U. N. A. M.

ESTUDIO CINETICO DE LA DESCARBOXILASA DEL ACIDO GLUTAMICO DE CEREBRO DE RATON

TRABAJOS DE TESIS QUE PRESENTA EL M. EN C. ALEJANDRO BAYON CASO PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS QUIMICAS

(ESPECIALIDAD BIOQUIMICA)

1 9 7 7 .

PROLOGO, INTRODUCCION Y ADVERTENCIA

A thorough kinetic analysis is an essential part of the characterization of any enzyme. Yet, too often such studies are either omitted from research publications or are presented in a very superficial manner. This is unfortunate but somewhat understandable.
Many biologists study enzymes but are intimidated by the apparent complexity of enzyme kinetics.

I. H. Segel

DE LOS MODELOS. Hemos estado y seguimos estando instalados en el "estado de gracia" del sentido común y en la "paz espiritual" de la lógica, a pesar de que es evidente que el uno y la otra han puesto invisibles y sutiles trabas para nuestro acercamiento a la verdad. La comprensión cabal de los fenómenos biológicos solo puede darse desde el pensamiento matemático, entendiendo éste en términos de los procedimientos usados para convertir una idea en un modelo y éste en una ecuación que pueda ser probada experimentalmente ó, de forma reciproca, para desarrollar ecuación y modelo a partir de datos experimentales. Sin embargo ningún modelo es definitivo. Cada uno de ellos recrea el mito de Sísifo. Por desalentador que parezca estamos limitados a y por los modelos. Ellos son el principio y fin de lo que llamamos ciencia? Son nuestra idea del universo. Más allá están el sentido común y la obscuridad.

DE LA CINETICA ENZIMATICA. Dentro de la complejidad funcional de los sistemas nerviosos algo de luz se ha posado sobre los sistemas capaces de transmitir información de una neurona a otra. De los probables mensajeros primarios en la comunicación sináptica, el ácido γ-amino butírico ha dado evidencia de un papel relevante en las acciones de tipo inhibitorio. Sin embargo los mecanismos que regulan su síntesis no han sido aún esclarecidos. Un intento de iniciar su dilucidación ha sido este estudio del comportamiento de la enzima que cataliza su producción. Algunos trabajos sobre las propiedades de la descarboxilasa del ácido glutámico habían sido realizados sin tomar en consideración que el instrumento necesario para la comprensión de esta actividad enzimática es el estudio cinético de su acción catalítica, incluyendo éste el análisis de dependencia de substrato (Glutamato) y activador (PLP) en un amplio intervalo de sus concentraciones (Artículo 2), estudios completos de inhibición por análogos de cada uno de estos reactantes (Artículos 1 y 3) así como por el producto de la reacción (Artículo 3). Ningún estudio cinético incompleto puede dar información concluyente sobre el comportamiento de un sistema de reactivos-enzima-productos

DE ENTIDADES PURAS Y SISTEMAS BIOLOGICOS. Sin embargo, las propiedades cinéticas de un sistema biológico solo adquieren sentido cuando se las enmarca en el contexto de su posible papel funcional. Esto implica tanto el conocimiento del comportamiento del sistema catalítico puro, y el del sistema integrado en el ser biológico, como la reconstrucción gradual del segundo a partir del primero En este trabajo hemos abordado un procedimiento suplementario que consiste en el estudio cinético del sistema enzimático sin eliminar

componentes que pudieran coexistir con el in vivo (aunque ésto implique extremar los cuidados y aumentar la complejidad en los procedimientos metodológicos y analíticos).

Este acercamiento ha fructificado en la identificación de dos formas de actividad enzimática que pudieran no haberse detectado - usando preparaciones puras y que son imposibles de detectar en un sistema íntegro.

"El lógico más versado en su ciencia - abandona las leyes de la lógica en - cuanto discurre realmente"

SCHOPENHAUER.

(paper 1)

KINETICS OF BRAIN GLUTAMATE DECARBOXYLASE. INHIBITION STUDIES WITH N-(5'-PHOSPHOPYRIDOXYL) AMINO ACIDS[1].

Alejandro Bayón[2], Lourival D. Possani and Ricardo Tapia[3]

Departamento de Biología Experimental, Instituto de Biología, Universidad Nacional Autónoma de México. Apartado Postal 70-600, México 20, D. F. México.

Please send proofs and correspondence to Ricardo Tapia, at the above address .

Abbreviations used: PLP, pyridoxal 5'-phosphate; GAD, glutamate decarboxylase.

[1] This work was supported in part by Grant No. 043/76 from the Consejo Nacional de Ciencia y Tecnología, México, D. F.

[2] Recipient of fellowship from the División de Formación del Profesorado, UNAM.

[3] To whom correspondence should be sent.

Running title.

Inhibition of brain glutamate decarboxylase.

Abstract - Seven N-(5'phosphopyridoxyl) amino acids, reduced analogs of the glutamate-pyridoxal phosphate Schiff base, were synthesized and purified. All of them inhibited mouse brain glutamate decarboxylase activity. The four most potent inhibitors were the aminooxyacetate, GABA, cysteinesulfinate and glutamate derivatives, and the effect of these compounds was studied kinetically. The inhibition produced was in all cases mixed function with respect to glutamate and competitive with respect to pyridoxal phosphate. The inhibition kinetics were non-linear. These results are interpreted as indicating an ordered binding of pyridoxal phosphate and glutamate to the enzyme. Furthermore, they are consistent with previous findings suggesting the existence of two kinds of glutamate decarboxylase activity differing in their dependence on free pyridoxal phosphate.

Brain glutamate decarboxylase (L-glutamate-1-carboxylyase, EC 4.1.1.15, GAD) seems to play an essential role in the regulation of brain excitability (Tapia, 1975; Tapia et al., 1975; Tower, 1976). In spite of the existence of many compounds capable of inhibiting its activity (Roberts et al., 1964; Wu & Roberts, 1974), little is known on its kinetic and regulatory mechanisms. Previous studies from this laboratory (Tapia & Sandoval, 1971) have shown that the stable oxime pyridoxal-5'-phosphate-O-acetic acid may be a useful inhibitory compound for the elucidation of such mechanisms, because of its structural similarity to the substrate-pyridoxal phosphate Schiff base (Roberts et al., 1964). In the present study we describe the synthesis, purification and the kinetics of the effect on brain glutamate decarboxylase activity of the reduced oxime and several other N-(5'-phosphopyridoxyl)-amino acids, which are also structurally similar to the substrate-pyridoxal phosphate Schiff base.

MATERIALS AND METHODS

Materials. DL- [1-^{14}C] Glutamic acid, and [^{3}H] $NaBH_4$ were from New England Nuclear (Boston, Mass.). Sephadex G-10 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Apotryptophanase was obtained by dialysing a commercial preparation of E. coli tryptophanase (grade II, Sigma Chemical Co., St. Louis, Mo.) Pyridoxal phosphate (PLP) and other

chemicals for synthesizing its derivatives, except α, γ -diaminobutyric acid (Aldrich Chemical Co. Cedar Knolls, N. J.), were obtained from Sigma.

Synthesis and purification of N-(5'-phosphopyridoxyl) amino acids. The reduced oxime PLP-aminooxyacetic acid and the reduced Schiff bases of PLP with L-glutamic acid, GABA, L-aspartic acid, L-cysteinesulfinic acid, taurine and LD-α, γ -diaminobutyric acid were synthesized and characterized essentially as previously described for PLP-aminooxyacetic acid and PLP-α, γ -diaminobutyrate (Possani et al., 1977). $NaBH_4$ was added to incubated mixtures of the amino acid and PLP, and the reduced Schiff bases were purified by gel filtration in a Sephadex G-10 column (2.5x34 cm), as shown in Fig. 1 for glutamate-PLP. The spectra of all the derivatives were similar to spectrum II of inset in Fig. 1. Their ε_{325} ranged from 5.7×10^3 for the cysteinesulfinate derivative to 8.0×10^3 for the α, γ -diaminobutyrate derivative. The main side product was reduced PLP, which was identified as pyridoxine phosphate. This compound absorbs also at 325 nm but is eluted from the column much later than the phosphopyridoxyl derivatives. These spectrophotometric data were confirmed by the radioactivity profile after reduction with $[^3H]$ $NaBH_4$, which showed three peaks, corresponding to the phosphopyridoxyl derivative, reduced PLP, and $[^3H]$ $NaBH_4$ and its oxidation products. As expected, ninhydrin-positive reaction was observed only in the fractions containing lower molecular weight compounds (Fig. 1). Similar elution profiles to those of Fig. 1 were obtained with the other phosphopyridoxyl derivatives. The

insert Fig 1 about here

α, γ-diaminobutyrate derivative gave positive ninhydrin reaction, because of the presence of a second amino group, which indicates that α, γ-diamino butyrate reacted with PLP through only one of its amino groups, probably the γ-amino as judged by the color of the ninhydrin product. However, this was not ascertained.

Besides the spectrophotometric criteria already mentioned, thin layer chromatography showed only one spot for each pure compound, which was fluorescent. For all compounds, except for the diaminobutyrate derivative, the spot was ninhydrin negative. Furthermore, when the phenylhydrazine method of Wada & Snell (1961) was used to check the presence of unreduced Schiff base, it was found that the contamination of the purified derivatives with the unreduced compound was less than 1%.

