Purification, properties, and kinetics of adenylosuccinate synthetase from *Escherichia Coli*

by

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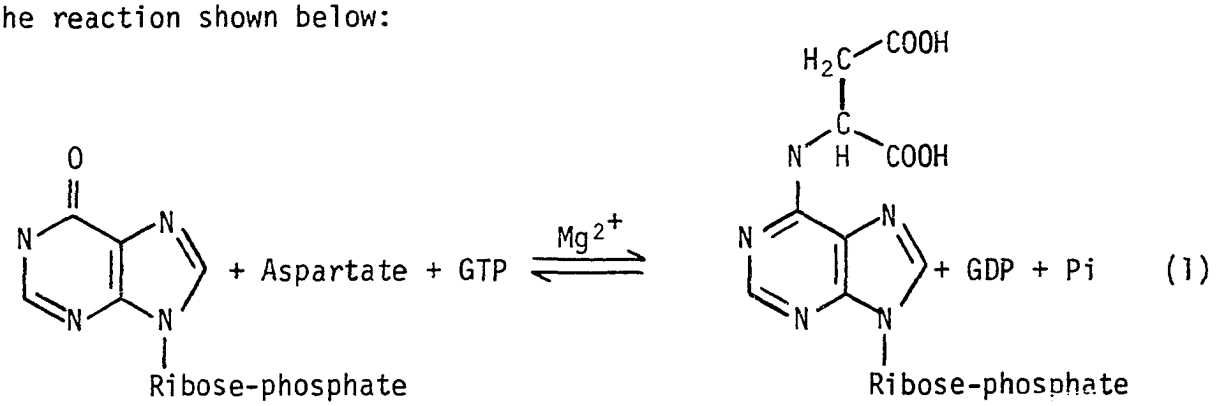
LIST OF ABBREVIATIONS

AMP	Adenosine-5'-monophosphate
DEAE	Diethylaminoethyl cellulose
EDTA	Ethylenediamine tetraacetate
GDP	Guanosine-5'-diphosphate
GMP	Guanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
IMP	Inosine-5'-monophosphate
KPi	Postassium phosphate
NaOH	Sodium hydroxide
^{18}O	Heavy oxygen
Pi	Inorganic orthophosphate
Thio-IMP	6-Mercaptopurine ribonucleoside-5'-phosphate
Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

Adenylosuccinate synthetase [IMP-L-aspartate ligase (GDP), EC 6.3.4.4] is an enzyme involved in the *de novo* biosynthesis of purines and catalyzes the reaction shown below:



IMP Adenylosuccinate

Purines are synthesized *de novo* by most organisms and the details of their biosynthesis have been determined by Buchanan and Greenberg and their coworkers (Buchanan and Sonne, 1946; Buchanan *et al.*, 1948; Sonne *et al.*, 1946, 1948; Greenberg, 1948, 1951; Goldthwait *et al.*, 1954; Greenberg and Jalniche, 1957). The various steps involved in their biosynthesis have been reviewed by Buchanan and Hartman (1959) and Balis (1968).

The synthesis of AMP from IMP and aspartate was first shown by Abrams and Bentley (1955) in rabbit bone marrow and by Lieberman (1956) in *Escherichia coli*. Carter and Cohen (1955, 1956) have shown that adenylosuccinate is an intermediate in the synthesis of AMP. Lieberman (1956) demonstrated adenylosuccinate could be reversibly formed from IMP and aspartate by a partially purified protein fraction from *E. coli*, and he suggested from ^{18}O incorporation studies that 6-phosphoryl-IMP was an intermediate in the reaction. From equilibrium-exchange studies of the

reaction, Fromm (1958) also suggested that 6-phosphoryl-IMP could be an intermediate. Miller and Buchanan (1962) studied the arsenolysis of adenylosuccinate and concluded from the absolute requirement for GDP that the mechanism involved a stepwise arrangement of all the substrates on the enzyme surface preceding product formation. No further studies have been made on the mechanism of the reaction.

Wyngaarden and Greenland (1963) have investigated the inhibition of the enzyme by nucleotides. AMP, adenylosuccinate, GMP, and GDP were shown to be effective inhibitors of the reaction. They suggested that AMP and GDP might function *in vivo* to control AMP biosynthesis.

Most recent studies on the enzyme have involved investigation of the structural requirements for binding at the IMP and aspartate sites on the enzyme. Baker (1967) has suggested that the ribose-phosphate moiety of IMP is most important for its binding. A large number of studies on substitutions in the ribose-phosphate moiety have been made by Baker and Hampton and their coworkers (Baker and Tanna, 1963, 1965a, b, c; Nichol *et al.*, 1967; Hampton and Chu, 1970). The major conclusion of these investigations is that although a purine moiety is required for binding at the IMP site, the phosphate group and the ribofuranose ring determine how well an analogue of IMP will bind to the enzyme.

The naturally occurring antibiotic hadacidin (N-formyl-N-hydroxyamino acetic acid), which blocks *de novo* purine synthesis, has been shown by Shigeura and Gordon (1962a, b) to strongly inhibit adenylosuccinate synthetase by competitively binding at the aspartate site with an apparent inhibition constant of 4.2×10^{-6} M as compared to the Michaelis constant of 1.5×10^{-4} M for aspartate.

The purpose of this study was to develop a better procedure for the purification of adenylosuccinate synthetase from *E. coli* B and to study the kinetic mechanism of the reaction catalyzed by the enzyme.

EXPERIMENTAL PROCEDURE

Materials

E. coli, strain B, cells, which had been grown on an enriched medium based on a casein hydrolysate and harvested at the three-fourths log stage, were obtained from Grain Processing Company, Muscatine, Iowa. GTP, GDP, IMP, streptomycin sulfate, 2-mercaptoethanol, HEPES, and L-aspartate were obtained from Calbiochem. Adenylosuccinate was synthesized from AMP and fumarate as described by Carter and Cohen (1956). β, γ-5'-Guanylylmethylene diphosphonate was a product of Miles Laboratories. Thio-IMP was supplied by P-L Biochemicals. N,N,N',N'-tetramethylethylenediamine and riboflavin were products of Canalco and the acrylamide used was supplied by Eastman Organic Chemicals and was recrystallized from chloroform as described by Loening (1967). All other chemicals were obtained from commercial sources and were reagent grade.

DEAE cellulose and DEAE cellulose paper (DE-81) were products of H. Reeve Angel and Co., Ltd. The cellulose was in the microgranular form and was supplied as a wet powder (DE-52). Sephadex G-100 was supplied by Pharmacia Fine Chemicals, Inc.

Methods

Standard assay The determination of adenylosuccinate synthetase activity during the purification was made by observing the increase in absorbance at 280 nm accompanying the conversion IMP to adenylosuccinate. The assays were carried out in a 1.0 ml total volume containing 20 mM HEPES, pH 7.7, 1 mM $MgSO_4$, 0.6 mM streptomycin sulfate, 5 mM aspartate,

0.15 mM IMP, and 0.06 mM GTP. The reactions were followed at 28° in a Cary model 15 recording spectrophotometer with a thermostatted cell housing using an extinction coefficient of 11.7×10^6 cm^2 per mole at 280 nm for the formation of adenylosuccinate from IMP (Atkinson *et al.*, 1964). Increase in absorbance due to the presence of aspartase in the initial steps of the purification was corrected for by using a blank reaction mixture minus the nucleotides. One unit of activity is defined as molarity of adenylosuccinate formed per minute.

Protein determination The protein concentration was determined by use of the Biuret method (Gornall *et al.*, 1949) with bovine serum albumin as a standard. Concentration is expressed as mg of protein per ml of solution. Specific activity is expressed as units of enzyme activity per mg of protein.

Analytical disc gel electrophoresis Purification of the enzyme followed by use of analytical disc gel electrophoresis as described by Ornstein (1964). A 7.5% acrylamide separating gel was used and a band of 10% sucrose was layered on top of it. The protein sample was made to 5% concentration in sucrose and layered above the 10% band. Electrophoresis was carried out at 5° with a current of 2.5 ma per tube (0.5 × 6.0 cm). Protein was stained with amido-schwartz dye and the excess dye was washed out with 7% acetic acid. The gels were stored in 7% acetic acid.

Nucleotide concentration and purity Concentrations of the nucleotides used in this study were determined from their characteristic ultraviolet absorption (P-L Circular OR-10, 1961). The purity of the

nucleotides was determined by chromatography on DEAE cellulose paper as described by Morrison (1968). The ammonium formate buffer solution described by Morrison was diluted 5:1 to obtain better resolution. The nucleotides were all found to be essentially pure as evidenced by ultraviolet examination of the chromatograms.

Stability constant determination In most previous studies on this enzyme, glycine at pH 8.0 was used as a buffer for the reaction. Owing to its structural similarity to aspartate and its poor buffering capacity at this pH, it would appear to be a poor choice as a buffer for this system. Since Lieberman (1956) has shown that Tris-Cl is an inhibitor of the reaction, one of the buffers described by Good *et al.*, (1966), HEPES, was chosen. The lack of a primary amino group and its pK of 7.55 make HEPES an ideal buffer for this system.

No data were available on the stability constants of the magnesium complexes with various nucleotides in HEPES buffer. Because of the necessity of controlling free magnesium concentration in the reaction mixture, the stability constants were determined by the method of Burton (1959), which involves measuring the spectral differences of 8-hydroxyquinoline and its magnesium complex in the presence and in the absence of other ligands. The determinations were made with adenyl phosphates, but should be valid for all nucleotides since the value of the stability constant depends essentially on the phosphate portion of the nucleotide (Walaas, 1959).

Spectral changes at 360 nm were measured on a Cary model 15 spectrophotometer. Values of Mg^{2+} concentration when Mg-ligand complex

concentration equals one-half the total ligand concentration were plotted against ligand concentration. Extrapolation to ligand concentration = 0 gave a value for the stability constant. Values in 20 mM HEPES, pH 7.7, 28°, were as follows: $MgATP^{2-}$, 100,000; $MgADP^{-}$, 10,000; and MgAMP, 110 M^{-1}. Binding of magnesium to aspartate, succinate, Pi, and streptomycin sulfate was also investigated. Aspartate and succinate had stability constants of less than 50 M^{-1} in 20 mM HEPES, while the stability constant with streptomycin and Pi was approximately 100 M^{-1}. The stability constant for $MgATP^{2-}$ was also determined in 20 mM HEPES, pH 7.7, containing 0.1 M tetraammonium bromide (O'Sullivan and Perrin, 1964), and was found to be 48,000 M^{-1}.

In carrying out the kinetic experiments these stability constants were used to fix the free magnesium concentration at 1 mM except for the experiments involving GDP, where the free magnesium concentration was held at 2.5 mM. The percentage of GTP in complex with magnesium was 98% or above in all experiments, even at the higher ionic strength used in the succinate inhibition experiments. Ninety-six percent of the GDP was in form of $MgGDP^{1-}$, while a maximum of 10% of the IMP is present as MgIMP at 1 mM free magnesium and 20% at 2.5 mM free magnesium.

Initial rate experiments Initial rate experiments were carried out at 28° in the Cary model 15 recording spectrophotometer with a thermostatted cell housing, using the 0 to 0.1 absorbance slide wire. The reactions were carried out in a volume of 1.0 ml containing 20 mM HEPES, pH 7.7, 0.8 mM streptomycin sulfate, either 1.0 or 2.5 mM (in the GDP inhibition experiments only) free magnesium (as magnesium sulfate), sufficient magnesium to

complex all ligands at the specific free magnesium concentration, aspartate, ATP, and IMP. In the inhibition studies, the given inhibitor and sufficient magnesium to complex it were also added. The reaction was initiated by addition of enzyme to the reaction mixture that had been incubated at 28° for ten minutes. The conversion of IMP to adenylosuccinate was then followed continuously at 280 nm for from 3 to 6 minutes. Initial velocity was expressed as molarity of adenylosuccinate formed per minute.

RESULTS AND DISCUSSION

Purification of adenylosuccinate synthetase

Adenylosuccinate synthetase was first purified by Lieberman (1956), but the resulting enzyme was not pure and no significant improvements in the preparation have been made since then. The first three steps described here are essentially the same as used by Lieberman, but the steps following the low pH treatment were worked out during this study.

