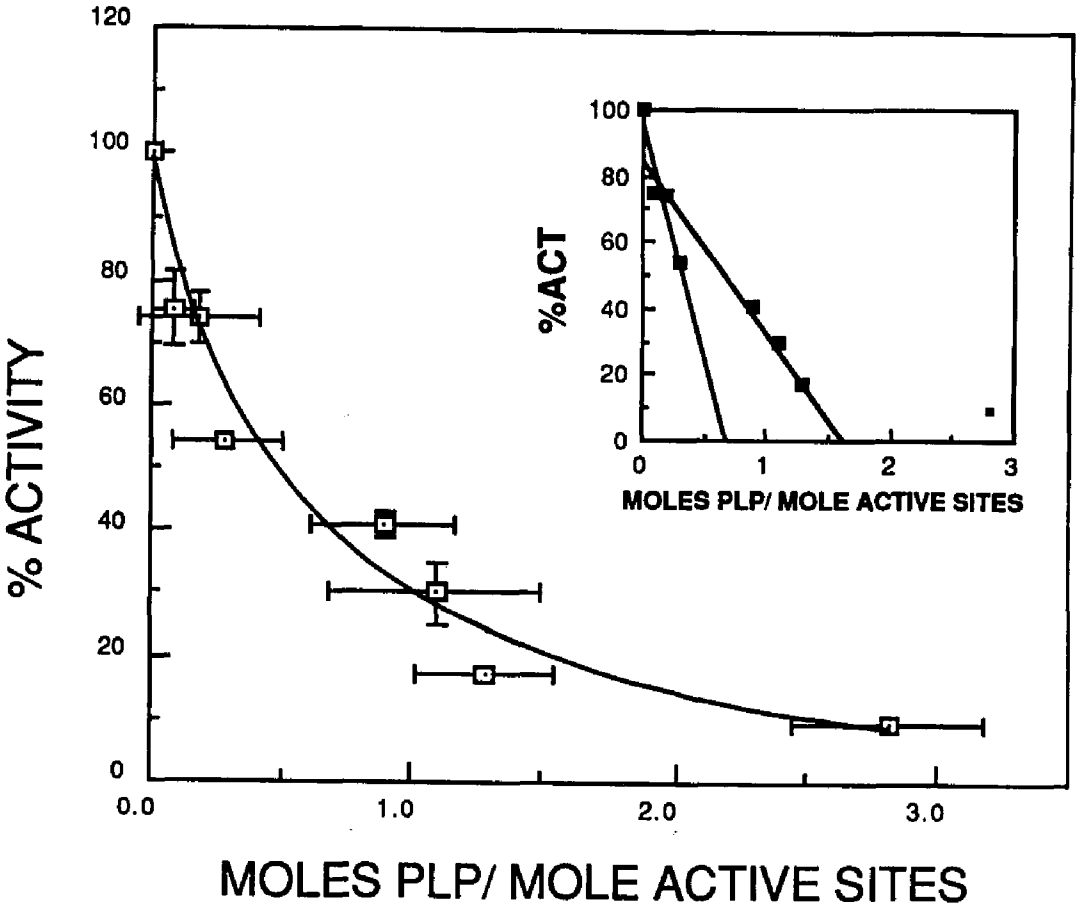


FIGURE 12. Number of moles of pyridoxal 5-phosphate incorporated per mole of YOPRTase active site during incubations of the enzyme with several PLP concentrations followed by the addition of tritiated sodium borohydride, *versus* YOPRTase activity. The inset shows two possible interpolations of the data in order to determine the number of "essential" lysines. The counting procedure and the enzyme assay procedure are described in "Materials and Methods".

However, modification of a lysine residue does not necessarily lead to inactivation. In order to determine the number of *essential* lysines modified one can extrapolate the initial part of the inactivation curve to zero activity, as shown in the inset to Figure 12. Since the data are not unambiguous, two possible fits are made to the early points in the curve. The result leads one to conclude that one to two of the lysines modified per subunit are essential for activity. The most obvious problem that arises is accounting for the large discrepancy between the moles/mole of YOPRTase modified lysines as determined by the amino acid analysis experiment, approximately 20 at a PLP concentration of 1.33 mM, and that quantity as determined in this experiment, approximately 3 at a concentration of 1 mM PLP.

7) ENZYMATIC COUPLED ASSAY EMPLOYING HPLC

Figure 13 shows the elution profiles for the incubation of reactants with ribokinase, PRibPP synthetase, orotate phosphoribosyltransferase and inorganic pyrophosphatase. The isocratic elution buffer provided complete resolution of AMP, orotate, ADP, OMP, and ATP which were found to elute at 4.2, 4.5, 5.3, 5.9 and 7.3 min respectively. Figure 14A is a plot of the time-dependent changes in peak heights for ATP, ADP, and AMP during an incubation with RKase in the presence and absence of ribose. AMP and ADP peak heights can be compared to ATP peak heights by dividing them by 3.0 and 2.4 respectively. In the absence of ribose there is a small amount of ADP formed due to ATPase activity in the partially purified ribokinase preparation. However upon adding ribose, large changes in AMP, ADP, and ATP concentrations occur. ADP is synthesized originally via the RKase reaction, then is utilized by contaminating myokinase. Figure 14B provides the reaction profile for an

FIGURE 13. HPLC elution profiles for the incubation of reactants wih ribokinase, PRiPP synthetase, YOPRTase and inorganic pyrophosphatase.

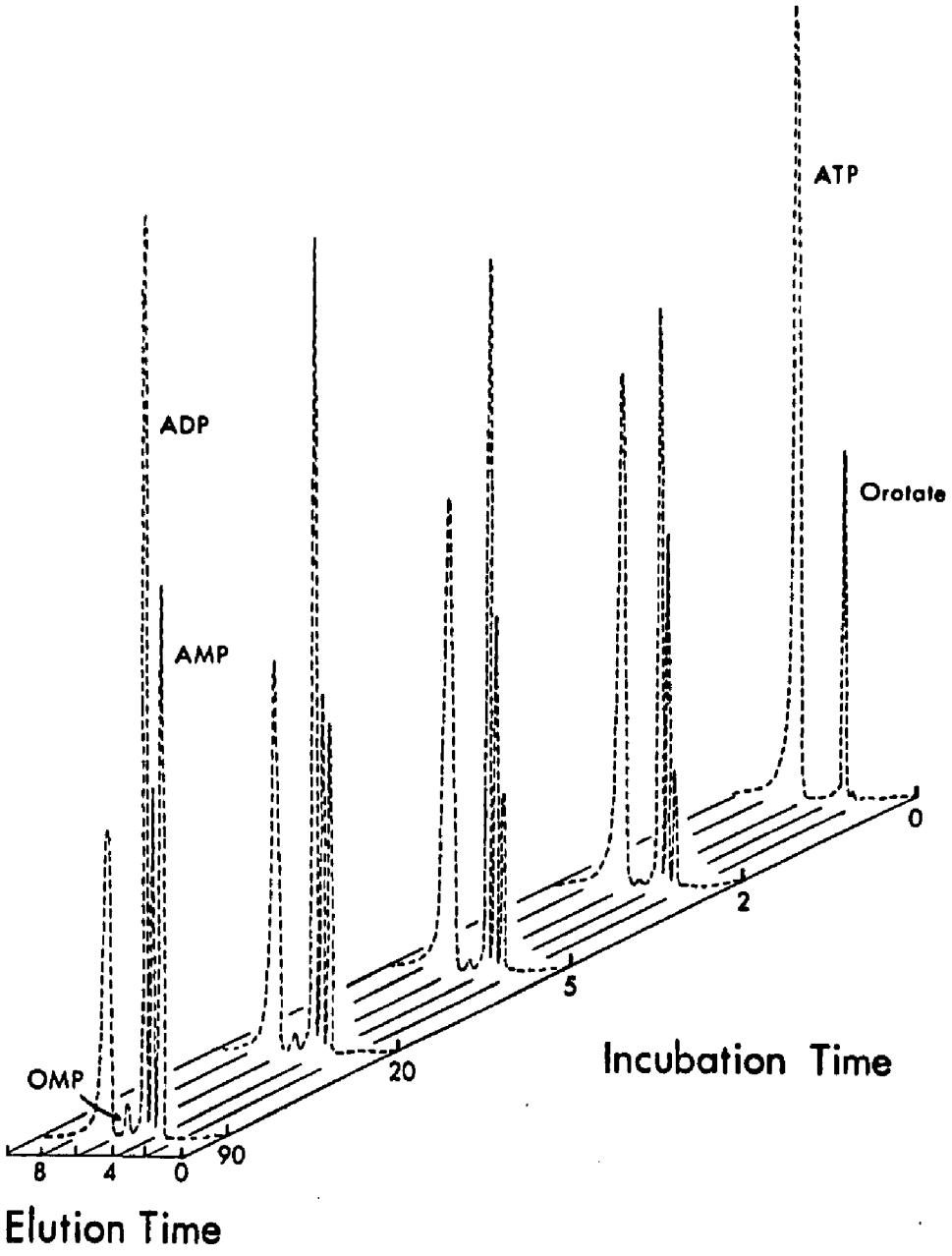
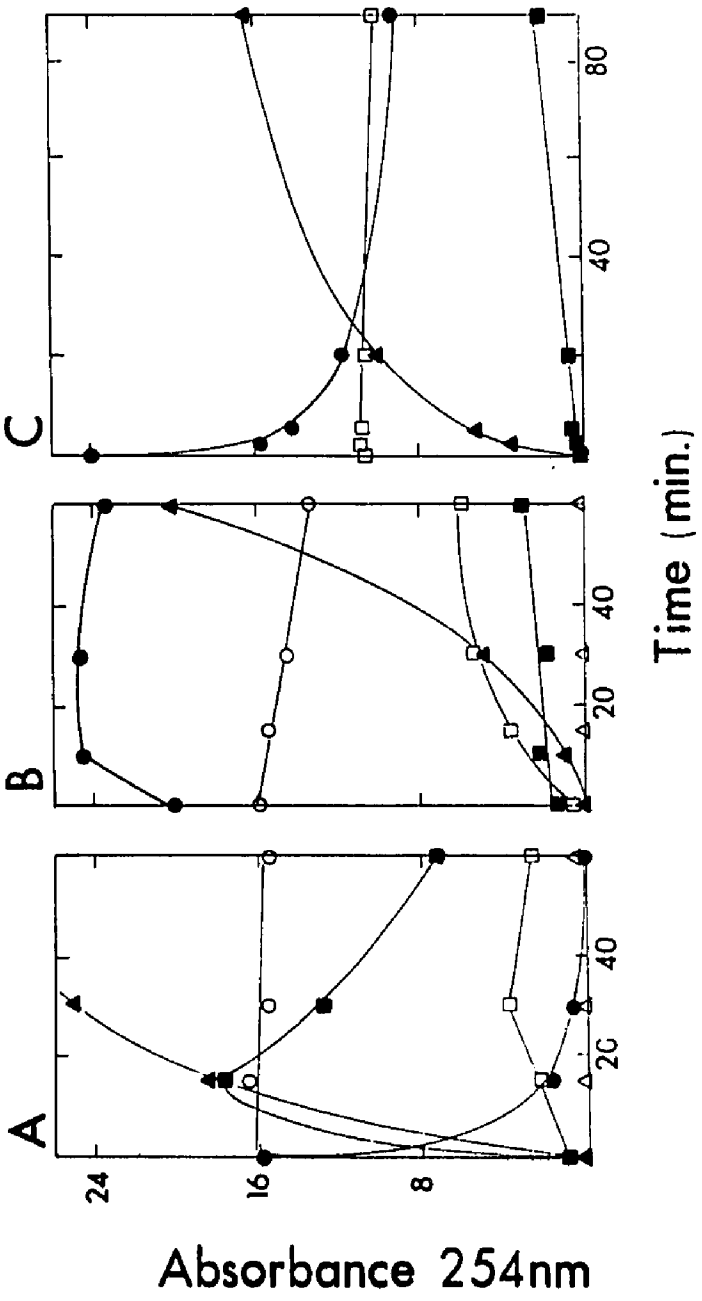

ATP
ADP
Orotate
AMP
OMP
0
2
5
20
90
8
4
0
Incubation Time
Elution Time

FIGURE 14. Time dependent changes in reactant concentrations during an incubation with ribokinase (RKase) and PRibPP synthetase (PRSase), or an incubation with those enzymes plus yeast orotate phosphoribosyl-transferase (YOPRTase) and inorganic pyrophosphatase (PPase). Reactant concentrations were determined from their absorbance at 254 nm when eluted from a quarternary amine HPLC column. A) RKase Assay: ATP concentration in the presence (●) and absence (○) of ribose, ADP concentration in the presence (■) and absence (□) of ribose, AMP concentration in the presence (▲) and absence (△) of ribose. B) RKase and PRSase incubated in the presence of ribose 5-phosphate: ATP concentration during RKase incubation (○), ADP concentration during RKase incubation (□), AMP concentration during RKase incubation (△), ATP concentration during PRSase incubation (●), ADP concentration during PRSase incubation (□), AMP concentration during PRSase incubation. C) Assay components for RKase, PRSase, YOPRTase and PPase: ATP (●), ADP (△), AMP (▲), orotate (□) and OMP (■). Assay and HPLC conditions were as described in "Materials and Methods".


A
B
C
24
16
8
20
40
40
80
Absorbance 254nm
Time (min.)

incubation with RKase in the presence of 100 μM R5P, 100 μM ATP, 1 mM $MgCl_2$, 50 mM TEA and 50 mM potassium phosphate, pH 8, as a test for PRSase activity in the RKase preparation. The synthesis of a small amount of ADP occurs but no AMP is formed. When PRSase is added to to this incubation mixture the amount of AMP formed is a direct measure of synthesized PRibPP. Figure 14C shows the reaction profile for an incubation mixture containing RKase, PRSase, YOPRTase and PPase activities and the substrates, ribose, ATP, orotate, and 1 mM $MgCl_2$. In Figure 14C we are following the approach to a steady state within this incubation system. The increasing concentrations of ADP and AMP are measures of the concentrations of ribose 5-phosphate and PRibPP, respectively, though allowances must be made for contaminating myokinase. However, the decrease in orotate and the increase in OMP concentrations are direct measures of the synthesis of OMP. Figure 13 shows the HPLC elution profiles as a function of time, for the incubation followed in Figure 14C. The excellent resolution of the reactant peaks is apparent.

8) QUATERNARY STRUCTURE DETERMINATION

a) Non-reacting Gel Experiments. Figure 15 is the calibration curve of the Bio-Sil TSK-250 HPLC gel filtration column used in this experiment. Over the loading concentration range of 0.25 to 5 μg/ml, YOPRTase remains in a dimeric configuration when loaded onto an HPLC gel filtration column with a running buffer consisting of 100 mM TEA, pH 6.8, alone or with 1 mM $MgCl_2$, as determined by its eluting position from the Bio-Sil column. When the buffer contained 100 mM TEA, pH 6.8, 1 mM $MgCl_2$ and 100 μM PRibPP, YOPRTase, again, remained in a dimeric configuration. Figure 16B shows the elution profiles. YOPRTase had an apparent

