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Evidence that Fungal MEP Proteins Mediate Diffusion of the Uncharged Species NH_3 across the Cytoplasmic Membrane

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Methylammonium and ammonium (MEP) permeases of *Saccharomyces cerevisiae* belong to a ubiquitous family of cytoplasmic membrane proteins that transport only ammonium (NH_4^+ + NH_3). Transport and accumulation of the ammonium analog [^{14}C]methylammonium, a weak base, led to the proposal that members of this family were capable of energy-dependent concentration of the ammonium ion, NH_4^+. In bacteria, however, ATP-dependent conversion of methylammonium to γ-*N*-methylglutamine by glutamine synthetase precludes its use in assessing concentrative transport across the cytoplasmic membrane. We have confirmed that methylammonium is not metabolized in the yeast *S. cerevisiae* and have shown that it is little metabolized in the filamentous fungus *Neurospora crassa*. However, its accumulation depends on the energy-dependent acidification of vacuoles. A Δ*vph1* mutant of *S. cerevisiae* and a Δ*vma1* mutant, which lack vacuolar H^+-ATPase activity, had large (fivefold or greater) defects in the accumulation of methylammonium, with little accompanying defect in the initial rate of transport. A *vma-1* mutant of *N. crassa* largely metabolized methylammonium to methylglutamine. Thus, in fungi as in bacteria, subsequent energy-dependent utilization of methylammonium precludes its use in assessing active transport across the cytoplasmic membrane. The requirement for a proton gradient to sequester the charged species $CH_3NH_3^+$ in acidic vacuoles provides evidence that the substrate for MEP proteins is the uncharged species CH_3NH_2. By inference, their natural substrate is NH_3, a gas. We postulate that MEP proteins facilitate diffusion of NH_3 across the cytoplasmic membrane and speculate that human Rhesus proteins, which lie in the same domain family as MEP proteins, facilitate diffusion of CO_2.

Methylammonium and ammonium permeases MEP1, MEP2, and MEP3 of *Saccharomyces cerevisiae* (35) and the ammonium and methylammonium transport B (AmtB) protein of enteric bacteria (64) are members of a unique family of cytoplasmic membrane transporters that are specific for ammonium (48). (We use ammonium to designate both the charged and uncharged species.) The MEP/Amt family (nomenclature, TC 2.49) occurs ubiquitously in bacteria, archaea, and eukarya (19, 36). Beginning with the pioneering studies of Hackette et al. (16), the activity of MEP/Amt proteins has been assessed by studying transport and accumulation of the ammonium analog methylammonium, which can be ^{14}C labeled. Based on studies with methylammonium it has been proposed that members of the MEP/Amt family transport the charged species NH_4^+ across the cytoplasmic membrane and concentrate it in an energy-dependent manner (19, 65).

We showed previously that enteric bacteria convert methylammonium to γ-*N*-methylglutamine in the ATP-dependent reaction catalyzed by glutamine synthetase and hence that methylammonium cannot be used to assess energy-dependent concentrative uptake (62). The same metabolic conversion occurs in other proteobacteria, including methylotrophic pseudomonads (2, 11, 12, 22–24, 51, 58), and in cyanobacteria (41) and plants (13). Contrary to a previous report (60), it also appears to occur in the gram-positive bacterium *Corynebacterium glutamicum* (39). In contrast, the fungi *S. cerevisiae* and *Penicillium chrysogenum* accumulate methylammonium in the absence of metabolism (16, 53).

When provided at high external concentrations, methylammonium accumulates in acidic compartments of fungi and other eukaryotes, thereby neutralizing these compartments and perturbing their function (15, 25, 45, 46, 61, 69). The same is true for ammonium and other weak bases (1, 32, 55, 66). We now present evidence that the charged species $CH_3NH_3^+$ is accumulated in acidic vacuoles of the yeast *S. cerevisiae* and the filamentous fungus *Neurospora crassa*, even when [^{14}C]methylammonium is provided at low external concentrations. Because acidification of vacuoles depends on the activity of the vacuolar H^+-ATPase (V-ATPase or V-type H^+-ATPase) (3, 4, 9, 27, 50), MEP-dependent sequestration of methylammonium is driven by an energy-requiring secondary process. Therefore, in fungi as in bacteria, the energy-dependent concentration of methylammonium does not provide evidence for its active transport across the cytoplasmic membrane. Interestingly, an *N. crassa* mutant (*vma-1*) that cannot acidify its vacuoles couples uptake of methylammonium to the energy-requiring secondary process used in bacteria, i.e., conversion to methylglutamine.

MATERIALS AND METHODS

Strains, media, and growth conditions. Growth experiments with *S. cerevisiae* strains 23344c, 31019b, and NCM3243 (Table 1) were performed as previously described (62) at 28°C. The minimal medium was medium 164 (21), which was supplemented appropriately (62). Medium 164 has an initial pH of 6.1 which does not change during growth. The carbon source was glucose, usually at a concentration of 3%, and the nitrogen source was as indicated. YPD medium contained yeast extract (1%), peptone (2%), and glucose (2%). To test the effect of ammonium or methylammonium concentration on the growth of *S. cerevisiae*,

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TABLE 1. Strains

Strain	Genotype	Source or reference
S. cerevisiae		
23344c	*MAT*α *ura3*	35
31019b	*MAT*α *ura3* Δ*mep1* Δ*mep2*::*LEU2* Δ*mep3*::*KanMX2*	35
NCM3243	*MAT*α *ura3* Δ*vph1*::*URA3*	This study
Ig43-1	**a**/α *his4*/+ *lys*/+ *met13*/+ *GLN1*/*GLN1*	A. P. Mitchell
Ig43-3	**a**/α *his4*/+ *lys*/+ *met13*/+ *gln1-103*/*gln1-103*	A. P. Mitchell
MM11	*MAT*α Δ*stv1*::*LYS2 ura3-52*	34
MM50	*MAT*α *leu2-1 ura3-52*	34
MM108	*MAT*α *his4 tfp1(vma1)*Δ*101*::*urA3 ura3-52*	34
MM112	*MAT*α *his3-*Δ*200 leu2 lys2* Δ*stv1*::*LYS2* Δ*vph1*::*LEU2 ura3-52*	34
N. crassa		
74A	Wild type	4
pvn2-53-19A	*vma-1*^{RIP2}	4

cells were first grown in medium 164 at pH 6.1 with glucose (3%) as the carbon source and 10 mM NH_4Cl or glutamate as the nitrogen source. Cells were then inoculated into medium 164 containing different concentrations of NH_4Cl or methylammonium and buffered at pH 7.5 or 7.0, respectively, by the addition of 50 mM Tris buffer. The nitrogen source for cells grown in the presence of methylammonium was 10 mM glutamate. Concentrations of NH_3 and CH_3NH_2 were calculated from those of ammonium and methylammonium using pK_a values of 9.25 and 10.6, respectively (18).

