Mechanisms of Manganese Transport Across Cell Membranes

Debaroti Addy
University Undergraduate Fellow, 1993-1994
Texas A&M University
Department of Biochemistry

APPROVED

Fellows Advisor E. D. Harris

Honors Director

Table Of Contents

Abstract

The uptake of manganese (Mn) by human fibroblast cells was studied to determine the chemical form of Mn used by the cells. These experiments tested the uptake of free Mn and Mn bound to the plasma protein, transferrin. Mn solutions were added to C-1474 human fibroblasts at varying concentrations and incubation times. The Mn concentrations ranged from 100 - 800 μmolar. At a two hour time point, uptake at 800 μmolar was 4-fold higher than at 100-400 μmolar and 2-fold higher than uptake at 600 μmolar. This suggested an inefficient system that requires high concentrations of Mn to trigger uptake. Removal of the albumin from the incubation medium and addition of EDTA (Ethylenedinitrilo-tetraacetic acid) to harvesting washes resulted in a reversal of the uptake seen at 800 μmolar. This suggested that Mn was not penetrating the cell in a free form. Next, the uptake of a transferrin (Tf)-Mn complex was tested. The K-562 erythrocyte tumor cell line was used to study uptake of this complex. Tf was used as a carrier protein because it has been shown to bind Mn (Scheuhammer et al. 1985). Complexes of Tf-Mn were formed by oxidizing Mn with ceruloplasmin. Initial studies showed 3-fold higher incorporation of free Mn than Tf-Mn complex. Iron (Fe) was also substituted for Mn to test the integrity of the Tf molecule. Under the same protocol, free Fe was incorporated ten times more readily than Tf-Fe. Adjustments with incubation medium and complex formation time are

needed to explore the possibilities of Mn uptake via Tf.

Introduction

Mn is an essential trace mineral. It is a required cofactor for several liver enzymes including P-enolpyruvate carboxykinase (Scrutton, 1986) and pyruvate carboxylase (Schramm, 1986). The physiologic concentration of Mn is less than 1μM\L which is only a fraction of the concentration of copper or zinc in the body. This low concentration is one of the reasons for the difficulty in determining the method of transport for Mn. Mn levels in biological systems are carefully regulated because deficiency or toxicity can easily result from any disturbance in homeostasis. Mn is also involved in the galactosyl transferase reactions which are associated with chondroitin sulfate formation and bone metabolism (Strause et al., 1986). Osteoporosis and chondrodystrophy are examples abnormalities that are associated with Mn deficiency (Asling et al., 1963). Studies of Mn transport have been increasing in recent years, however, a clear mechanism of transport for Mn from the plasma into cells has not been elucidated. The purpose of my studies was to determine the chemical form of Mn used by cells.

Mn is a cofactor for the enzyme arginase which is found in the in the liver and in the prickle cell layer of skin (Molokhia and Portnoy, 1969). Normal fibroblasts

(C-1474 cells) were studied in my experiments and were expected to show normal transport and metabolism of Mn. Previous unpublished studies from our lab have shown that free Mn was taken up by K-562 erythrocyte cells. Therefore, Mn was initially introduced to the fibroblasts in a free form.

Mn was also tested for binding to transferrin, an iron transport protein. It is possible that Mn and Fe share the same mechanism of transport because high levels of Mn have been shown to lead to deficient iron absorption in animals (Hartman et al., 1955). For optimal uptake of the Mn-Tf complex, it was tested in cells that were known to take up free Mn, the K-562 human erythrocyte tumor cell line.

Materials and Methods

Cell Culture Method:

The C-1474 fibroblasts were cultured under sterile conditions in DMEM (Dulbeccos' Modified Eagle's Medium) with 10% fetal calf serum at 37 °C. For each experiment they were separated into 3 ml petri dishes with 10 X 10^6 cells\ml; equalling 30,000 cells\ group.

Test of Unbound Mn:

Free ^{54}Mn and $MnCl_2$ were added to a solution of DPBS (Dulbecco's Phosphate

Buffered Saline at pH 7.4) and 3% bovine serum albumin. The unlabelled $MnCl_2$ was used to adjust concentrations of Mn from 100 μM to 800 μM. Specific activity was 1.78 μCi/μmol. The cells were then incubated with 3 mls of this Mn solution at 37°C. After incubation (time ranging from 30 min. to 2 hrs.) the supernatant medium was removed and the cells were washed twice with 2 mls of 0.15M sodium acetate to remove any Mn that had not been incorporated into the cells. Then 0.5 mls of 0.1M NaOH was added to each group in order to lyse the cells and free them from the surface of the petri dish. A plastic scraper was used to help remove the cells from the surface of the dishes. After scraping, the 0.5 mls was collected in omni vials. A protein assay was conducted on 10 μL of each sample and the rest was assayed for radioactivity on a Beckman gamma counter. The counter windows were set at (0-460) and (460-900).

