Biochemical Pharmacology, Vol. 25, pp. 639–642. Pergamon Press 1976. Printed in Great Britain.

THE EFFECT OF CASTRIX (2-CHLORO-4-DIMETHYLAMINO-6-METHYLPYRIMIDINE) ON THE DISTRIBUTION OF B_6 VITAMERS IN MOUSE BRAIN

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(*Received* 19 *May* 1975; *accepted* 9 *September* 1975)

Abstract—The effect of castrix (2-chloro-4-dimethylamino-6-methylpyrimidine) on the distribution of B_6 vitamers in mouse brain was determined. The levels of pyridoxal and pyridoxal phosphate decreased, and the levels of pyridoxamine and pyridoxamine phosphate increased at the time of occurrence of convulsions. The decrease in pyridoxal phosphate was almost equal to the increase in pyridoxamine phosphate. A similar effect of castrix was observed in the preconvulsive period of 10 min before the expected onset of convulsions. 4-Deoxypyridoxine, toxopyrimidine, semicarbazide, isonicotinic acid hydrazide or aminooxyacetic acid, at subconvulsant doses, had an anticonvulsant action against the convulsions induced by castrix and prevented the increase in pyridoxamine phosphate produced by castrix, while thiosemicarbazide or penicillamine which did not have an anticonvulsant action did not prevent the increase in pyridoxamine phosphate produced by castrix. These results suggest that convulsions induced by castrix probably occur as a result of the inhibition of synthesis of pyridoxal phosphate from pyridoxamine phosphate.

Castrix (2-chloro-4-dimethylamino-6-methylpyrimidine) produces severe convulsions in mice, which are prevented by vitamin B_6 [1]. The decrease in glutamic decarboxylase (L-glutamate-1-carboxy-lyase, EC 4.1.1.15) activity observed after the administration of a number of antivitamin B_6 has been related to occurrence of convulsions [2]. However, castrix does not inhibit the enzyme [3]. Some antivitamin B_6 inactivate pyridoxal (PL) or pyridoxal phosphate (PLP) by combining with PL or PLP, while some of them can be phosphorylated by PL kinase (ATP:pyridoxal 5-phosphotransferase, EC 2.7.1.35) and then compete with PLP [4]. Castrix, however, cannot react with PL or PLP, nor be phosphorylated by PL kinase, and the inhibitory action of castrix on PL kinase is only slight [3]. Thus, the mechanism of the antagonism between castrix and vitamin B_6 seems to be different from that of the other antivitamin B_6.

The present investigation was undertaken to determine the effects of castrix on the distribution of B_6 vitamers in brain.

MATERIALS AND METHODS

Chemicals. 4-Deoxypyridoxine (DOPN), DL-penicillamine (PA), thiosemicarbazide (TSC), semicarbazide (SC), isonicotinic acid hydrazide (INH) and B_6 vitamers were obtained from Nakarai Chemicals. Acid phosphatase was obtained from Boehringer Manheim. Castrix was obtained from Takeda Chemical Industries, Ltd. Aminooxyacetic acid (AOAA) was obtained from Sigma Chemicals. Toxopyrimidine (TXP) was obtained from Sankyo Pharmaceuticals.

Injections. Male dd mice weighing 15–20 g were used. All compounds were injected intraperitoneally. The solutions of drugs were prepared daily in water, the pH being adjusted to 7·0 immediately before use. The final concn of drugs was adjusted so that the required dosage was administered at 1·0% of the body weight. The dose of castrix was 2·5 mg/kg.

Extraction, separation and determination of B_6 *vitamers.* Mice given castrix alone or castrix together with TSC or PA were sacrificed in the first convulsion at 30–60 min after the injection, and other groups of mice without occurrence of convulsions were sacrificed within 10 min after the onset of convulsions in mice given castrix alone, except where specified. Different forms of vitamin B_6 were extracted from the brain by the method of Bain and Williams [5] and separated by ion exchange chromatography on Amberlite CG 120 according to the method of Loo and Badger [6] except for the use of 20 ml of hot (about 50°) buffer for the elution of pyridoxine (PN). The eluate of each compound was collected in one fraction. Since in this method pyridoxine phosphate (PNP) and PLP were eluted in the same fraction, both phosphate esters were hydrolyzed to PN or PL by the addition of acid phosphatase (1·7 mg/5 ml) and then separated by rechromatography. This rechromatography was sometimes omitted because only a trace of PNP was present in mouse brain and interfered only negligibly with the estimation of PLP. Hydrazides such as INH, SC or TSC have been shown to form hydrazones of PL or PLP in the tissue of hydrazide-treated animals [5], and these hydrazones or the complex formed between PL and PA or AOAA were found to interfere with the microbiological assay of B_6 vitamers. These compounds were eluted in the PLP, pyridoxamine phosphate (PMP), PL, PN or pyridoxamine (PM) fractions according to Loo and Badger's method. Therefore, as shown in Table 1, these compounds were removed from the PLP, PMP or PL fractions by a modification of Loo and Badger's method or by ion-exchange rechromatography after an appropriate treatment. Some compounds eluted in PN or PM fractions were not removed. The

