The Role of Zinc in Brain Excitability : The Effect of Disturbed Zinc Metabolism on Brain Excitability and Relationship of Brain Carbonic Anhydrase (Zinc Metalloemzyme) to Brain Excitability

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INTRODUCTION

In 1951, Tokuoka [25] conducted caudal resection of the pancreas in patients with genuine epilepsy and demonstrated remission of the seizures in most of the cases, suggesting the possibility of participation of abnormal zinc metabolism in the appearance of epileptic seizures. Experiments have been carried out in our department in order to elucidate the mechanism of such phenomenon. Concerning the effect of zinc metabolism on brain excitability, Nagasue (1957) [16] [17] of our department demonstrated the elevation of the electroshock seizure threshold as determined by cardiazol in zinc deficient sucking mice, and depression of the threshold in zinc excessive sucking mice. He has also speculated the possible participation of carbonic anhydrase as zinc enzyme in the changes of brain excitability.

However, the method using cardiazol is not entirely satisfactory to measure the brain excitability. As Takahashi et al. (1961) [22] [23] stated, the problem of brain excitability should be studied from the point of the seizure threshold and the intensity of seizures as a more reasonable approach. From this viewpoint, Woodbury-Davenport's [28] apparatus of electrical stimulation is extremely useful. It is therefore desirable to study the brain excitability in animals with disturbed zinc metabolism more accurately with this apparatus.

Carbonic anhydrase, containing zinc as an important component, is known to change its activity according to the content of zinc. [9] This zinc metalloenzyme has an intense catalytic action on the reversible reaction of $CO_2+H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO^-{}_3$. [12] Several investigators [3] [10] [13] [15] have already studied the relationship between this enzyme and brain excitability. A good agreement has been demonstrated between the activity of this enzyme in the brain and the level of brain excitability. No report is available, however, concerning the carbonic anhydrase activity in the state of disturbed zinc metabolism.

In this respect, the relationship between zinc metabolism and the brain

excitability should be studied, especially in reference to the activity of brain carbonic anhydrase.

EXPERIMENTAL MATERIALS AND METHODS

1. Animals

Mice of dd strain were maintained on the Oriental Solid Chow (water 7%, crude protein 24.2%, crude ash 6.2%, crude fiber 4.5%, soluble nitrogen-free extract 52.6%) together with vegetable and water.

Zinc deficient mice. According to the method described by Nishimura (1953),[18] the newly born mice were divided into two groups within 12 hours after birth. The first group was left with their own mothers as controls, while the second group was nurished by foster mothers 2 weeks after dilivery. Since zinc content in the milk is markedly decreased in later stage of lactation, the foster sucking mice show a characteristic appearance for zinc deficiency later than 10 days after birth. Nishimura[18] confirmed that the zinc content of these zinc deficient sucklings was decreased to 2/3 of the controls. Experiments were performed at 15 days of age.

Zinc excessive mice. Mice of dd strain of 17-23g were used. Zinc chloride, 0.05ml of 0.1 % solution with pH adjusted to 2.0 by the addition of HCl, was subcutaneously injected daily for 10 days in some animals, while tap water in which zinc chloride was dissolved to make 0.02% solution was given for drinking for 4 weeks in others, in order to prepare the zinc excessive animals. Food and water was given ad libitum.

ep-mouse. ep-mouse, a convulsive strain of mouse discovered by Imaizumi (1959),[7] was used as the mouse with enhanced brain excitability.[24] In this mouse a convulsive seizure has been induced by successive and alternating movements causing loss of postural equilibrium. The animals were bred in this laboratory and adult mice of both sexes, at least 12 weeks old, were used.

