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Role of Metabolic Energy in the Transport of β-Galactosides by *Streptococcus lactis*[1]

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Streptococcus lactis (ATCC 7962) accumulated thiomethyl-β-galactoside (TMG) and other galactosides against concentration gradients when the cells were supplied with a metabolizable substrate, such as glucose. The accumulated TMG was free and not phosphorylated. In the absence of glucose, TMG rapidly entered the cell to a concentration equal to that of the medium. Agents that uncouple oxidative phosphorylation abolished active transport but not the carrier-facilitated entry of TMG. Evidence that the transport carriers were functional in the absence of glucose or in the presence of uncoupling agents included the demonstration of counterflow, which depends on competitive inhibition for the carrier for exit.

Active transport of a sugar across a membrane against a concentration gradient requires the expenditure of energy, and inhibition of energy-yielding reactions of the cell would be expected to block accumulation. Inhibition of oxidative phosphorylation has been found to abolish the accumulation of the nonmetabolizable sugar thiomethyl-β-galactoside (TMG) in *Escherichia coli* (16). However, these inhibitors do not affect the simple entry and hydrolysis of *o*-nitrophenyl-β-galactoside (ONPG) which depends on carrier activity and not on accumulation (2, 10). Such studies imply that energy is essential only for accumulation but is not required for "facilitated diffusion" (or carrier-mediated transport). Winkler and Wilson (23) using metabolic inhibitors provided evidence that coupling with metabolic energy reduces the affinity of the carrier for its substrate in the exit direction. Recent studies with energy-uncoupled mutants have supported these conclusions (24, 25).

An experimental difficulty with *E. coli* is that it stores appreciable quantities of endogenous substrate which can be utilized to provide energy in the absence of added substrate. Thus, a correlation between utilization of exogenous substrate and transport is not feasible. In an attempt to circumvent these problems, a study was initiated with *Streptococcus lactis*. This organism is capable of transporting β-galactosides (3, 13), but it does not possess significant stores of utilizable endogenous substrate (19).

In this communication, studies are reported on the transport of several galactose analogues by *S. lactis* 7962. It was shown that this organism could only accumulate sugars against a concentration gradient when the cells were supplied with a metabolizable substrate. Accumulation was abolished by agents that uncouple phosphorylation from oxidation, but carrier activity was not impaired.

MATERIALS AND METHODS

Chemicals. D-[*1*-^{3}*H*]fucose was bought from Amersham Searle; thiomethyl-[^{14}C]-β-galactopyranoside (^{14}C-TMG) from New England Nuclear Corp.; thioisopropyl-[^{14}C]-β-D-galactopyranoside (^{14}C-IPTG) from Schwartz BioResearch, Inc.; thio-β-D-[^{3}H]-digalactopyranoside (^{3}H-TDG) and carbonylcyanidefluoromethoxyphenylhydrazone (CCFP) were gifts from E. P. Kennedy. Chloramphenicol was donated by Parke, Davis & Co. Nonradioactive TMG and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Calbiochem, and TDG, IPTG, and D-fucose were from Mann Research Laboratories. Pentachlorophenol was bought from Eastman Organic Chemicals.

Growth of S. lactis. Cells of *S. lactis* (ATCC 7962) were grown to mid-exponential phase at 37 C in 250-ml flasks with side arms without agitation in 200 ml of the complex medium of Citti et al. (1) which consists of: yeast extract (Difco), 10 g; Tryptone (Difco), 10 g; gelatin (Difco), 2.5 g; sodium chloride, 4 g; ascorbic acid, 0.5 g; sodium acetate, 1.5 g; water to a final volume of 1,000 ml; the solution was neutralized with sodium hydroxide, and D-galactose was added after autoclaving to a concentration of

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1%. The culture was inoculated by adding 20 ml of an overnight culture (stationary phase) to 200 ml of fresh medium.

The cells were harvested by centrifugation for 10 min at 12,000 × g at 4 C and washed with 30 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, the buffer used in all experiments. The washed pellet was suspended in 1 to 3 ml of phosphate buffer plus chloramphenicol (50 μg/ml). The absorbance of the cell suspension was determined in a Klett-Summerson colorimeter (no. 42 filter), and the dry weight of the cells was calculated from the previously determined relationship: 1 ml of cells at 100 Klett units is equal to 0.165 mg of dry weight.

