Studies on the Mechanism of Inhibition of Glutamine Synthetase by Methionine Sulfoximine*

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ABSTRACT: Glutamine synthetase (from sheep brain) is markedly inhibited by methionine sulfone and by methionine sulfoximine. In contrast to methionine sulfone, methionine sulfoximine inhibits the enzyme irreversibly. The rate at which irreversible inhibition is established is decreased by glutamate and unaffected by ammonia (or hydroxylamine), but a mixture of both glutamate and ammonia can effectively prevent inhibition.

Inhibition by methionine sulfoximine requires the presence of adenosine triphosphate and Mg^{2+} (or Mn^{2+}) and is associated with the cleavage of adenosine triphosphate to adenosine diphosphate. The inhibited enzyme was separated by gel filtration and shown to contain close to 8 moles each of a phosphorylated derivative of methionine sulfoximine and adenosine triphosphate, which are tightly bound to the enzyme but which can be released by brief heating at 100° or by treatment with perchloric acid. The phosphorylated derivative of methionine sulfoximine can be converted by treatment with acid or by several phosphatases to equimolar quantities of inorganic phosphate and methionine sulfoximine. Studies on the stability of methionine sulfoximine phosphate to acid indicate that the phosphoryl moiety is not attached to the α-amino or α-carboxyl group. The findings indicate that the irreversible inhibition of glutamine synthetase by methionine sulfoximine is associated with its phosphorylation and the tight binding to the enzyme of methionine sulfoximine phosphate and adenosine diphosphate.

Methionine sulfoximine and several other methionine derivatives are known to act as antagonists of glutamic acid in certain systems and to be inhibitors of glutamine synthetase. Methionine sulfoximine was first isolated from nitrogen trichloride treated protein and was later identified as the factor responsible for the occurrence of seizures in dogs fed NCl_3-treated flour (Bentley *et al.*, 1951). Subsequent studies established that methionine sulfoximine exhibits convulsant activity in a number of animal species (see, for example, Proler and Kellaway, 1962; Peters and Tower, 1959). Administration of methionine sulfoximine to animals produces irreversible inhibition of cerebral glutamine synthetase. While it cannot be definitely concluded that the convulsant activity of methionine sulfoximine is related solely to its effect on glutamine synthetase, it seems probable that inhibition of this metabolically important enzyme would lead to significant physiological effects. The ability of methionine sulfoximine to inhibit glutamine synthetase was first reported by Pace and McDermott (1952) in studies on extracts of sheep brain acetone powder. This observation has been amply confirmed and extended by a variety of studies on the effects of methionine sulfoximine on glutamine synthesis and related phenomena (see, for example, Koloušek and Jiráček, 1959; Peters and Tower, 1959; Sellinger and Weiler, 1963; Tews and Stone, 1964; Warren and Schenker, 1964; Lamar and Sellinger, 1965; Ronzio and Meister, 1967; Sellinger, 1967).

Our interest in the inhibition of glutamine synthetase by methionine sulfoximine developed from earlier work in this laboratory on the specificity of glutamine synthetase and its relationship to substrate conformation at the active site of this enzyme (Kagan *et al.*, 1965; Khedouri and Meister, 1965; Kagan and Meister, 1966a, b; Wellner *et al.*, 1966; Gass and Meister, 1968; Meister, 1968). Thus, it was postulated that methionine sulfoximine, which can assume a conformation analogous to that postulated for glutamate, attaches to the glutamate and ammonia binding sites of the enzyme (Ronzio and Meister, 1968). In the course of this work, we have found that methionine sulfoximine, in the presence of the enzyme, ATP, and metal ions (Mg^{2+} or Mn^{2+}), is converted into a phosphorylated derivative, which becomes tightly attached to the enzyme thus inhibiting it irreversibly. The present communication gives the details of these studies.

Experimental Section

Materials

L-Methionine (Schwartz BioResearch, Inc.), L-methionine sulfone, DL-methionine *dl*-sulfoximine (Nutritional Biochemicals Corp.), and L-methionine *dl*-sulfoximine (Calbiochem) were obtained from the sources indicated. D-Methionine and L-methionine were prepared by enzymatic resolution of DL-methionine (Greenstein, 1954). L- and D-methionine *dl*-sulfoxide

* From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received October 25, 1968.* Supported in part by the National Science Foundation and the National Institutes of Health, U. S. Public Health Service. Preliminary accounts of this work have appeared (Ronzio and Meister, 1967, 1968; Rowe *et al.*, 1968).

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and L- and D-methionine *dl*-sulfoximine were prepared as described by Bentley *et al.* (1951). L-Methionine *d*-sulfoxide and L-methionine *l*-sulfoxide were prepared from L-methionine *dl*-sulfoxide by separation as the picrates (Lavine, 1947). L-Methionine sulfone was also prepared by the method of Toennies and Kolb (1939).

AMP, ADP, ATP (Sigma Chemical Corp.), [β,γ-^{32}P]ATP, [8-^{14}C]ATP, and L-[^{14}C-methyl]methionine (Schwartz BioResearch, Inc.) were used in these studies. ADP was purified by paper electrophoresis at pH 3.6 followed by elution of ADP from the paper with water. The labeled ATP preparations contained less than 3% of ADP and P_i. The [β,γ-^{32}P]ATP contained 98.2–99.9% of ^{32}P in the β- and γ-phosphoryl positions as determined by hydrolysis for 7 min in 1 N HCl at 100°; no radioactivity was detected in the AMP moiety. *p*-Bis[2-(5-phenyloxazolyl)]benzene and 2,5-diphenyloxazole were obtained from the New England Nuclear Corp. Sephadex G-50 and Dowex 50 (8X) were obtained, respectively, from Pharmacia and BioRad.

Preparations of radioactive ADP (^{14}C and β-^{32}P) were obtained from [^{14}C]ATP and [β,γ-^{32}P]ATP, respectively, by use of the glutamine synthesis reaction. The following reaction mixture was incubated for 2.5 hr at 37°: enzyme (0.14 μM), $MgCl_2$ (0.02 M), sodium glutamate (0.05 M), NH_4Cl (0.1 M), imidazole-HCl buffer (pH 7.2, 0.02 M), 2-mercaptoethanol (0.025 M), and labeled ATP (0.05–0.1 mM); carrier ADP (5 mM) was added and the mixture was subjected to paper electrophoresis at pH 3.7. The ADP was eluted from the paper with water; electrophoresis was repeated if necessary. No radioactive ATP could be detected in the final ADP product.

