CHANGES IN THE RESTRICTION OF MOLECULAR ROTATIONAL DIFFUSION OF WATER-SOLUBLE SPIN LABELS DURING FATTY ACID STARVATION OF YEAST

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ABSTRACT Yeast mutants lacking fatty acid synthetase activity (*fas*$^-$) die when deprived of saturated fatty acid under conditions which are otherwise growth-supporting. The spin label technique is used to show that restriction of molecular rotational diffusion of spin label molecules dissolved in aqueous zones increases several fold under conditions of fatty acid starvation while the apparent physical state of cellular hydrocarbon zones remains essentially unchanged. We focus attention on the cellular aqueous interior as the potential site of alteration under selective starvation conditions. Correspondences exist between restriction of molecular motion of water soluble spin labels dissolved in the cell and loss of cell viability. The correspondences to changes in the molecular motion of hydrocarbon soluble spin labels are much less or are not detectable.

INTRODUCTION

Saturated fatty acid-requiring mutants (*fas*$^-$) of yeast have recently been shown to die if deprived of fatty acid under otherwise growth-supporting conditions. However, if protein synthesis is blocked in these cells during fatty acid starvation the cells remain largely viable (7).

A similar phenomenon called "unbalanced growth" has long been known in other fungi. For example, the conidia of inositol-requiring mutants of *Neurospora* (15) and biotin auxotrophs of *Aspergillus* (17) die if permitted to germinate in the absence of their respective requirements. Both of these mutations, like the fatty acid mutation, affect lipid synthesis. Biotin is required for fatty acid synthesis and inositol is an important component of phospholipids. Since lipids are important membrane components, it has been suggested that membrane abnormalities arising during the starvation period might account for the rapid cell death observed (5, 7). In the present study, this possibility is investigated by the use of the spin label technique in a yeast fatty acid auxotroph deficient both in fatty acid synthetase (*fas*$^-$) and in fatty acid desaturation (*ole*$^-$). This mutant is not able either to synthesize long-chain fatty acids or to desaturate them and is therefore dependent upon the exogenous supply of both.

A variety of techniques has been employed to show a relation between a physical measurement carried out on membranes or membrane lipids and some functional aspects of the membrane preparation (16). In general, these measurements have been in close agreement with biological function. As the fatty acid composition is altered, there are generally coincident changes in membrane physical state and in physiological parameters. It is generally believed that many membrane-associated enzymes have their activities modulated by membrane hydrocarbon zones. This emphasis on membrane physical state has given rise to the general concept that many cell functions are modified by membrane physical state.

The aqueous regions of cell organelle interiors are spatially separated from the general cellular cytoplasm by membranes, much as the cytoplasm is separated from the cell exterior by the plasma membrane. Clearly this spatial organization is vital to cell integrity.

The physical state of the cytoplasm has been of interest to a number of investigators in the past several years (3), but the general techniques available for measurements have not been convenient or adequately sensitive to carry out comparative measurements as a function of controlled cellular perturbation.

Recently the technique of using a paramagnetic ion or ion-complex in conjunction with an organic spin label has made possible measurements in regard to cytoplasmic physical state (13). This technique uses two paramagnetic species which have differential permeability so that only the signal from the cell interior or cell aqueous cytoplasm is seen. The removal of all signal from outside the cell makes possible measurements which emanate only from the cell interior.

We show that fatty acid starvation, in the mutants used here, results in negligible perturbation of cell membranes, less than $\pm 10\%$, under conditions of up to a six-fold increase in rotational motion measurements of a spin label localized in the cell interior. The comparative difference (6/0.1) of about 60, illustrates that the cytoplasm can be drastically altered while the cell hydrocarbon zones including membranes remain essentially unaltered with regard to spin label motion.

MATERIALS AND METHODS

Strain

The strain BW1 1C (*fas*1, *ole*1 a) has been previously described (7). This strain lacks fatty acid synthetase (*fas*1) as well as fatty acid desaturation (*ole*1) and is therefore completely dependent upon exogenous supply for both saturated and unsaturated fatty acids. This strain is respiratory sufficient (ρ^+, grande) and is able to utilize nonfermentable carbon sources. The respiratory deficient (ρ^-, petite) clone of this strain was identified by its inability to utilize either ethanol or glycerol as a carbon source. The single mutant strains *fas*1 *Ole*$^+$ ρ^+ and *Fas*$^+$ *ole*1 ρ^+ have also been previously described (7).

