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DATED...11th May.....1971.

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THE UNIVERSITY OF ALBERTA

KINETIC STUDIES OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE AND ITS INHIBITION BY ANTICANCER DRUGS

BY

© SAMUEL YUP-WO CHU

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, 1971

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 "KINETIC STUDIES OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE AND ITS INHIBITION BY ANTICANCER DRUGS", submitted by Samuel Y. Chu in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date 10th May, 1971.

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External Examiner

ABSTRACT

5'-Phosphoribosyl-formylglycineamide (FGAR) is one of the intermediates in the pathway of purine biosynthesis de novo. The enzymatic utilization of this intermediate is mediated by the enzyme 5'-phosphoribosyl-formylglycineamidine (FGAM) synthetase, which the anticancer drugs, azaserine and 6-diazo-5-oxo-L-norleucine (DON), are known to inhibit. To gain a better understanding of how the anticancer drugs act and how this enzymic reaction is controlled, experiments were carried out to synthesize the substrate FGAR and to partially purify the enzyme from Ehrlich ascites carcinoma cells for kinetic mechanism and inhibition studies.

FGAR was synthesized via direct phosphorylation of the 5' position of the linear ribonucleoside, N(N-formylglycyl)-ribofuranosylamine, with phosphoryl chloride in triethyl-phosphate, without protecting the 2',3' hydroxyl groups of the ribose.

A 56-fold purification of the FGAM synthetase was achieved through sonication, acid treatment, ammonium sulfate fractionation and Sephadex treatment. Both magnesium and potassium ions are required as activators.

Because of the number of rate equations which must be derived for all of the possible mechanisms of three or more substrate enzymic reactions, and their complexity, product inhibition studies of these enzymes have not commonly been performed. A simplified procedure was therefore developed to derive these rate equations. With this procedure,

product inhibition and dead-end inhibition patterns for the various possible three and four substrate enzymic mechanisms were obtained.

On the basis of initial rate, product inhibition and isotope exchange studies, a fully ping-pong kinetic mechanism is proposed for this tumor enzyme. The substrate L-glutamine binds to the enzyme first, while FGAR is the last substrate adding to the enzyme. The K_m values for glutamine, ATP and FGAR are 1.10×10^{-4} M, 1.5×10^{-3} M and 1.11×10^{-4} M, respectively. Ammonium chloride can replace glutamine as the nitrogen substrate. The K_m for ammonium chloride is 7.5×10^{-3} M at pH 8.0 and its V_{max} is about forty times less than that of the glutamine system.

Sulfhydryl reagents such as the mercurials inhibited the enzyme when glutamine was substrate and this inhibition could be reversed by glutathione or 2-mercaptoethanol. The antitumor drugs, azaserine and DON, also prevent the enzyme from utilizing glutamine, but stimulate rather than inhibit the utilization of ammonium chloride by the enzyme. Glutamine, but not other substrates or ammonium chloride, could protect the enzyme against the inhibition by these reagents. It appears that glutamine and ammonium chloride bind at different sites on the enzyme. The K_i values for azaserine and DON were found to be 2.3×10^{-6} M and 4.0×10^{-7} M, respectively, and these values are significantly lower than those found for the pigeon liver enzyme or that of other

normal cells; this difference may explain the selective toxicity of these drugs.

Acknowledgements

I would like to express my deepest appreciation and graditude to Dr. J. F. Henderson, my supervisor, for his invaluable guidance and patience during the course of this study. I would like to thank Dr. A. R. P. Paterson, Dr. J. S. Colter and members of the Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry for providing abundant interests and opportunities to learn.

The helpful discussions with Dr. W. A. Bridger, Dr. L. W. Brox and Dr. B. Paul are deeply appreciated. I sincerely thank Dr. R. J. Crawford of the Department of Chemistry, who has given me the inspiration and encouragement in taking up graduate studies and has also given me valuable advice concerning the synthetic part of the present work.

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List of Abbreviations

FGAR	5'-Phosphoribosyl-formylglycineamide
FGAM	5'-Phosphoribosyl-formylglycineamidine
AIR	5'-Phosphoribosyl-aminoimidazole
Pi	Orthophosphate
ATP	Adenosine 5'-triphosphate
K_m	Michaelis constant
K_i	Inhibition constant
V_{max}	Maximum velocity
EDTA	Disodium ethylenediaminetetraacetate
Tris	Tris(hydroxymethyl)aminomethane
DON	5-Diazo-5-oxo-L-norleucine
PP-ribose-P	5-Phosphoribosyl-pyrophosphate
PRA	5'-Phosphoribosylamine
GAR	5'-Phosphoribosyl-glycineamide
Carboxy-AIR	5'-Phosphoribosyl-aminoimidazole carboxylate
SAICAR	5'-Phosphoribosyl-aminoimidazole succinocarboxamide
AICAR	5'-Phosphoribosyl-aminoimidazole carboxamide
FAICAR	5'-Phosphoribosyl-formamidoimidazole carboxamide
IMP	Inosinate
TCA	Trichloroacetic acid
n.m.r.	Nuclear magnetic resonance
t.l.c.	Thin-layer chromatography
u.v.	Ultraviolet
i.r.	Infrared

I INTRODUCTION

One of the most complex medical and biological problems man faces today is cancer, which is really a group of many diseases. Malignant cells have in common, the ability to invade tissues locally or by distant metastasis and to disregard normal controls on cellular growth; these factors may lead to a patient's death. It is generally believed that cancer results from alterations in the control of cellular metabolism and from defects in the control of cell division.

Recent reviews on the subject of carcinogenesis (1-3) have pointed out that hundreds of chemicals, many viruses, and various forms of radiation, are capable of initiating the carcinogenic process. The actions of all these carcinogenic agents are thought by many to be related to deoxyribonucleic acid (DNA) synthesis and structure. Viruses may initiate the synthesis of DNA and may interact with the host DNA (4, 5), whereas chemical carcinogens may initiate the neoplastic transformation through interaction with cellular constituents, particularly DNA (6). Some of these chemicals inhibit the synthesis of DNA or ribonucleic acid (RNA) (7, 8), and some also interact with cell protein in a variety of tissues (9-11).

Basic research on the regulation of cell division, membrane function, the regulation of gene function, and the control of cellular metabolism, appear to be the most

promising approaches to a real understanding of the nature of cancer.

Purine nucleotide metabolism is important to cell growth for several reasons. Purine nucleotides are required for nucleic acid synthesis; they are the principal coenzymes in energy metabolism; there exist several pathways for the biosynthesis of purine nucleotides, and the pathways of purine nucleotide metabolism interact with those of amino acid and carbohydrate metabolism.

Although there is at present no reason to believe that purine nucleotide metabolism is the key area in the neoplastic conversion, studies of the control of purine nucleotide metabolism are relevant to cancer control in several ways. Studies in this area will contribute to our understanding of the mechanisms of metabolic regulation in general, and this should advance our understanding of the molecular basis of neoplasia. Control mechanisms may also throw light upon the sites of action of some drugs of present interest because many cancer chemotherapeutic agents are purine analogs. For instance, a purine analog, 6-mercaptopurine, has been useful for the treatment of childhood leukemia. However, the metabolism of these purine analogs and especially their modes of action are not well established. A full understanding of the basis of the actions of antitumor drugs would be facilitated by a greater knowledge of purine

nucleotide metabolism and specifically the enzymic reactions involved in both normal as well as abnormal growth. Studies of these aspects would consequently lead to useful information and may also provide new rationales for the design of effective anticancer drugs.

Most cells can synthesize purine nucleotides by the so-called purine de novo pathway (12), which utilizes non-purine precursors. In this pathway, purine nucleotides are synthesized from glycine, bicarbonate, formate, ribose, glutamine and aspartate by a series of ten enzymes. The end-product of this pathway is inosine 5'-monophosphate (IMP), which is further converted to adenosine 5'-monophosphate (AMP) and guanosine 5'-monophosphate (GMP). Alternative pathways synthesize these purine nucleotides directly from the corresponding purine base and 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P), using the purine phosphoribosyl-transferases (13, 14). Adenine phosphoribosyltransferase converts adenine to AMP, while hypoxanthine-guanine phosphoribosyl-transferase converts hypoxanthine to IMP and guanine to GMP. In a third pathway, purine bases may be converted first to their nucleosides through the phosphorylase reaction involving the participation of ribose-1-phosphate or deoxyribose-1-phosphate (15), or through dephosphorylation of nucleotides. These in turn are phosphorylated to their nucleotides (16-18). Once AMP and GMP are formed, they may be further phosphorylated to the di- and triphosphate level where

they are used either as co-enzymes or to synthesize nucleic acids. The monophosphates may also be subjected to catabolic processes which usually involve an initial dephosphorylation to the nucleoside level. AMP and GMP may also be converted back to IMP in one step reactions catalyzed by AMP deaminase or GMP reductase. Figure 1 illustrates the pathways of purine nucleotide biosynthesis and interconversion which are believed to occur in animal cells.

The existence of the pathway of purine nucleotide biosynthesis de novo in mammals was established by the use of purine-free diets. No ill effects were observed if mice or men were kept on this diet for extended periods. Although this biosynthetic pathway was recognized as early as the mid-nineteenth century, the complete pathway was only elucidated in the last two decades and details of the specific reactions involved still remain unclear. Much of the early work on this biosynthetic pathway was carried out using radioactive tracer techniques in the pigeon, since pigeon and other birds excrete uric acid (Figure 2) instead of urea. Investigation of this pathway was largely done by J. M. Buchanan and G. R. Greenberg and their associates. By administering ^{14}C labeled compounds to pigeons and degrading the excreted labeled uric acid, Sonne, Buchanan and Delluva (19, 20) showed that CO_2 and bicarbonate were precursors of the C-6 atom of uric acid; formate was the

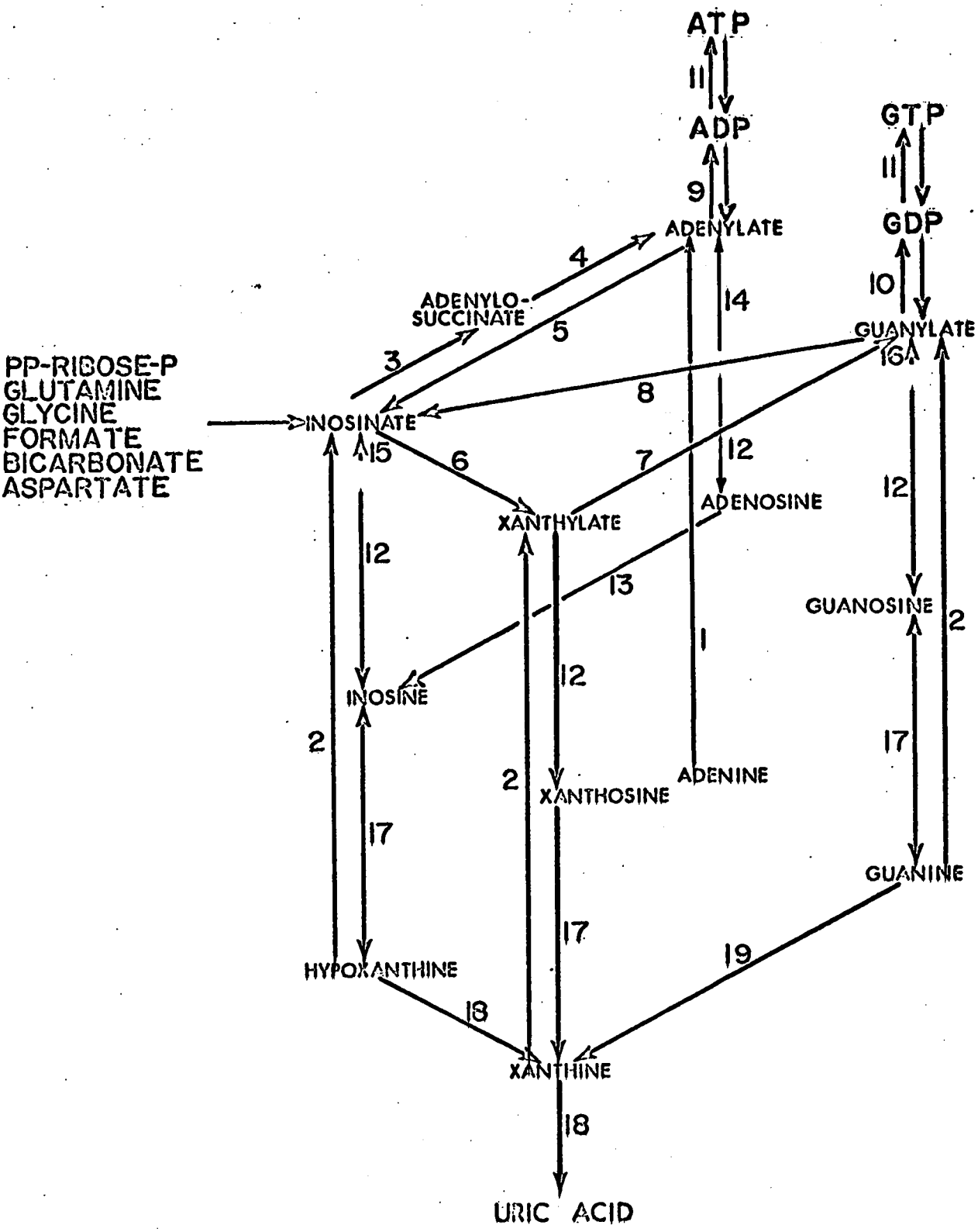
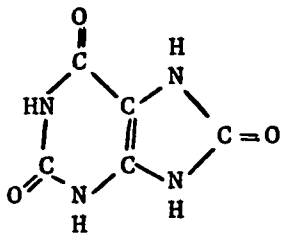
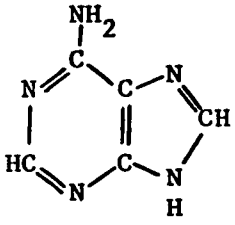


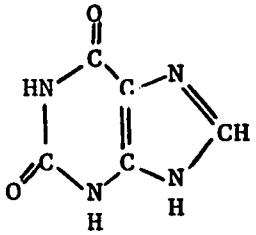
Figure 1. Purine ribonucleotide biosynthesis and interconversion.



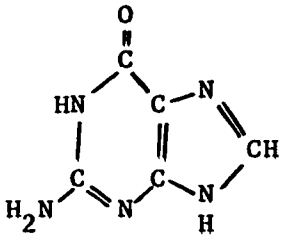
Uric acid



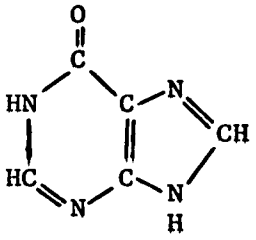
Adenine



Xanthine



Guanine



Hypoxanthine

Figure 2. Structural formulae of some common purines.

precursor for the C-2, C-8 atoms; the carboxyl carbon of glycine was a precursor of the C-4 atom, and the methylene carbon of glycine was a precursor of the C-5 atom.

The origins of the nitrogen atoms in purines were not easily determined owing to the rapid distribution throughout the amino acid pool of the ^{15}N-labeled precursors. However, following the isolation of radioactive hypoxanthine from pigeon liver extracts and determination of the rate of incorporation of different precursors and the positions in the ring which they labeled, Buchanan and Greenberg and their colleagues (12, 21-27) showed that the N-3 and N-9 atoms of the purine ring were derived from glutamine, and N-1 from aspartic acid. The N-7 atom was found to be derived from glycine itself. The overall results of these studies are outlined in Figure 3.

By using cell-free systems, these workers characterized the intermediates and individual reactions of the pathway. The end-product of this biosynthetic pathway was found by Greenberg (27) to be IMP. The synthesis of IMP requires ten enzymic reactions (12), and Figure 4 gives the outline of these reactions, while the individual enzymes involved are given in Table 1. Eight reactions involve C-N bond synthesis while there is one breakage of a C-N bond and in only one case is a C-C bond formed. ATP is involved in four steps as energy source while the first reaction uses PP-ribose-P. Folic acid is involved in two reactions and there are no

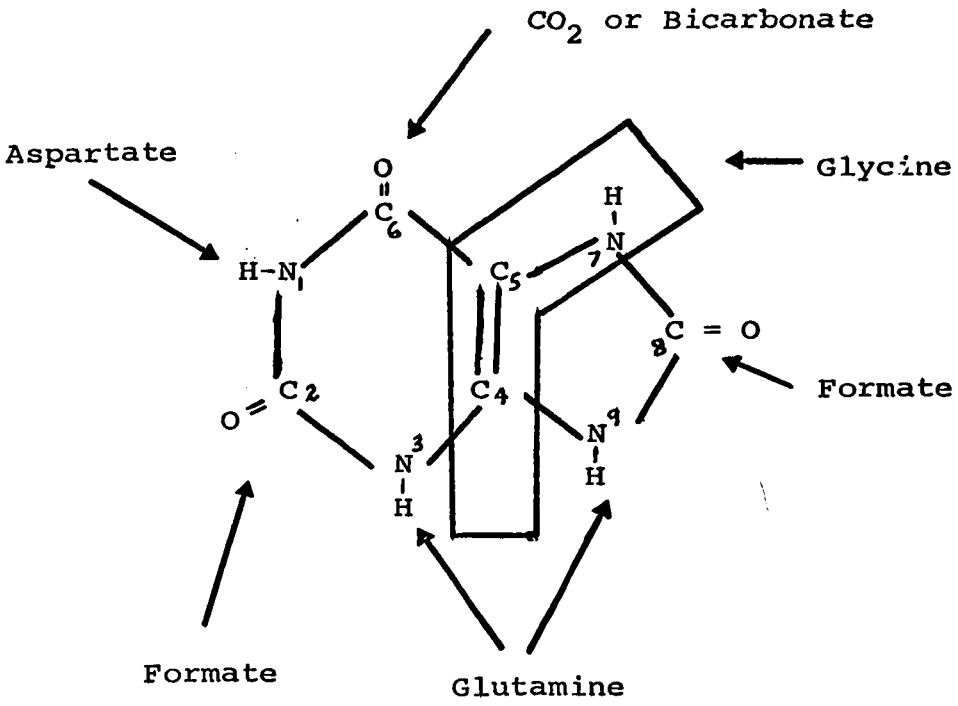


Figure 3. Precursors of purine ring atoms.

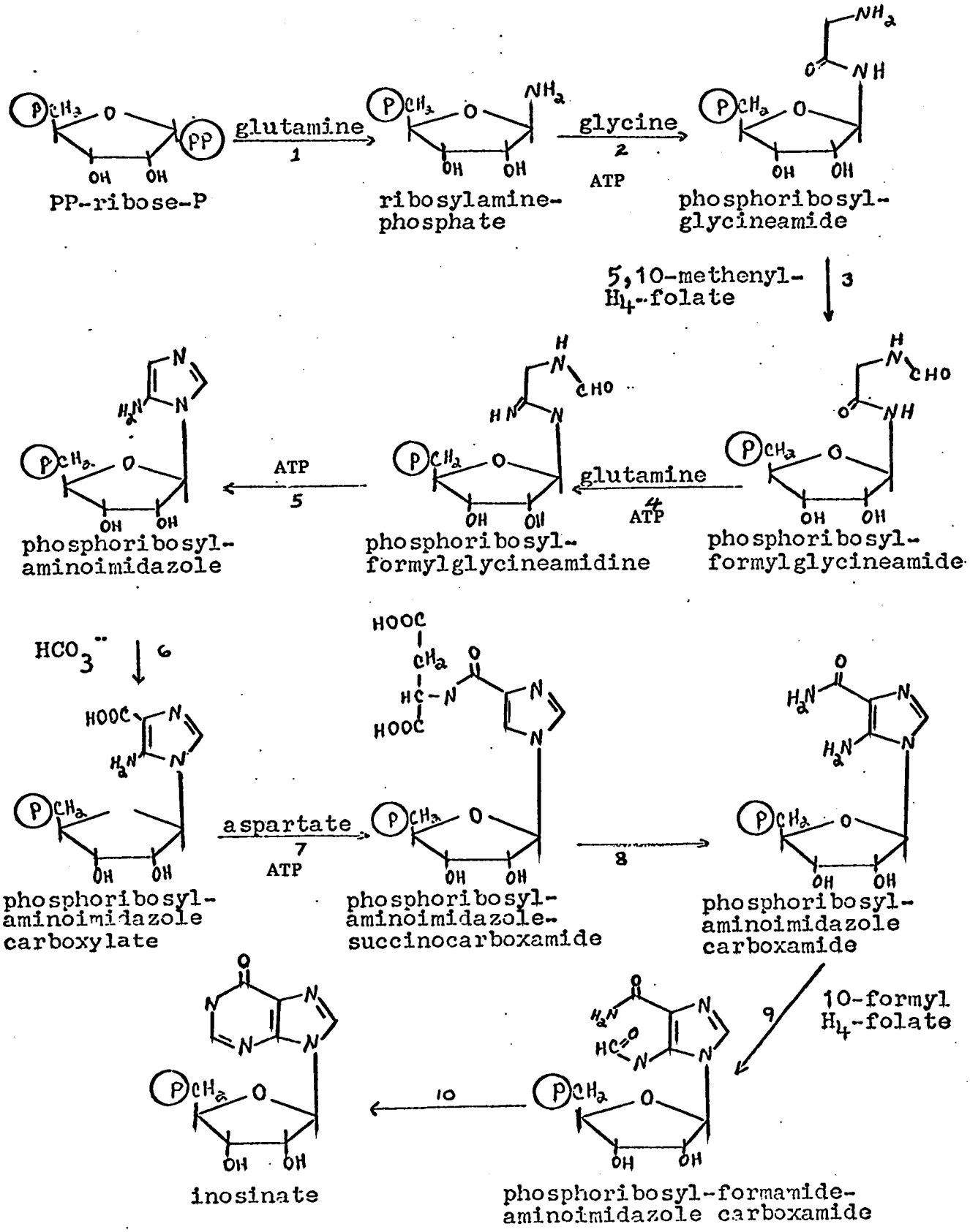


Figure 4. Enzymic reactions of purine ribonucleotide biosynthesis de novo.

TABLE 1: Enzymes of purine ribonucleotide biosynthesis de novo

Step	Enzyme Commission Number	Systematic Name	Trivial Name
1.	2.4.2.14	Ribosylamine-5-phosphate: pyrophosphate phosphoribosyl-transferase (glutamate-amidating)	Phosphoribosyl-pyrophosphate amidotransferase
2.	6.3.1.3	Ribosylamine-5-phosphate: glycine ligase (ADP)	Phosphoribosyl-glycineamide synthetase
3.	2.1.2.2	5'-Phosphoribosyl-N-formyl-glycineamide:tetrahydrofolate 5,10-formyltransferase	Phosphoribosyl glycineamide-formyltransferase
4.	6.3.5.3	5'-Phosphoribosyl-formyl-glycineamide:L-glutamine amido-ligase (ADP)	Phosphoribosyl-formylglycineamidine synthetase
5.	6.3.3.1	5'-Phosphoribosyl-formyl-glycineamidine cycle-ligase (ADP)	Phosphoribosylamino-imidazole synthetase
6.	4.1.1.21	5'-Phosphoribosyl-5-amino-4-imidazolecarboxylate carboxy-lyase	Phosphoribosylamino-imidazole carboxylase
7.	6.3.2.6	5'-Phosphoribosyl-4-carboxy-5-aminoimidazole:L-aspartate ligase (ADP)	Phosphoribosylamino-imidazole-succino-carboxamide synthetase
8.	4.3.2.2	Adenylosuccinate AMP-lyase	Adenylosuccinate lyase
9.	2.1.2.3	5'-Phosphoribosyl-5-formamide-4-imidazolecarboxamide:tetra-hydrofolate 10-formyltransferase	Phosphoribosyl-aminoimidazole-carboxamide formyltransferase
10.	3.5.4.10	IMP 1,2-hydrolase (decyclizing)	IMP cyclohydrolase

oxidation-reduction reactions. Several reactions are irreversible, but a certain degree of reversibility is encountered in the last five steps. A summary of these reaction types is given in Table 2.

The pathway of purine biosynthesis de novo has been shown to be controlled or at least potentially controllable by feedback inhibition mechanisms in bacteria, avian liver and various mammalian cells, and by repression in bacteria and perhaps mammalian cells as well. In the case of feedback inhibition, the synthesis of 5-phosphoribosylamine (PRA) from PP-ribose-P and glutamine by PP-ribose-P amidotransferase is inhibited by purine nucleotides and some purine nucleotide analogs. LePage and co-workers, using ascites tumor cells in vitro (28) and in vivo (29); McFall and Magasanik, using L cells in culture (30); and Henderson, using Ehrlich ascites tumor cells in vitro (31, 32); obtained evidence for this feedback inhibition. Recent studies have shown that a subline of the Ehrlich ascites tumor (33, 34) and two lines of cultured human skin fibroblasts (35, 36) have purine biosynthetic pathways which are less sensitive than normal to feedback inhibition. Control by repression was observed for PP-ribose-P amidotransferase and 5'-phosphoribosyl-formylglycineamidine (FGAM) synthetase when Aerobacter aerogenes was grown in the presence of adenine and guanine (37). The repression

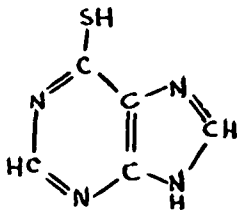
TABLE 2: Types of reactions in the pathway of purine ribonucleotide biosynthesis *de novo*

Reaction	Bond Formed or Broken	Reaction Type	Reversible
Phosphoribosyl pyrophosphase amidotransferase	carbon-nitrogen	glutamine amide transfer phosphoribosyltransferase	No
Phosphoribosyl-glycineamide synthetase	carbon-nitrogen	amide synthesis	Yes
Phosphoribosyl-glycineamide formyltransferase	carbon-nitrogen	one-carbon transfer	No
Phosphoribosyl-formylglycineamide amidotransferase	carbon-nitrogen	glutamine amide transfer	No
Phosphoribosyl-aminoimidazole synthetase	carbon-nitrogen	cyclization	No
Phosphoribosyl-aminoimidazole carboxylase	carbon-carbon	CO_2 fixation	Yes
Phosphoribosyl-aminoimidazole succinocarboxamide synthetase	carbon-nitrogen	aspartate amino transfer	Yes
Adenylosuccinate lyase	carbon-nitrogen	displacement	Yes
Phosphoribosyl-aminoimidazole carboxamide transformylase	carbon-nitrogen	one-carbon transfer	Yes
Inosinicase	carbon-nitrogen	cyclization	Yes

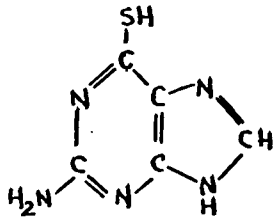
of Bacillus subtilis PP-ribose-P amidotransferase, adenylosuccinate lyase, 5'-phosphoribosyl-aminoimidazole carboxamide (AICAR) transformylase, and IMP cyclohydrolase by adenosine and guanosine has also been reported (38, 39). Derepression of bacteria (40) and mouse spleen infected with murine leukemia virus (41), has also been reported.

Purine nucleotide analogs inhibit this pathway by mimicking the effects of natural purine nucleotides on PP-ribose-P amidotransferase (42, 43). Examples of such antimetabolites are 6-mercaptopurine, 6-thioguanine and 6-methylmercaptopurine ribonucleoside (Figure 5), which are converted into the active nucleotide analogs in cells. Antimetabolites may also inhibit the synthesis of substrates or co-factors of this biosynthetic pathway. PP-ribose-P synthesis is inhibited by 3'-deoxyadenosine triphosphate, formycin triphosphate and other ATP analogs (42, 43); methionine sulfoximine inhibits glutamine synthesis (44); and aminopterin and amethopterin inhibit folic acid reductase. The amino acid antimetabolites azaserine (O-diazoacetyl-L-serine) and 6-diazo-5-oxo-L-norleucine (DON), with their structural resemblance to glutamine (Figure 6), act as competitive inhibitors of PP-ribose-P amidotransferase and FGAM synthetase.

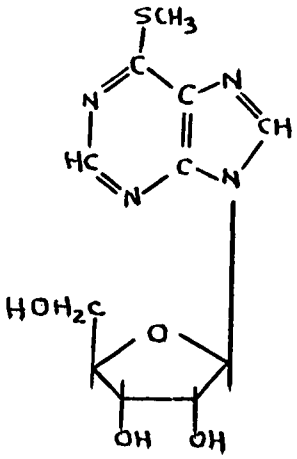
Both azaserine and DON are of chemotherapeutic interest. They show marked growth inhibitory effects of several experimental solid tumors, including Sarcoma 180 (45, 46).



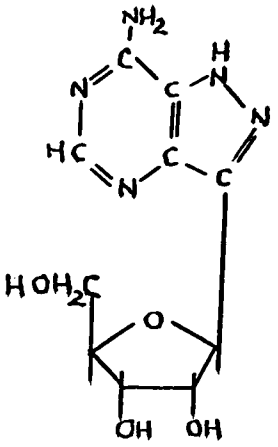
6-Mercatopurine



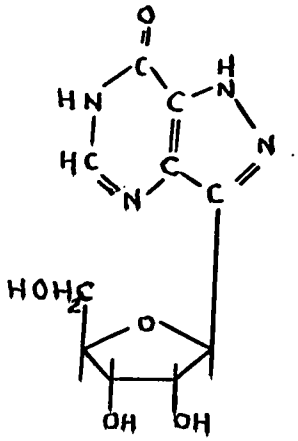
6-Thioguanine



Me6MPR



Formycin A



Formycin B

Figure 5. Structural formulae of some purine antimetabolites.

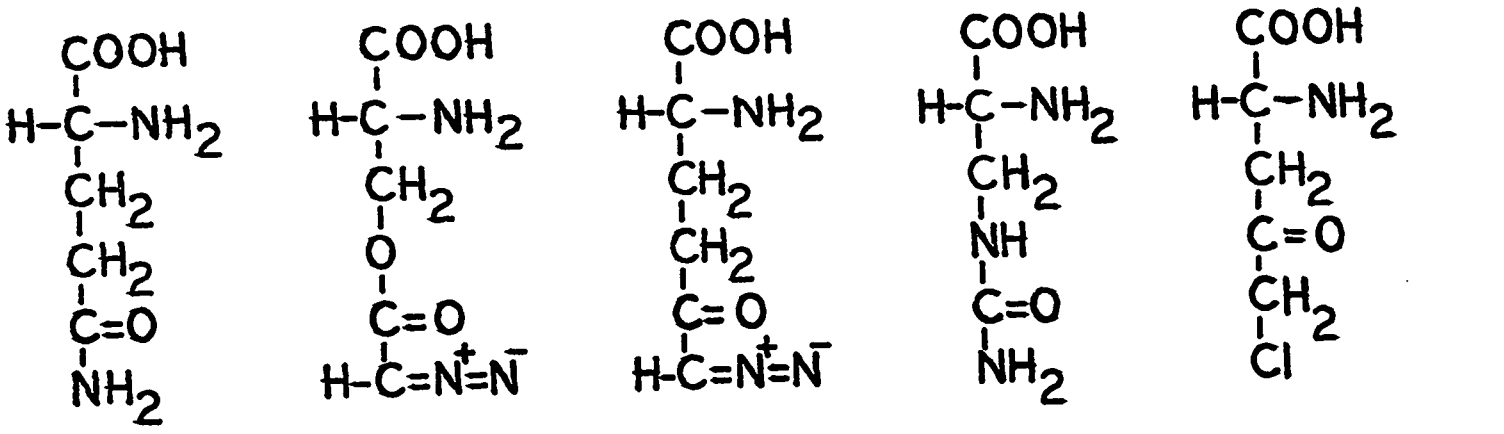


Figure 6. Structural formulae of L-glutamine and glutamine antimetabolites.

Both azaserine and DON inhibit the synthesis of purine nucleotides de novo by inhibiting FGAM synthetase (47-50); azaserine is known to react with a sulfhydryl group of the enzyme to yield a co-valently formed derivative (51, 52). DON, however, on dose basis, is forty times more effective than azaserine.

FGAM synthetase (5'-phosphoribosyl-formylglycineamide:L-glutamine amido-ligase [ADP], EC 6.3.5.3) activity was first demonstrated in 1957 when Levenberg and Buchanan incubated 5'-phosphoribosyl-formylglycineamide (FGAR) with a particular preparation from an alcohol fractionation of pigeon liver, and glutamine, ATP, and magnesium and potassium ions. They obtained a new intermediate which was identified as FGAM (44). This reaction is represented in Figure 7. FGAM synthetase catalyzes the irreversible transfer of the amide group of glutamine to FGAR to form the corresponding amidine. This enzyme has been purified from Salmonella typhimurium (53), and has a molecular weight of 135,000 daltons. Recently Mizobuchi and Buchanan obtained a highly purified enzyme from chicken liver; its molecular weight was determined to be 133,000 daltons (54). Magnesium ion is required for the reaction, and low concentrations of heavy metals markedly inhibit the enzyme. The equilibrium of the reaction is very far in the direction of FGAM synthesis and no exchange was detected between $^{32}P_i$ and ATP (55, 56).

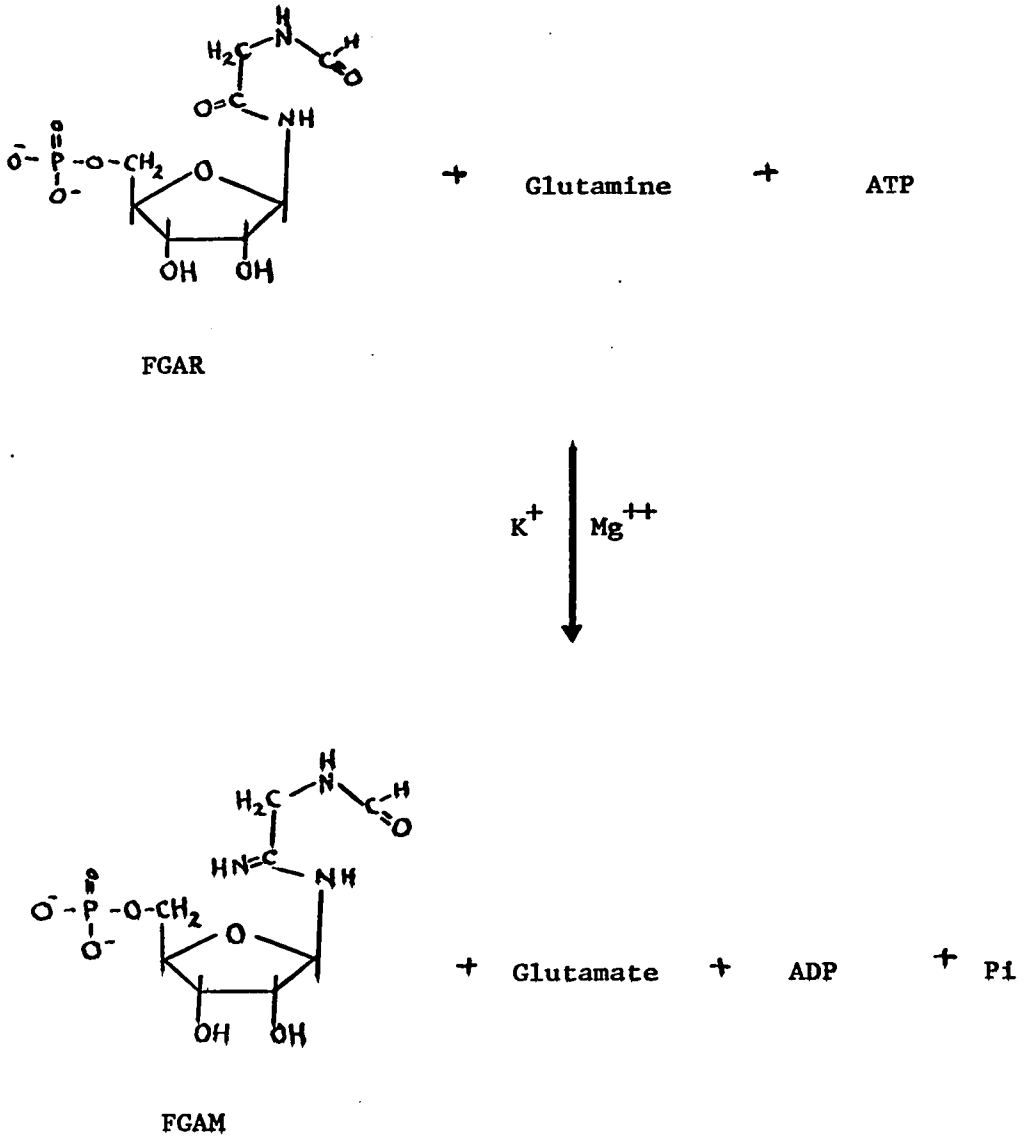


Figure 7. The 5'-phosphoribosyl-formylglycineamidine synthetase reaction.

Ammonia can replace glutamine as an alternative substrate in the chicken liver enzyme system, and it was concluded that it was probably bound to a site on the enzyme different from the site where glutamine binds (57). However, the Salmonella enzyme does not appear to use ammonia (51).

The amide nitrogen atom of glutamine is utilized not just for purine biosynthesis but also in many other areas of metabolism, such as in synthesis of cytidine triphosphate, glucosamine, histidine, p-aminobenzoic acid, asparagine, diphosphopyridine nucleotide and other compounds (58, 59). Table 3 lists the known glutamine amide transfer reactions. In most cases, ammonia can replace glutamine as substrate and in some cases hydroxylamine can also serve as the nitrogen donating substrate. The synthesis of an activated enzyme-bound intermediate is required for the reaction of glutamine (59) in the case of the glutamyl-dependent carbamyl phosphate synthetase, asparagine synthetase, and xanthosine monophosphate (XMP) aminase reactions. Another common feature of amidotransferases is that they are inhibited by antimetabolites of glutamine such as azaserine, DON, 2-amino-4-oxo-5-chloro-pentanoic acid (59) and albizzin (57) (Figure 6) when glutamine is used as the substrate.

The recent discovery that asparaginase suppressed the growth of certain lymphomas in animals (60-62), and its use in clinical trials led to the thought that L-asparagine is

TABLE 3: Glutamine amide transfer reactions

Amide-group acceptor	Product	Enzyme Commission Number
Phosphoribosyl pyrophosphate	phosphoribosylamine	2.4.2.14
Phosphoribosyl-formyl-glycineamide	Phosphoribosyl-formyl glycineamidine	6.3.5.3
Xanthylate	Guanylate	6.3.5.3 6.3.4.1
Uridine triphosphate	Cytidine triphosphate	6.3.4.2
Deamido-nicotinamide-adenine dinucleotide	Nicotinamide-adenine dinucleotide	6.3.5.1
Phosphodeoxyribulosyl-formimino-phosphoribosyl-aminoimidazolecarboxamide	Phosphodeoxyribulosyl amidino-phosphoribosyl-aminoimidazolecarboxamide	
Fructose 6-phosphate	Glucosamine 6-phosphate	
CO_2 + ATP	Carbamyl phosphate	
Aspartate	Asparagine	
Chorismate	o-Aminobenzoate	
Chorismate	p-Aminobenzoate	

required for growth in certain tumors. Indeed asparagine synthetase activities of a variety of normal mouse tissues were relatively low while a number of leukemias known to be resistant to therapy with asparaginase exhibited moderate to high asparagine synthetase activities. In contrast, leukemias known to be sensitive to treatment with asparaginase exhibited either no or remarkably low levels of asparagine synthetase (63). The synthesis of asparagine requires glutamine, and a depletion of this amino acid through its use in other reactions may result in a decreased availability of this metabolite for asparagine formation. Studies, then, on these glutamine-utilizing reactions may provide some information on the mechanism of asparaginase therapy. However, neither a detailed knowledge of the glutamine amide transfer reactions nor a complete picture of the control of the purine biosynthetic pathway is yet available. Detailed kinetic studies of the enzymes of the purine biosynthetic pathway have not previously been carried out.

Studies in this laboratory on adenine phosphoribosyltransferase (64-71) have emphasized very strongly the importance of studies of the kinetic mechanism of an enzyme reaction for a full understanding of both its control (allosteric or otherwise) and the effect of drugs on the reaction. The latter type of study cannot be considered to be complete, and cannot be interpreted in depth, without a

knowledge of the kinetic mechanism and kinetic parameters of the reaction.

In the pathway of purine biosynthesis de novo there are several types of phenomena that require kinetic studies. Several of these enzymes are, directly or indirectly, sites of action of anticancer drugs. Thus, phosphoribosyl pyrophosphokinase is inhibited by certain ATP analogs; PP-ribose-P amidotransferase is inhibited by some other purine nucleotide analogs; FGAM synthetase and PP-ribose-P amidotransferase are inhibited by glutamine analogs such as azaserine and DON; and phosphoribosyl-glycineamide (FGAR) formyltransferase and phosphoribosyl-aminoimidazole carboxamide (AICAR) formyltransferase are inhibited indirectly by folate analogs. Determination of the kinetic mechanisms of these reactions, and studies of the effects of drugs on these reactions, would give valuable insights into the mechanisms of these drug effects.

