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COUNTERFLOW EFFLUX OF THIAMIN IN *ESCHERICHIA COLI*

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A resting cell of *Escherichia coli* lacking thiamin kinase incorporated external thiamin with an energy-dependent counterflow efflux (C-efflux). This C-efflux could be separated from an energy-dependent exit by a selective inhibition of exit by $2 \cdot 10^{-2}$ M NaN_3. The extracellular thiamin could be replaced by thiamin diphosphate, resulting in the same rate of C-efflux, but the rate of C-efflux of intracellular thiamin diphosphate against the external thiamin was markedly low. This low rate of C-efflux of thiamin diphosphate could explain the higher accumulation of the compound than that of free thiamin in the thiamin-kinase-defective mutant as well as in its wild-type parent. Basic characteristics of free thiamin uptake and exit in *E. coli* W mutant were compared with those reported in K 12 mutant: a marked difference existed in the rate of exit. The low rate of exit in *E. coli* W 70-23-102 was inferred as the reason for the absence of an overshoot phenomenon of thiamin uptake in this strain.

Introduction

Escherichia coli cells take up and accumulate thiamin via a typical process of active transport and then phosphorylate the accumulated thiamin immediately [1]. Hence a similar mechanism to a group translocation system has been proposed for the membrane transport and phosphorylation of thiamin in *E. coli* by Kawasaki et al. [2,3]. Although this hypothesis is accepted widely [4], it is still under serious investigation [5], mainly because the mechanism by which thiamin diphosphate can be accumulated to a higher intracellular concentration than that of free thiamin is unknown. On the other hand, the following observations seem to indicate a membrane transport of thiamin without phosphorylation. 1. The system is a binding protein transport system [6]. 2. Free thiamin accumulates in thiamin-kinase-defective mutants of *E. coli* [7]. 3. Thiamin diphosphate accumulates through the transport system in which thiamin diphosphate and free thiamin compete with each other, and thiamin [^{32}P]diphosphate accumulates without dephosphorylation [8].

In order to elucidate how the intracellular level of the phosphorylated form of thiamin can increase to a much higher level than free thiamin, we used a thiamin-kinase-defective mutant of *E. coli* W (thiamin$^-$); 70-23-102. This mutant could accumulate free thiamin at a normal rate of uptake [9]. Even in this strain 70-23-102, however, the maximum accumulation of free thiamin (approx 160 nmol $\cdot$ g^{-1}) was far below that of thiamin diphosphate (approx. 700 nmol $\cdot$ g^{-1}), and the latter could be attained in both the parent (70-23) and the mutant (70-23-102). The reason for this difference in the level of accumulation of free thiamin and the diphosphate was studied, and found to be the different rate of counterflow efflux, not the rate of exit, between free thiamin and thiamin diphosphate. Thus there are two

Abbreviations FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone, DCCD, dicyclohexylcarbodiimide.

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types of efflux mechanisms in the thiamin transport system of *E. coli*.

Materials and Methods

Strain used and its growth condition. A thiamin auxotroph of *E. coli* W, strain 70-23-102, was provided for us by H. Nakayama [7] and it has defects in phosphohydroxymethylpyrimidine kinase (EC 2.7.4.7) and thiamin monophosphokinase (EC 2.7.1.89), and 70-23-107, given us from the same origin, has defects in phosphohydroxymethylpyrimidine kinase and thiamin monophosphate kinase (EC 2.7.4.16). These strains were grown in Davis and Mingioli's minimal medium [10] of double-strength supplemented with $1 \cdot 10^{-8}$ M thiamin diphosphate for approx. 14 h to the early stationary phase of growth with vigorous shaking at 37°C.

