Valinomycin-Induced Uptake of Potassium in Membrane Vesicles from *Escherichia coli*

(K-uptake mutants/active transport)

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ABSTRACT Osmotically shocked *Escherichia coli* and membrane vesicle ghosts from *E. coli* cells have lost the ability to accumulate potassium by active transport. The addition of valinomycin to the membrane ghosts restores the capacity to accumulate radioactive ^{42}K and ^{86}Rb by a temperature- and energy-dependent process. Membrane vesicles prepared from mutants of *E. coli* altered in potassium transport show defects in the valinomycin-stimulated accumulation of ^{42}K that are related to the defects in the intact cells.

Valinomycin is a cyclic depsipeptide antibiotic (1, 2) that acts by greatly increasing the permeability of membranes, specifically to potassium ions (3). Membranes whose permeability to potassium is altered by valinomycin include bacterial (3–5), erythrocyte (6–8), mitochondrial (9), and artificial black lipid membranes (10). The change is highly specific: only permeability to potassium and the related alkaline cations rubidium and cesium is affected (7, 10). The membranes remain impermeable to protons, sodium, lithium, and other ions in the presence of valinomycin. Evidence from relatively *thick* artificial membranes shows that valinomycin acts not by forming channels or pores through which potassium can travel, but by forming one-to-one complexes with K$^+$ ions (8) which can then diffuse across the hydrophobic lipid membranes, with the K$^+$ sheltered from the membrane within the cyclic folds of the valinomycin molecule (2). With mitochondria, at least, valinomycin facilitates the *net* accumulation of potassium (9) and this finding has led to models of valinomycin as a prototype for natural carriers of potassium in cell membranes. In this paper we present a similar finding with membranes prepared from cells of *Escherichia coli*—that is, valinomycin-facilitated uptake of potassium in an energy-dependent and apparently concentrative manner.

E. coli has a number of advantages in the study of potassium transport because this organism possesses a transport system with high affinity for K (11, 12), is able to maintain very large concentration gradients for K (13), and most of all because of the extensive knowledge of the genetics and metabolism of this organism. Lubin (14) first isolated and characterized specific potassium transport mutants in *E. coli* strain B. Later other potassium mutants were isolated in other *E. coli* strains (14–18) and *Bacillus subtilis* (14, 19), and a number of mutants of *E. coli* K-12 with alterations in potassium transport have recently been isolated. A total of eight genes affecting potassium transport have been identified. One set of four closely linked *kdp* genes ("K^+-dependent"; 15) and two other genes (*trkA, trkD; "t*ransport of K^+") affect primarily uptake of potassium, while mutations in the *trkB* and *trkC* genes result in a defect in the retention of potassium (Epstein, in preparation). All these mutational defects appear to be specific for potassium because the mutants are normal in the ability to transport β-galactosides and proline (unpublished data). If we are to pursue the model from mitochondrial studies that valinomycin can participate as part of a *natural* potassium accumulation mechanism, then it is crucial to show that mutants with altered potassium metabolism are altered in their response to valinomycin. This is one of the chief conclusions from this report.

A technique has been devised by Kaback (20–26) for isolating membrane vesicles from *E. coli* that lack both the soluble internal components of the cell and most of the components of the outer cell wall (20, 23). These vesicles are somewhat analogous to erythrocyte "ghosts" and have a demonstrated capacity to accumulate by *active transport* a large variety of amino acids (25), sugars (26), and even a cation, manganese (27). In this report we show that the vesicles accumulate potassium only if valinomycin or similar antibiotic carriers are added. Furthermore, valinomycin-stimulated uptake is dependent upon an exogenously added energy source, preferably D-lactate, and is altered in vesicles prepared from the potassium transport mutants.

MATERIALS AND METHODS

Vesicles prepared from *E. coli* strain B/1,5 were used in all experiments except those using mutant strains. The mutant strains of *E. coli* K-12 are all derivatives of strain FRAG-1, which is wild type for potassium transport: FRAG-5 (*kdp-ABC5*), a deletion mutant (15) that is defective in a transport system which has high affinity for potassium and whose synthesis is repressed by growth in media containing excess potassium (15); 2K110 (*kdpABC5 trkB110*), which carries in addition a defect in the retention of potassium such that the cells can no longer maintain their normal cell potassium when that in the growth medium falls below 2 mM; and 2K401 (*kdpABC5 trkA401*), which is severely defective in potassium uptake and requires a potassium concentration of at least 40 mM in order to achieve rapid growth rates. Detailed descriptions of these mutants and their properties appear elsewhere (15; Epstein, in preparation).

