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A mi familia

Mi agradecimiento a todas las personas que han laborado conmigo, cada una de ellas me ha enseñado algo valioso en la vida.

FOSFATO DE PIRIDOXAL Y GLUTAMATO DESCARBOXILASA EN PARTICULAS SUBCELULARES DE CEREBRO DE RATON Y SU RELACION CON CONVULSIONES.

MANUSCRITO QUE PRESENTA EL MEDICO CIRUJANO MIGUEL ANGEL PEREZ DE LA MORA COMO REQUISITO PARA OBTENER EL GRADO DE DOCTOR EN BIOQUIMICA

Se adjuntan sobretiros de otros dos trabajos previamente publicados por el candidato al grado, uno de los cuales llena el requisito, pedido por el reglamento del Doctorado en Bioquímica, de haber publicado un trabajo como primer autor en una revista Internacional.

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PYRIDOXAL PHOSPHATE AND GLUTAMATE DECARBOXYLASE IN SUBCELLULAR PARTICLES OF MOUSE BRAIN AND THEIR RELATIONSHIP TO CONVULSIONS.

ABREVIATURAS EMPLEADAS:

AchE	Acetylcholine Esterase.
CNS	Central Nervous System.
GABA	γ-Aminobutyric acid.
GAD	Glutamic acid Decarboxylase.
PLP	Pyridoxal phosphate.
PLPGH	Pyridoxal phosphate-γ-glutamyl hydrazone.

RESUMEN

En el presente trabajo se estudió la distribución subcelular del fosfato de piridoxal (PLP) en el cerebro del ratón, así como el efecto de la γ-glutamil-hidrazona del fosfato de piridoxal (PLPGH)—una droga convulsivante que disminuye los niveles de PLP y la actividad de la descarboxilasa glutámica (GAD) en el cerebro completo- tanto sobre los niveles de PLP como sobre la actividad de la GAD en partículas subcelulares de cerebro de ratón. Los principales hallazgos fueron los siguientes:

1) el PLP se encontró localizado preferentemente en el sobrenadante y en la fracción mitocondrial cruda. Dos terceras partes de la cantidad presente en la última fracción fueron encontradas en la subfracción que contiene mitocondrias puras, y el resto en la fracción sinaptosomal. Después de romper el sinaptosoma mediante choque osmótico, el PLP se encontró tanto en las mitocondrias intrasinaptosomales como en el axoplasma. 2) El tratamiento con PLPGH disminuyó los niveles de PLP en varias fracciones, siendo este efecto mucho más notable en las fracciones solubles que en las particuladas. Después del rompimiento osmótico de los sinaptosomas se observó que el PLP disminuye específicamente en el exoplasma. 3) La administración de PLPGH produjo también una inhibición de la actividad de la GAD -cuando la actividad de enzima fue medida en ausencia de PLP en el medio de incubación- en la mayoría de las fracciones estudiadas. En general, esta inhibición fue mayor en aquellas fracciones en las cuales los niveles de PLP fueron más afectados. En los sinaptosomas, esta correlación entre la disminución de los niveles de PLP y la inhibición de la GAD ocurrió única-

mente en el axoplasma. 4) La activación de la GAD por PLP exógeno fue mucho mayor en aquellas fracciones de los animales tratados con PLPGH que exhibieron una inhibición importante de la GAD, que en las fracciones correspondientes de los animales control. 5) No se observaron cambios ultraestructurales en ninguna de las partículas subcelulares de los animales tratados, en comparación con sus controles correspondientes.

Estos resultados indican que la disminución de la concentración de PLP y de la actividad de la GAD, previamente observada en el cerebro completo, ciertamente tiene lugar en el sinaptosoma, y por lo tanto apoyan la hipótesis de que el papel del PLP en los mecanismos que controlan la excitabilidad neuronal puede deberse, al menos en parte, a su acción regulatoria sobre la actividad de la GAD, la cual a su vez determina la velocidad de síntesis del ácido γ-aminobutírico en las terminaciones nerviosas.

Abstract.- The subcellular distribution of pyridoxal phosphate (PLP) in mouse brain, as well as the effect of pyridoxal phosphate-γ-glutamyl hydrazone (PLPGH) -a convulsant drug which decreases both PLP levels and glutamate decarboxylase (GAD) in whole brain- upon both PLP concentration and GAD activity in subcellular fractions, was studied. An electron microscopic evaluation of the subcellular particles of control and PLPGH-treated animals was also carried out. The main findings were the following:
1) PLP was localized mainly in the supernatant and crude mitochondrial fraction; two thirds of the amount present in the latter were located in the subfraction containing pure mitochondria, and the remainder in the synaptosomal fraction. After osmotic disruption of synaptosomes, PLP was found in both the intrasynaptosomal mitochondria and the axoplasm. 2) PLPGH treatment decreased PLP levels in several fractions, being this effect much more notable in the soluble fractions than in the particulate ones. After osmotic disruption of the synaptosomes, a specific decrease of PLP in the axoplasm was observed. PLPGH produced also an inhibition of GAD activity -when this enzyme was assayed in the absence of PLP- in most of the fractions studied. In general, this inhibition was greater in those fractions in which PLP levels were more affected. In synaptosomes, this correlation between the decrease of PLP levels and that of GAD activity occurred only in the axoplasm. The activation of GAD by exogenous PLP was much greater in those fractions from PLP-treated animals which displayed large GAD inhibition, than in the corresponding fractions from

control animals. 3) No ultrastructural changes were detected in the subcellular fractions from treated animals as compared to their corresponding controls. The present results show that the decrease of both PLP concentration and GAD activity, previously found in whole brain, actually takes place in the synaptosomes, and support the hypothesis that the role of PLP in the excitability controlling-mechanisms can be explained, at least in part, by its regulatory action on GAD activity, which determines the rate of GABA synthesis at the nerve endings.