The mutant *S. lactis* 7962-6 was kindly donated by W. E. Sandine (12).

Assays. The uptake of the various sugars was measured by exposing cells to ^{14}C-TMG, separating the cells from the medium by filtration on membrane filters (0.60-μm pore size, Millipore Corp.), and finally counting the cells on the filter in a liquid scintillation vial (23). The intracellular concentration of radioactive compound was calculated from the relationship between absorbance and intracellular water, which was determined from the sucrose space by the method described previously (8); 1 ml of cells at 100 Klett units (filter no. 42) equals 0.24 μliter of cell water. Counterflow was measured as described previously for *E. coli* (23).

RESULTS

Cells of *S. lactis* 7962 grown to exponential phase were incapable of accumulating the nonmetabolizable β-galactoside TMG in the absence of a metabolizable substrate but were capable of rapidly transporting this sugar into the cell until the concentration in the intracellular water equilibrated with that in the external medium (Fig. 1). Further evidence that membrane carriers were active under these conditions will be presented in a later section.

When glucose was supplied to the cells as an energy source, TMG was accumulated against a large concentration gradient (Fig. 1). Similar results were obtained with TDG (Fig. 2), the methyl pentose D-fucose (Fig. 3), as well as with IPTG (*not shown*). TMG was not appreciably phosphorylated by the cells, as less than 1% of the intracellular TMG was found to be in the anionic form when tested by chromatography on Dowex-1 (formate; reference 7).

Several sugars were tested for their capacity to supply metabolic energy for TMG accumulation. Of the sugars tested at pH 7, glucose was the most effective, whereas lactose gave less than 10% of the effect of glucose. The small effect of lactose may be due to the small contamination of commercial lactose (Sigma) by glucose. Arginine, an energy source for *S. lactis* (19), supported TMG accumulation to concentration one-third that reached with glucose (Fig. 1). TDG, ONPG, fucose, maltose, and melibiose were inactive. Although maltose was ineffective at pH 7.0, this sugar supported TMG accumulation at pH 4.0 to a concentration sixfold over that in the medium previously reported by Desai and Goldner (3).

The relative affinity of a sugar for the carrier (compared to that of TMG) was determined by measuring the inhibition of ^{14}C-TMG uptake by the added sugar. TMG uptake was measured in the presence of an inhibitor or, alternatively, the cells were allowed to accumulate ^{14}C-TMG, and its exit was measured after the addition of inhibitor. In the latter case, the

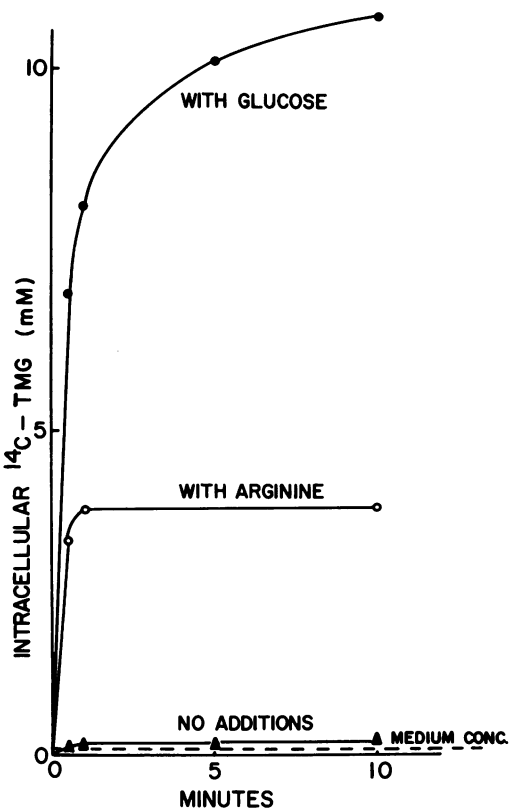

FIG. 1. *Dependence of TMG accumulation on glucose or arginine addition. Each tube contained: 0.1 ml of washed exponential-phase Streptococcus lactis 7962 cells at 7,800 Klett units; either 0.1 ml of 100 mM D-glucose or 0.1 ml of 100 mM arginine, as indicated; and 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, to a volume of 0.9 ml. After 5 min of incubation at 25 C, the reaction was started by the addition of 0.1 ml of 1.025 mM ^{14}C-TMG at 4.9 μCi/μmole. Portions of 0.2 ml were removed at intervals; the cells were collected on membrane filters, washed with 5.0 ml of buffer, and counted.*

