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EFFECTS OF PHLORETIN AND SYNTHETIC ESTROGENS ON β-GALACTOSIDE TRANSPORT IN *ESCHERICHIA COLI*

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SUMMARY

1. The effects of phloretin, phloridzin, diethylstilbestrol and hexestrol on facilitated diffusion of β-galactosides across *Escherichia coli* cells were compared to previously observed effects on monosaccharide transfer across human erythrocytes. These diphenolic agents inhibit β-galactoside transport. Phloretin inhibits noncompetitively and K_i values indicate relative specificity for galactoside as compared to α-methylglucoside or L-leucine transport. The inhibition is completely reversed by washing after treatment up to 30 min, but irreversible increases in cell permeability follow prolonged treatment. The pH influences phloretin but not diethylstilbestrol inhibition in the range 5.8–7.4.

2. Phloretin and diethylstilbestrol protect the galactoside transport mechanism from inhibition by *N*-ethylmaleimide. When *E. coli* are treated with metabolic inhibitors the effects of phloretin and diethylstilbestrol on inhibition of galactoside transfer and on protection of the essential sulfhydryl group of the transport mechanism from *N*-ethylmaleimide are markedly increased. Metabolic inhibitors also increase cellular uptake of [^{14}C]diethylstilbestrol. From studies with [^{14}C]diethylstilbestrol an upper limit of approx. 55000 β-galactoside carrier sites per cell was calculated. Concentrations of the diphenolic agents in the range $5 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ M stimulate galactoside transport.

3. The evidence suggests that the bacterial facilitated diffusion is a useful model for similar sugar transfer in human erythrocytes and other cells.

INTRODUCTION

Hexoses are transferred across human erythrocytes by facilitated diffusion, a mechanism believed to involve mobile membrane carriers[1–3]. Similar carriers probably mediate the insulin-sensitive facilitated diffusion of hexoses across mammalian adipose cells[4] and skeletal muscle membranes[5]. Despite extensive study and flux measurements with these cells, the biochemical nature of the carriers remains unknown. In contrast, β-galactoside transport in *Escherichia coli* involves a specific membrane component which has been at least partially characterized. The transport is dependent on a functioning *y* gene in the lactose operon[6], and the protein product of this gene,

presumably the membrane carrier or a portion thereof, has been identified and partially purified by FOX AND KENNEDY[7]. In the presence of the specific membrane protein β-galactosides are actively accumulated by respiring cells[6] or transferred via facilitated diffusion across cells treated with metabolic inhibitors[8–11].

The present studies were undertaken to evaluate the usefulness of the bacterial transport mechanism as a model for facilitated diffusion of hexoses in the mammalian cells listed above. The general features of facilitated diffusion, including relative specificity for the compounds transported, saturation kinetics and countertransport, are observed in the mammalian[1,4,5,12] and bacterial[8–11] mechanisms. All these sugar mechanisms involve essential sulfhydryl groups and can be inhibited by appropriate reagents, *e.g.* *N*-ethylmaleimide. In this report we demonstrate that phloretin, its β-glucoside phloridzin, and certain synthetic estrogens, all known previously to inhibit hexose transfer in mammalian cells[1,12], inhibit the bacterial transfer, and many of the features of the inhibition are remarkably similar to those in human erythrocytes.

MATERIALS AND METHODS

Cultures

E. coli strain ML 308 (constitutive for the lactose operon) was grown aerobically at 37° in Medium 63, a defined medium[13] containing 0.2 % succinate as carbon source. This growth medium was used unmodified in studies on leucine transport; for β-galactoside transport, cultures were grown in the same medium containing in addition 0.1 % casamino acids (Difco) *plus* 0.01 % yeast extract (Difco); for α-methylglucoside transport growth was in the same medium as for galactosides except that 0.2 % glucose replaced the succinate. Cultures were harvested in the log phase of growth, washed once, and suspended in a standard "incubation medium", Medium 63 (pH 7.0) containing 0.2 % succinate and 100 μg of chloramphenicol per ml. Suspensions were kept at 2–5° prior to use.

