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CARRIER-MEDIATED TRANSPORT OF THIAMINE IN BAKER'S YEAST

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SUMMARY

1. The characteristics of a system of [^{14}C]thiamine uptake were investigated in baker's yeast (*Saccharomyces cerevisiae*).

2. [^{14}C]Thiamine uptake by the system was found to be an energy- and temperature-dependent process which has an optimal pH at 4.5.

3. Most of [^{14}C]thiamine taken up existed intracellularly in free form, which accumulated against a large concentration difference.

4. The apparent K_m for [^{14}C]thiamine uptake was $1.8 \cdot 10^{-7}$ M, and the uptake of labeled thiamine was inhibited by several thiamine analogues, except for oxythiamine.

5. Short-chain fatty acids (C_2–C_6) strongly inhibited [^{14}C]thiamine uptake.

6. Caproate, which has a most pronounced inhibitory effect on [^{14}C]thiamine uptake, brought about the exit of [^{14}C]thiamine taken up.

7. Pyrithiamine inhibited significantly [^{14}C]thiamine exit caused by caproate, whereas oxythiamine did not.

8. It was discussed whether the uptake of thiamine in baker's yeast occurs by a carrier-mediated active process.

INTRODUCTION

Evidence has been accumulated which shows that the uptake of thiamine in microorganisms occurs by active transport[1–3]. Suzuoki[4] studied the transport and accumulation of thiamine in baker's yeast (*Saccharomyces cerevisiae*). His experiments were performed by the thiochrome fluorescence method for measuring the disappearance of thiamine from the medium during an incubation of yeast cells with a relatively large amount of thiamine. It was found that the yeast cells accumulate thiamine in the presence of exogenous glucose, and that this process is inhibited by metabolic inhibitors such as F^-, iodoacetate and N_3^-.

With *Escherichia coli* other investigators[3,5] have shown that the cells take up [^{14}C]thiamine against a concentration gradient regardless of the presence of glucose, and that the transported thiamine is accumulated as thiamine phosphates in the cells.

The present work was performed to characterize the thiamine transport process in baker's yeast using a membrane filter assay technique as a measure of thiamine uptake. The data show that the uptake of thiamine in baker's yeast occurs by a carrier-mediated active process.

MATERIALS

Organism

Fresh baker's yeast (*S. cerevisiae*) was obtained from the Oriental Yeast Company.

Chemicals

[^{14}C]Thiamine ([thiazole-2-^{14}C]thiamine hydrochloride, 18.9 Ci/mole), was obtained from the Radiochemical Centre, England (98% pure as determined by paper and thin-layer chromatography). Pyrithiamine hydrobromide and oxythiamine hydrochloride were the products of Sigma Chemical Co. Chloroethylthiamine* and dimethialium** were generous gifts from Sankyo Co., Ltd (Tokyo) and Tadeka Chemical Industries, Ltd (Osaka), respectively. All other chemicals were purchased from commercial suppliers. Aqueous solutions of acetic, propionic, *n*-butyric, *n*-valeric and *n*-caproic acid were prepared as 1 M solutions, adjusted to pH 5.0 with KOH.

METHODS

Assay of [^{14}C]*thiamine*

Yeast cells were washed once with cold water and were resuspended in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose. The cell suspension with an absorbance at 560 nm of 0.14 showed an average of 0.10 mg dry weight/ml. 5 ml of the cell suspensions were preincubated for 15 min at 37 °C and the uptake was then initiated by adding 50 μl of 0.1 mM [^{14}C]thiamine (18.9 Ci/mole); the incubation was continued at 37 °C with constant shaking. At appropriate intervals 1-ml samples were filtered on membrane filter (TM-1, 0.65 μm pore size, Toyo Roshi Co.), followed by one wash with 10 ml of 0.05 M potassium phosphate buffer (pH 5.0). The filters were dried, put into counting vials containing 10 ml of Bray's solution[6], and counted in a Packard Model 3375 Tri-Carb scintillation spectrometer.

Uptake of radioactive thiamine at 0 °C was performed in the same manner as described above at 37 °C, except that the cell suspension was placed in an ice–water bath 30 min before addition of labeled thiamine, as well as during the subsequent incubation period.

The rate of [^{14}C]thiamine uptake at 37 °C is expressed as nmole [^{14}C]thiamine taken up per mg dry weight after subtracting the uptake at 0 °C from that at 37 °C, unless otherwise indicated.

Chromatographic analysis

Yeast cells were incubated in a [^{14}C]thiamine uptake medium for 1 and 15 min at 37 °C, respectively. After reaction periods of 1 and 15 min, 5 and 1 ml, respectively, were independently collected on membrane filters, and washed with 50 and 10 ml of 0.05 M potassium phosphate buffer (pH 5.0), respectively. Each filter was dipped into 5 ml of 0.01 M acetate buffer (pH 4.5) and heated for 15 min at 85 °C to extract

* 3-2′-Methyl-4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride.