Our understanding of drug action and the basis of its selectivity would be furthered by the study of enzyme reactions which are similar to those inhibited by drugs, but which are in fact, not sensitive to their action. Thus, FGAM synthetase is more sensitive to azaserine than is PP-ribose-P amidotransferase. Adenylosuccinate synthetase is inhibited by aspartate analogs, but succinyl-AICAR synthetase is not.

The rates of all the reactions of the purine biosynthetic pathway are of course controlled by the concentrations of their substrates and products. A knowledge of the relative importance of these factors and of their interaction, depends in part on knowing the order in which these add to or leave the enzyme, and the enzyme mechanisms and dissociation constants.

Previous studies on the control of purine nucleotide synthesis de novo have been conducted mainly with the first enzyme of the pathway, viz. PP-ribose-P amidotransferase. Since studies on this biosynthetic pathway and its control have often been investigated by means of the accumulation of FGAR in the presence of azaserine, PP-ribose-P amidotransferase may not necessarily be the only enzyme whose rate is of concern in these experiments. The other enzymes leading to FGAR formation, viz. phosphoribosyl-glycineamide (GAR) synthetase, GAR formyltransferase and FGAM synthetase should be investigated. Few studies, however, have been made on these and other enzymes of the purine biosynthetic pathway, and those enzymes which have been studied were mainly from avian or bacterial sources. The present study is carried out with the FGAM synthetase from Ehrlich ascites tumor cells. Recent studies in comparative enzymology have shown that enzymes performing the same function may vary considerably from organism to organism. For studies of therapeutic relevance, the mammalian cell enzyme is a better

system to look at than that from other sources. Hitchings (72) has recently pointed out that metabolic specificity may be the result of a series of molecular sieves of different degrees and kinds, and that when the system is dealing only with normal metabolites, the variations are not apparent; a foreign substance such as a drug or an antimetabolite may however produce quite individual results. FGAM synthetase was chosen for kinetic investigation in the present study partly because of the chemotherapeutic importance of this enzyme, as mentioned above, and partly because it catalyzes a reaction which involves glutamine amide transfer and requires ATP. Detailed knowledge regarding the formation of C-N bonds and the role of ATP are lacking, not only in this specific reaction, but in other areas of cellular metabolism as well.

In the present studies, a new chemical synthesis of the substrate, FGAR, is described because it is not available commercially and the enzymic synthesis of this compound is not amenable to large scale preparations. FGAM synthetase was isolated and partially purified from Ehrlich ascites carcinoma cells. Initial rate, product inhibition, and isotope exchange studies were carried out and a kinetic mechanism was proposed for this enzymic reaction. The effects of some antitumor drugs and other reagents on this enzyme were also studied.

II CHEMICAL SYNTHESIS OF 5'-PHOSPHORIBOSYL-N-FORMYLGLYCINEAMIDE

A. Introduction

5'-Phosphoribosyl-N-formylglycineamide (V) (Figure 8) is one of the intermediates in the pathway of purine ribonucleotide biosynthesis de novo (49, 73). This pathway is of great importance in providing ATP and GTP, precursors of the nucleic acids. The enzyme which uses V as substrate is the site of action of the antitumor antibiotic azaserine, and the action of this compound and the binding of substrates to this enzyme have been studied in detail by Buchanan et al. (51-54, 56, 74). The enzymatic synthesis of this compound (75) requires the preparation of particular enzymes, and is not easily amenable to large-scale preparations. The chemical synthesis of V has been reported (76), but the yield was low, and the method used is not readily scaled up. A new route (Figure 8) was investigated to synthesize this ribonucleotide in quantities which will permit more extensive studies of its metabolism. The overall yield is 20%.

B. Experimental

1. Methods

Paper chromatography was carried out on Whatman No. 1 paper. The solvent systems used were: (A) 1-butanol:acetic acid:water (2:1:1), (B) 1-propanol:water (6:4), (C) 95% ethanol:water (77:23). Phosphate-containing compounds

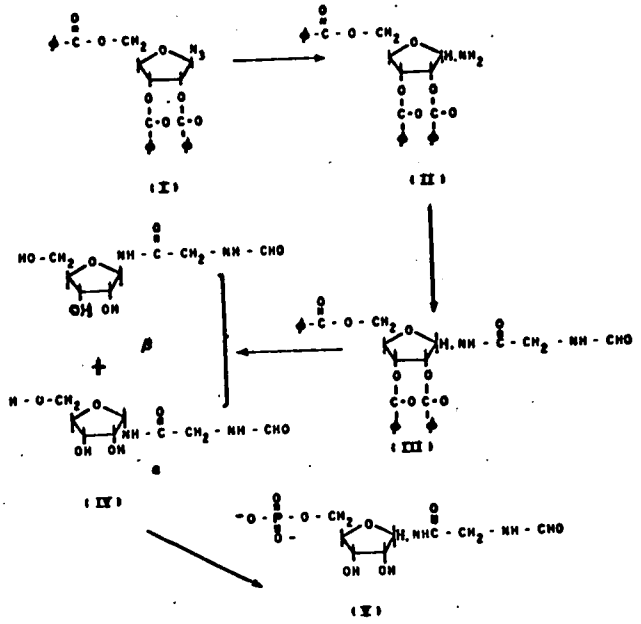


Figure 8. Reaction scheme for synthesis of FGAR.

were detected with the ammonium molybdate reagent (77). The t.l.c. was done on silica gel, and compounds were detected by their ultraviolet absorption or with a sulfuric acid spray. The i.r. spectra were determined with a Perkin-Elmer 137B spectrophotometer. The n.m.r. measurements were performed by the Department of Chemistry and Faculty of Pharmacy, University of Alberta, with Varian A-60 instrument. Microanalyses were performed by George I. Robertson, Jr., 73 West End Avenue, Florham Park, New Jersey. Melting points were determined on a Kofler micro hot stage.

The enzymatic conversion of formylglycineamide ribonucleotide to 5-aminoimidazole ribonucleotide was carried out by incubation of the former with adenosine triphosphate, L-glutamine, KCl, $MgSO_4$, phosphoribosyl-formylglycineamidine synthetase (partially purified from Ehrlich ascites tumor cells), and phosphoribosyl-aminoimidazole synthetase (partially purified from chicken liver); the general procedure of Flaks and Lukens (78) was followed. The ribonucleotide product was determined by the Bratton-Marshall test for diazotizable amines (79).

2. Preparation of 2,3,5-tri-O-benzoyl-N-(formylglycyl)-α,β-D-ribofuranosylamine

2,3,5-Tri-O-benzoyl-β-ribofuranosyl azide (1 g) (76) was dissolved in 40 ml of anhydrous acetone; 50 mg of platinum oxide were added, and the mixture was hydrogenated with a Parr hydrogenation apparatus at room temperature at a

pressure of 10 lb/sq in. for 45 min. Anhydrous magnesium sulfate was added and the solution filtered. The filtrate was added at once to a flask containing 400 mg of formylglycine (80) dissolved in 50 ml of acetone. To this solution, 600 mg of N,N'-dicyclohexylcarbodiimide were added and the mixture was allowed to stand at room temperature with occasional shaking for 2.5 h. The insoluble urea derivative was collected by filtration and the filtrate was concentrated to 20 ml and again filtered. The filtrate was kept at 0°C overnight. After removing the residue, the filtrate was evaporated to a gum at room temperature. Ethyl acetate (5 ml) was added and the solution was applied to a column of silica gel (2 x 30 cm). The column was eluted with 2 ml fractions of ethyl acetate. A compound which absorbed u.v. light after t.l.c. (R_f 0.5) in ethyl acetate was collected (80 to 160 ml) and the solution was concentrated to 20 ml and set aside at 0° for 2-3 h. The residue was filtered and the filtrate was evaporated under high vacuum to a dry residue: 740 mg (67%), m.p. 58-63°C, $[\alpha]_D^{20}$ + 42° (1% methanol). The t.l.c. gave a single spot detected by u.v. absorption (R_f 0.5; ethyl acetate), but a minor spot with R_f 0.42 also appeared when the H_2SO_4 spray was used. The i.r. spectroscopy of the product in chloroform gave absorption maxima at 3400, 1680, and 1520 cm^{-1} which were characteristic of the secondary amide group. Analysis showed this compound to be reasonably pure (90% based on N).

Further purification of the compound was unsuccessful but this preparation was acceptable in the subsequent reaction.

Anal. Calcd. for $C_{29}H_{26}O_9N_2$: C, 63.4; H, 4.73; N, 5.10. Found: C, 62.61; H, 5.11; N, 5.70.

3. Preparation of N-(N-formylglycyl)-α,β-D-ribofuranosylamine

2,3,5-Tri-O-benzoyl-N-formylglycyl-α-β-D-ribofuranosylamine (550 mg, 1 mmole) was dissolved in 1 ml of dry methanol and sodium methoxide (from 0.01 g sodium in 5 ml methanol) added at room temperature. After 10 h the solution was evaporated to a small volume (about 1.5 ml) and filtered. Methanol (10 ml) was added to the filtrate and this was passed through a Dowex-50 column (1 x 1.5 cm, H^+ form, methanol washed), and the column was washed with 20 ml of methanol. The combined methanol fractions were evaporated to dryness, dissolved in 25 ml of water, and extracted once with 30 ml of ether. The water layer was lyophilized, giving 140 mg of product (66%): $[\alpha]_D^{20}$ + 18.6° (1% methanol). The compound is hygroscopic. The i.r. examination of the compound in a KBr disc showed no ester-carbonyl band at 1720 cm^{-1}, but a hydroxyl band at 3425 cm^{-1} was present. The t.l.c. showed only a single spot (R_f 0.71 in methanol) as detected by the sulfuric acid spray.

Anal. Calcd. for $C_{18}H_{14}N_2O_6$: C, 41.05; H, 5.90; N, 11.98. Found: C, 41.03; H, 6.20; N, 12.00.

4. Preparation of 5'-phosphoribosyl-formylglycineamide

N-(N-Formylglycyl)-D-ribofuranosylamine (48 mg) was dissolved in 2 ml of triethylphosphate and the mixture was kept at 0°C. Phosphorous oxychloride (0.2 ml) was added slowly with stirring. The reaction mixture was then kept at this temperature for 1.5 h with stirring. Ether (100 ml) was added to the reaction mixture to precipitate the N-(N-formylglycyl)-D-ribose 5'-phosphordichloridate which was then collected by centrifugation. The product was washed once with 40 ml of ether and dissolved in 0.5 ml of cold water containing 0.1 ml of triethylamine. The aqueous solution was then adjusted to pH 8.5 with 1 N HCl. Barium bromide (1 M, 0.5 ml) was added and the barium salt was precipitated by addition of 5 volumes of cold 98% ethanol. The barium salt was centrifuged after 1 h at 0°C to give 90 mg. Paper chromatography of the salt showed one major product together with other phosphorylated compounds. The barium salt was dissolved in 5 ml of water, passed through a 1 x 1.5 cm column of Dowex-50 (H^+ form) and the column was washed once with 10 ml of water. The combined effluents were adjusted with ammonium hydroxide to pH 8 and applied onto a column (1 x 12 cm) of Dowex-1-X8 in the formate form. The column was washed with 20 ml of water and eluted with 0.05 M ammonium formate, pH 5. Fractions from 110 to 250 ml were collected and neutralized to pH 8 with ammonium hydroxide (these were enzyme substrates and contained organic

phosphate). Again this fraction was absorbed onto a column (1 x 4 cm) of Dowex-1-X8 in the chloride form. The column was washed with 150 ml of distilled water and the product was eluted with 0.24 M lithium chloride. Fractions from 2.0 ml to 20 ml were collected and lyophilized. The residue was washed twice with 4.5 ml of methanol:acetone (3:2) and dried under vacuum to give 28 mg of the lithium salt of N-(N-formylglycyl)-D-ribofuranosylamine-5'-phosphate (V) (yield 40%).

5. Separation of the α and β anomers of FGAR

The anomers of the lithium salt of FGAR were separated from each other by employing anion exchange column chromatography.

30 Mg of the lithium salt of FGAR was dissolved in 10 ml of distilled water and a small amount of Dowex 50 (H^+ form) was added to the solution to remove the lithium ion. The resin was removed by centrifugation and the supernatant was adjusted to pH 8.0 - 8.5. This was then absorbed onto a column (1 x 7.5 cm) of Dowex-1 X8 in the formate form. The column was washed with 20 ml of distilled water and then eluted with 0.05 M ammonium formate pH 6.0. Two peaks (A and B) that contained approximately equal amounts of organic phosphate were obtained (Figure 9).

Fractions of A and B were then pooled separately and worked up to yield the lithium salt as above. The product of fraction A was not a substrate of FGAM synthetase, and gave R_f values of 0.26, 0.31, 0.15 in solvent systems A, B and C respectively, while the product obtained

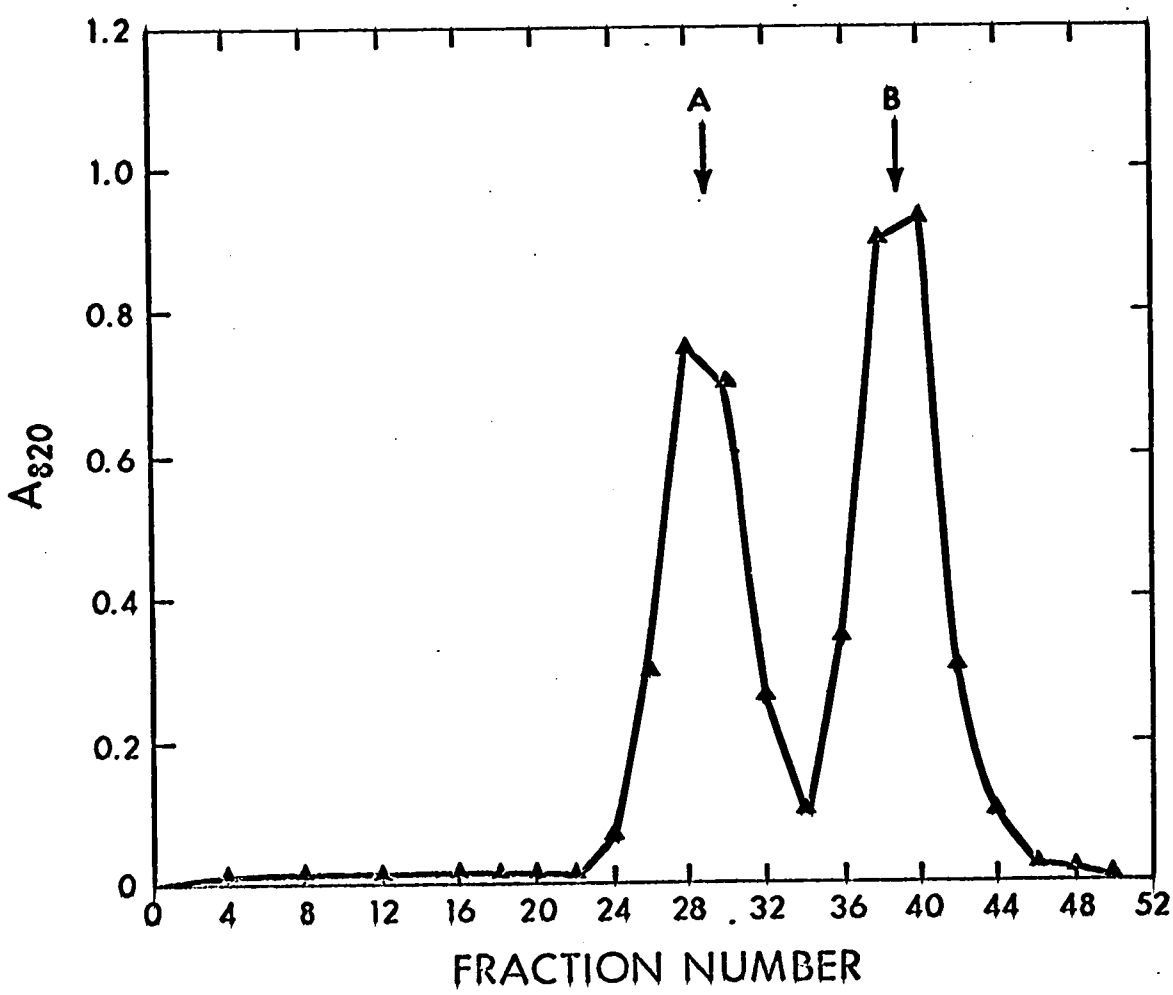


Figure 9. Elution of anomers of FGAR from Dowex-1-X8 formate. Organic phosphate was determined in 0.15 ml sample from each 10 ml fraction.

from peak B was a substrate for FGAM synthetase and gave R_f values of 0.26, 0.42, 0.35 in solvent systems A, B and C, respectively. Presumably the product obtained from peak B (enzyme active) was the β anomer and it is this form of FGAR that was used in the subsequent studies.

C. Results and discussion

Hydrogenation of 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl azide (I) in acetone with Adam's catalyst at room temperature for 45 min resulted in complete loss of the azide infrared absorption maximum, 2120 cm^{-1}, and gave a compound which presumably was the 2,3,5-tri-O-benzoyl-β-D-ribofuranosyl amine (II). Baddiley et al. (81) have reported the isolation of the hydrochloride salt of II using ethyl acetate as solvent. The solvent used here has the advantage of requiring a smaller amount of catalyst, and of reducing the time of reaction; a longer time of hydrogenation in ethyl acetate can result in migration of the 2-benzoyl residue to the 1-position, forming 3,5-di-O-N-benzoyl-D-ribofuranosylamine (81). Furthermore, acetone is a better solvent for the formylglycine which is used in the subsequent reaction.

Freshly prepared II reacted readily with formylglycine at room temperature in the presence of N,N'-dicyclohexyl-carbodiimide to give 2,3,5-tri-O-benzoyl-N-(N-formylglycyl)-α,β-D-ribofuranosylamine (III) in 67% yield.

Debenzoylation of III with sodium methoxide in anhydrous methanol gave N-(N-formylglycyl)-D-ribofuranosylamine (IV)

in good yield. The nuclear magnetic resonance spectrum of this compound in deuterated water with sodium 2,3-di-methyl-2 silapentane-5-sulfonate as reference standard showed peaks corresponding to 18 protons, with two proton singlets at 1.85τ (formyl protons); doublets of 4.3τ (anomeric proton of β-IV) and 4.6τ (anomeric proton of α-IV); plus a series of multiplets from 5.61 to 6.4τ corresponding to the 2, 3, 4, and 5 protons of ribose and two glycine protons. Either anomer, however, should exhibit but one formyl proton singlet and one anomeric proton doublet plus a simplified region for the remaining hydrogens to give a 9 proton spectrum. Thin-layer chromatography on silica gel showed only a single spot, and microanalysis indicated the empirical formula of IV (α or β form). We conclude, therefore, that product IV contains a mixture of α and β forms in approximately equal proportions. This conclusion was also based on the results of integration of proton signals. It has been noted by Baddiley et al. (81) that anomerization occurred immediately after hydrogenolysis of the azide to amine in the synthesis of N-(benzyloxy-carbonylglycyl)-α,β-D-ribofuranosylamine, rather than in the subsequent steps.

Direct phosphorylation of IV with phosphoryl chloride in triethylphosphate for 1 1/2 h at 0°C gave its 5'-phosphate (formylglycineamide ribonucleotide) (V). The direct phosphorylation of the linear ribonucleoside without protecting the 2',3'-hydroxyl groups of the ribose extends the

usefulness of the method of Yoshikawa *et al.* (82) of phosphorylation of unprotected nucleosides of 5' nucleotides with phosphoryl chloride and trialkylphosphate. The direct phosphorylation of unprotected nucleosides to their 5' nucleotides in different solvents has also been reported by Imai *et al.* (83).

This compound (V) gave the same R_f value (Table 4) as the authentic radioactive compound obtained from Ehrlich ascites tumor cells by the method of Henderson (31), and with values reported by others (84-86). On two chromatograms (solvents B and C) two zones were present, possibly due to the anomeric forms of compound V as noted by Westby and Gots (85) and Goldthwait *et al.* (84). The one with the higher R_f value had also been reported by Moore and LePage (86). Compound V also gave a negative ninhydrin test, but a positive test was obtained after acid hydrolysis to glycineamide ribonucleotide. The Schiff periodate test for *cis*-glycols was positive. Determination of the organic phosphate content using the method of Ames and Dubin (87), and ribose content by measuring spectrophotometrically the consumption of periodate (88), indicated their molar ratio to be 1.00:1.10 (Table 5). Phosphate analysis indicated this compound to be 92% pure, with impurities undoubtedly due to inorganic material. Compound V, 48.4%, can be converted to 5-amino-1-β-D-ribofuranosyl imidazole-5'-phosphate in the presence of adenosine triphosphate,

TABLE 4: Paper chromatography of synthetic and authentic radioactive formylglycineamide ribonucleotide

	R_f of compounds*		
	Solvent A	Solvent B	Solvent C
Formylglycineamide ribonucleotide	0.26	0.31, 0.42	0.15, 0.35
Formylglycineamide ribonucleotide-^{14}C	0.26	0.32, 0.43	0.15, 0.34
Ribose-5-PO_4	0.30	0.47	0.48

*Solvent systems are described in the Experimental section.

TABLE 5: Analysis of formylglycineamide ribonucleotide for organic phosphate and ribose content

	Experimental	Calculated
Organic phosphate	2.73 μmole/mg	2.97 μmole/mg
Ribose	2.76 μmole/mg	2.97 μmole/mg
Organic phosphate/ ribose	1.00/1.01	1.00/1.00

L-glutamine, magnesium ion, and an enzyme preparation containing phosphoribosyl-formylglycineamidine synthetase and phosphoribosyl-aminoimidazole synthetase. This result indicates that β and α anomers were synthesized in approximately equal amounts.

The anomers were further separated by employing anion exchange chromatography.

D. Summary

A new and convenient synthetic route to formylglycineamide ribonucleotide, an intermediate in the pathway of purine ribonucleotide biosynthesis de novo, is described. The method utilizes direct phosphorylation of the 5'-position of the linear riboside without protection of the 2',3'-hydroxyl groups of the ribose, and gives a good yield of mixed α and β anomers. These anomers could be separated from each other by employing anion exchange chromatography. This route is suitable for synthesis of the natural product in quantities required for biological studies.

III. PURIFICATION AND PROPERTIES OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE

A. Introduction

Studies of the pathway of purine biosynthesis de novo and its control have often been investigated by measurements of the accumulation under various conditions of one of its intermediates, FGAR, in the presence of azaserine (86, 89-92). Azaserine inhibits the enzyme FGAM synthetase, which catalyzes the conversion of FGAR to FGAM as illustrated in Figure 7; it reacts with a sulfhydryl group of the enzyme to yield a co-valently bound complex (51, 52).

Purification of FGAM synthetase from pigeon liver and Salmonella typhimurium has been reported (78), and recently Mizobuchi and Buchanan obtained a highly purified enzyme from chicken liver (54). Magnesium ion is required for the reaction, and low concentrations of heavy metals markedly inhibit the enzyme. The equilibrium of the reaction is very far in the direction of FGAM synthesis, and no exchange between $^{32}P_i$ and ATP has been found (55, 56). The amide group of glutamine is ordinarily the source of the amidine group of FGAM, but in the chicken liver system ammonium chloride could also serve as the nitrogen donor (74).

In this Chapter, the partial purification of this enzyme from Ehrlich ascites carcinoma cells is described. AIR synthetase activity was found also to be associated with this enzyme preparation; this enzyme catalyzes the

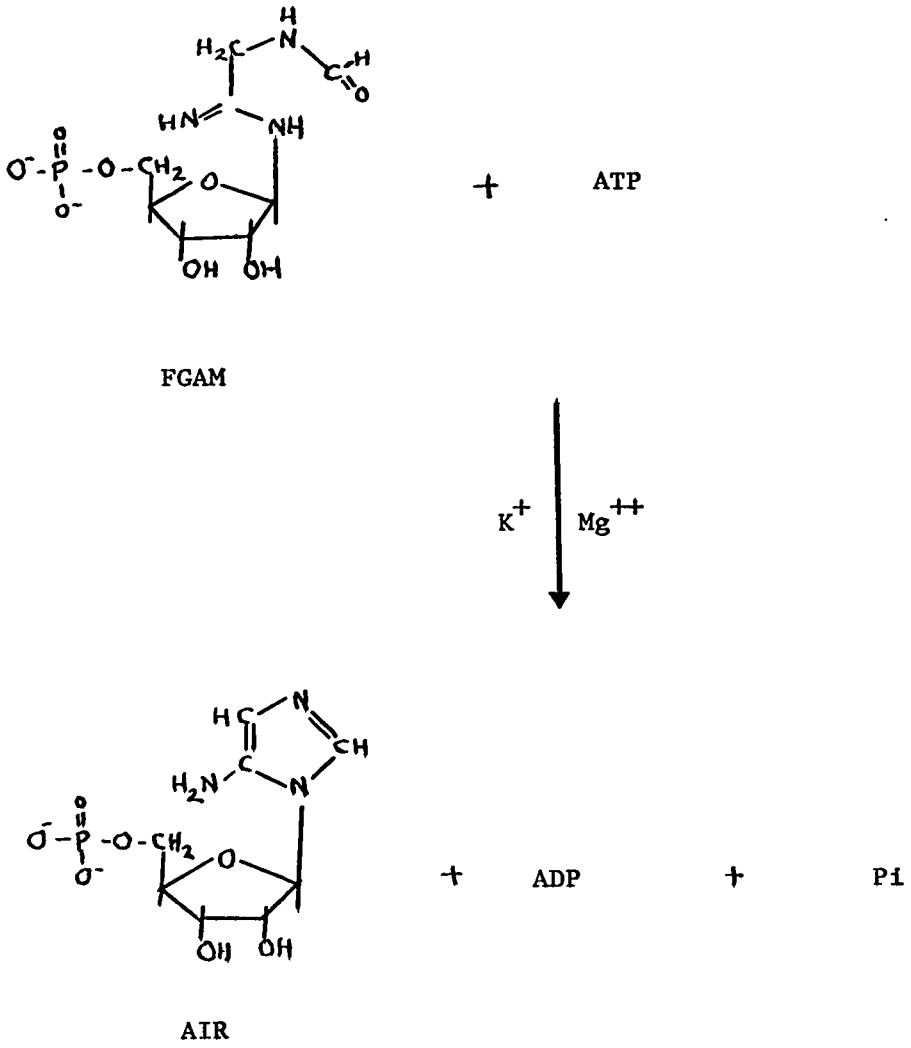


Figure 10. The 5'-phosphoribosyl-aminoimidazole synthetase reaction.

cyclization of FGAM (Figure 10). Both FGAM synthetase and AIR synthetase require the presence of potassium ion for activity. The former enzyme, however, requires less potassium ion than the latter for maximum activity. This result made it possible to assay the FGAM synthetase activity in this enzyme preparation. Ammonium chloride can replace potassium ion as the monovalent cation activator for this enzymic reaction. It can also serve as the nitrogen donating substrate.

B. Materials and methods

1. Chemicals

The previously synthesized FGAR (lithium salt) was used in these studies. Disodium ATP was purchased from Calbiochem and solutions of it were adjusted to pH 7.8-8.0 in the cold with sodium hydroxide. Disodium EDTA was obtained from Eastman. L-Glutamine was obtained from Calbiochem. Other chemicals were of reagent grade.

An AIR synthetase preparation was prepared from pigeon liver by ammonium sulfate fractionation according to the procedure of Flaks and Lukens (78). This fraction was dialyzed for 2 hours against 0.01 M potassium phosphate buffer pH 7.4 containing 0.1 M potassium chloride.

Sephadex and Sephadex derivatives were products of Pharmacia Fine Chemicals.

FGAR concentrations were determined by their phosphate content using the method of Ames and Dubin (87) with AMP as standard.

2. Enzyme source

ICR Swiss mice, 18 to 22 g, were inoculated intraperitoneally with Ehrlich ascites carcinoma cells. Six or seven days after implantation, tumor cells were removed by Pasteur pipette after laparotomy, and ascitic fluids were discarded after centrifugation. These tumor cells were used as source of enzyme.

3. Enzyme assays

a. Assay A

Enzyme activity was assayed by the method of Mizobuchi and Buchanan (54), in which FGAM was converted to AIR in the presence of ATP, magnesium ion, potassium ion and an excess of pigeon liver AIR synthetase. The amount of AIR formed was then determined by the Bratton-Marshall reaction (79). The reaction mixture contained in 0.3 ml a final concentration of 33.3 mM Tris-HCl buffer pH 8.0; 1.0 mM glutamine; 0.5 mM FGAR; 10.0 mM ATP; 10.0 mM $MgCl_2$; 0.1 M KCl; 0.05 ml of pigeon liver AIR synthetase and finally the enzyme preparation. The amount of pigeon liver AIR synthetase used was shown to be in excess since there was no appreciable change in the amount of AIR formed over a range of 0.01 to 0.05 ml of the AIR synthetase solution. As a control, the tumor cell enzyme preparation was omitted from the reaction mixture. The reaction mixture was

incubated for 30 min at 37°C and then chilled in an ice bath. 0.1 Ml of a 20% solution of TCA which had been adjusted to pH 1.4 with 1.33 M potassium phosphate was added to stop the reaction, and the protein precipitate was centrifuged down. The following solutions were mixed with the supernatant solution at room temperature: first, 0.05 ml of 0.1% sodium nitrite; after 3 min, 0.05 ml of 0.5% ammonium sulfamate; and after another 3 min, 0.05 ml of 0.1% N-1-(naphthyl)ethylenediamine dihydrochloride. The mixture was then allowed to stand for 30 min at room temperature to permit complete development of the color. After a further centrifugation for 3 min at 2000 rpm, the absorbance at 500 nm was read using a Beckman monochromator coupled with Gilford 220 absorbance indicator and Gilford 210 automatic cuvette positioner. The extinction coefficient of the colored derivative was determined by Levenberg and Buchanan to be 24,500 (93).

b. Assay B

To assay for the presence of both FGAM synthetase and AIR synthetase activities in the same enzyme preparation, the above procedure (Assay A) was employed but the external AIR synthetase was omitted.

c. Assay C (Two-stage incubation)

To assay the FGAM synthetase activity in an enzyme preparation that possessed both FGAM synthetase and AIR synthetase activities, the following procedure was

adopted. The enzyme preparation was first incubated as in Assay B with the substrates, ATP, FGAR, glutamine, magnesium chloride and Tris-HCl buffer pH 8.0, together with 20 mM KCl, for 30 min at 37°. After the incubation period, the reaction was stopped by adding 0.02 ml of 20% TCA, kept in an ice bath for 5 min, and then 0.01 ml of 2 N KOH, 0.05 ml of 1 M Tris-HCl buffer pH 8.0 and 0.02 ml of 2 M KCl were added to adjust the pH to 7.9-8.1 and to provide a higher concentration of potassium ion. Then 0.05 ml of pigeon liver AIR synthetase was added and the solution was further incubated for 30 min at 37°. After this incubation, the AIR formed was determined as described in Assay A.

A unit of enzyme activity was defined as that amount catalyzing the formation of 1 micromole of FGAM per min under the assay conditions. Specific activities are given as microunits of enzyme activity per mg of protein. Protein concentration was determined by the biuret-phenol reagent (94), standardized against bovine serum albumin.

C. Results

1. Enzyme purification

Ehrlich ascites tumor cells, 16 ml packed-cell-volume, were suspended in 60 ml of 0.01 M potassium phosphate buffer pH 7.4 containing 0.1 mM EDTA and 0.3 mM glutamine, and disrupted by sonic oscillation 3 times for 30 sec.

This suspension was centrifuged for 20 min at 12,000 x g. The supernatant fraction (Fraction I) was stirred, brought to pH 5.2 by dropwise addition of 1 N HCl, and then centrifuged for 15 min at 12,000 x g. The clear, acidic supernatant was adjusted with 1 N KOH to pH 6.8 (Fraction II) and then fractionated with ammonium sulfate. The precipitate which formed at 33% of saturation was discarded, whereas that forming between 33% to 50% of saturation contained FGAM synthetase activity. This precipitate was collected by centrifugation for 10 min at 8,000 x g and dissolved in 2.5 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 containing 5% glycerol (Fraction III). This preparation was desalted by employing Sephadex (G-25-fine) gel filtration. The Sephadex column (2.0 x 20 cm) was equilibrated with 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 containing 5% glycerol. The protein fraction that contained FGAM synthetase activity was eluted at the void volume and concentrated by treatment with dry Sephadex G-75. This step also removed low molecular weight proteins. After 15 min the gel was removed from the mixture by centrifugation. This process was repeated once and the protein fraction (Fraction IV) was frozen and stored. The enzyme was stable for about a week and then gradually lost its activity.

Table 6 summarizes the purification procedure. A 56-fold purification was achieved for enzyme Fraction IV. In response to the problem of enzyme instability, emphasis was placed on the speed of this procedure, and the entire

TABLE 6: Purification of FGAM synthetase from Ehrlich ascites tumor cells

	Fraction	Volume (ml)	μ Units	Protein (mg)	Specific activity μ units/mg	Yield %	Purification fold
I	12,000 x g	76.0	75,000	1,392.00	54	100.0	1.00
II	pH 5.2	66.0	63,500	164.00	398	82.0	7.35
III	$(NH_4)_2SO_4$	2.5	35,700	12.40	2,880	47.6	53.40
IV	G-75	3.0	27,800	8.80	3,060	40.8	56.00
V	G-150	1.0	925	1.80	498	2.5*	9.05
VI	DEAE Sephadex	1.0	1,180	0.95	1,040	3.2*	19.30

*This is the calculated value. In the actual experiment 6 mg of dialyzed Fraction III were applied onto the column.

process could be completed in 6-7 h. Purification procedures were carried out at 2-4°C.

Potassium phosphate buffer was used in the purification of FGAM synthetase in Salmonella typhimurium, pigeon liver (78) and in the chicken liver (54). It was reported that this buffer solution stabilized the enzyme (54). However this buffer solution was not employed in the last steps of the present purification for the following reasons: (a) the effect of potassium ion on the FGAM synthetase could be studied; (b) the presence of Pi would complicate the kinetic assay procedure since product inhibition by Pi would be involved.

Using assay procedure B in which external AIR synthetase was omitted from the incubation mixture, the presence of AIR synthetase activity in this enzyme preparation was demonstrated. Table 7 shows that through the above purification steps, AIR synthetase was always present. Addition of external AIR synthetase did not increase the total amount of AIR formation in Assay A. The fact that two enzyme activities were indeed present was demonstrated by employing the two-stage incubation procedure (Assay C).

2. Attempts at further purification

a. Sephadex G-150 gel filtration

6 Mg (1.2 ml) of the desalted Fraction III was put onto a Sephadex G-150 column (2.5 x 50 cm), which had

TABLE 7: Presence of AIR synthetase activity in partially purified FGAM synthetase preparations

Fraction	FGAM (nmoles)	
	Assay A	Assay B
I	1.04	1.01
II	1.34	1.38
III	4.70	4.69
IV	4.82	4.87

been previously equilibrated with 0.1 M potassium phosphate buffer pH 6.5 containing 1 mM glutamine, 2 mM magnesium chloride and 5% glycerol. The column was eluted with the same buffer; the elution pattern is shown in Figure 11. Both FGAM synthetase and AIR synthetase activities were found in the same peak and these fractions were pooled and concentrated by precipitation with 60% ammonium sulfate saturation. The precipitate was dissolved in 1.0 ml of the same eluting buffer (Fraction V). Table 6 shows that a marked loss of enzyme activity occurred after the gel filtration, probably due to the long time required for elution.

b. CM-Sephadex column chromatography

0.5 Ml of Fraction V was dialyzed against the eluting buffer of step (a) above for 30 min and the protein was then applied to a CM-Sephadex column (1 x 15 cm) which had been equilibrated with the same buffer. Elution was carried out with the same buffer and the first protein peak also possessed both FGAM synthetase and AIR synthetase activities. This material had only 20% the specific activity of Fraction V.

c. DEAE-Sephadex column chromatography

6 Mg (1.2 ml) of the desalted Fraction III was applied to DEAE-Sephadex column (2 x 15 cm), which had previously been equilibrated with the buffer used in step (a) above. The column was then eluted with same buffer, and the elution pattern is shown in Figure 12. Again both FGAM

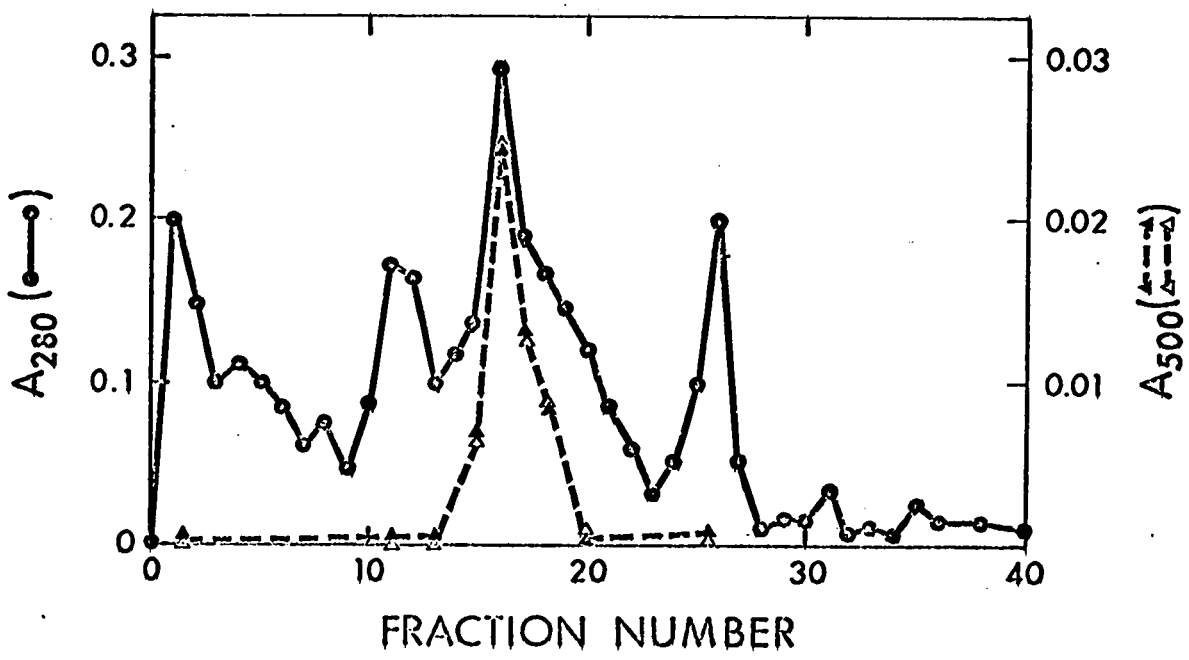


Figure 11. Gel filtration of FGAM synthetase on Sephadex G-150. 0.15 Ml of each 6 ml fraction was assayed for enzyme activity by Assays A (▲) and B (△); the incubation period was 1 h.

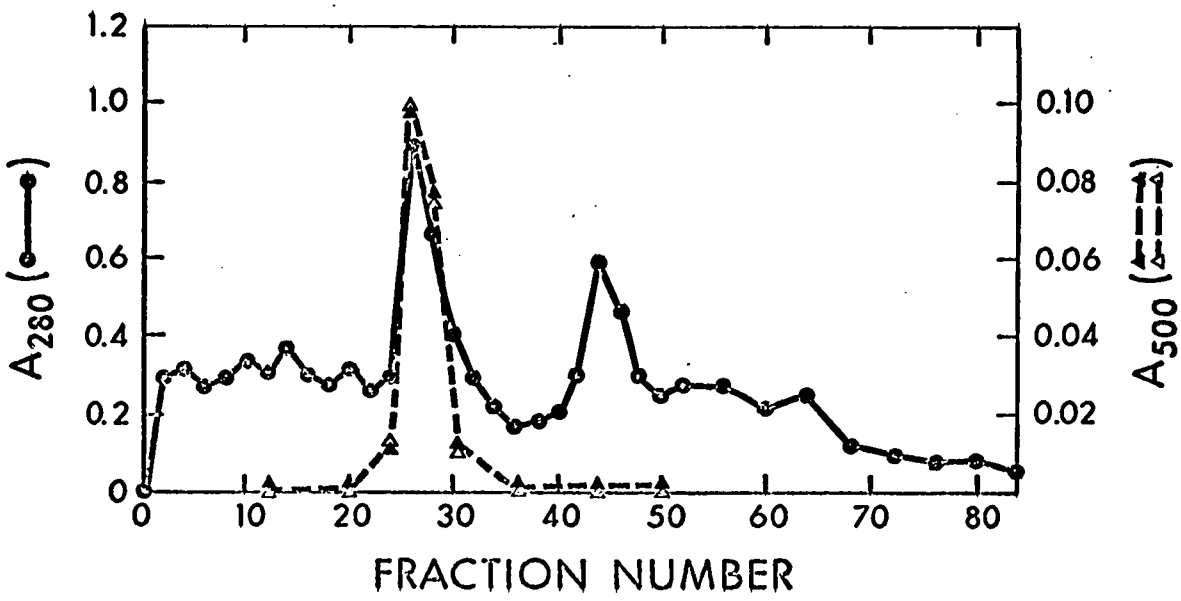


Figure 12. Column chromatography of FGAM synthetase on DEAE Sephadex A-50. 0.15 Ml of 3.2 ml fraction was assayed for enzyme activity by Assays A (▲) and B (△); the incubation period was 1 h.

synthetase and AIR synthetase activities were found in the same peak; these fractions were pooled and concentrated with 60% ammonium sulfate saturation. The precipitate was dissolved in 1.0 ml of the same eluting buffer (Fraction VI). Again a great loss of enzyme activity resulted (Table 6).

