Biochemical Pharmacology, Vol. 34, No. 23, pp. 4145–4150, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

CHELIDONIC ACID AND OTHER CONFORMATIONALLY RESTRICTED SUBSTRATE ANALOGUES AS INHIBITORS OF RAT BRAIN GLUTAMATE DECARBOXYLASE

TERENCE G. PORTER and DAVID L. MARTIN*
Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A.

(*Received* 30 *January* 1985; *accepted* 23 *April* 1985)

Abstract—Twenty conformationally restricted analogues of glutamate including benzoic acids, hydroxybenzoic acids, pyridine dicarboxylic acids, and pyran dicarboxylic acids were tested as inhibitors of glutamate decarboxylase from rat brain. Chelidonic acid, 2,6-pyridine dicarboxylic acid, chelidamic acid, gallic acid, and 3,4-dihydroxybenzoic acid were the most potent inhibitors of the enzyme, and generally the aromatic analogues were much more potent inhibitors than their aliphatic counterparts. An intercarboxylate distance of 0.75 nm appears optimal for substrate competition, indicating that glutamate binds to the active site in an extended conformation. At least one carboxyl group can be replaced by a phenolic hydroxyl without greatly affecting inhibition. The degree of inhibition was also influenced by the aromatic structure, particularly with respect to the atom bridging the dicarboxylate carbons. Kinetic analysis of the inhibition by chelidonic acid and chelidamic acid showed that these compounds were competitive with glutamate with K_i values of 1.2 and 33 μM respectively. Consistent with this result, chelidonic acid also inhibited the glutamate-dependent formation of apoenzyme. Chelidonic acid itself did not promote formation of apoenzyme and did not react with free pyridoxal-P. The effects of different classes of glutamate decarboxylase inhibitors are discussed in relation to the formation of apoenzyme and its reactivation by pyridoxal-P. As one of the most potent inhibitors of glutamate decarboxylase known, chelidonic acid may be of value in studies of the regulation of γ-aminobutyric acid synthesis.

A number of inhibition studies have been performed on glutamate decarboxylase (L-glutamate 1-carboxylyase, EC 4.1.1.15, GAD), the enzyme responsible for γ-aminobutyric acid (GABA) synthesis in brain [1–3]. However, interpretation of the mode of action of these inhibitors is often complicated by the complex catalytic mechanism of the enzyme. During catalysis in the presence of its cofactor, pyridoxal-P, GAD undergoes a cycle of inactivation and reactivation [4–6]. Inactivation is thought to result from an abortive transamination reaction similar to that described for other pyridoxal-P-dependent amino acid decarboxylases [7–9] that converts enzyme-bound pyridoxal-P into pyridoxamine-P and L-glutamate into succinic semialdehyde instead of the normal product, GABA. In the absence of free pyridoxal-P, this leads to inactivation of the enzyme [4], as holoenzyme (containing bound pyridoxal-P) is converted into inactive apoenzyme. However, in the presence of free pyridoxal-P, the apoenzyme that is formed is reconstituted to active holoenzyme, thereby completing a cycle of apo- and holoenzyme interconversion [6, 10].

Interaction at any stage of this cycle will lead to a modification of enzyme activity. Inactivation-promoters such as ATP inhibit the enzyme by enhancing the rate of apoenzyme formation or by inhibiting reactivation of the apoenzyme by pyridoxal-P and are characterized by their slow action and their requirement for high concentrations of glutamate to inhibit [5, 11]. Many of the most potent inhibitors of GAD known to date are not very closely related to glutamate in structure and appear to inhibit the enzyme by interacting with pyridoxal-P either when it is free or when it is bound to the enzyme as a Schiff base. These inhibitors include carbonyl-trapping reagents such as semicarbazide, arylhydrazides, and aminooxyacetic acid and possibly the mercapto compounds, mercaptopropionate and thiomalate [3, 12]. As investigational tools, these compounds have an obvious drawback in that they can affect other pyridoxal-P requiring enzymes. On the other hand, aliphatic analogues of glutamate which do not interact with pyridoxal-P are poor inhibitors of GAD and are only effective at millimolar concentrations [1].

