Mechanism of Action of Showdomycin

Part IV Interactions between the Mechanisms for Transport of Showdomycin and of Various Nucleosides in *Escherichia coli*

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¹⁴C-Labelled showdomycin was rapidly taken up by *Escherichia coli* K-12 cells. The showdomycin uptake was highly temperature dependent, sensitive to azide and *N*-ethylmaleimide, but was only partially inhibited by treatment with high concentration of iodoacetic acid.

The uptake of showdomycin was inhibited by a wide variety of nucleosides but not by purine and pyrimidine bases, nucleotides, ribose or ribose-5-phosphate. The inhibition of showdomycin uptake by adenosine was of a competitive type.

Since nucleosides inhibited the uptake of showdomycin but did not facilitate its efflux, they must play a role of inhibitors to the entry of the antibiotic into cells.

Removal of extracellular showdomycin by washing, or inhibition of its subsequent entry into cells by the addition of nucleosides or sulfhydryl compounds resulted in a rapid decrease in the intracellular level of the antibiotic during subsequent incubation.

In a previous communication,¹⁾ it was shown that in a cell-free extract of *E. coli*, showdomycin (SHM) reversibly inhibited both ribonucleotide reductase system and dTMP synthetase system. Since the antibiotic preferentially inhibited both the *de novo* syntheses of deoxyribonucleotides and the DNA synthesis *in vivo*,²⁾ it seemed likely that the reversible inhibition of these enzyme systems would be a possible cause of these inhibitions *in vivo*. The inhibitory action of SHM *in vivo* was reversed not only by the addition of a sulfhydryl compound but also by that of a nucleoside,²⁾ whereas in the cell-free system the inhibitory action on these enzyme systems was reversed by a sulfhydryl

compound but not by a nucleoside.¹⁾ This suggests that the mechanism for the reversal of the SHM-inhibition by nucleosides differs from that by the sulfhydryl compound. To discern these mechanisms and to answer the question why nucleosides cannot reverse the inhibitory action of SHM in a cell-free system, the effect of nucleosides on the transport of SHM into *E. coli* K-12 cells was examined, using ¹⁴C-labelled SHM (SHM-¹⁴C). The results are presented here.

MATERIALS AND METHODS

Preparation of ¹⁴C-labelled SHM. It has been informed³⁾ that ¹⁴C can be incorporated into the maleimide moiety of SHM by using either acetate-l-

1) Y. Komatsu and K. Tanaka, *Agr. Biol. Chem.*, **35**, 526 (1971).

2) Y. Komatsu and K. Tanaka, *ibid.*, **34**, 891 (1970).

3) R.J. Suhadolnik and R.M. Ramer, Abstracts of Papers, 156th National Meeting Amer. Chem. Soc., Atlantic City, N.J., September, 1968, Abstract 26.

^{14}C or acetate-2- ^{14}C .

SHM- ^{14}C was obtained by the incubation of *Streptomyces showdoensis* with sodium acetate-2- ^{14}C as follows. *Streptomyces showdoensis* N₂-209-56⁴⁾ was maintained on Bennett's agar slant (1% D-glucose, 0.2% Bacto Yeast Extract, 0.1% Bacto Beef Extract, 0.2% Difco Casamino Acid and 1.5% agar). Bennett's medium

FIG. 1. Paper Chromatograms of Culture Broth, Butanol-extract and Eluate from Silicic Acid Column.

The sample was mixed with non-labelled SHM reference, applied to a strip of Whatman 3MM paper (2.5×40 cm) and chromatographed with solvent B (*n*-butanol-ethanol-water (50:15:35)) for 16 hr by the ascending method. A spot of the reference was located under a UV lamp, then the chromatograms were cut into pieces (0.5cm) and counted with a liquid scintillation spectrometer in toluene phosphor solution.

