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# Hydroxyethylthiazole Uptake in *Escherichia coli*: General Properties and Relationship Between Uptake and Thiamine Biosynthesis

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Uptake of $^{35}$S-hydroxyethylthiazole (4-methyl-5-hydroxyethylthiazole) by *Escherichia coli* intact cells was studied. Hydroxyethylthiazole was taken up in the presence and absence of glucose at the same rate. The uptake was almost proportional to a hydroxyethylthiazole concentration gradient up to 0.1 mM with no tendency of saturation, and reached a steady state within 2 min. When the cells were treated with 1 mM *N*-ethylmaleimide, about 50% inhibition of hydroxyethylthiazole uptake was observed. Hydroxyethylthiazole uptake was stimulated by the addition of hydroxymethylpyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine), and this effect was further enhanced in the presence of glucose. For full activation of hydroxyethylthiazole uptake, 1 μM hydroxymethylpyrimidine was necessary in the presence of glucose. The rate of hydroxyethylthiazole uptake was almost linear up to 60 min in the presence of hydroxymethylpyrimidine and glucose. Hydroxymethylpyrimidine monophosphate and its pyrophosphate could not stimulate the uptake. Thiamine and 2-amino-hydroxyethylthiazole were inhibitory on hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose. *N*-ethylmaleimide and 2,4-dinitrophenol were also inhibitory. No stimulatory effect of hydroxymethylpyrimidine on hydroxyethylthiazole uptake was observed in mutant cells lacking either thiaminephosphate pyrophosphorylase or hydroxymethylpyrimidine monophosphate kinase. The possibility of direct participation of thiamine-synthesizing enzymes in hydroxyethylthiazole uptake was discussed.

The pathway of thiamine biosynthesis from its thiazole and pyrimidine moieties in *Escherichia coli* is well understood (12). The uptake of thiamine in *E. coli* was recently found to proceed by active transport (5, 9, 14), and regulation of thiamine uptake was found to be involved in repression and derepression of the carrier synthesis (4). The uptake of each moiety of thiamine and its regulation have not been studied in detail. It has been reported, based on the experiments with nonlabeled compounds (3), that a mutant of *E. coli* W resistant to 2-amino-hydroxyethylthiazole, an antimetabolite of hydroxyethylthiazole (4-methyl-5-hydroxyethylthiazole), lacks the ability to take up hydroxyethylthiazole and the antimetabolite as well from the medium, suggesting a possible carrier-mediated uptake of hydroxyethylthiazole in *E. coli*.

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This paper describes some general properties of the hydroxyethylthiazole uptake system of *E. coli* and presents evidence for direct participation of the thiamine-synthesizing enzyme system in the regulation of hydroxyethylthiazole uptake.

## MATERIALS AND METHODS

**Materials.** $^{35}$S-Hydroxyethylthiazole was prepared and purified from $^{35}$S-thiamine (Radiochemical Center, England) by the method of Williams et al. (15). Hydroxymethylpyrimidine (2-methyl-4-amino-5-hydroxymethylpyrimidine), hydroxyethylthiazole, and 2-amino-hydroxyethylthiazole were kind gifts from S. Yurugi, Takeda Pharmaceutical Industries, Ltd., Osaka. Hydroxymethylpyrimidine was converted to its phosphate and pyrophosphate by the method previously described (13). All other chemicals used were of analytical grade.

**Cultivation of E. col.** *E. coli* K-12 cells were cultured in a minimal medium (2) containing 0.2% glucose. The other two mutants of *E. coli* auxotrophic

for thiamine were grown in minimal medium supplemented with 0.01 μM thiamine; KG1673 (7) is a mutant of *E. coli* K-12 deficient in thiamine phosphate pyrophosphorylase (EC 2.5.1.3), and W70-23 (12) is a mutant of *E. coli* W lacking hydroxymethylpyrimidine monophosphate kinase (EC 2.7.4.7).

