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CATION REPAIR OF TOLUENE-TREATED *ESCHERICHIA COLI* ML35 CELLS AND THE TRANSPORT OF *O*-NITROPHENYL-D-β-PYRANOSIDE-GALACTOSIDE ACROSS THE REPAIRED MEMBRANE

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SUMMARY

The *Escherichia coli* ML35 mutant strain, containing constitutive β-galactosidase but not the "M protein" required for transport of β-galactosides into the cell, showed *O*-nitrophenyl-D-β-pyranoside-galactoside (ONPG) hydrolysis *in vivo* after treatment with toluene. In the presence of divalent and polyvalent cations, toluene-treated ML35 cells rapidly lost their ability to hydrolyse ONPG. The rate of repair of the toluene-treated ML35 cells by Mg^{2+} was found to depend on the cation concentration and the temperature of the repair system. In the presence of 0.05 M $MgCl_2$, the toluene-treated ML35 cells completely lost their ability to hydrolyse ONPG within 10 min at 35°. At the stage at which ONPG hydrolysis was no longer observed in repaired toluene-treated ML35 cells, addition of ATP to the ONPG assay system led to increased ONPG hydrolysis *in vivo*. Under these conditions, TMG and formaldehyde markedly inhibited ONPG hydrolysis in repaired toluene-treated cells but not in unrepaired toluene-treated or sonicated ML35 cells. The temperature dependence of ONPG hydrolysis in repaired toluene-treated ML35 cells and the K_m of ONPG hydrolysis of these cells indicated the possibility of the presence of a masked active transport system in the ML35 cell membrane.

INTRODUCTION

β-Galactosides are rapidly transferred across the cell membrane of strains of *Escherichia coli* which contain a functioning *y* gene in the *lac* operon. This transport in and out of the cell is mediated by a specific protein, the product of the *y* gene (called "M protein" or β-galactoside permease). In the absence of a functioning carrier, the cell is relatively impermeable to galactosides. *E. coli* ML35 cells contain a large excess of β-galactosidase but do not have the suitable permease for transfer of β-galactosides[1]. It has been assumed that transfer of β-galactosides into ML35 cells can only occur by simple passive diffusion[2] and that this strain does not have a functioning *y* gene in the lactose operon.

Abbreviations: CTAB, cetyltrimethylammonium bromide; ONPG, *O*-nitrophenyl-D-β-pyranoside-galactoside; TMG, thiomethyl-β-galactoside.

A convenient standard method for detecting cryptic enzymes in bacteria such as *E. coli* ML35 is treatment with toluene[3]. Toluene treatment alters the permeability barrier of such cells towards β-galactosides without disrupting the β-galactosidase activity within the cells.

This paper describes the effect of cations (Mg^{2+}, Ca^{2+}, spermine) on the repair of the damaged membrane of toluene-treated *E. coli* ML35 cells. In addition, different agents, such as thiomethyl-β-galactoside (TMG) and formaldehyde, which affect active transport of β-galactosides into *E. coli* cells[4,5], and ATP, which increases carrier-mediated transport of *O*-nitrophenyl-D-β-pyranoside-galactoside (ONPG)[6], were found to affect ONPG hydrolysis by $MgCl_2$-repaired toluene-treated ML35 cells. A possible mechanism for ONPG transport across the repaired cell membrane is discussed.

MATERIALS AND METHODS

Chemicals

ONPG (Sigma), TMG (Sigma), formaldehyde (BDH, Analar), ATP, crystalline sodium salt, Grade II (Sigma), spermine·3HCl (Calbiochem) and toluene (BDH, Analar).

Test organism

E. coli ML35 strain (constitutive β-galactosidase, negative permease, i^-, z^+, y^-)[1] was grown on nutrient broth (Difco) at 37°. The cells were harvested in the early exponential phase by centrifugation in the cold (10000 × *g* for 10 min at 4°), washed twice in glass-distilled water and suspended in 0.05 M Tris buffer (pH 7.6) at a density of $8 \cdot 10^8$ cells per ml.