Transport studies

Transport studies were at 30° and duplicate or triplicate samples were obtained in each instance. Steady state influx of *o*-nitrophenylgalactoside was estimated by the rate of hydrolysis to *o*-nitrophenol, as described previously[8,10]. Initial influx of [^{14}C]thiomethylgalactoside (New England Nuclear; specific radioactivity, 8.54 mC/mmole) was estimated as described previously[10] by rapid filtration of three sequential samples at 15–20 sec intervals through Millipore filters. Alternatively, a single sample was filtered at 20 sec and the results were comparable to the foregoing in the inhibitor studies reported below. Influx of α-[^{14}C]methylglucoside (Amersham/Searle; specific radioactivity, 3 mC/mmole) was estimated similarly, using the single 20-sec point. L-[^{14}C]Leucine (New England Nuclear; specific radioactivity, 248 mC/mmole) was tested similarly except that the filters were washed with 0.14 M NaCl at 23° rather than 2°–5°, to minimize leakage of amino acids[14]. Phloretin and phloridzin (K and K Laboratories) were dissolved in 50 % ethanol, and diethylstilbestrol (Nutritional Biochemicals), and hexestrol (Mann) were dissolved in absolute ethanol. For transport studies the ethanolic solutions of these compounds were diluted 1/100 in the bacterial suspensions (final ethanol concentrations 0.5 % or 1.0 %), control suspensions were treated with ethanol alone, the cells were then incubated for 5 min at 30°, and the

assay started by addition of a β-galactoside. To control for entry of *o*-nitrophenylgalactoside *via* mechanisms other than the specific galactoside transport, portions of the cells were first treated with 2.0 mM *N*-ethylmaleimide for 20 min at 25°, the reaction terminated with a 1.5-fold excess of β-mercaptoethanol, and the suspension assayed with *o*-nitrophenylgalactoside. The entry rate following *N*-ethylmaleimide treatment was approx. 25 % of that observed with untreated cells (Fig. 5) and was subtracted from the latter value.

Protection experiments

β-Galactosides protect essential –SH groups in the transport protein from inactivation by *N*-ethylmaleimide[7,10]. Protection was estimated experimentally and calculated as described previously[10]. The general procedure for testing phloretin and other compounds was as follows. Cell suspensions were shaken at 25° in the presence of the following reagents added in the sequence listed: sodium azide, 20 mM for 5 min; phloretin (0.1 mM), other test compound, or vehicle alone for 5 min (final ethanol concentrations were 2.5 % in these protection experiments); 0.5 mM *N*-ethylmaleimide or water control for 1 min; 1.0 mM β-mercaptoethanol to terminate the reaction. The cells were then washed and assayed for residual galactoside transport as described previously[10]. When thiomethylgalactoside was tested for protection the bacteria were shaken initially with 5 mM thiomethylgalactoside for 10 min prior to addition of azide. The values for percentage inhibition of transport owing to treatment with *N*-ethylmaleimide were calculated. If *a* is the percentage inhibition in the absence of the test compound and *b* is the percentage inhibition in its presence, then protection $= (a—b)/a \times 100$ (see ref. 10).

[^{14}C]*Diethylstilbestrol uptake*

[^{14}C]Diethylstilbestrol (Amersham/Searle; specific radioactivity 54 mC/mmole) in ethanol was added to bacterial suspensions and the cells harvested by centrifugation through liquid silicone, as described previously[11]. *E. coli* ML 30 (inducible for the lactose operon) was also tested, and cultures were grown as described above except that 0.5 mM isopropylthiogalactoside was added as inducer where appropriate.

RESULTS

Effects on o-nitrophenylgalactoside entry

The effects of phloretin and a number of related compounds were studied on *o*-nitrophenylgalactoside entry in the presence of 20 mM sodium azide. In the presence of azide entry estimated by the rate of hydrolysis to *o*-nitrophenol is a function of the carrier-mediated transfer and is independent of energy coupling[7,8]. Various concentrations of each compound were tested, and representative experiments are illustrated in Fig. 1. Diethylstilbestrol, hexestrol, phloretin and phloridzin all inhibited facilitated entry of *o*-nitrophenylgalactoside, and the mean concentrations (M) required for 50 % inhibition (data in Fig. 1 *plus* 3 additional experiments) were, respectively, $7.4 \cdot 10^{-6}$, $1.2 \cdot 10^{-5}$, $1.3 \cdot 10^{-5}$, and $3 \cdot 10^{-4}$. The comparable concentrations (M) reported for 50 % inhibition of hexose transfer across human erythrocytes are, respectively, $6.3 \cdot 10^{-6}$, $1.4 \cdot 10^{-5}$, $1 \cdot 10^{-5}$ and $8.3 \cdot 10^{-4}$ (see ref. 15). Values for the first three compounds thus correspond fairly closely in the two cell types. Phloridzin was relatively more effective

in *E. coli*, suggesting the possibility of liberation of its aglucone, phloretin, perhaps *via* the phospho-β-glucosidase described by FOX AND WILSON[16]. The curve for phloridzin in Fig. 1 is also noteworthy for the small increase of 10 % in *o*-nitrophenylgalactoside entry at approx. $5 \cdot 10^{-6}$ M. Such increments in transport at low concentrations of the compounds tested were observed repeatedly and are discussed below (DISCUSSION).

