BBA 66098

COMPARISON OF SOME PHYSICOCHEMICAL AND CATALYTIC PROPERTIES OF GLUTAMATE DECARBOXYLASE FROM VARIOUS *ESCHERICHIA COLI* K-12 SOURCES

MEIR LUPO AND YEHESKEL S. HALPERN
Department of Bacteriology, The Hebrew University–Hadassah Medical School, Jerusalem (Israel)

(Received September 26th, 1969)
(Revised manuscript received February 2nd, 1970)

SUMMARY

A wide variation was found in the specific activity of L-glutamate decarboxylase (EC 4.1.1.15) among genetically related *Escherichia coli* K-12 strains grown under different conditions.

Results are presented showing that the K_m values for two substrates, L-glutamate and L-glutamate-γ-methyl ester, the K_i values for five competitive inhibitors of the L-glutamate decarboxylase reaction, and the absorption spectrum, heat stability and electrophoretic mobility of L-glutamate decarboxylase, were practically identical when tested with four different preparations from strains CS101 and CS7B grown on glucose or succinate at different temperatures.

It is concluded that L-glutamate decarboxylase obtained from different strains grown under different conditions represents the same molecular species, and that the wide variation in specific activity observed is due to differences in the amount of enzyme formed by the different cultures.

INTRODUCTION

In 1962 HALPERN[1] made the observation that the glutamate decarboxylase (EC 4.1.1.15) activity of *Escherichia coli* was affected by growth conditions.

Wild-type cells, unable to utilize glutamate as the carbon source (Glu^-), grown on minimal salts medium with glucose at 37° or with succinate at 30°, exhibited high enzymic activity. The same strain, when grown on succinate at 37° exhibited a much lower enzymic activity. The acquisition of the ability to utilize glutamate as the sole carbon source, by mutation, was generally accompanied by a decrease in L-glutamate decarboxylase activity, the effect of growth conditions on L-glutamate decarboxylase levels in the mutants remaining the same as in the wild type strains.

The control of L-glutamate decarboxylase activity may be exerted by regulation of the rate of enzyme synthesis, *i.e.* different amounts of enzyme are produced

by different strains under different growth conditions, or at the level of enzyme structure, *i.e.* different strains under different growth conditions produce structurally different species of enzyme molecules differing in activity.

We have recently shown[2] that L-glutamate decarboxylase obtained from three interrelated *E. coli* K-12 strains, each grown under three different conditions, exhibited identical antigenic patterns. However, the immunological study has not excluded the possibility of structural alterations not affecting the antigenic properties of the molecule. The present study provides evidence for the catalytic identity of the enzyme in the different L-glutamate decarboxylase preparations.

MATERIALS AND METHODS

Bacterial strains

E. coli K-12 Hfr CS101 is a methionine auxotroph not capable of utilizing glutamate as carbon source (Glu^-).

E. coli K-12 F^- CS7B is a genetic recombinant obtained by conjugation between CS7, a glutamate utilizing mutant (Glu^+) derived from CS101, and F^- P678 (Glu^-) as the recipient. CS7B was selected for its ability to utilize glutamate as carbon source.

Growth conditions

The minimal medium of DAVIS AND MINGIOLI[3] from which citrate was omitted, was used in this study. Cells were grown in 250 ml conical flasks containing 100 ml medium or 2 l flasks containing 1 l medium supplemented with 25 μg/ml L-methionine and carbon source (0.25% glucose or 1% succinate, as indicated). Cultures were grown in a shaking water-bath.

Preparation of cell-free extracts

Cells were harvested in the stationary phase of growth and treated as previously described[2]. Extracts were dialyzed overnight in the cold against 0.067 M phosphate buffer (pH 6.0).

Determination of enzymic activity

Enzymic activity in cell-free extracts was determined manometrically as previously described[2]. One enzyme unit is defined as the amount releasing 1 μl CO_2 per h under the assay conditions.

Purification of L-glutamate decarboxylase

(1) Heat treatment. The crude bacterial extract, obtained as above, was heated at 63° for 4 min. The precipitate formed was discarded after centrifugation.

