Datura innoxia懸濁培養におけるK+-およびNa+-取り込み活性

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K^+- and Na^+-Uptake Activities of Suspension Culture of *Datura innoxia*.

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ABSTRACT

Using intact cells and protoplasts of suspension culture of *Datura innoxia*, some properties of K^+- and Na^+- uptake activities were characterized as follows:

1. Optimal pHs of K^+- or Na^+-stimulated H^+-release activity and K^+- or Na^+-uptake activity were pH 7.5.

2. K^+-Uptake activity was inhibited by K^+-ionophore. While, Na^+-uptake activity was inhibited by K^+- and H^+- ionophores.

3. Halfway addition of Na^+ to K^+-uptake mixture did not influence on K^+-uptake activity. But similar addition of K^+ to Na^+-uptake mixture caused the release of Na^+.

4. Of tested anions, HCO_3^-, NO_3^-, and Cl^- were more stimulative on K^+-uptake activity, which was osmotic pressure sensitive.

Introduction

Active transport of monovalent cations is driven by some energy forms which are defined by Peter Mitchell ($\Delta\bar{\mu}H^+ = F\Delta\psi - 2.3\,RT\Delta\,pH$: F, Faraday constant; R, gas constant; T, absolute temperature. MITCHELL, P. 1976). For example, Na^+/K^+-ATPase transorts Na^+ and K^+ by the hydrolysis of ATP and generates the ion-gradients across the nerve cell membrane. And Na^+- or K^+-channel induces a passive flow of the ion to dissipate its gradient according to the stimuli or signals (STRYER, L. 1988).

For plant cells, most studied monovalent cation is K^+, whose role is especially crucial on mechanical movement of stomatal guard cells (SCHROEDER, J. I. et al. 1984, ASSMANN, S. M. et al. 1985, SCHIMAZAKI, K. et al. 1986). While, Na^+ is less studied. Na^+ distribution across and within cell is interested in the case of halophytes and salt torelance (MILLS, D. Yt al. 1985, BLUMWALD, E. & POOLE, R. J. 1987).

In this study, we examined the property of K^+-transport system comparing with that of Na^+ using intact cells and protoplasts of *Datura innoxia* suspension culture. Existence of K^+-transport system in the cell was suggested.

Materials and methods

1. Cultivation and maintenance of suspension culture

Suspension culture of *Datura innoxia* was supplied from the Laboratory of Plant Breeding of our faculty. Used culture medium was the same as that of reported (MURASHIGE, T. & SKOOG, F. 1962), except that NH_4NO_3 was replaced by NZ-amine (type A, Wako Chemical; 2 g/l). Rotary shaking culture (100 rpm) was done at 25°C in 40 ml of culture medium using 100 ml Elren-Meyer flask. Once every week, 5 ml of the culture was inoculated to the new medium and cultured.

2. Preparation of intact cells.

Three-day old suspension culture was centrifuged (1,000 rpm, 3 ml). Cells were resuspended in sterilized 0.35 M mannitol (Wako Chemicals) and rotary shaken (100 rpm) at 25°C overnight. The cells were collected again by centrifugation (1,000 rpm, 3 min), and washed twice with the same mannitol solution. The cells (intact cells) were alive as far as judged by the staining with fluorescein diacetate (Sigma). Fluorescence formed by catalysis of the esterase inside the cell was observed.

3. Preparation of protoplasts.

Four-day old suspension culture (5 ml) was mixed with equal amount of lytic mixture (Cellulase RS, Yakult, 2%; Macerozyme R70, Yakult, 1%; Pectolyase Y23, Seinshin Chemicals, 0.02%; sorbitol, 0.7 M; pH was adjusted to 5.8 with 1 N HCl or 5%

KOH; sterilized by filtration through membrane filter, pore size 0.45 μm), and incubated at 25°C for four hours with gentle shaking. The mixture was filtrated through nylon mesh (mesh size 77 μm) and centrifuged (1,000 rpm, 3 min). Precipitated protoplasts was suspended in 0.35 M mannitol and washed twice with 0.35 M mannitol by centrifugation (1,000 rpm, 3 min). Protoplast suspension (2 ml) was layered on 20 % sucrose (5 ml) and centrifuged (1,000 rpm, 5 min). Protoplasts were recovered from the interface between mannitol and sucrose, and resuspended in 0.35 M mannitol.