To verify that phloretin inhibits the membrane carrier rather than internal β-galactosidase, toluene-treated and intact cells were compared (Fig. 2). *o*-Nitrophenylgalactoside hydrolysis in intact cells was inhibited approx. 70 % by $1 \cdot 10^{-4}$ M phloretin at initial *o*-nitrophenylgalactoside concentrations up to 1.0 mM. In contrast, phloretin slightly increased *o*-nitrophenylgalactoside hydrolysis in cells rendered permeable by toluene at all *o*-nitrophenylgalactoside concentrations tested. Phloridzin yielded similar results.

Energy metabolism decreases phloretin inhibition of *o*-nitrophenylgalactoside entry. In 3 experiments the mean K_i for phloretin observed with cells treated with 20 mM azide was $1.3 \cdot 10^{-5}$ M, whereas in the absence of azide the K_i was $3.0 \cdot 10^{-5}$ M. Inhibition by diethylstilbestrol was affected similarly. The experiment illustrated in Fig. 3 demonstrates that up to $2 \cdot 10^{-5}$ M diethylstilbestrol failed to inhibit *o*-nitrophenylgalactoside entry in the absence of azide, whereas $1 \cdot 10^{-5}$ M diethylstilbestrol inhibited approx. 50 % in the presence of azide. In succeeding sections evidence is presented to show that azide, in addition, increases total uptake of [^{14}C]diethylstilbestrol by *E. coli* and enhances the ability of the compound to protect the transport mechanism from inhibition by *N*-ethylmaleimide. Fig. 3 also shows that in the presence of azide a small increase of 4 % in *o*-nitrophenylgalactoside entry was ob-

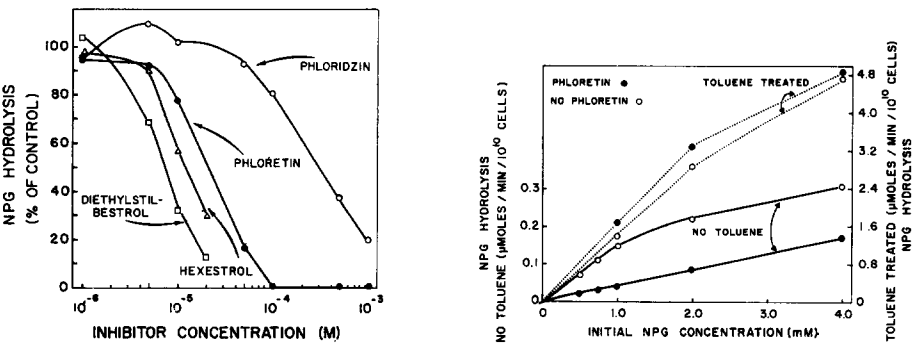


Fig. 1. Effects of various diphenolic compounds on *o*-nitrophenylgalactoside (NPG) hydrolysis. Washed suspensions of ML 308 in incubation medium pH 7.0 (MATERIALS AND METHODS) were tested for *o*-nitrophenylgalactoside hydrolysis at 30° in the presence of 20 mM sodium azide, the various concentrations of diphenols shown, and 2 mM *o*-nitrophenylgalactoside. Data for diethylstilbestrol and hexestrol are means of 2 experiments. Curves for phloretin and phloridzin represent single experiments. All values have been corrected for residual hydrolysis after *N*-ethylmaleimide treatment (MATERIALS AND METHODS).

Fig. 2. Effect of toluene treatment on inhibition of *o*-nitrophenylgalactoside (NPG) hydrolysis by phloretin. Washed suspensions of strain ML 308 were tested as described in Fig. 1 and MATERIALS AND METHODS. For toluene treatment 5 ml of suspension were mixed vigorously for 1 min with 3 drops of toluene, stored at 2° for 10 min, shaken at 37° for 10 min, and finally stored at 2° until assayed. Note that the scale of the right-hand ordinate (toluene treated cells) is 8-fold that of the left-hand ordinate. Results of 2 experiments were similar and 1 experiment is illustrated.

served with $1 \cdot 10^{-7}$ M diethylstilbestrol. This small increase is related to that observed with phloridzin (Fig. 1) and is discussed below (DISCUSSION).

