Action Patterns of Feedback Modifiers on Equilibrium Exchanges and Applications to Glutamine Synthetase (Escherichia coli W)*

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SUMMARY

Theoretical and experimental results are presented to illustrate the applicability of equilibrium rate measurements by isotope exchange to the understanding of the effect of modifiers on enzymic catalysis. A table summarizes the expected theoretical patterns for a two-substrate, two-product system with random substrate addition and ternary complex interconversion. Such patterns are used as a first approximation model for interpreting rate measurements with Escherichia coli glutamine synthetase for the glutamate \( \rightleftharpoons \) glutamine, ATP \( \rightleftharpoons \) P\(_i\), NH\(_3\) \( \rightleftharpoons \) glutamine, and O\(_{\text{glutamate}}\) \( \rightleftharpoons \) O\(_{\text{Pi}}\) exchanges. These measurements with nine different modifiers showed strikingly different inhibitory patterns, including apparent complete block of chemical interconversion, and differential interference with various substrate-binding and release steps. A new type of modifier action by GDP and AMP is reported, in which the glutamate \( \rightleftharpoons \) glutamine exchange is unimpaired but the ATP \( \rightleftharpoons \) P\(_i\) exchange is completely inhibited. This is interpreted as a cryptic catalysis in which the modifier prevents dissociation of the ATP, but still allows the rapid chemical interconversion of bound substrates and rapid exchange between the NH\(_3\)-glutamine and glutamate-glutamine pools, as well as \( ^{15} \)O between glutamate and P\(_i\).

Gaining an adequate understanding of the complex and intricate action of enzyme modifiers promises to be an intriguing but difficult task. A principal purpose of this paper is to give theoretical and experimental information that illustrates the value of measurement of exchange rates at equilibrium for revealing pertinent facets of modifier action. Some theoretical approaches with a model system are also summarized. For the experimental portion, glutamine synthetase from Escherichia coli was chosen for study.

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The exchange measurements may suffice to show whether the modifier effects primarily the chemical interconversion step or the binding and release steps of particular substrates. With glutamine synthetase, the studies have revealed a new type of modifier action in which all substrates are bound, chemical interconversion is not blocked, but release of one substrate pair is hindered.

The value of equilibrium rate measurements for study of the catalytic mechanism has gained increasing recognition, and is illustrated for glutamine synthetase by the preceding paper (1). The potential importance of such measurements as probes of modifier action has been shown by Silverstein with alcohol dehydrogenase (2) and by Silverstein and Sulebele with glutamate dehydrogenase (3). These and the present studies illustrate only some of the modes of application of exchange rate data to the understanding of modifier action.

Glutamine synthetase from E. coli appeared to be a particularly appropriate enzyme for these studies because of the elegant studies from Stadtman's laboratory (4, 5), demonstrating cumulative feedback of a number of modifiers. The principal effects they observe in initial velocity studies are: \( l \)-alanine, AMP, and carbamyl phosphate appear to mainly inhibit \( V_{\max} \); glycine, CTP, and \( l \)-tryptophan are partially competitive with \( l \)-glutamate; glucoseamine 6-phosphate and \( l \)-histidine are partially competitive with ammonia; and ADP and GDP partially compete with ATP. These effects were monitored by either a forward biosynthetic assay based on P\(_i\) release or by a transferase assay with glutamine, ADP, arsenate, and hydroxylamine, but were noted before the important relation between the enzyme's state of adenylylation and its metal ion specificity, activity, and response to pH and ionic strength, and sensitivity to modifiers were adequately recognized (6). The partial suppression of activity reported for some of these modifiers has been found to be artificial after more thorough characterization of the enzyme; more recent work reveals that most modifiers under initial velocity assay conditions have the potential capacity to inhibit activity 100% although their reported (4, 5) cumulative inhibitory behavior still holds true.

