A KINETIC STUDY OF THE *IN VIVO* INCORPORATION OF ^{65}Zn INTO THE RAT HIPPOCAMPUS¹

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Abstract

Previous autoradiographical studies utilizing ^{65}Zn demonstrated an apparent concentration of ^{65}Zn in the mossy fiber boutons of the hippocampus. To examine the speciation of the ^{65}Zn pool found in this neuronal pathway, we investigated the *in vivo* incorporation of systemic ^{65}Zn into rat hippocampus compared with other brain regions. We were especially interested in kinetically assessing the zinc associated with three previously identified cytosolic zinc-binding species found in the hippocampus. The hypothesis that two of these cytosolic zinc-binding species, a metallothionein-like protein and a putative zinc-glutathione complex, may be responsible for the sequestration of zinc in the hippocampus was tested. It was confirmed that the $t_{1/2}$ of hippocampal zinc is longer than other brain regions that were studied. Furthermore, we observed that ^{65}Zn is incorporated into three cytosolic zinc-binding species in the hippocampus as resolved using Ultrogel AcA 34 gel permeation chromatography. One of these species, the putative zinc-glutathione complex, accumulates zinc more slowly than the other species.

The data suggest that the putative zinc-glutathione complex may represent an important ^{65}Zn pool in the hippocampus. This finding is in accordance with our hypothesis that a zinc-binding species, specifically, the putative zinc-glutathione complex, may be responsible for the sequestration of zinc in the hippocampal mossy boutons.

Direct evidence for the presence of zinc in the hippocampus is provided by autoradiographical studies showing an apparent concentration of ^{65}Zn in the mossy fiber boutons of the hippocampus (von Euler, 1962; Hassler and Söremark, 1968). The zinc found in the mossy fiber boutons is believed to be either cytosolic or vesicular (Ibata and Otsuka, 1969; Haug, 1967, 1975).

There is little information available concerning normal zinc metabolism in the brain. Questions regarding zinc transport into the central nervous system, the intracellular localization of zinc, and the incorporation of zinc into proteins remain unanswered. Additionally, there is only limited information available on the turnover of zinc pools in the brain (Kasarskis, 1981), which is the subject of this study.

Since there is a very slow accumulation and turnover of ^{65}Zn in the hippocampus, in the order of weeks

(Dencker and Tjälve, 1979; Kasarskis, 1981), we evaluated the incorporation of ^{65}Zn into the rat brain from 2 to 10 weeks after the initial systemic injection of ^{65}Zn . Our study included: (1) a comparison of the $t_{1/2}$'s of whole body zinc to whole brain zinc; (2) a comparison of the $t_{1/2}$'s of hippocampal zinc to that of several brain regions; (3) the subcellular distribution of ^{65}Zn in the hippocampus compared with the cerebellum; and (4) the isolation of cytosolic zinc-binding species and a kinetic assessment of the zinc associated with these species in the hippocampus and the cerebellum.

The speciation of the zinc pool associated with the mossy fiber boutons has not been studied until recently (Sato et al., 1984). This current study utilizing ^{65}Zn was designed to provide additional insight into the possible identification of this zinc pool. Due to the relative selectivity of the ^{65}Zn for the hippocampal mossy fiber boutons, it is believed that this experimental approach allows one to identify those zinc pools that are associated with this neuronal pathway.

Materials and Methods

Experimental animals. Male Long-Evans hooded rats (Blue Spruce Farms, Altamont, NY), weighing 200 to

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225 gm, were used in this study. Animals were housed in plastic cages with filter tops, given free access to commercial rat chow (Charles River) and tap water, and maintained on a 12-hr light-dark cycle.

Experimental protocol. Twenty rats were injected intraperitoneally with a single dose of 250 μCi of $^{65}\text{ZnCl}_2$, 2.0 Ci/gm of ZnCl_2 . Rats, four animals per time point, were killed at 2, 4, 6, 8, and 10 weeks after injection. Anesthetized animals were perfused with a 0.9% NaCl solution made with deionized distilled water, neutralized to pH 7.0. The hippocampus and cerebellum were dissected by the method of Glowinski and Iverson (1966).

Determination of ^{65}Zn . Prior to dissection, the radioactivity in the whole rat was determined using a 5-inch well, γ -scintillation counter. A phantom weighing 300 gm, containing 1.25 μCi of ^{65}Zn , was used as a standard. Whole brain regions, subcellular fractions, and column fractions were counted using a Beckman 4000 γ -scintillation counter. Counting efficiency was 20%.

Subcellular fractionation method. We used a modification of a differential centrifugation method developed by Gray and Whittaker (1962). To maximize the recovery of cytosolic zinc, we used hypo-osmotic shock of both pellet 1 (nuclei, unbroken cells, and large synaptosomes) and pellet 2 (mitochondria, synaptosomes) to release occluded cytoplasm trapped within the synaptosomes. This method is described in detail elsewhere (Sato et al., 1984).

Materials. Column chromatography was performed using Ultrogel AcA 34 (LKB), and columns (1.6 $\times$ 70 cm, Pharmacia) were calibrated with blue dextran (Pharmacia) and 3-OH-tyramine (Sigma). $^{65}\text{ZnCl}_2$ was purchased from New England Nuclear.

