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DISSOCIATION OF A GALACTOSE TRANSPORT SYSTEM BY WARM-WATER TREATMENT

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SUMMARY

When *E. coli* A is warmed for several minutes at 48°, while suspended in 0.1 M Tris buffer (pH 7.3) containing 10 mM $MgCl_2$, its viability is retained undiminished and its growth is initiated without lag. Little macromolecular substance is released from the cells into the medium, although an appreciable release of soluble nucleotide derivatives and peptidic materials does occur. Galactose uptake, which is usually biphasic, is dissociated by warm-water treatment. The extremely rapid, respiration-independent primary phase is retained, while the slow, respiration-dependent secondary phase is reduced. The secondary phase can be restored by incubating the treated cells in the chloramphenicol-containing uptake medium in air, but not in an atmosphere of N_2. As a result of warm-water treatment, the cells may become depleted locally of ATP or its metabolic equivalent, which is needed to activate and sustain the uptake system. However, this interpretation could not be verified by supplementing the treated cells with ATP, $MgCl_2$ or the warm-water extract. Further analysis revealed that the cells contain a constant amount of phosphorylated galactose during transport and that it is free galactose which accumulates. Since it occurs during the secondary phase of uptake, dephosphorylation of the transport intermediate may be coupled to respiration, and it may be this process which is sensitive to warm-water treatment.

INTRODUCTION

Galactose uptake by *Escherichia coli* A appears to be a complex process which is localized at the cell surface[1]. The continuing interest in this process is directed toward analyzing its complexity, isolating the components and locating their sites of action. These problems were approached by applying warm water treatment[2], which, like osmotic shock[3,4], is known to act on the cell surface without breaking the cell membrane. Warm-water treatment is capable of selective and sequential attack on the outer surface of the cell envelope[5]. The cell surface is weakened by depleting it of bivalent cations and stressed by raising the temperature to 48°. The extent of release of material can be varied by controlling the cell turgor against the weakened and stressed cell wall by adjusting the osmotic pressure of the suspen-

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ding medium to predetermined levels in a decreasing sequence. In 100 mM buffer, at pH 7.3, a lipopolysaccharide–protein complex is the principal, if not only, macromolecular substance released by warm-water treatment[27]. In 20 mM buffer, or less, other proteins are released, including a galactose-binding protein[2]. The liberation of most macromolecular material is prevented by the presence of 10 mM $MgCl_2$ during warm-water treatment, although some small molecules are released. The experiments to be described are concerned with the effect of warm-water treatment in the presence of $MgCl_2$ on the ability of *E. coli* A to take up galactose.

MATERIALS AND METHODS

The conditions for growing cultures of *E. coli* A in a lactic acid–inorganic salts medium have been described[6]. Viability and growth rate were determined in solid and liquid medium, respectively, using an enriched medium consisting of 0.5 % each of glucose, yeast extract and peptone, and 1.5 % of agar as indicated. Galactose uptake was determined using the lactic acid–inorganic salts medium supplemented with chloramphenicol (100 μg/ml) and 20 mM D-[1-^{14}C]galactose[6]. In certain uptake experiments, partial anaerobiosis was imposed by either vigorously bubbling commercial-grade N_2 through the uptake medium or by stopping the agitation of the cell suspension in the presence of air. An O_2 deficit was presumed to result from the metabolic activity of the high cell densities that were employed (8–10 mg/ml dry wt.), which could not be relieved when O_2 was limiting. Galactose 6-phosphate was separated from galactose on Dowex-1 (formate)[6]. Warm-water treatment[5] involved suspending the cells in 0.1 M Tris buffer (pH 7.3) for 1 h at room temperature, centrifuging and resuspending them in the same buffer at 48° for 20 min or less, and, finally, centrifuging and resuspending them in either the growth medium or the galactose uptake medium. In most of these experiments, 10 mM $MgCl_2$ was included in the suspending medium during warm-water treatment at 48° to prevent the release of macromolecular material[5]. The extracts obtained by warm-water treatment were analyzed chromatographically using DEAE-cellulose[2] and assayed by the following tests: (a) spectrophotometrically at 257 or 269 mμ, (b) the Folin–Denis reagent[7], (c) the biuret reagent[8], and (d) precipitation by 6 % trichloroacetic acid[2].

The galactose-negative culture used for these experiments, *E. coli* A, lacks both galactokinase (EC 2.7.1.6) and galactose-1-phosphate uridyl transferase (EC 2.7.7.10). It is able to grow in a glucose (or a lactic acid) medium, but not in a medium containing α-methylglucoside or galactose as the carbon source. The latter sugars are taken up by a common transport system, but they are not metabolized. Unlike earlier observations, uptake did not end after only a few minutes, but continued at a constant rate for at least 30 min. Continued uptake seemed to result from more effective agitation and, consequently, more efficient aeration of the uptake medium.

