Uptake of glycine betaine and its analogues by bacteroids of *Rhizobium meliloti*

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Bacteroids isolated from alfalfa nodules induced by *Rhizobium meliloti* 102F34 transported glycine betaine at a constant rate for up to 30 min. Addition of sodium salts greatly increased the uptake activity, whereas other salts or non-electrolytes had less effect. The apparent K_m for glycine betaine uptake was $8.3 \mu\text{M}$ and V was about $0.84 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ in the presence of 200 mM-NaCl which gave maximum stimulation of the transport. Supplementing bacteroid suspensions with various energy-yielding substrates, or ATP, did not increase glycine betaine uptake rates. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the respiratory inhibitor potassium cyanide strongly inhibited glycine betaine uptake, but arsenate was totally inactive. Glycine betaine transport showed considerable structural specificity: choline, proline betaine, γ -butyrobetaine and trigonelline did not competitively inhibit the system, although choline and proline betaine were transported by bacteroids. Both a high-affinity activity and a low-affinity activity were found for choline uptake. These osmoprotective compounds might have a significant role in the maintenance of nitrogenase activity in bacteroids subjected to salt stress.

Introduction

Recent work has shown that *Rhizobium meliloti* is able to overcome growth inhibition caused by osmotic stress by uptake of osmoprotectants such as glycine betaine or proline betaine from the medium (Bernard *et al.*, 1986; Gloux & Le Rudulier, 1989). Both compounds are accumulated in osmotically stressed cells. In *R. meliloti*, choline also functions as an osmoprotectant, but only because it is oxidized to glycine betaine (Le Rudulier *et al.*, 1984a; Smith *et al.*, 1988). The transport of this compound is catalysed by three kinetically distinct transport systems: a high- and a low-affinity activity constitutively expressed, and a second high-affinity activity induced by choline (Pocard *et al.*, 1989).

Glycine betaine and proline betaine can partially restore the symbiotic nitrogen fixation ability of young nodulated alfalfa (*Medicago sativa*) subjected to salt stress (Pocard *et al.*, 1984). This suggests an important role for betaines as osmoprotective molecules in the nodules. Interestingly, *M. sativa* synthesizes proline betaine (Sethi & Carew, 1984) and we have recently

shown that proline betaine is also present in salt-stressed nodules of this plant (K. Gloux & D. Le Rudulier, unpublished). In addition, evidence has been obtained that phosphatidylcholine acts as a precursor of glycine betaine in other plants (Hitz *et al.*, 1981; Giddings & Hanson, 1982).

Despite the attention given to transport systems in bacteroids, to date there are no published studies characterizing betaines or choline transport activities in these symbiotic cells. This report describes glycine betaine uptake by bacteroids of *R. meliloti* under osmotic stress and in the presence of different energy-yielding substrates. Results of experiments investigating proline betaine and choline transport are also presented.

Methods

Organism and culture conditions. *Rhizobium meliloti* 102F34 was kindly supplied by R. C. Valentine (University of California, Davis, CA, USA). It was maintained on solid mannitol/salts/yeast extract (MSY) medium of O'Gara & Shanmugan (1976). Batch cultures were grown aerobically at 30°C in MSY for 24 h with shaking at 150 r.p.m. Cell growth was measured as optical density at 420 nm. The cells were harvested at $\text{OD}_{420} = 1.5$ by centrifugation ($7000 g$ for 10 min), washed in $25 \text{ mM-potassium phosphate buffer}$, pH 7.4, and resuspended at a cell density of $2 \times 10^9 \text{ cells ml}^{-1}$.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MDH, malate dehydrogenase.