Our studies reported here used a highly adenylylated form of glutamine synthetase from E. coli W which is very sensitive to added feedback modifiers. Quite distinctive effects of various modifiers have been observed, leading to some interesting and

1 E. R. Stadtman, private communication.
relatively clearcut conclusions about each modifier's mode of action.

**EXPERIMENTAL PROCEDURE**

Materials and procedures used in these experiments have been described previously (1). These include preparation of electrophoretically pure glutamine synthetase from *E. coli* W, and its characterization as highly adenylylated (E₁₀). Substrates were obtained and purified as described. Separation of substrates and modifiers from a reaction mixture was carried out by formate gradient elution from DEAE-cellulose (formate). [³C]Glutamate and ATP were products of Schwarz BioResearch, and feedback modifiers were either Sigma grade I or Mann Research Laboratories analyzed products.

Variation of modifier concentration without perturbations in pH, ionic strength, and other solution parameters was accomplished by making up modifier at maximal concentration in one portion of reaction solution and diluting aliquots of this solution with reaction solution which did not contain modifier. A similar procedure was employed in the previous study (1) to vary substrate levels without varying other conditions.

**RESULTS**

**Choice of Equilibrium Reaction Conditions**—The data of the preceding paper (1) allowed selection of substrate concentrations below saturation and below levels for certain substrate interactions at which exchange reactions are suppressed. Use of substrate levels below saturation favored detection of any competitive modifier interactions. Ionic strength was kept above 0.2 M, so that variation of added modifiers would have little or no effect on total ionic strength. The inhibitory effects which imidazole, as an analog of histidine, can exert upon adenylylated subunits was avoided by use of β,β-dimethylglutarate buffer at pH 6.50. Adenylylated subunits exhibit maximal activity near this pH (6).

Manganese ion was used at 1 mM concentration, with magnesium ion added equal to total nucleotide di- and triphosphates. As explained earlier (1), this procedure assured that nucleotides existed primarily as the magnesium complexes with low free Mg⁺⁺ (≤1 mM); high levels (60 mM) of free Mg⁺⁺ are reported to inhibit noncompetitively the activity of Mn⁺⁺-activated subunits especially near and above pH 7.

The reaction mixture used in the presence of added feedback modifiers contained: 0.5 mM NH₄, 5 mM glutamine, 1 mM glutamate, 10 mM Pi, 1 mM ATP, and 4 mM ADP. Measurements of [³C]glutamate ⇌ glutamine and [⁵⁺]P₁ ⇌ ATP exchanges were carried out at 37°C with purified glutamine synthetase, E₁₀, at pH 6.50 ± 0.05.

**Feedback Modifier Effects at Equilibrium**—Fig. 1 presents the effects observed with increasing levels of AMP and GDP. Both nucleotides have similar and pronounced inhibitor effects: the P₁ ⇌ ATP exchange is suppressed, while the glutamate ⇌ glutamine exchange is depressed only slightly by high AMP concentrations and remains largely unchanged in the presence of GDP.

Reciprocal plots of these data indicate that the P₁ ⇌ ATP is completely inhibited at infinite modifier concentration in both cases. Estimated apparent Kᵢ values for inhibition of P₁ ⇌ ATP are 10 and 1.5 mM with AMP and GDP, respectively.

The effects of increasing concentrations of L-alanine upon the equilibrium exchanges is presented in Fig. 2A. Shown in Fig. 2B for these results is a plot of 1/i, where i is the fraction of inhibition of a given exchange, versus the reciprocal of modifier concentration. The data show complete suppression of both exchanges by added L-alanine, the glutamate ⇌ glutamine somewhat more strongly than the P₁ ⇌ ATP exchange. The plot of Fig. 2B emphasizes the clearly biphasic nature of inhibition of both exchanges. Such biphasic behavior had been observed in initial velocity data, which also indicated that L-alanine is mainly a Vᵢ₀ inhibitor (4, 5). For the data of Fig. 2, the estimated apparent Kᵢ for alanine at low alanine concentration is 5.0 and 20 mM for the glutamate ⇌ glutamine and P₁ ⇌ ATP exchanges, respectively. At higher alanine concentration, the estimated apparent Kᵢ values are 57 and 200 mM.