E. coli B cells, which were supplied as a frozen paste, were thawed and suspended in a volume in ml of cold 5 mM KPi buffer, pH 7.0, equal to the weight of the frozen cells in grams. The suspension was passed through a French pressure cell which had been precooled to 5°. This and all subsequent steps are carried out at 5° unless otherwise noted. The pressure in the cell was maintained at 20,000 psi during cell lysis. The ruptured cells were diluted to a volume approximately equal to three times the wet weight of cells used in grams, and then centrifuged at 16,000 × g for 15 minutes. The cellular debris was discarded and the supernatant cell free extract was treated immediately.

Ten ml of a 11% solution of streptomycin sulfate solution for each 100 ml of cell free extract was added slowly with stirring to the cell free extract. After 10 minutes the solution was centrifuged at 16,000 × g for 20 minutes to remove the precipitate. The supernatant streptomycin fraction was then immediately adjusted to pH 5.4 with 0.1 M acetic acid with constant stirring. The solution was then placed in a 37° constant temperature bath and maintained at 37° for 15 minutes with constant stirring. It was then cooled in an ice bath, and the milky precipitate was removed by

centrifugation at 16,000 × g for 15 minutes. The pH of the low pH fraction was then adjusted to 6.8 with 1 M KOH.

Forty-five grams of ammonium sulfate for each 100 ml of the low pH fraction was then slowly added with stirring. After 15 minutes the precipitate was collected by centrifugation at 16,000 × g for 20 minutes. The supernatant was discarded and the precipitate was dissolved in 0.08 M KPi buffer, pH 7.0, containing 5 mM 2-mercaptoethanol and 1 mM EDTA. In the following steps the term buffer will refer to the specified concentration of KPi at pH 7.0 containing 5 mM 2-mercaptoethanol and 1 mM EDTA. The dissolved ammonium sulfate fraction was then dialyzed against 0.05 M buffer.

DEAE was prepared by washing with 0.5 N HCl followed by 0.5 N NaOH and then converted to the phosphate form by equilibrating with 1 M phosphate followed by extensive washing with water. The resin was then equilibrated by stirring with 0.08 M buffer. A column was poured with the equilibrated DEAE and several volumes of 0.08 M buffer were washed through. The dialyzed ammonium sulfate fraction was washed onto the column at a concentration of 100 mg of protein per 10 ml of column volume, and the column was washed with the 0.08 M buffer until the 280 nm absorbance of the effluent was less than 0.05. A linear gradient between 0.08 and 0.20 M buffer was then used to elute the enzyme. The total volume of the gradient was approximately ten times the column bed volume.

The column effluent was collected in a refrigerated fraction collector using a drop counter and 15 ml volumes were collected. The fractions were assayed for activity as described under "Methods" and the active fractions were pooled and concentrated by dialysis against a ten-fold excess of saturated ammonium sulfate containing 0.10 M buffer. The precipitated

protein was collected by centrifugation at 16,000 × g for 15 minutes and then dissolved in a minimal volume of 0.10 M buffer.

A Sephadex G-100 column (2.5 × 100 cm) was prepared and equilibrated with 0.10 M buffer. The concentrated protein solution from the DEAE step was applied to the column. Two ml fractions were collected in a refrigerated fraction collector and the active fractions were pooled and concentrated as described for the DEAE step. The precipitated enzyme was dissolved in Tris-Cl buffer, 0.37 M, pH 8.0, containing 2 mM N,N,N',N'-tetramethylethylenediamine, 5 mM 2-mercaptoethanol, and 1 mM EDTA and then dialyzed against several changes of this buffer to remove the ammonium ions.

The final step in the purification involves the use of a Canalco preparative disc gel electrophoresis apparatus. The standard high pH gel formulations recommended in the Prep-Disc Instruction Manual were used. A 10% acrylamide separating gel (4 cm in height) and 3.5% spacer gel (2 cm in height) were used and the protein sample (50 to 100 mg/run) was layered on top of the spacer gel under the electrode buffer. Bromophenol blue was added to the sample as a tracking dye. The only change from the recommended buffer systems involved the addition of 5 mM 2-mercaptoethanol and 1 mM EDTA to the elution buffer.

During the initial part of the electrophoresis, the current was maintained at 2.5 ma and then was increased to 5 ma after the protein had entered the gel. Elution buffer was pumped through the apparatus at a rate of from 10 to 30 ml per hour, depending on the stage of separation. The higher flow rate was established after the tracking dye had eluted from the gel. The eluent was collected in a refrigerated fraction collector and the fractions with the highest enzymic activity were pooled. An analytical

disc gel of the enzyme after preparative disc gel electrophoresis along with a densitometer scan of the gel is shown in Figure 1. About 90% of the protein appears to be in the larger band. The densitometer tracing doesn't show the true ratio of concentrations since the larger peak is considerably compressed relative to the smaller one.

The results of a typical preparation are shown in Table 1. A 150-fold

Table 1. Purification of adenylosuccinate synthetase

Enzyme fraction	Volume	Protein	Specific activity	Relative purification
	ml	mg/ml	units/mg	
Cell free extract[a]	1100	39.0	0.58	1
Streptomycin step	1150	34.0	1.01	1.75
Low pH fraction	1100	22.0	5.7	9.80
Ammonium sulfate precipitate	400	54.5	12.4	21.4
DEAE fraction	1900	2.0	52.5	90.5
Sephadex fraction	200	1.8	89.0	153

[a]Based on 390 grams of *E. coli* B frozen cells.

purification is obtained through the Sephadex step. A 3-4 fold purification occurs upon preparative electrophoresis but large amounts of enzyme have not been carried through this step in the procedure. The inclusion of 2-mercaptoethanol and EDTA in the buffers used in the purification was found to prevent loss of activity. Without these compounds present, a very low recovery of enzyme occurs after the column steps.

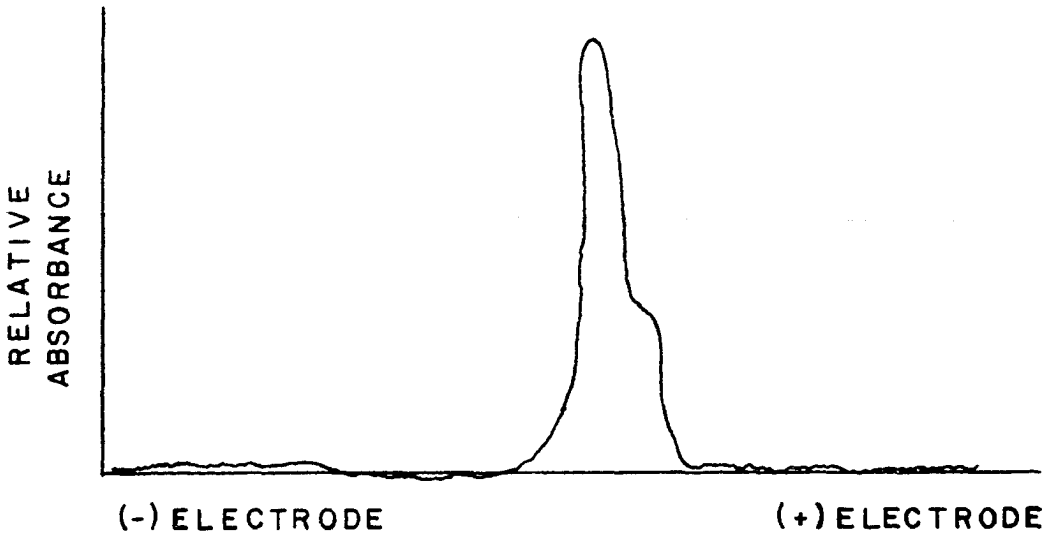


Figure 1. A photograph of an analytical disc gel of a purified adenylosuccinate synthetase preparation along with a densitometer tracing of the gel

Migration of the protein was toward the (+) electrode and the gel was stained for protein with amido-schwartz dye.

The purification described by Lieberman (1956) gives a 65-fold purification, but the assay is based on a stopped time assay which may give low values for the enzyme activity in the early steps of the purification due to the presence of adenylosuccinase. This would increase the apparent purification seen in that procedure. No direct comparisons can be made between the two procedures since different conditions are used for determination of specific activity.

Molecular weight of adenylosuccinate synthetase

The molecular weight of the enzyme was estimated by use of a Sephadex G-100 column (2.5 × 100 cm) as described by Andrews (1964). The column was calibrated with various proteins of known molecular weight as described previously (Rudolph and Fromm, 1970). The molecular weight was determined to be approximately 56,000 from the data shown in Figure 2, which is a plot of elution volume (tube number) versus the log of the molecular weight.

Initial velocity experiments

It is clear from a cursory examination of the literature that few attempts have been made to study the kinetics of three-substrate enzyme systems. It had been suggested (Fromm, 1967) that such systems may be segregated into two types (sequential and ping-pong) from initial rate studies. A choice may finally be made from among the various mechanisms in the two basic classes of enzyme and substrate interactions by using competitive inhibitors as well as product inhibitors.

Two basic experimental kinetic protocols may be used to investigate a three-substrate enzyme mechanism. In the procedure used by Frieden (1959) in studies on glutamate dehydrogenase, one substrate was held at a

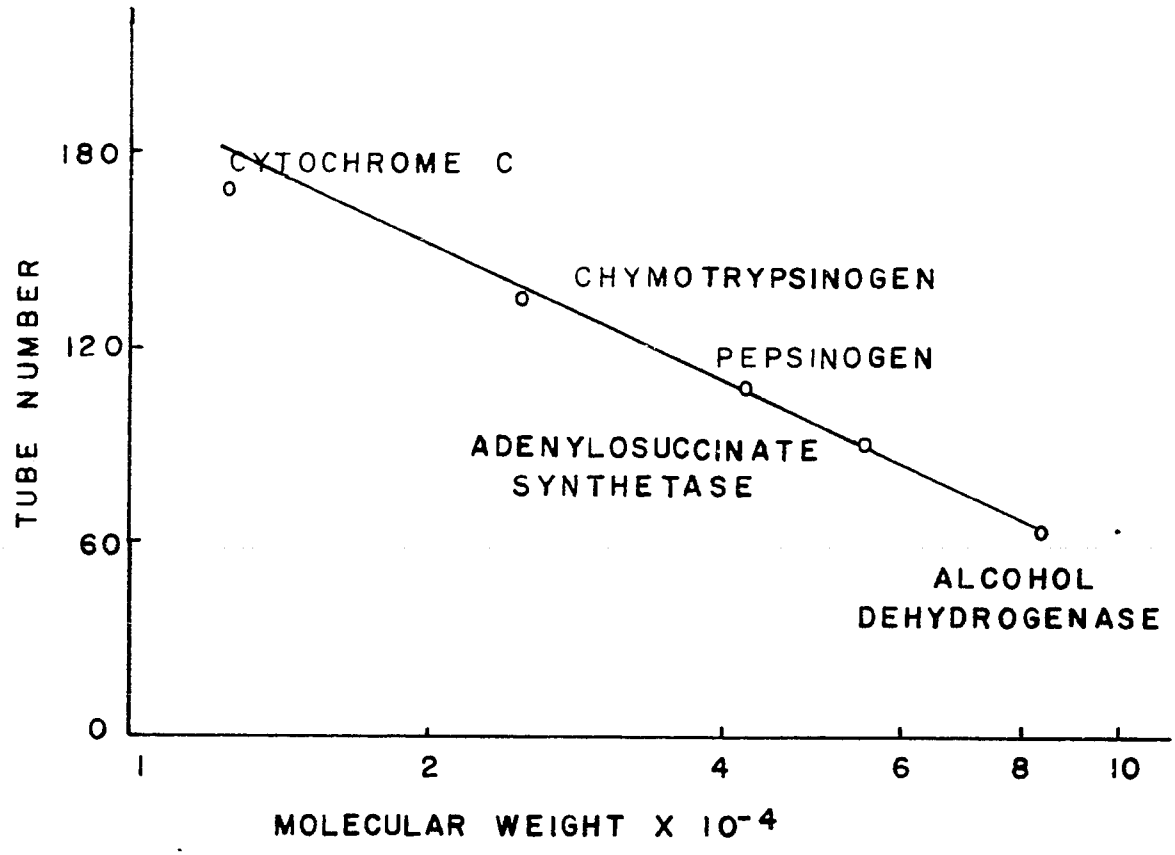


Figure 2. A plot of elution volume (tube number) versus the log of the molecular weight for various standard proteins and adenylosuccinate synthetase

The column (Sephadex G-100, 2.5 × 100 cm.) was calibrated with 5 mg of each of the standard proteins shown. They were applied in a 1 ml volume containing 5% sucrose and eluted with 0.1 M Tris-Cl buffer containing 5 mM 2-mercaptoethanol and 1 mM EDTA. Ten mg of adenylosuccinate synthetase was applied to the column.

constant level while the other two substrates were varied, as in a two-substrate system.

