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L-Glutamate 1-Carboxylyase and 4-Aminobutyrate: 2-Oxoglutarate Aminotransferase in Succulent Plants

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The results are given which have been obtained in the prospective study of the glutamate decarboxylase in 23 plant species belonging to 6 botanical familes of succulent plants. L-glutamate 1-carboxylyase is found in all the species of *Cactaceae, Euforbiaceae, Liliaceae* and *Amarilidaceae* tested. On the other hand, it has not been found in the species of *Crasulaceae* and *Aizoaceae* which have been studied. The optimum pH (5.7-6.0) and the Michaelis-Menten constant (1.6 and 4.7×10^{-3} M for *Cereus tortuosus* and *Opuntia vulgaris* respectively) agree with the data published for this enzyme in plants. In the experimental conditions, the coenzyme molecule (pyridoxal-5-phosphate) remains firmly bound to the protein. By means of «salting out» a 20- fold purified fraction has been obtained from *Opuntia vulgaris* stem.

Aminotransferase activities have been investigated in *Opuntia vulgaris* (stem and fruit), *Cereus tortuosus* (stem) *and Agave americana* (flowers, leaves and scape). Transaminating activity has been found between 4-aminobutyrate and 2-oxoglutarate in all those materials. Pyruvate and oxaloacetate could not replace 2-oxoglutarate as keto-acid acceptors. The best results have been obtained — according to its apparent intramitochondrial location — with preparations of ultrasonically disrupted mitochondria. The inverse transaminating reaction — glutamate: succinic semialdehyde aminotransferase — has also been clearly shown.

These results seem to confirm that the alternative 4-aminobutyrate by-pass system exists in most of the succulent plants studied, whose well-known hypoxic metabolism gives especial interest to the knowledge of its physiological role.

L-glutamate 1-carboxylyase (EC4.1.1.15) is found widely distributed in animals (14, 18) and plants (24, 15) as well as in micro-organisms (9, 20, 27). The concentration of 4-aminobutyrate (4-AB), a reaction product, is especially remarkable in brain (2, 1, 21), tubers, rhizomes and seeds (19, 7). The location in brain of this non-proteinogenetic amino acid has given rise to numerous papers which advocate a direct action in the transmission of the nervous system (3, 8). However, this hypothesis does not explain its presence in a wide diversity of organisms, so that at the present time and without disregarding the possible neurohormonal action of this compound, 4-aminobutyrate tends to be considered as a metabolite closely related to the Krebs cycle.

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4-aminobutyric (4-AB) acid can act as a donor of amino groups in a transamination reaction, catalysed by 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19), which relates it to the citric acid cycle. 2-oxoglutarate is the acceptor keto-acid, glutamic acid and succinic semialdehyde being formed. It is not necessary to point out the interest of this reaction which produces glutamic acid — from which in turn 4-AB is reproduced by decarboxylation — and which can lead to the formation of succinic acid by means of a coupled reaction. This sequence represents on the one hand a cycle coupled to that of Krebs and, on the other, constitutes an alternative metabolic pathway which relates glutamate to 2-oxoglutarate. Even if this by-pass is normally of a secondary action, it could eventually play an important role in some particular physiological conditions.

Therefore, the study of the reactions related with glutamate in succulent plants of well-known hypoxic metabolism due to the relatively small surface exposed to the atmospheric exchange, could contribute to clarify some aspects of the metabolism of amino acids in them. The results previously obtained in *Agave americana* (16) advised to extend the study to other plants of similar characteristics.

In this paper is shown that the 4-aminobutyrate by-pass is highly operative in most of the succulent plants studied, whose peculiar physiology increases the interest of the role which the 4-AB system can play as an alternative patway of the tricarboxylic acid cycle. In spite of having found traces of 4-aminobutyrate in *Crasulaceae*, its special and characteristic glycolitic and glyconeogenic metabolism justifies the fact that the functioning of the 4-AB system is not required.