With CTP, a suppression of both exchanges occurs as shown in Fig. 3. Interestingly, after some irregular but reproducible initial effects at low CTP concentrations, the ratio of exchange rates remains nearly equal. For the range of CTP from 2 to 10 mM, reciprocal plots of 1/i versus 1/i indicate an apparent Kᵢ for CTP of 8 mM for inhibition of both the glutamate ⇌ glutamine and the P₁ ⇌ ATP exchanges.

Fig. 4 contrasts the effects observed with increasing glycine and L-histidine. With glycine both exchanges are strongly suppressed, however the ratio of the (glutamate ⇌ glutamine) to

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**Fig. 1.** The effects of AMP and GDP on glutamate ⇌ glutamine and P₁ ⇌ ATP exchange reactions at chemical equilibrium. Experimental conditions are described in the text.

**Fig. 2.** The effects of L-alanine in equilibrium exchanges.
(P₁ ↔ ATP) rates decrease with increasing glycine concentration. On the other hand, L-histidine suppresses the P₁ ↔ ATP exchange more strongly than the glutamate ↔ glutamine, and neither inhibition appears potentially complete from extrapolations to infinite L-histidine concentration. Reciprocal plots of these data are presented in Fig. 5; they contrast the strong and potentially complete inhibitory effects of glycine to the differential and partial effects observed with L-histidine. The y-intercept of the plot for L-histidine is near 2.0, indicating only about 50% over-all inhibition at infinite [L-histidine]. The apparent Kᵢ values for glycine estimated from the reciprocal plots are 20 and 40 mM for the glutamate ↔ glutamine and the P₁ ↔ ATP exchanges, respectively. Similar values for histidine are 10 and 67 mM.

Glucosamine 6-phosphate and L-tryptophan produce rather variable, weak, and incomplete inhibitions of both exchanges (Fig. 6). The irregular behavior at low modifier concentrations, perhaps indicative of multiple site or subunit interactions, or of dual roles for modifiers as activators and inhibitors, were quite accurately reproducible and thus are not the result of single experiment artifacts.
Finally, the responses of exchange rates to added carbamyl phosphate are somewhat mixed, as shown in Fig. 7. Both exchanges are suppressed evenly and strongly, however the P₁ ⇌ ATP exchange is 100% inhibited over-all but the glutamate ⇌ glutamine is only partially suppressed at high levels of modifier. The reciprocal plot also shows the slightly stronger differential inhibition of P₁ ⇌ ATP than glutamate ⇌ glutamine exchanges. The apparent Kᵢ values for carbamyl-P for the glutamate ⇌ glutamine and P₁ ⇌ ATP exchanges are 3 and 7 μM, respectively.

**Effects of AMP and GDP on Transferase and Partial Reactions** —The interesting differential effects observed with these modifiers (Fig. 1) were probed further. First, to determine whether the inhibition of P₁ ⇌ ATP exchange results from blocking dissociation of ATP, P₆, or both, an equilibrium exchange reaction with [¹⁸O]glutamate was carried out. Table I presents these results. The modifiers decrease the P₁ ⇌ ATP considerably with no inhibitory (and even slight stimulatory) effects on the [¹⁸O]glutamate ⇌ P₁ exchange. If the bound modifiers block dissociation of both P₁ and nucleotide, both P₁ and ATP and [¹⁸O]glutamate ⇌ P₁ should be suppressed. If only nucleotide dissociation is blocked, the [¹⁸O]glutamate ⇌ P₁ should be altered little. The latter is observed.

