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TRANSPORT OF THIAMINE AND 4-METHYL-5-HYDROXYETHYLTHIAZOLE BY *SALMONELLA TYPHIMURIUM*

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The transport of thiamine and 4-methyl-5-hydroxyethylthiazole (MHET), its thiazole moiety, was studied using whole cells of *Salmonella typhimurium*. It was found that the bacteria possessed an active transport system for thiamine that had K_m 0.21 μM and V_{max} 33 nmol · min^{-1} · (mg dry wt. cells)$^{-1}$. Transport of thiamine was glucose dependent, whereas MHET uptake was dependent on both glucose and 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHMP), the pyrimidine moiety of thiamine. Uptake of both thiamine and MHET was severely curtailed by cyanide, azide, *N*-ethylmaleimide and carbonyl cyanide *m*-chlorophenylhydrazone. Oxythiamine inhibited thiamine, but not MHET, uptake and thiamine slightly inhibited MHET uptake. 2-Methyl-4-amino-5-methoxymethylpyrimidine and 4-amino-5-hydroxymethylpyrimidine were unable to replace MAHMP as stimulators of MHET uptake, but 2-methyl-4-amino-5-aminomethylpyrimidine was marginally effective in this regard. Similar results were obtained with attempts to replace MAHMP as a growth requirement for a *pur*D mutant of *Salmonella typhimurium*. MHET uptake showed saturation kinetics only in the presence of MAHMP, and is not otherwise actively transported.

Introduction

The de novo pathway for thiamine biosynthesis in bacteria remains incompletely known. Although biosynthetic studies have been done with yeast [1–3], *Bacillus subtilis* [4] and *Escherichia coli* [6–8], most of the recent work has been done with *Salmonella typhimurium* [8–10]. The studies of Newell and Tucker with this organism [11–14] revealed that the pyrimidine moiety of thiamine, 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHMP), is derived from aminoimidazole ribotide (AIR), an intermediate in purine biosynthesis. We had been doing isotope incorporation and nutritional experiments on the conversion of

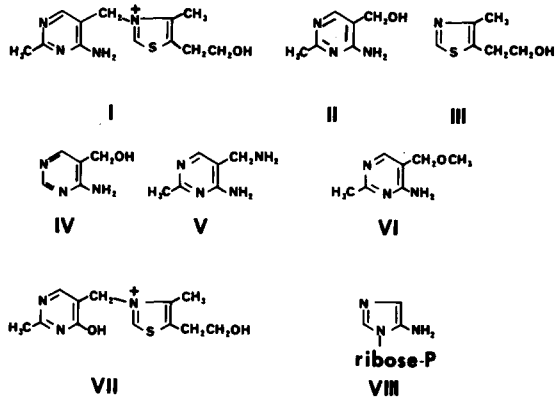

Fig. 1. Structures of thiamine, its moieties and derivatives. I, thiamine; II, 2-methyl-4-amino-5-hydroxymethylpyrimidine (MAHMP); III, 4-methyl-5-hydroxyethylthiazole (MHET); IV, 4-amino-5-hydroxymethylpyrimidine (AHMP); V, 2-methyl-4-amino-5-aminomethylpyrimidine (MAAMP); VI, 2-methyl-4-amino-5-methoxymethylpyrimidine (MAMMP); VII, oxythiamine; VIII, aminoimidazole ribotide.

Abbreviations, see Fig. 1.

aminoimidazole ribotide to MAHMP in *S. typhimurium* and we needed information on the transport of thiamine and its two moieties into the cells. Thiamine transport and that of its thiazole moiety, 4-methyl-5-hydroxyethylthiazole (MHET), had been studied previously only in *E. coli* [15–18] and yeast [19], but no information was available regarding the uptake of MAHMP into any organism. We, therefore, began an investigation of the uptake of thiamine and its moieties by *S. typhimurium* and this article describes our findings for thiamine and MHET uptake. Fig. 1 shows structural formulae for thiamine, its moieties and derivatives.

