Effect of phosphonic analogues of glutamic acid on glutamate decarboxylase[1]

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Summary. Among the phosphonic analogues of glutamic acid, only 4-amino-4-phosphono butyric acid, the compound which shows the highest affinity for pyridoxal phosphate, inhibits competitively both *Escherichia coli* and rat brain glutamate decarboxylases. Phosphinothricin, 2-amino-4-(methylphosphino)butyric acid, is a strong inhibitor of the mammalian enzyme.
Key words. E.coli; rat brain; glutamate, phosphonic analogues; glutamate decarboxylase; pyridoxal phosphate.

The phosphonic analogues of α-aminocarboxylic acids show a strong affinity for pyridoxal 5'-phosphate[2] (PLP); some of these analogues behave as antagonists in metabolic reactions involving PLP-dependent enzymes. A typical example is the inhibitory activity of 1-amino-ethylphosphonic acid on alanine racemase[3], knowledge of which prompted the synthesis of the new antibiotic alafosfalin[4]; another example is the potent inhibition of rat liver tyrosine transaminase by the phosphonic analogue of tyrosine[5]. Glutamate decarboxylase (GAD, EC 4.1.1.15) is a widely distributed pyridoxal-phosphate protein which catalyzes the synthesis of 4-aminobutyric acid. The bacterial enzyme has a high substrate specificity, practically limited to L-glutamate and L-glutamine[6], whereas the brain enzyme also acts on L-cys L-cysteine sulphinate and L-aspartate[7]. We have investigated the effect of the three phosphonic analogues of glutamic acid (table) and of some related compounds on bacterial *(Escherichia coli)* and mammalian (rat brain) glutamate decarboxylases and on free pyridoxal phosphate.

The formation of a Schiff base between the amino group of the various analogues and the formyl group of PLP was measured using a spectrophotometric method, as described earlier[2]. The apparent dissociation constants K_d listed in the apparent dissociation constants K_d listed in the table:

$$K_d = \frac{[\text{PLP}]\,[\text{amino acid}]}{[\text{aldimine}]}$$

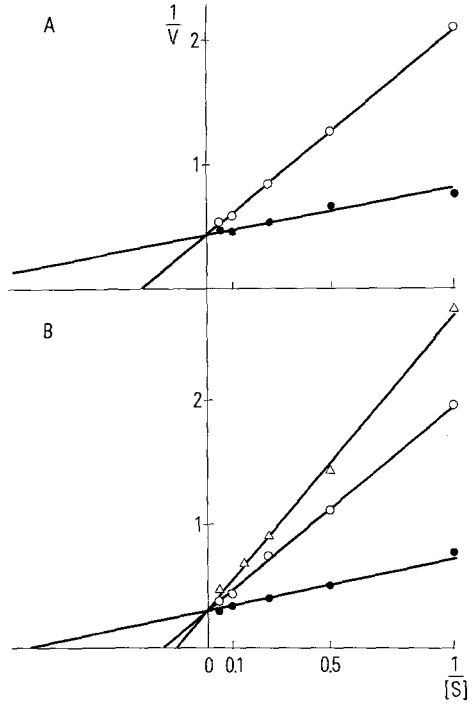
confirm the results obtained in our previous paper with other molecules. Compound 2, the α phosphonic analogue of glutamic acid, reacts with PLP at a faster rate than the dicarboxylic compound; this behavior may be related to the differences of $pK_{(NH_3+)}$ existing between the two series of amino acids[2]. On the other hand, when a phosphonic group replaces the γ-COOH of glutamic acid, giving compound 3, the affinity for PLP is greatly diminished; this unfavorable effect of $-PO_3H_2$ in the γ position was also observed with compound 4, since this diphosphonate gives a K_d ranging between the values determined for glutamic acid and compound 2; the presence at the end of the molecule of a strongly acidic group probably induces conformational changes of the carbon chain resulting in a lowered reactivity of the amino group. Compounds 2 and 4, which showed a higher affinity for PLP than compound 1, may then be expected to inhibit glutamate decarboxylase.

The enzymatic activity of *E. coli* glutamate decarboxylase (Sigma Chem. Co, St.Louis, Mo, USA) and of a partially purified rat brain enzyme[8] was measured by determining the rate of release of $^{14}CO_2$ from 1-|^{14}C|-L-glutamate (Amersham), using a technique slightly modified from Fonda[6]. Assays, in duplicate, were carried out at 30 °C in small conical plastic Eppendorf tubes containing the enzyme solution, PLP ($5 \cdot 10^{-4}$ M), and the potential inhibitor (0–30 mM) in a final volume (50 µl) of buffer (0.1 M pyridine-HCl, pH 4.22 for the bacterial enzyme; 0.1 M phosphate, pH 6.8 for the brain enzyme). A second vessel containing hyamine hydroxide was introduced into the tubes before the reaction was started by adding labeled glutamate (1–20 mM). Blanks without substrate were run in parallel. After an incubation period, CO_2 was finally re-

Affinity of glutamic acid and its phosphonic analogues for pyridoxal 5'-phosphate

Compound	Structure	K_d [mM]
1	HOOC–CH_2–CH_2–CH(COOH)(NH_2)	6.6
	L-Glutamic acid	
2	HOOC–CH_2–CH_2–CH(PO_3H_2)(NH_2)	2.7
	DL-4-Amino-4-phosphonobutyric acid	
3	H_2O_3P–CH_2–CH_2–CH(COOH)(NH_2)	14.3
	DL-2-Amino-4-phosphonobutyric acid	
4	H_2O_3P–CH_2–CH_2–CH(PO_3H_2)(NH_2)	4.3
	DL-1-Aminopropane-1,3-diphosphonic acid	

Experimental conditions as described in Cassaigne et al.[2]. Phosphate buffer, pH 8. Compounds 2, 3 and 4 were obtained from Prof. Isbell (Agricultural Univ., College Station, Texas). Compound 3 is obtainable from Calbiochem-Behring (San Diego, Ca).



