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Participation of Oxygen in the Bacterial Transformation of 2,4,6-Trinitrotoluene

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Abstract—The exposure of *Bacillus cereus* ZS18 cell suspensions to 2,4,6-trinitrotoluene (TNT) in the absence of other oxidizable substrates increases oxygen uptake, exceeding the basal level of respiration of the bacterium 1.5- and 2-fold with 50 and 100 mg/liter of TNT, respectively. The interaction of both living and to less extent dead bacterial cells with TNT results in the accumulation of superoxide anion ($O_2^{\dot{-}}$) in the extracellular medium, which was revealed by the EPR spectroscopy. The accumulation of $O_2^{\dot{-}}$ decreased by 50-70% in the presence of Cu,Zn-superoxide dismutase of animal origin. In the presence of living bacterial cells, the level of TNT decreased progressively, yielding hydroxylaminodinitrotoluenes together with $O_2^{\dot{-}}$. In the presence of heat-killed cells, a moderate decrease in TNT was observed, and the appearance of $O_2^{\dot{-}}$ was not accompanied by the production of any detectable TNT metabolites. Chelating agents inhibited the transformation of TNT and decreased the formation of $O_2^{\dot{-}}$. The demonstrated generation of $O_2^{\dot{-}}$ during the interaction of TNT with $K_4[Fe(CN)_6]$ together with the observed effects of chelating agents suggest the participation of iron in the one-electron reduction of TNT and the functioning of an extracellular redox cycle with the involvement of molecular oxygen.

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Key words: 2,4,6-trinitrotoluene, aerobic transformation, superoxide anion

Nitroaromatic compounds that are widely used as explosive substances and pesticides, due to their high stability together with toxic and mutagenic effects [1], constitute a group of important ecologically dangerous pollutants [2-4]. The toxicity of trinitrotoluene and its metabolites has been demonstrated using different test systems from bacterial cells to mammals [5-8].

Most microorganisms are capable of transforming the 2,4,6-trinitrotoluene (TNT) molecule in some way. The most usual pathway of microbial transformation of this compound under aerobic conditions is a consecutive two-electron mechanism resulting in the transformation of the nitro-group into an amino group through the nitroso and hydroxylamino groups. The most complete reductive transformation of TNT yielding triaminotoluene was revealed only in obligate anaerobic microorganisms [9, 10].

Under aerobic conditions, isomeric hydroxylaminodinitrotoluenes (HADNTs) are formed as the main metabolites of TNT in most microorganisms [11, 12] without their subsequent transformation to monoamino derivatives. Representatives of the genera *Lactobacillus* [13] and *Pseudomonas* [14] are capable of reducing TNT nearly stoichiometrically, yielding HADNTs.

One of the known enzymes of nitroreduction, oxygen-sensitive nitroreductase, reduces NO_2-groups under aerobic conditions by a one-electron mechanism yielding nitro anion radicals that can be oxidized by molecular oxygen producing the original TNT and the superoxide anion [15]. However, there are no data on the extracellular formation of the superoxide anion in the TNT–bacterial cell system.

The subject of the present work was the process taking place during the contact of TNT with bacterial cells (living or dead) under aerobic conditions.

MATERIALS AND METHODS

Microorganisms. In the present work we used representative strains of TNT-transforming microorganisms *Bacillus cereus* ZS18 and *Pseudomonas* sp. EN1582 isolat-

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Abbreviations: ADNT) aminodinitrotoluene; HADNT) hydroxylaminodinitrotoluene; $O_2^{\dot{-}}$) superoxide anion; SOD) Cu,Zn-superoxide dismutase; TNT) 2,4,6-trinitrotoluene.
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ed previously from waste products of the oil and gas industry [14].