**Assay of $^{35}$S-hydroxyethylthiazole uptake.** *E. coli* cells were cultured at 37 C and were harvested at absorbancy of 0.35 at 560 nm. Harvested cells were washed once with minimal medium and suspended in the medium to a final absorbancy of 0.35 at 560 nm. Five milliliters of the prepared cell suspension was preincubated for 5 min at 37 C in the presence of 100 μg of chloramphenicol per ml and 0.4% glucose unless otherwise stated. The uptake was initiated by the addition of 50 μliters of 0.1 mM $^{35}$S-hydroxyethylthiazole (72 mCi/mM), and the incubation at 37 C was continued with constant shaking. At appropriate intervals, the cells in 0.5 ml were quantitatively filtered on a membrane filter (type HA, 0.45 μm pore size; Millipore Corp.), followed by one wash with 10 ml of minimal medium. The filters, removed immediately from suction apparatus, were dried, put into counting vials containing 10 ml of Bray solution (1), and counted in a Packard Tri-Carb scintillation counter model 3320. The rate of $^{35}$S-hydroxyethylthiazole uptake was expressed as micromoles of $^{35}$S-hydroxyethylthiazole taken up per gram dry weight unless otherwise stated.

**Chromatographic analysis.** The fate of $^{35}$S-hydroxyethylthiazole after uptake by *E. coli* cells was studied by paper chromatography analysis. After exposing the cells to $^{35}$S-hydroxyethylthiazole as described above, five samples (ten samples in the absence of hydroxymethylpyrimidine) of a 1-ml reaction mixture were independently collected on membrane filters (Millipore Corp.) at indicated periods, followed by one wash with 10 ml of minimal medium, and the filters from the same reaction period were combined in 10 ml of 0.01 M sodium acetate buffer, pH 4.5. The cell suspensions in the buffer were heated for 15 min at 85 C and were then centrifuged for 15 min at 3,000 × *g*. The supernatant liquids were evaporated independently by Taiyo concentrator model TC-8 at 37 C and were each dissolved in 0.1 ml of distilled water. Each 0.05 ml of the concentrated samples was spotted on Toyo filter paper (no. 50, 1 by 20 cm) and developed by an ascending method in a solvent system of isopropyl alcohol:0.5 M sodium acetate buffer (pH 4.5):water, 65:15:20 (vol/vol/vol) (13). After being developed at room temperature, the paper was cut into 1-cm lengths, and each strip was counted in 10 ml of Bray solution.

## RESULTS

**General properties of the $^{35}$S-hydroxyethylthiazole uptake system of E. coli.** The time course of $^{35}$S-hydroxyethylthiazole uptake by intact cells of *E. coli* K-12 in the presence and absence of 0.4% glucose is shown in Fig. 1. The rate of uptake was almost equal regardless of the presence of glucose, and the uptake proceeded for 2 min linearly and then reached a steady state. The rate of $^{35}$S-hydroxyethylthiazole uptake at 0 C was approximately 35% of that at 37 C.

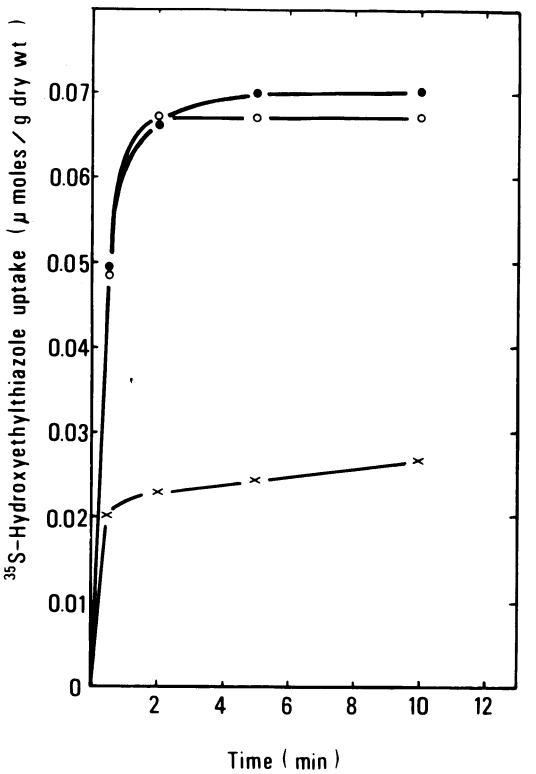


FIG. 1. *Time course of $^{35}$S-hydroxyethylthiazole uptake in the presence and absence of glucose and effect of temperature. Cell suspensions prepared as described in the text were incubated with 1 μM $^{35}$S-hydroxyethylthiazole in the presence of 0.4% glucose at 37 (●) and 0 C (×) or in the absence of glucose at 37 C (○), and the uptake was determined as described in the text.*

The effect of $^{35}$S-hydroxyethylthiazole concentration on the rate of uptake is shown in Fig. 2. Uptake of $^{35}$S-hydroxyethylthiazole was almost proportional to the concentration up to 0.1 mM with no tendency towards saturation.