Since the discovery that some antimetabolites of pyridoxal-5'-phosphate (PLP) produce convulsions (Holtz and Palm, 1964; Massieu, Ortega, Syrquin and Tuena, 1962; Purpura, Berl, González-Monteagudo and Wyatt, 1960; Rindi and Ferrari, 1959) and the publication of papers communicating that some children with convulsions could be adequately treated with pyridoxine (Bessey, Adam and Hansen, 1957; Coursin, 1969; Hunt, Stokes, McCrory and Stroud, 1954), data on the possible role of vitamin B_6 in the regulation of CNS excitability have been accumulating. This role seems to be related to the activity of glutamate decarboxylase (GAD, EC 4.1.1.15), because of the sum of the following data: 1) A decrease of brain PLP concentration by inhibition of pyridoxal kinase (Tapia, Pérez de la Mora and Massieu, 1969) or by a dietary deficiency of pyridoxine (Minard, 1967) results in a correlative decrease of GAD activity. 2) A decrease of GABA formation, because of GAD inhibition, results in the appearence of convulsions, independently of the total brain GABA levels (Tapia and Awapara, 1967). 3) The inhibition of GAD activity *in vivo* seems to be specific for the production of convulsions (Tapia and Pasantes, 1971).

These results, taken together, strongly suggest that brain PLP concentration exerts a regulatory action on GAD activity, which in turn is responsible for the control of nervous excitability through the synthesis of GABA, a very probable inhibitory transmitter at central synapses (Baxter, 1970; Curtis, Duggan, Felix and Johnston, 1970; Krnjević and Schwartz, 1967; Obata, Ito, Ochi and Sato, 1967; Obata and Takeda, 1969; Roberts and Kuriyama, 1968).

Subcellular studies have shown that GAD is concentrated in nerve endings (synaptosomes) (Fonnum, 1968; Salganicoff and De Robertis, 1965; Weinstein, Roberts and Kakefuda, 1963). It is at this subcellular site where GAD inhibition may result in a physiologically significant decrease of GABA concentration (Tapia and Awapara, 1967) and therefore, where the correlative decrease of PLP concentration and GAD activity must occur in order to account for the increased brain excitability. In the present work, we have studied PLP concentration and GAD activity in brain subcellular fractions from mice treated with a convulsant drug -pyridoxal phosphate-γ-glutamyl hydrazone (PLPGH)- that decreases PLP concentration and GAD activity in the whole brain (Tapia et al.,1969). An electron microscopic evaluation of the brain subcellular particles was carried out in the control and treated animals in order to check for possible morphological alterations of nerve endings due to the treatment or the convulsive activity.

MATERIALS AND METHODS

Adult albino mice (local strain) were used throughout this work. DL-[1-^{14}C]-glutamic acid was obtained from New England Nuclear (Boston, Mass.). PLPGH was synthesized as previously described (Tapia and Awapara, 1967; 1969). All other chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo.).

In all experiments PLPGH was injected intraperitoneally at a dose of 205 μmoles/kg. Control animals were similarly treated with 0.9 % NaCl. Terminal tonic-clonic convulsions appeared at 30-45 minutes after the administration of the hydrazone. At the moment of tonic convulsions the animals were

decapitated, the brain quickly removed and pooled in a chilled homogenizer until enough tissue (usually six to seven brains, cerebellum excluded) was collected for subcellular fractionation. A 10 % homogenate in 0.32 M sucrose was prepared in a teflon-glass homogenizer under gentle conditions (six strokes at approximately 850 rev/min).

Subcellular fractionation. In some experiments a high-speed pellet and supernatant were obtained by centrifuging the homogenate at 100,000 g for 30 minutes in a Spinco Model L centrifuge.

The primary fractions (nuclei, mitochondria, microsomes and supernatant) were obtained following the procedure of De Robertis, Pellegrino de Iraldi, Rodríguez de Lores Arnaiz and Salganicoff (1962). In some experiments the mitochondrial fraction was subfractionated in a discontinuous sucrose density gradient, as described by Gray and Whittaker (1962). In most of the experiments, however, a different gradient, which in our hands yielded a purer synaptosomal fraction than the Gray and Whittaker procedure, was used. This gradient consisted of 5 ml of 1.4 M sucrose, 6 ml of 1.3 M sucrose, 4 ml of 1.2 M sucrose and 4 ml of 0.8 M sucrose. After layering the resuspended crude mitochondrial fraction on the top of the gradient, it was centrifuged for 2.5 hr at 53,500 g in a SW-25.1 Spinco rotor.

In other experiments the crude mitochondrial fraction was osmotically shocked and subfractionated, according to Whittaker, Michaelson and Kirkland (1964). All the subfractions obtained were manually separated with a Pasteur pipette, and in some cases diluted with about one third of their volume of water, and centrifuged at 100,000 g for 30

minutes. Pellets containing each fraction or subfraction were resuspended in cold 0.32 M sucrose and assayed. Care was taken to mantain all material and tissue at 0-4° throughout all the fractionation steps.

Pyridoxal phosphate determination. For PLP extraction, each fraction or subfraction was treated with enough concentrated cold perchloric acid to obtain a 1 N final concentration, and centrifuged at 12,000 g at 1-4° (Servall centrifuge). One ml of the neutralized supernatant was used for PLP assay, according to the apotryptophanase procedure of Wada, Morisue, Sakamoto and Ichihara (1957), as modified by Minard (1967). The apotryptophanase was obtained by dialysing a commercial preparation from E. coli (Sigma Chemical Co., grade II).

Enzymatic determinations. GAD activity was assayed by measuring the $^{14}CO_2$ production from [1-^{14}C]-glutamic acid, as previously described (Tapia and Awapara, 1969). All fractions and subfractions were treated with 0.4 % Triton X-100 (final concentration) for 15 min before the incubation period. When PLP was added to the incubation mixture, its final concentration was 1.76×10^{-4} M. The radioactivity was counted in a Nuclear Chicago liquid scintillation counter.

