Effects of Thiamin on Vitamin B₆ Synthesis in Yeasts

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The effects of exogenous thiamin on the growth yield and vitamin B₆ content of 18 strains of yeasts and a few strains of bacteria were examined. The addition of thiamin hardly affected the growth yield of the yeasts tested, except for two strains *Saccharomyces uvarum* strain 4228 and *Saccharomyces uvarum* IFO 0751. In contrast, the vitamin B₆ content of all the yeasts tested, except *Pichia membranaefaciens* IFO 0189, decreased markedly in the presence of thiamin. In *S. uvarum* IFO 1265, the synthesis of vitamin B₆ was maximally inhibited by the addition of thiamin (1.5 nmol ml⁻¹) to the growth medium without affecting cell growth, whereas the amounts of cellular vitamin B₆ increased in the presence of the thiamin antagonist, pyriethiamin or oxythiamin, at concentrations that did not affect growth. When [4'-¹⁴C]pyridoxine.HCl (0.5 µg ml⁻¹) was added to the growth medium at least 54% of the added isotope was incorporated into the cells during 24 h incubation. In the presence of thiamin (15 nmol ml⁻¹), at least 32% of the added isotope was incorporated. The metabolism of [4'-¹⁴C]pyridoxine.HCl to inactive forms having no vitamin B₆ activity was not stimulated by the addition of thiamin. Thus, vitamin B₆ synthesis in many yeasts was affected by thiamin, whereas, in bacteria, growth yield and vitamin B₆ content were not affected by thiamin.

INTRODUCTION

The relation between added thiamin in the growth medium and vitamin B₆ requirements for yeast growth has been studied in detail in *Saccharomyces uvarum* strain 4228 (*Saccharomyces carlsbergensis* strain 4228), an organism widely used in the assay of vitamin B₆ (Atkin *et al.*, 1943; Rabinowitz & Snell, 1947; Fukui, 1953). This yeast does not require vitamin B₆ as an essential growth factor, except when thiamin is added to the culture medium (Rabinowitz & Snell, 1951; Chiao & Peterson, 1956). Exogenous thiamin greatly depresses the vitamin B₆ content of this yeast, possibly by inhibiting biosynthesis (Kawasaki & Yamada, 1965; Kishi, 1969); the severe deficiency of vitamin B₆ in the yeast was manifest in a marked reduction in the rate of nicotinic acid biosynthesis by inhibiting the synthesis of 3-hydroxyanthranilic acid from 3-hydroxykynurenine (Kawasaki *et al.*, 1969). Nakamura *et al.* (1974) reported that thiamin depressed the respiratory activity, the content of unsaturated fatty acids, especially palmitoleic acid (Nishikawa *et al.*, 1974*a*) and the level of sterols (Nishikawa *et al.*, 1974*b*) in the yeast, and that these effects of thiamin were abolished by the addition of pyridoxine to the growth medium. Haskell & Snell (1965) have also observed that deficiency of vitamin B₆ caused a marked decrease in the content of palmitoleic acid of the yeast, *Hanseniaspora valbyensis*. The facts strongly support the presumption that the externally added thiamin causes primarily the inhibition of vitamin B₆ biosynthesis, followed by various abnormalities in vitamin B₆ metabolism, so that *S. uvarum* 4228 cannot grow normally without the addition of pyridoxine, pyridoxal, and pyridoxamine.

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Abbreviations: Deoxythiamin, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-ethyl-4-methylthiazolium chloride hydrochloride; OMP, 2-methyl-4-amino-5-hydroxyethylpyrimidine.

These results are restricted to *S. uvarum* 4228, and the effect of thiamin on the synthesis of vitamin B₆ in other yeasts is unclear. However, Schultz *et al.* (1940) and Schultz & Atkin (1947) observed that, in the absence of exogenous vitamin B₆, thiamin inhibited the growth of yeasts other than *S. uvarum* 4228, suggesting that the inhibition of vitamin B₆ biosynthesis by thiamin may be common to all yeasts. In the present paper, we have examined the effects of thiamin on the synthesis and metabolism of vitamin B₆ in diverse yeast species and a few bacterial species.