Yeast Media

The mutant strains were routinely maintained on 1% yeast extract, 2% peptone, and 2% dextrose (YEPD) containing 1% Tween 80 and 1% Tween 40 (YEPD + TW) as a fatty acid supplement. This mixture of Tween detergents contained adequate amounts of both saturated and

unsaturated fatty acids to support growth of strain BW1 1C as well as both of the single mutant strains.

The composition of complete defined medium has been described elsewhere (6). This medium was supplemented with purified (99% pure) myristic and oleic acids (Hormel Institute, Austin, Minn.) both at 10^{-3} M solubilized with 3% Tergitol NP40. For fatty acid starvation experiments both fatty acids were omitted. Cycloheximide, when added, was at a concentration of 100 μg/ml which is sufficient to block cytoplasmic protein synthesis in these strains (7).

Growth Conditions for Starvation Experiments

Cells of strain BW1 1C were cloned and the clones were streaked onto YEPD + TW plates and allowed to grow up at 30°C. Log phase cells from this culture were harvested by centrifugation and washed twice with starvation medium. The cells were then resuspended in starvation medium at a density of 2×10^6 cells/ml. As a control, cells from the same starting log phase culture were transferred to medium containing fatty acid (synthetic complete with oleic and myristic acids; 3% Tergitol NP40). The cultures were maintained at 35°C.

Cells were harvested at intervals of starvation by centrifugation, washed three times with distilled water, pelleted, and stored in capped tubes on ice for further analysis.

Viability was determined by plating cells onto YEPD + TW plates at known dilutions throughout the starvation experiments. Plates were counted after 3 days incubation at 30°C, and each point on the survival curves in Fig. 1 represents the average of at least two plates. The viability of cells was also monitored by staining with methylene blue as previously described (7).

All cultures were assayed for revertants at the beginning and end of each experiment by plating onto unsupplemented medium. Cell concentrations for spin label measurements were determined with a hemocytometer.

Spin Label Addition to Cells

5N10 was added to thick suspensions of yeast directly in ethanol. The final concentration of ethanol was kept less than 1%. 5N10 was used at 10^{-4} M. Samples were mixed thoroughly in small tubes by vortexing and were then transferred into 50 μl capillaries, torch sealed, and were immediately used for electron spin resonance analysis. TEMPONE and PCA were added directly from stock cultures of 10^{-2} M aqueous 0.5 M $NiCl_2$. $K_3Fe(CN)_6$ was added to all samples at 10^{-6} M immediately before placing the samples into capillaries as an antireductant to prevent yeast samples from reducing the spin labels. No spectral differences could be seen plus and minus $K_3Fe(CN)_6$ except loss of signal intensity occurred in the absence of the antireductant. All cell preparations were concentrated by centrifugation except where noted. The strains used in the present analysis show near normal growth in 1 M $NiCl_2$ so probably experience no drastic toxicity from 0.5 M $NiCl_2$.

Spin Labeling

Electron spin resonance (ESR) measurements were carried out on a Japan electron-paramagnetic resonance spectrometer, Jes Me IX (Japan Electronics Mfg. Agency, Inc., Chicago, Ill.), X-band spectrometer. All measurements were carried out at room temperature.

The spin labels used were: 4-butyl-2,2-dimethyl-4-pentyl oxazolidine-*N*-oxyl (5N10); 4-decyl-2,2, 4-trimethyl oxazolidine-*N*-oxyl (2N12); 4-(6-cholesterol)-2,2-dimethyl oxazolidine-*N*-oxyl (6NC); 2,2-dimethyl-4-hexyl-4-(undecanoic acid) oxazolidine-*N*-oxyl (12NS); and 2,2,6,6-tetramethyl piperidine-*N*-oxyl (TEMPONE). Syntheses and purification procedures for these are published (10, 20) as is also the case for 3-carboxyl-2,2,6,6-tetramethyl pyrrolidine-*N*-oxyl (PCA) (18). The synthesis of 2,2,6,6-tetramethyl-4-phosphopiperidine-*N*-oxyl was carried out according to the method of Baer and McArthur (1). Diphenyl phosphoryl chloride (Aldrich Chemical Co., Milwaukee, Wisc.) was condensed with TEMPOL in dry pyridine. The reaction

product was reacted with 6 M $Ba(OH)_2$ for 8 h. The final product was purified by column chromatography using 200–300 mesh silica gel with methanol:ethyl acetate:acetic acid (9:1:1) as moving phase. The spin labels were purified and assayed for purity by thin-layer chromatography. The molecular motion term, rotational correlation time (τ_c), has been previously described in its use with spin labels (12, 14). The three spin labels used for most of the work represent three general structures—5N10 is an oxazolidine, TEMPONE a piperidine, and PCA a pyrrolidine—and they have slightly different numerical values for the hyperfine tensor and g-value tensor terms (Table I). We use an equation for τ_c