In the studies reported below, a procedure outlined previously (Fromm, 1967) was used. This experimental protocol has been used in this laboratory in studies on Coenzyme A-linked aldehyde dehydrogenase (Rudolph *et al.*, 1968). It involves varying one substrate while holding the other two at a fixed level in the general concentration range of their Michaelis constants. This experiment is then repeated, but at different concentrations of the fixed substrates, maintaining the ratio of the concentrations of the two fixed substrates constant. The other two substrates are then varied as described for the first one, making a total of three experiments necessary to determine if the mechanism is sequential or ping-pong.

The possible mechanisms for three substrate enzymes assuming analogy with observed two substrate mechanisms are summarized in Appendix I. These equations have been presented elsewhere (Fromm, 1967, Dalziel, 1969) and are included here for convenient reference. The patterns of the initial rate experiments for the individual mechanisms are shown in Table 2. With sequential mechanisms, whether ordered, partially ordered, or random (Mechanisms I to V), the lines on the Lineweaver-Burk (1934) plots all intersect either on or to the left of the $1/v$ axis. For a ping-pong type mechanism (Mechanisms VI to X), one or more of the three sets of data should yield parallel lines on the reciprocal plots.

The reciprocal plots from the initial rate study of adenylosuccinate synthetase are shown in Figures 3, 4, and 5. The lines all intersect to the left of the $1/v$ axis, thus eliminating all ping-pong mechanisms from further consideration. Mechanism III in which substrates A and B can add

Table 2. Initial rate patterns for the various three substrate mechanisms[a]

Mechanism[b]	Plot		
	1/A	1/B	1/C
I	I[c]	I	I
II	I	I	I
III	I	I	0[d]
IV	I	I	I
V	I	I	I
VI	P[e]	P	P
VII	I	I	P
VIII	P	I	I
IX	I	I	P
X	P	I	I

[a]Based on the experimental protocol described by Fromm (1967).

[b]The numbers refer to the mechanisms listed in Appendix I.

[c]I refers to a Lineweaver-Burk (1934) plot in which the lines intersect to the left of the $1/v$ axis.

[d]0 refers to a Lineweaver-Burk (1934) plot in which the lines intersect on the $1/v$ axis.

[e]P refers to a Lineweaver-Burk (1934) plot in which the lines are parallel.

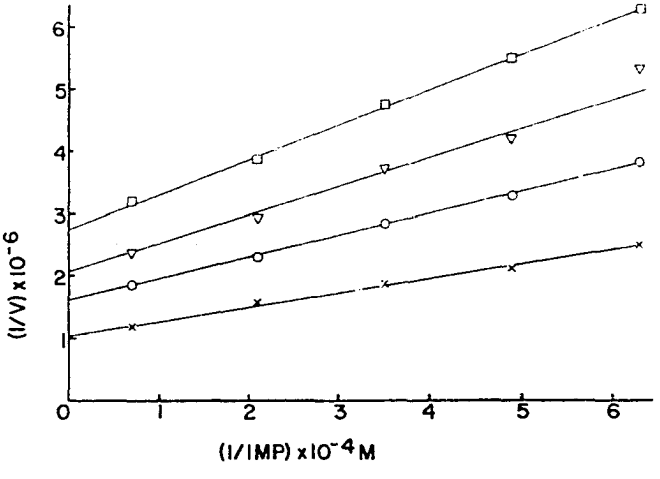


Figure 3. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP

The respective concentrations of aspartate and GTP were:

□, 0.31 and 0.0244 mM ▽, 0.42 and 0.0325 mM

○, 0.62 and 0.0487 mM ×, 1.25 and 0.0975 mM.

The IMP concentration was varied from 17.8 to 143.0 μM.

Other experimental details are described under "Experimental Procedure".

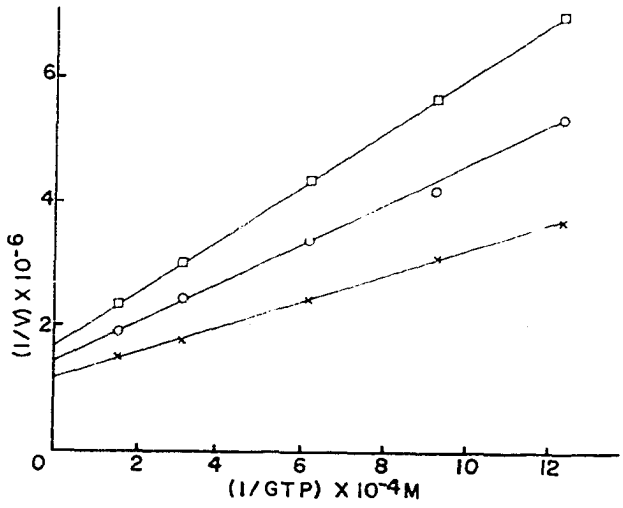


Figure 4. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP

The respective concentrations of aspartate and IMP were:

□, 0.42 and 0.050 mM ○, 0.62 and 0.075 mM

×, 1.25 and 0.150 mM.

The GTP concentration was varied from 8.1 to 65.0 μM.

Other experimental details are described under "Experimental Procedure".

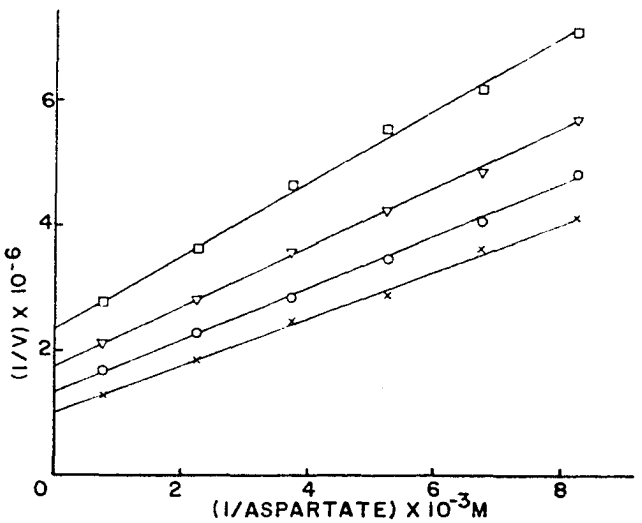


Figure 5. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate

The respective concentrations of GTP and IMP were:

□, 0.027 and 0.0375 mM ▽, 0.037 and 0.050 mM

○, 0.054 and 0.075 mM ×, 0.109 and 0.150 mM.

The aspartate concentration was varied from 0.12 and 1.31 mM.

Other experimental details are described under "Experimental Procedure".

randomly to the enzyme and substrate C is the obligatory third substrate is also eliminated since none of the plots converge on the $1/v$ axis.

Some further information can be obtained from the initial rate experiments. The slopes and intercepts of the initial rate reciprocal plots can be replotted against the reciprocal of the concentration of one of the other substrates. The expected replots for the various mechanisms are given in Table 3. For the sequential mechanisms, the rate equations for Mechanisms III, IV, and V predict that one or more of the slope replots will intersect the origin. When the data from Figures 3, 4, and 5 were treated in this manner, all replots had nonzero intercepts on the vertical axis. Thus, the initial rate data exclude all mechanisms except Mechanisms I and II.

Competitive inhibition studies

It has been suggested (Fromm, 1967) that a choice may be made among the various three-substrate mechanisms by use of competitive inhibitors. The following experiments were undertaken to distinguish between the possible sequential mechanisms.

Succinate was found to be a competitive inhibitor of aspartate as shown in Figure 6. Figures 7 and 8 show that succinate is a noncompetitive inhibitor with respect to both IMP and GTP.

Hampton (1960) has reported the inhibition of adenylosuccinate synthetase by thio-IMP and it has been shown to inhibit the enzyme from other sources (Atkinson *et al.*, 1964; Salser *et al.*, 1960). The binding of this compound to the enzyme has been studied by Baker and Tanna (1965a, c) and it was shown to be an effective inhibitor at relatively low

Table 3. Graphical method for differentiating between various three-substrate mechanisms[a]

Mechanism[b]	Substrate A		Substrate B		Substrate C	
	Slope	Intercept	Slope	Intercept	Slope	Intercept
I	N[c]	N	N	L[d]	N	N
II	N	N	N	N	N	N
III	NO[e]	N	NO	N	N	A[f]
IV	NO	N	N	L	N	L
V	N	N	NO	L	N	N
VI	L	L	L	L	L	L
VII	L	L	L	L	L	N
VIII	L	N	L	L	L	L
IX	L	L	L	L	L	N
X	L	N	L	L	L	L

[a]The normal Lineweaver-Burk (1934) plots for each substrate are made by the procedure described by Fromm (1967) and the slopes and intercepts for the different fixed concentrations are determined and then plotted against the reciprocal of one of the fixed concentrations.

[b]The numbers refer to the mechanisms listed in Appendix I.

[c]N refers to nonlinear replots with nonzero intercepts on the y axis.

[d]L refers to linear replots with nonzero intercepts on the y axis.

[e]NO refers to nonlinear replots which intersect the origin.

[f]A refers to a case which the reciprocal plot intersects on the axis.

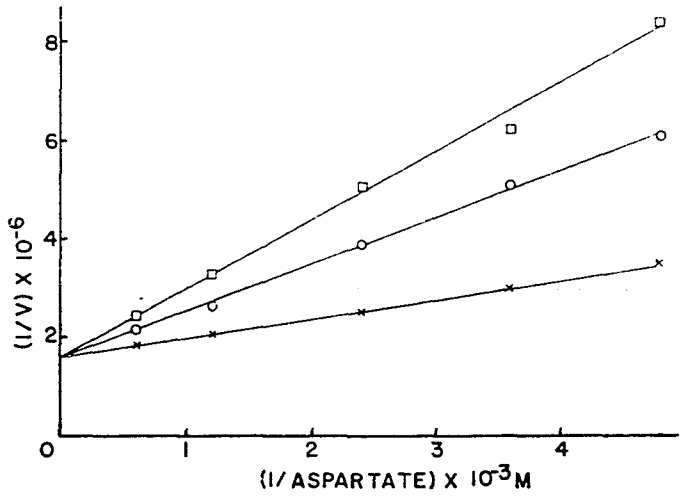


Figure 6. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of succinate

GTP and IMP concentrations were held constant at 0.033 and 0.15 mM respectively, and aspartate was varied from 0.208 to 1.67 mM. Succinate concentrations were none (X), 10 (O), and 20 mM (□). Other experimental details are described under "Experimental Procedure".

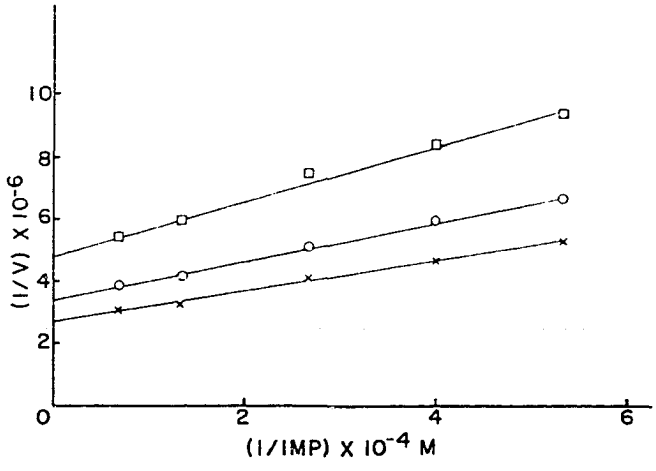


Figure 7. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of succinate

GTP and aspartate concentrations were held constant at 0.033 and 0.375 mM respectively, and IMP was varied from 0.019 to 0.15 mM. Succinate concentrations were none (X), 10 (○), and 20 mM (□). Other experimental details are described under "Experimental Procedure".

