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Regulation of Glutamine Synthetase

VI. Interactions of Inhibitors for *Bacillus licheniformis* Glutamine Synthetase

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The relationships of five feedback inhibitors for the *Bacillus licheniformis* glutamine synthetase were investigated. The inhibitors were distinguishable by differences in their competitive relationship for the substrates of the enzyme. Mixtures of L-glutamine and adenosine-5'-monophosphate (AMP) or histidine and AMP caused synergistic inhibition of glutamine synthesis. Histidine, alanine, and glycine acted antagonistically toward the L-glutamine inhibition. Alanine acted antagonistically toward the glycine and histidine inhibitions. Independence of inhibitory action was observed with the other pairs of effectors. Possible mechanisms by which the inhibitors may interact to control glutamine synthesis are discussed. The low rate of catalysis of the glutamyl transfer reaction by the *B. licheniformis* glutamine synthetase can be attributed to the fact that L-glutamine serves both as a substrate and an inhibitor for the enzyme. Effectors which act antagonistically toward the L-glutamine inhibition stimulated glutamotransferase activity. The stimulation was not observed when D-glutamine was used as substrate for the glutamyl transfer reaction.

Previous investigations (2, 9) showed that microbial glutamine synthetases can be effectively controlled when several end products of glutamine metabolism are simultaneously present. The glutamine synthetase of *Escherichia coli* can be partially inhibited by any one of eight end products. These eight inhibitors appear to be independent in their action on the *E. coli* enzyme. With the glutamine synthetases of *Bacillus cereus* and *B. licheniformis*, certain inhibitors appear to act independently, and others act synergistically or antagonistically toward each other. This study was undertaken to characterize the interactions of the inhibitors for the *B. licheniformis* enzyme and to determine how such interactions might function in the overall regulatory process.

MATERIALS AND METHODS

Enzyme assays. B. licheniformis glutamine synthetase was prepared as described in the accompanying paper (3). Activity of the enzyme was measured by following either the production of orthophosphate (Pi) in the biosynthetic reaction or the formation of γ-glutamylhydroxamate in the glutamyl transfer reaction (2).

The standard reaction mixture for the biosynthetic assay contained 7 mM $MnCl_2$ and 22 mM imidazole-chloride buffer in a final volume of 0.6 ml at a final *p*H of 7.0. The variable concentrations of glutamate, NH_4Cl, and adenosine triphosphate (ATP) are given in the figures and tables. Reactions were initiated by addition of enzyme and were incubated in open tubes at 37 C. Proportionality of Pi liberation to enzyme concentration is observed up to 0.25 μmole of Pi per 15 min of incubation. Controls lacking glutamate were included in each incubation, and corrections were made for the small amounts of Pi liberated from ATP in the absence of substrate.

The standard reaction mixture for the glutamyl transfer assay contained 20 mM potassium arsenate, 3 mM $MnCl_2$, 60 mM NH_2OH HCl (neutralized with KOH), and 21 mM imidazole-chloride buffer in a final volume of 1.0 ml at final *p*H of 7.0. The variable concentrations of glutamine and adenosine diphosphate (ADP) are given in the figures and tables. Reactions were initiated by addition of enzyme and were incubated in open tubes at 37 C. Glutamylhydroxamate formation is proportional to enzyme concentration in the range of 0 to 3 μmoles per 30 min of incubation.

Inhibition studies. Compounds tested as inhibitors were added to the reaction mixtures prior to the introduction of enzyme. Controls for each inhibitor were included in the experiments with both the biosynthetic and glutamyl transfer assays. With the biosynthetic assay, the control tube contained the inhibitor but lacked glutamate. With the glutamyl transfer assay, the inhibitor was added and glutamine was omitted. The results of inhibition analyses are expressed as per cent initial activity, i.e., 100 times

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the fractional activity observed in the presence of inhibitor.

Chemicals. Adenosine-5′-monophosphate (AMP) and carbamylphosphate (carbamyl-P; dilithium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals were obtained as previously described (2, 3), or were reagent grade.

RESULTS

Inhibition of the biosynthetic reaction. Figure 1 shows the inhibitory response of the *B. licheniformis* glutamine synthetase to five potential end products of glutamine metabolism. Over the concentration range tested, L-glutamine and AMP were the most effective; each caused inhibition of greater than 90%. Limited degrees of inhibition were obtained with saturating concentrations of L-alanine, glycine, and L-histidine. Carbamylphosphate (carbamyl-P) also caused slight inhibition (Table 1), but this compound was not studied in detail since the Pi formed by its spontaneous decomposition interfered with the biosynthetic assay. Other experiments showed that some analogues of L-alanine (i.e., β-alanine, D-alanine, and L-serine) are also inhibitory, but less so than L-alanine or glycine. The inhibition caused by 5′-AMP is relatively specific since neither 3′-AMP nor 5′-guanosine-monophosphate (GMP) is an inhibitor of the enzyme. As

