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THE INHIBITION *IN VITRO* OF RAT CEREBRAL GLUTAMINE SYNTHETASE BY AN EXOTOXIN FROM *PSEUDOMONAS TABACI*

C. LAMAR, JR., S. L. SINDEN and R. D. DURBIN*

The Department of Medicine, University of Wisconsin School of Medicine and the Veterans Administration Hospital, Madison, Wis., U.S.A.

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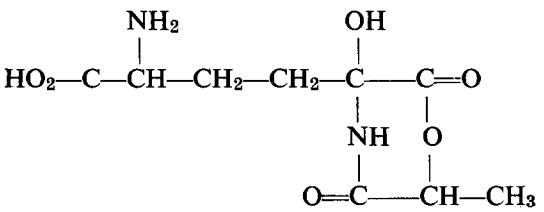
Abstract—A toxin elaborated by *Pseudomonas tabaci*, which causes wildfire disease in tobacco, has recently been shown to cause convulsions in mice and rats. Its formula is thought to be lactyl diamino hydroxy pimelic acid. It was shown that the toxin *in vitro* is a competitive inhibitor of cerebral glutamine synthetase. Preincubation studies with the toxin indicate that it is irreversibly bound to the glutamine synthetase molecule in the presence of ATP and Mg^{2+}. The action of the toxin *in vitro* on glutamine synthetase resembles in many respects the effects of methionine sulfoximime.

A TOXIN elaborated by the bacterium *Pseudomonas tabaci*, which produces chlorotic halos in the wildfire disease of tobacco, was initially tentatively identified as α-lactyl-amino-β-hydroxy-ε-aminopimelic acid (LPA).[1–4]



Work by Stewart[5] indicates that the acceptance of the structure shown above as being that of the wildfire toxin should be made with reservations. Braun[2] found that LPA at low concentrations inhibited the growth of *Chlorella vulgaris* and that this inhibition could be prevented by the addition of L-methionine to the medium. This amino acid failed to reverse the chlorotic halos produced by LPA on tobacco leaves. He noted similarities in the structure between LPA and methionine sulfoximine (MSO) and showed that MSO produced identical chlorotic lesions on tobacco leaves. Sinden and Durbin[6] have shown that chlorotic lesions caused by MSO and LPA on tobacco leaves could be prevented by the simultaneous administration of glutamine. Sinden *et al.*[7] have recently reported that LPA, administered i.p. to mice and intracisternally to rats, produces convulsive seizures indistinguishable from those seen after MSO administration.

* Pioneering Research Laboratory, ARS, USDA Department of Plant Pathology, University of Wisconsin, Madison, Wis.

Initially MSO was produced in wheat flour inadvertently when it was treated with NCl_3, a procedure employed in an attempt to "improve" the loaf by causing the introduction of air holes into it.[8] It was identified as a convulsant[9, 10] and as capable of inhibiting growth of bacteria[11] and Ehrlich ascites tumor cells.[12] Growth inhibition could be reversed by the addition of small quantities of glutamine. Sellinger and Weiler[13] have reported that MSO competitively inhibits rat cerebral glutamine synthetase (GS) activity *in vitro*.

Considerable information is available on the mechanism of glutamine formation by GS. Krishnaswamy *et al.*[14, 15] showed that, in the presence of ATP and Mg^{2+} and in the absence of ammonia, labeled glutamate was firmly bound to the GS molecule. After the addition of ammonia, glutamine was released.

Lamar and Sellinger[16] and Sellinger[17] provided evidence that MSO was bound to the enzyme in a manner not removable by dialysis. Ronzio and Meister[18] showed that MSO is bound to the active site of GS, probably through the imino moiety of MSO. They were able to recover phosphorylated MSO when the enzyme MSO complex was degraded by acid hydrolysis.[19]

In this report, evidence is presented that LPA inhibits cerebral glutamine synthetase *in vitro*. Aspects of this inhibition, which are similar to those reported for MSO, are compared.

MATERIALS AND METHODS

Materials. L-Glutamic acid and L-methionine sulfoximine were products of Calbiochem, Los Angeles, Calif. ATP and L-glutamohydroxamate were purchased from Sigma Chemical Co., St. Louis, Mo., and 2,3-dimercaptopropanol (BAL) was purchased from Mann Research Laboratories, New York, N.Y. Sephadex was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J. All other reagents were the best commercial grade available. Proteins were determined by the method of Lowry *et al.*[20] with crystalline serum albumin as a standard.

