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Active Transport of Manganese in Isolated Membranes of *Escherichia coli*

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Accumulation of manganese was measured in subcellular membrane vesicles isolated from *Escherichia coli*. Accumulation of ^{54}Mn by vesicles in 0.5 M sucrose is stimulated by glucose and D-lactate and is inhibited by metabolic poisons such as dinitrophenol, *m*-chlorophenyl carbonylcyanide hydrazone, valinomycin, and nigericin. Manganese uptake by vesicles requires 10 mM calcium, which is not required for uptake of manganese by intact cells. The calcium requirement is specific and cannot be replaced by magnesium, sodium, or potassium. Strontium can replace calcium but is somewhat less effective than calcium. The uptake of manganese is via a manganese-specific system which shows saturation kinetics with manganese with a K_m of 8×10^{-6} M and a V_{max} of 4 nmoles per min per g (wet weight) at 25 C. Magnesium and calcium do not compete for uptake. The accumulated manganese can be released from the vesicles by lipid active agents such as toluene, and can be exchanged for external manganese.

In the preceding paper (18), a specific manganese active transport system in intact cells of *Escherichia coli* was described. In the present paper, the same transport system was studied in isolated subcellular membrane vesicles from *E. coli*.

Procedures for isolating membrane vesicle "ghosts" from *E. coli* have been developed by Kaback and collaborators (1, 10-14), and their chemical composition (14) and abilities to actively accumulate amino acids (11-14) and sugars (1, 10) have been described. I recently used these vesicles in studies of the action of colicin E1 (2). The vesicles are less complicated than the cells since they lack the cell wall, ribosomes, and soluble enzymes, and contain less than 5% as much deoxyribonucleic acid and ribonucleic acid and 15% as much protein as do cells (14). The ability of the membrane vesicles to actively take up manganese provides strong evidence for a manganese transport system in *E. coli* because one can study the properties of manganese transport in the absence of possible interference or binding by many cellular materials.

MATERIALS AND METHODS

Organisms and media. *E. coli* strain B was maintained on tryptone agar slants at 4 C and grown in synthetic medium A (3) for preparation of vesicles.

Isolation of membrane vesicles. Vesicles were prepared from frozen cells of *E. coli* B by the method described by Kaback (10), and were stored at −70 C at 10 mg/ml (dry weight) in 0.5 M K-PO_4 (*p*H 6.6), 14 mM glucose, and 10 mM $MgSO_4$. Storage of the cells at −70 C before lysozyme-ethylenediaminetetraacetate (EDTA) treatment does not appear to drastically affect vesicle formation or function. Membranes prepared from cells frozen for up to 6 months accumulate ^{54}Mn, as shown in this report, and ^{14}C-proline (2) to similar extents as vesicles prepared from fresh unfrozen cells.

Assay of manganese transport by the vesicles. Vesicles stored at −70 C were thawed, centrifuged, and washed twice at 4 C with 20 volumes of 0.5 M sucrose and 14 mM glucose. Finally they were suspended in 0.5 M sucrose and 14 mM glucose at 10 mg/ml (dry weight) and incubated at room temperature (25 C) for 15 min. Carrier-free ^{54}Mn (10^{-9} M; New England Nuclear Corp., Boston, Mass.) was added 1 to 2 min after other additions were made; 0.05-ml samples were removed, diluted 40-fold in the incubation medium without radioactive manganese, and rapidly filtered through membrane filters (HA type; Millipore Corp., Bedford, Mass.). The dilution serves to stop the accumulation reaction and as a washing step. The filters were suspended in 10 ml of a dioxane-based scintillation fluid and counted in a Packard liquid scintillation counter (model 3375) as described in the preceding paper (18). Further washings were avoided for convenience, since washing lowers the ^{54}Mn retained on the filters by only 1 to 2% and would not affect the conclusions drawn.

Chemicals. All reagents used were reagent grade available from commercial sources. Deionized water was used throughout the experiments. Deoxyribonuclease, EDTA, sucrose, and lysozyme used for vesicle preparation and resuspension were obtained from

Sigma Chemical Co., St. Louis, Mo. Valinomycin and *m*-chlorophenyl carbonylcyanide hydrazone (CCCP) were obtained from Calbiochem, Los Angeles, Calif. Nigericin was the gift of R. L. Harned, Commercial Solvents Corp., Terre Haute, Ind.

