BBA 75754

LOCAL AND NON-LOCAL INTERACTIONS OF FLUXES MEDIATED BY THE GLUCOSE AND GALACTOSIDE PERMEASES OF *ESCHERICHIA COLI*

ARTHUR L. KOCH

Department of Microbiology, Indiana University, Bloomington, Ind. 47401 (U.S.A.)

(Received May 3rd, 1971)

SUMMARY

1. When α-methyl glucoside is transported *via* the glucose permease of *Escherichia coli*, there are two levels of effects on the transport of galactosides *via* the galactoside permease. The first is direct because it is immediate and responds to the external concentration and not to the instantaneous internal concentrations and appears to result from competition for some membrane component common to the transport *via* the glucose and galactoside permeases. The second effect acting in addition to the first is indirect in that it requires several minutes for the full inhibition to be observed when low concentrations are used and responds to the internal concentration. Glucose gives the first response, but does not give the second as quickly as does α-methyl glucoside. Neither azide nor succinate aid or prevent this second effect.

2. Under the usual growth conditions that result in high glucose permease, *i.e.* growth on glucose, galactosides have little inhibitory effect on α-methyl glucoside uptake although α-methyl glucoside effectively inhibits galactoside transport. However, under chemostat growth with glucose limitation, inhibitions are readily demonstrated. Having high levels of both permeases is not the sole requirement for observing interaction between fluxes because chemostat growth at fast dilution in a limiting mixture of glucose *plus* succinate results in bacteria with high levels of both permeases, but little interaction of either flux with the other.

INTRODUCTION

When genetically competent bacteria are grown so that they have produced a transport system for each of two different classes of carbohydrates, interactions between fluxes mediated by the two permease systems can sometimes be demonstrated. Thus, when *Escherichia coli* strain ML308, which constitutively produces galactoside permease, is grown in glucose so that the level of glucose permease is high, it is found that the addition of glucose (or α-methyl glucoside) greatly inhibits galactoside entry[1–5]. High levels of both permeases are necessary for this inhibition to be observed. Since, in addition, the influx of glucose increased the efflux of galactoside only when high levels of both permeases were present, and the same kinds of interaction appeared

Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PEP, phosphoenolpyruvate; TMG, thiomethyl-β-D-galactopyranoside.

to function for the transport through the galactoside permease by fluxes through the maltose system, it appeared[3] that the interaction was a local one resulting from the function of some common element (the "transporter"[6]) which could accept a single carbohydrate molecule at a time from any of several permease molecules which could have different substrate specificities.

Later on, we attempted[4] to use this presumed local interaction as a probe to investigate how the membrane enlarges during growth by shifting the medium so that first, one kind of permease and then another kind of permease was laid down. This experimental approach was based on the assumption that there would only be an interaction between the two transports if the respective permeases were located contiguously on or within the cytoplasmic membrane. As part of this study, two kinds of experiments were presented consistent with this local interaction hypothesis. These results were antagonistic to the alternate hypothesis which would assume that the interaction results from alterations in the compositions of the cytoplasm resulting from the action of one transport process which in turn inhibits or stimulates the other flux at all transport sites on the cytoplasmic membrane. Such inhibition might result either by direct diffusion and binding to the second site or by depletion of cellular energy reserves needed for active transport such as ATP or phosphoenolpyruvate (PEP) or actions of proton pumps.

The first kind of experiment we presented in evidence for a local effect was the demonstration that the effect of α-methyl-D-glucopyranoside on the galactoside permease limited *in vivo* hydrolysis of the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was immediate (*i.e.* it is expressed in only a few seconds). The inhibition did not take several minutes to develop as does the internal pool of α-methyl-D-glucopyranoside and α-methyl-D-glucopyranoside 6-phosphate (see refs. 1, 5, 7 and 8). The second kind of experiment showed that the degree of inhibition of galactoside transport by high concentrations of α-methyl-D-glucopyranoside progressively increased as the glucose permease level of the cells was increased by more extensive growth in glucose-containing media. This was consistent with a local hypothesis because the half-inhibitory concentrations (K_i) for the fraction of the galactoside transport, which was inhibitable by α-methyl-D-glucopyranoside, did not change during the course of induction of the glucose permease[9]. The K_i would be excepted to decrease with increasing glucose permease induction for any non-local interaction involving the accumulation of an inhibitor or the depletion of an energy store. But the K_i should be constant for any model postulating a qualitative change in proportion of types of sites on the bacterial membrane. Moreover, this half-inhibitory concentration (10 μM) was much less than the wide range of concentrations (29–210 μM) of α-methyl-D-glucopyranoside which are reported to half-saturate transport by the glucose permease in the intact cells[1–5]. If the effect had been internal, due to the build-up of a pool of α-methyl-D-glucopyranoside and α-methyl-D-glucopyranoside 6-phosphate or depletion of some energy reserve, then a partially induced cell for glucose permease should have been more completely inhibited in its galactoside transport by increasing the α-methyl-D-glucopyranoside concentration well above 200 μM because this would further increase the internal steady-state level.

However consistent these facts are (and others given in previous publications[3,4,9]) with a local hypothesis, the experimental facts uncovered by WINKLER AND WILSON[5] were very much in disagreement with the simple interpretation of local interaction.

If the interaction be not local, our use of the permease function as a probe for membrane synthesis[4,9] would be meaningless. In our view, the strong points in their data relevant to the local interaction hypothesis are: First, the galactosides, thiomethyl-β-D-galactopyranoside (TMG) and thiodigalactoside do not inhibit α-methyl-D-glucopyranoside uptake. Second, α-methyl-D-glucopyranoside influx, unlike glucose influx, does not speed but rather slows TMG efflux. They did, however, confirm our observation that glucose influx does speed efflux of TMG in glucose grown cells. Third, cells preloaded with α-methyl-D-glucopyranoside do not transiently take up [^{14}C]TMG in the presence of azide and iodoacetate, whereas cells preloaded with non-radioactive TMG do. Fourth, they found that low concentrations of α-methyl-D-glucopyranoside inhibited the initial rate of TMG uptake, but only after several minutes preincubation with α-methyl-D-glucopyranoside; although they reported that higher concentrations of α-methyl-D-glucopyranoside or glucose did have an immediate effect. It is irrelevant for the present discussion that WINKLER AND WILSON[5] subscribed to a model of a limited amount of a "common factor" involved in the permease–substrate complex moving across the cell membrane instead of a "transporter" or "carrier" actually bridging the gap. Their model is still a local model and it also is apparently contradicted by these same four observations. We leave counter arguments to try to make these four findings consistent with local "common factor" involved in the transport mechanism to DISCUSSION and proceed to describe experiments to test the local hypothesis more fully.