L-[^{14}C-Methyl]methionine *dl*-sulfoximine (1.6×10^6 cpm/mμmole) was synthesized from L-[^{14}C-methyl]-methionine by the procedure of Bentley *et al.* (1951). The crude product, which contains methionine sulfoxide, was purified by chromatography on a column of Dowex 50 (H^+; 1.0×7.5 cm diameter). A solution containing about 1 mM of product was adjusted to pH 1.5 by addition of HCl and then applied to the column. After washing the column with 50 ml of water the column was eluted with 0.05 M sodium citrate buffer (pH 2.5). Methionine sulfoxide was eluted between 45 and 75 ml of effluent; methionine sulfoximine was then eluted with sodium citrate buffer of pH 4.5 (prepared by adding 5 M NaOH to the pH 2.5 citrate buffer). The product emerged from the column between 130 and 180 ml of effluent. The fractions containing methionine sulfoximine were pooled and diluted by adding two volumes of water; the pH was adjusted to 1.5 by addition of HCl. This solution was applied to another column of Dowex 50 (H^+) which was then washed with 50 ml of water. Elution of methionine sulfoximine was carried out with 1 N NH_4OH and the effluent was concentrated by evaporation *in vacuo* at 50°. The labeled L-methionine sulfoxide obtained as a by-product in this procedure was desalted in the same way. L-[^{14}C-Methyl]methionine sulfone was prepared from L-[^{14}C-methyl]methionine sulfoxide by the procedure of Toennies and Kolb (1939).

Glutamine synthetase was isolated from sheep brain as described (Ronzio, Rowe, and Meister, submitted).

The authors are indebted to Dr. Gerhard Schmidt for samples of alkaline phosphatase (calf intestine) and acid phosphatases (yeast, prostate). We are grateful to Dr. R. T. Simpson and Dr. Bert L. Vallee for the *E. coli* phosphatase. The calf intestine phosphatase exhibited an activity of 10.1 units/mg in 0.1 M Tris-HCl buffer (pH 9) at 37° using *p*-nitrophenyl phosphate as substrate. (A unit is defined as the amount of enzyme that hydrolyzes 1 mmole of this substrate/min; this applies to the several phosphatases used here.) The yeast acid phosphatase exhibited an activity of 2.4×10^{-3} unit/mg in 0.1 M sodium acetate buffer (pH 4). The prostate acid phosphatase exhibited an activity of 1.41 units/mg in sodium acetate buffer (pH 5.6). The *E. coli* phosphatase exhibited an activity of 43.4 units/mg in 0.02 M Tris-HCl buffer (pH 8.0).

Methods

Synthetase activity was determined by the γ-glutamyl hydroxamate assay method previously described (Wellner and Meister, 1966), except that NaCl-free hydroxylamine was used. Manganous ions at a concentration greater than 5 μM produce appreciable inhibition of γ-glutamyl hydroxamate synthesis; for this reason, when the enzyme solution to be assayed contained $MnCl_2$, the concentration of manganous ion was adjusted so that it did not exceed 5 μM. In some experiments, the amount of γ-glutamyl hydroxamate formed was determined after 7.5-min incubation; all of the results are expressed in terms of micromoles of hydroxamate formed per 15 min.

Measurements of absorbancy were carried out with a Zeiss Model PMQ II spectrophotometer using Quartz cells with a 1-cm light path.

Paper electrophoresis was performed at 20 ± 3° with the following buffer systems: 0.5 N acetic acid, 0.4 N formic acid (pH 2.5), 0.5 N acetic acid (pH 2.6), 0.075 M ammonium formate (pH 3.6), 0.05 M ammonium acetate (pH 5.5), 0.05 M potassium phosphate (pH 7.5), and 0.05 M ammonium bicarbonate (adjusted to pH 9.0 by adding ammonium hydroxide). Whatman No. 3MM paper was used; the paper was prepared prior to use by washing with 5% formic acid containing 2 mM EDTA followed by water. The effective length of the paper strips was 40 cm; the potential gradient was 25–40 V/cm. Picric acid and caffeine were used as reference compounds. At pH 2.6, methionine sulfoximine phosphate moves as an anion and is clearly separated from methionine sulfoximine, methionine sulfoxide, methionine sulfone, ADP, and P_i (Figure 1).

Paper chromatography (ascending) was carried out with the following solvent systems: solvent I, isobutyric acid–water–concentrated ammonium hydroxide (66:33:1, v/v); solvent II, concentrated formic acid–*sec*-butyl alcohol–water (70:15:15, v/v); solvent III, 95% ethanol–1 N ammonium acetate (pH 5.5) (70:30, v/v); solvent IV, *n*-butyl alcohol–acetic acid–water (50:25:25, v/v); and solvent V, *t*-butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15, v/v). The R_F values for

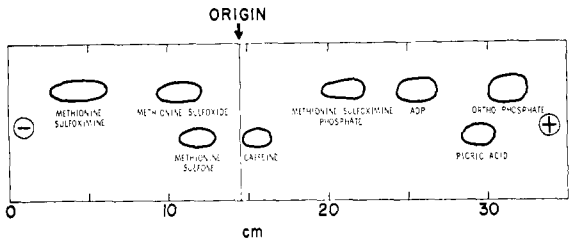

FIGURE 1: Paper electrophoretic separation of methionine derivatives, ADP, and P_i at pH 2.6 (see the text).

methionine sulfoximine, methionine sulfoximine phosphate, and P_i were, respectively, I: 0.64, 0.26, 0.31; II: 0.13, 0.09, 0.26; III: 0.37, 0.14, 0.29; IV: 0.24, 0.14, 0.39; and V: 0.27, 0.15, 0.70. The position of inorganic phosphate and compounds containing phosphate was determined after chromatography by spraying the chromatograms with ammonium molybdate according to the method of Bandurski and Axelrod (1952). The adenosine compounds were located by examination of the dried chromatogram under ultraviolet light; amino acids were located by the ninhydrin procedure.

Determinations of radioactivity (^{14}C and ^{32}P) were carried out with a Nuclear-Chicago liquid scintillation counter using double-isotope technique. Liquid samples (5–50 μl) were counted in dioxane solution (Bray, 1960). Radioactivity on dry Whatman 3MM paper strips (0.5–1.0 cm wide, 1.8 cm long) was measured in a toluene solution containing 0.05 g of *p*-bis[2-(5-phenyloxazolyl)]-benzene and 4 g of 2,5-diphenyloxazole per l. All determinations of radioactivity were made with a precision of $\pm 5\%$.

P_i was determined by the Leloir and Cardini (1957) modification of the method of Fiske and Subbarow (1925).

The quantitative ninhydrin procedure of Rosen (1957) was employed for the determination of amino acid.

