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L-ASPARAGINE BIOSYNTHESIS AND ITS CONTROL

by

Ting-Chao Chou

A DISSERTATION PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL OF YALE UNIVERSITY IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FROM THE DEPARTMENT OF PHARMACOLOGY

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ABSTRACT

The amount of L-asparagine and L-aspartic acid production by intact cells was determined by an enzymatic assay. L-Asparaginase resistant murine tumor cells produced 1.0-1.6 nmoles L-asparagine/mg of cells/hour while sensitive tumor cells produced less than 0.1 nmole /mg of cells/hour. The newly synthesized L-aspartic acid was largely localized in the cells; the newly synthesized L-asparagine, however, distributed both in the cells and in the medium. The L-asparagine analogs DONV (0.5 mM) and CONV (0.05 mM) inhibited L-asparagine production 86% and 95% respectively; no concurrent inhibition of L-aspartic acid production was observed.

The purification (305-fold) and characterization of L-asparagine synthetase from one of the L-asparaginase resistant tumors, 6C3HED-RG1, is described. Initial velocity studies and product inhibition studies suggest that L-glutamine interacted with free L-asparagine synthetase and released L-glutamic acid, the aminated enzyme then reacted with ATP to release pyrophosphate and form an aminated and adenylated enzyme. The resultant modified enzyme then appears to react with L-aspartic acid to release AMP and L-asparagine. The proposed mechanism was supported by alternate substrate studies and reversibility studies. Dead-end inhibition studies with L-asparagine analogs indicated that DONV competed with the L-glutamine or L-asparagine site on the free enzyme, and that CONV interacted with two or more stable enzyme forms.

L-Asparagine synthetase catalyzed a reaction with three substrates and four products, a Ter-Quad system according to Cleland's notation. Mathematical approaches have been used to analyze the possible kinetic patterns and mechanisms in Cleland's graphical model. Several general equations were derived to express the overall number of patterns and mechanisms at any number of substrates and products; and to express the specific number of patterns and mechanisms at any number of substrates, products and stable enzyme forms. The number of different mechanisms at S substrates, P products and n stable enzyme forms is given by

$$M(n;S,P) = \frac{1}{n}\sum_{d}\phi(d)\begin{pmatrix}\frac{S}{d}-1\\ \\ \frac{n}{d}-1\end{pmatrix}\begin{pmatrix}\frac{P}{d}-1\\ \\ \frac{n}{d}-1\end{pmatrix}$$

where d is summed over the common divisors of S, P and n; and ϕ is Euler's totient function.

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THIS THESIS IS DEDICATED TO

MY MOTHER, SEN-MEI CHEN CHOU

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
PP	Pyrophosphate
L-Asn	L-Asparagine
L-Asp	L-Aspartic acid
L-Gln	L-Glutamine
L-Glu	L-Glutamic acid
DONV	5-Diazo-4-oxo-L-norvaline
CONV	5-Chloro-4-oxo-L-norvaline
BONV	5-Bromo-4-oxo-L-norvaline
D-DONV	5-Diazo-4-oxo-D-norvaline
ONV	4-Oxo-L-norvaline
TCA	Trichloroacetic acid
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
DTT	Dithiothreitol

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I. INTRODUCTION

The discovery by Kidd in 1953 (1) of a tumor-inhibitory factor in guinea-pig serum and its identification by Broome in 1961 (2-5) as L-asparaginase has resulted in the development of intense interest in the mechanism of action of this enzyme and the metabolism of L-asparagine (6-9).

It has been shown by many investigators that only certain types of leukemic cells are sensitive to L-asparaginase (1,3-5,10-16). These results, coupled with the independent finding that some leukemic cells require L-asparagine in tissue culture (4, 17-20), led to the suggestion that the capability of cells to synthesize L-asparagine is inversely related to the sensitivity of the tumor to L-asparaginase. The major goals for this thesis are the investigation of L-asparagine biosynthesis and its control as well as development of theoretical methods for analyzing this type of problem, especially multiple-substrate reactions. Some specific goals have been achieved and others partially fulfilled depending upon the intrinsic properties of the problems and the individual priorities of solving such problems. The objectives are:

A. Studies on intact cells:

1. Devise a rapid assay method for L-asparagine production by intact cells.

2. Further studies of L-asparagine production by L-asparaginase-sensitive and resistant tumor cells.

3. Apply the above method as a potential test of sensitivity to L-asparaginase.

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4. Study the distribution of newly synthesized L-asparagine (and L-aspartic acid) in the cells and in the medium.

5. Study the uptake and incorporation of exogenous L-asparagine (and L-aspartic acid) by cells.

6. Analyze the effect of L-asparagine and its analogs on the production of L-asparagine by cells.

B. Studies on L-asparagine synthetase:

1. Study L-asparagine biosynthesis in cell-free extracts (105,000 x g supernatant fraction) from various sources.

2. Purification of L-asparagine synthetase from tumors resistant to L-asparaginase.

3. Study the physical and catalytic properties of L-asparagine synthetase.

4. Kinetic investigations of L-asparagine synthetase and deduction of the kinetic mechanism (reaction sequence) of this enzyme.

5. Study the mechanisms of inhibitions of L-asparagine biosynthesis by L-asparagine and its analogs.

C. Pattern analysis on a biochemical model:

1. Analyze the possible number of patterns and mechanisms in enzyme catalyzed reactions with multiple substrates and products.

2. Derive general formulae to express the over-all number of patterns and mechanisms with any given number of substrates and products.

3. Derive general formulae to express the specific number of patterns and mechanisms at any given number of stable enzyme forms and at any given number of substrates and products.

4. Use a different approach (mathematical induction) independently to solve the above problems.

5. Apply the derived general formulae for stepwise deduction of kinetic mechanisms (reaction sequences).

6. Present the mathematical implications of the derived general formulae and the relationship of general formulae derived from two independent approaches.

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The results obtained from the studies on L-asparagine production in intact cells have been presented at the ASPET Fall meeting, 1968 (21), and a manuscript for publication is in preparation. A portion of the preliminary results obtained from the studies on L-asparagine synthetase has been presented at the FASEB meeting in 1968 (22) and the First Graduate Research Conference in Genetics and Cell Biology, 1969 (23).

II. REVIEW OF THE LITERATURE

A. HISTORICAL BACKGROUND

Since the discovery in 1806 of asparagine in asparagus shoots by Vauguelin and Robiquet, (24),its significance in plants has been repeatedly investigated. Asparagine was found to accumulate in the seedling axis, in which most growth takes place (25). Little was known about the biosynthesis of L-asparagine before the 1950's. Mardashev and Lestrovaya in 1951 (26) suggested that asparagine is formed by amide nitrogen transfer from glutamine to aspartic acid. Re-investigation of this work failed to confirm the synthesis of asparagine and led to alternate explanations (27). Webster in 1955 (28) reported that preparations of wheat germ catalyzed β-aspartyl hydroxamate formation from L-aspartate and hydroxylamine and demonstrated the incorporation of ^{14}C-aspartic acid into asparagine in the presence of magnesium, ATP and ammonia, However, the incorporation was extremely low and net synthesis of L-asparagine was not observed. Levintow in 1957 (29) showed conversion of the amide nitrogen of glutamine

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to the amide nitrogen of asparagine in growing cultures of HeLa cells. Cells grown in the presence of ^{15}N-amide labeled glutamine incorporated significant quantities of the isotope into the asparagine of protein. Experiments carried out with $^{15}NH_4Cl$ revealed that significant quantities of the isotope were not incorporated into the amide group of asparagine. It is unlikely that a permeability barrier to externally supplied ammonia exists, since, when HeLa cells were grown on media containing $^{15}NH_4Cl$, the intracellular ammonium ion pool became enriched with ^{15}N. The total concentration of ammonium ion plus ammonia is believed to be exceedingly low in mammalian cells and probably also in other cells. At physiological conditions, it is probable that asparagine biosynthesis involves a more direct utilization of the amide nitrogen atom of glutamine.

An alternative pathway for asparagine biosynthesis was discovered by Blumental-Blodschmidt et al (30), who demonstrated that ^{14}C-atoms from ^{14}C-labeled HCN supplied to several types of seedlings became incorporated almost exclusively into the amide-C of asparagine. Subsequent work indicated that many other plant species incorporated ^{14}C-cyanide almost entirely into a dipeptide, γ-glutamyl-β-cyano-L-alanine, in which β-cyanoalanine was the primary labeled product. Ressler in 1963 (31) showed that β-cyano-L-alanine-4-^{14}C provides the amide carbon of asparagine in certain species of Lathyrus and Vetch. L-Asparagine synthetase has been purified

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10-fold from Lactobacillus arabinosus by Ravel and co-workers (32), and 21-fold from Streptococcus bovis by Burchall (33). In both laboratories, it was found to be in accord with the reaction:

$$\text{L-aspartic acid} + NH_3 + \text{ATP} \xrightarrow{\text{cation}^{++}} \text{L-asparagine} + \text{AMP} + \text{PP} \qquad \text{(II-1)}$$

S. bovis asparagine synthetase has a requirement for Mg^{++} and Mn^{++} cannot be substituted, whereas Mn^{++} is more active than Mg^{++} with the L. arabinosus enzyme.

Arfin (34) reported asparagine synthesis from aspartic acid and glutamine in the liver of thirteen to seventeen day old chicken embryos. This reaction was dependent upon both the mitochondrial and supernatant fractions. Studies in our laboratory, however, showed that the 105,000 x g supernatant fraction alone was needed to catalyze L-asparagine synthesis when a proper concentration of ATP is applied. General interest in L-asparagine metabolism in mammalian cells, however, was greatly increased by the important observations of Kidd (1) and Broome (2-5).

1. Kidd's Phenomenon: In 1953, Kidd (1) reported experiments initially designed to study immunological responses to cancer. He wanted to see if antiserum taken from rabbits immunized with mouse leukemia would affect the growth of the leukemia in the mice. In addition to injecting tumor bearing mice with rabbit antiserum, he injected guinea-pig serum as a source of complement to augment antibody reactions. To his surprise, even with guinea-pig serum injection alone, the leukemia of these

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mice regressed and in some instances permanent cures were obtained. He reached three important conclusions concerning his observations. First, he showed that among the sera from guinea-pigs, rabbits, horses and human subjects only guinea-pig serum was effective. Since the therapeutic effect was still present in the guinea-pig serum which had been heated at 56°C for 30 minutes, the effect apparently was not mediated by complement. Second, he established that the guinea-pig serum apparently affected only malignant cells, producung no toxic side effects to normal tissues. Third, he found that the guinea-pig serum affected some transplanted leukemias but not others. The unaffected group included all the newly transplanted leukemias treated, this at first made it appear that the only responsive leukemias were those that had had a long history of transplantation.

2. Broome's Discovery: While the findings of Kidd were still a fascinating puzzle and were not widely appreciated at the time, Broome in 1961 (2) made a breakthrough. He noted that Clementi in 1922 (35) reported that the blood of guinea-pigs contained an asparagine destroying enzyme which was absent from the blood of many other animals tested. Broome then began experiments that left no doubt that L-asparaginase in the guinea-pig serum wasthe antileukemic factor responsible for the phenomenon observed by Kidd. The strength of Broome's conclusions lay in his use of evidence from two independent experimental approaches. First, he showed one of the same trans-

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planted leukemias studied by Kidd that the antileukemia property of guinea-pig serum could not be distinguished from L-asparaginase activity by a variety of physical and chemical methods, indicating by these criteria the possible identity of these activities (3). Second, he showed that this line of leukemia needed a supply of L-asparagine in order to grow in culture (4).

3. L-Asparagine synthetase activity and the therapeutic effect of L-asparaginase: Independent of Broome's work, attempts to define unusual nutritional requirements of neoplastic cells in culture led to the discovery by Neuman and McCoy (17) that the Walker 256 tumor in culture required L-asparagine to sustain growth in culture. Similarly, Haley, Fischer and Welch (18) seeking to replace the requirement for enzymatically digested casein in the growth of L5178Y lymphoblastic leukemia cells in culture, found that L-asparagine was one of the essential factors contributed by this material. It is important to note that most normal and neoplastic cells, as typified by those that will grow in Eagle's medium (which does not contain asparagine), do not have an absolute nutritional requirement for this amino acid (19).

The logical conclusion drawn from the above three sections is that L-asparaginase sensitive tumor cells may either lack the ability to make L-asparagine or are deficient in this capacity. The confirmation of this hypothesis required the demonstration of L-asparagine synthesis in mammalian systems.

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4. A survey on the literature of L-asparagine synthesis: The first report on a mammalian system was by Patterson and Orr (36) who indicated that an enzyme preparation from Jensen sarcoma had a requirement for adenosine triphosphate (ATP), glutamine and aspartic acid. Recently, they have purified the enzyme from the Novikoff hepatoma and showed the alternative utilization of ammonia at a high pH (37). Broome and Schwartz (38) first reported that, in a whole cell system, sensitive tumors have a reduced ability to make L-asparagine when compared to sublines selected for resistance to L-asparaginase. This kind of study has been extended in more detail in this laboratory (21). Numerous reports have appeared correlating the therapeutic effect of L-asparaginase against experimental neoplasms with low or unmeasureable levels of L-asparagine synthetase in cell-free preparations (36,38,39). Low or intermediate levels of synthetase activity are found in extracts of normal tissues, with significantly higher levels in testes and brain (39). L-Asparagine is a potent feedback inhibitor of the enzyme from the Novikoff hepatoma and the 6C3HED-R lymphoma (14,37,40), and recent work in this laboratory indicates a competitive relationship with the L-glutamine or NH_3 site (23). Interestingly, L-asparagine synthetase from guinea-pig liver is reported to be insensitive to feedback inhibition by L-asparagine (41). The amount of synthetase activity in certain tissues increases in response to metabolic stress, such as treatment with L-asparaginase (40), feeding a diet deficient in L-asparagine and the presence of a rapidly

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growing tumor (42). However, a reciprocal relationship between the growth of an L-asparaginase sensitive 6C3HED tumor and the concentration of L-asparagine in the plasma has been reported (43). Therefore, L-asparagine synthetase may either undergo an adaptive change or no change due to high constitutive synthetase activity in resistant tumors, or a deficiency of this enzyme in sensitive tumors. From a hemodialysis study and a broad survey of patients there appears to be rigorous homeostatic control of the plasma concentrations of L-asparagine in a wide variety of disease states in man (6,44). The locus and mechanism of this homeostatic control is still under study (45). While a detailed study of mammalian L-asparagine synthetase is still lacking, this enzyme isolated from Escherichia coli has recently been characterized to a great extent and the reaction sequence been clarified (46). However, the coliform L-asparagine synthetase differs both in physical and kinetic properties when compared with the enzyme isolated from 6C3HED-RG1 lymphoma cells as will be described later. For a more detailed review of L-asparaginase and L-asparagine metabolism, consult a review article written by Cooney and Handschumacher (6).

B. CLELAND'S KINETIC THEORY

Cleland has proposed a general method to analyze possible mechanisms of enzyme-catlyzed reactions with more than one substrate or product (47). This general method has been widely used (48). Since L-asparagine synthetase catalyzes a reaction (in the presence

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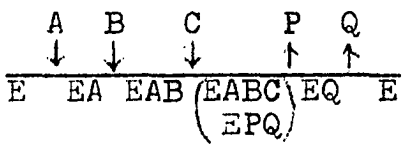
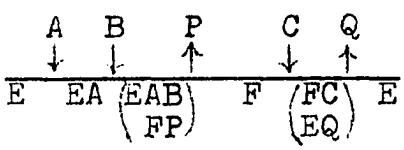
of Mg^{++} ion) with L-aspartic acid, L-glutamine and ATP as substrates giving L-glutamic and, AMP PP and L-asparagine as products, according to Cleland's designation the reaction is a Ter-Quad system. Attempts have been made to apply Cleland's theory to L-asparagine synthetase isolated from 6C3HED-RG1 lymphoma cells in this dissertation. For the convenience of later discussion, some of Cleland's nomenclature and rules are briefly presented here.

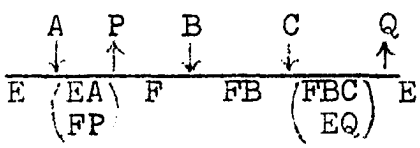
1. Nomenclature: According to Cleland (47) an enzyme catalyzed reaction with three substrates and two products is designated a Ter-Bi system which shows three patterns in graphical presentation if the reaction is a non-random sequence with a central complex(es):



(ordered Ter-Bi) (II-2)



(Bi-Uni-Uni-Uni ping pong) (II-3)



(Uni-Uni-Bi-Uni ping pong) (II-4)

in which A, B, C .... refer to substrates; P, Q, R .... refer to products,and E, F, G .... refer to stable enzyme forms which are incapable of a unimolecular reaction with the liberation of a substrate or product. EA, EAB .... etc. are the transitory complexes of the enzyme. Transitory complexes which cannot participate in bimolecular reaction steps with

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substrates or products, but can only undergo unimolecular degradation with release of substrates or products are called cen ral complexes; e.g. (EABC), (FP) .... etc. Of the three possible patterns in the Ter-Bi system, if the sequence of (II-3) is started at F it becomes (II-4) and so these two ping pong mechanisms are really the same and give rate equations of identical form (47). The number of kinetically important substrates or products in a mechanism was designated by the syllables Uni, Bi, Ter, Quad ..... The order of addition of substrates and release of products within the reaction sequence was described as follows: Mechanisms where all substrates must add to the enzyme before any products are released was designated "sequential". Such mechanisms were called "Ordered" if the substrates add in an obligatory order and the products leave similarly, and "Random" if the substrates do not react in obligatory order and alternate reaction sequences exist. When one or more products are released before all substrates have added to the enzyme, the enzyme will exist in two or more stable forms between which it oscillates during the reaction. Such mechanisms were called "Ping Pong". Isomerization of transitory enzyme forms may form a part of the reaction sequence without changing the rate equations. If isomerization of stable enzyme forms is a part of the reaction sequence, however, additional terms are present in the rate equations, and such mechanisms were designated "Iso Ordered", "Iso Random", and "Iso Ping Pong" to indicate the stable form isomerization.

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2. Rate equations: Steady-state rate equations are derived by writing an expression for the rate of change of concentration with time for each enzyme form in the mechanism. These expressions include rate constants for individual steps as well as the concentrations of reactants.

In the steady-state these rates of change are assumed to be very small compared with the rate at which the reactant concentration is changing, and are thus set equal to zero, giving a series of linear equations that express the concentrations of the various enzyme forms. Simultaneous solution of these equations yields distribution equations for the concentration of various enzyme forms, from which specific rate equations for the overall reaction are easily obtained. Although this procedure is straightforward in theory, it is always very laborious in practice. The method of King and Altman allows the steady-state rate equations for mechanisms of considerable complexity to be written down in terms of the individual rate constants without going through complex algebraic expansions of the large number of determinants (65). By defining one "inhibition constant" for each reactant in the mechanism, Cleland has been able to transform rate equations expressed in terms of rate constants into equations expressed entirely in terms of measureable kinetic constants (47,48).

3. The rules for prediction of reaction patterns: Cleland (47) has proposed rules for predicting initial velocity patterns. Two possible patterns are observed when reciprocal velocities are plotted against reciprocal

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substrate concentrations: if no irreversible step intervenes between the points of combination of the two substrates in the reaction sequence, the reciprocal plots will intersect to the left of the vertical axis; but if an irreversible step occurs in the reaction sequence between the times of combination of the two substrates, the reciprocal plots are parallel. An irreversible step would be release of a product not present initially, or the addition of a saturating amount of substrate. It is also possible to predict by inspection the inhibition patterns expected for any given mechanism (47). The slope of a reciprocal plot is affected whenever the inhibitor combines with the same enzyme form as the variable substrate, or whenever the points of combination of the inhibitor and the variable substrate with the enzyme in the reaction sequence are not separated by an irreversible step. The intercept is affected whenever the inhbitor combines with a different enzyme form than the variable substrate, and saturation with the variable substrate cannot overcome the inhibition. The latter can occur only in mechanisms without a central complex such as the Theorell-Chance mechanism (49). Isotope exchange studies have also been useful to identify the order of addition and release of reactant (48).

III. MATERIALS AND METHODS

A. MATERIALS

1. Chemicals and Radiochemicals: The following asparagine analogs were prepared by Dr. P. K. Chang and

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R. E. Handschumacher in this Department: 5-diazo-4-oxo-L-norvaline (DONV) (50); 5-diazo-4-oxo-D-norvaline (D-DONV); 5-chloro-4-oxo-L-norvaline (CONV); 5-bromo-4-oxo-L-norvaline (BONV) and L-β-aspartyl hydrazide. L- and D- aspartic acid, L- and D- asparagine and all other amino acids, reduced nicotinamide adenine dinucleotide (NADH). β-Cyanoalanine, L-2,4-diaminobutyric acid, L-methionine DL-sulfoximine, L-norvaline, L-cysteine sulfinic acid were obtained from Calbiochem Inc. DL-β-Methyl aspartic acid, reduced glutathione, p-chloromecuribenzoic acid (Na) were obtained from Nutritional Biochemicals Corp. N-Acetyl-L-aspartic acid was obtained from Sigma Chemical Co. and 5,5'-dithiobis-(2-nitrobenzoic acid).(DTNB) was obtained from Aldrich Chemical Co. All mono-, di- and tri-nucleosides were obtained from P. L. Biochemicals Inc. Uniformly labeled ^{14}C-L-aspartic acid (154 mc/mmole) and ^{14}C-L-asparagine (150 mc/mmole) were obtained from New England Nuclear Corp.

2. Enzymes: Glutamate-oxaloacetate transaminase and malate dehydrogenase were purchased from Boehringer & Suehne, G. m. b. H., Germany. L-Asparaginase was kindly supplied by Squibb & Sons, New Jersey.

3. Cell Culture Medium: Eagle's Minimal Essential Medium with Earle's salts (Grand Island Biochemical Co.) contained vitamins, inorganic salts, dextrose, phenol red and the following concentrations of amino acids (mg/liter): L-arginine HCl, 21.1; L-cystine, 12.0; L-histidine HCl H_2O, 10.5; L-isoleucine, 26.2; L-leucine, 26.2; L-lysine

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HCl, 36.5; L-methionine, 7.5; L-phenylalanine, 16.5; L-threonine, 23.8; L-tryptophan, 4.0; L-tyrosine, 18.1 and valine, 23.4 (51). Before use the medium was supplemented to give a final concentration of 2 mM glutamine, 10 units/ml heparin, and 0.05mM L-aspartic acid.

4. Cell Counting and Weighing: Cell number and volume were determined with a Coulter Electronic Cell Counter (Hialeah, Florida). Model B, with a size distribution plotter Model H. The aperature tube pore was 100μ. Cell samples were diluted in 0.9% saline. Packed cells were obtained by centrifugation at 800 x g for 5 minutes and were weighed. Extracellular fluid remaining on the cell-pack was assumed to be 20% of the total weight and was subtracted from the measured value.

5. Measurement of Radioactivity: All determinations of radioactivity were made in a Packard Tri-Carb Liquid Scintillation Spectrometer using a liquid scintillation phosphor of the following composition: 2, 5-diphenyloxazole (Pilot Chemicals, Inc.), 4 g; P-bis2-(5-phenyloxazolyl)-benzene (Pilot Chemicals, Inc.) 50 mg; Cab-O-Sil (Cabot Corp.) 15 g; toluene 1000 ml, and absolute ethanol, 500 ml.

6. Tumor Lines and Sources of Enzyme Preparation: The tumor cells (and host animal) used in these studies were P815Y leukemic mast cells (AKD_2F_1 mice), L5178Y lymphoblastic leukemic cells (AKD_2F_1 mice), and Sarcoma 180 cells (Swiss mice) which were routinely maintained in this laboratory. The L-asparaginase sensitive and its resistant subline of 6C3HED lymphosarcoma cells (C3H mice), designated

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6C3HED-S and 6C3HED-R respectively, were kindly provided by Dr. J. D. Broome and L-asparaginase resistant 6C3HED-RG1 cells of C3H/J mice were kindly provided by Dr. J. G. Kidd. The Novikoff hepatoma was maintained in Holtzman albino rats and kindly provided by Dr. A. B. Novikoff. The cell line used for routine whole-cell studies was the P815Y mastocytoma. It was selected because of its rapid and reproducible growth as well as its relatively high rate of L-asparagine biosynthesis. The 6C3HED-RG1 tumor was used as a source for the purification and characterization of L-asparagine synthetase.

B. METHODS

1. Tumor Transplantation: Ascites fluid from mice was collected and centrifuged at 800 x g for 5 min. The supernatant fluid was removed and the packed cells washed three times with Fischer's medium (52) which was kindly provided by Dr. Glenn A. Fischer of this department. Solid tumors excised free of connective and necrotic elements, were minced with scissors and pressed with a porceline pestle through a mortar shaped stainless steel sieve (40 mesh) which was kindly provided by Dr. J. G. Kidd. Tumors were transplanted by the intraperitoneal route for whole cell studies and the subcutaneous route in both groins for the preparation of L-asparagine synthetase. Each mouse received 1 to 2 million cells suspended in 0.1 ml of Fischer's medium supplemented with 10 per cent horse serum. All procedures were performed asceptically.

2. Assay of L-Aspartic Acid and L-Asparagine Production

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by Intact Cells: Tumor cells from ascites fluid were collected by centrifugation and washed three times at 25° with Eagle's medium containing 10 units per ml of heparin. Unless otherwise stated, each incubation contained 4 ml of a suspension of cells (10 mg/ml) in the same medium supplemented with L-aspartic acid (0.05 mM) and L-glutamine (2 mM). The cell suspension was incubated in precleaned glass scintillation counting vials at 37° with moderate shaking. One ml of the suspension was withdrawn at zero time and various intervals after incubation, pipetted into capped polycarbonate Spinco tubes and immediately heated for 10 min in a boiling water bath. After centrifugation at 105,000 x g for 1 hour, the supernatant fluid was assayed for L-aspartic acid and L-asparagine content according to the enzymatic method of Cooney and Handschumacher (43,44). In this method the conversion of L-aspartic acid to oxaloacetate by glutamate-oxaloacetate transaminase, and reduction of the keto acid by malic dehydrogenase with consequent oxidation of NADH is employed. The amount of L-asparagine was determined after adding a small amount of purified L-asparaginase into the same assay mixture to covert it to L-aspartic acid. The net increase of L-aspartic acid and L-asparagine at various times after incubation was calculated by subtracting the zero time values. Reproducibility was within 5% if cell suspensions of 20 mg/ml were used. Up to 25 assays could be performed in 2 hours. Each sample remained in the same cuvette throughout the entire assay procedure.

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3. Purification of ^{14}C-L-Aspartic Acid and ^{14}C-L-Asparagine: Dowex 1-x8 (200-400 mesh) anionic exchange resin in the chloride form (J. T. Backer Chemical Co.) was converted to formate form by the following procedure: The resin was washed three times with 5 volumes of distilled water. The resin was packed in a column (0.9 cm x 30 cm) and 2 N NaOH passed through until no chloride was detectable in the effluent. The column was washed with distilled water until the effluent reached a neutral pH, and conditioned with at least six column volumes of an equal part mixture of 2 N sodium formate and 2 N formic acid. The column was then washed with distilled water until effluent was free of taste. ^{14}C-L-asparagine was neutralized and adsorbed on the column and eluted with distilled water. The radioactive peaks in the fractions were monitored and the purity of ^{14}C-L-Asparagine was checked with high voltage electrophoresis as described in METHODS. ^{14}C-L-Aspartic acid was neutralized and adsorbed onto the column and the impurities eluted with distilled water. ^{14}C-L-Aspartic acid was then eluted with 0.05 N formic acid. Radiopurity as high as 99.8% was obtained by these methods.

4. Incorporation of ^{14}C-L-Aspartic Acid and ^{14}C-L-Asparagine into Acid-Insoluble Fractions: Uniformly labeled ^{14}C-L-asparagine (150 μc/μmole, 0.1 μmole) was added to the incubating cell suspension at zero time. After various periods of incubation, a portion of the cell suspension was heated in a boiling water bath for 10 min and centrifuged at 105,000 x g for 30 min. The supernatant fraction was assayed for

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radioactivity, L-asparagine content and the specific activity of the L-asparagine pool calculated. To the other portion of the incubated cell suspension, one volume of 5% TCA was added. The acid-insoluble residue was washed three times with 3 ml of 5% TCA containing 10 mM of unlabeled L-asparagine on a Millipore filter (0.22 μ pore diameter). The radioactivity in the insoluble residue was determined in a liquid scintillation counter and with appropriate correction for quenching. The values of incorporation were obtained from the following formula:

$$\frac{\text{cpm in acid-insoluble fraction at the end of incubation / mg of cells}}{\text{cpm / nmole at the end of incubation}} -$$

$$\frac{\text{cpm in acid-insoluble fraction at the beginning of incubation / mg of cells}}{\text{cpm / nmole at the end of incubation}} =$$

$$\frac{\text{net incorporation of L-Asn or L-Asp (nmole) into acid-insoluble fraction / mg of cells}}{\text{time unit}} \qquad \text{(III-1)}$$

The incorporation of ^{14}C-L-aspartic acid into acid-insoluble residue was similarly determined.

5. Assay of L-Asparaginase Activity: L-Asparaginase activity was assayed either with an enzymatically coupled oxidation of reduced pyridine nucleotide (43,44), or assayed with Nessler's reagent (53).

6. L-Asparagine Synthetase Preparations: The detailed procedure of purification of L-asparagine synthetase from 6C3HED-RG1 will be described on page 53. The chick-embryo liver L-asparagine synthetase was obtained from 14 to 17 day chick embryos (fertile leghorn eggs were purchased

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from Hall Brothers Hatchery, Inc., Wallingford, Conn.) The embryo livers, not including the gall bladders, were removed and homogenized with 3 volumes of Tris-HCl Buffer 0.1 M pH 8.0 in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15,000 x g for 10 min and the resulting supernatant fraction was further centrifuged in a Spinco ultracentrifuge (model L, Specialized Instrument Corp.) at 105,000 x g for 60 min. The synthetase activity was localized at the final supernatant fraction. The rat-embryo liver L-asparagine synthetase was obtained by a similar procedure except that 18 to 20 day embryos from Sprague Dawley rats were used.

7. Assay of L-Asparagine Synthetase Activity: In the standard assay procedure, the reaction mixture contained L-aspartic acid, 1.5 mM; ^{14}C-L-aspartic acid, 1.5 x 10^6 dpm (154 mcuries/mole); L-glutamine, 20 mM; ATP, 10 mM; $MgCl_2$, 12 mM; dithiothreitol, 5 mM; enzyme and Tris-HCl buffer, 0.1 M (pH 7.8) in a final volume of 1.0 ml. After incubation at 37°C with a mild shaking for 30 min, the reaction was stopped by heating in a boiling water bath for 10 min. The boiled mixture was then centrifuged at 10,000 x g for 10 min and 50 µl of the supernatant fluid was spotted on a filter paper sheet for high voltage electrophoresis as described in the following section. The percent conversion of ^{14}C-L-aspartic acid to ^{14}C-L-asparagine was calculated with Programa 101 electronic calculator (Olivetti-Underwood).

8. High Voltage Electrophoresis: A portion (50 µl) of the boiled reaction mixture was spotted on a Whatman 3-MM

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paper sheet and electrophoresis (cold-plate electrophoresis apparatus, Savant Instruments, Inc.) was performed at 30 volts/cm for 90 min in 0.15 M sodium phosphate buffer, pH 7.0. ^{14}C-L-Asparagine remained at the origin while ^{14}C-L-aspartic acid migrated toward the anode. The location of ^{14}C-L-asparagine was confirmed by treatment of a portion of the sample with a highly purified L-asparaginase obtained from E. coli (EC-II). After this treatment radioactivity originally localized at the origin migrated to the position of ^{14}C-L-aspartic acid. The relative positions of some of the components in the reaction mixture are shown in Figure 1. Unlabeled compounds were used to localize the radioactivity or ultraviolet absorbance of the corresponding compounds. The paper sheet was cut into small square pieces and the radioactivity counted with a liquid scintillation counter. The percent conversion of ^{14}C-L-aspartic acid into ^{14}C-L-asparagine was calculated and this percentage was multiplied by the total amount of L-aspartic acid (unlabeled and labeled) at the beginning of the incubation to give the net amount of L-asparagine that was synthesized.

9. Protein Determination: The method of Lowry et al (54) was used to determine the protein concentration. Dessicated bovine albumin, fraction V (Nutritional Biochemicals Corp.) was used as a protein standard.

10. Measurement of Sulphydryl Groups: Ellman's method (55) was used to determine sulfhydryl groups. Buffer blanks were used in control experiments, and the reaction mixture contained 0.1 ml of 5,5'-dithiobis-

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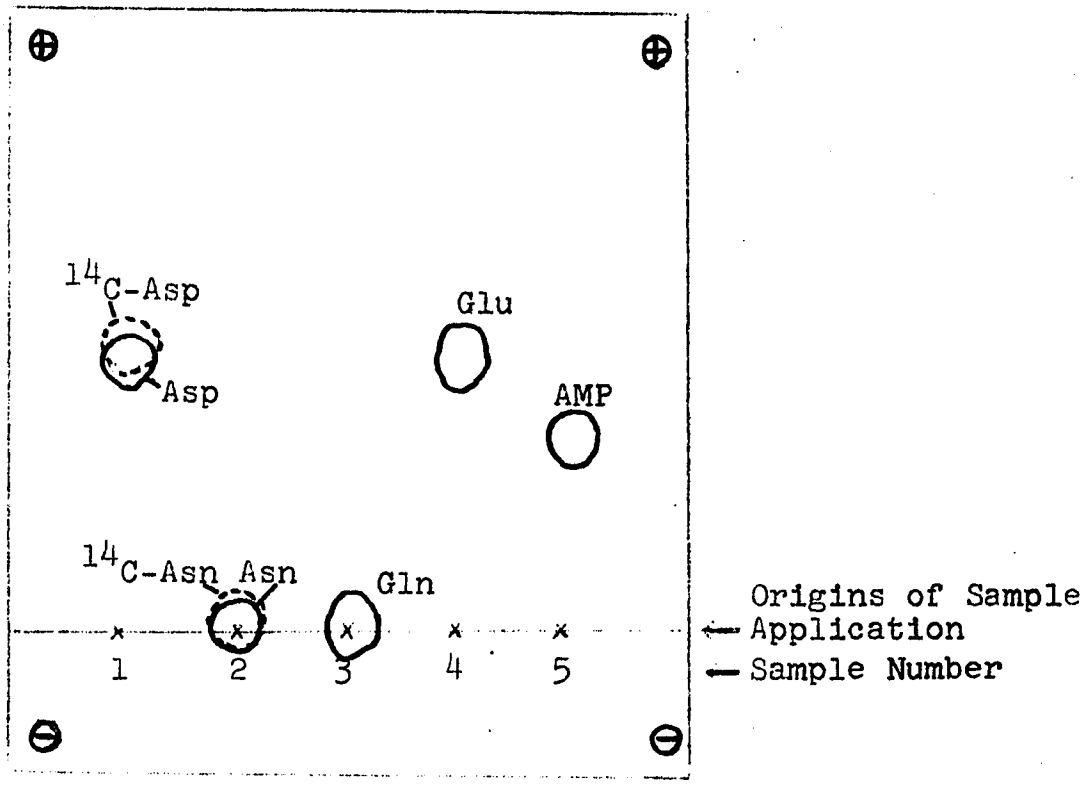

Figure 1. The relative positions of components in the L-asparagine synthetase reaction mixture after high voltage electrophoresis.

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(2-nitrobenzoic acid) (DTNB, 10^{-2} M) in potassium phosphate buffer, 1.0 M (pH 7.5); 0.05 ml each of the sulphydryl compound (5 mM), the other reagents or distilled water, and 3 ml of potassium phosphate buffer 1.0 M, pH 7.0. The absorbance was read at 410 mμ with a Beckman double beam spectrophotometer (model DB) 5 min after mixing all constituents. Whenever incubation (37°C, 30 min) was used, DTNB was added 5 min before reading the absorbance.

IV. EXPERIMENTAL RESULTS AND DISCUSSION

A. L-ASPARAGINE BIOYSNTHESIS IN INTACT CELLS

1. L-Aspartic Acid and L-Asparagine Production in Murine Tumor Cells: The optimal production of L-asparagine by the tumor cells tested required supplementation of the incubation medium with L-glutamine (2 mM). Only a minor increase in the production of asparagine was observed when L-aspartic acid (0.05 mM) was added (Table 1). In some experiments with L-asparaginase sensitive lines an apparent loss of free L-asparagine from the incubation mixture was observed. This may be attributed to lower biosynthesis and higher utilization of L-asparagine. It was found that cell suspensions of 10 mg/ml or less were optimal for L-aspartic acid and L-asparagine production; higher concentrations of cells caused the decrease of the production of these two amino acids, particularly after one hour (Figure 2).

Figure 3 shows the time course of the production of L-aspartic acid and L-asparagine in various murine tumor cells. L5178Y lymphoblastic leukemic cells, P815Y leu-

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TABLE 1.

Net Production of L-Aspartic Acid and L-Asparagine by P815Y Cells in Eagle's Medium With or Without Supplementation With L-Glutamine and L-Aspartic Acid

	L-aspartic acid		L-asparagine	
	30 min	60 min	30 min	60 min
	nmole per mg of cells		nmole per mg of cells	
Eagle's Medium	0.06	-0.03	0.07	-0.03
Eagle's Medium +0.05M L-aspartic acid	-0.07	0.23	0.23	0.16
Eagle's Medium +2.0mM L-Glutamine	1.29	2.38	0.62	1.00
Eagle's Medium +2.0 mM L-Glutamine +0.05 mM L-aspartic acid	1.26	2.37	0.67	1.25

Figures are the average of two assays in which 1% cell suspensions were used. Detailed procedures are described in METHODS.

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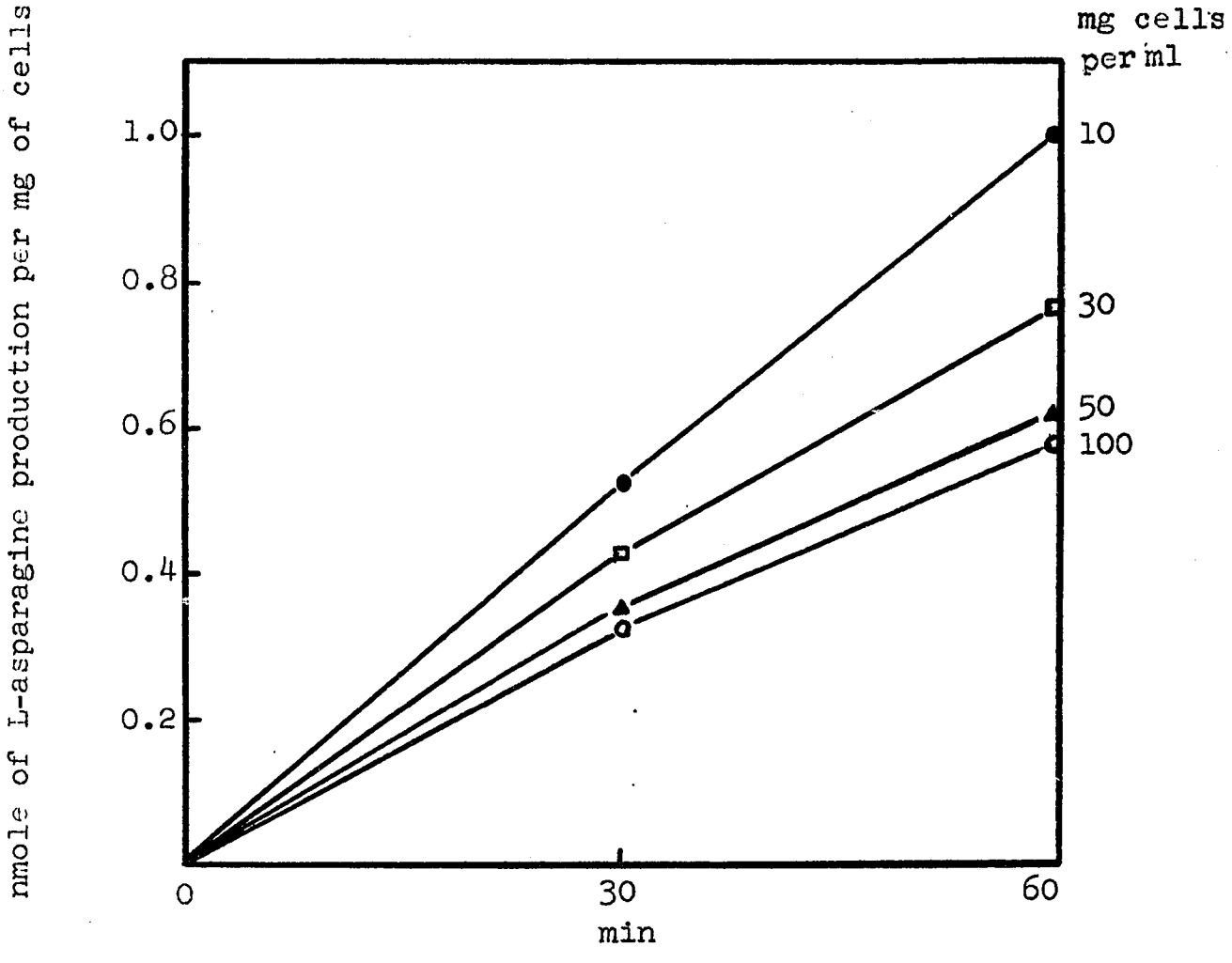

Figure 2. L-Asparagine production at different concentrations of P815 cells. The assay procedure is as described in METHODS. Cell concentrations are: 10 mg/ml (●——●), 30 mg/ml (□——□), 50mg/ml (▲——▲) and 100 mg/ml (○——○).