Results

Whole body zinc $t_{1/2}$ versus whole brain zinc $t_{1/2}$. The whole body and whole brain ^{65}Zn contents over the 10-week experiment are illustrated in Figure 1. The $t_{1/2}$'s for whole body zinc and whole brain zinc are 11.9 and 3.1 weeks, respectively. The whole brain ^{65}Zn content represents less than 0.1% of the total body content at all times. The standard deviation did not exceed 10% of the mean value, except at the 4-week time point. The variability at the 4-week time point was 50% because one rat failed to incorporate ^{65}Zn .

Estimated $t_{1/2}$ of zinc in rat brain regions. A comparison of the data from which the relative $t_{1/2}$'s of six brain regions are calculated is shown in Figure 2. The brain regions are divided into two groups; the striatum, mid-brain, and cerebellum have shorter $t_{1/2}$'s for ^{65}Zn compared with the hippocampus, cortex, and brainstem. The zinc of the hippocampus has the longest $t_{1/2}$ of all of the regions studied. The standard deviation did not exceed 15% of the mean except at the 4-week time point. The variability at the 4-week time point was 50% because one rat failed to incorporate ^{65}Zn .

The subcellular distribution of ^{65}Zn . Using a subcellular fractionation method that maximized cytosolic zinc recovery, we determined the subcellular distribution of ^{65}Zn in the hippocampus and cerebellum (see Table I). The cerebellum was used as a control region because both quantitatively and qualitatively it was shown to contain

Figure 1. Zinc kinetics in whole body and whole brain. Following the experimental protocol described under "Materials and Methods," we determined the $t_{1/2}$'s of whole body (A) and whole brain (B) zinc. These data are the means of four rats per time point $\pm$ SD. The correlation coefficient was -0.97 for whole brain and -0.99 for whole body data.

lower levels of zinc compared with the hippocampus. Initially, we found that a greater percentage of the total ^{65}Zn content was associated with the cytosolic fraction (S_3) of the hippocampus compared with the cerebellum. This difference persisted until the 6-week time point. Also, substantially more ^{65}Zn was incorporated into pellet 2 (P_2) and pellet 3 (P_3) of the hippocampus relative to the same fractions of the cerebellum, a difference which was apparent throughout this study. Additionally, at the 2-week time point, more of the total ^{65}Zn was incorporated into pellet 1 (P_1) of the cerebellum compared with the hippocampus. However, the cerebellar P_1 ^{65}Zn pool had diminished by the 4-week time point and thereafter was smaller than that of the hippocampus. The $t_{1/2}$'s of zinc associated with P_1 , P_2 , and P_3 were impossible to determine because the loss of ^{65}Zn from these subcellular fractions did not follow an exponential decay curve. However, the loss of ^{65}Zn from the cytosol did follow an exponential and was estimated at 3.0 weeks for the hippocampus and 2.6 weeks for the cerebellum.

The cytosolic distribution of ^{65}Zn in the hippocampus and the cerebellum. To examine the cytosolic zinc pool more closely, we looked at the distribution of cytosolic ^{65}Zn on Ultrogel AcA 34, shown in Figure 3. Qualitatively, three major peaks of radioactivity were observed in the hippocampus corresponding to three endogenous zinc species, peaks 1, 2, and 3. Over time, the radioactive zinc associated with peaks 1 and 2 fell, both having a $t_{1/2}$ of

2.2 weeks. The ^{65}Zn associated with peak 3 was slow in reaching a steady state. Consequently, although total cytosolic ^{65}Zn in the hippocampus was falling at 6 weeks after injection, peak 3 appeared to have incorporated more counts at 6 weeks than at 2 weeks after the injection. Peak 3 was the most prominently labeled cytosolic species at 6 weeks after injection. The levels of ^{65}Zn associated with peak 3 began to fall at 8 weeks after injection. It, too, had a $t_{1/2}$ of 2.2 weeks, estimated from the peak value obtained at 6 weeks.

In contrast to the hippocampus, the cerebellum dis-

Figure 2. Zinc kinetics in rat brain regions. Following the experimental protocol described under "Materials and Methods," we determined that $t_{1/2}$'s of zinc found in several brain regions. These data are reported as the means of four rats per time point $\pm$ SD, except for the data from the cerebellum and the hippocampus, which are the average of four rats per time point. The correlation coefficients for this study ranged from -0.94 to -0.99 .

plays only two peaks of ^{65}Zn corresponding to the two endogenous zinc-binding species, peaks 1 and 2. As observed in the hippocampus, the radioactive zinc associated with peaks 1 and 2 of the cerebellum fell, having a $t_{1/2}$ of 2.4 weeks and 2.3 weeks, respectively. More importantly, peak 3 was never as extensively labeled as it was in the hippocampus.

