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EFFECTS OF pH AND MONOVALENT CATIONS ON THE POTASSIUM ION EXIT FROM THE MARINE BACTERIUM, *VIBRIO ALGINOLYTICUS*, AND THE MANIPULATION OF CELLULAR CATION CONTENTS

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In the presence of an iso-osmotic concentration (0.4 M) of LiCl, the exit of cellular K^+ and concomitant entry of Li^+ in the marine bacterium, *Vibrio alginolyticus*, were enhanced by an increase in the medium pH, with an optimum at about pH 9.6. In addition to alkaline pH, the K^+ exit in the NaCl medium required the presence of a weak base such as diethanolamine, ethanolamine or methylamine, which is permeable to the membrane in its unprotonated form. No net entry of Na^+ was detected in this case and the amine accumulated in exchange for K^+. The K^+ exit observed at alkaline pH could be explained by the function of a K^+/H^+ antiporter. Once the cells were loaded with the amine, their exposure to the NaCl medium in the absence of loaded amine induced the entry of Na^+. In RbCl or CsCl medium, fast entry of Rb^+ or Cs^+ and exit of K^+ were observed at neutral pH (7.5), and the rate of K^+ exit increased with the medium pH. From these results, we established a simple method for the replcement of cellular cations with a desired cation (Li^+, Na^+, K^+, Rb^+ or Cs^+). The present method was found to be applicable also to *Escherichia coli*.

Introduction

Growing cells of bacteria accumulate a high concentration of K^+ and extrude Na^+ [1] and these cations have been shown to play an important role in the regulation of intracellular pH [2–6] and in the active transport of nutrients [7–11]. Intact cells of marine bacteria, like extremely halophilic bacteria [12], require both Na^+ and K^+ for the active transport of amino acids [8,13]. To characterize the transport systems of K^+ and Na^+ and their relationship to the active transport of amino acids, it is necessary to deplete cellular K^+ or to control the internal cation concentration of cells without damaging the transport systems. Although the use of membrane vesicles enables the manipulation of intravesicular cation contents [9,14], membrane vesicles lack K^+ uptake [15–18] and are not pertinent to the elucidation of K^+ uptake system and its roles in the active transport of amino acids.

There are several ways to deplete intact cells of internal K^+. Exposure of the cells to hypotonic conditions [19,20] and the treatment of the cells with inhibitors such as 2,4-dinitrophenol [21] and ionophores [22] have been employed. The former causes plasmolysis of the cells and K^+ is required for the deplasmolysis [23]. Since the latter uses inhibitors, complete removal of inhibitors from the cells is a prerequisite and ionophores are usually ineffective in the case of Gram-negative bacteria. We found that the exposure of the cells of a Gram-negative marine bacterium, *Vibrio al-*

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

ginolyticus, to alkaline pH in the presence of isoosmotic concentrations of monovalent cation induces the exit of cellular K^+. In this case, the K^+ exit was greatly influenced by the species of monovalent cation and buffer. Therefore, we studied the conditions which control the entry of monovalent cations such as Li^+, Na^+, Rb^+ and Cs^+ in exchange for K^+. Based on these results, we established a novel and simple method for the replacement of cellular cations with a desired cation. Recently, we have utilized this method for the manipulation of internal cation contents of *V. alginolyticus* and we have succeeded in demonstrating that K^+ is required for the generation of a pH gradient [6] and an Na^+ electrochemical gradient [13,24]. The present method was also found to be applicable to *Escherichia coli.* This paper deals with monovalent cation movements in *V. alginolyticus,* which constitute the basis for the manipulation of cellular cation contents.

Materials and Methods

Media and growth conditions

V. alginolyticus 138-2 was grown aerobically with glycerol as a sole carbon source in the presence of 50 mM Tris-HCl (pH 7.5) as previously described [6]. *E. coli* ML 308-225 ($i^-Z^-Y^+a^+$), kindly supplied by Dr. H.R. Kaback, was grown with the medium of Davis and Mingiolli [25] except that glucose was replaced by 37 mM sodium succinate. Cells were harvested by centrifugation at the late exponential phase of growth.