2. Determination of the brain excitability

Woodbury-Davenport's apparatus[28] for electrical stimulation was used to determine the brain excitability. Stimulation was given by application of the current on the cornea through a silver plate, using electroshock seizure threshold (EST) and the duration of maximal electroshock seizures (MES) as index. EST was expressed as the intensity of electrical current necessary to cause minimal clonic seizure of the head and anterior limbs upon electrical stimulation with 60 cycle/sec. for 0.2 sec.[22] In zinc deficient mice, the frequency of appearance of seizures more intense than EST upon stimulation with various intensities of electrical current was obtained and statistical analysis was carried out according to Litchfield-Wilcoxon (1949)[11] to calculate the EST_{50} as the intensity of

the current to cause minimal electroshock seizure in one half of the animals. For the determination of MES, electrical current 7 times as intense as the EST, 100 mA in zinc deficient animals and 50 mA in zinc excessive animals, were used for stimulation. Such stimulation caused tonic flexion or extension and clonic seizure. Since the intensity of seizure was best expressed by the tonic extension,[22] attention was particularly focussed on the period of tonic extension.

Since brain excitability, especially EST, was readily influenced by the temperature,[27] experimental animals were kept in a room in which temperature was maintained as constant as possible, within the range of 21-24°C for the determination of brain excitability.

3. Determination of carbonic anhydrase activity

Animals were killed by decapitation. Fresh brain tissue was taken out and homogenized in ice-cold distilled water in a glass homogenizer. An aliquot of the homogenate was used for the determination of carbonic anhydrase activity with Nishimura's modification(1963)[19] of Maren's pH changing method(1963),[12] which is based on the measurement of the time required for neutralization of an alkaline solution by the formation of carbonic acid from carbon dioxide and water. Carbonic anhydrase activity was expressed as $\frac{To-Te}{Te}$, where To represents the time required for the non-enzymological reaction to reach the end point, and Te corresponds the value in the presence of carbonic anhydrase. In a preliminary experiment of in vivo perfusion, absence of a significant difference in the carbonic anhydrase activity in the brain might permit to disregard the influence of carbonic anhydrase activity in red blood cells.

4. Determination of the water content of the brain

The brain tissue was dried in a desiccator at 110°C for 12 hours and the difference in weight was used to show the water content.

EXPERIMENTAL RESULTS

A. The effect of zinc intoxication on brain excitability and brain carbonic anhydrase activity

1. External appearance and behavior

Zinc deficient mice. Zinc deficient mice at the 15th day after birth showed disturbance in the development of body hair, bright colored skin, formation of folds, and localized rosary-like swelling in the tail, as shown in Fig. 1. The mobility of the hind limbs, with the thickened skin, was restricted. As shown in Fig. 2, the body weight-histogram in zinc deficient mice demonstrated a narrower distribution, 80% being within the range of 4.5-5.5g.

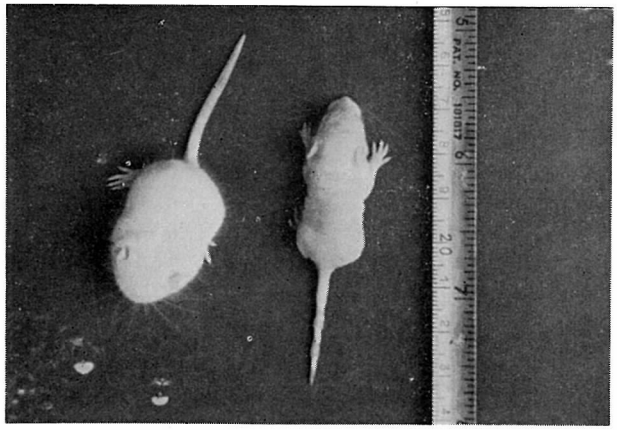

Fig. 1. External appearance of zinc deficient mice. Note rosary-like swelling in the tail and retardation of hair development.

