The Effect of Zinc on Brain Enzymes with Special Reference to Enzymohistochemical Changes in Ammon's Horn

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INTRODUCTION

The effect of zinc on the central nervous system is scarcely understood. In 1967, Fuchimoto et al.[3] demonstrated in mice that systemically or intracerebrally injected zinc markedly lowered the electroshock seizure threshold and intracerebral injection of a larger dose of zinc induced a tonic type of seizures. On the other hand, Peters[16][17] reported that, in patients with some types of porphyria, zincuria occurred simmultaneously with the manifestation of neurological symptoms including seizures, which were markedly improved by administration of chelating agents such as EDTA. These observations suggest that zinc has some effects on function of the central nervous system, with special reference to seizure activity.

It is widely presumed that zinc is related to catalytic actions in the biological system; zinc constitutes essential components of metalloenzymes and acts on enzyme activity as zinc-enzyme complex.[22] Therefore, it is very likely that zinc exerts some effects on function of the central nervous system by acting on the enzyme system.

On the other hand, Otsuka & Kawamoto[13] and Fujii[4] showed in mice that systemically or intracerebrally injected ^{65}Zn had a characteristic localization in the hippocampus and dentate gyrus (henceforth referred to as Ammon's horn according to Pribram & Kruger).[18]

Ammon's horn is rich in enzymes such as acid phosphatase, succinic dehydrogenase, cholinesterase, monoamine oxidase and cytochrome oxidase,[5][12][14] while it is closely related to seizure activity.[5] Thus, studies on the effect of zinc on enzymes in Ammon's horn will find a clue to elucidate the effect of zinc on function of the central nervous system, especially seizure activity.

In the present study, the effect of zinc on enzymes in Ammon's horn is histochemically studied and its relationship to the behavioral changes produced by zinc is discussed.

MATERIAL and METHODS

Male dd-mice, 20 g in weight, were used. The animals were maintained on Oriental Chow with free access to food and water.

Treatments of animals

Zinc, as the chloride, was dissolved in 0.9 % NaCl solution and was given subcutaneously in a daily dose of 50 γ for 5 days. Injection volume was 0.1 ml. Intracerebral injection was performed by a modification[3] of the method of Haley and McCormic. Zinc chloride was dissolved in Ringer's solution at a concentration of 2 mM. Injection volume was 0.02 ml. Twenty-four hours and 4-5 days after intracerebral injection, the animals were killed by decapitation. In controls, the same volume of 0.9 % NaCl solution or Ringer's solution was administered.

Autoradiographic procedures

In order to determine the intracerebral distribution of administered zinc, a trace dose of $^{65}ZnCl_2$ was injected subcutaneously or intracerebrally. The brain specimens obtained were fixed in 100 % alcohol solution and its paraffin sections were managed with "stripping film" (Fuji ET-2E) according to inverting method.

Histochemical procedures

After decapitation, the brain was removed as rapidly as possible and a slice through the center of Ammon's horn was made. The brain specimens obtained were fixed or frozen. Frozen sections were cut 15 μ thick in a cryostat.

The enzymes chosen for study were cytochrome oxidase, succinic dehydrogenase, diphosphopyridine nucleotide (DPN)- and triphosphopyridine nucleotide (TPN)-diaphorase, cholinesterase, acid phosphatase and monoamine oxidase.

Cytochrome oxidase was studied using Burstone method.[15] L-hydroxy-2-naphthoic acid was used with n-phenyl-p-phenylene diamine. Cytochrome C was added to the incubation medium (5 mg/ml total volume of incubation medium). Fresh frozen sections were incubated for about 60 minutes.

Succinic dehydrogenase activity was demonstrated by the technique of Nachlas et al.[15] The incubation medium consisted of equal parts of the following two solution : a ; 5 ml of 0.2 M phosphate buffer (pH 7.6) and 5 ml of 0.2 M sodium succinate, b ; 10 ml of aqueous solution of nitro-BT (1 mg/ml). Fresh frozen sections were incubated for 20 minutes.

