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EXPERIMENTAL ARTICLES

Nitroxyl Compounds in Bacteria: Search for Functions

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Abstract—A method was developed for incorporation of two deuterium atoms into the reactive group of a new type of nitroxyl compounds from *Micrococcus luteus* (a lysodektose which can be transformed into a long-living free radical). This compound was found to persist in the cells treated with gentamycin and chloramphenicol, while treatment with gramicidin C, ampicillin, benzyl viologen, furadonin, and mercury chloride resulted in a drastic decrease of its intracellular content. Cultivation of *M. luteus* in a medium with EDTA resulted in accumulation of much higher amounts of lysodektose (up to 300%), and this phenomenon is interpreted as an indication of the possible siderophore function of the compound. Since the *M. luteus* genome was recently sequenced, this may help to understand the fate and role of lysodektose in bacterial metabolism.

Key words: nitroxyl, radical, bacteria, *Micrococcus luteus.*

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Derivative of nitrogen oxides (hydroxylamines, nitroxyl radicals, and nitrones) are well known to chemists due to their unique ability to react with heavy metals, capture free radicals, and carry out addition reactions [1]. The concept of ageing as oxidative degradation somewhat overwhelmed the importance and positive role of free radicals in key biochemical reactions. The role of nitrogen oxides in the biological processes is widely recognized [2]; the information about natural organic nitrones and nitroxides is, however, scarce, limited to several dozens of microbial and protistan secondary metabolites used as antibiotics and other biologically active compounds [3, 4]. In the Institute of Biochemistry, Russian Academy of Sciences, new hydroxylamine compounds, lysodektose and ammonigenin, were discovered, which can transform to long-living nitroxyl radicals [5, 6]. The interest in natural nitroxyl radicals is rapidly increasing due to the discovery of the capacity of NO to form complexes with cyclic GMP [7] or a phytotoxin [8, 9] with formation of a new signal product. The ability of bacteria to synthesize nitroxide was revealed not only in the process of nitrate reduction, but as a result of NO synthase activity [10]. The ability of alkyl hydroxylamines to affect the activity of various enzymes, including viral proteins, was demonstrated, as well as the role of the proprietary (bacterial) NO in formation of the virulence of pathogenic bacteria [11]. We therefore believe that investigation of nitroxyl radicals is important for molecular microbiology. In order to improve our understanding of the role of these compounds in microbial physiology, the nitroxyl radical forms were investigated against the background of antibiotics and metabolic toxins.

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MATERIALS AND METHODS

In the work, Fleming's strains *Micrococcus luteus* 2665 and NCIMB 13267 grown in nutrient broth were used. To obtain the lysodektose preparation [5], the biomass was collected at the early stationary phase (24 h), treated with 50% ethanol, and centrifuged. The extract was then cleaned on a Dowex-1 (Cl^-) column to remove the anionogenic contaminants and on a Dowex 50W (Na^+) column to remove heavy cations. The extract was then treated with chloroform to remove most of the ethanol from the upper stratified phase. The material in the lower aquatic phase was concentrated by lyophilization and cleaned by gel-permissive chromatography on a Sephadex G-10 column. The lysodektose content was determined from the spectrum of the electron paramagnetic resonance (EPR spectrum) of its radical form. The latter is produced in small amounts (approx. 10%) when in contact with oxygen of the air and is completely revealed by treatment with an oxidant (0.01 M $K_3Fe(CN)_6$). For transition to the radical state, pH of the reaction mixture should exceed 7. In an acidic environment, lysodektose exists in a hydroxylamine form and is not oxidized by oxygen. The EPR signal was measured at 20°C in an RE-1306 spectrometer in a 0.1-ml flat quartz cuvette, with Mn^{++} as an external standard. The signals of the nitroxyl radicals are located between the 3rd and 4th lines of the standard. Phosmidomycin was synthesized by Dr. I. Wissner (Giessen University, Germany); other antibiotics used were commercially available preparations.

