may imply that some coenzymes are more sensitive to destruction or that they are more important for cell metabolism.

1 To whom reprint request should be addressed.
2 The authors thank Dr J. Behrend, Makor Company, Israel, for a generous gift of verrucarin A and roridin A.
3 Ichinone, M., and Kurata, H., in: Trichothecenes: Chemical, Biological and Toxicological Aspects, p. 72. Ed. Y. Ueno. Development in Food Science Series, No. 4. Elsevier Scientific Publishers, N.Y. 1983.
4 Joffe, A.Z. in: Microbial Toxins, vol. VII. p. 139. Eds S. Kadis, A. Ciegler and S.J. Ajl. Academic Press, New York 1971.
5 Ueno, Y., Adv. Nutr. Sci. *3* (1980) 301.
6 Yagen, B., Joffe, A.Z., Horn, P., Mor, N., and Lutsky, I.I., in: Mycotoxins in Human and Animal Health, p. 329. Eds J.V. Rodricks, C.W. Hesseltine and M.A. Mehlman. Pathotox Publishers, Park Forest South, Ill 1977.
7 McLaughlin, C.S., Vaughan, M.H., Campbel, I.M., Wei, C.M., Stafford, M.E., and Hansen, B.S., in: Mycotoxins in Human and Animal Health, p. 263. Eds W. Rodricks, C.W. Hesseltine and M.A. Mehlman. Pathotox Publishers, Park Forest South, Ill 1977.
8 Yarom, R., More, R., Eldor, A., and Yagen, B., Toxic. appl. Pharmac. *73* (1984) 210.
9 Yarom, R., Sherman, Y., More, R., Ginsburg, I., Borinski, R., and Yagen, B., Toxic. appl. Pharmac. *75* (1984) 60.
10 Segal, R., Milo-Goldzweig, I., Joffe, A.Z., and Yagen, B., Toxic. appl. Pharmac. *70* (1983) 343.
11 Rottem, S., Yagen, B., and Katznell, A., FEBS Lett. *175* (1984) 189.
12 Schoental, R., FEBS Lett. *61* (1976) 111.
13 Schoental, R., Biochem. Soc. Trans. *18* (1980) 147.
14 Wertlieb, D.M., and Guttman, H.N., J. Protozool. *10* (1963) 109.
15 Wei, R., Strong, F.M., Smalley, E.B., and Schnoes, H.K., Biochem. biophys. Res. Commun. *45* (1971) 396.
16 Jarvis, B.B., Yatawara, C.S., Greene. S.L., and Vrudhula, V.M., Appl. envir. Microbiol. *48* (1984) 673.
17 Bryant, M.C., in: Antibiotics and Their Laboratory Control. Laboratory Aids Series. Ed. F.J. Baker. Butterworths, London 1968.
18 Schappert, K.T., and Khachatourians, G.G., Appl. envir. Microbiol. *45* (1983) 862.
19 Burmeister, H.R., and Hesseltine, C.W., Appl. Microbiol. *20* (1970) 437.

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Occurrence of thiaminase II in *Saccharomyces cerevisiae*[1]

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Summary. It was found that cell-free extracts of *Saccharomyces cerevisiae* contain thiaminase II which hydrolyzes thiamine and thiamine analogs. The possible involvement of this enzyme and thiamine-synthesizing enzymes in thiamine production from thiamine antagonists is discussed.
Key words. Thiaminase; thiamine; thiamine antagonist; *Saccharomyces cerevisiae.*

Saccharomyces cerevisiae can grow in the presence of two thiamine antagonists, pyrithiamine and oxythiamine, although its growth is inhibited by each antagonist alone[2]. This phenomenon was explained as being due to thiamine production from pyrithiamine and oxythiamine, which was demonstrated using cell suspensions and a crude extract of *S. cerevisiae*[2]. However, the precise enzymatic mechanism of thiamine synthesis from these two thiamine antagonists in yeast cells remained to be established, although 2-methyl-4-amino-5-hydroxymethylpyrimidine(hydroxymethylpyrimidine), the pyrimidine moiety of pyrithiamine, and 4-methyl-5-β-hydroxyethylthiazole (hydroxyethylthiazole), the thiazole moiety of oxythiamine, are supposed to be precursors for thiamine synthesis.

We report in this communication that cell-free extracts of *S. cerevisiae* contain an enzyme hydrolyzing thiamine antagonists as well as thiamine, which gives the first evidence for the existence of thiaminase II (EC 3.5.99.2) in *S. cerevisiae*. Although thiaminase II has been reported from various bacteria[3], its physiological function is unknown. We also describe here that a recently isolated yeast mutant[4] deficient in hydroxyethylthiazole kinase (EC 2.7.1.50) cannot grow with pyrithiamine and oxythiamine, which suggests that, in addition to thiaminase II, other enzymes are involved in the reversal of pyrithiamine-induced growth inhibition of *S. cerevisiae* by oxythiamine.