DPN- and TPN- diaphorase were studied using nitro-BT method.[15] The brain was fixed in 10 % neutral formalin for about 24 hours. Frozen sections were cut. These sections were incubated for 10 minutes in DPN- diaphorase and for 20 minutes in TPN- diaphorase. The following concentration of reagents[10] was used : 0.5 ml of 0.1 % nitro-BT, 0.5 ml of 0.1 M phosphate buffer (pH 7.2-7.4), 5 mg DPNH or TPNH and 0.5 ml of distilled water.

Cholinesterase activity was demonstrated by the modification of Koelle method.[15] Fresh frozen sections were incubated for 90 minutes in a solution containing acetylthiocholine, copper glycinate, Na_2SO_4, $MgCl_2$ and malate buffer (pH 6.0), saturated with copper thiocholine. The sections were rinsed for 30 minutes to inhibit the non-specific cholinesterase with 10^{-6} M di-isopropyl fluorophosphate in 24 % Na_2SO_4 before incubation.

Acid phosphatase activity was determined after Takeuchi and Tanoue method.[15] Cold acetone paraffin sections were incubated for 24 hours in a solution containing 2 % sodium-β-glycerophosphate, 0.2 M acetate buffer (pH 5.0) and 2 % lead acetate. Then they were exposed to the sun in ammoniacal silver nitrate solution for 30 minutes.

Monoamine oxidase was stained using the method of Uono & Tanabe.[21] Fresh brain slices ca. 5 mm in thickness were fixed in 30 % ethanol for about 30 minutes, then immersed in 0.01 M KCN solution for 10—20 minutes. The slices were incubated in the medium made up of equal parts of the following solution for 4 hours: 0.5 % potassium tellurite, 0.1 % tyramine and acetate buffer (pH 7.4). After fixation in ice-cold formalin for 24 hours, the frozen sections were cut and substituted in gold chloride solution.

In each enzyme, control sections were incubated in the mixture without substrate.

RESULTS

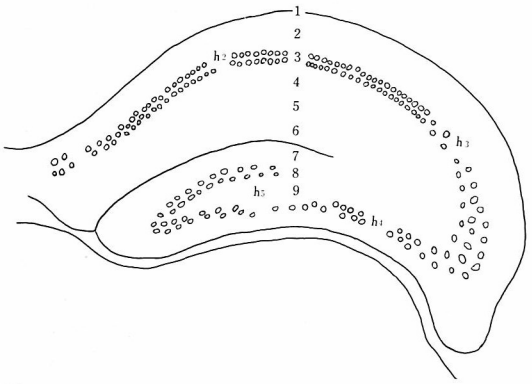
Behavioral changes

Subcutaneous injection of zinc produced an increase in excitability. However, spontaneous occurrence of seizures was not observed. After intracerebral injection of 2 mM of zinc, the animals had running or hopping movements lasting for 10—20 seconds. Thereafter, the animals apparently resumed their normal activity. In 3—5 days after injection, about half of the animals afresh had the running or hopping movements, which were followed by tonic extensor seizures and death. Intracerebral injection of 3 mM of zinc produced the extensor seizures and death in almost all cases.

Autoradiographic findings

Forty-eight hours after systemic injection, a larger dose of ^{65}Zn was accumulated in Ammon's horn compared to other parts of the brain. Intracerebrally injected ^{65}Zn was also accumulated in Ammon's horn, although it was weakly localized in 24 and 48 hours. As shown in Fig. 1, Ammon's horn consists of the alveus, stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum and stratum moleculare of the hippocampus, and the stratum moleculare, stratum granulosum and stratum multiforme of the dentate gyrus.[5][12][13] It has been histochemically shown that the stratum multiforme and the marginal part of

the stratum radiatum in contact with the stratum pyramidale, i. e. the layer of mossy fibers, give a strongly positive reaction for zinc. High density of the ^{65}Zn grain was observed in the stratum granulosum, stratum multiforme and the layer of mossy fibers (Fig. 2). Conspicuous localization in the stratum multiforme and the layer of mossy fibers in h_4 and h_5 areas has been presented by Fujii in this laboratory.[4] In contrast with the observation of Otsuka & Kawamoto,[13] the localization of ^{65}Zn was not so manifest in the stratum pyramidale.