A, 10-hr culture broth; B, butanol-extract; C, eluate from silicic acid column.

was also used for the seed culture. The culture medium for the production of SHM was as follows: 2% D-glucose, 0.4% urea, 0.1% K_2HPO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% KCl, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and tap water (pH 5.5). One loop from an agar slant of the *Streptomyces* was inoculated to 15ml of Bennett's medium and incubated at 28°C on a reciprocal shaker; after 22.5hr, 2ml of this seed culture was added to a flask containing 50ml of the medium for production. A solution (0.4ml) of sodium acetate-2- ^{14}C (250 $\mu\text{Ci}/4.9\mu\text{moles/ml}$) was added to the flask just after the inoculation. Culture was continued for another 10hr. As shown in Fig. 1-A, the radioactive carbon from acetate-2- ^{14}C was fairly well incorporated into SHM, paper chromatographic analysis showing that approximately 14% of the total radioactivity of added acetate-2- ^{14}C was located at the same *R_f* value as a SHM reference. After removal of the mycelium by centrifugation, the supernatant fluid was lyophilized, then extracted three times with 15ml of methanol. The extracts were combined and evaporated to about one-tenth volume under reduced pressure. To the concentrated solution 15ml of *n*-butanol was added and the methanol was exhaustively evaporated off under reduced pressure. After removal of the precipitate by centrifugation, the solution containing SHM (Fig. 1-B) was added to a column of silicic acid, 1×10cm in benzene. The column was washed with 100ml of benzene-acetone (9:1), and then with 200ml of benzene-acetone (4:1). SHM was eluted with benzene-acetone (1:4). The fractions containing SHM- ^{14}C were combined and evaporated to dryness.

Paper chromatographic analysis showed that over 98.1% of the total radioactivity of this preparation was due to SHM (Fig. 1-C). Antimicrobial assay employing *E. coli* K-12 showed that approximately 522,906 cpm of the preparation corresponded to 1 μmole of SHM.

Measurement of the uptakes of SHM- ^{14}C and other labelled nucleosides by E. coli K-12 cells. The uptakes of SHM- ^{14}C and other labelled nucleosides were measured by the technique of rapid chilling and rapid Millipore filtration.⁵⁾ A cell suspension of *E. coli* K-12 was prepared as previously reported⁶⁾ and stored in an ice-bath. The suspension (1~2ml), prewarmed

5) R.N. Peterson and A.L. Koch, *Biochim. Biophys. Acta*, **126**, 129 (1966).

6) Y. Komatsu and K. Tanaka, *Agr. Biol. Chem.*, **32**, 1021 (1968).

4) M. Mayama, H. Nagata and K. Motokawa, Annual Report of Shionogi Research Laboratory, No. 18, 13 (1968).

for 6 min at a given temperature, was pipetted into tubes containing SHM- ^{14}C or a labelled nucleoside, with additions as stated in the legends to the tables and figures. After a given time, the reaction mixture was diluted 3-fold by the rapid addition of the ice-cold minimal medium. The contents were then immediately filtered through a Millipore filter (type HA, 0.45μ pore size). Tubes and filters were washed three times with the ice-cold medium and then the filters were transferred to counting vials. They were dried at 100°C for 30 min and then counted with a liquid scintillation spectrometer using toluene phosphor solution (5ml).

Chemicals and reagents. Purified iodoacetic acid was obtained from Dr. N. Yoshida; cytosine arabinoside was purchased from the Upjohn Company; pseudouridine was purchased from Calbiochem; *N*-ethylmaleimide was purchased from Nakarai Chemicals Ltd.; all the labelled nucleosides and sodium acetate-2- ^{14}C were purchased from Schwarz BioResearch Corp.; unlabelled purine and pyrimidine bases, nucleosides and nucleotides were purchased from Sigma Chemical Co., Boehringer Mannheim and Schwarz BioResearch Corp.

RESULTS

Effect of adenosine on the inhibition by SHM of the incorporation of uracil- ^{14}C into nucleic acids

To differentiate reversing effect of adenosine (Ar) on the inhibitory action of SHM from the reviving effect of the nucleoside on cells repressed by SHM, *E. coli* B-3 cells were preincubated with SHM and/or Ar, then rapidly collected on Millipore filters and resuspended in a medium of desired composition; the their activity for synthesizing nucleic acids was followed. The results are shown in Fig. 2. The cells pretreated with SHM in the presence of Ar retained essentially full activity for synthesizing nucleic acids during the subsequent incubation (curve b), whereas those pretreated with SHM alone had a markedly depressed activity (curve c). The subsequent addition of Ar did not reverse the inhibitory effect of the pretreatment with SHM (curve d) and also the pretreatment with the nucleoside

Fig. 2. Effect of Adenosine on the Inhibition by SHM of the Incorporation of Uracil- ^{14}C into Nucleic Acids in *E. coli* B-3 Cells.