The transport system that is being studied with D-galactose as the principal non-metabolizable substrate, appears to be the glucose system[9]. Uptake is inhibited by D-glucose, but not by D-fructose or D-fucose. The latter two sugars enhance uptake to some extent. Galactose uptake is inhibited competitively by α-methylglucoside, 2-deoxyglucose and 3-*O*-methylglucose. Each of three sugars that were tested, α-methylglucoside, β-methylglucoside and galactose, was recovered as its 6-phosphate derivative. Since uptake apparently depends on a phosphorylative

mechanism, it was not surprising that D-glucuronic acid is also a competitive inhibitor of uptake. At 20 mM, maltose and lactose are not competive inhibitors, but modest noncompetitive inhibitors. At 2 and 20 mM, melibiose is not inhibitory at all, and thiomethylgalactoside is not inhibitory at 2.5 mM. Furthermore, this system does not appear to be any one of the four systems previously observed to transport galactose[10]: The *E. coli* A system is probably not the galactose permease[10] because its apparent K_m of accumulation is 20 mM compared with 0.1 mM for the galactose permease and because, at equal molar concentrations, α-methylglucoside inhibits uptake by 55–91 %, depending on the pH of the medium, whereas a 1000-fold excess inhibits the galactose permease only 15 %. The *E. coli* A system is probably not the methylgalactoside permease[10,11] because its K_m differs from the value of 10 μM reported for the methylgalactoside permease and because D-fucose is not a substrate as it is for the methylgalactoside permease. The *E. coli* A system is probably neither of the thiomethylgalactoside permeases[12,13] because thiomethylgalactoside, lactose and melibiose are not competitive inhibitors. *E. coli* A does not hydrolyze *o*-nitrophenyl-β-galactoside, unless previously induced.

RESULTS

The presence of 10 mM $MgCl_2$ prevents the liberation of most macromolecular material, but allows some small molecules to be released by warm-water treatment[5]. Chromatographic analysis of an extract prepared in the presence of $MgCl_2$ revealed two peaks of material that absorbed ultraviolet light and were not precipitated by trichloroacetic acid (Fig. 1). These materials were presumably nucleotide derivatives[3,14]. One of these peaks also coincided with reactivity toward the Folin–Denis reagent. A broad band of peptidic material was also observed, which reacted with both the biuret reagent and the Folin–Denis reagent. The identification of these

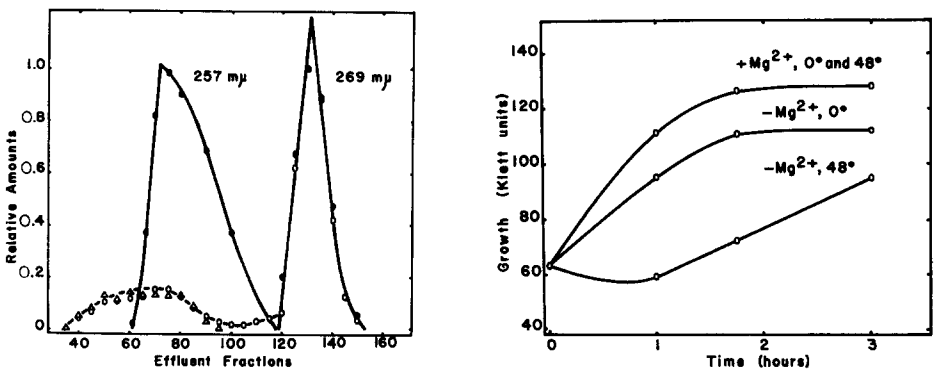


Fig. 1. Chromatographic analysis of a warm-water extract prepared in the presence of 10 mM $MgCl_2$ (6 min at 48°). Traces of macromolecular material were precipitated with 6 % trichloroacetic acid and removed by centrifugation before the extract was chromatographed on DEAE-cellulose[2]. Material was eluted using a 0–1 M NaCl gradient. The effluent was analyzed for material absorbing ultraviolet light (● ; λ_{max} as indicated) and reacting with the Folin–Denis reagent (○) and the biuret reagent (△).

Fig. 2. Effect of warm-water treatment in the presence of 10 mM $MgCl_2$ (6 min at 48°) on the rate of growth of *E. coli* A.

compounds is in progress because it might provide some useful clues to the reactions that occur during warm-water treatment.

