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Hydrogen Sulfide (H_2S) is a Mediator of Nitric Oxide (NO) Signaling Functions in Bacteria

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Abstract: *Gasotransmitters NO, CO and H_2S are the key signaling molecules in both animals and plants. They are generated and regulated enzymaticaly in all biological systems. It is now clear that these compounds act in concert to coordinate the cell responses, but exactly how this interaction is achieved is not known. H_2S has effects on the accumulation of both reactive chemical species ROS and RNS and can give rise to the other reactive species. We focus on the genetic and physiological evidences of an interaction between nitric oxide and hydrogen sulfide in the bacterial cell – in regulation of DNA repair gene expression and biofilm formation. The crystalline dinitrosyl iron complexes NO-29 and NO-33 with thiourea as the ligands and tetranitrosyl iron complexes with thiosulfate and tetrazole ($TNIC_{thio}$, $TNIC_{tetrazo}$) were studied first in pure solutions and in the combination with H_2S. According to the results of our genetic, physiological and EPR studies we concluded that H_2S is not acting as a typical genetic signal in bacteria, but it interacts with the true NO signals to ensure the NO-signaling mechanisms to arrange of stress challenges properly.*

Keywords: *Escherichia coli; Nitric oxide; Hydrogen sulfide; Interaction; Signaling functions*

1. INTRODUCTION

The history of amazing gas biology of NO started at the fall of the 20th century with a discovery, studies and identification an endothelium relaxing factor and a unique endogenous signaling molecule – nitric oxide (NO) [1-4]. Ten years later the two other environmental gases CO and H_2S were found to have the great inputs in aerobic metabolism, as well. They are generated enzymatically and produced endogenous messengers ("gasotransmitters") [5, 6], and their effects are not dependent on specific membrane receptors. A novel concept of "gasotransmitter" has been formulated recently. These are small molecules of endogenous gases with important physiological functions. They are toxic at high doses, but at the physiological doses they play the outstanding regulatory and signaling role. In 2006 H_2S has been denominated on a "clandestine microbial messenger" [7].

We and others have found that in *Escherichia coli (E. coli)* NO protects bacteria against oxidative and nitrosative stresses [8, 9], acts as a signaling molecule in their control. The genetic responses to the nirtosative stress are controlled by the OxyR [10] and the SoxRS [11] DNA repair pathways. We first provided the experimental evidences that NO functions as an activator of the global regulatory network – the DNA SOS repair response [12] and the "Quasi- Ada" DNA- repair response to alkylating agents in *E. coli* cells [13].

NO is a water soluble free radical with a short half-life in aerobic conditions. Being highly reactive and unstable, NO occurs inside the cells in the form of NO-donors – S-nitrosothiols and dinitrosyl iron complexes (DNICs), which are more stable and ensure NO transport *in vivo*.

DNICs are the most spread and important cellular NO- donors [14 - 16]. Such complexes were first observed and identified in all biological systems by their specific EPR signals [17, 18]. The mechanisms of DNIC appearance in the bacterial cells are not quite clear.

In *E. coli* L-cysteine is the major source of endogenous H_2S production through the combined action of two H_2S-generating enzymes [19]. In other bacterial species the main mechanisms of H_2S production are differ from that of the above in *E. coli* [20].

The reductive and nucleophile properties of H_2S are the most important characteristics of its chemistry that define its biological actions [21, 22]. At physiological pH H_2S exists largely in the form of the hydrosulfide anion = thiolate ion [HS^-]. While H_2S itself is a relatively low reactive one, the HS^- is a powerful nucleophile that can react with oxidants to give a diversity of sulfur complexes [23]. As a thiol, H_2S can undergo 1-electron or 2-electron oxidation to a radical [HS•] or the disulfide [S^0], respectively.

Bacteria produce both NO and H_2S, which can act as "a universal defense against antibiotics in bacteria" [20] via the Fenton reaction and the antioxidant enzymes activation [10, 20, 24].

Amazing similarities between the chemical effects of NO and sulfide *in vitro* and especially their joint regulatory effects in the cells proposed a cross talk between these species. A detailed comparative study on the potential molecular-genetic mechanisms of NO and H_2S cross-talk *in vivo* is absent. H_2S potentates the signaling effects of NO, so that both these gases can act synergistically. In *E. coli* mutants deficient in H_2S production the higher amounts of NO have been synthesized [7].

