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AMMONIA ACCUMULATION IN ACETATE-GROWING YEAST

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During growth on acetate, the pH of yeast cultures rises from 5.8 to around 7–8 in the stationary phase. This was found to result from acetic acid uptake and accompanying H^+ loss. In addition, acetate-growing yeast were found to accumulate ammonia. The influence of pH on ammonia transport and accumulation was studied with the analogue [^{14}C]methylamine with the following results. (a) Methylamine uptake kinetics from 0.1–50 mM were consistent with a single-component uptake system (NH_4^+ permease) at pH values more acidic than 6.5, and with a two-component system (NH_4^+ permease and NH_3 diffusion) above pH 7.5. (b) Equilibrium accumulation of methylamine was found to increase with increasing pH. (c) Methylamine efflux from methylamine-loaded cells increased as the external pH decreased. It was concluded from measurements of the internal pH under various culture conditions that the accumulation of ammonia in acetate-growing alkaline cultures resulted from the sum of two processes: (1) an energy-driven NH_4^+ transport; and (2) NH_3 diffusion dependent on the ΔpH.

Introduction

The utilization of acetate by growing yeast has received very limited attention in the past. This is surprising, since acetate has universally been used in preparing sporulation media. A possible reason for this lack of attention is the belief that most yeast strains are unable to grow on acetate, or do so only after considerable lags [1]. Chu et al. [2] have shown that during the lag period there is a destruction of ATP by reaction with acetate.

In this paper we have investigated the growth of yeast on acetate media particularly with respect to ammonia * uptake. We have found that acetate-grown cells accumulate ammonia up to very high levels. Our results suggest that (a) two different factors contribute to ammonia balance in yeast, i.e., NH_4^+ active transport and NH_3 diffusion, and (b) ammonia overaccumulation is brought about only under conditions in which the second process becomes predominant.

Materials and Methods

Organism and growth conditions. A diploid wild-type strain (S18) of *Saccaromyces cerevisiae*

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* The term ammonia as used in this text does not define the state of protonation but rather, the sum of protonated and unprotonated forms present. The specific protonated states are described as NH_3 or NH_4^+.

was used throughout the study. Strain 1278b, supplied by Dr. E. Dubois, was also used for comparative purposes. Yeast were grown with aeration at 30°C in a chemically defined medium containing yeast nitrogen base (Difco) lacking amino acids and ammonium sulfate, supplemented with 2% glucose or 150 mM acetate and 10 mM ammonium sulfate or 20 mM glutamate. The pH of the culture was routinely adjusted to 5.5. During growth, cell density was determined turbidimetrically in diluted (1/6) culture samples at 640 nm.

Determination of intracellular ammonia. Samples (50 ml) from exponentially growing cultures were collected on 1.2 μm pore filters, washed twice with 10 ml distilled water, and frozen in liquid nitrogen. Frozen filters were ground in 3 ml 3 M $HClO_4$ and 0.4 ml 0.4 M Tris, sonicated and centrifuged at $5000 \times g$ 15 min. The supernatants were neutralized with KOH, and after resedimenting the precipitate by centrifugation, used for ammonia determination [3].

Uptake and enzyme assays. For initial methylamine uptake rate experiments, glucose + ammonia or acetate + ammonia exponentially growing yeast were suspended in 2% glucose or 150 mM acetate and 10 mM sodium phosphate buffer at the pH values indicated, and incubated with shaking at 30°C for 2 h. After adjusting the pH, methylamine uptake was started by the addition of [^{14}C]methylamine (130000–22000 cpm/μmol), and samples (0.9 mg dry weight) were withdrawn at 1-min intervals, diluted into 5 ml of incubation medium, filtered (1.2 μm pore size), and washed twice with 5 ml of the incubation medium. The radioactivity on the filters was determined by liquid scintillation counting. Unless otherwise indicated, the methylamine concentration was 0.5 mM.

In the equilibrium accumulation experiments, uptake was also determined by the disappearance of [^{14}C]methylamine from the incubation media, after centrifugation (15 s) of the samples.

Acetate uptake was assayed in exponentially growing glucose + ammonia yeast, resuspended in 20 mM sodium phosphate buffer (pH 5.5) and incubated with aeration for 1 h at 30°C. Uptake was initiated by the addition of [^{14}C]acetate (10 mM, 0.454 μCi/μmol) and samples were withdrawn at 1-min intervals, filtered, washed and counted for radioactivity as described above.