Exponentially growing *E. coli* B-3 cells (O.D. at $660\text{m}\mu=0.07$) were preincubated with both or either of SHM ($10\mu\text{M}$ final concentration) and Ar ($200\mu\text{M}$) at 37°C for 12min. The cells were then rapidly collected on Millipore filters, resuspended in an equal volume of a medium containing $16\mu\text{M}$ uracil-2- ^{14}C ($1.25\mu\text{Ci}/\mu\text{mole}$) and either or neither SHM ($10\mu\text{M}$) or Ar ($200\mu\text{M}$), and subsequently incubated at 37°C . The incorporation of uracil-2- ^{14}C into nucleic acids was determined as follows: 1-ml aliquots was removed at intervals, an equal volume of ice-cold 10% trichloroacetic acid (TCA) was added, the precipitates formed were collected on Millipore filters and counted in the usual manner.

a, normal cells; b, cells preincubated with both SHM and Ar, and subsequently incubated in their absence; c, cells preincubated with SHM, and subsequently incubated in the absence of the antibiotic; d, cells preincubated with SHM, and subsequently incubated with Ar; e, cells preincubated with Ar, and subsequently incubated with SHM; f, cells preincubated with SHM, and subsequently incubated with the antibiotic.

did not reverse the inhibitory effect of the subsequent addition of the antibiotic (curve e). The complete repression of the incorporation of uracil into nucleic acids by the subsequent addition of SHM ruled out a possibility of any contamination with SHM-resistant cells (curve f). Thus, the inhibition by SHM could be reversed by the simultaneous addition of Ar with SHM but not by a single addition of the nucleoside before or after the treatment with SHM. This suggests that the reversal by Ar of the SHM-inhibition involves the mechanisms for uptake of SHM and the nucleoside by the cells.

Furthermore, removal of the extracellular SHM resulted in a significant revival of the incorporation of uracil into nucleic acids (curve c) and the addition of Ar showed no

effect on this revival (curve d). These observations suggest that the cells retain in themselves the ability to overcome the repression by SHM-pretreatment.

Rate of SHM- ^{14}C uptake into *E. coli* K-12 cells

Figure 3 shows the rate of uptake observed when the labelled antibiotic was incubated with exponentially growing *E. coli* cells. The rate of SHM- ^{14}C uptake was rapid at the beginning but became gradually slower. The initial rate of SHM uptake was calculated as 2.38×10^{-18} moles per cell during the first 1-min incubation with $10 \mu\text{M}$ SHM- ^{14}C in the medium at 37°C . This value was approximately twice that for uridine (1.21×10^{-18} moles/cell/min), deoxycytidine (1.41×10^{-18} moles/cell/min) and deoxyadenosine (0.99×10^{-18}

FIG. 3. Dependence of Total Uptake of SHM- ^{14}C into *E. coli* K-12 Cells with Time.

The cell suspension (7.52×10^8 cells/ml) was incubated at 37°C in the presence of $2.2 \mu\text{M}$ or $10 \mu\text{M}$ SHM- ^{14}C (522,906cpm/ μmole). 1-ml aliquots were removed after the indicated periods and prepared for counting as described in Materials and Methods.

○, $10 \mu\text{M}$ SHM- ^{14}C ; ●, $2.2 \mu\text{M}$ SHM- ^{14}C .

FIG. 4. Effect of Temperature on the Uptake of SHM- ^{14}C by *E. coli* K-12 Cells.

After the cell suspension (5.9×10^8 cells/ml in Exp. I and 7.52×10^8 cells/ml in Exp. II) had been allowed to equilibrate for 6 min at the desired temperature, 1-ml samples of the cell suspension were added to tubes containing 6,135 cpm of SHM- ^{14}C ($10 \mu\text{M}$ final concentration) and incubated at that temperature respectively for 4 min. The cells were collected on a Millipore filter, washed, and the total radioactivity of cells was determined. For further explanation see text.

moles/cell/min) and four times that of deoxyguanosine (0.58×10^{-18} moles/cell/min). The level of total uptake of SHM- ^{14}C by the cells reached a plateau of approximately 5×10^{-18} moles/cell. However, this level was lower (0.89×10^{-18} moles/cell) when incubation was carried out with a smaller amount of the antibiotic ($2.2 \mu\text{M}$).