Nodule production. Plants were obtained by clonal propagation of shoot cuttings from a single plant of *Medicago sativa* L. (cv. Europe). The cuttings were maintained on water for 1 week and then on the minimal salts medium of Sirois & Peterson (1982) with 5 mM-KNO₃ for 2 weeks. At this stage, the roots were rinsed with distilled water and the plants were transferred to an aeroponic device (plastic tank, 250 litres) containing 10 litres of nitrogen-free salts solution (Sirois & Peterson, 1982) and a mist generator (Defensor 505). Shoots and roots were separated by a plastic cover-plate with 3 cm diameter holes fitted with notched rubber stoppers. Each stopper supported one plant through a 1.0 cm diameter hole containing one Eppendorf microtube filled with agar. The plants were immediately inoculated by adding a washed suspension of bacteria (5 ml for each plant) into the plastic tank. Two weeks after inoculation the plastic cover supporting the plants was transferred to a tank (65 litres) containing 20 litres of Sirois and Peterson medium. The bottom of this tank was fitted with an aeration tube, and air was provided to the root system at 3.7 litres min⁻¹. The roots were 30% immersed in the medium. Four-week-old nodules were harvested and used immediately for bacteroid preparation. Nodules obtained from plants grown through this two-step procedure showed a two to three times greater acetylene reduction activity than nodules collected from plants maintained in the aeroponic device for 4 weeks. During the whole process, plants were grown in a greenhouse with 16 h of light each day; the nutrient solution was renewed weekly. Natural light was supplemented with 400 W Phytochrome lamps. Day and night temperatures were approximately 27 °C and 20 °C, respectively.

Bacteroid isolation. Nodules (3–4 g fresh wt) were surface-sterilized with calcium hypochlorite (0.5%, pH 7.0) for 5 min and thoroughly rinsed with sterile distilled water. The nodules were then crushed using a mortar and pestle in the mixture described by Bergersen & Turner (1970). After filtration, the homogenate was centrifuged at 270 g for 5 min to remove insoluble polyvinylpyrrolidone and plant cell debris. Bacteroids were separated by a second centrifugation (6800 g, 10 min) and washed twice with 50 mM-potassium phosphate buffer (pH 7.4) containing sucrose (300 mM) and magnesium sulphate (2 mM) as described by Rigaud *et al.* (1973). They were finally resuspended in 25 mM-potassium phosphate buffer (pH 7.4) to a final concentration of 40 mg dry wt ml⁻¹.

Determination of oxygen consumption. Bacteroid suspensions (2 ml) containing 0.25 mg protein were incubated in the chamber of a Hansatech oxygen electrode at 25 °C with 10 mM-succinate as energy-yielding substrate. Glycine betaine and NaCl were added at 10 mM and 50–600 mM, respectively. Results are expressed as nmol O₂ consumed min⁻¹ (mg protein)⁻¹.

Uptake experiments. Transport activities of bacteroids were determined immediately upon completion of the bacteroid isolation procedure. The method used was that described for transport assays in free-living cells (Perroud & Le Rudulier, 1985). Individual uptake experiments (final volume 0.5 ml) were done aerobically at 25 °C, at least in duplicate, and each experiment was repeated with two or three independent bacteroid preparations. Unless otherwise indicated, the substrate concentration in the assay mixture (25 mM-potassium phosphate buffer, pH 7.4) was 50 µM for [¹⁴C]glycine betaine and [¹⁴C]choline and 10 µM for [¹⁴C]proline betaine. Salts, osmotic agents, energy-yielding substrates or inhibitors were added to the bacteroid suspension 5 min before addition of ¹⁴C-labelled substrate. In competition experiments, bacteroids were added to a mixture of labelled substrate and unlabelled analogues. The reaction was stopped by rapid filtration through a cellulose nitrate filter (0.45 µm pore size), which was rinsed twice with 3 ml of the corresponding assay medium. Radioactivity accumulated by the bacteroids was measured after 30 min incubation. During this period uptake was linear at the concentrations used. The filters were solubilized in scintillation vials

containing 2.5 ml of ACS scintillant, (Amersham) and radioactivity was determined about 24 h later in a liquid-scintillation counter.

