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SOME PROPERTIES OF L-GLUTAMIC DECARBOXYLASE IN MOUSE BRAIN

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Abstract—A simple modification has been made of a previously reported apparatus by which it is possible to study any reactions in which radioactive carbon dioxide is evolved in various gas atmospheres.

L-Glutamic acid decarboxylase activity of acetone powders from mouse brain can be stabilized indefinitely when pyridoxal-P and GSH are added during the preparation. The enzyme in homogenates of the acetone powder is protected against inactivation during preincubation by GSH, or cysteine, or mercaptoethanol, and pyridoxal-P. It is sensitive to oxygen and assays are run routinely in an atmosphere of purified nitrogen alone or in mixture with carbon dioxide.

Orthophosphate was found to be a weak competitive inhibitor of the decarboxylase activity of homogenates at 0·1 M concentration and a noncompetitive inhibitor at 0·2 M. Under the standard test conditions the enzyme activity was not inhibited by 1×10^{-3} M concentrations of D-glutamic acid and several other compounds structurally related to glutamic acid, nor by straight chain aliphatic monocarboxylic acids from acetic to valeric; of the dicarboxylic acids from oxalic to pimelic, only oxalic was weakly inhibitory. The decarboxylase activity was inhibited to a varying extent by different sulfhydryl reagents. *p*-Hydroxymercuribenzoate was found to be a potent, noncompetitive inhibitor. 1,2-Naphthoquinone-4-sulfonic acid and 1-nitroso-2-naphthol-3,6-disulfonic acid (nitroso R salt) are relatively strong competitive inhibitors. The latter two compounds probably interact with a sulfhydryl group of the enzyme as well as with other groups of the active site. Several amino-naphthol-sulfonic acids were found to be weak inhibitors. Diethylstilbestrol disulfate was a weak competitive inhibitor of the decarboxylase activity, and estradiol disulfate was considerably more inhibitory and noncompetitive.

A series of experiments with hydroxylamine and with substituted hydroxylamines indicated that the free amino group is necessary for inhibition. The inhibition was decreased in compounds in which the hydroxyl group was replaced by an amino group (hydrazine) or the hydroxyl hydrogen was replaced by an uncharged constituent. On the other hand, substitution of this hydrogen with groups which are more acidic than oximes increased the inhibitory potency. Aminooxypropionic acid, a competitive inhibitor of the enzyme, was the most potent of these substances tested, 50% inhibition being found at a concentration of $4{\cdot}5 \times 10^{-7}$ M. DL-α-Hydrazinophenylpropionic and DL-α-hydrazinophenylacetic acids were much more effective competitive inhibitors than a large number of other hydrazine derivatives with a variety of substituents, but not containing an acidic function. Experiments with hydroxylamine and α-hydrazinophenylacetic acid showed that the *rate* of loss of enzymic activity during preincubation of the brain homogenate with these agents was less than in absence of inhibitor, a finding

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consistent with the interpretation that the major mode of inhibition by carbonyl-trapping agents is by combination with the holoenzyme in such a way as to block the catalytic site while the coenzyme remains attached to the apoenzyme.

It was found that the D-isomers of penicillamine and cysteine are better inhibitors of the decarboxylase activity than the L-isomers.

Some preliminary data are given on the solubilization of the decarboxylase and fractionation with ammonium sulfate. The results of the present study are discussed in relation to findings obtained with other B_6 enzymes.

THE ENZYME L-glutamic acid decarboxylase occurs in uniquely high concentrations in the central nervous system of various vertebrate species. Distribution studies made of the enzyme in various areas of monkey and rabbit brain and spinal cord have shown it to be located almost entirely in the gray matter. The decarboxylase is a pyridoxal-P-requiring enzyme, its activity being reduced in vitamin B_6 deficiency and rapidly restored to normal upon refeeding the vitamin to the deficient animals. The enzyme increases progressively with development in whole brain or in specific areas of brain of various species studied, showing typical sigmoidal temporal relationships. The data suggest that the decarboxylase may be the rate-limiting enzyme that under normal circumstances determines the level of γ-aminobutyric acid in a particular area of the central nervous system. Insofar as γ-aminobutyric acid may serve an important function as a regulator of neuronal activity and is the key member of an important series of metabolic reactions in the central nervous system, a knowledge of the properties of the brain decarboxylase is of considerable interest.[1-3] To date there has been little information about the properties of the brain enzyme because of the lability of the various preparations. A recent study has been made of the L-glutamic decarboxylase of *Escherichia coli*.[4-6] The purpose of the present work was to obtain further information about the crude brain enzyme which might be useful in its purification, in the understanding of its mode of action, and possibly in altering its activity *in vivo*.

EXPERIMENTAL

Enzyme. Acetone powder from mouse brain was used as a source of enzyme. When the acetone powders were prepared as described previously,[7] the preparations lost activity at an appreciable rate even during storage in a vacuum desiccator at −20°. It was found in the course of the study that the addition of approximately 10 mg of GSH and 5 mg of pyridoxal-P to each 700-ml portion of acetone (−20°) in the Waring Blendor prior to suspending approximately 75 mouse brains therein gave preparations that were stable indefinitely. Many of the experiments were performed with a batch of acetone powder made from 1400 mouse brains.

Substrate. Uniformly labeled L-glutamic acid-^{14}C or L-glutamic acid-1-^{14}C prepared by synthesis and resolution* (0·2 mc contained in 2 ml of water) was diluted to a final volume of 150 ml with suitable phosphate or bicarbonate buffers. A 40-ml sample of the buffer was then mixed with 30 ml of 0·5 M L-glutamic acid (pH 6·5), the pH adjustment of which was achieved with alkali containing the same cation as the buffer. To the mixture was then added 2 ml of a solution of pyridoxal-P containing either 5 or 1·5 mg/ml. Smaller quantities of similarly prepared substrate mixtures were prepared for special studies. When not in use the substrate mixtures were stored frozen. Variations in substrate will be discussed in the text in the specific sections dealing with them.

* Purchased from California Corp. for Biochemical Research, Los Angeles, Calif.

Apparatus. Apparatus similar to that previously described[3] was employed for the measurement of $^{14}CO_2$ liberated in the glutamic decarboxylase assays. When aerobic experiments were performed, the apparatus differed from that employed previously only by having no stopcock on the cross arm connecting the incubation flask and the attached counting vial containing the Hyamine base. For experiments with bicarbonate buffers carried out in N_2-CO_2 atmosphere it was necessary to employ a cross arm with a large-bore three-way stopcock. The gassing was so contrived that the gas passed into the incubation flask and out into the atmosphere without entering that half of the cross arm to which the counting vial was attached. The stopcock was then turned so that the incubation flask was sealed off from the counting vial and the outside atmosphere during the desired period of incubation. At the end of the experiment the stopcock was turned to establish communication between the incubation flask and the vial, and the CO_2 was allowed to diffuse into the Hyamine base. A newly designed cross arm, C, illustrated in Fig. 1, has been found suitable for all experiments other

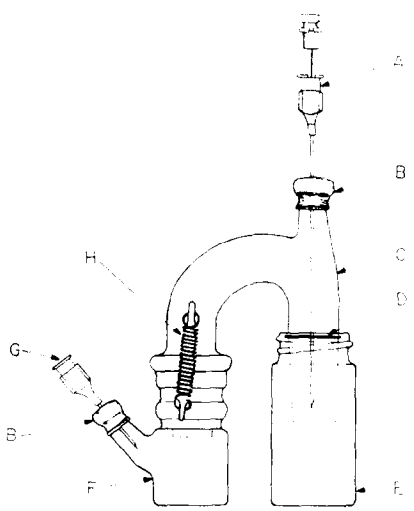


FIG. 1. Apparatus employed for measurement of $^{14}CO_2$ liberated in glutamic decarboxylase assay; see text.

than those using bicarbonate buffers. A 3·5-inch, 22-gauge spinal needle, A, is inserted in stopper B. This needle has a stylet which is gas-tight. The desired gas atmosphere is established by allowing gas to flow in through a needle, G, in stopper B and out through the spinal needle with the stylet removed. After a suitable period of gassing, G is removed and the stylet in the needle in A is rapidly replaced. Under the conditions employed, essentially complete diffusion and absorption of CO_2 by Hyamine was achieved within 30 min although, routinely, diffusion was allowed to take place for 90 min. Other parts of the apparatus in Fig. 1 are D, neoprene "O" ring; E, vial employed for counting; F, reaction flask; and H, Warburg spring.

