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EFFECT OF NH_4^+ ON NITROGENASE ACTIVITY IN NODULE BREIS AND BACTEROIDS FROM *PISUM SATIVUM* L.

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Summary

Nodule breis and bacteroid preparations were made from *Pisum sativum* L. (cv. Trapper) inoculated with a single strain of *Rhizobium leguminosarum*. The detached nodules were triturated under helium flow. The resultant breis could support C_2H_2 reduction in *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid buffer (Tes) without any additions for over an hour. NH_4^+ was found to inhibit C_2H_2 reduction and H_2 evolution. The inhibition was not dependent on the counterion and was evident immediately after the addition of NH_4^+ to the reaction mixture. L-Methionine-D,L-sulfoximine, added to inhibit assimilation of NH_4^+, had no effect on the inhibition. Addition of pyruvate enhanced the rate of C_2H_2 reduction in breis and partially overcame the inhibition by NH_4^+. Pyruvate was found necessary for measurable activity in bacteroid preparations. When ATP and an ATP-generating system were used in breis the effect of NH_4^+ was not observed.

Introduction

NH_4^+ has been shown to repress the synthesis of nitrogenase [1,2] in asymbiotic nitrogen-fixing bacteria and to inhibit the rate of C_2H_2 reduction [3,4]. A direct inhibition of nitrogen and acetylene reduction in cell-free extracts of *Azotobacter vinelandii* has also been reported [5], but effects on purified nitrogenase remain to be established in most organisms. Under conditions where free-living *Rhizobia* have been induced to fix nitrogen, repression by

Abbreviation: Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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NH_4^+ has been shown [6]. Tubb [7] interpreted his results as evidence for partial repression or inhibition depending on the method of measurement. Scowcroft et al. [8] found inhibition of nitrogenase activity by NH_4^+, which could be overcome by O_2. In symbiotic associations NH_4^+ has also been shown to decrease nitrogen fixation in intact plants [9—11] and in detached nodules [12—15].

However, inhibition of nitrogenase activity by NH_4^+ has not been demonstrated in nodule breis or bacteroid preparations [13—16]. In cell-free *Rhizobium* extracts no effect of NH_4^+ was found [17]. This has led to speculation that in the symbiotic system the inhibition of nitrogen fixation is not by NH_4^+ itself, but by a product of its further assimilation [14]. This study was undertaken to examine the effects of NH_4^+ on nodule breis and bacteroids of *Pisum sativum* L.

Materials and Methods

Plant Culture. Seeds of *P. sativum* L. (cv. Trapper) were planted 2 cm deep in 9.5 cm plastic pots of Turface (Wyandotte Chemicals Ltd, Wyandotte, MI). After 3 days the pots were inoculated with 3 ml of rhizobial culture (10^9 cells/ml), which had been incubated for 3 days at 28°C in a mannitol/yeast extract medium, pH 7.3 [18]. The bacterial strain used was a mutant selected for resistance to streptomycin (50 μg/ml) and tetracycline (0.25 μg/ml). The plants were grown in a controlled environment cabinet (Enconaire System Ltd, Winnipeg, Canada) with a 16 h photoperiod and a day/night temperature of 20/15°C. The mean irradiance of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ was provided by Sylvania cool white fluorescent and incandescent lamps. The watering regime consisted of N-free nutrient solution three times a week and water on other days. The nutrient solution was modified from that used by Wilson and Reisenauer [19] with 2 $\mu g \cdot ml^{-1}$ Fe (Sequestrene 330 Fe, CIBA-GEIGY).

Preparation of nodule breis. Nodules from 31—35-day-old plants were removed and weighed. The nodules (300—400 mg) were triturated in a glove box under helium flow, using a mortar and pestle. The grinding medium (1 ml/70 mg nodules) consisted of 80 mM Tes buffer pH 7.1/1 mM $MgCl_2$/200 mM ascorbate/2% (w/v) poly(ethylene glycol) grade 4000/5 mM sodium diethyldithiocarbamate/5 mM dithiothreitol. When bovine serum albumin was used its concentration was 1% (w/v). The homogenate was filtered through 2 Miracloth (Chicopee Hills, Inc. Milltown, NJ) discs in a 10 ml syringe and used in brei assays.