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

but remove the numerical dependency of the K term, which depends on the values of the tensors, and call the resulting value an empirical motion parameter, R_i (4), where $R_i = \tau_c/K$,

$$R_i = W_o[(h_o/h_{-1})^{1/2} - 1]. \quad (2)$$

W_o is the first derivative midline width, h_o is the first derivative midline height, and h_{-1} is the high field first derivative line height.

Hyperfine coupling constant (A_N) and g-value constant (g_N) measurements were carried out using a previously calibrated ^{15}N-TEMPONE sample (11) contained in a small capillary, ID 0.05 mm, inserted into the regular sample capillaries, ID 0.9 mm.

Electron Microscopy

For thin sectioning, yeast cells in the presence or absence of 0.5 M $NiCl_2$ were fixed for 2 h in 6% glutaraldehyde, brought to pH 7.2 with Tris buffer, and stained for 2 h in 1% osmium tetroxide in the same buffer. The samples were dehydrated through a graded series of acetone in water, embedded, and polymerized overnight at 60°C. Sections were cut on an LKB-Huxley ultramicrotome with glass knives and post-stained for 30 min in 1% uranyl acetate and 3 min in 0.4% lead acetate. Samples were viewed with an RCA EMU-3H electron microscope.

RESULTS

Cell Growth and Competence

Cells of the strain BW1 1C (*ole*1, *fas*1) lose viability if starved for fatty acids under otherwise growth-supporting conditions (Fig. 1). A previous study demonstrated that the phenomenon of cell death during fatty acid starvation was a property of the *fas*$^-$ mutation. Cells deficient only in fatty acid desaturase (*ole*$^-$) do not lose viability if starved for fatty acid. However, if protein synthesis is blocked by the addition of cycloheximide (100 μg/ml) at the start of fatty acid starvation, a 30–60% survival rate is maintained (Fig. 1). Under any given set of starvation conditions, respiratory-deficient cells die less rapidly than do respiratory-sufficient cells (Fig. 1).

Cellular Localization of Hydrocarbon Spin Labels

The data shown in Table I illustrate the solvent dependency of A_N and g_N values. 5N10 is a substituted hydrocarbon having the empirical formula, $C_{14}H_{28}NO$. The nitroxyl group has approximately the polarity of a ketone site. 5N10 partitions between methyl oleate and water by a factor of 60 ± 5 in favor of the oleate phase (Table II). The A_N of 5N10 in the various cell preparations ranged from 14.3 to 14.4 G, the

