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THE TRANSPORT OF β-GALACTOSIDES ACROSS THE MEMBRANE OF PERMEASELESS *ESCHERICHIA COLI* ML35 CELLS AFTER TREATMENT WITH CETYLTRIMETHYLAMMONIUM BROMIDE

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SUMMARY

The cationic detergent cetyltrimethylammonium bromide (CTAB) did not lyse or affect the viability of *Escherichia coli* ML35 cells at low concentrations (1–3 μg CTAB per $8 \cdot 10^8$ cells per ml). Under these conditions the CTAB-treated ML35 cells were able to hydrolyse *O*-nitrophenyl-D-β-pyranoside-galactoside (ONPG) and effect respiration on lactose. These CTAB-treated ML35 cells were also able to grow on minimal medium containing lactose as sole source of carbon and energy. The effect of CTAB on ML35 cells was temperature and pH dependent. The ONPG hydrolysis by CTAB-treated ML35 cells could be inhibited by NaN_3 and dinitrophenol but not by NaF. ATP markedly enhanced ONPG hydrolysis by starved CTAB-treated ML35 cells, and thiomethyl-β-galactoside (TMG) or formaldehyde inhibited ONPG hydrolysis by CTAB-treated ML35 cells. However, TMG, formaldehyde and ATP did not influence the β-galactosidase activity of sonicated ML35 cells. The involvement of a masked active transport system of β-galactosides into CTAB-treated ML35 cells is discussed.

INTRODUCTION

It is not uncommon to observe stimulation of metabolic processes by chemical agents at low concentrations and depression or inhibition of these processes by the same agents at higher concentrations.

Although detergents show bacteriolytic action at given concentrations, it has been shown that some detergents at concentrations lower than inhibiting values can stimulate metabolism[1]. A common feature of the antibacterial action of ionic detergents is their ability to cause leakage of cellular constituents and other disturbances in normal cell membrane function[1–8]. However, LEIVE[9] found that brief treatment of *Escherichia coli* cells with a low concentration of EDTA altered the permeability of these cells without affecting their viability.

The cationic detergent cetyltrimethylammonium bromide (CTAB) at relatively high concentrations (around 10 μg CTAB per 10^9 bacterial per ml) causes the release of

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

different cellular constituents from both Gram-positive and Gram-negative bacteria[2,3,10] and morphological changes visible in the electron microscope[11] and reduces the negative charge of the bacteria[12,13].

During comparative studies of the effects of CTAB and other detergents and the Prymnesium toxin on intact normal and mutant bacterial strains, as well as on spheroplasts and protoplasts[14], the interesting effect of sublethal concentrations of CTAB on the cell membrane of *E.coli* ML35, described in this paper, was discovered. Due to its peculiar genetical constitution the *E. coli* ML35 mutant is unable to transport and accumulate β-galactosides from the medium. Treatment with sublethal concentrations of CTAB rendered the cell capable of utilizing β-galactosides. This experimental technique is a valuable tool for investigating the mode of β-galactoside transport into cells as described in this paper.

MATERIALS AND METHODS

The cryptic *E. coli* ML35 strain (constitutive β-galactosidase, negative permease, i^-, z^+, y^-) was grown at 37° on nutrient broth (Difco). These cells do not grow on minimal medium containing lactose (0.2 %) but grow when glucose (0.2 %) is substituted for lactose. Cells were harvested in the early exponential phase of growth, washed once in the cold (4–6°) with glass-distilled water (the same results were obtained when the cells were washed with water at 23°) and resuspended at a density of $8 \cdot 10^8$ cells per ml. ML35 cells were treated with CTAB at various concentrations in 0.05 M Tris buffer (pH 7.6) or in 0.02 M phosphate buffer (various pH's) at 35°, or in minimal medium[15] (pH 7) in 0.4 % glucose or 0.2 % lactose at 37°.

O-Nitrophenyl-D-β-pyranoside-galactoside (ONPG) hydrolysis in the cells was tested in 1-ml aliquots of the CTAB-treated ML35 cells to which 1 ml of 6.6 mM ONPG was added giving final concentrations of 3.3 mM ONPG. The ONPG hydrolysis *in vivo* has been shown to be proportional to the transport rate, since there is a large excess of β-galactosidase which catalyses immediate hydrolysis of ONPG after it enters the cell[19]. The hydrolysis of ONPG was terminated after 10 min of incubation at 23° (unless stated otherwise) by addition of 3 ml 1 M Na_2CO_3. The absorbance was measured after centrifugation in a Klett–Summerson photometer (Filter 42). Controls of ONPG hydrolysis with untreated ML35 cells were also carried out in all experiments. The absorbance of untreated cells was subtracted in each case (except for Fig. 3 where it is shown graphically) from the absorbance obtained for treated cells in the presentation of results.