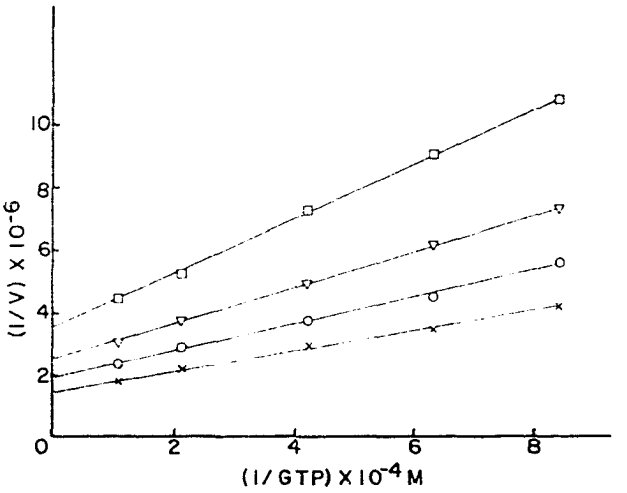


Figure 8. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of succinate

IMP and aspartate concentrations were held constant at 0.15 and 1.0 mM respectively, and GTP was varied from 0.012 to 0.095 mM. Succinate concentrations were none (X), 12.5 (○), 25 (▽), and 50 mM (□). Other experimental details are described under "Experimental Procedure".

concentrations. The effect of thio-IMP with respect to IMP is shown in Figure 9. The data clearly indicate that it is a competitive inhibitor of IMP. Figures 10 and 11 show that thio-IMP is a noncompetitive inhibitor with respect to both GTP and aspartate.

The enzyme seemed to be insensitive to inhibition by most nonguanyl nucleotide di- and triphosphates (Wyngaarden and Greenland, 1963), and the use of monophosphates as inhibitors of GTP presented the problem of possible binding to the IMP site. Because of this, the β, γ-methylene diphosphonate-substituted derivative of GTP, guanylyl methylene diphosphonate, which is not a substrate for the reaction, was tested as an inhibitor of GTP. Figure 12 indicates that it is a competitive inhibitor for GTP. The inhibition relative to IMP and aspartate was once again noncompetitive as shown in Figures 13 and 14.

The rate equations for the effect of competitive inhibitors on the various mechanisms are shown in Appendix II along with the interactions of the inhibitor with the enzyme and the substrates. The predicted inhibition patterns for the various mechanisms are shown in Table 4.

The data for this enzyme show that a competitive inhibitor for a particular substrate will be noncompetitive relative to the other two substrates. As is shown in Table 4, only Mechanisms II and IV would give rise to this set of inhibition patterns. The data are totally incompatable with the ordered mechanism (Mechanism I) and Mechanism IV has been ruled out from the initial rate studies, so the competitive inhibition study supports the choice of the rapid-equilibrium fully random mechanism (Mechanism II) for adenylosuccinate synthetase.

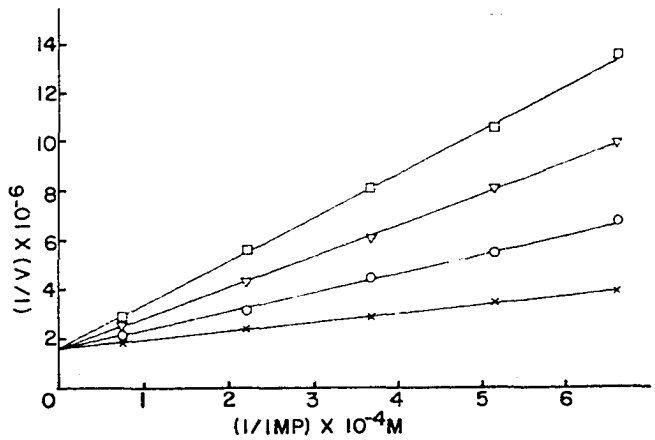


Figure 9. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of thio-IMP

Aspartate and GTP concentrations were held constant at 1.25 and 0.048 mM respectively, and IMP was varied from 0.015 to 0.136 mM. Thio-IMP concentrations were none (X), 0.012 (○), 0.024 (▽), and 0.048 mM (□). Other experimental details are described under "Experimental Procedure".

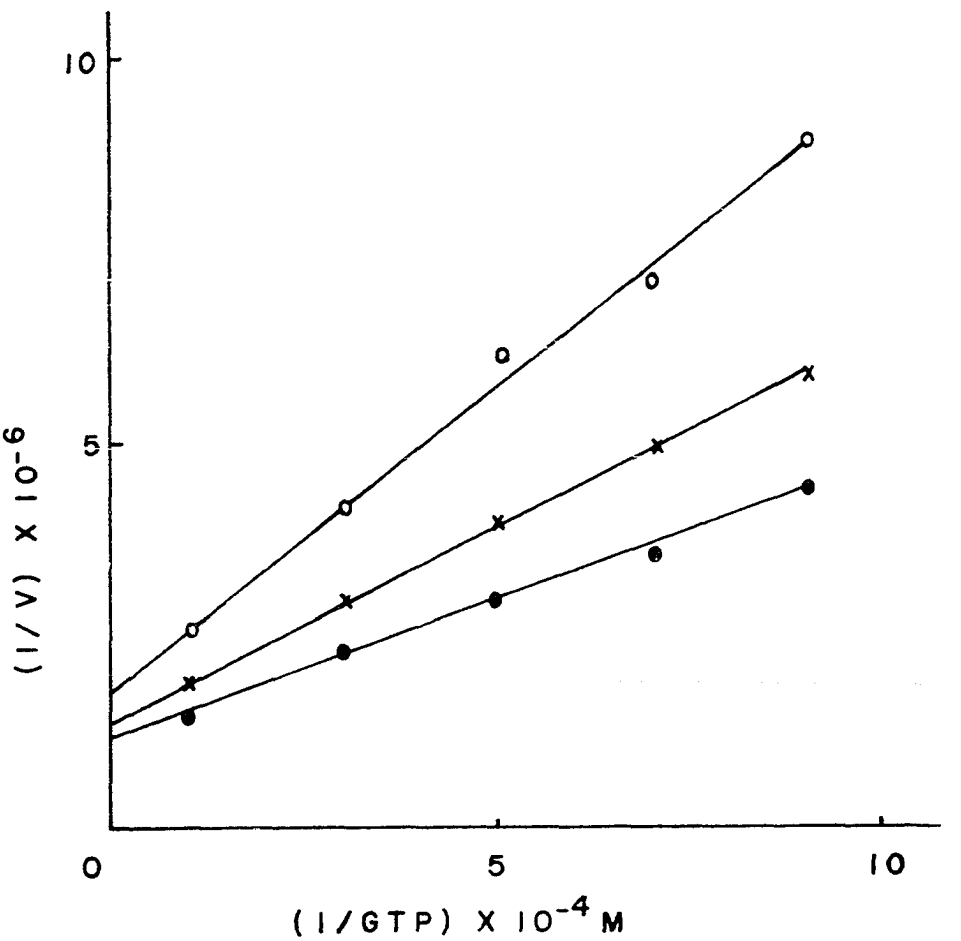


Figure 10. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of thio-IMP

IMP and aspartate concentrations were held constant at 0.15 and 5.0 mM respectively, and GTP was varied from 0.01 to 0.09 mM. Thio-IMP concentrations were none (●), 0.019 (X), and 0.057 mM (o). Other experimental details are described under "Experimental Procedure".

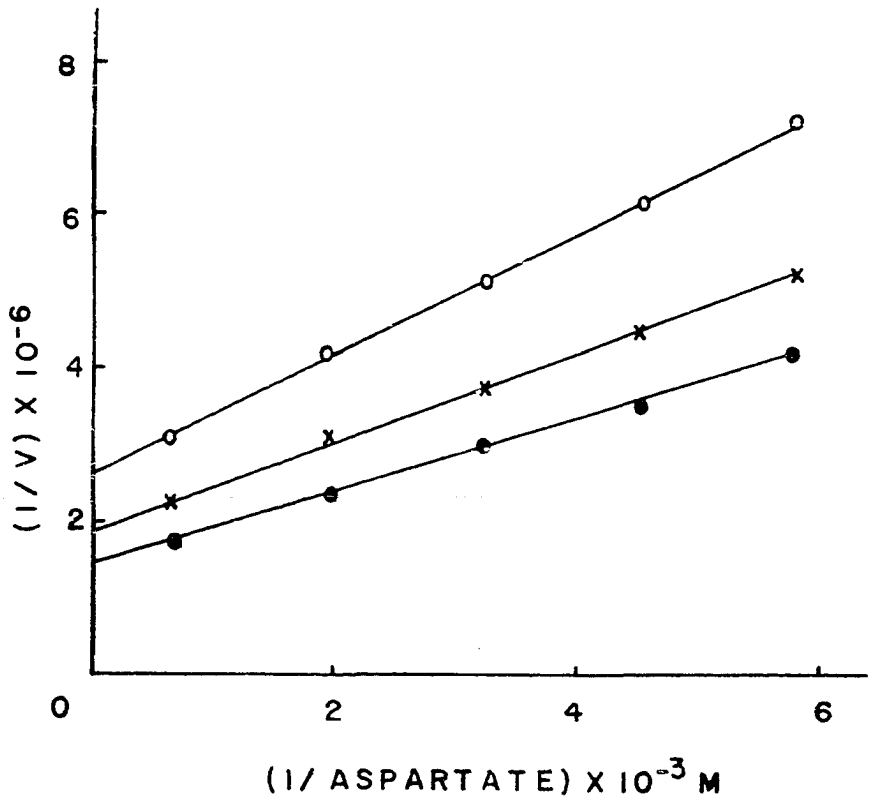


Figure 11. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of thio-IMP

GTP and IMP concentrations were held constant at 0.095 and 0.06 respectively, and aspartate was varied from 0.17 to 1.56 mM. Thio-IMP concentrations were none (●), 0.014 (X), and 0.041 mM (○). Other experimental details are described under "Experimental Procedure".

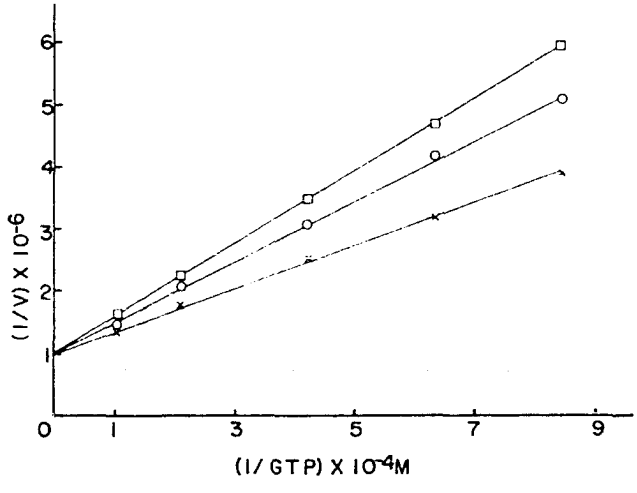


Figure 12. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of β,γ-5'-guanylyl methylene diphosphonate

IMP and aspartate concentrations were held constant at 0.15 and 5.0 mM respectively, and GTP was varied from 0.012 to 0.095 mM. β,γ-5'-Guanylyl methylene diphosphanate concentrations were none (X), 0.031 (○), and 0.062 mM (□). Other experimental details are described under "Experimental Procedure".