MATERIALS AND METHODS

Strains and assay conditions were as previously described[2–5,9]. The hydrolysis of ONPG by cell suspensions and lysates was carried out in a Cary Model 16 manual spectrophotometer with a constant temperature chamber and cell housing. All measurements were at 420 nm. This instrument was equipped with an interface, and a Houston "Omnigraphic" XT recorder. The extra photometric accuracy in absorbance measurements over previously available apparatus was essential for the experiments to be presented because it was necessary to accurately measure small changes in the total light passing through very turbid bacterial suspensions. By having bacteria in the reference pathway of the dual beam instrument or using a high zero suppression, the change due to ONPG hydrolysis could be accurately measured. We have also adapted the magnetic mixing cuvette holder manufactured for a Zeiss PMQ spectrophotometer so that it can be mounted into the Cary, so that measurements can be made while reagents were added to the 2 cm × 2 cm cuvette. This continuous mixing permits accurate measurements to be resumed quickly after additions to the cuvette instead of waiting 20–30 sec for the fluctuations on the recorder due to the light-scattering component of the signal to subside. This transient response can be largely overcome because the cells are partially oriented at all times by stirring due to the magnet. Radioactive assays at a final concentration of 4.54 μM of [^{14}C]TMG and 2.27 μM α-methyl-D-glucopyranoside uptake were performed at 15° as described previously[3,4,9] unless otherwise noted.

All bacteria were grown at 37° and *in vivo* ONPG hydrolysis and enzyme assays were performed at 28°. All permease assays were carried out in the presence of 50 μg/ml chloramphenicol.

RESULTS

Initial time course of the α-methyl-D-glucopyranoside and glucose inhibition of in vivo ONPG hydrolysis of glucose-grown cells

To measure the speed with which α-methyl-D-glucopyranoside can inhibit the *in vivo* hydrolysis requires more sophisticated apparatus than required to measure effects on the steady-state rates of cellular hydrolysis. The rate of hydrolysis measured in cultures with turbidity corresponding to absorbance of less than 1 is so slow, measurements must be made over a protracted time to measure the rate. In these circumstances, processes that influence the rate can be quite slowly acting and the speed with which they act not be measurable. Higher concentrations of cells leads to a higher blank value due to cell turbidity. Moreover, because the cells are rod-shaped, when a reagent is added and mixed, there is transient absorbance due to an altered distribution of orientations of the cells by the flow pattern established by the mixing operation.

By magnetic stirring together with high zero suppression or balancing against a blank containing bacteria, the results shown in Figs. 1–3 were obtained. Fig. 1 is a photograph of the chart recording itself while Figs. 2 and 3 are tracings from the recordings of another experiment. For convenience, the absorbance axis has been

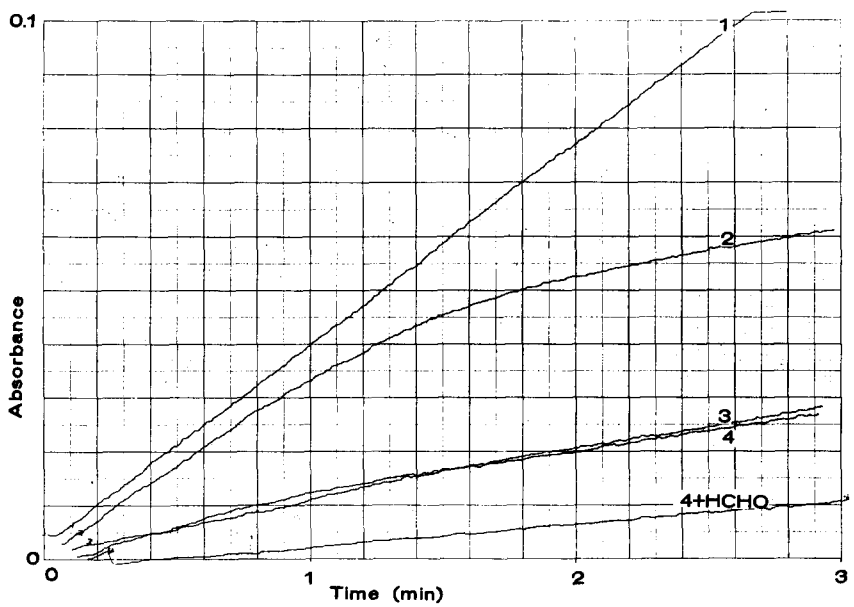

Fig. 1. Recording of kinetics of α-methyl-D-glucopyranoside inhibition of *in vivo* ONPG hydrolysis. A culture of ML308 growing in glucose–M9 medium with a 52-min doubling time at 37° was harvested, washed, and the hydrolysis of ONPG by these cells at a concentration of 0.099 mg/ml in a stirred cuvette was followed: 1, control, the slope corresponds to a permease level of 198 μmoles/g per min; 2, α-methyl-D-glucopyranoside added to a final concentration 40 μM at the same time the ONPG was added, the initial slope corresponds to 180 μmoles/g per min and the final slope to 48 μmoles/g per min; 3, as in Curve 2, but α-methyl-D-glucopyranoside added 3 min before ONPG, the slope corresponds to 42 μmoles/g per min; 4, α-methyl-D-glucopyranoside added to 2500 μM simultaneously with ONPG, the slope corresponds to 47 μmoles/g per min; 4 + HCHO, formaldehyde added to 10 mM at the end of the first 3 min of Run 4 and the recorder rezeroed.

displaced from the original recording; zero on the time axis is the time α-methyl-D-glucopyranoside was injected into the cuvette. These figures show that the actions of α-methyl-D-glucopyranoside in inhibiting galactoside transport is very rapid at high concentrations confirming the previous results of BONIFACE AND KOCH[4]. But at lower concentrations, the effect is not instantaneous. Rather, it takes several minutes for a steady-state level of inhibition to become established. These figures also confirm that 10 μM α-methyl-D-glucopyranoside causes about 50% of the maximal inhibition and that only a small portion of the *in vivo* hydrolysis rate above the permease inhibited control is non-inhibitable by high concentrations of α-methyl-D-glucopyranoside. It has been shown previously that formaldehyde inhibits permease function without

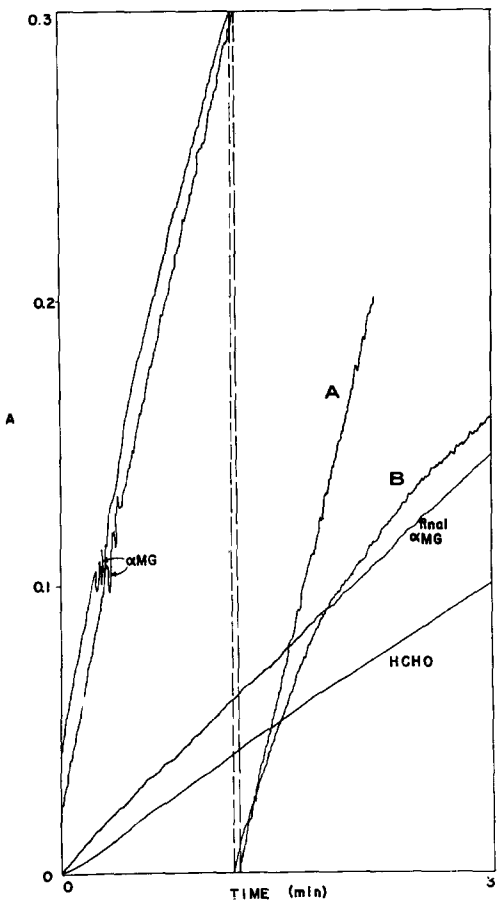

Fig. 2. The effect of low concentrations of α-methyl-D-glucopyranoside on *in vivo* ONPG hydrolysis. Cells growing in glucose containing medium at a 52-min doubling time were used. In Curve A, 25 μM α-methyl-D-glucopyranoside, and in Curve B, 50 μM α-methyl-D-glucopyranoside were introduced at the indicated points. The recorder was offset at the point indicated by the vertical lines. The curve labeled final α-methyl-D-glucopyranoside is for the experiment of Curve B but with the time displaced by 4 min and the ordinate rezeroed. The experiment of Curve A reached the same final degree of inhibition but only after 7 min. The initial slope after 25 μM α-methyl-D-glucopyranoside was added measured in the next 30 sec is 90% of the preinhibition rate. αMG = α-methyl-D-glucopyranoside.