Table 1. The methods to remove interfering substances from B_6 vitamers

Interfering substance	Fraction (ml) PLP (18)	PIC (15)	PMP (25)	(10)	PL (25)	PN (20)	PM (25)	Method to remove interfering substance
Isonicotinic acid hydrazone of PLP					*	*		Rechromatography of PL fraction after reduction with $NaBH_4$†
Isonicotinic acid hydrazone of PL						*	*	
Semicarbazone of PLP	*	*						Rechromatography of PLP fraction after hydrolysis by acid phosphatase‡
Semicarbazone of PL						*	*	
Thiosemicarbazone of PLP		*	*					Buffer volume of PIC fraction was increased to 30 ml
Thiosemicarbazone of PL						*	*	
Thiazolidine of PLP and PA	*							Rechromatography of PLP fraction after hydrolysis by acid phosphatase§
Thiazolidine of PL and PA				*	*			The volume of 0·1 M acetate buffer, pH 5·0 was increased to 20 ml
Complex of PLP with AOAA	*							Rechromatography of PLP fraction after hydrolysis by acid phosphatase‖
Complex of PL with AOAA			*¶					

PIC = pyridoxic acid.

* Indicates the fraction which was interfered.

† 0·5 ml of 5 N KOH was added to 12·5 ml of PL fraction, and 7 mg $NaBH_4$ was added during mixing. After 30 minutes at 30° the mixture was added to 0·5 ml of 4 N HCl and 0·3 ml of 1 M acetate buffer, pH 3·5, and was applied to a column of Amberlite CG 120. The column was rinsed with 10 ml each of water, 0·1 M acetate buffer, pH 5·0, and 0·1 M phosphate buffer, pH 6·0 to elute reduced isonicotinic acid hydrazone of PLP, and then PL was eluted as PN borate complex with 25 ml of 0·1 M phosphate buffer, pH 6·0.

‡ 5 ml of PLP fraction adjusted to pH 4·5 was incubated with 1·7 mg of acid phosphatase at 37° for 2 hr. After addition of 1·0 ml of 0·01 M acetate buffer, pH 3·5, the mixture was applied to the column. After rinsing with 10 ml each of water and 0·1 M acetate buffer, pH 5·0, PLP was eluted as PL with 25 ml of 0·1 M phosphate buffer, pH 6·0. The semicarbazone of PL remained on the column.

§ The same method as the separation of PLP from the semicarbazone of PLP was used except that the volume of 0·1 M acetate buffer, pH 5·0 was increased to 20 ml.

‖ The same method as the separation of PLP from the semicarbazone of PLP.

¶ Since the complex of PL with AOAA was about seven times less active in promoting growth than PL, and the amount of the complex formed in brain may be very small compared with the amount of PMP, it will interfere negligibly with the microbiological assay of PMP.

B_6 vitamers in the eluates were individually assayed microbiologically with *Saccharomyces carlsbergensis* (ATCC 9080) against its own vitamer as reference standard which was dissolved in saturated perchlorate and treated on the column in the same way. Phosphate esters were hydrolyzed by acid phosphatase (0·34 mg/ml) before the assay. The assay method was modified from that of Chiao and Peterson [7]: the concentration of each component except hydrolyzed casein and sugar was half that of their basal medium. Thiamine was added at 500 μg/l.

