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A novel mechanism of cation/substrate cotransport: Na^+/H^+/adenosine cotransport in *Vibrio parahaemolyticus*

Yoshie Okabe, Yuki Sakai-Tomita, Yuki Mitani, Masaaki Tsuda and Tomofusa Tsuchiya

Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama (Japan)

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Adenosine is actively transported with Na^+ in *Vibrio parahaemolyticus* (Sakai,Y., Tsuda, M., Tsuchiya, T. (1987) Biochim, Biophys. Acta 893, 43–48). The proton conductor carbonylcyanide *m*-chlorophenylhydrazone, CCCP, strongly inhibited active transport of adenosine at pH 8.5 as well as at pH 7.0. This seemed peculiar because the driving force, an electrochemical potential of Na^+, is established by the Na^+-extruding respiratory chain at pH 8.5 in this organism, although it is established by the function of the Na^+/H^+ antiporter at pH 7.0. This suggested that H^+ might be involved in the adenosine transport. We detected H^+ uptake induced by adenosine influx in *V. parahaemolyticus* cells in the presence of Na^+, but not in its absence, suggesting the occurrence of Na^+/H^+/adenosine cotransport. We isolated formycin A-resistant mutants which showed defective adenosine transport. The mutation resulted in simultaneous losses of Na^+ uptake and H^+ uptake induced by adenosine. In revertants from these mutants the Na^+ uptake and H^+ uptake were restored simultaneously. The frequencies of reversion were in the order of 10^{-7}, indicating that the mutations were single mutations; namely that Na^+/adenosine cotransport and H^+/adenosine cotransport took place via the same carrier. Thus, we conclude that adenosine is transported by the novel mechanism of Na^+/H^+/adenosine cotransport in *V. parahaemolyticus*.

Introduction

Cation/substrate cotransport is one of the major mechanisms of active transport in cell membranes. Two cations are known to be utilized as coupling cations for nutrient transport, H^+ and Na^+.

In *Escherichia coli*, which is one of the best characterized microorganisms, the respiratory chain extrudes H^+, and an electrochemical potential of H^+ is established across cytoplasmic membrane, which is the driving force for H^+ coupled cotransport, ATP synthesis, flagellar rotation, and so forth. Thus circulation of H^+ across membranes plays a fundamental role in energy transduction in membranes [1]. Several H^+/substrate cotransport systems are known in *E. coli*, of which the lactose transport system is the best characterized [2–4]. Uptake of a substrate transported by this mechanism is strongly inhibited by an H^+ conductor. The electrochemical potential of H^+ is converted to that of Na^+ via the Na^+/H^+ antiporter [5]. Therefore, formation of an electrochemical potential of Na^+ is sensitive to an H^+ conductor. The electrochemical potential of Na^+ thus established drives Na^+/substrate cotransport. Uptake of a substrate that is transported by this mechanism is also strongly inhibited by an H^+ conductor [6]. The melibiose transport system is one of the best characterized Na^+/substrate cotransport systems [7]. This system possesses unique properties with respect to cation coupling, utilizing Na^+, H^+ or Li^+ as a coupling cation depending on the substrate transported [7,8]. Similar versatility of cation coupling has not, to our knowledge, been reported for other transport systems.

In *Vibrio parahaemolyticus*, which is a slightly halophilic marine bacterium, as well as in *V. alginolyticus* [9], the respiratory chain extrudes Na^+ under alkaline conditions [10]. Thus an electrochemical potential of Na^+ is directly established. The respiratory chain

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Mops, 4-morpholinepropanesulfonic acid; Tricine, tris(hydroxymethyl)methyl-glycine; TMAH, tetramethylammonium hydroxide.

Correspondence: T. Tsuchiya, Department of Microbiology, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan.

also extrudes H^+, and an electrochemical potential of Na^+ is also established via the Na^+/H^+ antiporter [11]. Therefore, the electrochemical potential of Na^+ in *V. parahaemolyticus* is only partly sensitive to an H^+ conductor at alkaline pH levels. Under neutral conditions, however, Na^+ extrusion by the respiratory chain does not take place, and the electrochemical potential of H^+ established by the respiratory chain is converted to that of Na^+ by the function of the Na^+/H^+ antiporter. Therefore, formation of an electrochemical potential of Na^+ is sensitive to an H^+ conductor at neutral pH. Thus the effect of an H^+ conductor at various pH levels could be a good means of distinguishing the Na^+-coupled process from the H^+-coupled process in *V. parahaemolyticus*, as well as in *V. alginolyticus*. An H^+ conductor should completely inhibit systems driven by an H^+ electrochemical potential, while systems driven by an Na^+ electrochemical potential should be only partly inhibited.