RESULTS

Titration with potassium ferricyanide demonstrated exact correspondence between the EPR signal and the

lysodektose content in the samples. The lysodektose concentration in *M. luteus* cytoplasm was thus estimated to be close to 10^{-3} M. Qualitative measurement became possible due to the simultaneous registration of the ferricyanide absorption spectrum (with the maximum at 440 nm) and the EPR signal of the radical form of lysodektose.

Analysis of the chemical structure and of some properties of lysodektose suggests that its functions include involvement in cell wall formation, participation in metal chelation (siderophore functions), regulation of activity of metal-containing enzymes, and participation in the antioxidant processes due to its spin trap features.

Double deuteration of lysodektose. The possibility to convert hydroxylamine to nitrone and back to hydroxylamine makes it possible to incorporate one hydrogen atom to the methyl group with 100% efficiency and to introduce the second atom in the oxidation–reduction cycle with 66% efficiency. We realized this possibility according to the following scheme. First, the lysodektose solution in the hydroxylamine form was supplemented with $K_3Fe(CN)_6$ (up to 0.01 M until the temporary disappearance of the EPR signal). The reaction mixture was then rapidly passed through an anion-exchange minicolumn (1 ml) to remove ferricyanide; dry sodium boron deuteride ($NaBD_4$) was then added to the solution. After 20 min, the reaction mixture was passed through a fresh anion-exchange column and a new portion of $K_3Fe(CN)_6$ was added to reveal the radical form. The initial EPR signal of lysodektose in the radical form is a sextet (Fig. 1, *1*). After one oxidation–reduction cycle with boron deuteride, the EPR signal became a quintet (Fig. 1, *2*); after the second oxidation–reduction cycle, it became a broad quartet (Fig. 1, *3*), corresponding to the substitution of two protons adjacent to the R–N–O*(CH_3) group by deuterium. Thus, significant introduction of a hydrogen isotope into lysodektose was demonstrated in the course of reduction of its nitrone form to the hydroxylamine one. These results confirm our suggestion [6] that in the course of oxidation of the hydroxylamine form of lysodektose, it is converted to the nitrone form via formation of a nitroxyl radical. Due to their ability to react with free radicals, these forms of lysodektose probably participate in the antioxidant processes.

The experimental data suggest also the possibility of tritium introduction into lysodektose in the case of $NaBD_4$ used as a reducing agent instead of $NaBT_4$. The radioactively labeled lysodektose preparation will make it possible to monitor the transformations of this compound in the cell.

Attempts were made to obtain a deuterium-substituted phosmidomycin and its methylated derivatives using the same procedure. However, unlike deuterolysodektose, deuterium of boron deuteride was not incorporated into these antibiotics, probably due to the rapid hydrolysis of their nitrone form resulting in production of an aldehyde and hydroxylamine, as it occurs with some known spin traps of nitrone origin [12]. The radical form of phosmidomycin is, however, formed in the course of oxidation (Fig. 1, *4*) and may be oxidized further.

Chelation and reduction of gold by lysodektose. Iminoxyls are known to form complexes with Lewis acids (for example, palladium, indium, or gallium halogenides) in aprotic solvents [1, 13, 14]. This is especially interesting in relation to the recently discovered ability of *M. luteus* to transform elemental gold Au^0 from small to larger granules and the detection of a gold-binding protein [15, 16]. Direct interaction of Au^0 and $[AuCl_4]^-$ with the lysodektose radical and effect of gold on lysodektose content in growing *M. luteus* cells were therefore investigated.

While on contact with the lysodektose radicals, a solution of Au^0 nanoparticles changes its configuration somewhat (changes color from red to blue), the EPR spectrum of lysodektose suffers no qualitative changes [5]; on contact with $[AuCl_4]^-$, however, the signal disappears immediately due to the oxidation of the radicals by a chloroaurate ion (the yellow solutions turns red). Both elemental gold and its chloride had no effect of the hydroxylamine form of lysodektose. Thus, lysodektose does not directly participate in the first stage (dissolution of gold); it may, however, participate in the second stage, when gold is reduced back to Au^0 forming catalytically active particles. The phenomenon of gold nanoparticles catalyzing the oxidation of some carbon compounds by molecular oxygen, which was discovered over 20 years ago [17] and specified in recent works [18], certainly confirms the concept of Levchenko et al., concerning the possible involvement of gold in bacterial oxidation of natural methane [19] and probably in oxidation of other organic compounds.