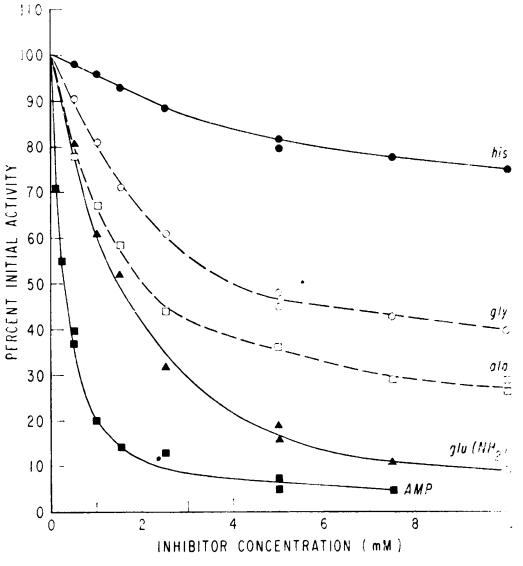

FIG. 1. *Inhibition of Bacillus licheniformis glutamine synthetase. The standard biosynthetic assay was used with 7.5 mM L-glutamate, 7.5 mM NH_4Cl, 7.5 mM ATP, and inhibitor (as indicated). Abbreviations: his = L-histidine, gly = glycine, ala = L-alanine, glu(NH_2) = L-glutamine, and AMP = adenosine-5′-monophosphate.*

TABLE 1. *Effects of various inhibitors on the biosynthetic reaction with L- and D-glutamate as substrate*[a]

Inhibitor	Per cent initial activity	
	L-Glutamate	D-Glutamate
L-Glutamine	13	14
D-Glutamine	100	98
L-Alanine	37	26
Glycine	48	51
L-Histidine	83	72
AMP	20	21
Carbamyl-P	86	

[a] ATP and D- and L-glutamate were present at 7.5 mM. The concentrations of all inhibitors was 5 mM, except AMP (1 mM). The concentration of NH_4Cl was 7.5 mM in the experiments with L-glutamate and 50 mM with D-glutamate. Otherwise, the standard biosynthetic assay was used.

reported earlier (3), cytidine triphosphate (CTP) and other nucleotide triphosphates may either activate or inhibit the enzyme, depending upon the relative concentrations of ATP and Mn^{++} present.

Other potential end products of glutamine metabolism, including glucosamine-6-phosphate, uridine-5′-diphosphate-*N*-acetylglucosamine, anthranilic acid, L-tryptophan, nicotinamide adenine dinucleotide, and *p*-aminobenzoic acid, did not inhibit the glutamine synthetase when tested at 5 mM concentrations; nor did various other amino acids such as L-isoleucine, L-methionine, and L-phenylalanine.

The limited degrees of inhibition obtained with saturating concentrations of L-alanine, glycine, and L-histidine are similar to the effects of these compounds and other products of glutamine metabolism on the activity of *E. coli* glutamine synthetase; the latter enzyme is subject to partial inhibition by saturating concentrations of any one of eight separate end products (9). On the other hand, the *B. licheniformis* enzyme is very susceptible to inhibition by glutamine, whereas the *E. coli* enzyme is not.

Since glutamine is the immediate product of the biosynthetic reaction, the inhibition by glutamine could be a simple case of product inhibition. However, this possibility is contraindicated by the results of studies on the effects of the D and L isomers of glutamate and glutamine. Both D- and L-glutamate can serve as substrates for the biosynthetic reaction, and presumably give as products D- and L-glutamine, respectively (4). This assumption is supported by the fact that both D- and L-glutamine are substrates in

the glutamyl transfer reaction. In view of these observations, it appears significant that L-glutamine is an effective inhibitor of the biosynthetic reaction when either L- or D-glutamate is the substrate; yet D-glutamine is not an inhibitor of the reactions with either substrate (Table 1). These results suggest that the inhibitory action of L-glutamine is not attributable to its direct competition with glutamate at the catalytic site as would be expected in the case of simple product inhibition. If this were true, then D-glutamate, which may also be a product of the biosynthetic reaction as well as a substrate for the reverse (glutamyl transfer) reaction, should also be an inhibitor. From these considerations, it appears that L-glutamine inhibits by virtue of its reaction at a site on the enzyme other than the catalytic site, i.e., at an allosteric site that is specific for L-glutamine and which cannot be occupied by D-glutamine.

Kinetics of inhibition. To determine the nature of the inhibitory effects of the five feedback inhibitors, double reciprocal plots of the reaction velocities versus substrate concentrations were made for data obtained in the presence and absence of each of the various inhibitors. In general, such kinetic analyses are difficult to interpret since the double reciprocal plots do not yield straight lines. Thus, a plot (Fig. 2B) of the data obtained for glutamate in the absence of any inhibitor yields a bimodal curve, with one linear segment representing the data obtained at the higher range of glutamate concentrations. This segment intersects the ordinate and, when extrapolated to the abscissa, yields an apparent K_m of about 7.7 mM. On the assumption that this linear segment represents the binding of glutamate to a particular site, it would appear that histidine and glutamine are competitive inhibitors with respect to glutamate for this site; the straight line portions of the double reciprocal plots of data obtained in the presence and absence of these two inhibitors converge at the ordinate (Fig. 2B). By the same reasoning, AMP, glycine, and alanine show mixed competitive, noncompetitive relationships with respect to glutamate (Fig. 2A). Although the double reciprocal plots suggest the existence of a competitive relationship between glutamine and glutamate, this is not necessarily in contradiction to the conclusion discussed above that glutamine is an allosteric inhibitor rather than an immediate end product inhibitor. It is well established that many feedback inhibitors exhibit a competitive type of kinetics with respect to specific substrates, even when they are bound to sites other than the substrate binding site (8). In such instances, it is assumed that reaction of the inhibitor at its specific allosteric site induces a conformational