During studies on the adenosine transport system of *V. parahaemolyticus*, which we previously reported to be an Na^+/substrate cotransport system [12], we unexpectedly observed that H^+ conductors severely inhibited adenosine transport at pH 8.5. As reported here, further studies showed that active transport of adenosine in *V. parahaemolyticus* is driven by a novel mechanism, namely Na^+/H^+/substrate cotransport.

Materials and Methods

V. parahaemolyticus AQ3334 [10] was used as parental strain. Adenosine transport negative mutants, FR12, FR22 and FR41 were isolated as formycin A-resistant mutants. For this, cells of AQ3334 grown in medium S [13] supplemented with 0.5% polypeptone were introduced into 20 tubes, diluted with medium S plus 40 mM glycerol and 100 μM formycin A, and shaken at 37°C for 24 h. Then they were diluted 10^6-fold with medium S, and spread on 20 agar plates containing medium S plus 40 mM glycerol. After incubation at 37°C for 24 h, the colonies that appeared on the plates were replica-plated onto agar plates containing medium S supplemented with 10 mM adenosine or 40 mM glycerol. Colonies that grew very poorly on the adenosine plates and normally on the glycerol plates were picked up. The mutants thus obtained grew well in the presence of 100 μM formycin A. Spontaneous revertants were obtained from mutants FR12, FR22 and FR41 as cells that grew normally on plates containing medium S supplemented with 10 mM adenosine. These revertants showed restored sensitivity to formycin A. Revertants FR12R1, FR12R2, FR22R1 and FR41R1 were derived from FR12, FR12, FR22 and FR41, respectively.

For transport assays, cells were grown aerobically at 37°C in medium S supplemented with 0.5% polypeptone, and 3 mM adenosine when it was necessary to induce the adenosine transport system. Cells were harvested in the late exponential phase of growth, washed twice with buffer consisting of 0.2 M Mops-Tris (pH 7.5) and 10 mM $MgSO_4$, and suspended in the same buffer. Assay mixtures for adenosine transport consisting of 0.2 M Mops-Tris (pH 7.5)/10 mM $MgSO_4$/20 mM Tris-lactate/2 mM NaCl and cells (about 0.1 mg cell protein/ml). After preincubation at 25°C for 3 min, transport was initiated by addition of [^{14}C]adenosine (0.2 μCi/μmol, final 0.1 mM). Samples were taken at intervals, filtered, washed and counted [6].

For measurement of Na^+ uptake, cells were washed twice with buffer consisting of 0.2 M Mops-TMAH (pH 7.5) and 5 mM $MgSO_4$, and suspended in the same buffer. Uptake of Na^+ was measured in assay mixture consisting of 0.2 M Mops-TMAH (pH 7.5)/0.1 mM NaCl and cells (2–3 mg cell protein/ml), using a Na^+-selective electrode, as described previously [7].

For measurement of H^+ uptake, cells were washed twice with 0.2 M choline-Cl and suspended in the same solution. Uptake of H^+ was measured in assay mixture consisting of 0.15 M choline-Cl/50 mM NaCl (unless otherwise stated) and cells (about 1 mg cell protein/ml), using an H^+-electrode, as described previously [7].

Protein contents were determined by a published procedure [14] with bovine serum albumin as standard. [U-^{14}C]Adenosine was purchased from Amersham International. The Na^+-electrode was from Radiometer Co., Copenhagen.

Results

Properties of the adenosine transport system

To determine the substrate specificity in the adenosine transport system, we first tested whether other nucleosides inhibited adenosine transport. As shown in Fig. 1, excess guanosine, cytidine or uridine inhibited adenosine transport appreciably. Very strong inhibition of [^{14}C]adenosine uptake was observed with excess unlabeled adenosine. Judging from the extent of inhibition, adenosine seemed to be the best substrate, followed by guanosine, uridine and cytidine.

