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Effects of Toluene on *Escherichia coli*

ROBERT W. JACKSON AND J. A. DEMOSS

Department of Biology, Revelle College, University of California, San Diego, La Jolla, California

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ABSTRACT

JACKSON, ROBERT W. (University of California, San Diego, La Jolla), AND J. A. DEMOSS. Effects of toluene on *Escherichia coli*. J. Bacteriol. 90:1420–1425. 1965.—When toluene is added at appropriate levels to exponentially growing cultures of *Escherichia coli*, a time-dependent loss of turbidity is observed which is concurrent with a loss of material to the medium and with unmasking of β-galactosidase. In addition, the galactoside permease system is totally destroyed. Electron micrographs confirm the indications that the cells are not being lysed by toluene, although the cytoplasm collapses to the interior of the cell. Included in the material lost from the cell after toluene treatment is 85% of the total ribonucleic acid (RNA), the principal source of which appears to be the ribosomes. The loss of RNA is temperature-dependent. Protein is also lost to the medium as a function of both temperature and available toluene. Up to 25% of the total protein is found in the medium, the precise amount depending on the level of toluene employed. Zone centrifugation studies of extracts from treated cells indicate that toluene elicits a rapid disaggregation of ribosomes that is terminated, at any stage, by disruption of the cells. The disaggregation is temperature-dependent and does not occur at 4 C. It appears to be distinct from the actual degradation of ribosomal RNA and is accompanied by an accumulation of small particles during the initial phases of treatment at 21 C. Toluene added to crude extracts of normal *E. coli* cells is unable to cause detectable ribosome destruction.

Toluene has been employed for many years by microbiologists to sterilize cultures and to maintain solutions in a sterile condition. Furthermore, it has been employed as an unmasking agent in the assay of a variety of enzymes, e.g., β-galactosidase (Herzenberg, 1959), arabinose isomerase (Dobrogosz and DeMoss, 1963), and alkaline phosphatase (Levinthal, 1962), in bacteria in which it apparently alters the accessibility of these enzymes, present in whole cells, to exogenous substrates. Despite its history of long and extensive use, the nature of the effects of toluene has received scant attention. Scholz et al. (1959) noted that yeast cells treated with toluene exhibited large increases in the cellular content of hexosephosphates. They suggested that toluene causes a partial breakdown of intracellular compartments concerned with glycolytic reactions. Aside from this report, little attention appears to have been given to the mechanism(s) whereby toluene elicits cell death and causes enzyme unmasking, or to a possible relationship between these phenomena.

Preliminary evidence indicated to us that toluene-treated *Escherichia coli* cells remain essentially intact and retain at least some of their metabolic capabilities, and that β-galactosidase is not solubilized, but remains with the cell pellet. Therefore, toluene-elicited death, as well as the unmasking of enzymes, did not seem to be a simple matter of disrupting the cell or of wholly denaturing it. We report in this paper the results of some of our investigations into the effect of toluene on the structure and function of *E. coli*.

MATERIALS AND METHODS

Cultures and extracts. Except where a specific strain is noted, parallel experiments were conducted with each of *E. coli* strains ML-308, K-12, and the K-12 derivatives 200-PS and W-3000. The bacteria were grown in minimal salts medium (Sypherd and Strauss, 1963) supplemented with 0.5% glycerol. The culture flasks were aerated by shaking at approximately 76 rev/min in a New Brunswick Gyrotory water bath.

For the preparation of extracts, cell cultures were centrifuged at 12,000 × *g* for 10 min. The cells were then suspended in the original volume of minimal salts medium and centrifuged at 12,000 × *g* for 10 min. The resulting washed pellet was suspended in approximately 5 ml of appropriate buffer and broken in the French pressure cell at approximately 4,000 psi. The suspension of broken cells was centrifuged at 20,000 × *g* for 20 min, and the resulting supernatant fluid was used as the extract.

Toluene treatment. Cell suspensions were shaken with toluene in a New Brunswick Gyrotory water

bath at approximately 76 rev/min. The toluene treatment of cell extracts was accomplished in a tube with intermittent 60-sec immersions in a water bath and 30-sec vigorous stirrings with a vortex mixer.

Analytical procedures. β-Galactosidase was assayed according to the method of Lederberg (1950).

