The Uptake of Divalent Manganese Ion by Mature Normal Human Red Blood Cells

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ABSTRACT At physiological pH and concentrations of Mn^{++} in excess of 5×10^{-4} M, study of the Mn^{++} ion movement into human red cells is complicated by physicochemical alterations of the ion itself. At concentrations below 5×10^{-4} M, the rate of uptake bears a linear relationship to the Mn^{++} concentration. The permeability constant for inward movement of Mn^{++} is 2.87 ± 0.13 (S.E.) $\times 10^{-9}$ cm./sec. The rate is not influenced by the addition of metabolic substrates such as glucose or adenosine or the metabolic inhibitors iodoacetate or fluoride. Co^{++}, Ca^{++}, and Mg^{++} do not appear to compete with Mn^{++} for entry, but at high concentrations relative to Mn^{++}, they reduce the rate of entry. Ca^{++} is far more effective than Co^{++} or Mg^{++} in this regard. The permeability constant for outward Mn^{++} movement is 1.38 ± 0.21 (S.E.) $\times 10^{-9}$ cm./sec., about half of that for entry. This slower rate of outward movement is consistent with the finding that 40 to 60 per cent of the Mn^{++} taken up by the red cells is non-ultrafilterable. Less than 5 to 10 per cent of the Mn^{++} appears to be bound to the stroma. It is concluded that entry and exit of Mn^{++} is a process of passive diffusion involving no carriers, transport, or metabolic linkage.

The many studies on the transport of Na^+ and K^+ across a variety of biological membranes stand in direct contrast to the few comparable observations on divalent cations. Among the several significant contributions to the latter problem are those of Soloway, Welsh, and Solomon (1), as well as those

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of Flückiger and Keynes (2) and Keynes and Lewis (3) who studied Ca^{++} transport in nerve, and those of Gilbert and Fenn (4), Harris (5), and Shanes and Bianchi (6) on the Ca^{++} fluxes in muscle. Most of the investigators using animal cells have concluded that Ca^{++} movement into and out of nerve and muscle cells was governed by processes of passive diffusion and cell membrane binding, although Gilbert and Fenn (4) presented evidence for an active extrusion of Ca^{++} from frog muscle. Saltman *et al.* (7–10) also found the uptake of Fe^{++}, Cu^{++}, and Zn^{++} by rat liver slices to be a passive process. On the other hand, the uptake of Sr^{++} by barley root investigated by Epstein (11), as well as the uptake of Mn^{++} and Mg^{++} by yeast cells described by Rothstein and coworkers (12–14) appears to be dependent on the operation of an active transport system.

Utilizing red blood cells, Jandl and Simmons (15) have studied the effects of polyvalent cations on membrane reactions, particularly the phenomena of hemolysis and agglutination. Jandl and colleagues (16) have also studied the incorporation of iron into reticulocytes. However, the mechanism of entry and exit of the ions through the membranes, the role of metabolism, and other factors in ion distribution, have not been explored in erythrocytes.

Mg^{++} is the divalent ion of greatest current physiological interest. It plays an essential role as an activator of many enzymes. However, chemical methods for measurement of Mg^{++} in biological systems present many difficulties and the only usable isotope, Mg^{28}, has a half-life of only 21 hours. For these reasons, Mn^{++} was used as a substitute in the present studies. Mn^{++} can usually replace Mg^{++} as an enzyme activator (17, 18); it has a similar binding affinity for most ligands (19), it is transported into the yeast cell by the same mechanism as Mg^{++} (13, 14), analytical assays can be made more readily, and it has a satisfactory isotope with a 310 day half-life (Mn^{54}). During the course of the studies to be reported in this paper, it became apparent that the use of this ion was accompanied by a number of serious problems. Unexpected (to us) difficulties with oxidation of stock solutions of Mn^{++} had to be taken into account before reproducibility of experiments was possible. Questions concerning the valence and other aspects of the chemical state of manganese within cells can be raised. Nevertheless, useful data were obtained concerning Mn^{++} uptake and release by red cells, the effects of metabolic factors, the role of cation binding, and the influence of other cations on these processes. From these results, it can be concluded that Mn^{++} entry and exit involve only passive diffusion through the erythrocyte membrane, and that the distribution of Mn^{++} is influenced by binding or other chemical reactions within the cell.