Our previous data suggested that the adenosine transport system in *V. parahaemolyticus* was partly inducible [12]. Actually, we observed about 2-fold higher uptake of adenosine by cells grown in the presence of adenosine than by cells grown in its absence (data not shown).

We measured Na^+ uptake in cells elicited by influx of adenosine, and other nucleosides. Uptake of Na^+ occurred not only with adenosine, but also with guanosine, uridine, cytidine (Table I) and formycin A, an antibiotic and analog of adenosine (data not shown). The initial velocities of Na^+ uptake elicited by each

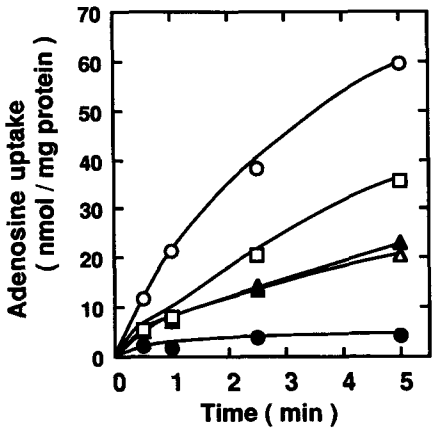

Fig. 1. Effects of nucleosides on adenosine uptake. Uptake of adenosine was measured in cells grown in the presence of adenosine. Each nucleoside (1 mM) was added 1 min before addition of [^{14}C]adenosine (0.1 mM). When the effect of unlabeled adenosine was tested, no correction for change in specific activity of radioisotope was made to show decrease in [^{14}C]adenosine uptake. Symbols: ○, control; ●, adenosine; △, guanosine; ▲, uridine; □, cytidine.

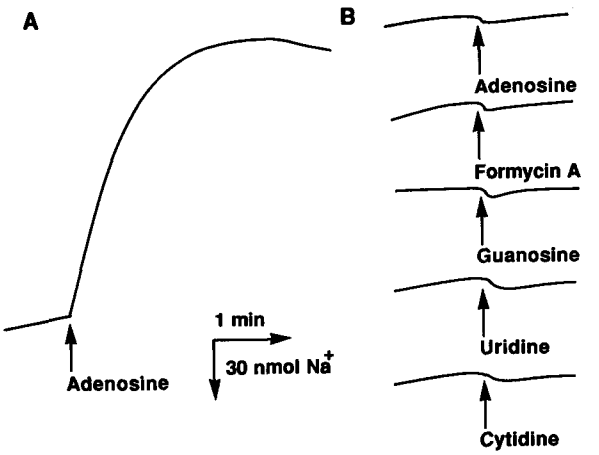

Fig. 2. Effects of nucleosides on Na^+ uptake elicited by adenosine influx. Uptake of Na^+ was measured with an Na^+-selective electrode. In (A), adenosine at 0.1 mM was added at the time indicated by an arrow. Upward deflection represents uptake of Na^+. In (B), cells were preincubated with 0.4 mM adenosine, and nucleosides were added at 0.1 mM concentration at the times indicated by an arrow.

nucleoside were about twice as high in cells grown in the presence of adenosine as in cells grown in its absence. Thus, the observed Na^+/adenosine, Na^+/guanosine, Na^+/uridine and Na^+/cytidine cotransports all seemed to be mediated by the same transport system, the adenosine system. This idea was supported by the finding that the Na^+ uptakes elicited by addition of adenosine, formycin A, guanosine, uridine or cytidine to cell suspensions were all prevented equally by preincubation of the cells with excess adenosine (Fig. 2). The Na^+ uptake induced by adenosine was also prevented by preincubation of the cells with excess guanosine, uridine, cytidine or formycin A (data not shown).

We then tested the effect of an H^+ conductor, CCCP, on uptake of adenosine. If an electrochemical potential of Na^+ is the sole driving force for active transport of adenosine, CCCP should have different effects on adenosine transport at different pH levels, because an electrochemical potential of Na^+ is established by different mechanisms at different pH levels in *V. parahaemolyticus*, as described earlier. In fact, very strong inhibition at pH 6.5 and partial inhibition at pH 8.5 by CCCP of α-aminoisobutyric acid transport, which is an Na^+-substrate cotransport system, have been reported [9]. Unexpectedly, however, adenosine transport in *V. parahaemolyticus* was strongly inhibited by CCCP not only at pH 7.0 but also at pH 8.5 (Fig. 3). Similar results were obtained with other H^+ conductors (data not shown). These results suggest that H^+ is also involved in active transport of adenosine.