** 3-2′-Methyl-4′-aminopyrimidyl-(5′)-methyl-4,5-dimethyl thiazolium chloride hydrochloride

intracellular thiamine and its phosphates, and were then centrifuged for 20 min at $3000 \times g$ to remove denatured proteins and filters. The supernatants were lyophilized independently and dissolved in 0.1 ml each of distilled water. Each 8 μl of the concentrated samples were spotted on Toyo filter paper strip (No. 50, 2 cm $\times$ 40 cm) and developed by ascending chromatography in a solvent system containing isopropyl-alcohol–0.5 M acetate buffer (pH 4.5)–water (65:15:20, by vol.). Radioactivity on papers was measured by the autoscanner, actigraph II, Nuclear Chicago.

RESULTS

Uptake of [^{14}C]thiamine and effect of temperature

The time course of [^{14}C]thiamine uptake by yeast cells in the presence of 0.1 M glucose is shown in Fig. 1. The uptake was linear for about 20 min, reaching a maximum at 30 min, after which time the level of intracellular thiamine remained constant. The absence of [^{14}C]thiamine uptake at 0 °C is also shown in Fig. 1. The absorbance of the cell suspensions used in the experiment was 0.11 at 560 nm which corresponds to 81 μg dry weight/ml. The rate of labeled thiamine uptake was proportional to the cell suspension, at least up to 0.2 mg dry weight under the conditions employed.

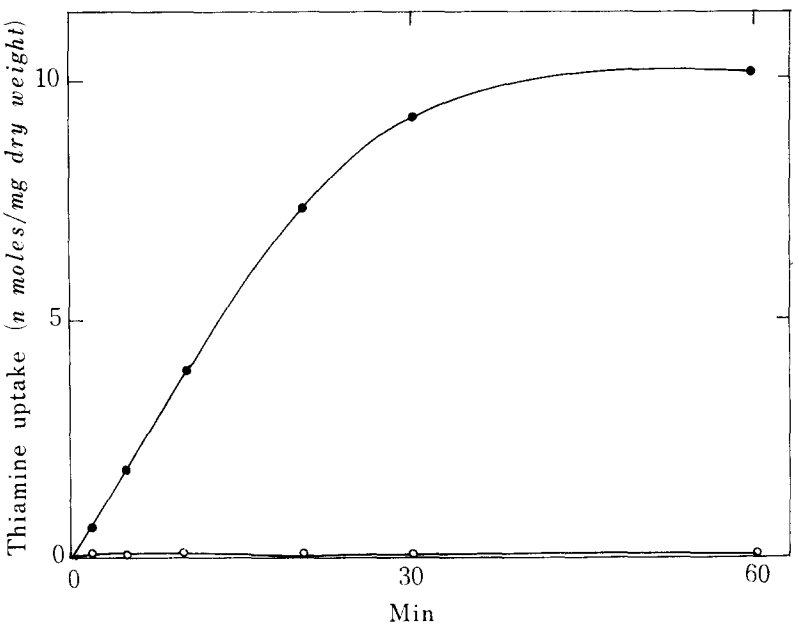

Fig. 1. Time course of [^{14}C]thiamine uptake and effect of temperature. 10 ml of yeast cell suspensions (81 μg dry weight/ml) in 0.05 M potassium phosphate buffer (pH 5.0) containing 0.1 M glucose, were preincubated for 15 min at 37 °C, and then [^{14}C]thiamine was added to the medium at 1 μM concentration, followed by further incubation at 37 °C (●) and 0 °C (○), respectively. The uptake of [^{14}C]thiamine was measured as described in Methods at the indicated times.

pH dependence of [^{14}C]thiamine uptake

The uptake of labeled thiamine at various pH values was conducted for 5 min at 37 °C. As shown in Fig. 2 the rate of [^{14}C]thiamine is maximal at pH 4.5.

