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Influence of Oxidative and Nitrosative Stress on Accumulation of Diphosphate Intermediates of the Non-mevalonate Pathway of Isoprenoid Biosynthesis in Corynebacteria and Mycobacteria

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Abstract—Artificial generation of oxygen superoxide radicals in actively growing cultures of *Mycobacterium tuberculosis*, *Myc. smegmatis*, and *Corynebacterium ammoniagenes* is followed by accumulation in the bacterial cells of substantial amounts of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP) – an intermediate of the non-mevalonate pathway of isoprenoid biosynthesis (MEP) – most possibly due to the interaction of the oxygen radicals with the 4Fe–4S group in the active center and inhibition of the enzyme (E)-4-oxy-3-methylbut-2-enyl diphosphate synthase (IspG). Cadmium ions known to inhibit IspG enzyme in chloroplasts (Rivasseau, C., Seemann, M., Boisson, A. M., Streb, P., Gout, E., Douce, R., Rohmer, M., and Bligny, R. (2009) *Plant Cell Environ.*, **32**, 82-92), when added to culture of *Myc. smegmatis*, substantially increase accumulation of MEcDP induced by oxidative stress with no accumulation of other organic phosphate intermediates in the cell. *Corynebacterium ammoniagenes*, well-known for its ability to synthesize large amounts of MEcDP, was also shown to accumulate this unique cyclodiphosphate in actively growing culture when NO at low concentration is artificially generated in the medium. A possible role of the MEP-pathway of isoprenoid biosynthesis and a role of its central intermediate MEcDP in bacterial response to nitrosative and oxidative stress is discussed.

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Key words: non-mevalonate pathway, nitrosative stress, mycobacteria

Reactive oxygen species (ROS) and NO, generated by cells of the eukaryotic immune system, are very important in struggle against bacterial infections [1-5]. Among most possible targets of the oxygen radicals and NO are Fe–S centers of the bacterial enzymes [6-8]. In particular, two bacterial enzymes of this Fe–S type function in the terminal part of the non-mevalonate pathway of isoprenoid biosynthesis (see Scheme 1) – IspG (GcpE, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase) and IspH (LytB, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase) [9-11]. Functioning of this pathway is indispensable for bacterial growth of many species [12] including pathogenic *Mycobacterium tuberculosis* [13].

Earlier the generation of superoxide radicals and other ROS in some bacteria and plants was shown to be followed by accumulation in the bacterial cells and chloroplasts of an intermediate of non-mevalonate pathway of isoprenoid biosynthesis (methyl-erythritol-4-phosphate pathway, MEP) – 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP). A reason for this is suggested to be the inhibiting effect of superoxide radicals on 4Fe–4S active centers of IspG, catalyzing the utilization of this compound, resulting in reduced rate of transformation of MEcDP into (E)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP) [14-16]. The largest amounts of MEcDP were detected in the case of oxidative stress in culture of *Corynebacterium ammoniagenes*, much smaller – in culture of *Myc. smegmatis* [14] while in slow growing culture of *Myc. tuberculosis* MEcDP has not been detected at all. The question arises of the reason for this phenomenon: is it due to special resistance of the microbe to the