3. Catalytic properties

The enzyme preparation (Fraction IV) catalyzed the reaction of FGAR, ATP, and L-glutamine to form FGAM, ADP, Pi, and glutamate; both magnesium ion and potassium ion were required. FGAM was identified by its conversion to AIR, glutamate by paper chromatography, Pi by its reaction with ammonium molybdate. The stoichiometry of the reaction was not studied, however. Table 8 shows that the omission of any one of the substrates resulted in no FGAM formation.

A linear relationship of FGAM formation was obtained with respect both to time of incubation (Figure 13a) and to enzyme concentration (Figure 13b), indicating that the enzyme preparation contained negligible activities of interfering enzymes under the assay conditions.

Figure 14 shows the effect of pH on the activity of FGAM synthetase; the optimal activity for this enzyme was found to be at about pH 8.0.

TABLE 8: Substrate requirements for FGAM synthetase activity

Conditions	FGAM (nmoles)
FGAR, ATP, Mg^{++}, K^{+}, glutamine (complete system)	10.7
FGAR, K^{+}, ATP, Mg^{++}	< 0.1
FGAR, ATP, glutamine, K^{+}	< 0.1
Glutamine, ATP, Mg^{++}	< 0.1
Glutamine, Mg^{++}, K^{+}	< 0.1
ATP, Mg^{++}, K^{+}	< 0.1

Enzyme activity was measured by Assay A.

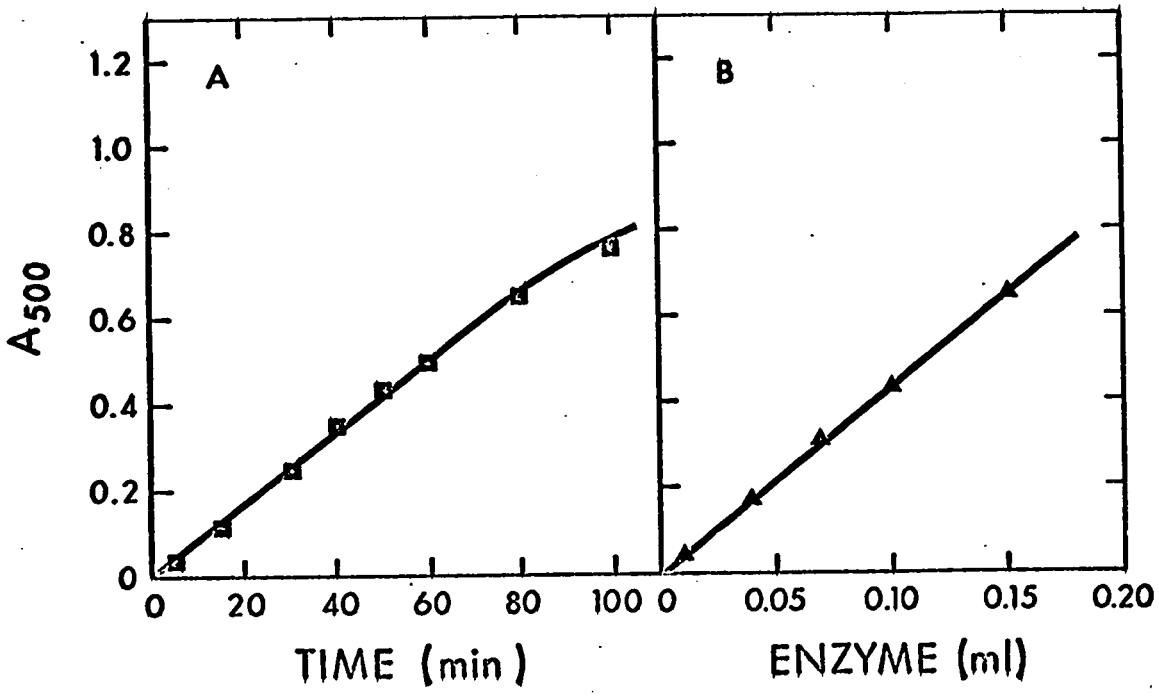


Figure 13. Effects of (A) incubation time, and (B) enzyme concentration on the assay of FGAM synthetase activity by Assay A.

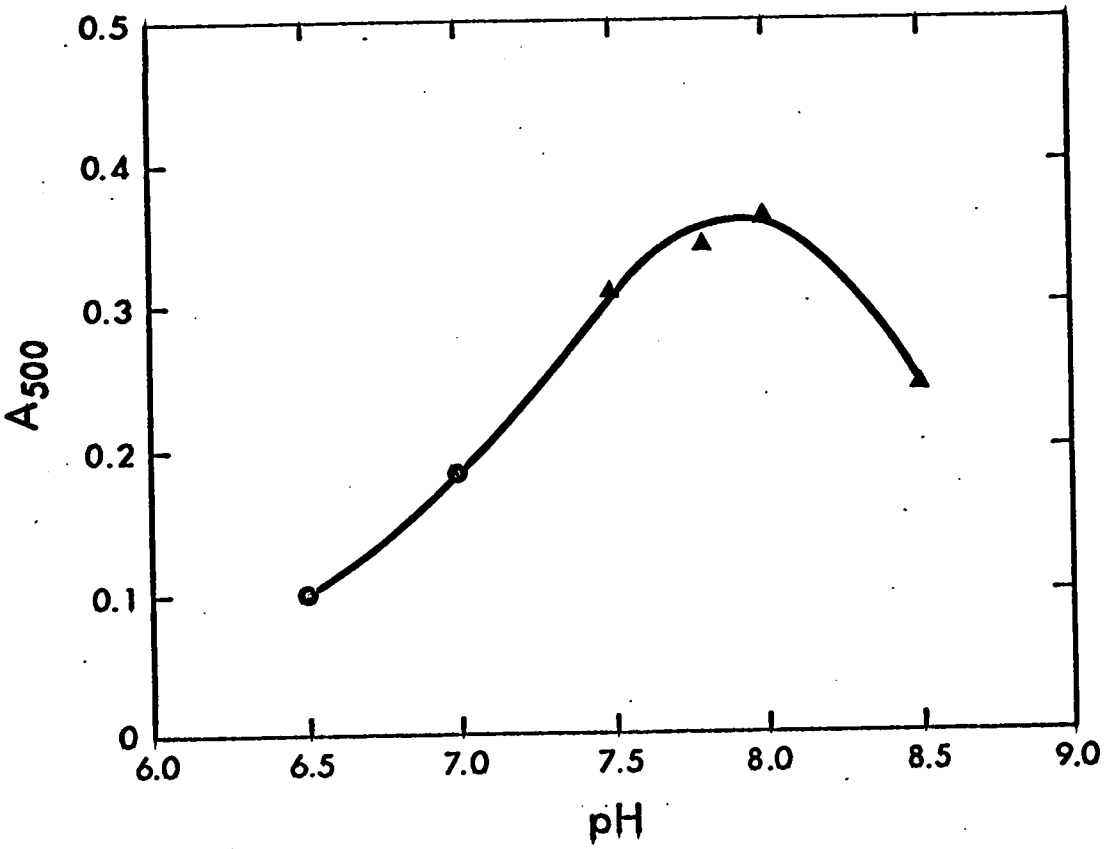


Figure 14. Effect of pH on the activity of FGAM synthetase. 0.05 M Tris-HCl buffers (▲); 0.05 M sodium hydrogen maleate-NaOH buffers (●). Assay A was used.

4. Protection by substrates of enzyme against thermal inactivation

When the enzyme preparation (Fraction IV) was incubated at 50° for 5 min, a loss of up to 98% of enzyme activity was found. However, incubating the enzyme with $MgCl_2$, KCl, and substrates FGAR, ATP and glutamine protected the enzyme completely from denaturation by this heat treatment; omission of the substrates ATP, FGAR and glutamine resulted in a 90% loss of enzyme activity (Table 9). When the enzyme was incubated with $MgCl_2$, KCl and glutamine, 92% protection of enzyme activity against heat inactivation was obtained. Under similar conditions but in the absence of K^+ ion, a loss of up to 60% of enzyme activity was found. Neither ATP or FGAR alone or in combination could protect the enzyme against this heat treatment.

5. Effect of potassium ion on enzyme activity

Potassium ion is required in a fairly high concentration as an activator of AIR synthetase (78) while the possibility of such a requirement for the FGAM synthetase had not been investigated. To determine if potassium ion was required for this enzyme activity, the two-stage incubation (Assay C) procedure was used in the presence of various amounts of KCl in the first stage. At the same time another set of experiments were carried out under similar conditions as above but the second incubation step was omitted. In this

TABLE 9: Protection of FGAM synthetase activity against thermal inactivation

Additions	Relative residual activity (%)
Glutamine, FGAR, ATP, $MgCl_2$, KCl	100
$MgCl_2$, Glutamine, K^+	92
Glutamine, K^+	94
Glutamine, Mg^{++}	40
ATP, Mg^{++}, K^+	12
FGAR, Mg^{++}, K^+	15
FGAR, ATP, K^+, Mg^{++}	25
MgCl, K^+	10
K^+	12
Mg^{++}	4
none	2

Reaction mixtures (1 ml) contained 0.05 M sodium hydrogen maleate-NaOH buffer pH 6.5, enzyme (Fraction IV) and 2 μmoles each addition. The mixtures were incubated at 50° for 5 min and then chilled. 0.1 Ml portions were assayed by Assays A, B and C.

way the potassium ion effect on tumor AIR synthetase could also be studied. Enzyme activity was also determined by Assay A to test if the two-stage incubation procedure could give the same amount of enzyme activity. Figure 15 shows that FGAM synthetase required potassium ion for full activity, but the concentration required was less than that required for AIR synthetase activity; FGAM synthetase required 10 mM KCl for maximum activity while that for AIR synthetase was 75 mM. At 50 mM KCl no appreciable amount of AIR synthetase activity was detected.

As shown in Table 10, TCA treatment completely abolished the enzyme activity in this two-stage incubation procudure. The presence of the trichloroacetic ion did not interfere with the conversion of FGAM to AIR during the second incubation step and it was also shown that no appreciable increase of absorbance was observed after 15 minutes of the second incubation period.

6. Effect of ammonium chloride on enzyme activity

Ammonium chloride can replace glutamine as the nitrogen donor for the chicken liver FGAM synthetase (74). It not only can act as the nitrogen donor for the tumor cell enzyme, but it can also replace potassium ion as the monovalent cation activator (Figure 16). Approximately equal molar concentrations of NH_4Cl and KCl gave the same effect.

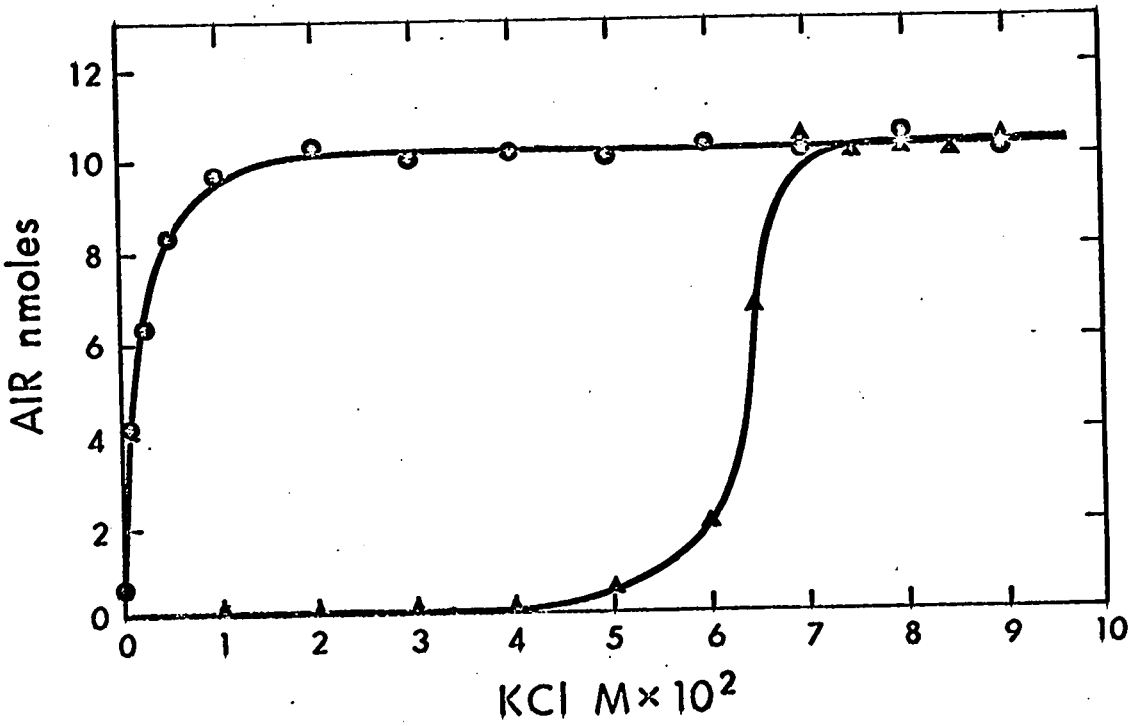


Figure 15. Effect of potassium ion on FGAM synthetase and AIR synthetase activities. FGAM synthetase activity was measured by Assay C (●), and AIR synthetase was measured by Assay B (▲).

TABLE 10: Comparison of Assay A and Assay C

Conditions	AIR (nmoles)
Assay C	10.7
Assay A	10.9

Enzyme was incubated with substrates and 100 μM KCl (Assay A), or with substrates and 20 μM KCl (Assay C). After 30 min incubation the reaction was stopped by addition of TCA, and AIR was measured in Assay A. In Assay C, the TCA extract was neutralized and incubated for 30 min with pigeon liver AIR synthetase and 88 mM KCl; AIR was then measured.

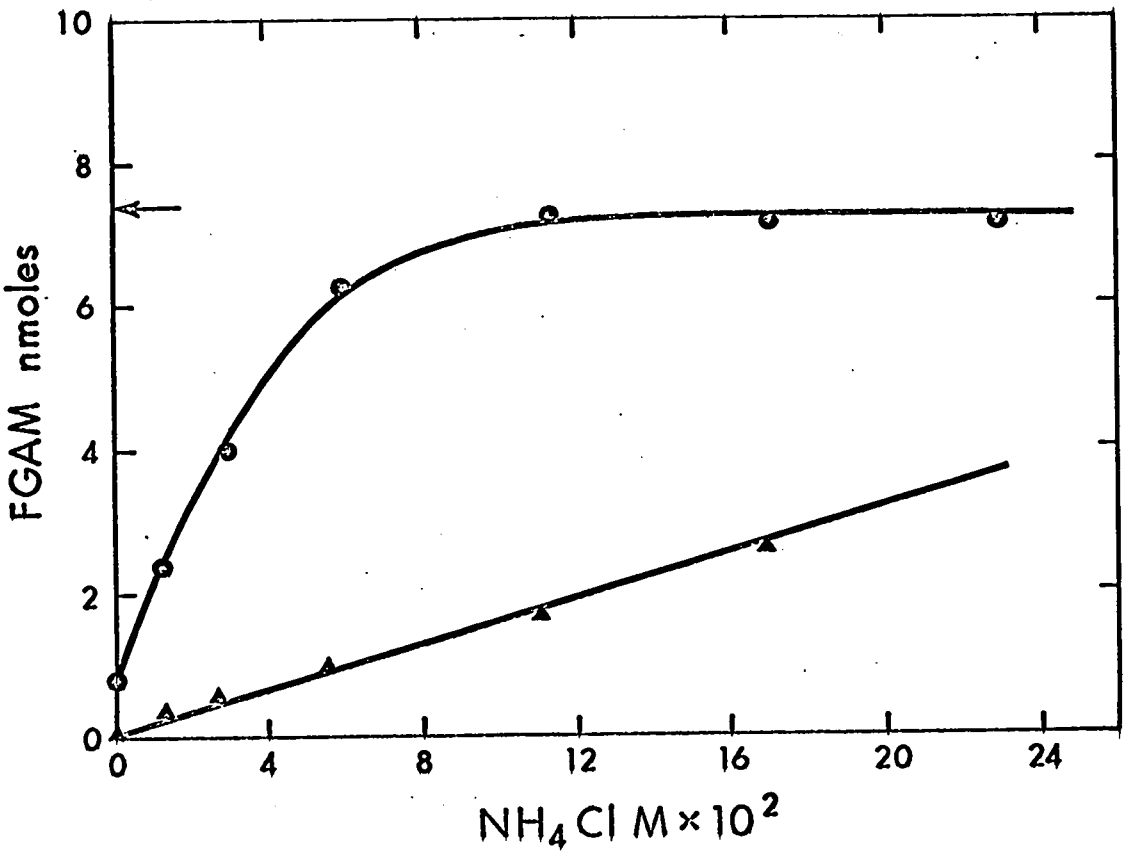


Figure 16. Effect of NH_4Cl on enzyme activity. Enzyme activity was assayed by Assay C under the following conditions: with 4.3×10^{-4} M glutamine but in the absence of K^+ (●); in the absence of 36 mM K^+ but in the absence of glutamine (▲). (↑), indicates the amount of enzyme activity in the presence of glutamine and K^+.

D. Discussion

AIR synthetase activity was found to be associated with the FGAM synthetase preparation from Ehrlich ascites tumor cells. The presence of both enzyme activities in the preparation was demonstrated if the external pigeon liver AIR synthetase was omitted from the assay mixture (Assay A), and the activity of each of these two enzymes could be demonstrated separately by the two-stage incubation procedure. Attempts to purify the enzyme further were unsuccessful due to the instability of the enzyme. Kinetic and drug inhibition studies on FGAM synthetase with this enzyme preparation would be complicated by the presence of AIR synthetase activity. One-stage incubation procedures such as Assay A are not suitable for kinetic studies because ATP is also a substrate for the AIR synthetase reaction, and a double requirement of ATP might give parabolic 1/v vs 1/ATP plots. In order to overcome this problem, different assay conditions were investigated to assay only the FGAM activity in this enzyme preparation and the two-stage incubation procedure (Assay C) was found to suit this purpose.

The fact that FGAM synthetase was eluted in the exclusion volume on Sephadex G-100 and in the early fractions of Sephadex G-150 gel filtration implies that this protein has a molecular weight of around 150,000 daltons. The molecular weight of the chicken liver FGAM synthetase was found to be 133,000 daltons (54).

The requirement for potassium ion or ammonium ion as activator places this enzyme in a large group of such enzymes. Recently Suelter has reviewed this subject (95), and the monovalent cation was suggested to exert a role by maintaining a specific protein conformation necessary for optimum catalytic activity (96); Melchior (97) and Lowenstein (98) in contrast have proposed that these cations interact with the substrates. Glutamine plus KCl gave 92% protection of FGAM synthetase against heat inactivations while in the absence of the KCl, up to 60% of the enzyme activity was lost; KCl alone gave no protection, however. These results suggest that potassium ion seems to be playing a role in maintaining the integrity of the enzyme.

The inability to separate FGAM synthetase and AIR synthetase activities in the purification procedure used might suggest that they are present as an aggregate or complex, although the possibility also exists that these two enzymes may just possess similar physical properties and hence be difficult to separate. The present studies show that the enzyme behaved differently during purification from that reported by Levenberg and Buchanan (44). In the pigeon liver system, the FGAM synthetase is found in the 0-35% ammonium sulfate saturation fraction while the AIR synthetase is located in the 45-65% ammonium sulfate saturation fraction. In an experiment that was carried out on the tumor cell acetone powder extract under similar conditions of pH, buffer

and enzyme concentration as for pigeon liver enzyme system, the 0-35% ammonium sulfate saturation fraction of tumor cell extract showed no FGAM synthetase activity. When this fraction was incubated with the 50-60% ammonium sulfate saturation fraction of the tumor cell extract, no AIR formation was detected. On the other hand, 35-45% and 45-50% ammonium sulfate saturation fractions of this tumor cell acetone powder extract had FGAM synthetase as well as AIR synthetase activities. Recently FGAM synthetase has been purified from chicken liver (54) but because of the assay procedure employed, one could not estimate the amount of AIR synthetase activity in that enzyme preparation.

Enzyme aggregates catalyzing two or more reactions within the same biosynthetic pathway are known. In the pathway of purine nucleotide biosynthesis de novo, recent studies with pigeon liver extracts have suggested that the enzymes of the first four reactions in this pathway may exist as one large macromolecular complex, although this can be split into its component parts by appropriate treatments (99). The last two enzymes of this pathway also form a single large unit in Salmonella typhimurium (85) and in chicken liver (100). In the pathway of pyrimidine biosynthesis, a complex of aspartate transcarbamylase and carbamyl phosphate synthetase has been extracted from baker's yeast (101). In tryptophan biosynthesis, both N-(5'-phosphoribosyl)anthranilate isomerase and indole-3-glycerophosphate synthetase have been

found in aggregate form in the mutant try I of Neurospora crassa (102). An enzyme aggregate is also involved in the biosynthesis of aromatic amino acids in Bacillus subtilis (103).

The presence of a multifunctional enzyme complex or aggregate in a biosynthetic pathway may be of biological importance. In cases where the product of one of the enzymes in the aggregate is an intermediate common to two biosynthetic pathways, the enzyme complex or aggregate may provide a preferential channeling of the common substrate into one pathway (104, 105). In cases of enzyme aggregate consisting of enzymes involved in the same pathway, it may have a function in localizing an inhibitor or activator to the control enzyme or in the utilization of a catalytic site for one enzyme and a regulatory site for the other (102, 103).

Ehrlich ascites carcinoma cells possess a high rate of growth and metabolism. The presence of possible enzyme aggregates in biosynthetic pathways, particularly that concerned with purine metabolism, may be advantageous. The intermediate generated in one step will at once be utilized due to its localization within the region where the subsequent reaction occurs.

E. Summary

FGAM synthetase was purified 56-fold from Ehrlich ascites carcinoma cells by a procedure involving acid

treatment, ammonium sulfate precipitation and Sephadex gel filtration. The presence of glutamine, magnesium chloride, and potassium chloride protected the enzyme against thermal denaturation. However, the same treatment in the absence of potassium chloride resulted in a loss of 60% of the enzyme activity, while potassium chloride alone provided no protection. The enzyme preparation was found to contain AIR synthetase activity as well. Further attempts to separate the two enzymes were unsuccessful. Both FGAM synthetase and AIR synthetase required potassium ion as activator but the former required a lower concentration for maximal activity. Based on the different requirements of these two enzymes for potassium ion, FGAM synthetase could be assayed separately in the presence of AIR synthetase. Ammonium ion could replace potassium as the monovalent cation activator, and could also serve as the nitrogen donor for the reaction.

The possibility that both FGAM synthetase and AIR synthetase are present as an aggregate in the tumor cells has been discussed.

IV. A SIMPLIFIED METHOD FOR DERIVATION OF STEADY-STATE RATE EQUATIONS FOR THREE-SUBSTRATE--THREE-PRODUCT AND THREE-SUBSTRATE--FOUR-PRODUCT ENZYME REACTIONS

A. Introduction

Enzyme kinetics is a major tool for the elucidation of mechanisms of enzyme catalysis. As more and more complicated enzyme reactions receive attention, the problem of deriving steady-state rate equations for possible mechanisms becomes more acute. Attempts have been made to interpret kinetic data without having to derive complete rate equations, and these methods are suitable for many situations. Cleland (115), for example, has dealt with the prediction by inspection of patterns of graphed kinetic data for various mechanisms. More recently, Wong and Hanes (151), and Dalziel (152) have reported new criteria for distinguishing enzyme mechanisms involving three substrates, which also do not require derivation of rate equations.

Complicated reaction systems, however, such as those which involve random or partially random mechanisms, still require derivation of rate equations for best interpretation of kinetic data. Likewise, the presence of modifiers also requires that rate equations be derived in order to distinguish among various possible mechanisms (153). Several attempts (154-157) have been made to simplify the derivation of steady-state rate equations for complex enzyme reactions, but it is still quite a task to produce all of the equations required to analyze product and dead-end inhibition data from

three or four-substrate reactions, for example.

If one considers the six major classes of enzymes, and ignores water as a substrate, it is apparent that in general, oxidoreductases and transferases comprise two-substrate reactions with two or sometimes three products; hydrolases and isomerases are one-substrate--one-product reactions; lyases are one-substrate--two-product reactions. These are the reaction types whose kinetic mechanisms have been studied the most. More and more attention, however, is now being given to the ligases, most of which are three-substrate--three-product reactions. One important sub-group in this class, however, is that termed "C-N ligases with glutamine as amido-N donor", which now comprises eleven enzyme reactions which have three substrates and four products.

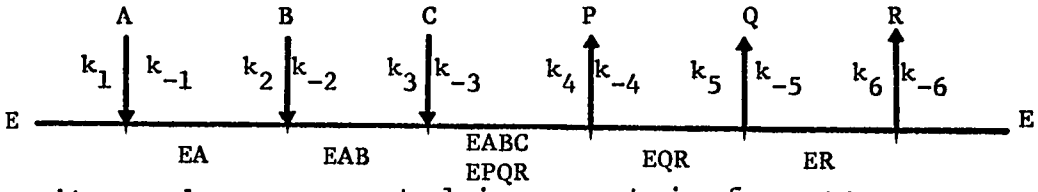
The present study is an attempt to facilitate kinetic studies of three-substrate reactions with three or four products. A simplified procedure is presented for deriving steady-state rate equations which, although also applicable to simpler reactions too, is most useful for analysis of product and dead-end inhibition studies of these more complicated cases. Through the use of this method, product and dead-end inhibition equations and patterns have been derived for 16 different three-substrate reaction mechanisms; these are presented in detail in the Appendix. Throughout this study, an attempt has been made to obtain practical results which can easily be applied to the initial velocity

data obtained in kinetic studies of enzyme mechanisms.

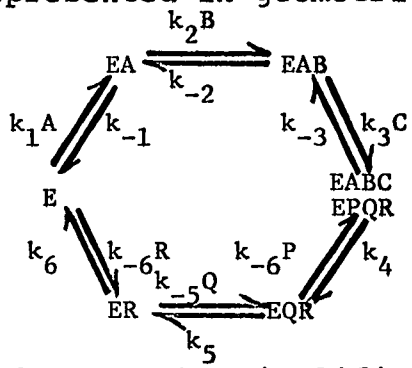
B. Theory

Most individual rate constants, e.g., k_1, k_2, k_{-1}, k_{-2}, etc., in a full steady-state description of an enzyme reaction, are difficult if not impossible to evaluate, and hence have little or no practical use. Indeed, in most systems of derivation they are amalgamated into kinetic constants, e.g., K_A, K_Q, etc., in Cleland's system (158), or $\emptyset_1$, $\emptyset_2$, etc., in Dalziel's system (157), before equations are actually put to use. In the present system individual rate equations are not used even in the initial phases of the derivation.

Taking an ordered ter ter enzymic reaction system as illustrated, the mechanism may be written according to Cleland's system as follows:

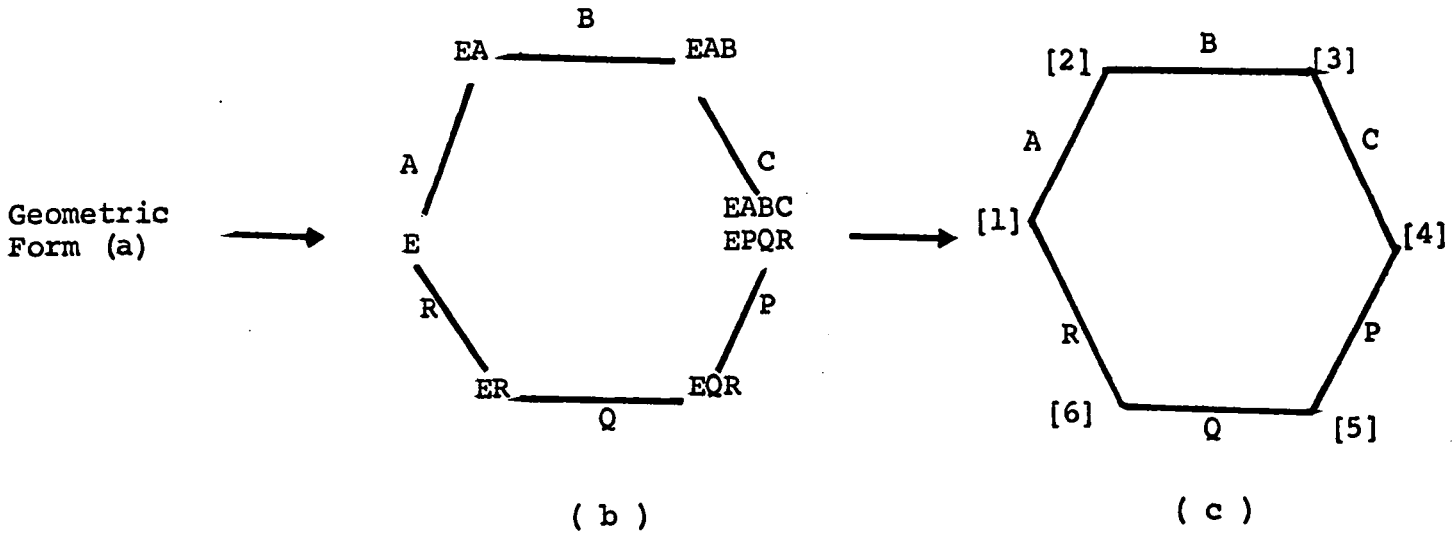


or it can be represented in geometric form as:



(a)

This geometric form can be simplified as shown in Scheme I.

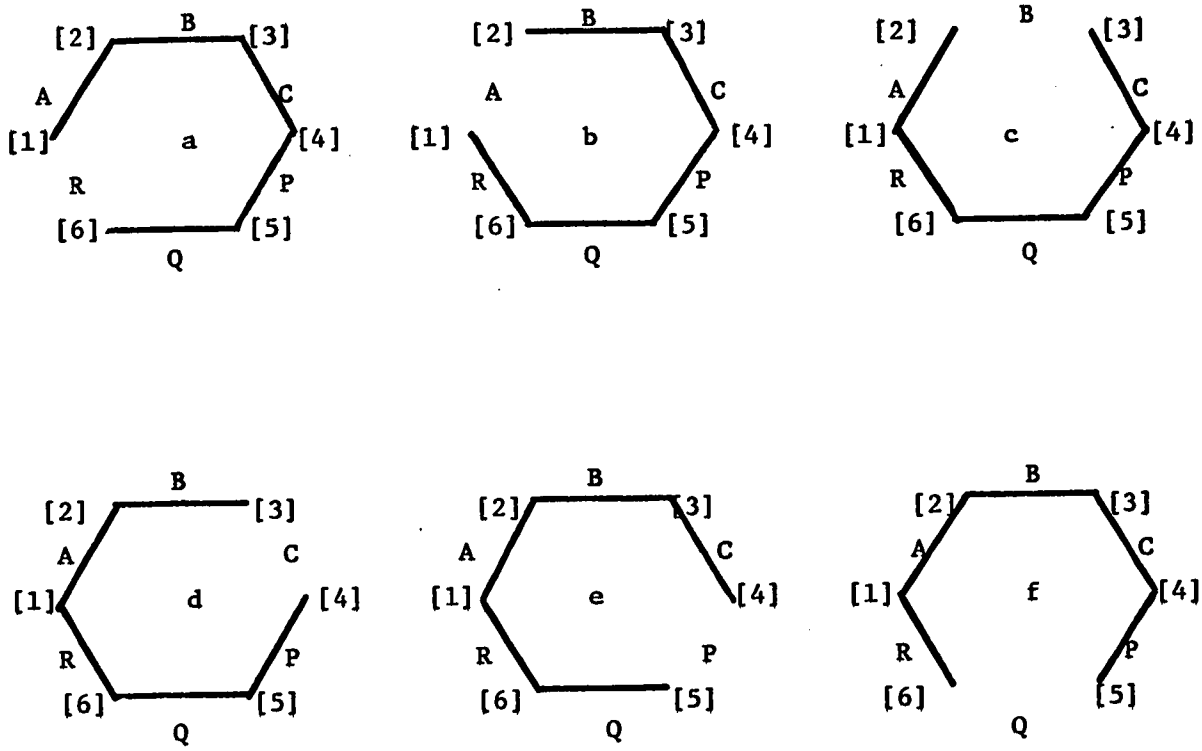

Geometric
Form (a)
B
EA
EAB
A
C
EABC
EPQR
E
R
P
ER
EQR
Q
(b)
[2]
B
[3]
A
C
[1]
[4]
R
P
[6]
Q
[5]
(c)

SCHEME I

The simplified form (c) contains the essential features of the ter ter mechanism, the order of addition of substrates and of release of the products. The number of enzyme forms, represented by the corners of the polygon, remains the same. Each corner of the polygon represents an enzyme form, and is numbered. In Figure (c), [1] is the free enzyme, [2] is the EA complex, etc. A, B, C, P, Q , and R represent the substrates and products and are so written on each side of the polygon in the same order as they associate with or dissociate from the enzyme. The only difference between this simplified form (c) and the ordinary reaction scheme is that no kinetic constants are included. It is based on this simplified graphical representation that the rate equations are devised.

The procedure for such derivation is described in the following steps. The ordered ter ter mechanism is used to illustrate the method.

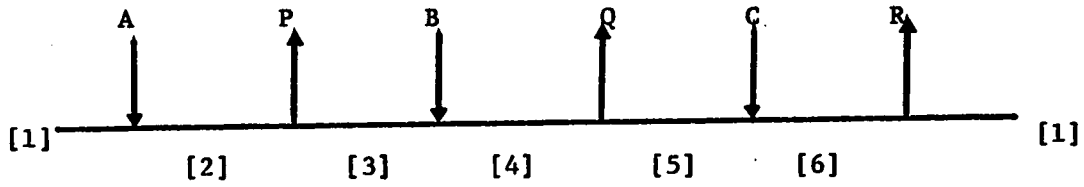
1. A model of the mechanism under consideration is depicted first. Construct a series of polygons whose number equals the sum of the number of substrates; the number of the sides of each should also equal the same sum. Thus for the ter ter mechanism, six hexagons are drawn, as shown in Scheme II. In each polygon in the series, a different side is left open, in a manner similar to the procedure of King and Altman (154).


[2]
B
[3]
A
C
[1]
a
[4]
R
P
[6]
[5]
Q
[2]
B
[3]
A
C
[1]
b
[4]
R
P
[6]
[5]
Q
[2]
B
[3]
A
C
[1]
c
[4]
R
P
[6]
[5]
Q
[2]
B
[3]
A
C
[1]
d
[4]
R
P
[6]
[5]
Q
[2]
B
[3]
A
C
[1]
e
[4]
R
P
[6]
[5]
Q
[2]
B
[3]
A
C
[1]
f
[4]
R
P
[6]
[5]
Q

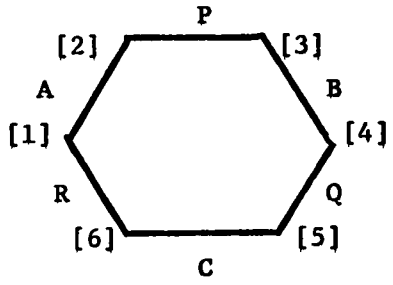
SCHEME II

Each corner of the polygon represents an enzyme form as mentioned above and is numbered in the same order in each of the polygons. A, B, C, P, Q, and R represent the substrates and products as described before and are written on the side of the polygon in the order required for mechanism under consideration. The six possible open-sided hexagonal figures are denoted a, b, c, d, e, and f.

The ping-pong ter ter mechanism may be considered as a further example.



The notation on one of the hexagons would be written as:



A ter quad mechanism will require seven heptagons, a through g, with A, B, and C as substrates and P, Q, R, and S as products.

2. The rate equation for a ter ter enzymic reaction, based on steady-state assumptions, and without specifying kinetic constants, will be:

$$\frac{v}{E_o} = \frac{[ER] - [E]R}{[E] + [EA] + [EAB] + [EABC] + [EQR] + [ER]}$$

or

$$\frac{v}{E_o} = \frac{[6] - [1]R}{[1] + [2] + [3] + [4] + [5] + [6]}$$

where v = velocity, E_o = total enzyme concentration. [1], [2] . . . etc, are concentrations of different enzyme forms. Similarly for a ter-quad reaction, the rate equation is

$$\frac{v}{E_o} = \frac{[7] - [1]S}{[1] + [2] + [3] + [4] + [5] + [6] + [7]}$$

3. To obtain the terms which represent the concentration of each enzyme form (or corner of the polygon) in the rate equation, all the terms which appear on pathways leading to the enzyme form (corner) in question, and which are represented by closed sides of each polygon, are multiplied together, with the following restrictions: (a) if the path leading to the enzyme form goes clockwise, product terms are denoted "Unity", and (b) if the path goes counterclockwise, substrate terms are denoted "Unity". For each enzyme form such terms derived from each hexagon are summed.

Thus in Scheme II, for free enzyme, E , designated by corner [1], the terms derived from each hexagon are as follows:

(a) PQ

(b) BC

(c) C

(d) 1

(e) 1

(f) P

and E = PQ + BC + C + P + 1 + 1.

In a similar manner, terms for other enzyme forms are determined and tabulated as shown in Table 11.

4. The numerator of the rate equation is the difference between the terms equivalent to the last enzyme form, ER or corner [6], and the terms equivalent to the first enzyme form, E or corner [1], multiplied by the last product, R. In the ordered ter ter mechanism:

$$(ABC + BCR + CR + R + R + PR) - R\ (PQ + BC + C + 1 + 1 + P)$$
$$= ABC - PQR$$

5. The denominator of the rate equation is equal to the sum of the terms in Table I, except that no term is written more than once.

6. In the full rate equation, each term in both numerator and denominator are multiplied by Θ_n, with n different for each term, to denote the different group of rate constants which in ordinary schemes for the enzyme mechanism would be

TABLE 11: Kinetic terms of an ordered ter ter mechanism

	[1]	[2]	[3]	[4]	[5]	[6]
a	PQ	APQ	ABPQ	ABCPQ	ABCQ	ABC
b	BC	PQR	BPQR	BCPQR	BCQR	BCR
c	C	AC	PQR	CPQR	CQR	CR
d	1	A	AB	PQR	QR	R
e	1	A	AB	ABC	QR	R
f	P	AP	ABP	ABCP	ABC	PR

associated with each expression. The full expression for the ordered ter ter mechanism is given in Equation I, where v = velocity, and E_o = total enzyme concentration.

$$\frac{v}{E_o} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 + \theta_4 A + \theta_5 C + \theta_6 P + \theta_7 Q + \theta_8 AB + \theta_9 AP + \theta_{10} AC + \theta_{11} BC + \theta_{12} CR + \theta_{13} PQ + \theta_{14} PR + \theta_{15} QR + \theta_{16} ABC + \theta_{17} ABP + \theta_{18} APQ + \theta_{19} BCR + \theta_{20} CQR + \theta_{21} PQR + \theta_{22} ABCP + \theta_{23} ABCQ + \theta_{24} ABPQ + \theta_{25} BPQR + \theta_{26} BCQR + \theta_{27} CPQR + \theta_{28} ABCPQ + \theta_{29} BCPQR} \qquad \text{Equation I}$$

7. Initial velocity. In the absence of products, the double reciprocal form of the initial rate equation based on Equation I is Equation II.

$$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC} \qquad \text{Equation II}$$

where ψ_o . . . etc are arbitrary kinetic constants. This is similar to the form used by Fromm (109) to express Dalziel's initial rate equation (159).