Chemicals. Epinephrine hydrochloride, sodium 4,5-dioxybenzene-1,3-disulfonate (Tiron), superoxide dismutase from bovine erythrocytes, and xanthine oxidase from milk were from Sigma (USA); xanthine from Chemapol (Czechia); sodium azide from Labtekh (Russia); 2,2′-bipyridyl from Reanal (Hungary); EDTA from Reakhim (Russia). The mixture of HADNT isomers as well as analytical standards 2-aminodinitrotoluene (2-ADNT) and 4-ADNT were synthesized earlier [13]. The chromatographically pure preparation of TNT was twice recrystallized from ethanol.

Transformation of TNT by cell suspensions. The bacteria were cultivated until the late exponential growth phase in medium A of the following composition (g/liter): glucose, 5; $MgSO_4$, 0.25; Na_2HPO_4, 4.5; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.6 (without TNT). The cells were pelleted by centrifugation (9000*g*, 15 min), washed twice with 30 mM Na_2HPO_4/20 mM KH_2PO_4 (pH 7.0), and resuspended in buffer containing TNT so that the absorption (A_{600}) constituted 0.1, 0.5, or 1.0. In experiments with dead cells, the washed cell suspension was incubated in boiling water (100°C, 15 min). The loss of vitality by the cells was monitored by their inability to grow on agarized medium A and in meat-peptone broth. In the experiments with chelating agents, the cells were washed with buffer containing EDTA (0.1 M) or 2,2′-bipyridyl (1 mM). TNT was added before autoclaving to achieve a final concentration of 100 mg/liter. Flasks (250-ml), each containing 25 ml of the incubation mixture, were incubated at 28°C under static aerobic conditions.

Oxygen uptake by cell suspensions was measured by Warburg's manometric method every 20 min for 2 h. The cells grown in medium A without TNT were pelleted by centrifugation (15 min, 9000*g*) and washed twice with 50 mM K,Na-phosphate buffer, pH 7.0. The oxygen uptake by the incubation mixtures containing cell suspensions (A_{600} = 2.0) was measured in the same buffer containing 50 or 100 mg/liter of TNT.

Transformation of TNT in the presence of ferrocyanide ($K_4[Fe(CN)_6]$) was performed in 50 mM phosphate buffer containing 100 mg/liter of TNT. The reaction was initiated by the addition of $K_4[Fe(CN)_6]\cdot 3H_2O$ (10^{-2} mM). The formation of ferricyanide ($K_3[Fe(CN)_6]$) was monitored by increase in absorbance at 320 nm.

HPLC analysis of the products of TNT transformation in cell-free incubation mixtures was performed using a Series 200 chromatograph (Perkin Elmer, USA) in the reversed-phase mode on a Phenomenex column (150 × 4.6 mm C18) using an absorbance detector at 254 nm. The products were eluted in the isocratic mode with the solvent system acetonitrile–water (40 : 60 v/v) at 0.5 ml/min and 30°C. The retention time (min) was 16.0 for TNT, 7.9 for 2HADNT, 7.7 for 4HADNT, 9.84 for 2ADNT, and 11.1 for 4ADNT.

Decrease in TNT concentration (100 mg/liter) after the addition of bacterial cells depending on the density of the bacterial suspension

Strain	Initial decrease in TNT with addition of cells into the incubation mixture (mg/liter)		
	Absorption of the cell suspension (A_{600})		
	0.1	0.5	1
	Gram-positive bacteria		
Bacillus sp. NE1	4.5 ± 0.20	5.0 ± 0.50	6.1 ± 0.35
Bacillus subtilis JH642	5.7 ± 0.25	6.8 ± 0.10	7.5 ± 0.15
Bacillus cereus ZS18	4.7 ± 0.10	6.8 ± 0.25	8.4 ± 0.10
	Gram-negative bacteria		
Pseudomonas putida ZS41	4.5 ± 0.20	7.9 ± 0.10	9.5 ± 0.05
Pseudomonas sp. ZS50	3.9 ± 0.05	6.4 ± 0.05	7.8 ± 0.02
Pseudomonas sp. ZS81	4.0 ± 0.1	7.2 ± 0.05	8.2 ± 0.05

