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 MECHANISM OF ADENINE PHOSPHORIBOSYLTRANSFERASE  
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STUDIES OF THE KINETIC MECHANISM, BINDING OF  
SUBSTRATES, AND CATALYTIC MECHANISM OF ADENINE  
PHOSPHORIBOSYLTRANSFERASE

BY

RONALD EDWARD ALFRED GADD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES  
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1969

UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES OF THE KINETIC MECHANISM, BINDING OF SUBSTRATES, AND CATALYTIC MECHANISM OF ADENINE PHOSPHORIBOSYLTRANSFERASE", submitted by Ronald Edward Alfred Gadd in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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## ABSTRACT

The kinetic mechanism of adenine phosphoribosyltransferase, the enzyme responsible for the synthesis of adenylate (AMP) and pyrophosphate from adenine and phosphoribosylpyrophosphate (PP-ribose-P), is of the ordered bi bi type; pyrophosphate, in addition to acting as a normal product inhibitor, also acts as a dead end inhibitor binding to free enzyme. This mechanism is consistent with the observed product inhibition patterns, isotope exchange pattern between adenine-<sup>14</sup>C and AMP, and protection by pyrophosphate against enzyme inactivation by p-hydroxymercuribenzoate (PMB). A parallel pattern of lines for double reciprocal plots of initial velocity against substrate concentration has, in the past, been regarded as evidence for ping pong bi bi mechanisms, but an apparently parallel pattern of lines may also be observed for ordered bi bi mechanisms when the dissociation constant for the first substrate is much smaller than its Michaelis constant. The dissociation constant of PP-ribose-P was determined by measuring the rate of enzyme inactivation by heat and PMB in the absence and presence of PP-ribose-P, and was found to be much smaller than the Michaelis constant for PP-ribose-P. That the dissociation constant of PP-ribose-P is very small suggests that the release of PP-ribose-P from the enzyme-PP-ribose-P complex is slow, and this is compatible with the

observation that no reverse reaction or isotope exchange between pyrophosphate-<sup>32</sup>P and PP-ribose-P was detected.

The groups both on the enzyme and on the substrates, PP-ribose-P and adenine, which are involved in binding to enzyme were investigated. Both the 5-phosphate and 1-pyrophosphate moieties of PP-ribose-P are involved in binding. The 5-phosphate moiety may bind to the enzyme by anionic-cationic interactions or by hydrogen or charge-transfer bonding; in the two latter cases the 5-phosphate would probably act as an electron donor. An anionic group on the 1-pyrophosphate moiety is also involved in binding, and magnesium ion which is chelated to the 1-pyrophosphate moiety may also act as a link in binding pyrophosphate to the enzyme. The ribofuranose moiety does not make a major contribution to binding, and its most important function may be to keep the 5-phosphate and 1-pyrophosphate moieties at a critical distance apart.

The ionization constant and heat of ionization for the group on the enzyme which is involved in PP-ribose-P binding were determined from plots of pH versus the negative logarithm of its Michaelis constant. From these data and from substrate protection against enzyme inactivation by protein reagents, an  $\epsilon$ -amino group of lysine was postulated as being involved in PP-ribose-P binding, but a sulfhydryl group cannot be ruled out. It is apparent that the enzyme contains

a sulfhydryl group that is protected by PP-ribose-P against modification by PMB and that modification of this sulfhydryl group by PMB abolishes enzyme activity.

The 6-amino group, and 3- and 7-nitrogens of adenine are involved in binding; the first as an electron acceptor and the last two as electron donors. Those positions of adenine at which there is probably a lack of bulk tolerance include the 6-amino, 2-carbon and the 3-, 7- and 9-nitrogen positions. The ionization constant and heat of ionization for the group on the enzyme which was involved in adenine binding were determined from plots of pH versus the negative logarithm of the Michaelis constant of adenine. From these data an  $\epsilon$ -amino group of lysine or a phenolic hydroxyl group was implicated in adenine binding, but a choice between these two possibilities could not be made.

The inhibition of adenine phosphoribosyltransferase by nucleosides was investigated, and some were found to be very potent inhibitors. Although no direct evidence was presented, the inhibition patterns obtained for adenine 6'-deoxy- $\beta$ -D-allofuranoside and the structural requirements for inhibition by nucleosides suggest that they do not bind to those sites normally occupied by the substrates, adenine and PP-ribose-P, but to some other site which may or may not be a regulatory site.

The catalytic mechanism of adenine phosphoribosyltrans-

ferase was investigated, and proton removal from the 9-NH group of adenine by imidazole was suggested to be the rate-determining step; this would be followed by a more rapid nucleophilic attack of the 9-nitrogen on the anomeric carbon of PP-ribose-P. This mechanism is compatible with the effect of  $^2\text{H}_2\text{O}$  on the maximum velocity, and the inversion of configuration of  $\alpha$ -PP-ribose-P to  $\beta$ -AMP. The ionization constant and heat of ionization for a group on the enzyme which may be involved in the catalytic mechanism were determined from plots of pH versus the logarithm of the maximum velocity; this group was tentatively identified as the imidazole of histidine.

### Acknowledgements

I would like to express my appreciation to Dr. J. F. Henderson, my supervisor, for his assistance and patience during this work. I would also like to thank Dr. A. R. P. Paterson, Dr. J.S. Colter and the staff members in the McEachern Laboratory and Department of Biochemistry for having provided many opportunities to study and to learn. I also wish to acknowledge the National Cancer Institute of Canada for their financial support over the past two years. Finally I would like to express my appreciation to Sally, my wife, for providing the encouragement to complete this program.



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Abbreviations of Kinetic Constants (a)

$K_A$	Michaelis constant for PP-ribose-P
$K_{iA}$	Product inhibition constant for PP-ribose-P
$K_B$	Michaelis constant for adenine
$K_{iB}$	Product inhibition constant for adenine
$K_{iiB}$	Inhibition constant for adenine acting as a competitive inhibitor of 2,6-diaminopurine
$K_P$	Michaelis constant for pyrophosphate
$K_{iP}$	Product inhibition constant for pyrophosphate
$K_{iiP}$	Inhibition constant for pyrophosphate binding to free enzyme.
$K_Q$	Michaelis constant for AMP
$K_{iQ}$	Product inhibition constant for AMP
$K_{eq}$	Equilibrium constant
$V_1$	Maximum velocity in the forward direction
$V_2$	Maximum velocity in the reverse direction
A	PP-ribose-P
B	Adenine
P	Pyrophosphate
Q	AMP
I	Inhibitor

(a) Kinetic constants for Mechanism II are given in terms of rate constants in Table IV.

## Abbreviations

AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
EDTA	disodium ethylenediaminetetraacetate
GMP	guanosine 5'-monophosphate
IMP	inosine 5'-monophosphate
PMB	p-hydroxymercuribenzoate
PP-ribose-P	$\alpha$ -D-1-pyrophosphorylribofuranosyl-5-phosphate
Tris	tris (hydroxymethyl) aminomethane
XMP	xanthosine 5'-monophosphate
PP <sub>i</sub>	pyrophosphate

## I INTRODUCTION

Purine nucleotides function in cellular physiology as structural units in macromolecules and as coenzymes. The bulk of cellular purine nucleotides are in the polymers deoxyribonucleic acid and ribonucleic acid, and smaller amounts of purine nucleotides are involved in polysaccharide, lipid and protein syntheses, phosphorylation reactions, and as the principal coenzymes of energy metabolism.

Purine nucleotides may be synthesized in cells by three alternative pathways. In the first, called biosynthesis de novo, the purine ring is synthesized from non-purine precursors such as glutamine, glycine, bicarbonate and other small molecules (23). The end product of this pathway, inosine 5'-monophosphate (IMP), may be converted by other enzymes to guanosine 5'-monophosphate (GMP), xanthosine 5'-monophosphate (XMP) and adenosine 5'-monophosphate (AMP), as shown in Figure 1.

In the second pathway purine bases are converted to nucleosides which are in turn converted to nucleotides. Purine nucleoside phosphorylase(s) catalyzes the synthesis of nucleosides or deoxynucleosides from purine bases and ribose 1-phosphate or deoxyribose 1-phosphate, respectively (43). The adenosine triphosphate (ATP)-dependent conversion of adenosine to AMP has been demonstrated in yeast and several animal tissues (24,80,115), and Tarr (125) in 1964

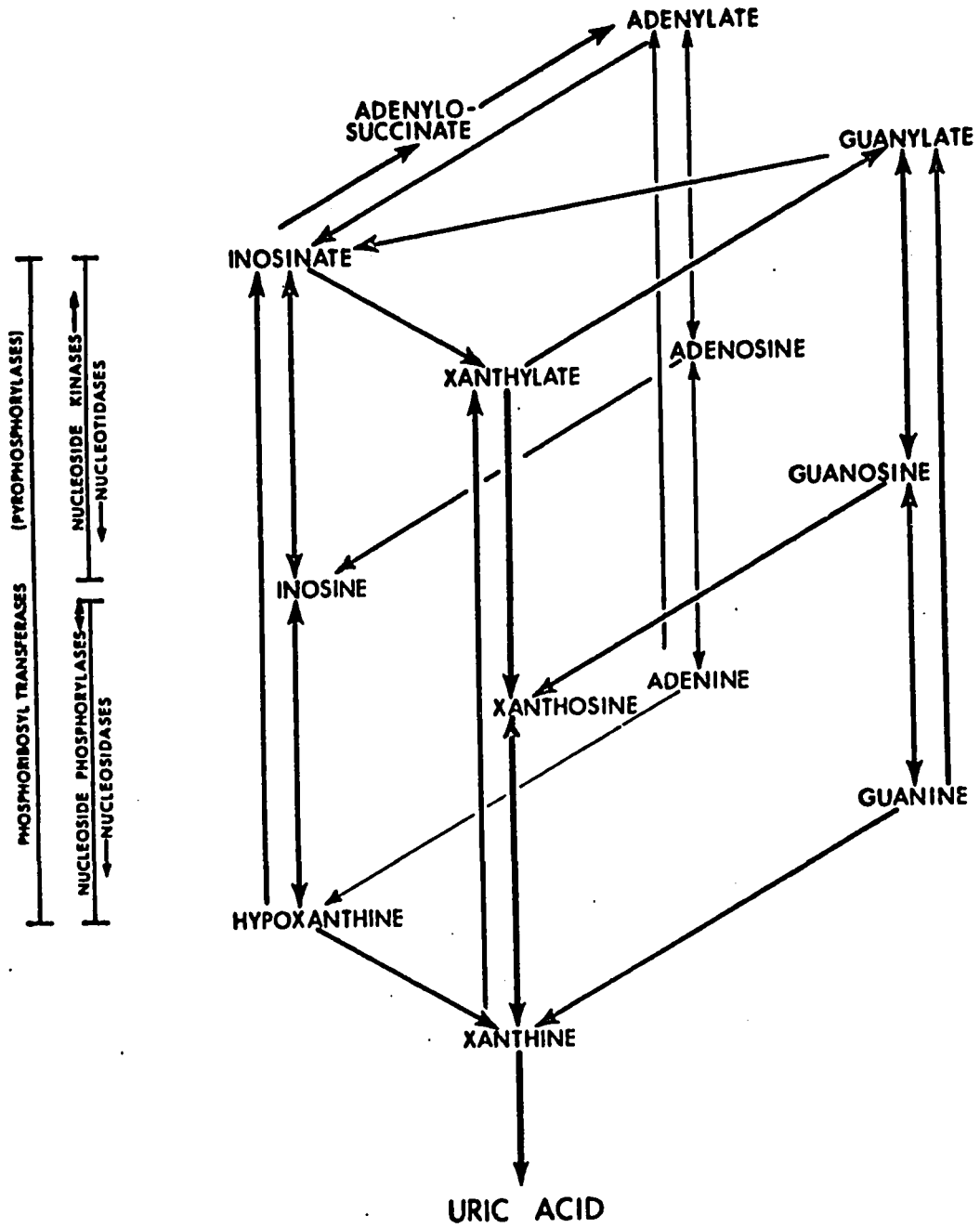


Figure 1. Purine ribonucleotide synthesis and interconversions.

reported guanosine kinase activity in salmon milt. Pierre, Kimball and LePage (107), in 1967, provided data supporting the existence of separate inosine and guanosine kinases in crude extracts of mouse tumors.

In the third pathway, adenine, hypoxanthine and guanine react directly with  $\alpha$ -D-1-pyrophosphorylribofuranosyl-5-phosphate (PP-ribose-P) to give the corresponding purine nucleotides and pyrophosphate (75,79) without going through a nucleoside intermediate. The enzymes responsible for these reactions, shown in Figure 2, are called purine phosphoribosyltransferases. Evidence for the existence of this pathway was first presented by Goldwasser (46,47) in 1953 and 1954. In a preliminary note in 1954, Kornberg, Lieberman and Simms (76) also presented evidence for the direct conversion of adenine to AMP, and suggested that the source of the ribose 5-phosphate moiety required was PP-ribose-P. The first detailed study of adenine phosphoribosyltransferase was reported in 1955 by the same authors (79), who partially purified this enzyme from yeast and showed that for each mole of adenine and PP-ribose-P used, one mole each of AMP and pyrophosphate was produced. This reaction is shown in Figure 3. These investigators also demonstrated that different enzymes were responsible for the conversion of adenine to AMP and of hypoxanthine to IMP. In the same year Korn et al. (75) also observed that extracts of pigeon

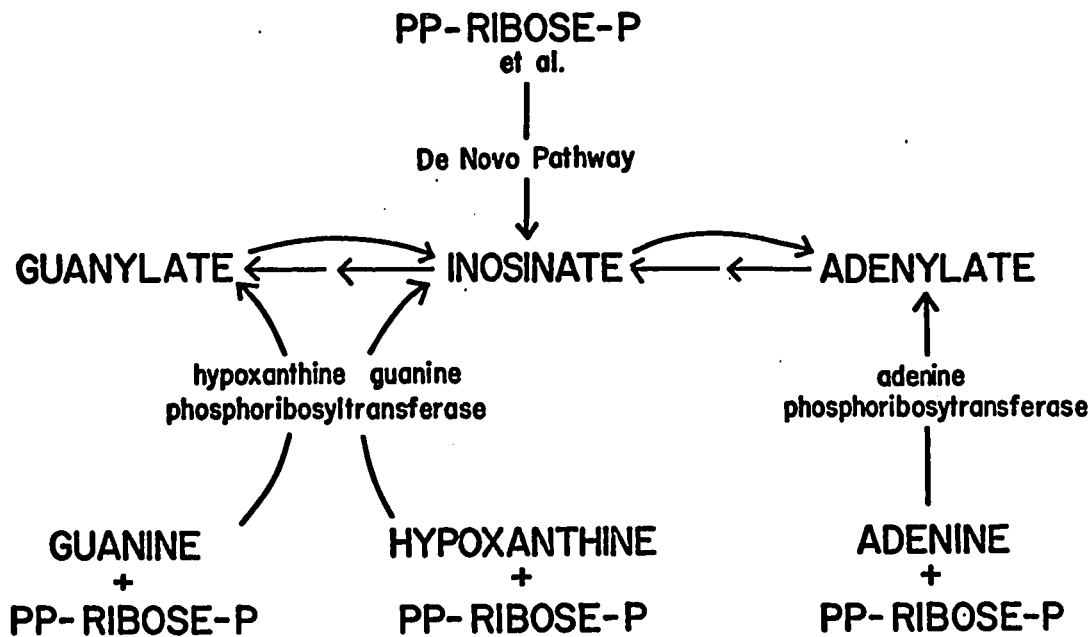


Figure 2. Purine phosphoribosyltransferases.



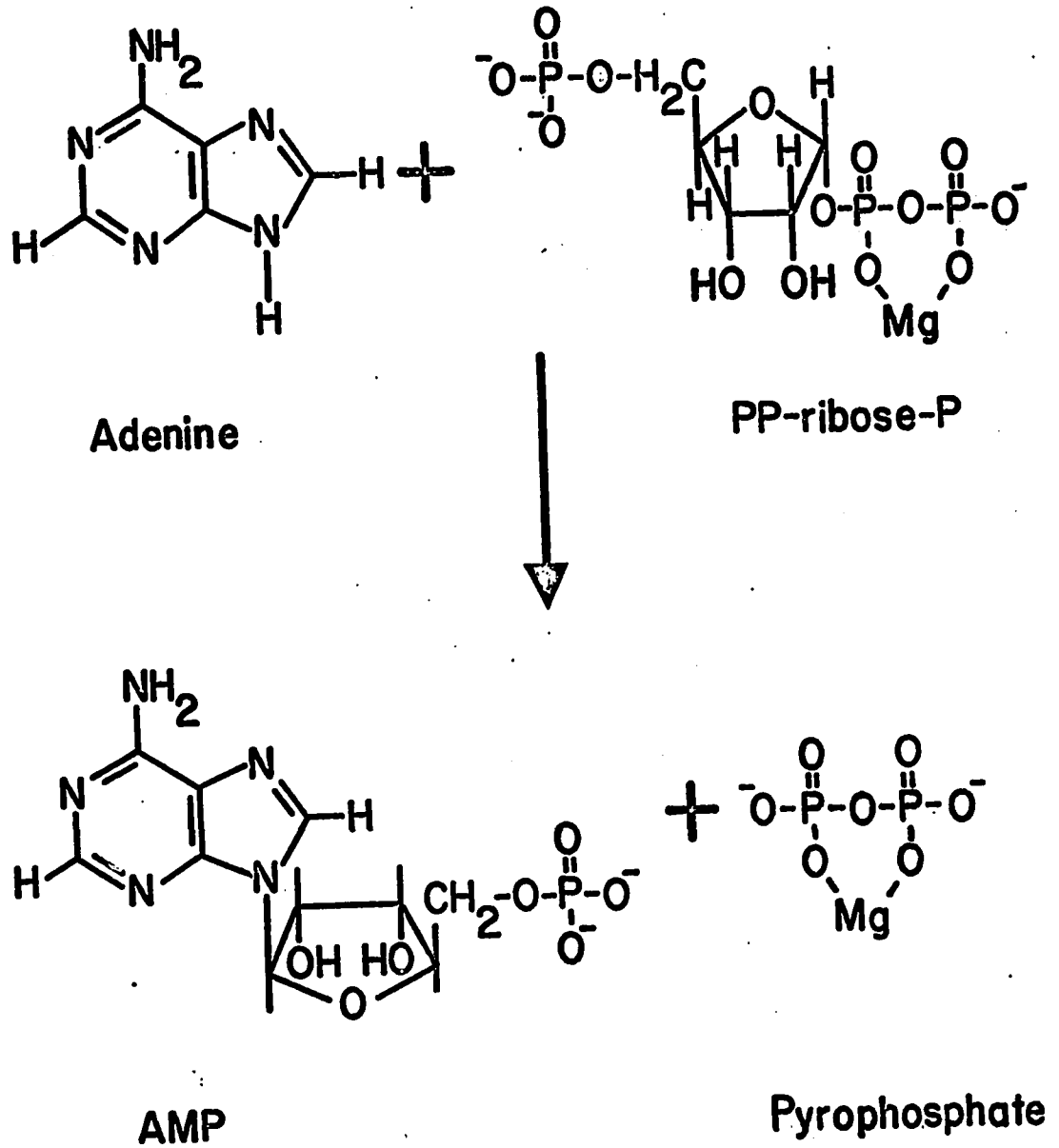


Figure 3. The adenine phosphoribosyltransferase reaction.

liver converted hypoxanthine to IMP in the presence of PP-ribose-P. Henderson et al. (57), from kinetic data, and Kelley et al. (71), from genetic data, have presented evidence that hypoxanthine and guanine phosphoribosyltransferase activities are catalyzed by a single enzyme. Kalle and Gots (69), in 1961, purified 20-fold from Salmonella typhimurium an enzyme which had an absolute requirement for xanthine as the purine in a phosphoribosyltransferase reaction. In contrast, xanthine is utilized very poorly in animal cells, and Kelley et al. (71) observed that human mutants who lack hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) also failed to synthesize XMP from xanthine and PP-ribose-P. This observation suggests that hypoxanthine-guanine phosphoribosyltransferase is also responsible for the conversion of xanthine to XMP. At the present time, therefore, only two phosphoribosyltransferases appear to be responsible for the conversion of adenine, hypoxanthine, guanine and xanthine to their respective nucleotides in mammalian cells.

The exact roles of adenine and hypoxanthine-guanine phosphoribosyltransferases in cell function are not known, and may in fact vary in different cell types; the most obvious possible function, however, is for the utilization by cells of exogenously supplied or dietary purines. The purine phosphoribosyltransferases may also be important in

the metabolism of purine nucleosides, since these nucleosides may be cleaved to free purine bases which may then react with PP-ribose-P by means of the appropriate phosphoribosyltransferase. Evidence presented by LePage and Junga (89) in 1963 suggests that this is the pathway by which thioguanosine is converted to the corresponding ribonucleotide by ascites tumor cells.

Since RNA may be degraded to free bases, purine phosphoribosyltransferases may also be involved in the reutilization of these degradation products. In fact Friedkin and Kalckar (43) have suggested that in most mammalian cells nucleotide synthesis from purine bases and purine nucleosides occurs principally by purine phosphoribosyltransferases; Lieberman and Ove (91), Brockman et al. (20), and Brockman (19) have presented evidence that purine phosphoribosyltransferases are the principal enzymes involved in purine nucleotide formation from free purine bases in Ehrlich ascites tumor cells.

Although purine phosphoribosyltransferases are almost certainly involved in nucleotide synthesis, their importance relative to the de novo pathway is not known. Human mutants whose erythrocytes have only 20 per cent of normal adenine phosphoribosyltransferase activity appear to be healthy (60). Possibly the best evidence for the importance of purine phosphoribosyltransferases is that male children who completely

lack hypoxanthine-guanine phosphoribosyltransferase suffer from a neurological disease called the Lesch-Nyhan syndrome; it is characterized by self-destructive mutilation and mental retardation (56). Kelley et al. (72) have also shown that partial or complete deficiency of hypoxanthine-guanine phosphoribosyltransferase activity is associated with excessive purine synthesis and hyperuricemia in some patients. They propose that the normal function of this enzyme is required for the regulation of purine biosynthesis. Whether a similar metabolic disorder may be associated with a complete deficiency of adenine phosphoribosyltransferase is not known.

Whatever the physiological roles of the purine phosphoribosyltransferases may be, they are definitely known to play important pharmacological roles in the activation of drugs in cancer chemotherapy. Many of the purine base analogs used in cancer chemotherapy act only after conversion to the nucleoside monophosphate, which may be either the pharmacologically active form or an intermediate in the formation of the active form of the drug. For example, cells which lack hypoxanthine-guanine phosphoribosyltransferase are resistant to 8-azaguanine and 6-mercaptopurine (19,20), and cells which lack adenine phosphoribosyltransferase are resistant to 2,6-diaminopurine (91).

Adenine phosphoribosyltransferase (adenosine monophos-

phate: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7, and also called adenylate pyrophosphorylase) may be regarded as a member of a larger class of enzymes which transfer the 5-phosphoribosyl moiety of PP-ribose-P to a nitrogen atom of a variety of substrates (Table I). Until recently little information concerning the mechanisms of these reactions has been available, and the information that was available was somewhat contradictory. In 1955 Kornberg, Liberman and Simms (79) reported that isotope exchange between adenine and AMP did not occur unless pyrophosphate was present, and on the basis of this observation a reaction mechanism which involved a ternary enzyme-PP-ribose-P-adenine complex was proposed. More recently the form of double reciprocal plots of initial velocity versus substrate concentration led several groups (31,64) to conclude that the mechanism involved a binary enzyme-substrate complex. In the present study the kinetic mechanism of adenine phosphoribosyltransferase was investigated further in order to explain these anomalous observations, and a kinetic model which is consistent with all the available kinetic data has been proposed. Those amino acid residues at the active site which are necessary for the binding of substrates and the catalytic activity of the enzyme have been investigated by the use of protein reagents, and ionization constants have been determined from plots of the negative logarithm of the Michaelis constants and the loga-

TABLE I: Ribonucleotide Phosphoribosyltransferases

5-Phosphoribosyl-1-Pyrophosphate + Base $\longrightarrow$	
Ribonucleotide + Pyrophosphate	
<u>Base</u>	<u>Ribonucleotide</u>
1. Adenine	Adenylic acid
2. Guanine Hypoxanthine	Guanylic acid Inosinic acid
3. Xanthine	Xanthylic acid
4. Glutamine, Ammonia	Phosphoribosylamine
5. Orotic acid	Orotidylic acid
6. Uracil	Uridylic acid
7. 2,4-Diketopyrimidines 2,6-Diketopurines	Uridylic and Orotidylic acids Uric acid and Xanthine 3-ribonucleotides
8. Nicotinic acid	Nicotinic acid ribonucleotide
9. Nicotinamide	Nicotinamide ribonucleotide
10. Quinolinic acid	Nicotinic acid ribonucleotide
11. Imidazoleacetic acid	Imidazoleacetic acid ribo- nucleotide
12. Histamine	Histamine ribonucleotide
13. Anthranilic acid	N-(5'-phosphoribosyl)- anthranilic acid
14. ATP	1-(5'-phosphoribosyl)ATP

rithm of the maximum velocity versus pH. From these data a possible catalytic mechanism for adenine phosphoribosyltransferase has been proposed.

Since many purine base analogs used in cancer chemotherapy are active only after conversion to the nucleotide form, it would be desirable to be able to predict which analogs are likely to be substrates and thereby effectively compete with adenine for binding to adenine phosphoribosyltransferase. Those atoms or groups of PP-ribose-P and adenine which are involved in their binding to the enzyme were investigated by a structure-activity study. It is hoped that this type of study may permit selection or design of drugs which are likely to be rapidly converted to their nucleotide form by adenine phosphoribosyltransferase and which may be pharmacologically active against cancer.

## II METHODS AND MATERIALS

### A. Introduction

In 1966 Hori and Henderson (63) purified adenine phosphoribosyltransferase 65-fold from Ehrlich ascites tumor cells by a procedure involving treatment with acid, carboxymethyl Sephadex, diethylaminoethyl Sephadex, and precipitation with  $(\text{NH}_4)_2\text{SO}_4$ . Their preparation was used in the present study. Stock solutions of the enzyme preparation were prepared every week by dissolving 5 mg of the lyophilized enzyme preparation in 1 ml of 0.15 M Tris-HCl buffer, pH 7.4, containing 20% (w/v)  $(\text{NH}_4)_2\text{SO}_4$ . This solution was kept at 5° and no appreciable loss of activity was observed during storage for seven days.

### B. Chemicals

Adenine-8- $^{14}\text{C}$  (30.7 mC per mmole) and AMP-8- $^{14}\text{C}$  (43 mC per mmole) were obtained from Nuclear-Chicago; sodium pyrophosphate- $^{32}\text{P}$  (4,500 mC per mmole at the time of purchase) and heavy water,  $^2\text{H}_2\text{O}$  (99.7% deuterium oxide) from New England Nuclear; and 2,6-diaminopurine-2- $^{14}\text{C}$  (5.5 mC per mmole) from Calbiochem. D-Ribose, ribose 5-phosphate, fructose 6-phosphate, glucose 6-phosphate, ribulose 1,5-diphosphate, glucose 1,6-diphosphate, fructose 1,6-diphosphate, 6-phosphogluconic acid, adenosine 5'-phosphoramidate, adenosine 5'-monoacetate, adenosine 2'-monophosphate, adenosine 3'-monophosphate, adenosine 2',3'-cyclicmono-



phosphate, 5'-O-trityl-adenosine, p-hydroxymercuribenzoate, and adenine were obtained from Sigma. Sodium borohydride was obtained from Fisher Scientific;  $\alpha$ -D-ribose 1-phosphate and  $\beta$ -D-ribose 1-phosphate, from Calbiochem; N-bromosuccinimide and 4-amino-1H-tetrazole, from Aldrich; trinitrobenzenesulfonic acid, 2-chloro-3,5-dinitropyridine, 2-hydroxy-5-nitrobenzyl bromide, N-acetylimidazole and 5-dimethylaminonaphthalene-1-sulfonyl chloride, from Pierce; AMP from P-L laboratories. Ribose 1,5-diphosphate was kindly supplied by Dr. G. Tener and the aliphatic diol diphosphates were kindly supplied by Dr. R. M. Barker. The dimagnesium salt of PP-ribose-P was obtained from P-L Biochemicals and from Sigma. The sodium salt of PP-ribose-P was prepared by dissolving the dimagnesium salt in distilled water containing that amount of disodium ethylenediaminetetraacetate (EDTA) calculated to complex the amount of magnesium ion present. This preparation was used in all experiments, and it was stable indefinitely when kept frozen at  $-20^{\circ}$ . The PP-ribose-P obtained from Sigma contained 61% dimagnesium PP-ribose-P by weight while that obtained from P-L Biochemicals contained 71% dimagnesium PP-ribose-P by weight.

Diazonium-1H-tetrazole was prepared from 5-amino-1H-tetrazole and sodium nitrite by the method of Horinishi (66).

#### C. Assays of enzyme activity

##### 1. Conversion of adenine to AMP

a. Spectrophotometric method

Five  $\mu\text{g}$  of enzyme preparation was added to 10 x 100 mm test tubes containing 0.1 mM PP-ribose-P, 1 mM  $\text{MgSO}_4$ , 0.05 mM to 1 mM adenine, and 0.10 M Tris-HCl buffer, pH 7.4, in a total volume of 1 ml. Assay mixtures were incubated for 22 minutes at 37° in a water bath shaking at 50 strokes per minute, and the incubation was terminated by immersing the tubes in an ethanol-dry ice bath until the contents were frozen. Unreacted adenine was removed by four extractions with 2-ml portions of water-saturated 1-butanol (38,63). Residual butanol was removed by two extractions with 1-ml portions of diethyl ether, and residual traces of ether were expelled by aeration. The absorbance of AMP was measured at 260  $\mu\text{m}$  in 1 ml cuvettes in a Gilford spectrophotometer. The lower limit of detection of this assay procedure was 1  $\mu\text{mole}$  of AMP.

b. Radioisotope method

One  $\mu\text{g}$  of enzyme preparation was added to test tubes containing 0.003 mM to 0.02 mM PP-ribose-P, 1 mM  $\text{MgSO}_4$ , 0.0003 mM to 0.002 mM adenine-<sup>14</sup>C and 0.10 M Tris-HCl buffer, pH 7.4, in a total volume of 0.5 ml. The assay mixtures were incubated for 12 minutes at 30° in a water bath shaking at 50 strokes per minute, and the incubation was terminated by the addition of 3 ml of water-saturated 1-butanol. The reaction rate was constant over this incubation

period (63). Unreacted adenine was removed by four extractions with 3-ml portions of water-saturated 1-butanol (38, 63). The aqueous phase which remained after butanol extraction was transferred to a glass counting vial, 4.5 ml of Bray's (18) phosphor solution was added and radioactivity was measured in a Nuclear-Chicago liquid scintillation spectrometer at 89% efficiency. Quenching was measured by channels-ratio. The composition of Bray's phosphor solution is: naphthalene, 60 gm; 2,5-diphenyloxazole, 4 gm; 1,4-di [2-(5-phenyloxazolyl)]benzene, 0.2 gm; methanol, 100 ml; ethylene glycol, 20 ml; and p-dioxane to make 1 liter. The lower limit of detection of this assay procedure was 5  $\mu$ moles of AMP- $^{14}\text{C}$ , which gave approximately 300 cpm. This value is more than 200 cpm above the blank background of 50 to 100 cpm observed for most of the reaction mixtures. The amount of AMP- $^{14}\text{C}$  produced in these reaction mixtures was calculated from a graph which was constructed by plotting  $\mu$ moles of adenine- $^{14}\text{C}$  versus cpm. The cpm were obtained by transferring various amounts of adenine- $^{14}\text{C}$  to counting vials containing 4.5 ml of Bray's phosphor solution.

At an adenine- $^{14}\text{C}$  concentration of  $2 \times 10^{-7}$  M the four extractions with 3-ml portions of water-saturated 1-butanol used in the standard procedure removed 98.97% of the residual adenine, at  $9 \times 10^{-7}$  M adenine they removed 99.55% of the residual adenine, and at  $3.2 \times 10^{-6}$  M adenine they removed 99.75% of the residual adenine. In experiments in

which adenine-<sup>14</sup>C concentrations were increased to between 10<sup>-5</sup> M and 10<sup>-4</sup> M, the number of extractions was increased to seven or eight in order to keep the concentration of unreacted adenine as low as possible.

Figure 4 shows that AMP is also extracted into butanol from solutions whose pH is less than 7.0. When enzyme assays were done at such pH's, 0.05 ml of 2 M Tris-HCl buffer, pH 8.0, was added before extraction in order to raise the pH to between 7.6 and 8.0. No pH dependence was observed for adenine extraction.

## 2. Conversion of AMP to adenine

Six µg of enzyme preparation was added to test tubes containing 0.05 mM to 10 mM tetrasodium pyrophosphate, 2 mM MgSO<sub>4</sub>, 0.3 mM to 0.4 mM AMP-<sup>14</sup>C, and 0.1 M Tris-HCl buffer, pH 7.4, in a total volume of 0.1 ml. The assay mixtures were incubated for 30 minutes at 30° and the incubation was terminated by the addition of 0.02 ml of 4 M formic acid. One-tenth ml of this solution and 3 µg of carrier adenine were applied to Whatman 3MM paper which was developed by ascending chromatography for 20 hours in 1-butanol: glacial acetic acid:H<sub>2</sub>O (25:15:10) (127). Chromatograms were dried, the areas containing adenine were cut out and placed in counting vials, 18 ml of toluene phosphor solution (toluene containing 4.0 gm of 2,5-diphenyloxazole, and 100 mg of 1,4-di[2-(5-phenyloxazolyl)]benzene, per liter)

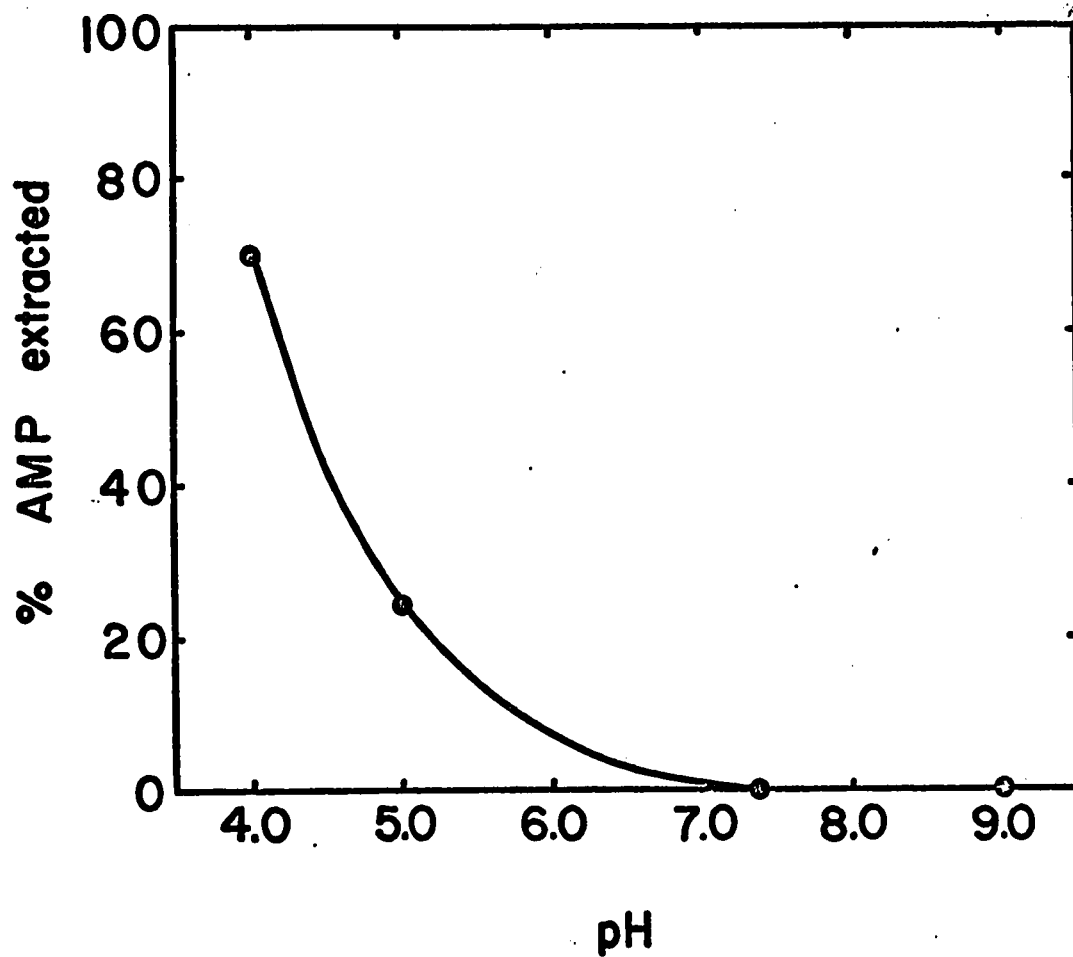


Figure 4. Extraction of AMP as a function of pH.  
Concentration was  $10^{-4}$  M.

were added to each vial and radioactivity was measured in a Nuclear-Chicago liquid scintillation spectrometer (106,111) at 60% efficiency. Quenching was controlled by channels-ratio. The lower limit of detection of this assay procedure was 100  $\mu$ moles of adenine-<sup>14</sup>C, which gave approximately 4,400 cpm above the blank background of 9,000 cpm. The high blank background values were due to the 0.4% of impurities in the AMP-<sup>14</sup>C.

3. Conversion of 2,6-diaminopurine to 2,6-diaminopurine ribonucleoside 5'-monophosphate

Six  $\mu$ g of enzyme preparation was added to test tubes containing 0.1 mM PP-ribose-P, 1.1 mM MgSO<sub>4</sub>, 2 mM to 5 mM 2,6-diaminopurine-<sup>14</sup>C and 0.1 M Tris-HCl buffer, pH 7.4, in a total volume of 0.1 ml. The reaction mixtures were incubated for 30 minutes at 30° in a water bath shaking at 50 strokes per minute and the incubation was terminated by the addition of 0.02 ml of 4 M formic acid. The reaction rate was constant over the incubation period. One-tenth ml of the reaction mixture was applied to Whatman 3MM paper which was developed by ascending chromatography for 5 hours in 5% disodium phosphate. Because carrier 2,6-diaminopurine nucleoside 5'-monophosphate was not available, the paper chromatograms were cut into 1-cm segments and the radioactivity of each was measured in toluene phosphor solution as described previously. The lower limit of detection of

this assay procedure was 200  $\mu$ moles of 2,6-diaminopurine ribonucleoside 5'-monophosphate- $^{14}\text{C}$ , which gave approximately 1,200 cpm above the blank background which was approximately 3,000 cpm. The high blank background values were due to 0.09% of impurities in the 2,6-diaminopurine- $^{14}\text{C}$ . Two radioactive areas were detected in chromatograms of reaction mixtures containing substrates and enzyme, whereas only one radioactive area was present in chromatograms of control mixtures containing substrate but no enzyme. The product which was formed only in the presence of enzyme had an  $R_f$  value of 0.50, and was presumed to be the ribonucleotide of 2,6-diaminopurine. Blair (13) in 1967 reported that the adenine phosphoribosyltransferase of L-strain mouse fibroblasts converted 2,6-diaminopurine to the corresponding nucleoside monophosphate, and that this compound had an  $R_f$  of 0.49 in 5%  $\text{Na}_2\text{HPO}_4$  saturated with isoamyl alcohol. The presence of isoamyl alcohol has relatively little effect on  $R_f$ 's in this system, but markedly retards the rate of development.

4. Isotope exchange between pyrophosphate- $^{32}\text{P}$  and PP-ribose-P

Four  $\mu\text{g}$  of enzyme preparation was added to test tubes containing 0.06 mM PP-ribose-P, 2 mM  $\text{MgSO}_4$ , 1 mM sodium pyrophosphate- $^{32}\text{P}$  (diluted 1:90 with non-radioactive pyrophosphate), from  $10^{-7}$  M to  $10^{-3}$  M adenine and 0.10 Tris-

HCl buffer, pH 7.4, in a total volume of 1 ml. The reaction mixtures were incubated for 20 to 100 minutes at 30° in a water bath shaking at 50 strokes per minute. Upon completion of the incubation, 0.5 ml of the reaction mixtures were applied to 5 x 15 mm columns of Dowex-1-Cl. Pyrophosphate was eluted with 20 ml of 0.135 M LiCl, and PP-ribose-P was then eluted with 5 ml of 0.5 M LiCl. The 0.5 M LiCl eluent was dried in vacuo, taken up in 1 ml of distilled water, transferred to counting vials containing 9.0 ml of Bray's phosphor solution, and <sup>32</sup>P radioactivity measured. The lower limit of detection for this assay procedure was 20 μmoles which gave approximately 200 cpm above the blank background of 300 cpm.

The completeness of the separation of pyrophosphate and PP-ribose-P on columns of Dowex-1-Cl was confirmed by the following procedure. One-half ml of a 1 mM solution of pyrophosphate and 0.5 ml of a 0.1 mM solution of PP-ribose-P, both in the presence and absence of 2 mM magnesium ion, were placed on 5 x 15 mm columns of Dowex-Cl. The columns were then eluted with four 5-ml portions of 0.135 M LiCl, followed by 5 ml of 0.50 M LiCl. The samples were dried in vacuo, and dissolved in 1 ml of H<sub>2</sub>O; pyrophosphate and the pyrophosphate moiety of PP-ribose-P were hydrolyzed to orthophosphate by heating in a boiling water bath for 15 minutes following the addition of 1 ml of 1 N HCl. Orthophosphate



was determined spectrophotometrically by the method of Ames and Dubin (4). Both in the presence and absence of magnesium ion, 97% of the pyrophosphate was eluted in the first 10 ml of 0.135 M LiCl; the remaining 3% was eluted with an additional 5 ml of the same solution. Both in the presence and absence of magnesium ion, 72% to 77% of the PP-ribose-P applied was retained on the column after elution with 20 ml of the lower ionic strength LiCl solution, and all the PP-ribose-P remaining was eluted with 5 ml of 0.5 M LiCl. The loss of PP-ribose-P was probably due to hydrolysis during the chromatographic procedure.

### III KINETICS OF ADENINE PHOSPHORIBOSYLTRANSFERASE

#### A. Introduction

##### 1. Uses of kinetic models

An enzyme-catalyzed reaction may be divided into three main phases: the formation of enzyme-substrate complexes; a rearrangement of these complexes to form enzyme-product complexes; and the dissociation of the latter to yield products and free enzyme. A model describing at least the first and third of these phases can be deduced from kinetic analyses of the reaction by (a) initial velocity measurements in the absence of products; (b) initial velocity measurements in the presence of products; and (c) measurements of the initial velocities of isotope exchanges. The rate constants of the individual steps in the reaction can sometimes be calculated if it is possible to study the reaction from both directions, and if both the concentration of the enzyme and the number of active centres per enzyme molecule are known. It is more common for kinetic analyses to be performed on only partially purified enzymes for which the molecular weight, enzyme concentration, and number of active centres per molecule, may not be known. In such cases only kinetic constants such as Michaelis and dissociation or inhibition constants for the substrates and products, the apparent maximum velocity, and the equilibrium constant, can be determined. Each of these is a function of two or

more individual rate constants. If the enzymatic reaction under study is irreversible, even some of the kinetic constants can only be determined with difficulty.

Information gained from kinetic analysis of an enzyme reaction may be used in several ways. In favorable cases the rate-limiting step in the reaction may be identified, and the presence of a covalently-linked enzyme-substrate intermediate may be suggested. When the enzyme under investigation is one of a series in a pathway it may be possible to predict from its equilibrium constant the direction in which metabolites will flow along the pathway. The possibility that a particular enzyme is the rate-controlling step in the pathway may also be evaluated. It may be possible to extrapolate from the kinetic model and constants to a prediction of the behavior of the enzyme in the intact cell. This must be done with caution, however, because little is known about the kinetics of enzymes at their intracellular concentrations and at intracellular concentrations of substrates, products, activators and inhibitors. Kinetic studies in vitro may be of use for predicting and understanding the actions of antimetabolites or other drugs in vivo, although the physiological disposition and metabolism of the drugs must of course be taken into account. Finally, these results may aid in the understanding of other enzymes which catalyze similar reactions.

