# β-GLUCOSIDE PERMEASES AND PHOSPHO β-GLUCOSIDASES IN AEROBACTER AEROGENES: RELATIONSHIP WITH CRYPTIC PHOSPHO β-GLUCOSIDASES IN ENTEROBACTERIACEAE*

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Enterobacteriaceae show marked differences in the number of β-glucosides used as carbon sources.[1] These differences were linked to possible evolutionary relationships between *E. coli–Shigella* on the one hand and *Citrobacter–Salmonella* one the other.[2–4] *E. coli* wild-type strains (β-gl$^-$) are unable to utilize β-glucosides. From most strains, β-gl$^+$ mutants can be isolated which ferment salicin and other aryl β-glucosides, but not cellobiose. *Citrobacter* wild-type strains and mutants of various *Salmonella* species ferment cellobiose, and some strains show a weak fermentation of salicin and arbutin.[3, 4] With the exception of *E. coli*,[5, 6] no data are yet available on the catabolism of β-glucosides by these genera. Among Enterobacteriaceae, *Aerobacter aerogenes* can utilize the widest range of substrates (β-methyl glucoside, aryl β-glucosides, and cellobiose).[1, 2] Therefore, investigations on the mechanisms involved in the catabolism of β-glucosides by *A. aerogenes* could be useful in the elucidation of similar mechanisms in genera with a narrower substrate range. The present investigation indicates the existence of a complex system for the utilization of β-glucosides, consisting of two permeases, a transphosphorylase, and two enzymes which split only phosphorylated β-glucosides.

*Materials and Methods.—Bacterial strains:* The strain *A. aerogenes* $A_1$ is a collection strain from our Department. The strain *A. aerogenes* 2002 (gua$^-$, arg$^-$) and its derivatives 2050 and 2070, described by Tanaka and Lin,[7] were obtained from Dr. E. Lin. The strain *E. coli* K12 β-gl$^-$ and its β-gl$^+$ mutants have been described previously.[5] The strain *E. coli* $H_{23}$ (cellobiose$^+$, salicin$^-$, arbutin$^-$) was received from Dr. F. Ørskov. The *Citrobacter* strain B was received from Dr. F. Kauffmann and strain 532 from Dr. E. Ewing. The *S. typhimurium* strain 489 (ile$^-$, pur$^-$, met$^-$) was received from Dr. G. Dubnau; from this strain two spontaneous cellobiose-fermenting mutants were obtained ($C_1^+$ and $C_2^+$). $S_1^+$, a spontaneous mutant of $C_1^+$, in addition also ferments salicin and arbutin, and whole cells split PNP-glu constitutively.

*Culture media:* For enzyme and permease determinations the bacteria were grown in medium A,[8] supplemented with 0.2% yeast extract, 0.5% Na succinate, and the required growth factors — medium AY.[5] For cell-free extracts the cells were grown overnight by aeration in medium LB — 1% yeast extract, 2% tryptone (Difco), 0.1% NaCl, adjusted to pH 7.0 with NaOH.

*Active uptake of β-glucosides:* The active uptake of $C^{14}$-thiophenyl β-glucoside (TPG) was determined under the conditions previously described for *E. coli*,[5] but in the absence of chloramphenicol. "Chasing" experiments were performed with $2 \times 10^{-4}$ *M* of the labeled compound and $10^{-2}$ *M* of the cold compound.

*Enzyme determinations:* The determination of the splitting of *p*-nitrophenyl β-glucoside (PHP-glu), *o*-nitrophenyl β-glucoside (ONP-glu), phenyl β-glucoside, and salicin was made, as previously described,[5] by measuring the liberation of the aglycone. One unit of enzyme corresponds to the liberation of 1 mμmole aglycone/min. Phosphatase determinations were made in a volume of 0.15 ml containing: 5 μmoles of the tested phosphate

ester, 2.5 μmoles tris buffer pH 7.2, and 50–100 U of enzyme. One unit of enzyme liberates 1 mμmole $P_i$/min. Phosphate determinations were made by the method of Fiske and SubbaRow,[9] glucose determinations with "Glucostat" (Worthington), and protein determinations by the method of Lowry *et al.*[10]