4. Measurement of H^+-release

Assay mixture (total 12 ml, stirred at 25°C) contained 0.35 M mannitol and intact cells (1.5 mg dry weight) or protoplasts (4 mg protein). After initial pH of the mixture was adjusted to desired value with 0.1 % NH_4OH (0-20 μl), salt (final 10mM) was added. Decrease of the pH of the mixture was monitored for one minute, subtracted the value of control experiments with assay mixture without salt or cells (protoplasts), and expressed as pH per mg dry weight or mg protein per min.

5. Measurement of K^+- or Na^+-uptake.

Assay mixture (total 12 ml, at 25°C) contained 0.35 M mannitol and intact cells (1.5 mg dry weight) or protoplasts (4 mg protein). pH was adjusted with 0.1 % NH_4OH. By the addition of salt (final 10 mM), assay was started. At intervals (10 sec), an aliguot of 500 μl was sampled out and filtrated through membrane filter (pore size 0.45 μm, washed repeatedly with 0.33 N HNO_3 and 0.35 M mannitol). The filter was washed four times with total 10 ml of 0.35 M mannitol (at 25°C), suspended in 5 ml of 0.33 N HNO_3, and kept at room temperature for overnight. Concentration of K^+ or Na^+ in the 0.33 N HNO_3 was determined with flame photometer using standard solution, and expressed as ppm per mg dry weight or mg protein.

Dry weight of intact cells was determined with the cell suspension in which mannitol was replaced with water by centrifugation (1,000 rpm, 3 min). The cell suspension (0.5 ml) was dried at 105°C overnight, cooled to room temperature for 30 min in dessicator, and weighed.

Protein concentration of protoplasts suspension was determined according to the method of Lowry et al. (LOWRY, T. H. et al. 1951) using bovine serum albumin as a standard.

7. Chemicals

Monensin was purchased from Calbiochem-Behring. Valinomycin and nigericin were from Sigma. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone) was from Boehringer Mannheim. Other used reagents were the best grade commercially obtainable.

Results and Discussion

1. Cation stimulated H^+-release activity.

In the stimulation for opening of guard cells of stoma, H^+-release is observed in accompanied with the uptake of K^+ into the cells (SHIMAZAKI, K. et al. 1986). According to stimuli (for example, blue light) cytoplasmic membrane ATPase generates electrochemical gradient of H^+ using the chemical energy of ATP (SZE, H. 1985). K^+ enters into the cells through K^+-specific channels down the electrochemical gradient of H^+ ($\Delta\bar{\mu}$ H^+ in chemiosmotic theory). At first we measured cation stimulated H^+-release activity using intact cells and protoplasts of *Datura innoxia* suspension culture. As shown in Table 1, of cations tested, Ca^{2+}, K^+ and Na^+ were stimulative on H^+-release (decrease of medium pH) from intact cells and protoplasts. Minus values of intact cells meant that pH of the assay mixture increased by the addition of these salts compared with their controls. Optimal pH of K^+- or Na^+-stimulated H^+-release was pH 7.5 both in intact cells (Fig. 1a) and protoplasts (Fig. 1b).

As this study was focused on K^+ and Na^+, some properties of their uptake systems were then examined.

2. Effect of pH on K^+-or Na^+-uptake activity.

As K^+- or Na^+-stimulated H^+-release activity (whose optimal pH was 7.5) was observed, the effect of pH on K^+- or Na^+-uptake activity of intact cells and protoplasts was examined. Although intact cells had wider active range of pH than protoplasts, optimal pH for their uptake activities were pH 7.5 (Fig. 2). Although the ATPase activity was not examined in this study, K^+-stimulated H^+-release activity and K^+-uptake activity coincided well to above K^+-transport system.