Protein Assay:

Ten μliters from each sample was assayed for protein content using the BCA Protein Assay Reagent. A standard curve was created by measuring the absorbencies of known concentrations of a purified protein (egg albumin). Then the absorbencies of the unknown samples were plotted against this curve and the protein concentrations of the unknowns were extrapolated from the graph. The protein concentration of each

sample (mg/ml) was used to equalize the differences in gamma radiation counts (CPM-counts per million) due to protein differences. Uptake of Mn into the cells was expressed in CPM\mg protein.

Formation of Mn-Tf Complex:

Previous studies suggest that Mn^{2+} must be oxidized to Mn^{3+} to bind Tf. In these studies the binding of Mn to Tf was catalyzed by ceruloplasmin which serves as an oxidative agent. Ceruloplasmin's action as an oxidizing agent has been observed in unpublished studies by Keith Schrader, another Honors Fellow. The Mn, Tf, and ceruloplasmin were incubated at 37 °C for two hours in a solution of HEPES (N-2-hydroxethylpiperazine-N^1-2-ethanesulfonic acid) and sodium bicarbonate, pH 7.4. This solution was then applied to a Sephadex G-75 column to separate free Mn from the complex of Mn and Tf. Samples were eluted at two min. intervals and measured for radioactivity on a gamma radiation counter. The Gamma counter window settings were (0 to 460) and (460 to 900).

Incubation of K-562 cells with Tf-Mn Complex:

Since the C-1474 cell line did not show significant uptake of free Mn, it was decided to test the Mn-Tf complex on a cell line that was known to take up free Mn,

the K-562 cell line. A Mn-Tf complex was created by incubating ^{54}Mn, human apo-transferrin, and ceruloplasmin in a solution of HEPES and sodium bicarbonate. The incubation was conducted for 5 days at 4°C. The complex was then dialyzed overnight against DPBS at pH 7.4 with 2 changes of medium. The K-562 cells (1 X 10^6 cells per group) were incubated with either the Tf-^{54}Mn complex solution or a solution of free ^{54}Mn. Both solutions contained comparable amounts of radioactivity. After one hour at 37°C, the cells were washed with DPBS and 1mM EDTA (ethyldiaminetriaminic acid) to remove any unincorporated ^{54}Mn. The cells were then measured for ^{54}Mn content on a Beckman gamma counter with window settings at (0-460) and (460-900).

This procedure was also repeated with ^{59}Fe and Tf to determine if the 5 day incubation period affected the integrity of the Tf molecule. The samples were measured for radioactivity on the Beckman gamma counter with window settings at (0-1000) and (460-900).

Results

Normal (C-1474) fibroblasts were used in all studies with free ^{54}Mn. The first experiments were conducted using 5 different concentrations of free ^{54}Mn from 100 μmolar to 800 μmolar. Incubation time was 2 hours. Uptake of the ^{54}Mn was minimal

at the lower concentrations and then increased dramatically after 600 µmolar. Maximal uptake was seen at 800 µmolar and was nearly 4-fold higher than uptake seen at 100, 200, and 400 µmolar. See figure A.