*Enzyme assays.*Ordinarily, 0·36 ml of buffer containing substrate prepared as above was added to the incubation flask followed by 0·2 ml of water or solution containing

appropriate amounts of various materials being tested for effect on enzyme activity. The apparatus was brought to 37° in a Dubnoff incubator, and the reaction was started by the injection of 0·2 ml of enzyme preparation through the serum stopper on the incubation flask from a 1-ml tuberculin syringe calibrated to deliver exactly 0·2 ml. Usually the enzyme preparation was a homogenate containing 5 mg mouse brain acetone powder prepared by suspending 25 mg of mouse brain powder per ml of ice-cold distilled water and homogenizing in a ground-glass homogenizer. After it was found that enzyme in this form was sensitive to O_2, the water used for homogenization was gassed thoroughly with purified N_2 prior to preparation of homogenate. At all times after preparation the homogenate was kept on ice. After incubation for suitable periods of time, usually 15 or 30 min, 0·1 ml of 8 N sulfuric acid was injected into the reaction vessel, and the flasks were left in the incubator for 90 min, at which time the counting vial was removed and the radioactivity measured in the Packard Tri-Carb liquid scintillation counter, model 314-DC or 314-A as previously described.[5] Blank values (obtained when the incubations were performed as above except that enzyme was omitted or inactivated enzyme was employed) were subtracted from those obtained from vessels containing active enzyme. All experiments, including blanks, were always performed in duplicate and in many instances in triplicate.

RESULTS

Experiments on stabilization of enzyme

Experiments were carried out in which homogenates of mouse brain acetone powder in distilled water (5 mg/ml) were incubated aerobically in distilled water or buffer at 37° prior to the aerobic measurement of activity in sodium phosphate buffer (0·0263 M, pH 6·2) containing 50 μg pyridoxal-P. The activities of the homogenate incubated for 15, 30, and 60 min were 40, 23, and 21%, respectively, of those found for the preliminary homogenate tested immediately after preparation. When the preliminary incubation was performed in 0·01 M GSH contained in 0·05 M sodium phosphate buffer (pH 6·2) there was no inactivation of the enzyme. The activity of inactivated enzyme could not be restored by performing the assay in the presence of 0·01 M GSH. In similar experiments it was shown that when preincubation took place with 5×10^{-4} M pyridoxal-P the enzyme could be protected against inactivation to the extent of approximately 80%. Pyridoxal and pyridoxamine were completely ineffective. Also a combination of both GSH and pyridoxal-P in these concentrations gave complete protection of the enzyme against inactivation during a 30-min preincubation. Mercaptoethanol (0·01 M) was as effective as GSH in protecting the enzyme. As a result of these findings, GSH and pyridoxal-P were added during the preparation of acetone powders as described in the previous section, and stabilization of the activity was achieved over a prolonged period.

Of a large variety of substances tested only GSH, cysteine, mercaptoethanol and pyridoxal-P showed protective action. No protection against inactivation was given by preincubation with 0·01 M concentrations of the following substances: glycine, L-alanine, L-isoleucine, L-leucine, L-valine, L-serine, L-threonine, L-aspartic acid, D-glutamic acid, D,L-α-methylglutamic acid, L-asparagine, L-glutamine, D,L-α-methylglutamine, L-arginine, L-lysine, D,L-δ-hydroxylysine, D,L-ornithine, L-cystine, L-methionine, L-phenylalanine, L-tyrosine, L-histidine, L-proline, L-tryptophan, γ-aminobutyric acid, and α-ketoglutaric acid.

In some experiments in which activity was measured immediately after preparation of the homogenate it was noted that the activity in the presence of GSH was higher than in the controls. This enhancement of activity probably was attributable to the protection of a sensitive sulfhydryl group on the enzyme against oxidation by dissolved oxygen (Table 1). When an atmosphere of pure N_2 or 95% N_2–5% CO_2 was established

TABLE 1. EFFECT OF GAS ATMOSPHERE AND GSH ON GLUTAMIC ACID DECARBOXYLASE ACTIVITY OF BRAIN ACETONE POWDER

Experiment no.	Atmosphere	Addition (0·01 M)	Enzyme activity (μmole/hr/100 mg acetone powder)
1	Air		14·9
	Air	GSH	18·4
	N_2*		18·0
2	Air		15·1
	O_2		7·5
	N_2		18·4
	95% N_2–5% CO_2†		19·5
3	Air		16·1
	Air	GSH	19·1
	N_2		18·4
	N_2	GSH	19·3
	O_2		8·3
	O_2	GSH‡	12·5
	O_2	GSH§	13·5

* Purified N_2.
† Bicarbonate buffer employed, final pH 6·2 to 6·3.
‡ GSH present when flask was gassed with O_2.
§ GSH added after flask was gassed with O_2.

before the addition of enzyme, the activity invariably was higher than when the experiment was performed in air. When pure O_2 was employed the activity was reduced to approximately 50% of that found in air. The oxidation of —SH groups by O_2 has been discussed and pertinent literature cited.[8] Because of the report that albumin stabilizes bacterial glutamic decarboxylase,[6] incubations were made of 2, 4, 6, and 8 mg of enzyme powder for 30 min in absence or presence of 0·1% crystalline bovine serum albumin (Armour) and of 5 mg of enzyme powder for 15, 30, 45, 60, and 90 min in the presence or absence of albumin. The incubations were performed in $9{\cdot}1 \times 10^{-4}$ M potassium bicarbonate buffer in a 95% N_2– 5% CO_2 atmosphere (pH 6·2) in the presence of 15 μg of pyridoxal-P. Under these conditions the albumin produced no enhancement of activity over that observed in its absence. The difference in behavior to stabilization by albumin of the brain and bacterial enzymes may possibly be attributable to the relatively large quantities of protein impurities already present in the brain preparation. The bacterial decarboxylase is activated by chloride.[9] When assays were performed in bicarbonate buffer, as above, constant activity was observed when the chloride content was varied between $1{\cdot}1 \times 10^{-4}$ and $1{\cdot}1 \times 10^{-2}$ M. The following cations, when tested as sulfates at a concentration of 1×10^{-5} M, had no effect on enzymatic activity: Al^{3+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}. Ammonium ion was without effect on enzymatic activity even at 0·01 M concentration.

Buffer ion effects

During experiments with the bacterial glutamic decarboxylase it was noted that the activity in the acetate buffer ordinarily used was lower than that found in pyridine buffer. Acetate in high concentrations was found to be a competitive inhibitor of the bacterial enzyme ($K_1 = 0{\cdot}2$ M).[4] Although measurements of activity of the brain enzyme are made routinely in phosphate buffer, it appeared possible that phosphate or other buffer anions also might decrease the affinity of the enzyme for the substrate. Experiments were performed in which activities measured in different buffers at pH 6·2 were compared with the activity in phosphate. Buffers of the indicated concentrations showed the following percentages of the activity found with 0·0263 M phosphate: citrate (0·0263 M) 72; maleate (0·0132 M) 81; arsenate (0·0132 M) 87; imidazole (0·0132 M) 86. In the absence of buffer other than substrate, adjusted to pH 6·2, the activity was 83% of that in phosphate buffer. In a number of experiments it was found that either potassium bicarbonate or potassium phosphate gave 20% greater activity than the corresponding sodium salts. However, potassium ion in excess of 1×10^{-4} M gave no further activation. Somewhat greater activity was observed in experiments