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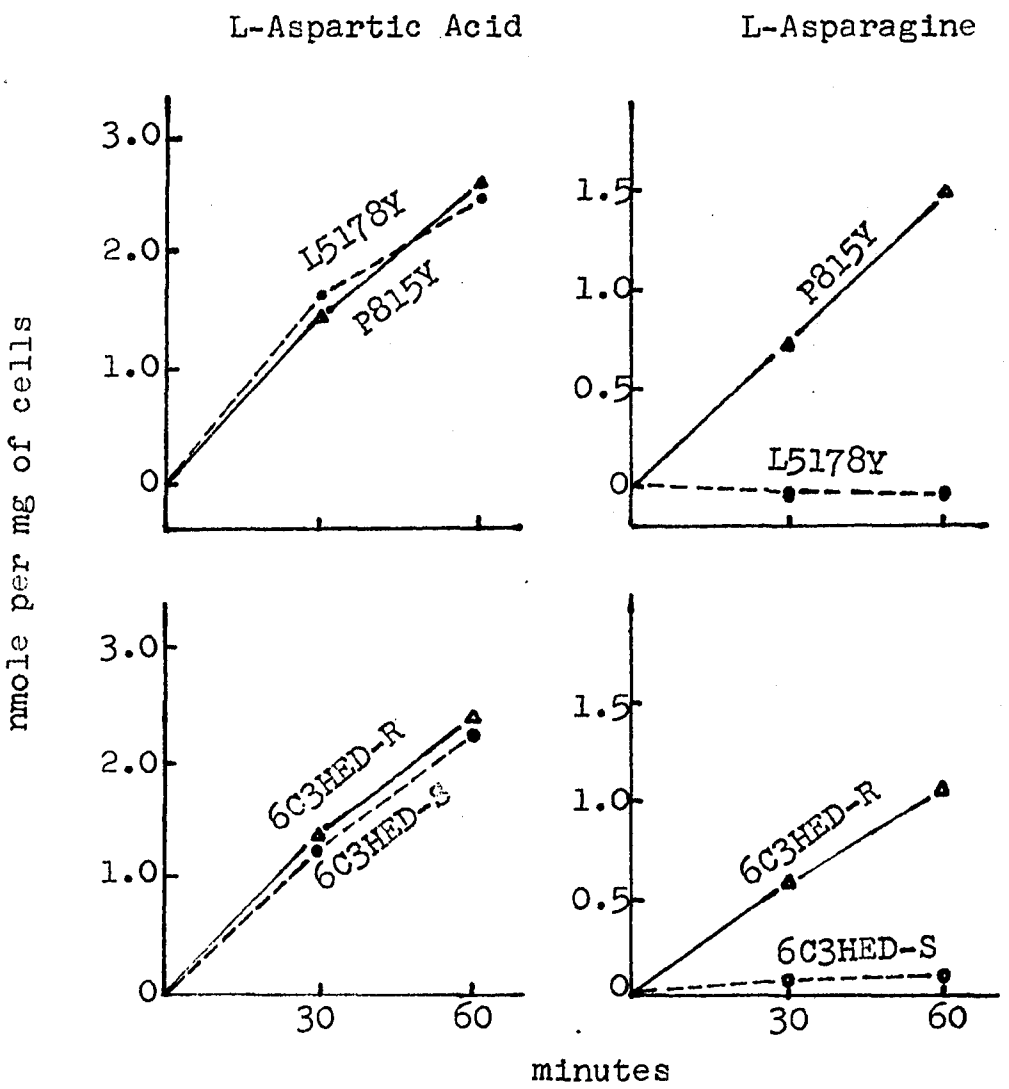

Figure 3. Time course of L-aspartic acid and L-asparagine production in murine tumor cells. One percent cell suspensions were used for all experiments as described in METHODS.

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kemic mast cells or 6C3HED-S lymphosarcoma cells synthesized similar amounts of L-aspartic acid (ca. 2.3 nmole/mg/hr). L-Asparagine biosynthesis in these same cell lines, however, was quite different. L-Asparaginase-sensitive cells such as 6C3HED-S and L5178Y were relatively unable to synthesize L-asparagine (i.e. less than 0.1 nmole/mg/hr), while the L-asparaginase-resistant cells (P815Y leukemia) synthesized large amounts of L-asparagine (i.e. 1.4 nmole/mg/hr).

The L-asparagine biosynthesis in sensitive and resistant tumor cells grown in the same strain of mice was also studied. Prolonged treatment of L-asparaginase-sensitive 6C3HED-S cells with sublethal doses of L-asparaginase causes the emergence of an L-asparaginase-resistant subline, 6C3HED-R cells (38). Again, only the L-asparaginase-resistant subline elaborated significant amounts of L-asparagine (Fig. 3). Similar results have been seen by Broome and Schwartz by using a different assay method (38). Other resistant tumor lines such as the 6C3HED-RG1 tumor or Sarcoma 180 in mice or the Novikoff hepatoma in rats produced significant amounts of L-asparagine (Table 2), a result consistant with the data from cell-free extracts that L-asparaginase-resistant tumor cells can synthesize this amino acid except in the case of extracts from the P815Y tumor which are discussed later in this thesis (Table 8) (23).

2. <u>The Distribution of Newly Synthesized L-Aspartic Acid and L-Asparagine in Cell Suspensions</u>: When the incubation

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TABLE 2

L-Aspartic Acid and L-Asparagine Production in Various Tumor Cells[a]

Tumor Cells	Host Strain	Net Production of L-Aspartic Acid	Net Production of L-Asparagine	L-asparaginase Sensitivity[c]
		nmoles/mg of cells/hr		
Mouse				
P815Y	AKD_2F_1	3.51±0.27[b]	1.33±0.09[b]	Resistant
6C3HED-S	C3H	1.82[d]	0.02[d]	Sensitive
6C3HED-R	C3H	2.21	1.15	Resistant
6C3HED-RG1	C3H/HEJ	2.11	1.32	Resistant
L5178Y	AKD_2F_1	2.64	-0.18	Sensitive
Sarcoma 180	Swiss	3.01	0.54	Resistant
Rat				
Novikoff hepatoma	Holtzman albino	3.67	1.38	Resistant

[a] In each experiment tumor cells from 2 to 5 animals were pooled. One percent cell suspensions were used.

[b] Figures represent the mean value ± standard error. (14 assays were performed)

[c] P815Y and L5178Y cells were tested in tissue culture in Fischer's medium (see ref.52). The sources of other L-asparaginase-sensitive and resistant cells were indicated in MATERIALS AND METHODS.

[d] Average values from two experiments.

mixture of P815Y cells (1% suspension) was separated into the supernatant medium and the cells by centrifugation at 800 x g for 5 min at 25%, the newly synthesized L-aspartic acid was largely localized in the cells compared to the medium (326:1); the newly synthesized L-asparagine, however, was more evenly distributed in the cells and in the medium (8:1). (Fig. 4). A similar pattern of distribution was observed with the 6C3HED-R, 6C3HED-RG1 and Novikoff hepatoma cells.

3. Effects of L-Asparagine Analogs and Structurely Related Compounds on L-Asparagine and L-Aspartic Acid Production in P815Y Cells: Since L-asparaginase is believed to exert its therapeutic effect by eliminating L-asparagine from the vascular space, it may also be desirable to find compounds which may inhibit L-asparagine biosynthesis within cancer cells. Many asparagine analogs, i.e. 5-diazo-4-oxo-L-norvaline (DONV), 5-chloro-4-oxo-L-norvaline (CONV) and bromo-4-oxo-L-norvaline (BONV) have been synthesized (56) and their effects on L-asparagine and L-aspartic acid biosynthesis has been studied. One of these, DONV, had no effect on L-aspartic acid biosynthesis but was a good inhibitor of L-asparagine biosynthesis. It exhibited a dose-inhibition relationship when its concentration was increased from 0.1 mM to 0.5 mM (Fig. 5).

Table 3 indicates the inhibition of L-asparagine and L-aspartic acid production in P815Y cells by asparagine analogs. The L-form DONV inhibited L-asparagine biosynthesis quite selectively, however the D-isomer of DONV had no

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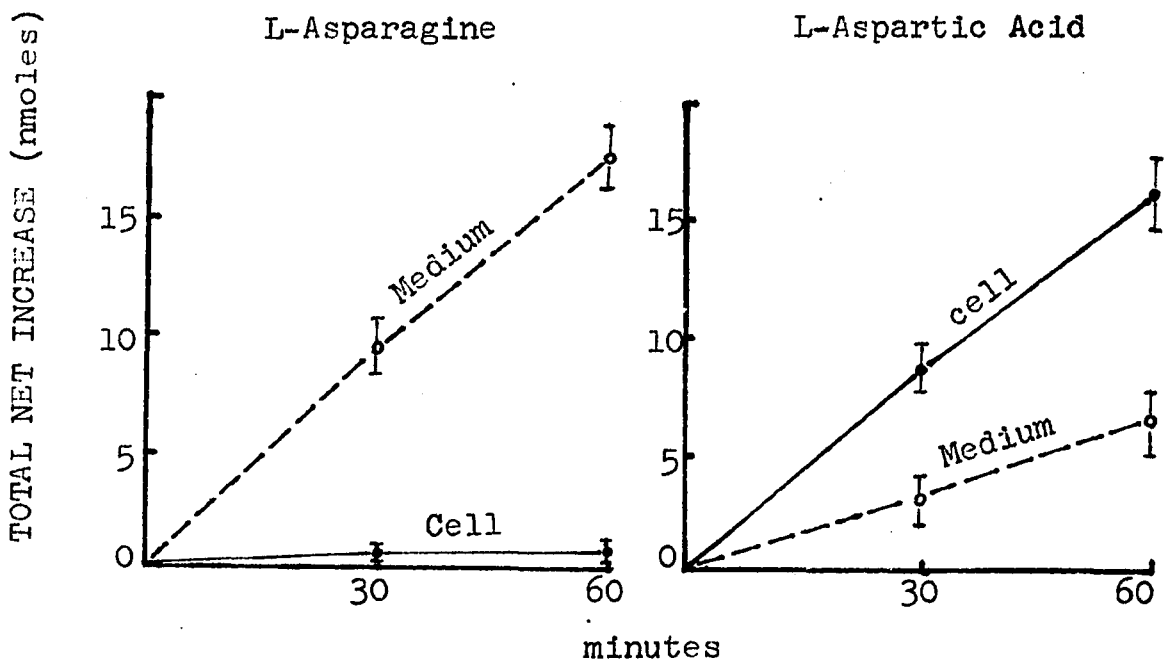

Figure 4. Distribution of newly synthesized L-aspartic acid and L-asparagine in P815 cells and the medium. Each point on the chart represents the value in a 2.0 ml sample. At zero time, the total L-aspartic acid in the medium (2.0 ml) was 100 nmoles, and in the cells (16mg) was 18.6 nmole; total L-asparagine in the same medium was 10.2 n-mole and in the cells was 2.4 nmole. All values were obtained from the average of four experiments ± standard error.

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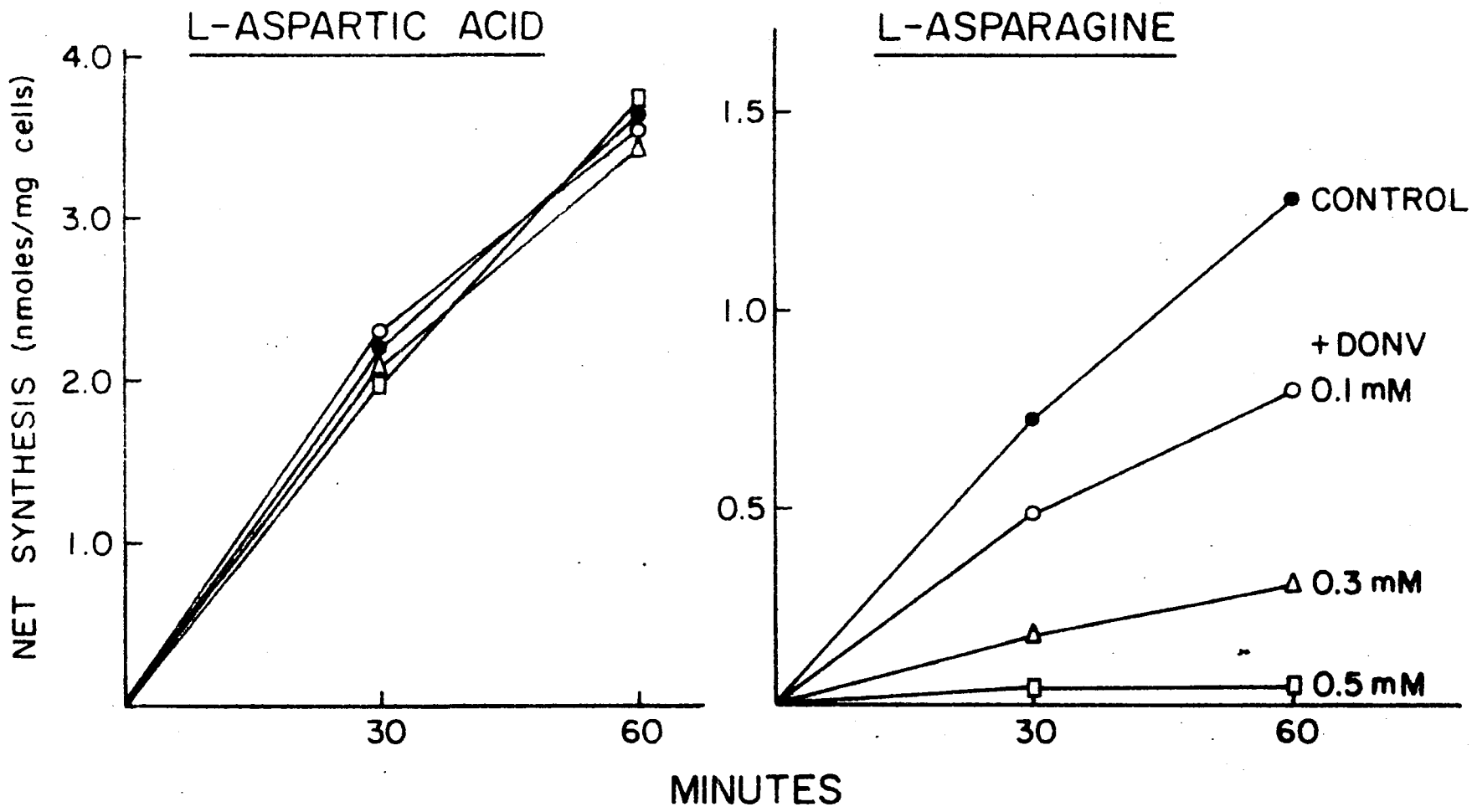

Figure 5. Inhibition of L-aspartic acid and L-asparagine production in P815Y cells by DONV. All figures represent net synthesis in the incubation mixture (cells and medium).

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TABLE 3

Inhibition of L-Aspartic Acid and L-Asparagine Production in P815Y Cells by 4-oxo-norvaline Analogs of Asparagine

Compound	Concentration	Percent Inhibition[a]			
		Without preincubation		With preincubation[b]	
		L-aspartic acid	L-asparagine	L-aspartic acid	L-asparagine
5-Diazo-4-oxo-L-norvaline	$1x10^{-4}$	0	51	12	40
	$5x10^{-4}$	5	88	23	92
5-Diazo-4-oxo-D-norvaline	$5x10^{-4}$	0	0	0	0
5-Chloro-4-oxo-L-norvaline	$1x10^{-6}$	2	9	5	2
	$1x10^{-5}$	0	62	0	20
	$5x10^{-5}$	0	95	3	100
5-Bromo-4-oxo-L-norvaline	$1x10^{-5}$	0	23	11	19
	$1x10^{-4}$	52	78	67	86
5-Hydroxy-4-oxo-L-norvaline	$5x10^{-4}$	0	0	0	0

[a] Percent inhibition of the net increase of L-aspartic acid and L-asparagine were measured at the end of one hour incubation. One percent cell suspensions were used and the reaction next was assayed as described in METHODS.

[b] The cell suspensions were preincubated for 30 min with the inhibitors and glutamine but without aspartic acid.

effect on either L-asparagine or L-aspartic acid biosynthesis. CONV was an even more potent inhibitor. BONV was only slightly more potent as an inhibitor of L-asparagine biosynthesis than the L-form DONV; but it also caused some inhibition of L-aspartic acid biosynthesis. The hydrolysis product of these L-form analogs, 5-hydroxy-4-oxo-L-norvaline, did not affect the biosynthesis of either amino acid at the concentrations tested.

Other compounds with structures related to asparagine were also tested for their effects on L-asparagine and L-aspartic acid production by suspension of P815Y cells. The results were summarized in Table 4. L-Asparagine was the most potent inhibitor of its own biosynthesis. At 10^{-4} M L-asparagine, almost no net increase of L-asparagine could be observed in P815Y cell suspensions. The concentration for 50% inhibition was 2.5×10^{-5} M (2 5 nmole/ml) which was equivalent or somewhat lower than the concentration in the plasma or ascites fluid of the animals (Table 5 and Fig. 6). These results suggest that in whole cells the metabolic effect of the feedback inhibition by L-asparagine is important. With the preparation of asparagine synthetase from 6C3HED-RG1, a potent product inhibition was observed (Fig. 6). In contrast, D-asparagine, L-aspartic acid or D-aspartic acid at the level of 5×10^{-4} M had no effect on either L-asparagine or L-aspartic acid production by intact P815Y cells. The glutamine analog, 6-diazo-5-oxo-L-norleucine (1×10^{-4} M) which does not inhibit L-asparaginase (50) was a potent inhibitor of L-asparagine biosynthesis

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TABLE 4

Inhibition of L-Aspartic Acid and L-Asparagine Production in P815Y Cells by Aspartic Acid or Asparagine Analogs

	Concentration (M)	Percent Inhibition[a]	
		L-Aspartic Acid	L-Asparagine
L-Asparagine	$1x10^{-4}$	0	99
D-Asparagine	$5x10^{-4}$	0	3
L-Aspartic acid	$5x10^{-4}$	0	0
D-Aspartic acid	$5x10^{-4}$	5	0
4-Oxo-L-norvaline	$5x10^{-4}$	7	0
L-Norvaline	$5x10^{-4}$	0	4
6-Diazo-5-oxo-L-norleucine	$1x10^{-4}$	0	98
L-Aspartic-β-chloroethylamine	$1x10^{-4}$	0	0
	$5x10^{-4}$	4	14
DL-β-Methyl aspartic acid	$5x10^{-4}$	0	0
S-Methyl-L-cysteine sulfoximine	$5x10^{-4}$	0	8
S-Carbamyl-L-cysteine	$5x10^{-4}$	0	5
L-Cysteine sulfinic acid	$5x10^{-4}$	0	2
L-2,4-Diaminobutyric acid	$5x10^{-4}$	2	10

[a] Percent inhibition of the net increases of L-aspartic acid and L-asparagine were measured at the end of one hour incubation. Compounds were added to the one percent cell suspensions at the beginning of the incubations.

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TABLE 5

Level of L-Asparagine and L-Aspartic Acid in Solid Tumor, Ascites Fluid and Plasma of Animals

	L-Asparagine	L-Aspartic Acid
	(μg/gm of tumor)	
Solid Tumor[a]		
6C3HED-R	36	35
6C3HED-S	26	49
	(nmoles/ml)	
Ascites fluid[b]		
6C3HED-RG1	51	45
P815Y	44	37
	(nmoles/ml)	
Plasma[c]		
Mouse	29	10
Rat	77	23
Dog	56	3
Man	51	3

[a] A freshly removed tumor was weighed and immediately boiled with distilled water (1g/2ml) for 15 min and then homogenized with Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105,000 x g for 60 min. The resulting supernatant was assayed for L-aspartic acid and L-asparagine with an enzymatic method (43,44).

[b] Ascites fluid was separated from tumor cells by centrifugation at 800 x g for 5 min and boiled for 15 min. The boiled fluid was then centrifuged at 105,000 x g for 60 min. The resulting supernatant was assayed with an enzymatic method (43,44).

[c] The data were kindly provided by Cooney and Handschumacher (44).

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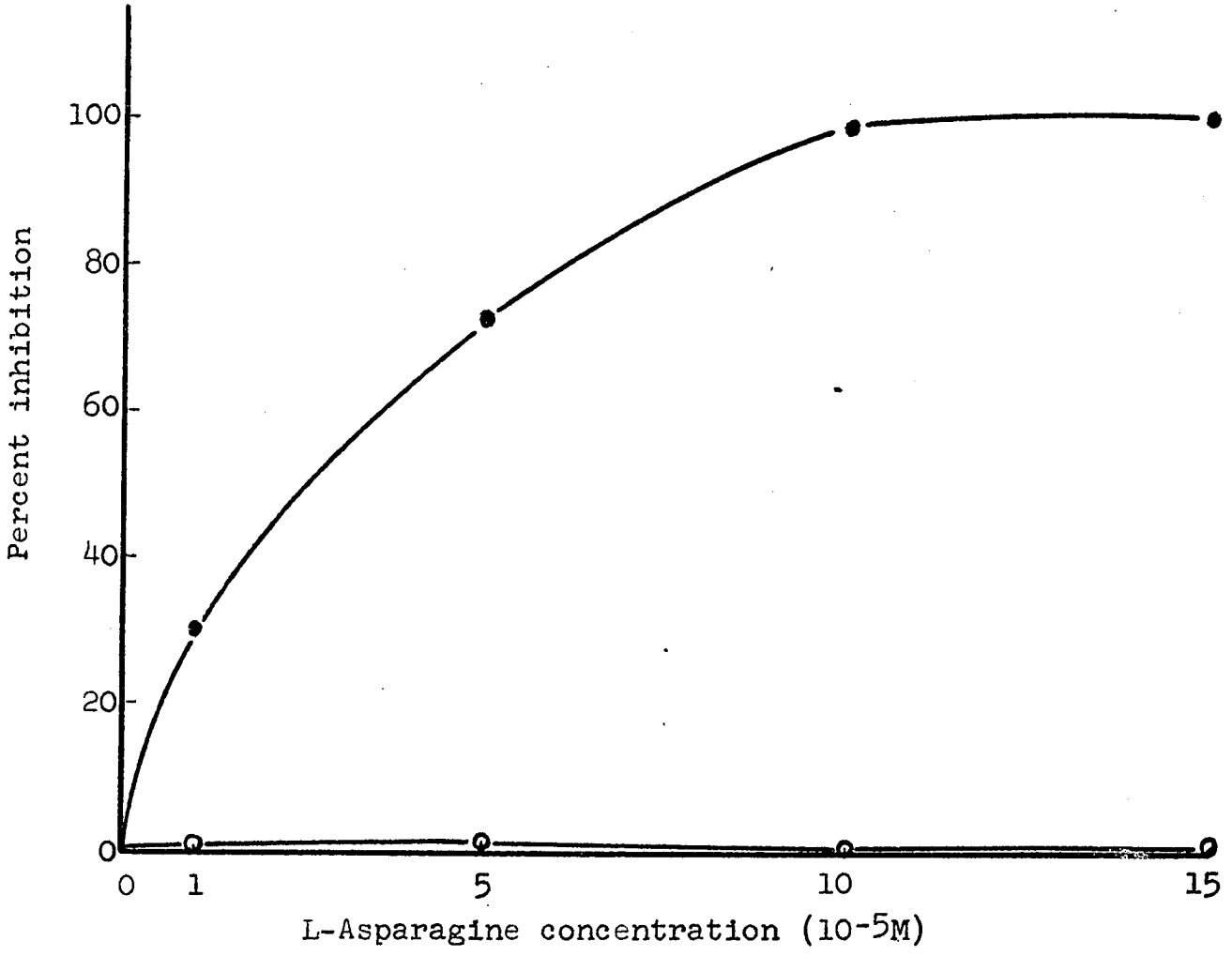

Figure 6. Effects of L-asparagine on L-asparagine and L-aspartic acid production. Effect on L-asparagine production (•——•), effect on L-aspartic acid production (o——o). P815Y cell suspensions (1 mg/ml) were used with the assay procedure described in METHODS.

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in the whole cell system (98% inhibition), a result consistant with the postulated role of L-glutamine as the amide donor in this reaction. Aspartic-β-chloroethylamine was a relative weak inhibitor of L-asparagine biosynthesis. S-Carbamyl-L-cysteine had been shown by Adamson and Fabro (57) to have minimal antitumor activity but this analog did not inhibit L-asparagine or L-aspartic acid biosynthesis in this system.

4. Incorporation of L-Asparagine and L-Aspartic Acid into the Acid-Insoluble Fraction: When tracer amounts of uniformly labeled ^{14}C-L-asparagine (3 nmoles/ml) were added to a 1% suspension of P815Y or 6C3HED-RG1 cells, the total radioactivity in the medium decreased while the total amount of radioactivity in the cells increased with time. Thus, L-asparagine is in a state of efflux concurrent with active inward transport presenting a dynamic bidirectional flux of the amino acid across the cell membrane. The specific activity of the asparagine in the medium decreased even more sharply while in the cells it increased (Fig. 7). Under these conditions, about 0.40and 0.45 nmoles of L-asparagine were incorporated into the acid-insoluble fractions of 6C3HED-RG1 and P815Y cells per mg per hour respectively (Fig. 8). When uniform labeled ^{14}C-L-aspartic acid was added to the same cell suspensions, at least 98% of the radioactivity remained in the medium with no appreciable increase in the radioactivity in the cells during a one hour incubation. Unmeasurable amounts of radioactivity were incorporated from ^{14}C-aspartic acid into the

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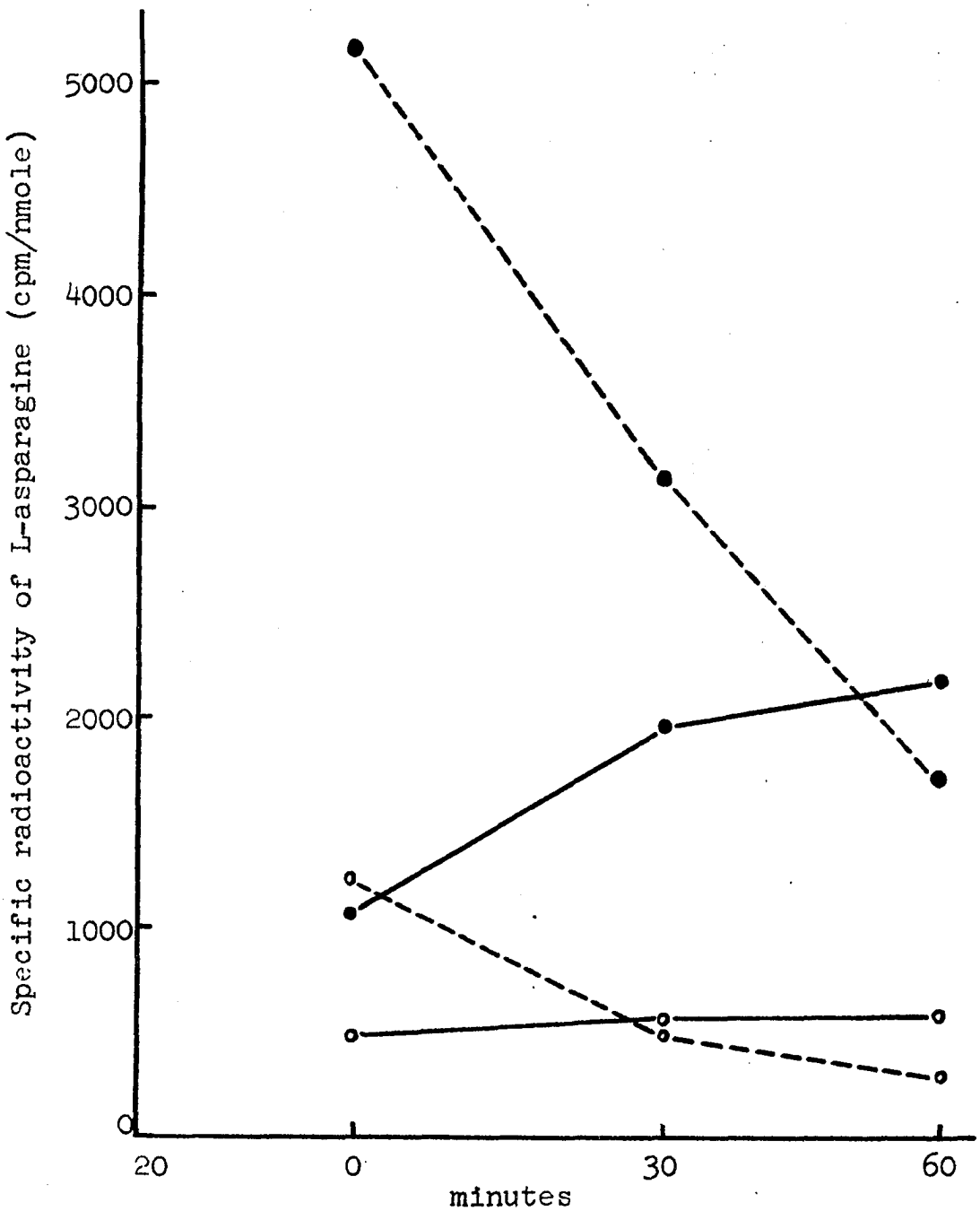

Figure 7. Time course of the change of specific radioactivity of L-asparagine in P815Y cells and the medium. Specific radioactivity in the cells: •——•, 10 mg/ml cell suspension; o——o 100 mg/ml cell suspension. Specific radioactivity in the medium: •- - - -• , 10 mg/ml cell suspension; o- - - -o , 100 mg/ml cell suspension. Exogenous ^{14}C-L-asparagine was added 20 min before separation of cells from medium and heating the samples in a boiling water bath at zero time.

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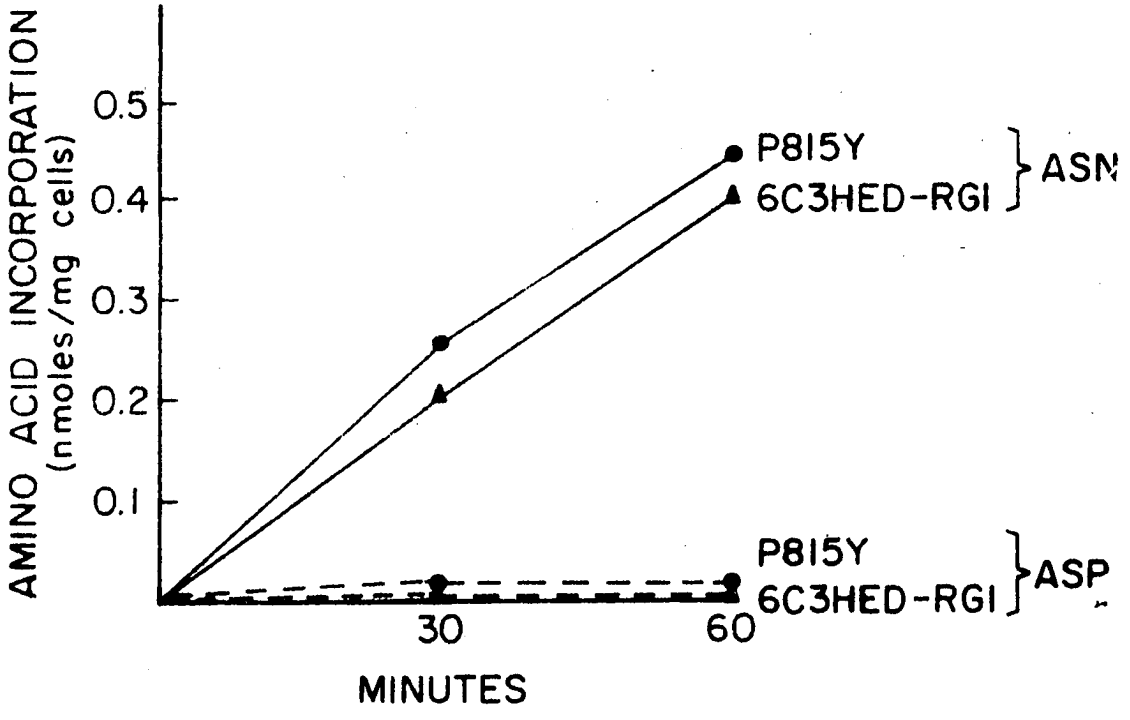

Figure 8. Incorporation of L-asparagine and L-aspartic acid into the acid-insoluble fraction of P815Y and 6C3HED-RGl cells. Uniformly labeled ^{14}C-L-asparagine or ^{14}C-L-aspartic acid was added to 1% cell suspensions. Incorporation of amino acids was calculated by measuring the changes in specific activities of the L-asparagine and L-aspartic acid in the cells and determining radioactivity in the acid-insoluble residue as described in METHODS.

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acid-insoluble fractions of both the P815Y and 6C3HED-RG1 cells (Fig. 8). Addition of L-asparaginase (0.7 IU/ml) to 1% suspension of P815Y cells at the beginning or at the end of one hour incubation caused a 23% increase in the sum of L-aspartic acid and L-asparagine produced by the system (in the cell plus in the medium). These results suggested that the elimination of newly released L-asparagine in the medium caused a significant increase of the production of L-asparagine (or L-aspartic acid) by the cells.

5. Discussion: Since both L-aspartic acid and L-asparagine production can be assayed in every sample, it is advantageous to monitor the production of L-aspartic acid as a measure of cell's normal condition and viability. All of the tumor cells studied produced similar amounts of L-aspartic acid (Table 2). Since the net increase of L-aspartic acid and L-asparagine after incubation of cell suspensions were measured by subtracting the zero time values, the result represents the algebraic sum of their biosynthesis, incorporation and metabolism. By measuring ^{14}C-L-asparagine incorporation into the acid-insoluble fraction and coupling this with the direct assay of L-asparagine, the specific radioactivity of L-asparagine throughout the incubation period could be approximated. On this basis, P815Y cells incorporated at least 0.45 nmole of L-asparagine into the acid-insoluble fraction in one hour (Fig. 8), in addition to the net production of 1.40 nmole of L-asparagine in the whole system during the same

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period. No detectable amount of exogenous ^{14}C-L-aspartic acid entered P815Y cells or was incorporated into acid-insoluble fractions (Fig. 8).

Although L-asparagine biosynthesis has been studied in both neoplastic tissue extracts or partially purified enzyme preparations, the present studies indicate that the relative L-asparagine synthetase activity in tissue extracts may not necessarily reflect the real L-asparagine producing capability of the intact cells. In at least one case there was not a consistent relationship between the capability of cells to produce L-asparagine and the L-asparagine synthetase in the cell-free extract (Table 2). A cell-free extract (105,000 x g supernatant fraction) from P815Y cells showed low L-asparagine synthetase activity, comparable to extracts from the sensitive 6C3HED-S line; however, intact P815Y cells, an asparaginase resistant form of leukemia in mice, produced as much as L-asparagine as other L-asparaginase resistant tumor cells. Possible explanations are that the homogenization procedure destroyed cell compartmentation and may thus have disturbed a regulatory mechanism. Alternatively, the L-asparagine synthetase in the different types of cells might have different properties or catalyze L-asparagine biosynthesis by a different reaction; or, L-asparaginase synthetase in P815Y cells might be bound to cell membranes or organelles and not extracted into the supernatant fraction. The possibility that P815Y cells might contain L-asparaginase was excluded by the direct assay. Experiments in which equal amounts of extracts

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from P815Y and 6C3HED-RG1 cells were mixed did not indicate the presence of inhibitory factors in the extracts of the P815Y cells. When different cell fractions such as centrifugation fractions, ammonium sulfate precipitating fractions or gel filtration fractions from P815Y tumor were pooled, the amount of L-asparagine synthesized was not significantly different from that of the synthetase active fraction alone. This indicates that there is no measureable specific inhibitory factor for L-asparagine biosynthesis in the cell other than the products of its own synthesis. The possibility that P815Y cells incorporated more L-asparagine into protein than other cells was also excluded by the isotopic studies. These unusual relationships are further discussed in the next section with evidence in support of the third possibility, different cellular distributions of synthetase activity.

The possibility still remains that there is another pathway in the tissue to provide L-asparagine for protein synthesis. It is possible that a transamidation reaction takes place after L-aspartic acid has been attached on transfer-RNA which is specific for L-asparagine, thus no free L-asparagine step for protein synthesis is necessary. This has been reported by Nierenberg and coworkers (58) as an alternative synthetic pathway for glutamine. If L-asparagine is formed by a similar pathway, there would be virtually no free L-asparagine detectable, but no results in support of this concept has been reported.

Direct assay of L-asparagine and L-aspartic acid showed that practically all newly synthesized L-aspartic acid was

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localized in the cells while the newly synthesized L-asparagine distributed in both the cells and the medium with an approximately 8-fold higher concentration in the cells. These findings were consistent with the results obtained from using tracer amounts of ^{14}C-L-aspartic acid or ^{14}C-L-asparagine; in which ^{14}C-L-aspartic acid was excluded from the P815Y cells while ^{14}C-L-asparagine was concentrated by the cells from the medium. The fact that a certain fraction of L-asparagine is released into the medium from the cell may be related to the finding that some cultured mammalian cells have a population-dependent requirement for some amino acids including L-asparagine (19).

Tissue slices from normal rat liver, kidney, spleen or brain liberated virtually no L-asparagine into the incubation medium under the conditions of these assays possibly due to their higher metabolism of this amino acid. This is of interest since it has been shown that each of these tissues has measurable amounts of asparagine synthetase in cell-free extracts. No detectable amount of L-asparaginase has been found in the tumor cells used for these studies but L-asparaginase activity was detected in the rat and guinea-pig liver as reported by others (40,59). Hsu (27) reported the formation of L-glutamine from L-asparagine and L-glutamic acid in animal tissues including pigeon, rat or rabbit liver, pigeon brain and rabbit kidney. However, ammonium chloride could be utilized for the same purpose in lieu of L-asparagine. The excess production and

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accumulation of L-asparagine and L-aspartic acid in the neoplastic cells might be related to a lack of proper regulation of this process in the malignant cell.

The rate of L-asparagine biosynthesis in many human leukemic cells assayed by the method presented appeared to be low* but this may be complicated by the low viability of cells taken from patients for these assays. Further modification of the conditions to study human leukemic cells is under study but preliminary results have not been encouraging. Hopefully an improved method would give results comparable or complementary to those obtained in a test currently employed for detecting potential sensitivity to L-asparaginase by measuring valine and uridine incorporation in the presence and absence of L-asparagine (61-63).

