Regulation of Glutamine Synthetase

II. Patterns of Feedback Inhibition in Microorganisms

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The feedback inhibition of glutamine synthetase was investigated by use of partially purified enzyme preparations from Salmonella typhimurium, Micrococcus sodonensis, Pseudomonas fluorescens, Bacillus cereus, Bacillus licheniformis, Clostridium pasteurianum, Rhodospirillum rubrum, Neurospora crassa, Candida utilis, and Chlorella pyrenoidosa. Inhibition analyses indicated that the enzyme of each organism can be effectively regulated with mixtures of end products from the diverse pathways of glutamine metabolism. When tested individually, tryptophan, histidine, alanine, glycine, glutamine, 5'-adenylate (AMP), cytidine-5'-triphosphate, carbamyl phosphate, and glucosamine-6-phosphate gave limited inhibition. In most cases, the inhibitors were independent in their action, and cumulative degrees of inhibition were obtained with mixtures of these end products. In contrast, with the glutamine synthetases of the two Bacillus species, the simultaneous presence of AMP and histidine (or AMP and glutamine) gave inhibition greater than the sum of the amounts of inhibition caused by either inhibitor alone. Also, alanine and carbamyl phosphate acted synergistically to inhibit the enzyme from N. crassa. The remarkable similarity in the overall patterns of end-product inhibition observed with the enzymes from different sources indicates that these diverse organisms have evolved comparable mechanisms for the regulation of glutamine metabolism. Nevertheless, the enzymes from different sources do differ significantly in their physical and catalytic properties, as was demonstrated by dissimilarities in their purification behaviors, specificity for nucleotide substrate, ability to catalyze the glutamyl transfer reaction, and ability to utilize Mn++ and Mg++ as activators for the biosynthetic reaction.

A variety of mechanisms for feedback control of enzymes catalyzing branched biosynthetic pathways have been recognized (for review see 27). These include (i) the elaboration of isoenzymes which individually are inhibited by different end products and (ii) several mechanisms in which a single form of the enzyme is effectively inhibited only when more than one of the end products are simultaneously present. Our present knowledge does not permit the conclusion that different microorganisms utilize a single mechanism for the control of a particular enzyme. In some cases (5), closely related species may exhibit marked differences in the patterns of feedback control of an enzyme which presumably occupies the same relative position in their metabolism.

Glutamine synthetase represents a complex metabolic branch-point and thus imposes special requirements for an effective means of control. The amide nitrogen of glutamine may be utilized in diverse pathways leading to the formation of purines (2), cytidine triphosphate (Hulbert and Chaikraborty, Federation Proc. 20:361, 1961), tryptophan (8), histidine (18), carbamyl phosphate (23), glucosamine-6-phosphate (12), pyridine nucleotides (24), and p-aminobenzoic acid (29). In addition, the α-amino group of glutamine is a potential source of nitrogen for the transaminative production of several α-amino acids (15).

From studies with the glutamine synthetase of Escherichia coli (30), evidence was presented for a unique type of multiple end-product control, viz., cumulative feedback inhibition. When tested individually, alanine, glycine, histidine, tryptophan, adenosine-5'-monophosphate (AMP), cytidine-5'-triphosphate (CTP), carbamyl phosphate, or glucosamine-6-phosphate caused limited degrees of inhibition. Cumulative degrees of
inhibition were obtained when various mixtures of these end products were tested. Further studies (Woolfolk and Stadtman, in press; Shapiro and Stadtman, unpublished data) have indicated that these eight (inhibitors) react at separate sites on the E. coli enzyme.

Somewhat different patterns of control have been suggested in preliminary studies with the glutamine synthetases of other microorganisms. For example, Kohlhaas et al. (10) reported that nicotinamide adenine dinucleotide (NAD), CTP, and guanosine-5' monophosphate (GMP) were feedback inhibitors for the enzyme from Saccharomyces cerevisiae. For the glutamine synthetase of Lactobacillus arabinosus (25), the product of the reaction in the synthetic direction, glutamine, serves as a potent inhibitor. To determine to what extent these various regulatory mechanisms are general in nature, the patterns of feedback control of the glutamine synthetase of 10 diverse microbial types were determined and are reported in the present study. The organisms selected for our investigation include aerobic, anaerobic, and photosynthetic bacteria, a mold, a yeast, and a green alga.