Toluene treatment of E. coli ML35 cells

Toluene (2%, v/v) was added to the ML35 cell suspension in Tris buffer in a closed tube (1.2 cm × 17 cm) and rotated (60 rev./min) for 6 min and then centrifuged (2000 × *g* for 2 min). The upper phase (containing the toluene) was discarded. The amount of toluene remaining in the cell phase is not known. These cells were considered "toluene treated" without further treatment.

Sonication

ML35 cells ($8 \cdot 10^8$ cells per ml) suspended in 0.05 M Tris buffer (pH 7.6) were sonicated in a Branson sonifier (4 kcycles for 1 min at 4°).

Hydrolysis of ONPG by E. coli ML35 cells

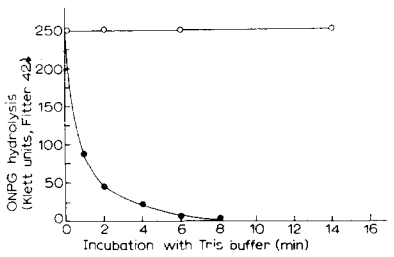
A mixture of final volume 2 ml containing 3.3 mM ONPG and $1.6 \cdot 10^8$ toluene-treated ML35 cells in 0.025 M Tris buffer (pH 7.6), as well as other additives as noted, was incubated for 10 min at 23° (except when indicated otherwise). (ONPG hydrolysis by toluene-treated ML35 cells was found to be proportional to the time of incubation up to 30 min at every temperature tested in the range of 8–35°.) Incubation was terminated by addition of 3 ml 1 M Na_2CO_3. After centrifugation (10000 × *g* for 10 min), the absorbance of the supernatant was determined in a Summerson–Klett colorimeter (Filter 42). The results given represent the reading *minus* the absor-

bance of a blank of the incubation mixture not containing cells. Under the conditions of this test untreated ML35 cells showed no observable ONPG hydrolysis even after 30 min of incubation at 23°.

RESULTS

Effect of cations on the ability of toluene-treated E. coli ML35 cells to hydrolyse ONPG

The ability of toluene-treated ML35 cells to hydrolyse ONPG was not reduced by repeated washing in glass-distilled water before the toluene treatment. However, when washed twice in 0.05 M $MgCl_2$ before being treated with toluene, such cells rapidly (within 10 min at 35°) lost their ability to hydrolyse ONPG (Fig. 1).



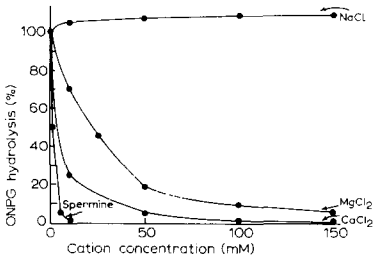


Fig. 1. Effect of pretreatment with $MgCl_2$ on the ability of toluene-treated *E. coli* ML35 cells to hydrolyse ONPG. Harvested ML35 cells were washed twice in 0.05 M $MgCl_2$ (●) or in glass-distilled water (○) and then suspended in Tris buffer, treated as described in MATERIALS AND METHODS with toluene and tested for ONPG hydrolysis after different times of incubation at 35°.

Fig. 2. Effect of different cations on ONPG hydrolysis by toluene-treated *E. coli* ML35 cells. Toluene-treated ML35 cells were incubated at 35° for 10 min in 0.05 M Tris buffer (pH 7.6) in the presence of the listed cation at the given concentrations. ONPG hydrolysis was carried out as described in MATERIALS AND METHODS.

Similarly, the ONPG hydrolysis by toluene-treated cells was reduced markedly when incubated with different concentrations of Ca^{2+}, Mg^{2+} or spermine at 35° (Fig. 2). Na^+ (even at high concentrations) had no effect on ONPG hydrolysis by the toluene-treated ML35 cells (Fig. 2).