(2) First $(NH_4)_2SO_4$ fractionation. The supernatant of the heat-treated fraction was brought to 33% satn. with $(NH_4)_2SO_4$ by slow addition of solid $(NH_4)_2SO_4$ with continuous stirring. The mixture was further stirred for 5 h in the cold, centrifuged and the precipitate discarded.

(3) Second $(NH_4)_2SO_4$ fractionation. The supernatant was brought to 50% satn. with $(NH_4)_2SO_4$ by dropwise addition of a saturated solution. The mixture was stirred overnight in the cold, the precipitate was collected and dissolved in 0.45 M

citrate–phosphate buffer (pH 5.0). This fraction was incubated for 20 min at 37° and the precipitate discarded.

(4) DEAE-cellulose treatment. The supernatant was dialyzed for 4–5 h against 0.067 M phosphate buffer (pH 6.0) and adsorbed on DEAE-cellulose, previously washed several times with the same buffer. The mixture was stirred for 1 h in the cold, centrifuged and the DEAE-cellulose washed twice with 0.067 M phosphate buffer (pH 6.0). The enzyme was eluted from the anion-exchanger by the addition of 0.45 M citrate–phosphate buffer (pH 5.0) containing 0.5 M NaCl. This procedure resulted in a 15-fold purification of the enzyme, yielding a L-glutamate decarboxylase preparation with a specific activity of 107 141 units/mg protein, as shown in Table I.

TABLE I

PURIFICATION OF L-GLUTAMATE DECARBOXYLASE FROM STRAIN CS7B GROWN ON GLUCOSE AT 37°

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Specific activity (units/mg protein)*	*Total activity* $\times 10^{-5}$ *(units)*	*Yield (%)*
Crude extract	25.0	14.00	7 375	25.6	100.0
(1) Supernatant after 4 min at 63°	20.0	6.60	16 905	22.4	87.5
(2) Supernatant after 33% $(NH_4)_2SO_4$ satn.	15.0	2.50	29 300	11.0	43.0
(3) Supernatant after 50% $(NH_4)_2SO_4$ satn. and incubation for 20 min at 37°	2.3	7.50	59 533	10.3	40.0
(4) DEAE-cellulose eluate	2.0	0.48	107 141	1.02	4.0

Determination of spectrum

The spectrum of purified L-glutamate decarboxylase was determined at pH 5.0 in a Cary 14 recording spectrophotometer at room temperature. The effect of glutamate analogs on the spectrum was determined after incubation of the enzyme for 20 min at 37° in the presence of 0.1 M of the analog.

The effect of the two substrates, L-glutamate and L-glutamate-γ-methyl ester (at a final concentration of 0.1 M), was examined by their addition, immediately before reading, to enzyme preincubated with buffer only. Blanks contained all the ingredients of the reaction except for the enzyme.

Immunoprecipitation

Precipitation of L-glutamate decarboxylase with anti-L-glutamate decarboxylase serum was carried out as described earlier[2].

Immunoelectrophoresis

Glass slides (50 mm × 75 mm) were coated with 4.0 ml of 1% Noble agar solution in veronal buffer, ionic strength 0.025 (pH 8.2). Cell-free extract containing 40 000 L-glutamate decarboxylase units/ml was placed in the holes, and electrophoresis was run for 90–120 min with a voltage gradient of 4 V/cm (30 V per slide), in an electrophoresis bath containing veronal buffer, ionic strength 0.05 (pH 8.2). After completion of the run, anti-L-glutamate decarboxylase serum was placed in the main trough, and the slides were developed for 48 h at room temperature in a moist atmosphere.

Excess antigen and antibody were thoroughly washed away, and the agar was dried and stained with 1% amido black in 7% acetic acid.

Determination of inhibition constants

The apparent K_i for various L-glutamate decarboxylase inhibitors was determined by the method of Dixon as described by WEBB[4].