RESULTS

Accumulation of manganese by the vesicles. Addition of ^{54}Mn at the very low concentration of 10^{-9} M to the vesicles suspended in 0.5 M sucrose, 10 mM glucose, and 10 mM $CaCl_2$ resulted in accumulation of manganese in the vesicles (Fig. 1). The rate of uptake is linear over a period of 20 to 30 min, after which it reaches a steady state with 50% of the radioactive manganese remaining free in the medium and 50% taken up by vesicles. Since the vesicles occupy at best a volume-to-volume ratio of 1:20 in the mixture, this indicates about a 20-fold greater concentration of manganese inside the vesicles (on the assumption that the manganese accumulated remains free rather than bound or precipitated in some form inside the vesicles). Although no attempt has been made so far to directly measure the free manganese inside the vesicles, most of the manganese accumulated is free or at least loosely bound as indicated by (i) the exchangeability of part of the accumulated ^{54}Mn with external manganese and (ii) the release of the accumulated ^{54}Mn by lipid-active agents like toluene (Fig. 1). Toluene appears to make membranes leaky to small molecules (9, 19) and does not cause visible lysis. When either manganese at 10^{-4} M or toluene at 1% (v/v) was added before ^{54}Mn, the accumulation of the radioactive manganese was completely inhibited; when added after 45-min accumulation of ^{54}Mn, there was rapid release of radioactive manganese from the vesicles (Fig. 1). Manganese chloride at 10^{-3} M does not cause the release of ^{54}Mn byeond that released by 10^{-4} M $MnCl_2$.

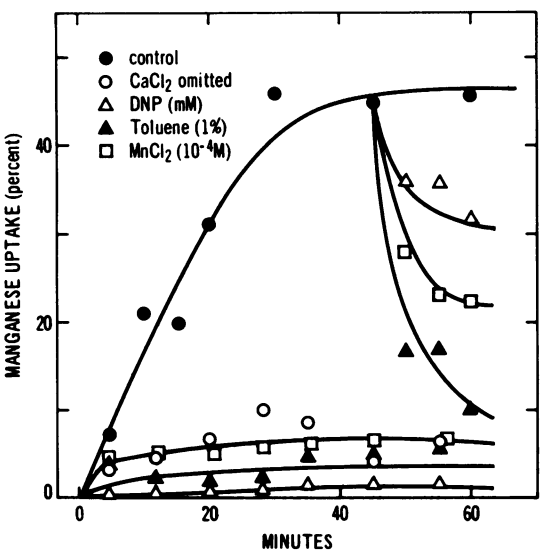

FIG. 1. *^{54}Mn accumulation by isolated membrane vesicles of Escherichia coli. Vesicles (10 mg/ml, dry weight) suspended in 0.5 M sucrose and 14 mM glucose in the presence or absence of 10 mM $CaCl_2$ were distributed in 0.5-ml samples and incubated at room temperature. An 0.5-μCi amount of ^{54}Mn (10^{-9} M) per ml was added and, at the indicated times, 0.05-ml samples were diluted, filtered, and counted. Dinitrophenol (DNP), toluene, or $MnCl_2$ were added either at −1 or +45 min. Data are presented as the fraction of total radioactivity remaining on the filter after correction for background (fraction of radioactivity remaining on the filter when the same incubation mixture with ^{54}Mn is filtered in the absence of added vesicles).*

Accumulation of manganese by membrane vesicles shows energy dependence such as is typical of other active transport systems in the vesicles (1, 11, 12, 14). Dinitrophenol completely inhibited ^{54}Mn accumulation (Fig. 1 and Table 1). Other metabolic inhibitors, including CCCP, valinomycin, and nigericin, also prevented ^{54}Mn accumulation (Table 1). Barnes and Kaback (1) reported that valinomycin inhibits lactose accumulation by the vesicles and Kaback (*unpublished data*) and Bhattacharyya (*unpublished data*) also found valinomycin inhibition of ^{14}C-proline accumulation by vesicles. The accumulation of ^{54}Mn is only moderately dependent upon an exogenous energy source. Vesicles suspended in sucrose plus 10 mM $CaCl_2$ showed essentially the same rate of uptake of 10^{-9} M ^{54}Mn in the presence or absence of glucose or D-lactate. How-