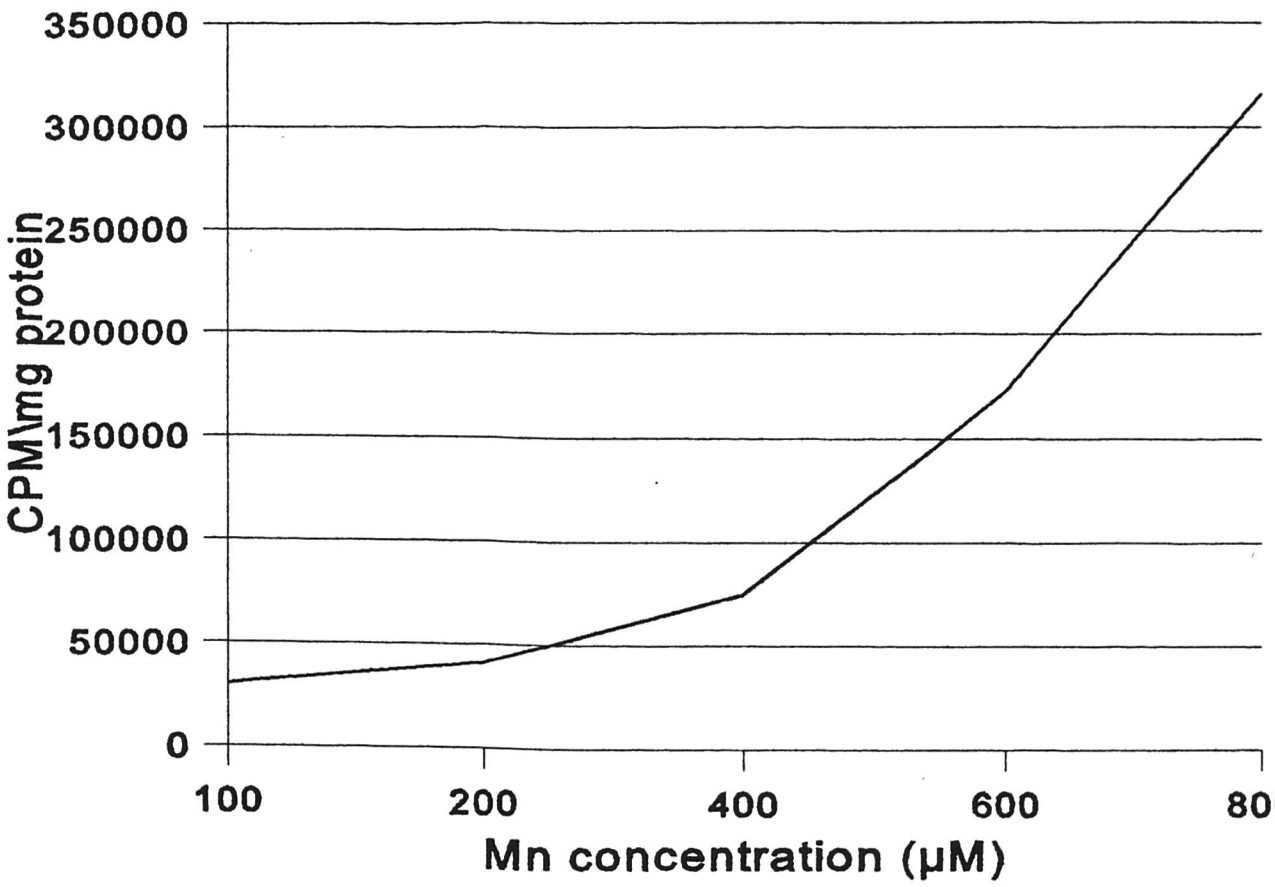

Fig. A. Uptake of free Mn at varying concentrations by C-1474 fibroblasts. *Cells were incubated for 2 hrs. in DPBS with 3% BSA and varying concentrations of Mn from 100µM to 800µM. Uptake was maximal at 800µM. Uptake is shown in CPM\mg protein.*

A time course from 30 min. to 2 hrs. revealed the highest ^{54}Mn incorporation to be at 800 µmolar concentrations regardless of incubation time. The 2 hour incubation was used in future experiments for consistency. See figure B.