The uptake of C^{14}-leucine, C^{14}-uracil, and β-methyl-C^{14}-thiogalactoside by cells was measured by filtering the cells directly onto 0.45-μ filters (Millipore Filter Corp., Bedford, Mass.) and placing the dried filters into vials containing 10 ml of scintillation fluid (4 g of 2,5-diphenyloxazole and 100 mg of 1,4-di[2-(5-phenyloxazolyl)]-benzene per liter of toluene). The vials were assayed in a Model 725 Nuclear Chicago scintillation counter. Ribonucleic acid (RNA) and protein were precipitated with 5% trichloroacetic acid, and the precipitates were filtered and assayed for radioactivity by the same procedure.

For zone centrifugation analysis, cell extracts were centrifuged through 5 to 20% sucrose gradients in tris(hydroxymethyl)aminomethane (Tris)-Mg^{2+} buffer at *p*H 7.4 (0.02 M Tris, 0.01 M magnesium acetate, 0.01 M potassium chloride, and 1 ml of β-mercaptoethanol per liter of solution) for 90 min at 39,000 rev/min in the Spinco SW 39 rotor. An amount (0.2 ml) of the extract was layered on top of gradients consisting of 2.1 ml of each sucrose solution. Eight drop fractions were collected and diluted to 2.5 ml with distilled water. The RNA content was then estimated by reading the absorbancy at 260 mμ.

RESULTS

Treatment of cultures of *E. coli* with toluene leads to a dramatic decrease in cell viability. For example, when added to fresh cultures of *E. coli* containing approximately 5×10^9 viable cells per milliliter, toluene at a level of 1.5 μliters per milliliter of culture lowered the viable cell count to approximately 5×10^2 cells per milliliter. At a level of 50 μliters per milliliter of culture, the level approximating the usual conditions for unmasking enzymes, there were no viable cells observed.

The addition of toluene to cultures of *E. coli* also resulted in changes in turbidity which depended on the amounts added (Fig. 1). When added to exponentially growing cultures at 37 C at levels between 1.5 and 10 μliters per milliliter of culture at a turbidity of 125 ± 15 Klett units (filter no. 54), toluene caused a fairly rapid decline in turbidity, which leveled off in from 60 to 90 min. Above this range, the turbidity underwent no further increase, but did not decline. Below this range, turbidity continued to increase, and at 0.6 μliter per milliliter the rate of increase slowed perceptibly, then essentially returned to its initial value, indicating that a large number of cells were undamaged and continued to grow.

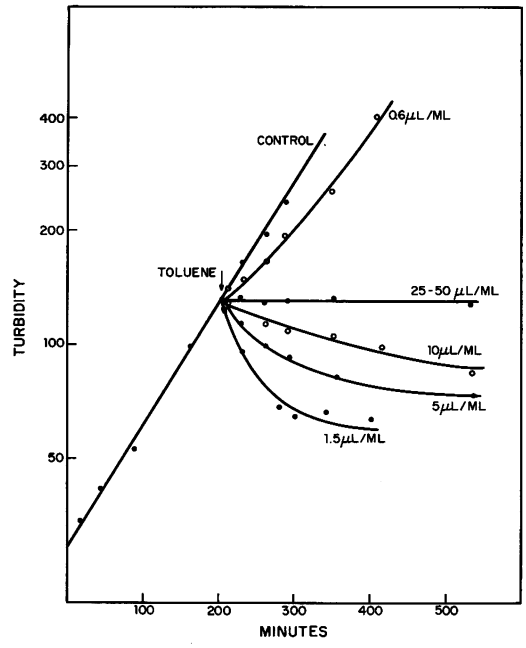

FIG. 1. *Changes in turbidity (Klett units, filter no. 54) of exponentially growing Escherichia coli after addition of various levels of toluene to the culture.*

During the course of the decline in turbidity, there appeared in the medium increasing amounts of material absorbing at 260 mμ, and a progressive unmasking of the enzyme β-galactosidase occurred. Furthermore, both the rate and the extent of the unmasking appeared to be functions of the level of toluene used. At 50 μliters per milliliter of culture, unmasking was maximal and occurred within seconds; but, at 1.5 μliters per milliliter, unmasking was approximately 50% of maximum and paralleled the decreasing turbidity. The addition of further toluene, after the maximal turbidity loss at lower levels is attained, did not bring about a further decline of turbidity but did cause further unmasking of β-galactosidase.

