INHIBITION OF AMINO ACID AND SUGAR TRANSPORT BY SHOWDOMYCIN*

S. Roy-Burman, Y. H. Huang and D. W. Visser

Department of Biochemistry, School of Medicine
University of Southern California, Los Angeles, California 90033

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SUMMARY. Showdomycin inhibits the uptake of sugars and amino acids in Escherichia coli B cells. The data show that inhibition of transport by the alkylating action of showdomycin is a primary explanation for its inhibitory effects on growth of E. coli. The inhibitory effects are reversed completely by preincubation with cysteine or common nucleosides excepting guanosine, deoxyguanosine and pseudouridine. Uridine produces a two- to three-fold increase of 2-deoxyglucose and α-methyl-D-glucoside transport into E. coli cells. Analysis of products accumulated from 2-deoxyglucose shows a greater increase of free sugar than the sugar phosphate.

INTRODUCTION. The diverse effects of the nucleoside antibiotic, showdomycin (SHM), on biological systems may be related directly to the alkylating property of its maleimide structure which is known to react specifically with sulfhydryl groups (1,2,3). The biological activities of SHM include: its radiosensitization of Escherichia coli (4), its induction of volume changes in mitochondria (5), its inhibitory action on protein synthesis, nucleic acid synthesis and growth in E. coli (6,7,8), and its selective inhibitory effect on certain enzymes (3). Unlike most nucleoside analogs, it is likely that SHM exerts its inhibitory effects on growth of bacteria and tumors (9,10) without prior conversion to the nucleotide level because it is not a substrate for nucleoside kinase or nucleoside phosphorylase in cell-free preparations of Ehrlich ascites cells (3).

An interesting finding which relates the nucleoside structure of SHM to its biological activity has been reported by Nishimura and Komatsu (7), who found that the growth-inhibiting activity of SHM on E. coli is reversed specifically by most nucleosides, whereas purines, pyrimidines, ribose or deoxyribose are inactive as reversing agents. This finding, in context with the observation that transport of certain sugars (11) and amino acids (12) are inhibited by the alkylating agent, N-ethylmaleimide (NEM), prompted this investigation of the effects

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of SHM on sugar and amino acid uptake in whole cells. The results show that inhibition of transport mechanisms by SHM in E. coli occurs at concentration levels of SHM which are similar to those which inhibit growth, and that nucleosides have the unique ability to prevent inhibition of transport by SHM.

EXPERIMENTAL PROCEDURES. Materials: Showdomycin was obtained as a gift from Dr. Haruo Nishimura of Shionogi Research Laboratory, Fukushima-Ku, Osaka, Japan. Uniformly ^{14}C-labeled glucose, mannose and α-methyl-D-glucoside were purchased from Calbiochem. 2-Deoxyglucose-1-^{14}C was obtained from New England Nuclear Corp.

Bacteria: E. coli B (Hill) cells were grown in minimal medium (13) for 18 hrs at 37°, harvested by centrifugation, and the sedimented cells were washed twice with ice-cold medium A.* The washed cells were suspended in medium A to give 3 optical density units per ml at 660 mμ. Aliquots from this suspension were used for uptake studies. All experiments were carried out with fresh cells within 2 hours from the time of harvest.

Assay for uptake: Unless otherwise noted, the standard reaction mixture (1 ml) contained 0.2 mM substrate and E. coli B cell suspension (equivalent to 0.4 - 0.5 mg dry weight) in medium A. The substrate was ^{14}C-glucose (1.6 X 10^4 cpm/μmole), ^{14}C-mannose (7.6 X 10^3 cpm/μmole), ^{14}C-α-methyl-D-glucoside (2.3 X 10^4 cpm/μmole), or ^{14}C-2-deoxyglucose (1.3 X 10^5 cpm/μmole). After incubation at 37° in the presence of the radioactive sugar for 1 to 10 min, the reaction mixture was chilled, diluted immediately with 5 ml of ice-cold medium A and filtered through a Millipore filter (0.45-μ pore size). The filters were washed twice with 5 ml portions of ice-cold medium A, dried, glued to planchets and counted on a gas-flow counter. A zero time control was included for each set of experiments where the radioactive substrate was added to the ice-cold reaction mixture and immediately diluted, filtered and washed. The zero time control, usually 30 to 60 cpm, was subtracted to obtain the data presented in the tables.

The standard reaction mixture (1.0 ml) for leucine uptake contained 0.04 mM leucine (5 X 10^6 cpm/μmole) and E. coli B cell suspension (equivalent to 0.4 to 0.5 mg dry weight) in medium A. Prior to addition of leucine the reaction mixtures were preincubated with 200 μg of chloramphenicol for 20 min at 37°. After addition of leucine the incubations were carried out for 1 min at

* Minimal medium described by Davis and Mingioli (13) without glucose.