Lactic dehydrogenase activity (EC 1.1.1.27) was measured according to Bergmeyer, Bernt and Hess (1963); succinic dehydrogenase (EC 1.3.99.1) by the method of Slater and Bonner (1952), as modified by De Robertis et al., (1962), but with a 5 min incubation period; AchE (EC 3.1.1.7) according to Ellman, Courtney, Andres and Featherstone (1961) and Mg^{++}-ATPase (EC 3.6.1.3) following the method of Jäernfelt (1961)

and measuring the inorganic phosphate according to Sumner (1944). Protein was measured according to Lowry, Rosebrough, Farr and Randall (1951). The "t" Student test was used for the statistical evaluation of results.

Electron microscopy. The fixation of the subcellular particles was accomplished by adding some drops of the fractions to a solution containing phosphate-buffered 1 % osmium tetroxide, pH 7.4, for 2 hr at 4° (Palade, 1952). After centrifugation and dehydration in graded cold ethanol the pellets were embedded in Epon-812 for 24 hrs at 60°C. Thin sections in the silver range were obtained with a Reichert OmU-2 ultramicrotome and collected in uncovered copper grids for further staining with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Venable and Coggeshall, 1965). The cutting orientation was parallel to the direction of sedimentation, in such a way that the whole thickness of the fixed pellet could be surveyed in a single section. The grids were examined in a Philips EM-200 electron microscope equipped with a 30 µ objective aperture.

RESULTS

Separation of the crude mitochondrial fraction according to Gray and Whittaker (1962), as well as subfractionation after osmotic shock (Whittaker et al., 1964) gave results similar to those previously reported from both the ultrastructural (see for example Fig. 1a) and the biochemical viewpoints: lactic dehydrogenase was located mainly in the supernatant and the axoplasm (subfraction O); succinic dehydrogenase in the crude mitochondrial fraction, pure mitochondria (subfraction C) and intraterminal mitochondria

(subfraction I); ATPase in damaged synaptosomes (subfraction II) and in subfraction I and AchE in the crude mitochondria and synaptosomes (subfraction B). The protein distribution observed in these experiments was also similar to that previously reported (Gray and Whittaker, 1962; Whittaker, 1964; Whittaker et al., 1964).

Centrifugation of the crude mitochondrial fraction in the four layers discontinuous sucrose density gradient (0.8, 1.2, 1.3 and 1.4 M) described in Methods resulted in four bands. The first was above 0.8 M sucrose, the second between 0.8 M and 1.2 M sucrose, the third in the interphase between 1.2 M and 1.3 M sucrose and the four band above 1.4 M sucrose. Occasionally some particulate material was found in band 4, reaching the bottom of the tube. When this occurred the whole 1.4 M sucrose layer was taken as a single fraction. These subfractions were designated 1, 2, 3 and 4, respectively, from top to bottom. There were no differences between treated and control animals with regard to the gross appearence of the gradients after centrifugation.

Electron microscopically, fraction 1 consisted of a practically pure population of large myelin fragments (Fig. 2). As observed in figure 3, most elements in fraction 2 were well preserved synaptosomes, with numerous synaptic vesicles and intraterminal mitochondria. Postsynaptic membranes adhered to synaptosomes were frequently seen in this fraction. Membrane fragments and only very few free mitochondria contaminated this preparation. In contrast, the synaptosomal fraction B obtained with the Gray and Whittaker's gradient contained numerous free mitochondria (compare Figs. 1a and 1b).

In fraction 3 synaptosomes mixed with mitochondria were observed (Fig. 4). Fraction 4 was composed of free well preserved mitochondria; synaptosomes or other contaminants were rarely seen in this fraction (Fig. 5).

From the biochemical point of view (Table 1), proteins were located mainly in fraction 2 (52%); fraction 3 was next with 20%. Succinic dehydrogenase, as expected, was highly concentrated in fractions 3 and 4, rich in mitochondria. In contrast, and also in agreement with the morphological findings, AchE was located mainly in subfractions 2 and 3, especially in the former. GAD was concentrated in the synaptosomal fraction 2; subfractions 1 and 4 contained only a small amount of GAD. There was no relation between the presence of PLP in the incubation medium and the distribution of GAD in the various subfractions (Table 1).

Subcellular distribution of PLP. Before the experiments with PLPGH, PLP subcellular distribution in brain of normal mice was studied in detail. PLP concentration in the homogenate was 0.89 µg/g. Among the primary fractions, PLP was distributed mainly in the crude mitochondrial fraction (36%) and in the supernatant (38%); the nuclear fraction contained 21%, and only 5% was found in the microsomal fraction (mean values of 5 experiments; recovery was 92%). When the crude mitochondrial fraction was subfractionated in the two layers gradient of Gray and Whittaker, 0-14% of the recovered PLP was found in the myelin band, 33% in the synaptosomal band and 60% in the mitochondrial pellet (mean values of 5 experiments; recovery was 87%).

Table 1.- Percent distribution (total recovered = 100) of protein, SDH, AchE and GAD activities in the subfractions of the crude mitochondrial fraction

Subfraction	Morphology	Protein	SDH	AchE	GAD	
					Without PLP	With PLP
1	Myelin	15.4	2.1 (0.14)	11.0 (0.71)	6.6 (0.42)	4.3 (0.27)
2	Synaptosomes	51.8	10.9 (0.21)	63.2 (1.22)	67.9 (1.31)	72.4 (1.39)
3	Synaptosomes and mitochondria	20.4	41.5 (2.04)	18.7 (0.92)	17.4 (0.85)	15.2 (0.74)
4	Mitochondria	12.4	45.5 (3.67)	7.1 (0.59)	8.1 (0.65)	8.1 (0.64)

Absolute values of the crude mitochondrial fraction were: protein, 48.6 mg/g original tissue; SDH, 390 U/g original tissue; AchE, 268 umoles/h/g original tissue; GAD without PLP, 13.4 umoles/h/g original tissue and 30.7 umoles/h/g original tissue with PLP (1.76×10^{-4}M final concentration).
Recoveries for protein and enzymes ranged from 66% to 89%.
Mean values of 2 to 3 experiments for SDH, AchE and GAD, and 6 experiments for protein.
Figures in parentheses are relative specific activities = $\frac{\text{\% of recovered activity}}{\text{\% of recovered protein}}$

PLP distribution in the four-layers gradient is shown in Table 2. In agreement with the results of the two-layers gradient, PLP was found mainly in subfractions 3 (36 %) and 4 (40 %), mixed mitochondria and synaptosomes, and pure mitochondria, respectively. About 20 % of PLP was distributed in the synaptosomal fraction 2.

