EFFECT OF PYRIDOXAL ADMINISTRATION ON THE CONTENTS OF PYRIDOXAL PHOSPHATE AND γ-AMINOBUTYRIC ACID IN MOUSE BRAIN

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Summary Many mice treated with a high single dose of PL died in convulsions. The convulsions were very similar to those induced with some anti B₆ in several ways: (1) a long latent period occurs before convulsions; (2) PLP levels in brain decrease; (3) GABA levels in brain, especially in synaptosomal fraction, decrease. The possibility that a deranged GABA metabolism in nerve ending may be a major cause of the convulsions is discussed.

Since the discovery that some anti B₆ produce convulsions (1–3) and the publication of papers reporting that some children with convulsions could be adequately treated with PN (4–6), data on the possible role of B₆ in the regulation of central nervous system excitability have been accumulating. This role seems to be related to the activity of GAD [EC 4.1.1.15] on the basis of the following data; (1) A decrease of PLP brain levels by inhibition of pyridoxal kinase [EC 2.7.1.35] (7) or by a dietary deficiency of B₆ (8) result in a correlative decrease in GAD activity. (2) A decrease of GABA formation in the nerve endings (9, 10), because of the GAD inhibition, results in a physiological significant decrease of GABA concentrations at this site and in the appearance of convulsions (10). Altogether these results strongly suggest that the concentration of PLP in the brain exerts a regulatory action on the activity of GAD, which in turn is responsible for the control of nervous excitability through the synthesis of GABA, a presumptive inhibitory transmitter at central synapses (11, 12).

Abbreviations used: PL, pyridoxal; anti B₆, antivitamin B₆; PLP, pyridoxal phosphate; GABA, γ-aminobutyric acid; PN, pyridoxine; GAD, glutamate decarboxylase; PM, pyridoxamine; PNP, pyridoxine phosphate; GABA-T, GABA: α-ketoglutarate aminotransferase; PMP, pyridoxamine phosphate.
On the other hand, it has been reported that a large dose of B₆ induces tonic convulsions which precede death in animals and that PL is about twice as toxic as PN or PM (13), though there is currently no single theory which can explain the facts regarding the toxic action of B₆ vitamers.

The possibility exists that B₆ at high dosage levels acts in a manner similar to that of some of the anti B₆. The existence of such a possibility dictated our present experimental approach to the problem, which has been to compare the B₆-induced convulsions with seizures caused by various well known anti B₆ in the belief that the results so obtained might shed light on the biochemical mechanisms involved in the B₆-induced seizures. Since many experiments indicated the possible implication of GABA metabolism in anti B₆-induced convulsions as shown above, particular emphasis in the present studies has been placed on this aspect of the research.

MATERIALS AND METHODS

**Materials.** B₆ vitamers were purchased from Nakarai Chemicals. Acid phosphatase was obtained from Boeringer Mannheim Chemicals.

**Animals.** DDY mice (weighing about 20 g) were used in this experiment.

**Administration of drugs.** Solutions of B₆ vitamers were prepared in 0.9% saline, the pH being adjusted to 7.0 immediately before use. The final concentration of the drugs was adjusted so that the required dosage was administered in a volume equivalent to 1% of the body weight of the animals. Injections were intraperitoneal, and the injected animals were kept in a laboratory with minimal background noise.

**Determination of B₆ vitamers.** Brain extract to be assayed were prepared by homogenizing about 400 mg of brain with 9 vol. of cold 1N perchloric acid, centrifuging and reextracting the residue twice with 3 vol. of cold 0.2 N perchloric acid, and neutralizing the combined extracts with 5N KOH to pH about 6.0. Seven milliliter of the extract obtained was applied to a cationic exchange column (Amberlite CG-120, 1 × 3.5 cm) and elution was performed according to the procedure of Loo and Badger (14). The vitamers in the eluates were individually assayed microbiologically with Saccharomyces carlsbergensis (ATCC 9080) as previously reported (15). Phosphate esters were hydrolyzed by acid phosphatase (0.5 mg/ml eluate) before assay. Since the PLP fraction contained PNP, the hydrolyzed product (PL and PN) was separated again with the same column, and the each one was assayed with the yeast.