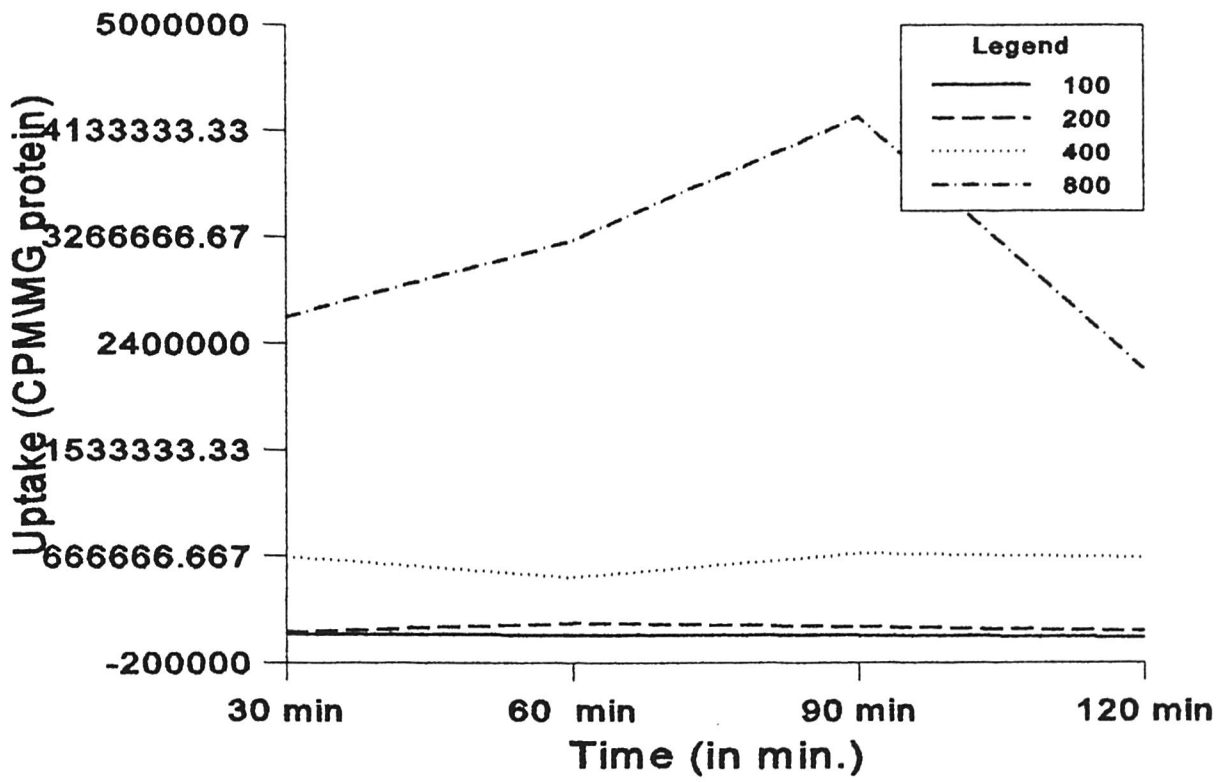

Fig. B. Uptake of free Mn by C-1474 fibroblasts over a 2 hr. time course. *Cells were incubated in DPBS with 3% BSA and varying concentrations of Mn from 100μM to 800μM. Throughout the time course, uptake at 800μM was highest. Uptake is shown in CPM\mg protein.*

Keeping a constant concentration of 800 μmolar, the fibroblasts were tested for ^{54}Mn uptake at 4 °C and 37 °C. Albumin was removed from the incubation medium of half the groups to see if this might have an effect on ^{54}Mn transport. The groups with 3% bovine serum albumin showed significant uptake at 800 μmolar and 37°C, however, the groups without albumin showed no significant uptake at 4 or 37°C. These results indicated that albumin might be as acting as a binding protein

to facilitate the transport of Mn into the fibroblasts. Further tests with and without albumin were repeated with 400 and 800 µmolar concentrations of ^{54}Mn. Once again, uptake was seen only in the groups with 3% albumin and more significantly at 800 µmolar than 400 µmolar. See figure C.