Determination of superoxide anion by EPR. Superoxide anion was determined using the spin trap sodium 4,5-dioxybenzene-1,3-disulfonate (Tiron) that interacts with $O_2^{\cdot-}$ in alkaline medium yielding the EPR-detectable stable radical Tiron-semiquinone [16]. The EPR spectra were recorded at room temperature (25°C) on an ESP-300 spectrometer (Bruker, Germany) with microwave power 50 mW, microwave frequency 9.8 GHz,

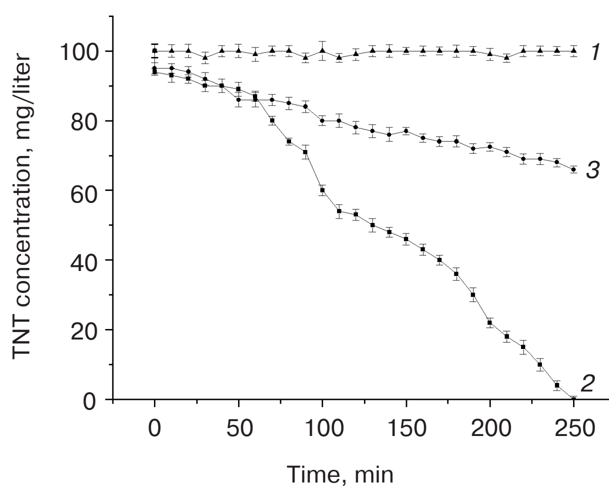

Fig. 1. Decrease in TNT content in the medium during the incubation with *Bacillus cereus* ZS18 (A_{600} = 1.0) in phosphate buffer: *1*) TNT without cells; *2*) living cells with TNT; *3*) heat-killed cells with TNT.

and modulation amplitude 0.5 G. Tiron (50 mM) was added to the investigated solutions after the removal of the cells, and the pH value of the resulting solution was adjusted to 9.5-10.0 using 20% NaOH solution. The samples (100 µl) were placed into glass capillaries. Xanthine–xanthine oxidase reaction was used as the standard system for generation $O_2^{\dot{-}}$. The reaction mixture contained 10 ml of 0.05 M phosphate buffer, 1.52 g of xanthine, and 0.5 U of xanthine oxidase (3.125 mg protein).

Determination of superoxide anion by the adrenochrome method. The investigated cell-free incubation mixture (2 ml) was supplemented with 200 µl of 0.1% aqueous epinephrine hydrochloride solution. After 15 min of incubation, the absorption of the solution at 480 nm was measured on a Lambda 35 spectrophotometer (Perkin Elmer) using the cell-free incubation mixture as the reference solution. The content of $O_2^{\dot{-}}$ was calculated using the absorption coefficient for adrenochrome of 4020 $M^{-1}\cdot cm^{-1}$ [17, 18].

RESULTS

Previously, TNT was shown to be moderately affected by randomly chosen microorganisms, yielding isomeric forms of HADNT as the main products of its transformation. The content of compounds containing at least one completely reduced nitro group (ADNT) did not exceed 3-10% of the initial concentration of TNT [14]. There are two representative strains isolated previously from waste product of the oil and gas industry (oil sludge), *Bacillus cereus* ZS18 and *Pseudomonas* sp. EN1582, that can grow in a medium containing TNT and transform its high concentration yielding isomeric HADNT as major metabolites.

Screening of a number of strains including gram-positive and gram-negative microorganisms demonstrated that the presence of the cell biomass in an incubation mixture or in a growth medium resulted in some decrease in TNT content (table). This decrease (4-9 mg/liter) could be due to the sorption of TNT by the bacterial cells or by its transformation within the corresponding concentrations. After the removal of the cells and subsequent extraction of the incubation mixtures with ether or chloroform, no TNT was revealed in the extracts.