S. cerevisiae strain NCM3243 (Δ*vph1*::*URA3*), which carries a deletion of 64% of the *VPH1* gene (identical to that in BJ6717 [33]), was constructed from wild-type strain 23344c (aka NCM3018; Leu^+ Ura^-) (35) as follows. First, the *LEU2* marker in plasmid pΔ*vph1*::*LEU2* (33), generously provided by E. W. Jones, was replaced with the *URA3* marker in plasmid pJES1256 as described below to yield plasmid pJES1276. Then the 4.0-kb fragment of plasmid pJES1276, which carries Δ*vph1*::*URA3*, was amplified by PCR and introduced into strain 23344c by the lithium acetate method (20). Mutants resulting from recombination of the fragment into the chromosome were selected on minimal medium containing 50 mM NH_4Cl as the nitrogen source and lacking uracil. Several Ura^+ clones were analyzed by PCR and Southern blot and one that showed the correct replacement of *VPH1* was chosen.

Plasmid pJES1276 was constructed in the following several steps. Plasmid pΔ*vph1*::*LEU2* was cleaved with *Xho*I and *Eco*RI, which yielded two fragments of 2.9 kb, one of 1.2 kb, and one of 0.3 kb. The two 2.9-kb fragments were purified from an agarose gel and ligated. The resulting plasmid, pJES1274, carries the 5′ end of *VPH1* followed by a portion of the *LEU2* marker. pJES1274 was linearized with *Eco*RI and made blunt ended with the Klenow fragment of DNA polymerase I. It was then ligated to the 1.2-kb *Hin*dIII fragment from plasmid pJES1256, also made blunt ended, which carries the *URA3* marker, to yield pJES1275. Finally, the 0.3-kb *Xho*I-*Bam*HI fragment of pΔ*vph1*::*LEU2* made blunt ended, which carries a portion of the 3′ end of *VPH1*, was ligated into pJES1275 which had been linearized with *Bam*HI and made blunt ended. The resulting plasmid, pJES1276, carries the Δ*vph1*::*URA3* deletion and insertion. The correct orientation of cloned fragments was confirmed by sequencing.

S. cerevisiae strains MM50 (wild type), MM108 (Δ*vma1*), MM11 (Δ*stv1*), and MM112 (Δ*vph1* Δ*stv1*) (34) (Table 1) were kindly provided by M. Manolson. They were grown in YPD medium containing 2% glucose because MM108 and MM112 grow very poorly in minimal medium, even at a low pH. *N. crassa* strains 74A (wild type) and pvn2-53-19A (*vma-1*^{RIP2}) (4) (Table 1) were kindly provided by B. J. Bowman. They were maintained on slants in Vogel's minimal medium N (7). They were grown in liquid culture in a modified Vogel's medium in which the usual nitrogen sources (ammonium nitrate at 25 mM) were replaced. Ammonium chloride or proline (10 mM) was the nitrogen source and glucose (2%) was the carbon source. Mycelia were grown from conidia inoculated to an initial density of 2×10^6 conidia/ml into 1-liter flasks containing ~150 ml of the appropriate medium (optical density at 600 nm [OD_{600}] of ~0.13). Cultures were grown at 30°C in an orbital shaker (~175 rpm) and mycelia were harvested by filtration when the OD_{600} reached 0.5.

Preparation of genomic DNA for analysis of putative Δ*vph1* strains of *S. cerevisiae*. Genomic DNA was prepared by a miniscale extraction procedure as follows. (i) A 2-ml sample of an overnight culture grown in YPD medium at 28°C (OD_{600} of ≈4) was harvested by centrifugation and washed with 1 ml of H_2O. (ii) The cell wall was digested at 37°C for 40 min in 0.1 ml of lysis buffer (1 M sorbitol, 100 mM EDTA, 4 mM β-mercaptoethanol, 0.5 mg of lyticase [Sigma]/ml). (iii) Spheroplasts were lysed by the addition of 0.1 ml of 0.1 M Tris-HCl (pH 8.0)–10 mM EDTA–2% sodium dodecyl sulfate, vigorous vortexing, and incubation at 65°C for 10 min. (iv) Proteins were precipitated by the addition of 0.1 ml of 5 M potassium acetate and incubation for 1 h on ice. They were removed by centrifugation at high speed for 10 min at 4°C. (v) DNA was precipitated by the addition of 1 volume of isopropanol to the supernatant and centrifugation for 20 min. Pellets were washed with 70% ethanol, air dried, and suspended in 0.2 ml of 10 mM Tris-HCl (pH 7.5)–1 U of RNase A. Typically, 2 ml of culture yielded 50 μg of genomic DNA.

Transport assays. *S. cerevisiae* strains 23344c, 31019b, and NCM3243 were grown in medium 164 with proline (10 mM) as the nitrogen source, unless otherwise indicated. Strains MM50, MM108, MM11, and MM112 were grown in YPD–2% glucose. Cells were harvested at an OD_{600} of 0.5. Assays of [^{14}C]methylammonium transport were performed as described previously (62) at 28°C and pH 6.1. The initial concentration of [^{14}C]methylammonium was 6 μM and the assay buffer contained 50 mM HEPES (pH 6.1), 72 mM NaCl, and 2% glucose.