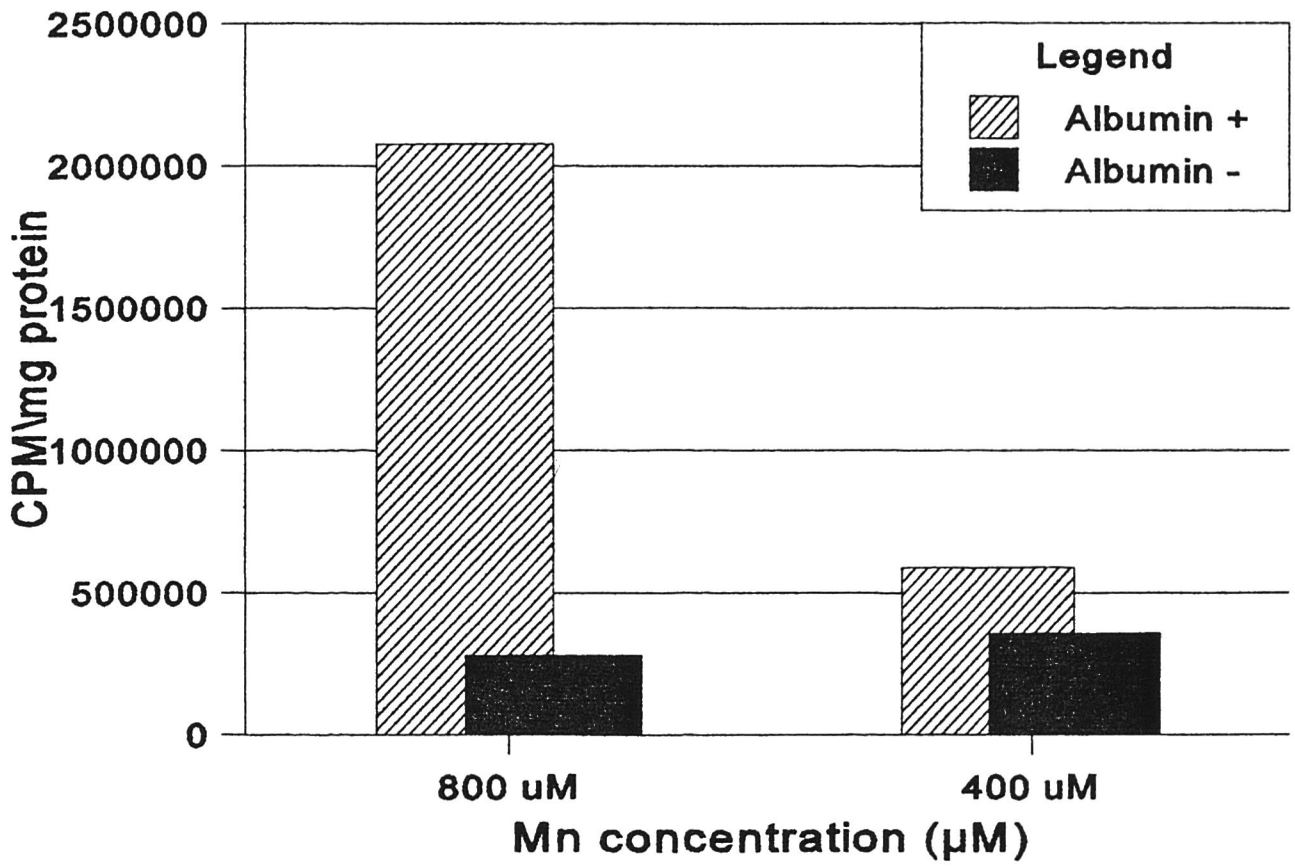

Fig. C. Uptake of free Mn by C-1474 fibroblasts +\- albumin in the incubation medium. *Cells were incubated in DPBS +\- BSA and 800µM or 400µM Mn for 2 hrs. Highest uptake is seen with 3% BSA at 800µM Mn. Uptake is shown in CPM\mg protein.*

To test if the ^{54}Mn was actually entering the cells in complex to albumin, EDTA was added to the harvesting washes to remove any ^{54}Mn that might be

adhering to the surface of the fibroblasts. Albumin was included the incubation medium of all groups. The ^{54}Mn concentrations used were 400 and 800 μmolar. Incubation temperature was also varied with half the groups at 4°C and the other half at 37 °C. No significant uptake was seen in any of the groups indicating that the free ^{54}Mn was not actually entering the fibroblasts.

At this point, a new method was used to introduce the ^{54}Mn to cells in culture. Since free ^{54}Mn did not seem to be entering the cells, a plasma protein was complexed with the ^{54}Mn to see if it would enhance uptake. Albumin was not included in the incubation medium of these experiments. Transferrin was the carrier protein of choice because it is thought to bind ^{54}Mn in plasma. (Rabin et al., 1993) K-562 cells were used in this experiment because they have been shown to take up free ^{54}Mn in unpublished studies by other members of our lab. The complex of Tf-Mn was incubated with three groups while three other groups were incubated with a solution of free ^{54}Mn. All groups were incubated for one hour at 37°C. The results revealed more free ^{54}Mn was taken in than Tf-^{54}Mn complexes. See figure D.