Gel filtration was carried out as follows. Columns (0.8 cm × 18 cm) were prepared from deaerated Sephadex G-50 and equilibrated with the appropriate buffer at 22–25°. The enzyme solution (0.25–0.6 ml) was added to the top of the column and elution was carried out with the appropriate buffer with a flow rate of 0.3–0.5 ml/min. Fractions of ~0.35 ml were collected in 0.5-ml tubes at 0°. In these studies, protein emerged from the column between 2.5 and 4 ml of effluent while low molecular weight compounds emerged between 6.0 and 11.5 ml. The concentration of enzyme was determined by measuring γ-glutamyl hydroxamate synthesis or by the method of Lowry *et al.* (1951). When enzyme activity was determined, the aliquot taken for assay was sufficiently small so that the maximum Mn^{2+} concentration was 5 μM. Separate experiments showed that Mn^{2+} at a concentration of 5 μM produced less than 6% inhibition using the standard assay procedure. Enzyme-bound nucleotide, labeled with either ^{32}P or ^{14}C, was measured by determining the amount of radioactivity present in 2.5–50-μl aliquots of each fraction. The binding of unlabeled nucleotide in the protein fraction was measured after precipitating the protein at 0° by addition of perchloric acid (final concentration 7.5%); the absorbancy at 257 mμ was measured (Wellner and Meister, 1966). In a number of experiments the binding of nucleotide was studied under equilibrium conditions similar to those employed by Hummel and Dreyer (1962). In these studies the column was equilibrated with 1.6–150 μM nucleotide, 0.02 M $MnCl_2$, 0.005 M Tris-HCl buffer (pH 7.2), and 0.15 M KCl; the bound nucleotide could also be determined from the difference between the trough following the protein peak and the initial equilibration nucleotide concentration.

TABLE I: Effect of Various Methionine Derivatives on Glutamine Synthetase Activity.

Compound Added[a]	Inhibn (%)
L-Methionine	0
D-Methionine	0
L-Methionine *l*-sulfoxide	14
L-Methionine *d*-sulfoxide	11
D-Methionine *dl*-sulfoxide	7
L-Methionine sulfone	67
L-Methionine *dl*-sulfoximine	76
D-Methionine *dl*-sulfoximine	0

[a] The methionine derivative was added to the standard γ-glutamyl hydroxamate assay system (Wellner and Meister, 1966), and the reaction was started by addition of the enzyme. The reaction mixtures contained initially sodium L-glutamate (0.05 M), NH_2OH (0.10 M), NaATP (0.01 M), $MgCl_2$ (0.02 M), imidazole-HCl buffer (pH 7.2, 0.05 M), 2-mercaptoethanol (0.025 M), methionine derivative (0.025 M), and enzyme (10 mμM); after incubation at 37° for 15 min, the formation of γ-glutamyl hydroxamate was measured.

Results

Inhibition of Glutamine Synthetase Activity by Various Methionine Derivatives. The data given in Table I summarize the effects of adding a number of methionine derivatives to the standard γ-glutamyl hydroxamate assay system. Under these conditions, L-methionine *dl*-sulfoximine and L-methionine sulfone produced marked inhibition of synthetase activity. Much less inhibition was observed with L-methionine *l*-sulfoxide and L-methionine *d*-sulfoxide; little or no inhibition was observed with D-methionine *dl*-sulfoxide, D-methionine *dl*-sulfoximine, L-methionine, and D-methionine. The inhibition by L-methionine sulfone was examined as a function of L-glutamate concentration and the data were plotted according to the method of Lineweaver and Burk (1934). The findings (Figure 2) are consistent with a mixed type of inhibition (Dixon and Webb, 1964). A markedly different result was obtained when similar studies were carried out with L-methionine *dl*-sulfoximine (Figure 3). These findings

are consistent with irreversible inhibition, and this was demonstrated by subsequent studies which are described below. It is of interest that at relatively low concentrations of L-methionine *dl*-sulfoximine (curve 2, Figure 3) the reciprocal plot yields a straight line which intersects the curve for the uninhibited enzyme (curve 1) at the ordinate. This suggests that glutamate competes with methionine sulfoximine under these conditions. When the enzyme was preincubated with L-methionine sulfone in the presence of ATP and magnesium ions prior to dilution and assay, there was only slight, perhaps negligible, inhibition. On the other hand, when the enzyme was preincubated in this manner with L-methionine sulfoximine, marked inhibition was observed. The experiments described in Figure 4 demonstrate that there is substantial difference between the type of inhibition produced by methionine sulfone and that caused by methionine sulfoximine. In the experiments represented by curve 1 (Figure 4), the enzyme was incubated with 0.04 M L-methionine sulfone in the presence of ATP and magnesium ions; after 7.5 and 32 min, samples of the reaction mixture were added to a large volume of assay solution containing 0.005 M L-glutamate in which the synthetase activity was determined. There was little if any irreversible inhibition of the enzyme. In the experiments described by curve 2 (Figure 4), the enzyme was incubated with 0.001 M L-methionine sulfoximine, ATP, and magnesium ions; at various intervals samples were removed and added to a large volume of a solution containing ATP, magnesium ions, and 0.1 M L-glutamate. This mixture was incubated for 10 min, after which assay of glutamine synthetase activity was carried out in the presence of 0.05 M L-glutamate. As indicated in curve 2, the inhibition of the enzyme increased with duration of preincubation. In a similar experiment (curve 3) in which the 10-min incubation with glutamate prior to assay was omitted and in which a lower concentration (0.005 M) of L-glutamate was used, inactivation of the enzyme during preincubation occurred more rapidly. However, after 32-min preincubation, inactivation of the enzyme was substantially the same in the experiments described by curves 2 and 3. The difference between the rates of inactivation in these two experiments provides additional evidence that glutamate competes with methionine sulfoximine.

Many experiments in this laboratory have confirmed the observation that L-methionine sulfoximine in the presence of ATP and either manganous or magnesium ions irreversibly inactivates glutamine synthetase. There is an absolute requirement for ATP and one of these metal ions for irreversible inactivation. For example, 98–100% of the activity is inhibited when the enzyme is incubated at pH 7.2 and 37° for 15 min with 0.005 M L-methionine *dl*-sulfoximine, 0.01 M ATP, and 0.02 M magnesium chloride (or 0.002 M manganese chloride); no inhibition occurs if metal ion or ATP is omitted. A number of procedures have been employed in an effort to reactivate the methionine sulfoximine inactivated enzyme. For example, incubation of the inactivated enzyme for 1 hr at 37° with 0.05 M L-glutamate, 0.01 M ATP, 0.02 M magnesium chloride,

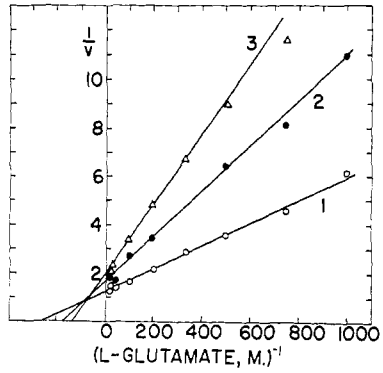

FIGURE 2: Inhibition of glutamine synthetase activity by methionine sulfone. The reaction mixtures contained sodium L-glutamate (0.001–0.045 M, as indicated), NH_2OH (0.10 M), NaATP (0.01 M), $MgCl_2$ (0.02 M), imidazole-HCl buffer (pH 7.2; 0.05 M), 2-mercaptoethanol (0.025 M), L-methionine sulfone (curve 1, none; curve 2, 0.001 M; curve 3, 0.002 M), and enzyme (8.5 mμM); incubated at 37° for 15 min; volume, 1 ml. Ordinate: (μmoles of γ-glutamylhydroxamate formed)$^{-1}$. Abscissa: (L-glutamate concentration (M))$^{-1}$.