Cell-free extracts for enzyme assays were obtained by cell disruption in a MSK cell homogenizer. NADP-glutamate dehydrogenase was assayed as described in Ref. 4.

Measurement of intracellular pH. Internal pH and intracellular aqueous space were measured as described by Rottenberg [5] through the distribution of [^{14}C]propionic acid (53 mCi/mmol) at a concentration of 17 μM unless otherwise indicated. The intracellular volume was determined by using $^{3}H_2O$ for the total aqueous space and subtracting the space occupied by [^{14}C]poly(ethylene glycol) [5]. 1 mg of cells (dry weight) is equivalent to 5.42 μl of intracellular aqueous space.

Determination of culture medium acetate, ammonia and glutamate. The utilization of acetate, ammonia and glutamate during growth on these carbon and nitrogen sources was followed in culture media samples obtained after removal of the yeast cells by filtration. The assays for ammonia, acetate and glutamate were carried out following Refs. 3,6,7.

Results and Discussion

Time-course of acetate, ammonia and glutamate utilization in acetate cultures

Acetate utilization and pH changes of the media during yeast growth on two different nitrogen sources are shown in Fig. 1. It can be seen that, regardless of the nitrogen source, the amount of acetate used during growth is approximately the same, except for a slightly smaller utilization in glutamate cultures that possibly arises from the use of glutamate as additional carbon source (around 125 μmol/ml in acetate + ammonia and 100 μmol/ml in acetate + glutamate). As reported earlier [8], accompanying acetate utilization, a pH increase is observed which is also the same in both cultures. This suggests that the alkalization of the media is independent of the type of nitrogen source and therefore is a consequence of acetate utilization itself.

The time-courses of ammonia and glutamate utilization are also shown in Fig. 1. It is apparent from the differences in utilization of the two nitrogen sources (around 12 μmol/ml ammonia in NH_4^+ cultures versus 5 μmol ammonia (α-amino) in glutamate), that ammonia is being taken up to a