Conformationally restricted substrate analogues are often potent enzyme inhibitors, and it has been reported that 4,5-dihydroxyisophthalic acid isolated from *Streptomyces toyocaensis* and closely related compounds are good inhibitors of GAD from bovine brain [13, 14]. As more specific inhibitors of GAD would be extremely useful as tools to study the regulation and role of GABA in the nervous system, this study was undertaken to examine a number of commercially available, conformationally restricted analogues of glutamate as inhibitors of rat GAD, to obtain information on the structural requirements for substrate binding and competition, and to characterize the mode of action of the most potent of these compounds. Some of this work has appeared in a preliminary account [15].

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Materials. D,L-[1-^{14}C]Glutamate was obtained from New England Nuclear (Boston, MA) and purified as described previously [16]. Pyridoxal 5'-phosphate (pyridoxal-P), 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), 2-aminoethylisothiouronium bromide (AET), and protein standards were purchased from the Sigma Chemical Co. (St. Louis, MO.) Inhibitors were obtained from the Aldrich Chemical Co. (Milwaukee, WI), and all other reagents and chromatographic media were from previously cited sources [17].

Enzyme assay. GAD activity was measured by trapping and counting $^{14}CO_2$ from purified DL-[1-^{14}C] glutamate as described previously [18, 19].

Preparation of enzyme. GAD was partially purified by a modification of the method of Spink *et al.* [17]. All procedures were performed at 4°, and all buffers contained 30 μM pyridoxal-P and 1 mM AET. Briefly, Wistar rats were killed by decapitation, and whole brains including cerebellum but excluding brain stem were rapidly removed. A 10% homogenate was prepared in a solution containing 30 μM pyridoxal-P, 1 mM AET, and 100 μM phenylmethylsulfonyl fluoride (pH 7.2) and centrifuged at 18,000 *g* for 45 min. Phenyl-Sepharose that had been pre-equilibrated with 5 mM phosphate buffer, pH 7.0, was then added (20% v/v), and the mixture stirred gently with an overhead stirrer for 45 min at 4°. The phenyl-Sepharose was separated by vacuum filtration and washed thoroughly with phosphate buffer. GAD was eluted by stirring the phenyl-Sepharose with an equal volume of 5 mM phosphate buffer, pH 7.2, containing 1% Triton X-100, and the phenyl-Sepharose was removed by vacuum filtration. The elution was repeated, and the combined filtrates were applied to a column (4 × 6 cm) of DEAE-Sephacel that had been equilibrated with the 5 mM phosphate buffer. After a thorough wash, the enzyme was eluted with 400 mM sodium chloride in 5 mM phosphate buffer directly onto a column of hydroxylapatite (1.5 × 20 cm) that had been equilibrated with the 5 mM phosphate buffer. GAD was then eluted with a linear potassium phosphate gradient (5–300 mM). The peak activity fractions were combined and concentrated by using an Amicon ultrafiltration unit fitted with a PM 10 membrane and then applied to an Ultrogel column (60 × 1.2 cm) previously equilibrated with 50 mM phosphate buffer, pH 7.0. Peak activity fractions were pooled, concentrated, and stored frozen at −20° in 40% sucrose. GAD can be stored in this manner for at least 1 year without loss of activity.

Gel filtrations, rechromatography on phenyl-Sepharose, and non-denaturing electrophoresis. Gel filtration was performed in 50 mM phosphate buffer, pH 7.0, on an Ultrogel column (60 × 1.2 cm) previously calibrated for determination of molecular weight with ribonuclease, ovalbumin, aldolase, and L-glutamate dehydrogenase. Rechromatography on phenyl-Sepharose (with a descending phosphate and ascending glycerol gradient) and non-denaturing polyacrylamide gel electrophoresis were performed as described previously [17, 20].

Inhibition studies. To study a large number of test compounds, an initial screening test similar to that described by Taberner *et al.* [3] was performed. The substrate, L-glutamate, was present at approximately half K_m (0.5 mM), and the enzyme activity was determined with or without 1 mM inhibitor at 37° in 100 mM HEPES containing 1 mM AET and 20 μM pyridoxal-P (pH 7.0). Inhibition was expressed as a percentage of the control which contained no inhibitor. More complete kinetic analysis was performed on the most potent inhibitors at glutamate concentrations of 1 to 10 mM. Values for the apparent K_m and the inhibition constant (K_i) were obtained by fitting the competitive inhibition equation to the data by an adaption of the method of Eisenthal and Cornish-Bowden [21].

