Proc. Nat. Acad. Sci. USA
Vol. 68, No. 4, pp. 781-785, April 1971

Regulation of Rat Liver Glutamine Synthetase: Activation by α-Ketoglutarate and Inhibition by Glycine, Alanine, and Carbamyl Phosphate

SURESH S. TATE AND ALTON MEISTER

Department of Biochemistry, Cornell University Medical College, New York, N.Y. 10021

Communicated January 25, 1971

ABSTRACT **Rat liver glutamine synthetase ($s_{20,w}$ 15.0 S; MW about 352,000) resembles ovine brain glutamine synthetase in that it (*a*) has 8 subunits, (*b*) acts on both L- and D-glutamate and certain glutamate analogs (e.g., β-glutamate, *cis*-cycloglutamate, and α-methyl-L-glutamate), and (*c*) is irreversibly inhibited by L-methionine-S-sulfoximine. The liver enzyme (but not the brain enzyme) is (*a*) markedly activated by α-ketoglutarate and less so by citrate, and (*b*) inhibited noncumulatively by glycine and alanine, in the presence of Mn^{++} but not Mg^{++}; inhibition increases with increasing concentrations of glutamate. These regulatory phenomena seem to be correlated with metabolically related enzymes, e.g., glutamine transaminase. Both liver and brain glutamine synthetases are inhibited by carbamyl phosphate (with Mn^{++} but not with Mg^{++}), which provides a means for controlling glutamine for pyrimidine biosynthesis. Addition of Mn^{++} to the Mg^{++}-synthetase system, even at Mg^{++}: Mn^{++} ratios of 1000, markedly inhibits synthesis by both brain and liver enzymes. This finding, and the fact that Mn^{++} promotes sensitivity to the negative effectors, indicates that Mn^{++} plays a central role in the regulation of glutamine synthetase. Properties of the glutamine synthetases that have been isolated from mammalian, plant, and bacterial cells are compared. They are similar with respect to subunit size, substrate specificity, inhibition by methionine sulfoximine, and Mn^{++}-sensitive inhibition by glycine, alanine, and carbamyl phosphate, but differ in certain other regulatory phenomena and in subunit structure.**

Glutamine synthetase catalyzes a reaction of considerable significance in the nitrogen metabolism of many cells. Therefore, the synthesis of glutamine is an important pathway for the utilization of ammonia. Furthermore, glutamine serves as a building block of protein, as a nitrogen donor in various biosynthetic pathways, and as a source of ammonia (1). It would be expected that glutamine synthetase would be regulated by mechanisms related to the functions of glutamine in a particular cell type. Stadtman (2), Holzer (3), and their colleagues have carried out extensive studies on the regulation of *Escherichia coli* glutamine synthetase and have found that this enzyme is subject to modulation by mechanisms involving covalent attachment of adenylyl residues and cumulative feedback inhibition by the end products of glutamine metabolism. No such regulatory mechanisms have been found for the ovine brain glutamine synthetase, which has been studied in this laboratory (4); this is not surprising since the function of the brain enzyme, which must certainly be quite different from that found in *E. coli*, seems to be closely associated with the metabolism of glutamate and γ-aminobutyrate, which are probably neurotransmitters. The activity of the brain enzyme is known to be affected by the relative concentrations of various nucleotides and metal ions (5–8), and it has been suggested that metal-ion interactions may be of significance in the control of this enzyme (8). In the present work, we have purified rat liver glutamine synthetase and have examined some of its properties, especially in relation to regulatory phenomena.

MATERIALS

Unlabeled and [^{35}S]L-methionine-*SR*-sulfoximine were prepared by Dr. S. L. N. Rao of this laboratory. [^{14}C-methyl]L-methionine-*SR*-sulfoximine was obtained as described (9, 10). Ovine brain and pea glutamine synthetases were prepared and kindly made available to us by Drs. W. Bruce Rowe and Y. Tsuda of this laboratory. The unlabeled nucleotides, amino acids, and α-ketoglutarate were obtained from Sigma Chemical Corp.