To derepress synthesis of glucose permeases, cells were grown overnight in YPD at 28°C and were then inoculated into modified YPD containing only 0.05% glucose at an OD_{600} of 0.1 and grown to an OD_{600} of 0.5. Cells were harvested, washed, and suspended in assay buffer lacking glucose. At this step, cells were kept on ice. Assays of D-[1-^{14}C]glucose transport were performed essentially as for [^{14}C]methylammonium. Cells were warmed for 20 min at 28°C and transport was initiated by adding D-[1-^{14}C]glucose (specific activity, 0.59 Ci/mol) to a final concentration of 170 μM.

For testing the effect of weak bases on transport, *S. cerevisiae* cells were washed with 50 mM HEPES, pH 7.5, and then incubated at 28°C for 20 min in the same buffer or buffer containing 200 mM NH_4Cl or 1 mM chloroquine (pK_a values of 8.1 and 10.2) (45), as indicated. Cells were then washed with 50 mM HEPES, pH 7.5, suspended in assay buffer, and used immediately for transport assays.

Harvested mycelia of *N. crassa* were washed with and suspended in a 1/2 volume of chilled assay buffer. Mycelial suspensions were stored on ice until they were used for transport assays, usually within 3 h. Prior to assaying transport of [^{14}C]methylammonium, mycelial suspensions were warmed to 30°C for 30 min. Assays were initiated by adding [^{14}C]methylammonium (50 or 10 Ci/mol for mycelia grown with ammonium chloride or proline, respectively, as the nitrogen source) to a final concentration of 4 μM. Samples (0.5 ml) were filtered at appropriate intervals and washed. Radioactivity was measured by liquid scintillation counting and transport was normalized to mycelial dry weight, which was 0.7 to 0.9 mg/ml.

Analysis of ^{14}C-labeled products. ^{14}C-labeled products accumulated by *S. cerevisiae* or *N. crassa* were analyzed as previously described (62). After the indicated times of exposure to [^{14}C]methylammonium, samples of *S. cerevisiae* (0.5 ml of cells at an OD_{600} of 1) were filtered, washed, suspended in 1 ml of water, and boiled for 20 min. Insoluble material was removed by centrifugation. More than 90% of the radiolabel initially present in cells was extracted by this means. Control experiments demonstrated that methylammonium and methylglutamine were stable to the extraction procedure (data not shown).

Mycelia of *N. crassa* were grown with proline as the nitrogen source. At an OD_{600} of 0.5 they were concentrated approximately twofold by partial filtration. [^{14}C]methylammonium (50 Ci/mol) was added to 4 ml of concentrated mycelial suspension to a final concentration of 5 μM, and 1-ml samples were filtered at appropriate intervals and washed. Filtered samples were placed in 50% ethanol and boiled for 30 min. The radioactivity extracted from each sample was determined by liquid scintillation counting and the same amount (~2,400 cpm) was analyzed chromatographically. After chromatography, radioactivity on thin-layer plates was assessed by autoradiography and/or phosphorimaging.

RESULTS

Transport and accumulation of [^{14}C]methylammonium by *S. cerevisiae*. As reported previously (8, 35), wild-type *S. cerevisiae* 23344c grown in synthetic medium with proline as the nitrogen (N) source accumulated [^{14}C]methylammonium (initial external concentration of 6 μM) slowly over a long time (30 to 40 min), whereas strain 31019b, which lacks all three MEP proteins, did not (Fig. 1A). As previously reported (52), concentration by the wild-type strain appeared to be at least 1,000-

