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EXPERIMENTAL ARTICLES

Assessing the Toxic Effect of 2,4,6-Trinitrotoluene on Cells of *Escherichia coli* K12 by Flow Cytofluorometry

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Abstract—The magnitude of transmembrane potential $\Delta\psi$ in cells of *Escherichia coli* K12 was determined by the method of flow cytofluorometry for different phases of growth. It was large in the log phase, whereas in the lag and stationary phases, the population was shown to consist of two subpopulations with low and large values of $\Delta\psi$ in cells. In the presence of 200 mg/l of 2,4,6-trinitrotoluene (TNT), this bimodal distribution of $\Delta\psi$ over the population was observed during the entire growth period until TNT was almost completely eliminated from the cultivation medium (to a concentration of 18–20 mg/l). The mean value of $\Delta\psi$ in cells of the population grown in the presence of TNT was substantially smaller than that in controls due to the larger fraction of the subpopulation with a low value of $\Delta\psi$. Upon elimination of TNT, the distribution of $\Delta\psi$ in cells of the culture became unimodal and close to that in the control culture in the early log phase of growth. These findings are discussed from the standpoint that considers heterogeneity of the culture of *Escherichia coli* K12 as a mechanism of its adaptation to the presence of xenobiotics.

Key words: flow cytofluorometry, *Escherichia coli* K12, 2,4,6-trinitrotoluene, toxicity.

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The presence of 200 mg/l of 2,4,6-trinitrotoluene (TNT) was shown in our previous studies to inhibit the growth and reduce the number of colony-forming units of *Escherichia coli* K12. TNT was observed to suppress redox processes (the reduction system) in cells and to affect the morphological and physiological features of cells. Concurrently, the transformation of this xenobiotic by the culture switches from nitroreduction to denitration [1]. All these effects, including changes in the TNT transformation pathways, may be caused by TNT affecting energy accumulation by bacterial cells.

The goal of this work was to assess the TNT influence on the transmembrane potential $\Delta\psi$, which is the electrical component of the proton potential $\Delta\bar{\mu}\,H^+$ difference on the cytoplasmic membrane of the bacterial cell.

MATERIALS AND METHODS

The object of study, strain *Escherichia coli* K12, was obtained from the collection of the Chair of Microbiology, Kazan State Medical Academy.

The inoculum was grown for 16–18 h at 30°C in 100-ml flasks on nutrient broth under forced aeration on a shaker (120 rpm) and introduced into the medium to a final concentration of 3.4×10^6 cells/ml into 250-ml flasks with a synthetic medium composed of the following (g/l): $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.25; NaCl, 0.5; glucose, 3.0; TNT, 0.2; and phosphate buffer (0.2 M KH_2PO_4/Na_2HPO_4, pH 7.0), 4 vol %.

TNT was determined by a method based on its reaction with sodium sulfite in alkaline medium [2].

The magnitude of transmembrane potential $\Delta\psi$ in bacterial cells was determined in relative fluorescence units (RFU) by the flow cytofluorometry technique [3]. Cells of the grown culture were washed free of incubation medium by centrifugation and suspended in 0.06 M phosphate buffer to a density of approximately 2×10^6 cells/ml. The potential-sensitive fluorochrome 3,3'-dihexyl-oxacarbocyanine iodide ($DiOC_6(3)$) was added to a 1-ml aliquot of the bacterial suspension to a final concentration of 2 μM, and cells were stained at room temperature for 15 min in the dark. The controls were intact cells with membranes deenergized by heating at 60°C for 5 min. The viability of heated cells was tested by inoculating the suspension into nutrient broth and determining the permeability of the cytoplasmic membrane to the fluorescent dye propidium iodide (PI). To determine the permeability of the cytoplasmic membrane, PI was added to a 1-ml aliquot of the bacterial