Preparation of membrane vesicles

Vesicles were prepared from *E. coli* B by the ethylenediaminetetraacetate (EDTA)–lysozyme method of Kaback (22, 23)

Abbreviation: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

as previously described (27, 28), and stored at 10 mg (dry wt) per ml at −70°C in 0.5 M potassium phosphate buffer (pH 6.6) plus 10 mM $MgSO_4$.

Uptake experiments

Frozen vesicles were thawed at room temperature, diluted 1:20 with 0.25 M sucrose–10 mM Tris·HCl (pH 7.0)–5 mM sodium phosphate buffer (pH 7.0)–5 mM $MgSO_4$, homogenized, and centrifuged. The pellet was suspended again in the same solution and the process of homogenization and centrifugation was repeated once or twice more. The accumulation of radioactivity by washed vesicles was measured as described earlier (27, 28) except that 0.25 M sucrose–10 mM Tris·HCl (pH 7.0)–5 mM sodium phosphate buffer (pH 7.0)–5 mM $MgSO_4$ was used for both reaction incubation and dilution before Millipore filtration. Radioactivity was counted in a Nuclear-Chicago gas-flow counter.

Antibiotics and inhibitors were used as solutions in ethanol and added so as to make the final concentration of ethanol in the incubation mixture not more than 2%. This concentration of ethanol was later found to inhibit proline uptake by the vesicles in some experiments, and in recent experiments the concentration of ethanol was reduced to 0.2%.

Flame photometry

Measurements of net chemical potassium were made with an IL model 143 Flame Photometer (Instrumentation Laboratories, Boston, Mass.). After preparation of vesicles and exposure to 0.3 μg/ml valinomycin and (or) potassium in various concentrations for 2 min, 2-ml samples of vesicles were centrifuged for 15 min at 25°C at 18,000 × g in a Sorvall RC2B centrifuge, and the pellets were resuspended in 5 ml of deionized water. After heating of the suspension for 5 min in a boiling water bath and (or) the addition of detergent and the addition of a Li internal standard (1% v/v of 1.5 M LiCl), the potassium content was measured in the flame photometer.

Materials

Valinomycin and CCCP (*m*-chlorocarbonylcyanide phenylhydrazone) were purchased from Calbiochem. Nigericin (7, 9) was kindly supplied by R. L. Harned, Commercial Solvents Corp., Terre Haute, Ind. Radioactive ^{42}K was purchased from International Chemical and Nuclear Corp., Irvine, Calif., and ^{86}Rb was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS AND DISCUSSION

Valinomycin-stimulated accumulation of ^{42}K and its dependence upon an external energy source and the external potassium concentration are shown in Fig. 1. In the absence of valinomycin, 2–3% of the radioactive potassium remains on the filters (Fig. 1*A*); this retention of ^{42}K does not increase with time of incubation and corresponds approximately to the [^{14}C]inulin space determined in separate experiments (unpublished data). The addition of valinomycin at 0.3 μg/ml, however, causes a rapid increase in the accumulated ^{42}K until after 1–2 min about 13–15% of the potassium is retained by the filters. Adding 0.1 mM nonradioactive potassium (10-fold excess) at the same time as the ^{42}K has essentially no effect on the extent of ^{42}K accumulation after subsequent valinomycin addition (Fig. 1*A*). However, 1 mM nonradioactive KCl decreases the extent of ^{42}K accumulation about one-third and 5 mM KCl reduces the accumulation to that seen without valinomycin. The 2–3% of the radioactivity remaining on the
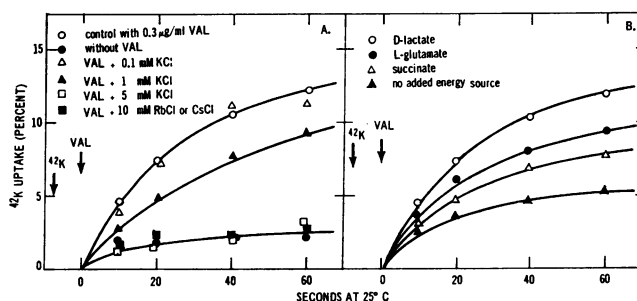