As shown in Fig. 4, the SHM uptake mechanism was strongly dependent on temperature. This may be an indication that the process requires a high activation energy. Above 52°C the capacity to take up SHM- ^{14}C fell markedly. The presence of an active transport mechanism for SHM was supported by the observation that azide, a reagent inhibiting the production of ATP in cells, significantly inhibited the uptake of SHM- ^{14}C (Table I). At $10 \mu\text{M}$ SHM- ^{14}C , the rate of

TABLE I. EFFECT OF AZIDE ON THE UPTAKE OF SHM- ^{14}C BY *E. coli* K-12 CELLS

After the cell suspension (6.85×10^8 cells/ml) had been preincubated with the cited amount of azide at 37°C for 6 min, 1-ml aliquots were transferred to tubes containing 6135 cpm of SHM- ^{14}C ($10 \mu\text{M}$ final concentration) and incubated at 37°C for additional 4 min. The cells were collected on a Millipore filters, washed, and its total radioactivity was determined. For further explanation see text.

NaN_3 (mM)	Uptake of SHM- ^{14}C (cpm/ml cells)
0	1588
20	814
39	656
77	460

uptake was reduced to about a half and a third of the control value by 20 and 77mM azide, respectively. It appears, therefore, that SHM is at least partially, actively transported by *E. coli* K-12 cells. N-Ethylmaleimide (NEM) and iodoacetic acid (IA) also affected certain steps of the SHM uptake mechanism, as described in details later.

Specificity of SHM uptake in *E. coli* K-12 cells

To examine the possibility that a nucleoside

FIG. 5. Effect of Nucleosides on the Uptake of SHM- ^{14}C by *E. coli* K-12 Cells.

The cell suspensions ($5.3 \sim 7.4 \times 10^8$ cells/ml) were allowed to equilibrate at 37°C for 6 min, 1-ml samples of the cells were then transferred to incubation tubes containing 6,135 cpm of SHM- ^{14}C ($10 \mu\text{M}$ final concentration) and cited amounts of the nucleoside being tested. After a 4-min incubation, cells were washed and its total radioactivity was determined. The values are expressed as percent uptake of the untreated controls. For further explanation see text.

Abbreviation: Ar, adenosine; Adr, deoxyadenosine; Gr, guanosine; Gdr, deoxyguanosine; Hr, hypoxanthine riboside (inosine); Nr, xanthosine; Ur, uridine; Udr, deoxyuridine; Cr, cytidine; Cdr, deoxycytidine; Tr, thymine riboside; Tdr, deoxythymidine; ψUr , pseudouridine; BUdr, 5-bromodeoxyuridine; IUr, 5-iodouridine; IUdr, 5-iododeoxyuridine; CAra, cytosine arabinoside.

interferes with the uptake of SHM into cells, the effect of various nucleosides on the initial rate of SHM- ^{14}C uptake was determined. Figure 5 shows the results of experiments in which the initial rate of SHM- ^{14}C uptake was determined in the presence of various nucleo-

FIG. 6. Competitive Inhibition of the Uptake of SHM-¹⁴C by Ar.

Various concentrations of SHM-¹⁴C (522,906 cpm/ μ mole) were added to cells (6.1×10^8 cells/ml) in the presence (○) or absence (●) of 14.6 μ M Ar. After a 3-min incubation at 37°C, cells were washed and the total radioactivity of the cells was determined. For further explanation see text.

Fig. 7-A. Effect of NEM on the Uptakes of SHM-¹⁴C and Cdr-¹⁴C.

sides. Nucleosides were added at concentrations 0.85~547 times that of SHM-¹⁴C (10 μ M). The uptake of the SHM-¹⁴C preparation into the cells was diluted by the authentic SHM-¹²C.

It can be seen that Ar and deoxyadenosine (Adr) and a wide variety of pyrimidine nucleosides inhibited the uptake of SHM-¹⁴C by about, or more than, 90% at an inhibitor ratio of 17:1. Guanosine (Gr), deoxyguanosine (Gdr), xanthosine (Xr) and inosine (Hr) required higher concentrations (:20~60 times that of Ar) to reduce the rate of SHM-¹⁴C uptake appreciably. Pseudouridine (ψ Ur) was

FIG. 7-B. Effect of IA on the Uptakes of SHM-¹⁴C and Labeled Nucleosides.