The addition of the thermally treated bacteria into the incubation mixture resulted in a less pronounced, but detectable decrease in TNT concentration (Fig. 1). However, the treated cells were incapable of proliferating. During the prolonged incubation of such cells with TNT, neither HADNT nor ADNT were detected in the incubation mixture (Fig. 2).

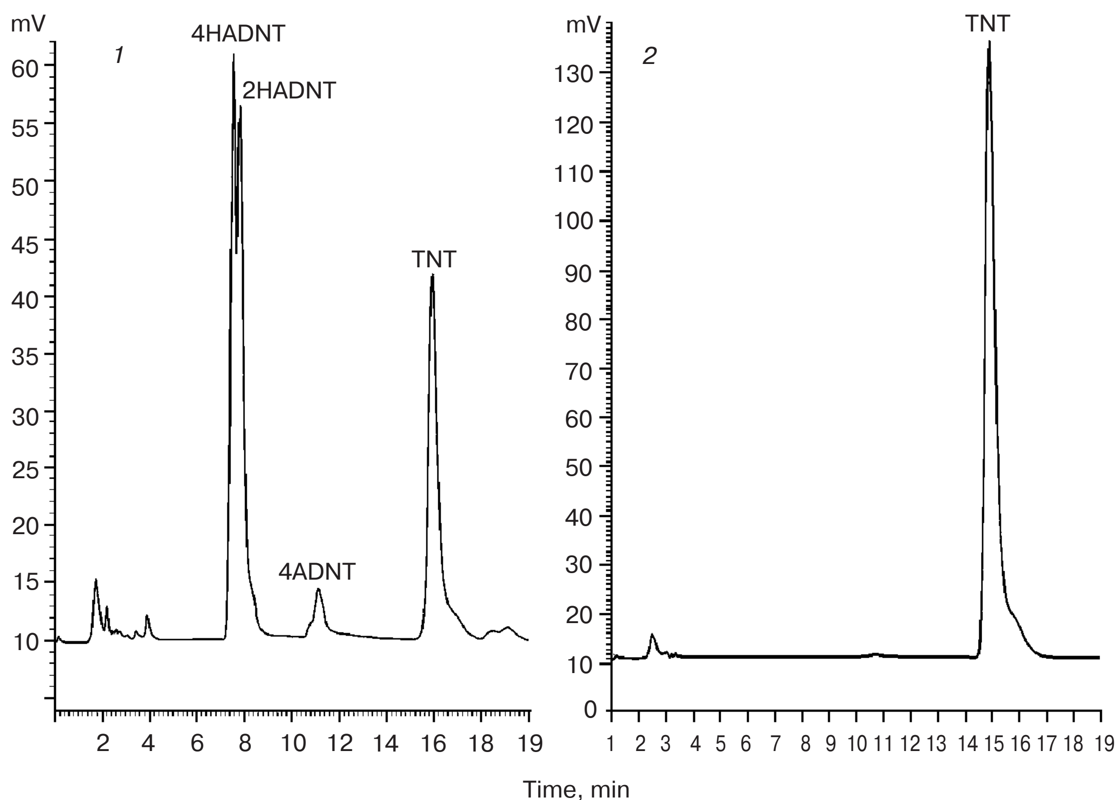

Fig. 2. HPLC analysis of incubation mixtures containing TNT (100 mg/liter) after 3 h of incubation with *Bacillus cereus* ZS18: *1*) living cells; *2*) heat-killed cells.

