EFFECT OF ATP AND CHLORIDE IONS ON GLUTAMIC DECARBOXYLASE FROM BRAIN, *E. coli*, AND CARROT ROOT

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ATP inhibits glutamic decarboxylase from brain and *E. coli*, yet it is without effect on the same enzyme from carrot root. Increased concentrations of pyridoxal 5'-phosphate decrease the inhibitory effect of ATP on brain glutamic decarboxylase yet do not decrease but rather increase the degree of inhibition of *E. coli* glutamic decarboxylase by ATP. Inorganic phosphate significantly decreases the inhibitory effect of ATP on glutamic decarboxylase from both sources. Chloride ions competitively inhibit brain glutamic decarboxylase, noncompetitively the enzyme from carrot root, and slightly activate *E. coli* glutamic decarboxylase.

In earlier papers[1,2] from this Laboratory we have reported on the inhibition of glutamic decarboxylase (EC 4.1.1.15) by ATP. Šorm and Turský[3] and later Susz, Haber, and Roberts[4] observed the inhibition of brain glutamic decarboxylase by chloride ions. ATP and chlorides inhibit this enzyme *in vitro* at concentrations similar to those at which they are present in the nervous tissue and act as potential physiological regulators of glutamic decarboxylase in the nervous system. We have examined the effect of these compounds on glutamic decarboxylase preparations from *E. coli* and carrot root and compared this effect with the inhibitory action of ATP and chlorides on brain glutamic decarboxylase preparations purified more than those used in our preceding experiments[1-3].

EXPERIMENTAL

Chemicals. ATP and CTP disodium salt were from Reanal, AMP and GTP from Koch-Light, hyamine hydroxide 10 X from Packard, D,L-glutamic acid-[1-^{14}C], 23·6 mCi/mmol from the Radiochemical Centre Amersham, pyridoxal 5'-phosphate from Fluka, liquid scintillator SLS 31 from Spolana, Neratovice, L-glutamic acid and the remaining A.R. Chemicals from Lachema, Brno.

Preparation of enzymes. An effort was made to prepare all enzymes free of contaminants cleaving ATP.

Brain glutamic decarboxylase was obtained from rat brain. The brains were homogenized with cold redistilled water at a ratio of 1 : 7 (w/v), 5 min at 3000 revolutions, in a glass homogenizer

TABLE I

Purification of Glutamic Decarboxylase from Rat Brain

Stage of purification	Protein mg	Total activity μmol . min^{-1}	Specific activity[a]	Yield %
Homogenate	380·7	1·65	4·34	100
Extract	102·12	0·84	8·16	50·6
Extract after heating	54·45	0·6	11·1	36·4
Ethanol fraction	5·8	0·28	17·1	17·2

[a] In nmol . min^{-1} . mg protein^{-1}.

TABLE II

Purification of Glutamic Decarboxylase from Carrot Root

Stage of purification	Specific activity[a]	Stage of purification	Specific activity[a]
Homogenate	27	DEAE-cellulose 0·2M-NaCl	230
Extract	62	DEAE-cellulose 0·4M-NaCl	140
Ammonium sulfate precipitate 0—60%	194	Ammonium sulfate precipitate 0—60% from DEAE fractions	303
Lyophilized dialysate	103		

[a] In nmol . min^{-1} . mg protein^{-1}.

TABLE III

Effect of ATP on Glutamic Decarboxylase (GAD) from Different Sources

ATP mM	GAD, activity %		
	brain	*E. coli*	carrot
0	100	100	100
0·5	40	80·8	101·4
1·0	30	79	107
2·0	27·5	67·7	113

TABLE IV

Effect of Chloride Ions on Glutamic Decarboxylase (GAD) from Different Sources

NaCl mM	GAD, activity %		
	brain	*E. coli*	carrot
0	100	100	100
50	79·4	125	—
100	60·9	129	70
200	46	143	60·5

with a teflon pestle. The homogenate was centrifuged 60 min at 76000 *g* and 2°C. The supernatant was decanted off and treated with 1M sodium phosphate buffer, pH 6, to a final phosphate concentration of 0·05M. This solution was heated 10 min at 50°C. The precipitate was centrifuged off. The supernatant was treated with ethanol at 0°C (concentration 0—15%) and at −2°C (concentration 15—35%). Decarboxylase activity was found in the fraction precipitated at 15—35% saturation with ethanol. This fraction was dissolved in 0·1M Tris-acetate buffer at pH 6·5 and was used for the determination of the properties of brain glutamic decarboxylase. The purification procedure is summarized in Table I.