Cellular volumes. Intracellular aqueous volumes were determined by the [³H]water/[¹⁴C]dextran technique of Stock *et al.* (1977). Values were 6.9 ± 0.7 and 5.2 ± 0.3 µl (mg protein)⁻¹ for bacteroids resuspended in 25 mM-potassium phosphate buffer and the same buffer with 0.2 M-NaCl added, respectively.

Radioisotopes. [^{methyl-14}C]Choline (2.15 MBq µmol⁻¹) and [^{carboxy-14}C]dextran (18.5 MBq g⁻¹) were purchased from Amersham and New England Nuclear, respectively. [³H]Water (370 MBq ml⁻¹) and L-[U-¹⁴C]proline (9.62 MBq µmol⁻¹) were from CEA (France). [^{methyl-14}C]Glycine betaine was prepared from [^{methyl-14}C]choline as described by Perroud & Le Rudulier (1985). [¹⁴C]Proline betaine was synthesized from L-[U-¹⁴C]proline by the method of Corti (1949) as modified by Ikutani (1968). Analyses of purity were done as previously described (Gloux & Le Rudulier, 1989).

Protein determinations. Total protein was assayed by the Lowry method using BSA as the standard.

Results and Discussion

Oxygen uptake by bacteroids: effects of NaCl and glycine betaine

The preparations were shown to be free from mitochondria by the method described by Salminen & Streeter (1987). Malate dehydrogenase (MDH) activity of bacteroids that had been passed through a French pressure cell [16000 p.s.i. (about 110 MPa)] was tested with two inhibitors of mitochondrial MDH activity (Waters *et al.*, 1985). After 70 min pre-incubation with 1 mM-iodoacetate or 1 mM-PMSF, MDH rates of 2.21 and 2.17 µmol min⁻¹ (mg protein)⁻¹ were observed, whereas the untreated preparation gave a rate of 2.12 µmol min⁻¹ (mg protein)⁻¹. These rates were comparable to the rates reported for MDH from bacteroid forms of *Bradyrhizobium japonicum* (Salminen & Streeter, 1987), indicating that our bacteroid preparations were free of mitochondria. This was confirmed by examination of the bacteroid preparations by electron microscopy. In addition, virtually no contamination by free-living bacteria could be detected.

It is well-known that supplementing bacteroid suspensions with organic acids stimulates respiratory activity. However, to our knowledge, only one report deals with bacteroids of *R. meliloti* (Bekki *et al.*, 1987). We have verified that the low respiratory activity supported by endogenous reserves [5 nmol O₂ min⁻¹ (mg protein)⁻¹] was greatly increased by succinate, maximal stimulation being observed with 10–20 mM substrate [65 nmol O₂ min⁻¹ (mg protein)⁻¹]. Addition of other carbon sources, including malate, glucose, fructose or sucrose, only slightly enhanced respiration activity [7–9 nmol O₂ min⁻¹ (mg protein)⁻¹], whereas fumarate had no effect. All subsequent experiments on the effects of salt stress on

respiration activity were done in the presence of 10 mM-succinate (Fig. 1). Addition of 50–100 mM-NaCl inhibited O_2 consumption by only 20% and 24%, respectively; 370 mM-NaCl was necessary to reach 50% inhibition. When a concentration as high as 600 mM-NaCl was added, O_2 consumption was still 34% of the initial rate. Similar effects of NaCl on bacteroid respiration have been observed with bacteroids isolated from nodules of *M. sativa* inoculated with *R. meliloti* strain 2011, and nodules of *M. ciliaris* inoculated with *R. meliloti* strain ABS 7 (Bekki *et al.*, 1987). The results are also in agreement with previously unpublished results obtained with free-living cells of *R. meliloti* strain 102F34 in which 50% inhibition of O_2 consumption was observed when 440 mM-NaCl was added to the incubation medium. Addition of 10 mM-glycine betaine to a succinate-free medium did not stimulate bacteroid respiration either in the absence or the presence of 400 mM-NaCl (results not shown). This contrasts with the case of free-living cells of *R. meliloti* where, in low-salt medium, glycine betaine is used as an energy-yielding substrate (Bernard *et al.*, 1986), and thus stimulates O_2 consumption 2-fold. One possible explanation for this lack of stimulation in bacteroids might be loss of glycine betaine uptake activity during the differentiation process. Therefore, bacteroids were assayed for their ability to take up [*methyl*- ^{14}C]glycine betaine.