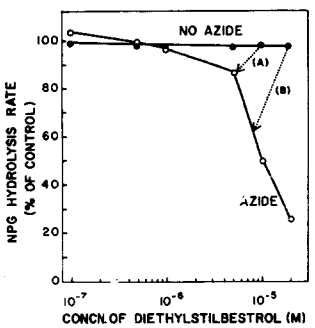
Lineweaver–Burk analysis

The mechanism of inhibition by phloretin was studied further at *o*-nitrophenylgalactoside concentrations of 1–10 mM, and the results plotted by the method of LINEWEAVER AND BURK[17]. Fig. 4 illustrates the mean results of 3 experiments which indicate a non-competitive type of inhibition, a K_i for phloretin of $1.3 \cdot 10^{-5}$ M, and a K_t for *o*-nitrophenylgalactoside of $1.8 \cdot 10^{-3}$ M in cells not treated with phloretin.

Phloretin inhibition observed with cells treated for less than approximately one hour was completely reversed by washing. In 5 experiments suspensions of ML 308, grown separately, were treated with $1 \cdot 10^{-4}$ M phloretin for approx. 20 min at 25°. Rates of *o*-nitrophenylgalactoside entry assayed within this time were 15 ± 9% (mean ± S.D.) of that observed with non-treated controls. Cells were then centrifuged and washed twice with incubation medium at 5°, and on reassay the *o*-nitrophenylgalactoside entry was 102 ± 19% of the control value. In addition, the effects of washing on phloretin, phloridzin or diethylstilbestrol inhibition could be evaluated in the "protection" experiments described below. Quantification of protection (MATERIALS AND METHODS) involves a comparison of cells treated or not treated with these compounds and subsequently washed. Complete reversibility of the inhibition was observed uniformly in the protection experiments.

Specificity of inhibition

The effects of phloretin on influx of *o*-nitrophenylgalactoside, thiomethylgalactoside, α-methylglucoside and L-leucine were examined at various substrate



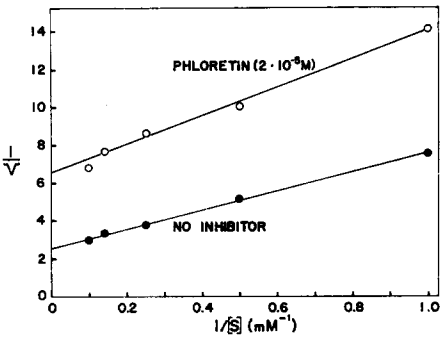


Fig. 3. Effects of sodium azide on diethylstilbestrol inhibition of *o*-nitrophenylgalactoside (NPG) hydrolysis. Cells of strain ML 308 were tested as described in Fig. 1 and MATERIALS AND METHODS with (○—○) or without (●—●) 20 mM sodium azide. Data are means for duplicate determinations using one bacterial culture. In this same experiment both azide-treated and untreated cells were tested for uptake of [^{14}C]diethylstilbestrol using various specific radioactivities. The cells were shaken at 30° for 1 min and then centrifuged through liquid silicone. The broken lines connect points of equal uptake, *i.e.* $3.6 \cdot 10^{-4}$ (A) and $7.7 \cdot 10^{-4}$ (B) μmoles/10^{10} cells.

Fig. 4. Lineweaver–Burk plots of *o*-nitrophenylgalactoside (NPG) entry rate in the absence (●—●) or presence (○—○) of phloretin ($2 \cdot 10^{-5}$ M). The velocity (v) is in units of μmoles of *o*-nitrophenylgalactoside hydrolyzed per min per 10^{10} cells. Conditions for the assay are described in Fig. 1. Each point represents the mean value of 3 experiments with separately grown cultures of ML 308.

concentrations and the results plotted by the method of LINEWEAVER AND BURK[17]. Sodium azide was not used in these assays. As indicated in Table I, phloretin inhibited each influx mechanism but was considerably more effective against the galactosides. The K_i values with α-methylglucoside and L-leucine, respectively, were at least 4- and 8-fold those for the galactosides. Inhibition was non-competitive in each instance, and the plots resembled those in Fig. 3.

TABLE I

VALUES OF K_i FOR PHLORETIN INHIBITION OF INFLUX OF VARIOUS COMPOUNDS IN *E. coli* ML 308

Cells were tested for *o*-nitrophenylgalactoside hydrolysis as described in Fig. 1 except that azide was omitted. Influx of thiomethylgalactoside, α-methylglucoside and L-leucine was estimated as described in MATERIALS AND METHODS. All studies were at 30° and various concentrations of each substrate were tested in the presence or absence of $1 \cdot 10^{-4}$ M phloretin. K_t values were obtained by plotting according to the method of LINEWEAVER AND BURK[17] and K_i values were calculated for the non-competitive inhibition observed with each. Values shown are means of 2 experiments except for thiomethylgalactoside which is the mean of 3 experiments.