No significant difference in the rate of $^{35}$S-hydroxyethylthiazole uptake was observed in the range of pH 6.15 to 7.60 (data not shown).

The effect of *N*-ethylmaleimide on $^{35}$S-hydroxyethylthiazole uptake is shown in Table 1. The uptake was inhibited about 55% when the cells were treated with 1.0 mM *N*-ethylmaleimide for 5 min before the initiation of the uptake, whereas 30% inhibition was observed when the uptake was initiated with the simultaneous addition of *N*-ethylmaleimide and $^{35}$S-hydroxyethylthiazole.

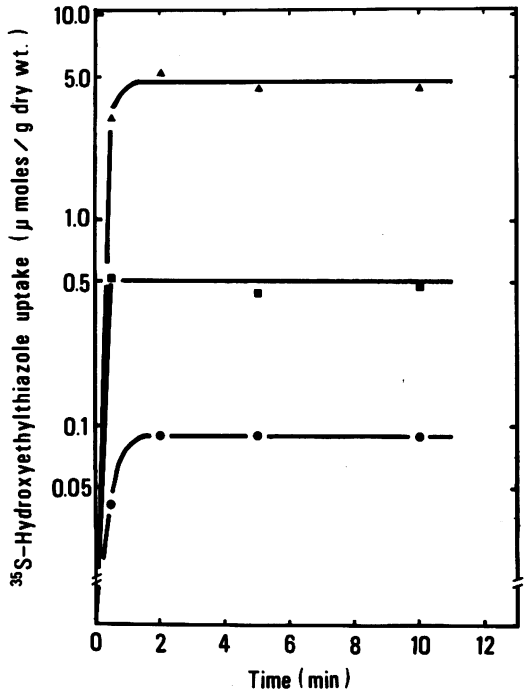


FIG. 2. *Effect of $^{35}$S-hydroxyethylthiazole concentrations on the rate of uptake. Samples of cell suspensions of E. coli K-12 were incubated with 1 (●), 10 (■), and 100 μM (▲) hydroxyethylthiazole, respectively, and the uptake was assayed as described in the text.*

TABLE 1. *Effect of N-ethylmaleimide on $^{35}$S-hydroxyethylthiazole uptake by E. coli K-12 cells*[a]

| Time of 1.0 mM *N*-ethylmaleimide addition | Inhibition of control (%) | |
|---|---|---|
| | 30 s | 5 min |
| Before preincubation ..... | 45.7 | 57.8 |
| After preincubation ..... | 22.8 | 29.8 |

[a] Cell suspensions were preincubated for 5 min with or without 1.0 mM *N*-ethylmaleimide in the presence of 0.4% glucose. Radioactivity taken up by the cells was determined 30 s and 5 min after initiation of the uptake reaction.

**Effect of hydroxymethylpyrimidine on $^{35}$S-hydroxyethylthiazole uptake.** The effect of hydroxymethylpyrimidine on $^{35}$S-hydroxyethylthiazole uptake was studied, because these moieties are known to be necessary for thiamine biosynthesis in *E. coli* (12). The rate of $^{35}$S-hydroxyethylthiazole uptake was remarkably increased by the addition of 0.1 mM hydroxymethylpyrimidine (Fig. 3). The stimulatory effect of hydroxymethylpyrimidine was further enhanced in the presence of 0.4% glucose, and the rate of uptake was almost linear up to 60 min under this condition. The addition of hydroxymethylpyrimidine had no effect upon uptake at 0 C. The effect of hydroxymethylpyrimidine concentration on $^{35}$S-hydroxyethylthiazole uptake is shown in Fig. 4. In the presence of 0.4% glucose, about 1 μM hydroxymethylpyrimidine was necessary for maximal activation of $^{35}$S-hydroxyethylthiazole uptake without practical stimulatory effect at 0.01 μM hydroxymethylpyrimidine. The effect of hydroxymethylpyrimidine was observed immediately after addition to the uptake mixture (Fig. 5).