RESULTS

The effect of castrix on the distribution of B_6 *vitamers in mouse brain*

Mice were given castrix and sacrificed in the first convulsion, and B_6 vitamers in the brain were assayed (Table 2). Total amounts of vitamin B_6 did not change after the injection of castrix. While the levels of PL and PLP decreased significantly, the levels of PM and PMP increased significantly after the injection of castrix. The decrease in PLP was almost equal to the increase in PMP. Only trace amounts of PNP and PN (0·11 ± 0·09 and 0·02 ± 0·01 nmoles/g wet wt tissue, respectively) were present in mouse brain as reported previously [5], and they appeared not to be affected by castrix.

The effect of castrix was determined also in the preconvulsive period of 10 min before the expected first convulsion or after 10 or 20 min of the first convulsion, and as shown in Fig. 1 an inverse relationship between the concentrations of brain PLP and PMP was demonstrated.

The effect of antivitamin B_6 *on the distribution of* B_6 *vitamers in brain of castrix treated mice*

The toxic action of castrix was protected not only by vitamin B_6 but also by some antivitamin B_6 at their subconvulsive doses [3]. Therefore, the effect of some antivitamin B_6 on the action of castrix was also assessed.

The effect of antivitamin B_6 *which protected mice from castrix-induced convulsions.* When mice were injected with DOPN, TXP, SC or INH alone at subconvulsive doses, the brain PLP level decreased but the PMP level did not change significantly as shown in Table 2. In DOPN or TXP treated mice the increase in PL was observed, and in SC or INH treated mice the bulk of the PLP was recovered as its hydrazone (data were not shown). AOAA at a subconvulsive dose did not alter the PLP level, but decreased markedly the PMP level. Only a trace of PLP complex with AOAA was found (data were not shown). On the other hand, when mice were given castrix together with one of the antivitamin B_6 at a subconvulsive dose the convulsions, and the increase in the PMP level which was observed after the treatment with castrix alone did not occur as shown in Table 2.

The time course of the effect of DOPN on the level of PLP or PMP in castrix-treated mice is shown in Fig. 2. The increase in the PMP level did not occur

Table 2. The effect of antivitamin B_6 on the distribution of B_6 vitamers in brain of castrix-treated mice

Experiment	Treatment	Dose (mg/kg)	No. of mice: treated	No. of mice: convulsing	Total B_6 (as PN.HCl)	PLP	PMP	PL	PM
1	Control	—	20	0	100 ± 5 (15·18 ± 0·83)	100 ± 4 (7·11 ± 0·28)	100 ± 5 (8·12 ± 0·37)	100 ± 14 (0·36 ± 0·05)	100 ± 15 (0·07 ± 0·01)
1	Castrix	2·5	20	20	101 ± 5[a]	89 ± 6[a]	110 ± 3[a]	78 ± 14[a]	143 ± 43[a]
2	DOPN	50	5	0	93 ± 5	65 ± 5[c]	103 ± 3[c]	241 ± 15[c]	257 ± 114[b]
3	TXP	25	5	0	92 ± 3	89 ± 2[f]	101 ± 1[c]	139 ± 6[g]	44 ± 44[f]
4	SC	50	4	0		40 ± 3[a]	95 ± 6[c]	112 ± 13[c]	
5	AOAA	30	4	0	87 ± 2	103 ± 8[c]	73 ± 3[a]	88 ± 11[c]	111 ± 44[c]
7	TSC	5	5	0	-	85 ± 5[d]	105 ± 3[c]	75 ± 8[f]	-
8	PA	50	4	0		98 ± 4[c]	109 ± 7[c]	94 ± 15[c]	129 ± 21[c]
2	Castrix + DOPN*	2·5 ± 50	5	0	96 ± 3	83 ± 2[a,g]	94 ± 2[b,g]	237 ± 13[c,h]	343 ± 71[c,h]
3	Castrix + TXP*	2·5 + 25	5	0	99 ± 4	86 ± 6[a,h]	99 ± 3[c,h]	120 ± 27[c,h]	47 ± 18[b,h]
4	Castrix + SC*	2·5 + 50	9	0	—	38 ± 4[a,h]	100 ± 3[c,h]	106 ± 16[c,h]	-
5	Castrix + AOAA†	2·5 + 30	4	0	94 ± 9	98 ± 2[c,h]	79 ± 6[a,h]	86 ± 20[c,h]	100 ± 30[c,h]
6	Castrix + INH*	2·5 + 60	8	0		56 ± 4[a]	100 ± 4[c]	105 ± 13[c]	-
7	Castrix + TSC*	2·5 + 5	9	9		86 ± 6[a,i]	109 ± 3[a,i]	80 ± 8[a,i]	-
8	Castrix + PA*	2·5 + 50	5	5		81 ± 5[a,i]	110 ± 3[a,i]	76 ± 8[a,i]	124 ± 12[c,i]

* Castrix and antivitamin B_6 were administered simultaneously.