Preparation of bacteroids. Bacteroids were isolated by transferring the brei obtained from 500—600 mg nodules into a capped centrifuge tube under helium flow and centrifuging at 2000 × *g* for 10 min. The mitochondrial fraction was obtained by centrifuging the supernatant fluid from the bacteroid preparation at 40 000 × *g* for 2 min. The bacteroid and mitochondrial pellets were then resuspended in fresh grinding medium. All transfers and resuspensions were carried out under helium flow.

Acetylene reduction assay. All reactions were carried out in 1.5 × 10 cm test tubes capped with serum stoppers. The final volume of the reaction mixture was 1.5 ml consisting of 0.5 ml brei or bacteroid preparation and 1 ml Tes buffer with other additions as indicated in figure and table legends. All additions

were buffered to pH 7.1. L-Methionine-D,L-sulfoximine, an inhibitor of glutamine synthetase [20], was used at 10 mM final concentration. KCl up to 20 mM had no detectable effect on C_2H_2 reduction but was always included in the controls. The O_2 concentration in the gas phase (10.5 ml) above the reaction mixture was 2% for the breis and 1% for the bacteroids. These were found to be the optimal concentrations in our assay system. The tubes were placed in a Buchler Evapomix shaker at 30°C and shaken for 2 min at 300 rev./min. The assay was then started by injecting C_2H_2 to give a final saturating concentration of 5% (v/v) in the gas phase. Gas samples (0.25 ml) were withdrawn at 10 or 15 min intervals and assayed in a gas chromatograph. At the conclusion of the experiment the pH of the tubes was checked to ensure that it had not changed.

Results

Addition of ascorbate to Tes buffer grinding medium was found to be necessary for activity. The rate of C_2H_2 reduction was further enhanced by poly(ethylene glycol) which gave higher activity and better yield than solid poly(vinyl pyrrolidone). Poly(ethylene glycol) prevents proteins from binding to tannins [21] and adds to the osmotic potential of the medium stabilizing the bacteroids. Inclusion of 0.3 M sucrose in this grinding medium resulted in lower activity of the breis, and sucrose was, therefore, omitted from the medium. Sodium diethyldithiocarbamate, a copper chelator, added as protection against poly(phenol oxidase), and the sulfhydryl reagent dithiothreitol did not increase activity but were included to promote stability.

The breis could sustain linear rates of C_2H_2 reduction in Tes buffer reaction mixture, without any additions, for over an hour. A lag period was sometimes seen in the beginning of the experiment. Increasing the speed of shaking seemed to minimize the lag period, and sampling at 15, 30 and 45 min ensured that the linear portion was used for calculation of the rates.

The results of Fig. 1 show that NH_4Cl decreased the rate of C_2H_2 reduction and that the inhibition increased with the concentration of NH_4Cl. The mean rate (±S.E.) of the control (no NH_4Cl) samples was 1.23 ± 0.30 μmol/g nodule fresh wt. per h. The magnitude of the inhibition varied greatly among preparations, but in all cases increasing the NH_4Cl concentration also increased the degree of inhibition. This inhibition was significant ($P < 0.05$) at NH_4Cl concentrations of 4 mM or higher. The same inhibition was seen in nodule breis from peas inoculated with a commerical inoculum (data not shown) indicating that it was not restricted to the strain used.

This inhibition was not dependent on the counterion (Table I). In an experiment where 10 mM NH_4Cl inhibited the C_2H_2 reduction by 31%, the same degree of inhibition was obtained with 5 mM $(NH_4)_2SO_4$.