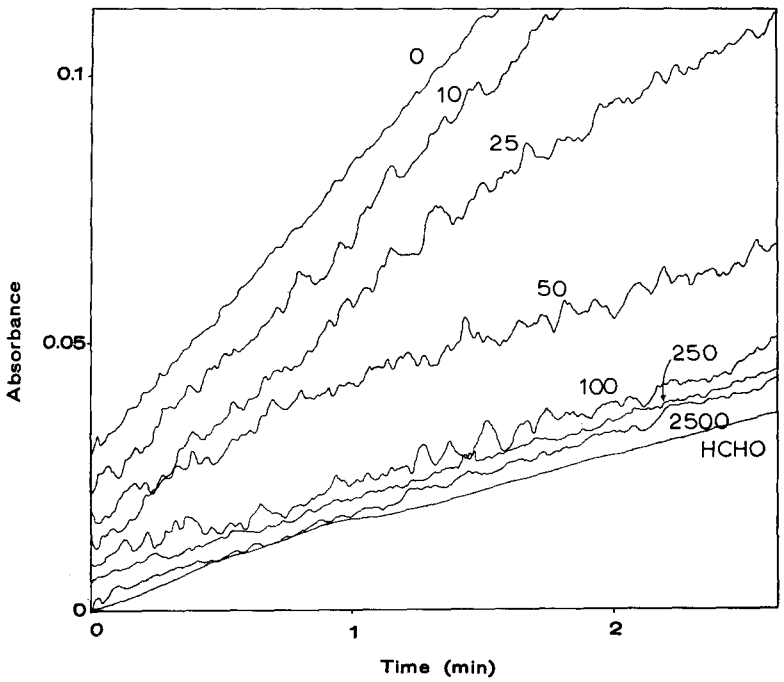

Fig. 3. Kinetics of α-methyl-D-glucopyranoside inhibition of ONPG hydrolysis in glucose-grown ML308. Bacteria with a doubling time of 46 min were harvested, washed, and resuspended at a final concentration of 0.107 mg/ml. The final concentration of α-methyl-D-glucopyranoside in μM is shown on the figure. ONPG and the α-methyl-D-glucopyranoside were added simultaneously at $t = 0$. The ordinate scale has been shifted for convenience in display. The control without α-methyl-D-glucopyranoside corresponds to net *in vivo* hydrolysis of 161 μmoles/g per min. The control with formaldehyde corresponds to 40 μmoles/g per min. The extra noise in this figure compared with Fig. 2 depends on turbulence patterns which sometimes developed in the stirred cuvette.

interfering with passive diffusion or cryptic entry or with the action of enzyme released to the outside of the permeability barrier of the cell[2,3]. Therefore, a formaldehyde control is regularly run to correct for such processes.

From many experiments of the kind shown in Figs. 1–3, it has become clear that there is an immediate effect as well as a delayed one. As can be seen from Fig. 4, there is a particular concentration of α-methyl-D-glucopyranoside (of about 12 μM) where there is almost no immediate effect, but where a very severe steady-state inhibition will develop. This is exactly the same finding as reported by WINKLER AND WILSON[5] for the uptake of [^{14}C]TMG: immediate inhibition at high concentrations and delayed inhibition at concentrations in the range of 10–100 μM.

The establishment of the steady-state inhibition of *in vivo* hydrolysis of ONPG, even at the lowest concentration of α-methyl-D-glucopyranoside giving detectable inhibition, is established well before uptake of α-methyl-D-glucopyranoside comes to its steady-state plateau of accumulation where entry is balanced by exit, achievement of plateau concentrations of α-methyl-D-glucopyranoside takes approximately 20 min at all concentrations at 28° (Fig. 5).

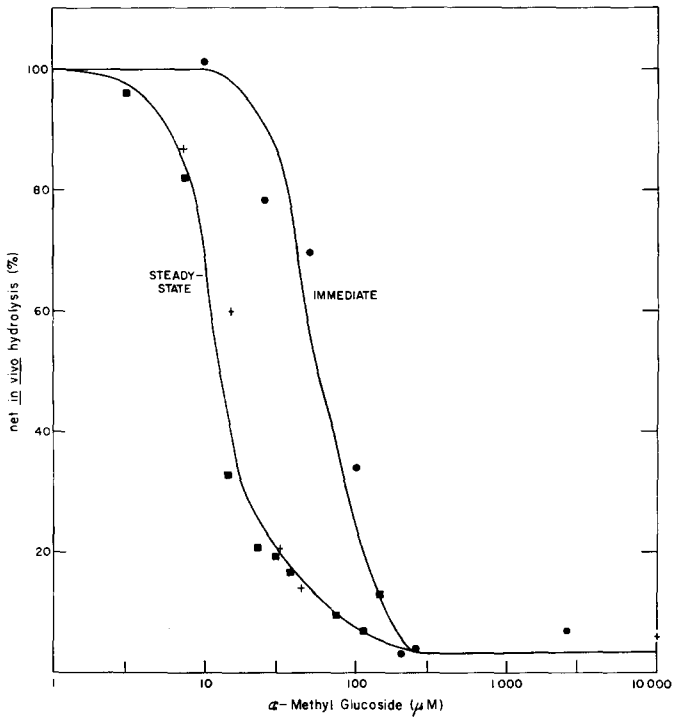

Fig. 4. Immediate and steady-state inhibition of ONPG *in vivo* hydrolysis by α-methyl-D-glucopyranoside. This figure has been constructed from experiments such as shown in Figs. 1–3 as well as other steady-state measurements in unstirred cuvettes at lower cell densities. The initial slope is the tangent of the recording at the time of α-methyl-D-glucopyranoside addition. All data refer to glucose-grown ML308 where the uninhibited control had net *in vivo* hydrolysis rates of 160–180 μmoles/g per min.

WINKLER AND WILSON[5] noted that glucose has an immediate effect on [^{14}C]-TMG uptake, and similarly we find that even at glucose concentrations giving less than maximal inhibition of galactoside entry, the effect is displayed within a very few seconds (see Table I). It is to be noted that the maximum immediate inhibition produced by glucose is less than that produced by α-methyl-D-glucopyranoside (see also ref. 5 and Fig. 3 of ref. 10). This was the first indication that we made which suggested that the action of α-methyl-D-glucopyranoside in interacting with galactoside transport was more complex than the initial action of glucose itself.