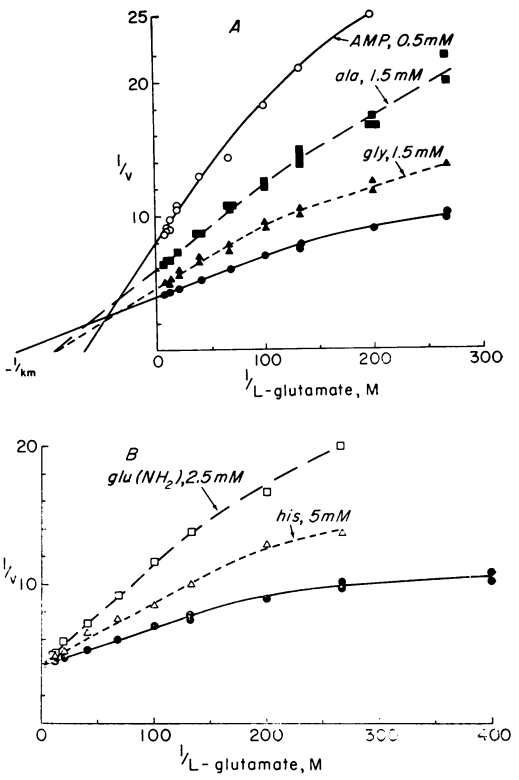

FIG. 2. *Double reciprocal plots of initial velocity against glutamate concentration in the presence and absence of inhibitors. The standard biosynthetic assay was used with 50 mM NH_4Cl, 7.5 mM ATP, glutamine synthetase (27 μg of protein), glutamate (solid circles), and inhibitors (as indicated). V = millimicromoles of Pi formed per 7.5 min. See legend to Fig. 1 for other abbreviations.*

change in protein structure that in effect reduces the apparent affinity of the enzyme for its substrate at the catalytic site, without altering the V_{max} of the enzyme.

Differential responses of the various inhibitors to the different substrates is further indicated by the data in Table 2, which summarize the effects of each inhibitor when just one or two of the three substrates is present at less than saturating concentrations. The data show that the inhibitions by glycine, histidine, AMP, and glutamine were more pronounced when both glutamate and ammonia were present at low concentrations. Alanine was unique in that it was less inhibitory at low concentrations of ammonia. The inhibition by either alanine or glycine was less pronounced when the ATP concentration was lowered, whereas the reverse was true for glutamine and AMP, and the effect of histidine was independent of the ATP concentration. Thus, no two of the

TABLE 2. *Reversal of the glutamine synthetase inhibition with excess substrate*

Inhibitor	Per cent initial activity with different substrates limiting[a]				
	No substrate limiting (A)	Low glutamate (B)	Low NH_4Cl (C)	Low NH_4Cl and glutamate(D)	Low ATP (E)
Alanine.....	49	36	54	35	66
Glycine.....	69	58	65	47	80
Histidine....	96	93	92	81	96
L-Glutamine	77	49	65	18	65
AMP.....	53	41	41	25	29

[a] Standard biosynthetic assay with: (A) 100 mM L-glutamate, 50 mM NH_4Cl, and 7.5 mM ATP; (B) 7.5 mM L-glutamate, 50 mM NH_4Cl, and 7.5 mM ATP; (C) 100 mM L-glutamate, 7.5 mM NH_4Cl, and 7.5 mM ATP; (D) 7.5 mM L-glutamate, 7.5 mM NH_4Cl, and 7.5 mM ATP; and (E) 100 mM L-glutamate, 50 mM NH_4Cl, and 2.5 mM ATP. The concentrations of inhibitors were 5 mM except for AMP (1 mM).

five inhibitors responded in an identical fashion to variations in the concentrations of all three substrates. Apparent similarity in behavior of AMP and glutamine as indicated by the data of Table 2 is not supported by the data of Fig. 2 which shows that the two inhibitors are distinguishable on the basis of the kinetics of their inhibitory effects. These results suggest that the five inhibitors exert their effects by interacting differently with the enzyme. Although other possibilities are not excluded, the simplest interpretation is that they react at separate sites on the enzyme.

Inhibition with pairs of inhibitors. If any two substances are completely independent in their action, the presence of one will not influence the capacity of the other to inhibit the enzyme. In other words, the residual enzyme activity that remains in the presence of one added compound will be susceptible to further inhibition by the second compound to the same fractional extent as is observed by the second when it acts alone on uninhibited enzyme.