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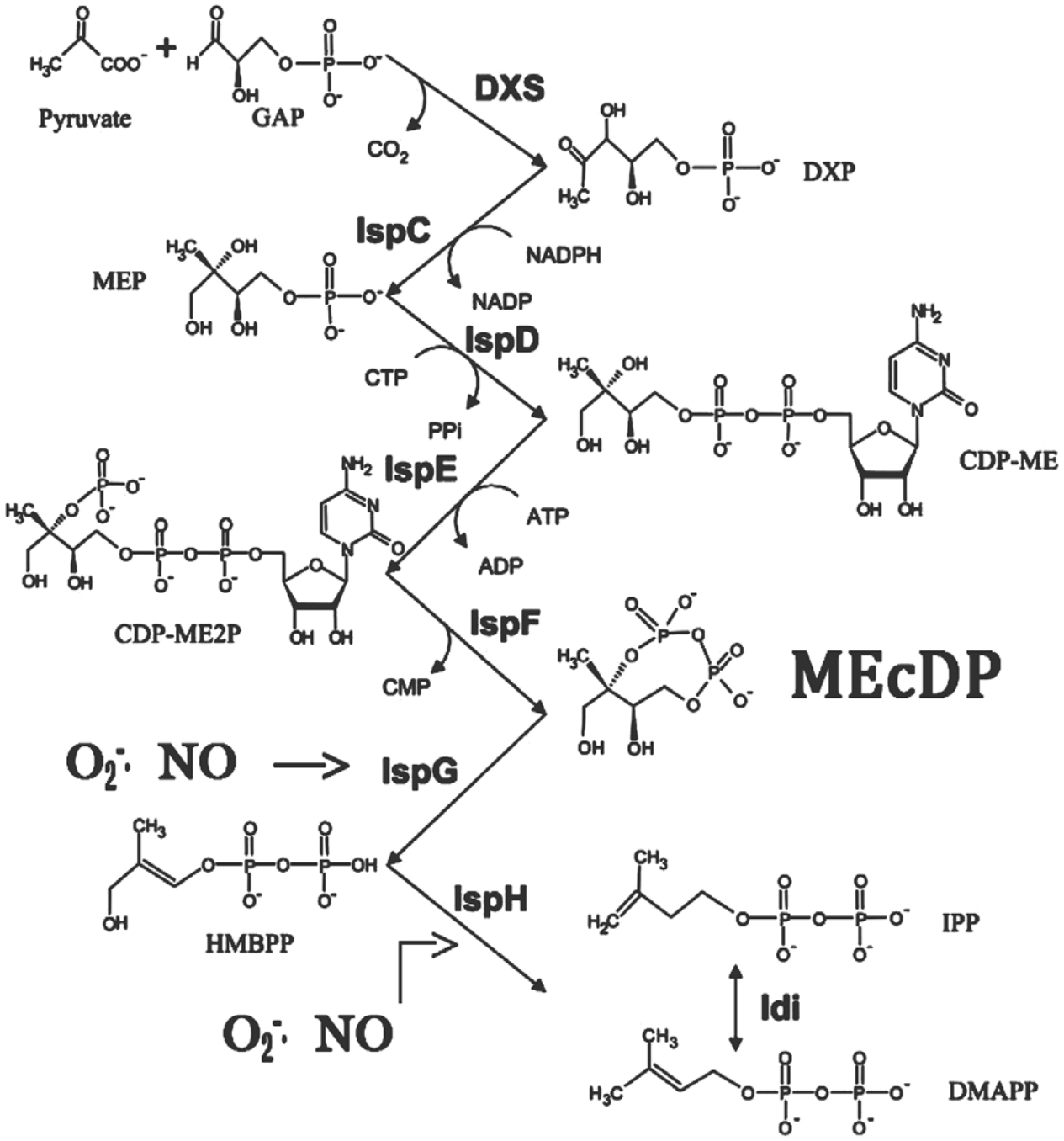


Non-mevalonate pathway of isoprenoid biosynthesis and possible loci of action of products of oxidative and nitrosative stress

Scheme 1

active oxygen species due for example to resistance of the tuberculosis enzyme IspG responsible for MEcDP, and can that be an explanation for elevated resistance of these bacteria to oxidative stress in the host macrophages [17]?

As to NO, though Fe–S centers of proteins are recognized targets of nitrosative stress [18-20], there is now no indications in the literature on the influence of NO on Fe–S enzymes of the MEP pathway.

In this work we investigated the effect of artificial generation of both ROS and NO on accumulation of MEcDP and other diphosphate intermediates of MEP pathway of isoprenoid synthesis in mycobacteria and corynebacteria in actively growing cultures. The role of significant accumulation of MEcDP in actively growing bacterial cultures in response to low concentrations of NO and ROS is discussed.

MATERIALS AND METHODS

The following bacterial strains were used in this study: *Myc. smegmatis* strain mc^{2}155, *Myc. tuberculosis* H37Rv, *C. ammoniagenes* ATCC 6872, and *Micrococcus luteus*, Flemming strain 2665.

Mycobacterium smegmatis was grown at 37°C on an orbital shaker (250 rpm) in 200 ml of the rich medium containing per liter: 10 g peptone, 5 g NaCl, 3 g yeast extract supplemented with 0.05% (v/v) Tween 80, pH 7.0, in 750 ml flasks. *Corynebacterium ammoniagenes* and *M. luteus* were grown at 30°C on an orbital shaker (250 rpm) in 200 ml of the rich medium containing per liter: 10 g peptone, 5 g NaCl, 5 g yeast extract, pH 7.5, in 750 ml flasks. Concentration of materials added during cultivation, time of additions, and time of harvest are given in the text and figure legends. In experiment with *M. luteus* transformed by pMind-gcpE plasmid, the cells were cultivated in medium supplemented with kanamycin (10 μg/ml). At the mid log phase tetracycline was added to 0.02 μg/ml, and at the end of log phase glucose (to 1%) and benzyl viologen (BV) dihydrochloride (to 50 μg/ml) were added. Biomass was pelleted by centrifugation after 20 h cultivation from the addition of BV. The control strain of *M. luteus* was also transformed by pMind plasmid (without the *gcpE* gene).