The effect of pre-accumulated α-methyl-D-glucopyranoside

On the non-local hypothesis where it is assumed that the effect of α-methyl-D-glucopyranoside on galactoside transport does not arise from competition with a galactoside for some common factor needed for entry but rather arises from internal inhibition by accumulated pools, it would be expected that the inhibition of ONPG entry should be maintained after dilution until the α-methyl-D-glucopyranoside and α-methyl-D-glucopyranoside 6-phosphate leaves the cell. The exit process is known to be quite slow since the half-life for this process must be similar to the time to reach the steady-state when α-methyl-D-glucopyranoside is added to a culture. Fig. 6 shows

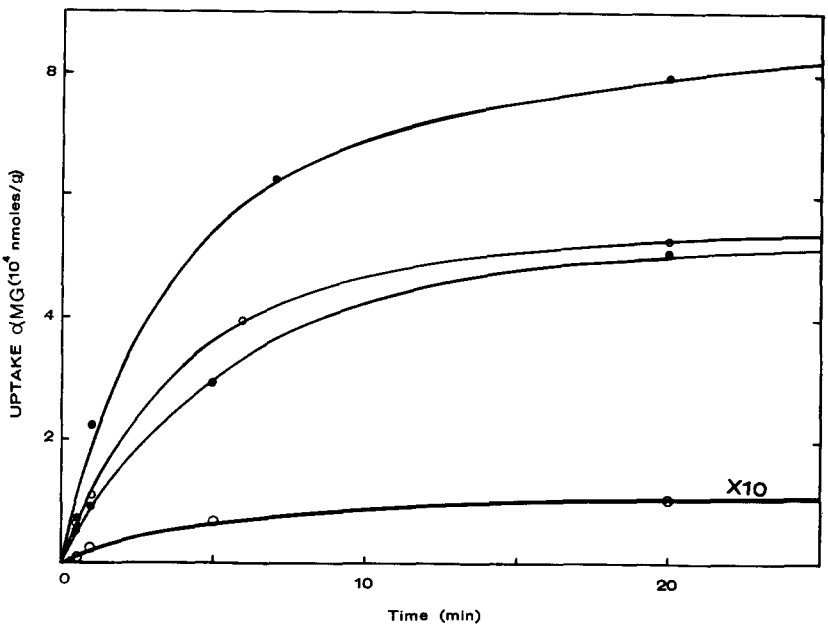

Fig. 5. Kinetics of α-methyl-D-glucopyranoside uptake at 28°. The concentration of α-methyl-D-glucopyranoside was from top to bottom: 5000, 1000, 200 and 0.25 μM. The uptake for the owest concentration has been expanded 10 times. αMG = α-methyl-D-glucopyranoside.

TABLE I

INITIAL GLUCOSE INHIBITION OF GLUCOSE-GROWN ML308 CELLS

	In vivo hydrolysis of ONPG (*μmoles/g per min* ± *S.E.*)	
Uninhibited controls	178 ± 3	
13.9 μM glucose	100 ± 11	Transient rate established within 5 sec, relieved in 30 sec by glucose consumption or uptake
1390 μM glucose	74 ± 4	Rate established in less than 5 sec
2500 μM α-methyl-D-glucopyranoside	36	
10 mM formaldehyde control	25	

experiments where concentrated suspensions of cells were pre-equilibrated with the indicated concentration of α-methyl-D-glucopyranoside at 28° for 10 min; this would have resulted in all cases in maximal inhibition of entry if a galactoside had been present. Then the samples were diluted 50-fold directly into M-9 buffer containing ONPG in a thermostatted cuvette which was being stirred magnetically in the spectrophotometer. It was expected that after re-equilibration, there would be no steady-state inhibition of ONPG hydrolysis below 200 μM preincubation concentration which results in a final concentration of 4 μM.

However, in these experiments, the cell concentration during the preincubation was sufficiently very high so that uptake lowered the external concentration of α-methyl-D-glucopyranoside significantly, thus the steady-state inhibition at the end

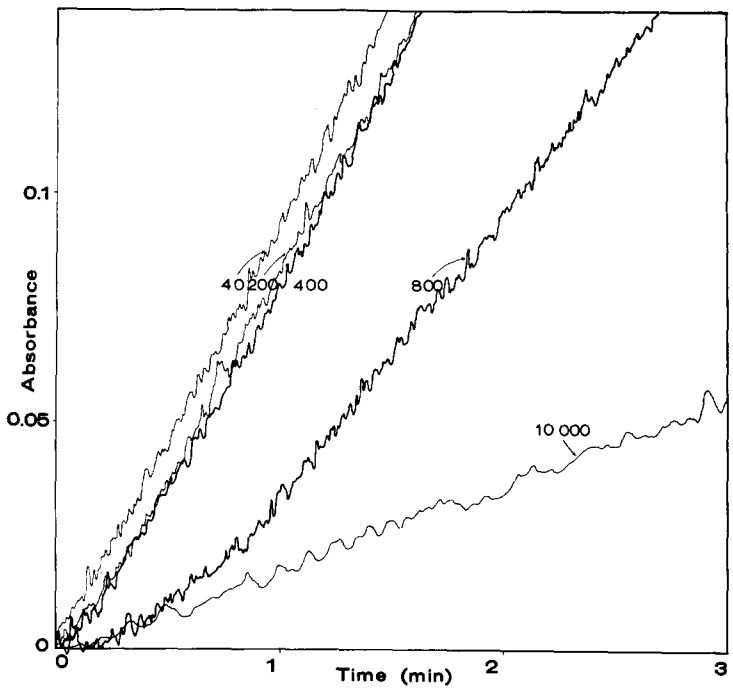

Fig. 6. Effect of preincubation with α-methyl-D-glucopyranoside. Glucose-grown cells of ML308 were washed, concentrated and preincubated with the indicated μM concentrations of α-methyl-D-glucopyranoside for 7–10 min at 28°, then the cells were diluted 50-fold into M-9 containing ONPG, and the recording was started. There the cell concentration was 0.086 mg/ml. Shown are tracings of recordings, shifted vertically for convenience.

of these experiments was less than expected for either 400 or 800 μM preincubation at a lower concentration of bacterial cells. If uptake were negligible, the final concentration would be 8 and 16 μM in these two cases respectively, which should have given nearly 50% and nearly 100% of the maximal inhibition but instead gave 0 or 30% of the maximal inhibition. This consideration shows that external concentration is more significant than the corresponding internal one in determining inhibition of galactoside uptake. Other experiments (not shown), have directly measured the decrease in inhibition of *in vivo* ONPG hydrolysis by α-methyl-D-glucopyranoside by extremely concentrated suspensions of bacteria after a time expected for the depletion of the external medium of the α-methyl-D-glucopyranoside. Depletion of α-methyl-D-glucopyranoside from the medium is not a significant factor in any other experiment reported here.