Effect of metabolic toxins on the synthesis and transformations of lysodektose. In an attempt to determine the functions of lysodektose in bacterial cells, EPR and NMR investigation was carried out of the ethanol extracts from five *M. luteus* strains (from an English, German, and Israeli collections), as well as from 14 mutant strains (collection of the Institute of Biochemistry, Russian Academy of Sciences), selected for resistance to lysozyme. H-NMR (after drying of the ethanol extracts and their transition to heavy water) revealed some differences between the strains. For example, in strain NCIMB, more pronounced signals were revealed at chemical shifts of 1.97 ppm; triplets at 2.4 and 3.93 ppm; and reduced signals at 2.26 ppm, as compared to the Fleming strain. EPR signals were revealed in all strains in the presence of an oxidant (potassium ferricyanide); when calculated fro the same biomass, their value for different strains varied not more than twofold.

Cultivation in the medium with 50% D_2O or 1 M NaCl for 20 h did not impair bacterial growth and had no effect on lysodektose synthesis.

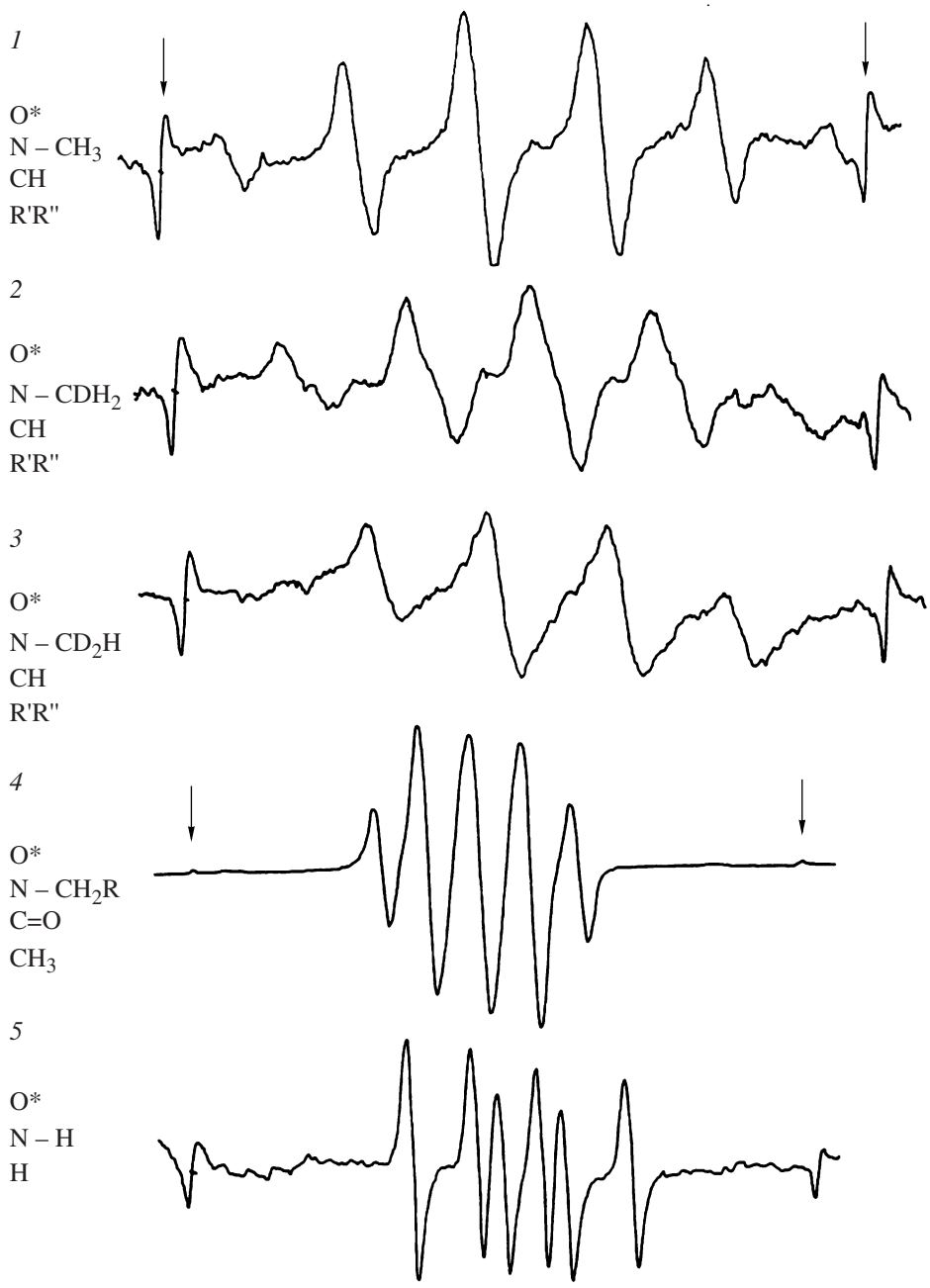

Fig. 1. EPR spectra of the radical forms of natural nitroxyl compounds (the 3rd and 4th lines of the external standard Mn^{++} are indicated by arrows): lysodektose (*1*), mono-deutero-lysodektose (*2*), ld-deutero-lysodektose (*3*), methyl-phosmidomycin (*4*), and hydroxylamine (*5*).

Experiments for detection of the metabolic toxin affecting biosynthesis or destruction of lysodektose were carried out in two variants. The *M. luteus* culture (Fleming strain) was grown in nutrient broth to the mid-exponential phase (variant 1) or to the stationary phase (variant 2); then, the tested compound was added, and the incubation was continued for another 17 h with subsequent measurement of the optical density. The cells