The criteria enumerated above leave little doubt on the identity of the synthesized compounds. This was further confirmed by elementary analysis for the glutamate, GABA and cysteinesulfinate derivatives, which gave the expected proportions of C, H, and N.

Enzyme Preparation. Adult albino mice (local strain) were used throughout. Brains, without cerebellum, were quickly removed after cervical dislocation and homogenized either in water (10% w/v) or, only for the initial experiment (Table I), in 0.5% Triton-X-100 (20% w/v). The homogenates were centrifuged at 100,000 g for one h, at 0-4°C (Beckman L5-65 centrifuge), and the supernatants were used as source of the enzyme. The enzyme is purified about 2.5 fold by this procedure. A freshly prepared supernatant was used for each set of experiments.

Enzyme assay. GAD activity was determined by measuring the $^{14}CO_2$ released from [1-^{14}C] glutamic acid as previously described (Tapia & Awapara, 1969), with slight modifications. The volume of the enzyme preparation was reduced to 0.2 ml and the total incubation volume to 1 ml. The reaction was started by the addition of the enzyme. 3 N Sulfuric acid was used instead of trichloroacetic acid to stop the reaction and release the $^{14}CO_2$ from the medium. The incubation time was in all cases 20 min. At this time the reaction was still linear under all conditions studied. A blank flask stopped at zero time was included for each substrate concentration in each experiment.

Determination of endogenous PLP. In order to calculate the actual concentration of PLP in the incubation mixtures, its endogenous level in the enzyme preparation was measured by the apotryptophanase method of Wada et al. (1961), as modified by Minard (1967). Similar results were found when the preparation was deproteinized with perchloric acid before the assay and when the supernatant was used directly for the PLP determination.

RESULTS

The effect on GAD activity of the N-(5'-phosphopyridoxyl)-amino acids synthesized is shown in Table 1. All compounds, except the

diaminobutyrate derivative, inhibited GAD activity in supernatants of both water and Triton-X-100 homogenates. The effect of the four most potent PLP-derivatives, namely aminooxyacetate-PLP, GABA-PLP, cysteinesulfinate-PLP and glutamate-PLP, was kinetically studied at broad ranges of glutamate and PLP concentrations. Figure 2 shows Lineweaver-Burk plots at varying glutamate and with saturating PLP concentrations, at several fixed inhibitor concentrations. Secondary slope and intercept replots are shown in the insets. The primary plots indicate that the four PLP derivatives behave as mixed function inhibitors with respect to glutamate. The same type of inhibition pattern was observed when glutamate concentration was increased up to 5-fold with respect to the maximum used in Fig. 2. The secondary plots for the PLP-aminooxyacetate are hyperbolic. For the other derivatives these replots seem to be straight lines at the inhibitor concentration range used.

Double reciprocal plots at varying PLP and fixed saturating glutamate concentrations in the presence of the inhibitors are non linear. The slope of these curves increases with PLP concentration, approaching competitive behavior (Fig. 3).

DISCUSSION

The reduced PLP-amino acid Schiff bases that we have synthesized were designed as structural analogs of the PLP-glutamate Schiff base, its amino acid and PLP moieties resembling the glutamate and PLP molecules, respectively. Since the carbon-nitrogen double bond has been reduced, dissociation of the two components is prevented, making these compounds a powerful tool for studying the glutamate-PLP kinetic relationship in the GAD catalyzed reaction.

The kinetic studies with the four PLP derivatives used show that they are competitive inhibitors with respect to PLP, but display mixed function inhibition with respect to glutamate. This behavior may be interpreted in two ways. A first explanation would be that ordered binding of PLP and glutamate takes place on the enzyme. This explanation is supported by the following observations (see Segel, 1975): The decrease of apparent V_{max} by the inhibitors (Fig. 2) cannot be overcome by saturation with glutamate, indicating that the substrate and the inhibitor combine with different enzyme forms. Besides the effect on V_{max}, the slope of the curves also changes in the presence of the inhibitors (Fig. 2), indicating that the inhibitor binds before glutamate to an enzyme form reversibly connected with the form binding glutamate. Since PLP competes with the inhibitors for the same enzyme

form (there is an effect on the slope but no effect on V_{max}, Fig. 3), it follows that PLP also binds before glutamate. This behavior implicates that the ordered binding occurs in non-rapid equilibrium, since in ordered rapid equilibrium saturating substrate should overcome the effect on V_{max}.

This is the simplest explanation for the primary inhibition pattern shown in Figs. 2 and 3, and we shall elaborate it in the context of the enzyme kinetics in absence of inhibitors (Bayón et al., accompanying paper). Nevertheless, an alternative kinetic model could also account for this pattern. Mixed function inhibition with respect to glutamate by the PLP derivatives, which compete with PLP, could also be explained if an enzyme-inhibitor-glutamate complex could form in addition to the enzyme-inhibitor complex, assuming a rapid equilibrium random bireactant mechanism[1] (Segel, 1975). This is an interesting but unlikely possibility because steric hindrance makes difficult that both glutamate and the PLP-amino acids share the same site of the enzyme, and consequently two separate sites, one for glutamate binding and the other for inhibitor binding (allosteric site), would have to be postulated.

The secondary plots in Fig. 2, together with the curved primary plots in Fig. 3, provide an additional and valuable piece of information. Non linear-hyperbolic slope or intercept replots arise from alternate paths

to product formation, that is, at least two productive enzyme species exist, independently of the kinetic model underlying this behavior (Segel, 1975). Two types of kinetic explanations could account for this inhibition pattern. One of them would require that the velocity equation include terms containing inhibitor concentration in the numerator, in addition to those in the denominator, meaning that at least one enzyme-inhibitor-glutamate complex is formed and that it is an alternate productive species (this could be due to either partial inhibition or alternate activation of the enzyme with the PLP derivatives, Segel, 1975). Nevertheless, this explanation also implicates the existence of an allosteric site for the PLP derivatives, as previously discussed. The second kinetic explanation is that the two productive enzyme species are in fact two different, not interconvertible enzyme activities, one of them resistant to the inhibition produced by the PLP derivatives (Segel, 1975). This interpretation is also in agreement with the fact that increasing inhibitor concentrations cannot decrease the enzyme activity to zero but only to a fixed limit velocity represented by the theoretical asymptotes of both the intercept replot in Fig. 2A, and all the primary curves in Fig. 3 (see also Tapia & Sandoval, 1971). In this regard, it is noteworthy that only when the inhibition values are at least 50%, a hyperbolic shape of replots is observed (Fig. 2, insets).

Since the PLP derivatives approach competitive behavior with respect to PLP, the activity of GAD not inhibited by these compounds should be

independent of free PLP concentration. This possibility of the existence of two enzyme activities, differing in their dependence on free PLP, already proposed by our group (Tapia & Sandoval, 1971), will be further studied and discussed in the subsequent paper (Bayón et al., accompanying paper) on the basis of a kinetic analysis in the absence of inhibitors.

Acknowledgements - The authors are indebted to Dr. T. P. King from The Rockefeller University for the elementary analysis of the compounds.

REFERENCES

Bayón A., Possani L. D. & Tapia R., J. Neurochem., accompanying paper.

Minard F. N. (1967) J. Neurochem. 14, 681-692.

Possani L. D., Bayón A. & Tapia R. (1977) Neurochemical Res. In press.

Roberts E., Wein J. & Simonsen D. G. (1964) Vitamins and Hormones 22, 503-559.

Segel I. H. (1975) Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems 959 pp. John Wiley & Sons, New York (see particularly chapters: 9-DD, 9-F, 6-A, 9-H and 4-C).

Tapia R. (1975) in Handbook of Psychopharmacology (Iversen L. L., Iversen S. D. & Snyder S. H., eds.) Vol. 4, pp. 1-58. Plenum Press, New York.

Tapia R. & Awapara J. (1969) Biochem. Pharmac. 18, 145-152.

Tapia R. & Sandoval M. E. (1971) J. Neurochem. 17, 2051-2059.

Tapia R., Sandoval M. E. & Contreras P. (1975) J. Neurochem. 24, 1283-1285.

Tower D.B. (1976) in GABA In Nervous System Function (Roberts E., Chase T. N. & Tower D. B., eds.) pp. 461-478. Raven Press, New York.

Wada H., Morisue T., Sakamoto Y. & Ichihara K. (1957) J. Vitaminol. 3, 183-188.

Wada H. & Snell E.E. (1961) J. biol. Chem. 236, 2089-2095.

Wu J.-Y. & Roberts E. (1974) J. Neurochem. 23, 759-767.