More critical to the question of whether the external or internal concentration of α-methyl-D-glucopyranoside effects the inhibition of galactoside entry is the lag before the inhibition decrease from almost 100% to the less inhibited steady-state rate. It can be seen that even at a 400 μM preincubation concentration, dilution resulted in no more than a few seconds lag. Concentrations of 800 μM did inhibit *in vivo* ONPG hydrolysis for about 30 sec before the steady-state inhibition characteristic of the final concentration of α-methyl-D-glucopyranoside was established. The conclusion must be drawn that either the internal pool of α-methyl-D-glucopyranoside or

α-methyl-D-glucopyranoside 6-phosphate or some prior energy depletion do play some role in the inhibitory processes but not an exclusive role.

The effect of temperature on both the lag in establishing the steady-state rate of inhibition after α-methyl-D-glucopyranoside addition and the lag after the dilution of α-methyl-D-glucopyranoside is about the same and corresponds to a Q_{10} of about 2.5. This estimate is only approximate since it is based on measurements of only one batch of cells at two temperatures only (15° and 28°). A similar temperature coefficient applies as well to β-galactosidase activity and to the *in vivo* hydrolysis of ONPG.

TABLE II

PROPERTIES OF SUCCINATE-GROWN, GLUCOSE SHIFTED AND GLUCOSE-GROWN ML308

Data	*0.2% succinate-growing cells*	*Succinate then 3/4 doubling in glucose + succinate*	*0.2% glucose-growing cells*
Doubling (min)	60	49	48
β-Galactosidase (*in vitro* ONPG) (μmoles/g per min)	8700	5800	3000
Galactoside permease ([^{14}C]TMG) (nmoles/g per 4 min)	1550	1020	772
Net *in vivo* ONPG (μmoles/g per min)	456	305	190
Glucose permease (^{14}C-labeled α-methyl-D-glucopyranoside) (nmoles/g per min)	414	1240	1760
Maximal inhibition of galactoside permease by α-methyl-D-glucopyranoside			
([^{14}C]TMG 4 min uptake, %)	9	3	25
(ONPG hydrolysis rate, %)	20	37	82
Inhibition of glucose permease by 0.9 mM TMG (^{14}C-labeled α-methyl-D-glucopyranoside per 4 min uptake, %)	3	−10	0

Property of cells in transition from growth on succinate to growth on glucose

Cells of ML308 grown in succinate as a carbon source have higher β-galactosidase and galactoside permease, but lower glucose permease[9,10] than do cells grown on glucose as a carbon source. These facts are reconfirmed in Table II. In addition, it can be seen that the properties of succinate-grown cells which are then allowed to grow 75% of a generation in the presence of a mixture of succinate and glucose are intermediary in character. Presumably, the decreased levels of the products of the lactose operon is a reflection of catabolite repression due to the glucose. The increased

TABLE III

PROPERTIES OF CARBON-LIMITED CHEMOSTAT GROWN CULTURES OF ML308

	*Doubling time and limiting carbon source input concentration**				
	41 min *0.02 % glucose*	*196 min* *0.02 % succinate–* *0.01 % glucose*	*175 min* *0.02 % glucose* *6-phosphate*	*10.6 h* *0.01 % glucose–* *0.02 % succinate*	*4.3 h* *0.04 % succinate*
β-Galactosidase					
In vitro ONPG	—	13 300	11 700	11 900	9300
Galactoside permease					
[^{14}C]TMG uptake 4 min	—	2130	4000	1960	890
ONPG *in vivo* hydrolysis	99**	595	503	441	511
Glucose permease					
^{14}C-labeled α-methyl-D-glucopyranoside uptake 4 min	1880	1175	332	306	301
Maximal inhibition of galactoside permease by α-methyl-D-glucopyranoside					
TMG uptake, %	44	14	—	13	—
ONPG *in vivo* hydrolysis, %	57	18	18	14	14
Inhibition of 4 min α-methyl-D-glucopyranoside uptake by 0.9 mM TMG, %	49	24	—	25	13

* Residual carbon source content in the chemostat cultures in all cases is several orders of magnitude below the input concentrations and is essentially zero during measurement.

** Measurement not valid due to energy starvation (see text).

TABLE IV

GLUCOSE-LIMITED CHEMOSTAT ML308 AT DIFFERENT FAST GROWTH RATES

	Doubling time (h):				
	1.66	*2.24*	*2.24*	*0.942*	*0.54*
β-Galactosidase (μmoles/g per min)	6030	12 800	10 500	8200	3300
Galactoside permease net *in vivo* hydrolysis (μmoles/g per min)	246	479	440	400	—
Glucose permease (^{14}C-labeled α-methyl-D-glucopyranoside) (nmoles/g per 4 min)	3450	2200	2720	3000	1420
Inhibition of 4 min α-methyl-D-glucopyranoside uptake by 0.9 mM TMG (%)	64	7	20	35	20

level of glucose permease appears to be a specific inductive response of the glucose permease[4,5*].

A variety of control measurements were performed in addition to those presented in Table II. Since these results were observed in all cases, we can summarize them here, understanding that they apply as well to the data of Tables III and IV. In all cases, 10 mM glucose or α-methyl-D-glucopyranoside blocks or dilutes out essentially completely the uptake of 2.45 μM ^{14}C-labeled α-methyl-D-glucopyranoside. Uptake of α-methyl-D-glucopyranoside is linear well beyond 4 min and stimulated 2.5–5-fold by 0.2 M azide. The uptake of [^{14}C]TMG at 4 min is about one-half that predicted by direct proportionality with the 30 sec uptake. This corresponds to a half time for the uptake process of 1.5 min at 15°. Non-isotopic TMG at a final concentration of 0.9 mM lowers uptake in 4 min to 1/3 of that without carrier. From this, a K_m for uptake of 400 μM can be calculated. These two rough calculations are in good agreement with the half time of 1.5 min previously observed[3] at this temperature, and the K_m for uptake of 500 μM quoted by KEPES[6]. Another generally observed result was that the inhibition of TMG uptake by glucose or α-methyl-D-glucopyranoside is greater when measured over 30 sec than when measured over 4 min. This means that glucose and α-methyl-D-glucopyranoside have a greater effect on the initial velocity than on the steady-state plateau suggesting that there is also an inhibition of the exit process, so that both entry and exit are decreased in the presence of these agents. Also regularly observed is that saturation concentrations of glucose initially cause less inhibition of ONPG *in vivo* hydrolysis than does α-methyl-D-glucopyranoside.

* In strain ML, the inductive effect of glucose during growth raises the apparent v_{max} of uptake and the apparent K_m does not change (ref. 5 and KOCH, unpublished). In strain K-12, the apparent K_m decreases while v_{max} remains constant (KEPES, personal communication). The measurements in the present paper were made at concentrations well below any literature estimate of K_m and are therefore estimates of v_{max}/K_m or the second order rate constant for the interaction of the substrate with the permease part of the transport system times the concentration of permease. Therefore, it is likely to be a measure of the concentration of permease molecules in the cell membrane, independent of the strain differences between ML and K-12. Probably, the results with K-12 imply that some other stage in the transport system limits the maximum rate of transport.

An important confirmation in Table II is that there is little or no reciprocal inhibition by TMG of α-methyl-D-glucopyranoside uptake as previously reported by WINKLER AND WILSON[5] in glucose grown cells.