METHODS

Organisms. The organisms with IFO numbers were obtained from the Institute for Fermentation, Osaka, Japan, and those with OUT numbers from the Department of Fermentation Technology, Osaka University, Suita, Japan. *Saccharomyces uvarum* 4228 is ATCC 9080. *Bacillus subtilis* (NRRL B558) was obtained from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, U.S.A. *Escherichia coli* K12 and *Klebsiella pneumoniae* were obtained from the Research Institute for Microbial Disease, Osaka University, Suita, Osaka, Japan. Stock cultures of yeasts were maintained on agar slants prepared from 3% (w/v) malt extract, 0.5% (w/v) yeast extract and 1.5% (w/v) agar (pH 5.0); bacteria were maintained on nutrient agar slants. Inoculated slants were incubated at 30 °C for 24 to 72 h (yeasts) or at 37 °C for 24 h (bacteria). Stock cultures were stored at 4 °C, and sub-cultured at 2 to 4 week intervals.

Growth medium. The vitamin B₆-free basal medium used in the cultivation of yeasts and bacteria was that of Chiao & Peterson (1956) and contained (l⁻¹): 50 g glucose, 550 mg KH₂PO₄, 425 mg KCl, 125 mg CaCl₂·2H₂O, 125 mg MgSO₄·7H₂O, 2.5 mg MnSO₄·H₂O, 2.5 mg FeCl₃·6H₂O, 4 g hydrolysed casein (vitamin-free Casamino acids; Difco), 8 µg biotin, 2.5 mg calcium pantothenate, 25 mg inositol, 2.5 mg nicotinic acid, 5 g potassium citrate monohydrate and 1 g citric acid monohydrate. The pH was adjusted to 5.0. The medium was freshly prepared at double-strength, brought to the desired volume with water and other experimental additions, and all its components were autoclaved together at 115 °C for 5 min. The medium was slightly modified from that used for the microbiological assay by Atkin *et al.* (1943). For the microbiological assay, the medium was steamed for 10 min at 100 °C (Atkin *et al.*, 1943; Rabinowitz & Snell, 1947). For investigating the requirement of *S. carlsbergensis* 4228 for vitamin B₆ in the presence of thiamin, the culture medium was steamed for 15 min at 100 °C (Rabinowitz & Snell, 1951). In our experiments, the culture medium was autoclaved at 115 °C for 5 min. An uninoculated control medium was always incubated under experimental conditions, and no bacterial contamination was ever detected. The growth yield of the yeasts was not affected by changing the conditions of sterilization, or by changing the concentration of calcium pantothenate from 2.5 µg ml⁻¹ to 2.5 mg ml⁻¹.

Culture conditions. A starter culture (100 ml), inoculated with one loopful of organisms from an agar slant, was incubated aerobically by shaking at 80 r.p.m. for 72 h until reaching the late-stationary phase of growth. Yeasts were incubated at 30 °C and bacteria at 37 °C. The cells were harvested by centrifugation, washed three times with sterilized 0.9% (w/v) NaCl, and resuspended in the same medium sterilized at a concentration of 1 mg (dry wt) ml⁻¹. A portion (20 ml) of the cell suspension and sterile water (30 ml) was added to sterile double-strength basal medium (50 ml) with or without thiamin. HCl, and the inoculated medium incubated with shaking for 24 or 48 h. The final concentration of thiamin. HCl was 15 nmol ml⁻¹ except where otherwise noted. The concentration of thiamin given is based on the amount added to the medium. However, the loss of thiamin in the medium by autoclaving under the sterilizing condition used was less than 5%. The growth of organisms was measured turbidimetrically at 610 nm. Colorimeter readings were converted to dry weight cells by using a previously constructed calibration curve relating transmission to the dry weight of cells.