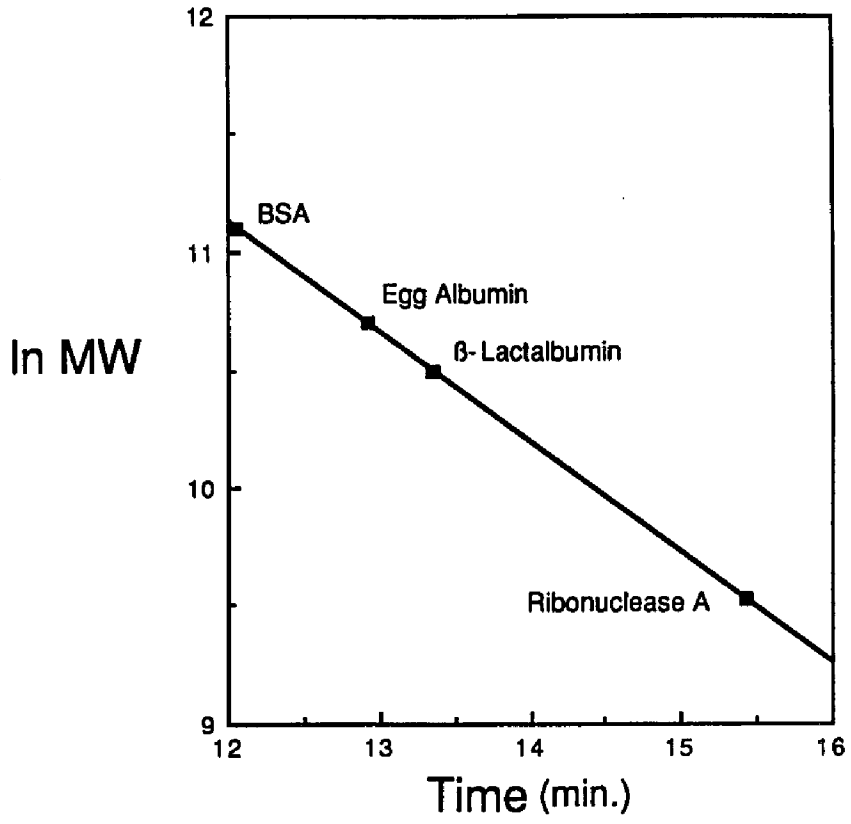
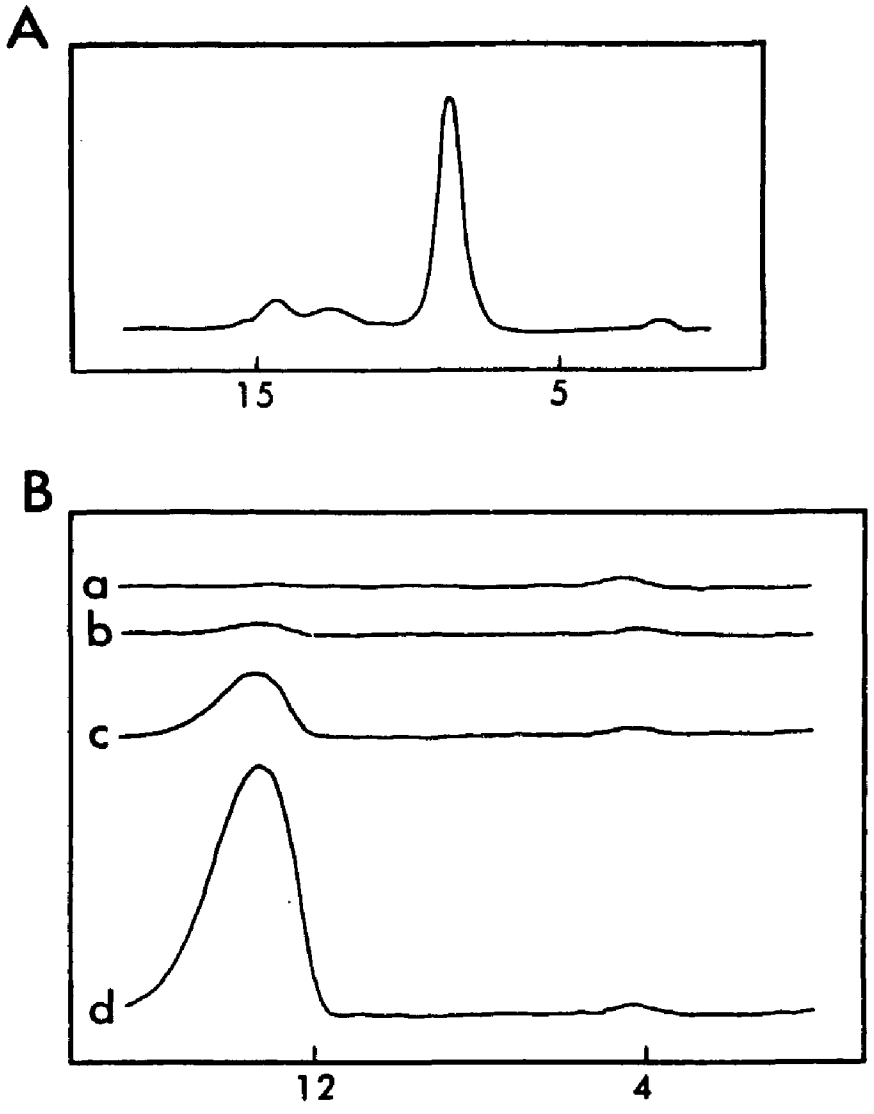


FIGURE 15. Calibration curve for Bio-Sil TSK-250 HPLC gel filtration column. The natural logs of the standards' MW were plotted against the standards' elution times.

FIGURE 16. Elutions of YOPRTase through the Bio-Sil TSK-250 HPLC gel filtration column. A) Sample of YOPRTase (10 µl sample of a 0.52 mg/ml stock solution), using absorbance at 254 nm for detection. Because no reaction coil was used for this run, YOPRTase eluted at approximately 8 min. B) Nonreacting gel experiment. A 50 µl aliquot of YOPRTase (E), eluted with a 100 mM triethanolamine (pH 6.8) solution, and using a reaction coil to allow enzymatic activity to mark point of elution. a) 1 µg/ml E after heat denaturation, b) 0.25 µg/ml E, c) 1 µg/ml E, and d) 5 µg/ml E. C) Reacting gel experiment. A 50 µl aliquot of YOPRTase (E), eluted with a 100 mM triethanolamine (pH 6.8) solution containing 100 µM PRiPP, 100 µM orotate and 1 mM $MgCl_2$, a reaction coil was in place and enzymatic activity marked the point of elution. a) 0.25 µg/ml E, b) 1 µg/ml E, and c) 5 µg/ml E.


A
15
5
B
a
b
c
d
12
4
C
a
b
c
12
4

molecular weight of 37.1 x 10^3, within a 95% confidence interval of 35.8 x 10^3 to 38.5 x 10^3, when eluted with buffer alone or with buffer and $MgCl_2$ (the elution times for YOPRTase in the presence and absence of $MgCl_2$ were the same, within experimental error, and the data were combined). When eluted with buffer containing PRibPP and $MgCl_2$, YOPRTase had an apparent molecular weight of 38.9 x 10^3 within a 95% confidence interval of 36.8 x 10^3 to 41.1 x 10^3. The difference in the molecular weights is significant at the 95% level of certainty. The 1,800 difference in apparent MW cannot be accounted for entirely by the binding of two PRibPP molecules (PRibPP MW = 383) per YOPRTase dimer, suggesting that hydrodynamic changes are occurring, possibly due to conformational changes or changes in bound water. When YOPRTase was injected at a loading concentration of 0.1 μg/ml, the eluting peak (not shown) was barely distinquishable from the baseline. This loading concentration for YOPRTase was examined only in 100 mM TEA, pH 6.8, with no additions. The apparent molecular weight remained 37.1 x 10^3.

b) Reacting Gel Experiments. The results of the reacting gel experiment with a running buffer consisting of 100 μM PRibPP, 100 μM orotate, 1 mM $MgCl_2$ and 100 mM TEA, pH 6.8 are shown in Figure 16C. These data were not amenable to a straightforward interpretation. The problem arises in choosing a point on the elution profile that measures the elution time for YOPRTase. It is unlikely that linear kinetics have been achieved under the conditions described, so the centroid position cannot be assumed to give the correct value (Ackers, 1976). The failure to to achieve linear kinetics is suggested by the lack of change in the depletion region that preceded the product peak in the elution profiles of the reacting gel experiments, as enzyme concentration was increased (not shown). The depletion region is a negative area of UV absorbance, relative to the baseline, that is a measure of the amount of UV absorbing substrate utilized to form product.

c) Experiments Using Higher YOPRTase Concentration. Figure 16A shows the result of an experiment in which YOPRTase was injected at a concentration of 0.54 mg/ml and observed directly by its absorbance at 254 nm. Since YOPRTase did not appear to undergo concentration dependent changes in apparent molecular weight, an experiment was performed at an YOPRTase concentration that allowed observation of eluting protein at 254 nm. A 200 µl aliquot of 60 µg/ul YOPRTase was injected onto a column equilibrated with 100 mM TEA pH, 6.8. The column was then equilibrated with 10 µM OMP, 1 mM $MgCl_2$ and 100 mM TEA, pH 6.8, and YOPRTase aliquots were injected again. The elution time for YOPRTase was 9.28 ± 0.03 min with buffer alone and 9.20 ± 0.05 min when OMP and $MgCl_2$ were included (not shown). These values cannot be used to interpolate an apparent molecular weight from the previous calibration curve because no reaction line was used in this last experiment. However, the elution times for YOPRTase with and without OMP and $MgCl_2$, are essentially the same, within experimental error, and one can say that there is no significant change in the apparent molecular weight when OMP and $MgCl_2$ are added to the running buffer.

9) [1-^{14}C]PRibPP SYNTHESIS

a) Synthesis and Purification. The batch of [1-^{14}C]PRibPP synthesized and used for the gel filtration experiment with YOPRTase was approximately 95% labeled PRibPP, as determined by the HPLC assay discussed below. The remaining 5% of radioactivity is most likely due to labeled R5P. The batch prepared for use in the flow dialysis PRibPP binding experiment was only 60% in labeled PRibPP as determined by the HPLC assay. This was probably due to a poor connection between the DEAE column and the gradient reservoir which resulted in buffer prematurely leaking onto the

column, spoiling the gradient.

b) HPLC Assay of Labeled PRibPP. Figure 17 shows the elution profile of a sample containing orotate, OMP, and UMP when loaded onto the C_{18} HPLC column. OMP eluted at 2.98 min; orotate at 4.2 min; and UMP at 4.7 min, as determined by injections of single component, standard solutions. Figure 18A is the elution profile for the injection of an aliquot from the incubation mixture containing [^{14}C]PRibPP but no enzyme. The major peak of radioactivity was found to elute at 3.0 min. Figure 18B shows the elution profile for a duplicate incubation mixture sample that was heated for 2 min in a boiling water bath, prior to removing an aliquot for injection. The major peak of radioactivity now elutes at 3.5 min, probably due to the breakdown of [^{14}C]PRibPP to [^{14}C]R5P]. When YOPRTase and ODCase activities were added to the incubation mixture the major peak of radioactivity was found to elute at 5.0 min, the region where UMP elutes, as shown in Figure 19A. This experiment was repeated, with samples being heated in a boiling water bath for two min. prior to removing an aliquot for injection. The elution profile is shown in Figure 19B. The major peak of radioactivity elutes at 4.7 min, again, the region where UMP elutes. Using this assay it was found that the [1-^{14}C]PRibPP preparation used for the flow dialysis binding experiment had only 60% of the total radioactivity incorporated into UMP. Therefore only 60% of the radioactivity of this batch was associated with PRibPP.

10) GEL FILTRATION OF YOPRTase-[1-^{14}C]PRibPP COMPLEX

Figure 20 shows the result of the application of a 300 µl volume containing [^{14}C]PRibPP, 49 µg of YOPRTase, 2 µg of pyrophosphatase, 1 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8, to a molecular sieve column. The elution profile shows that a

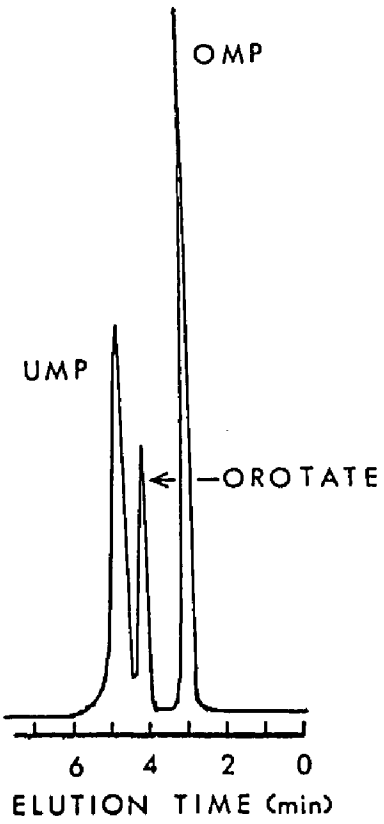


FIGURE 17. Reversed phase high pressure liquid chromatography separation of the UV absorbing components of the YOPRTase assay solution.

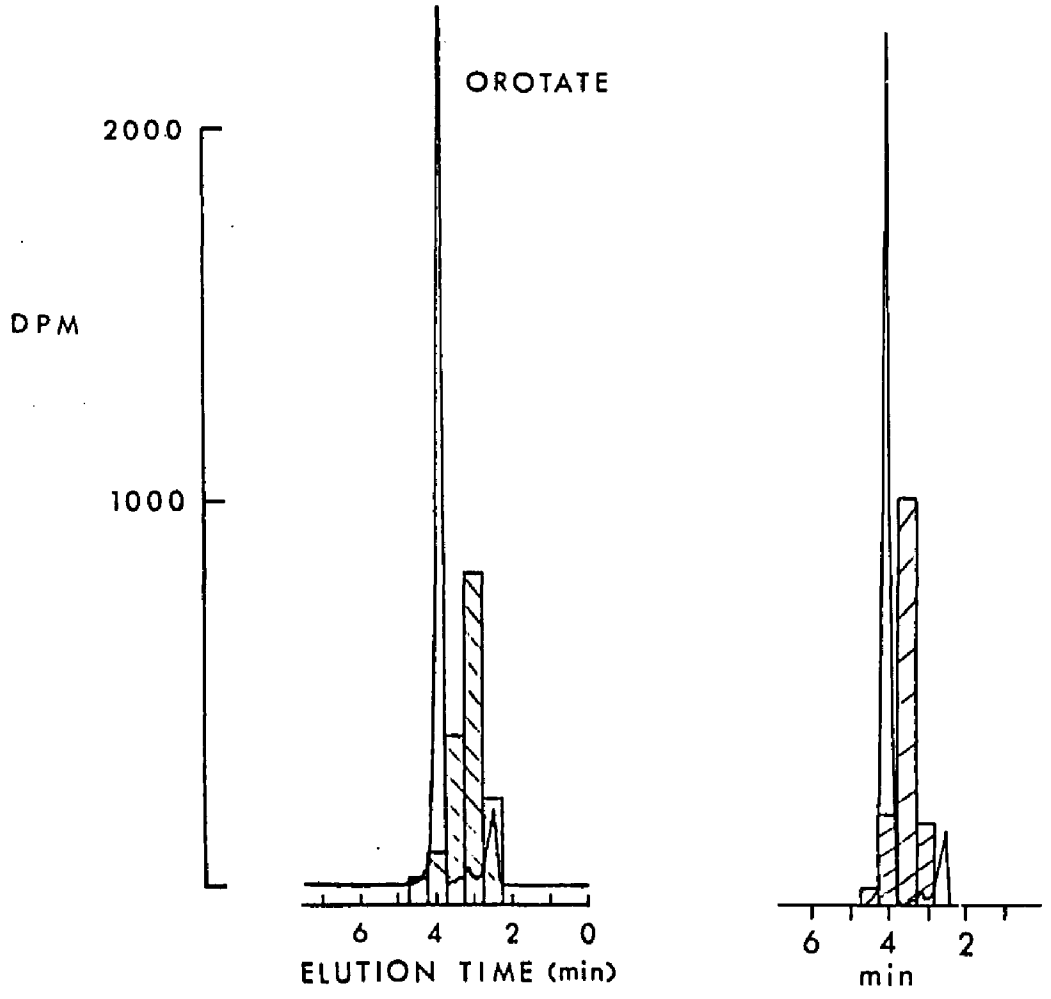


FIGURE 18. Reversed phase high pressure liquid chromatography separation of the UV absorbing components of the YOPRTase-YODCase assay solution prior to the addition of the enzymes. [^{14}C]PRibPP has been added and its elution position identified through the use of radioactivity counting procedures described in "Materials and Methods". DPM are indicated by the bars. A) Assay mixture was filtered prior to injection. B) Assay mixture was heated in boiling water for two min. and then filtered prior to injection.

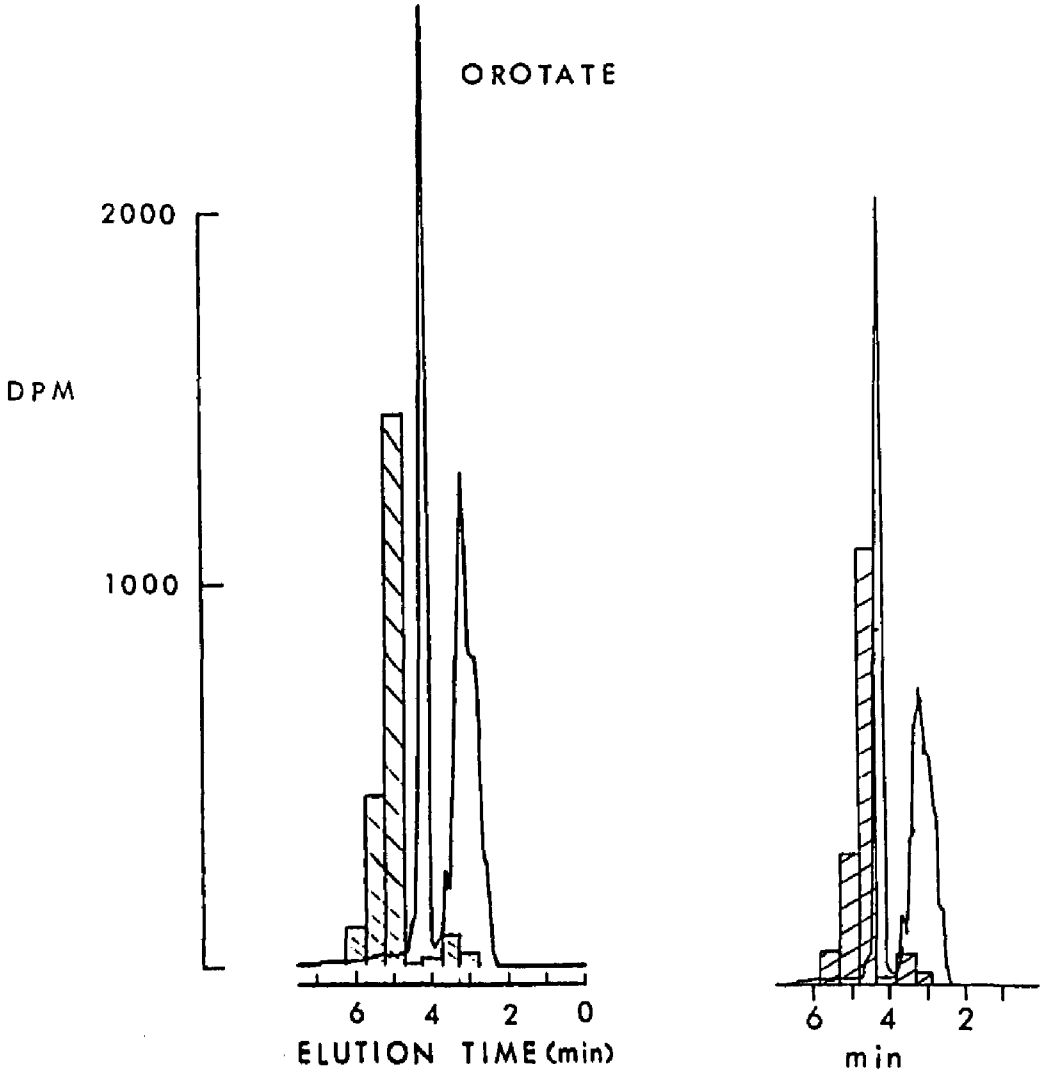


FIGURE 19. Reversed phase high pressure liquid chromatography separation of the UV absorbing components of the YOPRTase-YODCase assay solution after the addition of the enzymes. [^{14}C]PRibPP has been added and its elution position identified through the use of radioactivity counting procedures described in "Materials and Methods". DPM are indicated by the bars. A) Assay mixture was filtered prior to injection. B) Assay mixture was heated in boiling water for two min. and then filtered prior to injection.