Preparation of buffers

Diethanolamine, ethanolamine, methylamine and Tris buffers were prepared by titrating with HCl, and Tricine and Hepes buffers were prepared by titrating with NaOH or LiOH. The pH value of each buffer was adjusted so as to give a desired pH in the reaction mixture and the buffer concentration was expressed as the total concentration of amine used.

Measurement of K^+ exit and monovalent cation entry by a filtration method

Harvested cells of *V. alginolyticus* were washed twice with 0.4 M NaCl using a volume equal to half that of the growth medium and suspended in 0.4 M NaCl at the final concentration of 40–60 mg cell protein per ml, unless otherwise noted. Protein was determined by the method of Lowry et al. [26] with bovine serum albumin as a standard. The concentrated cell suspension was stored on ice until use. The reaction was started at 25°C by the addition of 2 μl of the cell suspension to 100 μl of the reaction mixture described in the figure legends. At time intervals, the reaction was terminated by the addition of 1.5 ml 0.4 M choline chloride/10 mM Tris-HCl (pH 7.2) and by the immediate filtration on Schleicher and Schull membrane filter OE67 (0.45 μm pore size). The filter was washed three times with 1.5 ml each of the above buffered choline chloride solution within 15 s. The filter was immersed in 2.5 ml 5% trichloroacetic acid and then K^+, Na^+, Li^+, Rb^+ and Cs^+ contents were determined by flame photometry using a Perkin-Elmer 403 atomic absorption spectrophotometer.

The intracellular cation concentrations were calculated using the values 3.3 μl internal water space/mg cell protein for *V. alginolyticus* [27] and 5.4 μl/mg cell protein for *E. coli* [28].

Measurement of internal pH

The methylamine uptake was followed by the filtration method as described above in the presence of 20 μM of [^{14}C]methylamine (61.9 Ci/mol) and the internal pH was calculated as described by Rottenberg [29].

Measurement of K^+ exit by K^+ electrode

The concentrated cell suspension was added to 3.0 ml of an appropriate salt solution at 25°C. The reaction mixture was adjusted to desired pH by adding 0.15 ml 1.0 M buffer solution and then the release of cellular K^+ was monitored by use of a K^+ electrode (F2312K, Radiometer, Copenhagen, Denmark) with a reference calomel electrode (K701, Radiometer). The concentrations of K^+ in the reaction mixture were calculated based on the calibration curve prepared under the identical conditions with standard KCl solution.

Standard procedure for the replacement of cellular cations with a desired cation

Harvested cells of *V. alginolyticus* were suspended in 0.4 M chloride salt of a desired cation

containing 50 mM diethanolamine hydrochloride (pH 8.5) using a volume equal to half that of the growth medium. After incubation for 10 min at 25°C, it was centrifuged and the cells were again treated as above. Finally, the cells were washed twice with and suspended in 0.4 M of the above salt with 50 mM Hepes buffer at pH 7.0.

In the case of *E. coli*, 0.14 M chloride salt of a desired cation along with 50 mM diethanolamine hydrochloride (pH 9.3) was used as the incubation medium and the cells were finally washed twice with, and suspended in, 0.14 M salt/50 mM Hepes (pH 7.0).

Chemicals

Tris, Tricine, Hepes and other amines were obtained from Nakarai Chemicals. [^{14}C]Methylamine hydrochloride was from New England Nuclear. Other reagents used were of analytical grade.

Results

Exit of cellular K^+ and entry of Li^+ or Na^+

When the cells of the marine bacterium, *V. alginolyticus,* were suspended in an iso-osmotic concentration (0.4 M) of LiCl or NaCl at neutral pH, a slow exit of cellular K^+ was detected at 25°C. Fig. 1A shows the K^+ exit and Li^+ entry with the cells suspended in 0.4 M LiCl containing 50 mM buffer. The initial rates of K^+ exit and Li^+ entry were calculated to be about 0.03 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ at pH 7.5. In the presence of diethanolamine hydrochloride or Tricine-LiOH at pH 8.9, the rates of K^+ and Li^+ flows increased about 13-fold (0.40 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) and the cellular K^+ was completely replaced with Li^+ after 20 min incubation. The sum total concentration of cellular K^+ and Li^+ was kept constant (470–500 mM) throughout the cation movements. Since the re-uptake of released K^+ is negligible, especially where the initial velocity of cation flows are concerned, the K^+ exit apparently proceeded in proportion to the Li^+ entry.