† Aminooxyacetic acid was administered 1 hr prior to injection of castrix. Values are expressed as per cent of the control in each experiment. The values of control and castrix alone groups in experiments 2–8 are not shown as the values are not significantly different from those in experiment 1. The absolute values of controls, expressed as nmoles/g wet wt tissue (mean ± S.E.), are given in parentheses.

[a] $P < 0.005$; [b] $P < 0.01$; [c] $P < 0.025$; [d] $P < 0.05$; [e] $P > 0.05$ against control in each experiment. [f] $P < 0.005$; [g] $P < 0.01$; [h] $P > 0.05$ against corresponding antivitamin B_6 alone. [i] $P > 0.05$ against castrix alone in each experiment.

at any time equivalent to the onset of castrix convulsion, 10 min before it or 20 min after it.

The effect of antivitamin B_6 which did not protect mice from castrix-induced convulsions. PA and TSC at their subconvulsive doses did not protect against castrix convulsions [3], but rather shortened the time to onset of convulsions. As shown in Table 2 these antivitamin B_6 did not affect significantly the distribution of B_6 vitamers in brain of castrix-treated mice.

The effect of PM on the distribution of B_6 vitamers in brain of castrix-treated mice

When mice were given PM at a dose of 3·0 mg/kg together with castrix, about half the mice were protected from the convulsions. When mice were given only PM at this dose, total amounts of vitamin B_6 in brain increased by 13%, which was equal to 0·24% of the PM injected, and the levels of both PLP and

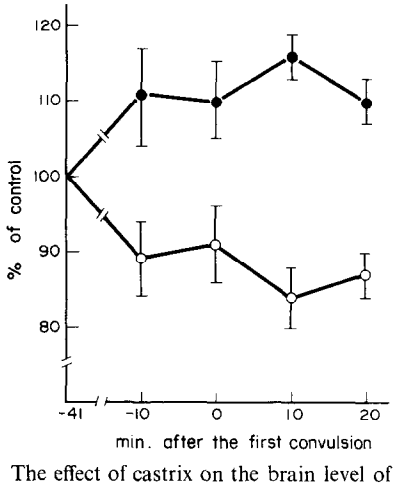

Fig. 1. The effect of castrix on the brain level of PLP and PMP. ○, PLP; ●, PMP. The absolute value of the control: PLP, 7·14 ± 0·26 nmoles/g wet wt tissue; PMP, 8·26 ± 0·22 nmoles/g wet wt tissue. Each point represents mean ± S.E. of five mice. The first convulsion occurred 41 ± 5 min after the injection of castrix.

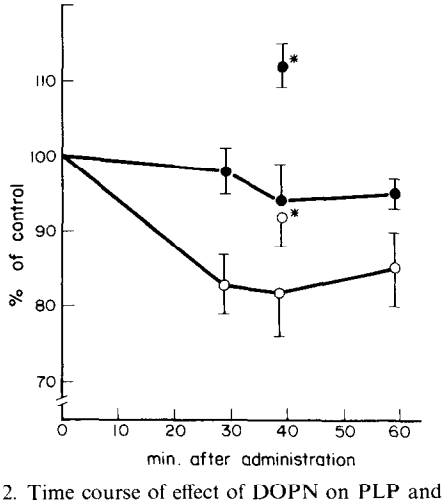

Fig. 2. Time course of effect of DOPN on PLP and PMP levels in brain of castrix-treated mice. ○, PLP; ●, PMP. The absolute value of the control: PLP, 7·07 ± 0·29 nmoles/g wet wt tissue; PMP, 7·98 ± 0·63 nmoles/g wet wt tissue. Each point represents mean ± S.E. of five mice. Mice were given DOPN (50 mg/kg) and castrix (2·5 mg/kg) simultaneously or only castrix (*). Mean convulsion time of mice given only castrix was 39 ± 3 min.