Compound transported	K_t (*mM*)	K_i (*mM*)
o-Nitrophenylgalactoside	2.00	0.03
Thiomethylgalactoside	1.60	0.05
α-Methylglucoside	0.37	0.20
L-Leucine	0.001	0.41

Prolonged treatment of *E. coli* with phloretin increased the membrane permeability and released internal β-galactosidase to the medium. Cells were shaken with $5 \cdot 10^{-4}$ M phloretin for up to 4 h at 30° and samples were tested for *o*-nitrophenylgalactoside hydrolysis at various times. Within the first h phloretin decreased the hydrolysis by 67 %, but subsequently the rate of hydrolysis increased progressively to twice that of the controls at 4 h. Fig. 5 illustrates the results of a subsequent experiment in which the cells were treated similarly for 6 h. Again, at 28 min the usual inhibition of *o*-nitrophenylgalactoside hydrolysis was observed, and the residual hydrolysis after *N*-ethylmaleimide treatment, *i.e.*, non-specific entry, was similar for phloretin treated and untreated cells. After 6 h *o*-nitrophenylgalactoside hydrolysis was markedly increased in the treated suspensions. The activity was not sensitive to *N*-ethylmaleimide and on centrifugation much remained in the supernatant solution. The treated cells in the pellet were washed by centrifugation and again tested with *o*-nitrophenylgalactoside. Although the total rate of hydrolysis was then similar to the control cells, fully 70 % was resistant to *N*-ethylmaleimide, as compared to 25 % in controls, indicating increased membrane permeability. Owing to these observations, the exposure of cells to phloretin and related compounds was limited to less than 20–30 min in all inhibition studies.

Prior studies indicate that phloretin inhibits growth of several bacterial species, including *E. coli*[18]. To estimate the phloretin concentration required for bacteriostasis, growth of ML 308 in liquid medium was followed *via* the absorbance (Fig. 6). 50 % inhibition of the growth rate was observed 30 min after addition of 0.09 mM phloretin. In excess of 0.1 mM a decrease in optical density with time was observed and suggested the possibility of cell lysis.

Effects of pH

LEFEVRE AND MARSHALL[15] reported that human erythrocytes bound phloretin maximally at approx. pH 5–6. As the pH was increased from 6 to 8 both the binding of phloretin and its ability to inhibit glucose transfer markedly decreased. In contrast, the binding and inhibition of sugar transfer observed with diethylstilbestrol was not dependent on pH in this range[15]. The effects of pH upon inhibition of *o*-nitrophenylgalactoside entry by each of these compounds were studied with *E. coli* ML 308, and the results are illustrated in Fig. 7. *o*-Nitrophenylgalactoside hydrolysis (entry) in the absence of the inhibitors was maximal at approx. pH 6.8. Phloretin inhibition of the entry was greatest at approx. pH 5.8 and decreased progressively as pH increased

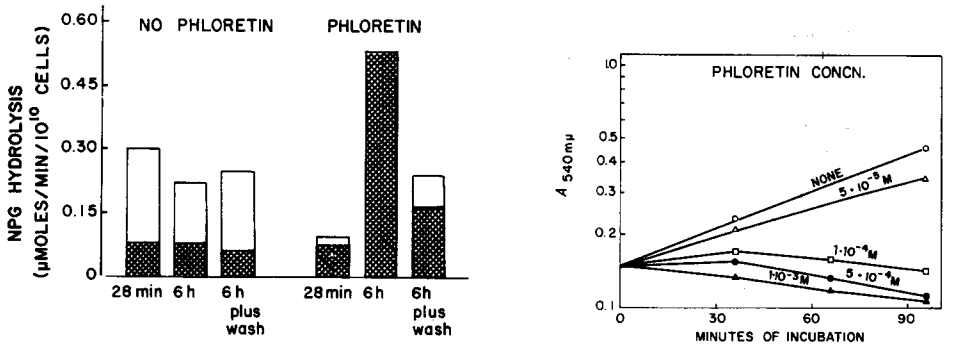


Fig. 5. Effects of time of exposure to phloretin on *o*-nitrophenylgalactoside (NPG) hydrolysis. *o*-Nitrophenylgalactoside hydrolysis rate was estimated in the presence of 20 mM azide as described in Fig. 1. The shaded area in each bar represents the residual *o*-nitrophenylgalactoside hydrolysis rate after treatment with *N*-ethylmaleimide as described in MATERIALS AND METHODS.