In using the procedures described in this paper to evaluate inhibitors of asparagine biosynthesis, it is essential that they not interfer with the enzyme assay used in the determination of L-aspartic acid and L-asparagine. Although L-DONV was a specific inhibitor of L-asparaginase (50), the total L-asparagine could still be determined by prolonging the time to allow the complete conversion of L-asparagine to L-aspartic acid in the assay mixture. Of all L-asparagine analogs studied, none was a significant inhibitor or a substrate for glutamate-oxaloacetate transaminase or malate dehydrogenase and

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none interfere with the assay of L-aspartic acid and L-asparagine significantly (Table 6) except β-cyanoalanine which caused absorbance changes equivalent in amounts to those produced by L-asparagine. This may relate to the observation that β-cyanoalanine is a precursor of L-asparagine biosynthesis in certain plants (31). Later experiments by Cooney and Jackson in this laboratory indicated that L-asparaginase catalyzed a reaction which converted β-cyanoalanine to L-aspartic acid. Table 6 also indicates that the analogs used had no significant contamination of L-aspartic acid or L-asparagine and that the enzymatic assay is very specific for both L-aspartic acid and L-asparagine. Both the incubation medium for the whole cells and the incubation mixture for cell-free extracts contained relatively high concentrations of L-glutamine (2 mM and 20 mM respectively). These could minimize the effect of DONV and L-asparagine because our kinetic studies indicated that DONV or L-asparagine competed for the L-glutamine site (see Figs. 16, 22).

B. STUDIES ON L-ASPARAGINE SYNTHETASE

1. L-Asparagine Synthetase Activity in 105,000 x g Supernatant Fractions from Various Sources: L-Asparagine synthetase activity was detected in the cell-free extracts (105,000 x g supernatant) from various sources including embryonic livers of chick and rat, as well as L-asparaginase resistant tumors such as Novikoff hepatoma of the rat, 6C3HED-R and 6C3HED-RG1 lymphosarcomas of the mouse (approx. 2.2 nmoles of L-asparagine synthesized per mg of protein per hr.

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TABLE 6

Interference by Various Analogs With the Enzymatic Coupling Assay of L-Aspartic Acid and L-Asparagine[a]

Assay mixture containing 0.048 mM of L-aspartic acid 0.035 mM of L-asparagine and 0.500 mM of the following analogs:	Absorbance change equivalent to	
	L-aspartic acid (mM)	L-asparagine (mM)
Control[b]	0.047	0.035
D-Aspartic acid	0.050	0.034
D-Asparagine	0.046	0.035
DONV	0.047	0.035
CONV	0.046	0.034
BONV	0.046	0.035
ONV	0.050	0.035
S-Carbamate cysteine	0.051	0.038
S-Methyl systeine sulfoximine	0.051	0.036
Cysteine sulfinic acid	0.051	0.036
DL-β-Methyl aspartate	0.049	0.036
Aspartic-β-chloroethylamine	0.047	0.039
β-Cyanoalanine	0.049	0.123

[a]Assay procedure was exactly the same as those for L-aspartic acid and L-asparagine as described in METHODS with the exception that the know concentration of analogs (0.5 mM) was added to the assay mixture.

[b]In the control experiment, no analogs were added to the assay mixture.

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at 0.15 mM of L-aspartic acid in the assay mixture). However, L-asparaginase sensitive tumors such as 6C3HED-S lymphosarcoma and L5178Y lymphoblasts showed little enzymatic activity (less than 0.2 nmoles of L-asparagine synthesized per mg of protein at 0.15 mM of L-aspartic acid in the assay mixture). A notable exception was the supernatant enzyme preparation from L-asparaginase resistant P815Y mast cell tumor which had low synthetase activity comparable to that from L-asparaginase sensitive tumors. The results are summarized in Table 7 . Further studies indicated that the majority of the synthetase activity in the homogenate from P815Y cells was associated with the particulate fraction rather than in the 105,000 x g supernatant fraction (Table 8). It is still not clear, however, why the overall synthetase activity in the crude homogenate is much lower than that from other L-asparaginase resistant tumor sources. Since a soluble enzyme preparation from 6C3HED-RG1 showed the highest synthetase activity, this tumor was used as an enzyme source for further purification.

2. Some Preliminary Studies on L-Asparagine Synthetase in Embryonic Livers and 6C3HED-RG1 Tumor: The preliminary studies on L-asparagine synthetase preparation obtained from 13-16 day chick embryo liver (105,000 x g supernatant fraction) showed the synthetase activity was 5.8 nmole/mg protein/hr. In this crude preparation the apparent Km for L-aspartic acid was 2.0×10^{-3} M. CONV

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TABLE 7

L-Asparagine Synthetase Activity in the 105,000 x g Supernatant from Various Sources

105,000 x g supernatant fraction	L-asparagine synthetase activity (nmole/mg of protein/hr)
Chick embryo liver (15th day)	2.20±0.08
New-born chick liver	<0.01
Rat embryo liver (14th day)	2.80±0.09
New-born rat liver	<0.01
Adult rat liver	<0.01
Novikoff Hepatoma	2.55±0.09
P815Y	0.20±0.02
6C3HED-S	0.17±0.03
6C3HED-R	4.84±0.23
6C3HED-RG1	12.86±0.46
L5178Y	<0.01
L1210	3.39±0.20
S-180	2.63±0.15

Two to three mg of protein from a 105,000 x g supernatant fraction was used in each assay tube. The assay mixture contained L-aspartic acid (0.15 mM), ^{14}C-L-aspartic acid 1.5 x 10^6 dpm, specific activity 154 mcuries/mole, L-glutamine (20 mM), ATP (10 mM), $MgCl_2$ (10 mM), dithiothreitol (5 mM) and Tris-HCl buffer (0.1M, pH 8.0) to a final volume of 1.0 ml. After incubation at 37°C the reaction was stopped by heating in a boiling water bath for 10 min. The percent conversion of ^{14}C-L-aspartic acid to ^{14}C-L-asparagine was measured by high voltage electrophoresis on Whatman 3 MM filter paper at 30 volts/cm for 90 min in sodium phosphate buffer (0.1 M, pH 7.0), followed by liquid scintillation counting. Figures represent the mean value ± standard error. Note that the unlabeled L-aspartic acid used in this assay was only one tenth that used in the standard assay to ensure a higher percent conversion of L-aspartic acid to L-asparagine. All figures obtained from at least 3 assays.

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TABLE 8

Localization of L-Asparagine Synthetase in Murine Tumor Cell Fractions

Tumor	nmoles of L-asparagine produced/mg of cells/hr	Crude Homogenate	105,000 x g Supernatant
		nmoles of L-asparagine synthesized/ mg of protein/hr	
6C3HED-RG1	1.23	9.2±1.5[a]	45.7±1.7
L1210	1.08	4.0±0.6	10.5±0.8
S180	0.54	4.9±0.6	9.0±0.8
P815Y	1.19	2.2±0.5[b]	0.7±0.4

The procedures for homogenation of tumor cells, ultracentrifugation of enzyme preparations and the measurement of L-asparagine production by whole cells were described in METHODS. The values were mean ± standard error (4 assays).

[a] 15,000 x g supernatant fraction of 6C3HED-RG1 showed a value of 22.4 ± 1.8.

[b] 15,000 x g supernatant fraction of P815Y showed a value of 4.9 ± 0.8.

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(0.1 mM) inhibited L-asparagine synthetase by 78%. When L-glutamine (20 mM) was replaced with the same concentration of NH_4Cl, only 14.5% of the enzymatic activity with glutamine was observed. When Mg^{++} (10 mM) was replaced with Mn^{++} 13.5% of the original enzymatic activity was observed. The same concentration of Zn^{++}, Sr^{++}, Cu^{++} and Ag^{++} gave no enzymatic activity. Even with a 105,000 x g supernatant preparation, the enzyme was labile to mild purification procedures such as Sephadex gel filtration. Dialyzing the same enzyme preparation against Tris-HCl buffer pH8.0, 0.1M, 4°C for two hours caused the loss of 85% of enzymatic activity; however, only 40% of the enzymatic activity was lost if 2-mercaptoethanol (5 mM) or substrates (Asp 0.015 mM, ATP 1 mM, Glu 2 mM and Mg^{++} 1 mM) were added to the dialysate. The enzyme was fully protected during dialysis, when dithiothreitol (1 mM) and substrates (same concentration as above) were added to the dialysate.

The preliminary studies on L-asparagine synthetase preparation obtained from 14-21 day rat embryo livers (105,000 x g supernatant fraction) indicated that the enzymatic activity was 6.6 nmole L-asparagine/mg protein/hr and more than 95% of the radioactivity that remained at the origin (L-asparagine position) after high-voltage electrophoresis moved as aspartic acid after treatment with purified asparaginase. The synthetase activity dropped to 4.4 nmole/mg protein/hr in new-born rat liver preparations and 0.6 nmole/mg protein/hr in 5 day old rats. No net L-asparagine formation could be demonstrated in preparations

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from adult rat liver. The amount of electrophoretically immobile radioactivity increased with the age of the animal. However, the percentage of this material that was not digestable by L-asparaginase increased from >10% in new-born animals to <99% in animals one month old.

The preliminary studies on L-asparagine synthetase preparation obtained from 6C3HED-RG1 tumor (38-48% ammonium sulfate precipitated fraction of 105,000 x g supernatant or the step 2 enzyme preparation described on p. 53) indicated that the reaction required ATP (optimal 7-10 mM), Mg^{++} (optimal 10-12 mM) and L-glutamine (optimal 15-20 mM) and L-aspartic acid (optimal 2-3 mM). The electrophoretic chromatogram of the reaction mixture after incubation (with step 3 enzyme preparation) showed UV absorption in the AMP and ATP regions. L-Glutamine can be replaced by NH_4Cl but the latter is only 85 to 88 percent as efficient an amido donor as L-glutamine under the standard assay conditions. The pH-activity profile suggested that ammonia rather than ammonium ion is the reactive species (Fig. 9). None of the following cations could substitute for Mg^{++} as an activator: Mn^{++}, Zn^{++}, Cd^{++}, Co^{++}, Ca^{++}, Ba^{++}, Sn^{++}, Cu^{++}, Hg^{++}, Cu^{+}, Hg^{+}, Na^{+}, and K^{+}. Many of these cations were inhibitory when optimal Mg^{++} was present. None of the following nucleosides could substitute for ATP and give more than 46% of the optimal reaction rate: i.e. GTP, UTP, CTP, TTP, ADP, GDP, UDP, CDP, AMP, GMP, UMP, CMP, TMP and IMP. All of them, to various degrees inhibited the reaction. The following compounds, at the concen-

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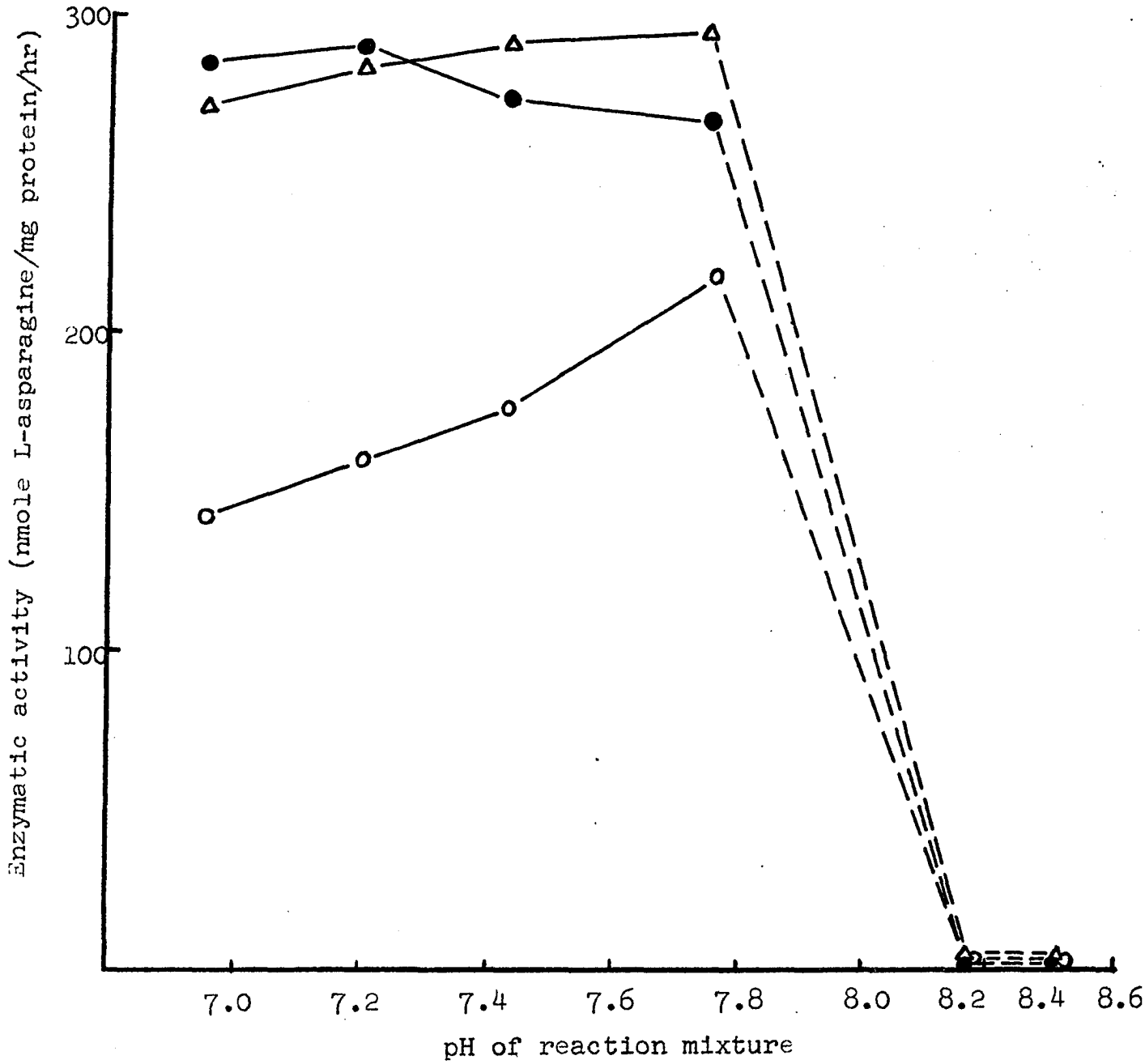

Figure 9. pH-enzymatic activity profile of L-asparagine synthetase isolated from 6C3HED-RG1 tumor. Reaction mixture with 20mM L-glutamine (•), 20mM NH_4Cl (o) and 10mM L-glutamine + 10mM NH_4Cl (▲). Sephadex G100 gel filtered step 3 enzyme preparation and Tris-HCl buffer 0.2M were used.

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tration of 2 mM had little or no inhibition on L-asparagine biosynthesis either in the presence of DTT (5 mM) or in the absence of DTT: D-asparagine, 2-amino asparagine, meso-diamino succinic acid, L-2,4-diaminobutyric acid, L-β-aspartyl hydrazide and β-cyanoalanine. A preliminary study of step 3 enzyme preparation using gel filtration indicated that the molecular weight of the enzyme is about 94,000 (Fig. 10).

3. Purification of L-Asparagine Synthetase from 6C3HED-RG1 Tumor: 6C3HED-RG1 cells were injected subcutaneously into both sides of groins of C3H/J mice with 1,000,000 cells in 0.1 ml on each side. 10 to 12 days after transplantation, the solid tumor mass, about 2 gm per mouse, was collected. All subsequent procedure steps were performed at 4°C.

Step 1. Preparation of cell extracts: The tumors were minced with scissors and homogenized in 2 volumes of cold Tris-HCl buffer 0.1 M, pH 7.8, with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 15,000 x g for 10 min and the resulting supernatant fraction was further centrifuged at 105,000 x g for 60 min in the Spinco ultracentrifuge. The supernatant fluid was used for further purification.

Step 2. The first ammonium sulfate precipitation: A saturated ammonium sulfate solution was made in Tris-HCl buffer (0.1 M, pH 7.8), and unless other wise stated, contained dithiothreitol (3 mM). The saturated ammonium sulfate solution was gradually added to the supernatant

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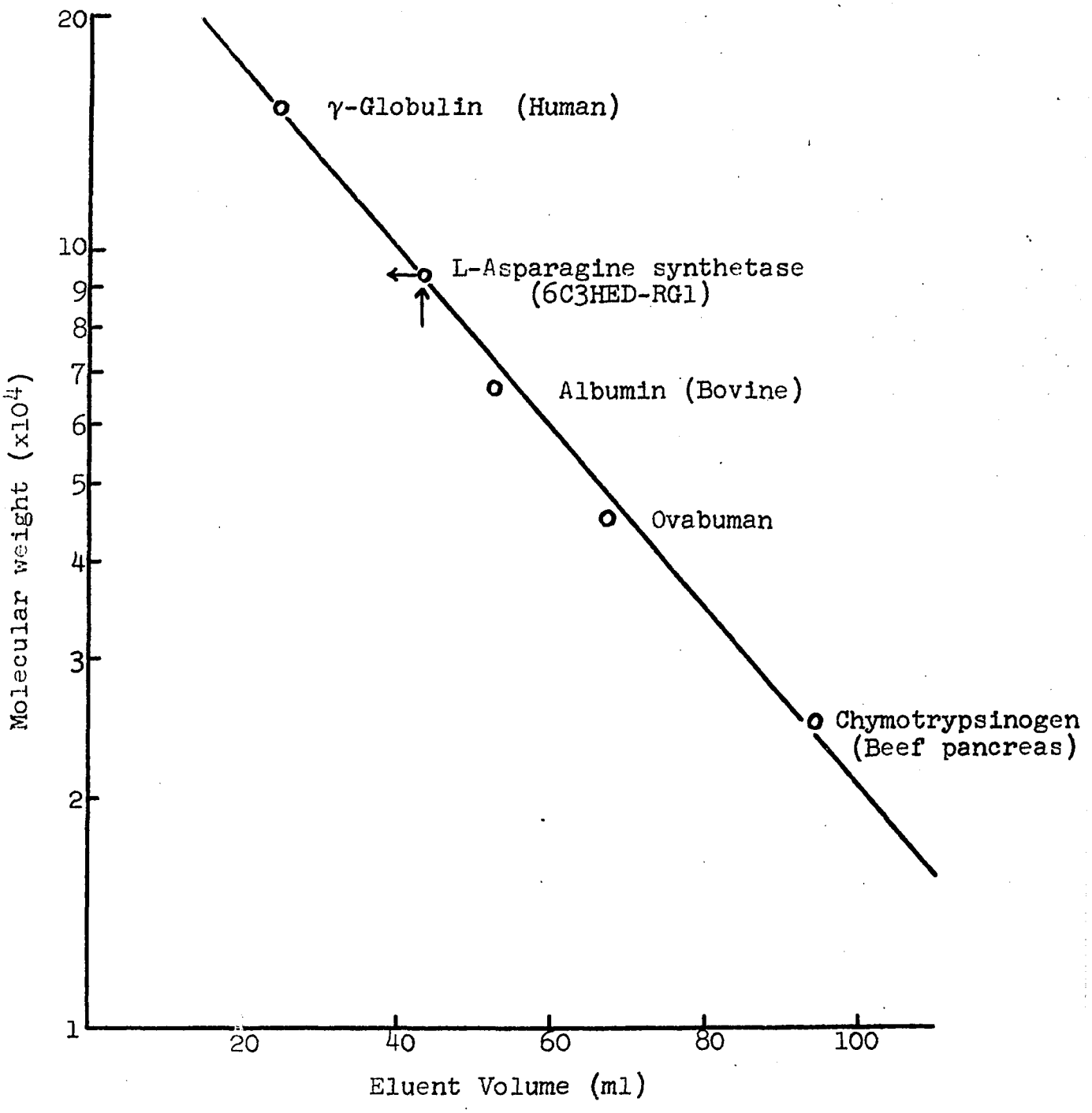

Figure 10. Plot of the elution volume against molecular weight for proteins used as standards and L-asparagine synthetase of 6C3HED-RG1 tumor. Sephadex G100 (Pharmacia) was swelled and equilibrated with Tris- HCl buffer pH 7.8 (0.1M) and packed into a column (2.5x4 5 cm). Adsorbed samples were eluted with the same buffer. The relative protein content of fractions was scanned with an automatic UV Scanner.

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fluid from step 1 with mild stirring at 4°C. The precipitate that formed between 38 to 48 percent ammonium sulfate saturation was collected by centrifugation at 15,000 g for 10 min.

Step 3. DEAE Sephadex A-50 chromatography: The precipitate from step 2 was dissolved (20 mg of protein per ml) in Tris-Buffer 0.1 M, pH 7.8 containing glycerol (10%) dithiothreitol (3 mM), EDTA (0.01 mM), $MgCl_2$ (0.2 mM) L-aspartic acid (0.0015 mM), L-glutamine (0.2 mM) and ATP (0.01 mM). The same buffer mixture was used for equilibrating the DEAE Sephadex A-50 anion exchanger (Pharmacia) column. After the DEAE gel had been swelled in distilled water at 90°C for 5 hours, the gel was treated repeatedly with 0.5 N NaOH and then with 0.5 N HCl to remove trace amounts of heavy metals. This treated gel was then washed with double distilled water and packed into a column (2.5 cm x 45 cm) and equilibrated with the buffer mixture. The enzyme solution (about 5 ml) was adsorbed onto the column and eluted with a linear continuous NaCl gradient between 0 and 1N NaCl. The solutions used in the preparation of the gradient contained the same buffer as used for equilibration. The flow rate was 10 ml per hour. A typical elution profile is shown in Figure 11 . The fractionated enzyme was unstable (see p. 59) and the next step was performed within one day.

Step 4. The second ammonium sulfate precipitation: The peak fraction of L-asparagine synthetase activity in step 3 was fractionated with 43 to 46% $(NH_4)_2SO_4$ saturation as described above. The resulting precipitate was

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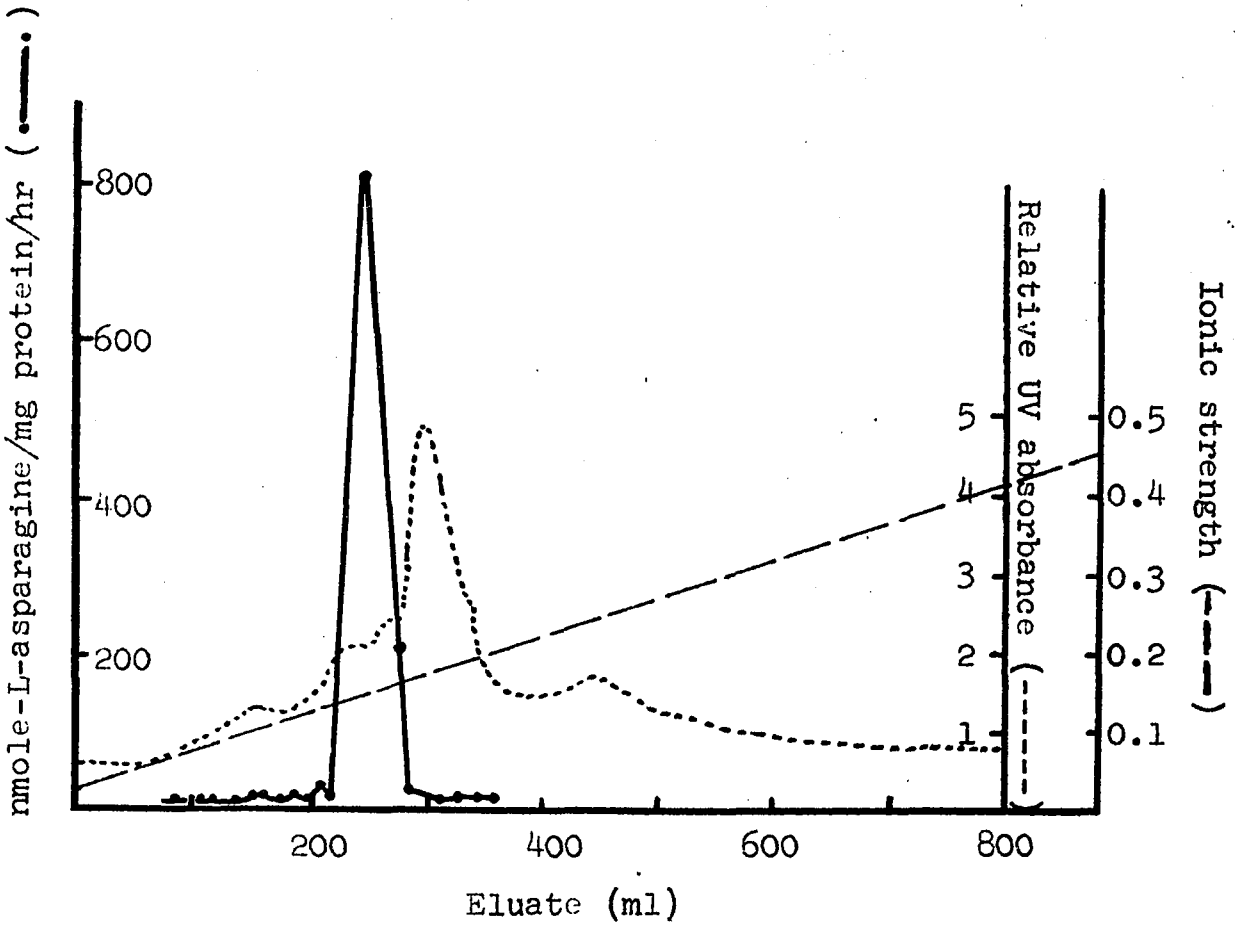

Figure 11. DEAE Sephadex A-50 chromatography of 6C3HED-RG1 L-asparagine synthetase preparation.

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dissolved in the buffer mixture to make a solution with about 1 mg of protein per ml and immediately processed according to the next step.

Step 5. Sephadex G100 gel filtration: A Sephadex G100 (Pharmacia) column (0.90 cm x 60 cm) was equilibrated with the buffer mixture as described above. The enzyme solution with residual ammonium sulfate was passed through the column and eluted with the buffer mixture. The peak fractions were pooled and used for study within 24 hours. Table 9 shows the L-asparagine synthetase activity of fractions obtained at various steps in the purification procedure. The overall enrichment is 305-fold from crude homogenate.

4. Stability of L-Asparagine Synthetase from the 6C3HED-RG1 Tumor. Many other procedures for enzyme purification and stabilization have been tested but with variable degrees of success. The 105,000 x g supernatant preparation was stable for at least three months if stored at -10°C. It lost all enzymatic activity after heating to 55°C or higher temperatures for 5 min. Lowering the pH to 5.3 for 5 min caused the loss of 67% of enzymatic activity. Up to 50% of this loss could be prevented or reversed if dithiothreitol (5 mM) and substrates (0.1 mM) were present during or after the treatment. When the step 3 enzyme preparation was dialyzed against Tris-HCl buffer (0.1 M, pH 8.0) for 3 hours in the absence of DTT, 80% of the enzymatic activity was lost. This activity could be restored to 70% of the original activity by adding reducing

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TABLE 9

Purification of L-Asparagine Synthetase from 6C3HED-RG1 Cells

Fraction step	Volume	Protein Concentration	Specific activity[a]	Total enzyme activity[a]	Yield
	ml	mg/ml	nmole L-asparagine/hour		%
1a. Crude homogenate	120	34.2	9	36.936	-
1b. 105,000 x g supernatant	95	8.8	41	34.276	92.8
2. Ammonium sulfate 38-48% saturation	10	10.6	302	31.950	86.5
3. DEAE Sephadex A-50	91.3	0.12	1720	18.837	51.0
4. Ammonium sulfate 43-46% saturation	3	0.93	2630	7.387	20.1
5. Sephadex G100	8.3	0.26	2750	5.910	16.0

a. All the assay conditions are the same as those indicated in METHODS.

agents with sulfhydryl groups such as mercaptoethanol (10 mM) or dithiothreitol (3 mM). Step 1b enzyme preparation was rather stable to dialysis and the apparent synthetic activity was actually increased by adding dithiothreitol to the dialysate during dialysis (Fig. 12). Precipitating the enzyme protein with 60% acetone resulted in a loss of 82% of the enzymatic activity even in the presence of dithiothreitol (3 mM), substrates (0.1 mM) and at low temperatures (-5°C ~ -15°C). Further purification after step 3 (DEAE Sephadex A-50) was difficult since instability increased with purification. This was even true when the procedures were performed in the presence of dithiothreitol (5 mM), glycerol (10%), substrates and cofactors (0.1 mM).

Diluted, partially purified enzyme solution at a concentration of 0.5 mg of protein/ml or lower was unstable in storage at -10°C to -15°C. Addition of crystalline bovine albumin (5mg/ml) to the enzyme preparation doubled the storage half-life of enzymatic activity. The half-life for the protected enzyme preparation from step 3 was about one week at -15°C.

Dialysis of the first ammonium sulfate fractionated enzyme preparation (step 2) against sodium phosphate buffer 0.1 M, pH 7.8) for 2 hours caused a 50% loss of enzyme activity. Upon adding calcium phosphate gel to the equilibrated preparation, the enzymatic activity resided in the supernatant fraction and 2.5-fold purification from the preceding step could be achieved despite the loss of total activity.

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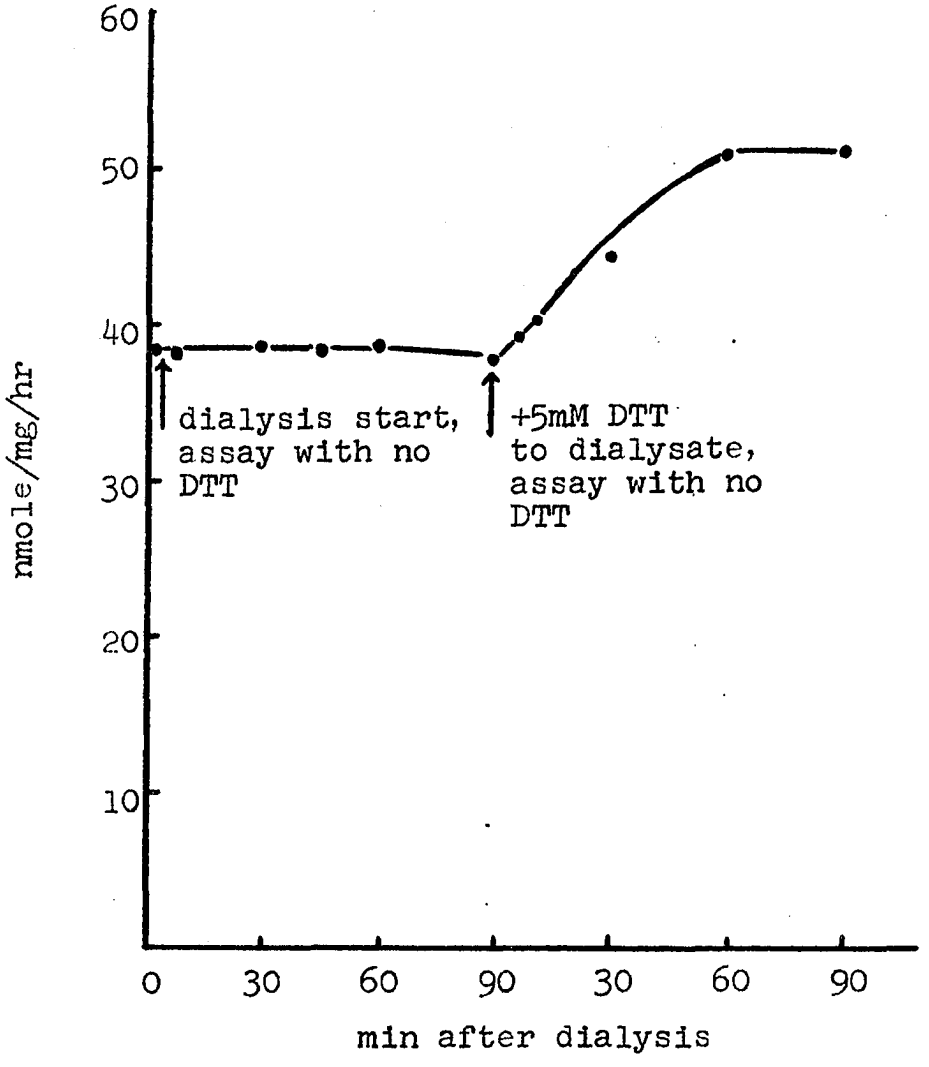

Figure 12. Activation of L-asparagine synthetase by dithiothreitol. 5 ml of 105,000 x g supernatant of 6C3HED-RG1 preparation (20 mg protein/ml) was dialyzed against 300 ml of Tris-HCl buffer 0.1M pH 7.8. After 90 min, DTT was added to the dialysate to make a final concentration of 5mM. Samples (0.1 ml) were taken from the dialysis bag for L-asparagine synthetase assay at various times. No additional DTT was added to the standard assay mixture.

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5. Kinetic Studies

a. Initial Velocity Studies: Initial velocity studies involve varying the concentration of one substrate at various fixed levels of one of the others with no products present initially and with the third substrate concentration held constant. For the L-asparagine synthetase system, there are three initial velocity patterns, depending on which pair of substrates is varied. Since ATP, AMP and PP all form complexes with Mg^{++} (64), we assumed throughout that ATP-Mg^{++}, AMP-Mg^{++} and PP-Mg^{++} are the active species in the enzymatic reaction. When ATP was a variable substrate, it was varied with the same molarity of Mg^{++} ion with 2 mM Mg^{++} excess in the reaction mixture. When L-glutamine and L-aspartic acid (Fig. 13) or L-aspartic acid and ATP (Fig. 14), or L-glutamine and ATP (Fig. 15) as a pair of variable substrates, all three double-reciprocal plots (Lineweaver-Burk plots) showed a parallel pattern indicating three ping-pong mechanisms involved in the reaction sequence.

b. Product Inhibition Studies: Inhibition studies provide a powerful tool for deducing kinetic mechanisms. The most important inhibitors are the products themselves. Theoretically, all products will give inhibition effects since the denominator of a full rate equation contains not only those terms which are seen in an initial velocity study, but also others which include the concentration of both substrates and products. Product inhibition experiments were performed by varying the concentration of one

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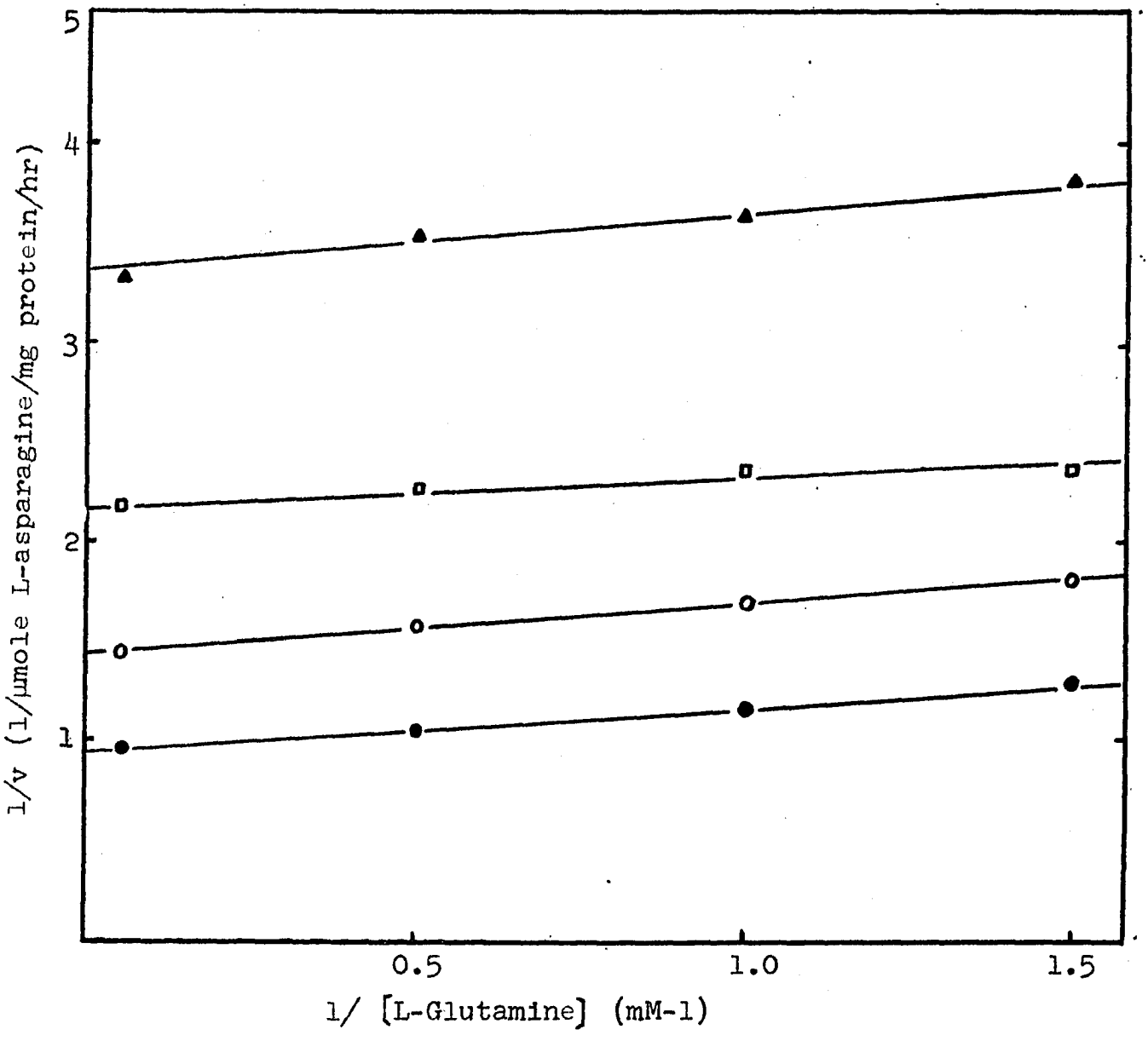

Figure 13. Double-reciprocal plots of initial reaction velocity against L-glutamine concentration for various concentrations of L-aspartic acid. The concentrations of L-aspartic acid were 0.22 mM (▲——▲), 0.33 mM (▫——▫), 0.67 mM (○——○) and 3.0 mM (●——●). A saturating level of ATP (10 mM) and step 3 enzyme preparation were used in the standard assay mixture.

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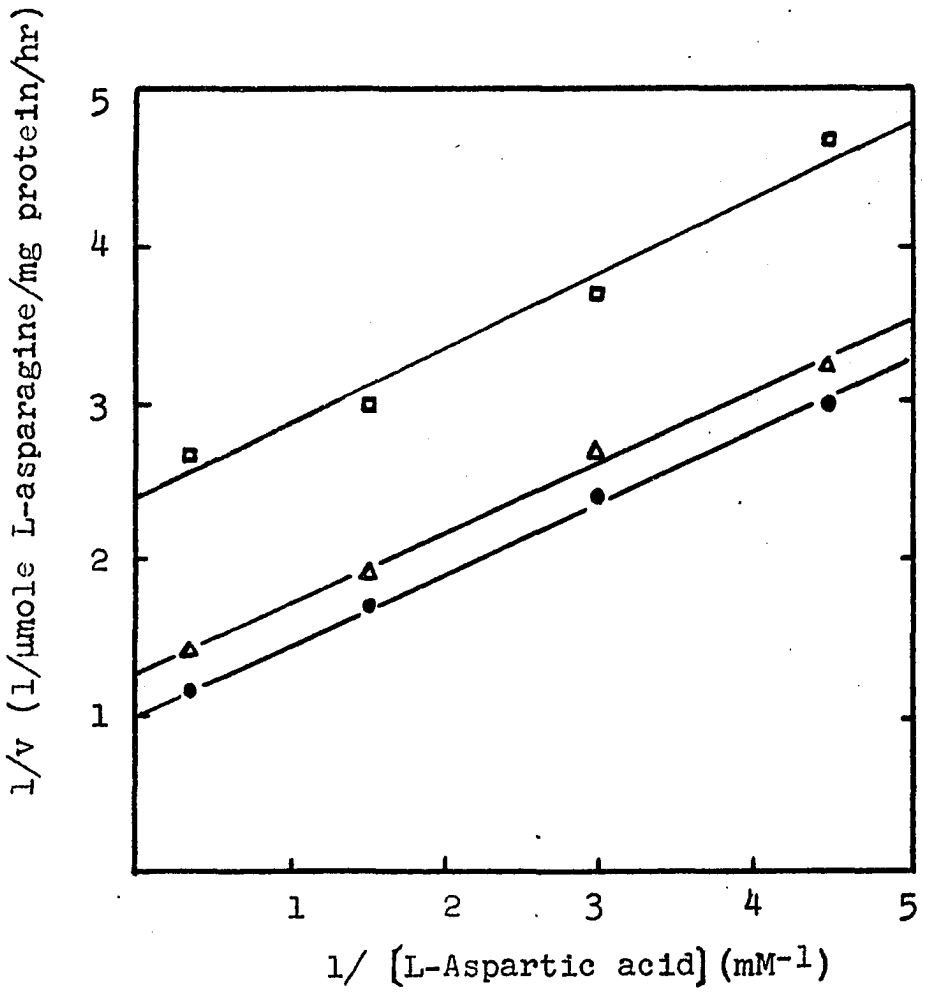

Figure 14. Double-reciprocal plots of initial reaction velocity against L-aspartic acid concentration for various concentrations of ATP. The concentrations of ATP were 0.6 mM (▫——▫), 3.0 mM (△——△) and 10.0 mM (●——●). A saturating level of L-glutamine (20 mM) and step 3 enzyme preparation were used in the standard assay mixture.