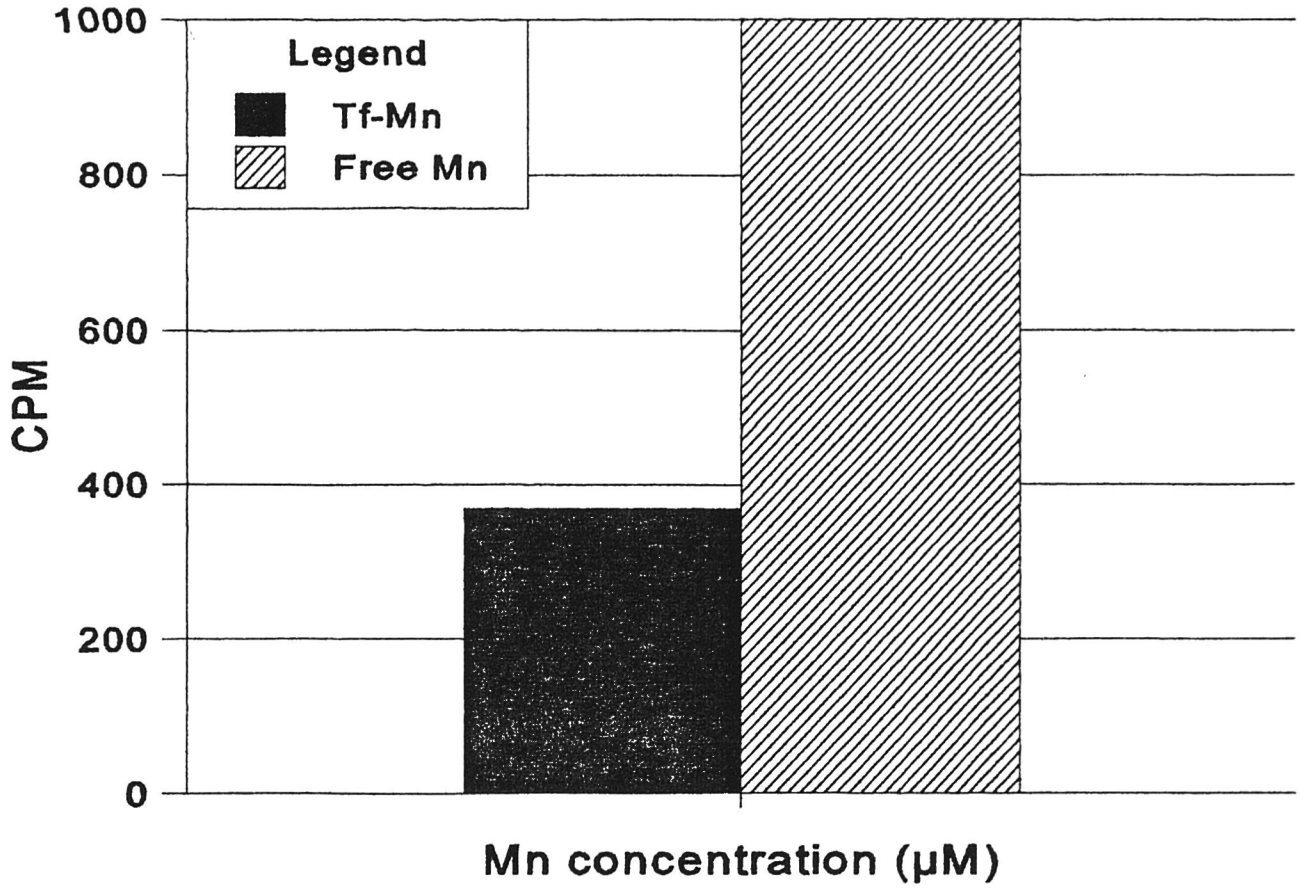

Fig. D. Uptake of a Tf-Mn complex or free Mn by K-562 erythrocytes. *K-562 cells were incubated or 1 hr. with either Tf-Mn or free Mn. Free Mn entered the cells 3-fold more readily than the Tf-Mn complex. Uptake is expressed in CPM.*

To test the integrity of the Tf protein the same protocol was used to test uptake of iron (Fe) via Tf. This uptake system has been well characterized and was expected to reveal if the Tf molecule had maintained its structural integrity throughout the experiment. The results revealed that the K-562 cells took up free ^{59}Fe more readily than complexed ^{59}Fe. See figure E.