After the cell suspensions ($6.85 \sim 7.52 \times 10^8$ cells/ml) had been allowed to equilibrate at 37°C for 6 min, 1-ml samples of the cells were transferred to incubation tubes containing 6,135 cpm of SHM-¹⁴C (10 μ M final concentration) 36,371 cpm deoxycytidine-2-¹⁴C (20 μ M), 4,265 cpm uridine-2-¹⁴C (10 μ M), 31,576 cpm deoxyadenosine-8-¹⁴C (20 μ M) or 32,009 cpm deoxyguanosine-8-¹⁴C (20 μ M), and cited amounts of NEM or IA, then, incubated at 37°C for a further 4 min. The cells were washed and its total radioactivity was determined. For further explanation see text.

found to inhibit the uptake at high concentrations (above 70 times that of Ar). Cytosine arabinoside (CAra) produced the least inhibition among nucleosides tested. The uptake of SHM- ^{14}C was not inhibited by any of natural purine and pyrimidine bases, nucleoside mono-, di- and triphosphates, ribose or ribose-5-phosphate. These results indicate that SHM is transported by the system which is unique for nucleosides and does not overlap with the mechanism for the transport of other types of compounds. The marked inhibition of SHM- ^{14}C uptake by adenine- or pyrimidine nucleosides may indicate the presence of a common factor in the mechanism by which SHM and these nucleosides enter the cells. On the other hand, the requirement of high concentrations of other nucleosides (Gr, Gdr, Xr, Hr, Ur or CAra) to inhibit the SHM uptake may indicate that they have reduced affinities for the SHM transport mechanism and/or that the mechanism of uptake of these nucleosides differ from that for SHM uptake.

It has been possible to discern further evidence for a common factor and also for specific differences in the mechanisms by which SHM and individual nucleosides enter the cells. From the Lineweaver-Burk plot in Fig. 6 it can be seen that the inhibition of the SHM- ^{14}C uptake by Ar is of a competitive type, suggesting that the two molecules share one common transport site.

The SHM-transport mechanism may not be identical with those of deoxycytidine (Cdr) and uridine (Ur) since each transport system showed a different sensitivity to the action of a thiol reagent NEM and to that of IA. This result is shown in Fig. 7, in which the rates of the uptakes of SHM- ^{14}C , Cdr- ^{14}C , Ur- ^{14}C , Adr- ^{14}C and Gdr- ^{14}C in the presence of increasing concentrations of NEM or of IA are compared. Low concentrations of NEM almost completely depressed the rate of SHM- ^{14}C uptake, while the rate of Cdr- ^{14}C uptake could only be decreased to 40% the maximal values even by an excess amount of NEM

(Fig. 7-A). On the contrary, IA dramatically depressed the rate of Cdr- ^{14}C uptake and that of Ur- ^{14}C uptake, while that of SHM- ^{14}C uptake could only be decreased to about 60% of the maximal values even by an excess amount of IA (Fig. 7-B). This result suggests that perhaps more than one kind of site mediates the transport of this antibiotic. The portion resistant to IA was also found in the transports of Adr and of Gdr.

Since IA-treatment almost completely destroys Cdr uptake with significant SHM uptake, the inhibitory effect of Cdr on the uptake of SHM- ^{14}C without any uptake of this nucleoside into cells is to be examined by using cells treated with IA. The results

FIG. 8. Effect of Cdr on the Uptake of SHM- ^{14}C in the Presence or Absence of IA.

Various concentrations Cdr and $10\mu\text{M}$ (final concentration) SHM- ^{14}C (522,906 cpm/ μmole) were added to 1 ml of the cells (7.52×10^8 cells/ml) in the presence (●—●) or absence (○—○) of 6.8mM IA. After a 4-min incubation at 37°C , cells were washed and its total radioactivity was determined. The values are expressed as percent uptake of the nucleoside-untreated controls in the presence (963 cpm/ml cells) and absence (1,736 cpm/ml cells) of IA, respectively. For further explanation see text.

showed that Cdr still retained an almost full activity for inhibition of the uptake of SHM (Fig. 8); moreover, a greater reduction was seen at low inhibitor ratios than in the case of normal cells. From these results it is concluded that Cdr may interact with SHM transport without translocation into the inside of cells.