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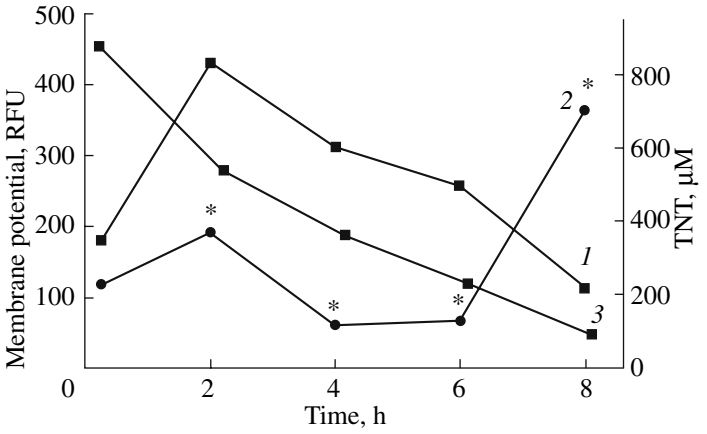

Fig. 1. Effect of 2,4,6-trinitrotoluene (TNT) on the membrane potential in cells of *E. coli* K12: (*1*) control cells; (*2*) "TNT cells"; (*3*) TNT content of the growth medium. Here and in subsequent figures, points with a statistically significant difference from controls at a level of $P < 0.001$ are denoted by "*".

suspension to a final concentration of 30 μg/ml, and cells were stained for 15 min in the dark. The samples were analyzed on a FacsCalibur flow cytometer (Becton and Dickinson, United States) at a rate of less than 1000 cells/s under laser excitation (wavelength, 488 nm). The readings of the membrane potential, expressed in relative fluorescence units, were taken from the green fluorescence channel (detector FL1, 530/30 nm BP filter), and fluorescence from propidium iodide from the red channel (detector FL3, 670 LP filter). The instrument was calibrated using standard fluorescent microspheres (Becton and Dickinson). The forward scatter of light (correlating with the cell size) was also measured in all assays. The size of cells was expressed in relative light scatter units (RLSU). No less than 30000 cell events were recorded in each test variant, and the results were analyzed on a Macintosh Quadra 650 workstation running the Cell Quest software (Becton and Dickinson) with use of the Kolmogorov–Smirnoff statistical test. In order to correctly compare $\Delta\psi$ in cells of different size, only normalized ("signal minus noise") histograms were used.

RESULTS AND DISCUSSION

The source of $\Delta\bar{\mu}_{H^+}$ is the respiratory chain of bacterial cells, and its magnitude can determine the value of its electric component $\Delta\psi$. The primary carrier of one proton and two electrons in *E. coli* cells is NAD^+ [4]. The NADH content of *E. coli* K12 cells decreases in the presence of TNT as a result of TNT-induced inhibition of the redox metabolism [5] and because some NADH is utilized in the formation of nitroreduction products [6]. It is then quite probable that the magnitude of $\Delta\bar{\mu}_{H^+}$ in bacterial cells will also fall under such conditions, which, in turn, can be accompanied by a decrease in the transmembrane potential $\Delta\psi$ in bacterial cells. The latter is most often measured by means of potential-sensitive fluorochromes, widely used in flow cytofluorometry [3, 7–11].

The time variation of $\Delta\psi$ in *E. coli* K12 control cells and in cells cultured in the presence of TNT (the "TNT cells") is plotted in Fig. 1. One can see that, in controls, $\Delta\psi$ attains a peak value after 2 h of growth and starts to gradually decrease afterwards. After 6 h of growth, its level continues to remain higher than that in cells of the early lag phase (0.25 h) and does not fall below this level until 8 h of growth.

The presence of TNT changes the time variation of cellular $\Delta\psi$. Likewise in controls, it goes up during the first two hours, but in controls, it increases by a factor of 2.4, while in TNT cells, this increase is only 1.6. After 4 and 6 h, $\Delta\psi$ becomes significantly smaller than in cells of the early lag phase. Unlike the controls, however, after eight hours of culturing, when the xenobiotic is virtually eliminated from the medium (its residual content drops to under 20 mg/l), $\Delta\psi$ increases threefold over its level in cells of the early lag phase.