Uptake of O_2 was measured polarographically (Oxygraph Model KM, Gilson) in a suspension of $8 \cdot 10^8$ cells per ml in 2 ml of 0.05 M Tris buffer (pH 7.6) in the absence or presence of either lactose, citruline or citrate in a final concentration of 0.2 %. The rate of O_2 uptake of each sample was measured for at least 4 min at 35°.

The presence of 260-nm-absorbing materials in the CTAB-treatment suspension mixture was measured spectrophotometrically (Perkin–Elmer ultraviolet Model 137) after the cells had been removed by centrifugation (10000 × *g* for 10 min) in the cold. The presence of β-galactosidase in the suspension mixture was tested by ONPG hydrolysis.

Sonicated *E. coli* ML35 cells were prepared by sonicating $8 \cdot 10^8$ cells per ml suspended in 0.05 M Tris buffer (pH 7.6) in a Branson sonifier for 1 min (4 kcycles) at 0°.

The detergents CTAB (British Drug Houses), sodium dodecyl sulphate (Light), Triton X-100 and Tween-80 (Baker Chemicals) and benzalkonium chloride (Assia, Israel) were used without further purification. Research grades of ONPG (Sigma), thiomethyl-β-galactoside (TMG) (Sigma), formaldehyde (BDH) and ATP, sodium salt, Grade II (Sigma) were used. All the other reagents were chemically pure.

RESULTS

Effect of CTAB on E. coli ML35 cells

E. coli ML35 cells ($8 \cdot 10^8$ cells per ml) were incubated in Tris buffer for 60 min at 35° with concentrations of CTAB progressing from 1 to 10 μg/ml. Fig. 1 shows that under these conditions CTAB concentrations below 3 μg/ml had no measurable effect on the viability of ML35 cells, while concentrations of 4 μg/ml and above caused lysis of the cells. At the low CTAB concentrations (below 3 μg/ml) no 260-nm-absorbing materials and β-galactosidase activity were detected in the suspension medium (Fig. 1).

In addition, ML35 cells incubated in the presence of 3 μg CTAB per ml in the minimal medium containing 0.2 % glucose showed a normal growth curve (Fig. 2).

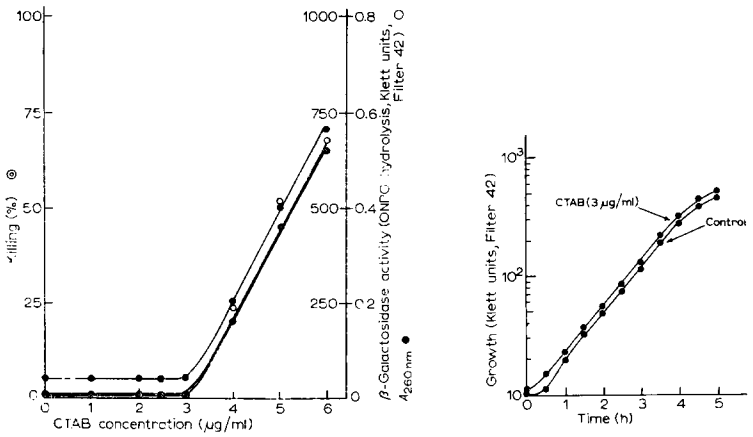

Fig. 1. Killing of *E. coli* ML35 cells and release of 260-nm-absorbing material and β-galactosidase from the cells by various concentrations of CTAB. ML35 cells were suspended in Tris buffer containing different concentrations of CTAB for 60 min at 35°. The percentage of killing was determined by viable counts on agar plates. Other determinations are as described in MATERIALS AND METHODS.

Fig. 2. Growth of *E. coli* ML35 cells on glucose in the presence of sublethal concentrations of CTAB. ML35 cells were grown on minimal medium containing 0.2% glucose with and without CTAB (3 μg/ml). Growth is expressed in the increase of absorbance as measured in a Klett photometer (Filter 42).

Fig. 3 shows that both ONPG hydrolysis and respiration on lactose were markedly increased, after a short latent period of about 10 min, in ML35 cells incubated with 2 μg CTAB per ml at 35°. Cells treated with CTAB for 30–40 min showed 4–5 times the respiration on lactose and 20 times the ONPG hydrolysis found in control cells. CTAB treatment of the cells had no effect on endogenous respiration.