Inactivation studies. The enzyme was prepared for use by dialyzing it against 50 mM potassium phosphate buffer containing 20 μM pyridoxal-P and 1 mM AET, pH 7.0, for 60 min at 25° to ensure that it was in the holoenzyme form. Free pyridoxal-P was then removed by exhaustive dialysis at 4° against 50 mM HEPES (pH 7.0) containing 1 mM AET.

Inactivation time courses were performed by a two-stage incubation procedure [5]. GAD was incubated first with 5 mM glutamate and a test compound when appropriate for various lengths of time at 37° to allow inactivation to occur. Then the remaining GAD activity was measured in a 5-min assay.

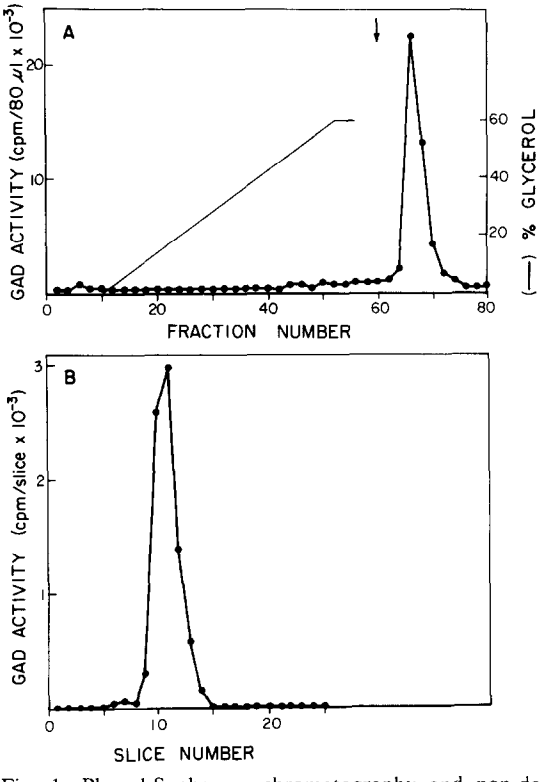


Fig. 1. Phenyl-Sepharose chromatography and non-denaturing gel electrophoresis of rat brain GAD. (A) Partially purified rat GAD was chromatographed on phenyl-Sepharose by the procedure which separates three forms of the enzyme from porcine and/or rat brain. The column was then eluted with 1% Triton X-100 (↓). Key: (●) GAD activity; and (–) glycerol concentration. (B) Non-denaturing gel electrophoresis of rat GAD. Gel slices (3 mm) were assayed for GAD activity (●).

Table 1. Purification of glutamate decarboxylase from rat brain

Purification step	Total protein (mg)	Total Activity (units)	Specific activity (units/mg × 10^3)	Purification (-fold)	Yield (%)
Homogenate	10,260	1.98	0.19	1.0	100
Supernatant	7,280	1.73	0.24	1.26	88
Phenyl-Sepharose batch separation	650	1.13	1.74	9.16	57
DEAE-Sephacel + hydroxylapatite	26.3	0.47	17.80	93.7	24
Ultrogel	9.6	0.34	35.40	186.0	17

One unit is the amount of enzyme that catalyzes the production of 1 μmole of CO_2 in 1 min under standard assay conditions.

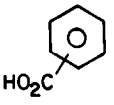
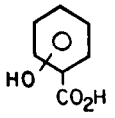
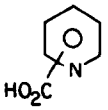
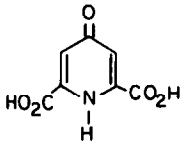
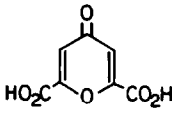
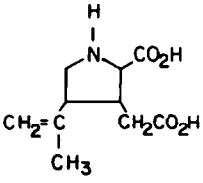
GROUP	STRUCTURE	SUBSTITUENT POSITIONS	INHIBITION (%)
Benzene carboxylic acids		1,2	34
		1,3	78
		1,2,3	78
		1,2,4	75
		1,3,5	72
		1,2,4,5	52
		1,2,3,4,5,6	14
Hydroxybenzoic acids		2,3	52
		3,4	90
		3,5	59
		3,4,5	92
Pyridine carboxylic acids		2,3	22
		2,4	79
		2,5	73
		2,6	90
		3,4	28
		3,5	39
chelidamic acid:			86
Pyran dicarboxylic acid			
chelidonic acid:			100
Pyrrolidine			
kainic acid:			5

Fig. 2. Inhibition of rat glutamate decarboxylase by conformationally restricted substrate analogues. Each compound was tested at a concentration of 1 mM against 0.5 mM L-glutamate in the presence of excess pyridoxal-P. Assay conditions are described in Materials and Methods.