**Table 1. Growth conditions for the aerobic organisms**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>Additions to basal medium*</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimurium</em> O200a</td>
<td>Bruce Ames, National Institute of Arthritis and Metabolic Diseases</td>
<td>Glucose, 0.4%</td>
<td>hr</td>
</tr>
<tr>
<td><em>Micrococcus sodenensis</em></td>
<td>J. J. Perry, Department of Bacteriology, North Carolina State University, Raleigh</td>
<td>Na acetate, 0.5%; biotin, 60 μg/ml</td>
<td>18</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> B. cereus 14B22</td>
<td>ATCC 9945a</td>
<td>Glucose, 0.4%; Glucose, 0.5%; biotin, 60 μg/ml; Glucose, 0.4%</td>
<td>16</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Department of Microbiology, University of Texas, Austin</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>ATCC 9950</td>
<td>Glucose, 0.1%; Sucrose, 0.4%; biotin, 60 μg/ml</td>
<td>24</td>
</tr>
<tr>
<td><em>Neurospora crassa</em> EM 5297</td>
<td>Martin Flavin, National Heart Institute</td>
<td>Glucose, 0.4%; yeast extract, 0.0025%; dehydrated nutrient broth, 0.16%</td>
<td>96</td>
</tr>
</tbody>
</table>

* Basal medium: K2HPO4, 3H2O, 13.9 g; KH2PO4, 5.3 g; NaCl, 2.5 g; K2SO4, 2.5 g; MgSO4·7H2O, 1 g; monosodium glutamate, 5 g; and tap water, 1,000 ml. The solutions of the phosphates, glutamate, and carbon sources were sterilized separately. Uninoculated medium was pH 7. The C. pyrenoidosa cultures were shaken in the dark in 4-liter flasks containing 600 ml of medium. The other organisms were grown in 20-liter carboys containing 10 liters of medium with forced aeration. The inocula were grown in the media described above.

**Materials and Methods**

**Organisms and culture media.** The conditions for growth of the aerobic organisms are given in Table 1. *Clostridium pasteurianum* ATCC 6013 was grown anaerobically at 30 C under conditions described by Carnahan and Castle (3) with 0.5% sodium glutamate as the nitrogen source. *Rhodospirillum rubrum* ATCC 11170 was grown photosynthetically with malate at 30 C under conditions described by Omerod et al. (20) with 0.5% sodium glutamate as the nitrogen source.

**Preparation of cell-free extracts.** Cell pastes after harvest by centrifugation were washed once with 0.01 M imidazole chloride buffer (pH 7) and were resuspended in 0.01 M imidazole buffer containing 0.01 M MnCl2 and 0.001 M β-mercaptoethanol. *Micrococcus sodenensis* cells were disrupted by 15-min treatment with a sonic oscillator (Branson Instruments, Inc., Stamford, Conn.). The other organisms were ruptured by passage through a French pressure cell. After either method of rupture, the cell debris was removed by centrifugation at 20,000 × g for 20 min. The resulting supernatant solutions were subjected to the purification procedures described in Table 2. The three basic fractionation steps employed were precipitation by addition of solid ammonium sulfate, by dropwise addition of 1 M acetic acid, and by dropwise addition of acetone. Precipitates were collected by centrifugation at 20,000 × g for 20 min and were immediately...
TABLE 2. Partial purifications and the activity measurements of the microbial glutamine synthetases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Fractionation of extract (precipitated at)</th>
<th>Glutamine synthetase activity</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A) With MgCl₂ (B) With MnCl₂ (C) Glutamyl transfer assay</td>
<td>B/A</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>pH 5.1-4.4; (NH₄)₂SO₄, 33-55% saturation</td>
<td>0.91 1.08</td>
<td>3.56</td>
</tr>
<tr>
<td><em>Micrococcus sodonensis</em></td>
<td>pH 4.9-3.9; acetone, 0-45% at pH 5.7</td>
<td>0.33 0.09</td>
<td>0.27</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>pH 5.1-4.4, twice</td>
<td>0.085 0.39</td>
<td>0.05</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>pH 5.1-4.4, twice</td>
<td>0.15 1.75</td>
<td>6.08</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>pH 5.1-4.4; acetone, 0-45% at pH 5.7;</td>
<td>0.66 1.92</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄, 50-70% saturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Clostridium pasteuri-</td>
<td>(NH₄)₂SO₄, 50-70% saturation; acetone;</td>
<td>0.14 0.81</td>
<td>21.1</td>
</tr>
<tr>
<td>nam*</td>
<td>0-45% at pH 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>pH 5.7-4.7; acetone, 0-45% at pH 5.7</td>
<td>0.26 0.75</td>
<td>18.8</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>pH 5.1-4.4; acetone, 0-45% at pH 5.7</td>
<td>0.13 0.81</td>
<td>39.8</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>(NH₄)₂SO₄, 30-50% saturation;</td>
<td>0.13 0.94</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄, 0-40% saturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td>pH 7-4.4</td>
<td>0.05 0.34</td>
<td>2.45</td>
</tr>
</tbody>
</table>

a Results expressed as micromoles of P₁ per minute per milligram of protein. Reaction mixture: cell-free extract, 0.0075 M ATP, 0.1 M glutamate, 0.05 M NH₄Cl, 0.02 M imidazole, and 0.005 M MnCl₂ (for A) or 0.05 M MgCl₂ (for B).
b Results expressed as micromoles of glutamylhydroxamate per minute per milligram of protein. Reaction mixture: cell-free extract, 0.1 M glutamine, 0.3 M NH₂OH, 0.02 M arsenate, 0.0004 M ADP, 0.003 M MnCl₂, and 0.02 M imidazole.

dissolved in the imidazole-MnCl₂ buffer described above. The fractionated extracts were stored at 4 °C. Under these conditions (manganese ion present), the glutamine synthetase preparations showed no appreciable losses in activity during 7 days of storage.