2,4-Dinitrophenol and *N*-ethylmaleimide were both inhibitory on $^{35}$S-hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose (Table 2). Complete inhibition was observed when the cells

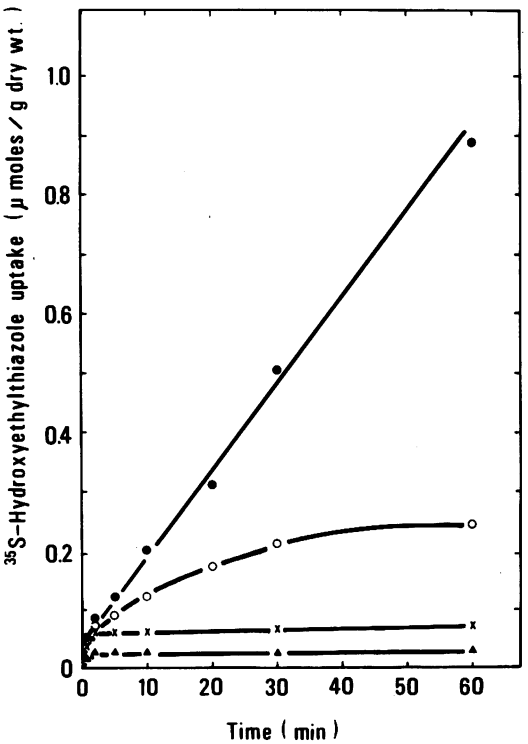


FIG. 3. *Effect of hydroxymethylpyrimidine and glucose on the rate of $^{35}$S-hydroxyethylthiazole uptake. Cell suspensions of E. coli K-12 were preincubated for 5 min with 0.1 mM hydroxymethylpyrimidine in the presence (●) or absence (○) of 0.4% glucose, and the uptake was followed by the addition of 1 μM $^{35}$S-hydroxyethylthiazole at 37 C. As controls, glucose alone was added to the uptake medium incubated at 37 C (×), and glucose plus 0.1 mM hydroxymethylpyrimidine were incubated in the uptake medium at 0 C (▲).*

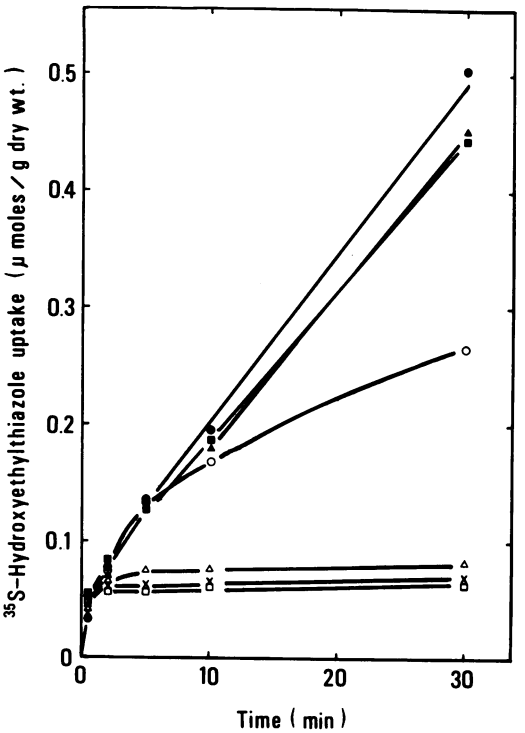


FIG. 4. *Effect of hydroxymethylpyrimidine concentrations on $^{35}$S-hydroxyethylthiazole uptake by E. coli K-12 cells. Cell suspensions were preincubated with varying concentrations of hydroxymethylpyrimidine in the presence of 0.4% glucose for 5 min at 37 C. The uptake was initiated by adding 1 μM $^{35}$S-hydroxyethylthiazole and then determined as described in the text. Hydroxymethylpyrimidine added was 1 nM (□), 10 nM (△), 0.1 μM (○), 1 μM (■), 10 μM (▲), and 0.1 mM (●), respectively. The control (×) contained no hydroxymethylpyrimidine.*

were treated with 1 mM *N*-ethylmaleimide for 5 min before initiation of the uptake.