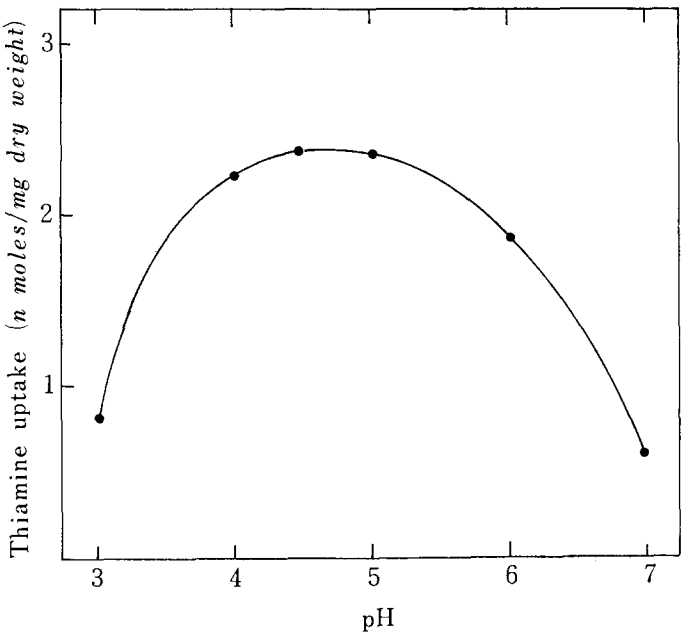

Fig. 2. Dependence of [^{14}C]thiamine uptake on pH. Standard experimental conditions described in Methods were used except for 0.05 M citrate–phosphate buffer of the indicated pH in place of 0.05 M potassium phosphate buffer (pH 5.0). The incubation was carried out for 5 min. Radioactivity taken up by the cells at 0 °C was subtracted from that taken up at 37 °C at each pH.

Intracellular state of transported [^{14}C]thiamine

It is well known that thiamine in the cell exists mainly in the form of its coenzyme, thiamine pyrophosphate, and that intracellular free thiamine is negligible. In *E. coli* it was characteristic that [^{14}C]thiamine accumulated as thiamine phosphates such as thiamine pyrophosphate and thiamine monophosphate even after incubation of 30 s (ref. 2). When the intracellular thiamine was extracted from yeast cells allowed to accumulate [^{14}C]thiamine for 1 and 15 min and subjected to paper chromatography, a major peak of the radioactivity was obtained with an R_F of 0.72, which corresponds to free thiamine (Figs 3A and 3B). The small peak (R_F=0.24) represents thiamine pyrophosphate. These results show that free thiamine exists mainly within the cells in a form chemically identical to that present extracellularly.

Establishment of concentration gradients

An important aspect of any transport study is to determine whether the transported molecules are taken up against a concentration gradient. The intracellular thiamine concentration was calculated from the results in Fig. 1 (30-min sample). On the basis of 2.1 μl of intracellular water per mg of dry yeast[7], the intracellular thiamine concentration was 10100-fold the external thiamine concentration.

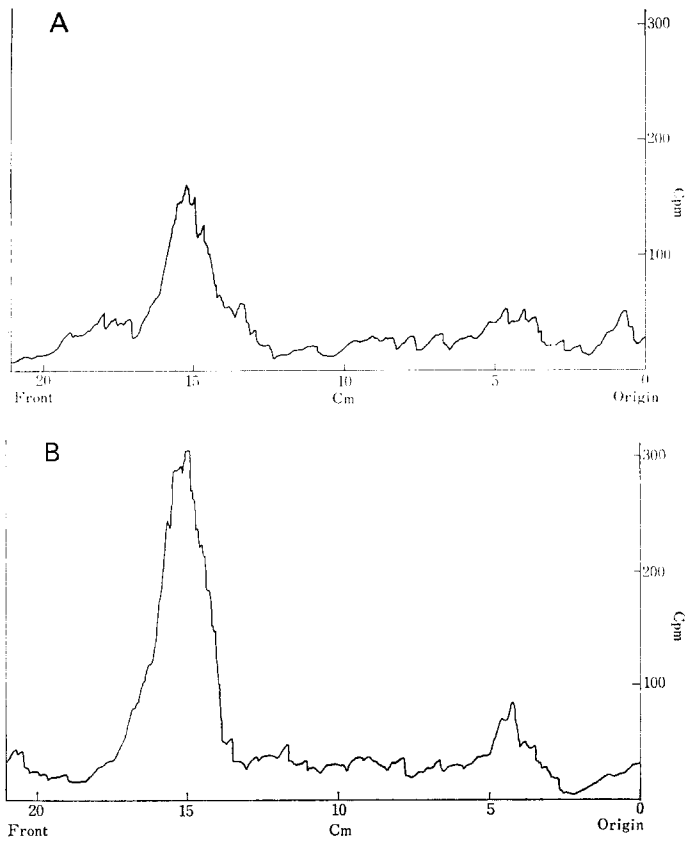

Fig. 3. Intracellular state of [^{14}C]thiamine transported. Samples of yeast cell suspensions (81 μg dry weight/ml) were exposed to 1 μM [^{14}C]thiamine for 1 min (A) and 15 min (B), respectively. Procedures used for chromatographic analysis are described in Methods.