**Biosynthetic assay of enzyme activity.** Glutamine synthetase catalyzes the amidation of glutamate, according to equation 1.

\[
\text{glutamate} + \text{NH}_4^+ + \text{adenosine-5'}\text{-triphosphate} \xrightarrow{\text{Mn}^{++} \text{Mg}^{++}} \text{glutamine} + \text{adenosine-5'}\text{-diphosphate} + \text{P}_1
\]  

(1)

Enzyme activity is conveniently estimated by measuring the amount of glutamate-dependent orthophosphate (P₁) liberated (1). The complete (assay) mixture contained cell extract, L-glutamic acid (monosodium), NH₄Cl, ATP, MnCl₂, and imidazole in a total volume of 0.3 or 0.6 ml at a final pH of 7.0. The concentrations of reactants are given in the appropriate tables. Incubations were carried out in open tubes at 30 or 37 °C, coinciding with the growth temperature used for the organism from which the extract was prepared. The reactions were initiated by the addition of enzymes. Proportionality of P₁ liberation to enzyme concentration is observed up to 0.25 μmole of P₁ per 15 min of incubation under the assay conditions. Controls lacking glutamate were included in each incubation employed, and corrections were made for the small amounts of P₁ liberated in the absence of substrate.

Net synthesis of glutamine was established by paper chromatography of deproteinized incubation mixtures using 1-butanol–water–acetic acid (4:4:1) as the developing solvent. The Rₚ values of glutamine and glutamic acid were 0.18 and 0.25, respectively.

**Glutamyl transfer assay of enzyme activity.** Glutamine synthetase also catalyzes a transfer of the γ-glutamyl moiety of glutamine to hydroxylamine according to equation 2.

\[
\text{glutamine} + \text{hydroxylamine} \xrightarrow{\text{ADP, AsO}_4^{2-}, \text{Mn}^{++}} \text{glutamylhydroxamate} + \text{NH}_4^+
\]  

(2)

For some studies, the amounts of γ-glutamylhydroxamate formed in this reaction were used to esti-
mate glutamine synthetase activity. The γ-glutamylhydroxamate after reaction with ferric chloride (14) was measured with a Klett-Summerson colorimeter equipped with a 540-mu filter, with authentic γ-glutamylhydroxamate as the standard. The complete assay mixture contained cell extract, L-glutamate, neutralized hydroxylamine hydrochloride, arsenate, ADP, MnCl₂, and imidazole, in a final volume of 1 ml at pH 7.0. The concentrations of reactants are described in the appropriate tables. Incubations were performed in open tubes at 30 or 37°C, the optimal growth temperature for the particular organism. The reactions were initiated by the addition of enzyme. Glutamylhydroxamate formation is proportional to enzyme concentration in the range of 0 to 3 μmoles per 15 min of incubation. Controls lacking glutamine or ADP and arsenate were included in all assays.

In experiments wherein magnesium was employed as metal activator, the cell extracts were freed from manganese either by exhaustive dialysis against 200 to 500 volumes of 0.01 M imidazole buffer or by precipitating the glutamine synthetase by one of the previously described methods and redissolving the carefully drained pellet in the imidazole buffer. Protein concentrations were estimated from absorbance at 260 and 280 μm (11).

**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 inhibitor in the tube containing the inhibitor but lacked glutamate. With the transfer assay, the inhibitor was added and glutamine was omitted. The results of inhibition analyses are expressed as per cent activity, i.e., 100 times the fractional activity observed in the presence of inhibitor. The reported values represent the average of two or more determinations.

**Chemicals.** ATP (dextrose), CTP (disodium), L-glutamine (grade III), and L-glutamic acid-mono-hydroxamate (γ) were obtained from Sigma Chemical Co., St. Louis, Mo. ADP (sodium salt) and 5'-adenyl acid were obtained from Pabst Laboratories, Milwaukuee, Wis. Carbamyl phosphate (dilithium salt, B grade) was obtained from Calbiochem, Los Angeles, Calif. All other chemicals were reagent grade.