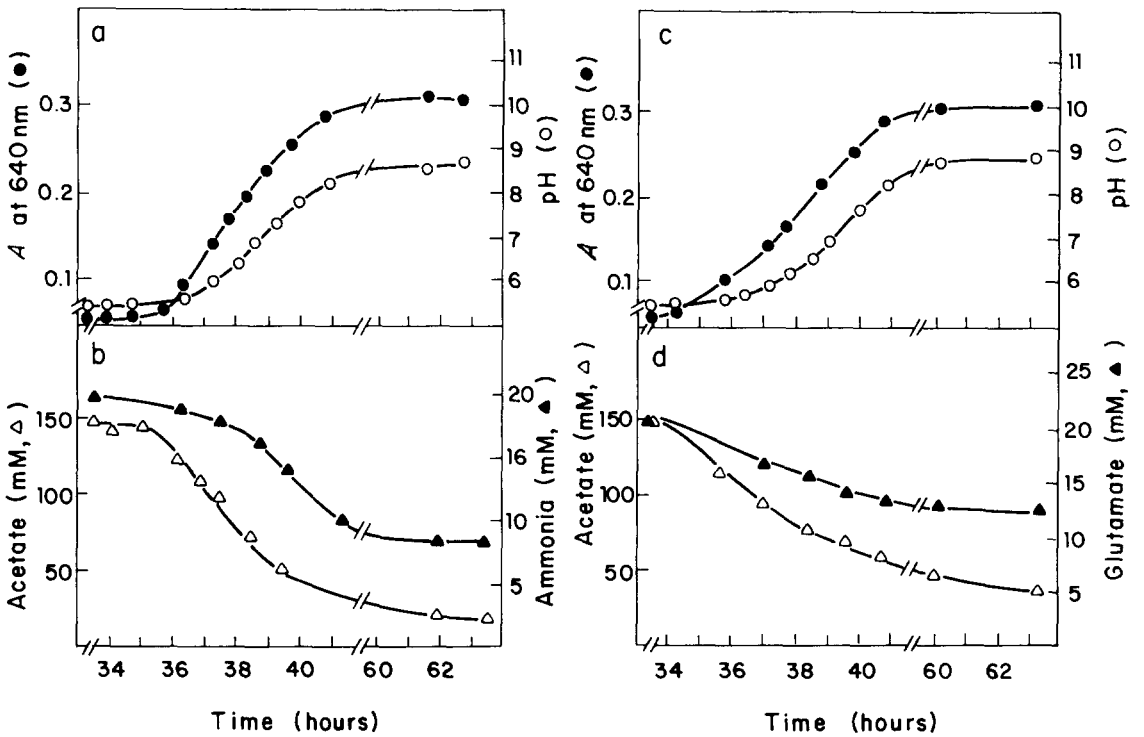

Fig. 1. Time-course for cell growth, nitrogen and carbon use, and pH of media in acetate cultures. The yeast were grown on 150 mM acetate and ammonium sulfate (a,b) or glutamate (c,d) as nitrogen sources.

larger extent than glutamate in acetate cultures. Since its use does not contribute to a rise in cell mass (final cellular densities of both cultures are equivalent), ammonia is probably accumulating within the cells. This has been confirmed after finding that yeast cells from acetate + ammonia cultures have a 40-times higher ammonia internal concentration than those from acetate + glutamate or glucose + ammonia cultures (unpublished data).

The origin of H^+ loss in acetate cultures

In most living cells or subcellular organelles, acetic acid is known to be taken up by the process of diffusion. We have also found that unrelated yeast strains (S18, 1278b) show non-saturation kinetics of acetate uptake, indicating that acetic acid is also freely diffusible in yeast (results not shown). This accounts for an earlier observation in which it was found that the extent of growth on acetate was strictly dependent on the initial pH of the media, low pH levels giving rise to a larger growth.

Therefore it is felt that the loss of H^+ in acetate growth media is due to the uptake of acetic acid. This is supported by two additional findings.

(1) The alkalization/growth quotient is a constant ratio, independent of the cation used in making the acetate salt. The use of four different cations (Na^+, K^+, Li^+, Ca^{2+}) gave rise to similar ratios during early exponential growth. During middle and late exponential phase, Li^+ cultures showed a retarded growth, while Ca^{2+} cultures were found to accumulate a precipitate ($CaCO_3$) that allowed for a smaller alkalization.

(2) The addition of 29 μmol/ml of acetic acid to mid-exponential culture media restored the initial culture pH. Since the amount of acetic acid used during growth to mid-exponential phase in acetate + ammonia cultures was 72 μmol/ml, a quantity much larger than the total amount of H^+ lost from the media during growth, the loss of acetic acid could easily account for the alkalization of the media. In addition, there is apparently a process of acid secretion masked by the acetic

acid-dependent alkalization, since otherwise there would be a much larger pH increase. This process of acid secretion has remained unobserved in the past [9].

The effect of pH on methylamine uptake

Roon et al. [10] have described an NH_4^+ transport system that operates in yeast, over a narrow pH range (5.5–7.5) with maximal activity at 6.5. Dubois and Grenson [11] reported the existence of a second NH_4^+ transport system operating at lower ammonia concentrations than the one initially discovered. Ammonia permeases have now been widely reported in microorganisms, and are energy-dependent [12].

On this basis, the cause of ammonia accumulation in acetate grown yeast could be one of the following: (a) a decrease in the ammonia utilization rate by cellular aminating processes with no alteration in the uptake rate; (b) activation of the ammonia permease(s) by acetate; and (c) a contribution of an additional or alternative transport system to ammonia uptake, specifically diffusion, provided that an adequate ΔpH exists (acid inside).

Condition (a) is consistent with the drop in NADP-glutamate dehydrogenase, the main aminating pathway in yeast, that takes place in these cells. However, the variations of NADP-glutamate dehydrogenase under a number of external ammonia concentrations allowed us to conclude that NADP-glutamate dehydrogenase decrease is a consequence, rather than a cause, of ammonia accumulation (unpublished data). Condition (b) is ruled out, since the activity of the ammonia permease in acetate grown cells is around 100-times lower than in glucose grown cells (7–32 nmol methylamine/min per mg dry weight for glucose cells versus 0.04 nmol/min per mg dry weight for acetate grown yeast). On the other hand, possibility (c) is more likely, since the rise in pH increases the proportion of the free diffusible species, NH_3, while lowering that of the charged (NH_4^+), carrier-transported species. The following results provide support for this last possibility.