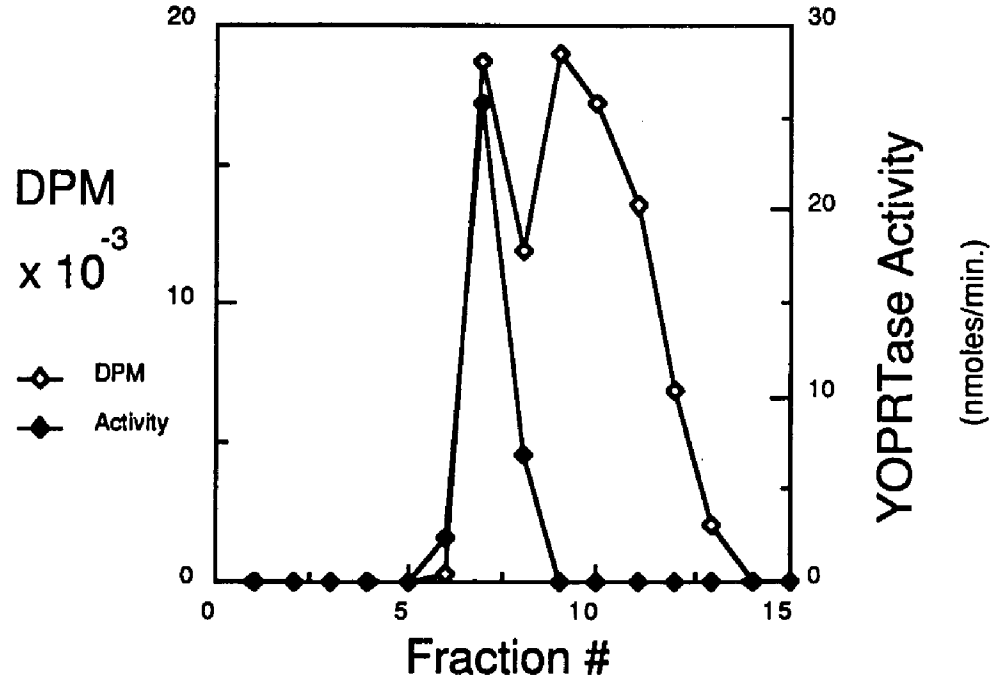


FIGURE 20. Gel Filtration Chromatography (Sephadex G-25) Fractionation of newly synthesized labeled (^{14}C)-PRibPP in the presence of YOPRTase. YOPRTase activity was located with the spectroscopic assay. Conditions of the chromatography experiment are described in "Materials and Methods". Fractions containing radioactivity were identified by Liquid Scintillation Counting as described in "Materials and Methods".

portion of the [^{14}C]PRibPP coeluted with the YOPRTase activity. The fraction of the total YOPRTase applied to the column that eluted in each fraction was estimated from the observed activity in 10 μM aliquots. Of the 49 mg of YOPRTase applied to the column, 3.5 μg eluted in fraction six; 36 μg in fraction seven; and 9.5 μg in fraction eight. These values corresponded to 0.18, 1.8, and 0.41 nmoles of YOPRTase active sites in the respective fractions. From the total DPM's in each fraction, the amount of [^{14}C]PRibPP in each fraction was determined to be 3 x 10^{-3}, 0.17, and 0.11 nmoles for fractions six, seven, and eight, respectively. The active site to [^{14}C]PRibPP ratios for the fractions are 60:1, 11:1, and 4:1 for fractions six, seven, and eight, respectively. Therefore YOPRTase is eluting faster than the coeluting [^{14}C]PRibPP. This suggests a simple equilibrium between YOPRTase bound PRibPP and free PRibPP as the YOPRTase moves down the column, or that an enzyme-substrate intermediate is rapidly hydrolyzed. In either case this is further evidence that [^{14}C]PRibPP has been synthesized. Bell and Koshland (1970) used this type of experiment, with similar results, to argue that ATP-PRTase formed a covalent phosphoribosyl-enzyme intermediate when incubated with ^{14}C-PRibPP. This was subsequently proved erroneous by Brashear and Parsons (1975). What Bell and Koshland had isolated was enzyme with tightly bound product formed from bound ATP and the added [^{14}C]PRibPP. Thus this type of experiment, as it stands, cannot be used as an argument for the existence of an enzyme substrate intermediate.

11) [1-^{13}C]PRibPP SYNTHESIS

It was found that the reaction to synthesize [1–^{13}C]PRibPP attained equilibrium after approximately 3 hr. A typical synthesis and isolation resulted in 0.4 ml of 14.5

mM [1-^{13}C]PRibPP. The [1-^{13}C]-spectrum of [1-^{13}C]PRibPP is shown in Figure 28.

12) FLOW DIALYSIS: [1-^{14}C]PRibPP BINDING STUDY

The flow dialysis experiment was performed in order to determine the dissociation constant for the binding of PRibPP to YOPRTase, and the number of binding sites. The procedure used was published by Colowick and Womack, (1969). The results of the flow dialysis experiments are shown in Figure 21. The plot of the calibration experiment is labeled A, the plot for the experiment with enzyme is labeled B and the plot of the corrected and normalized experimental values is labeled C. The last point on the calibration curve had to be extrapolated. Samples to which additions were made are marked by arrows, which are labeled with the approximate concentration of PRibPP in the upper chamber after each addition.

From curve A it can be seen that the addition of a 5 µl aliquot containing 0.45 M PRibPP, 1 M $MgCl_2$ and 50 mM Tris, pH 8, to the upper chamber as the 16th fraction collected, had no visible effect on the dialysis rate of labeled PRibPP from the apparatus. Therefore it is unlikely that binding of [^{14}C]PRibPP to the apparatus or membrane was occurring. This also illustrates that the increase in the dialysis rate of total PRibPP, due to its increased concentration in the upper chamber, is exactly compensated for by the decrease in specific activity of the [^{14}C]PRibPP. Thus there is no increase in the amount of labeled isotope eluting from the flow dialysis unit upon the addition of unlabeled PRibPP in the absence of enzyme.

The experiment was complicated by two factors. The [^{14}C]PRibPP sample was only 60% PRibPP with the other 40% of the [^{14}C] associated with what is most likely

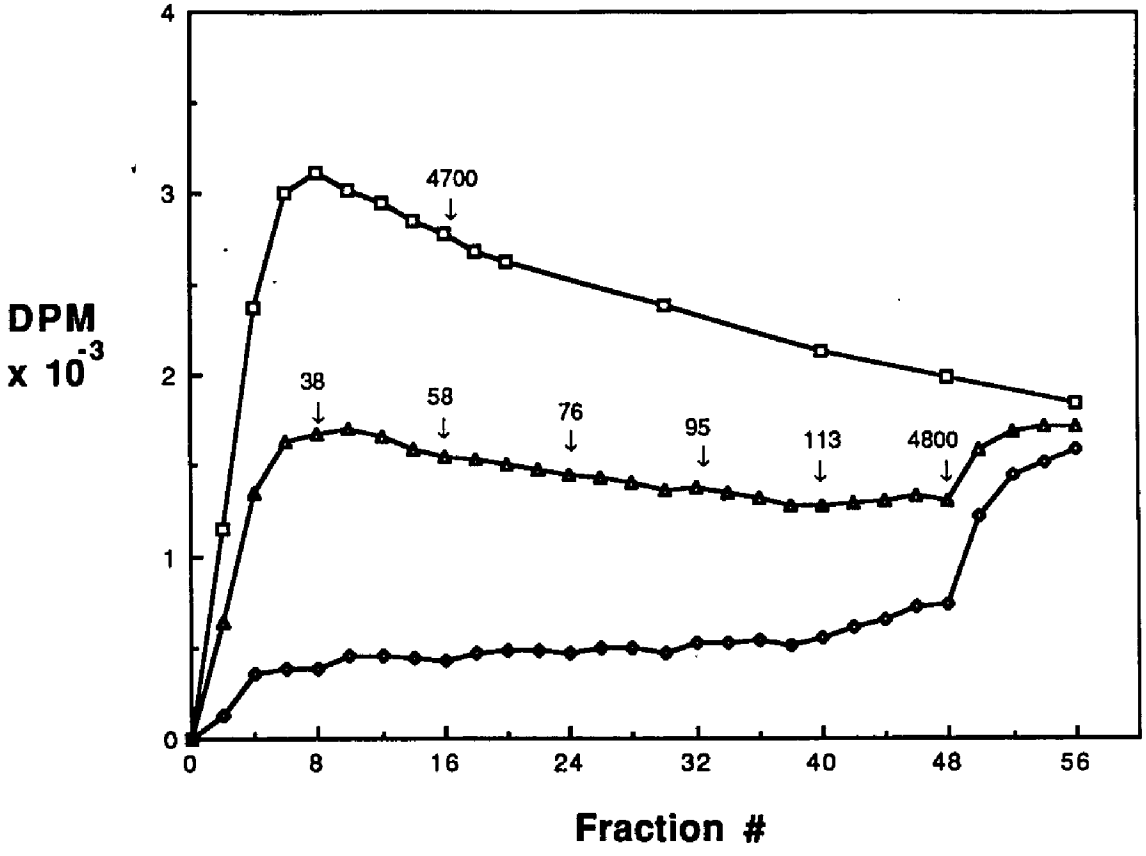


FIGURE 21. Flow dialysis measurements of the binding of ^{14}C-PRibPP to YOPRTase: control experiment (□) where the sample chamber contained ^{14}C-PRibPP but no enzyme, binding experiment (Δ) where sample chamber contained 200 µl ^{14}C-PRibPP and 250 µl of a 4 mg/ml solution of YOPRTase. The experiment was performed as discussed in "Materials and Methods". The arrows mark the additions of unlabeled PRibPP and are labeled with approximate concentrations.

R5P, the precursor and breakdown product of PRibPP (due to time constraints I had to use this batch). The other problem was that the loss of the [^{14}C]PRibPP from the upper chamber over the time of the experiment was much greater than that of the [^{14}C]glucose that Colowick and Womack used in their experiments. In their control study, it can be seen that the amount of [^{14}C]glucose in the effluent from the flow dialysis unit remained virtually constant during the experiment, once a steady state dialysis rate was reached. Greater than 98% of the initial steady state value was maintained in their experiment. However, in my experiment there was a constant and significant decrease in eluting [^{14}C]PRibPP CPM's as the experiment proceeded (Figure 21, curves A & B). This is probably due to the lower initial concentration of labeled ligand that I am using (due to a lower enzyme concentration); the lower specific activity of the ligand in my experiment; and to charge repulsion of the highly anionic PRibPP which would result in a faster dialysis rate.

These complications were dealt with by using the control experiment as a calibration standard. The assumption was made that PRibPP and R5P would dialyze from the upper chamber at approximately the same rate. If this assumption is valid, then one can subtract 40% of the CPM value of a given sample in the control experiment from the CPM value of the corresponding sample for the experiment with enzyme, thereby correcting for the presence of [^{14}C]R5P. The control experiment was used to determine normalization factors to correct for the loss of ligand over the course of the experiment. These factors were derived by dividing the CPM values of the calibration curve samples 10 through 56 (Figure 21, curve A), by the CPM value of sample number 8, which was taken as the initial steady state value. Each CPM value for the samples from the experiment with enzyme was then divided by the corresponding normalization factor. When these corrections were made, plotting the dialysis rate (CPM) *versus* sample number (curve C) produced a staircase-like plot

similar to one presented by Colowick and Womack (1969).

Given that the CPM value in the sixth fraction, after an addition of ligand, is a measure of the concentration of free ligand in the upper chamber, that the total amount of PRiPP added to the upper chamber is known, and that the CPM value obtained when there is excess unlabeled ligand can be taken as the measure of 100% free PRibPP, then the fraction of free PRibPP in the upper chamber can be calculated by dividing the measured CPM for a given PRibPP concentration by the CPM value at 100% free PRibPP. Using this value for free PRibPP and the known concentration of total PRibPP (corrected for the small increase in volume due to the additions) the concentrations of bound and free PRibPP can be obtained.

Figure 22 is a Scatchard plot (Scatchard, 1949) of bound substrate (B) *versus* bound/free substrate (B/F) based on the dialysis rate data obtained from curve C (fractions 6, 14, 22, 30, 38, 46, and 56). The data were fitted to the following equation:

$$B = n - (K_d \times (B/F)) \qquad (3)$$

Where Kd is the dissociation constant for the binding interaction of ligand with protein, and n is the concentration of protein binding sites for ligand. This analysis yielded a K_d value of 33.5 ± 4 μM and a binding site concentration of 112.3 ± 9 μM. Given that the YOPRTase concentration in the experiment was 55.6 μM the number of binding sites per YOPRTase dimer was determined to be 2.02 ± 0.16 sites.

13) [1-^{13}C]NMR OF YOPRTase-[1-^{13}C]PRibPP MIXTURE

These experiments were initiated as an attempt to characterize, spectroscopically,

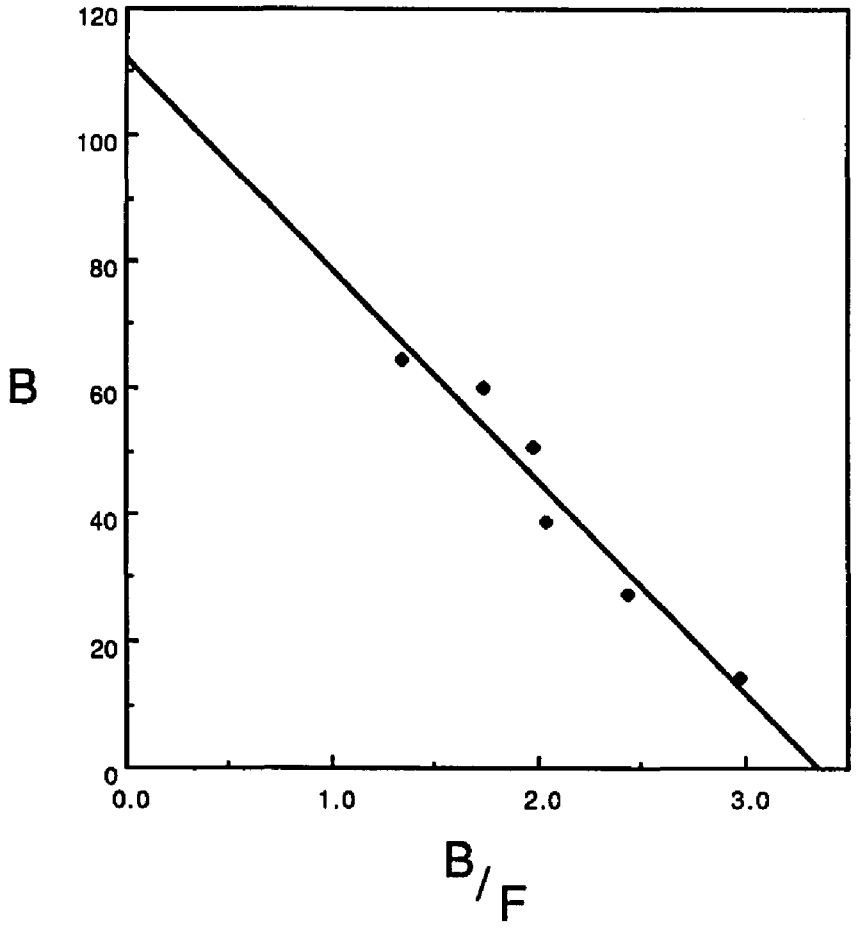


FIGURE 22. Scatchard plot of the data shown in Figure 21. B is the bound substrate and F is the free substrate. Both are μMolar in concentration.

a kinetically competent enzyme-substrate intermediate at the active site of YOPRTase. The ^{13}C-spectrum of the D-[1-^{13}C]ribose used for the synthesis of [1-^{13}C]PRibPP was acquired in order to characterize our starting material. The proton-decoupled ^{13}C-spectrum of D-[1-^{13}C]ribose (99.8% enriched) in 50% D_2O is shown in Figure 23. It was identical to the spectrum published by Serianni *et al.*, (1979a), proving that we had D-[1-^{13}C]ribose as our starting material. The four resonances that are observed can be explained by the equilibriums that exist between the pyranose and furanose ring configurations of ribose, and between the α and β configurations about the ribose anomeric carbon (C1). The resonance at 101.07 ppm downfield from TMS is the β-furanose form; the resonance at 96.37 ppm is due to the α-furanose form; the resonance at 93.93 ppm is due to the β-pyranose form; and the resonance at 93.61 ppm is due to the α-pyranose form. These assignments are from Serianni *et al.*, (1979a). Figure 24 shows the more complex natural abundance ^{13}C-spectra of D-ribose in 50% D_2O for comparison. Figure 25 is the ^{13}C-spectrum of the incubation mixture for the synthesis of [^{13}C]PRibPP (containing [^{13}C]ribose), prior to the addition of ribokinase and PRibPP synthetase. The two large peaks at 56.2 and 55.6 ppm may be due to TEA, which is at a high concentration relative to [1-^{13}C]ribose. The other smaller peaks upfield from the ribose resonances are most likely due to ATP and or buffer contaminants. Figure 26 is the ^{13}C-spectrum of a sample of the incubation mixture, removed three hr after the addition of enzyme and chelexed to quench the reaction. The resonances at 93.98 and 93.65 ppm have disappeared, signaling the loss of ribose, and a new one has arisen at 98.57 which is assumed to be [1-^{13}C]PRibPP. The resonances at 101.51 and 96 ppm correspond to the β and α-furanose forms of ribose 5-phosphate, respectively (Serianni *et al.*, 1979b).