MATERIALS AND METHODS

Blood was obtained from the antecubital vein of hematologically normal adult male human donors within 1 hour prior to starting each experiment. One mg. of heparin

was added to each 2 ml. sample of blood. The fresh whole blood was centrifuged at 1750 × *g* for 10 minutes in a 25 × 115 mm. pyrex test tube and the plasma, leukocyte-platelet layer, and upper 5.0 mm. of the red cell column were removed by aspiration. The remaining red cells then were washed three times with a saline-tris buffer of pH 7.4.

Preliminary study of the ionic activity of Mn^{++} in the presence of tris(hydroxymethyl)aminomethane at pH 7.4 by the titration technique of Martell and Frost (20) and the potentiometric method described by Kolthoff and Lingane (21) revealed no complexing of Mn^{++} by tris. Consequently, a saline-tris buffer, the use of which was described by Gourley (22), was chosen as a cell-suspending medium to avoid the interactions with Mn^{++} which occur in media containing organic acids, phosphates, or proteins. Bertinchamps and Cotzias (23) have discussed the protein-Mn^{++} interactions. The final buffer concentration was 0.05 M at pH 7.4. The buffering was adequate to restrict pH changes to less than 0.1 pH unit during the course of a 3 hour incubation period at 37°C.

The isotope Mn^{54} was added in the form of $MnCl_2$, diluted with appropriate amounts of carrier $MnCl_2$. Incubations were carried out in large (100 ml.) Warburg vessels, shaken in a 37°C. water bath. After predetermined times aliquots of the red cells were removed, and washed three times, with ice-chilled (0–4°C.) sodium chloride solution (0.154 M) to free them of any trapped intercellular radioactivity. The radioactivity of an aliquot of washed cells was measured in a well-type scintillation counter. That the washing procedure did not remove appreciable Mn^{54} from the cells was demonstrated by estimating the radioactivity in both the supernatant and in the washed cells. The Mn^{54} left in the washed cells was within 2 per cent of that lost from the supernatant, indicating that less than 2 per cent was lost in the washing procedure. Radioactivity measurements of the cells rather than of supernates were chosen as standard procedure because cell uptake could be measured with considerably greater sensitivity and accuracy in this way. Counting times were chosen to yield counting errors of less than 2 per cent (24).

Red cell counts, determined with the Coulter electronic blood cell counter (25, 26), and microhematocrit values were both found to provide satisfactory reference points for the Mn^{++} uptake. Standard deviations of the microhematocrit values and the red blood counts were 3 and 2.8 per cent respectively. Mn^{++} uptake was calculated on the basis of the hematocrit values and expressed as μg. Mn^{++}/ml. cell water. Ponder's value (27) of 0.65 gm. water/gm. RBC was taken as the normal for red cell water in whole blood and from our observations, 96.9 μ^3 was taken as the value for red cell mean corpuscular volume (MCV).[1]

The uptake of Mn^{++} was relatively slow so that long periods of incubation were often necessary. Bacterial contamination occurred with increasing frequency in flasks incubated for more than 5 hours, and in most flasks after 24 hours. Fortunately most measurements could be made in a 3 hour period, at a time when contamination was not a problem.

[1] Although the initial red cell mean corpuscular volume 91.03 $\mu^3 \pm 1.54$ (S.E.) ($n = 6$) in the saline-tris suspension agrees with the figure given by Brecher *et al.* (26), for the MCV in males, this volume increased during 3 hours of incubation to 96.9 $\mu^3 \pm 1.16$ (S.E.) ($n = 14$). Most of this increase in size occurred during the 1st hour of incubation, and to simplify the calculation, the larger value was used in all calculations based on red cell volume.

Ultrafiltration experiments were performed at 37°C. under a N_2 atmosphere utilizing the Toribara ultrafiltration apparatus (28) and 24/32 inch Nojax Visking casing. The casing was presoaked, warmed, and rinsed several times in distilled water to minimize metal binding by the casing itself, as suggested by Hughes and Klotz (29). Even then, however, significant amounts of Mn^{++} were bound by the sacs in the concentration range from 10^{-7} M to 10^{-4} M, necessitating use of the correction procedure for such binding outlined by Hughes and Klotz (29).

