Thiamin Accumulation and Growth Inhibition in Yeasts

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Thiamin caused depression of growth, a marked decrease in cellular vitamin B₆ content and cytochrome oxidase activity in *Saccharomyces* yeasts growing in a vitamin B₆-free medium under aerobic conditions but had practically no effect in *Kluyveromyces*, *Schizosaccharomyces* and *Candida* spp. Pyridoxine added concomitantly with thiamin permitted the thiamin-sensitive yeasts to grow normally with increased activity of cytochrome oxidase. δ -Aminolaevulinate also caused the increase in cytochrome oxidase activity but growth was only partially improved by the addition of this precursor of haem biosynthesis. These phenomena were similar to those found previously in *Saccharomyces carlsbergensis* strain 4228 (ATCC 9080) (Nakamura *et al.*, 1981). Thiamin-sensitive yeasts accumulated thiamin more than 24-fold when compared with the thiamin-insensitive cells. Thiamin transported into the thiamin-sensitive yeasts was recovered only in the non-esterified form. Thiamin added to the growth medium was also accumulated by growing cells of the thiamin-sensitive yeasts especially during the lag and early exponential phases of growth. Pyridoxine did not affect either thiamin accumulation or the intracellular form of the transported thiamin.

INTRODUCTION

Thiamin inhibits the growth of *Saccharomyces carlsbergensis* strain 4228 under aerobic conditions when added to a vitamin B₆-free medium. As reported previously (Nagai *et al.*, 1974; Nakamura *et al.*, 1974, 1976, 1980, 1981; Nishikawa *et al.*, 1974, 1977, 1978), the thiamin-induced growth depression is accompanied by the following sequence of events in the cells: a marked decrease in the vitamin B₆ content, a pronounced lowering of the activity of δ -aminolaevulinate synthase, the absence of δ -aminolaevulinate, porphyrins and cytochromes, marked decreases in the activities of respiratory chain enzymes and other haem-containing enzymes, and an extreme reduction of respiration and a significant alteration in lipid composition. Pyridoxine or other vitamin B₆ compounds added concomitantly with thiamin completely abolish these effects of thiamin, and cells grow normally with normal profiles of the above cellular activities (Nagai *et al.*, 1974; Nakamura *et al.*, 1974, 1980, 1981; Nishikawa *et al.*, 1974, 1977). The addition of δ -aminolaevulinate has the same effects as that of pyridoxine except that growth is not completely restored (Nakamura *et al.*, 1981). The activities of some pyridoxal phosphate-dependent enzymes other than δ -aminolaevulinate synthase may also be reduced by the thiamin-induced vitamin B₆ deficiency, and this may lead to disturbance in the metabolism of amino acids, glycogen and polyamines. This possible effect of thiamin may be responsible for the growth repression observed.

The effect of thiamin on the growth of *S. carlsbergensis* 4228 is observed at low concentrations not exceeding physiological demands (less than 1 ng thiamin.HCl per ml of medium) (Rabinowitz & Snell, 1951). However, thiamin is known to be transported in large amounts into *S. cerevisiae* (Iwashima *et al.*, 1973; Suzuoki, 1955) and the accumulation of thiamin could account for the above-mentioned pronounced effects.

The present paper deals with the close relationship between thiamin accumulation and its effects and with the generality of the thiamin effects in *Saccharomyces* yeasts.

METHODS

Yeasts. *Saccharomyces carlsbergensis* strain 4228 [ATCC 9080, IFO (Institute for Fermentation, Osaka, Japan) 0565], *S. cerevisiae* [ATCC 7753], *S. sake* Kyokai no. 7, and *S. oviformis* [IFO 0262] were used to determine whether the thiamin effects can be observed generally in *Saccharomyces* yeasts. *Kluyveromyces fragilis* [IFO 0288], *K. lactis* [IFO 1090], *Schizosaccharomyces pombe* [IFO 0346] and *Candida utilis* [IFO 1086] were also used for comparison.