METHODS

Glutamine synthetase activity was measured by the γ-glutamylhydroxamate procedure (6) except that 0.1 M imidazole · HCl buffer (pH 7.2) was used. The reaction was also monitored by phosphate determinations (11) in reaction mixtures containing NH_4Cl (40 mM). The Mn^{++}-dependent activity was generally measured in the presence of 2 mM $MnCl_2$. Protein was determined by the method of Lowry *et al.* (12) with crystalline bovine serum albumin as standard.

Polyacrylamide gel electrophoresis was performed (13) on 4% gels in a buffer (pH 7.2) composed of Tris (0.06 M)–citric acid (0.02 M)–Na_2EDTA (1 mM)– glycerol (5%, v/v), and on 6% gels in a continuous buffer system containing Tris (0.05 M; adjusted to pH 8.5 with acetic acid)–sodium dodecyl-sulfate (0.1%, w/v). The gels were prepared as described (14) and stained with Coomassie blue.

Isolation of rat liver glutamine synthetase

The enzyme was isolated from fresh rat liver by a modification of the method used for isolation of ovine brain glutamine synthetase (15). The livers (50 units/g) were homogenized in a Waring Blendor and extracted with 4 vol of 0.15 M KCl containing 5 mM 2-mercaptoethanol and 1 mM EDTA (pH 7.2). The cell-free extract was obtained and processed as described (15) except that DEAE-cellulose chromatography was performed in imidazole–EDTA buffers, followed by chromatography on Sephadex G-100. The final preparation was about

300-fold purified; overall yield, 20%; specific activity, 134 units/mg. Details of this procedure will appear in a subsequent publication.

RESULTS

Properties of rat liver glutamine synthetase

The enzyme preparation was apparently homogeneous as judged by gel electrophoresis and analytical ultracentrifugation [$s_{20,w}$, 15.0 S in 0.15 M KCl–5 mM 2-mercaptoethanol–1 mM EDTA (pH 7.2); protein concentration, 0.5 mg/ml]. The enzyme exhibited morphology in the electron microscope similar to that of ovine brain glutamine synthetase and thus appears to have the octameric structure proposed by Haschemeyer (16–18) for the brain enzyme. We are indebted to Drs. P. Trotta and R. H. Haschemeyer for the ultracentrifugation and electron microscopic studies. Gel electrophoresis in sodium dodecylsulfate was used to determine the molecular weight of the monomeric units of the enzyme (14, 19); trypsinogen, ovalbumin monomer and dimer, bovine serum albumin monomer and dimer, and aspartate β-decarboxylase (20) were used as standards. The dissociated monomers of the rat liver, ovine brain, and pea glutamine synthetases moved as single discrete bands on the gels. A molecular weight of 44,000 ± 2,000 was obtained for the monomer of rat liver enzyme; the values for the brain and pea enzymes are 49,000 ± 3,000 and 45,000 ± 2,000, respectively. Recent equilibrium ultracentrifuge studies (S. Wilk and R. Haschemeyer, unpublished data) on the ovine brain enzyme indicate that the molecular weight of the native enzyme is about 400,000, a value consistent with the view that the enzyme is composed of eight polypeptide chains of molecular weight about 49,000. These studies indicate that earlier molecular weight estimates of 500,000 and 525,000 (16) are too high, probably because of aggregation, which could not be detected under the conditions employed. Since the rat liver enzyme also appears to be octameric, its molecular weight probably is about 352,000.