Mycobacterium tuberculosis was grown aerobically at 37°C in shaker flasks (200 ml medium in 500 ml flasks, agitation at 200 rpm) in Sauton's medium supplemented with 0.5% albumin, 0.2% glucose, and 0.085% NaCl in the presence of 0.05% Tween 80. Initial cell concentration was 10^5-10^6 cells/ml. Cells were grown eight days until the optical density at 600 nm reached 0.7 (amount of cells was determined by the Most Probable Number method (MPN), $2.4\cdot10^7$ cells/ml). At this moment the culture was supplemented with glucose (to 1%) and BV (to 12.5 μg/ml). Cultivation was continued for four days until the optical density at 600 nm reached 4.8 (MPN, $9.5\cdot10^9$ cells/ml).

Bacterial cells from 400 ml culture were collected by centrifugation, suspended in 15 ml ethanol, incubated with periodic stirring for 30 min, centrifuged, and resuspended again in 15 ml 70% ethanol (15-20 min incubation) and finally centrifuged to discard the pellet. The combined extract was evaporated to dryness in a stream of air in a Petri dish and then treated with 0.7 ml of 30% D_2O supplemented with SDS (0.7%) and EDTA (50 mM) for ^{31}P-NMR investigation using a Bruker AM300 spectrometer (121.49 MHz, the ^{31}P chemical shifts are referred to the signal of 75% H_3PO_4 in D_2O (0.0 ppm, external standard)).

A source of nitric oxide, 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO) (Sigma, USA), was dissolved in 10 mM KOH immediately before addition into growing bacterial culture at the mid log phase to concentration 90-100 μM and cultivation further continued for 22 h.

Nitrosoglutathione was prepared by mixing equimolar solutions of reduced glutathione and sodium nitrite, and after 5 min the mixture was added to mid log phase culture and cultivation continued for 10 h.

Mycobacterium tuberculosis Rv2868c/gene (synonyms – gcpE and IspG) was cloned into *M. luteus*. The *gcpE* gene was amplified by PCR using a primers 5′-GAC**CTGCAG**AACCCGATTAGGCTTTTCCA-3′ and 5′-G**ACTAGT**CGACATCTGCGAACTCCCTT-3′ (*Pst*I and *Spe*I restriction sites are shown in bold). The purified PCR product was ligated into pGEM-T vector (Promega, USA). The ligated product pGEM-*gcpE* was transformed into *E. coli* strain TG1, and selected colonies were examined by PCR. Thereafter, pGEM-*gcpE* and pMind vectors [21] were digested with the restriction enzymes *Pst*I and *Spe*I, and the *gcpE B* fragment was ligated into pMind. The ligated product pMind-*gcpE* was transformed into *E. coli* strain TG1, and the sequence of the cloned gene was confirmed. pMind-*gcpE* was transformed into *M. luteus*.

RESULTS AND DISCUSSION

Benzyl viologen (BV) as well as methyl viologen (paraquat) is known to facilitate generation of superoxide radicals in aerobically grown bacterial cells [22]. Earlier we demonstrated that BV-induced oxidative stress is followed in many aerobic bacteria by accumulation of MEcDP [14, 15]. As a rule, BV was added to bacterial cultures at the end of logarithmic or beginning of stationary phase simultaneously with glucose to increase substantially the yield of MEcDP. The growth to high densities of culture was required for determination of phosphates by

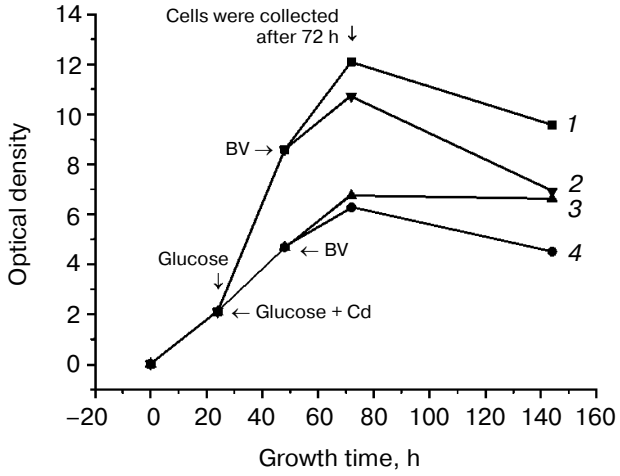


Fig. 1. Influence of benzyl viologen (BV) and cadmium ions on growth (optical density of culture, A_{600}) of *Myc. smegmatis*. Additions to the basic medium: *1*) BV; *2*) glucose + BV; *3*) glucose + Cd^{2+}; *4*) Cd^{2+} + BV.