In addition to oxidation, reactive cysteines in the family of protein tyrosine phosphatases (PTPs) can undergo the other modifications by the signaling gasotransmitters – NO and H_2S. The first results on S-nitrosylated PTPs were announced, but the number of PTPs known to undergo Cys S-nitrosylation or sulfhydration is very limited, though the use of S- nitrosylation in PTPs as the protective mechanism against oxidative stress should be of high medicine perspective.

The present study is a part in our purposeful search for the novel water-soluble crystal cationic nitrosyl iron complexes with thiourea, promising for basic and practical medicine. Complex application of NO donors and H_2S is a new direction in our investigations.

2. MATERIALS AND METHODS

2.1. NO-donating Agents

The cationic crystalline dinitrosyl iron complexes (DNICs) with thiourea NO-29, $[Fe(SC(NH_2)_2)_2(NO)_2]_2SO_4 \cdot H_2O$ and NO-33, $[Fe(SC(NH_2)_2)_2(NO)_2]Cl \cdot H_2O$ [25, 26], and tetranitrosyl iron complexes (TNICs) with thiosulfate $Na_2[Fe_2(S_2O_3)_2(NO)_4] \cdot 4H_2O$ ($TNIC_{thio}$) and tetrazole ligand $[(n\text{-}C_3H_7)_4N]_2[Fe_2S_2(NO)_4]$ ($TNIC_{tetrazo}$) were synthesized at the Institute of Problems of Chemical Physics RAS, [27].

The other chemicals and reagents were purchased from Sigma–Aldrich (USA). At physiological pH hydrogen sulfide (H_2S) exists largely in the form of hydrosulfide anion (HS^-), with a little amount of S^{2-} and dissolved uncharged gas. In the experimental work the term "sulfide" we used for the aqueous solution (pH 7,4) of Na_2S, which in aerobic conditions is the sum of $H_2S + HS^- + S^{2-}$. The relative amounts of the three species at the equilibrium depend on pH, ionic strength, temperature and the "side" reactions.

Na_2S stock solutions (200 mM) were prepared fresh before each experiment by dissolving anhydrous Na_2S in a strong buffer (TRIS or phosphate buffer 1 M at pH 7.4) and diluted further in 100 mM TRIS or phosphate buffer pH 7.4 Incubation mixtures of NO donors with Na_2S were obtained by adding appropriate volumes of the stock solutions directly to the incubation buffer to achieve final concentrations.

2.2. Bacterial Strains and β- galactosidase (β-gal) Assay

All experiments with the gene expression were performed with the wild type and the mutant *E. coli* strains. A level of the sfiA-gene expression of the SOS- regulon was studied with *E. coli* PQ37 with the [sfiA::lacZ] operon fusion and a deletion in the chromosomal lac operon, so that β- galactosidase activity was strictly dependent on the sfiA expression. A sfiA gene expression was monitored as described by Quillardet et al. [28]. The *E. coli* PQ37 was kindly provided by M. Hofnung (Pasteur Institute, Paris).

The isogenic *E. coli* TN530 wt [soxS::lacZ] and TN531 ΔsoxR [soxS::lacZ] strains were kindly provided by Nunoshiba and were used for the soxS gene expression according to [29]. *Ps. aeruginosa* PAO1 (the clinical isolate) was used in the biofilm experiments [30].

The quantitative level of β-gal activity in the cells was determined according to Miller [31]. Briefly, an overnight *E. coli* culture was diluted 1:50 into LB medium and grown for 3,5 hours to OD600=0.3-

0.4, which corresponded to the early log-phase of growth. Cells were treated with NO donors and/or H_2S for 45 min at 37°C and further incubated in the presence of the chromogen *o*-nitrophenyl-β-*D*-galactopyranoside (ONPG) for 30 min. As the positive controls, 26.3 μM 4-nitroquinoline oxide, 4NQO, was used for *E. coli* PQ37 and 0.5 mM menadione for *E. coli* TN530. The β-gal activity was measured by PD-303UV digital spectrophotometer (Apel Co. Ltd., Japan), at 420 nm. To calculate the β-gal activity (E), an equation E = 1000*OD420/t, where OD420 is the optical density at 420 nm and t is the time of incubation with the chromogen, was used. The B-buffer composition was as in [31].

The LB liquid medium was used for the bacterial growth.