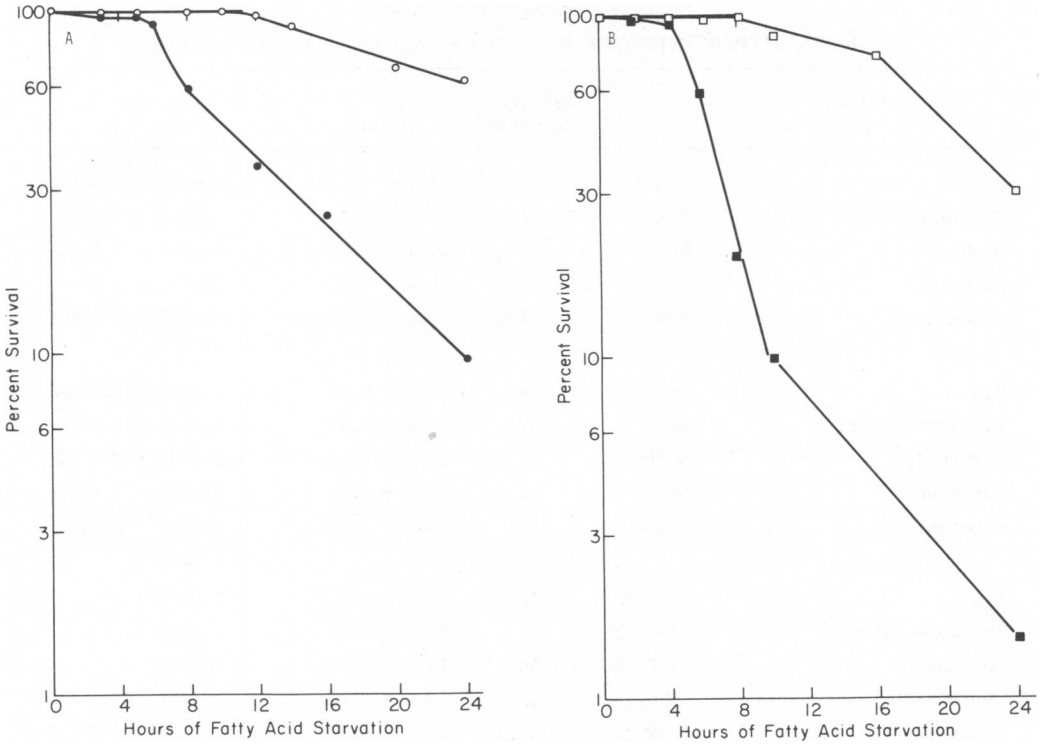
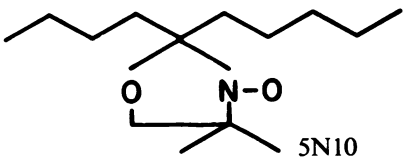


FIGURE 1 Survival of fatty acid starved cells of strain BW1 1C (*ole*1, *fas*1) at 35°C. (*A*) shows data from the respiratory deficient (ρ^-) strain. ○, cycloheximide 100 μg/ml, no fatty acid; ●, no fatty acid. (*B*) respiratory sufficient (ρ^+) strain. □, cycloheximide, 100 μg/ml, no fatty acid; ■, no fatty acid.

same as in an aqueous dispersion of yeast phospholipids (Table I) (8). Yeast cells grown on glucose have phospholipids in the various membrane fractions and may also contain some neutral lipids at various cell sites. The 5N10 molecule is hydrocarbon soluble and probably localizes at all cellular sites where hydrocarbons exist. The same general statements go for all the hydrocarbon spin labels.

Rotational Motion of Hydrocarbon Spin Labels

Spin labels 5N10, 2N12, 6NC, and 12NS (methyl ester) were used to probe the membranes of both the ρ^+ and ρ^- strains. 5N10 was used most extensively:



5N10

TABLE I

ISOTROPIC HYPERFINE COUPLING AND g-VALUES FOR SPIN LABELS

Solvent	Label	Dielectric constant	A_N (gauss)	g_N
H_2O	PCA	78	16.1 ± 0.05	2.0052 ± 0.0001
Ethylene glycol	PCA	37	15.8 ± 0.05	2.0053 ± 0.0001
Methanol	PCA	34	15.4 ± 0.05	2.0054 ± 0.0001
Pyridine	PCA	12	14.4 ± 0.05	2.0057 ± 0.0001
n-hexane	PCA	1.9	14.1 ± 0.05	2.0059 ± 0.0001
H_2O	5N10	78	16.1 ± 0.05	2.0052 ± 0.0002
Ethylene glycol	5N10	37	15.8 ± 0.05	2.0053 ± 0.0002
Methanol	5N10	34	15.4 ± 0.05	2.0054 ± 0.0002
Pyridine	5N10	12	14.5 ± 0.05	2.0056 ± 0.0002
n-hexane	5N10	1.9	14.2 ± 0.05	2.0059 ± 0.0002
H_2O	TEMPONE	78	16.3 ± 0.05	2.0054
Ethylene glycol	TEMPONE	37	16.1 ± 0.05	2.0055
Methanol	TEMPONE	34	15.7 ± 0.05	2.0056
Pyridine	TEMPONE	12	14.8 ± 0.05	2.0058
n-hexane	TEMPONE	1.9	14.5 ± 0.05	2.0060
Whole cells	PCA	-	15.9-16.0 ± 0.1	2.0052 ± 0.0002
Whole cells	5N10	-	14.3-14.4 ± 0.1	2.0059 ± 0.0002
Whole cells	TEMPONE	-	15.7-16.0 ± 0.1	2.0056 ± 0.0002
Whole cell phospholipids	5N10	-	14.3-14.4 ± 0.1	2.0059 ± 0.0002

Values for the isotropic hyperfine constant (A_N) and g-value constant (g_N) are given in a variety of solvents illustrating the correlation between dielectric constants of solvents and the A_N and g_N values. Values for A_N and g_N in the cell preparations are also given. g_N measurements for the cell preparations show an error factor one-third of the range of the measurement, so are useful only in determining gross differences. The yeast phospholipid sample was extracted from the ρ^+ strain after growth on palmitate and oleate. The phospholipids were prepared as previously reported (8).

