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INVESTIGATION OF GLUAMATE DECARBOXYLASE ACTIVITY AND SOME OF ITS PROPERTIES IN SQUASH (Cucurbitaceae) FAMILY WITH A pCO_2 MEMBRANE ELECTRODE

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ABSTRACT

Potentiometric gas electrodes are suitable to investigate the kinetic properties and activities of enzymes. Relative activities and some properties of glutamate decarboxylase were studied in squash family using a pCO_2 membrane electrode. Crude plant homogenates were used in order to avoid time consuming purification steps and possible conformational changes. L-Glutamate quantities in the crude extracts were estimated from V-[S] curves by extrapolation. No direct correlation between these quantities and enzyme activities was observed. Rather similar values of Km were obtained for squash family which are in the same order of magnitude as those of other higher plants.

INTRODUCTION

There is a growing interest in purification and studying the properties of glutamate decarboxylase (GAD) RC. 4.1.1.15) which is found in relatively large quantities in animals, plants and bacteria. However. many efforts for its purification have met with failure because of the lability of GAD. Like most of the other amino acid decarboxylases, it requires pyridoxal phosphate (PLP) as coenzyme. The enhancing effect of PLP on the bacterial enzyme was indicated by Shukuya and Schwart (1960). Suzs et. al. (1966) succeeded to isolate and purify GAD from mouse brain, its richest source, by stabilyzing it with PLP and aminoethylisothiuronium bromide. Lawson and Quinn (1967) studied glutamate, arginine and histidine decarboxylases in E. Coli. GAD was also isolated from human brain (Strausbauch, 1970), barley (Inatomi, 1975), wheat leaf (Weinberger, 1952), sunflower cotyledons (Smith, 1961) and recently from potato tubers (Satyanarayan, 1985) and squash (Melius, 1966, Matsumoto, 1986).

These studies have indicated that the enzymes purified from different sources showed different properties. In most of these works, GAD was isolated from a single source and its activity was measured by volumetric or nuclear techniques. There has been no report of a systematic comparative study of a given plant family, probably due to the difficulties encountered in purification of the enzyme. It would be rather interesting to investigate the properties of GAD and to correlate relative enzyme activities with substrate concentrations in a plant family. Schales et. al. (1946) studied the distribution of the enzyme in 34 different plants and found that the squash family had the highest content of the enzyme. Therefore, squash family (cucurbitaceae familia) was chosen for the investigation to be reported here.

The best comparison would be among the purified enzymes. However, the isolation of GAD is usually possible with rather low yield which does not allow physicochemical measurements. On the other hand, the purification steps are time consuming and might produce conformational changes in the enzyme protein causing shifts on Km values and optimum pH. To avoid these difficulties. crude enzyme homogenates were used in our experiments.

The potential use of the pCO_2 electrode in analytical and biochemical applications was thoroughly evaluated by Guilbault and Shu (1972) and Jensen and Rechnitz (1979) have shown that dynamic properties of this electrode are very suitable for rate measurements.

GAD catalyzes the decarboxylation of L-glutamic (GA) acid to produce CO_2 and γ-aminobutyric acid (GABA) as shown below:

$$\text{L-glutamic acid} \xrightarrow[\text{PLP}]{\text{GAD}} \gamma\text{-aminobutyric acid} + CO_2 \quad (1)$$

In the present work, we used a pCO_2 membrane electrode to detect the CO_2 produced in Eqn (1).

EXPERIMENTAL

Apparatus

A Brown Melsungen homogenizer and a Sorvall centrifuge was used to prepare the plant homogenates. Potentiometric measurements were taken with a Tacussel model TS 80 milivoltmeter in conjunction

with a Tacussel pCO_2 diffusion electrode, type pCO2-1. Measurements were performed in a 20 ml double jacketed cell at 37 + 0.1°C, temperature control was maintained by means of a Julabo constant temperature circulator.