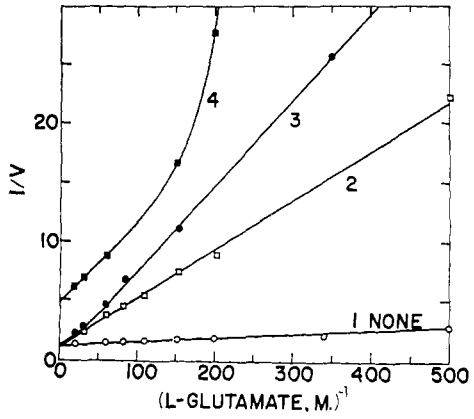

FIGURE 3: Inhibition of glutamine synthetase activity by methionine sulfoximine. The reaction mixtures contained sodium L-glutamate (0.002–0.05 M, as indicated), NH_2OH (0.10 M), NaATP (0.01 M), $MgCl_2$ (0.02 M), imidazole-HCl buffer (pH 7.2, 0.05 M), 2-mercaptoethanol (0.025 M), L-methionine *dl*-sulfoximine (curve 1, none; curve 2, 0.002 M; curve 3, 0.005 M; curve 4, 0.015 M), and enzyme (7.1 mμM); incubated at 37° for 15 min; volume, 1 ml. Ordinate: (μmoles of γ-glutamylhydroxamate formed)$^{-1}$. Abscissa: (L-glutamate concentration (M))$^{-1}$.

and 0.1 M ammonium chloride did not restore detectable synthetase activity. Chromatography of the inactivated enzyme on DEAE-cellulose and precipitation of the enzyme with 60% ethanol likewise did not restore synthetase activity. Other procedures were also used including, for example, treatment of the inactivated enzyme with 6 M urea, dialysis (against 50 mM Na-EDTA in 50 mM imidazole-HCl (pH 7.2) buffer for 24 hr), and gel filtration; the results were similar.

As indicated in Figure 5, neither ammonia nor hydroxylamine (curves 2A and 2B) decreased the rate at which the enzyme was inhibited by methionine sulfoximine in the presence of ATP and magnesium ions. However, as stated above, addition of L-glutamate

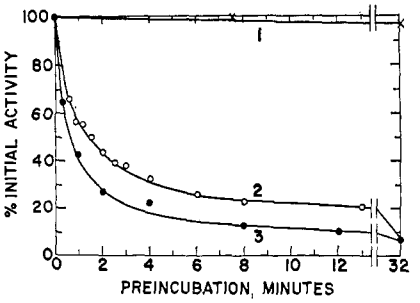

FIGURE 4: Effect of preincubation of enzyme with inhibitor on the inhibition of glutamine synthetase by methionine sulfone and methionine sulfoximine. Curve 1: a mixture (volume, 0.1 ml) containing enzyme (2.27 μM), NaATP (0.01 M), $MgCl_2$ (0.02 M), 2-mercaptoethanol (0.01 M), KCl (0.15 M), imidazole-HCl buffer (pH 7.2, 0.1 M), and L-methionine sulfone (0.04 M) was incubated at 37° for 0–32 min. A sample (0.0025 ml) was taken at each time interval and added to 1 ml of standard assay solution (containing 0.05 M sodium L-glutamate) and incubated for 15 min; the γ-glutamyl hydroxamate formed was then determined. Curve 2: a mixture (volume, 0.1 ml) containing enzyme (2.27 μM), NaATP (0.01 M), $MgCl_2$ (0.02 M), 2-mercaptoethanol (0.01 M), KCl (0.15 M), imidazole-HCl buffer (pH 7.2, 0.1 M), and L-methionine *dl*-sulfoximine (0.001 M) was incubated for 0–32 min. A sample (0.0025 ml) was taken at each time interval and added to 0.5 ml of a solution containing NaATP (0.01 M), $MgCl_2$ (0.02 M), sodium L-glutamate (0.1 M), 2-mercaptoethanol (0.05 M), and imidazole-HCl buffer (pH 7.2; 0.05 M). This mixture was incubated for 10 min, at which time 0.5 ml of a solution containing NH_2OH (0.2 M), $MgCl_2$ (0.02 M), NaATP (0.01 M), and imidazole-HCl buffer (pH 7.2; 0.05 M) was added; after 15 min the reaction was terminated and the γ-glutamyl hydroxamate formed was determined. Curve 3: this experiment was identical with that described in curve 1, except that L-methionine *dl*-sulfoximine (0.005 M) was used in place of L-methionine sulfone and the concentration of L-glutamate used in the assay was 0.005 M.

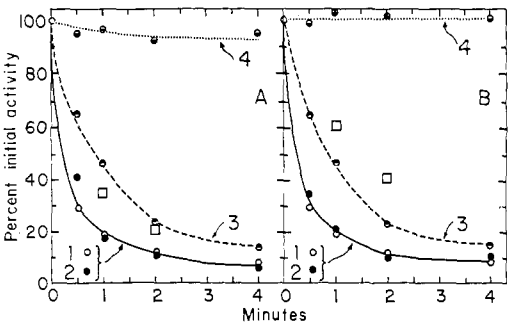

FIGURE 5: Effect of glutamate, ammonia, and hydroxylamine on the inhibition of glutamine synthetase by methionine sulfoximine. (A) Glutamate and NH_3: curve 1 (○—○—○—○): a reaction mixture (0.1 ml) containing enzyme (3.8 μM), $MgCl_2$ (0.02 M), 2-mercaptoethanol (0.01 M), and imidazole-HCl buffer (0.05 M, pH 7.2) was placed at 37° for 5 min. Then a solution (0.002 ml) containing L-methionine sulfoximine (0.25 M) and NaATP (0.5 M) was added, and the mixture was incubated at 37°. At the intervals indicated in the figure, samples (0.0025 ml) were removed and added to a final volume of 1 ml of standard assay solution; activity was determined from the amount of γ-glutamyl hydroxamate formed in 15 min. Curve 2 (●—●—●—●): the conditions were the same as for curve 1 except that NH_4Cl (0.05 M) was present in the reaction mixture. Curve 3: the conditions were the same as for curve 1 except that L-glutamate (0.05 M) was present in the reaction mixture. Curve 4: the conditions were the same as for curve 1 except that both NH_4Cl (0.05 M) and L-glutamate (0.05 M) were present in the reaction mixture. (□—□—□) The conditions were the same as for curve 4 except that the reaction mixture contained L-glutamate (0.005 M) and NH_4Cl (0.005 M). (B) Glutamate and NH_2OH: curves 1–4 (□—□—□) same as in A except that NH_2OH was added in place of NH_4Cl.