Heat inactivation

Four ml of 0.067 M phosphate buffer (pH 6.0) were prewarmed to 64° for 5 min, and 1.0 ml enzyme solution prewarmed to 37° was added. The mixture was incubated at 64°. Samples were taken at various time intervals and stored on ice. The samples were centrifuged, and residual enzymic activity in the supernatant was determined. These experiments were performed at several protein concentrations in order to eliminate possible concentration effects.

RESULTS

Determination of spectrum

The spectrum of purified L-glutamate decarboxylase obtained from strain CS7B grown on succinate at 30° is shown in Fig. 1. As can be seen from Fig. 1, only

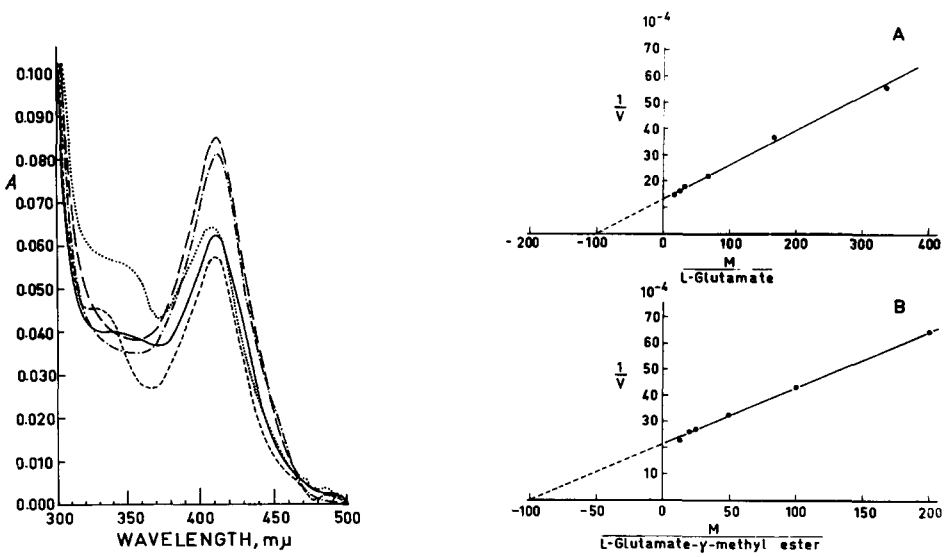

Fig. 1. The effect of glutamate and glutamate analogs on the spectrum of L-glutamate decarboxylase from strain CS7B grown on succinate at 30°. Protein concentration, 990 μg/ml. All compounds tested were added to a final concentration of 0.1 M. — — —, no additions (control); —·—, glutaric acid; · · · · ·, L-glutamic acid; ———, L-glutamic acid-γ-methyl ester; - - - - -, DL-α-methyl glutamic acid.

Fig. 2. Determination of affinity constants (Lineweaver–Burk plot). A. Cell-free extract of strain CS7B grown on succinate at 37°. Protein concentration, 355 μg/ml, in a total volume of 2.0 ml. B. Cell-free extract of strain CS7B grown on succinate at 30°. Protein concentration, 56 μg/ml, in a total volume of 2.0 ml. For other experimental details see MATERIALS AND METHODS. v = μl CO_2 per h.

TABLE II

COMPARISON OF THE MAXIMAL VELOCITIES AND AFFINITY CONSTANTS FOR THE DECARBOXYLATION OF L-GLUTAMATE AND L-GLUTAMATE-γ-METHYL ESTER IN THE PRESENCE OF DIFFERENT L-GLUTAMATE DECARBOXYLASE PREPARATIONS

Crude cell-free extracts were used; for other experimental details see MATERIALS AND METHODS.