H^+ uptake elicited by adenosine

To determine whether H^+/adenosine cotransport occurs, we tested whether H^+ uptake occurred when adenosine was added to the cell suspension. As shown in Fig. 4, we observed considerable uptake of H^+ elicited by adenosine. We also detected H^+ uptake elicited by formycin A and by cytidine, but not by

TABLE I

Comparison of initial rates of Na^+ uptake elicited by nucleoside influx

Substrate	Na^+ uptake [a] (nmol/min per mg protein)		
	A [b]	B [c]	B/A
Adenosine	4.4	9.3	2.1
Guanosine	4.4	9.3	2.1
Uridine	3.8	7.6	2.0
Cytidine	2.8	5.2	1.9

[a] Initial velocity of Na^+ uptake measured after addition of each substrate to the cell suspension.
[b] Cells grown in the absence of adenosine.
[c] Cells grown in the presence of 3 mM adenosine.

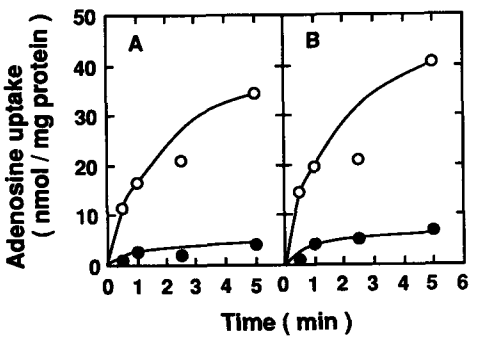

Fig. 3. Inhibition of adenosine transport by CCCP. Transport of [^{14}C]adenosine was measured at pH 7.0 (A) or 8.5 (B), and in the absence (○) or presence (●) of 10 μM CCCP. The buffers used were 0.2 M Mops-Tris (A) and 0.2 M Tricine-TMAH (B).

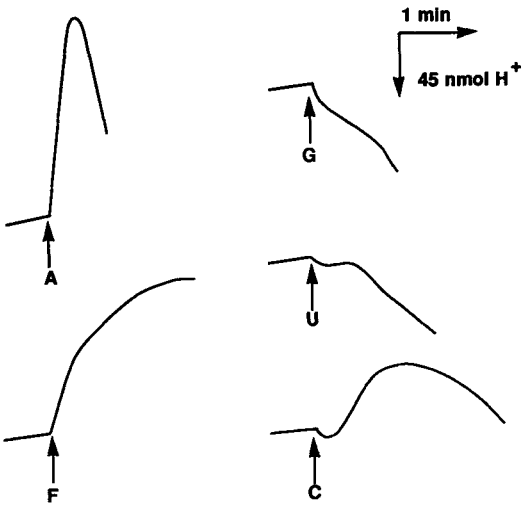

Fig. 4. Uptake of H^+ elicited by influx of nucleosides. Uptake of H^+ was measured with an H^+-electrode. At the times indicated by arrows, nucleosides (A, adenosine; F, formycin A; G, guanosine; U, uridine; C, cytidine) were added at 0.05 mM. Upward deflection represents uptake of H^+.

guanosine or uridine. In contrast, efflux of H^+ was observed when guanosine or uridine was added. This would be due to a membrane potential (interior positive) established by Na^+/guanosine and Na^+/uridine cotransport, as observed in Na^+/melibiose cotransport of *E. coli* [15]. The acidification observed 0.5 to 1 min after the addition of nucleosides (except formycin A) (Fig. 4) would be due to metabolism of the nucleosides. These results indicate that H^+/guanosine cotransport and H^+/uridine cotransport do not take place, or take place very inefficiently. The observed H^+ uptake elicited by adenosine, formycin A or cytidine would not be due to protonation and deprotonation of the substrates, because such H^+ uptake was not observed with guanosine or uridine. Thus, *V. parahaemolyticus* cells clearly possess an H^+/adenosine (and H^+/formycin A and H^+/cytidine) cotransport system. However, these results did not show whether Na^+/adenosine cotransport and H^+/adenosine cotransport are mediated by the same system or by different systems. If H^+/adenosine cotransport is mediated by the Na^+/adenosine cotransport carrier, then H^+ uptake elicited by adenosine should be inhibited by other substrates of the Na^+/adenosine carrier. In fact, we found that it was inhibited by guanosine, cytidine, uridine and formycin A (data not shown). Furthermore, we observed about 2-fold greater H^+ uptake elicited by adenosine in cells grown in the presence of adenosine than in cells grown in its absence (data not shown), as observed with Na^+ uptake elicited by adenosine. Similar results were obtained with formycin A (data not shown). If Na^+, H^+ and adenosine are transported by the same carrier, their transport must be by the novel mechanism of Na^+/H^+/adenosine cotransport.