Growth conditions. Cultivation of the cells in a synthetic medium containing 5% (w/v) glucose as carbon source and estimation of growth were similar to those employed previously for *S. carlsbergensis* strain 4228 (Nakamura *et al.*, 1980, 1981) except that the amount of added δ -aminolaevulinic acid hydrochloride was doubled to 170 μ g per ml of medium. Inoculum size was 0.4 μ g dry cells per ml and an increased inoculum size (20 μ g dry cells per ml) was also used when indicated.

Cellular vitamin B₆ content. The extraction of intracellular vitamin B₆ and its microbioassay with *S. carlsbergensis* strain 4228 were carried out as described previously (Nakamura *et al.*, 1980).

Cytochrome oxidase activity. Cell extracts were prepared and the activity of cytochrome oxidase was determined as described previously (Nakamura *et al.*, 1980). Protein was measured by the Lowry method using bovine serum albumin as the standard. One unit of the enzyme was defined as the amount which causes the oxidation of 1 nmol cytochrome *c* per min. Specific activity was expressed as units per mg protein.

Thiamin uptake by yeast. Determination of thiamin uptake by the cells was performed by a modification of the method of Iwashima *et al.* (1973). The cells harvested at the early-exponential phase of growth were washed three times with chilled deionized water and suspended in 50 mM-potassium phosphate buffer (pH 5.0) containing 0.1 mM-glucose at a final concentration of 100 μ g dry cells per ml. The cell suspension (5 ml) was incubated at 30 °C for 15 min with gentle shaking. Thiamin uptake was initiated by adding 0.3 μ mol (0.28 μ Ci) of [¹⁴C]thiamin ([thiazole-2-¹⁴C]thiamin, HCl, 14.0 Ci mol⁻¹). Incubation was continued for 60 min with constant shaking and the uptake of radioactivity into the cells was measured at 0, 10, 30 and 60 min after the addition of [¹⁴C]thiamin. At each time, 1 ml portions of the reaction mixture were withdrawn and the cells were collected on a membrane filter (TM-1, 0.65 μ m pore size, Toyo Roshi Co, Osaka, Japan) were washed once with 10 ml of 50 mM-potassium phosphate buffer (pH 5.0). After drying, the radioactivity was measured in 10 ml of Bray's solution (Bray, 1960) with a Horiba liquid scintillation spectrometer model L2-500. A control experiment was carried out in the absence of cells and the radioactivity adsorbed by the membrane was subtracted from that of the main experiment. Incubation of the cells with non-labelled thiamin was carried out to check for the proliferation of the cells during the incubation.

Intracellular state of transported thiamin. The cells incubated with [¹⁴C]thiamin for 60 min under the above conditions were collected from 1 ml of the reaction mixture and were washed on the membrane filter with 10 ml 50 mM-potassium phosphate buffer (pH 5.0). For comparison, an experiment with a shorter incubation time (5 min) was also done using cells from 5 ml of the reaction mixture. In the case of thiamin-insensitive yeasts, many more cells were collected because of their low thiamin uptake. Each filter was heated (85 °C, 10 min) in 5 ml 10 mM-sodium acetate buffer (pH 4.5) and the resulting extracts were then centrifuged for 10 min at 10000 *g* to remove denatured protein and cells. The supernatants were freeze-dried and dissolved in 100 μ l distilled water. The concentrated samples (15 μ l each) were spotted on Toyo no. 50 filter paper (2 $\times$ 40 cm) and developed by ascending chromatography with three different solvents: (A) isopropyl alcohol/0.5 M-acetate buffer (pH 4.5)/water (65:15:20, by vol.), (B) *n*-propyl alcohol/0.5 M-acetate buffer (pH 4.5) (60:40, v/v) and (C) *n*-propyl alcohol/1 M-acetate buffer (pH 5.0)/water (65:15:20, by vol.). Radioactivity on the paper was measured with a Packard radiochromatogram scanner model 385.