TABLE 1. *Effect of various compounds on rat liver glutamine synthetase*

Compound added	Relative activity* With Mn^{++}†	With Mg^{++}‡
None (control)	100 (100)	100 (100)
α-Ketoglutarate (20)§	160 (122)	173 (127)
Citrate (20)	114 (108)	140 (113)
Glycine (20)	42 (90)	99 (93)
L-Alanine (20)	52 (85)	105 (95)
D-Alanine (20)	37 (63)	81 (94)
L-Serine (20)	61 (92)	114 (120)
β-Alanine (20)	57 (94)	105 (100)
AMP (10)	93 (97)	99 (96)
CTP (4)	*77*	113
DPN (10)	*110* (*108*)	120 (107)
Carbamyl phosphate(10)	*31* (*23*)	88 (95)
D-Glucosamine-6-phosphate (9)	*94* (*107*)	111 (97)

* Values given in parentheses were obtained with ovine brain glutamine synthetase; control values, 27 (Mn^{++}) and 115 (Mg^{++}) units/mg enzyme.

† Activity was determined in mixtures containing 0.1 M imidazole·HCl (pH 7.2), 10 mM L-glutamate, 10 mM ATP, 2 mM $MnCl_2$, 20 mM 2-mercaptoethanol, and 40 mM NH_4Cl (or 125 mM NH_2OH in studies indicated by italics); P_i or hydroxamate was determined. Control value, 20 units/mg.

‡ Activity was determined in mixtures containing 125 mM NH_2OH, 50 mM L-glutamate, and 50 mM $MgCl_2$; otherwise as given above. Control value, 82 units/mg.

§ Concentration given in parentheses (mM).

The substrate specificity of rat liver glutamine synthetase is similar to that of the ovine brain enzyme. The apparent K_m value for L-glutamate is 5×10^{-3} M in the presence of either Mg^{++} or Mn^{++}; the corresponding V_{max} values are 160 and 33 units per mg, respectively. Like the ovine brain enzyme, the rat liver enzyme acts on D-glutamate, only on the L-isomer of α-methylglutamate, and on various other glutamate analogs, including β-glutamate, *cis*-cycloglutamate, and *threo*-γ-methyl-L-glutamate (21). It may thus be concluded that the conformation of L-glutamate at the active site is similar to that deduced for the brain enzyme, i.e., L-glutamate attaches in an extended conformation in which its α-hydrogen atom is directed away from the enzyme (21).

Previous studies (9, 10, 22) showed that the convulsant methionine sulfoximine is an irreversible inhibitor of the brain enzyme by virtue of its ability to attach to the glutamate- and ammonia-binding sites of the enzyme; thus, when methionine sulfoximine is incubated with enzyme, ATP, and metal ions, it is converted to methionine sulfoximine phosphate, which is tightly attached to the enzyme. Of the four optical isomers of methionine sulfoximine, only L-methionine-*S*-sulfoximine inhibits ovine brain (23), mouse brain (24), and liver (24) glutamine synthetases and causes convulsions in mice (24). Rat liver glutamine synthetase is also inhibited irreversibly by methionine sulfoximine, and methionine sulfoximine phosphate was identified in the present work as the enzyme-bound product. Experiments were performed in which the enzyme was incubated with either [^{35}S]- or [methyl-^{14}C]L-methionine sulfoximine in the presence of ATP and metal ions. After gel filtration or extensive dialysis, about 4 mol of methionine sulfoximine phosphate was bound per mol of completely inactivated enzyme. A maximum of 4.6 mol of methionine sulfoximine phosphate was bound per mol of enzyme, as determined by equilibrium dialysis.

Regulation of rat liver glutamine synthetase

A large number of compounds were tested as possible effectors of the enzyme's activity; the effects of some of these on Mn^{++}- and Mg^{++}-dependent glutamine synthetase activity are given in Table 1. (Comparative data on ovine brain glutamine synthetase are included.) The liver enzyme is significantly activated by α-ketoglutarate, and less so by citrate, in the presence of Mg^{++} or Mn^{++}; α-ketoglutarate increased activity by about two-fold (Fig. 1). Other compounds including isocitrate, pyruvate, glyoxylate, phenylpyruvate, and succinate had no effect. A survey of amino acids revealed that glycine, L-alanine, and L-serine inhibited the Mn^{++}-dependent activity, while the Mg^{++}-dependent activity was not affected by these compounds. It is curious that the rat liver enzyme is inhibited by D-alanine and β-alanine; indeed, D-alanine inhibits more than L-alanine. L-Aspartate, L-asparagine, L-histidine, and γ-aminobutyrate (each 20 mM) did not significantly affect the activities. Of the nucleotides examined, CTP inhibited in the presence of Mn^{++}; with Mg^{++} there was slight stimulation. It may be relevant that the ovine brain enzyme can use GTP and CTP, in place of ATP, in glutamine synthesis with Mn^{++} but not Mg^{++} (8). DPN^+ activated the liver