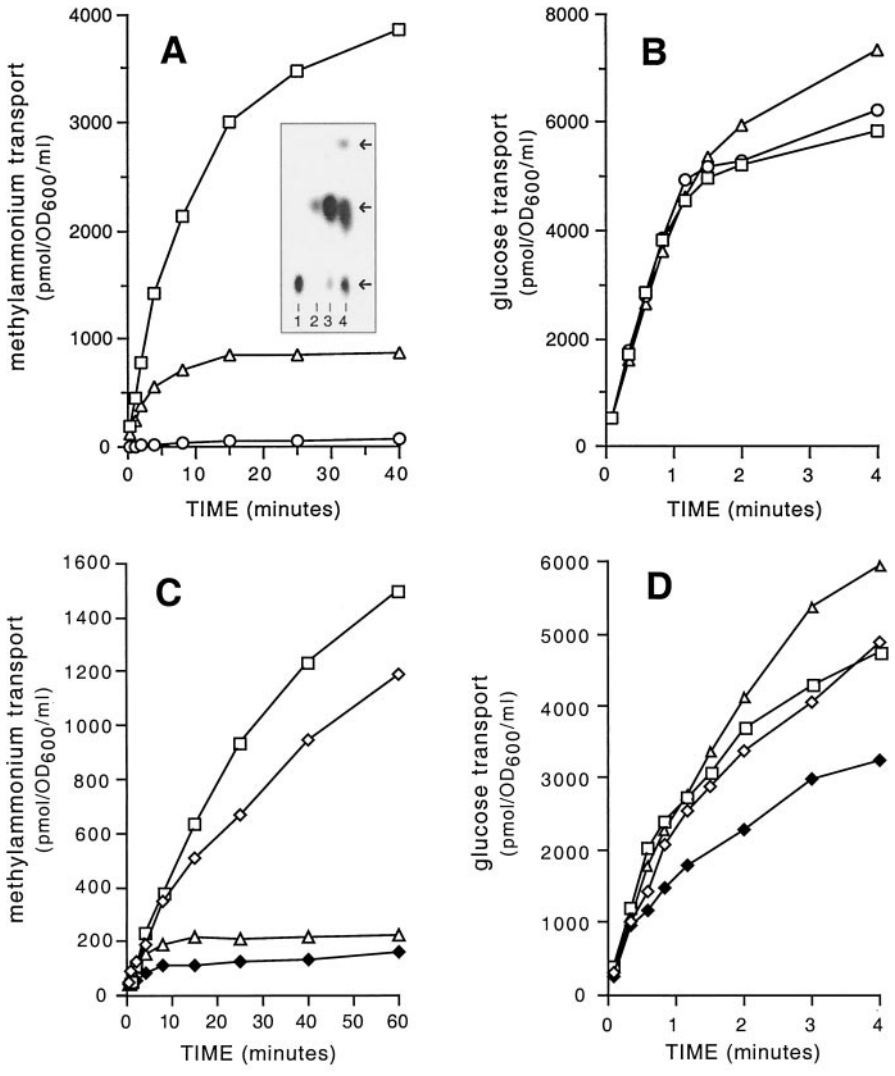

FIG. 1. Transport of methylammonium (A and C) and glucose (B and D) by *S. cerevisiae* (see Materials and Methods). Strains for panels A and B were 23344c (wild type; squares), 31019b (Δ*mep1* Δ*mep2* Δ*mep3*; circles), and NCM3243 (Δ*vph1*; triangles), and strains for panels C and D were MM50 (wild type; squares), MM11 (Δ*stv1*; open diamonds), MM108 (Δ*vma1*; triangles), and MM112 (Δ*vph1* Δ*stv1*; closed diamonds). (A) The initial concentration of [^{14}C]methylammonium was 6 μM. Cells were grown in minimal medium with proline (10 mM) as the N source. The degree of concentration of methylammonium by strain 23344c was calculated assuming a cell volume of 70 μm^3 and 10^7 cells/ml/OD_{600} (59). Inset, ^{14}C-labeled products accumulated intracellularly 25 min after exposure to [^{14}C]methylammonium. Products were separated by thin-layer chromatography and subjected to autoradiography. Lane 1, [^{14}C]methylglutamine; lane 2, [^{14}C]methylammonium; lane 3, wild type; lane 4, Δ*vph1*. (B) The initial concentration of D-[1-^{14}C]glucose was 170 μM. Cells were grown in modified YPD containing 0.05% glucose. (C) As for panel A except cells were grown in YPD–2% glucose. (D) As for panel B.

fold (see legend to Fig. 1). The wild-type strain also accumulated [^{14}C]methylammonium when grown with glutamate, glutamine, or NH_4Cl as the N source (activity, 170 to 270 pmol/ml/OD_{600}/min depending on the N source). Extraction and chromatography of ^{14}C-labeled products indicated that ≤2% of the methylammonium was metabolized, apparently to methylglutamine (Fig. 1A inset, lane 3). In agreement with this, a mutant strain, Ig43-3, that lacks glutamine synthetase (*gln1-103* lesion) (40; A. Mitchell, personal communication) accumulated [^{14}C]methylammonium as rapidly and to the same extent as its congenic parent strain, Ig43-1 (both grown on glutamine as the N source) (data not shown).

Effect of weak bases on accumulation of methylammonium. At higher concentrations than those we employed in transport assays, the weak base methylammonium is commonly used to determine the pH difference across biological membranes (17, 28, 56). Whereas the uncharged species (CH_3NH_2) can diffuse across membranes in an unmediated manner, the charged species ($CH_3NH_3^+$) cannot. Hence, partitioning of methylammonium across a membrane allows for an estimation of the pH gradient. We wondered whether accumulation of [^{14}C]methylammonium in our transport experiments might be due to sequestration of the charged species into acidic compartments. To test this, we first determined whether at high concentrations the weak bases ammonium and chloroquine, which are known to accumulate in acidic compartments and increase the luminal pH (6, 44–46), would interfere with the accumulation of [^{14}C]methylammonium. Treatment of wild-type *S. cerevisiae* strain

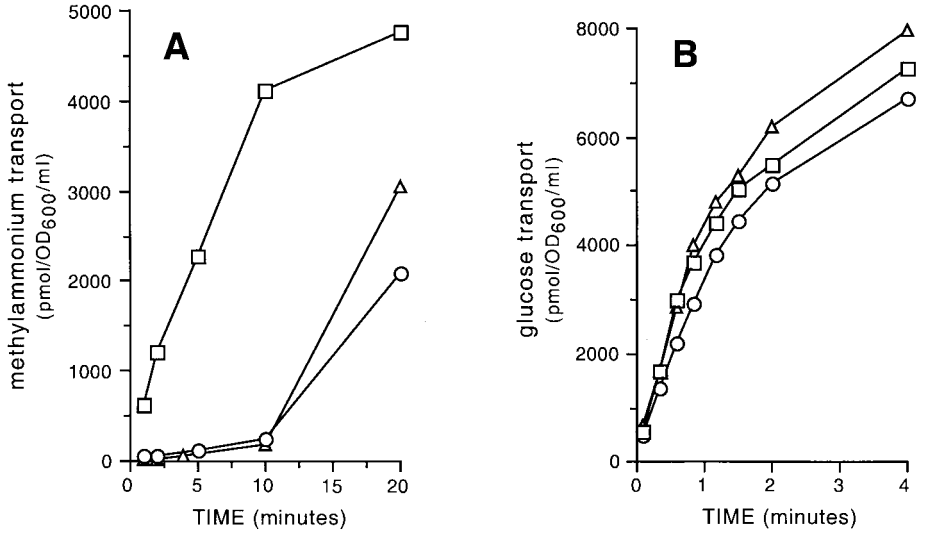

FIG. 2. Effect of weak bases on the accumulation of methylammonium (A) and glucose (B) by *S. cerevisiae* (see Materials and Methods). Transport by strain 23344c (wild type) was assessed after exposure to buffer at pH 7.5 (squares) or to buffer containing 1 mM chloroquine (2 μM unprotonated form; triangles) or 200 mM NH_4Cl (3.5 mM NH_3; circles). (A) The initial concentration of [^{14}C]methylammonium was 6 μM. Cells were grown in minimal medium with proline (10 mM) as the N source. (B) The initial concentration of D-[1-^{14}C]glucose was 170 μM. Cells were grown in modified YPD containing 0.05% glucose.

23344c at pH 7.5 with ammonium (200 mM) for 20 min prevented accumulation of [^{14}C]methylammonium (Fig. 2A). The same was true for treatment with chloroquine (1 mM), which is structurally unrelated to ammonium and methylammonium. In both cases, accumulation resumed 10 min later and in fact was restored to normal (data not shown). The latter may be accounted for by reacidification of vacuoles and other acidic compartments because the assay buffer contained 2% glucose as the energy source. In agreement with the above interpretations, transport and metabolism of D-[1-^{14}C]glucose were not affected by treatment with ammonium or chloroquine (Fig. 2B).