Discussion

In these studies, we injected rats with a single systemic dose of radioactive zinc. In interpreting the data, we assumed that the radioactive zinc fully mixed with the endogenous pool and thus reflected the endogenous zinc

TABLE I

The subcellular distribution of ^{65}Zn

The cerebella and the hippocampi, pooled from four rats per time point, were fractionated into subcellular components as described under "Materials and Methods." These data are presented as the average ^{65}Zn content of the various subcellular components. The endogenous zinc levels did not change over time (data not shown).

	Weeks after Injection				
	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
Hippocampus					
Homogenate	120,620	66,400	54,240	36,040	23,123
Pellet 1 (P_1)	25,908	13,589	12,478	13,090	6556
Pellet 2 (P_2)	24,294	10,693	12,364	5805	3825
Pellet 3 (P_3)	8787	7182	3712	2338	1763
Cytosol (S_3)	47,016	33,666	18,907	12,572	7250
Recovery (%)	88	98	93	94	84
Cerebellum					
Homogenate	95,240	37,120	28,160	22,240	13,389
Pellet 1 (P_1)	38,873	12,690	9258	9987	4869
Pellet 2 (P_2)	7696	4851	6865	2968	1662
Pellet 3 (P_3)	4844	1595	1496	1022	436
Cytosol (S_3)	31,579	12,774	8264	6608	3891
Recovery (%)	87	86	92	93	81

pool. Also, in evaluating our data, we had to consider whether the systemic injection of $125\text{ }\mu\text{g}$ of zinc that was used in this study could influence the brain zinc pool. This is a highly unlikely event since zinc homeostasis in the brain is probably under tight regulation, making it quite difficult to alter brain zinc levels by raising the systemic levels of zinc to this degree.

In comparing the $t_{1/2}$ for whole body zinc with whole brain zinc, we observed that these two zinc pools behave independently from each other, that is, their $t_{1/2}$'s are quite different. In comparing the $t_{1/2}$ for zinc in various brain regions, differences were also observed. The hippocampal zinc pool had the longest $t_{1/2}$ of any of the brain regions studied at 3.7 weeks, a value which is comparable to the $t_{1/2}$ reported by Kasarskis (1981)—3.8 weeks.

We attempted to identify the subcellular component that might account for the longer $t_{1/2}$ of hippocampal zinc. Most of the data obtained from the kinetic study on the subcellular distribution of ^{65}Zn in the hippocampus and the cerebellum are complex and, therefore, difficult to interpret. For example, in examining the hippocampal P_1 ^{65}Zn pool, we initially observed that the ^{65}Zn content decreased between 2 and 4 weeks after injection, remained almost unchanged at 4 to 8 weeks after injection, and finally decreased again at the 10-week time point. An explanation for the complexity is that several ^{65}Zn pools are probably contributing to the overall $t_{1/2}$'s that were determined for each subcellular component. In an earlier study (Sato et al., 1984), it was determined that the cytosolic zinc pools of the hippocampus were perhaps important in the sequestration of zinc. Therefore, we focused on examining these cytosolic zinc pools. In assessing our data on the cytosolic distribution of ^{65}Zn -labeled binding species, we were especially interested in the fate of two ^{65}Zn -labeled binding species, peaks 2 and 3. Peak 2, a metallothionein-like species, and peak 3, a putative zinc-glutathione complex, characterized in an earlier study (Sato et al., 1984), are believed to be re-

Figure 3. The cytosolic distribution of ^{65}Zn in the hippocampus and the cerebellum. The cytosol, prepared by the subcellular fractionation method described under "Materials and Methods," was loaded onto Ultrogel AcA 34 columns. Zinc-binding species were eluted with 50 mM Tris, 0.9% NaCl, pH 7.4, at 4°C . The distribution of endogenous zinc was not changing over time (data not shown). The data for the hippocampus and the cerebellum are shown as solid and open circles, respectively.

sponsible, in part, for the sequestration of zinc in the hippocampus. It has already been demonstrated using autoradiography that ^{65}Zn is preferentially accumulated in the mossy fiber layer of the H_3 and H_5 portion of the hippocampus (Hassler and Söremark, 1968). We proposed that the ^{65}Zn might be associated with the metallothionein-like protein or the zinc-glutathione complex and, therefore, expected one or both of these zinc-binding species to be heavily labeled. Additionally, it has been proposed that this hippocampal zinc pool turns over very slowly (Dencker and Tjälve, 1979). From these data, we would predict that the zinc pool of interest would be long-lived. We found that peak 3, the zinc-glutathione complex, fit both of these criteria. Peak 3 is not only the most heavily labeled species at 6 weeks after injection, but also the ^{65}Zn associated with it was slow to reach a steady state compared with the other zinc-binding species. The actual $t_{1/2}$ of the zinc associated with the zinc-glutathione complex is not different from other zinc-binding species. Overall, the $t_{1/2}$'s of all of the cytosolic zinc-binding species are relatively similar, 2.2 to 2.4 weeks. Coupled with our earlier studies, these data, although indirect, suggests that the putative zinc-glutathione complex, peak 3, may be responsible for the sequestration of zinc in the mossy fibers. This finding supports our hypothesis that a zinc-binding species may be responsible for the sequestration of zinc in the hippocampus and provides information concerning the speciation of the zinc pool.

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