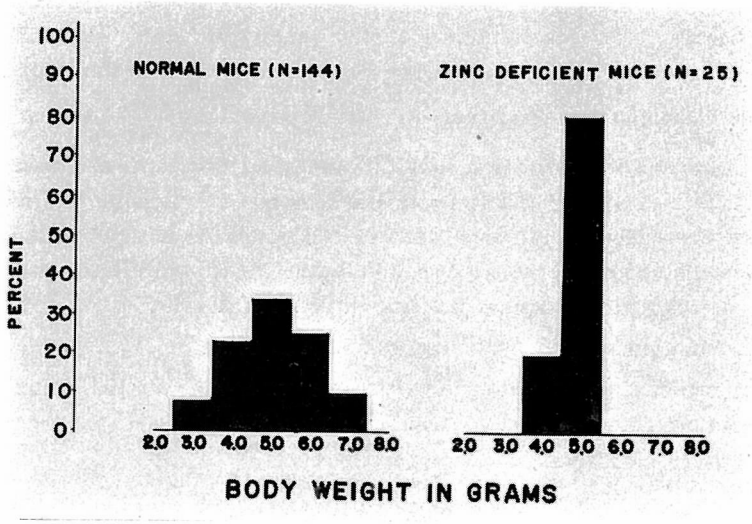

Fig. 2. Body weight of normal and zinc deficient mice at 15 days of age.

Zinc excessive mice. Under the conditions of the present experiment, a slight decrease in body weight was the only change characterizing zinc excessive mice as compared with the controls. Neither skin change nor diarrhea was noted. Upon completion of the zinc excessive state, the movement became active with occasional jumping on hind limbs.

2. Brain excitability

Zinc deficient mice. The brain excitability of zinc deficient mice is shown in Table 1. The EST_{50} in normal mice was 11.6 mA (12.6-10.6 mA of 95%

Table 1. Brain excitability of zinc deficient mice

Group	EST mA		§ MES sec
	EST 50	95% Confidence limits	
Zinc deficiency	13.1 (20)	15.2−11.3	* 9.6±2.2 (15)
Control	11.6 (51)	12.6−10.6	11.7±3.3 (43)

Experimental animals, at 15 days of age, weigh 3.8-6.0g.
§ The duration of the tonic extensor phase was measured as the most representative index of MES.
* P <0.05 Numbers of experimental animals are shown in parentheses.

confidence limits), being higher than 8 mA reported by Vernadakis and Woodbury in CF♯I mice.[27] Difference in species and in the condition of breeding might explain such changes. The EST_{50} in zinc deficient mice, 13.1 mA (15.2-11.3 mA), was definitely higher than in the controls. Concerning MES, both normal and zinc deficient mice showed tonic extension in almost all animals upon stimulation with 100 mA for 0.2 sec. However, clonic phase of seizure was indistinct and tonic flexion was somewhat incomplete. Duration of tonic extensor phase alone was therefore selected for the measurement. As shown in Table I, MES was 9.6±2.2 sec. in zinc deficient mice, indicating a significant shortening from the corresponding value in control mice ($p<0.05$). In zinc deficient mice, the brain excitability was characterized by elevation of seizure threshold and decrease in the intensity of seizure.

Zinc excessive mice. The EST in zinc excessive mice prepared by subcutaneous administration of zinc chloride showed a highly significant decrease, 6.5±0.18 mA as compared with 6.9±0.30 mA in the controls as shown in Table 2. MES,

Table 2. Brain excitability of zinc excessive mice prepared by subcutaneous injection of zinc.

		Zinc excess	Control
EST mA		* 6.5±0.18 (19)	6.9±0.30 (17)
MES sec	TF	2.0±0.21 (12)	1.9±0.20 (12)
	TE	13.6±2.1 (12)	12.6±1.4 (12)
	CL	3.0±0.75 (12)	3.5±0.94 (12)

Zinc chloride, 0.05ml of 0.1% solution, was subcutaneausly injected daily for 10 days. Experimental animals were 20-25g in weight; average weight was 22.8g in zinc excessive mice and 22.5g in control mice.
TF : Tonic flexor phase TE : Tonic extensor phase CL : Clonic phase
* p <0.005 Numbers of experimental animals are shown in parentheses.