The properties of *E. coli* glutamic decarboxylase were examined with a commercial preparation of Koch-Light. This preparation did not cleave ATP and was used as such without any additional purification.

Glutamic decarboxylase from carrot root. Carrot roots were homogenized in redistilled water at a ratio of 1 : 1 (w/v) 3 min. The homogenate was allowed to stand 60 min at 4°C. It was centrifuged afterwards 50 min at 65000 *g* and 2°C. The supernatant was decanted off and treated with solid ammonium sulfate to 60% saturation. The thus saturated solution was set aside for 16 h

TABLE V
Effect of Some Nucleotides and of Adenosine on Glutamic Decarboxylase from Brain and *E. coli*

Additions of 1 mM	GAD, activity %		Additions of 1 mM	GAD, activity %	
	brain	*E. coli*		brain	*E. coli*
0	100	100	UTP	50	86
ATP	30	79	GTP	53	72
AMP	100	91	CTP	73	81
Adenosine	100	100			

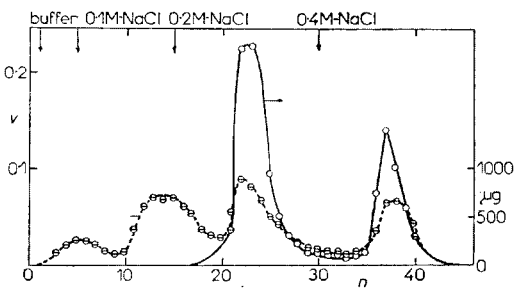

FIG. 1
Chromatography of Glutamic Decarboxylase from Carrot on DEAE Cellulose
⊖ Protein in mg . ml^{-1}, ○ enzyme activity v in μmol . min^{-1} . mg protein^{-1} . n number of fractions.

at 4°C. The precipitate was centrifuged off during 60 min at 8000 *g* and 2°C. The sediment was dissolved and its solution dialyzed against redistilled water containing 1·2 mM cysteine, 16 h at 4°C. The desalted solution showing enzymatic activity and at the same time a high ATP-ase activity was used for an additional purification of the enzyme on DEAE-cellulose. Before being applied to the DEAE-cellulose column (0·9 . 15 cm), the solution was made 0·01M in sodium phosphate and its pH was adjusted to 6·5. The purification was allowed to proceed at 4°C and a discontinuous gradient of sodium chloride in the phosphate buffer described above was used. Glutamic decarboxylase was displaced from the column by 0·2 and 0·4M sodium chloride in two separate peaks (Fig. 1). The fractions of the first peak and the fractions of the second peak were pooled separately. The material contained in the first peak cleaved 7%, the material in the second peak 17% of 1 mM ATP in 10 min. Since precipitation with ammonium sulfate to 60% saturation removed the ATPase activity from both fractions during the preparation of the enzyme, both peaks obtained by chromatography on DEAE-cellulose were pooled before the treatment with ammonium sulfate. The protein precipitated was separated by centrifugation at 43000 *g* for 10 min. The sediment was dissolved in redistilled water, dialyzed, and used as the enzyme preparation. The process of purification of glutamic decarboxylase from carrot root is summarized in Table II.

Determination of decarboxylase activity. The decarboxylase activity was determined by the isotope method[5] and by the measurement of the radioactivity of CO_2 liberated from DL-glutamic acid [1-^{14}C]. We used the apparatus shown in Fig. 2 for this purpose.