8. Product inhibition. In the presence of the product, those terms containing that particular product alone or in combination with substrates should be included in the initial rate equation. For instance, for the ordered ter ter mechanism in the presence of product P, the denominator terms of P, AP, ABP, ABCP should be included in the initial rate equation:

$$\frac{v}{E_o} = \frac{\theta_1 ABC}{\theta_3 + \theta_4 A + \theta_5 C + \theta_6 P + \theta_8 AB + \theta_9 AP + \theta_{10} AC + \theta_{11} BC + \theta_{16} ABC + \theta_{17} ABP + \theta_{22} ABCP}$$

Equation III

or

$$\frac{E_o}{v} = \psi_o(\phi_o + P) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3 + P) + \frac{\psi_4}{AB} + \frac{\psi_5}{BC}(\phi_5 + P) + \frac{\psi_6}{ABC}(\phi_6 + P)$$

Equation IV

Here the notations $\psi_o \ldots$, $\theta_o \ldots$, are arbitrary kinetic constants.

9. Dead-end inhibition. In the presence of dead-end inhibitors, the denominator terms which are affected are those referring to the particular enzyme form or forms to which the inhibitor binds. Thus if a dead-end inhibitor that binds to free enzyme E or form [1] is present, the denominator terms 1, BC, C in the initial rate equation are affected. If the full rate equation is represented instead of that for the initial rate, other terms in form [1] such as PQ and P should be considered. For the ordered ter ter mechanism, the initial rate equation in the presence of a dead-end inhibitor that binds with form [1] will be

$$\frac{v}{E_o} = \frac{\theta_1 ABC}{\theta_3 + \theta_3\left(\frac{I}{K_i}\right) + \theta_4 A + \theta_5 C + \theta_5 C\left(\frac{I}{K_i}\right) + \theta_8 AB + \theta_{10} AC + \theta_{11} BC + \theta_{11} BC\left(\frac{I}{K_i}\right) + \theta_{16} ABC}$$

Equation V

or

$$\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A}\left(1+\frac{i}{K_i}\right) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}\left(1+\frac{i}{K_i}\right) + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}\left(1+\frac{i}{K_i}\right)$$

Equation VI

Here i is the concentration of the inhibitor, and K_i is the inhibition constant.

10. If a compound acts as a dead-end competitive inhibitor with respect to 2 substrates or 1 substrate and 1 product or any other combination, the rate equation can easily be obtained by considering the affected terms as found in Table 11. Similarly, when a product inhibitor (for instance P) acts also as a dead-end inhibitor with respect to substrate A, the rate equation can easily be derived by considering the affected corners (here [1]) in Table 11 as:

$$\frac{E_o}{v} = \psi_o(\phi_0+P) + \frac{\psi_1}{A}(1+\frac{P}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3+P) + \frac{\psi_4}{AB}(1+\frac{P}{K_i}) + \frac{\psi_5}{BC}(\phi_5+P) + \frac{\psi_6}{ABC}(\phi_6+P)(1+\frac{P}{K_i})$$

Equation VII

C. Discussion

The procedure outlined above appears to be simpler and quicker for deriving the rate equation than those previously reported, and kinetic information can easily be obtained. Initial rate equations in the presence of dead-end inhibitors, for instance, can be derived easily as outlined in step 9 above. Dead-end enzyme complexes have been found in enzymic reactions (160 - 162), and Rudolph and Fromm (111) have used competitive or dead-end inhibitors to elucidate kinetic mechanism. Hurst (163, 164) also has

reported an analysis of dead-end inhibition patterns for a ping-pong bi bi mechanism; complex mathematical analysis, however, was required. Rate equations in the presence of dead-end and product inhibitors or products that act also as dead-end inhibitors can be derived according to step 10. The equations derived by this method were used in the kinetic studies of the mechanism of FGAM synthetase, as described below.

V. KINETIC MECHANISM OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE. I. INITIAL RATE STUDIES

A. Introduction

Little information is available concerning the mechanism of FGAM synthetase reactions and kinetic studies of this enzyme have not previously been carried out. Mizobuchi and Buchanan (54) have purified the enzyme from chicken liver and have characterized both enzyme-glutamyl (74) and FGAR-ATP-enzyme complexes (56). They proposed a mechanism in which the overall reaction is broken down into partial reactions operating in a highly coordinated manner with one another (56), although the exact nature of this coordination is not understood. In the present study, initial velocity data suggest a fully ping-pong mechanism. Initial rate studies in which ammonium chloride was used as the nitrogen donor were consistent with a partially ping-pong mechanism.

B. Materials and methods

1. Chemicals

The FGAM synthetase used was Fraction IV of the enzyme preparation described above (Chapter III). AIR synthetase was the ammonium sulfate fraction of pigeon liver prepared through the procedure of Flaks and Lukens (78). This fraction was dialyzed for 2 hours against 0.01 M potassium phosphate buffer pH 7.4 containing 0.1 M potassium chloride.

2. Methods

The lithium salt of FGAR was converted to its free acid by shaking with a small amount of Dowex 50 (H^+ form) before use and its concentration was determined by its phosphate content using the method of Ames and Dubin (87) with AMP as standard.

Initial rate experiments were carried out by incubating FGAM synthetase for 30 min at 37° and pH 8.0, with various amount of substrates. Enzyme activity was assayed by the two-stage incubation procedure (Chapter III). Velocity (v) was expressed as millimicromoles of FGAM formed under the assay conditions. When ammonium chloride was used as the nitrogen donor, glutamine was omitted from the reaction mixture. In this case, the first incubation period was extended to one h. The rate of synthesis of FGAM was linear for this incubation period at the enzyme concentration used.

C. Results

Initial rate studies of three substrate reactions have usually been carried out by holding the concentration of one substrate at a constant level and varying the concentrations of the other two substrates as in a two-substrate system (106-108). Recently Fromm reported a procedure (109) in which one of the substrates is varied while holding the other two fixed in the general concentration ranges of their Michaelis constants. This experiment is then repeated at

different concentrations of the fixed substrates, while maintaining the ratio of their concentrations constant. Using this procedure, a total of three sets of experiments can give enough information to determine if the enzyme mechanism is sequential or ping-pong. Experiments using this protocol have been carried out with the coenzyme A-linked aldehyde dehydrogenase (110), and with adenylosuccinate synthetase (111). Initial rate equations for three substrate-three product (109) and three substrate-four product enzymic reactions have been derived in Chapter IV. The initial rate kinetic patterns for three substrate reactions obtained from Lineweaver-Burk plots (112) are presented in Table 12 One or more of the three sets of data should give parallel double reciprocal plots if the emzymic mechanism is ping-pong or partially ping-pong. However, all three plots should give intersecting reciprocal plots if the mechanism is sequential, whether it is ordered, partially ordered or random.

Figures 17-19 show that parallel patterns were obtained for experiments in which glutamine was used as the nitrogen donor. These results eliminate sequential mechanisms. A non-intersecting double reciprocal plot was obtained when the experiment was carried out with low concentrations of glutamine (about equal to its Michaelis constant) while the other substrates were varied (Figure 20).

Initial rate kinetics using ammonium chloride as the nitrogen donor were also studied. The graphs shown in

TABLE 12: Patterns obtained in initial rate studies of ter quad reaction

Mechanism and initial rate equation	Kinetic patterns: Substrate varied A	B	C
1. Ordered ter quad: $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	N[1]	N	N
2. Random ter quad: $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AC} + \frac{\psi_5}{AB} + \frac{\psi_6}{BC} + \frac{\psi_7}{ABC}$	N	N	N
3. Partially random (AB random): $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{C} + \frac{\psi_2}{AC} + \frac{\psi_3}{BC} + \frac{\psi_4}{ABC}$	N	N	C[2]
4. Partially random (BC random): $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{B} + \frac{\psi_2}{C} + \frac{\psi_3}{BC} + \frac{\psi_4}{ABC}$	N	N	N
5. Partially random (AC random): $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{C} + \frac{\psi_3}{AB} + \frac{\psi_4}{BC} + \frac{\psi_5}{ABC}$	N	N	N
6. Fully ping-pong: $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U[3]	U	U
7. Partially ping-pong (BC ordered): $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	N	N
8. Partially ping-pong (AB ordered): $\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	N	N	U

1. non-competitive
2. competitive
3. un-competitive

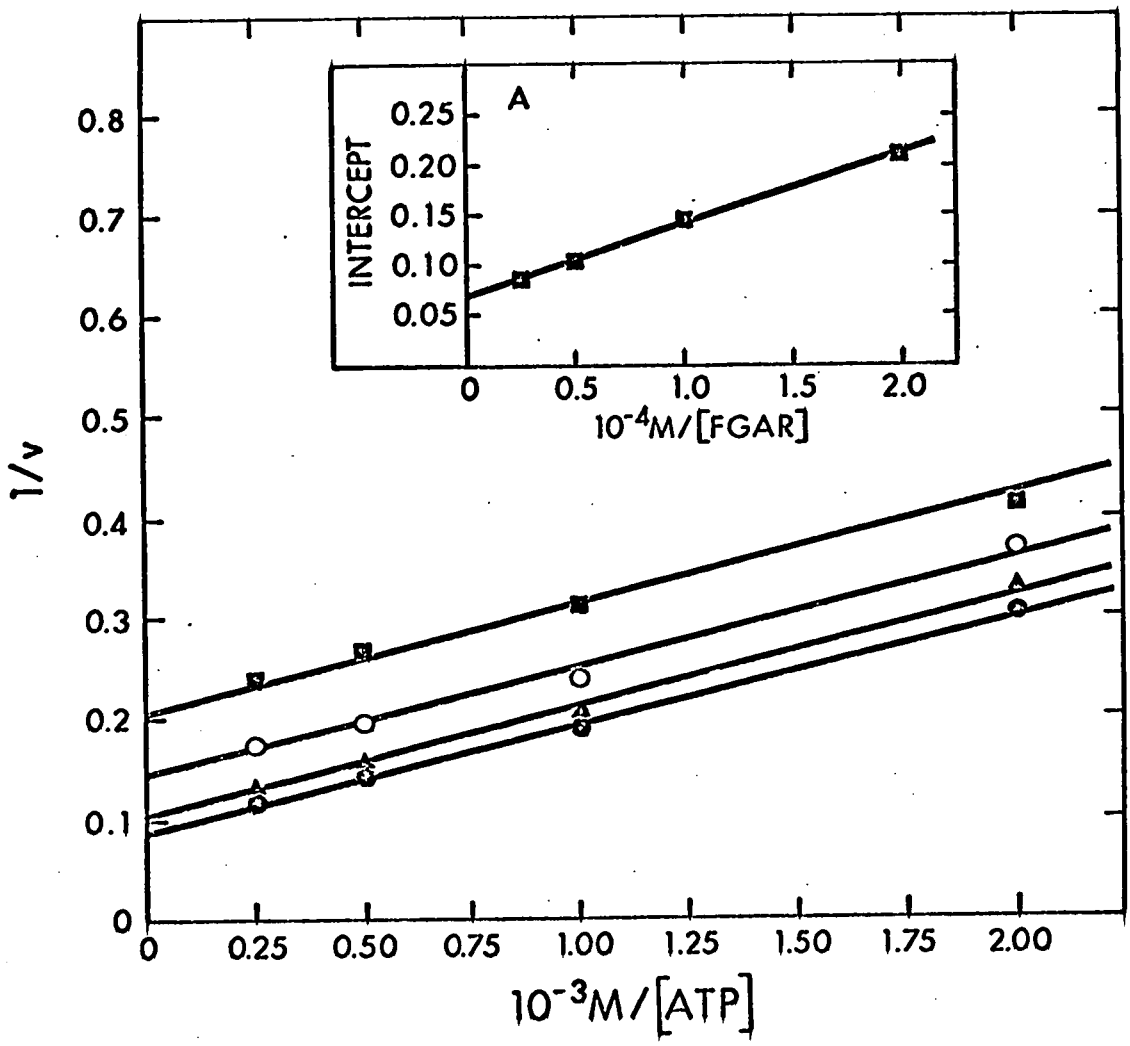


Figure 17. Double reciprocal plot of initial velocity against ATP concentration. The concentrations of FGAR and glutamine, respectively, were: 5.0×10^{-5} M and 8.3×10^{-5} M (▣); 1.0×10^{-4} M and 1.66×10^{-4} M (○); 2.0×10^{-4} M and 3.32×10^{-4} M (▲); 4.0×10^{-4} M and 6.64×10^{-4} M (●). Enzyme amount: 0.312 mg.

Insert: Replot of intercepts against the reciprocal of FGAR concentration.

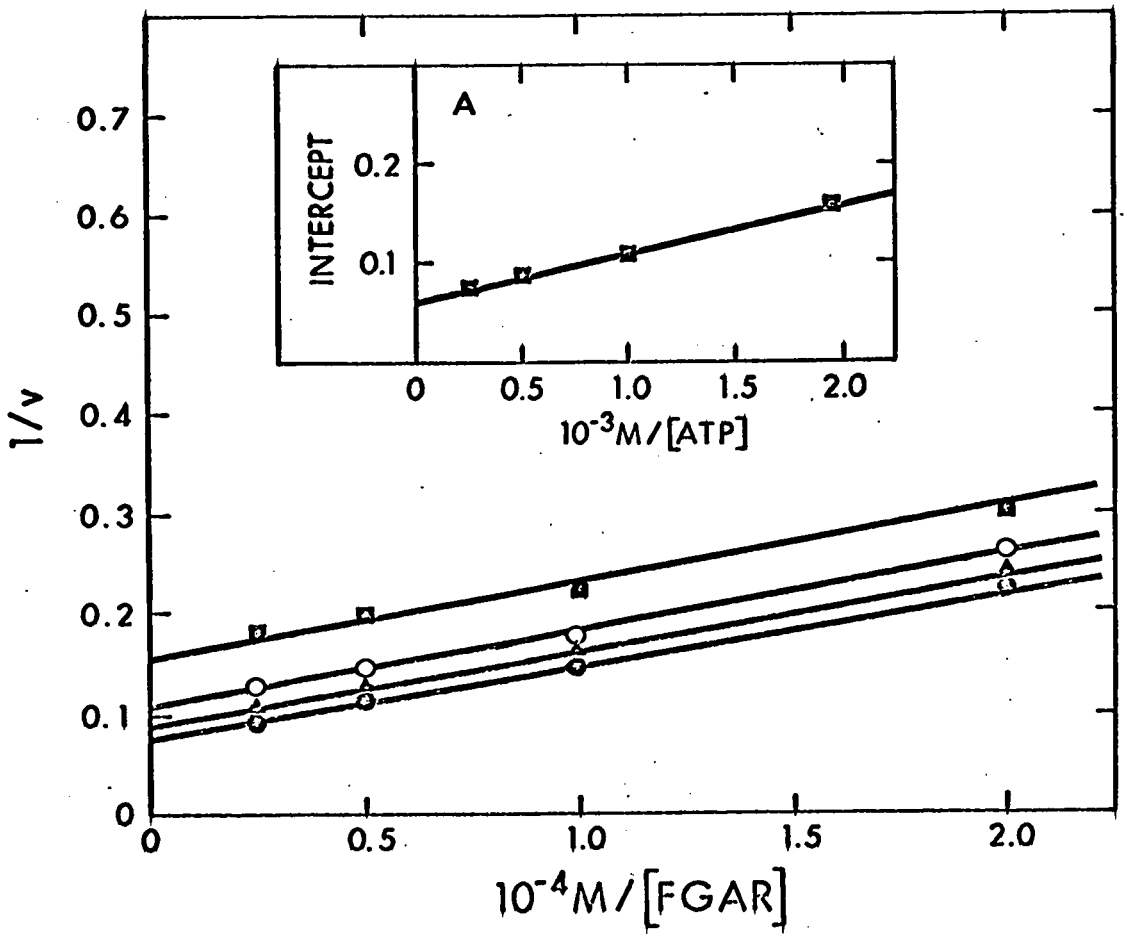


Figure 18. Double reciprocal plot of initial velocity against FGAR concentration. The concentrations of ATP and glutamine, respectively, were: 5×10^{-4} M and 8.3×10^{-5} M (□); 1.0×10^{-3} M and 1.66×10^{-4} M (○); 2.0×10^{-3} M and 3.32×10^{-4} M (▲); 4.0×10^{-3} M and 6.64×10^{-4} M (●). Enzyme amount: 0.370 mg.

Insert: Replot of intercepts against the reciprocal of ATP concentration.

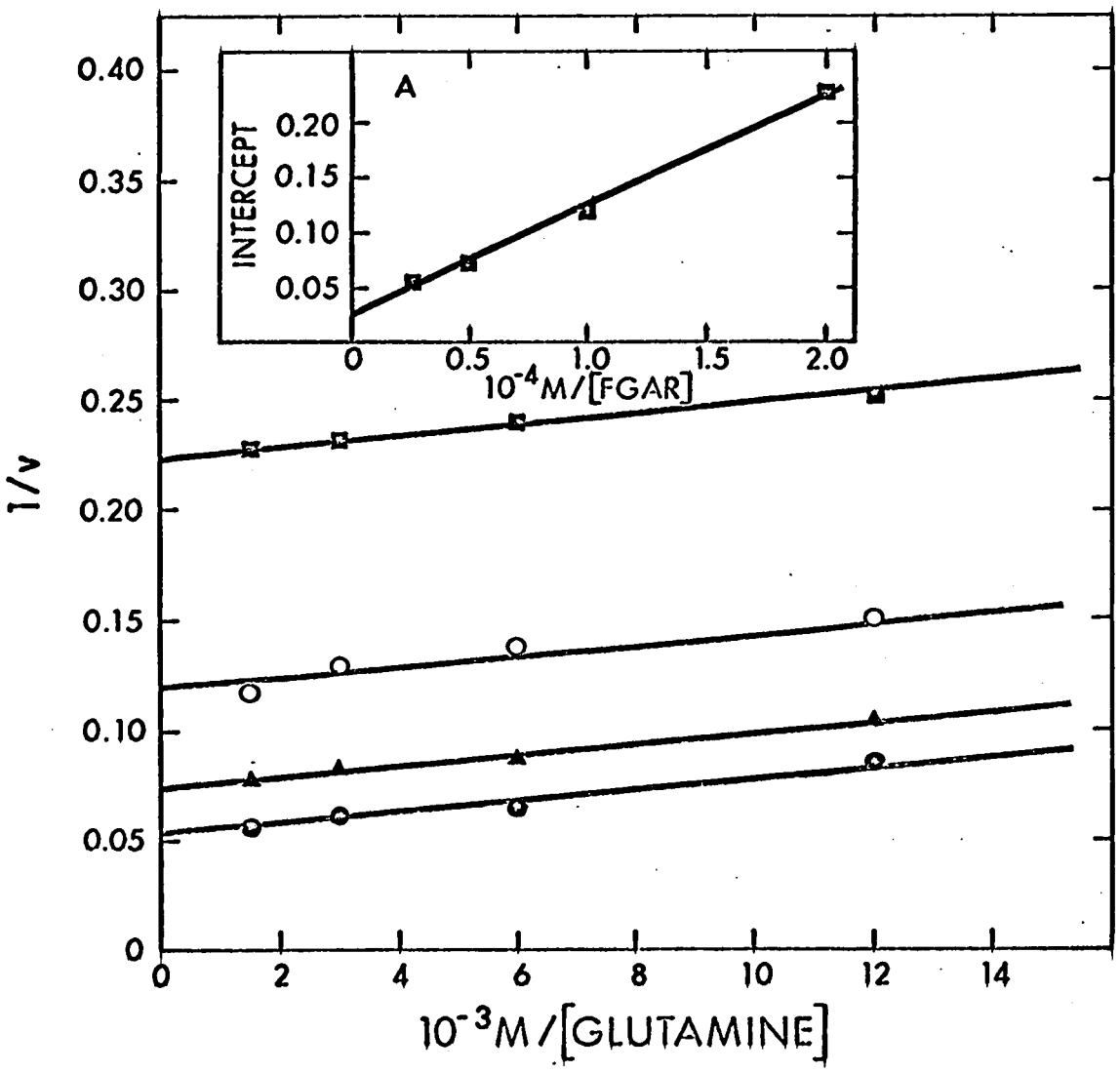


Figure 19. Double reciprocal plot of initial velocity against glutamine concentration. The concentrations of FGAR and ATP, respectively, were: 5×10^{-5} M and 5×10^{-4} M (▣); 1×10^{-4} M and 1×10^{-3} M (○); 2×10^{-4} M and 2×10^{-3} M (▲); 4×10^{-4} M and 4×10^{-3} M (●). Enzyme amount 0.82 mg.

Insert: Replot of intercepts against the reciprocal of FGAR concentration.

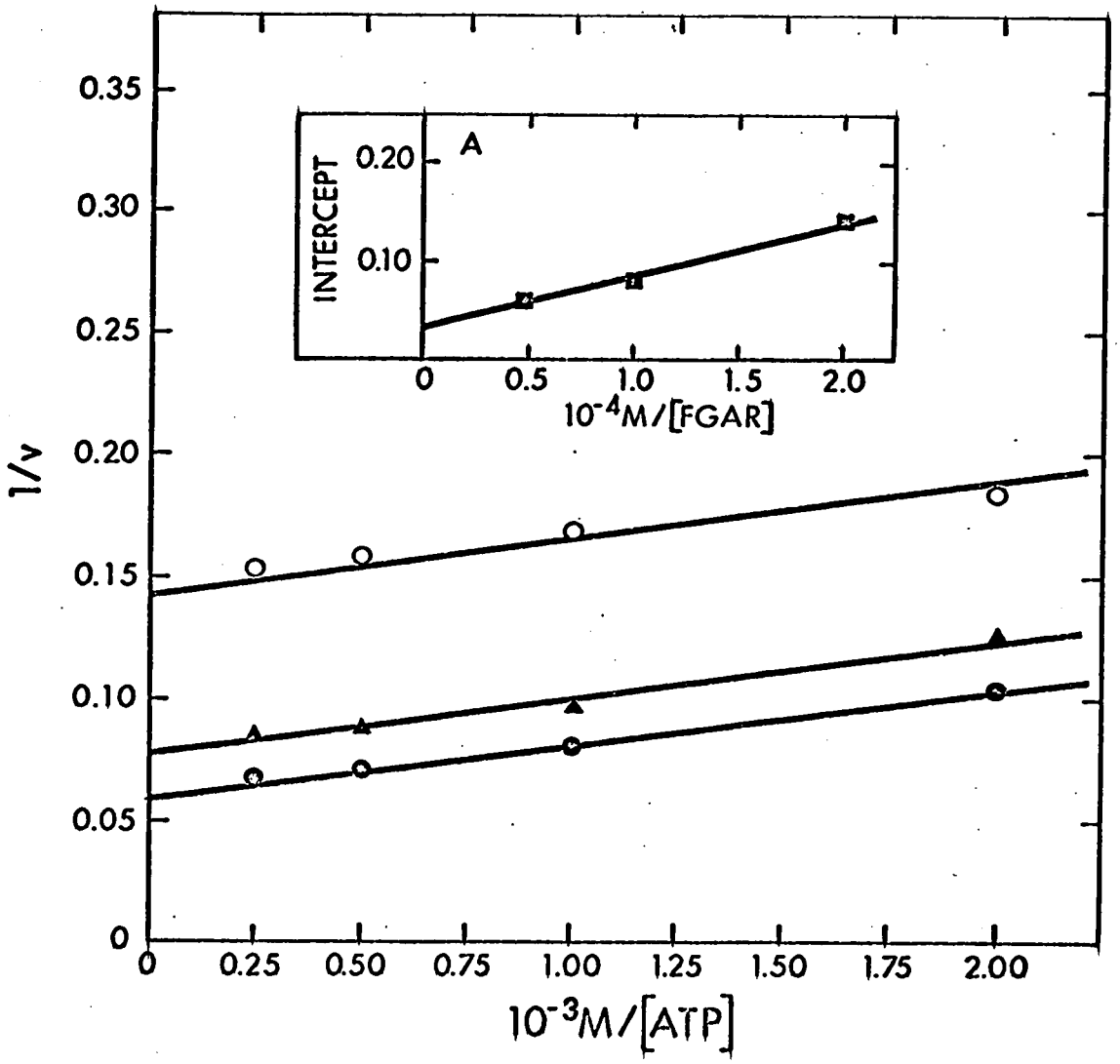


Figure 20. Double reciprocal plot of initial velocity against ATP concentration. Glutamine concentration was held at constant level of 1.4×10^{-4} M. The concentrations of FGAR were: 1×10^{-4} M (O); 2×10^{-4} M (▲); 4×10^{-4} M (●). Enzyme amount: 1.06 mg.

Insert: Replot of intercepts against the reciprocal of FGAR concentration.

Figures 21 and 22 intersect to the left of the 1/v axis when ammonium chloride and ATP were used as the varied substrates, respectively. A parallel double reciprocal plot was obtained, however, when FGAR was used as the varied substrate (Figure 23). These results suggest a partially ping-pong mechanism for the reaction; again sequential mechanisms were eliminated.

When glutamine was used as the nitrogen donor, the experimental data fit a steady state rate equation of the form:

$$\frac{E_o}{v} = \psi_o + \frac{\psi_A}{A} + \frac{\psi_B}{B} + \frac{\psi_C}{C}$$

where A, B, C are the substrates and $\psi_o, \psi_A, \ldots$ are the kinetic constants.

When ammonium chloride was used, the data obtained fit a steady-state rate equation of the form:

$$\frac{E_o}{v} = \psi_o + \frac{\psi_A}{A} + \frac{\psi_B}{B} + \frac{\psi_C}{C} + \frac{\psi_{AB}}{AB}$$

The Michaelis constant (K_m) for a substrate is calculated from $\frac{\psi_n}{\psi_o}$ where n = A, B, or C. $\frac{\psi_o}{E_o}$ can be obtained from a replot of intercepts, while $\frac{\psi_n}{E_o}$ can be obtained from the slope or replot of slopes of any particular variable substrate. The maximum velocity is $\frac{1}{\psi_o}$ at unit enzyme concentration.

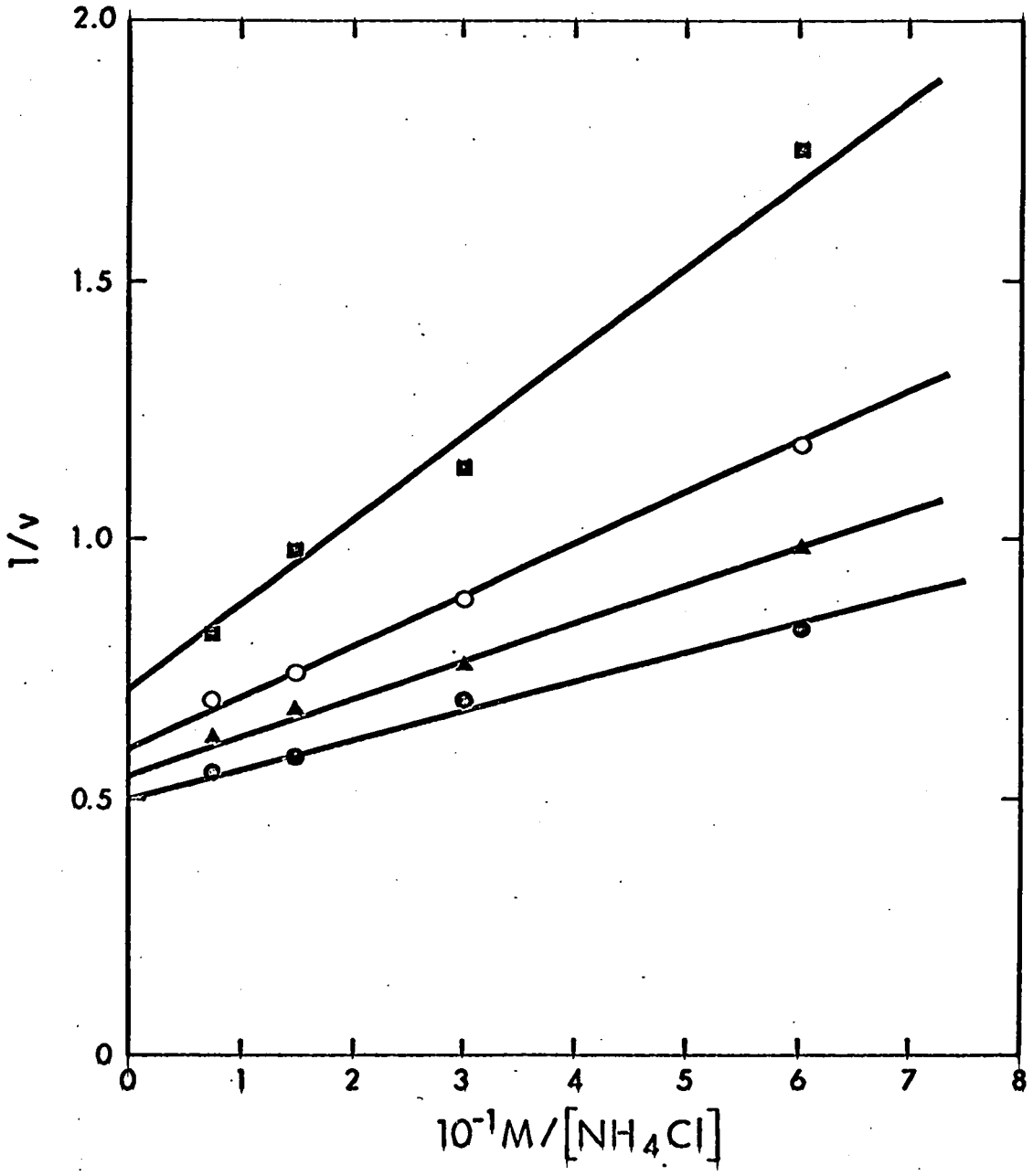


Figure 21. Double reciprocal plot of initial velocity against NH_4Cl concentration. The concentrations of FGAR and ATP, respectively, were: 5×10^{-5} M and 5×10^{-4} M (■); 1×10^{-4} M and 1×10^{-3} M (O); 2×10^{-4} M and 2×10^{-3} M (▲); 4×10^{-4} M and 4×10^{-3} M (●). Enzyme amount: 0.86 mg.

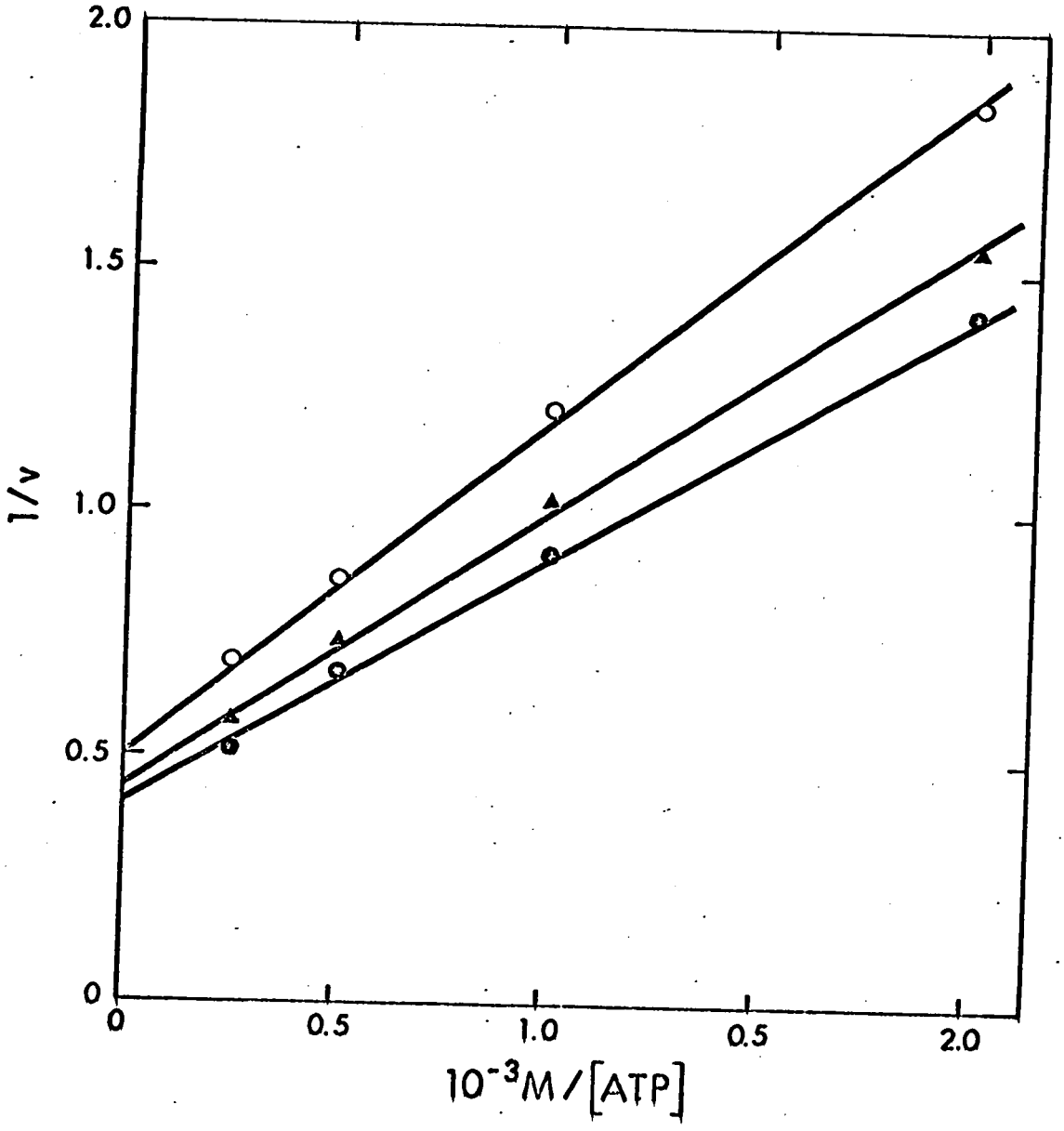


Figure 22. Double reciprocal plot of initial velocity against ATP concentration. The concentrations of FGAR and NH_4Cl, respectively, were: 1×10^{-4} M and 3.32×10^{-2} M (O); 2×10^{-4} M and 6.64×10^{-2} M (▲); 4×10^{-4} M and 1.328×10^{-1} M (⊙). Enzyme amount: 1.24 mg.

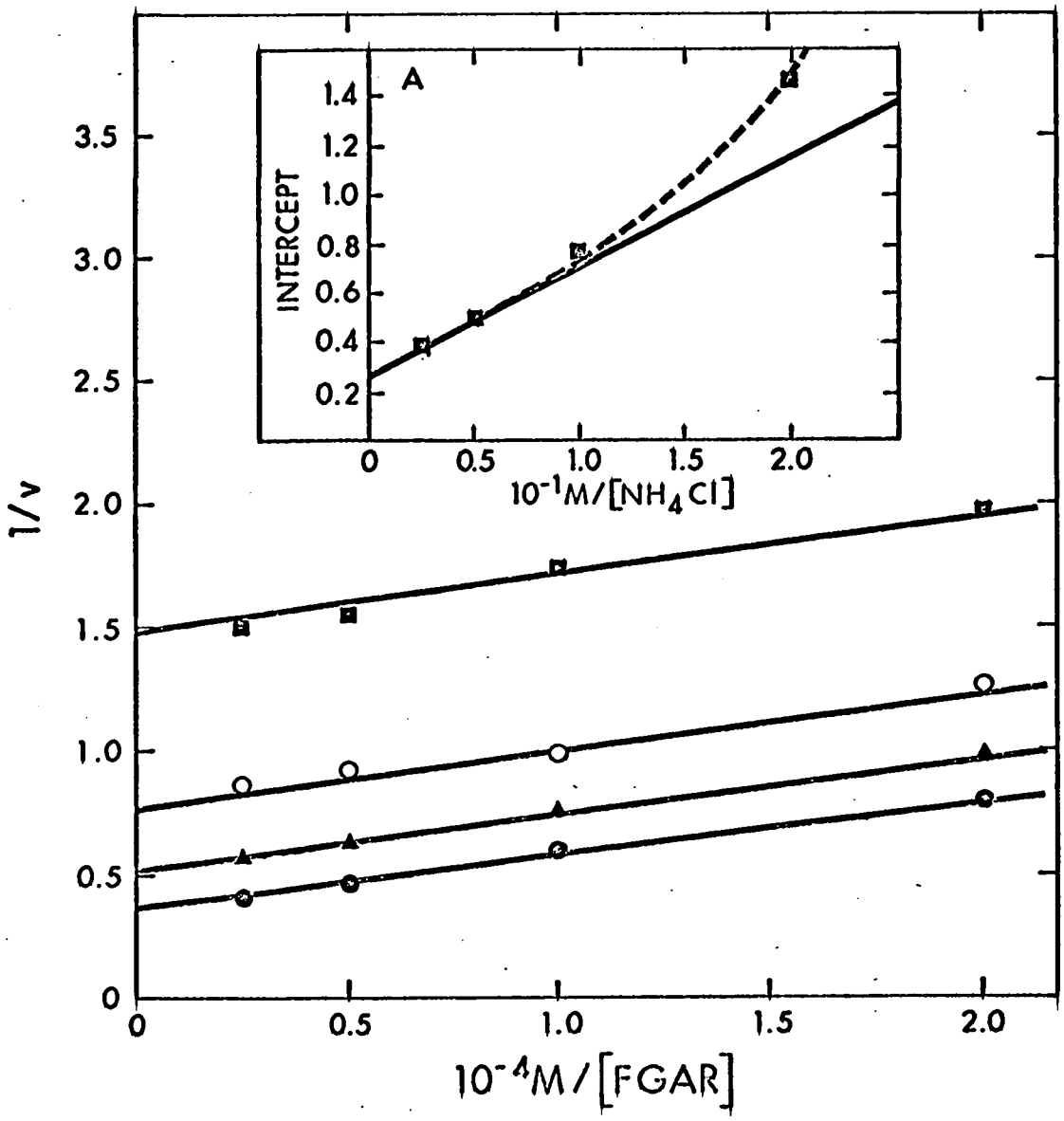


Figure 23. Double reciprocal plot of initial velocity against FGAR concentration. The concentrations of ATP and NH_4Cl, respectively, were: 5×10^{-4} M and 1.66×10^{-2} M (■); 1×10^{-3} M and 3.32×10^{-2} M (O); 2×10^{-3} M and 6.64×10^{-2} M (▲); 4×10^{-3} M and 1.320×10^{-1} M (●). Enzyme amount: 1.64 mg.

Insert: Replot of intercepts against the reciprocal of NH_4Cl concentration.

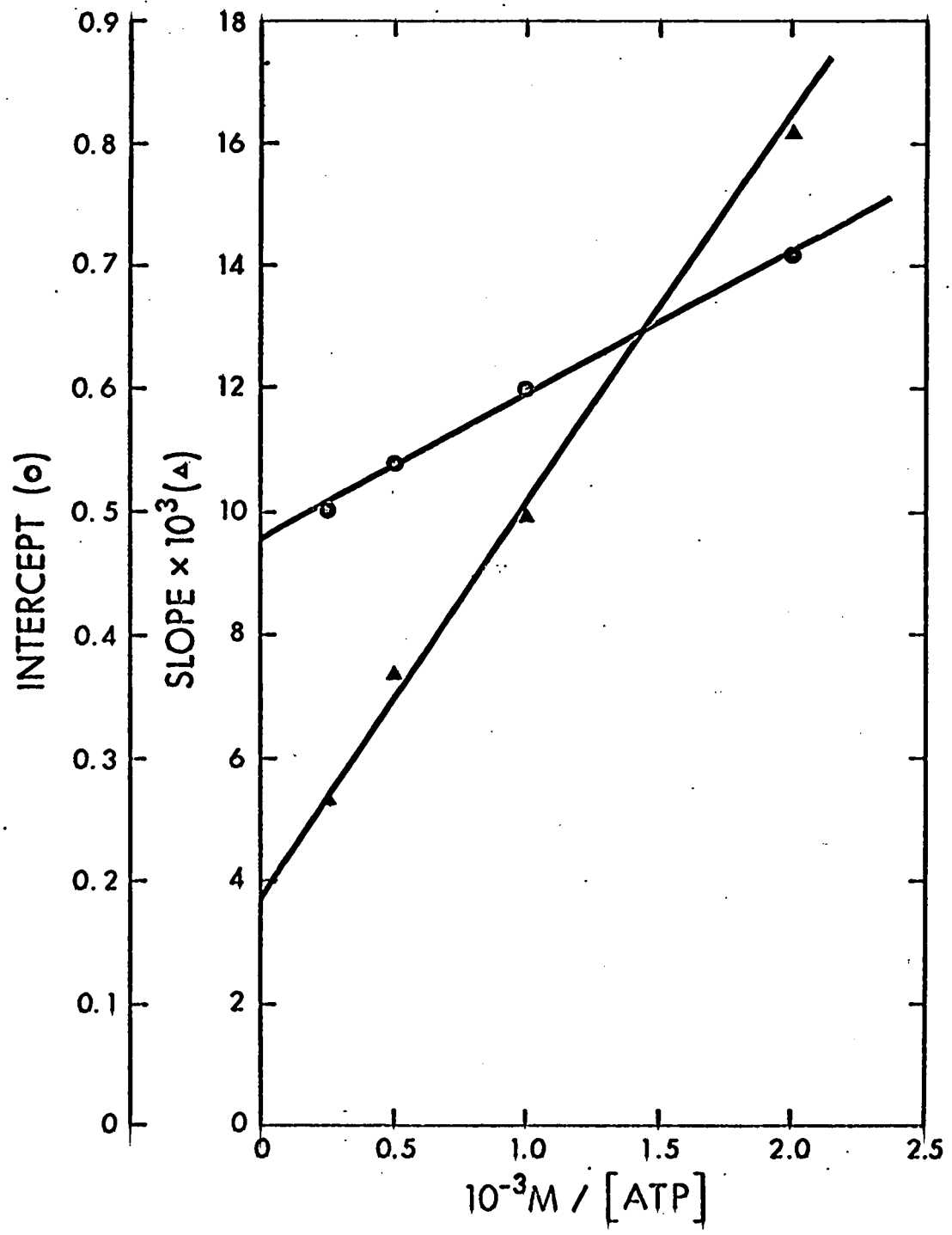


Figure 24. Replot of intercepts (O) and slopes (▲) from Figure 21 against the reciprocal of ATP concentration.