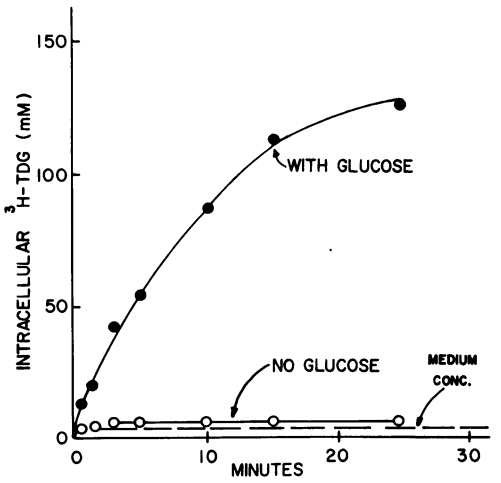

FIG. 2. *Accumulation of TDG. The experiment was performed as in Fig. 1; 0.1 ml of 20 mM ^{3}H-TDG at 2 μCi/μmole was added to start the reaction.*

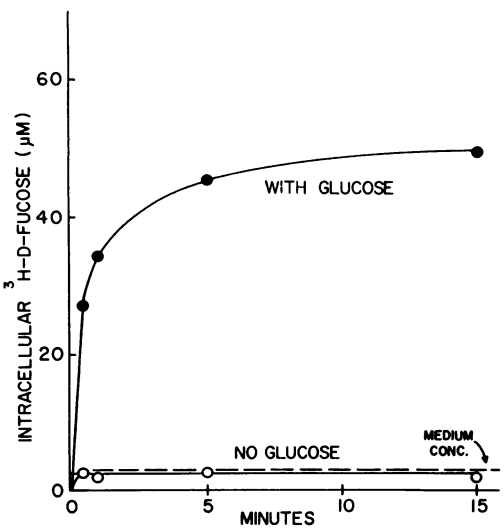

FIG. 3. *Accumulation of D-fucose. The experiment was performed as in Fig. 1. The reaction was started by adding 0.1 ml of 25 μM D-[^{3}H]fucose at 200 μCi/μmole.*

steady-state level is maintained by continuous influx and efflux and interference with influx leads to a rapid fall in intracellular level. Galactose (10 mM) strongly inhibited TMG uptake (Fig. 4) and rapidly reduced previously accumulated TMG to a very low level (*not shown*). Galactose added at a concentration of 1 mM to cells preincubated with ^{14}C-TMG also produced a rapid release of the radioactive sugar (Fig. 4). This inhibitory effect of galactose appeared to be a direct one on the membrane carrier for TMG and not on metabolism of the energy source. Added galactose was found to have no significant effect on the uptake or metabolism of glucose in this organism. Because of the high affinity of the carrier for galactose, it was impossible from these data to determine whether this sugar can act as an energy donor for TMG accumulation.

Although the carrier responsible for TMG transport showed a very weak affinity for lactose, this sugar was capable of entering the cells and supporting growth. This was shown by transferring galactose-grown cells to the rich medium (1) supplemented with various sugars at a concentration of 1%. The following growth rates were observed (doubling time in minutes): no sugar, 308; lactose, 84; galactose, 48; glucose, 36; gluconate, 300. Cells grown in rich medium containing 30 mM arginine (doubling