Hippocampus
1. Alveus
2. Stratum oriens
3. Stratum pyramidale
4. Stratum radiatum
5. Stratum lacunosum
6. Stratum moleculare

Dentate gyrus
7. Stratum moleculare
8. Stratum granulosum
9. Stratum multiforme

Fig. 1. Architecture of Ammon's horn.

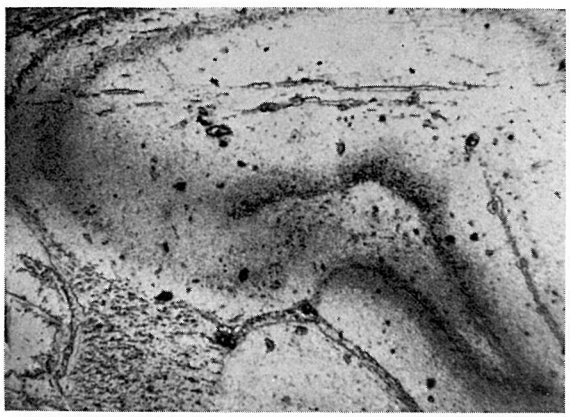


Fig. 2. Autoradiograph of Ammon's horn following intracerebral injection of ^{65}Zn. Four days after injection. The ^{65}Zn grains are localized in the stratum granulosum, stratum multiforme and the layer of mossy fibers.

Histochemical findings

The effect of zinc on enzyme activities is summarized in Table 1.

Table 1. Histochemical changes of enzyme activities in Ammon's horn following administration of zinc

	Cytochrome oxidase		Succinic dehydrogenase		DPN-diaphorase		TPN-diaphorase		Cholinesterase		Acid phosphatase		Monoamine oxidase	
	C	Zn	C	Zn	C	Zn	C	Zn	C	Zn	C	Zn	C	Zn
Hippocampus														
Alveus	−	−	−	−	−	−	−	−	−	−	−	−	−	−
St. oriens	++	+	++	+	+++	+	+	±	++++	++++	−	−	++	++
St. pyramidale	−	−	−	−	−	−	−	−	−	−	+++	+++	−	−
St. radiatum	+	±	+	±	++	±	+	±	+++*	±*	+*	+*	+	+
St. lacunosum	−	−	−	−	−	−	−	−	−	−	−	−	−	−
St. moleculare	+++	++	+++	++	++++	+	+++	+	++	++	−	−	+++	++
Dentate gyrus														
St. moleculare	+++	++	+++	++	++++	+	+++	+	++	++	−	−	++	++
St. granulosum	−	−	−	−	−	−	−	−	−	−	+++	+++	−	−
St. multiforme	+	±	+	+	+	±	+	±	+	±	−	−	++	++

Since the changes produced by systemic injection of zinc were almost the same as the changes in 4–5 days after intracerebral injection, only the latter is presented in this table.
* The layer of mossy fibers. C : Control Zn : Zinc intoxication

Cytochrome oxidase

In controls, the highest activity was detectable in the stratum moleculare of the hippocampus and dentate gyrus. The activity was moderate in the stratum oriens and stratum radiatum, while other layers showed low or no activity (Fig. 3). In zinc intoxication, the enzyme activity was slightly or moderately weaker than that in controls (Fig. 4).

Succinic dehydrogenase

The distribution of this enzyme was essentially the same as that of cytochrome oxidase (Fig. 5). This is consistent with the observation of Ortmann[12] and Otsuka & Umetani.[14] Zinc intoxication produced a slight or moderate decrease in the enzyme activity (Fig. 6).