Role of V-ATPase in accumulation of [^{14}C]methylammonium. The pH of the large yeast vacuole is estimated to be between 5.5 and 6.2, which is approximately 0.8 to 1.5 pH units lower than that of the cytosol (49, 50, 68). Acidification of the vacuole is maintained by a specific vacuolar H^+-ATPase (V-ATPase), the assembly and function of which involve several proteins (9, 14, 42). Disruption of the *VPH1* gene, which codes for a subunit of the V-ATPase required for its assembly, results in the loss of ATPase activity and a defect in acidification of vacuoles (33, 49, 50). Introduction of the Δ*vph1* lesion into our wild-type strain decreased accumulation of [^{14}C]methylammonium by ~80%, with little effect on the initial rate of uptake (Fig. 1A). The same was true for disruption of the *VMA1* gene (34), which codes for a catalytic subunit of the ATPase (Fig. 1C). Effects of the Δ*vma1* lesion were studied in a different wild-type background. Like accumulation of [^{14}C]methylammonium in wild-type strains, residual accumulation in the Δ*vph1* and Δ*vma1* strains depended on the presence of glucose in the buffer (data not shown). It may be due to accumulation into other acidic compartments, as has been described for entrapment of fluorescent dyes (32) and/or to increased metabolism (see below). As was the case for cells treated with weak bases, the Δ*vph1* and Δ*vma1* strains showed no defect in the transport and metabolism of D-[1-^{14}C]glucose (Fig. 1B and D, respectively). A strain that carried both the Δ*vph1* lesion and a Δ*stv1* lesion (34), which disrupts a gene homologous to *VPH1*, also showed a large decrease in accumulation of [^{14}C]methylammonium (Fig. 1C). However, the double mutant strain, which grew poorly even in enriched medium, showed a defect in transport and metabolism of D-[1-^{14}C]glucose (Fig. 1D). The Δ*stv1* lesion alone had no effect on accumulation of either [^{14}C]methylammonium or D-[1-^{14}C]glucose.

The fact that the initial rate of [^{14}C]methylammonium uptake was little affected by the Δ*vph1* or Δ*vma1* alleles is in agreement with the view that these lesions do not alter the expression or activity of MEP proteins, which appear to be localized to the cytoplasmic membrane (38; G. Fink, personal communication). Although the Δ*vph1* strain grew slower than the wild type or the Δ*mep* triple mutant on various nitrogen sources (Table 2), it showed no particular growth defect at low ammonium concentrations at pH values below 7. This provided

TABLE 2. Growth of *S. cerevisiae* at pH 6.1 on different nitrogen sources

Nitrogen source[a]	Concentration (mM) of nitrogen source	Doubling time (min)		
		Wild type[b]	Δ*mep1* Δ*mep2* Δ*mep3*[b]	Δ*vph1*
Glutamate	10	160	160	230
Proline	10	200	200	410
Arginine	2.5	160	170	180
Urea	5	270	270	450
NH_4Cl	20	160	175	205
	5	160	290	210
	1	175	>700	215

[a] Cells were grown in medium 164 with glucose (3%) as the carbon source and different nitrogen sources at the concentrations indicated. Strains were 23344c (wild type), 31019b (Δ*mep1* Δ*mep2* Δ*mep3*) (35), and NCM3243 (Δ*vph1*).

[b] Final ODs of strains 23344c and 31019b were 13 and 13 on glutamate, 12 and 13 on proline, 11.5 and 11.5 on arginine, 14 and 13.5 on urea, 16 and 16 on 20 mM NH_4Cl, 10 and 7 on 5 mM NH_4Cl, and 1.3 and 0.4 on 1 mM NH_4Cl.

TABLE 3. Effect of pH on the growth of *S. cerevisiae* at different ammonium concentrations

$(NH_4^+ + NH_3)$ concentration (mM)	Doubling time (min) at pH:[a]			
	5.5	6.5	7.5	8.0
7	165	165	220	420[b]
70	165	170	1,200[b]	NG[c]

[a] Cells were grown in medium 164 with glucose (3%) as the carbon source and ammonium as the sole nitrogen source. At pH values of 7.5 and 8.0, 50 mM Tris was added to the medium for buffering. The wild-type strain was 23344c.
[b] Initial doubling time.
[c] NG, no growth.

an independent line of evidence that the MEP proteins were expressed and localized normally in the Δ*vph1* strain and had normal physiological function.

Interestingly, the Δ*vph1* strain showed increased metabolism of [^{14}C]methylammonium (Fig. 1A). Although methylglutamine accounted for <2% of the ^{14}C-labeled material in wild-type or Δ*mep* strains, methylglutamine and an unidentified new product accounted for ~20% of the ^{14}C-labeled products in the Δ*vph1* strain (Fig. 1A inset, lane 4). Similarly, the unidentified product accounted for up to 20% of the ^{14}C-labeled product in the Δ*vma1* and Δ*vph1* Δ*stv1* strains, which were grown in enriched medium (data not shown).

Perturbation of growth of wild-type *S. cerevisiae* by NH_3. Different synthetic media for *S. cerevisiae* contain high concentrations of ammonium [usually 35 mM $(NH_4)_2SO_4$ = 70 mM $(NH_4^+ + NH_3)$] (Difco manual of dehydrated culture: media and reagents for microbiological and clinical laboratory procedures, Difco Laboratories, Detroit, Mich.). Given that NH_3 can perturb the pH of vacuoles, we wondered whether this could contribute to the slow growth of yeast at neutral and higher pH values. As indicated in Table 3, raising the concentration of ammonium from 7 to 70 mM had no effect on growth at pH values of 6.5 or lower (medium 164 with citrate-phosphate buffer and 3% glucose as the carbon source). However, at pH values of 7.5 or higher, it markedly increased the doubling time. For a given concentration of ammonium $(NH_4^+ + NH_3)$ only the concentration of the uncharged species, NH_3, increases with increasing pH and hence NH_3 must be responsible for growth inhibition. The decrease in growth rate (increase in doubling time) at pH 7.5 was progressive for concentrations of ammonium between 7 and 70 mM (Fig. 3A).