Reagents

All the reagents were of analytical grade. Solutions were prepared with deionized water. Pyridoxal phosphate solutions were stored in the refrigerator in coloured bottles and renewed every week. Glutamic acid standard solutions were prepared weekly in 0.1 M phosphate buffer, pH = 5.8, and also stored in the refrigerator. Fresh plants, except squirting cucumber, were purchased from the local markets. Squirting cucumbers were picked up from the green house of the Science Faculty of Ankara University. They were kept in the refrigerator until use (never more than a week). Enzyme activity depends on the freshness and ripeness of the vegetables. Therefore, young and fresh ones were used for the analyses. For each variety, instead of a whole plant, mixtures of small pieces from many plants were used to obtain average values.

Preparation of Crude Homogenates

Plants were washed with tapwater before use. 100 g of plant tissue from mesocarb layer was taken and homogenized in parts at 1300 rpm for 2-3 minutes with cold 0.05 M phosphate buffer, pH = 5.8, containing 0.012 mM PLP, 1 mM 2. mercaptoethanol and 1/1000 (v/v) Triton X-100 in a glass homogenizer with a teflon pestle. During homogenization, homogenizer was kept in crashed ice. Crude extract, filtered through cheese cloth, was centrifuged at 12 000 rpm for 20 minutes and the volume of the filtrate was recorded.

Procedure

2 mL of 2.3 10^{-3} M PLP and a series of aliquots ranging from 0.1 to 4 mM of 6.10^{-2} M glutamic acid was placed in a 20 mM double jacketed cell kept at 37°C and the volume was filled up to 15 mL with 0.1 M phosphate buffer, (pH = 5.8). The solution was stirred with a magnetic stirrer and pCO_2 electrode was placed above the solution. After thermal equilibrium was reached (about 15 minutes), 2 mL of crude enzyme homogenate (kept at 37°C for 10 minutes) was injected and potential changes were recorded every minute for 15 minutes. Since Tacussel pCO_2 electrode is a diffusion type electrode and sample solutions conta-

ined 0.17/1000 v/v Triton X-1000, electrode was always kept above the solution (~2 mm from the surface). This also provides the advantage of avoiding the contamination of the membrane and clogging of its pores by particles in the solution (i.e. proteins etc.). Initial rates were plotted against glutamic acid concentrations. For estimating GAD activities, glutamic acid concentrations giving maximum velocity were chosen.

RESULTS AND DISCUSSION

Initial rates, used for plotting V versus [S], were calculated from linear portion of time-pCO_2 electrode response curves, an example of which is given in Fig. 1. Possible presence of L-glutamic acid in plant homogenates was considered and verified by the evolution of carbon dioxide in the absence of added glutamic acid standards. Therefore, glutamic acid contents of the homogenates by the time of analysis were estimated by extrapolation of V-[S] curves (Fig. 2, 3) and given in Table 1.

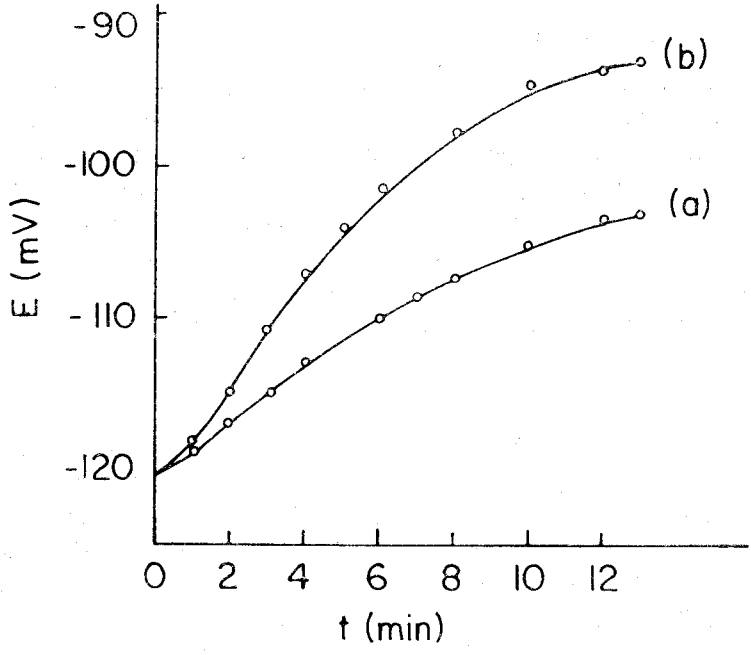

Fig. 1. Two typical time-pCO_2 electrode response curves for the reaction of glutamate decarboxylation: a) [GA] = 4.10^{-4} M, b) [GA] = 1.1 10^{-3} M.