DPN- and TPN-diaphorase

Pattern of the distribution of both enzymes was closely similar to that of cytochrome oxidase and succinic dehydrogenase. The highest activity was detectable in the stratum moleculare, while the activity was moderate in the

stratum oriens, stratum radiatum and stratum multiforme (Fig. 7). Zinc intoxication produced a marked decrease in the activity. The decrease was equally found in each layer (Fig. 8). There was no significant decrease in 24 hours after intracerebral injection.

The above changes in the oxidative enzymes were not specific in Ammon's horn, since the enzyme activities were decreased in other parts of the brain. In addition, the degree of the changes produced by systemic injection of zinc was almost the same as that produced by intracerebral injection.

Cholinesterase

The highest activity was detectable in the stratum oriens and moderate activity in the layer of mossy fibers and the stratum moleculare (Fig. 9). In contrast with the observation of other investigators,[9)7)] it is noteworthy that the layer of mossy fibers gave a stronger positive reaction for cholinesterase. Zinc intoxication produced a characteristic change in 4—5 days after intracerebral injection, although there was no change in 24 hours. The most manifest change was observed in the layer of mossy fibers in h_3, h_4 and h_5 areas. As shown in Fig. 10, the enzyme activity was diminished and/or faint in this layer. Another change was a slight decrease in the stratum multiforme.

Acid phosphatase

The highest activity was detectable in the stratum pyramidale and stratum granulosum. The activity was low in the layer of mossy fibers. There was no difference between control and zinc intoxication.

Monoamine oxidase

High or moderate activity was detectable in the stratum oriens, stratum moleculare and stratum multiforme. Zinc intoxication did not produce any significant change in the activity.

DISCUSSION

Although it has been shown in rat liver[6)] and rabbit heart[20)] that zinc ion inhibits mitochondrial respiration at very low concentration, the effect of zinc on brain respiration is not obvious. The present study showed that systemically or intracerebrally injected zinc inhibited more or less the activities of cytochrome oxidase, succinic dehydrogenase, DPN-and TPN-diaphorase. The oxidative processes in tissues are often complex chain phenomena and are controlled by the functioning of enzymes.[23)] The investigated enzymes act in a chain of oxidation-reduction processes; succinic dehydrogenase catalyzes the oxidation of succinic acid in TCA cycle and cytochrome oxidase oxidizes reduced cytochrome C at the end of the respiratory chain, while DPN-and TPN-diaphorase catalyze the oxidation of reduced coenzyme I and II (DPNH and TPNH).[23)] Therefore,

the observed inhibition of the enzyme activities indicates that brain respiration is inhibited by administration of zinc.

About the mode of action of zinc on the respiratory chain, Skulachev et al.[20] showed in rabbit heart that zinc ion caused a stronger inhibition of the activities of mitochondrial DPNH and succinate oxidase than that of succinic dehydrogenase and cytochrome oxidase, and they concluded that low concentrations of zinc ion inhibited the electron transfer in the middle part of respiratory chain. The present observation that zinc inhibited the activity of DPN-diaphorase more than those of succinic dehydrogenase and cytochrome oxidase is in good agreement with the observation of Skulachev et al.

It has been shown that metabolic energy in the brain is principally obtained from oxidative metabolism and brain function is very sensitive to anoxia.[19] Therefore, the inhibition of brain respiration by zinc probably causes some disturbances in brain function.

Direct causal relationship between the inhibition of brain respiration and brain excitability or the initiation of seizures is not yet established. However, it is presumed that the inhibition of oxidation in the brain may be related to the trigger mechanisms of seizures, since the disturbance of oxidative metabolism such as anoxia or cyanic poisoning can induce seizures.[19] In comparison with intracerebral injection, systemic injection of zinc produced almost the same degree of inhibition of the oxidative enzymes but failed to induce seizures. Therefore, it seems that the observed inhibition of the oxidative enzymes produced by zinc is insufficient to explain the neurochemical mechanisms which underlie the initiation of seizures.