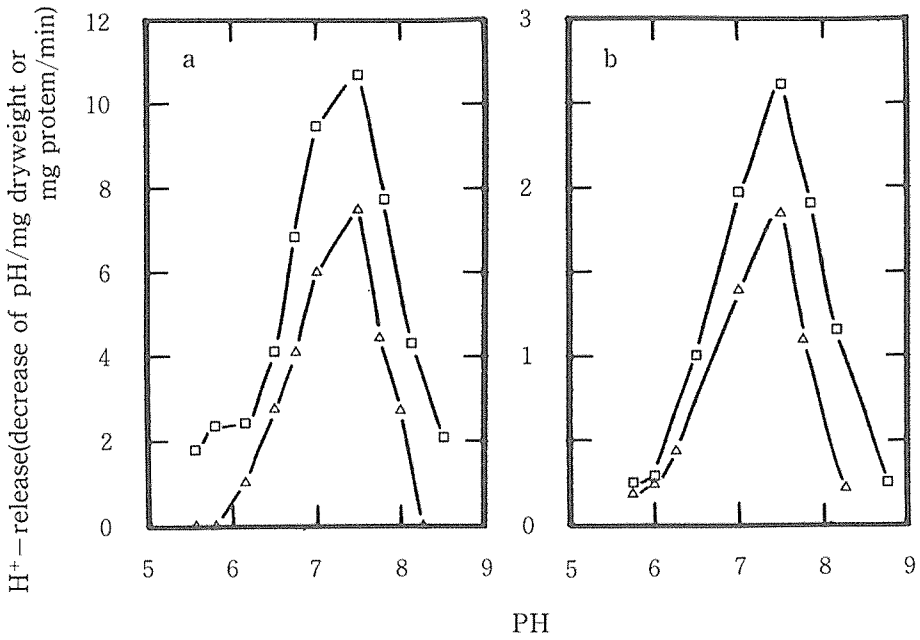

Fig. 1 Effect of pH on K^+- or Na^+-stimulated H^+-release activity of intact cells (a) and protoplasts (b). Initial pH of the assay mixture was adjusted to indicated pH with 0.1 % NH_4OH. KCl(□) or NaCl(△) was added to the mixture to final 10 mM. Other condition was the same as that of Table 1.

3. Effect of ionophores on K^+- or Na^+-uptake activity.

To know whether K^+ and Na^+ were transported actively or passively, effect of various ionophores on these activities was examined. In the presence of some ionophores, these activities were decreased (Table 2). For K^+-uptake activity, valinomycin (K^+-ionophore) and nigericin (K^+/H^+-ionophore) were most effective. FCCP (H^+-ionophore) was less effective. These inhibition pattern of K^+-uptake activity also suggested that K^+ was actively transported via K^+-specific channel or K^+/H^+-antiporter. Ionophore inhibition pattern of Na^+-uptake activity was more complicated than that of K^+, because Na^+-ionophore (monensin) was less inhibitory than K^+- and/or H^+-ionophore (nigericin, valinomycin and FCCP). H^+-gradient and/or K^+-gradient might be involved in the Na^+-uptake system. Na^+ is less important cation for *Datura innoxia* cells than in the case of halophytes. So, in the presence of enough amunt of K^+, Na^+ might be released from the cells (via K^+-system ?).

4. Relationship between K^+- and Na^+-uptake activity

In the previous section, Na^+-uptake activity was

Table 1 Effect of various cations on H^+-release from intact cells and protoplasts of *Datura innoxia*.

salts	relative activity (%) intact cels	protoplasts
KCl	100	100
NaCl	70.1	71.2
$CaCl_2$	130.1	198.5
$BaCl_2$	44.1	105.3
$MgCl_2$	11.1	35.9
$ZnCl_2$	−17.5	21.4
$CuCl_2$	−17.5	9.2
$MnCl_2$	−66.7	93.1

Assay mixture (total 12 ml, at 25°C) coxtained 0.35 M mannitol, 1.5mg dry weight of intact cells or 4mg protein of protoplasts. Their initial pH was adjusted to pH7.5 with 0.1% NH_4OH. Salts were added to the mixture to final 10mM (as a concentration of cation). 100% for intact cells was 11.4pH/mg dry weight/min and for protoplast was 2.6pH/mg protein/min.