on the other hand, failed to show a significant difference between two groups. Similar findings were also obtained upon oral administration. As shown in Table 3, EST was 6.3±0.16 mA in zinc excessive mice, while the corresponding value was 6.6±0.21 mA in the controls, indicating a significant decrease. No significant difference was observed in MES. Although a decrease in body weight was noted after administration of a large amount of zinc,[21] the dose administered in the present experiment did not cause such change, precluding the possibility that the depression of seizure threshold was due to changes in body weight. Summarizing the results mentioned above, depression of seizure threshold was seen in the state of zinc excess, but the intensity of seizure was unchanged.

Table 3. Brain excitability of zinc excessive mice prepared by oral administration of zinc.

		Zinc excess	Control
EST mA		* 6.3±0.16 (10)	6.6±0.21 (10)
MES sec	TF	1.5±0.18 (12)	1.4±0.15 (12)
	TE	12.3±2.5 (12)	13.0±1.2 (12)
	CL	4.0±1.1 (12)	4.0±1.0 (12)

Zinc chloride, dissolved in drinking water at a concenration of 0.02%, was given for 4 weeks. Experimental animals were 17-23g in weight; average weight was 19.4g in zinc excessive mice and 20.8g in control mice.
TF : Tonic flexor phase TE : Tonic extensor phase Cl : Clonic phase
* $p<0.005$ Numbers of experimental animals are shown in parentheses.

3. Carbonic anhydrase activity in the brain

The carbonic anhydrase activity in 100 mg of fresh brain tissue is shown in Table 4 and 5. The activity was 29.2±3.3 units in zinc deficient mice, representing a definite decrease as compared with the level in control mice, 36.2±6.3 ($p<0.005$). Since no difference was found in the water content of the brain, a significant difference was also found in the activity per unit dry weight. On the other hand, in zinc excessive mice, activity of 92.5 units was found in the mice given evcessive zinc subcutaneously as compared with 93.8 units in the controls and 120.2 units were demonstrated in the mice given excessive zinc orally as compared with 118.1 units in the controls, showing absence of significant difference between zinc excessive mice and the controls. Water content in the brain showed no significant difference.

B. Activity of brain carbonic anhydrase in ep-mice

The activity of carbonic anhydrase in each part of the ep-mice brain is shown

Table 4. Activity of brain carbonic anhydrase in zinc deficient mice. The enzymatic activity was assayed by Nishimura's modification of Maren's method.

	Water content (%)	Carbonic anhydrase activity (activity unit/100mg w. w.)
Zinc deficiency	82.3±0.48 (4)	*29.2±3.3 (10)
Control	82.0±0.79 (8)	36.2±6.3 (16)

* $P<0.005$ Numbers of experimental animals are shown in parentheses.

Table 5. Activity of brain carbonic anhydrase in zinc excessive mice

Group	Water content (%)	Carbonic anhydrase activity (activiy unit/100mg w. w.)
Subcutaneous injection		
Zinc excess	76.6±0.40 (5)	92.5±12.2 (10)
Control	76.9±0.17 (5)	93.8±13.6 (10)
Oral administration		
Zinc excess	77.2±0.41 (5)	120.2±21.1 (8)
Control	77.1±0.42 (5)	118.1±12.0 (8)

Zinc, as the chloride, was given subcutaneously in a daily dose of 50mg for 10 days or orally at a concentration of 0.02% in drinking water for 4 weeks.
Numbers of experimental aminals are shown in parentheses.

in Fig. 3. The most striking change was demonstrated in the cerebellum ; the enzymatic activity of ep-mice was about 40% higher than that of dd-mice. Another significant change was observed in the cerebrum. Although it has been shown that the seizure activity of ep-mice originates in the limbic system,[8] carbonic anhydrase activity in the hippocampus and dentate gyrus of ep-mice was not significantly changed. The midbrain, pons and medulla oblongata showed the highest activity but there was no change between ep-mice and dd-mice.