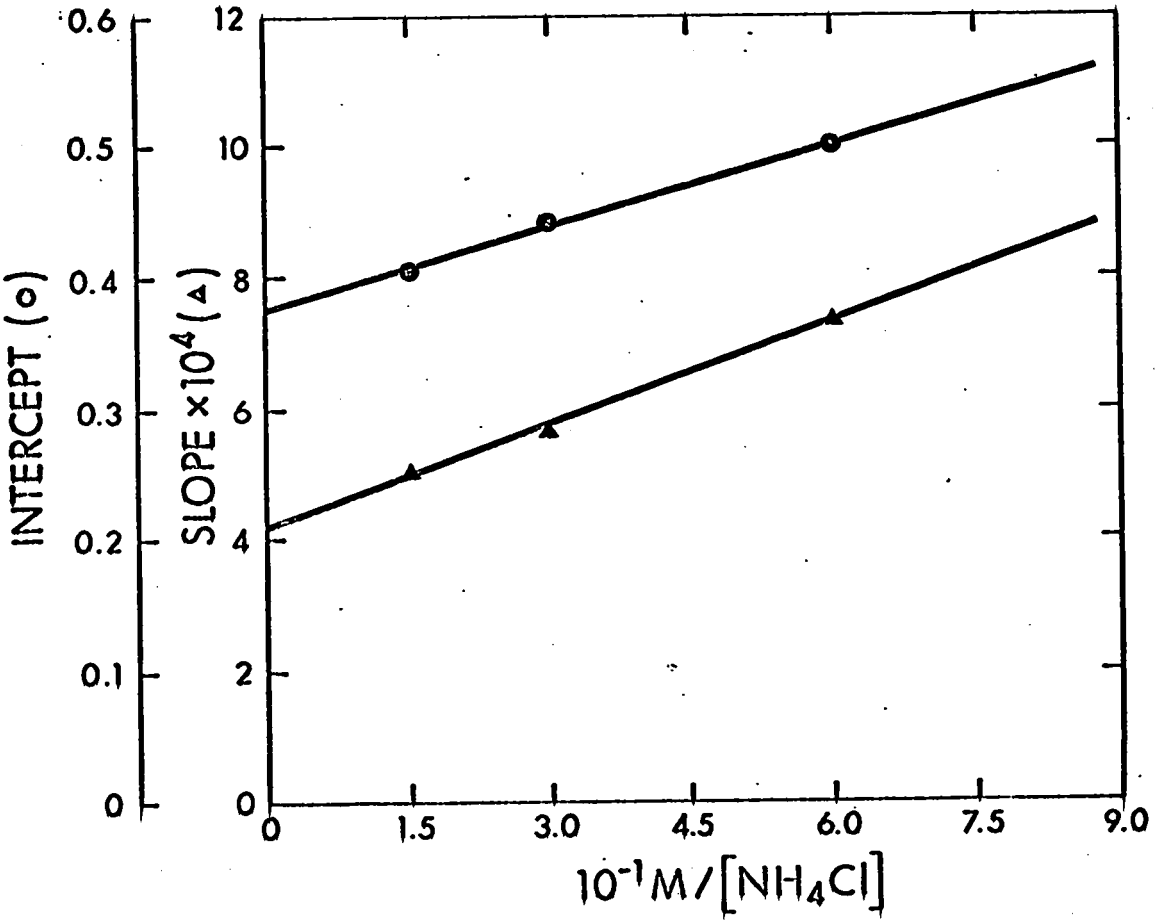


Figure 25. Replot of intercept (⊙) and slope (▲) from Figure 22 against the reciprocal of NH_4Cl concentration.

In the system in which glutamine was used as the nitrogen donor, the K_m values were calculated to be: L-glutamine, 1.10×10^{-4} M; ATP, 1.50×10^{-3} M; and FGAR, 1.11×10^{-4} M. V_{max} values ranged from 1.43 to 1.67 millimicromoles of FGAM formed per min per milligram of protein.

When ammonium chloride was used as nitrogen donor, the K_m values were calculated to be: ammonium chloride, 7.5×10^{-3} M; ATP, 1.12×10^{-3} M; and FGAR 1.00×10^{-4} M. V_{max} values ranged from 37 to 41 micromicromoles of FGAM formed per min per milligram of protein.

D. Discussion

The data obtained from initial rate studies of the FGAM synthetase reaction (in the case when glutamine is the nitrogen donor) are consistent with a kinetic mechanism. which involves only binary enzyme-substrate complexes. These data are, however, not compatible with linear mechanisms in which any two or all three substrates are bound to the enzyme simultaneously (113-115).

Little information was obtained concerning the order of addition of the substrates. However, in the case when ammonium chloride was used as the nitrogen donor, FGAR must be the last substrate which adds to the enzyme because only when FGAR was varied, was a non-intersecting plot (Figure 23) obtained (Table 12). Although a non-intersecting double reciprocal plot could also be obtained when the first

substrate was varied in a three-substrate partially ping-pong mechanism (in the case where addition of B and C is ordered (Table 12), FGAR could not be the first substrate in this reaction because FGAR would have to be converted to a product; since ATP and NH_4^+/glutamine would not have yet added to the enzyme, this would be impossible. The observation that glutamine protects the enzyme against heat inactivation provides indirect evidence that glutamine is the first substrate to bind.

The replotting technique of Rudolph and Fromm (111) was applied here. In general, sequential addition of substrates gave more than one non-linear intercept or slope replots using the above experimental procedure. In this study, only one non-linear replot (Figure 23) was obtained. In the case of a partially ping-pong mechanism, non-linearity can only be found in the intercept replot while the reciprocal plot is parallel. This was indeed the case. An experiment was also carried out by the ordinary method in which ATP and FGAR were varied at a fixed low (Michaelis) concentration of glutamine (Figure 20), and the results indicated a ping-pong mechanism since non-intersecting reciprocal plots were obtained. These results served to verify the usefulness of the experimental protocol outlined by Fromm (109) for initial rate studies for three substrate reactions.

The K_m value for glutamine is much smaller than the K_m value for ammonium chloride. However, under the experimental

conditions, the pH of the reaction mixture was 8.0 and the free ammonia concentration at this pH was about 5% of the total concentration of ammonium chloride. Free ammonia rather than ammonium chloride might well be the true substrate under these experimental conditions. Taking this fact into consideration, the K_m for ammonia was calculated to be 4.0×10^{-4} M which is within the same range as the K_m for glutamine.

E. Summary

The kinetic mechanism of the action of FGAM synthetase from Ehrlich ascites carcinoma cells was investigated by initial velocity studies. In the case when glutamine was used as the nitrogen donating substrate, the data obtained are consistent with a fully ping-pong mechanism. Whereas if ammonium chloride is used as the nitrogen donating substrate in place of glutamine, a partially ping-pong mechanism was suggested. The K_m values for glutamine, ATP, and FGAR are 1.10×10^{-4} M, 1.5×10^{-3} M and 1.11×10^{-4} M, respectively. These values are comparable to those obtained using ammonium chloride as the nitrogen donating substrate where the K_m values for ATP and FGAR are 1.12×10^{-3} M and 1.00×10^{-4} M, respectively. The K_m value for ammonium chloride is 7.5×10^{-3} M. The ratio of V_{max} values towards ammonium chloride and glutamine is 1/40. Free ammonia rather than ammonium ion might be the true substrate under these experimental conditions.

VI. KINETIC MECHANISM OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE. II. PRODUCT INHIBITION STUDIES

A. Introduction

Kinetic evidence was described above which suggested a fully ping-pong mechanism for the Ehrlich ascites tumor cell FGAM synthetase (Chapter V). Based also on initial rate studies using ammonium chloride as nitrogen donor, and substrate protection studies against heat denaturation of the enzyme, a reaction scheme was proposed in which glutamine binds to the free enzyme while FGAR was the last substrate involved in the reaction.

Further elucidation of the kinetic mechanism of this enzymic reaction was carried out by product inhibition studies as reported in this Chapter. The use of product inhibition studies to distinguish different mechanisms has long been known to be a useful tool in studying the mechanisms of two substrate systems (115, 116). In Chapter IV, a number of product and dead-end inhibition patterns have been presented. The data obtained from these studies support the ping-pong mechanism as proposed in Chapter V. The order of addition and release of substrates and products was defined.

B. Materials and methods

1. Chemicals

L-Glutamine-^{14}C with a specific activity of 219 mCi/mM was purchased from New England Nuclear Corporation, Boston,

Mass., and was diluted to give the specific activity indicated in the Figure legends. Other materials and the enzyme preparation were the same as described in previous chapters.

2. Enzyme assay

Enzyme activity was assayed by the two-stage incubation procedure described in Chapter III. When the product FGAM was used as inhibitor, the enzyme preparation was incubated at 37° with ATP, FGAR, glutamine-^{14}C, 33.3 mM Tris-HCl buffer pH 8.0, 10.0 mM $MgCl_2$ and 20 mM KCl in a final volume of 0.3 ml. After 30 min, the reaction was stopped by addition of 0.02 ml of 20% TCA, held in an ice bath for 2-3 min and then neutralized by the addition of 0.01 ml of 2N KOH. After centrifuging the solution for 3 min in a clinical centrifuge, 0.02 ml aliquots of the supernatant were chromatographed on Whatman 3 MM paper together with 5 µl of a carrier solution consisting of 10 mM glutamine and 10 mM glutamate. Descending chromatography was carried out in isopropanol-formic acid-water (40:2:10 by volume). Glutamate (R_f 0.47) was detected with ninhydrin; these spots were cut out and transferred to counting vials containing 18 ml of toluene scintillation phosphor solution (toluene containing 4.0 gm of 2,5-diphenyloxazole and 100 mg of 1,4-di[2-(5-phenyloxazolyl)]benzene per litre) and the radioactivity was measured in a Nuclear Chicago Liquid Scintillation counter.

Product inhibition studies were carried out by varying the concentrations of one substrate at several fixed levels of one of the products while the concentrations of the other two substrates were held constant. Velocity is expressed as millimicromoles of FGAM formed under the assay condition except in the case when FGAM was the product inhibitor. In this case the velocity was expressed as the counts per min of glutamate produced under the assay conditions.

3. Synthesis of FGAM

FGAM was synthesized enzymatically from FGAR by the procedure described by Lukens and Flaks (78), with the following modifications. The incubation mixture contained, in a final volume of 10 ml, the following: 20 μmoles of FGAR, 200 μmoles of ATP, 20 μmoles of L-glutamine, 200 μmoles of $MgCl_2$, 500 μmoles of Tris-HCl buffer pH 8.0 and 500 μmoles of KCl together with 4 ml of the dialyzed 0-43% ammonium sulfate saturation fraction of pigeon liver extract (78). After incubating for 45 min at 37°, the reaction was stopped and deproteinized by adjusting the solution to pH 12-13 with KOH; it was then held in an ice bath for two min. After centrifugation, the supernatant was adjusted to pH 8.5-9.0 with N HCl. This solution was absorbed onto a column (1 x 15 cm) of Dowex-1-X8 in the formate form. The column was then washed with 100 ml of distilled water. Elution was carried out with 0.1 M ammonium formate buffer

pH 5.0. The early fractions contained FGAM which was monitored by its organic phosphate content. The identity of FGAM was confirmed by its reaction with AIR synthetase. These fractions were pooled and freeze-dried to yield the ammonium salt which was then converted to the lithium salt by the procedure described above for the preparation of FGAR. The product was free from Pi, glutamate or glutamine.

C. Results and discussion

In the analysis of inhibition patterns, Cleland's nomenclature was used (108). Thus inhibition will be called competitive, uncompetitive or non-competitive, respectively, when the presence of product alters the slope, the intercept or both slope and intercept in double reciprocal plots of velocity versus concentration of the variable substrate.

In the presence of the product glutamate, the inhibition patterns were as shown in Figures 26-28. These are non-competitive, competitive and uncompetitive, respectively, when glutamine, ATP and FGAR were the variable substrates. A competitive product inhibition can only result if the product combines with the same enzyme form as the substrate. In the present case, the competitive plot implies either that glutamate is released just before the addition of ATP or that glutamate is the last product of the reaction while ATP is the first substrate. If the latter possibility is true, we should however expect an uncompetitive inhibition

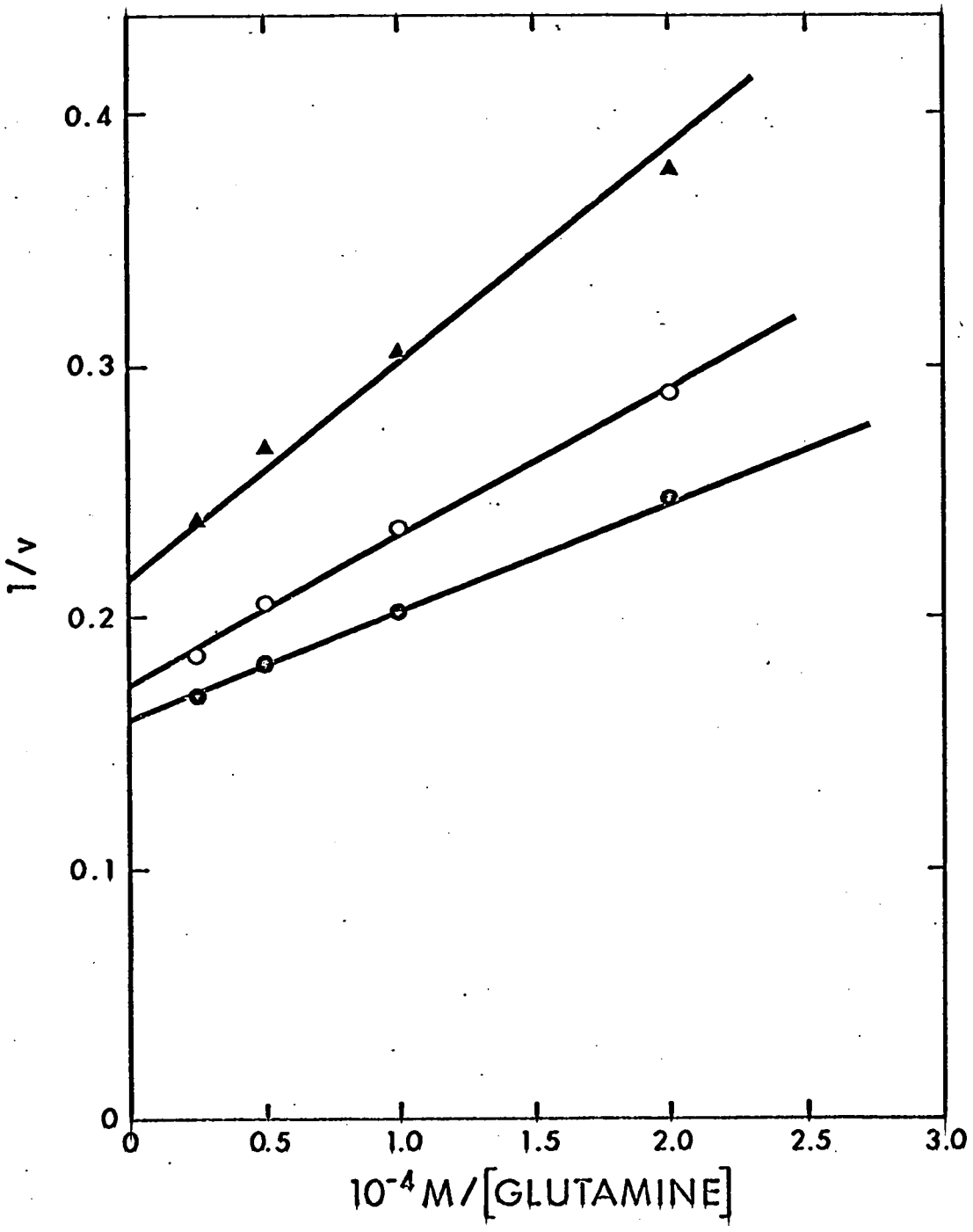


Figure 26. Product inhibition by glutamate. Double reciprocal plot of initial velocity against glutamine concentration at a FGAR concentration of 4×10^{-4} M and an ATP concentration of 4×10^{-3} M. Glutamate concentrations were: 0 (●); 5×10^{-3} M (○); and 2.5×10^{-2} M (▲).

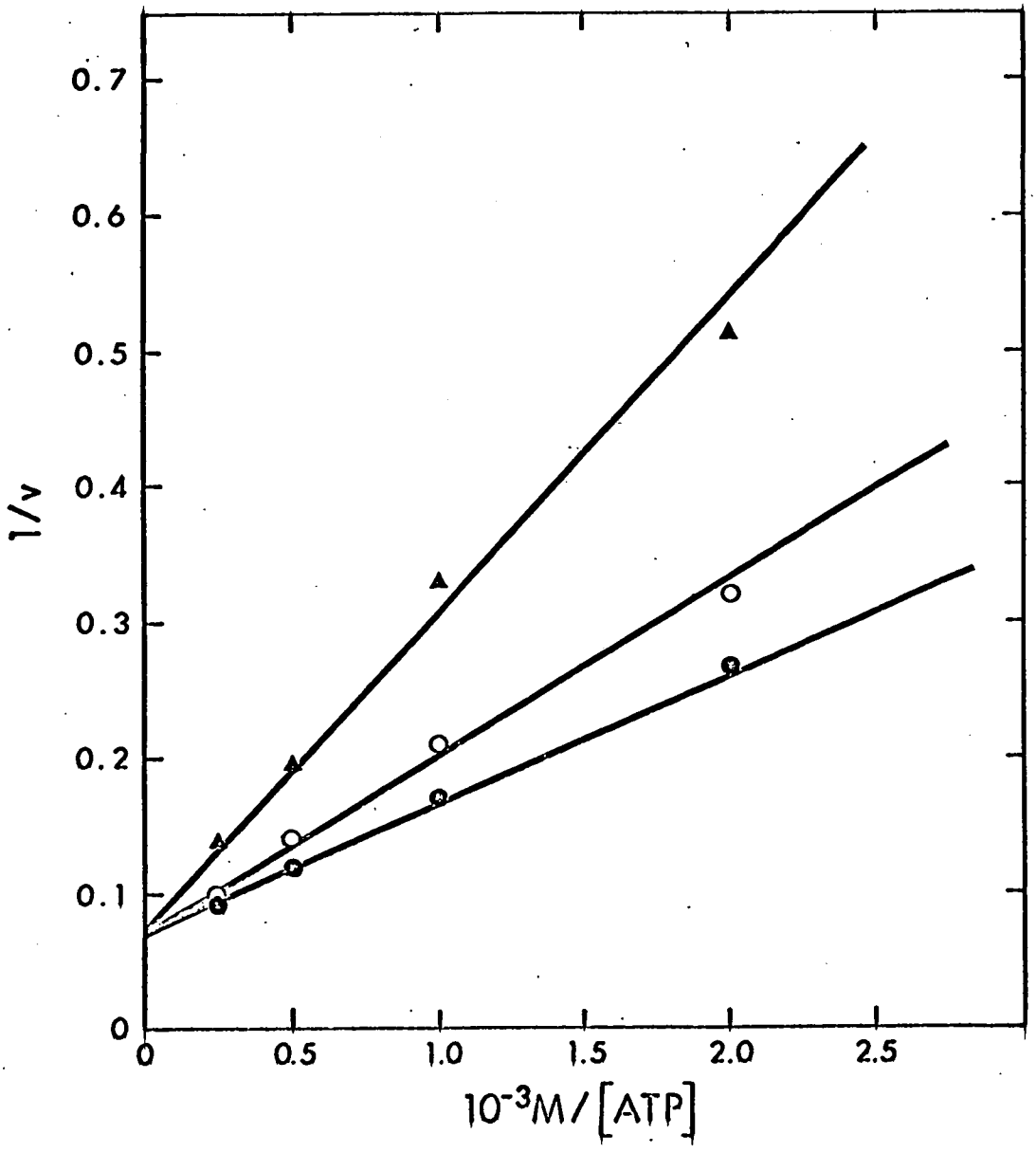


Figure 27. Product inhibition by glutamate. Double reciprocal plot of initial velocity against ATP concentration at a FGAR concentration of 4×10^{-4} M and a glutamine concentration of 4×10^{-4} M. Glutamate concentrations were: 0 (⊙); 5×10^{-3} M (O); and 2.5×10^{-2} M (Δ).

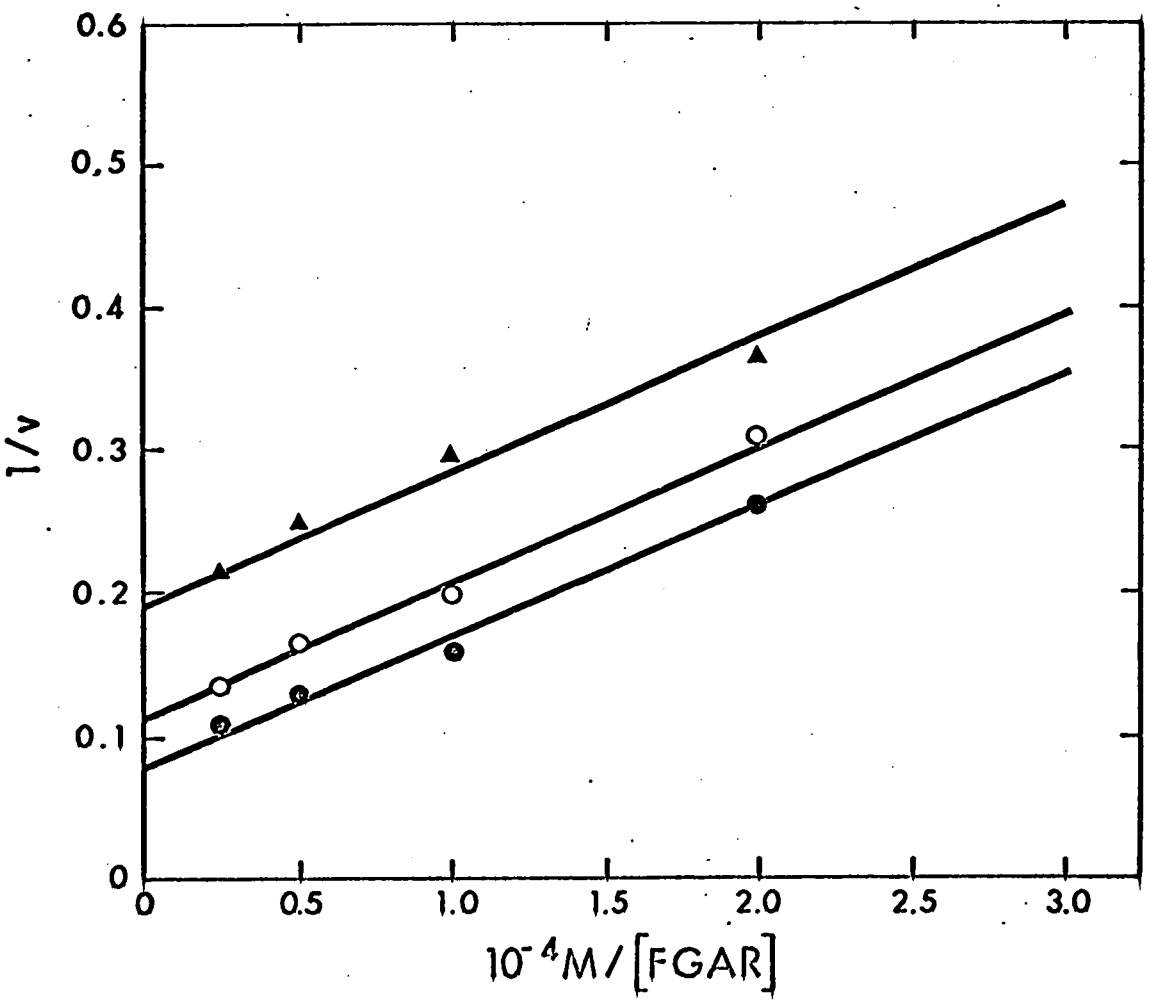
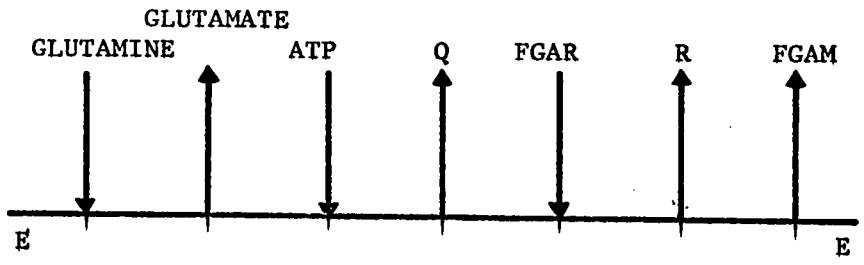
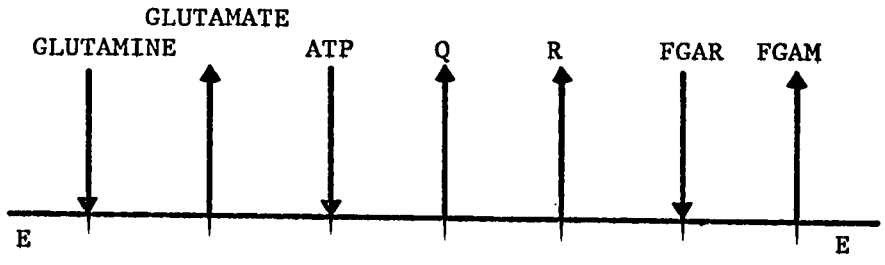


Figure 28. Product inhibition by glutamate. Double reciprocal plot of initial velocity against FGAR concentration at an ATP concentration of 4×10^{-3} M and a glutamine concentration of 4×10^{-4} M. Glutamate concentrations were: 0 (●); 5×10^{-3} M (○); and 2.5×10^{-2} M (▲).

pattern by glutamate with respect to glutamine. Furthermore, FGAM cannot be released before the addition of glutamine. The only alternative is that glutamine binds with the free enzyme and that glutamate is released before the addition of the second substrate, ATP. Initial rate studies and substrate protection studies against enzyme inactivation by heat have also suggested that glutamine is the first substrate of the reaction. Product inhibition by FGAM with respect to glutamine is competitive (Figure 32) and this result confirms that glutamine is the first substrate in this reaction. According to the product inhibition patterns for three-substrate four-product systems shown below (Appendix), only two out of the ten possible mechanisms would be consistent with the above data. They are both of the fully ping-pong type and are represented as follows:



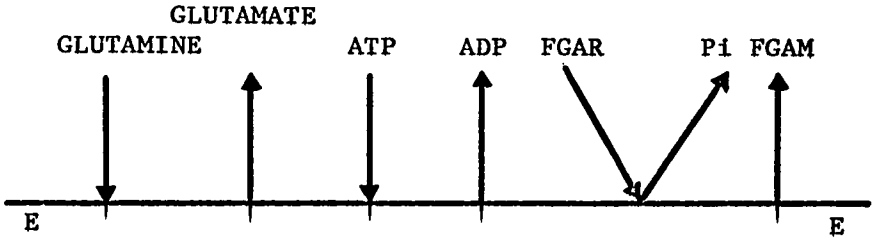
Mechanism 4-I



Mechanism 4-II

where Q and R are either ADP or Pi.

Inhibition by Pi gave competitive inhibition (Fig. 29-31) with respect to FGAR but uncompetitive inhibition with respect to ATP and glutamine. These data agree well with the patterns expected for Mechanism 4-II, in which Q is ADP and R is Pi. The competitive inhibition pattern obtained between Pi and FGAR was not consistent with Mechanism 4-I. However if both the conversion of the FGAR-ammonia-Pi-enzyme complex to the enzyme-product complex, and the release of Pi from this complex are rapid so that no kinetically significant substrate-enzyme was formed (as in the Theorell-Chance Mechanism for alcohol dehydrogenase (117), the competitive inhibition by Pi with respect to FGAR would be expected (108, 116). A fairly high concentration of Pi (20 mM) was required to give any appreciable amount of inhibition. The data obtained so far are consistent either with Mechanism 4-II or with the following mechanism:



Mechanism 4-III

Product inhibition by FGAM was uncompetitive with respect to both ATP and FGAR (Figures 33, 34). These data are consistent with Mechanism 4-III but not with Mechanism 4-II. ADP gave uncompetitive inhibition with respect to glutamine and non-competitive inhibition with respect to ATP (Figure 35, 36). These results are also consistent with Mechanism 4-III.

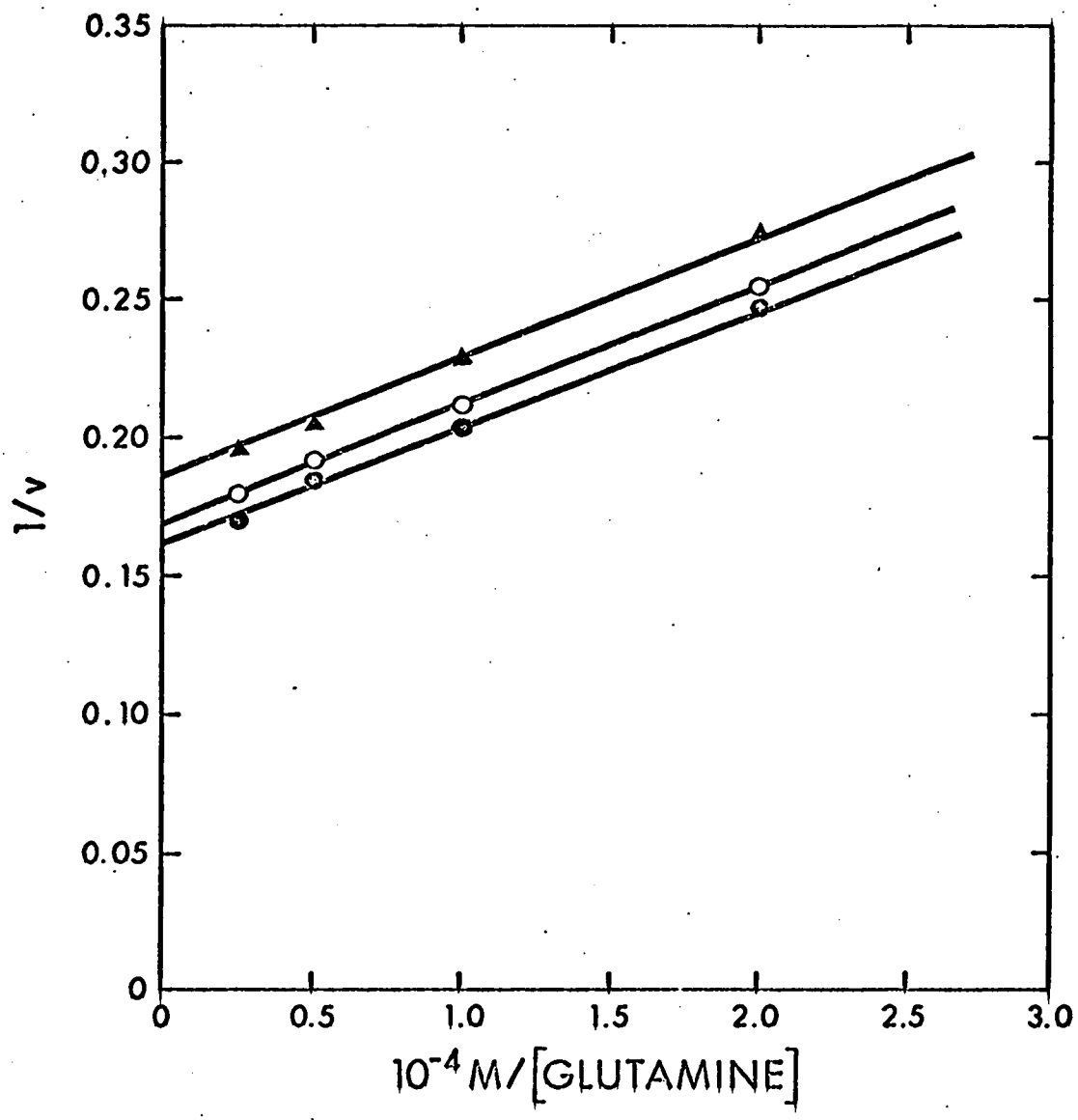


Figure 29. Product inhibition by phosphate. Double reciprocal plot of initial velocity against glutamine concentration at FGAR concentration of 4×10^{-4} M and an ATP concentration of 4×10^{-3} M. Phosphate concentrations were: 0 (●); 3.3×10^{-2} M (○); and 1.6×10^{-1} M (▲).

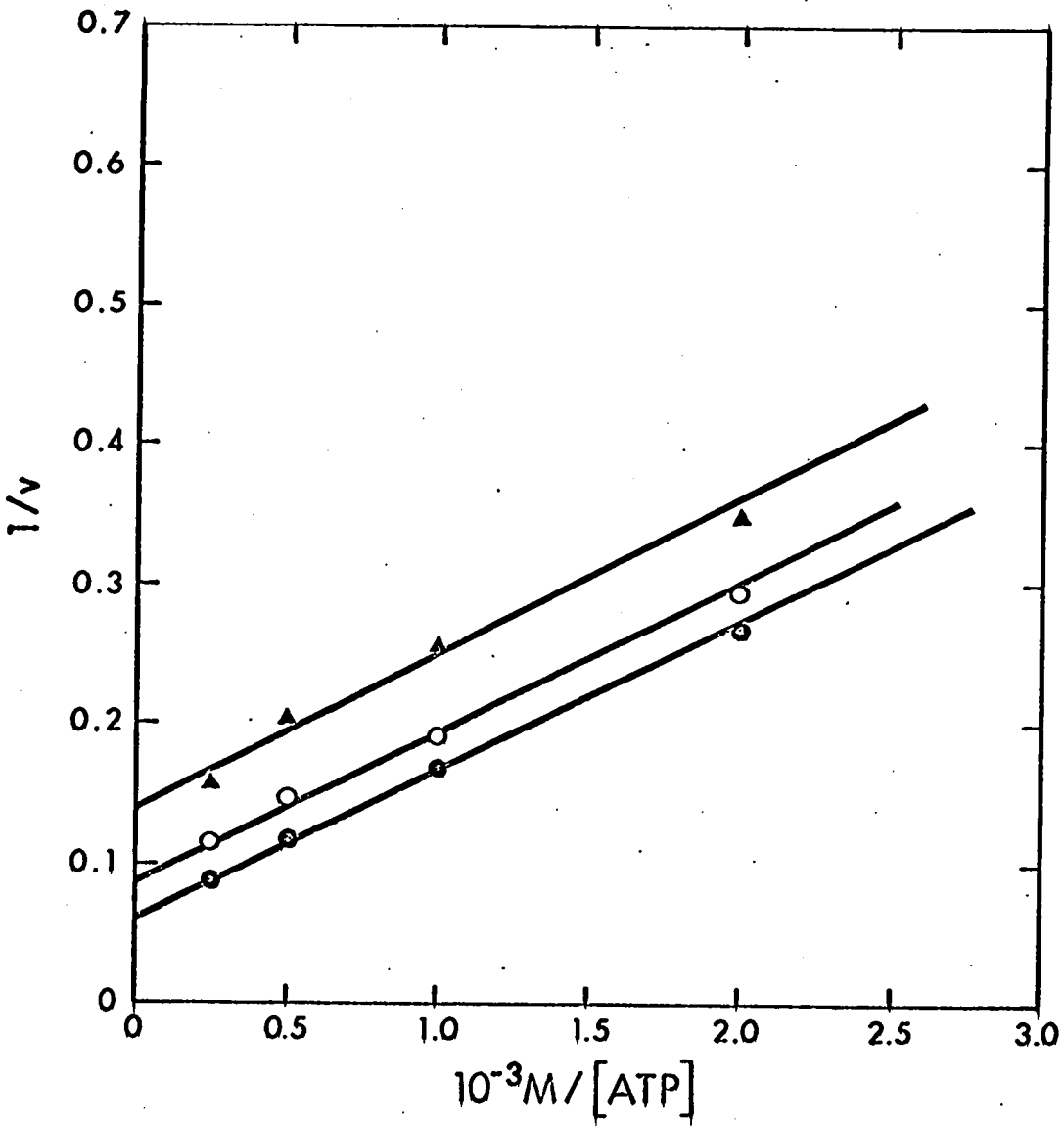


Figure 30. Product inhibition by phosphate. Double reciprocal plot of initial velocity against ATP concentration at a FGAR concentration of 4×10^{-4} M and a glutamine concentration of 4×10^{-4} M. Phosphate concentrations were: 0 (⊙); 2.2×10^{-2} M (○); and 5.5×10^{-2} M (▲).

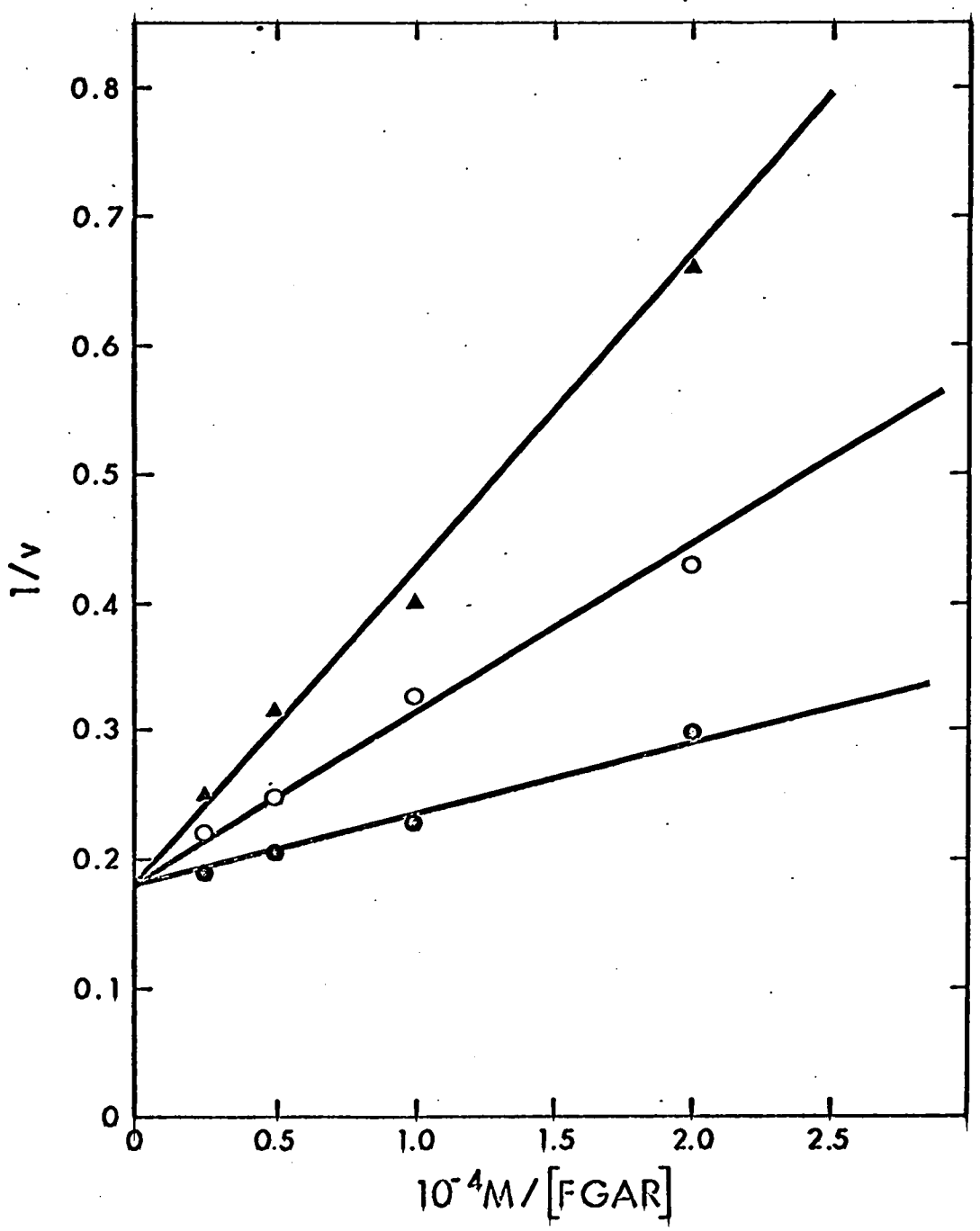


Figure 31. Product inhibition by phosphate. Double reciprocal plot of initial velocity against FGAR concentration at an ATP concentration of 4×10^{-3} M and a glutamine concentration of 4×10^{-4} M. Phosphate concentrations were: 0 (⊙); 2.2×10^{-2} M (○); and 5.5×10^{-2} M (▲).