Energy requirement for [^{14}C]thiamine uptake

Many microbial transport systems are dependent upon, or stimulated by the presence of an energy source. As shown in Table I, after 5 min of incubation at 37 °C, yeast cells took up 14 times more labeled thiamine in the presence of glucose than in its absence. The preincubation of yeast cells with arsenate or CN^- caused a marked reduction in the amount of [^{14}C]thiamine taken up. 2,4-Dinitrophenol also inhibited thiamine uptake. Furthermore, the uptake was markedly inhibited by *N*-ethylmaleimide which has been suggested to inhibit "carrier" activity in the transport system in *E. coli*[8]. The results suggest that the process of thiamine permeation in baker's yeast is an energy-dependent process, in agreement with the results reported previously[4]. Cirillo[9] has defined active transport as energy-dependent transport which results in the accumulation of a solute against a large concentration difference.

Effect of [^{14}C]thiamine concentration on the rate of uptake

The rate of [^{14}C]thiamine uptake increased with increasing external vitamin concentration, tending to become saturated at high extracellular thiamine concen-

TABLE I

EFFECT OF GLUCOSE AND METABOLIC INHIBITORS ON [^{14}C]THIAMINE UPTAKE

Yeast cell suspensions (81 μg dry weight/ml) were preincubated for 15 min at 37 °C with or without addition as indicated. [^{14}C]thiamine was added to the medium at 1 μM concentration, followed by further incubation for 5 min at 37 °C. The uptake of [^{14}C]thiamine was measured as described in Methods.

Addition (mM)	*[^{14}C]thiamine uptake (nmoles/mg dry weight)*	*% Inhibition*
None	1.72	—
−Glucose	0.122	92.9
+Sodium arsenate (10)	1.08	36.9
+KCN (2)	0.877	48.9
+2,4-dinitrophenol (0.4)	0.606	64.7
+*N*-Ethylmaleimide (0.1)	0.127	92.6

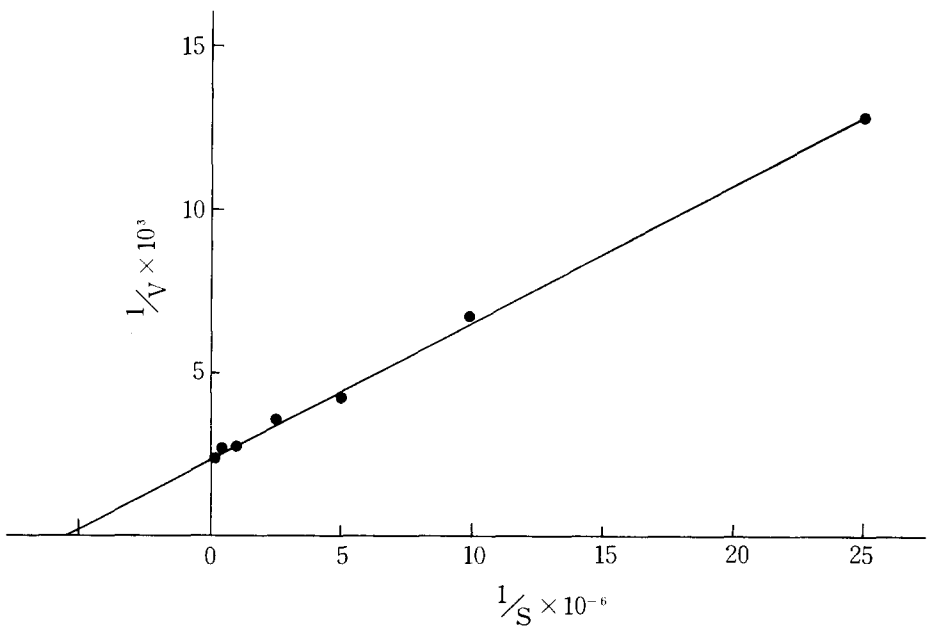

Fig. 4. Lineweaver–Burk plot of [^{14}C]thiamine uptake. After preincubation for 15 min at 37 °C yeast cell suspensions (74 μg dry weight/ml) were incubated with varying concentrations of [^{14}C]thiamine for 1 min at 37 °C and 0 °C, respectively. Radioactivity taken up at 0 °C was corrected from that taken up at 37 °C at each [^{14}C]thiamine concentration.

trations. Fig. 4 shows that apparent K_m calculated from a Lineweaver–Burk plot of the data is $1.8 \cdot 10^{-7}$ M.

Effect of short-chain fatty acids on [^{14}C]thiamine uptake

In earlier reports it has been demonstrated that acetate and other short-chain fatty acids inhibit the uptake of phosphate in yeast[10] and that of amino acid in *Bacillus subtilis*[11], similar to the effect of 2,4-dinitrophenol.