S. cerevisiae was grown in 10 l of thiamine-free Wickerham's synthetic medium[2] for 15 h at 30 °C with shaking, harvested, and washed once with distilled water. The washed yeast cells were suspended in 20 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA, sonicated for 20 min at 10 kc, and then centrifuged at 15,000 × g for 20 min. The supernatant solution was treated with $^1/_{10}$ volume of 0.8% protamine sulfate solution, and the suspension was centrifuged at 15,000 × g for 20 min. The supernatant solution was then brought to 40% saturation with solid ammonium sulfate. The precipitate formed was removed by centrifugation at 15,000 × g for 20 min, and the supernatant solution was brought to 80% saturation by the addition of ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1mM EDTA. The solution was dialyzed against the same buffer and it was used as enzyme

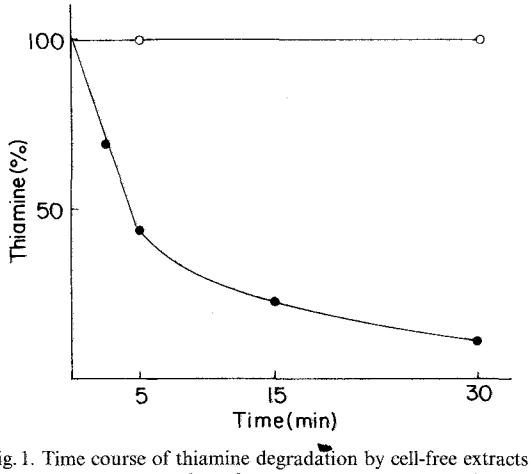

Fig. 1. Time course of thiamine degradation by cell-free extracts of *Saccharomyces cerevisiae:* ●—●, complete; ○—○ complete but boiled enzyme.

solution. The reaction mixture for the enzyme assay contained 50 mM potassium phosphate buffer (pH 7.5), 2 μM thiamine, 1 mM dithiothreitol and the enzyme solution (2.1 mg protein) in a total volume of 2 ml. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 1 ml of 10% metaphosphoric acid. After centrifugation at 2600 × g for 15 min thiamine left in the supernatant was determined by the thiochrome method[6]. As shown in the figure 1, thiamine was decomposed time-dependently by cell-free extracts prepared from *S. cerevisiae*. The reaction proceeded almost linearly for the initial 5 min. Optimal pH and temperature for the reaction were 7.5 and 37 °C, respectively (data not shown).

Table 1 shows the enzyme reaction products formed from thiamine and several thiamine analogs including thiamine antagonists. The reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 2 μM thiamine or thiamine analog, 1 mM dithiothreitol and the enzyme solution (2.1 mg protein) in a total volume of 2 ml was incubated for 1 h at 37 °C. The reaction was stopped by heating at 85 °C for 15 min after adjusting the pH of the mixture to 4.5. The supernatant obtained by centrifugation at 2600 × g for 15 min was lyophilized and dissolved in 0.1 ml of distilled water. 5 μl of the concentrated sample was spotted on a Toyo filter paper strip (No. 50, 2 cm × 40 cm), and developed by ascending chromatography in a solvent system containing isopropyl alcohol-0.5 M sodium acetate buffer (pH 4.5)-water (65:15:20, by vol). Hydroxymethylpyrimidine (Rf: 0.75) and hydroxyethylthiazole (Rf. 0.96) on the paper were detected by bioautography using *Escherichia coli* 70–17 and 26–43, which require the pyrimidine and thiazole moiety of thiamine for growth, respectively.

Thiamine analogs used were 3–2′-methyl-4′-aminopyrimidyl-(5′)-methyl-4,5-dimethylthiazolium chloride hydrochloride (dimethialium), pyrithiamine hydrobromide, oxythiamine hydrochloride and 3–4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-hydroxyethylthiazolium chloride hydrochloride (2-northiamine). Both hydroxymethylpyrimidine and hydroxyethylthiazole were identified as reaction products when thiamine was used as a substrate. Hydroxymethylpyrimidine was also formed from dimethialium and pyrithiamine which are thiamine analogs modified at the thiazole portion of thiamine. On the other hand, hydroxyethylthiazole was detected as a reaction product from oxythiamine and 2-northiamine which are modified at the pyrimidine portion of thiamine. 2-Northiamine was degraded to hydroxyethylthiazole and a compound stimulatory to *E. coli* 70–17 with a R_f value of 0.69 which is smaller than that of hydroxymethylpyrimidine. This product was proved to be 4-amino-5-hydroxymethylpyrimidine(2-norhydroxymethylpyrimidine) which is known to support the growth of *E. coli* 70–17[7], since its R_f value coincided with that of authentic 2-norhydroxymethylpyrimidine and the two compounds were inseparable when co-chromatographed.