The synthetic yeast medium commercialized by Difco, yeast nitrogen base without amino acids and ammonium, which is identical to synthetic medium SD, is acidic (pH 4.5) and not buffered (67; Difco manual). In this medium increasing the concentration of ammonium had no effect on growth rate (not shown). At 72 h, when the cells had reached stationary phase, the pH had dropped to 1.5 and no more than 15 mM ammonium had been used. This was sufficient for maximum yield. Because higher concentrations of ammonium are not required, providing them simply causes difficulty if one wishes to buffer the medium to a higher pH.

Perturbation of growth of wild-type *S. cerevisiae* by methylamine. Although methylammonium is known to inhibit growth of *S. cerevisiae*, its effects might be different from those of ammonium for a number of reasons; these include the difference in its pK_a value, the difference in its partition coefficient between water and organic solvents, and its failure to be significantly metabolized in the yeast cytoplasm. Methylammonium progressively inhibited growth of wild-type *S. cerevisiae* at concentrations between 10 and 200 mM (Fig. 3B). Inhibition appeared to be biphasic. Effects at low concentrations were more severe than those of ammonium, whereas effects at high concentrations were less severe. As reported previously (8, 52), growth of the Δ*mep* triple mutant was markedly less sensitive to inhibition by methylammonium than growth of wild-type *S. cerevisiae*. There was little inhibition up to 60 mM, but above that the decrease in growth rate as a function of concentration (slope of the curve in Fig. 3B) was the same as for the wild

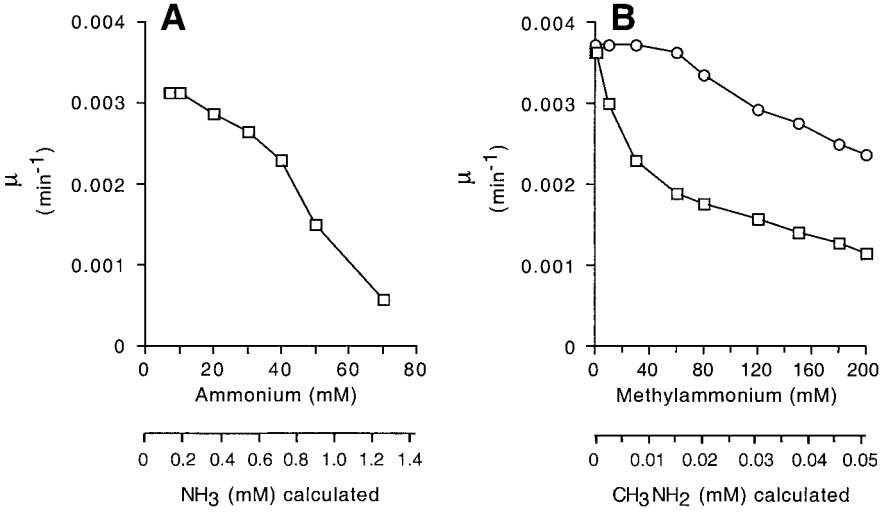

FIG. 3. Effect of different concentrations of ammonium (A) or methylammonium (B) on the growth rate constant μ of *S. cerevisiae*. (A) Strain 23344c (wild type) was grown in medium 164 buffered with 50 mM Tris, pH 7.5, with glucose (3%) as the carbon source and NH_4Cl as the sole nitrogen source. (B) Strains 23344c (wild type; squares) and 31019b (Δ*mep1* Δ*mep2* Δ*mep3*; circles) were grown in medium 164 buffered with 50 mM Tris, pH 7.0, with glucose (3%) as the carbon source and glutamate (10 mM) as the sole nitrogen source.

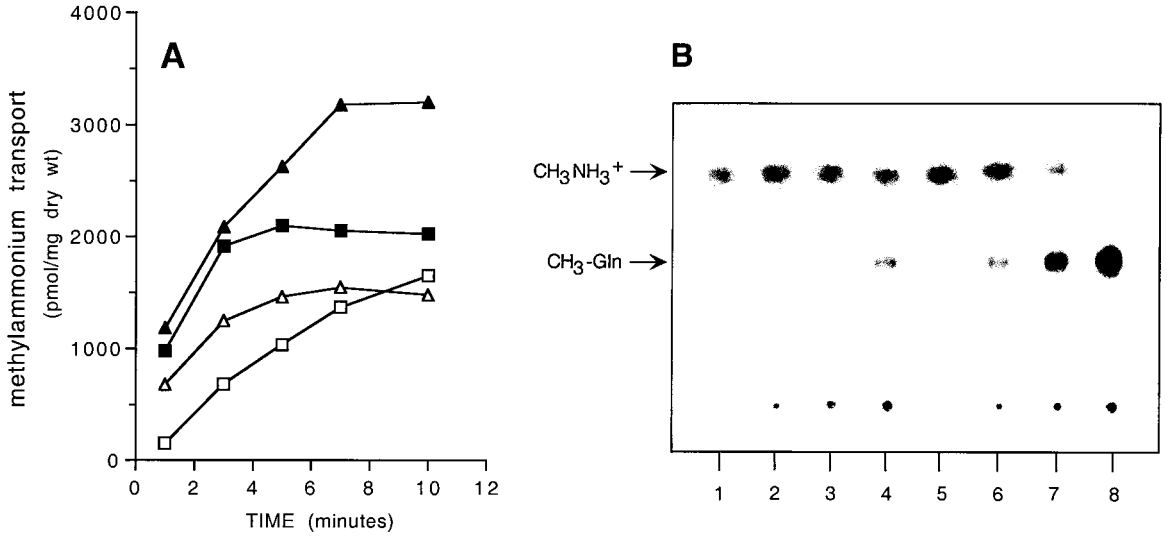

FIG. 4. Transport of methylammonium by *N. crassa* (A) and characterization of the products accumulated (B) (see Materials and Methods). (A) The initial concentration of [^{14}C]methylammonium was 4 μM. Strains 74A (wild type; squares) and pvn2-53-19A (*vma-1*; triangles) were grown in modified Vogel's minimal medium with glucose (2%) as the carbon source and 10 mM NH_4Cl (open symbols) or 10 mM proline (closed symbols) as the sole nitrogen source. (B) Components of cell extracts (~2,400 cpm) were separated by thin-layer chromatography and subjected to autoradiography. Lanes 1 to 4, strain 74A (wild type); lanes 5 to 8, strain pvn2-53-19A (*vma-1*). Extracts were prepared 1 min (lanes 1 and 5), 4 min (lanes 2 and 6), 10 min (lanes 3 and 7), and 25 min (lanes 4 and 8) after exposure to [^{14}C]methylammonium (initial concentration, 5 μM). Cells were grown in Vogel's minimal medium with proline (10 mM) as the N source. Positions of [^{14}C]methylammonium ($CH_3NH_3^+$) and [^{14}C] methylglutamine (CH_3-Gln) are indicated on the left.

type. Growth inhibition of the mutant at high concentrations may be a function of unmediated diffusion of the uncharged species CH_3NH_2 across the cytoplasmic membrane. Presumably, both CH_3NH_2 and NH_3 diffuse across vacuolar membranes, whose composition differs from that of the cytoplasmic membrane (e.g., differences in lipid composition [5, 57, 63]), in an unmediated manner.