*Enzymatic preparation of phosphorylated β-glucosides:* Crude preparations of phosphorylated β-glucosides were obtained by incubation for 2 hr at 37° of 2 ml 0.1 *M* phosphate donor (see *Results*), 1 ml phosphotransferase (2500–3000 U), 2 ml 0.1 *M* phosphate acceptor, and 1 ml 0.05 *M* tris HCl buffer pH 7.2. The reaction was stopped by addition of 20 ml acetone–ethanol 1:1 v/v, incubation for 2 hr at 37°, evaporation to dryness, and resuspension of the residue in 2 ml $H_2O$. This procedure did not always completely inactivate the phosphotransferase. An alternative method consists in inactivation of the enzyme by addition of dioxan to a final concentration of 15%, repeated extraction with ethyl ether, evaporation, and resuspension in $H_2O$.

*Cell-free extracts:* Washed cells, 50 mg dry weight/ml in 0.075 *M* phosphate buffer, were sonicated for 1 min in a "Sonifer" sonicator. After centrifugation for 20 min at 9000 × *g*, protamine sulfate was added to the supernatant to a final concentration of 0.4%; after centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant. Each fractionating step was held for 2 hr at 4° and pH 7.2, followed by centrifugation and dialysis. The fraction precipitating between 45 and 60% $(NH_4)_2SO_4$ was further purified by chromatography on a DEAE-Sephadex column as described in Figure 1.

*Abbreviations:* TPG, thiophenyl β-glucoside; PNP-glu, *p*-nitrophenyl β-glucoside; ONP-glu, *o*-nitrophenyl β-glucoside; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; F-1,6-P, fructose-1,6-diphosphate; PEP, phosphoenol pyruvate.

*Results.—Enzyme induction:* As previously reported for *E. coli*,[5] the hydrolysis of aryl β-glucosides by intact cells is induced by β-methyl glucoside, thiomethyl β-glucoside, TPG, phenyl β-glucoside, and salicin. In addition, cellobiose (0.5%) and lactose (1%) also act as inducers. Lactose is a weak inducer and splitting of β-glucosides can be detected only after induction of β-galactosidase. Lactose cannot be replaced by other inducers of β-galactosidase such as thiomethyl β-galactoside or melibiose. With PNP-glu as substrate, cells fully induced by salicin have an activity of 420 U/mg protein; cells induced by cellobiose, 280 U; cells induced by lactose, 63 U; while noninduced cells have an activity of 6 U.

*Active uptake of β-glucosides:* Prior investigations[5] showed that β-glucosides fermenting mutants of *E. coli* form an inducible stereospecific β-glucoside permease with high affinity for aryl β-glucosides and low affinity for cellobiose and α-methyl glucoside. While extraction with acetone–ethanol gave no indication for the accumulation of β-glucosides in a phosphorylated form, Fox and Wilson,[6] by using a different extraction method, show that β-glucosides are accumulated by the β-glucoside permease in a phosphorylated form, and that the phosphorylation occurs through the PEP-dependent kinase system of Kundig, Ghosh, and Roseman.[11] In *A. aerogenes*, by using $C^{14}$-TPG as substrate, it was found that TPG is accumulated by two inducible β-glucoside permeases. One, induced by aryl and alkyl β-glucosides, with high affinity for aryl β-glucosides and low affinity for cellobiose (β-glucoside permease I), is similar to the β-glucoside permease previously described in *E. coli*. A second permease induced by cellobiose and lactose (β-glucoside permease II) has a high affinity for both aryl β-glucosides and cellobiose. Chasing experiments (Table 1) showed that the uptake of TPG by permease I is increased by glucose, while glucose decreases the uptake by

TABLE 1. *Uptake of $C^{14}$-TPG by noninduced cells and cells induced by salicin, cellobiose, and lactose.*

| Addition ($10^{-2}$ M) | Non-induced | Induced | | |
|---|---|---|---|---|
| | | Salicin | Cellobiose | Lactose |
| — | 100 (8)* | 100 (38) | 100 (43) | 100 (27) |
| Phenyl β-glucoside | 75 | 31 | 33 | 41 |
| Cellobiose | 108 | 102 | 21 | 28 |
| Lactose | 91 | 93 | 97 | 95 |
| Glucose | 78 | 168 | 43 | 57 |
| α-Methyl glucoside | 74 | 53 | 41 | 56 |

Cells induced by growth in medium AY with $10^{-2}$ M salicin, $1.5 \times 10^{-2}$ M cellobiose, or $2.5 \times 10^{-2}$ M lactose. Concentration of TPG $2 \times 10^{-4}$ M (15,000 cpm per ml). Accumulation of TPG was determined with 500-μg cells.