37°, chilled, diluted immediately with 3 ml ice-cold medium A and filtered through Millipore filters (0.45-μ pore size). The filters were washed four times with 4 ml portions of ice-cold medium A. The zero-time controls and assay procedures were the same as described for sugar uptake.

Assay of accumulated products in E. coli B cells: The reaction mixtures (20 ml) were incubated with the ^{14}C-labeled sugar under the standard assay conditions used for uptake studies. After 1 min, the reaction mixtures were chilled and 30 ml ice-cold medium A was added and centrifuged. The sediment was washed twice with 50 ml medium A at 0° C. Cells were extracted according to the method described by Anraku (12), and the extract analyzed by descending paper chromatography on Whatman No. 3 MM paper in a solvent system containing butanol : acetic acid : water (2:1:1; v/v/v). This system separates completely the respective free sugars from their phosphorylated derivatives.

Table 1: Effect of SHM on the uptake of sugars in E. Coli B in the presence and absence of uridine.

Addition (mM)	Incubation (min)	Sugar uptake (%)			
		Glucose	Mannose	α-Methyl-D-glucoside	2-Deoxy-glucose
None	10	100(126)*	100(123)*	100 (25)*	100(7.6)*
SHM (0.02)	10	19	18	68	54
SHM (0.1)	10	1	2	37	8
Uridine (0.5); SHM (0.02)**	10	94	108	258	300
Uridine (0.5)	10	95	-	303	298
None	1	100 (27)*	100 (19)*	-	-
SHM (0.1)	1	0	0	-	-

The standard assay procedure was used, except that the reaction mixtures were preincubated with the indicated amounts of SHM or uridine for 10 min at 37° prior to addition of labeled sugar.

* Values in the parentheses represent mμmoles of substrate incorporated.

** The reaction mixture was preincubated for 10 min at 37° with uridine; SHM was added and the preincubation continued for an additional 10 min.

\- Not determined.

RESULTS. The uptake of glucose or mannose by E. coli B whole cells is inhibited more than 80% in the presence of 0.02 mM SHM and both sugars are inhibited completely at 0.1 mM antibiotic (Table 1). Deoxyglucose, α-methyl-D-glucoside and leucine transport are less sensitive to inhibition by SHM (Tables 1 and 2). These effects are not due to lysis of the cells by SHM, since about 90% of the cells remain viable after preincubation with 0.1 mM SHM for 10 min.

Preincubation of cells with uridine prior to addition of SHM prevents complevely the inhibitory effects (Table 1). The protective effect of uridine on glucose and mannose uptake is involved directly in the transport process, since

Table 2: Effect of nucleosides on the inhibitory action of SHM on glucose, α-methyl-D-glucoside and leucine uptake.

Addition (mM)	Glucose uptake(%)		α-Methyl-D-glucoside uptake (%)		Leucine uptake(%)	
	SHM added		SHM added		SHM added	
	None	(0.02 mM)	None	(0.02 mM)	None	(0.06 mM)
None	100(102)*	10	100(25)*	68	100(0.27)*	38
Uridine (0.5) * *	106	102	290	273	416	404
Cytidine (1.0)* *	104	98	-	-	370	416
Deoxythymidine (1.0)	102	118	-	-		-
Adenosine (1.0)* *	100	100	-	-	264	271
5-Hydroxyuridine (2.0)	100	98	-	-	307	288
5-Hydroxydeoxy-uridine (2.0)	110	100	-	-	300	-
Guanosine (1.0)	103	11	188	55	200	48
Deoxyguanosine (1.0)	100	8	-	-	-	-
Pseudouridine (1.0)	94	10	105	60	99	31

Uptake was measured after 10 min incubation at 37° under standard assay conditions, except that prior to the addition of ^{14}C-glucose or ^{14}C-α-methyl-D-glucoside, the reaction mixtures were preincubated for 10 min at 37° with ribonucleosides or deoxyribonucleosides at the concentrations indicated, then SHM (0.02 mM) was added and the preincubation was continued for an additional 10 min.

The reaction mixture for leucine uptake was preincubated with the indicated amount of nucleosides and SHM for 10 min at 37°, prior to the addition of ^{14}C-leucine. Uptake was measured after 1 min incubation at 37° under standard assay conditions described in the text.

* Values in the parentheses represent mμmoles sugar incorporated.

* * Corresponding deoxyribonucleosides gave similar results.

\- Not determined.

uridine alone does not alter uptake of either sugar (Table 1). In contrast, the uptake of leucine, 2-deoxyglucose and α-methyl-D-glucoside is increased two- to three-fold by preincubation in the presence of uridine and SHM does not depress these increased levels of uptake (Tables 1 and 2).