Thiamin accumulation in growing cells. A thiochrome method was used to determine the cellular content of thiamin. This should be equal to the sum of the amount of thiamin synthesized in the cells and that taken up from the growth medium. Portions of cells (2 mg dry wt) were quickly collected on filter membranes at appropriate cultivation times and washed once with chilled 50 mM-potassium phosphate buffer (pH 5.0). Thiamin in the cells was extracted in 10 ml boiling 25 mM-sulphuric acid for 20 min. Protein was removed by centrifugation after adding 2.5 ml 10% (w/v) TCA and the supernatant was adjusted to pH 4.5 with 4 M-sodium acetate. After adsorption on a column of Permutit, thiamin was eluted with 0.1 M-HCl containing 25% (w/v) KCl and converted to thiochrome with potassium ferricyanide (Airth & Foerster, 1970). The amount of thiochrome was determined fluorometrically using a Shimadzu spectrophotofluorometer. Free and free plus phosphorylated (total) thiamin was estimated before and after taka-diastase [a final concentration of 0.15% (w/v)] treatment, respectively.

Chemicals. Vitamin-free Casamino acids were purchased from Difco, cytochrome *c* (horse heart), δ -aminolaevulinic acid hydrochloride and bovine serum albumin were from Sigma. [¹⁴C]Thiamin was obtained from Amersham (97% pure as determined by paper chromatography and TLC) and taka-diastase was from Sankyo Co.,

Tokyo, Japan. All other chemicals used were of analytical reagent grade or of the highest purity available commercially.

RESULTS AND DISCUSSION

Effects of thiamin and pyridoxine on growth of yeasts

Eight yeast species belonging to *Saccharomyces*, *Kluyveromyces*, *Schizosaccharomyces* and *Candida* were examined for growth response to thiamin and pyridoxine under aerobic conditions. The growth of *S. cerevisiae*, *S. sake* and *S. oviformis* was also inhibited by adding thiamin to the vitamin B₆-free medium as shown previously for *S. carlsbergensis* strain 4228 (Nakamura *et al.*, 1974, 1980) (Fig. 1). Thiamin caused an elongation of the lag phase of growth, a lowering of growth rate and of maximum growth level. These thiamin-sensitive species (all of them belonged to *Saccharomyces*) were designated as Type I yeasts. The growth inhibition caused by thiamin was completely abolished by concomitant addition of pyridoxine. However, in contrast to pyridoxine, δ -aminolaevulinate did not bring about the normal growth profile; the prolonged lag phase was not improved although the rate and maximum level of growth were considerably increased in all Type I yeasts (data not shown). The growth of the other four yeasts was not inhibited by thiamin (designated as Type II yeasts).

Effect of thiamin on cellular vitamin B₆ content

Cellular vitamin B₆ content of Type I yeasts was high during early growth (the mid-exponential phase) and decreased rapidly when the cells were cultivated without thiamin or pyridoxine (designated as control cells) (Table 1). Kawasaki & Yamada (1965) observed a similar pattern of time-course change in vitamin B₆ content of *S. carlsbergensis* strain 4228. The cells grown with added thiamin (designated as thiamin-grown cells) of Type I yeasts contained markedly lower amounts of vitamin B₆ throughout the growth period. The difference of vitamin B₆ content between the control and the thiamin-grown cells was most marked at the mid-exponential phase of growth. In Type II yeasts, thiamin exerted little effect on cellular vitamin B₆ content during early growth. These results suggest a close relationship between the decrease in the vitamin B₆ content at the early growth phase and the growth depression.

Fig. 1. Effects of thiamin and pyridoxine on growth of various yeasts. Thiamin hydrochloride and pyridoxine hydrochloride were added to culture medium at final concentrations of 1 μ g and 20 ng per ml, respectively. Culture conditions were the same as described in Methods. (a) *S. carlsbergensis*, (b) *S. cerevisiae*, (c) *S. sake*, (d) *S. oviformis*, (e) *K. fragilis*, (f) *K. lactis*, (g) *Schiz. pombe* and (h) *C. utilis*. The cells were grown in the absence of thiamin and pyridoxine (control cells) (○), with thiamin (thiamin-grown cells) (●) and with both thiamin and pyridoxine (thiamin and pyridoxine-grown cells) (△).