In the gradient used after osmotic shock (Table 2), it was observed that synaptic vesicles (subfraction D) and membranes (subfractions E-G) contained only negligible amounts of PLP. Most PLP present in the parent fraction W_s was recovered in the damaged synaptosomes (subffaction H, 50 %), and axoplasm (subfraction O, 27 %).

Since PLP is a low molecular weight substance, which theoretically could easily diffuse out or into the subcellular particles, several experiments were done to assess its possible redistribution during the separating procedures. When the homogenate in 0.32 M sucrose was centrifuged at 100,000 g for 30 min, to obtain a high speed-pellet and the corresponding supernatant, 42 % of PLP (6 experiments) was obtained in the pellet. This value did not differ gratly from that observed in the particulate fractions obtained after separation of the primary fractions (sum of nuclear, crude mitochondrial and microsomal fractions, 53 %). When the high speed-pellet was washed twice with 0.32 M sucrose and recentrifuged at 100,000 g, 100 % of the particulate PLP remained in the pellet (2 experiments)

In other experiments, the homogenate was maintained for several hours at 0-4º and the high speed-pellet was obtained from aliquots taken at 1, 2, 4 and 6 hr (2 experiments). No

- Distribution of protein and PLP in the subfractions of the crude mitochondrial fraction

ion	Protein		PLP		
	mg/g original tissue	% distrib. (total recovered=100)	ug/g original tissue	%distrib. (total recovered=100)	Relative distribution
1)	5.6 (3)	15	0.007 (5)	2.4±0.7	0.16
;osomes)	18.9 (6)	52	0.062 (6)	21.3±2.6	0.41
;osomes ;ochondria)	7.4 (6)	20	0.105 (6)	36.5±6.6	1.82
1ondria)	4.5 (6)	13	0.116 (6)	39.8±6.7	3.06
(% of crude 1rial frac-	76 [75-93]			95 [65-120]	
) ; pellet 1otic shock)	19.5 (4)	47	0.11 (3)	44 [42-50]	0.94
supernatant 1otic shock)	22.4 (4)	53	0.14 (3)	56 [50-58]	1.06
(% of 1itochon- ction)	90 [87-95]			71 [66-84]	
sm)	5.67 (4)	25	0.041 (3)	27 [24-32]	1.08
ic es)	1.83 (4)	8	0.008 (3)	5 [2-7]	0.62
	0.88 (4)	4	0* (3)	0	0
nes)	1.69 (4)	7	0* (3)	0	0
	2.75 (4)	12	0.005 (3)	3 [2-4]	0.25
-d somes)	5.98 (4)	26	0.075 (3)	50 [45-54]	1.92
-ondria)	4.29 (3)	18	0.022 (3)	15 [10-23]	0.83
	99 [97-100]			130 [119-142]	

-re mean values of the number of experiments shown in parentheses.
than three experiments were done, the S.E.M. for the percent distri- given. Otherwise the range is indicated in brackets.

1istribution = $\frac{\text{\% of recovered PLP}}{\text{\% of recovered protein}}$

significant change in the percent distribution of PLP in the pellet was observed during the experimental period.

When a known amount of PLP (100 μg/g of original tissue) was added to a homogenate and the latter was subjected to the centrifugation procedure to obtain the primary fractions, 94 % of the recovered coenzyme was found in the soluble fraction (supernatant) (2 experiments). PLP was also added to the top or the bottom layers of the sucrose density gradients and they were centrifuged for 2 hr at 53,500 g in the absence of tissue, to assess the diffusion of the coenzyme during centrifugation. Practically no diffusion was observed.

Effect of PLPGH-induced convulsions on the morphology of brain subcellular particles. Figure 6 shows that neither PLPGH administration nor the convulsive activity had any appreciable effect upon the morphology of the synaptosomes isolated in fraction 2 of the four-layers gradient. The structure of myelin and mitochondria was also unaffected by PLPGH treatment.

Effect of PLPGH on protein content of brain subcellular fractions. The adminstration of PLPGH did not modify either the protein content of the homogenates or the protein distribution in the high speed pellet and supernatant, in the primary fractions, and in the subfractions obtained from the crude mitochondrial fraction before and after osmotic shock.

Effect of PLPGH on the concentration of PLP in subcellular fractions. As can be seen in Table 3, the adminstration of PLPGH to mice produced the previously reported decrease of PLP in whole brain (Tapia et al., 1969). The decrease of PLP was larger in the high speed supernatant than in the pellet, in which the changes were not statistically significant. When

3.- Effect of PLPGH treatment on PLP concentration in the high-speed pellet and supernatant and in the primary fractions

.on	Control	Treated*	% of control	p
nate	0.971 ± 0.076 (9)	0.548 ± 0.016 (9)	56.4	< 0.001
peed	0.424 ± 0.021 (9)	0.354 ± 0.03 (10)	83.5	> 0.5
peed atant	0.466 ± 0.1 (10)	0.112 ± 0.035 (10)	23.9	< 0.01
ondria fraction)	0.330 ± 0.045 (8)	0.261 ± 0.021 (8)	79.1	> 0.1
omes + atant	0.373 ± 0.052 (6)	0.083 [0 - 0.227]+ (6)	22.3	—

gures are ug/g of original tissue. Mean values of the number of nents shown in parentheses, ± S.E.M.

ficed at the moment of tonic convulsions (30-45 min after injection 5 umoles/kg of PLPGH).

of values; the zero value was obtained in two experiments.

the effect of PLPGH was studied in the primary fractions, a similar difference between the changes in particulate and soluble PLP was found (Table 3).