Second, to probe how these modifiers can differentially maintain glutamate ⇌ glutamine exchange, partial reaction systems were designed to test for transferase activity and for NH₃ ⇌ glutamate and glutamate ⇌ glutamine exchanges, in the presence and absence of AMP and GDP. Results with the transferase systems indicate that AMP very poorly substitutes for or mimics ADP (100% with the complete system). Somewhat similar results were obtained with the partial exchange reactions. The NH₃ ⇌ glutamate exchange responded very strongly to added GDP or ATP (30% above the level with the complete system); AMP alone had little effect but with added P₁ apparently can mimic the effects of GDP and the other substrates in the complete system quite well. The effects upon the glutamate ⇌ glutamine exchange were less dramatic but appreciable. GDP with or without added P₁ stimulated glutamate ⇌ glutamine to about 50 to 60% of maximal exchange activity; AMP alone had no effect, but AMP plus P₁ produced about 20% activity. These data must be interpreted cautiously, however, as it has been difficult to exclude possible slight contamination by ADP of the AMP and GDP stock solutions. If these modifiers can decrease the Kᵢ for ADP or ATP appreciably, small contaminations of ADP or ATP could effectively produce the complete system for exchange or reaction by the enzyme. Additional interpretations of these data are given under “Discussion” in relation to theoretical mechanisms of modifier action presented.

**DISCUSSION**

Our results show that equilibrium isotope exchange rate measurements can give considerable insight into feedback modifier action. One obvious feature of the data is that the modifiers apparently have distinctly different modes of action. The variety of control effects appears unusual. This may reflect different metabolic needs with a requirement for a variety of mechanisms to transmit the control action of many inhibitors to a single catalytic site. Before further discussion, it may be useful to summarize the effects of the modifiers in simple qualitative terms, without specifying details or interpreting the mode of action. Such a summary is given in Table II, where the relative “strength” of inhibition denotes a low apparent Kᵢ.

The interpretation of the feedback modifier effects depends on the catalytic mechanism: whether ordered or random binding occurs, whether partial reactions occur, and whether covalent intermediates with the enzyme are formed. As presented in the preceding paper, for the adenylylated form of glutamine synthetase, substrate binding is not ordered, no covalent intermediates or partial reactions can be detected, and any exchange depends upon the presence of all substrates. The latter is particularly important, as it means that if the binding of any single substrate to the catalytic site is blocked by a modifier, all exchanges will be inhibited. Such an inhibitory pattern will be observed, whether the velocity-substrate concentration relationships follow the simple Michaelis-Menten pattern or more complex patterns, e.g. cooperative effects. Understanding the type of relationship that may result, however, is aided by detailed knowledge of the enzyme catalyzed reaction pathway, which can be based on the usual Michaelis-Menten relationships as a first approximation.

Theoretical patterns of modifier action for a sequence involving two reactants and two products in a nonordered combination, with interconversion through ternary complexes, are summarized

**Table I**

**Effects of AMP and GDP on [¹⁸O] exchange between glutamate and P₁ catalyzed by glutamine synthetase**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Exchanges</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>GDP</td>
</tr>
<tr>
<td>P₁ ⇌ ATP</td>
<td>[¹⁸O]Glu ⇌ P₁ (¹⁸O) atom % excess in P₁</td>
</tr>
<tr>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

*For one oxygen transferred per micromole of P₁ ⇌ ATP exchanged this enrichment is calculated to be 0.035 atom per cent excess.

**Table II**

**Summary of modifier effects on equilibrium exchanges catalyzed by glutamine synthetase**

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Inhibition of</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁ ⇌ ATP</td>
<td>Gln ⇌ Gln</td>
</tr>
<tr>
<td>AMP</td>
<td>Strong, complete</td>
</tr>
<tr>
<td>GDP</td>
<td>Strong, complete</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>Strong, complete</td>
</tr>
<tr>
<td>CTP</td>
<td>Strong, complete</td>
</tr>
<tr>
<td>Glycine</td>
<td>Strong, complete</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>Moderate, partial</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>Weak, variable</td>
</tr>
<tr>
<td>Carbamyl-P</td>
<td>Strong, complete</td>
</tr>
</tbody>
</table>
Summary of modifier effects on equilibrium exchange kinetics

Values are calculated from Equations 3 and 4 with initial conditions as given in the text.