To determine whether the various inhibitors are independent in their action, the capacity of each substance to inhibit the enzyme was measured in the presence and absence of 5 mM concentrations of each of the other four inhibitors. To simplify presentation of the data, the residual activity with 5 mM concentrations of each single compound was arbitrarily set equal to 1.0. The fraction of this residual activity that remained when each of these partially inhibited enzyme systems was further supplemented with varying concentrations of a second inhibitor was then determined. When tested in this manner, it is evident that, if the second inhibitor acts completely independently of the first inhibitor, then the fractional inhibition caused by any concentration of the second will be the same in the presence and absence of the first inhibitor. Thus, the curves obtained by plotting the fractional activity, V/V_0, against the concentration of the second inhibitor, in the presence and absence of the first inhibitor, will be superimposable. As can be seen from the data plotted in Fig. 3, by these criteria, alanine and glycine appear to act completely independently of AMP. In contrast, the presence of glutamine or histidine renders AMP more effective as an inhibitor. Thus, in the absence of glutamine or histidine, 50% inhibition ($V/V_0 = 0.5$) was obtained with 0.73 mM AMP. However, in the presence of 5 mM concentrations of glutamine or histidine, 50% inhibition of the residual activities was obtained with 0.055 or 0.31 mM concentrations of AMP, respectively. These results show that AMP and glutamine or AMP and histidine are synergistic feedback inhibitors for the glutamine synthetase. The synergism was not observed when D-glutamine, L-asparagine, or L-isoglutamine was substituted for L-glutamine. Also, neither 3'-AMP nor 5'-GMP would substitute for 5'-AMP as a co-effector to act synergistically with glutamine or histidine.

In comparable studies, the extent to which glutamine acts independently of alanine, glycine,

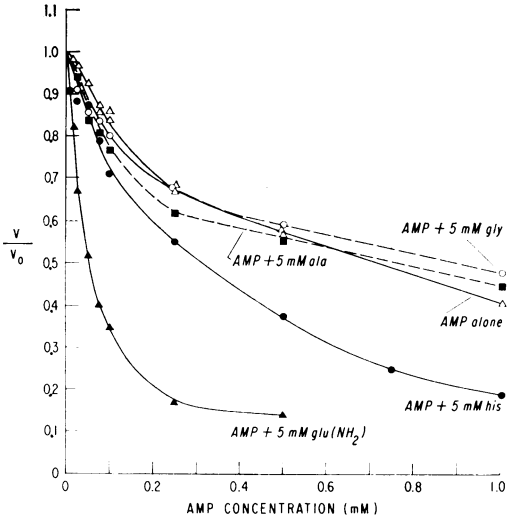

FIG. 3. *Inhibition of glutamine synthetase activity by AMP in the presence and absence of other inhibitors. The standard biosynthetic assay mixture was used with 7.5 mM glutamate, 50 mM NH_4Cl, 7.5 mM ATP, and inhibitors (as indicated).*

and histidine was determined. The fractional inhibition produced by glutamine in the presence of any of the latter three amino acids was significantly less than that observed when glutamine was tested alone (Fig. 4). A probable explanation for this antagonism is that the reaction of alanine, glycine, or histidine at their specific inhibitor sites in some way interferes with the reaction of glutamine at its site.

A pronounced antagonism exists also between alanine and glycine, and histidine appears to be slightly antagonistic with alanine (Fig. 5). In contrast, glycine and histidine appeared to act independently (Fig. 6). Thus, these data illustrate three forms of interaction between the 10 possible pairs of inhibitors: pairs which act independently of each other, pairs which act synergistically with each other, and pairs which act antagonistically toward each other.

Inhibition of the glutamotransferase activity. Compounds were tested as inhibitors of the glutamyl transfer reaction catalyzed by *B. licheniformis* glutamine synthetase (Fig. 7). In some cases, these results differ qualitatively from those obtained with the biosynthetic reaction. For example, 5 mM histidine caused a marked stimulation in glutamotransferase activity in contrast to its marked inhibitory effect on the biosynthetic reaction (Fig. 1). Alanine and glycine which were potent inhibitors of the biosynthetic reaction caused only a slight inhibition of the glutamyl

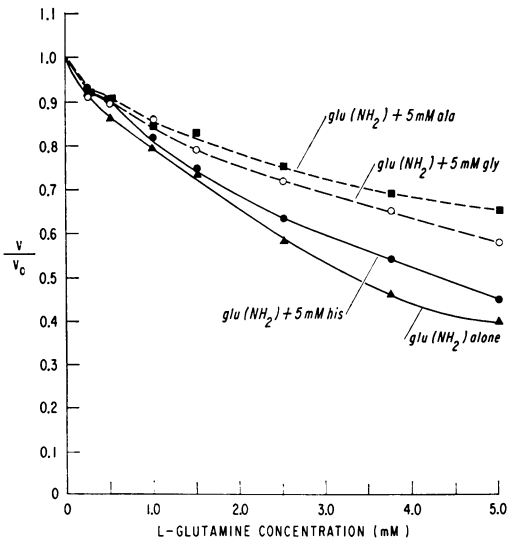

FIG. 4. *Inhibition of glutamine synthetase activity by glutamine in the presence and absence of other inhibitors. The standard biosynthetic assay mixture was used with 7.5 mM L-glutamate, 50 mM NH_4Cl, 7.5 mM ATP, and inhibitors (as indicated).*