Materials and Methods

Organism and growth conditions. The organism used for transport studies throughout this work was a tryptophan-requiring auxotroph of *Salmonella typhimurium*, strain number 23592, from the American Type Culture Collection. This organism was selected at it is the strain we use in our biosynthetic experiments. It was grown on the salts medium of Davis and Mingioli [20] supplemented with 0.2% glucose and 25 mg/l tryptophan, at 37°C under vigorous aeration. Growth was monitored by measuring absorbance at 560 nm in a Bausch and Lomb Spectronic 20 spectrometer in 1 cm cuvettes. Cells were harvested at a density corresponding to an absorbance of 0.7–0.9. Stock cultures were maintained on nutrient agar slants under sterile mineral oil at 4°C. For growth stimulation experiments, a kanamycin-resistant *pur*D mutant of *S. typhimurium* obtained from Dr. J. Roth of the University of Utah, was used.

Uptake assays. Harvested cells were washed once with minimal medium and then resuspended in the same medium containing 0.4% glucose (unless otherwise indicated) to an absorbance of 0.5 at 560 nm, equivalent to 0.35 mg dry weight cells per ml. Suspensions (10 ml) of cells were equilibrated to 37°C in a water bath and the uptake was initiated by addition of the radioactive substrate. Incubation was continued at 37°C with shaking and 1 ml samples were withdrawn at timed intervals. The samples were rapidly filtered through 25 mM polycarbonate membrane filters (BioRad 0.45 μm pore size) and washed with 10 ml minimal medium (also at 37°C). The filters were then removed and placed in a scintillation vial containing 5 ml of scintillation fluid and counted. Inhibitors, antagonists and other test compounds were preincubated with the cell suspension for 5 min prior to the addition of the radioactive substrate.

Radioactive counting. The samples were counted in a Beckman LS230 liquid scintillation counter. The scintillation fluid contained 3.5 g diphenyloxazole (PPO), 25 mg 1,4-bis[2-(5-phenyloxazole)] benzene (POPOP), 50 g naphthalene and *p*-dioxane to a total volume of 500 ml.

Chemicals. [*thiazole*-2-^{14}C]Thiamine, specific activity 24.3 mCi/mmol, was purchased from Amersham Corporation. Radioactive MHET was obtained from this by using a modification of the bisulfite cleavage reaction of Williams et al. [21]. Radioactive thiamine (25 μCi) in 1 ml water was mixed with 1 ml 5 M sodium bisulfite and the mixture heated on a boiling water bath for several hours, at 4.5. At the end of this procedure, the mixture was brought to pH 12 and the solution was reduced to dryness. The residue was extracted continuously with chloroform in a Soxhlet apparatus for 3 h. The chloroform extract was evaporated to dryness and the residue was purified by thin-layer chromatography on a silica gel G_{254} precoated Polygram plate as previously described [9]. The MHET-containing band was extracted with hot ethanol (3 × 10 ml). After filtration and evaporation, this procedure yielded [2-^{14}C]MHET, 8.27 μCi.

Oxythiamine, sodium azide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), dinitrophenol, *N*-ethylmaleimide and amino acids were from Sigma Chemical Company. MAHMP was kindly provided by Takeda Chemical Industries Ltd., Osaka, Japan, for which we are deeply indebted. 2-Methyl-4-amino-5-aminomethylpyrimidine (MAAMP), 2-methyl-4-amino-5-methoxymethylpyrimidine (MAMMP) and unlabelled MHET were all provided by Dr. E. Rogers of Merck, Sharp and Dohme Inc. 4-Amino-5-hydroxymethylpyrimidine (AHMP) was synthesized as described by Bellion and Lash [22].

All other common chemicals were obtained from commercial suppliers and were of reagent grade.

Results

Time-course plots for the uptake of both [^{14}C]thiamine and [^{14}C]MHET are shown in Fig. 2. Thiamine uptake was dependent on the presence of glucose, whereas MHET uptake was not stimulated by glucose alone. Maximal uptake of MHET was observed only in the presence of glucose and MAHMP.