<table>
<thead>
<tr>
<th>Role of modifier</th>
<th>R and ( R' ) versus [M]</th>
<th>1/i and 1/i' versus 1/[M]*</th>
<th>y intercepts</th>
<th>Relative slopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E/R' )</td>
<td>As M ( \to ) ( \infty )</td>
<td>( 1/i )</td>
<td>( 1/i' )</td>
<td>( 1/i' &gt; 1/i )</td>
</tr>
<tr>
<td>( k/k' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (G)*</td>
<td>( 0.45x^2 )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 1.15x )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Decrease (G)</td>
<td>( 0.82x )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>( k )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (G)</td>
<td>( 1.4 )</td>
<td>( 0.2 R_0 )</td>
<td>( 0.2 R'_0 )</td>
<td>1.25</td>
</tr>
<tr>
<td>( k' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (G)</td>
<td>( 0.29x )</td>
<td>( 0.25 R_0 )</td>
<td>( 0.88 R'_0 )</td>
<td>1.34</td>
</tr>
<tr>
<td>( k'' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)*</td>
<td>( 0 )</td>
<td>0</td>
<td>0.73 ( R_0 )</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 3.90x )</td>
<td>( 0.87 R_0 )</td>
<td>( 0.22 R'_0 )</td>
<td>8.26</td>
</tr>
<tr>
<td>( k''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( \infty )</td>
<td>( 0.81 R_0 )</td>
<td>0</td>
<td>5.49</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 3.80x )</td>
<td>( 1.12 R_0 )</td>
<td>( 0.29 R'_0 )</td>
<td>1.66</td>
</tr>
<tr>
<td>( k''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( 0 )</td>
<td>0</td>
<td>1.01 ( R'_0 )</td>
<td>1.44</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 3.80x )</td>
<td>( 0.30 R_0 )</td>
<td>( 0.04 R'_0 )</td>
<td>&lt;0</td>
</tr>
<tr>
<td>( k'''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( \infty )</td>
<td>( 1.14 R_0 )</td>
<td>0</td>
<td>&lt;0</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 3.80x )</td>
<td>( 1.12 R_0 )</td>
<td>( 0.29 R'_0 )</td>
<td>&lt;0</td>
</tr>
<tr>
<td>( k''''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( 0 )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 1.60x )</td>
<td>( 0.61 R_0 )</td>
<td>( 0.40 R'_0 )</td>
<td>&lt;0</td>
</tr>
<tr>
<td>( k'''''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( 0 )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 1.60x )</td>
<td>( 0.61 R_0 )</td>
<td>( 0.40 R'_0 )</td>
<td>&lt;0</td>
</tr>
<tr>
<td>( k''''''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( 0 )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 1.60x )</td>
<td>( 0.61 R_0 )</td>
<td>( 0.40 R'_0 )</td>
<td>&lt;0</td>
</tr>
<tr>
<td>( k'''''''' )</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decrease (S)</td>
<td>( 0 )</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Increase (G)</td>
<td>( 1.60x )</td>
<td>( 0.61 R_0 )</td>
<td>( 0.40 R'_0 )</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

* 1/i = \( V_0/(V_0 - V) \) (cf. Fig. 5).
* G = gradually, S = sharply.
* 80% maximum inhibition of a given set of k's.
* x = value without modifier.
* Modifier effectively removes active enzyme.
* Cannot be distinguished from competitive modifiers.