Fig. 1. Valinomycin-dependent accumulation of ^{42}K: inhibition by alkali cations and energy requirements. *A*. Vesicles from *E. coli* B/1,5 were suspended at 5 mg (dry weight) per ml in sucrose–Tris·HCl–Na–PO$_4$–MgSO$_4$ with 10 mM lithium D-lactate as energy source. Nonradioactive salts were added first, then 10^{-5} M ^{42}K, followed by valinomycin (0.3 μg/ml). *B*, as in *A* except that lactate, glutamate, or succinate was added at 10 mM.

filters in the absence of valinomycin includes ^{42}K in the external fluid which remains on the filters, since the filters were not washed in these experiments. 10 mM RbCl or CsCl completely inhibits the accumulation of ^{42}K by vesicles. These experiments provide the first suggestion that valinomycin-induced ^{42}K uptake is a saturable function of potassium concentration, with a half-saturation constant (K_m) of about 2 mM K^+. The inhibition constants (K_i) for Rb$^+$ and Cs$^+$ on potassium accumulation are of the same order of magnitude (data not shown). In the experiment in Fig. 1*A*, valinomycin-induced ^{42}K uptake was also completely inhibited by 10^{-5} M *m*-chlorophenyl carbonylcyanide hydrazone (CCCP), a potent "proton conductor" and uncoupler of oxidative phosphorylation, and with 5 × 10^{-4} M irehdiamine A (29) and 1% (v/v) toluene, agents which cause general cell leakiness.

Potassium accumulation by the vesicles is stimulated by the addition of an external energy supply (Fig. 1*B*). In the absence of an added energy source, valinomycin stimulated accumulation of ^{42}K about 2-fold. D-Lactate, which has been found by Kaback, Milner, and Barnes (25, 26) to be the preferred energy source for membrane accumulation of amino acids and lactose, stimulated ^{42}K accumulation most extensively of those energy sources we have tried. L-Glutamate and succinate (Fig. 1*B*) and citrate and glucose (data not shown) are also stimulatory, but to a lesser extent.

If valinomycin is serving as a carrier for the metabolically active accumulation of alkali cations, then Rb$^+$ should not only be an inhibitor of potassium accumulation but also a substrate for the accumulation system. When using radioactive ^{86}Rb, we find that uptake by the vesicles is strictly dependent upon the addition of valinomycin and is saturable, with a K_m of about 2 mM Rb$^+$ (Figs. 2 and 4 and unpublished data). Most types of experiments in this paper have been carried out independently using ^{42}K and ^{86}Rb with similar results. In Fig. 2, one can see that the extent and kinetics of ^{86}Rb uptake are approximately the same whether valinomycin or ^{86}Rb is added first (Fig. 2*A*). Even a 5-min delay between the time of adding valinomycin and the time of adding radioactivity does not affect the time course—which shows that valinomycin is not "consumed" or irreversibly tied up by the vesicles. As also seen in Fig. 2*A*, the addition of 50 mM KCl or 10 μM CCCP or 1% toluene just before the addition of valinomycin and ^{86}Rb completely eliminates the valino-
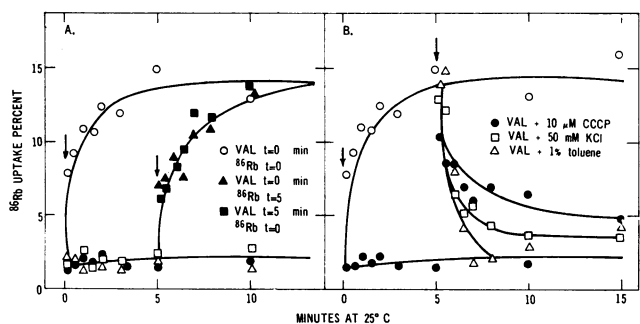
FIG. 2. Kinetics of valinomycin-stimulated ^{86}Rb accumulation. *A.* 0.1 mM ^{86}Rb and (or) 0.3 μg/ml valinomycin were added at *t* = 0 or 5 min as indicated. CCCP (10 μM), 50 mM KCl, or 1% toluene was added to aliquots 30 sec before the addition of ^{86}Rb and valinomycin at *t* = 0. *B.* Control (O) and CCCP (●) data are repeated from Fig. 2*A* and, in addition, data are shown from additional aliquots which had valinomycin and ^{86}Rb added at *t* = 0, followed by CCCP, KCl, or toluene at 5 min.

