Response of *Rhizobium fredii* P220 to osmotic shock: interrelationships between K^+ , Mg^{2+} , glutamate and homospermidine

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The response of *Rhizobium fredii* P220, a salt-tolerant strain of soybean rhizobia, to osmotic shock was investigated by using non-growing washed cells. Rapid changes in K^+ , Mg^{2+} , glutamate and homospermidine were observed in strain P220 cells subjected to sudden changes in the osmolarity of incubation buffer. Osmotic upshock resulted in elevation of cellular K^+ and glutamate, and reduction in cellular homospermidine and Mg^{2+} . When the cells were transferred to upshock buffer lacking K^+ , the reduction in Mg^{2+} was totally blocked, but the elevation of glutamate and the reduction in homospermidine were only partially repressed. Osmotic downshock resulted in the opposite phenomenon: there was an elevation of homospermidine and Mg^{2+} , and a rapid fall of K^+ and glutamate. When the cells were transferred to downshock buffer lacking Mg^{2+} , the elevation of homospermidine was partially repressed, but the decrease in K^+ and glutamate was not repressed at all. Lowering of the cellular K^+ by treatment with ionophores nigericin and monensin resulted in a slight decrease in glutamate and a slight increase in homospermidine and Mg^{2+} , possibly due to a pH effect caused by the K^+ – H^+ exchange. Raising the cellular Mg^{2+} content by treatment with ionophore A23187 brought about an increase in homospermidine. The homospermidine content of Mg^{2+} -deficient cells grown with low- Mg^{2+} medium reduced to 35% of those grown with the basal medium. These results indicate that in *R. fredii*, K^+ strictly controls Mg^{2+} flux during osmotic shock whereas the reverse is not true, and that glutamate and homospermidine essentially escape direct control by K^+ . We also suggest that Mg^{2+} , which has no effect on the pool size of glutamate, is one of the factors which regulate homospermidine content in rhizobial cells.

Keywords: *Rhizobium fredii*, soybean rhizobia, homospermidine, glutamate, osmotic shock

INTRODUCTION

The ability of soil micro-organisms to maintain their internal osmotic activity through regulation of their cellular component(s) is of profound functional significance because soil micro-organisms may be subjected to a wide range of environmental ionic strengths. The accumulation of these components, referred to as compatible solutes or osmoprotectants, contributes to the maintenance of turgor pressure and prevention of dehydration of the cells in hypertonic environments. It has

been proposed that many amino acids, polyols, betaines, K^+ etc., act as compatible solutes in various micro-organisms (Brown, 1990). As regards *Rhizobium* and *Bradyrhizobium*, agronomically important soil micro-organisms which form nitrogen-fixing root nodules on the host legumes, there are several reports indicating that glutamate and K^+ play a central role in osmoregulation. Bernard *et al.* (1986) suggested the involvement of glycine betaine in osmoregulation of *R. meliloti*, although Botsford (1984) noticed that added glycine betaine did not relieve the growth inhibition of *R. meliloti* under salt stress. Recently, we observed the accumulation of glutamate and K^+ under salt-stressed conditions in *R. fredii*, a new group of fast-growing rhizobia capable of producing

Abbreviation: homospd, homospermidine.

effective nodules on a wild soybean (Fujihara & Yoneyama, 1993). In *R. fredii* P220, a highly salt-tolerant strain, we also found that Mg^{2+} and homospermidine (homospd), a major polyamine in *Rhizobium*, might be closely associated with osmoregulation, since the cellular levels of Mg^{2+} and homospd were also regulated critically in response to the external medium osmolarity.

Homospd, an analogue of triamine spermidine, is an organic polycation detected ubiquitously in the soil environment (Fujihara & Harada, 1989a) and its occurrence has been demonstrated in a wide variety of micro-organisms (Tait, 1985). We reported earlier that all the rhizobia and bradyrhizobia tested contained homospd as a major polyamine, occupying over 80% of total polyamines under normal growth conditions (Fujihara & Harada, 1989b), although the functional role of this triamine in micro-organisms remained undetermined. To ascertain whether the cellular homospd content in rhizobia is controlled by a mechanism related to osmoregulation, we carried out a short-term experiment in which non-growing cells of strain P220 were subjected to a rapid change in external buffer osmolarity. We also investigated the interrelationships between K^+ , Mg^{2+} , glutamate and homospd by using ionophores which have high selectivity for inorganic monovalent or divalent cations and transport them through bacterial cell membranes.