Reconstitution of holoenzyme. To prepare apoenzyme, GAD was preincubated with the test compounds for 30 min at 30° and then applied to a Sephadex G-25 column (30 × 1.2 cm) that had been equilibrated with 10 mM phosphate buffer (pH 7.2) containing 1 mM AET. Following elution with the same buffer, the fractions were assayed for protein and enzyme activity. Reactivation of apoenzyme with pyridoxal-P was determined by preincubating the samples in the presence or absence of 20 μM pyridoxal-P for 10 min at 30° before assay. The effect of each compound was expressed as a change in specific activity relative to a control which went through all procedures in the absence of the effectors.

Absorbance experiment. The reactivity of chelidonic acid with free pyridoxal-P was observed by monitoring its effect on the absorbance spectrum of pyridoxal-P in the region 300–450 nm as described previously [3].

RESULTS

Purification of the enzyme. A summary of a typical purification procedure is shown in Table 1. This procedure could be completed in 3 days and provided enzyme that was purified over 150-fold in 20–30% yield. The enzyme eluted as a single peak during gel filtration on Ultrogel at an elution volume corresponding to a molecular weight of 90,000. Rechromatography on phenyl-Sepharose produced a single activity peak which was eluted by 1% Triton X-100 (Fig. 1A), and the enzyme activity ran as a single discrete band during non-denaturing polyacrylamide gel electrophoresis (Fig. 1B).

Inhibition studies. The results of the initial screening of potential inhibitors are shown in Fig. 2. Under these conditions, all of the compounds tested inhibited GAD to some degree. Chelidonic acid, 2,6-pyridine dicarboxylic acid, 3,4,5-trihydroxybenzoic acid (gallic acid), and 3,4-dihydroxybenzoic acid were the most potent, each inhibiting by more than 90%. Under the same reaction conditions, the aliphatic analogues, glutarate and succinate, inhibited GAD by 30 and 20% respectively. Kinetic analysis (Fig. 3) showed that chelidonic acid was competitive with glutamate with a K_i of 1.2 μM. Chelidamic acid was also competitive with a K_i of 33 μM. The apparent K_m of the enzyme was 1.1 mM.

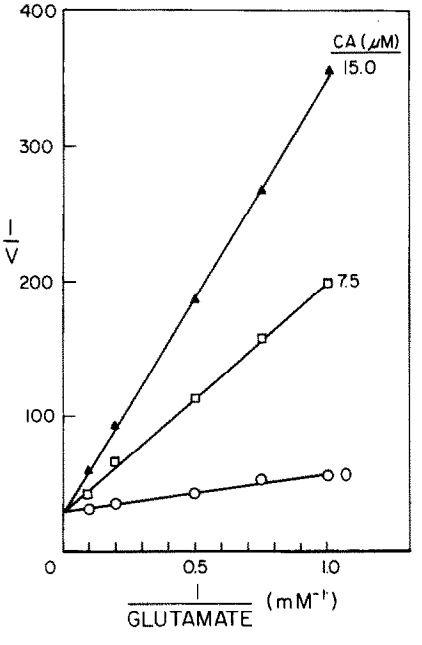


Fig. 3. Reciprocal plot of the effect of chelidonic acid on GAD activity. The units of $1/v$ are $\mu moles^{-1}$ min.

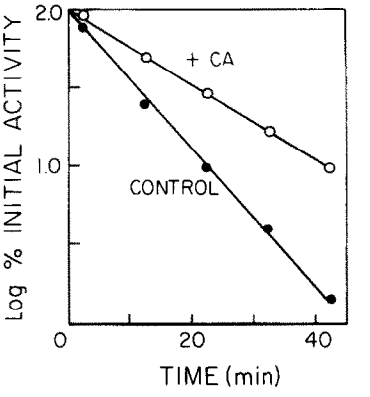


Fig. 4. Retardation of glutamate-promoted inactivation of GAD by chelidonic acid. GAD was preincubated in the absence of pyridoxal-P with (●) 5 mM glutamate or (○) 5 mM glutamate + 7.5 μM chelidonic acid for the indicated time period. The remaining activity was then measured in a short (5 min) assay. Data points are the means of triplicate values of all which agreed within 5% of the mean.

