Effect of Exogenous Fatty Acids on Biotin Deprived Death of *Saccharomyces cerevisiae*

Shoji SHIMADA,*¹ Hiroshi KURAISHI*² and Ko AIDA

The Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan

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The effect of exogenous fatty acids on cell growth and death of the biotin-requiring yeast *Saccharomyces cerevisiae* BA-1 was examined with respect to the mechanism of synthetic pathway of fatty acid under biotin starvation. At a growth temperature of 30°C, exogenous unsaturated fatty acids, such as palmitoleic, oleic, linoleic, and linolenic acids which promote the cell growth and suppress death effectively, were incorporated intactly into the cellular fatty acids, whereas the saturated fatty acid, palmitic acid, which supports growth but somewhat inhibits death, was once incorporated, and about 60% of incorporated palmitic acid was found to be desaturated. However, at an elevated temperature of 36°C, even palmitic acid showed similar effects to unsaturated fatty acids in cell growth and death; followed by an increased desaturation of palmitic acid. Thus the data indicate that palmitic acid, as well as unsaturated fatty acids directly compensate for the deficiency of endogenously synthesized fatty acids caused by biotin starvation.

Auxotrophic microbes die in some cases if permitted to grow in the absence of required substances. This phenomenon has been termed unbalanced growth death and clearly been observed with yeast cells which require biotin, pantothenate, or inositol for growth.^{1~3)} Death from unbalanced growth occurs when the biotin-requiring yeast is incubated in medium which lacks biotin but contains aspartic acid.⁴⁾ This death was accompanied by inhibition of *de novo* fatty acid synthesis from acetyl-CoA,⁵⁾ and could be prevented by the addition of fatty acids, especially unsaturated acids to the medium.⁶⁾ Therefore, it has been suggested that death may result from defective cell membranes.⁶⁾ In fatty acid mutants of *Saccharomyces cerevisiae* (fas 1 and fas 1, ole), it has likewise been reported that fatty acid starvation results in a high percentage of dead cells.⁷⁾

In the present work, the effects of various exogenous fatty acids on the growth and death

of cells were investigated in relation to changes in cellular fatty acid content and composition. And, fatty acids were examined especially in regard to the relationship between the compensation system for deficiency of cellular fatty acids caused by biotin starvation and the mechanism of synthetic pathway of fatty acid. Moreover, the effect of saturated fatty acid on cell death was examined in connection with both temperature and desaturation activity.

MATERIALS AND METHODS

Yeast strain. The strain of *Saccharomyces cerevisiae* BA-1 used in the present investigation was the same as that described previously.⁴⁾

Media. The basal medium (pH 4.5) had the following composition per liter: glucose, 10 g; Na₂HPO₄, 2.2 g; citric acid, 2.0 g; KCl, 0.6 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·6H₂O, 0.1 g; H₃BO₃, 500 µg; FeCl₃·6H₂O, 200 µg; MnSO₄·H₂O, 200 µg; Na₂MoO₄·2H₂O, 200 µg; KI, 100 µg; CuSO₄·5H₂O, 40 µg; inositol, 2.0 mg; pyridoxine·HCl, 400 µg; thiamine·HCl, 400 µg; calcium pantothenate, 400 µg. Ammonium sulfate and sodium aspartate were used as the sole source of nitrogen at concentrations of 1.057 (Am medium) and 2.5 g/liter (Asp medium), respectively. The preculture medium used to obtain the biotin-deficient cells was the Am medium containing 0.08 µg/liter of biotin, and the Asp medium used for observation of cell death was

*¹ Present address: Laboratory of Plant Physiology, The Institute of Physical and Chemical Research (Rikagaku Kenkyusho), Wako-shi, Saitama 351, Japan.