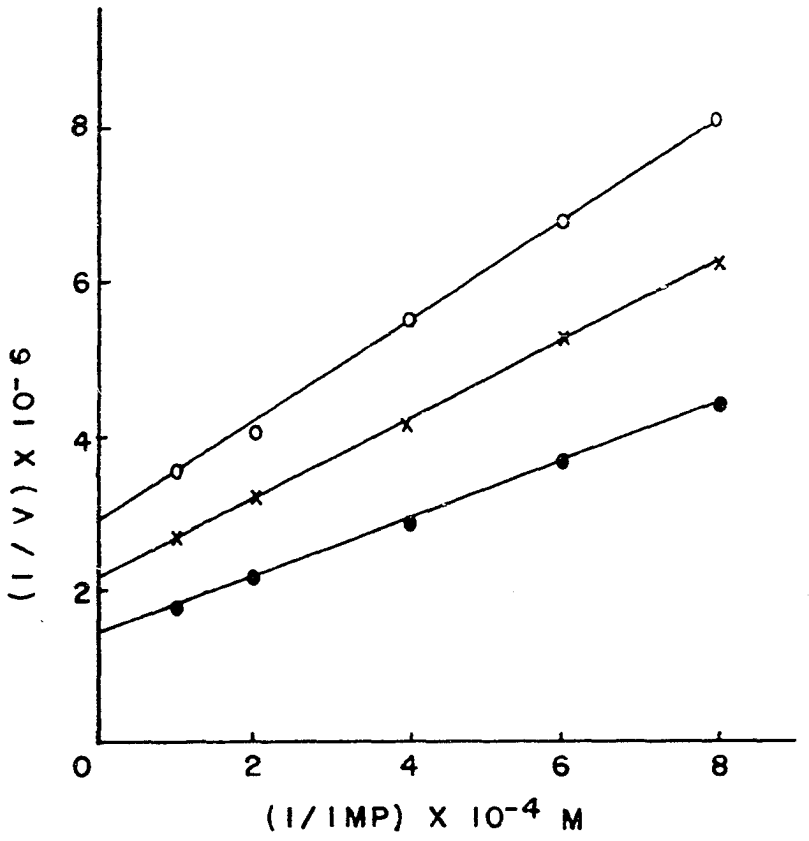


Figure 13. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of β,γ-5'-guanylyl methylene diphosphonate

Aspartate and GTP concentrations were held constant at 5.0 and 0.047 mM respectively, and IMP was varied from 0.012 to 0.10 mM. β,γ-5'-Guanylyl methylene diphosphonate concentrations were none (●), 0.083 (X), and 0.166 mM (○). Other experimental details are described under "Experimental Procedure".

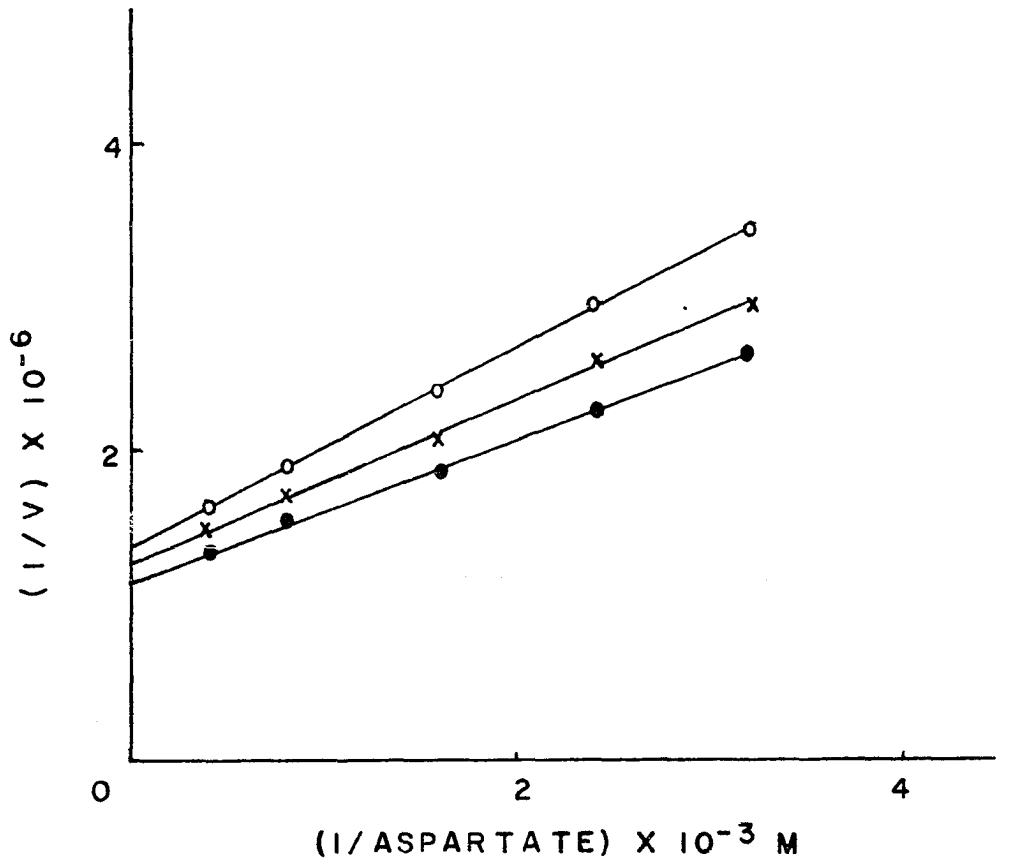


Figure 14. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of β,γ-5'-guanylyl methylene diphosphonate

GTP and IMP concentrations were held constant at 0.095 and 0.15 mM respectively, and aspartate was varied from 0.31 to 2.5 mM. β,γ-5'-Guanylyl methylene diphosphonate concentrations were none (●), 0.031 (X), and 0.062 mM (O). Other experimental details are described under "Experimental Procedure".

Table 4. Competitive inhibition patterns for various three-substrate mechanisms[a]

Mechanism[b]	Competitive inhibitor for substrate	1/A plot	1/B plot	1/C plot
I	A	C[c]	N[d,e]	N[d,e]
	B	U[f]	C	N[g]
	C	U	U	C
II	A	C	N	N
	B	N	C	N
	C	N	N	C
III	A	C	N	C[h]
	B	N	C	C[i]
	C	U	U	C
IV	A	C	N	N
	B	U	C	N
	C	U	N	C

[a]The various interactions of the competitive inhibitor is given in Appendix II along with the inhibited rate equation.

[b]The numbers refer to the mechanisms listed in Appendices I and II.

[c]C refers to a Lineweaver-Burk (1934) plot which shows competitive inhibition.

[d]N refers to a Lineweaver-Burk (1934) plot which shows noncompetitive inhibition.

[e]If EI reacts with B to form EIB, the plots would be nonlinear.

[f]U refers to a Lineweaver-Burk (1934) plot which shows uncompetitive inhibition.

[g]If EAI reacts with C to form EAIC, the plot would be nonlinear.

[h]If EIB reacts with C to form EIBC, the plot would noncompetitive.

[i]If EIA reacts with C to form EIAC, the plot would be noncompetitive.

Table 4. (Continued)

Mechanism	Competitive inhibitor for substrate	1/A plot	1/B plot	1/C plot
V	A	C	N	N
	B	N	C	N
	C	N	N	C
VI	A	C	U	U
	B	U	C	U
	C	U	U	C
VII	A	C	N[j]	U
	B	U	C	U
	C	U	U	C
VIII	A	C	U	U
	B	U	C	N[k]
	C	U	U	C
IX	A	C	N	U
	B	N	C	U
	C	U	U	C
X	A	C	U	U
	B	U	C	N
	C	U	N	C

[j]If EI reacts with B to form EIB, the plot would be nonlinear.

[k]If E'I reacts with C to form E'IC, the plot would be nonlinear.

Product inhibition studies

Further confirmation of the conclusions reached from the competitive inhibition study was obtained from a product inhibition study. If the possible abortive complexes are considered, analogous to the complexes formed in the competitive inhibition study, the rate equations for Mechanism II with a product present are the same as derived for competitive inhibition with P, Q, or R substituting for I. Thus, if the product binds at only one site, the same patterns should be observed with the product inhibitors as were observed with the competitive inhibitors. Because adenylosuccinate is derived from both IMP and aspartate, it seemed probable that it could bind at more than one site, causing a change in the type of inhibition observed. The product inhibition patterns for adenylosuccinate are shown in Figures 15, 16, and 17. Since it is competitive for IMP and noncompetitive relative to aspartate and GTP, apparently the adenylosuccinate binds significantly only to the IMP site and aspartate can bind even in the presence of adenylosuccinate indicating that the two sites may be spatially separated.

The effect of GDP with respect to the three substrates was also studied and the data is shown in Figures 18, 19, and 20. It was found to be competitive with GTP and noncompetitive relative to the other two substrates. The effects of these two products are in agreement with the conclusions reached from the competitive inhibition experiments.

Inhibition with Pi was studied relative to GTP and it was found to be noncompetitive with respect to GTP. The concentration required for significant inhibition (10 mM Pi), along with its probable binding at the IMP site at these high concentrations, weaken any conclusions that might be drawn

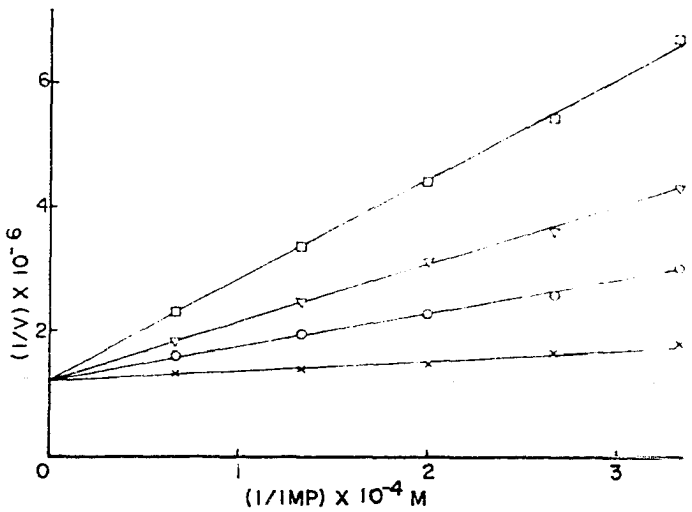


Figure 15. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of adenylosuccinate

GTP and aspartate concentrations were held constant at 0.057 and 2.5 mM respectively, and IMP was varied from 0.03 to 0.15 mM. Adenylosuccinate concentrations were none (X), 0.011 (○), 0.023 (▽), and 0.045 mM (□). Other experimental details are described under "Experimental Procedure".

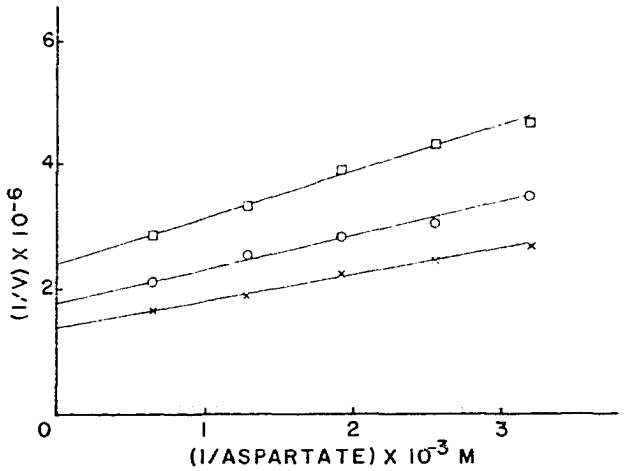


Figure 16. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of adenylosuccinate GTP and IMP concentrations were held constant at 0.057 and 0.060 mM respectively, and aspartate was varied from 0.31 to 1.56 mM. Adenylosuccinate concentrations were none (X), 0.008 (○), and 0.023 mM (□). Other experimental details are described under "Experimental Procedure".