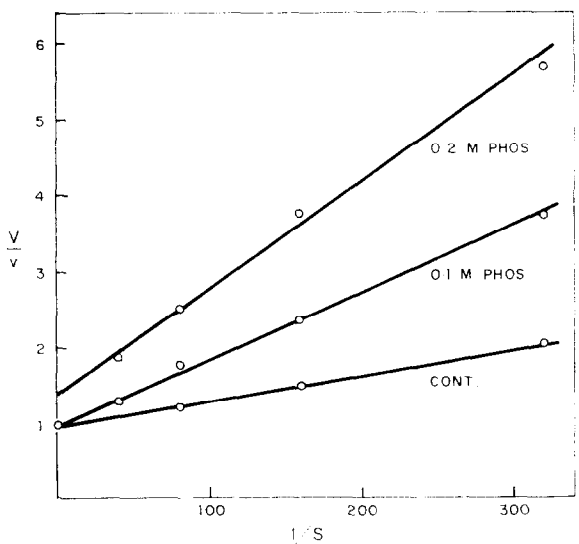


FIG. 2. Effect of phosphate on reaction velocity at various substrate concentrations. It proved to be most convenient for comparative purposes in this and subsequent inhibition studies to express the velocities (15-min incubation) as ratios of the reaction rates observed (v) to those found in control tubes containing 0·0246 M substrate (V), a concentration giving maximal rates under conditions employed. It can be shown from the classical Michaelis-Menten relationships that the uninhibited reaction plots of the reciprocals of these ratios (V/v) versus the reciprocals of the molar substrate concentrations (1/S) should give straight lines with an intercept of one. The presence of a competitive inhibitor should only increase the slope. In all other similar plots in subsequent figures the ordinate and abscissa are in the same units as in this figure.

with bicarbonate buffer than with phosphate. Increasing the bicarbonate concentration at a constant pH of 6·2 did not alter activity. For example, identical activities were obtained with the following gas mixtures and potassium bicarbonate concentrations: $1{\cdot}82 \times 10^{-3}$ M (10% CO_2–90% N_2); $9{\cdot}1 \times 10^{-3}$ M (50% CO_2–50% N_2): and $1{\cdot}64$ 10^{-2} M (90% CO_2–10% N_2).

In $9{\cdot}1 \times 10^{-4}$ M (5% CO_2–95% N_2) potassium bicarbonate buffer, phosphate proved to be weakly inhibitory. The results in Fig. 2 showed that phosphate is a weak competitive inhibitor at 0·1 M concentration and not competitive at the 0·2 M concentration. Because phosphate buffer is easier to use than bicarbonate, it was felt that the relative effects of various inhibitors could be studied in the usual phosphate concentration of 0·0263 M without introducing too great an error.

In experiments in which activity was measured at different values of pH employing either bicarbonate or phosphate buffer, the maximal activity of the enzyme was found at pH 6·4, the same value as that reported previously for this enzyme.[10]

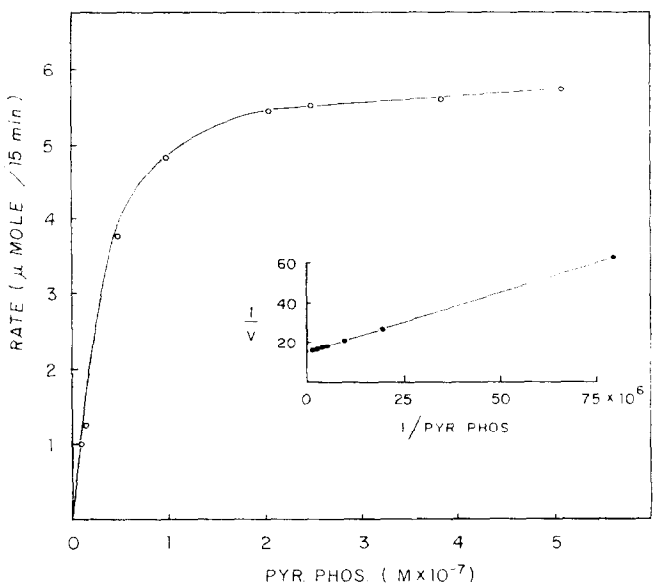


FIG. 3. Reaction velocity as a function of concentration of pyridoxal-P. When the homogenate made from the acetone powder was assayed immediately, it showed no activation by pyridoxal-P. When the homogenate (25 mg/ml) was incubated in distilled water under N_2 for 2·5 hr at 37°, over 90% of the original activity was lost, and the residual activity was increased maximally approximately fourfold by the addition of excess pyridoxal-P. The results show the increase in activity over that found in the absence of added coenzyme as a function of the concentration of added pyridoxal-P, and the reciprocal plot of the data is shown in the insert.

The decarboxylase as a B_6 enzyme

The decarboxylase is a B_6 enzyme, requiring pyridoxal-P for activity.[7] When mouse brain acetone powder is prepared in the absence of added pyridoxal-P, and when no pyridoxal-P is added during assay, the homogenates of the powder usually show, at most, 20 to 40% of the maximal activity attainable in the presence of excess coenzyme. If acetone powder is prepared with pyridoxal-P and GSH present, little or no activation often results when pyridoxal-P is added in excess during the assay. Separate experiments showed that the maximal extent of activation by pyridoxal-P of a particular acetone powder preparation is the same for the decarboxylase which goes into solution in water or buffer and for that remaining with the insoluble residue. The results in Fig. 3 show the activity as a function of coenzyme concentration when a

partially resolved enzyme preparation was employed for the determination of reaction rate.

Effect on the decarboxylase of compounds related in structure to L-*glutamic acid and of several aliphatic mono- and dicarboxylic acids*

D-Glutamic acid is not decarboxylated by the enzyme and even at a concentration of 5×10^{-3} M is not an inhibitor of the decarboxylation of L-glutamic acid. L-Aspartic acid inhibited only to the extent of 12, 8, and 6%, respectively, at concentrations of 1×10^{-2}, 5×10^{-3}, and 1×10^{-3} M; and D-aspartic acid had no effect at these concentrations. Likewise, the following substances closely related structurally to L-glutamic acid were not inhibitory at 1×10^{-3} M concentrations in the standard assay system: DL-β-hydroxyglutamic acid, α,γ-diaminoglutaric acid (mixture of isomers), DL-α-methylglutamic acid, and DL-α-methylglutamine.

Acetic, propionic, butyric, and valeric acids did not inhibit the enzyme at 1×10^{-3} M concentration, nor did the following dicarboxylic acids: malonic, succinic, glutaric, adipic, and pimelic. Of the dicarboxylic acids tested, only oxalic acid proved to be very weakly inhibitory, the following percentage inhibitions being observed at the molar concentrations noted: 1×10^{-2}, 42; $7{\cdot}5 \times 10^{-3}$, 34; 5×10^{-3}, 32; $2{\cdot}5 \times 10^{-3}$, 21; 1×10^{-3}, 17.

Inhibition by substances which react with sulfhydryl groups

Previous results have shown that the bacterial[5] glutamic decarboxylases probably are sulfhydryl enzymes. The oxygen sensitivity of the brain decarboxylase and its protection by GSH and mercaptoethanol suggested that the enzyme also contains a sensitive sulfhydryl group, the integrity of which is related to maintenance of enzymatic activity. Experiments were carried out with several reagents which can react with sulfhydryl groups in proteins. It was shown that 1×10^{-4} M N-ethylmaleimide, a substance that forms addition compounds with sulfhydryl groups,[11] produced inhibition to the extent of 83%. When the inhibitor was added to an incubation mixture containing 1×10^{-3} M GSH, no inhibition was observed, a finding consistent with the known instantaneous reaction of GSH sulfhydryl groups with N-ethylmaleimide and the slower reaction of protein sulfhydryl groups.[12] Both of the alkylating agents, iodoacetic acid and iodoacetamide, were relatively weakly inhibitory, producing 39 and 38% inhibition, respectively, at 1×10^{-3} M concentration. Mercuric chloride produced 55% inhibition at 1×10^{-3}, 10% at 1×10^{-4}, and none at 1×10^{-5} M and lower concentrations. Preliminary experiments also showed that PMB,* a mercaptide-forming reagent, and 1,2-naphthoquinone-4-sulfonate, a substance that can form adducts with sulfhydryl groups, were inhibitory. The effects of PMB (1×10^{-4} M) and naphthoquinone sulfonate (1×10^{-3} M) could be prevented only partially by GSH, approximately 50% of the original activity being found even when the inhibitor was added last to an incubation mixture containing 1×10^{-3} M GSH. The effects of the latter two inhibitory substances were not decreased by increasing threefold the concentration of pyridoxal-P present during the incubation. The lower inhibitory potency of mercuric chloride relative to mercuribenzoate could possibly be attributed

* *p*-Hydroxymercuribenzoate.

to a greater extent of reaction of the former substance with nonenzymatic impurities in the enzyme suspension.