*² Present address: Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3, Saiwai-cho, Fuchu-shi, Tokyo 183, Japan.

without biotin.⁴⁾ When the effects of supplemental fatty acids on growth and death were examined, each fatty acid was added at the concentration of 0.2 mM into Asp medium without biotin. For labeling experiments, an isotope was added to Asp medium containing the corresponding unlabeled fatty acid (0.2 mM) at the time of transfer; these contained 2 μ Ci of [$1-^{14}$ C]palmitic acid or 2 μ Ci of [$10-^{14}$ C]oleic acid per 100 ml.

Growth condition of transfer culture. The biotin-deficient cells cultured in the above Am medium at 30°C for 32 hr were collected by centrifugation, washed once with sterile deionized water, resuspended in the same medium, and inoculated into various biotin-free media to examine the appearance of dead cells.

Measurement of growth. Growth was measured as described previously,⁴⁾ but when exogenous fatty acid was added to Asp medium, it was determined by estimating the protein content of yeast cells by the Folin-Ciocalteu technique of Lowry *et al.*⁹⁾

Counting dead cells. Dead cells were counted under a microscope as described previously.⁴⁾

Extraction and analysis of fatty acids. Cellular fatty acids were extracted and converted into the corresponding methyl esters as described previously,⁹⁾ but when exogenous fatty acid was added to Asp medium, cells were collected by centrifugation, washed with water, and washed once with 1% Triton X-100. Fatty acid esters were separated by gas-liquid chromatography with a Shimadzu GC-4BPF gas chromatograph equipped with a flame ionization detection system, using a column with 10% diethylene glycol succinate (DEGS) on Chromosorb W and N₂ as the carrier gas at 165°C. Methyl esters of fatty acids were identified by comparing their retention time with those of known fatty acid methyl esters in mixtures. The area under each component-fatty acid peak was measured by multiplying the peak height by the width at one-half the height. The quantity of individual fatty acids was determined by calculating the peak area and compared with the internal standard, pentadecanoic acid (C_{15:0}). Gas-liquid chromatographic analysis of the radio-active esters was performed using a Shimadzu GC-5AP₃T with thermal conductivity detector in combination with a combustion tube (copper oxide, 830°C) and a proportional counter. The operating conditions were: DEGS (10%) on Chromosorb column; 165°C ([$1-^{14}$ C]palmitic acid), 180°C ([$10-^{14}$ C]oleic acid) temperature; 26.5 ml/min He carrier gas.

Chemicals. [$1-^{14}$ C]Palmitic acid of 53.2 mCi/mmol and [$10-^{14}$ C]oleic acid of 41.4 mCi/mmol were purchased from the Daichi Pure Corp., Tokyo. The two acids were shown to be 99% pure by radio gas-liquid chromatography.

RESULTS

Effect of biotin on cell growth and death

Baker's yeast was cultured in a biotin-deficient (0.08 μ g/liter) medium containing ammonium sulfate to obtain living biotin-deficient cells. The biotin-deficient cells were harvested after 32 hr of cultivation and transferred to Asp medium with and without biotin. Figure 1 shows the effect of biotin on the yeast cell growth and death. There was little or no difference in the optical density in the two media at 3 hr after transfer, and moreover, no dead cells were observed in either of the above media (Fig. 1). However, although some increase in turbidity was noted, rapid cell death occurred when the cells were cultured in Asp

FIG. 1. Growth (A) and Death (B) of Baker's Yeast Cells in Asp Medium with (○—○) and without (●—●) Biotin.

Biotin to a final concentration of 2.0 μ g/1000 ml was added to Asp medium at zero time.

medium without biotin for 32 hr. On the other hand, cell death was almost completely prevented, and cell growth was normally resumed at the end of 8-hr incubation in the presence of biotin.

Effect of various fatty acids on cell growth and death

Each of the eight fatty acids (myristic, palmitic, palmitoleic, stearic, oleic, elaidic, linoleic, and linolenic acids) was individually added to the biotin-free Asp medium, and cell growth and the percentage of dead cells were determined after 8 hr of transfer culture. As shown in Fig. 2, longer-fatty acids with 14 carbon atoms or more promoted growth and palmitoleic acid was the most effective for growth. This effect was near that of biotin. However, fatty acids with 18 carbon atoms, such as stearic and elaidic acids, suppressed cell growth more than that of control cells without fatty acid. On the other hand, the prevention of cell death by biotin starvation was strongly observed with palmitoleic, linoleic, and linolenic acids. Oleic, elaidic, and myristic

FIG. 2. Effect of Addition of Different Fatty Acids to Biotin-free Asp Medium on Growth (□) and Death (■).