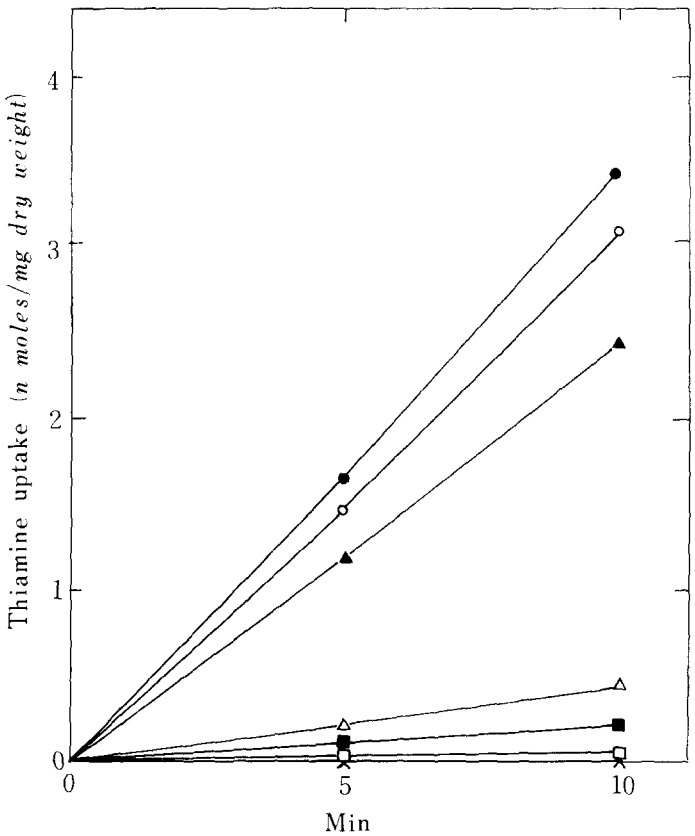

Fig. 5. Effect of short-chain fatty acids on [^{14}C]thiamine uptake. Yeast cell suspensions (81 μg dry weight/ml) were preincubated for 15 min at 37 °C in the uptake medium with or without 20 mM fatty acid. The other conditions for the uptake were the same as described in Methods. ●, control; ○, potassium acetate; ▲, KCl; △, potassium propionate; ■, potassium butyrate; □, potassium valerate; ×, potassium caproate.

As shown in Fig. 5, [^{14}C]thiamine uptake by yeast cells was also inhibited strongly by preincubation of the cells with *n*-fatty acids (C_2–C_6). The inhibition by fatty acids increased with chain length of the acids. Although an inhibitory effect of potassium acetate was less than that of KCl at 20 mM, the inhibition was found to be mainly due to acetate itself at concentrations higher than 40 mM (Fig. 6). These fatty acids reduced the rate of oxygen consumption in yeast cells (Table II). The inhibition of [^{14}C]thiamine uptake by fatty acids cannot be ascribed only to a decrease in the rate of glucose oxidation by the cells because the inhibition of the uptake was much more sensitive to fatty acids, except for acetate, than that found in the rate of oxygen consumption (Fig. 5 and Table II).

Among the fatty acids tested, caproate was most effective as an inhibitor of [^{14}C]thiamine uptake by yeast cells. Fig. 7 shows the effect of caproate concentration on [^{14}C]thiamine uptake when it was added simultaneously with [^{14}C]thiamine

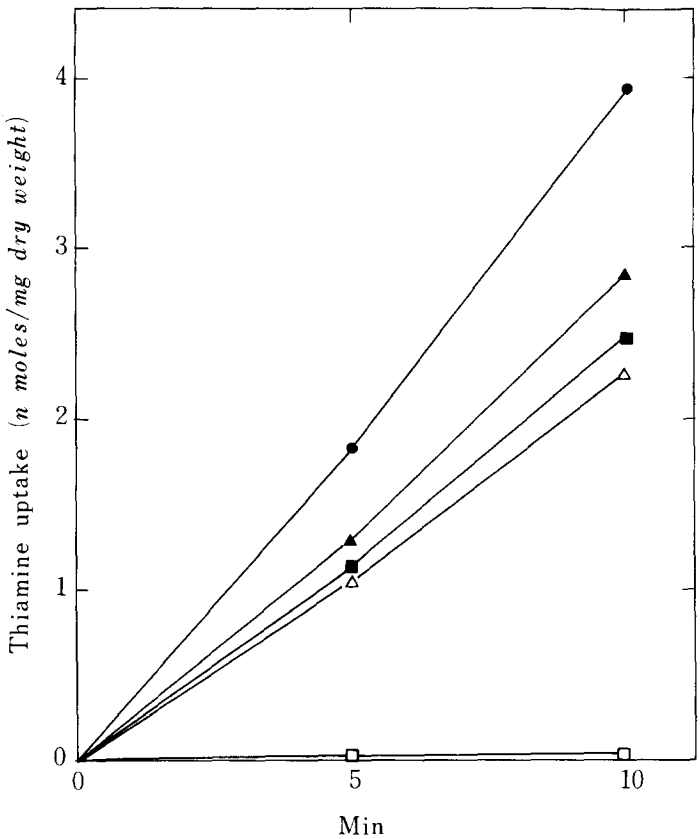

Fig. 6. Inhibitory effect of acetate on [^{14}C]thiamine uptake. Experimental conditions were the same as described in the legend for Fig. 5. ●, control; ▲, KCl at 40 mM; △, KCl at 0.1 M; ■, potassium acetate at 40 mM; □, potassium acetate at 0.1 M.

