EFFECTS OF THIAMINE AND PYRIDOXINE ON THE CONTENT AND COMPOSITION OF STEROLS IN SACCHAROMYCES CARLSBERGENSIS 4228

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SUMMARY: The level of sterols in S. carlsbergensis 4228 cells grown aerobically on a synthetic medium fortified with thiamine was significantly low compared with that in the control cells. The levels of free and esterified sterols in the thiamine-cells were 60% and 10% of the corresponding sterol levels in the control cells, respectively. Analysis by gas-liquid chromatography of non-saponifiable lipids extracted from the cells revealed that the amounts of squalene, lanosterol and two unidentified sterols were higher than those in the control cells and that ergosterol and zymosterol, major sterols in the control cells, were not present. These effects of thiamine on the content and composition of sterols were abolished by the addition of pyridoxine to the medium.

It has been known that the growth of Saccharomyces carlsbergensis 4228 (ATCC 9080) on a synthetic medium is depressed by thiamine unless pyridoxine is added to the medium (1). In the preceding communication (2), we reported that the cells growing in the presence of thiamine and absence of pyridoxine ("thiamine-cells") exhibited a markedly low respiration even under aerobic conditions. We have also reported (3) that the level of unsaturated fatty acids in "thiamine-cells" was about one fourth the level in the control cells which were grown in the absence of both thiamine and pyridoxine, and that the addition of pyridoxine to the medium abolished these effects of thiamine. Since sterols, as well as unsaturated fatty acids, are known to be important components of membraneous systems of yeast cells, it seemed reasonable to expect that thiamine could affect the level of sterols in the cells as well.

This communication reports that addition of thiamine significantly decreases the sterol content of the yeast and that ergosterol, the most predominant sterol in the control cells, is absent in "thiamine-cells".

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MATERIALS AND METHODS

Growth of yeast. The cultivation conditions for Saccharomyces carlsbergensis 4228 (ATCC 9080) and the growth measurement were described in the preceding paper (2).

Extraction of lipids. Nonsaponifiable lipids were extracted with petroleum ether after the treatment of cells with 1 g of KOH in 5 ml of 70% methanol at 80° for 2.5 hrs under nitrogen. Pyrogallol was added to prevent the oxidation. Pooled petroleum ether extracts were washed three times with water and dried over anhydrous sodium sulfate. Extraction of free and esterified sterols were done according to the method of Monner and Parks (4). An aliquot of the washed petroleum ether extract was dried up and applied to a thin layer chromatographic plate (Silica Rider 5B). After development with 2% methanol in benzene, sterol ester fraction (Rf = 0.58-0.67), L-fraction corresponding to free lanosterol (0.09-0.14) and E-fraction corresponding to free ergosterol (0.03-0.09) were scraped from the plate and lipids were eluted with 5 ml of benzene-methanol (1:1). The eluate containing the sterol esters were dried up and dissolved in 0.1 ml of benzene and saponified with 20% KOH in methanol in the presence of pyrogallol.

Analysis of nonsaponifiable lipids by gas-liquid chromatography. Analyses of free sterols and squalene were done with a Shimadzu GC-5A gas-liquid chromatograph equipped with a hydrogen flame ionization detector. A glass column (4 mm X 1.5 m) packed with 1.5% silicone SE-30 adsorbed on Chromosorb W (60-80 mesh) was used at 235° or 240° with the use of nitrogen as carrier gas at flow rate of 50 ml per min. To determine the sterol content, the part of recorder tracings of peak areas were excised and weighed. Cholestane or cholestanol was used as an internal standard. Lanosterol and cholestane were purchased from Sigma Chemicals Co. and squalene, cholestanol and ergosterol were from Nakarai Chemicals Ltd., Kyoto, Japan. Zymosterol was kindly supplied by Dr. N. Ariga, Gifu University, Japan.

RESULTS

The yeast cells grown in the presence and absence of thiamine were harvested at the middle logarithmic growth phase and nonsaponifiable lipids were extracted from them. As revealed in the analysis by gas-liquid chromatography, the total amount of nonsaponifiable lipids of "thiamine-cells" was much smaller than that of the control cells (Figure 1). Zymosterol and ergosterol were almost completely absent in "thiamine-cells", and the amount of squalene and of lanosterol were higher than those of the control cells. This fact seems to suggest that the biosynthesis of ergosterol is inhibited at certain step(s) after lanosterol in the cells growing in the presence of thiamine.