Effect upon gross morphology. The results indicated above are consistent with the notion that toluene causes disruption of *E. coli* cells. However, when partitioning of unmasked β-galactosidase between the soluble and sedimentable phases (material centrifuged at 12,000 × *g* for 10 min) of toluene-treated (50 μliters per milliliter) cells was examined, it was found that essentially all the enzyme activity was sedimentable. Moreover, when soluble β-galactosidase in extracts of fully induced W-3000 cells was brought together with toluene-treated uninduced cells, there was neither a sedimentable enzyme activity nor a loss of soluble activity. These results suggested that un-

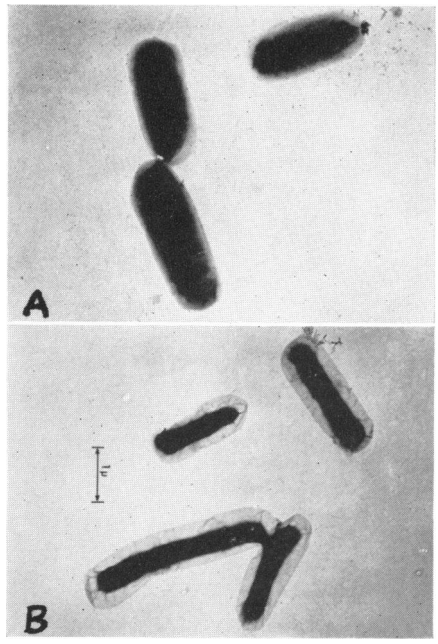

FIG. 2. *Electron micrographs of normal (A) and toluene-treated (B) Escherichia coli cells (20,000 ×). Cells were washed with minimal salts medium, followed by half-strength minimal salts and two distilled-water washes.*

masking of β-galactosidase was not the result of disrupting the cell, and that the enzyme was not simply released from the cell merely to attach itself again to sedimentable cell components.

That toluene-treated cells were not disrupted was confirmed by microscopic examination. With phase-contrast microscopy of cells treated with both 1.5 and 50 μliters per milliliter, no gross change in morphology was observed nor was there any apparent decrease in cell number or increase in cellular debris. Electron micrographs of cells (Fig. 2) treated with 50 μliters per milliliter also failed to reveal significant amounts of cellular debris, but did reveal that the cells appear to be essentially intact with perhaps slight shrinkage and a collapsing of the cytoplasmic contents to the center of the cell.

Loss of selective permeability. Inasmuch as toluene-treated cells remained intact but were rendered more accessible to the substrate of β-galactosidase, the effect of toluene on galactoside permease was examined. Cultures of ML 308 at 4 C were allowed to concentrate C^{14}-thiomethylgalactoside (TMG) to a maximum, and then toluene was introduced into the culture. The radioactivity remaining with the cells was determined. Figure 3 shows that concentrated TMG was rapidly lost from the cells. With 50 μliters of toluene per milliliter of culture, the reaction appeared to be instantaneous and was virtually complete as rapidly as a point could be taken; at 1.5 μliters per milliliter the rate was somewhat slower. When TMG and toluene were added simultaneously, the cells were unable to concentrate TMG. These results clearly indicate that toluene-treated cells have lost the ability to concentrate substrates.

However, if cell inviability is due solely to a loss of selective permeability and the ability to concentrate substrates, the possibility exists that toluene-treated cells can be encouraged to metabolize and respire and eventually to repair themselves. This possibility was examined by exposing treated cells to a variety of media and extracts of normal cells. Although treated cells could oxidize certain substrates and the terminal respiratory chain appeared to be intact, cell repair was never observed. Nor were treated cells able to carry out protein synthesis, as judged by the uptake of C^{14}-amino acids. Hence it appears that damage incurred by toluene treatment extends to macromolecular synthesis.

Effect on macromolecules. The inability to carry out macromolecular synthesis could result from a loss of significant portions of the synthesizing machinery, as suggested by the appearance of 260-mμ absorbing material in the medium surrounding treated cells. That material was further

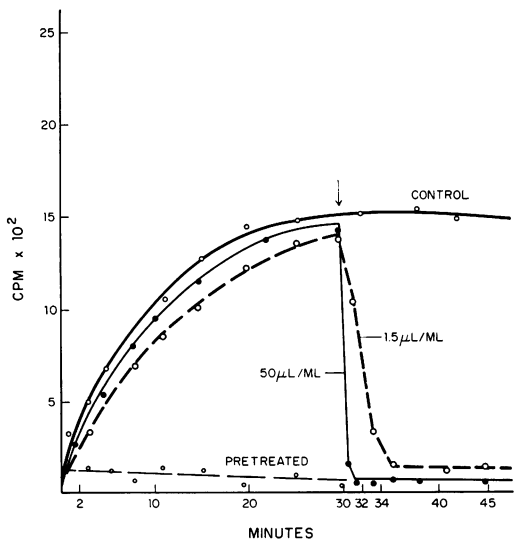

FIG. 3. *Destruction of the galactoside permease system by toluene. Exponentially grown cells were chilled to 4 C and allowed to concentrate C^{14}-TMG to a maximum before toluene was added (arrow indicates time at which toluene was added). Samples of cells (1 ml) were filtered and counted. Pretreated cells were treated with both levels of toluene immediately prior to addition of C^{14}-TMG.*