TABLE II

EFFECT OF SHORT-CHAIN FATTY ACIDS ON OXYGEN UPTAKE

Oxygen uptake was measured manometrically using Warburg's apparatus. Yeast cell suspensions (0.16 mg dry weight/ml) in 0.05 M potassium phosphate buffer (pH 5.0) were put into Warburg flasks with 0.3 ml of 4 M KOH in the centre well. Glucose (0.1 M) and fatty acid (20 mM) were added from the side arm after preincubation for 15 min at 37 °C. After then oxygen uptake was measured for 1 h at 37 °C.

Addition (mM)	*Oxygen uptake (μl)*	*% Inhibition*
None	91.4	—
Acetate (20)	72.4	20.8
Propionate (20)	43.0	53.0
Butyrate (20)	47.4	48.1
Valerate (20)	55.8	38.9
Caproate (20)	60.6	33.7

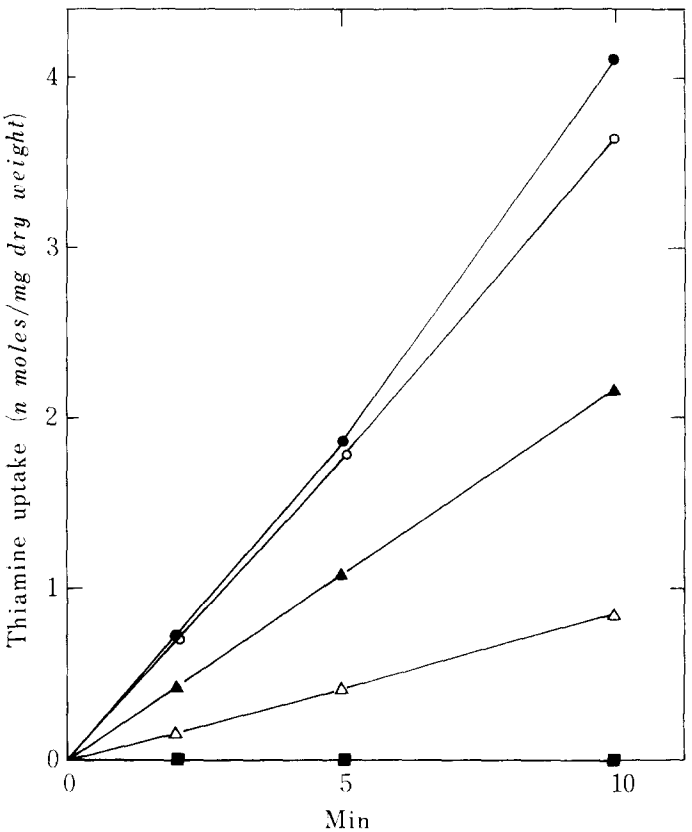

Fig. 7. Effect of concentration of caproate on [^{14}C]thiamine uptake. After preincubation for 15 min at 37 °C yeast cell suspensions (74 μg dry weight/ml) in the uptake medium were exposed simultaneously to 1 μM [^{14}C]thiamine and various concentrations of potassium caproate as indicated. ●, control; ○, 2 mM; ▲, 5 mM; △, 10 mM; ■, 20 mM potassium caproate.

into the uptake medium. The labeled thiamine uptake was inhibited completely by caproate at a concentration of 20 mM.

[^{14}C]Thiamine exit in the presence of caproate

When caproate was added to the uptake medium at 5 min after incubation at 37 °C, the amount of [^{14}C]thiamine decreased (Fig. 8). Since this exit phenomenon was not observed at 0 °C, the process seemed to be temperature dependent. From these results the loss of intracellular [^{14}C]thiamine in the presence of caproate was assumed not to be due to simple diffusion but to the function of the "carrier" for thiamine which is supposed to be located in the cell membrane.