Fig. 3 shows the effects of varying concentrations of MAHMP on MHET uptake in the presence of glucose. MAHMP has no effect below 0.01 μM and reaches its maximum effect at 1 μM.

Substrate concentration

Both thiamine and MHET uptake systems showed dependence on substrate concentration and both were saturable, although for MHET this was only true if MAHMP were present. Thiamine gave K_m and V_{max} values of 0.27 μM and 39 nmol/min per g cell dry weight were obtained for thiamine transport and for MHET uptake the values were 0.043 μM and 33 nmol/min per g cell dry weight, respectively.

Effects of other compounds on uptake

(a) Oxythiamine. This substance was found to inhibit severely thiamine uptake, but to have essentially no effect on MHET uptake by the cells (Fig. 4).

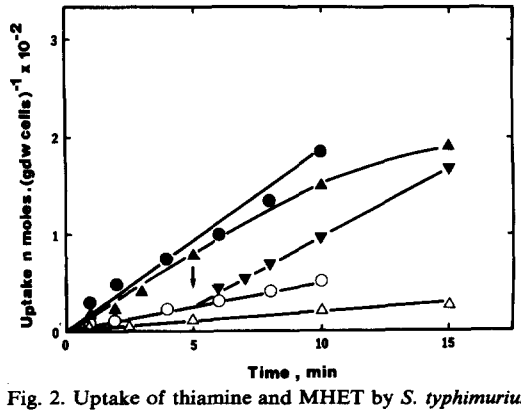

Fig. 2. Uptake of thiamine and MHET by *S. typhimurium* as a function of time. Symbols: △, thiamine; ○, MHET + glucose; ▲, MHET + glucose + MAHMP; ●, thiamine + glucose; ▼, MAHMP added to MHET + glucose incubation at time indicated by arrow. Glucose concentration was 0.4%, MHET was 0.1 μM, thiamine was 0.1 mM, and MAHMP was 0.1 mM.

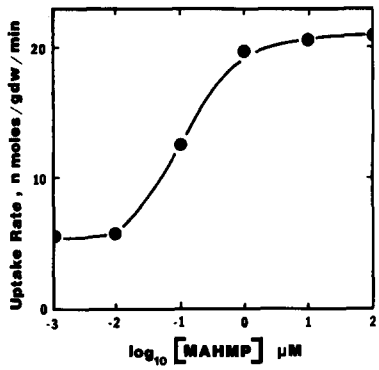

Fig. 3. Uptake of MHET by *S. typhimurium* as a function of MAHMP concentration. Incubations were performed as described in the text with the concentration of MHET at 0.1 μM.

(b) Metabolic inhibitors. *N*-Ethylmaleimide, CCCP, cyanide and azide all very strongly inhibited uptake of both thiamine and MHET (Fig. 5).

(c) Thiamine and other pyrimidines. Thiamine only slightly inhibited MHET uptake in the presence of MAHMP. 2-Methyl-4-amino-5-methoxymethylpyrimidine and 4-amino-5-hydroxymethylpyrimidine (AHMP) were unable to stimulate MHET uptake, but a slight stimulation was observed from the 5-aminomethyl analog (MAAMP) (Fig. 6).

Effect of temperature and pH

Uptake of both thiamine and MHET was temperature dependent, there being essentially no ob-

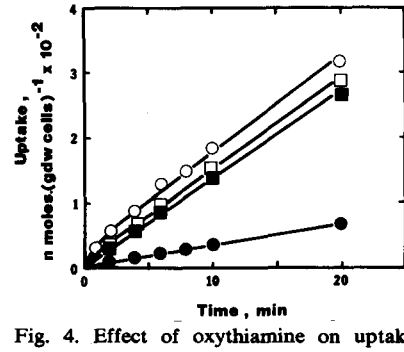

Fig. 4. Effect of oxythiamine on uptake of thiamine and MHET by *S. typhimurium*. Symbols: ○, thiamine; □, MHET; ■, MHET + oxythiamine; ●, thiamine + oxythiamine. Glucose was present in all incubations at 0.4%. MAHMP was present in MHET incubations at 0.1 mM. Oxythiamine was used at 0.1 mM. Thiamine was at 0.1 mM and MHET at 0.1 μM.