Bioassay of vitamin B₆. The amount of vitamin B₆ in the cells or in the culture supernatant was determined using *S. uvarum* 4228 and with pyridoxine hydrochloride as a standard (Atkin *et al.*, 1943; Rabinowitz & Snell, 1947). Prior to the determination, samples were acidified with 0.1 M-HCl, autoclaved at 121 °C for 3 h, and the pH was adjusted to 5.0 with 4 M-sodium acetate. The amount of vitamin B₆ determined was expressed as an equivalent weight of pyridoxine hydrochloride.

Determination of vitamin B₆ metabolites. The effect of thiamin on vitamin B₆ metabolism was studied by following degradation of ¹⁴C-pyridoxine. Metabolites of vitamin B₆ in cells were extracted with chilled 5% (v/v) perchloric acid [100 ml (g dry wt cells)⁻¹]. The extract was neutralized with KOH, stored at 0 to 4 °C overnight to precipitate potassium perchlorate, and then lyophilized after removal of the precipitates by centrifugation. The supernatant from the culture fluid was directly lyophilized. The lyophilized samples from 100 ml of culture fluid were dissolved in 0.05 M-ammonium formate (final volume 20 ml) containing cold authentic vitamin B₆ metabolites (2 mg each of pyridoxine 5'-phosphate, pyridoxal 5'-phosphate, pyridoxamine 5'-phosphate and pyridoxamine, 1 mg each of pyridoxal and pyridoxine, 0.8 mg each of 4-pyridoxic acid and 4-pyridoxic acid lactone, and 0.02 mg 4-pyridoxic acid 5'-phosphate), and the pH was adjusted to 4.25 or 3.20.

Fig. 1. The separation of vitamin B₆ metabolites by chromatography on Dowex 50W-X8. The pH values of the samples applied to the column were 4.25 (a) and 3.20 (b). The elution buffer used in (a) was 0.05 M-ammonium formate. The unlettered arrow in (b) indicates the position at which 0.05 M-ammonium formate was changed to 0.5 M-ammonium formate, details are given in Methods. PN, pyridoxine; PM, pyridoxamine; PL, pyridoxal; PNP, pyridoxine 5'-phosphate; PMP, pyridoxamine 5'-phosphate; PLP, pyridoxal 5'-phosphate; PiC, 4-pyridoxic acid; PiC-Lac, 4-pyridoxic acid lactone.

The column chromatography procedure of Johansson *et al.* (1968) with slight modification was used to fractionate vitamin B₆ metabolites. A column (0.9 × 40 cm) containing Dowex 50W-X8 (200 to 400 mesh) was buffered with 0.05 M-ammonium formate at pH 4.25, and 10 ml samples (described above) layered on the column. Vitamin B₆ metabolites from the column were eluted first with 0.05 M-ammonium formate at pH 4.25 until 4-pyridoxic acid lactone had been eluted. Elution was continued with a gradient, formed by adding 0.5 M-ammonium formate (pH 7.3) to 100 ml 0.5 M-ammonium formate (pH 4.25) in a closed chamber. The eluate was collected in 5 ml fractions at a flow rate of about 0.5 ml min⁻¹ and monitored by measuring A_{325} . A typical elution pattern is shown in Fig. 1. Elution of 4-pyridoxic acid and 4-pyridoxic acid 5'-phosphate was also detected by measuring their blue fluorescence at the elution pH. 4-Pyridoxic acid lactone was measured by its blue fluorescence after the pH of the fraction was adjusted to 9.0. Pyridoxal and pyridoxal 5'-phosphate were detected colorimetrically by the phenylhydrazine reaction (Wada & Snell, 1961). In the column chromatography procedure of Johansson *et al.* (1968), pyridoxine was eluted before pyridoxamine. Under our experimental condition, pyridoxine was eluted after pyridoxamine. This was confirmed both by elution of authentic ¹⁴C-pyridoxine and by conversion of the pyridoxine fraction to 4-pyridoxic acid using acid KMnO₄ oxidation (Fujita *et al.*, 1955). When the pH of the sample applied to the column was 3.20 (Fig. 1b), pyridoxine 5'-phosphate was not separated from pyridoxal 5'-phosphate. However, when the sample pH was 4.25 (Fig. 1a), pyridoxine 5'-phosphate was clearly separated from pyridoxal 5'-phosphate, although the latter was not separated from 4-pyridoxic acid 5'-phosphate. Therefore, fractionation of the metabolites was performed at both pH values. Pyridoxine 5'-phosphate was fractionated under the conditions of Fig. 1(a). The amount of pyridoxal 5'-phosphate was determined by subtracting the amount of 4-pyridoxic acid 5'-phosphate fractionated under the condition of Fig. 1(b) (the first peak) from the total amounts of 4-pyridoxic acid 5'-phosphate and pyridoxal 5'-phosphate fractionated under the condition of Fig. 1(a) (the first peak). Separation of vitamin B₆ metabolites other than pyridoxine 5'-phosphate and pyridoxal 5'-phosphate was performed under the condition of Fig. 1(b). Radioactivity in fractions was measured by liquid scintillation counting. Ethanol (5 ml) and a portion (10 ml) of a mixture that contained PPO (5 g), dimethyl POPOP (0.1 g) and toluene (1 l) was added to a sample (0.3 ml) of each fraction.