(0.05 M) did decrease the rate at which inhibition was established under these conditions (curves 3A and 3B). On the other hand, when both L-glutamate (0.05 M) and ammonium chloride (0.05 M) were added together (curve 4A), there was relatively little inhibition. Similar results were observed when L-glutamate and hydroxylamine were added together (curve 4B). Little or no protection against inhibition was observed when 0.05 M L-glutamine was added; with 0.25 M L-glutamine about half as much protection was obtained as found with 0.05 M L-glutamate alone.

Binding of Methionine Sulfoximine and Nucleotide to the Enzyme. In previous studies (Ronzio and Meister, 1968; Rowe *et al.*, 1968) evidence was obtained that the irreversible inhibition of glutamine synthetase by methionine sulfoximine in the presence of ATP and metal ion is associated with the binding of close to 8 moles each of methionine sulfoximine and ADP per mole of enzyme. These findings have been confirmed and extended by a series of experiments involving gel filtration and other procedures. In the present studies, somewhat larger columns of Sephadex G-50 were employed in the gel filtration studies than were used in earlier experiments reported from this laboratory (Wellner and Meister, 1966). Under the conditions of the previously reported experiments, it was possible to demonstrate the binding of ATP and ADP to the enzyme in the presence of manganese ions, but no binding of nucleotide was observed when manganese ions were replaced by magnesium ions. Under the present conditions of gel filtration, no binding of nucleotide was observed with either manganese or magnesium ions unless the column buffer contained both nucleotide and metal ions (Table II; expt 1 and 2). When a mixture containing enzyme, ATP, manganese ions, and methionine sulfoximine was subjected to gel filtration using a column buffer containing ATP and manganese ions, 7.4 moles of methionine sulfoximine was bound per mole of enzyme (Table II; expt 3) and the binding of nucleotide was about the same as that found in the absence of methionine sulfoximine (Table II; expt 2). It is of considerable interest, however, that appreciable amounts of nucleotide remained attached to the enzyme in the presence of methionine sulfoximine even when the column did not contain ATP and metal ion (Table II; expt 4). As shown in expt 5, little or no methionine sulfoximine became bound to the enzyme in the absence of ATP. Although binding of ATP in the presence of magnesium ions could not be demonstrated under the conditions of expt 6 (nor was nucleotide bound in the presence of Mg^{2+} under the conditions of gel filtration used earlier (Wellner and Meister, 1966)), addition of meth-

TABLE II: Binding of Methionine Sulfoximine and Nucleotide to the Enzyme.

Expt No.	Components of Mixture Applied to Column[a]	Column Buffer	Binding (moles/mole of enzyme)	
			Nucleotide	Methionine Sulfoximine
1[b]	ATP, Mn^{2+}		~0.5	
2	ATP, Mn^{2+}	ATP, Mn^{2+}	19	
3	ATP, Mn^{2+}, methionine sulfoximine	ATP, Mn^{2+}	18	7.4
4	ATP, Mn^{2+}, methionine sulfoximine		10	8.9
5	Mn^{2+}, methionine sulfoximine			~1
6	Mg^{2+}, ATP		0	
7	Mg^{2+}, ATP, methionine sulfoximine		6.1	7.9

[a] The mixtures (volume, 0.2 ml) contained enzyme (9–12 μM), KCl (0.15 M), EDTA (25–35 μM), and buffer (Tris-HCl buffer, pH 7.2, 0.005 M) plus the components listed in the table: NaATP (60–112 μM), $MnCl_2$ (0.002 M), $MgCl_2$ (0.02 M), L-[^{14}C]methionine sulfoximine (0.005 M). Under these conditions the enzyme was completely inhibited. The column buffer contained Tris-HCl (pH 7.2, 0.005 M), KCl (0.15 M), and, where indicated in the table, NaATP (60–112 μM) and $MnCl_2$ (0.002 M). The mixtures were incubated for 15 min at 37° and then applied to the column at 22–26°; fractions (0.3–0.4 ml) were collected at 0° and aliquots of these were removed for determinations of radioactivity, nucleotide, and protein. [b] [8-^{14}C]ATP was used.

ionine sulfoximine to reaction mixtures containing magnesium ions and ATP led to the binding of substantial amounts of nucleotide as well as methionine sulfoximine (Table II; expt 7).

Studies with L-[^{14}C-methyl]methionine sulfone similar to those described in Table II showed virtually no binding of this inhibitor to the enzyme.

In the earlier studies (Wellner and Meister, 1966) a maximum of about 16 moles of ATP was bound per mole of enzyme and the maximal amount of ADP bound was somewhat greater. The ratio (ADP:ATP) for maximal binding varied from 1.2 to 1.8 and appeared to parallel the corresponding ratio of the γ-glutamyl transfer activities observed in the presence of ADP and ATP. The enzyme preparation used in the present studies exhibited a ratio of 1.2 with respect to its ADP- and ATP-stimulated γ-glutamyl transferase activities. The maximal binding of ATP observed in the experiments described in Table II is not far from the average value of 16 observed previously.

Ultracentrifugal and electron microscope studies have shown that ovine brain glutamine synthetase is composed of eight apparently identical subunits (Haschemeyer, 1965, 1966). The present and earlier studies provide clear experimental evidence that glutamine synthetase can bind considerably more than 8 moles of nucleotide. It therefore seemed of interest to examine the binding of ATP as a function of ATP concentration. This was carried out under conditions similar to those employed in expt 2 (Table II) using a column buffer containing ATP and magnesium ions. Figure 6 gives the details of the gel filtration experiment summarized in expt 2 (Table II). When a series of experiments of this type were carried out with various concentrations of ATP, the binding of ATP was found to follow a curve which exhibited a sharp change in slope at a binding value close to 7.5 moles of ATP/mole of enzyme (Figure 7). In these studies, the maximal binding of ATP was approximately 17 moles/mole of enzyme. However, the ATP concentration required for maximal binding was about ten times higher than that required for the binding of about 8 moles of ATP/mole of enzyme.