Effect of sulfhydryl compounds on the uptake of SHM-¹⁴C by E. coli cells

Table II shows the results of experiments in which the initial rate of SHM-¹⁴C uptake by *E. coli* K-12 cells was determined in the presence of various sulfhydryl compounds. It can be seen that these compounds apparently inhibited the uptake of the antibiotic by cells. However, as clearly shown in Fig. 9, the antibiotic rapidly reacted with these

sulfhydryl compounds non-enzymatically. These results suggest that sulfhydryl-modified SHM compounds have reduced affinities for the SHM transport mechanism, resulting in the reduction in the rate of its uptake.

Efflux of SHM by E. coli cells

Figure 10-A shows the effect of the addition of Ar or mercaptoethanol (M-SH) on the pool of SHM-¹⁴C in *E. coli* K-12 cells. In less than 7 min the pool of radioactive material(s)

TABLE II. EFFECT OF SULFHYDRYL COMPOUNDS ON THE UPTAKE OF SHM-¹⁴C BY *E. coli* K-12 CELLS

In Experiment I, the cell suspension (1.76×10^9 cells/ml) was allowed to equilibrate at 37°C for 10 min, 1-ml samples of the cells were then transferred to incubation tubes containing 6,135 cpm SHM-¹⁴C ($10 \mu\text{M}$) with or without $400 \mu\text{M}$ (final concentration) of M-SH. After a 4-min incubation at 37°C, cells were washed and its total radioactivity was determined.

In Experiment II, the cell suspension (9.3×10^8 cells/ml) was allowed to equilibrate at 37°C for 6 min, 1-ml samples of the cells were then transferred to incubation tubes containing 6,135 cpm SHM-¹⁴C ($10 \mu\text{M}$) and $171 \mu\text{M}$ (final concentration) of L-cysteine, L-glutathione, or dithiothreitol. After a 4-min incubation at 37°C, cells were washed and its total radioactivity was determined. For further explanation see text.

Exp.	Sulfhydryl compounds (μM)	Uptake of SHM- ¹⁴ C (cpm/ml cells)
I	None	2239
	Mercaptoethanol (383)	33
II	None	2269
	Cysteine (171)	33
	Glutathione (171)	91
	Dithiothreitol (171)	20

FIG. 9. Paper Chromatograms of SHM-Derivatives.

Reaction tubes contained 6,135 cpm of SHM-¹⁴C ($11.7 \mu\text{M}$ final concentration) and, L-cysteine (Cys), L-glutathione (G-SH), or dithiothreitol (DTT) each $200 \mu\text{M}$ final concentration. Reaction was carried out at 5°C for 10 min in aqueous solution. The reaction mixtures and a reference SHM-¹⁴C solution (each $90 \mu\text{l}$) were applied on strips of Whatman 3MM paper ($1.2 \times 40 \text{cm}$), and chromatographed with solvent B (see the legend to Fig. 1) for 19hr by the ascending method. The chromatograms were cut into pieces and they were counted with a liquid scintillation spectrometer in toluene phosphor solution.

FIG. 10-A. Effect of M-SH and Ar on the Plateau Level of Accumulation of SHM- ^{14}C by *E. coli* K-12 Cells.

within cells reached a steady state. The level of this pool was rapidly reduced when Ar or M-SH was added to the suspending medium. The half-time of efflux was less than 3 min when a 34-fold excess of Ar or M-SH was added to the cells after the plateau level had been attained. However, it must be discerned whether these compounds inhibit the entry of SHM- ^{14}C into cells or whether they facilitate its efflux from cells. This result is shown in Fig. 10-B. *E. coli* K-12 cells were incubated with SHM- ^{14}C at 37°C for 10 min to be loaded with the labelled antibiotic, then rapidly washed on Millipore filters and resuspended in an equal volume of a minimal medium which contained no SHM. Efflux of SHM- ^{14}C was followed by counting the radioactivity of the cells which had been collected at intervals on Millipore filters. Radioactivity did leak from cells significantly during the subsequent incubation at 37°C in the medium containing no SHM. The subsequent addition of Ar and M-SH had no effect on this leakage. This is a direct demonstration of the ability of the cells to discharge SHM.

It seems reasonable to conclude that nucleosides and sulfhydryl compounds inhibit the

FIG. 10-B. Effect of M-SH and Ar on the Efflux of SHM- ^{14}C by *E. coli* K-12 Cells.