Assay of uptake, exit and counterflow efflux of thiamin. Uptake of [^{14}C]thiamin was determined using primarily the same method as described previously [6]. The rate of C-efflux and the rate of exit were measured by counting the remaining [^{14}C]-thiamin in preloaded *E coli* cells after an outflow reaction with and without external non-radioactive thiamin, respectively. Cells were preloaded with [^{14}C]thiamin for 30 to 60 min at 37°C in the same reaction mixture as that of uptake reaction to reach a steady state of uptake. They were collected by filtration on a membrane filter (0.45-μm pored) or by cold centrifugation (15 000 × g, 5 min, 2°C) and washed by filtration or by cold centrifugation, respectively, with the same volume of cold 0.1 M sodium phosphate buffer, pH 7.0 The preloaded cells were suspended in 0.033 M Tris-HCl buffer of pH 7.4 to the cell density of 5 mg · ml^{-1} at 0–2°C to minimize the decrease of preloaded [^{14}C]thiamin. Preliminary experiments proved that no decrease of cellular thiamin occurred for at least 60 min at this high cell concentration and temperature. This concentrated cell suspension was used as a stock of preloaded cells. The preloaded cells were dispersed rapidly into 50-times the volume of a prewarmed 37°C reaction mixture which contained all ingredients except the preloaded cells In the case of C-efflux (which accompanies exit), the prewarmed mixture contained non-radioactive thiamin at the concentration of $2 \cdot 10^{-7}$ M together with 0.4% glucose, and 0.1 M sodium phosphate buffer of pH 7 0. In the exit reaction, no thiamin was added in the dispersion buffer. At each time indicated, portions (4-ml) were withdrawn and filtered immediately as described above. Cells and filter were then washed by filtration with 4 ml of cold 0.1 M sodium phosphate buffer of pH 7.0. To follow the exit activity, the amount of [^{14}C]thiamin in the cell was subtracted from the value without 37°C incubation (time 0). For the activity of C-efflux, differences between the amount of intracellular [^{14}C]thiamin in the reactions with and without external thiamin were plotted. The content of intracellular H_2O of *E coli* was assumed to be 2.55 ml per g dry weight [11]. The amount of cells was determined by an absorbance measurement at 420 nm. [^{14}C]Thiamin was counted after the addition of a toluene-based scintillator.

Chemical determination of thiamin and its phosphates. Thiamin was determined by fluorometry after BrCN oxidation following the method previously described [6]. To separate the free thiamin from the phosphates, the BrCN oxidation products were shaken for 1 min with 3 ml *n*-butanol twice, extracting only the non-phosphorylated oxidation product or free thiochrome.

Chemicals. [*thiazole*-2-^{14}C]Thiamin hydrochloride with the specific activity of 900 MBq/mmol was obtained from Amersham, U.K., and the radiochemical purity was 97% or above. Neopyrithiamin hydrobromide (95% purity), thiamin monophosphate chloride and thiamin diphosphate chloride were products of Sigma Chemicals. FCCP was the product of Boehringer Mannheim, GmbH.

Results

Counterflow efflux of free thiamin accumulated in E. coli 70-23-102. In an earlier study by Nakayama and Hayashi [7] the accumulation of free thiamin in *E coli* W, 70-23-102 was pointed out. While detailed characteristics of the uptake system of this mutant will be reported elsewhere [9], some kinetic parameters of the uptake reaction of this mutant are summarized in Table I. An evident change in the thaimin uptake system accompanying the defect in thiamin kinase was the disappearance of the low-affinity uptake reaction. As a result of this change, reduced uptake activity (V_{in}) was observed when the uptake

TABLE I

[^{14}C]THIAMIN UPTAKE REACTIONS IN *E. COLI* W MUTANTS

	Thiamin monophosphokinase	Type of thiamin accumulated	K_m (nM)	V_{in}		Maximum uptake	
				nmol/g per min	μmol/l cell H_2O per min	μmol/g	μmol/l cell H_2O
E. coli W 70–23	+	mainly thiamin diphosphate	800	110	(43.1)	0.70	(270)
E. coli W 70–23	+	mainly thiamin diphosphate	30	30	(11.8)	0.70	(270)
E. coli W 70–23–102 [a]	–	free thiamin	0.8	31	(12.2)	0.16	(63)

[a] See Ref. 9.

TABLE II

FACTORS AFFECTING THE INTRACELLULAR LEVEL OF THIAMIN IN *E. COLI*

V_{in} and V_{out} values are expressed as μmol min^{-1} per l cell H_2O (X2 55 for nmol · g^{-1} · min^{-1}).