Kinetic models do, of course, have their limitations. They are based on rate equation, and entirely different enzyme mechanisms can sometimes lead to the same rate equations. Possible models may therefore be excluded by the use of these equations, but the correctness of a model cannot be proven conclusively. A kinetic model also gives little insight into the actual chemical mechanism of the reaction, and gives no information as to the function of the protein as a catalyst. Whether the protein simply improves collision probabilities between reactants by binding them, or promotes the reaction by altering the electronic nature of the substrates, or distorts substrate bonds by undergoing conformational changes subsequent to their binding, or a combination of these, is not answered.

## 2. Early studies on the kinetic mechanism of adenine phosphoribosyltransferase

The early studies by Kornberg, Lieberman and Simms (79) established that the adenine phosphoribosyltransferase from yeast did not catalyze isotope exchange between adenine and AMP unless pyrophosphate was present, and that the reaction was essentially irreversible. On the basis of these data Imsande and Handler (67) proposed that the mechanism of the yeast enzyme involved a ternary enzyme-PP-ribose-P-adenine complex. More recently, Hori and Henderson (64) proposed on the basis of initial velocity plots and

some preliminary product inhibition studies that only binary enzyme-substrate complexes were involved. Dean, Watts and Westwick (31), who partially purified adenine phosphoribosyltransferase from human erythrocytes, also proposed a binary complex type of mechanism on the basis of initial velocity data, and Henderson et al. (60) in 1968 obtained similar initial velocity plots for mutant adenine phosphoribosyltransferases from human erythrocytes.

Studies of quinclinate phosphoribosyltransferase by Packman and Jacoby (104,105) produced initial velocity data which were compatible with either ternary enzyme-substrate complexes or with binary complexes, depending on the conditions of the experiment and on the enzyme preparation used. Results of isotope exchange experiments were compatible only with the involvement of ternary enzyme-substrate intermediates. These data therefore raised the possibility that the apparent kinetic mechanism of adenine phosphoribosyltransferase might also change from one set of experimental conditions to another.

In order to clarify these apparent contradictions and inconsistencies and to establish a more certain kinetic model for the adenine phosphoribosyltransferase of Ehrlich ascites carcinoma cells, more detailed initial velocity, product inhibition, isotope exchange and substrate binding experiments were undertaken.

## B. Initial velocity studies

In initial velocity experiments the initial rate of the reaction is measured when the concentration of one substrate is varied at several fixed levels of the other substrate in the absence of added products. Initial rates are measured to avoid inhibition by products that would accumulate if the reaction proceeded for a longer period of time; to avoid depletion of the substrates, since the steady state rate equations employed in these kinetic analyses assume that substrate concentrations do not change; and to ensure that neither the enzyme nor the substrates undergo inactivation or degradation due to instability at the pH, ionic strength and temperature of the reaction.

For bireactant enzyme reactions three basic mechanisms are possible; these are shown schematically in Figure 5. The terms "ping pong bi bi", "ordered bi bi" and "random bi bi" were introduced by Cleland in 1963 (26) and will be used throughout. In this discussion A and B will be called the first and second substrates, respectively, and P and Q will be called the first and second products, respectively. The kinetic data of Hori and Henderson (64) are not compatible with the random bi bi mechanism, nor are studies of substrate binding by Hori and Henderson (64) and Murray and Wong (103). These workers have shown by studies of the effects of ligands on rates of heat inactivation, that

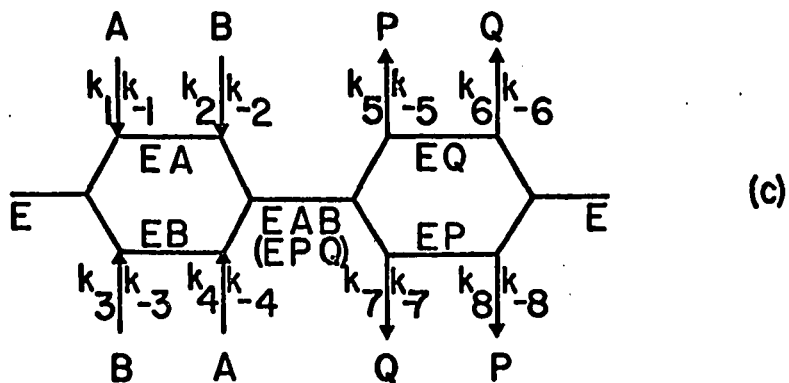
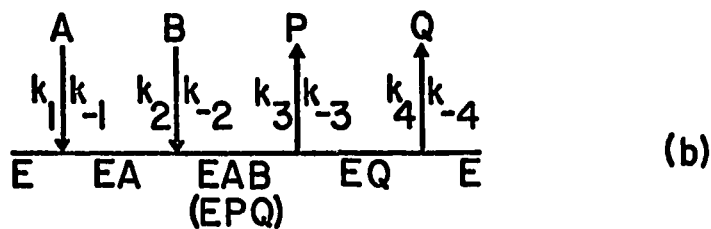
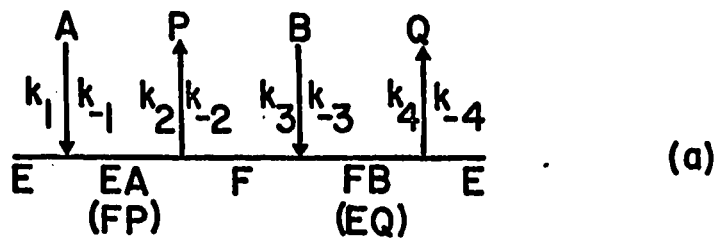


Figure 5. Kinetic models for two-substrate reactions. (a), ping pong bi bi mechanism; (b), ordered bi bi mechanism; and (c), random bi bi mechanism.

PP-ribose-P and AMP bind to free enzyme but that adenine does not do so. Stonehill and Balis (122) showed that adenine-<sup>14</sup>C did not bind to the adenine phosphoribosyltransferase of Escherichia coli. These results not only rule out the random bi bi model, but establish PP-ribose-P as A, adenine as B, pyrophosphate as P and AMP as Q; these indentifications are supported by the product inhibition studies reported by Hori and Henderson (64).

The steady state rate equations for the ordered bi bi and ping pong bi bi mechanisms in the absence of products were derived by the methods of King and Altman (73) and of Cleland (26), and are given as Equations (1) and (3), respectively. Equations (2a) and (4a) give the double reciprocal forms of these rate equations when substrate A (PP-ribose-P) is varied at fixed levels of substrate B (adenine). The first term in both equations defines the slope, while the second term defines the intercept. Note that in the ordered bi bi mechanism both the slope and the intercept terms change with variation of the second substrate, B, whereas in the ping pong bi bi mechanism only the intercept term changes with variation in the second substrate. A pattern of lines intersecting to the left of the vertical axis is therefore to be expected for sequential (i.e., random or ordered bi bi) mechanisms when reciprocal initial velocities are plotted against reciprocal substrate concen-



Rate Equations for Initial Velocity of Bi Bi Mechanisms.

Ping Pong Bi Bi Mechanism

$$\frac{v}{E_0} = \frac{V_1^{AB}}{K_A^B + K_B^A + AB} \quad (1)$$

$$\frac{E_0}{v} = \frac{K_A}{V_1^A} + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (2a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1^B} + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (2b)$$

Ordered Bi Bi Mechanism

$$\frac{v}{E_0} = \frac{V_1^{AB}}{K_{iA}K_B + K_A^B + K_B^A + AB} \quad (3)$$

$$\frac{E_0}{v} = \frac{K_A}{V_1^A} \left( 1 + \frac{K_{iA}K_B}{K_A^B} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (4a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1^B} \left( 1 + \frac{K_{iA}}{A} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (4b)$$

trations, whereas a pattern of parallel lines is to be expected for ping pong bi bi mechanisms. Equations (2b) and (4b) give the double reciprocal forms of the rate equations when the second substrate, B, is varied at fixed levels of the first substrate, A. In this situation the slope term for a sequential mechanism is again a function of the fixed substrate while for a ping pong bi bi mechanism the slope term is independent of this substrate. Again, a pattern of intersecting lines is to be expected for sequential mechanisms while a pattern of parallel lines is to be expected for ping pong mechanisms.

On the basis of the pattern of lines obtained in double reciprocal plots, therefore, it should be possible to make a distinction between sequential and ping pong mechanisms. It has not always been recognized, however, that it may be difficult experimentally to distinguish between lines which really are parallel and those which deviate only slightly from parallel. Lines whose slopes differ from each other by 10% or less cannot be distinguished from parallel by the graphical methods used in this study. This figure approaches the statistical variation between replicate analyses encountered in the methods used here. Even if these difficulties could be resolved, however, another problem remains. The terms which define the slope in the ordered bi bi mechanism are  $\frac{K_{iA}K_B}{K_A} + 1$ , when A is the varied substrate, and

$\frac{K_{iA}}{A} + 1$ , when B is the varied substrate. If  $\frac{K_{iA}K_B}{K_A}$  and  $\frac{K_{iA}}{A}$  have values of less than 0.1 at the lowest concentrations of A and B used, there would be a difference of less than 10% between the slopes of any of the two lines when the second substrates were changed. Such a small difference would not be detected by the usual graphical method of analysis. It will be shown later that this condition does in fact exist.

Double reciprocal plots of initial velocity against substrate concentration were constructed from measurements of initial velocity, as described above, at pH values between 5.5 and 11.0. In every case patterns of apparently parallel lines were obtained. Figures 6 and 7 are examples of these data, obtained at pH 6.9. The double reciprocal plots shown in Figure 6 are similar to those observed at pH 7.4 by Hori and Henderson (64) with the same enzyme, those of Dean, Watts and Westwick (31) with the enzyme from human erythrocytes, and those of Henderson et al. (60) with mutant enzymes from human erythrocytes. The enzyme mechanism must therefore be either ping pong bi bi or ordered bi bi with a slope term which deviates very little from unity.

### C. Product inhibition studies

In product inhibition experiments initial velocities are measured when the concentration of one substrate is varied at several fixed levels of one of the products while the concentration of the other substrate is held constant.

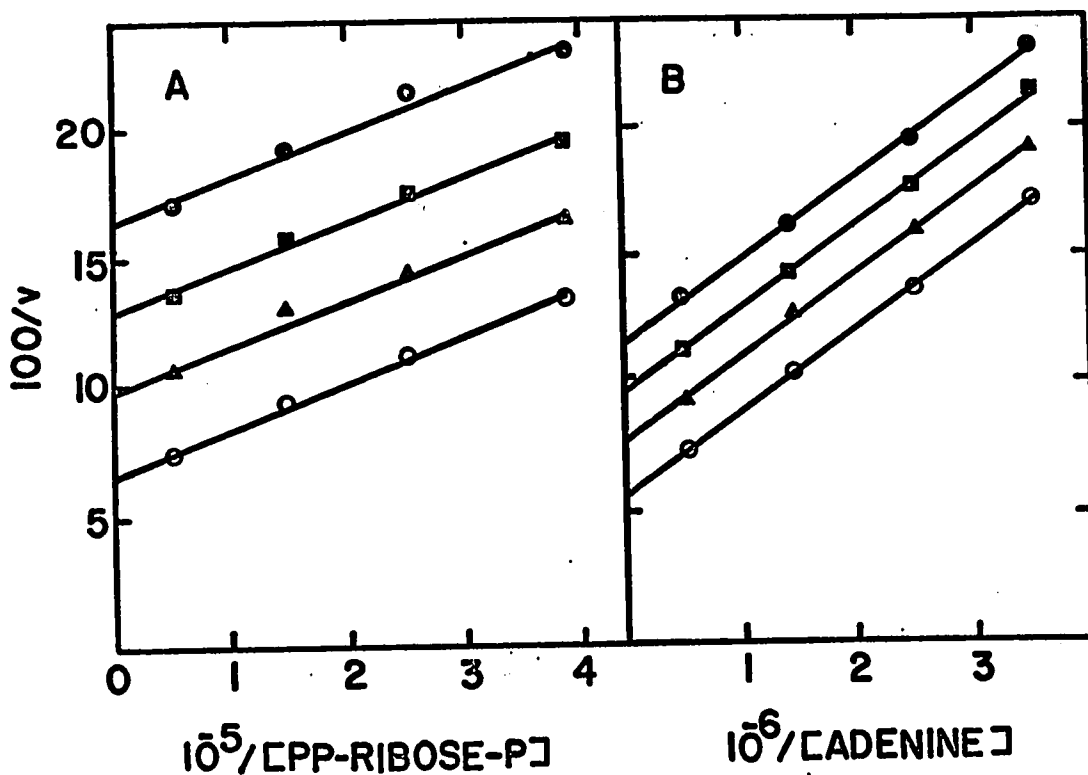


Figure 6. Effects of substrate concentrations on initial velocity. A, double reciprocal plot of initial velocity against PP-ribose-P concentration. Adenine concentrations were held constant at  $2.8 \times 10^{-7}$  M (●),  $4 \times 10^{-7}$  M (■),  $7 \times 10^{-7}$  M (▲), and  $2 \times 10^{-6}$  M (○). B, double reciprocal plot of initial velocity against adenine concentration. PP-ribose-P concentrations were held constant at  $2.8 \times 10^{-6}$  M (●),  $4 \times 10^{-6}$  M (■),  $7 \times 10^{-6}$  M (▲), and  $2 \times 10^{-5}$  M (○).  $v$  is expressed as micromicromoles of AMP per min.

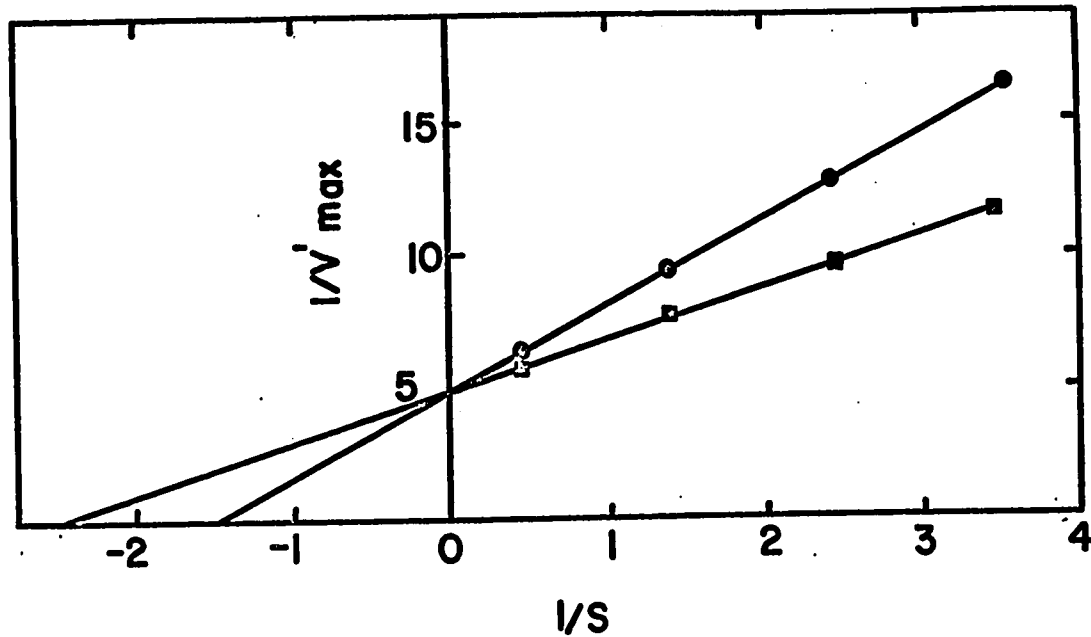


Figure 7. Secondary plot of reciprocals of apparent maximum velocities obtained from the ordinates of Figure 6 against reciprocals of molar concentrations ( $1/S$ ) of PP-ribose-P (■) and adenine (●). The units for PP-ribose-P concentration are  $10^{-5}$  M, and for adenine,  $10^{-6}$  M. The units of  $V'_{max}$  are micromicromoles of AMP per min.

Because initial velocities are used in this type of experiment, products other than the one added to the reaction mixture are not present in significant concentrations. Double reciprocal plots of velocity versus concentration of variable-substrate are then examined to see whether the presence of the product altered the slope only (competitive inhibition), the vertical intercept only (uncompetitive inhibition), or both the slope and the vertical intercept (noncompetitive inhibition). The terminology used is that of Cleland (27).

The rate equation for inhibition by any product is obtained from the full rate equation for the entire mechanism, Equations (5) and (8), by setting the concentration of the product not under study equal to zero. The rate equations for both ping pong  $bi\ bi$  and ordered  $bi\ bi$  mechanisms and the double reciprocal forms of these rate equations with either product P present or product Q present are given on pages 35 and 36. Table II gives the inhibition patterns expected for random, ordered and ping pong  $bi\ bi$  mechanisms as well as the product inhibition patterns actually observed by Hori and Henderson (64) and in the present work.

Hori and Henderson (64) observed that inhibition by product Q was competitive with respect to substrate A at a concentration of substrate B equal to 10 times its Michaelis constant and was noncompetitive with respect to substrate B at a concentration of substrate A equal to its Michaelis

Rate Equations for Initial Velocity of Bi Bi Mechanisms  
with Products Present.

Ping Pong Bi Bi Mechanism

$$\frac{v}{E_0} = \frac{V_1 AB}{K_A B + K_B A + AB + \frac{K_{iA} K_B P}{K_{iP}} + \frac{K_{iB} K_A Q}{K_{iQ}} + \frac{K_{iA} K_B PQ}{K_{iP} K_Q}} \quad (5)$$

$$+ \frac{K_B AP}{K_{iP}} + \frac{K_A BQ}{K_{iQ}}$$

Product P Present

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left( 1 + \frac{K_{iA} K_B P}{K_A K_{iP} B} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} + \frac{K_B P}{K_{iP} B} \right) \quad (6a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{K_{iA} P}{K_{iP} A} + \frac{P}{K_{iP}} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (6b)$$

Product Q Present

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left( 1 + \frac{Q}{K_{iQ}} + \frac{K_{iB} Q}{K_{iQ} B} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (7a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{K_{iB} K_A Q}{K_B K_{iQ} A} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} + \frac{K_A Q}{K_{iQ} A} \right) \quad (7b)$$

Rate Equations for Initial Velocity of Bi Bi Mechanisms  
with Products Present.

Ordered Bi Bi Mechanism

$$\frac{v}{E_0} = \frac{V_1 AB}{K_{iA}K_B + K_A B + K_B A + AB + \frac{K_{iA}K_B K_Q P}{K_P K_{iQ}} + \frac{K_{iA}K_B Q}{K_{iQ}} + \frac{K_{iA}K_B P Q}{K_P K_{iQ}} + \frac{K_Q K_B A P}{K_P K_{iQ}} + \frac{K_A B Q}{K_{iQ}} + \frac{A B P}{K_{iP}} + \frac{K_{iA}K_B P Q}{K_{iB} K_P K_{iQ}}} \quad (8)$$

Product P Present

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left( 1 + \frac{K_{iA}K_B}{K_A B} + \frac{K_{iA}K_B K_Q P}{K_A K_P K_{iQ} B} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} + \frac{K_Q K_B P}{K_P K_{iQ} B} + \frac{P}{K_{iP}} \right) \quad (9a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{K_{iA}}{A} + \frac{K_{iA}K_Q P}{K_P K_{iQ} A} + \frac{K_Q P}{K_P K_{iQ}} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} + \frac{P}{K_{iP}} \right) \quad (9b)$$

Product Q Present

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left( 1 + \frac{K_{iA}K_B}{K_A B} + \frac{Q}{K_{iQ}} + \frac{K_{iA}K_B Q}{K_A K_{iQ} B} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (10a)$$

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{K_{iA}}{K} + \frac{K_{iA}Q}{K_{iQ} A} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} + \frac{K_A Q}{K_{iQ} A} \right) \quad (10b)$$



TABLE II: Product Inhibition Patterns for Bi Bi Mechanisms

Mechanism	Inhibitory Product	Variable Substrate			
		A (PP-ribose-P)		B (Adenine)	
		<u>Low B</u>	<u>Sat. B</u>	<u>Low A</u>	<u>Sat. A</u>
Random Bi Bi	P or Q	NC	NC	NC	NC
Ordered Bi Bi	P	NC	UC	NC	NC
	Q	C	C	NC	-
Ping Pong Bi Bi	P	NC	-	C	C
	Q	C	C	NC	-
Observed Product Inhibition Pattern for Adenine Phosphoribosyltransferase	P	NC(a)	NC	NC(a)	NC
	Q		C(a)	NC(a)	

(a) Hori and Henderson (64).

The abbreviations used are: C, competitive; UC, uncompetitive; NC, noncompetitive; -, no inhibition; A, PP-ribose-P; B, adenine; P, pyrophosphate; Q, AMP.

constant. These observations were used to exclude a random bi bi mechanism since inhibition by Q would be expected to be noncompetitive with respect to both substrates. However, product inhibition by Q will not distinguish between an ordered bi bi mechanism and a ping pong bi bi mechanism. In addition, Hori and Henderson (64) observed that product P was a noncompetitive inhibitor with respect to substrate A and to substrate B at non-saturating levels of the non-varied substrates. These observations are not expected for a ping pong bi bi mechanism, and it was therefore suggested that in addition to acting as a product inhibitor, P also acts as a "dead-end" inhibitor with enzyme form E-Q. A "dead-end" inhibitor is defined as a compound which can reversibly combine with one or more enzyme forms to give an enzyme-inhibitor complex which cannot undergo further conversion to other enzyme forms in the reaction sequence. Products binding to enzyme forms other than those to which they normally bind act both as product and "dead-end" inhibitors.

The most important experiments for deciding between ping pong and sequential mechanisms are the use of pyrophosphate as product inhibitor when substrate A is varied at saturating levels of substrate B, and when substrate B is varied at saturating levels of substrate A. In the former case there should be no inhibition for a ping pong mechanism, whereas the inhibition should be uncompetitive

for an ordered bi bi mechanism. When substrate B is varied at saturating levels of substrate A the inhibition should be competitive for a ping pong bi bi mechanism and noncompetitive for an ordered bi bi mechanism. These experiments had not been done by earlier workers.

Figure 8 shows that pyrophosphate was a noncompetitive inhibitor with respect both to PP-ribose-P when the concentration of adenine was 100 times its Michaelis constant, and to adenine when the concentration of PP-ribose-P was 50 times its Michaelis constant. The product inhibition patterns observed in these experiments are not compatible with those expected (Table II) for a ping pong bi bi kinetic model, but more closely resemble those expected for an ordered bi bi mechanism. Inhibition by pyrophosphate was found to be noncompetitive with respect to substrate A (PP-ribose-P) at saturating levels of substrate B (adenine), although the kinetic expressions predict that this inhibition should be uncompetitive. Further studies were undertaken to examine this discrepancy.

#### D. Isotope exchange studies

Isotope exchange studies are one of the most useful techniques for selecting a kinetic model. The quantitative use of this method was first proposed by Boyer (16,17) in 1959 but only since the introduction by Cleland (25), in 1967, of relatively simple methods for deriving the

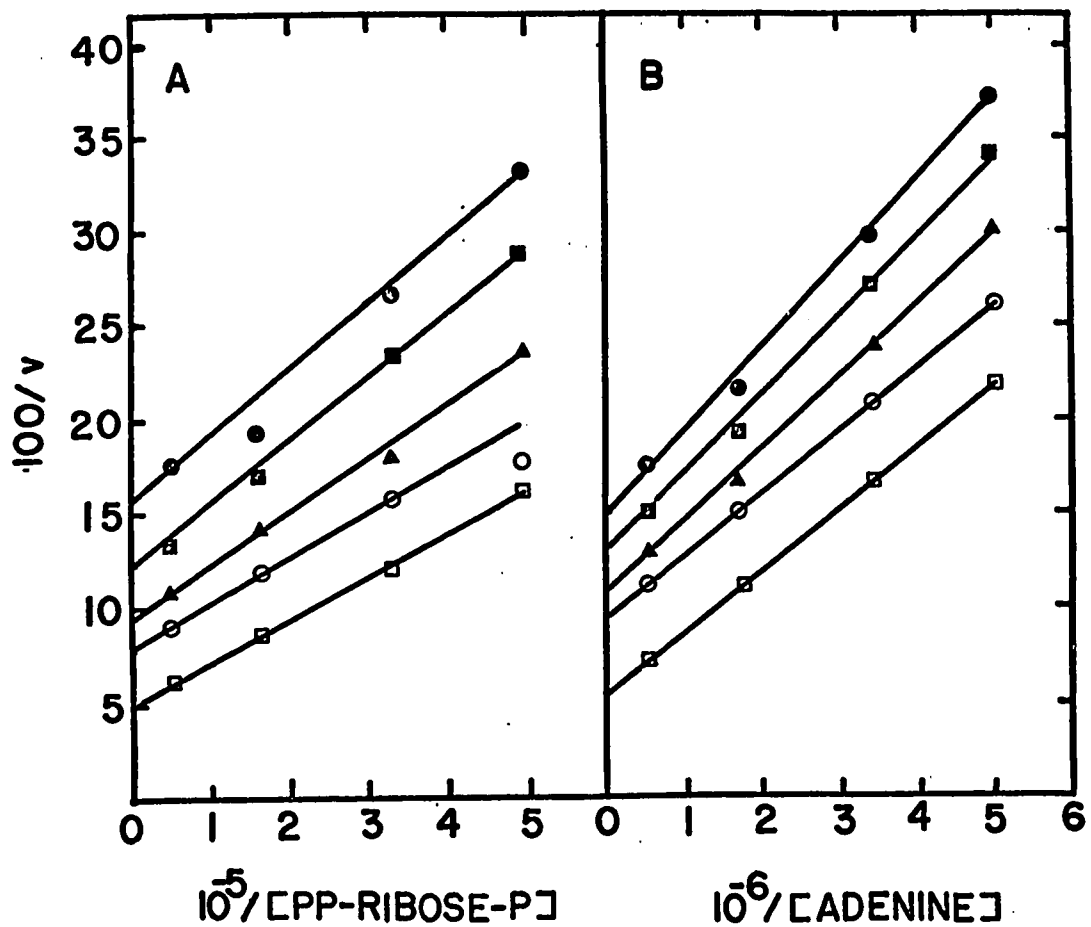


Figure 8. Product inhibition by pyrophosphate. A, double reciprocal plot of initial velocity against PP-ribose-P concentration at an adenine concentration of  $10^{-4}$  M. The concentrations of pyrophosphate are: 0 ( $\square$ ),  $10^{-4}$  M ( $\circ$ ),  $4 \times 10^{-4}$  M ( $\triangle$ ),  $7 \times 10^{-4}$  M ( $\blacksquare$ ), and  $3 \times 10^{-4}$  M ( $\bullet$ ). B, double reciprocal plot of initial velocity against adenine concentration at a PP-ribose-P concentration of  $s \times 10^{-4}$  M. In each case an amount of magnesium ion equal to that of pyrophosphate was added in addition to that normally present during assay.  $v$  is expressed as micromicromoles of AMP per min.

appropriate equations has this type of kinetic experiment become more widely used. The rate equations for isotope exchange reactions for both ordered bi bi and ping pong bi bi mechanisms are given on page 42. Equation (11) is the rate equation for isotope exchange between substrate A and product P, and Equation (12) is the rate equation for isotope exchange between substrate B and product Q; both are for a ping pong bi bi mechanism. For either reaction isotope exchange occurs independently of the second substrate or second product. Equation (13) is the rate equation for isotope exchange between substrate B and product Q for an ordered bi bi mechanism. Because the equation is of the form 
$$\frac{aBPQ}{b(B+Q+BQ) + cP(B+Q+BQ) + dP^2(Q + BQ)}$$
, it is apparent that no exchange will take place in the absence of product P. The rate of exchange will furthermore increase to a maximum as P increases, but will then go to zero at infinite concentrations of product P. A similar equation can be derived for isotope exchange between substrate A and product P. In this case substrate B must be present for isotope exchange to occur, and at infinite concentrations of substrate B the rate of isotope exchange will go to zero. For random bi bi mechanisms complete inhibition of the rate of isotope exchange between substrate B and product Q by product P would not be observed, and in fact some isotope

Rate Equations for Isotope Exchange Reactions.

Ping Pong Bi Bi Mechanism

Exchange between A and P

$$\frac{v}{E_0} = \frac{V_{1AP}}{\frac{K_A K_{iP}^A}{K_{iA}} + K_{AP} + \frac{K_{AP}}{K_{iA}}} \quad (11)$$

Exchange between B and Q

$$\frac{v}{E_0} = \frac{V_{2BQ}}{\frac{K_Q K_{iB}^Q}{K_{iQ}} + K_{QB} + \frac{K_{QB}}{K_{iQ}}} \quad (12)$$

Ordered Bi Bi Mechanism : Exchange Between B and Q

$$\frac{v}{E_0} = \frac{V_{1BPQ}}{\frac{K_{iA} K_B K_{Eq}}{K_A} \left[ K_{iA} K_B + K_{AB} + \frac{K_{ABQ}}{K_{iQ}} + \frac{K_{iA} K_B Q}{K_{iQ}} \right] + \frac{P K_{iA} K_B K_{Eq}}{K_A K_P K_{iQ}}} \quad (13)$$

$$\left[ 2K_{iA} K_B K_Q + K_A K_Q B + \left( \frac{K_{iA} K_B}{K_{iB}} + \frac{K_A K_Q}{K_{iQ}} \right) BQ + K_{iA} K_B \left( 1 + \frac{K_Q}{K_{iQ}} \right) Q \right]$$

$$+ \frac{P^2 K_{iA} K_{iA} K_B K_B K_Q K_{Eq}}{K_A K_P K_P K_{iQ} K_{iQ}} \left[ K_Q + Q + BQ \right]$$

exchange should be observed in the absence of product P (20). For random bi bi mechanisms similar patterns should also be observed for isotope exchange between substrate A and product P. As a consequence of this, isotope exchange studies may be used to detect a small degree of randomness in an apparently ordered mechanism. As much as 10% randomness in a predominantly ordered reaction would be difficult to detect by initial velocity studies.

Isotope exchange studies were carried out by varying the concentration of product P (pyrophosphate) from  $10^{-5}$  M to  $10^{-2}$  M at constant concentrations of substrate B (adenine- $^{14}\text{C}$ ) and product Q (AMP). For pyrophosphate concentrations between  $10^{-4}$  M and  $10^{-2}$  M an amount of magnesium ion equal to that of the pyrophosphate was added in addition to that normally present during the assay. The reaction mixtures were incubated for 12 minutes, the incubations terminated by the addition of water-saturated 1-butanol, and unreacted adenine was removed as described previously. Figure 9 shows the rate of isotope exchange between adenine- $^{14}\text{C}$  and AMP as a function of pyrophosphate concentration. No isotope exchange was observed in the absence of pyrophosphate, but as the concentration of pyrophosphate was increased, the rate of exchange reached a maximum and then decreased. Because the magnesium salt of pyrophosphate began to precipitate at a concentration of  $2 \times 10^{-2}$  M, it was not possible to

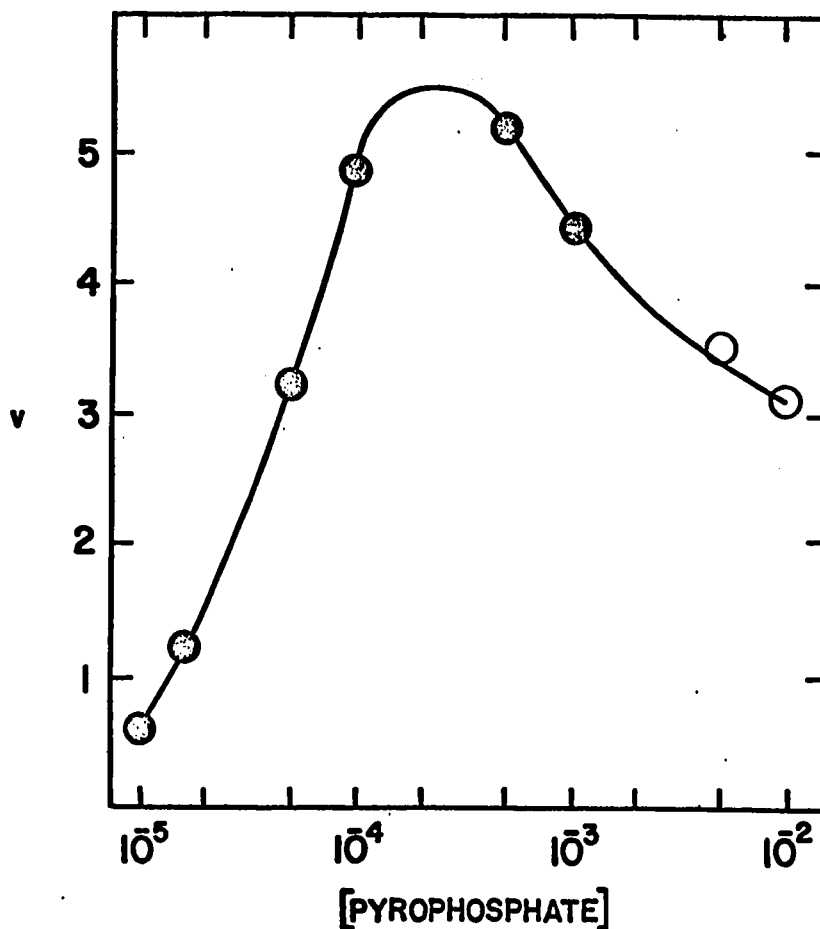


Figure 9. Isotope exchange between adenine-<sup>14</sup>C and AMP. Plot of initial velocity against pyrophosphate concentration at an adenine-<sup>14</sup>C concentration of  $10^{-6}$  M and AMP concentration of  $10^{-5}$  M. The concentrations of pyrophosphate at which the magnesium salt of pyrophosphate began to precipitate out of solution are indicated by open circles. From  $5 \times 10^{-3}$  M to  $10^{-2}$  M pyrophosphate an amount of magnesium ion equal to that of pyrophosphate was added in addition to that normally present during assay. v is expressed as micromicromoles of AMP per min.



determine if the exchange rate went to zero at very high concentrations of pyrophosphate. Although it was therefore not possible to see if there was a small degree of randomness in the reaction, these data are consistent only with a reaction mechanism which is predominantly, if not solely, ordered.

E. Reversal of the reaction: initial velocity and isotope exchange studies

The reverse reaction was assayed by following the conversion of AMP-<sup>14</sup>C and pyrophosphate to adenine-<sup>14</sup>C and PP-ribose-P. In this assay 6 µg of enzyme preparation was used per reaction mixture in an effort to obtain a measurable velocity. The concentration of pyrophosphate was varied from  $5 \times 10^{-5}$  M to  $10^{-3}$  M, the concentration of MgSO<sub>4</sub> was  $2 \times 10^{-3}$  M to  $5 \times 10^{-4}$  M. The reaction mixtures were incubated for 30 minutes at 30° and adenine-<sup>14</sup>C measured as described previously.

The rate of isotope exchange between pyrophosphate and PP-ribose-P was measured by following the rate of incorporation of pyrophosphate-<sup>32</sup>P into PP-ribose-P. In this assay 4 µg of the enzyme preparation was used per reaction mixture, the concentrations of pyrophosphate and PP-ribose-P were held constant at  $10^{-3}$  M and  $2 \times 10^{-5}$  M, respectively, and the concentration of adenine was varied up to 1 mM. The reaction mixtures were incubated at 30° for various times from 20 minutes to 100 minutes, and <sup>32</sup>P in PP-ribose-P measured as

described earlier.

Table IV gives the maximum velocities for the forward and reverse reactions, and the maximum rates of isotope exchange between adenine and AMP in the presence of pyrophosphate, and between pyrophosphate and PP-ribose-P in the presence of adenine. An upper limit is given for both the reverse reaction and the isotope exchange between pyrophosphate and PP-ribose-P because the actual rates were at or below the limits of detection of the methods used. Because (a) no reverse reaction could be detected, (b) no isotope exchange could be detected between pyrophosphate and PP-ribose-P, (c) isotope exchange between adenine and AMP was readily observed, and (d) the rate of the forward reaction was readily observable, the rate constant  $k_{-1}$  for mechanism I in Figure 10 must be very small compared to the other rate constants. If such is the case, the dissociation constant  $K_{iA}$ , ( $k_{-1}/k_1$ ), would also probably be very small.  $K_{iA}$  appears in the term in the rate equations for ordered bi bi reactions which determine the deviation from parallel of lines in double reciprocal plots. If it is sufficiently small, this deviation might be undetectable.

#### F. Substrate dissociation constants.

Because the adenine phosphoribosyltransferase reaction is not really reversible, substrate dissociation constants for PP-ribose-P and adenine could not be obtained

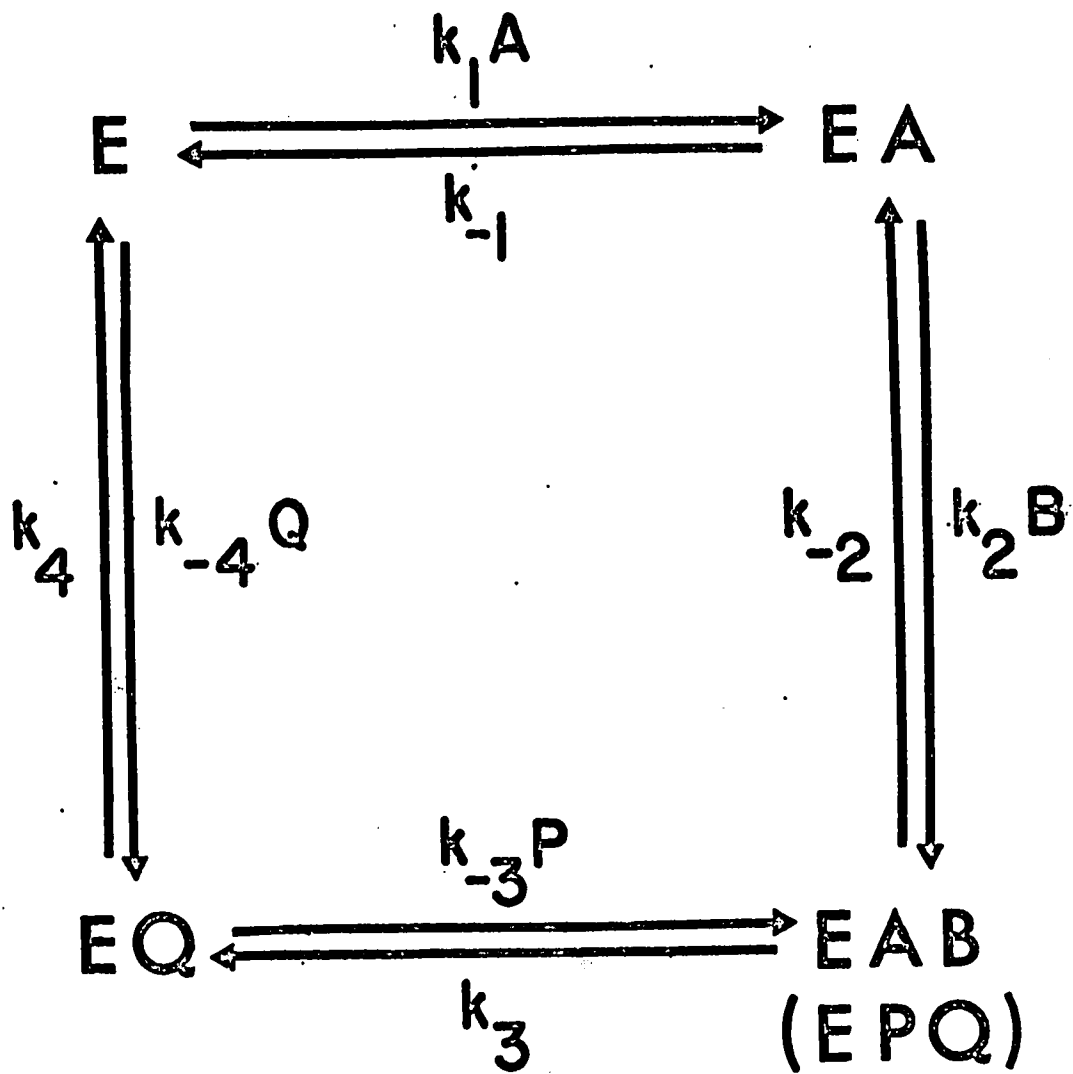


Figure 10. Kinetic Mechanism I.

by using these substrates as product inhibitors in the reverse reaction. In 1964 Mildvan and Leigh (100) proposed an alternative method for the determination of the dissociation constants of enzyme-substrate complexes which depends on the alteration by a substrate of the rate of inactivation of the enzyme by an irreversible inhibitor. The major advantage of this method is that a highly purified enzyme is not required provided that the contaminants present do not alter the rate of the enzyme-catalyzed reaction and do not remove appreciable quantities of the inhibitor. Adenine phosphoribosyltransferase is particularly suited to this method since the enzyme is readily inactivated by *p*-hydroxymercuribenzoate (PMB), and this inactivation is retarded in the presence of PP-ribose-P.

The rate equations for inactivation by PMB are given on page 49. Provided that the concentration of PMB is so much greater than the concentration of enzyme that it does not change significantly during the incubation, the apparent rate of enzyme inactivation may be represented by a first-order expression, Equation (14). Although the concentration of groups in the reaction mixtures which may react with PMP are not known, for reasons discussed later it is believed that the concentration of PMB does not change significantly during the incubation. Equation (15) is the integrated form of this equation; it predicts that plots of  $\log [E]_0/[E]$

Rate Equations for Inactivation of Adenine Phosphoribosyltransferase by PMB.

$$-\frac{d[E]}{dt} = k_{app}[E][PMB] \quad (14)$$

$$\log \frac{[E]_0}{[E]} = \frac{k_{app}t}{2.3}[PMB] \quad (15)$$

$$k_{app} = \frac{kK_{iA}}{K_{iA} + [PP\text{-ribose-P}]} \quad (16)$$

$$\frac{1}{k_{app}} = \frac{1}{k} + \frac{[PP\text{-ribose-P}]}{kK_{iA}} \quad (17)$$

$[E]_0$  - total enzyme activity at  $t = 0$

$[E]$  - enzyme activity at  $t > 0$

$t$  - time (sec.)

$k$  - rate of enzyme inactivation ( $\text{mole}^{-1} \text{sec}^{-1}$ )

versus PMB concentration, at a constant time and at several concentrations of PP-ribose-P, are linear and pass through the origin. The apparent rate of inactivation,  $K_{app}$ , may be calculated from the slopes of these plots. The relationship between  $k_{app}$ , the concentration of PP-ribose-P, and the dissociation constant ( $K_{iA} = k_{-1}/k_1$ ) for PP-ribose-P is given in Equations (16) and (17). A plot of  $k_{app}^{-1}$  versus PP-ribose-P concentration gives, on the abscissa, the negative value of the dissociation constant,  $-K_{iA}$ , for PP-ribose-P

Figure 11 shows the plots of  $\log [E]_0/[E]$  versus PMB concentration at several concentrations of PP-ribose-P. It should be noted that there was a rapid initial rate of inactivation followed by a slower rate of inactivation. In the absence of added PP-ribose-P the rate constant for the rapid initial rate of inactivation was calculated to be  $8.82 \times 10^{-3} \text{ mole}^{-1} \text{ sec}^{-1}$ , and the rate constant for the slower rate of inactivation was calculated to be  $1.75 \times 10^{-3} \text{ mole}^{-1} \text{ sec}^{-1}$ . Figure 12 shows the replots of  $k_{app}$  versus PP-ribose-P concentration from both the rapid initial inactivation and the slower inactivation segments of the first-order plots. The dissociation constants calculated from these replots would be expected to be similar, and were calculated to be  $1.85 \times 10^{-7} \text{ M}$  and  $1.30 \times 10^{-7} \text{ M}$ , respectively. In a separate experiment a dissociation constant of  $1.80 \times 10^{-7} \text{ M}$  was obtained.