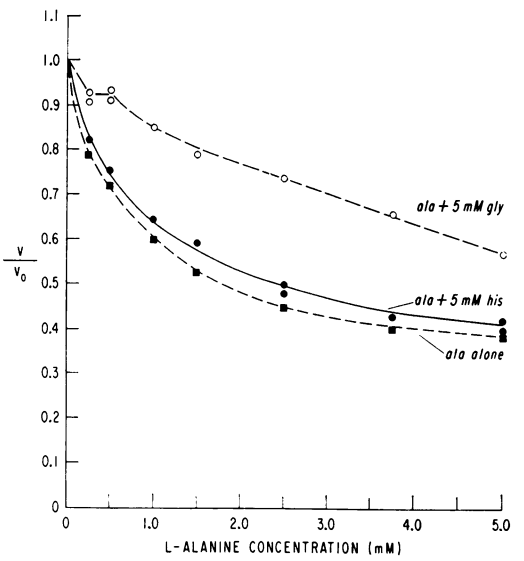

FIG. 5. *Inhibition of glutamine synthetase activity by alanine in the presence and absence of other inhibitors. The standard biosynthetic assay mixture was used with 7.5 mM L-glutamate, 50 mM NH_4Cl, 7.5 mM ATP, and inhibitors (as indicated).*

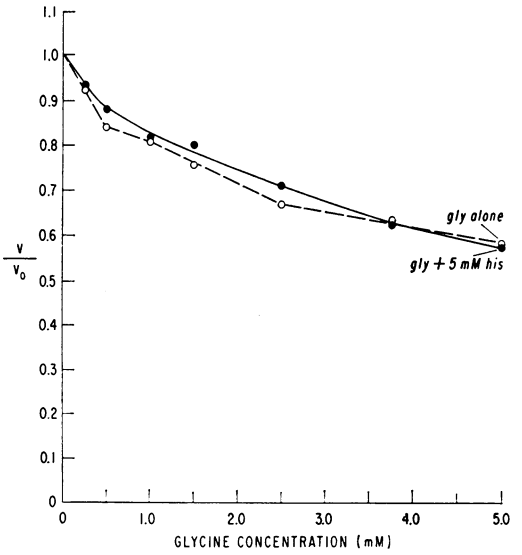

FIG. 6. *Inhibition of glutamine synthetase activity by glycine in the presence and absence of histidine. The standard biosynthetic assay mixture was used with 7.5 mM L-glutamate, 50 mM NH_4Cl, 7.5 mM ATP, and inhibitors (as indicated).*

transfer reaction. On the other hand, AMP served as an effective inhibitor of both reactions. Noteworthy is the fact that a slight stimulation in activity was caused by 10 mM glutamate. Table 3

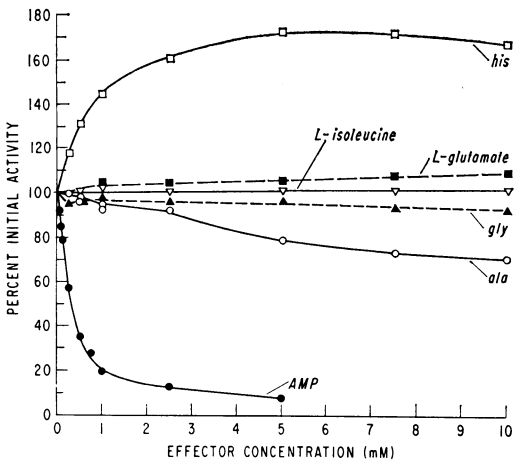

FIG. 7. *Effects of the inhibitors on the glutamyl transfer reaction catalyzed by the Bacillus licheniformis glutamine synthetase. The standard glutamyl transfer reaction mixture was used with 30 mM L-glutamine, 2 mM ADP, and effectors (as indicated).*

shows that extremely high levels of glutamate (600 mM) caused a marked stimulation of glutamotransferase activity. These effects appear to be specific since comparable concentrations of isoleucine did not appreciably stimulate or inhibit either the biosynthetic or the glutamyl transfer reactions.

The stimulation of hydroxamate formation by histidine and glutamate is apparently attributable to glutamine synthetase activity, since there was no detectable reaction when glutamate or histidine was tested in the absence of ADP, arsenate, or glutamine (Table 3). Therefore, these effectors stimulated the production of γ-glutamylhydroxamate from glutamine. This conclusion is also supported by the finding that glutamylhydroxamate was the only hydroxamate spot detected on thin layer chromatograms (3) of reaction mixtures containing glutamine, a mixture of glutamine and glutamate, or a mixture of glutamine and histidine.