Inactivation and reconstitution studies. Recent studies have established that compounds can affect GAD activity by influencing the rates of interconversion of the holo- and apoenzyme [5, 11]. Thus, the effects of chelidonic acid on this aspect of the mechanism of GAD were examined. Chelidonic acid

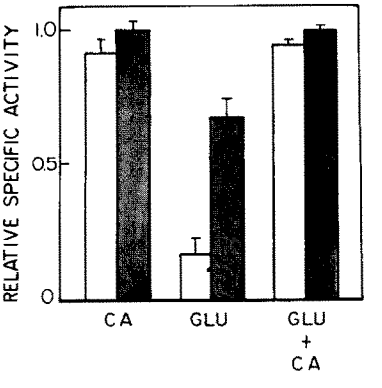


Fig. 5. Effect of chelidonic acid on apoGAD formation. GAD was preincubated for 30 min with 1 mM chelidonic acid (CA), 5 mM glutamate (GLU), and 5 mM glutamate + 1 mM chelidonic acid (GLU + CA). At the end of this period, the incubation mixture was passed over Sephadex G-25 and then assayed in the absence (open bar) or presence (stippled bar) of 20 μM pyridoxal-P. Results are presented as the specific activity relative to a control which went through all procedures in the absence of the effectors.

significantly reduced the rate of inactivation of GAD by glutamate (Fig. 4). Since inactivation by glutamate results from the production of apoenzyme through a branch in the catalytic pathway, a simple competitive inhibitor is expected to have exactly this effect. The expected reduction in the rate of inactivation as calculated with the usual equation for competitive inhibition (57%) agreed closely with the observed reduction (50%). Furthermore, inhibition by chelidonic acid was immediate and constant; the enzyme was inhibited to the same extent 1 and 30 min after adding the inhibitor (results not shown). In contrast, inhibition by compounds that affect the interconversion of apo- and holoenzyme increases with time of incubation over a period of 20 min [5, 11].

Further evidence that chelidonic acid does not inhibit GAD by promoting the formation of apoenzyme was obtained by incubating the enzyme with inhibitor and then passing it over a Sephadex G-25 column to remove the inhibitor and any released cofactor. If apoenzyme was produced, this treatment would lead to a reduction in the specific activity of the enzyme, but no such reduction in specific activity was observed when the enzyme was incubated with chelidonic acid (Fig. 5). In contrast, there was an 80% decrease in specific activity when the enzyme was incubated with glutamate before chromatography on Sephadex G-25 and more than 60% of this lost activity could be recovered by the addition of free pyridoxal-P to the enzyme after Sephadex treatment. Thus, apoenzyme was formed by incubation with glutamate. However, when the incubation was carried out in the presence of chelidonic acid and glutamate, there was virtually no loss of specific activity, indicating that chelidonic acid inhibits glutamate-dependent apoenzyme formation. These results are entirely in accord with a mechanism in which chelidonic acid merely blocks entry of glutamate into the active site.

Chelidonic acid had no effect on the absorbance of free pyridoxal-P in the 300–450 nm region even when present in 20-fold excess over the cofactor (data not shown).

DISCUSSION

The enzyme isolation procedure provided a rapid and convenient method to produce reasonable quantities of a partially purified (>150-fold) preparation of rat brain GAD suitable for use in these studies. In addition to rapidity, the purification scheme had several advantages over published procedures for purifying the enzyme from mouse [21], rat, and human [2, 22] brain. By avoiding the use of synaptosomal preparations [23] and an acid/heat precipitation step [2, 22], higher yields were obtained. Further, the introduction of a phenyl-Sepharose batch separation step introduced a higher degree of purification at an early stage and allowed a larger quantity of brain to be processed.