Chemicals. Deoxythiamin was a generous gift from Dr Eiichi Hiraoka. Pyrithiamin and oxythiamin were obtained from Sigma, OMP from Takeda Chemical Industries, pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate from Chugai Pharmaceutical Co, [4-hydroxymethyl-¹⁴C]pyridoxine hydrochloride (1.3 mCi mmol⁻¹; 48 MBq mmol⁻¹) from Daiichi Pure Chemicals Co, and Dowex 50W-X8 from Dow Chemical Co. 4-Pyridoxic acid 5'-phosphate was synthesized from pyridoxal 5'-phosphate by oxidation with silver oxide (Wada & Snell, 1961).

RESULTS

Effects of thiamin on the growth yield and vitamin B₆ content of organisms

The growth yield and vitamin B₆ content of yeasts of *Saccharomyces* strains after incubation for 24 h and 48 h in medium with and without added thiamin is shown in Table 1. In *S. uvarum* 4228 and *S. uvarum* IFO 0751, cell yields were greatly decreased (by 61% and 69% after 24 h incubation, respectively) by addition of thiamin to the growth medium. Except for these two yeasts, the effect of thiamin on growth of the yeasts tested was slight. However, the amounts of vitamin B₆ were greatly reduced in the presence of thiamin in all *Saccharomyces* strains tested. In other *Saccharomyces* strains (not shown in Table 1), the inhibition of vitamin B₆ production by thiamin for 24 and 48 h incubation, respectively, was 90 and 93% in *S. cerevisiae* IFO 0305, 58 and 19% in *S. cerevisiae* IFO 1234, 63 and 61% in *S. cerevisiae* IFO 1346, 40 and 68% in *S. montanus* IFO 0044, 90 and 92% in *S. delbrueckii* IFO 0421, and 37 and 59% in *S. rouxii*

Table 1. *Effects of thiamin on the growth yield and vitamin B₆ content of Saccharomyces strains*

Cell yields [mg dry wt cells (100 ml culture)⁻¹] and vitamin B₆ content were determined in the absence and presence of thiamin (15 nmol ml⁻¹). The vitamin B₆ content of the media or the cultures is expressed as pyridoxine·HCl equivalents [μg (100 ml culture)⁻¹]. The numbers in parentheses are the percentage of the reduction of vitamin B₆ produced per mg dry wt cells by thiamin.

Organism	Medium	Incubation time (24 h)			Incubation time (48 h)		
		Cell yield	Vitamin B ₆ content		Cell yield	Vitamin B ₆ content	
			Medium	Culture		Medium	Culture
<i>S. uvarum</i> 4228	Basal	490	0.01	1.62	580	0.02	1.85
	Thiamin	190	0.01	0.39 (38)	330	0.02	0.36 (66)
<i>S. uvarum</i> IFO 0295	Basal	490	0.17	5.45	560	0.64	8.74
	Thiamin	410	0.04	2.29 (50)	600	0.16	3.36 (64)
<i>S. uvarum</i> IFO 0751	Basal	520	0.01	1.01	550	0.02	1.12
	Thiamin	160	0	0.18 (42)	360	0.01	0.16 (78)
<i>S. uvarum</i> IFO 1265	Basal	460	0.75	21.85	705	6.54	23.76
	Thiamin	400	0.18	5.58 (71)	675	0.08	7.95 (65)