Glycine betaine uptake by bacteroid preparations: effects of salts and osmolarity

In *R. meliloti* 102F34, the glycine betaine uptake system is constitutive and strongly stimulated by increased

Fig. 1. Influence of NaCl concentration on the rate of O_2 consumption by bacteroids of *R. meliloti*. Measurements were made as described in Methods; incubation mixtures contained bacteroids (0.25 mg protein) and 10 mM-succinate. Initial O_2 concentration was assumed to be 253 μM at 25 °C. ---, NaCl concentration giving 50% inhibition of respiration. Each point is the mean of at least three independent determinations; error bars indicate SD.

osmolarity (Bernard *et al.*, 1986). Similarly, bacteroids isolated from *M. sativa* nodules were able to transport [^{14}C]glycine betaine at a very low rate in no-salt medium [6.5 pmol min⁻¹ (mg protein)⁻¹] and at a much higher rate in the presence of NaCl (Fig. 2). Maximal stimulation (120-fold) was observed with 200 mM-NaCl. Uptake was linear for at least 30 min under all conditions. After 30 min, the intracellular concentration of glycine betaine in bacteroids treated with 200 mM-NaCl was approximately 4.5 mM, which is 90 times higher than the concentration in the medium. In bacteroids not treated with NaCl, the intracellular concentration was approximately 27 μM compared to 50 μM in the incubation mixture. These results suggest the existence of passive uptake in the absence of NaCl, whereas active uptake occurs in the presence of NaCl. The maximal velocity of glycine betaine uptake was nearly 15-fold higher in free-living cells of *R. meliloti* grown at 200 mM-NaCl compared to bacteroids incubated under the same conditions. This agrees with the observation of San Francisco & Jacobson (1985), who reported that uptake rates for succinate and malate were 20–25-fold higher in cultured cells compared to bacteroids of *B. japonicum* strain USDA 3278. Since we have recently characterized a periplasmic glycine betaine binding protein in *R. meliloti* grown in high-salt medium (unpublished result), one possible explanation for this large decrease in uptake

Fig. 2. Time-course of [*methyl*- ^{14}C]glycine betaine uptake by bacteroids of *R. meliloti*. Incubation mixtures (0.5 ml) contained 50 μM -[*methyl*- ^{14}C]glycine betaine and bacteroids (0.1 mg protein) in 25 mM-potassium phosphate buffer (pH 7.4) with 0 (○), 50 (△), 100 (□), 200 (●), 300 (▲), or 400 (■) mM-NaCl. The NaCl was added to the bacteroid suspension 5 min before addition of radioactive substrate. The points shown are the mean values of duplicate assays from three different experiments; error bars indicate SD. The insert shows the effect of NaCl concentration (mM) on the initial rate of glycine betaine uptake [nmol min⁻¹ (mg protein)⁻¹].

activity could be that the periplasmic binding protein might be lost during bacteroid differentiation or isolation. This possibility is currently under study.