Biotin-deficient cells grown in 300 ml of Asp medium with fatty acid (0.2 mM) for 8 hr at 30°C were collected by centrifugation, washed with 1% Triton X-100, and growth and death were determined.

acids reduced death by 66, 57, and 54%, respectively, as compared with the control level. But, palmitic and stearic acids had a partial inhibitory effect on cell death, and they reduced death by 40 and 25%, respectively.

Effect of biotin on the content and composition of fatty acid

Changes in cellular fatty acid content and composition were examined after introduction of biotin-deficient cells into Asp medium with and without biotin (Table I). The total content of cellular fatty acids in both groups of cells increased with time for the first 3.5 hr after transfer. The fatty acid content of cells in the biotin-supplemental medium continued to increase during the subsequent 4.5 hr period, while the fatty acid content of cells without

FIG. 3. Separation of Fatty Acid Methyl Esters by Gas-liquid Chromatography.

Cells were grown in Asp medium with and without biotin for 8 hr at 30°C.

TABLE I. FATTY ACID CONTENT AND COMPOSITION OF WHOLE CELLS GROWN IN THE ASP MEDIUM WITH AND WITHOUT BIOTIN

Cells were grown for 32 hr in Am medium at 30°C, washed, and transferred to Asp medium with and without biotin. Samples were removed at the times indicated, and the cell fatty acid content and composition were determined.

Biotin (μ g/1000 ml)	Culture period (hr)	Fatty acid content (mg/100 mg protein of cells)							Total
		C _{12:0}	C _{14:0}	C _{14:1}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	
0	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	1.84
	3.5	0.21	0.14	0.40	0.16	1.34	Trace	0.81	3.06
	8.0	0.10	0.06	0.19	0.07	0.82	Trace	0.37	1.61
2	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	1.84
	3.5	0.05	0.11	0.05	0.70	1.50	0.16	1.14	3.71
	8.0	0.03	0.09	0.04	0.70	1.70	0.26	1.52	4.34

biotin was below the original level during the same period. This fact indicates that cells cultured in the absence of biotin became deficient in cellular fatty acids. Figure 3 shows that cells from Asp medium with or without biotin contain mainly laurate, myristate, myristoleate, palmitate, palmitoleate, stearate, and oleate, and that significant quantitative changes in fatty acid composition may be observed with a lapse of time in the biotin deficient and sufficient conditions. Table I seems to show that the amount of C₁₆₋₁₈ fatty acids in cells incubated in biotin-sufficient Asp medium increased remarkably by 8 hr after transfer. The level was more than that of cells at zero time, but there was no increase in C₁₂₋₁₄ fatty acids. In contrast, the fatty acid content in cells cultured in biotin-free Asp medium decreased in saturated acids and oleic acid.

Effect of supplemental fatty acids on cell fatty acid composition

The effect of supplemental fatty acids on fatty acid composition of cells was examined under biotin-deficient conditions. The results were summarized in Table II. It appears that unsaturated fatty acids, such as palmitoleic and oleic acids, were incorporated intactly into cells at 30°C. A similar observation was made after the addition of linoleic or linolenic acid, which has not been contained in the yeast strain used here. In general, it was found that exogenous unsaturated fatty acids as growth

factor resulted in increased concentrations of cellular unsaturated fatty acid with the same chain-length. For example, cells grown on exogenous oleic acid were richer in C_{18:1} acid than in other acids of cells in 3.5 hr of incubation. And, cells grown on linoleate and linolenate contained extremely large amounts of corresponding fatty acids. Judging from these data, the accumulation of unsaturated fatty acid derived directly from exogenous unsaturated fatty acid is predominant in cells grown on unsaturated fatty acids. In the addition of saturated fatty acids, such as myristate, palmitate, and stearate, the first two were found to be incorporated into cells and accumulated in cellular fatty acids within 3.5 hr; thereafter no further incorporation was observed, whereas the latter was found to be incorporated into cells only to a small extent. In addition, it appeared that cells were capable of converting saturated fatty acids into monounsaturated fatty acids, such as myristate to myristoleate and palmitate to palmitoleate.