Table 1. *Effect of thiamin on cellular vitamin B₆ content in yeasts*

Cells were grown with or without added thiamin as described in Methods and in the legend of Fig. 1. Samples of the culture corresponding to approximately 200 mg of dry weight cells were withdrawn at the indicated growth phase and washed three times with 100 ml each of ice-cold deionized water. Vitamin B₆ was extracted from the cells and assayed using *S. carlsbergensis* strain 4228 (ATCC 9080) as described in Methods.

Yeast	Vitamin B ₆ content [ng pyridoxine.HCl (mg dry cells) ⁻¹]					
	Control cells*			Thiamin-grown cells*		
	Mid-exponential phase	Late-exponential phase	Stationary phase	Mid-exponential phase	Late-exponential phase	Stationary phase
Type I						
<i>S. carlsbergensis</i>	12	6.6	2.6	0.61	1.6	0.77
<i>S. cerevisiae</i>	7.0	5.2	3.1	0.95	1.0	0.79
<i>S. sake</i>	27	11	2.3	1.2	0.89	0.71
<i>S. oviformis</i>	80	46	21	6.0	2.7	1.3
Type II						
<i>K. fragilis</i>	19	19	20	22	17	5.4
<i>K. lactis</i>	11	11	5.2	10	4.3	3.1
<i>Schiz. pombe</i>	12	24	13	13	22	6.2
<i>C. utilis</i>	9.2	8.0	11	10	8.7	5.9

* See the text and legend for Fig. 1.

Effects of thiamin, pyridoxine and δ -aminolaevulinate on the activity of cytochrome oxidase in the cells

Cytochrome oxidase activity in the control cells of the yeasts, except for *Schiz. pombe* and *C. utilis*, increased with growth (Table 2). In all Type I yeasts, cytochrome oxidase activity was markedly lower when the cells were grown with thiamin, as observed previously in *S. carlsbergensis* strain 4228 (Nakamura *et al.*, 1974, 1976, 1980). The low level of activity was maintained during cultivation with the exception of *S. oviformis* where activity increased during the stationary phase. However, this increased activity was still much lower than the activity in the control cells. The thiamin effect was prevented by the concomitant addition of pyridoxine, indicating that the decrease in the activity of cytochrome oxidase resulted from the thiamin-induced lowering of vitamin B₆ content as discussed earlier in the case of *S. carlsbergensis* strain 4228 (Nakamura *et al.*, 1974, 1976). The addition of δ -aminolaevulinate also caused the prevention of the thiamin effect as observed in *S. carlsbergensis* strain 4228 (Nakamura *et al.*, 1981).

The cytochrome oxidase activity level in Type II yeasts was not affected by thiamin. In *Schiz. pombe*, a significant decrease (one-half of the activity found in control cells) was observed at the mid-exponential phase. However, the oxidase activity of the thiamin-grown cells of *Schiz. pombe* was still high compared even with the control cell activities of the other yeasts. Also, the rate of thiamin-induced reduction of the activity in this yeast was much smaller than those in Type I yeasts. These observations suggest that the thiamin effects found previously in *S. carlsbergensis* strain 4228 occur in *Saccharomyces* yeasts so far as tested under the mechanism described in the introductory statement.

Form of thiamin transported into yeast cells

Cells of *S. cerevisiae* accumulate added thiamin in large amounts and it is found predominantly in the non-esterified form (Suzuoki, 1955; Iwashima *et al.*, 1973). The results of paper chromatography of the extracts from *S. carlsbergensis* and *K. lactis* incubated with [¹⁴C]thiamin for 60 min are shown in Fig. 2. The transported radioactivity in *S. carlsbergensis* (Type I yeast) was detected in one peak corresponding to non-esterified thiamin which had an

Table 2. *Effects of thiamin, pyridoxine and δ -aminolaevulinate on the activity of cytochrome oxidase in yeasts*
 Cultivation of cells, preparation of cell extracts and assay of cytochrome oxidase were carried out as described in Methods.