To determine whether or not the stimulation of glycine betaine uptake is specific to NaCl, other electrolytes or non-electrolytes were added to the incubation medium at concentrations osmotically equivalent to 200 mM-NaCl (Table 1). NaCl and Na₂HPO₄ greatly enhanced uptake (120- and 70-fold stimulation respectively), whereas LiCl and KCl had much less effect (only 15- and 8-fold stimulation, respectively). Moreover, adding organic compounds (sucrose or mannitol) to the incubation medium led to only a 5-fold increase in uptake. These results differ from those obtained previously in free-living cells of *R. meliloti* (Pocard, 1987), where the stimulation observed with NaCl (300 mM) was only four times that of the control cells. Furthermore, in cultured cells KCl (300 mM) had the same effect as NaCl, and sucrose (460 mM) gave a stimulation 2.7-fold higher than NaCl. Whether glycine betaine uptake by bacteroids is coupled to Na⁺ is not known, but these experiments show that the stimulation of uptake is not simply a consequence of elevated external osmotic pressure.

Kinetics of glycine betaine transport

The concentration dependence of glycine betaine uptake was studied by varying the substrate concentration (0.5–100 µM) and measuring the velocity of uptake over 30 min by bacteroids incubated in 200 mM-NaCl (Fig. 3). Glycine betaine uptake was a saturable process; it showed Michaelis–Menten kinetics, and from the data the apparent K_m was calculated to be about 8.3 µM with a value for V of 0.84 nmol min⁻¹ (mg protein)⁻¹. There was

Fig. 3. Kinetics of [methyl-¹⁴C]glycine betaine uptake by bacteroids of *R. meliloti*. Glycine betaine uptake was measured as described in Methods; incubation mixtures contained bacteroids (0.1 mg protein) in 25 mM-potassium phosphate buffer (pH 7.4) with 200 mM NaCl. The points shown are the mean values of three determinations. The insert is a Lineweaver–Burk plot of the reciprocal initial rate of glycine betaine uptake (nmol⁻¹·min·mg protein) against the reciprocal of concentration (µM⁻¹).

no significant difference between cultured cells and bacteroids in apparent K_m but V , as previously mentioned, was much lower in bacteroids than in free-living cells. Most of the studies on metabolite uptake by bacteroids have given apparent K_m values in the micromolar range, e.g. 40 µM for succinate and malate in bacteroids of *B. japonicum* strain USDA 110 (Reibach & Streeter, 1984) and 10–15 µM for the same substrates in bacteroids of *B. japonicum* strain USDA 3278 (San Francisco & Jacobson, 1985).

Effect of energy-yielding substrates and inhibitors on glycine betaine uptake

We attempted to define the conditions for maximal glycine betaine uptake by incubating the bacteroids with various oxidizable carbon substrates or ATP, in the absence or presence of 200 mM-NaCl. In both conditions, supplying bacteroids with 20 mM-succinate, which greatly enhanced O₂ consumption, surprisingly had no effect on glycine betaine uptake. Sucrose, fructose, glucose (20 mM) or ATP (2 mM) did not have any significant effect on uptake, whereas pre-incubation with fumarate and malate led to a small reduction in the accumulation of glycine betaine (7% and 12%, respectively; results not shown).

Potassium cyanide (KCN; 0.5 mM), a respiratory inhibitor, greatly reduced (81%) glycine betaine uptake by bacteroids in the presence of 200 mM-NaCl. The protonophore carbonyl cyanide *m*-chlorophenylhydra-

Table 1. Effect of various solutes on [methyl-¹⁴C]glycine betaine uptake by bacteroids of *R. meliloti*

Incubation mixtures contained 50 µM-[methyl-¹⁴C]glycine betaine and bacteroids (0.1 mg protein) in 25 mM-potassium phosphate buffer (pH 7.4) with added electrolytes or non-electrolytes at concentrations osmotically equivalent to 200 mM-NaCl. Addition of osmolytes was done as described in Fig. 2. Mean results of duplicates from two separate experiments are shown ± SD.