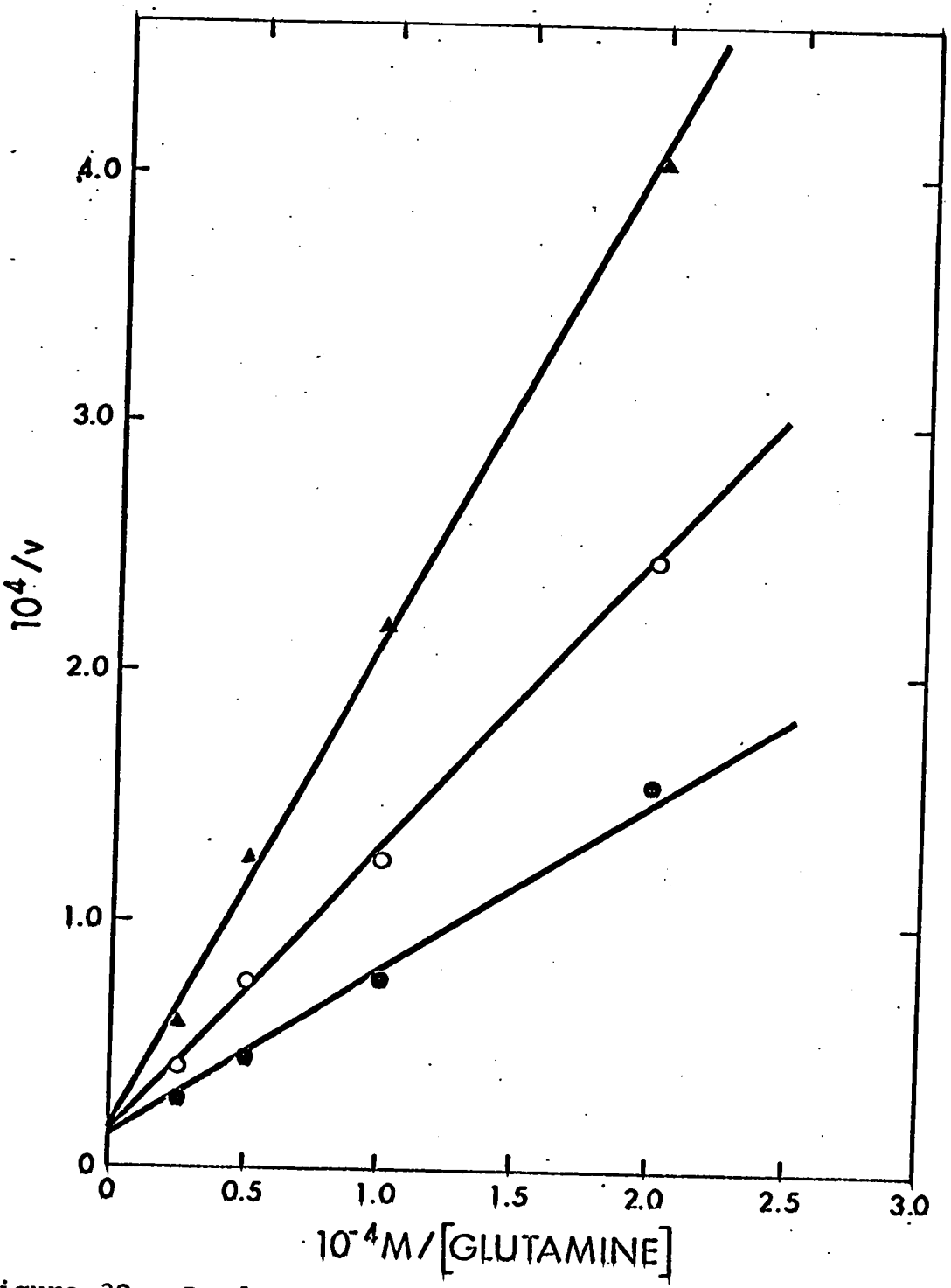


Figure 32. Product inhibition by FGAM. Double reciprocal plot of initial velocity against glutamine-^{14}C (6.1 mCi/mmole) at a FGAR concentration of 4×10^{-4} M and an ATP concentration of 4×10^{-3} M. FGAM concentrations were: 0 (●); 5.04×10^{-5} M (○); and 1.26×10^{-4} M (▲).

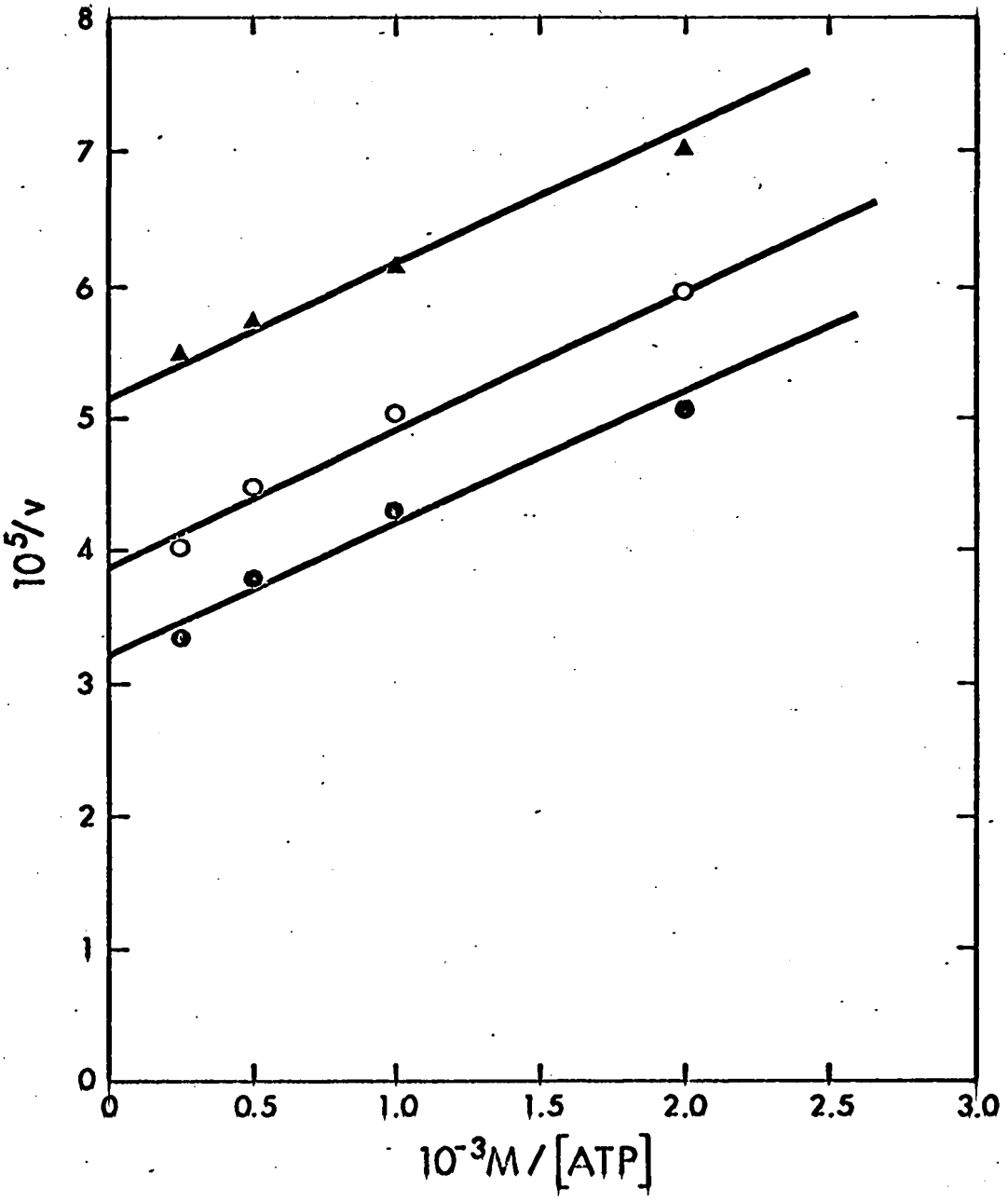


Figure 33. Product inhibition by FGAM. Double reciprocal plot of initial velocity against ATP concentration at a FGAR concentration of 4×10^{-4} M and a glutamine-^{14}C (6.1 mCi/mmole) concentration of 4×10^{-4} M. FGAM concentrations were: 0 (⊙); 5.04×10^{-5} M (O); and 1.26×10^{-4} M (▲).

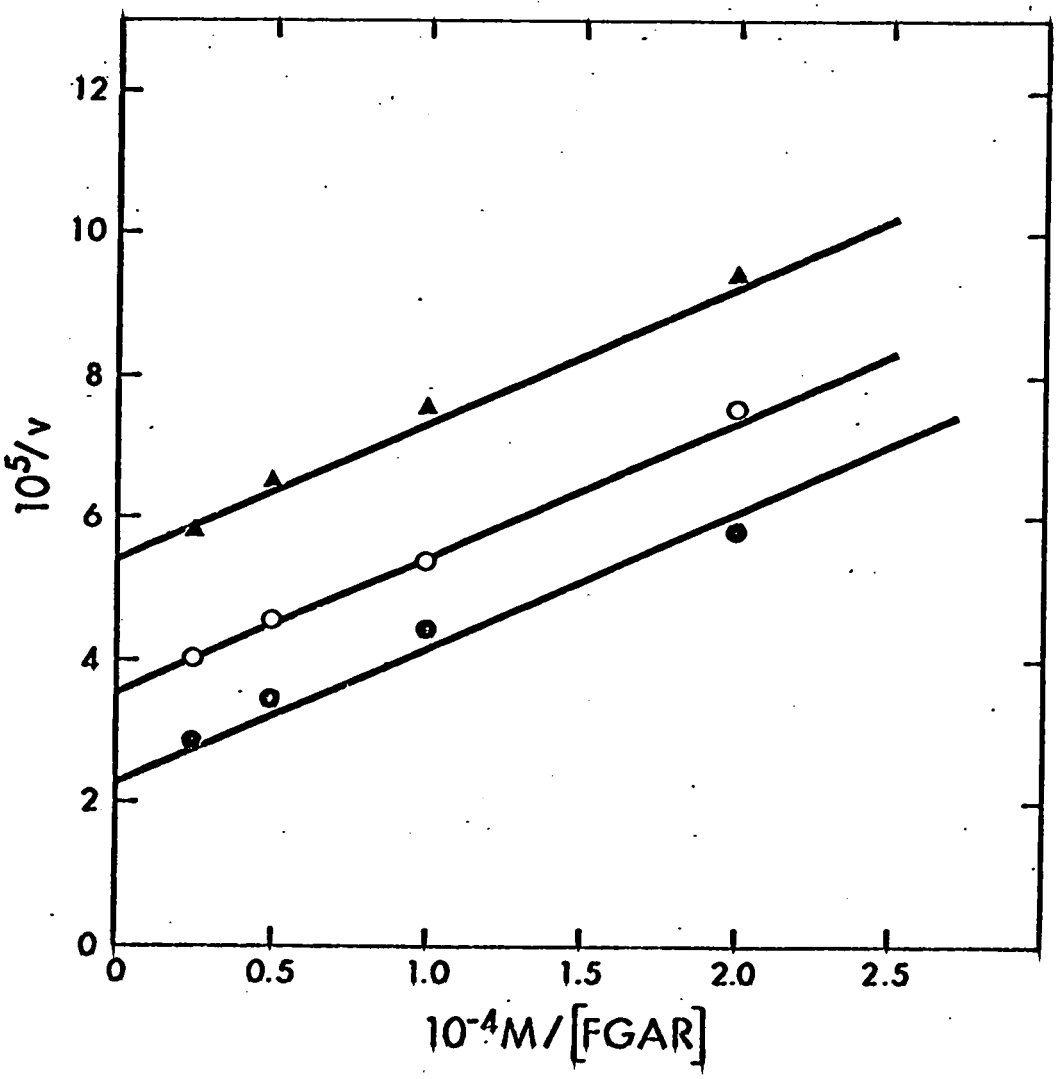


Figure 34. Product inhibition by FGAM. Double reciprocal plot of initial velocity against FGAR concentration at an ATP concentration of 4×10^{-3} M and a glutamine-^{14}C (6.1 mCi/m mole) concentration of 4×10^{-4} M. FGAM concentrations were: 0 (⊙); 5.04×10^{-5} M (O); and 1.26×10^{-4} M (▲).

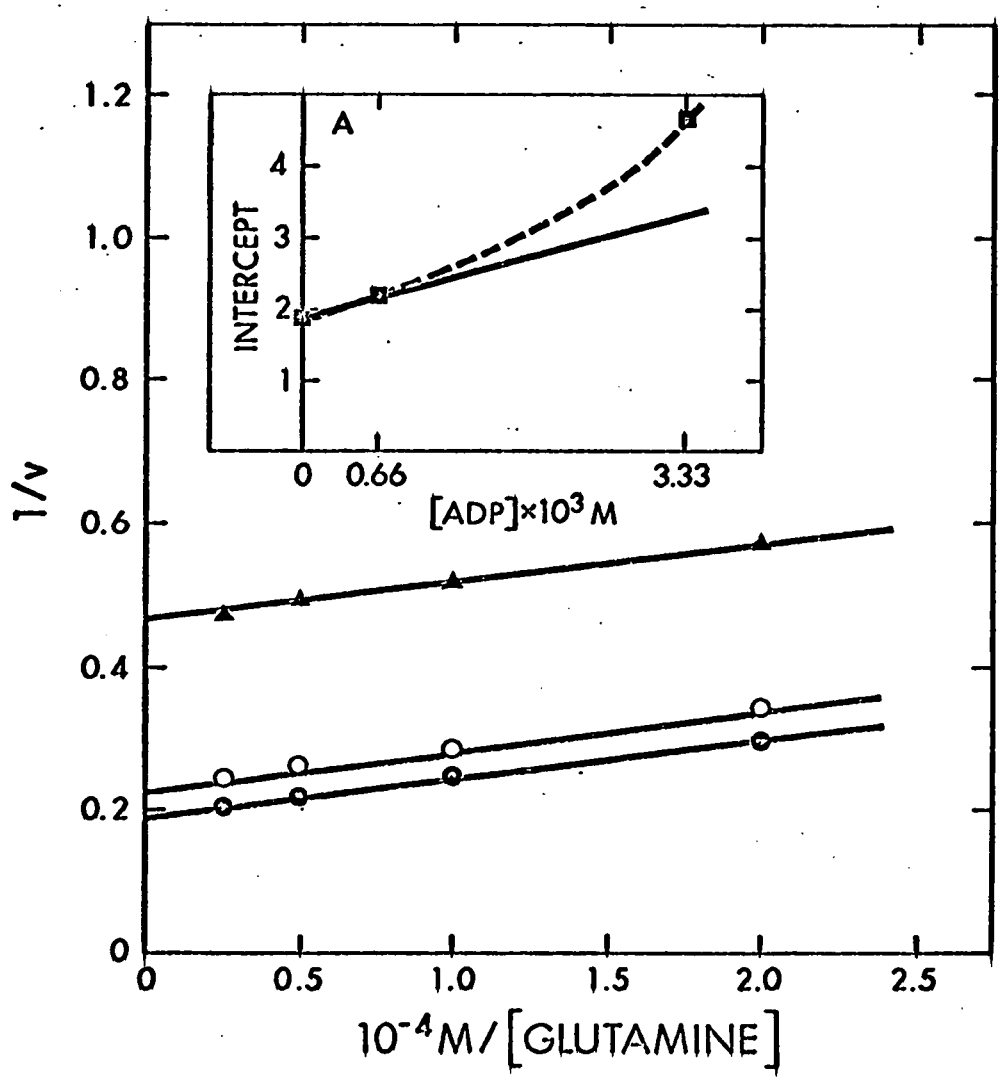


Figure 35. Product inhibition by ADP. Double reciprocal plot of initial velocity against glutamine concentration at a FGAR concentration of 4×10^{-4} M and an ATP concentration of 4×10^{-3} M. ADP concentrations were: 0 (●); 6.6×10^{-4} M (○); and 3.33×10^{-3} M (▲).

Insert: Replot of intercepts against ADP concentration.

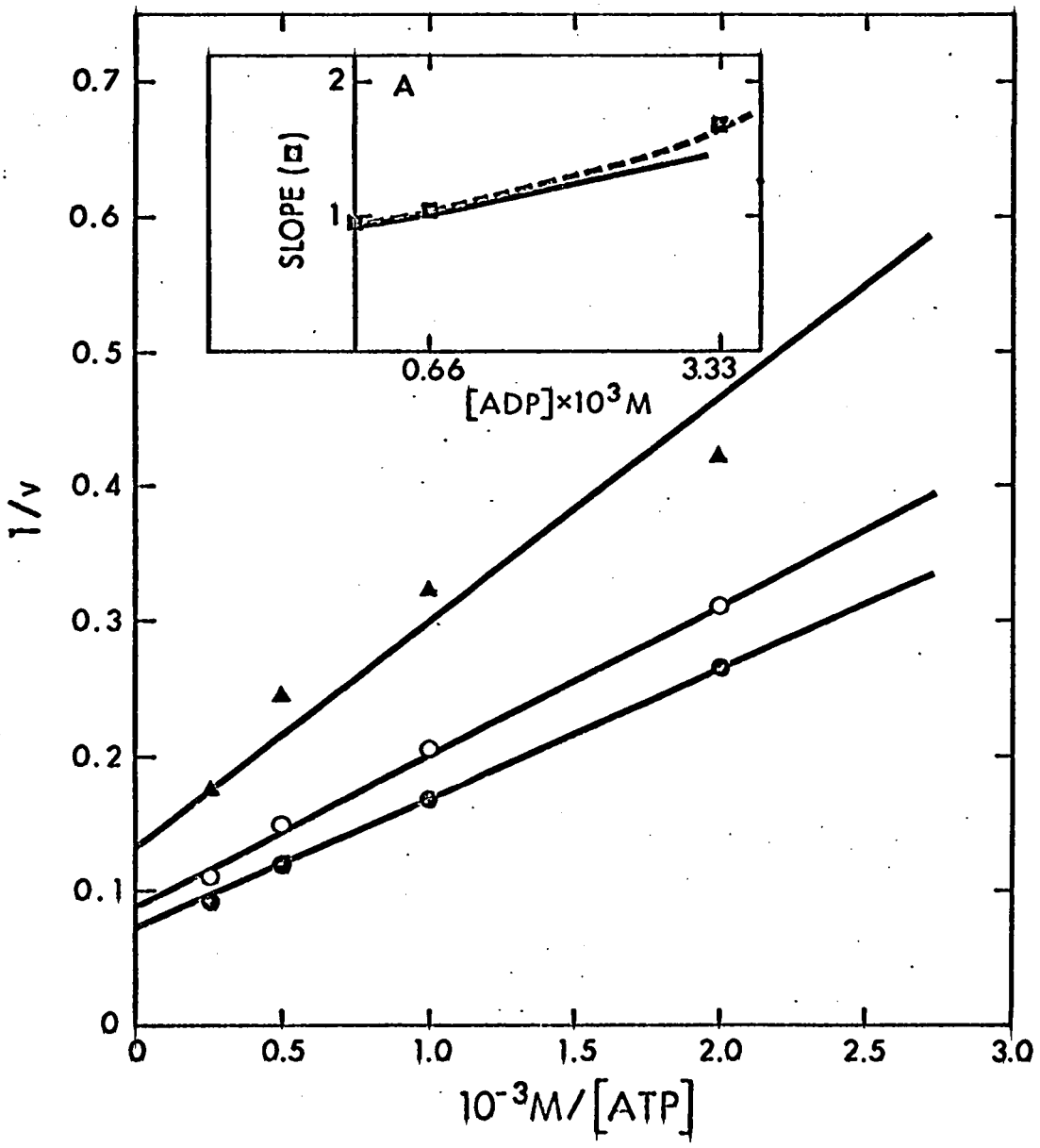
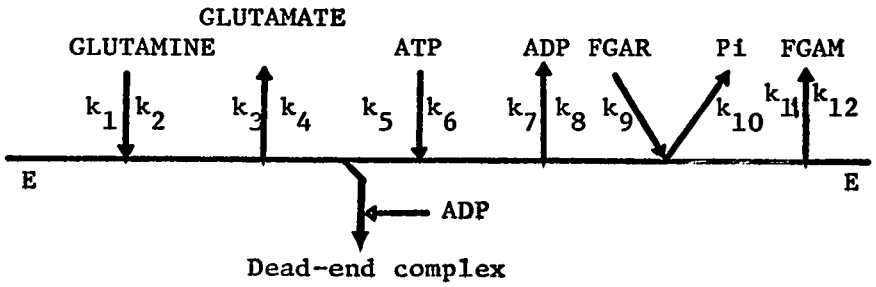


Figure 36. Product inhibition by ADP. Double reciprocal plot of initial velocity against ATP concentration at a FGAR concentration of 4×10^{-4} M and a glutamine concentration of 4×10^{-4} M. ADP concentrations were: 0 (●); 6.6×10^{-4} M (O); and 3.33×10^{-3} M (▲).

Insert: Replot of slopes against ADP concentration.

ADP gave non-competitive inhibition with respect to FGAR (Figure 37), and this observation is contrary to what one would expect from the above mechanism. However, if ADP acts both as a product and as a dead-end inhibitor such that it forms a dead-end complex with the ammonia-enzyme intermediate as illustrated in Mechanism 4-IV, the above result could be explained. Parabolic intercept and slope replots were obtained for Figures 35, 36, respectively, and these results provide further evidence for the presence of this dead-end complex (118).



Mechanism 4-IV

All the data obtained from product inhibition studies fit the following steady-state rate equation:

$$\frac{v}{E_o} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 AB + \theta_4 AC(1 + \frac{Q}{K_i}) + \theta_5 BC + \theta_6 CP + \theta_7 AQ(1 + \frac{Q}{K_i}) + \theta_8 PQ + \theta_9 ABC + \theta_{10} ACP + \theta_{11} ABR + \theta_{12} ABQ + \theta_{13} BCS + \theta_{14} APQ + \theta_{15} AQR(1 + \frac{Q}{K_i}) + \theta_{16} CPS + \theta_{17} BRS + \theta_{18} PRS + \theta_{19} PQR + \theta_{20} PQS + \theta_{21} QRS(1 + \frac{Q}{K_i}) + \theta_{22} PQRS + \theta_{23} BQRS + \theta_{24} APQR + \theta_{25} ABQR}$$

where A, B, C are substrates and P, Q, R. S. are products. $\theta_1 - \theta_{25}$ are kinetic constants.

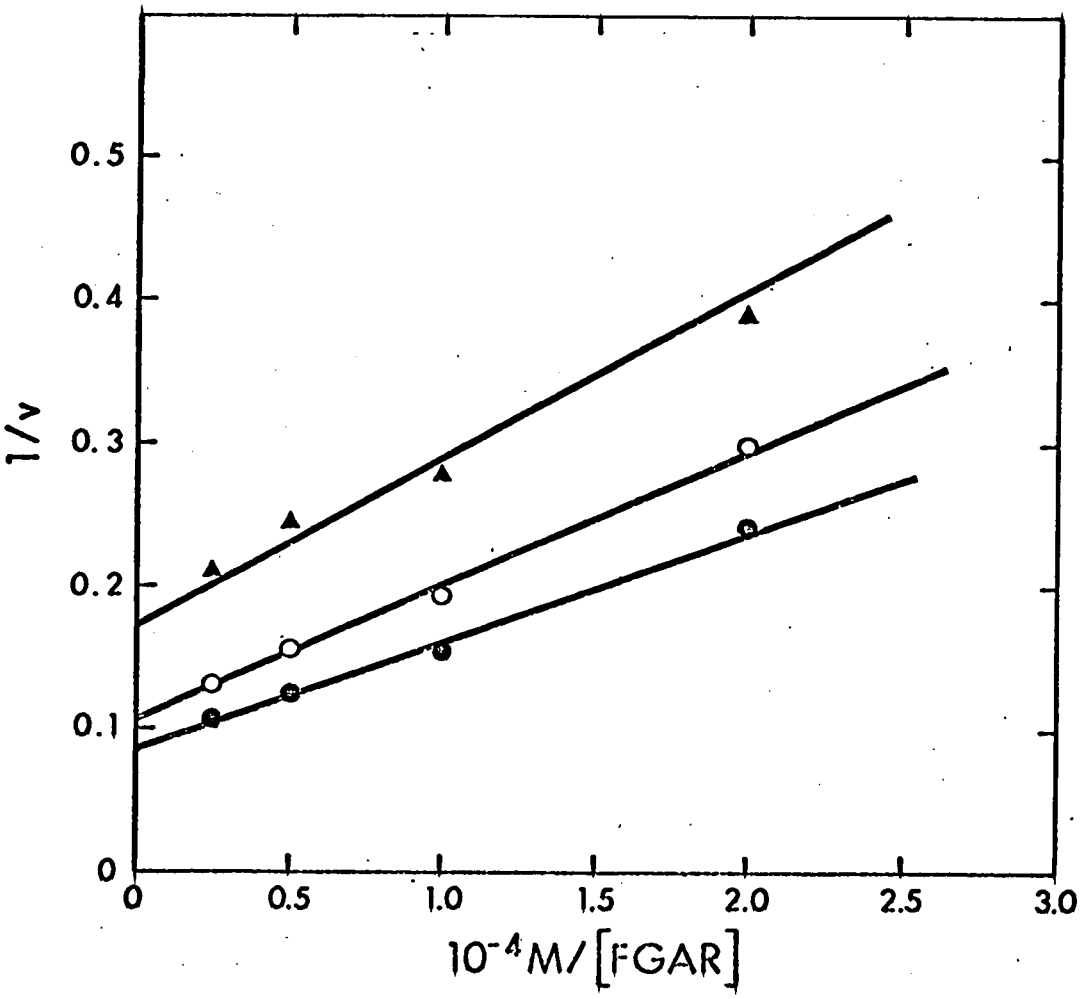


Figure 37. Product inhibition by ADP. Double reciprocal plot of initial velocity against FGAR concentration at an ATP concentration of 4×10^{-3} M and a glutamine concentrations of 4×10^{-4} M. ADP concentrations were: 0 (●); 6.6×10^{-4} M (○); and 3.33×10^{-3} M (▲).

The inhibition constants are given below:

$$K_{ia} = \frac{k_2}{k_1} = \frac{\theta_{18}}{\theta_{24}} \qquad K_{ib} = \frac{k_6}{k_5} = \frac{\theta_{15}}{\theta_{25}} \qquad K_{ic} = \frac{k_8}{k_9} \qquad K_{ip} = \frac{k_3}{k_4} = \frac{\theta_{15}}{\theta_{24}}$$

$$K_{iq} = \frac{k_7}{k_8} \qquad K_{ir} = \frac{k_{11}}{k_{10}} = \frac{\theta_3}{\theta_{11}} \qquad K_{is} = \frac{k_{11}}{k_{12}} = \frac{\theta_5}{\theta_{13}}$$

Only the inhibition constants for FGAM and Pi could be calculated. From the replots of slopes in Figure 32 and Figure 31 against product concentration, the K_{iFGAM} and K_{iPi} were found to be 1.32×10^{-4} M and 6.45×10^{-3} M, respectively.

Recent studies have shown the danger of deducing the kinetic mechanism of an enzymic reaction based on initial rate studies alone. Apparently parallel patterns of lines for double reciprocal plots of initial velocity against substrate concentrations, have been observed for ordered mechanisms such as hypoxanthine-guanine phosphoribosyltransferase (119) and adenine phosphoribosyltransferase (120). Moffet and Bridger have reported a reaction with a covalent enzyme-substrate intermediate which did not exhibit ping-pong kinetics (121). Product inhibition data, however, have been regarded as better support for the choice of kinetic mechanisms. In the present studies, the data obtained from the product inhibition experiments agreed well with the data obtained from initial rate studies. All of these data are consistent with a ping-pong kinetic mechanism for the FGAM synthetase reaction, as illustrated by Mechanism 4-IV. Results of isotope exchange studies reported in the

following Chapter provid further evidence which supports the proposed ping-pong mechanism.

Two general types of mechanisms have been proposed for ATP-linked reactions. They involve either phosphorylated intermediates (122) or concerted mechanisms in which all substrates interact simultaneously on the enzyme to yield products without giving rise to co-valently linked phosphorylated intermediates (123-125). The ping-pong mechanism proposed here requires the presence of co-valent intermediates. However, the presence of a phosphorylated FGAR derivative does not seem possible because FGAR is the last substrate binding to the enzyme. Similarly, since glutamate is released before the addition of ATP, a phosphorylated glutamyl compound could not possibly be formed. However, the participation of a phosphoenzyme intermediate is indicated. in the ping-pong mechanism proposed here, and a phosphorylated enzyme complex has also been suggested for the chicken liver FGAM synthetase (56).

Quite a number of other enzymes form phosphorylated intermediates. Evidence has been presented that in nucleoside diphosphokinase (126, 127) and ATP-citrate lyase (128), phosphoenzyme intermediates may be involved, and a phosphorylated enzyme intermediate has also been reported for succinyl coenzyme A synthetase (129). Employing pre-steady-state kinetics, Bridger, Millen and Boyer (130) observed that the formation of phosphorylated enzyme from ATP and succinyl CoA

synthetase gave the product, Pi, at a rate equal to or greater than the net catalytic rate. The postulation of a Theorell-Chance-like mechanism in the present system also suggests that the release of Pi from the enzyme is rapid.

D. Summary

Product inhibition studies were carried out with FGAM synthetase and evidence was obtained to support a fully ping-pong mechanism. Glutamine binds to the free enzyme and glutamate is then released before the addition of ATP. A Theorell-Chance-like mechanism was suggested in the later steps of the reaction such that no kinetically significant FGAR-ammonia-Pi-enzyme complex was obtained; the liberation of Pi from the enzyme complex was therefore rapid. Kinetic evidence was also obtained which suggests the formation of a phosphoenzyme intermediate, whereas phosphorylated FGAR or glutamyl compounds were not found. A dead-end ammonia-ADP-enzyme complex was also postulated. The inhibition constants for FGAM and Pi were calculated to be 1.32×10^{-4} M, and 6.45×10^{-3} M, respectively.

VII. KINETIC MECHANISM OF PHOSPHORIBOSYL-FORMYL-GLYCINEAMIDINE SYNTHETASE. III. ISOTOPE EXCHANGE STUDIES

A. Introduction

The results of the initial rate and product inhibition studies described in Chapters V and VI are consistent with a ping-pong mechanism in which kinetically significant amounts of a FGAR-ammonia-Pi-enzyme complex are not formed. A dead-end ADP-ammonia-enzyme complex was also postulated.

In this chapter, isotope exchange studies were carried out with FGAM synthetase and evidence was obtained to support the previously proposed ping-pong mechanism.

B. Materials and methods

1. Chemicals

ATP-γ-^{32}P with a specific activity of 10.5 Ci/mM on 26th Aug 1970 and ^{32}Pi with a specific activity of 500 mCi/mM on 4th Dec 1970, were purchased from New England Nuclear Corp., Boston, Mass., and were diluted to give the specific activities indicated in the Tables. Other chemicals and materials used were the same as described in Chapters V and VI.

2. Radioactive assays

Incubations were carried out in the presence of various amounts of substrates and enzymes (Fraction IV) for 30 min

at 37°. Details of the conditions are described in Table or Figure legends. Experiments were done in triplicate.

Glutamate-^{14}C was separated from glutamine-^{14}C by means of paper chromatography, and radioactivity was determined as described in Chapter VI. In ATP-γ-^{32}P-Pi exchange experiments, the reaction was stopped by adding 0.1 ml of 95% ethanol. The radioactive Pi formed was isolated by chromatography on Dowex-1-(Cl) according to the methods of Mizobuchi, Kenyon and Buchanan (56). 0.05 Ml of the eluant was taken for radioactivity measurements in Bray's phosphor (131) solution (Composition: 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 litre).

In the ^{32}Pi-ATP exchange experiments, ^{32}Pi and radioactive ATP were separated by means of the isobutyl alcohol-benzene procedure for phosphomolybdate (132). An aliquot of 50 µl of the reaction mixture was transferred to a test-tube containing 1.5 ml of 2.5% ammonium molybdate in 0.5N H_2SO_4 solution. This was then extracted 4 times with 2 ml of water-saturated isobutyl alcohol:benzene (1:1 v/v) to remove ^{32}Pi. 0.1 Ml of the aqueous layer was taken for radioactivity determinations in Bray's phosphor.

3. Assay of glutaminase activity

Glutaminase activity was assayed by ammonia formation. After incubating the enzyme preparation with glutamine for

30 min at 37°, the reaction was terminated by addition of 0.1 ml of Nessler reagent. The ammonia liberated was estimated colorimetrically at 420 nm against standard ammonium chloride solutions.

C. Results and discussion

Isotope exchange between ^{32}Pi and ATP can occur in the proposed ping-pong mechanism for FGAM synthetase both when all substrates and products are present and when both glutamine and glutamate are omitted from the incubation. In no case, however, can ^{32}Pi exchange with ATP in the absence of FGAR. On the other hand, if the reaction proceeds by a sequential mechanism, exchange between ^{32}Pi and ATP cannot occur in the absence of glutamine and glutamate. Table 13 shows that ^{32}Pi exchanged with ATP when all the substrates and products were present, and when only glutamine and glutamate were absent. No exchange was observed in the absence of FGAR. These data are in agreement with the proposed reaction mechanism (Mechanism 4-IV). The requirement for FGAR for exchange indicated that Pi cannot be released before addition of FGAR. In the previous Chapter, Pi was found to inhibit the reaction competitively with FGAR which indicates that Pi may be released before the addition of FGAR. The present isotope exchange data rule out this possibility and the inhibition pattern observed earlier may be the result of the formation of a kinetically

TABLE 13: Isotope exchange between ^{32}Pi and ATP

Omissions	ATP nmoles	ATP counts/min
None	10.8	403
FGAR and FGAM	0.403	15
FGAR	0.350	13
Glutamine and glutamate	9.7	361

Enzyme (0.65 mg) was incubated for 20 min at 37° with 27.5 mM Tris-HCl buffer pH 8.0, 10 mM $MgCl_2$, 50 mM KCl, 2.4 mM ATP, 0.35 mM FGAR, 0.24 mM glutamine, 12 mM glutamate, 1.6 mM ADP, 51 mM ^{32}Pi (6.1 μCi/mmole), and 0.1 M FGAM in a final volume of 0.25 ml.

insignificant FGAR-ammonia-Pi-enzyme complex. Furthermore, ATP must bind to the enzyme before the addition of FGAR since even in the presence of all substrates and products with the exception of FGAR, no exchange between ^{32}Pi and ATP was observed.

When the enzyme was incubated with glutamine-^{14}C, radioactive glutamate was formed (Table 14). The formation of glutamate was not affected by the presence of FGAR or ATP. This was not a result of glutaminase activity because no free ammonia was detected in this incubation mixture. This result shows that glutamine is the first substrate binding to the free enzyme and this event is followed then by the release of glutamate. No significant increase in glutamate formation was observed over a range of 5×10^{-5} M to 4×10^{-4} M glutamine (Figure 38). This result is expected since the enzyme could not turn over in the absence of all the other substrates. This result also shows a lack of glutaminase activity in the enzyme preparation because if this activity were present, we would expect a significant increase in glutamate formation unless the K_m for glutaminase is well below 5×10^{-6} M.

The release of ^{32}Pi from ATP-γ-^{32}P required the presence of both FGAR and glutamine. Table 15 shows that no ^{32}Pi was formed in the absence of FGAR and glutamine. This result confirms the conclusions reached from ^{32}Pi-ATP exchange studies.

All the data obtained in the present studies are consistent with the ping-pong mechanism proposed in

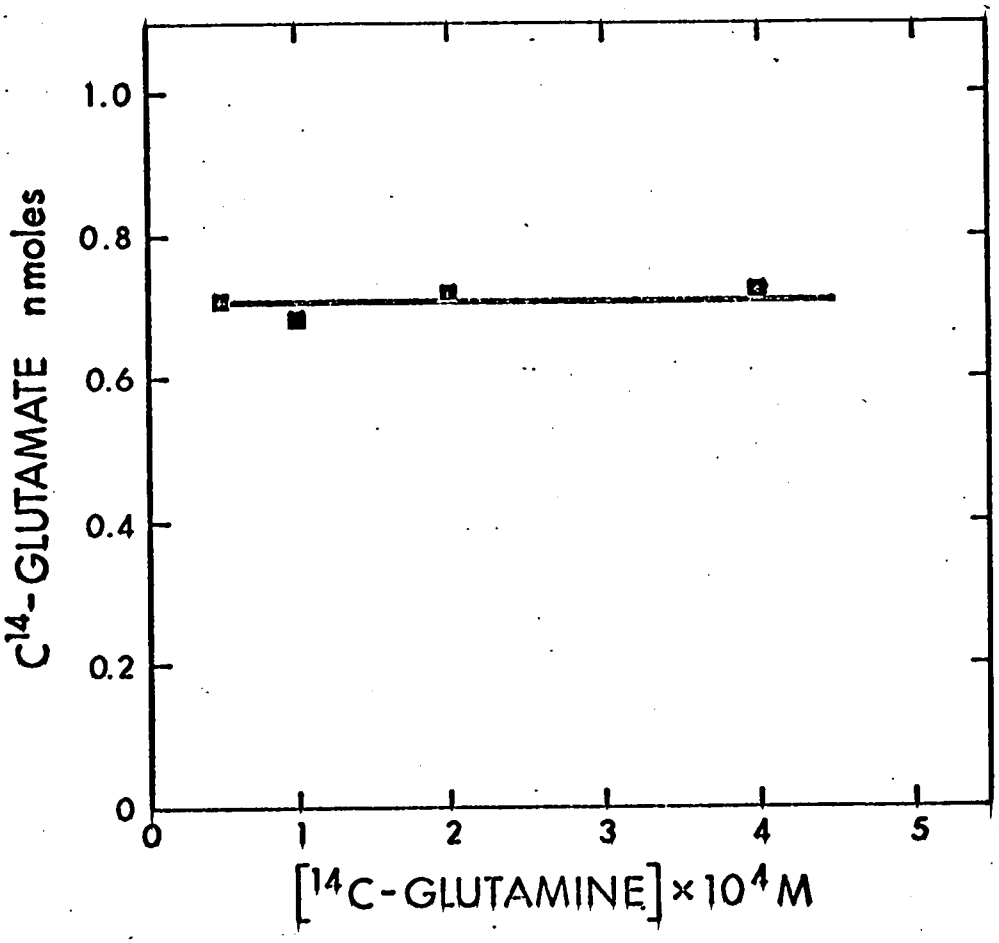


Figure 38. Glutamate formation in the absence of ATP and FGAR as a function of glutamine concentration. Enzyme (0.12 mg) were incubated 30 min at 37° in 0.3 ml of 33.3 mM Tris-HCl buffer pH 8.0, 10 mM $MgCl_2$, 20 mM KCl and glutamine-^{14}C (6.1 mCi/mmole) as indicated.

TABLE 14: Effects of ATP and FGAR on glutamate formation

Additions	Glutamate (nmoles)	Glutamate (counts/min)
Glutamine-^{14}C	0.715	672
Glutamine-^{14}C and ATP	0.620	467
Glutamine-^{14}C and FGAR	0.730	702

Enzyme (0.12 mg) was incubated for 30 min at 37° with 33.3 Tris HCl buffer pH 8.0, 10 mM $MgCl_2$, 20 mM KCl and the indicated additions in a final volume of 0.2 ml. Concentrations of glutamine-^{14}C (6.1 mCi/mmole), ATP, and FGAR were 2×10^{-4} M, 4 mM and 0.4 mM, respectively.