**Effect of hydroxymethylpyrimidine monophosphate and pyrophosphate on $^{35}$S-hydroxyethylthiazole uptake.** Hydroxymethylpyrimidine is known to be metabolized to its phosphate and pyrophosphate by different kinases involved in thiamine biosynthesis (12). The effect of these phosphates on $^{35}$S-hydroxyethylthiazole uptake was therefore compared with that of hydroxymethylpyrimidine. Hydroxymethylpyrimidine mono- and pyrophosphate at 1 μM were not effective on the uptake in the presence of glucose (Fig. 6). The cell membrane of *E. coli* is supposedly impermeable to hydroxymethylpyrimidine mono- and pyrophosphate (10, 11).

**Effect of 2-amino-hydroxyethylthiazole and thiamine on $^{35}$S-hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose.** It is apparent that $^{35}$S-hydroxyethylthiazole uptake is regulated through synthetic reactions for thiamine and its phosphates. To establish the role of this physiological regulation in *E. coli*, the effect of an antimetabolite of hydroxyethylthiazole, 2-amino-hydroxyethylthiazole (3), and of thiamine was studied. $^{35}$S-Hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose was inhibited by 2-amino-hydroxyethylthiazole added after the uptake was started (Fig. 7). A complete inhibition of the uptake was brought about at a concentration of 10 μM, suggesting the inhibition by the antimetabolite of hydroxyethylthiazole kinase (3) and subsequently of thiamine biosynthesis. To confirm this result, thiamine was added to the uptake reaction mixture at 0.1 mM during the preincubation time. The addition of thiamine resulted in 43.0% inhibition of the uptake in the presence of hydroxy-

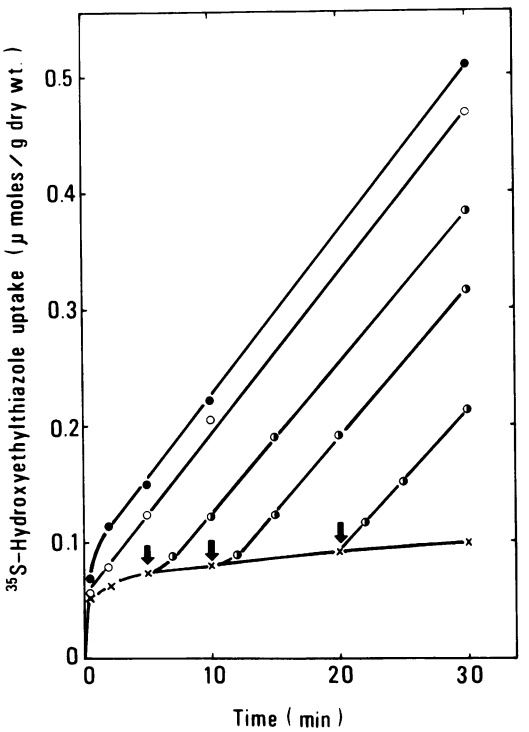


FIG. 5. *Effect of timing of the addition of hydroxymethylpyrimidine on $^{35}$S-hydroxyethylthiazole uptake. Cell suspensions of E. coli K-12 prepared as described above were preincubated with 0.4% glucose for 5 min at 37 C. At the times shown by arrows, 10 μM hydroxymethylpyrimidine was added (◑). Symbols: ●, uptake shown after preincubation with hydroxymethylpyrimidine; ○, uptake shown by simultaneous addition of hydroxymethylpyrimidine and $^{35}$S-hydroxyethylthiazole.*

TABLE 2. *Effect of 2,4-dinitrophenol and N-ethylmaleimide on $^{35}$S-hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose*[a]

| Addition (1.0 mM) | $^{35}$S-hydroxyethylthiazole uptake (μmol/g dry wt) | Inhibition (%) |
|---|---|---|
| None | 0.122 | |
| 2,4-Dinitrophenol | | |
| Before preincubation | 0.054 | 55.7 |
| After preincubation | 0.064 | 47.5 |
| *N*-Ethylmaleimide | | |
| Before preincubation | 0 | 100 |
| After preincubation | 0.052 | 57.3 |

[a] Uptake was assayed 10 min after incubation with 1 μM $^{35}$S-hydroxyethylthiazole in the presence of 10 μM hydroxymethylpyrimidine and 0.4% glucose. Inhibitors indicated in the table were added to the reaction medium before or after a 5-min preincubation. Radioactivity taken up by cells at 0 C was subtracted.