**RESULTS**

It is obvious that the presence of multiple forms of glutamine synthetase in a given organism could be most decisively demonstrated by examination of crude unfraccionated extracts, since any fractionation procedure applied to cell-free extracts might conceivably result in the selective destruction of one or more isoenzymes. Unfortunately, the unambiguous demonstration of glutamine synthetase activity in crude extracts of some organisms is not possible, owing to the presence of phosphatase activities that catalyze the liberation of P₁ from ATP and thereby cause interference in the biosynthetic assay procedure. Moreover, the possible presence of amidohydro-

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tive activities of the transfer and synthetic reactions could not be compared because of the presence of an amidohydrolase which catalyzed the deamination of glutamine or the formation of glutamylhydroxamate from glutamine and hydroxylamine. The latter reaction, in contrast to the glutamyl transfer reaction catalyzed by glutamine synthetase, is not dependent on ADP or arsenate. By means of an acid precipitation step (pH 4.4 to 3.5) amidohydrolase preparations were obtained which were free from glutamine synthetase. However, the reciprocal removal of amidohydrolase activity from glutamine synthetate preparations was not accomplished. Thus, this interfering activity made it impractical to measure the glutamotransferase activity (Tables 2 and 7) or to test glutamine as a feedback inhibitor of the biosynthetic reaction catalyzed by the glutamine synthetase of \textit{P. fluorescens} (Table 3).

Comparisons of potential end products as feedback inhibitors. Results of analyses in which potential end products were tested as feedback inhibitors are presented in Table 3. This survey included seven of the eight compounds previously found to be inhibitory for the glutamine synthetase of \textit{E. coli} (30), as well as glutamine, which was reported to inhibit the enzyme from \textit{L. arabinosus} (25). The assay conditions employed to demonstrate the inhibitions were either limiting glutamate with excess NH$_4$Cl or limiting NH$_4$Cl with excess glutamate, as indicated. As seen in Table 3, the glutamine synthetase of each organism is inhibited by more than one end product. Moreover, each enzyme exhibits a unique regulation pattern which differs from the others in respect to the extent of inhibition caused by the end products. However, certain similarities are apparent in the inhibition patterns of the glutamine synthetases of closely related organisms. This similarity is seen with the two \textit{Bacillus} species, and the inhibitory response of the enzyme from \textit{S. typhimurium} resembles that previously reported for \textit{E. coli} glutamine synthetase (30). In addition to the compounds reported in Table 3, \textit{p}-amino benzoic acid, antranilic acid, methionine, isoleucine, GMP, and NAD were either noninhibitory or only slightly inhibitory, causing less than 5% diminution in activity.

As is also shown in Table 3, different degrees of inhibition were obtained depending on whether MnCl$_2$ or MgCl$_2$ was used in the assay system. With many of the enzymes studied, inhibitions caused by alanine and glycine were greater when Mn$^{2+}$ was the activator, whereas the converse was often true of the AMP inhibition. The choice of metal activator even more dramatically influenced the manner in which CTP affected the activities of the glutamine synthetases of the

<p>| Table 3. Influence of end products of glutamine metabolism on the activities of the microbial glutamine synthetases$^a$ |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Ala$^b$ (5mM)</th>
<th>Gln$^b$ (5mM)</th>
<th>Trp$^b$ (5mM)</th>
<th>AMP$^b$ (5mM)</th>
<th>CTP$^b$ (2mM)</th>
<th>GluNH$_2$$^b$ (10mM)</th>
<th>His$^e$ (15mM)</th>
<th>GA-6-P$^d$ (1mM)</th>
<th>AMP$^e$ (5mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Salmonella typhimurium}</td>
<td>65 (58)</td>
<td>80 (68)</td>
<td>77 (95)</td>
<td>83 (67)</td>
<td>79 (86)</td>
<td>78 (94)</td>
<td>67 (85)</td>
<td>83 (85)</td>
<td></td>
</tr>
<tr>
<td>\textit{Micrococcus sadenonis}</td>
<td>84 (30)</td>
<td>85 (26)</td>
<td>98 (26)</td>
<td>75 (53)</td>
<td>79 (46)</td>
<td>96 (26)</td>
<td>58 (80)</td>
<td>75 (80)</td>
<td></td>
</tr>
<tr>
<td>\textit{Bacillus licheniformis}</td>
<td>28 (53)</td>
<td>51 (75)</td>
<td>95 (38)</td>
<td>85 (60)</td>
<td>67 (26)</td>
<td>85 (104)</td>
<td>104 (68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{B. cereus}</td>
<td>34 (53)</td>
<td>51 (75)</td>
<td>96 (36)</td>
<td>78 (60)</td>
<td>67 (26)</td>
<td>85 (104)</td>
<td>104 (68)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>51 (49)</td>
<td>59 (50)</td>
<td>88 (85)</td>
<td>88 (85)</td>
<td>78 (79)</td>
<td>85 (60)</td>
<td>85 (104)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Clostridium pasteurianum}</td>
<td>19 (60)</td>
<td>38 (62)</td>
<td>90 (79)</td>
<td>84 (84)</td>
<td>78 (113)</td>
<td>75 (15)</td>
<td>80 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Rhodospirillum rubrum}</td>
<td>49 (31)</td>
<td>68 (42)</td>
<td>90 (85)</td>
<td>88 (84)</td>
<td>72 (85)</td>
<td>77 (87)</td>
<td>75 (94)</td>
<td>84 (84)</td>
<td></td>
</tr>
<tr>
<td>\textit{Candida utilis}</td>
<td>88 (99)</td>
<td>83 (100)</td>
<td>98 (79)</td>
<td>79 (64)</td>
<td>69 (98)</td>
<td>69 (98)</td>
<td>85 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Neurospora crassa}</td>
<td>87 (98)</td>
<td>87 (90)</td>
<td>100 (95)</td>
<td>(--)$^d$</td>
<td>86 (94)</td>
<td>95 (94)</td>
<td>58 (80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Chlorella pyrenoidosa}</td>
<td>71 (90)</td>
<td>72 (90)</td>
<td>100 (99)</td>
<td>73 (85)</td>
<td>87 (94)</td>
<td>88 (88)</td>
<td>80 (80)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Abbreviations: Ala = L-alanine; Gly = glycine; Try = L-tryptophan; AMP = 5'-adenylic acid; CTP = cytidine-5'-triphosphate; GluNH$_2$ = L-glutamine; His = L-histidine; GA-6-P = D-glucosamine-6-phosphate.