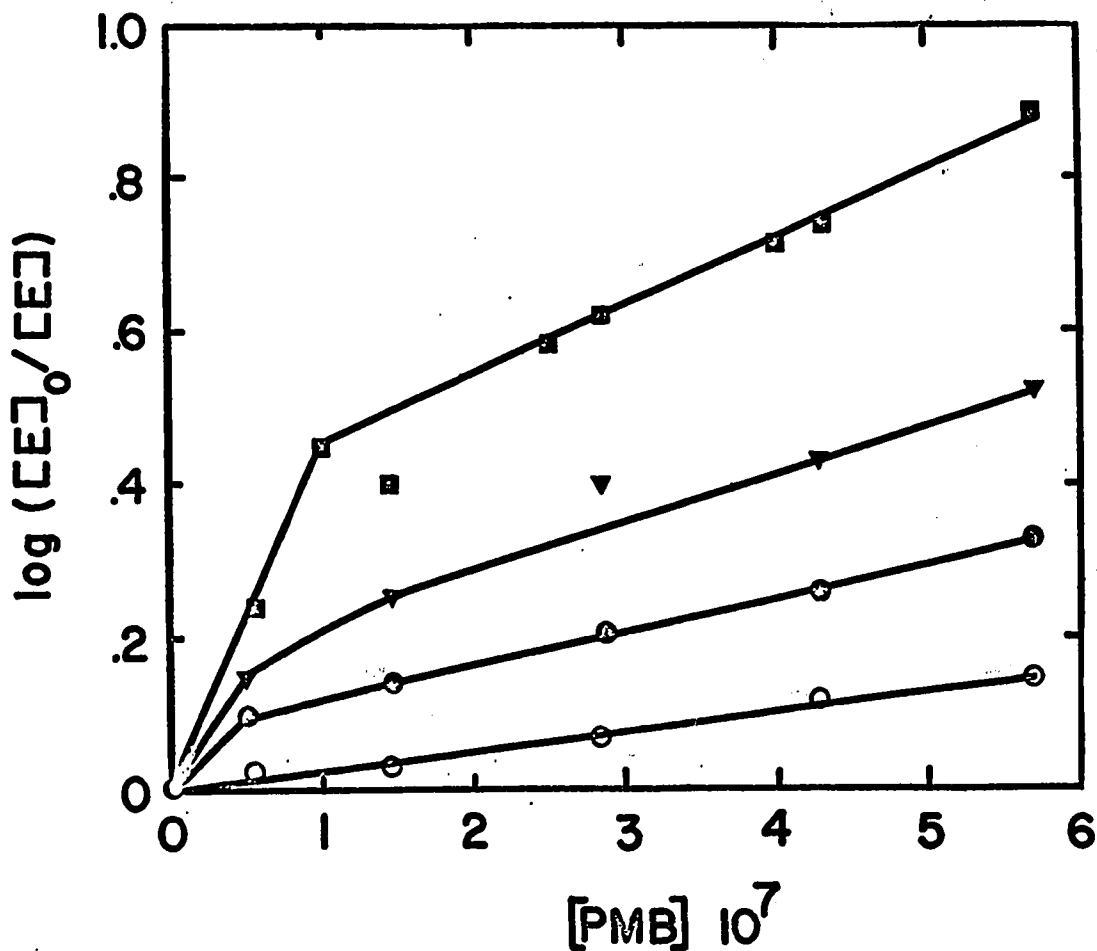


Figure 11. Enzyme inactivation by PMB. Plot of  $\log [E]_0/[E]$  against PMB concentration. The concentrations of PP-ribose-P are: 0 (■),  $8 \times 10^{-8}$  M (▼),  $2 \times 10^{-7}$  M (●), and  $3 \times 10^{-7}$  M (○). After a 20 min incubation the reaction mixtures were completed by the addition of  $10^{-4}$  M PP-ribose-P and  $2 \times 10^{-6}$  adenine- $^{14}$ C and assayed by the usual radioisotope method.

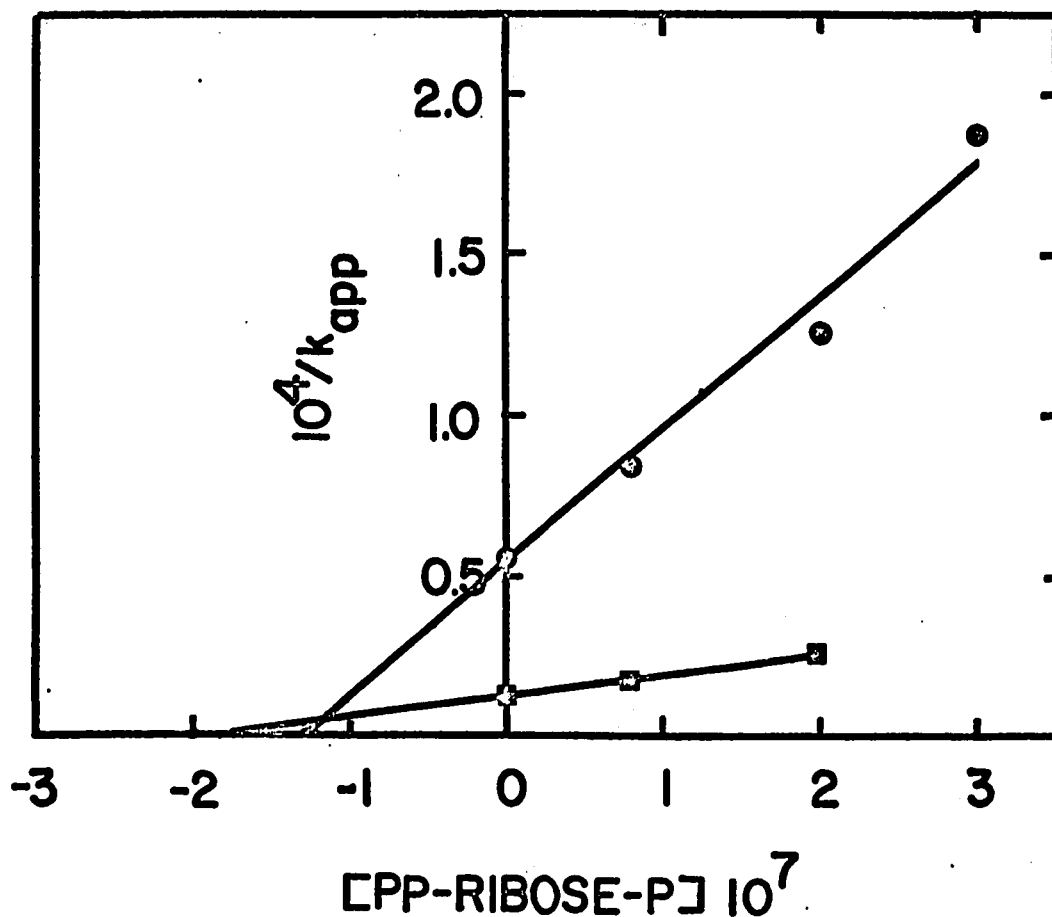


Figure 12. Secondary plots of the reciprocals of the apparent rates of inactivation obtained from the slopes of Figure 11 against molar concentrations of PP-ribose-P. The reciprocals of the apparent rates of inactivation obtained from the first portion of the slopes of Figure 11 are designated by (■) and those from the second portion of the slopes by (●).  $k_{app}$  is expressed as  $\text{moles}^{-1} \text{sec}^{-1}$ .



The biphasic character observed for plots of  $\log [E]_0 / [E]$  versus PMB concentration deviated from the expected result, and the following possibilities were considered:

(a) The concentration of PMB was limiting, and pseudo first-order inactivation was therefore not achieved. (b) The enzyme contains two sulfhydryl groups which are necessary for activity and which can be protected by the binding of PP-ribose-P to one catalytic site. They would have to react with PMB at different rates. (c) The enzyme contains two catalytic sites, each containing a sulfhydryl group which can be protected by PP-ribose-P. They again would have to react with PMB at different rates.

Possibility (a) can be readily ruled out because the rate of inactivation would be expected to be slow at low PMB concentrations and increase as the PMB concentration is increased, if the concentration of PMB were not sufficient to cause first-order inactivation kinetics. This was not observed. Possibility (c) is tentatively excluded on the grounds that no other evidence is available which suggests that this enzyme has multiple active sites. The interpretation favored is (b), that the enzyme contains one catalytic site and two sulfhydryl groups which have different rates of reaction. PP-ribose-P must prevent the reaction of PMB with them either sterically or by causing conformational changes in the enzyme which makes them unavailable

for reaction with PMB.

Frieden (42), in 1963, proposed a similar technique for the determination of substrate-enzyme dissociation constants which depends on the alteration by the substrate of the rate of enzyme inactivation by heat. He has shown that the thermal inactivation of glutamate dehydrogenase (42) is a first order process, and that for such a situation the apparent rate of inactivation,  $k_{app}$ , can be calculated from plots of the logarithm of enzyme velocity versus time of heating. As in the case of PMB inactivation,  $k_{app}$  is related to the dissociation constant ( $K_{iA}$ ) and the concentration of PP-ribose-P by Equation (17). This method was used in order to check the values obtained by the method of Mildvan and Leigh.

Adenine phosphoribosyltransferase was incubated at 37° for various lengths of time with and without added PP-ribose-P, and then assayed by the usual radioisotope method. Figure 13 is a plot of log velocity versus time and shows that the thermal denaturation of this enzyme consisted of two first-order processes. Because the thermal denaturation was initiated by transferring the reaction mixtures from an ice bath at 0° to a shaking water bath at 37°, the zero time cannot be accurately determined and consequently the rate of denaturation cannot be accurately calculated from the first portion of the curves in Figure 13. After 10 minutes, however, the plots are linear and it may be postulated that

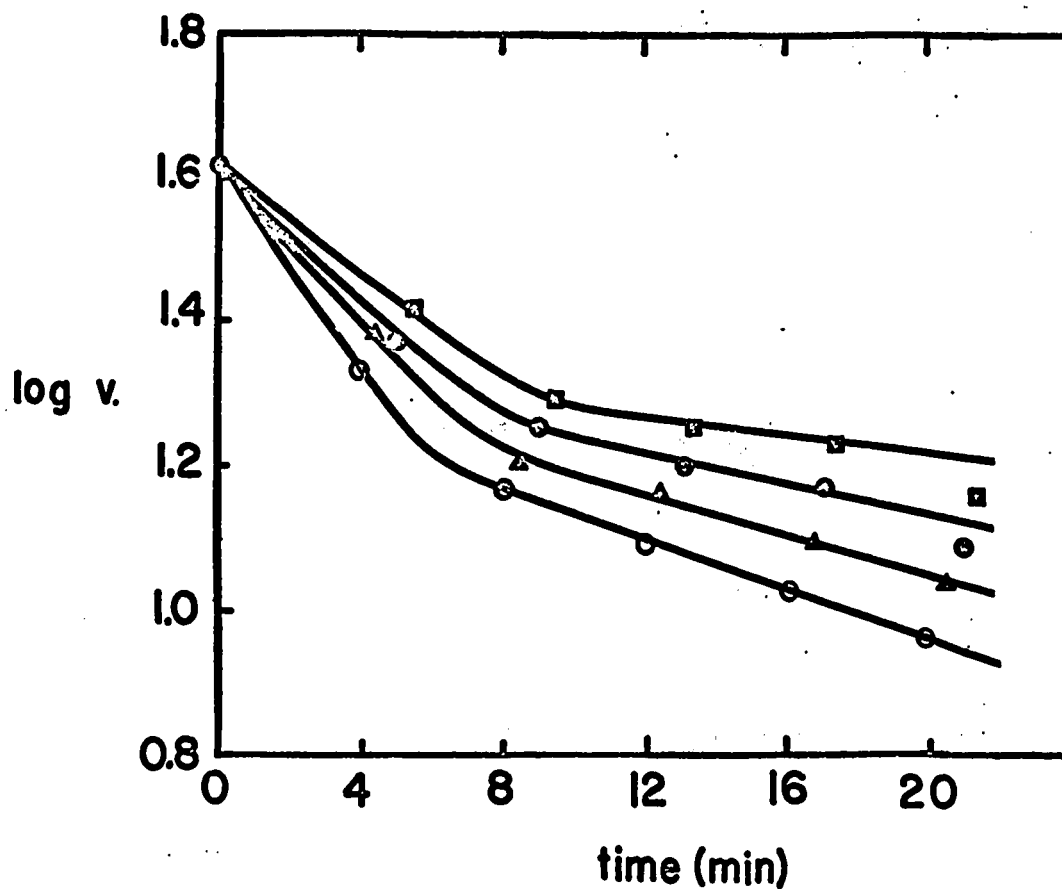


Figure 13. Enzyme inactivation by heat. Plot of log v against time of incubation (min) at 37°. The concentrations of PP-ribose-P are: 0 (O),  $1.7 \times 10^{-7}$  M ( $\Delta$ ),  $2.5 \times 10^{-7}$  M ( $\bullet$ ), and  $7.0 \times 10^{-7}$  M ( $\blacksquare$ ). The reaction mixtures were completed by the addition of  $10^{-5}$  M PP-ribose-P and  $10^{-6}$  M adenine-<sup>14</sup>C and assayed by the normal radioisotope method. v is expressed as micromicromoles of AMP per minute. 2.5  $\mu$ g of enzyme preparation were used per reaction mixture.

from 10 to 20 minutes thermal denaturation is a first order process. The apparent rates of inactivation were determined using this portion of the plots and from these data (Figure 14) the dissociation constant for PP-ribose-P was calculated to be  $3.8 \times 10^{-7}$  M. It is of interest to note that for both thermal and PMB inactivation a rapid initial rate of inactivation occurred followed by a slower rate of inactivation. At the present time the significance of this observation is not known.

Although the dissociation constant obtained by the method of Frieden is two-fold higher than that obtained by the method of Mildvan and Leigh, both values are much lower than the Michaelis constant for PP-ribose-P. A dissociation constant of  $2.2 \times 10^{-7}$  M for PP-ribose-P was obtained by averaging the three values obtained by PMB inactivation measurements and the one value obtained by those of thermal inactivation. The Michaelis constant for PP-ribose-P is  $6 \times 10^{-6}$  M and the ratio of  $K_{iA}$  to  $K_A$  is therefore 0.036. Because  $K_{iA}$  is much smaller than  $K_A$ , a change of only 6 to 7 per cent in the slopes of the initial velocity plots would occur and this would not be detected by graphical analysis.

Although it is not necessary to know the dissociation constant for the second substrate, adenine, in order to choose among the proposed kinetic models, this value was determined to make as complete as possible the list of

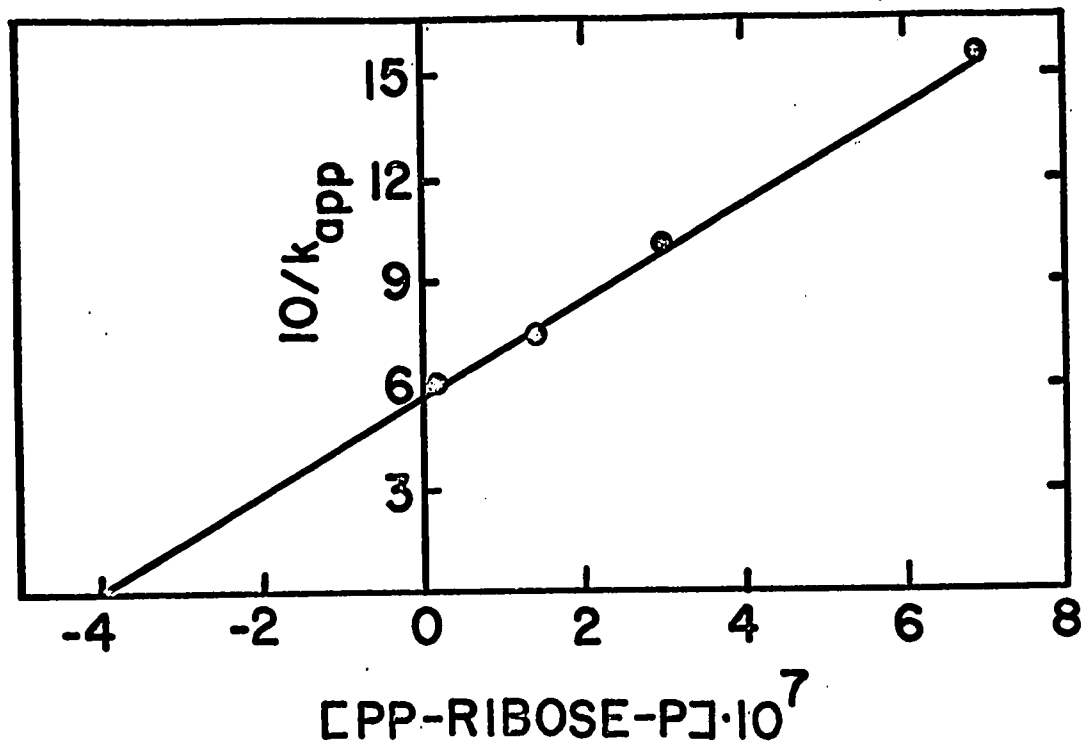


Figure 14. Secondary plot of the reciprocals of the apparent rates of inactivation obtained from the slopes of Figure 13 against molar concentrations of PP-ribose-P.  $k_{app}$  is expressed as  $\text{min}^{-1}$ .

kinetic constants known. The dissociation constant for adenine was determined by using this substrate as an inhibitor during the formation of 2,6-diaminopurine ribonucleotide from PP-ribose-P and the alternative substrate, 2,6-diaminopurine-<sup>14</sup>C. Webb (132) has shown that for competitive inhibition by alternative substrates the equations for normal competitive inhibition are applicable if only one of the reactions is determined. The rate equation (Equation 18-a) for competitive inhibitors of 2,6-diaminopurine (substrate C) may therefore be used, and the inhibition constant for adenine ( $K_{iiB}$ ) obtained merely by replotting slopes versus adenine concentration. At the concentration of PP-ribose-P used,  $K_{iA}/A$  approaches zero to give equation 18-b. The experimental results are shown in Figure 15, as is the replot of slopes. The inhibition constant for adenine was calculated to be  $1.1 \times 10^{-5}$  M, and the apparent Michaelis constant ( $K_C$ ) for 2,6-diaminopurine was  $2.5 \times 10^{-3}$  M; the latter is close to the value of  $1.8 \times 10^{-3}$  M obtained by Blair (13) for the L-cell enzyme. The inhibition constant for adenine,  $K_{iiB}$ , is equal to  $k_{-2}/k_2$ , and since  $k_{-1}$  is very small the inhibition constant for adenine would be approximately equal

$$\text{to } K_{iB}, \frac{k_{-1} + k_{-2}}{k_2}.$$

#### G. Binding of pyrophosphate to free enzyme

Hori and Henderson (64) found that pyrophosphate

Rate Equations for Initial Velocity with 2,6-Diamino-  
purine-<sup>14</sup>C as Second Substrate and Adenine as Inhibitor.

$$\frac{E_0}{v} = \frac{K_C}{V_1 C} \left( 1 + \frac{K_{iA}}{A} + \frac{B}{K_{iiB}} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (18a)$$

Since  $A = 350 K_{iA}$

$$\text{then } \frac{K_{iA}}{A} = \frac{1}{350} = 0.00285$$

$$\text{therefore } \frac{E_0}{v} = \frac{K_C}{V_1 C} \left( 1 + \frac{B}{K_{iiB}} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (18b)$$

C - 2,6-diaminopurine

$K_C$  - Michaelis constant for 2,6 -diaminopurine

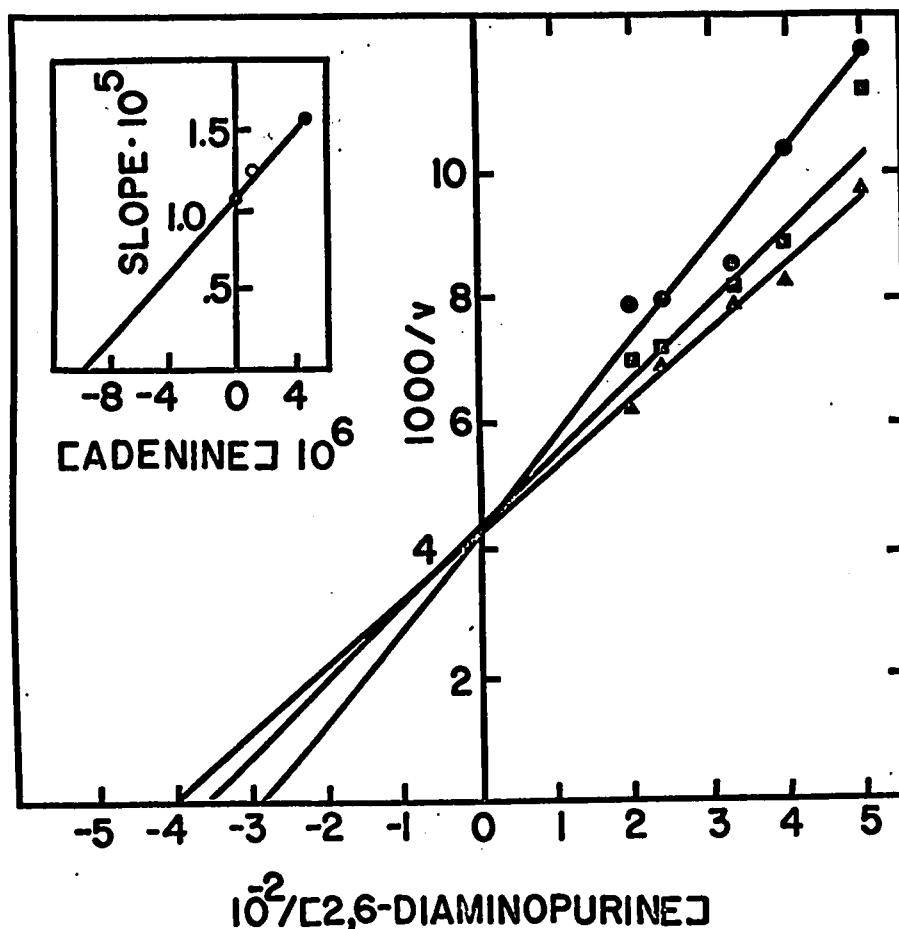


Figure 15. Inhibition by adenine of nucleotide synthesis from 2,6-diaminopurine. Double reciprocal plot of initial velocity against 2,6-diaminopurine concentration at a PP-ribose-P concentration of  $10^{-4}$  M. The concentrations of adenine are:  $10^{-6}$  M ( $\blacktriangle$ ),  $10^{-6}$  M ( $\blacksquare$ ), and  $5 \times 10^{-6}$  M ( $\bullet$ ).  $v$  is expressed as micromicromoles of 2,6-diaminopurine ribonucleotide per min. The insert represents the secondary plots of slopes against molar concentrations of adenine. The units of slope have no dimensions.



partially protected adenine phosphoribosyltransferase against inactivation by heating. The present study has found that this product will also decrease the rate of enzyme inactivation by PMB.  $10^{-3}$  M Pyrophosphate and  $10^{-5}$  M PP-ribose-P afforded 57 and 100 per cent protection, respectively, against inactivation by  $5 \times 10^{-6}$  M PMB. These results clearly demonstrate that pyrophosphate binds to the free enzyme as well as to the enzyme form E-Q; a kinetic mechanism based on these observations is shown in Figure 16. Although it cannot be unequivocally demonstrated that the binding to free enzyme is at the active site, data will be presented later which suggests that this is so.

The possibility was considered that pyrophosphate acted as a dead-end inhibitor binding to free enzyme and that this inhibition might explain the anomalous product inhibition data observed above. The double reciprocal form of the rate equation for an ordered bi bi mechanism with P acting as a dead-end inhibitor binding to free enzyme is given on page 63. The product inhibition patterns predicted by these equations are given in Table III together with those which were actually observed. The latter are consistent with the mechanism shown in Figure 16.

When substrate A is varied at saturating levels of substrate B, with P acting both as product and as dead-end inhibitor (Equation 20-a), the slopes are equal to

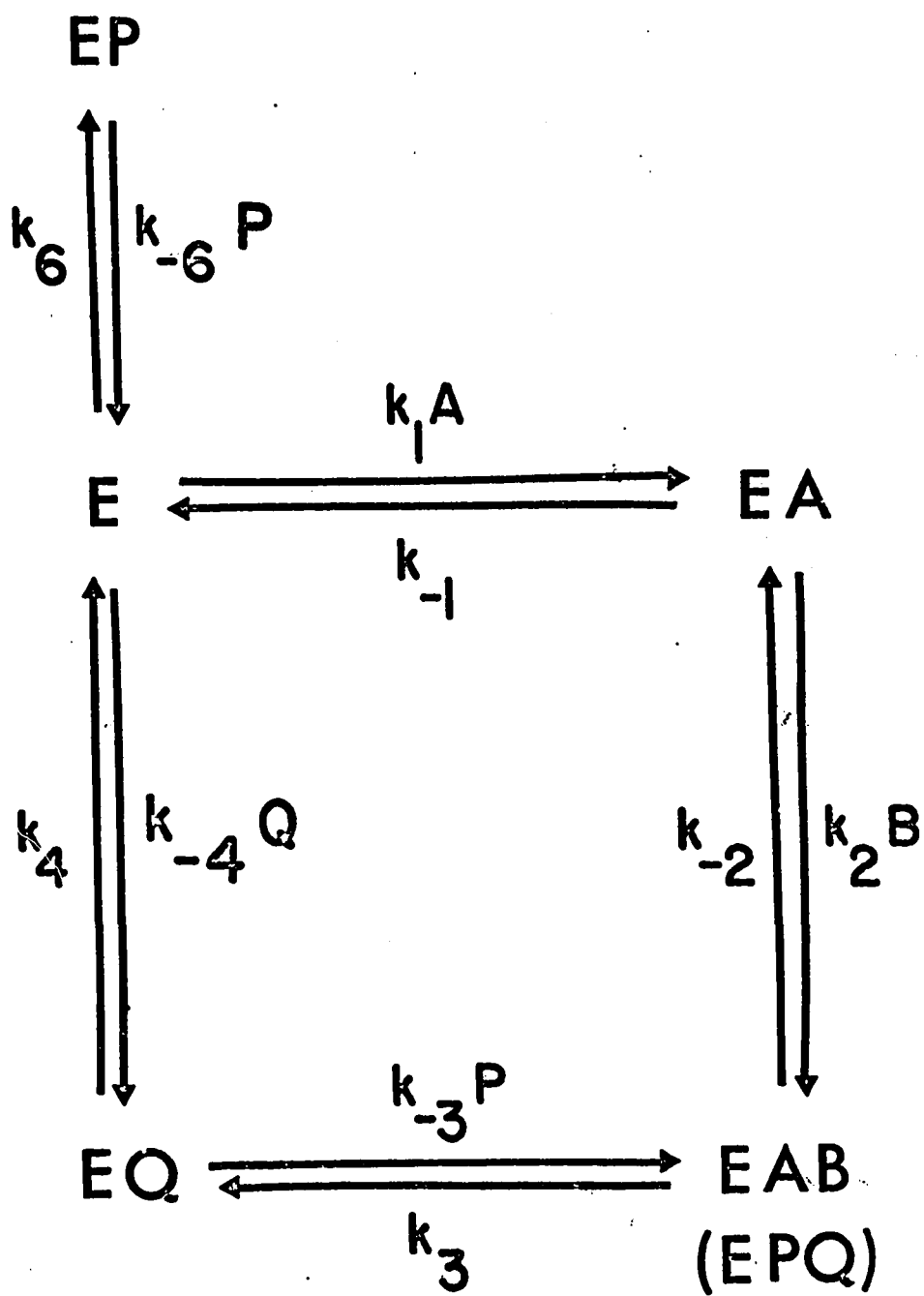


Figure 16. Kinetic mechanism II.

Rate Equations for Initial Velocity of Mechanism II.

(19)

$$\frac{v}{E_0} = \frac{V_1 AB}{K_{iA}K_B \left(1 + \frac{P}{K_{iiP}}\right) + K_A B \left(1 + \frac{P}{K_{iiP}}\right) + K_B A + AB + \frac{K_{iA}K_B K_Q P}{K_P K_{iQ}}}$$

$$\left(1 + \frac{P}{K_{iiP}}\right) + \frac{K_{iA}K_B Q}{K_{iQ}} + \frac{K_{iA}K_B P Q}{K_P K_{iQ}} + \frac{K_Q K_B A P}{K_P K_{iQ}} + \frac{K_A B Q}{K_{iQ}} + \frac{A B P}{K_{iP}}$$

$$+ \frac{K_{iA}K_B P Q}{K_{iB} K_P K_{iQ}}$$

Product P Present

Substrate A varied

(20a)

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left[ \frac{K_{iA}K_B}{K_A B} \left(1 + \frac{P}{K_{iiP}}\right) + 1 + \frac{P}{K_{iiP}} + \frac{K_{iA}K_B K_Q P}{K_A K_P K_{iQ} B} \left(1 + \frac{P}{K_{iiP}}\right) \right]$$

$$+ \frac{1}{V_1} \left[ 1 + \frac{K_B}{B} + \frac{K_Q K_B P}{K_P K_{iQ} B} + \frac{P}{K_{iP}} \right]$$

Substrate B varied

(20b)

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left[ \frac{K_{iA}}{A} \left(1 + \frac{P}{K_{iiP}}\right) + 1 + \frac{K_{iA}K_Q P}{K_P K_{iQ} A} \left(1 + \frac{P}{K_{iiP}}\right) + \frac{K_Q P}{K_P K_{iQ}} \right]$$

$$+ \frac{1}{V_1} \left[ 1 + \frac{K_A}{A} \left(1 + \frac{P}{K_{iiP}}\right) + \frac{P}{K_{iP}} \right]$$

TABLE III: Product Inhibition Patterns for Ordered Bi Bi Mechanisms

Mechanism	Inhibitory Product	Variable Substrate			
		A (PP-ribose-P)		B (Adenine)	
		Low B	Sat. B	Low A	Sat. A
Ordered Bi Bi	P	NC	UC	NC	NC
	Q	C	C	NC	-
Ordered Bi Bi with P acting as dead end inhibitor with free enzyme	P	NC	NC	NC	NC
	Q	C	C	NC	-
Observed Product Inhibition Pattern for Adenine Phosphoribosyltransferase	P	NC (a)	NC	NC (a)	NC
	Q		C (a)	NC (a)	

(a) Hori and Henderson (64).

The abbreviations used are: C, competitive; UC, uncompetitive; NC, noncompetitive; -, no inhibition; A, PP-ribose-P; B, adenine; P, pyrophosphate; Q, AMP.

$\frac{K_A}{V_1 A} (1 + \frac{P}{K_{iiP}})$ , where  $K_{iiP}$ ,  $(k_{-6}/k_6)$ , is the inhibition constant of P with free enzyme. A replot of slope versus pyrophosphate concentration (Figure 17) gives on the abscissa the negative value of the inhibition constant,  $K_{iiP} = 1.55 \times 10^{-3}$  M. Similarly, when substrate B is varied at saturating levels of substrate A, with P acting both as product and as dead-end inhibitor (Equation 20-b), the intercepts are equal to  $\frac{1}{V_1} (1 + \frac{P}{K_{iP}})$ , where  $K_{iP}$  is equal to  $(k_3 + k_4)/k_{-3}$ . A replot of intercept versus pyrophosphate concentration (Figure 17) gives, on the abscissa, the negative value of  $K_{iP}$ ,  $-4.7 \times 10^{-4}$  M. This is in very good agreement with the value of  $5 \times 10^{-4}$  M obtained for  $K_{iP}$  by Hori and Henderson (64). Finally, when substrate B is varied at saturating levels of substrate A (Equation 20-b), the slope reduces to  $\frac{K_B}{V_1 B} (1 + \frac{K_Q P}{K_P K_{iQ}})$ . A replot of slope versus pyrophosphate concentration (Figure 17) gives on the abscissa the negative value of  $\frac{K_P K_{iQ}}{K_Q}$ . Since  $K_{iQ}$  is known, the ratio of  $K_Q$  to  $K_P$  was calculated to be  $1.21 \times 10^{-2}$ .

One consequence of the dead-end binding of P to free enzyme is that terms of  $P^2$  are introduced into the rate equations (Equations 19 and 20). Inhibition patterns and replots of slope versus concentration of P should conse-

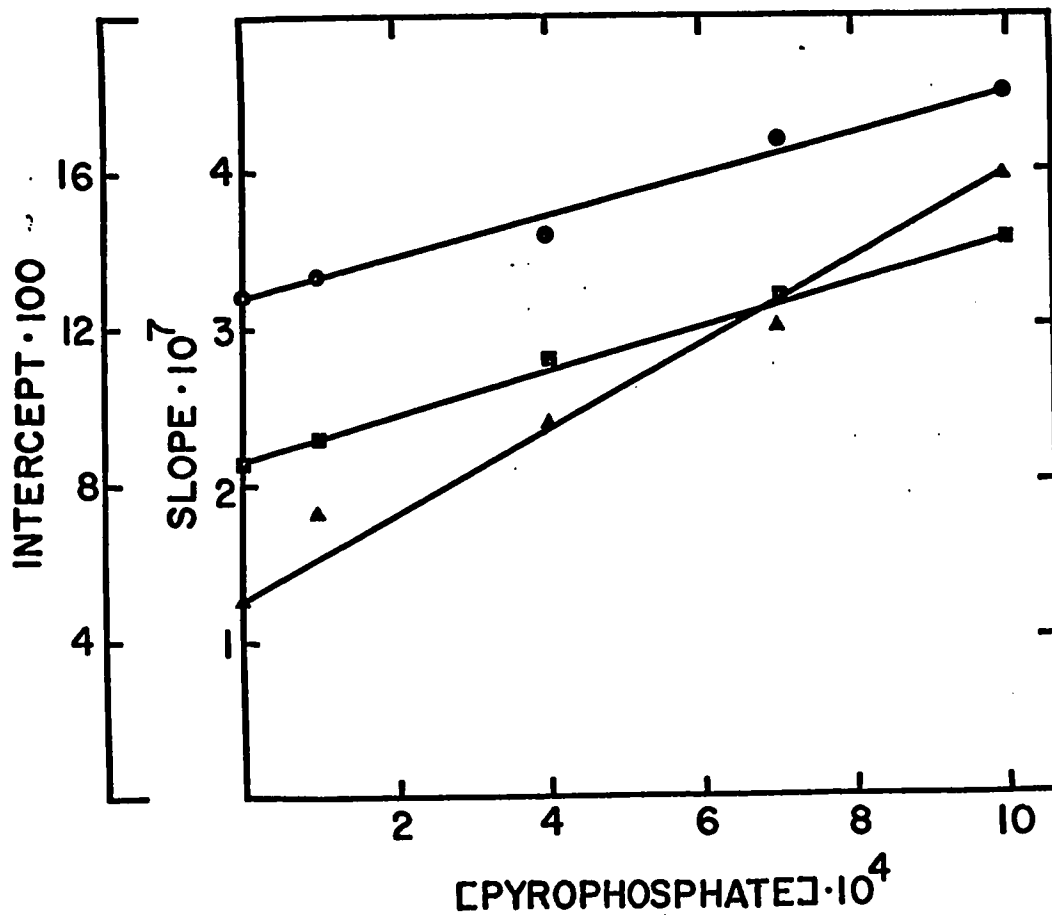


Figure 17. Secondary plots of slopes and intercepts from Figure 8 against concentrations of pyrophosphate; slopes (■) and intercepts (●) obtained from double reciprocal plots of initial velocity against PP-ribose-P concentration, and slopes (▲) from double reciprocal plots of initial velocity against adenine concentration.

quently be parabolic instead of linear. The slope term for such a case is a complicated function of inhibitor concentration, and may be represented by the general equation  $\text{slope} = 1+a+bP+cP^2$ . If  $bP$  is much greater than  $cP^2$ , however, the replot of slope versus  $P$  will approach linearity. The maximum deviation from linearity would occur when the concentration of the non-variable substrate is at a maximum while the concentration of  $P$  is at a maximum. When  $A$  is the variable substrate,  $bP = 6.8 \times 10^{-1}$ , and  $cP^2 = 9.5 \times 10^{-3}$ . There would therefore be only a 1.5% deviation from linearity and this could not be detected by the usual graphical means. Similarly, when  $B$  is the variable substrate,  $bP = 4.1 \times 10^{-1}$ , and  $cP^2 = 9.5 \times 10^{-3}$ ; a 2.3% deviation from linearity would result and this again could not be detected. Due therefore to somewhat unusual ratios of kinetic constants for the adenine phosphoribosyltransferase reaction, the expected parabolic plots of initial velocity versus substrate concentration at various concentrations of  $P$  are not observed, even though  $P$  acts as a dead-end inhibitor with free enzyme. These data are consistent with an ordered  $bi$   $bi$  mechanism for adenine phosphoribosyltransferase in which product  $P$  also binds to free enzyme as a dead-end inhibitor.

#### H. Discussion

Hori and Henderson (64), in 1966, published initial velocity studies and preliminary product inhibition studies

which were consistent with a ping pong bi bi mechanism for adenine phosphoribosyltransferase involving binary enzyme-substrate complexes. They obtained a minimum value for the equilibrium constant of 290; this is in agreement with the qualitative observation of Kornberg, Lieberman and Simms (79) that the equilibrium greatly favors AMP synthesis. Although an equilibrium constant cannot be calculated from the kinetic constant obtained in this study, the observations that no reverse reaction could be detected, and that the dissociation constant for PP-ribose-P is low, suggest that the equilibrium greatly favors synthesis of AMP. No explanation was however given by Hori and Henderson for the observation of Kornberg, Lieberman, and Simms (79) that pyrophosphate was necessary for isotope exchange between adenine and AMP. The present investigation shows that the mechanism is not of the ping pong type but instead is ordered bi bi. This type of mechanism is consistent with the observed product inhibition pattern, when pyrophosphate also combines with free enzyme to give dead-end inhibition, and also with the observed isotope exchange pattern between adenine and AMP. The principle difference between the conclusions of this study and those of Hori and Henderson (64) and of Dean, Watts, and Westwick (31) is the recognition that apparently parallel lines may be produced in double reciprocal plots for ordered reactions when the kinetic constant  $K_{iA}$  is very small, and that this



in fact does happen in the adenine phosphoribosyltransferase reaction.

In 1968 Henderson et al. (57) proposed that hypoxanthine-guanine phosphoribosyltransferase is an ordered reaction containing a ternary enzyme-substrate complex even though initial velocity data were consistent with a mechanism involving binary enzyme-substrate complexes. Product inhibition and isotope exchange studies indicated, however, that the enzyme reaction is sequential. The product inhibition studies also indicated that product P, pyrophosphate, may bind to free enzyme as a dead-end inhibitor. In 1958 Hartman and Buchanan (54) found that PP-ribose-P amidotransferase (EC 2.4.2.14) did not catalyze exchange between pyrophosphate-<sup>32</sup>P and PP-ribose-P in the absence of glutamine. In this reaction ribose 5-phosphate is transferred from PP-ribose-P to the amide group of glutamine to give phosphoribosylamine, pyrophosphate and glutamic acid. On the basis of this observation a mechanism was proposed which involved a ternary enzyme-substrate complex and a direct nucleophilic attack by the amide nitrogen of glutamine on carbon-1 of PP-ribose-P; the latter is made strongly electrophilic by the pyrophosphate group. Packman and Jacoby, as discussed above, also have obtained data consistent with an ordered mechanism for quinolinate phosphoribosyltransferase. In 1963 Martin (96) obtained an intersecting pattern for double reciprocal plots

of initial velocity versus substrate concentration for phosphoribosyl-ATP pyrophosphorylase. In this reaction ribose 5-phosphate is transferred from PP-ribose-P to the 1-nitrogen of ATP to give N-1-(5'-phosphoribosyl)-ATP and pyrophosphate. In addition they observed isotope exchange between pyrophosphate and PP-ribose-P, and between ATP and N-1-(5'-phosphoribosyl)-ATP, in the absence of other products and substrates. On the basis of these isotope exchange studies a ping pong bi bi mechanism was postulated, but no explanation was given for the anomalous initial velocity studies.

The  $K_A$ ,  $K_B$  and  $K_{iP}$  values shown in Table IV are similar to those reported by Hori and Henderson (64), and the Michaelis constants are also similar to those reported by Atkinson and Murray (5), Murray and Wong (103) and Murray (101) for the Ehrlich ascites tumor enzyme. Blair (13) obtained a Michaelis constant for PP-ribose-P of  $3 \times 10^{-4}$  M with 2,6-diaminopurine as the second substrate and  $6.9 \times 10^{-5}$  M with adenine as the second substrate. Because the Michaelis constant for the first substrate, PP-ribose-P, is a function of the maximum velocity, a change of the second substrate from adenine to an alternative substrate such as 2,6-diaminopurine might affect the maximum velocity, and if this occurs a change in  $K_A$  would also be expected. The apparent Michaelis constant for 2,6-diaminopurine was  $1.8 \times 10^{-3}$  M, and that for adenine  $3 \times 10^{-5}$  M. This work was done with the

TABLE IV: Kinetic Data for Mechanism II

<u>Kinetic Parameter</u>	<u>Rate Constants</u>	<u>Values</u>
$V_1 E_0$	$\frac{k_3 k_4}{k_3 + k_4}$	27.2 $\mu\text{moles min.}^{-1}$ (a)
$V_2 E_0$	$\frac{k_{-1} k_{-2}}{k_{-1} + k_{-2}}$	< 0.5 $\mu\text{moles min.}^{-1}$ (a)
$K_A$	$\frac{k_3 k_4}{k_1 (k_3 + k_4)}$	$6 \times 10^{-6}$ M
$K_{iA}$	$\frac{k_{-1}}{k_1}$	$2.2 \times 10^{-7}$ M
$K_B$	$\frac{k_4 (k_{-2} + k_3)}{k_2 (k_3 + k_4)}$	$9 \times 10^{-7}$ M
$K_{iB}$	$\frac{k_{-1} + k_{-2}}{k_2}$	(b)
$K_P$	$\frac{k_{-1} (k_{-2} + k_3)}{k_{-3} (k_{-1} + k_{-2})}$	(c)
$K_{iP}$	$\frac{k_3 + k_4}{k_{-3}}$	$4.7 \times 10^{-4}$ M
$K_Q$	$\frac{k_{-1} k_{-2}}{k_{-4} (k_{-1} + k_{-2})}$	(c)
$K_{iQ}$	$\frac{k_4}{k_{-4}}$	$3 \times 10^{-5}$ M (b)
$K_{iiP}$	$\frac{k_{-6}}{k_6}$	$1.55 \times 10^{-3}$ M
$K_{Eq}$	$\frac{k_1 k_2 k_3 k_4}{k_{-1} k_{-2} k_{-3} k_{-4}}$	290 (b)
$K_{iiB}$	$\frac{k_{-2}}{k_2}$	$1.1 \times 10^{-5}$ M

TABLE IV (continued)

<u>Kinetic Parameter</u>	<u>Rate Constants</u>	<u>Values</u>
Rate of isotope exchange between adenine- $^{14}\text{C}$ and AMP	(e)	$5.2 \mu\text{moles min.}^{-1}$ (a)
Rate of isotope exchange between pyrophosphate- $^{32}\text{P}$ and PP-ribose-P	(f)	$<0.05 \mu\text{moles min.}^{-1}$ (a)

(a) Per  $\mu\text{g}$  of enzyme preparation.

(b) Not determined but approximately equal to the value for  $K_{iiB}$ .

(c) Not determinable for this mechanism.

(d) Hori and Henderson (64).

(e) Rate equation (13).

(f) Rate equation not derived but form similar to equation (13).

enzyme isolated from a strain of L-cells. The Michaelis constants reported by Dean, Watts, and Westwick (31) for the human erythrocyte adenine phosphoribosyltransferase are  $6.5 \times 10^{-5}$  M for PP-ribose-P and  $2 \times 10^{-6}$  M for adenine. Henderson et al. (60) obtained Michaelis constants between 1.0 and  $2.6 \times 10^{-6}$  M for adenine and between 1.9 and  $8.1 \times 10^{-6}$  M for PP-ribose-P for mutant adenine phosphoribosyltransferases of human erythrocytes. In all cases the Michaelis constant for PP-ribose-P was higher than that of adenine.