	Type of intracellular thiamin	V_{in}	V_{out}	
			Exit	Counterflow efflux
E. coli W				
70-23	mainly thiamin diphosphate	11.8 [c]	6.5 [d]	0.21 [d]
70-23-102	free thiamin	12 2	5.5 [e]	6.7 [e]
E. coli K12				
KG 33 [a]	mainly thiamin diphosphate	43	0.94	
KG 1676 [b]	free thiamin	6.4	14.3	

[a] Calculated from Refs. 1 and 2.
[b] Calculated from Ref. 5.
[c] This is for the high-affinity (K_m = 3 · 10^{-8} M) uptake system.
[d] This cell contained mainly [^{14}C]thiamin diphosphate in the amount of 114 μmol per l cell H_2O (290 nmol · g^{-1}).
[e] This cell contained 59 μmol per l cell H_2O (150 nmol g^{-1}) of [^{14}C]thiamin.

TABLE III

PARTIAL INHIBITION OF THIAMIN UPTAKE BY HIGH CONCENTRATION OF INTRACELLULAR THIAMIN DIPHOSPHATE IN *E. COLI* 70-23-102

	Level of preloading [a]		K_m (nM)	V_{in} [c]
	thiamin	thiamin diphosphate		
Control		9.0 [b]	0.77	12 2
Thiamin preloaded cell				
I	32.2		3.3	11.4
II	35.7		4.3	12.2
III	43.9		2 8	12.2
Thiamin diphosphate preloaded cell				
I		56.9	0.77	9.4
II		276	5.0	5.1

[a] μmol per l cell H_2O (X2 55 for nmol · g^{-1}).
[b] Endogenous thiamin diphosphate.
[c] μmol · min^{-1} per l cell H_2O (X2 55 for nmol · g^{-1} · min^{-1}).

reaction was carried out at a high thiamin concentration of, for example, 10^{-5} M. In $2 \cdot 10^{-7}$ M or less, however, strains 70-23 and 70-23-102 had similar rates of uptake (V_{in}) with a lowered maximum uptake in 70-23-102 (Table I). The mechanism of this low maximum uptake of free thiamin in 70-23-102 (63 μmol per l cell H_2O vs. 270 μmol per l cell H_2O in the parent 70-23) was studied. First, an exit reaction of thiamin was characterized in the mutant and compared with that of the parent 70-23. The initial rate of exit (V_{out}) is shown in Table II, and the low rate of exit in this mutant seemed to be inadequate to counterbalance the rate of uptake in attaining the low steady-state level, because the parent 70-23 reached a much higher cellular level with similar values for uptake and exit (Table II). We looked for evidence showing a reduction of the initial uptake activity in highly preloaded cells (i.e., by a feedback inhibition). When the cells of 70-23-102 were preloaded with non-radioactive thiamin to a concentration close to that of steady state cells (approx 40 μmol per l cell H_2O), practically no reduction of uptake activity was observed with a slight and non-effective elevation (3–5-times) in K_m (Table III). In the case of thiamin diphosphate prepacking, a substantial decrease of V_{in} was observed in highly loaded cells (Table III) Hence there remained a possibility that the uptake reaction was counter-balanced by some other outflow mechanism of thiamin in 70-23-102 cells. This was proved to be the case when the efflux rate was measured in the presence of high concentration of extracellular thiamin. The rate of outward flow of preloaded [^{14}C]thiamin was markedly enhanced when the non-radioactive thiamin was added to the concentration of $2 \cdot 10^{-7}$ M (Fig. 1A).