2.3. Quantification of NO Releasing

To measure the concentration of NO (in nM) generated in solutions by NO-donors, the sensor electrode "amiNO-700" of "in NO Nitric Oxide Measuring System" (Innovative Instruments, Inc., Tampa, FL, USA) was used [25, 26]. The NO level was recorded for ~500 sec (with the pace of 0.2 sec) in aqueous solution of NO donor (pH 7,4) with or without H_2S.

2.4. Plankton Cell Culture and Biofilm Processing

The basic protocol of the method described in [32]. The plankton cell growth was determined by OD_{600} value. 0.1% (wt/v) crystal violet was used for 10 min to stain the attached cells. Unattached dye was rinsed away by washing two times with distilled water and the stained biomass was dissolved with 1:4 (v/v) mixtures of acetone and ethanol. After 15 min, the OD_{570} was measured to quantify the biofilm biomass. The "biofilm productivity" values were assessed according to the equation: $P=OD_{570}/OD_{600}$.

2.5. EPR Study

The EPR study with *E. coli* cells were performed according to the standard methodology which our research team used [11,12].

X-band EPR spectra were recorded with the Radiopan spectrometer (Poland) under the following conditions: temperature 77 K, microwave power 5 mW, modulation amplitude 0.5 mT. The intensity of the EPR signals was expressed in the relative units.

2.6. Statistical Analysis

Results are presented as the mean values of at least four experiments and SEM.

Statistical analysis of the experimental results was performed using the Microsoft Excel and OriginPro 7.0 software packages. Asterisk indicates a statistically significant difference in the experimental datum relative to the 0 conc. in the control, at the P value=0.05.

3. RESULTS

3.1. Signaling Activity of the Novel NO-29 and NO-33 Donors

In vitro the novel NO-donors with the cationic ligand thiourea – NO-29 and NO-33 generated free NO in the presence or absence of hydrogen sulfide. The process had a small period of initiation of about 10 sec. We didn't observe any significant changes in the kinetics of NO generation after H_2S addition, except the little opposite H_2S influence on the initial stage of the process (Fig. 1).

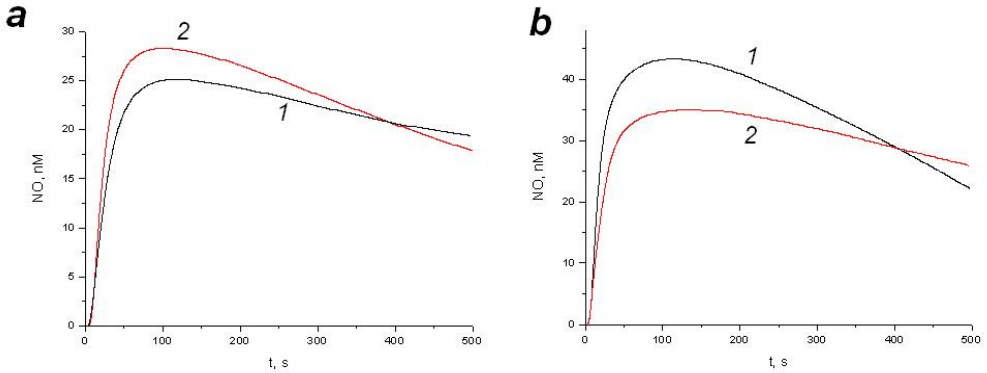

Fig1. *The kinetics of NO (nM) generated by NO-29 (a) and NO-33 (b) donors ($4 \cdot 10^{-6}$ M) in aqueous solutions at pH 7.4 and T=25°C and anaerobic conditions, being studied alone (1) or in combination with the hydrogen sulfide [1:1] (2)*

The experimental findings demonstrate a high signaling activity of the new NO-donors without any additional stimulation. In both test systems they activated the reporter gene expression up to 4-5 folds over the spontaneous level.