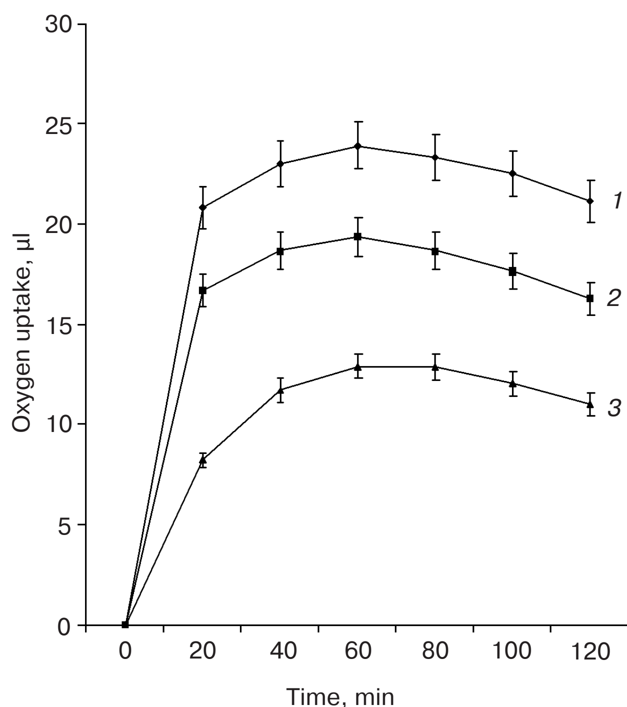

Fig. 3. Oxygen uptake in the *Bacillus cereus* ZS18 (A_{600} = 2.0)–TNT system in the absence of exogenous respiratory substrate: *1*, *2*) in the presence of TNT, 50 and 100 mg/liter, respectively; *3*) in the absence of TNT.

Investigation of the role of oxygen in the system containing different TNT concentrations and the washed cells of *B. cereus* ZS18 showed that the addition of TNT increased the oxygen uptake 1.5-2-fold compared to that of the control (basal respiration in the absence of glucose and TNT) (Fig. 3). The respiratory poison (NaN_3) blocking completely both the substrate-induced (glucose) and basal respiration at concentration of 1 mg/ml did not affect the oxygen uptake in the presence of TNT (data not shown).

The level of the non-respiratory consumption of oxygen suggests participation of reactive oxygen species in the transformation of TNT. In fact, an instant formation of $O_2^{\dot{-}}$ was detected after the addition of the cells into the incubation mixture containing TNT using the reaction of superoxide-dependent transformation of epinephrine to adrenochrome.

Since the xanthine-oxidase reaction is known as a classic example of producing $O_2^{\dot{-}}$ during xanthine oxidation, it was used as the control system generating superoxide anion and for the standard spectra of Tiron-semiquinone (the product of the interaction of Tiron with $O_2^{\dot{-}}$). The comparison of the standard spectra with those obtained using the TNT–bacterial cells system is presented in Fig. 4. It is seen that the initial step of the TNT transformation is actually connected with generation of $O_2^{\dot{-}}$ that was steadily detected in all TNT + cells systems in the presence of Tiron as the spin trap (Fig. 4). The production of the superoxide anion was started directly at the moment of the addition of the cell suspension in the phosphate buffer containing TNT. The concentration of $O_2^{\dot{-}}$ in most cases was maximal (18-20 mM) after 15-60 min of the incubation. Subsequently, the accumulation in the mixture of HADNTs resulted in a decrease in the EPR-signal of Tiron-semiquinone. The complete conversion of TNT into HADNTs ceased the generation of $O_2^{\dot{-}}$ (Fig. 4).

To exclude the involvement of enzymes in the investigated process, the actively growing *B. cereus* ZS18 and *Pseudomonas* sp. EN1582 cells were tested before and after 15 min of incubation in boiling water. The cells treated in this way did not grow on nutrient media without TNT, this indicating the lethal effect. However, the thermal treatment of the cells, independently of Gram reaction, did not prevent formation of superoxide anion,