The unusual response of the glutamotransferase activity of the *B. licheniformis* enzyme to various effectors may be related to the fact that, unlike the enzymes from other sources, glutamine serves both as a substrate and an inhibitor for the enzyme. As noted earlier, this dual role of glutamine is best explained by the assumption that the *B. licheniformis* enzyme possesses a specific allosteric inhibitor site as well as a catalytic site for glutamine. This is supported by the fact that L-glutamine but not D-glutamine is an inhibitor of the biosynthetic reaction (Table 1), whereas both isomers are substrates for the

TABLE 3. *Effect of glutamate and related compounds on the glutamyl transfer reaction catalyzed by Bacillus licheniformis glutamine synthetase*

Changes in standard assay mixture[a]		Glutamyl-hydroxamate formed per 30 min
Added	Omitted	
		mmoles
		1.53
	ADP or arsenate	0
L-Glutamate, 600 mM		11.9[b]
L-Glutamate, 50 mM	Glutamine or ADP or arsenate	0
L-Histidine, 5 mM		3.11
L-Histidine, 5 mM	Glutamine or ADP or arsenate	0
D-Glutamate, 50 mM		2.35
D-Glutamate, 50 mM	Glutamine or ADP	0
L-Aspartate, 50 mM		2.64
L-Aspartate, 50 mM	Glutamine or ADP	0
L-Asparagine, 50 mM		2.10
L-Asparagine, 50 mM	Glutamine or ADP	0
L-Isoglutamine, 50 mM		1.53
L-Isoglutamine, 50 mM	Glutamine	0.02

[a] Standard glutamyl transfer reaction mixture with 30 mM L-glutamine, 2 mM ADP, and glutamine synthetase (0.15 mg of protein).

[b] Calculated from determination made for a shorter incubation period.

transfer reaction (Table 4). If this view is correct, it follows that the glutamotransferase activity is always partially inhibited when L-glutamine is the substrate. Such inhibition may account for the relatively low glutamotransferase activity of the *B. licheniformis* enzyme as compared to the enzyme derived from eight other microorganisms studied (2). Many of the unusual effects of various compounds on the glutamyl transfer reaction could be explained if the *B. licheniformis* enzyme is partially inhibited by L-glutamine under the standard transfer assay conditions. Then, the inherent capacity of certain ligands to cause partial inhibition of the enzyme, as observed in the biosynthetic reaction, might, in case of the transferase activity, be more or less offset by the activation which results from their antagonistic action against the relatively stronger L-glutamine inhibition. For example, the activation of transferase activity by histidine (Fig. 7) could be a

TABLE 4. *Effects of the inhibitors on the glutamyl transfer reaction with* L- *and* D-*glutamine as substrates*[a]

Effector	Per cent initial activity	
	L-Glutamine	D-Glutamine
None	100	100
Alanine	80	13
Glycine	94	52
Histidine	164	79
AMP	59	84
L-Glutamate	156	61

[a] Concentrations were as follows: L- and D-glutamine, 30 mM; ADP, 2 mM; alanine, glycine, and histidine, 5 mM; AMP, 0.25 mM; and L-glutamate, 50 mM. Otherwise the standard glutamyl transfer conditions were used.

manifestation of the antagonism that exists in the interactions of histidine and L-glutamine (Fig. 4); i.e., activation could be due to the alleviation of a strong L-glutamine inhibition by the less effective inhibitor histidine. Unfortunately, a quantitative evaluation of these effects is not possible since the conditions for measuring the transferase activity are not the same as those used in the biosynthetic assay. However, by the same reasoning, the very strong inhibitory effect of AMP in the transferase reaction could be an expression of the synergistic action of AMP and L-glutamine in inhibiting the enzyme (Fig. 3). Moreover, the slight inhibition of the glutamotransferase activity caused by alanine and glycine could reflect a balance of their inhibitory roles and their ability to act antagonistically toward the L-glutamine inhibition (Fig. 4).

If the atypical effects of various ligands on the transferase activity are related to their antagonistic and synergistic actions with respect to L-glutamine inhibition, then more normal responses to this ligand should be obtained when D-glutamine replaces L-glutamine as a substrate in the transfer assay. This follows from the fact that, in contrast to L-glutamine, D-glutamine is not an inhibitor of the enzyme (Table 1). The data in Table 4 show that a normal inhibition pattern is obtained when D-glutamine is the substrate for the transferase reaction. Thus, histidine, alanine, and glycine, which either stimulate the reaction or cause only slight inhibition when L-glutamine is the substrate, all cause significant inhibition of the reaction when D-glutamate is the substrate. On the other hand, AMP is a less effective inhibitor when D-glutamine is the substrate.

Effects of glutamate. The effects of glutamate are complicated and deserve special consideration. Glutamate stimulates the glutamyl transfer reaction when L-glutamine is the substrate, but it inhibits the reaction when D-glutamine is the substrate (Tables 3 and 4). Stimulation of the reaction with L-glutamine occurs also with various glutamate analogues including D-glutamate, L-aspartate, and L-asparagine; however, isoglutamine, the only compound tested with a substituent on the α-carboxyl group, is without effect (Table 3). Glutamate inhibition of the reaction with D-glutamine could be due simply to competition by these two compounds for the catalytic substrate binding site. The ability of an excess of glutamate to reverse partially the L-glutamine inhibition of the biosynthetic reaction, as noted previously (Table 2), could also be explained by direct competition of these compounds at the catalytic site. However, these interpretations do not account for the fact that glutamate and its catalytically inactive analogues (Table 3) stimulate the transfer reaction when L-glutamine is the substrate. Among other possibilities, the stimulatory effects of these compounds would be explained if they compete with L-glutamine for binding at its allosteric inhibitor site, either directly or as a consequence of induced conformational changes caused by their interaction at a second allosteric (activating) site.