Strain	*Growth conditions*		K_m *(M)*		v_{max} *(µl CO_2 per h per mg protein)*	
	Carbon source	*Temp.*	L-*Glutamate*	L-*Glutamate-γ-methyl ester*	L-*Glutamate*	L-*Glutamate-γ-methyl ester*
CS101	Glucose	37°	$7.0 \cdot 10^{-3}$	$1.9 \cdot 10^{-2}$	$8.6 \cdot 10^{2}$	$7.9 \cdot 10^{2}$
CS7B	Glucose	37°	$9.6 \cdot 10^{-3}$	$2.0 \cdot 10^{-2}$	$8.4 \cdot 10^{3}$	$6.4 \cdot 10^{3}$
	Succinate	37°	$1.0 \cdot 10^{-2}$	$2.8 \cdot 10^{-2}$	$1.1 \cdot 10^{3}$	$9.2 \cdot 10^{2}$
		30°	$8.6 \cdot 10^{-3}$	$1.0 \cdot 10^{-2}$	$5.9 \cdot 10^{3}$	$4.0 \cdot 10^{3}$

glutamate and glutamate analogs with a free α-amino group caused a change in the spectrum of the enzyme, while glutaric acid, although capable of attachment to the enzyme (see Table IV), did not influence its spectrum. The same pattern of spectral shifts due to glutamate and its analogs was found with L-glutamate decarboxylase preparations from strain CS7B grown on glucose or succinate at 37°.

Determination of affinity constants

We have observed that in addition to glutamate, L-glutamate-γ-methyl ester is also decarboxylated in the presence of L-glutamate decarboxylase. We determined the affinity constants of four different L-glutamate decarboxylase preparations for the two substrates, L-glutamate and L-glutamate-γ-methyl ester. The results are summarized in Fig. 2 and Table II.

No differences were found between the preparations tested in their affinity for either substrate, whereas their specific activities varied greatly (see Table II). However, when crude extracts of strains CS101 and CS7B showing a 6-fold difference in specific activity of L-glutamate decarboxylase were purified as described in MATERIALS AND METHODS (see also Table I and ref. 2), the final specific activities of the purified L-glutamate decarboxylase preparations of the two strains were practically identical (approx. 90 000 units/mg protein). The γ-methyl ester of L-glutamic acid had a higher K_m value than glutamate, and its decarboxylation rate was lower than that of glutamate.

The possibility existed that the decarboxylation of L-glutamate-γ-methyl ester was due to some other enzyme, different from L-glutamate decarboxylase. To test this point, immunoprecipitation with anti-L-glutamate decarboxylase serum was performed as previously described[2]. The results are shown in Table III. The residual decarboxylating activity in the supernatant, after precipitation with anti-L-glutamate decarboxylase serum, was practically identical for both glutamate and the γ-methyl ester.

It can therefore be assumed that L-glutamate decarboxylase is responsible for the decarboxylation of both L-glutamic acid and L-glutamic acid-γ-methyl ester. This conclusion is further supported by the results presented in Table II showing that the

TABLE III

IMMUNOPRECIPITATION OF CELL-FREE EXTRACT FROM STRAIN CS7B GROWN ON GLUCOSE AT 37° WITH ANTI-L-GLUTAMATE DECARBOXYLASE SERUM

Different amounts of cell-free extract were incubated with equal amounts of anti-L-glutamate decarboxylase serum diluted 1:5, in a total volume of 1.0 ml. After precipitation in the cold the supernatant was examined for residual decarboxylase activity. The concentrations of substrates in the decarboxylation assay were :60 mM L-glutamate, 80 mM L-glutamate-γ-methyl ester. For further experimental details see MATERIALS AND METHODS and ref. 2.

L-Glutamate decarboxylating activity after incubation with normal rabbit serum (control) (units)	*Residual glutamate decarboxylating activity in supernate after precipitation with anti-L-glutamate decarboxylase serum (units)*	*% of control*	*L-Glutamate-γ-methyl ester decarboxylating activity after incubation with normal rabbit serum (control) (units)*	*Residual L-glutamate-γ-methyl ester decarboxylating activity in supernate after precipitation with anti-L-glutamate decarboxylase serum (units)*	*% of control*
3 029	187	6	2299	133	6
6 058	1485	24	4376	1016	23
12 567	7190	57	8523	4742	55

decarboxylating activities towards glutamate and the γ-methyl ester were similarly affected by growth conditions.