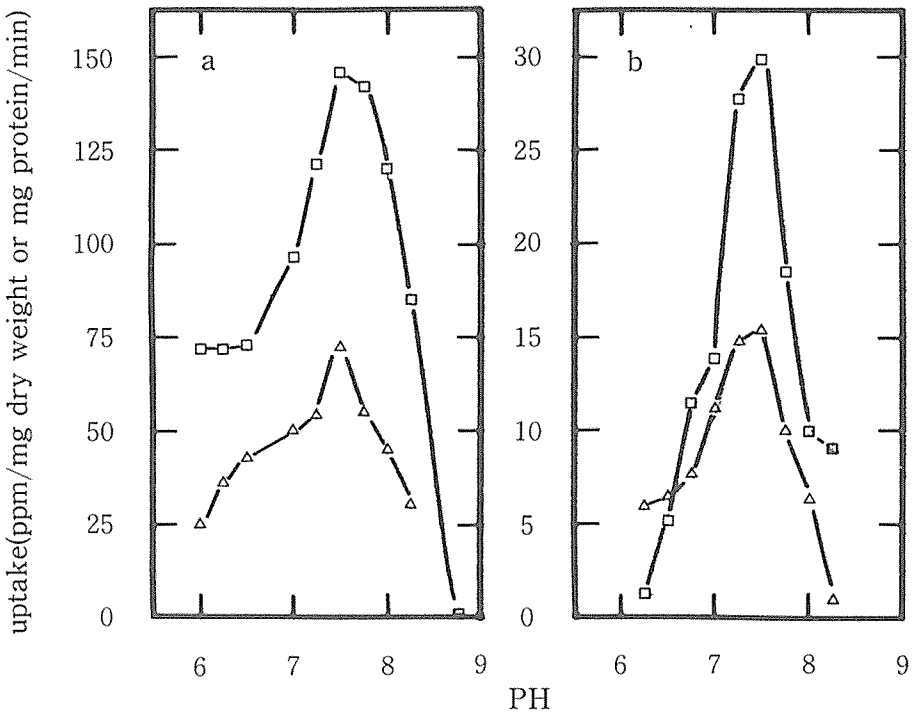

Fig. 2　Effect of pH on K^+- or Na^+-uptake activity of intact cells and protoplasts.
Assay mixture (total 5 ml, at 25°C) contained 0.35 M mannitol and intact cells (a, 1.5 mg dry weight) or protoplasts (b, 4 mg protein). pH was adjusted to indicated value with 0.1 % NH_4OH. By the addition of KCl(□) or NaCl(△) to 10 mM, the assay was started. At indicated time, 500 μl was sampled out and filtrated through membrane filter (pore size 0.45 μm). K^+ or Na^+ concentration on the filter was determined as described in Materials and methods.

inhibited by K^+-ionophore rather than Na^+-ionophore. This suggested the involvement of K^+-gradient on Na^+-uptake system. K^+- or Na^+-uptake activity was measured in the presence of the same concentration of other cation (10 mM, Na^+ or K^+). K^+-uptape (or Na^+-uptake) experiment was started as usual, at 35 seconds later, Na^+ (or K^+) was added, and the uptake assay was continued. Na^+-addition was not effective on K^+-uptake activity (Fig. 3). While, Na^+-uptake activity was abolished by the halfway addition of K^+, and transported Na^+ was released from the protoplasts (Fig. 3).

5. Effect of anions and osmotic pressure on K^+-uptake activity.

The effect of anions on K^+-uptake activity was examined (Table 3). There were some discrepancies in the activities between intact cells and protoplasts (as in the case of Table 1). The order of effectiveness of anions in the K^+-uptake as a counter anion was $HCO_3^- \geqq NO_3^- > BrO_3^- \geqq Cl^- > HPO_4^{2-} = SO_4^{2-}$. That was essentially the same as those anions in dissipating the

Table 2　Effect of various ionophores on K^+- or Na^+- uptake activity of protoplasts.

ionophores	residual activity (%)	
	K^+	Na^+
none	100	100
valinomycin	55.0	47.4
monensin	95.6	80.7
nigericin	69.5	62.8
FCCP	83.8	60.9

Ionophores were added to final 1.0 %. 100 % for K^+-uptake was 39ppm/mg protein/min and for Na^+- uptake was 10ppm/mg protein/min.

electrical potential generated by H^+-ATPase and K^+-transport ($SCN^- > NO_3^- > Cl^-$, Br^-, I^-; SZE, H. 1985), and those of anions in relative rate of dissipation (relative permeabilities of anions; $SCN^- > NO_3^- = Cl^-$, $Br^- > SO_4^{2-} = HPO_4^{2-}$) in tonoplast membrane vesicles (KAESTMER, K. H. & SZE, H. 1987).

6. Effect of osmotic pressure on K^+-uptake activity.

It is known that K^+ is transported into guard cells to regulate or maintain their turgor pressure (SCHNABL, H. & RASCHKE, K. 1980). When the concentration of osmotic stabilizer (mannitol) was increased from 0.1 to 0.8 M, K^+-uptake activity of intact cells was constant from 0.1 to 0.35 M. Above 0.4 M, the activity increased and attained to plateau above 0.6 M (Fig. 4).