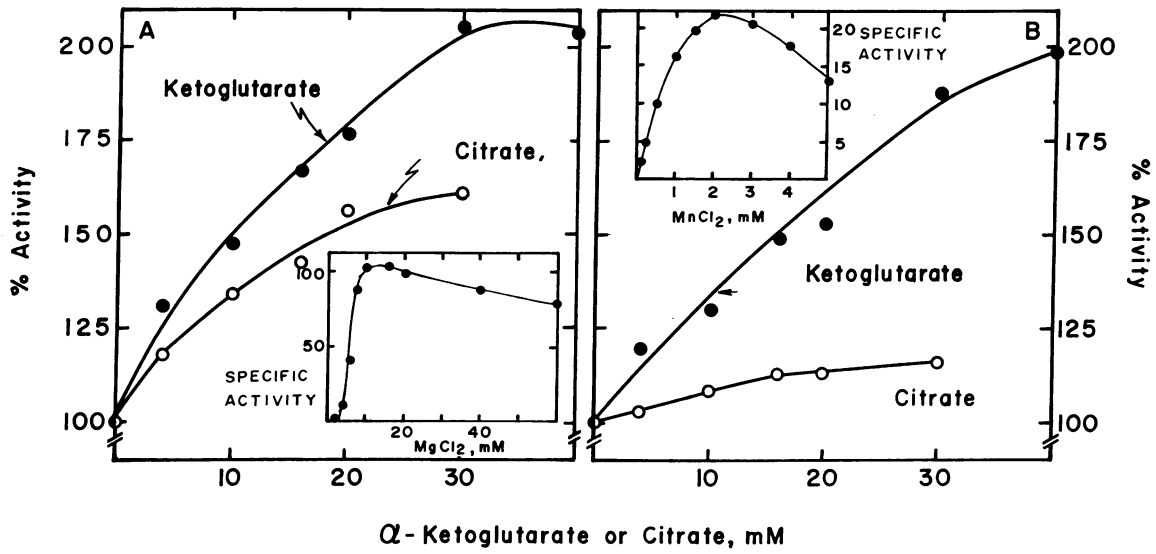

FIG. 1. Effect of α-ketoglutarate and citrate on the activity of rat liver glutamine synthetase. The synthesis of γ-glutamylhydroxamate was measured in solutions containing 0.1 M imidazole·HCl buffer (pH 7.2), 20 mM 2-mercaptoethanol, 50 mM L-glutamate, 10 mM ATP, 125 mM NH_2OH, either 50 mM $MgCl_2$ (*A*) or 2 mM $MnCl_2$ (*B*), and α-ketoglutarate and citrate as shown. The insets show the effect of varying either $MgCl_2$ or $MnCl_2$ concentration on the activity in the absence of the effectors.

enzyme to a small extent, while AMP, UTP, and UMP had no effect. Carbamyl phosphate markedly inhibited the liver and brain enzymes in the presence of Mn^{++}; the Mg^{++}-dependent activity was less affected. D-Glucosamine-6-phosphate did not significantly affect the activities. The effect of increasing concentrations of glycine, L-alanine, and D-alanine on the activity of the liver enzyme at saturating concentrations of glutamate and ATP is described in Fig. 2*A*. Inhibition is maximal at about 70%. When either L-alanine was added to mixtures containing saturating concentrations of glycine, or the reverse, there was no further inhibition. Inhibition by glycine and alanine increases as glutamate concentration increases (Fig. 2*B*) and is independent of ammonia concentration over the range 2–20 mM.