^{31}P-NMR. Detectable amounts of MEcDP were registered in bacteria after 1 h of contact with BV + glucose in aerobic medium, and the maximal concentration was reached by 24 h, the concentration of BV 50 μg/ml being optimal for cultures coming to the end of log phase [14].

We investigated the effect of BV on accumulation of organic diphosphates in mycobacteria and corynebacteria in actively growing cultures. Results for *Myc. smegmatis* are presented in Figs. 1 and 2. Addition of glucose (1%) at the beginning of the log phase (optical density about 2 at 600 nm in peptone-yeast extract medium) stimulated growth rate by 30-60% (Fig. 1, curve *1* – typical one from four experiments). The ^{31}P-NMR spectrum of the material extracted by alcohol at the end of log phase of this culture shows (Fig. 2, spectrum *1*) signals in a range from −5 to −15 ppm assigned to organic diphosphates: terminal β-phosphate of organic diphosphates (from −6 to −8 ppm); α-phosphates of organic diphosphates (from −9 to −11 ppm); and phosphates of dinucleotides like hexose-nucleoside-diphosphate (from −11 to −13 ppm). A duplet typical for MEcDP and centered at chemical shift −14.7 ppm was not noted in this case.

Addition of BV (25 μg/ml) at the late log phase (Fig. 1, curve *2*) inhibited the growth rate in glucose-containing medium only about 10-30% and in spite of oxidative stress the final biomass was not changed or decreased no more than 10-15% of control (no BV). This cell biomass contains substantial amount of MEcDP (Fig. 2, spectrum *2*: multiplet centered at −10 ppm and duplet at −14.7 ppm). A signal at −7.5 ppm assigned to terminal β-phosphate of organic diphosphates is noteworthy reduced.

Recent investigation of the MEP pathway of isoprenoid biosynthesis in plant chloroplasts revealed that infusion of Cd^{2+}-containing solutions in leaves of some plants [16] induced accumulation of MEcDP in their chloroplasts, supposedly because of incorporation of cadmium ions in the apoenzyme IspG by binding to cysteines in the active center, thus substituting iron for cadmium in the active center of IspG.

We also observed an inhibitory effect of addition of 0.15 mM $CdCl_2$ simultaneous with 1% glucose at the beginning (1/3) of logarithmic phase on the growth of *Myc. smegmatis*. Figure 1 (curve *3*) illustrates 30-70% inhibition when 0.15 mM $CdCl_2$ + glucose was added as compared with the control (supplemented with glucose only), and this inhibition depends on culture density, rate, and phase of growth in each experiment (not shown). Figure 2 (spectrum *3*) indicates that growth in cadmium-containing medium brings accumulation of MEcDP similar to the effect of oxidative stress (Fig. 2, spectrum *2*). Subsequent addition of 0.15 mM $CdCl_2$ and 25 μg/ml BV (+ glucose) (Fig. 1, curve *4*) causes very high accumulation of MEcDP in the cells (Fig. 2, spectrum *4*), so that concentration of MEcDP is greater than the concentration of other phosphates extracted from the cells by alcohol (for better presentation, signals in spectrum *4* of