This preparation did not contain the high molecular weight form of the enzyme reported in mouse brain [24], since the enzyme eluted as a single activity peak during chromatography on Ultrogel at an elution volume corresponding to a molecular weight of 90,000. Moreover, the preparation was devoid of the other two low molecular weight forms of the enzyme found in rat brain [20], since the enzyme was eluted as a single peak during rechromatography on phenyl-Sepharose under conditions that resolve the multiple forms of rat brain GAD and ran as a single band during non-denaturing polyacrylamide gel electrophoresis. Weakly hydrophobic species of GAD are not retained well by phenyl-Sepharose at low ionic strength and may have been lost during the initial batch separation on phenyl-Sepharose. The apparent K_m of the partially purified preparation was 1.1 mM, identical to that of the purified enzyme [22].

All of the conformationally restricted analogues of glutamate inhibited GAD and were generally more potent than aliphatic analogues such as glutarate and succinate. The order of potency was influenced by the aromatic structure as well as the type, number, and position of the ring substituents. Generally, the benzene carboxylic acids and pyridine dicarboxylic acids showed very similar trends of inhibition. Analogues with two carboxylic groups separated by a chain of three atoms, such as 1,3-benzene dicarboxylic acid (isophthalic acid), 1,2,3- and 1,2,4,-benzene tricarboxylic acids, and 3,4- and 2,4-pyridine dicarboxylic acids, were roughly equipotent, inhibiting by 70–80%. Reducing the separating chain to two atoms significantly reduced inhibition; 1,2-benzene dicarboxylic acid (phthalic acid) and 2,3- and 3,4-pyridine dicarboxylic acids inhibited by only 20–30%. Increasing the number of carboxylate groups beyond three also decreased inhibition as seen with 1,2,4,5-benzene tetracarboxylic acid and benzene hexacarboxylic acid (mellitic acid). Hydroxybenzoic acids were good inhibitors of rat GAD as has been reported for the bovine brain enzyme [14]. Thus, at least one of the carboxyl groups can be replaced by a phenolic hydroxyl without greatly affecting inhibition. The strong inhibition (90%) by 3,4,5-trihydroxybenzoic acid (gallic acid) and 3,4-dihydroxybenzoic acid together with the reported potency of 4,5-dihydroxyisophthalic acid [13, 14] also indicates that two phenolic hydroxyl groups in positions 3 and 4 from a carboxyl group imparts a very strong affinity for the enzyme.

The atom bridging the dicarboxylate carbons (the "central" atom) also exerts a strong influence on the degree of inhibition. Thus, 2,6-pyridine dicarboxylic acid and chelidamic acid are more powerful inhibitors than isophthalic acid, suggesting that a nitrogen atom in the central position improves binding to the enzyme. It is of interest that 2,6-pyridine dicarboxylic acid, a potent inhibitor of GAD, has neurotoxic effects [25]. Two other compounds known to have excitatory and neurotoxic effects, quinolinic acid and kainic acid, are poor inhibitors of GAD. Replacement of nitrogen with oxygen as the central atom strengthened binding even further. Thus, the most potent inhibitor among the compounds tested was the pyran dicarboxylic acid, chelidonic acid, which fully inhibited the enzyme under these conditions, and kinetic analysis showed it to be more than an order of magnitude more potent than chelidamic acid. The marked effect of the central atom might be attributable to differences in hydration and elec-

tronegativity, which are important factors in the combination of an inhibitor and an enzyme [26].

For aliphatic dicarboxylate anions in solution, the exact conformation needed for combination with an enzyme is not readily predicted, since the effects of internal rotation, electrostatic repulsion, and steric hinderance between movable groups must be considered. However, these effects are minimized by the rigid benzene ring, allowing more accurate predictions [27]. The stronger inhibition of GAD by isophthalic acid over phthalic acid is doubtlessly attributable to the greater distance between the carboxylate groups in isophthalic acid (0.745 vs 0.425 nm [27]) and suggests that glutamate binds to the active site of GAD in a fully extended conformation.

These results with GAD are qualitatively similar to the results of a study of bovine liver glutamate dehydrogenase (GDH) [27]. For GDH, the intercarboxylate distance for maximum inhibition is also 0.75 nm. A notable difference between the enzymes is the very weak inhibition of GDH by 2,6-pyridine dicarboxylic acid and chelidonic acid, both strong inhibitors of GAD. This difference may impart a high degree of specificity of chelidonic acid for GAD over GDH *in vivo*.