$^b$ Assay system: 0.0075 M glutamate, 0.05 M NH$_4$Cl, 0.0075 M ATP, 0.02 M imidazole, and metal activator (as specified below). The values to the left refer to activity measurements when 0.005 M MnCl$_2$ was used on the activator. The values in the parentheses refer to activity measurements when 0.05 M MgCl$_2$ was used on the activator.

$^c$ Assay system: 0.1 M glutamate, 0.001 M NH$_4$Cl, 0.0075 M ATP, 0.02 M imidazole, and 0.005 M MnCl$_2$.

$^d$ CTP and AMP served as substrates for P$_1$ production.
higher protists, *N. crassa*, *Candida utilis*, and *Chlorella pyrenoidosa*. When the assay system contained MgCl₂, CTP served as an inhibitor of the enzymes from these three organisms, whereas, with MnCl₂, CTP stimulated P₁ production. Other experiments with *N. crassa* have shown that the apparent stimulation by CTP is due to the ability of CTP to replace ATP as a substrate for glutamine synthetase. For example, in the absence of ATP, P₁ is released from CTP only in the presence of glutamate and ammonia. Moreover, net synthesis of glutamine (as determined by paper chromatography) could be demonstrated in both the CTP- and ATP-dependent reactions. Table 4 gives results of additional investigations on these roles of ATP and CTP. In the assay containing MnCl₂, CTP was slightly more effective than ATP as substrate. The activities were additive when a mixture of 7.5 mM ATP and 2 mM CTP was used. However, with a saturating level of ATP (15 mM), additional CTP (6 mM) did not stimulate P₁ production. Thus, it would appear that the glutamine synthetase of *N. crassa* is a single enzyme which, when activated by Mn⁴⁺, requires high levels of ATP or CTP for maximal activity. Further evidence to support the view that the nucleotides are substrates for the same enzyme was obtained in other experiments, which showed that histidine caused comparable degrees of inhibition of the ATP- and CTP-dependent activities. When the reaction mixture contained MgCl₂, CTP was much less effective than ATP as substrate. Monder (16) has reported that only Mn⁴⁺ activates mammalian glutamine synthetase when

uridine, inosine, or guanosine triphosphates are used, whereas either Mn⁴⁺ or Mg⁴⁺ activates the mammalian enzyme when ATP is the nucleotide substrate.

As shown in Table 3, CTP also stimulated P₁ production by the extracts of *C. pasteurianum*. However, this activity does not appear to be a consequence of CTP acting directly as a substrate for glutamine synthetase. Other experiments have shown that CTP is ineffective as a substrate for P₁ production when ATP is omitted from the reaction mixture containing the *C. pasteurianum* extract. *N. crassa* extracts catalyzed the liberation of P₁ from AMP. This latter activity appeared to be unrelated to glutamine synthetase, since it was not dependent on glutamate or ATP.

**Effects of combinations of inhibitors.** In an attempt to characterize the patterns of multiple end-product regulation for the glutamine synthetases, experiments were conducted with several pairs of inhibitors (Table 5). For purposes of comparison, values were calculated on the assumption that each of the two inhibitors acts on a different form of the enzyme, or that both act independently on a single form of the enzyme. Certain pairs of inhibitors obviously do not act on separate enzymes, since the calculated sum of the individual inhibitions exceeds 100% (negative values). With the majority of the other pairs tested, the experimental values are in closer agreement with the values calculated by assuming the two inhibitors interact with a single form of the enzyme. The data which more closely agree with the enzyme multiplicity predictions were obtained when one inhibitor of the pair caused a small degree of inhibition. In these situations, the differences between the two theoretical values are small; therefore, the margin of experimental error prevents one from distinguishing between the assumptions. Histidine and AMP acted synergistically on the glutamine synthetases of *B. licheniformis* and *B. cereus* in causing inhibitions which were decidedly greater than either of the calculated values (Table 5). This synergistic action, wherein the simultaneous presence of two end products results in greater inhibition than the fractional inhibitions caused by either end product alone, has been observed with enzymes other than glutamine synthetase (4, 21), and has been termed cooperative feedback inhibition. However, to avoid confusion with the cooperative effects often seen in the homologous interactions of feedback inhibitors, it would appear desirable to use another term; hence, we suggest the term synergistic feedback inhibition. The glutamine synthetases of the *Bacillus* species were also susceptible to synergistic feedback inhibition with