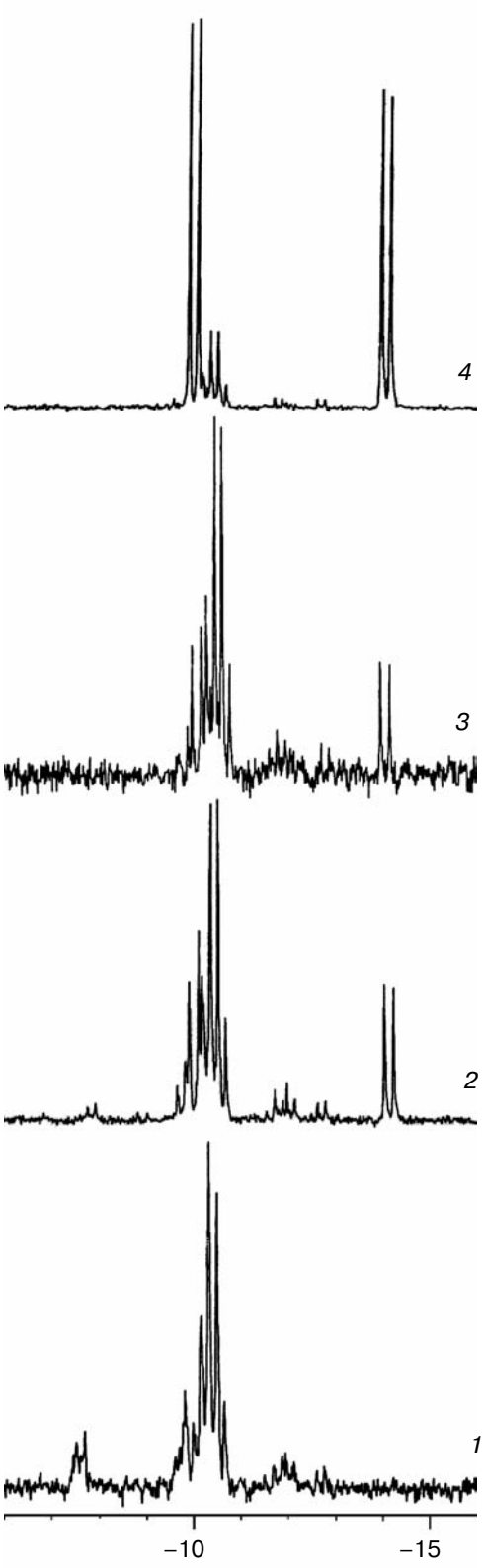


Fig. 2. ^{31}P-NMR spectra of ethanol extract of *Myc. smegmatis* cells grown on medium supplemented with glucose (*1*), glucose + BV (*2*), glucose + Cd^{2+} (*3*), glucose + BV + Cd^{2+} (*4*).

Fig. 2 are reduced to 1/6). Note that no substantial amount of glyceraldehyde phosphate, deoxy-xylulose phosphate, methylerythritol phosphate, and other precursors of MEcDP in the MEP-pathway of isoprenoid biosynthesis (CDP-ME, CDP-ME2P) are detected by ^{31}P-MNR under these experimental conditions. Two explanations for this are possible: elevated stationary concentration of these compounds is followed by sharp elevation of their metabolism, or the rate of their transformation into MEcDP is so high compared to the rate of their biosynthesis that even substantial inhibition of MEcDP utilization by IspG has no influence upon the amount of these products in the cells. It is important to note that under conditions of high rate of MEcDP accumulation, a certain part of the culture retains its ability to grow, and the number of colony-forming cells on solid medium is more than 10^7 per ml (not shown). As the MEP pathway is indispensable for bacterial growth, we assume that under this condition some cells continue to synthesize IspG enzyme or they can restore the inhibited enzyme molecules and thus diminish negative effect of oxidative and cadmium stress [2, 19, 23]. As to the nature of organic diphosphates registered on Fig. 2, we argue that a signal at chemical shift −7.5 ppm (Fig. 2, spectrum *1*) can be assigned to terminal phosphorus of diphosphates located beyond IspG in the MEP pathway, and it is drastically reduced or disappears during oxidative or cadmium stress, respectively. We shall discuss this possibility later.

Contrary to *Myc. smegmatis* and many related bacteria, accumulation of MEcDP under oxidative stress was never registered in *Myc. tuberculosis*. A possible reason for this might be an unusual resistance of the tuberculosis IspG enzyme to active oxygen species that make the whole bacterium more resistant to oxidative stress [17] and help it to survive within a macrophage. To check this suggestion, we transferred a plasmid bearing the *Myc. tuberculosis* IspG gene into bacterium *M. luteus* (see "Materials and Methods") and incubated it under conditions of oxidative stress. We expected that no MEcDP would accumulate in the *M. luteus* mutant if the transferred enzyme is really resistant to oxygen radicals, but if this enzyme is sensitive to superoxide as the enzyme of micrococci, then MEcDP will accumulate in amounts typical for the wild strains of *M. luteus*. Thus, in the mutant micrococci we observed accumulation of MEcDP under oxidative stress as in a wild strain [14] (data not shown), and that is why we conclude that the *Myc. tuberculosis* IspG enzyme is sensitive to oxidative stress. So, now we returned to search for conditions causing MEcDP accumulation in the *Myc. tuberculosis* bacterium, and this time we used early phase of active growth of the *Myc. tuberculosis* culture. Accumulation of MEcDP in *Myc. tuberculosis* cells treated with BV as compared with *Myc. smegmatis* is presented in Fig. 3. Contrary to our earlier experiments, when we added BV and glucose to the late logarithmic or even to stationary phase when biomass was rather high, now we made additions to actively growing culture (7-8 days of culture, optical density about 0.6 at 600 nm) and the amount of added BV was reduced to 12.5 μg/ml so that not to kill the cells, and we let them grow for another 4 days in glucose-supplemented medium before being taken for NMR analysis (Fig. 3, spectrum *1*). We made three independent experiments to prove the accumulation of MEcDP and present the best spectrum in Fig. 3. We clearly see two signals of MEcDP (duplet at −14 and −10 ppm). Contrary to *Myc. smegmatis* (Fig. 2, spectrum *2*), extract from *Myc. tuberculosis* does not show NMR signals of dinucleotides (chemical shifts at −10/−11 ppm) or other metabolically important organic diphosphates like NAD(P)$^+$ and NAD(P)H. In general, alcoholic extract from *Myc. tuberculosis* looks much poorer than that of *Myc. smegmatis*. Besides definite signals of MEcDP, only signals of orthophosphate and a small monophosphate component at chemical shifts 0 or −1 ppm in *Myc. tuberculosis* versus 21 components (in the range of chemical shifts from +5 to −15 ppm) in *Myc. smegmatis* are observed. Because the negatively charged MEcDP is clearly extracted from the cells, the suggestion of possible different extractability of the compounds due to complex cell wall structure of these relative organisms is not realistic. The difference is most possibly explained by lower level of metabolism in general and lower level of phosphates in slowly growing *Myc. tuberculosis* bacterium.

