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Membrane Permeability and the Loss of Germination Factor from *Neurospora crassa* at Low Water Activities

GISELA CHARLANG AND N. H. HOROWITZ

Division of Biology, California Institute of Technology, Pasadena, California 91109

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Neurospora crassa conidia incubating in buffer at low water activities (a_w) release a germination-essential component as well as 260-nm absorbing and ninhydrin-positive materials, regardless of whether an electrolyte or non-electrolyte is used to reduce a_w. Chloroform and antibiotics known to increase cell-membrane permeability have a similar effect. This suggests that membrane damage occurs in media of low a_w and that an increase in permeability is responsible for the release of cellular components. The damage caused in media of low a_w is nonlethal in most cases, and the conidia recover when transferred to nutrient medium.

Living organisms have a high and, within narrow limits, apparently irreducible requirement for water. Most microorganisms are unable to grow in media whose water activity (a_w) is below 0.9. A few unusual bacteria, the extreme halophiles (7, 18), grow at a_w 0.75. Growth of some fungi has been reported at a_w 0.62 (18), but the growth is so slow even under ideal conditions of temperature and nutrition that its biological significance is doubtful. Some very dry natural environments, such as locales in the dry valleys of Antarctica where the prevailing a_w is 0.45 or less at 0 C, are abiotic (6). In many other regions of the world, also, water is the life-limiting factor. Yet little is known about what determines the water requirements of microorganisms, or what cellular responses result when these requirements are not met.

Charlang and Horowitz (3) have presented evidence for the existence of a substance in conidia of *Neurospora crassa* which is essential for their germination and which is lost from the cells in media of low a_w. The substance, called "germination factor" (GF), can be extracted from conidia or mycelium. When added to media of low a_w, it shortens the long germination lag that is observed in these media. GF activity is found in extracts of other fungi, as well—e.g., *Aspergillus nidulans* and *Penicillium chrysogenum*—but not yeast; *Escherichia coli* contains a trace (unpublished data from this laboratory). GF is not present in complete medium (see composition under Materials and Methods).

GF loss occurs in media of low a_w regardless of whether an electrolyte or non-electrolyte is used to reduce a_w. In this paper, we present evidence that a nonlethal alteration of the permeability of the cell membrane in such media is responsible for the loss of GF, along with other molecules. A similar, but usually lethal, effect is obtained when antibiotics known to damage the membrane specifically are used.

MATERIALS AND METHODS

Wild-type *N. crassa*, strain 74A, was grown on slants or in 125-ml flasks of complete medium [Vogel minimal medium N (19) supplemented with 0.5% yeast extract, 0.25% casein hydrolysate, and 1.5% agar] in the manner previously described (3).

Experimental procedures. Conidia from 4-day-old cultures were harvested by suspension in distilled water and filtered through four layers of cheesecloth to remove mycelial debris. They were washed once and suspended in 0.067 M sodium phosphate buffer, pH 6.0, before being counted in a haemocytometer. A total of 10^8 conidia/ml were incubated in buffer at low a_w or in buffer with added antibiotics or chloroform, at 30 C for up to 48 h with shaking. At various time intervals, samples were taken, and the conidia were removed by centrifugation and Millipore filtration. The cell-free solutions were tested for GF activity, 260-nm absorbance, and ninhydrin reaction.

The a_w of the buffer (initially 0.998) was lowered to 0.938, 0.908, or 0.843 by adding NaCl or glycerol. The required amounts of solutes were calculated from the tables of Robinson and Stokes (17), Scott (18), and Ingram (7).

Certain antibiotics have been shown to damage the cell membrane with a resulting increase in permeability (5). Of these, we used nystatin (8, 9, 10) at 5 μg/ml, polymyxin B (5, 8, 15) at 50 μg/ml, and tyrocidine (12) at 20 μg/ml. Nystatin was decomposed photochemically (10) before GF assays were carried out. Nystatin and polymyxin were obtained from Sigma Chemical

Co., and tyrocidine was obtained from Nutritional Biochemicals Corp.

Assays. The GF assay was essentially the one previously described (3). Samples to be tested were added to 50 ml of complete medium plus NaCl at a_w 0.934. Inoculation was with 10^2 conidia/ml, and the flasks were incubated with shaking at 30 C for 90 h.

The maximum absorbance in the 250- to 260-nm range was read from spectral curves obtained on a recording Cary 15 spectrophotometer. The method of Moore and Stein (13) was used for determinations of ninhydrin-positive material.

RESULTS

Experiments at low a_w. Loss of GF from conidia is correlated with loss of 260-nm absorbing and ninhydrin-positive materials. This is shown in Table 1 for solutions of lowered a_w and in Table 2 for substances known to damage the cell membrane. Germination does not occur during 48-h exposure to any of the solutions listed in these tables, except for the tyrocidine case discussed below.