Three experiments showing the effect of different concentrations of PMB in inhibiting the enzyme are summarized in Fig. 4, and data from one of a number of experiments showing that the inhibition is not competitive with substrate are given in

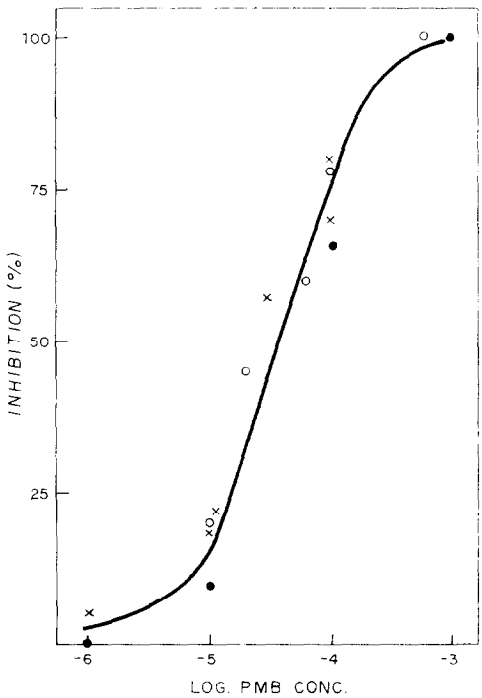


FIG. 4. Inhibition of the brain enzyme as a function of the molar concentration of PMB.* Symbols ●, ○, × represent 3 different experiments.

Fig. 5. The inhibition by PMB was not dependent on the order of addition of the reagents, the same inhibition being observed whether PMB was added to the complete substrate mixture immediately before or after the addition of enzyme or whether the PMB was mixed with the enzyme prior to the addition of substrate.

Simple sulfhydryl compounds and proteins containing free sulfhydryl groups can form conjugates with quinones[8] by addition of the sulfhydryl groups to reactive double bonds or carbonyl groups; or the quinones may act as oxidizing agents of the sulfhydryl groups. In the present experiments it was found that 1,2-naphthoquinone-4-sulfonate was an inhibitor of the decarboxylase (see Fig. 11). The fact that the inhibition is strictly competitive (Fig. 6) suggests that the bonds formed between the enzyme and inhibitor are readily dissociable. An experiment was performed in which the final concentration of this inhibitor was 5×10^{-4} M and the substrate, $5{\cdot}6 \times 10^{-2}$ M. In this experiment the complete incubation mixture with or without inhibitor was

to stand at 0° for 10 min. The GSH was added to some of the flasks (final concentration, 1×10^{-2} M) and incubation was performed at 37° for 30 min. Under these conditions it was found that in the absence of GSH the 1,2-naphthoquinone-4-sulfonate was inhibitory to the extent of 65%, whereas in the presence of GSH the degree of inhibition was reduced to 29%. The fact that only incomplete protection was afforded by a 20-fold excess of GSH over inhibitor is consistent with the idea that the affinity of the 1,2-naphthoquinone-4-sulfonate for the enzyme depends on interaction with other groups on the enzyme, in addition to sulfhydryl.

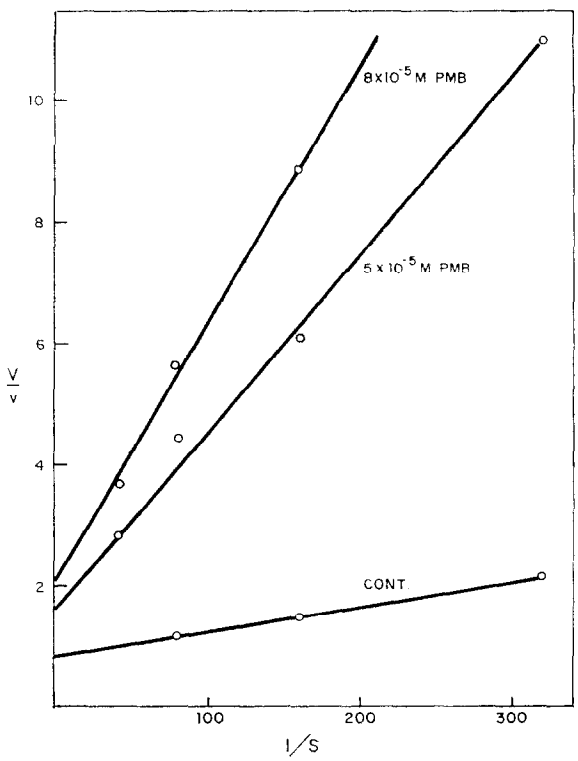


FIG. 5. Reciprocal plots for two concentrations of PMB at various substrate concentrations (units same as in Fig. 2).

A compound closely related to 1,2-naphthoquinone-4-sulfonate, 1-nitroso-2-naphthol-3,6-disulfonic acid (nitroso R salt), previously was found to be a good inhibitor of cystathionase, also a B_6 enzyme.[13] In this compound there exists an equilibrium between the nitrosophenol and quinone-oxime forms. It was suggested that this substance might react with an essential sulfhydryl group on the enzyme and the aldehyde group of the coenzyme of cystathionase.[13] In the present study this compound was found to be a more potent competitive inhibitor of the decarboxylase than the naphthoquinone sulfonate (Fig. 7, and see Fig. 11). The inhibition was only partially prevented by GSH and was not influenced by increasing the amount of pyridoxal-P from 15 to 50 μg per flask.

A further analysis was made of the structural requirements for inhibition by the nitroso R salt by study of the action of structurally related substances. It was found that 1-nitroso-2-naphthol and 2-nitroso-1-naphthol inhibited to the extent of 27 and 28%, respectively, at 1×10^{-3} M concentrations, while nitroso R salt consistently gave

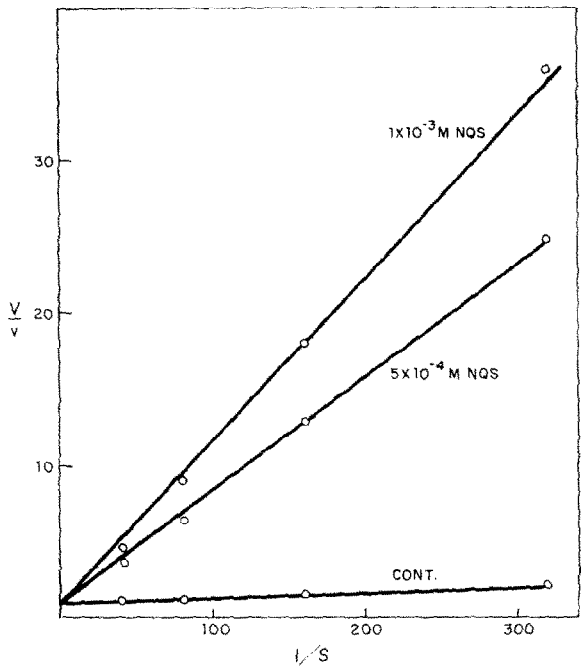


FIG. 6. Reciprocal plots for two concentrations of 1,2-naphthoquinone-4-sulfonate (NQS) at various substrate concentrations (units same as in Fig. 2).