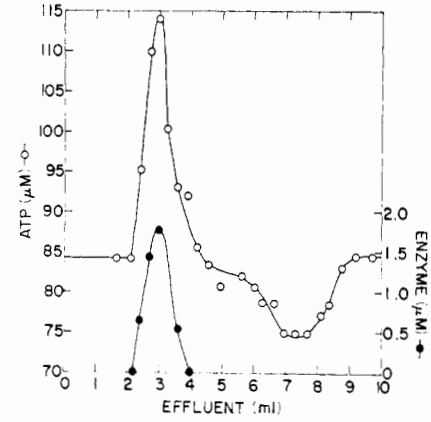

FIGURE 6: Binding (equilibrium) of nucleotide to the enzyme. A mixture (volume, 0.2 ml) containing enzyme (8.6 μM), $MnCl_2$ (0.002 M), NaATP (85 μM), KCl (0.15 M), EDTA (0.025 mM), and Tris-HCl buffer (pH 7.2, 0.005 M) was incubated at 37° for 15 min and then placed on a column of Sephadex G-50 as described in the text. Elution was carried out at 22–24° with a solution containing ATP (85 μM), KCl (0.15 M), Tris-HCl buffer (pH 7.2, 0.005 M), and $MnCl_2$ (0.002 M). Aliquots of the fractions were taken for determinations of enzyme and nucleotide.

TABLE III: Characterization of Enzyme-Bound Compounds after Incubation of the Enzyme with [β,γ-^{32}P]ATP and [^{14}C]Methionine Sulfoximine.

Expt No.	Isotope	Total Radioactivity Bound (cpm)	Methionine Sulfoximine Derivative (cpm)	ADP (cpm)	Moles Bound/Mole of Enzyme: Methionine Sulfoximine Derivative	Moles Bound/Mole of Enzyme: ADP
1[a]	^{14}C	1.72×10^4	1.69×10^4		7.9	
	^{32}P	1.24×10^4	0.60×10^4	0.64×10^4	7.9	8.6
2[b]	^{14}C	4.96×10^4	4.30×10^4		6.8	
	^{32}P	2.53×10^6	1.22×10^6	1.23×10^6	7.4	7.4

[a] The reaction mixture contained enzyme (8.5 μM), $MgCl_2$ (0.02 M), [β,γ-^{32}P]NaATP (1.67 mM; 370 cpm/mμmole of phosphate), L-[^{14}C]methionine *dl*-sulfoximine (5 mM; 1240 cpm/mμmole), KCl (0.1 M), imidazole-HCl buffer (pH 7.2, 0.01 M), and NaEDTA (0.1 mM) in a final volume of 0.30 ml. The mixture was incubated at 37° for 30 min after which time less than 2% of the initial enzyme activity remained. It was then added to the top of a Sephadex G-50 column equilibrated with ammonium bicarbonate buffer (pH 8, 0.05 M) containing NaEDTA (0.1 mM). The column was eluted with the same buffer and aliquots of the fractions collected were used for determinations of protein and radioactivity. The protein peak (2.55 mμmoles) contained 20.2 mμmoles of ^{14}C (calculated as methionine sulfoximine) and 41.2 mμmoles of ^{32}P. A sample of the pooled fractions containing the enzyme was heated at 100° for 1 min and then cooled in ice. The precipitated protein was removed by centrifugation and the supernatant solution was lyophilized; the residue was dissolved in a small amount of distilled water and subjected to paper electrophoresis in the presence of carrier methionine sulfoximine, ATP, ADP, and P_i. The areas of the paper strip corresponding to methionine sulfoximine, methionine sulfoximine derivative, ATP, ADP, and P_i were cut out and used for determinations of radioactivity. Less than 1% of the ^{32}P was found as P_i and ATP. [b] The reaction mixture contained enzyme (5.8 μM), $MgCl_2$ (0.02 M), [β,γ-^{32}P]NaATP (0.41 mM; 31,600 cpm/mμmole of phosphate), L-[^{14}C]methionine *dl*-sulfoximine (5 mM; 1240 cpm/mμmole), and imidazole-HCl buffer (pH 7.2, 0.05 M) in a final volume of 1.0 ml. The mixture was incubated at 37° for 30 min after which time less than 5% of the initial enzyme activity remained. The sample was then subjected to gel filtration with a buffer consisting of Tris-HCl (pH 7.2, 0.05 M). The protein peak (5.21 mμmoles) contained 40.5 mμmoles of ^{14}C and 83.5 mμmoles of ^{32}P. The remainder of the experiment was carried out as described in expt 1, except that carrier AMP was also added; on paper electrophoresis, there was complete separation of ADP, methionine sulfoximine phosphate, and AMP. Less than 1% of the ^{32}P was found as P_i; 4% of the ^{32}P was present in ATP.

Studies on the Enzyme-Bound Methionine Sulfoximine Derivative. The experiments described above show that L-methionine sulfoximine inhibits glutamine synthetase irreversibly, that such inhibition requires ATP and metal ion, and that it is associated with the binding to the enzyme of both methionine sulfoximine and nucleotide. When gel filtration studies were carried out with ATP labeled with ^{32}P in the β- and γ-phosphoryl moieties and [^{14}C]methionine sulfoximine, the inactivated enzyme obtained by gel filtration was found to contain ^{14}C equivalent to about 8 moles of methionine sulfoximine and ^{32}P equivalent to about 16 moles of ^{32}P per mole of enzyme (Ronzio and Meister, 1968). Additional studies which confirm this finding are given in Table III. In these experiments, the inactivated enzyme was heated at 100° for 1 min; after cooling, the precipitated protein was removed by centrifugation and the supernatant solution was found to contain virtually all of the radioactivity. Similar results were obtained when the inactivated enzyme was treated with 4% perchloric acid or with 1 N NaOH. However, deproteinization with 60% ethanol did not release the radioactivity. Analysis of the supernatant solutions (obtained after deproteinization by heating or with perchloric acid) by paper electrophoresis revealed the presence of equimolar amounts of ADP and a new compound containing about equimolar amounts of ^{14}C and ^{32}P; little or no radioactivity was found in the areas of methionine sulfoximine, P_i, ATP, and AMP. Treatment of the new compound with 6 N HCl at 100° for 2 hr gave equimolar amounts of [^{32}P]P_i and [^{14}C]methionine sulfoximine; these were identified and separated by paper electrophoresis (Figure 1). The methionine sulfoximine phosphate derivative was distinguished from inorganic phosphate and methionine sulfoximine by paper chromatography in five solvent systems (see above, Methods). In addition, as described in Figure 8, methionine sulfoximine phosphate (A) was effectively separated from methionine sulfoximine (C) and methionine sulfone (B) by chromatography on a column of Dowex 50. In a separate experiment methionine sulfoxide was found to emerge from the column with methionine sulfone. The new compound (A) emerged as a symmetrical peak before the other compounds were eluted from the column. The radioactive methionine sulfoximine