DISCUSSION

In the present study a rather long term experiment was carried out from the view point of disturbance in zinc metabolism. It is clearly demonstrated that

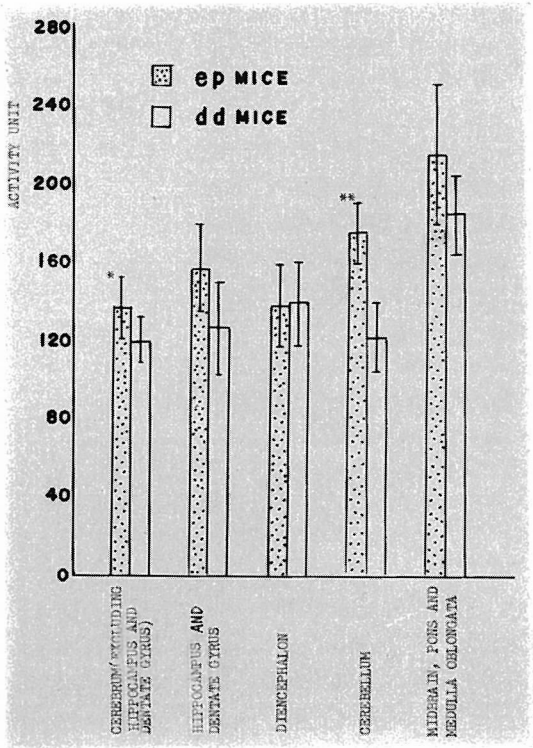

Fig. 3. Activity of brain carbonic anhydrase in ep-mice. Each bar represents the mean of 5-9 animals. The small horizontal lines at the tops of the bars denote the standard deviations.
* $P<0.05$
** $P<0.01$

the disturbance in zinc metabolism may influence the excitability of the brain in response to electrical stimulus; zinc deficiency caused an elevation of seizure threshold, while zinc excess caused a depression, in agreement with Nagasue's study[16) 17)] on the changes of seizure threshold using cardiazol. On the other hand, the intensity of seizures as expressed by the duration of maximal electroshock seizures showed a decrease in zine deficiency but failed to show a significant change in zinc excess.

Since the body weight of experimental animals might influence the result of measurement of brain excitability with Woodbury's apparatus,[28)] giving some restriction on the experimental condition, following points should be considered in the evaluation of these results.

First, experiments for zinc deficiency were carried out at the developmental stage, when the manifestation of zinc deficiency appears rapidly and intensely. During this period, however, excitability and metabolism of the brain exhibit a

profound change. Concerning the brain excitability, EST of mice decreases slowly but markedly until the 18th day after birth followed by a slow rise, probably in relation to the development of excitatory and inhibitory system of the brain.[27] According to Millichap (1957),[15] on the other hand, appearance of tonic pattern is insufficient at this stage. In rats the appearance of tonic pattern is said not to appear until the 15th day after birth. Concerning the metabolism of the brain, the type of metabolic process changes from anaerobic to aerobic state, together with the changes in various chemical components.[14] Effects on zinc metabolism at this stage, therefore, cannot be regarded as the same with those at the mature stage. The results of the present experiments in zinc deficient mice should be regarded as the effect on the development of seizure activity to be accurate. Zinc deficiency therefore appears to delay the development of seizure activity. In zinc excess, on the other hand, no significant difference was noted in MES. Effect of further zinc excess on MES remains to be determined.

