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Methodological Studies on the Uptake of Zinc by 3T3 Cells[1] (38920)

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Studies with cell and tissue culture models should yield considerable information about basic transfer processes of trace elements at the cellular level. Mammalian cells grown in culture are generally placed in a medium containing a source of energy, essential amino acids, minerals, and blood serum, a milieu which simulates in many respects the circulating plasma in blood vessels of the whole animal. Either the amino acids or serum proteins in the culture media contain ligand groups which can combine with ions of the trace elements to form metallochelates; and it is possible that these forms of metal chelates affect the uptake or transfer of the trace elements. Although some nutritional studies have been carried out to determine the essentiality of certain trace elements for mammalian cells, there have been no reports on methodological studies of the effect of amino acids and/or serum on the uptake of trace elements by the cell. The object of the experiments reported herein was to shed some light on this question. The trace element used in these studies was zinc, and the radioactive tracer was ^{65}zinc.

Material and Methods. Cell culture. The 3T3 cell line of mouse fibroblast cell (Clone 4A) was obtained from Dr. Robert W. Holley, The Salk Institute.[3] The cells were grown at 37° in 5 ml Dulbecco's Modified Medium[4] (DMM) (1), pH 7.3–7.4, and incubated in an atmosphere of 5% CO_2 and air so that the pH was maintained between 7.3–7.4. The medium contained a final concentration of 5% calf serum (Gibco) and 25 units of penicillium plus 25 μg of streptomycin[5] per ml. The Zn content of the serum used was 1.3 μg per ml. No trace element except iron was added to the DMM. Sixty mm Nunc-tissue culture dishes were seeded with 1×10^5 3T3 cells for the growth studies.

Experimental procedures. In the Zn uptake studies, cells grown for 3–5 days to a density of $7–9 \times 10^5$ 3T3 cells per dish were used. Before the treatment with zinc, the used medium was aspirated from the dishes and replaced with 5 ml of new media. For the studies on the influence of different Zn concentrations and on incubation time, DMM with 5% calf serum was used, while for the other experiments in which the composition of DMM was varied the media were made up from the individual ingredients. The incubation time usually was one or 2 hr for the Zn uptake studies.

After incubation with Zn, the dishes were set in an icebath and the medium was aspirated immediately. The cells were washed a total of six times with ice-cold Tris buffered saline, pH 7.3. The second, third, and fourth washing solutions also contained 0.01 *M* EDTA. The measurement in a gamma counter of the last wash solution showed less than 100 cpm of ^{65}Zn. After the washing procedure, the cells were trypsinized with 4 ml of 0.05% trypsin in Ca and Mg free Tris buffered saline (Trypsin-TD) and were incubated for 20 min at 37°. The cell number per dish was counted with a hemocytometer, and the radioactivity of the cells per dish was measured in a gamma counter. The recorded impulses were corrected for background and expressed as percentage of the ^{65}Zn dose. The data were expressed as zinc uptake, and all data in the

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[4] Purchased from Grand Island Biological Co., New York, MEM Powder Catalog No. H-16.

[5] Gibco Catalog No. 514L.

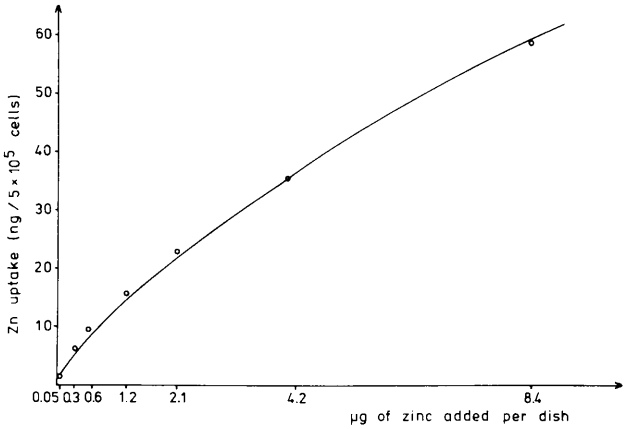

FIG. 1. Influence of increasing zinc additions on zinc uptake. Each datum is the mean of three replications.

figures and tables were corrected to a uniform cell number of 5×10^5 cells. Each treatment was replicated three times.