were then precipitated by centrifugation and the biomass was treated with an equal volume of ethanol. The EPR spectra of ethanol extracts were determined in the presence of 0.01 M potassium ferricyanide. Thus, both the degree of cell poisoning and the changes in lysodektose metabolism were assayed simultaneously. In the second variant, the synthesis of major biopolymers was expected to cease, while a number of enzymes still retained activity; addition of various substrates (glucose, sodium glutamate, or hydroxylamine) in the presence of toxins could therefore indicate the pathway of lysodektose biosynthesis. We used a similar approach

Table 1. Effect of toxins on the exponential-phase *M. luteus* culture after 17 h of incubation (EPR is the value of the second component of the EPR signal, mm, in ethanol extracts in the presence of ferricyanide per unit of optical density)

Antibiotic	Concentration, μg/ml	$OD_{600} \times 0.1$	EPR
Without additions	0	1.200	1000
Adriablastin	5	1.158	648
″	50	0.335	441
Ampicillin	1	0.163	30
″	5	0.162	18
Benzyl viologen	10	0.741	475
Benzylpenicillin	7	0.234	214
Gramicidin C	2	1.122	713
″	30	0.172	0
Gentamycin	4	0.406	804
Isoniazid	100	0.904	781
Cloxacillin	3	1.185	928
″	30	0.367	405
Nalidixic acid	50	0.956	312
Penicillin	12	1.142	876
Polymyxin M	2	1.198	242
″	30	0.707	394
Streptomycin	30	1.014	686
Sulfanilamide	500	1.169	727
Phosmidomycin	150	0.906	747
Furadonine	500	0.516	338
Furacillin	50	1.003	560
″	100	0.759	440
Chloramphenicol	10	0.510	843
Quinacillin	3	1.184	886
″	30	1.134	814

previously in order to determine a new, nonmevalonate pathway of isoprenoid biosynthesis [20]. For example, in the presence of glucose, *M. luteus* synthesized 2-C-methylerythrol-2,4-cyclo-pyrophosphate (MEC), while simultaneous action of the generators of superoxide radicals inhibited the enzymes responsible for further MEC metabolism.