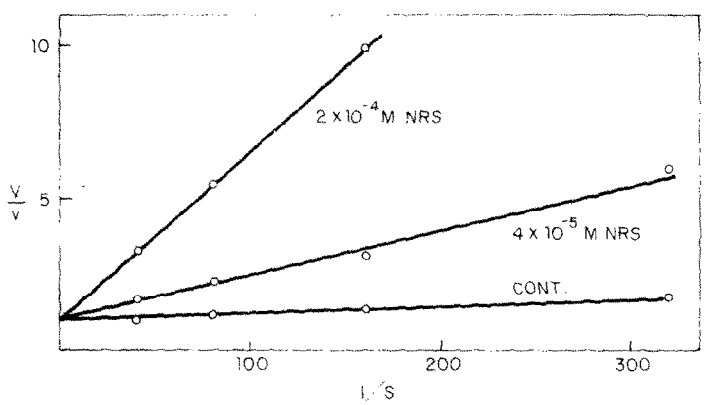


FIG. 7. Reciprocal plots for two concentrations of nitroso R salt (NRS) at various substrate concentrations (units same as in Fig. 2).

approximately 90% inhibition at this concentration. This indicated that the sulfonic acid groups are important in the attachment of the nitroso R salt to the enzyme. 2-Naphthol-3,6-disulfonic acid gave only 10% inhibition at this concentration, showing the requirement for the quinone structure. In another experiment it was shown that

nitroso R salt gave 63% inhibition at 2×10^{-4} M concentration whereas 1-nitroso-2-naphthol-6-sulfonate (naphthol green B) gave only 10% inhibition at the same concentration. The latter result shows that the sulfonic acid group in the 3 position of the nitroso R salt is the more important of the two sulfonic acid groups for the inhibition.

Experiments with a large number of substituted naphthalene derivatives revealed no substance that inhibited to an extent comparable to the above quinones. The most effective compounds of this group, giving approximately 40% inhibition at 1×10^{-3} M, were: 1-amino-2-naphthol-4-sulfonic acid; 7-amino-1-naphthol-3,6-disulfonic acid; 8-amino-1-naphthol-5,7-disulfonic acid; and 8-amino-1-naphthol-3,6-disulfonic acid. In the case of these latter substances the nature of the inhibition was not determined.

Inhibition by estrogen sulfates

It has been reported that estrogen sulfates inhibit the kynurenine transaminase of rat kidney by competition with pyridoxal-P for the coenzyme binding site on the apoenzyme.[14] Diethylstilbestrol disulfate, estradiol disulfate, and estrone sulfate were tested.* Experiments with 10^{-3} M concentrations of these compounds showed that estradiol and diethylstilbestrol disulfates both were more potent inhibitors than the estrone sulfate. The inhibition was not decreased by increasing the levels of pyridoxal-P.

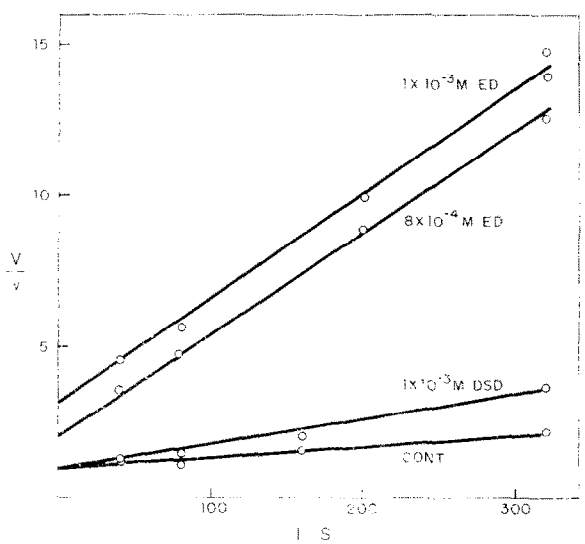


FIG. 8. Reciprocal plots for estradiol (ED) and diethylstilbestrol (DSD) disulfates (units same as in Fig. 2).

Diethylstilbestrol disulfate appeared to be a very weak competitive inhibitor whereas estradiol disulfate was considerably more inhibitory and not competitive with substrate (Fig. 8). In the case of L-glutamic decarboxylase of brain, therefore, the latter compound does not appear to compete either with substrate or coenzyme. Other experiments showed that preincubation of the enzyme with 4×10^{-4} M estradiol disulfate

* Kindly given to us by Dr. Merle Mason of the University of Michigan, Ann Arbor, Mich.

for as long as 60 min did not increase the extent of inhibition over that found when the measurement was performed immediately.

Inhibition of the decarboxylase by hydroxylamine and related compounds

By far the largest group of effective inhibitors of B_6 enzymes is found among those reagents that can combine with the aldehyde group of the coenzyme. As seen from Fig. 9, the kinetics are complex in reactions catalyzed by B_6 enzymes when activity

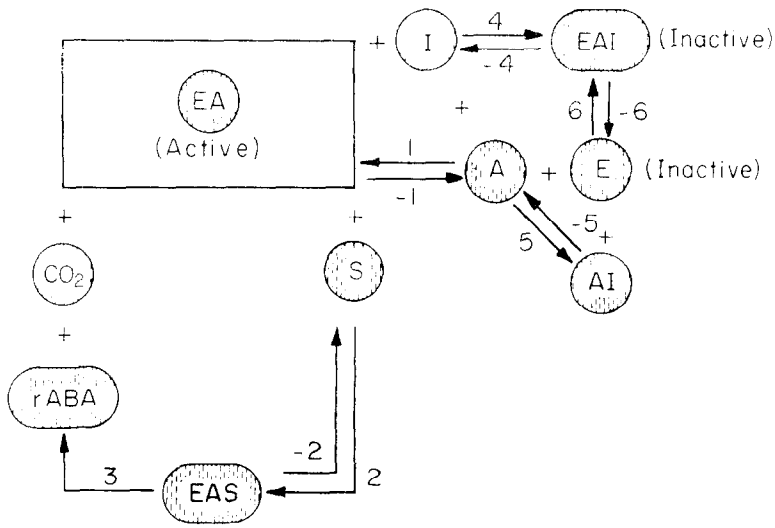


FIG. 9. Minimal number of reactions involved in action of glutamic decarboxylase (E) on substrate (S) in absence and presence of inhibitors (I). A, pyridoxal-P; and γABA, γ-aminobutyric acid.

is measured in the presence of both added coenzyme and reagents that can react with free and enzyme-bound coenzyme.

Experiments with a bacterial glutamic decarboxylase, which under the conditions employed was fully saturated with bound coenzyme, showed that hydroxylamine was strictly competitive with substrate.[15] The results in Fig. 11 show the degree of inhibition of the brain decarboxylase when the assay was performed in the presence of the usual concentration of pyridoxal-P, 8×10^{-5} M. However, the amount of free hydroxylamine present in any assay mixture is less than the amount added because the inhibitor reacts with the free coenzyme to form an oxime. (The amount of hydroxylamine bound to the holoenzyme can be considered negligible by comparison with that found in the free form or combined with free coenzyme.) Therefore, in the plots shown subsequently the concentrations of hydroxylamine and other substances cited include both free and coenzyme-bound reagent. Theoretically, under the usual conditions of assay, the maximal concentration of hydroxylamine or other carbonyl-trapping agent that could be bound would be 8×10^{-5} M, the concentration of coenzyme employed.