Thus, in *Myc. tuberculosis* (as well as in many other bacteria) oxidative stress induced even by low concentration of the redox cycling drug BV provokes accumulation during the active phase of growth of MEcDP – the central metabolite of the MEP pathway of isoprenoid biosynthesis.

Our data on the accumulation of MEcDP in response to superoxide as well as the data of other investigators [16] makes us think that the most likely target of active oxygen is 4Fe–4S active center of enzyme IspG, though the similar center of IspH is also affected (the fact that accumulation of HMBPP is not observed could be explained by simultaneous inhibition of HMBPP utilization and reduction of HMBPP synthase IspG).

The Fe–S centers of enzymes are sensitive to attack by ROS and NO [6, 20], so we investigated the influence of NO-generating compounds on the phosphate composition in bacterial cells of interest. First we checked the influence of NO upon the culture of *C. ammoniagenes* noted for its prompt response to oxidative stress [14]. In control experiments, a culture of the corynebacterium growing on the peptone–yeast extract medium at the middle of log phase was supplemented with substrates: glucose (0.5%) and lactate (0.11%). Biomass ($A_{600} > 20$) was extracted and analyzed after 20 h incubation as usual (see "Materials and Methods"). Results are presented as ^{31}P-NMR spectra in Fig. 4 (spectrum *1*). In the case of control cells of *C. ammoniagenes*, we see α-phosphates of organic diphosphates (chemical shifts from −9 to