The enzyme preparation (0·1 ml) and 0·2 ml of buffer (containing pyridoxal-5'-phosphate and the inhibitor examined) were added to the reaction vessel (*a*). In experiments with brain decarboxylase, 0·1M Tris acetate buffer at pH 6·5 was used. *E. coli* decarboxylase was examined in 0·1M pyridine hydrochloride buffer at pH 5·0, carrot decarboxylase in 0·1M phosphate buffer at pH 5·6. Hyamine hydroxide 10 X (0·5 ml), a base used to absorb CO_2 liberated, was placed in the vessel for scintillation measurement. The entire vessel was filled up with nitrogen. The incubation was allowed to proceed in the water bath of a Warburg apparatus. The vessel was thermostated at 37°C for 5 min and the reaction was then started by the addition of 40 µl of substrate containing

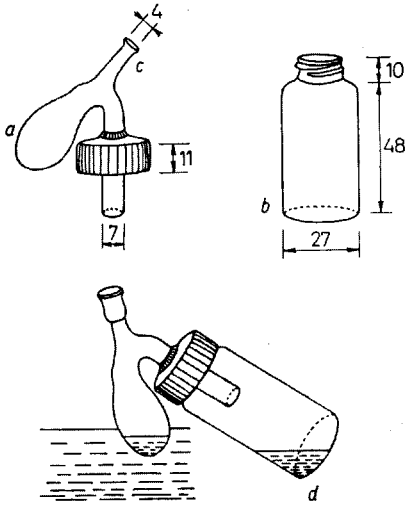

FIG. 2
Apparatus for Isotope Determination of Glutamic Decarboxylase
See text for details.

0·1 μCi of D,L-glutamate [1-^{14}C]. The final substrate concentration was 40 mM with specific activity 7·35 μCi of D,L-glutamate/mmol of L-glutamate in most cases. Decarboxylation was allowed to proceed 10 min and the enzymatic reaction was then discontinued by the addition of 0·2 ml of 2M sulfuric acid. The contents of the vessels were mixed for additional 60 min in the water bath. The scintillation liquid (8 ml) was added to vessel (*b*) containing hyamine. The blank value (without the enzyme) was determined for each experiment. The results were expressed in terms of a standard (0·001 μCi of isotope). The activity was measured in Tricarb 3320 Scintillation Spectrometer. The protein content was determined by the method of Lowry and coworkers[6].

RESULTS

The effect of ATP on glutamic decarboxylase from brain, *E. coli*, and carrot root is summarized in Table III.

ATP did not affect glutamic decarboxylase from carrot, weakly inhibited *E. coli* decarboxylase, and strongly suppressed the activity of brain glutamic decarboxylase. Since inorganic phosphate decreased the inhibitory effect of ATP on brain decarboxylase and on the enzyme from *E. coli*, the effect of ATP on carrot decarboxylase was determined at a final concentration of phosphate equal 5 mM.

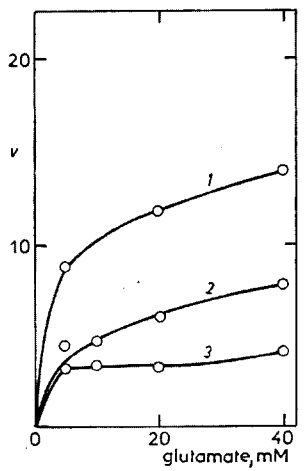

FIG. 3

Rate of Decarboxylation of Glutamic Acid by Partly Purified Brain Enzyme as Function of Glutamate Concentration without and with ATP

v Enzyme activity in nmol . min^{-1} . mg protein^{-1}. *1* Control, *2* 0·5 mM-ATP, *3* 1 mM-ATP.