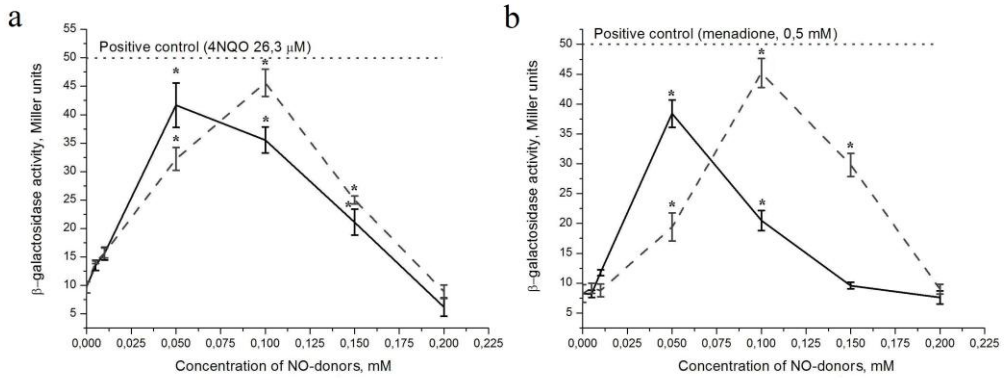

Fig2. *Kinetics of the sfiA (SOS regulon, a) and the soxS (SoxRS regulon, b) gene expression, induced by NO-29 (solid line) and NO-33 (dashed line) donors in E. coli PQ37, a, and E. coli TN530, b, respectively*

0.05 mM concentration was optimal for NO-29, while a twofold higher concentration was optimal for NO-33. Induction of the DNA – repair genes with the combination of the chemicals – NO-donor and H_2S – increased the gene expression additionally, maximum 1.5-2.0 fold, depending the ratio of NO:H_2S. The optimum effects were achieved at the ratio NO:H_2S = 1:10 **(Fig. 3-4)**.

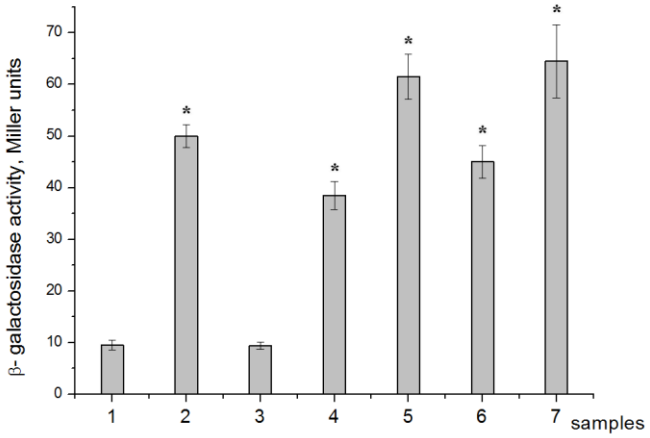

Fig3. *Expression of the soxS gene (SoxRS regulon) in the E. coli TN530 [soxS::lacZ] and H_2S influence: 1 – intact control; 2 – menadione (positive control) 0.5 mM; 3 – H_2S 0.5 mM; 4 –NO-29 0.05 mM; 5 – NO-29 0.05 mM + H_2S 0.5 mM (1:10); 6 – NO-33 0.1 mM; 7 – NO-33 0.1 mM + H_2S 1.0 mM*

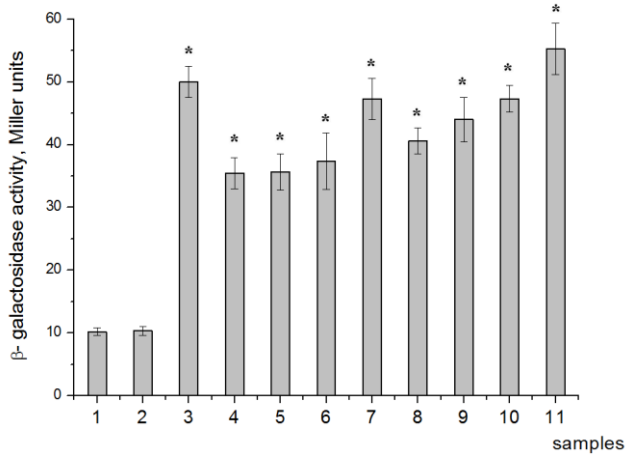

Fig4. *Expression of the sfiA gene (SOS regulon) in the E. coli PQ37 [sfiA::lacZ] and H_2S influence: 1 – intact control; 2 – H_2S 0.5 mM; 3 – 4NQO (positive control) 26.3 μM; 4 –NO-29 0.05 mM; 5 – NO-29 0.05 mM + H_2S 0.005 mM (10:1); 6 – NO-29 0.05 mM + H_2S 0.05 mM (1:1); 7 – NO-29 0.05 mM + H_2S 0.5 mM (1:10); 8 – NO-33 0.1mM; 9 – NO-33 0.1 mM + H_2S 0.01 mM (10:1); 10 – NO-33 0.1 mM + H_2S 0.1 mM (1:1); 11 – NO-33 0.1 mM + H_2S 1.0 mM (1:10)*

Hence, H_2S potentates the signaling effects of NO-donating agents, and both reactants can act synergistically.