Data from the other spin labels will be only briefly described.

Data from 5N10-labeled yeast are given in Fig. 2. Throughout the starvation period for both ρ^+ and ρ^- strains R_i measurements remain stable. The degree of restriction of spin label motion offered by cellular hydrocarbon zones is typical of that of growing yeast cells. In 5N10 spectra, the general spectral character did not undergo any detectable modification during the starvation period.

TABLE II

SOLUBILITY AND MOTIONAL PROPERTIES OF SPIN LABELS

Spin Label	Partition ratio oleic acid/water	R_i
5N10	60 ± 5	
TEMPONE	1.7 ± 0.1	
PCA (at pH 7.0)	1.0 ± 0.2 *	
PCA (at pH 3.0)	0.05‡ or less	
PCA in 10% PVP		0.13
PCA in 50% sucrose		0.15
PCA in 40% lysozyme		0.22
PCA in 50% sucrose, 10% BSA		0.15

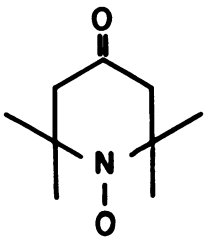
Partition ratios for 5N10, TEMPONE, and PCA between methyl oleate and water are given. R_i values of PCA in aqueous media containing different solutes are also given.

*Represents an upper limit; PCA may partition less than this value.

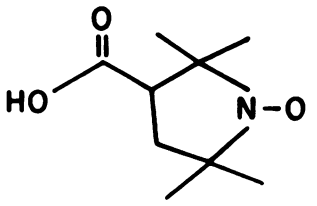
‡At the PCA concentration used on cells; this value is modified as different concentrations are used.

Cellular Localization of Water-Soluble Spin Labels

Measurements used TEMPONE and PCA:



TEMPONE



PCA

Samples containing known concentrations of yeast cells yielded a protected volume of 16 μm^3/cell and 20 μm^3/cell, respectively (see Fig. 3 and legend). 0.5 M $NiCl_2$ was in all these samples to quench signal outside the cells. The diameters of the spherical-equivalent protected volumes are 3.13 μm and 3.36 μm. The actual diameter of the yeast cells used here as determined by electron microscopy is estimated to be on the order of 3–3.5 μm. The micrographs show a variety of sizes, buds, and quasi-spherical shapes. The micrographs represent sections of all cell positions. Phase