Since the reverse reaction for adenine phosphoribosyltransferase could not be demonstrated,  $V_2$ , the maximum velocity in the reverse direction,  $K_p$ , the Michaelis constant for pyrophosphate, and  $K_Q$ , the Michaelis for AMP, could not be obtained. Because all of these kinetic constants and the concentration of the enzyme are required in order to calculate the individual rate constants, the rate-limiting step of adenine phosphoribosyltransferase could not be determined by these methods.

#### IV ROLE OF MAGNESIUM ION IN THE ADENINE PHOSPHORIBOSYLTRANSFERASE REACTION

##### A. Introduction

Kornberg, Lieberman and Simms (79) showed in their study of adenine phosphoribosyltransferase that magnesium ion is required for the activity of this enzyme. Hori and Henderson (64) subsequently demonstrated that divalent calcium, manganese and magnesium ions also supported high initial rates of this reaction, whereas divalent cobalt, nickel and zinc ions supported lower but still substantial rates. The mode of action of magnesium ion in the adenine phosphoribosyltransferase reaction has been investigated in the present study.

Vallee and Coleman (128) have proposed that metal ions exert their effects on enzyme reactions either through interaction with the enzyme itself, or through interaction with substrate(s) or cofactor(s). In the former case the metal ion may either be bound so firmly to the protein that it remains associated with it throughout isolation and purification procedures, or it may be removed and may have to be added back to restore full activity. In this case the concentration of metal ion required for activity would be expected to be proportional to the concentration of enzyme. In the other situation the concentration of metal ion required would be expected to be proportional to the concentration of

substrate or cofactor.

The role of the metal ion in the enzyme-metal-ion-substrate complex may be to participate in the binding of the substrate to the enzyme, to activate the substrate in the enzyme-substrate complex or both. Vallee and Coleman (128) have proposed further that metal ions may activate enzymatic reactions by maintaining the secondary, tertiary or quaternary structures of the proteins or by transferring electrons in oxidation-reduction reactions. Dixon and Webb (36) have suggested two ways in which metal ions may produce an apparent activation even though no metal ion is involved in the enzyme-substrate interaction. Metal ions may form complexes with a product to remove it from the system and thus prevent product inhibition, or they may participate in the removal of an inhibitor due to the formation of an inhibitor-metal-ion complex.

For those cases in which the metal ion acts as a link between the substrate and the enzyme, Dixon and Webb (36) have suggested four possible sequences of binding. (a) The active enzyme-metal-ion-substrate complex may be formed directly from the three components in a trimolecular reaction. (b) The metal ion may form an enzyme-metal-ion complex which then binds the substrate. (c) The metal ion may combine with the substrate to give a substrate-metal-ion complex which binds to the enzyme as the true substrate. (d) The

enzyme-metal-ion-substrate complex may be formed by a random addition employing both sequences (b) and (c). It is also conceivable that the substrate may first bind to free enzyme and that the metal ion then binds to this complex. The fate of the metal ion after catalysis is not considered in any of these proposals. It may remain associated with the enzyme after catalysis, it may be released back into the medium, or it may remain associated with the products.

B. Magnesium ion binding to PP-ribose-P

The possibility was considered first that magnesium ion bound to PP-ribose-P and that this complex was then bound as the true substrate to adenine phosphoribosyltransferase. That magnesium could bind to PP-ribose-P had previously been implied by the observation that the rate of its hydrolysis at pH 7.4 was markedly increased in the presence of this ion (77), and by the commercial availability of the dimagnesium salt of PP-ribose-P; a very rough estimate of the association constant for the magnesium-ion-PP-ribose-P complex had been published (15). Further evidence has been found for this interaction.

Figure 18 shows the titration curve for PP-ribose-P in the presence and absence of magnesium ion. Fructose 1,6-diphosphate was titrated as a control in order to verify the validity of the method. In the absence of magnesium ion three distinct ionization constants, at pH 2.05, 2.95 and



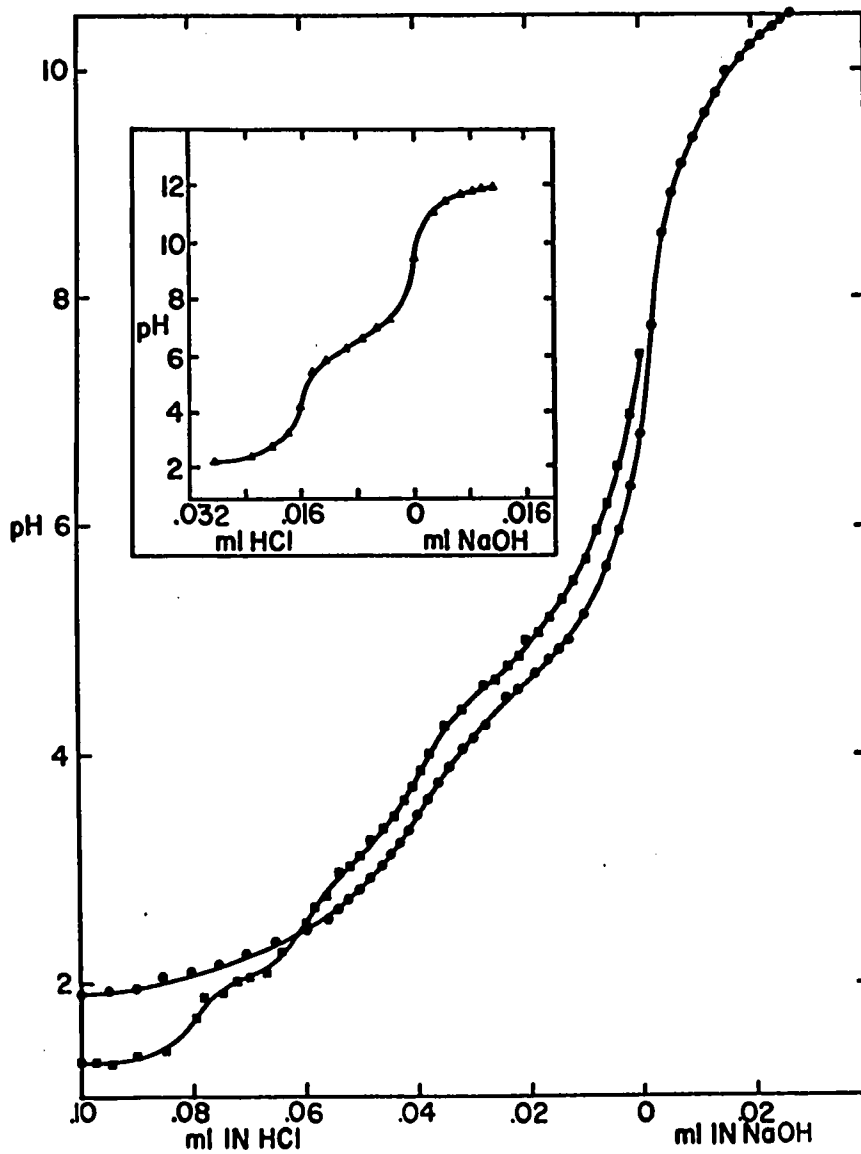


Figure 18. Titration curve for PP-ribose-P at a concentration of  $10^{-2}$  M in the absence of magnesium ion (■) and in the presence of  $10^{-2}$  M magnesium ion (●). The insert is the titration curve for fructose 1,6-diphosphate (▲) in the absence of magnesium ion.

4.70, were observed for PP-ribose-P, while in the presence of magnesium ion only one ionization constant, at pH 4.70, was observed. Because magnesium ion masks two ionization constants, these data suggest that magnesium ion binds to the pyrophosphate moiety of PP-ribose-P. Five ionization constants would be expected to be observed for PP-ribose-P but it is possible that the 5-phosphate moiety and the  $\beta$ -phosphate of the pyrophosphate moiety have one ionization constant that is the same.

If PP-ribose-P were the only ligand to which magnesium ion is bound in this system, then the magnesium ion concentration required for optimum enzyme activity should increase if the PP-ribose-P concentration were changed from  $10^{-5}$  to  $5 \times 10^{-3}$  M at a constant enzyme concentration. This hypothesis was tested and the results are shown in Figures 19 and 20. The optimum magnesium ion concentration was found to be  $10^{-3}$  M for a PP-ribose-P concentration of  $10^{-5}$  M; this increased 35-fold to  $3.5 \times 10^{-2}$  M when the PP-ribose-P concentration was  $5 \times 10^{-3}$  M (a 500-fold increase). The disproportionality of these increases will be discussed later, but this increase in magnesium ion requirement is generally consistent with the proposition that PP-ribose-P is the ligand to which magnesium ion binds.

The possibility that magnesium ion may chelate not only to the pyrophosphate group to form the active substrate

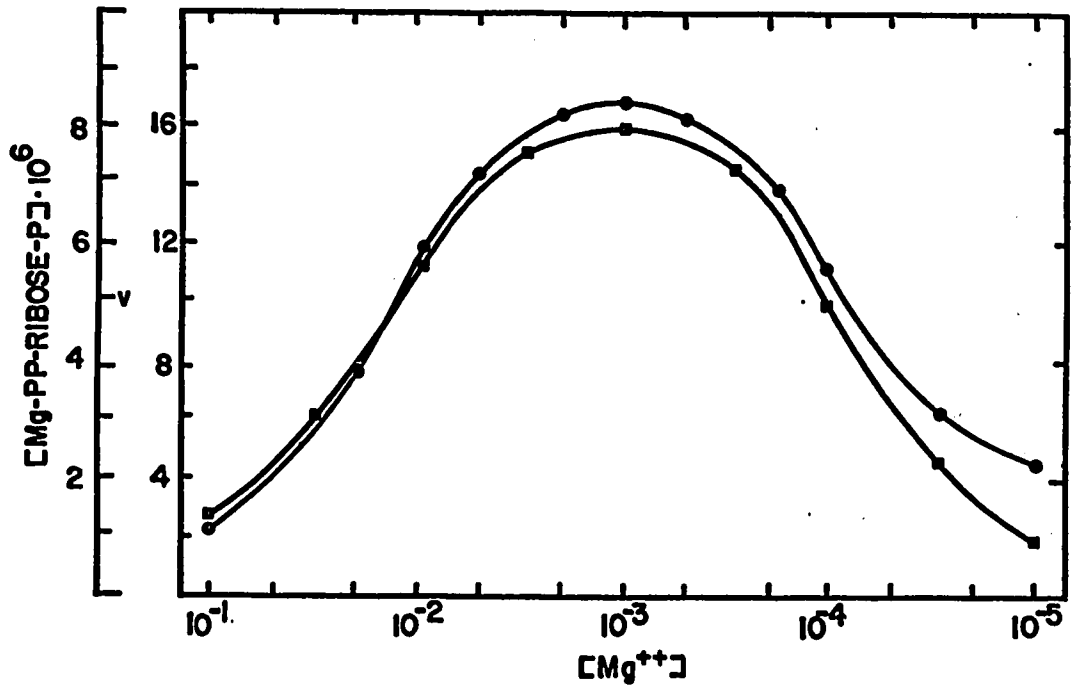


Figure 19. Concentration of Mg-PP-ribose-P (■) and initial velocity (●) as functions of magnesium ion concentration. The concentration of PP-ribose-P was  $10^{-5}$  M and that of adenine was  $2 \times 10^{-6}$  M.  $v$  is expressed as micromicromoles of AMP per minute.

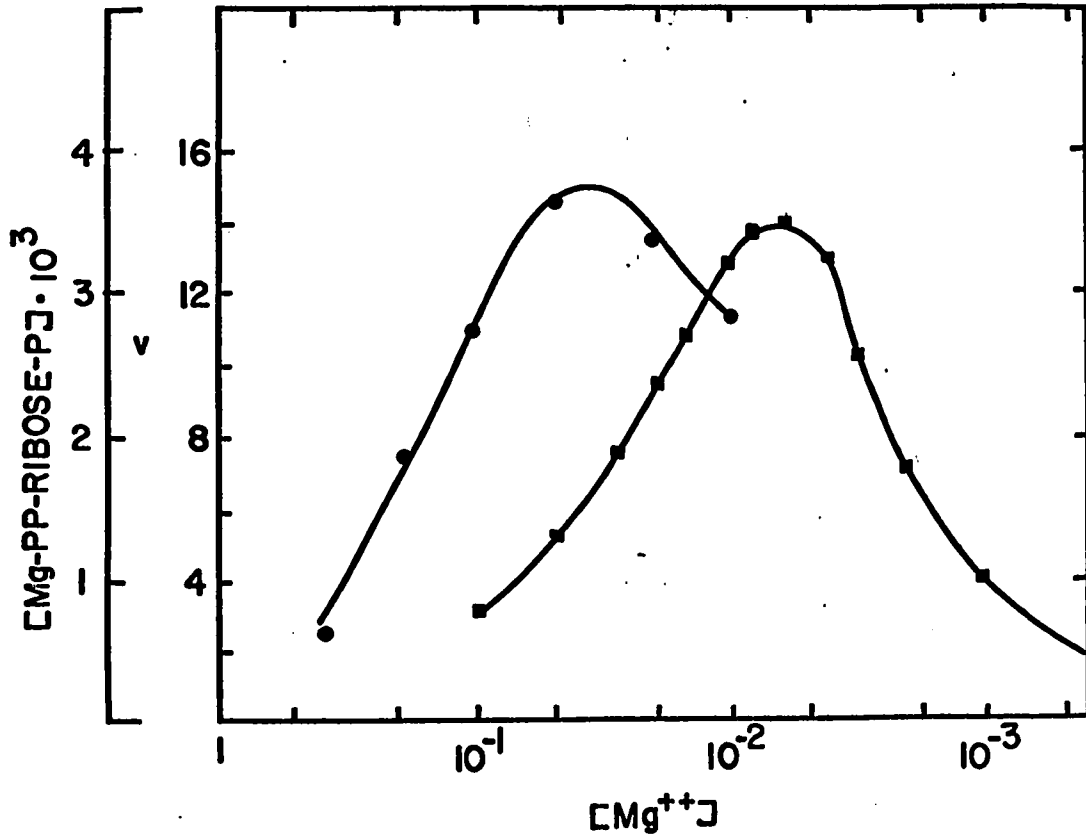
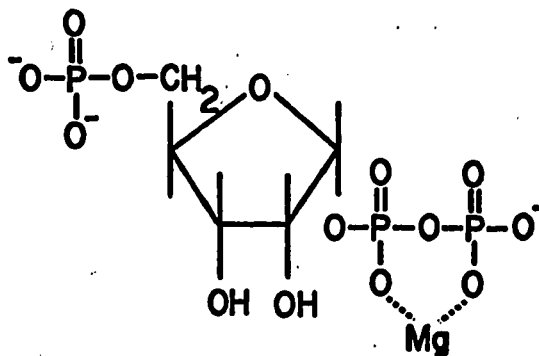
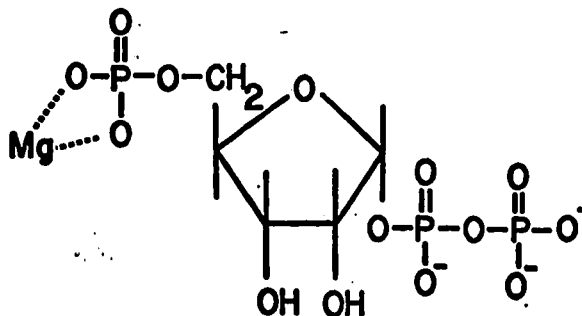


Figure 20. Concentration of Mg-PP-ribose-P (■) and initial velocity (●) as functions of magnesium ion concentration. The concentration of PP-ribose-P was  $5 \times 10^{-3}$  M and that of adenine was  $2 \times 10^{-6}$  M.  $v$  is expressed as micromicromoles of AMP per min.

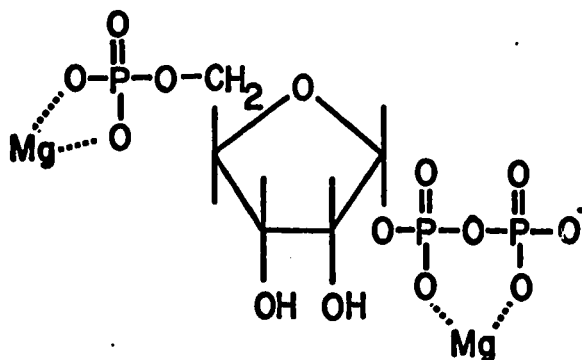
but also to the 5-phosphate group to form an inactive compound is suggested by the observations of Hori and Henderson (63) and the present work which showed that as the magnesium ion concentration was increased, the enzyme activity increased to a maximum and then declined. That PP-ribose-P can chelate two magnesium ions is suggested by the observation that PP-ribose-P is obtained from commercial sources as the dimagnesium salt. The stability constant for the pyrophosphate moiety of PP-ribose-P with magnesium ion is known only to be greater than  $10^3$  (15); that of the 5'-phosphate moiety of AMP, which is structurally similar to the 5-phosphate moiety of PP-ribose-P, is  $10^2$  (15). Epp, Ramasarma and Wetter (40), using infrared spectroscopy, and Happ and Morales (52), using nuclear magnetic resonance spectroscopy, showed that magnesium ion does not bind to the adenine moiety of adenosine phosphate, and the stability constant for magnesium ion with AMP must therefore be the stability constant for the magnesium-ion-5'-phosphate complex. If only the form of PP-ribose-P in which magnesium ion is bound to the 1-pyrophosphate group (Mg-PP-ribose-P) (Figure 21) is the catalytically active form of the substrate, then an inactive form of PP-ribose-P (Mg-PP-ribose-P-Mg) might be produced as the magnesium ion concentration is increased; a parallel decrease in enzyme activity due to a decrease in the concentration of the active substrate would result. Figure 22 shows the



Mg-PP-RIBOSE-P

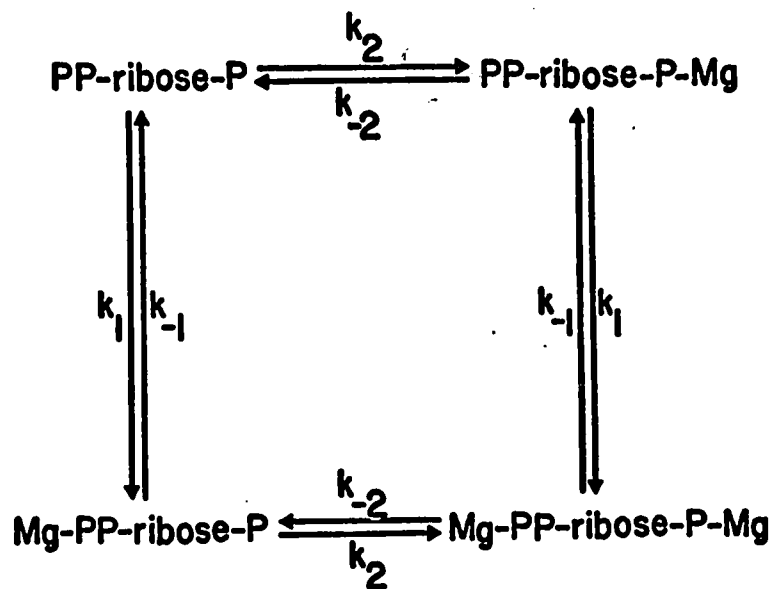


PP-RIBOSE-P-Mg



Mg-PP-RIBOSE-P-Mg

Figure 21. Structural formulae for complexes of magnesium ion and PP-ribose-P.



$$K_1 = \frac{k_1}{k_{-1}} = \frac{[\text{Mg-PP-ribose-P}]}{([\text{Mg}] - [\text{Mg-PP-ribose-P}])([\text{PP-ribose-P}] - [\text{Mg-PP-ribose-P}])}$$

$$K_2 = \frac{k_2}{k_{-2}} = \frac{[\text{PP-ribose-P-Mg}]}{([\text{Mg}] - [\text{PP-ribose-P-Mg}])([\text{PP-ribose-P}] - [\text{PP-ribose-P-Mg}])}$$

$$[\text{Mg-PP-ribose-P}] = [\text{Mg}] + [\text{PP-ribose-P}] + 1/K_1$$

$$\frac{-\sqrt{([\text{Mg}] + [\text{PP-ribose-P}] + 1/K_1)^2 - 4[\text{Mg}][\text{PP-ribose-P}]}}{2}$$

Figure 22. Interconversions of magnesium-ion-PP-ribose-P complexes.

proposed scheme in which Mg-PP-ribose-P is the active form of the substrate while PP-ribose-P, PP-ribose-P-Mg and Mg-PP-ribose-P-Mg are all catalytically inactive forms. Both PP-ribose-P (64) and Mg-PP-ribose-P bind to free enzyme, but it is not known whether Mg-PP-ribose-P-Mg also binds to free enzyme.

Figure 19 shows a plot of enzyme activity and of Mg-PP-ribose-P concentration as a function of magnesium ion concentration. This graph was calculated for a PP-ribose-P concentration of  $10^{-5}$  M using stability constants of 80 for the magnesium-ion-5-phosphate complex and  $10^4$  for the magnesium-ion-1-pyrophosphate complex. These stability constants were chosen in order to construct the curve of Mg-PP-ribose-P concentration which most closely approximates the observed curve of enzyme activity as a function of magnesium ion concentration. A stability constant of  $10^4$  for the magnesium-ion-1-pyrophosphate complex is not unreasonable since the complex of free pyrophosphate with magnesium ion has a stability constant of  $25 \times 10^4$  (15).

Figure 20 shows a plot of enzyme activity and of Mg-PP-ribose-P concentration as a function of magnesium ion concentration constructed as before but for a PP-ribose-P concentration 500-fold higher than that used above. That concentration of magnesium ion for which the maximum amount of PP-ribose-P is in the form of Mg-PP-ribose-P is calculated



to be  $6 \times 10^{-3}$  M. The concentration of magnesium ion required for maximum activity, at this PP-ribose-P concentration, was experimentally determined (Figure 20) to be  $3.5 \times 10^{-2}$  M, however. That is, a 6-fold greater concentration of magnesium ion was actually required for maximum activity than that predicted.

Those curves constructed from stability constants and those obtained experimentally are, however, similar in several respects. As the concentration of magnesium ion is increased, the enzyme activity and Mg-PP-ribose-P concentration increase to a maximum and then decrease. As the concentration of PP-ribose-P is increased, the concentration of magnesium ion required for maximum enzyme activity and for the maximum concentration of Mg-PP-ribose-P is increased. And as the concentration of PP-ribose-P is increased, the range of magnesium ion concentrations that will give maximum enzyme activity and maximum concentrations of Mg-PP-ribose-P is decreased, and much sharper peaks result. These similarities do not explain the observation that at very high PP-ribose-P concentrations a higher concentration of magnesium ion is actually required for maximum enzyme activity than that predicted from the curves constructed from stability constants. It is possible that sodium ion, which was  $2 \times 10^{-2}$  M at  $5 \times 10^{-3}$  M tetrasodium PP-ribose-P, may compete with magnesium ion for the 1-pyrophosphate moiety of PP-

ribose-P. Although stability constant for the Na-PP-ribose-P complex have not been reported, Bock (15) has reported that the stability constant for the Na-pyrophosphate complex is 220 at zero ionic strength, and 10 at 1.0 ionic strength. For these concentrations of sodium ion and PP-ribose-P, 78% of PP-ribose-P would be in the form of the Na-PP-ribose-P complex for a stability constant of 220, 48% for a stability constant of 50, and 25% for a stability constant of 10. At  $10^{-5}$  M PP-ribose-P not enough sodium ion was present to complex with PP-ribose-P. It is therefore possible that sodium ion may compete with magnesium ion for the 1-pyrophosphate moiety of PP-ribose-P and the concentration of magnesium ion required for maximum activity would thus be increased. Because PP-ribose-P is only between 61% and 71% pure, reaction mixtures containing  $5 \times 10^{-3}$  M PP-ribose-P may also contain up to  $1.95 \times 10^{-3}$  M pyrophosphate which could bind significant amounts of the added magnesium ion. These data suggest, therefore, that magnesium ion does bind to PP-ribose-P to give a Mg-PP-ribose-P complex which is the true substrate of adenine phosphoribosyltransferase and that Mg-PP-ribose-P is an inactive form of the substrate. The possibilities that PP-ribose-P and Mg-PP-ribose-P-Mg are competitive inhibitors with respect to Mg-PP-ribose-P were also considered.

C. Magnesium ion binding to free enzyme

The possibility was also considered that magnesium ion might also bind directly to the enzyme. In 1966 Hori and Henderson (64) demonstrated that PP-ribose-P, both in the absence and presence of magnesium ions, protected adenine phosphoribosyltransferase against heat inactivation, while magnesium ions alone had no protective effect. The protection by PP-ribose-P was much greater in the presence of magnesium ion than in its absence. Table 5 shows the effect of magnesium ions and PP-ribose-P, both alone and in combination, on the extent of inactivation of the enzyme by PMB. EDTA was added to all reaction mixtures in order to chelate any endogenous magnesium ion present, and a magnesium ion concentration of  $10^{-3}$  M was obtained by adding an amount of magnesium ion equal to that of the added EDTA in excess of  $10^{-3}$  M magnesium ion. At  $5 \times 10^{-6}$  M PMB, magnesium ion alone gave no significant protection against inactivation, PP-ribose-P gave 60% protection and PP-ribose-P in the presence of magnesium ion gave complete protection. When the concentration of PMB was increased to  $10^{-3}$  M, PP-ribose-P gave almost no protection and PP-ribose-P in the presence of magnesium ion gave 67% protection. These results suggest that the Mg-PP-ribose-P complex binds to the enzyme much more tightly than does PP-ribose-P and that magnesium ion does not bind to free enzyme. It should be pointed out,

TABLE V: Effect of Magnesium Ion on PP-ribose-P Protection Against Enzyme Inactivation by PMB

<u>PMB</u> <u>(M)</u>	<u>PP-ribose-P</u> <u>(10<sup>-5</sup> M)</u>	<u>Mg<sup>2+</sup></u> <u>(10<sup>-3</sup> M)</u>	<u>Enzyme Activity</u> <u>(% of Control)</u>
5 x 10 <sup>-6</sup>	-	-	12
5 x 10 <sup>-6</sup>	-	+	17
5 x 10 <sup>-6</sup>	+	-	60
5 x 10 <sup>-6</sup>	+	+	100
10 <sup>-3</sup>	-	-	0
10 <sup>-3</sup>	+	-	9
10 <sup>-3</sup>	+	+	67

Two µg of enzyme preparation per reaction mixture was incubated with PMB for 20 min at 30° in 0.1 M Tris-HCl buffer, pH 7.4, in the absence or presence of PP-ribose-P and magnesium ion; EDTA was added first to remove endogenous magnesium ion as described in the text. The reaction mixtures were completed and enzyme activity was assayed by the radioisotope method; the concentration of adenine-14C was 2 x 10<sup>-6</sup> M.

however, that if magnesium ion binds to a group other than that modified by PMB, protection against enzyme inactivation might not be observed. In these experiments the results were expressed as per cent inhibition because the inactivation process was not linear with time and consequently rates of inactivation could not be calculated.

#### D. Discussion

The lack of protection of adenine phosphoribosyltransferase against heat inactivation by magnesium ion reported by Hori and Henderson (64), and the lack of protection against PMB inactivation by magnesium ion demonstrated in these experiments, suggest that magnesium ion does not bind to free enzyme; these experiments also show that PP-ribose-P in the presence of magnesium ion gives more protection against inactivation than does PP-ribose-P alone. The observations that magnesium ion masks two ionization constants of PP-ribose-P and that a higher magnesium ion concentration was required for optimum enzyme activity when the concentration of PP-ribose-P was increased from  $10^{-5}$  M to  $5 \times 10^{-3}$  M suggest that magnesium does bind to PP-ribose-P. Studies of product inhibition by pyrophosphate at limiting and non-limiting magnesium ion concentrations were interpreted by Hori and Henderson (64) to indicate that PP-ribose-P and pyrophosphate both react in the form of their magnesium ion complexes. Kornberg, Lieberman and Simms (77), in 1955,

reported that the hydrolysis of PP-ribose-P is markedly increased in the presence of magnesium ion, and Bock (15), in 1960, reported a minimum value for the stability constant between magnesium ion and PP-ribose-P. These data further suggest that PP-ribose-P is the ligand to which magnesium ion is bound and that the magnesium salt of PP-ribose-P is the true substrate. Because PP-ribose-P was also bound to free enzyme but was catalytically inactive, it is conceivable that magnesium ion might also bind to this complex to give the catalytically active enzyme-substrate-magnesium-ion complex. From the presently available data it is not possible to determine whether the role of magnesium ion is to act as a link between enzyme and substrate, to partially neutralize the negative charges of the pyrophosphate moiety, to participate in the catalysis, or a combination of these.

Hori and Henderson (63) and the present work have shown that as the magnesium ion concentration is increased, the enzyme activity reaches a maximum and then declines. The present work suggests that this is due to magnesium ion binding to the 5'-phosphate group of PP-ribose-P to give a catalytically inactive substrate. Because metal ion-ligand complexes such as  $(ADP)_2Mg^{4-}$  have been proposed to occur (15), the possibility of similar PP-ribose-P complexes and their effects on the various reaction parameters should not be ruled out. At the present time, however, it is difficult to

assess the effects of such complexes on the reaction. It should be noted that at PP-ribose-P concentrations between  $10^{-6}$  M and  $10^{-4}$  M, the optimum concentration of Mg-PP-ribose-P was calculated to be at a maximum at a magnesium ion concentration of  $10^{-3}$  M. Between these concentrations 83  $\pm$  0.5% of the PP-ribose-P was calculated to be in the Mg-PP-ribose-P form.

Although a function for magnesium ion in the catalysis cannot be demonstrated from the data available, the observation that free PP-ribose-P does bind to free enzyme to give a catalytically inactive complex suggests a possible catalytic function. Numerous workers including Kosower (83), Ingram (68), and Bruice and Benkovic (21,22) have postulated that the main function of divalent cations, in substrates containing pyrophosphate, is to neutralize the negative charges of the pyrophosphate moiety. Ingram (86) and Bruice and Benkovic (21,22) have also suggested that neutralization of the negative charges of the pyrophosphate moiety may facilitate the approach of a nucleophile. In addition Bruice and Benkovic (21,22) have suggested that the chelated species may be a better leaving group in nucleophilic reactions than the more highly ionized species. These authors suggest that pyrophosphate, in displacement reactions, should possess a low net charge to facilitate its expulsion and that this may be attained through complexation with a divalent cation or a positively charged protein functional group.

## V BINDING OF PP-RIBOSE-P TO ADENINE PHOSPHORIBOSYLTRANSFERASE

### A. Introduction

Kornberg, Lieberman and Simms (76) and Remy, Remy and Buchanan (112) first isolated and characterized  $\alpha$ -D-ribose 5-phosphate 1-pyrophosphate in 1955; their identification was confirmed by the chemical synthesis of PP-ribose-P from ribose by Tener and Khorana (126) in 1958. The present study has attempted to establish those aspects of the structure of PP-ribose-P which are required for binding to adenine phosphoribosyltransferase, and to identify those amino acids in this enzyme which may be involved in their binding.

One method by which those parts of a substrate which are involved in binding to enzyme can be determined is to measure the ability of a series of substrate analogs to inhibit enzyme activity. The inhibition constants obtained from such kinetic studies are, or are closely related to, enzyme-inhibitor dissociation constants. Such data must be interpreted with caution, however, because it is sometimes difficult to determine whether a change in binding is directly due to a particular structural modification, or is due to a secondary effect of the modification. Webb (134) and Baker (6) have suggested that the interaction energy,  $\Delta F$ , between an enzyme and an inhibitor may be a more significant measure of binding than the inhibition constants, and have calculated this energy from the inhibition constant by the equation  $\Delta F = RT \ln K_i$ .



Figure 23 shows the relationship between inhibition constant and the free energy of binding. This Figure shows that a doubling of the inhibition constant from  $10^{-5}$  M to  $2 \times 10^{-5}$  M results in only approximately a 6% decrease in the free energy of binding, and a doubling of the inhibition constant from  $10^{-3}$  M to  $2 \times 10^{-3}$  M results in only approximately a 9.5% decrease in the free energy of binding. On these grounds Baker (7) considers a two-fold increase in inhibition constant to be insignificant.

In order to determine those structural features of PP-ribose-P which are involved in the binding of this substrate to adenine phosphoribosyltransferase, the inhibition constants of a number of analogs of PP-ribose-P were determined. Those groups on the enzyme which were involved in binding were investigated by analyzing the effects of pH on the Michaelis constant for PP-ribose-P, and by studying the effects of PP-ribose-P on rates of inactivation of the enzyme by reagents which react with various amino acids. On the basis of the studies described in the previous chapter, it will be assumed throughout that the actual substrate of adenine phosphoribosyltransferase is the complex of magnesium ion with the pyrophosphate moiety of PP-ribose-P.

#### B. Inhibition by analogs of PP-ribose-P

The effects on the initial velocity of the adenine phosphoribosyltransferase reaction of eighteen PP-ribose-P

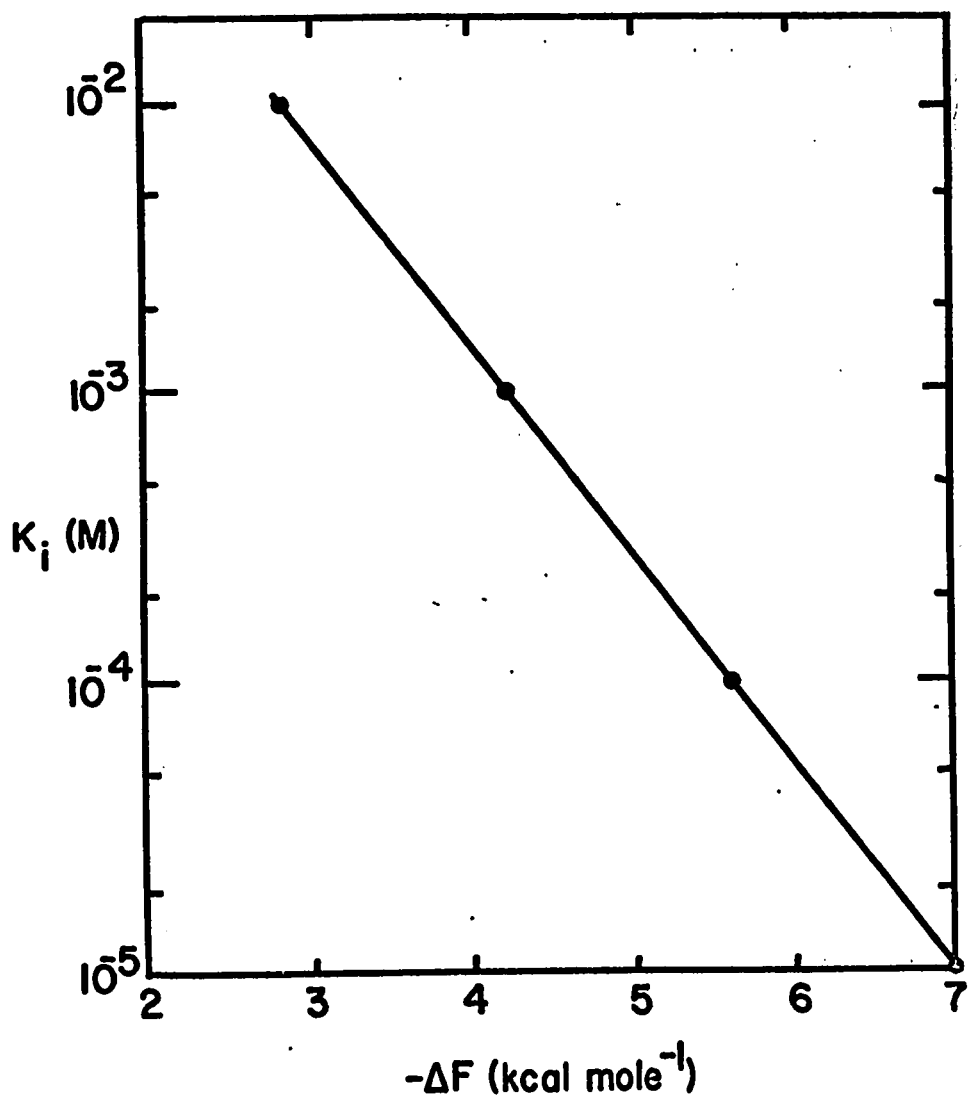


Figure 23. Relationship between free energy of binding and inhibition constant.

analogs were measured using the standard radioisotope assay with  $6 \times 10^{-6}$  M PP-ribose-P (equal to its Michaelis constant),  $9 \times 10^{-6}$  M adenine (equal to 10 times its Michaelis constant), and  $10^{-3}$  M test compound.

For those compounds which produced more than 20% inhibition in this preliminary test, the initial rates of the reaction were measured at various concentrations of PP-ribose-P in the absence of inhibitor and at two or more concentrations of inhibitor; the concentration of adenine was constant at  $9 \times 10^{-6}$  M. In all cases the inhibition was competitive with respect to PP-ribose-P, and no inhibition was detected when the concentration of adenine was varied in the presence of  $7 \times 10^{-4}$  M and  $10^{-3}$  M 1,5-pentanediol diphosphate at a concentration of PP-ribose-P,  $6 \times 10^{-5}$  M, equal to 10 times its Michaelis constant.

As examples of these data the plots for experiments with ribose 1,5-diphosphate (Figure 24) and with 1,5-pentanediol diphosphate (Figure 25) are given. The slope of each line was obtained from these plots and plotted against inhibitor concentration; extrapolation to zero gives, on the abscissa, the negative value of the inhibition constant. The rate equations for inhibition for PP-ribose-P analogs which bind only to free enzyme are given on page 98.

An approximate value of the inhibition constant was calculated by the method of Webb (131) from the measured

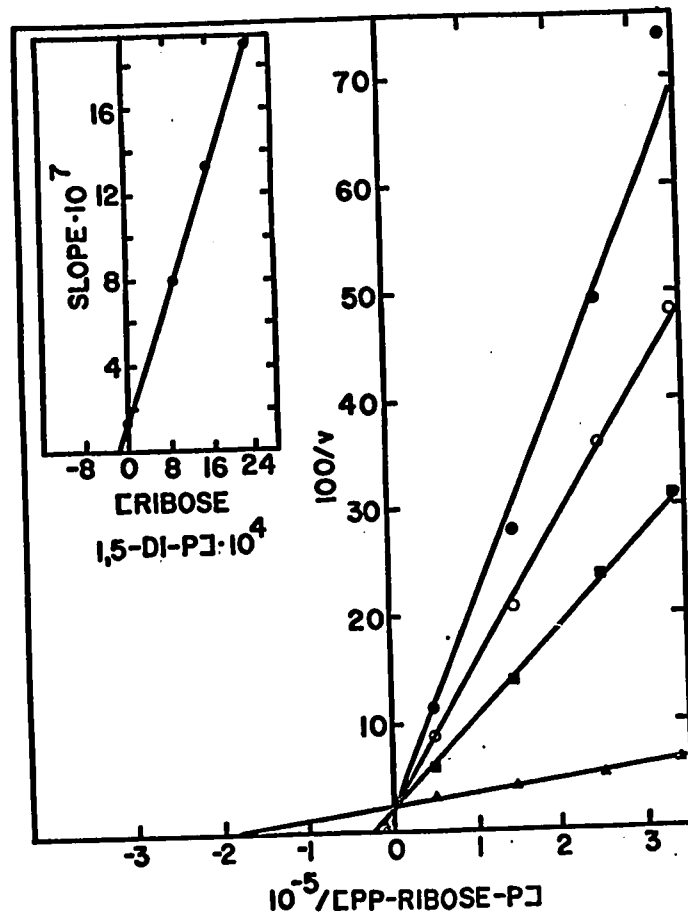


Figure 24. Inhibition by ribose 1,5-diphosphate. Double reciprocal plot of initial velocity against PP-ribose-P concentration at an adenine concentration of  $9 \times 10^{-6}$  M. The concentrations of ribose 1,5-diphosphate are:  $0$  ( $\blacktriangle$ ),  $7 \times 10^{-4}$  M ( $\blacksquare$ ),  $1.4 \times 10^{-3}$  M ( $\circ$ ), and  $2.1 \times 10^{-3}$  M ( $\bullet$ ).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of ribose 1,5-diphosphate.

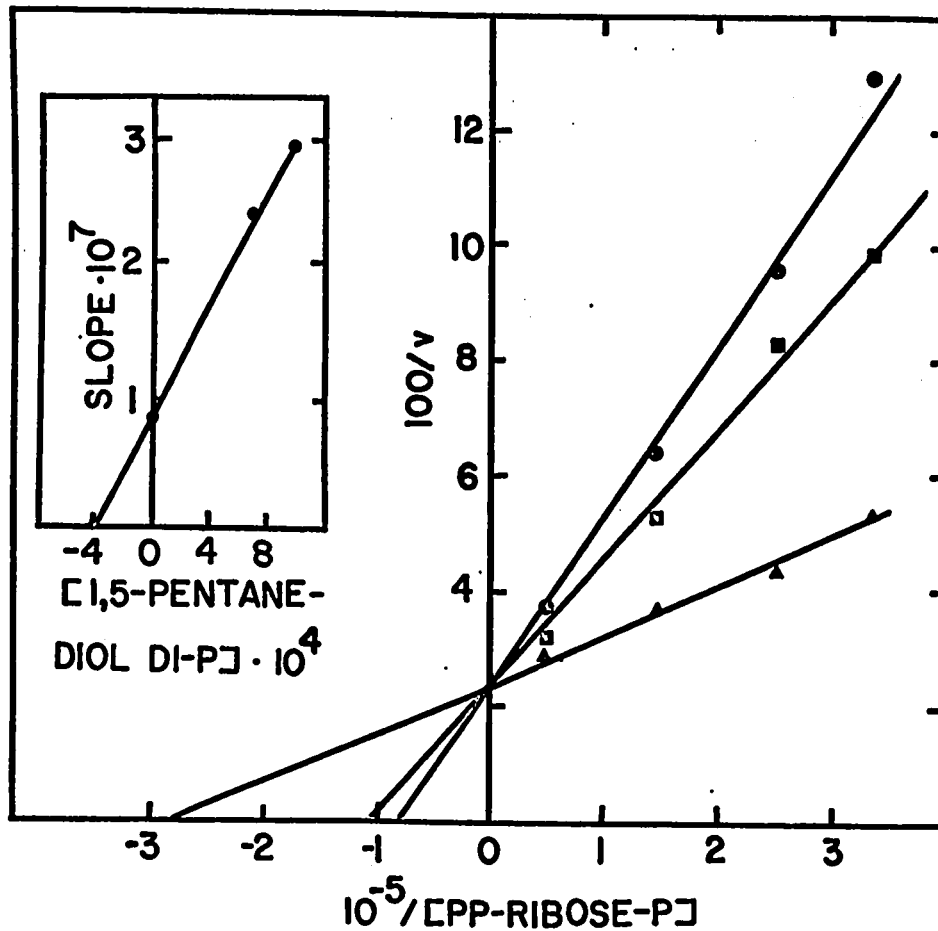


Figure 25. Inhibition by 1,5-pentanediol diphosphate. Double reciprocal plot of initial velocity against PP-ribose-P concentration at an adenine concentration of  $9 \times 10^{-6}$  M. The concentrations of 1,5-pentanediol diphosphate are:  $0$  ( $\Delta$ ),  $7 \times 10^{-4}$  M ( $\blacksquare$ ) and  $10^{-3}$  M ( $\bullet$ ).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of 1,5-pentanediol diphosphate.