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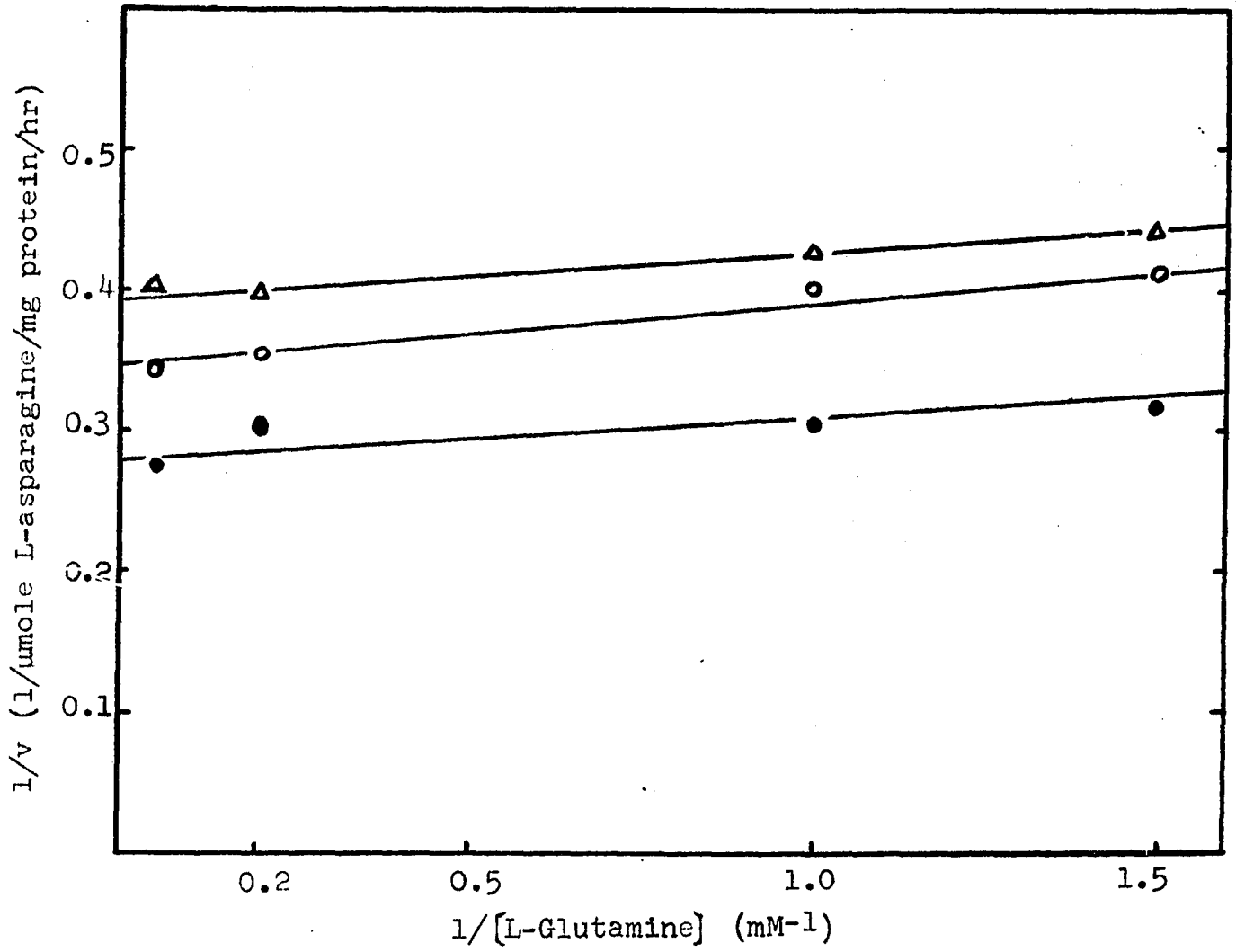

Figure 15. Double-reciprocal plots of initial reaction velocity against L-glutamine concentration for various concentrations of ATP. The concentrations of ATP were 0.67 mM (△——△), 1.0 mM (○——○) and 2.0 mM (●——●). L-Aspartic acid (3 mM) was used in the standard assay mixture. Step 5 enzyme preparation was used.

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substrate at various fixed levels (including zero) of one of the products. The concentration of the other substrates (either saturating or non-saturating) are held constant, and initial velocities were measured.

Double-reciprocal plots of the initial reaction velocity against L-glutamine concentration demonstrated that the inhibition was competitive with respect to L-asparagine (Fig. 16) and the inhibition was uncompetitive with respect to both AMP and PP (Fig. 17). The Ki, Asn slope (Gln) was about 0.4 mM. The Ki values for AMP and PP in Figure 17 were obtained from experiments with non-saturating level of the substrates. Using non-saturating level of substrates is in some cases beneficial since it may magnify product inhibitory effects. With respect to L-aspartic acid, L-asparagine acted as an uncompetitive inhibitor, PP acted as a competitive inhibitor whereas AMP acted as a non-competitive inhibitor (Fig. 18). Again, non-saturating level of substrate (e.g. ATP) was used. No inhibition could be observed with 10 mM of L-glutamic acid when L-glutamine, L-aspartic acid or ATP was a variable substrate.

c. Reversibility Studies: Since low level of substrates i.e. L-glutamine 0.2 mM, ATP 0.01 mM, $MgCl_2$ 0.2 mM, L-aspartic acid 0.0015 mM were added to the buffer to protect the enzyme during the purification procedure, quantitative study of the reverse reaction was not possible. However, the preliminary experiments indicated that the conversion of L-asparagine into L-aspartic acid was strongly

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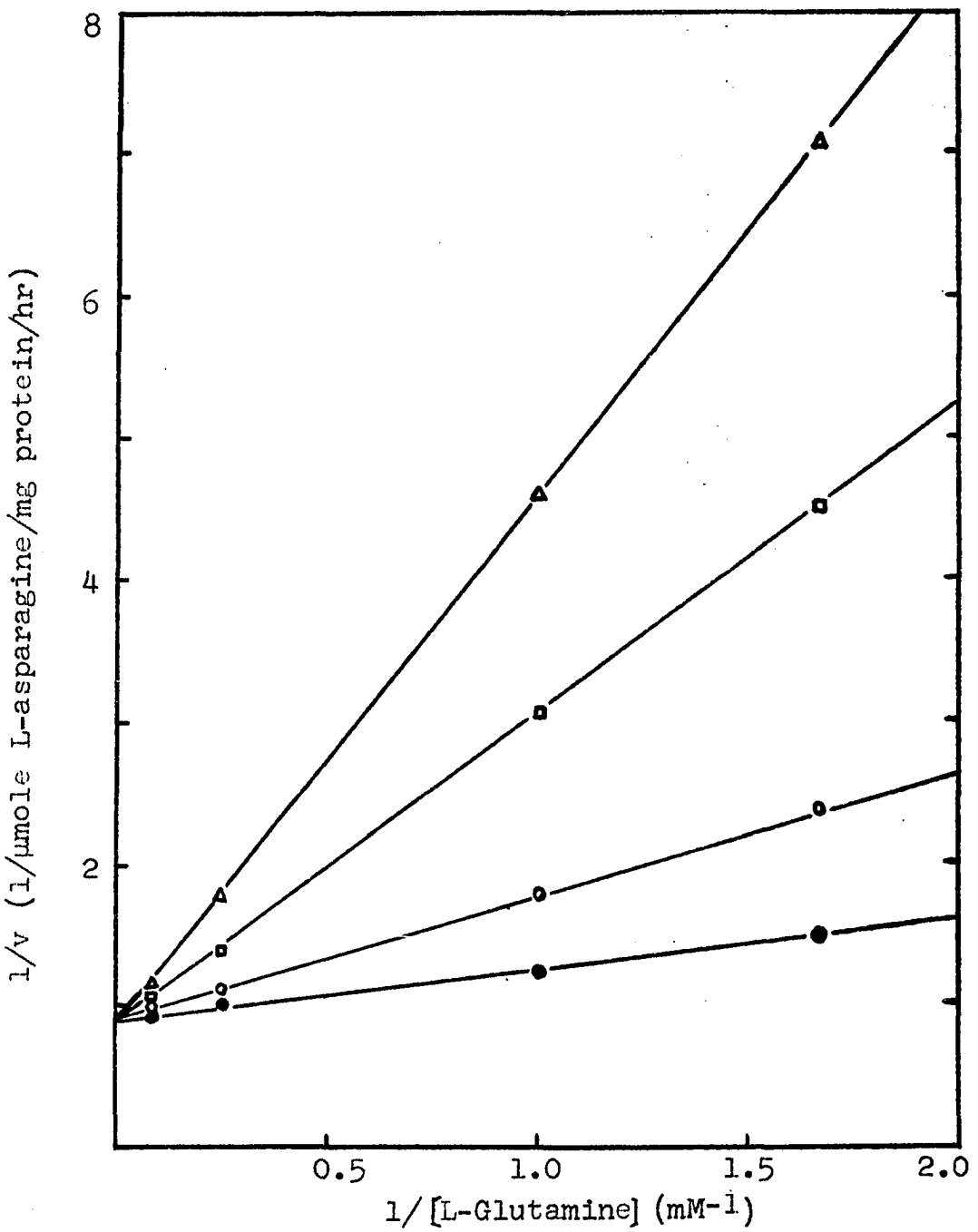

Figure 16. Double-reciprocal plot of initial reaction velocity against L-glutamine concentration to show the effect of L-asparagine. The concentrations of L-asparagine were 0.40mM (△——△), 0.20 mM (▫——▫), 0.06 mM (○——○) and 0 mM (●——●) respectively. The assays were carried out in the presence of DTT (3mM) and step 3 enzyme preparation was used. V_{max} = 1.1 μmoles/mg/hr; $K_{i\ Asn}^{Gln}$ (slope) = 1.0×10^{-4}M.

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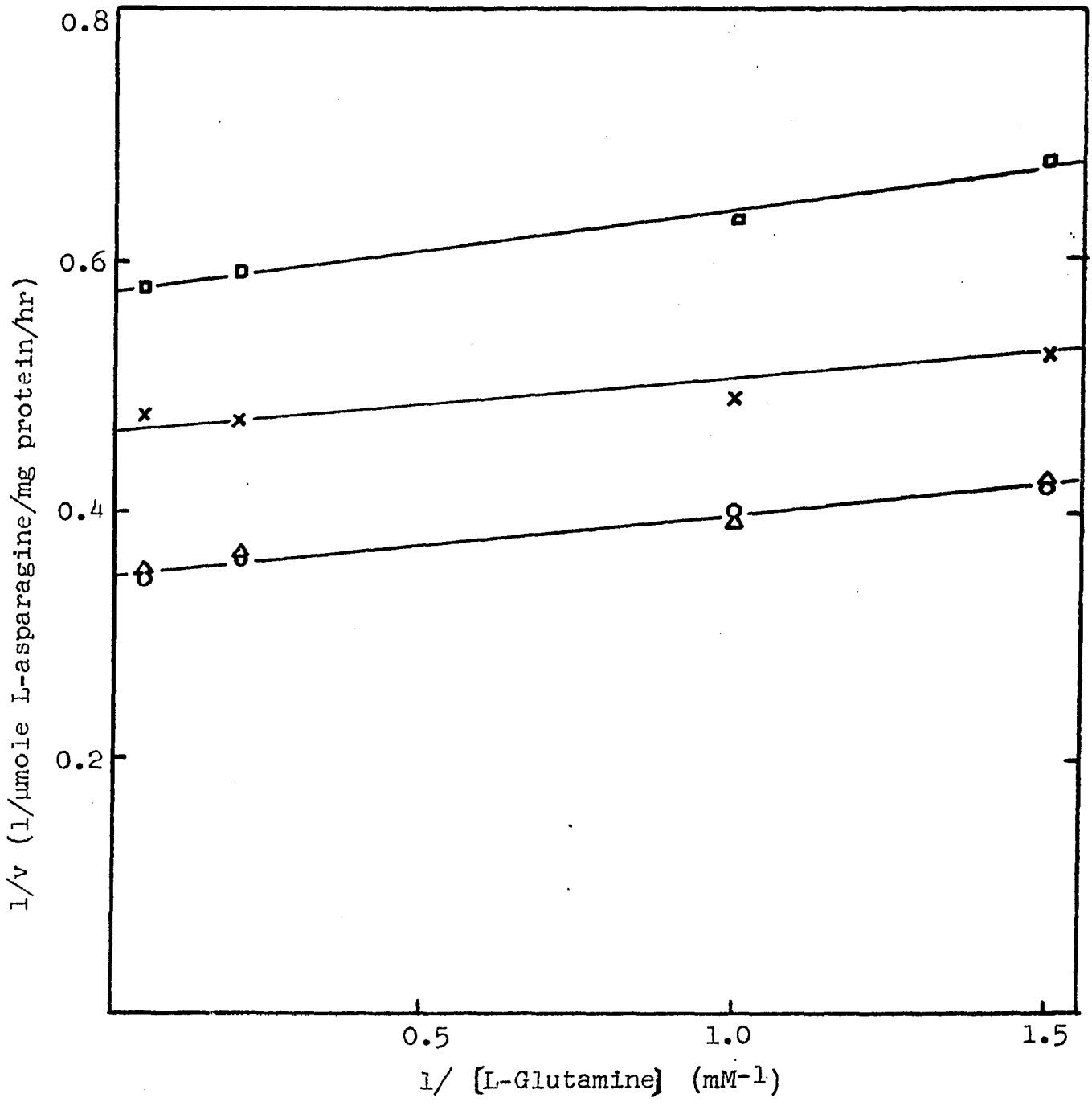

Figure 17. Double-reciprocal plots of initial reaction velocity against L-glutamine concentration to show the effects of the products. Control (o——o), plus 5.0 mM of L-glutamic acid (▲——▲), plus 5.0 mM of AMP (×——×), plus 5.0 mM of PP (□——□). All experiments were carried out at 1.0 mM of ATP, 3.0 mM of L-aspartic acid, and step 5 enzyme preparation was used.

$K_{i\ AMP}^{Gln} = 1.5 \times 10^{-2}$ M, $K_{i\ PP}^{Gln} = 7.5 \times 10^{-3}$ M. $V_{max} = 2.9$ umole/mg protein/hr.

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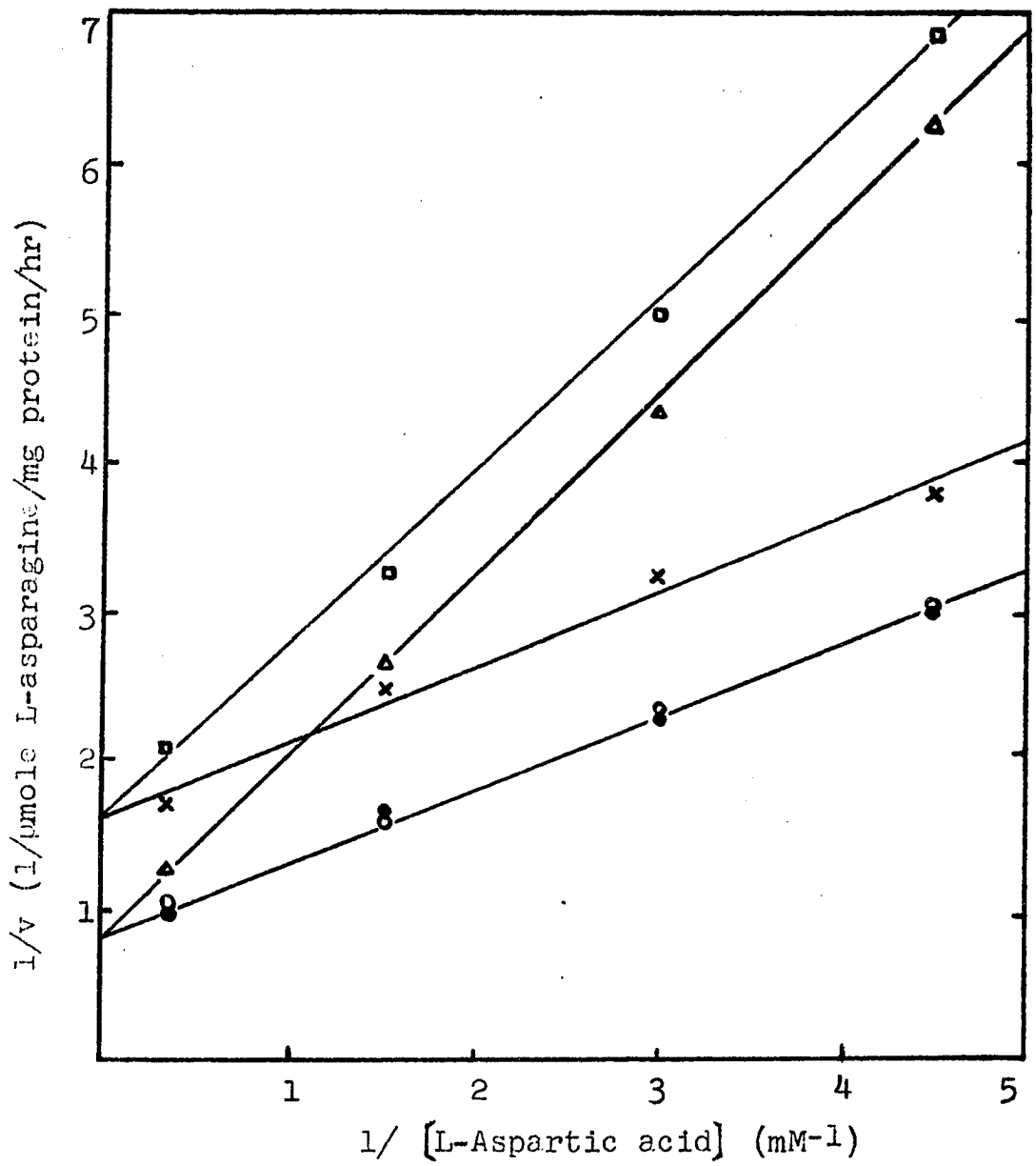

Figure 18. Double-reciprocal plots of initial reaction velocity against L-aspartic acid concentration to show the effects of the products. Control (•——•), plus 5mM L-glutamic acid (o——o), plus 1.0mM of L-asparagine, (x——x), plus 10mM of PP (Δ——Δ) and plus 10mM of AMP (□——□). All experiments were performed at 1mM ATP 20mM L-glutamine and step 3 enzyme preparation. V_{max} = 1.3 μmole/mg protein/hr. Ki^{Asp}_{Asn} = 1.0×10^{-3} M; Ki^{Asp}_{PP} = 7.0×10^{-3} M; $Ki^{Asp}_{AMP\ (slope)}$ = 8.3×10^{-3} M and $Ki^{Asp}_{AMP\ (int)}$ = 1.0×10^{-2} M.

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inhibited by L-glutamine but not inhibited by either L-aspartic acid or ATP (Table 10).

d. Alternate Substrate Studies: NH_4Cl appeared to be an alternate substrate for L-asparaginase isolated from 6C3HED-RG1 tumor, although it is not as good substrate as L-glutamine under standard assay conditions (Fig. 9). Initial reaction velocity studies with NH_4Cl as a variable substrate showed an intersecting pattern with respect to ATP in double-reciprocal plots (Fig. 19). Product inhibition studies showed that L-asparagine was nearly a competitive inhibitor with respect to NH_4Cl and pyrophosphate was a non-competitive inhibitor with respect to NH_4Cl (Fig. 20). These results when compared with results obtained from L-glutamine as a substrate (Fig. 16), provided valuable information concerning the question of whether both substrates react with the same enzyme form as well as the information concerning the reaction sequence (see p. 80).

e. A Mathematical Approach of Deducing Kinetic Mechanisms: For enzyme catalyzed reactions with a low number of substrates and products, most investigators apparently have deduced the kinetic mechanism from kinetic data with random trial of fits. For enzymatic reactions with higher complexity, the necessity of a step by step mathematical approach becomes more acute. Although enzyme catalyzed reactions with four or more substrates or products, at least at the present time, are rarely known; the author feels that a theoretical aspect of analysis on reaction patterns is still desirable. Therefore, a general formula

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TABLE 10

Reversibility of the Reaction Catlayzed by L-Asparagine Synthetase Isolated from 6C3HED-RG1 Tumor[a]

Amount of reactants added to the reaction system[b] (mM)							Rate of conversion of L-asparagine to L-aspartic acid[d]
Substrate			Product				nmole/mg protein/hr
Gln	ATP[c]	Asp	Glu	AMP	PP	Asn	
–	–	–	–	–	–	0.2	28
–	–	–	10	10	10	0.2	146
–	–	1.5	10	10	10	0.2	169
–	10	–	10	10	10	0.2	149
20	10	1.5	10	10	10	0.2	10
20	10	1.5	–	–	–	0.2	18
20	–	1.5	10	10	10	0.2	41

a. The rate for the forward reaction at 20 mM Gln, 10 mM ATP 12 mM Mg^{++} and 1.5 mM ASP, was 374 nmole Asp converted to Asn per mg protein per hour.

b. As indicated in the text (see p. 65), small amounts of normal substrates were added in the enzyme preparations.

c. Whenever 10 mM of ATP was added, 12 mM of Mg^{++} was added.

d. Percent conversion of ^{14}C-L-asparagine into ^{14}C-L-aspartic acid was measured with the same way as those for forward reaction as described in METHODS. After high voltage electrophoresis, L-asparagine remained at origin while L-aspartic acid migrated toward the anode.

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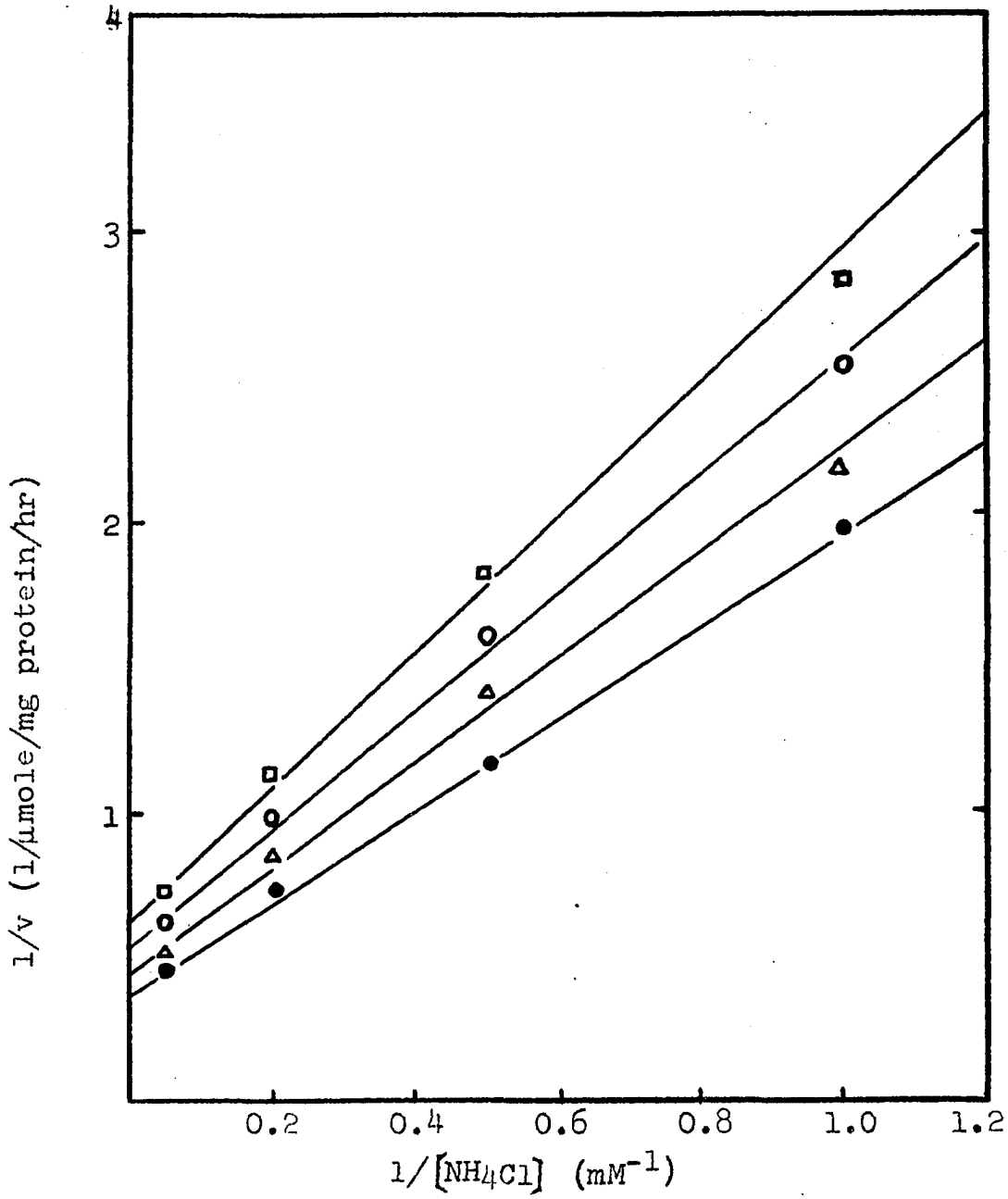

Figure 19. Double-reciprocal plots of initial reaction velocity against NH_4Cl (alternate substrate) concentration for various concentrations of ATP. The concentrations of ATP were 0.67mM (▫——▫), 1.0mM (○——○), 2.0mM (△——△) and 10.0mM (●——●). The reaction mixture (in Tris-HCl buffer pH 7.8, 0.1M) contained L-aspartic acid (3.0mM), ^{14}C-L-aspartic acid (154 mc/mmole, 1 mc/ml), DTT (3mM), variable levels of NH_4Cl, different levels of ATP plus Mg^{++} with 2mM excess of Mg^{++} and step 5 enzyme preparation. V_{max} = 2.63 μmole/mg protein/hr. K_{NH_4Cl} = 3.9×10^{-3} M.

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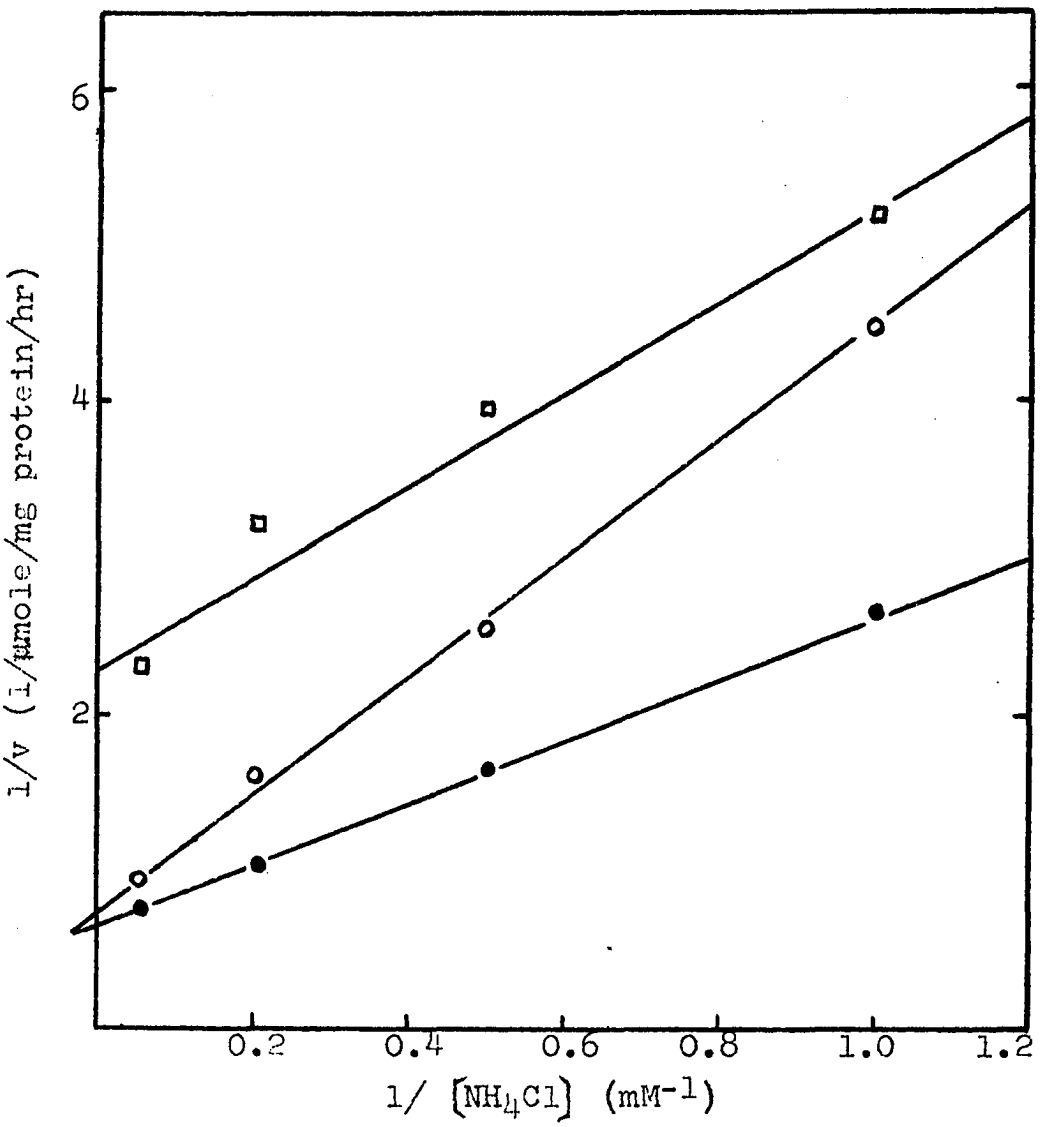

Figure 20. Double-reciprocal plots of initial reaction velocity against NH_4Cl (alternate substrate) concentration to show the effects of L-asparagine and pyrophosphate. Control (•——•), plus 1.0mM asparagine (o——o) and plus 5.0mM PP (▫——▫). The reaction mixture (in Tris-HCl buffer pH 7.8, 0.1M) contained L-aspartic acid (0.667 mM), ^{14}C-L-aspartic acid (154 mc/mmole, 1 μc/ml), DTT (3mM), ATP (1.0mM), Mg^{++} (3.0mM), inhibitor, step 5 enzyme preparation and variable concentrations of NH_4Cl. V_{max} =1.54 μmole/mg protein/hr. $Ki\ {}^{NH4Cl}_{Asn\ (slope)}$ = 1.1 x 10^{-3} M; $Ki\ {}^{NH4Cl}_{PP\ (int)}$ = 2.0 x 10^{-3} M.

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for calculating the number of possible arrangements and mechanisms at any given number of substrates, products and stable enzyme forms has been derived. For details, please see Section V of this dissertation. Kinetic studies can show consistency or inconsistancy of the experimental data with possible mechanisms. In order to be sure that one has correctly determined the mechanism it is necessary to examine every possible mechanism that is at all plausible, and reject those that do not fit (48). Cleland has proposed a method of prediction of initial velocity and inhibition patterns by inspection (47,48). In the following pages this method together with a general sequential pattern analysis will be applied to L-asparagine synthetase purified from the 6C3HED-RG1 tumor.

Since L-asparagine synthetase catalyzes a reaction with three substrates (i.e., L-aspartic acid, L-glutamine and ATP) and four products (i.e., L-asparagine, L-glutamic acid, AMP and PP), according to Cleland it is a Ter-Quad system. If consideration is limited to arrangements in which no alternate reaction pathway exists, stable enzyme forms do not isomerize and a central complex is always present, the number of reaction patterns can be expressed by the following equations with the designation that S and P are the number of substrates and products respectively and n is the number of stable enzyme forms; d is summed over the common divisor of S,P and n; d_1 is the divisor of the largest common divisor of S and P; and is Euler's totient function. (see section V for details).

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The number of overall sequential patterns (linear)

$$A(S,P) = \frac{[(S-1)+(P-1)]!}{(S-1)!\,(P-1)!} = \binom{S+P-2}{S-1,P-1} = \sum_{v=1}^{n} \binom{S-1}{v-1}\binom{P-1}{v-1} \qquad \text{(IV-1)}$$

(see p. 109)

The number of linear sequential patterns at a given number of stable enzyme forms

$$A(n;S,P) = \binom{S-1}{n-1}\binom{P-1}{n-1} \qquad \text{(IV-2), (see p. 110)}$$

The number of overall sequences (linear)

$$B(S,P) = \binom{S+P-2}{S-1,P-1}\, S!\, P! \qquad \text{(IV-3)}$$

The number of overall (circular) mechanism patterns

$$M(S,P) = \frac{1}{S+P}\sum_{d_1}\phi(d_1)\,\frac{\binom{S+P}{d_1}!}{\binom{S}{d_1}!\,\binom{P}{d_1}!} \qquad \text{(IV-4), (see p. 114)}$$

The number of circular mechanism patterns at a given number of stable enzyme forms

$$M(n;S,P) = \frac{1}{n}\sum_{d}\phi(d)\binom{\frac{S}{d}-1}{\frac{n}{d}-1}\binom{\frac{P}{d}-1}{\frac{n}{d}-1} \qquad \text{(IV-5)}$$

(see p. 112 or Appendix V)

The number of overall mechanisms (circular)

$$N(S,P) = \frac{S!\,P!}{S+P}\sum_{d_1}\phi(d_1)\,\frac{\binom{S+P}{d_1}!}{\binom{S}{d_1}!\,\binom{P}{d_1}!} \qquad \text{(IV-6)}$$

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For a Ter-Quad system where S=3 and P=4; n=1 3, we may substituite these numbers into above equations. From equation (IV-1), we get

$$A(S,P) = \binom{5}{2} = \frac{5!}{3!2!} = 10$$

or more specifically,we get

$$\sum_{i=1}^{3} \binom{S-1}{i-1}\binom{P-1}{i-1} = \binom{2}{0}\binom{3}{0}+\binom{2}{1}\binom{3}{1} + \binom{2}{2}\binom{3}{2}$$

$$= \quad 1 \quad + \quad 6 \quad + \quad 3 \quad = 10$$

Therefore, the Ter-Quad system has ten total patterns of arrangements of which one pattern (the first term) comes from arrangement with one stable enzyme form; six patterns (the second term) and three patterns (the third term) come from the arrangements with two and three stable enzyme forms,respectively.

However, since all substrates or products are of different identities, from equation (IV-4), we get

$$B(S,P)= \binom{S+P-2}{S-1,P-1} S!\ P! = 10 \times 3 \times 4 = 1440$$

From equation (IV-4), we get

$$M(S,P) = \frac{1}{3+4} \times 1 \times \frac{\left(\frac{3+4}{1}\right)!}{\left(\frac{3}{1}\right)!\left(\frac{4}{1}\right)!}$$

$$= \frac{1}{7} \times 1 \times \frac{7!}{3!\ 4!} = 5$$

or more specifically, when we correlate equation (IV-4) to

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equation (IV-5), we get

$$M(S,P) = \sum_{n} M(n;S,P) = \sum_{n=1}^{3} M(n;3,4)$$

$$= \frac{1}{1} \times 1 \times \binom{2}{0}\binom{3}{0} + \frac{1}{2} \times 1 \times \binom{2}{1}\binom{3}{1} + \frac{1}{3} \times 1 \times \binom{2}{2}\binom{3}{2}$$

$$= 1 \qquad + 3 \qquad + 1 = 5$$

Therefore, the Ter-Quad system has five patterns of mechanisms of which one pattern comes from a mechanism with one stable enzyme form; three patterns and one pattern comes from mechanisms with two and three stable enzyme forms respectively. Since all substrates or products have different identities, from equation (IV-6), we get

$$N(S,P) = M(S,P)\ S!\ P! = 5 \times 3! \times 4! = 720$$

It would be a tremendous labor to analyze 1440 different arrangements or 720 different mechanisms for a Ter-Quad system. A short-cut would be to disregard the difference among substrates and products at the beginning. In other words, we consider the "pattern" first and then try to fit some of the kinetic data and eliminate many improbable patterns. After a mechanism pattern has been proposed, the composition of each stable enzyme form can be determined.

The usefulness of equations (IV-1) to (IV-6) are proportional to the number of substrates, products, as will be discussed in section V of this dissertation. When S and P

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are low, the number of patterns of either linear sequential patterns or mechanism patterns can be carefully figured out with a graphical enumeration without difficulty. The method of graphical counting is rather empirical, however, the equations provide a rapid and unambiguous proof of graphical counting.