**Subcellular fractionation.** Animals were decapitated and the brain were quickly removed and pooled in a chilled homogenizer until enough tissue (usually from 5–6 brains) was collected for subcellular fractionation. A 10% homogenate in 0.32 M sucrose was prepared using a Teflon-glass homogenizer under mild conditions (5 strokes at approximately 1,000 rpm). The primary fractions (nuclei, crude mitochondria, microsomes, and supernatant) and the subfractions (myelin, synaptosomes, and mitochondria) of the crude mitochondrial fraction were obtained by the
procedure of Gray and Whittaker (16). Pellets containing each fraction or subfraction were resuspended in cold water and used for assay of GABA and glutamate. Care was taken to maintain all material and tissue at 0–4°C throughout all the fractionations.

**Determination of GABA.** The homogenate or the suspensions of its subcellular fractions were homogenized in the same volume of 2N perchloric acid and centrifuged at 17,000 × g for 15 min. The obtained extracts were neutralized with 5 N KOH to pH about 4 and passed through a Dowex-50-H+ column (0.6 × 4 cm). The column was washed with 10 ml of water and the amino acids were eluted with 6 ml of 2 N NH₄OH. The eluates were evaporated to dryness under vacuum. The residue was resuspended in 0.1 ml of water, and the 20 µl of the suspension was spotted on Toyo filter paper No. 51. The chromatograms were run (ascendent chromatography, 80% phenol), dried for 24 hr and fully treated with 0.2% ninhydrin. The ninhydrin color was developed by heating the paper in an oven at 80°C for 20 min. The spot corresponding to GABA or glutamate was cut out and extracted with 5 ml of 60% ethanol, and the absorbance of the extract was determined at 575 nm (17).

**Determination of enzyme activities.** GAD activity in brain homogenates was determined by measuring GABA formation from glutamate. Homogenates were prepared in 9 vol. of 1/15 M phosphate buffer, pH 6.2. After addition of 0.15 ml of 1.1 M Na glutamate to 1.5 ml of the homogenate, incubation was carried out at 37°C for 20 min. GABA content in the reaction mixture was determined by the procedure described above.

The activity of GABA-T [EC 2.6.1.19] was measured by determining glutamate production from α-ketoglutarate. Homogenates were prepared in 9 vol. of 0.1 M phosphate buffer, pH 8.0. The incubation mixture contained 0.15 ml of 0.5 M GABA solution, 0.45 ml of the same buffer, and 0.75 ml of the homogenate. After the addition of 0.15 ml of 0.1 M α-ketoglutarate solution, incubation was carried out at 37°C for 20 min. The reaction mixture was heated at 100°C for 10 min and centrifuged at 12,000 rpm for 10 min. Glutamate in the supernatant was estimated using the enzymatic procedure described by Schousboe et al. (18).