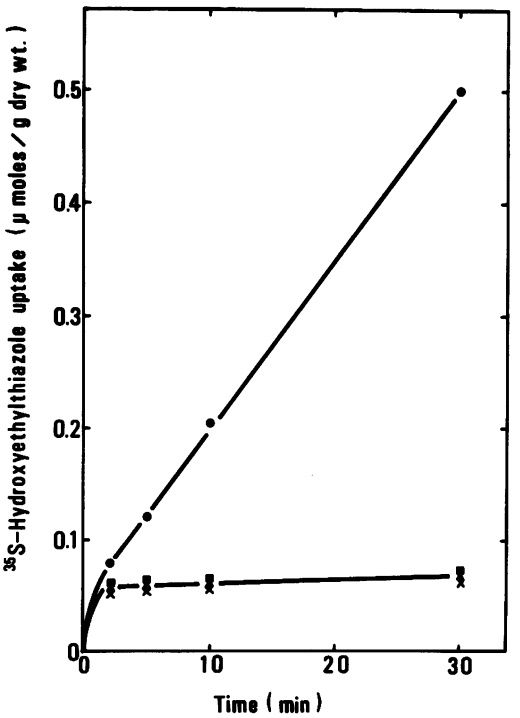


FIG. 6. *Effect of hydroxymethylpyrimidine phosphates on the rate of $^{35}$S-hydroxyethylthiazole uptake. E. coli K-12 cells were preincubated with 1 μM concentrations of hydroxymethylpyrimidine (●) and its monophosphate (■) and pyrophosphate (▲) in the presence of 0.4% glucose, and uptake was initiated by the addition of $^{35}$S-hydroxyethylthiazole. Control (×) contained no hydroxymethylpyrimidine or its phosphates.*

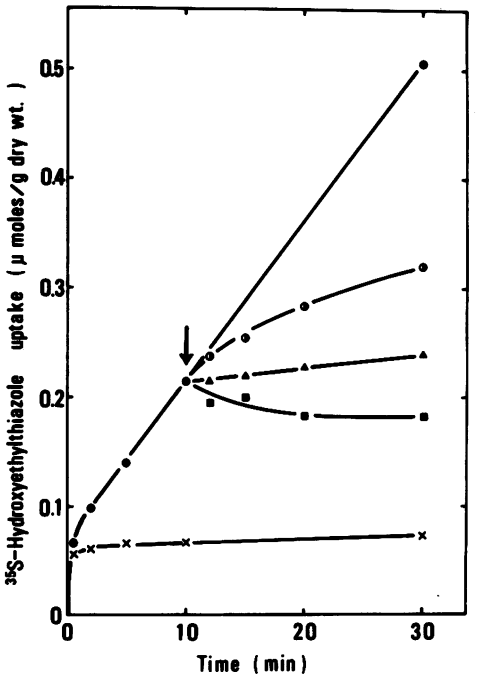


FIG. 7. *Effect of thiamine and 2-amino-hydroxyethylthiazole on $^{35}$S-hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine and glucose. At the time indicated by the arrow, 1 μM (◑), 10 μM (▲) and 0.1 mM (■) 2-amino-hydroxyethylthiazole were added, respectively, to the uptake medium containing 10 μM hydroxymethylpyrimidine and 0.4% glucose (●). The control (×) contained no hydroxymethylpyrimidine or glucose.*

methylpyrimidine and glucose but not in the absence of these compounds (data not shown).

**Effect of hydroxymethylpyrimidine on $^{35}$S-hydroxyethylthiazole uptake by mutants of E. coli auxotrophic for thiamine.** To further establish the physiological relationship between hydroxyethylthiazole uptake and thiamine biosynthesis, two mutants were used: KG 1673, a mutant of strain K-12 deficient in thiamine monophosphate pyrophosphorylase, and W70-23, a mutant of strain W deficient in hydroxymethylpyrimidine monophosphate kinase. $^{35}$S-Hydroxyethylthiazole uptake was not stimulated by the addition of hydroxymethylpyrimidine in both mutant cells (Fig. 8). These results indicate that the condensation of hydroxyethylthiazole monophosphate and hydroxymethylpyrimidine pyrophosphate to form thiamine monophosphate is necessary for the stimulation of $^{35}$S-hydroxyethylthiazole uptake in *E. coli*.