The typical DEAE fractionation of the [1-^{13}C]PRibPP incubation mixture resulted in three peaks. The first peak (ribose) is washed off the column with water leaving

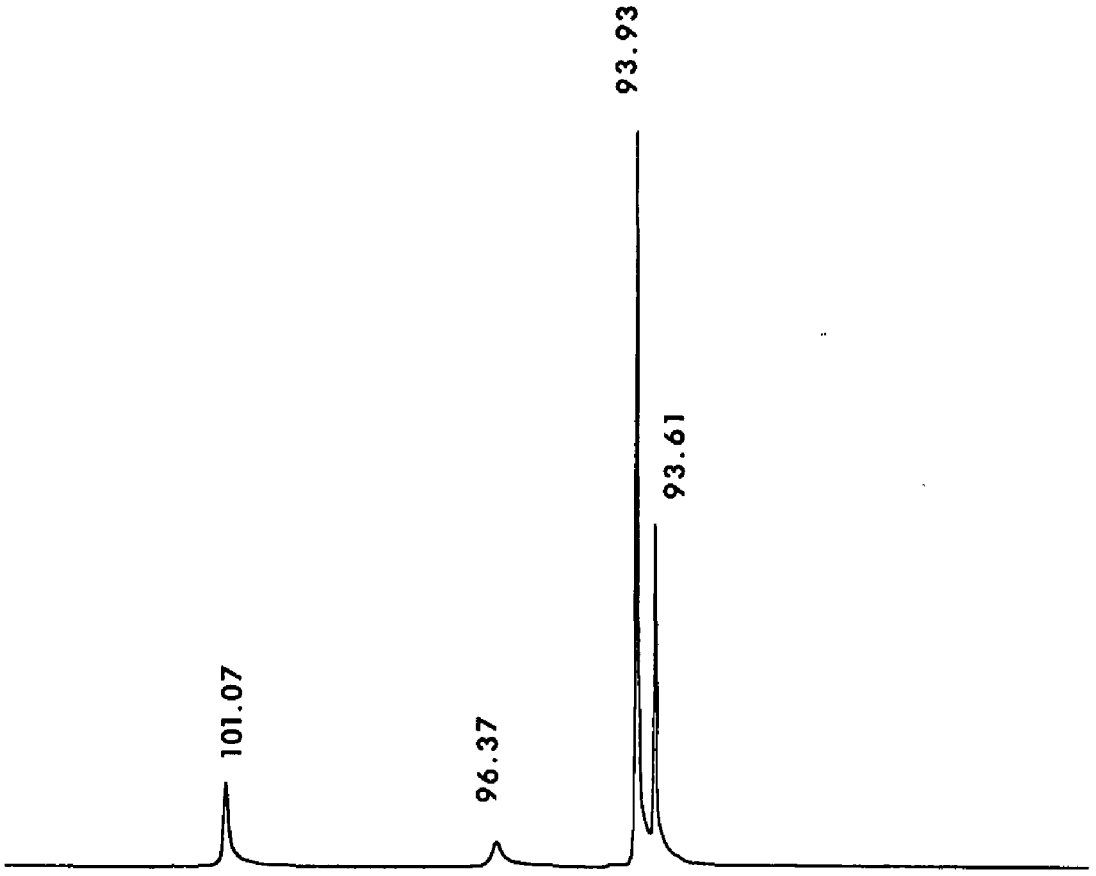


FIGURE 23. ^{13}C-NMR spectrum of enriched ^{13}C-ribose purchased from Omicron Chemicals. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

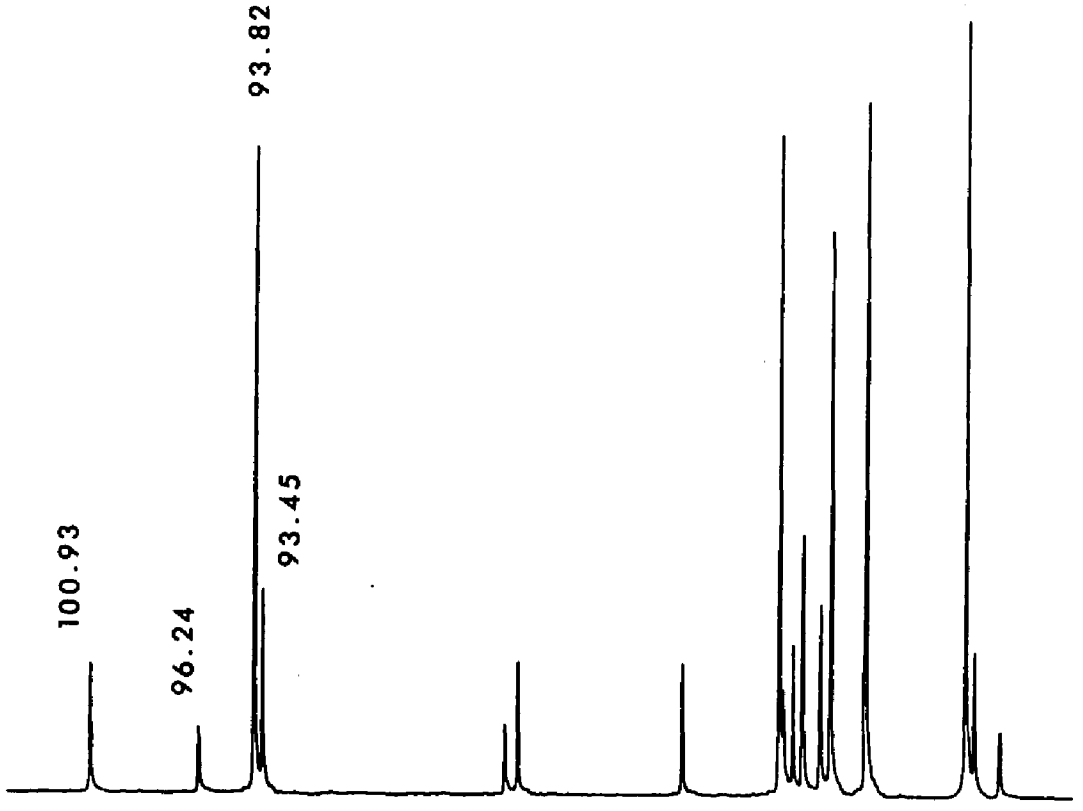


FIGURE 24. Natural abundance ^{13}C-NMR spectrum of ribose purchased from Sigma Chemicals. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

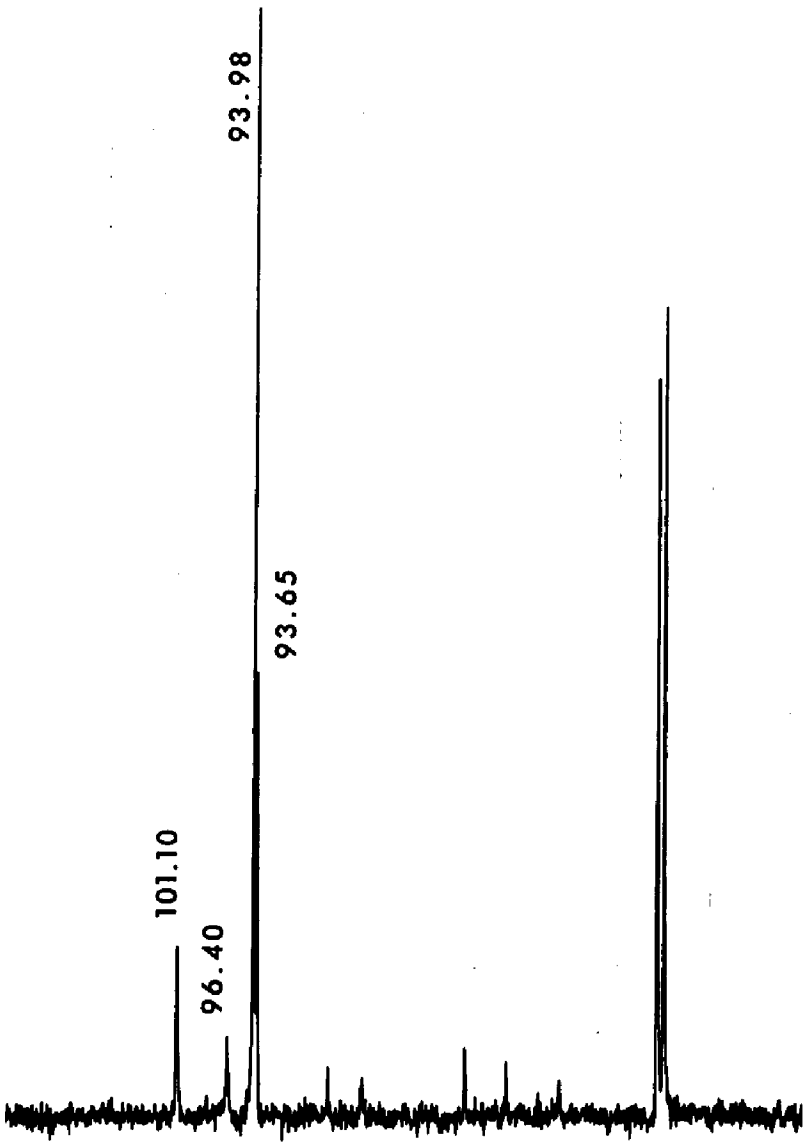


FIGURE 25. ^{13}C-NMR spectrum of enriched ribose included in an incubation solution containing ATP and TRIS buffer (pH 7.5). Resonance assignments and conditions of the experiments are described in " Materials and Methods".

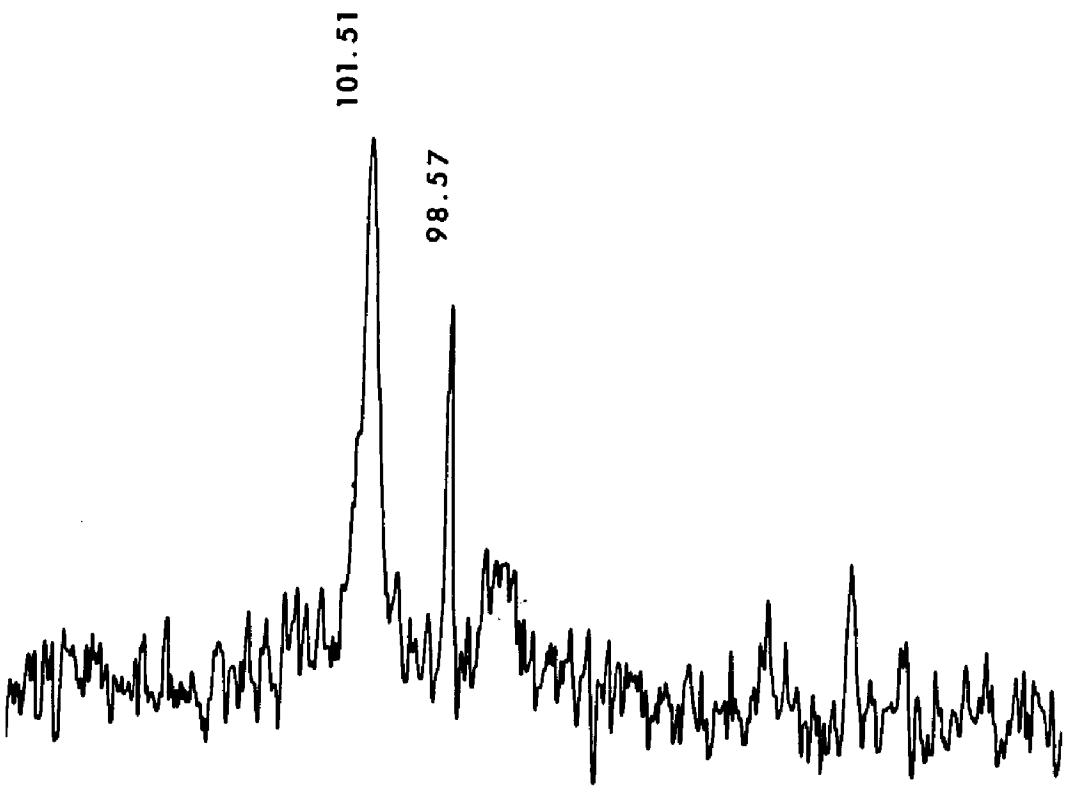


FIGURE 26. ^{13}C-NMR spectrum of enriched ribose included in an incubation solution containing ATP, ribokinase and PRibPP synthetase, and subsequent to a 3 hr incubation. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

ribose 5-phosphate and PRibPP to be eluted in the salt gradient. The less anionic R5P eluted first in the gradient, followed by PRibPP. Figure 27 is the ^{13}C-spectrum of a sample (in 30% D_2O) of the first peak to elute in the salt gradient. As expected only two peaks are seen in the anomeric carbon region of the spectrum, because the phosphorylation of C5 prevents the pyranose ring configuration of ribose from forming. The peak which appears at 66.58 ppm is of unknown origin. Based on its chemical shift, it is likely to be due to an alcoholic carbon. Perhaps it is [1-^{13}C]ribulose which could result from the presence of contaminanting D-ribose-5-P isomerase in either the RKase or PRSase preparations. The peaks which appear at 101.48 and 96.74 ppm are due to the β and α-furanose forms of R5P respectively (Serianni *et al.*, 1979). These investigators reported chemical shifts of 102.5 and 97.5 for the respective β and α configurations about the ribose C1 carbon at pH 5.5. Figure 28 shows a ^{13}C-natural abundance spectrum of ribose 5-phosphate in 50% D_2O, for comparison. The β and α forms of the anomeric carbon have resonances at 101.18 and 96.34 ppm respectively. Figure 29 is the ^{13}C-spectrum of a sample from the second DEAE peak eluted in the salt gradient, which is [1-^{13}C]PRibPP. It is a doublet with peaks at 98.6 and 98.5 ppm and a J_{cop} coupling constant of 5 Hz. This is well within the range of J_{cop} values of 2.3 to 10.5 reported in a compilation by Stothers (1972) and close to values of 5.5 and 4.7 Hz reported by Serianni *et al.*, (1979b) for α and β ribose-5-P respectively. The chemical shift of this doublet matches that of the doublet found furthest downfield in the ^{13}C-natural abundance spectrum of PRPP (Figure 30). This resonance can be assigned to C1 based on the [^{1}H]NMR spectra of PRibPP, in which the H1' proton resonates furthest downfield (Syed *et al.*, 1987). The other evidence that we have actually synthesized [1-^{13}C]PRibPP is the enzymatic activity observed spectrophotometrically upon addition of an aliquot of the concentrated and desalted second DEAE peak to an assay mixture containing YOPRTase and ODCase fig