In the presence of 1–3 μg CTAB per ml, ML35 cells grew in the minimal medium containing lactose as sole carbon and energy source (Fig. 4). The generation time is

about 45–50 min at each of the CTAB concentrations, but increased detergent concentration enhanced the yield of the cells and shortened the lag period. In spite of the marked enhancement of respiration on lactose, the CTAB-treated cells were found not to respire on 0.4 % citrate or 0.2 % citruline when measured polarographically (Gilson Oxygraph). The presence of the cryptic enzyme for these two substrates in ML35 cells was not tested. However, since ML35 cells can grow on different intermediates of the tricarboxylic acid cycle and on arginine, it is logical to suppose that enzymes for these substrates are present cryptically in the strain.

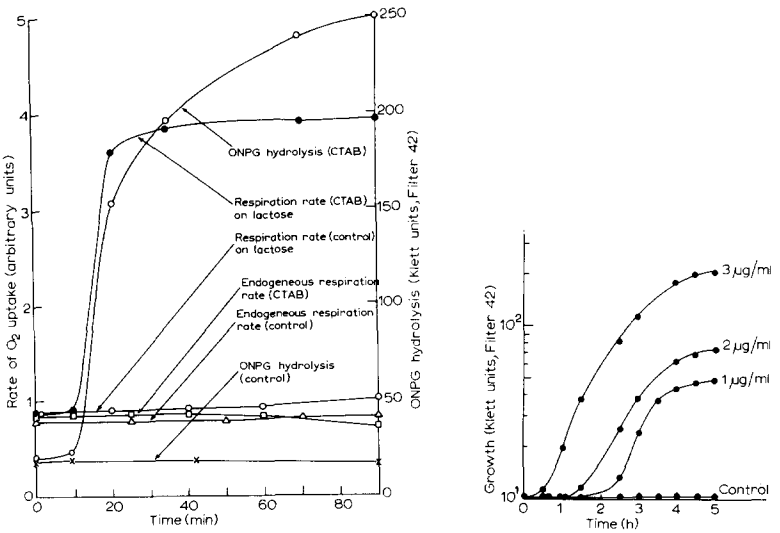

Fig. 3. Respiration on lactose, endogenous respiration and ONPG hydrolysis in CTAB-treated *E. coli* ML35 cells. For determination of respiration on lactose and ONPG hydrolysis, ML35 cells were incubated for different times in Tris buffer with 2 µg CTAB at 35°. At different times of incubation, aliquots of the treated or untreated cells were tested for their ability to hydrolyse ONPG and for their respiration.

Fig. 4. Growth of CTAB-treated *E. coli* ML35 cells on lactose. Washed ML35 cells were inoculated to a final concentration of $8 \cdot 10^8$ cells per ml in minimal medium containing 0.2 % lactose and various concentrations of CTAB into 250-ml Erlenmeyer flasks with side arms. The cultures were shaken at 37° and growth followed by measuring absorbance in a Klett photometer (Filter 42).

SALTON[2] showed that the lytic effect of CTAB on different bacterial strains is temperature dependent. Therefore, ML35 cells were incubated with 2 µg CTAB per ml at different temperatures and their subsequent ability to hydrolyse ONPG was tested. It was found that below 10° there was no detectable effect on the cells, while progressive increases in the temperature of the CTAB treatment led to a progressively increasing ability to hydrolyse ONPG (Fig. 5).

The effect of 2 µg CTAB/ml at different pH's (6–8) on the permeability of ML35 cells towards ONPG is shown in Fig. 6. Increased pH of the CTAB treatment led to increased hydrolysis of ONPG.

Cationic detergents are known to be more active in the alkaline range. BAKER *et al.*[1] suggest that the cationic detergents are more effective at a high pH because of the greater ability of dissociated molecules to enter the bacterial cells. It has also been

suggested[16] that at low pH's the H^+ protects the cells from the toxic cationic detergents.

Of the detergents tested on ML35 cells at sublethal concentrations (including the anionic detergent sodium dodecyl sulphate, the nonionic detergents Trition X-100 and Tween-80, and the cationic detergent benzalkonium chloride), only CTAB caused increased hydrolysis of ONPG.