The viability of *E. coli* A, as measured by colony counts, was not reduced by warm-water treatment at 48°, whether performed in the presence or absence of Mg^{2+}. In liquid medium, a lag in growth initiation did not occur as a consequence of warm-water treatment, unless Mg^{2+} was withheld during treatment (Fig. 2). A similar study revealed that the stability of the galactose uptake system depended on both the pH of the suspending medium and the presence of Mg^{2+} (Table I). At 0°, either low pH (pH 5.3) or Mg^{2+} stabilized the system nearly as well as a combination of low pH and Mg^{2+}. At 48°, low pH was a more important contributor than Mg^{2+} to the preservation of the uptake system. Evidently, growth and uptake rely on pH-dependent bonds involving bivalent cations, which, once broken, may be recovered to some extent.

TABLE I

EFFECT OF pH AND Mg^{2+} DURING WARM-WATER TREATMENT ON GALACTOSE UPTAKE BY *E. coli* A

In this experiment, the cell suspension was supplemented with 10 mM $MgCl_2$ while it stood for 1 h at room temperature before warm-water treatment. This modification was intended to moderate the effect of warm-water treatment in order to reveal the separate effects of pH and Mg^{2+} more clearly.

Pretreatment of cells			*Relative uptake of galactose*
pH	*Mg^{2+} concn. (mM)*	*Temperature*	
7.3	0	0°	0.68
	0	48°, 12 min	0.63
	10	0°	0.98
	10	48°, 12 min	0.70
5.3	0	0°	0.93
	0	48°, 12 min	0.82
	10	0°	1.00
	10	48°, 12 min	0.81

The lost uptake capacity of treated cells could not be restored by adding 20 mM $MgCl_2$ to the uptake medium or by returning the warm-water extract of treated cells, even at twice its nominal concentration. Furthermore, at a concentration of 4 mM, ATP was not effective for restoring the lost uptake capability. The treatment of SCARBOROUGH *et al.*[15], which depletes the lactose system of *E. coli* ML308 to reveal a requirement for ATP, did not elicit any change in the galactose system of *E. coli* A.

The influence of temperature on the galactose uptake system was studied at pH 7.3 in the presence of $MgCl_2$ (Fig. 3). At 68°, galactose uptake was completely abolished within 8 min, however, at 48°, galactose uptake was reduced, but never abolished. The loss in uptake capability proceeded at a constant rate until about one-half of the initial capacity was lost. The rate of loss of function resembled the characteristic rate of release of material from the cell surface[5], and it correlated with the rate of release of materials that absorbed ultraviolet light and reacted

with the Folin–Denis reagent. Clearly, in the presence of 10 mM $MgCl_2$, a substantial loss of function can occur without a corresponding loss of macromolecular material. This observation contrasts with the release of HPr, a component of the phosphotransferase system[16], and several nutrient-binding proteins[2,17–19] that occurs in the absence of Mg^{2+} or in the presence of EDTA.

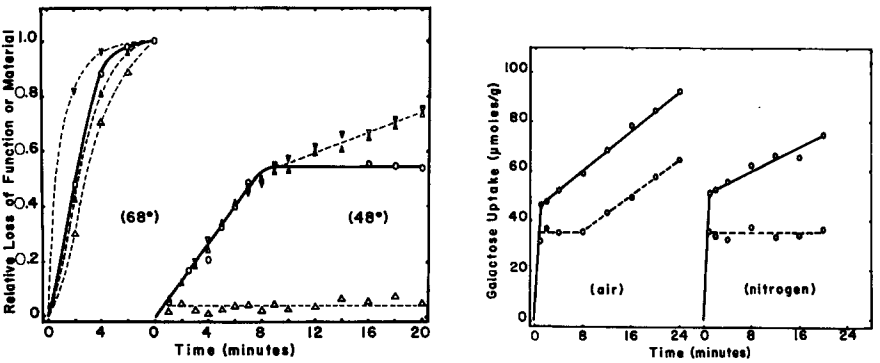


Fig. 3. Effect of temperature and time in the presence of 10 mM $MgCl_2$ on the loss of galactose uptake capability and on the release of cellular materials. (○), galactose uptake; (△), materials absorbing at 258 mμ; (▽), reacting with the Folin–Denis reagent; (△), and precipitating with 6 % trichloroacetic acid.

Fig. 4. Effect of warm-water treatment (12 min at 48°) and partial anaerobiosis on the kinetics of uptake of galactose by *E. coli* A. The atmosphere during uptake is indicated in parentheses. ———, untreated cells, - - - - -, treated cells.