TABLE IV: Hydrolysis of Methionine Sulfoximine Phosphate by Various Phosphatases.[a]

Phosphatase (units)	Conditions	% Hydrolysis	Molar Ratio P_i/Methionine Sulfoximine
Yeast acid (5.6×10^{-4})	pH 4.0; 0.1 M acetate	100	1.1
Prostate acid (1.9×10^{-2})	pH 5.6; 0.1 M acetate	100	0.9
Calf intestine alkaline (1.67×10^{-2})	pH 9.0; 0.1 M Tris-HCl	100	1.1
E. coli alkaline (1.85×10^{-2})	pH 8.0; 0.02 M Tris-HCl, 2 M NaCl	7	0.7
E. coli alkaline (1.85)	pH 8.0; 0.02 M Tris-HCl, 2 M NaCl	100	0.9

[a] The general procedure was as follows: enzymatically prepared [^{14}C,^{32}P]methionine sulfoximine phosphate (0.75 mμmole) was incubated with the phosphatase for 30 min in 0.06 ml at 37°. At the end of incubation 30 μg of unlabeled P_i and 27 μg of unlabeled methionine sulfoximine were added, the reaction mixture was spotted on moist Whatman 3MM paper, and paper electrophoresis was performed at pH 2.6 as described under Methods. The paper was dried and cut into 0.5 × 4.0 cm strips which were counted in a scintillation counter. In the studies with *E. coli* phosphatase, all of the radioactivity remained at the origin after electrophoresis; this was eluted with water and, after reelectrophoresis, satisfactory separations were obtained. Controls in which phosphatase was omitted showed less than 4% hydrolysis.

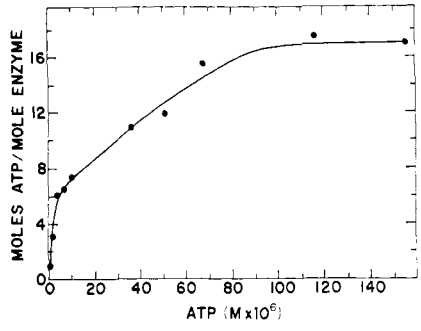

FIGURE 7: Effect of ATP concentration on binding of ATP to the enzyme. The procedure was similar to that used in Figure 6. Mixtures (volume, 0.4 ml) containing enzyme (12.5 μM), $MnCl_2$ (0.002 M), NaATP (as indicated), KCl (0.15 M), EDTA (0.035 mM), and Tris-HCl (pH 7.2, 0.005 M) were applied at 22–23° to columns of Sephadex G-50; elution was carried out with buffers consisting of Tris-HCl (pH 7.2, 0.005 M), NaATP (as indicated), $MnCl_2$ (0.002 M), and KCl (0.15 M). Aliquots of the fractions were taken for determinations of enzyme and nucleotide.

phosphate obtained from the inactivated enzyme was subjected to paper electrophoresis at various values of pH; the mobilities (relative to picric acid) were 0.37, 1.0, 1.2, and 1.4 at values of pH of 2.6, 5.5, 7.5, and 9.0, respectively. These findings indicate that the new derivative is considerably more acidic than methionine sulfoximine.

When the methionine sulfoximine phosphate derivative was incubated with several phosphatases under the conditions described in Table IV, equimolar amounts of methionine sulfoximine and inorganic phosphate were formed. As indicated above, methionine sulfoximine phosphate was completely converted into P_i and methionine sulfoximine on treatment with 6 N HCl at 100° for 2 hr. However, complete hydrolysis in 1 N HCl at 100° required about 3 hr, and, under these conditions, 50% hydrolysis occurred in about 80 min; thus, the phosphate derivative is relatively stable to acid.

Studies on the Inhibition of Glutamine Synthetase by Methionine Sulfoximine in the Presence of ADP and Mn^{2+}. In the course of these studies it was observed that incubation of the enzyme with methionine sulfoximine, ADP, and Mn^{2+} led to irreversible inhibition. Inhibition in the presence of ADP was observed only with Mn^{2+} and not with Mg^{2+}. Such inhibition was less than 10% of that observed with ATP at low (10^{-6}–10^{-4} M) nucleotide concentrations; at higher (10^{-2}, 10^{-3} M) concentrations of ADP, 50–80% inhibition of the enzyme was observed. Although inhibition by methionine sulfoximine in the presence of ADP was considerably less than with ATP, the observation seemed to require an explanation. After careful study of this phenomenon we have concluded that the inhibition observed with ADP is probably associated with conversion of ADP into ATP catalyzed by adenylate kinase present as a contaminant in the enzyme preparation. However, we cannot conclusively exclude the possibility that the conversion of ADP into ATP and AMP is a catalytic property of the enzyme itself. Although purified preparations of glutamine synthetase do not exhibit adenylate kinase activity when tested by spectrophotometric methods, under the conditions of the present studies in which large amounts of the enzyme have been used, sufficient adenylate kinase can be detected in the presence of Mn^{2+} by radioisotope procedures to account for the present results. For example, when the enzyme (8.5 μM) was incubated for 2 hr at 37° with [^{14}C]- and [β-^{32}P]ADP (0.276 mM), and the mixture was then analyzed by the paper electrophoretic method (at pH 3.6: after adding carrier ATP, ADP, and AMP), 75% of the initial radioactivity was recovered, and the final concentrations of ATP, ADP, and AMP were, respectively, 0.04, 0.087, and 0.059 mM; the AMP did not contain ^{32}P. Additional evidence for the conversion of ADP

into ATP was obtained in studies in which the enzyme was preincubated with ADP and Mn^{2+} prior to addition of methionine sulfoximine; inhibition was established much more rapidly than in controls in which methionine sulfoximine, ADP, and Mn^{2+} were added together initially. Finally, it was shown by gel filtration studies analogous to those described above that [^{14}C]methionine sulfoximine is bound to the enzyme in the presence of ADP and Mn^{2+}. In two representative experiments in which the enzyme was incubated with ADP, Mn^{2+}, and [^{14}C]methionine sulfoximine, under conditions similar to those described in Table II and in which the enzyme was 50% inhibited, 8.9 and 7.9 moles of [^{14}C]methionine sulfoximine were bound per mole of inhibited enzyme. Studies carried out as described in Table III showed that the [^{14}C]-methionine sulfoximine derivative released from the enzyme by heating was identical with that obtained in the experiments in which ATP was added initially.