The general equations presented above make it possible to count the number of patterns without really counting. In some enzyme kinetic studies, where complete sets of kinetic data are not obtainable due to 1) extremely high Ki value for one or more of the products, 2) nonenzymatic interaction among reactants such as precipitate formation, or 3) the limitation of the assay method etc. It is sometimes possible to deduce the kinetic mechanism from an incomplete set of kinetic data since the relationship among the reactants (substrates or products) are multiple. To deal with this more complicated situation, the general equations presented here will also be helpful. For example, a Ter-Quad system consists of 10 possible patterns of arrangements and 5 possible patterns of mechanisms. From our kinetic data (Table 11) there are three parallel patterns in double-reciprocal plots of initial velocity studies indicating at least three ping-pong's (and therefore at least three stable enzyme forms) involved in the reaction sequence. From Equations IV-2 and VI-5 it is clear that at three stable enzyme forms (n=3) there are three patterns of

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TABLE 11

A Summary of Kinetic Studies on L-Asparagine Synthetase Isolated from 6C3HED-RG1 Tumor

Kinetic studies	Parameters	Pattern in double reciprocal	Figure
Initial velocity studies	Asp vs Gln	parallel	13
	Gln vs ATP	parallel	15
	Asp vs ATP	parallel	14
Product inhibition studies	Asp vs pp	competitive	18
	Asp vs AMP	noncompetitive	18
	Asp vs Asn	uncompetitive	18
	Gln vs Asn	competitive	16
	Gln vs AMP	uncompetitive	17
	Gln vs PP	uncompetitive	17
Deadend inhibition studies	Asp vs DONV	uncompetitive	21
	Asp vs CONV	noncompeitive	21
	Gln vs DONV	competitive	22
	Gln vs CONV	noncompetitive	22
Alternate substrate studies	NH_4Cl vs ATP	Intersecting	19
	NH_4Cl vs Asn	competitive	20
	NH_4Cl vs PP	noncompetitive	20

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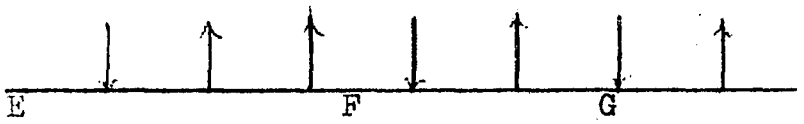
arrangements and all of them are really the same pattern of mechanism. Therefore, analyzing one pattern of them would mean analyzing all of them and no possibility of leaving out any pattern of mechanism. This can be graphically presented as the following:

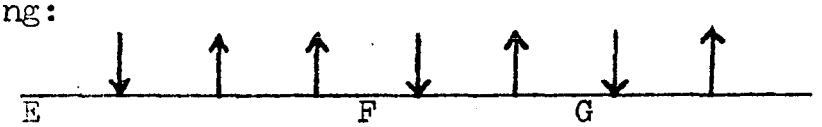


(IV-7)

(IV-8)

(IV-9)

Our initial velocity studies indicated that the double-reciprocal plots with Asp versus ATP, Gln versus ATP and Asp versus Gln, all showed parallel patterns indicating these three pairs of ping-pongs (Table 11). Let us arbitrarily take one pattern of them, IV-7 , for analysis. In the sequence, there are three input spaces for three different substrates. The permutation would be $P_3^3=3!=6$, and this permutation can be graphically presented as the following:



(IV-7)

ATP	Asp	Gln	(IV-7 a)
ATP	Gln	Asp	(IV-7 b)
Asp	ATP	Gln	(IV-7 c)
Asp	Gln	ATP	(IV-7 d)
Gln	Asp	ATP	(IV-7 e)
Gln	ATP	Asp	(IV-7 f)

In our kinetic data (Table 11), two competitive product

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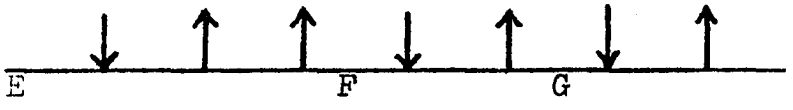
inhibitions, namely Gln versus Asn and Asp versus PP, indicating that Gln and Asp react with the same stable enzyme form, and that Asp and PP react with another stable enzyme form. Therefore, the relative positions of Asn and PP in IV-7 a to IV-7 f can be determined and the remaining two spaces for products will be either Glu or AMP. So, the number of remaining possible mechanisms is $P_3^3 \times P_2^2 = 6 \times 2 = 12$, as shown below:



(IV-7)

ATP	(Glu AMP)	PP	Asp	Asn	Gln	(AMP Glu)	(IV-7 á)
ATP	(Glu AMP)	Asn	Gln	PP	Asp	(AMP Glu)	(IV-7 b́)
Asp	(Glu AMP)	(AMP Glu)	ATP	Asn	Gln	PP	(IV-7 ć)
Asp	(Glu AMP)	Asn	Gln	(AMP Glu)	ATP	PP	(IV-7 d́)
Gln	(Glu AMP)	PP	Asp	(AMP Glu)	ATP	Asn	(IV-7 é)
Gln	(Glu AMP)	(AMP Glu)	ATP	PP	Asp	Asn	(IV-7 f́)

All but one of the above 12 possible mechanisms were excluded by the experimental results summarized on Table 11. Mechanisms IV-7 á and IV7 f́ were inconsistent with the results obtained from product inhibition studies on Asp versus Asn as well as Asp versus AMP. Mechanisms IV-7 b́ and IV-7 ć were inconsistent with the results obtained from the product inhibition study of Gln versus PP. Mechanism IV-7 é was not consistent with the results obtained from the alternate substrate initial velocity study on NH_4Cl

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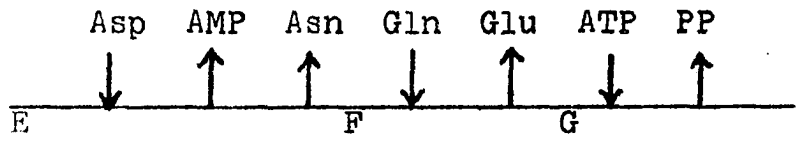
versus ATP. The upper sequence of mechanism IV-7d′ was inconsistent with the results obtained from product inhibition studies on Asp versus AMP and Gln versus AMP. All kinetic data obtained appeared to be consistent with the lower sequence of mechanism IV-7d′ which is given by:



(IV-10)

Among three stable enzyme forms (i.e., E,F and G) it is possible by using elementary knowledge of chemical structures of reactants to judge which one is the free enzyme form. In mechanism IV-10, if E is the free enzyme form and Asp reacts with E and sequencially releases AMP and Asn, AMP and Asn appear from nowhere and thus the assumption that E is the free enzyme form is apparently not plausible. If G is the free form, and ATP reacts with G and releases PP to form adenylated enzyme, then Asp adds on the adenylated enzyme and this is followed by the release of AMP and Asn. The first few steps seems plausible, however, there is no reason for releasing Asn as mentioned above, without input of an amide function. The most plausible explanation would be that F in mechanism IV-10 is the free enzyme form, in which Gln reacts with F and releases Glu to form aminated enzyme, then ATP adds to the aminated enzyme and releases PP to form aminated, adenylated enzyme. Asp then reacts with this aminated, adenylated enzyme to release AMP and Asn, and form the original free enzyme. These processes recycle again and again as the reaction

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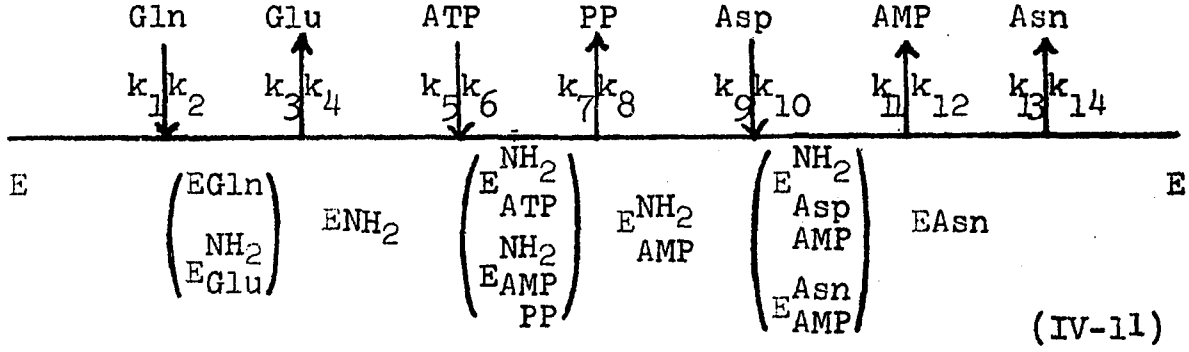
proceeds. Therefore, the following schematic presentation of kinetic mechanism of L-asparagine synthetase is proposed:



(IV-11)

According to Cleland's notation (IV-11) is a Penta Uni-Bi Ping-pong mechanism. (IV-11) can be written into stepwise mechanisms:

$$\mathrm{Gln} + \mathrm{E} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EGln} \rightleftharpoons \mathrm{E}^{NH_2}_{Gln} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{ENH_2} + \mathrm{Glu} \qquad \text{(IV-12)}$$

$$\mathrm{ENH_2} + \mathrm{ATP} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E}^{NH_2}_{ATP} \rightleftharpoons \mathrm{E}^{NH_2}_{AMP \cdot PP} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \mathrm{E}^{NH_2}_{AMP} + \mathrm{PP} \qquad \text{(IV-13)}$$

$$\mathrm{E}^{NH_2}_{AMP} + \mathrm{Asp} \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \mathrm{EAsp}^{NH_2}_{AMP} \rightleftharpoons \mathrm{E}^{Asn}_{AMP} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{EAsn} + \mathrm{AMP} \qquad \text{(IV-14)}$$

$$\mathrm{EAsn} \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} \mathrm{E} + \mathrm{Asn} \qquad \text{(IV-15)}$$

To deduce the rate equation of above mechanism the method of King and Altman (65) will be applied. For simplicity, the following notation was made:

A=Gln, B=ATP, C=Asp, P=Glu, Q=PP, R=AMP, S=Asn, F=ENH_2 and G=$E^{NH_2}_{AMP}$.

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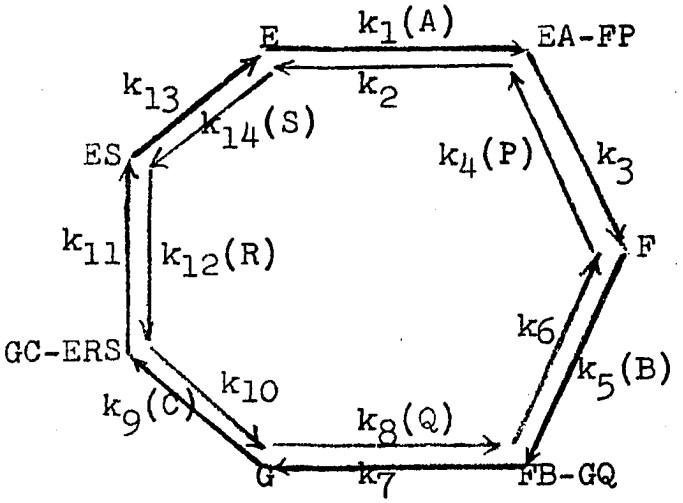
Therefore, (IV-1_2) to (IV-15) can be written into a heptagonal form:



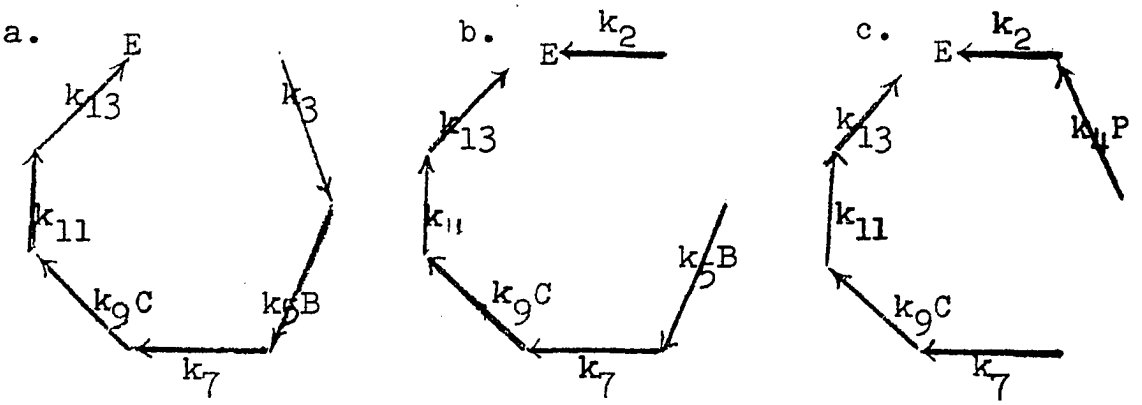
(IV-16)

The enzyme species, m = 7 and the reversible steps, n = 7, according to King and Altman (65):

$$\frac{m!}{(n-1)!(m-n+1)!} = \binom{m}{n-1} = \binom{7}{6} = \binom{7}{1} = 7$$

Therefore, every enzyme species has 7 terms each of which contain the product of 6, (n-1), rate constants and concentration factors.

For E (free enzyme) containing species, the following schematic presentation can be obtained:



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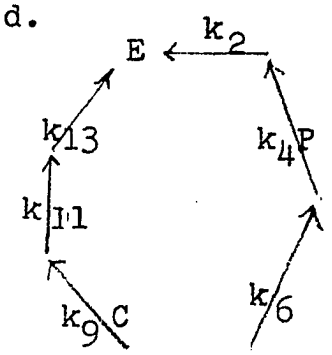
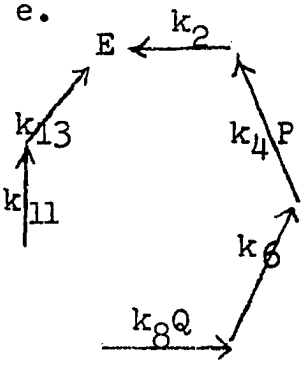
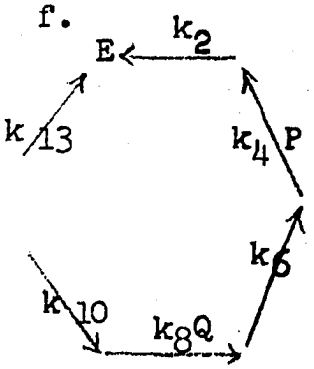
d.



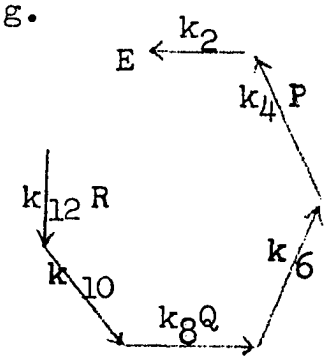
e.



f.



g.



(IV-17a~g)

The schemes of (EA+FP), F, FB+GQ, (GC+ERS), and ES enzyme-containing species are shown in Appendix I.

The relative concentration of each enzyme-containing species is proportioned to the summation of 7 terms:

$$Et = \sum_{i=1}^{n} EXi = \left\{ (E)+(EA-FP)+(F)+(FB-GQ)+G+(GC-ERS)+(ES) \right\}$$

Thus, the relative concentration of E enzyme-containing species is:

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$$\frac{(E)}{(Et)} \propto \{k_3k_5k_7k_9k_{11}k_{13}BC + k_2k_5k_7k_9k_{11}k_{13}BC$$
$$+ k_2k_4k_7k_9k_{11}k_{13}PC + k_2k_4k_6k_9k_{11}k_{13}PC$$
$$+ k_2k_4k_6k_8k_{11}k_{13}PQ + k_2k_4k_6k_8k_{10}k_{13}PQ$$
$$+ k_2k_4k_6k_8k_{10}k_{12}PQR\}$$
$$\propto \{(k_2+k_3)k_5k_7k_9k_{11}k_{13}BC + k_2k_4(k_6+k_7)k_9k_{11}k_{13}PC$$
$$+ k_2k_4k_6k_8(k_{10}+k_{11})k_{13}PQ + k_2k_4k_6k_8k_{10}k_{12}PQR\} \quad \text{(IV-18)}$$

The relative concentration of other enzyme-containing species, i.e., (EA-FP), F, (FB-GQ), G, (GC-ERS) and ES are shown in Appendix II.

Since the net rate of production of product (i.e., S or L-asparagine) is

$$\frac{d(S)}{dt} = \frac{\{k_{13}(ES) - k_{14}(E)(S)\}\ Et}{(E)+(EA+FP)+(F)+(FB+GQ)+(G)+(GC+ERS)+(ES)} \quad \text{(IV-19)}$$

substitute the relative enzyme concentrations of each enzyme containing species into (IV-19) and a full rate equation can be obtained.

The numberator of the rate equation will be:

$$\{(k_2+k_3)k_5k_7k_9k_{11}k_{13}k_{14}BCS + k_2k_4(k_6+k_7)k_9k_{11}k_{13}k_{14}PCS\ PCS$$
$$+k_2k_4k_6k_8(k_{10}+k_{11})k_{13}k_{14}PQS + k_1k_3k_5k_7k_9k_{11}k_{13}ABC$$
$$-(k_2+k_3)k_5k_7k_9k_{11}k_{13}k_{14}BCS - k_2k_4(k_6+k_7)k_9k_{11}k_{13}k_{14}PCS\ S$$
$$-k_2k_4k_6k_8(k_{10}+k_{11})k_{13}k_{14}PQS - k_2k_4k_6k_8k_{10}k_{12}k_{14}PQRS\}\ Et\ Et$$
$$= \{k_1k_3k_5k_7k_9k_{11}k_{13}ABC - k_2k_4k_6k_8k_{10}k_{12}k_{14}PQRS\}\ Et \quad \text{(IV-20)}$$

Similarly, the denominator of the rate equation can be obtained (see Appendix III for details).

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Therefore, the derived full rate equation is:

$$v = \frac{\left\{k_1k_3k_5k_7k_9k_{11}k_{13}ABC - k_2k_4k_6k_8k_{10}k_{13}k_{14}\ PQRS\right\}\ Et}{}$$

$$\begin{aligned}
&k_1k_3k_5k_7(k_{10}+k_{11})k_{13}\ AB \\
&+k_1k_3(k_6+k_7)k_9k_{11}k_{13}\ AC \\
&+k_1k_3k_6k_8(k_{10}+k_{11})k_{13}\ AQ \\
&+(k_2+k_3)k_5k_7k_9k_{11}k_{13}\ BC \\
&+k_2k_4(k_6+k_7)k_9k_{11}k_{13}\ PC \\
&+k_2k_4k_6k_8(k_{10}+k_{11})k_{13}\ PQ \\
&+k_1k_5k_9(k_3k_7k_{11}+k_3k_7k_{13}+k_3k_{11}k_{13}+k_7k_{11}k_{13})\ ABC \\
&+k_1k_3k_5k_8(k_{10}+k_{11})k_{13}\ ABQ \\
&+k_1k_3k_5k_7k_{10}k_{12}\ ABR \\
&+k_1k_4(k_6+k_7)k_9k_{11}k_{13}\ ACP \\
&+k_1k_4k_6k_8(k_{10}+k_{11})k_{13}\ APQ \\
&+k_1k_3k_6k_8k_{10}k_{12}\ AQR \\
&+(k_2+k_3)k_5k_7k_9k_{11}k_{14}\ BCS \\
&+(k_2+k_3)k_5k_7k_{10}k_{12}k_{14}\ BRS \\
&+k_2k_4(k_6+k_7)k_9k_{11}k_{14}\ PCS \\
&+k_2k_4k_6k_8k_{10}k_{12}\ PQR \\
&+k_2k_4k_6k_8(k_{10}+k_{11})k_{14}\ PQS \\
&+k_2k_4(k_6+k_7)k_{10}k_{12}k_{14}\ PRS \\
&+(k_2+k_3)k_6k_8k_{10}k_{12}k_{14}\ QRS \\
&+k_1k_3k_5k_7k_9k_{12}\ ABCR
\end{aligned} \qquad \text{(IV-21)}$$

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$$+k_1k_3k_5k_8k_{10}k_{12} \quad ABQR$$

$$+k_1k_4k_6k_8k_{10}k_{12} \quad APQR$$

$$+(k_2+k_3)k_5k_7k_9k_{12}k_{14} \quad BCRS$$

$$+(k_2+k_3)k_5k_8k_{10}k_{12}k_{14} \quad BQRS$$

$$+k_2k_4(k_6+k_7)k_9k_{12}k_{14} \quad PCRS$$

$$+k_4k_8k_{12}k_{14}(k_2k_6+k_2k_{10}+k_6k_{10}) \quad PQRS$$

(IV-21) is a rate equation expressing the reaction velocity in terms of reactant concentrations and the rate constants in individual steps. Cleland (47) has proposed a method to transform rate equation in King's method into a rate equation expressed entirely by kinetic constants and concentration factors, by introducing the equilibrium constant, and by defining for each reactant one inhibition constant in addition to the Michaelis-Menten constant observed when all other reactants are saturating. The relationship between rate constants for individual steps and kinetic constants is shown in Appendix IV.

f. Dead-end Inhibition Studies: The L-asparagine analogs DONV or CONV have been shown to be potent inhibitors of L-asparagine production in whole cell suspensions (Table 3). Studies on a cell-free extract from 6C3HED-RG1 indicated that both analogs inhibited L-asparagine synthetase only in the absence of a reducing agent - dithiothreitol (DTT) (see the next subsection). Since the enzyme purification procedure required the addition of reducing agent such as dithiothretol or β-mercaptoethanol to stabilize

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the enzyme, dead-end inhibition by these two analogs were studied with step 1b enzyme preparations (in the absence of DTT). DONV appeared to be an uncompetitive inhibitor, and CONV a noncompetitive inhibitor with respect to L-aspartic acid (Fig. 21). With respect to L-glutamine, DONV acted as a competitive and CONV acted as a noncompetitive inhibitor (Fig. 22). These results provided information concerning the enzyme forms with which these analogs combine (see p. 82).

g. Effect of a Reducing Agent on Kinetic Properties of L-Asparagine Synthetase: A sulfhydryl group appeared to be essential to L-asparagine synthetase since the enzymatic activity was inhibited 73% by preincubation with p-chloromercuribenzoate ($5x10^{-5}M$). This inhibition could be reversed by adding $5x10^{-3}M$ of diethiothreitol (DTT) to the assay mixture (Fig. 23). The same concentration of cupric chloride exhibited the same degree of inhibition but the recovery of enzyme activity by DTT was not as complete (Fig. 24). A reduced state was favorable to the enzyme since the addition of di thiothreitol (5mM) to the reaction mixture accelerated the reaction by 20% (Fig. 12). In the presence of DTT, L-glutamine became a better substrate, as indicated by a 4-fold reduction of the Km. The affinity of L-aspartic acid as a substrate for the enzyme was not significantly changed by DTT (Table 12). The affinity of L-asparagine as an inhibitor of the enzyme, however, decreased 2.5-fold with respect to either L-glutamine or L-aspartic

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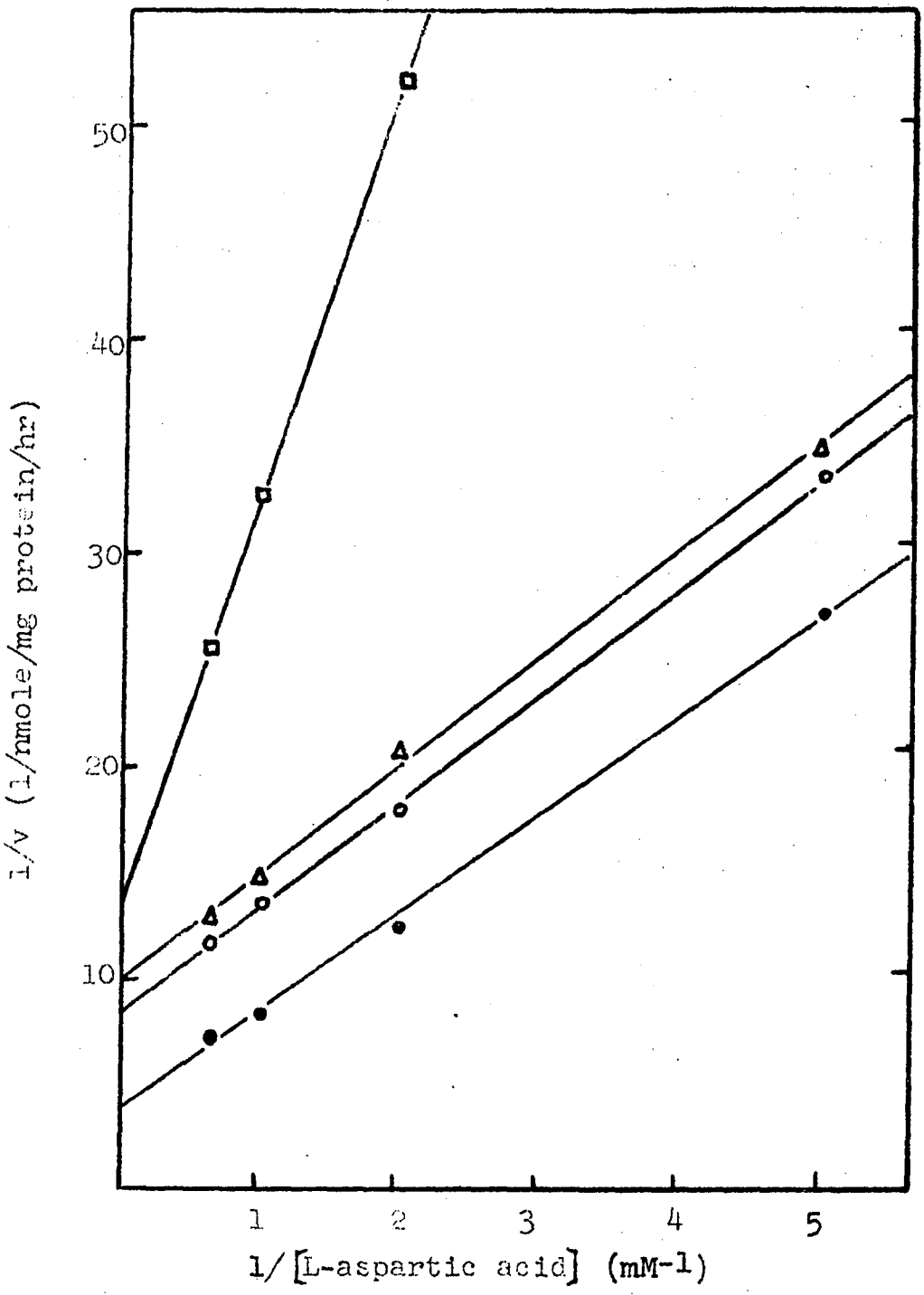

Figure 21. Double-reciprocal plots of initial reaction velocity against L-aspartic acid concentration to show the effects of CONV, DONV and asparagine. Control (•——•), plus 0.2mM CONV (▫——▫), plus 0.5mM DONV (▵——▵) and plus 0.2mM asparagine (○——○). All the experiments were performed in the absence of DTT and at a low concentration of L-glutamine (1.5mM). Step 1b enzyme preparation was used. Kinetic parameters are: V_{max} = 25 nmoles/mg protein/hr; K_{Asp} = 1.4 x 10^{-3}M; $Ki^{Asp}_{DONV(int)}$ = 2.9 x 10^{-4}M; $Ki^{Asp}_{Asn(int)}$ = 1.6 x 10^{-4}M; $Ki^{Asp}_{CONV(int)}$ = 7.2 x 10^{-5}M; $Ki^{Asp}_{CONV(slope)}$ = 6.2 x 10^{-5}M.

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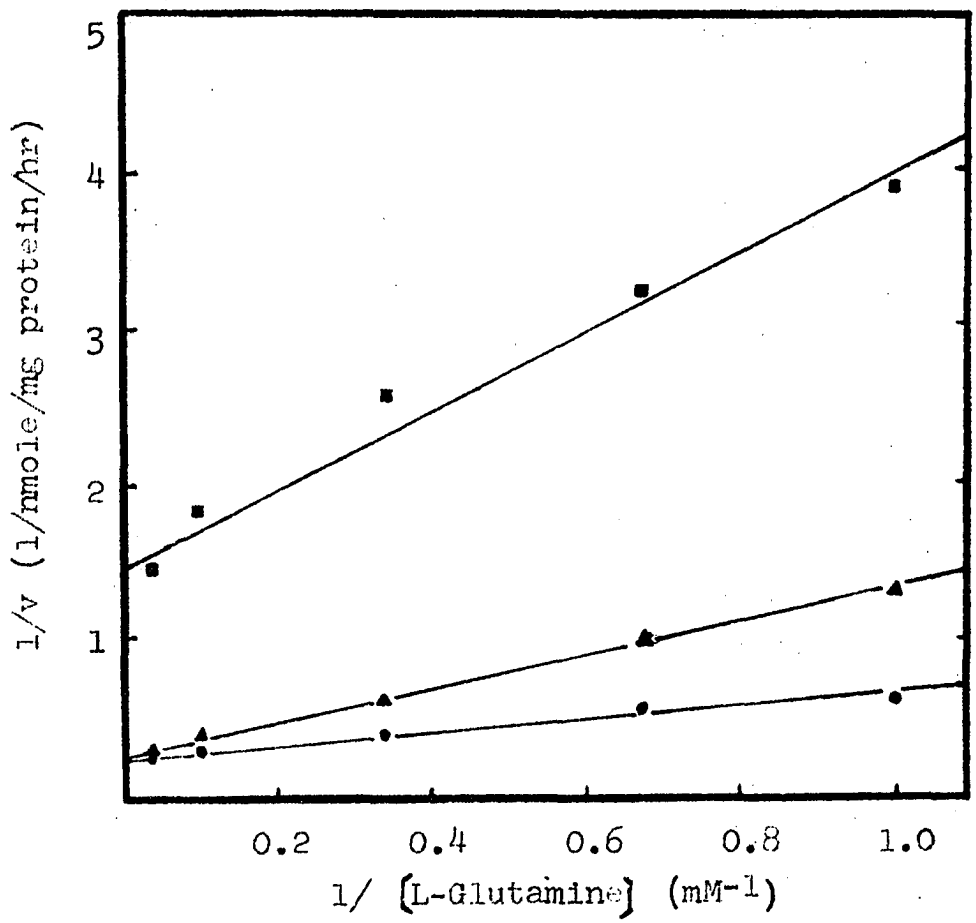

Figure 22. Double-reciprocal plots of initial reaction velocity against L-glutamine concentration to show the effects of DONV and CONV. Control (•——•), plus 0.5mM DONV (▲——▲), and plus 0.2mM CONV (■——■). All the experiments were performed in the absence of DTT and other reactants at standard assay concentrations as shown in METHODS. Step 1b enzyme preparation was used. Kinetic parameters are: V_{max} = 37 nmole/mg protein/hr; K_{Gln} = 1.8×10^{-3}M; $Ki^{Gln}_{CONV(int)} = 2.9 \times 10^{-5}$M; $Ki^{Gln}_{CONV(slope)} = 4.8 \times 10^{-5}$M; $Ki^{Gln}_{DONV(slope)} = 4.3 \times 10^{-4}$M.

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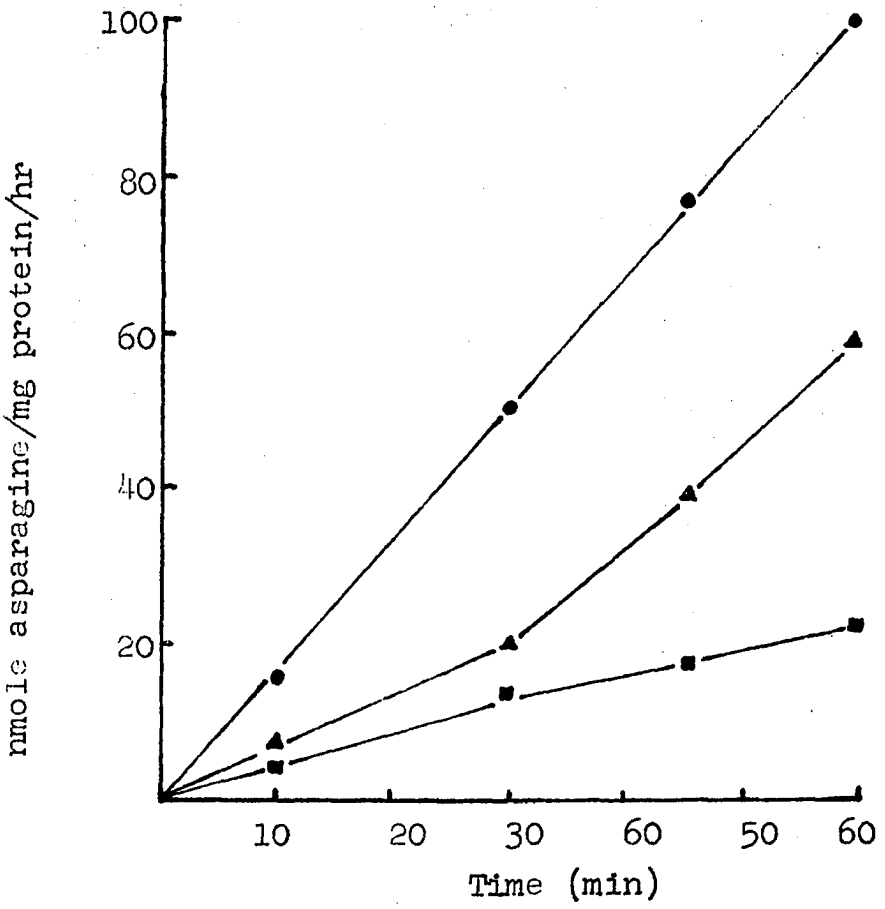

Figure 23. Inhibition of L-asparagine synthetase by p-chloromercuribenzoate and the reactivation of the enzyme by dithiothreitol. DTT free step 2 enzyme preparation from 6C3HED-RG1 tumor was used. (●——●), the enzyme was preincubated for 30 min in the absence of the inhibitor and substrates, and then the enzymatic activity was assayed in the presence of DTT (5×10^{-3}M) at various times. (▲——▲), the enzyme was preincubated for 30 min in the presence of the inhibitor (5×10^{-5}M) and in the absence of substrates, and then the enzymatic activity was assayed in the presence of DTT (5×10^{-3}M) at various times. (■——■), the enzyme was preincubated with the inhibitor (5×10^{-5}M) and in the absence of substrates, and then the enzymatic activity was assayed in the absence of DTT at various times.

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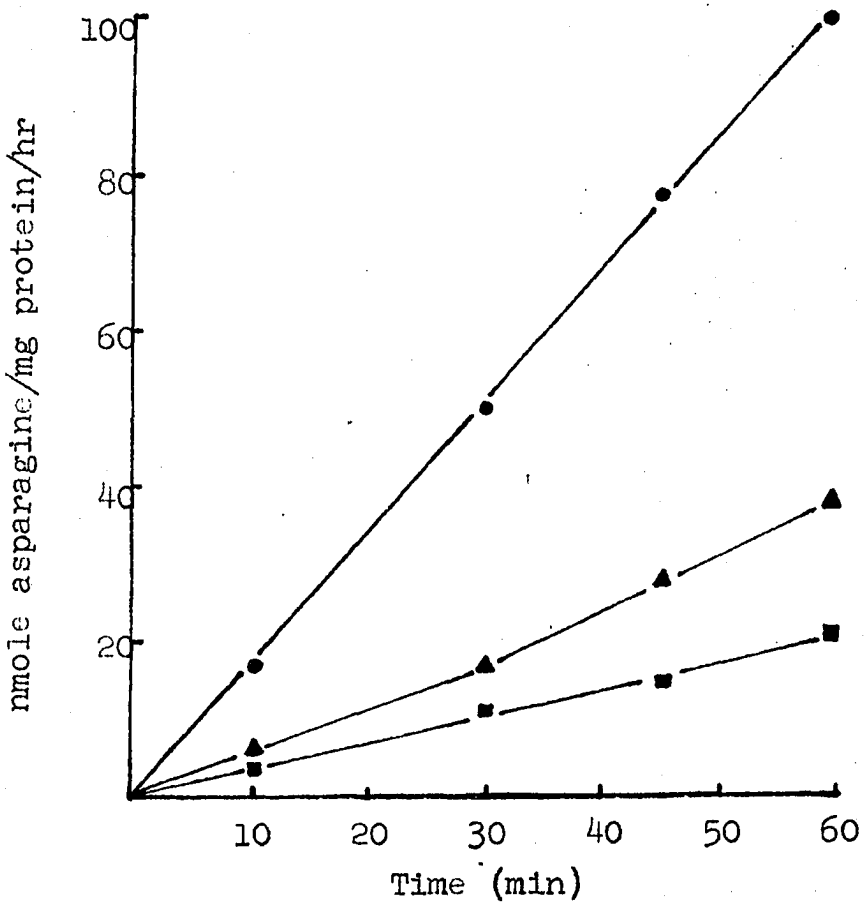

Figure 24. Inhibition of L-asparagine synthetase by cupric chloride and the reactivation of the enzyme by dithiothreitol. The same conditions as Figure 23 were used except $CuCl_2$ (5×10^{-5}M) was used as an inhibitor. •——•, Control experiment. ▲——▲, the enzyme was preincubated with $CuCl_2$ and then assayed in the presence of DTT at various times. ■——■, the enzyme was preincubated with $CuCl_2$ and then assayed in the absence of DTT at various times.

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TABLE 12

Effect of Reducing Agent on Kinetic Properties of L-Asparagine Synthetase[a]

		Without dithiothreitol		With dithiothreitol (3×10^{-3}M)	
		Km[b]	Ki[c]	Km	Ki
Substrate	Gln	2.5×10^{-3}	-	$6.\times10^{-4}$	-
	Asp	1.1×10^{-3}	-	1.0×10^{-3}	-
	Asn	-	1.7×10^{-4}(Gln)[d] 2.2×10^{-4}(Asp)	-	4.4×10^{-4}(Gln) 6.1×10^{-4}(Asp)
Inhibitor	DONV	-	4.3×10^{-4}(Gln) 3.3×10^{-4}(Asp)	-	not inhibitory[e]
	CONV	-	2.9×10^{-5}(Gln) 7.3×10^{-5}(Asp)	-	not inhibitory[e]

a. A dithiothreitol-free enzyme preparation (105,000 x g supernatant from 6C3HED-RG1 precipitated with 38-48% ammonium sulfate saturation) was used for the standard assay (see METHODS).

b. Km was obtained by the negative reciprocal of the intercept on the horizontal, x, axis of Lineweaver-Burk plot (66).

c. Ki was calculated by either of the following formula when applicable:

$$Ki = \frac{[I]}{\frac{\text{Slope i}}{\text{Slope o}} - 1} \quad \text{or} \quad Ki = \frac{[I]}{\frac{\text{Yint,i}}{\text{Yint,o}} - 1}$$

where I is the molar concentration of inhibitor. Slope i and Slope o are the slopes of Lineweaver-Burk plot in the presence and absence of inhibitor respectively. Yint, i and Yint, o are the intercepts on ordinate, y, axis of Lineweaver-Burk plot in the presence and absence of inhibitor respectively.

d. Compounds in the parentheses are variable substrates.

e. Not inhibitory at DONV (1×10^{-3}M) and CONV (2×10^{-4}M).

acid as a variable substrate. The most dramatic effect of DTT was the complete protection against the inhibitory action of DONV ($1x10^{-3}M$) or CONV ($2x10^{-4}M$) (Table 12). These results suggest that a portion of the catalytic center has been changed by the reducing agent, possibly by the breakage of an intermoleuclar or intramolecular disulfide bond. This explanation is further supported by the results in Figure 25. This figure shows the time course of L-asparagine biosynthesis by the synthetase which is inhibited by DONV in the absence of DTT. If DTT (5mM) was added to the reaction mixture, the inhibited enzyme was restored to full activity with the same slope as the dithiothreitol enhanced control enzyme. A similar phenomenon was observed with L-asparagine ($4x10^{-4}M$) as an inhibitor, but with less restoration of enzyme activity by DTT (Fig. 26). The possibility of direct interaction between DTT and DONV was excluded by an experiment which incubated DTT with DONV for 20 min, neither the total amounts of sulfhydryl group of the DTT nor the total amounts of DONV were changed.

6. Comparison of Properties of L-Asparagine Synthetases from Various Sources: L-Asparagine synthetase activity has been purified to various degrees and studied to various extents from various sources (Table 13). Although all of them (Table 13) appeared to be catalyzing the reaction which splits ATP to AMP and PP, with conversion of Asp to Asn, the properties of L-asparagine synthetase from various sources are quite different. These are manifested by

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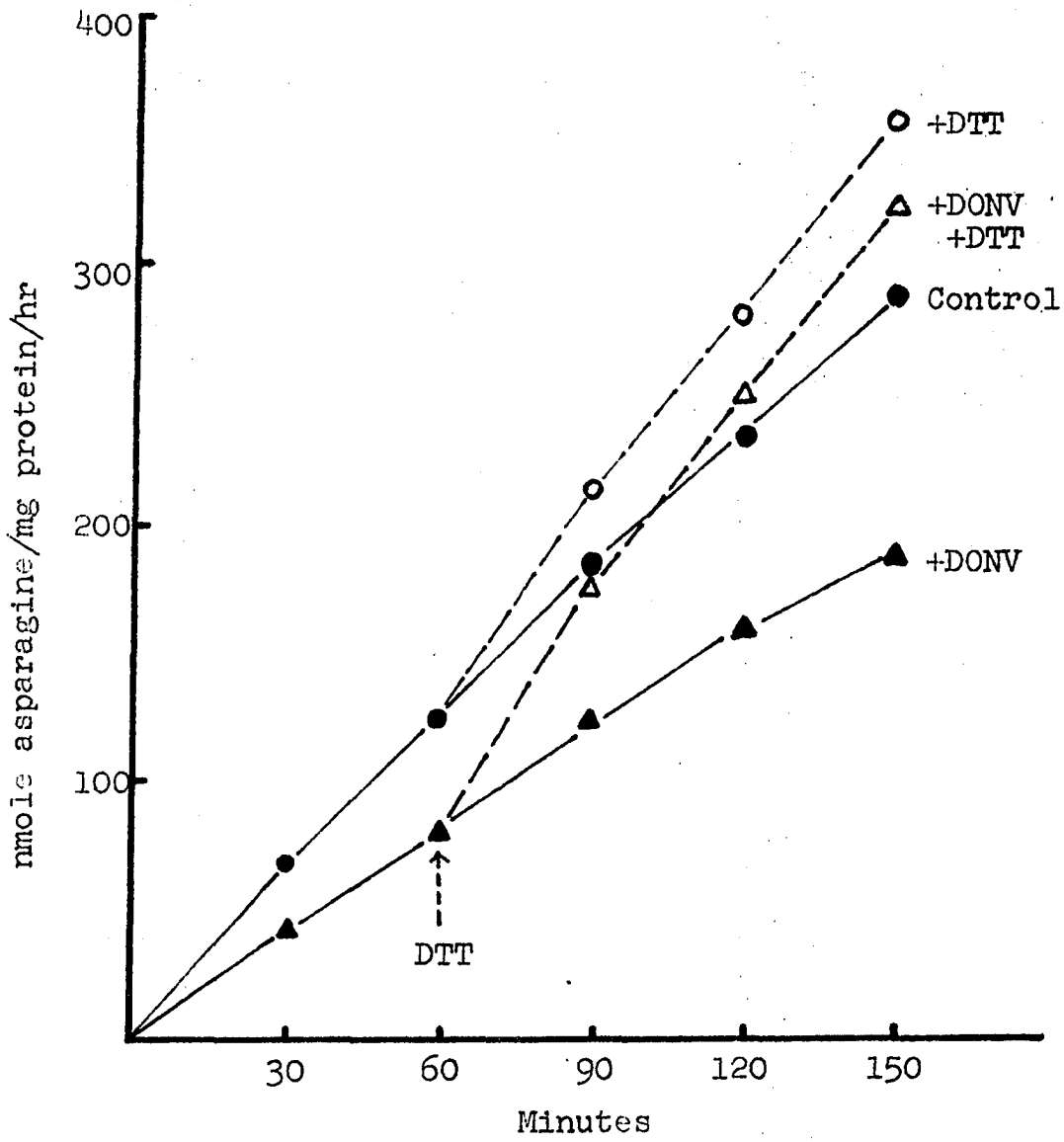

Figure 25. Activation of L-asparagine synthetase and reactivation of DONV inhibited L-asparagine synthetase by dithiothreitol. A step 2 enzyme prepared without DTT from 6C3HED-RG1 tumor was used. After 60 min of incubation, 5mM of dithiothreitol (DTT) was added to both control and DONV (1mM) inhibited incubation mixtures which contained no DTT. L-Asparagine biosynthesis was assayed with high-voltage electrophoresis as described in METHODS.