Hemoglobin electrophoresis experiments were carried out in veronal buffer at pH 8.5, using a Beckman Spinco paper electrophoresis apparatus. Analysis of the Mn^{++} movement was made through study of radioautograms prepared from the paper strips. High-speed centrifugation was performed at 4°C. in a Servall hi-speed centrifuge.

A number of technical difficulties attended the use of Mn^{++} in the studies reported here. The first problems were encountered in studies involving a wide range of Mn^{++} concentrations. At very low concentrations, study of the uptake of the cation may present problems, if a large proportion of added Mn^{54} disappears from the suspension, or if glass-absorbed Mn^{54} from a previous experiment desorbs into the suspension. This problem was controlled by siliconizing[2] glassware and by careful cleaning procedures. At higher ion concentrations, two difficulties were noted. Some hemolysis occurred, especially in experiments of long duration, amounting, for example, to 3.5 per cent of the cells after exposure to 4.5×10^{-3} M $MnCl_2$ for 3 hours. Also, aggregation and precipitation of the Mn^{++} occurred in the presence of red cells at ion concentrations above 5×10^{-4} M, giving rise to very erratic and non-reproducible results. After incubation for a few hours, precipitates could not be seen in the supernates, but Mn^{54} appeared in a colloidal fraction that came down with the cells upon centrifugation and then could be washed away from the cells. After 24 hours of standing, a clearly apparent white precipitate could be found in the supernates. Because of these difficulties, the concentration range of Mn^{++} was limited in the present studies to 1×10^{-5} to 5×10^{-4} M. In this range, hemolysis was negligible, the results were reproducible, and the Mn^{++} that disappeared from the medium could be found in the cellular fraction. These concentrations of Mn^{++} were sufficiently higher than the most recently determined values for red cell manganese content (0.024 μg./ml. RBC) obtained through neutron activation analysis by Bowen (30), to permit study of net ion movement by measurement of radioactivity alone.

Another type of difficulty evidenced itself as progressive day to day change in the rate of uptake of Mn^{++} by fresh red cells, despite apparent constancy of conditions. This difficulty was finally traced to changes occurring in the 0.1 M $MnCl_2$ stock solution when it was exposed to air. For example, if the stock solution was left for 3 days in air, the rate of erythrocyte uptake of Mn^{++} was only 20 per cent as high as the uptake from the same solution freshly made and kept under a N_2 atmosphere. A parallel decrease in the hemolytic activity of high concentrations of Mn^{++} is shown in Fig. 1. Polarographic determinations at −1.51 v. of the Mn^{++} of the stock solution indicated a 10 per cent reduction in concentration after 24 hours, but no further change thereafter. That some oxidative reaction was involved in these changes in

[2]Dow Corning 200 fluid (viscosity grade 350 centistokes).

Mn^{++} solutions was indicated by the fact that the changes did not occur in a N_2 atmosphere. Although, with respect to time, the polarographic measurements correlated well with the changes in uptake and the hemolysis studies, it is apparent that the 10 per cent decrease in Mn^{++} ion cannot alone account for the much larger reductions in rates of uptake or in hemolytic activity. To eliminate this problem, fresh $MnCl_2$ solutions were prepared for each experiment and kept under an atmosphere of N_2. The experiments and washing procedures were also carried out in an atmosphere of N_2. In short term experiments of 1 hour or less no differences in the rate of uptake were observed in air as compared with an N_2 atmosphere. The N_2 atmosphere alone did not cause hemolysis.

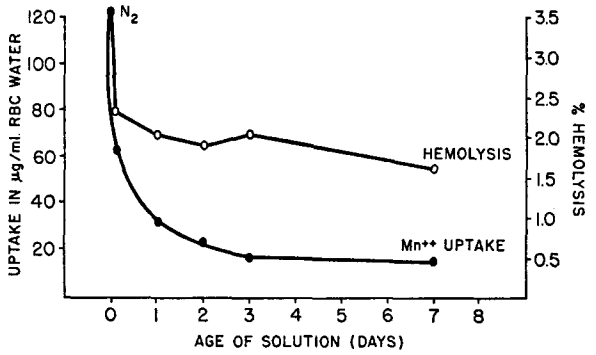

FIGURE 1. 3 hour uptake of Mn^{54} from diluted stock solutions of varying age and hemolysis produced by these solutions. The concentration of carrier Mn^{++} was 4.5×10^{-3} moles/liter. Note that the uptake and hemolysis at zero time are represented by three points; the upper, marked N_2, indicates the uptake and hemolysis in a solution freshly prepared under a nitrogen atmosphere and with the experiment conducted under nitrogen. The lower points at zero time represent the uptake and hemolysis in a solution prepared simultaneously but in contact with ambient oxygen tension of air. The other experiments, at varying time intervals, were also conducted under ambient atmospheric conditions.