Role of V-ATPase in accumulation of [^{14}C]methylammonium by *N. crassa*. Although there have been no biochemical or genetic studies of MEP proteins in *N. crassa*, its genome carries at least two genes that code for such proteins (*Neurospora* Genome Project, University of New Mexico [http://biology.unm.edu/biology/ngp/home.html]). As is the case for *S. cerevisiae*, the vacuole of *N. crassa* is acidic and its pH has been estimated to be ~6 (30). However, unlike the case for *S. cerevisiae*, disruption of the function of the vacuolar ATPase by the *vma-1^{RIP2}* mutation apparently caused no decrease in the accumulation of [^{14}C]methylammonium (Fig. 4A). This was true whether mycelia were grown on 10 mM proline or 10 mM ammonium chloride as the N source, although accumulation by mycelia grown on proline was about twice that by mycelia grown on ammonium chloride. The *vma-1* mutant of *N. crassa* showed profoundly increased metabolism of [^{14}C]methylammonium with respect to the congenic wild-type strain (Fig. 4B and Table 4). Whereas the ratio of [^{14}C]methylglutamine to [^{14}C]methylammonium in the wild-type strain increased from 0.1 after 10 min to 0.3 after 25 min of exposure, this ratio in the *vma-1* mutant strain increased from 0.7 to 3 over the same interval (Table 4). Presumably, increased metabolism in the mutant strain can be accounted for by a longer residence time of methylammonium in the cytosol, where it can be assimilated by glutamine synthetase. We do not know why a higher proportion of [^{14}C]methylammonium was converted to methylglutamine in the *vma-1* mutant strain of *Neurospora* than in the Δ*vma1* strain or other vacuolar ATPase mutants of *S. cerevisiae*. However, we note that *vma1* mutant strains of the two organisms differ in a number of respects (4). For example, vacuoles of the *Neurospora* mutant, which is much more highly branched than its parent, often appear to be distorted and multilamellar, whereas vacuoles of *S. cerevisiae* appear to be morphologically normal.

Lack of involvement of the MEP proteins of *S. cerevisiae* in growth on alternative nitrogen sources. Marini and colleagues proposed that the MEP proteins of *S. cerevisiae* were needed to recover ammonium that leaked from cells during growth on nitrogen sources other than ammonium (35). However, we found that the Δ*mep* triple mutant showed no growth defect on a variety of alternative nitrogen sources at pH 6.1 (Table 2),

TABLE 4. Quantitative analysis of ^{14}C-labeled products accumulated by *N. crassa* during transport of [^{14}C]methylammonium

Time after exposure (min)[a]	Normalized intensity (%) of indicated product accumulated by:[b]					
	Wild type			*vma-1*		
	$CH_3NH_3^+$	CH_3-Gln	Origin	$CH_3NH_3^+$	CH_3-Gln	Origin
1	93	2	5	92	4	4
4	87	4	9	77	16	7
10	80	8	12	52	37	11
25	60	17	23	18	57	25

[a] Thin-layer chromatography plates were analyzed with a Molecular Dynamics PhosphorImager. Samples were as shown in Fig. 4B (see Materials and Methods). Strains were 74A (wild type) and pvn2-53-19A (*vma-1*) (4).

[b] $CH_3NH_3^+$, [^{14}C]methylammonium; CH_3-Gln, [^{14}C]methylglutamine; origin, ^{14}C-labeled products that were not resolved in our system.

although it had the expected defects at external ammonium concentrations of ≤5 mM. Thus, in our hands growth tests also failed to provide evidence that the MEP proteins concentrate ammonium in an energy-dependent manner.

DISCUSSION

Function of the MEP proteins of fungi. In *S. cerevisiae*, transport of [^{14}C]methylammonium across the cytoplasmic membrane depends on the three MEP proteins, and accumulation of [^{14}C]methylammonium provided the strongest evidence that these proteins catalyze energy-dependent concentration of their substrates. However, we have now shown that accumulation appears to depend on energy-dependent sequestration of [^{14}C]methylammonium into vacuoles and other acidic compartments (Fig. 1 and 2) and hence does not provide evidence for energy-dependent concentrative transport across the cytoplasmic membrane. Accumulation of [^{14}C]methylammonium was decreased more than 80% by mutations that decrease or eliminate vacuolar ATPase activity (Fig. 1) and was completely eliminated by weak bases, which are known to neutralize acidic compartments (Fig. 2). Neither affected transport or metabolism of D-[1-^{14}C]glucose.

The second line of evidence that MEP proteins mediated concentrative uptake of their substrates was that *mep* mutants showed impaired growth on nitrogen sources other than ammonium (reported previously [35] but data were not shown). Growth defects and cross-feeding of ammonium to other strains were attributed to the inability of *mep* mutants to accumulate the ammonium they leaked during catabolism of alternative nitrogen sources. We were unable to confirm the growth defects previously reported. Rather, we found that the Δ*mep* triple mutant showed no defect in either growth rate or cell yield on several alternative nitrogen sources (Table 2).