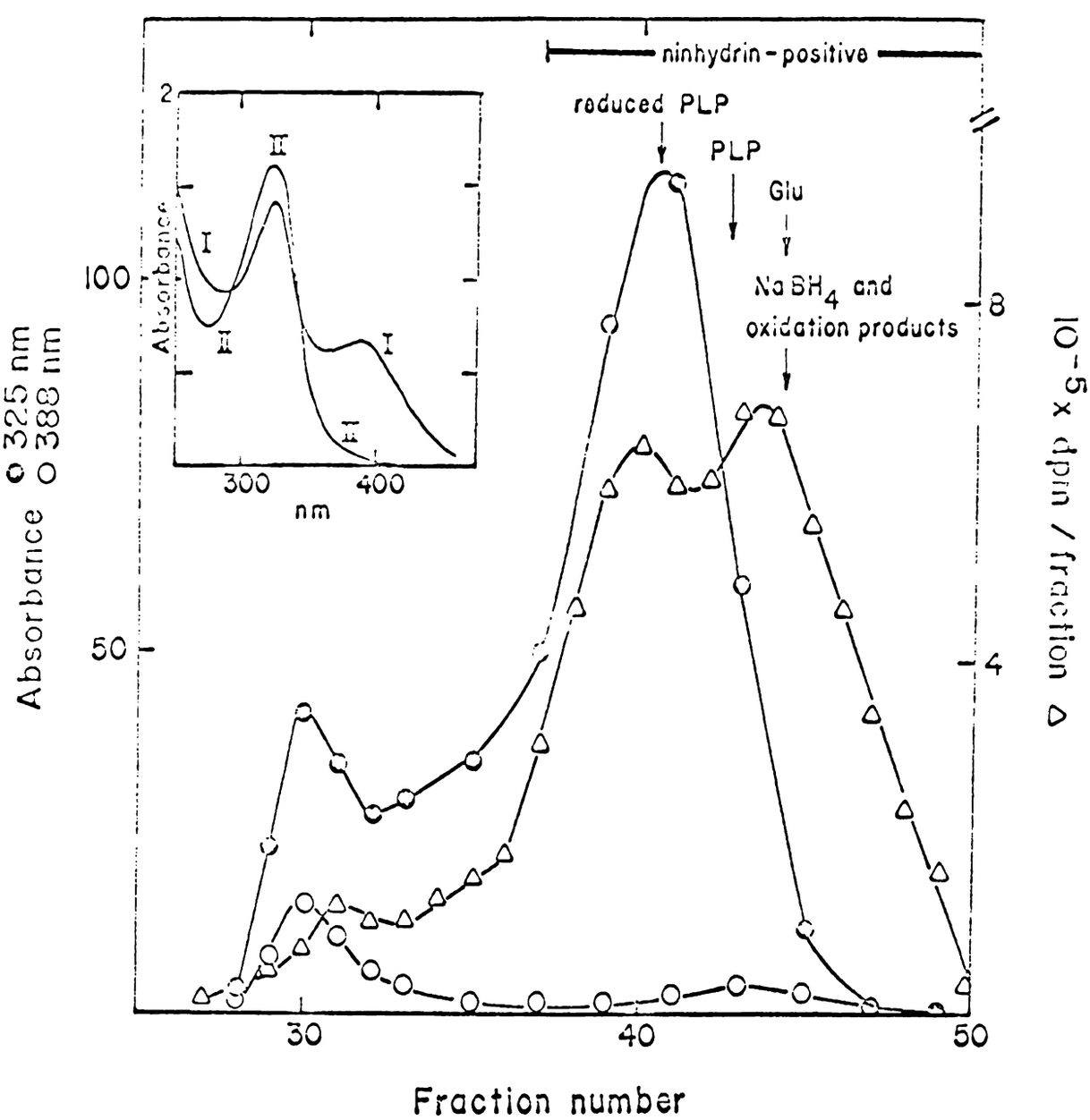
Footnote to the text (page 10):

[1] This alternative possibility arises because kinetic patterns at varying substrates for non-rapid equilibrium ordered bireactant and rapid equilibrium random bireactant mechanisms cannot be distinguished (Segel, 1975). Complete product inhibition studies could help to make the distinction, but in the present case they are precluded because one of the products is the volatile CO_2.

Table 1. Effect of the N-(5'-phosphopyridoxyl) amino acids synthesized on glutamate decarboxylase activity of supernatants of Triton-X-100 and water homogenates of mouse brain

Pyridoxal phosphate derivative (1 mM) (R=5'-phosphopyridoxyl)	% of control values	
	Triton supernatant	Water supernatant
R-Glutamate	75 ± 8	79 ± 4
R-γ-Aminobutyrate	53 ± 0	60 ± 5
R-Aspartate	79 ± 1	92
R-Cysteinesulfinate	63 ± 6	66 ± 5
R-Taurine	85 ± 4	79
R-Aminooxyacetate	34 ± 2	38 ± 3
R-α,γ-Diaminobutyrate	91 ± 8	98 ± 5

Homogenates were prepared and centrifuged at 100,000 g as described in Materials and Methods. Glutamate concentration was 33 mM and PLP concentration was 0.1 mM. The figures are mean values of three to four experiments ± S.D. (only two experiments when no S.D. is shown). Absolute control values were (μmoles/h/g original tissue): Triton supernatant, 65.0; water supernatant, 39.0.


ninhydrin-positive
reduced PLP
PLP
Glu
NaBH4 and oxidation products
Absorbance
325 nm
388 nm
100
50
8
4
10⁻⁵ x dpm / fraction
30
40
50
Fraction number
2
Absorbance
I
II
300
400
nm

s/i
0.5
0.15
0.1
[I]
1/v
INTERCEPT
SLOPE
a
b
c
d

17. C

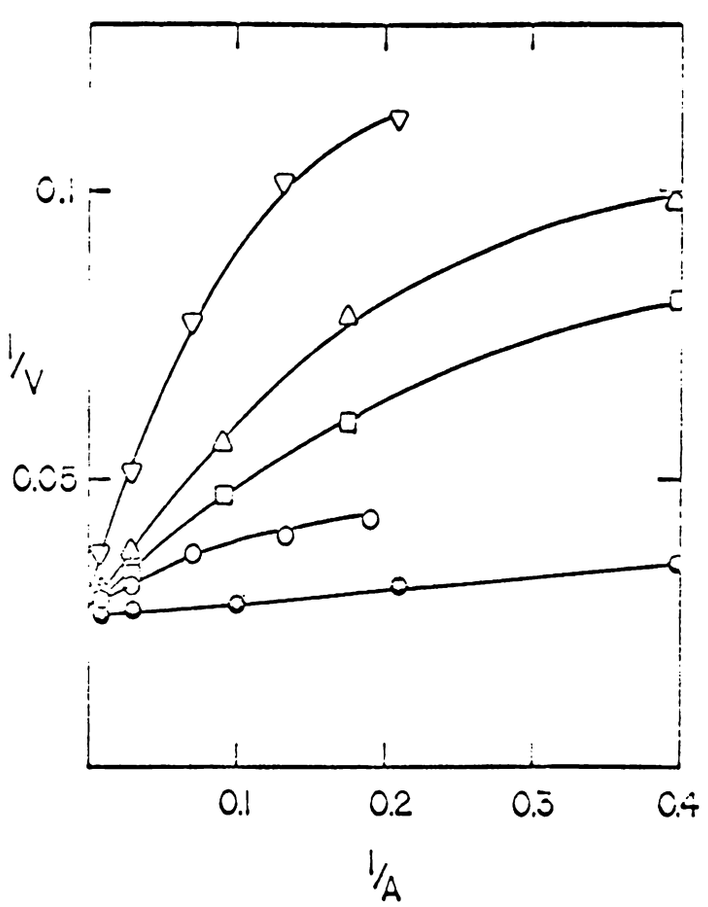


Figure Legends:

Fig. 1. Sephadex G-10 separation of a glutamate-pyridoxal phosphate reaction mixture after partial reduction with (^{3}H) $NaBH_4$. Fractions of 1.6 ml were collected and their spectra, radioactivity and ninhydrin reaction were determined. The elution peaks of the unreacted PLP and glutamate (Glu), as well as those of the reduced PLP and other side products, are indicated by arrows. N-(5'-phosphopyridoxyl) glutamate is eluted in fractions 28-33, but unreduced Schiff base is also present in these fractions, as indicated by the absorbance at 388 nm. Inset shows the spectrum (in 0.2 M potassium phosphate buffer, pH 7.2) of fraction 28 (spectrum I), after 2.5-fold dilution. This fraction was further reduced with $NaBH_4$ and passed again through the column. Fractions containing the reduced derivative were pooled and the spectrum was then obtained without dilution (spectrum II). The disappearance of the peak at 388 nm shows the absence of detectable contaminant unreduced Schiff base.

Fig. 2. Enzyme activity as a function of glutamate concentration in the presence of N-(5'-phosphopyridoxyl)-amino acids; 1/v *vs* 1/S plots. PLP concentration was in all cases 0.1 mM. S is glutamate concentration (mM), v is enzyme activity expressed as μmoles of glutamate decarboxylated/h/g of original tissue. Each point is the mean value of 3-4 independent determinations. The phosphopyridoxyl-amino acids used as inhibitors were: *a*, N-(5'-phosphopyridoxyl)-aminooxyacetate;

b, N-(5'-phosphopyridoxyl)-γ-aminobutyrate; c, N-(5'-phosphopyridoxyl) cysteinesulfinate; d, N-(5'-phosphopyridoxyl) L-glutamate. The derivatives were tested at the following concentrations (mM): 0.00 (○); 0.01 (◐); 0.1 (□); 0.38 (◪); 0.5 (△); 1.0 (▲). Insets are secondary plots of slopes (expressed as 10^2 x S/v) and intercepts (expressed as 10^2 x 1/v) vs inhibitor concentrations (I) (mM).