Zn solutions. In each experiment, with the exception of the study on the influence of different Zn concentrations, 0.2 ml of a Zn solution containing 0.2 μCi ^{65}Zn and 0.6 μg Zn were added to each dish. The Zn compounds were ^{65}Zn-$ZnCl_2$ in a solution of pH 6.3, or ^{65}Zn-$ZnCl_2$ treated with the organic ligand under study. ^{65}Zn-$ZnCl_2$ was mixed with L-histidine-HCl in a molar ratio of 1:5 or 1:10, 1:50, 1:500, 1:5000, respectively; and the pH of these solutions was adjusted to 7.0 with diluted NaOH. In the experiment studying the influence of different Zn compounds on Zn uptake (Table III), ^{65}Zn-$ZnCl_2$ was treated with glycine, L-histidine-HCl, L-threonine, L-valine, fumarate, and ethylenediamine (EDA) in a 1:2 molar ratio.

For the compounds ^{65}Zn-Zn-citrate, ^{65}Zn-Zn-ethylenediaminetetraacetate (EDTA), ^{65}Zn-Zn-nitrilo-triacetate (NTA), and ^{65}Zn-Zn albumin, ^{65}Zn-$ZnCl_2$ and the ligand were mixed in a molar ratio of 1:1. In all solutions, the pH was adjusted to 7.0 with diluted NaOH. The Zn compounds were added to the dishes in a 0.8% NaCl solution.

Results and Discussion. Zn concentration and Zn uptake. In an initial study, the influence of increased amounts of Zn on Zn uptake was tested. ^{65}Zn-$ZnCl_2$ was added to an average of 9×10^5 cells in amounts of 0.05, 0.3, 0.6, 1.2, 2.1, 4.2, or 8.4 μg. Zn uptake was measured after an incubation period of 4 hr. The mean results are shown in Fig. 1. The relationship between zinc concentration in μg and zinc uptake in ng does not appear to be linear. In fact, if the Zn uptake is expressed as a percentage of added Zn, the percent uptake clearly decreased with increasing amounts of Zn; these findings suggest a saturation phenomenon. These data are similar to Zn uptake by excised intestinal mucosal strips reported by Sahagian *et al.* (2). In subsequent studies, 0.6 μg Zn was added to each dish.

Incubation time and Zn uptake. In two experiments, the influence of incubation time of zinc on Zn uptake by the cells was studied. Cells were grown for 4 days to a mean cell density of 9.2×10^5 cells per dish, after which the fluid was changed and new medium was added. The cells were then incubated with ^{65}Zn-$ZnCl_2$ for 2, 4, 6, 8, 12, or 16 hr. The course of the Zn uptake is shown in Fig. 2 (line defined by circles). The data indicate that there may be an initially maximum uptake; however, subsequent studies by R. Munoz and G. Matrone (unpublished data) show that Zn uptake is linear with time up to 4 hr. In the following studies with 3T3 cells, which were nearly confluent, Zn incubation was limited to 1 or 2 hr.

Zn uptake by confluent cells which were trypsinized and then replated is very different from that of nearly confluent cells not replated (Fig. 2, line defined by triangles). In this study, 3T3 cells were cultured for 12

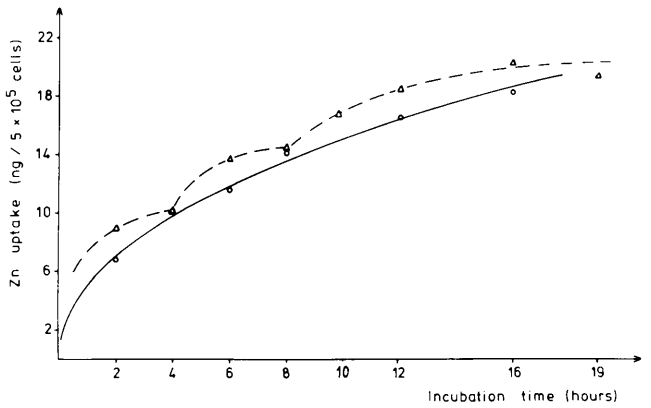

FIG. 2. Influence of incubation time on zinc uptake. ○, nearly confluent cells without replating; Δ, trypsinized cells newly plated out. Each datum is the mean of three replications.