*Preparation of LPA.** The toxin was produced and purified by following the procedures of Woolley *et al.*[3] with minor modifications. A highly pathogenic isolate of *P. tabaci* (isolate No. 5, I.C.P.B., Dept. of Bacteriology, University of Calif., Davis) was grown in 15-l. fermentation tanks at 24° in Woolley's synthetic medium. The tanks were aerated (0·5 C.F.M.) and stirred (365) rpm. When the maximum toxin concentration was reached at 4–5 days, the cells were removed by centrifugation, and Norit A (10 g/l.) was added after adjustment of the pH to 4·0 with concentrated HCl.

The supernatant was concentrated to dryness in a rotary evaporator. Methanol was added (100 ml/l. of original filtrate) to the dried culture filtrate. After cooling, the methanol extract was filtered (Whatman No. 40 filter paper) and the alcohol filtrate was concentrated to facilitate storage at − 20°. Prior to use, aliquots of the alcohol concentrate were adjusted to pH 7·0 with NaOH, evaporated to dryness and redissolved in 0·01 M Tris buffer, pH 7·2. Insoluble material was removed by centrifugation. The concentration was adjusted to contain 10 μmoles/ml of LPA by dilution.

Sinden *et al.*[7] have published complete details on the methods used to measure the concentrated pure product and to assay its activity on a weight basis. The crude

* Fermentations were conducted in the Pilot Plant, Department of Biochemistry, University of Wisconsin (NIH Grant PO7-FR0026).

product obtained was purified on a PA-28 resin exchange column of the Beckman 120B amino acid analyzer. Toxin concentrations are expressed in micromoles of glycine equivalents. Two-tenths n-mole produced a visible chlorotic lesion on tobacco leaves. The crude extract contained, as identified on a Beckman 120B amino acid analyzer, the following compounds (μmoles/ml): LPA, 5·9; serine, 5·1; threonine, 2·7; tyrosine, 2·6; and traces of glutamic acid, valine and isoleucine.

The majority of experiments were conducted with partially purified preparations obtained in the manner described. The results of inhibition experiments were confirmed with purified crystalline LPA.

Glutamine synthetase preparations. Male Holtzman rats (120–150 g) were decapitated and the cerebral cortex was homogenized (20%, w/v) in water with a glass homogenizer and a tight-fitting teflon pestle. All procedures were carried out at 4°. The homogenate [sp. act. (enzyme units/mg protein): 0·9] was centrifuged at 15,000 *g* for 10 min in a Sorvall refrigerated centrifuge, model RC2B. The resulting supernatant (post-lysosomal supernatant) was centrifuged at 140,000 *g* for 1 hr in a Spinco model L ultracentrifuge. The microsomal pellet was resuspended in half the original volume of 0·1 M Tris, pH 7·2, and was used as the enzyme source in most cases [sp. act. (enzyme units/mg protein) 16·0]. Inhibition experiments were repeated with enzyme purified further by eluting GS from the microsomal pellet by means of homogenization in 0·14 M NaCl [sp. act. (enzyme units/mg protein)=35·5].

Glutamine synthetase assay. The procedure of Sellinger and De Balbian Verster[21] was followed, except that half volumes were used. One unit of enzyme activity was defined as the amount of enzyme causing the formation of 1 μmole L-glutamohydroxomate per hr.

Preincubation studies. (Additional data are available in the Results section.)

A. One set of studies involved the preincubation of certain ingradients of the usual assay mixture followed by the addition of the remaining components of the reaction mixture and completing the assay in the usual manner (see Table 1).

B. A second type of study involved the preincubation of certain constituents followed by the passage of the mixture through identical G-50 Sephadex columns (1·5 × 8·0 cm). The resin was equilibrated at 4° with 0·01 M Tris buffer, pH 7·4. The void volume of the columns was 5·2 ml and 2·5 ml of the effluent following the void volume was collected and used as the enzyme source in the usual assay. Table 2 describes the constituents of the preincubation mixture and the conditions The concentrations of ATP and Mg^{2+} were increased 6-fold to correspond with the quantity of enzyme used in the preincubation mixture, which was calculated to be sufficient for six assays. The results are an average of three experiments. Similar, but separate, experiments were conducted using toxin treated with 0·4 M $NaHCO_3$ for 3 hr at room temperature, followed by neutralization with HCl. Equal amounts of precombined $NaHCO_3$ and HCl were added to the control toxin. It has been shown[1, 4] that this treatment (alkali inactivation) results in the production of a compound that fails to cause chlorosis on tobacco leaves. It has been postulated that this treatment results in the rupturing of the lactyl bond of LPA. However, since the exact structure has not been accepted, this assumption remains tentative.