These cations, when incubated with sonicated ML35 cells, had no effect on the expression of β-galactosidase activity. Furthermore, repaired toluene-treated ML35 cells (which ceased hydrolysing ONPG after 10 min of exposure to 0.05 M $MgCl_2$ at 35°) again showed the original level of ONPG hydrolysis after sonification.

Fig. 3 shows that in the presence of 0.05 M $MgCl_2$, toluene-treated ML35 cells lose 100% of the capacity to hydrolyse ONPG after about 10 min of incubation at 35°, while at 25° ONPG hydrolysis ceased completely only after 2 h. At 0° the ONPG-hydrolysing capacity was hardly reduced even after a 3-h incubation with the $MgCl_2$.

These temperature-dependent kinetics suggest that the repair by $MgCl_2$ of the toluene-damaged cell membrane is not simply a matter of binding of cations to the acidic groups of the membrane but probably involves the action of some enzymatic repair system.

Factors affecting ONPG hydrolysis by repaired toluene-treated ML35 cells

To test the dependence of the repair process on energy supply, ATP was added to the ONPG-hydrolysing system. It was found that ATP addition not only did not accelerate the loss of the ONPG-hydrolysing capacity but actually prolonged the period required for complete loss of ONPG-hydrolysing capacity at 35° (Fig. 3). In addition, repaired toluene-treated ML35 cells (after incubation with $MgCl_2$ for 10 min at 35°) showed an increasing capacity to hydrolyse ONPG after addition of ATP for different times to the ONPG incubation mixture (Fig. 4).

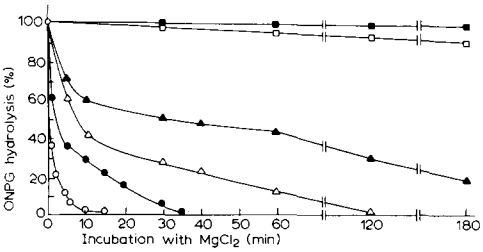


Fig. 3. ONPG hydrolysis in the presence or in the absence of ATP by toluene-treated *E. coli* ML35 cells, repaired by $MgCl_2$ at different temperatures. Toluene-treated ML35 cells were incubated with 0.05 M $MgCl_2$ at various temperatures. At different times, aliquots were taken for determination of ONPG hydrolysis in the presence and absence of 5 mM ATP as described in MATERIALS AND METHODS. ○, 35°; △, 25°; □, 0°; white symbols, without ATP; black symbols, with ATP.

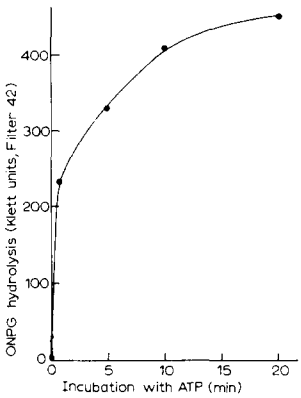


Fig. 4. Effect of incubation with ATP for different times on ONPG hydrolysis of repaired toluene-treated *E. coli* ML35 cells. Toluene-treated ML35 cells were first incubated with 0.05 M $MgCl_2$ for 10 min at 35° and then incubated at 23° for different times with 5 mM ATP. ONPG hydrolysis was determined as described in MATERIALS AND METHODS. (At 0 time a sample without ATP was tested.)

The question arose as to whether the ATP interfered in some way with the repair process of the cations or whether it was active in facilitating the transport of ONPG into the cells whose membranes were being repaired. At the concentration of ATP used (5 mM), only a small portion of Mg^{2+} (50 mM) can be bound by the added ATP. ATP has been shown to enhance ONPG transport into *E. coli* strains having a functional *y* gene in their *lac* operon[6,7].

In order to study the involvement of a possible carrier-mediated transport of ONPG into repaired toluene-treated ML35 cells, two of the agents known to decrease the carrier-mediated transport of β-galactosides without significantly affecting the activity of β-galactosidase were tested.