Fig. 6. Effects of various concentrations of phloretin on growth of strain ML 308. Bacteria were grown aerobically on Medium 63 (no supplements of casamino acids or yeast extract were added) at 37° with sodium succinate (0.2 %) as carbon source. The doubling time of the untreated control was approx. 60 min. The figure illustrates one experiment representative of two performed.

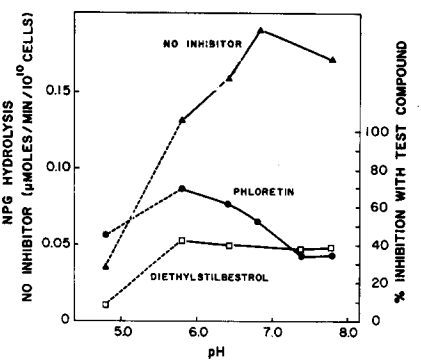


Fig. 7. Effects of pH on *o*-nitrophenylgalactoside (NPG) hydrolysis and on inhibition by phloretin or diethylstilbestrol. Cells of ML 308 were tested for *o*-nitrophenylgalactoside hydrolysis in the presence of 20 mM azide as described in Fig. 1, except that potassium phosphate (0.1 M) of the indicated pH was used in place of the usual potassium phosphate of the incubation medium (MATERIALS AND METHODS). To test at pH 4.8 potassium acetate (0.1 M) was used in place of the phosphate. The left-hand ordinate refers to hydrolysis in the absence of the test compounds, the right-hand ordinate to the % inhibition with either phloretin ($2 \cdot 10^{-5}$ M) or diethylstilbestrol ($8 \cdot 10^{-6}$ M). Each point is the mean value of two experiments with separately grown cultures.

TABLE II

PROTECTION OF GALACTOSIDE TRANSPORT BY PHLORETIN AND DIETHYLSTILBESTROL FROM INHIBITION BY *N*-ETHYLMALEIMIDE

Protection was estimated as described in MATERIALS AND METHODS, using phloretin ($1 \cdot 10^{-4}$ M), diethylstilbestrol ($2 \cdot 10^{-5}$ M), phloridzin ($1 \cdot 10^{-3}$ M) or thiomethylgalactoside ($5 \cdot 10^{-3}$ M). Galactoside transport was assayed by [^{14}C]thiomethylgalactoside uptake in expt. 4 (ref. 10) and by *o*-nitrophenylgalactoside hydrolysis in the remaining experiments.

Expt.	*Compound*	*N-Ethylmaleimide inhibition* (%)		*Protection* (%)
		No compound (*a*)	*Compound* (*b*)	$(a-b)/a \times 100$
1	Phloretin	50.1	18.5	63.1
	Thiomethylgalactoside	50.1	43.7	14.4
	Phloretin *plus* thiomethylgalactoside	50.1	19.2	61.9
2	Phloretin	48.3	19.7	59.7
	Thiomethylgalactoside	48.3	37.2	23.0
	Phloretin *plus* thiomethylgalactoside	48.3	16.7	65.5
3	Diethylstilbestrol	37.9	8.5	77.5
	same *minus* azide	40.8	36.6	10.5
4	Diethylstilbestrol	51.6	31.5	39.0
	same *minus* azide	47.0	36.6	22.2
5	Phloridzin	40.4	25.5	36.9

to 7.4. In contrast, diethylstilbestrol inhibition remained unchanged from pH 5.8 to 8.0. The effects of pH on inhibition of *o*-nitrophenylgalactoside entry are thus similar to those reported with human erythrocytes.

Protection experiments

Phloretin, phloridzin and diethylstilbestrol all protected the essential –SH groups of the galactoside transport mechanism against *N*-ethylmaleimide (Table II). The protection was marked and consistent only when energy metabolism of the cells was inhibited, as with sodium azide. Table II shows that protection by diethylstilbestrol in 2 experiments was 10.5 % and 22.2 % in the absence of azide, as compared to 77.5 % and 39.0 %, respectively, in the presence of azide. The three compounds tested were much more effective than thiomethylgalactoside in protecting the transport, as indicated by the values in Table II and prior studies with thiomethylgalactoside[10]. Addition of thiomethylgalactoside to phloretin did not appear to increase protection significantly.