(a) Uptake kinetics. We have investigated the relative contributions of carrier-mediated transport and diffusion on methylamine uptake at various pH values. Methylamine (pK' 10.6) has been shown to be transported by the ammonia carrier,

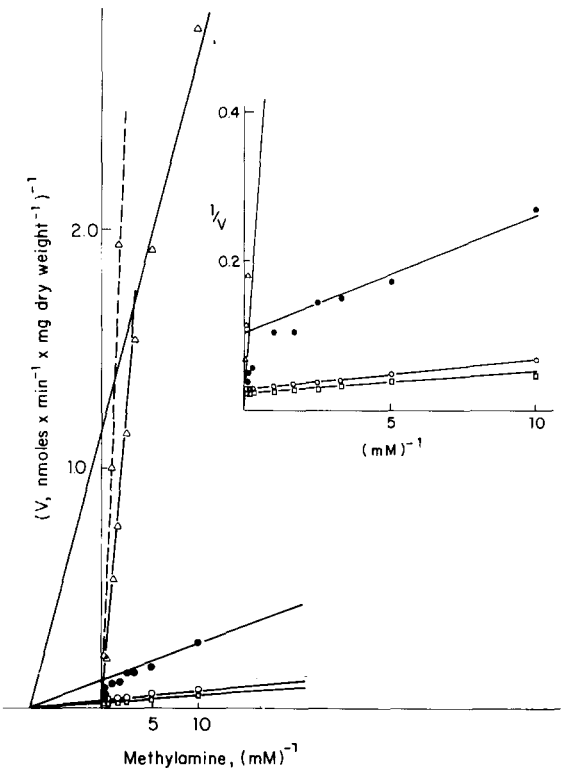

Fig. 2. Effect of pH on methylamine uptake. The assay conditions were those described in Materials and Methods, and methylamine concentrations were varied as indicated on the abscissa. The K_m obtained from these plots is the same for all pH conditions, approx. 0.14 mM. pH: △, 8.5; ●, 7.5; ○, 6.5; □, 5.5. The trace △------△ is a theoretical estimate of the uptake rate at pH 8.5 after subtracting the activity of the 0.14 mM K_m transport system. The inset shows the higher concentration range points at a larger scale.

but unlike ammonia, it is not metabolized by yeast cells [10]. The dependence on substrate concentration of the initial uptake rates of methylamine at pH values 5.5–8.5, is shown in Fig. 2. Uptake at pH 5.5 and 6.5 show saturation kinetics, and the double-reciprocal plots in Fig. 2 give straight lines, consistent with the presence of only one transport system over the concentration range 0.1–50 mM with an affinity of 0.13–0.14 mM for methylamine. The Lineweaver-Burk plot of the results is best fitted by two components. The high-affinity component, although much less active (a 35-times lower V_{max}) is still present; however, at methylamine concentrations higher than 0.3 mM, a low-affinity component predominates. This component is thought to represent simple diffusion of the free

methylamine. The dashed line representative of the process has been calculated after subtracting the pH 8.5 permease activity from the total uptake.

A two-component double-reciprocal plot is also found at pH 7.5, but in this case the high-affinity component is predominant at concentrations up to 1 mM. At higher concentrations, uptake is mediated by diffusion: when subtracting the activity of the permease at pH 7.5, the uptake rates in the range 1–50 mM methylamine fit the same theoretical (dashed) diffusion line.

(b) Accumulation ratios. The total accumulation of methylamine at different pH levels is shown in Table I. Because of the long incubation times required to obtain equilibrium, the molarity of the buffer used was increased in order to prevent unacceptable pH drifts. Equilibrium was attained after different incubation periods, longer with increasing pH. In all cases, the cells were unable to maintain the in/out ratio recorded in Table I for periods longer than 1 h (with pH 3, the retention period is much shorter). This is reflected in the discrepancy between the in/out values at 1 h and 3 h, pH 6.5.

The results from Table I show that the internal/external ratios vary with pH and that larger accumulations are observed at higher pH values. This is consistent with the finding that internal ammonia concentration greatly increases in cells grown on alkaline-buffered glucose media and on acetate media (Table II). The effect of increasing pH on methylamine and ammonia accumulation supports the concept that a diffusion process is involved.