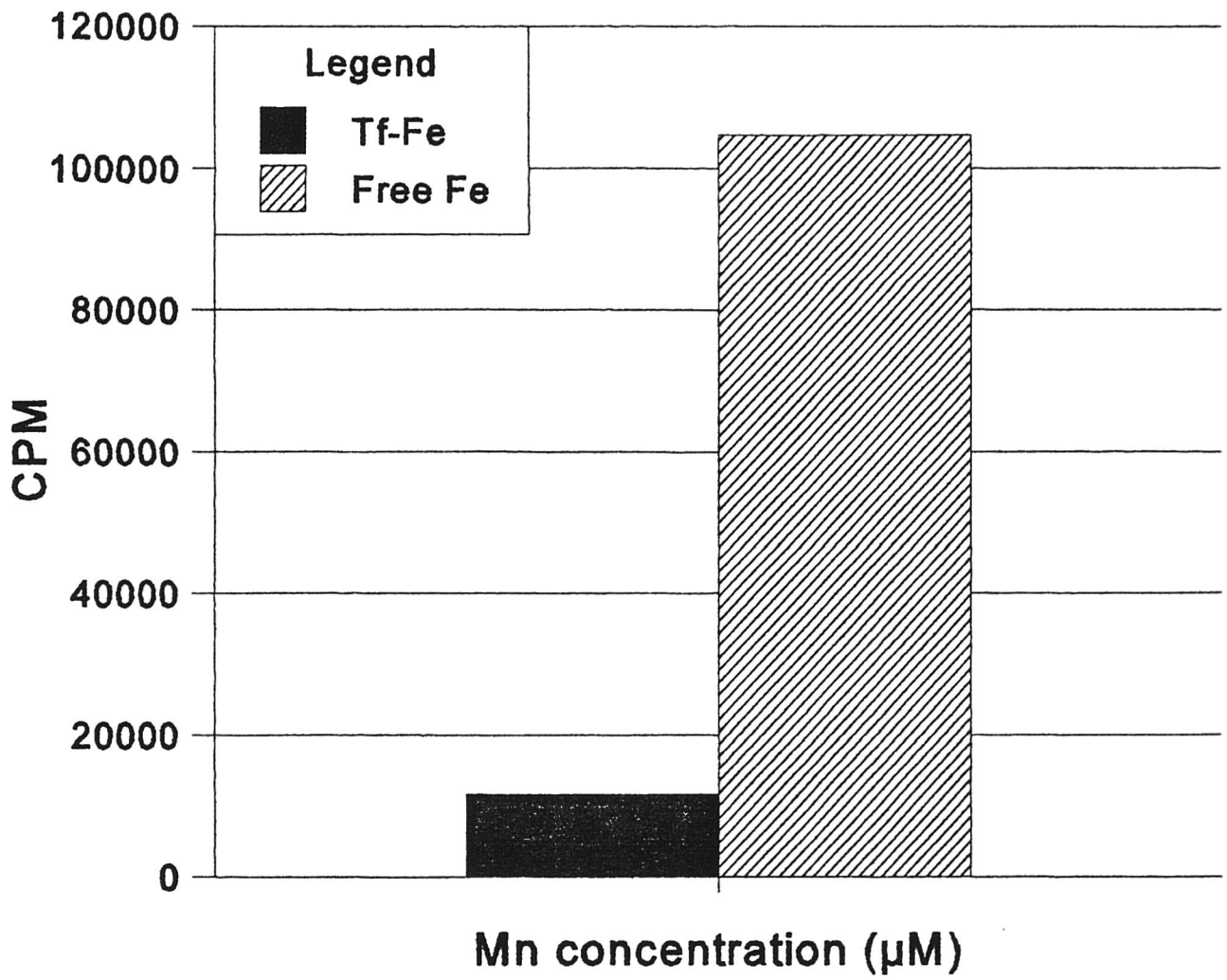

Fig. E. Uptake of a Tf-Fe complex or free Fe by K-562 erythrocytes. *K-562 cells were incubated for 1 hr. with either Tf-Fe or free Fe. Free Fe entered the cells 10-fold more readily than the Tf-Fe complex. Uptake is expressed in CPM.*

Discussion

Previous studies of Mn metabolism suggest two main methods of transport: incorporation of the free ion and incorporation of a Mn-protein complex. Free ^{54}Mn was shown by Rabin et al. to be taken up by the CNS as a free ion that was critically affected by plasma protein binding (1992). Other researchers have

suggested albumin, transferrin, and transmanganin as possible plasma carriers of Mn. A 1985 study by Scheuhammer et al. demonstrated the binding of ^{54}Mn to Tf rather than albumin. They indicated that about 50% of Mn in plasma was found in association with Tf while only 5% was associated with albumin. Protein complex transport of Mn and Fe transport were found by Davidson et al. in their study of lactotransferrin in human milk(1989). Their studies found receptor mediated uptake of lactoferrin-Mn in brush border membrane vesicles of the small intestine of rhesus monkeys. In addition, human neuroblastoma cells have been characterized as expressing a Tf receptor by which Tf-Mn complexes are incorporated into the cell (Suarez et al., 1992).

The results of my experiments indicate that Mn does not enter fibroblast cells in a free form. At high concentrations, it appears to be adhering to the cell membrane of fibroblasts but is not penetrating the cell. Previous studies have indicated that free Mn enters K-562 cells. Therefore, it is possible that the shape of the cells affects the transport of Mn. The fibroblast is a surface adhering cell that has 180° exposure to the surrounding medium. K-562 cells, on the other hand, are free floating and have 360° access to the surrounding medium.

In the experiments with the K-562 cells and the Tf-Mn, it is possible that the Tf-Mn was not presented under the appropriate physiologic conditions or that the 5

day complex formation incubation may have damaged the Tf protein. The uptake of Tf-Mn in neuroblastoma cells was tested with a Tf-Mn complex that had been formed over a 30 min. time period (Suarez et. al., 1993). In that experiment, a 20-fold excess of hydrogen peroxide was used to oxidize the Mn from 2+ to 3+. Uptake of the Tf-Mn complex by the neuroblastoma cells was successful and yielded a K_D of 13 +\- 1 nM and a receptor number of 11,000 +\- 2,000 per cell.

Another reason for the lack of Tf-Mn uptake could have been the lack of albumin in the incubation medium. Without albumin in the media, the cells were exposed to non-physiologic conditions which may have made a difference in the recognition of the Tf-Mn complex. Since albumin appeared to be helping Mn adhere to the surface of fibroblasts in my previous experiments, it is possible that the K-562 cells needed it to recognize the Tf-Mn complex. A shorter complex formation period may be sufficient and less stressful for the Tf-Mn.

One interesting point that challenges the protein complexed theory of Mn transport is the uptake of free Mn seen in K-562 cells. The uptake seen in groups of K-562 cells incubated with complexes of Tf-Mn may not have been uptake of Tf-Mn. It may have actually been incorporation of free Mn that had disassociated from the Tf. This is suggested by a Sephadex G-75 column chromatography that a was performed on the Mn-Tf solution (sol. A) after it had been incubated with the K-562

cells. The chromatography revealed an initial peak of the Mn and Tf complex and then a second peak that represented the presence of some free Mn. See figure F.