Analysis of the effect of antibiotics of the exponentially growing cells (Table 1) revealed a pronounced inhibition of bacterial growth by gramicidin C, ampicillin, cloxacillin, and benzylpenicillin. The content of detected nitroxyl radicals in the cells also decreased. However, while gentamycin and chloramphenicol caused significant growth inhibition, the content of the radicals did not decrease; nalidixic acid, benzyl viologen, and polymyxin M suppressed radical formation, but not cell growth.

In the case of the stationary-phase culture with the highest cell density (Table 2), addition of up to 1% glutamate and a number of toxins resulted in a certain increase in the content of nitroxyl radicals; on the other hand, mercury ($HgCl_2$), gold ($AuCl_4^-$), iodine acetate, gramicidin C, and to a lesser extent benzyl viologen and furadonine cause a decrease in the radical content.

A significant increase of the lysodektose content could have indicated the toxic action against the enzyme directly responsible for lysodektose metabolism. Unfortunately, none of the toxins exhibited such an effect. The action of the metabolic toxins as inhibitors of lysodektose accumulation may be explained either by cell wall disruption and lysodektose leakage (gramicidin C is known to create pores in the membrane, while penicillins impair the biosynthesis of cell walls biosynthesis and promote cell lysis), or by direct oxidative decomposition of lysodektose (redox mediators and gold chloride). The first hypothesis is confirmed by the fact that only cell incubation with penicillin resulted in detection of trace amounts of lysodektose in cell-free culture liquid. In the case of heavy metal poisoning, lysodektose probably actively participates in protection of the organism and is consumed for neutralization (reduction) of the toxin.

Only cultivation in the medium with Na-EDTA (up to 0.3–0.6 mM) after complete cessation of growth resulted in a threefold increase in the lysodektose concentration in bacterial cells; this result may be explained as a reaction to the deficiency of iron (or other bivalent ions) in order to overcome it by secretion of a factor with an affinity to iron (Fig. 2).

DISCUSSION

Our results concerning lysodektose content in *M. luteus* cells under different cultivation conditions demonstrated that this is a constitutive compound important to this bacterial species. However, even in closely related microorganisms its functional role is possibly played by metabolites of a different structure, with the nitroxyl group bound to a substitute other than lysodektose. It should be mentioned that even the presence of a double bond or a heteroatom at any of the carbon atoms close to the nitrogen atom results in a very short-lived nitroxyl radical, which was possibly undetectable under the experimental conditions. Thus, although no long-lived nitroxyl radical was detected in other bacteria, this does not rule out the existence of nitroxyl intermediates and compounds with the nitrone group, including those structurally similar to those found in *M. luteus.*

Can a complex of the physicochemical characteristics of an isolated compound lead to a conclusion concerning its function in the cell?

The reaction capacity of NO radicals was previously thoroughly studied [21]. Involvement of hydroxylamines in the formation of complexes with hemoglo-

bin was demonstrated. Diallyl hydroxylamines and their radical were less capable of complex formation, but acted as spin traps in the nitrone form. On the other hand [22], bacterial cells possess the enzymatic system (peroxyredoxin–alkylhydroxiperedoxide reductase of *Salmonella typhymurium*), which catalyze decomposition of highly active, and therefore, dangerous nitroxyl compounds (similar to peroxynitryl). These systems make it possible for bacteria both to decrease the content of nitroxyl compounds formed in the metabolism of intracellular nitrites to the physiologically required level and to decompose peroxynitryl produced by the host cells as an antibacterial agent.

Together with our foreign colleagues, we applied to an international center for sequencing the *M. luteus* DNA. We are presently expecting to achieve a greater understanding of the role of nitroxyles.

In *M. luteus* cells, two enzymes were found which may be related to lysodektose. These are monoamine oxidase and the transmembrane nitrite reductase. In spite of their multidirectional action, both enzymes catalyze formation of a (possibly short-lived) hydroxylamine; its fate is probably directly related to the fate of lysodektose, another hydroxylamine. In the future, genetic inactivation of these enzymes may determine their relation to lysodektose.