These studies demonstrate a clear mechanistic difference between compounds such as chelidonic acid which compete directly for glutamate and other types of inhibitors. The retarding effect of chelidonic acid on substrate-promoted inactivation is similar to that observed with chloride and glutarate [11]. Inactivation by glutamate requires the binding and decarboxylation of the substrate at the active site, and competitive inhibitors such as chelidonic acid will limit glutamate binding and reduce the rate of inactivation. Inactivation-promoters such as ATP actually increase the rate of inactivation [5, 11], an effect opposite that of substrate competitors. Further, compounds such as aminooxyacetic acid that inhibit by interaction with pyridoxal-P can be used to form apoenzyme [19]. Chelidonic acid does not react with free pyridoxal-P and prevents apoenzyme formation. As one of the most potent inhibitors of GAD known, chelidonic acid may be useful in studies of the regulation of GABA synthesis, and its pharmacological potential is currently under investigation.

Acknowledgements—Supported by Grant MH35664 from the National Institute of Mental Health, USPHS/DHHS.

REFERENCES

1. J-Y.Wu and E. Roberts, *J. Neurochem.* **23**, 759 (1974).
2. J. M. Blindermann, M. Maitre, L. Ossola and P. Mandel, *Eur. J. Biochem.* **86**, 143 (1978).
3. P. V. Taberner, M. J. Pearce and J. C. Watkins, *Biochem. Pharmac.* **26**, 345 (1977).
4. L. P. Miller, D. L. Martin, A. Mazumder and J. R. Walters, *J. Neurochem.* **30**, 361 (1978).
5. M. P. Meeley and D. L. Martin, *Cell. molec. Neurobiol.* **3**, 39 (1983).
6. M. P. Meeley and D. L. Martin, *Cell. molec. Neurobiol.* **3**, 55 (1983).
7. A. Novogrodsky and A. Meister, *J. biol. Chem.* **239**, 879 (1964).
8. B. S. Sukhareva and A. E. Braunstein, *Mol. Biol. Moscow* **5**, 302 (1971).
9. M. H. O'Leary and R. L. Baughn, *J. biol. Chem.* **252**, 7168 (1977).
10. T. G. Porter and D. L. Martin, *J. Neurochem.* **43**, 1464 (1984).
11. D. L. Martin and S. B. Martin, *J. Neurochem.* **39**, 1001 (1982).
12. E. Roberts and D. G. Simonsen, *Biochem. Pharmac.* **12**, 113 (1963).
13. A. Endo, N. Kitahara, H. Oka, Y. Miguchi-Fukazawa and A. Terahara, *Eur. J. Biochem.* **82**, 257 (1978).
14. A. Endo and N. Kitahara, *J. Antibiot., Tokyo* **34**, 1351 (1981).
15. T. G. Porter, S. B. Martin and D. L. Martin, *Abstr. Soc. Neurosci.* **8**, 819 (1982).
16. L. P. Miller and D. L. Martin, *Life Sci.* **13**, 1023 (1973).
17. D. C. Spink, S. J. Wu and D. L. Martin, *J. Neurochem.* **40**, 1113 (1983).
18. B. Seligman, L. P. Miller, D. E. Brockman and D. L. Martin, *J. Neurochem.* **30**, 371 (1978).
19. S. B. Martin and D. L. Martin, *J. Neurochem.* **33**, 1275 (1979).
20. D. C. Spink and D. L. Martin, in *Glutamate, Glutamine and GABA in the Central Nervous System* (Eds. L. Hertz, E. Kvamme, E. G. McGeer and A. Schousbor), p. 129. Alan R. Liss, New York (1983).
21. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974).
22. M. Maitre, J. M. Blindermann, L. Ossola and P. Mandel, *Biochem. biophys. Res. Commun.* **85**, 885 (1978).
23. J-Y. Wu, T. Matsuda and E. Roberts, *J. biol. Chem.* **248**, 3029 (1973).
24. J-Y. Wu, E. Wong, K. Saito, E. Roberts and A. Schousboe, *J. Neurochem.* **27**, 653 (1976).
25. A. C. Foster, J. F. Collins and R. Schwarcz, *Neuropharmacology* **22**, 1331 (1983).
26. K. S. Rogers, M. R. Boots and S. G. Boots, *Biochim. biophys. Acta* **258**, 343 (1972).
27. W. S. Caughey, J. D. Smiley and L. Hellerman, *J. biol. Chem.* **224**, 591 (1957).