TABLE 1. *Inhibition of manganese uptake by metabolic poisons*

Inhibitor[a]	Concn	^{54}Mn uptake (%)	
		5 min	20 min
First expt			
None		6.0	18.5
Dinitrophenol	10^{-3} M	3.4	3.1
	5×10^{-3} M	2.6	3.0
CCCP	10^{-5} M	5.0	9.4
	10^{-4} M	4.9	8.1
Second expt			
None		3.7	19.3
Valinomycin	0.3 μg/ml	1.9	2.1
Nigericin	0.3 μg/ml	1.7	1.8

[a] Inhibitors were added 30 sec before the addition of ^{54}Mn to 10 mg of vesicles per ml in 0.5 M sucrose, 14 mM glucose, and 10 mM $CaCl_2$.

ever, endogenous energy reserves in the vesicles may suffice for a small amount of manganese accumulation. In the presence of 10^{-6} M manganese, glucose and D-lactate stimulated the rate of manganese uptake two- to threefold (*data not shown*).

Calcium stimulation of manganese accumulation by the vesicles. When calcium was omitted from the incubation medium, manganese accumulation was inhibited by at least 80% (Fig. 1). This requirement for calcium is peculiar to membrane vesicles since, under the same conditions and in the same medium, there was no stimulatory effect of calcium on the manganese uptake by whole cells of *E. coli* (*data not shown*). Furthermore, 10 mM Ca^{2+} does not stimulate but rather causes a 50% inhibition of the rate of ^{14}C-proline uptake by the vesicles (*data not shown*). The optimal calcium concentration for the stimulation of manganese accumulation by the vesicles is about 10 mM (Table 2). The requirement for calcium is highly specific: cations such as magnesium, sodium, or potassium, or anions like sulfate, phosphate, or chloride do not stimulate manganese uptake (Table 3). Strontium, however, can replace calcium for stimulation of ^{54}Mn uptake, but is less effective than calcium (Table 3).

TABLE 2. *Optimal concentration of calcium for the stimulation of ^{54}Mn accumulation in vesicles*

Calcium concn[a] (mM)	^{54}Mn uptake (%)	
	5 min	20 min
0	2.7	4.2
0.1	2.1	3.9
1	4.0	6.9
10	5.8	13.7
100	3.9	11.4

[a] Vesicles suspended in 0.5 M sucrose and 14 mM glucose were supplemented with $CaCl_2$ as indicated, and then 10^{-9} M ^{54}Mn was added.

TABLE 3. *Specificity of calcium stimulation*

Additions[a]	^{54}Mn uptake (%)	
	5 min	20 min
None	1.2	1.9
$CaCl_2$	4.5	20.1
$SrCl_2$	3.1	12.7
$MgCl_2$	1.3	2.5
$MgSO_4$	1.2	2.1
KCl	1.1	2.7
NaCl	1.4	2.9
Na-PO_4 buffer (*p*H 7.0)	1.1	3.1

[a] The basic medium contained 0.5 M sucrose and 14 mM glucose. All additions were to a final concentration of 10 mM.

Effect of external concentration of manganese. The initial rate of manganese accumulation by the vesicles (Fig. 2) is dependent on the external concentration of manganese and shows Michaelis-Menten kinetics with a K_m of 8×10^{-6} M and a V_{max} of 0.1 nmoles per mg of vesicles per 5 min of incubation.

Specificity of manganese accumulation by the vesicles. Another property of active transport processes is their high degree of specificity toward substrates. ^{54}Mn accumulation by the vesicles shows high specificity. Magnesium, sodium, and potassium at concentrations up to 10 mM (Table 4) neither inhibit nor stimulate ^{54}Mn accumulation in the presence of 10 mM $CaCl_2$. Slight inhibitions of ^{54}Mn uptake by 0.1 M KCl, NaCl, or $MgCl_2$ may be a nonspecific effect rather than one directly on transport. Cobalt and iron, which competitively inhibit ^{54}Mn accumulation by intact *E. coli* (18), are also inhibitory for the vesicles (Table 4).

