INOSITOL-LESS DEATH IN YEAST RESULTS IN A SIMULTANEOUS INCREASE IN INTRACELLULAR VISCOSITY

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ABSTRACT Inositol auxotrophs of yeast developing on isositol-deficient medium continue protein synthesis for 4–6 h, lose viability rapidly after 6 h, and show an increase in cytoplasmic viscosity as measured by spin label rotational motion. Cycloheximide prevents the rapid loss of cell viability, stops protein synthesis, and simultaneously prevents an increase in cytoplasmic viscosity. From these observations, we infer that intracellular translational diffusion is upset as a consequence of inositol starvation. Cell death may be caused by a modified intracellular diffusion environment.

INTRODUCTION

Starvation for required nutrients causes death in fungi in a limited number of cases. The conidia of inositol auxotrophs of *Neurospora crassa* (8) and conidia of biotin auxotrophs of Aspergillus (11) die when allowed to germinate in the absence of their dietary requirements. Thymidine-requiring mutants of yeast die when starved for the auxotrophic requirement (1), as do saturated fatty acid-requirers (fas^-) of this same organism (3). In contrast to these, most other known auxotrophs of fungi, including unsaturated fatty acid requirers of yeast (ole^-), retain high viability for many hours under starvation conditions (4). The fas^- and ole^- mutants of yeast are particularly interesting in that both requirements are related to the synthesis of appropriate membrane components, whereas only the fas^- mutants die when deprived of the specific growth requirement.

Spin labeling experiments have been carried out on the fas^- and ole^- mutants of yeast (4). For the fas^- mutants, in the absence of growth supplements, it was found that restriction to rotational diffusion of water-soluble spin labels dissolved in the cell cytoplasm undergoes a marked increase concomitant with cell death. This increased restriction to rotational motion can be interpreted as an increase in the cytoplasmic viscosity. The ole^- mutants, which remain viable during starvation, showed no such change in cytoplasmic viscosity. Furthermore, for both the fas^- and ole^- mutants, spin labels localized in the hydrocarbon zones of membranes showed no detectable

alteration in rotational motion during starvation. These experiments suggest that the viscosity of aqueous cytoplasm may be a sensitive indicator of, and critically related to, those events that accompany cell death during unbalanced growth.

The experiments described here were carried out to investigate further this apparent relationship between cell death and increased cytoplasmic viscosity. It was found that inositol-requiring mutants of yeast die rapidly when starved for inositol. Over the same time interval the cytoplasmic viscosity inferred from spin label rotation increases dramatically. Cycloheximide, when present during starvation, largely prevents the loss of viability and eliminates the rise in cytoplasmic viscosity. These data are analyzed in terms of altered diffusional properties for cellular components resulting from unbalanced growth during starvation.

METHODS

Strain

The inositol-requiring strain MC13 (*inol*-13 *lys* 2 X) has been previously described (2).

Growth Conditions

All growth trials were at 30°C.

Media

The strain was maintained on YEPD (2% glucose, 2% bactopeptone, 2% agar, 1% yeast extract). Complete synthetic medium contained the following components: 2% glucose, Difco vitamin-free yeast nitrogen base (Difco Laboratories, Detroit, Mich.) (6.7 g/liter), biotin (2 μg/liter), calcium pantothenate (400 μg/liter), folic acid (2 μg/liter), niacin (400 μg/liter), *p*-aminobenzoic acid (200 μg/liter), pyridoxine hydrochloride (400 μg/liter), myo-inositol (2.0 mg/liter), lysine (20 mg/liter), arginine (10 mg/liter), methionine (10 mg/liter), threonine (60 mg/liter), tryptophan (10 mg/liter), leucine (10 mg/liter), histidine (10 mg/liter), adenine (10 mg/liter), and uracil (10 mg/liter). Inositol-less medium was identical to complete medium, except for the omission of inositol.

Inositol Starvation

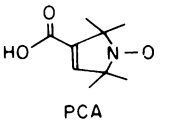
Strain MC13 was tested for growth in liquid synthetic medium inoculated from an overnight YEPD log phase preculture. Preculture cells were washed twice in minimal medium and inoculated at a density of approximately 10^6 cells/ml in complete synthetic medium with inositol or in complete medium without inositol. For the cycloheximide experiments, cells were washed, inoculated in complete medium, and allowed to grow until optical density increased perceptibly. Cycloheximide at a concentration of 100 μg/ml (sufficient to block protein synthesis completely) was added and the cells were harvested 30 min later, washed twice in minimal medium plus cycloheximide, and resuspended in cycloheximide-containing inositol-less medium at 10^6 cells/ml. Cell growth was followed by measurement of optical density and by plating for viable cells.