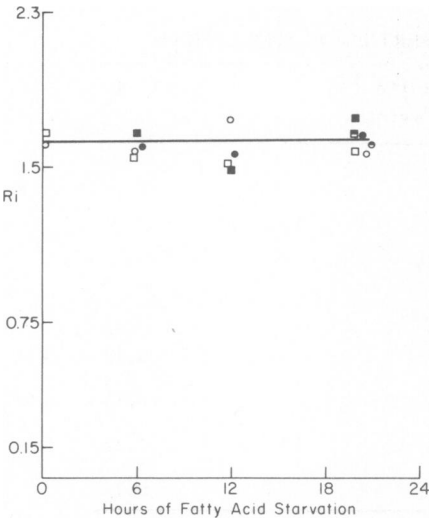


FIGURE 2

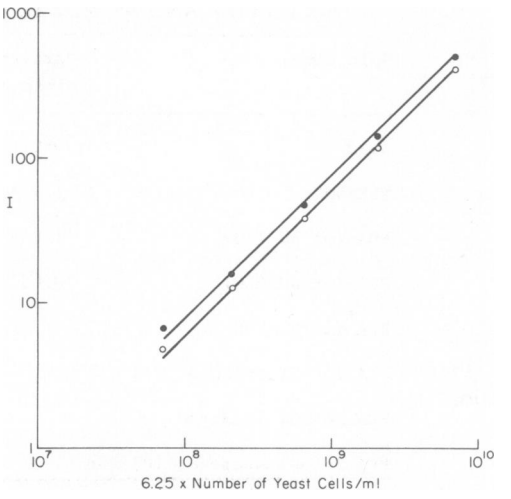


FIGURE 3

FIGURE 2 Rotational motion of 5N10 in normal and fatty acid starved cells. ○, ρ^-, fatty acid supplemented; ●, ρ^-, no fatty acid; ⊖, ρ^-, cycloheximide 100 μg/ml, no fatty acid. □, ρ^+, fatty acid supplemented; ■, ρ^+, no fatty acid; ⊟, ρ^+, cycloheximide 100 μg/ml, no fatty acid. Standard deviation (SD) of the mean fell between 0.17 and 0.42 for five samples of ρ^+ and between 0.15 and 0.37 for three samples of ρ^-.

FIGURE 3 Signal intensity of TEMPONE and PCA signals as a function of cell concentration. ○, TEMPONE; ●, PCA. I, signal intensity and equals the expression $W_1^2 h_1 / g$ where W_1 is the first derivative linewidth of the low field line, h_1 is the low field first derivative line height, and g is instrument gain. Both TEMPONE and PCA are used at 10^{-2} M. The highest values shown, 400 for TEMPONE and 500 for PCA, represent 10 and 12.5% of the total signal. This highest concentration is the thickest suspension that could be pulled into a capillary. The dilution shown covers a range of a factor of 100.

microscopy employing a micrometer also yielded estimates of cell diameter in the same range.

The inclusion of TEMPOPHOSPHATE with yeast cells followed by the addition of 0.5 M $NiCl_2$ caused less than 1% of the signal expected of equivalent concentrations of TEMPONE or PCA to remain. The phosphate-containing spin label is expected to be impermeable to yeast cells (or very slightly permeable). Since nickel is able to remove the TEMPOPHOSPHATE signal, the latter apparently has the same general access to the yeast cells as does nickel. Upon standing, an increase in the TEMPOPHOSPHATE signal was noted. This was probably due either to slow leakage or to an anion transport system.

TEMPONE partitions between hydrocarbons and water; however, it appeared to be predominantly localized in water zones in the yeast cells, as is evidenced by the A_N values shown in Table I.

The PCA molecule has high water solubility and partitions between methyl oleate and water at neutral pH favoring the aqueous phase by at least a factor of 20 (Table II).

At acid pH PCA partitions about equally between water and methyl oleate. The A_N and g_N values of PCA indicate that its residence in the cell preparations was almost entirely in an aqueous polar environment (Table I). The PCA therefore samples the aqueous cytoplasm, but is not necessarily randomly dispersed within the aqueous cytoplasm. The PCA could be more concentrated at surfaces and less concentrated in other zones.

Proteins and other biopolymers are positioned in cells in a manner which we cannot duplicate in vitro in the laboratory. However, to arrive at some approximations of the possible effects of increased concentrations of intracellular molecules, PCA was analyzed in the presence of several solutes (Table II). All these solutes, ranging from polyvinylpyrrolidine to sucrose or lysozyme, decrease the rotational motion of PCA. The magnitude of the decrease, however, is small compared with the effects of starvation on the motion of PCA in the cell interior.

Rotational Motion of Water-Soluble Spin Labels

The spin labels TEMPONE and PCA, in the presence of 0.5 M $NiCl_2$, were used in the analysis of both the ρ^+ and ρ^- cells under the same conditions of fatty acid depletion as with the analysis employing 5N10. The paramagnetic ion Ni^{++} broadens nitroxide spectra by mechanisms which affect all three hyperfine lines equally. Therefore, Ni^{++} broadening does not change the rotational correlation time τ_c or the empirical parameter R_i. This independence of R_i on Ni^{++} concentration is expected theoretically and has been verified in our laboratory.

TEMPONE, a small spin label which partitions between methyl oleate and water, with somewhat higher solubility in the methyl oleate, shows a dramatic change in motional freedom during the starvation process (Fig. 4). The ρ^+ strain confers a more rapid rise in restriction to spin label motion than does the ρ^- strain as starvation proceeds. This comparison parallels the relative growth rates since the ρ^+ grows faster. The ρ^+ strain, after 6 h of starvation, reaches close to its maximum in restriction to TEMPONE motion and correspondingly the survival has also drastically dropped. The drop in survival of the ρ^- strain proceeds more slowly than the ρ^+ strain as does the rate of change in restriction to spin label motion with starvation. There is a general correspondence between the loss of viability and increase in restriction to spin label motion.