(c) Ammonia efflux from ammonia-loaded cells. If the media pH determines the extent of ammonia accumulation through NH_3 diffusion and pH-dependent distribution, the efflux from ammonia-loaded cells should be pH dependent. Table I shows that this is indeed the case. Following equilibrium accumulation at pH 6.5, a pH shift towards acid or alkaline values had opposite effects. Whereas a decrease in the medium pH to 3 gave rise to a rapid loss of methylamine from the cells, an increase in the pH brought about a transient release of methylamine followed by reuptake that led, after 1 h, to an amine accumulation larger than that of the pH 6.5 cells. This initial release of methylamine also occurred after the addition of a cation (K^+, Na^+, $Tris^+$) or small amounts (20 μM) of ammonium sulfate, and probably reflects the cation exchange activity in yeast [13,14].

TABLE I

ACCUMULATION OF METHYLAMINE

(A) Equilibrium accumulation of methylamine at various pH. Glucose + ammonia growing yeast were transferred to incubation media containing 2% glucose and different buffers. After 2 h incubation [^{14}C]methylamine (40 μM) was added and its uptake followed as indicated in Materials and Methods. The results show the methylamine in/out concentration ratios at equilibrium and the times between brackets are the times needed to reach equilibrium. *(B) Accumulation of methylamine in methylamine-loaded yeast after a pH transition.* Yeast were allowed to accumulate methylamine at pH 6.5 until equilibrium. The pH was then changed to the values indicated and methylamine accumulation allowed to proceed to equilibrium.

	A Accumulation at equilibrium						B Accumulation following a transition to:			
pH:	3.0	4.0	5.0	6.5	7.5	8.5	3.0	6.5	7.5	8.5
50 mM Tris 50 mM KCl	56 (45 min)			241 (1 h)	306 (1 h)	320 (1.5 h)				
100 mM Tris 100 mM KCl				326 (1 h)				119 (3 h)	184 (3 h)	199 (3 h)
100 mM PO_4HK_2/ PO_4HK	69 (1 h)	208 (1 h)	251 (1 h)	304 (1 h)						
				309 (1 h)			88 (3 h)	168 (3 h)		

TABLE II

INTERNAL pH AND AMMONIA OR METHYLAMINE ACCUMULATION UNDER DIFFERENT CULTURE CONDITIONS

Ammonia accumulation and internal pH were measured in exponentially growing yeast (Expts. 1, 3, 5, 7, 8, 9). In Expts. 2, 4 and 6, exponentially growing acetate + ammonia or glucose + ammonia yeast were resuspended in acetate or glucose incubation media of the indicated composition, and after 2 h incubation used for the determination of methylamine equilibrium accumulation and internal pH. Methylamine and propionic acid concentrations used are as indicated.

Expt.	Medium composition	Culture pH	Ammonia in/out	Methylamine in/out	Internal pH	Methylamine concentration (mM)	Propionic acid concentration (μM)
1	2% acetate (pH 5.5)/ 10 mM ammonium sulfate	7.3	3.4				
		8.55			7.34		4.9
		8.9			7.39		29.5
2	2% acetate (pH 7.5)	7.5			6.67		16.3
3	2% acetate/0.2 M Pipes (pH 5.5)	5.7	0.52				
4	2% acetate (pH 3.5)/ 50 mM Tris/50 mM KCl	3.5			< 3		29.5
5	2% glucose (pH 5.5)/ 10 mM ammonium sulfate	3.5	0.061				
		3.2			6.09		4.9
6	2% glucose (pH 3.5)/ 50 mM Tris/50 mM KCl	3.5		60	6.16	1.8	
				12.26		17	
7	2% glucose/0.2 M Pipes (pH 5.5)/ 10 mM ammonium sulfate	5.1	0.73		6.74		17
8	2% glucose (pH 5.5)/ 20 mM glutamate	5.2			6.56		4.9
9	2% glucose/0.2 M Pipes (pH 7.5)/ 10 mM ammonium sulfate	7.2	1.93		7.13		17

The driving force for methylamine accumulation

Methylamine is concentrated around 300-fold in normally growing yeast (Table I medium pH 5–8.5) and higher values have been reported [10]. This concentration ratio is more than can be due to a pH gradient driving the diffusion of NH_3: with medium pH 7.5 and 8.5 the internal pH levels required would be 5.02 and 6.01 [15] and not the 7.1–7.4 found (Table II). This indicates that an additional active uptake process is operating.