Inhibition of [^{14}C]thiamine uptake by analogues

In order to establish the specificity of the uptake system, the effect of several structurally related compounds on [^{14}C]thiamine uptake was investigated (Table III). The analogues were added to the uptake medium simultaneously with [^{14}C]thiamine,

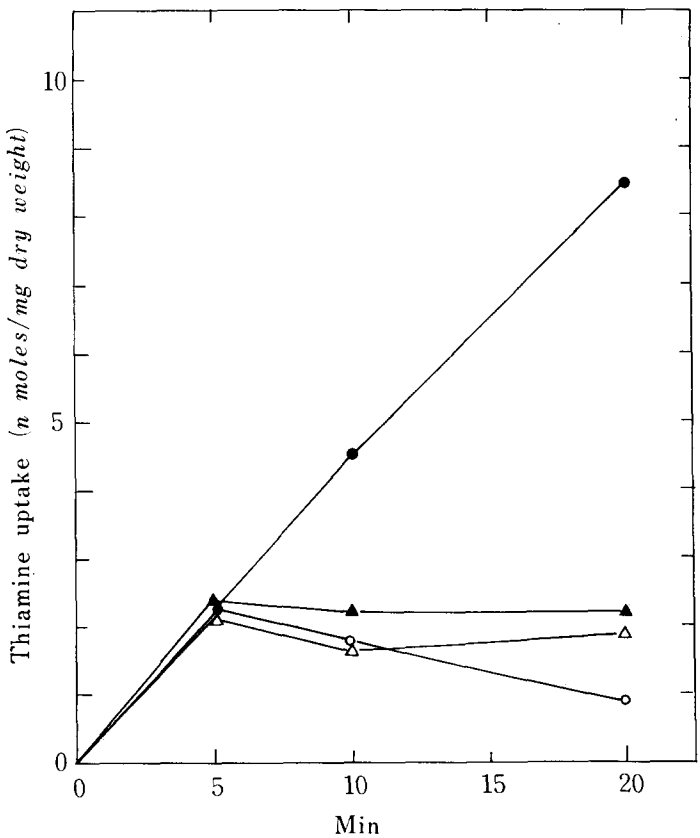

Fig. 8. Effect of adding caproate on the time course of [^{14}C]thiamine uptake. Yeast cell suspensions (81 μg dry weight/ml) in the uptake medium were preincubated for 15 min at 37 °C. After the addition of 1 μM [^{14}C]thiamine following procedures were carried out as indicated. ●, control incubated at 37 °C; ○ and ▲, 20 mM potassium caproate was added to the uptake medium at 5 min after incubation at 37 °C and the incubation was continued for another 15 min at 37 °C and 0 °C, respectively; △, 20 mM potassium caproate was added to the uptake medium at 5 min after incubation at 37 °C and the incubation was continued for another 5 min at 37 °C and thereafter at 0 °C.

followed by incubation for 5 min at 37 °C, at a ratio of analogues to thiamine of 1:1, inhibition of the uptake of labeled thiamine was 42.2% with pyrithiamine, 37.7% with chloroethylthiamine and 38.7% with dimethialium, respectively. On the other hand, oxythiamine was without effect up to a ratio of 25:1. These results indicate that the thiamine uptake system in baker's yeast is mediated by a transport system which displays structural specificity for thiamine and closely related compounds.

Pyrithiamine, furthermore, inhibited [^{14}C]thiamine exit from the cells by approx. 50% in the presence of caproate, as opposed to oxythiamine under the same conditions (Fig. 9).

TABLE III

INHIBITION OF [^{14}C]THIAMINE UPTAKE BY THIAMINE ANALOGUES

Yeast cell suspensions (74 μg dry weight/ml) in the uptake medium were exposed simultaneously to 1 μM [^{14}C]thiamine and analogue at concentration indicated. After incubation for 5 min at 37 °C [^{14}C]thiamine taken up by cells was measured as described in Methods.

Addition (μM)	*[^{14}C]thiamine uptake (nmoles/mg dry weight)*	*% Inhibition*
None	2.00	—
Pyrithiamine (1)	1.16	42.2
Pyrithiamine (5)	0.300	85.0
Oxythiamine (5)	2.01	0
Oxythiamine (25)	2.04	0
Chloroethylthiamine * (1)	1.25	37.7
Chloroethylthiamine (5)	0.462	76.9
Dimethialium ** (1)	1.23	38.7
Dimethialium (5)	0.508	74.6

* 3-2′-Methyl-4′-aminopyrimidyl-(5′)-methyl-4-methyl-5-chloroethyl thiazolium chloride hydrochloride.

** 3-2′-Methyl-4′-aminopyrimidyl-(5′)-methyl-4,5-dimethyl thiazolium chloride hydrochloride.

DISCUSSION

An earlier report by Suzuoki[4] described the phenomenon that thiamine uptake by yeast is closely correlated with carbohydrate and phosphate metabolism, and he suggested that thiamine would be trapped on the cell membrane by a surface enzyme such as thiamine pyrophosphokinase (EC 2.7.6.2) as one of the possible mechanisms of thiamine transport in baker's yeast.