These results clearly show that the enzymatic degradation of thiamine and thiamine analogs is catalyzed by thiaminase II present in the cytosol of *Saccharomyces cerevisiae*. Table 2 shows the substrate specificity of yeast thiaminase II. The enzyme activity was determined as described above, except for the reaction time, which was 5 min. Dimethialium, 2-northiamine and α-hydroxyethylthiamine as well as thiamine were determined by the thiochrome method. It was found that dimethialium is a better substrate than thiamine, where as 2-northiamine was degraded more slowly. α-Hydroxyethylthiamine was hardly hydrolyzed by yeast thiaminase II.

Table 3 shows the effect of thiamine or dimethialium added to the growth medium on thiaminase II activity. Yeast cells were grown in 500 ml of Wickerham's synthetic medium with or without 1 μM thiamine or dimethialium. Preloading of yeast cells with thiamine or dimethialium was done by addition of thiamine or dimethialium at a final concentration of 1 μM into the culture grown without thiamine at 30 min before the end of cultivation. After cultivation for 15 h at 30 °C by shaking, yeast cells were harvested, washed once with distilled water, and then sonicated as described above. The supernatant obtained by centrifugation of the sonic extract was used as enzyme solution after dialysis against 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA. It appeared to be interesting that thiaminase II activity in the crude extract from *S. cerevisiae* grown in the presence of 1 μM thiamine was decreased to approximately 13% of that of control cells. However, the enzyme activity was not affected by 1 μM dimethialium added to the growth medium. Furthermore, thiaminase II activity in the extract of yeast cells preloaded with thiamine or dimethialium after cell growth was about the same as that of control cells. These results suggest that a decrease in thiaminase II activity caused by exogenous thiamine cannot be due to the inactivation of the enzyme by thiamine or other thiaminase substrates, as previously described for bacterial

Table 1. Degradation products of thiamine and thiamine analogs by cell-free extracts of *Saccharomyces cerevisiae*

Substrate	Product
Thiamine	Hydroxymethylpyrimidine, hydroxyethylthiazole
Dimethialium	Hydroxymethylpyrimidine
Pyrithiamine	Hydroxymethylpyrimidine
Oxythiamine	Hydroxyethylthiazole
2-Northiamine	2-Norhydroxymethylpyrimidine, hydroxyethylthiazole

Table 2. Activity of thiaminase II against thiamine and thiamine analogs

Substrate	Thiaminase II activity (pmol/mg/min)
Thiamine	144.8
Dimethialium	175.2
2-Northiamine	76.0
α-Hydroxyethylthiamine	7.6

The enzyme activity is expressed as the amount of thiamine or thiamine analog degraded per mg of protein per min. The enzyme boiled for 10 min was used for the blank experiment. Values are means from 2 experiments.

Table 3. Effect of thiamine or dimethialium added to the growth medium on thiaminase II activity

Cells	Thiaminase II activity (pmol/mg/min)
Grown without thiamine or dimethialium	121.8
Grown with thiamine (1 μM)	16.0
Grown with dimethialium (1 μM)	126.0
Preloaded with thiamine (1 μM)	125.2
Preloaded with dimethialium (1 μM)	128.4

Values are means from 2 experiments.

Table 4. Effect of pyrithiamine and oxythiamine on the growth of *Saccharomyces cerevisiae* and a hydroxyethylthiazole kinase deficient mutant of *S. cerevisiae*

Addition	Growth (optical density at 560 nm)	
	S. cerevisiae	Hydroxyethylthiazole kinase deficient mutant
None	0.480	0.450
Pyrithiamine (1 μM)	0.030	0.015
Oxythiamine (10 μM)	0.050	0.045
Pyrithiamine (1 μM) plus oxythiamine (10 μM)	0.470	0.015

Values are means from 2 experiments.

thiaminase I[8,9]. In addition, since the decrease in the enzyme activity was not observed when hydroxymethylpyrimidine or hydroxyethylthiazole was added to the growth medium (data not shown), it was presumed that thiamine or thiamine pyrosphosphate accumulated in cells acts as a corepressor to repress thiaminase II synthesis in *S. cerevisiae*.

Table 4 shows the effect of pyrithiamine and oxythiamine on the growth of *S. cerevisiae* and a hydroxyethylthiazole kinase deficient mutant of *S. cerevisiae*[4]. 5 ml of thiamine-free Wickerham's synthetic medium with or without thiamine antagonist was inoculated with a washed cell suspension of *S. cerevisiae* or its mutant equivalent to 0.016 mg (dry weight) and incubated at 30 °C without shaking. Growth after 18 h was then measured turbidimetrically at 560 nm. It was found that the reversal of pyrithiamine-induced growth inhibition of *S. cerevisiae* by oxythiamine was impaired in a hydroxyethylthiazole kinase deficient mutant of *Saccharomyces cerevisiae*. The results show that in *S. cerevisiae* enzymes synthesizing thiamine from hydroxymethylpyrimidine and hydroxyethylthiazole[5] are essentially involved, in addition to thiaminase II in the production of thiamine from pyrithiamine and oxythiamine, resulting in the relief of growth inhibition by these two thiamine antagonists.