Fig. 1B shows the K^+ and Na^+ flows in 0.4 M NaCl containing 50 mM buffer. The rate of K^+ exit was very slow at pH 7.5, which increased about 18-fold (0.18 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) in the presence of diethanolamine hydrochloride at pH 8.9. K^+ exit, however, was not induced by Tricine-NaOH even at pH 8.9. In contrast to the case of Li^+, no net entry of Na^+ was detected in any of the cases, and cellular Na^+ was maintained at 60–80 mM.

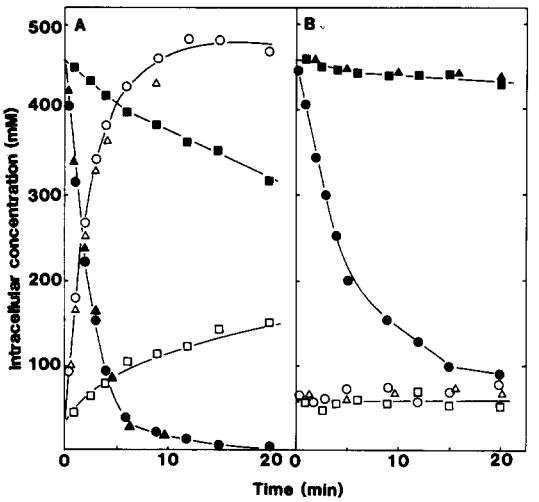

Fig. 1. Time courses of K^+ exit and Li^+ or Na^+ entry with the marine bacterium, *V. alginolyticus,* suspended in 0.4 M LiCl (A) or NaCl (B) containing 50 mM buffer at pH 7.5 and 8.9. The reaction mixture contained 0.4 M LiCl or NaCl, 0.8–1.2 mg cell protein/ml, and 50 mM each of Tris-HCl (pH 7.5) (■, □), diethanolamine hydrochloride (pH 8.9) (●, ○), or Tricine-LiOH (in A) or Tricine-NaOH (in B), pH 8.9 (▲, △). The cellular K^+ (closed symbols) and Li^+ or Na^+ (open symbols) were determined by the filtration method. In A, the cells were washed twice with and suspended in 0.4 M LiCl before use. The results are presented as intracellular concentration in mM.

Fig. 2 shows the effects of pH and the species of buffer on the K^+ exit in LiCl or NaCl medium. The initial rate of K^+ exit increased by the increase in medium pH with the optimum at about 9.6. In the LiCl medium, Tris-HCl gave nearly the same results as diethanolamine hydrochloride when compared at the same pH. Thus, the rate of K^+ exit in the LiCl medium was primarily dependent on the medium pH and was unaffected by the species of buffer.

On the other hand, the K^+ exit in the NaCl medium was very slow with Tris-HCl even at pH 9.4, and diethanolamine hydrochloride was required for the effective exit of K^+. Therefore, the effects of several amine buffers on the K^+ exit in the NaCl medium were examined. In the presence of 50 mM each of diethanolamine, ethanolamine,

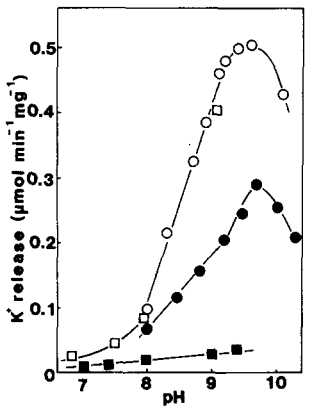

Fig. 2. Effects of pH and the species of buffer on the K^+ exit from *V. alginolyticus*. The reaction mixture contained 0.4 M LiCl (open symbols) or 0.4 M NaCl (closed symbols), 1.3–1.6 mg cell protein/ml, and 50 mM each of Tris-HCl (squares) or diethanolamine hydrochloride (circles) at the indicated pH. The rate of K^+ efflux was monitored by a K^+ electrode. In the experiments with LiCl medium, the cells were washed twice with 0.4 M LiCl before use. The results are presented as $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