Determination of inhibition constants

We tested a number of glutamate analogs for their effect on the L-glutamate decarboxylase reaction. Compounds found to exert an inhibitory effect were further studied to determine the type of inhibition and the inhibition constants with the different L-glutamate decarboxylase preparations. The results are summarized in Fig. 3 and Table IV.

TABLE IV

INHIBITION CONSTANTS OF L-GLUTAMATE DECARBOXYLASE INHIBITORS DETERMINED WITH FOUR DIFFERENT L-GLUTAMATE DECARBOXYLASE PREPARATIONS

For experimental details see Fig. 3.

Strain	*Growth conditions*		*Inhibition constant (M)*				
	Carbon source	*Temp.*	*α-Keto-glutarate*	*α-Methyl glutarate*	*DL-α-Methyl glutamate*	*Glutaric acid*	*Hydroxyl-amine hydro-chloride*
CS101	Glucose	37°	$2.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-2}$	$3.3 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$	$4.5 \cdot 10^{-5}$
CS7B	Glucose	37°	$2.2 \cdot 10^{-2}$	$5.5 \cdot 10^{-3}$	$3.3 \cdot 10^{-2}$	$2.3 \cdot 10^{-2}$	$4.0 \cdot 10^{-5}$
	Succinate	37°	$1.8 \cdot 10^{-2}$	$8.5 \cdot 10^{-3}$	$3.3 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$	$3.0 \cdot 10^{-5}$
		30°	$1.0 \cdot 10^{-2}$	$1.5 \cdot 10^{-2}$	$2.6 \cdot 10^{-2}$	$1.8 \cdot 10^{-2}$	$1.5 \cdot 10^{-5}$

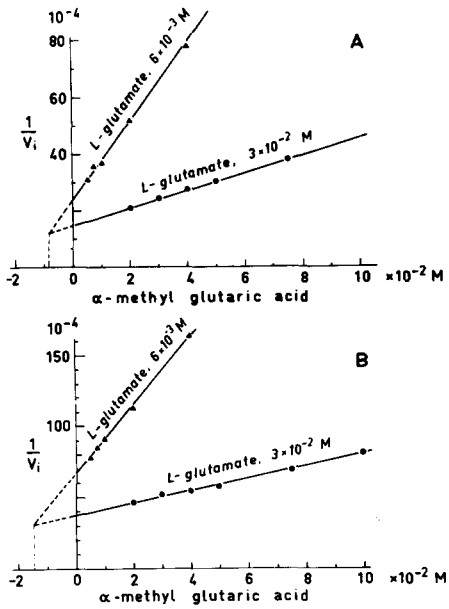

Fig. 3. Determination of inhibition constants (Dixon's plot). A. Cell-free extract of strain CS7B grown on succinate at 37°. Protein concentration, 355 μg/ml, in a total volume of 2.0 ml. B. Cell-free extract of strain CS7B grown on succinate at 30°. Protein concentration, 56 μg/ml, in a total volume of 2.0 ml. For other experimental details see MATERIALS AND METHODS. v = μl CO_2 per h.

Similar K_i values were obtained for each inhibitor when tested with the different L-glutamate decarboxylase preparations. The variation observed did not exceed the limits of experimental error. All inhibitions were of the competitive type.

The following analogs had no effect on glutamate decarboxylation in the presence of L-glutamate decarboxylase: D-glutamate; L-glutamine; DL-methionine sulfone; DL-methionine-*dl*-sulfoxide; DL-methionine-*dl*-sulfoximine; L-glutamic acid-γ-ethyl ester; L-glutamic acid-γ-hydrazide; γ-benzyl-L-glutamate; acetyl-L-glutamate; *p*-aminobenzoyl-L-glutamate; DL-norvaline; γ-amino butyrate; and α-amino iso butyrate.