A more exact representation, with appropriate rate constants, is shown by Equation 2.

\[
\begin{align*}
A & \xrightarrow{k_1} EA \\
B & \xrightarrow{k_2} EB \\
A + B & \xrightarrow{k_3} A + B \\
A + B & \xrightarrow{k_4} A + B
\end{align*}
\]

(2)

The typical modifier effects for both competitive and noncompetitive interactions may be assessed by use of the general equations expressing rates of exchange for the system of Equation 2 (7, 8). The more general equations for random substrate-binding order can be made quite specific, defining ordered binding sequences, according to the relative magnitudes of the individual rate con-
REACTION COORDINATE

Fig. 8. A typical reaction profile for a two-substrate, two-product, random system.

stants. For the scheme of Equation 2 above, the rate of A ⇌ P exchange is defined as R and equals

\[
R = E_i \left[ 1 + \frac{k'(k_{-s} + k_{s}Q)}{k_{-s}k_{s} + k_{-s}(k_{-s} + k_{s}Q)} \right] \left[ 1 + \frac{1}{k} \left( 1 + \frac{k_s}{k} + \frac{k_{s}K_{s}K_{-s}K_{-s}}{k_{-s}K_{-s}K_{s}K_{s}} \right) \right]
\]

and the rate of B ⇌ Q exchange is defined as \( R' \) and equals

\[
R' = E_i \left[ 1 + \frac{k'(k_{-s} + k_{s}P)}{k_{-s}k_{s} + k_{-s}(k_{-s} + k_{s}P)} \right] \left[ 1 + \frac{1}{k} \left( 1 + \frac{k_s}{k} + \frac{k_{s}K_{s}K_{-s}K_{-s}}{k_{-s}K_{-s}K_{s}K_{s}} \right) \right]
\]

For the equilibrium exchange kinetics discussed here, modifiers are distinguished as those which compete or do not compete with substrates, i.e., their effects are dependent or independent of substrate concentration. To describe the various effects that a modifier may exert on the enzyme and the catalytic reaction pathway, consideration of the reaction profile for a model system at equilibrium is helpful. For the random binding mechanism of Equation 2, assuming rate constants as given below, the reaction profile of Fig. 8 is quite reasonable. The alternative pathways allow binding of one reactant independent of the presence of the other. Thus the preferred order of substrate binding is kinetically determined.

Those modifiers which do not compete for substrate-binding sites directly can be considered to alter the reaction profile of Fig. 8 in some manner, either by raising the activation energy barrier between two intermediates or a set of intermediates. In this process, \( K_{eq} \) for the over-all reaction is not altered, \( \Delta G_{eq} \) is constant in order that the Haldane relationship, Equation 5, for this system, is not violated.

\[
K_{eq} = \frac{V_i K_A K_Q}{V_i K_A K_Q}
\]

For a basic system with unequal exchange rates (\( R \neq R' \)) and without rate-limiting covalent interconversion (\( k \) and \( k' \) not the only rate-limiting constants) the following conditions were chosen.

\[
E_i = 1
\]

\[
k = 10, k' = 5
\]

\[
k_1 = k_{-1} = k_4 = k_{-4} = k_6 = k_{-4} = k_7 = k_{-7} \cong 2
\]

\[
k_2 = k_{-2} = k_3 = k_{-3} = k_2 = k_{-2} = k_4 = k_{-4} \cong 1
\]

so that

\[
K_1 = K_3 = K_4 = K_6 = K_7 = K_1 = K_2 = K_4 \cong 1
\]

Also, because random binding order is operative,

\[
K_A = K_1 = K_3 = K_6 = K_7 - K_1
\]

\[
K_B = K_2 = K_1 = K_2 = K_4 = K_1
\]

Such assumptions reduce the equations for \( R \) and \( R' \) to forms that can be used readily to predict inhibition kinetics. Initial substrate levels are chosen as slightly above their \( "K_m values," \) as \( A = B = P = Q = 2 \). Under this set of conditions, one may calculate that

\[
R = 0.230
\]

\[
R' = 0.137
\]

\[
R/R' = 1.69
\]

Table III presents the various manners in which these rates, their ratio, and the percentage inhibition (i) of each varies with modifier levels, comparing the patterns obtained for various possible modifier roles. Reciprocal plots and the patterns obtained thereby provide additional diagnostic tests to distinguish among different cases. These patterns have been used for interpretation of the present experimental data for glutamine synthetase.