When conidia are incubated in buffer at a_w 0.998, there is some loss of 260-nm absorbing and ninhydrin-positive materials into the medium, but there is no detectable loss of GF. At low a_w, however, GF is released along with larger amounts of 260-nm absorbing and ninhydrin-positive substances (Table 1). It should be noted that the loss of materials from the cells is both a_w- and time-dependent.

All of the solutions (except buffer) listed in

TABLE 1. *Amounts of GF activity, 260-nm absorbing and ninhydrin-positive materials released from conidia at different levels of a_w*

Suspending medium	Molal concentration of solute (NaCl or glycerol)	a_w of suspending medium	GF activity[a]			OD at 260 nm[b]			Ninhydrin-positive material[c]		
			6 h[d]	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Buffer		0.998	0.0	0.0	0.0	0.13	0.23	0.33	0.014	0.014	0.025
Buffer plus NaCl	1.78	0.938	1.5	5.0		0.20	0.24		0.068	0.12	
	2.57	0.908	6.3	10.5	18.3	0.195	0.30	0.74	0.075	0.164	0.242
	4.15	0.843	6.3	9.4	15.4	0.190	0.33	0.84	0.06	0.151	0.259
Buffer plus glycerol	3.32	0.938	0.0	2.7	2.2	0.12	0.275	0.68	0.039	0.045	0.05
	5.0	0.908	1.3	5.0	10.8	0.17	0.33	1.06	0.05	0.078	0.156
	8.75	0.843	1.9	8.6	47.3	0.16	0.77	3.30	0.092	0.28	0.99

[a] GF bioassay of 0.25 ml of incubation mixture. Amounts are expressed in mg of mycelial dry weight, average of two flasks.
[b] Peak absorbance in 250 to 260 nm range.
[c] Expressed in mM amino acid equivalents based on a leucine standard.
[d] Time of exposure.

TABLE 2. *Amounts of GF activity, 260-nm absorbing and ninhydrin-positive materials released from conidia by antibiotics or chloroform*

Suspending medium	Concentration of antibiotic (μg/ml)	GF activity[a]			OD at 260 nm[b]			Ninhydrin-positive material[c]		
		3 h[d]	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h
Buffer		0.0	0.0	0.0	0.13	0.13	0.23	0.012	0.014	0.014
Buffer plus nystatin	5	trace	4.0	57.7	1.43	2.2	4.9	2.26	2.28	3.21
Buffer plus polymyxin B	50		45.0	69.5		4.3	11.1		2.9	6.5
Buffer plus tyrocidine	20	46.4	1.8	1.2	2.83	1.43	0.58	1.35	0.36	0.067
Buffer plus chloroform	saturated	72.2[e]			6.5[e]			3.05[e]		

[a] GF bioassay of 0.25 ml of incubation mixture. Amounts expressed in mg dry weight (average of 2 flasks).
[b] Peak absorbance in 250 to 260 nm range.
[c] Expressed in mM amino acid equivalents based on a leucine standard.
[d] Time of exposure.
[e] Exposure was for 1 h.

Table 1 cause extensive plasmolysis of conidia. No, or at best only partial, recovery from plasmolysis occurs during a 48-h exposure to the NaCl solutions. It thus appears that the alteration in permeability that is induced by these solutions is not so extensive as to permit free entry of NaCl into the cells. Although plasmolyzed, the cells are nevertheless fully viable, as shown by subsequent plate counts on sorbose medium (2). In the case of glycerol solutions, there is complete recovery from plasmolysis within 48 h at a_w 0.938 and a_w 0.908, and the cells show full viability when plated out. In glycerol at a_w 0.843, however, viability is reduced by about 40%, and recovery from plasmolysis is abnormal. The cells are enlarged and have a transparent center with the protoplasm distributed around the periphery. In contrast, normal conidia tend to be uniformly dense.

The data of Table 1 parallel these observations in that they show that glycerol is more damaging than NaCl at high concentrations. At low concentrations (high a_w), the reverse is true. Our previous growth studies showed that glycerol is less toxic than NaCl at water activities in the neighborhood of 0.9 or higher (3). For example, *N. crassa* 74A will grow very slowly in complete medium plus glycerol at a_w 0.898, but it does not grow at all at the same water activity with NaCl.

Experiments with antibiotics and chloroform. The experiments summarized in Table 2 were performed to test the hypothesis that loss of GF from conidia in media of low a_w results from altered permeability of the cell membrane. A number of antibiotics are known to be specifically active against membranes. Nystatin and tyrocidine have been shown to inhibit germination and growth of *N. crassa* by irreversibly damaging the cell membrane (8, 10, 12). Polymyxin B is known to breach the osmotic barrier, especially in gram-negative bacteria (15), probably by inducing a reorientation of membrane lipids (5). The effect of polymyxin B on *N. crassa* cell membranes has not been studied in detail, but Kinsky (8) has shown that it does inhibit both germination and growth. Chloroform causes extensive damage to plasma membranes (5). It was used in our experiments to induce maximum leakage.