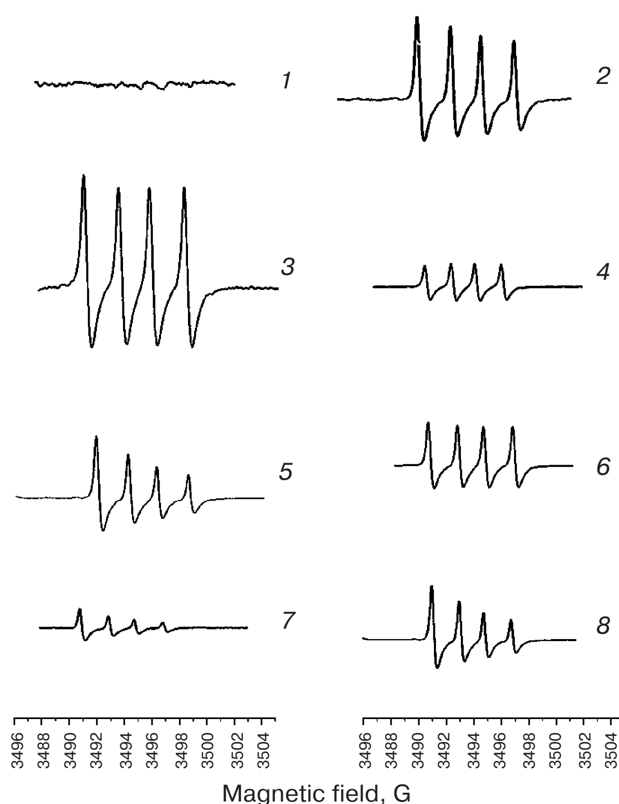

Fig. 4. Typical spectra of the Tiron–superoxide adduct: *1*) living bacterial cells in phosphate buffer without TNT (control); *2*) xanthine oxidase reaction; *3*, *4*) living and thermally treated cells of *B. cereus* ZS18 (A_{600} = 1.0) in phosphate buffer with TNT (100 mg/liter), respectively; *5*) variant *3* in the presence of Cu,Zn-superoxide dismutase; *6*, *7*) variant *3* after preliminary treatment with EDTA or 2,2′-bipyridyl, respectively; *8*) non-biological transformation of TNT initiated by $K_4[Fe(CN)_6]$. The spectra were recorded 15 min after the addition of the cells into the incubation mixtures (*1-7*) or after the start of the reaction (*2*, *8*) at 25°C (microwave power, 50 mW; frequency, 9.8 GHz; modulation amplitude, 0.5 G).

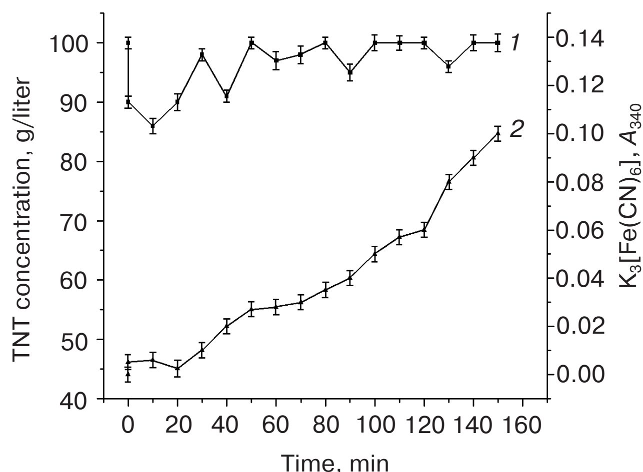

Fig. 5. Dynamics of TNT transformation (*1*) and $K_3[Fe(CN)_6]$ formation (*2*) during the abiotic reaction of TNT with $K_4[Fe(CN)_6]$.

although decreased sharply its concentration compared to living cells (Fig. 4).

The addition of a commercial preparation of Cu,Zn-superoxide dismutase (SOD) from bovine erythrocytes into the incubation mixture (400 U/ml) decreased the superoxide anion concentration by 50-70% (Fig. 4).