Table 2. *Effects of thiamin on the growth yield and vitamin B₆ content of various yeasts and bacteria*

Cell yields [mg dry wt cells (100 ml culture)⁻¹] and vitamin B₆ content were determined in the absence and presence of thiamin (15 nmol ml⁻¹) after 24 h incubation. The vitamin B₆ content of the cultures is expressed as pyridoxine·HCl equivalents [μg (100 ml culture)⁻¹]. Inhibition by thiamin is expressed as a percentage of the reduction of vitamin B₆ produced per mg dry wt cells.

Organism	Medium	Cell yield	Vitamin B ₆ content of culture	Inhibition (%)
Yeasts				
<i>Debaryomyces hansenii</i> IFO 0019	Basal	320	8.58	
	Thiamin	320	1.98	77
<i>Hansenula anomala</i> OUT 6080	Basal	500	11.14	
	Thiamin	690	2.93	81
<i>Pichia membranaefaciens</i> IFO 0189	Basal	180	3.96	
	Thiamin	180	5.23	-32
<i>Torulopsis colliculosa</i> IFO 0381	Basal	540	38.61	
	Thiamin	560	3.63	91
Bacteria				
<i>Bacillus subtilis</i> NRRL B558	Basal	430	17.85	
	Thiamin	440	18.92	-4
<i>Escherichia coli</i> K 12	Basal	60	4.10	
	Thiamin	64	6.93	-58
<i>Klebsiella pneumoniae</i>	Basal	200	9.86	
	Thiamin	280	14.26	-3

IFO 0495. The decrease of growth yields of these yeast strains after 24 h incubation in the presence of thiamin was 8 to 17%. The decrease in growth yields of many yeasts became less marked as the incubation period was prolonged. In contrast, the inhibition of vitamin B₆ synthesis in many yeasts increased as the incubation period was prolonged. Thus, the reduction of vitamin B₆ content could not be ascribed to autolysis of the yeast cells.

Similar data for yeasts other than *Saccharomyces* and for bacteria are shown in Table 2. Growth yields of the yeasts listed in Table 2 were not affected by the presence of thiamin in the growth medium. In contrast, the content of vitamin B₆ was significantly reduced in the presence of thiamin for all yeasts other than *Pichia membranaefaciens*. Similar effects of thiamin on other yeast strains (*Candida utilis* IFO 0619, *Kluyveromyces marxianus* IFO 0219, *Schizosaccharomyces pombe* IFO 0346 and *Schwanniomyces occidentalis* OUT 6181; not described in Table 2) were also observed. Neither the decrease of growth yield nor the decrease of vitamin B₆ content were apparent in the three bacteria tested.

Relation between growth and vitamin B₆ content of *S. uvarum* IFO 1265

To investigate the differential effect of thiamin on growth yields and vitamin B₆ synthesis, these effects were examined in *S. uvarum* IFO 1265 in which vitamin B₆ synthesis, but not growth yield, was affected by the presence of thiamin in the growth medium. The effects of various amounts of thiamin on the growth yield and vitamin B₆ biosynthesis are shown in Fig. 2. The addition of only 0.3 nmol thiamin. HCl ml⁻¹ markedly inhibited vitamin B₆ biosynthesis; maximum inhibition was produced by the addition of 1.5 nmol thiamin. HCl ml⁻¹. However, no decrease of the growth yield was produced by thiamin at the concentrations used.