No conidia survive after a 1-h exposure to chloroform-saturated buffer, and values for released GF, 250-nm absorbing, and ninhydrin-positive material are high (Table 2). In nystatin-supplemented buffer, viability of conidia decreases gradually to nearly zero in 24 h. The opposite trend is shown in amounts of material released. Polymyxin B has a similar effect, but there were some survivors after 24 h, and they had germinated in the incubation mixture. It appears that the damage caused to the sensitive fraction of the population was extensive, judging from the large quantities of material released.

The reaction of tyrocidine is unusual. At a concentration of 20 μg and 10^7 conidia/ml, the antibiotic is lethal, viability being reduced to less than 1% after a 2-h exposure. With 10^8 conidia/ml, the same tyrocidine concentration causes an immediate and rapid loss of 260-nm absorbing and ninhydrin-positive material, as well as GF. But after 1 h, the reaction is reversed, and the released material begins to disappear from the medium. After 3 h, the conidia germinate. At 6 h, only traces of GF activity, about half of the 260-nm absorbing and 20% of the ninhydrin-positive materials, remain in the medium; at the same time, germination is essentially complete. After 24 h, no further growth has occurred, but the quantities of intracellular material in the medium have been further reduced. Because germination does not occur in unsupplemented buffer, it appears that tyrocidine—or, more likely, an impurity in the commercial preparation—provides nutrient to the conidia.

Dialysis results. Mach and Slayman (12) found that 44% of the 260-nm absorbing material released by *Neurospora* mycelium during a

TABLE 3. *Amounts of nondialyzable 260-nm absorbing material released from conidia at different levels of a_w or by chloroform*

Suspending medium	Time (h)	OD[a] before dialysis	OD[a] after dialysis	Nondialyzable material (% of total)
Buffer only at a_w 0.998	6	0.192	0.036	18.2
	24	0.194	0.032	16.5
	48	0.230	0.043	18.7
Buffer plus NaCl at a_w 0.843	6	0.159	0.023	14.5
	24	0.250	0.035	14.2
	48	0.415	0.051	12.3
Buffer plus glycerol at a_w 0.843	6	0.338	0.076	22.5
	24	0.791	0.135	17.1
	48	4.69	0.350	7.47
Buffer plus excess chloroform	1	7.86	2.49	31.8

[a] Peak absorbance in 250 to 260 nm range.

2-min exposure to an inactivating concentration of tyrocidine was nondialyzable. We dialyzed 260-nm absorbing material released from conidia exposed to low a_w or chloroform for 24 h at 4 C against four changes of water. The results, presented in Table 3, show that increasing absolute amounts of nondialyzable material are correlated with increased length of exposure to low a_w. The fraction of nondialyzable material in the total 260-nm absorbing material decreases with time. It thus appears that, as an index of damage, the absolute amount rather than the percentage of nondialyzable material is more revealing. On the other hand, with a short exposure to a highly damaging compound like chloroform, one observes the release of a large percentage of nondialyzable material, as Mach and Slayman did with tyrocidine.

DISCUSSION

Exposure of *Neurospora* conidia to media of low a_w results in the release of a germination-essential component, together with 260-nm absorbing and ninhydrin-positive materials. Similar results are obtained regardless of whether an electrolyte or a non-electrolyte is used to reduce a_w. We conclude that reduction of a_w, and not specific solute toxicity, is responsible. Because membrane-specific antibiotics also induce a release of GF and other materials from conidia, it is likely that the loss of intracellular substances at low a_w results from alteration of the cell membrane.

Membrane damage at low a_w may explain the water requirements of many microorganisms, and resistance to such damage may explain xerotolerance. Hypertonic solutions of NaCl appear to cause membrane damage in blue-green algae (1), and high sucrose concentrations make *E. coli* permeable to nucleoside triphosphates (4). The loss of putrescine from *E. coli* growing at moderately elevated osmolarities, however, is not the result of membrane damage (14). Preliminary experiments with *A. nidulans* and *P. chrysogenum* in our laboratory have shown that these species are much more xerotolerant than *N. crassa*: their conidia retain GF and germinate at a_w <0.9. Since their GF is readily diffusible—as shown by its rapid loss from conidia in chloroform-saturated buffer—there is an evident correlation between ability to germinate at low a_w and resistance to permeability changes.

Specific toxicities may also be manifested at high solute concentrations, superimposed on the effects of low a_w. The results presented in this paper show that at the lowest water activities tested, glycerol begins to be lethal, but NaCl does not. In our earlier experiments on the effects of reduced a_w on germination and growth, sucrose was more inhibitory than NaCl, glycerol, or glucose (3). Specific solute toxicities have also been observed in yeast (11, 16).

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