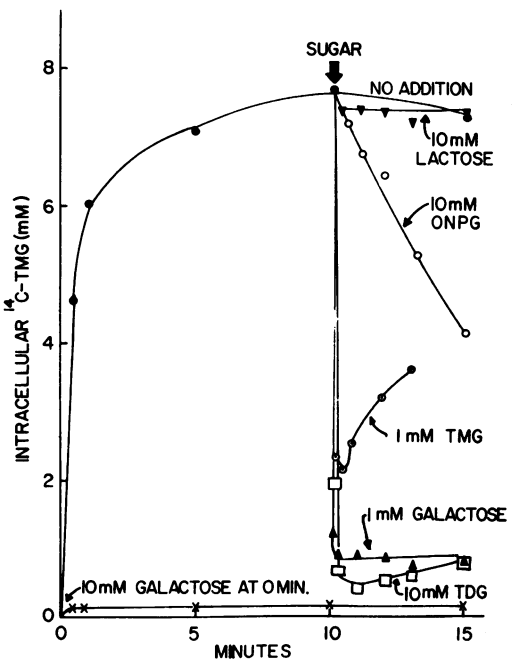

FIG. 4. *Effect of various sugars on TMG accumulation. Each vessel contained: 0.1 ml of washed Streptococcus lactis 7962 cells at 1,000 Klett units, 0.1 ml of 100 mM glucose, and 0.5 to 0.7 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, to a final volume of 1.0 ml. After 5 min of incubation at 25 C, 0.1 ml of 1 mM ^{14}C-TMG at 5 μCi/μmole was added to start the reaction. Portions of 0.1 were filtered at the indicated intervals. In parallel tubes, 0.1 ml of 10 or 100 mM sugar was added at 10 min, as indicated by the arrow. Portions of 0.1 ml were removed for cell filtration at the indicated times.*

time 80 min) showed very low TMG transport activity. When galactose was added to the growth medium, the cells showed a sixfold increase in TMG accumulation. Lactose induced transport to one-half that of galactose; IPTG, TMG, D-fucose and glucose had no inducing effect.

Effect of uncoupling agents. The addition of agents that uncouple oxidation from phosphorylation (2×10^{-6} M CCFP or 2×10^{-5} M pentachlorophenol) was found to abolish active transport of TMG by *S. lactis*. The inhibitors did not, however, affect the initial rate of entry of the galactoside, and there was rapid equilibration of the concentrations inside and out.

The activity of the carrier for TMG was apparently not dependent on sulfhydryl groups, as addition of *p*-chloromercuribenzoate (1 mM) had no effect on accumulation. Formaldehyde (10 mM) also had no effect.

Evidence for the presence of membrane carriers in energy-uncoupled cells. TMG entered cells rapidly in the absence of glucose (Fig. 1). That this entry was carrier-mediated was shown by the inhibition by chemical analogues known to competitively block active transport. Figure 5 shows that the rate of entry of TMG into glucose-deprived cells was reduced by galactose as well as by thiodigalactoside and D-fucose.

Further evidence for the presence of functional carriers was demonstrated by the competitive inhibition for the carrier on the inner surface of the membrane (counterflow; Fig. 6). Cells were preloaded with 20 mM nonradioactive TMG, separated from the medium, and placed in 0.5 mM ^{14}C-TMG. The radioactive sugar entered the cell but was "trapped" on the inside, because the nonradioactive sugar molecules, present in high concentrations, occupied most of the sites for exit. Thus, accumulation of the radioactive TMG occurred. The competition for exit persisted only for a brief period of time, as the inhibiting sugar molecules were transported out of the cell on the membrane carriers. Equilibration of internal and external TMG was finally established. This phenomenon, known as counterflow, is a characteristic property of membrane carriers uncoupled from energy (25). The activity of the carriers was not abolished by uncoupling agents that inhibit active accumulation. When CCFP was present in concentrations ranging from 5×10^{-7} to 5×10^{-5} M, neither the initial rate of entry of ^{14}C-TMG nor the position of the counterflow peak was altered.