3.2. The Bacterial Biofilm Formation

A level of the bacterial biofilms absolutely depended upon the cell genotype, the structure and dose of NO-donors, and the ratio of NO:H_2S. The productivity of the bacterial biofilm formation ($P=OD_{570}/OD_{600}$) was higher in *E. coli* soxR mutant than in the *E. coli* wt, due to the higher level of nitrosative stress in the mutant cells. 0.05 mM NO-29 treatment of the cells decreased the productivity of the bacterial biofilm in both strains 1.6-2.0 fold (Fig. 5, samples 2). In the experiments with 0.01 mM $TNIC_{thio}$ the level of biofilm production was equal to control. Low sublethal concentrations of NO-donors inhibited the biofilm formation in *E. coli* and *P. aeruginosa* by a factor of 1.5–2.0 relative to the control, which corresponded to the activity of antibiotic cyprofloxacine (CF), as an inhibitor of biofilm formation (a positive control) (Fig. 5-7). The productivity of NO-29 0.05 mM is 4.6 fold higher in *Ps. aeruginosa* (3.7), as compared with the same productivity in *E. coli* (0.8). On the other hand, the biofilm induction by 0.05 mM NO-29 is 1.5 fold higher than that of the same concentration of $TNIC_{thio}$ in *Ps. aeruginosa*. Then H_2S addition further decreased the productivity of biofilm formation (Fig 5, samples 4). The cell treatment with 0.01-0.1 mM pure hydrogen sulfide solutions didn't affect the productivity of biofilm formation (Fig. 5-7). A correction of $TNIC_{thio}/H_2S$ ratio from 1:1 to 1:10 increased the bacterial dispersion in *E. coli* PQ37 [Fig. 6].

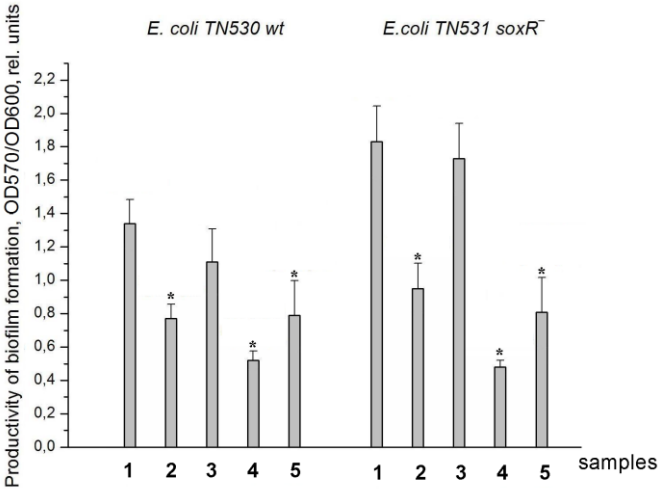

Fig5. *Hydrogen sulfide decreases a "productivity of biofilm formation" induced by NO-29 in E. coli TN530 wt and TN531soxR mutant strain: 1 – intact control; 2 – NO-29 0.05 mM; 3 – H_2S 0.5 mM; 4 - NO-29 0.05 mM + Na_2S 0.5 mM; 5 – CF 0.05 μM. For details, see Materials and Methods*