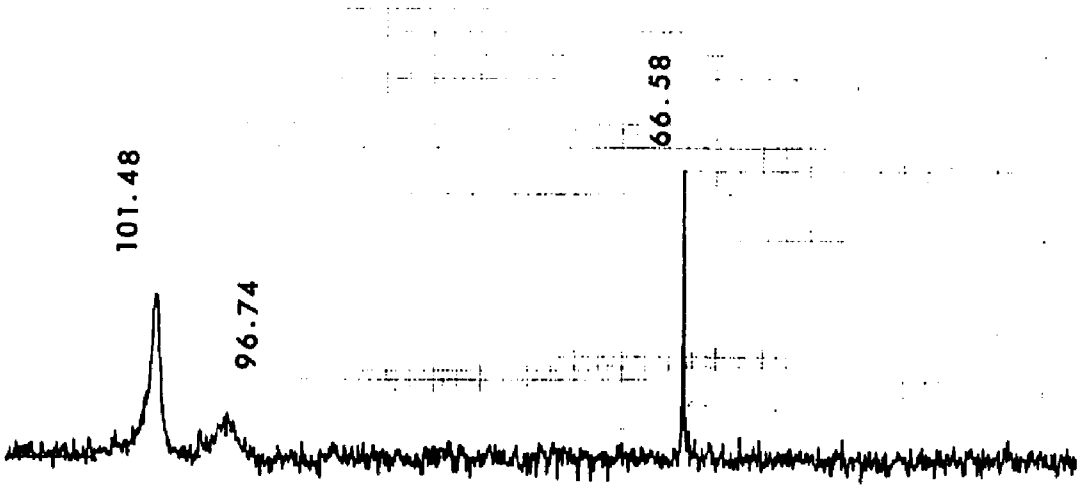


FIGURE 27. ^{13}C-NMR spectrum of the first peak that elutes during a DEAE cellulose chromatography separation of the incubation mixture whose spectrum is shown in Figure 26. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

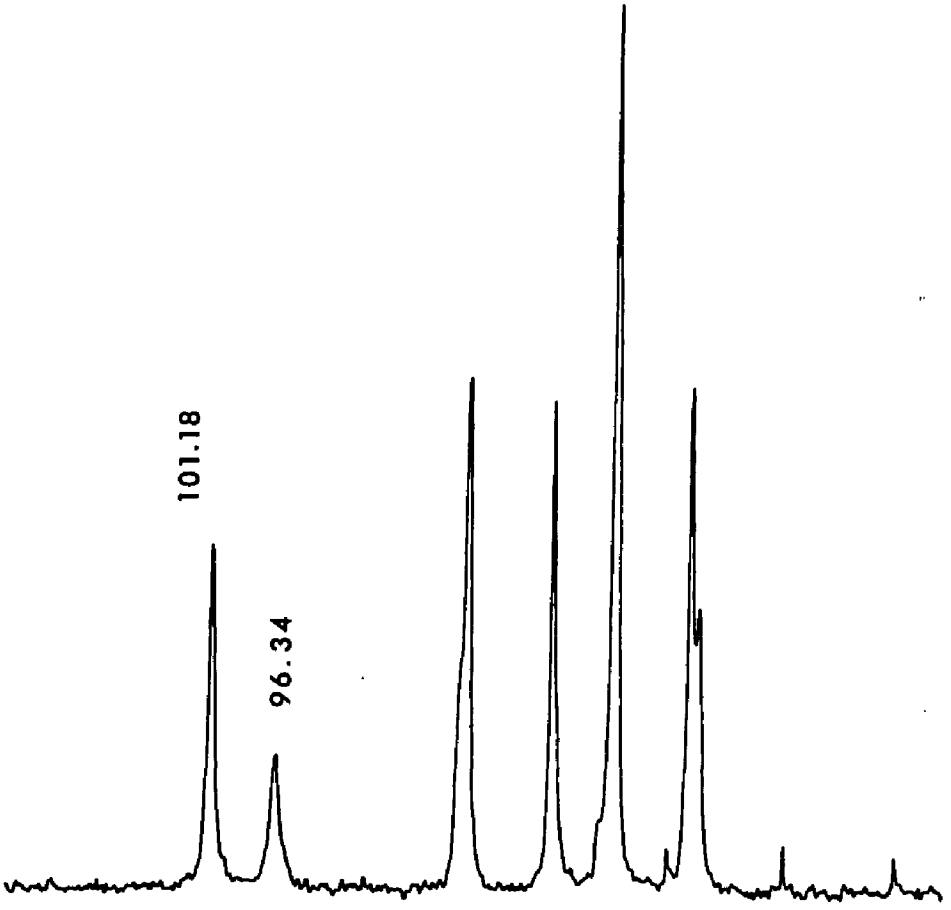


FIGURE 28. ^{13}C-NMR spectrum of ribose 5-phosphate, purchased from Sigma Chemicals. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

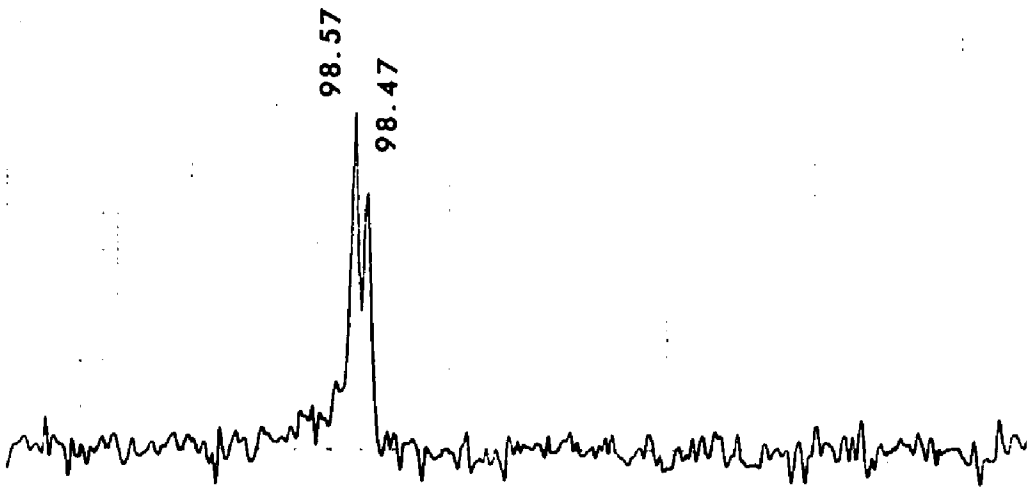


FIGURE 29. ^{13}C-NMR spectrum of the second peak that elutes during a DEAE cellulose chromatography separation of the incubation mixture whose spectrum is shown in Figure 26. Resonance assignments and conditions of the experiments are described in "Materials and Methods".

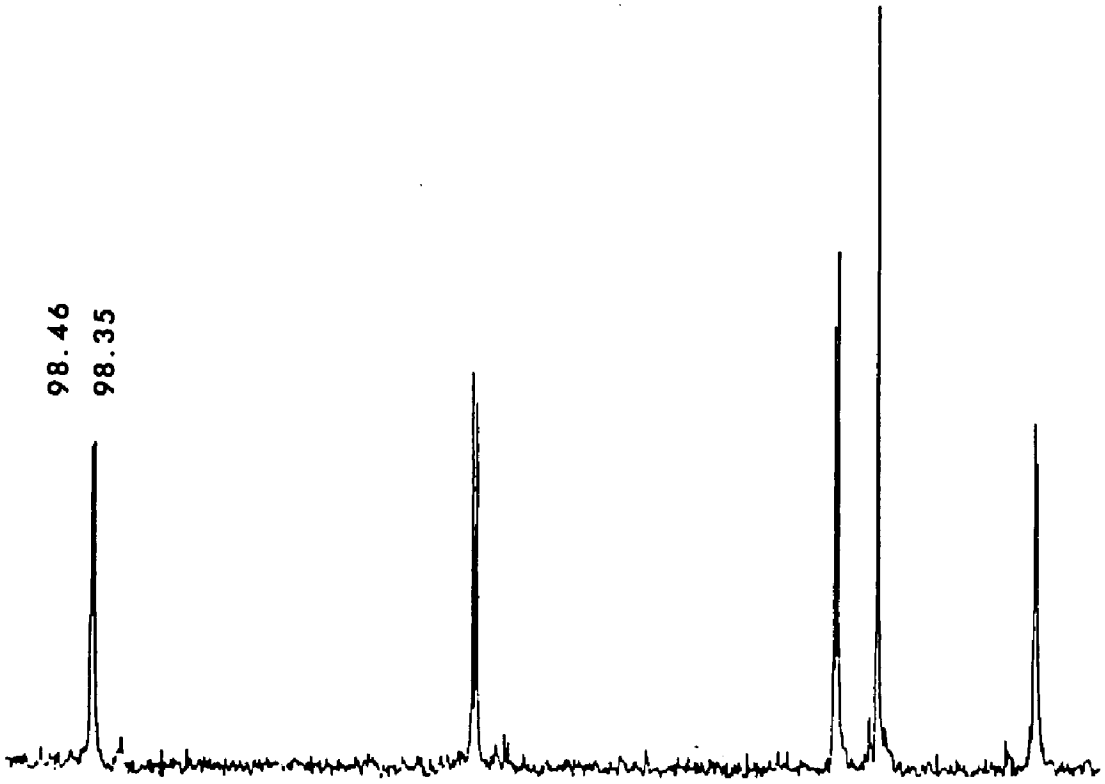


FIGURE 30. Natural abundance ^{13}C-NMR spectrum of PRibPP purchased from Sigma Chemicals. Resonance assignments and conditions of the experiments are described in " Materials and Methods".

actvities. This assay was used to determine that the concentration of the [1–^{13}C]PRibPP used for the following experiments was 14.5 mM in a volume of 0.4 ml.

A ^{13}C-spectrum of 4 mM [1-^{13}C]PRibPP in 20 mM KPi, pH 7.5, is shown in Figure 31. The doublet has chemical shifts of 98.53 and 98.43 ppm. When this solution was made 6 mM in Mg^{2+} these resonances shifted approximately 0.1 ppm upfield to 98.45 ppm and 98.35 ppm (not shown). Figure 32 is the ^{13}C-spectrum of a 0.325 ml volume containing 0.55 mM YOPRTase (1.10 mM active sites), 1.8 mM [1-^{13}C]PRibPP, 4 mM Mg^{2+}, and 20 mM KPi, pH 7.5. As originally designed this experiment should have involved an excess concentration of enzyme active sites over the PRibPP concentration. When no signal was observed above the noise in the spectrum after 10 hr of accumulations, more [1-^{13}C]PRibPP was added. Another 10 hr of accumulations still failed to yield any peaks besides TMS, while the YOPRTase had begun to denature and precipitate out of solution. The sample was then frozen and taken to Hunter College where the spectrum shown was obtained after the addition of more Mg^{2+} to yield the concentrations stated above. Figure 32 also shows an expansion of the spectral region containing the anomeric carbons. There are three fairly well resolved peaks and possibly a fourth which is not as clear. The resonance at 101.1 is probably due to β-[1-^{13}C]R5P, from the breakdown of [1-^{13}C]PRibPP. The peak at 97.5 is most likely due to [1-^{13}C]PRibPP, and is possibly an enzyme bound form of this substrate with the 1 ppm upfield chemical shift the result of local environment effects, Palmer *et al.*, (1982). Due to the noise in the spectrum, it is not possible to make a definite statement, however, there seems to be a broad peak at 96.4 ppm which could be due to α-[1-^{13}C]R5P from the breakdown of [1-^{13}C]PRibPP. This leaves the remaining peak in the anomeric carbon region, the one that appears at 94.97 ppm, to be asssigned. This peak may be due to an enzyme-substrate complex.

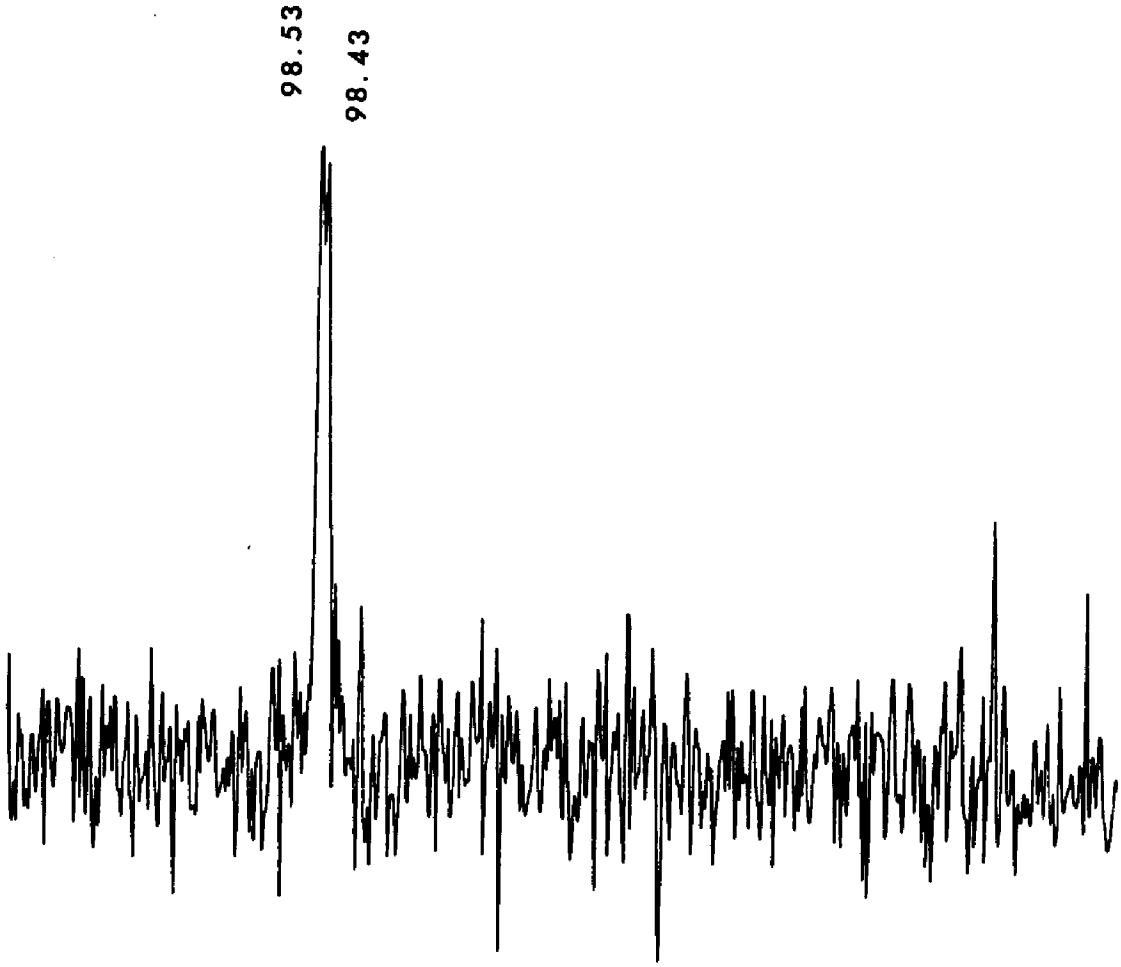
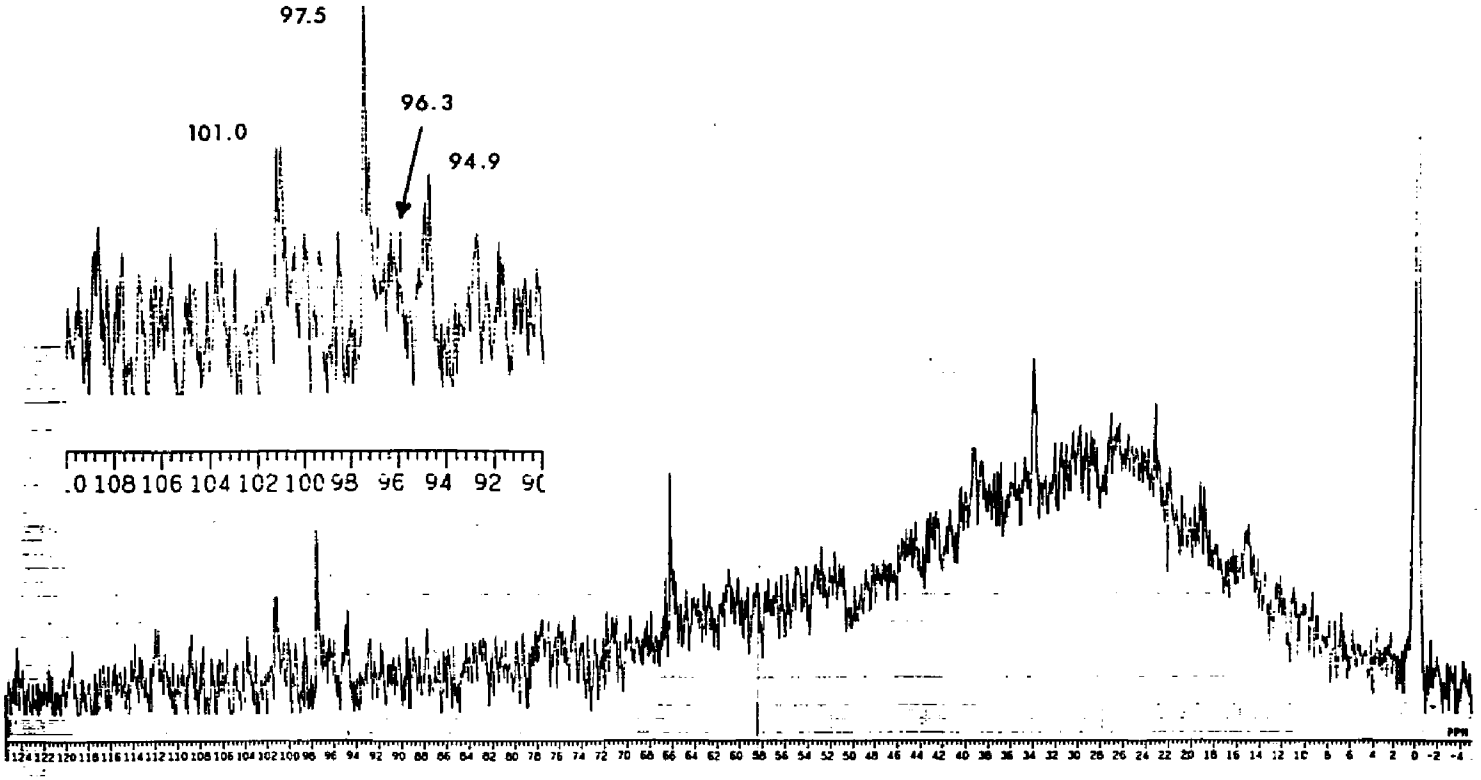


FIGURE 31. ^{13}C-NMR spectrum of 4 mM [^{13}C]PRibPP in 20 mM phosphate buffer (pH 7.5), synthesized enzymatically and purified as described in "Materials and Methods". Resonance assignments and conditions of the experiments are described in "Materials and Methods".