Fig. 3. Enzyme activity as a function of PLP concentration in the presence of N-(5'-phosphopyridoxyl)-amino acids; 1/v vs 1/A plots. Glutamate concentration was in all cases 33 mM. A is PLP concentration (μM), v is as defined in Fig. 2. Symbols represent the phosphopyridoxyl derivative used (at a 0.1 mM concentration): no derivative (○); N-(5'-phosphopyridoxyl)-aminooxyacetate (▽); N-(5'-phosphopyridoxyl)-γ-aminobutyrate (△); N-(5'-phosphopyridoxyl)-cysteinesulfinate (□); N-(5'-phosphopyridoxyl)-glutamate (○). Each point is the mean value of 3 independent determinations. Endogenous PLP concentration was measured in all cases. This value, as well as that of PLP contaminating the inhibitors (determined as described in Methods) were taken into account for calculating the final PLP concentrations in the assay medium.

KINETICS OF BRAIN GLUTAMATE DECARBOXYLASE. INTERACTIONS WITH GLUTAMATE, PYRIDOXAL 5'-PHOSPHATE AND GLUTAMATE-PYRIDOXAL 5'-PHOSPHATE SCHIFF BASE[1]

Alejandro Bayón[2], Lourival D. Possani, Mauricio Tapia and Ricardo Tapia[3].

Departamento de Biología Experimental, Instituto de Biología, Universidad Nacional Autónoma de México, Apartado Postal 70-600, México 20, D. F. México.

Please send proofs and correspondence to Ricardo Tapia, at the above address.

Abbreviations used: PLP, pyridoxal 5'-phosphate; GAD, glutamate decarboxylase.

[1]This work was supported in part by grant No. 043/76 from the Consejo Nacional de Ciencia y Tecnología, México, D. F.

[2]Recipient of fellowship from the División de Formación del Profesorado, Universidad Nacional Autónoma de México.

[3]To whom correspondence should be sent.

Running title:

Glutamate decarboxylase-substrate-coenzyme interactions.

Abstract - The kinetic behavior of glutamate decarboxylase from mouse brain was analyzed in a wide range of glutamate and pyridoxal 5'-phosphate concentrations, approaching three limit conditions: (I) in the absence of glutamate-pyridoxal phosphate Schiff base; (II) when all glutamate is trapped in the form of Schiff base; (III) when all pyridoxal phosphate is trapped in the form of Schiff base. The experimental results in limit condition (I) are consistent with the existence of two different enzyme activities, one dependent and the other independent on free pyridoxal phosphate. The results obtained in limit conditions (II) and (III) give further support to this postulation. These data show that the free pyridoxal phosphate-dependent activity can be abolished when either all substrate or all cofactor are in the form of Schiff base. The free pyridoxal phosphate-independent activity is also abolished when all substrate is trapped as Schiff base, but it is not affected by the conversion of free pyridoxal phosphate into the Schiff base. A kinetic and mechanistc model for brain glutamate decarboxylase activity, which accounts for these observations as well as for the results of previous dead end inhibition studies, is postulated. Computer simulations of this model, using the experimentally obtained kinetic constants, reproduced all the observed features of the enzyme behavior. The possible implications of the kinetic model for the regulation of the enzyme activity are discussed.

End of abstract. -

GABA is an inhibitory transmitter in neuromuscular junctions of some invertebrates and most probably also in the central nervous system of vertebrates (Krnjević, 1974; Curtis, 1975; Otsuka, 1976). This amino acid is synthesized by α-decarboxylation of L-glutamate, catalyzed by glutamate decarboxylase (L-glutamate-1-carboxylyase, EC 4.1.1.15; GAD). In mammals, GAD is present only in nervous tissue and, in contrast to other vitamin B_6-dependent enzymes, is particularly sensitive to deficiencies of pyridoxal 5'-phosphate (PLP), both *in vitro* (Roberts *et al.*, 1964; Tapia *et al.*, 1969a, 1970) and *in vivo* (Minard, 1967; Tapia *et al.*, 1969b; Pérez de la Mora *et al.*, 1973). This dependence on PLP represents probably a regulatory control of the physiological action of GABA (Pérez de la Mora *et al.*, 1973; Tapia & Pasantes, 1971).

Previous studies from this laboratory (Tapia & Sandoval, 1971) have shown that the oxime N-(5'-phosphopyridoxylene) aminooxyacetic acid, a compound structurally similar to the glutamate-PLP Schiff base, is a selective inhibitor of brain GAD. From the results of inhibition studies with this PLP derivative as compared to those obtained with aminooxyacetic acid, it was postulated that two types of GAD activity are present in brain, one dependent and the other independent on free PLP concentration (Tapia & Sandoval, 1971; see also Bayón *et al.*, accompanying paper).

In the present paper we have analyzed this hypothesis more directly, be means of a kinetic study of the GAD dependence on PLP and glutamate concentrations. Furthermore, this approach led us to study the effects of the formation of the glutamate-PLP Schiff base on GAD activity. The results of these experiments support the above hypothesis and allow us to propose a kinetic model of GAD activity that provides a mechanism for its regulation.

MATERIALS AND METHODS

Chemicals, determination of endogenous PLP, and enzyme preparation and assay, were as described in the preceding paper (Bayón et al., accompanying paper).

For the experiments requiring very low PLP concentrations, an enzyme preparation diluted 10-fold was used. A curve of activity vs enzyme concentration at saturating substrate and PLP was obtained in order to test for possible effects of endogenous inhibitors or activators. Linearity was observed over a wide range of enzyme concentrations (including those used for the kinetic experiments), indicating that no such effects were present. When required, the endogenous PLP levels were taken into account for calculating the PLP concentration in the assay medium.

The dissociation constant of the glutamate-PLP Schiff base (Ko) was determined spectrophotometrically (Pye-Unicam 1800 spectrophotometer with a constant temperature cell holder) under the same experimental conditions of the GAD assay (pH 6.3, 37° C). The absorption of several mixtures of PLP (at fixed 0.1 mM concentration) and glutamate (varied from 16.5 to 82.5 mM) was read at 410 nm. The absorption coefficient of PLP at this wavelength was determined. Knowing this value, and maintaining glutamate concentration in great molar excess with respect to PLP, the following equation can be established:

$$\frac{1}{\Delta A} = \frac{Ko}{\Delta A_{max}} \cdot \frac{1}{S} + \frac{1}{\Delta A_{max}}$$

where S is glutamate concentration; ΔA is the difference in absorption between a given concentration of PLP alone and after the addition of varying amounts of glutamate; and ΔA_{max} is the value of ΔA when all PLP has been converted into Schiff base. From a plot of $1/\Delta A$ versus $1/S$ the value of Ko can be determined.

RESULTS AND DISCUSSION

In order to analyze the GAD dependence on PLP and glutamate concentrations, it was first necessary to determine to what extent the glutamate-PLP Schiff base is formed in the assay medium. In fact, PLP-amino acid Schiff bases are readily formed in solutions (Finseth &

Sizer, 1967), and it has been described that the non-enzymatic formation of substrate-activator complexes may affect enzyme activity (London & Steck, 1969).

For this purpose, the dissociation constant of the Schiff base glutamate-PLP (Ko) was determined as described in Materials and Methods. The value obtained (6.24×10^{-2}M, see Fig. 1, inset) is similar to those reported for other amino acid-PLP Schiff bases (Finseth & Sizer, 1967) and it was used for calculating, with the aid of a computer, the amount of Schiff base formed at varying glutamate and PLP concentrations (Fig. 1). These data thus indicate the glutamate and PLP concentration range at which the Schiff base is significantly formed, and permitted to define three limit conditions for studying the enzyme behavior: (I), when Schiff base concentration is zero; (II), when all glutamate present is in the form of Schiff base; and (III), when all PLP present is in the form of Schiff base.

Limit condition (I). Absence of glutamate-PLP Schiff base. This limit condition is approached when glutamate-PLP Schiff base (SA) concentration approaches zero, and free PLP (A) and free glutamate (S) concentrations approach total PLP (AT) and total glutamate (ST) concentrations, respectively.

In order to study experimentally the enzyme behavior in this limit condition we have measured its activity at glutamate and PLP concentrations

low enough to yield less than 0.1% of Schiff base formed with respect to both total glutamate and total PLP (see Fig. 1). The results of these experiments are shown in Fig. 2. Lineweaver-Burk plots, varying PLP at several fixed glutamate concentrations, are non-linear and approach horizontal asymptotes as PLP concentration is lowered (Fig. 2a). Double reciprocal plots, varying glutamate at several fixed PLP concentrations, yield a family of straight lines (Fig. 2b); the secondary plot of the slopes of these curves *vs* 1/PLP is non linear and approaches an horizontal asymptote at low PLP concentrations (Fig. 2b').

The hyperbolic shape of plots in Figs. 2a and 2b', both approaching horizontal asymptotes and intersecting the ordinate axis above the origin, is consistent with the existence of two different GAD activities, one dependent and the other independent on free PLP concentration. A mathematical demonstration of this conclusion is shown in the Appendix to this paper. In view of previous independent evidence on this point (Tapia and Sandoval, 1971), and since other alternative interpretations, as discussed in the preceding paper (Bayón *et al.*, accompanying paper), are unlikely, the subsequent kinetic analysis of the data was made on the basis of the existence of two separate enzyme activities.