**RESULTS AND DISCUSSION**

The administration of 1.8 mmol/kg PL caused symptoms of “running fit,” followed by convulsions which culminated eventually in the death of the animals. The mode of seizures was very similar to that caused by administration of anti B₆ such as tiosemicarbazide, isonicotinic hydrazide, and toxopyrimidine. When the amount of PL administered was doubled (3.6 mmol/kg), the percentage of animals suffering the convulsions was increased and the time to the onset of convulsions was reduced (Table 1), through the mode of the convulsions was slightly different from that caused by anti B₆.
The effect of the convulsant dose of PL (1.8 mmol/kg) on the contents of B₆ vitamers was determined at the mean convulsion time (about 60 min) (Table 2). PL administration induced a remarkable decrease of PLP concentration, but did not influence the PMP concentration. Moreover, the effect of PL on PLP levels was studied using both non-convulsant and convulsant dosage levels (Fig. 1). In both cases, PL significantly decreased brain PLP levels in mice one hour after the administration, and the degree of decrease in PLP levels increased markedly with increased doses of PL. Administration of the same dose of PN or PM as that of PL did not induce convulsions but also decreased PLP levels determined one hour after the administrations, though the degree of decrease was smaller than that of PL (Table 2). The fact that B₆ administration is accompanied by a fall in the PLP levels is an unexplainable feature, in agreement with the preliminary report of Bain and Williams (19). However, it is conjectured that this is due to a derangement of the phosphorylating mechanism by the existence in excess of B₆, especially PL, although there is no direct evidence to support this supposition.

**Effect of PL administration on brain GAD activity**

If free PLP was decreased by inhibition of net synthesis of PLP, it would be expected that the activity of B₆ enzymes with relatively low affinity for PLP *in vivo* (such as GAD) would be affected under these conditions. However, when the effect of
Effect of PL, 0.018, 0.18 and 1.8 mmol/kg, administered intraperitoneally, on the PL and PLP contents of brain. PLP contents are expressed as per cent of the control (untreated) mice. PL and PLP in brain were determined 60 min after PL administration.

The convulsant dose of PL was studied in whole brain, no decrease in GAD and GABA-T activities was observed (data not shown). Interpretation of this contradictory result was complicated by the possibility that the real GAD activity in brain apparently returned to the normal level during preparation of the enzyme solution and incubation for the enzyme assay. In fact, it was found that PLP content in brain of mice treated with PL rapidly increased to normal or higher levels during brain homogenizing and incubating for GAD assay because of synthesis of PLP from the large amount of PL in brain (data not shown). From these results and many previous reports that there is a correlation between the levels of PLP and the activity of GAD (9, 10, 20, 21), it is postulated that the real GAD activity at the onset of PL-induced convulsion must have been remarkably inhibited.

Effect of PL administration on GABA level in whole brain

The cause of anti B6-induced convulsion has been ascribed to the drop in the levels of GABA in brain (22), though the correlation between GABA levels and convulsions does not hold true in some instances (10, 23). The current investigation was carried out to determine whether GABA levels could be decreased by PL (PN or PM) administration.

All three compounds significantly decreased brain GABA content in mice one hour after administration of 1.8 mmol/kg which was correspond to convulsant dose of PL (Table 3). PL was more potent than PN, and PM was the least potent. Moreover, the degree of GABA decrease by PL increased markedly with increased doses of PL (Fig. 2A). The fate of GABA level, following the administration of 1.8 mmol/kg PL, is illustrated in Fig. 2B. There was a significant decrease in the GABA level during the 60 min period immediately following the administration at which mice were frequently attended by convulsions, but little further change occurred during the subsequent 4 hr.
Table 3. Effect of B6 vitamers administration on GABA and glutamate levels in whole brain. Values represent means±S.D. for number of experiments in parentheses.

|        | Control | PL (1.8 mmol/kg) | PL (3.6 mmol/kg) | PN (1.8 mmol/kg) | PM (1.8 mmol/kg) |
|--------|---------|------------------|------------------|------------------|------------------|
| GABA* (µmol/g) | 2.72±0.26 | 1.68±0.17* | — | 1.87±0.22* | 2.05±0.11* |
| (µmol/g)   | (15)    | (8)             | —               | (5)              | (5)              |
| Glutamate* (µmol/g) | 8.80±0.73 | 8.96±0.32 | 7.92±0.32 | — | — |

* GABA and glutamate were determined at the mean convulsion time, 60 min after 1.8 mmol/kg B6 vitamer administration and 30 min after 3.6 mmol/kg PL administration, respectively. * Significantly different from controls p<0.001.