* Uptake of TPG μmoles/gm.

permease II. In contrast to *E. coli* glucoside permease, β-glucoside permease I has relatively high affinity for α-methyl glucoside. β-Glucoside permease I produces a higher accumulation of $C^{14}$-TPG at 4° (maximal accumulation 53 μmoles/gm dry weight) than at 28° (38 μmoles/gm), while β-glucoside permease II shows a higher accumulation at 28°. The two permeases differ also in their $K_m$ values for the uptake of TPG. At 4° the $K_m$ for uptake by permease I is $3 \times 10^{-5}$ M and $2 \times 10^{-4}$ M by permease II.

In order to determine whether the PEP-dependent kinase system is coupled with the active uptake of TPG, the *A. aerogenes* wild-type strain 2002 and its pleiotrophic carbohydrate-negative mutants[7] 2050 (enzyme $I^-$) and 20070 ($HPr^-$) were tested in this respect. The wild-type strain is salicin$^+$ and cellobiose$^+$ and forms both inducible permeases. The mutants 2050 and 2070 are salicin$^-$ and cellobiose$^-$ and unable to form both permease I and permease II. A partial revertant of the mutant 2070 (2070 $S^+$), which ferments salicin but not cellobiose, becomes inducible for permease I but is unable to form permease II. It appears, therefore, that the coupling with the PEP-dependent kinase system is required for the accumulation of β-glucosides by both permeases.

In order to establish whether β-glucoside permease II is present in cellobiose-fermenting strains of other Enterobacteriaceae, the uptake of $C^{14}$-TPG and chasing by cellobiose was tested in cellobiose-fermenting strains of *E. coli*, *Citrobacter*, and *Salmonella*. The *E. coli* strain $H_{23}$ accumulates 5.5 μmoles/gm TPG (extr. concentration $2 \times 10^{-4}$ M); the internal concentration of TPG does not decrease by addition of $10^{-2}$ M cellobiose. The accumulation of TPG and affinity for cellobiose does not increase by growth in AY medium with 0.5 per cent cellobiose. In contrast to *E. coli*, the *Citrobacter* strains B and 532 (7.5 and 8.1 μmoles/gm) and the cellobiose-fermenting mutants *S. typhimurium* 489 $C_1^+$ and $C_2^+$ (5.3 and 6.5 μmoles/gm) accumulate TPG constitutively through a permease of type II, with high affinity for cellobiose. The constitutive accumulation of cellobiose by β-glucoside permease II increases to 28 μmoles/gm in the mutant 489 $S_1^+$, which, in addition, also ferments salicin and arbutin.

*Enzymatic activity of cell-free extracts:* Crude sonic extracts of noninduced cells and of cells induced by β-methyl glucoside, cellobiose, and lactose split PNP-glu, ONP-glu, phenyl β-glucoside, and salicin. While whole cells show a 70- to 90-

TABLE 2. *Splitting of ONP-glu by the 45–60 per cent ammonium sulfate fraction of sonic extracts.*

| Addition ($2 \times 10^{-2}$ *M*) | Non-induced | Inducer: β-Methyl glucoside | Inducer: Cellobiose | Inducer: Lactose |
|---|---|---|---|---|
| — | 42 | 83 | 72 | 56 |
| G-6-P | 231 | 479 | 451 | 324 |
| G-1-P | 242 | 461 | 423 | 328 |