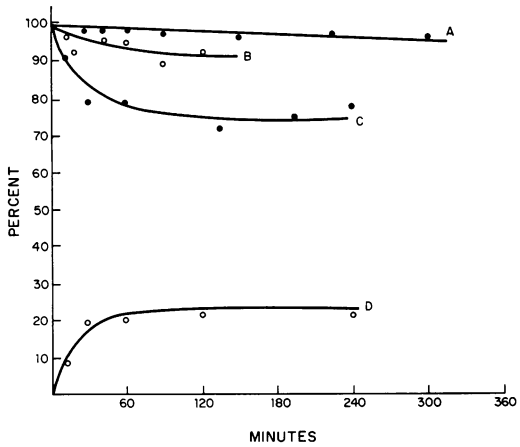

FIG. 4. *Loss of protein (C¹⁴-leucine label) from cells after toluene treatment at 37 C. Data are expressed as per cent of label in untreated cells at zero time. (A) Untreated control. (B) Label remaining with cells after treatment at 1.5 μliters of toluene per ml of culture. (C) Label remaining with cells after 50 μliters of toluene per ml of culture. (D) Trichloroacetic acid-precipitable label in medium surrounding cells subsequent to addition of 50 μliters per ml.*

characterized by growing cultures in the presence of C¹⁴-leucine or C¹⁴-uracil to label protein or RNA, respectively. The radioactivity of cells and of trichloroacetic acid-precipitable material in the medium during the course of toluene treatment was then determined. Figures 4 and 5 show the loss of protein and RNA from treated cells. The protein lost (Fig. 4) accounted for approximately 8% at a maximum of the total protein when cells were treated with 1.5 μliters per milliliter of culture. However, at 50 μliters per milliliter, the protein lost approached 25% of the cellular protein. This was reflected almost precisely by an increase in the medium of trichloroacetic acid-precipitable protein. At 4 C there was negligible loss, even at levels of 50 μliters of toluene per milliliter of culture.

The total loss of RNA was independent of the levels of toluene; however, the rate of loss was much faster at 50 than at 1.5 μliters per milliliter (Fig. 5). In both cases, the maximal loss accounted for nearly 85% of the cellular RNA. This loss was not reflected by similar amounts of trichloroacetic acid-precipitable RNA in the medium, although a small amount was found in the medium at very early stages of toluene treatment. As with protein, the loss was negligible at 4 C.

Origin of released RNA. Since the level of RNA lost upon toluene treatment approximates that expected if the ribosomes were the source of the RNA, extracts of treated ML 308 cells were examined by means of zone centrifugation.

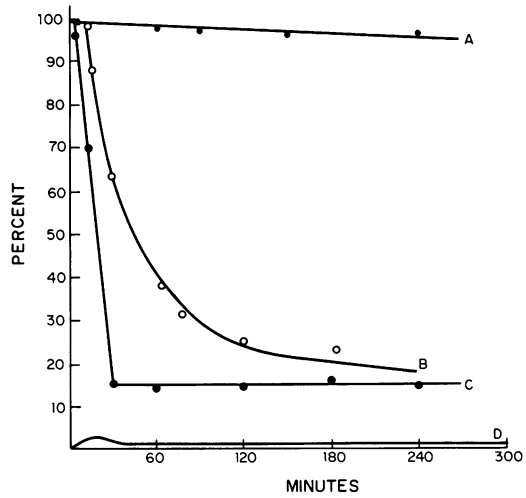

FIG. 5. *Loss of RNA (C¹⁴-uracil label) from cells after toluene treatment at 37 C. Data are expressed in per cent of label found in untreated control at zero time. (A) Untreated control. (B) Label remaining with cells after treatment with 1.5 μliters of toluene per ml of culture. (C) Label remaining in cells subsequent to addition of 50 μliters of toluene per ml of culture. (D) Trichloroacetic acid-precipitable label in the medium surrounding the cells after treatment with 50 μliters per ml.*