RESULTS

Pattern of Mn^{++} Uptake by Red Cells

Fig. 2 illustrates the pattern of uptake of Mn^{54} by erythrocytes from solutions having concentrations of carrier Mn^{++} from 10^{-5} to 5×10^{-4} M. The curve indicates the slow rate of uptake. Although the curve shows a tendency to level off between 5 and 9 hours from the start of the incubation, there is a slight continuing uptake for as long as measurements are feasible, in this case, 27 hours. The flattening of the curve after 12 hours may represent establishment of an equilibrium distribution but in view of the slow continued uptake for as long as 27 hours, and the complications of bacterial contamination and cell changes after 5 hours, no attempt was made to study the equilibrium state.

Although it was not feasible to study equilibria, the rates of uptake could be determined readily in a reasonable time. Apart from a small, rapid, initial increment, Mn^{++} uptake was linear for 4 to 5 hours, as shown in Fig. 3 at all concentrations used in the range of 1×10^{-5} M to 5×10^{-4} M. This allowed estimates of uptake rates from the slopes of the curves. The influence of Mn^{++} concentration on this rate illustrated in Fig. 4, indicated a simple proportionality at least up to a concentration of 2×10^{-4} M.

In order to compare the rates of uptake under a variety of conditions, the data were calculated in terms of permeability constants, using the Fick equation as outlined by Davson (31) and modified to take into account a signifi-

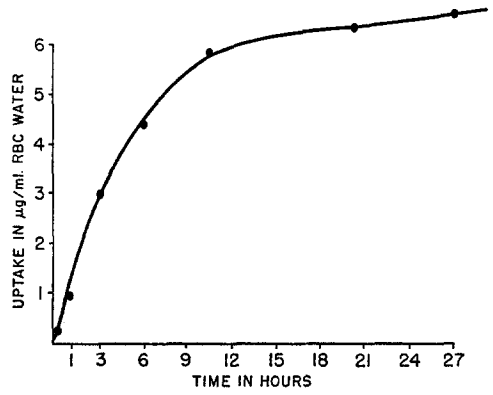

FIGURE 2. Uptake of Mn^{54} by red blood cells over a 27 hour period of incubation. The concentration of carrier Mn^{++} was 1.4×10^{-4} moles/liter of saline-tris buffer.

cant decrease in the external concentration of the ion as outlined by Mullins *et al.* (32).[3] Estimations of the outward permeability constant (K_{out}) were made by first incubating erythrocytes in Mn^{54}, washing and resuspending them in saline-tris buffer, and then observing the loss of radioactivity from the cell mass. These permeability constants are given in Table I. The repro-

[3] The equation utilized in these calculations was as follows:—

$$K = \frac{1}{b} \cdot \frac{V}{At} \cdot 2.303 \log \frac{C_{out}}{C_{out} - C_{in.}\, b}$$

Where K = permeability constant in centimeters/sec.

$b = \dfrac{\text{extracellular volume + volume cell water}}{\text{extracellular volume}}$

V = 96.9 μ^3

A = surface area, 163 μ^2 after Ponder (33).

C_{out} = initial extracellular Mn^{++} concentration (counts/ml. supernatant)

C_{in} = intracellular concentration at time (t) in counts/ml. cell water

t = time in seconds

ducibility of these measurements is evidenced by the relatively small standard error for K_{in} of 2.87 $\pm$ 0.13 $\times$ 10^{-9} cm./sec. for forty-two determinations.