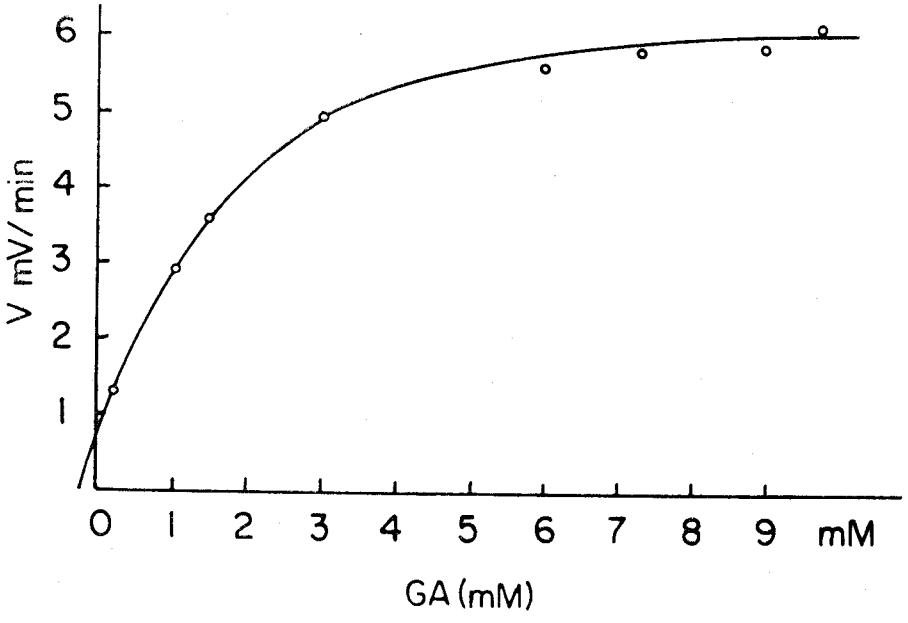

Fig. 2. V-[GA] curve for squash GAD.

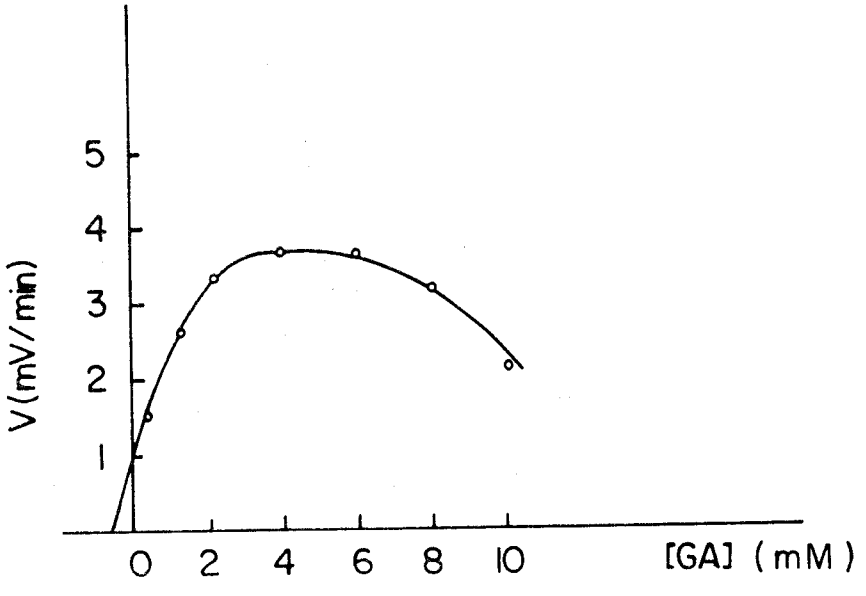

Fig. 3. V- [GA] curve for cucumber GAD.