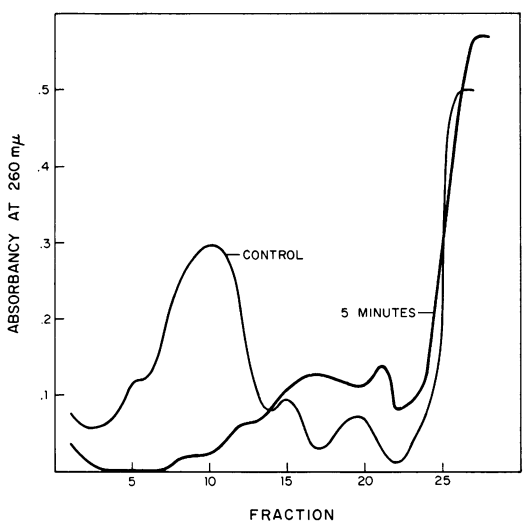

FIG. 6. *Disaggregation of ribosomes in cells after addition of 50 μliters of toluene per ml of culture at 37 C. Cells were washed with minimal salts, pelleted, and then taken up in buffer and broken in a French pressure cell. Extracts were centrifuged through sucrose gradients. See Materials and Methods for details.*

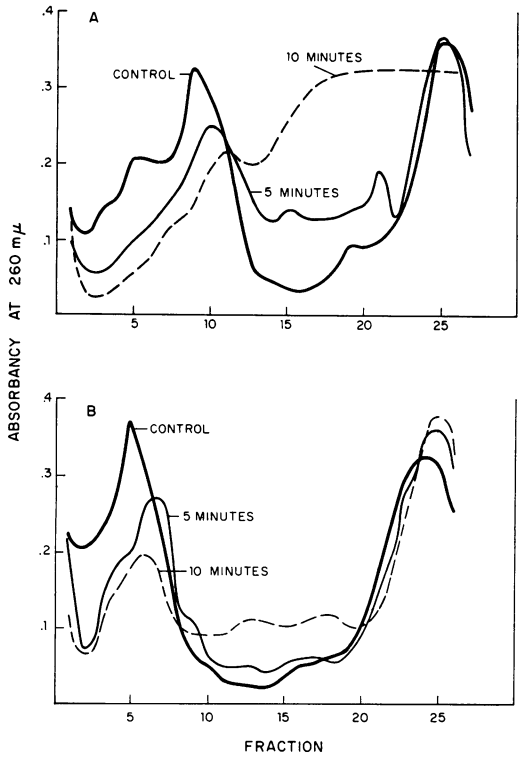

FIG. 7. *Disaggregation of ribosomes in cells treated with 50 μliters of toluene per ml of culture at 21 C. (A) Extracts prepared as in Fig. 6 and Materials and Methods. Accumulated subparticles begin to decline at about 20 min and require in excess of 60 min to disappear entirely. (B) Extracts from A above have been placed at 37 C for 15 min, then centrifuged through sucrose gradients. Toluene-elicited subparticles have disappeared but further disaggregation of ribosomes does not take place.*

As seen in Fig. 6, the normal ribosome profile was rapidly abolished in cells treated at 37 C with 50 μliters of toluene per milliliter of culture. Within 15 min, there was no evidence whatever of particulate fractions. With cells treated at 21 C, the process was slowed sufficiently to observe that there was a progressive decrease in the 100*S* and 70*S* ribosomes, which was essentially complete within 30 min. During this time there was a concurrent increase in smaller particles, which reached a maximum in approximately 10 min and declined thereafter, requiring in excess of 60 min for total disappearance (Fig. 7A). When cells were treated at 4 C, there was no observable change in the ribosomal profile.

Disruption of the toluene-treated cells terminates the disaggregation of the ribosomes, but not the degradation of the accumulated smaller particles. When the extracts used for the profiles in Fig. 7A were incubated at 37 C for 15 min, there was a significant decrease in the lighter particles, but not in those in the 100*S* to 70*S* region (Fig. 7B). This appeared to be true whether the extracts were made from washed treated cells or from treated cells resuspended in toluene-containing medium.

Direct treatment of crude extracts of normal cells with various levels of toluene did not produce a significant disaggregation of the ribosomes.

Attempts were made to demonstrate a toluene-elicited ribosome-disaggregating factor by preparing various fractions from treated cells and mixing these fractions with ribosomes from both normal and treated cells. However, in all cases, these tests failed to produce evidence for such a factor.