methylamine, triethanolamine, Tris and Tricine buffers at pH 9.3, the initial rates of K^+ exit were 0.23, 0.15, 0.15, 0.06, 0.04 and 0.04 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively, as measured by the K^+ electrode. Thus, in addition to alkaline pH, the presence of diethanolamine, ethanolamine or methylamine was required for the effective exit of K^+ in the NaCl medium.

When the cells were incubated at 4°C in the presence of 50 mM diethanolamine hydrochloride at pH 8.9, the rate of K^+ exit in the LiCl or NaCl medium decreased to less than 0.01 $\mu mol\ min^{-1} \cdot mg^{-1}$ and more than 85% of cellular K^+ was retained after 30 min. Thus, the K^+ exit observed at alkaline pH was dependent on the incubation temperature.

Exit of K^+ and entry of membrane-permeable amine in the NaCl medium

We previously demonstrated that methylamine can be used for the determination of pH gradient, inside acidic, in *V. alginolyticus* and that such a pH gradient is collapsed by the addition of a high concentration (50 mM) of diethanolamine or ethanolamine [24]. Thus, these amines can be transported across the cell membrane in their unprotonated forms by passive diffusion and accumulated in the protonated forms due to the relative acidity of the cell interior. Since no appreciable change in the turbidity of cell suspension was detected during the K^+ exit in the NaCl medium, entry of these membrane-permeable amines was expected. To determine the amine entry under the present experimental conditions, ^{14}C-labeled methylamine was employed. As shown in Fig. 3, methylamine was accumulated in accordance with the K^+ exit, whereas internal Na^+ was slightly extruded at first and then maintained at about 50–60 mM. The sum total concentration of cellular K^+, Na^+ and methylamine was maintained between 470 and 500 mM. Judging from the pK_a value of methylamine (10.6 at 25°C), most of the methylamine was protonated within the cells. Thus the protonated methylamine apparently acted as a counter cation and exchanged for K^+ in the ratio 1 : 1. As will be discussed later, these results could be understood by considering that the K^+ exit was mediated by a K^+/H^+ antiporter functioning at alkaline pH.

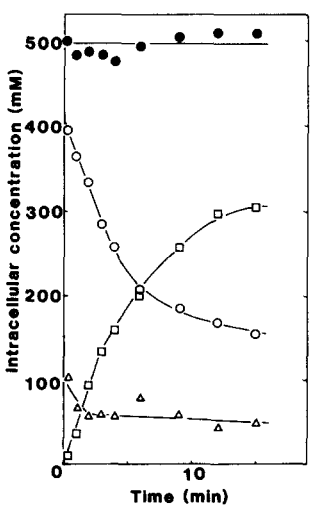

Fig. 3. Time courses of K^+ exit and methylamine entry in NaCl medium. The reaction mixture contained 0.4 M NaCl, 0.8 mg cell protein/ml, 25 mM Tricine-NaOH, 50 mM methylamine hydrochloride and 0.5 μCi [^{14}C]methylamine (61.9 Ci/mol) at pH 8.9. The cellular K^+ (○), Na^+ (△) and methylamine (□) were determined by the filtration method. Closed circles represent the sum total concentration of K^+, Na^+ and methylamine.