Table 1. Estimated glutamic acid quantities* and relative GAD activities in squash family

Plant	mg GA per g of plant tissue	Relative GAD activity △E(mV)/ G plant/ 10min
Squash (Cucurbita pepo)	3.10^{-3}	37
Cucumber (Cocumis sativus)	7.10^{-3}	17
— (Cucumis flexiosus)	12.10^{-3}	13
Water mellon (Citrullus lanatus)	11.10^{-3}	12
Mellon (Cucumis melo)	2.10^{-3}	14
Squirting cucumber (Ecballium elaterium)	16.10^{-3}	18

* These values show the glutamic acid quantities for 1 g of plant tissue, calculated from the data measured in plant homogenates at the time of analysis. They may be different from the quantities originally present in the fresh tissues.

For measuring enzyme activity it was necessary to calibrate pCO_2 electrode with standard enzyme solutions. Since pure GAD was not available, the GAD activities in Table 1 are expressed as calculated potential changes, △E (mV) during the first ten minutes of the decarboxylation reaction per g of plant tissue. Activity measurements were performed as explained in the experimental section. Glutamate concentrations were kept as 6 mM because of the substrate inhibition observed for cucumber and mellon at higher concentrations (Figures 3 and 5). The substrate inhibition was also observed by Satyanarayan and Nair (1985) for potato GAD. However, this point needs further justification, preferably with puiified enzymes.

A direct correlation between glutamate quantities and GAD activities was not observed. However, from the results in Table 1, it can be seen that, though some minor discrepancy exists, glutamate quantities are relatively larger when GAD activities are lower. These glutamate quantities measured in the homogenates at the time of analysis don't give the quantities originally present in the plants but is expected to be proportional to them. Satyanarayan (1985) pointed out that the amount of holoenzyme present in the cells was controlled by a complex mechanism in which L-glutamate and free PLP levels and decarboxylation dependent transamination play important roles. Glutamic acid metabolism in mammalian brain is better studied and it is known that ATP and P_i concentrations also control GAD activation-inactivation cycle. Less is known about plant GAD.

Km Values

Substrate concentrations for Lineweaver-Burk graphs were corrected according to the estimated glutamate concentrations coming from

homogenates. In Fig. s 4 and 5 two typical 1 / V-1 / S graphs plotted for squash and cucumber are shown. From these graphs the apparent Km values of the enzyme was calculated to be 2.6 for squash and cucumber.

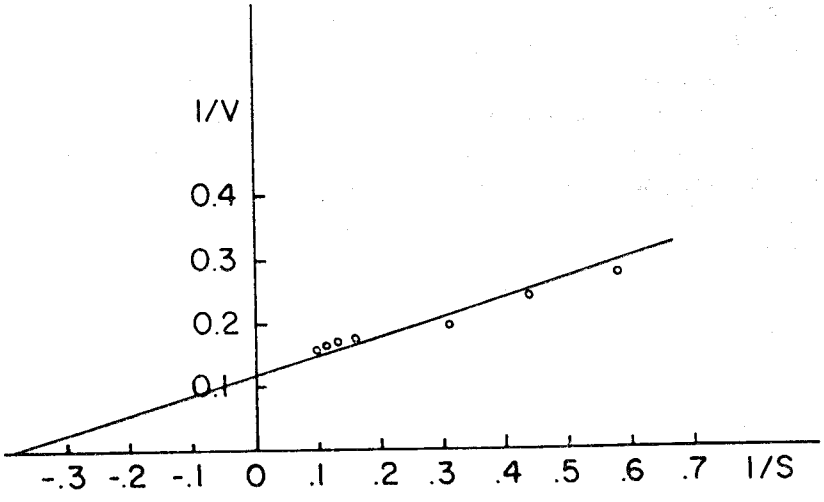

Fig. 4. Lineweaver-Burk plot for squash GAD.