Sonic extracts were made from cell suspensions of 50 mg/ml, in 0.075 *M* phosphate buffer, pH 7.4. Cells were grown in LB medium with 0.5% β-methyl glucoside, 0.5% cellobiose, or 1% lactose. The activity of the 45–60% ammonium sulfate fraction is expressed in mμmoles *o*-nitrophenol/min liberated by 1 mg protein.

fold increase of enzymatic activity by growth in the presence of β-methyl glucoside and cellobiose, the increase of activity of their cell-free extracts is only two- to threefold (Table 2). The enzyme(s) from extracts of noninduced cells has a low affinity for cellobiose, and this affinity does not increase after growth in cellobiose medium. The nature of the enzymatic splitting of cellobiose is still unknown. After fractionation with $(NH_4)_2SO_4$, the highest activity was found in the 45–60 per cent fraction. The activity of both crude extracts and the 45–60 per cent $(NH_4)_2SO_4$ fraction increases 5–8 times by the addition of $10^{-2}$ *M* G-6-P, G-1-P, F-1,6-P, α-glycerophosphate, and AMP and to a lesser extent by addition of ADP, ATP, *p*-nitrophenyl phosphate, and PEP. In contrast to α-glycerophosphate, β-glycerophosphate is a very weak activator. Detectable activation was obtained with $2 \times 10^{-4}$ *M* G-6-P, $8 \times 10^{-4}$ G-1-P, and $1.5 \times 10^{-3}$ α-glycerophosphate.

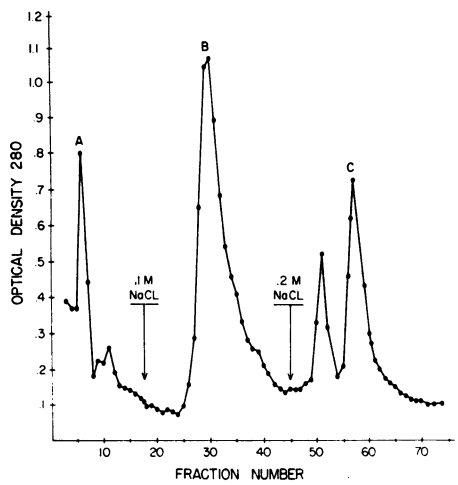


FIG. 1.—Chromatography on DEAE-Sephadex column (125-cm length, 2-cm i.d.). Elution with 0.05 *M* tris HCl buffer, pH 7.4, and stepwise addition of 0.1 *M* and 0.2 *M* NaCl. Input 230 mg protein of the 45–60% ammonium sulfate fraction from sonic extracts of cells induced by 0.5% β-methyl glucoside.

Further data on the enzymes involved in the splitting of β-glucosides were obtained by DEAE-Sephadex chromatography (Fig. 1). No single peak showed detectable splitting of aryl β-glucosides. Activity was, however, obtained by combining peaks *A* and *B* or *A* and *C* upon addition of G-6-P, F-1,6-P, or α-glycerophosphate. In contrast to crude extracts, G-1-P was much less active. Peak *A* contains a phosphatase-transphosphorylase, referred to as gl-phosphotransferase. Peaks *B* and *C* contain two distinct enzymes referred to as phospho β-glucosidases *A* and *B*. Phospho β-glucosidases *A* and *B* split β-glucosides only after they were phosphorylated by the gl-phosphotransferase.

*gl-Phosphotransferase:* The protein found in peak *A* has phosphatase as well as phosphotransferase activity.

In the presence of suitable acceptors (glucose, α- and β-glucosides) it acts mainly as a transferase and was therefore called gl-phosphotransferase. At pH 7.2 and 37°, 1 mg of enzyme protein splits 9.3 μmoles *p*-nitrophenyl phosphate/minute. If this activity is arbitrarily assigned a value of 100, the activities with other substrates are: α-glycerophosphate, 124; AMP, 73; F-1,6-P, 65; G-6-P, 57; ADP, 43; ATP, 38; G-1-P, 12; β-glycerophosphate, 2; and bis *p*-nitrophenyl phosphate, 2. Transferase activity was determined by comparing the quantity of $P_i$ liberated from phosphate esters in the presence and absence of equimolar concentrations of acceptors (Table 3). It was found that free glu-