These present results suggest that an alteration in GABA metabolism, more specifically the low GABA levels caused by inhibition of GAD, may be a major cause of PL-induced convulsions but not a result of the convulsions.

**Effect of PL administration on GABA level in synaptosomes**

Some experiments suggest a lack of correlation between GABA levels in whole brain and convulsions, but they do not rule out the possibility that GABA content in a subcellular site is a critical factor. For example, the GABA in the nerve endings may be directly involved in the production of convulsions (10) because the GABA-synthesizing enzyme, GAD, is located predominantly in the nerve endings (24).

When the effect of PL was studied in the primary subcellular fraction of brain, the decrease of GABA content was found in the 105g supernatant and in the crude
Table 4. Effect of PL administration on GABA and glutamate levels in subcellular fraction of brain. Values represent means ± S.D. for number of experiments in parentheses.

|                     | 10^6 Supernatant | Crude mitochondria | Synaptosomes | Myelin + Mitochondria |
|---------------------|------------------|--------------------|--------------|-----------------------|
| Control (6)         | 1.36 ± 0.03      | 0.797 ± 0.025      | 0.394 ± 0.009 | 0.270 ± 0.070         |
| PL (1.8 mmol/kg) (3)| 0.95 ± 0.02*     | 0.514 ± 0.028*     | 0.277 ± 0.003* | 0.205 ± 0.043         |
| PL (3.6 mmol/kg) (3)| 0.99 ± 0.05*     | 0.619 ± 0.026*     | 0.316 ± 0.012* | 0.248 ± 0.059         |

GABA and glutamate were determined at the mean convulsion time, 60 min after 1.8 mmol/kg PL administration and 30 min after 3.6 mmol/kg PL administration, respectively. * Significantly different from controls p<0.001.

mitochondrial fraction (Table 4). In view of the heterogeneity of the crude mitochondrial fraction, the content of GABA in the subfractions of this fraction was studied in more detail. Only a significant decrease of GABA was observed in synaptosomes after the PL treatment, whereas other subfraction (myelin and mitochondria) did not seem to be affected (Table 4). Although the degree of GABA decrease by PL was not different between in the 10^6 g supernatant (cytosol) and in the synaptosomes (nerve endings), the decrease of GABA level in the latter fraction may be the major factor in the seizure mechanism of PL-induced convulsion because GAD is located mainly in synaptosomes and the reaction product, GABA, is an inhibitory neurotransmitter substance as described above. By contrast, the glutamate level in each fraction was hardly affected by PL administration (Table 4), showing that glutamate might not be an important factor involved in the production of PL-convulsions.

From the present results and our previous report (10), it may be concluded that after the treatment of mice with PL the following sequence of events leading to the appearance of convulsions occurs in the nerve endings; inhibition of net synthesis of PLP → decrease of PLP levels → inhibition of GAD → decreased rate of GABA synthesis → decrease of GABA levels → convulsions.