If this is the case, then H^+ uptake caused by adenosine would require Na^+ or would be stimulated by Na^+. Therefore, we next tested the effect of Na^+ on the H^+ uptake. As shown in Fig. 5, the H^+ uptake induced by adenosine was greatly stimulated by Na^+. In the absence of Na^+, H^+ uptake was very small. It should be pointed out that the assay mixture contained a micromolar level of Na^+ even when no Na^+ was added (data not shown). Half-maximal stimulation was observed at 4 mM Na^+. Similar, but smaller, stimulation by Na^+ of H^+ uptake elicited by formycin A was also observed, and again the stimulation was half-maximal at 4 mM Na^+. Stimulation of substrate-induced H^+ uptake by Na^+ has not been observed in the melibiose transport system of *E. coli*, which uses either Na^+ or H^+ as a coupling cation [7,15]: on the other hand, addition of Na^+ to the assay medium reduced melibiose-induced H^+ uptake [16], and addition of sufficient Na^+ induced efflux of H^+ [7,15]. Thus, the type of Na^+-coupling and H^+-coupling in the adenosine transport system of *V. parahaemolyticus* is different from that in the melibiose system of *E. coli*.

Na^+ uptake and H^+ uptake in mutants and revertants

The above results support the idea of Na^+/H^+/adenosine cotransport in *V. parahaemolyticus*. We tried to confirm this idea by analyzing mutants of the adenosine transport system and their revertants. We first isolated many formycin-A-resistant mutants, and obtained many adenosine transport-negative mutants from them, such as mutant FR12. Cells of FR12 grew well even in the presence of 100 μM formycin A, although wild-type AQ3334 cells did not. The mutant FR12 cells did not grow well in minimal medium supplemented with adenosine as a sole source of carbon (Table II), and showed no uptake of [^{14}C]adenosine (data not shown). We obtained many such adenosine transport-negative mutants. We also obtained revertants from such mutants as cells that grew normally

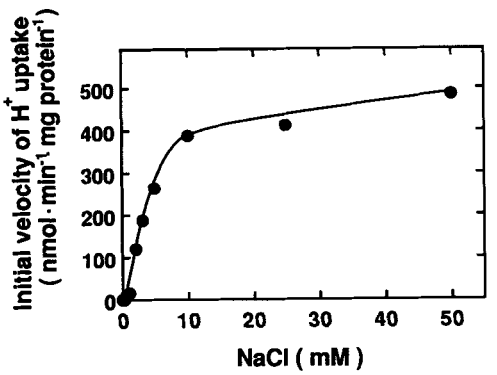

Fig. 5. Dependence on Na^+ of H^+ uptake elicited by adenosine influx. The initial velocity of H^+ uptake caused by adenosine was measured at various concentrations of NaCl. The total concentration of choline-Cl plus NaCl was maintained at 0.2 M.

TABLE II

Properties of wild-type, mutant and revertant cells

Strain	Growth on:			Reversion frequency
	glycerol	glycerol + formycin A	adenosine	
AQ3334	+ [a]	− [b]	+	
FR12	+	+	−	$1.0 \cdot 10^{-7}$
FR12R1	+	−	+	
FR12R2	+	−	+	
FR22	+	+	−	$2.7 \cdot 10^{-7}$
FR22R1	+	−	+	
FR41	+	+	−	$3.8 \cdot 10^{-7}$
FR41R1	+	−	+	

[a] +, cells grew well.
[b] −, cells did not grow or grew very poorly.