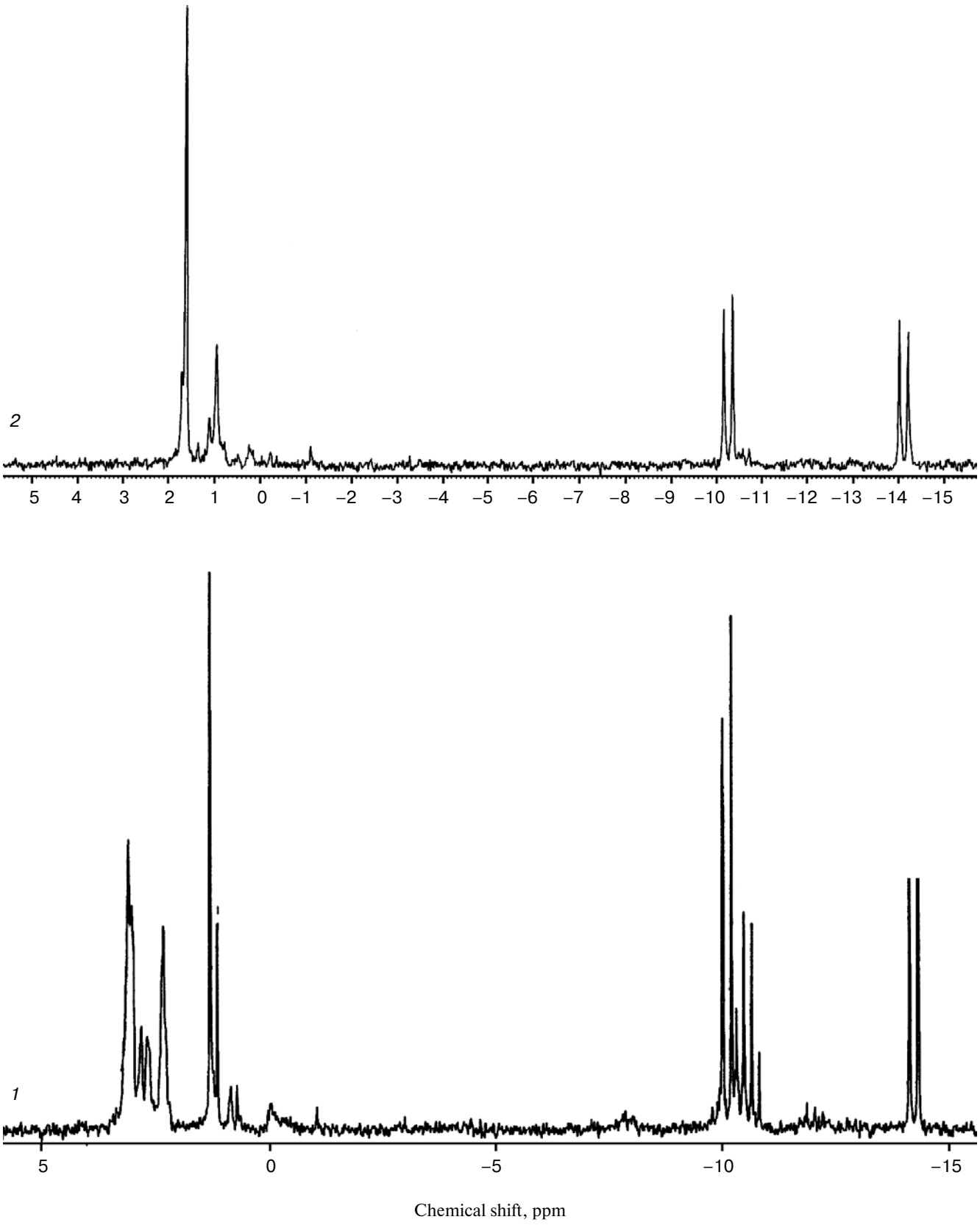


Fig. 3. ^{31}P-NMR spectra of ethanol extract of the *Myc. tuberculosis* cells (*2*) in comparison with that of *Myc. smegmatis* (*1*).