Perhaps the most striking result of the present study is the inhibitory pattern given by GDP. As noted in Fig. 1B, this modifier can completely block the Pi ⇌ ATP exchange with almost no effect on the glutamate ⇌ glutamine exchange. Such a pattern is similar to that illustrated in Table III, Cases 2e or 2f. The pattern is that anticipated for a modifier that markedly slows the dissociation, or slows both the dissociation and association reactions of one substrate pair. The slight initial increase in the glutamate ⇌ glutamine exchange could, by analogy with the patterns summarized in Table III, be indicative of preferential slowing of the dissociation reaction for Pi or ATP, or for ADP and ATP.

Most importantly for the GDP effect, although the Pi ⇌ ATP exchange is completely blocked at higher GDP concentrations, there must be bound Pi and ATP at the catalytic site to allow the unhindered glutamate ⇌ glutamine exchange to continue. An enzyme with Pi, ADP, or ATP release blocked cannot catalyze any net reaction, but the equilibrium measurements reveal a catalytic capacity hidden from initial velocity studies, con-
conveniently designated as a "cryptic catalysis." A possible mode of action of GDP is indicated in Fig. 9. The inhibition of the $P_i \rightleftharpoons ATP$ exchange by AMP and GDP could result from interference in the dissociation of either the ATP or $P_i$. The continuation of the $O_{\text{glutamate}} \rightleftharpoons O_{P_i}$ exchange at nearly the same rate (Table I) in the presence of the modifiers is not compatible with an inhibition of $P_i$ disassociation. Thus these modifiers likely interfere selectively with ATP dissociation. That $>1$ oxygen goes to glutamate for each $P_i \rightleftharpoons ATP$ exchanges argues that $P_i$ on-off is faster than ADP on-off, although this is not the only explanation.

Initial velocity studies have indicated that GDP is largely competitive with ADP or ATP and that AMP acts noncompetitively (4, 5). A competition of GDP with glutamate binding at the catalytic site is clearly not compatible with the continued glutamate $\rightleftharpoons$ glutamine exchange observed at high GDP concentrations (Fig. 1B). Additional nucleotide could overcome the GDP effect by displacing the GDP selectively from a binding site spatially distinct from the catalytic site. Thus ADP and ATP may bind to both a catalytic and to a control site. AMP may not be displaced similarly, thus accounting for its apparent noncompetitive relation with substrates under initial velocity conditions. The effect of AMP may be interpreted similarly to that of GDP, with a prominent feature being the cryptic catalysis.

However, the lowering of the glutamate $\rightleftharpoons$ glutamine observed at higher AMP concentration is suggestive of some competitive effect on the $P_i$ or ATP binding at equilibrium, as illustrated in Section 1 of Table III.

The complete inhibition of the exchanges by higher L-alanine concentrations, taken together with the initial velocity data indicating that alanine is largely a "$V_{max}$" inhibitor (5), would appear to be consistent with blocking of $k$ and $k'$ steps. However, for such an effect, there should be equal relative effects on both exchanges. Instead, as noted in Fig. 2B, the glutamate $\rightleftharpoons$ glutamine exchange is inhibited more strongly, with marked decrease in the ratio of the glutamate $\rightleftharpoons$ glutamine to $P_i \rightleftharpoons$ ATP exchanges as alanine concentration is increased. In terms of the analyses given in Table III, such behavior is consistent with alanine action as a competitive inhibitor of the glutamate $\rightleftharpoons$ glutamine exchange. The biphasic nature of the L-alanine inhibition, suggests that relationships other than a simple 1:1 mass action effect are operative. Multiple sites or interactions may exist. Alanine may noncompetitively block glutamate or glutamine association, which is difficult to distinguish from a competitive mode of action.