TMG, a nonmetabolized galactoside, competes with the ONPG for the same carrier[4]. Fig. 5 shows the effect of TMG on ONPG hydrolysis by repaired toluene-treated ML35 cells at the stage of cell repair when ATP is necessary for ONPG hydrolysis. At a concentration of 10 mM TMG, which is 3 times higher than that of the ONPG present in the assay system, the ONPG hydrolysis was decreased

by 40 %; at a concentration of 50 mM TMG the inhibition of ONPG hydrolysis was 87 %. No effect of the TMG (at 100 mM) was found on the hydrolysis of ONPG by unrepaired toluene-treated cells or by sonicated cells. Under the condition of this test untreated ML35 cells showed no observable ONPG hydrolysis. It was therefore impossible to test the effect of TMG on the uptake of ONPG by these cells.

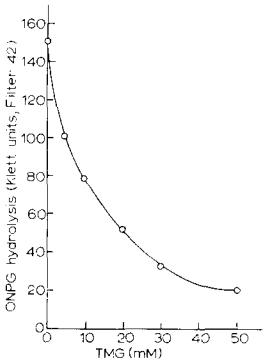


Fig. 5. Effect of TMG on ONPG hydrolysis by repaired toluene-treated *E. coli* ML 35 cells in the presence of ATP. Toluene-treated ML35 cells were first incubated with 0.05 $MgCl_2$ for 10 min at 35°. ONPG hydrolysis by these cells incubated with 5 mM ATP and different concentrations of TMG was tested as described in MATERIALS AND METHODS.

TABLE I

EFFECT OF FORMALDEHYDE ON ONPG HYDROLYSIS BY REPAIRED AND UNREPAIRED TOLUENE-TREATED *E. coli* ML35 CELLS AND BY SONICATED ML35 CELLS

Toluene-treated ML35 cells were incubated with 0.05 M $MgCl_2$ for 10 min at 35°. These cells (with 5 mM ATP), unrepaired toluene-treated cells and sonicated cells were incubated with and without 3.3 mM formaldehyde in 0.15 M Tris buffer (pH 7.6) for 5 min at 23°. The ONPG hydrolysis was then determined as described under MATERIALS AND METHODS.

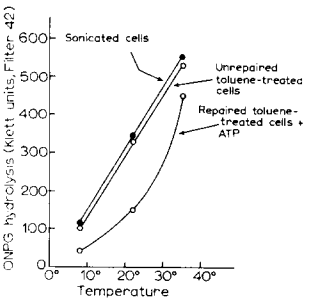
Percent inhibition of ONPG hydrolysis by formaldehyde		
Repaired toluene-treated cells	*Unrepaired toluene-treated cells*	*Sonicated cells*
80	20	20

KOCH[5] showed that formaldehyde rapidly stops the carrier (permease) action with only a small effect on the β-galactosidase activity. The effect of formaldehyde on repaired cells at the stage where ATP is required for ONPG hydrolysis is shown in Table I. It can be seen that ONPG hydrolysis by these cells was reduced by 80 % under these conditions, while the formaldehyde inhibition of β-galactosidase activity of sonicated cells or of unrepaired toluene-treated cells was only about 20 %. KOCH[5] showed that formaldehyde has a slight secondary effect in breaking down the permeability barrier. This could be the reason that formaldehyde did not completely inhibit ONPG hydrolysis in the repaired cells.

Fig. 6 shows the temperature dependence of ONPG hydrolysis in the range of 8–35° by repaired and unrepaired toluene-treated ML35 cells. It can be seen that the curve of temperature dependence of repaired cells is exponential, while

that of unrepaired cells is linear. Sonicated ML35 cells also show a linear temperature dependence.