Effect of saturated fatty acids on cell growth and death at 36°C

Biotin-deficient cells cultured in Am medium were harvested at 32 hr and allowed to grow at either 30 and 36°C in Asp medium containing a saturated fatty acid, such as palmitic and stearic acids for the purpose of studying their effect on cell growth and death. In medium without fatty acid, cell growth was inhibited

TABLE II. EFFECTS OF EXOGENOUS FATTY ACIDS ON FATTY ACID COMPOSITION OF WHOLE CELLS

Fatty acid added (0.2mM)	Culture period (hr)	Fatty acid composition (mg/100 mg protein of cells)							
		C _{12:0}	C _{14:0}	C _{14:1}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}
C _{14:0}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.06	0.54	0.42	0.12	0.80	Trace	0.32	
	8.0	0.02	0.48	0.38	0.10	0.68	Trace	0.24	
C _{16:0}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.09	0.09	0.12	0.50	1.35	Trace	0.47	
	8.0	0.08	0.10	0.14	0.24	1.29	Trace	0.37	
C _{16:1}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.08	0.18	0.06	0.45	3.91	Trace	0.47	
	8.0	0.05	0.09	0.05	0.16	1.52	Trace	0.26	
C _{18:0}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.10	0.10	0.20	0.13	1.13	0.06	0.59	
	8.0	0.11	0.09	0.20	0.15	1.35	0.08	0.79	
C _{18:1}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.12	0.16	0.15	0.24	1.24	Trace	2.20	
	8.0	0.05	0.14	0.08	0.15	0.91	Trace	2.53	
C _{18:2}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.07	0.16	0.05	0.33	0.57	Trace	0.33	3.04
	8.0	0.05	0.20	0.09	0.33	0.72	Trace	0.35	2.39
C _{18:3}	0	0.03	0.07	0.05	0.27	0.81	0.05	0.56	
	3.5	0.09	0.25	0.07	0.55	0.82	Trace	0.58	3.60
	8.0	0.06	0.16	0.07	0.31	0.73	Trace	0.36	1.71

at an elevated temperature of 36°C (control cells) and reduced to two-thirds of the control level at 30°C (Table III), whereas at 36°C, cell growth in the presence of palmitic acid was clearly promoted to about twice that of cells incubated at 30°C, and cell death was inhibited more than at 30°C. By contrast, in stearate at 36°C, the cell growth level was almost the same as that at 30°C. In addition, this growth was as well as that of control cells at 30°C. The findings imply that in both the presence and absence of stearate, cell growth was inhibited by the elevated temperature. The temperature elevation may thus result in the prevention of cell death.

Uptake and incorporation of [1-¹⁴C]palmitic acid and [10-¹⁴C]oleic acid by yeast

The uptake and incorporation of [1-¹⁴C]-palmitic acid from growth medium to cells were examined in palmitic acid-containing Asp medium at both 30 and 36°C to further study

the effect of fatty acids on cell growth and death. In experiments shown in Figs. 4-I and II, labeled palmitic acid was desaturated by yeast and converted to C₁₆ and C₁₈ monounsaturated fatty acids at both 30 and 36°C. Table IV shows the effect of incubation temperature on the relative rate of desaturation of [1-¹⁴C] palmitic acid incorporated into yeast cells grown in Asp medium. It is evident that radioactive palmitate was converted into C₁₆ monounsaturated fatty acid (palmitoleic acid) in 72.8% yield at 36°C, whereas it was converted into this component in 61.3% yield at 30°C. With respect to the distribution of radioactivity in conversion of palmitate to C₁₈ fatty acid, a yield of only about 7% was observed. This fact indicates that more than 60% of palmitic acid, once incorporated, was converted into the corresponding monounsaturated acid and accumulated in the cellular fatty acids and only a small amount was elongated. Moreover, this result suggests that

FIG. 4. Incorporation and Elongation of [1- 14 C]-palmitic Acid by Growing Cultures of Yeast at 30°C (I) and at 36°C (II).