The simplest approach for this analysis is to consider each curve in Fig. 2a as composed by two straight lines: an horizontal asymptote, which represents the reciprocal velocity of the free PLP-independent enzyme

activity, and a positive slope line, corresponding to the contribution of the free PLP-dependent activity. The experimental curves can be resolved into its two components by subtracting the contribution of the free PLP-independent activity (the velocity obtained from the horizontal asymptotes) from the total activity. The differences obtained correspond to the velocities contributed by the PLP-dependent activity, and should give straight lines with positive slope when plotted as Lineweaver-Burk plots, provided that the 1/v values given by the asymptotes are correctly chosen. This procedure provides a trial and error method for estimating the position of both the asymptote (dashed lines in Fig. 2a) and the positive slope component (Fig. 2c) for each curve, allowing the kinetic analysis of each of the two enzyme activities.

Insert Fig. 2 about here

The behavior of the free PLP-independent activity is thus displayed by plotting the intercepts of the estimated horizontal asymptotes (which are the reciprocal limit velocities, V_{lim}, of the hyperbolic experimental curves) vs the reciprocal of the corresponding glutamate concentrations. The resulting plot shows simple Michaelis-Menten unireactant kinetics (Fig. 2a'). The kinetics of the free PLP-dependent activity is shown in Figs. 2c and 2d (Fig. 2d is obtained by replotting the data in Fig. 2c, varying glutamate at fixed PLP concentrations). Their corresponding

secondary plots of slope vs reciprocal glutamate and PLP concentrations are shown in the insets. The observed pattern is consistent with either rapid equilibrium random bireactant kinetics or non-rapid equilibrium ordered bireactant kinetics, but dead end inhibition studies with reduced Schiff base analogs (Bayón et al., accompanying paper) strongly favor the latter possibility.

On the above basis, the velocity equations of the two enzyme activities can be summed in order to obtain the total velocity equation:

$$v_1 = \frac{[S]\ Vmax_1}{Ks_1 + [S]} \tag{4}$$

$$v_2 = \frac{[A]\ [S]\ Vmax_2}{Kia\ \ Ks_2 + [S]\ Ka + [A]\ Ks_2 + [A]\ [S]} \tag{5}$$

$$v_T = v_1 + v_2 \tag{6}$$

where v is initial velocity, (S) and (A) are as previously defined and the subindexes refer to each of the enzyme activities (subindex 1 for the free PLP-independent, subindex 2 for the free PLP-dependent and subindex T for total activity);the kinetic constants follow Cleland's nomenclature (see Segel, 1975) and their values, obtained from the plots in Figs. 2a', 2c and 2d (Segel, 1975) are: $Ks_1 = Ks_2 = 5 \times 10^{-4}$ M; Ka = Kia = 9×10^{-7} M; the ratio $Vmax_2/Vmax_1$ is 2.76.

Although these equations describe essentially the dependence of GAD activity on PLP and glutamate, further information on this point was obtained by studying the enzyme behavior at high PLP and glutamate concentrations. When glutamate and PLP concentrations are such that substantial amounts of Schiff base are formed, the corresponding equilibrium equation (expressed in the form of equations (1) to (3) in the legend to Fig. 1) must be considered in addition to the velocity equations (4) to (6). As already mentioned, in order to study the enzyme behavior in the presence of Schiff base effects, limit conditions (II) and (III) have to be established.

Limit condition (II). All glutamate is in the form of Schiff base. This limit condition is defined by $(S) = 0$ and $(SA) = (ST)$ and it is experimentally approached when the $(SA) / (ST)$ ratio approximates to one, as (AT) is increased at fixed (ST) (see Fig. 1). If this condition is assumed in equations (1) to (6), the total velocity equation becomes $v_T(\text{lim II}) = 0$. Fig. 3a shows the experimental velocity curves obtained by increasing (AT) at several fixed (ST). As (AT) increases all curves reach a peak velocity and then bend downwards. The shift to the right of the peak velocities with increasing fixed (ST) is due to the difficulty for trapping a substantial fraction of (ST) in (SA), when (ST) is high (see Fig. 1). As a consequence of this fact, and because of the limited solubility of PLP, the predicted limit velocity (zero) was attained only at low (ST) (in the order of 10^{-4} M) when (AT) was increased to 0.5 M (these data were obtained in three independent experiments, but they are not shown

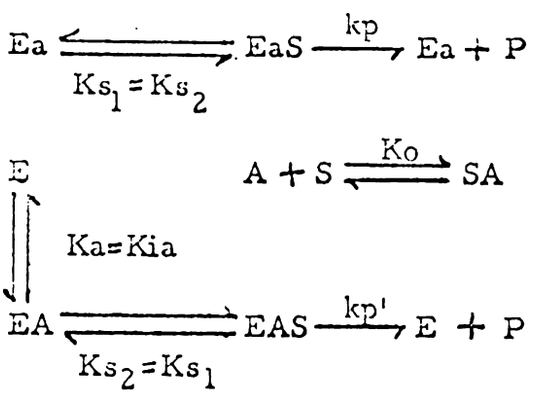
in Fig. 3a). When velocity curves were obtained by increasing (ST) at several fixed (AT), the decrease of activity at high (AT) is reflected in a shift to the right of the velocity curves when fixed (AT) is high enough to trap a significant fraction of (ST) in (SA) (Fig. 3b and 3c).

Limit condition (III). All PLP is in the form of Schiff base. This limit condition is defined by $(A) = 0$ and $(SA) = (AT)$ and, analogously to limit condition (II), it is experimentally approached when the $(SA) / (AT)$ ratio approximates to one as (ST) is increased at fixed (AT) (see Fig. 1). If condition (III) is assumed in equations (1) to (6), the total velocity equation becomes v_T (lim III) $= v_1$. When the (ST) concentrations necessary to attain this condition are well above the K_s value (as in the present case, see Fig. 1), then v_T (lim III) $= Vmax_1$ at any (AT). Figs. 3b and 3c show the experimental velocity curves obtained by increasing (ST) at several fixed (AT). The decrease of activity observed, approaching the limit velocity at increasing (ST), can only be observed at low (AT) for reasons analogous to those discussed for limit condition (II). This behavior is reflected in the curves of Fig. 3a as a lower activity of the initial points of curves VI-VII as compared to the initial points of curve V. Although the limit velocity could not be experimentally attained, its value can be estimated from the ordinate

of the asymptote to curve 1 in Fig. 3b. A reasonable estimate for this value is the one shown as a dashed line in the same figure. Since this asymptote represents $Vmax_1$, its difference with respect to the maximal attained velocity (in Fig. 3c) will represent $Vmax_2$. The calculated ratio $Vmax_2/Vmax_1$ is 2.88, which is in excellent agreement with those obtained from inhibition studies, 2.73 (calculated from Fig. 4 in Tapia & Sandoval, 1971) and from the analysis of limit condition (I), 2.76 (see above). These results give further support to the kinetic model we propose.

Computer simulation of GAD behavior: The kinetic model represented by equations (1) to (6) accounts for all the experimental results of both the present and the preceding papers. Fig. 4a shows a computer simulation of the behavior of the model. The experimentally determined constants were used, and the substrate and cofactor curves shown cover the concentration ranges experimentally assayed. When compared with the curves in Figs. 3b and 3c (and indirectly with 3a), the simulated curves show the adequacy of the model. Another advantage of this simulation is its capability to resolve theoretically the enzyme behavior into its two components and to show graphically their properties, implicit in equations (1) to (6). When v_1 is zero, only the free PLP-dependent activity is simulated, showing that it is inhibited at high PLP or glutamate concentrations (Fig. 4b). On the other hand, if v_2 is zero, the free PLP-independent activity is displayed (Fig. 4c), and it can be seen that it is inhibited only by high PLP concentrations.

Kinetic and mechanistic model of GAD activity. In spite of considerable research carried out in order to explore GAD properties (Roberts & Simonsen, 1963, Tapia et al., 1970; Wu & Roberts, 1974; Miller & Martin, 1976), no kinetic model of the GAD catalyzed reaction was available. On the basis of the results discussed in the preceding sections, and in the light of inhibition studies (Bayón et al., accompanying paper; Tapia & Sandoval, 1971), we postulate the following kinetic model, which accounts for equations (1) to (6).



All symbols have been defined in the above sections, except: P, reaction products; kp and kp', forward rate constants for product formation of the free PLP-independent and free PLP-dependent enzyme activities, respectively; Ea and E, the catalytic sites responsible for the free PLP-independent and the free PLP-dependent enzyme activities, respectively. Since PLP is a necessary cofactor for the GAD catalyzed reaction, it has to be postulated that the free PLP-independent catalytic site possesses tightly bound PLP (Tapia & Sandoval, 1971), which is represented by index 'a' in Ea. Other implications of the model, which are in

full agreement with the experimental findings, are the following:

(1) The binding of the glutamate-PLP Schiff base to the free PLP-independent catalytic site would be precluded because of the presence of tightly bound PLP. As a consequence, the trapping of glutamate in the Schiff base at high PLP concentrations would result in a decrease of GAD activity, but the trapping of PLP at high glutamate concentrations would not affect the activity. Furthermore, the PLP-amino acids used in the preceding paper (Bayón et al., accompanying paper) would not inhibit this activity.