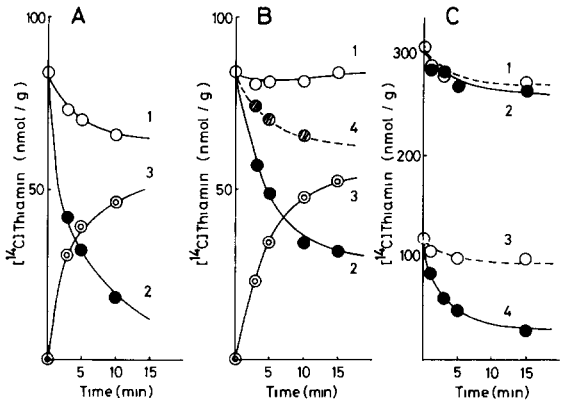

Fig. 1 Differentiation of C-efflux from exit and inactivity of cellular thiamin diphosphate in C-efflux A *E coli* W 70-23-102 cell was preloaded with [^{14}C]thiamin and the C-efflux and exit reactions were followed as in Materials and Methods 1, intracellular [^{14}C]thiamin in thiamin-free buffer; 2, intracellular [^{14}C]thiamin in 0 2 μM thiamin-containing buffer. The amount of net C-efflux (3) was calculated by subtracting the amount of 2 from that of 1 B Preloading of [^{14}C]-thiamin and the exit and C-efflux reactions were carried out as in Materials and Methods except for the addition of 2 10^{-2} M NaN_3 to both the reaction mixture of exit and C-efflux 1, cellular [^{14}C]thiamin in thiamin-free buffer, 2, cellular [^{14}C]thiamin in 0 2 μM thiamin-containing buffer, 3, net C-efflux calculated from 1 and 2, 4, the same reaction as 1, but NaN_3 was excluded C, Preloading of *E coli* W 70-23 and 70-23-102 cells was carried out for 60 min, but in the case of 70-23 a [^{14}C]thiamin concentration of 10^{-7} M and a reaction period of 4 min were adopted in order to limit the amount of accumulation 1, cellular [^{14}C]thiamin diphosphate in thiamin-free buffer, 2, cellular [^{14}C]thiamin diphosphate in 0 2 μM thiamin-containing buffer, in the cell of 70-23, 3, cellular [^{14}C]thiamin in thiamin-free buffer; 4, cellular [^{14}C]thiamin in 0 2 μM thiamin-containing buffer, in the cell of 70-23-102

In order to differentiate counterflow efflux from the exit, we defined the amount of outward flow of preloaded [^{14}C]thiamin, which was dependent on the presence of extracellular thiamin, as the counterflow efflux (C-efflux) Hence the amount of exit (which was observed without the addition of external thiamin) was subtracted from the total amount of outward flow of [^{14}C]thiamin in the extracellular media containing non-radioactive thiamin, to calculate the C-efflux (Fig. 1A, curve 3). During the course of C-efflux, 70-23-102 cells continued to take up extracellular non-radioactive thiamin to maintain the same level of total cellular thiamin This was ascertained by determining total cellular thiamin chemically by fluorometry (data not shown).

The exit process of preloaded [^{14}C]thiamin was inhibited nearly completely by $2 \cdot 10^{-2}$ M NaN_3 (Fig. 1B). $1 \cdot 10^{-2}$ M Na_2HAsO_4 in Tris-maleate buffer showed a similar inhibition against the exit (data not shown). However, the C-efflux was still active under those conditions, suggesting strongly independent routes for the two outflow processes. Those two processes of exit and C-efflux were different not only in sensitivity to metabolic inhibitors but also in dependency on external thiamin, on pH and on temperature (see below).

Another important difference between C-efflux and exit was the rate of outward flow of cellular thiamin diphosphate. As shown in Fig. 1C and Table II, the exit rates of free thiamin and thiamin diphosphate were nearly the same. The rate of C-efflux of thiamin diphosphate was, however, markedly low. The inactivity of thiamin diphosphate in the C-efflux reaction was measured using thiamin-kinase-positive parent 70-23 because this strain could convert cellular [^{14}C]thiamin to its diphosphate immediately and nearly quantitatively after incorporation. The same inactivity of thiamin diphosphate in the C-efflux seemed to be applicable also in the mutant 70-23-102, since this mutant could accumulate thiamin diphosphate precisely to the parent cell level of 276 μmol per l cell H_2O (Table III). These results seem to explain the mechanism by which a thiamin-kinase-positive cell can accumulate a huge amount of thiamin diphosphate. The results shown in Fig. 1C mean that intracellular thiamin diphosphate is not exchangeable with external thiamin. On the other hand, thiamin-thiamin exchange occurs without affecting, at least directly, the cellular thiamin level. However, the same initial rate of uptake was observed in both cases of the uptake reactions with and without accompanying C-efflux (Table III, thiamin-preloaded cell) (V_{in} with C-efflux was not small because only [^{14}C]thiamin uptake was measured, instead of the sum of [^{14}C]thiamin uptake and cold thiamin outflow). Accordingly, the effective v_{in} with the C-efflux was actually smaller than the rate of inflow of thiamin without C-efflux. Thus, the activity of C-efflux seemed to be the reason for the different levels of thiamin and its diphosphate in 70-23-102 cells under the experimental conditions. The same result suggested that the membrane carriers responsible for the C-efflux and the exit were different in reactivity to intracellular thiamin diphosphate.