Effect of diethanolamine concentration on the K^+ exit in the NaCl medium

As shown in Fig. 4, the rate and extent of K^+ exit increased by the increase in the concentration of diethanolamine and the cellular K^+ reached a steady-state level after 20 min. Assuming that the exchange ratio of K^+ to the protonated diethanolamine is the same as in the case of methylamine, the internal pH at the steady-state level may be estimated from the equilibrium distribution of diethanolamine between the inside and outside of the cells. The internal pH in the presence of 5, 10, 20 and 50 mM diethanolamine was calculated to be pH 7.8, 7.6, 7.7 and 7.8, respectively. At the steady-state level, the internal pH in the absence of diethanolamine as measured from the equilibrium distribution of a trace amount of [^{14}C]methylamine was about 7.8. These results suggested that the K^+/H^+ antiporter ceased to function at the internal pH of about 7.8. Since diethanolamine distributes according to the pH gradient, this might be the reason why relatively high concentration of diethanolamine (50 mM) was required to replace most of cellular K^+.

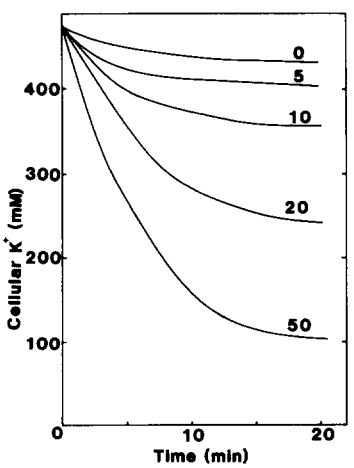

Fig. 4. Effect of diethanolamine concentration on the K^+ exit in the NaCl medium. The reaction mixture contained 0.4 M NaCl, 1.0 mg cell protein/ml and various concentrations of diethanolamine hydrochloride (pH 8.9) as indicated in the figure (shown in mM). The total buffer concentration was adjusted to 50 mM by the addition of Tricine-NaOH (pH 8.9). The K^+ exit was monitored by a K^+ electrode.

Entry of Na^+ to the amine-loaded cells

Once the cells were loaded with the permeable amine, the exposure of these cells to the NaCl medium in the absence of loaded amine resulted in the entry of Na^+. Fig. 5 shows the effect of diethanolamine concentration on the Na^+ entry with the diethanolamine-loaded cells. The initial rate and extent of Na^+ entry were dependent on the concentration of diethanolamine in the external medium. In the absence of diethanolamine, a fast entry of Na^+ was observed and the internal Na^+ concentration reached to about 460 mM. As was expected, the Na^+ entry was not detected in the presence of 50 mM diethanolamine hydrochloride. The diethanolamine-loaded cells initially contained about 150 mM Na^+, and Na^+ was rather slightly extruded from the cells to keep a steady-state level of Na^+ (60–80 mM). Although not

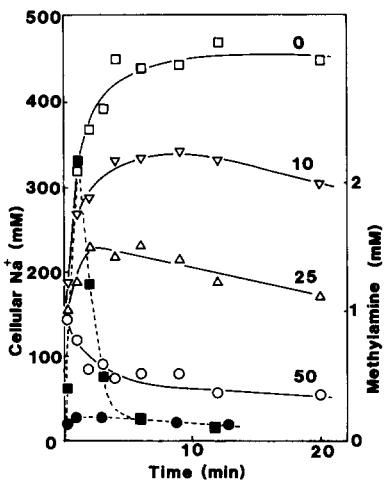

Fig. 5. Effect of diethanolamine concentration on the Na^+ entry to the diethanolamine-loaded cells. The diethanolamine-loaded cells were prepared by the standard procedure for the replacement of cellular cations, except that the cells were finally suspended in the incubation medium (0.4 M NaCl containing 50 mM diethanolamine hydrochloride, pH 8.5). The reaction mixture contained 0.4 M NaCl, 0.8 mg cell protein/ml, and various concentrations of diethanolamine hydrochloride (pH 8.5) as indicated in the figure (shown in mM). The total buffer concentration was adjusted to 50 mM by the addition of Tricine-NaOH (pH 8.5). The cellular Na^+ was determined by the filtration method. In separate experiments, 20 μM [^{14}C]methylamine was added to the reaction mixture and its accumulation was followed in the presence of 50 mM Tricine-NaOH (■) or 50 mM diethanolamine hydrochloride (●) and the results are presented as intracellular concentration in mM.

shown here, essentially the same results were obtained with the methylamine-loaded cells. These results indicated that, after the treatment of cells at alkaline pH in the presence of permeable amine, a simple incubation of the cells with 0.4 M NaCl in the absence of the amine could effectively replace cellular cations with Na^+.