In view of the heterogeneity of the high speed pellet and of the crude mitochondrial fraction, we studied in more detail the PLP concentration in the subfractions of the crude mitochondrial fraction. Table 4 shows that only a small decrease of PLP in synaptosomes (subfraction 2) was observed after PLPGH treatment, whereas other fractions were not affected. However, when the crude mitochondrial fraction was osmotically shocked and centrifuged at 10,000 g to obtain a pellet (W_p) and the corresponding supernatant (W_s), a statistically significant decrease of PLP was found in fraction W_s (Table 5). The isolation of the components of this fraction in a sucrose density gradient (subfractions D-G were not studied in view of their negligible PLP content) indicated that the change of PLP concentration found in fraction W_s was due to a selective decrease of this coenzyme in the axoplasm of synaptosomes (subfraction O) (Table 5).

Effect of PLPGH on GAD activity of brain subcellular particles. In some experiments the previous PLPGH-induced GAD inhibition (40 %) in the whole homogenate was found. Likewise, PLPGH treatment resulted in a 66.3 % inhibition (mean value of four experiments) of GAD in the high speed pellet after centrifugation of the homogenate, while no activity was detected in three of four experiments in the corresponding supernatant fraction. The addition of PLP to the incubation mixtures reversed completely the inhibition of GAD in both the homogenate

Subfraction	Control	Treated	% of control	p
Mitochondria (crude fraction)	0.307 ± 0.017 (6)	0.293 ± 0.021 (6)	95.4	>0.5
1 (myelin)	0.007 ± 0.002 (5)	0.007 ± 0.001 (6)	100	>0.5
2 (synaptosomes)	0.062 ± 0.005 (6)	0.048 ± 0.01 (6)	77.4	>0.1
3 (synaptosomes and mitochondria)	0.105 ± 0.028 (6)	0.093 ± 0.012 (6)	88.6	>0.5
4 (mitochondria)	0.116 ± 0.021 (6)	0.103 ± 0.02 (5)	88.8	>0.5

See notes in Table 3 for units and other indications.

Subfraction	Control	Treated	% of control	p
W_p (10000 g pellet after osmotic shock)	0.189 ± 0.056 (5)	0.144 ± 0.003 (5)	76.2	>0.1
W_s (10000 g supernatant after osmotic shock)	0.136 ± 0.015 (5)	0.087 ± 0.001 (5)	64.0	<0.02
O (axoplasm separated by gradient centrifugation of W_s)	0.066 ± 0.013 (6)	0.035 ± 0.006 (7)	53.0	>0.05
H (damaged synaptosomes, separated by gradient centrifugation of W_s)	0.055 ± 0.018 (5)	0.047 ± 0.009 (5)	85.5	>0.5
I (intraterminal mito-chondria, separated by gradient centrifugation of W_s)	0.049 ± 0.008 (6)	0.045 ± 0.010 (5)	91.8	>0.5

See notes in Table 3 for units and other indications.

and the supernatant fraction.

When GAD activity was measured in the crude mitochondrial fraction and its subfractions (Table 6), GAD inhibition (35-58 %) was observed in the crude mitochondrial fraction and in the subfractions 2 and 3, which contain synaptosomes, while the activity of this enzyme was practically unaffected in subfraction 4, containing purified mitochondria. The results obtained with myelin and mitochondrial subfractions were equivocal because of the low activity of the enzyme.

In the experiments of density gradient centrifugation after osmotic shock of the crude mitochondrial fraction, a 60-75 % inhibition of GAD activity in fractions W_p and W_s was obtained after PLPGH treatment (Table 7). When fraction W_s was further subfractionated and the resulting subfractions were assayed for GAD activity, 40 % inhibition was found in the axoplasm (subfraction O). No significant changes were detected in the other subfractions.

Addition of PLP to incubation mixtures reversed in great proportion the inhibition of GAD in most of the fractions (Tables 6 and 7), being this effect more notable in the soluble fractions. The ratio of GAD activity +PLP/-PLP, which indicates the degree of activation of GAD by exogenous PLP, was much larger and statistically significant in those fractions in which GAD inhibition was detected, in comparison to the control values (Tables 6 and 7).

DISCUSSION

Subcellular fractionation. The electron microscopic evaluation of the subcellular particles, together with the

	−PLP	+PLP	+PLP/−PLP	−PLP	+PLP	+PLP/−PLP
Mitochondria (crude fraction)	13.45 ± 1.44 (3)	30.78 [25.98 – 35.58]§ (2)	2.23 [2.19 – 2.26]§	7.43 ± 0.71‡ (3)	24.93 ± 2.24 (3)	3.35 ± 0.21 (3)
1 (myelin)	0.60 ± 0.14 (3)	1.16 ± 0.18 (3)	1.91 ± 0.28 (3)	0.48 ± 0.16 (3)	0.83 ± 0.10 (3)	1.73 ± 0.89 (3)
2 (synaptosomes)	6.14 ± 0.52 (6)	19.20 ± 0.68 (6)	3.13 ± 0.27 (6)	2.31 ± 0.24* (6)	14.14 ± 0.93† (6)	6.23 ± 0.41 (6)
3 (synaptosomes and mitochondria)	1.62 ± 0.18 (6)	4.03 ± 0.33 (6)	2.48 ± 0.19 (6)	0.68 ± 0.08‡ (4)	2.66 ± 0.16‡ (4)	3.90 ± 0.42 (4)
4 (mitochondria)	0.91 ± 0.18 (6)	2.14 ± 0.35 (6)	2.36 ± 0.09 (6)	0.68 ± 0.16 (4)	1.32 ± 0.14 (6)	1.93 ± 0.29 (4)

The figures are umoles/h/g original tissue. Mean values of the number of experiments shown in parentheses, ± S.E.M. PLP concentration in the incubation medium, when added was 1.76 x 10^{-4} M