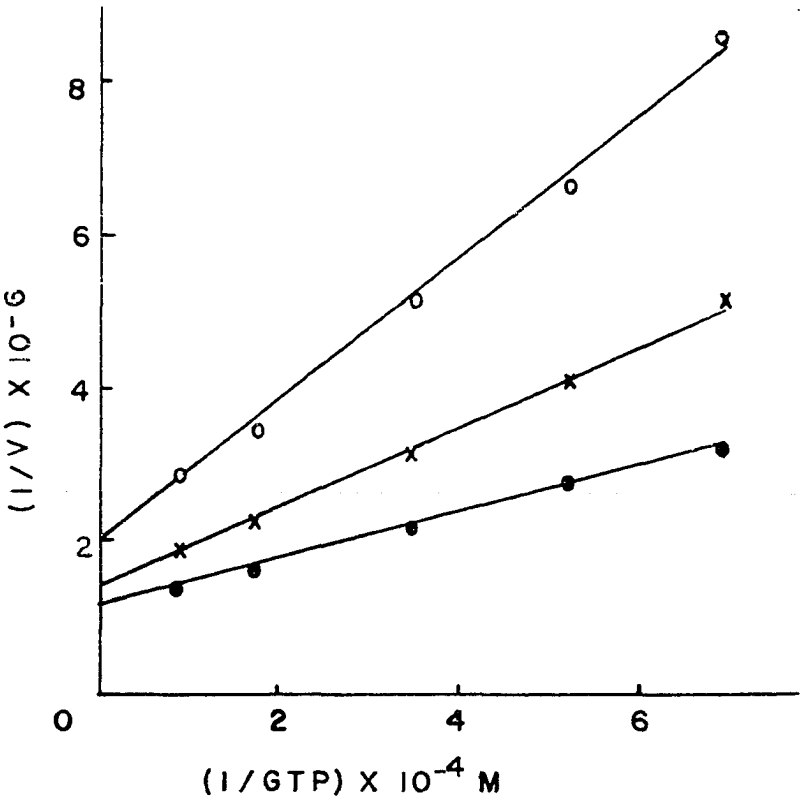


Figure 17. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of adenylosuccinate

IMP and aspartate concentrations were held constant at 0.06 and 1.25 mM respectively, and GTP was varied from 0.014 to 0.114 mM. Adenylosuccinate concentrations were none (●), 0.014 (X), and 0.034 mM (o). Other experimental details are described under "Experimental Procedure".

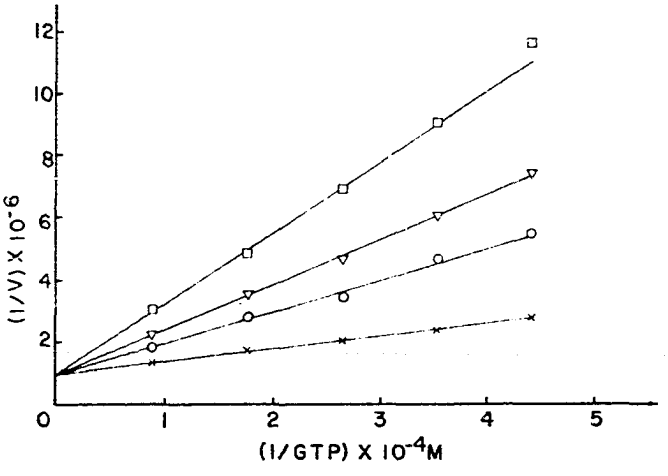


Figure 18. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of GTP in the presence and absence of GDP

Aspartate and IMP concentrations were held constant at 2.5 and 0.04 mM respectively, and GTP was varied from 0.023 to 0.114 mM. GDP concentrations were none (X), 0.015 (○), 0.030 (▽), and 0.061 mM (□). Other experimental details are described under "Experimental Procedure".

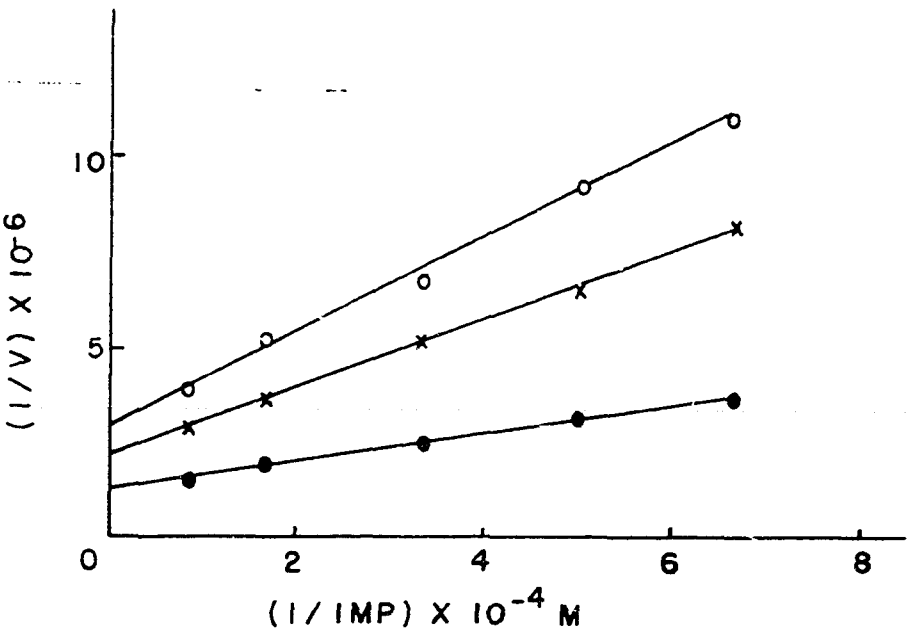


Figure 19. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of IMP in the presence and absence of GDP

Aspartate and GTP concentrations were held constant at 2.5 and 0.057 mM respectively, and the IMP was varied from 0.015 to 0.12 mM. GDP concentrations were none (●), 0.023 (X), and 0.046 mM (○). Other experimental details are described under "Experimental Procedure".

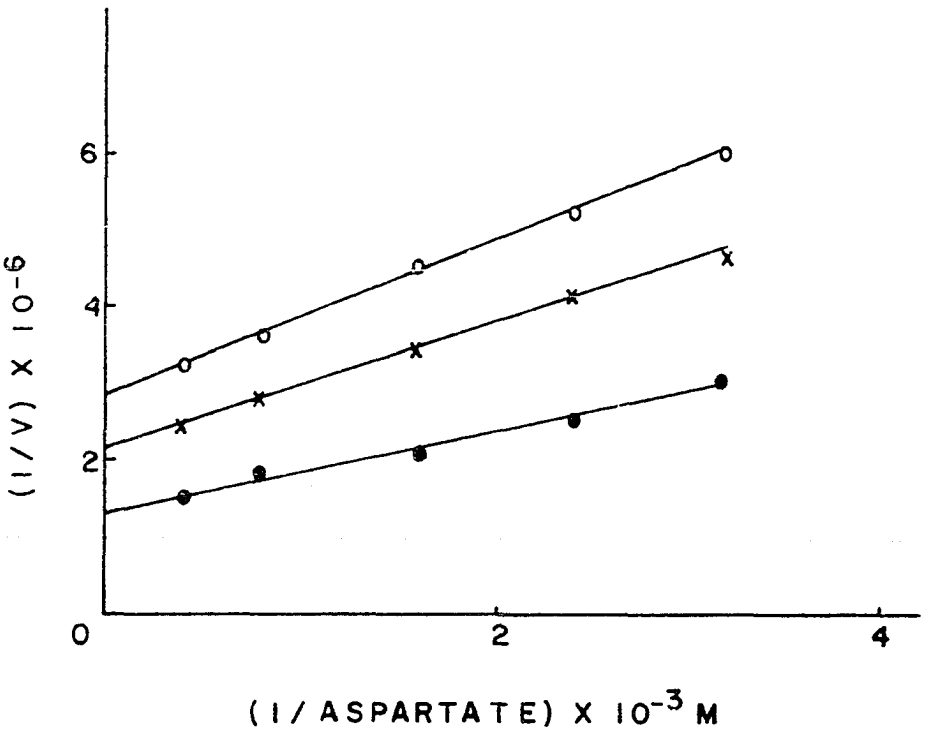


Figure 20. Plot of reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of aspartate in the presence and absence of GDP

GTP and IMP concentrations were held constant at 0.15 and 0.057 mM respectively, and aspartate was varied from 0.31 to 2.50 mM. GDP concentrations were none (●), 0.018 (X), and 0.037 mM (○). Other experimental details are described under "Experimental Procedure".

from these experiments. The problem encountered with the Pi inhibition study indicates the value of the competitive inhibition approach.

Determination of kinetic constants

It was not possible to determine values for the inhibition constants from the data above because of the obvious complexity of the rate equations. Apparent inhibition constants were calculated as shown in Table 5. A summary of the inhibition studies is also shown in Table 5.

Determination of Michaelis constants from the initial rate data is complicated by the fact that a (substrate)^2 term occurs in the denominator of some of the equations derived from the initial rate equations which are used for the slope and intercept replots. In the case of the rapid equilibrium, fully random mechanism (Mechanism II), for example, when a 1/A experiment is performed, the slope term will be $\emptyset_1 + \emptyset_4/C + \emptyset_5/xC + \emptyset_7/xC^2$ and the intercept term will be $\emptyset_0 + \emptyset_2/xC + \emptyset_3/C + \emptyset_6/xC^2$, since $B = xC$ where x is a constant for the particular experiment. Both equations describe a parabolic behavior for the replot. In order to determine the Km values, an extrapolation to infinite substrate concentration is made and $\emptyset_o/E_o$ and $\emptyset_{substrate}/E_o$ are determined and the Km for a particular substrate is calculated from $\emptyset_{substrate}/\emptyset_o$. The Michaelis constants were calculated to be: GTP, 1×10^{-5} M; IMP, 2×10^{-5} M; and aspartate, 3.5 10^{-4} M.

Table 5. Inhibition of adenylosuccinate synthetase by competitive and product inhibitors

Inhibitor	Type of inhibition relative to			Apparent inhibition constant[a]
	Aspartate	GTP	IMP	
Succinate	Competitive	Noncompetitive	Noncompetitive	7.5 mM
Thio-IMP	Noncompetitive	Noncompetitive	Competitive	10 μM
β-γ-5'-Guanylyl methylene diphosphonate	Noncompetitive	Competitive	Noncompetitive	80 μM
GDP	Noncompetitive	Competitive	Noncompetitive	12 μM
Adenylosuccinate	Noncompetitive	Noncompetitive	Competitive	5 μM

[a]Calculated from the equation $K_{app} = (I)S_o/(S_I - S_o)$ where K_{app} is the apparent inhibition constant, I is the concentration of the inhibitor, S_o is the slope of the uninhibited line from the competitive inhibition plot, and S_I is the slope of the inhibited line on the same graph for a particular I.

The equation is derived by combining the slope terms of the reciprocal plots for the inhibited and uninhibited Michaelis-Menten equations as follows:

$$S_I = (Km/Vm)(1 + (I)/K_{app}) \text{ and } S_o = Km/Vm$$

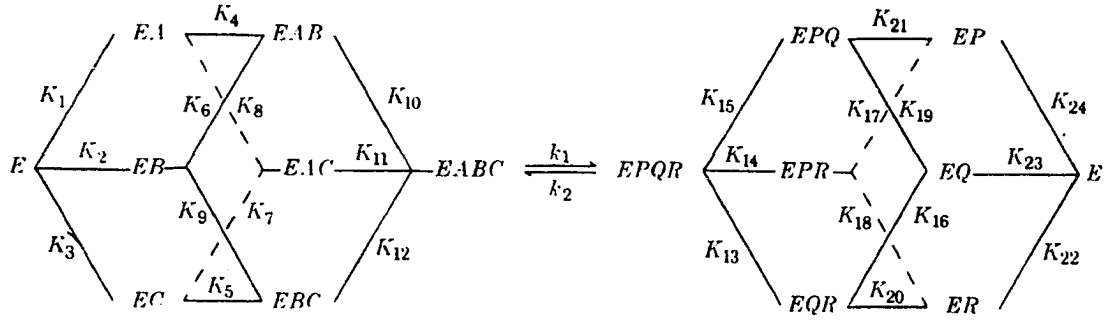
$$S_I = S_o(1 + (I)/K_{app})$$

$$K_{app} = (I)S_o/(S_I - S_o)$$

GENERAL DISCUSSION

The purification of adenylosuccinate synthetase presented in this study represents a significant improvement over previous reported preparations. Hopefully, physical and chemical studies can be carried out on the protein to determine more of its properties.

The kinetic experiments described in this study serve to verify the usefulness of studying the kinetic mechanisms of three-substrate enzyme systems by use of competitive inhibitors of substrates. These investigations, when combined with the initial rate findings for Coenzyme A-linked aldehyde dehydrogenase (Rudolph *et al.*, 1968) indicate that a choice can be readily made between ping-pong and sequential mechanisms by use of the experimental protocol outlined above. In the case of adenylosuccinate synthetase, it is clear that only one pathway of enzyme and substrate interaction is consistent with the experimental results, *i.e.* the fully random mechanism (Mechanism II) which is depicted in Scheme I.