METHODS

Bacterial strain and growth condition. *R. fredii* P220, a fast-growing strain originally isolated from Panama (Hernandez & Focht, 1984), was obtained from the Laboratory of Soil General Microbiology (National Institute of Agro-Environmental Sciences, Tsukuba, Japan), and maintained on yeast/mannitol agar slopes at 4 °C. Strain P220 cells were grown in a basal medium (pH 6.8) containing 0.06% yeast extract (Difco), 0.05% K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, and 0.5% sucrose (all w/v). Cells in the early stationary phase were inoculated in 1 l Ehrlenmeyer flasks containing 200 ml of a basal medium with or without addition of 0.3 M NaCl, constantly shaken at 30 °C, and harvested by centrifugation at the early stationary phase of growth. Cell growth was monitored by periodic reading of OD_{660} with a Shimadzu UV-160 spectrophotometer. Viable cell counts were made by recording the number of colonies formed on yeast-mannitol agar plate after dilution of the cultured cells with distilled water.

Osmotic upshock and downshock experiment. The cell peller harvested from the basal medium without addition of NaCl was suspended in 10 mM HEPES buffer containing 0.1 mM EDTA, pH 7.0 (basal HEPES buffer), and centrifuged at 8000 g for 10 min at room temperature. This washing was repeated twice to remove nutrients around the cells. The washed cells were then transferred into the buffer which consisted of basal HEPES buffer, 1 mM $MgSO_4$, 1 mM KCl, and 0.6 M sucrose, which will be referred to as 'upshock' buffer. The cells harvested from the basal medium containing 0.3 M NaCl were washed twice with the basal HEPES buffer containing 0.6 M sucrose, and transferred into the buffer which consisted of basal HEPES buffer, 1 mM $MgSO_4$, and 1 mM KCl, which will be referred to as 'downshock' buffer. After incubation at 30 °C with gentle shaking, the cells were harvested by centrifugation at 8000 g at 4 °C, and washed twice with an ice-cold basal HEPES buffer isotonic to the incubation buffer. The resulting cell pellet was

divided into two portions. An aliquot of the cell pellet was immediately subjected to acid extraction for the analysis of cellular components. Another aliquot of the pellet was resuspended in 0.1 M NaOH and served for protein estimation.

Ionophore treatment. Washed cells which had been grown with basal media containing 0.3 M NaCl were used in this experiment. These cells were suspended in a basal HEPES buffer containing 0.6 M sucrose and 1 mM $MgSO_4$, and incubated at 30 °C for 60 min in the presence or absence of each ionophore. Ionophores nigericin, monensin and A12387, dissolved in ethanol, were added to final concentrations of 2 μM , 50 μM , and 10 μM , respectively. These experiments were done in triplicate.

Analyses. Polyamines were extracted from the cells with 0.5 M perchloric acid and derivatized with benzoyl chloride as described by Flores & Galston (1982). Separation and quantification of polyamine derivatives were carried out using a Shimadzu LC-9A HPLC equipped with a UV detector under the following conditions: column, Shim-pack CLC-ODS (6 $\times$ 150 mm); column temperature, 45 °C; mobile phase, 64% (v/v) methanol solution; liquid flow rate, 0.8 ml min⁻¹; detection, 254 nm. Diaminohexane was used as an internal standard for quantitative analysis of polyamines. For the analysis of extracellular polyamines, the supernatant obtained from the cell suspension after incubation was made alkaline (pH > 11) with the addition of solid KOH, and saturated with Na_2SO_4 . Polyamines were extracted from the solution with n-butanol by mechanical shaking. After evaporation of n-butanol, the residue was dissolved in 0.5 M perchloric acid and subjected to polyamine analysis as above. Determination of K^+ and Mg^{2+} in acid extract was performed by an atomic absorption spectrophotometer (Shimadzu AA-670). Cellular glutamate was colorimetrically determined in combination with glutamate dehydrogenase and diaphorase as described in detail by Beutler (1985). Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin as a standard.

Chemicals. Homospd and aminobutylhomospd (aminobutyl-homospd) were chemically synthesized according to the method described by Okada *et al.* (1979). Putrescine, diaminohexane, benzoylchloride and standard solutions of K^+ , Mg^{2+} and Na^+ were purchased from Wako Pure Chemical Industries. The glutamate assay kit was a product of Boehringer Mannheim GmbH. Dye reagent for protein assay was obtained from Bio-Rad. Ionophores nigericin, monensin and A23187 were purchased from Sigma. All other organic and inorganic reagents used were of analytical grade.