The response to NH_4^+ addition was very rapid (Fig. 2). L-Methionine-D,L-sulfoximine was included in the reaction mixtures to inhibit assimilation of NH_4^+. Good linearity was found between 10 and 30 min, which suggested that the inhibition was immediate following NH_4^+ addition. If the inhibition depended on assimilation of NH_4^+, a time lag should have existed. Student's *t*-test indicated that the rates of C_2H_2 reduction, (see legend of Fig. 2) of the samples receiving NH_4^+, either at the start of the experiment or after 10 min

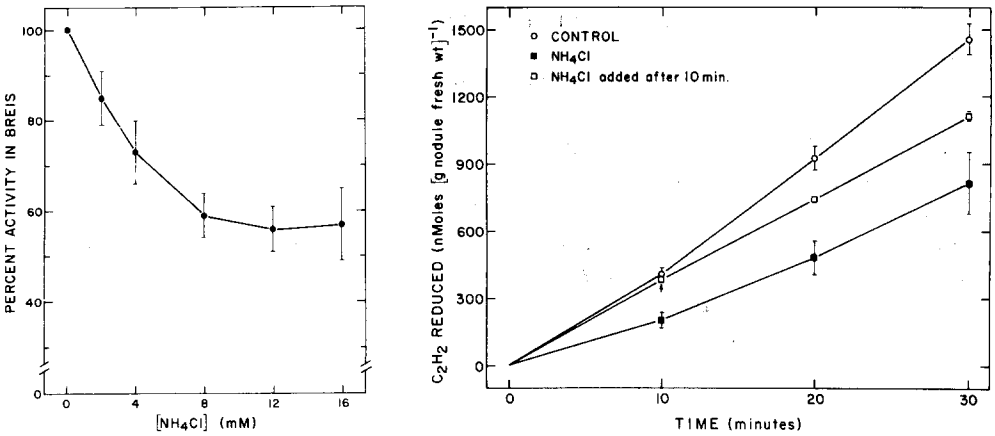

Fig. 1. The relative rates of C_2H_2 reduction by nodule breis as a function of NH_4Cl concentrations. Each point represents a mean of three to six experiments. The bars represent 2-times S.E. of the mean.

Fig. 2. Acetylene reduced by breis as a function of time. L-Methionine-D,L-sulfoximine was 10 mM. The bars represent 2-times S. E. of duplicate samples. The regression coefficients (rates) for the lines from 10—30 min were 52.4 ± 3.0 (ηmol C_2H_2/min) (○———○), 30.7 ± 5.8 (■———■), and 36.5 ± 0.9 (□———□).

were significantly different from the control value ($P < 0.01$).

Comparison of the effect of NH_4^+ on C_2H_2 reduction and H_2 evolution (Table II) showed that in a brei where C_2H_2 reduction was inhibited by 44% the H_2 evolution also decreased by 51%. Due to the standard error in the control samples in the H_2 measurements the difference between the two percentages was not regarded as significant.

Pyruvate increased the rate of C_2H_2 reduction over the rate of samples in a buffer reaction mixture (Table III). In one experiment NH_4^+ inhibited the rates of buffer samples by 18% and with pyruvate samples the inhibition was only 8%. In another experiment where NH_4^+ concentration was doubled its effect in buffer samples was 66%, whereas the inhibition in samples with pyruvate was

TABLE I

EFFECT OF NH_4Cl AND $(NH_4)_2SO_4$ ON THE RATE OF ACETYLENE REDUCTION IN BREIS

The reaction mixture consisted of 0.7 ml 71 mM Tes Buffer/0.5 ml brei/0.1 ml 150 mM L-methionine-D, L-sulfoximine/0.2 ml of the addition given below. The final volume was 1.5 ml of 60 mM Tes, pH 7.1. Means ± S. E. of duplicate samples.