In the usual reciprocal plots the inhibition by hydroxylamine was found to be strictly competitive with substrate (Fig. 10). Measurements also were made of the

inhibition produced by $7{\cdot}5 \times 10^{-5}$ M hydroxylamine at different concentrations of added pyridoxal-P. At 8×10^{-5}, $1{\cdot}6 \times 10^{-4}$, and $2{\cdot}4 \times 10^{-4}$ M concentrations of the coenzyme the inhibitions were 30, 9, and 0·5%, respectively. It would not appear that pyridoxal-P oxime is a particularly potent inhibitor since, if it is assumed in the experiment above that at $1{\cdot}6 \times 10^{-4}$ M concentration of the coenzyme virtually all

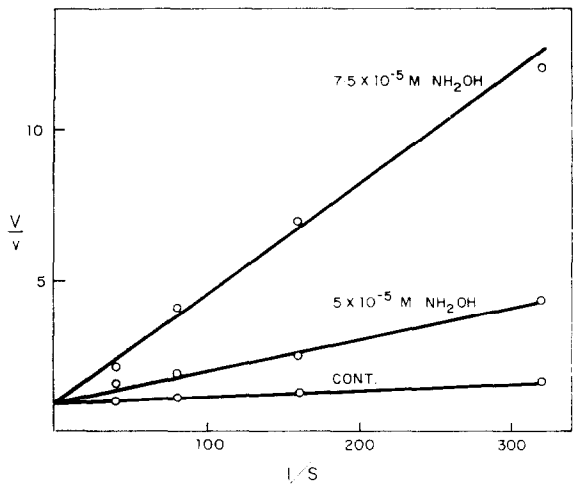
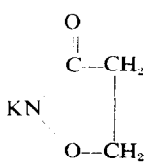
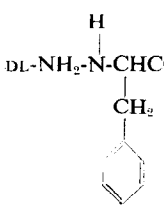
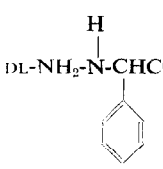
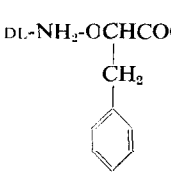


FIG. 10. Reciprocal plots for hydroxylamine (units same as in Fig. 2).

of the hydroxylamine is converted to the oxime, then only 9% inhibition would be achieved when approximately equimolar concentrations of the oxime and the free coenzyme are present. Pyridoxal oxime was found to be a very weak inhibitor of the enzyme, only 18% being produced at 1×10^{-3} M concentration, at which concentration hydroxylamine itself produced complete inhibition. The fact that hydroxylamine is competitive with substrate strongly supports the idea that it competes with substrate for the aldehyde group of the coenzyme. Oximes generally are weakly acidic compounds (see References 16 and 17 for some recent data). However, the oxime of pyridoxal-P would be expected to be much more acidic than an oxime of an ordinary aldehyde or ketone, since there is a strong electromeric displacement of electrons through the system of conjugated double bonds toward the pyridine ring nitrogen.[18] The importance of the acidity of the aldehyde derivative is emphasized by the results summarized in Table 2. Hydrazine and derivatives of hydroxylamine in which the hydroxyl hydrogen is replaced by various uncharged constituents were less inhibitory than hydroxylamine itself in the standard test system (group 1). Those compounds in which there is an acidic group are either stronger inhibitors of the enzyme or approximately equipotent with hydroxylamine (group 2). Substitution of one of the hydrogens of the amino group of hydroxylamine (methoxyamine) destroyed all inhibitory activity. Likewise, removal of amino and carboxyl groups by cyclization of aminooxypropionic acid (3-isoxazolidinone) reduced the inhibition remarkably (group 3). In the case of the latter compound it is probable that some hydrolysis to aminooxypropionic acid may have taken place during the incubation so that no inhibition may have been given by the test substance itself. The latter results emphasize the requirement for the aldehyde-binding group in this class of reagents. In

TABLE 2. INHIBITION OF GLUTAMIC DECARBOXYLASE BY SOME CARBONYL-TRAPPING AGENTS

Group	Substance tested	Percent inhibition 10^{-3} M	10^{-4} M	10^{-5} M
1*	NH_2H	100	70	10
	NH_2ONH_2	75	37	5
	NH_2OCH_3	65	15	0
	NH_2OCH_2— 	62	16	0
	$NH_2OCH_2CH_2CH(COOCH_2CH_3)_2$	90	17	0
2*	NH_2OCH_2COOH	100	100	95
	$NH_2OCH_2CH_2COOH$	100	100	97
(-)	$NH_2O\overset{H}{\underset{CH_3}{C}}COOH$	100	99	68
(-)	$NH_2O\overset{H}{\underset{CH_3}{C}}COOH$	100	96	32
	$NH_2O\overset{CH_3}{\underset{CH_3}{C}}COOH$	100	75	6
3*		60	8	0
	CH_3NHOH	0	0	0
4†		100	82	21
		100	80	24
		100	74	14

* All the hydroxylamine derivatives in groups 1–3 were supplied by Dr. M. J. Vander Brook of the Upjohn Company, Kalamazoo, Mich.

† Compounds in group 4 were given by Dr. John Biel of the Lakeside Laboratories, Milwaukee, Wis.

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Fig. 11 are shown the inhibitions produced by various concentrations of hydroxylamine and of the four most potent inhibitors in Table 2, and the results are compared with those obtained for nitroso R salt and 1,2-naphthoquinone-4-sulfonic acid, compounds discussed previously.

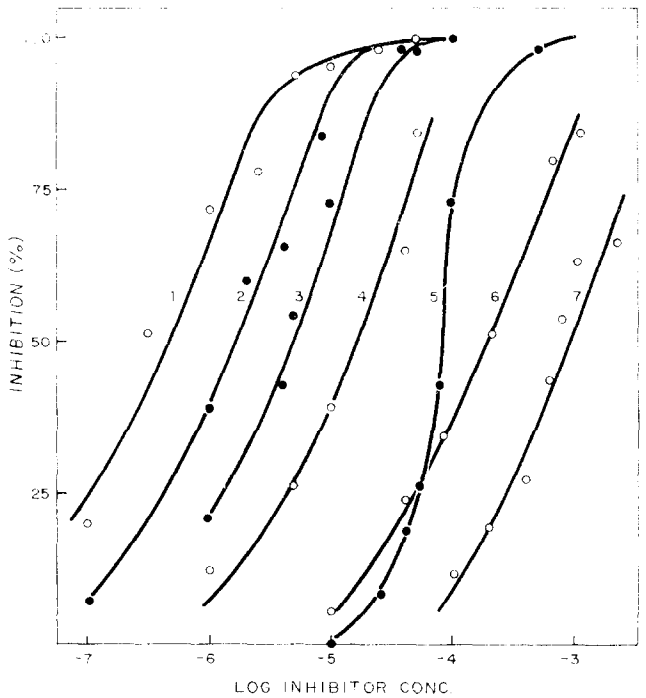

FIG. 11. Inhibition of brain glutamic decarboxylase by various molar concentrations of several inhibitors: 1, aminooxypropionic acid; 2, aminooxyacetic acid; 3, (+) 2-aminooxypropionic acid; 4, (—) 2-aminooxypropionic acid; 5, hydroxylamine; 6, nitroso R salt; 7, 1,2-naphthoquinone-4-sulfonic acid.

DL-α-Aminooxy- and DL-α-hydrazinophenylpropionic acids and DL-α-hydrazinophenylacetic acid also were relatively potent inhibitors of the decarboxylase (group 4, Table 2). The results in Fig. 12 and 13 show that both the aminooxy- and hydrazinophenylpropionic acids are competitive inhibitors of the enzyme.

The following 18 of approximately 50 other hydrazine derivatives* tested gave the indicated inhibitions of 60% or more at concentrations of 1×10^{-3} M: N-2-pyridyl-N-β-hydrazinoethylamine, 81; 2-pyridylmethyl hydrazine, 80; 2-(N-methyl-N-phenyl)-aminoethylhydrazine, 78; 2-(benzhydryloxy)ethylhydrazine, 77; 10-(β-hydrazinoethyl)phenothiazine, 77; 3-tropylhydrazine, 76; 2-hydrazinomethylthiophene, 75; 1-(3,4,5-trimethoxy)phenyl-2-hydrazinopropane, 74; 2-(N-methyl-N-benzyl)aminoethylhydrazine, 73; 3-hydrazinomethylpyridine, 73; 1-phenyl-2-hydrazinoethanol, 73; 1-phenyl-3-hydrazinopropene, 72; 3-hydrazinoquinoline, 72; 1-(4-methoxy)phenyl-2-hydrazinopropane, 69; O-benzylhydroxylamine, 68; N-2-pyridyl-N-β-hydrazinoethylamine, 68; hydroxyethylhydrazine, 62; 2,2′-dipryridyl-β-hydrazinoethylamine, 62.