Rate Equations for Initial Velocity in the Presence of Dead-end Inhibitors Binding to Free Enzyme.

$$\frac{E_0}{v} = \frac{K_A}{V_{1A}} \left( \left[ 1 + \frac{I}{K_i} \right] + \frac{K_{iA}K_B}{K_{AB}} \left[ 1 + \frac{I}{K_i} \right] \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (21a)$$

$$\frac{E_0}{v} = \frac{K_A}{V_{1B}} \left( \left[ 1 + \frac{K_{iA}K_B}{K_{AB}} \right] \left[ 1 + \frac{I}{K_i} \right] \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right) \quad (21b)$$

Since  $K_A = 20 \cdot K_{iA}$  and  $B = 10 K_B$

then  $\frac{K_{iA}K_B}{K_{AB}} = \frac{1}{200} = 0.005$

therefore  $\frac{E_0}{v} = \frac{K_A}{V_{1A}} \left( 1 + \frac{I}{K_i} \right) + \frac{1}{V_1} \left( 1 + \frac{K_B}{B} \right)$  (22)

Inhibition Constants as a Function of Fractional Inhibition for Compounds Binding to Free Enzyme.

$$i = 1 - v_i/v = (v - v_i)/v \quad (23a)$$

$$1 = \frac{\left( \frac{V_{1AB}}{K_{iA}K_B + K_{AB} + K_B A + AB} \right) - \left( \frac{V_{1AB}}{K_{iA}K_B \left( 1 + \frac{I}{K_i} \right) + K_{AB} \left( 1 + \frac{I}{K_i} \right) + K_B A + AB} \right)}{\left( \frac{V_{1AB}}{K_{iA}K_B + K_{AB} + K_B A + AB} \right)} \quad (23b)$$

$$K_i = \frac{I (1 - i)}{i \left( 1 + \frac{K_B A}{K_{iA}K_B + K_{AB}} + \frac{AB}{K_{iA}K_B + K_{AB}} \right)} \quad (24)$$

fractional inhibitions for those compounds which inhibited the reaction less than 20%. Into the expression for fractional inhibition,  $i = (1 + v_i/v) = (v - v_i)/v$ , were substituted the expressions for  $v$  and  $v_i$  appropriate for this enzyme reaction and for competitive inhibitors with respect to PP-ribose-P; when solved for  $K_i$  this expression is given in Equation 24. This method becomes increasingly inaccurate as the fractional inhibition decreases, but for these weak inhibitors the  $K_i$ 's calculated from double reciprocal plots are also of limited accuracy.

The structural formulae for the classes of PP-ribose-P analogs tested are given in Figure 26, and their dissociation constants relative to that of PP-ribose-P are given in Table VI. D-ribose,  $\alpha$ -D-ribose 1-phosphate,  $\beta$ -D-ribose 1-phosphate and 6-phosphoglucono- $\gamma$ -lactone (the predominant form of 6-phosphogluconic acid in neutral aqueous solution (108)) did not bind to free enzyme. Ribose 5-phosphate bound to free enzyme more than 15 times more effectively than did ribose or the 1-phosphate derivatives of ribose. Ribose-, fructose-, and glucose-diphosphates bound approximately 20 times more effectively than did the corresponding 5- and 6-phosphate derivatives. PP-ribose-P bound 600 times more tightly to the enzyme than did the most effective inhibitor, ribose 1,5-diphosphate. These results suggest that the following structural features of PP-ribose-P are involved in binding to the

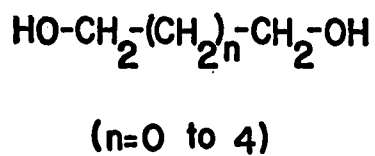
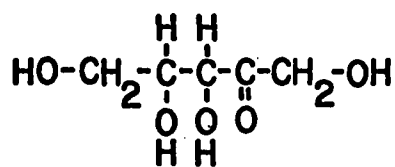
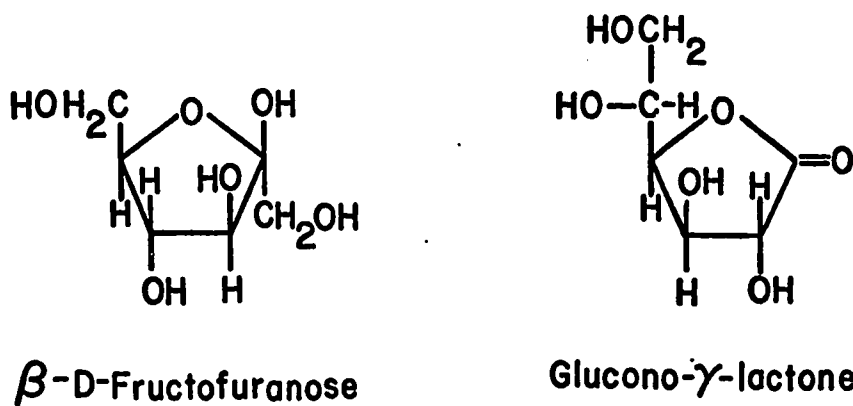
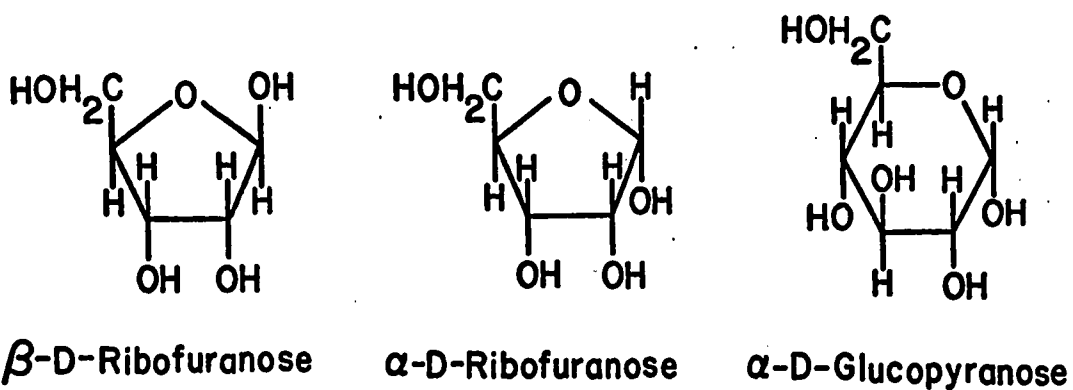


Figure 26. Structural formulae for classes of PP-ribose-P analogs.



TABLE VI: Inhibition Constants for Analogs of PP-ribose-P

Compound	$K_i$ (mM)	$K_i$ analog/ $K_i$ PP-ribose-P
PP-Ribose-P	0.0002	(1)
D-Ribose	> 50	> 250,000
$\alpha$ -D-Ribose 1-phosphate	> 50	> 250,000
$\beta$ -D-Ribose 1-phosphate	> 50	> 250,000
D-Ribose 5-phosphate	3.3	16,500
D-Fructose 6-phosphate	5.1	25,500
D-Glucose 6-phosphate	5.1	25,500
D-Ribose 1,5-diphosphate	0.12	600
D-Ribulose 1,5-diphosphate	0.41	2,050
D-Fructose 1,6-diphosphate	0.20	1,000
D-Glucose 1,6-diphosphate	0.42	2,100
6-Phosphogluconic acid	> 50	> 250,000
Ethylene glycol diphosphate	24	120,000
1,3-Propanediol diphosphate	1.2	6,000
1,4-Butanediol diphosphate	0.45	2,250
1,5-Pentanediol diphosphate	0.39	1,950
1,6-Hexanediol diphosphate	0.38	1,900
2-Pentanone 1,5-diphosphate	0.26	1,300

The inhibition constants were determined as described in the text.

enzyme.

1. The di-ionized phosphate at the 5-carbon position appears to be involved in binding since ribose 5-phosphate bound approximately 15 times more effectively than did D-ribose,  $\alpha$ -D-ribose 1-phosphate and  $\beta$ -D-ribose 1-phosphate. Whether this group binds through an anionic-cationic bond or as an electron donor in a hydrogen bond cannot be evaluated. At the magnesium ion concentration used, there would not be a significant concentration of the Mg-PP-ribose-P-Mg complex present.

2. The pyrophosphate moiety on the 1-carbon position appears to make the largest contribution to binding since PP-ribose-P bound 600 times more tightly to the enzyme than did ribose 1,5-diphosphate, and 16,500 times more tightly than did ribose 5-phosphate. The pyrophosphate moiety could be bound to the enzyme through the magnesium ion which chelates to the pyrophosphate moiety, through the ionized group on the pyrophosphate moiety, or through both. An ionized group on the pyrophosphate moiety appears to be involved in binding since ribose-, fructose- and glucose-diphosphates bound approximately 20 times more effectively than did the corresponding 5- and 6-phosphate derivatives. At the magnesium ion concentration used, none of these compounds would be in the form of their magnesium ion complexes. An alternate explanation for the observation that PP-ribose-P bound

600 times more tightly to the enzyme than did ribose 1,5-diphosphate is that the binding of a mono-ionized pyrophosphate is favored over that of a di-ionized phosphate group.

3. The distance between the ionized groups at the 1- and 5-carbon positions may also be important in binding since of the aliphatic diol diphosphates tested those with a maximum distance of approximately 9.3 A or greater between phosphorous atoms (Figure 27) were the most effective inhibitors. The maximum distance between the phosphorous atoms of glucose-, fructose- and ribose-diphosphates were estimated from CPK atomic models; only for glucose 1,6-diphosphate (8.8 A) is this distance significantly less than 9.3 A. The different distances between phosphate groups may explain the observation that ribose 1,5-diphosphate (9.2 A) and fructose 1,6-diphosphate (10.5 A) bound 2- and 3-fold, respectively, more effectively than did glucose 1,6-diphosphate. Other explanations are, however, possible.

4. Although the contribution to binding by the ribofuranose moiety is much less than that of the 1-pyrophosphate and 5-phosphate moieties, ribose- and fructose-diphosphates, both containing a furanose ring, bound 2- to 3-fold more effectively than did glucose-diphosphate, which contains a pyranose ring. In addition, replacement of the hydrogens on the 2-carbon of 1,5-pentanediol diphosphate by a keto group to give 2-pentanone 1,5-diphosphate resulted in a

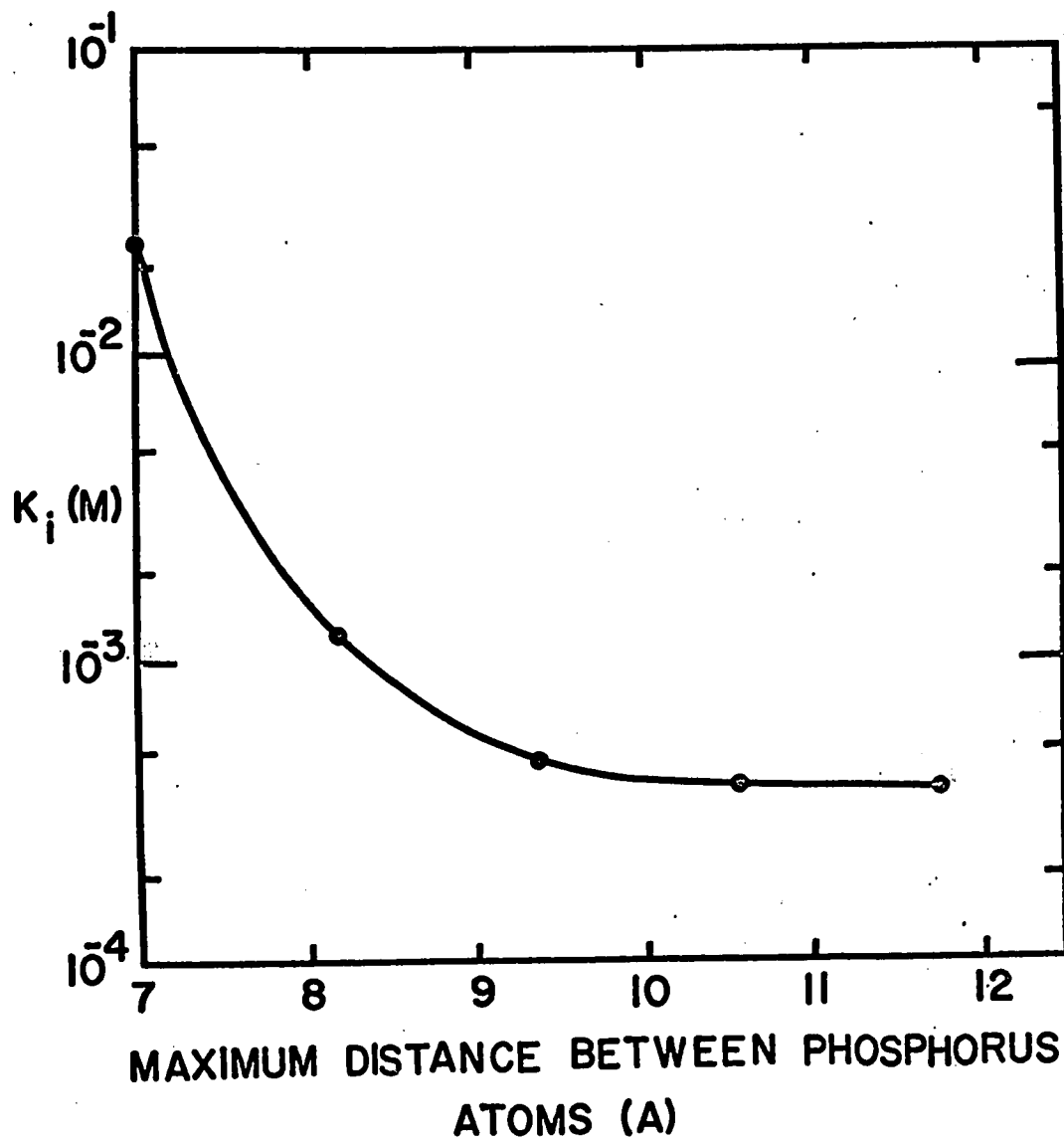


Figure 27. Relationship between inhibition constants and maximum distance between phosphorus atoms of aliphatic diol diphosphates.

50% increase in binding, as indicated by inhibition constants. The significance of a 2-fold or less increase in inhibition constant is, however, doubtful.

C. The effect of pH on the Michaelis constant of PP-ribose-P

Enzymes are generally active only over a limited range of pH, and in most cases a definite optimum pH is observed. That there is such an optimum pH may be due to an effect of pH on the maximum velocity, an effect on the affinity of enzyme for substrate, or an effect on the stability of the enzyme. All of these effects on enzymes are due to changes in the state of ionization of the free enzyme, the enzyme-substrate complex, or the substrate. Dixon (32, 33) and Laidler (87) have shown that the ionization constants of amino acid side chains involved in substrate binding or in the catalytic mechanism may be determined from plots of the negative logarithm of the Michaelis constant ( $pK_M$ ) versus pH. It must be remembered, however, that groups which do not ionize at all, and groups which do not ionize over the pH range studied, may be involved in substrate binding, and that their presence will not be indicated by these plots. The ionization constants ( $K_a$ 's) of the amino acid side chains may also be altered due to interaction with neighboring groups, thus making a positive identification solely on the basis of these data somewhat tenuous.

From double reciprocal plots similar to those shown in

Figure 6, the Michaelis constants for PP-ribose-P were determined at various pH values from 5.5 to 10.7. The negative logarithm of the Michaelis constants ( $pK_A$ ) were plotted as a function of pH as described by Laidler (87) and Dixon (32). Dixon (32) has shown that these plots consist of sections of straight lines with integral slopes joined by short curved parts. The intersections of straight portions of different slopes correspond to ionization constant values; inflections concave to the pH axis correspond to ionization constants for enzyme or substrate, whereas those convex to the pH axis correspond to ionization constants for the enzyme-substrate complex. The shape of these curves is such that the graph misses the intersection point of the straight segments by a vertical distance of 0.3 units. The graph of pH versus  $pK_A$ , as shown in Figure 28, shows two ionization constants at 30°, 9.35 and approximately 6. The ionization constant of 6 is due to ionization of the enzyme-substrate complex and its significance will be discussed in the section on the catalytic mechanism. The ionization constant of 9.35 is believed to be that of some group on the free enzyme associated with PP-ribose-P binding because PP-ribose-P does not ionize at this pH. The Michaelis constant for PP-ribose-P is equal to  $(k_3k_4)/k_1(k_3 + k_4)$ , which reduces to  $V_1/k_1$  since  $V_1$  is equal to  $(k_3k_4)/(k_3 + k_4)$ . It will be shown later that  $\log V_1$  does not change as a function of pH in this region, and the

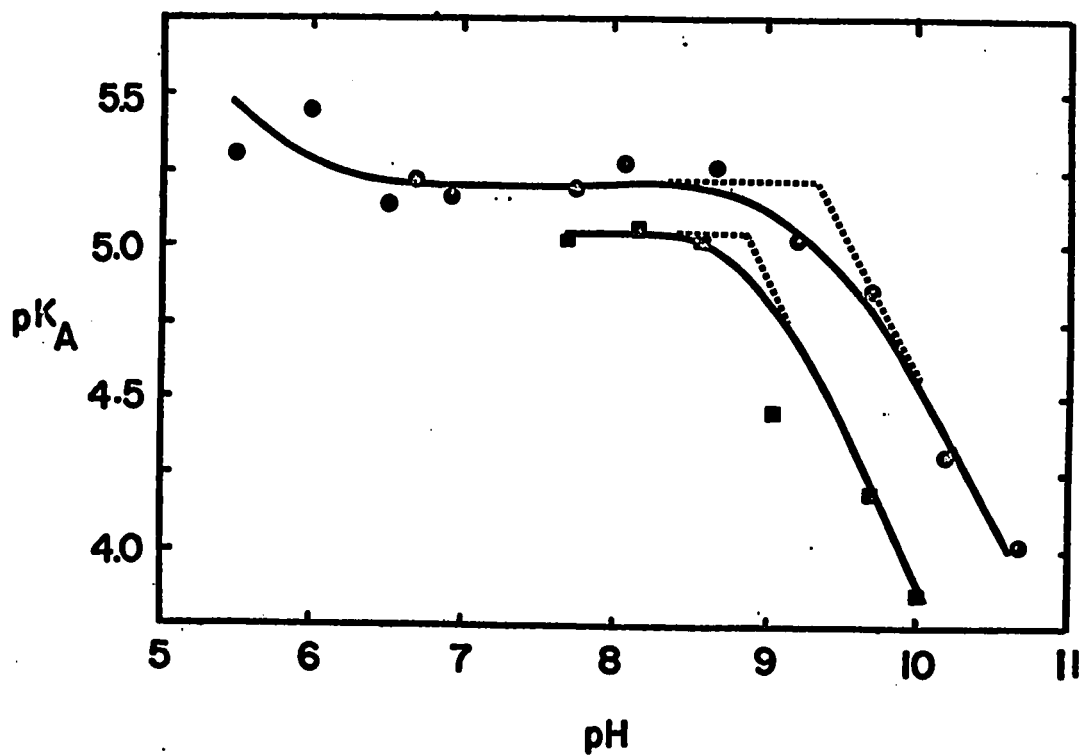


Figure 28. Effect of pH on the negative logarithm of the Michaelis constant for PP-ribose-P at 30° (●) and 40° (■).

enzyme group that is ionizing at a pH 9.35 must therefore be associated with  $k_1$ , the rate constant which describes the addition of PP-ribose-P to free enzyme.

The  $pK_a$  value of 9.35 obtained from these plots is close to those found in proteins for the sulfhydryl group of cysteine, 7.2 to 10.2, the  $\alpha$ -amino group of N-terminal residues, 7.6 to 8.4, and the  $\epsilon$ -amino group of lysine, 9.4 to 10.6 (34). Caution, however, must be exercised before any one of these groups can be chosen solely on the basis of its  $pK_a$  value. Webb (135) and Benesch and Benesch (11) have shown that the  $pK_a$  of sulfhydryl groups is markedly dependent on vicinal ionic groups. For example, a sulfhydryl group near a positively charged group usually has a  $pK_a$  value from 7.2 to 8.5, a sulfhydryl group in no electric field, a  $pK_a$  value from 8.2 to 9.2, and a sulfhydryl group near a negatively charged group, a  $pK_a$  value from 9.2 to 10.2. Similar changes of ionization constants have been observed for other amino acids.

Dixon and Webb (33) have suggested that valuable clues to the identity of the group responsible for a particular ionization may be obtained by determining its heat of ionization. This is done by determining the ionization constant at two or more temperatures and using the equation  $\Delta H = -2.303 RT^2 (dpK_a/dT)$ . From the data presented in Figure 28 a heat of ionization of  $+18,800 \text{ cal mole}^{-1}$  was calculated for the group of adenine phosphoribosyltransferase which ionizes at pH 9.35.



This value is much higher than the heats of ionization of any of the amino acid residues reported by Steinhardt and Beychok (121). Both  $\epsilon$ - and  $\alpha$ -amino groups have heats of ionization of approximately  $+13,000 \text{ cal mole}^{-1}$  (121) while sulfhydryl groups have heats of ionization of approximately  $+6,500 \text{ cal mole}^{-1}$  (11). As Lindley (93) has pointed out, if two groups with different heats of ionization are vicinal in a protein molecule, the apparent heats of ionization of the two groups will approach an intermediate value. This observation however, does not explain the very high heat of ionization observed for the group on adenine phosphoribosyltransferase which ionizes at pH 9.35. It is of interest, furthermore, that Dyson and Noltman (37) obtained heats of ionization, for the same group in phosphoglucoisomerase, of  $4,100 \text{ cal mole}^{-1}$  in the reverse reaction and  $11,300 \text{ cal mole}^{-1}$  in the forward reaction; on the basis of the average of these values,  $7,700 \text{ cal mole}^{-1}$ , they inferred that there was a histidine residue at the active site.

Although at the present time no definitive explanation can be postulated for the high heat of ionization observed for adenine phosphoribosyltransferase, there are several technical difficulties which may affect the measurement of this value. The ionization constants determined (Figure 28) may deviate 0.05 pH units from the true value due to uncertainties in the graphical analysis; a deviation of up to

±20% in the heat of ionization could result. The accuracy of the determination of the pH values of the buffers used, which were measured before the reaction, depends on the standard buffer solution used in calibrating the pH meter and on the accuracy of the pH meter used. In this case the accuracy of the standard buffer was ±0.02 pH units and the limit of accuracy of the pH meter was ±0.01 pH units; these could produce a deviation up to ±10% in the experimentally determined heat of ionization. Finally, a variation of temperature during the incubations of only 0.25 degree could result in a deviation of ±13% in the calculated heat of ionization. If all of these errors were in the same direction, the error in the calculated heat of ionization would be ±8,100 cal mole<sup>-1</sup>. It is not inconceivable, therefore, that the true heat of ionization may be within the range of literature values for α- and ε-amino groups and close to the heats of ionization for sulfhydryl groups that are vicinal to amino groups not involved in substrate binding. These possible errors would have little effect on the determination of ionization constants, however; they would deviate by no more than ±0.08 pH units.

In summary, the pK<sub>a</sub> value of 9.35 obtained from plots of pK<sub>a</sub> versus pH is much higher than those normally found for the α-amino group of N-terminal residues in proteins but close to those values for the ε-amino group of lysine and for sulf-

hydryl groups near a negatively charged group or in no electric field. The heat of ionization for this group was calculated to be between +10,700 and +26,900 cal mole<sup>-1</sup>, the lower value of which is close to the literature values for  $\alpha$ - and  $\epsilon$ -amino groups but much higher than those for sulfhydryl groups near a negatively charged group or in no electric field. The heat of ionization is close to that expected for a sulfhydryl group near an amino group; however, the  $pK_a$  value is much higher than would be expected for a sulfhydryl group near a positively charged amino group. These data therefore suggest but do not prove, that the group of  $pK_a$  9.35 is an  $\epsilon$ -amino group of lysine.

D. The effect of protein reagents on the activity of adenine phosphoribosyltransferase

So called "group-specific" protein reagents have often been used in attempts to identify amino acid residues at the active sites of enzymes. The term "group-specific" is somewhat of a misnomer, however, since it implies that a particular reagent modifies a single type of functional group of a protein to the exclusion of all other types of functional groups. This is in fact the exception rather than the rule.

Substrate or inhibitor protection against inactivation by protein reagents may be used to implicate certain functional groups in the catalytic mechanism or in substrate

binding, but this type of experiment may be beset with problems of interpretation. Protection by substrates does not provide adequate evidence by itself that the functional group in question is part of the active site since substrate binding may slow down or prevent reaction of the reagent with neighboring or distant groups by steric hindrance or by causing protein conformational changes which make the particular functional group in question unavailable for modification. Protection of the enzyme by substrates against inactivation by various protein reagents may however permit a choice to be made among several functional groups which have been implicated in substrate binding or in the catalytic mechanism on the basis of independent evidence.

1. Sulfhydryl reagents.

Because the data presented above suggested that a sulfhydryl group of cysteine, and N-terminal  $\alpha$ -amino group, or an  $\epsilon$ -amino group of lysine might be involved in PP-ribose-P binding, substrate protection against enzyme inactivation by various protein reagents was investigated in a further attempt to decide which of these was involved in PP-ribose-P binding. Protection against inactivation by p-hydroxymercuribenzoate was measured by incubating various reaction mixtures containing the enzyme preparation for 20 minutes at 30° in the presence of PMB, after which the reaction mixtures were completed and assayed by the normal radioisotope

method. Table VII shows the protection by PP-ribose-P and analogs of PP-ribose-P against inactivation by PMB. In all cases magnesium ion was present during the first incubation period. PP-ribose-P at  $10^{-5}$  M (approximately  $2 K_A$  and  $50 K_{iA}$ ) protected the enzyme against PMB inactivation, as did the competitive inhibitors, ribose 1,5-diphosphate and fructose 1,6-diphosphate; magnesium ion was not required for protection by the latter. These observations are consistent with the inhibition studies and suggest that these PP-ribose-P analogs bind to the PP-ribose-P binding site.

Ribose 5-phosphate did not protect the enzyme against PMB inactivation, whereas substantial protection was observed by pyrophosphate. Together, ribose 5-phosphate and pyrophosphate protected the enzyme to a degree more than the sum of the individual degrees of protection. If ribose 5-phosphate and pyrophosphate together may be regarded as a PP-ribose-P analog, it may be postulated that the pyrophosphate moiety bound to the enzyme first and that this caused a conformational change in the protein which permitted the ribose 5-phosphate moiety to bind. These observations may also suggest that pyrophosphate does bind to the active site of the free enzyme.

In a previous chapter it was suggested that adenine phosphoribosyltransferase contains two sulfydryl groups, both of which are prevented by PP-ribose-P from reacting

TABLE VII: Protection by PP-ribose-P and Analogs of PP-ribose-P Against Enzyme Inactivation by PMB

PP-ribose-P ( $10^{-5}$ M)	Additions				PMB (M)	Enzyme Activity (% of Control)
	PP <sub>i</sub> ( $10^{-3}$ M)	R 5-P ( $10^{-3}$ M)	R 1,5-diP ( $10^{-3}$ M)	F 1,6-diP ( $10^{-3}$ M)		
-	-	-	-	-	$5 \times 10^{-6}$	17
+	-	-	-	-	$5 \times 10^{-6}$	100
-	-	-	-	-	$10^{-3}$	0
+	-	-	-	-	$10^{-3}$	67
-	-	-	+	-	$5 \times 10^{-6}$	64
-	-	-	-	+	$5 \times 10^{-6}$	52
-	-	+	-	-	$5 \times 10^{-6}$	17
-	+	-	-	-	$5 \times 10^{-6}$	63
-	+	+	-	-	$5 \times 10^{-6}$	91

Two  $\mu$ g of enzyme preparation per reaction mixture was incubated with PMB for 20 min at 30° in 0.1 M Tris-HCl buffer, pH 7.4, in the presence of the additions indicated. The reaction mixtures were completed and enzyme activity was assayed by the radioisotope method; the concentrations of adenine and magnesium ion were  $2 \times 10^{-6}$  M and  $10^{-3}$  M, respectively. The abbreviations used are R 5-P, ribose 5-phosphate; R 1,5-diP, ribose 1,5-diphosphate; and F 1,6-diP, fructose 1,6-diphosphate.

with PMB. Stadtman (120) and Storey (123) have suggested that inhibition by sodium arsenite may be used to demonstrate the presence of sulfhydryl groups which are close enough to form a thioarsenite derivative. Sodium arsenite at concentrations of  $10^{-3}$  and  $10^{-4}$  M, incubated with the enzyme but without substrates for 20 minutes at  $30^{\circ}$ , did not inhibit the enzyme. These observations suggest that the two sulfhydryl groups are not vicinal, but are not unequivocal proof of this. Webb (137) points out that substituted arsenoxides may react with some vicinal sulfhydryl groups with which sodium arsenite will not react, and also that adjacent protein cationic groups may prevent these reagents from reacting with vicinal sulfhydryl groups.

Table VIII shows the reversal of PMB inactivation by glutathione and 2-mercaptoethanol. At a PMB concentration of  $5 \times 10^{-5}$  M, at which 100% inactivation was observed, neither  $10^{-4}$  M glutathione nor  $10^{-4}$  M 2-mercaptoethanol significantly reversed the inactivation. At  $10^{-7}$  M PMB, at which only 53% inactivation occurred,  $10^{-4}$  M 2-mercaptoethanol completely reversed the inactivation. Webb (136) has suggested that, although PMB will react with other functional groups, sulfhydryl groups are the primary sites of mercurial binding and that no other protein functional groups can effectively compete with sulfhydryl groups for mercurials. Because the enzyme was inhibited by very dilute solutions of

TABLE VIII: Reversal of Enzyme Inactivation Caused by PMB

PMB (M)	Reduced Glutathione ( $10^{-4}$ M)	2-Mercaptoethanol ( $10^{-4}$ M)	Enzyme Activity (% of Control)
0	-	-	100
$5 \times 10^{-5}$	-	-	0
$5 \times 10^{-5}$	+	-	8
$5 \times 10^{-5}$	-	+	6
$10^{-7}$	-	-	47
$10^{-7}$	-	+	99

Four  $\mu$ g of enzyme preparation per reaction mixture was incubated with PMB for 20 min at 30° in 0.1 M Tris-HCl buffer, pH 7.4. Either glutathione or 2-mercaptoethanol was added and the incubation was continued for 20 min at 30°. The reaction mixtures were completed and enzyme activity was assayed by the radioisotope method; the concentrations of adenine, magnesium ion and PP-ribose-P were  $8 \times 10^{-7}$  M,  $10^{-5}$  M and  $10^{-5}$  M, respectively.



PMB ( $10^{-7}$  M) and the inactivation was reversed by 2-mercaptoethanol, it is believed that cysteine was the amino acid modified by PMB. The observation that less activity became recoverable as the percent inactivation increased may have several explanations. As the inactivation progresses a larger number of sulfhydryl groups may be modified and this may cause denaturation of the protein. It is also possible that at high PMB concentrations groups other than sulfhydryls may be modified.

## 2. Sodium borohydride

Grazi et al. (50) have shown that for aldolase in which the enzyme forms a Schiff base with the substrate, borohydride reduces the Schiff base to a stable enzyme-substrate bond. Inactivation by the reagent is therefore accelerated in the presence of substrate, and the possibility that this might also occur in adenine phosphoribosyltransferase was investigated. Table IX shows the effect of sodium borohydride on enzyme activity and the effect of substrates on the rate of inactivation by sodium borohydride.

These experiments were carried out at pH 7.0 and 0° for 20 minutes; 0.02 ml of 1 M sodium borohydride, followed immediately by 0.01 ml of 2 M acetic acid, were added twice to reaction mixtures containing buffer, enzyme and the substrates indicated in the table in a total volume of 0.40 ml. The reaction mixtures were completed and assayed by the normal

TABLE IX: Inactivation of Adenine Phosphoribosyltransferase by Sodium Borohydride

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<u>Addition</u>	<u>Enzyme Activity (% of Control)</u>
None	21
Mg <sup>++</sup>	19
PP-ribose-P	20
PP-ribose-P + Mg <sup>++</sup>	28

---

Two  $\mu\text{g}$  of enzyme preparation per reaction mixture was incubated with sodium borohydride for 20 min at  $0^\circ$  in 1 M Tris-HCl buffer, pH 7.4, in the presence of the additions indicated. The reaction mixtures were completed and enzyme activity assayed by the radioisotope method; the concentrations of adenine, magnesium ion and PP-ribose-P were  $10^{-5}$  M,  $10^{-3}$  M and  $10^{-4}$  M, respectively.

radioisotope method. Acetic acid was added during the reduction, as suggested by Horecker et al. (61), in order to keep the pH of the reaction mixtures constant. Because magnesium ion, PP-ribose-P and PP-ribose-P plus magnesium ion, did not alter the rate of borohydride reduction, it is not likely that a Schiff base intermediate is involved in the enzymatic reaction.

Light and Sinha (92) have shown that sodium borohydride selectively reduces two disulfide bonds in trypsinogen and trypsin, and the possibility that sodium borohydride inactivated adenine phosphoribosyltransferase by reduction of a disulfide bond essential for activity was investigated. Free sulfhydryl groups in reduced and nonreduced enzyme preparations were estimated by the method of Ellman (39) in which the protein is reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) and the optical density at 412 m $\mu$  measured. A 25% increase in free sulfhydryl groups was found on reduction, which suggests that sodium borohydride reduced one or more disulfide bonds which are essential for enzymatic activity; it must be recalled, however, that the enzyme preparation used was not pure.

### 3. Other protein reagents

Protection by substrates against enzyme inactivation by other protein reagents was also investigated. Those amino acids which are modified by these reagents, according

to the literature, are listed in Table X.

Reaction mixtures containing enzyme and various combinations of substrates were incubated for 20 minutes at 30° in the presence and absence of protein reagents. The reaction mixtures were then completed and assayed for enzyme activity by the normal radioisotope method. Table XI shows the effects of these protein reagents on enzyme activity.

A concentration of 18 mM N-acetylimidazole was required to decrease enzymatic activity by 50%, and PP-ribose-P did not protect against this inactivation. This reagent has been shown to acylate phenolic hydroxyl groups,  $\alpha$ - and  $\epsilon$ -amino groups, and sulfhydryl groups (28,113). A sulfhydryl group does not appear to be the group modified since PP-ribose-P did not alter the rate of inactivation. Whether acylation of tyrosine, lysine, or an N-terminal amino acid was responsible for the reduction of enzymatic activity cannot be determined from these data.

Trinitrobenzene sulfonic acid completely inactivated the enzyme, but in the presence of  $10^{-6}$  M PP-ribose-P, 31% protection was observed. This reagent has been reported to react preferentially with all free primary amino groups (28), but Kotaki, Horada and Yagi (85) have shown that it also reacts with sulfhydryl groups at neutral or alkaline pH. Inactivation of the enzyme by this reagent may therefore be due to modification of a sulfhydryl or amino group. Almost

TABLE X: Amino Acids Modified by Protein Reagents as Reported in the Literature

Reagent	Amino acids modified (a)								Ref.
	Free amino groups	Tyrosine	Cysteine	Serine	Methionine	Tryptophan	Histidine	Arginine	
N-acetyl imidazole	+	+	+						28,113
Trinitrobenzene sulfonic acid	+		+						28,85
Dansyl chloride	+	+	+	+			+		28,49
Salicylaldehyde	+								28,99, 139
Ethyl thioltri-fluoroacetate	+								28,45
2-Chloro-3,5-dinitropyridine	+								117
2-Hydroxy-5-nitrobenzylbromide			+		+				28
Diazonium-1-H-tetrazole	+	+	+	+	+	+	+	+	66
N-Bromosuccinimide	+	+	+	+	+	+	+	+	28,118

(a) The rates of modification depend on the reaction conditions.

TABLE XI: Protection by PP-ribose-P Against Enzyme Inactivation by Other Protein Reagents

Reagent	Conc. (M)	Buffer	pH	PP-ribose-P ( $10^{-5}$ M)	Enzyme Activity (% of Control)
N-Acetylimidazole	$1.8 \times 10^{-2}$	A	7.4	-	46
	$1.8 \times 10^{-2}$	A	7.4	+	48
Trinitrobenzene-sulfonic acid	$3.1 \times 10^{-3}$	A	7.4	-	0
	$3.1 \times 10^{-3}$	A	7.4	+	31
5-Dimethylaminonaphthalene-1-sulfonyl chloride	$1.8 \times 10^{-3}$	A	10.3	-	21
	$1.8 \times 10^{-3}$	B	10.0	-	11
	$1.8 \times 10^{-3}$	B	8.0	-	63
Salicylaldehyde	$7.2 \times 10^{-3}$	A	7.4	-	1
Ethyl thioltrifluoroacetate	(10% (v/v))	A	10.3	-	5
	(10% (v/v))	B	10.0	-	9
	(10% (v/v))	B	8.0	-	11
2-Chloro-3,5-dinitropyridine	$2.5 \times 10^{-3}$	A	10.3	-	1
	$2.5 \times 10^{-3}$	B	10.0	-	8
	$2.5 \times 10^{-3}$	B	8.0	-	5
2-Hydroxy-5-nitrobenzylbromide	$4.3 \times 10^{-3}$	A	6.8	-	62
	$8.6 \times 10^{-3}$	C	6.8	+	0
	$8.6 \times 10^{-3}$	C	6.8	-	0
Diazonium-1-H-tetrazole	$7.2 \times 10^{-3}$	A	7.4	-	60

Two  $\mu$ g of enzyme preparation per reaction mixture was incubated with the protein reagents indicated and at the pH indicated for 20 min at 30° in the absence and presence of PP-ribose-P. The reaction mixtures were completed and enzyme activity assayed by the radioisotope method; the concentrations of adenine and magnesium ion were  $10^{-6}$  M and  $10^{-3}$  M respectively. The buffers used were: A, Tris-HCl 0.1 M; B, Carbonate 0.1 M; and C, Tris-Glycine-Histidine 0.1 M.

complete inactivation was obtained by incubating the enzyme preparation with 5-dimethylamino-naphthalene-1-sulfonyl (dansyl)chloride, salicylaldehyde, ethyl thioltrifluoroacetate and 2-chloro-3,5-dinitropyridine, all of which react with amino groups (28,117). In addition to amino groups, dansyl chloride has been shown to react with phenolic hydroxyls, sulfhydryls and imidazoles (49), and ethyl thioltrifluoroacetate has been shown to participate in extensive disulfide exchange (45). Salicylaldehyde has been shown to react with primary amines to form a Schiff base (99,139) but the specificity of this reagent and of 2-chloro-3,5-dinitropyridine has not been investigated.

Complete inactivation was obtained with 8.6 mM 2-hydroxy-5-nitrobenzyl bromide; it has been reported to alkylate cysteine, methionine and tryptophan rapidly in acidic or neutral media, and in addition to alkylate tyrosine in alkaline media (28). Since the reaction was carried out at pH 6.8, tyrosine would not be alkylated; cysteine is not likely to have been modified since PP-ribose-P did not protect against inactivation. These observations imply that inactivation was due to alkylation of either methionine or tryptophan.

Diazonium-1H-tetrazole is reported to complex with histidine and tyrosine to produce intensely colored products; however, Howard and Wild (66), in 1957, reported that this reagent can react equally as well with many other functional

groups, but without producing spectral changes. For example, diazonium-1H-tetrazole can react with the  $\epsilon$ -amino group of lysine,  $\alpha$ -amino groups, guanidino groups, indole groups, and can also form unstable S-azo derivatives with sulfhydryl groups, and oxidize aliphatic hydroxyl groups to aldehydic groups. Diazonium-1H-tetrazole was found to inhibit the enzyme 40% but because of the large number of amino acid residues that can be modified, interpretation is difficult if not impossible.

In summary, the plot of pH versus the negative logarithm of the PP-ribose-P Michaelis constant suggested that a protein functional group which ionizes at pH 9.35 is involved in PP-ribose-P binding; it was tentatively identified as an N-terminal  $\alpha$ -amino group, a lysine  $\epsilon$ -amino group or a cysteine sulfhydryl group. On the basis of the value of the heat of ionization this group was tentatively identified as lysine. PP-ribose-P protection against enzyme inactivation by trinitrobenzene sulfonic acid may also indicate that the group involved in binding of this substrate is the  $\epsilon$ -amino of lysine. Because this reagent may also react with sulfhydryl groups and because of substrate protection against enzyme inactivation by PMB, the protein group modified by trinitrobenzene sulfonic acid cannot, however, be positively identified as a lysine residue. It is unlikely that both a lysine and cysteine are involved in PP-ribose-P binding



since, if this were the case, two ionization constants would be expected to be observed in plots of pH versus the negative logarithm of the Michaelis constant for PP-ribose-P. PP-ribose-P did protect the enzyme against inactivation by PMB but it cannot be determined from the evidence presented whether this would occur because the sulfhydryl group is involved in PP-ribose-P binding, whether the enzyme undergoes a conformational change due to PP-ribose-P binding, or simply due to steric hindrance after PP-ribose-P binding.

#### E. Discussion

The evidence presented is consistent with the involvement of the magnesium-ion-1-pyrophosphate moiety, the 5-phosphate moiety, and the ribofuranose ring in substrate binding to enzyme. It is not possible from these data to determine if anionic-cationic interactions, hydrogen bonding or charge-transfer bonding between the enzyme and the ionized 5-phosphate and 1-pyrophosphate moieties are involved in substrate binding. The ribofuranose ring is the least important moiety involved in binding and its most important role may be to keep the 5-phosphate and magnesium-ion-1-pyrophosphate groups at a critical distance apart.

Baker (6) has postulated that di- and mono-ionized phosphate groups may complex with amino acid side chains through anionic-cationic, charge-transfer and hydrogen bonding. In charge-transfer and hydrogen bonding, phosphate

groups generally act as electron donors but they may also act as electron acceptors via the ester oxygen. The only protein groups to which phosphate may not complex are the di-carboxylic amino acids, the aliphatic amino acids and phenylalanine. It could conceivably bind, therefore, to any one of thirteen other amino acids, to the polyamide backbone of the protein, or to any N-terminal amino group. Those amino acids to which metal ions have been shown to bind are histidine in some heme proteins with iron, and cysteine in carboxypeptidase A and carbonic anhydrase with zinc (128). The pyrophosphate moiety of PP-ribose-P could bind, therefore, to either an amino or a sulfhydryl group on the enzyme through magnesium ion. Although the actual amino acid side chains involved in the binding of substrates and inhibitors or the type of interaction cannot be determined, these structure-activity studies may be useful in the design of inhibitors of adenine phosphoribosyltransferase.

At pH values substantially below 9.35 the amino group of lysine would be protonated and thus carry a positive charge; in this form it could be involved in binding the ionized phosphate group of PP-ribose-P through an anionic-cationic type of bond, whereas substantially above pH 9.35 the amino group would have no net charge and PP-ribose-P may lose one point of attachment with the enzyme. The sulfhydryl group of cysteine would be protonated at pH values substantially below

pH 9.35 and could be involved in a hydrogen bond with the substrate, whereas substantially above pH 9.35 the sulfur group would carry a net negative charge which could result in anionic-anionic repulsion between PP-ribose-P and the enzyme.

It is not likely that either aspartic or glutamic acids are close to the PP-ribose-P binding site since anionic-anionic repulsion would be expected to inhibit substrate binding. The enzyme does apparently contain a disulfide bond which is essential for activity, and the inactivation by 2-hydroxy-5-nitobenzyl bromide suggests that modification of a methionine or tryptophan residue abolished activity. It is not likely that either of these residues are close to the binding site of PP-ribose-P since this substrate did not prevent or retard enzyme inactivation by this reagent.

## VI BINDING OF ADENINE TO ADENINE PHOSPHORIBOSYLTRANSFERASE

### A. Introduction

In order to identify those groups or atoms of adenine which are required for binding of this substrate to adenine phosphoribosyltransferase, the inhibition constants of a number of analogs of adenine were determined. Groups on the enzyme which were involved in adenine binding were investigated by analyzing the effects of pH on the Michaelis constant for adenine.

### B. Inhibition by analogs of adenine

Approximately 75 analogs of adenine were tested for possible inhibitory effects using the procedure described in the previous chapter; initial velocities were determined by the usual radioisotope method. The inhibition constants for these analogs were determined as described previously; Equations (25) and (26) are the double reciprocal forms of the rate equation for competitive inhibitors of adenine which bind only to the enzyme-PP-ribose-P complex, and Equation (28) is the corresponding expression for fractional inhibition. As examples of these experiments the plots for 6-N-hydroxylaminopurine (Figure 29), 7-aminoimidazo(4,5-d)pyridazine (Figure 30) and 4-amino-5-imidazolecarboxamide (Figure 31) are given. The structural formulae for those analogs with changes in the purine ring are given in Figure 32.