As a measure of protein synthesis during inositol starvation, uptake of [^{14}C]lysine into trichloroacetic acid (TCA)-precipitable material was measured in whole cells. Isotope (0.1 μCi/ml) was added to synthetic complete medium with or without inositol at the time cells were transferred from the preculture. At intervals, 0.5-ml samples were withdrawn from the

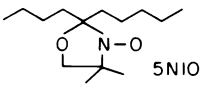
culture and mixed with 0.5 ml of 20% TCA. The samples were allowed to stand for 10 min in a water bath at 60°C and were then filtered with membrane filters (HAWP 02500) and washed with 20 vol of cold 5% TCA. The filters were dried and counted by liquid scintillation. [^{14}C]lysine, sp act 348 mCi/mmol, was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.).

Spin Labeling

Two spin labels having widely different solubility properties were used. The water-soluble spin label, 3-carboxyl-2,2,5,5-tetramethylpyrroline-*N*-oxyl (PCA), was synthesized according to the procedure of Rosantsev (12). The lipid-soluble spin label, 4-butyl-2,2-dimethyl-4-pentyloxazolidine-*N*-oxyl (5N10) was synthesized by the general procedure of Keana et al. (5) and was purified as previously described (15). 5N10 has slight solubility in water, but under the conditions of our experiments, its signal originated from molecules in the membrane and other cellular hydrocarbon zones (4). The structures of PCA and 5N10 are shown below.



PCA



5N10

5N10 was added to the yeast samples from concentrated stock solutions at 0.1 M in ethanol. The final concentration used was 10^{-4} M. PCA was added to the yeast samples from an aqueous stock containing 10^{-2}M spin label and 2 M $NiCl_2$, to give final concentrations of 10^{-3}M PCA and 0.2M $NiCl_2$. The use of $NiCl_2$ to eliminate spin label signals from medium outside the cells has been described and characterized in several previous reports (4, 7, 9, 14). Yeast cells continue to grow at their normal rate in medium containing 0.2 M $NiCl_2$

Electron Spin Resonance (ESR) Analysis

All ESR measurements were carried out at X-band microwave frequencies on a Japan Electron Optics Laboratory spectrometer, model JES-ME-1X (JEOL USA, Electron Optics Div., Medford, Mass.). Samples were analyzed at room temperature (22 ± 2°C).

For comparative measurements of rotational motion, an empirical motional parameter, R_i, was employed. This parameter is linearly related to the rotational correlation time, τ_c, according to Eqs. 1 and 2:

$$\tau_c = KW_0[(h_0/h_{-1})^{1/2} - 1], \quad (1)$$

$$R_i = W_0[(h_0/h_{-1})^{1/2} - 1], \quad (2)$$

where W_0, h_0, and h_{-1} are the midfield line width, the midfield line height, and the high field line height, respectively, measured from first-derivative spectra of the nitroxide triplet absorption (6). The numerical constant, K, which depends on the spin label tensor parameters and the spectrometer frequency, is eliminated in the expression for R_i. The values of R_i were reproducible within ±10%.

RESULTS

Fig. 1 shows the viability of yeast cells as a function of time for different treatments used in the present studies. The closed squares show that yeast cell auxotrophs for

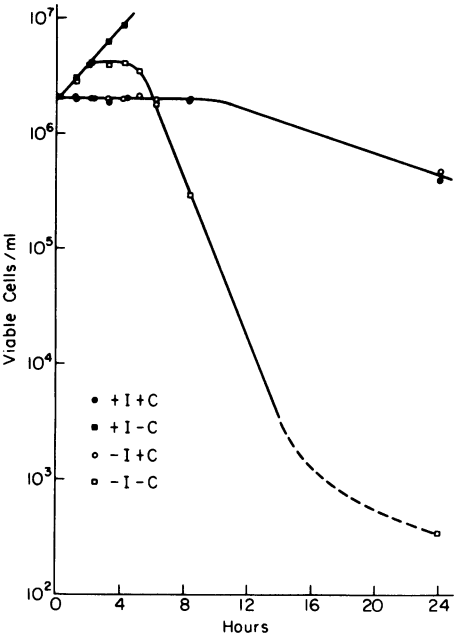


FIGURE 1 Cell viability under different conditions of inositol (I) starvation with and without added cycloheximide (C). Culture conditions are described in Methods.

inositol have a normal growth, with a doubling time of approximately 2 h, and viability when supplemented with inositol. The open squares show that, when deprived of inositol, the cells start losing viability at about 6 h and continue to lose viability rapidly for the next several hours. The open circles and closed circles show that, when cycloheximide is included in the growth medium, loss of viability is largely prevented whether inositol is included in the culture medium or not. Cycloheximide is a protein synthesis inhibitor of eukaryotic cells and largely prevents the synthesis of macromolecules.