TABLE 3. *Hydrolysis and transfer reactions catalyzed by gl-phosphotransferase.*

| Addition ($2.5 \times 10^{-2}$ *M*) | G-6-P | | *p*-Nitrophenyl-P | | α-Glycero-P |
|---|---|---|---|---|---|
| | $P_i$ | Glucose | $P_i$ | *p*-Nitro phenol | $P_i$ |
| — | 92 | 87 | 140 | 146 | 180 |
| Glucose | 13 | — | 11 | 89 | 9 |
| ONP-glu | 12 | 142 | 14 | 85 | 11 |
| Salicin | 44 | 130 | 60 | 112 | 39 |
| PNP-glu | 48 | 112 | 66 | 117 | 59 |
| Phenyl β-gluc. | 41 | 132 | 56 | 108 | 35 |
| β-Methyl gluc. | 27 | 118 | 47 | 128 | 28 |
| TPG | 14 | 151 | 27 | 104 | 17 |
| α-Methyl gluc. | 19 | 136 | 58 | 119 | 38 |

The hydrolytic and transfer activity was determined with 0.05 ml substrate, 0.05 ml acceptor, 0.05 ml gl-phosphotransferase, 0.05 ml tris HCl buffer, pH 7.2, 0.05 *M*. The final concentrations of the donor and acceptor were $2.5 \times 10^{-2}$ *M*.

cose as well as α- and β-glucosides can act as acceptors. Very weak or no detectable phosphate transfer was observed with galactose, lactose, *o*-nitrophenyl β-galactoside, fructose, sucrose, serine, adenosine, *p*-nitrophenol, and cellobiose.

In the presence of appropriate acceptors, the release of glucose from G-6-P can be stimulated by as much as 150 per cent. This could be explained by a competition in acceptor activity between the glucose liberated from G-6-P and the added phosphate acceptor. The presence of acceptors, however, decreases the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate.

Chromatography of the transfer products followed by autoradiography with $C^{14}$-TPG as acceptor indicated that the nature of the transfer products depends also on the phosphate donor. With G-6-P as phosphate donor (incubation for 1 hr at 37° and chromatography in butanol–pyridine–water 6:4:3), in addition to the initial spot for TPG ($R_f$ 0.74), there appear three new spots with $R_f$ values of 0.082, 0.13, and 0.19. With α-glycerophosphate as donor, only a single new spot ($R_f$ 0.13) was detected. Crude preparations of phosphorylated β-glucosides were obtained with G-6-P, α-glycerophosphate, and F-1,6-P as phosphate donors; PNP-glu, ONP-glu, phenyl β-glucoside, and salicin as acceptors; and inactivation of the enzyme by acetone:ethanol or dioxan. These compounds act as substrates for phospho β-glucosidases (Table 4). The best results were obtained with G-6-P as phosphate donor.

The gl-phosphotransferase preparation has the following properties: It retains 78 per cent of its activity after heating for three minutes at 100°, but is

TABLE 4. *Splitting of ONP-glu and ONP-glu phosphate.*

| | ONP-glu | ONP-glu and G-6-P | ONP-glu, G-6-P, and gl-phospho transferase | ONP-glu-P |
|---|---|---|---|---|
| (*A*) *A. aerogenes* phospho β-glucosidases | | | | |
| A | 0 | 0 | 53 | 43 |
| B | 0 | 0 | 36 | 31 |
| (*B*) Ammonium sulfate fraction* (45–60%) | | | | |
| *A. aerogenes* A 1 | 43 | 254 | 262 | 225 |
| *A. aerogenes* 2002 | 24 | 102 | 218 | 209 |
| *E. coli* β-gl⁻ | 0 | 15 | 158 | 161 |
| *E. coli* β-gl⁺ $c_1$† | 3 | 18 | 128 | 178 |
| *S. typhimurium* 489 | 0 | 5 | 73 | 82 |
| *S. typhimurium* 489 $S_{1+}$ † | 4 | 9 | 79 | 94 |
| *Proteus mirabilis* | 0 | 0 | 28 | 26 |
| *Ps. aeruginosa* | 0 | 0 | 0 | 0 |
| *Staph. albus* | 0 | 0 | 0 | 0 |
| *Staph. aureus* | 0 | 0 | 0 | 0 |

The enzymatic activity was expressed in mμmoles *o*-nitrophenol per mg protein. ONP-glu was in a concentration of $10^{-2}$ *M* and G-6-P $2 \times 10^{-2}$ *M*. The concentration of gl-phosphotransferase was 150 U. ONP-glu phosphate was prepared with G-6-P as phosphate donor.