Rate Equations for Initial Velocity in the Presence of Dead-end Inhibitors Binding to Enzyme-PP-ribose-P Complex.

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{I}{K_i} + \frac{K_{iA}}{A} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right) \quad (25)$$

Since  $A = 10$   $K_A = 200$   $K_{iA}$

then  $\frac{K_{iA}}{A} = \frac{1}{200} = 0.005$

therefore  $\frac{E_0}{v} = \frac{K_B}{V_1 B} \left( 1 + \frac{I}{K_i} \right) + \frac{1}{V_1} \left( 1 + \frac{K_A}{A} \right)$  (26)

Inhibition Constants as a Function of Fractional Inhibition for Compounds Binding to Enzyme-PP-ribose-P Complex.

$$i = 1 - v_i/v = (v - v_i)/v \quad (27a)$$

$$i = \frac{\left( \frac{V_1 AB}{K_{iA} K_B + K_A B + K_B A + AB} \right) - \left( \frac{V_1 AB}{K_{iA} K_B + K_A B + K_B A \left( 1 + \frac{I}{K_i} \right) + AB} \right)}{\left( \frac{V_1 AB}{K_{iA} K_B + K_A B + K_B A + AB} \right)} \quad (27b)$$

$$K_i = \frac{I (1 - i)}{i \left( \frac{K_{iA}}{A} + \frac{K_A B}{K_B A} + 1 + \frac{B}{K_B} \right)} \quad (28)$$

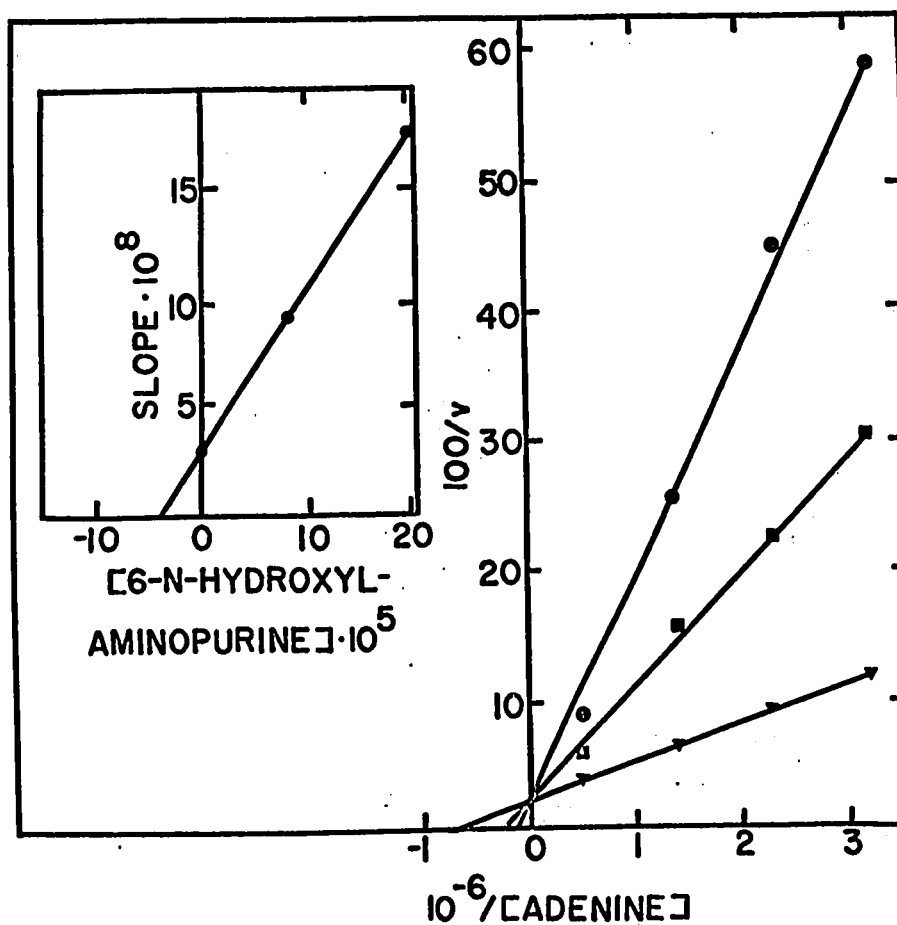


Figure 29. Inhibition by 6-N-hydroxylaminopurine. Double reciprocal plots of initial velocity against adenine concentration at a PP-ribose-P concentration of  $6 \times 10^{-5}$  M. The concentrations of 6-N-hydroxylaminopurine are: 0 ( $\Delta$ ),  $8 \times 10^{-5}$  M ( $\blacksquare$ ), and  $2 \times 10^{-4}$  M ( $\odot$ ).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of 6-N-hydroxylaminopurine.

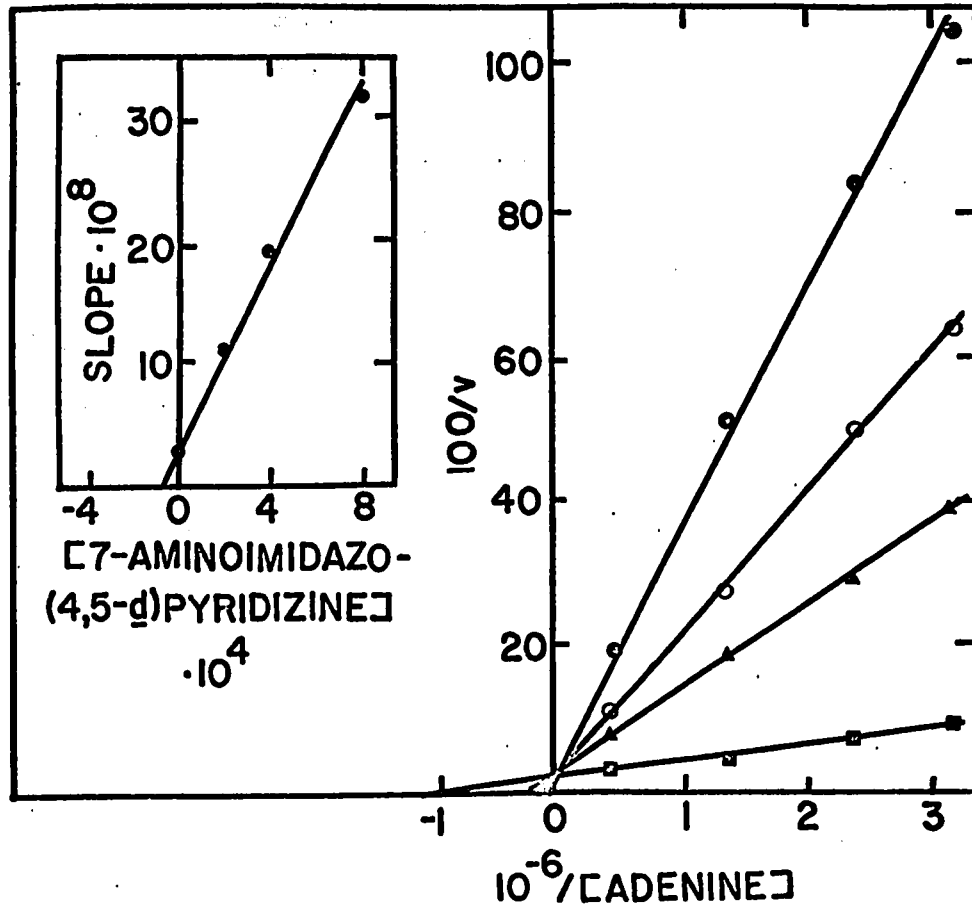


Figure 30. Inhibition by 7-aminoimidazo(4,5-d)pyridazine. Double reciprocal plot of initial velocity against adenine concentration at a PP-ribose-P concentration of  $6 \times 10^{-5}$  M. The concentrations of 7-aminoimidazo(4,5-d)pyridazine are: 0 ( $\blacksquare$ ),  $2 \times 10^{-4}$  M ( $\blacktriangle$ ),  $4 \times 10^{-4}$  M ( $\circ$ ), and  $8 \times 10^{-4}$  M ( $\odot$ ).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of 7-aminoimidazo(4,5-d)pyridazine.

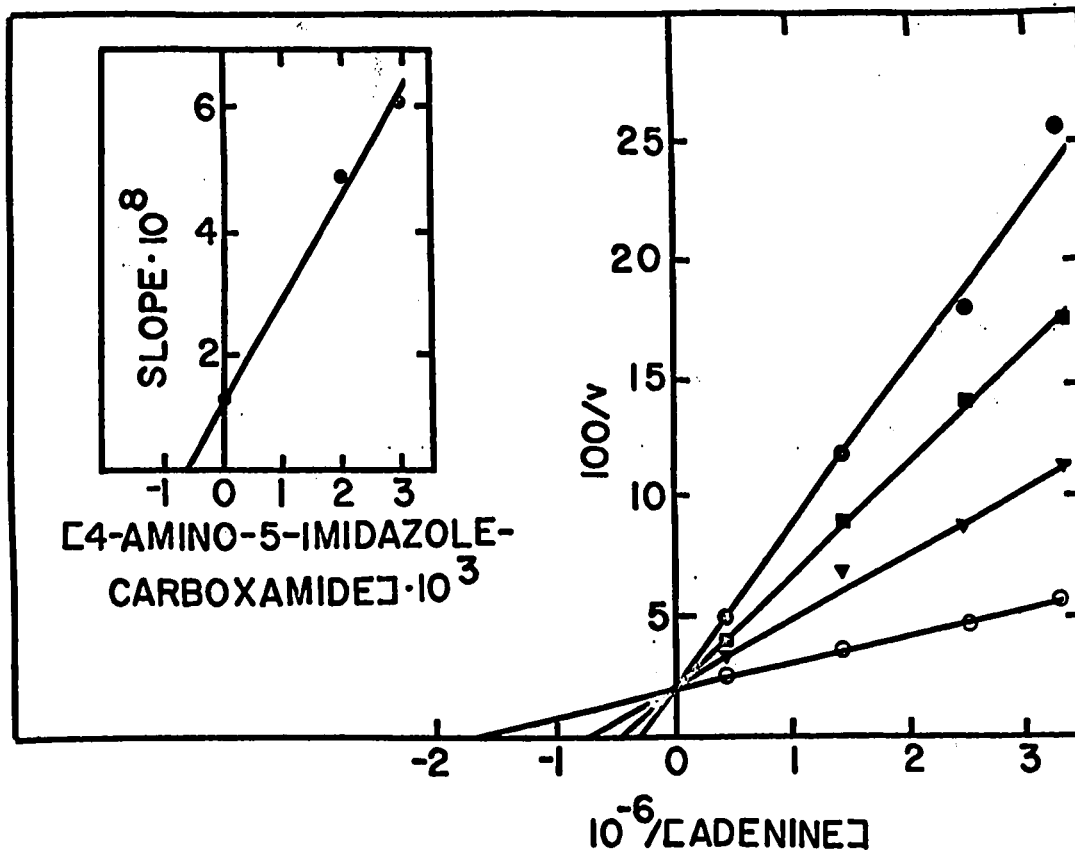


Figure 31. Inhibition by 4-amino-5-imidazolecarboxamide. Double reciprocal plots of initial velocity against adenine concentration at a PP-ribose-P concentration of  $6 \times 10^{-5}$  M. The concentrations of 4-amino-5-imidazolecarboxamide are: 0 (O),  $10^{-3}$  M ( $\Delta$ ),  $2 \times 10^{-3}$  M ( $\blacksquare$ ), and  $3 \times 10^{-3}$  M ( $\bullet$ ).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of 4-amino-5-imidazolecarboxamide.



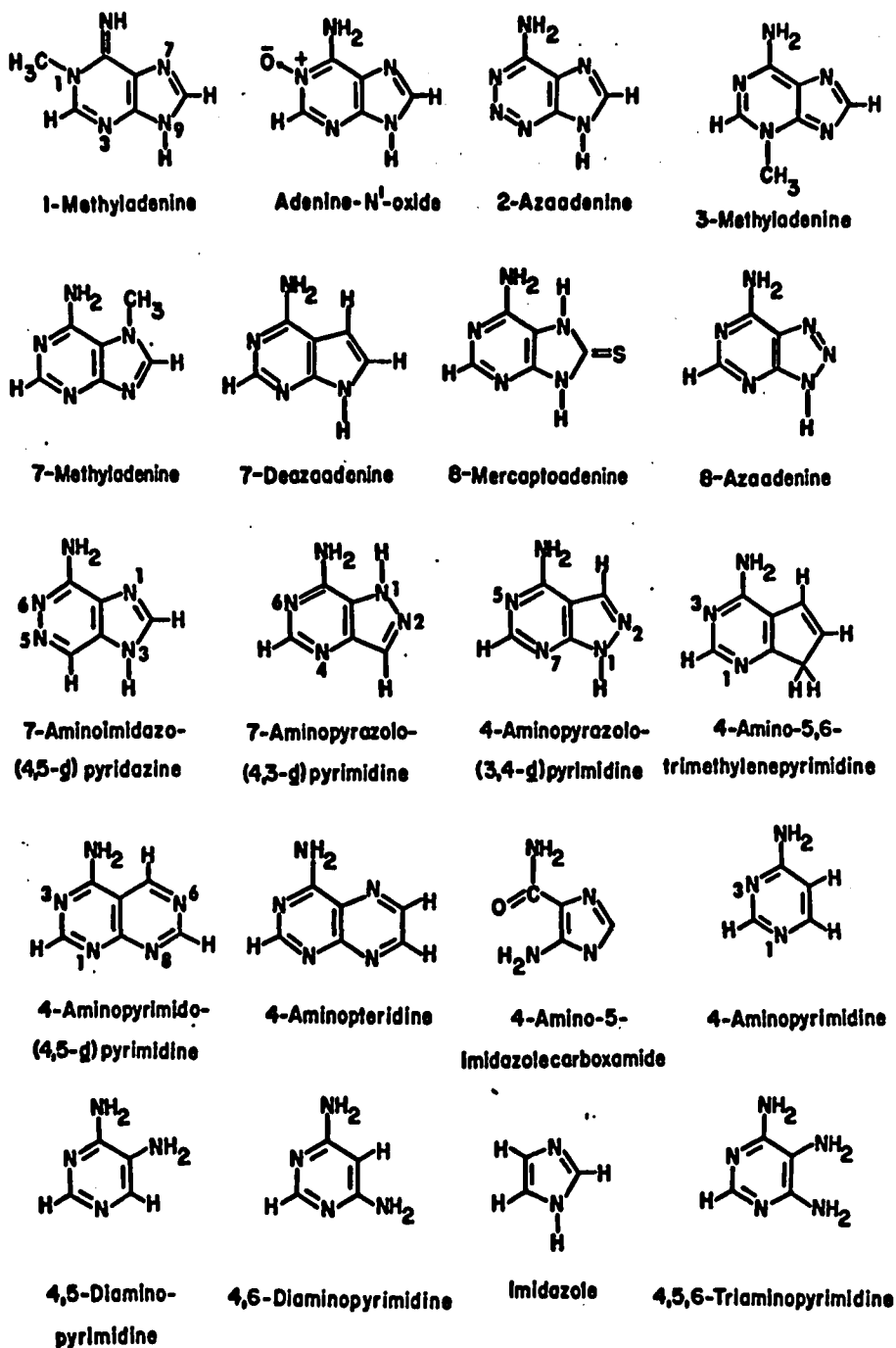


Figure 32. Structural formulae of adenine analogs with changes in the purine ring.

### 1. Ionization of analogs of adenine

Because ionization of substrates or inhibitors may greatly affect their binding to the enzyme, it is necessary to know the net charge on these analogs before comparing dissociation constants. Table 12 gives the literature values of ionization constants for some of the analogs used in this study and for compounds which are structurally similar to some of the analogs used; it may be seen that at the pH at which these studies were conducted (pH 7.4) most analogs would be expected to carry no net charge.

6-Chloro-, 6-mercapto-, 6-cyano-, and 8-azaadenine would, however, be at least partially in the anionic form due to ionization of the 9-NH group, and the possibility was considered that their inhibition constants might vary with the extent of ionization. Figure 33 shows that the negative logarithm of the inhibition constant of 6-chloropurine was not appreciably affected by changes of pH, and 6-chloropurine must therefore bind equally well to the enzyme whether the 9-nitrogen is in the neutral or anionic state. For 6-carboxypurine and 6-trimethylammoniumpurine the substituents at the 6-position would be completely ionized under the conditions of these experiments.

### 2. The 6-carbon substituent

Table 13 gives the inhibition constants for those analogs of adenine for which the substituent on the 6-carbon

TABLE XII: Ionization Constants for Some Adenine Analogs and Related Compounds  
as Reported in the Literature

Compound	pK <sub>a</sub> (a)		
	Anion (mono)	Anion (di)	Cation (mono)
Purine	8.93		2.39
Adenine	9.81		4.22
6-Methylpurine	9.02		2.60
6-Chloropurine	7.82		<2
6-Cyanopurine	6.88		0.3
6-Hydroxypurine	8.94	12.10	1.98
6-Methoxypurine	9.16		2.21
6-Mercaptopurine	7.77	10.87	2.5
6-Methylmercaptopurine	8.74		0
6-N-Hydroxylaminopurine	9.83	>12	3.80
6-Hydroxyamidinopurine	9.4		2.0
6-Aminoamidinopurine	10		5.12
6-Methylaminopurine	9.77		4.18
6-Dimethylaminopurine	10.5		3.87
2,6-Diaminopurine	10.77		5.09
2-Hydroxyadenine	8.99		4.51

TABLE XII (Continued)

Compound	pK <sub>a</sub> (a)		
	Anion (mono)	Anion (di)	Cation (di)
2-Methyladenine			5.1
7-Methylpurine			2.29
8-Methylpurine	9.37		2.85
8-Carboxypurine	2.91	9.37	0
8-Aminopurine	9.36		4.68
8-Mercaptopurine	6.64	11.16	< 2.5
8-Azapurine	4.87		2.12
8-Hydroxypurine	8.24	> 12	2.58
9-Methylpurine			2.36
4-Aminopyrimidine			5.71
4,5-Diaminopyrimidine			6.03
4,6-Diaminopyrimidine			6.01
4,5,6-Triaminopyrimidine			5.78
4-Aminopteridine			3.56
4-Aminopyrazolo (3,4-d) - pyrimidine	10.84		4.59
7-Aminopyrazolo (4,3-d) - pyrimidine	10.2		5.0

(a) These ionization constants were obtained from references 2,3,10,97 and 115.

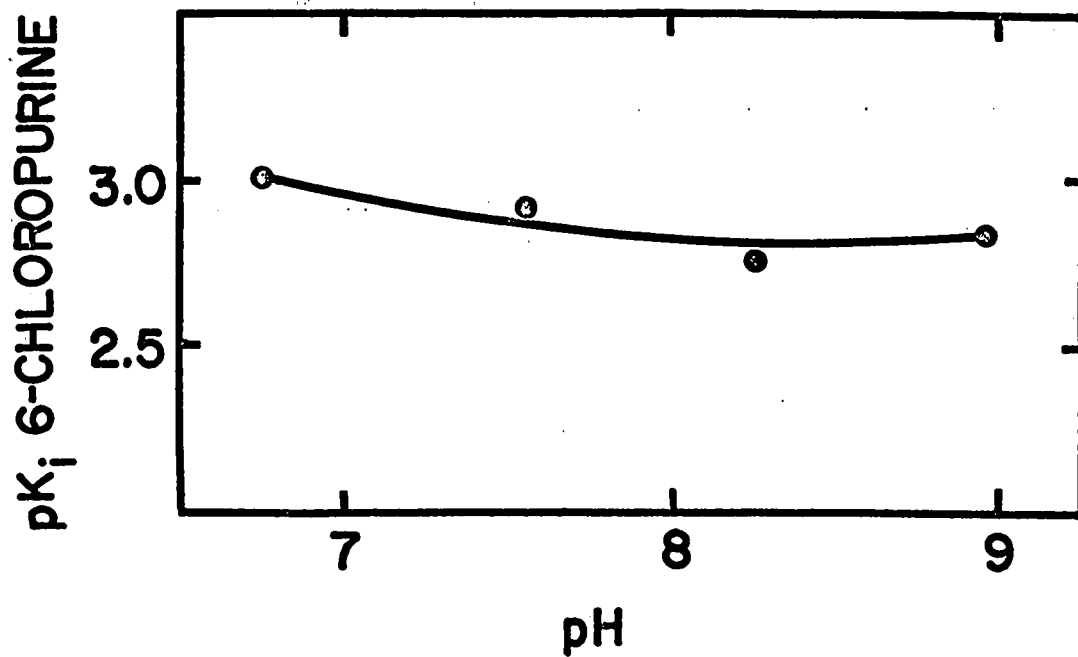


Figure 33. Effect of pH on the negative logarithm of the inhibition constant of 6-chloropurine ( $pK_a = 7.8$ ).

TABLE XIII: Inhibition Constants for Analogs of Adenine with Changes of the 6-Carbon Substituent

Compound	Source (a)	$K_i$ (mM)	$K_i$ Analog/ $K_i$ Adenine
Adenine	M	0.011	(1.0)
6-Methylaminopurine	M	0.31	28.2
6-Dimethylaminopurine	J	12	1,090
6-Trimethylammoniumadenine	A	> 12	> 1,090
6-Phenylaminopurine	K	< 1.2	> 0.9
6-Benzylaminopurine	I	> 1.2	< 2.8
6-Carboxypurine	J	> 1.2	< 2.8
6-Cyanopurine	J	> 1.2	< 2.8
6-Furfurylaminopurine	I	1.2	109
6-(2,2-Dimethylhydrazino)- purine	A	> 12	< 50
6- $\beta$ -Hydroxyethylaminopurine	J	1.2	109
6-n-Hexylaminopurine	M	0.88	80
6-N-Hydroxyaminopurine	M	0.040	3.6
Purine	L	0.40	36

TABLE XIII (Continued)

Compound	Source (a)	$K_i$ (mM)	$K_i$ Analog/ $K_i$ Adenine
6-Methylpurine	J	0.67	61
6-Chloropurine	M	0.66	60
6-Bromopurine	A	2.8	255
6-Iodopurine	M	1.0	91
6-Hydroxypurine	M	> 50	> 4,540
6-Mercaptopurine	J	> 50	> 4,540
6-Methoxypurine	M	> 12	> 1,090
6-Ethoxypurine	I	> 12	> 1,090
6-Methylmercaptopyrimidine	J	> 12	> 1,090

The inhibition constants were determined as described in the text.

(a) The compounds are coded as to their sources as follows:

- |   |                         |
|---|-------------------------|
| A - Cancer Chemotherapy National Service Center | H - Abbott              |
| B - Dr. A. Hampton                              | I - Aldrich             |
| C - Dr. C. G. Smith                             | J - Calbiochem.         |
| D - Dr. G. A. LePage                            | K - Mann                |
| E - Dr. J. A. Montgomery                        | L - Nutritional Biochem |
| F - Dr. R. K. Robins                            | M - Sigma               |
| G - Cyclo Chemicals                             |                         |

has been altered or replaced by another group. The inhibition constants, for those compounds in which the 6-carbon amino group has simply been replaced by another group, were at least 36-fold greater than that of adenine, thus suggesting that the 6-amino group is involved in the binding of this substrate to the enzyme. It should be noted that uncharged amino groups may complex with enzymes either as electron acceptors or as electron donors in a hydrogen bond or charge-transfer type of bond. A role as an electron donor may be ruled out in this case since the electron donors (6), 6-chloro-, 6-bromo-, and 6-iodopurine, are poorer inhibitors than 6-methylpurine and purine, compounds whose 6-substituents cannot act as electron donors. The decrease in inhibition constants as successive hydrogens of the 6-amino groups are replaced by methyl groups also suggests that this group is involved in substrate binding as an electron acceptor, either in a hydrogen bond or in a charge-transfer bond.

### 3. Bulk tolerance at the 6-carbon position

The bulk tolerance of the enzyme for groups at the 6-carbon position of adenine was investigated by determining the inhibition constants of analogs in which one of the hydrogen atoms of the 6-amino group was replaced by another group. None of the compounds tested complexed with the enzyme as effectively as did the substrate, and it is



apparent from the inhibition constants of Table 13 that the inhibition constants increase as the bulk of the 6-amino group is increased by substitution, and as the flexibility of the substituting group is decreased.

From these data the 6-amino group of adenine appears to be involved in substrate binding as an electron acceptor, and there is also apparently some lack of bulk tolerance at this position. Ronca and Succelli (114) obtained similar results with adenosine deaminase for base analogs with alterations of the 6-carbon substituent, but ascribed the lack of inhibition by 6-methyl-, 6-methoxy-, 6-methylthio- and 6-dimethylaminopurine to the low polarity of these 6-carbon substituents. Although this may certainly be a factor contributing to the poor inhibition by these analogs, it is believed that enough analogs have been tested in the present study to show that this is not the only or the most important factor in inhibitor binding to adenine phosphoribosyltransferase.

#### 4. The $\pi$ -electron cloud of the pyrimidine moiety

Baker (6) has suggested that the  $\pi$ -cloud of the pyrimidine ring of purine is electron deficient and therefore can accept electrons from a donor group on the enzyme. Substitution of an electron donating amino group at the 6-position, however, would be expected to change the  $\pi$ -cloud to an electron rich system which could donate electrons to an

acceptor group on the enzyme. If the  $\pi$ -electron system were an electron donor, 6-methylpurine would be expected to be a stronger inhibitor than 6-chloropurine, whereas if the  $\pi$ -cloud were an electron acceptor, 6-chloropurine would be expected to be the stronger inhibitor. It is apparent from the data of Table 13 that there was no difference in the inhibition constants of these two analogs, thus suggesting that the  $\pi$ -cloud of the pyrimidine ring of adenine is not involved in binding the substrate to the enzyme.

The cyano and carboxyl groups of 6-cyano- and 6-carboxypurine should likewise withdraw electrons from the  $\pi$ -cloud of the pyrimidine moiety, while the 6-benzylamino group of 6-benzylaminopurine should donate electrons to the  $\pi$ -cloud. No significant differences between the inhibition constants were observed for these analogs. The inhibition constants of adenine N<sup>1</sup>-oxide and 1-methyladenine are also approximately the same (see below). If the  $\pi$ -cloud of the pyrimidine ring were acting as either a donor or acceptor, 1-methyladenine would be expected to be a much weaker inhibitor than adenine N<sup>1</sup>-oxide, since methylation of the 1-nitrogen abolishes aromaticity of the pyrimidine ring.

5. The 1-nitrogen, 2-carbon and 3-nitrogen groups  
of adenine

a. The 1-nitrogen

Of all the analogs with changes at the 6-carbon

position tested, only 6-hydroxypurine (hypoxanthine) and 6-mercaptapurine failed to inhibit the enzyme. Both of these compounds are almost completely in the tautomeric keto and thione forms (94), respectively, and consequently bear a double bond between the 1-nitrogen and 6-carbon atoms of the purine nucleus. Furthermore in both of these compounds the 1-nitrogen is no longer basic but has become weakly acidic (114). Although this tends to suggest that the 1-nitrogen may be involved in substrate binding, 6-methoxy and 6-methylmercaptapurine, which have basic 1-nitrogens, are also very weak inhibitors.

Table 14 gives the inhibition constants for analogs of adenine with changes at the 1-nitrogen, 2-carbon and 3-nitrogen positions of the purine ring. The inhibition constants of both adenine  $N^1$ -oxide and 1-methyladenine were approximately six-fold greater than that of adenine. The observation does not necessarily suggest that the 1-nitrogen of adenine is involved in substrate binding as an electron donor, however, since there could be lack of bulk tolerance at this position; the 6-amino group of adenine is also changed to a 6-imino group in 1-methyladenine, and there is a partial positive charge on the 1-nitrogen in adenine- $N^1$ -oxide which would make it unavailable as an electron donor. 2-Fluoroadenine complexed to the enzyme almost as effectively as did adenine, whereas the basicity of its 1-nitrogen is

TABLE XIV: Inhibition Constants for Analogs of Adenine with Changes of the Pyrimidine Moiety

Compound	Source (a)	$K_i$ (mM)	$K_i$ Analog/ $K_i$ Adenine
Adenine N <sup>1</sup> -oxide	I	0.070	6.4
1-Methyladenine	M	0.060	5.4
2-Aminoadenine	J	4.6	418.0
2-Fluoroadenine	E	0.016	1.4
2-Methyladenine	A	0.34	30.9
2-Thioadenine	J	4.6	418.0
2-Hydroxyadenine	L	> 4.6 < 12.0	> 418.0 < 1,090
2-Methylthioadenine	A	> 4.6 < 12.0	> 418.0 < 1,090
2-Azaadenine	A	0.027	2.45
3-Methyladenine	A	> 12.0	> 1,090.0
7-Aminoimidazo (4,5-d) pyridazine	H	0.070	6.36
4-Amino-5-imidazolecarboxamide	J	0.65	59.0
Imidazole	M	> 100.0	> 9,080.0

The inhibition constants were determined as described in the text.

(a) The code for the source is given in Table XIII.

much weakened. The interpretation of other changes at the 2-position which increase the basicity of the 1-nitrogen is complicated by lack of bulk tolerance at the 2-position, and further studies with a compound such as 1-deazaadenine would be useful.

b. 2-Carbon

2-Fluoroadenine and 2-azaadenine bind almost as well as adenine, and the 2-CH of adenine therefore does not appear to be an important factor in this process. Other substituents bind increasingly poorly as their bulk increases, except for 2-hydroxy- and 2-methyladenine. 2-Methyladenine binds more tightly than would be expected from the size of the methyl group; this suggests that the adjacent portion of the enzyme may be hydrophobic. 2-Hydroxyadenine will be discussed in the next section.

c. The 3-nitrogen

No inhibition by 3-methyladenine was detected; this may indicate either a lack of bulk tolerance at this position or binding to the enzyme of the 3-nitrogen. Interchanging 3-nitrogen and 2-carbon positions (as in 7-aminoimidazo(4,5-d)pyridazine) led to a six-fold loss in binding. Because the basicity of the 1-nitrogen is not weakened (2), the observation that 7-aminoimidazo(4,5-d)pyridazine bound three-fold less tightly than 2-fluoroadenine and 2-azaadenine suggests that the 3-nitrogen is involved in binding as an

electron donor. Since 2-hydroxyadenine has a relatively acidic 3-NH group (110), an involvement of the 3-nitrogen in substrate binding may explain why 2-hydroxyadenine was a weaker inhibitor than would be predicted from the size of this group and lack of bulk tolerance at the 2-carbon position.

The complete removal of the 1,2 and 3 positions of adenine as in 4-amino-5-imidazoloecarboxamide, resulted in a 60-fold decrease in binding to adenine phosphoribosyltransferase. The fact that this compound did bind to the enzyme, however, indicates that its amide  $\text{NH}_2$  group occupied the position normally occupied by the 6-amino group of adenine. Models show that there is no difficulty in achieving this configuration. The change of the aromatic 3-nitrogen of adenine to an amino group in this compound may be an important cause of its decreased affinity compared to adenine.

Although recent nuclear magnetic resonance (29) and X-ray crystallographic studies (55,86,124,129) suggest that the naturally occurring purine nucleotides are in the anti conformation (Figure 34) in solution, it is possible that PP-ribose-P and the purine base could be oriented in the syn conformation when bound to the enzyme. Klee and Mudd (74) and CPK atomic models show that there is very little steric hindrance to rotation about the glycosidic bond from the anti to the syn conformations. Therefore the lack of

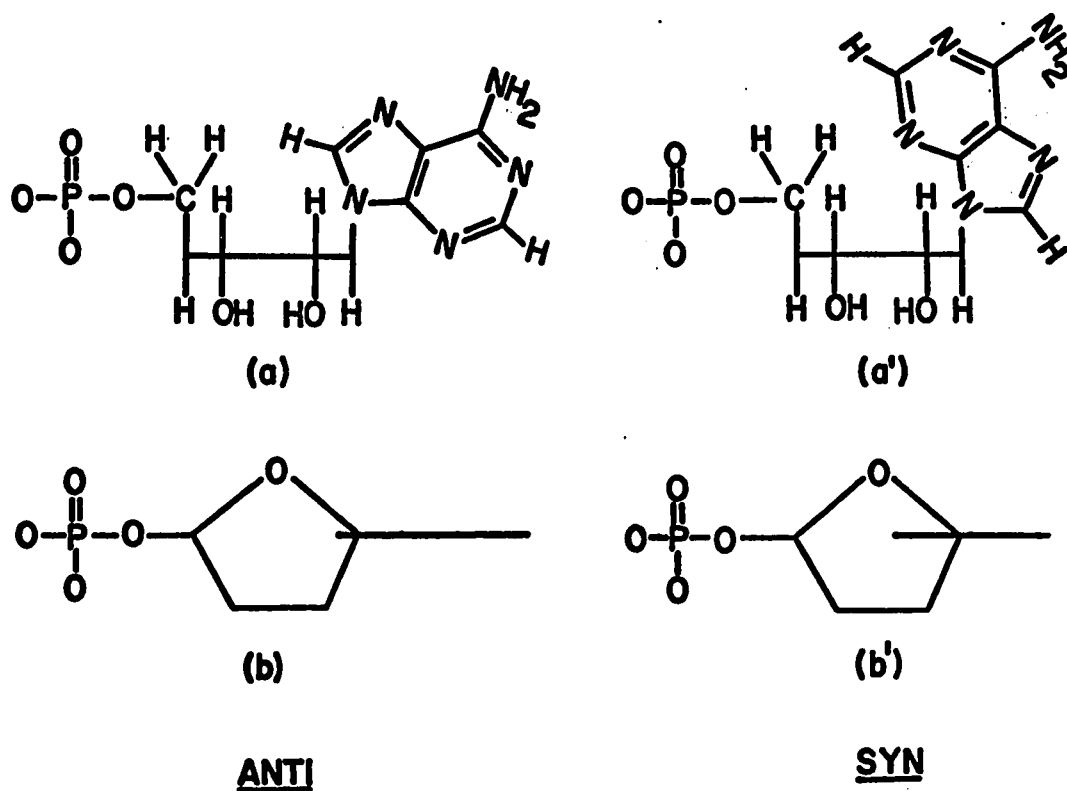


Figure 34. Syn and anti conformations of AMP; (a) and (a'), ribose ring perpendicular to the plane of the paper; and (b) and (b'), ribose ring in the plane of the paper.

inhibition by 3-methyladenine may be due to either lack of bulk tolerance of the enzyme for groups at the 3-nitrogen position or to the conformation of PP-ribose-P with respect to the purine base analog.

6. The imidazole moiety

Table 15 gives the inhibition constants for analogs of adenine with changes in the ring atoms of the imidazole moiety and with changes of substituents on the imidazole ring.

a. The 7-nitrogen

7-Methyladenine was an inhibitor and complexed to the enzyme 1/20th as well as adenine; no inhibition was observed for 7- $\alpha$ -D-ribofuranosyladenine, however. These results may indicate that the 7-nitrogen of adenine complexes with the enzyme as an electron donor, and that there is a lack of bulk tolerance at this position. If the 7-nitrogen were an electron donor, a correlation between basicity and inhibitory activity would be expected. Note that 8-mercaptoadenine, which has an acidic 7-NH group, and 8-bromoadenine, which has a base weakened 7-nitrogen (26) are both weaker inhibitors than adenine and 8-methyladenine. The weaker inhibition by 8-bromoadenine compared with that observed for 8-methyladenine is probably not due to lack of bulk tolerance at the 8-carbon position since these substituents are approximately the same size. A bromo group has a Van der Waal's radius of 1.95 A (133), and a methyl group a radius



TABLE XV: Inhibition Constants for Analogs of Adenine with Changes in the Imidazole Moiety

Compound	Source (a)	$K_i$ (mM)	$K_i$ Analog/ $K_i$ Adenine
7-Methyladenine	G	0.24	21.8
7- $\beta$ -D-Ribofuranosyladenine	A	>50.0	4,540.0
7-Deazaadenine	C	0.90	81.8
8-Methyladenine	A	0.026	2.36
8-Bromoadenine	G	0.39	35.5
8-Mercaptoadenine	I	0.80	72.7
8-Azaadenine	L	2.8	254.0
9-Methyladenine	G	14.0	1,270.0
4-Aminopyrazolo (3,4-d) pyrimidine	A	0.068	6.18
7-Aminopyrazolo (4,3-d) pyrimidine	F	0.16	14.5
4-Aminopteridine	G	0.12	10.9
4-Aminopyrimido (4,5-d) pyrimidine	I	0.073	6.6
4-Amino-5,6-trimethylenepyrimidine	A	1.2	109.0
4-Aminopyrimidine	G	4.8	437.0
4,5-Diaminopyrimidine	G	0.75	68.0
4,6-Diaminopyrimidine	G	17.0	1,545.0
4,5,6-Triaminopyrimidine	M	6.4	582.0

The inhibition constants were determined as described in the text.

(a) The code for the source is given in Table XIII.

of  $1.57 \times 2.27 \text{ \AA}$  (133).

That the 7-nitrogen is complexed to the enzyme as an electron donor is also indicated by the 81-fold loss in binding when the 7-nitrogen of adenine is replaced by a CH group as in 7-deazaadenine. This is also confirmed by the observation that 4,5-diaminopyrimidine bound to the enzyme 7-fold more effectively than did 4-aminopyrimidine; the latter lacks a nitrogen in the position of the 7-nitrogen of adenine.

If a basic 7-nitrogen is involved in adenine binding, 7-aminopyrazolo(4,3-d)pyrimidine, which has an acidic 1-NH group corresponding to the 7-nitrogen of adenine, would be expected to have an inhibition constant similar to that of 7-deazaadenine. This was not observed; the inhibition constant of 7-deazaadenine was approximately 6-fold higher than that of 7-aminopyrazolo(4,3-d)pyrimidine. If one assumes that the plane of the base analog can rotate with respect to the binding site of adenine, as shown in Figure 35, the 7-amino group of 7-aminopyrazolo(4,3-d)pyrimidine could still overlap that binding site normally occupied by the 6-amino group of adenine, the 4-nitrogen could overlap that site normally occupied by the equivalent atom in adenine, and the basic 2-nitrogen of 7-aminopyrazolo(4,3-d)pyrimidine could overlap that binding site normally occupied by the 7-nitrogen of adenine. This 2-nitrogen could therefore be an electron donor and permit this analog to bind more tightly than would

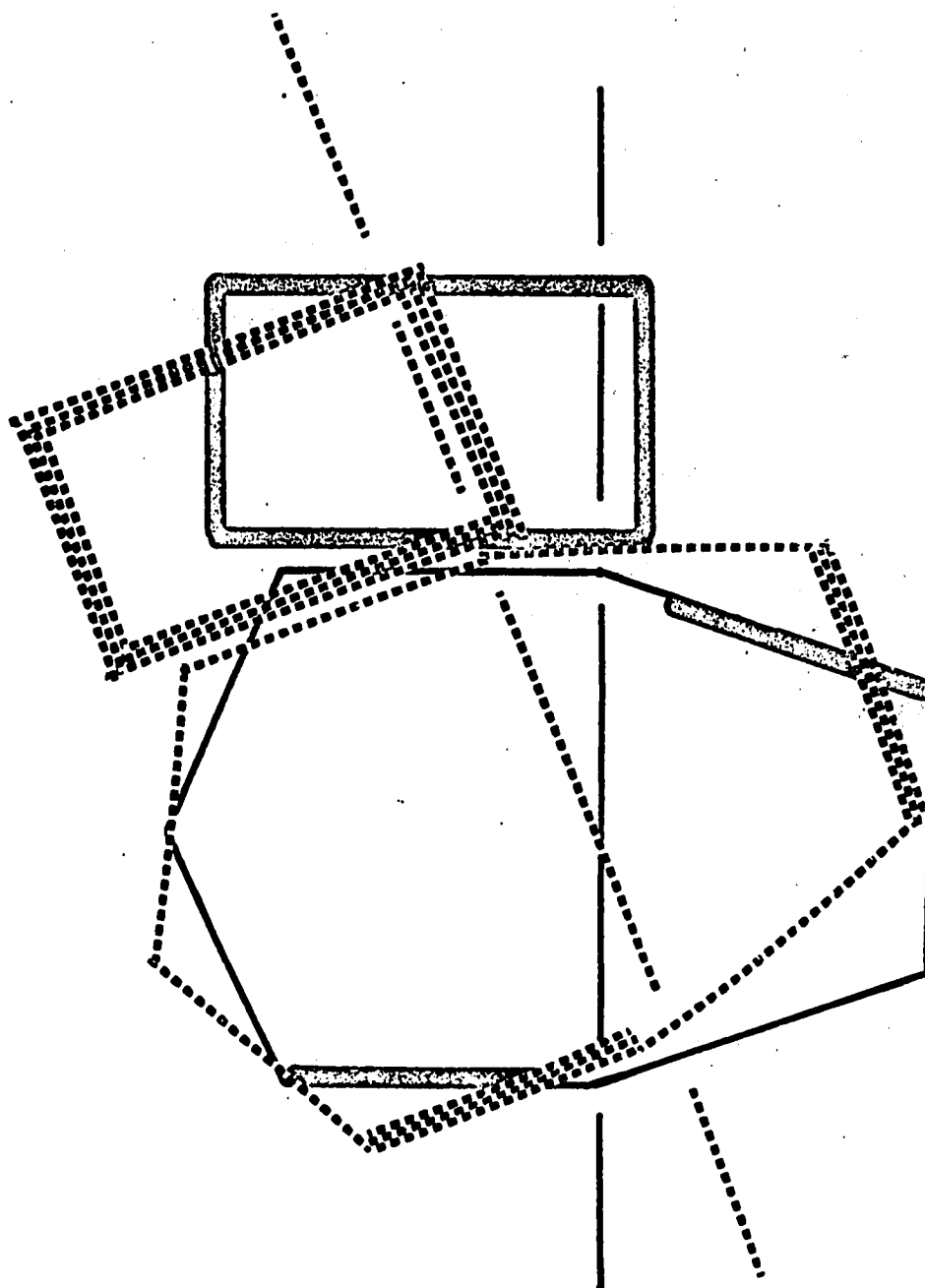


Figure 35. Proposed binding of pyrazolopyrimidine analogs. The axis of the pyrazolopyrimidine outline (.....) is shown rotated approximately  $25^\circ$  from the axis of the adenine outline (—). The heavy lines indicate the atoms believed to be involved in binding.

be predicted for a compound without a basic 7-nitrogen.

A similar rotation of the plane of the base for 4-aminopyrazolo(3,4-d)pyrimidine would permit the basic 2-nitrogen of this analog to overlap that site normally occupied by the 7-nitrogen of adenine. For such a situation, however, the acidic 1-NH group of 4-aminopyrazolo(3,4-d)pyrimidine would be situated overlapping that site normally occupied by the 8-CH moiety of adenine. The observation that 7-aminopyrazolo(4,3-d)pyrimidine was not a stronger inhibitor than 4-aminopyrazolo(4,3-d)pyrimidine suggests that the 8-CH group of adenine is not involved in substrate binding to the enzyme.

b. The 8-carbon

Replacement of the 8-CH group of adenine by nitrogen, as in 8-azaadenine, results in a 250-fold increase in the inhibition constant with respect to that of adenine, and a 3-fold increase in the inhibition constant with respect to that of 7-deazaadenine. The insertion of a nitrogen atom at the 8-CH position may decrease binding because it leads to decreased basicities of the 1-, 3-, and 7-nitrogen atoms. 1,2,3-Triazoles are weaker bases than imidazoles (2, 7).