We have previously proposed[3,4,9-11] that the cell consists of a mosaic of carbohydrate transport sites and that a particular site might have only one kind of permease, possibly in multiple copies, or it might have several different kinds. It was hypothesized that interaction of fluxes would only be possible *via* those sites possess-

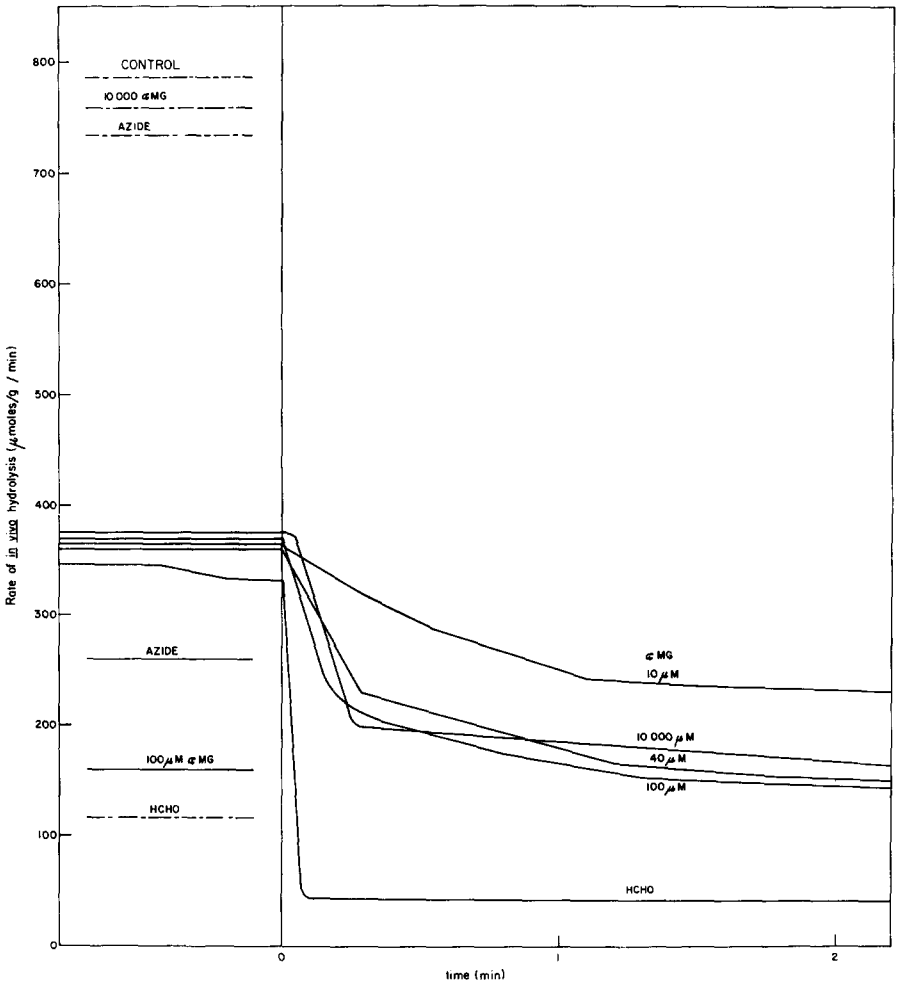

Fig. 7. The response of transient cells grown first in succinate and then one generation in glucose to α-methyl-D-glucopyranoside and azide. The control values for succinate-grown organisms are shown by the dash-dotted lines. The steady-state values for the control cells and cells grown 1 generation in 0.2% glucose culture are 669 and 295 μmoles/g per min; the maximum inhibition by α-methyl-D-glucopyranoside is 3% and 59%; the inhibition by 2 mM N_3^- is 7% and 26%. The rates were measured as described in the text. The initial transients had a half-life of 5 sec or faster, even though this is not evident in this type of plot. 10 μM of α-methyl-D-glucopyranoside gave a final inhibition of 33% and since this is very nearly one half of 59%, 10 μM is very nearly the half-inhibitory concentration of α-methyl-D-glucopyranoside. There was a lag in growth on shift to glucose; over the first generation, the average doubling time was 68 min. αMG = α-methyl-D-glucopyranoside.

ing different kinds of permeases. On this basis, the above observations all could be interpreted to mean that glucose grown cells had an abundance of sites only transporting glucose (or α-methyl-D-glucopyranoside) and only a minor proportion of sites transporting solely galactosides. With this hypothesis, the inhibition of TMG on α-methyl-D-glucopyranoside uptake should have been greater in the cells grown on succinate and then those grown on glucose. That result was certainly not observed, and seems to strongly argue against the local interaction hypothesis.

On the other hand, the initial kinetics of inhibition by α-methyl-D-glucopyranoside on a transient phase culture are more consistent with a local interaction hypothesis. Cells previously growing in succinate were grown for one generation in glucose alone with no succinate. Measurements of the rate of hydrolysis of ONPG by this culture are shown in Fig. 7; an attempt has been made to show the rate directly, and not the integral as measured by the absorbance *vs.* time curves such as in Figs. 1–3. It can be seen that the growth in glucose lowers the galactoside permease level and raises the degree of inhibition by α-methyl-D-glucopyranoside or by azide. The rates were constructed from the rates for successive increments of absorbance of 0.1. The rates have been plotted with lines shown through the rate for the 0.1 *A* interval at the time when the absorbance was at the half-way point of the interval. Actually, this representation does not show how fast the initial inhibition by α-methyl-D-glucopyranoside is. The inhibition is achieved in less than 5 sec. This is true even with low levels of α-methyl-D-glucopyranoside. All the other transients were at least that fast. Thus, these cells which are only partially induced for glucose permease show the immediate phase, but the delayed response is much less marked.

The intermediate steady-state response with 10 μM α-methyl-D-glucopyranoside also confirms our previous observation that the half-inhibitory concentration is the same in the bacteria partially induced for glucose permease as in the fully induced glucose bacteria[9], which is one of the strongest bits of evidence in favor of a local interaction.

Properties of chemostat grown cells

Another way to simultaneously achieve high levels of both the glucose permease and the galactoside permease is to grow the cells in chemostat culture. We have previously reported[4] that glucose-limited chemostat cultures of ML308 have high levels of galactoside permease (and β-galactosidase). This phenomenon has also been extensively studied (KOCH AND HSIE, unpublished). Therefore, a number of chemostat cultures in apparatus of a design described in ref. 12 with ML 308 limited in various ways and growing with various doubling times were studied. The properties of cells from steady-state cultures are shown in Table III. Succinate alone, glucose and succinate supplied at a low dilution rate, or glucose 6-phosphate all give high levels of the lactose operon products and low glucose permease, and therefore, correspond to catabolite non-repressive conditions.