(2) The observed inhibition of free PLP-dependent activity at high substrate or cofactor concentrations is a consecuence of the non-enzymatic formation of the glutamate-PLP Schiff base. The kinetic reasons for this type of inhibitions have been amply analyzed and discussed by London & Steck (1969), but steric factors could also account for these effects. It is possible that the Schiff base connot enter the free PLP-dependent catalytic site, and therefore the inhibition would be due to the removal of free cofactor or of substrate because of their trapping in the form of Schiff base. That the Schiff base cannot bind to the enzyme is suggested by the ordered nature of the kinetics of the free PLP-dependent activity. In this regard it is noteworthy that, among the reduced PLP-derivatives studied kinetically in the preceding paper (Bayón et al., accompanying paper),

N-(5'-phosphopyridoxyl) glutamate has apparently the lowest affinity for the enzyme, in spite of being the closest structural analog to the glutamate-PLP Schiff base.

(3) The kinetic model implies that the two types of GAD activity are independent and cannot be interconverted by changing substrate or cofactor concentrations, but the model gives no information on the physical relations between the two activities. At least three possibilities can be envisaged: the two catalytic sites responsible for them might be located in the same enzyme molecule, in different enzyme subunits or in different enzyme molecules. Because of the latter possibility, a relatively crude enzyme preparation was used in these studies, but we emphasize that the possible effect of endogenous inhibitors or activators was excluded in control experiments (see Materials and Methods). Fractionation and purification studies in order to elucidate the relations between the two activities are presently in progress (Possani et al., 1977).

The available evidence from experiments in vivo indicates that under physiological conditions the level of PLP in brain determines GAD activity, both in enzyme preparations from whole brain (Minard, 1967; Tapia et al., 1969b; Tapia & Pasantes, 1971) and in isolated nerve endings (Pérez de la Mora et al., 1973). The properties of the kinetic model postulated in the present paper suggest that only a fraction of the enzyme activity is susceptible for regulation by PLP concentration in vivo, the other providing a basal

minimum rate of GABA synthesis.

The non enzymatic formation of PLP-glutamate Schiff base may also represent a control mechanism of GAD activity *in vivo*, since the trapping of PLP would result in a decrease of the free PLP-dependent activity. Furthermore, the formation of Schiff bases of PLP with other amino acids present in brain, such as cysteine sulfinate, taurine, aspartate and GABA itself (Bayón *et al.*, accompanying paper), might also contribute to this regulatory mechanism. Other amino compounds present in brain could also participate in this regulation by trapping PLP. For example, it has been reported that norepinephrine and ATP inhibit brain GAD only when added free PLP is present (Haber, 1973; Miller & Martin 1976). Interestingly, norepinephrine and dopamine, putative central neurotransmitters, have been shown to form complexes with PLP, non enzymatically (Neary *et al.*, 1972).

Acknowledgements - The authors wish to thank Mr. Rolando Lara for valuable discussions and Mr. Gustavo Rodc for excellent technical assistance.

Appendix.- Although the analysis of independent enzyme activities coexisting in a preparation is commonly carried out by solving the experimental curves in their components by iterative trial and error methods (Segel, 1975), in the present case a more direct approach can be used. Two separate enzyme activities, one dependent and the other independent on free PLP concentration, are described by the velocity equations (4) to (6) in the paper. When $Ks_1 = Ks_2$ and Kia = Ka (as it is in the present case, see text), and defining $Vmax_2/Vmax_1 = \gamma$, the following equations can be written:

Substituting eq. (4) and (5) in (6) and rearranging:

$$v_T = \frac{Vmax_1 \frac{[S]}{Ks} \cdot \left(1 + \frac{[A]}{Ka} + \frac{\gamma [A]}{Ka}\right)}{\left(1 + \frac{[S]}{Ks}\right)\left(1 + \frac{[A]}{Ka}\right)} \qquad (7)$$

Rearranging for S and obtaining the reciprocal:

$$\frac{1}{v_T} = \frac{Ks\left(1 + \frac{[A]}{Ka}\right)}{Vmax_1\left(1 + \frac{[A]}{Ka} + \frac{\gamma [A]}{Ka}\right)} \cdot \frac{1}{[S]} + \frac{1}{Vmax_1} \cdot \frac{\left(1 + \frac{[A]}{Ka}\right)}{\left(1 + \frac{[A]}{Ka} + \frac{\gamma [A]}{Ka}\right)} \qquad (8)$$

This equation describes a family of straight lines intersecting on the horizontal axis (see Fig. 2b). It may be shown (Segel, 1975) that slope and intercept replots vs $1/\Delta A$ are hyperbola intersecting the ordinate above the origin (see Fig. 2b').

When eq. (7) is rearranged for A and the reciprocal is obtained, we have:

$$\frac{1}{v_T} = \frac{Ka \quad \left(1 + \frac{[S]}{Ks}\right)}{Vmax_1 \quad \frac{[S]}{Ks}\left(1 + \gamma + \frac{Ka}{[A]}\right)} \cdot \frac{1}{[A]} + \frac{1}{Vmax_1} \cdot \frac{\left(1 + \frac{[S]}{Ks}\right)}{\frac{[S]}{Ks}\left(1 + \gamma + \frac{Ka}{[A]}\right)} \qquad (9)$$

When A equals infinite:

$$\frac{1}{v_T\,[A]=\infty} = \frac{1}{Vmax_1} \cdot \frac{\left(1 + \frac{[S]}{Ks}\right)}{\frac{[S]}{Ks}\,(1 + \gamma)} \qquad (10)$$

Defining:

$$\Delta\left(\frac{1}{v_T}\right) = \frac{1}{v_T} - \frac{1}{v_{T\,[A]=\infty}} \qquad (11)$$

Substituting eq. (9) and (10) in (11), simplifying terms and rearranging:

$$\frac{1}{\Delta\left(\frac{1}{v_T}\right)} = \frac{Vmax_1}{Ka} \cdot \frac{(1+\gamma)^2}{\gamma} \cdot \frac{([S]/Ks)}{\left(1 + \frac{[S]}{Ks}\right)} \cdot [A] + \frac{Vmax_1}{\gamma} \cdot (1+\gamma) \cdot \frac{([S]/Ks)}{\left(1 + \frac{[S]}{Ks}\right)} \qquad (12)$$

This equation represents a family of straight lines in $1/\Delta\left(\frac{1}{v_T}\right)$ vs [A] plots, and demonstrates that $1/v_T$ vs $1/[A]$ curves (eq. 9) are rectangular hyperbola intersecting the ordinate above the origin (see Figs. 2a). Their horizontal asymptotes may be calculated from the $1/v_T$ values when [A] is zero. Although [A] = 0 gives an indetermination in eq. (9), in eq. (7) it gives:

$$v_T = \frac{Vmax_1 \ [S]/Ks}{1 + \frac{[S]}{K_s}} \qquad (13)$$

or:

$$\frac{1}{v_T} = \frac{Ks}{Vmax_1} \cdot \frac{1}{[S]} + \frac{1}{Vmax_1} \qquad (14)$$

In equation (14) the $1/v_T$ values are the ordinates of the horizontal asymptotes to the hyperbola in $1/v_T$ vs $1/[A]$ plots of equation (9), at different [S], and it is also the velocity equation of the free PLP-independent enzyme activity (see Fig. 2a').

REFERENCES

Bayón A., Possani L. D. & Tapia R., J. Neurochem., accompanying paper.

Curtis D. R. (1975) in Metabolic Compartmentation and Neurotransmission (Berl S., Clarke D. D. & Schneider D., eds.) pp. 11-36. Plenum Press, New York.

Finseth F. & Sizer I. W. (1967) Biochem. biophys. Res. Commun. 26, 625-630

Haber B. (1973) Texas Rep. Biol. Med. 31, 311-319.

Krnjević K. (1974) Physiol. Rev. 54, 418-540.

London W. P. & Steck T. L. (1969) Biochemistry 8, 1767-1779.

Miller L. P. & Martin D. L. (1976) Life Sci. 19, 281-288.

Minard F. N. (1967) J. Neurochem. 14, 681-692.

Neary J. T., Meneely R. L., Grever M. R. & Diven W. F. (1972) Arch. Biochem. Biophys. 151, 42-47.

Otsuka M. (1976) in GABA in Nervous System Function (Roberts E., Chase T. N. & Tower D. B., eds.) pp. 245-249. Raven Press, New York.

Pérez de la Mora M., Feria-Velasco A. & Tapia R. (1973) J. Neurochem. 20, 1575-1587.

Possani L. D., Bayón A. & Tapia R. (1977) Neurochemical Res. In press.

Roberts E. & Simonsen D. G. (1963) Biochem. Pharmac. 12, 113-134.

Roberts E., Wein J. & Simonsen D. G. (1964) Vitamins and Hormones 22, 503-559.

Segel I. H. (1975) Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems 959 pp. John Wiley & Sons, New York (see particularly chapters: 2-L, 4-C and 9-C).

Tapia R., Pérez de la Mora M. & Massieu G. H. (1969a) Biochem. Pharmac. 16, 1211-1218.