There may be some lack of bulk tolerance at the 8-CH position since 8-methyladenine is a weaker inhibitor than adenine; the 8-methyl group would not be expected to have a

base-weakening effect on the 7-nitrogen.

c. The 9-nitrogen

The 9-NH group does not appear to be involved in the binding of adenine to the enzyme because ionization of the 9-NH group of 6-chloropurine did not appreciably affect the inhibition constant, and because the inhibition constants for 7-deazaadenine and 4-amino-5,6-trimethylenepyrimidine were similar; replacement of the 9-NH group of 7-deazaadenine with a 9-CH<sub>2</sub> group therefore does not affect binding. Finally, the inhibition constant for 4,6-diaminopyrimidine was 4-fold higher than that of 4-aminopyrimidine. 4,6-Diaminopyrimidine would be expected to be a stronger inhibitor than 4-aminopyrimidine if the 9-NH group of adenine were involved in binding, since an amino group may act as an electron acceptor. Methylation of the 9-nitrogen gives a compound which was a much weaker inhibitor than either 7- or 8-methyladenine. Since the data suggest that the 9-NH group of adenine is not involved in binding, the poor inhibition observed is probably due to a lack of bulk tolerance at this position. This would be expected since a glycosidic bond is formed between the 1-carbon of PP-ribose-P and the 9-nitrogen of adenine, and hence adenine and PP-ribose-P would have to be very close to each other on the enzyme surface.

d. The imidazole  $\pi$ -electron cloud

If the  $\pi$ -electron system of the imidazole

moiety of purine were involved in complexing with the enzyme, it would be expected to be acting as an electron donor, since imidazole is a  $\pi$ -excessive heteroaromatic (1). 7-Deazaadenine is also a  $\pi$ -excessive heterocycle, although its  $\pi$ -electron density is lower than in adenine. The fact that the non-aromatic 4-amino-5,6-trimethylenepyrimidine bound almost as effectively as did 7-deazaadenine suggests that the  $\pi$ -cloud does not serve as an electron donor in binding.

4-Aminopteridine and 4-aminopyrimido(4,5-d)pyrimidine have pyrazine and pyrimidine rings, respectively, in place of the imidazole ring of adenine. Since both pyrazine and pyrimidine rings are  $\pi$ -deficient heteroaromatics (1), their  $\pi$ -clouds would be expected to act only as electron acceptors in a  $\pi$ -electron type of interaction; if the  $\pi$ -cloud of the imidazole moiety of adenine were involved in binding as an electron donor, both of these compounds would be expected to be no better inhibitors than 7-deazaadenine or 4-amino-5,6-trimethylenepyrimidine. This result was not observed, and since both 4-aminopteridine and 4-aminopyrimido(4,5-d)pyrimidine have a basic nitrogen situated close to that position normally occupied by the 7-nitrogen of adenine, it is suggested that both of these basic nitrogens are involved in binding.

## 7. Summary

Those moieties of adenine which are probably

involved in binding to the enzyme and those positions of adenine at which there is probably a lack of bulk tolerance may be summarized as follows:

- (a) Those groups that probably complex with enzyme include the 6-amino group, the 3-nitrogen and the 7-nitrogen. The first complexes as an electron acceptor and the last two as electron donors.
- (b) Those groups that probably do not complex with the enzyme are the 1-nitrogen, 2-CH, 8-CH, and 9-NH groups, and the  $\pi$ -electron clouds of the pyrimidine and imidazole moieties. Those groups for which no information is available include the 4-, 5- and 6-carbon atoms.
- (c) Those positions of adenine at which there is probably a lack of bulk tolerance include the 6-amino, 2-carbon, and the 3-, 7- and 9-nitrogen positions.

It is apparent from the inhibition studies that most modifications of adenine result in compounds whose inhibition constants are much larger than that of adenine. Modification of only one of the groups which have been postulated to be involved in adenine binding reduces but does not abolish inhibition, but modification of two or three of these groups completely abolishes inhibition.

C. The effect of pH on the Michaelis constant of adenine

The effect of pH on the Michaelis constant of aden-

ine ( $K_B$ ) was determined and plotted as described in the previous chapter. The graph of pH versus  $pK_B$ , as shown in Figure 36, shows two ionization constants at 30°, 10.25 and 5.95. The ionization constant of 5.95 is due to ionization of the enzyme-substrate complex; the significance of this will be discussed in the chapter on the catalytic mechanism. The ionization constant of 10.25 is believed to be that of some group on the enzyme-PP-ribose-P complex associated with adenine binding, because adenine has ionization constants of 4.22 and 9.81, and the latter is associated with ionization of the 9-NH group. Since ionization of the 9-NH group of 6-chloropurine does not appreciably affect the inhibition constant, it is unlikely that ionization of the 9-NH group of adenine would affect its Michaelis constant.  $K_B$  is equal to  $k_4(k_{-2}+k_3)/k_2(k_3+k_4)$  and this reduces to  $V_1(k_{-2}+k_3)/k_2k_3$ . It will be shown later that  $\log V_1$  does not change as a function of pH in this region, and the group that is ionizing at pH 10.25 must therefore be associated with  $(k_{-2}+k_3)/k_2k_3$ . Since  $k_3$  is also contained in the velocity expression, which does not change, the group that is ionizing at pH 10.25 must therefore be associated with either  $k_{-2}$  or  $k_2$ , the rate constants which describe the release and addition, respectively, of adenine to the enzyme.

The  $pK_a$  value of 10.25 obtained from these plots is close to those found in proteins for the phenolic hydroxyl



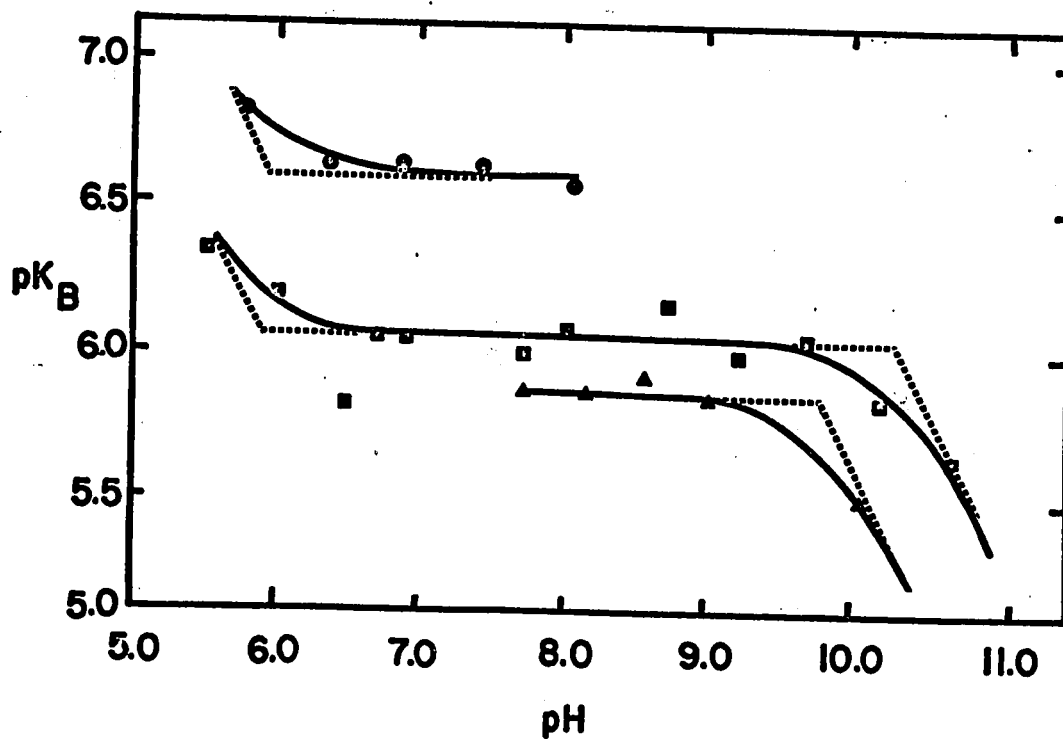


Figure 36. Effect of pH on the negative logarithm of the Michaelis constant of adenine at 10° (●), 30° (■) and 40° (▲).

group of tyrosine, 9.8 to 10.4, and for the  $\epsilon$ -amino group of lysine, 9.4 to 10.6 (34). The observation that only one ionization constant was observed in these plots suggests that other groups involved in substrate binding do not ionize over the pH range studied.

The heat of ionization of this group was calculated from the ionization constants determined at 30° and 40° by the method described previously, and found to be +20,100 cal mole<sup>-1</sup>. This is much higher than the heat of ionization reported by Steinhardt and Beychok (121) for any of the amino acid residues normally found in proteins. The  $\epsilon$ -amino group of lysine has a heat of ionization of approximately +13,000 cal mole<sup>-1</sup>, and the phenolic hydroxyl group of tyrosine a heat of ionization of approximately +6,000 cal mole<sup>-1</sup>. The same sources and magnitudes of errors encountered in the estimation of the heat of ionization of the group involved in PP-ribose-P binding would also apply for this heat of ionization. The cumulative error was estimated to be  $\pm 43\%$  which would be equal to  $\pm 8,700$  cal mole<sup>-1</sup>. The heat of ionization could therefore be as low as 11,400 cal mole<sup>-1</sup> if all errors were in the same direction. This value is close to the literature values for the heats of ionization for amino groups but much higher than those reported for phenolic hydroxyl groups. However, for phenolic hydroxyl groups close to amino groups heats of ionization up to 9,500 cal mole<sup>-1</sup> might be

observed. Although these data suggest that the group which ionizes at pH 10.25 is an amino group, a phenolic hydroxyl group cannot be ruled out.

D. Protection by adenine against enzyme inactivation by protein reagents.

Protection by adenine against inactivation by protein reagents was investigated in order to obtain information about the binding site for this substrate. N-Bromosuccinimide reacts most rapidly with tryptophan but also with tyrosine and histidine (28), and side reactions with methionine, cysteine, cystine, lysine and arginine may also occur (118). PP-ribose-P did not protect the enzyme against inactivation by this reagent (Table 16) thus suggesting that the group modified was not at the PP-ribose-P binding site; however, a combination of fructose 1,6-diphosphate and adenine did protect the enzyme against inactivation. Fructose 1,6-diphosphate was used because it is an analog of PP-ribose-P but is not a substrate. Since adenine cannot bind to free enzyme, protection by this combination suggests both that fructose 1,6-diphosphate permits adenine to bind, and that one of the amino acids cited above is situated at or near its binding site. Because lysine and tyrosine may be modified by this reagent, and ionize near pH 10.25, it is not possible to decide from these data which group is involved.

TABLE XVI: Protection by Adenine Against Enzyme Inactivation by N-Bromosuccinimide and PMB

Reagent	Conc. (M)	Additions		Enzyme Activity (% of Control)
		Adenine ( $10^{-5}$ M)	F 1,6-diP ( $10^{-3}$ M)	
N-Bromosuccinimide	$1.1 \times 10^{-2}$	-	-	10
	$1.1 \times 10^{-2}$	+	-	14
	$1.1 \times 10^{-2}$	+	+	50
PMB	$5 \times 10^{-6}$	-	+	52
	$5 \times 10^{-6}$	+	+	48

Two  $\mu$ g of enzyme preparation per reaction mixture was incubated with the protein reagent indicated for 20 min at 30° in 0.1 M Tris-HCl buffer, pH 7.4, in the presence of the additions indicated. The reaction mixtures were completed and enzyme activity was assayed by the radioisotope method; the concentrations of pp-ribose-p and magnesium ion were  $10^{-5}$  M and  $10^{-3}$  M, respectively.

Table 16 also shows that adenine did not significantly enhance or decrease the protection by fructose 1,6-diphosphate against enzyme inactivation by PMB although the enzyme-fructose-1,6-diphosphate complex permits adenine to bind to the enzyme.

#### E. Discussion

The plot of pH versus the negative logarithm of the Michaelis constant of adenine suggests that a protein functional group which ionizes at pH 10.25 is involved in adenine binding; it was tentatively identified as a lysine  $\epsilon$ -amino group or a tyrosine phenolic hydroxyl group. Neither the calculated heat of ionization nor adenine protection against enzyme inactivation by N-bromosuccinimide permits a choice to be made between these two possible amino acids.

At pH values substantially below 10.25 the  $\epsilon$ -amino group would be protonated and could not be involved in binding to the 6-amino group of adenine as an electron donor. Both above and below pH 10.25 the  $\epsilon$ -amino group of lysine could act as an electron acceptor with the 3- and 7-nitrogen atoms of adenine, but binding of adenine would not be expected to be affected by changes in pH. It is therefore unlikely that the group on the enzyme that ionizes at pH 10.25 is an  $\epsilon$ -amino group of lysine.

At pH values substantially less than 10.25 the phenolic hydroxyl group of tyrosine would carry no net charge and

could act as an electron acceptor with the 3- and 7-nitrogen atoms of adenine and as an electron donor with the 6-amino group. Substantially above pH 10.25 the phenolic hydroxyl would be ionized and carry a net negative charge and could not act as an electron acceptor with the 3- and 7-nitrogen atoms of adenine but could still act as an electron donor with the 6-amino group. The Michaelis constant of adenine would therefore not be expected to be altered by varying the pH in this region if the phenolic group of tyrosine were involved in binding to the 6-amino group of adenine, but such alteration would be expected if the phenolic group of tyrosine were involved in binding to one of the 3- or 7-nitrogen atoms.

Although it is not possible to predict, from a structure-activity type of study, precisely to which amino acid residue in the enzyme the 6-amino group of adenine binds, a few may be ruled out. Anionic residues, such as glutamic and aspartic acids, may be ruled out since 6-carboxypurine inhibited almost as effectively as did 6-phenylamino-, 6-benzylamino-, 6-furfurylamino- and 6-cyanopurine. The lack of inhibition by 6-trimethylammonium adenine may indicate the presence of a cationic protein residue; however a more likely explanation for its inactivity is a lack of bulk tolerance at this position. In addition, any amino acid residue which cannot act as an electron donor may also be ruled out. For example,

the  $\epsilon$ -amino group of lysine, which is protonated at pH values substantially below pH 9.4, could not act as an electron donor, and neither could cysteine, the aliphatic amino acids and arginine (6).

The 9-NH group of adenine is not involved in binding, and ionization of this group in 6-chloropurine did not affect the affinity of the enzyme for this analog. This suggests that there is no cationic group on the enzyme or on the first substrate, PP-ribose-P, which is close to the 9-NH group of adenine when it is bound to the enzyme. Although the 1-pyrophosphate group of PP-ribose-P would be expected to be close to the 9-NH group, the negative charge on the  $\alpha$ -phosphate and one of those on the  $\beta$ -phosphate of the pyrophosphate moiety are neutralized by chelation with magnesium ion. The negative charge on the  $\beta$ -phosphate is probably too far removed from 9-NH group of adenine to repel this group if it were ionized.

VII INHIBITION OF ADENINE PHOSPHORIBOSYLTRANSFERASE  
BY PURINE NUCLEOSIDES AND NUCLEOTIDES

A. Introduction

Murray (101) in 1966 obtained evidence which he interpreted to mean that AMP, ADP, ATP and GMP inhibit adenine phosphoribosyltransferase by competing with PP-ribose-P for free enzyme. Murray (102) also showed that in addition to acting as an inhibitor, ATP at concentrations less than 250  $\mu$ M stimulated adenine phosphoribosyltransferase activity and could reverse inhibition by AMP and GMP. Hori and Henderson (64) obtained evidence from protection against heat inactivation and from kinetic studies that AMP does bind to free enzyme, but Henderson and Miller (59) and Hori, Gadd and Henderson (62) also obtained evidence from kinetic studies that AMP, GMP and deoxy-AMP also bind to at least one other enzyme form. Hori, Gadd and Henderson (62) showed that low concentrations of AMP, GMP and deoxy-AMP stimulated enzyme activity as much as 30 per cent. In addition to inhibition by nucleotides, Henderson and Gadd (58) showed that some nucleosides inhibit adenine phosphoribosyltransferase, and presented evidence which suggests that these nucleosides were not strictly competitive inhibitors with respect to either substrate, PP-ribose-P or adenine, since they inhibit both at high concentrations of PP-ribose-P and of adenine. A number of nucleosides and nucleotides were tested for



inhibitory activity to determine if those groups involved in binding were the same as those involved in the binding to the enzyme of the substrates, adenine and PP-ribose-P.

B. Inhibition by AMP analogs.

Analogues of AMP were tested for inhibition of adenine phosphoribosyltransferase by the method described previously for PP-ribose-P analogs. Table 17 gives the dissociation constants for the nucleotide analogs tested. These  $K_i$  values were calculated from fractional inhibitions assuming that the analogs bind only to free enzyme, and they should therefore be regarded only as apparent dissociation constants which may be lower than the true dissociation constants. At the pH at which these analogs were tested the phosphate groups would be completely ionized. Because adenosine 5', 3'-phosphouridine, adenosine 5'-phosphoramidate and AMP bound almost equally well, and more than 250 times as tightly as did adenosine 5'-acetate and 5'-O-trityl-adenosine, one negative charge at the 5' position appears to be necessary for binding. Because adenosine 5',3-phosphouridine and AMP had the same inhibition constant, there appears to be no lack of bulk tolerance at the 5' position of AMP. Those compounds in which the phosphate group has been moved to a position other than 5', as in adenosine 2'-monophosphate, adenosine 3'-monophosphate and adenosine 2',3-cyclicmonophosphate, were still inhibitors, but the inhibition constants were

TABLE XVII: Inhibition Constants for Nucleotides and Nucleotide Analogs

Compound	$K_i$ (mM)	$K_i$ Analog/ $K_i$ AMP
Adenosine 5'-monophosphate	0.030 (a)	(1.00)
Adenosine 2'-monophosphate	0.200	6.57
Adenosine 3'-monophosphate	0.219	7.13
Adenosine 2',3'-cyclic monophosphate	0.512	17.10
Adenosine 5'-acetate	> 7.50	> 250.00
Adenosine 5'-phosphoramidate	0.037	1.23
5'-O-Trityl-adenosine	> 7.50	> 250.00
Adenosine 5',3'-phosphate-uridine	0.028	0.94

(a) Hori and Henderson (64).

The inhibition constants were calculated from fractional inhibition (Equation 24) assuming that the nucleotides and nucleotide analogs bind to free enzyme only.

6- to 17-fold greater than that observed for AMP. These results may suggest that the ribose moiety of these analogs rotates about the glycosidic bond to bring the phosphate close to that position normally occupied by the 5'-phosphate moiety of AMP.

### C. Inhibition by nucleosides

Approximately 53 nucleosides were tested as inhibitors of adenine phosphoribosyltransferase. These included nucleoside analogs with alterations in the purine moiety only, nucleoside analogs with alterations in the sugar moiety only, and nucleoside analogs with changes in both the purine and the sugar moieties. The per cent inhibitions of these nucleosides are listed in Table 18 and their structural formulae are given in Figure 37.

As an example of inhibition by nucleosides, Figures 38 and 39 show in double reciprocal plots the effects of adenine 6'-deoxy- $\beta$ -D-allofuranoside on initial velocity versus substrate concentration. Figure 38 shows that adenine 6'-deoxy- $\beta$ -D-allofuranoside was a competitive inhibitor with respect to adenine at a concentration of PP-ribose-P ( $6 \times 10^{-5}$  M) which was equal to 10 times its Michaelis constant. Figure 39 shows that adenine 6'-deoxy- $\beta$ -D-allofuranoside was a noncompetitive inhibitor with respect to PP-ribose-P at a concentration of adenine ( $9 \times 10^{-6}$  M) which was equal to 10 times its Michaelis constant. These results suggest that

TABLE XVIII: Inhibition by Nucleoside Analogs

Compound	Source (a)	% Inhibition	
		[adenine] = $9 \times 10^{-7} M$	[adenine] = $9 \times 10^{-6} M$
Adenosine	M	31	0
2-Azaadenosine	A	95	82
2,6-Diaminopurine $\beta$ -D-ribofuranoside	B	3	
2-Fluoroadenosine	A	70	
Isoinosine	A	9	9
6-Chloropurine $\beta$ -D-ribofuranoside	B	0	
6-Ethylthiopurine $\beta$ -D-ribofuranoside	A	0	0
6-N-Hydroxylaminopurine $\beta$ -D-ribofuranoside	F	5	
6-Iodopurine $\beta$ -D-ribofuranoside	A	4	4
6-Mercaptopurine $\beta$ -D-ribofuranoside	A	0	

TABLE XVIII (Continued)

Compound	Source (a)	% Inhibition	
		[PP-ribose-P] = $6 \times 10^{-5}$ M [adenine] = $9 \times 10^{-7}$ M	[PP-ribose-P] = $6 \times 10^{-6}$ M [adenine] = $9 \times 10^{-6}$ M
6-Methylaminopurine $\beta$ -D- ribofuranoside	M	0	
6-Methylmercaptapurine $\beta$ -D- ribofuranoside	A	0	
8-Azaadenosine	A	7	
8-Bromoadenosine	F	15	
8-Methoxyadenosine	F	0	
2-Aminopurine $\beta$ -D-ribofur- anoside	A	0	0
Guanosine	M	11	
Inosine	M	19	
Xanthosine	M	17	
Cytidine	M	1	
Uridine	M	3	

TABLE XVIII (Continued)

Compound	Source (a)	% Inhibition	
		[PP-ribose-P] = $6 \times 10^{-5}$ M [adenine] = $9 \times 10^{-7}$ M	[PP-ribose-P] = $6 \times 10^{-6}$ M [adenine] = $9 \times 10^{-6}$ M
Adenine 3'-deoxy-3'-amino- $\beta$ -D-ribofuranoside	A	79	63
Adenine $\beta$ -L-ribofuranoside	D	57	
Adenine 4'-deoxy-4'-mercapto- $\beta$ -D-ribofuranoside	D	6	
Adenine 5'-deoxy- $\beta$ -D-allofuranoside	D	88	
Adenine 6'-deoxy- $\beta$ -D-allofuranoside	D	88	70
Adenine $\beta$ -D-arabinofuranoside	D	0	
Adenine $\beta$ -D-fructofuranoside	A	58	58
Adenine $\beta$ -D-galactopyranoside	A	32	32
Adenine $\beta$ -D-glucopyranoside	A	57	57

TABLE XVIII (Continued)

Compound	Source (a)	% Inhibition	
		[PP-ribose-P] = $6 \times 10^{-5}$ M [adenine] = $9 \times 10^{-7}$ M	[PP-ribose-P] = $6 \times 10^{-6}$ M [adenine] = $9 \times 10^{-6}$ M
Adenine 2'-deoxy-2'-ethyl-thio- $\beta$ -D-xylofuranoside	D	10	
Adenine 2'-deoxy- $\beta$ -D-ribofuranoside	M	41	0
Adenine 3'-deoxy- $\beta$ -D-ribofuranoside	A	12	
Adenine $\beta$ -D-lyxofuranoside	D	4	
Adenine $\beta$ -D-xylofuranoside	B	79	66
9-(2-(Bis-(2-chloroethyl)-amino)ethyl)adenine	A	17	
9-pentyladenine	E	28	
9- $\omega$ -valeryladenine	A	0	
9-(Tetrahydro-2-furyl)-adenine	A	59	
9- $\omega$ -valeronitriloadenine	A	46	

TABLE XVIII (Continued)

Compound	Source (a)	% Inhibition	
		[PP-ribose-P] = $6 \times 10^{-5} M$ [adenine] = $9 \times 10^{-7} M$	[PP-ribose-P] = $6 \times 10^{-6} M$ [adenine] = $9 \times 10^{-6} M$
Deoxyguanosine	M	42	3
Deoxyuridine	M	3	
Deoxycytidine	J	2	
Deoxyinosine	M	16	
6-Mercaptopurine 6'-deoxy- β-D-allofuranoside	A	6	
6-Mercaptopurine β-D-xylo- furanoside	D	3	
6-Mercaptopurine 5'-deoxy- β-D-allofuranoside	D	59	
6-Mercaptopurine β-D-ara- binofuranoside	D	32	
6-Dimethylaminopurine 3'- deoxy-3'-amino-β-D-ribo- furanoside	A	0	



TABLE XVIII (Continued)

Compound	Source (a)	% Inhibition	
		[PP-ribose-P] = $6 \times 10^{-5}$ M [adenine] = $9 \times 10^{-7}$ M	[PP-ribose-P] = $6 \times 10^{-6}$ M [adenine] = $9 \times 10^{-6}$ M
1-Methyl-4-aminopyrazolo- (3,4-d) pyrimidine	A	0	16
1-Methyl-4-n-butylamino- pyrazolo (3,4-d) pyrimidine	A	0	0

The standard radioisotope method was used with the concentrations of substrates as indicated,  $10^{-3}$  M test compound and  $10^{-3}$  M magnesium ion.

(a) The code for the source is given in Table XIII.

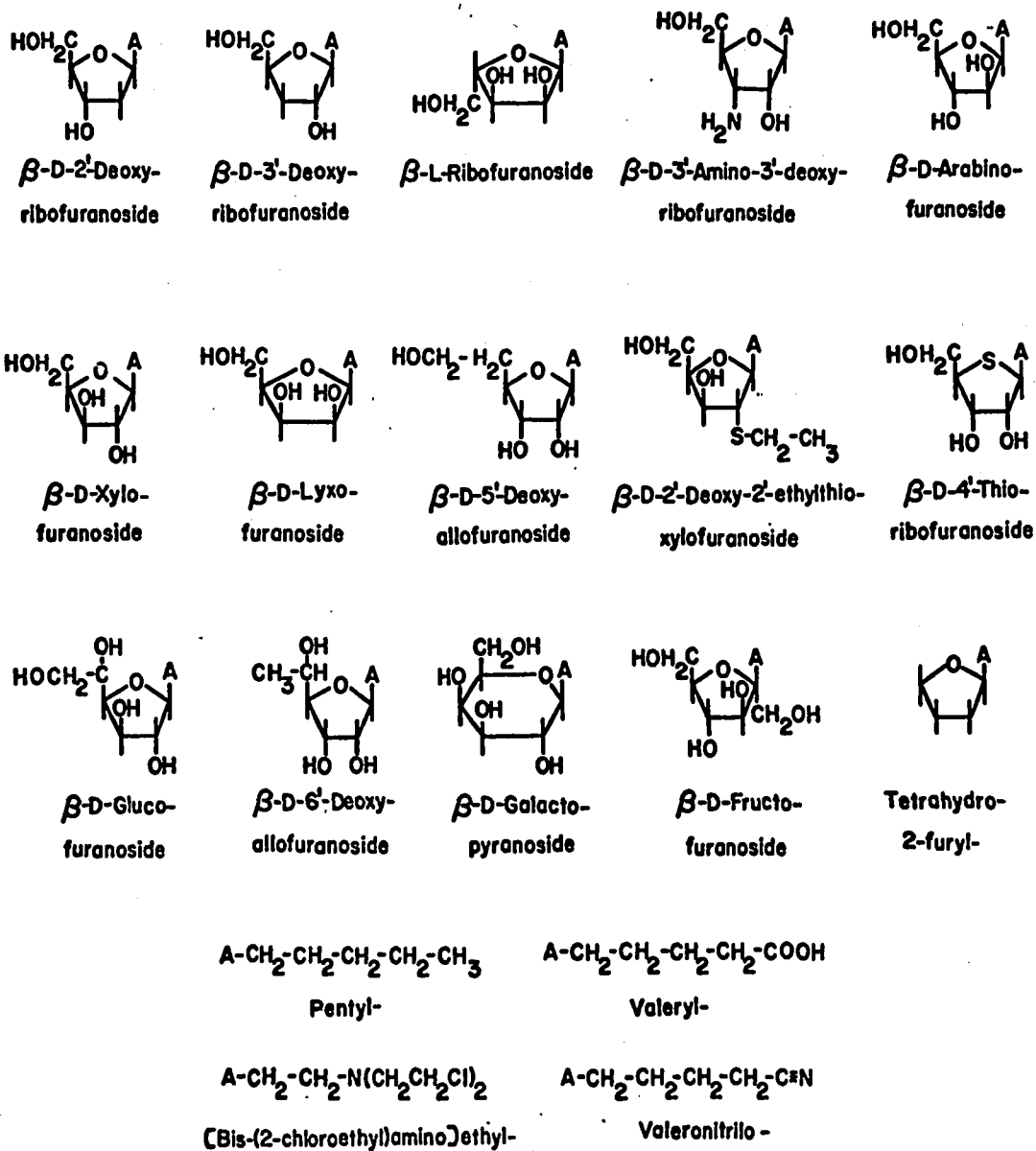


Figure 37. Structural formulae of nucleoside analogs. "A" represents either a purine or a pyrimidine.

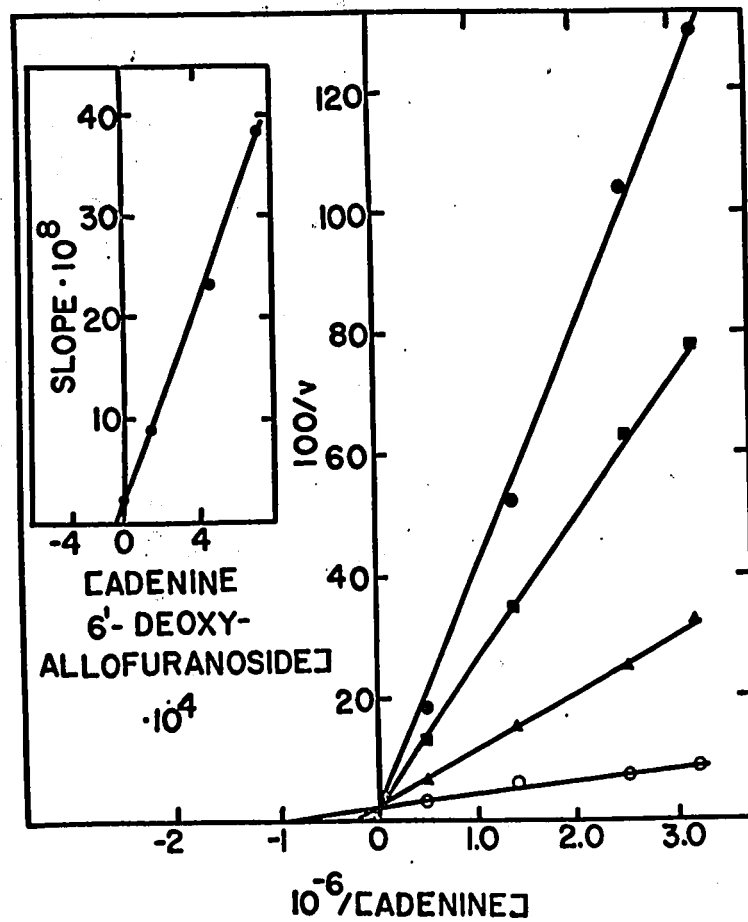


Figure 38. Inhibition by adenine 6'-deoxyallofuranoside. Double reciprocal plot of initial velocity against adenine concentration at a PP-ribose-P concentration of  $6 \times 10^{-5}$  M. The concentrations of adenine 6'-deoxyallofuranoside are: 0 (○),  $1.5 \times 10^{-4}$  M (▲),  $4.5 \times 10^{-4}$  M (■), and  $7 \times 10^{-4}$  M (●).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes against molar concentrations of adenine 6'-deoxyallofuranoside.

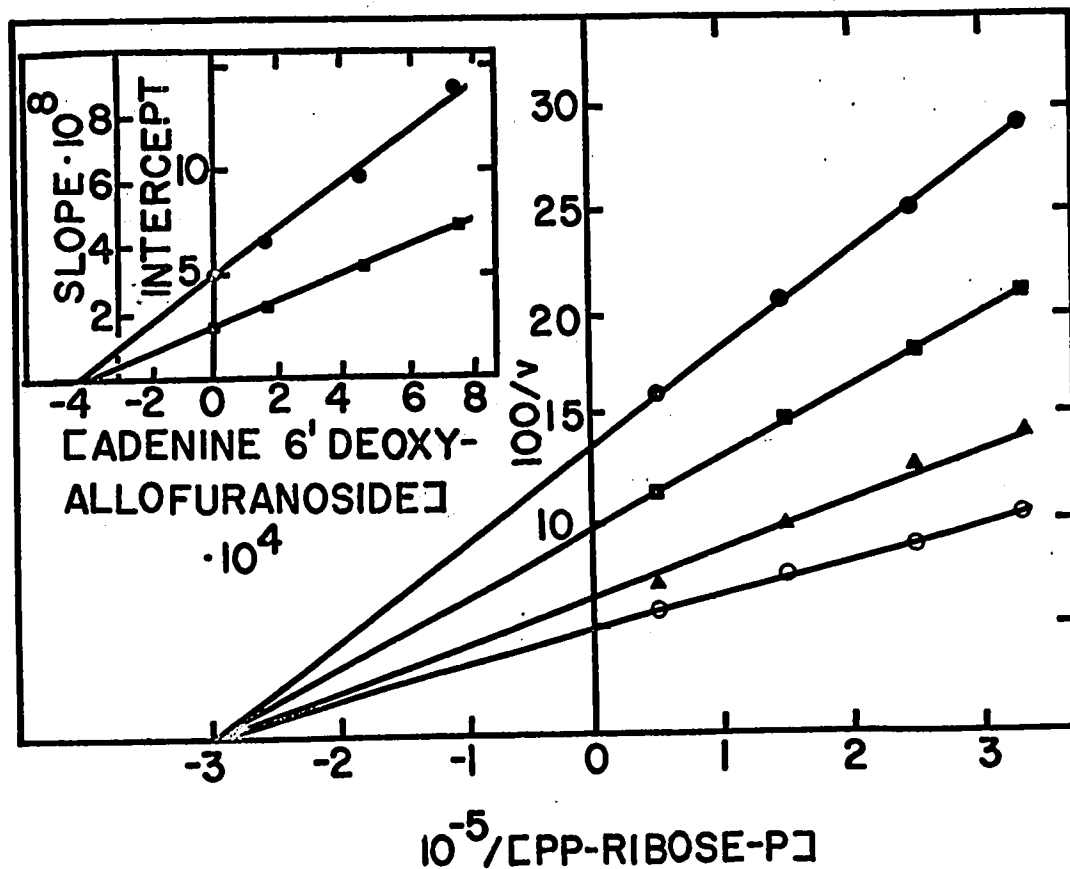


Figure 39. Inhibition by adenine 6'-deoxyallofuranoside. Double reciprocal plot of initial velocity against PP-ribose-P concentration at an adenine concentration of  $9 \times 10^{-6}$  M. The concentrations of adenine 6'-deoxyallofuranoside are: 0 (○),  $1.5 \times 10^{-4}$  M (△),  $4.5 \times 10^{-4}$  M (□), and  $7 \times 10^{-4}$  M (●).  $v$  is expressed as micromicromoles of AMP per min. The insert represents the secondary plot of slopes (□) and intercepts (●) against molar concentrations of adenine 6'-deoxyallofuranoside.

Rate Equations for Initial Velocity in the Presence of Dead-end Inhibitors Binding to the Enzyme-PP-ribose-P Complex and Free Enzyme.

$$\frac{v}{E_0} = \frac{V_i^{AB}}{K_{iA}K_B \left(1 + \frac{I}{K_i}\right) + K_A B \left(1 + \frac{I}{K_i}\right) + K_B A \left(1 + \frac{I}{K_{ii}}\right) + AB} \quad (29)$$

Substrate B varied

$$\frac{E_0}{v} = \frac{K_B}{V_1 B} \left[ \frac{K_{iA}}{A} \left(1 + \frac{I}{K_i}\right) + 1 + \frac{I}{K_{ii}} \right] + \frac{1}{V_1} \left[ 1 + \frac{K_A}{A} \left(1 + \frac{I}{K_i}\right) \right] \quad (30)$$

Substrate A varied

$$\frac{E_0}{v} = \frac{K_A}{V_1 A} \left[ \frac{K_{iA}K_B}{K_A B} \left(1 + \frac{I}{K_i}\right) + 1 + \frac{I}{K_i} \right] + \frac{1}{V_1} \left[ 1 + \frac{K_B}{B} \left(1 + \frac{I}{K_{ii}}\right) \right] \quad (31)$$

adenine 6'-deoxy- $\beta$ -D-allofuranoside binds to two forms of the enzyme, free enzyme and the enzyme-PP-ribose-P complex, and the rate equations for such a situation are given on page 177.  $K_i$  is the inhibition constant with free enzyme and  $K_{ii}$  is the inhibition constant with the enzyme-PP-ribose-P complex. Equations 30 and 31 are the double reciprocal forms of the rate equations when the concentrations of one substrate is varied at several fixed concentrations of the inhibitor while the concentration of the other substrate is held constant. The first term in both equations defines the slope while the second term defines the intercept. The slope terms in both equations may be rearranged to the following forms:

$$\frac{K_A}{V_1 A} \left(1 + \frac{K_{iA} K_B}{K_A B}\right) \left(1 + \frac{I}{K_i}\right) \text{ when A is varied, and } \frac{K_B}{V_1 B} \left(1 + \frac{K_{iA}}{A}\right) \left(1 + \frac{I}{K_{ii}}\right) \text{ when B is varied.}$$

Since both  $\frac{K_{iA} K_B}{K_A B}$  and  $\frac{K_{iA}}{A}$  are less than  $10^{-2}$ , the slope terms may be reduced to  $\frac{K_A}{V_1 A} \left(1 + \frac{I}{K_i}\right)$  and  $\frac{K_B}{V_1 B} \left(1 + \frac{I}{K_{ii}}\right)$ , and both  $K_i$  and  $K_{ii}$  may be determined from

plots of slope versus inhibitor concentration. These values are  $4.2 \times 10^{-5}$  M for  $K_{ii}$ , and  $4.2 \times 10^{-4}$  M for  $K_i$ . Adenine 6'-deoxy- $\beta$ -D-allofuranoside therefore bound more effectively to the enzyme-PP-ribose-P complex than to free enzyme. The

intercept terms are  $\frac{1}{V_1} \left[1 + \frac{K_B}{B} \left(1 + \frac{I}{K_{ii}}\right)\right]$  when substrate A is

varied, and  $\frac{1}{V_1} \left[ \frac{K_A}{A} \left( 1 + \frac{I}{K_i} \right) \right]$  when substrate B is varied. As the concentration of inhibitor was altered, the term  $\frac{1}{V_1} \left[ 1 + \frac{K_B}{B} \left( 1 + \frac{I}{K_{ii}} \right) \right]$  was altered 150% while the term  $\frac{1}{V_1} \left[ 1 + \frac{K_A}{A} \left( 1 + \frac{I}{K_i} \right) \right]$  was altered by only 14.5%. Therefore when the concentration of substrate B was varied at several concentrations of inhibitor I, and the concentration of substrate A was 10 times its Michaelis constant, a change in the intercept would be difficult to detect by graphical analysis and the inhibition would appear to be competitive. Similar results were obtained with adenosine.

Table 18a shows the per cent inhibition for nucleoside analogs with changes in the base moiety only. Adenosine was only a moderately effective inhibitor while 2-azaadenosine and 2-fluoroadenosine were much stronger inhibitors. Other changes in the purine moiety, such as replacement of the 6-amino group by hydroxyl-, iodo-, ethylthio-, mercapto-, methylamino- or hydroxylamino- groups; replacement of the hydrogen on the 2-carbon by amino- or hydroxyl- groups; or replacement of the hydrogen on the 8-carbon by methoxy- or bromo- groups, almost completely abolished inhibitory activity. Because nucleosides appear to be able to compete more effectively with adenine for the enzyme-PP-ribose-P complex than with PP-ribose-P for free enzyme, the purine moiety may

be binding to the same site as adenine. If this were the case, however, adenosine would be expected to be the most potent inhibitor. In addition, steric hindrance by PP-ribose-P would be expected to prevent binding of these nucleoside analogs. It is therefore unlikely that the binding site for the purine moiety of these analogs is the same as that of adenine.

Table 18b shows the per cent inhibitions for nucleoside analogs with changes in the sugar moiety only. 9-(tetrahydro-2-furyl)-, 9-valeronitrilo-, 9-(3'-amino-3'-deoxyribofuranosyl)-, 9-(6'-deoxy- $\beta$ ,D-allofuranosyl)-, 9-allofuranosyl-, 9-xylofuranosyl-, 9-(L-ribofuranosyl)-, 9-glucofuranosyl- and 9-fructofuranosyladenine were more potent inhibitors than was adenosine. Because it has been previously shown that a 2'-, 3'- or 5'-phosphate group was necessary for binding of nucleotide analogs to the enzyme, and that the unsubstituted sugar, D-ribofuranose, did not bind to the PP-ribose-P binding site, these nucleoside analogs would not be expected to be effective competitive inhibitors of PP-ribose-P for free enzyme. When the inhibitors bind to the enzyme-PP-ribose-P complex, the sugar moiety should be prevented from binding to the PP-ribose-P binding site.

Table 18c shows the per cent inhibitions for nucleoside analogs with changes in both the purine and sugar moieties. Of those compounds tested, only deoxyguanosine, 6-mercapto-



purine homoribofuranoside and 6-mercaptapurine arabinofuranoside were as effective inhibitors as adenosine.

#### D. Discussion

The work of Henderson and Miller (59) and Hori, Gadd and Henderson (62) with nucleotides, and the present work with nucleosides, show that both classes of compounds bind to at least two enzyme forms. Both nucleosides and nucleotides bind to free enzyme but it is unlikely that nucleosides bind to those sites normally occupied by the substrates. AMP is known to bind to a product binding site, and therefore those groups on the enzyme which are involved in binding adenine and the ribose 5-phosphate moiety of PP-ribose-P are probably also involved in AMP binding. Because the 5-phosphate group is apparently necessary for AMP binding, it is doubtful that the nucleosides bind to those sites normally occupied by AMP or by adenine and PP-ribose-P. In addition, the structural requirements observed for inhibition by the nucleosides is not consistent with their binding to these sites. Nucleosides also bind to the enzyme-PP-ribose-P complex, but the 9-substituent of the nucleosides could not bind to the already occupied PP-ribose-P binding site, and the purine moiety would not be expected to bind to the adenine binding site because of the bulky group on its 9-position. At the present time not enough analogs have been tested to make a critical evaluation of those groups and

atoms of nucleosides that are involved in binding.

Although no direct kinetic evidence is available at the present time, it seems unlikely that these nucleoside analogs bind to either the PP-ribose-P or adenine binding sites. Inhibition by these nucleosides may be the result of binding to a site which is close enough to the substrate binding sites that the bound nucleosides sterically prevent adenine and PP-ribose-P binding, or to some allosteric site.

## VIII CATALYTIC MECHANISM OF ADENINE PHOSPHORIBOSYLTRANSFERASE

### A. Introduction

At the present time little is known about the basis of the catalytic activity of enzymes. A number of theories have been advanced to explain this property of certain proteins, but Koshland and Neet (82) have pointed out that enzyme catalyzed reactions are  $10^9$  to  $10^{12}$  times more rapid than predicted by theoretical calculations. The kinetic model of an enzyme reaction describes the order of substrate addition and of product release, but may give little or no information concerning the actual catalytic process. A ping pong bi bi kinetic model implies the existence of an enzyme-bound E-S' complex, but its existence must be confirmed by isolation. S' may be defined as that part of substrate S that remains associated with enzyme. Once isolated, however, the complex may be degraded and the particular amino acid side chain to which the intermediate, S', is bound may be determined. This may show that the amino acid side chain in question is involved in a displacement reaction on the substrate, and any catalytic mechanism proposed must be consistent with this observation. Such intermediates cannot be isolated in ordered bi bi kinetic mechanisms, and the involvement of particular amino acid residues must therefore be determined by other means.

Amino acid functional groups which directly participate

in the reaction may be divided into three classes: those that are involved only in the binding of substrates and products to the enzyme; those that are involved only in the actual catalytic mechanism; and those that are involved in both substrate binding and the catalytic mechanism. In practice it is doubtful if such a clear distinction between the roles of a particular amino acid functional group may be made.

Amino acids involved in the catalytic mechanism have been identified by ionization constants determined from variation in maximum velocity as a function of pH. Histidine has been implicated by such studies, for example, in the catalytic mechanism of chymotrypsin (8) and phosphoglucose isomerase (37). The identification of particular amino acids solely on the basis of the kinetically determined ionization constant may be somewhat uncertain, however, since ionization constants of amino acids may be changed as much as 1 pH unit upon incorporation into a protein. Heat of ionization has been used as confirmatory evidence in the identification of an amino acid residue.

Particular amino acid functional groups may also be implicated in the catalytic mechanism by showing a correlation between their disappearance and a loss of enzyme activity upon treatment of the enzyme with a protein reagent. The difficulties encountered in the use of this technique

have been discussed previously.