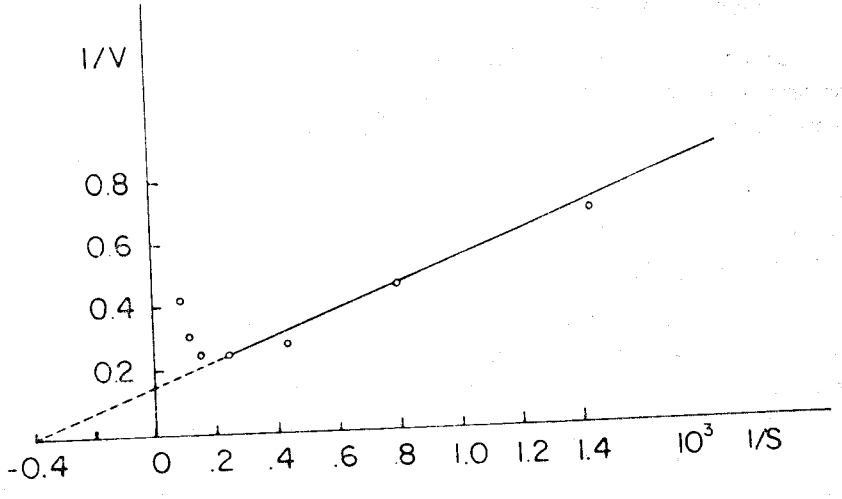

Fig. 5. Lineweaver-Burk plot for cucumber GAD.

As seen in Table 2, similar Km values (between 1.9 and 4.0) were obtained for squash family plants. These Km values are also comparable to those found for GAD's of barley roots (Inatomi, 1975), wheat leaf (Weinberger, 1952), field bean (Ambe, 1963), carrot (Schales, 1946b), and crude enzyme of mouse brain (Suzs, 1966). Km value (2.6 mM) for crude squash homogenate in this work is lower than that (8mM) of Matsumoto et. al. (1986) given for the purified enzyme. Similar differences in Km values were observed in a previous work (Suzs, 1966) for mouse brain GAD. This may be considered as a further support for the suggestion of Suzs et. al. (1966) Purification steps might cause conformational changes on the enzyme protein. Since we could not find Km values of other members of the squash family GAD's in the literature, it was not possible to compare our results.

Table 2. Km values of squash family (Cucurbitaceae familia), obtained in the present work.

Source	Km (mM)
Cucurbita pepo	2.6
Cucumis sativus	2.6
Cucumis flexiosus	2.8
Cucumis melo	4.0
Citrullus lanatus	2.7
Ecballium elaterium	1.9

Temperature Effect

Crude plant extracts diluted with 0.05 M phosphate buffer and kept at certain temperature for ten minutes in the presence of 0.012 mM PLP were then cooled down to 37°C, transferred into the measuring cell and after injection of glutamic acid standard solutions potential drops were recorded for 15 minutes. The results for squash GAD, given in Fig. 6, show that the enzyme activity is effected by heat even after exposure for ten minutes.

In the experiments, after the heat treatment at 46° and 52°C for ten minutes, following glutamic acid injection, the reaction is slow at the beginning but after 5 minutes it speeds up catching the 37°C values in case of 46°C and going over it in case of 52°C. Matsumoto et. al. (1986). stated the optimal temperature for squash GAD as 60°C, but at this temperature a decrease in activity was observed in this work. It was also noticed that PLP plays an important role in restoring enzyme acti-