The partial loss in function suggested that either the cell population might be heterogeneous with respect to its sensitivity to warm-water treatment or the galactose system might consist of two pools of uptake with differing susceptibilities toward warm-water treatment. The cell population did seem homogeneous, when treated at 48° in the presence of $MgCl_2$, because not only were the cells completely viable, but their growth proceeded without lag at a rate that was identical with the rate of growth of the untreated cells (Fig. 2). For these reasons, two pools of uptake which are dissociated by warm-water treatment seemed preferable.

Cells which had been treated at 48° for 12 min were compared with untreated cells for their ability to take up galactose. This comparison was made in air and in an atmosphere of N_2. Also determined was the extent of phosphorylation of the galactose that was taken up. Galactose uptake proved to be biphasic (Fig. 4). The primary phase was completed within 1 min and it was apparently independent of respiration, while the secondary phase proceeded at a constant rate over an extended period of time. The velocity of the secondary phase was reduced by one-half as a result of partial anaerobiosis produced by vigorously bubbling N_2 through the uptake medium.

The primary and secondary phases were dissociated by warm-water treatment. Without affecting the fast, respiration-independent primary phase of uptake, warm-water treatment inactivated the slow, respiration-dependent secondary phase of uptake. The effect of warm-water treatment was not permanent, however, because the operation of the secondary phase could be restored by incubating the treated

cells for 8 min in the uptake medium in air, but not in N_2. The effect of sublethal heat treatment has already been shown to be a temporary interruption of respiration[20].

Since chloramphenicol was present in the uptake medium, reactivation of the transport system probably did not involve protein synthesis, but may have depended on redistribution of preexisting substances, resynthesis of metabolites or reconstitution of altered structures.

As expected for a phosphorylative mechanism of sugar transport[16], the amount of galactose 6-phosphate recovered from the cells remained substantially unchanged during the transport process, while the amount of galactose increased with time (Table II) (*cf.* ref. 21). Pretreatment at 48° did not alter the ratio of galactose/

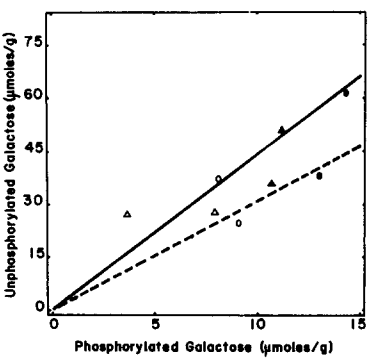
TABLE II

EFFECT OF WARM-WATER TREATMENT AND ANAEROBIOSIS ON THE EXTENT OF PHOSPHORYLATION OF THE GALACTOSE THAT WAS TAKEN UP BY *E. coli* A

Warm-water treatment 12 min at 48°.

Atmosphere during uptake	*Pretreatment of cells*	*Uptake (μmoles/g)*		*Ratio galactose/ galactose 6-phosphate*
		Galactose 6-phosphate	*Galactose*	
Air	0° (a)*	13.0	38.2	2.7
	0° (b)	14.3	61.4	4.3
	48°, 12 min (a)	9.1	24.6	2.7
	48°, 12 min (b)	8.1	37.1	4.6
N_2	0° (a)	10.7	35.9	3.4
	0° (b)	11.2	51.0	4.6
	48°, 12 min (a)	7.9	27.7	3.5
	48°, 12 min (b)	3.7	27.2	7.3

* (a) and (b) represent composites of 1–8-min samples and 12–24-min samples, respectively, which were obtained in Fig. 3.



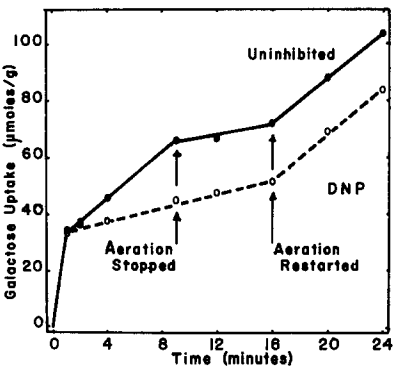


Fig. 5. Correlation between accumulation of galactose and amount of phosphorylated galactose recovered from *E. coli* A (data from Table II). ●, uptake in air by untreated cells; ○, uptake in air by warm-water treated cells. ▲, uptake in N_2 by untreated cells; △, uptake in N_2 by warm-water treated cells. Uptake time: - - - - -, 1–8-min composites; ———, 12–24-min composites.