on adenosine (Table II). These revertants showed formycin A sensitivity. The frequencies of reversion were in the order of 10^{-7}, suggesting that the mutations in mutants such as FR12 were single mutations. As seen in Fig. 6, both Na^+ uptake and H^+ uptake elicited by adenosine were lost in FR12, and the cells also showed no Na^+ uptake when guanosine, uridine or cytidine was added (data not shown). A revertant, FR12R1, showed simultaneous restoration of Na^+ uptake and H^+ uptake elicited by adenosine (Fig. 6). Restorations of Na^+ uptake and H^+ uptake were also observed in another revertant FR12R2 isolated independently (data not shown). Both revertants showed

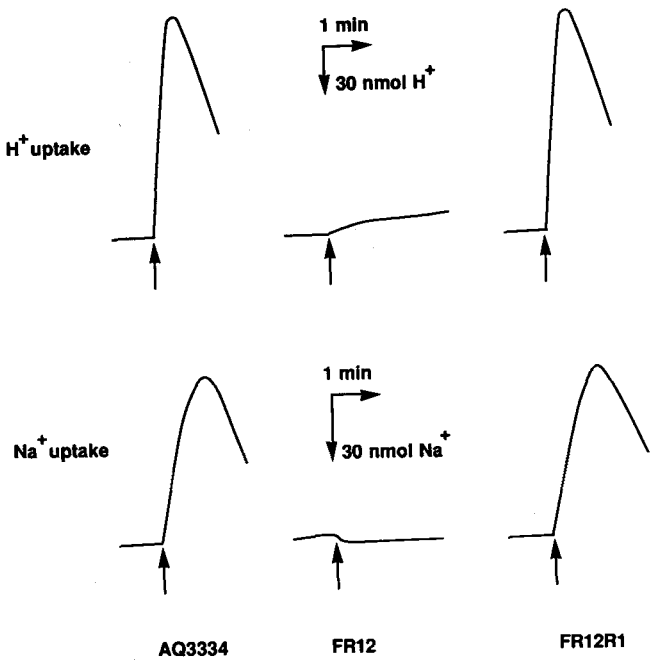

Fig. 6. Disappearance and reappearance of H^+ uptake and Na^+ uptake elicited by adenosine in a mutant and revertant. The uptakes of H^+ and Na^+ were measured with ion-selective electrodes in wild-type (AQ3334), mutant (FR12) and revertant (FR12R1) cell suspensions under standard conditions. Adenosine at 0.05 mM was added to cell suspensions at the times indicated by arrows.

normal adenosine uptake (data not shown). Similar results were obtained on adenosine uptake, Na^+ uptake and H^+ uptake in two other formycin-A-resistant mutants, FR22 and FR41, and their revertants, FR22R1 and FR41R1 (data not shown). Thus the uptakes of Na^+, H^+ and adenosine are clearly mediated by a single transport system.

Discussion

The present results show that *V. parahaemolyticus* cells possess a Na^+/H^+/adenosine cotransport system. This is a novel mechanism of cotransport in biological membranes. This is the first clear example of a Na^+/H^+/substrate cotransport system. About 15 years ago, the melibiose system was found as the first example of an Na^+/substrate cotransport system of *E. coli* [6,7,15], but later an Na^+/glutamate cotransport system [17,18], Na^+/proline cotransport system [19,20] and Na^+/serine (threonine) cotransport system [21] were also found in *E. coli*, indicating that Na^+/substrate cotransport is one of the main mechanisms of active transport in *E. coli*. Therefore, we think that other Na^+/H^+/substrate cotransport systems may be found in *Vibrio* and certain organisms. We have reported that *V. parahaemolyticus* cells possess an Na^+/serine cotransport system [10]. This system may also be an Na^+/H^+/substrate cotransport system, because we observed very strong inhibition of serine uptake by CCCP at pH 8.5, and also H^+ uptake induced by serine in *V. parahaemolyticus* (unpublished observations). Most transport systems in *V. alginolyticus* are reported to be Na^+-coupled [11], so some of them may be Na^+/H^+/substrate cotransport systems. However, in *E. coli* B, glutamate was proposed to be transported by an Na^+/H^+/glutamate [22,23] rather than an Na^+/glutamate cotransport mechanism [17,18], but later at least two cation-coupled glutamate transport systems were found in these cells, an Na^+-coupled and an H^+-coupled system [24]. Thus, it seems possible that the proposed Na^+/H^+/glutamate cotransport is a mixture of Na^+/glutamate and H^+/glutamate cotransport. As we reported previously, an outwardly directed H^+ gradient drives inwardly directed glutamate transport in the presence of Na^+ [17]. It has been reported that glutamate transport in membrane vesicles of *Bacillus stearothermophilus* is most likely cotransported with Na^+ and H^+ [25]. However, it is not clear whether this glutamate transport is mediated by a single transport system or a mixture of two (or more) systems.