*$p < 0.001$ (difference from control value)

†$p < 0.01$ (difference from control value)

‡$p < 0.02$ (difference from control value)

§ Range of values

Subfraction	Control			Treated		
	-PLP	+PLP	+PLP/-PLP	-PLP	+PLP	+PLP/-PLP
W_p (10,000 g pellet after osmotic shock)	4.84 ± 0.35 (4)	13.15 ± 0.77 (4)	2.72 ± 0.11 (4)	1.56 ± 0.30* (4)	11.43 ± 1.20 (4)	7.35 ± 0.90* (4)
W_s (10,000 g supernatant after osmotic shock)	7.21 ± 0.56 (4)	17.43 ± 0.76 (4)	2.42 ± 0.14 (4)	1.96 ± 0.15* (3)	13.81 ± 0.76+ (3)	7.05 ± 0.48* (3)
O (axoplasm separated by gradient centrifugation of W_s)	1.50 ± 0.20 (7)	6.97 ± 0.59 (7)	4.65 ± 0.54 (7)	0.78 ± 0.12+ (6)	5.63 ± 0.43 (7)	7.24 ± 0.71* (6)
H (damaged synaptosomes separated by gradient centrifugation of W_s)	0.38 ± 0.11 (4)	0.90 ± 0.17 (5)	2.38 ± 0.43 (4)	0.32 ± 0.09 (4)	0.70 ± 0.07 (4)	2.21 ± 0.62 (4)
I (intraterminal mitochondria separated by gradient centrifugation of W_s)	0.33 ± 0.05 (5)	0.65 ± 0.13 (5)	1.99 ± 0.56 (5)	0.31 ± 0.07 (5)	0.61 ± 0.13 (5)	1.95 ± 0.72 (5)

See note in table 6 for units and other indications

* $p < 0.001$

\+ $p < 0.02$

measurement of some enzymatic markers (lactic dehydrogenase, Mg^{++}-ATPase, succinic dehydrogenase and AchE) indicated that the fractions obtained according to Gray and Whittaker (1962) and to Whittaker _et al_. (1964) correspond to the fractions obtained by these authors (see Whittaker, 1964). Although subfraction B of Gray and Whittaker is a good source of synaptosomes, we tried to improve its purity by eliminating the contaminating mitochondria. By using the four layers gradient described in Materials and Methods, we obtained a fraction (subfraction 2) which according to morphological and biochemical studies (Fig. 1b and Table 1) consists mainly of synaptosomes and some membranes (synaptic membranes?), and was practically free of mitochondria, which in our hands were abundant in the synaptosomal band of Gray and Whittaker (see Fig. 1a). Fraction 3 of our gradient was similar to the latter synaptosomal band, although the contamination with mitochondria was larger (compare Figs. 1a and 4). The distribution of succinic dehydrogenase, AchE and GAD in the four layers gradient (Table 1) was in excellent agreement with the morphological observations.

Subcellular distribution of PLP. The bimodal distribution of PLP mainly in the crude mitochondrial and soluble fractions (see text in Results) is similar to that observed for the alanine and aspartate aminotransferases, as well as for GAD (Fonnum, 1968; Salganicoff and De Robertis, 1965; van Kempen, van den Berg, van der Helm and Veldstra, 1965). The localization of PLP in the different subfractions obtained from the crude mitochondrial fraction by the two different gradients used, as well as its distribution in the fractions

obtained after osmotic shock of the crude mitochondrial fraction (Table 2) also follows the distribution pattern of aspartate aminotransferase (Fonnum, 1968). These results suggest that the subcellular distribution of PLP in brain depends largely upon the distribution of the most abundant B_6-enzymes. Therefore, it seems probable that a considerable amount of the coenzyme measured in these experiments was bound to these enzymes and subsequently liberated by the perchloric acid extraction. However, the presence of free PLP could be , as discussed below, of physiological significance. γ-Aminobutyrate aminotransferase, which is considered to be a mitochondrial enzyme (Salganicoff and De Robertis, 1965; van Kempen _et al_., 1965) would appear also responsible for some of the PLP present in the subfractions rich in mitochondria (Table 2). Similar results to those obtained in the present paper have been reported in guinea pig brain (Loo and Mack, 1971).

Effect of PLPGH-induced convulsions on the structure of subcellular particles. In the present paper no ultrastructural changes that could be ascribed to PLPGH treatment were detected in any of the studied fractions (Fig. 6). Some structural modifications of synaptosomes have been observed in rats, after methionine sulphoximine-induced convulsions (De Robertis, Sellinger, Rodríguez de Lores Arnaiz, Alberici and Zieher, 1967). Differences between the mechanism of action of the latter drug and that of PLPGH could account for these different observations.

Effects of PLPGH on GAD activity and PLP content in subcellular fractions. The decrease of PLP levels by PLPGH

administration was more notable in the soluble fractions than in the particulate ones. This result could be explained by the known inhibitory action of PLPGH upon the activity of pyridoxal kinase (Tapia and Awapara, 1969; Tapia et al., 1969), which is a soluble enzyme (Loo and Whittaker, 1967). Thus, the inhibition of pyridoxal kinase would produce a decrease in free PLP, primarily in the soluble fractions, and secondarily in the particle-bound PLP. The concentration of the latter would be dependent mainly on the equilibrium between the free PLP and the PLP bound to the particulate enzymes. Accordingly, the lack of effect of PLPGH on PLP concentration in particulate fractions could be due to the fact that aminotransferases, which most probably possess tightly bound PLP in vivo (Tapia and Pasantes, 1971), are mostly particulate (van Kempen et al., 1965).