Characteristics of counterflow efflux reaction. The initial rate of C-efflux thus obtained in the presence of a saturating concentration of external thiamin ($2 \cdot 10^{-7}$ M) was dependent sigmoidally on the concen-

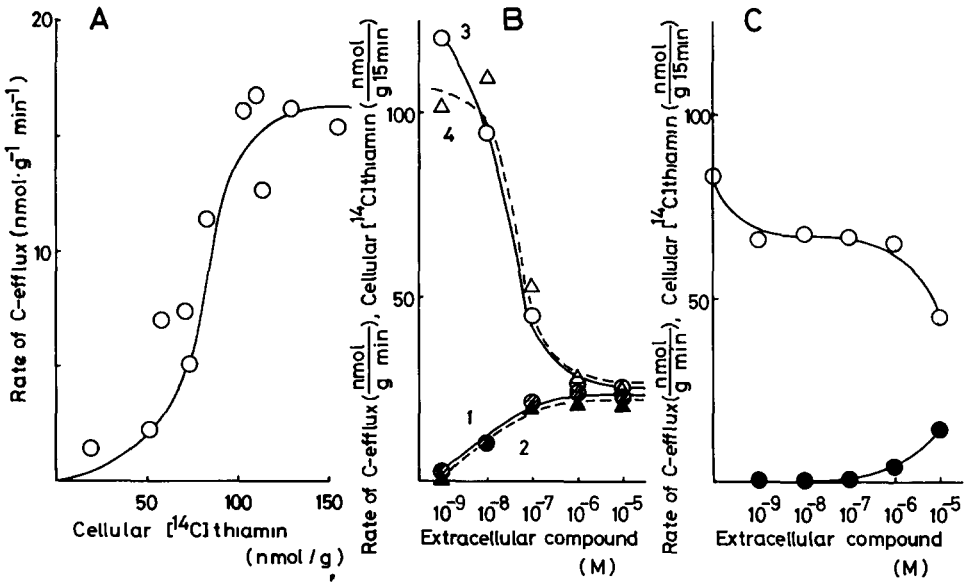

Fig. 2. Effect of thiamin concentration on C-efflux. A. 70-23-102 cells were preloaded with [^{14}C]thiamin to various concentrations by 3 10^{-8} M–$2 \cdot 10^{-6}$ M [^{14}C]thiamin for 30 s–30 min. The exit and C-efflux reactions were carried out for 2 min and the difference was plotted. B, C. The preloaded cell was prepared and contained 116 (B) or 103 (C) nmol · g^{-1} [^{14}C]thiamin. This preloaded cell was dispersed into each concentration of either non-radioactive thiamin (B, curve 1 and 3), thiamin diphosphate (B, curve 2 and 4) or pyrithiamin (C). After 2 min (for the rate measurement, closed or hatched circle or triangle, net C-efflus is plotted) or 15 min (for the extent measurement, open circle or open triangle, cellular [^{14}C] thiamin is plotted) of incubation, the cells were collected by filtration and washed as in Materials and Methods

tration of preloaded cellular [^{14}C]thiamin, as shown in Fig. 2A. The intracellular concentration for the half maximal velocity of C-efflux was calculated to be $2 \cdot 10^{-5}$ M. The exit rate of the same strain was also dependent sigmoidally on the cellular thiamin (Fig. 3C, see below) and nearly the same K_m was also obtained.

As we defined above, the C-efflux was dependent on the presence of external thiamin. The stimulatory effect of external thiamin saturated at approx. $2 \cdot 10^{-7}$ M (Fig. 2B) and the K_m value for half-maximal stimulation of C-efflux was calculated to be $2 \cdot 10^{-8}$ M, when measured by the cell preloaded with a saturating concentration (more than 100 nmol $\cdot$ g^{-1}) of intracellular [^{14}C]thiamin. When the external thiamin was replaced by its diphosphate, the rate of C-efflux was much the same (Fig. 2B). This K_m value for the C-efflux was 25-times higher than the K_m value for the uptake reaction (Table I).