Variations with different enzyme preparations. For reasons that are not yet understood, the glutamotransferase activities of different preparations of *B. licheniformis* respond differently to the presence of various effectors. Thus, with the enzyme preparation of Fig. 7, alanine and glycine cause only slight inhibition of the transferase activity; however, with another preparation of the synthetase obtained from a different batch of cells, alanine and glycine actually stimulated the glutamotransferase activity (Fig. 8). With still a third preparation (not shown), a stimulation of glutamotransferase activity was achieved with 5 mM glycine, but not with 5 mM alanine. It is noteworthy that glutamate stimulated and AMP inhibited the glutamyl transfer reaction of all three enzyme preparations, whereas isoleucine was essentially without effect.

Although the basis of this differential behavior is not understood, it appears significant that comparable effects have been observed with the glutamine synthetase from *E. coli* and have been shown to reflect differences in the cultural conditions. Thus, the nature and extent of the effects of individual ligands on the *E. coli* enzyme is dependent upon the age of the culture, the nitrogen source, and the degree of aeration during growth (Kingdon and Stadtman, *unpublished data*). In view of these results, it appears possible

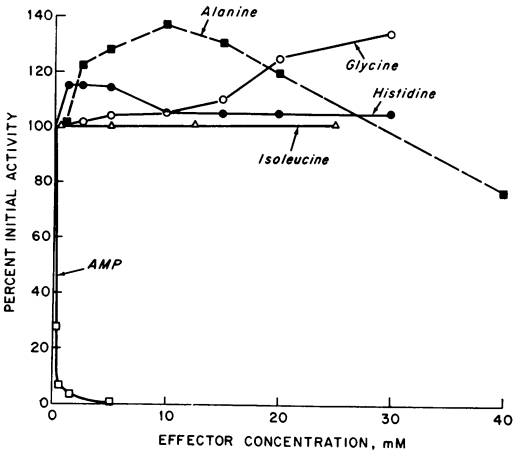

FIG. 8. *Effects of the inhibitors on the glutamyl transfer reaction catalyzed by a second preparation of Bacillus licheniformis glutamine synthetase. The standard glutamyl transfer reaction mixture was used with 30 mM L-glutamine, 0.4 mM ADP, and effectors (as indicated).*

that variations in the cultural conditions (e.g., in the age of the cultures) used in the present study may be responsible for the different behaviors of the various enzyme preparations.

DISCUSSION

The present study shows that the glutamine synthetase of *B. licheniformis* is inhibited by a number of potential end products of glutamine metabolism. As with the *E. coli* enzyme, some of these compounds appear to act independently of each other and collectively produce cumulative effects. However, in contrast to the *E. coli* enzyme, this basic pattern of cumulative feedback inhibition is modified by a substantial interaction between certain pairs of inhibitors as manifested by marked degrees of synergism or antagonism in their combined effects. Figure 9 summarizes some of the properties of the *B. licheniformis* glutamine synthetase that are disclosed by the present study. This figure is not intended to represent an enzyme model but merely illustrates some of the interactions that occur between various ligands. The available data suggest that the enzyme possesses a separate binding site for each of the five metabolic inhibitors, histidine, AMP, L-glutamine, alanine, and glycine. In addition, there are probably separate binding sites for each substrate, D- or L-glutamate, ATP, and NH_4^+; these substrate sites are probably shared by D- or L-glutamine, ADP, and hydroxylamine, respectively. Synergistic effects are exerted by combinations of histidine and AMP or by AMP and L-glutamine (Fig. 9), whereas antagonism exists between histidine and alanine, L-glutamine and glycine, alanine and glycine, alanine and glutamine, and histidine and glutamine. Obviously, it is possible to account for all of the observed ligand effects by appropriate assumptions as to the nature and number of binding sites for each ligand, and as to the direction of influences resulting from the interactions of these sites. Many of the antagonistic and synergistic effects could also be readily explained by the allosteric transition hypothesis of Monod, Wyman, and Changeaux (5), especially as it has been amplified to include nonrestrictive binding of ligands to both allosteric forms of the enzyme (7). On the other hand, without further modification, it would appear difficult for this hypothesis to account for some of the observations; for example, alanine and glycine both antagonize the effect of L-glutamine and are at the same time antagonistic to each other, yet alanine but not glycine is antagonistic with respect to histidine. Similarly, strong synergism exists between histidine and AMP and between AMP and L-glutamine, yet histidine and L-glutamine act antagonistically toward each other.