As another approach to specificity, I have attempted to measure the accumulation of ^{45}Ca and ^{28}Mg by the vesicles. Neither calcium nor mag-

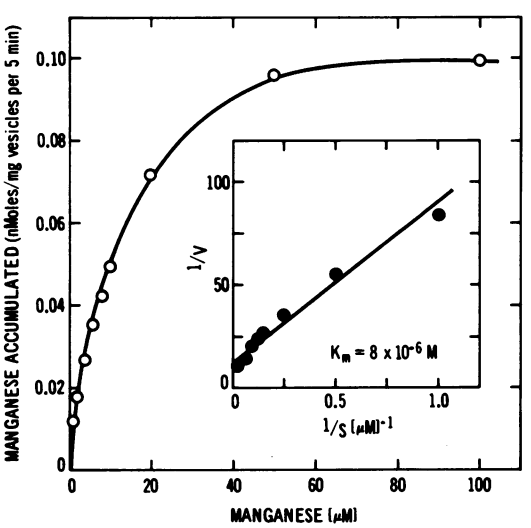

FIG. 2. *Kinetics of manganese uptake by membrane vesicles. To 0.2-ml samples of vesicles (10 mg/ml, dry weight) suspended in 0.5 M sucrose, 14 mM glucose, and 10 mM $CaCl_2$, 0.01 ml of different $MnCl_2$ solutions were added to make the final concentrations as indicated. After the addition of ^{54}Mn (1.25 μCi/ml), samples were removed at 5 and 10 min. Since the rate of uptake is linear during the 10-min incubation, the 10-min values were divided by 2 and averaged with the 5-min values so as to provide the rate for each concentration as nanomoles per 5 min.*

TABLE 4. *Specificity of manganese transport in E. coli vesicles*

Additions[a]	Concn (mM)	^{54}Mn uptake (%)	
		5 min	20 min
First expt			
No addition		5.8	13.7
$-CaCl_2$		2.7	4.2
KCl	10	5.8	11.3
	100	5.7	7.7
$MgCl_2$	1	5.7	15.5
	10		13.3
	100	5.2	12.4
NaCl	1	8.9	19.8
	10	5.3	16.8
	100	6.2	13.6
		15 min	30 min
Second expt			
No addition		25.6	40.8
$-CaCl_2$		8.6	10.7
$CoCl_2$	1	16.7	25.5
$FeCl_2$	1	6.0	14.3

[a] The basic medium contained 0.5 M sucrose, 14 mM glucose, and 10 mM $CaCl_2$. In the first expt. 10^{-9} M ^{54}Mn was added; in the second expt. 10^{-6} M ^{54}Mn was added.

nesium is accumulated under my experimental conditions, and calcium does not stimulate the uptake of magnesium.

DISCUSSION

Isolated membrane vesicles like intact cells of *E. coli* can accumulate manganese, and the process shows properties characteristic of active transport, e.g., energy dependence, saturation kinetics, and specificity. However, there are some differences between the two systems, the most striking difference being the apparent requirement for calcium in the membrane vesicles. One possible explanation for this effect might be that Ca^{2+} stabilizes the membrane vesicles. However, mere stabilization would not explain the specific requirement for calcium by the vesicles for manganese uptake since, under the same conditions, proline uptake is not stimulated by calcium. On the contrary, there is about a 50% inhibition of ^{14}C-proline uptake in the presence of 10 mM $CaCl_2$. Also, magnesium does not replace the calcium requirement. Apart from a stabilizing effect of calcium on the vesicles, calcium might be involved in some more specific way with the manganese uptake system in *E. coli*. It is of interest that a calcium-activated adenosine triphosphatase has been reported in the membranes of *E. coli* (4), and in mitochondria it has been shown that calcium can stimulate manganese uptake possibly by activating the carrier for manganese transport (B. Chance, *unpublished data*; 15). The stimulation of manganese transport by calcium in the vesicles bears an analogy to sodium-stimulated concentrative uptake of amino acids, sugars, and other compounds in animal cells (17, 20), and of glutamate in *E. coli* (5). The failure to detect a calcium requirement in intact cells may be due to the binding of calcium loosely on or within the cell wall, effectively raising the calcium concentration in the vicinity of the manganese transport system. Such loose binding of ^{45}Ca has been measured (Silver, *unpublished data*). However, the extraordinarily high concentration (10 mM) of calcium required by the vesicles does not stimulate manganese uptake by intact cells but actually is slightly inhibitory (18).