The influence of a number of metabolic substrates and inhibitors was tested, including: glucose in concentrations of either 5 or 18 $\times$ 10^{-3} M, 5 $\times$ 10^{-3} M

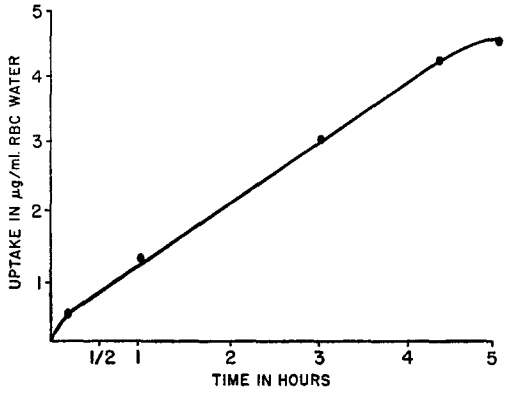

FIGURE 3. Uptake of Mn^{54} by red blood cells over a 5 hour period of incubation. The concentration of carrier Mn^{++} was 1.4 $\times$ 10^{-4} moles/liter of saline-tris buffer.

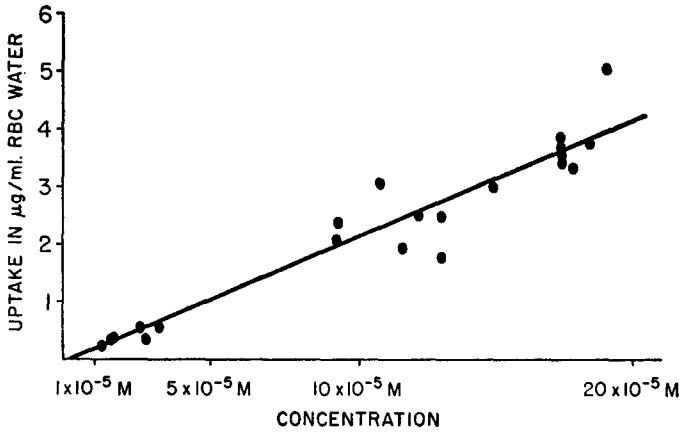

FIGURE 4. Relationship between uptake of Mn^{54} after 3 hours of incubation and concentration of carrier Mn^{++} in the medium. By the method of least squares: $Yx = 2.04 + 0.212\ (x - 9.935)$. The standard error of estimate of this slope was 0.995.

adenosine, 5 $\times$ 10^{-3} M sodium iodoacetate, and 5 $\times$ 10^{-3} M, 2.5 $\times$ 10^{-2} M, or 1.25 $\times$ 10^{-1} M sodium fluoride. In no case, except with the highest concentration of sodium fluoride did these agents exert any effect on the rate of uptake of Mn^{++} as expressed by the permeability constant, shown in Table I. The inhibitory action of the highest fluoride concentrations is not surprising, for such concentrations are known to affect not only the metabolism of red cells but their permeability to K^+ as well (34–36).

The effect of temperature on Mn^{++} uptake was assessed by simultaneous studies at 37°C., 26°C., and 4°C. An Arrhenius plot of the three rates yielded a straight line relationship with a slope equivalent to an energy of activation of 13,000 cal./mol.

Other divalent cations can influence the rate of Mn^{++} uptake as shown in Table II. Of the three tested, Ca^{++} had the most effect, Co^{++} was somewhat less effective, and Mg^{++} had the least effect. These ions are required in concentrations many times higher than those of the Mn^{++}. For example, Ca^{++} produces 50 per cent inhibition of Mn^{++} uptake at 1×10^{-3} M; Co^{++} in a

TABLE I

PERMEABILITY CONSTANTS

	K	Standard error	No. of observations (N)
	(*cm./sec.*)		
K_{in} control	2.87×10^{-9}	±0.13	42
K_{out} control	1.38×10^{-9}	±0.21	5
K_{in} NaF			
5×10^{-3} M	2.54×10^{-9}	±0.23	5
2.5×10^{-2} M	2.54×10^{-9}	±0.23	5
1.25×10^{-1} M	1.63×10^{-9}	±0.42	2
K_{in} iodoacetate			
5×10^{-3} M	2.97×10^{-9}	±0.57	3
K_{in} glucose			
$5\text{–}18 \times 10^{-3}$ M	2.36×10^{-9}	±0.42	6
K_{in} adenosine			
5×10^{-3} M	2.79×10^{-9}		1