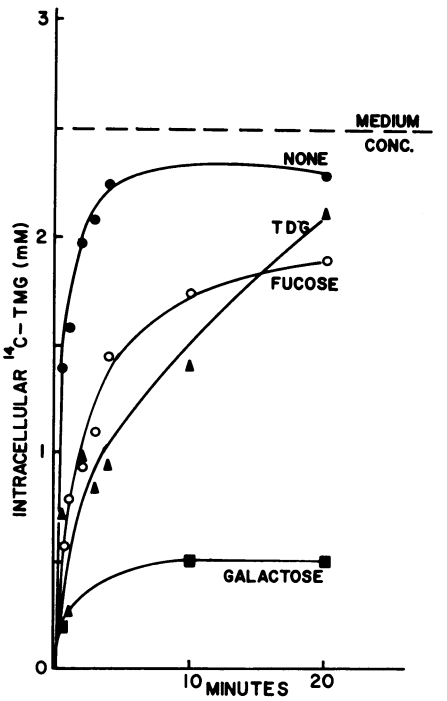

FIG. 5. *Effect of various sugars on TMG equilibration. Each tube contained: 0.2 ml of washed exponential-phase Streptococcus lactis 7962 cells at 8,000 Klett units; either 20 mM D-fucose, 20 mM TDG, or 10 mM galactose, as indicated; and 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, to a volume of 0.9 ml. The reaction was started by adding 0.1 ml of 25 mM ^{14}C-TMG at 0.1 μCi/μmole. Samples of 0.1 ml were removed at intervals, and the cells were filtered and counted as in Fig. 1.*

That transport carriers function even in the absence of an energy source was further indicated by comparing the activities of *S. lactis* 7962 with the mutant *S. lactis* 7962-6 which cannot ferment galactose (12). The mutant cells were capable of glycolysis but could not accumulate TMG in the presence of glucose. In the absence of glucose, the mutant cells required more than 30 min to achieve an intracellular concentration equal to that of the medium (1 mM), whereas the wild type reached equilibration within 2 min. The mutants thus appeared to lack transport carrier.

Efflux of TMG. The observation that cells did not accumulate β-galactosides without an added metabolizable sugar, yet appeared to have functional transport carriers, led to the expectation that exit of sugars was faster in cells that were incapable of accumulation. This

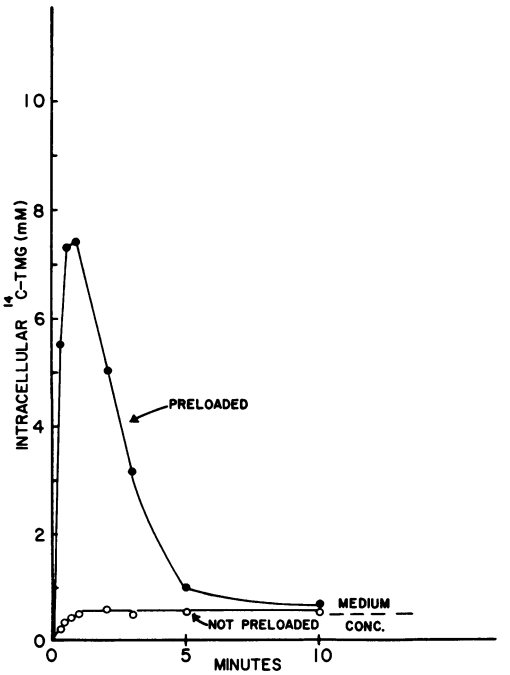

FIG. 6. *Counterflow. The experiment was performed essentially as described previously (23). Each tube contained: 0.5 ml of washed Streptococcus lactis cells at 4,500 Klett units, 0.4 ml of 100 mM TMG for preloading, and 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, to a volume of 2.0 ml. After 30 min of incubation at 25 C, the cells were centrifuged at 24,000 × g for 15 min at 4 C, and the sides of the centrifuged tubes were swabbed. The reaction was started by quickly mixing the cells with 3.0 ml of 0.5 mM ^{14}C-TMG at 1 μCi/μmole in phosphate buffer. Portions of 0.2 ml were removed at intervals; the cells were collected on membrane filters, washed with 5.0 ml of phosphate buffer, and counted.*

was found to be the case when efflux of accumulated β-galactoside was measured in the presence and absence of glucose (Table 1). Exit was more rapid from the cells in the absence of glucose (i.e., unable to accumulate sugars) than in the presence of glucose. Addition of uncoupling agent to cells in the presence of glucose increased the rate of exit. The inhibitor also slightly increased the exit rate from cells in the absence of added energy source, probably indicating an incomplete uncoupling of active transport in the absence of an added energy source.