* From sonic extracts of uninduced cells.

† Constitutive.

inactivated by $2 \times 10^{-3}$ *M* lauryl sulfate and 15 per cent dioxan. It is not inhibited by $10^{-2}$ *M* fluoride, EDTA, or inorganic phosphate. It has a broad pH optimum between 6.0 and 7.2. These properties differentiate this enzyme from other transferases described in Enterobacteriaceae.[12]

*Phospho β-glucosidases:* The activity of phospho β-glucosidases A and B was tested in the following systems: (*a*) gl-phosphotransferase, $2.5 \times 10^{-2}$ *M* ONP-glu, PNP-glu, phenyl β-glucoside or salicin, and $2.5 \times 10^{-2}$ *M* of a phosphate donor, in most instances G-6-P; (*b*) crude preparations of enzymatically phosphorylated β-glucosides (Table 4); (*c*) PNP-glu phosphate (synthesized by Dr. F. Fox). Phospho β-glucosidases A and B are unable to split free β-glucosides.

Phospho β-glucosidase A is contained in peak *B* (Fig. 1). The highest activity was obtained with the system containing G-6-P and gl-phosphotransferase. In this system the activity of the enzyme is as follows: ONP-glu, 56 U/mg protein; phenyl β-glucoside, 49 U; PNP-glu, 31 U; and salicin, 4 U. It retains 72 per cent of its activity after incubation for 20 minutes at 49°, but is inactivated after storage for 2–3 weeks in the frozen state. It is more stable when kept at 4° in the presence of 30 per cent $(NH_4)_2SO_4$. Phospho β-glucosidase B is contained in peak *C*. In contrast to phospho β-glucosidase A, it splits salicin and is more heat-sensitive. The splitting of aryl β-glucosides in the presence of G-6-P and gl-phosphotransferase is: salicin, 40 U/mg protein; phenyl β-glucoside, 38 U; ONP-glu, 34 U; PNP-glu, 16 U. Preliminary data obtained with phosphorylated phenyl β-glucoside (prepared with F-1,6-P as phosphate donor) showed that G-6-P is an end product of the reaction. Phospho β-glucosidase B is inactivated by incubation for five minutes at 49°. Higher temperature

sensitivity for splitting of salicin in comparison with other β-glucosides was also found in *E. coli*.[5] In the frozen state phospho β-glucosidase B is more stable than phospho β-glucosidase A.

*Phospho β-glucosidases in other Enterobacteriaceae:* In order to determine the presence of phospho β-glucosidases in other Enterobacteriaceae, this activity was tested in the following systems: (*a*) β-glucosides; (*b*) β-glucosides + G-6-P; (*c*) β-glucosides + gl-phosphotransferase from *A. aerogenes* and G-6-P; (*d*) crude preparations of phosphorylated β-glucosides. The activity obtained after the addition of G-6-P alone is an indirect indicator for the phosphotransferase activity of the extract. Data obtained with ONP-glu are given in Table 4. It appears that all tested strains of Enterobacteriaceae possess a constitutive phospho β-glucosidase, but lack to different degrees the ability to phosphorylate β-glucosides. No cryptic phospho β-glucosidase activity was found in the tested *Pseudomonas aeruginosa* and *Staphylococcus* strains. In comparison with the addition of G-6-P alone, the addition of G-6-P + gl-phosphotransferase produces no further increase in the activity of extracts from noninduced cells of the strain *A. aerogenes* $A_1$, but produces a twofold increase in extracts of the strain 2002. A low phospho β-glucosidase activity was obtained after addition of G-6-P to *E. coli* and *S. typhimurium* extracts and their 45–60 per cent ammonium sulfate fraction. This activity increases seven- to tenfold by addition of gl-phosphotransferase from *A. aerogenes*. The tested *Proteus mirabilis* extract shows no detectable activity in the presence of G-6-P and a relatively low activity after addition of gl-phosphotransferase from *A. aerogenes*. When tested with ONP-glu in the presence of gl-phosphotransferase and G-6-P, no significant difference was found between the activity of the 45–60 per cent $(NH_4)_2SO_4$ fraction of wild-type extracts of *E. coli* and *S. typhimurium* and their constitutive aryl β-glucosides fermenting mutants. When tested with different β-glucosides, the activity of the 45–60 per cent fraction from the *E. coli* K12 wild-type extract is: phenyl β-glucoside, 184 U/mg protein; ONP-glu, 158 U; PNP-glu, 76 U; and salicin, 3 U; a substrate specificity similar to that of phospho β-glucosidase A from *A. aerogenes*. In extracts obtained from induced or constitutive β-$gl^+$ cells there appears, in addition, a phospho β-glucosidase of type B, which also splits salicin (67–83 U/mg protein in the presence of G-6-P and gl-phosphotransferase).