Competitive inhibition of *Escherichia coli* (A) and rat brain (B) glutamate decarboxylases by 4-amino-4-phosphonobutyric acid (30 mM, ○) and 2-amino-4-(methylphosphino)butyric acid (10 mM, △). Control curve (●) |S|=glutamic acid, mM. The points shown in the figure are the means of three experiments, each one carried out in duplicate.

leased by H_2SO_4, trapped with hyamine hydroxide and estimated using a scintillation counter (Intertechnique).

Compound 2, which obviously cannot be a substrate, was the only compound showing inhibitory properties; K_i values obtained through Lineweaver-Burk plots (fig.) were one order of magnitude greater than the K_m of both *E.coli* GAD (K_m = 0.9 mM; K_i = 10 mM) and rat brain GAD (K_m = 1.3 mM; K_i = 10.5 mM). Our results indicate that binding of the α phosphonic group at the active site of both enzymes is weakened by steric hindrance.

Compound 3, despite the presence of an α-aminocarboxylic group, was neither a substrate nor an inhibitor. Negative results were also obtained with 4 and with two phosphonic analogues of aspartic acid, 3-amino-3-phosphonopropionic and 2-amino-3-phosphonopropionic acids: the brain enzyme which recognizes L-aspartic acid as a substrate was not inhibited by the two last compounds tested at a concentration 30-fold higher than that of the substrate. Moreover, the inertness of compound 3 may emphasize the importance of the γ-carboxylic group of glutamic acid for enzyme recognition. We have investigated the role of this group by studying the effect on decarboxylase activity of 2-amino-4-(methylphosphino)-butyric acid, also known as phosphinothricin[9], homocysteic acid and norvalin, compounds differing from glutamic acid by the replacement of its γ-COOH group by $-PO(OH)(CH_3)$, $-SO_3H$ and $-CH_3$ respectively. The strict specificity of *E.coli* GAD towards the γ-carboxyl group was confirmed by the lack of inhibitory effect of the above-mentioned substances; this may be related to the recent observation of Vospel et al.[10] that this acidic group is involved in a linkage with an arginine residue in the catalytically active form of *E.coli* GAD. Unlike the bacterial enzyme, brain GAD was able to recognize other acidic groups in the same position: 2-amino-4-(methylphosphino) butyric acid was a potent competitive inhibitor with a K_i of 2.2 mM, a value very close to the K_m for glutamate; L-cysteic and L-homocysteic acids, used in a molar ratio |inhibitor|/|substrate|=20, led to inhibition values of 60 and 40% respectively. L-Norvaline was inert towards the two enzymes. It may be emphasized that all the inhibitory molecules tested possess an ω-mono-anionic group like the substrate, a fact which may explain the inertness of 3 and 4 towards brain GAD. However, work in an other field has shown that compound 3 is able to interfere with the neuronal glutamate receptor[11].

The dialanyl derivative of phosphinothricine is a natural compound which exerts its antibiotic properties against several microorganisms through the action of phosphinothricin on glutamine synthetase[9]; phosphinothricin is also a potent inhibitor of rat liver glutamine synthetase[12]. We have shown here another target site of this compound in eucaryotic enzymatic system.

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Number of dopamine neurons predicts prolactin levels in two inbred mouse strains

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Summary. Mice of the BALB/cJ strain have more dopamine neurons than mice of the CBA/J strain. We now report that BALB/cJ mice have less circulating and pituitary prolactin than CBA/J mice, a relationship expected from the difference in tuberoinfundibular dopamine neuron number.
Key words. Prolactin; inbred mouse strains; dopamine neurons; hypothalamus; pituitary.

Mice of the BALB/cJ strain display greater activity of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) in brain compared to another inbred strain of mice, CBA/J[1,2]. Histological analysis using antibodies to tyrosine hydroxylase to localize catecholamine cells has revealed that the difference in TH activity between these two strains is due entirely to a difference in number of dopaminergic cells. Mice of the BALB/cJ strain have 20–50% more TH staining neurons in each population of dopamine cells in the brain (i.e., A9, A10, A11, A12, A13, A14) compared to CBA/J mice[3,4]. The activity of TH per dopamine cell, as calculated from the activity of TH measured in vitro and the number of TH staining cells, is not different between the two strains[3]. The possibility that the release of dopamine may be greater in brains of BALB/cJ mice compared to CBA/J mice is supported by biochemical studies showing a greater dopamine synthesis and turnover rate in the BALB/cJ strain[5].

The difference in dopamine cell number raises the question of how these strains of mice may differ in physiological processes and behaviors, especially those involving dopamine neurons. Fink and Reis[6,7] compared these two mouse strains with respect to locomotor activity and behavioral responses to drugs which act via dopamine neurons. Their studies support the hypothesis that the level of dopaminergic neurotransmission in BALB/cJ mice is greater than in CBA/J mice. We have extended those findings by examining another process that is dependent upon dopamine neurons in the central nervous system, the regulation of prolactin release.

Methods. Male and female mice of the BALB/cJ and CBA/J strain were obtained from the Jackson Laboratory (Bar Har-