Among the inhibitors studied, only CTP gave equal relative inhibition of both exchanges so that the exchange ratios remained close to constant. In terms of the analyses given in Table III (e.g., Case 1a) this is consistent with action of CTP as a modifier that completely blocks substrate interconversions at $k$ and $k'$. Initial velocity data had indicated a partially competitive effect with glutamate. Were this the primary and exclusive role of CTP under the conditions used here, a preferential decrease in the glutamate $\rightleftharpoons$ glutamine exchange rate would have been anticipated. Thus, CTP effectively removes active enzyme from the reaction solution.

The inhibitory pattern of glycine could result largely from complete blocking of substrate interconversions, but the slightly greater effect of the glutamate $\rightleftharpoons$ glutamine exchange indicates that at least one other factor is operative. This would correspond to the effect expected if there was some competitive effect on the glutamate $\rightleftharpoons$ glutamine exchange. Initial velocity data indicate some competition between glycine and glutamate (5).

Perhaps the most interesting feature of the L-histidine effect is that it clearly cannot be a competitive inhibitor of $P_i \rightleftharpoons ATP$ and likely not of the glutamate $\rightleftharpoons$ glutamine exchange because of apparent lack of potentially complete inhibition as histidine concentration is increased. Partial effects on both exchanges not overcome by substrate are indicated, for example as in the patterns of Case 2c in Table III.

The pattern of carbamyl phosphate action is not the result of inhibition of substrate interconversion, but could be taken as suggestive of a competitive effect on the $P_i \rightleftharpoons$ ATP exchange. However, if as indicated in Fig. 7, nearly complete inhibition of the $P_i \rightleftharpoons$ ATP but incomplete inhibition of the glutamate $\rightleftharpoons$ glutamine exchange results, a competition for the binding site of either $P_i$ or ATP at the catalytic site is ruled out. Some residual catalysis appears to remain, implying that some $P_i$ and ATP remain bound so as to allow substrate interconversions to occur. Thus a mechanism as in Case 2b of Table III is possible although mixed effects are likely operative. Residual activity by unadenylated subunits less sensitive to bound modifier may also explain these effects.

With both glucosamine-6-P and L-tryptophan, the small and somewhat erratic patterns noted do not lend themselves to simple interpretations. It should be mentioned, however, that although over-all only minor effects on the $P_i \rightleftharpoons$ ATP and glutamate $\rightleftharpoons$ glutamine exchanges were observed, the modifiers might still have considerable effect such as on the initial velocity of associations and dissociations of other substrates not tested in these experiments, namely $NH_3$ and ADP. The cooperative binding of L-tryptophan noted elsewhere via other techniques (5, 9) is suggested in Fig. 7, especially in the glutamate $\rightleftharpoons$ glutamine exchange. The variable but reproducible responses to glucosamine-6-P, L-tryptophan, and to some extent with AMP and CTP, at low modifier levels have been observed also in a somewhat similar fashion with L-alanine under initial velocity conditions (5).

The measurements of exchange rates reported in this paper obviously reflect only a few of those that could be made to probe the effect of each modifier. Other exchange measurements could include $NH_3 \rightleftharpoons$ glutamine, $ADP \rightleftharpoons$ ATP, and several $O$ exchanges in addition to the $O_{\text{glutamate}} \rightleftharpoons O_{P_i}$, namely $O_{\text{glutamine}} \rightleftharpoons O_{\text{ATP}}, O_{\text{ATP}} \rightleftharpoons O_{P_i},$ $O_{\text{glutamine}}$ and $O_{\text{glutamine}} \rightleftharpoons O_{\text{ATP}}$. Variation in substrate levels
not used in the present studies, may give an important additional means of distinguishing between modifier modes of action.

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Action Patterns of Feedback Modifiers on Equilibrium Exchanges and Applications to Glutamine Synthetase (Escherichia coli W)

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