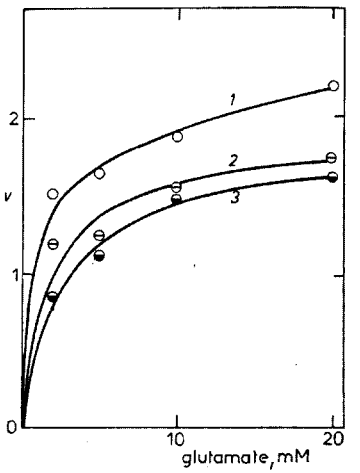

FIG. 4

Rate of Decarboxylation of Glutamic Acid by Enzyme from *E. coli* as Function of Substrate Concentration without and with ATP

v Enzyme activity in nmol . min^{-1} . mg protein^{-1}. *1* Control, 2 1 mM-ATP, *3* 2 mM-ATP.

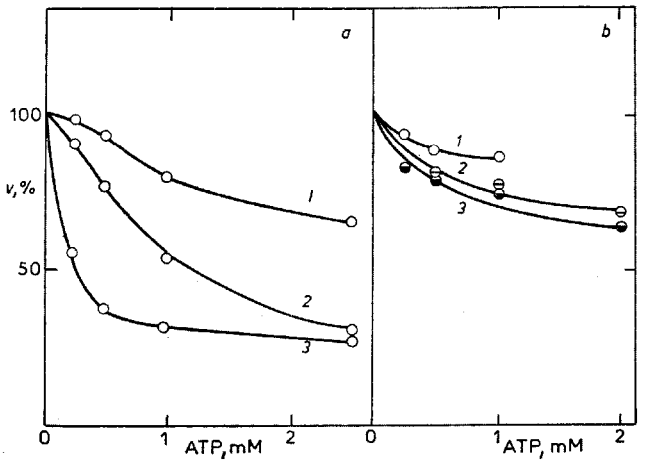

FIG. 5

Activity of Glutamic Decarboxylase as Function of ATP Concentration at Different Concentrations of Pyridoxal 5'-Phosphate

P-5'-P Pyridoxal 5'-phosphate, *v* enzyme activity. Concentration of P-5'-P *a*: *1* 375 μM, *2* 75 μM, *3* 15 μM, *b*: *1* 0, *2* 16 μM, *3* 40 μM.

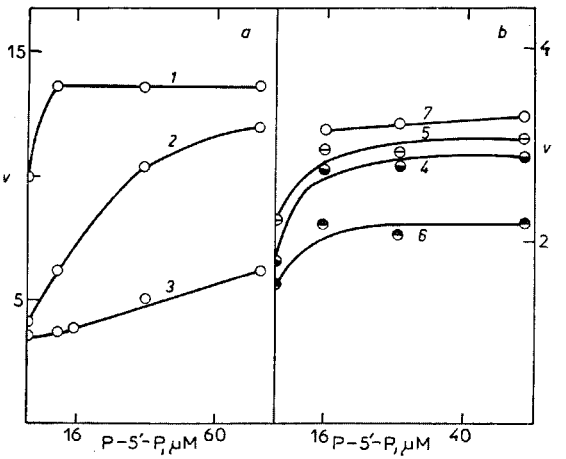

FIG. 6

Rate of Decarboxylation of Glutamic Acid as Function of Pyridoxal 5'-Phosphate Concentration; Effect of Inorganic Phosphate on Inhibitory Action of ATP

a Brain enzyme, *b* enzyme from *E. coli*. P-5'-5 pyridoxal 5'-phosphate, P_i inorganic phosphate, *v* enzyme activity in nmol . min^{-1} . mg $protein^{-1}$. *a*: *1* control, *2* 1 mM-ATP + 20 mM P_i, *3* 1 mM-ATP, *b*: *4* control, *5* 1 mM-ATP + 20 mM P_1, *6* 1 mM-ATP, *7* 20 mM-P_1.

The results obtained with partly purified preparations of brain glutamic decarboxylase were similar to those obtained with the brain extract. ATP did not compete for the binding site either with the substrate (Fig. 3) or with the coenzyme (Fig. 6).

The inhibition of *E. coli* glutamic decarboxylase is considerably weaker compared to the inhibition of brain decarboxylase in the presence of ATP and is not competitive either (Fig. 4).