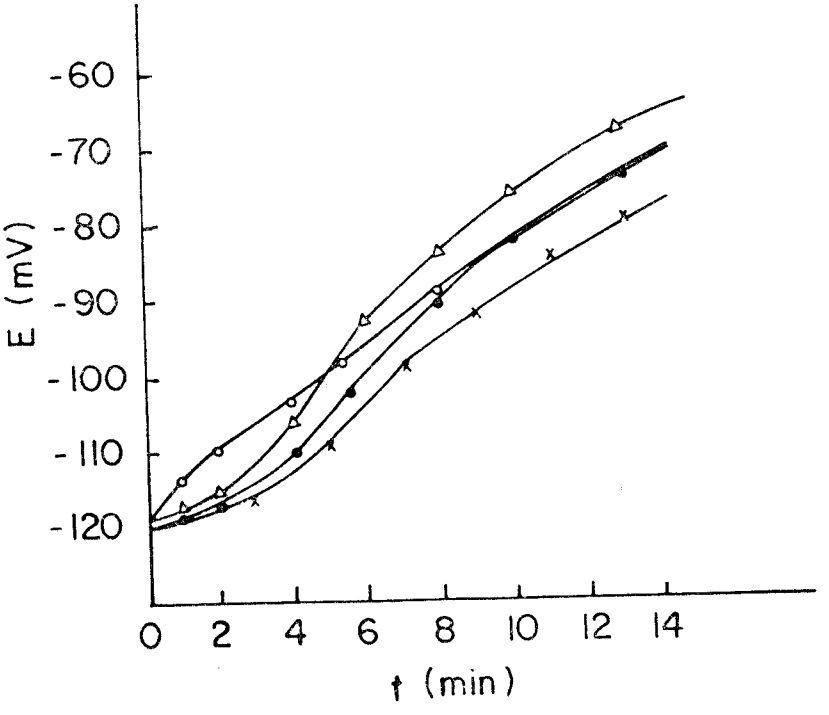

Fig. 6. Effect of temperature on GAD activity: a) o—o—o at 37°C, b) ●—●—● at 46°C, c) △—△—△— at 52°C, d) x—x—x at 60°.

vity at elevated temperatures. Tests performed at 60°C in the presence and absence of PLP showed that in the latter case loss of activity is more severe than the former one. (See Fig. 7).

Enzyme Stability

Enzyme homogenates, stored in the refrigerator at 5°C containing 0.012 mM PLP and 1mM 2. mercaptoethanol, lost only about 2 percent of its activity during the first 24 hours and 25-30 percent after 5 days.

Effect of Cl^- Anions

In previous works (Shukuya, 1960, and Suzs, 1966), it was reported that Cl^- anions have inhibited the activity of the brain GAD but not that of the bacterial enzyme. Analysis with cucumber GAD showed that