It should be pointed out that the observed initial rates of adenosine uptake, Na^+ uptake and H^+ uptake are different. Perhaps this is due to differences in assay conditions for each measurement. At present it is difficult to measure uptake of adenosine, Na^+ and H^+ simultaneously. Also, it is difficult to carry out quanti-

tative analysis of the Na^+/H^+/adenosine cotransport with whole cells because adenosine is metabolized. It is desirable to analyze the transport system using right-side-out membrane vesicles. We have been trying to prepare such vesicles of *V. parahaemolyticus*, but we have not yet succeeded in such attempts.

We have observed versatility in cation coupling in the adenosine transport system of *V. parahaemolyticus*: with adenosine as substrate, Na^+ and H^+ couple simultaneously, and similar coupling is observed with formycin A and cytidine. However, with guanosine or uridine as substrate, transport is coupled with Na^+, but not with H^+. We also found versatility in cation coupling in the melibiose transport system of *E. coli*, Na^+, H^+ or Li^+ being used as a coupling cation, depending on the substrate transported [7]: with melibiose as substrate, either Na^+ or H^+ acts as a coupling cation, Na^+ being used preferentially. But with methyl β-D-thiogalactoside as substrate, Na^+ or Li^+ is used, but H^+ is not, and with methyl α-D-galactoside as substrate Na^+, H^+ or Li^+ is used. The adenosine transport system of *V. parahaemolyticus* is the second example of versatility in combinations of cations and substrates. Like the melibiose transport system of *E. coli* [26–29], the adenosine transport system of *V. parahaemolyticus* would be suitable for studies on structure–function relationships in a transport carrier from the viewpoint of cation coupling.

The finding of an Na^+/H^+/substrate cotransport system in the cell membrane is interesting with regard to the evolution of cation/substrate cotransport system. Three types of cation/substrate cotransport system have been reported: namely, cotransport systems utilizing (1) H^+, (2) H^+ or Na^+, and (3) Na^+ as the coupling cation. In some cases Na^+ can be replaced by Li^+. The Na^+/H^+/substrate cotransport system using H^+ and Na^+ is a fourth type. This seems to be a more complicated type than systems (1) to (3) above. At present, no conclusions are possible about the order of appearance of these four types during evolution, or their relationships. Structural analyses of many cotransport systems and comparison of these systems would be valuable for providing an insight into the evolution of cotransport systems.

It has been believed that H^+ is the major coupling cation for energy-transducing systems in membranes of microbial organisms, as well as of mitochondria and chloroplasts. Now it is becoming clear that Na^+ also plays an important role in energy-transduction in membranes. In *Propionigenium modestum*, a decarboxylation-coupled Na^+ pump [30] and Na^+-coupled ATPase [31] have been reported. Similar Na^+-coupled ATPase has been found in *Methanococcus voltae* [32], *V. alginolyticus* [33] and *Methanobacterium thermoautotrophicum* [34]. In *V. parahaemolyticus*, we found that oxidative phosphorylation occurred in mutants lacking H^+-translocating ATPase [35]. We observed Na^+-driven ATP synthesis in these mutants. The wild-type cells have an H^+-coupled oxidative phosphorylation system in addition to the Na^+-coupled one. Thus cells of *V. parahaemolyticus* seem to possess an Na^+-coupled ATP synthase. If this is the case, Na^+ as well as H^+ is a major coupling cation in energy transducing systems in the membrane of *V. parahaemolyticus*. Since both Na^+ and H^+ are extruded by the respiratory chain, the circulations of Na^+ and H^+ across the cytoplasmic membrane are of primary importance in membrane bioenergetics in *Vibrio*.

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