Materials and Methods

The tests carried out in the prospective study of L-glutamate 1-carboxylyase have been made by electrophoretic identification (12) of 4-AB, the reaction product, using crude extracts obtained by grinding the plant material in a Blendor type apparatus (40 g of plant in 200 ml of KH_2PO_4-Na_2HPO_4 buffer 0.1 M, pH 5.7) suitably cooled. The homogenate is filtered through cloth. For the determination of the optimum pH of decarboxylating activity, the extracts were obtained in the same conditions, but substituting and overlaping the phosphate buffer by other suitable ones. These were prepared according to GOMORI (10) and the final pH checked in a Polymetron 45-B pH meter. In all cases incubations were carried out for 30 min. to 2 hours at 35°C in thermostatically-controlled baths fitted with mechanical shakers, working with substrate concentrations of 8.8×10^{-3}M and coenzyme (pyridoxal-5-phosphate) concentrations of 1.7×10^{-4}M.

The quantitative determinations of the decarboxylating activity were carried out manometrically in a Warburg apparatus (Braun SL-65) using extracts obtained as described above. The determination of proteins has been made according to the method of LOWRY *et al.* (13). On all ocasions work was carried out at a temperature of 35°C and with the following shaking conditions: amplitude 3.5 and 150 o.p.m. The flasks contained 2 ml of extract, 0.2 ml of pyridoxal-5-phosphate (PLP) (0.4 μMoles) and 1 ml of phosphate buffer 0.1 M, pH 5.7. In the sidearm there were 0.5 ml of sodium glutamate solutions of suitable molarity to give final concentrations of 13.6, 9.7, 5.8, 2.9, 1.4 and 0.7×10^{-3}M. The results of the experiments have been corrected with the simultaneous readings of the termobarometer and the blanks.

The tests of aminotransferase activities have been carried out with homogenates obtained with citric acid-borax-phosphate buffer 0.5M pH 8.3 (26), as well as with

extracts purified by dialysis with buffer 5×10^{-4}M (in a microdialyser designed for this purpose), extracts purified by «salting out» (with ammonium sulphate, pH 8.3 between 20 and 50 % saturation) and with isolated mitochondria according to the method of MILLERO *et al.* (17), after disruption in a ultrasonic apparatus MSE-60. The transaminating activities have been shown by electrophoretic identification of the resulting amino acids. The succinic semialdehyde used in the reaction with glutamate was prepared by us according to the method of WITT and HOLZER (28) and taken immediately before use to the pH of the reaction. Incubations were carried out for 30 min. to 2 hours at 35°C in baths with mechanical shaking. The concentrations of the different substrates used were of 5 to 10×10^{-3}M. The concentration of PLP was also in this case 1.7×10^{-4}M.

Products. The amino acids and coenzymes were supplied by Sigma and Nutritional Biochemical. The ninhydrin was purchased from BDH and the pyridine from Riedel and Haën. The remaining products and solvents were from national firms.

Results

Occurrence of L-glutamate 1-carboxylyase

Table 1 shows the decarboxylating activity found in the species tested by the semiquantitative electrophoretic method.

Table I. *Activity of L-glutamate 1-carboxylyase in some species of succulent plants.* The determinations have been carried out by semi-quantitative paper electrophoresis. Intensities of the spots of 4-AB formed are approximately represented.

CACTACEAE		EUFORBIACEAE	
Opuntia vulgaris	6+	*Euphorbia mogador*	3+
Opuntia ficus indica	6+	AMARILIDACEAE	
Opuntia microdasys albispina	4+	*Agave americana*	6+
Cereus tortuosus	6+	CRASULACEAE	
Cereus geometisans	5+	*Crassula portulacea*	—
Cereus peruvianus	5+	*Sedum altissimun*	—
Echinopsis multiplex	2+	*Sedum pachyphyllum*	—
Ferocactus albispinus	1+	*Echeveria calva*	—
Mammillaria elegans	3+	*Cotyledon ausana*	—
Pilocereus euphorbioides	1+	AIZOACEAE	
Trichocereus santiaguensis	2+	*Faucaria tigrina*	—
LILIACEAE			
Aloe brovifolia	1+		
Lapiedra martinezi	4+		
Gasteria linguata	2+		
Hawortia attenuata	1+		

Determination of the optimum pH

The determination of the optimum pH for the decarboxylation of glutamate has been made in the most active species, values being obtained in all cases which vary from 5.7 to 6.0 (Fig. 1).