The cells treated with PCA (Fig. 5) show the same general effects on restriction to PCA motion as for restriction to TEMPONE motion. PCA partitions more favorably into aqueous regions than does TEMPONE and is probably a more reliable indicator of the state of aqueous regions than is TEMPONE. PCA shows about the same initial rate of increase in restriction of motion as does TEMPONE. PCA differs in that, even in the ρ^+ strain, the restriction to motion continues to increase after the first 6 h.

Electron Microscope Examination

Thin-section electron micrographs of both ρ^+ and ρ^- cells of strain BW1 1C revealed no detectable differences between cells starved 12 h and fatty acid-supplemented cells.

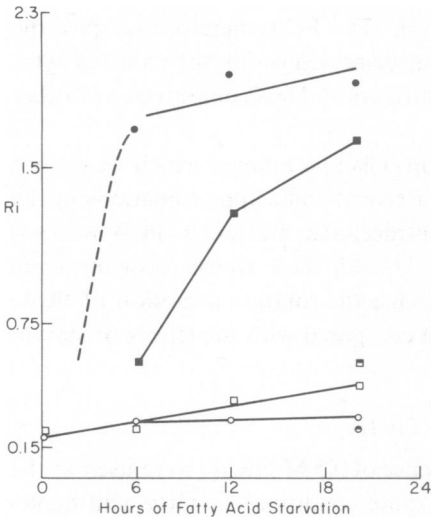


FIGURE 4

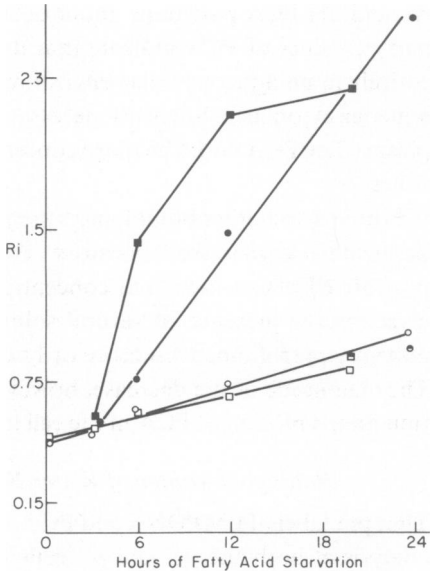


FIGURE 5

FIGURE 4 Rotational diffusion of TEMPONE in normal and starved cells. Symbols are the same as shown in Fig. 2 except for the spin label used.

FIGURE 5 Rotational diffusion of PCA in normal and starved cells. Symbols are the same as in Fig. 2 except for the spin label used.

At 12 h more than 99% of the cells are dead. Electron micrographs of cells starved 20 h, at which time more than 99.9% of the cells are dead, also failed to reveal any ultrastructural changes. The addition of 0.5 M $NiCl_2$ does not give any detectable alteration in the cells. The viscosity changes implied by changes in spin label motion cannot be accounted for by gross structural changes or by selective deformation due to nickel treatment. The ρ^+ and ρ^- strains of BW1 (*ole*1, *fas*1) grow in 1 M $NiCl_2$, so it is not surprising that 0.5 M $NiCl_2$ causes no obvious structural changes. Inspection of a large number of sections failed to reveal any consistent differences between starved and unstarved cells and cells in the presence and absence of $NiCl_2$.

DISCUSSION

The rapid loss of viability in fatty acid synthetase-deficient (*fas*$^-$) cells starved for fatty acid is a characteristic not shared by most other auxotrophic mutants starved for their auxotrophic requirement. For example, amino acid or adenine auxotrophs starved for their respective requirements stop growing but remain completely viable for 8–10 h with very gradual viability loss thereafter (7). Recently thymineless death has been reported in yeast (2). Other auxotrophic mutants in the fungi which share this characteristic loss of viability with yeast fatty acid synthetase mutants are all defective in some aspect of lipid synthesis. For example, inositol requirers in several species of fungi (9, 15) die if starved for inositol. Inositol is an important component of the

phospholipids in the fungi. Since the lipids affected in all of these mutants are important structural components of cell membranes, it has been suggested that loss of viability might be related to abnormalities arising in the membranes (5, 7).