FIGURE 32. ^{13}C-NMR spectrum of 2 mM ^{13}C-enriched PRibPP in 20 mM phosphate buffer (pH 7.5), and in the presence of 1 mM YOPRTase. Resonance assignments and conditions of the NMR experiments, which were performed at the CUNY NMR Facility at Hunter College, are described in "Materials and Methods".


97.5
96.3
101.0
94.9
.0 108 106 104 102 100 98 96 94 92 90

Unfortunately the control experiment to obtain the YOPRTase ^{13}C-natural abundance spectrum was unsuccessful due to denaturation of the enzyme. Only 3 mg out of an original 7 mg were recovered from the experiment with YOPRTase and [1–^{13}C]PRibPP. Only 1 mg out of the 3 mg was recovered from the attempt to obtain the natural abundance ^{13}C-spectra of YOPRTase alone.

DISCUSSION

1) YOPRTase Purification

Three modifications have been introduced to the YOPRTase purification procedure in this laboratory. Sephacryl S-200 was used instead of Sephadex G-100 in the molecular sieve chromatography stage. Sephacryl S-200 can be used at higher hydrostatic pressures and faster flow rates, significantly reducing the time required for this step.

Removing NaCl as a buffer component for the molecular sieve chromatography, eliminated the need for dialysis prior to the DEAE cellulose chromatography. This appears to have resulted in the loss of the minor peak of YOPRTase activity that usually elutes from the DEAE column prior to the major peak (Umezu et al.,, 1971). This suggests that the minor peak is a proteolytic fragment of YOPRTase that retains activity.

The additon of an OMP Sepharose affinity chromatography step introduced a purification step that is more specific for YOPRTase binding than is Blue Sepharose chromatography. These changes resulted in a purification procedure that consistently yielded YOPRTase of high purity.

2) PRibPP SYNTHETASE PURIFICATION

PRibPP synthetase was needed for the enzymatic synthesis of [^{14}C]PRibPP and [^{13}C]PRibPP. Switzer and Gibson's purification procedure for this enzyme (Switzer

and Gibson, 1978) was straightforward, providing an excellent yield of highly purified enzyme. The HPLC assay for PRibPP synthetase proved to be a good replacement for Switzer and Gibson's [^{14}C]ATP assay.

3) RIBOKINASE PURIFICATION

This enzyme was also required for the enzymatic synthesis of PRibPP. The purification procedure developed in this laboratory yielded ribokinase with a specific activity lower than that obtained by Schimmel *et al.*, (1974). However, our procedure was simpler and yielded a preparation that was not adversely affected by freezing and thawing.

4) STUDIES WITH 5-AZAOROTATE

5-Azaorotate was found to be a substrate for YOPRTase, with a K_m of 75.5 μM and a V_{max} of 13.8 μmol/min./mg of protein. Victor *et al.*, (1979a) showed that YOPRTase utilizes a Ping Pong mechanism. That has been found to be true for its utilization of 5-azaorotate. The net formation of the putative nucleotide product, 5-azaorotidine 5'-monophosphate was not observed when the reaction was followed by HPLC. The formation of a transient peak, early in the reaction, suggests that a nucleotide product is formed, but is quickly broken down to a UV invisible product. This is probably due to ring opening of the 5-azaorotate 5'-monophosphate product that is the likely product. These results are contrary to Cihak and Sorm's findings from their studies of 5-azaorotic acid utilization in E. coli and mouse liver extracts (Cihak and

Sorm, 1972; Cihak *et al.*, 1968). These investigators claim to have observed the formation of 5-azaorotidine 5'-monophosphate and 5-azauridine 5'-monophosphate when they followed the reaction using labeled 5-azaorotic acid, along with paper chromatography to separate labeled products. Further evidence that any nucleotide product is quickly broken down in our system, is the observation that the YOPRTase reaction with 5-azaorotic acid proceeds with zero-order kinetics, after an initial velocity period where the kinetics are more complex. The failure to observe product inhibition may be due to the breakdown of the nucleotide product to a compound that does not bind to YOPRTase. The breakdown may be analogous to the breakdown of 5-azauridine 5'-monophosphate to ribosyl N-formylbiuret, and ribosylbiuret, reported by Cihak *et al.*, (1964). Cihak and Sorm (1972) found that when 5-azaorotic acid is injected intraperitoneally into mice, their utilization of orotic acid is decreased and they develop orotic aciduria.

Cha and his colleagues reported that replacing the C5 carbon of orotic acid with nitrogen enhances binding to OPRTase 22-fold (Niedzwicki *et al.*, 1984). This is not supported by our finding that YOPRTase has a higher Km for 5-azaorotic acid than for orotic acid. In the absence of true dissociation constants the Km value can be used as a measure of a substrate's binding affinity. The values of Km for 5–azaorotic acid and orotic acid are 76 and 38 μM respectively, suggesting that 5-azaorotic acid has only half the affinity for YOPRTase that orotic acid displays. The value Kcat/Km is the second order rate constant for a substrate (at low substrate concentrations) and a measure of a substrate's specificity. The Kcat/Km values for 5-fluororotic acid, orotic acid and 5–azaorotic acid are, 5.2×10^5, 2.5×10^5, and 6.1×10^4, respectively. Thus 5–azaorotic acid has only a quarter of the specificity of orotic acid for YOPRTase, while 5–fluoroorotic acid has twice the specificity of orotic acid. Cha and his colleagues have stated that increased acidity of the pyrimidine ring (the ability of one of

the ring amine protons to dissociate, the carboxyl group is completely ionized at pH 8) is responsible for the enhanced binding of analogs such as 5–fluororotic acid to OPRTase. However our findings suggest that some other factor may be playing a role, since 5–azaorotic acid is 95% dianion while orotic acid is only 3% dianion at pH 8 (Niedzwicki *et al.*, 1984). Therefore increased acidity of the ring system has not led to enhanced binding (as measured by Km) of 5–azaorotic acid in our system. Our suspicion is that 5–azaorotic acid's enhanced ability to chelate metal ions causes its metal complex to predominate in solution, and we propose that this complex is inhibitory. We don't have a pKa value for 5-Fluoroorotic acid, however it is likely to be close to the pKa of 5–fluorouracil which is 7.98 (Brown, 1970). Introduction of the carboxyl group seems to have little affect on the ring acidity since uracil and orotic acid have pKas of 9.5 and 9.45 respectively (Brown, 1970). Therefore 5–fluoroorotic acid is likely to be only 51% ionized and not as extensively complexed with metal ion as 5–azaorotic acid. Thus, 5-fluoroorotic acid's intermediate acidity between orotic acid and 5–azaorotic acid may make it a better substrate than both. Steric considerations do not appear to play role in the differing specificities of 5–azaorotic acid and 5–fluororotic acid.

5) STUDIES WITH URACIL 6-ALDEHYDE

The inactivation of YOPRTase by uracil 6-aldehyde is most likely the result, at least in part, of Schiff base formation between the aldehyde and lysine residues at the active site of YOPRTase. This is supported by the partial reversibility of the inactivation, the finding that sodium borohydride reduces the extent of the reversibility, and the protection of YOPRTase from inactivation by substrates. The protection

studies suggest that the putative modified lysine(s) is at the binding site of PRibPP, and most likely is interacting with the pyrophosphate group. This conclusion comes from the observation that PRibPP–Mg^{2+} provides the greatest amount of protection (80%). Also, the relatively small pyrophosphate–Mg^{2+} complex provides a significant amount of protection (50%), almost as much as that provided by OMP in the presence of Mg^{2+} (56%). Another possibility is that PRibPP–Mg^{2+} interacts with two lysines *via* its pyrophosphate group and its 5' phosphate group. Only PRibPP-Mg^{2+} would be able to protect both simultaneously. Orotate also provides significant protection (59%) in the absence of $MgCl_2$, however when metal is added its ability to protect diminishes to 37%.

The finding that orotate provided greater protection against inactivation by uracil 6-aldehyde, in the absence of $MgCl_2$ (59% activity recovered) than in its presence (37% activity recovered) runs counter to the proposal of Dodin *et al.*, (1982) that an orotate-Mg complex is the true substrate for YOPRTase. A dissociation constant of 1 x 10^{-4} has been reported for Mg^{2+} binding to orotate (Davidenko and Zinich, 1979). Using this dissociation constant in an equilibrium calculation, one finds that in a solution of 1 mM Mg^{2+} and 1 mM orotate, 0.73 mM orotate-Mg^{2+} complex is formed. When the 23% level of YOPRTase activity in the presence of 5 mM uracil 6-aldehyde and the absence of of any protecting substrate, is subtracted from the YOPRTase activity levels in the presence of 5 mM uracil 6-aldehyde, and 1mM orotate or 5 mM uracil 6-aldehyde, 1 mM orotate and 1 mM Mg^{2+}, we get 36% and 14% respectively. These are the degrees of protection attributable to orotate alone or orotate and Mg^{2+}, respectively. Thus when approximately 73% of the orotate in solution is tied up in a complex with Mg^{2+}, orotate's ability to protect against inactivation by uracil 6-aldehyde is decreased by 61%. Also, one can calculate an expected level of YOPRTase activity of 33% in the presence of 0.23 mM orotate (free orotate) and 5 mM uracil

6–aldehyde, which is not far from the observed value of 37%. Given that the error in this type of experiment is between 5 and 10%, there is an excellent correspondence between the amount of orotate complexed with Mg^{2+} and the loss of orotate's ability to protect against inactivation. It should be noted that this argument against an orotate-Mg^{2+} complex as the substrate for YOPRTase is weakened by the fact that orotate may not bind to the enzyme in a kinetically relevant manner in the absence of PRibPP.

Both the inactivation and the reactivation of YOPRTase with uracil 6–aldehyde are slow, especially when compared with the same reactions with PLP. Yet, with relatively high concentrations of uracil 6–aldehyde and long incubations, YOPRTase can be brought to levels of apparent zero activity. This suggests that uracil 6–aldehyde may be highly specific for active site lysine(s), due to its similarity to the YOPRTase substrate orotic acid.

Mr. Dean Cuebas synthesized the uracil 6-aldehyde used in these studies. Unfortunately I was not able to repeat the observed inactivation of YOPRTase with material that I synthesized. However, recently Mr. Sungyung Chung has successfully repeated the inactivation with uracil 6-aldehyde he has synthesized, and will be carrying on these studies.

6) CHEMICAL MODIFICATION OF YOPRTase LYSINE RESIDUES WITH PLP

The finding that uracil 6–aldehyde inactivated YOPRTase, probably by Schiff base formation with lysine residues of YOPRTase, led to the decision to perform chemical modification experiments with a classical chemical modifier of lysine residues, pyridoxal 5'-phosphate (PLP). These experiments have shown that PLP modifies YOPRTase lysines. The competition experiments provide evidence that lysines susceptible to PLP modification are at the active site of YOPRTase and are involved in

binding PRibPP–Mg^{2+}. The literature is full of studies identifying lysine as an active site residue in enzymes that bind organophosphates. One example is the study of the inactivation of bovine glutamate dehydrogenase by PLP, performed by Piszkiewicz and Smith (1971). They proposed that PLP was interacting with or near a site responsible for binding the pyrophosphate group of NADH.

Substrate protection studies with 1 mM PLP again showed that PRibPP-Mg^{2+} provides the greatest protection against inactivation. Interestingly, $MgCl_2$ appeared to have conflicting roles when PLP is the inactivating reagent. While its addition increased the protection provided by PRibPP from 49% recovered to 110% recovered, its addition had the opposite effect upon the protection afforded by OMP, decreasing the activity recovered from 48% for OMP alone to 29% for OMP in the presence of 1 mM $MgCl_2$. This is probably due to enhanced PLP binding in the presence of $MgCl_2$ due to the formation of PLP-Mg^{2+} complexes.

An apparently constant feature of these studies of YOPRTase with inactivating reagents is the failure to recover the full YOPRTase activity after an inactivating incubation followed by dialysis or ultrafiltration. Also it was observed in the competition studies that despite PRibPP's direct competition with PLP for a binding site it could only reverse inhibition by PLP to 50% of the original activity. Two possible explanations occur to me. PLP could interact with at least two types of lysine. The modification of one type could be readily reversible while the other could be extremely slow. An exhaustive dialysis experiment would be required to test this, and would require a relatively large amount of enzyme. The other possibility is that PLP modifies lysine reversibly while modifying another type of amino acid irreversibly. PLP can also form covalent bonds with histidine and cysteine residues. The attachment to histidine results in the destruction of that residue in the presence of light, rendering the PLP inactivation irreversible (Rippa and Pontremoli, 1969). The binding to protein

thiol groups is usually reversible, however, the loss of free thiols has been correlated with the irreversible inactivation of pig lactate dehydrogenase by PLP (Gould & Engel, 1982). These reports lead one to speculate that the irreversible loss of 50% of the YOPRTase may be due to one of these types of interactions. Sloan and Strauss (1984) have provided evidence that YOPRTase has essential histidines at the active site, and it has been suggested that sulfonated derivatives of orotic acid interact with sulfhydryl groups. The amino acid analysis provided no information on this point. Also, loss of any residues due to the reduction of an enzyme–substrate by sodium borohydride would not be apparent in the amino acid analysis. Though the amino acid analysis has provided dramatic evidence for the modification of YOPRTase lysines, it not clear how much weight should be placed on the number (21) of modified lysines it suggests.

That question is highlighted by the results of the experiment examining the incorporation of tritium labeled PLP into YOPRTase. This experiment suggests that one to two essential lysines per YOPRTase subunit are modifed by PLP, and that a maximum of approximately three lysines per subunit are incorporated at a PLP concentration of 2 mM.

7) ENZYMATIC COUPLED ASSAY EMPLOYING HPLC

The development of this HPLC assay method was initiated to reduce our dependence on time consuming radioactive assays. The method has enabled us to follow the purification of ribokinase, and PRibPP synthetase activities. It has also allowed us to follow the *in vitro* synthesis of OMP from ribose and ATP using the enzymes ribokinase, PRibPP synthetase, YOPRTase and pyrophosphatase.

8) QUATERNARY STRUCTURE DETERMINATION

Elution gel chromatography is a simple yet rigorously exact method for studying protein subunit association (Valdes and Ackers, 1979). It is nondenaturing and, when HPLC gel filtration columns are used, it is rapid and requires relatively little enzyme (Furman and Neet, 1982). The experiment reported here is an example of small zone transport (Valdes and Ackers, 1979; Ackers, 1976) wherein a small sample volume (relative to the column volume) is loaded onto the column.

The experiment has established that YOPRTase has an apparent molecular weight of $37.1 \times 10^3 \pm 1.4 \times 10^3$ in the presence of 100 mM TEA and 1 mM $MgCl_2$ over the loading concentration range 0.1 μg/ml to 5 μg/ml. When PRibPP is added to the buffer, YOPRTase has an apparent molecular weight of $38.9 \times 10^3 \pm 2.2 \times 10^3$. The difference is significant and may be due to a conformational change occurring when YOPRTase binds PRibPP.

YOPRTase was also injected at a concentration of 0.54 mg/ml and observed directly *via* its absorbance at 254 nm. This experiment was performed in the presence and absence of OMP and $MgCl_2$. YOPRTase remained in a dimeric configuration in the presence of OMP and $MgCl_2$.

This study has provided no evidence for the existence of a monomer-dimer equilibrium under conditions similar to those of the usual YOPRTase assay mixture. The possibility that an equilibrium exists between an inactive monomer and an active dimer does not seem likely. If any monomer was formed in the reported experiment, it was irreversibly inactivated. If this were not the case, once it mixed with the post-column assay mixture a reequilibration would have occured to form some active dimer and the formation of product (OMP) would have marked the presence of YOPRTase.