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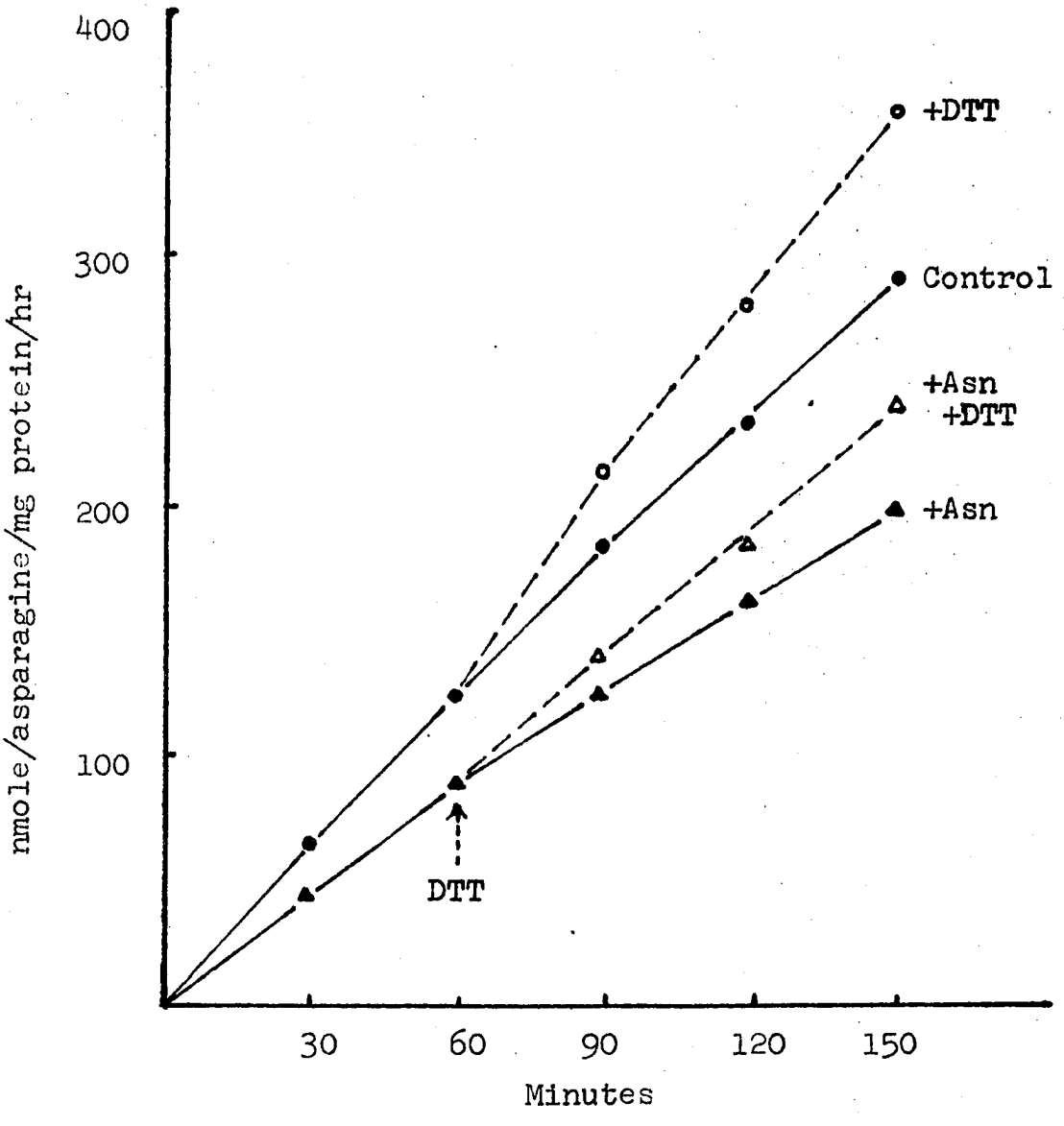

Figure 26. Activation of L-asparagine synthetase and reactivation of L-asparagine inhibited L-asparagine synthetase by dithiothreitol. The same experimental conditions as in Figure 25 were used except that L-asparagine (0.4mM) was used as the inhibitor.

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TABLE 13

A. Properties of L-Asparagine Synthetases from Various Sources[a]

Sources	Purification[b]	Specific Activity (nmole/mg/hr)	Amido donor	Activator[c]	Stabilization by reducing agent	pH optimal[d]	References
Microorganism:							
Lactobacillus arabinosus	13x	31,600	NH_4Cl (NH_2OH) not Gln	Mn^{++} Mg^{++} (1:0.7)	β-mercaptoethanol	8.2	(32)
Streptococcus bovis	21x	23,000	NH_4Cl (NH_2OH)	Mg^{++} (not Mn^{++})	enzyme 50% inhibited by p-hydroxymercuribenzoate, $10^{-2}M$	7.2	(33)
Escherichia coli	370x	27,240	NH_4Cl (NH_2OH) not Gln	Mg^{++} (not Mn^{++})	β-mercaptoethanol	8.4	(46)
Avian:							
Chick-embryo liver	2x		Gln (not NH_4Cl)	Mg^{++} (not Mn^{++})	NA	7.5	(34)
	2x		Gln	Mg^{++}	β-mercaptoethanol	8.0	This dissertation (Pp. 47-50)
					DTT		

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TABLE 13 (contd)

A. Properties of L-Asparagine Synthetases from Various Sources[a]

Sources	Purification[b]	Specific Activity (nmole/mg/hr)	Amido donor	Activator[c]	Stabilization by reducing agent	pH optimum[d]	References
Mammalian:							
Guinea-pig liver	S	15-40	Gln	Mg^{++} (not Mn^{++})	NA[e]	7.5	(41)
Guinea-pig liver and other tissues (small intestines, spleen etc.)	18X from S[g]	230[f]	$(NH_4)_2SO_4$ or Gln+K_2SO_4 but not Gln plus NH_4Cl(??)	Mg^{++}	NA	7.7.8.0	(41)
Mouse liver and spleen, and tumors	S[g]	0-876[g]	Gln NH_4Cl (1:0.72)	Mg^{++}	NA	7.6,8.0	(40)
Mouse normal tissues and tumors	S	0-156	Gln	Mg^{++}	DTT	7.6	(39)
Mouse tumor (6C3HED-RG1)	309x	2,700	Gln NH_4Cl (1:0.87)	Mg^{++} (not Mn^{++}	DTT	7.6,8.0	This dissertation (Pp. 51-53)

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TABLE 13 (contd)

A. Properties of L-Asparagine Synthetases from Various Sources[a]

Source	Purification[b]	Specific Activity (nmole/mg/hr)	Amido donor	Activator[c]	Stabilization by reducing agent	pH[d] optimal	References
Rat Jensen sarcoma	pH5 enzyme	NA	Gln NH_4Cl	Mg^{++} Mn^{++} (1:0.2)	Glutathione	8.0	(36)
Rat Novikoff hepatoma	108x	5,360	Gln	Mg^{++} Mn^{++} (1:0.88)	NA	8.0	(37)
Rat embryo liver	S		Gln NH_4Cl	Mg^{++} Mn^{++}	β-mercaptoethanol DTT	7.8	This dissertation (Pp.50-51)

B. Kinetic Parameters of L-Asparagine Synthetases from Various Sources[a]

Source	Kinetic Constants (M)	Kinetic Pattern Studies: Initial Velocity	Kinetic Pattern Studies: Product Inhibition[h]	References
Microorganism: *Lactobacillus arabinosus*	Asp, $Km=4.2\times10^{-3}$ ATP, $Km=2.0\times10^{-3}$ Mn^{++}, $Km=3.5\times10^{-3}$	NA	Asn vs Asp noncompetitive Asn inhibit Asp dependent PP-ATP exchange	(32)
Streptococcus bovis	Asp, $Km=2.6\times10^{-2}$ ATP, $Km=4.0\times10^{-3}$ NH_4Cl, $Km=4.0\times10^{-3}$ Mg^{++}, $Km=4.5\times10^{-2}$	NA	Asn, Ki (Asp) $=1.8\times10^{-4}$	(33)

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TABLE 13 (contd)

B. Kinetic Parameters of L-Asparagine Synthetases from Various Sources[a]

Source	Kinetic Constants (M)	Kinetic Pattern Studies		References
		Initial Velocity	Product Inhibition[h]	
Escherichia coli	Asp, Km-3.3×10^{-4} ATP, Km=6.3×10^{-4}	Asp vs NH_4Cl, parallel ATP vs NH_4Cl, parallel ATP vs Asp, intersecting	Asn, Ki(Asp)=1.2×10^{-4} AMP, Ki(ATP)=1.5×10^{-3} Asn vs Asp, competitive ATP vs AMP, competitive Asp vs AMP, noncompetitive ATP vs Asn, noncompetitive Asn inhibit Asp-dependent PP-ATP exchange	(46)
Avian:				
Chick-embryo liver	Asp, Km=2.0×10^{-3}	NA	Asn, Ki(Asp)=1.1×10^{-4} DONV, Ki(Asp)=1.8×10^{-4}	unpublished observations
Mammalian:				
Guinea-pig liver	Asp, Km=9.0×10^{-4} ATP, Km=3.0×10^{-4}	NA	Asn, Ki(Asp)=1.5×10^{-4} (with Gln as substrate) Asn, Ki(Asp)=1.0×10^{-1} (with $(NH_4)_2SO_4$ as substrate)	(41)
Rat Novikoff hepatoma	Asp, Km=5.8×10^{-4} ATP, Km=1.1×10^{-4} Gln, Km=1.1×10^{-3} NH_4Cl, Km=1.2×10^{-2} Mg^{++}, Km=2.4×10^{-2}	NA	Asn vs Asp, noncompetitive	(37)

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TABLE 13 (contd)
B. Kinetic Parameters of L-Asparagine Synthetases from Various Sources[a]

Source	Kinetic Constants (M)	Kinetic Pattern Studies			References
		Initial Velocity	Product Inhibition[h]	Analog Inhibition[h]	
Mouse tumor (6C3HED-RG1)	Asp, Km=1.2×10^{-3} ATP, Km=7.8×10^{-4} Gln, Km=6.5×10^{-4} NH_4Cl, Km=3.9×10^{-3}	See Table 11	Asn, Ki(Asp)=2.5×10^{-4} Asn, Ki(Gln)=1.0×10^{-4} AMP, Ki(Asp)=9.4×10^{-3} AMP, Ki(Gln)=1.5×10^{-2} PP, Ki(Asp)=7.0×10^{-3} PP, Ki(Gln)=7.5×10^{-3} Asn, Ki(NH_4Cl)=1.1×10^{-3} PP, Ki(NH_4Cl)=2.0×10^{-3}	DONV, Ki(Asp)=2.9×10^{-4} DONV, Ki(Gln)=4.3×10^{-4} CONV, Ki(Asp)=6.7×10^{-5} CONV, Ki(Gln)=3.8×10^{-5}	This dissertation

a. Different experimental conditions were used by various authors, data obtained from different authors can, therefore, not be directly compared. For convenience, all enzymes specific activities are transformed to nmole/mg protein/hr, and all kinetic constants are presented in molar units.
b. All values represent folds of purification from crude homogenate. S represents 105,000 x g supernatant fraction.
c. The ration in the parentheses represent the ratios of activity as an activator of the enzyme when replaced by each other.
d. When optimum pH of the reaction was not determined, the pH used in the enzyme assay was presented.
e. Data not available.
f. The 105,000 x g supernatant fractions from liver, small intestine and occasionally spleen were pooled for further purification.
g. Supernatant fractions of 10,000 rpm (Sorvall SS-34 rotor) were used for most experiments. The highest value, 876, obtained from 105,000 x g supernatant.
h. Superscription of Ki is inhibitor, subscription of Ki is the variable substrate (in parentheses) or alternate substrate. The Ki values may have been obtained from nonsaturation level of other substrates. The values are affected by reducing agents.

differences in the amido donors and divalent cation activators. Apart from large differences in Km or Ki values, the product inhibition patterns or initial velocity patterns which underlie the basic kinetic mechanism (reaction sequence) are also different. For example, L-asparagine is an uncompetitive inhibitor with respect to L-aspartic acid with 6C3HED-RG1 enzyme, a competitive inhibitor with respect to L-aspartic acid with E. coli enzyme and a noncompetitive inhibitor with respect to L-aspartic acid in both L. arabinosus and Novikoff hepatoma enzymes (Table 13).

7. Discussion: Since L-asparagine synthetases differ in both physical and kinetic properties from one source to another, caution should be used when extrapolations are being made. Even with the same tumor, the data obtained from a cell-free extract may not reflect events in a whole cell system (Tables 7,8).

The only metabolic pathways of L-asparagine known at present are its utilization for protein synthesis and its conversion to L-aspartic acid by hydrolysis or to α-keto succinamic acid by transamination. Thus, we may

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expect that L-asparagine has a role as a feed-back inhibitor. This in fact has been shown for L-asparagine synthetases from various sources (32,33,37,46). In whole cell systems of P815Y tumor, the concentration of L-asparagine for 50% inhibition of L-asparagine production (p. 33) is much lower than that of Ki values of L-asparagine in L-asparagine synthetase from various sources. It seems reasonable to expect that L-asparagine synthesis in intact cells is under more effective control by L-asparagine than that in a cell-free system. The capability of viable cells to concentrate L-aspargine from the medium (p. 27) may be a major factor in this feed-back regulation. Another regulatory effect of L-asparagine includes repression of L-asparagine synthetase synthesis. Although a relatively high concentration of L-asparagine was required for this effect, this effect was observed in Lactobacillus arabinosus (32) and Escherichia coli (46). However, this repression effect was not observed in Streptococcus bovis (33). In 6C3HED-Ror-s tumors, no induction of L-asparagine synthetase synthesis by administration of substrates has been observed (40). Reducing agents such as DTT greatly modified the kinetic properties of L-asparagine synthetase (Table 12). It is not known whether the redox states or the naturally occurring reducing agents in the cells play any role in the control of L-asparagine biosynthesis. In coliform bacteria asparagine synthetase (46), the reaction sequence appeared to be a Bi-Uni-Uni-Bi ping pong mechanism in which L-aspartic

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acid and ATP added in a random order, followed by the release of PP and the addition of ammonia and then L-asparagine and AMP left the enzyme in a random order. This is different from the Penta Uni-Bi ping pong mechanism deduced from 6C3HED-RG1 enzyme (p. 82), in which L-glutamine interacts with free enzyme and releases L-glutamic acid; the aminated enzyme then reacts with ATP to release PP and form an aminated, adenylated enzyme. The resultant modified enzyme then appears to react with L-aspartic acid to release AMP and L-asparagine (scheme IV-11). Studies on L-asparagine synthetases isolated from E. coli (46), L. arabinosus (32) and Novikoff hepatoma (37) have suggested that the reactions may proceed through an enzyme-bound β-aspartyladenylate intermediate. The mechanism proposed for 6C3HED-RG1 as described above did not exclude this possibility. Thus, we may speculate that adenylated (and aminated) enzyme reacts with L-aspartic acid to form β-aspartyladenylated enzyme (an enzyme complex containing activated L-aspartic acid), the activated L-aspartic acid then reacts with enzyme-bound activated amido group at the juxtaproximal locus leading to the formation of L-asparagine. The fact that L-glutamine is a better substrate than ammonium chloride under the standard assay conditions may be explained by ionization of ammonia (pK_a 9.3). Figure 9 suggests that nonionized ammonia is the active amido donor. An alternative explanation would be an induced configuration fit of L-glutamine to the enzyme to facilitate the formation of active ammonia.

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V. PATTERN ANALYSIS OF MULTIPLE SUBSTRATE REACTIONS

A. L-ASPARAGINE SYNTHETASE AS A MULTIPLE SUBSTRATE ENZYME

L-Asparagine synthetases from various sources have been shown to catalyze reactions with L-glutamine (or ammonium chloride, or hydroxamine), L-aspartic acid and ATP as substrates and L-glutamic acid, L-asparagine, AMP and PP as products (p. 94). All these reactions require a divalent cation (Mg^{++} or Mn^{++}) as an activator (Table 13). The synthetases with L-glutamine as a substrate will be a Ter-Quad system according to Cleland, whereas the synthetases with ammonia as a substrate will be Ter-Ter system assuming that the leaving of H_2O is not a controlling step (47).

B. MULTIPLE-SUBSTRATE ENZYME REACTION AS A MODEL FOR PATTERN ANALYSIS

When a reaction with more than one substrate or product is under consideration, there will be a certain number of possible sequences of addition of substrates or release of products. A product can be released from the enzyme before all substrates have been added on the enzyme; among these ping pong mechanisms are those catalyzed by various pyridoxol phosphate requiring amino-transferases (67) and the dehydrogenation reactions catalyzed by some flavoproteins (68). Products may release after all substrates have been added to the enzyme, among these ordered mechanisms are those catalyzed by a variety of NAD- and NADP-requiring dehydrogenases (69,70). In many cases, all possible binary enzyme-substrate complexes are formed rapidly and reversibly. In

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this case, the magnitude of any dissociation constant for a reactant is unaffected by the prior attachment of any other reactant to the enzyme, and the only slow steps are those interconverting higher complexes such as ternary complexes. Among the reactions obeying this mechanism are those catalyzed by a kinase such as creatine kinase. In a special case, a central complex may not exist, this is the so called Theorell-Chance mechanism as reported for lactic dehydrogenase of mammalian heart (49,71).

All ordered mechanisms (with the release of products after all substrates have been added) have only one sequential pattern. The ping pong mechanisms may have many patterns depending on how many substrates, products and stable enzyme forms are involved. Random mechanisms can be deduced by alternative explanations of kinetic data for contradicting linear (non-random) conditions. The Theorell-Chance mechanism has long been considered as an exceptionally rare case (47). Therefore, pattern analysis in the following subsections will be centered on the linear pattern analysis of which only ordered mechanisms and ping pong mechanisms will be considered. For L-asparagine synthetases which catalyze a reaction with L-glutamine, L-aspartic acid and ATP as substrates and L-glutamic acid, L-asparagine, AMP and PP as products, the sequencial patterns are shown as the following:

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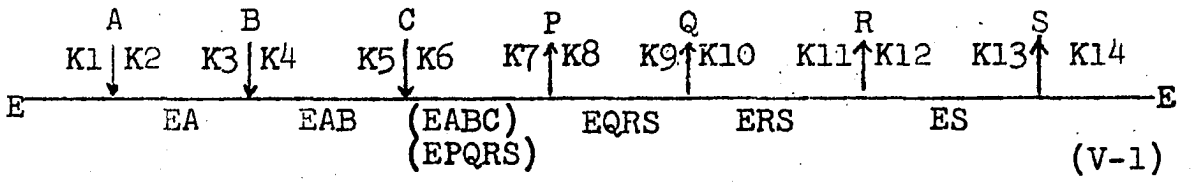
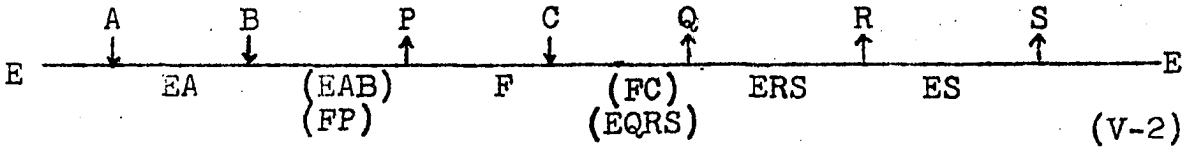


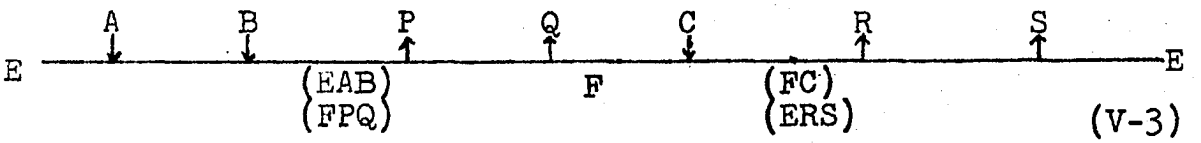
(V-1)

ordered Ter-Quad



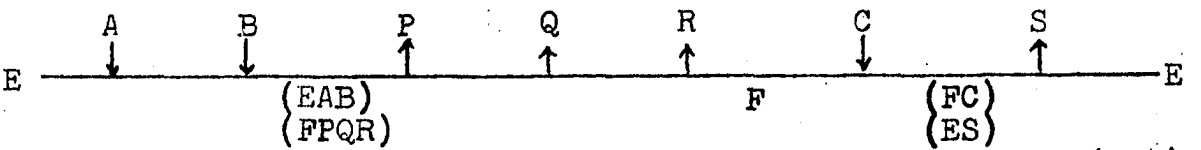
(V-2)

Bi-Uni-Uni-Ter ping pong



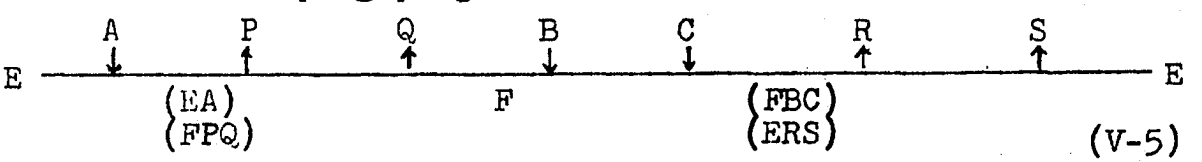
(V-3)

Bi-Bi-Uni-Bi ping pong



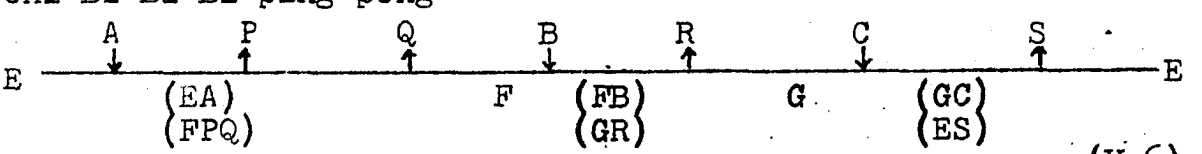
(V-4)

Bi-Ter-Uni-Uni ping pong



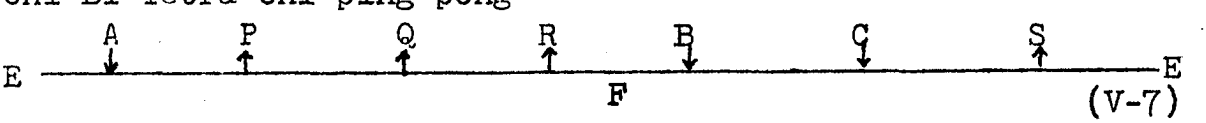
(V-5)

Uni-Bi-Bi-Bi ping pong



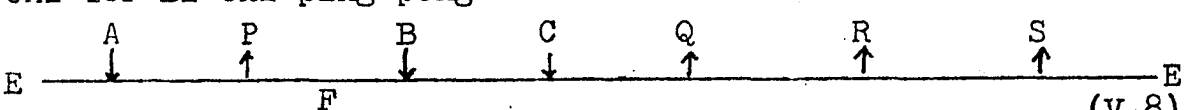
(V-6)

Uni-Bi-Tetra Uni ping pong



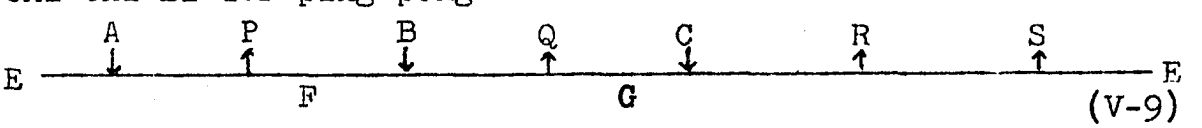
(V-7)

Uni-Ter-Bi-Uni ping pong



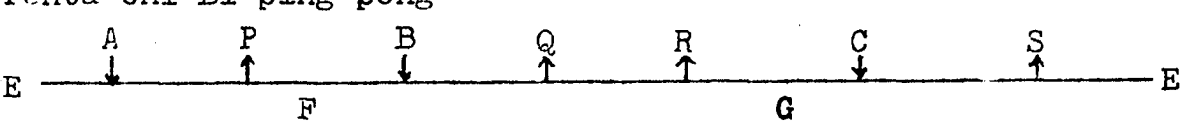
(V-8)

Uni-Uni-Bi-Ter ping pong



(V-9)

Penta Uni-Bi ping pong



Uni-Uni-Uni-Bi-Uni-Uni ping pong (V-10)

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Where A, B, and C are substrates, P; Q; R and S are products; E, F and G are stable enzyme forms; EA, EAB....are transitory complexes; and (EABC), (EPQRS); (FP)....are central complexes. For definitions please see references (47,48) or (pp. 9-13).

C. NUMBER OF SEQUENTIAL PATTERNS AND MECHANISM PATTERNS IN THE ARRANGEMENT OF ENZYME CATALYZED REACTIONS WITH MULTIPLE SUBSTRATES AND PRODUCTS

Summary: Cleland's model of enzyme catalyzed reactions with multiple substrates and products is subjected for pattern analysis. General equations are derived to express: 1) the overall number of sequential patterns and mechanism patterns at any given number of substrates and products; 2) the number of sequential patterns and mechanism patterns at any given number of substrates, products and stable enzyme forms.

Introduction: Cleland (47) has proposed a general method to analyze possible mechanisms of enzyme-catalyzed reactions with more than one substrate or product. This general method has seen considerable application (48). In his original paper (47), discussion was extended to the number of possible sequential patterns and mechanism patterns in the graphical sequence. If consideration is limited to 1) ordered and ping pong mechanisms in which no alternate reaction pathway exists; 2) free stable enzyme forms do not isomerize and 3) a central complex is always present; it appears that the total number of possible

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patterns of both sequences and mechanisms at a given number of stable enzyme forms may be expressed by the mathematical equations devised below rather than by the laborious and empirical method of graphical counting.

Analysis: Any enzyme-catalyzed reaction starts with the addition of the first substrate and ends with the release of the last product and recycles as a loop. When a linear sequence contains multiple substrates (S, inputs) and products (P, outputs), the overall patterns of sequential arrangements can be interpreted as the number of permutations of (S-1)+(P-1) things of which S-1 are alike and the other P-1 are alike, therefore, A (S,P),

$$\text{The number of overall sequential patterns} = \frac{[(S-1)+(P-1)]\ !}{(S-1)!(P-1)!} = \binom{S+P-2}{S-1,P-1} \qquad \text{(V-11)}$$

of which S-1 and P-1 are the numbers of substrates and products, respectively, that can be freely arranged.

Equation (V-11) gives the overall number of patterns in sequences containing all possible number of stable enzyme forms. However, it gives no information about the number of patterns at a given number of stable enzyme forms. Therefore, the author intends to further analyze this problem.

In Cleland's reaction model (47), whenever an output (↑ or product) is immediately followed by an input (↓ or substrate) in the sequence (let this pair be represented by ↑↓), there will be a new stable enzyme form. The

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possible number of stable enzyme forms should be equal to or less than the lower limit of ↓'s or ↑'s. In practice the number of ↑↓ pairs in a given sequence is equal to the number of stable enzyme forms or the number of ping pongs*, and also equal to the number of competitive inhibitions when all product inhibition experiments are carried out. The sequence of ↓'s and ↑'s is completely determined at a given number of ↑↓ pairs and the indices of ↑'s and ↓'s involved in each ↑↓ pair. At n number of ↑↓'s, the ↑'s can be chosen in $\binom{P-1}{n-1}$ different ways and the ↓'s in $\binom{S-1}{n-1}$ ways. Since any choice of (n-1) indices of ↑'s may be combined with any choice of ↓'s indices; the number of sequential patterns for n ↑↓'s in the sequence is therefore

$$A(n;S,P)=\binom{S-1}{n-1}\binom{P-1}{n-1} \qquad \text{(V-12)}$$

The number of overall patterns equals the sum of patterns of every possible given number of stable enzyme forms. Thus, by correlation of (V-11) and (V-12) we get

$$\frac{(S+P-2)!}{(S-1)!(P-1)!} = N_1 + N_2 + \ldots . N_n = \sum_{j=1}^{n} N_j = \sum_{j=1}^{n} \binom{S-1}{j-1}\binom{P-1}{j-1} \qquad \text{(V-13)}$$

* Since all enzyme-catalyzed reactions start with an input and end with an output and recycle. From the end recycles to the beginning there is always an output followed by an input (↑ ↓). However, in Cleland's notation (47), ping pong sequence at both ends, although common to all enzyme-catalyzed reactions, are not mentioned as such.

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of which S, $P \geq n \geq 1$; N_1, N_2 ..... Nn are the number of sequential patterns for 1, 2 .....n number of the stable enzyme forms ($\uparrow\downarrow$'s) respectively.

Since all enzyme-catalyzed reactions proceed as a loop and each stable enzyme form can be a starting point of a loop, several sequential patterns involving a given number of stable enzyme forms may actually have the same mechanism* and give rate equations of identical form (47). If the number of substrates (S), products (P) and stable enzyme forms (n) have no common divisor other than one, the following relationship should be true. At a given number of stable enzyme forms, the number of mechanism patterns equals the number of sequential patterns divided by the number of stable enzyme forms. Therefore, each term in the middle of equation (V-13) divided by the corresponding number of stable enzyme forms of that term will give the number of mechanism patterns in that specific term. Based on this reasoning, equation (V-14) can be established in which the overall number of mechanism patterns equals the summation of the partitions of them at all number of stable enzyme forms. Thus,

$$\text{The number of overall mechanism patterns} = \sum_{j=1}^{n} M_j = \frac{N_1}{1} + \frac{N_2}{2} + \ldots \frac{Nn}{n} = \sum_{j=1}^{n} \frac{N_j}{j}$$

* For instance, among ten overall sequential patterns of a Ter-Quad system (V-1 V-10) only five of them are really different mechanism patterns. The sequential patterns (V-2) and (V-7); (V-3) and (V-5); (V-4) and (V-8); and (V-6), (V-9) and (V-10) are really the same mechanism patterns.

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$$= \sum_{j=1}^{n} \frac{\binom{S-1}{j-1}\binom{P-1}{j-1}}{j} \qquad \text{(V-14)}$$

of which S, $P \geq n \geq 1$; M_1, M_2 ..... Mn (or $\frac{N_1}{1}$, $\frac{N_2}{2}$....$\frac{N_n}{n}$) are the number of mechanism patterns for 1, 2 ..... n number of stable enzyme forms respectively.

In the case where S and P have no common divisors, the number of mechanism patterns (ring arrangement patterns) is

$$\frac{1}{S+P}\binom{S+P}{S,P} = \frac{S+P-1}{SP}\binom{S+P-2}{S-1,P-1}$$

$$= \left(\frac{1}{S} + \frac{1}{P} - \frac{1}{SP}\right)\binom{S+P-2}{S-1,P-1}$$

$$\leq 1 \times \binom{S+P-2}{S-1,P-1}$$

But when S, P and n have a common divisor other than one, sequences consisting a symmetric repeating intra-arrangement may occur: therefore, equation (V-14) will only give an approximate value,or a non-integer,and a further mathematical treatment is necessary. In this respect, the author would like particularly to thank Professor Leonard J. Savage of the Yale Statistics Department for deriving of an equation (Appendix V) which can be transformed into equation (V-15).

$$M(n;S,P) = \frac{1}{n}\sum_{d} \phi(d)\binom{\frac{S}{d}-1}{\frac{n}{d}-1}\binom{\frac{P}{d}-1}{\frac{n}{d}-1} \qquad \text{(V-15)}$$

of which M(n;S,P) is the number of mechanism patterns at S

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number of substrates, P number of products and n number of stable enzyme forms; d is summed over the common divisors of n, S and P; and ϕ is Euler's totient function. the number of positive integers less than d and prime to d, is given by

$$\phi(d) = d\left(1-\frac{1}{p_1}\right)\left(1-\frac{1}{p_2}\right)\ldots\ldots\ldots \qquad \text{(V-16)}$$

where p_1, p_2 ..... are the different prime factors of d. $\phi(1)=1$ is a convention (72). The following is a ϕ table:

d	1	2	3	4	5	6	7	8	9	10	11	12
ϕ(d)	1	1	2	2	4	2	6	4	6	4	10	4

(IV-17)

Upon checking the mathematical literature, it was later found by Savage and Riordan (personal communication)that closlyrelated problems have been analyzed by Barton and David in "Runs in a ring" ,Biometrika, 45, 1958, pp 572-578;and in "Combinatorial Chance", 1962, pp 131-134,Charles Griffin and Co. Ltd.,London. In fact, equation (V-15) is almost explicitly implied in the above two references. Since the solution of the ring problem by Savage is specifically related to the purpose of this dissertation and I found it easier to read then that of Barton and David, the solution by Savage is thereby given in Appendix V.

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According to the necklaces problem quoted by E. Lucas, Theorie des numbres, Paris, 1891, pp. 501-503 from C. Moreau (73), the number of circular permutations of n objects of specification $(n_1, n_2 \ldots\ldots, n_m)$ is given by

$$N_1 (n_1, n_2, \ldots\ldots n_m) = \frac{1}{n} \sum \phi (d_1) \frac{(n/d_1)!}{\prod_{j=1}^{m} \left(\frac{n_j}{d_1}\right)!} \qquad \text{(V-18)}$$

where d_1 is a divisor of the greatest common divisor of n_1 n_2, ... n_m.

This can be applied to an enzyme system to calculate the overall number of mechanism patterns at all possible numbers of stable enzyme forms (but not the number of mechanism patterns at a given number of stable enzyme forms). Thus equation (V-18) is transformed to

$$M (S,P) = \frac{1}{S+P} \sum \phi(d_1) \frac{\left(\frac{S+P}{d_1}\right)!}{\left(\frac{S}{d_1}\right)!\left(\frac{P}{d_1}\right)!} \qquad \text{(V-19)}$$

where M (S,P) is the overall number of mechanism patterns at S number of substrates and P number of products and d_1 is a divisor of the greatest common divisor of S and P.

By correlation of equations (V-15 and (V-19), we get

$$\sum_{n} \frac{1}{n} \sum_{d} \phi (d) \binom{\frac{S}{d} - 1}{\frac{n}{d} - 1} \binom{\frac{P}{d} - 1}{\frac{n}{d} - 1}$$

$$= \frac{1}{S+P} \sum \phi (d_1) \frac{\left(\frac{S+P}{d_1}\right)!}{\left(\frac{S}{d_1}\right)!\left(\frac{P}{d_1}\right)!} \qquad \text{(V-20)}$$

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Based on the equations presented in this subsection, the number of sequential patterns and mechanism patterns in some enzymatic reaction systems are summarized in Table 14 .

Some of the above general equations have been applied for pattern analysis in a Ter-Quad system which is a typical example of L-asparagine synthetase catalyzed reactions. (Section V-B-e).

Discussion: According to Cleland (47), a Ter-Bi system shows three sequential patterns, of which only two are really different mechanism patterns. For a Ter-Ter system there are six possible patterns of which only four are different mechanisms; for a Quad-Ter system there are ten patterns, only five are different; for a Quad-Bi system there are four patterns, three of them are different; for a Quad-Quad system there are twenty total patterns of which only nine are different. Thus, all the numbers above stated by Cleland are in agreement with the number obtained from equations presented in this section (Table 14), except that in Quad-Quad systems our number of different mechanisms is ten instead of nine. Further graphical analysis in this laboratory indicated that his value of "nine" was apparently a miscounting of the graphical solution.

Thus, we have general equations derived from a widely used biochemical model, and the parameters involved can be extended infinitely.

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TABLE 14

Number of Patterns of Sequences and Mechanisms in Enzyme-Catalyzed Reactions With More Than One Substrate and Product (Numbers Obtained from Deduced Equations)

Reaction system Substrate Product	Highest number of stable enzyme form (a)	Number of sequential patterns at each given number of stable enzyme form (b)							Number of mechanism patterns at each given number of stable enzyme form (c)							Overall number of sequential patterns (d)	Overall number of mechanism patterns (e)
		N_1 (f)	N_2	N_3	N_4	N_5	N_6	N_7	M_1 (g)	M_2	M_3	M_4	M_5	M_6	M_7		
Bi-Bi	2	1	1						1	1						2	2
Bi-Ter (Ter-Bi)	2	1	2						1	1						3	2
Bi-Quad (Quad-Bi)	2	1	3						1	2						4	3
Ter-Ter	3	1	4	1					1	2	1					6	4
Ter-Quad (Quad-Ter)	3	1	6	3					1	3	1					10	5
Quad-Quad	4	1	9	9	1				1	5	3	1				20	10
Ter-Quint (Quint-Ter)	3	1	8	6					1	4	2					15	7
Quad-Quint (Quint-Quad)	4	1	12	18	4				1	6	6	1				35	14
Quint-Quint	5	1	16	36	16	1			1	8	12	4	1			70	26
Sex-Sex	6	1	25	100	100	25	1		1	13	34	26	5	1		252	80
Hept-Hept	7	1	36	225	400	225	36	1	1	18	75	100	45	6	1	924	246

TABLE 14 (contd)

Number of Patterns of Sequences and Mechanisms in Enzyme-Catalyzed Reactions With More Than One Substrate and Product

(a) The highest number of stable enzyme forms is equal to the lower limit of the number of substrates or products.

(b) According to equation V-12.

(c) According to equation V-15.

(d) According to equations V-11 or V-13.

(e) According to equations V-14 or V-19.

(f) N_1, N_2, N_3 ..... is the number of sequential patterns at one, two, three ..... stable enzyme forms respectively.

(g) M_1, M_2, M_3 ..... is the number of mechanism patterns at one, two, three ..... stable enzyme forms respectively.

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In practice, enzyme catalyzed reactions with four or more substrates or products, at least at the present time, are rarely known.

However, similar analysis may be useful for multiple enzyme complexes. For simple reactions with low number of substrates and products, most investigators apparently have deduced kinetic mechanisms from experimental data with random trials of fits. For reactions with high complexity the necessity of a step by step mathematical approach becomes more acute.

Knowing the number of all possible mechanisms by no means gives the direct information of what the actual mechanisms are. The deduction of a reaction mechanism relys on kinetic data obtained. Therefore, the equations presented help deduce complicated mechanisms and give an accurate and rapid check for the number of graphical presentations but do not substitute for them. The equations give not only the number of overall patterns of sequences (V-11, V-13) and mechanisms (V-14, V-19) but also give the number of patterns of sequences (V-12) and mechanisms (V-15) at any possible number of stable enzyme forms. Many biological systems are believed to be involved in inputs and outputs (such as stimuli and responses) or other vectors such as facilitations and inhibitions. It may be of interest to take advantage of the universal feature of equations (V-12) and (V-15) or similar analysis to study the sequential and mechanism patterns in various systems. At this stage, this kind of application remains to be explored.

Although knowledge of the physical or biochemical

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nature of pharmacological receptors are still at a primitive stage, attempts have been made to extrapolate the kinetics of enzyme systems to pharmacological receptor systems (74). Thus, as far as the formal theory goes, the following may be used interchangeably: enzyme and receptor, substrate and drug, alternate substrate and agonist, inhibitor and antagonist, velocity and effect, maximal velocity and efficacy, Michaelis constant and affinity (reciprocal). At this stage the application of pattern analysis to pharmacological receptor systems also remains to be explored.