Large cells and cell aggregates, sensed by the cytometer as single cells, can contain a large number of molecules of the potential-sensitive dye $DiOC_6(3)$ and be falsely interpreted by the instrument as cells with elevated $\Delta\psi$. To take this into account, the size of control and TNT cells was also determined (Fig. 2a) and the membrane potential was normalized with respect to cell dimensions (Fig. 2b). It can be seen from Fig. 2a that the early effect of TNT consists in a reduction of the cell size. It is not until after 6 h of cultivation that TNT cells reach the size of control cells in the early lag phase; and they outgrow them only after 8 h, at a moment when the xenobiotic is almost completely eliminated from the medium. This pattern of size variation of TNT cells in *E. coli* K12, obtained in this study from forward light scattering data, is in agreement with the results of earlier micrometric measurements of the size of TNT cells in *E. coli* K12 and *Bacillus subtilis* SK1 [5, 12].

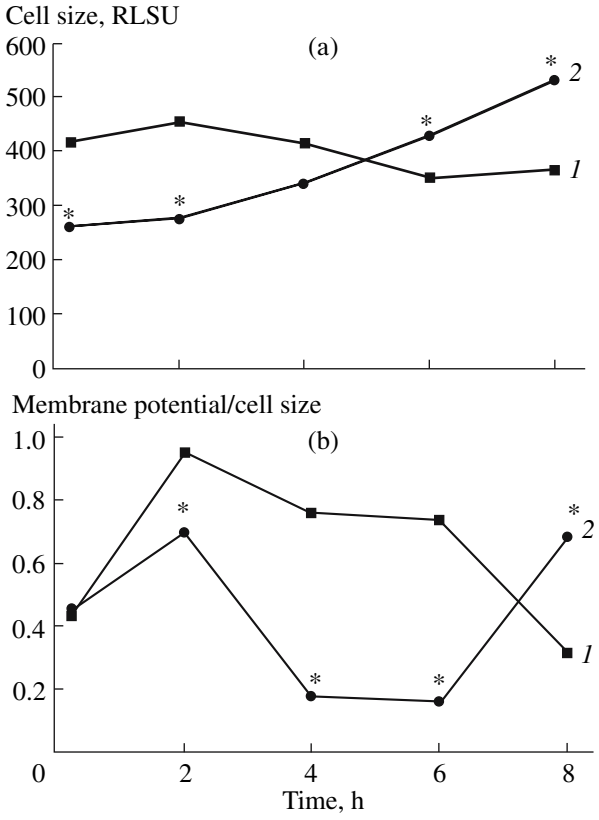

Fig. 2. Effect of TNT on (a) size of cells and (b) cellular membrane potential normalized by cell size in *E. coli* K12: (*1*) control cells; (*2*) TNT cells. Cell size is given in relative light scatter units (RLSU).

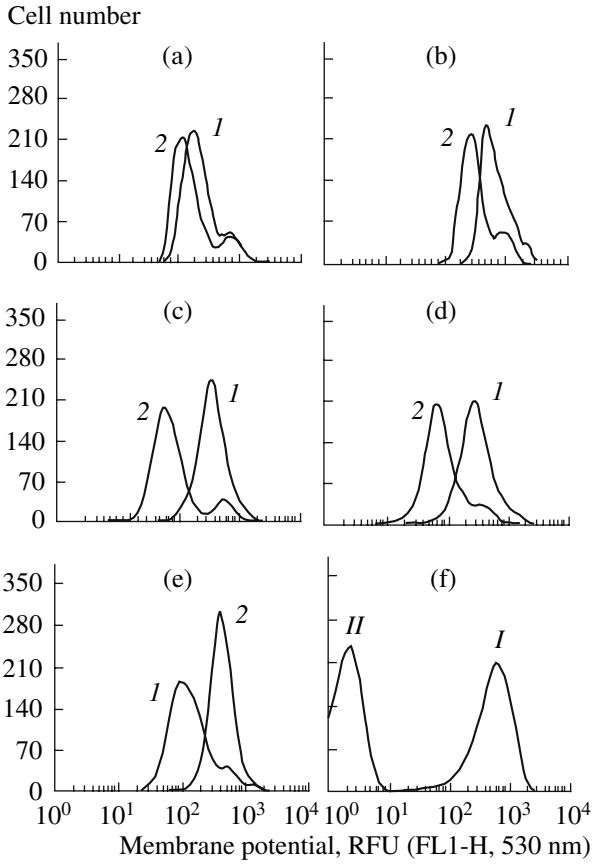

Fig. 3. Histograms of cellular membrane potential in a culture of *E coli* K12 after (a) 0.25, (b) 2, (c) 4, (d) 6, and (e) 8 h of cultivation: (*1*) control culture cells and (*2*) TNT cells. (f) Control for staining of cells with deenergized membranes: (*I*) log-phase cells; and (*II*) log-phase cells heated at 60°C for 5 min. The Y-axis is the number of cells, and the X-axis is the membrane potential in relative fluorescence units (RFU) of the dye $DiOC_6(3)$.