In conclusion, it was demonstrated that *Saccharomyces cerevisiae* contains thiaminase II together with thiamine-synthesizing enzymes. It is well known that yeast not only synthesizes thiamine de novo but takes up and accumulates thiamine from the medium. The possible role of thiaminase II in the regulation of thiamine metabolism in *S. cerevisiae* remains to be clarified.

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2 Iwashima, A., Yoshioka, K., Nishimura, H., and Nosaka, K., Experientia *40* (1984) 582.
3 Wittliff, J. L. and Airth, R. L., Meth. Enzymol. *18* (1970) 234.
4 Iwashima, A., Nosaka, K., Nishimura, H., and Kimura, Y., J. gen. Microbiol. *132* (1986) 1514.
5 Nose, Y., Ueda, K., Kawasaki, T., Iwashima, A., and Fujita, T., J. Vitam. *7* (1961) 98.
6 Fujita, A., Meth. Enzymol. *2* (1955) 622.
7 Uematsu, T., Vitamins *34* (1966) 555 (in Japanese).
8 Suzuki, K., and Ooba, J., J. Biochem. *72* (1972) 1053.
9 Agee, C. C., and Airth, R. L., J. Bact. *115* (1973) 957.

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Synthesis of an analog of human calcitonin gene related peptide, [Asu^{2,7}]-h-CGRP

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Summary. The analog of h-CGRP, des-Ala-deamino-dicarba-h-CGRP, was synthesized by the combination of the conventional solution and the solid phase peptide synthesis methods. This analog showed stronger and longer-lasting hypocalcemic and hypophosphatemic activities than the natural hormone.
Key words. h-CGRP analog; synthesis; Ca and Pi lowering effects.

Calcitonin Gene Related Peptide (CGRP) was identified in 1983 as an alternative gene product of the calcitonin gene in the rat by Rosenfeld et al.[1]. In man, complementary DNA encoding the precursor of CGRP was recognized by Steenbergh et al.[2] in messenger RNA extracted from medullary thyroid carcinoma. The amino acid sequence of human CGRP was also derived from medullary thyroid carcinoma extracts[3]. Subsequently, additional CGRP genes were identified in rat and man by Rosenfeld et al.[4] and Steenbergh et al.[5], respectively. The peptides all consist of 37 amino acids with disulfide bridges between Cys 2[6] and Cys 7, and their remaining amino acid sequences are highly homologous.

Several biological effects of CGRP have been reported including hypocalcemia and hypophosphatemia[7], hypotension, an increase of heart rate[8] and inhibition of gastric acid secretion[9].

Tippins et al.[10] have reported that destruction of the disulfide bridge at the amino-terminus abolished biological activity, but the modification of the middle and carboxyl-terminal regions of this hormone (such as acylation of Lys 24 or Lys 35 and substitution of Val 22 and Asn 25 with Met and Ser, respectively) did not alter the biological activity. Therefore, the amino-terminal portion, especially the S-S linkage of the peptide, appears to be important for biological activity.

The present paper deals with the synthesis of an analog in which the amino-terminal amino acid(Ala) and the amino group of Cys 2, and the S-S bond of the natural peptide were replaced by a hydrogen atom and by an ethylene linkage, respectively (fig. 1).

Recently, solid phase peptide synthesis has made it possible to synthesize peptides of 30 to 40 amino acids very rapidly. However, a peptide which contains a fragment linked with ethylene instead of the S-S bond is much more troublesome to synthesize by this method. Therefore, the synthesis of the analog was carried out using a conventional solution method for the preparation of amino-terminal fragment [1], which corresponded to the sequence (2–8) of the natural hormone. Fragment [1] was coupled to the solid phase carboxyl-terminal linear fragment (9–37)-resin. All the functional groups in the amino acid residues were protected by groups which are used commonly in solid phase synthesis. Fragment [1] was synthesized as shown in figure 2. Each fragment was purified by the conventional procedures, and applied to the next step after confirming the homogeneity by thin layer chromatography and by amino acid analysis. Compound [5] was converted to the p-nitrophenyl ester at the ω-position of the Asu residue with p-nitrophenyl trifluoroacetate[11]. After removal of the amino protecting group (Boc), [4] was treated with pyridine at 50 °C under high dilution condition (1 m mol/l). The crude cyclized product [3] was treated with anhydrous HF at 0 °C for 1 h in the presence of anisole, followed by column chromatography on CHP-20P (Mitsubishi Chem-