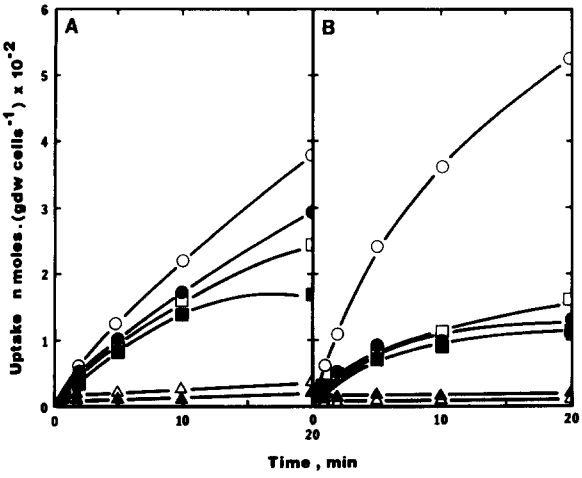

Fig. 5. Effects of inhibitors and uncouplers on thiamine (A) and MHET (B) uptake by *S. typhimurium*. Symbols: ○, no inhibitor added; ●, cyanide; □, azide; ■, 2,4-dinitrophenol; △, *N*-ethylmaleimide; ▲, carbonyl cyanide *m*-chlorophenylhydrazone. All inhibitors were present at 1 mM. Glucose was included in all incubations at 0.4%. MAHMP was in all MHET incubations at 0.1 mM. The concentration of thiamine used was 0.1 mM; that of MHET 0.1 μM.

servable uptake at 2°C. Thiamine uptake showed a very broad pH profile with the maximum occurring at 6.8–7.0. A similar result was obtained for MHET uptake in the presence of MAHMP. In the absence of the latter, no pH dependence could be discerned.

Growth experiments

To determine if the effects of pyrimidine derivatives on MHET uptake were related to their

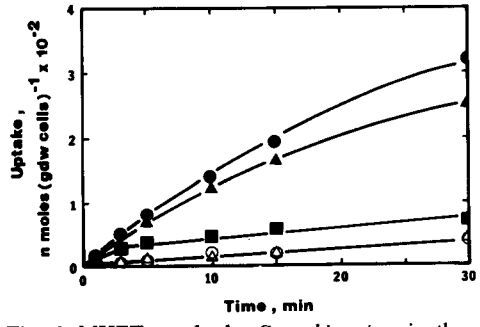

Fig. 6. MHET uptake by *S. typhimurium* in the presence of different pyrimidines and thiamine. Symbols: ●, MAHMP; ▲, MAHMP + thiamine; ■, MAAMP; ○, AHMP; △, 2-methyl-4-amino-5-hydroxymethylpyrimidine. All additions were at 0.1 mM, glucose was present in all incubations at 0.4% and MHET concentration was 0.1 μM.

TABLE I

GROWTH RESPONSE OF *SALMONELLA TYPHIMURIUM purD* (*kan*^{r})

Additions to basal medium [a]	Absorbance (540 nm)	
	36 h	72 h
Thiamine + adenine	0.60	0.60
Thiamine	0.03	0.03
Adenine	0	0
Adenine + MAHMP	0.72	0.72
Adenine + MAAMP	0.11	0.50
Adenine + AHMP	0.06	0.06
Adenine + MAMMP	0.05	0.12

[a] Basal medium was that of Davis and Mingioli [20] supplemented with kanamycin (100 ng/ml); thiamine and pyrimidines were added to a final concentration of 5 μg/ml. Media were inoculated with 0.1 ml taken from a 1 : 100 dilution of a fully-grown liquid culture. Samples were incubated with shaking at 37°C. Absorbance measurements were made in a Bausch and Lomb Spectronic 20 using 10 mm cuvettes at 540 nm.

ability to replace MAHMP biosynthetically, effects of these compounds on the growth of a kanamycin-resistant *purD* mutant of *S. typhimurium* was tested. The kanamycin resistance property of this strain was useful in these experiments as kanamycin prevented growth of anything other than the test organism. The results, Table I, show that the aminomethyl analog (MAAMP) was able to promote good growth whereas the MAMMP and AHMP were unable to support growth.