SCHEME I

The replotting technique used to differentiate between the various sequential mechanisms may prove to be of general value for distinguishing between different sequential mechanisms for three substrate systems. As

Table III indicates, no additional information can be obtained in the case of ping-pong mechanisms using this procedure; however, each sequential mechanism considered has a unique replot pattern. The nonlinearity of some of the replots is caused by varying both of the fixed substrates in a constant ratio as described for the determination of the Michaelis constants. It may be necessary to vary the fixed substrates over a wide concentration range in order to determine whether a replot is truly linear or nonlinear. The experimental difficulty inherent in this procedure will be a function of the rate constants of the system. Therefore, it is felt that evidence for a given mechanism which is obtained by the replotting procedure outlined here should be tested using the approach involving competitive substrate inhibitors and, if possible, product inhibitors.

The mechanisms of enzymic reactions that involve synthesis of amide bonds have been extensively investigated in recent years (Hartman and Buchanan, 1958; Miller and Buchanan, 1962; Krishnaswamy *et al.*, 1962; Snoke and Bloch, 1955; and Mizobuchi *et al.*, 1968). Reactions similar to the one described in this study include glutamine synthetase (Krishnaswamy *et al.*, 1962), glutathione synthetase (Snoke and Bloch, 1955), and a number of enzymes involved in the biosynthesis of purines (Buchanan and Hartman, 1959). All these cases involve the cleavage of a nucleoside triphosphate to a diphosphate and inorganic phosphate.

Two general types of interactions for this class of enzymes have been proposed. Krishnaswamy *et al.*, (1962) suggested the formation of a phosphorylated intermediate in the glutamine synthetase reaction and have since presented evidence for this proposal including the ability of β-aminoglutaryl phosphate to react with ADP to form β-aminoglutarate

(Khedouri *et al.*, 1964) and ATP as well as the isolation of a phosphorylated methionine sulfoxide derivative (Ronzio and Meister, 1968). Lieberman (1956) also suggested the possibility of 6-phosphoryl-IMP as an intermediate in adenylosuccinate synthesis from the fact that 6-^{18}O-IMP gives rise to ^{18}O-labelled phosphate. Hartman and Buchanan (1958) and Graves and Boyer (1962), on the other hand, have proposed that for adenylosuccinate synthetase and other enzymes, all three substrates interact at the enzyme surface in a concerted fashion without formation of new covalent-linked compounds as obligatory intermediates. This suggestion is based on the observation that all three products are required to obtain reversal of the reaction and no partial reactions are observed even with arsenate replacing phosphate in the reaction (Miller and Buchanan, 1962). The absence of partial reactions does not necessarily exclude the formation of covalent intermediates since the requirement for GDP in the reverse reaction could be concerned with the formation of the proper protein conformation for reaction to occur. The mechanism proposed in this paper would be consistent with either idea and no further conclusions can be drawn without a detailed study of the catalytic steps of the reaction.

The exclusion of ping-pong mechanisms from consideration would rule out the type of mechanism that has been considered for glutamine synthetase assuming release of products after formation of covalent intermediates. However, if no products were released until all substrates were present on the enzyme surface, the random mechanism would not be inconsistent with this idea.

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The author is especially appreciative of the support and encouragement given him by his parents.

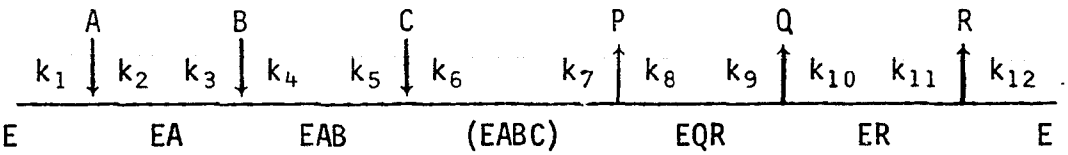
APPENDIX I

Mechanisms and Rate Equations for Enzyme Systems Utilizing Three Substrates

The shorthand notation of Cleland (1963) is used in describing these mechanisms and the rate equations are given in terms of Ø's as suggested by Dalziel (1957). These rate equations have appeared elsewhere (Fromm, 1967; Dalziel, 1969) but are included here for clarity.

Quaternary complex mechanisms

I. Ordered Ter Ter



The rate equation for this mechanism is:

$$E_o/v = Ø_o + Ø_1/A + Ø_2/B + Ø_3/C + Ø_4/(A)(B) + Ø_5/(B)(C) + Ø_6/(A)(B)(C). \quad (2)$$

E_o represents total enzyme concentration and v, initial velocity. A, B, and C represent substrates and R, Q, and P are taken to be their respective products. These definitions apply to all the mechanisms depicted here. The Ø values are defined as:

$$Ø_o = 1/k_7 + 1/k_9 + 1/k_{11},\ Ø_1 = 1/k_1,\ Ø_2 = 1/k_3,\ Ø_3 = (k_6 + k_7)/k_5k_7,$$
$$Ø_4 = k_2/k_1k_3,\ Ø_5 = k_4(k_6 + k_7)/k_3k_5k_7,\ Ø_6 = k_2k_4(k_6 + k_7)/k_1k_3k_5k_7$$

II. Random Ter Ter (Rapid Equilibrium) In this and all rapid equilibrium mechanisms included here, it is assumed that all steps in the sequence which are in equilibrium (steps that are represented by K's) equilibrate rapidly relative to the rate limiting step.

$E + A = EA, K_1$	$EC + B = EBC, K_5$	$EB + C = EBA, K_9$
$E + B = EB, K_2$	$EB + A = EAB, K_6$	$EAB + C = EABC, K_{10}$
$E + C = EC, K_3$	$EC + A = EAC, K_7$	$EAC + B = EABC, K_{11}$
$EA + B = EAB, K_4$	$EA + C = EAC, K_8$	$EBC + A = EABC, K_{12}$

$$EABC \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EPQR$$

All equilibria occurring after the formation of EPQR are omitted since they are not kinetically significant. This mechanism is depicted in Scheme I in the General Discussion.

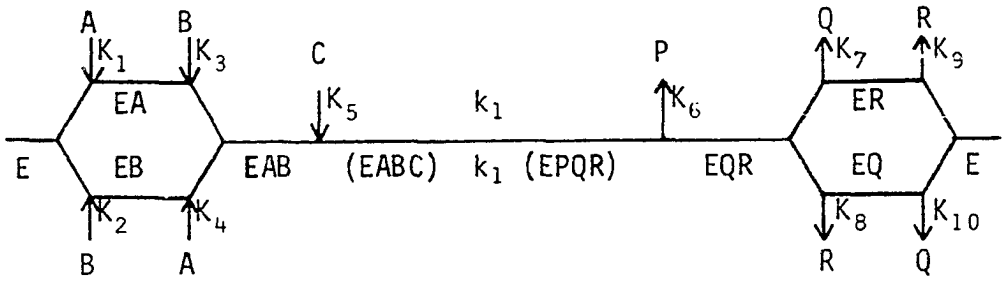
The rate equation for this mechanism is:

$$E_o/v = \emptyset_o + \emptyset_1/A + \emptyset_2/B + \emptyset_3/C + \emptyset_4/(A)(B) + \emptyset_5/(A)(C) + \emptyset_6/(B)(C) + \emptyset_7/(A)(B)(C). \quad (3)$$

where

$$\emptyset_o = 1/k_1,\ \emptyset_{12} = K_{12}/k_1,\ \emptyset_2 = K_{11}/k_2,\ \emptyset_3 = K_{10}/k_1,\ \emptyset_4 = K_9K_{12}/k_1,\ \emptyset_5 = K_7K_{11}/k_1,\ \emptyset_6 = K_8K_{11}/k_1,\ \emptyset_7 = K_1K_8K_{11}/k_1$$

III. Random AB (Rapid Equilibrium)



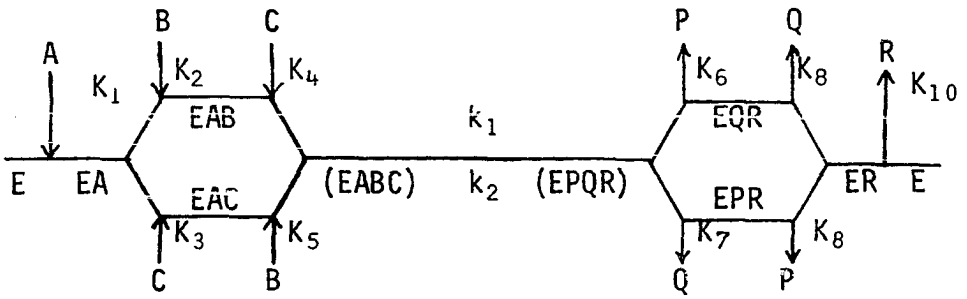
The rate equation for this mechanism is:

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{C} + \frac{\emptyset_2}{(A)(C)} + \frac{\emptyset_3}{(B)(C)} + \frac{\emptyset_4}{(A)(B)(C)} . \qquad (4)$$

where

$$\emptyset_o = \frac{1}{k_1} , \quad \emptyset_1 = \frac{K_5}{k_1} , \quad \emptyset_2 = \frac{K_4K_5}{k_1} , \quad \emptyset_3 = \frac{K_3K_5}{k_1} , \quad \emptyset_4 = \frac{K_1K_3K_5}{k_1}$$

IV. Random BC (Rapid Equilibrium)



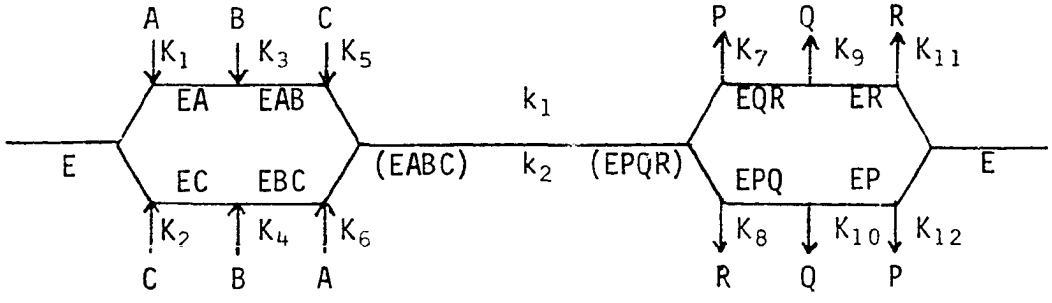
The rate equation for this mechanism is :

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{B} + \frac{\emptyset_2}{C} + \frac{\emptyset_3}{(B)(C)} + \frac{\emptyset_4}{(A)(B)(C)} . \qquad (5)$$

where

$$\emptyset_0 = \frac{1}{k_1} \ , \ \emptyset_1 = \frac{K_5}{k_1} \ , \ \emptyset_2 = \frac{K_4}{k_1} \ , \ \emptyset_3 = \frac{K_2K_4}{k_1} \ , \ \emptyset_4 = \frac{K_1K_2K_4}{k_1}$$

V. Random AC (Rapid Equilibrium)



The rate equation for this mechanism is:

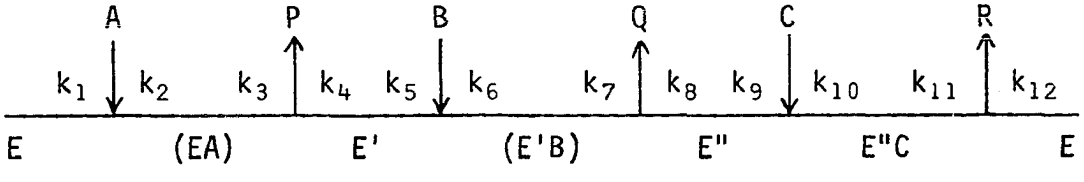
$$\frac{E_0}{v} = \emptyset_0 + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{C} + \frac{\emptyset_3}{(A)(B)} + \frac{\emptyset_4}{(B)(C)} + \frac{\emptyset_5}{(A)(B)(C)} \qquad (6)$$

where

$$\emptyset_0 = \frac{1}{k_1} \ , \ \emptyset_1 = \frac{K_6}{k_1} \ , \ \emptyset_2 = \frac{K_5}{k_1} \ , \ \emptyset_3 = \frac{K_4K_6}{k_1} \ , \ \emptyset_4 = \frac{K_3K_5}{k_1} \ , \ \emptyset_5 = \frac{K_1K_3K_5}{k_1}$$