Given that the AmtB protein of enteric bacteria, which is homologous to the MEP proteins, fails to concentrate either methylammonium or its natural substrate ammonium (62), the most parsimonious interpretation of the results in fungi is that MEP proteins, like AmtB, facilitate methylammonium and ammonium diffusion. Acidic trapping of the charged species $CH_3NH_3^+$ in fungal vacuoles implies that the substrate for the MEP proteins is the uncharged species CH_3NH_2. By analogy, their natural substrate would be NH_3. This is in accord with previous findings that MEP/AmtB proteins are required for growth at low ammonium concentrations only at low pH values (62), that is under conditions in which the uncharged species NH_3 is limiting. Under other circumstances, i.e., higher ammonium concentrations or higher pH values, NH_3 can apparently diffuse across the cytoplasmic membrane in an unmediated manner fast enough to support optimal growth. In fact, if both ammonium concentration and pH are high, NH_3 can diffuse across fungal membranes fast enough to inhibit growth, presumably by neutralization of vacuoles and/or other acidic compartments. This may contribute to slow growth of fungi in standard minimal media at pH values of ≥7 (Fig. 3) (49).

Mutant strains of *S. cerevisiae* and *N. crassa* that cannot acidify their vacuoles metabolized larger amounts of [^{14}C]methylammonium to γ-*N*-methylglutamine than their parent strains (Fig. 1 and 4; Table 4). This was particularly true of the *N. crassa* mutant, which showed no defect in the accumulation of the ^{14}C label (Fig. 4; see Results). Presumably metabolism occurs because methylammonium remains in the cytoplasm long enough to be assimilated in an energy-dependent manner by glutamine synthetase. Thus, the fungal mutant strains behave like bacteria, which lack vacuoles.

Quantitative aspects of ammonium and methylammonium transport. We noted previously that enteric bacteria require the AmtB protein for optimal growth when the external concentration of the uncharged species NH_3 falls to ~50 nM or below (total concentration of ammonium, 10 μM at pH 7 or 1 mM at pH 5 [62]). *S. cerevisiae* appears to require the MEP proteins at a concentration of NH_3 that is 100-fold higher (5 μM NH_3 = 1 mM ammonium at pH 7.1 [62]). The requirement for porters in *S. cerevisiae* at a higher NH_3 concentration is likely due to several factors. (i) The volume of *S. cerevisiae* is 70 times larger than that of enteric bacteria, whereas its surface area is only 16 times larger (59). Thus, its surface/volume ratio is only 1/5 that of enteric bacteria. (ii) Differences in membrane composition between *S. cerevisiae* and enteric bacteria (5, 26) may reduce the rate of unmediated diffusion of NH_3 in *S. cerevisiae*. (iii) Sequestration of NH_3 into acidic vacuoles in *S. cerevisiae* may reduce its rate of assimilation into the central intermediates of nitrogen metabolism by cytosolic glutamine synthetase and glutamate dehydrogenase.

Although the pH of the yeast vacuole is at most 0.6 units below that of the buffered medium we used for transport assays (49), [^{14}C]methylammonium was concentrated 1,000-fold rather than the expected 4-fold. Additional concentration, which occurred slowly, may be due to homeostatic mechanisms that allow continued acidification of vacuoles as weak base is accumulated. Whereas concentration of [^{14}C]methylammonium was from 6 μM to 6 mM, vacuoles are known to accumulate basic amino acids and cations to far higher concentrations of several hundred millimolar (29, 43, 47, 54).

Function of the human Rhesus antigen-associated protein RhAG and its homologue from kidney RhCG (RhGK). Marini and colleagues found that the human Rhesus antigen-associated protein (RhAG) and the Rhesus antigen itself, which are prominent on the surface of red blood cells, show sequence relatedness to MEP/Amt proteins (36). In fact, however, Rh proteins bear sequence relatedness to MEP proteins over only a single domain, whereas MEP/Amt proteins are related to one another across three domains (E. Soupene, unpublished observations). The nematode *Caenorhabditis elegans* contains members of both the MEP and Rh subfamilies (36).

Marini and colleagues recently proposed that RhAG and its kidney homologue RhCG (31) (RhGK) actively transport NH_4^+ (37). Their conclusion rests on the ability of the human proteins to complement the growth defects of the Δ*mep* triple mutant of *S. cerevisiae* at low ammonium concentrations. However, their evidence appears self-contradictory. Cloned RhAG and RhCG allowed the Δ*mep* triple mutant to form small colonies in 5 to 7 days at low ammonium concentrations; they did not allow it to take up [^{14}C]methylammonium, presumably because complementation was partial. Unexpectedly, cloned RhAG and RhCG made the Δ*mep* triple mutant resistant to methylammonium under conditions in which both it and wild-type *S. cerevisiae* were sensitive. The ad hoc interpretation of the latter finding was that the Rh proteins actively export methylammonium. In summary, the incompatible results were

that RhAG and RhCG allow the Δ*mep* triple mutant to grow at low ammonium concentrations but make it resistant to methylammonium.

As discussed in Results, MEP proteins themselves confer sensitivity to methylammonium, and in fact their name derives from this property. The Δ*mep* triple mutant was selected for resistance to growth inhibition by methylammonium (8) and is more resistant than the wild type over a wide range of concentrations (Fig. 3B). In assessing the effect of cloned Rh proteins on the methylammonium resistance of triple *mep*Δ, Marini et al. used 200 rather than the usual 100 mM methylammonium, an unusual circumstance under which the Δ*mep* triple mutants like the wild type, is sensitive. It is likely that sensitivity is caused by unmediated diffusion of methylammonium (specifically CH_3NH_2) across the cytoplasmic membrane (see second phase of inhibition in Fig. 3B). Hence the effects of overexpressed RhAG and RhCG may be due to a perturbation of the yeast cytoplasmic membrane that causes a decrease in unmediated CH_3NH_2 diffusion.

Marini et al. (37) fail to consider our evidence that Amt/MEP proteins are specific for the uncharged species CH_3NH_2 and NH_3 rather than the charged species $CH_3NH_3^+$ and NH_4^+ and that they increase the rates of diffusion of the neutral species rather than actively transporting them (62). Although the Rh complex of red blood cells- and other members of the Rh subfamily- may also mediate diffusion of NH_3, this seems unlikely physiologically (31). Rather, we speculate that the Rh complex may be the postulated protein facilitator for diffusion of CO_2 (10), which like NH_3 is a gas that also crosses membranes passively. Further assessment of the substrate specificities of Rh and MEP proteins and of the variety of their physiological roles in different organisms and tissues seems warranted.

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