As shown by the dotted lines in Fig. 5, a trace amout of [^{14}C]methylamine was transiently accumulated and then released during Na^+ entry in the absence of diethanolamine. At the peak of the accumulation (1 min), the internal pH was calculated to be about 6.3, giving a pH gradient of 2.2 units, inside acidic. Such a large pH gradient may be created by the bulk release of unprotonated diethanolamine from the cells by passive diffusion. Indeed, a large transient accumulation of [^{14}C]methylamine was not observed in the presence of 50 mM diethanolamine (Fig. 5). Thus, the entry of Na^+ seems to be driven by the large pH gradient, inside acidic, possibly via a Na^+/H^+ antiporter working in reverse.

Exit of K^+ and entry of Rb^+ or Cs^+

Although not shown here, a fast exit of K^+ was observed at neutral pH in RbCl or CsCl medium. At pH 7.5, the initial rate of K^+ exit in RbCl medium was 0.21 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, whereas that of Rb^+ entry was faster than the K^+ exit, amounting to 0.49. As a result, a transient increase in the cellular cation contents was observed in the initial phase of cation flows. At pH 8.9, the rate of K^+ exit increased to 0.49 and the Rb^+ entry to 0.53. In the CsCl medium, the K^+ exit and Cs^+ entry proceeded at nearly the same rate and the initial rates of flows at pH 7.5 and 8.9 were 0.20 and 0.49, respectively. The cellular K^+ was completely replaced with Rb^+ or Cs^+ after 20 min incubation at pH 7.5. Thus, although the K^+ exit was enhanced by the increase in medium pH, the fast entry of Rb^+ or Cs^+ and K^+ exit occurred even at neutral pH, which was in marked contrast to the case of Li^+ or Na^+.

Since Rb^+ is taken up by the K^+ transport system of *V. alginolyticus* [6], the initial fast entry of Rb^+ over K^+ exit may be due to the uptake of Rb^+ via the K^+ transport system. Although Cs^+ could not replace K^+ in the K^+ transport system when assayed at low concentration (10 mM), we observed that the K^+ uptake was inhibited in the presence of 0.4 M CsCl and Cs^+ entered into the cells instead of K^+ (data not shown). Thus, Cs^+ seems to be able to substitute for K^+ at high concentrations.

Replacement of cellular cations with a desired cation

From the described results above, it is apparent that the cellular cations can be replaced with a desired cation such as Li^+, Na^+, Rb^+ or Cs^+ by simply incubating the cells under appropriate conditions. A standard procedure for the manipulation of cellular cation contents was prepared as described in Materials and Methods. As shown in our previous paper [13], when the K^+ depletion and cation loading were performed at pH 9.3, the cellular activity of α-aminoisobutyric acid uptake

TABLE I

INTRACELLULAR CATION CONCENTRATIONS OF *V. ALGINOLYTICUS* and *E. COLI* AFTER REPLACEMENT WITH A DESIRED CATION

V. alginolyticus and *E. coli* were treated by the standard procedure for the replacement of cellular cations and intracellular cation concentrations were determined. [X^+] represents the species of replacing cation and results are expressed in mM.

Salt used	*V. alginolyticus*			*E. coli*		
	[X^+]	[Na^+]	[K^+]	[X^+]	[Na^+]	[K^+]
NaCl	480		< 1	260		< 1
KCl	570	< 1		220	< 1	
LiCl	520	< 1	3	260	< 1	8
RbCl	460	3	8	260	1	5
CsCl	360	3	1	270	4	1

was significantly reduced except for the case of Na^+. Thus, the standard procedure for *V. alginolyticus* was performed at pH 8.5. This method was also found to be applicable to *E. coli*. In this case, 0.14 M chloride salt containing 50 mM diethanolamine hydrochloride (pH 9.3) was employed as the incubation medium. Table I shows the intracellular cation concentrations of *V. alginolyticus* and *E. coli* treated by the standard procedure. The washed cells of *V. alginolyticus* normally contain about 480 mM K^+ and 80 mM Na^+. These cellular cations were effectively replaced with the monovalent cation used for the treatment. When 0.4 M KCl was used, cellular cations were replaced with K^+ and Na^+ was completely removed from the cells.