**Intracellular form of $^{35}$S-hydroxyethylthiazole after uptake by E. coli cells.** The fate of $^{35}$S-hydroxyehtylthiazole after the entry

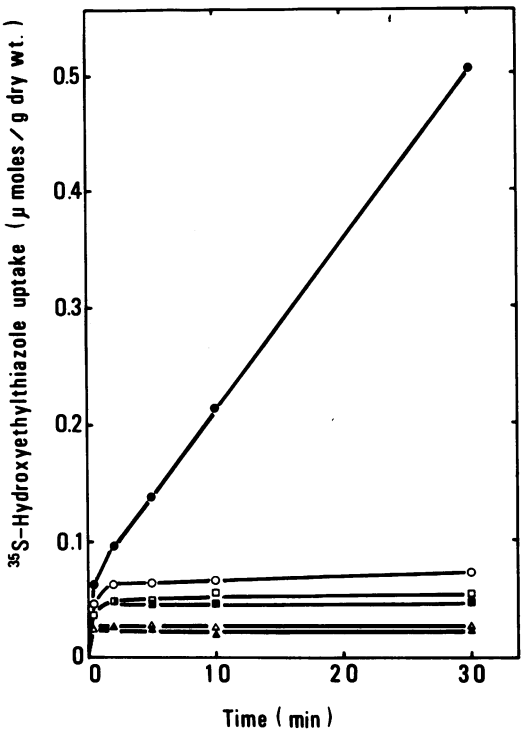


FIG. 8. *$^{35}$S-hydroxyethylthiazole uptake by E. coli mutants in the presence and absence of hydroxymethylpyrimidine. Cell suspensions of each strain were preincubated with 0.4% glucose in the presence (solid marks) or absence (open marks) of 10 μM $^{35}$S-hydroxyethylthiazole. Symbols: K-12, ●, ○; KG1673, ■, □; W 70-23, ▲, △.*

into the cell in the presence and absence of hydroxymethylpyrimidine was followed by paper chromatography analysis. Most of the radioactivity was detected as free hydroxyethylthiazole, and a small peak of radioactivity which corresponded to hydroxyethylthiazole monophosphate was obtained in the absence of hydroxymethylpyrimidine but in the presence of 0.4% glucose (Fig. 9). In the presence of hydroxymethylpyrimidine and glucose, radioactivity corresponding to the $R_F$ of thiamine pyrophosphate on the paper increased with increase of incubation time, whereas the radioactivity at the spot of free hydroxyethylthiazole remained constant.

## DISCUSSION

The rate of $^{35}$S-hydroxyethylthiazole uptake in *E. coli* was not dependent on the presence of glucose and was almost proportional to the hydroxyethylthiazole concentration gradient up to 0.1 mM with no tendency towards saturation (Fig. 1 and 2). Considering an apparent $K_m$ value of 1 μM for thiamine uptake (5), these results suggest that the uptake of hydroxyethylthiazole in *E. coli* is passive in its nature. This assumption was supported by a constant rate of the uptake at a range of pH 6.15 to 7.60 (data not shown). Two different mechanisms of the passive transport system are known: facilitated diffusion, whereby a substrate molecule is equilibrated across the cell membrane by a protein carrier(s), and simple diffusion, whereby a substrate molecule is physically diffused across the cell membrane. The fact that $^{35}$S-hydroxyethylthiazole uptake was inhibited by *N*-ethylmaleimide indicates that this solute was equilibrated across the cell membrane mediated by a protein carrier(s). From the study of thiamine synthesis from hydroxyethylthiazole and hydroxymethylpyrimidine by cell suspensions of 2-amino-hydroxyethylthiazole-resistant mutants, it has been suggested (3) that hydroxyethylthiazole uptake by *E. coli* cells might involve facilitated diffusion.

It has been reported (4–6) that thiamine uptake by *E. coli* cells proceeds by a physiological mechanism involving active transport and that mutant cells auxotrophic for thiamine monophosphate (KG 1675) can take up thiamine actively as free $^{14}$C-thiamine (8).