*Discussion.*—The active uptake of β-glucosides occurs through two distinct permeases: β-glucoside permease I which is an aryl β-glucoside permease and β-glucoside permease II which is probably a cellobiose permease with high affinity for alkyl and aryl β-glucosides. A permease with properties similar to permease I was previously found in β-$gl^+$ mutants of *E. coli*,[5] and a constitutive permease of type II was found in *Citrobacter* and cellobiose-fermenting mutants of *S. typhimurium*. One could conceive that *A. aerogenes* is closer to a hypothetical common ancestor which possessed both permeases, while *E. coli* retained only permease I and *Citrobacter–Salmonella* permease II. This could reflect differences in their phylogenetic evolution.[2] Further studies with cellobiose-fermenting strains are required for the verification of this hypothesis.

In Enterobacteriaceae the splitting of aryl β-glucosides occurs through enzymes designated as phospho β-glucosidases. These enzymes were found independently

during the present investigation and by Fox and Wilson[6] in *E. coli*. A similar mechanism is also implied in the catabolism of lactose in *Staphylococcus*.[13, 14] It appears that the hydrolysis of β-glucosides by Enterobacteriaceae is the result of a two-step reaction. The first step consists of the phosphorylation of β-glucosides, followed by their hydrolysis by phospho β-glucosidases A and B. In *A. aerogenes* the phosphorylation can occur by two distinct mechanisms. One is through substrate-specific, membrane-bound enzyme(s) II of the PEP-dependent kinase system.[6] The data obtained with mutants in the PEP-dependent kinase system lacking enzyme I or the HPr protein indicate that the active uptake of β-glucosides is coupled with their phosphorylation by this kinase system. A second mechanism of phosphorylation could be through the soluble gl-phosphotransferase. The high constitutive activity of the gl-phosphotransferase and the relatively low concentration of G-6-P required for the phosphorylation of β-glucosides indicate a possible physiologic role of this transferase in the catabolism of β-glucosides. It is possible that the two transfer systems have different functions. The PEP-dependent kinase could function in the phosphorylation of external β-glucosides, while the gl-phosphotransferase acts in the phosphorylation of still undefined internal substrates of the constitutive phospho β-glucosidases. A rather unexpected finding was the presence of constitutive phospho β-glucosidases in several Enterobacteriaceae, independently of their ability to phosphorylate β-glucosides. The physiologic role of the cryptic phospho β-glucosidases (if any) is unknown.

*Summary.*—In *A. aerogenes*, β-glucosides are accumulated by two permeases. One, induced by alkyl and aryl β-glucosides, has a high affinity for aryl β-glucosides, but low affinity for cellobiose. The second, induced by cellobiose and lactose, has a high affinity for both cellobiose and aryl β-glucosides. β-Glucosides are split in a phosphorylated form by a system which includes a phosphotransferase and two enzymes called phospho β-glucosidases. Constitutive, cryptic phospho β-glucosidases were found in all strains of Enterobacteriaceae investigated so far, but not in *P. aeruginosa* and *Staphylococcus*.

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