The transport of ONPG has been shown to be rate limiting for the overall process resulting in the hydrolysis of ONPG by intact *E. coli* ML308 cells which contain a functional *y* gene[4]. The effect of ONPG concentration on the rate of its



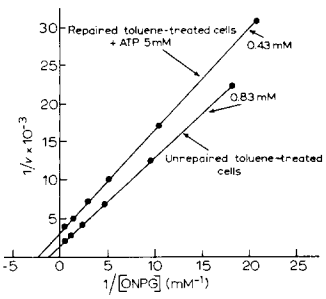


Fig. 6. Effect of temperature on ONPG hydrolysis by repaired and unrepaired toluene-treated *E. coli* ML35 cells and by sonicated *E. coli* ML35 cells. Toluene-treated ML35 cells were incubated with 0.05 M $MgCl_2$ for 10 min at 35°. ONPG hydrolysis by repaired cells in the presence of 5 mM ATP and by unrepaired ML35 cells as well as sonicated ML35 cells was determined after 10 min at different incubation temperatures.

Fig. 7. Dependence of rate of ONPG hydrolysis by repaired and unrepaired toluene-treated *E. coli* ML35 cells on ONPG concentration. Toluene-treated ML35 cells were incubated with 0.05 M $MgCl_2$ for 10 min at 35°. ONPG hydrolysis by repaired cells in the presence of 5 mM ATP as well as by unrepaired toluene-treated cells and sonicated cells was carried out in the presence of different ONPG concentrations under the conditions described in MATERIALS AND METHODS. The K_m values were calculated by the method of Lineweaver and Burk.

hydrolysis by toluene-treated ML35 cells before and after repair is shown in Fig. 7. It can be seen that the Michaelis constant (K_m) for the repaired toluene-treated cells at the stage where ATP is required for ONPG hydrolysis (0.43) was slightly but significantly different from that of the toluene-treated cells before repair (0.83).

DISCUSSION

The results show that spermine, Ca^{2+} or Mg^{2+} is able to repair the damage caused by toluene to the cell membrane of *E. coli* ML35 cells as detected by the entry of ONPG into the cell. The kinetics of membrane repair by $MgCl_2$, studied in detail, consists of two stages. In the first stage, 95 % of the ability to hydrolyse ONPG was lost within 3–5 min at 35°; the residual activity continued to decrease for another 5–7 min (second stage).

Although it has been assumed that *E. coli* ML35 cells possess all the attributes of simple passive diffusion of β-galactosides and do not possess a functioning *y* gene in the *lac* operon[2], the results seem to indicate that the entry of ONPG into the toluene-treated ML35 cells after partial repair of the membrane by $MgCl_2$ is a carrier-mediated process. This suggestion is based mainly on the following findings.

(1) When ATP was added to the ONPG assay system after the toluene-treated cells had been incubated for different times with $MgCl_2$, the hydrolysis of ONPG by the toluene-treated cells increased mainly at the second stage of repair. Addition of ATP did not alter the ONPG hydrolysis in unrepaired toluene-treated cells or

in sonicated cells. Although the role of ATP in the transport of ONPG into *E. coli* cells is as yet uncertain, it was shown that ATP increases ONPG hydrolysis in cells under mild osmotic shock 5–6-fold[7,8].

(2) TMG, a competitive inhibitor for the β-galactoside transport mediated by M protein[4], decreased the ability of the toluene-treated cells to hydrolyse ONPG *in vivo* at the stage of membrane repair at which ATP enhances ONPG hydrolysis. On the other hand, TMG did not affect the ONPG hydrolysis by sonicated cells or by unrepaired toluene-treated cells.

(3) Formaldehyde, an agent used for testing nonspecific transport into cells[5], markedly decreased ONPG hydrolysis in toluene-treated cells in the second stage of membrane repair and hardly affected the β-galactosidase activity of unrepaired toluene-treated cells or sonicated cells.