* Supplied by Dr. John Biel, Lakeside Laboratories, Milwaukee, Wis.

In spite of structural diversity of the above compounds, their inhibitory potency was similar and none was so inhibitory as hydroxylamine. The inhibitory action of these substances was not altered markedly by any of the groups present in addition to the carbonyl-reacting hydrazine moiety. It is interesting that none of the compounds above has any acidic groups, the presence of which is apparently a requirement for maximal effectiveness of this class of compounds.

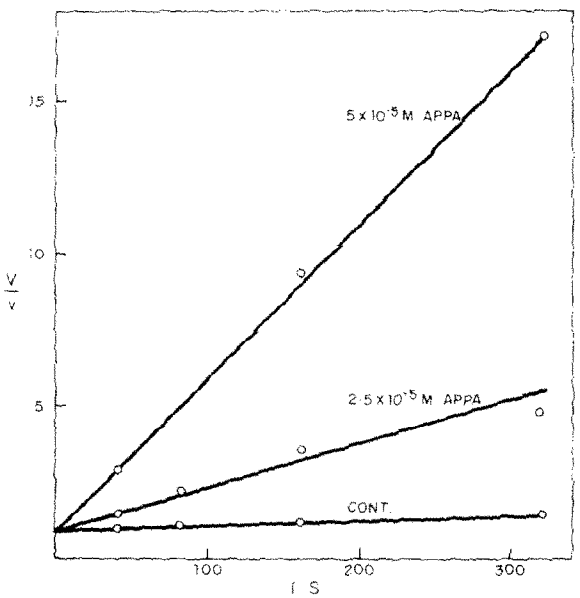

FIG. 12. Reciprocal plots for DL-α-aminooxyphenylpropionic acid (APPA). Same units as in Fig. 2.

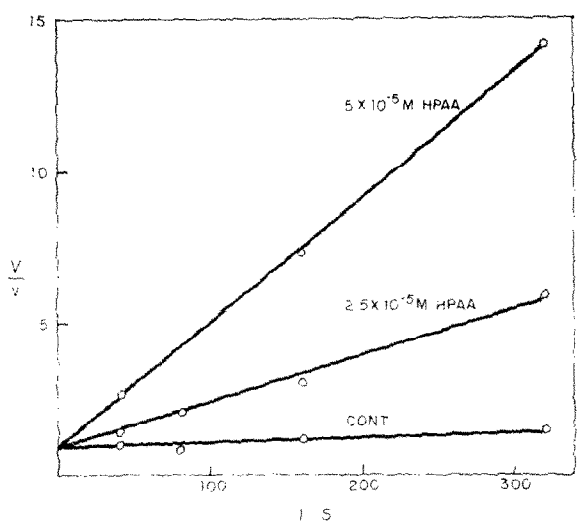

FIG. 13. Reciprocal plots for DL-α-hydrazinophenylacetic acid (HPAA). Same units as in Fig. 2.

It can be inferred from the kinetics cited previously that the most potent of the inhibitory carbonyl-trapping reagent compete with substrate for the active site on the enzyme by forming reversible complexes. The spectral data obtained with the use of purified bacterial glutamic decarboxylase suggested that in the presence of hydroxylamine the oxime of enzyme-bound pyridoxal-P-is formed.[5] It is not possible to show such complex formation directly in the crude enzyme preparations employed in the present studies. However, in keeping with the kinetic data, it was shown that the rate of decline of activity of the brain enzyme incubated for various periods of time in buffer alone prior to measurement of activity was greater than when the preincubation was performed in the presence of hydroxylamine or α-hydrazinophenylacetic acid. Two-tenths ml of a homogenate containing 5 mg of mouse brain acetone powder was added to incubation flasks containing either 0·2 ml of water or a solution of hydroxylamine or α-hydrazinophenylacetic acid in amounts that gave final concentrations of 5×10^{-5} M after dilution with substrate. The flasks were gassed with nitrogen and incubated for various times at 37° prior to the addition of 0·36 ml of substrate mixture for measurement of activity. There was a decline in activity with time of preincubation in all the samples, the rates being approximately the same in the control flasks and in those containing inhibitor for the first 30 min, as shown by the relatively constant percentages of the control activity observed in the flasks containing inhibitor (Fig. 14).

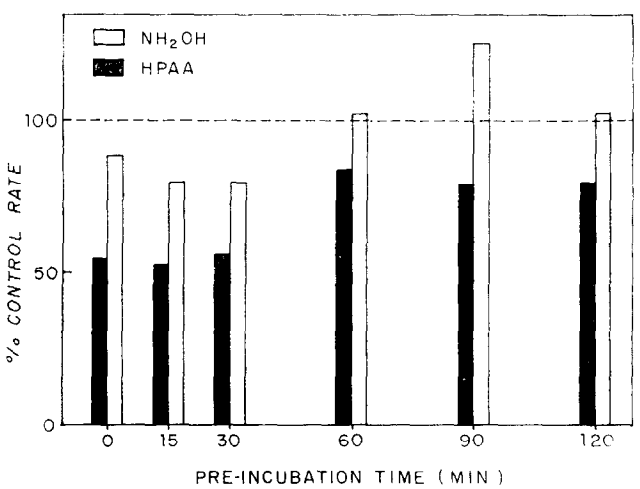

FIG. 14. Influence of time of "preincubation" on relative rates of inactivation of the enzyme in the absence and presence of hydroxylamine and DL-α-hydrazinophenylacetic acid (HPAA).

However, at the later times the rate of decline of activity was greater in the samples in buffer alone, as reflected in the higher percentages of the control activity observed in the presence of inhibitor after 60 to 120 min of preincubation. Actually, the samples containing hydroxylamine had slightly more activity than the controls at the later times. These and previous results are in keeping with the interpretation that the main mode of inhibition by the carbonyl-trapping reagents studied is by combination with the holoenzyme in such a way as to block the catalytic site while the coenzyme remains attached to the apoenzyme. The inactivation of the enzyme preincubated in buffer

alone, which can be prevented by pyridoxal-P or GSH, probably occurs because the dissociation of the coenzyme from the apoenzyme is followed by changes in the protein.

Some recent studies have shown that hydroxylamine can react with components of the nucleic acids,[19, 20] particularly the pyrimidine bases.[20] However, it appears unlikely that the inhibitory effects on B_6 enzymes are mediated through interaction with any site of the coenzyme other than the aldehyde group, since the concentrations of hydroxylamine required to demonstrate the other reactions ranged between 0·5 and 2 M.

The D-*isomers of penicillamine and cysteine are more inhibitory than the* L-*isomers*

The specificity of the decarboxylase for the L-isomer of glutamic acid and the differences observed between the inhibitory activity of the two isomers of 2-(aminooxy)-propionic acid (curves 3 and 4, Fig. 11) focused interest on the asymmetry of the active site. Penicillamine and cysteine were interesting substances to study in this regard. Both the D- and L- forms of these aminothiols can react with pyridoxal-P in solution to form thiazolidine derivatives,[21, 22] and both these substances were more inhibitory than the other D- and L- amino acids which we tested. It was found that the D-isomers were significantly more potent (Fig. 15).