To investigate the role of the cell surface and particularly the structures containing multivalent metal ions in the decrease in TNT concentration and the formation of $O_2^{\dot{-}}$, the cells were treated with chelating agents (EDTA and 2,2′-bipyridyl) before their addition to the TNT-containing incubation mixture. The treatment of the cells with both EDTA and 2,2′-bipyridyl decelerated the transformation of TNT and decreased significantly the level of superoxide anion production (Fig. 4).

The formation of $O_2^{\dot{-}}$ and the initial decrease in TNT with subsequent restoration of its original concentration during the interaction of TNT with $K_4[Fe(CN)_6]$ in the absence of the cells (Fig. 5) suggest the participation of iron ions in this process. The oscillatory character of the TNT concentration curve in this case can be due to the reoxidation of the formed nitro anion radical of TNT by oxygen, this being supported by the formation of $O_2^{\dot{-}}$ (Fig. 4). Besides, the shift in the Fe(II)↔Fe(III) equilibrium in the TNT–ferro-/ferricyanide system during this abiotic redox reaction is also possible.

DISCUSSION

The polynitroaromatic compound TNT possesses a pronounced electrophilicity that decreases during the consecutive reducing of the nitro groups (mainly in 2nd and 4th positions). Conversely, the oxygenase attack, being characteristic for the initiation of aerobic decomposition of most aromatic compounds, does not take place in the case of TNT [15]. Previously, aminodinitrotoluenes (ADNTs) were revealed by thin layer and gas chromatography as the first stable metabolites of TNT. HADNTs were first revealed by HPLC analysis as the main metabolites of TNT in the fungus *Phanerochaete chrysosporium* [11]. Subsequently, these products of the four-electron reduction of TNT nitro groups appeared to be its key metabolites in most gram-positive and gram-negative bacteria [13, 14].

In spite of the interest of investigators in the mechanisms of TNT transformation, some important aspects of the interaction of TNT with the microbial cell are poorly described in publications devoted to this stable xenobiotic.

Measuring the TNT concentration directly at the moment of the contact of the bacterial cells with this compound (while incubating cell suspensions with TNT) revealed some decrease in TNT (4-9% of the initial dose). The contact of the cells with TNT resulted in the production of superoxide anion that was detected by EPR spectroscopy using Tiron as the spin trap and also by the colorimetric method based on the conversion of epinephrine into adrenochrome. The methods of the detection of $O_2^{\dot{-}}$ with the use of such molecular detectors as ferricytochrome *c*, epinephrine, and spin traps are not strictly specific for $O_2^{\dot{-}}$ [19]. So we used the known method of enzymatic dismutation using SOD to confirm the presence of superoxide anion and to determine its concentration.

According to the results of the manometric experiments, the oxygen uptake during the incubation of the cell suspensions in the presence of TNT increases 1.5-2-fold compared to the level of endogenous oxidation without TNT. It is important that the increase in the oxygen uptake was detected in the absence of any exogenous respiratory substrate. These data together with the instant character of the response reaction in the beginning of the contact of the cells with TNT suggest the non-enzymatic nature of the observed phenomenon. This assumption was supported by experiments with the dead cells, namely the reduction of 20% of the original TNT together with the appearance of the EPR signal as the result of its contact with thermally treated bacterial cells.

The presented data suggest that TNT and oxygen are involved in the redox cycle (Fig. 6). Due to a high electrophilicity, the TNT molecule can be reduced by a one-electron mechanism in the presence of nonspecific reductants, including those associated with components of the cell surface containing multivalent metal ions (iron or manganese). The nitro anion radical formed as the result of the one-electron reduction is unstable and reoxidizes with oxygen, yielding the superoxide anion.