concentration of 3×10^{-3} M and Mg^{++} in concentrations between 8 and 60×10^{-3} M give the same 50 per cent inhibition. The Mn^{++} concentration in these systems is only 1 to 4×10^{-5}. Thus, the relative concentrations of these ions are between 50/1 for Ca^{++} and 1000/1 for Mg^{++}. It seems doubtful, therefore, that these other divalent ions are competing with Mn^{++} for entry into the cells. Rather, it is more likely that the Ca^{++}, Co^{++}, and Mg^{++} act directly on the red cell membrane to reduce its permeability to Mn^{++}.

The State of Mn^{++} in the Red Cell

MN^{++} BINDING BY THE RED CELL MEMBRANE Cells containing the amount of Mn^{54} taken up in 3 hours of incubation were washed free of external Mn^{++}, hemolyzed either with 20 volumes of distilled water or by the freeze-thaw method, and centrifuged at $17,000 \times g$ for 20 minutes to separate the stroma from supernatant hemolysate. This method of separating stroma from hemolysates is justified by the work of Danon *et al.* (37) who succeeded in recovering

95 to 100 per cent of red cell ghosts, prepared by hemolysis in hypotonic aqueous solution, by centrifuging at 3,500 × *g* for 5 minutes. Our own observations showed that no stromal particles could be seen on phase microscopic examination of the supernatants removed for measurement of radioactivity. Nearly all (96.6 per cent ± 2.4 per cent, S.D.) the radioactivity was found in the supernatant rather than in the stroma separated by centrifugation. This indicates that possibly 5 per cent of the Mn^{++} was bound to the erythrocyte stroma. This amount correlates well with the 5 to 10 per cent of

TABLE II

EFFECT OF INHIBITORS ON Mn^{++} UPTAKE

(Mn^{++}) 1×10^{-5} to 4×10^{-5} M

Ion concentration		3 hr. uptake of Mn^{++} per cent of control
	M	
Mg^{++}	1.2×10^{-6}	100
	1.2×10^{-5}	100
	1.2×10^{-4}	100
	1×10^{-3}	100
	3×10^{-3}	100
	8×10^{-3}	100
	6×10^{-2}	30
Ca^{++}	1.2×10^{-4}	70
	1.0×10^{-3}	48
	1.2×10^{-3}	44
	2×10^{-3}	17
	1.3×10^{-2}	4
Co^{++}	1.1×10^{-4}	100
	3.0×10^{-3}	49
	5.0×10^{-3}	35

the Mn^{++} taken up rapidly by intact red cells in the first 10 minutes as illustrated in Fig. 3, and suggests that the early uptake is related to binding by the membranes, whereas the greater part of the uptake, 90 to 95 per cent, represents actual movement of Mn^{++} into the interior of the cells. Confirmatory evidence for this concept was obtained through experiments, in which Mn^{++} uptake by stroma of red cells that had been hemolyzed in the reaction vessel, before exposure to Mn^{54}, was compared with uptake of this ion by a similar initial volume of intact cells. Under these conditions, the stromal Mn^{++} uptake was 3 per cent of the amount taken up by a like initial volume of whole cells. Fig. 5 illustrates one of these experiments.

ULTRAFILTRATION In an attempt to assess the proportion of Mn^{++} taken up by red cells that might be accounted for by intracellular binding of the ion, ultrafiltrates were prepared from hemolysates of cells that had taken up varying amounts of Mn^{++}. In addition, hemolysates were prepared directly

and incubated with Mn^{++}, following which ultrafiltration was carried out. Over a wide range of Mn^{++} concentrations ranging from 1×10^{-7} to 1×10^{-3} M, 40 to 60 per cent of the amount taken up by the intact cells or added to hemolysates was found to be non-ultrafilterable. Graphic representation of these results is shown in Fig. 6, in which the concentration of bound (non-ultrafilterable) Mn^{++} per mole of Hb is plotted against the negative logarithm