Uptake of [^{14}C]diethylstilbestrol

The preceding observations demonstrate that diethylstilbestrol inhibits transport of β-galactosides and protects the essential –SH groups from *N*-ethylmaleimide. Inasmuch as both effects are markedly increased when energy metabolism is inhibited, it was of great interest to examine the uptake of [^{14}C]diethylstilbestrol in the presence and absence of metabolic inhibitors. *E. coli* cells were incubated with the compound at various carrier concentrations from $3.6 \cdot 10^{-9}$ to $2.0 \cdot 10^{-5}$ M, and the cells were sepa-

TABLE III

UPTAKE OF [^{14}C]DIETHYLSTILBESTROL BY *E. coli*

Suspensions of *E. coli* (absorbance at 540 mμ approx. 50) in incubation medium were shaken with [^{14}C]diethylstilbestrol in the absence or presence of sodium azide (20 mM) or 2,4-dinitrophenol (1 mM) for 1 min at 30° and then centrifuged through liquid silicone[11].

Expt.	*Strain*	*Concentration of diethylstilbestrol added (M)*	[^{14}C]*Diethylstilbestrol uptake (pmoles/10^{10} cells)*		
			No inhibitor	*Azide*	*2,4-Dinitrophenol*
1	ML 308	$3.6 \cdot 10^{-9}$	0.08		0.19
2	ML 308	$8.0 \cdot 10^{-7}$	15.6	42.8	38.4
3	ML 308	$1 \cdot 10^{-7}$	2.2	5.0	
		$1 \cdot 10^{-6}$	19.0	69.0	
		$1 \cdot 10^{-5}$	363	1012	
		$2.0 \cdot 10^{-5}$	770	1450	
4	ML 30, uninduced	$3.6 \cdot 10^{-9}$	0.11	0.22	0.23
	ML 30, induced	$3.6 \cdot 10^{-9}$	0.10	0.20	0.19

rated from the ambient medium by centrifugation through liquid silicone[11]. The results in Table III demonstrate that cells treated with sodium azide (20 mM) or 2,4-dinitrophenol (1 mM) contained more than twice the quantity of diethylstilbestrol observed with untreated controls at all the carrier concentrations tested. No clear evidence was obtained for saturation of the uptake processes in the concentration range tested. Induction of *E. coli* ML 30 failed to affect uptake of diethylstilbestrol significantly (Table III).

The foregoing results suggest that metabolic inhibitors increase diethylstilbestrol uptake and thereby augment the inhibition of galactoside transport. Examination of the data in Fig. 3, however, suggests that total uptake of diethylstilbestrol is not the only factor. In this experiment diethylstilbestrol uptake and inhibition of *o*-nitrophenylgalactoside entry in the presence or absence of azide were compared. The broken lines in Fig. 3 connect points at which total uptake of the compound was equal in azide-treated and untreated cells. At these points of equal uptake no inhibition of entry was observed in azide-free cells, whereas marked inhibition of entry was noted in azide-treated suspensions.

DISCUSSION

The present results demonstrate that phloretin and related compounds inhibit the facilitated diffusion of β-galactosides across *E. coli*, and the inhibition is in many respects similar to that of hexose transport across human erythrocytes[1]. Similar concentrations of phloretin, diethylstilbestrol and hexestrol are required for 50% inhibition in both cell types. The effects of pH, the reversibility on washing, and the irreversible changes which follow prolonged exposure or relatively high concentrations of the diphenolic compounds are similar for both types of cells. Countertransport has been observed with human erythrocytes[21] and the bacterial mechanism[10,11]. More recently, kinetic methods have been developed to estimate the ratio of the reaction

rate constant for formation (or for dissociation) of the carrier-substrate complex at the outer as compared to the inner face of the membrane[22]. Studies with both cell types indicate that the reaction rate constants are significantly greater at the outer as compared to the inner face[22].

LEFEVRE[1] has summarized observations on the effects of a large series of these diphenolic compounds on human erythrocytes. The inhibition of hexose transport was characterized by determination of a coefficient, m, defined by the equation:

$$\frac{\text{Control transfer rate}}{\text{Inhibited transfer rate}} = 1 + \frac{[I]^m/K_i}{1 + [P]/K}$$

where $[I]$ and $[P]$ are concentrations of inhibitor and transferred sugar, respectively, and K_i and K are the dissociation constants of the inhibitor-inhibitor site and sugar-transport site, respectively. The coefficient m can be interpreted as the average number of inhibitor molecules required to block one reactive site, and the values observed averaged typically about 1.5 (ref. 1). A similar analysis of the present data (Fig. 1) yields values of m for phloretin, diethylstilbestrol, hexestrol and phloridzin, respectively, of 1.8, 1.9, 2.1 and 1.3. Thus in the bacterial cells approx. 2 molecules of each inhibitor, with the exception of phloridzin, are required to inactivate a transport site*.