On the other hand, more rapidly growing chemostat cultures with either 0.02% glucose or 0.01% glucose *plus* 0.02% succinate as a carbon and energy source have an increased amount of glucose permease. In the case of the 196-min glucose–succinate culture, lactose operon products are abundant and the inhibition of α-methyl-D-glucopyranoside on galactoside permease is lower. This is similar to the intermediate transient culture of Table II. Quite different, however, is the very rapidly growing

glucose chemostat culture shown in the first column of the table. These cells have an apparent low level of galactoside transport, an extremely high level of glucose permease, and α-methyl-D-glucopyranoside uptake is inhibited by TMG, as contrasted with all other conditions that we and WINKLER AND WILSON[5] have tested.

The low level of apparent galactoside permease in this case is an artifact and is due to energy depletion of these very rapidly metabolizing cells[10]. Low levels of glucose did stimulate the *in vivo* hydrolysis rate, but an accurate measurement of the permease level was not possible because of the concomitant glucose inhibition of function. The inhibition of α-methyl-D-glucopyranoside uptake is not an artifact of energy supply because azide was observed to have its usual effect of increasing uptake 2.5-fold.

To extend these results, another chemostat with glucose as the sole carbon source was set up. The dilution rate was altered several times and each time after 4–5 doubling times, samples of cells were taken from the chemostat and the various assays performed. These results are shown in Table IV. It can be seen that in the first sample taken, very high glucose permease and moderate amounts of galactoside permease were present. Perhaps the levels of these products were not at steady-state yet. (The chemostat inoculum had been prepared in 0.04% limiting glucose.) But very clearly, a high degree of inhibition of α-methyl-D-glucopyranoside transport by TMG was observed. When the dilution rate of the chemostat was decreased, the β-galactosidase level rose and the inhibition decreased but was significantly greater than zero at all flow rates. At the highest flow rate shown, the culture was no longer growing under carbon-limited chemostat conditions but were washing out as the turbidity had fallen slightly.

Energy requirement for the indirect effect

It has recently been demonstrated[10] that metabolic energy is needed for all of the transport processes studied here. It has been long known that TMG accumulation is prevented by energy poisons such as azide, while the *in vivo* hydrolysis of ONPG is only slightly decreased by energy poisons. In contrast, α-methyl-D-glucopyranoside accumulation is even stimulated by energy poisons. It has been found that cells can be sufficiently depleted of energy reserves so that none of these processes take place until a metabolized carbon source has been supplied. This depletion can be accomplished by allowing the cells to attempt to accumulate α-methyl-D-glucopyranoside in the presence of azide and no carbon source. Subsequently none of the three transports can be demonstrated in the cell suspension after they have been washed to remove the azide and α-methyl-D-glucopyranoside. Such cells fully recover when supplied with a trace of glucose. This finding makes it reasonable to retain the possibility that there is some common energy requirement for transport (perhaps PEP or heat-resistant protein phosphate of the phosphotransferase system of ROSEMAN AND KUNDIG[13]).

Previous work[5] showed that azide inhibited *in vivo* hydrolysis slightly, and the same percentage inhibition was found in the presence of α-methyl-D-glucopyranoside as in control cells. We therefore tried the obverse experiment with an agent that increases the energy supply. Succinate grown ML308 cells were allowed to grow 2 generations in glucose *plus* succinate. The properties of the washed cells are presented in Table V. Measurements were made in the magnetically stirred cuvette. This was

TABLE V

TEST FOR ENERGY DEPLETION DUE TO α-METHYL-D-GLUCOPYRANOSIDE PUMPING

Succinate growing cells, allowed to grow 2 generations in glucose plus succinate	*μmoles/g per min* ± *S.E.*
In vitro hydrolysis	
β-Galactosidase	6460
In vivo hydrolysis	
Control	456 ± 19
10 mM α-methyl-D-glucopyranoside	198 ± 10
10 mM α-methyl-D-glucopyranoside + 0.2% succinate	210 ± 9
0.2% succinate	424 ± 1.5
10 mM formaldehyde	37.5 ± 5.5
500 μM TDG	33
1390 μM glucose	247 ± 12
1390 μM glucose + 10 mM α-methyl-D-glucopyranoside	230 ± 23

done so that there would be adequate aeration to permit the succinate to be metabolized. It can be seen that an energy source like an energy poison does not reverse the effect of high concentrations α-methyl-D-glucopyranoside on the *in vivo* hydrolysis of ONPG.

Dominance of glucose over α-methyl-D-glucopyranoside in inhibiting ONPG transport

Fig. 8 shows that although glucose inhibits *in vivo* hydrolysis of ONPG to a smaller degree initially than does α-methyl-D-glucopyranoside a mixture of the two give the smaller inhibition instead of the larger. First, it should be noted that 100 (Curve F) or 10000 μM α-methyl-D-glucopyranoside (Curve G), 5000 μM glucose (Curve E), or a mixture of 100 μM α-methyl-D-glucopyranoside and 50 μM glucose (Curve D) all lead to a final rate of ONPG hydrolysis of between 42 and 48 μmoles/g per min, well below the uninhibited control of 273 (Curve A), but significantly above the formaldehyde control of 24 (Curve H). The kinetics of attainment of this final degree of inhibition is different with the different inhibitory situations. The full inhibition is essentially instantaneous with the high concentration of α-methyl-D-glucopyranoside (Curve G); it takes a minute to develop for the smaller concentration (100 μM) of α-methyl-D-glucopyranoside (Curve F); a high concentration of glucose gives instantaneous partial inhibition at a higher rate (96 μmoles/g per min) than given by α-methyl-D-glucopyranoside (Curve E). Later and quite abruptly the inhibition becomes more severe; 50 μM glucose (Curve C) initially gives the same initial effect as 5000 μM glucose (Curve E). However, with the lower concentration the inhibition is completely relieved in a few minutes when the glucose is taken up by the cells. This is discussed more fully below. The interesting point is the intraction between 50 μM glucose and 100 μM α-methyl-D-glucopyranoside (Curve D). This mixture initially results in a rate of hydrolysis of 96 indistinguishable from that of glucose alone. When the glucose is consumed or taken up, the more severe inhibition due to the 100 μM α-methyl-D-glucopyranoside alone is expressed. Other controls in the same experiment are not shown, but demonstrated that this dominance of glucose effect is also exhibited in the presence of azide and fluoride; although it must be noted in passing that 50 μM glucose is more quickly taken up in the presence of azide. This is consistent with the well-known stimulation of α-methyl-D-glucopyranoside uptake by azide.