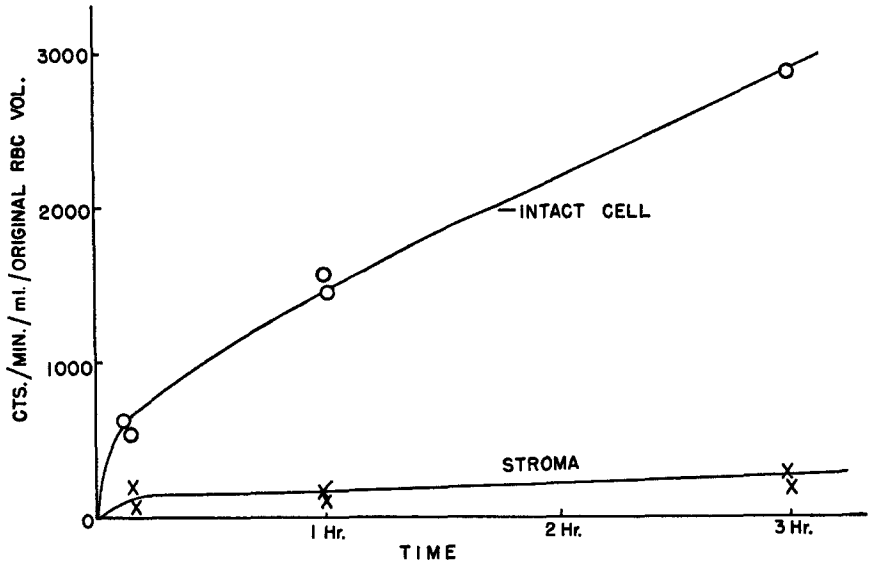

FIGURE 5. Comparison of uptake of Mn^{54} by intact red blood cells with uptake by stroma over a 3 hour period of incubation. The stroma was prepared from the same red cell sample and represented an identical initial volume of cells.

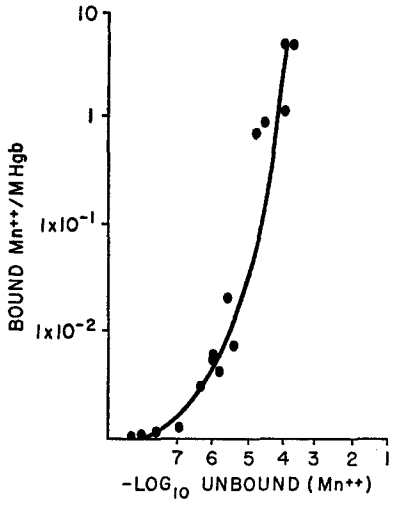

FIGURE 6. Effect of varying concentration of free Mn^{++} on the ratio of non-ultrafilterable Mn^{++}/mole of hemoglobin. Note that the graph of the function fails to parallel the abscissa at higher concentrations of Mn^{++}.

of the free (ultrafilterable) Mn^{++} concentration. The values for bound Mn^{++} were referred to hemoglobin concentration because of the accuracy and ease of measuring this protein, and because hemoglobin is present in red cells in many times the concentration of other intracellular proteins or potential ligands. Saturation of binding sites would have been suggested by a tendency for the curve of Fig. 5 to level off parallel to the abscissa; no such tendency was noted within the concentration range studied.

ELECTROPHORETIC STUDIES Paper electrophoresis of cell hemolysates containing Mn^{++} demonstrated that all the radioactive Mn^{++} moved toward the cathode in a fashion identical with the movement of Mn^{++} in a solution of $MnCl_2$ alone placed on a parallel strip of paper. The failure of Mn^{++} to move toward the anode with the hemoglobin or with any other negatively charged molecule does not exclude binding to protein but simply indicates that if such binding did occur, it was weaker than the force exerted by the electrical field in the electrophoresis apparatus.

EFFLUX OF MN^{++} The permeability constant for efflux of Mn^{++} was $1.38 \pm 0.21 \times 10^{-9}$ cm./sec. or about half of the rate of influx of 2.8×10^{-9} cm./sec. The factor of $\frac{1}{2}$ in permeability constants is consistent with the 50 per cent fraction of filterable Mn^{++} found in the hemolysate.