Addition	Concentration (mM)	C_2H_2 reduced	
		μmol/g nodule fresh wt. per h	%
KCl	10	2.29 ± 0.03	100
NH_4Cl	10	1.58 ± 0.07	69
$(NH_4)_2SO_4$	5	1.57 ± 0.09	69
K_2SO_4	5	2.13 ± 0.06	93

TABLE II

EEFECT OF NH_4Cl (15 mM) ON C_2H_2 REDUCTION AND H_2 EVOLUTION IN BREIS

The reaction mixtures were as in Table I. Means ± S. E. of duplicate samples.

Gas phase	Gas measured	μmol/g nodule fresh wt. per h	
		KCl	NH_4Cl
He : O_2, 5% C_2H_2	C_2H_4	1.69 ± 0.05 (100%)	0.94 ± 0.02 (56%)
He : O_2	H_2	1.52 ± 0.21 (100%)	0.74 ± 0.01 (49%)

only 36%. Thus, pyruvate cut the inhibition by NH_4^+ in half. Bovine serum albumin had no detectable effect on the inhibition. Pyruvate was chosen as the organic acid because it gave consistent results. Although succinate could give higher rates it also often gave lower rates than the buffer controls. This inhibitory effect of succinate has also been reported by Houwaard [15]. We have recently also used malate which gives 3—4-times higher rates of C_2H_2 reduction than pyruvate, without affecting the pattern of NH_4^+ inhibition.

In breis where the inhibition by NH_4^+ was 35 and 14% (Table IV) in buffer reaction mixtures, no inhibition was seen in samples with ATP and an ATP-generating system. In Expt. II, ATP with an ATP-generating system gave lower rates than the buffer reaction mixtures, but still no effect of NH_4^+ was evident. This variable effect of ATP on the rate of C_2H_2 reduction did not seem to depend on the concentration of ATP used.

Only marginal activity could be measured in bacteroid preparations in the absence of exogenous substrates. Addition of pyruvate to the reaction mixture gave measurable linear rates of C_2H_2 reduction. Mitochondria further increased this rate: in an experiment where the rate with pyruvate was 74.4 ± 7.6 nmol/g nodule fresh wt. per h (mean ±S.E.), the rate with pyruvate and mitochondria was 119.1 ± 0.5. The presence of mitochondria did not appear to change the inhibition by NH_4^+. Fig. 3 shows that NH_4^+ inhibited C_2H_2

TABLE III

EFFECT OF PYRUVATE AND NH_4Cl ON C_2H_2 REDUCTION IN BREIS

The final concentration of pyruvate in the reaction mixture (1.5 ml) was 20 mM. The concentration of NH_4Cl was 7.5 mM in Expt. I and 15 mM in Expt. II. L-methionine-D,L-sulfoximine was 10 mM. Expt. I had 1% (w/v) bovine serum albumin in the grinding medium. Means ± S.E. of duplicate samples.

Experiment	μmol C_2H_4/g nodule fresh wt. per h			
	Buffer		Pyruvate	
	KCl	NH_4Cl	KCl	NH_4Cl
I	2.50 ± 0.03 (100%)	2.05 ± 0.03 (82%)	2.91 ± 0.08 (100%)	2.69 ± 0.06 (92%)
II	1.13 ± 0.08 (100%)	0.38 ± 0.01 (34%)	1.39 ± 0.02 (100%)	0.89 ± 0.12 (64%)

TABLE IV

EFFECT OF ATP AND ATP-GENERATING SYSTEM ON THE NH_4Cl INHIBITION OF C_2H_2 REDUCTION

The ATP concentrations were 1 mM in Expt. I and 3 mM in Expt. II. Each tube with ATP received 45 μmol creatine phosphate and 600 μg creatine phosphokinase. The concentration of NH_4Cl was 7.5 mM. Expt. I was without, Expt. II with 10 mM L-methionine-D,L-sulfoximine. Other details as in Table I. Means ± S.E. of duplicate samples.