Pyrithiamin, a structural analogue of thiamin, is known to inhibit approx 50% of the uptake activity in *E coli* K 12 when added in 100-times concentration [1]. In *E. coli* W 70-23-102, the inhibition of uptake was very weak; 20% inhibition by the addition of 300-times concentration [9]. The effect of pyrithiamin on C-efflux was similarly weak, approx. 200-times concentration of pyrithiamin gave a comparable rate of C-efflux (Fig. 2C).

The optimal temperature for C-efflux was observed at approx. 42°C (Fig 3A). The results of up-

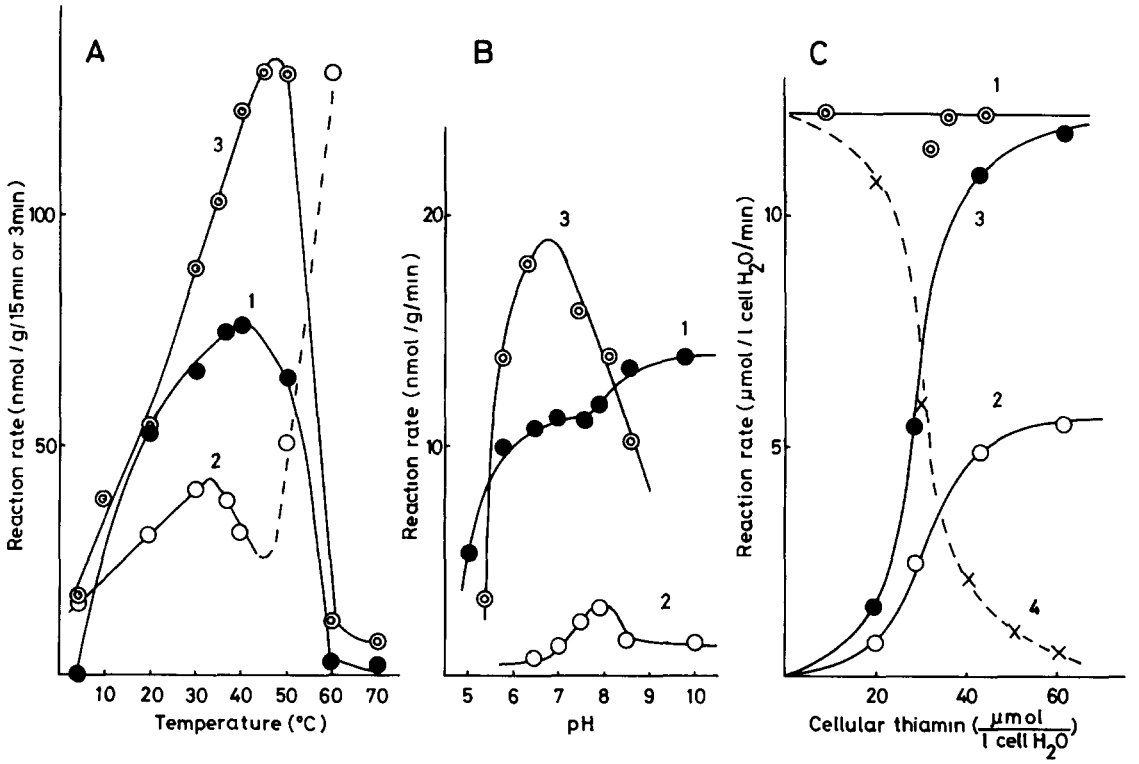

Fig. 3. Effect of temperature (A) and pH (B) on C-efflux and its possible physiological role in *E coli* W 70-23-102 (C). A The preloaded cell was prepared and contained 135 nmol $\cdot$ g^{-1} [^{14}C]thiamin. The procedures for exit and C-efflux were as described for 15 min at each temperature indicated. The uptake was assayed for 3 min. 1, net C-efflux, 2, exit, 3, uptake. B. 1 and 2. The preloading of cells was carried out as in Materials and Methods. The stock of preloaded cells was dispersed into prewarmed 0.1 M Tris-maleate buffer of each pH containing glucose with or without non-radioactive thiamin. The reaction mixture was incubated for 3 min or 10 min at 37°C to measure the C-efflux plus exit, or exit, respectively. 3 The uptake was assayed in 0.1 M Tris-maleate buffer of each pH containing $4 \cdot 10^{-6}$ M [^{14}C]thiamin and 0.4% glucose for 5 min at 37°C or 4°C 1, net C-efflux; 2; exit; 3, uptake C 1, V_{in} was re-plotted from Table III; 2, the rate of exit, obtained in the experiments of Fig. 2A, was plotted, 3, the sum of curve 2 and the rate of C-efflux in Fig 2A; 4, the difference between curve 1 and curve 3