DISCUSSION

These studies demonstrate that *S. lactis* 7962 accumulated TMG as the free sugar in a manner similar to that found in *E. coli*. The natural substrate for this transport system in galactose-grown cells appeared to be galactose, as the carrier possessed its greatest affinity for galactose and almost no affinity for lactose. Furthermore, galactose was a good inducer of TMG transport, whereas lactose was only one-third as active as an inducer. The galactose derivative 6-deoxy-D-galactose (D-fucose) seemed to share this carrier, as it was actively transported, and D-fucose shows inhibition of TMG entry.

The absence of energy coupling, although abolishing accumulation, did not lead to loss of carrier activity. In the absence of glucose, TMG entry was rapid and inhibited by galactose and TDG. In contrast, the transport-negative mutant showed extremely slow entry of the sugar in the presence or absence of glucose. Additional evidence for the presence of membrane carriers in uncoupled cells was the demonstration of counterflow. As in *E. coli*, uncoupling of *S. lactis* increased exit rate. All of these data are consistent with the view that energy uncoupling increases the affinity for exit, having relatively little effect on entry. Thus, removal of glucose abolishes active transport while the membrane carriers are still able to facilitate the equilibration of internal and external concentrations of sugar.

Galactoside carrier activity in *E. coli* has been reversibly reduced under special conditions. Reactivation on addition of glucose was

TABLE 1. *Efflux of ^{14}C-TMG from Streptococcus lactis 7962*[a]

Additions	Time for 50% loss of intracellular ^{14}C-TMG (sec)
A. None	48
B. Glucose	271
C. CCFP	22
D. Glucose + CCFP	51

[a] For preloading, each vessel contained: 0.4 ml of washed *S. lactis* 7962 cells at 4,800 Klett units, 0.4 to 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$, for a final volume of 2.0 ml; 0.4 ml of 100 mM glucose in vessels B and D; and 2 μliters of 10^{-2} M CCFP in 95% ethanol in vessels C and D. To achieve a starting intracellular ^{14}C-TMG concentration of about 10 mM, 0.2 ml of 1 mM ^{14}C-TMG at 5 μCi/μmole was added to vessel B; vessels A, C, and D received 0.6 ml of 10 mM ^{14}C-TMG at 0.05 μCi/μmole. After incubation for 30 min at 4 C, efflux was measured by diluting the cells 1:100 with buffer which contained 2.5×10^{-6} M CCFP in the case of vessels C and D. Samples (10 ml) were filtered and counted. All manipulations were carried out at 4 C.

described by Koch (11), and reactivation on addition of adenosine triphosphate (ATP) was described by Scarborough et al. (17) and by West (20). It seems likely that these effects are unrelated to the uncoupling phenomenon which is the subject of this paper.

Energy requirement for active transport. The profound effect in anaerobic cells of agents that inhibit phosphorylation or that uncouple oxidative phosphorylation is of considerable interest. In 1964, Whittam et al. (21) showed that oligomycin inhibited the sodium transport in the human erythrocyte, a cell without oxidative phosphorylation. Harold and Baarda (5) found that a variety of uncoupling agents blocked the utilization of metabolic energy for membrane transport by *S. faecalis*, a fermentative organism. Pavlasova and Harold (15) showed that β-galactoside accumulation in *E. coli* under anaerobic conditions was abolished by uncouplers without affecting carrier activity and without altering the steady-state level of ATP in the cell. These authors attribute the effect of uncouplers to the dissipation of proton gradients across the cell membrane, in accordance with the chemiosmotic theory of Mitchell (14). The present observations on *S. lactis* 7962 add further evidence that the source of energy for accumulation of certain sugars is a high-energy compound or high-energy state. This high-energy intermediate may be generated either by oxidative phosphorylation directly or from anaerobic metabolism via ATP.

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