Yeast	Cytochrome oxidase specific activity [nmol (min) ⁻¹ (mg protein) ⁻¹]									
	Control cells*				Thiamin and pyridoxine- grown cells*				Thiamin and ALA- grown cells†	
	Mid- expo- nential phase	Late- expo- nential phase	Stationary phase	Mid- expo- nential phase	Late- expo- nential phase	Stationary phase	Mid- expo- nential phase	Late- expo- nential phase	Stationary phase	Stationary phase
Type I										
<i>S. carlsbergensis</i>	21	24	42	0.81	0.54	0.30	18	24	25	12
<i>S. cerevisiae</i>	22	39	52	0.43	0.61	0.25	14	13	43	16
<i>S. sake</i>	21	37	68	0.79	0.78	0.24	19	19	44	9.1
<i>S. oviformis</i>	18	23	52	0.28	0.32	3.6	18	18	57	46
Type II										
<i>K. fragilis</i>	89	105	212	82	108	118	—	—	—	—
<i>K. lactis</i>	21	25	41	24	26	37	—	—	—	—
<i>Schiz. pombe</i>	230	100	93	118	88	127	—	—	—	—
<i>C. utilis</i>	90	71	67	92	67	63	—	—	—	—

—, Not determined.

* See the text and the legend for Fig. 1.

† Cells grown with both thiamin and δ -aminolaevulinate (ALA).

Fig. 2. Paper chromatography of radioactivity in the cell extracts from *S. carlsbergensis* (a) and *K. lactis* (b) incubated with [^{14}C]thiamin. Cell suspensions (100 μg dry cells per ml) prepared as described in the text were incubated at 30 $^{\circ}\text{C}$ for 60 min with [^{14}C]thiamin (60 μM). Paper chromatography was done using solvent A as described in Methods. TMP denotes thiamin monophosphate and TPP denotes thiamin pyrophosphate.

R_F value of 0.72. This was confirmed by experiments using the other two solvents (solvents B and C). A similar result was obtained also in the other yeasts belonging to Type I (data not shown).

Thiamin transported into *K. lactis* (Type II yeast) was detected as thiamin pyrophosphate (TPP) and thiamin monophosphate (TMP) in addition to non-esterified thiamin (Fig. 2b). Some other unknown radioactive substances were also found. In the other three yeasts belonging to Type II, transported thiamin was mainly found in the non-esterified form. However, in contrast to the Type I yeasts, appreciable amounts of radioactive TPP were also detected (data not shown).

Thiamin accumulation in yeast cells

The time course of [^{14}C]thiamin uptake by the cells was followed for each yeast. The radioactivity was transported into the cells in both yeast types without appreciable lag. The levels of thiamin attained after 60 min incubation are shown in Table 3. The results clearly indicate that the accumulation in cells of Type I yeasts was much higher than that in Type II yeasts (more than 24-fold). The possibility that thiamin became attached to the cell surface of Type I yeasts by some ionic interaction was excluded since washing of the cells with 0.1 M-HCl or 0.3 M-KCl caused no appreciable reduction of the radioactivity (data not shown). The intracellular concentration of accumulated thiamin in *S. carlsbergensis* was calculated from the result in Table 3 to be 190 mM assuming that cellular water space in the yeast cells is 2.1 μl per mg dry weight (Okada & Halvorson, 1964). This concentration is about 9500 times that of exogenously remaining thiamin. A similar result was obtained by Iwashima *et al.* (1973) in *S. cerevisiae*. Such a level of thiamin accumulation in Type I yeasts is extraordinarily high compared with that in *Escherichia coli* (Kawasaki *et al.*, 1969; Kawasaki & Esaki, 1971) or in other bacteria (Henderson & Zevely, 1978; Nishimune, 1973).

Experiments were then performed to explore the relationship between the capacity of thiamin accumulation and the thiamin-induced vitamin B₆ deficiency which has been suggested as the cause of haem deficiency and growth depression.