Ping-Pong mechanisms

VI. Hexa Uni Ping-Pong



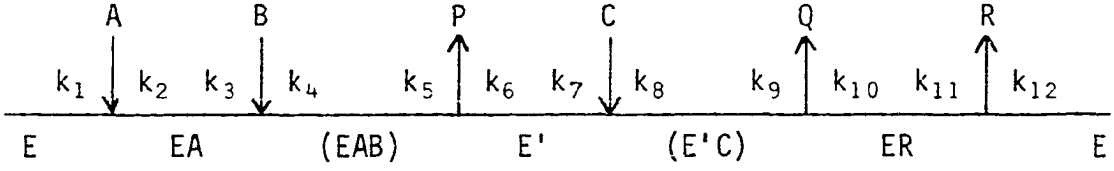
The rate equation for this mechanism is:

$$\frac{E_0}{v} = \emptyset_0 + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} \qquad (7)$$

where

$$\emptyset_o = \frac{1}{k_3} + \frac{1}{k_7} + \frac{1}{k_{11}}, \quad \emptyset_1 = \frac{(k_2+k_3)}{k_1k_3}, \quad \emptyset_2 = \frac{(k_6+k_7)}{k_5k_7}, \quad \emptyset_3 = \frac{(k_{10}+k_{11})}{k_9k_{11}}$$

VII. Ordered Bi Uni Uni Bi Ping-Pong



The rate equation for this mechanism is:

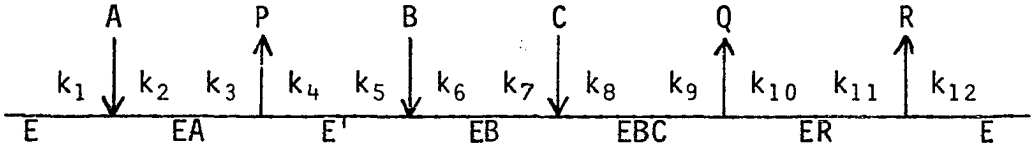
$$\frac{E_o}{v} = \emptyset_0 + \frac{\emptyset_1}{A} = \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(A)(B)} \tag{8}$$

where

$$\emptyset_o = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}}, \quad \emptyset_1 = \frac{1}{k_1}, \quad \emptyset_2 = \frac{(k_4+k_5)}{k_3k_5}, \quad \emptyset_3 = \frac{(k_8+k_9)}{k_7k_9},$$

$$\emptyset_4 = \frac{k_2(k_4+k_5)}{k_1k_3k_5}$$

VIII. Ordered Uni Uni Bi Bi Ping-Pong



The rate equation for this mechanism is:

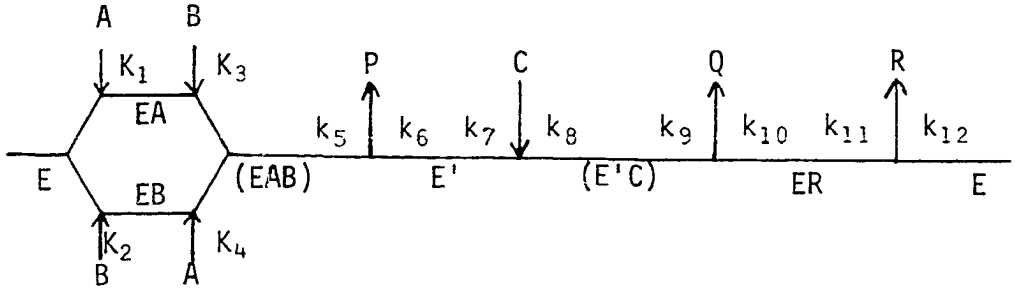
$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(B)(C)} \tag{9}$$

where

$$\emptyset_o = \frac{1}{k_3} + \frac{1}{k_9} + \frac{1}{k_{11}}, \quad \emptyset_1 = \frac{(k_2+k_3)}{k_1k_3}, \quad \emptyset_2 = \frac{1}{k_5}, \quad \emptyset_3 = \frac{(k_8+k_9)}{k_7k_9},$$

$$\emptyset_4 = \frac{k_6(k_8+k_9)}{k_5k_7k_9}$$

IX. Random Bi Uni Uni Bi Ping-Pong



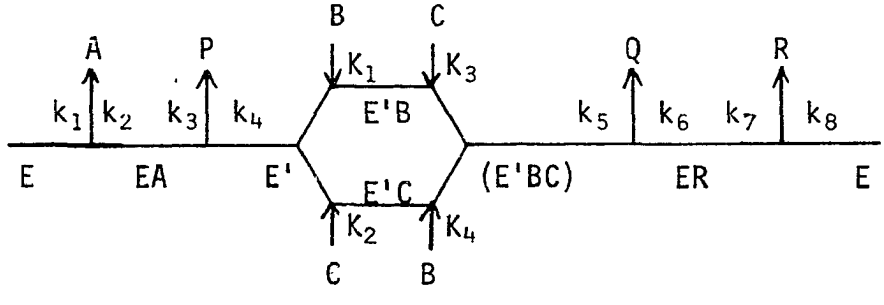
The rate equation for this mechanism is:

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(A)(B)} \qquad (10)$$

where

$$\emptyset_o = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}}\ ,\ \emptyset_1 = \frac{K_4}{k_5}\ ,\ \emptyset_2 = \frac{K_3}{k_5}\ ,\ \emptyset_3 = \frac{(k_8+k_9)}{k_7k_9}\ ,\ \emptyset_4 = \frac{K_1K_3}{k_5}$$

X. Random Uni Uni Bi Bi Ping-Pong



The rate equation for this mechanism is:

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(B)(C)} \qquad (11)$$

where

$$\phi_0 = \frac{1}{k_3} + \frac{1}{k_5} + \frac{1}{k_7}\ ,\ \phi_1 = \frac{(k_2+k_3)}{k_1 k_3}\ ,\ \phi_2 = \frac{K_4}{k_5}\ ,\ \phi_3 = \frac{K_3}{k_5}\ ,\ \phi_4 = \frac{K_1 K_3}{k_5}$$

APPENDIX II

Rate Equations Describing the Effect of Competitive Inhibitors on the Three-Substrate Mechanisms of Appendix I

The equations describing the effects of competitive inhibitors on Mechanisms I, II, IV, V, and VII have been presented by Fromm (1967) and, although they are summarized in Table IV, will not be presented here. The inhibited rate equations for Mechanisms III, VI, VIII, IX, and X are presented below. The Ø's are the same as defined in Appendix I, and the assumed additional interactions in the presence of the inhibitors are given with each equation. The competitive inhibitor, I, is assumed to interact at the same enzymatic site as does the substrate it is competitive with.

III. Random AB (Rapid Equilibrium)

A. Inhibitor for A:

$$E + I = EI \quad K_i$$

$$EI + B = EIB \quad K_{ii}$$

$$EB + I = EIB \quad K_{iii}$$

$$EIB + C = EIBC \quad K_{iv}$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{C} + \frac{\emptyset_2}{(A)(C)}\left(1 + \frac{(I)}{K_{iii}} + \frac{(I)(C)}{K_{iii}K_{iv}}\right) + \frac{\emptyset_3}{(B)(C)}$$

$$+ \frac{\emptyset_4}{(A)(B)(C)}\left(1 + \frac{(I)}{K_i}\right) \qquad (12)$$

B. Inhibitor for B:

$$E + I = EI \; K_i$$

$$EI + A = EIA \; K_{ii}$$

$$EA + I = EIA \; K_{iii}$$

$$EAI + C = EAIC \; K_{iv}$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{C} + \frac{\emptyset_2}{(A)(C)} + \frac{\emptyset_3}{(B)(C)}\left(1 + \frac{(I)}{K_{iii}} + \frac{(I)(C)}{K_{iii}K_{iv}}\right) + \frac{\emptyset_4}{(A)(B)(C)}\left(1 + \frac{(I)}{K_i}\right) \tag{13}$$

C. Inhibitor for C:

$$EAB + I = EABI \; K_i$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{C}\left(1 + \frac{I}{K_i}\right) + \frac{\emptyset_2}{(A)(C)} + \frac{\emptyset_3}{(B)(C)} + \frac{\emptyset_4}{(A)(B)(C)} \tag{14}$$

IV. Hexa Uni Ping-Ping

A. Inhibitor for A:

$$E + I = EI \; K_1$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A}\left(1 + \frac{(I)}{K_1}\right) + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} \tag{15}$$

B. Inhibitor for B:

$$E' + I = E'I \; K_1$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B}\left(1 + \frac{(I)}{K_1}\right) + \frac{\emptyset_3}{C} \tag{16}$$

C. Inhibitor for C:

$$E'' + I = E''I \; K_1$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C}\left(1 + \frac{(I)}{K_1}\right) \tag{17}$$

VIII. Ordered Uni Uni Bi Bi Ping-Pong

A. Inhibitor for A:

$$E + I = EI \; K_1$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A}\left(1 + \frac{(I)}{K_1}\right) + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(B)(C)} \tag{18}$$

B. Inhibitor for B:

$$E' + I = E'I \; K_1$$

$$E'I + C = E'IC \; K_2$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B}\left(1 + \frac{(I)}{K_1} + \frac{(I)(C)}{K_1K_2}\right) + \frac{\emptyset_3}{C}$$

$$+ \frac{\emptyset_4}{(B)(C)}\left(1 + \frac{(I)}{K_1} + \frac{(I)(C)}{K_1K_2}\right) \tag{19}$$

C. Inhibitor for C:

$$E'B + C = E'BC \; K_1$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C}\left(1 + \frac{(I)}{K_1}\right) + \frac{\emptyset_4}{(B)(C)} \tag{20}$$

IX. Random Bi Uni Uni Bi Ping-Pong

A. Inhibitor for A:

$$E + I = EI \quad K_i$$

$$EI + B = EIB \quad K_{ii}$$

$$EB + I = EIB \quad K_{iii}$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A}\left(1 + \frac{(I)}{K_{iii}}\right) + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(A)(B)}\left(1 + \frac{(I)}{K_i}\right) \tag{21}$$

B. Inhibitor for B:

$$E + I = EI \quad K_i$$

$$EA + I = EIA \quad K_{ii}$$

$$EI + A = EIA \quad K_{iii}$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B}\left(1 + \frac{(I)}{K_{ii}}\right) + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(A)(B)}\left(1 + \frac{(I)}{K_i}\right) \tag{22}$$

C. Inhibitor for C:

$$E' + I = E'I \quad K_i$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C}\left(1 + \frac{(I)}{K_i}\right) + \frac{\emptyset_4}{(A)(B)} \tag{23}$$

X. Random Uni Uni Bi Bi Ping-Pong

A. Inhibitor for A:

$$E + I = EI \quad K_i$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A}\left(1 + \frac{(I)}{K_i}\right) + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(B)(C)} \tag{24}$$

B. Inhibitor for B:

$$E' + I = E'I \; K_i$$

$$E'C + I = E'IC \; K_{ii}$$

$$E'I + C = E'IC \; K_{iii}$$

$$\frac{E_o}{v} = \emptyset_o + \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B}\left(1 + \frac{(I)}{K_{ii}}\right) + \frac{\emptyset_3}{C} + \frac{\emptyset_4}{(B)(C)}\left(1 + \frac{(I)}{K_i}\right) \qquad (25)$$

C. Inhibitor for C:

$$E' + I = E'I \; K_i$$

$$E'B + I = E'IB \; K_{ii}$$

$$E'I + B = E'IB \; K_{iii}$$

$$\frac{E_o}{v} = \frac{\emptyset_1}{A} + \frac{\emptyset_2}{B} + \frac{\emptyset_3}{C}\left(1 + \frac{(I)}{K_{ii}}\right) + \frac{\emptyset_4}{(B)(C)}\left(1 + \frac{(I)}{K_i}\right) \qquad (26)$$