Phloretin is a competitive inhibitor of hexose transport in human erythrocytes, whereas diethylstilbestrol and hexestrol inhibit non-competitively[1]. In the bacterial transport studied here all three compounds inhibit non-competitively. A possible explanation of these observations, in accord with prior suggestions of BOWYER AND WIDDAS[19] and LEFEVRE[1], is that the diphenolic compounds do not act by direct displacement of the sugar from the transport site, but by attaching to adjacent inhibitor sites, and the type of inhibition depends on the number of adjacent sites involved per transport site. Thus the m value observed for phloretin inhibition of the erythrocyte transport was 1.0 (ref. 1), whereas the m values for diethylstilbestrol and hexestrol were 1.4 and 1.6, respectively. In the *E. coli* transport experiments the m values for all three compounds were approx. 2. It is conceivable that m values in excess of one, *i.e.* where more than one molecule of inhibitor is required to inactivate a transport site, are associated with non-competitive inhibition.

Phloridzin was a much poorer inhibitor than the other compounds tested above, and similar observations have been reported for human erythrocytes[1]. Moreover, low concentrations of phloridzin increased the β-galactoside transport (Fig. 1). Confirmation of the stimulatory effect was obtained in 3 experiments with *E. coli* ML 308 grown with glucose as the carbon source in place of succinate. Both phloridzin and phloretin were poorer inhibitors of β-galactoside transport in these cells, and $5 \cdot 10^{-6}$ to $1 \cdot 10^{-5}$ M phloridzin increased *o*-nitrophenylgalactoside entry by 30–40 % as compared to increases of 10–15 % in succinate-grown cells. Phloridzin also increased [^{14}C]thiomethylgalactoside accumulation by approx. 18 % in three additional experiments with succinate-grown cells. The stimulatory effect of phloridzin has not been reported previously and merits further study.

* Although the data could be analyzed in terms of other models, *e.g.* that of allosteric transitions[20], we have restricted the analysis in this report to that presented by LEFEVRE[1] in order to compare directly his prior results with the present observations.

Although both the *E. coli* and the human erythrocyte transport mechanisms involve essential sulfhydryl groups, substrates protect the bacterial mechanism from reaction with *N*-ethylmaleimide[6,7,10], whereas substrates enhance the reaction with *N*-ethylmaleimide in erythrocytes[23]. Interestingly, phloretin and the related diphenols acted like the substrates to protect against *N*-ethylmaleimide in *E. coli* (Table II), and phloretin enhanced reaction with *N*-ethylmaleimide in erythrocytes (M. J. GUY AND D. SCHACHTER, unpublished observations). In *E. coli* metabolic inhibitors increased the protection of the transport sulfhydryl groups by phloretin and diethylstilbestrol. Energy uncoupling also increased the inhibition of the transport by these diphenols. Whether this resulted from altered bacterial metabolism of the test compounds or from changes in the transport mechanism is unknown. Gross binding of [^{14}C]diethylstilbestrol to bacterial cells was increased on treating with azide or 2,4-dinitrophenol, but this failed to account for the enhanced inhibition of transport.

Studies of [^{14}C]diethylstilbestrol uptake permit a rough estimate of the maximal number of β-galactoside transport sites per cell. With $5 \cdot 10^{-6}$ M to $2 \cdot 10^{-5}$ M diethylstilbestrol (Fig. 3) the percent inhibition of transport was directly proportional to the amount of inhibitor bound, and 100% inhibition required 110000 molecules bound per cell. If 2 molecules are required to inactivate each transport site (see above), the data indicate a maximum of approx. 55000 sites per cell or 11000 sites per μm^2 of cell surface*. FOX *et al.*[24] have estimated a lower limit of approx. 10000 sites per cell. The density of glucose carrier sites in human erythrocytes has been estimated as 11400 per μm^2 cell surface[25], a value close to the upper limit calculated for the *E. coli* cells in these experiments.

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* Although the precise localization of [^{14}C]diethylstilbestrol within or on the bacterial cell is not known, it appears that the bulk of the uptake represents binding to particulate components. Suspensions of ML 308 were sonicated and a particulate preparation, sedimented by centrifugation at 100000 × *g* for 60 min, was incubated with [^{14}C]diethylstilbestrol. The binding observed (μmoles diethylstilbestrol per mg protein) was sufficient to account for the bulk of the uptake by intact cells.

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