Biotin-deficient cells were grown in 150 ml of Asp medium with 7.69 mg (0.2 mM) palmitic acid containing 3 μ Ci (0.0564 μ mol) of [1- 14 C]palmitic acid for 8 hr at 30°C, collected by centrifugation, washed with 1% Triton X-100, and the fatty acid composition was determined. (A) radioactivity, (B) mass distribution.

FIG. 5. Incorporation of [10- 14 C]oleic Acid by Growing Culture of Yeast at 30°C.

Biotin-deficient cells were grown in 150 ml of Asp medium with 8.47 mg (0.2 mM) oleic acid containing 3 μ Ci (0.0722 μ mol) of [10- 14 C]oleic acid for 8 hr at 30°C, collected by centrifugation, washed with 1% Triton X-100, and the fatty acid composition was determined. (A) radioactivity, (B) mass distribution.

the higher activity of palmitic acid desaturation at 36°C than at 30°C may be correlated with the much lower number of dead cells at 36°C. On the other hand, in the experiment shown in Fig. 5, oleic acid was found to be incorporated intactly into cells and accumulated in cellular fatty acids as such. These findings are in good agreement with the results presented in Table II.

DISCUSSION

As shown in Fig. 1 when biotin-deficient cells grown for 32 hr were transferred into medium supplemented with aspartic acid as the sole nitrogen source, rapid death and a high percentage of dead cells were observed. Thus, this phenomenon has been termed "unbalanced growth death." Recently, Mizunaga *et al.*⁶⁾ showed that death due to unbalanced growth was completely suppressed by the addition of palmitoleic, oleic, and linoleic acids under biotin starvation. In the present studies, we also obtained similar results to Mizunaga *et al.*⁶⁾ by adding palmitoleic, oleic, and linoleic acids. In addition, we obtained results showing that the addition of the saturated fatty acid palmitic acid suppressed cell death strongly when the growth temperature was elevated to 36°C (Table III). In cell growth, however, we obtained some different results (Fig. 2) from Mizunaga *et al.*⁶⁾ Exogenous palmitoleic acid

TABLE III. EFFECT OF TEMPERATURE ON GROWTH AND CELL DEATH OF YEAST

Cells were grown for 32 hr in Am medium at 30°C, washed, and transferred to two 50-ml portions of Asp medium with fatty acid (0.2 mM). One portion was incubated at 30°C and the other at 36°C. After 8 hr, the cell growth and death ratios of yeast cells were determined as described in the text.

Fatty acid added (0.2 mM)	Growth temp. (°C)	Growth (mg protein)		Death (%)	
		0 hr	8 hr	0 hr	8 hr
None	30	10.0	24.3	0	85
	36	10.0	16.5	0	18
C _{16:0}	30	10.0	28.8	0	47
	36	10.0	50.5	0	18
C _{18:0}	30	10.0	19.8	0	62
	36	10.0	17.0	0	22

showed the most promotive effect in cell growth as biotin, whereas other fatty acids did not show such an effect. As shown in Table II, it appears that exogenous palmitoleic acid acts as a substitute for the predominant component in cellular fatty acids. Therefore, this effect by palmitoleic acid implies that it may compensate directly for the deficiency of the predominant component in fatty acids, such as endogenously synthesized palmitoleic acid. Similarly at 36°C, palmitic acid obviously acts as a substitute for endogenously synthesized unsaturated fatty acid in the yeast cells used here. The findings are in accord with the opinion expressed by Mizunaga *et al.*⁶⁾ As a whole, the survival data after addition of either unsaturated or saturated fatty acid shows that the cell death caused by biotin starvation may be at least associated with deficiency of fatty acids or fatty acid-containing lipids. It also appears that failure to synthesize the predominant component of fatty acids is involved in the death of the biotin-deficient cells.