Osmolyte	Concn (mM)	Glycine betaine uptake [nmol (30 min) ⁻¹ (mg protein) ⁻¹]	Stimulation* (-fold)
None		0.2 ± 0.01	0
NaCl	200	23.2 ± 0.6	120
Na ₂ HPO ₄	175	13.8 ± 0.2	70
LiCl	200	3.1 ± 0.05	15
KCl	200	1.6 ± 0.05	8
Sucrose	300	1.1 ± 0.15	5
Mannitol	350	1.1 ± 0.05	5

* Compared to the value obtained in the absence of osmolyte.

zone (CCCP; 10 μ M), which inhibits the formation of a pH gradient by *B. japonicum* bacteroid suspensions (Bhandari & Nicholas, 1985), strongly inhibited glycine betaine uptake (96% inhibition). However, arsenate (5 mM), which can compete with phosphate so that very little functional ATP is formed, was without any inhibitory effect on uptake. These results confirm that glycine betaine accumulation in bacteroids of *R. meliloti* is via an active transport system and suggest that direct utilization of ATP by the glycine betaine transport mechanism is unlikely. However, the results also suggest that at least a portion of the glycine betaine taken up may be coupled to proton movement. We are aware of the difficulties in interpreting these results and determining the driving force. A more detailed analysis of the components of glycine betaine uptake system, in both free-living and bacteroids of *R. meliloti*, is needed. Such studies are in progress in our laboratory.

Specificity of glycine betaine uptake

The specificity of the glycine betaine transport system was determined by experiments in which various structural analogues at 10 times and 100 times the glycine betaine concentration were allowed to compete with uptake of [methyl- ^{14} C]glycine betaine. The relative accumulation of [^{14} C]glycine betaine was determined by reference to untreated controls (Table 2). As expected, unlabelled glycine betaine was the most effective competitor (89% and 98% inhibition at 0.5 and 5 mM, respectively). When used at 0.5 mM, all other betaines were without effect on glycine betaine uptake. Similarly, non-methylated compounds, such as glycine and proline, did not cause any inhibition. Higher concentrations of

competitors (5 mM) were also ineffective except in the case of proline betaine and trigonelline. However, when present in 100-fold excess, proline betaine had only a slight inhibitory effect (25% inhibition) on glycine betaine transport. The strong inhibition (82%) observed with trigonelline was almost certainly the consequence of the toxicity of this compound at the concentration used, because whereas a concentration of 3 mM-trigonelline allows good growth of *R. meliloti*, 6 mM kills the cells. Thus, it seems unlikely that proline betaine and trigonelline are transported by the same system as glycine betaine. Therefore, the glycine betaine uptake system in *R. meliloti* bacteroids possesses considerable structural specificity. Work with *E. coli* has also shown a strong specificity for glycine betaine transport system; the purified periplasmic binding protein binds glycine betaine with high affinity but has no affinity for either choline or proline (Barron *et al.*, 1987). While proline betaine was not tested by these authors, we have shown that proline betaine uptake by *R. meliloti* is not likely to be dependent on a periplasmic binding protein (Gloux & Le Rudulier, 1989).

Uptake of the glycine betaine analogues choline and proline betaine

Because both proline betaine and choline are transported by free-living cells of *R. meliloti* (Pocard *et al.*, 1989; Gloux & Le Rudulier, 1989), and since they are not competitors of glycine betaine transport by *R. meliloti* bacteroids, measurements of uptake by bacteroid suspensions were done using 50 μ M-[methyl- ^{14} C]choline and 10 μ M-[^{14} C]proline betaine. Uptake of both compounds was nearly constant for about 30 min. At low osmolarity, the rate of proline betaine uptake was similar to that of glycine betaine while the rate of choline uptake was about 9-fold greater. Addition of 200 mM-NaCl caused an approximately 25- and 2-fold stimulation of proline betaine and choline uptake, respectively (Table 3).