Experiment	μmol C_2H_4/g nodule fresh wt. per h			
	Buffer		ATP + gen. syst.	
	KCl	NH_4Cl	KCl	NH_4Cl
I	2.09 ± 0.01 (100%)	1.35 ± 0.06 (65%)	2.38 ± 0.03 (100%)	2.86 ± 0.10 (120%)
II	2.75 ± 0.18 (100%)	2.36 ± 0.02 (86%)	1.67 ± 0.11 (100%)	1.77 ± 0.09 (106%)

reduction in bacteroid preparations and that this inhibition increased with NH_4^+ concentration. The mean rate of the control samples was 86.8 ± 21.4 nmol/g nodule fresh wt. per h. The replication between different bacteroid preparations was superior to that obtained with nodule breis. The inhibition was significant ($P > 0.05$) at NH_4Cl concentration of 4 mM or higher.

The activities reported here were rather low. Our more recent data has shown increased rates, especially with malate. Malate required 3% O_2 for optimal activity in our bacteroid system, giving rates in the micromolar range. In an experiment where the rate of C_2H_2 reduction with pyruvate was 156 ± 12

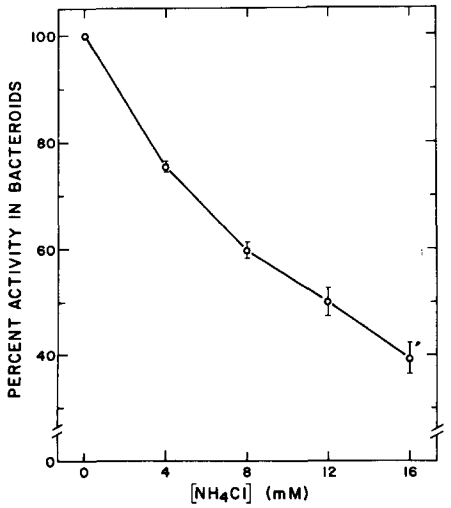

Fig. 3. The relative rates of C_2H_2 reduction by bacteroids as a function of NH_4Cl concentration. Bovine serum albumin at 1% (w/v) final concentration was used in the grinding medium. Pyruvate was 20 mM, L-methionine-D,L-sulfoximine 10 mM, and 0.1 ml mitochondria (about 50 μg mitochondrial protein) were added to the reaction mixture. The points represent the means of six experiments except at 16 mM NH_4Cl (three experiments). The bars represent 2-times standard errors.

nmol/g nodule fresh wt. per h in the KCl control tubes and 79 ± 10 nmol (51% of the control) with NH_4^+, the corresponding rates with malate were 2.38 ± 0.13 μmol/g nodule fresh wt. per h and 1.56 ± 0.03 μmol (66% of the control) with NH_4^+. Other experimental details were as in the legend to Fig. 3. Thus, the inhibition by NH_4^+ was not a consequence of low activity.

Discussion

Metabolic regulation of nitrogenase activity in the plant-*Rhizobium* symbiosis is still poorly understood. The effects of combined nitrogen on intact plants are well documented [9,12,22]. The lapse of a few days between application of nitrogen and observation of the effects make the interpretation of the data difficult in terms of what effects can be contributed to NH_4^+ itself and which are brought about by its subsequent assimilation. A decrease in leghemoglobin content has been reported following application of 100 mM NH_4Cl [10]. Evidence for reduction of photosynthesis following 100 mM NH_4Cl treatment has also been presented [22]. This may be due to uncoupling of photosynthetic electron transport chain [23].

The use of detached nodules was thought to ensure that free NH_4^+ did actually reach the nodules, and an effect on the C_2H_2 reduction was evident within a few hours [14]. The nodules still consist of relatively large amounts of plant host material which could conceivably remove some of the free NH_4^+ or exert other forms of control over the bacteroids in situ. In nodule brei and bacteroid preparations the structural integrity of the host is destroyed allowing a more direct uptake by the bacteroids of the solutes provided. Thus, nodule breis and bacteroid preparations would seem to offer the best opportunity for observing direct, short-term effects of NH_4^+ on the symbiotic partner.