Discussion

The results clearly demonstrate that cells of *S. typhimurium* possess an active transport system for thiamine, resembling that described for *E. coli* [15]. It is energy dependent, saturable, shows a temperature and pH dependence and is inhibited by energy poisons, *N*-ethylmaleimide and by its analog, oxythiamine. The internalized form of thiamine in *S. typhimuirum* has been shown by Newell and Tucker [12] to be thiamine pyrophosphate as it is also in *E. coli* [15]. In *E. coli* thiamine transport was shown to be mediated by a repressible thiamine-binding protein [17] and it is likely that a similar system exists in *S. typhimurium*.

By contrast, MHET is not actively transported into cells of *S. typhimurium*. Its uptake is very slow alone and is not markedly stimulated by glucose; neither is any pH dependence apparent. However, its uptake is significantly enhanced by the presence of MAHMP and under these conditions features of an active transport system can be observed such as glucose dependence, saturation kinetics and inhibition by energy poisons. It is now clear that these features are seen as a consequence not of MHET active transport, but of the active transport of MAHMP as shown in the following article [22]. MAHMP stimulation of MHET uptake is at a maximum above 1 μM and no stimulation is observed below 0.01 μM. These data fit well with the K_m for MAHMP uptake of 0.07 μM [22]. Furthermore, the apparent K_m and V_{max} values for MHET uptake are close to those determined for MAHMP. Thus, MHET is only taken into the cells when there is sufficient MAHMP present with which to react to produce phosphorylated thiamine derivatives. It has been suggested for *E. coli* [18] that MHET enters cells by facilitated diffusion as its entry was inhibited by *N*-ethylmaleimide. However, since *N*-ethylmaleimide inhibits MAHMP uptake [22], this is not necessarily the case and simple diffusion could also be involved. It is also possible that the cells have an active transport system for MHET that also binds and transports MAHMP such that MAHMP can be transported independently of MHET but not vice versa.

Of the other pyrimidine analogs tested, only the aminomethyl analog showed any degree of stimulation of MHET uptake. It is, therefore, possible that this substance can be converted into MAHMP by *S. typhimurium* as has been suggested for yeast [23]. By contrast, the demethyl analog of MAHMP was entirely unable to stimulate MHET uptake. Thus, it is unlikely that this material, AHMP, can be taken up and methylated by the cells to produce MAHMP. These findings are of interest concerning the biosynthesis of MAHMP from aminoimidazole ribotide, which involves a ring enlargement, a methylation and addition of two other carbons, the details being unknown. It is likely, in light of our data, that methylation is an early step in the process, occurring before ring expansion, such that AHMP would not be an intermediate. This idea receives support from growth experiments using a *pur*D mutant of *S. typhimurium*. Such mutants are auxotrophic for purines and MAHMP since the lesion occurs before 5-aminoimidazole ribotide, the branch-point compound. It was found that when MAAMP replaced either thiamine or MAHMP, a slow growth was achieved, where as AHMP was essentially unable to support growth as was the methoxymethyl derivative.

This is in contrast to the results obtained by White [24], in which AHMT was able to support the growth of an *E. coli pur*I mutant. However, AHMT was not methylated; instead it was converted to a demethylthiamine, which the cells could apparently use in lieu of thiamine. This means that the attachment of the methyl group to the ring must be an early step in the pathway between aminoimidazole ribotide and MAHMP in *E. coli* also, but that in *E. coli*, the systems for uptake and utilization of MAHMP are less specific than those in *S. typhimurium* and can recognize and use AHMP.

Acknowledgements

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