FIG. 3. Valinomycin-dependent [42]K accumulation: differences with mutants. Vesicles were prepared from cells of wild-type *E. coli* (strains K-12 and B) and mutant strain 2K110 by the lysozyme–EDTA procedure and from strain FRAG-5 by the penicillin method. Li^{+} D-lactate (10 mM) was added as energy source at −30 sec. *A.* To 5 mg (dry weight) of vesicles per ml in sucrose–Tris·HCl–Na-PO₄–MgSO₄, 10⁻⁴ M [42]K was added, followed by 0.3 μg/ml valinomycin. Nigericin at 0.3 μg/ml was added to a portion of the wild-type K-12 control 1.5 min after 0.3 μg/ml valinomycin was added to start the reaction. *B*, to 10 mg/ml vesicles in 0.5 M K-phosphate (pH 6.6) plus 10 mM MgSO₄, [14C]proline was added at 10⁻⁵ M at *t* = 0 to start the reaction. Valinomycin (0.3 μg/ml) was added at −30 sec to part of the *E. coli* B control.

mycin-stimulated uptake. The mechanisms operating here are different in each case: KCl is presumedly functioning as a competitive inhibitor of rubidium uptake, CCCP is acting as an energy poison and toluene is destroying the integrity and functional activity of the membranes. The control data and CCCP curve from Fig. 2*A* are reproduced in Fig. 2*B* along with additional data which shows that adding CCCP, KCl or toluene after 5 min of incorporation of ^{86}Rb causes the rapid loss of radioactive rubidium from the vesicles, again by three different mechanisms: exchange with KCl, leakage with toluene and presumedly carrier-mediated loss with CCCP. Dinitrophenol acts on the vesicles in a similar way to CCCP (data not shown).

Mutants

Although the evidence so far presented supports the model of valinomycin-stimulated active transport of potassium in membranes prepared from *E. coli* cells, there is no indication of a relationship between valinomycin-induced uptake and the normal potassium metabolism of the cells. Therefore, vesicles were prepared from potassium-dependent mutants defective in different genes and tested both for their ability to accumulate radioactive proline (as a measure of the overall intactness and functional activity of the vesicles) and for their ability to accumulate [42]K or [86]Rb (Figs. 3 and 4). Vesicles from wild-type *E. coli* K-12 and from the potassium mutants are indistinguishable from vesicles from *E. coli* strain B with regard to the initial rate and extent of uptake of ^{14}C-proline under our standard conditions (Fig. 3*B*). However, although the vesicles from the wild-type K-12 strain showed valinomycin stimulated [42]K accumulation similar to that of *E. coli* B vesicles, the mutant vesicles showed altered properties (Fig. 3*A*). The initial rate of uptake of [42]K with 2K110 vesicles was essentially the same as with the wild type, but after about 1 min the accumulated [42]K was rapidly lost from the vesicles. The reduced uptake in FRAG-5 is unexpected since the deletion mutation in this strain affects genes that are not expressed in high potassium medium when grown (Epstein, in preparation). FRAG-5 vesicles prepared later by lysozyme–EDTA were indistinguishable from wild-type vesicles in their response to valinomycin. Two additional points can be seen in Fig. 3: (*i*) Nigericin, an antibiotic known to reverse the specific potassium transport effects of valinomycin, causes
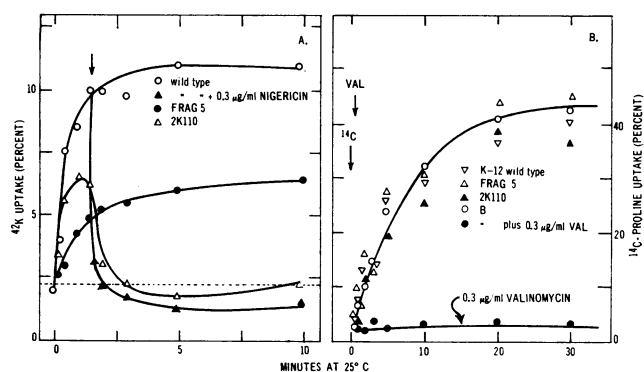
an immediate and rapid loss of the accumulated [42]K (Fig. 3*A*). (*ii*) Valinomycin inhibits the active transport of [14C] proline by the vesicles. Valinomycin also inhibits the D-lactate-dependent accumulation of lactose (26) and manganese (27) by membrane vesicles. In experiments whose data are not shown, it was determined that vesicles prepared and suspended in 0.5 M sodium rather than potassium phosphate buffer (K phosphate was generally used in the [14C]proline experiments) were resistant to valinomycin inhibition of proline uptake. The addition of 1 mM K^{+} to these "Na^{+}-vesicles" restored sensitivity of [14C]proline accumulation to inhibition by valinomycin. Kaback (personal communication) has made similar observations and found valinomycin inhibition of the accumulation of other amino acids.