The driving force for steady-state ammonia or methylamine accumulation by microorganisms has been reported to be due to the membrane potential (*Clostridium pasteurianum* [16] and *Neurospora* [17] reviewed in Ref. 18). This conclusion arises from (a) the correlation between TPP^+ and methylamine in/out gradients, (b) the effect of K^+ and K^+-specific ionophores on reversing methylamine accumulation, (c) the very small effect of CCCP (5 μM) on methylamine accumulation [14], and (d) ammonia-induced depolarization measured with microcapillary electrodes [17].

The results shown in Table III indicate that the accumulation of methylamine in our yeast strain is also pH-dependent but is larger than that of TPP^+ reported by two independent research groups [19,20]. Hence, the membrane potential, if correctly reflected by the TPP^+ accumulation, would be smaller than that needed to drive methylamine transport. On the other hand, the total $\Delta\bar{\mu}H^+$ makes up a driving force which is much larger [20] and, except at alkaline pH, able to account for the methylamine accumulation. The uptake of K^+ in yeast has been suggested to be driven by the protonmotive force [18].

Our results suggest that the steady-state level of

TABLE III

MEMBRANE POTENTIALS CALCULATED FROM METHYLAMINE EQUILIBRIUM ACCUMULATION

The membrane potentials were calculated from the experimentally determined in/out accumulation ratios and the Nernst equation, and are shown in the MA^+ lines. TPP^+ lines show the membrane potentials reported by Refs. 19 and 20 calculated on the basis of TPP^+ distribution. All values are mV. [19], [20] are the reference numbers.

Conditions	pH								
	3	4	4.5	5	5.5	6.5	7.0	7.5	8.5
TPP^+ 20 mM buffers			−45 [20]	− 54 [20]		−68 [20]	−82 [20]		
TPP^+ 45 mM Tris			−66 [19]			−114 [19]		−150 [19]	
MA^+ 100 mM PO_4HK_2/PO_4H_2K	−105	−136		−141		−146			
MA^+ 50 mM Tris/50 mM KCl	−102					−140		−146	−147
MA^+ 100 mM Tris/100 mM KCl						−148			
TPP^+ 20 mM buffers/ 200 mM KCl			−35 [20]			−44 [20]	−50 [20]		

methylamine accumulation in yeast is due to at least two processes: (1) an energy-driven uptake of NH_4^+ catalyzed by a specific permease(s); and (2) the diffusion and pH-dependent distribution of NH_3. These two processes acting in opposite directions at acid medium pH, would cause ammonia cycling, with the final methylamine (ammonia) balance depending on the relative rates of both. At alkaline pH, both processes would proceed in the same direction and the final accumulation of methylamine would be increased.

The contribution of NH_3 diffusion to ammonia balance is clearly indicated by (a) the predominance of this transport mechanism over NH_4^+ uptake above 1 mM at pH levels greater than 7.5 and (b) the difference in ammonia accumulation between acetate + ammonia cultures (see first line Table II) and pH 7.5 glucose + ammonia cultures (Expt. 9, Table II) that correlates with the inverse of ΔpH. Additionally, the pH transition experiments (Table I) are also consistent with this hypothesis, since an accelerated efflux is promoted by an increased in/out pH ratio, and vice-versa.

The following considerations suggest that, in yeast, NH_3 diffusion could play a significant role in a number of physiological conditions. Ammonium sulfate concentrations in standard cultures are around 10 mM, that is, around 500-times higher than the methylamine concentration used for the equilibrium distribution experiments. When using these high concentrations, methylamine accumulation ratios drop with increasing methylamine concentration (Expt. 6, Table II) and ammonia is not accumulated (Expt. 5, Table II). This probably reflects the fact that NH_4^+ and CH_3-NH_3^+ uptake are ammonia- and methylamine-inhibited, although the K_i for methylamine is probably higher than that for ammonia [10,11]. However, with a rise in the medium pH, there is an accumulation of ammonia within the cells that can be seen either in glucose (Expts. 5, 7, 9, Table II) or acetate (Expts. 1, 3, Table II) cultures, in spite of the very low permease activity (Fig. 2). This accumulation probably reflects the contribution of NH_3 diffusion, a process lacking genetic or regulatory control, and is due to occur whenever the medium pH and ammonia concentration make NH_3 diffusion significant vs. NH_4^+ transport. These conditions are probably met in ammonia-supplemented standard acetate-based sporulation cultures, and an uncontrolled ammonia accumulation drived by NH_3 diffusion could be involved in the well-documented inhibition of sporulation by ammonia [21].

Acknowledgments

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