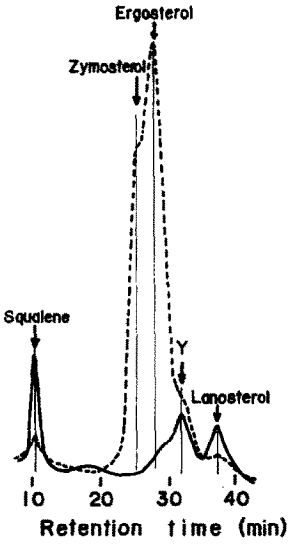

Figure 1. Gas-liquid chromatograms of nonsaponifiable lipids from S. carlsbergensis 4228 cells harvested at the middle logarithmic growth phase (A_{610} for the control cells, 2.65 ; A_{610} for "thiamine-cells", 3.15). Comparable amounts of the samples of the control cells (-----) and "thiamine-cells" (———) were injected. Column temperature was 235°.

As reported in the preceding papers (2,3), the effects of thiamine on the cell growth and on the fatty acid composition were abolished when pyridoxine was added to the medium. This was also the case for the content of sterols as shown in Table I. The content of sterols in the cells growing in the presence of pyridoxine was not affected by the addition of thiamine. The data in Table I also show that thiamine decreased more markedly the content of sterol esters (about one tenth) than the content of free sterols (about six tenths).

Table I. Effects of thiamine and pyridoxine on the contents of squalene and sterols in the cells of S. carlsbergensis

Cells*	Contents of lipids (mg/g dry cells)			
	Squalene	Sterols		
		Total	Free	Esterified
Control	0.7	10.8	3.8	7.0
+ Thiamine (1 μg/ml)	1.9	2.8	2.3	0.5
+ Pyridoxine (0.02 μg/ml)	0.8	14.2		
+ Thiamine + Pyridoxine	0.9	12.7		

* Cells were harvested at the early logarithmic growth phase (A_{610} : 0.9—1.3). Methods for the analysis were described in the text.

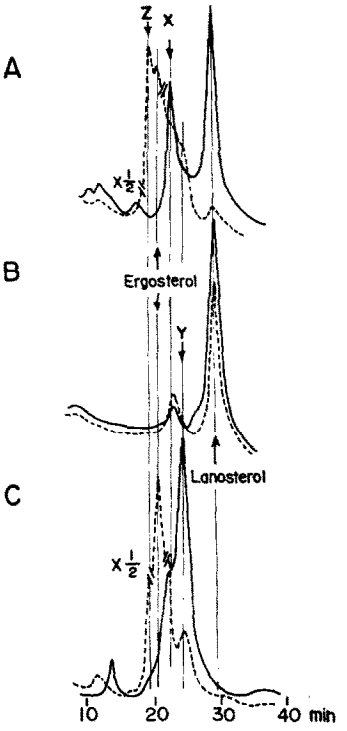

Figure 2. Gas-liquid chromatograms of sterol fractions separated by thin layer chromatography. Sterol esters were saponified as described in the text. Sterol samples of adequate amounts were injected for the analyses of ester-, L- and E-fractions from the control cells (-----). For the analyses of ester-, L- and E-fractions, 10, 0.5 and 1.5 times as much sterol samples from "thiamine-cells" (——) as those from the control cells were injected, respectively. A: Sterols obtained after the saponification of sterol esters. B: L-fraction. C: E-fraction. Column temperature was 240°. The detector sensitivity was decreased to one half at indicated times in the analyses of the sterols from the control cells (A and C).

The gas-liquid chromatographic profiles of free and esterified sterols of the control cells and "thiamine-cells" are shown in Figure 2. Ergosterol, which was seen as the main free sterol in the control cells, was absent in "thiamine-cells". Instead, an unidentified sterol (Y) with retention time of 24 min was the main sterol in the E-fraction of "thiamine-cells". The major sterols in the sterol ester fraction of "thiamine-cells" were lanosterol and another unidentified sterol (X) with retention time of 22 min, whereas those of the control cells were zymosterol (indicated by arrow Z) and ergosterol.

It is noteworthy that, the data in Figure 2 also show that the compositions of free and esterified sterols were completely different from each other in the control cells as well as in "thiamine-cells". The different sterol composition between free and esterified sterols was seen on the yeast cells during the re-

spiratory adaptation (5) as was observed by Parks et al. (6), suggesting the different physiological roles of both forms of sterols.

The effect of thiamine on the contents and the compositions of sterols were observed in all the growth phases. However, the level of sterols in "thiamine-cells" increased slightly and the small peaks corresponding to zymosterol and ergosterol appeared as the cultivation proceeded until the stationary phase was attained.

When "thiamine-cells" were incubated with glucose and pyridoxine, rapid increases in the level of sterol, especially of zymosterol and ergosterol, were observed within a few hours accompanied with an appearance of respiratory activity and with increases in the level of unsaturated fatty acids (data to be published).