DISCUSSION

The loss of turbidity by cultures of *E. coli* after addition of toluene apparently reflects some change in their optical properties. This is presumably due in part to a loss of material from the cell. That this explanation is insufficient, however, is indicated by the fact that, with levels higher than 25 μliters of toluene per milliliter of culture, no turbidity loss is observed, even though an acute loss of intracellular matter occurs.

The loss of material from toluene-treated cells presents an interesting and complex state of affairs. The complete and very rapid destruction of the galactoside permease, even by quite low levels of toluene (1.5 μliters per milliliter of culture), with the resultant rapid efflux of concentrated substrate (TMG), indicates that the cell is opened up to the free passage of small molecules. A similar loss of tryptophan permease activity has been noted by Burrous (Ph.D. Thesis, University of Illinois, 1962). It seems reasonable to suppose that all permease activities are similarly affected, indicating a generally deleterious effect on the cellular membrane. However, disruption of the membrane cannot be considered complete, since the terminal respiratory chain seems to be intact.

The loss of cellular protein depends not only on the level of toluene, but also on the temperature at which the treatment is performed. The temperature dependency could be the result of lower solubility of toluene in cellular lipid and lipoprotein at low temperatures, and thus, indirectly, could, in actuality, be a further reflection of toluene-level dependency. It should be noted in this regard that the destruction of galactoside permease by toluene occurs at 4 C; thus, not all of the effects of toluene are temperature-dependent. However that may be, higher levels of toluene bring about higher losses of protein from the cell

and may indicate that larger "holes" are made in the cytoplasmic membrane.

Toluene-elicited RNA loss from *E. coli*, like protein loss, is temperature-dependent, but, unlike protein loss, it is independent of toluene levels within the range used in the experiments reported here. The rate of loss of RNA from the cells, however, is affected by the levels of toluene and presumably reflects the length of time required for the whole population of cells to absorb sufficient toluene at the low levels to elicit the effect. Temperature dependency, in this case, as with protein loss, may have a wholly trivial explanation based on lowered solubility of toluene in lipids and lipoproteins. On the other hand, since the lost RNA appears to be largely of ribosomal origin and since it appears in the surrounding medium as acid-soluble material, the possibility exists that toluene elicits an enzymatic degradation of ribosomes followed by degradation of ribosomal RNA, and that it is the enzymatic action that is temperature-dependent.

The results of zone centrifugation studies of extracts of treated cells indicate that the disaggregation of ribosomes is a distinct reaction from degradation of RNA, since between 15 and 30 min are required at 37 C for the maximal loss of cellular RNA to occur. However, the time for complete disaggregation of normal ribosomes is much less than that. This notion is supported by the fact that the smaller particles which accumulate during the early stages of 21 C toluene treatment continue to degrade in extracts of treated cells at 37 C, but the particles in the 100*S* and 70*S* region do not.

Of the various mechanisms that can be imagined for the toluene-elicited disaggregation of ribosomes, all fall into two classes which involve either a disaggregating enzyme or the direct denaturation by toluene. All attempts to demonstrate a disaggregating factor have thus far met with failure. This could indicate that such a factor is very unstable or that an inhibitor is released upon cell disruption. Alternatively, it may suggest that the peculiar organization of the cell is required for the factor to be effective. This latter explanation could also be important to a mechanism involving denaturation by toluene, inasmuch as toluene is unable to effect disaggregation of ribosomes in extracts.

Whether the mechanism ultimately proves to involve an enzyme or simply a denaturation, the study of toluene-elicited ribosomal subparticles could provide some important information regarding the structure of ribosomes and their organization in the cell.

The unmasking of β-galactosidase by toluene presents a further curiosity, namely, that a level sufficient to destroy galactoside permease (1.5 μliters per milliliter of culture), to produce a seven orders of magnitude reduction in viable count, and presumably to render the cells freely permeable to small molecules, results only in approximately a half-maximal unmasking of the enzyme. It has already been noted that the masking of β-galactosidase is only partially a question of impermeability of the substrates (Lederberg, 1950). Hence, the results with toluene lend some weight to the notion that masking reflects some peculiarity of organization of the cell rendering the enzyme inaccessible to the substrate (Bonner, 1955).

With regard to the lethal effect of toluene, it must be said that either the loss of the ability to concentrate substrates or the loss of ribosomes would be sufficient to explain the inviability of toluene-treated cells. Still, the treated cell retains the machinery to provide itself with energy, and thus may find some utility in the study of terminal oxidation reactions and of membrane-bound activities.

ACKNOWLEDGMENTS

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