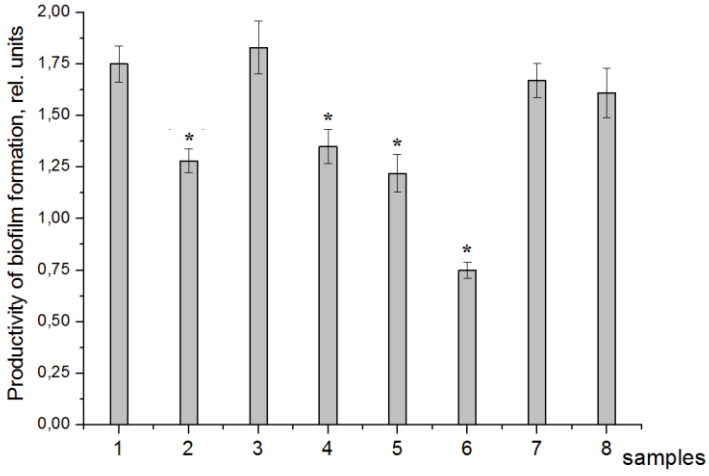

Fig6. *Hydrogen sulfide decreases a "productivity of biofilm formation" induced by $TNIC_{thio}$ in E. coli PQ37: 1 – intact control; 2 – CF 0.025 μM; 3 – $TNIC_{thio}$ 0.01 mM; 4 – $TNIC_{thio}$ 0.05 mM; 5 – $TNIC_{thio}$ 0.01 mM +H_2S 0.01 mM (1:1); 6 – $TNIC_{thio}$ 0.01 mM + H_2S 0.1 mM (1:10); 7 – H_2S 0.01 mM; 8 – H_2S 0.1 mM*

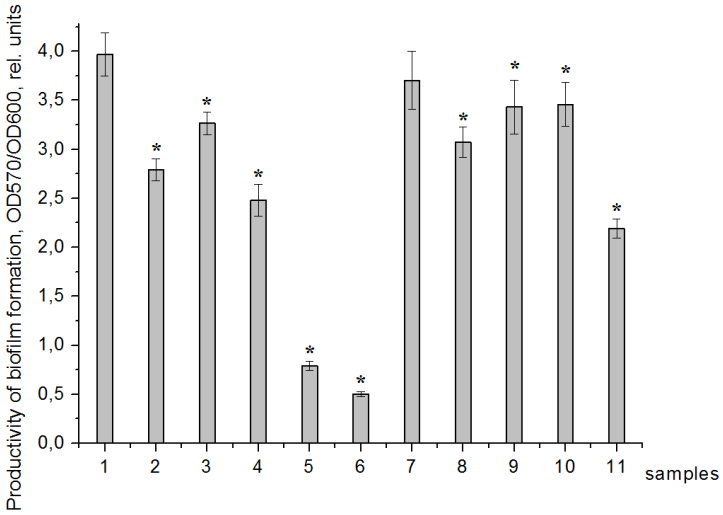

Fig7. *Hydrogen sulfide decreases a "productivity of biofilm formation" induced by $TNIC_{thio}$ and NO-29 in Pseudomonas aeruginosa: 1 – intact control; 2 – CF 0.05 μM; 3 – $TNIC_{thio}$ 0.025 mM; 4 – $TNIC_{thio}$ 0.05 mM; 5 – $TNIC_{thio}$ 0.025 mM+H_2S 0.25 mM (1:10); 6 – $TNIC_{thio}$ 0.05 mM+H_2S 0.5 mM (1:10); 7 – NO-29 0.05 mM; 8 – NO-29 0.05 mM +H_2S 0.5 mM (1:10); 9 – H_2S 0.5 mM; 10 – $TNIC_{tetrazo}$ 0.05 mM; 11 – $TNIC_{tetrazo}$ 0.05 mM + H_2S 0.5 mM (1:10). For details, see Materials and Methods*

3.3. The EPR Signals

Fig. 8 depicts the EPR signals recorded in *E. coli* PQ37 after the respective treatments. The signal **c** corresponded to the DNICs with the thiol containing ligands ("broad" thiol signal, g_{aver}=2.03 ($g_{\perp}$=2.04, $g_{||}$=2.014)), while the signal **d** corresponded to the DNICs with the persulfide containing ligands (the "narrower" persulfide signal) g_{aver}=2.03 (g_{I}=2.032 and g_{II}=2.020). The amplitude of *c* and *d* signals was nearly identical. The narrower persulfide signals were generated mostly upon the cell treatment with the combination of both NO-29 and H_2S agents (1:10).