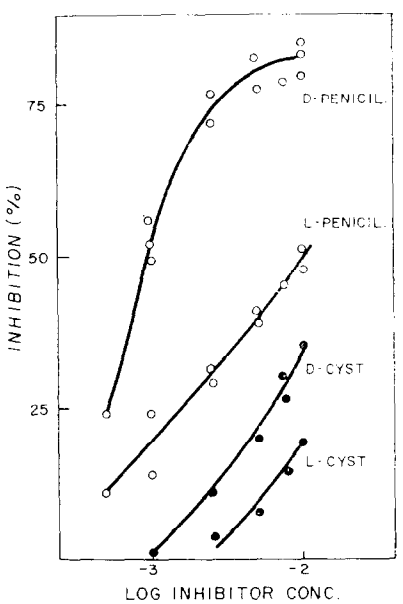

FIG. 15. Inhibition of the enzyme by various molar concentrations of D- and L-isomers of penicillamine and cysteine.

It was shown previously that L-cysteine is the most inhibitory of the α-amino acids found in proteins[10] and that DL-penicillamine is a competitive inhibitor of brain glutamic decarboxylase, which upon injection into animals decreases the activity of the enzyme *in vivo*.[23]

Solubilization of the enzyme and some preliminary purification steps

In a typical experiment 1 g of mouse brain acetone powder was suspended in 80 ml of an ice-cold solution, 0·01 M with respect to KCl and GSH, which previously had been adjusted to pH 6·4 and gassed with N_2 in the small cup of the Waring Blendor. The suspension was centrifuged for 15 min at 17,000 × *g*. The supernatant fluid was brought to 45% saturation with ammonium sulfate by slow addition of the salt while kept under N_2 and maintaining the pH at 6·8. The precipitate was removed and the supernatant brought to full saturation with ammonium sulfate. The original suspension, the supernatant fluid, and the solutions obtained on redissolving the two precipitates were analyzed for activity and protein content; 51% of the activity and 27·5% of the protein in the powder were recovered in the original extract. Seventy-two per cent of the activity and 45% of the protein of the extract were brought down in the 45% ammonium sulfate precipitate, giving approximately a threefold increase in specific activity over the original suspension. Less than 1% of the activity and 21% of the protein was found in the precipitate obtained upon saturation with ammonium sulfate. Work is in progress on the subfractionation of the solutions of the 45% precipitate.

DISCUSSION

Various aspects of the chemistry of pyridoxal-P, the type of electron shift which probably occurs in decarboxylation, and related subjects have been reviewed recently.[24] The spectral properties of crystalline phosphorylase are consistent with the existence of a substituted aldimine structure at physiological pH values and of the free Schiff base form at more acid values.[25] In three enzymes, phosphorylase,[26] and cystathionase and glutamic-aspartic transaminase,[27] the aldehyde group of the coenzyme must lie close to the ε-amino group of a lysine residue or be bonded to it, since ε-N-pyridoxyl lysine was isolated in all cases after reduction with sodium borohydride at an acid pH. It has been suggested from a comparison of the spectral properties of phosphorylase with adducts obtained between pyridoxal-5-phosphate and various aminothiols that there also may be a peptide-bound cysteinyl residue bonded to the carbonyl carbon of pyridoxal-P through its sulfhydryl group.[22, 28] The postulate of this dual interaction of the carbonyl carbon requires that the amino and sulfhydryl groups lie close together, but does not require that the amino acid residues containing these groups lie even on the same peptide chain. Previously, from experiments with L-cysteine-sulfinic and L-cysteic acid decarboxylases, it was suggested that the pyridoxal-P moiety was linked to sulfhydryl groups of the protein,[29] and it was concluded from work with a bacterial amino acid decarboxylase that a protein sulfhydryl group is bound to and protected by the aldehyde group of the coenzyme.[30] It also has been suggested that the imino group formed between the aldehyde group of the coenzyme and the protein can be converted directly to a Schiff base of a substrate amino acid upon the carbon of the pyridoxal-P aldehyde group,[31] so that hydrolysis to the free aldehyde group need not take place at any time during enzymatic action.

Because a crude enzyme preparation was employed in the present study it was not possible to do direct studies, such as those mentioned above,[26, 27] showing the proximity of the aldehyde group of the coenzyme and an ε-amino group of a lysine residue of the apoenzyme. However, the results obtained on the inhibition of the decarboxylase by carbonyl-trapping agents (see Table 2) are consistent with the presence of a cationic

group in the vicinity of the aldehyde group. It was found that the most potent inhibitors were those that could form derivatives possessing acidic groups. The acidity of the oxime formed from hydroxylamine and the aldehyde group of the coenzyme would be expected to be enhanced by the proximity of an electron-attracting cationic group. The affinity of hydroxylamine for the holoenzyme would be increased over that for the free coenzyme because of the electrostatic attraction of the negatively charged oximino group to the adjacent positively charged group, once the oximino bond is formed with the enzyme-bound coenzyme. Consistent with this hypothesis were the findings that hydrazine and several derivatives of hydroxylamine in which the hydroxyl hydrogen is replaced by uncharged constituents were less inhibitory than hydroxylamine itself, and that aminooxyacetic and aminooxypropionic acids were the most potent of all the inhibitors tested. The substitution of both α-hydrogens of aminooxyacetic acid with methyl groups gave a compound, 2-(aminooxy)-2-methylpropionic acid, with approximately the same inhibitory potency as that of hydroxylamine, the enhancing effect of the presence of the carboxyl group apparently being offset by the inhibitory steric effects of the methyl groups. DL-α-Aminooxyphenylpropionic acid, DL-α-hydrazinophenylpropionic, and DL-α-hydrazinophenylacetic acids were found to be much more effective competitive inhibitors than a large number of other hydrazine derivatives with a variety of substituents, but not containing an acidic function. Interestingly, the results in Table 2 and Fig. 11 show that the (+) form of 2-(aminooxy)propionic acid is a more effective inhibitor than the (−) form. This compound eventually may prove useful in an analysis of the asymmetry of the active site of the enzyme once a highly purified preparation becomes available. Similarly, the finding that the D-isomers of penicillamine and cysteine are significantly more inhibitory than the L-isomers should prove useful in this respect.

The sulfhydryl nature of the brain enzyme seems well established by the data reported in this paper. The competitive nature of the inhibitions by 1,2-naphthoquinone-4-sulfonate and nitroso R salt suggests that a sulfhydryl group may be present at or near the active site of the enzyme.

The effects of administering carbonyl-trapping agents to animals would be expected to be much more complicated than in enzyme studies *in vitro*. In any assessment of the mechanism of action of carbonyl-trapping agents *in vivo* a variety of possibilities must be considered by which the amount of pyridoxal-P available for interaction with the apoenzyme could be limited as well as the direct combination with enzyme-bound pyridoxal-P. Thus, in addition to the structure of the enzyme itself, such factors as ease of penetration of tissue and cell barriers, rate of detoxication, and the nature of the major ionic forms present at physiological pH values might play important roles in determining which B_6 enzymes in the various tissues would be inhibited, and to what extent. Furthermore, a number of the substances that can inhibit various B_6 enzymes because of their ability to combine with the aldehyde group of the coenzyme also can form derivatives with pyridoxal which inhibit the formation of pyridoxal-P by inhibiting pyridoxal phosphokinase, the enzyme that converts pyridoxamine, pyridoxal, or pyridoxine to the 5-phosphate esters by interaction with ATP.[32–34] Derivatives of carbonyl reagents and pyridoxal and pyridoxal-P actually have been found to exist in the tissues and body fluids of animals treated with these substances.[35] The administration of isonicotinic hydrazide to mice and dogs resulted in the appearance of the corresponding hydrazones of pyridoxal and pyridoxal-P in the liver,

increase in the contents of pyridoxine and pyridoxal, and decrease in the levels of pyridoxal and pyridoxamine phosphates. In view of this discussion it is interesting that, upon administration to animals of hydroxylamine or aminooxyacetic, potent competitive inhibitors of both the glutamic decarboxylase and γ-aminobutyric acid transaminase[36, 37] of brain, only the transaminase was inhibited and there were marked elevations of γ-aminobutyric acid content in the brains of the treated animals. One of the simplest possible explanations, which is being explored, is that the two enzymes are present in different cell types or in different intracellular sites in the same cells and that the reagents penetrate to the regions containing the transaminase, but not to those wherein the decarboxylase is located.

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