Other common nucleosides excepting guanosine, deoxygunosine and pseudouridine also reverse the inhibitory effect of SHM on glucose transport (Table 2). Purines, pyrimidines, or the 5'-phosphate derivatives of the nucleosides are not effective, whereas the nucleoside analogs, 5-hydroxyuridine and 5-hydroxydeoxyuridine, are as effective as uridine. The inhibitory effect produced by SHM on the uptake of α-methyl-D-glucoside and leucine is prevented by preincubation with the same nucleosides which produce stimulation, guanosine and deoxyguanosine being the exception. These two nucleosides stimulate transport but do not protect against the inhibitory effect of the antibiotic. Pseudouridine neither stimulates uptake nor protects against the inhibitory effect of SHM.

A comparison of the inhibitory effect of SHM and NEM on glucose transport and the influence of uridine and cysteine on these inhibitions are shown in Table 3. Inhibition of glucose uptake by SHM or NEM occurs at similar levels, and inhibition is almost complete at 0.1 mM concentration of either. The inhibitory effect of 0.02 mM SHM is more pronounced when the cells are preincubated with SHM prior to addition of glucose. For example, inhibition does not occur when 0.02 mM SHM and glucose are added simultaneously, whereas preincubation with the same amount of SHM produces 90% inhibition. The inhibitory effect of SHM or NEM on glucose uptake is protected completely by preincubation in the presence of cysteine, whereas the inhibitory effect of SHM, but not that of NEM, is protected completely by preincubation with uridine. Unlike cysteine, uridine is less effective when it is added simultaneously with SHM. The inhibitory effect of SHM on transport is irreversible as indicated by the failure of cysteine or uridine to reverse inhibition of transport after the cells are preincubated with SHM (Table 3).

DISCUSSION. The data provide good evidence that inhibition of transport mechanisms by SHM may be the primary explanation for its inhibitory effects on growth and protein and nucleic acid synthesis in *E. coli* (6,7). Firstly, the concentration levels which cause inhibition of growth (7) and nucleic acid synthesis (6) are comparable to those which inhibit transport mechanisms

Table 3: Comparative effects of SHM and NEM on glucose uptake.

Addition (mM)		Glucose uptake (%)
Before preincubation	After preincubation	
None	None	100
SHM (0.02)	None	10
None	SHM (0.02)	100
SHM (0.1)	None	0
Uridine (1.0)	SHM (0.1)*	93
Cysteine (0.5)	SHM (0.1)*	90
None	SHM (0.1)	23
Uridine (0.2)	SHM (0.1)	109
Uridine (0.2); SHM (0.1)	None	7
SHM (0.1)	Uridine (0.2)	5
Cysteine (0.5)	SHM (0.1)	90
Cysteine (0.2); SHM (0.1)	None	100
SHM (0.1)	Cysteine (0.2)	5
NEM (0.1)	None	0
Uridine (1.0)	NEM (0.1)*	1
Cysteine (0.5)	NEM (0.1)*	90
None	NEM (0.1)	18
Uridine (1.0)	NEM (0.1)	5
NEM (0.1); cysteine (0.2)	None	100

Glucose uptake was measured after 10 min incubation at 37° under standard assay conditions except that prior to the addition of ^{14}C-glucose all reaction mixtures were preincubated for 10 min with or without SHM, NEM, Urd or cysteine, as indicated in left-hand column. Compounds indicated in right-hand column were added at the same time as ^{14}C-glucose.

* After preincubation with uridine or cysteine, SHM or NEM was added and preincubation was continued for another 10 min prior to the addition of ^{14}C-glucose.

(Tables 1 and 2). Secondly, nucleosides which are effective for reversing SHM-induced inhibition of transport (Table 2) are those which also restore growth. Thirdly, pseudouridine, guanosine or deoxyguanosine, which do not protect against inhibition of glucose or amino acid uptake, are also uniquely ineffective for abolishing the inhibitory effect of SHM on growth of E. coli B. Our observations regarding the inability of guanosine and deoxyguanosine to reverse the inhibitory effect of SHM on E. coli B growth differ from those of Nishimura and Komatsu (7), who found that nucleosides, including guanosine and

Table 4: Influence of SHM and uridine on the nature of cellular products formed from α-methyl-D-glucoside and deoxyglucose.

Substrate	Addition (mM)	Radioactivity accumulated (%)			
		Total	Free sugar	Phosphorylated derivative	Other
α-Methyl-D-glucoside	None	100 (240)*	32.4	67.2	<1
α-Methyl-D-glucoside	SHM (0.1)	100 (98)*	34.1	65.8	<1
2-Deoxyglucose	None	100 (98)*	31.0	66.8	2.2
2-Deoxyglucose	Uridine (0.5)	100 (170)*	44.5	51.2	4.3

The reaction mixtures (20 ml) were incubated for 1 min at 37° under standard assay conditions for sugar uptake. Prior to the addition of radioactive sugar the mixtures were preincubated for 10 min at 37° with SHM or uridine at concentrations indicated. Other conditions of the reaction mixture and the details of analysis of accumulated products are as described in the text.