DISCUSSION

In the preceding communication (3), we reported that thiamine reduced the level of unsaturated fatty acids in S. carlsbergensis cultivated without added pyridoxine. The data presented in this communication unequivocally indicated that the level of sterols was also depressed in "thiamine-cells". These phenomana could be explained in terms of direct or indirect actions of thiamine on certain component(s) which is involved in the biosynthesis of both unsaturated fatty acids and ergosterol. Karst and Lacroute (7) recently reported the isolation of some mutant strains of S. cerevisiae which require sterol for growth. All of these mutants required unsaturated fatty acids for growth in addition. Other mutants of S. cerevisiae, ole 2 and ole 3, required an unsaturated fatty acid and methionine for growth and did not synthesize ergosterol (8). Very recently, Bard et al. (9) reported that the primary defect of these ole mutants were lesions in porphyrin biosynthesis and that porphyrin intermediates satisfied the requirement for fatty acids and methionine. In this connection, it has been reported that the electron transferring system for fatty acid desaturation contains cytochrome b_5 as a component (10,11), and that P_{450} is involved in lanosterol demethylation (12). The requirement of pyridoxal 5'-phosphate for the synthesis of heme at the step of δ-aminolevulinate synthesis has been established (13). Considering these reports, together with our findings of low respiration and low cytochrome level in "thiamine-cells" (2), it seems attractive to explain that the depression in the levels of unsaturated fatty acids and sterols by thiamine is caused by an inhibitory effect of thiamine on the biosynthesis of pyridoxal 5'-phosphate, hence, on the biosyntheses of heme and porphyrin.

Until recently, ergosterol had been considered as an essential sterol for yeast. But, Proudlock et al. (14) demonstrated that ergosterol is not essential

for anaerobic growth of yeast. The sterol specificity of sterol-requiring mutants was studied by Karst and Lacroute, but it was not stringent (7). The absence of ergosterol has been reported on the nystatin-resistant mutant (15-17). It is interesting that ergosterol was absent especially at the early logarithmic growth phase in "thiamine-cells", and that lanosterol and unidentified sterols, X and Y, were present in the cells. The elucidation of the chemical structures of these unidentified sterols is in progress.

The data presented here and in the preceding communications (2,3) would make a significant contribution to understanding of the mechanisms for growth inhibition by thiamine and its restoration by pyridoxine. It must be emphasized that "thiamine-cells" could be useful for the research on the biogeneses of ergosterol and mitochondria in yeast, since ergosterol formation and respiratory adaptation occur within a few hours after the addition of pyridoxine to the medium.

REFERENCES

1. Rabinowitz, J. C. and Snell, E. E. (1951) Arch. Biochem. Biophys. 33, 472-481.
2. Nakamura, I., Nishikawa, Y., Kamihara, T. and Fukui, S. (1974) Biochem. Biophys. Res. Commun. in press.
3. Nishikawa, Y., Nakamura, I., Kamihara, T. and Fukui, S. (1974) Biochem. Biophys. Res. Commun. in press.
4. Monner, D. A. and Parks, L. W. (1968) Anal. Biochem. 25, 61-69.
5. Nagai, J., Kawamura, S. and Katsuki, H. Manuscripts in preparation.
6. Parks, L. W., Anding, C. and Ourisson, G. (1974) Eur. J. Biochem. 43, 451-458.
7. Karst, F. and Lacroute, F. (1973) Biochem. Biophys. Res. Commun. 52, 741-747.
8. Bard, M. (1972) J. Bacteriol. 111, 649-657.
9. Bard, M., Woods, R. A. and Haslam, J. M. (1974) Biochem. Biophys. Res. Commun. 56, 324-330.
10. Oshino, N., Imai, Y. and Sato, R. (1966) Biochim. Biophys. Acta 128, 13-28.
11. Tamura, Y., Yoshida, Y., Kumaoka, H. and Sato, R. (1973) Seikagaku (in Japanese) 45, 580-580.
12. Wada, F., Hirata, K. and Sakamoto, Y. (1969) J. Biochem. (Tokyo) 65, 171-175.
13. Lascelles, J. (1964) Tetrapyrrole Biosynthesis and Its Regulation, pp. 45-46, Benjamin, New York.
14. Proudlock, J. W., Wheeldon, L. W., Jollow, D. J. and Linnane, A. W. (1968) Biochim. Biophys. Acta 152, 434-437.
15. Woods, R. A. (1971) J. Bacteriol. 108, 69-73.
16. Parks, 1. W., Bond, R. T., Thompson, E. D. and Starr, P. R. (1972) J. Lipid Res. 13, 311-316.
17. Fryberg, m., Oehlschlager, A. C. and Unrau, A. M. (1974) Arch. Biochem. Biophys. 160, 83-89.