However, if irreversibly inactivated monomer was a readily attained form, YOPRTase would be rapidly inactivated as the dimer dissociated to monomer. Thus YOPRTase does not undergo substrate dependent associations, unlike UMP synthase (Traut and Jones 1977; 1979).

Mayer has suggested that an equilibrium exists between monomeric and dimeric forms of YOPRTase. He based this proposal on experiments in which YOPRTase was subjected to cross-linking with glutaraldehyde or dimethyl suberimidate then examined by SDS gel electrophoresis (Mayer, 1983). On the SDS gels, he observed bands which he attributed to YOPRTase monomer and cross-linked dimer. He also stated that the presence of $MgCl_2$ promoted the formation of the dimeric form of YOPRTase. The results of my study do not conflict with the data reported by Mayer, however, they do contradict his interpretation. In the present study there is no evidence for the formation of monomer, at concentrations of YOPRTase as low as 0.1 μg/ml, under the reported experimental conditions. I propose the following reinterpretation of Mayer's work. His cross-linking and SDS gel electrophoresis studies indicated an equilibrium between cross-linked and noncross-linked dimer. The addition of $MgCl_2$ to YOPRTase incubated with the cross-linking reagents shifted the equilibrium towards the formation of cross-linked dimer.

Mr Sungyung Chung has also used the nonreacting gel filtration technique to examine the quaternary structure of yeast HGPRTase (Mayer, L., Ashton, R. W., Chung, S. H., Ali, L. Z., and Sloan, D. L., submitted to Arch. Biochem. Biophys.). His results provide an interesting contrast with the findings for YOPRTase. In the absence of substrate, HGPRTase was found to elute primarily as a dimer, with a small amount of the monomer form also eluting. When PRibPP and $MgCl_2$ were added to the elution buffer the equilibrium shifted and the monomer became the major form of the eluting enzyme. The PRTases's diverse kinetic mechanisms (Victor *et al.*, 1979a;

Ali and Sloan 1982; Hanna *et al.*, 1983) would thus appear to be paralleled by their varied association-dissociation responses to the presence and absence of substrates.

9) [1-^{14}C]PRibPP SYNTHESIS

The procedure for the synthesis of [^{14}C]PRibPP was reported by Goitein *et al.*, (1978). An HPLC assay developed in this laboratory has confirmed the formation of [^{14}C]PRibPP and has been useful in quantifying the labeled components ([^{14}C]PRibPP *vs* [^{14}C]R5P) of the preparation.

10) GEL FILTRATION OF YOPRTase-[1-^{14}C]PRibPP COMPLEX

This experiment has demonstrated the binding of [^{14}C]PRibPP to YOPRTase, providing further evidence for the synthesis of [^{14}C]PRibPP, and its competence as a ligand for YOPRTase.

11) [1-^{13}C]PRibPP SYNTHESIS

The synthesis of [1-^{13}C]PRibPP is reported for the first time to our knowledge. It is a straightforward procedure, yielding a product of high [1-^{13}C]-spectroscopic purity (Figure 27).

12) FLOW DIALYSIS: [1-^{14}C]PRibPP BINDING STUDY

Colowick and Womack (1969) first reported the use of flow dialysis to determine the dissociation constant and the number of binding sites involved in an enzyme-substrate interaction. This method was chosen by us to study the binding interaction of PRibPP with YOPRTase because it is rapid, yet accurate (Colowick and Womack 1969). This is important when one is working with a substrate as labile as PRibPP. An equilibrium dialysis experiment can require up to 12 hours for equilibrium across the membrane to be established. Such an experiment, performed at room temperature, could not provide quantitative results due to hydrolysis of PRibPP. The technique is based on the following principles: when ligand is mixed with enzyme, chemical equilibrium is established within seconds: the equilibrium concentration of free ligand is directly proportional to the rate of dialysis across the membrane: a steady state is reached, where ligand entering the lower chamber is equal to ligand being removed in the effluent. The steady state is achieved when rates for ligand entering and leaving the lower chamber are approximately equal. The concentration of ligand in the effluent is then a true measure of the concentration of free ligand in the upper chamber. The rate of change in the concentration of ligand in the lower chamber is given by the following equation:

$$\partial N/\partial t = (S \times D) - [N \times (f/V)] \qquad (4)$$

Where N is the concentration of ligand in the lower chamber, S is the concentration of ligand in the upper chamber, D is a diffusion constant whose value depends on the ligand as well as the apparatus, f is the flow rate of buffer passing through the lower chamber, and V is the volume of the lower chamber. The time course for the approach to the steady state is given by the integrated form of the

equation:

$$N_{(t)}/V=[(S \times D)/f] \times [1-\exp^{-(f \times t/V)}] + [N_{(t=0)}/V] \times [\exp^{-(f \times t/V)}] \quad (5)$$

Where t is the time after an addition of ligand to the upper chamber. When labeled substrate is first added $N_{(t=0)} = 0$ and the second term drops out. When f x t = 12 or greater and V=3 ml, then $\exp^{-(f \times t/V)}$ is less than or equal to 0.018, and greater than 98% of the steady state will have been attained. Using this equation, it was calculated that the steady state condition is reached after 1.9 min for a flow rate of 6.5 ml/min. This is equivalent to the passage of 12 ml of buffer through the lower chamber. For subsequent additions of ligand to the upper chamber the time course to achieve the steady state is given by:

$$N/V=[(S \times D)/f] \times [1-\exp^{-(f \times t/V)}] + (S/V) \times [\exp^{-(ft/V)}] \quad (6)$$

At the steady state the second term is negligible and can be ignored. Again the steady state is achieved after the flow of about 12 ml. Since 2 ml fractions were collected, the CPM value of every sixth fraction collected after the addition of ligand was used in the calculations to determine the concentration of free ligand.

The justification for the correction used to normalize the experimental values for the dialysis rate (CPM) is as follows. If equation 2 is rearranged, after dropping the second term, which is negligible at the steady state, it can be seen that it has the form of a first order rate equation:

$$N = S \times [\{(D \times V)/f\} \times \{1 - \exp^{-(f \times t/V)}\}] \quad (7)$$

At any given time t the ratio N/S has a value that is independent of the starting concentration S and is dependent only on time, when all other factors are held constant. Therefore it is valid to use the normalization factors determined from the control experiment to adjust the CPM measurements from the experiment with enzyme, despite the differing levels of free ligand in the two experiments.

This experiment yielded a value of 33.5 ± 4 μM for the dissociation constant (Kd) of the binding equilibria between YOPRTase and PRibPP. This value is only slightly smaller than the Km value of 38 μM determined for PRibPP by kinetic analysis (Victor *et al.*, 1979a). The YOPRTase dimer was found to have 2.02 ± 0.2 equal and independent binding sites. This is far from unexpected since YOPRTase exists as a dimer of identical subunits, and does not display any type of cooperativity (Victor *et al.*, 1979a). Ali and Sloan (1982) used flow dialysis to determine a Kd value of 2.0 ± 0.2 μM for the PRibPP binding interaction with HGPRTase, an order of magnitude stronger binding than in the case of YOPRTase. This may reflect a situation in which YOPRTase, which utilizes a ping pong mechanism (Victor *et al.*, 1979a), is using binding energy to cleave the pyrophosphate, and may be storing some of the binding energy in some form, perhaps in conformational changes within an enzyme-substrate intermediate. Whereas HGPRTase, which utilizes an ordered Bi Bi mechanism, uses a greater amount of the binding energy to hold PRibPP rigidly at the enzyme active site. Jencks (1975) has discussed the possible ways that enzymes can use the free energy made available by binding interactions with substrates. Ali and Sloan (1982) also found the number of binding sites per HGPRTase dimer to be 1.9 ± 0.2. At the time they had no evidence for the dissociation of HGPRTase to monomer in the presence of substrate.

13) [1-^{13}C]NMR OF YOPRTase-[1-^{13}C]PRibPP MIXTURE

It is fortunate that the resonances of the anomeric carbons of carbohydrates fall in a window in the ^{13}C-spectrum roughly centered about 100 ppm where the amino acid residues of proteins do not resonate (Allerhand *et al.*, 1979). Without a natural abundance ^{13}C-spectra of YOPRTase in the absence of [^{13}C]PRibPP, we can only speculate as to the nature of the resolved peaks. However, it is probable that the resonances occurring between 94 and 102 ppm are due to the anomeric carbons of the phospho-derivatives of [1-^{13}C]ribose. The resonance at 95.0 ppm in the ^{13}C-spectrum of YOPRTase incubated with [1-^{13}C]PRibPP does not correspond to the chemical shifts for [1–^{13}C]ribose 5-phosphate or [1-^{13}C]PRibPP (98.5 ppm). It is unlikely that it is due a protein carbon resonance since it lies in the anomeric carbon window discussed in "Results". It is probable that it is due to an intermediate arising from the YOPRTase-[1–^{13}C]PRibPP interactions. The proposed intermediate could be YOPRTase-[PRibPP] or YOPRTase-[PRib]. The latter seems most likely as a simple noncovalent binding interaction between YOPRTase and PRibPP is unlikely to result in chemical shifts > 1 (Palmer *et al.*, 1982). Yet, the resonance at 95.0 ppm is very likely due to the central carbon atom of a hemiacetal function, similar to the C-1 carbons of PRibPP and R5P, since it lies within the anomeric carbon region. Such an intermediate could arise by an attack on the C-1 carbon of PRibPP by a carboxylate oxygen atom from a nearby aspartic or glutamic residue. A frontside attack by this residue following an S_N1 dissociation of PPi from PRibPP is consistent with the carbocation transition state suggested by Parson's studies (Goitein *et al.*, 1978) and with the inversion of configuration that occurs in the reaction catalyzed by YOPRTase. Unfortunately the experiments performed to date are not sufficient to make more definitive statements. The spectrum obtained has too much noise to be able to use the

J_{cop} splitting of the C–1' carbon [1-^{13}C]PRibPP as a probe for the nature of a possible complex. However, an experiment with higher concentrations of YOPRTase and [1-^{13}C]PRibPP should readily yield this information.

The results do not exclude the possibility that other residues beside ASP or GLU could be involved, since unexplained resonances occur outside the anomeric carbon region. A bond to nitrogen (histidine) or to sulfur (cysteine) would result in larger chemical shifts (Ray and Harper, 1983; Gamcsik et al, 1983). None of these possibilities can be excluded as there are several resonances in the spectrum which can be assigned to enzyme or an enzyme-substrate complex only when a ^{13}C-natural abundance spectrum for YOPRTase is in hand. A ^{13}C-difference spectrum between YOPRTase with [1–^{13}C]PRibPP and YOPRTase alone would be extremely desirable.

To further illustrate the dilemma, the large, well resolved peak at 66.2 ppm could be assigned to threonine based on a tentative assignment by Howarth and Lian (1984) of threonine 45 of ribonuclease A to a resonance at 68.6 ppm. On the other hand, Gamcsik *et al.*, (1983) have demonstrated that the formation of a hemithioacetal enzyme-substrate complex by the attack of CYS 25 thiolate of papain at the aldehydic carbon of [1-^{13}C]glycinal, results in a dramatic chemical shift of 126 ppm from 200.9 for the aldehyde tc 74.9 for the hemithioacetal. A third possibility is that this is a contaminant rising well above the noise relative to [1-^{13}C]PRibPP and any complexes, due to the dilution of [1-^{13}C]PRibPP among the complexes, and breakdown to [1–^{13}C]R5P. Thus it is clear that much more work needs to done, starting with obtaining the ^{13}C-natural abundance spectrum of YOPRTase, before the observed resonances can be assigned.

CONCLUSION

The work reported herein has provided evidence to support the possible existence of a putative enzyme substrate intermediate in the reaction catalyzed by YOPRTase. A hypothetical transition state that could lead to such an intermediate is illustrated in Figure 33. The proposal that such an intermediate should exist is a consequence of the finding that YOPRTase catalyzes the formation of OMP from PRibPP and orotate in the presence of Mg^{2+} *via* a Bi Bi Ping Pong kinetic mechanism (Victor *et al.*, 1979a). This is supported by my findings that 5-azaorotate is utilized by YOPRTase *via* such a mechanism, in a metal dependent reaction, with a Km of 75.5 ± 0.1 μM. [^{14}C]PRibPP was synthesized, and used to show that PRibPP binds to two equal and independent sites on the YOPRTase dimer with an intrinsic K_d of 33.3 ± 4 μM. [^{13}C]PRibPP was synthesized, and used in preliminary [^{13}C]NMR studies. The [^{13}C]NMR spectrum of an incubation of YOPRTase with [^{13}C]PRibPP shows a peak at 95 ppm which is consistent with a ^{13}C-phosphoribosyl-YOPRTase intermediate. Further work is needed to confirm this. Studies with uracil 6–aldehyde, a novel modifier of lysine residues, and PLP have provided evidence for the presence of lysine or lysines at the active site, that could be involved in the binding of the PRibPP-Mg^{2+} complex. PLP appears to be modifying one to two essential lysines per YOPRTase subunit, based on the incorporation of tritium when PLP treated YOPRTase was reduced with [^{3}H]$NaBH_4$. Finally, nonreacting HPLC gel filtration techniques were used to show that YOPRTase is a dimer with an apparent MW of 37,100 ± 1,400 in a solution of 100 mM ionic strength, pH 6.8, and at protein concentrations of 0.1 to 5.0 μg/ml. When PRibPP and $MgCl_2$ were added to the buffer, the apparent MW increased to 38,900 ± 2,200, suggesting a conformational change in YOPRTase when PRibPP-Mg^{2+} binds, with no change its quaternary structure. When OMP and $MgCl_2$ were added to the buffer there was no change in YOPRTase's quaternary structure.

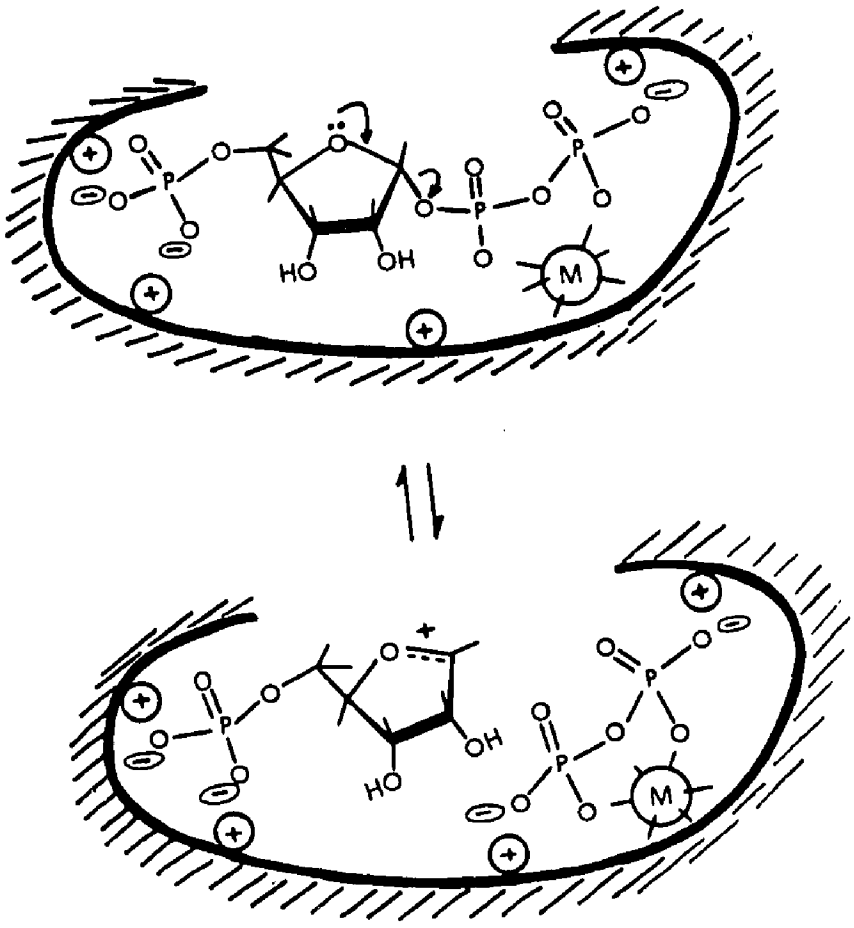


FIGURE 33. Hypothetical topograhy of the YOPRTase active site. Illustrated herein are the positions where positively charged entities, such as lysine, might be required, and a mechanism through which the Phosphoribosyl-YOPRTase complex might be formed.

APPENDIX I

The following programs were written in Microsoft BASIC for an NEC 8201 laptop computer. The programs would require minor modifications to transfer them to a different machine.