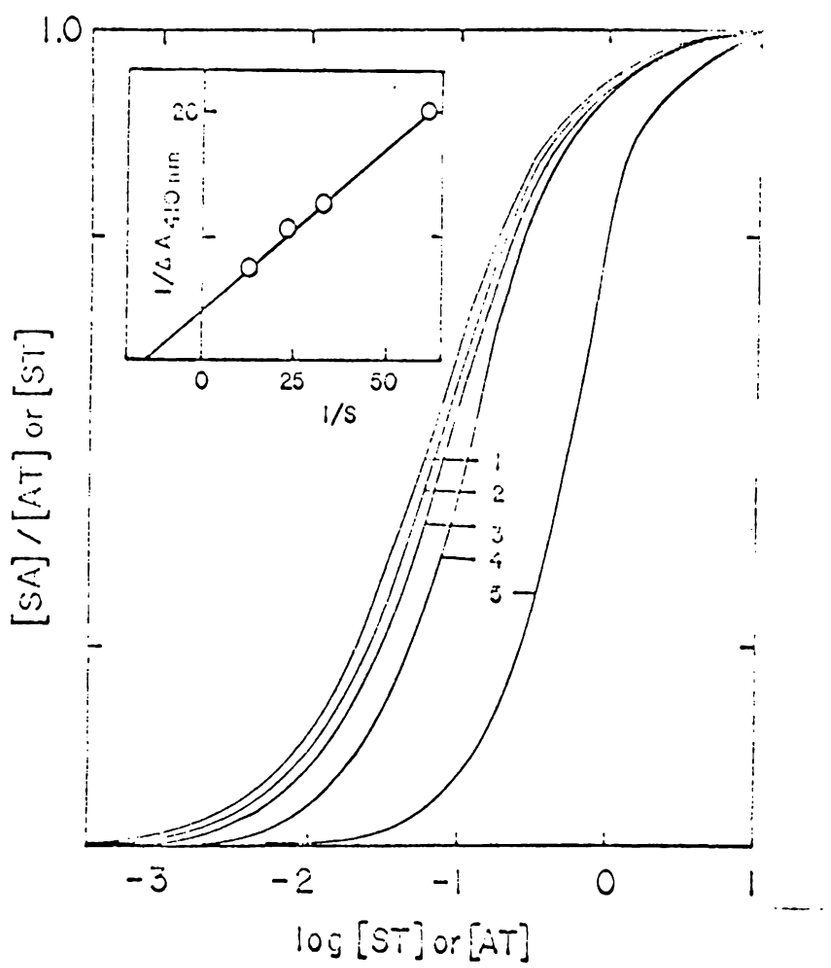
Tapia R., Pérez de la Mora M. & Massieu G. H. (1969b) Ann. N. Y. Acad. Sci. 166, 257-266.

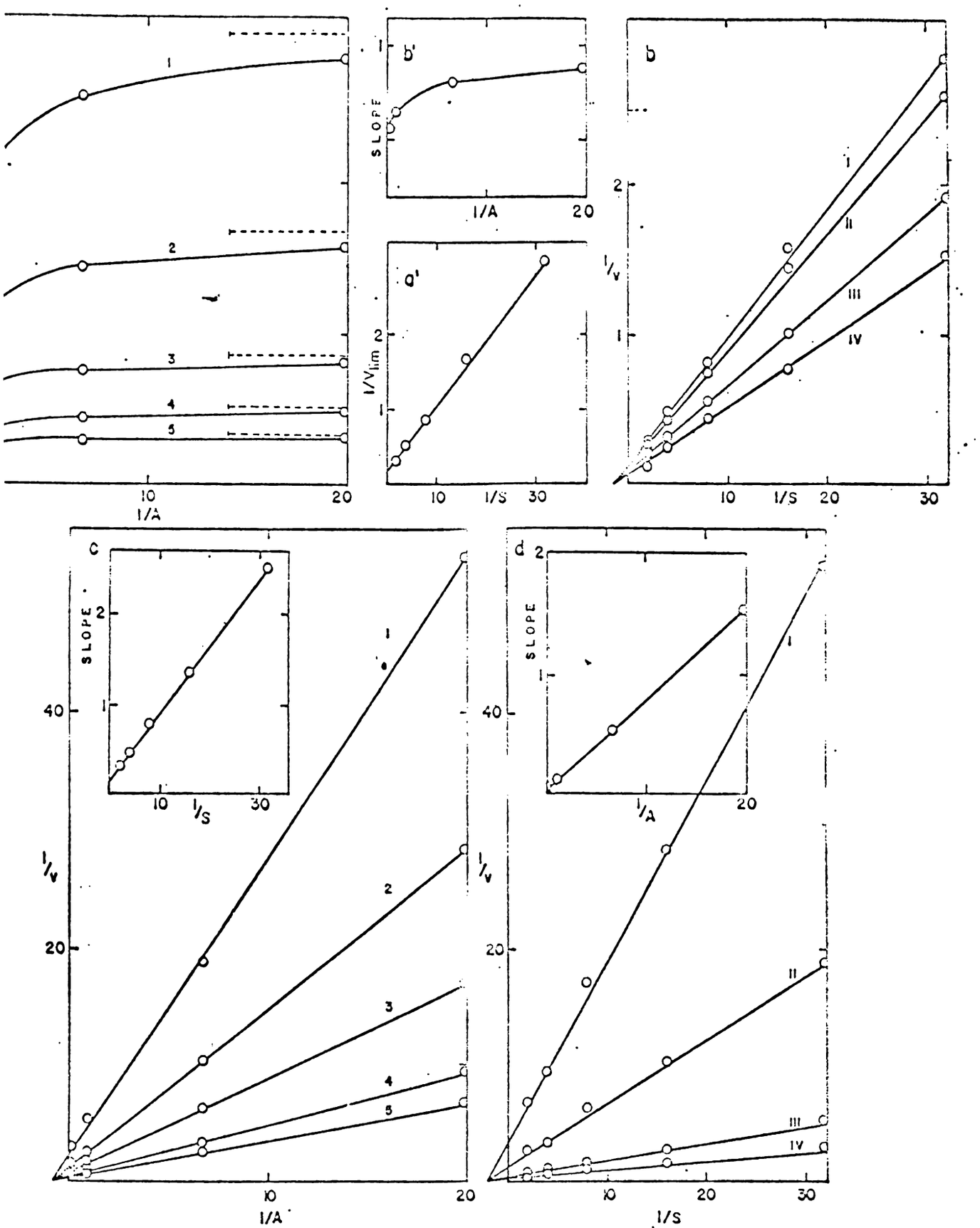
Tapia R., Pasantes H. & Massieu G. H. (1970) J. Neurochem. 17, 921-925.

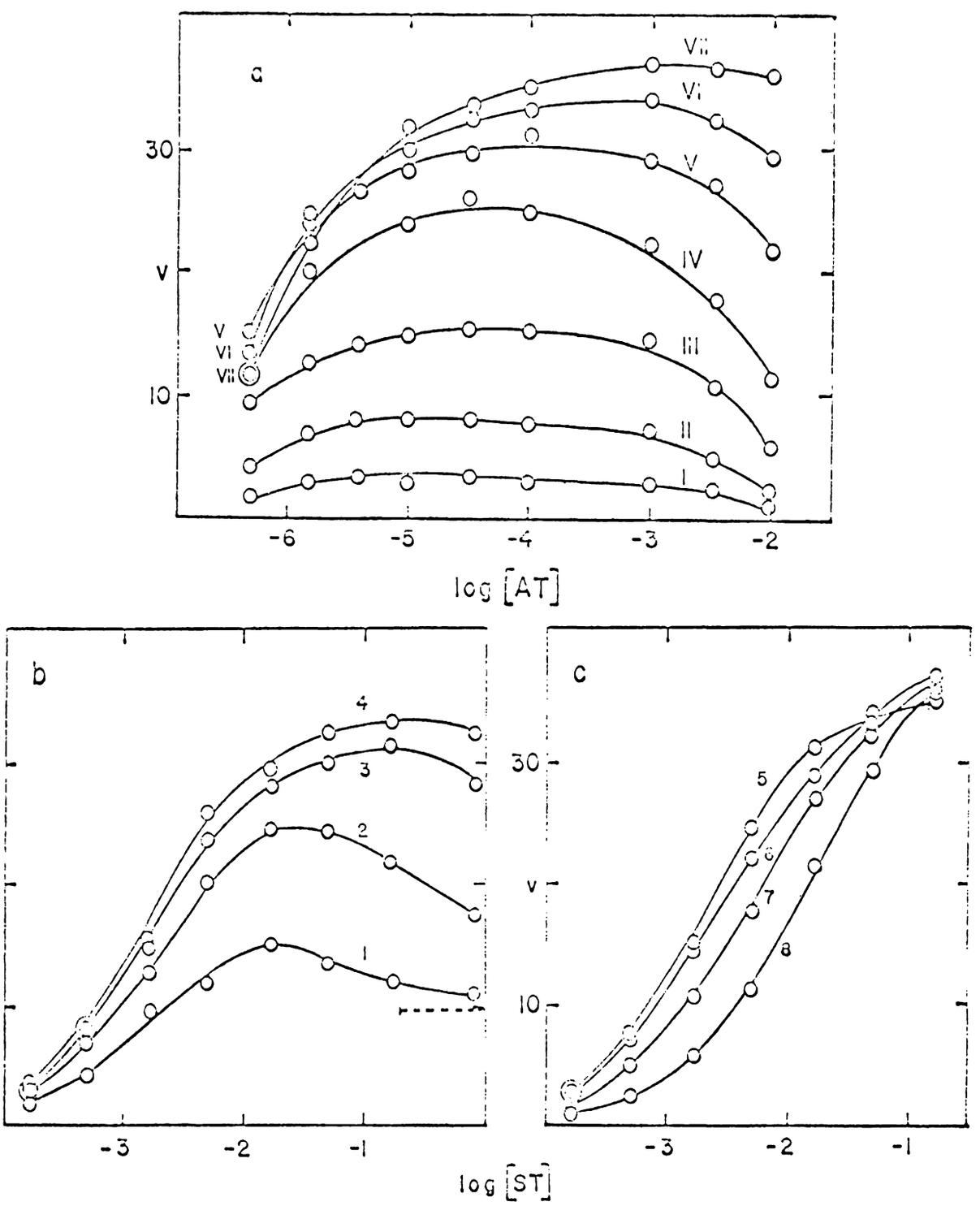
Tapia R. & Pasantes H. (1971) Brain Res. 29, 111-122.