Any catalytic mechanism proposed for adenine phosphoribosyltransferase must be consistent with the following requirements:

1. Both substrates must add to the enzyme before any of the products are released.
2. The products are AMP and pyrophosphate, not orthophosphate.
3. The glycosidic bond is inverted from the  $\alpha$ -configuration of PP-ribose-P to the  $\beta$ -configuration of AMP.
4. The carbon-oxygen bond of the ester linkage between the 1-carbon and the pyrophosphate moiety must ultimately be broken.
5. The proton at the 9-nitrogen position of adenine must be removed.

B. Expansion of the kinetic model

The kinetic model previously shown in Figure 16 contains only the rate constants which describe the addition of substrates and the release of products. There is no consideration in that model of the actual catalytic process in which the bond between carbon 1 of PP-ribose-P and the oxygen is broken, the proton on the 9-nitrogen of adenine is removed, and the bond between the 9-nitrogen of adenine and the 1-carbon of PP-ribose-P is formed. Figure 40 shows an expanded

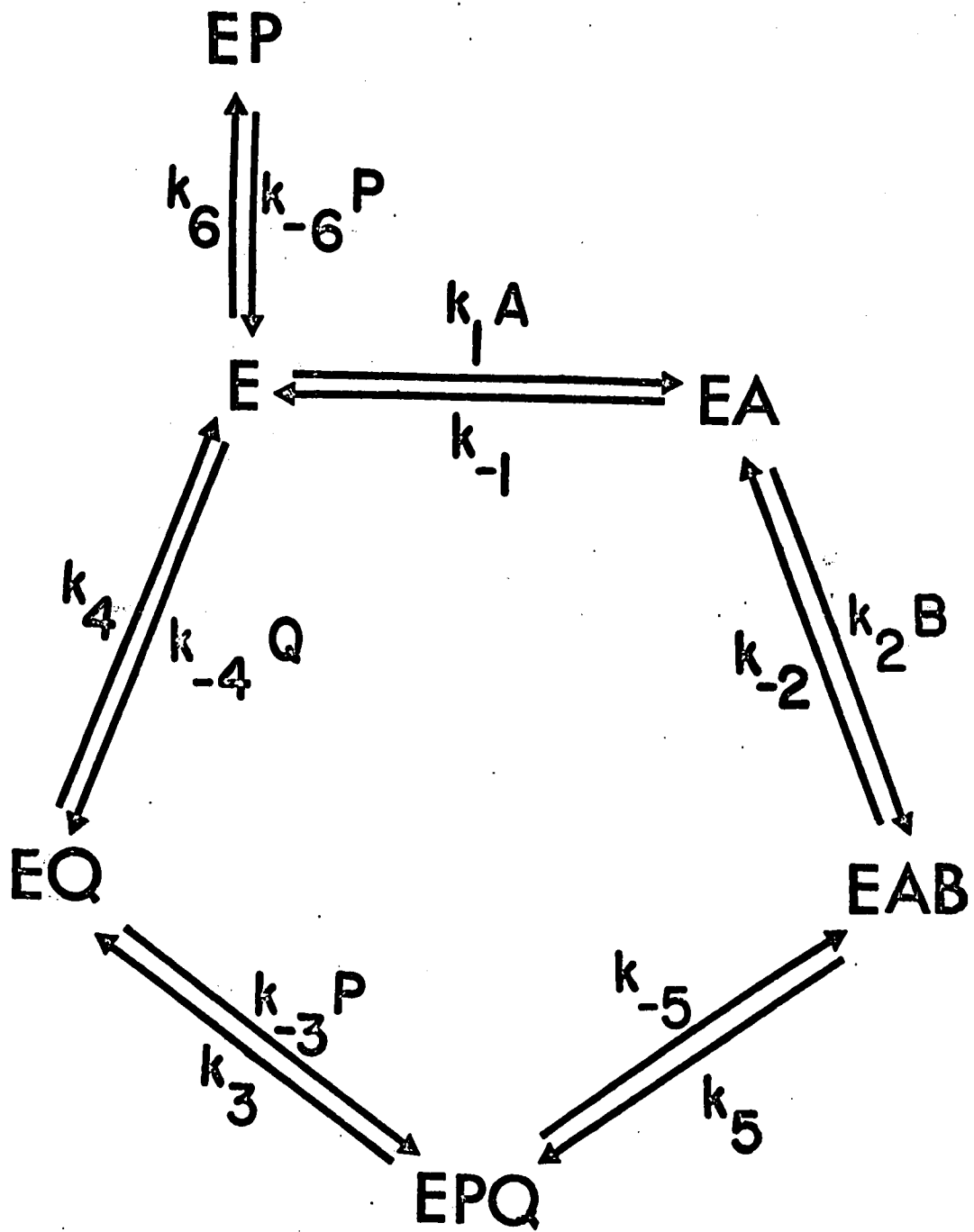


Figure 40. Expanded kinetic mechanism.

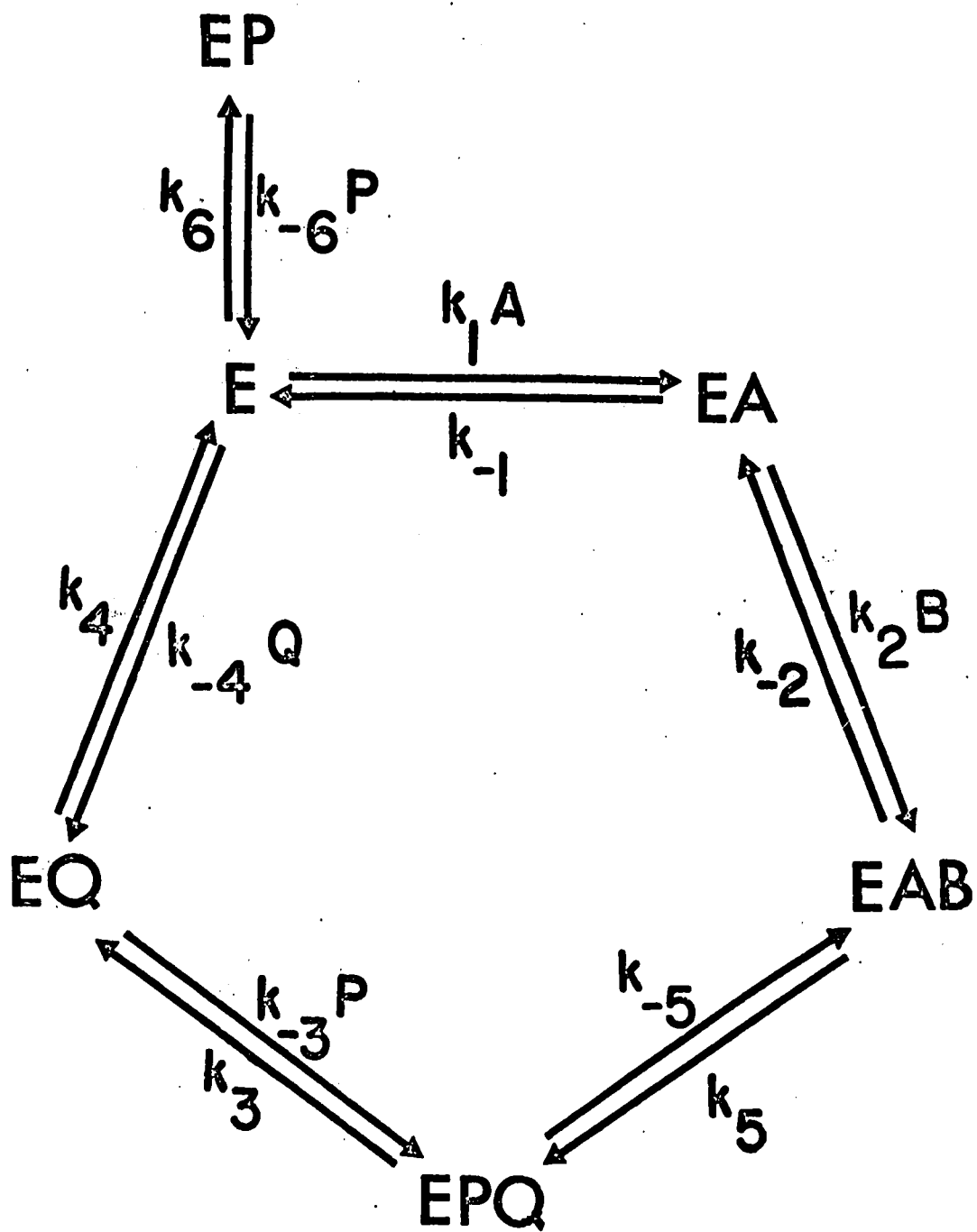


Figure 40. Expanded kinetic mechanism.

form of the kinetic model in which two rate constants have been introduced to describe kinetically the chemical conversion of PP-ribose-P and adenine to AMP and pyrophosphate. Because there is one bond broken, one bond made and one proton removed, at least three pairs of rate constants or three enzyme isomerizations should be included in the kinetic model; the two shown are used for simplicity to describe the catalytic mechanism as a whole.

The derivation of the rate equation for the expanded model gives rise to one that is identical, in terms of kinetic constants, to that derived for the simpler model. Most of the kinetic parameters, however, including  $K_A$ ,  $K_B$ , etc. are now more complex functions of the individual rate constants. The only kinetic constants that remain unchanged in terms of rate constants are  $K_{iA}$ ,  $K_{iQ}$ , and  $K_{iip}$ . The maximum velocity in the forward direction is now a function not only of the dissociation of the products, pyrophosphate and AMP, but also a function of the rate of molecular interconversion. As in the simpler case,  $K_A$  and  $K_B$  contain the  $V_1$  term.

### C. Effect of pH on maximum velocity

Maximum velocities at various pH values between 5.5 and 11 were obtained from double reciprocal plots of initial velocity versus substrate concentration. Figure 41 shows the effect of pH on  $V_1$  at 10°, 30° and 37.5°.



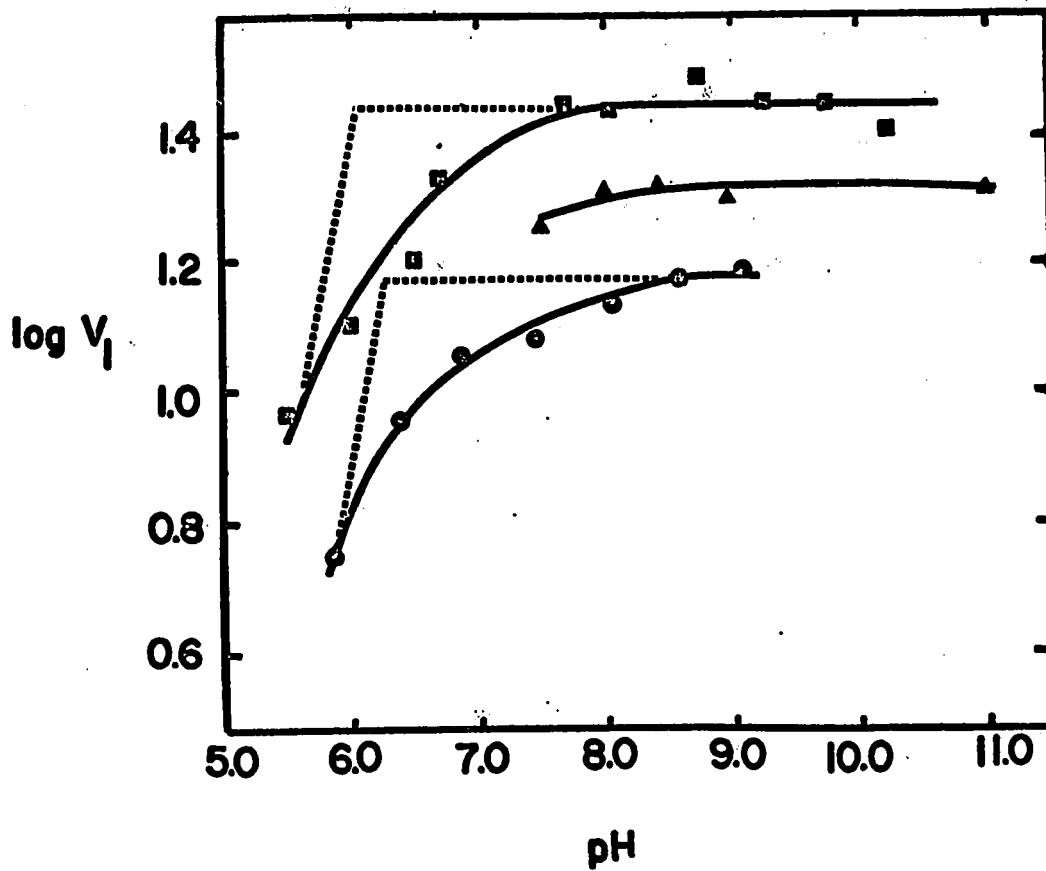


Figure 41. Effect of pH on the logarithm of  $V_1$  at 10° (●), 30° (■), and 37.5° (▲).

The velocities observed at one pH but at different temperatures may not be directly compared since at the lower temperature higher concentrations of the enzyme preparation were used in order to obtain a measurable velocity. At 30° the apparent ionization constant is 6.05 and at 10° the apparent  $pK_a$  is 6.25. The ionization constants were not determined at higher temperatures since these would shift the  $pK_a$  to lower pH values, and at higher temperatures and lower pH values the enzyme is unstable. At temperatures below 10° the initial velocity became too slow to measure. The  $pK_a$  values obtained are close to those found in other proteins at 25° for the imidazole group of histidine, 5.6 to 7.0, and that of N-terminal  $\alpha$ -amino residues, 6.5 to 8.5, but much higher than that of carboxyl groups, 3.0 to 4.7 (34). Because PP-ribose-P has an ionization constant much lower than this value ( $pK_a = 4.7$ ) and adenine has  $pK_a$ 's of 4.2 and 9.8, the ionization constant determined ( $pK_a = 6.05$  at 30°) is believed to be due to the ionization of an amino acid residue on the enzyme and not to the substrates.

The apparent heat of ionization calculated from the change in ionization constant between 10° and 30° according to the equation  $\Delta H = -2.303RT^2 (dpK_a/dT)$  was calculated to be +6,140 cal mole<sup>-1</sup>. This value is very close to the +6,900 to +7,500 cal mole<sup>-1</sup> reported for the imidazole group of histidine, and significantly different from that for  $\alpha$ -amino

groups, +10,000 to +13,000 cal mole<sup>-1</sup>, and that for carboxyl groups, -1,500 cal mole<sup>-1</sup> to +1,500 cal mole<sup>-1</sup> (34).

The same sources of errors encountered in the estimation of the heats of ionization for those groups involved in PP-ribose-P and adenine binding would also be encountered in determining the heat of ionization calculated above. If all of these errors were additive, then a maximum error up to ±87% could result, although this might seem somewhat unlikely. Because the ionization constant is lower than that usually observed for α-amino groups, and higher than that usually observed for carboxyl groups, and because the heat of ionization would be required to deviate by 63% or more from the experimentally calculated heat of ionization to be in the region of those values observed for α-amino or carboxyl groups, it is suggested that the group in question is in fact a histidine residue.

Because  $V_1$  is equal to  $(k_3k_4)/(k_3 + k_4)$  in the simple kinetic model, and to  $(k_3k_4k_5)/(k_3k_4 + k_4k_{-5} + k_5k_4 + k_5k_3)$  in the expanded model, a decrease in  $V_1$  as the pH is lowered may be attributed to a decrease in  $k_3$ ,  $k_4$  or  $k_5$ , or to an increase in  $k_{-5}$ . Decreases in  $k_3$  and  $k_4$  would reflect slower product release whereas slower catalysis would be due either to a decrease in  $k_5$  or to an increase in  $k_{-5}$ .

Because  $V_1$  is a function of pH at the lower pH values and because both  $K_A$  and  $K_B$  contain  $V_1$  terms, plots of pH

versus  $pK_A$  and  $pK_B$  were expected to have the upward inflections in the region of pH 6 which were observed in Figures 28 and 36.  $K_B$  is equal to  $V_1(k_{-2} + k_3)/(k_2k_3)$  for the simple kinetic model and equal to  $V_1(k_3k_5 + k_3k_{-2} + k_{-2}k_{-5})/k_2k_3$  for the expanded model. As  $V_1$  decreases, therefore,  $K_B$  would also decrease. The negative logarithm of  $K_B$  would then increase and give an upward inflection in the  $pK_B$  versus pH plot (Figure 36). Because  $K_A$  is also a function of  $V_1$  ( $K_A = V_1/k_1$ ) for both the simple and expanded kinetic models,  $K_A$  would also decrease and the increase in its negative logarithm would thus give an upward inflection in the  $pK_A$  versus pH plot (Figure 28).

It is concluded from these experiments, therefore, that there is a group in the enzyme-PP-ribose-P-adenine complex which ionizes at pH 6.05 at 30° C and has a heat of ionization of approximately +6,000 cal mole<sup>-1</sup>. This group is believed to be an amino acid residue at the active site. Although the presence of ionized substrates and ionized amino acid residues can affect the ionization constant and heat of ionization of adjacent amino acid residues, these results at least suggest the involvement of a histidine residue in either the catalytic mechanism or in the release of products. A distinction between these two possible roles cannot be made because the enzymatic reaction is not reversible.

D. Effect of  $^2\text{H}_2\text{O}$  on the maximum velocity

During the catalytic process the proton at the 9-nitrogen position of adenine must be removed and a glycosidic bond must be formed. The possibilities were considered either that the removal of the proton was the rate-determining step, or that the nucleophilic attack by the 9-nitrogen on the 1-carbon of PP-ribose-P was the rate-determining step. Comparison of reaction rates in  $\text{H}_2\text{O}$  and  $^2\text{H}_2\text{O}$  has often been used to distinguish between these two types of possibilities. The removal of the proton by a general base is the rate-determining step in general base catalysis. A nucleophilic attack may occur subsequent to this proton removal, but it must be faster than the attack by the base. For those cases in which the rate-limiting step is the attack by the nucleophile and subsequent breakdown of the intermediate, a proton may be lost or gained, but its transfer is faster than the nucleophilic attack.

The "isotope effect" may be defined as the ratio of the rate of the reaction in  $\text{H}_2\text{O}$  to that in  $^2\text{H}_2\text{O}$ . Wiberg (138) has, on theoretical grounds, postulated that general base catalysis and general acid catalysis should proceed faster in  $\text{H}_2\text{O}$  than in  $^2\text{H}_2\text{O}$ . Bender, Pollik and Neveau (9) observed that general-base-catalysed reactions in which the base was imidazole gave an isotope effect greater than two, whereas in nucleophilic reactions catalyzed by imidazole no isotope

effect was observed. If the identity of the nucleophile is unknown, however, an observed isotope effect may be somewhat difficult to interpret since in nucleophilic reactions catalyzed by carboxylate ions isotope effects may vary from 1.1 to 1.9. In addition isotope effects much less than 2 have been observed in some cases of general base catalyzed hydrolysis. Solvation effects can give rise to isotope effects from 1.0 to 1.5, but in the case of imidazole these are essentially nil (9).

Maximum velocities and Michaelis constants were determined from double reciprocal plots of initial velocity versus substrate concentration at pH 8.10 in  $\text{H}_2\text{O}$  and in  $^2\text{H}_2\text{O}$ . Bender et al. (8) observed that the  $\text{pK}_a$ 's of protein functional groups are  $0.5 \pm 0.2$  pH units higher in  $^2\text{H}_2\text{O}$  than in  $\text{H}_2\text{O}$ . Because the pH chosen was 2 pH units above that group on the enzyme which affects the  $V_1$  term, enzyme ionization should not affect the observed maximum velocities.

Tris-HCl buffer, 0.15 M, was dried over NaOH in vacuo and then taken up in a volume of  $^2\text{H}_2\text{O}$  equal to the volume of  $\text{H}_2\text{O}$  removed. The apparent  $\text{p}^2\text{H}$  was measured with a pH meter and, as suggested by several groups (44,95,119), 0.4 units were then added to this value to obtain the true  $\text{p}^2\text{H}$  value. Dried  $\text{MgSO}_4$  was dissolved in  $^2\text{H}_2\text{O}$ , and the substrates were prepared by diluting concentrated  $\text{H}_2\text{O}$  solutions of PP-ribose-P and of adenine in  $^2\text{H}_2\text{O}$ . The amount of  $\text{H}_2\text{O}$  introduced into

the reaction mixtures was below 1% of the total water.

Figure 42 shows the secondary plots obtained from double reciprocal plots of initial velocity versus substrate concentration. The ratio of  $V_1(\text{H}_2\text{O})/V_1(^2\text{H}_2\text{O})$  was found to be 1.84, that of  $K_A(\text{H}_2\text{O})/K_A(^2\text{H}_2\text{O}) = 1.55$ , and that of  $K_B(\text{H}_2\text{O})/K_B(^2\text{H}_2\text{O}) = 1.70$ . An isotope effect on  $K_A$  and  $K_B$  was expected because both Michaelis constants contain  $V_1$  terms. Although the isotope effect was found to be slightly less than the 2 or greater expected for base catalyzed reaction by imidazole (9), the value obtained is close enough to 2 to be consistent with a mechanism involving proton transfer to imidazole as the rate-limiting step. Bernhard (12) has postulated that in general base catalysis the efficiency of the general base is dependent not only on its ability to accept a proton but also on its ability to transfer the acquired proton either back to the substrate or to some other proton acceptor such as the aqueous solvent, in order to regenerate the original catalyst. In addition, Bernhard suggests that the optimal rate for enzyme catalysis in aqueous solution should be obtained when the base strength of the base is equal to the acid strength of its conjugate acid, that is when the  $\text{p}K_a$  is near neutrality; of the potential acid-base catalysts in proteins, the imidazole group of histidine would appear to be the most efficient.

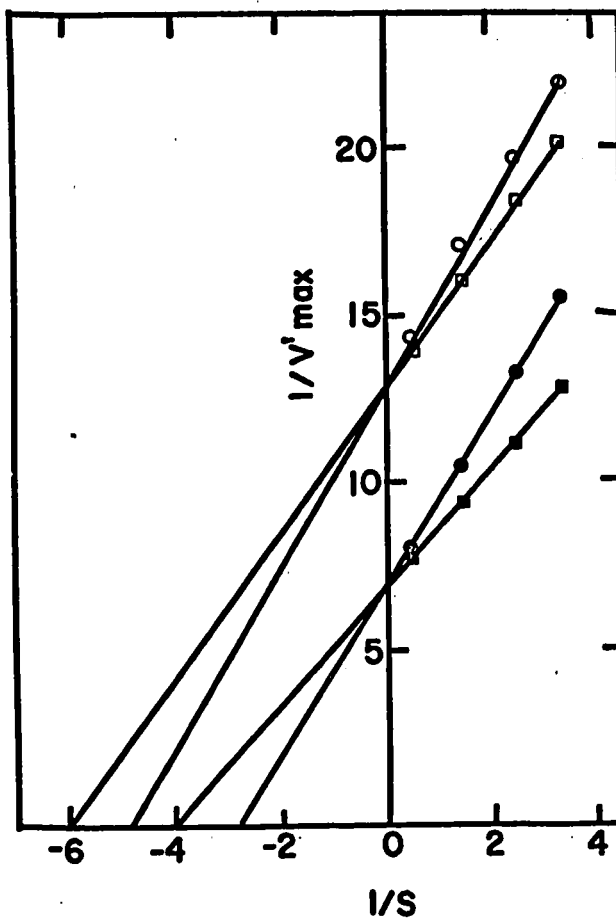


Figure 42. Secondary plots of reciprocals of apparent maximum velocities in  $^2\text{H}_2\text{O}$  ( O , □ ) and  $\text{H}_2\text{O}$  ( ● , ■ ) against reciprocals of molar concentrations (1/S) of PP-ribose-P ( □ , ■ ) and adenine ( O , ● ). The units for PP-ribose-P concentration are  $10^{-5}$  M, and for adenine,  $10^{-6}$  M. The units of  $V'$  max are micromicromoles of AMP per min and were obtained from double reciprocal plots of initial velocity against substrate concentration.



#### E. Adenine analogs as substrates

A number of adenine analogs were tested for their ability to serve as substrates, using the spectrophotometric assay described above. The maximum velocities for the adenine analogs were not determined in these studies. One compound, 2,6-diaminopurine, was assayed by using radioactive 2,6-diaminopurine-<sup>14</sup>C as previously described, and it is the only analog of adenine for which the Michaelis constant was determined. Those analogs which were shown to serve as substrates are 2-fluoroadenine, 4-aminopyrazolo(3,4-d)pyrimidine, 2-azaadenine, 6-chloropurine, 6-methylpurine and purine (Table 19). Flaks, Erwin and Buchanan (41) in 1957 reported that the condensation of 4-amino-5-imidazolecarboxamide and PP-ribose-P to yield 4-amino-5-imidazolecarboxamide ribonucleotide was catalyzed by the same enzyme which caused the formation of AMP from adenine and PP-ribose-P. Haavik (51) found that adenine phosphoribosyltransferase from calf liver catalyzes the conversion of 4-aminopyrazolo(3,4-d)pyrimidine, 2-fluoroadenine and 8-azaadenine to their respective nucleotides.

Adenine and 2-fluoroadenine were converted to their respective nucleotides at the same apparent maximum rate; this was 4-fold faster than the rate for 8-azaadenine, and 12-fold faster than the rate for 4-aminopyrazolo(3,4-d)pyrimidine (51). This order of rate of nucleotide synthesis

TABLE XIX: Alternate Substrates of Adenine Phosphoribosyltransferase

Substrate	Conc. (M)	Δ Absorbance (260 mμ)
Adenine	$10^{-4}$	0.266
2-Fluoroadenine	$10^{-4}$	0.296
4-Aminopyrazolo (3,4-d) pyrimidine	$10^{-3}$	0.113
2-Azaadenine	$4 \times 10^{-4}$	0.042
6-Chloropurine	$2 \times 10^{-3}$	0.016
6-Methylpurine	$2 \times 10^{-3}$	0.037
Purine	$2 \times 10^{-3}$	0.020

(a) The assay used was that described for the spectrophotometric measurement of the conversion of adenine to AMP. The change in absorbance was determined by subtracting the absorbance obtained for reaction mixtures not containing enzyme preparation from the absorbance obtained for reaction mixtures containing enzyme preparation.

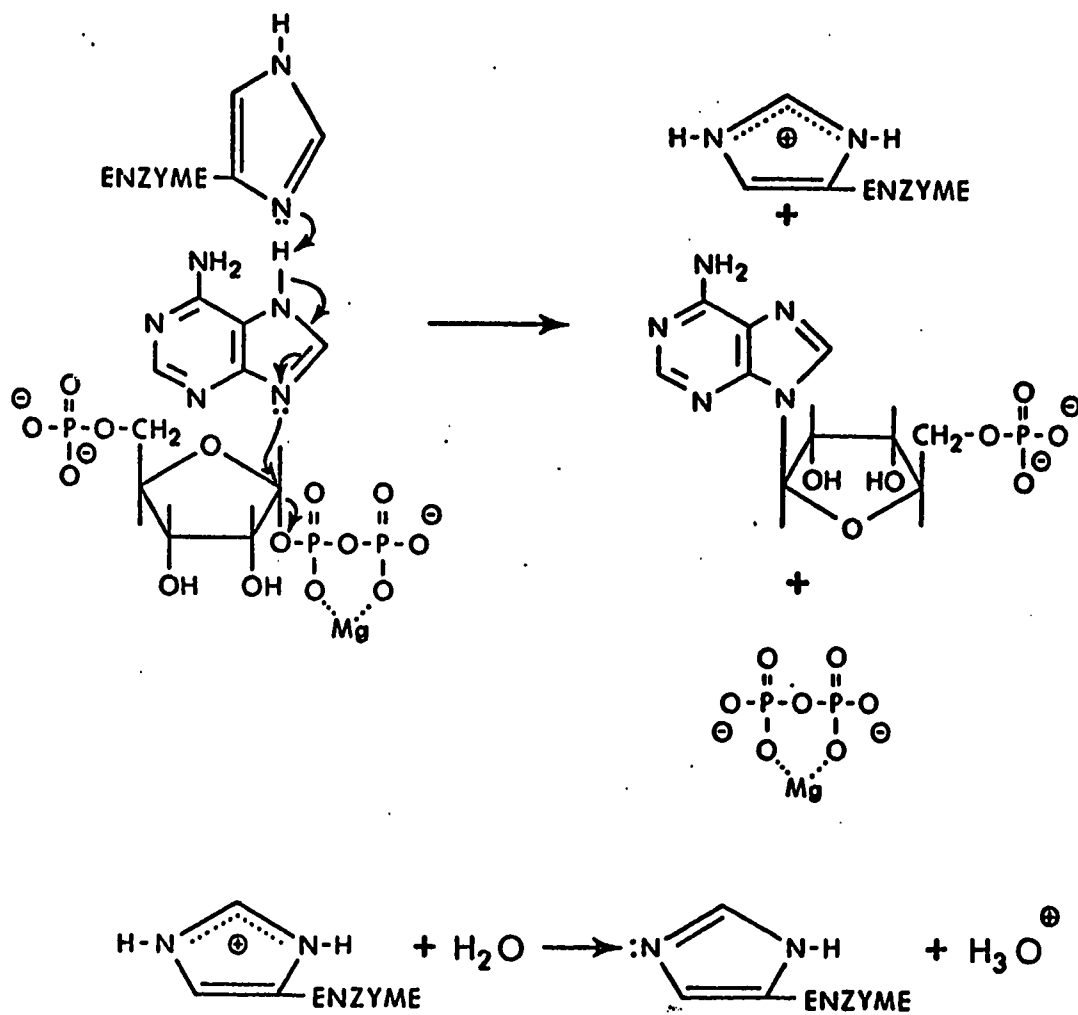
does not correlate with inhibition constants observed in the present study; adenine and 2-fluoroadenine have approximately the same  $K_i$  value which is 6-fold lower than that of 4-aminopyrazolo(3,4-d)pyrimidine and 254-fold lower than the  $K_i$  for 8-azaadenine. Because the maximum velocity is equal to  $(k_3k_4)/(k_3 + k_4)$  in the simple kinetic model and  $(k_3k_4k_5)/(k_3k_4 + k_4k_{-5} + k_5k_4 + k_5k_3)$  in the expanded model, the slower rate of nucleotide synthesis for 4-aminopyrazolo(3,4-d)pyrimidine and 8-azaadenine must be due to a slower release of products or a slower catalytic step than that of adenine and 2-fluoroadenine. Since these adenine analogs bind less effectively to the enzyme than adenine, it would not be expected that their nucleotides would bind more effectively than AMP; it is therefore reasonable to conclude that the slower rate of nucleotide synthesis is due to a slower catalytic step.

Maximum velocities of nucleotide synthesis from these alternative substrates were not determined, and because it is not certain that the rates reported by Haavik were true maximum velocities, no attempt has been made to interpret these data in any detail. A more detailed study of the relationship between structure and maximum rate of nucleotide synthesis by such compounds might, however, give valuable insights into the catalytic mechanism of adenine phosphoribosyltransferase.

## F. Discussion

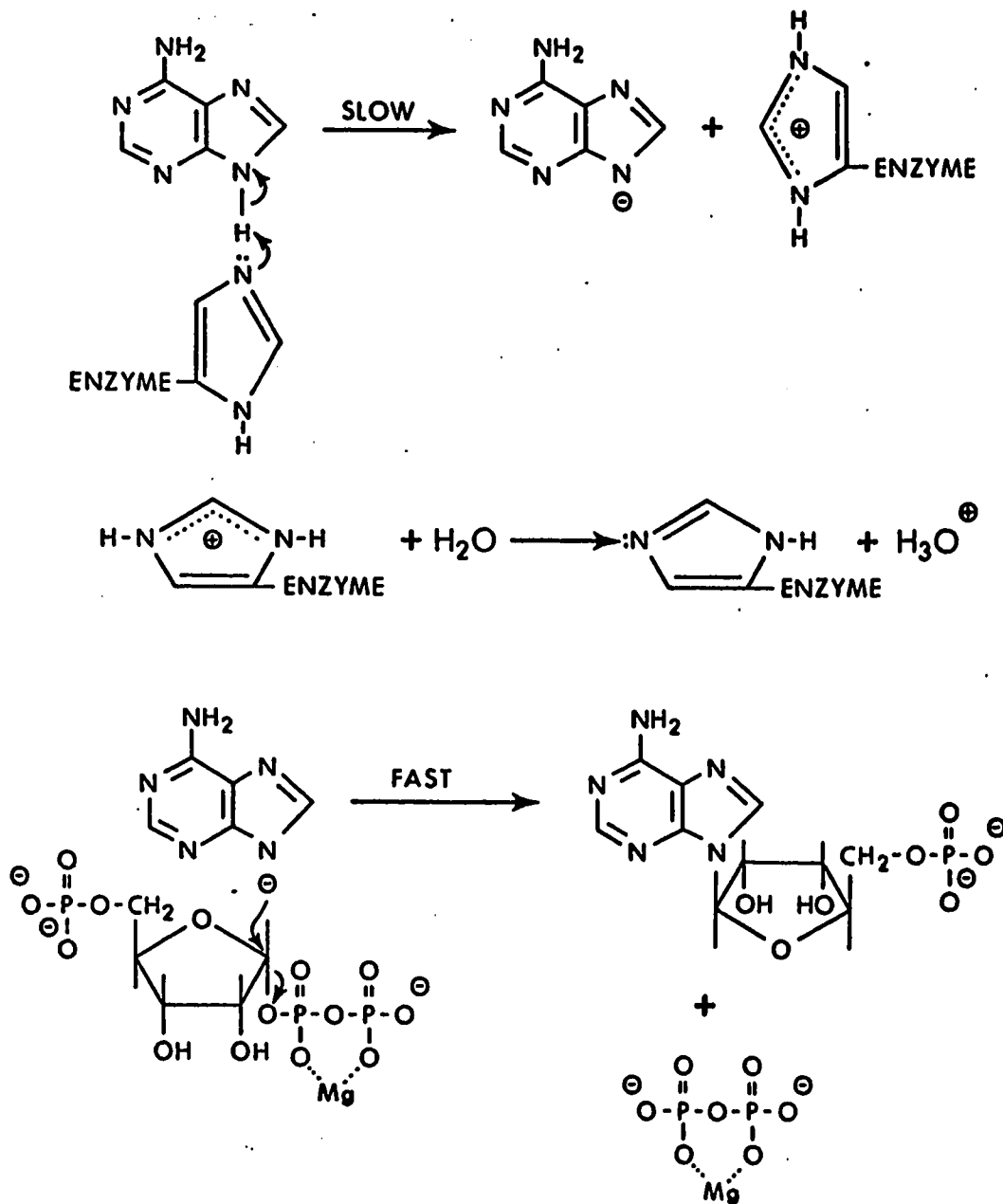
Any mechanism for adenine phosphoribosyltransferase must explain the inversion of the glycosidic bond from the  $\alpha$ -configuration of PP-ribose-P to the  $\beta$ -configuration of AMP. Both Glaser (48) and Koshland (81) have suggested that reactions involving inversion of glycosidic bonds probably involve backside nucleophilic attacks by the acceptor on the anomeric carbon. Inversion of configuration involving an enzyme-bound intermediate would require at least three displacement reactions. Kosower (83) has suggested that displacement reactions on five-membered rings are not fast and should not be favored because of steric hindrance by the 5-carbon phosphate to a rearward approach by adenine. CPK atomic models (Ealing Corporation) show that if adenine and PP-ribose-P are in the anti conformation to each other no steric hindrance due to the 5-carbon phosphate would be expected. Kosower (83) has further suggested that the adenine phosphoribosyltransferase reaction proceeds by way of an enzyme-bound 5-phosphoribosyl 1-carbonium or 4-oxonium ion followed by electrophilic attack leading to inversion (Figure 43-a). If such were the case it is difficult to explain why pyrophosphate is not released from the enzyme before the second substrate is bound, although the anionic-cationic attraction between pyrophosphate and the oxonium or carbonium ion might prevent its release from the enzyme until the





(b)

Figure 43. (Continued)



(c)

Figure 43. (Continued)

oxonium or carbonium ion were neutralized. The mechanism still seems unlikely, however, because secondary carbonium ions are somewhat unstable and would be expected to undergo solvolysis quite readily, the products being ribose 5-phosphate and pyrophosphate. In addition, anions such as chloride ion would be expected to readily attack the carbonium ion to give products other than those observed for adenine phosphoribosyltransferase.

Pullman and Pullman (109) have calculated resonance energies for adenine and the tautomeric form of adenine which has an acidic 7-NH group. From these data it can be calculated that only  $0.16 \text{ kcal mole}^{-1}$  is required for their interconversion. Because the binding energy for adenine is calculated to be  $6.9 \text{ kcal mole}^{-1}$ , there is enough energy involved in binding to move this hydrogen from the 9- to the 7-nitrogen. If this were to occur, then a concerted mechanism (Figure 43-b) could be postulated. This type of mechanism does not seem likely, however, since 4-aminopyrazolo(3,4-d)pyrimidine cannot undergo this tautomeric shift of the 9-nitrogen proton and is a substrate.

The mechanism favored is shown in Figure 43-c. In this mechanism both substrates must bind to the enzyme before catalysis takes place, inversion of the glycosidic bond takes place, C-O bond cleavage and not O-P bond cleavage occurs, and the products are AMP and pyrophosphate. The involvement



of histidine as a general base in the rate determining step is consistent with the observed effects of pH and  $^2\text{H}_2\text{O}$  on the maximum velocity. Removal of the proton on the 9-nitrogen of adenine should facilitate attack by this atom on the anomeric carbon of PP-ribose-P, the latter having been made strongly electrophilic by the pyrophosphoryl group. Anionic-anionic repulsion between the now charged adenine and the pyrophosphate moiety of PP-ribose-P would have been expected if the negative charges on the pyrophosphate moiety had not been partially neutralized through chelation with magnesium ion. This role of magnesium ion in catalysis may therefore be postulated, and is consistent with the observation that the complex of PP-ribose-P with free enzyme was catalytically inactive. The possibility that bond stretching might contribute to the catalytic efficiency of adenine phosphoribosyltransferase was suggested, though by no means proven, by the evidence presented above (Chapter V) that a conformational shift occurred following binding of the pyrophosphate moiety, and that this is required for binding of the ribose 5-phosphate moiety. If this conformational change did occur, and if it resulted in a stretching of the 1-C-O bond, the breakdown of the intermediate adenine-PP-ribose-P complex to AMP might be accelerated.

## IX GENERAL CONCLUSIONS

The general conclusions of this study of adenine phosphoribosyltransferase are illustrated schematically in Figure 44. This figure shows that PP-ribose-P, the first substrate, binds to the enzyme through its 1-pyrophosphate and 5-phosphate moieties. The magnesium-ion-pyrophosphate complex is postulated to bind first resulting in a conformational change of the enzyme which permits binding of both the 5-phosphoryl moiety and the second substrate, adenine. Adenine is bound to the enzyme through its 6-amino group, 3-nitrogen and 7-nitrogen atoms. The catalytic process is postulated to involve removal of the proton from the 9-nitrogen of adenine by an imidazole moiety, followed by a nucleophilic attack by the 9-nitrogen on the anomeric carbon of PP-ribose-P to give pyrophosphate and AMP. Pyrophosphate is the first product released, followed by AMP; free enzyme is thus released.

Much less is known about the amino acid residues in the enzyme which are involved in substrate binding. The amino acid residues which are involved in PP-ribose-P binding are represented by HA-, HB- and HC-, one of which is postulated to be an  $\epsilon$ -amino group of lysine; the possibility that a sulfhydryl group of cysteine is involved in binding cannot, however, be ruled out. Either lysine and cysteine could bind to the 5-phosphoryl moiety, the magnesium ion chelated

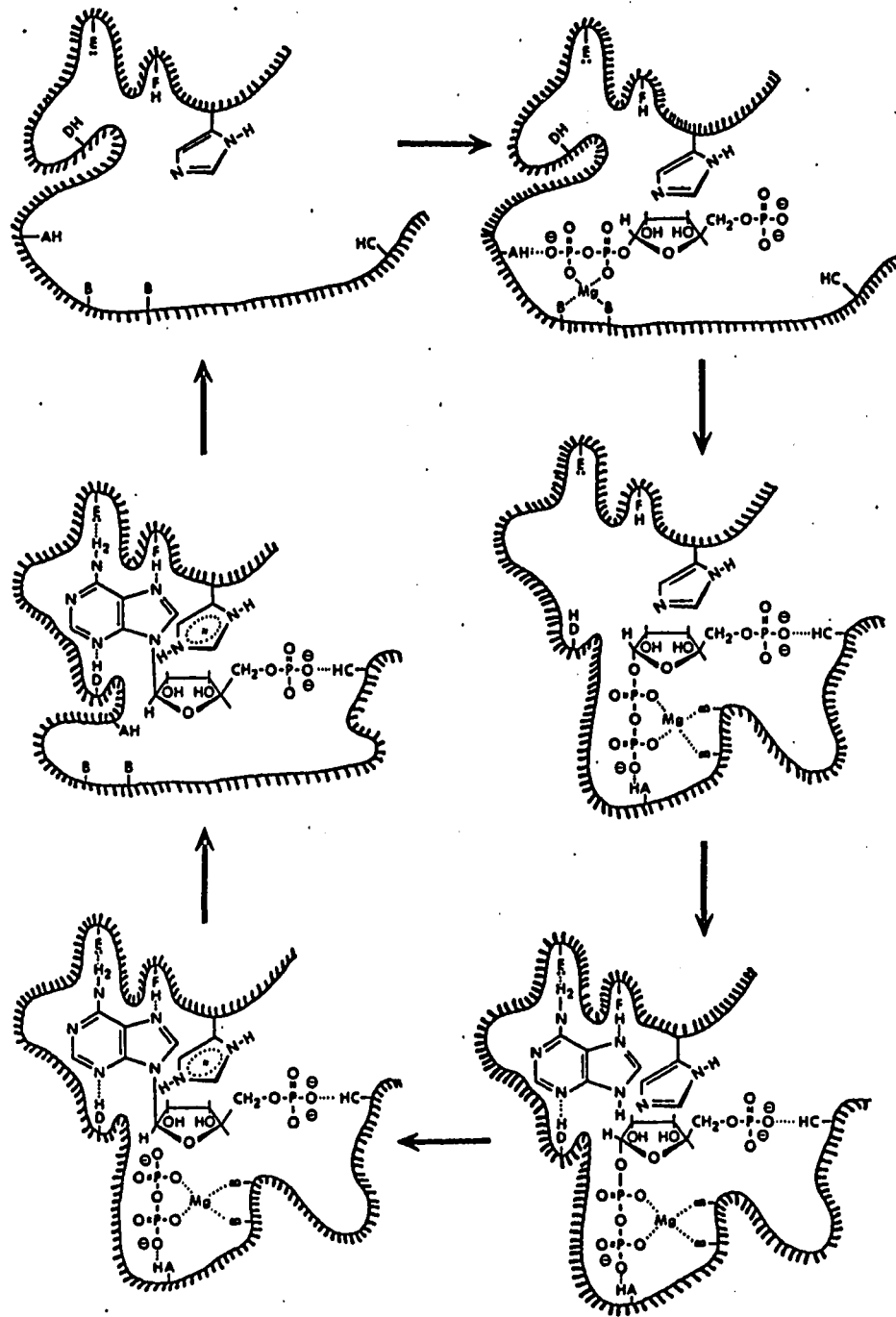


Figure 44. Proposed mechanism of adenine phosphoribosyltransferase.

to the 1-pyrophosphoryl moiety, or the anionic group on the 1-pyrophosphoryl moiety. The amino acid residues which are involved in binding adenine are represented by HD-, E-, and HF-, of which E- acts as an electron donor and HD- and HF- act as electron acceptors. A phenolic hydroxyl group of tyrosine is postulated to be involved in binding to either the 3- or the 7-nitrogen of adenine. E- could not be an anionic residue such as glutamic or aspartic acid. The possibility was considered that the imidazole moiety may also be involved in binding to the 3-nitrogen of adenine.

No consideration has been given to the release of products in this model; if an anionic group were situated close to the 1-carbon of PP-ribose-P, pyrophosphate could be dislodged by anionic repulsion as it gains a negative charge upon cleavage of the C-O bond.

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