**Table 4. Utilization of ATP and CTP by the glutamine synthetase of Neurospora crassa**

<table>
<thead>
<tr>
<th>Reaction mixture*</th>
<th>P₁ produced³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m M</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>5</td>
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<td>MnCl₂</td>
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<td>MnCl₂</td>
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<tr>
<td>MnCl₂</td>
<td>0</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0</td>
</tr>
</tbody>
</table>

* Plus 0.1 m glutamate, 0.05 m NH₄Cl, 0.02 m imidazole, and enzyme.

Micromoles per 15 min per milligram of protein. Calculated from determinations made with appropriate dilutions of enzyme preparation.

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### Table 5. Theoretical and observed inhibitions of microbial glutamine synthetases with pairs of end products

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Alanine</th>
<th>Glycine</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Gly⁺</td>
<td>+Try⁺</td>
<td>+AMP⁺</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>(52) 45</td>
<td>(50) 42</td>
<td>(54) 48</td>
</tr>
<tr>
<td><em>Micrococcus spondonensis</em></td>
<td>(72) 69</td>
<td>(82) 82</td>
<td>(63) 59</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>(14) -21</td>
<td>(27) 27</td>
<td>(11) -34</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>(17) -17</td>
<td>(33) 30</td>
<td>(12) 30</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>(30) 10</td>
<td>(45) 39</td>
<td>(42) 34</td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>(7) -43</td>
<td>(17) 9</td>
<td>(18) 12</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>(33) 17</td>
<td>(44) 39</td>
<td>(43) 37</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>(73) 71</td>
<td>(86) 86</td>
<td>(70) 67</td>
</tr>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td>(51) 43</td>
<td>(71) 71</td>
<td>(52) 44</td>
</tr>
</tbody>
</table>

---

* The lower numbers are activities observed when the pairs of inhibitors were present simultaneously. The numbers in parentheses are theoretical values calculated with the assumption that each inhibitor of the pairs acts independently and concurrently on the same enzyme, i.e., the product of the activity percentages observed when the inhibitors were tested individually (see Table 3). The italicized numbers are the theoretical values calculated with the assumption that each inhibitor acts on a separate enzyme, i.e., 100% minus the sum of the inhibition percentages obtained when the inhibitors were tested individually (see Table 3).

⁺ glutamate, 0.0075 M; NH₄Cl, 0.05 M; with 0.0075 M ATP; 0.02 M imidazole (pH 7); and glutamine synthetase preparation.

⁺⁺ Glutamate, 0.1 M; NH₄Cl, 0.001 M, with other components as in footnote b.
a mixture of AMP and glutamine. This latter synergism can be more lucidly demonstrated when lower levels of the co-effectors are employed (Hubbard and Stadtman, unpublished data).

With several pairs of inhibitors (Table 5), the experimental activities were considerably higher than either of the calculated values. These antagonistic effects were most often observed when alanine or glycine served as one of the inhibitors. This antagonism is most readily visualized by assuming that the two ligands act on a single form of the enzyme. One possible explanation is that the two end products are mutually competitive for the same site. Alternatively, the occupation of one site by its ligand could interfere with the binding of the second ligand. Regardless of the reason, the varying degrees to which two inhibitors act antagonistically for the various glutamine synthetases illustrate differences in the interaction of the inhibitors with the enzymes from different sources.

Further investigations showed that the microbial glutamine synthetases could be inhibited 53 to 94% with a mixture of six end products of glutamine metabolism (Table 6). These data cannot be directly compared with those presented in Tables 3 and 5, since different assay conditions were used. However, it would seem likely that certain of these enzymes would have been more effectively inhibited had other end products of glutamine metabolism been included in the inhibitor mixtures. In any event, these data and the data of Table 5 indicate that the simultaneous presence of a physiological excess of several end products causes greater inhibition than when only one or two are present in excess. It therefore appears evident that mechanisms involving some degree of cumulative feedback inhibition are operative in most of the organisms examined.

End products of glutamine metabolism also inhibit the glutamyl transfer reaction catalyzed by the microbial glutamine synthetases. The data in Table 7 show that these activities are inhibited to varying degrees by alanine, AMP, and carbamyl phosphate. Again, the results from assays with pairs of inhibitors generally favor the assumption that with each organism inhibitors are acting on a single form of the enzyme. N. crassa represents the exceptional case, in that alanine and carbamyl phosphate acted synergistically in inhibiting the glutamotransferase activity. Additional experiments have shown that the biosynthetic reaction

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**Table 6. Effects of a mixture of six end products on the activities of the glutamine synthetases**

<table>
<thead>
<tr>
<th>Source of glutamine synthetase prepn</th>
<th>Per cent activity in the presence of</th>
<th>Ala + Glu + Thr + His + AMP + GA + P (3 mm each)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><em>Micrococcus sodonensis</em></td>
<td>47</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction mixture: 0.0075 m glutamate, 0.001 m NH₄Cl, 0.0075 m ATP, 0.005 m MnCl₂, 0.02 m imidazole, and enzyme.