It is interesting to find that exogenous linoleic and linolenic acids were taken up by the yeast cell and were capable of replacing endogenously synthesized unsaturated fatty acids to prevent cell death. Although these fatty acids are not detected in cells, they were incorporated into cellular fatty acids, in a manner similar to the case of oleic acid (Table II), and retained there as the most predominant component in fatty acids. At the same time, it was evident that they could quantitatively compensate for the deficiency of cellular fatty acids caused by

biotin starvation. Both linoleic and linolenic acids probably contribute to the functionality of cell membrane, because they are structurally *cis*-typed unsaturated fatty acids and have a common Δ^9 -double bond as palmitoleic acid. Moreover, a point of interest is the effect of palmitic acid on cell growth (Table III). At 36°C, although exogenous palmitic acid was once desaturated in cells, it had a great promotive effect on cell growth, as well as palmitoleic acid at 30°C (Fig. 2, Table III). Perhaps this effect of palmitic acid results in the prevention of cell death. This phenomenon in the presence of palmitic acid at 36°C is considered similar to the phenomenon of palmitoleic acid at 30°C. But the reason for the promotive effects by both palmitic and palmitoleic acids yet remains obscure. In light of observations that the extent of incorporating palmitic acid in cellular fatty acids did not differ appreciably between 30 and 36°C (data not shown) and that the extent of desaturation of palmitic acid in cells increased as the temperature was elevated to 36°C (Table IV), it seems likely that the increase in desaturation rate is associated with an increase in the growth temperature.

The cell death reported here was also found by Henry⁷⁾ and Mizunaga *et al.*⁶⁾ using the fatty acid mutants of *S. cerevisiae*. Henry pointed out that changes at the cell surface might be closely associated with death, because the detergent Tergitol NP 40 accelerated viability loss during the period of fatty acid starvation.⁷⁾ In addition, a somewhat similar observation was made by Mizunaga *et al.*⁶⁾ that

TABLE IV. DESATURATION OF [1-¹⁴C]PALMITIC ACID BY YEAST AT 30°C AND 36°C

Cells were grown for 32 hr in Am medium at 30°C, washed, and transferred to two 150-ml of Asp medium with 7.69 mg (0.2 mm) palmitic acid containing 3 μ Ci (0.0564 μ mol) of [1-¹⁴C]palmitic acid. One portion was incubated at 30°C and the other at 36°C. After 8 hr, the cells were collected by centrifugation, and the fatty acid composition of cells and radioactivity of fatty acids were determined.

Growth temp. (°C)	Radioactivity cpm/mg protein of cells (% distribution)			Desaturation (conversion ratio)
	C _{16:0}	C _{16:1}	C _{18:1}	C _{16:1} + C _{18:1} /C _{16:0}
30	4067 (31.1)	8015 (61.3)	1000 (7.6)	2.21
36	1946 (20.6)	6886 (72.8)	623 (6.6)	3.80

Ca and Mg ions in cells decreased rapidly after a 3-hr period of biotin starvation. As a result, the cell surface structure may change irreversibly under these conditions, and the cells lose viability. Recently, under biotin-deficient conditions, cell death was reduced when DNA synthesis was stopped by the addition of hydroxyurea (HU), although protein and RNA syntheses in cells were not affected by this drug.¹⁰⁾ This finding indicates that a relationship between cell death and DNA synthesis, as well as protein synthesis under biotin deficiency. Therefore, the cell death described here is rather the result of some metabolic "unbalance," involving lipid synthesis containing fatty acid, and macromolecular synthesis in cells which occurs when cells are deficient in cellular fatty acids caused by biotin starvation.

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