In the case of the dead cells, this reaction does not couple with subsequent transformation of TNT by the traditional pathway of nitro group reduction independ-

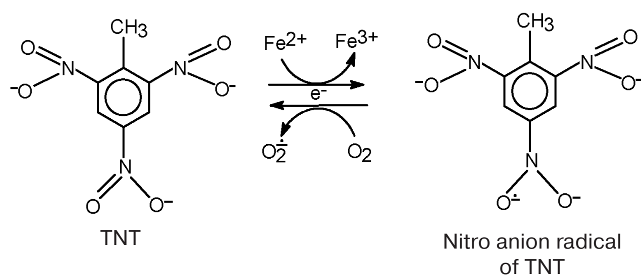

Fig. 6. Hypothetical scheme of extracellular redox cycle functioning in the initial step of TNT transformation by aerobic bacteria.

ently of the presence of glucose as the potential electron donor. In the experiments with the living cells (*B. cereus* ZS18 and *Pseudomonas* sp. EN1582), their incubation with TNT in the presence of glucose was accompanied with subsequent transformation of TNT into isomeric HADNTs as the key metabolites.

Iron is transported from the environment by the low molecular weight chelating agents binding Fe(III) [20, 21]. The mechanism of the consumption of iron from the extracellular medium includes reduction of the chelated Fe(III) on the surface of the cell by the reductases of the cell membrane [22]. We assume that the reduced iron associated with the cell surface can be involved in the redox cycle of the one-electron reduction of TNT. This fragment of the cycle was demonstrated in this work using the model TNT + $K_4[Fe(CN)_6]$ (Fig. 5). The source of electrons for the reduction of TNT can be also the structural non-heme iron not bound to chelator that is present on the cell surface of microorganisms of different taxonomic levels [23].

Thus, the main role in the initiation of the processes yielding superoxide anion in the cell–TNT system is probably played by the surface structures of the living and to a certain degree dead cells due to the presence of functional elements containing multivalent metal ions (presumably iron). This is supported by the significant suppression of TNT transformation and generation of $O_2^{\dot{-}}$ after the treatment of the cells with EDTA and especially the reagent specific to iron ions, 2,2′-bipyridyl, which can bind the reduced iron on the surface of the cells.

Among a few examples of the formation of extracellular superoxide anion by microorganisms, there is an interaction of *Escherichia coli* cells with the herbicide paraquat. This reagent is easily reduced to a stable oxygen-sensitive cation radical that reacts with oxygen yielding superoxide anion [24, 25]. The generation of the superoxide anion in the presence of paraquat was shown in experiments with microorganisms, chloroplasts, lung microsomes, and homogenates of the liver, kidneys, and lungs. Although the authors consider that the cation radical is formed intracellularly with its subsequent diffusion into the environment and reoxidation by oxygen, the extracellular functioning of the redox cycle cannot be excluded, as we suggest for TNT.

The possibility of one-electron reduction of TNT is discussed in the literature mainly in connection with the reaction catalyzed by the oxygen-sensitive nitroreductase using NAD(P)H as the original electron donor, and the uncoupling of the two-electron flow occurs at the flavin level [26]. NAD(P)H is the original reductant also for the one-electron reduction of TNT in the molecular mechanism suggested for TNT-induced oxidative stress, where the reductase domain of the neuronal NO-synthase is responsible for the formation of the nitro anion radical and then superoxide [27].

The extracellular accumulation of $O_2^{\dot{-}}$ in the early stage of microbial transformation of TNT demonstrated in the present work is of interest in connection with the high toxicity of this radical that causes oxidation of membrane lipids, DNA fragmentation, development of inflammation, and damage to vessels [19, 28]. These phenomena are partially connected with the toxicity of the superoxide anion, the main role likely being played by the formation of secondary reactive oxygen species, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) [28].

Since the revealed formation of superoxide anion by cell suspensions in contact with TNT is common not only for representatives of the genera *Bacillus* and *Pseudomonas*, but also for a wide circle of tested microorganisms (data not shown), it is likely that the microflora of humans and higher animals can also promote the extracellular generation of superoxide anion under the action of this xenobiotic, and this can make a significant contribution to the development of toxic and genotoxic effects of TNT.

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