○ None, ● with M-SH, ▲ with Ar

A. 15ml of the cell suspension (5.6×10^8 cells/ml) was added to a tube containing $11.7\mu\text{M}$ (final concentration) SHM- ^{14}C (522,906 cpm/ μmole) and incubated at 37°C . At 7 and 8 min after the beginning of incubation, 4-ml aliquots of the cell suspension were transferred to a tube containing M-SH ($400\mu\text{M}$ final concentration) and to one containing Ar ($400\mu\text{M}$ final concentration), respectively. Separate aliquots (1ml) of the suspension were used to follow uptake over each time interval. The assays were performed as described in Materials and Methods.

B. The cell suspension (6.8×10^8 cells/ml) was allowed to equilibrate at 37°C for 6 min. 11 ml samples of the cells was incubated with $12.7\mu\text{M}$ (final concentration) SHM- ^{14}C (522,906 cpm/ μmole) at 37°C for 10 min. Cells were then washed on Millipore filters, resuspended in an equal volume of fresh medium and then subsequently incubated at 37°C . Efflux was determined as follows: 1-ml aliquots were removed at intervals, the cells were collected on Millipore filters and counted in the usual manner. 1-ml aliquots of the cell suspension were used to find the initial SHM level before the measurement of efflux.

entry of SHM, though independent mechanisms, but they do not affect its efflux from cells, resulting in the decrease in its intracellular concentration.

Irreversible binding of SHM to the cellular macromolecule(s)

Although most of the uptake of SHM- ^{14}C by *E. coli* cells from the medium was reversible (Fig. 10-A and B), a significant amount of the intracellular SHM- ^{14}C was incorporated into the cellular acid-insoluble fraction (Table III). In addition, loss of the radioactivity

TABLE III. INCORPORATION OF SHM- ^{14}C INTO ACID-INSOLUBLE COMPONENTS OF *E. coli* K-12 CELLS AT 37°C

The cell suspension (8.5×10^8 cells/ml) was incubated with $13.4 \mu\text{M}$ SHM- ^{14}C (522,906 cpm/ μmole), in the presence or absence of inhibitor (M-SH or Ar), at 37°C for 10 min. The incorporation of SHM- ^{14}C into cold TCA-insoluble components of the cells was determined as follows; an equal volume of ice-cold 10% TCA solution was added to the cell suspensions, the precipitates were collected on Millipore filters, and counted in the usual manner. The incorporation of SHM- ^{14}C into hot TCA-insoluble components of cells was determined as follows; the cold TCA-insoluble materials in 5% TCA solution were heated at 90°C for 30 min, and the precipitates were collected on Millipore filters and counted. For further explanation see text.

Fraction	Addition	None	M-SH (cpm/ml cells)	Ar
Whole cells		2239	33	131
Cold 5% TCA-Insoluble		626	14	34
Hot 5% TCA-Insoluble		569	14	36

incorporated into the cellular macromolecule on the subsequent addition of Ar or M-SH was insignificant (Fig. 11). It may be reasonable to assume that SHM is not incorporated into the cellular nucleic acids since only insignificant amounts of the radioactivity was released from the cellular acid-insoluble fraction on heating at 90°C in 5% trichloroacetic acid (Table III).

DISCUSSION

Since the SHM uptake mechanism was highly temperature dependent (Fig. 4), and was sensitive to azide (Table I), this antibiotic

FIG. 11. Effect of M-SH and Ar on the Incorporation of SHM- ^{14}C into the Hot TCA-Insoluble Fraction.

The cold cell suspension (6.8×10^8 cells/ml) was allowed to equilibrate at 37°C for 6 min, 5-ml samples of the cells were then transferred to an incubation tube containing 36,708 cpm of SHM- ^{14}C ($14 \mu\text{M}$ final concentration) and incubated at 37°C. At 11 and 12 min after the beginning of incubation, 1-ml aliquots of the cell suspension were transferred to a tube containing Ar ($384 \mu\text{M}$ final concentration) and to one containing M-SH ($392 \mu\text{M}$ final concentration), respectively. The assay conditions were the same as those indicated in Table III.

may be transported through the cell membrane in a process dependent upon the metabolic energy.

The uptake of SHM was inhibited by a wide variety of nucleosides (Fig. 5) but not by purine and pyrimidine bases, nucleotides, ribose and ribose-5-phosphate. This suggests that the transport system of SHM is unique for nucleosides and does not overlap with mechanisms for transport of other types of compound.