TABLE 15: Effects of glutamine and FGAR on phosphate formation

Additions	Pi (nmoles)	Pi (counts/min)
ATP-γ-^{32}P and FGAR	0.057	12
ATP-γ-^{32}P and glutamine	0.456	38
ATP-γ^{32}P, FGAR and glutamine	13.90	2,950

Enzyme (0.46 mg) was incubated for 30 min at 37° with 33.3 mM Tris-HCl buffer pH 8.0, 10 mM $MgCl_2$, 20 mM KCl and the indicated additions in a final volume of 0.3 ml. Concentrations of ATP-γ-^{32}P (9 mCi/mmole), FGAR and glutamine were 5 mM, 0.4 mM and 0.4 mM, respectively.

Chapter VI. The only other kinetic mechanism that could give these results is a partially pong-pong mechanism in which glutamine binds to the free enzyme followed by the release of glutamate; an ordered addition of ATP and FGAR follows, and finally release of the product occurs. Kinetic evidence obtained previously, however, rules out this possibility.

D. Summary

Further elucidation of the kinetic mechanism of FGAM synthetase was carried out through isotope exchange studies. The data obtained are in agreement with those obtained from initial rate and product inhibition studies. No exchange was obtained between ^{32}Pi and ATP in the absence of FGAR, but the presence of glutamine and glutamate was not required. Glutamine-^{14}C was bound to the enzyme and glutamate released in the absence of the other substrates. All the data obtained in these studies are consistent with the proposed ping-pong mechanism for this enzyme reaction.

VIII. INHIBITION OF 5'-PHOSPHORIBOSYL-FORMYLGLYCINEAMIDINE SYNTHETASE BY ANTITUMOR DRUGS AND OTHER REAGENTS

A. Introduction

It has been shown that FGAM synthetase is inhibited by the antitumor drugs, azaserine and DON (47-50). A single cysteine group on the Salmonella typhimurium enzyme was reported to react specifically with azaserine to form a co-valently bound complex (51, 52).

Ammonium chloride can replace glutamine as the nitrogen donor for the chicken liver enzyme (74) as well as for the Ehrlich ascites tumor cell enzyme (Chapter III). The K_m value for ammonium chloride was found to be 75 times greater than that for glutamine and the V_{max} value was forty times slower (Chapter V). In Salmonella typhimurium, this enzyme does not appear to use ammonium chloride or ammonia as a source of nitrogen for the reaction (51). Recently, Schroeder, Allison and Buchanan (57) demonstrated that reagents such as iodoacetamide and azaserine abolished the utilization of glutamine as the nitrogen donor but stimulated the utilization of ammonium chloride in the chicken liver enzyme reaction. They suggested that ammonium chloride might bind to a site on the enzyme quite different to that to which glutamine bound.

In recent years, attention has been given to the field of comparative enzymology. It is thus of interest to

investigate the effect of sulfhydryl reagents, azaserine and DON, on the tumor cell enzyme. Hitchings (72) pointed out that variations between species may not be apparent when dealing with natural substrates of a particular enzyme reaction; however, a foreign substrate, such as a drug or an anti-metabolite may produce quite individual result with enzymes from different species.

The effects of sulfhydryl specific reagents on the Ehrlich ascites tumor cell FGAM synthetase are reported here. Substrate protection against inhibition by various protein reagents have also been carried out with the FGAM synthetase to illustrate its different binding sites for ammonium chloride and glutamine. The effects of the antitumor drugs on the utilization of glutamine and ammonium chloride by the tumor cell enzyme were also investigated.

B. Materials and methods

Iodoacetate (Eastman) and iodoacetamide (Sigma) were recrystallized before use. Glutathione (reduced form) was purchased from Sigma. 2-Mercaptoethanol was a product of Fisher Scientific. p-Hydroxymercuribenzoate (PMB) and p-chloromercuribenzene sulfonate sodium salt (PCMBS) were purchased from Sigma. Both azaserine and DON were supplied by the Cancer Chemotherapy Natnional Service Centre, National Cancer Institute, U.S.A. Other chemicals, and the enzyme preparation, were the same as described in Chapter V.

Enzyme activity was assayed by Assay A, as described in Chapter III. When glutamine was the nitrogen donor, the assay was designated as "Glutamine Assay". When ammonium chloride was used as the nitrogen donor, glutamine was omitted from the reaction mixture and the assay was designated as "Ammonia Assay". The concentration of ammonium chloride used in this assay was 5×10^{-2} M. Preincubation studies were carried out by first incubating the enzyme preparation in 0.01 M sodium hydrogen maleate buffer pH 6.8 with the inhibitor for 10 min at 37°. After this period, small aliquots were removed for assay so as to avoid further inhibition by the inhibitor. Controls were carried out in which the inhibitor was omitted in the preincubation period. Substrate protection against enzyme inactivation by various reagents was studied by preincubating the enzyme preparation with the specific inhibitor and substrate simultaneously for 10 min; small aliquots were then removed for assay. In each case the substrates or reagents used in the preincubation were diluted to such an extent that their effects on the assay were negligible.

Kinetic studies of drug inhibition were carried out by measuring enzyme activity at varying concentrations of glutamine while the concentrations of the other substrates were held constant; either a fixed level of drug or enzyme preparation, which has been preincubated with drugs, was also present. Velocity is expressed as millimicromoles of FGAM formed under the assay conditions.

C. Results

1. Inhibition by sulfhydryl reagents

Figure 39 shows the effects of organic mercurial compounds on FGAM synthetase. FGAM Synthetase was inhibited both by p-hydroxymercuribenzoate (PMB) and p-chloromercuri-benzene sulfonate (PCMBS). Concentrations of 6.5×10^{-5} M for PMB and 7.5×10^{-5} M for PCMBS were found to give 50% inhibition of enzyme activity. Although other protein functional groups do react with PMB, sulfhydryl groups are regarded as the primary site of mercurial binding (133, 134). Substrate protection studies against enzyme inactivation by PMB were carried out to determine if sulfhydryl groups may be involved in substrate binding. Table 16 shows that glutamine at 1×10^{-4} M (its K_m) protected the enzyme against PMB inactivation. At a PMB concentrations of 2×10^{-4} M, where 89% of the enzyme activity was inhibited, incubation in the presence of glutamine resulted in only 18% loss of activity. Other substrates were ineffective in this respect. Table 17 shows the effects of the reversal of PMB inhibition by glutathione and 2-mercaptoethanol. At a PMB concentration of 3.2×10^{-4} M at which a 97% loss of enzyme activity occurred, both glutathione and 2-mercaptoethanol could significantly reverse this inhibition. A single cysteine of Salmonella typhimurium FGAM synthetase was reported to react co-valently with azaserine (51, 52),

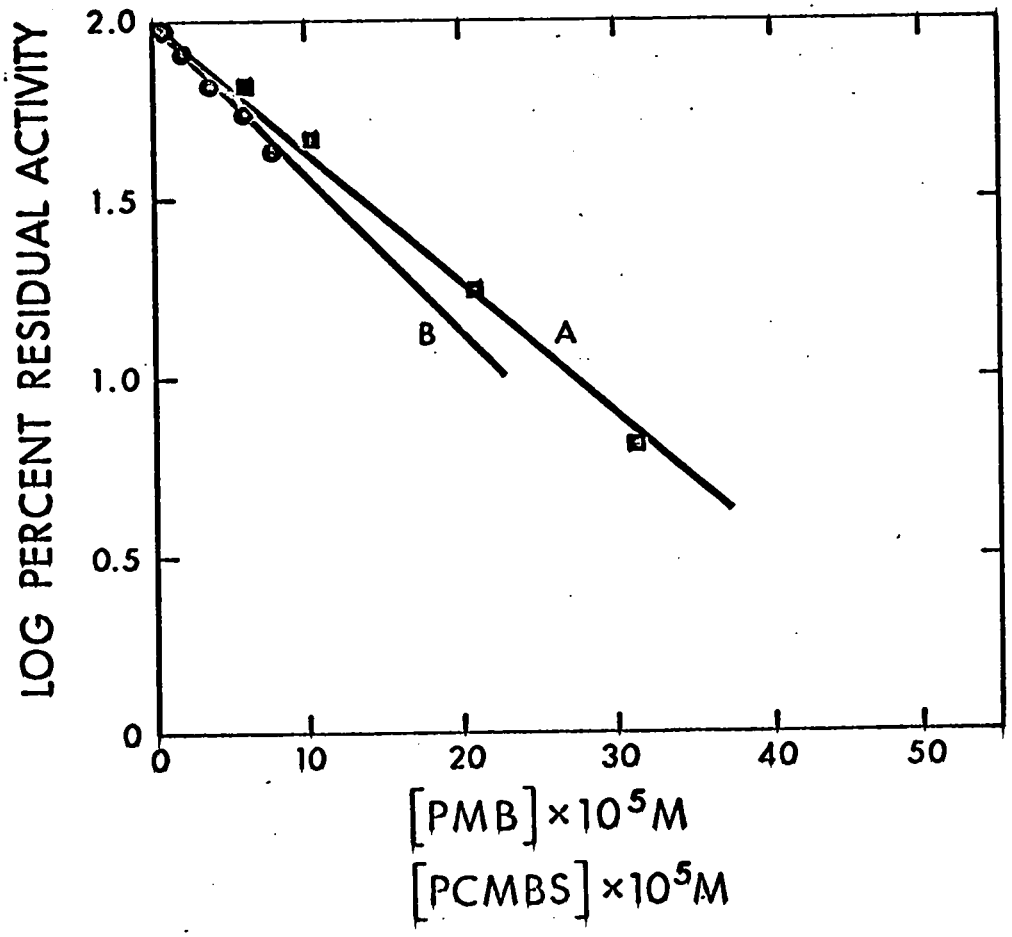


Figure 39. Effects of PMB and PCMBS on FGAM synthetase activity. Enzyme (1.47 mg) was incubated with PMB or PCMBS in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 at 37° for 10 min. 20 µl samples were removed and assayed (glutamine assay). A, PMB; B, PCMBS.

TABLE 16: Protection by substrates against enzyme inactivation by PMB

PMB (M)	Glutamine 1×10^{-4} M	ATP 1×10^{-3} M	FGAR 1×10^{-4} M	Residual activity (%)
0	-	-	-	100
2×10^{-4}	-	-	-	11.0
2×10^{-4}	+	-	-	82.0
2×10^{-4}	-	+	-	12.0
2×10^{-4}	-	-	+	10.0
4.5×10^{-4}	-	-	-	0.1
4.5×10^{-4}	+	-	-	15.0
4.5×10^{-4}	-	+	-	2.0
4.5×10^{-4}	-	-	+	1.0

Enzyme (1.65 mg) was incubated for 10 min at 37° with PMB in 0.15 mg of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 and the conditions indicated. 20 µl samples were removed for enzyme assays (Glutamine Assay).

TABLE 17: Reversal of enzyme inactivation caused by PMB

PMB (M)	Glutathione 1.85×10^{-4} M	2-mercaptoethanol 2.1×10^{-4} M	Residual activity (%)
0	-	-	100
1.6×10^{-4}	-	-	18
1.6×10^{-4}	+	-	37
1.6×10^{-4}	-	+	42
3.2×10^{-4}	-	-	3
3.2×10^{-4}	+	-	16
3.2×10^{-4}	-	+	21

Reaction mixtures (0.05 ml) contained enzyme (0.89 mg), 10 mM sodium hydrogen maleate-NaOH buffer pH 6.8 and the indicated amounts of PMB. The mixtures were incubated at 37° for 10 min. Glutathione or 2-mercaptoethanol or water was then added and the mixtures were further incubated for 10 min at 37°. 10 µl samples were removed for enzyme assay (Glutamine Assay).

and the present results also suggest the involvement of a sulfhydryl group in the active centre for glutamine binding.

The effects on enzyme activities of alkylating reagents such as iodoacetate and iodoacetamide are shown in Figure 40. Both iodoacetate and iodoacetamide inhibited the utilization of glutamine by the enzyme. The inhibitions followed pseudo-first order kinetics. A concentration of 3.8×10^{-4} M iodoacetate or 1.3×10^{-4} M iodoacetamide gave 50% inhibition. Glutamine, but not ammonium chloride, ATP, or FGAR, was found to protect the enzyme against inactivation by these reagents (Table 18). It should be noted that these compounds react with arginine and aromatic amines, but such reactions are generally slower than those with sulfhydryl groups (135). These alkylating agents gave the same effect as those obtained by PMB, which provides further evidence that an essential sulfhydryl group is involved in the binding of glutamine. Table 19 shows the effects of iodoacetate and iodoacetamide on the utilization of ammonium chloride by FGAM synthetase. In the case of iodoacetamide, a stimulation (10%) rather than inhibition was obtained. The inability of ammonium chloride to protect the enzyme so that it could utilize glutamine in the presence of sulfhydryl reagents, and the stimulating effect of iodoacetamide on the enzyme in utilizing ammonium chloride, suggest strongly that ammonium chloride and glutamine bind at different sites on the enzyme.

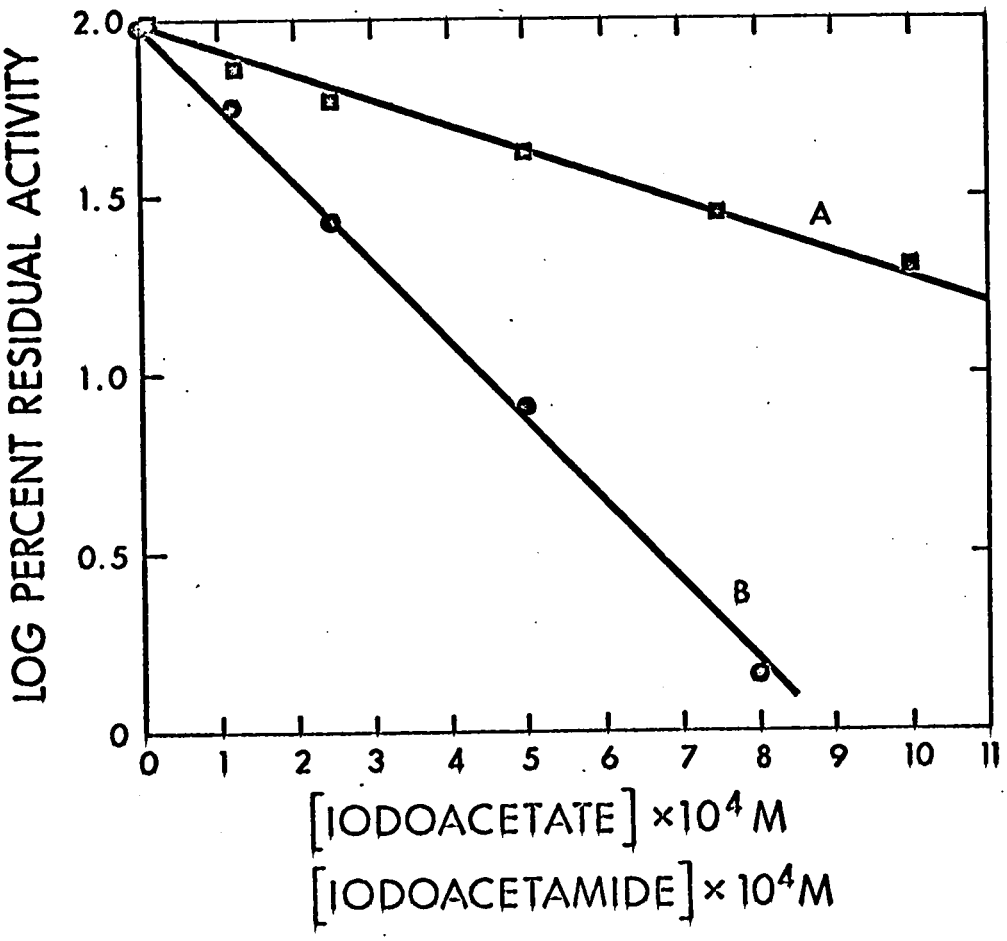


Figure 40. Effects of iodoacetate and iodoacetamide on FGAM synthetase activity. Enzyme (1.56 mg) was incubated with iodoacetate or iodoacetamide in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 at 37° for 10 min. 20 µl samples were removed and assayed (glutamine assay). A, iodoacetate; B, iodoacetamide.

TABLE 18: Protection by substrates against enzyme inactivation by iodoacetate

Iodoacetate 5×10^{-4} M	Iodoacetamide 2×10^{-4} M	Glutamine 1×10^{-4} M	ATP 1×10^{-3} M	FGAR 1×10^{-4} M	NH_4Cl 20 mM	Residual activity (%)
-	-	-	-	-	-	100
+	-	-	-	-	-	42
+	-	+	-	-	-	81
+	-	-	+	-	-	45
+	-	-	-	+	-	42
+	-	-	-	-	+	48
-	+	-	-	-	-	23
-	+	+	-	-	-	65
-	+	-	+	-	-	25
-	+	-	-	+	-	28
-	+	-	-	-	+	24

Enzyme (1.50 mg) was incubated for 10 min at 37° with iodoacetate or iodoacetamide in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 and the conditions indicated. 20 µl samples were removed for enzyme assays (Glutamine Assay).

TABLE 19: Effects of various reagents on FGAM synthetase activity when NH_4Cl was the nitrogen donor

Additions	Residual activity (%)
none	100
Iodoacetate (5×10^{-4} M)	76
Iodoacetamide (2.5×10^{-4} M)	110
Azaserine (8×10^{-6} M)	121
DON (1.8×10^{-6} M)	114

Enzyme (2.12 mg) was incubated for 10 min at 37° with the indicated conditions in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8. 20 µl samples were removed for enzyme assays (Ammonia Assay).

2. Inhibition by azaserine and DON

The effects of azaserine and DON on the utilization of glutamine by the enzyme are shown in Figure 41. Azaserine at 3×10^{-6} M and DON at 6×10^{-7} M gave 50% inhibition. Competitive inhibition patterns were obtained when glutamine was the varied substrate in the presence of azaserine or DON (Figures 42, 43). The K_i values for azaserine and DON were found to be 2.3×10^{-6} M and 4.0×10^{-7} M, respectively. If the enzyme was first preincubated with the drugs, the inhibition patterns were changed to non-competitive types (Figures 44, 45). Competitive inhibition is a result of the reversible combination of this inhibitor with the substrate binding site and non-competitive inhibition may result from the binding of inhibitor to a site on the enzyme other than that to which the substrate binds. Irreversible inhibition, however, is characterized by a progressive increase with time and the velocity constant of the reaction is affected. A competitive type of irreversible inhibition, may occur however (136) and is the result of the binding of the irreversible inhibitor to the substrate binding site. A change of kinetic pattern from competitive to non-competitive after a short preincubation period may indicate that azaserine and DON first react reversibly with the glutamine binding site of the enzyme followed by a rapid irreversible inhibition step to give an inactive enzyme.

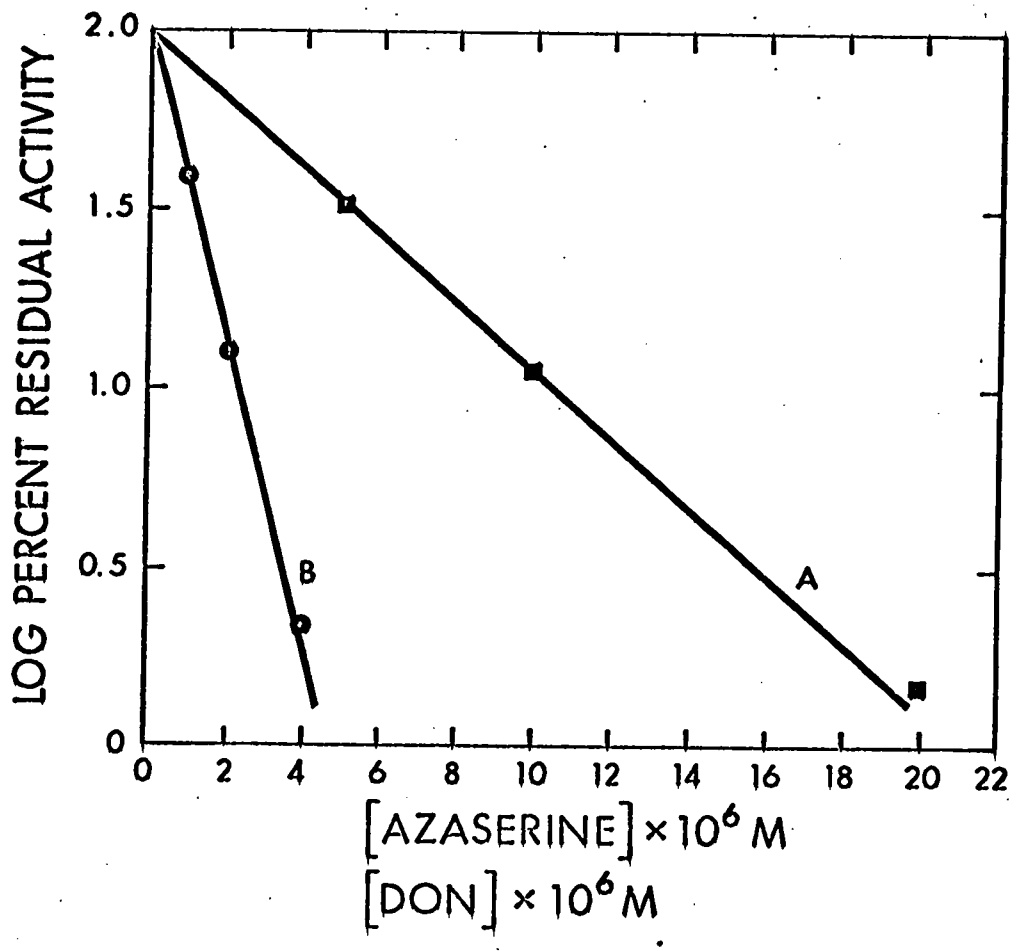


Figure 41. Effects of azaserine and DON on FGAM synthetase activity. Enzyme (1.61 mg) was incubated with azaserine or DON in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 at 37° for 10 min. 20 µl samples were removed and assayed (glutamine assay). A, azaserine; B, DON.

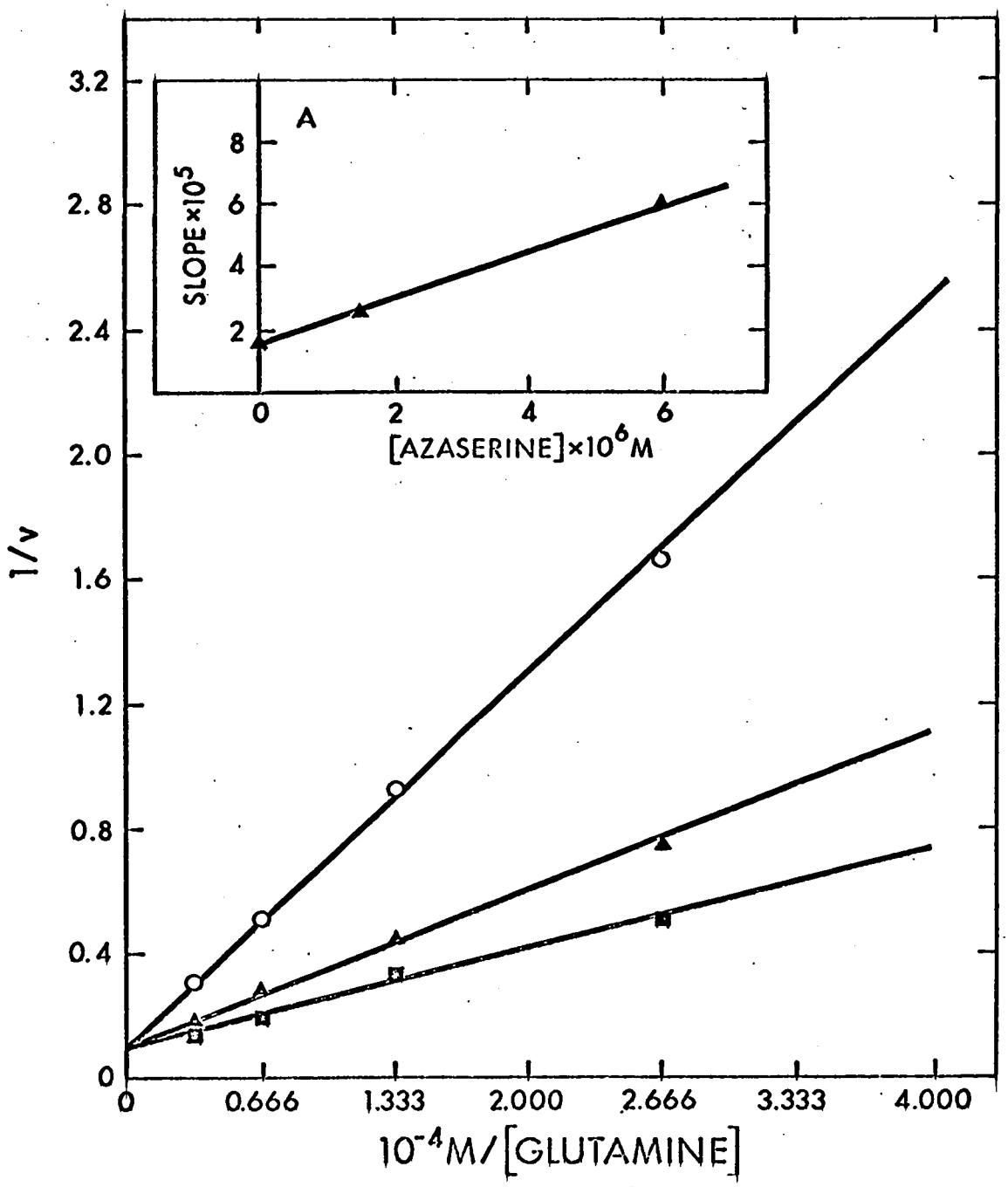


Figure 42. Inhibition by azaserine. Double reciprocal plot of initial velocity against glutamine concentration at a FGAR concentration of 3.75×10^{-4} M and an ATP concentration of 3.75×10^{-3} M. Azaserine concentrations were: 0 (□); 1.5×10^{-6} M (▲); and 6×10^{-6} M (O).

Insert: Replot of slopes against azaserine concentration.

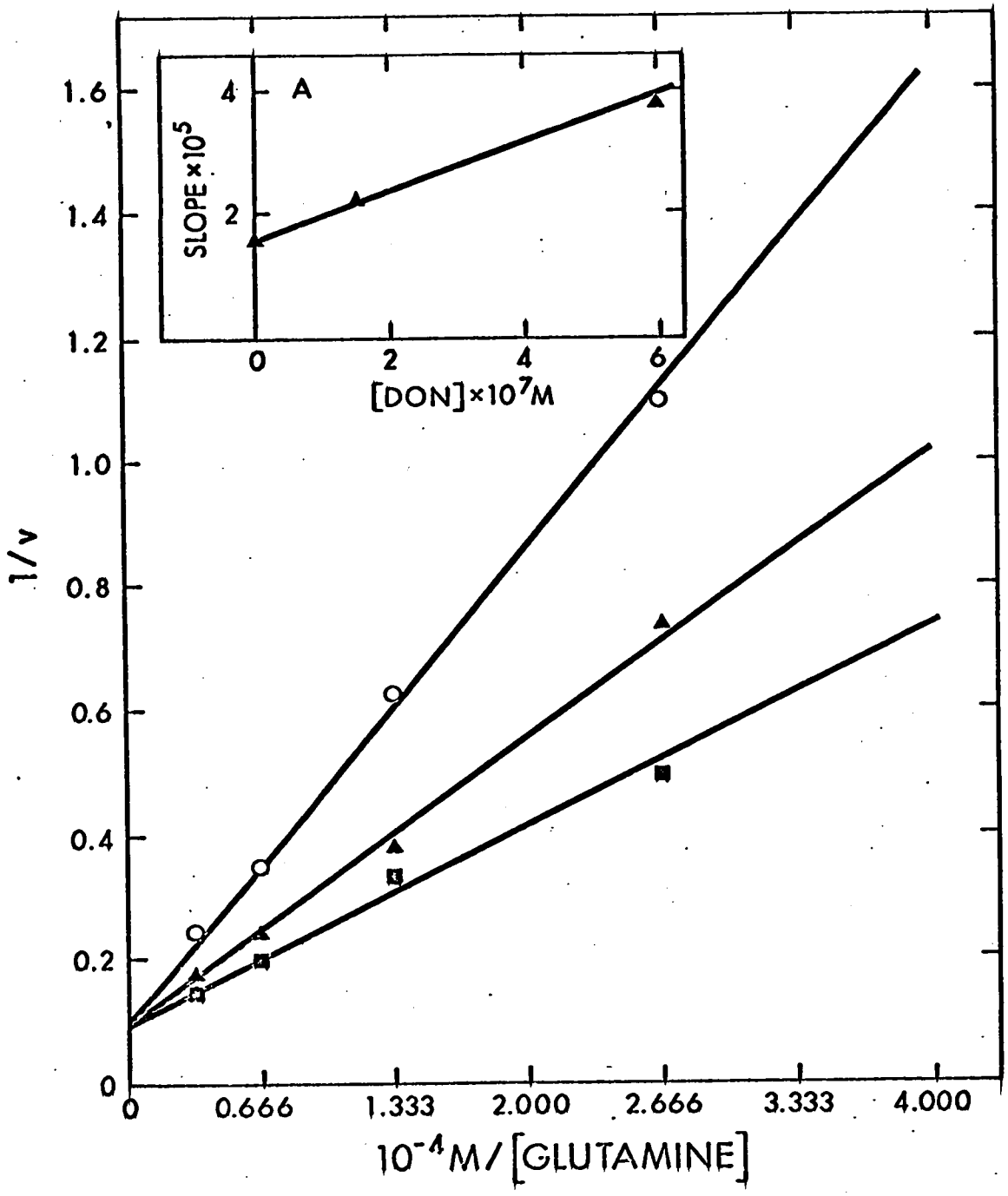


Figure 43. Inhibition by DON. Double reciprocal plot of initial velocity against glutamine concentration at a FGAR concentration of 3.75×10^{-4} M and an ATP concentration of 3.75×10^{-3} M. DON concentrations were: 0 (□); 1.5×10^{-7} M (▲); and 6×10^{-7} M (O).

Insert: Replot of slopes against DON concentration.

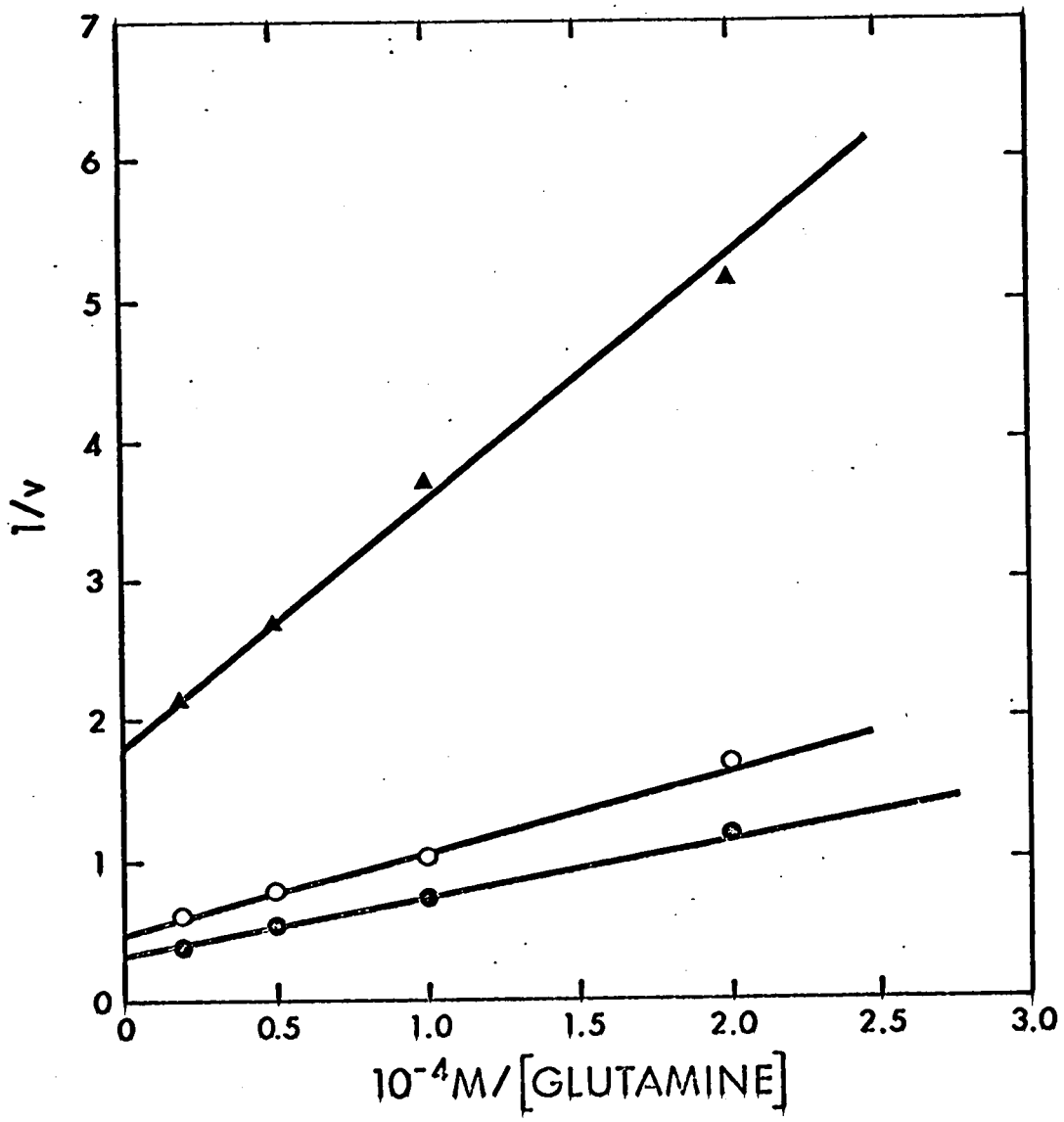


Figure 44. Inhibition by azaserine. Double reciprocal plot of initial velocity against glutamine concentration at an ATP concentration of 4×10^{-3} M and a FGAR concentration of 4×10^{-4} M. Enzyme was preincubated with azaserine in 2 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 at 37° for 5 min. 75 µl samples were removed for initial rate studies. Concentrations of azaserine were: 0 (◎); 2.4×10^{-6} M (O); and 1.2×10^{-5} M (▲).

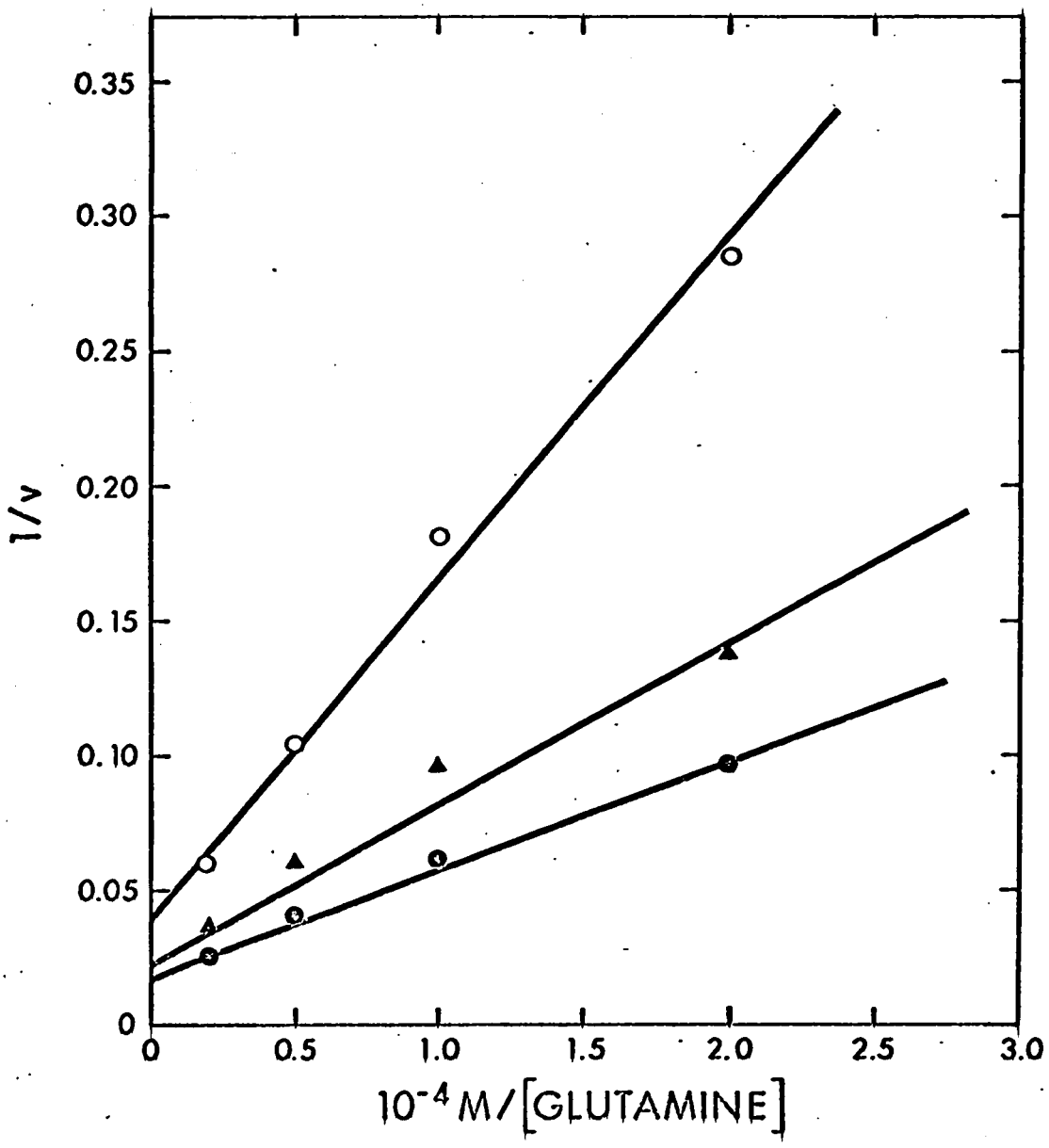


Figure 45. Inhibition by DON. Double reciprocal plot of initial velocity against glutamine concentration at an ATP concentration of 4×10^{-3} M and a FGAR concentration of 4×10^{-4} M. Enzyme was preincubated with DON in 2 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 at 37° for 5 min. 75 µl samples were removed for initial rate studies. Concentrations of DON were: 0 (●); 4×10^{-7} M (▲); and 8×10^{-7} M (○).

Only glutamine could protect the enzyme against inhibition by azaserine and DON (Table 20), and these drugs stimulated rather than inhibited the utilization of ammonium chloride by the enzyme (Table 19).

D. Discussion

The tumor cell FGAM synthetase was inhibited by sulfhydryl specific reagents and the glutamine antimetabolites, azaserine and DON when glutamine was substrate. Of all the substrates, only glutamine could give protection against enzyme inhibition by these reagents. Glutathione and 2-mercaptoethanol could reverse the enzyme inhibition by PMB. Azaserine has been found to react with a sulfhydryl group on the Salmonella typhimurium enzyme to form a co-valently linked complex (51, 52) and the data obtained from the present studies also suggest that the tumor cell enzyme possesses a sulfhydryl group at its active centre for glutamine binding. The lack of protection provided by ammonium chloride against enzyme inhibition by these reagents when glutamine was substrate indicates also that glutamine and ammonium chloride (ammonia) bind to different sites on the enzyme. The antitumor drugs, azaserine and DON, irreversibly compete with glutamine for the same binding site on the enzyme.

The stimulatory effects of these drugs and alkylating agents such as iodoacetamide on the utilization of ammonium

TABLE 20: Protection by substrates against enzyme inactivation by azaserine and DON

Azaserine 8×10^{-6} M	DON 1.8×10^{-6} M	Glutamine 1×10^{-4} M	ATP 1×10^{-3} M	FGAR 1×10^{-4} M	NH_4Cl 2×10^{-2} M	Residual activity (%)
-	-	-	-	-	-	100
+	-	-	-	-	-	16.5
+	-	+	-	-	-	32
+	-	-	+	-	-	17
+	-	-	-	+	-	21
+	-	-	-	-	+	23
-	+	-	-	-	-	14
-	+	+	-	-	-	31
-	+	-	+	-	-	18
-	+	-	-	+	-	19
-	+	-	-	-	+	21

Enzyme (1.64 mg) was incubated for 10 min at 37° with azaserine or DON in 0.15 ml of 0.01 M sodium hydrogen maleate-NaOH buffer pH 6.8 and the indicated conditions. 20 µl samples were removed for enzyme assays (Glutamine Assay).

chloride by the enzyme was of interest. When these reagents react with the enzyme, certain groups are introduced into the enzyme and its conformation will be altered (137); a facilitation of the binding of ammonia or the amide nitrogen (after its release from glutamine) in an "induced fit" (138) manner may result. The small inhibitory effect of iodoacetate on the enzyme when ammonium chloride was substrate could be due to the effect of introducing the charged carboxylate group into the enzyme.