RESULTS

Effect of LPA on GS activity. Glutamine synthetase activity was inhibited 50 per

cent in the presence of a 0·3 mM concentration of LPA. At the same concentration, MSO inhibited GS activity by 25 per cent. When 0·3 mM concentrations of both inhibitors were present, enzyme activity was inhibited by 55 per cent. It is apparent that the effect of the two inhibitors is not additive; this suggests that both inhibitors are acting at the same site. The results of kinetic studies are represented in the manner of Lineweaver and Burk[22] and are shown in Fig. 1. The concentration of LPA was

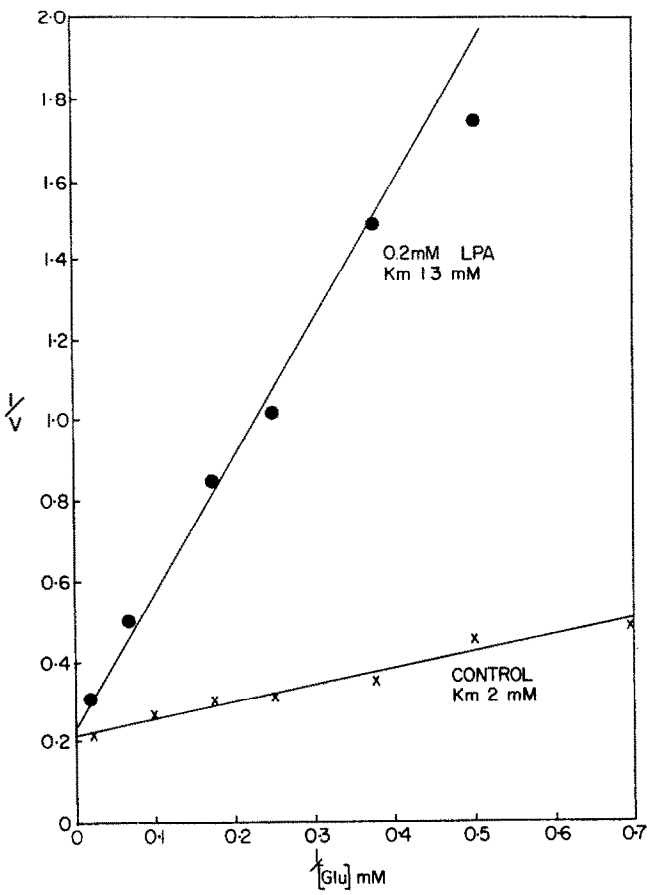


FIG. 1. Lineweaver–Burk plot of the inhibition of glutamine synthetase activity *in vitro* by LPA (0·2 mM). The enzyme preparation used was from the cerebral microsomal pellet. The reaction mixture contained: L-glutamate at various concentrations; ATP, 13 mM; Mg^{2+}, 20 mM; Tris buffer, pH 7·2, 66 mM; BAL 0·5 mM; and neutralized hydroxylamine, 50 mM.

0·2 mM and units are expressed as per milliliter of the enzyme preparation used. The lines plotted through the points at the higher substrate concentrations fall in a pattern characteristic of competitive inhibition. At low glutamate concentrations the points fell below the line plotted through the higher substrate concentrations. This consistent finding was interpreted as indicating that at low substrate concentrations LPA was affecting the enzyme in a different manner than at high substrate concentrations.

Fig. 2 shows the results of GS activity at three substrate and three inhibitor concentrations. The data are plotted according to Dixon and Webb[23] and indicate competitive inhibition with respect to glutamate. The K_i for the inhibitor was calculated to be $2{\cdot}6 \times 10^{-5}$ M.