* Values in the parentheses represent mμmoles of total sugar extracted from the cells.

deoxyguanosine, reversed the inhibitory effect of SHM on growth of E. coli Umezawa. Finally, the transport of glucose and amino acids is intimately associated with membrane proteins (14,15) and the membranes are the first to be exposed to this antibiotic.

Since thiol compounds protect against the inhibitory effects of SHM (6,7, Table 3), it may be concluded that SHM acts primarily as an alkylating agent of enzymes or membrane proteins which are associated with transport in E. coli. Thus, the mechanism of action of SHM may be considered to be similar to that of NEM, which is known to inhibit transport of sugars by alkylation of sulfhydryl groups of membrane proteins (12).

An interesting difference between the actions of NEM and SHM is apparent in the ineffectiveness of nucleosides to reverse the inhibitory effects of NEM on glucose uptake (Table 3), whereas most nucleosides overcome completely the inhibitory effect of SHM. This protective action of nucleosides on SHM inhibition appears to be distinct from that provided by cysteine. The action of cysteine can be explained by its chemical interaction with SHM (3), whereas the common nucleosides do not react with SHM.

Komatsu and Tanaka (6) suggested that the N-glycosyl linkage may be a structural requirement for the protective effect of SHM inhibition by nucleo-

sides. However, the inability of guanosine and deoxyguanosine to protect inhibition of growth and transport by SHM indicates that the structure of the purine or pyrimidine moiety may also be important. Evidence that the protective action of nucleosides on SHM inhibition is exerted by the unchanged nucleosides and not by their phosphorylated derivatives is apparent from the protective effect of 5-hydroxydeoxyuridine which is not phosphorylated by E. coli B cells (16) and by the ineffectiveness of purines, pyrimidines, ribose or deoxyribose. Furthermore, it is unlikely that ribose-5-phosphate or deoxyribose-5-phosphate, resulting from phosphorolysis of the protective nucleoside, is involved in reversing inhibition of glucose uptake because guanosine and deoxyguanosine are non-protective and at the same time are susceptible to nucleoside phosphorylase action (17). Thus, it is reasonable to conclude that the intact nucleoside interacts at sites on the cell surface in some manner which prevents the structurally related nucleoside antibiotic from reacting with susceptible sulfhydryl groups.

When cells are incubated in the absence of SHM with ^{14}C-labeled α-methyl-D-glucoside or deoxyglucose for 1 min and then analyzed for accumulated radioactive products, one-third of the total radioactivity is present as free sugar, and the remainder as the 6-phosphate derivative (Table 4). SHM does not change this pattern of accumulated products, suggesting that the antibiotic interferes specifically with the transport of these sugars rather than with reactions occurring subsequent to transport.

It has been suggested that glucose and α-methyl-D-glucoside are transported mainly via the phosphoenolpyruvate-phosphotransferase system in which sugars are phosphorylated at the 6-position in E. coli and released into the cell as such (14,15,18). Uridine causes a two- to three-fold increase of deoxyglucose and α-methyl-D-glucoside transport. At these conditions, analysis of accumulated products from deoxyglucose shows a greater increase of free sugar than the sugar phosphate. These results suggest that nucleosides may activate the transport, or release, of free deoxyglucose. Alternatively, the release of pentose phosphates by nucleoside phosphorylases may provide substrates, energy sources or activators of transport mechanisms, thereby changing the pattern of accumulated products. The inability of pseudouridine

the molecular weight range of reovirus proteins is 40,000-150,000.

Lodish has reported that mild HCHO treatment of bacteriophage f2-RNA considerably enhanced specific initiation and translation of f2-RNA cistrons in E. coli extracts owing to a partial disruption of the secondary structure (7). In the experiments described here, a similar stimulation of polypeptide synthesis was observed after treatment of the reovirus ssRNA with HCHO. This suggests that secondary structure in the reovirus ssRNA may exert a regulatory function in translation.

In the experiments described here the optimal Mg^{++} concentration was 11 mM with either 80S or 60S and 40S ribosomal subunits. Other investigators have demonstrated a requirement for ribosomal factors in the initiation of hemoglobin chains in rabbit reticulocyte cell-free systems at lower Mg^{++} concentrations (9, 10). Preliminary experiments in our own laboratory suggest that at 5 mM Mg^{++}, reovirus ssRNA-directed polypeptide synthesis is stimulated by the addition of a 0.5 M KCl wash of crude ribosomes (9, 10). Further work is underway and will be reported elsewhere.

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