take and exit are also plotted. The different optimal temperatures for the three reactions might imply different kinds of restricting factors for those reactions. The optimal pH values for uptake, exit and C-efflux in the strain 70-23-102 were pH 6.8, 8.0 and 10 or more alkaline, respectively, in Tris-maleate buffer (Fig. 3B). The observation of enhanced C-efflux in the alkaline region indicated that the C-efflux could not be the result of preventing recapture of [^{14}C]thiamin leaving from the preloaded cells in the exit rection. The same observation also indicated that the activity of C-efflux was being affected not only by the activity of the uptake carrier (in the sense that C-efflux was dependent on the extracellular thiamin), but also by some other factor(s) such as H^+ gradient across the transporting membrane.

A close relationship between uptake and net C-efflux remaining in the presence of $2 \cdot 10^{-2}$ M $NaN_3$was observed when the effect of inhibitors was studied. Both processes were inhibited by $1 \cdot 10^{-5}$ M FCCP (uptake, 51% inhibition; C-efflux, 51% inhibition), $1 \cdot 10^{-2}$ M arsenate in Tris buffer (uptake, 65%; C-efflux, 59%), and $2 \cdot 10^{-3}$ M DCCD (uptake, 74%; C-efflux, 30%). Thus C-efflux seemed to be an energy-dependent process. The exit reaction inhibited by $2 \cdot 10^{-2}$ M NaN_3 was activated significantly by CN^-, while C-efflux was rather indifferent to those inhibitors.

Discussion

The amount of C-efflux measured by the above method accompanied an inherent limitation; the specific radioactivity of cellular [^{14}C]thiamin declines along with the progress of C-efflux. Thus the apparent decline of C-efflux rate seen at a longer incubation period does not actually mean a lowered C-efflux rate. An appropriate correction for the dilution of radioactivity of cellular [^{14}C]thiamin should be made. Hence we focused our attention on the initial rate of C-efflux in this work.

The rate of C-efflux, together with that of exit, seemed to respond sigmoidally to the cellular thiamin concentration (Fig. 2A, 3C). The mechanism of these reactions is unknown so far, but a positive cooperativity in the postulated membrane carriers for those processes could be inferred. In this connection it would be of interest to point out that thiamin-binding protein exhibits a cooperativity kinetics between its binding sites [12].

Concerning the physiological significance of C-efflux in the growing conditions of *E. coli*, the role in the strain 70-23-102 was deduced as follows: V_{in} of cells containing various cellular concentrations of thiamin was found to be constant (Fig. 3C, curve 1). The sum of the rate of exit and the rate of C-efflux of cells containing various levels of thiamin gave a curve like curve 3, Fig. 3C. When the difference between curve 1 and curve 3 was plotted (curve 4, Fig. 3C), this final curve could explain not only the time course of free thiamin uptake in 70-23-102 [9] but also the maximum uptake of 63 μmol per molecule H_2O (Table I) fairly well. This fact seems to suggest that C-efflux might participate in determining cellular thiamin level at various concentrations in 70-23-102. The growth medium for this mutant contained $1 \cdot 10^{-8}$ M of thiamin diphosphate and this was also proved to be above the lowest limit for C-efflux (Fig. 2B). Thus, this mutant seems to be furnished with the mechanism to discard (by exchange) even a small amount of free thiamin, which cannot be used by this mutant. In the parent cell, where the incorporated thiamin is destined to be converted into its diphosphate, the rate of C-efflux was too low to be distinguished from the exit (Table II, Fig. 1C). In this case, a feedback inhibition for the uptake seems to be working together with the exit to attain the equilibrium (Table III).

The main reason for the absence of overshoot phenomenon, which has been observed in *E. coli* K 12 mutants [5], would be the relatively low rate of exit in strain 70-23-102 (Table II), but a lowered K_m for cellular thiamin of the C-efflux reaction in those K 12 mutants could also be possible.

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