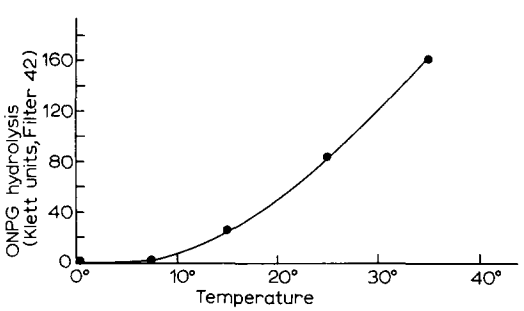

Fig. 5. Effect of temperature of CTAB treatment on the ability of *E. coli* ML35 cells to hydrolyse ONPG *in vivo*. ML35 cells were incubated in Tris buffer with 2 μg CTAB per ml at different temperatures for 30 min. Details of ONPG hydrolysis assay are given in MATERIALS AND METHODS.

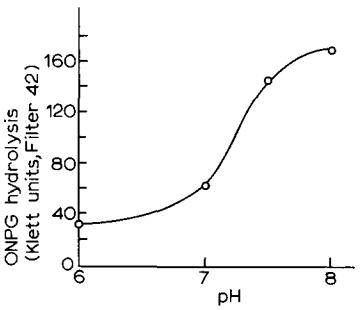

Fig. 6. Effect of pH of CTAB treatment on the ability of *E. coli* ML35 cells to hydrolyse ONPG *in vivo*. ML35 cells were incubated in sodium phosphate buffer at different pH's with 2 μg CTAB per ml for 30 min. ONPG hydrolysis by these cells was determined as described in MATERIALS AND METHODS.

Factors affecting ONPG hydrolysis by CTAB-treated E. coli ML35 cells

Having established that sublethal concentrations of CTAB at a given temperature and pH markedly enhance the ability of ML35 cells to hydrolyse ONPG *in vivo*, it was of interest to investigate the mechanism by which the galactoside penetrates the ML35 cells which have a nonfunctional *y* gene and which are not capable of galactoside transport into the cell under normal conditions. The factors known to affect galactoside transport into *E. coli* strains having a functional *y* gene were therefore tested for their effect on ONPG hydrolysis in CTAB-treated ML35 cells.

Energy poisons. Active transport and accumulation of β-galactosides in *E. coli* cells (y^+) was demonstrated to be inhibited by the energy poisons NaN_3 and dinitrophenol but not by NaF (ref. 17). ONPG hydrolysis by CTAB-treated ML35 cells incubated in NaN_3 or dinitrophenol was reduced to about 10 % of full activity, while the ability to hydrolyse ONPG in the presence of NaF was hardly affected (Table I). However, NaF and NaN_3, when combined, produce a further decrease in ONPG hydrolysis.

ATP. It was suggested by SCARBOROUGH *et al.*[18] that ATP is involved in the transport of β-galactosides in *E. coli*. Table II shows no effect on the ONPG hydrolysis by ML35 cells treated with CTAB immediately after harvesting. On the other hand, when ML35 cells were incubated in Tris buffer at 35° for 3 h before CTAB treatment (causing starvation), the intensity of ONPG hydrolysis was greatly reduced unless ATP was added to the ONPG system.

SCARBOROUGH *et al.*[18] also showed that K^+ and ATP synergistically stimulate the accumulation of ONPG in *E. coli* ML308 (i^-, z^+, y^+, a^+) cells, but that K^+ alone (at 5 mM) reduces the rate of ONPG transport.

TABLE I

EFFECT OF NaN_3, NaF AND DINITROPHENOL ON ONPG HYDROLYSIS BY CTAB-TREATED *E. coli* ML35 CELLS

ML35 cells ($8 \cdot 10^8$/ml) were incubated in Tris buffer with 2 μg CTAB per ml for 20 min at 35°. The treated cells were incubated for a further 20 min at 35° in the presence of different concentrations of dinitrophenol, NaN_3, NaF or with both of the last two. The ONPG hydrolysis by the treated cells was determined as described under MATERIALS AND METHODS.

Additions to assay medium	*Concn. (mM)*	*Percent of OPNG hydrolysis under control conditions*
None	–	100
NaN_3	10	15
NaF	10	92
NaN_3+NaF	10+10	4
Dinitrophenol	0.005	72
	0.01	40
	0.05	12
	0.1	3

TABLE II

ACTIVATION OF ONPG HYDROLYSATES BY ATP

ML35 cells were starved by incubation in Tris buffer for 3 h with shaking. These cells and freshly harvested ML35 cells were incubated in Tris buffer with 2 μg CTAB per ml for 30 min at 35°. ONPG hydrolysis after 10 min of incubation at 23° was tested in the presence of different concentrations of ATP.