Scarcely any information is at present available concerning the biological significance of zinc in the brain and the mode of action of zinc on the metabolic process is also unknown. As the role of zinc in the brain on brain excitability, carbonic anhydrase should be mentioned first. According to Ashby's hypothesis (1952),[3] this enzyme removes excessive CO_2 produced as the result of metabolic process and quickly restores the excitability of neurons. Activity of this enzyme in the brain shows a good agreement with the level of brain excitability.[3][10][13][15] Studies using a specific inhibitor of carbonic anhydrase, acetazolamide, resulted in a marked shortening of MES but EST scarcely showed any change,[29] suggesting the influence of carbonic anhydrase of the brain on the intensity of seizures. In the present experiment, decrease in the carbonic anhydrase activity of the brain was found in zinc deficient mice with a difference in MES, while no significant change was seen in zinc excessive mice without a change in MES, indicating a relationship between carbonic anhydrase of the brain and the intensity of seizures. Concerning the carbonic anhydrase activity in conditions with disturbed zinc metabolism, no decrease in carbonic anhydrase activity was found in the lens of rabbit maintained on zinc free diet.[4] Ratio between carbonic anhydrase and hemoglobin in red blood cells of zinc deficient rats was reported to show no significant difference from the control level.[6] Environmental deficiency of zinc therefore does not appear to influence the carbonic anhydrase activity in mature animals. This does not conflict with the fact that the zinc contained in the molecule of carbonic anhydrase resists exchange.[26] The decrease in carbonic anhydrase activity in zinc deficient sucklings therefore may be reasonably explained as follows, although a direct of zinc deficiency cannot be ruled out. In the developmental stage of the brain, zinc deficiency inhibits the maturity by delaying the transition of brain metabolism from anaerobic to

aerobic type. Since carbonic anhydrase of the brain appears with the aerobic metabolism and its activity parallels with oxygen consumption, [1) 2) 19)] inhibition of maturity is naturally expected to be associated with the decrease is carbonic anhydrase activity. The decrease in carbonic anhydrase activity in the brain of zinc deficient sucking mice prepared according to Nishimura therefore may be the secondary effect via aerobic metabolism.

The interpretation that changes in carbonic anhydrase activity is secondary to chemical changes in the brain may be supported by the present findings in ep-mice. Carbonic anhydrase activity of ep-mice was markedly higher in the cerebellum, which has no direct causal relations with the seizure activity. In contrast, there was no significant change in the hippocampus and dentate gyrus, which are closely connected with the seizure activity in animals.[8)] Therefore, a characteristic increase in carbonic anhydrase activity in the cerebellum of ep-mice is considered to be caused by their special quality that the seizure is induced by loss of postural equilibrium.

Although an excessive zinc is known to inhibit carbonic anhydrase in vitro,[20)] a decrease in the activity of this enzyme did not take place in the present experiment, probably due to the failure of introduction of sufficient amount of zinc into the brain to inhibit carbonic anhydrase due to the presence of blood-brain barrier.

Although the mechanism of the depression of EST in zinc excess is not clear at present, Fuchimoto [5)] tried to explain such phenomenon with changes in the distribution of electrolytes in the brain. Studies into this problem are under investigaton in this laboratory.

SUMMARY

Influence of disturbed zinc metabolism on brain excitability measured by Woodbury-Davenport's apparatus and carbonic anhydrase activity in the brain was studied in zinc deficient sucking mice prepared according to Nishimura and in mature mice with zinc intoxication due to a long term administration of zinc chloride. On the other hand, relationship of brain carbonic anhydrase to brain excitability was examined. The observations may be summarized as follows :

1) In zinc deficiency EST showed a rise and MES showed a shortening. In zinc excess EST showed a depression but MES failed to show a significant change.

2) Carbonic anhydrase activity in the brain decreased in zinc deficiency but failed to show a change in zinc excess, being compatible with the present concept that this enzyme is related to MES.

3) Carbonic anhydrase activity in the brain of ep-mice was higher than that

of dd-mice. The increase in the cerebellum was most prominent, while that in the hippocampus and dentate gyrus was not significant. This suggests that brain carbonic anhydrase is of secondary importance in the neurochemical mechanism of seizures.

4) The findings in zinc deficient sucklings prepared according to the method of Nishimura probably represents the effect of zinc on the development of seizure activity. Zinc deficiency appears to delay the development of seizure activity.

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