RESULTS

Effects of osmotic upshock on cellular contents of K^+ , Mg^{2+} , glutamate and polyamines

Washed cells of *R. fredii* P220 which had been grown in basal medium were transferred into 10 mM HEPES buffer (pH 7.0) with or without addition of 0.6 M sucrose, and the cellular contents of K^+ , Mg^{2+} , glutamate and homospd were determined (Fig. 1). The total protein content of the cells remained constant during the 180 min incubation period, and no growth was observed in the HEPES buffer as the OD_{660} of the suspension remained constant. Transfer of the cells to the upshock buffer resulted in a rapid increase in cellular K^+ and glutamate, which reached a maximum within 30 min of upshock treatment. The pool size of glutamate in *Rhizobium* species, when grown

Fig. 1. Changes of K^+ , Mg^{2+} , glutamate and polyamine contents in *R. fredii* P220 cells after osmotic upshock. Cells, grown in basal medium and washed twice with basal HEPES buffer, were transferred into the upshock buffer, which consisted of basal HEPES buffer, 1 mM KCl, 1 mM $MgSO_4$ and 0.6 M sucrose, and incubated at 30 °C. After washing with ice-cold HEPES buffer, isotonic to the incubation buffer without addition of KCl and $MgSO_4$, the cells were analysed for K^+ (a), Mg^{2+} (b), glutamate (c), and polyamines (d) as described in Methods. Open symbols, cells incubated in upshock buffer. Filled symbols, cells incubated in downshock buffer. In (d), $\circ$ and $\bullet$ represent homospd, and $\square$ and $\blacksquare$ represent putrescine. Data are means of duplicate experiments.

Table 1. Effect of K^+ removal from the upshock buffer

R. fredii P220 cells grown in basal medium were transferred into the low-osmolarity or high-osmolarity (upshock) buffer as shown in Fig. 1 with or without addition of 1 mM KCl, and incubated at 30 °C for 120 min. After washing the cells, the contents of K^+ [$\mu\text{mol (mg protein)}^{-1}$] and of Mg^{2+} , glutamate and homospd [all nmol (mg protein) $^{-1}$] were determined. The results are means $\pm$ SD from three incubations.

Incubation buffer	K^+	Mg^{2+}	Glutamate	Homospd
Low osmolarity	0.84 ± 0.06	168.2 ± 3.9	42.8 ± 16.3	157.9 ± 2.1
Upshock	1.60 ± 0.09	54.7 ± 3.6	613.2 ± 65.9	100.9 ± 3.7
Upshock $-K^+$	0.97 ± 0.01	178.3 ± 7.5	299.8 ± 32.6	135.9 ± 2.9

in normal yeast media, is generally very small (Hua *et al.*, 1982; Yelton *et al.*, 1983). In the present study, the glutamate content of *R. fredii* P220 cells grown in the basal medium did not exceed 50 nmol (mg protein) $^{-1}$. During the first 30 min of incubation in the upshock buffer, the pool size of glutamate in strain P220 increased approximately 20-fold (Fig. 1). In contrast to the increase in the cellular K^+ and glutamate, cellular Mg^{2+} and

homospd contents decreased after osmotic upshock. Although the changes in Mg^{2+} and homospd content were not as rapid as those of K^+ and glutamate, the phenomena were reproducibly observed regardless of the sort of osmotic agents added (see below). As for putrescine, a polyamine generally known to rapidly fluctuate in response to various abiotic stresses or stimuli (Slocum *et al.*, 1984), its cellular content remained at less

Fig. 2. Effects of nigericin and monensin on cellular K⁺, Mg²⁺, glutamate and homospd contents. Strain P220 cells were grown with basal medium containing 0.3 M NaCl, washed and suspended in a buffer which consisted of basal HEPES buffer, 0.6 M sucrose and 1 mM MgSO₄, pH 7.0, and incubated at 30 °C for 60 min in the presence or absence of the ionophores nigericin and monensin, used at final concentrations of 2 µM and 50 µM, respectively. Data are means ± SD from triplicate incubation samples, expressed as a percentage of the values without addition of ionophore. The mean values without ionophores were: K⁺, 1.77 µmol (mg protein)⁻¹; Mg²⁺, 83.3 nmol (mg protein)⁻¹; glutamate, 576.6 nmol (mg protein)⁻¹; homospd, 65.5 nmol (mg protein)⁻¹.