ATP concentrations in the assay medium (mM)	*ONPG hydrolysis (Klett units, 420 nm)*	
	Fresh cells	*Starved cells*
0	150	20
0.5	150	100
1	155	150
2	155	155
5	155	155

It was found that ONPG hydrolysis by fresh CTAB-treated ML35 cells was decreased markedly (80–90 %) when 5 mM KCl was added to the ONPG system, although no synergistic effect of K^+ and ATP was found.

TMG. The nonmetabolized galactoside TMG (which competes with ONPG for galactoside transport mediated by "M protein" (ref. 19) decreased the ONPG hydrolysis by CTAB-treated ML35 cells (Fig. 7). In the presence of 3.3 mM ONPG, increasing amounts of TMG (from 0.1 to 0.5 mM) in the assay system led to decreasing ONPG hydrolysis. However, even in the presence of 0.1 M TMG, ONPG hydrolysis by sonicated or by toluene-treated [21] *E. coli* ML35 cells was not affected.

Formaldehyde. Since it stops the action of the carrier system, formaldehyde is considered to be a useful agent for testing non-specific transport into cells[20]. Table III shows that ONPG hydrolysis of CTAB-treated ML35 cells incubated with formaldehyde (3.3 mM) decreased by 80 %, while the β-galactosidase activity of sonicated or toluene-treated ML35 cells was only reduced by about 20 %.

TABLE III

EFFECT OF FORMALDEHYDE ON ONPG HYDROLYSIS IN *E. coli* ML35 CELLS TREATED WITH CTAB OR TOLUENE OR AFTER SONICATION

Sonicated and toluene-treated *E. coli* ML35 cells as well as cells treated with CTAB (2 μg/ml for 20 min at 35°) were mixed in 0.15 M Tris buffer (pH 7.6) with 3.3 mM formaldehyde, and the degree of ONPG hydrolysis determined immediately. The 100% ONPG hydrolysis was determined for each respective case in the absence of formaldehyde.

Percent inhibition of ONPG hydrolysis by formaldehyde		
Sonicated cells	*Toluene-treated cells*	*CTAB-treated cells*
17	20	80

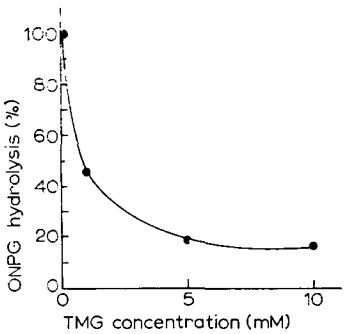

Fig. 7. Effect of TMG on ONPG hydrolysis by CTAB-treated *E. coli* ML35 cells. ML35 cells were suspended in Tris buffer in the presence of 2 μg CTAB/ml for 20 min. ONPG hydrolysis by the treated cells was determined in the presence of different concentrations of TMG.

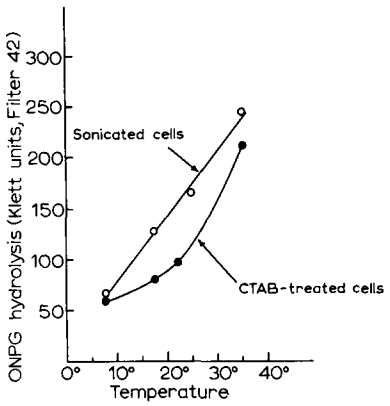

Fig. 8. Effect of temperature on ONPG hydrolysis by CTAB-treated *E. coli* ML35 cells. For CTAB treatment ML35 cells were suspended in Tris buffer in the presence of 2 μg CTAB/ml for 20 min. ONPG hydrolysis at different temperatures was tested with CTAB-treated ML35 cells, as described in MATERIALS AND METHODS, and with sonicated ML35 cells using 0.2-ml aliquots of the cell suspension (containing $1.6 \cdot 10^8$ cells per ml) instead of the usual aliquot measure.

Temperature. When tested at different temperatures, the coefficient rate of ONPG hydrolysis by CTAB-treated ML35 cells was about 4 times lower in the range of 8–17° than that in 23–35°. On the other hand, the coefficient rate of ONPG hydrolysis by sonicated ML35 cells was constant throughout the range of 8–35° (Fig. 8).