The inhibition of the SHM uptake by Ar was of competitive type (Fig. 6), suggesting that the two molecules may share one common transport site. Although it has not yet been examined whether the SHM uptake is inhibited by other nucleosides in a purely competitive manner, in general, their capacity to

inhibit the initial rate of SHM uptake seems to be evidence for either the affinity of the test compounds for SHM transport sites or their binding to a site(s) close to but different from the transport site. The uptake of SHM was largely inhibited by both the ribosides and 2'-deoxyribosides of adenine and of a wide variety of pyrimidine bases. This suggests that substitution of 2'-hydroxyl group of the ribose moiety for hydrogen in the nucleosides is fairly well tolerated by the transport site. However, inversion of that group is not tolerated by the site, since cytosine arabinoside (the epimer of cytidine) did not inhibit the uptake of SHM.

Gr, Gdr, Xr and Hr were all less inhibitory to SHM uptake. This suggests that, although the nature of the aglycone moiety in nucleosides is generally not particularly critical for the binding to the SHM transport system, one exception is the minor tolerance for the substitution of 6-amino group for hydroxyl group in the adenine moiety. The site is tolerant to a bulky group at the position 5 in the uracil moiety, since 5-bromodeoxyuridine, 5-iodouridine and 5-iododeoxyuridine inhibited the SHM uptake quite strongly.

Although SHM bears a similar structural relationship to ψ Ur and can even be viewed as ψ Ur which has lost an—NH group in the contraction to a five-membered ring,⁷⁾ ψ Ur was less inhibitory for the SHM uptake. This forms a striking contrast to the fact that Ur bearing also a similar structural relationship to SHM⁷⁾ markedly inhibited the uptake of the antibiotic.

Since Cdr was a potent inhibitor of the uptake of SHM even when the uptake of the nucleoside had been repressed completely by the addition of IA (Fig. 8), the inhibition by the nucleoside of the uptake of SHM must occur at an outer site of the cell membrane. The site should be resistant to IA, but may

be sensitive to NEM⁸⁾ (Fig. 7-A). IA may affect rather an inner site of the cell membrane where the translocation of Cdr is involved. The mechanism of SHM uptake may involve at least two kinds of transport sites, one labile to IA, the other stable to the reagent (Fig. 7-B).

Since Ar inhibited the uptake of SHM into cells fairly strongly but did not facilitate its efflux from cells (Fig. 5 and Fig. 10-B), the nucleoside must play a role of an inhibitor to the entry of the antibiotic into cells. A wide variety of sulfhydryl compounds also inhibit the entry of the antibiotic, though by a different mechanism from that of nucleosides (Table II and Fig. 10-B). An intact stereospecific structure at the maleimide moiety of SHM may be required for the transport of SHM because a variety of sulfhydryl-modified SHM compounds were all inert in the transport system (Table II and Fig. 9). This may be a reason why sulfhydryl compounds can induce an effect similar to that of nucleosides on the entry of SHM though they have no similarity in structure with nucleosides. These inhibitory effect on the entry of SHM into cells may be a major source of the reversal of the SHM-inhibition by nucleosides and sulfhydryl compounds.

Removal of the extracellular SHM by washing or inhibition of subsequent entry of SHM by the addition of nucleosides or sulfhydryl compounds resulted in a rapid decrease in the intracellular level of the antibiotic during the subsequent incubation (Fig. 10-A and B). Since the reaction with SHM and its targets may be essentially reversible,^{1,9)} the decrease in the concentration of SHM may induce the consequent dissociation of the antibiotic from the targets, resulting in the revival of the targets.

The reversal of the SHM-inhibition and the

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recovery of the cell from the SHM-repression by nucleosides could not be explained by the results obtained in experiments with cell-free systems.¹¹ The data on the transport of SHM obtained in this paper, now make it possible to elucidate these mechanisms. The mechanism of action seems to be most logically explained by the assumption that the antibiotic behaves as a nucleoside analogue at entry through the cell membrane, but subsequently behaves as a derivative of maleimide.

It is of interest to note that a minor but

significant amount of SHM has been shown to become a stably bound part of cellular proteins (or other macromolecules), however the antibiotic may not be incorporated into nucleic acids.

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