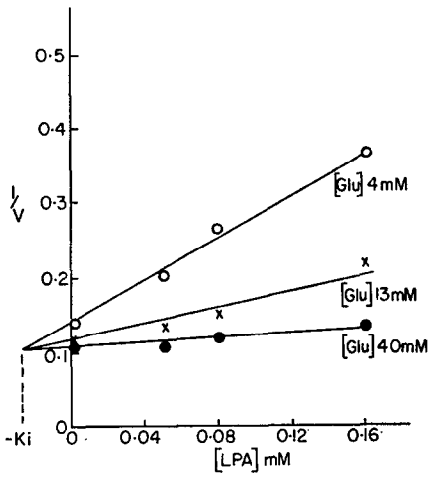


FIG. 2. The inhibition of glutamine synthetase activity by 0·04, 0·08 and 0·16 mM concentrations of LPA at 40, 13 and 4 mM concentrations of glutamate. The representation is by the Dixon and Webb method.[23] The reaction mixture is described in Fig. 1. The enzyme preparation used was resuspended rat cerebral microsomal pellet. Units are expressed per milliliter of enzyme solution (see text). The affinity constant (K_i) for LPA was calculated to be $2{\cdot}6 \times 10^{-5}$ M.

Preincubation experiments. Two types of experiments were designed to assess the importance of ATP, Mg^{2+} and glutamate on the inhibitory properties of LPA. Previous work has shown that for MSO to be maximally effective, both Mg^{2+} and ATP must be present in the preincubation mixture.[17] The results of preincubating the enzyme with the inhibitors in the presence of various ingradients are shown in Table 1.

TABLE 1. ACTIVITY OF GLUTAMINE SYNTHETASE AFTER PREINCUBATION IN PRESENCE OF INHIBITOR AND ATP, Mg^{2+} AND GLUTAMATE

Preincubation components*	+ LPA (0·2 μM)	+ MSO (0·4 μM)
1) GS + ATP + Mg^{2+}	100†	100†
2) GS + ATP + Mg^{2+}	10 (0–30)‡	5(0–12)‡
3) GS + ATP + Mg^{2+} + Glu	50(40–85)	36(20–40)
4) GS + ATP	82(77–90)	94(90–100)
5) GS + Mg^{2+}	86(50–90)	96(90–98)
6) GS	78(76–82)	88(80–92)

* Concentrations, final (mM): ATP, 13; Mg^{2+}, 20; glutamate, 40. Tris. 66 mM, pH 7·2, and BAL, 0·5 mM were also present in all instances.

† Inhibitor not present during preincubation but added at time of assay. Value of 100 per cent activity ascribed and results compared accordingly.

‡ In the parentheses are included the range of the 3 values obtained.

Number one represents the enzyme activity after preincubation in the presence of ATP and Mg^{2+}, but in the absence of inhibitor. The inhibitor was then added at the start of the assay. The amount of activity when the toxin was added at the initiation of the reaction was given a value of 100 per cent and the other results were compared accordingly. It can be seen that preincubation in the presence of ATP and magnesium almost completely inactivates the enzyme. The presence of glutamate partially protects the enzyme. When either ATP or Mg^{2+} or both are absent, there is a variable but slight degree of inactivation. Of significance is the parallelism in the results between the effects of MSO and LPA.

In companion experiments (Table 2), six aliquots of cerebral GS were preincubated separately in the presence of ATP and Mg^{2+}. Samples 2, 3 and 4 contained LPA;

TABLE 2. EFFECT OF CHROMATOGRAPHY ON COLUMNS OF SEPHADEX G-50 ON REVERSING THE INHIBITION OF GLUTAMINE SYNTHETASE PREINCUBATED UNDER VARIOUS CONDITIONS

Contents* of pre incubation mixture	Per cent activity† remaining after passage through Sephadex G-50 column
1) GS, ATP, Mg^{2+}, Tris buffer	100
2) All of No. 1 + LPA	5(0–12)‡
3) All of No. 1 + LPA§	65(50–70)
4) All of No. 1 + LPA and glutamate	68(60–80)
5) All of No. 1 + MSO	15 (5–20)
6) All of No. 1 + MSO and glutamate	40(28–50)

* Concentrations of components of preincubation mixture (mM): ATP, 70; Mg^{2+}, 120; Tris buffer, pH 7·2, 260; LPA, 0·12; glutamate, 60; MSO, 1·5. (Concentrations were adjusted according to the enzyme activity applied to the column, i.e. sufficient for 6 assays.)

† As determined in usual GS assay, aliquot of post-void volume effluent as enzyme source. Results are in units/mg protein, the control being assigned a value of 100 per cent and other values reported as per cent of control.