A 50% inhibition cannot be achieved even at high ATP concentrations (Fig. 5).

The inhibitory effect of ATP on brain glutamic decarboxylase and *E. coli* glutamic decarboxylase can be affected by pyridoxal 5'-phosphate to a different degree. Increasing concentrations of pyridoxal phosphate strongly decrease the inhibition of the brain decarboxylase by ATP whereas the enzyme from *E. coli* is even more inhibited by ATP at high concentrations of pyridoxal 5'-phosphate (Fig. 5).

On the other hand, similarly to brain glutamic decarboxylase the enzyme from *E. coli* is inhibited not only by ATP but also by other nucleoside triphosphates. AMP, which does not inhibit brain glutamic decarboxylase, shows a slight inhibitory effect on the decarboxylase from *E. coli* (Table IV).

Inorganic phosphate had the same influence on the inhibitory effect of ATP on glutamic decarboxylase from brain and *E. coli*. A 20 mM concentration of inorganic

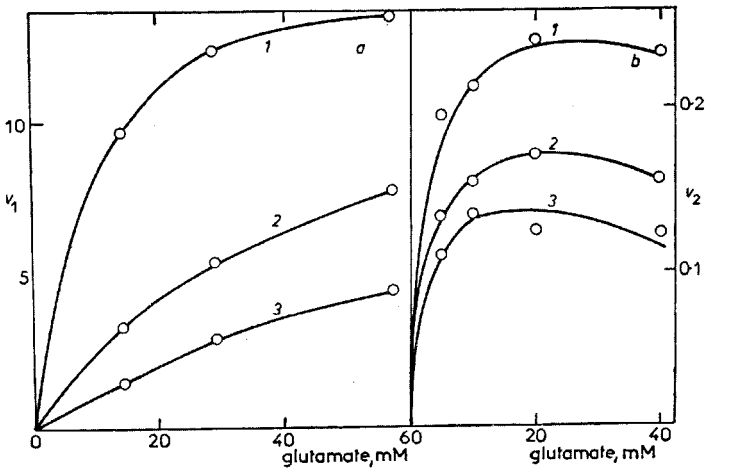

FIG. 7

Rate of Decarboxylation of Glutamic Acid as Function of Glutamate Concentration without and with NaCl

a Brain enzyme, v_1 enzyme activity in nmol . min^{-1} . mg $protein^{-1}$. *b* Carrot enzyme, v_2 enzyme activity in μmol . min^{-1} . mg $protein^{-1}$. *a* *1* control, *2* 0·115 M-NaCl, *3* 0·23 M-NaCl, *b*: *1* control, *2* 0·1 M-NaCl, *3* 0·2 M-NaCl.

phosphate showed no effect on the activity of the enzyme from brain. This concentration however, significantly reduced the inhibitory effect of ATP. Fig. 6 shows the dependence of the activity of glutamic decarboxylase on the increasing concentration of pyridoxal 5'-phosphate under the conditions of control experiments, in the presence of ATP, and in the presence of ATP and of 20 mM inorganic phosphate. Inorganic phosphate reduced the inhibitory effect of ATP on the decarboxylases from both sources.

E. coli glutamic decarboxylase was slightly activated by inorganic phosphate. The inhibitory effect of ATP in the presence of inorganic phosphate was even more decreased than the effect of ATP on brain glutamic decarboxylase.

The effect of chloride ions on glutamic decarboxylase is summarized in Table V. Chloride ions slightly activated the enzyme from *E. coli*, in accordance with recorded data[7]. Enzyme from carrot roots was inhibited by chloride ions. This inhibition was noncompetitive as regards the substrate; by contrast, the inhibition of brain glutamic decarbxylase by chlorides was competitive (Fig. 7).

DISCUSSION

In our preceding studies[1,2] on the effect of ATP on brain glutamic decarboxylase we arrived at conclusions which are in agreement with the results of the present investigation: ATP does not compete either with the substrate or the coenzyme for the binding site on the decarboxylase. After saturation of the enzyme with pyridoxal phosphate, an additional increase of the latter decreases the inhibitory action of ATP. Inorganic phosphate at concentrations which do not affect the activity of glutamic decarboxylase also decrease the inhibitory action of ATP.