When S=P, equation (V-11) becomes $\binom{2S-2}{S-1}$ and equation (V-13) becomes $\binom{2S-2}{S-1}=\sum_{j=1}^{n}\binom{S-1}{j-1}^2$, and we get $N_1 = Nn$, $N_2 = Nn-1$ ..... Thus, it is a symmetric distribution of values (Table 14). Assuming in each case that all possible sequential arrangements have the same probability, the distribution curve of N_1, N_2, N_3 .....Nn, will be sharper when S or P or both increase. In equation (V-13) a symmetric curve is obtained when S=P (Fig. 27) while an asymmetric curve is obtained when S≠P. The distribution of values obtained from equation (V-14) is always an assymetric one (Fig. 28).

D. PATTERN ANALYSIS OF ENZYME-CATALYZED REACTIONS BY MATHEMATICAL INDUCTION.

In the preceding subsection, general equations were deduced to express the number of possible sequential patterns and different mechanism patterns at a

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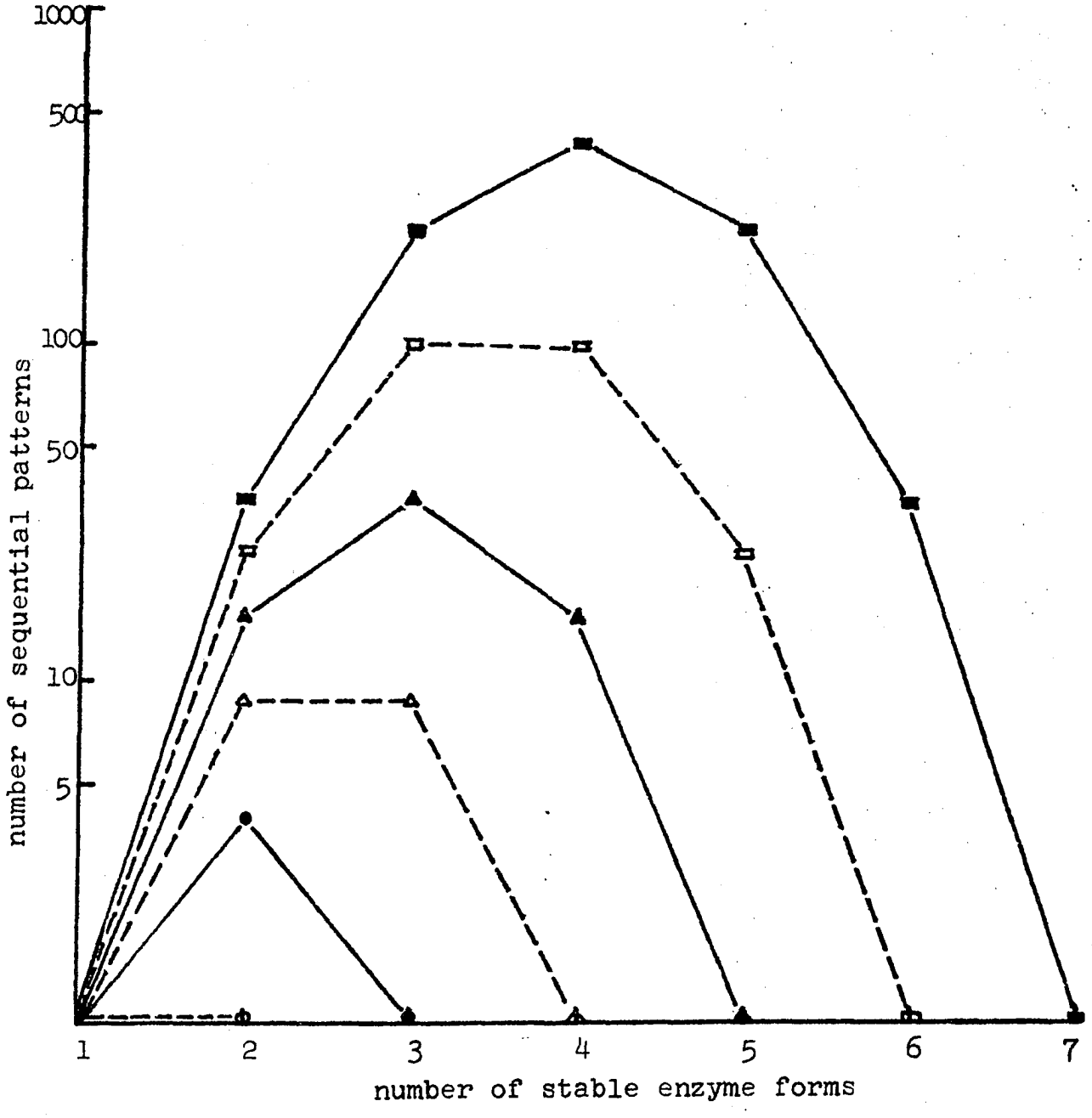

Figure 27. Relationship among number of sequential patterns stable enzyme forms and substrates when the number of substrates equals products. Reaction systems are: Bi-Bi ○---○, Ter-Ter ●——●, Quad-Quad △---△, Quint-Quint ▲——▲, Sext-Sext □---□, Hept-Hept ■——■.

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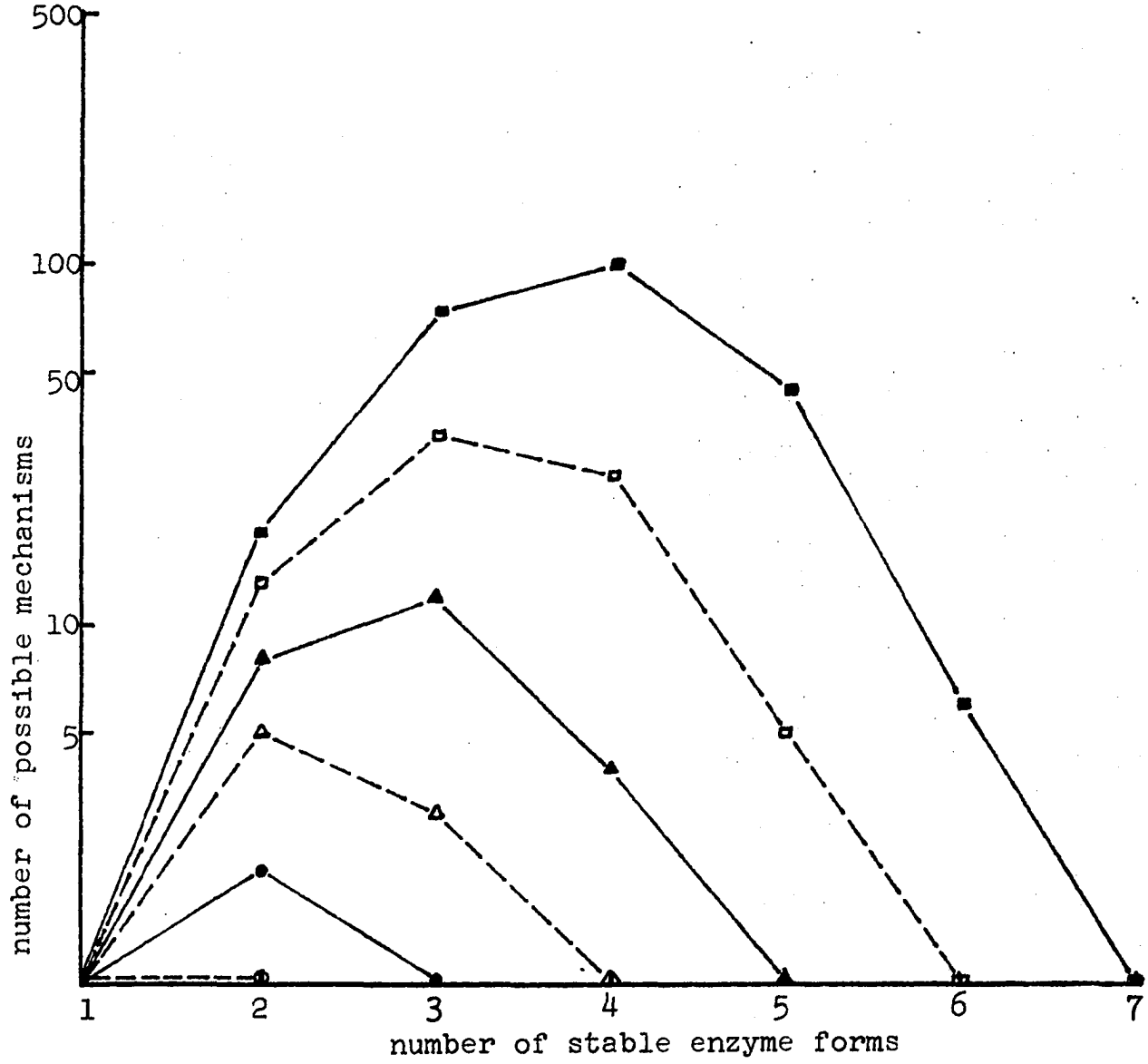

Figure 28. Relationship among number of different mechanism patterns, stable enzyme forms and substrates when the number of substrates equals products. Reaction systems are: Bi-Bi o---o, Ter-Ter •——•, Quad-Quad ▲---▲, Quint-Quint ▲——▲, Sext-Sext ▫--- ▫, Hept-Hept ■——■.

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given number of stable enzyme forms. It appears that the same biochemical model, under the same conditions described on p.108, can be analyzed with a different approach, namely - mathematical induction.

Analysis: If there are S number of substrates and P number of products and each of them are different identities, the number of overall arrangements (permutations) of them will be:

$$\text{The number of overall arrangements} = P^{(S-1)+(P-1)}_{(S-1)+(P-1)} = P^{S+P-2}_{S+P-2} = (S+P-2)! \qquad (V\text{-}21)$$

Since we are concerned only with the patterns of arrangements, from now on, we will disregard the differences among substrates or among products, and consider all substrates as inputs, all products as outputs. The overall patterns of sequential arrangements can be interpreted as the number of permutations of (S-1) + (P-1) things of which S-1 are alike and the other P-1 are alike. Therefore,

$$\text{The number of overall sequential patterns} = \frac{[(S-1)+(P-1)]!}{(S-1)!\,(P-1)!} = \binom{S+P-2}{S-1,P-1} \qquad (V\text{-}11)$$

of which S-1 and P-1 are the numbers of substrates and products respectively that can be freely arranged.

For further analysis, it is more convenient to set s=S-1, p=P-1, thus equation (V-11) can be presented as:

$$\text{The number of overall sequential patterns} = \frac{(s+p)!}{s!p!} = \binom{s+p}{s,p} \qquad (V\text{-}22)$$

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If $s \geq p$ (and from now on, until $p > s$ is mentioned), the highest number of an output is followed by an input (let this pair be represented by$\uparrow\downarrow$) will be P (or p+1). Therefore,

The maximum number of $\uparrow\downarrow$ pairs

= the maximum number of stable enzyme forms

= P (or p+1) (V-23)

of which p can be referred as the number of ping pong, if the ping pong at both ends of a linear sequence is disregarded.

The extra number of s after the maximum number of $\uparrow\downarrow$ pairing = s-p = S-P (V-24)

The total number of arrangements, A_o, with the highest number of $\uparrow\downarrow$ pairs (P$\uparrow\downarrow$) will be:

$$A_o = \frac{[p + (s-p)]\,!}{p\,!\,(s-p)\,!} = \frac{s!}{p!\,(s-p)!} = \binom{s}{p} \qquad \text{(V-25)}$$

The total number of arrangements, A_2 with the next highest number of $\uparrow\downarrow$ pairs (P-1)$\uparrow\downarrow$, will be:

$$A_1 = \frac{[(p+1)+(s-p+2)]\,!}{(p-1)!\,(s-p+2-1)!} - A_o\left[\frac{p!}{(p-1)!}\right]$$

$$= \frac{(s+1)!}{(p-1)!\,(s-p+1)!} - \frac{s!}{(s-p)!\,(p-1)!} = \binom{p}{1}\left[\binom{s+1}{p} - \binom{s}{p}\right]$$

$$= \binom{s}{p-1} \qquad \text{(V-26)}$$

The term $A_o\left[\frac{p!}{(p-1)!}\right]$ is the number which is redundant to the arrangements with the highest number of $\uparrow\downarrow$ pairs.

The total number of arrangements, A_2, with the third highest number of $\uparrow\downarrow$ pairs, $[(P-2)\uparrow\downarrow]$, will be:

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$$A_2 = \frac{[(p-2)+(s-p+4)]!}{(p-2)(s-p+4-2)!2!} - A_o\left[\frac{p!}{(p-2)!2!}\right] - A_1\left[\frac{(p-1)!}{(p-2)!}\right]$$

$$= \frac{(s+2)!}{(p-2)!(s-p+2)!2!} - \frac{(s+1)!}{(p-2)!(s-p+1)!1!1!} + \frac{s!}{(p-2)!(s-p)!2!}$$

$$= \binom{p}{2}\left[\binom{s+2}{p} - \binom{2}{1}\binom{s+1}{p} + \binom{s}{p}\right] \qquad \text{(V-27)}$$

of which, $A_o\left[\frac{p!}{(p-2)!2!}\right]$ and $A_1\left[\frac{(p-1)}{(p-2)!}\right]$ are the numbers which are redundant to the arrangements with the highest and the next highest number of ↑ ↓ pairs respectively.

Similarly, the total number of arrangements containing the fourth highest number of ↑↓ pairs, $[(p-3)\uparrow\downarrow]$), can be represented by equation (V-28).

$$A_3 = \frac{(s+3)!}{(p-3)!(s-p+3)!3!} - A_o\left[\frac{P!}{(p-3)!3!}\right] - A_1\left[\frac{(p-1)!}{(p-3)!2!}\right] - A_2\left[\frac{(p-2)!}{(p-3)!}\right]$$

$$= \frac{(s+3)!}{(p-3)!(s-p+3)!3!} - \frac{(s+2)!}{(p-3)!(s-p+2)!2!1!} + \frac{(s+1)!}{(p-3)!(s-p+1)!1!2!}$$

$$- \frac{s!}{(p-3)!(s-p)!3!}$$

$$= \binom{p}{3}\left[\binom{s+3}{p} - \binom{3}{1}\binom{s+2}{p} + \binom{3}{2}\binom{s+1}{p} - \binom{s}{p}\right] \qquad \text{(V-28)}$$

The above equations can be extended to the general case:

$$A_n = \frac{(s+n)!}{(p-n)!(s-p+n)!n!} - A_o\left[\frac{p!}{(p-n)!n!}\right] - A_1\left[\frac{(p-1)!}{(p-n)!(n-1)!}\right]$$

$$- A_2\left[\frac{(p-2)!}{(p-n)!(n-2)!}\right] - A_3\left[\frac{(p-3)!}{(p-n)!(n-3)!}\right] - \cdots - A_{n-1}\left[\frac{(p-(n-1))!}{(p-n)!}\right]$$

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$$= \frac{(s+n)!}{(p-n)!(s+p-n)!n!} - \frac{(s+n-1)!}{(p-n)!(s-p+n-1)!1!(n-1)!}$$

$$+ \frac{(s+n-2)!}{(p-n)!(s-p+n-2)!2!(n-2)!}$$

$$- \frac{(s+n-3)!}{(p-n)!(s-p+n-3)!3!(n-3)!} + \cdots + (-1)^{n-1} \frac{(s+1)!}{(p-n)!(s-p+1)!(n-1)!1!}$$

$$+ (-1)^{n} \frac{s!}{(p-n)!(s-p)!n!}$$

$$= \binom{p}{n}\left[\binom{s+n}{p} - \binom{n}{1}\binom{s+n-1}{p} + \binom{n}{2}\binom{s+n-2}{p} - \binom{n}{3}\binom{s+n-3}{p} + \cdots\cdots\right.$$

$$\left. + (-1)^{n-1}\binom{n}{n-1}\binom{s+1}{p} + (-1)^{n}\binom{n}{n}\binom{s}{p}\right] = \binom{s}{s+n-p}^{*} = \binom{s}{p-n}$$

where $s \geq p \geq n \geq o$ (V-29)

The detailed derivation of each term of equation (V-29) is shown in APPENDIX VI. When $p-n<o$, equation (V-29) becomes meaningless. Under this condition, any form of enzyme does not exist.

If all the above equations (V-25~V-29) appear to be correct, the number of overall patterns of arrangements (equation V-22) will equal the sum of patterns of arrangements at every possible given number of ↑↓ pairs. This is in fact proved by introducing p=n into the general case equation (V-29) since we get:

$$A_n = \frac{(s+p)!}{s!\ p!} - A_o - A_1 - A_2 \cdots\cdots - A_{n-1}$$

Therefore, $$\frac{(s+p)!}{s!\ p!} = \sum_{j=o}^{n} A_j \qquad \text{(V-30)}$$

* see Ref. 77, p.65

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Using the same reasoning as described on p. 111 , equation (V-31) can be established.

$$\text{Number of overall mechanism patterns} = \frac{A_0}{p+1} + \frac{A_1}{p} + \frac{A_2}{p-1} + \frac{A_3}{p-2} + \cdots\cdots + \frac{A_n}{p-(n-1)}$$

$$= \sum_{j=0}^{n} \frac{A_j}{p-(j-1)} \qquad \text{(V-31)}$$

of which s+1, p+1 and n+1 have no common divisor other than one, otherwise equation (V-31) may give only approximate value.

Discussion:

Since all enzyme-catalyzed reactions start with an input and end with an output, the enzyme form involved in both ends of linear sequence is purposely neglected during the reasoning processes in this subsection (e.g., s=S-1 and p=P-1). The neglected enzyme form is reintroduced when the necessity arises (e.g., equation V-31).

The salient feature of introducing the equations in this subsection is "recursion". For example, as we have obtained in the foregoing expressions of A_0, A_1, and A_2 we can derive an expression for A_3 by straightforward reasoning. Having obtained A_3, we can proceed to A_4 and so on. This method is due to Pascal (ref. 75 , p. 67).

Of particular interest is that equations obtained from mathematical induction in this subsection and those from deduction (V-C), at the first glance, have no apparent similarity. Upon further studies, it was found that a general expression of a partitional combination can be established, (V-F), which is essentially the same as those appeared in Ref. 77, p. 65.

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E. A MATHEMATICAL TRIANGLE DERIVED FROM A BIOCHEMICAL MODEL

Summary: Using enzyme-catalyzed reactions with multiple substrates as a model for pattern analysis, two general equations with no apparent similarities are obtained from two independent approaches. Both equations at a special case underlie a mathematical triangle which has the same form as the Pascal Triangle with the exception that all constituents are to the second power. Two formulae, each derived for a hypothetical condition, are given to calculate the probability of having a given number of stable enzyme forms in the enzyme system.

The Triangles: When S=P-1, 2, 3.....n are substituted into equations (V-13), we get the following relationships:

S	P	N_1	N_2	N_3	N_4	N_5	N_6	N_7 ..... N_n	$\sum_{j=1}^{n} N_j =$
1	1	$\binom{0}{0}^2$							$\binom{0}{0}$
2	2	$\binom{1}{0}^2$	$\binom{1}{1}^2$						$\binom{2}{1}$
3	3	$\binom{2}{0}^2$	$\binom{2}{1}^2$	$\binom{2}{2}^2$					$\binom{4}{2}$
4	4	$\binom{3}{0}^2$	$\binom{3}{1}^2$	$\binom{3}{2}^2$	$\binom{3}{3}^2$				$\binom{6}{3}$
5	5	$\binom{4}{0}^2$	$\binom{4}{1}^2$	$\binom{4}{2}^2$	$\binom{4}{3}^2$	$\binom{4}{4}^2$			$\binom{8}{4}$
6	6	$\binom{5}{0}^2$	$\binom{5}{1}^2$	$\binom{5}{2}^2$	$\binom{5}{3}^2$	$\binom{5}{4}^2$	$\binom{5}{5}^2$		$\binom{10}{5}$
7	7	$\binom{6}{0}^2$	$\binom{6}{1}^2$	$\binom{6}{2}^2$	$\binom{6}{3}^2$	$\binom{6}{4}^2$	$\binom{6}{5}^2$	$\binom{6}{6}^2$	$\binom{12}{6}$
⋮	⋮								
n	n	$\binom{n-1}{0}^2$	$\binom{n-1}{1}^2$	$\binom{n-1}{2}^2$	$\binom{n-1}{3}^2$	$\binom{n-1}{4}^2$	$\binom{n-1}{5}^2$	$\binom{n-1}{6}^2$ ..... $\binom{n-1}{n-1}^2$	$\binom{2(n-1)}{n-1}$

(V-32)

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When s=p=o, 1, 2, 3, 4.....n are substituted into equation (V-30) we get the following relationships:

ℓ	p	A_0	A_1	A_2	A_3	A_4	A_5	A_6	A_7 ..... A_n	$\sum_{j=1}^{n'} A_j =$
0	0	1								1
1	1	1	1							2
2	2	1	4	1						6
3	3	1	9	9	1					20
4	4	1	16	36	16	1				70
5	5	1	25	100	100	25	1			252
6	6	1	36	225	400	225	36	1		924
7	7	1	49	441	1225	1225	441	49	1	3432
⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮	⋮
n'	n'	1								

(V-33)

Triangles (V-32) and (V-33) are really the same and both have the same form as Pascal Triangle, except that each constituent number is squared. In both triangles, the square root of each number, except those at the ends, is the sum of the square roots of the two nearest numbers in the line above.

When n'=0, 1, 2, 3.....each is substituted into equation (V-30), A_o, A_1, A_2, A_3.....is obtained accordingly. A_o, A_1, A_2.....which is the number of sequential arrangements at the highest (i.e., p+1), the next highest (i.e., p) and the third highest (i.e., p-1) number of stable enzyme forms.....respectively can be written into another notation so that the number of stable enzyme forms can be directly related into equation (V-13). This is done by replacing n with p-j. Therefore,

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$$A_j = \binom{p}{p-j}\left[\binom{s+p-j}{p} - \binom{p-j}{1}\binom{s+p-j-1}{p} + \binom{p-j}{2}\binom{s+p-j-2}{p} - \binom{p-j}{3}\right.$$

$$\binom{s+p-j-3}{p} + \ldots\ldots\ldots (-1)^{p-j-1}\binom{p-j}{p-j-1}\binom{s+1}{p} + (-1)^{p-j}$$

$$\left.\binom{p-j}{p-j}\binom{s}{p}\right] = \binom{p}{j}\left[\sum_{i=0}^{p-j}(-1)^i\binom{p-j}{i}\binom{s+p-j-i}{p}\right] \qquad \text{(V-34)}$$

Where $s \geq p \geq j \geq o$; j is the number of ping-pongs (or the number of stable enzyme forms minus one, since the stable enzyme form involved in the first substrate and the last product are not considered; A_j is the number of sequential arrangements at j number of ping-pongs (or the number of stable enzyme forms minus one); p=P-1, which equals the number of products that can be freely arrnaged in the sequences; and s=S-1 which equals the number of substrates which can be freely arranged in the sequences.

Discussion: In the reaction model discussed in this paper, the reaction sequence is assumed to proceed from left to right (one direction). When a given number of stable enzyme forms is considered, a new dimension is introduced into the reasoning process, since in the sequence there are two parameters, inputs and outputs involved; and only when an output is followed by an input (i.e., ↑↓ pair) will there be a new stable enzyme form (47). This will not be true when an input followed by an output (i.e., ↓↑ pair) occurs. When we mention the number of sequential arrangements at given pairs of ↑↓ , ↑'s and ↓'s in ↑↓ pairs are self-contained in the arrangements.

Although N_1, N_2, N_3.....has the same value as A_o, A_1, A_2.....respectively in the triangles (V-32) and (V-37)

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this can only be attributed to the symetric conditions (i.e., S=P). In asymetric conditions, the order is reversed, that is, the first number in the N series (N_1) equals the last number in the A series, and the last number in N series equals the first number in the A series (A_o), and so on.

Since a binomial coefficient obtained from the Pascal Triangle is the number of different shortest zig-zag paths between two given corners in a network, the avenues and streets in New York City may well be a good model. Therefore, from a one dimentional city map as a model we get equation $An=(x+y)^n$. When x=y=1, the expansion of equations with n=0, 1, 2, 3.....sequentially we get all the numbers and sequences in the Pascal Triangle. With multiple substrate enzyme reactions as a model, a two dimentional factor is introduced into consideration. We get equation (V-13) or (V-30) which underlie the triangle with the second exponential power in all of its constituents. Thus, we have a model-equation-triangle relationship in both cases. In fact, the numbers in triangles (V-32) and (V-33) can be considered as the number of different shortest zig-zag paths from a given corner to another given corner in a network of streets with a return trip back to the original point. When the forward trip and the return trip are on the different planes, it means returning to the mirror image of the starting point.

If we assume in each case that all patterns of non-random sequential arrangements have the same probability, at a given number of substrates (S) and products (P), the

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probability, Pro, to have a given number of stable enzyme forms (n) is:

$$Pro = \frac{\binom{s-1}{n-1}\binom{p-1}{n-1}}{\binom{S+P-2}{S-1}} \qquad (V\text{-}35)$$

Where S, $P \geq n \geq 1$. The numerator on the right is equation (V-12) and the denominator on the right is equation (V-13). And

$$\sum_{i=1}^{n} Pro_i = 1 \qquad (V\text{-}36)$$

Where n=1, 2, 3.....n gives a distribution of probabilities. When S=P, equation (V_{-35}) becomes:

$$Pro = \frac{\binom{S-1}{n-1}^2}{\binom{2S-2}{S-1}} \qquad (V\text{-}37)$$

Similarly, the probability of the existence of a given number of stable enzyme forms at a given number of substrates and products can be calculated from equations (V-30) and (V-29). And similarly, the distribution of probabilities can be calculated.

If we assume in each case that all of the nonrandom mechanisms patterns have the same probability, at a given number of substrates (S) and products (P); the probability, Pro', to have a given number of stable enzyme forms (n) is:

$$Pro' = \frac{\frac{1}{n}\sum_{d} \phi(d)\binom{\frac{S}{d}-1}{\frac{n}{d}-1}\binom{\frac{P}{d}-1}{\frac{n}{d}-1}}{\frac{1}{S+P}\sum_{d_1} \phi(d_1)\frac{\left(\frac{S+P}{d_1}\right)!}{\left(\frac{S}{d_1}\right)!\left(\frac{P}{d_1}\right)!}} \qquad (V\text{-}38)$$

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Where S, P $\geq$ n $\geq$ 1. The numberator on the right is equation (V-15) in which d is summed over the common divisors of S, P and n; ϕ is Euler's totient function. The denominator on the right is equation (V-19) in which d_1 is a divisor of the greatest common divisor of S and P.

Since,

$$\sum_n \frac{1}{n} \sum_d \phi(d) \binom{\frac{S}{d}-1}{\frac{n}{d}-1} \binom{\frac{P}{d}-1}{\frac{n}{d}-1} = \frac{1}{S+P} \sum \phi(d_1) \binom{\frac{S+P}{d_1}}{\frac{S}{d_1}, \frac{P}{d_1}} \qquad (V-39)$$

therefore,

$$\sum_{i=1}^{n} Pro'_i = 1 \qquad (V-40)$$

where n=1, 2, 3.....n gives a distribution of probabilities.

The great issue raised by equation (V-35) and (V-38) is whether the enzyme systems in Nature follow any of the above two assumptions. The proof or disproof of the above hypotheses will need the accumulation of a huge amount of information which would require decades to come.

F. A COMBINATORIC FORMULA DERIVED FROM PATTERN ANALYSIS OF A BIOCHEMICAL MODEL

Two independent approaches are used for pattern analyses on enzyme catalyzed reactions with multiple substrates involved. An equation is obtained by correlating two resultant equations to express a general combinatoric notation, $\binom{n}{r}$, into a partitional form which is

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given by:

$$\binom{n}{r} = \sum_{i=o}^{n-r,\ m-r} (-1)^i \binom{m-r}{i} \binom{n+m-r-i}{m}$$

where n, m $\geq$ r $\geq$ o; n, m and r are integers.

Derivation:

Let set s=S-1 and p=P-1,thus, s and p are the number of substrates and products which can be freely arranged respectively.

Equation (V-12) can be written into:

$$A_j = \binom{s}{j} \binom{p}{j} \qquad (V-41)$$

Correlate equations (V-34) and (V-41) and we get:

$$A_j = \binom{p}{j} \left[\sum_{i=o} (-1)^i \binom{p-j}{i} \binom{s+p-j-i}{p} \right]$$

$$= \binom{p}{j} \binom{s}{j}$$

Therefore, when s, p $\geq$ j $\geq$ o

$$\binom{s}{j} = \sum_{i=o} (-1)^i \binom{p-j}{i} \binom{s+p-j-i}{p} \qquad (V-42)$$

if s = p,

$$\binom{s}{j} = \sum_{i=o}^{s-j} (-1)^i \binom{s-j}{i} \binom{2s-j-i}{s} \qquad (V-43)$$

Equation (V-42) can be transformed into a basic general notation

$$\binom{n}{r} = \sum_{i=o} (-1)^i \binom{m-r}{i} \binom{n+m-r-i}{m} \qquad (V-44)$$

Where $\binom{n}{r}$, the number of combinations of n different things taken r at a time, is expressed in a partitional form by introducing a number m, so that n, m $\geq$ r $\geq$ o; n,m and r

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are integers. There is a combinatoric formula in the literature (77) as shown below:

$$\sum_{v} (-1)^{v} \binom{a}{v} \binom{n-v}{r} = \binom{n-a}{n-r} \qquad (V-45)$$

which has the similar form as equation (V-44), because both can be transformed to each other by artificial enforcement of making the following dictionary:

Equation (V-45)		Equation (V-44)
v	⟷	i
a	⟷	m-r
n-a	⟷	n
r	⟷	m
∴ n	⟷	m+n-r

(V-46)

The natural limit for equation (V-44) is that $n, m \geq r \geq 0$, however, the natural limit for equation (V-45) is that $n \geqslant r \geqslant a \geqslant 0$, otherwise, both equations are the same.

In fact, equations (V-44) and (V-45) have been proved from completely different methods. The former has been proved by using an immaterial formula (77),

$$\binom{-a}{k} = (-1)^{k} \binom{a+k-1}{k} \qquad (V-47)$$

However, the latter has been derived from pattern analyses of a biochemical model. Each step of the derivations has

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its physical meaning.

G. AN APPLICATION OF A COMBINATORIC FORMULA

From equation (V-44), which is derived in this subsection, it is possible to prove the identity

$$\sum_{i=o} (-1)^i \binom{m}{i} \binom{n+m-i-1}{n} = \sum_{i=o} (-1)^i \binom{m}{i} \binom{n+m-i-1}{2m-1} \tag{V-48}$$

From the literature (73), it has been shown that

$$L(n,m) + mL(n,m-1) + \cdots + \binom{m}{k} L(n,m-k) + \cdots + L(n,o) = \binom{n+m-1}{n} \tag{V-49}$$

and

$$L(n,m) = \binom{n-1}{m-1} = \sum_{k=o} (-1)^k \binom{m}{k} \binom{n+m-k-1}{n} \tag{V-50}$$

where L(n,m) is the number of ways of putting n like objects into m different cells with no cells empty.

Equation (V-50) has a form similar to equation (V-44). If the following dictionary is made to correlate both of them

Equation (V-44)		Equation (V-50)
i	⟷	k
n	⟷	n-1
r	⟷	m-1
m-r	⟷	m
∴ m	⟷	2m-1

(V-51)

we get:

$$\binom{n-1}{m-1} = \sum_{i=o} (-1)^i \binom{m}{i} \binom{n+m-i-1}{2m-1} \tag{V-52}$$

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Therefore, equation (V-48) can be established. However, it should be noted that the natural limits are different in both ends of equation (V-48) as shown below:

$$\sum_{i=o}^{m-1} (-1)^i \binom{m}{i} \binom{n+m-i-1}{n} = \sum_{i=o}^{\substack{m \text{ or} \\ n-m}} (-1)^i \binom{m}{i} \binom{n+m-i-1}{2m-1} \qquad (V\text{-}53)$$

From equation (V-54) it is also possible to prove the identity

$$\sum_{i=o} (-1)^i \binom{k}{i} \binom{m+q-i-1}{q-i} = \sum_{i=o} (-1)^i \binom{k}{i} \binom{m+q-i-1}{q+k} \qquad (V\text{-}54)$$

It has been shown in the literature (73) that

$$\binom{m-k+q-1}{q} = \sum (-1)^i \binom{m+q-i-1}{q-i} \binom{k}{i} \qquad (V\text{-}55)$$

which has the similar form as equation (V-44). If the following dictinary is made to correlate both of them

Equation V-44		Equation V-55
n	⟷	m-k+q-1
r	⟷	q
m-r	⟷	k
∴ m	⟷	q+k

(V-56)

we get:

$$\binom{m-k+q-1}{q} = \sum (-1)^i \binom{k}{i} \binom{m+q-i-1}{q+k} \qquad (V\text{-}57)$$

Therefore, equation (V-54) can be established. However, it should be noted that the natural limits are different in both ends of equation (V-54), as shown below:

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$$\sum_{i=o}^{\substack{k \text{ or} \\ m-1}} (-1)^i \binom{k}{i} \binom{m+q-i-1}{q-i} = \sum_{i=o}^{\substack{k \text{ or} \\ m-k-1}} (-1)^i \binom{k}{i} \binom{m+q-i-1}{q+k} \qquad \text{(V-58)}$$

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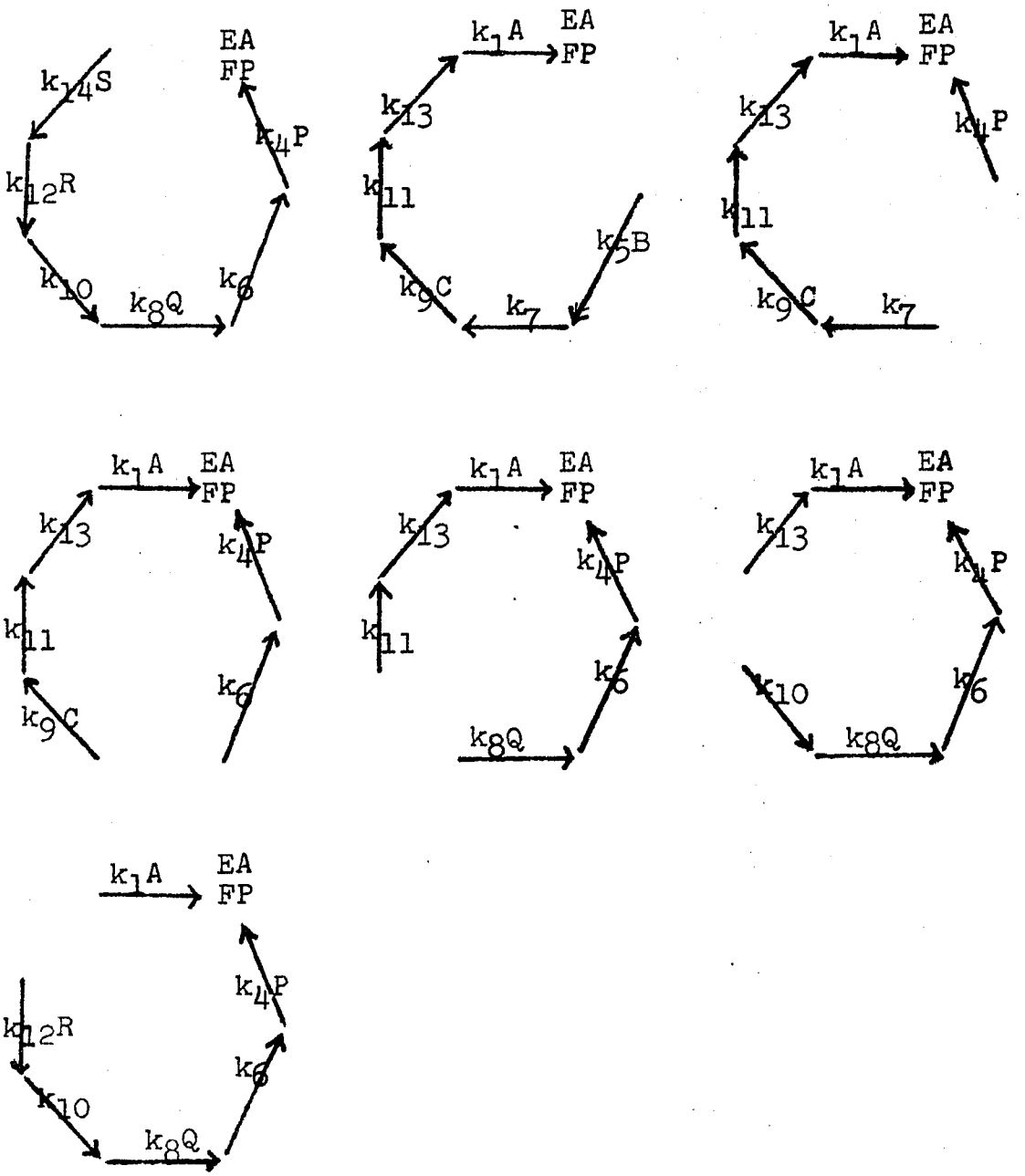
APPENDIX I

Schematic presentation of rate constants and concentration factors which are involved in each enzyme-containing species. King's method was applied to L-asparagine synthetase isolated from 6C3HED-RG1 tumor (see p. 83).