DISCUSSION

Mn^{++} did not prove to be as satisfactory as anticipated for studies of the factors affecting penetration of the membrane of the red blood cell by divalent cations. Its advantages in terms of ease of measuring its isotope and chemical analysis, as well as its similarity to Mg^{++} in physiological properties, is to a large measure offset by its complex behavior in solution, such as the aging effect on standing in air and the formation of colloidal aggregates and precipitates with higher concentrations of the ion in the presence of cells and hemolysates. These complications are undoubtedly related to known chemical properties of Mn^{++} solutions such as hydration, hydrolysis, and autooxidation to higher valence states (38, 39), followed in some cases by precipitation of $Mn(OH)_3$. Gurd and Wilcox (40) have pointed out the importance of considering some of the physicochemical transformations that transition metal ions in solution may undergo prior to interacting with protein molecules, and the present experience clearly emphasized this point.

The difficulties enumerated above were avoided by using fresh solutions of $MnCl_2$, by excluding O_2 from the system, and by limiting the Mn^{++} concentrations to the range 1×10^{-5} M to 5×10^{-4} M. Within these limitations, useful information was obtained concerning rates of entry and exit of Mn^{++}. All

the data are compatible with the concept of a simple diffusion of Mn^{++} into and out of the cell, with alteration of about half of the cellular ion into a non-diffusible form. None of the data suggests the existence of a specific transport system, or a membrane carrier, or involvement of metabolic processes.

The rates of entry and exit of Mn^{++} are very slow and within the limits of the concentrations used, a 50-fold range, the rate of entry was directly proportional to the concentration. Substrates such as glucose and adenosine or metabolic inhibitors, fluoride and iodoacetate in concentrations that block metabolism, had no effect. O_2 had no effect other than on the manganese ion itself. Other cations, Ca^{++}, Co^{++}, and Mg^{++}, did not compete for entry. The action of these ions is undoubtedly due to their direct effect on membrane permeability, like that seen in similar effects of these ions on cell permeability to other ions.

Parpart (41) pointed out the inhibitory effects of Ca^{++} on red cell permeability to Cl^- and $SO_4^=$, while Davson (42) and Levi (43) called attention to similar effects of this ion on K^+ permeability. Kahn (44) has studied inhibition by Ca^{++} of the active uptake of K^+ by cold-stored human red cells; he noted no inhibition of K^+ uptake by Mg^{++}. Bolingbroke and Maizels (45) recently described a relatively specific role for Ca^{++} in maintaining a low permeability of the red cell membrane to inorganic ions. The present studies suggest a high degree of specificity for Ca^{++} in reducing red cell permeability to Mn^{++}. It is 20 times as effective as Mg^{++}.

The site of cellular binding of Mn^{++}, based on the results of experiments involving the separation of stroma from hemolysate, seems to be different from that observed after the uptake of trivalent ions, such as Fe^{+++} and Co^{+++}, by red cells from a saline suspension, as studied by Jandl and Simmons (15). In the case of the trivalent ions it appears that most of the erythrocyte uptake represents membrane binding. In contradistinction, the uptake of divalent manganese represents ion movement into the cell with only 5 to 10 per cent bound to the membrane. Of the Mn^{++} which has moved into the cell, 40 to 60 per cent is converted to a non-ultrafilterable form and this is consistent with the back-exchange experiments which demonstrated a rate of efflux roughly one-half that for the influx of Mn^{++}. Neither our ultrafiltration experiments nor electrophoresis studies suggest a single specific intracellular ligand. Borg and Cotzias (46) have demonstrated Mn^{++} incorporation in manganese porphyrin compounds by erythrocyte precursors, but this seems unlikely to occur in mature red cells. Other possible binding sites include enolase or 2-phosphoglyceric acid, as pointed out by Malmstrom (17, 18), or binding to ATP. Finally, the intracellular formation of non-ultrafilterable Mn^{++} ionic polynuclear complexes, or precipitates, must be considered.

Saltman *et al.* (7–10) in their studies of the uptake of iron, zinc, and copper by rat liver slices, concluded that the cell membrane appeared to play a minor

role, whereas saturation of an intracellular binding site seemed to be the rate-determining factor. This conclusion was consistent with the low energy of activation of 770 cal./mol. In the case of Mn^{++} and the red cell, however, entry of the cation into the cell seems to be the rate-limiting factor during the 3 to 5 hour periods employed in the present study with no evidence of saturation of an intracellular binding site during this period. The energy of activation of 13,000 cal./mol. presumably represents the process of penetration of the membrane.

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