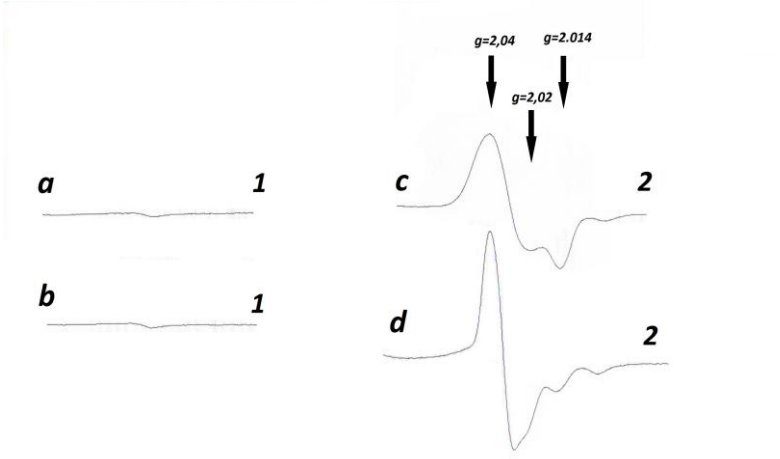

Fig8. *EPR spectra of E. coli PQ37 incubated in liquid LB-medium for the respective treatment: a, control; b, 0.5 mM H_2S; c, 0.05 mM NO-29; d, 0.05 mM NO-29 + 0.5 mM H_2S. The EPR spectra were recorded at 77 K. The spectrometer signal amplification (rel. units) is shown to the right*

4. DISCUSSION

The aim of our work is to study the signaling functions of the pure NO-donors and hydrogen sulfide and the combination of both in the defense DNA repair pathways and in the biofilm formation as the responses of the bacterial cell to stress. We didn't observe any signaling activity of the pure H_2S solutions in *E. coli* cells. The NO-29 and NO-33 donors were much more potent inducers of the sfiA and the soxS gene expression (4-6 fold surpass over the spontaneous level), as compared to the well studied $DNIC_{cis}$ and $DNIC_{gly}$ at ten fold higher concentrations [11, 12]. These NO donors are stable thermodynamically and might be beneficial in understanding of interaction between NO and H_2S [25, 26].

In aerobic experiments *in vitro* with NO-donors a complex of reactive nitrogen intermediates (RNI), ranging from nitric oxide radical to nitrite, as the products of NO oxidation was appeared. In physiological environments, a lot of additional compounds exist, including NO^-, S- nitrosothiols, peroxynitrite ($OONO^-$), and dinitrosyl iron complexes; all of these products initiate the SOS and the soxRS DNA repair pathway activation in *E. coli* sells [11, 33 - 36].

The hydrogen sulfide was a potent mediator of NO- signaling functions in *E. coli* DNA repair genes; the results of the combination cell treatment with NO and sulfide absolutely depended upon the molar ratios of the reactants [samples 5-6, Fig.6]. In our work the best ratio of the reactants was 10 fold excess of sulfide, and the earlier published data were correlated with ours [37, 38].

The responses of mammalian cell exposure to exogenous H_2S are less significant than those elicited by endogenous H_2S production. Physiological significance of H_2S is not just clear, but the high H_2S concentrations (1-10 μM) control the relaxation of blood vessels, inhibit inflammation, and modulate neuronal activity [39 - 41]. H_2S is much less known signaling molecule in bacteria than NO.

Numerous papers have provided experimental evidences for the multiple roles of NO and H_2S in the individual and the cooperative applications [38, 42 - 46]. What is clear - is that these compounds should work together to give a coordinated response in the cell, but the molecular mechanisms and the details of this interaction are not clear. The bacterial cells are the least investigated objects among the objects have been studied in this area.

In *E. coli* cells the narrow persulfide EPR-signals predominantly appeared after combined cell exposure to NO in complex with 10 fold excess of sulfide (Fig.8). The signals *a* and *b* are the controls. The signal *c* corresponds to DNIC-29 with thiol-containing ligand (the broad thiol 2.03 signal), while the signal *d* is the narrow one (the narrow persulfide 2.03 signal) is generated upon cell treatment with the combination of the both DNIC and sodium sulfide (1:10). The spectrum of the cells treated with DNIC-29 and sulfide (1:1) was similar with *c* and is not shown. The amplitude of *c* and *d* signals is nearly identical in both cases, but after the double integration, the concentration of the paramagnetic centers responsible for the narrow 2.03 signal appears to be significantly lower than that for the broad thiol 2.03 signal.