Fig. 3 is from one of our earlier experiments with vesicles from the mutant strains. In a series of experiments in which vesicles were prepared from FRAG-1, 2K110, and 2K401 on three independent occasions, the loss of accumulated potassium from strain 2K110 was not reproducible (see, for example, Fig. 4*B*). Fig. 4 shows the results from a more recent experiment to test the response of the mutant vesicles to various concentrations of valinomycin. With wild-type vesicles, both the initial rate of uptake and the apparent equilibrium amount of ^{86}Rb retained depend on the valinomycin concentration in the range 0.01–0.3 μg/ml (Fig. 4*A*). In *E. coli* B vesicles, the initial rate of uptake continued to increase as the valinomycin concentration was raised from 0.3 to 10 μg/ml. With vesicles from the mutant strains 2K110 and 2K401, higher concentrations of valinomycin were required to stimulate comparable accumulation (Fig. 4*B*) or even significant accumulation (Fig. 4*C*). In another experiment whose data are not shown, the addition of 50 mM RbCl, 10 min after the beginning of valinomycin-stimulated ^{86}Rb uptake, caused more rapid *exchange* of ^{86}Rb for nonradioactive rubidium with vesicles prepared from the retention mutant 2K110 than with wild-type K-12 or B vesicles. In summary,

TABLE 1. *Net potassium accumulation (flame photometry)*

	Vesicle-associated K (nmol/mg)
First expt., strain B; external K⁺ concn 1.0 mM	
No valinomycin	1.8
Valinomycin	3.9
"	3.6
Valinomycin plus dinitrophenol	1.5
Second expt., strain K-12; 0.5 mM K⁺ where added	
FRAG-1,	
neither valinomycin nor K⁺	<0.1
plus K⁺	1.1
plus K⁺ and valinomycin	2.5
2K110	
neither valinomycin nor K⁺	<0.1
plus K⁺	0.9
plus K⁺ and valinomycin	1.0

In the first experiment, *E. coli* B/1,5 vesicles were prepared in K phosphate buffer, washed, and resuspended in incubation medium plus 10 mM Li⁺ D-lactate and 1 mM KCl. 0.3 µg/ml valinomycin and 1.0 mM dinitrophenol were added where indicated. In the second experiment, *E. coli* K-12 vesicles were prepared in Na phosphate buffer, washed, and resuspended in incubation medium plus 14 mM glucose as energy source. 0.5 mM KCl was added only where indicated. Potassium was measured in the pellets from 10 mg (dry weight) of membrane vesicles (2 ml at 5 mg/ml) centrifuged in each sample.

the vesicles from potassium mutant strains appear to show defects in valinomycin-stimulated uptake *in vitro* related to their defects *in vivo* (15, Epstein, in preparation); the "retention" mutant 2K110 shows differences in retention and exchange of potassium; and the severely defective uptake mutant 2K401 yields vesicles severely defective in potassium uptake (Fig. 4C).