(EA+FP) Enzyme-containing species:



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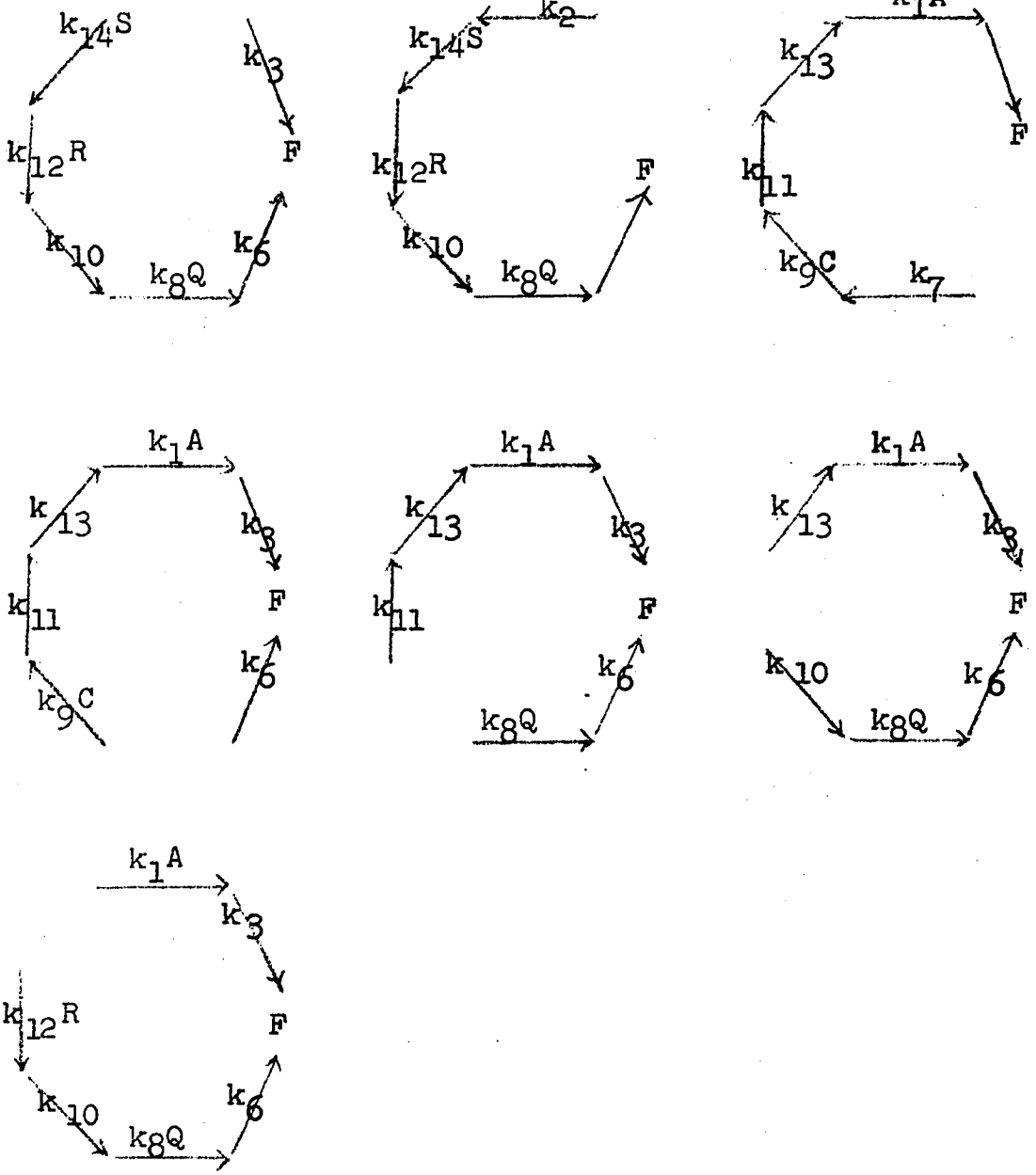
F enzyme-containing species:



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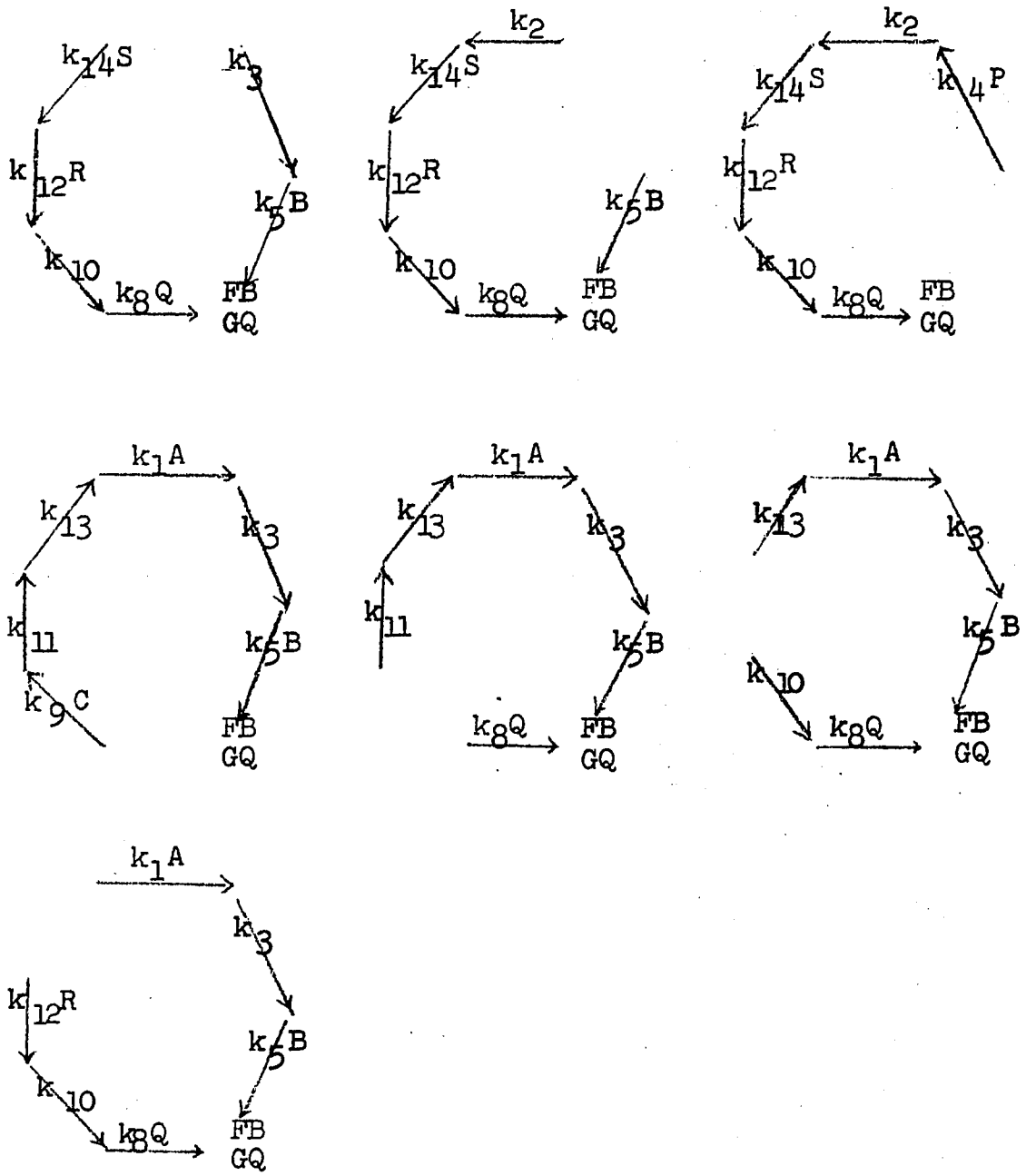
(FB+GQ) enzyme-containing species:



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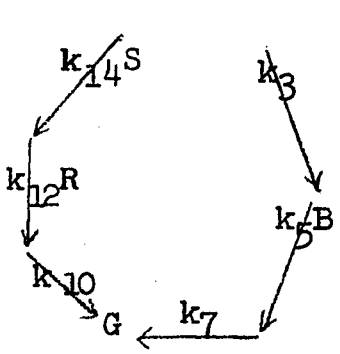
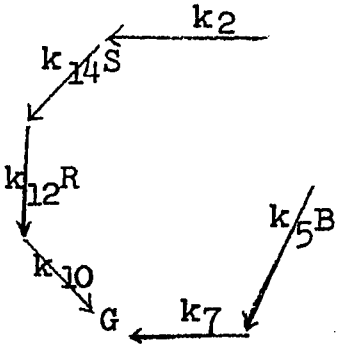
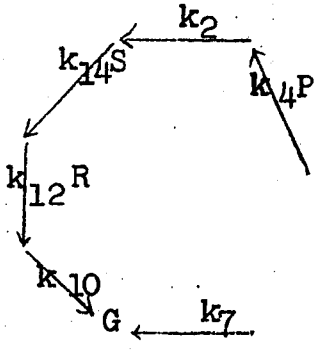
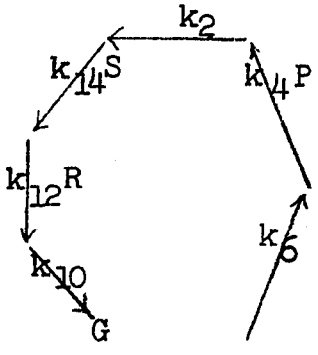
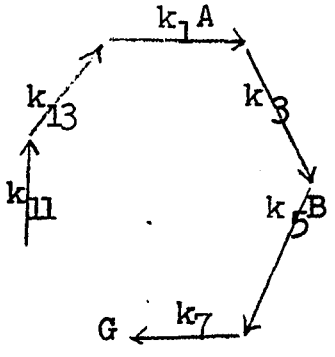
G enzyme-containing species:

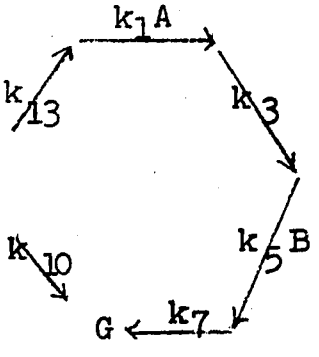


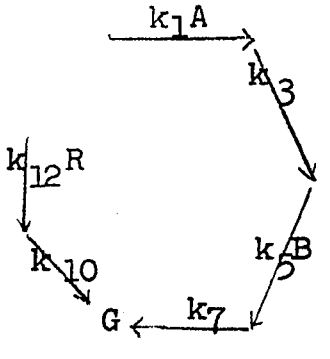












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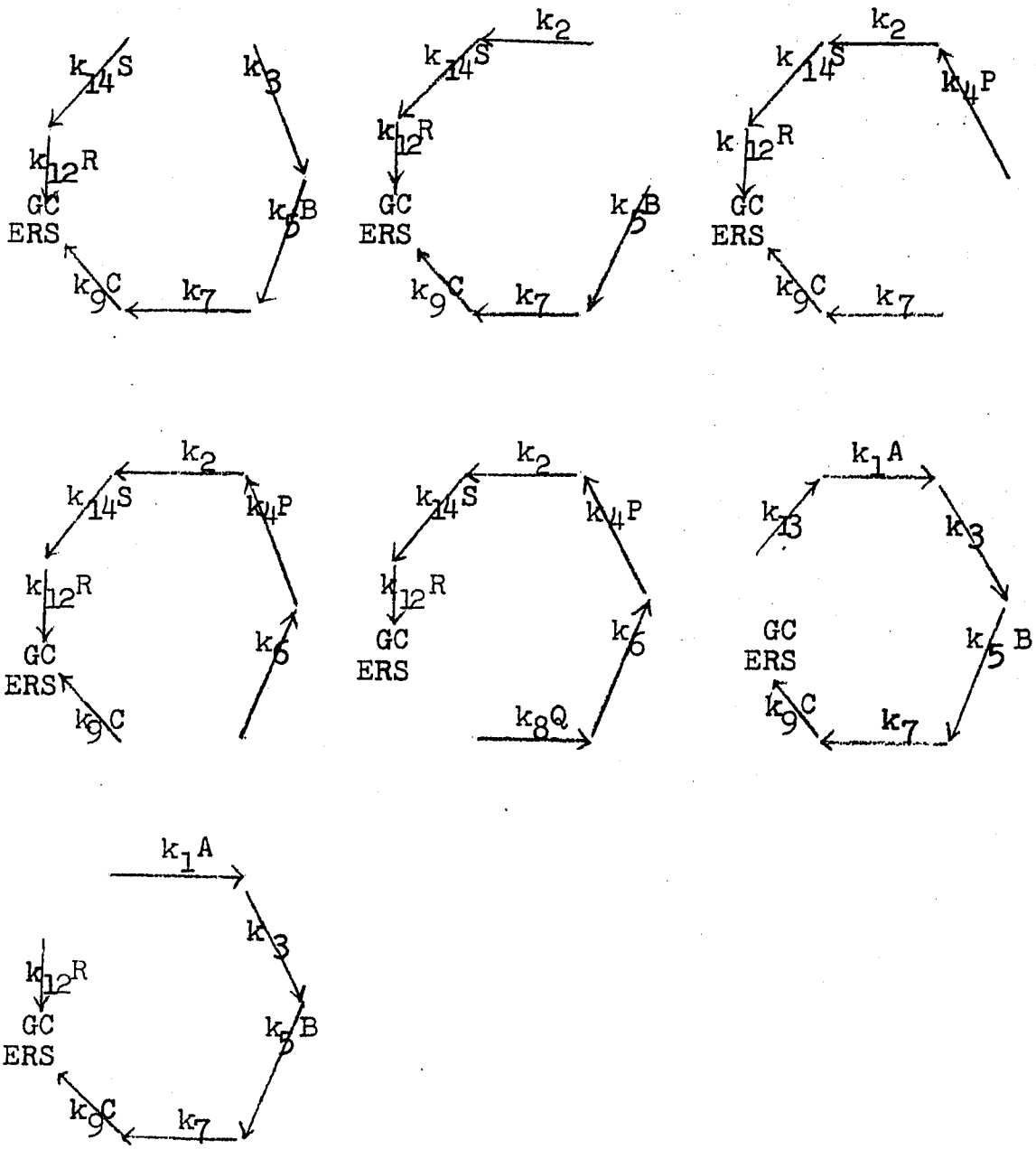
(GC+ERS) enzyme-containing species:



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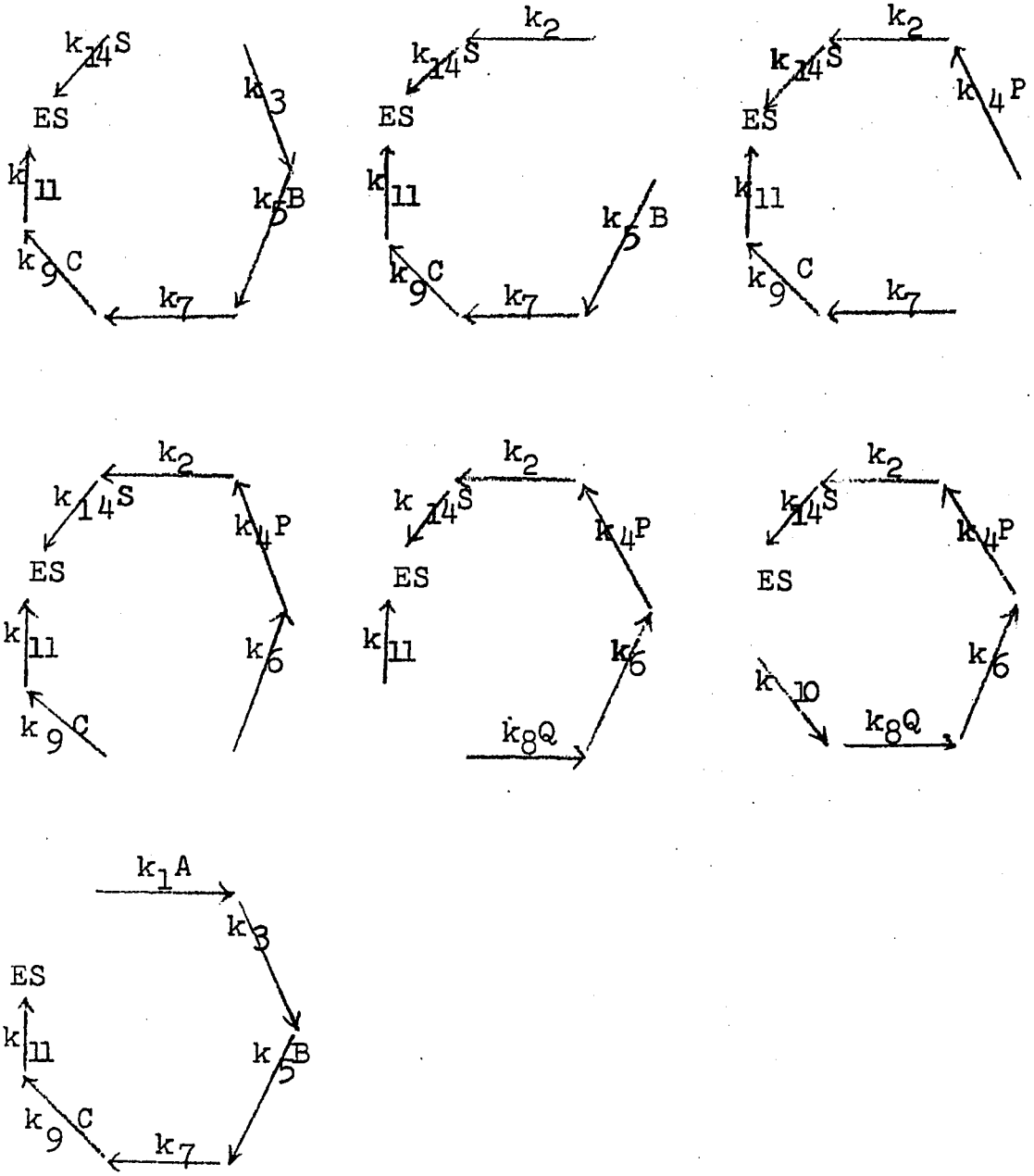
ES enzyme-containing species:



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APPENDIX II

The relative concentration of each enzyme-containing species in L-asparagine synthetase reactions. (see p. 85).

$$\frac{EA+FP}{Et} \propto \{ k_4k_6k_8k_{10}k_{12}k_{14}PQRS + k_1k_5k_7k_9k_{11}k_{13}\,ABC$$
$$+k_1k_4k_7k_9k_{11}k_{13}ACP + k_1k_4k_6k_9k_{11}k_{13}\,ACP$$
$$+k_1k_4k_6k_8k_{11}k_{13}APQ + k_1k_4k_6k_8k_{10}k_{13}\,APQ$$
$$+k_1k_4k_6k_8k_{10}k_{12}\,APQR \}$$
$$\propto \{ k_4k_6k_8k_{10}k_{12}k_{14}PQRS + k_1k_5k_7k_9k_{11}k_{13}\,ABC$$
$$+k_1k_4(k_6+k_7)k_9k_{11}k_{13}\,ACP$$
$$+k_1k_4k_6k_8(k_{10}+k_{11})k_{13}\,APQ$$
$$+k_1k_4k_6k_8k_{10}k_{12}\,APQR \}$$

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$$\frac{F}{Et} \propto \{ k_3k_6k_8k_{10}k_{12}k_{14}QRS + k_2k_6k_8k_{10}k_{12}k_{14}\ QRS$$
$$+k_1k_3k_7k_9k_{11}k_{13}AC + k_1k_3k_6k_9k_{11}k_{13}\ AC$$
$$+k_1k_3k_6k_8k_{11}k_{13}AQ + k_1k_3k_6k_8k_{10}k_{13}\ AQ$$
$$+k_1k_3k_6k_8k_{10}k_{12}\ AQR \}$$
$$\propto \{ (k_2+k_3)k_6k_8k_{10}k_{12}k_{14}\ QRS$$
$$+k_1k_3(k_6+k_7)k_9k_{11}k_{13}\ AC$$
$$+k_1k_3k_6k_8(k_{10}+k_{11})k_{13}\ AQ$$
$$+k_1k_3k_6k_8k_{10}k_{12}\ AQR \}$$

$$\frac{FB+GQ}{Et} \propto \{ +k_3k_5k_8k_{10}k_{12}k_{14}BQRS + k_2k_5k_8k_{10}k_{12}k_{14}\ BQRS$$
$$+k_2k_4k_8k_{10}k_{12}k_{14}PQRS + k_1k_3k_5k_9k_{11}k_{13}\ ABC$$
$$+k_1k_3k_5k_8k_{11}k_{13}ABQ + k_1k_3k_5k_8k_{10}k_{13}\ ABQ$$
$$+k_1k_3k_5k_8k_{10}k_{12}\ ABQR \}$$
$$\propto \{ (k_2+k_3)k_5k_8k_{10}k_{12}k_{14}\ BQRS$$
$$+k_2k_4k_8k_{10}k_{12}k_{14}\ PQRS$$
$$+k_1k_3k_5k_9k_{11}k_{13}\ ABC$$
$$+k_1k_3k_5k_8(k_{10}+k_{11})k_{13}\ ABQ$$
$$+k_1k_3k_5k_8k_{10}k_{12}\ ABQR \}$$

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$$\frac{G}{Et} \propto \{ k_3k_5k_7k_{10}k_{12}k_{14}BRS + k_2k_5k_7k_{10}k_{12}k_{14}\ BRS$$
$$+k_2k_4k_7k_{10}k_{12}k_{14}PRS + k_2k_4k_6k_{10}k_{12}k_{14}\ PRS$$
$$+k_1k_3k_5k_7k_{11}k_{13}AB + k_1k_3k_5k_7k_{10}k_{13}\ AB$$
$$+k_1k_3k_5k_7k_{10}k_{12}\ ABR \}$$
$$\propto \{ (k_2+k_3)k_5k_7k_{10}k_{12}k_{14}\ BRS$$
$$+k_2k_4(k_6+k_7)k_{10}k_{12}k_{14}\ PRS$$
$$+k_1k_3k_5k_7(k_{10}+k_{11})k_{13}\ AB$$
$$+k_1k_3k_5k_7k_{10}k_{12}\ ABR \}$$

$$\frac{GC+ERS}{Et} \propto \{ k_3k_5k_7k_9k_{12}k_{14}BCRS + k_2k_5k_7k_9k_{12}k_{14}\ BCRS$$
$$+k_2k_4k_7k_9k_{12}k_{14}PCRS + k_2k_4k_6k_9k_{12}k_{14}\ PCRS$$
$$+k_2k_4k_6k_8k_{12}k_{14}PQRS + k_1k_3k_5k_7k_9k_{13}\ ABC$$
$$+k_1k_3k_5k_7k_9k_{12}\ ABCR \}$$
$$\propto \{ (k_2+k_3)k_5k_7k_9k_{12}k_{14}\ BCRS$$
$$+k_2k_4(k_6+k_7)k_9k_{12}k_{14}\ PCRS$$
$$+k_2k_4k_6k_8k_{12}k_{14}\ PQRS$$
$$+k_1k_3k_5k_7k_9k_{13}\ ABC$$
$$+k_1k_3k_5k_7k_9k_{12}\ ABCR \}$$

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$$\frac{ES}{Et} \propto \{ \; k_3k_5k_7k_9k_{11}k_{14}BCS + k_2k_5k_7k_9k_{11}k_{14}\,BCS$$

$$+k_2k_4k_7k_9k_{11}k_{14}PCS + k_2k_4k_6k_9k_{11}k_{14}\,PCS$$

$$+k_1k_3k_5k_7k_9k_{11}ABC + k_2k_4k_6k_8k_{10}k_{14}\,PQS$$

$$+k_1k_3k_5k_7k_9k_{11}ABC \; \}$$

$$\propto \{ \; (k_2+k_3)k_5k_7k_9k_{11}k_{14}\,BCS$$

$$+k_2k_4(k_6+k_7)k_9k_{11}k_{14}\,PCS$$

$$+k_2k_4k_6k_8(k_{10}+k_{11})k_{14}\,PQS$$

$$+k_1k_3k_5k_7k_9k_{11}\,ABC \; \}$$

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APPENDIX III

The deviation and condensation of the denominator of the rate equation. The relative concentration of each enzyme-containing species appeared in Appendix II and IV-23 was substituted into the denominator of IV-24. There are 31 terms obtained, in which 4 of the terms with ABC concentration factors can be condensed into one term and 3 of the terms with PQRS concentration factors can be condensed into one term as shown below:

Terms in the denominator containing ABC concentration factors:

$$k_1k_5k_7k_9k_{11}k_{13}^{ABC} + k_1k_3k_5k_9k_{11}k_{13}^{ABC}$$
$$+k_1k_3k_5k_7k_9k_{13}^{ABC} + k_1k_3k_5k_7k_9k_{11}^{ABC} \qquad \text{(AIII-1)}$$
$$=k_1k_5k_9(k_7k_{11}k_{13}+k_3k_{11}k_{13}+k_3k_7k_{13}+k_3k_7k_{11})^{ABC}$$

Terms in the denominator containing PQRS concentration factors:

$$k_4k_6k_8k_{10}k_{12}k_{14}^{PQRS} + k_2k_4k_8k_{10}k_{12}k_{14}^{PQRS}$$
$$+k_2k_4k_6k_8k_{12}k_{14}^{PQRS} = k_4k_8k_{12}k_{14}(k_6k_{10}+k_2k_{10}+k_2k_6)^{PQRS} \qquad \text{(AIII-2)}$$

All other terms which are not condensable have been shown in the denominator of (IV-26).

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APPENDIX IV

Relationship between rate constants and kinetic constants in the proposed mechanism for L-asparagine synthetase. (The mechanism appeared in IV-1b.) Cleland's notations were used throughout (47).

$$V_1 = \frac{(\text{numerator}_1)}{(\text{coef ABC})} = \frac{k_3k_7k_{11}k_{13}}{k_3k_7k_{11}+k_3k_7k_{13}+k_3k_{11}k_{13}+k_7k_{11}k_{13}}$$

$$V_2 = \frac{(\text{numerator}_2)}{(\text{coef PQRS})} = \frac{k_2k_6k_{10}}{k_2k_6+k_2k_{10}+k_6k_{10}}$$

$$Ka = \frac{(\text{coef BC})}{(\text{coef ABC})} = \frac{k_{11}k_{13}(k_2+k_3)}{k_3k_7k_{11}+k_3k_7k_{13}+k_3k_{11}k_{13}+k_7k_{11}k_{13}}$$

$$Kb = \frac{(\text{coef AC})}{(\text{coef ABC})} = \frac{k_3(k_6+k_7)k_{11}k_{13}}{k_5(k_3k_7k_{11}+k_3k_7k_{13}+k_3k_{11}k_{13}+k_7k_{11}k_{13})}$$

$$Kc = \frac{(\text{coef AB})}{(\text{coef ABC})} = \frac{k_3k_7(k_{10}+k_{11})k_{13}}{k_9(k_3k_7k_{11}+k_3k_7k_{13}+k_3k_{11}k_{13}+k_7k_{11}k_{13})}$$

$$Kp = \frac{(\text{coef QRS})}{(\text{coef PQRS})} = \frac{(k_2+k_3)k_6k_{10}}{k_4(k_2k_6+k_2k_{10}+k_6k_{10})}$$

$$Kq = \frac{(\text{coef PRS})}{(\text{coef PQRS})} = \frac{k_2(k_6+k_7)k_{10}}{k_8(k_2k_6+k_2k_{10}+k_6k_{10})}$$

$$Kr = \frac{(\text{coef PQR})}{(\text{coef PQRS})} = \frac{k_2k_6k_{10}}{k_{14}(k_2k_6+k_2k_{10}+k_6k_{10})}$$

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$$Kia = \frac{(\text{coef CP})}{(\text{coef ACP})} = \frac{(\text{coef PQ})}{(\text{coef APQ})} = \frac{(\text{coef PQR})}{(\text{coef APQR})} = \frac{k_2}{k_1}$$

$$Kib = \frac{(\text{coef AQ})}{(\text{coef ABQ})} = \frac{(\text{coef AQR})}{(\text{coef ABQR})} = \frac{(\text{coef QRS})}{(\text{coef BQRS})} = \frac{k_6}{k_5}$$

$$Kic = \frac{(\text{coef ABR})}{(\text{coef ABCR})} = \frac{(\text{coef BRS})}{(\text{coef BCRS})} = \frac{(\text{coef PRS})}{(\text{coef PCRS})} = \frac{k_{10}}{k_9}$$

$$Kip = \frac{(\text{coef AC})}{(\text{coef ACP})} = \frac{(\text{coef AQ})}{(\text{coef APQ})} = \frac{(\text{coef AQR})}{(\text{coef APQR})} = \frac{k_3}{k_4}$$

$$Kiq = \frac{(\text{coef AB})}{(\text{coef ABQ})} = \frac{(\text{coef ABR})}{(\text{coef ABQR})} = \frac{(\text{coef BRS})}{(\text{coef BQRS})} = \frac{k_7}{k_8}$$

$$Kir = \frac{(\text{coef BCS})}{(\text{coef BCRS})} = \frac{(\text{coef PCS})}{(\text{coef PCRS})} = \frac{k_{11}}{k_{12}}$$

$$Kis = \frac{(\text{coef BC})}{(\text{coef BCS})} = \frac{(\text{coef PC})}{(\text{coef PCS})} = \frac{(\text{coef PQ})}{(\text{coef PQS})} = \frac{k_{13}}{k_{14}}$$

$$Keq = \frac{(\text{numerator}_1)}{(\text{numerator}_2)} = \frac{k_1 k_3 k_5 k_7 k_9 k_{11} k_{13}}{k_2 k_4 k_6 k_8 k_{10} k_{12} k_{14}}$$

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APPENDIX V

The following is a transcription of notes of proof by Professor Leonard J. Savage. (For the relationship between this problem and the enzyme catalyzed reactions, please see P. 112.)

The problem raised by Mr. Chou is to find the number $A(r,b;t)$ of distinguishable ways that r red and b black beads can be arranged in an oriented ring with t transitions from red to black. The answer is given here in terms of two special functions. $C(n,k)$ denotes, for non-negative integers n and k with $k \leq n$, the number of ways to choose n things k at a time. The function ϕ* has many remarkable properties, which can be found in any book on number theory, but only one will be used here:

[1] $$\sum_{d|x} \phi(d) = x,$$

where the summation extends over all the divisors of x, including 1 and x. For example

$$\sum_{d|24} \phi(d) = \phi(1) + \phi(2) + \phi(3) + \phi(4) + \phi(6) + \phi(8) + \phi(12) + \phi(24)$$
$$= 1 + 1 + 2 + 2 + 2 + 4 + 4 + 8$$
$$= 24.$$

As shall be shown, A can be expressed thus, in terms of C and ϕ,

[2] $$A(r,b;t) = \frac{1}{t} \sum_{d|r,b,t} \phi(d) C\left(\frac{r}{d} - 1, \frac{t}{d} - 1\right) C\left(\frac{b}{d} - 1, \frac{t}{d} - 1\right),$$

where d is summed over the common divisors of r, b, and t.

* See pp. 112-113.

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For example, $A(8,8;4) =$

$$\frac{1}{4}\left(\phi(1)C(7,3)C(7,3) + \phi(2)C(3,1)C(3,1) + \phi(4)C(1,0)C(1,0)\right)$$

$$= \frac{1}{4}(1 \times 35 \times 35 + 1 \times 3 \times 3 + 2 \times 1 \times 1)$$

$$= \frac{1}{4}(1225 + 9 + 2) = 309,$$

which would be troublesome to obtain by mere enumeration.

As in the example, the first term of [2] is typically almost as large as the whole sum. Indeed, 1 is often the only common divisor of r, b, and t, in which case the first term is the only term.

The equation [2] has probably been published elsewhere. For it is rather like a known formula for the number of distinguishable oriented rings with r red and b black beads, namely,

$$[3] \qquad \sum_t A(r,b;t) = \frac{1}{r+b} \sum_{d \mid r,b} \phi(d) C\left(\frac{r+b}{d}, \frac{r}{d}\right).$$

See, for example, page 162 of An Introduction to Combinatorial Analysis by John Riordan (published by John Wiley and Sons, New York, 1964). I was led to guess [2] on learning 3 and by studying work toward [2] by David T. C. Chou.

A demonstration of [2] follows. It demands somewhat more mathematical experience than has been presupposed in merely introducing and stating [2] .

Consider one of the oriented rings in question, and

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temporarily open it by introducing a white bead after a red one and before a black one. The number of these opened arrangements is the number of ways to partition the integers r and b both into t ordered positive terms, as is not hard to see. This number is

[4] $$B(r,b;t) = C(r - 1, t - 1)C(b - 1, t - 1);$$

for $C(r - 1, t - 1)$ is known, and easily shown, to be the number of ways to partition r into t positive terms.

Each opened arrangement has a well defined period p, which is the smallest number of red-black transitions by which the white bead must be displaced to come to a position homologous to its initial position. Necessarily, p is a divisor of t, and $d = t/p$ is a divisor of t, r, and b. If $D(p;r,b;t)$, or $D(p)$ for short, is the number of opened arrangements of period p, then the number of closed arrangements, which is the number A that we seek is

[5] $$A(r,b;t) = \sum_{p|t} \frac{D(p)}{p},$$

because each opened arrangement of period p gives the same closed arrangement as the $(p - 1)$ other opened arrangements that arise by shifting the original one.

The numbers $D(p)$ are not especially easy to compute, but they are closely related to simpler numbers $E(p)$, the number of opened arrangements whose period is a divisor of p. Of course,

[6] $$E(p) = \sum_{p'|p} D(p') .$$

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And, as is easy to see, $E(p) = 0$ unless t/p, which I shall call d, divides r, b, and t, in which case,

[7] $$E(p) = B(\tfrac{pr}{t}, \tfrac{pb}{t}; p)$$

$$= B(\tfrac{r}{d}, \tfrac{b}{d}; \tfrac{t}{d}) \ .$$

According to equations [7] and [4] , equation [2] says that

[7] $$A(r,b;t) = \frac{1}{t} \sum_{d|t} \phi(d) E(t/d) \ .$$

But, in view of [6] and [1] in that order,

$$\frac{1}{t} \sum_{d|t} \phi(d) E(t/d)$$

$$= \frac{1}{t} \sum_{d|t} \phi(d) \sum_{p|(t/d)} D(p)$$

$$= \frac{1}{t} \sum_{dp|t} \phi(d) D(p)$$

$$= \frac{1}{t} \sum_{p|t} D(p) \sum_{d|(t/p)} \phi(d)$$

$$= \frac{1}{t} \sum_{p|t} D(p) \frac{t}{p} = A(r,B;t) \ .$$

So equation [2] is indeed correct.

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APPENDIX VI

Substitution, rearrangement, transformation and generalization of equations in V-D subsection.

$$A_o = \frac{S!}{P!(S-P)!} \qquad \text{(AVI-1)}$$

$$A_1 = \frac{(S+1)!}{(P-1)!(S-P+1)!1!} - \frac{S!}{P!(S-P)!}\left[\frac{P!}{(P-1)!}\right]$$

$$= \frac{(S+1)!}{(P-1)!(S-P+1)!1!} - \frac{S!}{(S-P)!(P-1)!} \qquad \text{(AVI-2)}$$

$$A_2 = \frac{(S+2)!}{(P-2)!(S-P+2)!2!} - \left[\frac{S!}{(P!(S-P)!}\right]\left[\frac{P!}{(P-2)!2!}\right]$$

$$-\left[\frac{(S+1)!}{(P-1)!(S-P+1)!1!} - \frac{S!}{(S-P)!(P-1)!}\right]\left[\frac{(P-1)!}{(P-2)!}\right]$$

$$= \frac{(S+2)!}{(P-2)!(S-P+2)!2!} - \frac{S!}{(S-P)!(P-2)!2!} - \frac{(S+1)!}{(P-2)!(S-P+1)!}$$

$$+ \frac{S!}{(P-2)!(S-P)!}$$

$$= \frac{(S+2)!}{(P-2)!(S-P+2)!2!} - \frac{(S+1)!}{(P-2)!(S-P+1)!} + \frac{S!}{(P-2)!(S-P)!2!}$$

(AVI-3)

$$A_3 = \frac{(S+3)!}{(P-3)!(S-P+3)!3!} - \left[\frac{(S+2)!}{(P-2)!(S-P+2)!2!} - \frac{(S+1)!}{(P-2)!(S-P+1)!}\right.$$

$$\left. + \frac{S!}{(P-2)!(S-P)!2!}\right]$$

$$\left[\frac{(P-2)!}{(P-3)!}\right] - \left[\frac{(S+1)!}{(P-1)!(S-P+1)!} - \frac{S!}{(S-P)!(P-1)!}\right]\left[\frac{(P-1)!}{(P-3)!2!}\right]$$

$$-\left[\frac{S!}{P!(S-P)!}\right]\left[\frac{P!}{(P-3)!3!}\right]$$

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$$= \frac{(S+3)!}{(P-3)!(S-P+3)!3!} - \frac{(S+2)!}{(P-3)!(S-P+2)!2!} + \frac{(S+1)!}{(P-3)!(S-P+1)!} - \frac{S!}{(P-3)!(S-P)!2!}$$

$$- \frac{(S+1)!}{(P-3)!2!(S-P+1)!} + \frac{S!}{(P-3)!(S-P)!2!} - \frac{S!}{(P-3)!(S-P!3!}$$

$$= \frac{(S+3)!}{(P-3)!(S-P+3)!3!} - \frac{(S+2)!}{(P-3)!(S-P+2)!2!} + \frac{(S+1)!}{(P-3)!(S-P+1)!2!} - \frac{S!}{(P-3)!(S-P)!3!} \qquad \text{(AVI-4)}$$

$$A_4 = \frac{(S+4)!}{(P-4)!(S-P+4)!4!} - \left[\frac{(S+3)!}{(P-3)!(S-P+3)!3!} - \frac{(S+2)!}{(P-3)!(S-P+2)!2!} + \frac{(S+1)!}{(P-3)!(S-P+1)!2!} - \frac{S!}{(P-3)!(S-P)!3!} \right] \left[\left[\frac{(P-2)!}{(P-4)!2!} \right] - \left[\frac{(S+1)!}{(P-1)!(S-P+1)!1!} - \frac{S!}{(S-P)!(P-1)!} \right] \left[\frac{(P-1)!}{(P-4)!3!} \right] \right] - \left[\frac{S!}{P!(S-P)!} \right] \left[\frac{P!}{(P-4)!4!} \right]$$

$$= \frac{(S+4)!}{(P-4)!(S-P+4)!4!} - \frac{(S+3)!}{(P-4)!(S-P+3)!3!} + \frac{(S+2)!}{(P-4)!(S-P+2)!2!} - \frac{(S+2)!}{(P-4)!(S-P+2)!2!2!} - \frac{(S+1)!}{(P-4)!(S-P+1)!2!} + \frac{(S+1)!}{(P-4)!(S-P+1)!2!} - \frac{(S+1)!}{(P-4)!(S-P+1)!3!} + \frac{S!}{(P-4)!(S-P)!3!} - \frac{S!}{(P-4)!(S-P)!2!2!} + \frac{S!}{(P-4)!(S-P)!3!} - \frac{S!}{(P-4)!(S-P)!4!}$$

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$$= \frac{(S+4)!}{(P-4)!(S-P+4)!4!} - \frac{(S+3)!}{(P-4)!(S-P+3)!3!} + \frac{(S+2)!}{(P-4)!(S-P+2)!2!2!}$$

$$- \frac{(S+1)!}{(P-4)!(S-P+1)!3!} + \frac{S!}{(P-4)!(S-P)!\quad 4!} \qquad \text{(AVI-5)}$$

The "2!" (indicated by ↑) provides a valuable clue for generalization of the formula. Therefore,

$$A_o = \frac{S!}{P!(S-P)!} \qquad \text{(AVI-1)}$$

$$A_1 = \frac{(S+1)!}{(P-1)!(S-P+1)!1!} - \frac{S!}{(P-1)!(S-P)!1!} \qquad \text{(AVI-6)}$$

$$A_2 = \frac{(S+2)!}{(P-2)!(S-P+2)!2!} - \frac{(S+1)!}{(P-2)!(S-P+1)!1!1!} + \frac{S!}{(P-2)!(S-P)!2!} \qquad \text{(AVI-7)}$$

$$A_3 = \frac{(S+3)!}{(P-3)!(S-P+3)!3!} - \frac{(S+2)!}{(P-3)!(S-P+2)!2!1!} + \frac{(S+1)!}{(P-3)!(S-P+1)!1!2!}$$

$$- \frac{S!}{(P-3)!(S-P)!3!} \qquad \text{(AVI-8)}$$

$$A_4 = \frac{(S+4)!}{(P-4)!(S-P+4)!4!0!} - \frac{(S+3)!}{(P-4)!(S-P+3)!1!3!} + \frac{(S+2)!}{(P-4)!(S-P+2)!2!2!}$$

$$- \frac{(S+1)!}{(P-4)!(S-P+1)!1!3!} + \frac{S!}{(P-4)!(S-P)!0!4!} \qquad \text{(AVI-9)}$$

$$\vdots$$

$$A_n = \frac{(S+n)!}{(P-n)!(S-P+n)!0!n!} - \frac{(S+n-1)!}{(P-n)!(S-P+n-1)!1!(n-1)!}$$

$$+ \frac{(S+n-2)!}{(P-n)!(S-P+n-2)!2!(n-2)!}$$

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$$- \frac{(S+n-3)!}{(P-n)!(S-P+n-3)!3!(n-3)!} + \cdots\cdots\cdots\cdots - \cdots\cdots\cdots\cdots$$

$$+ \cdots\cdots\cdots\cdots$$

$$(-1)^{n-1} \frac{(S+1)!}{(P-n)!(S-P+1)!(n-1)!1!} + (-1)^{n} \frac{S!}{(P-n)!(S-P)!n!0!} \qquad \text{(AVI-10)}$$

Note that the algebraic sum of the determinator of each term is always equal to the algebraic sum of the numerator of each term.

The general case equation, (AVI-10), which is in permutation form can be transformed into combinatoric form. Thus:

$$\text{The first term of (AVI-10)} = \frac{(S+n)!}{(P-n)!(A-P+n)!n!} = \left[\frac{P!}{(P-n)!n!}\right]\left[\frac{(S+n)!}{(S-P+n)!P!}\right]$$

$$= \binom{P}{n}\binom{S+n}{P} \qquad \text{(AVI-11)}$$

$$\text{The second term of (AVI-10)} = \frac{(S+n-1)!}{(P-n)!(S-P+n-1)!1!(n-1)!}$$

$$= \left[\frac{P!}{(P-n)!n!}\frac{n!}{1!(n-1)!}\right]\left[\frac{(S+n-1)!}{(S-P+n-1)!\ P!}\right]$$

$$= \binom{P}{n}\binom{n}{1}\binom{S+n-1}{P} \qquad \text{(AVI-12)}$$

$$\text{The third term of (AVI-10)} = \frac{(S+n-2)!}{(P-n)!(S-P+n-2)!2!(n-2)!}$$

$$= \left[\frac{P!}{(P-n)!n!}\frac{n!}{2!(n-2)!}\right]\left[\frac{(S+n-2)!}{(S-P+n-2)\ P!}\right]$$

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$$= \binom{P}{n} \binom{n}{2} \binom{S+n-2}{P} \qquad \text{(AVI-13)}$$

The last term of (AVI-10) $= \dfrac{S!}{(P-n)!(S-P)!n!0!} = \left[\dfrac{P!}{(P-n)!n!}\right]\left[\dfrac{n!}{n!}\right]\left[\dfrac{S!}{(S-P)!P!}\right]$

$$= \binom{P}{n} \binom{n}{n} \binom{S}{P} \qquad \text{(AVI-14)}$$

Therefore, equation (V-29) can be obtained.

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