Dialysis experiments

Plant extracts obtained from *Cereus tortuosus* were dialyzed for 24 hours with a sodium-potassium buffer 5×10^{-4}M, pH 5.7, in a refrigerated chamber, and the dialyzed was tested in the following conditions: a. Without the addition of PLP; b. PLP added; c. With the addition of boiled undialyzed extract, and d. With the addition of boiled undialyzed extract and PLP.

Another test was carried out with undialyzed extract kept in the same conditions as the dialyzed one. The activities, found by means of electrophoresis are summarized in Table II.

Determination of the Km

The Michaelis-Menten constant and kinetics of the enzyme from *Cereus tortuo-*

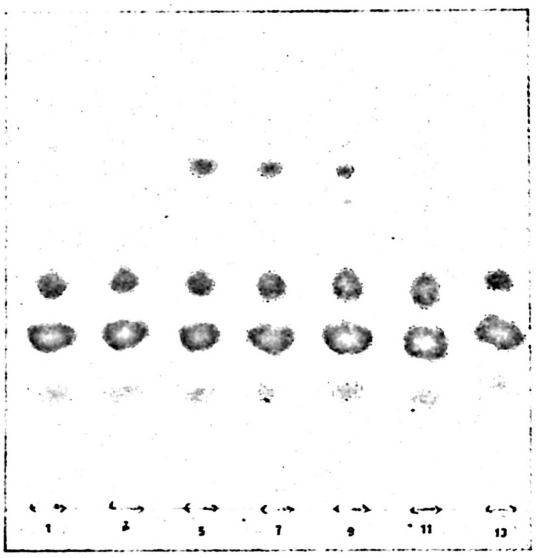

Fig. 1. *Activity of L-glutamate 1-carboxylyase at different pH. Electrophorogram obtained using extracts of* Opuntia vulgaris *(fruit).* The tubes contained 1.5 ml of buffer (acetate buffer for lower values; phosphate buffer for higher values of pH), 0.2 ml of PLP (0.4 μMoles), 0.3 ml sodium glutamate (20.4 μMoles) and 1 ml of enzyme extract. The final values of pH were 5.3, 5.7, 6.0, 6.4, 6.8, 7.2 and 7.4 respectively.

sus and *Opuntia vulgaris* have been determined by the manometric method.

As shown in Table III, similar values of Km have been found plotting the values according LINEWEAVER and BURK

Table II. *Activity of L-glutamate 1-carboxylyase in dialyzed extracts of* Cereus tortuosus. Dialysis was carried out for 24 hours with sodium potassium phosphate buffer 5×10^{-4} M pH 5.7, at a temperature of 4° C

Dialyzed	++
Dialyzed + PLP	+++
Dialyzed + boiled extract	++++
Dialyzed + PLP + boiled extract	++++
Extract kept the same as the dialyzed	+++++

Table III. *QCO_2 and Km values of L-glutamate 1-carboxylyase of* Cereus tortuosus *and* Opuntia vulgaris. $QCO_2 = \mu l\ CO_2$ in 1 min. × 60/mg protein per milliliter.

	Km	QCO_2
Cereus tortuosus	1.6×10^{-3} M	59.83
Opuntia vulgaris	4.7×10^{-3} M	25.20

(11) both using «normal» velocities and those obtained by the method of SCHALES and SCHALES (22, 23) for reactions with a rapid shift from the zero order to the first order (Fig. 2).