$^{35}$S-Hydroxyethylthiazole uptake in *E. coli* was remarkably stimulated by hydroxymethylpyrimidine (Fig. 3), and this effect was further enhanced in the presence of glucose. These observations indicate that further metabolism of hydroxyethylthiazole after its entry into the cell is playing an important role in its uptake across the cell membrane. In the presence of hydroxymethylpyrimidine and glucose, $^{35}$S-hydroxy-

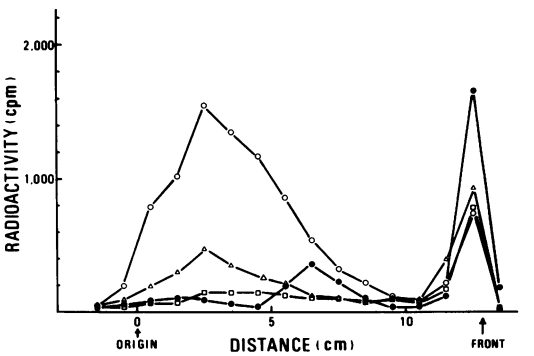


FIG. 9. *Intracellular forms of $^{35}$S-hydroxyethylthiazole taken up by E. coli K-12 in the presence or absence of hydroxymethylpyrimidine. The uptake medium containing cell suspensions, 0.4% glucose, and 1 μM $^{35}$S-hydroxyethylthiazole was incubated in the absence of hydroxymethylpyrimidine for 5 min (●) or in the presence of the same compound for 30 s (□), 2 min (△), and 20 min (○), respectively.*

ethylthiazole should be used for thiamine synthesis as the thiazole moiety of thiamine with hydroxymethylpyrimidine and, therefore, a constant supply of hydroxyethylthiazole should be necessary. The presence of intracellular forms of $^{35}$S-hydroxyethylthiazole after entry into the cells in the presence and absence of hydroxymethylpyrimidine also supports this assumption: accumulation of $^{35}$S-thiamine pyrophosphate was increased with increase of incubation time in the concomitant presence of hydroxymethylpyrimidine and glucose (Fig. 9).

To establish the physiological participation of the enzymes involved in thiamine synthesis in the hydroxyethylthiazole uptake system, two kinds of mutants auxotrophic for thiamine have been used. A mutant of *E. coli* K-12 (KG 1673), which is deficient in thiaminephosphate pyrophosphorylase, took up $^{35}$S-hydroxyethylthiazole at the same rate in the presence and absence of hydroxymethylpyrimidine. The other, a mutant of *E. coli* W (70-23) lacking hydroxymethylpyrimidine monophosphate kinase, showed, in addition, no stimulatory effect of hydroxymethylpyrimidine on $^{35}$S-hydroxyethylthiazole uptake. Thus, the stimulatory effect of hydroxymethylpyrimidine on hydroxyethylthiazole uptake is due to the stimulation of thiamine biosynthesis from hydroxyethylthiazole and hydroxymethylpyrimidine.

The rate of $^{35}$S-hydroxyethylthiazole uptake in the presence of hydroxymethylpyrimidine was remarkably increased by the addition of glucose, whereas glucose had no effect on the uptake rate in the absence of hydroxymethylpyrimidine. This stimulatory effect of glucose was decreased by the addition of 2,4-dinitrophenol. These results suggest that energy supplied from glucose is necessary for thiamine synthesis from hydroxyethylthiazole and hydroxymethylpyrimidine, and not for passage of hydroxyethylthiazole across the cell membrane. On the other hand, the inhibitory effect of *N*-ethylmaleimide on the uptake is supposed to be both on a carrier protein(s) and on the enzymes for thiamine synthesis.

From the results described above, the rate of hydroxyethylthiazole uptake is regulated by the enzymes which catalyze thiamine biosynthesis in *E. coli*. Inhibition of the uptake by thiamine or 2-amino-hydroxyethylthiazole also supports this possibility. This regulatory mechanism seems to be necessary and sufficient for living cells, because hydroxyethylthiazole is considered to be merely equilibrated across the cell membrane by a protein carrier(s) consuming no metabolic energy. Further studies are needed on the uptake of labeled hydroxymethylpyrimidine by *E. coli* to determine whether this uptake is regulated by the addition of hydroxyethylthiazole.

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