Fig. 6. Influence of 50 μM 2,4-dinitrophenol (DNP) and partial anaerobiosis on the secondary phase of galactose uptake by *E. coli* A. Partial anaerobiosis was produced by stopping the agitation of the cell suspension in air at the times indicated with arrows. ●—●, uninhibited cells; ○- - -○, cells inhibited with dinitrophenol.

galactose 6-phosphate, although the amount of galactose 6-phosphate and the rate of accumulation of galactose were both reduced. The maintenance of an active phosphorylating system was apparently required for sustained uptake of galactose because warm-water treated cells when incubated under N_2 lost some phosphorylated galactose, while retaining galactose unchanged. The correlation between the amount of galactose 6-phosphate recovered from the cells and the accumulation of galactose is noted in Fig. 5.

Further evidence for the dependence of the secondary phase of uptake on respiration was provided by the observation that uptake was inhibited by either partial anaerobiosis or 50 μM dinitrophenol (Fig. 6). No additional effect was noted from partial anaerobiosis in the presence of dinitrophenol. As in Fig. 4, the normal rate for the secondary phase was regained when aeration was resumed, whether in the presence or absence of dinitrophenol. At 500 μM dinitrophenol, the secondary phase could not be recovered by prolonged incubation in air (Fig. 7).

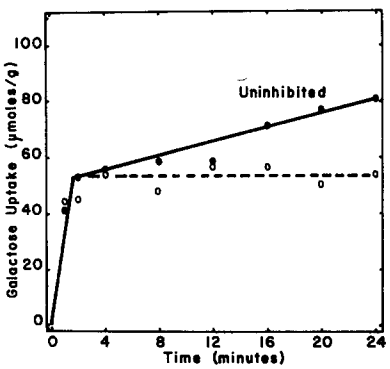


Fig. 7. Inhibition of the secondary phase of galactose uptake by 500 μM 2,4-dinitrophenol. ●—●, uninhibited cells; ○- - -○, cells inhibited with dinitrophenol.

DISCUSSION

The complexity of the process of sugar transport in *E. coli* is well established by the recognition of both a phosphotransferase system[16] and a class of protein that bind sugars in a highly specific manner[2,18]. In addition, the involvement of respiration in this process is evident[22]. Each of these factors can be rate-limiting under appropriate conditions. Perhaps, yet to be recognized is an additional factor that is required for freeing sugar from the phosphorylated transport intermediate. It has been assumed[23] that the product of sugar transport is the phosphorylated sugar, which is thought to accumulate during the transport process. At least in the case of *E. coli* A, this assumption is not valid because it is the free sugar that accumulates, while the amount of phosphorylated sugar remains constant during the uptake process (Fig. 4). Although the phosphotransferase reactions of ROSEMAN[23] provide a biochemical basis for at least a portion of the transport mechanism, they do not account for the respiration dependence of the overall process because phosphoenolpyruvate is presumably derived from glycolysis. Since the secondary phase of uptake is respiration dependent and it correlates with dephosphorylation of the transport

intermediate, a juxtaposition of these factors might be considered. Perhaps, dephosphorylation is coupled to respiration. This could account for what is observed here and for what has been observed by KEPES[22]. The involvement of both glycolysis and respiration in transport would provide for the push and the pull needed for uptake (*cf.* ref. 24). Still undetermined is the means by which low concentrations of N_3^- and dinitrophenol stimulate glucose transport[25], unless there are competing respiratory processes.

It may be profitable to seek membrane-bound phosphatases or phosphotransferases that function both in releasing sugar from the transport intermediate and in conserving the phosphoryl group. Highly specific acid phosphatases have been isolated from *E. coli* ML308 (see ref. 26), which exhibit specificities that approximate the specificity of the *E. coli* A transport system. For example, phosphatase IIc hydrolyzes glucose 6-phosphate and galactose 6-phosphate, but not fructose 6-phosphate[26], and the *E. coli* A transport system handles glucose and galactose, but not fructose[6].

The location and the isolation of the components of the transport system are related problems, and the solution of the latter problem may result from knowledge about their sites of action. For the reasons already enumerated[5], warm-water treatment offers some promise for the selective release of surface components, and, consequently, some idea about their intracellular location. Added discrimination is provided by the presence of $MgCl_2$ which prevents the release of macromolecular material while permitting the release of relatively small molecules. The possibility should still be considered that warm-water treatment, even in the presence of $MgCl_2$, causes other effects: macromolecules are displaced, but not released or they are displaced, degraded and released, and irreversible changes occur as the direct consequence of warm-water treatment or the release of degradative enzymes. Nevertheless, some useful clues to the structure and function of bacterial cell surfaces are likely from the analysis of extracts prepared by warm-water treatment and from the study of these modified cells.

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