Another group possibly related to lysodektose comprises several membrane proteins similar to transporters of heavy metal ions. For marine *M. luteus* strains, ability to excrete iron-binding compounds of a hydroxamic nature was reported [23]. The enzymes of cell wall synthesis are the third group potentially involved in lysodektose transformations.

The low sensitivity of the TLC-based chemical detection methods [24], which is ten times worse than that of the EPR method, hampers investigation of nitroxyls and their derivatives.

The idea that lysodektose and its analogues are bacterial "healing" agents is rather tempting. Similar to blood thrombocytes, which form a clot at the site of damage of a blood vessel, and thus close the wound, lysodektose probably exits the cell through a damaged membrane and transforms to the radical and nitrone forms on contact with oxygen; these forms then react with each other and with the components of the cell wall and seal the damaged site. Although individual reactions of such kind have been reported, the phenomenon as a whole has not been confirmed. In our experiments, simple mixing of the nitrone and radical forms of lysodektose was insufficient to reveal attachment of the radical to the nitrone; this, however, may result from our poor choice of reaction conditions. It is also possible that this process in vivo is catalyzed by some enzyme of the cell wall.

Table 2. Effect of toxins on the stationary-phase *M. luteus* culture after 17 h of incubation (EPR is the value of the second component of the EPR signal, mm, in ethanol extracts in the presence of ferricyanide and glucose (A) or glutamate (B) to 1%)

Antibiotic	Concentration, μg/ml	EPR-A	EPR-B
Ampicillin	130	60	–
″	170	–	180
$[AuCl_4]^-$	70	8	–
″	160	–	30
Benzyl viologen	100	32	–
″	100	–	50
Benzylpenicillin	70	12	–
Gramicidin C	90	6	–
″	100	–	15
Gentamycin	100	36	–
″	200	–	180
Doxycycline	100	–	135
Isoniazid	500	130	–
″	500	–	111
Iodine acetate	460	18	–
″	300	–	34
Kanamycin	110	43	–
″	180	–	120
Nalidixic acid	150	–	92
$HgCl_2$	100	12	–
″	100	–	20
Streptomycin	90	66	–
″	110	–	220
Tetracycline	90	40	–
″	130	–	240
Phosmidomycin	200	85	–
″	100	–	177
Furadonine	500	52	–
Chloramphenicol	100	70	–
″	110	–	210
Without additions	0	80	–
″	0	–	80

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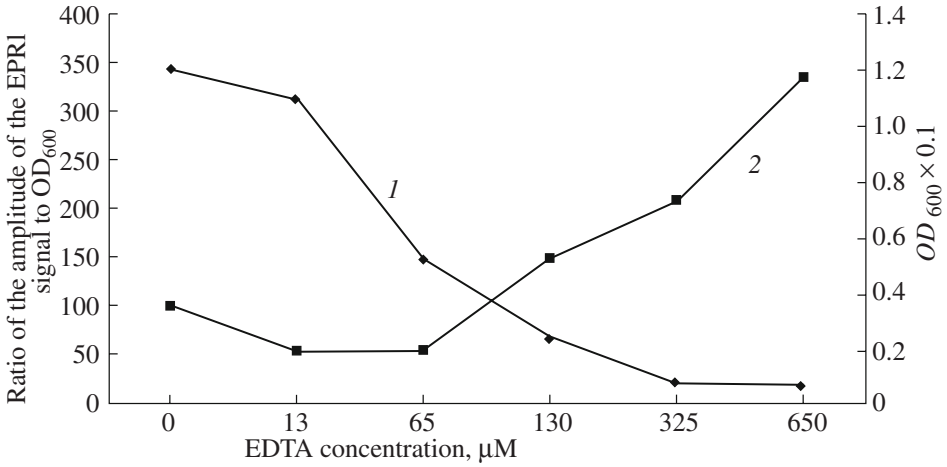

Fig. 2. Optical density of the culture (*1*) and the lysodektose radical content in *M. luteus* cells (*2*) depending on the concentration of the chelating agent for bivalent ions (EDTA).

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