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**Table 7. Effects of end products of glutamine metabolism on the glutamotransferase activities**

<table>
<thead>
<tr>
<th>Source of glutamotransferase prepn</th>
<th>Per cent activity in the presence of</th>
<th>Ala</th>
<th>AMP</th>
<th>CP</th>
<th>Ala + CP</th>
<th>Ala + AMP</th>
<th>AMP + CP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td></td>
<td>37</td>
<td>55</td>
<td>51</td>
<td>18 (19)</td>
<td>22 (20)</td>
<td>31 (28)</td>
</tr>
<tr>
<td><em>Micrococcus sodonensis</em></td>
<td></td>
<td>70</td>
<td>81</td>
<td>68</td>
<td>44 (48)</td>
<td>56 (57)</td>
<td>53 (55)</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td></td>
<td>32</td>
<td>70</td>
<td>85</td>
<td>30 (27)</td>
<td>20 (22)</td>
<td>56 (60)</td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td></td>
<td>9</td>
<td>98</td>
<td>59</td>
<td>6 (5)</td>
<td>9 (9)</td>
<td>58 (58)</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td></td>
<td>6</td>
<td>98</td>
<td>61</td>
<td>6 (4)</td>
<td>5 (6)</td>
<td>64 (60)</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td></td>
<td>52</td>
<td>95</td>
<td>47</td>
<td>33 (24)</td>
<td>52 (49)</td>
<td>47 (45)</td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td></td>
<td>64</td>
<td>99</td>
<td>71</td>
<td>24 (46)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>Chlorella pyrenoidosa</em></td>
<td></td>
<td>62</td>
<td>97</td>
<td>61</td>
<td>30 (37)</td>
<td>61 (60)</td>
<td>61 (59)</td>
</tr>
</tbody>
</table>

* CP, carbamyl phosphate. Inhibitor concentrations were 5 mm. Reaction mixture: 0.03 m glutamine, 0.06 m NH₄OH, 0.02 m arsenate, 0.004 m ADP, 0.003 m MnCl₂, 0.02 m imidazole, and cell extracts.

* The numbers to the left are the per cent activities observed in the presence of the pair of inhibitors. The numbers in parentheses are values calculated with the assumption that the two inhibitors act independently on a single form of the enzyme, i.e., 100 times the product of the fractional activities observed when the inhibitors were tested individually.
catalyzed by the *N. crassa* enzyme is also susceptible to this synergistic feedback inhibition caused by the alanine and carbamyl phosphate mixture.

**DISCUSSION**

The formation of glutamine may be regarded as the first enzymatic step of a highly branched pathway that leads ultimately to the biosynthesis of a variety of end products. Therefore, by analogy to the regulation of other branched pathways, it is not surprising that glutamine synthetase is subject to feedback inhibition by the ultimate end products of glutamine metabolism.

Previous studies have shown that the regulation of a given biosynthetic branched pathway may be achieved by distinctly different mechanisms in different organisms (5, 27). For example, the regulation of aspartokinase, which is the first common step in the biosynthesis of lysine, methionine, threonine, and isoleucine, involves the elaboration of multiple enzymes in *E. coli* (28), concerted feedback inhibition in *Rhodopseudomonas capsulatus* (5) and in *B. polymyxa* (22), a sequential feedback mechanism in *R. spheroides* (Datta, personal communication), and still different mechanisms in *Rhodospirillum rubrum* (5) and in *S. cerevisiae* (26). It is, therefore, somewhat surprising that the feedback control patterns of the glutamine synthetases from widely different organisms appear to be qualitatively very similar. In general, the patterns of inhibition are similar to the cumulative inhibition established in *E. coli* (30) Thus, each of the various end products is able by itself to cause only a limited degree of inhibition; however, as the number of end products that are present simultaneously is increased, there is a progressive increase in the extent of inhibition. Such a mechanism of inhibition constitutes an elegant means of metabolic control of those enzymes that catalyze the synthesis of an early precursor that is a common intermediate in the biosynthesis of several end products. For, as each of the various end products becomes present in excess, it causes a curtailing of the biosynthesis of the common precursor by an amount presumably required for the synthesis of the particular end product.

The drastic consequences that would be expected (28) if a physiological excess of one end metabolite could cause total inhibition of the common precursor are thus avoided. Yet, when all end products are present in more than adequate amounts, the synthesis of the common precursor is effectively stopped.

It is obvious that, if such a mechanism of control is to be effective, it must be supplemented with secondary feedback controls by each of the diverse end metabolites of the first unique step that is specifically concerned with its biosynthesis. By means of these secondary controls, the reduced supply of the common precursor that is available when a given end product is present in excess is diverted away from that particular end product and is thus made available only for the syntheses of those end products that are still required.