Our studies with radioactive thiamine at growth factor level confirmed that yeast cells accumulate thiamine in the presence of glucose, and that this process is inhibited by some metabolic inhibitors. Further, a radioactive method enabled us to investigate the kinetics, analogue inhibitions at initial phase of thiamine transport concentration difference at equilibrium and exit reactions of transported thiamine.

From the results obtained we conclude that the uptake of [^{14}C]thiamine by yeast cells has several characteristics of a carrier-mediated active process: the vitamin is taken up and retained against a concentration difference and the transport process appears to be energy dependent, is pH and temperature sensitive, displays structural specificity and saturation kinetics.

It should be noticed that the intracellular form of [^{14}C]thiamine accumulated by the system in baker's yeast was mainly free [^{14}C]thiamine, which differs from the case of *E. coli*[2] and Ehrlich ascites tumor cells[12] in which accumulation of thiamine phosphates was shown. With yeast cells a small amount of thiamine pyrophosphate was detected in the cells after thiamine was taken up, whereas no thiamine monophosphate was found (Fig. 3). These results indicate that free thiamine is accumulated and is subsequently phosphorylated to thiamine pyrophosphate by thiamine pyrophosphokinase; this phosphorylation is not involved in the accumulation of thiamine in baker's yeast.

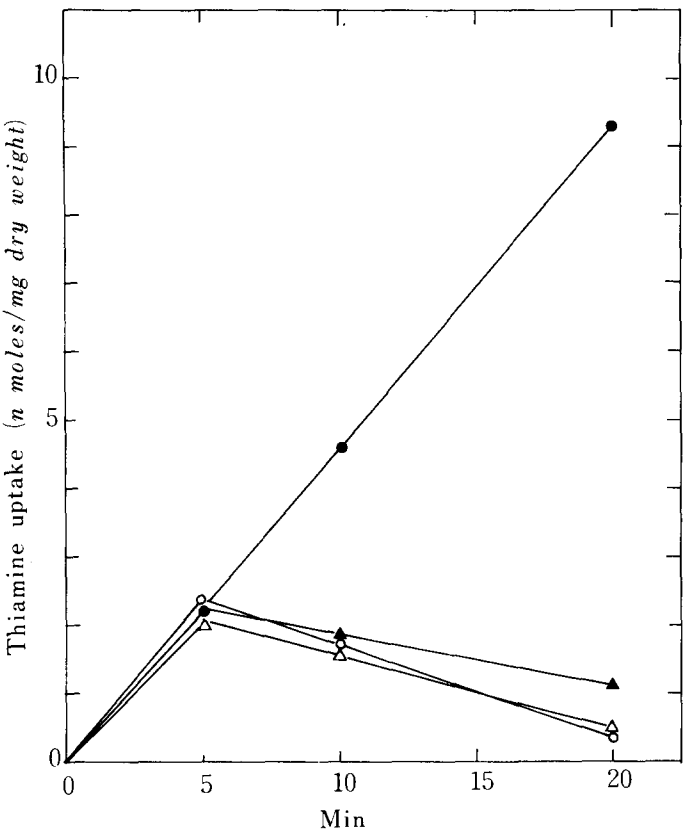

Fig. 9. Effect of thiamine analogues on [^{14}C]thiamine exit caused by caproate. Experimental conditions were the same as described in the legend for Fig. 8 except for the changes as indicated. All the incubations were carried out at 37 °C. ●, control; ○, 20 mM potassium caproate was added to the uptake medium at 5 min after incubation; ▲ and △, 25 μM each of pyrithiamine and oxythiamine was added, respectively, at the same time when caproate was added to the uptake medium.

In addition to various metabolic inhibitors, short-chain fatty acids strongly inhibited [^{14}C]thiamine uptake by yeast cells (Fig. 5). Although the precise mechanism for the inhibition by fatty acids remains to be clarified, as suggested in an earlier report[13] the fatty acids may either alter membrane structure (*e.g.* reduce its fluidity) or uncouple the connection between the electron transport chain and "carrier" required for the transport of thiamine into the cells. Among the fatty acids tested, caproate was most effective in inhibiting [^{14}C]thiamine uptake by yeast cells, and it brought about the exit of [^{14}C]thiamine from the cells when it was added to the uptake medium during [^{14}C]thiamine uptake. This exit process was temperature dependent and was inhibited significantly by pyrithiamine (Fig. 9), which also inhibits strongly the entry of [^{14}C]thiamine in yeast cells (Table III).

These findings suggest that the "carrier" for thiamine not only functions for the influx of thiamine under the physiological conditions in which energy is normally supplied, but is also responsible for the efflux of thiamine in the presence of caproate,

which may disorganize the energetic state of the cell, and particularly the cell membrane where the "carrier" functions. More studies will be required to elucidate the exact mechanism of the efflux of thiamine from the cell.

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