than 5 nmol (mg protein)⁻¹ during the 180-min incubation. Similarly, the cellular content of aminobutyl-homospd, a tetraamine detected exclusively in fast-growing type of rhizobia (Fujihara & Harada, 1989b), was not significantly different (data not shown). Since the upshock buffer contained no detectable polyamines after cell incubation, the decrease in cellular homospd following upshock is not due to the excretion of homospd from the cells.

In order to ascertain whether the rapid uptake of K⁺ in response to the osmotic upshock affects the cellular levels of glutamate, Mg²⁺ and homospd, the washed cells were transferred to the upshock buffer lacking K⁺ (Table 1). Removal of K⁺ from the upshock buffer resulted in a complete block of Mg²⁺ efflux from the cells under high osmolarity, indicating a strict control of Mg²⁺ flux by K⁺. On the other hand, the decrease in cellular homospd and glutamate accumulation under upshock were not completely blocked by the K⁺ removal, implying that cellular glutamate and homospd were not under the strict control of K⁺. Similar results were obtained in the cells treated with NaCl rather than sucrose as an osmotic agent, although the accumulation rate of K⁺ and glutamate in NaCl-upshock buffer was somewhat slow when compared with that in the sucrose-upshock buffer (data not shown).

Nigericin and monensin are ionophores that transport specific monovalent cations through bacterial cell membranes (Henderson *et al.*, 1969). To ascertain the influence of K⁺ on the cellular glutamate, Mg²⁺, and homospd in *R. fredii* P220, the effects of the addition of these ionophores

under the high osmotic condition were examined. As shown in Fig. 2, both agents lowered the cellular level of K⁺ by 15–20% under high osmolarity. The reduction of the cellular K⁺ was accompanied by a 22% decrease in glutamate, and an 25% and 19% increase in Mg²⁺ and homospd, respectively. Among the ionophores tested, valinomycin, a carrier of monovalent cations, showed no effect on cellular cation content (data not shown), possibly due to the impermeability of valinomycin or valinomycin-cation complex in rhizobial cell membranes.

Effects of osmotic downshock on cellular contents of K⁺, Mg²⁺, glutamate and polyamines

Washed cells of strain P220, grown in the basal medium containing 0.3 M NaCl, were transferred into the 10 mM HEPES buffer with or without addition of 0.6 M sucrose. The results of the downshock experiment were the opposite to those of the upshock experiment (Fig. 3). After the downshock, cellular K⁺ readily decreased and nearly all the glutamate, accumulated in the cells under high osmolarity, disappeared within 30 min. By contrast, cellular levels of Mg²⁺ and homospd increased immediately after the downshock, and levelled off after 60 min. A temporal accumulation of cellular putrescine was also observed. The putrescine content attained 29 nmol (mg protein)⁻¹ after 30 min, but rapidly decreased to the initial low level. Aminobutylhomospd content was very low, and showed no change by downshock (data not shown).

To determine whether Mg²⁺ uptake during the downshock is linked to the changes in other components, strain P220 cells grown in the basal medium containing 0.3 M NaCl were transferred to the downshock buffer lacking Mg²⁺ (Table 2). Both rapid excretion of K⁺ and a precipitous decrease in cellular glutamate due to the downshock occurred even though Mg²⁺ was not incorporated within the cells. The accumulation of homospd in response to the downshock was, however, partially repressed when Mg²⁺ was removed from the downshock buffer. The influence of Mg²⁺ influx on homospd content was further examined in the following experiment (Fig. 4). Under the high osmotic conditions, in which the contents of cellular Mg²⁺ and homospd remained low, as shown in Fig. 3, ionophore A23187 was added together with 1 mM MgSO₄ to the incubation buffer. Ionophore A23187 is an ion carrier showing especially high affinity for divalent cations such as Mg²⁺ and Ca²⁺ (Reed & Lardy, 1972). A23187 at 10 µM stimulated the incorporation of Mg²⁺ into the rhizobial cells under high osmolarity. After 60 min of incubation with A23187, the cellular Mg²⁺ level increased by approximately 44% as compared with that in non-treated cells. A23187 treatment did not change the cellular K⁺ and glutamate content, but increased the cellular homospd content by approximately 21%. These results imply that Mg²⁺ has no effect on the K⁺ flux and the pool size of glutamate during osmotic shock, but does affect the pool size of homospd. Ca²⁺ was found to be ineffective for homospd accumulation unlike Mg²⁺, because the homospd content was not affected even though the