Tapia R. & Sandoval M. E. (1971) J. Neurochem. 18, 2051-2059.

Wu J.-Y. & Roberts E. (1974) J. Neurochem. 23, 759-767.


1.0
[SA] / [AT] or [ST]
-3
-2
-1
0
1
log [ST] or [AT]
1
2
3
4
5
1/Δ A 410 nm
20
1
0
25
50
1/S


a
1
2
3
4
5
10
20
1/A
b'
SLOPE
1/A
20
a'
1/V_lim
2
1
10
1/S
30
b
1
II
III
IV
1/v
2
1
10
1/S
20
30
c
SLOPE
2
1
10
1/S
30
40
20
1/v
1
2
3
4
5
10
20
1/A
d
SLOPE
2
1
1/A
20
40
20
1/v
1
II
III
IV
10
20
30
1/S


a
VII
VI
V
IV
III
II
I
30
v
10
-6
-5
-4
-3
-2
log [AT]
b
4
3
2
1
-3
-2
-1
c
5
6
7
8
30
v
10
-3
-2
-1
log [ST]

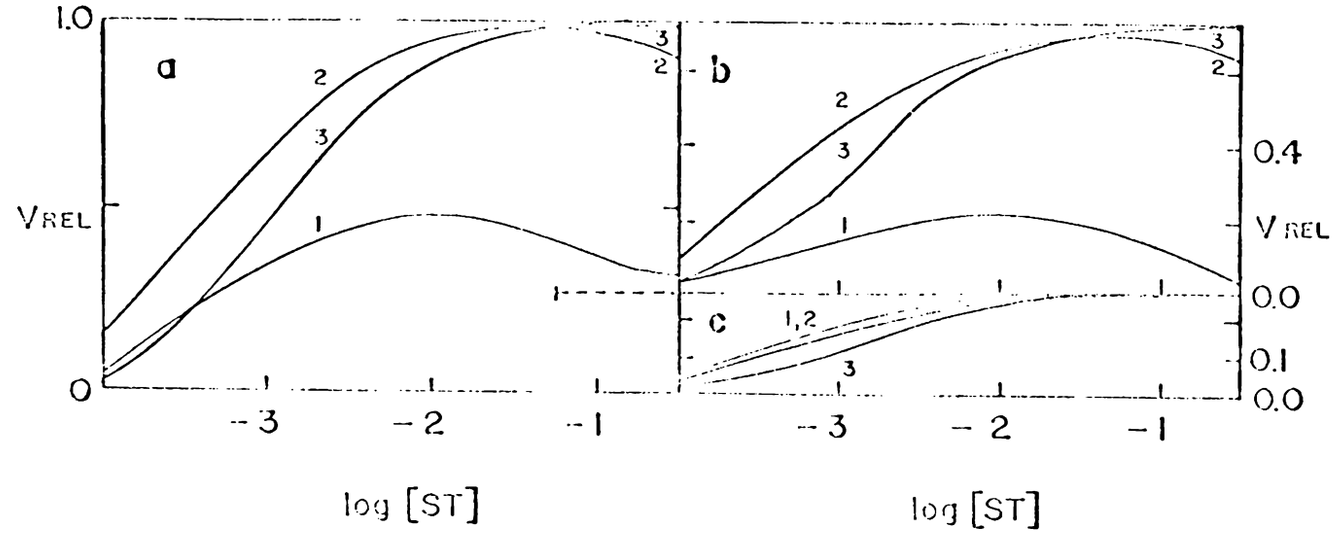

1.0
a
2
3
3
2
VREL
1
0
-3
-2
-1
log [ST]
b
2
3
3
2
0.4
VREL
1
0.0
c
1,2
1
3
0.1
0.0
-3
-2
-1
log [ST]

Figure legends:

Fig. 1. Glutamate-PLP Schiff base formation. Inset, determination of its dissociation constant (Ko). ΔA_{410} is defined in Methods and is expressed in absorbance units; S is glutamate concentration (M). The Ko value obtained from this plot (6.24×10^{-2} M) was used to obtain the curves of Schiff base formation at varying glutamate and PLP concentrations (main figure). Total glutamate and PLP concentrations are defined as: $[ST] = [S] + [SA]$ (equation 1) and $[AT] = [A] + [SA]$ (eq. 2), respectively, where $[A]$ = free PLP, $[S]$=free glutamate, and $[SA]$ = glutamate-PLP Schiff base. Since $Ko = [S][A]/[SA]$, Schiff base concentration was calculated from the quadratic equation:

$$[SA] = \frac{Ko + [AT] + [ST] \pm \sqrt{(Ko + [AT] + [ST])^2 - 4[AT][ST]}}{2} \quad \text{(eq.3)}$$

which resulted from substituting equations (1) and (2) in the equilibrium equations (since $[SA]$ cannot exceed $[ST]$ or $[AT]$, the larger positive root was not used). A FORTRAN IV program in a Burroughs 6700 computer (Centro de Servicios de Cómputo, UNAM) was used for calculating the values of $[SA]$. Results are expressed as the molar fraction of Schiff base of the total concentration of the fixed reactant, vs total molar concentration of the varied reactant (eq. (3) is symetrical for $[ST]$ and $[AT]$). Curves (1) to (5) result from different total concentrations of the fixed reactant: (1), up to 10^{-3} M; (2), 10^{-2} M; (3), $3.3. \times 10^{-2}$ M; (4), 10^{-1} M and (5), 1 M.

Fig. 2. Enzyme behavior in the absence of Schiff base. In all coordinates A is PLP μM concentration; S is glutamate mM concentration; v is enzyme activity expressed as μmoles of GABA/h/g of original tissue; and slopes are expressed in the corresponding v/S or v/A units. a shows 1/v vs 1/A plots at several fixed S concentrations (M): (1), 3.125×10^{-5}; (2), 6.25×10^{-5}; (3), 1.25×10^{-4}; (4), 2.5×10^{-4}; (5), 5×10^{-4}. The estimated asymptotes to each curve are in dashed lines and their ordinates were replotted in a' as 1/Vlim vs their corresponding 1/S values (see text). b shows the same data as in a, replotted as 1/v vs 1/S at several fixed A concentrations (M): (I), 5×10^{-8}; (II), 1.5×10^{-7}; (III), 1.05×10^{-6}; (IV), 10^{-5}. The slopes are plotted vs 1/A in b'. Data in c and d were obtained by subtraction of the velocities contributed by the PLP-independent activity (asymptotes) from the total observed velocities, as described in the text. The numbers on each curve represent fixed substrate and cofactor concentrations, as indicated above. The secondary slope replots are shown in the insets to c and d. The experimental data in a and b were obtained using an enzyme preparation diluted 10-fold in order to lower the endogenous level of PLP in the assay medium, but glutamate specific activity was increased 100-fold for increasing the sensitivity of the method. Each point is the mean value of 2 independent experiments.

Fig. 3. Enzyme behavior in the presence of glutamate-PLP Schiff base. v is defined in the legend to Fig. 2. AT and ST are expressed in molar concentrations. a shows v vs log [AT] curves, at several fixed [ST] (M): (I), 1.65×10^{-4}; (II), 4.95×10^{-4}; (III), 1.65×10^{-3}; (IV), 4.95×10^{-3}; (V), 1.65×10^{-2}; (VI), 4.95×10^{-2}; (VII), 1.65×10^{-1}. b and c show v vs log [ST] curves at several fixed [AT] (M): (1), 5×10^{-7}; (2), 1.5×10^{-6}; (3), 1.05×10^{-5}; (4) 3.38×10^{-5}, (5), 10^{-4}; (6), 10^{-3}, (7), 3.33×10^{-3}; (8), 10^{-2}. An estimation of the asymptote to curve 1 is the dashed line (see text). Each point represents the mean value of 3-4 independent experiments.

Fig. 4. Computer simulation of the kinetic model of GAD activity. A FORTRAN IV program was designed in order to calculate the theoretical relative velocities of the enzyme reaction (defined as $Vrel = v_T/Vmax_T$, where v_T is as previously defined and $Vmax_T = Vmax_1 + Vmax_2$) at varying [ST] and [AT], using equations (1) to (6) (legend to Fig. 1 and text) and the experimentally determined kinetic constants. Vrel vs log [ST] curves were plotted at three [AT] (M): (1), 5×10^{-7}; (2), $3.3. \times 10^{-5}$; (3), 10^{-1}. In a the whole model is displayed ($v_T = v_1 + v_2$). In b the free PLP-dependent component is obtained by making $v_2 = 0$, and consequently $v_T = v_1$. In c the free PLP-independent activity is displayed making $v_1 = 0$, and consequently $v_T = v_2$.