If, as it is apparent from the foregoing discussion, free PLP is decreased by inhibition of pyridoxal kinase, it would be expected that the activity of the soluble enzymes with relatively low affinity for PLP in vivo, such as glutamate and DOPA decarboxylase (Tapia and Pasantes, 1971), would be affected under these conditions. In accordance with this expectation, it was found that in all fractions sudied in the present paper there was a correlation between PLP concentration and GAD activity, as had been previously reported in whole brain (Tapia et al., 1969). In the soluble fractions, in which PLP was most decreased, GAD activity reached its lowest velue, while practically no changes were detected in those fractions (H, I, 1 and 4) in which PLP was not modified (Tables 4-6). The same correlation was found in

some fractions, such as the axoplasm, which displayed intermediary changes in both PLP concentration and GAD activity. The apparent lack of correlation between these parameters in the synaptosomal fraction (subfraction 2, Tables 4 and 6) can be explained on the basis of the differences in the subcellular localization of PLP and GAD in the synaptosome and the differential effect of PLPGH in the soluble and the particulate fractions: since GAD is located mainly in the axoplasm (Fonnum, 1968; Salganicoff and De Robertis, 1965; Weinstein et al., 1963; see also control values of Table 7), a decrease of its activity would be readily apparent in the whole synaptosome, and any change of GAD activity in mitochondria, in which this enzyme is present only in low amounts, would not be noticed. Precisely the opposite situation occurs with PLP: since this coenzyme is present in large amounts in the synaptosomal mitochondria (Table 2), and since PLPGH does not seem to affect the mitochondrial PLP (Tables 4 and 5), the decrease in the axoplasmic PLP could not be detected clearly in the whole synaptosome. That this is the case is indicated by the fact that, when the changes induced by PLPGH upon GAD activity and PLP concentration were measured in the different components of the synaptosome (axoplasm and mitochondria), the correlation between both parameters could be observed only in the axoplasm (Tables 5 and 7).

The correlative changes of GAD activity and PLP concentration just discussed, along with the relatively low affinity of GAD for its coenzyme *in vivo* (Tapia and Pasantes, 1971) and the previous findings of studies in whole brain

(Tapia *et al.* 1969), indicate that the inhibition of GAD activity due to PLPGH treatment is secondary to the decrease of PLP levels in the different subcellular particles. This is supported by the fact that PLP added *in vitro* reversed most of the inhibition in the fractions studied, as shown by the much larger activation due to PLP in those fractions in which GAD was inhibited, as compared to the control values (Tables 6 and 7). The larger effect of PLPGH upon GAD activity and PLP concentration in the supernatant fraction in comparison to the axoplasmic fraction is an interesting finding, since both fractions are soluble. If the primary event involved in GAD inhibition by PLPGH is actually a decrease in pyridoxal kinase activity, this result could mean that somatic pyridoxal kinase is more inhibited than the axoplasmic enzyme.

From the present results it can be concluded that the following previously suggested (Tapia *et al.*, 1969) sequence of events leading to the appearance of convulsions after PLPGH treatment indeed occurs in the nerve endings: pyridoxal kinase inhibition → decrease of PLP concentration → GAD inhibition → decreased rate of GABA synthesis → convulsions. According to this mechanism, the role of PLP in the excitability of the CNS would be accounted for by its regulatory effect on GAD activity at the nerve endings. A decrease of the critical concentration of PLP at these structures would result in a diminished rate of synthesis of GABA, with a consequent blocking of the GABA dependent inhibitory mechanisms at several neuronal nuclei, and the appearance of convulsions. That the inhibition of other B_6-enzymes might also contribute to the hyperexcitability

observed after PLPGH treatment seems to be ruled out by previous experiments from our laboratory, which indicate that GAD inhibition is specifically related to the convulsions produced by PLPGH (Tapia and Pasantes, 1971). The possibility that other unknown role of PLP in the CNS, independent of its coenzymatic function, might also be involved in the regulation of cerebral excitability, is still an open question.

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LEGENDS FOR FIGURES

Figure 1a. Subfraction B obtained according to Gray and Whittaker's method. M = free mitochondria (16,600 X).

Figure 1b. Subfraction 2 of the four layers gradient described in the present work (16,600 X).

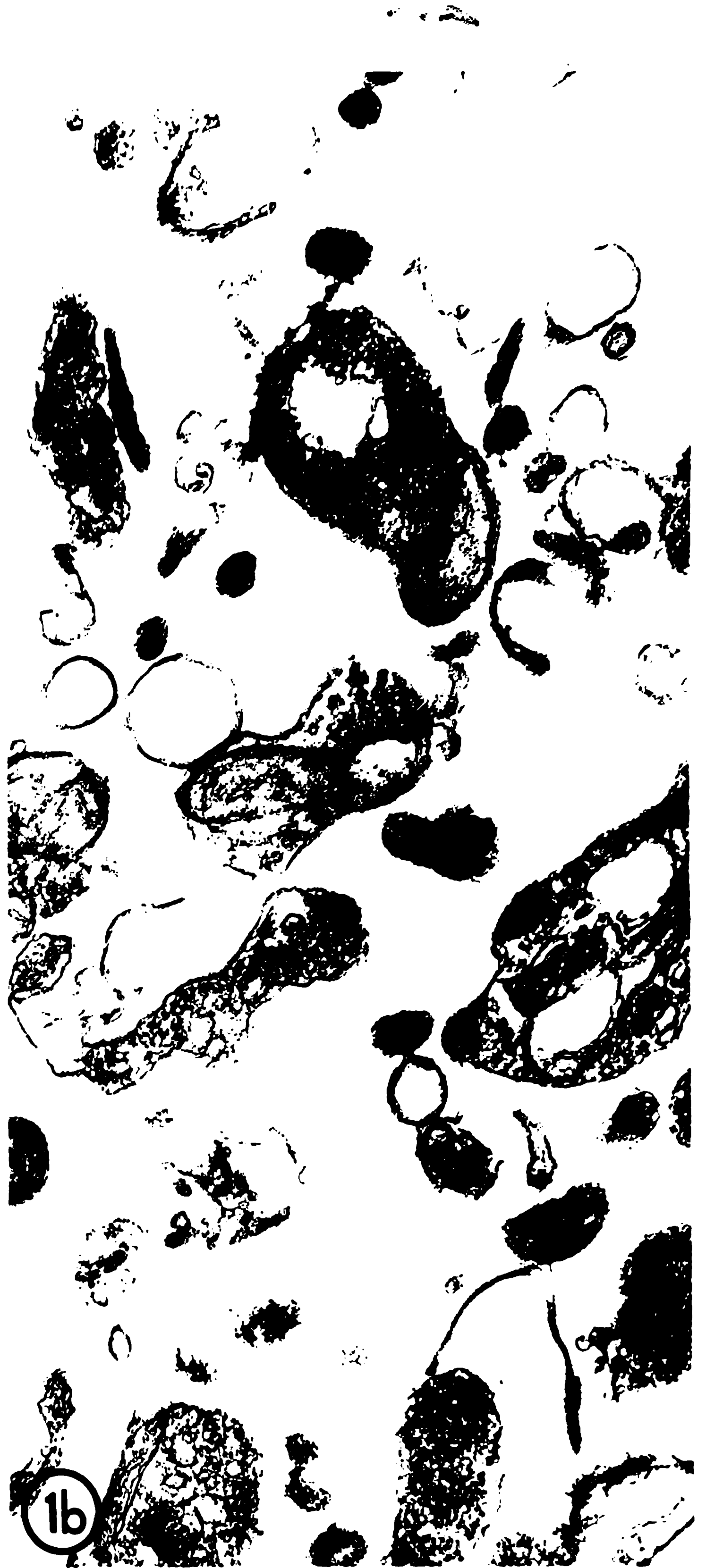
Figure 2. Subfraction 1, essentially composed of myelin (16,600 X).