Fig. 3. Changes of K^+ , Mg^{2+} , glutamate and polyamine contents in *R. fredii* P220 cells after osmotic downshock. Cells, grown in basal media containing 0.3 M NaCl and washed twice with basal HEPES buffer containing 0.6 M sucrose, were transferred into the downshock buffer which consisted of basal HEPES buffer, 1 mM KCl and 1 mM $MgSO_4$, and incubated at 30 °C. After washing with ice-cold HEPES buffer, isotonic to the incubation buffer without adding KCl and $MgSO_4$, the cells were analysed for K^+ (a), Mg^{2+} (b), glutamate (c), and polyamines (d) as described in Methods. Open symbols, cells incubated in upshock buffer. Filled symbols, cells incubated in downshock buffer. In (d), ○ and ● represent homospd, and □ and ■ represent putrescine. Data are means of duplicate experiments.

Table 2. Effect of Mg^{2+} removal from the downshock buffer

R. fredii P220 cells grown in basal medium containing 0.3 M NaCl were transferred into the high-osmolarity or low-osmolarity (downshock) buffer as shown in Fig. 3 with or without addition of 1 mM $MgSO_4$, and incubated at 30 °C for 120 min. After washing the cells, the contents of K^+ [$\mu\text{mol (mg protein)}^{-1}$] and of Mg^{2+} , glutamate and homospd [all nmol (mg protein) $^{-1}$] were determined. The results are means $\pm$ SD from three incubations.

Incubation buffer	K^+	Mg^{2+}	Glutamate	Homospd
High osmolarity	1.47 ± 0.12	58.1 ± 3.8	578.7 ± 16.3	45.3 ± 5.2
Downshock	0.66 ± 0.06	238.4 ± 9.6	12.2 ± 10.2	90.5 ± 1.7
Downshock – Mg^{2+}	0.57 ± 0.09	62.5 ± 3.3	18.4 ± 9.5	74.5 ± 1.2

cellular level of Ca^{2+} increased with the addition of A23187 together with 1 mM $CaCl_2$ (data not shown).

Polyamine content and viability of the cells grown under Mg^{2+} deficient conditions

To examine the influence of Mg^{2+} on cellular polyamine content, strain P220 cells were grown in the basal media with or without addition of magnesium (Table 3). The concentration of Mg^{2+} was 0.03 mM in the low- Mg^{2+}

medium, where yeast extract is the sole source of Mg^{2+} , while that in the basal medium was 0.66 mM. The Mg^{2+} content of the cells grown with the low- Mg^{2+} medium was 37% of those grown with the basal medium. The homospd content of the Mg^{2+} -deficient cells was found to be 35% of those grown with the basal medium. No significant differences were seen between two cultures, either in OD_{660} or in the total protein of the cells recovered from the culture medium (Table 3). However, the Mg^{2+} -deficient cells excreted considerable amounts of

Fig. 4. Effects of A23187 on the cellular K⁺, Mg²⁺, glutamate and homospd contents. Strain P220 cells were grown with basal media containing 0.3 M NaCl, washed and suspended in a buffer which consisted of basal HEPES buffer, 0.6 M sucrose and 1 mM MgSO₄, pH 7.0, and incubated at 30 °C for 60 min in the presence or absence of ionophore. Ionophore A23187 was used at a final concentration of 10 µM. Data are means ± SD from triplicate incubation samples, expressed as a percentage of the values without addition of ionophore. The mean values without A23187 were: K⁺, 1.52 µmol (mg protein)⁻¹; Mg²⁺, 80.5 nmol (mg protein)⁻¹; glutamate, 463.5 nmol (mg protein)⁻¹; homospd, 61.6 nmol (mg protein)⁻¹.