‡ In the parentheses are included the range of the 3 values obtained.

§ Preincubated at 2°; all others at 37°.

sample 3 was preincubated at 2° and the remainder at 37°. Samples 5 and 6 contained MSO. Glutamate was added to tubes 4 and 6. The final volumes were approximately 2·5 ml. Immediately after a 10-min preincubation, the tubes were cooled and the contents were passed through separate, identical G-50 Sephadex columns. An amount of the effluent (2·5 ml) after the passage of the void volume (5·2 ml) was collected and 0·2-ml portions were used as the enzyme source in the usual assay. The results are shown in Table 2. The activities are expressed in terms of protein content and are the average of three experiments. Arbitrarily assigning 100 per cent activity to the control (no inhibitor present), the tubes preincubated with LPA at 2° or in the presence of glutamate at 37° retained 65 per cent of the activity. The mixture preincubated with MSO in the presence of glutamate retained 40 per cent. In the absence of glutamate and at 37°, LPA was responsible for almost complete inactivation of the enzyme and MSO for 85 per cent loss. Kinetic studies could not be done on samples incubated in the absence of glutamate because of insufficient enzyme activity. Fig. 3 shows the result of kinetic studies done on the enzyme eluted from the Sephadex column, which was preincubated in the presence of LPA, glutamate, Mg^{2+} and ATP, and the control

(in the absence of LPA). Similar results have been reported when MSO was studied in the same manner.[16] It was concluded that LPA, like MSO, was bound to the GS molecule by an enzymatic mechanism requiring the presence of ATP and Mg^{2+} and that, as a result of this binding, the enzyme is irreversibly inhibited. Similar preincubation

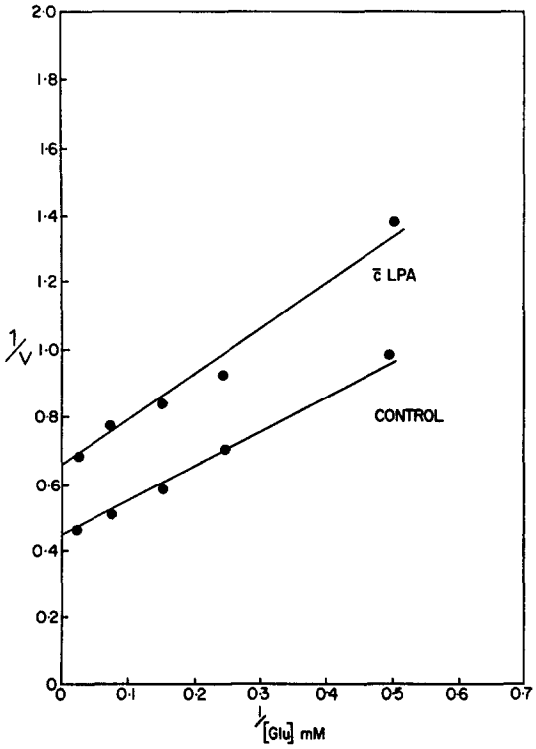


FIG. 3. Lineweaver–Burk pattern comparing glutamine synthetase activity in an enzyme preparation treated by preincubation and followed by passage of the mixture through a G-50 Sephadex column. The preincubation mixture of the control contained: ATP, 72 mM; Mg^{2+} 120 mM; and Tris buffer. pH 7·2, 260 mM. The LPA mixture contained all the above plus LPA, 0·12 mM and glutamate, 60 mM. These kinetic studies were done on samples 1 and 4 listed in Table 2.

experiments were performed to compare the inhibition caused by "alkaline-inactivated LPA"[4] with appropriate controls. Assays *in vitro*, conducted in the usual manner, showed that both the alkaline-treated and the control LPA (0·08 mM) inhibited GS to the same extent (30 per cent). However, after passage of the preincubated mixture through Sephadex columns, GS was inhibited 90 per cent by the control and 50 per cent by the alkaline-treated LPA. The results are expressed in Fig. 4 by the method of Lineweaver and Burk.[22] The results indicate that alkaline treatment modified the LPA molecule (possibly by breaking the lactyl bond as previously postulated).[4] The modified inhibitor could still inhibit the enzyme *in vitro*, but could no longer accomplish the irreversible binding to the GS molecule that was noted when preincubation studies were performed. Gel filtration then was able to remove, at least in part, the modified inhibitor from the enzyme molecule. The fact that the Sephadex

column treatment did not reverse the inhibition completely was ascribed to incomplete hydrolysis of the LPA by the alkali treatment. This assumption was proven when alkaline-hydrolyzed LPA was analyzed on the amino acid analyzer. The results showed that only 30 per cent of LPA had been converted to the "inactive" product.