Figure 3. Subfraction 2. Most elements are synaptosomes (16,600 X).

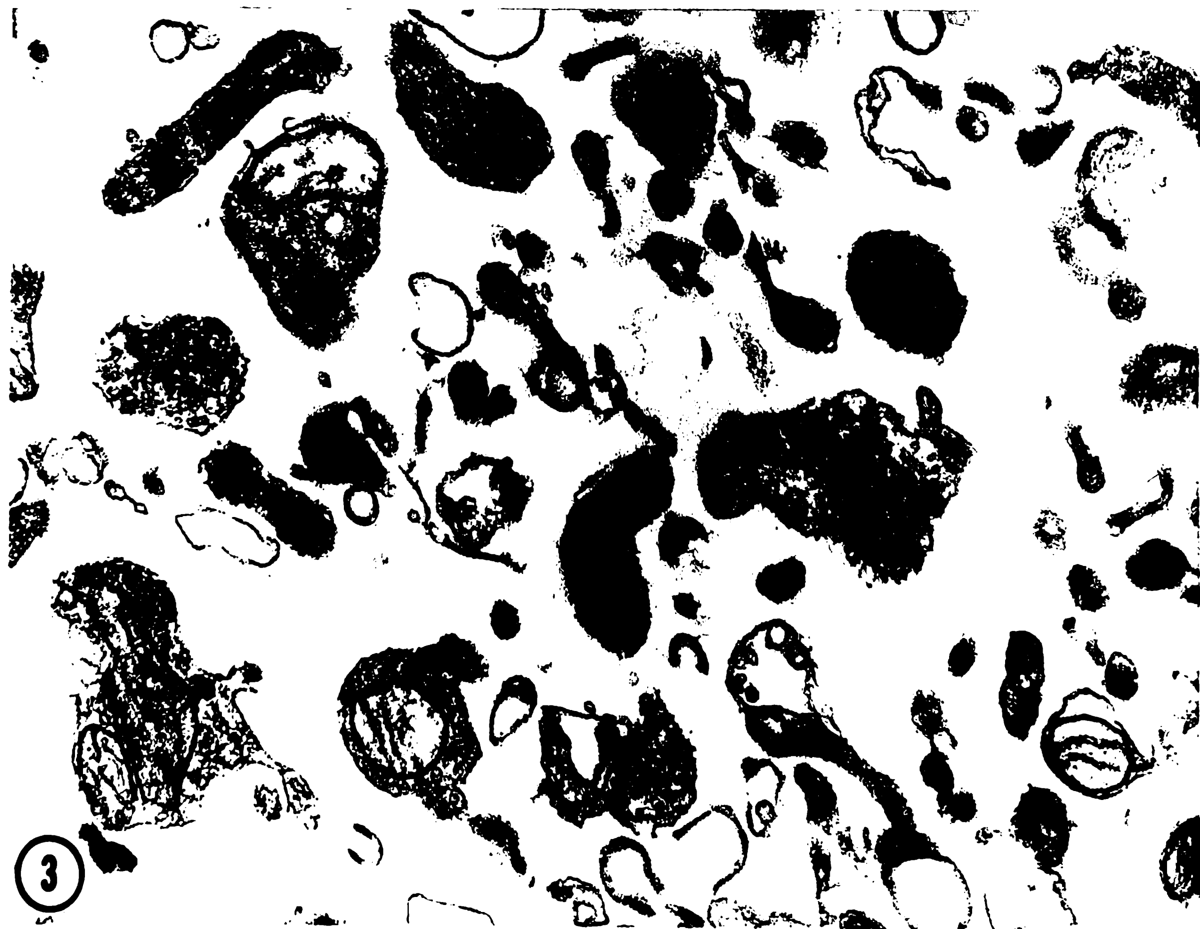
Figure 4. Subfraction 3. Some synaptosomes (S) are mixed with mitochondria (m) (16,600 X).

Figure 5. Subfraction 4. The majority of elements are free mitochondria (m), and occasionally few synaptosomes (S) are depicted (16,600 X).

Figure 6. Subfraction 2 from brain of animals treated with PLPGH (16,600 X). Inset.- Synaptosomes (asterisk) often remained attached to the post-synaptic elements (Ps) in both the control and treated animals (17,200 X).

M

1b

2


3

m