Earlier studies have shown that glutamine binds to the free enzyme, followed by the release of glutamate (Chapter V-VII). The free amide nitrogen (after its release from glutamine) is presumably enzyme-bound because no free ammonium ion was detected. It is proposed that glutamine binds to a sulfhydryl site on the tumor cell enzyme to form a γ-glutamyl-enzyme complex, and the amide nitrogen is then released and transferred to another site on the enzyme. After this transfer, an acid hydrolysis of the γ-glutamyl-enzyme complex by water follows to release the glutamate before the addition of other substrates.

The K_i values for azaserine and DON found for the tumor cell system (2.3×10^{-6} M and 4.0×10^{-7} M, respectively) were significantly lower than those obtained for the pigeon liver enzyme (47, 78). The K_i values for azaserine and DON for the pigeon liver enzyme were 3.4×10^{-5} M and 1.1×10^{-6} M, respectively. Indeed, tumor cells are more susceptible to these drugs than the normal tissues (45, 47, 86, 89).

E. Summary

The effects of azaserine and DON, and of sulfhydryl reagents on the activity of FGAM synthetase were studied. Glutamine protected the enzyme against inhibition by these reagents when glutamine was substrate, while the utilization of ammonium chloride was not inhibited. Glutathione and 2-mercaptoethanol could reverse the inhibitory effect of p-hydroxymercuribenzoate on the enzyme. These studies suggest that an essential sulfhydryl group is present in the active centre of the enzyme and presumably is the binding site for glutamine. Ammonium chloride or ammonia appears to bind to the enzyme at a site different from that to which glutamine binds.

Azaserine and DON compete with glutamine for the sulfhydryl group of the enzyme to form a co-valent intermediate. This inhibition could not be reversed by the addition of glutamine or other substrates. The K_i values for azaserine and DON were found to be 2.3×10^{-6} M and 4.0×10^{-7} M, respectively.

IX. GENERAL CONCLUSION

The mechanism of the reaction catalyzed by phosphoribosylformylglycineamidine synthetase is shown schematically in Figure 46. X, Y, and Z represent hypothetical sites on the enzyme to which ammonia or the free amide nitrogen (after its release from glutamine), ATP and FGAR are bound. A sulfhydryl group is postulated to be involved in glutamine binding. This sulfhydryl group reacts with the first substrate, glutamine, by nucleophilic attack on the γ carbonyl carbon of glutamine to form a thioester linkage; the amide nitrogen is released and bound to a nearby site (designated to be X), presumably by hydrogen bonding. During this process, a conformational change of the enzyme occurs permitting the binding of the second substrate, ATP. Before the addition of ATP a rapid acid hydrolysis of the thioester by water occurs, and glutamate is released. ATP reacts with the γ site to form a phosphoenzyme intermediate with the release of ADP. A phosphohistidine enzyme complex was found in the succinate thiokinase reaction (130, 139, 140), and a phospho-enzyme complex was also detected in the glyceraldehyde-3-phosphate dehydrogenase system (141). Whether a histidine group is involved in the tumor FGAM synthetase phospho-complex is not known.

FGAR then binds to the enzyme and is located in such a position that a simple concerted displacement reaction among ammonia, FGAR and the phospho-enzyme complex can occur.

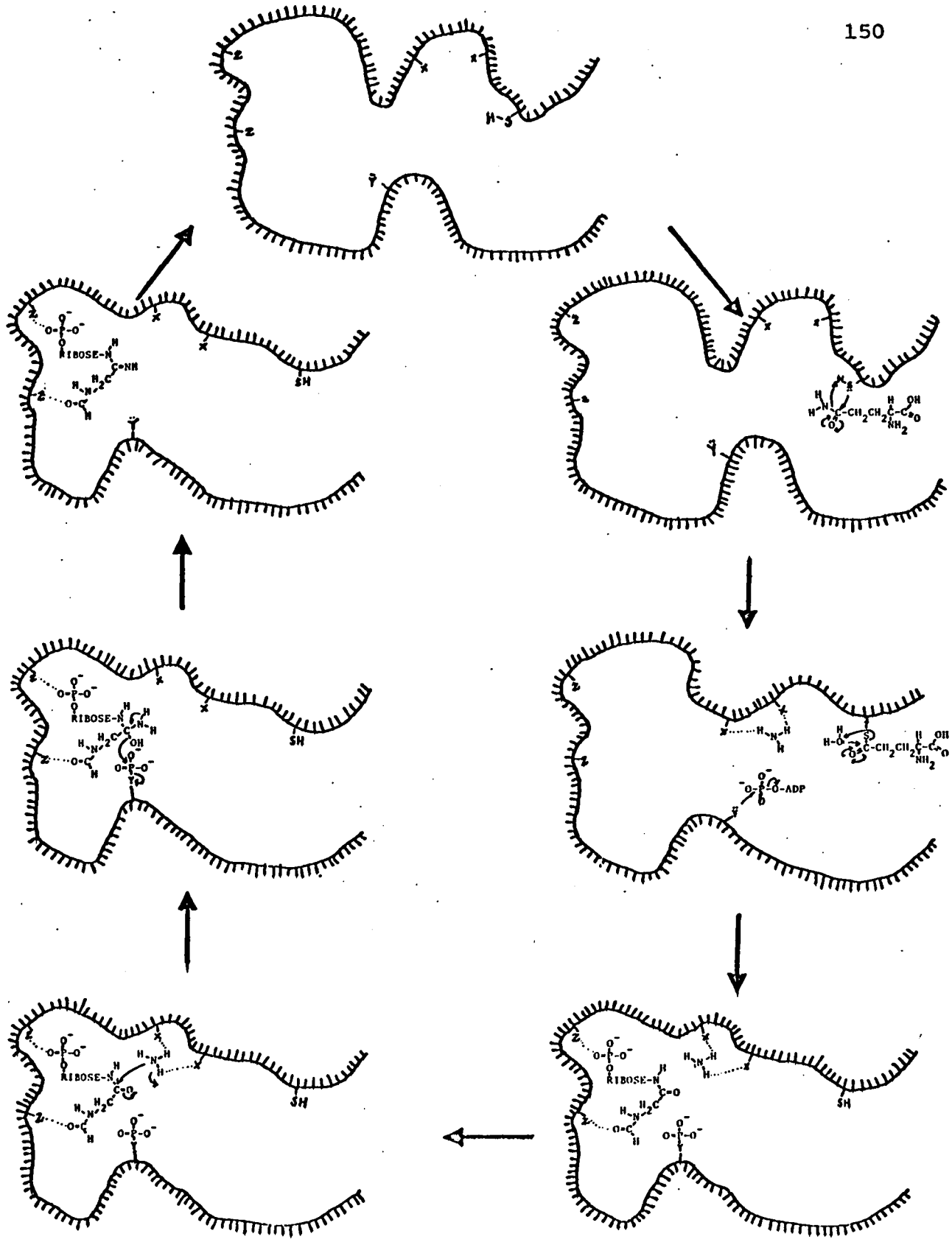


Figure 46. Proposed mechanism of phosphoribosyl-formylglycineamidine synthetase.

The electron rich ammonia reacts with the carbonyl carbon of FGAR to form a Schiff base and the hydroxyl group so formed is displaced to the electron deficient phosphoryl group of the enzyme to form Pi in a one step manner.

Evidence has been obtained that the enzymic decarboxylation of acetoacetate proceeds through an intermediate formation of a Schiff base (142); the β-carbonyl oxygen is released. In the mechanism proposed for FGAM synthetase, a transfer of the carbonyl oxygen to the phospho group is suggested. ^{18}O Experiments in other acyl or carbonyl transfer systems have provided evidence that an oxygen atom of the carbon compound is transferred to the γ-phosphate of ATP (123, 143-146). The release of Pi after the addition of FGAR and the requirement for the presence of FGAR for ^{32}Pi-ATP exchange indirectly supports this proposed scheme so that simple hydrolysis of the phospho-enzyme intermediate by water would be unlikely. After this displacement reaction Pi is released first followed by FGAM, and the free enzyme is thus formed.

Nothing is known about the amino acid residues involved in substrate binding and no consideration has been given to the role of magnesium and potassium ions in the above model. Several possible roles for the metal ion in ATP phosphotransferase reactions have been suggested by Jencks (147): "It can shield the negative charges of the phosphate group which would otherwise tend to repel the attack of the

electron pair of a nucleophile; increase the reactivity of the atom which is being attacked by withdrawing electrons; make the leaving group a better leaving group; act as a bridging group between the nucleophile and substrate; change the pK and reactivity of the nucleophile; change the geometric arrangement of the substrate in such a manner to facilitate the reaction." The magnesium ion involved in this reaction may possess some or all the above attributes. The requirement of equivalent or excess amount of magnesium ion over ATP for maximum enzyme activity may well indicate the formation of Mg-ATP substrate complex such that terminal phosphoryl group of ATP is activated towards nucleophilic attack. The potassium ion may simply play a role in maintaining the integrity of the enzyme.

Although the FGAM synthetase from Ehrlich ascites tumor cells possesses catalytic properties similar to those of the enzyme from other sources, it does have some individual properties. The K_i value for azaserine with the tumor cell enzyme is significantly lower than that obtained in other systems--an effect that may in part explain drug selectivity. Growth inhibition by azaserine in bacterial systems could be reversed by the addition of amino acids such as phenylalanine and tryptophan. This effect is not due to the compensation for the inhibition provided by the amino acids, but rather by the fact that the enzyme appears to have less affinity for the inhibitors (148, 149).

It is of interest to note that FGAM synthetase concentrations or activities differ tremendously in tumor cells compared to those in pigeon liver or other systems. FGAM synthetase activity was found in the present work to be one percent of that obtained from pigeon liver on the basis of unit weight of the tissue. The enzyme preparation in tumor cells and pigeon liver were estimated to be present in different amounts, even though assayed under somewhat different conditions (Dr. L. W. Brox, personal communication). The PP-ribose-P amidotransferase activities of various tumor cells are two to ten percent of that found in Aerobacter aerogenes in pigeon liver. Other studies have indicated that various kinds of tumor cells are much more sensitive to azaserine than are some normal tissues (45, 47, 86, 89). This is in accordance with the hypothesis that until the activity of an enzyme has been reduced to the point where it is limiting, inhibitors of the enzyme will not produce marked effects on metabolism (150).

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XI. APPENDIX

A. Rate equations

1. Ter quad mechanisms

There are ten possible mechanisms for a three-substrate--four-product enzyme system provided that no alternative pathways exist. These ten possible mechanism (I-X) and their simplified steady-state rate equations are presented in the following pages.

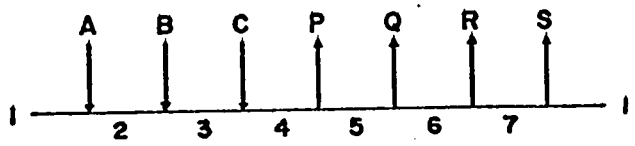
2. Ter ter mechanisms

For enzyme reactions involving three-substrate--three-products, there are six possible mechanisms if no alternative reaction pathways exist. Cleland (158) has worked out the full rate equations for these mechanisms. However for the consistency of presenting the inhibition patterns, these six mechanisms (XI-XVI) together with the simplified steady-state rate equations are also presented.

B. Product inhibition and dead-end inhibition patterns

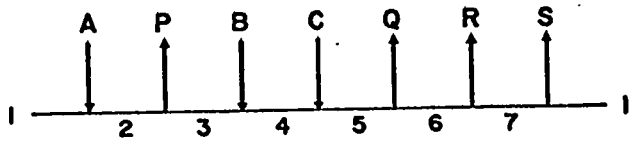
Kinetic patterns of three-substrate--four-product enzyme reactions in the presence of various products or dead-end inhibitors are presented in Tables 21 and 22. Similarly the kinetic patterns of product inhibition and dead-end inhibition patterns are presented in Tables 23 and 24. Cleland's nomenclature (108) was used in analyzing these inhibition patterns as described in Chapter VI.

Mechanism I .Ordered Ter Quad



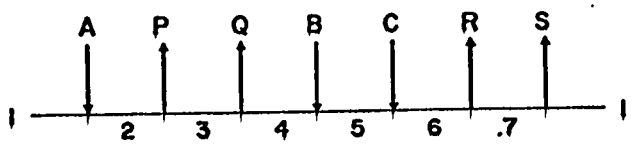
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 + \theta_4 A + \theta_5 C + \theta_6 P + \theta_7 S + \theta_8 AB + \theta_9 AC + \theta_{10} AP + \theta_{11} BC + \theta_{12} CS + \theta_{13} PQ + \theta_{14} PS + \theta_{15} RS + \theta_{16} ABC + \theta_{17} ABP + \theta_{18} APQ + \theta_{19} BCS + \theta_{20} CRS + \theta_{21} PQR + \theta_{22} PQS + \theta_{23} PRS + \theta_{24} QRS + \theta_{25} ABCP + \theta_{26} ABCQ + \theta_{27} ABCR + \theta_{28} ABPQ + \theta_{29} APQR + \theta_{30} CQRS + \theta_{31} BCRS + \theta_{32} PQRS + \theta_{33} ABCQR + \theta_{34} ABCPQ + \theta_{35} ABPQR + \theta_{36} BCQRS + \theta_{37} BPQRS + \theta_{38} CPQRS + \theta_{39} ABCPQR + \theta_{40} BCPQRS}$$

Mechanism II Uni Uni Bi Tri Ping Pong



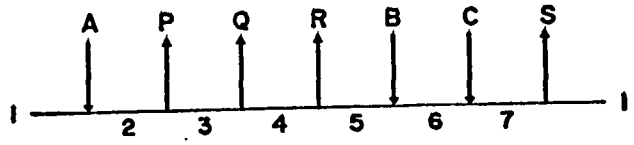
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 A + \theta_4 P + \theta_5 AB + \theta_6 AC + \theta_7 AP + \theta_8 AQ + \theta_9 BC + \theta_{10} CP + \theta_{11} PQ + \theta_{12} PS + \theta_{13} ABC + \theta_{14} ABQ + \theta_{15} ACP + \theta_{16} APQ + \theta_{17} BCS + \theta_{18} CPS + \theta_{19} PQR + \theta_{20} PQS + \theta_{21} PRS + \theta_{22} ABCQ + \theta_{23} ABCR + \theta_{24} ABQR + \theta_{25} APQR + \theta_{26} BCRS + \theta_{27} BQRS + \theta_{28} PQRS + \theta_{29} CPRS + \theta_{30} ABCQR + \theta_{31} BCQRS + \theta_{32} CPQRS}$$

Mechanism III Uni Bi Bi Bi Ping Pong



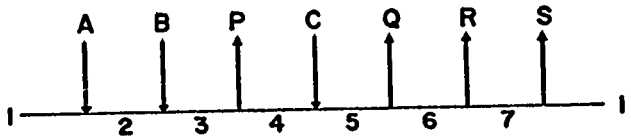
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 A + \theta_4 AB + \theta_5 BC + \theta_6 AC + \theta_7 AR + \theta_8 PQ + \theta_9 RS + \theta_{10} AQ + \theta_{11} ABC + \theta_{12} ABR + \theta_{13} APQ + \theta_{14} ACQ + \theta_{15} AQR + \theta_{16} BCP + \theta_{17} BRS + \theta_{18} BCS + \theta_{19} CPQ + \theta_{20} PQR + \theta_{21} PRS + \theta_{22} PQS + \theta_{23} QRS + \theta_{24} ABCP + \theta_{25} APQR + \theta_{26} ACPQ + \theta_{27} ABCR + \theta_{28} BPRS + \theta_{29} BCRS + \theta_{30} BCPS + \theta_{31} CPQS + \theta_{32} PQRS + \theta_{33} BCPRS + \theta_{34} CPQRS}$$

Mechanism IV Uni Tri Bi Uni Ping Pong



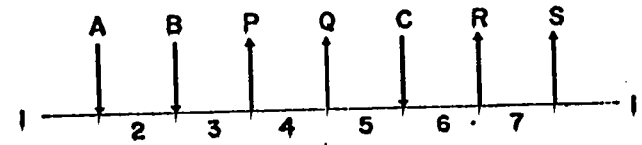
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 A + \theta_4 S + \theta_5 AR + \theta_6 AB + \theta_7 BC + \theta_8 RS + \theta_9 AC + \theta_{10} PS + \theta_{11} BS + \theta_{12} ABC + \theta_{13} AQR + \theta_{14} ACR + \theta_{15} BCP + \theta_{16} BPS + \theta_{17} BCS + \theta_{18} APQR + \theta_{19} ABCP + \theta_{20} ABCQ + \theta_{21} ACQR + \theta_{22} BCPQ + \theta_{23} BPQS + \theta_{24} BCPS + \theta_{25} CPQR + \theta_{26} PQRS + \theta_{27} ABCPQ + \theta_{28} ACPQR + \theta_{29} BCPQS + \theta_{30} CPQRS}$$

Mechanism V Bi Uni Uni Tri Ping Pong



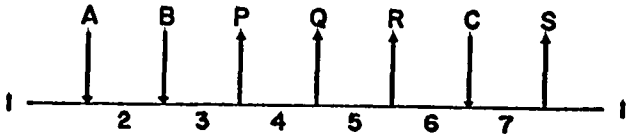
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 C + \theta_4 P + \theta_5 AC + \theta_6 AP + \theta_7 AB + \theta_8 BC + \theta_9 CS + \theta_{10} PS + \theta_{11} PQ + \theta_{12} APQ + \theta_{13} ABC + \theta_{14} ABP + \theta_{15} ABQ + \theta_{16} BCS + \theta_{17} CRS + \theta_{18} PQR + \theta_{19} QRS + \theta_{20} PRS + \theta_{21} PQS + \theta_{22} APQR + \theta_{23} ABPQ + \theta_{24} ABQR + \theta_{25} ABCQ + \theta_{26} ABCR + \theta_{27} BQRS + \theta_{28} CQRS + \theta_{29} PQRS + \theta_{30} ABPQR + \theta_{31} ABCQR + \theta_{32} BPQRS}$$

Mechanism VI Bi Bi Uni Bi Ping Pong



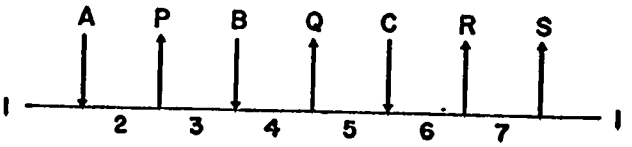
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 C + \theta_4 AC + \theta_5 BC + \theta_6 CP + \theta_7 PQ + \theta_8 RS + \theta_9 CS + \theta_{10} AB + \theta_{11} ACP + \theta_{12} APQ + \theta_{13} ABC + \theta_{14} ABQ + \theta_{15} BRS + \theta_{16} BCS + \theta_{17} CRS + \theta_{18} CPS + \theta_{19} PQS + \theta_{20} APQR + \theta_{21} ABQR + \theta_{22} ABCR + \theta_{23} ABCP + \theta_{24} ABPQ + \theta_{25} BQRS + \theta_{26} BCRS + \theta_{27} PQRS + \theta_{28} ABPQR + \theta_{29} BPQRS}$$

Mechanism VII Bi Tri Uni Uni Ping Pong



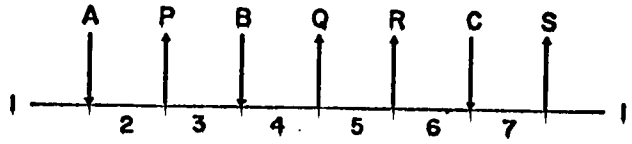
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 C + \theta_4 S + \theta_5 AB + \theta_6 AC + \theta_7 BC + \theta_8 BS + \theta_9 CP + \theta_{10} CS + \theta_{11} PS + \theta_{12} RS + \theta_{13} ACP + \theta_{14} CPQ + \theta_{15} PQR + \theta_{16} ABC + \theta_{17} QRS + \theta_{18} PRS + \theta_{19} ABR + \theta_{20} BRS + \theta_{21} BCS + \theta_{22} CPS + \theta_{23} PQS + \theta_{24} APQR + \theta_{25} ACPQ + \theta_{26} ABCP + \theta_{27} ABCQ + \theta_{28} ABQR + \theta_{29} BQRS + \theta_{30} CPQS + \theta_{31} PQRS + \theta_{32} ABPQR + \theta_{33} BPQRS + \theta_{34} ABCPQ}$$

Mechanism VIII Uni Uni Uni Uni Uni Bi Ping Pong



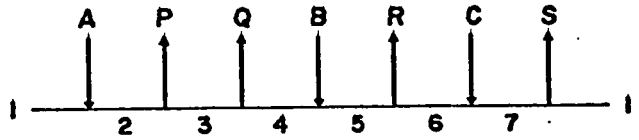
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 AC + \theta_4 AB + \theta_5 AQ + \theta_6 BC + \theta_7 CP + \theta_8 PQ + \theta_9 ABC + \theta_{10} ACP + \theta_{11} APQ + \theta_{12} AQR + \theta_{13} ABQ + \theta_{14} ABR + \theta_{15} BRS + \theta_{16} BCS + \theta_{17} CPS + \theta_{18} PRS + \theta_{19} PQR + \theta_{20} PQS + \theta_{21} APQR + \theta_{22} ABQR + \theta_{23} ABCR + \theta_{24} BCRS + \theta_{25} BQRS + \theta_{26} PQRS + \theta_{27} CPRS}$$

Mechanism IX Uni Uni Uni Bi Uni Uni Ping Pong



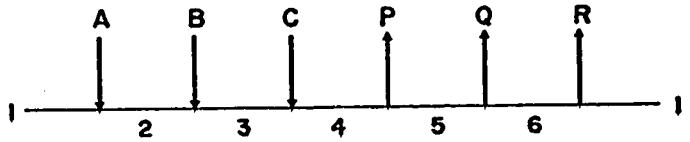
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 AC + \theta_4 AB + \theta_5 BC + \theta_6 CP + \theta_7 PS + \theta_8 BS + \theta_9 ABC + \theta_{10} PQR + \theta_{11} CPQ + \theta_{12} ACP + \theta_{13} ACQ + \theta_{14} AQR + \theta_{15} QRS + \theta_{16} BRS + \theta_{17} PRS + \theta_{18} ABR + \theta_{19} BCS + \theta_{20} CPS + \theta_{21} APQR + \theta_{22} PQRS + \theta_{23} ACPQ + \theta_{24} BQRS + \theta_{25} ABCQ + \theta_{26} CPQS + \theta_{27} ABQR}$$

Mechanism X Uni Bi Uni Uni Uni Uni Ping Pong



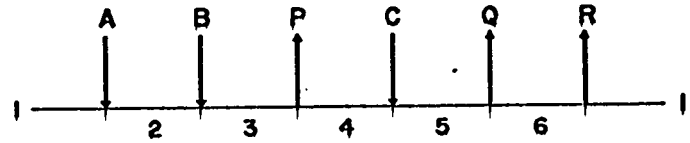
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQRS}{\theta_3 AB + \theta_4 AR + \theta_5 BC + \theta_6 AC + \theta_7 RS + \theta_8 BS + \theta_9 PQR + \theta_{10} BCP + \theta_{11} CPQ + \theta_{12} ABC + \theta_{13} QRS + \theta_{14} ACQ + \theta_{15} AQR + \theta_{16} ABR + \theta_{17} BRS + \theta_{18} PRS + \theta_{19} BPS + \theta_{20} PQS + \theta_{21} BCS + \theta_{22} APQR + \theta_{23} PQRS + \theta_{24} ABCR + \theta_{25} ACPQ + \theta_{26} BPRS + \theta_{27} CPQS + \theta_{28} BCPS}$$

Mechanism XI Ordered Ter Ter



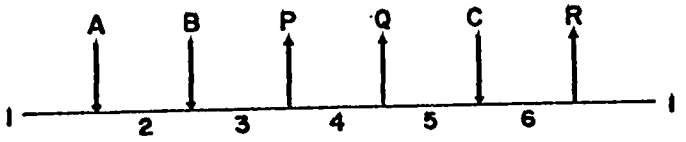
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 + \theta_4 A + \theta_5 C + \theta_6 P + \theta_7 R + \theta_8 AB + \theta_9 AP + \theta_{10} AC + \theta_{11} BC + \theta_{12} CR + \theta_{13} PQ + \theta_{14} PR + \theta_{15} QR + \theta_{16} ABC + \theta_{17} ABP + \theta_{18} APQ + \theta_{19} BCR + \theta_{20} CQR + \theta_{21} PQR + \theta_{22} ABCP + \theta_{23} ABCQ + \theta_{24} BPQR + \theta_{25} BCQR + \theta_{26} ABPQ + \theta_{27} CPQR + \theta_{28} ABCPQ + \theta_{29} BCPQR}$$

Mechanism XII Bi Uni Uni Bi Ping Pong



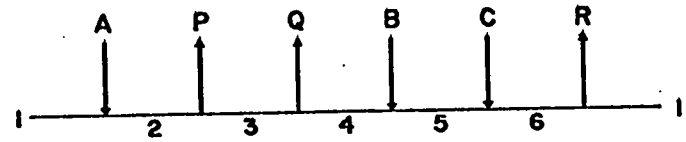
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 C + \theta_4 P + \theta_5 AB + \theta_6 AC + \theta_7 AP + \theta_8 BC + \theta_9 CR + \theta_{10} PR + \theta_{11} QR + \theta_{12} PQ + \theta_{13} CQR + \theta_{14} ABC + \theta_{15} ABQ + \theta_{16} ABP + \theta_{17} APQ + \theta_{18} BQR + \theta_{19} BCR + \theta_{20} PQR + \theta_{21} ABCQ + \theta_{22} ABPQ + \theta_{23} BCQR + \theta_{24} BPQR}$$

Mechanism XIII Bi Bi Uni Uni Ping Pong



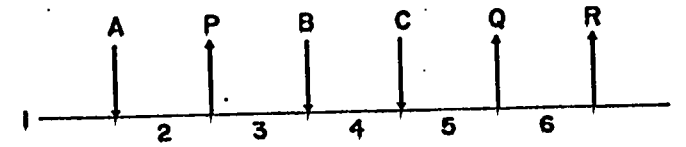
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 C + \theta_4 R + \theta_5 AB + \theta_6 AC + \theta_7 BC + \theta_8 BR + \theta_9 CR + \theta_{10} CP + \theta_{11} PQ + \theta_{12} PR + \theta_{13} QR + \theta_{14} ABC + \theta_{15} ABQ + \theta_{16} ACP + \theta_{17} APQ + \theta_{18} BCR + \theta_{19} BQR + \theta_{20} CPR + \theta_{21} PQR + \theta_{22} ABPQ + \theta_{23} ABCP + \theta_{24} BPQR}$$

Mechanism XIV Uni Bi Bi Uni Ping Pong



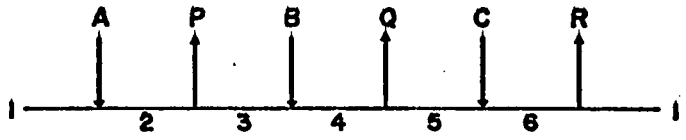
$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 A + \theta_4 R + \theta_5 AB + \theta_6 AC + \theta_7 BC + \theta_8 AQ + \theta_9 PQ + \theta_{10} PR + \theta_{11} BR + \theta_{12} QR + \theta_{13} ABC + \theta_{14} BCR + \theta_{15} BCP + \theta_{16} BPR + \theta_{17} PQR + \theta_{18} CPQ + \theta_{19} APQ + \theta_{20} ACQ + \theta_{21} ABCP + \theta_{22} ACPQ + \theta_{23} BCPR + \theta_{24} CPQR}$$

Mechanism XV Uni Uni Bi Bi Ping Pong



$$\frac{v}{E_0} = \frac{\theta_1 ABC - \theta_2 PQR}{\theta_3 A + \theta_4 P + \theta_5 AB + \theta_6 AC + \theta_7 AP + \theta_8 AQ + \theta_9 BC + \theta_{10} CP + \theta_{12} PQ + \theta_{13} QR + \theta_{14} ABC + \theta_{15} ACP + \theta_{16} APQ + \theta_{17} ABQ + \theta_{18} BCR + \theta_{19} BQR + \theta_{20} CPR + \theta_{21} PQR + \theta_{22} ABCQ + \theta_{23} BCQR + \theta_{24} CPQR}$$

Mechanism XVI Hexa Uni Ping Pong



$$\frac{v}{E_0} = \frac{\theta_1\ ABC - \theta_2\ PQR}{\theta_3 AB + \theta_4 AC + \theta_5 AQ + \theta_6 BC + \theta_7 BR + \theta_8 CP + \theta_9 PQ + \theta_{10} PR + \theta_{11} QR + \theta_{12}\ ABC + \theta_{13} ABQ + \theta_{14} ACP + \theta_{15} APQ + \theta_{16} BQR + \theta_{17} BCR + \theta_{18} CPR + \theta_{19} PQR}$$

TABLE 21: Product inhibition pattern: three-substrate--four-product enzyme reactions.

Product present	Mechanism	Initial rate equation	Kinetic patterns Substrate varied A	B	C
P	I	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+P)+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}(\phi_5+P)+\frac{\psi_6}{ABC}(\phi_6+P)$	N	N	N
P	II	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(\phi_4+P)+\frac{\psi_5}{ABC}(P)$	N	N	N
P	III	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}(\phi_1+P)+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{BC}$	N	U	U
P	IV	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}(\phi_1+P)+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{BC}$	N	U	U
P	V	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+P)+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}(P)+\frac{\psi_6}{ABC}(P)$	N	N	C
P	VI	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(\phi_4+P)$	N	N	U
P	VII	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(\phi_4+P)$	N	N	U
P	VIII	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(P)$	N	C	U
P	IX	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(P)$	N	C	U
P	X	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}(\phi_1+P)+\frac{\psi_2}{B}+\frac{\psi_3}{C}$	C	U	U

TABLE 21 (continued)

Product present	Mechanism	Initial rate equation	Kinetic patterns (Substrate varied) A	B	C
Q	I	$\frac{E_o}{v} = \psi_0(\phi_o+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{AB}+\frac{\psi_4}{BC}+\frac{\psi_5}{ABC}$	U	U	U
Q	II	$\frac{E_o}{v} = \psi_0(\phi_o+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{BC}(\phi_4+Q)$	U	N	N
Q	III	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+Q)+\frac{\psi_3}{C}+\frac{\psi_4}{BC}(\phi_4+Q)$	U	C	U
Q	IV	$\frac{E_o}{v} = \psi_0(\phi_o+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{BC}$	U	U	U
Q	V	$\frac{E_o}{v} = \psi_0(\phi_o+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{BC}$	U	U	N
Q	VI	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{AB}$	U	U	C
Q	VII	$\frac{E_o}{v} = \psi_0(\phi_o+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{AB}$	U	U	U
Q	VIII	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{BC}(Q)$	U	N	C
Q	IX	$\frac{E_o}{v} = \psi_0(\phi_o+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+Q)+\frac{\psi_3}{C}$	U	N	U
Q	X	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+Q)+\frac{\psi_3}{C}$	U	C	U
R	I	$\frac{E_o}{v} = \psi_0(\phi_o+R)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}+\frac{\psi_6}{ABC}$	U	U	U
R	II	$\frac{E_o}{v} = \psi_0(\phi_o+R)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{BC}$	U	U	U
R	III	$\frac{E_o}{v} = \psi_0(\phi_o+R)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+R)+\frac{\psi_4}{BC}(\phi_4+R)$	U	N	N

TABLE 21 (continued)

Product present	Mechanism	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
R	IV	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(\phi_2+R) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	C	U
R	V	$\frac{E_o}{v} = \psi_0(\phi_0+R) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
R	VI	$\frac{E_o}{v} = \psi_0(\phi_0+R) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
R	VII	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3+R) + \frac{\psi_4}{AB}$	U	U	C
R	VIII	$\frac{E_o}{v} = \psi_0(\phi_0+R) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3+R)$	U	U	N
R	IX	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3+R)$	U	U	C
R	X	$\frac{E_o}{v} = \psi_0(\phi_0+R) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(\phi_3+R) + \frac{\psi_4}{ABC}(R)$	U	N	N
S	I	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+S) + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}(\phi_6+S)$	C	N	N
S	II	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
S	III	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
S	IV	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC} + \frac{\psi_5}{AC}(\phi_5+S) + \frac{\psi_6}{ABC}(S)$	C	N	N
S	V	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+S)$	C	N	U

TABLE 21 (continued)

Product present	Mechanism	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
S	VI	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+S)$	C	N	U
S	VII	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+S) + \frac{\psi_5}{ABC}(S)$	C	N	N
S	VIII	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	C	U	U
S	IX	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AC}(S)$	C	U	N
S	X	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+S) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AC}(S)$	C	U	N

TABLE 22: Dead-end inhibition patterns: three-substrate--four-product enzyme reactions.

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
I	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i}) + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}(1+\frac{i}{K_i})$	C	N	N
I	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC}(1+\frac{i}{K_i}) + \frac{\psi_6}{ABC}$	U	C	N
I	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	C
I	[4]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
I	[5]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
I	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
I	[7]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
II	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{1}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
II	[2]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_I}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
II	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{1}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	C	N
II	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{1}{K_i}) + \frac{\psi_4}{BC}$	U	U	C
II	[5]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
II	[6]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
II	[7]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
III	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{1}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
III	[2]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
III	[3]	$\frac{E_o}{v} = \psi_0 (1+\frac{1}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
III	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{1}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}(1+\frac{1}{K_i})$	U	C	N

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns (Substrate varied) A	B	C
III	[5]	$\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{BC}$	U	U	C
III	[6]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
III	[7]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
IV	[1]	$\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
IV	[2]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
IV	[3]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
IV	[4]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
IV	[5]	$\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}(1+\frac{i}{K_i})$	U	C	N
IV	[6]	$\frac{E_o}{v} = \psi_o + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{BC}$	U	U	C
IV	[7]	$\frac{E_o}{v} = \psi_o(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
V	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i})$	C	U	U
V	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	C	U
V	[3]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
V	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{AB}$	U	U	C
V	[5]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
V	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
V	[7]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VI	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i})$	C	N	U
VI	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	C	U
VI	[3]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_2}{A} + \frac{\psi_3}{B} + \frac{\psi_4}{C} + \frac{\psi_5}{AB}$	U	U	U
VI	[4]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
VI	[5]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i}) + \frac{\psi_4}{AB}$	U	U	C
VI	[6]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VI	[7]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VII	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1 + \frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1 + \frac{i}{K_i})$	C	N	U
VII	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1 + \frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	C	U
VII	[3]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VII	[4]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VII	[5]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
VII	[6]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i}) + \frac{\psi_4}{AB}$	U	U	C
VII	[7]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns (Substrate varied) A	B	C
VIII	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1 + \frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	C	U	U
VIII	[2]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
VIII	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1 + \frac{i}{K_i}) + \frac{\psi_3}{C}$	U	C	U
VIII	[4]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_I}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
VIII	[5]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i})$	U	U	C
VIII	[6]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
VIII	[7]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
IX	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1 + \frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	C	U	U
IX	[2]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
IX	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1 + \frac{i}{K_i}) + \frac{\psi_3}{C}$	U	C	U
IX	[4]	$\frac{E_o}{v} = \psi_0(1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U

TABLE 22 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
IX	[5]	$\frac{E_o}{v} = \psi_0 (i + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
IX	[6]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i})$	U	U	C
IX	[7]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
X	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1 + \frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	C	U	U
X	[2]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
X	[3]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
X	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1 + \frac{i}{K_i}) + \frac{\psi_3}{C}$	U	C	U
X	[5]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
X	[6]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i})$	U	U	C
X	[7]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U

TABLE 23: Product inhibition patterns: three-substrate--three-product enzyme reactions.

Product present	Mechanism	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
P	XI	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+P)+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}(\phi_5+P)+\frac{\psi_6}{ABC}(\phi_6+P)$	N	N	N
P	XII	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+P)+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}P+\frac{\psi_6}{ABC}P$	N	N	C
P	XIII	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}(\phi_4+P)$	N	N	U
P	XIV	$\frac{E_o}{v} = \psi_0(\phi_0+P)+\frac{\psi_1}{A}(\phi_1+P)+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{BC}$	N	U	U
P	XV	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{BC}(\phi_4+P)+\frac{\psi_5}{ABC}P$	N	N	N
P	XVI	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}(\phi_2+P)+\frac{\psi_3}{C}+\frac{\psi_4}{AB}P$	N	C	U
Q	XI	$\frac{E_o}{v} = \psi_0(\phi_0+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}+\frac{\psi_4}{AB}+\frac{\psi_5}{BC}+\frac{\psi_6}{ABC}$	U	U	U
Q	XII	$\frac{E_o}{v} = \psi_0(\phi_0+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{AB}$	U	U	N
Q	XIII	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{AB}$	U	U	C
Q	XIV	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_3}{B}(\phi_2+Q)+\frac{\psi_3}{C}+\frac{\psi_4}{BC}(\phi_4+Q)$	U	C	C
Q	XV	$\frac{E_o}{v} = \psi_0(\phi_0+Q)+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{BC}(\phi^4+Q)$	U	N	N
Q	XVI	$\frac{E_o}{v} = \psi_0+\frac{\psi_1}{A}+\frac{\psi_2}{B}+\frac{\psi_3}{C}(\phi_3+Q)+\frac{\psi_4}{BC}Q$	U	N	C

TABLE 23 (continued)

Product present	Mechanism	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
R	XI	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+R) + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}(\phi_6+R)$	C	N	N
R	XII	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+R)$	C	N	U
R	XIII	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(\phi_4+R) + \frac{\psi_5}{AC}R + \frac{\psi_6}{ABC}R$	C	N	N
R	XIV	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC} + \frac{\psi_5}{ABC}R + \frac{\psi_6}{AC}R$	C	N	N
R	XV	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
R	XVI	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(\phi_1+R) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AC}R$	C	U	N

TABLE 24: Dead-end inhibition patterns: three-substrate--three-product enzyme reactions.

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
XI	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i}) + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}(1+\frac{i}{K_i})$	C	N	N
XI	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_5}{BC}(1+\frac{i}{K_i}) + \frac{\psi_6}{ABC} + \frac{\psi_4}{AB}$	U	C	N
XI	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	C
XI	[4]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
XI	[5]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
XI	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB} + \frac{\psi_5}{BC} + \frac{\psi_6}{ABC}$	U	U	U
XII	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i})$	C	N	U
XII	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	C	U
XII	[3]	$\frac{E_o}{V} = \psi_0(1+\frac{i}{K_I}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XII	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{AB}$	U	U	C

TABLE 24. (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
XII	[5]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XII	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XIII	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}(1+\frac{i}{K_i})$	C	N	U
XIII	[2]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	C	U
XIII	[3]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XIII	[4]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XIII	[5]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{AB}$	U	U	C
XIII	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{AB}$	U	U	U
XIV	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
XIV	[2]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
XIV	[3]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U

TABLE 24 (continued)

Mechanism	Dead-end inhibitor binds to enzyme forms:	Initial rate equation	Kinetic patterns (Substrate varied) A	B	C
XIV	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}(1+\frac{i}{K_i})$	U	C	N
XIV	[5]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{BC}$	U	U	C
XIV	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
XV	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	C	U	U
XV	[2]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
XV	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1+\frac{i}{K_i}) + \frac{\psi_3}{C} + \frac{\psi_4}{BC}(1+\frac{i}{K_i})$	U	C	N
XV	[4]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1+\frac{i}{K_i}) + \frac{\psi_4}{BC}$	U	U	C
XV	[5]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
XV	[6]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C} + \frac{\psi_4}{BC}$	U	U	U
XVI	[1]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A}(1+\frac{i}{K_i}) + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	C	U	U
XVI	[2]	$\frac{E_o}{v} = \psi_0(1+\frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U

TABLE 24 (continued)

Mechanism	Dead-end inhibitor binds tp enzyme forms:	Initial rate equation	Kinetic patterns: Substrate varied A	B	C
XVI	[3]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B}(1 + \frac{i}{K_i}) + \frac{\psi_3}{C}$	U	C	U
XVI	[4]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U
XVI	[5]	$\frac{E_o}{v} = \psi_0 + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}(1 + \frac{i}{K_i})$	U	U	C
XVI	[6]	$\frac{E_o}{v} = \psi_0 (1 + \frac{i}{K_i}) + \frac{\psi_1}{A} + \frac{\psi_2}{B} + \frac{\psi_3}{C}$	U	U	U