Net potassium measurements

Radioisotope experiments cannot distinguish between *net* accumulation of potassium stimulated by valinomycin and valinomycin-stimulated *exchange* of added radioactive potassium for intravesicular nonradioactive potassium under conditions of no net chemical accumulation or perhaps even potassium loss. For this purpose one needs measurements of total potassium by a technique such as flame photometry. Table 1 includes data from two experiments measuring net potassium accumulation in the presence of valinomycin. In the first experiment, vesicles from *E. coli* strain B were washed twice and resuspended in the standard incubation medium to which 1 mM potassium was added. In the absence of valinomycin or if dinitrophenol was added along with the valinomycin, 1.5–1.8 nmol of potassium sedimented per mg (dry weight) of vesicles in the centrifuge. However, in the presence of valinomycin, more than twice as much vesicular potassium was found. Because the vesicles in the first experiment in Table 1 had been prepared in 0.5 M K phosphate buffer and resuspended in the presence of 1.0 mM K⁺, vesicles were prepared in Na-phosphate buffer and potassium only was added where indicated. These "Na⁺-vesicles" contained no detectable potassium (<0.1 µmol/g dry weight; second experiment, Table 1). When 0.5 mM KCl was added, 0.9–1.1
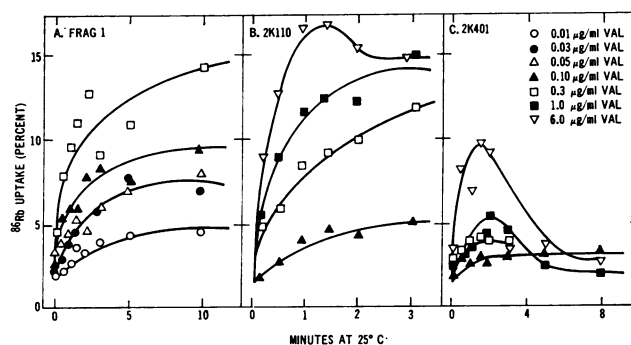

FIG. 4. Effect of varying the valinomycin concentration on wild-type and mutant vesicles. All three vesicle preparations were made by the lysozyme–EDTA procedure and suspended at 5 mg/ml (dry weight) with 10 mM Li⁺ D-lactate as energy source. 10^{-5} M ⁸⁶Rb was added and the reactions were started by the addition of valinomycin as indicated. Control experiments showed about the same [¹⁴C]proline accumulation with all three vesicle preparations. In this experiment the ethanol concentration was held at 0.2% (v/v).

nmol of K⁺ per mg dry weight was found with the membrane pellets. Upon the addition of valinomycin, only the vesicles from the wild-type K-12 strain showed an additional 2.4-fold increase in membrane-associated potassium. These experiments show *net* potassium accumulation stimulated by valinomycin; they do *not* demonstrate accumulation of potassium to a free internal concentration higher than the external concentration of potassium. To examine this question, one must take the data in Table 1 and recalculate it in terms of moles of potassium per liter of vesicle water. If we assume (as the most optimistic assumption) that the accumulated potassium is free in solution and that the 10 mg (dry weight) of vesicles contains 20 µl of intravesicular water and when pelleted in the centrifuge carry over an additional 2 µl of intervesicular water (ref. 24 and Kaback, personal communication), one calculates for the valinomycin-stimulated accumulation that the internal potassium may reach 1.8 (first experiment) or 2.4 (second experiment) times the external concentration. Since much of the accumulated potassium could be bound and not free, the data in Table 1 can be considered suggestive but not sufficient to establish concentration above the external concentration of free potassium.

E. coli cells accumulate and retain ⁴²K, but membrane vesicles prepared from them cannot accumulate ⁴²K unless valinomycin is added; next we asked at what stage in the preparation of the vesicles is the potassium-accumulation capacity lost, and whether it can be restored by the addition of a fraction released from the cells. The results from these experiments are preliminary and essentially negative, so they will be only listed briefly here: (*i*) Converting *E. coli* to spheroplasts does not eliminate the ability to accumulate ⁴²K, but subjecting the cells to the cold Tris–osmotic shock procedure of Neu and Heppel (30) entirely destroys the ability to accumulate potassium (data not shown). (*ii*) The addition of the osmotic shock fluid, or the residual "shockate" (after the 10 mM potassium present has been dialyzed away) to osmotically shocked cells does not restore activity. The addition of either fresh or dialyzed shockate to vesicles inhibits the valinomycin-dependent accumulation (in preparation).

After this paper had been submitted, a note appeared reporting the isolation of a valinomycin-like ionophore from mitochondria (31). The mitochondrial ionophore shows sodium–potassium discrimination similar to that of the untreated mitochondria and is apparently a cyclic peptide similar in size to valinomycin but different in amino-acid composition. A natural potassium ionophore as well as mutants defective in its synthesis, may also exist in *E. coli*,

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