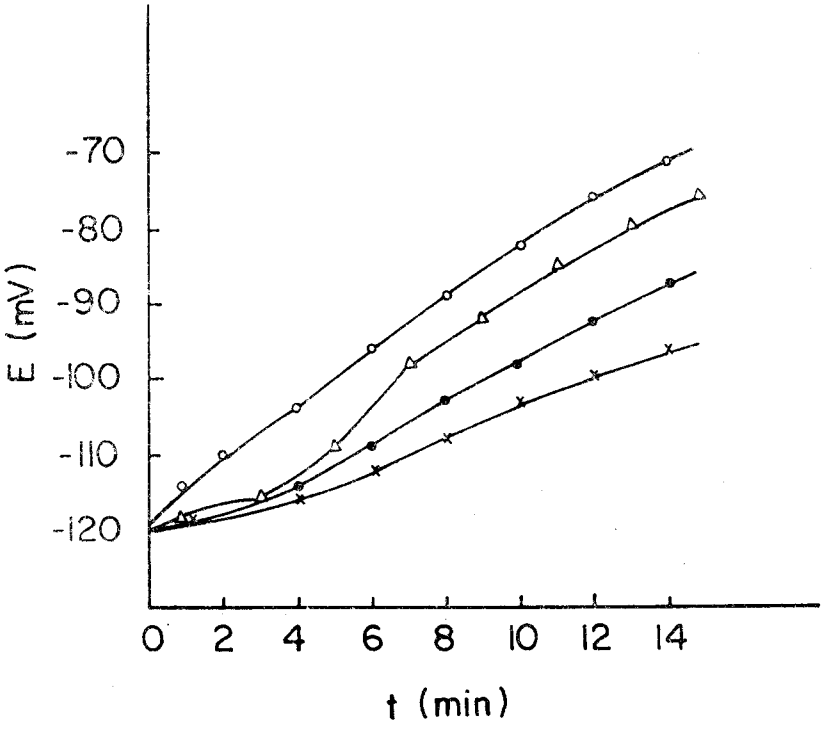

Fig. 7. Role of PLP in stabilyzing the enzyme activity at elevated temperatures. PLP concentration = 0.012 mM, GA concentration = 4.01^{-3} M. a) o—o control, at 37°C, b) △—△— kept at 60°C for ten minutes in the presence of PLP, c) ●—●— kept at 60°C for twenty minutes in the presence of PLP, d) x—x— kept at 60°C for ten minutes without PLP.

plant GAD was also inhibited by Cl^- anions at the concentration of 0.05 M (Fig. 8). Doubling the Cl^- concentration didn't change the degree of inhibition. For brain GAD the significance of Cl^- inhibition was explained (Suzs, 1966) by the terms of Cl^- ion fluxes during nerve activity which might play a role in the controle of GABA production from L-glutamate. Glutamate metabolism in animals, especially in brain, is the subject of numerous papers in recent literature, but less is known about the metabolism in plants.

CONCLUSION

pCO_2 membrane electrode is a useful tool for studying the kinetic properties of the enzymes that catalyse the reactions in which either

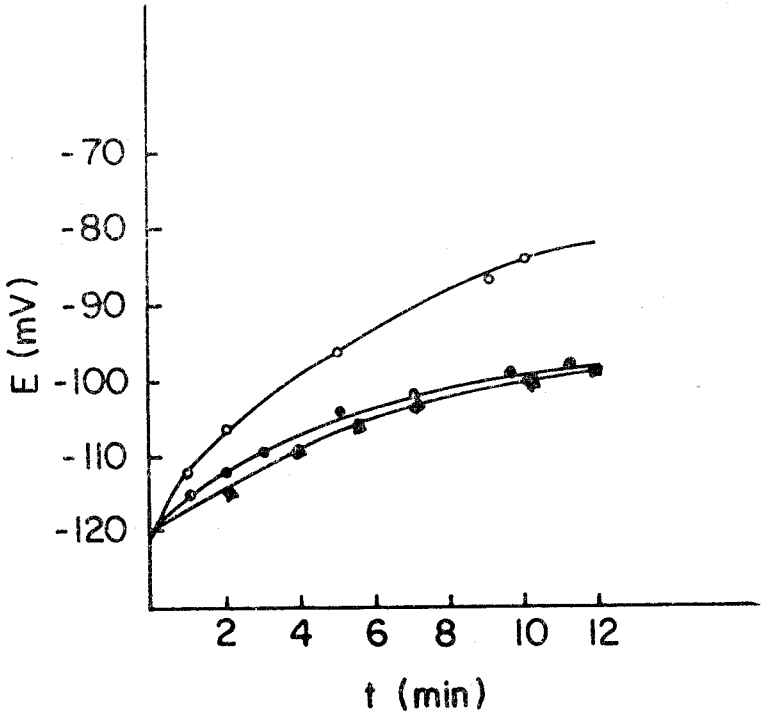

Fig. 8. Cl^- inhibition on GAD: a) o—o control (Cl^- absent), b) ●—● 0.05 M NaCl, c) .■.■ 0.1 M NaCl.

absorption or preferably evolution of CO_2 is involved. However, it needs calibration for enzyme activity measurements and reaches the equilibrium rather slowly. In this paper, results of the measurements taken for GAD of cucurbitaceae familia are presented.

Glutamic acid decarboxylases of Cucurbitaceae familia have similar Km values which are also in the same order of magnitude as those of other higher plant GADs. Km values of the crude and purified enzymes are different probably due to the conformational changes during purification steps. Heat effects the GAD activity even when it is exposed for short periods. Above 60 °C a decrease in enzyme activity is certain. Presence of PLP helps to stabilize the enzyme conformation at elevated temperatures. Cl^- ions and for some members of the family substrate concentrations over 6 mM cause inhibition.

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