Table 3. The effect of PM on the distribution of B_6 vitamers in brain of castrix-treated mice

	Dose (mg/kg)	No. of mice		Total B_6	PLP	PMP	PL	PM
		treated	convulsing					
Control	—	5	0	100 ± 3	100 ± 3	100 ± 3	100 ± 14	100 ± 38
PM	3·0	5	0	113 ± 4[a]	108 ± 4[b]	108 ± 5[c]	209 ± 14[a]	238 ± 38[a]
Castrix	2·5	5	5	100 ± 5[e]	86 ± 4[a]	116 ± 3[a]	88 ± 18[e]	138 ± 62[e]
Castrix + PM*	2·5 + 3·0	5	3	102 ± 2[e,h]	92 ± 4[b,g]	110 ± 4[a,g]	168 ± 20[e,f]	181 ± 67[d,h]

* Castrix and PM were administered simultaneously. Results are given as per cent of control (mean ± S.E.).
[a] $P < 0{\cdot}005$; [b] $P < 0{\cdot}01$; [c] $P < 0{\cdot}025$; [d] $P < 0{\cdot}05$; [e] $P > 0{\cdot}05$ against control. [f] $P < 0{\cdot}005$; [g] $P < 0{\cdot}05$; [h] $P > 0{\cdot}05$ against castrix only.
Amounts of PNP and PN increased negligibly after administration of PM, 3·0 g/kg.

PMP increased (Table 3). On the other hand, when mice were given PM together with castrix, total amounts of vitamin B_6 in brain did not increase significantly. The level of PMP was lower, and the level of PLP was higher than that in castrix-only treated mice.

DISCUSSION

Castrix decreased brain PL and PLP levels, and increased PM and PMP levels at the time of occurrence of convulsions. The decrease in PLP was much smaller than that observed at the time of convulsions induced by other antivitamin B_6 [5, 8–12], and was not associated with a rise in the PL level. The decrease in PLP was almost equal to the increase in PMP. A similar inverse relationship between the concentrations of PLP and PMP was observed also in the preconvulsive period of 10 min before the expected onset of convulsion and 10 or 20 min after the onset of convulsion. This suggests that the decrease in the PLP level concomitant with the increase in the PMP level may be relevant to the course of castrix convulsions.

DOPN, TXP, SC, INH and AOAA, each of which prevented castrix convulsions at their subconvulsive doses, were found to prevent the increase in the PMP level produced by castrix, while TSC and PA, which did not prevent castrix convulsions, did not prevent the increase in the PMP level produced by castrix.

These results indicate that convulsions probably occur as a result of the inhibition of synthesis of PLP from PMP by castrix and that if the inhibition is removed by a certain antivitamin B_6 at a subconvulsive dose, the convulsions do not occur. If an antivitamin B_6 removes completely the inhibition of the synthesis of PLP from PMP and its action is not affected by castrix, the distribution pattern of B_6 vitamers in castrix plus the antivitamin B_6-treated mice should be similar to that in the antivitamin B_6-only treated mice. This tendency was observed except in the case of DOPN (Table 2). The brain level of PLP in castrix plus DOPN-treated mice was significantly lower than that in castrix-treated mice, but higher than that in DOPN-treated mice. This seems to indicate that the action of DOPN was affected by castrix.

No regular correlation between the decrease of total PLP in brain and convulsions by carbonyl trapping agents has been shown [8, 9, 13]. Minard suggested that hydrazine convulsions resulted from a compartmental deficiency of PLP in brain [8]. The present data also confirm a lack of correlation between the decrease of total amounts of PLP and convulsions, and suggest the compartmentation of PMP $\leftrightharpoons$ PLP at a functionally active site. Furthermore, the change in the distribution of B_6 vitamers induced by castrix suggests that the decrease in the brain PLP level may be caused through the inhibition of PMP oxidase (pyridoxaminephosphate: oxygen oxidoreductase (deaminating) EC 1.4.3.5) rather than PL kinase: PLP synthesized from PMP, distinct from PLP synthesized by phosphorylation of PL, might have an important role in brain function. In fact the inhibitory action of castrix was observed on PMP oxidase activity in the supernatant of mouse brain homogenate: 50% inhibition was observed with 1 mM castrix at pH 10·2. A detailed study of the inhibition of castrix on purified PMP oxidase is in progress.

The mechanism of the antagonism by some antivitamin B_6 of castrix convulsion remains to be determined. The similar effect of PM and the antivitamin B_6 on the PMP level in castrix-treated mice suggests that PM and the antivitamin B_6 compete with castrix by the same mechanism.

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