DISCUSSION

Inhibition of rat liver glutamine synthetase by glycine and L-alanine is in accord with other work in this laboratory (25), which showed that glyoxylate and pyruvate are by far the most active α-keto acid substrates for highly purified rat liver glutamine transaminase. Thus, there may be substantial utilization of the α-amino group of glutamine for synthesis of alanine and glycine in liver; the reported inhibition by glycine and alanine of glutamine synthetases of other cells (2, 26) may reflect analogous phenomena. However, inhibition of liver glutamine synthetase by glycine and alanine is not cumulative as it is in *E. coli* (2, 27). The potentiation by glutamate of the glycine and alanine inhibitions seems to reflect the requirement of substrate for the binding of these ef-

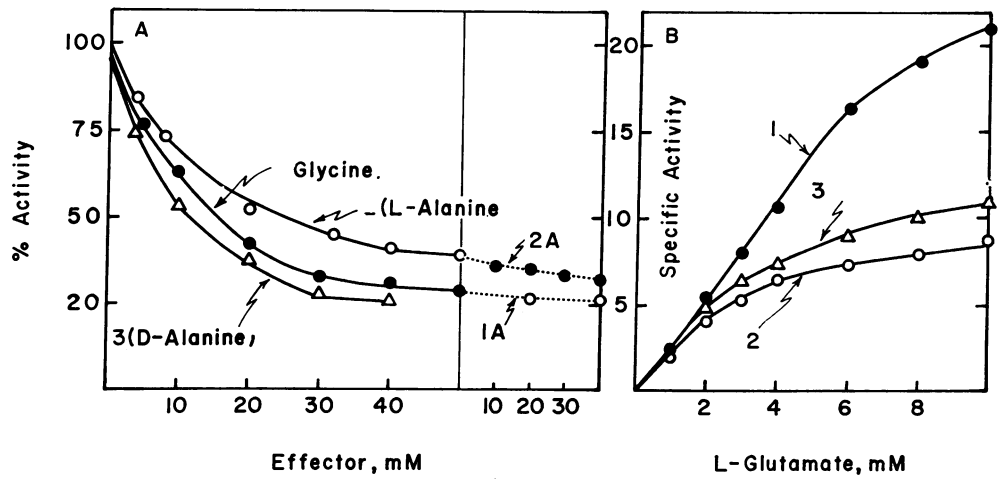

FIG. 2. Effect of glycine and alanine on rat liver glutamine synthetase activity. Assay solutions contained 10 mM ATP, 2 mM $MnCl_2$, and 40 mM NH_4Cl; inorganic phosphate was determined. (*A*) The concentrations of glycine and alanine were varied; L-glutamate, 10 mM. In *Curves 1A* and *2A*, L-alanine and glycine were added to solutions containing 50 mM glycine or 50 mM L-alanine, respectively. (*B*) The effect of L-glutamate concentration on the activity in the *absence* (*Curve 1*) and *presence* of 20 mM glycine (*Curve 2*) or 20 mM L-alanine (*Curve 3*).

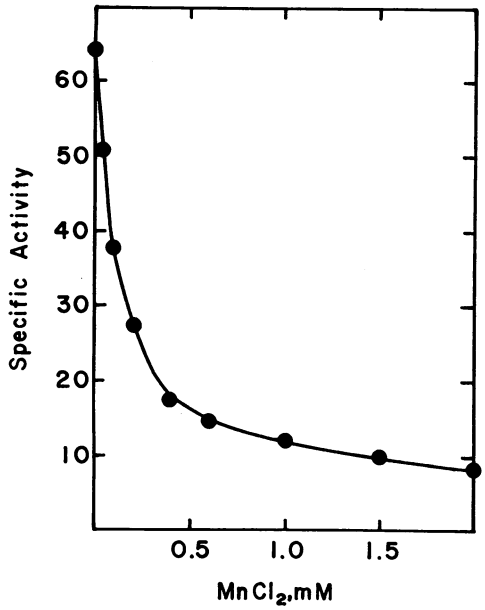

FIG. 3. Effect of $MnCl_2$ on the Mg^{++}-dependent activity. Glutamine synthetase activity was determined by the hydroxamate assay in solutions containing 50 mM L-glutamate, 10 mM ATP, 125 mM NH_2OH, and 100 mM $MgCl_2$.