Fig. 2 shows the uptake of radioactive lysine into TCA-precipitable material over the time interval when cells lose viability when starved for inositol. The open circles in Fig. 2 show that protein synthesis continues for the first 4–6 h in cells deprived of inositol and then stops. Little if any radioactive lysine is incorporated into TCA-precipitable material between 6 and 24 h in these experiments.

Fig. 3 shows spin label data taken on the same time scale as that for the experiments shown in Fig. 1. The data for the hydrocarbon spin label, 5N10, illustrate that there is no detectable change in the restriction to rotational diffusion in hydrocarbon and lipid zones of the yeast cells over the time periods studied. 5N10 partitions into hydrocarbon zones to such an extent that no intracellular aqueous signal is visible. Data for the small water-soluble spin label, PCA, illustrate that the restriction to rotational diffusion in the aqueous zones of the cytoplasm is highly dependent upon

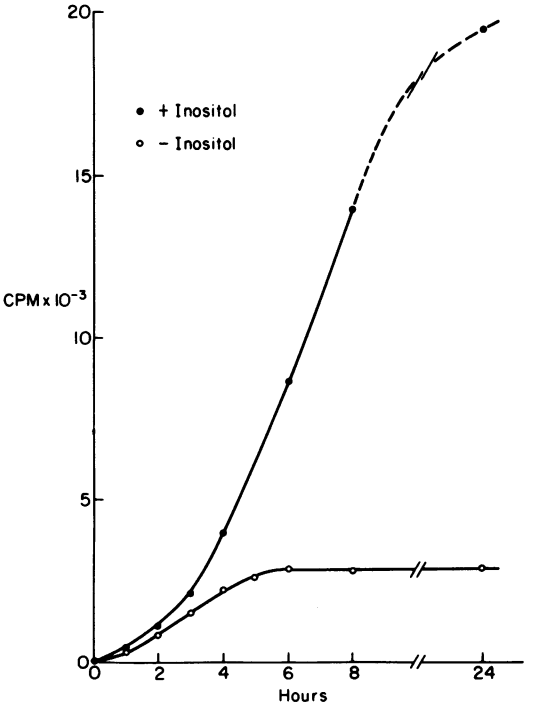


FIGURE 2 Uptake of [^{14}C]lysine by yeast cells with and without inositol supplementation. Labeling conditions are described in Methods.

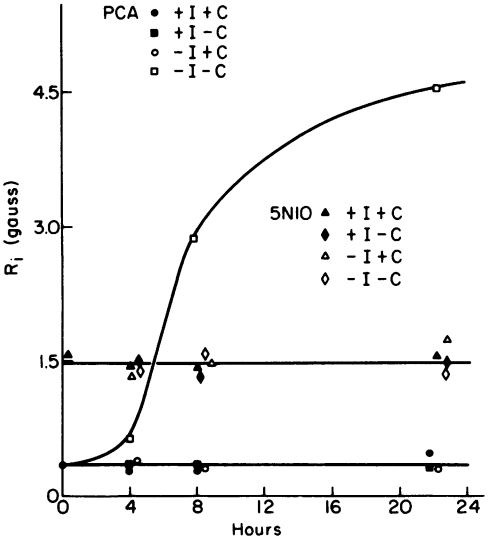


FIGURE 3 Empirical motion parameter (R_i) plotted *vs.* time under different growth conditions. The structures and properties of the spin labels PCA and 5N10 are described in the text. Abbreviations: C, cycloheximide; I, inositol.

the metabolic condition. Fig. 3 shows that starvation for inositol in the absence of cycloheximide is the only condition where the apparent cytoplasmic viscosity has a steep increase. When inositol is supplemented or cycloheximide is included in the growth medium, there is no detectable change in restriction to PCA motion in the aqueous cytoplasm. The increase in restriction to PCA motion occurs over the same approximate time scale as does loss of cell viability and the reduction in protein biosynthesis in the inositol auxotrophs starved for inositol.

DISCUSSION

Several conditions have now been described for which the interruption of synthesis of cell membrane or cell wall components results in both cell death and increased cytoplasmic viscosity. In all these cases, continued protein synthesis in the absence of continued synthesis of the cell surface components may be associated with cell death (3, 4, 8, 11). Takayama et al. (14) observed that the drug Isoniazid (isonicotinic acid hydrazide), which inhibits synthesis of the cell wall constituent mycolic acid but which does not inhibit protein synthesis, causes both cell death and increased cytoplasmic viscosity in *Mycobacterium tuberculosis*. Fatty-acid-requiring mutants (fas^-) of yeast lose viability and show increased cytoplasmic viscosity under starvation conditions, and both cell death and increased cytoplasmic viscosity can be prevented by inhibiting protein synthesis (4). In the experiments described here, inositol requirers of yeast show similar behavior, with conditions that lead to cell death also giving rise to increased cytoplasmic viscosity. In these mutants inositol is required for the synthesis of phosphatidylinositol, an essential requirement for membrane growth in this organism. Furthermore, the inhibition of protein synthesis during starvation prevents both cell death and the increase of cytoplasmic viscosity. It seems appropriate to discuss these results in terms of the effects that cytoplasmic viscosity may have on the balance of growth in the cell. Many conditions have been described, including thymine-less death in yeast (1), where unbalanced growth is thought to be the cause of decreased viability.