1) This program fits initial velocity data to a Bi Bi Ping Pong kinetic mechanism. Data are read from a data file and calculated values are stored in another data file.

```
500 'PING PONG
510 CLEAR
520 DIM V(100),A(100),B(100),S(4,5),Q(4),SM(4),SS(4)
530 PRINT "FIT TO PING PONG EQ."
540 INPUT "HOW MANY POINTS ";NP
550 JJ=0:P=NP-3
560 INPUT"TYPE(1) OR DATA FILE(2) DATA ";M
563 IF M=1 THEN GOTO 570
570 N=3:N1=N+1:N2=N+2
580 OPEN "DATA"FOR INPUT AS #1
586 GOTO 3010
590 'INPUT
601 INPUT#1 ,A(I),B(I),V(I)
640 Q(1)=V(I)^2/B(I)
650 Q(2)=V(I)^2/A(I)
660 Q(3)=V(I)^2
```

```
670 Q(4)=V(I)
680 GOTO 3080
690 KA=S(2,1)/S(3,1)
695 KB=S(1,1)/S(3,1)
700 JJ=J+1:NT=0:M=3
720 GOTO 3010
730 D=KA/A(I)+KB/B(I)+1
735 Q(1)=1/D
740 Q(2)=1/A(I)/D^2
745 Q(3)=1/B(I)/D^2
750 Q(4)=V(I)
755 GOTO 3080
760 CV=S(1,1)
770 KA=KA-S(2,1)/S(1,1)
780 KB=KB-S(3,1)/S(1,1)
790 AB=KA*KB
800 NT=NT+1
810 IF NT<5 THEN GOTO 3010
815 '
820 OPEN "NEWV" FOR OUTPUT AS #1
830 PRINT
#1,"_VCAL_____1/VCAL_____DIF"
1080 FOR I =1 TO NP
1090 X1 = CV/(KA/A(I)+KB/B(I)+1)
1100 DX=V(I)-X1
1110 X5=1/X1
```

```
1120 S2=0:S2=S2+DX^2
1130 PRINT#1,X1,1/X1,DX
1140 NEXT I
1145 CLOSE #1
2010 S2=S2/P
2020 S1=SQR(S2)
2030 FOR J=2 TO N1
2040 FOR K=1TO N
2050 S(K,J)=S(K,J)*SM(K)*SM(J-1)
2060 NEXT K
2070 NEXT J
2080 SA=S1*SQR(S(2,3))/S(1,1)
2090 SB=S1*SQR(S(3,4))/S(1,1)
2095 SV=S1*SQR(S(1,2))
2100 SK=S1*SQR(KB^2*S(2,3)+KA^2*S(3,4)+2     *KA*KB*S(2,4))/S(1,1)
2110 WA=1/SA^2:WB=1/SB^2:WV=1/SV^2:WC=1/
  SK^2:CX=SV/CV:WL=1/CX^2:VA=CV/KA
2150 CX=SV/CV
2190 EA=S1*SQR(S(1,2)+S(2,3)/KA^2+2*S(1,    3)/KA)/KA
2200 EB=S1*SQR(S(1,2)+S(3,4)/KB^2+2*S(1,    4)/KB)/KB
2210 RA=1/EA^2
2220 RB=1/EB^2
2230 CLOSE #1
2280 BEEP
2290 PRINT"Ka=";KA;" S.E.=";SA
2300 PRINT"Kb=";KB;" S.E.=";SB
```

```
2310 PRINT"Vmax=";CV;" S.E.=";SV
2320 PRINT"Sigma=";S1
2330 PRINT"VAR.=";S2
2335 '
2440 GOTO 3380
3010 FOR J=1 TO N2
3020 FOR K=1 TO N1
3030 S(K,J)=0
3040 NEXT K
3050 NEXT J
3060 FOR I =1TO NP
3070 ONMGOTO 590,640,730
3080 FOR J=1TO N1
3090 FOR K=1 TO N
3100 S(K,J)=S(K,J)+Q(K)*Q(J)
3110 NEXT K
3120 NEXT J
3130 NEXT I
3135 CLOSE #1
3140 FOR K=1TO N
3150 SM(K)=1/SQR(S(K,K))
3155 NEXT K
3160 SM(N1)=1
3170 FOR J=1TO N1
3180 FOR K=1TO N
3190 S(K,J)=S(K,J)*SM(K)*SM(J)
```

```
3200 NEXT K
3210 NEXT J
3220 SS(N1)=-1
3230 S(1,N2)=1
3240 FOR L=1 TO N
3250 FOR K=1TO N
3260 SS(K)=S(K,1)
3270 NEXT K
3280 FOR J=1TO N1
3290 FOR K=1TO N
3300 S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
3310 NEXT K
3320 NEXT J
3330 NEXT L
3340 FOR K=1TO N
3350 S(K,1)=S(K,1)*SM(K)
3360 NEXT K
3370 ON M GOTO 690,690,760
3380 OPEN"POINTS"FOR OUTPUT AS#1
3390
PRINT#1,"__V_____1/V_____A_____
_____1/A_
3399 FOR I=1TO NP
3400 PRINT#1,V(I),1/V(I),A(I),1/A(I)
3401 NEXT I
3410 CLOSE
```

```
3420 END
```

2) This program fits initial velocity data to a sequential kinetic mechanism.

```
500 'SEQUEN
510 CLEAR
520 DIM V(50),A(50),B(50),S(5,6),Q(5),SM(5),SS(5)
530 PRINT"Fit To Sequential Equation"
533 INPUT "How Many Points?";NP
540 JJ=0
560 '
563 M=1
565 OPEN"DATA" FOR INPUT AS #1
570 N=4:N1=N+1:N2=N+2:II=0
586 GOTO 3010
590 'PRINT"V";I;"=";
595 INPUT# 1,A(I),B(I),V(I)
640 Q(1)=V(I)^2/B(I)
650 Q(2)=V(I)^2/A(I)
660 Q(3)=V(I)^2
665 Q(4)=V(I)^2/A(I)/B(I)
670 Q(5)=V(I)
680 GOTO3080
690 KA=S(2,1)/S(3,1)
695 KB=S(1,1)/S(3,1)
697 KI=S(4,1)/S(1,1)
```

```
700 JJ=J+1:NT=0:M=3
720 GOTO3010
730 D=KA/A(I)+KB/B(I)+1+KI*KB/A(I)/B(I)
735 Q(1)=1/D
740 Q(2)=1/A(I)/D^2
745 Q(3)=(1+KI/A(I))/B(I)/D^2
750 Q(4)=1/A(I)/B(I)/D^2
753 Q(5)=V(I)
755 GOTO 3080
760 CV=S(1,1)
770 KA=KA-S(2,1)/S(1,1)
780 KB=KB-S(3,1)/S(1,1)
790 KI=KI-S(4,1)/S(1,1)/KB
800 NT=NT+1
810 IFNT<5THENGOTO3010
820 OPEN "NEWV"FOR OUTPUT AS#1
880 PRINT #1,SPACE$(1)+"CONC
A"+SPACE$(6)+"1/A"+SPACE$(9)+"CONC B"
940 FOR I=1 TO NP
950 X2= 1/A(I)
955 PRINT #1,USING "#####.#    #.###^^^^    #####.#";A(I),X2,B(I)
960 NEXT I
970 'LF 4
975 PRINT#1,CHR$(27)+CHR$(76)
980 PRINT#1 ,SPACE$(3)+"1/B"+SPACE$(8)+"EXPTL
V"+SPACE$(6)+"1/EXP V"
```

```
1000 FOR I=1 TO NP
1010 X4=1/B(I)
1020 X3=1/V(I)
1030 PRINT#1,USING"#.###^^^^    #.###^^^^    #.###^^^^"; X4,V(I),X3
1040 NEXT I
1060 PRINT#1,SPACE$(1)+"CALC V"+SPACE$(6)+"1/CALC
V"+SPACE$(7)+"V DIFF"
1080 FOR I=1 TO NP
1090 X1=CV/(KA/A(I)+KB/B(I)+1+KI*KB/A(I)/B(I))
1100 DX=V(I)-X1
1110 X5=1/X1
1120  S2=S2+DX^2
1130 PRINT#1,USING"#.###^^^^    #.###^^^^    ##.####"; X1,X5,DX
1140 NEXT I
2005 P=NP-N
2010  S2=S2/P
2020  S1=SQR(S2)
2030 FOR J=2TO N1
2040 FOR K=1TO N
2050  S(K,J)=S(K,J)*SM(K)*SM(J-1)
2060 NEXT K
2070 NEXT J
2075 CI=KI*KB/KA
2080 R=KI/KA
2085 RI=1/KI
2090 VA=CV/KA:VB=CV/KB
```

```
2095 SV=S1*SQR(S(1,2))
2100 CP=(1-KA/KI)
2110 SA=S1*SQR(S(2,3))/S(1,1)
2120 SB=S1*SQR(S(3,4))/S(1,1)
2130 EA=S1*SQR(S(4,5))/KB/S(1,1)
2140 Y=SQR(CI^2*S(2,3)+KI^2*S(3,4)+S(4,5)-2*KI*CI*S(2,4)-
2*CI*S(2,5)+2*KI*S(3,5))
2141 EB=S1*Y/KA/S(1,1)
2150 SR=EA/KI^2
2160 Y=CP^2*S(1,2)+RI^2*S(2,3)+RI^2*S(4,5)/CI^2-2*CP*RI*S(1,3)
2161 SP=S1*SQR(Y+2*CP*RI*S(1,5)/CI-2*RI^2*S(2,5)/CI)/S(1,1)^2
2170 CP=CP/S(1,1)
2175 PRINT#1, CHR$(27)+CHR$(76)
2290 PRINT#1, "Ka=";KA;" S.E.=";SA
2300 PRINT#1, "Kb=";KB;" S.E.=";SB
2310 PRINT#1, "Vmax=";CV;" S.E.=";SV
2320 PRINT#1, "SIGMA=";S1
2330 PRINT#1, "VAR.=";S2
2331 PRINT#1, "1/Kia=";RI;" S.E.=";SR
2332 PRINT#1, "VCPT=";CP;" S.E.=";SP
2340 CLOSE#1
2440 GOTO 3380
3010 FOR J=1 TO N2
3020 FOR K=1 TO N1
3030 S(K,J)=0
3040 NEXT K
```

```
3050 NEXT J
3060 FOR I=1 TO NP
3070 ON MGOTO 590,640,730
3080 FOR J=1 TO N1
3090 FOR K=1TO N
3100 S(K,J)=S(K,J)+Q(K)*Q(J)
3110 NEXTK
3120 NEXTJ
3130 NEXTI
3135 CLOSE#1
3140 FOR K=1TO N
3150 SM(K)=1/SQR(S(K,K))
3155 NEXTK
3160  SM(N1)=1
3170 FOR J=1TON1
3180 FOR K=1TON
3190  S(K,J)=S(K,J)*SM(K)*SM(J)
3200 NEXT K
3210 NEXT J
3220  SS(N1)=-1
3230  S(1,N2)=1
3240 FOR L=1TO N
3250 FOR K=1TO N
3260  SS(K)=S(K,1)
3270 NEXT K
3280 FOR J=1TO N1
```

```
3290 FOR K=1 TO N
3300  S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
3310 NEXT K
3320 NEXT J
3330 NEXT L
3340 FOR K=1 TO N
3350  S(K,1)=S(K,1)*SM(K)
3360 NEXT K
3370 ON MGOTO 690,690,760
3380 END
```

3) This program fits data to a straight line. It will then interpolate a value of the dependent variable that corresponds to an entered value for the independent variable. The program will also calculate a confidence interval. It will prompt the user for a Students' t value. The t value that is used is dependent upon the desired level of confidence and the number of degrees of freedom.

```
10 N=0:SX=0:SY=0:U=0:W=0:P=0:SD=0:VD=0:VS=0:VA=0
20 INPUT"Which file contains the data";F$
30 OPEN F$ FOR INPUT AS#1
40 IF EOF(1) THEN GOTO 90
50 INPUT#1,X,Y
60 N=N+1
70 SX=SX+X:SY=SY+Y:U=U+X^2:W=W+Y^2:P=P+(X*Y)
80 GOTO 40
```

```
90 CLOSE
100 A=N*U-(SX^2):B=N*W-(SY^2):C=N*P-SX*SY
110 SLOPE=C/A
120 ALPHA=(SY-(SLOPE*SX))/N
121 LPRINT,"X","Y","YI","RESIDUALS":LPRINT CHR$(13)
140 CLOSE:OPEN F$ FOR INPUT AS#1
150 IF EOF(1)THEN GOTO 220
160 INPUT#1,X,Y
170 YI=(SLOPE*X)+ALPHA
180 D=Y-YI
190 LPRINT,X,Y,YI,D
191 LPRINT CHR$(13)
200 SD=SD+(D^2)
210 GOTO 150
220 CLOSE
230 VD=(1/(N-2))*SD
240 VS=(N/A)*VD
250 VA=(U/A)*VD
260 LPRINT "SLOPE=";SLOPE:LPRINTCHR$(13)
270 LPRINT "INTERCEPT=";ALPHA:LPRINTCHR$(13)
280 LPRINT "VAR(delta)=";VD:LPRINTCHR$(13)
281 LPRINT "VAR(slope)=";VS:LPRINTCHR$(13)
282 LPRINT "VAR(intercept)=";VA:LPRINTCHR$(13)
283 INPUT"What is the Students' t value that you want to use";T
290 INPUT"Input Y value";Y
291 INPUT"How many replications is Y the avg. of";N1
300 K2=(T^2)*VD
```

```
310 X1=(SLOPE^2)-((N*K2)/A)
320 X2=(2*SLOPE)*(Y-(SY/N))
330 X3=(Y-(SY/N))^2-((1/N1)+1/N)*K2
340 V1=(X2+(SQR((X2^2)-4*X1*X3)))/(2*X1)
350 V2=(X2-(SQR((X2^2)-4*X1*X3)))/(2*X1)
351 X=(Y-ALPHA)/SLOPE
360 L2=V1+SX/N
370 L1=V2+SX/N
380 LPRINT "Y=";Y; "X=";L1;"<";X;"<";L2:LPRINTCHR$(13)
390 GOTO 290
```

4) This program calculates a spin-lattice relaxation rate from experimental data and a crude estimate of 1/T1. The algorithm is from Sass and Ziessow (1977). Data are read from a data file.

```
10 'PROGRAM FINDS 1/T1
15 CLEAR
20 INPUT "Estimated T1=";T1
24 DIM X(14),Y(14)
25 INPUT "How many points ";NP
26 T=-1*(1/T1)
27 OPEN "DATA" FOR INPUT AS #1
30 FOR I =1 TO NP
40 INPUT #1,X(I),Y(I)
50 NEXT I
```

```
60 CLOSE
69 ES=0:T2=0:SE=0:T3=0:MT=0:T4=0:ME=0:M1=0:TE=0:TS=0
70 FOR I=1TO NP
80 E=EXP((T)*X(I))
90 ES =ES +(E*E)
100 T2=T2+Y(I)*(X(I)*X(I))*E
110 SE=SE+E
120 T3=T3+(X(I)*X(I))*E
130 MT=MT+X(I)*Y(I)*E
140 T4=T4+(X(I)*X(I))*(E*E)
150 ME=ME+Y(I)*E
160 M1=M1+Y(I)
170 TE=TE+X(I)*(E)
180 TS=TS+X(I)*(E*E)
190 NEXT I
200 G=((NP*ES-SE*SE)*MT)+((ME*SE-M1*ES)*TE)+((M1*SE-NP*ME)*TS)
210 G1=((NP*TS-TE*SE)*MT)+((NP*ES-SE*SE)*T2)+((ME*TE-
M1*TS)*TE)+(ME*SE-M1*ES)*T3
211 G2=2*(M1*SE-NP*ME)*T4
212 G3=G1+G2
220 RR=T-(G/G3)
225 R1=R1+1
230 IF R1=5 THEN GOTO 255
240 T=RR
250 GOTO 69
255 BEEP
260 PRINT "T1=";-1/RR
270 END
```

APPENDIX II

Dr. C. Grubemeyer (New York Univ.) isolated a Salmonnella typhimurium clone that he believed to be overproducing clonal *E. coli* OPRTase. He asked us to confirm this with our standard spectrophotometric assay for OPRTase. Dr. Grubmeyer provided us with two controls, wild type *Salmonnella typhimurium* LT-2, and SA2434, a mutant lacking pyr E, the OPRTase gene. Two clones were examined SA2434#1 and SA2434#2. Both had been transfected, in separate experiments, with bacterial phage p22 that carried a plasmid containing the *E. Coli* pyr E gene.

The frozen cell samples sent by Dr. Grubmeyer were thawed and made up to approximately 5ml per gram of cells with the following buffer: 40 mM triethanolamine (pH 8), 1 mM EDTA, and 1 mM dithiothreitol. The cells were disrupted by sonification for four times 15s, with cooling on ice. The sonicates were centrifuged at 27,000 g for 30 minutes. The supernatants were decanted and assayed for OPRTase activity and protein concentration. The findings are presented in Table XI. Clearly, Dr. Grubemeyer has successfully cloned the *E. coli* pyr E gene into both the SA2434#1 and the SA2434#2 clones. However only the latter is an overproducer of OPRTase, with 74 times the OPRTase activity of wild type *Salmonnella typhimurium* LT-2.

Table XI

OPRTase Actvity in *Salmonella typhimurium* Containing Clonal *E. Coli* OPRTase

Cells	Protein (mg/ml)	Volume (ml)	Specific Activity (mmol/min./mg)	Total Activity
Wild Type	5.2	24	0.06	7.5
SA2434	9.6	43	0	0
SA2434#1	6.0	40	0.08	19.2
SA2434#2	5.2	42	4.44	970

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