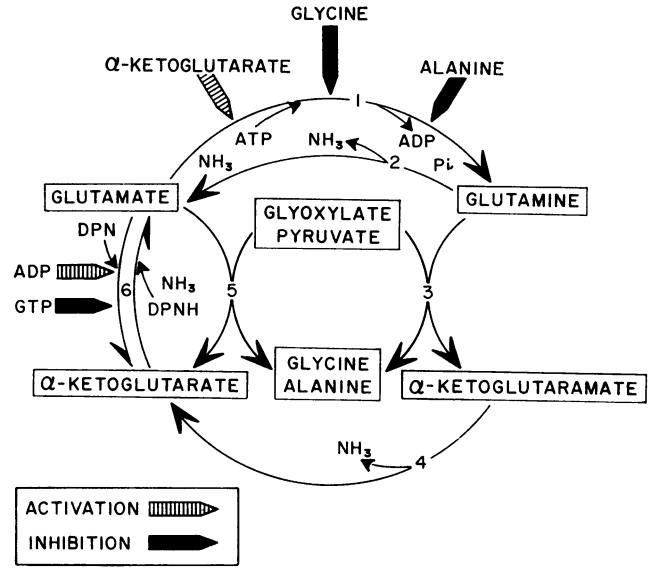

FIG. 4. Metabolic relationships between glutamine and other compounds in liver. (*1*) glutamine synthetase; (*2*) glutaminase; (*3*) glutamine transaminase; (*4*) ω-amidase; (*5*) glutamate transaminases; (*6*) glutamate dehydrogenase.

fectors. Glutamine-dependent carbamyl phosphate synthetases are present in liver and other mammalian tissues (28); inhibition of liver glutamine synthetase by carbamyl phosphate can thus provide the cell with a means for regulating the supply of glutamine for pyrimidine biosynthesis.

Our data show that the inhibitions almost always require Mn^{++}. In addition, the synthesis reaction, with both the liver and brain enzymes, is much slower in the presence of Mn^{++} than of Mg^{++}. Addition of Mn^{++} to the Mg^{++} synthesis system, even at Mg^{++}:Mn^{++} ratios of 1000, markedly inhibits the liver enzyme (Fig. 3); similar results were obtained with the brain enzyme. The mechanism by which Mn^{++} makes the liver enzyme sensitive to the negative effectors is not yet clear; it is notable, however, that the liver enzyme becomes sensitive to inhibition by glycine, L-alanine, and carbamyl phosphate, even in the presence of Mg^{++}, when traces of Mn^{++} are added. The adenylylated form of the *E. coli* enzyme is specifically activated for synthesis by Mn^{++} and this form is much more sensitive to most of the inhibitors; however, the unadenylylated form is much more senstive to inhibition by glycine and L-alanine than is the adenylylated form (2, 27, 29). *Bacillus subtilis* glutamine synthetase, although not adenylylated, is inhibited by glycine and L-alanine, but only with Mn^{++} (26).

The striking activation of the liver enzyme by α-ketoglutarate (with either Mn^{++} or Mg^{++}) seems to provide a cellular mechanism by which "excess" α-ketoglutarate produced by