Enzyme purification by salting-out

A 20 fold purified enzyme preparation is obtained from *Opuntia vulgaris* by salting-out with ammonium sulphate between 22 and 45 %. Kinetic values of the purified fraction were in good agreement with those found in the crude extracts.

Coenzyme binding. Activators and inhibitors

The addition of PLP to the undialyzed extracts only increases the decarboxylating activity very slightly. The possible ions

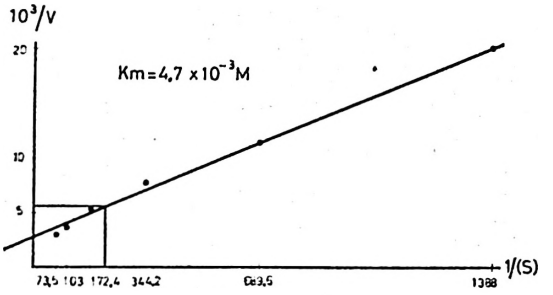

Fig. 2. *L-glutamate 1-carboxylyase of* Opuntia vulgaris. *Determination of the Km according to Schales and Schales (22).* Concentrations of substrate: 13.6, 9.7, 5.8, 2.9, 1.4 and 0.7×10^{-3} M.

requirement, suggested by the increase of activity observed on adding boiled extract to the dialyzed of *Cereus tortuosus* has been studied by treating with EDTA 10^{-3}, 10^{-4} and 10^{-5}M. Activity measurement showed no significant changes, however, as compared with that obtained without the addition of EDTA. On the other hand, the enzyme activity of the extract from *Opuntia vulgaris* is clearly increased by this compound (about 50 %) Also glutathion (1×10^{-3}M) produces a very similar activation and, against, it does not appear to have any effect on the enzyme from *Cereus tortuosus*. 2,3-dimercapto-propanol (2×10^{-3}M) inhibits the glutamate decarboxylyase of this latter species in a proportion around 40 %.

Activity is clearly enhanced when working in nitrogen atmosphere. As was to be expected, according with the results obtained with the enzyme from *Lupinus* (15), no effect has been observed on the velocity of the reaction when 4-AB is added, even at high concentrations.

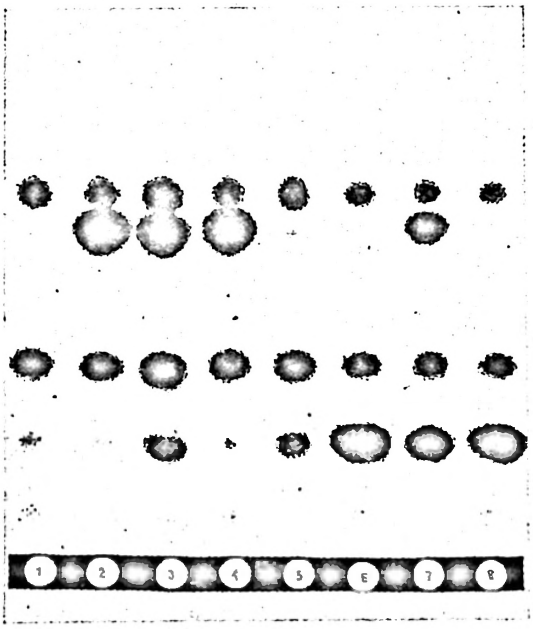

Fig. 3. *Agave americana. 4-aminobutyrate:2-oxoglutarate aminotransferase (tubes 1 to 5) and glutamate: succinic semialdehyde amino transferase (tubes 6, 7 and 8).*
Tube 1 only contained buffer (citric acid-borax-phosphate 0.5 M pH 8.3) and enzyme preparation; tubes 2 and 3 also contained the complete reaction system (25 μMoles of 4-AB, 10.2 μMoles of 2-oxoglutarate and 0.2 μMoles of PLP). In tube 4,2-oxoglutarate was omitted; tube 5, without 4-AB. Tubes 6 and 7 contained the complete system for the inverse reaction (enzyme extract, coenzyme, succinic semialdehyde — 50 μMoles — and sodium glutamate, 27.2 μMoles). In tube 8 succinic semialdehyde was omitted. Tubes 1, 2 and 6 were stopped at zero time by the addition of ethanol.