In contrast with the diffuse type of inhibition in the oxidative enzymes, the diminution of cholinesterase activity occurred only in the layer of mossy fibers in Ammon's horn, 4—5 days after intracerebral injection of zinc. The effect of zinc on cholinesterase activity is not yet known. Based on the observation that intracerebrally injected ^{65}Zn was localized in the layer of mossy fibers, the stratum multiforme and stratum granulosum at this time, however, it seems reasonable to consider that the diminution of cholinesterase activity in this region is caused by the inhibiting effect of zinc on this enzyme.

It should also be noted that intracerebrally injected zinc produced tonic extensor seizures including running or hopping movements, 4—5 days after intracerebral injection. The simultaneous occurrence of the diminution of cholinesterase activity and the seizures seems to suggest a close relationship between the two.

Although Ammon's horn is rich in cholinesterase, its functional significance is not clearly understood.[9] Mathisen & Blackstad[9] expressed the opinion that the mossy fiber system as a whole might be unassociated with cholinesterase, since the activity was lower in the layer of mossy fibers in rats. In mice, however, cholinesterase activity was considerably high in this layer, as shown in the present

study. This suggests that cholinesterase may play a significant role in the layer of mossy fibers in mice.

In spite of the lack of corroboration, it is widely presumed that cholinesterase serves cholinergic transmission in the central nervous system.[9][19][23] Mathisen & Blackstad[9] observed in rats that the histochemical localization of cholinesterase in Ammon's horn was not necessarily dependent on the presence of synaptic structures. Since the layer of mossy fibers consists of axons derived from the dentate cells,[5] cholinesterase in this layer is probably unassociated with synaptic transmission. However, if acetylcholine functions to facilitate the generation and propagation of the impulse along the axon, as well as across the synapse,[11] it seems likely that the inhibition of cholinesterase activity by zinc produces the abnormal accumulation of acetylcholine and facilitates the impulse transmission. The view that zinc in Ammon's horn may play an important role in the impulse transmission has been expressed by Euler[1] and Otsuka & Kawamoto.[13]

It is well known that Ammon's horn is closely connected with seizure activity ; Ammon's horn has a very low seizure threshold and the initiated seizure discharges readily spread to other adjacent areas and to the hypothalamus or become generalized.[5] If the changes of the impulse transmission can significantly affect electrical activity in Ammon's horn, it seems possible that the inhibition of cholinesterase activity by zinc initiates seizure discharges in Ammon's horn and consequently induces generalized seizures.

The decrease of cholinesterase activity in the layer of mossy fibers following intracerebral injection of zinc was recognized only in 4—5 days, when the injected zinc showed distinct localization in this layer. This suggests that the inhibition of cholinesterase activity needs a larger dose of zinc. On the other hand, the activities of the oxidative enzymes were also decreased in 4—5 days, while they remained unchanged in 24 hours. Therefore, it seems that the observed changes in enzyme activities have nothing to do with the neurochemical mechanisms of the tonic extensor seizures induced immediately after intracerebral injection of zinc, as demonstrated by Fuchimoto et al.[3]

SUMMARY

Changes in brain enzymes produced by zinc were histochemically studied to elucidate the effect of zinc on function of the central nervous system. Since systemically or intracerebrally injected ^{65}Zn showed a characteristic distribution in Ammon's horn, attention was focused on histochemical changes in this region. The observations may be summarized as follows :

1) The activities of cytochrome oxidase, succinic dehydrogenase, DPN-and TPN-diaphorase were inhibited by systemic or intracerebral injection of zinc.

Particulary the activities of DPN- and TPN-diaphorase were strongly inhibited. These findings indicate that zinc inhibits the oxidative process in the brain.