The earlier results reported with nodule breis and bacteroids [15,16] have raised the possibility that free NH_4^+ per se does not exert any control over nitrogen fixation in a symbiotic relationship.

Our data, however, would suggest that at least in *P. sativum* with our experimental procedures, NH_4^+ does indeed directly inhibit C_2H_2 reduction in breis and bacteroids. This effect was independent of the counterion and was evident within 10 min of the addition of NH_4^+. As the inhibition by NH_4^+ was also seen in the H_2 evolution it would seem to involve nitrogenase rather than being an artifact of the measuring system. The inhibition was also observed in nodule breis from peas inoculated with a commercial inoculum, suggesting that we are not dealing with a phenomenon restricted to our particular strain of *R. leguminosarum*.

The data with pyruvate and the ATP-generating system in breis might provide some clues to the perplexing difference between the results of other workers [14,16,24] and our efforts. Pyruvate partially overcame the inhibition by NH_4^+, and with ATP and the ATP-generating system no inhibition was seen. Customarily these additions are included in the reaction mixtures designed to maximize the rates of C_2H_2 reduction. Our data suggest that this could easily lead to negative results.

In breis, pyruvate cut the inhibition by NH_4^+ in half. This may also be the case with bacteroid preparations where concentration-dependent inhibition by

NH_4^+ was observed. Until we are able to obtain good C_2H_2 reduction in bacteroids without pyruvate, we can not establish the relationship between the effect of NH_4^+ and pyruvate in the bacteroid preparation.

The possibility that the effect of NH_4^+ in our system is mediated by its acting as an uncoupler of oxidative phosphorylation remains open. The data with ATP and an ATP-generating system are consistent with this. Pyruvate could act in a phosphoroclastic fashion providing a source of ATP for nitrogenase, and this would be independent of uncoupling. We shall attempt to resolve this question.

The variability we have observed in breis could well be due to the presence of metabolites, enzymes and cofactors from the host material. These undoubtedly contribute to the activity of the breis. Consistent with this was the greater reproducibility among different bacteroid preparations.

In contrast to earlier results reported with detached nodules [14] L-methionine-D,L-sulfoximine did not abolish the inhibition by NH_4^+. The use of L-methionine-D,L-sulfoximine should slow down assimilation of added NH_4^+ and ensure that the inhibiting agent is indeed NH_4^+ and not an assimilation product. We have detected accumulation of NH_4^+ in the presence of L-methionine-D,L-sulfoximine in breis fixing N_2.

In this paper, we have demonstrated inhibition of C_2H_2 reduction, as well as H_2 evolution, by NH_4^+ in breis and bacteroids. This inhibition is manifested very quickly after addition of NH_4^+. It is partially overcome by pyruvate. No effect is seen when ATP and ATP-generating system are included in the reaction mixture. The inhibition by NH_4^+ is consistent with the effects seen in free-living *Rhizobia*, detached nodules and intact plants. The idea of produced NH_4^+ being able to switch off nitrogenase under conditions leading to high endogenous levels is attractive from the standpoint of high energy cost of nitrogen fixation [22], but the regulatory mechanisms involved remain to be elucidated.

Laane et al. [16] found no effect of NH_4^+ on the energized state of the membrane or on C_2H_2 reduction in *Rhizobium leguminosarum*. The possibility remains that in our system the mode of action of NH_4^+ is analogous to that in *Azotobacter vinelandii* where it switches off the flow of reducing equivalents to nitrogenase by lowering the electrical gradient, ψ, across the membrane.

At present we do not know how well the added solutes, such as pyruvate, ATP and NH_4^+, are actually taken up by the bacteroids. If the membranes are leaky, they would allow for a ready entry of the solutes and would, perhaps, account for the discrepancy between the results of Laane et al. [16] discussed above and ours. From the standpoint of the NH_4^+ inhibition the integrity of the membranes is not critical, although it will certainly be so in the elucidation of the mechanism of this inhibition.

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