days during which time DMM with 10% calf serum was changed twice. The cells were trypsinized with 0.05% trypsin-TD solution, then 5×10^5 cells were plated per dish. The cells were immediately treated with ^{65}Zn-$ZnCl_2$, and Zn uptake was measured after every 2 hr for a period of 12 hr and also after 16 and 19 hr. The mean measurements are also shown in Fig. 2. A smooth curve for uptake was not obtained. Moreover, the cyclic-type rise in uptake after 2, 6, and 10 hr was significantly higher. This result could be due to the Zn requirement in certain phases of the cell cycle (3, 4).

Influence of amino acids and serum on Zn uptake—composition of the media and Zn uptake. Zn uptake by the cells might be influenced by the amino acids present in DMM and/or by the addition of the serum. To test this, 5 ml of one of the following media were used: (1) DMM without amino acids, (2) DMM with amino acids, (3) DMM without amino acids plus 2% or 0.2% calf serum, (4) DMM with amino acids plus 2% or 0.2% calf serum.

The incubation period of the cells (5.3×10^5 cells per dish) was 2 hr, and zinc was added as ^{65}Zn-Zn-L-$hist_2$ (1:10 molar ratio). The mean data for Zn uptake in percent of the Zn dose are listed in Table I. The highest Zn uptake was observed by the cells incubated with DMM without amino acids and without serum. The amino acids of DMM or the 2% serum clearly decreased the Zn uptake to a similar degree ($P < 0.01$). However, the addition of serum at a level of only 0.2% reduces the Zn uptake much less than 2%. With the addition of serum, small amounts of free amino acid, as well as serum proteins, get into the medium. The decreased Zn uptake can be explained by the presence of these chelating ligands in the serum.

Molar ratio of zinc to histidine and Zn uptake. To study the influence of an excess of amino acids on the Zn uptake, ^{65}Zn-Zn-L-$hist_2$ was added to the cells in different molar ratios of zinc to histidine. Cells with a cell density of 5.5×10^5 cells were incubated in 5 ml DMM with amino acids plus 0.2% calf serum or 5 ml DMM without amino acids, but plus 0.2% calf serum. In the Zn solutions added to the dishes, the molar

TABLE I. INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON Zn UPTAKE (% OF THE DOSE PER 5×10^5 CELLS).

Composition of the incubation medium	Zn uptake[a]
DMM without amino acids	10.91 ± 0.73[b]
DMM with amino acids	2.26 ± 0.17
DMM without amino acids	
+ 2% calf serum	2.07 ± 0.10
+ 0.2% calf serum	6.92 ± 0.54
DMM with amino acids	
+ 2% calf serum	2.17 ± 0.03
+ 0.2% calf serum	2.50 ± 0.06

[a] Each value is the mean of three replications.
[b] Standard error.

TABLE II. INFLUENCE OF THE MOLAR RATIO OF Zn:HISTIDINE IN DMM ON Zn UPTAKE (% OF THE DOSE PER 5 × 10^5 CELLS).

Zinc uptake[a]	Molar ratio of ZN:histidine 1:5	1:50	1:500	1:5000
DMM with amino acids + 0.2% calf serum	2.28 ± 0.03[b]	2.08 ± 0.10	1.70 ± 0.09	0.96 ± 0.02
DMM without amino acids + 0.2% calf serum	5.79 ± 0.34	3.53 ± 0.02	2.02 ± 0.20	1.18 ± 0.06

[a] Each value is the mean of three replications.
[b] Standard error.

ratio of zinc and histidine was widened from 1:5 to 1:50, 1:500, or 1:5000. The mean values for Zn uptake after 2 hr of incubation are listed in Table II. In DMM without amino acids, Zn uptake clearly decreases as the amount of histidine is increased ($P < 0.01$). In DMM with amino acids containing a fivefold excess of histidine, Zn uptake is significantly smaller than in the comparable solution of DMM without amino acids. Further increases in excess histidine also reduced Zn uptake to a lesser extent than in the absence of amino acids. Therefore, similar to the influence of amino acids in DMM, no stimulation of Zn uptake could be obtained by raising the amounts of histidine.