In the case of both fatty acid and inositol-requiring mutants, it has been shown that viability loss could be largely prevented by blocking protein synthesis (Fig. 2; ref. 7). Furthermore, it had previously been shown that mutants defective only in fatty acid desaturase activity (*ole*) do not die when deprived of the unsaturated fatty acids they require for growth (7). These mutants differ from the *fas*$^-$ strains in their ability to synthesize saturated fatty acids under starvation conditions; therefore, the synthesis of fatty acid-containing lipids is not totally interrupted. These observations led to the conclusion that death in fatty acid-starved cells resulted only in the presence of ongoing protein synthesis in the absence of fatty acids. ESR measurements of the two single mutants, *ole*1 and *fas*1, carried out on isolated ρ^+ genotypes support this argument. The *ole*1 mutation causes only a slight increase in restriction to PCA motion during fatty acid starvation whereas the *fas*1 mutation causes a drastic increase in the restriction to the motion of PCA under the same conditions.

The present study takes advantage of a double mutant defective in the synthesis of long-chain fatty acids (*fas*$^-$) as well as in the desaturation of fatty acids (*ole*$^-$). Therefore, in the absence of exogenous fatty acid, this mutant is unable to synthesize fatty acids or modify their degree of unsaturation. Under conditions of fatty acid starvation, therefore, the mutant would be expected either to produce membranes deficient in fatty acid-containing lipids or to stop the synthesis of membranes altogether. In the present study, spin labels which probe the cellular hydrocarbon zones show little if any change in physical environment as a consequence of fatty acid starvation.

The consequences of such a condition where new cell membrane cannot be made but the cell machinery for other metabolic functions is at least initially intact should be great. We use two water-soluble spin labels to examine molecular rotational diffusion in the aqueous interiors of both ρ^+ and ρ^- fatty acid auxotrophs under conditions of fatty acid starvation and supplementation. Both spin labels show that the barriers to molecular rotation are drastically increased in the starvation process.

The increase in these barriers to molecular rotation correlates temporally with the period of cell death during fatty acid starvation (Figs. 1, 3, 4). The ρ^+ strain, which begins to decline in viability somewhat before the ρ^- strain (Fig. 1), also shows an earlier initial increase in apparent viscosity (Figs. 3, 4). Furthermore, the more rapid rate of cell death observed in the ρ^+ strain (Fig. 1) correlates with its more rapid initial rate of increase in restriction to spin label motion compared with the ρ^- strain. The slight increase in R_i values observed in fatty acid-supplemented control cultures (Figs. 3, 4) occurs as the cultures approach and enter stationary phase. In stationary cultures of these fatty acid-requiring strains there is a gradual decline in viability.

One possibility is that synthetic processes, which continue in the absence of the normal increase in cell volume brought about by cell division, lead to an increase in intracellular viscosity. Indeed, this could explain the relative protection afforded cycloheximide-treated cells. In fact, protein and RNA synthesis in the cells not treated with

cycloheximide have been shown to continue at rates comparable to fully supplemented cells for approximately 3 h after the removal of fatty acid. Cell division, as measured by increase in cell number, in marked contrast, ceases at 1 h (7).

However, the increase in R_i does not occur in a linear fashion throughout the starvation period, as might be predicted from the above hypothesis. Rather, the increase in R_i starts approximately at the time when cells rapidly lose viability. The restriction of the molecular motion of the cytoplasmic probe might, therefore, not be brought about by a simple change in intracellular solute concentration but rather by some as yet not understood alteration in the structure of the cytoplasmic matrix. For example, aggregation of protein and other solutes at membrane interfaces would increase the solute concentration at the membrane interface regions.

Data given in Table I show that solute materials at fairly high concentration, such as 40% lysozyme, do not restrict PCA nearly so much as the yeast cell interior under starvation conditions for fatty acids. This suggests that simple increase in solute concentration is not a totally adequate explanation for the observed restriction in the motion of the probes during fatty acid starvation. However, proteins and other solutes in the contained volume of the cell may have greater effects on the rotational diffusion of small spin label molecules than they have in bulk volume.

An important question raised by the increase in R_i during starvation is whether this observed change in the physical state of cytoplasm is either the result or the cause of cell death. It is impossible to be sure in individual cells whether the increase in R_i slightly precedes, accompanies, or closely follows death. It is possible, therefore, that the observed change in R_i is the result of a change in "state" of the cytoplasmic matrix following death. It is interesting to note, in this regard, that Takayama et al. (19) observed an increased restriction of spin label motion in *Mycobacterium tuberculosis* when these cells were treated with the bacteriocidal agent isoniazid.

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