The time-course of growth and vitamin B₆ content of *S. uvarum* IFO 1265 is shown in Fig. 3. The growth curve was hardly changed by the addition of 15 nmol thiamin. HCl ml⁻¹; stationary phase was attained after 12 h incubation. However, the biosynthesis of vitamin B₆ was severely inhibited by thiamin, and the total amount of vitamin B₆ in the culture with added thiamin scarcely increased after 12 h incubation. The vitamin B₆ content in the inoculum cells was 31 ng (mg dry wt cells)⁻¹. After inoculation, the amount of cellular vitamin B₆ decreased for 8 h. The minimum content of vitamin B₆ reached in the absence and presence of thiamin was 10.7 ng and 3.8 ng (mg dry wt cells)⁻¹, respectively. Thus, during the early stages of growth, the yeasts utilized the vitamin B₆ introduced into the culture from the inoculum. We consider that the minimum essential level of vitamin B₆ for growth of the yeast is not known, but if the level is under 3.8 ng (mg dry wt cells)⁻¹, no inhibition of growth in the presence of thiamin would be expected.

Fig. 2. The relation between growth yield and vitamin B₆ content of *S. uvarum* IFO 1265 in media containing various amounts of thiamin. The media were inoculated with a 72 h culture [18 mg dry wt (100 ml medium)⁻¹] and then incubated at 30 °C for 24 h. Culture conditions are described in Methods. Growth yield of the yeast (○), total vitamin B₆ content in culture (△), and vitamin B₆ content in the culture medium (▲) are shown.

Fig. 3. Growth and vitamin B₆ content of *S. uvarum* IFO 1265 during incubation in the absence and presence of 15 nmol thiamin ml⁻¹. Culture conditions are described in Methods. Growth of the yeast (○, ●), cellular vitamin B₆ content (□, ■) and total amounts of vitamin B₆ in the cultures (△, ▲) were determined in the presence (open symbols) and absence (closed symbols) of thiamin.

Effects of thiamin analogues on the growth yield and vitamin B₆ content of S. uvarum IFO 1265

Effects of the thiamin analogues pyrithiamin, deoxythiamin and oxythiamin on the growth yield and vitamin B₆ content of *S. uvarum* IFO 1265 were examined (Table 3). Pyrithiamin did not affect the growth yield, but enhanced the vitamin B₆ biosynthesis. Deoxythiamin did not affect the growth yields or vitamin B₆ biosynthesis, whereas oxythiamin reduced both. OMP, the pyrimidine moiety of the thiamin molecule, showed the inhibitory activity similar to that of thiamin, that is, it did not reduce the growth yield of the yeast, but reduced the vitamin B₆ content. Its activity, however, was less than that of thiamin.

The effects of thiamin analogues were overcome by the inclusion of equimolar concentrations of thiamin (Table 3). The reduction of growth yield of the yeast by oxythiamin was countered by addition of thiamin. The inhibitory effect of OMP on vitamin B₆ biosynthesis was not additive to the effect of added thiamin. If the inhibitory action of OMP is attributed to the same function as that of thiamin, probably by means of its conversion to thiamin within the cells, the action should not be additive to the effect of added thiamin, because the inhibitory action of thiamin is maximum at a concentration of 20 nmol ml⁻¹.

Table 3. *Effects of thiamin and its analogues on the growth yield and vitamin B₆ content of Saccharomyces uvarum IFO 1265*

Cultures were inoculated to give 0.2 mg dry wt cells ml⁻¹ and incubated at 30°C for 24 h. Cell yields are expressed as mg dry wt cells (100 ml culture)⁻¹ and the vitamin B₆ content of the cultures as pyridoxine.HCl equivalents [µg (100 ml culture)⁻¹].

Addition (20 nmol ml ⁻¹)	Cell yield	Vitamin B ₆ content of culture
None	440	17.54
Thiamin	390	4.09
Pyrithiamin	420	32.40
Deoxythiamin	400	14.91
Oxythiamin	70	0.86
OMP	400	9.41
Thiamin + pyrithiamin	390	3.24
Thiamin + deoxythiamin	400	3.86
Thiamin + oxythiamin	390	3.92
Thiamin + OMP	400	4.18

Fig. 4. The relation between growth yield and vitamin B₆ content of *S. uvarum* IFO 1265 with various amounts of thiamin antagonists (a) pyrithiamin and (b) oxythiamin. Media were inoculated with a 72 h culture [20 mg dry wt (100 ml medium)⁻¹] and then incubated at 30 °C for 24 h. Culture conditions are described in Methods. Growth yield (○) and vitamin B₆ content in the cultures (●) in the presence of thiamin antagonists are expressed as the ratio (value observed in the presence of antagonist)/(value observed in the absence of antagonist).