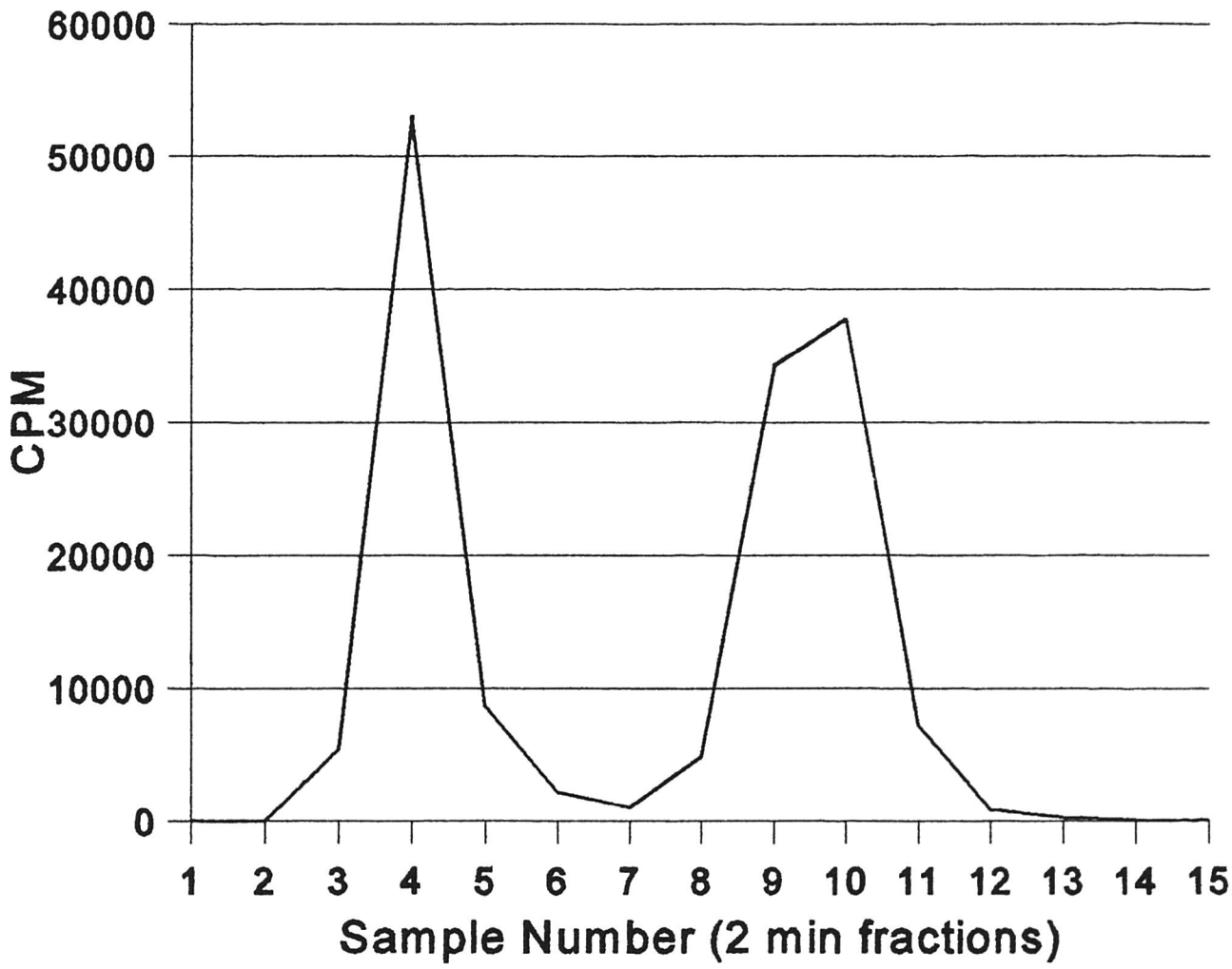

Fig. F. Sephadex G-75 column separation of the Tf-Mn complex post incubation with K-562 cells. *Two peaks were seen in the fractions. The first indicates the formation of a Tf-Mn complex and the second represents free Mn. Radioactivity is expressed in CPM.*

This corresponds with the levels of uptake seen in the respective groups. The uptake by the cells incubated with the sol. A was one half or less that of the cells

that were incubated with sol. B. The free Mn from the second peak in sol. A was about one half the level of the free Mn in the control solution made with free ^{54}Mn (sol B). These results suggest that perhaps only free Mn was entering the cells.

All of these studies indicate that Mn transport is being facilitated to varying degrees by transport proteins in different cell types. Studies regarding the effect of albumin in the uptake of the complexes and studies on the formation of the Tf-Mn complex are needed to determine the specific needs of this transport system. Further comparisons of Tf-Fe to Tf-Mn transport will also be helpful because of the increasing similarities between the two systems. Clearly, more work is needed to investigate the possibilities for Mn uptake across cell membranes.

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Appendix A

Listing of graphs and charts