Thiamin accumulation in yeast cells during cultivation

Thiamin has no inhibitory effect on the growth of *S. carlsbergensis* when added after the initiation of growth (Chiao & Peterson, 1956). This fact strongly suggests that thiamin exerts its effect at extremely early stages of growth. Hence, the cellular content of thiamin of Type I and Type II yeasts at such a growth phase was determined in the thiamin-supplemented culture. To obtain sufficient amounts of cells for measuring thiamin content during the lag phase of growth, the inoculum size was increased to 20 µg (dry weight) of cells per ml medium. In Type I yeasts, the free thiamin content began to increase immediately after cultivation had started and reached maximum levels before appreciable proliferation of cells occurred (Fig. 3), reflecting the high

Table 3. Thiamin accumulation in yeast

The cells were incubated with [¹⁴C]thiamin as described in the legend for Fig. 2 except that samples were withdrawn from the cell suspensions during the incubation at appropriate time intervals. The radioactivity of the cells on membrane filters was counted. The time course of the radioactivity transported into the cells was plotted. Accumulation of [¹⁴C]thiamin in the cells was calculated from the maximum levels attained. All the procedures were the same as described in Methods.

Yeast	[¹⁴ C]Thiamin accumulated [nmol (mg dry cells) ⁻¹]
Type I	
<i>S. carlsbergensis</i>	400
<i>S. cerevisiae</i>	259
<i>S. sake</i>	224
<i>S. oviformis</i>	225
Type II	
<i>K. fragilis</i>	6.6
<i>K. lactis</i>	3.3
<i>Schiz. pombe</i>	9.4
<i>C. utilis</i>	3.6

Fig. 3. Accumulation of thiamin in growing yeast cells at extremely early growth stage. Cells harvested at the stationary phase were inoculated at a final concentration of 20 µg dry cells per ml of the medium containing thiamin (1 µg per ml) and cultivated as described in Methods. Cells were harvested at the indicated time of cultivation, and free thiamin was extracted from the cells and determined as described in Methods. (a) *S. carlsbergensis*, (b) *S. cerevisiae*, (c) *S. sake*, (d) *S. oviformis*, (e) *K. fragilis*, (f) *K. lactis*, (g) *Schiz. pombe* and (h) *C. utilis*. Accumulation of free thiamin [nmol (mg dry cells)⁻¹] (○), growth [µg dry cells (ml)⁻¹] (●).

Table 4. *Effect of thiamin on cellular vitamin B₆ content in Saccharomyces carlsbergensis*

Cells were grown and harvested as described in Fig. 3 and vitamin B₆ content was measured as described in Table 1.

Cultivation time (h)	Vitamin B ₆ content [ng (mg dry cells) ⁻¹]	
	Control cells*	Thiamin-grown cells*
0	2.2	2.2
4	4.6	0.93
8	11	2.0
12	2.0	1.6

* See the text and the legend for Fig. 1.

capacity of thiamin accumulation of the cells shown in Table 3. In contrast, Type II yeasts showed a much lower content of free thiamin in this short period of cultivation.

Cellular content of vitamin B₆

The time-course of vitamin B₆ content in the thiamin-grown cells and the control cells was determined in a typical Type I yeast, *S. carlsbergensis* (Table 4). For comparison with the changes in thiamin content of the thiamin-grown cells shown in Fig. 3, a large inoculum was used. These results indicate that the rapidly accumulated thiamin (cf. Fig. 3*a*) inhibits the increase in the content of vitamin B₆ which was detected in the control cells.

Addition of pyridoxine to the medium used for thiamin uptake (2 µM) or to the thiamin-supplemented medium for growth (100 nM) had no effect on the amounts of thiamin uptake or the cellular thiamin content. Also the intracellular form of accumulated thiamin was not influenced. Therefore, the elimination of the thiamin effects by pyridoxine (Table 1 and Nagai *et al.*, 1974; Nakamura *et al.*, 1974, 1980; Nishikawa *et al.*, 1974, 1977) is neither the result of a decrease in the capacity of thiamin accumulation nor the result of an alteration in the form and the level of accumulated thiamin. This supports the hypothesis that accumulated thiamin exerts its effect through the decrease in cellular vitamin B₆ content.

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