According to the calculations [47], if the amplitudes of the wide and the narrow signals in the EPR spectra of mono- and binuclear forms of low-molecular and protein-bound dinitrosyl iron complexes with thiol-containing ligands are nearly similar, the concentration of the paramagnetic centers in the wide thiol signals would be higher than that of in the narrow persulfide ones. We suppose that in our case, the EPR-invisible "extra paramagnetic" centers (being formed in the samples with the DNICs with the –R-S-S- narrow persulfide signals) are further transformed into the binuclear diamagnetic EPR-silent centers due to a low level of thiol-containing ligands.

In 1973 the mononuclear form of DNIC (m- DNIC) with the protein-bound persulfide (R-S-S-) ligands, with a characteristic narrow EPR signal at g_{aver}= 2.03 ($g_{\perp}$= 2.35, $g_{||}$ = 2.02), became a new kind of DNICs in biosystems which was generated in a solution of nitrogenase from *Azotobacter vinelandii* [48]. The authors suggested that the appearance of m-DNICs with the persulfide ligands was initiated by the release of inorganic sulfur (sulfide) from decomposing of iron–sulfur clusters of nitrogenase; their interaction with thiol groups of the enzyme yields persulfides (disulfide anions, R-S-S-). In the presence of nucleophilic agents able to bind inorganic sulfur, DNICs with persulfide ligands are converted into m-DNICs with thiol groups of nitrogenase [35].

It was established that the destruction of the other iron-sulfur proteins in response to NO attack might also initiate the synthesis of m-DNICs with persulfide ligands, as well [49 - 52].

In 1999 we succeeded in recording a narrow intense EPR 2.03 signals after *E. coli* PQ37 treatment with NO-donor $DNIC_{cys}$, and these signals were correlated with enhanced expression of the *E. coli* sfiA gene [12]. Further we confirmed that the releasing of free iron and inorganic sulfur was the result of NO-catalyzed destruction of the Fe-S cluster of the FNR $[4Fe\text{-}4S]^{2+}$ anaerobic transcription regulator of the aidB gene expression in *E. coli* MV2176. Subsequent reconstruction of the Fe-S cluster was related with the synthesis of m-DNIC with persulfide ligands and enhanced level of the aidB gene expression [53].

It became evident that $^{\bullet}NO$ and H_2S can affect each other's functions, and it was suggested that $^{\bullet}NO$ and H_2S can interact directly to form the novel molecular entities, which could modulate and increase

the •NO and H_2S biological effects. An interaction of sodium nitroprusside and H_2S can form a nitrosothiol-like species, which has physiological effects similar to those of •NO [54]. Then it has been suggested that the interaction between S-nitrosothiols and Na_2S can lead to the formation of the simplest S-nitrosothiol (thionitrous acid, highly unstable HSNO) [55]. Nevertheless, it is unclear, whether the formation of HSNO could actually take place *in vivo*.

Recently, Cortese-Krott et al. found that if RSNOs were incubated with Na_2S in excess, the accumulation of a "yellow product" was obtained, which the authors identified as nitrosopersulfide - $SSNO^-$ [37]. $SSNO^-$ is unstable at physiological pH and upon decomposition it gives both • NO and polysulfides. So, H_2S could regulate not only •NO production from its own metabolite (RSNOs), but it could also affect its reactivity and signaling [56].

There is plenty of information concerning the signaling and physiological roles for NO–H_2S cross-talk, but precisely what this role it, is not clear.

Nitrite is a known bioactive oxidation product of nitric oxide which efficiently converted to S-nitrosothiols *in vivo*; the latter react with sulfide *in vitro*, leading to formation of thionitrite (SNO^-) and nitrosopersulfide ($SSNO^-$). The chemistry of interaction between all these components have been studied in detail [22], but now there is no evidence for the $SSNO^-$ generation *in vivo.*

The intensive investigation has been expanded on the NO and H_2S interactions in the mammalian cells mostly, but the similarities with the NO-mediated biological functions in the biosystems are striking.

We suppose that H_2S *per se* is not an ordinary signaling molecule in bacteria. In the combination with NO donors, H_2S reinforces the NO signaling mechanism as the source of sulfur and an additional •NO production. The initial stage of the NO initiated process of the iron-sulfur transcription regulator destruction and the formation of m-DNICs with the EPR specific persulfide signals. Subsequent reconstruction of the Fe-S clusters *in vivo* is enhanced during the combined cell treatment with NO and H_2S.

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