The similarity between the distribution of Δψ normalized by the cell size (Fig. 2b) and the unnormalized graphs of Δψ (Fig. 1) in controls and in the population of TNT cells rules out the possibility of a false-positive difference of Δψ arising from dissimilar size of cells in the compared populations.

Of significant interest is the following body of evidence relating to the TNT influence on the cellular metabolic status. Both the control and TNT populations of cells were found to be inhomogeneous with respect to Δψ. Both populations comprised subpopulations characterized by a high value of Δψ (referred to as high-Δψ cells) and subpopulations with a low value of Δψ (low-Δψ cells). Shown in Fig. 3 are histograms of cellular membrane potential for bacterial cultures grown with and without TNT (the left peaks in the histograms correspond to low-Δψ-cells and the right ones to high-Δψ-cells). It is noteworthy that, in the early lag phase, cells in the control and test cultures alike are already not uniform in their energy state (Fig. 3). Supposedly, in the lag phase it is only the cells with a Δψ value close to that of high-Δψ-cells that are ready to multiply. This supposition leads to an attractive explanation of a commonly observed difference between the total number of cells and the number of colony-forming cells determined in the same aliquot of a culture. In the log phase (time span between 2 and 6 h), high-Δψ-cells begin to gradually outnumber low-Δψ ones (Figs. 3b–3d). The histograms of Δψ become unimodal in shape and shift rightwards to higher values of the potential. The fraction of low-energized cells disappears. After 8 h of culturing (in the phase of decelerated growth and during the transition to the stationary phase), the histogram of Δψ once again becomes bimodal (Fig. 3e). Therefore, both in the stationary and early lag phases, the control culture consists of two subpopulations of cells characterized by different values of Δψ. Given that the inoculum used in our experiments was an aliquot of the stationary culture, it can be concluded that the similarity of the histograms in Figs. 3a and 3e attests to the correctness of the obtained data.

Histograms of Δψ for the culture of TNT cells, unlike those for cells of the control, are bimodal for populations aged 2 to 6 h. And it is only after 8 h of growth that the population comes to be represented by

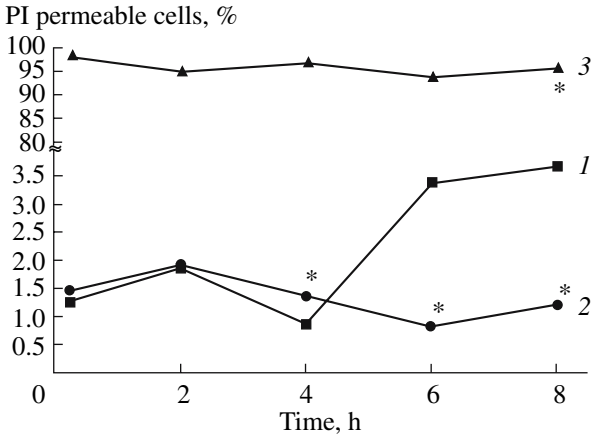

Fig. 4. Effect of TNT on permeability of *E. coli* K12 cells to propidium iodide (PI): (*1*) control cells, (*2*) TNT cells, and (*3*) control cells subjected to heating. The data were recorded with a FL3 detector, 670 nm.

high-$\Delta\psi$ cells alone, and is no longer heterogeneous in this respect. The histogram acquires a unimodal shape (Fig. 3e) concurrently with elimination of TNT from the culture medium and cell enlargement (Figs. 1 and 2).

The average values of $\Delta\psi$ in TNT cells over the cultivation period between 2 and 6 h happen to be smaller than the respective values for the control culture (Figs. 1 and 2b) because of the prevalence of low-$\Delta\psi$ TNT cells (the peaks in histograms arising from low-$\Delta\psi$ TNT cells are shifted leftwards (Figs. 3a–3d)).