On basis of their inhibitory capacities, AMP and L-glutamine appear to be potentially the most important compounds in the regulation of glutamine synthetase activity in *B. licheniformis*. Low concentrations of either compound cause substantial inhibition of the enzyme and at saturating concentrations each causes nearly complete inhibition. Moreover, these two compounds are synergistic in their action, so that together they are very much more inhibitory than would be expected from their independent inhibitory capacities. Their combined action results from the fact that the apparent affinity of the enzyme for AMP is increased by the presence of L-glutamine. This is clearly illustrated in Fig. 3; 0.7 mM AMP is required for 50% expression of its inhibitory potential in the absence of L-glutamine, but, in the presence of 5 mM L-glutamine, only

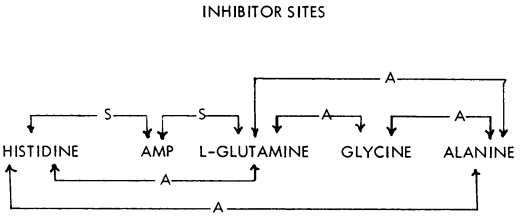

FIG. 9. *Inhibitors of Bacillus licheniformis glutamine synthetase. S, pair of inhibitors act synergistically. A, pair of inhibitors act antagonistically. Pairs not indicated are independent in their action.*

0.05 mM AMP is required. A similar but less pronounced increase in affinity of AMP for the enzyme is apparently the basis of the synergistic action of AMP and histidine. These synergistic effects are not restricted to *B. licheniformis*; other studies show that the glutamine synthetases of *B. cereus* (2) and *B. subtilis* (Hubbard and Stadtman, *unpublished data*) are synergistically inhibited by mixtures of AMP and histidine or of AMP and L-glutamine.

It appears significant from the standpoint of cellular regulation that the synergistic inhibitions of AMP and either glutamine or histidine are partially counteracted by high levels of substrates, e.g., glutamate, NH_4^+, or ATP.

These observations point to the existence of a delicately balanced mechanism for the regulation of glutamine metabolism in which AMP plays the dominant role. Its inhibitory influence is accentuated by histidine and L-glutamine on the one hand, and is restrained by substrates, including ATP, on the other. In particular, the appositive actions of ATP and AMP, whose concentrations must vary in a reciprocal manner, form the basis of effective regulation which is directly linked to the energy metabolism of the organism. The importance of the ratio of ATP to AMP in the cellular regulation of other metabolic processes has been emphasized by Atkinson (1). The regulation of glutamine synthetase by AMP is supplemented by the interactions of alanine and glycine which can independently cause partial inhibition of the enzyme. In addition, alanine and glycine can influence the AMP regulatory system through their antagonistic effects on the interactions of histidine and L-glutamine, respectively. It should be emphasized that the above considerations are only an attempt to reconcile in a general way the unusual responses of the *B. licheniformis* enzyme to various effectors; they do not necessarily describe normal physiological functions. From the standpoint of cellular regulation, it is possible to offer reasonable teleological explanations for almost any specific observation, and one could speculate further on hypothetical situations in which each of the various effector interactions could serve regulatory functions. Such arguments appear unwarranted in the absence of more detailed information concerning the role of glutamine in the biosynthesis of various compounds in *B. licheniformis* and especially information about the intracellular concentrations of various end metabolites and substrates during growth.

Ravel et al. (6) reported that the glutamine synthetase of *Lactobacillus arabinosus* does not catalyze the glutamyl transfer reaction. Their failure to detect glutamotransferase activity may be due to the fact that, like the enzymes from *B. licheniformis*, the *L. arabinosus* glutamine synthetase is very sensitive to inhibition by L-glutamine (6). Similar sensitivity to L-glutamine inhibition was observed, also, with the enzymes from other species of *Bacillus*, e.g., *B. cereus* (2) and *B. subtilis* (Hubbard and Stadtman, *Unpublished data*).

LITERATURE CITED

1. ATKINSON, D. E., 1966. Regulation of enzyme activity. Ann. Rev. Biochem. **35**:85–124.
2. HUBBARD, J. S., AND E. R. STADTMAN. 1967. Regulation of glutamine synthesis. II. Patterns of feedback inhibition in microorganisms. J. *Bacteriol.* **93**:1045–1055.
3. HUBBARD, J. S., AND E. R. STADTMAN. 1967. Regulation of glutamine synthetase. V. Partial purification and properties of glutamine synthetase from *Bacillus licheniformis*. J. Bacteriol. **94**:1007–1015.
4. MEISTER, A. 1962. Glutamine synthesis. p. 443–468. *In* P. D. Boyer, H. Lardy, and K. Myrback [ed.], vol. 6. Academic Press, Inc. New York.
5. MONOD, J., J. WYMAN, AND J. P. CHANGEUX. 1965. On the nature of allosteric transitions: a plausible model. J. Mol. Biol. **12**:88–118.
6. RAVEL, J. M., J. S. HUMPHREYS, AND W. SHIVE. 1965. Control of glutamine synthesis in *Lactobacillus arabinosus*. Arch. Biochem. Biophys. **111**:720–726.
7. RUBIN, M. M., AND J. P. CHANGEUX. 1966. On the nature of allosteric transitions: Implications of non-exclusive ligand binding. J. Mol. Biol. **21**:265–274.
8. STADTMAN, E. R. 1966. Allosteric regulation of enzyme activity. Advan. Enzymol. **28**:41–154.
9. WOOLFOLK, C. A., AND E. R. STADTMAN. 1964. Cumulative feedback inhibition in the multiple end product regulation of glutamine synthetase in *Escherichia coli*. Biochem. Biophys. Res. Commun. **17**:313–319.