Investigation of aminotransferase activities

With 4-AB as the amino acid donor. The transaminating activities have been studied in *Opuntia vulgaris* (stem and fruit), *Cereus tortuosus* (stem) and *Agave americana* (flowers, stem and scape) (Figure 3).

In all of these extracts 4-AB:2-oxoglutarate aminotransferase activity has been found. Pyruvate cannot replace 2-oxoglutarate as ketoacid in the experimental condition.

The determination of transaminase activity in the crude extracts is difficult due to the location of the enzyme and the preexistence and reactivity of the reaction products (glutamic acid and alanine). On the other hand, the aspartate present in the extract transaminates with 2-oxoglutarate. Therefore, the transaminase activity (4-AB:2-oxoglutarate) was investigated and clearly shown by using dialized extracts and the fraction obtained by salting-out with ammonium sulphate between 20 and 50 % saturation.

Completely satisfactory results have been obtained with preparations of mitochondria disrupted ultrasonically. The production of glutamate was evident and without any interference.

With other amino acids. The following aminotransferase activities have been shown also to occur in these tissue extracts:

Aspartic acid + 2-oxoglutarate ⟶
Glutamic acid + Oxaloacetate
Glutamic acid + Oxaloacetate ⟶
Aspartic acid + 2-oxoglutarate
Alanine + 2-oxoglutarate ⟶
Glutamic acid + Pyruvate
Glutamic acid + Pyruvate ⟶
Alanine + 2-oxoglutarate

In the crude extracts, the activities of these reactions are much higher than those found when using 4-AB as amino donor. The transamination between alanine and 2-oxoglutarate has been the most intense, the inverse step (transamination between glutamic acid and pyruvate) being, on the contrary, very poor. The transaminase reactions leading from glutamate to aspartate and viceverse showed always high activities, the sense of the reaction depending on the respective substrates present.

«Inverse reaction» (glutamate: succinic semialdehyde aminotransferase)

The transamination between glutamate and succinic semialdehyde to produce 4-AB and 2-oxoglutarate has been shown in the forementioned materials, using the same enzyme preparations. The activity was in all cases much higher than that corresponding to the «direct reaction».

Discussion

The results obtained corroborate the wide occurrence of L-glutamate 1-carboxylyase in plants. There are significant variations in the specific activity, according to the enzyme source, but its presence is constant in the different species studied. *Crasulaceae* and *Aizoaceae* are an exception, which can be explained by their peculiar carbohydrate metabolism. It must be pointed out that the 4-AB system is found to be especially operative in the plants which exhibit a smaller surface for oxygen exchange.

The values of optimum pH found, as well as the kinetic constants are in good agreement with the data previously published for glutamate decarboxylase of plant origin (24, 15). The sharp decrease of the activity confirms the results obtained by BEEVERS (4), the slope of the curve showing in all cases a rapid transition to a first order reaction. The —SH compounds of low molecular weight do not enhance the activity in the experimental conditions. Only glutathion appears to have slight activating effect of the enzyme from *Opuntia vulgaris*.

We have not found any inhibiting influence after the addition of EDTA. Due to the instability of the apoenzyme at the higher pH values needed to achieve a good chelatting effect, the interesting problem of the requirement of trace elements (25) remains unsolved.

The quantitative determination to the decarboxylating activity has shown the considerable relative specific activity of the species *Cereus tortuosus* and *Opuntia vulgaris*, confirming the results obtained in semiquantitative electrophoresis.