Discussion

Of the several methionine derivatives examined here, methionine sulfoximine and methionine sulfone are the most effective inhibitors of glutamine synthetase. The present studies show that inhibition by methionine sulfone is reversible and that this inhibitor does not remain attached to the enzyme.[1] On the other hand, the inhibition of glutamine synthetase by methionine sulfoximine is irreversible and is associated with the binding to the enzyme of close to 8 moles of this inhibitor/mole of enzyme. Since the enzyme is composed of eight apparently identical subunits (Haschemeyer, 1965, 1966), the findings are in accord with the view that 1 mole of methionine sulfoximine binds to an active site of each subunit. Inhibition by methionine sulfoximine and its binding to the enzyme require ATP and Mg^{2+} (or Mn^{2+}) and are associated with cleavage of ATP to ADP. The present studies also indicate that methionine sulfoximine is converted on the enzyme into a phosphorylated derivative. This derivative, which can be liberated from the enzyme by heating or by treatment with perchloric acid, is cleaved by strong mineral acid or by several phosphatases to yield equimolar quantities of inorganic phosphate and methionine sulfoximine. The relative stability of the methionine sulfoximine phosphate derivative to hydrolysis by 1 N HCl at 100° indicates that the phosphoryl moiety of this compound is probably not attached to the α-amino or α-carboxyl groups of methionine sulfoximine. Evidence that the phosphoryl group is attached to the sulfoximine nitrogen atom will be presented in a subsequent paper (Rowe, Ronzio, and Meister *et al.*, 1969).

The finding that glutamate decreases the rate at which methionine sulfoximine irreversibly inhibits the enzyme suggests that there are at least two steps in the inhibition of the enzyme. Thus, glutamate appears to compete with methionine sulfoximine for attachment to the enzyme; attachment of methionine sulfoximine is followed by its phosphorylation and irreversible inhibition of the enzyme. The observation that ammonia (or hydroxylamine) plus glutamate affords virtually complete protection against inhibition (Figure 5) is consistent with the hypothesis expressed earlier (Ronzio and Meister, 1968) that methionine sulfoximine can assume a conformation similar to that proposed for L-glutamate and thus can bind to the enzyme by attaching to both the glutamate and the ammonia binding sites, essentially as a bifunctional reagent. Although this interpretation requires further experimental test, it seems pertinent to note that the failure of ammonia to protect against inhibition in the absence of glutamate could indicate that binding of ammonia to the enzyme requires prior binding of glutamate. The binding site for ammonia may be made available by a conformational or electronic change in the enzyme induced by the binding of glutamate. Since essentially the same results were obtained with ammonia and hydroxylamine, it would appear that hydroxylamine also binds to the ammonia binding site of the enzyme. The markedly different reactivities of ammonia and hydroxylamine with D-glutamate (Levintow and Meister, 1953), α-aminoadipate (Wellner *et al.*, 1966), and other amino acid substrates of the enzyme (Meister, 1968) has led to the suggestion that hydroxylamine might react directly from solution as

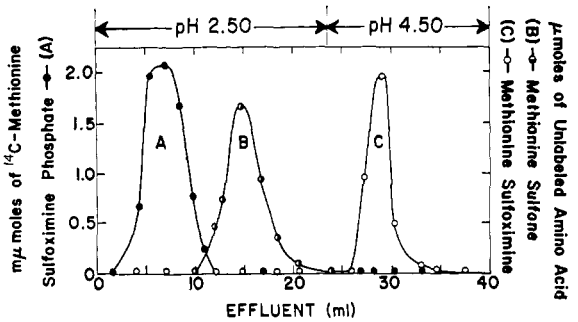

FIGURE 8: Separation of methionine sulfoximine phosphate (A) from methionine sulfoximine (C) and methionine sulfone (B). A mixture (volume, 0.5 ml) containing enzyme (7.7 μM), L-[^{14}C]methionine sulfoximine (5 mM), NaATP (10 mM), magnesium chloride (20 mM), KCl (0.15 M), EDTA (1 mM), and imidazole-HCl buffer (pH 7.2, 0.02 M) was incubated at 37° for 20 min. The enzyme was separated by gel filtration using a column buffer containing imidazole-HCl buffer (pH 7.2, 0.02 M) and EDTA (1 mM). The fractions containing the inhibited enzyme were combined, and a sample (1 ml) containing 8 mμmoles of ^{14}C (based on methionine sulfoximine) was mixed with 6 μmoles of DL-methionine sulfone and 6 μmoles of DL-methionine sulfoximine. The mixture was deproteinized with perchloric acid (final concentration, 4%), and centrifuged at 0°. The clear supernatant solution was diluted with ten volumes of water and applied to a Dowex 50 (H^+) column (0.7 × 2.5 cm) at 5°. The column was washed with 5 ml of water and elution was carried out 0.05 M sodium citrate buffer (pH 2.50). Fractions of about 2 ml were collected and used for determination of radioactivity and amino acids (ninhydrin method). After elution of methionine sulfone, a buffer of pH 4.50 was used.

[1] Evidence has been obtained that methionine sulfone interacts with the enzyme, ATP, and Mg^{2+} to yield a new compound, which is not phosphorylated and which does not bind to the enzyme; these studies will be described in a subsequent publication (W. B. Rowe and A. Meister, unpublished data, 1969).

well as from a site on the enzyme. However, other interpretations of these data (Meister, 1968) as well as of the present data are not excluded. For example, the binding of ammonia (or hydroxylamine) might increase the affinity of the enzyme for glutamate, thus making it a more effective antagonist toward methionine sulfoximine. Further study of this aspect of the mechanism of the reaction is needed.

In previous studies in this laboratory it was found that the enzyme can bind about 16 moles of ATP/mole of enzyme in the presence of Mn^{2+} (Wellner and Meister, 1966). However, under the conditions of equilibrium binding employed in the present work, nucleotide was not bound if nucleotide and metal ion were not present in the column buffer. When methionine sulfoximine was present (in addition to ATP and Mn^{2+} or Mg^{2+}), there was a substantial amount of nucleotide bound, indicating that nucleotide is bound more strongly in the presence of methionine sulfoximine. The data indicate that the inhibited enzyme contains tightly bound methionine sulfoximine phosphate and nucleotide and that this complex does not involve covalent linkages between methionine sulfoximine phosphate, nucleotide, and the enzyme. The formation of this complex seems to explain the irreversible inhibition of glutamine synthetase that results when methionine sulfoximine is incubated with the enzyme and also when it is administered to animals. The ability of glutamine synthetase to participate in the phosphorylation of methionine sulfoximine gives additional support to the view that an enzyme-bound γ-glutamyl phosphate intermediate is formed in the course of glutamine synthesis. The phosphorylation reaction described here thus seems to reflect an aspect of the normal reaction mechanism.

Acknowledgment

The authors are indebted to Mrs. Lois R. Manning for her skillful technical assistance.

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