A second difference between manganese transport in intact cells and in vesicles is in the kinetic parameters which describe the system. The K_m for the intact cells is 2×10^{-7} M Mn^{2+}, and that for the membrane vesicles is 8×10^{-6} M—a very significant difference for which I have no explanation. Silver, Johnseine, and King (18) report a V_{max} for the intact cells of 1 to 4 nmoles per min per 10^{12} cells. The data in Fig. 2 show a V_{max} of 0.1 nmole per 5 min per mg (dry weight). Assuming 1 g for the wet weight of 10^{12} *E. coli* cells and 5:1 as the ratio of wet weight to dry weight for the vesicles, I obtain values for the V_{max} of 1 to 4 nmoles per min per g (wet weight) for the intact cells and 4 nmoles per min per g (wet weight) for the membrane vesicles. It can be concluded that the vesicles retain essentially all of the ^{54}Mn accumulation capacity of the cells from which they are isolated. The vesicles also retain the ability to discriminate between manganese and magnesium, cobalt, or iron (Table 4), which is characteristic of this transport system in intact cells (18).

Evidence that the ^{54}Mn accumulation system is an "active transport" system rather than one mediating "facilitated diffusion" comes from: (i) the inhibition of uptake by metabolic poisons and (ii) the relatively "free" and exchangeable state of intravesicular manganese. Table 1 shows that dinitrophenol, CCCP, valinomycin, and nigericin, all of which are inhibitors of metabolic energy processes, inhibit the accumulation of ^{54}Mn. The mechanism of action of these substances is still unsettled (6–8, 16). Inhibition of ^{54}Mn uptake by dinitrophenol and CCCP cannot be explained by uncoupling of oxidative phosphorylation, since the vesicles apparently lack the oxidative phosphorylation machinery of the intact *E. coli* (Klein, Dahms, and Boyer, Fed.

Proc. **29**:341, 1970). However, CCCP has also been shown to inhibit active transport in *Streptococcus faecalis*, which lacks an oxidative phosphorylation apparatus (8). Harold and Baarda (8) explain these results in terms of an increase in the proton permeability of the cell membrane caused by the "uncouplers" and propose that many transport systems are "driven" by Mitchell's (6, 16) "proton pump." My experiments with dinitrophenol and CCCP are consistent with but have little to add to this explanation. Valinomycin and nigericin, however, are antibiotics which act by altering the permeability of the membrane to potassium ions or protons, or both (6–8, 15, 16). Nigericin may be acting on the vesicles by increasing proton permeability, effectively "short-circuiting" the proton extrusion pump. If this is the explanation, then other transport systems in the vesicles must be tied to proton conduction since nigericin also inhibits the accumulation of ^{14}C-proline and ^{42}K in the vesicles (Bhattacharyya, *unpublished data*). Valinomycin, which inhibits accumulation of ^{54}Mn (Table 1), actually stimulates the accumulation of ^{42}K by the vesicles (Bhattacharyya, *manuscript in preparation*), suggesting that the tie-in of potassium transport to the energy metabolism of the vesicles occurs at a different point from that for ^{54}Mn and proline. Kaback, Milner, and Barnes (1, 12) are studying the bioenergetic system of the vesicles and the relationship of D-lactate consumption to transport.

That the intravesicular manganese is relatively free and not bound is indicated by the results of experiments, such as that shown in Fig. 1, which demonstrate turnover (exchange) of internal for external manganese and ready release of ^{54}Mn from the membrane vesicles only with lipid-active agents such as toluene.

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