Table 2. Effect of unlabelled competitors on [methyl- ^{14} C]-glycine betaine uptake by bacteroids of *R. meliloti*

Uptake assays were done as described in Methods with 50 μ M-[methyl- ^{14} C]glycine betaine and bacteroids (0.1 mg protein) in 25 mM-potassium phosphate buffer (pH 7.4) with 200 mM-NaCl, and 0.5 or 5 mM competitor. Data are given as a percentage of the non-inhibited uptake rate, which was 25.2 ± 1.1 nmol (30 min) $^{-1}$ (mg protein) $^{-1}$.

Addition	Percentage inhibition at an inhibitor concentration of:	
	0.5 mM	5 mM
None	0	0
Glycine betaine	89 ± 2	98 ± 1
Choline	-6 ± 10	-25 ± 6
Proline betaine	0 ± 10	25 ± 4
γ -Butyrobetaine	-10 ± 7	-6 ± 2
Trigonelline	2 ± 6	82 ± 2
Glycine	1 ± 1	-9 ± 2
Proline	-9 ± 1	-10 ± 1

Table 3. Uptake of [methyl- ^{14} C]choline and [^{14} C]proline betaine by bacteroids of *R. meliloti*

Incubation mixtures contained 50 μ M-[methyl- ^{14} C]choline or 10 μ M-[^{14} C]proline betaine and bacteroids (0.1 mg protein) in 25 mM-potassium phosphate buffer (pH 7.4) with 0, 200 or 400 mM-NaCl. Data are means of results from two separate experiments $\pm$ SEM.

NaCl concn (mM)	Uptake [nmol (30 min) $^{-1}$ (mg protein) $^{-1}$]	
	Choline	Proline betaine
None	1.5 ± 0.1	0.16 ± 0.02
200	2.7 ± 0.2	3.95 ± 0.15
400	1.9 ± 0.1	Not determined

Under these conditions, after 30 min uptake, the total amount of both compounds that had been taken up was about six to nine times lower than that of glycine betaine. Some of the difference may be due to the fact that choline and proline betaine concentrations in the assay might have been less than optimal.

When choline uptake was studied as a function of substrate concentration over the range 5–150 μM , a non-linear Lineweaver–Burk plot was obtained with bacteroids maintained at low or high osmolarity. This suggests that a high-affinity activity as well as a low-affinity transport system might be present in *R. meliloti* bacteroids, and is consistent with results obtained with free-living cells (Pocard *et al.*, 1989). In 200 mM-NaCl (Fig. 4), the approximate K_m values for these activities were 10 and 100 μM , respectively with values for V of 27 and 125 $\text{pmol min}^{-1} (\text{mg protein})^{-1}$. Succinate (20 mM) or ATP (2 mM) did not stimulate these activities but the uptake was strongly inhibited by 0.5 mM-KCN and 10 μM -CCCP. However, a residual uptake activity (about 46% and 26%, respectively, of the non-inhibited assay) was always observed in the presence of KCN and CCCP. This suggests that a substantial amount of choline is taken up by passive diffusion, a process which also occurs in the case of malate and succinate uptake by bacteroids of *B. japonicum* (Reibach & Streeter, 1984).

It is of interest that isolated bacteroids of *R. meliloti* are able to transport glycine betaine, choline and proline betaine, all of which, are osmoprotective agents in free-living and symbiotic nitrogen-fixing bacteria (Le Rudulier & Bouillard, 1983; Pocard *et al.*, 1989). We have previously shown that nitrogenase activity of *Klebsiella pneumoniae*, which is completely inhibited under conditions of salt stress, is significantly restored in the presence of glycine betaine, proline betaine and other related compounds (Le Rudulier *et al.*, 1984b). Thus, one

Fig. 4. Kinetics of [methyl- ^{14}C]choline uptake by bacteroids of *R. meliloti*. Experimental conditions etc. as described in Fig. 3.

might expect a beneficial role for these molecules in the maintenance of nitrogenase activity of bacteroids subjected to salt stress. Further experiments are required to clarify this point.

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