Table 3. Polyamine content and viability of *R. fredii* P220 cells grown with low-Mg²⁺ medium

R. fredii P220 cells were inoculated into the basal yeast medium or the low-Mg²⁺ medium in which MgSO₄ was not added, and grown for 65 h at 30 °C. After the measurement of OD₆₆₀, an aliquot of the culture was diluted stepwise with distilled water, plated on the yeast/mannitol agar, and the numbers of the colonies formed on the plates were scored after 3–4 d incubation. Another aliquot of the culture was used for the analysis of cellular polyamines, Mg²⁺, K⁺ and protein. The Mg²⁺ concentrations of basal and low-Mg²⁺ medium were about 0.66 mM and 0.03 mM, respectively. Values are means ± SD from three cultures.

Property	Basal medium	Low-Mg ²⁺ medium
OD ₆₆₀	0.84	0.87
Cell protein [mg (ml medium) ⁻¹]	0.13 ± 0.01	0.14 ± 0.01
10 ⁻⁹ × Viable cells (ml medium) ⁻¹	1.91 ± 0.15	0.20 ± 0.03
Putrescine [nmol (mg protein) ⁻¹]	1.9 ± 0.5	2.0 ± 0.2
Homospd [nmol (mg protein) ⁻¹]	164.4 ± 5.2	57.0 ± 2.6
Aminobutylhomospd [nmol (mg protein) ⁻¹]	9.9 ± 0.4	3.3 ± 0.3
K ⁺ [µmol (mg protein) ⁻¹]	1.17 ± 0.11	0.87 ± 0.05
Mg ²⁺ [nmol (mg protein) ⁻¹]	116.2 ± 8.9	42.8 ± 7.1

slime materials as evidenced by the highly viscous culture medium after 65 h culture. The numbers of colony-forming cells on agar plates were examined for cells from basal and low-Mg²⁺ cultures after successive dilution with distilled water. Interestingly, fewer viable cells resulted from the low-Mg²⁺ culture than from the basal culture. If the cells from each culture were diluted with physiological saline (0.9 % NaCl), no significant differences in the cell numbers on agar plate were observed between the two cultures. This suggested that the cells from the low-Mg²⁺ culture were largely collapsed during dilution with distilled water. It appears that Mg²⁺- and homospd-deficient cells might have lost cell membranes or envelopes, and were therefore very fragile under low osmotic conditions. The slime formation in Mg²⁺- and homospd-deficient cells may be associated with an adaptive mechanism of these cells to preserve structural integrity in external low osmolality.

DISCUSSION

We previously reported that cellular levels of homospd and Mg²⁺ in growing *R. fredii* P220 were significantly affected by the external salinity (Fujihara & Yoneyama, 1993). The response of non-growing cells of strain P220 to the short-term downshock or upshock in the present experiment (Figs 1 and 3) implies that homospd and Mg²⁺, together with K⁺ and glutamate, are the cellular components closely associated with osmoregulation in *R. fredii*. The results obtained here provide further implications as to their possible interrelationships.

There is strong evidence that K⁺ plays a major role in bacterial osmoregulation, as well as in maintaining the structure of ribosomes (Epstein, 1986). It has also been established that K⁺ uptake under salt stress accompanies the accumulation of cellular glutamate as the counter-anion in several bacterial species, including *Rhizobium* (Tempest & Meers, 1970; Measures, 1975; Hua *et al.*, 1982; Yelton *et al.*, 1983; Cayley *et al.*, 1989). Measures (1975) reported that high concentrations of K⁺ enhanced the enzyme activity of glutamate dehydrogenase in cell-free systems prepared from several bacterial species, implying a possible relationship between K⁺ influx and enlargement of the glutamate pool size during osmotic upshock. The present data, however, do not support the idea that K⁺ influx is the cause of the glutamate accumulation within the cells, since there was still a substantial (sevenfold) increase in the glutamate pool even though K⁺ influx was completely blocked under high osmolality (Table 1). Hua *et al.* (1982) reported no stimulative effect of K⁺ on glutamate dehydrogenase activity in several *Rhizobium* species. Thus, it appears that in *R. fredii* cellular glutamate essentially escapes K⁺ control and there might be other mechanisms regulating the pool size of glutamate in response to osmotic change.

The decrease in the cellular glutamate caused by the treatment with ionophores nigericin and monensin (Fig. 2) might be related to a pH side-effect, since both these ionophores carry monovalent cations in exchange for H⁺ through bacterial membranes (Henderson *et al.*, 1969).