On the contrary to what has been found in the enzyme from *Lupinus albus* (15), pyridoxal-5-phosphate is firmly bound to the apoenzyme, in such a way that its addition to the reaction mixture only stimulates the activity very weakly.

The 4 - aminobutyrate: 2 - oxoglutarate aminotransferase activity has been clearly shown in the plants studied. As far as we know, only DIXON and FOWDEN (6) had been able to show this activity in mitochondrias isolated from peas. There certainly exist many experimental difficulties for the measurement of this activity, due to the analogy between substrates and products. On the other hand, glutamate is normally found in considerable amounts in the extracts. The demonstration of the transaminating activity of 4-AB in the fractions obtained by salting out with ammonium sulphate and mitochondria disruption, clearly establishes the functionning

of the 4-AB by-pass system in the plants studied and seems to corroborate its physiological role in them. It has not been possible to check the result obtained in peas by DIXON and FOWDEN (6) that pyruvate also acts as 4-AB amino acceptor. Of all the ketoacids tested, only 2-oxoglutarate showed activity.

The demonstration of the glutamate: succinic semialdehyde aminotransferase was easier than that of the direct transaminase due, on the one hand, to the lower concentration of 4-AB in the blanks and, on the other, to its lesser further metabolism in the experimental conditions. The finding of this activity in plants is a clear evidence of the alternative possibilities of the metabolic transformations of 4-AB in plants, depending on the substrates availability.

The results obtained suggest a different location of the decarboxylating and transminating enzymes (extra- and intramitochondrial respectively). This difference in location is interesting in the interpretation of its physiological role. Under normal conditions, the efficiency of a «distant system» appears to be small, when it is known that the great majority of the plant cells have an active tricarboxylic acid cycle and glutamate dehydrogenase; but in conditions that could affect the α-ketoglutarate dehydrogenase, of lead to a decreased activity of the Krebs cycle, the 4-AB by — pass system could be induced reaching a higher biological significance.

Resumen

Se presentan los resultados obtenidos en la labor prospectiva de la enzima descarboxilante del glutamato en 23 especies vegetales pertenecientes a 6 familias botánicas de plantas suculentas. La L-glutamato 1-carboxiliasa se encuentra en todas las especies de Cactáceas, Euforbiáceas, Liliáceas y Amarilidáceas ensayadas, no habiéndose encontrado, en cambio, en las especies de Crasuláceas y Aizoáceas que se han estudiado. El pH óptimo (5,7-6,0) y la constante cinética de Michaelis-Menten (1,6 y 4,7 × × 10^{-3}M para *Cereus tortuosus* y *Opuntia vulgaris*, respectivamente) coinciden satisfactoriamente con los datos publicados para esta enzima en orígenes vegetales. La proteína apoenzimática retiene fuertemente unida, en las condiciones experimentales, a la molécula coenzimática (piridoxal-5-fosfato). Por fraccionamiento salino, se ha conseguido purificar unas veinte veces la L-glutamato -1carboxiliasa procedente de *Opuntia vulgaris* (tallo).

Se han investigado actividades aminotransferásicas en *Opuntia vulgaris* (tallo y fruto), *Cereus tortuosus* (tallo) y *Agave americana* (flores, hojas y escapo). En todos estos materiales se ha encontrado actividad transaminante entre el 4-aminobutirato y el 2-oxoglutarato, no hallándose actividad apreciable cuando se utilizan piruvato y oxalacetato como cetoácidos aceptores. Los mejores resultados se han obtenido — dada su situación intramitocondrial aparente — con preparaciones de mitocondrias rotas al ultrasonido. También se ha puesto de manifiesto la reacción transaminante inversa, glutamato:semialdehido succínico aminotransferasa.

Estos resultados parecen confirmar la hipótesis de que el ciclo alternativo del 4-aminobutirato existe en la mayoría de las plantas suculentas investigadas, cuyo reconocido metabolismo hipóxico presta singular interés al conocimiento de su papel fisiológico.

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