TABLE 2. *Comparison of glutamine synthetases from various cells*

Properties	Rat liver	Ovine brain	Pea	*E. coli*	*B. subtilis*
No. of subunits (subunit mol. wt.)	8(44,000)	8(49,000)	8(45,000)	12(50,000)	12(50,000)
Approx. molecular weight	352,000	392,000	360,000	592,000	600,000
$s_{20,w}$ (S)	15.0	15.0	14.0	20.3	19.3
Specificity: D-Glutamate	yes	yes	yes	yes	yes
α-Methyl-L-glutamate	yes	yes	yes	yes	ND
cis-Cycloglutamate	yes	yes	yes	yes	ND
threo-γ-Methyl-L-glutamate	yes	yes	yes	yes	ND
Inhibition: L-Methionine-*S*-sulfoximine	yes	yes	yes	yes	ND
Glycine, alanine	yes*	slight*	yes*	yes	yes*
Carbamyl phosphate	yes*	yes*	slight*	yes	ND
Tryptophan, histidine	no	no	slight	yes	yes
AMP	no	no	yes	yes	yes
Glucosamine-6-phosphate	no	no	slight	yes	ND
Glutamine	no	no	ND	no	yes
Activation: α-Ketoglutarate	yes	slight	slight*	no	ND
Adenylylated form	no	no	no	yes	no
Literature references	†	(18, 21)†	(‡)†	(2, 31, 32)	(26)

* With Mn^{++} only. † Present work. ‡ Y. Tsuda and A. Meister, unpublished data. ND, no data available.

the citric acid cycle or by transamination of glutamate or glutamine can stimulate glutamine formation (Fig. 4). It is pertinent to note that the effect of α-ketoglutarate in increasing the glutamine formation in *E. coli* is mediated indirectly by stimulating deadenylylation to produce more active enzyme and by inhibiting adenylylation (2, 30).

Our studies indicate that about 4 mol of methionine sulfoximine is bound per mol of totally inactivated rat liver enzyme, which has eight subunits. Although this interesting finding requires further investigation, we may consider several possible explanations: (*a*) binding of inhibitor to half the active sites influences the remaining, sites so as to prevent them from combining with substrate, (*b*) during isolation half of the enzyme's sites are inactivated, and (*c*) the enzyme normally uses only four subunits for catalysis, the remainder having been modified through evolution to function in regulation. The inhibition by β-alanine, glycine, L- and D-alanine may reflect steric resemblance between these compounds and those that can bind to the glutamate site (e.g., D-glutamate, β-glutamate).

Finally, we may compare some properties of the glutamine synthetases that have been isolated from various sources (Table 2). The bacterial enzymes are dodecameric and have molecular weights of about 600,000, while the brain, liver, and pea enzymes have eight subunits and lower molecular weights; however, all of the enzymes have subunits of about the same size. In addition, they all exhibit qualitatively the same substrate specificity and are inhibited by L-methionine-*S*-sulfoximine. Thus, the active catalytic sites of these enzymes (and the mechanisms of the reactions catalyzed) are probably quite similar. That the enzymes vary with respect to control mechanisms is consistent with known differences in their intracellular functions. It is conceivable that brain glutamine synthetase has functions that relate to neurotransmission as well as to metabolism; possibly there are two types of glutamine synthetase in the brain. The *E. coli* enzyme is the only glutamine synthetase known to occur in an adenylylated form, and only the rat liver glutamine synthetase is markedly activated by α-ketoglutarate. Nevertheless, the inhibitions by glycine, L-alanine, and carbamyl phosphate are fairly constant phenomena, as is sensitivity to Mn^{++}. It seems probable that Mn^{++} plays a central role in the regulation of the activity of glutamine synthetase.

This work was supported in part by a grant from the National Institutes of Health, U.S. Public Health Service. We thank Miss Fang Leu for technical assistance.