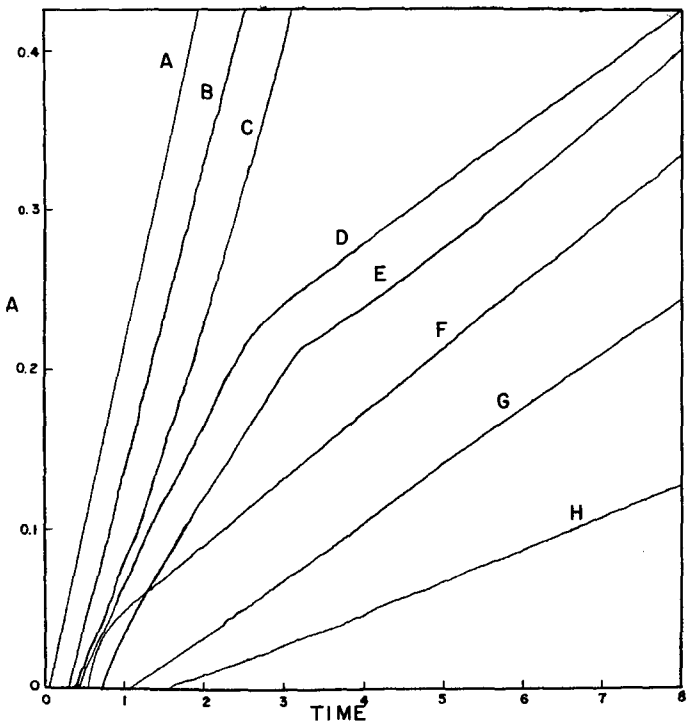

Fig. 8. Two phases of inhibition by glucose and α-methyl-D-glucopyranoside. A single batch of glucose-grown cells was used throughout. The following treatments were given starting a few seconds before the ONPG was added at A = 0. A, uninhibited control; B, azide; C, 50 μM glucoses D, 100 μM α-methyl-D-glucopyranoside + 50 μM glucose; E, 5000 μM glucose; F, 100 μM α-methyl-D-glucopyranoside; G, 10000 μM α-methyl-D-glucopyranoside; H, formaldehyde-inhibited control.

To measure the degree of inhibition by glucose accurately at low concentrations, either lower cell concentration may be used so that there is a long interval elapse before the glucose is consumed or the process can be measured through a longer light path. The former approach was used for the data reported in ref. 10. To demonstrate that the loss of the initial inhibitory action of 50 μM glucose is due to the depletion of the glucose, the second approach was employed. *In vivo* hydrolysis of ONPG was measured in a cuvette of 1 cm light path and again with the same batch of bacteria in a 5-cm cuvette. In both, the initial glucose concentration was the same and the same total amount of bacteria were in the light path. It was found that the slopes before glucose was added were identical and equal to the final slope after the glucose was consumed. The period of time when the transport was inhibited was about 5 times shorter with the 1-cm cell. The maximal degree of inhibition wich could be accurately measured with the longer cuvette was the same as that achieved initially by much larger concentrations of glucose.

Returning to Fig. 8, after a considerably longer interval glucose (Curve E) yields a similar maximal inhibition as does α-methyl-D-glucopyranoside (Curve G). Other experiments have shown that the break takes place at a time dependent on the time the cells have been exposed to glucose and are roughly independent of the dura-

TABLE VI

THE DELAYED SECOND INHIBITORY EFFECT OF GLUCOSE ON GLUCOSE-GROWN ML308

Time of break after ONPG hydrolysis (min)	*Control*	*Rate of in vivo hydrolysis of ONPG (μmoles/g per min)*	
		Immediate response	*Post-break response*
12	5000 μM glucose	186	120 (approx.)
3	5000 μM glucose for 12 min prior to ONPG addition	204	137
—	10000 μM α-methyl-D-glucopyranoside	82	—
13	10000 μM α-methyl-D-glucopyranoside + 5000 μM glucose	177	120 (approx.)

tion of the ONPG hydrolysis (Table VI). The break took place at the 12th min when the ONPG and 5000 μM glucose were added together. After a 10-min preincubation with glucose, the break took place after only 3 min of ONPG hydrolysis. The break was at the 13th min in the presence of 10000 μM α-methyl-D-glucopyranoside and 5000 μM glucose. In all cases the same prebreak slope characteristic of the maximal first phase glucose effect was observed. The same final slope as produced by high α-methyl-D-glucopyranoside concentration was observed after the break.

DISCUSSION

The experiments described here demonstrate in several different ways that the effects of both glucose and α-methyl-D-glucopyranoside on galactoside transport are multiple. The observations presented here are consistent with the assumption that there is a local competition that is very rapidly established and which is given by both glucose and α-methyl-D-glucopyranoside. In addition, a more severe inhibition develops very quickly if high concentrations of α-methyl-D-glucopyranoside are used and only after a several min lag at lower concentrations or after 10–13 min by glucose. If cells are preincubated in high concentrations of α-methyl-D-glucopyranoside and then diluted by a large factor, the inhibitory effect is largely abolished, not instantaneously, but much more rapidly than the time for the loss of α-methyl-D-glucopyranoside and α-methyl-D-glucopyranoside 6-phosphate.

Data from chemostat cultures show that it is possible to demonstrate the reciprocal inhibition of a galactoside on α-methyl-D-glucopyranoside uptake, but only in glucose-limited high dilution rate chemostat cultures which have high levels of both glucose permease and the constituent galactoside permease.

These various observations can be understood on the assumption that there is a local interaction dependent on the propinquity of the two different kinds of permease molecules on the membrane. The second indirect effect takes time to develop before it inhibits transport of galactoside. All of the observations of WINKLER AND WILSON[5] can be fitted into the scheme. The immediate effect is evidence for some common element retained locally at the membrane needed for galactoside and for glucose and

glucoside transport. These experiments do not permit the choice between the transporter of KEPES[6], the common factor of WINKLER AND WILSON[5], or a bound element of the phosphotransferase system or some other factor yet unnamed. However, for this immediate process the factor must be fixed to some quite small region of the membrane because its action responds immediately to the external concentrations.

The indirect effect may be due to depletion of some energy coupling but also may be exerted by some control mechanism of metabolism of the bacteria; if this is so, then this new delayed inhibition is yet a different glucose effect than those reported in the literature.

ACKNOWLEDGEMENTS

This investigation was supported by the National Science Foundation through GB 7022 and by the U.S. Public Health Service through Grant AI-09337.

REFERENCES

1 D. P. KESSLER AND H. V. RICKENBERG, *Biochem. Biophys. Res. Commun.*, 10 (1963) 482.
2 A. L. KOCH, *Ann. N.Y. Acad.*, 102 (1963) 602.
3 A. L. KOCH, *Biochim. Biophys. Acta*, 79 (1964) 177.
4 J. BONIFACE AND A. L. KOCH, *Biochim. Biophys. Acta*, 135 (1967) 756.
5 H. H. WINKLER AND T. H. WILSON, *Biochim. Biophys. Acta*, 135 (1967) 1030.
6 A. KEPES, *Biochim. Biophys. Acta*, 40 (1960) 70.
7 H. HAGIRA, T. H. WILSON AND E. C. C. LIN, *Biochim. Biophys. Acta*, 78 (1963) 505.
8 P. HOFFEE, E. ENGLESBERG AND F. LAMY, *Biochim. Biophys. Acta*, 79 (1964) 337.
9 A. L. KOCH AND J. BONIFACE, *Biochim. Biophys. Acta*, 225 (1971) 239.
10 A. L. KOCH, *J. Mol. Biol.*, 59 (1970) 000.
11 A. L. KOCH, *J. Theoret. Biol.*, 14 (1967) 103.
12 A. L. KOCH, *Adv. Microbial Physiol.*, 6 (1971) 147.
13 W. KUNDIG, F. KUNDIG, D. ANDERSON AND S. ROSEMAN, *J. Biol. Chem.*, 241 (1966) 3243.