In principle, it cannot be ruled out that the observed changes in the transmembrane potential in the test cultures could be brought about by permeability impairment of cytoplasmic membranes (CPM) caused by TNT or products of its metabolism. The results of CPM permeability assays are shown in Fig. 4. The fraction of cells in the control and test cultures stained by PI, i.e., cells with a permeable CPM [13], did not exceed 2–4%. By contrast, in the control culture exposed to heating and failing to form colonies, the fraction of stained cells was on average as high as 96%. Therefore, the observed decrease of $\Delta\psi$ in cells of the test cultures was not related to increased CPM permeability. Instead, changes in cellular $\Delta\psi$ could be explained by suppression of mechanisms responsible for maintaining the energy status of bacteria, i.e., by the impaired regulation of redox processes (in the reduction system) [5]. It is important that, in batch cultures, inhibition of the mechanisms maintaining high level of $\Delta\psi$ is not lethal. It has only a transient nature, and $\Delta\psi$ in TNT cells returns to its original value as soon as the xenobiotic is eliminated from the culture (Fig. 1).

The integrity of bacterial CPMs is one of the viability criteria for bacterial cells. The fact that the CPM permeability was not affected by TNT (or products of its metabolism) indicates that cells of *E. coli* K12 retained their viability despite the toxic action of this xenobiotic. However, as reported elsewhere, the permeability of the outer lipoprotein membrane definitely increased in the presence of TNT [5]. It cannot be ruled out, therefore, that targets of the toxic action of TNT reside on the outer side of CPM.

As shown earlier, the cells of *E. coli* K12 cultured in the presence of TNT not only become smaller but some of them also change morphologically, taking on a rounded, coccoid shape. The percent of coccoid cells was observed to decrease with the TNT content of the culture medium. Such heterogeneity of the culture is probably due to different susceptibility of cells to the toxic action of TNT [5]. The results reported here show that, under toxic contamination by TNT, the culture of *E. coli* K12 also exhibits heterogeneity with respect to $\Delta\psi$, consisting of two well-defined subpopulations of low-$\Delta\psi$ and high-$\Delta\psi$ TNT cells. We believe that this is not a particular effect caused by the presence of TNT in the medium. The control culture of *E. coli* K12 also proved to be heterogeneous with respect to cellular $\Delta\psi$. Not so strong as in the case of TNT cells, this heterogeneity is most prominent in the early lag phase and during the transition to the stationary phase (Figs. 3a and 3e). In the stationary phase, cells are exposed to conditions unfavorable for their growth or multiplication. The observed similarity of the population structures with respect to $\Delta\psi$ in the stationary and lag phases can be explained by similar types of metabolism in cells of the stationary phase and the same cells used as inoculum (lag phase). Population heterogeneity was previously shown in starving bacterial cultures, trying to sustain themselves by preserving one part of their population (represented by so-called cannibal cells) at the expense of lysed cells of the rest of the population [14, 15]. It appears that conditions unfavorable for cellular growth and multiplication, no matter what their nature, can trigger dissociative activities in the culture, forming a subpopulation of cells best resistant (adapted) to the conditions that happen to be lethal to most cells in the culture.

The results of this investigation support our conclusion made elsewhere that the capacity of a culture to completely eliminate TNT is due not so much to its cells having resistance to the toxic action of this xenobiotic but rather to the activation of adaptation mechanisms both at the level of the cell and the culture level. The toxic action of TNT shows up in morphological changes induced in a part of the culture cells, in suppressed redox metabolism, in changed strategy of xenobiotic transformation [1, 5, 12, 16], in repressed capacity of cells to accumulate energy, and in changed energy profile of the population. However, in spite of such manifest toxic effects, TNT is efficiently eliminated by the culture, bringing to norm not only the morphological and physiological characteristics of its cells [5] but also the transmembrane potential of cells and its population profile. This fact suggests that the toxic influence of the xenobiotic is overcome by switching the transformation strategy from nitroreduction, requiring consid-

erable energy, to denitration, which is probably less energy dependent.

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