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Table 3 Effect of various anions on K^+-uptake.

salts	relative activity (%) tntact cells protoplasts K^+	Na^+
KCl	100	100
$KHCO_3$	137.8	159.7
KNO_3	176.1	133.3
$KBrO_3$	26.6	119.4
K_2HPO_4	− 1.1	93.1
K_2SO_4	−41.1	91.7
$K_3C_6H_5O_7$ H_2O	40.0	137.5
$K_2C_4H_4O_6$ 1/2 H_2O	−62.2	56.9
$K_2B_4O_7$ $5H_2O$	−42.2	23.6

Salt was added to 10mM by the concentration of K^+. 100% for intact cells was 155ppm/mg dry weight/min, and that for protoplasts was 31ppm/mg protein/min (at pH 7.5). Other experimental condition was similar to that of Fig. 2.

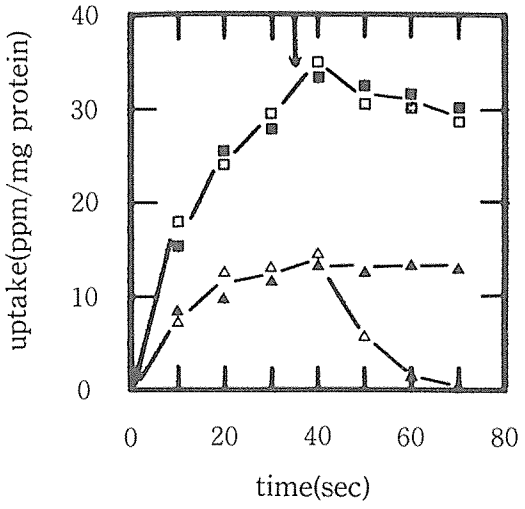

Fig. 3 Effect of Na^+ (or K^+) on K^+-uptake (or Na^+-uptake) activity of protoplasts.

Assay mixture were similar to that of Fig. 2, except that at 35 sec 10 mM NaCl was added to K^+-uptake mixture(□) or 10 mM KCl was added to Na^+-kptake mixture(△). Closed symbols were control without addition at 35 sec.

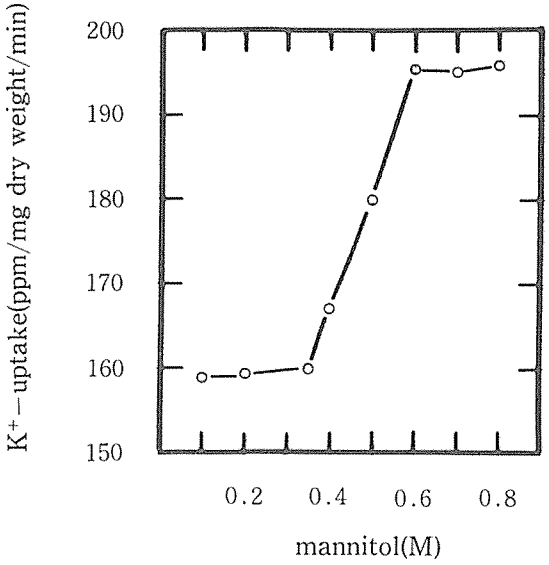

Fig. 4 Effect of osmotic pressure on K^+-uptake activity by intact cells.

Assay mixture (total 5 ml, at 25°C, pH 7.5) contained 0.1-0.8 M mannitol, 1.5 mg dry weight of intact cells, and 10 mM KCl. Other condition was similar to that of Fig. 2.

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Datura innoxia 懸濁培養における K^+-および Na^+-取り込み活性

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摘　要

Datura innoxia の懸濁培養細胞の細胞とプロトプラストを用いて，K^+ と Na^+ の取り込み活性について調べた結果，以下の事が明らかとなった．

K^+ もしくは Na^+ によって促進される H^+ 放出活性および K^+ もしくは Na^+ とりこみ活性の至適 pH は 7.5 であった．

K^+ とりこみ活性は K^+ イオノホアにより阻害された．

一方，Na^+ とりこみ活性は K^+ および H^+ イオノホアにより阻害された．

K^+ とりこみ活性測定時に途中で Na^+ を添加しても活性に変化やかったが，Na^+ とりこみ活性測定時に K^+ を同様に添加すると Na^+ が放出された．

調べたアニオンのうち，HCO_3^-，NO_3^-，および Cl^- が K^+ とりこみ活性に対して，より促進的であった．このとりこみ活性は浸透圧感受性であった．