As shown in Table I, essentially the same results were obtained with *E. coli*. Although not shown here, the K^+ exit from *E. coli* was enhanced by the increase in medium pH and the presence of membrane-permeable amine was required for the K^+ exit in the NaCl medium, especially up to the medium pH of 8.9. These results were essentially similar to those observed in the case of *V. alginolyticus*, except that the entry of Na^+ into the *E. coli* cells was observed at an external pH above 8.9.

Discussion

As shown in this paper, K^+ exit is greatly influenced by the species of monovalent cation in the external medium, suggesting the participation of specific cation transport systems in the cation movements. In the NaCl medium, the presence of membrane-permeable amine is required for the K^+ exit, where no net entry of Na^+ is detected (Fig. 1B) and the amine is accumulated in exchange for K^+ (Fig. 3). Thus, the K^+ exit observed at alkaline pH can be explained by the function of a K^+/H^+ antiporter that has been demonstrated in *E. coli* by Brey et al. [4]. The entry of unprotonated amine into the cells by passive diffusion and its protonation inside the cells actually allows the K^+ exit via the K^+/H^+ antiporter even in the absence of Na^+ entry. In this case, since the internal pH is decreased by the K^+ exit and the antiporter ceases to function at an internal pH of about 7.8, a relatively high concentration of permeable amine is required for the bulk release of cellular K^+ (Fig. 4). In the absence of permeable amine, the internal pH is effectively lowered with only a slight release of K^+ in the NaCl medium. Therefore, as has been suggested in the case of *E. coli* [4,30], it is possible for the K^+/H^+ antiporter to function as a regulator of cytoplasmic pH at alkaline external pH in *V. alginolyticus* under normal conditions.

As described in our previous paper [24], *V. alginolyticus* possesses a respiration-dependent primary Na^+ pump functioning at alkaline pH (8.5). Although not shown here, the cells of *V. alginolyticus* washed with 0.4 M NaCl or LiCl can generate the membrane potential of about -150 mV at pH 8.9 even in the absence of exogenous substrates, and Na^+ flow is always in the direction of extrusion. Thus, the steady-state level of internal Na^+ is maintained at about 60–80 mM in the medium of 0.4 M NaCl (Figs. 1B, 3 and 5). The results of Fig. 5 suggest that a large pH gradient, inside acidic, seems to be required for the induction of Na^+ entry.

In contrast to Na^+, Li^+ enters into the cells in proportion to the K^+ exit and the presence of permeable amine is not required (Fig. 1A). The reason for the apparent difference observed between Li^+ and Na^+ is not clear at present.

It is worthy of note that the K^+-depleted and cation-loaded cells prepared by the present method are not plasmolysed. The K^+-depleted cells prepared by hypotonic treatment [19] are plasmolysed and require K^+ for deplasmolysis [23]. In *V. alginolyticus*, the K^+-depleted and Na^+-loaded cells have 5-fold higher activity of K^+ uptake than the plasmolysed cells (unpublished results). Thus, the manipulation of cellular cations by the present method is of great advantage over the hypotonic treatment. We utilized the present method for the manipulation of internal cation contents of *V. alginolyticus* and we could clearly demonstrate the role of K^+ in the generation of a pH gradient (inside alkaline) [6] and an Na^+ electrochemical gradient [13,24].

The present method is essentially applicable to *E. coli* also (see Table I). Thus, a type of mechanism similar to that discussed above seems to be operative on the monovalent cation movements in *E. coli*. One remarkable exception is that the

primary Na^+ pump functioning at alkaline pH as demonstrated in *V. alginolyticus* [24] has not been documented in *E. coli.*

The present method for the manipulation of cellular cation contents may be widely applicable to the investigation of cation transport systems employing intact cells of Gram-negative bacteria.

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