1. Meister, A., *Biochemistry of the Amino Acids* (Academic Press, New York, 1965), Vol. 2, p. 621.
2. Stadtman, E. R., B. M. Shapiro, A. Ginsburg, A. Kingdon, and M. D. Denton, *Brookhaven Symposia in Biology*, **21**, 378 (1968); Stadtman, E. R., B. M. Shapiro, H. S. Kingdon, C. A. Woolfolk, and J. S. Hubbard, *Advan. Enzyme Regul.*, **6**, 257 (1968); Stadtman, E. R., A. Ginsburg, J. E. Ciardi, J. Yeh, S. B. Hennig, and B. M. Shapiro, *Advan. Enzyme Regul.*, **8**, 99 (1970).
3. Holzer, H., *Advan. Enzymol.*, **32**, 297 (1969).
4. Meister, A., *Harvey Lect.*, Series 63, 139 (1967–68).
5. Meister, A., *Enzymes*, **6**, 443 (1962).
6. Wellner, V. P., and A. Meister, *Biochemistry*, **5**, 872 (1966).
7. Greenberg, J., and N. Lichtenstein, *J. Biol. Chem.*, **234**, 2337 (1959).
8. Monder, C., *Biochemistry*, **4**, 2677 (1965).
9. Ronzio, R. A., and A. Meister, *Proc. Nat. Acad. Sci. USA*, **59**, 164 (1968).
10. Ronzio, R. A., W. B. Rowe, and A. Meister, *Biochemistry*, **8**, 1066 (1969).
11. Fiske, C. H., and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
13. Davis, B. J., *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
14. Weber, K., and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
15. Ronzio, R. A., W. B. Rowe, S. Wilk, and A. Meister, *Biochemistry*, **8**, 2670 (1969).
16. Haschemeyer, R. H., papers presented before the Division of Biological Chemistry, 150th and 152nd National Meetings, American Chemical Society, 1965 (Atlantic City, abstract 68) and 1966 (New York, N.Y., abstract 46), respectively.
17. Haschemeyer, R. H., *Trans. N.Y. Acad. Sci.*, **30**, 875 (1968).
18. Haschemeyer, R. H., *Advan. Enzymol.*, **33**, 71 (1970).
19. Shapiro, A. L., E. Vinuela, and J. V. Maizel, Jr., *Biochem. Biophys. Res. Commun.*, **28**, 815 (1967).
20. Bowers, W. F., V. B. Czubaroff, and R. H. Haschemeyer, *Biochemistry*, **9**, 2620 (1970).
21. Meister, A., *Fed. Proc.*, **27**, 100 (1968); *Advan. Enzymol.*, **31**, 183 (1968).
22. Rowe, W. B., R. A. Ronzio, and A. Meister, *Biochemistry*, **8**, 2674 (1969).
23. Manning, J. M., S. Moore, W. B. Rowe, and A. Meister, *Biochemistry*, **8**, 2670 (1969).
24. Rowe, W. B., and A. Meister, *Proc. Nat. Acad. Sci. USA*, **66**, 500 (1970).
25. Meister, A., *Enzymes*, **6**, 193 (1962) and work with J. L. Cooper to be published.
26. Deuel, T. F., A. Ginsburg, J. Yeh, E. Shelton, and E. R. Stadtman, *J. Biol. Chem.*, **245**, 5195 (1970); Deuel, T. F., and E. R. Stadtman, *J. Biol. Chem.*, **245**, 5206 (1970).
27. Woolfolk, C. A., and E. R. Stadtman, *Biochem. Biophys. Res. Commun.*, **17**, 313 (1964).
28. Hager, S. E., and M. E. Jones, *J. Biol. Chem.*, **242**, 5674 (1967); Nakanishi, S., K. Ito, and M. Tatibana, *Biochem. Biophys. Res. Commun.*, **33**, 774 (1968); Tatibana, M., and K. Ito, *J. Biol. Chem.*, **244**, 5403 (1969).
29. Shapiro, B. M., H. S. Kingdon, and E. R. Stadtman, *Proc. Nat. Acad. Sci. USA*, **58**, 642 (1967).
30. Shapiro, B. M., and E. R. Stadtman, *Biochem. Biophys. Res. Commun.*, **30**, 32 (1968); Shapiro, B. M., *Biochemistry*, **8**, 659 (1969).
31. Valentine, R. C., B. M. Shapiro, and E. R. Stadtman, *Biochemistry*, **7**, 2143 (1968); Shapiro, B. M., and A. Ginsburg, *Biochemistry*, **7**, 2153 (1968).
32. Weisbrod, R., Doctoral dissertation, Cornell University Medical College, 1971.