Influence of different Zn compounds on Zn uptake. In serum, zinc does not exist in the form of an aquo-complex but is bound to different bio-ligands. Therefore, the specific influence of certain complexing agents like amino acids, organic acids, and albumin on Zn uptake was tested. Because preliminary studies had shown that DMM with amino acids was less suitable for this kind of experiment, 3T3 cells were incubated with 5 ml DMM without amino acids plus only 0.2% calf serum. The 3T3 cells had a mean cell density of 7 × 10^5 cells. As presented in Table III, bio-ligands normally found in serum did not show a distinct influence on Zn uptake when tested individually at a molar concentration of 1:2 or 1:1 (Zn: ligand), except for the decreases noted with histidine and albumin. These two ligands are reported to be the most important specific ligands in serum (5, 6). The depressing effect of albumin on Zn uptake was greater than that observed for histidine. A clear decrease in Zn uptake was obtained with EDTA while the chelates NTA and EDA did not influence Zn uptake significantly. Chesters (4) observed a distinct inhibition of ^{3}H-thymidine incorporation into DNA by EDTA, an effect fully reversible by the addition of Zn; but this inhibition could not be obtained by NTA. The results reported by Weser and Brauer (7) showing the influence of various amino acids and EDTA on the uptake of Zn by rat liver nuclei (molar ratio approximately 1:2.9, Zn:ligand), were similar to those obtained in this study with 3T3 cells.

TABLE III. INFLUENCE OF DIFFERENT Zn COMPOUNDS IN DMM WITHOUT AMINO ACIDS PLUS 0.2% CALF SERUM ON Zn UPTAKE (% OF THE DOSE PER 5 × 10^5 CELLS).[a]

Ligand	Molar ratio Zn:ligand	Zn uptake[b]
Cl_2		5.04 ± 0.64[c]
Gly_2	1:2	4.24 ± 0.25
$Hist_2$	1:2	3.70 ± 0.13
Thr_2	1:2	4.56 ± 0.46
Val_2	1:2	4.54 ± 0.41
Albumin	1:1	3.51 ± 1.17
Citrate	1:1	4.94 ± 0.23
Fumarate	1:2	4.78 ± 0.31
EDTA	1:2	2.45 ± 0.56
NTA	1:1	4.16 ± 0.52
EDA	1:1	4.85 ± 0.44

[a] Incubation time was 1 hr. Molar ratio of 1:2 (Zn:ligand) was used.
[b] Each value is the mean of three replications.
[c] Standard error.

The data presented herein show that the uptake of Zn^{2+} ions by 3T3 cells is decreased by ligands in the medium. Both the concen-

tration of ligands and the type of ligand affect the magnitude of decrease in Zn uptake. The concentrations of amino acids present in Dulbecco's modified medium bring about a decrease in the uptake of Zn. Addition of 2% calf serum alone or in combination with amino acids decreased uptake to a similar extent. Further studies will be necessary to elucidate the uptake mechanism and to explain how the ligands interfere with this mechanism.

Summary. In the present work, experiments were conducted on the uptake of zinc by 3T3 cells.

(1) The percent Zn uptake gradually decreased with addition of increasing amount of zinc (0.05–8.4 μg).

(2) With the increase of the incubation period from 2 to 16 hr, Zn uptake by nearly confluent cells increases gradually; however, confluent cells which are newly replated show a distinct cyclic increase in the Zn uptake after 2, 6, and 10 hr.

(3) The amino acids of DMM and serum decrease Zn uptake.

(4) Histidine at a molar excess of 1:50, 1:500, and 1:5000 reduces Zn uptake in comparison to a treatment with ^{65}Zn-Zn-L-hist$_2$ at a molar ratio of 1:5.

(5) When zinc is added in the form of different Zn compounds, at a molar ratio of 1:2 or 1:1 (Zn:ligand), EDTA decreases the Zn uptake markedly. A small influence was shown also by albumin and histidine; however, other amino and organic acids at a molar ratio of 1:2 did not alter Zn uptake significantly.

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