2) Intracerebral injection of zinc diminished cholinesterase activity in the layer of mossy fibers in 4—5 days, when the injected ^{65}Zn showed distinct localization in this layer.

3) The activities of monoamine oxidase and acid phosphatase remained unchanged.

4) A little larger dose of zinc produced tonic extensor seizures with running or hopping movements in 4—5 days after intracerebral injection. Relationship between the behavioral changes and the histochemical changes in brain enzymes produced by zinc is discussed briefly.

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Explanations of figures

Fig. 3. Cytochrome oxidase in control (Burstone method).
Fig. 4. Cytochrome oxidase in zinc intoxication (Burstone method).
Fig 5. Succinic dehydrogenase in control (Nachlas method).
Fig. 6. Succinic dehydrogenase in zinc intoxication (Nachlas method).
Fig. 7. DPN-diaphorase in control (nitro-BT method).
Fig. 8. DPN-diaphorase in zinc intoxication (nitro-BT method).
Fig. 9. Cholinesterase in control (modified Koelle method).
Fig. 10. Cholinesterase in zinc intoxication (modified Koelle method). The activity almost disappared in the layer of mossy fibers.

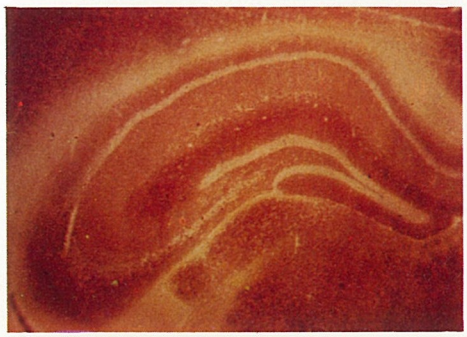

Fig. 3.

Fig. 4.

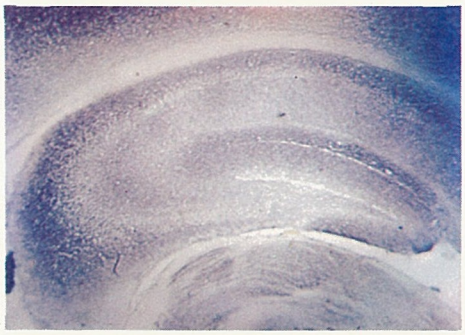

Fig. 5.

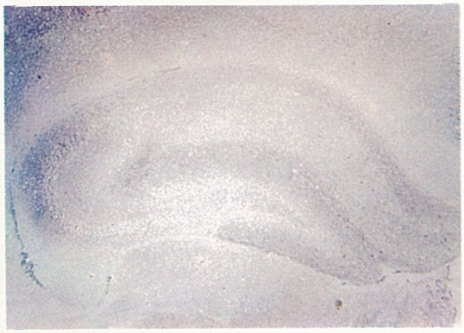

Fig. 6.

Fig. 7.

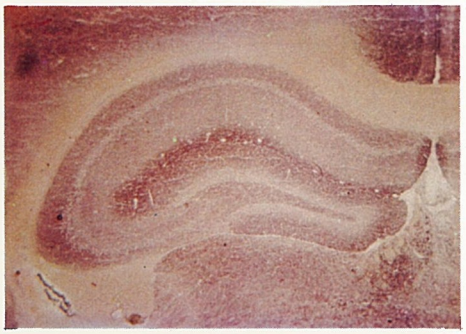

Fig. 8.

Fig. 9.

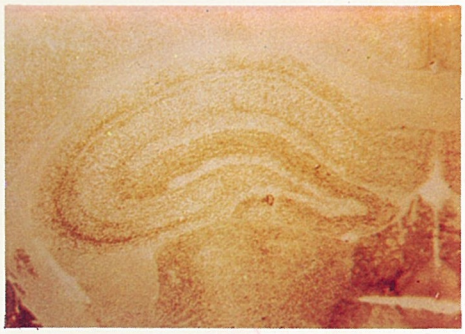

Fig. 10.