In the case of glutamine metabolism, the existence of secondary controls in some bacteria has been established for the first unique step involved in the biosynthesis of CTP (31), AMP (4), histidine (17), and tryptophan (8). Whereas it is reasonable to assume that similar secondary controls occur also in the biosynthesis of the other feedback inhibitors of glutamine synthetase, the existence of such controls remains to be demonstrated.

Although the present data suggest that each of the organisms studied elaborates only one glutamine synthetase that is susceptible to partial inhibition by the different end products, the possibility is not excluded that multiple forms of the enzyme are produced in some organisms. A particularly labile isoenzyme could have been destroyed during preparation of the cell-free extracts, or by the minimal purification procedure required to eliminate reactions that interfered with the enzyme assays. Also, the enzyme assay conditions employed might not be favorable for the detection of isoenzymes with different optimal requirements. Moreover, in those situations where the maximal inhibition obtained by a given inhibitor is very small, it is not possible with the relatively insensitive assay procedures employed to distinguish between the possibility that they act independently on a common enzyme (cumulative feedback inhibition) or are specific inhibitors of isoenzymes that are present in small quantities.

It should be emphasized that the significance of the observed inhibition patterns for the glutamine synthetases from different organisms can be properly evaluated only after the complete regulatory patterns for the biosynthesis of each of the multiple inhibitors is also established in these organisms. It remains to be determined whether the inhibition of glutamine synthetase by some of the metabolites is due to end-product inhibition, or if instead it is the manifestation of a modified sequential feedback mechanism in which the inhibitor is an intermediate rather than an ultimate end product. For example if glutamine is the physiological nitrogen donor in the biosynthesis of glucosamine-6-phosphate (GA-6-P), then would not *N*-acetyl-GA-6-P, UDP-*N*-acetyl-
glucosamine, or some other derivative be more appropriately regarded as the ultimate end product? If so, then the inhibition of glutamine synthetase by GA-6-P might be the expression of a sequential feedback control mechanism (9, 19) in which the last common intermediate (GA-6-P) of a branched pathway inhibits the first biosynthetic step leading to its formation. If this is true, then it follows that the first steps involved in the further metabolism of GA-6-P should be under feedback control by UDP-N-acetylglucosamine or other terminal end products.

The fact that glutamine is a strong inhibitor of the glutamine synthetase from some organisms, might also be attributable to the operation of a sequential feedback control system in these organisms. In the presence of secondary controls involving feedback inhibitors of the first steps that are uniquely concerned with the biosynthesis of the individual end products, it follows that the inhibitory action of an end product at its secondary site would spare the utilization of glutamine. This would result sequentially in the accumulation of glutamine, which in turn would lead to inhibition of glutamine synthetase, thereby controlling its own synthesis. Such a mechanism of sequential feedback inhibition of the glutamine synthetases of B. licheniformis and B. cereus could be reinforced by the synergistic inhibition caused by the simultaneous presence of glutamine and its end product AMP, thus providing for an even more delicately balanced system of control.

Although there is a remarkable qualitative similarity in the inhibition patterns of the glutamine synthetases from different organisms, it may be significant that there is considerable variation in the absolute effectiveness of any given inhibitor toward the different enzymes (see Table 3). It remains to be seen whether these differences reflect differences in the relative demands for glutamine in the biosynthesis of the various metabolites, or whether they may reflect fundamental variations in the overall regulatory patterns of the different organisms with efficient mechanisms for cellular regulation. By the same token, metabolic economy would suffer if a biosynthetic enzyme is inhibited by a metabolite whose metabolic fate is not in some way dependent upon that enzyme. Attention is therefore directed to the fact that alanine and glycine are among the most effective inhibitors of the glutamine synthetases from all of the organisms examined. Whereas these results suggest that glutamine may be intimately involved in the biosynthesis of alanine and glycine, a definite role of glutamine in the formation of these two amino acids has not been unequivocally established.

Another question is raised by the observation that carbamyl phosphate is an inhibitor for the glutamine synthetase of N. crassa. Davis (6) recently reported that ammonia, and not glutamine, was the source of nitrogen for the carbamyl phosphate synthetase which was demonstrable in cell-free extracts of this organism. The carbamyl phosphate produced by this enzyme was specifically utilized in the synthesis of arginine. On the other hand, physiological and genetic experiments (7) pointed toward a second mechanism for the formation of the carbamyl phosphate specifically utilized in pyrimidine biosynthesis. Since the present findings indicate a regulatory role for carbamyl phosphate in the synthesis of glutamine by N. crassa, it might be well to re-examine the role of glutamine in the synthesis of carbamyl phosphate in this organism. If it is not involved in the synthesis of the carbamyl phosphate used specifically for the synthesis of arginine, perhaps it is used for the synthesis of the aspartylphosphate that is specifically involved in the synthesis of the pyrimidine.

**Literature Cited**