Comparison of the electrophoretic mobilities of different L-glutamate decarboxylase preparations

Fig. 4. shows the electrophoretic mobilities of different L-glutamate decarboxylase preparations. The mobilities of the three L-glutamate decarboxylase preparations from strain CS7B were identical with each other and with that of the standard preparation of strain CS101.

Comparison of heat stability of different L-glutamate decarboxylase preparations

We observed that L-glutamate decarboxylase from *E. coli* K-12 is considerably heat stable. We took advantage of this finding and further tested the identity of the different L-glutamate decarboxylase preparations by comparing their heat stabilities at 64°. A typical heat-inactivation curve is presented in Fig. 5. Similar inactivation

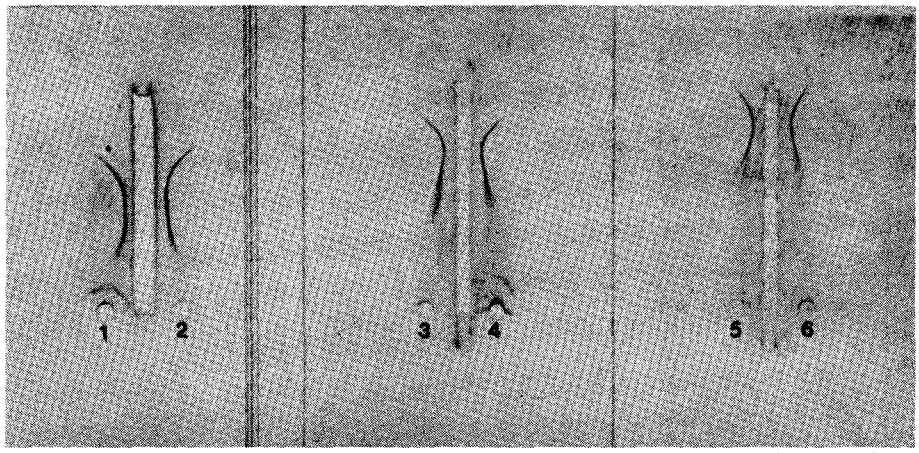

Fig. 4. Comparison of the electrophoretic mobilities of different L-glutamate decarboxylase preparations by immunoelectrophoresis. 1, cell-free extract of strain CS101 grown on glucose at 37°; 2, cell-free extract of strain CS7B grown on succinate at 30°; 3, cell-free extract of strain CS7B grown on glucose at 37°; 4, cell-free extract of strain CS7B grown on succinate at 37°; 5, cell-free extract of strain CS7B grown on succinate at 37°; 6, cell-free extract of strain CS7B grown on succinate at 30°. For other experimental details see MATERIALS AND METHODS.

curves were obtained with the four preparations tested. The half-lives extrapolated from the initial inactivation rates of the four L-glutamate decarboxylase preparations tested were very similar, ranging from 5.7 to 6.7 min.

DISCUSSION

In a previous work from this laboratory[2], we showed that L-glutamate decarboxylase from three genetically related *E. coli* K-12 strains, each grown under three different conditions, although exhibiting several-fold differences in specific activity, were antigenically identical.

The possibility that structural alterations at the active site, not affecting the antigenic properties of the molecule, were responsible for the differences in the specific activity of L-glutamate decarboxylase obtained from different strains grown under different conditions, was largely eliminated by the experiments reported in this study.

The salient findings were the following.

(a) As can be seen from Fig. 1, L-glutamate decarboxylase has a peak of absorption in the region of 410 mμ due to a Schiff-base formed between pyridoxal 5-

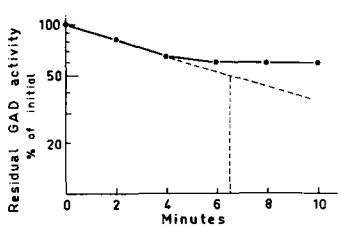

Fig. 5. Kinetics of heat inactivation of L-glutamate decarboxylase (GAD) at 64°. Cell-free extract of strain CS101 grown on glucose at 37°. Protein concentration in heated mixture, 1.8 mg/ml. For other experimental details see MATERIALS AND METHODS.

phosphate and an ε-amino group of lysine[5,6]. Addition of glutamate or glutamate analogs with a free α-amino group results in a decrease in the peak at 410 mμ and the appearance of a new peak in the region of 330 mμ. This transition is probably due to trans-schiffization occurring between pyridoxal 5-phosphate and the α-amino group of the analog[7].