(4) The temperature dependence (in the range of 8–35°) of the ONPG hydrolysis by toluene-treated cells with partially repaired membranes is exponential, while that of sonicated or unrepaired toluene-treated cells is linear. The exponential temperature dependence of ONPG hydrolysis by the repaired toluene-treated cells could be explicable in terms of energy required for activation of a carrier.

(5) The K_m of ONPG hydrolysis by membrane-repaired toluene-treated cells is slightly different from the K_m of β-galactosidase.

In the accompanying paper[9], it was shown that *E. coli* ML35 cells treated with sublethal concentrations of the cationic detergent cetyltrimethylammonium bromide (CTAB) under certain conditions were able to grow on minimal medium containing lactose as the sole source of carbon and energy. The ability of such CTAB-treated ML35 cells to hydrolyse ONPG was markedly increased in the presence of ATP (when the cells were starved) and was decreased by TMG and by formaldehyde. It was suggested that CTAB alters only the outer face of the cell membrane and that ONPG and lactose diffused freely up to the inner face when they are bound by a specific (carrier) protein and transported by it into the cell.

In line with the above theory, a possible mechanism for the transport of the ONPG into repaired toluene-treated ML35 cells might be the following: on addition of Mg^{2+} the toluene-damaged membrane starts sealing, first at the inner face of the damaged membrane and afterwards the outer face. At the first stage of repair, when the inner face is already sealed but the outer face is still unrepaired, diffusion of ONPG into the cells stops because of the barrier at the inner face. At this stage the ONPG transport is demonstrated only in the presence of ATP and inhibited markedly by TMG and formaldehyde. This phenomenon could be explained by the presence of a specific protein subunit, as described by STEIN[8], present in the cell membrane. At the second stage of repair when both faces of the membrane are already sealed by Mg^{2+}, neither ONPG nor ATP come into contact with the specific protein in the membrane and ONPG hydrolysis stops completely.

With respect to the Mg^{2+} repair mechanism, MAGER[10] and TABOR[11] have shown that divalent cations (Mg^{2+} and Ca^{2+}) and polycationic substances (lime spermine) stabilized osmotically sensitive spheres (such as spheroplasts and protoplasts) in hypotonic media. The results described here show that these cations are also able to repair the damage already done to the cell membrane by toluene. TABOR[11] suggested that the stabilization of the osmotically sensitive spheres resulted from the ability of the salts to bind acidic groups in the cell membrane. The strong

temperature dependence of the repair process in toluene-treated ML35 cells, however, indicates that this repair mechanism involves, in addition, certain processes like diffusion within the membrane phase, which requires energy activation, or phospholipid phase changes similar to those described by LUZZATI AND HUSSON[12].

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REFERENCES

1 G. N. COHEN AND J. MONOD, *Bacteriol. Rev.*, 21 (1957) 169.
2 H. H. WINKLER AND T. H. WILSON, *J. Biol. Chem.*, 241 (1966) 2200.
3 K. WALLENFELS, M. L. ZARNITZ, G. LAULE, H. BENDER AND M. KESER, *Biochem. Z.*, 331 (1959) 459.
4 H. V. RICKENBERG, G. N. COHEN, G. BUTTIN AND J. MONOD, *Ann. Inst. Pasteur*, 91 (1956) 829.
5 A. L. KOCH, *Biochim. Biophys. Acta*, 79 (1964) 177.
6 G. H. SCARBOROUGH, M. K. RUMLEY AND E. P. KENNEDY, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 951.
7 I. C. WEST, *FEBS Letters*, 4 (1969) 69 169.
8 W. D. STEIN, *J. Gen. Physiol.*, 54 (1969) 81.
9 S. ULITZUR, *Biochim. Biophys. Acta*, 211 (1970) 533.
10 J. MAGER, *Biochim. Biophys. Acta*, 36 (1959) 529.
11 C. W. TABOR, *Biochem. Biophys. Res. Commun.*, 2 (1960) 117.
12 V. LUZZATI AND F. HUSSON, *J. Cell Biol.*, 12 (1962) 207.