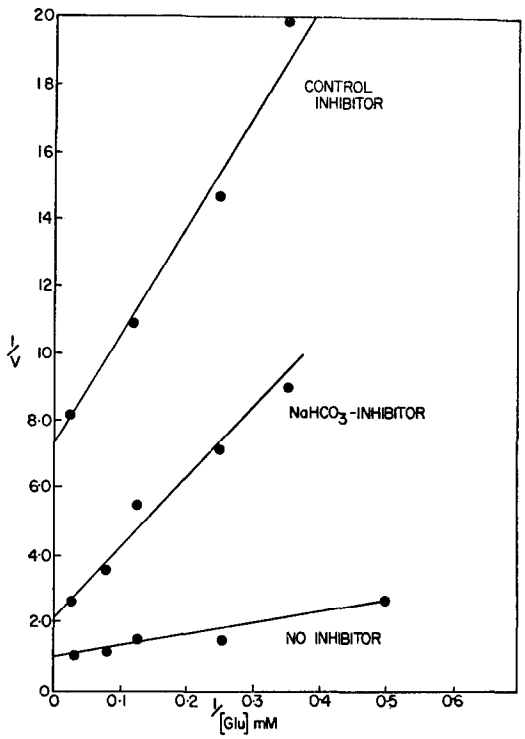


FIG. 4. Lineweaver–Burk pattern comparing glutamine synthetase activity in an enzyme preparation treated by preincubation and followed by passage of the mixture through G-50 Sephadex columns. The preincubation mixture contained: enzyme; ATP, 72 mM; Mg^{2+}, 120 mM; and Tris buffer, pH 7·2, 260 mM. The control inhibitor contained LPA, 0·08 mM. The $NaHCO_3$ inhibitor contained LPA which had been subjected to 0·4 M $NaHCO_3$ for 3 hr.

Nevertheless, the reduction of the inhibition by the alkaline hydrolysis treatment of LPA was of significant magnitude and was a consistent finding in four separate experiments.

DISCUSSION

The significance of this report is enhanced by the recent finding that LPA causes convulsions in mice and rats that are indistinguishable from those caused by MSO.[7]

Considerable evidence has been accumulated[16, 17] and definite proof has been reported[18] that MSO binds to the GS molecule at the active site. Data have been presented to show that MSO undergoes phosphorylation during the process.[19] The studies reported here on the mechanism of action of LPA showed that: 1) the inhibition of GS is competitive with glutamate *in vitro*; 2) the presence of both Mg^{2+} and ATP is required for maximum inactivation in preincubation experiments; and 3) glutamate partially protects the enzyme from this inactivation. Whatever the nature

of the LPA inactivation of GS, it is not reversed by the passage of the enzyme–inhibitor complex through a G-50 Sephadex column. When enzyme preparations partially inactivated by preincubation with ATP, Mg^{2+} and LPA (in the presence of glutamate) are compared kinetically with controls preincubated in the absence of LPA, the results indicate that a structural change has occurred in the enzyme. The similarity of the results obtained when LPA and MSO inhibition studies are compared indicates that LPA, like MSO,[18] is probably bound to the active site of the GS molecule. Assuming that the formula of LPA is correct, it is likely that the lactyl ring is the reactive center of the molecule. Accordingly, it can be postulated that breakage of the lactone bond of the inhibitor during the enzymatic reaction with GS could result n a covalent attachment of inhibitor to enzyme. The fact that previous alkaline treatment of LPA, which has been postulated to hydrolyze the lactyl ring,[4] results in a reversible inhibition after gel filtration further affirms this conclusion.

The study of two structurally dissimilar compounds, which inhibit GS *in vitro* in a similar manner and cause convulsions *in vivo*, promises to contribute insight into the cause of these experimental seizures. It has been shown that MSO forms an alkyl bond with the enzyme.[19] The evidence indicates that in many respects LPA acts in a manner similar to MSO. It seems permissible to speculate that the formation of this bond, if not with glutamine synthetase, then with some other as yet unknown enzyme, may be the fundamental cause of the seizure phenomena in this experimental model.

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