The optimum pH for glutamate dehydrogenase in the direction of glutamate formation is reported to be 7.7 in *Nitrosomonas europaea*, and the reaction rate of this enzyme declines sharply below pH 7 (Hooper *et al.*, 1967). It is possible that a rapid change in the intracellular pH severely affects the pool size of glutamate. Many transport systems involve H^+ as a coupling ion and there is evidence for a pump that effects the exchange of K^+ for H^+ (Epstein & Schultz, 1965). In *Escherichia coli*, osmotic upshift results in an increase of cytoplasmic pH (Castle *et al.*, 1986). Kroll & Booth (1981) confirmed that K^+ fluxes play a significant role in the regulation of internal pH. Efflux of K^+ out of the cells by downshock or by ionophore treatment in the present experiment might, therefore, lower the intracellular pH. In plants and microorganisms, elevation of cellular H^+ generally induces a rapid increase in cellular polyamines (Slocum *et al.*, 1984). Indeed, we previously showed that both homospd and Mg^{2+} in *R. fredii* P220 cells increased with lowering of the medium pH (Fujihara & Yoneyama, 1993). In the light of these observations, the K^+ effect on cellular Mg^{2+} and homospd as well as glutamate might be associated with the change in the intracellular H^+ concentrations caused by the K^+-H^+ exchange during osmotic shock. It is plausible that homospd, a highly alkaline compound, has a functional role in maintaining the intracellular pH or restoring the ionic imbalance caused by the rapid transport of K^+ .

There are reports on the effect of K^+ on polyamine metabolism. High concentrations of K^+ are known to repress ornithine decarboxylase in *E. coli* (Rubenstein *et al.*, 1972) and arginine decarboxylase in *Vibrio parahaemolyticus* (Yamamoto *et al.*, 1989), which are the enzymes associated with putrescine formation. Munro *et al.* (1972) reported a close relationship between K^+ uptake and putrescine excretion during osmotic upshift in *E. coli*. In *R. fredii*, however, K^+ did not affect the cellular level of putrescine. The pool size of putrescine in rhizobial cells remained very small during osmotic shock (Figs 1 and 3), and excretion of this amine from the cells did not occur.

The removal of Mg^{2+} from downshock buffer partially repressed the elevation of cellular homospd (Table 2), and the increase in the cellular Mg^{2+} level induced by ionophore A23187 treatment resulted in an increase of homospd content, with no change in cellular K^+ and glutamate (Fig. 4). These data imply that Mg^{2+} , which has no effect on the pool size of glutamate and K^+ flux, is one of the factors affecting cellular homospd content. The observation that strain P220 cells grown with low- Mg^{2+} medium contained less homospd than those grown with basal medium (Table 3) might support the possible involvement of Mg^{2+} in homospd production. Since the removal of K^+ from upshock buffer completely blocked the Mg^{2+} efflux (Table 1), while the removal of Mg^{2+} from downshock buffer had no effect on the K^+ efflux (Table 2), it is apparent that Mg^{2+} flux is strictly controlled by K^+ . The weak effect of K^+ on the cellular homospd pool, therefore, might be partially through the change in the cellular Mg^{2+} pool caused by the K^+ flux. The requirement for Mg^{2+} in spermidine biosynthesis is well known in *E.*

coli (Tabor & Tabor, 1964), but little is known regarding the homospd biosynthesis. Recently, Böttcher *et al.* (1993) suggested a synthetic pathway for homospd in root cultures of *Senecio vulgaris*. According to their scheme, homospd synthase does not require metallic ions. Also, no stimulation by Mg^{2+} has been described in homospd synthase from grass pea (Srivenugopal & Adiga, 1980) or photosynthetic bacteria (Tait, 1979). Thus, whether the participation of Mg^{2+} in rhizobial homospd production is the promotion of the enzyme activities remains to be determined.

The significant decrease in cell viability, possibly due to the leakage of cellular inclusions or cell rupture by downshock, observed in the Mg^{2+} - and homospd-deficient cells (Table 3) may offer another aspect of the role of homospd in rhizobial cells. The possible involvement of polyamines in membrane or cell-wall stability has been inferred from many *in vitro* and *in vivo* studies (Peter *et al.*, 1978; Schuber, 1989). In addition to the function of homeostatic buffering, homospd, newly formed in response to the osmotic downshift, might have a protective function for rhizobial cells exposed to low osmotic environments. With respect to this protective function the localization of homospd in cells will be investigated further.

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