DISCUSSION

At concentrations of CTAB lethal to bacterial cells (about 10 μg per 10^9 bacteria per ml), the detergent appears to rupture the cell membrane but leaves it flexible enough to re-form into presumably spherical bodies[8]. This is in contrast with the anionic detergent sodium dodecyl sulphate which completely disorganizes the cell membrane[8]. The primary site of action of CTAB and the bacterial cell membrane was suggested to be lipid components of the membrane, and lysis of the cells is a secondary osmotic lysis[22].

SALTON[2] found that 420 μg CTAB can be absorbed by 1 mg (dry wt.) of *E. coli* cells. At this rate, it appears that the concentration used in our study (2 μg CTAB per $8 \cdot 10^8$ ML35 cells per ml (=0.4 mg dry wt. per ml) corresponds to about 1 % of the maximal absorption of CTAB by these cells. At this low concentration, no 260-nm-absorbing materials were seen to leak from the cells, and the viability of the cells and their ability to grow on glucose, as well as their endogenous respiration, remained unaffected. Although it is not yet known whether other essential metabolites leave the cells, these observations suffice to indicate that the absorption of low CTAB concentrations does not cause loss of the capacity of the cell membrane to control transport of metabolites through the cell membrane in both directions.

Indirectly, these observations led us to the assumption that at the low concentration (1 % of saturation capacity) CTAB only combines with the acidic groups of the membrane phospholipids, located on the outer side of the cell membranes, but is not sufficiently concentrated to affect the phospholipids on the inner side of the membrane. When the outer layer of the membrane is affected by CTAB, and partially disorganized, compounds freely diffuse into the space between the two leaflets of the membrane but cannot diffuse further into the cells because of the presence of an unaffected "inner side" barrier of the membrane which prevents substances from entering or leaving the cells freely. This could be the reason why CTAB-treated *E. coli* ML35 cells were not able to effect respiration on citrate and citrulline.

There apperars to be a contradiction in the finding that ML35 cells, which lack a functional *y* gene and normally are not able to transport β-galactosides across the membrane, were capable of respiration on lactose substrate and hydrolysed ONPG *in vivo* after treatment with the low CTAB concentration.

A variety of experiments indicated that the entry of galactosides into the CTAB-treated ML35 was not simple diffusion through damaged membrane but a carrier-mediated process. The fact that CTAB-treated ML35 cells remain viable and grow as well as untreated cells militates against the presence of a sufficiently damaged membrane to allow free diffusion. Even more direct evidence was found in the fact that TMG successfully competes with the ONPG in transport into the cells as indicated by the lowering of ONPG hydrolysis *in vivo* in these CTAB-treated ML35 cells, but that the TMG did not influence ONPG hydrolysis in sonicated or toluene-treated ML35 cells in which the synthetic galactosides diffuse into the cell through the disrupted membranes. In addition, formaldehyde, which is a useful agent for testing nonspecific transport into cells[20], markedly lowered ONPG hydrolysis. The effect on ONPG hydrolysis of ATP addition to starved CTAB-treated cells suggests that the galactoside transport is an energy-mediated process. Additional evidence for the assumption that the transport of ONPG into the CTAB-treated cells is not a simple diffusion process is the different temperature coefficients for CTAB- and toluene-treated cells.

The genetic origin of the mutation in the *y* gene in the *lac* operon of ML35 cells is unknown. One could suggest that in the case of a point mutation, one subunit of the original "M protein" is formed and located within the bimolecular lipid layer of the cell membrane. STEIN[23] recently suggested that the carriers are built up of two subunits embedded in the bimolecular lipid layer. These subunits have the ability to bind a substrate, and when they are associated with the symmetrical protein at the opposite face, the substrate crosses the membrane by transfer from one subunit to the other.

Accepting this model, one could suggest that the transfer of the β-galactosides

into CTAB-treated ML35 cells is mediated by one of these masked subunits which is exposed by the action of CTAB on the outer layer of the cell membrane.

It is obvious that the above-mentioned hypothesis may not be the only possible one. For example, a more general hypothesis would be that in the absence of CTAB the tertiary and quaternary structure of the mutated polypeptide chains assume a nonfunctional arrangement, but that the presence of CTAB alters the molecular environment in such a way as to change the structure to a functionally active form. This would be independent of any suggestions as to the possible subunit structure of the permease.

In addition, one or two of the permeases of TMG usually present in *E. coli* cells might possibly have been so altered by the CTAB treatment that they could participate in transport of β-galactosides into the cell.

A clear-cut explanation of the β-galactoside transport into CTAB-treated cells will only be obtained after tests of various other *lac* operon mutant strains which were not available to me during this research.

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