Pyrithiamin (up to 100 nmol ml⁻¹) stimulated vitamin B₆ synthesis, but did not affect growth yields at these concentrations (Fig. 4). Oxythiamin, however, exhibited a similar effect at low concentration, but reduced both the growth yield and the vitamin B₆ content at higher concentrations; the decrease of vitamin B₆ content was correlated with the growth susceptibility of the yeast to oxythiamin. These effects of thiamin antagonists must be due to their ability to prevent the action of thiamin on the yeast.

Effects of thiamin on the metabolism of vitamin B₆ in S. uvarum IFO 1265

Saccharomyces uvarum IFO 1265 cells, preincubated for 72 h in thiamin-free basal medium, were washed with 0.9% (w/v) NaCl and inoculated (0.2 mg dry wt ml⁻¹) into basal medium containing [4'-¹⁴C]pyridoxine.HCl (0.5 µg ml⁻¹). To determine to what extent transported [4'-¹⁴C]pyridoxine was metabolized by the cells in the absence and presence of thiamin, the extracts of cells, and the culture medium, after 24 h incubation, were analysed for individual forms of vitamin B₆. The perchloric acid extracts of cells and the culture medium were fractionated by ion-exchange chromatography, and the distribution of isotope between pyridoxine, pyridoxal, pyridoxamine, pyridoxic acid and their phosphorylated forms was determined. The culture medium was also analysed after 48 h incubation. Each of the non-phosphorylated forms of vitamin B₆ accumulated intracellularly to similar levels (Table 4). Labelled pyridoxic acid was not detected in the cells and culture medium. The predominant phosphorylated form to be labelled was pyridoxic acid 5'-phosphate. Of the [4'-¹⁴C]pyridoxine added, 16% was catabolized to pyridoxic acid 5'-phosphate; the amounts of pyridoxic acid 5'-phosphate present in the cells and in the culture medium were about equal. At least 54% of the added isotope was incorporated into the cells, and 53% of the added isotope was catabolized to forms other than pyridoxine. The main intracellular phosphorylated form of ¹⁴C-labelled vitamin B₆ was pyridoxamine 5'-phosphate. Pyridoxamine 5'-phosphate was not recognized in the medium.

When thiamin was added to the basal medium at a concentration of 15 nmol ml⁻¹, the uptake of [4'-¹⁴C]pyridoxine by the yeast cells was reduced. After 24 h incubation, 65% of the added isotope was present as pyridoxine in the medium; thus, at least 32% of the added [4'-¹⁴C]-pyridoxine was incorporated into the cells, whilst 31% was catabolized to other forms. The distribution of isotope was hardly affected by the presence of added thiamin except that the excretion of pyridoxamine into the culture medium was reduced and pyridoxamine 5'-phosphate in the cells was increased by the presence of added thiamin. The percentage for [4'-¹⁴C]-pyridoxine catabolized to pyridoxic acid 5'-phosphate was 11% in the presence of thiamin.

Table 4. *Effect of thiamin on metabolism of ^{14}C -labelled pyridoxine by *Saccharomyces uvarum* IFO 1265*

The metabolism of ^{14}C -labelled pyridoxine was determined in the absence and presence of thiamin (15 nmol ml^{-1}). The results are expressed as a percentage of the pyridoxine added. The numbers in parentheses were determined after 48 h incubation, the remainder were determined after 24 h incubation. The total recovery of radioactivity was 96%.