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REFERENCES

1) MAKINO, K., KINOSHITA, T., and SASAKI, T. (1954): Atoxopyrimidine. Nature, 173, 34–35.
2) MASSIEU, G. H., ORTEGA, B. G., SYRQUIN, A., and TUENA, M. (1962): Free amino acids in brain and liver of deoxypyridoxine-treated mice subjected to insulin shock. J. Neurochem., 9, 143–151.
3) RINDI, G., and FERRARI, G. (1959): The γ-aminobutyric acid and glutamic acid content of brains of rats treated with toxopyrimidine. Nature, 183, 608–609.
4) Bessey, O. A., Adam, D. J. D., and Hansen, A. E. (1957): Intake of vitamin B6 and infantile convulsion: A first approximation of requirements of pyridoxine in infants. Pediatrics, 20, 33–44.
5) Hunt, A. D., Stokes, J., McCrory, W. W., and Stroud, H. H. (1954): Pyridoxine dependency: Report of a case of intractable convulsions in an infant controlled by pyridoxine. Pediatrics, 13, 140–145.
6) Courson, D. B. (1969): Vitamin B6 and brain function in animals and man. Ann. N. Y. Acad. Sci., 166, 7–15.
7) Tapia, R., Pérez, De La Mora, M., and Massieu, G. H. (1969): Correlative changes of pyridoxal kinase pyridoxal-5'-phosphate and glutamate decarboxylase in brain, during drug-induced convulsions. Ann. N. Y. Acad. Sci., 166, 257–266.
8) Minard, F. N. (1967): Relationships among pyridoxal phosphate, vitamin B6-deficiency, and convulsions induced by 1,1-dimethylhydrazine. J. Neurochem., 14, 681–692.
9) Pérez, De La Mora, M., Feria-Velasco, A., and Tapia, R. (1973): Pyridoxal phosphate and glutamate decarboxylase in subcellular particles of mouse brain and their relationship to convulsions. J. Neurochem., 20, 1575–1587.
10) Abe, M., and Matsuda, M. (1977): γ-Aminobutyric acid metabolism in subcellular particles of mouse brain and its relationship to convulsions. J. Biochem., 82, 195–200.
11) Curtis, D. R., Duggan, A. W., Felix, D., and Johnston, G. A. R. (1970): GABA bicuculline and central inhibition. Nature, 226, 1222–1224.
12) Obata, K., and Takeda, K. (1969): Release of γ-aminobutyric acid into the fourth ventricle induced by stimulation of the cat's cerebellum. J. Neurochem., 16, 1043–1047.
13) Kraft, H. G., Fierig, L., and Hotony, R. (1961): Zur pharmakologie des vitamin B6 und seiner derivate. Arzneimittel Forsch., 11, 922–929.
14) Loo, Y. H., and Badger, L. (1969): Spectrofluorimetric assay of vitamin B6 analogues in brain tissue. J. Neurochem., 16, 801–804.
15) Takahashi, Y., and Matsuda, M. (1975): Separate determination of vitamin B6 complex. Jikeikai Med. J., 22, 13–19.
16) Gray, E. G., and Whittaker, V. P. (1962): The isolation of nerve endings from brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat., 96, 79–88.
17) Kay, R. E., Harris, D. C., and Entenman, C. (1956): Quantification of the ninhydrin color reaction as applied of paper chromatography. Arch. Biochem. Biophys., 63, 14–25.
18) Schousboe, A., Wu, J. Y., and Roberts, E. (1973): Purification and characterization of the 4-aminobutyrate-2-ketoglutarate transaminase from mouse brain. Biochemistry, 12, 2688–2873.
19) Bain, J. A., and Williams, H. L. (1960): Inhibition in the nervous system and gamma-aminobutyric acid, ed. by Roberts, E., Pergamon Press, Oxford, p. 275.
20) Takahashi, Y., and Matsuda, M. (1976): Effects of penicillamine on the contents of B6 vitamers of the mouse brain. J. Nutr. Sci. Vitaminol., 22, 375–379.
21) Abe, M., and Matsuda, M. (1976): A correlation between changes in γ-aminobutyric acid metabolism and seizures induced by antivitamin B6. J. Biochem., 80, 1165–1171.
22) Killam, K. F., and Bain, J. A. (1957): Convulsant hydrazides I: in vitro and in vivo inhibition of vitamin B6 enzymes by convulsant hydrazides. J. Pharmacol. Exp. Ther., 119, 255–262.
23) Wood, J. D., and Peesker, S. J. (1973): The role of GABA metabolism in the convulsant and anticonvulsant actions of aminoxyacetic acid. J. Neurochem., 20, 379–387.
24) Salganicoff, L., and De Robertis, E. (1965): Subcellular distribution of the enzymes of the glutamic acid, glutamine and γ-aminobutyric acid cycles in rat brain. J. Neurochem., 12, 287–309.