Brain glutamic decarboxylase attains in the presence of ATP the properties of an enzyme with a homotropic, negative, and cooperative effect as evidenced by the value of $[S]_{10}/[S]_{10}$ higher than 81, by the graphical plot of $1/V$ *versus* $1/[S]$ with a concavity directed downwards, and by the value of Hill's coefficient equal 0·5 (ref.[2]). We assume that the binding of ATP changes the conformation of the enzyme which is modified both by pyridoxal phosphate and by inorganic phosphate.

The inhibitory effect of ATP on *E. coli* glutamic decarboxylase is considerably weaker; the effect of inorganic phosphate on the inhibition is retained, the modifying effect of pyridoxal phosphate is altered.

Chloride ions competitively inhibit the enzyme from brain. The enzyme from carrot is also inhibited by chloride ions; the evaluation of the inhibition by Woolf's method ($V/[S]$ *versus* $[S]$) suggests noncompetitive inhibition, which is less probable, the graphical plot of $1/v$ *versus* $1/[S]$ indicates noncompetitive inhibition with substrate inhibition.

The glutamic decarboxylases from brain, plants, and microorganisms share many properties in common. They all are pyridoxal phosphate enzymes. Their pH-optimums

are shifted toward the acidic pH-range. The mechanism of action described for the *E. coli* enzyme is most likely the same for decarboxylases from various sources. The binding of the aldehyde group to the ω-amino group of lysine and the SH-group of the apoenzyme leads to an aldamine which is converted into a Shiff's base at acidic pH. In the presence of substrate, a Schiff's base is formed by the reaction of the amino group of glutamate with pyridoxal phosphate[8]. Braunstein and coworkers presented evidence of a hexamer structure of *E. coli* glutamic decarboxylase[9]. The allosteric properties of glutamic decarboxylase from pea[10] and brain[1,2] are most likely connected with the oligomer structure of these decarboxylases, too.

The similar characteristics of enzymes catalyzing the same chemical reaction follow from the character of evolution. Profound structural differences developed as a result of successful mutations even between enzymes whose function did not change in the process of evolution. These differences do not affect, however, the most engaged parts of the molecules, the active centers. We may adduce by way of example cytochrome *c* in whose molecule approximately 50% of amino acids have been replaced in the process of divergence starting with prokaryots and eukaryots and ending with higher mammals[11].

Changes affecting also the functional parts of the molecules can be expected to involve such catalysts whose function underwent changes in the process of evolution. Glutamic decarboxylase is one of these enzymes. The glutamic decarboxylase of *E. coli* is an inducible enzyme whose quantity increases at acidic pH and in the presence of glutamate as the main nutrient[12,13]. *E. coli* growing under different conditions do not need glutamic decarboxylase since they can make suffice even a small quantity of the active enzyme[14]. Glutamic decarboxylase plays most likely an important role in maintaining the glutamate level of plants, which is essential for transamination reactions[15], and in the catalysis of an alternate pathway of α-ketoglutarate degradation[16]. In mammalian tissues glutamic decarboxylase is found in the nervous system only. There it converts in one reaction glutamate acting as a stimulator into 4-aminobutyric acid acting as an inhibitor. The mediator function of 4-aminobutyric acid has been generally accepted[17].

The results of this study show that the enzyme from brain reacts most sensitively to the action of the two physiological potential regulators of glutamic decarboxylase activity. We assume that this is a result of the development of a specialized function of brain glutamic decarboxylase in the nervous system.

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Note added in proof: Wu and Roberts (J. Neurochem. *23*, 759 (1974)) did not observe any significant inhibition of purified brain glutamic decarboxylase even at 10 mM ATP. We repeated their experiments with a partly purified enzyme preparation and found that their negative results were due to the high inorganic phosphate in medium (50 mM) and 200 μM pyridoxal phosphate.