Fractions of pyridoxine metabolites	^{14}C Recovered (%)			
	In thiamin-free culture		In thiamin-supplemented culture	
	In medium	In cells	In medium	In cells
Pyridoxine	41.9 (40.6)	1.14	64.8 (63.0)	0.96
Pyridoxal	13.2 (15.9)	1.41	13.0 (14.3)	0.95
Pyridoxamine	15.0 (13.6)	1.15	0.9 (1.7)	0.69
Pyridoxic acid	0 (0)	0	0 (0)	0
Pyridoxine 5'-phosphate	3.2 (1.7)	0.61	1.0 (1.3)	0.36
Pyridoxal 5'-phosphate	0.6 (2.2)	0.88	0.6 (2.5)	0.66
Pyridoxamine 5'-phosphate	0 (0)	1.73	0 (0)	2.09
Pyridoxic acid 5'-phosphate	7.0 (9.0)	9.07	3.5 (4.3)	7.74
Total	80.0 (83.0)	16.0	83.8 (87.1)	13.0

DISCUSSION

The added thiamin did not stimulate the excretion of vitamin B_6 into the medium in yeast cultures (Table 1). Thus, the decrease of the amounts of vitamin B_6 in the cultures implied a decrease of intracellular vitamin B_6 .

We next considered the effect of thiamin on the metabolism of vitamin B_6 . Shane & Snell (1976) have reported that pyridoxine is taken up actively by *S. carlsbergensis* 4228. When [$4\text{'-}^{14}\text{C}$]pyridoxine ($0.5\text{ }\mu\text{g ml}^{-1}$) was added to *S. uvarum* IFO 1265 in thiamin-free medium, at least 54% of the added [$4\text{'-}^{14}\text{C}$]pyridoxine was incorporated into the cells and 30% of the incorporated [$4\text{'-}^{14}\text{C}$]pyridoxine was metabolized to pyridoxic acid 5'-phosphate. On the other hand, in the thiamin-supplemented medium, at least 32% of the added [$4\text{'-}^{14}\text{C}$]pyridoxine was incorporated into the cells, and 35% of the incorporated [$4\text{'-}^{14}\text{C}$]pyridoxine was metabolized to pyridoxic acid 5'-phosphate. The rate of metabolism of [$4\text{'-}^{14}\text{C}$]pyridoxine to inactive metabolites in the presence of thiamin was only 1.2-fold more than that in the absence of thiamin. This result makes it unlikely that the decrease in vitamin B_6 content of the culture in the presence of added thiamin is due to the stimulation of metabolism of vitamin B_6 to inactive metabolites. It is reasonable to suggest that vitamin B_6 synthesis was inhibited by added thiamin.

Oxythiamin, pyrithiamin and deoxythiamin are well-known thiamin antagonists (Rogers, 1970). The growth of *S. uvarum* IFO 1265 was inhibited by oxythiamin ($10\text{ to }100\text{ nmol ml}^{-1}$), but was not inhibited by pyrithiamin at the concentration used. The activity of oxythiamin as a thiamin antagonist is attributed to the inhibition of thiamin diphosphate (TDP)-mediated enzymes after its conversion to oxythiamin diphosphate (Eich & Cerecedo, 1954; Datta & Racker, 1961). Pyrithiamin diphosphate also inhibits the TDP-mediated enzyme, but the principal enzyme target of pyrithiamin is thiamin kinase (Eich & Cerecedo, 1954; Johnson & Gubler, 1968). Deoxythiamin does not inhibit the TDP-mediated enzyme, but inhibits active membrane transport of thiamin, so that micro-organisms that do not need thiamin are unaffected by deoxythiamin (Hiraoka *et al.*, 1961; Hiraoka & Shimada, 1961; Kishi & Hiraoka, 1967). The results in Table 3 are consistent with this.

The evidence for the mode of action of thiamin antagonists obtained in this paper shows that vitamin B_6 biosynthesis is stimulated when the activity of intracellular thiamin is suppressed by addition of thiamin antagonists at concentrations that do not affect growth.

The inhibition of vitamin B_6 biosynthesis by thiamin was not recognized for *Escherichia coli* K12, *Bacillus subtilis* NRRL B558 and *Klebsiella pneumoniae* under the same conditions as those used in the yeast experiments. Therefore, this phenomenon may be specific to yeast. A study of the function of thiamin in vitamin B_6 synthesis in yeasts is presently in progress.

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