Glutaric acid, which lacks a free α-amino group, binds to the enzyme (see Table IV) without affecting its spectrum.

Our finding that the spectra of different L-glutamate decarboxylase preparations are identical and show the same pattern of spectral shifts in the presence of glutamate and glutamate analogs indicates that in all these preparations the pyridoxal 5-phosphate is bound to the L-glutamate decarboxylase protein in the same manner, forming a protonated Schiff's-base[8].

(b) No significant differences between the L-glutamate decarboxylase preparations tested were found in regard to the K_m values for the two substrates, L-glutamate and L-glutamate-γ-methyl ester (see Table II). That the same enzyme, L-glutamate decarboxylase, is involved in the decarboxylation of both L-glutamic acid and L-glutamic acid-γ-methyl ester has been demonstrated by immunoprecipitation with anti-L-glutamate decarboxylase serum (see Table III).

(c) Further comparison of the different L-glutamate decarboxylase preparations in a study of the kinetics of glutamate decarboxylation in the presence of glutamate analogs (inhibitors) showed that all L-glutamate decarboxylase preparations tested gave very similar respective K_i values (see Table IV).

The five inhibitors examined may be divided into the following three groups, on the basis of their chemical structure.

1. Compounds structurally related to glutamate but lacking a free amino group (glutaric acid and its derivatives). These compounds cannot form a Schiff base with the coenzyme, pyridoxal 5-phosphate. Their inhibitory effect must therefore be due to competition with glutamate for the substrate binding site on the enzyme molecule.

2. Compounds derived from glutamate, possessing a free amino group (DL-α-methyl glutamate) and which may therefore compete with glutamate for either pyridoxal 5-phosphate or the substrate binding site.

3. Hydroxylamine, a carbonyl reagent, which is devoid of any structural similarity to glutamate and probably competes with glutamate for the pyridoxal 5-phosphate only.

Our finding that the respective K_i values of these chemically different inhibitors, obtained with different L-glutamate decarboxylase preparations, were practically identical, strongly argues against structural differences among the various L-glutamate decarboxylase preparations examined.

(d) Two other criteria used for comparison between the different L-glutamate decarboxylase preparations were electrophoretic mobility and heat stability at 64°. The results support our conclusion that L-glutamate decarboxylase from the different sources examined is of identical molecular structure. The wide variation in specific activity observed is therefore most likely due to differences in the amount of enzyme formed by the different cultures.

We have recently mapped a regulatory gene determining the level of L-glutamate decarboxylase activity. These studies will be published elsewhere.

ACKNOWLEDGMENTS

We wish to express our thanks to Drs. D. Sulitzeanu and I. Flechner for many valuable suggestions, and to Mrs. Avivith Even-Shoshan for technical assistance.

REFERENCES

1 Y. S. HALPERN, *Biochim. Biophys. Acta*, 61 (1962) 953.
2 M. LUPO, Y. S. HALPERN AND D. SULITZEANU, *Arch. Biochem. Biophys.*, 131 (1969) 621.
3 B. D. DAVIS AND E. S. MINGIOLI, *J. Bacteriol.*, 60 (1950) 17.
4 J. L. WEBB, *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York, 1963, p. 149.
5 B. S. SUKHAREVA, *Biochem. Biophys. Res. Commun.*, 25 (1966) 585.
6 J. A. ANDERSON AND H. WANG CHANG, *Arch. Biochem. Biophys.*, 110 (1965) 346.
7 E. H. CORDES AND W. P. JENKINS, *Biochemistry*, 1 (1962) 773.
8 R. SHUKUYA AND G. W. SCHWERT, *J. Biol. Chem.*, 235 (1960) 1653.