What changes may be introduced into the metabolic state of a cell by changes in the local viscosity? Two possible factors may be called the "recognition time" and the "arrival time." Of these, the recognition time is dependent upon the rotational motion of molecules in the cell and is directly related to the rotational correlation time, a quantity measured in spin label studies. As will be seen in the subsequent analyses, the probability that a molecule will assume the appropriate orientation in its encounter with another species is independent of viscosity, to a first approximation. On the other hand, the arrival time, a measure of the number of collisions per second on a specific surface, is directly related to the viscosity. We are led to suggest that a metabolite in limiting concentration, which is near the minimum allowable concentration at the normal viscosity, may well be below adequate concentration at an elevated viscosity and this may result in local starvation, hence unbalanced growth, in the cell.

The Problem of Recognition

If a molecule, for example a repressor, or a transfer RNA molecule, must present a particular element of its surface to a specific combining zone—for example the precise sequence of the operator gene or the codon on the messenger RNA—then it presumably does so by diffusing linearly into the near neighborhood of the specific combining zone and then, by rotational diffusion, randomly presenting its surface until the match is made and the hydrogen bonds (or London dispersion forces) stop the rotation and bind the rotating molecule firmly.

The time for this recognition (t_R) may be estimated by supposing that, in rotational diffusion about one axis, an angle 2π must be covered. Then if θ is the rotational diffusion coefficient, we have $\bar{\theta}^2 = 2\theta t$ for the mean square angle traversed in a time (t). Using 2π as the angle, we have $4\pi^2 = 2\theta t_R$ and $t_R = 2\pi^2/\theta$. The value of θ for a sphere, radius a, is $\theta = kT/8\pi\eta a^3$. Thus $t_R = 16\pi^3\eta a^3/kT$. Since the rotational correlation time $\tau_c = 4\pi\eta a^3/3kT$, we see that $t_R = 12\pi^2\tau_c$. Thus the recognition time and the rotational correlation time are closely related.

The actual problem of recognition involves presenting the specific surface successfully before linear diffusion can move the two surfaces apart. In a time t linear diffusion accounts for a mean square distance $\bar{X}^2 = 2Dt$, where D is the linear diffusion coefficient. In a time t_R, with $D = kT/6\pi\eta a$, we have $\bar{X}^2 = (2kT/6\pi\eta a)t_R$ and, substituting $t_R = 16\pi^3\eta a^3/kT$, we have $\bar{X}^2 = 2kT/6\pi\eta a \cdot 16\pi\eta a^3/kT = (16\pi^2/3)\, a^2$, independent of both the viscosity and the temperature but dependent on the radius. Recognition is thus a hard problem for a large molecule. Nevertheless, recognition, though directly related to the rotational correlation time, is probably *not* the factor that stops the cell at high viscosity. Seen intuitively, this means that the slower rotational diffusion is matched by equivalently slower linear diffusion.

The Number of Collisions per Second: Arrival Time

One method of estimating this number of collisions is an adaptation from Smoluchowski (10, 13). If a large surface has a hemispherical sensitive area of radius R and this radius includes the radius of colliding molecules, then the collision frequency, ϕ, for molecules with diffusion constant D and concentration C is $\phi = 2\pi RDC$. Setting $D = kT/6\pi\eta R$ as an approximation, we obtain $\phi = kTC/3\eta$. If a metabolite is in limiting concentration, then C is just able to provide the necessary number ϕ to keep the cell going. Increasing the viscosity by a factor of 10 or so, as is observed, would reduce ϕ to below the sustaining rate. This may well be damaging to the cell.

We suggest that the decreased diffusion of small molecules and unbalanced growth resulting from this effect should be considered as a possible cause of cell death. Altered diffusion properties for enzyme substrates and enzyme products could significantly affect the balance of synthetic and degradative metabolic pathways. For multi-enzyme pathways, feedback inhibition of an early enzyme by reaction products of a later enzyme should depend on the diffusion properties of the molecules involved. Further studies designed to test this hypothesis may help elucidate the role of cytoplasmic viscosity in the balance and regulation of cell growth under normal conditions.

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