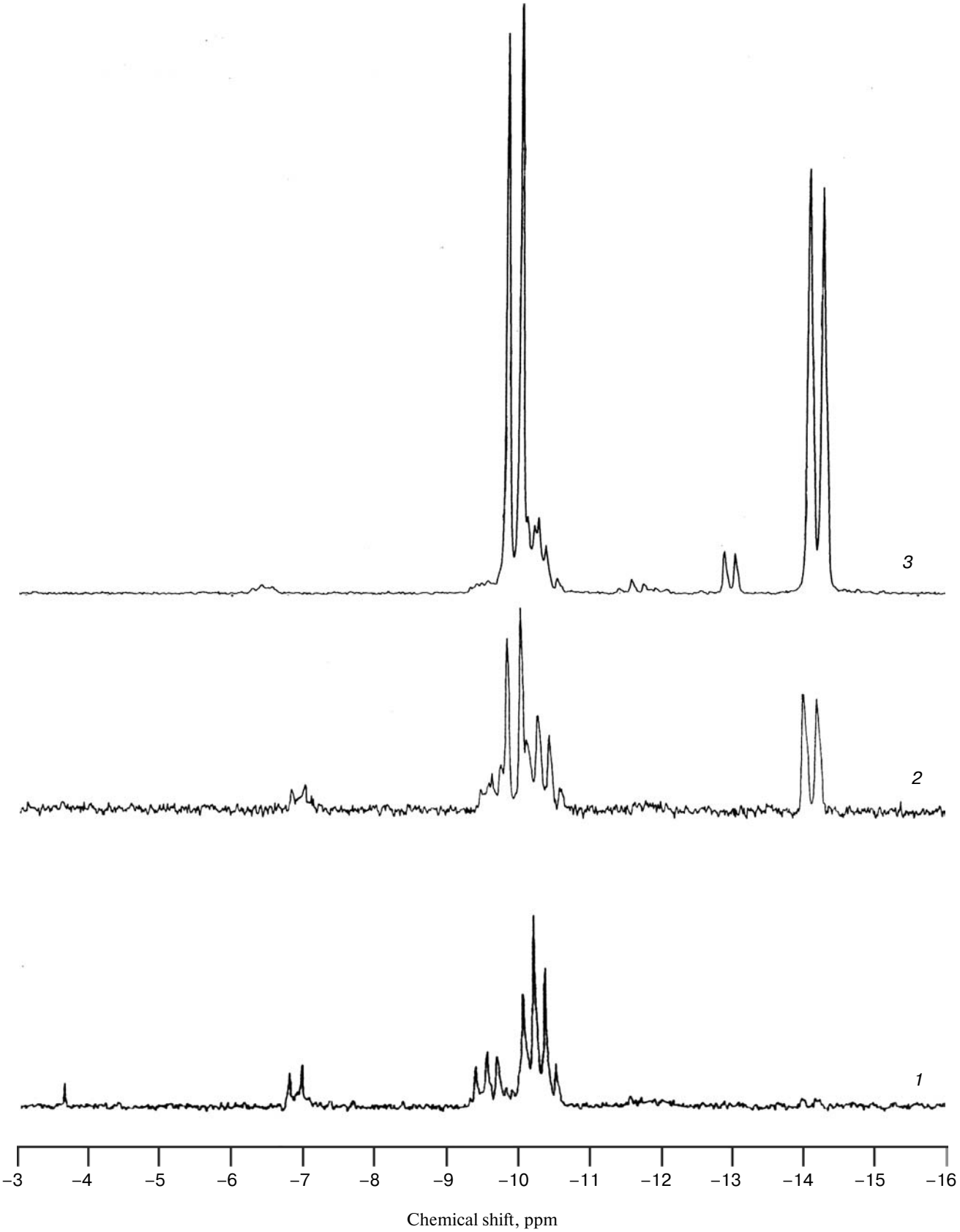


Fig. 4. ^{31}P-NMR spectra of the ethanol extract of *C. ammoniagenes* cells grown on medium with no addition of oxidative stress generator (*1*) or supplemented with the NO generator DETA/NO (*2*), or supplemented with benzyl viologen (*3*).

−11 ppm), terminal β-phosphates of organic diphosphates (chemical shifts from −6 to −8 ppm), and a small signal at −3/−4 ppm (possibly a terminal γ-phosphate of organic triphosphate). As to the unique signal of cyclodiphosphate MEcDP at chemical shift −14 ppm, its split peak could be seen as a trace signal. On addition of artificial NO generator (DETA/NO) at low concentration (90 μM) together with glucose (0.5%) and lactate (0.11%) at the middle of logarithmic phase, a substantial increase after 20 h incubation of the signal of MEcDP as compared with control cells is seen (Fig. 4, spectrum *2*). Spectrum *2* in Fig. 4 illustrates a clear signal typical of MEcDP (duplets at −14 and −10 ppm), while in control cells only trace signals could be observed (Fig. 4, spectrum *1*). Thus, this is the first demonstration of MEcDP accumulation in bacterial cells when they are cultivated under the influence of NO. Certainly, the registered amounts of MEcDP is far from the potential of the tested microorganism, and in spectrum *3* of Fig. 4 we see the response of *C. ammoniagenes* to BV-induced oxidative stress, when the concentration of MEcDP becomes very large (signal amplitudes were reduced by 4-fold for convenience of presentation).

So we suggest that Fe–S enzymes of the non-mevalonate pathway of isoprenoid biosynthesis responsible for transformation of MEcDP into the subsequent products (IspG and IspH) are controlled in bacteria not only by superoxide radicals, but also by nitric oxide NO. Of course, the degree of inhibition by these agents may differ greatly for the two enzymes and significantly depend on the individual microorganism.

In Fig. 5 the effect of nitric oxide on a growing culture of *Myc. smegmatis* is illustrated. We made five independent experiments. In an attempt to attribute the phenomenon also to mycobacteria, we added DETA/NO (0.1 mM, 22 h) to growing culture of *Myc. smegmatis*, or another NO generator nitrosoglutathione (0.5 mM, 10 h), but we could not register accumulation of MEcDP by ^{31}P-NMR in extract from these cells. However, there is an intriguing feature in the *Myc. smegmatis* (grown in the NO-supplemented medium) NMR spectra in the range of chemical shifts from −6 to −10 ppm. A signal of the terminal β-phosphate of organic diphosphates at about −7.5 ppm (Fig. 5, spectrum *1*) pronounced in control cells, "disappeared" under the influence of nitric oxide (cultivation in presence of DETA/NO) and is replaced by a larger and split peak at chemical shift −6.8 ppm (and possibly corresponding to it also split peak at chemical shift −9.7 ppm; Fig. 5, spectrum *2*). Also, when NO was generated by nitrosoglutathione (Fig. 5, spectrum *3*), another split and larger peak appeared at chemical shift −7.0 ppm simultaneously with a second multiplet at chemical shift −9.7 ppm. These results could be explained without contradiction for *Myc. smegmatis* under particular experimental conditions by suggestion of preferential inhibition by NO of the enzyme IspH. So, if

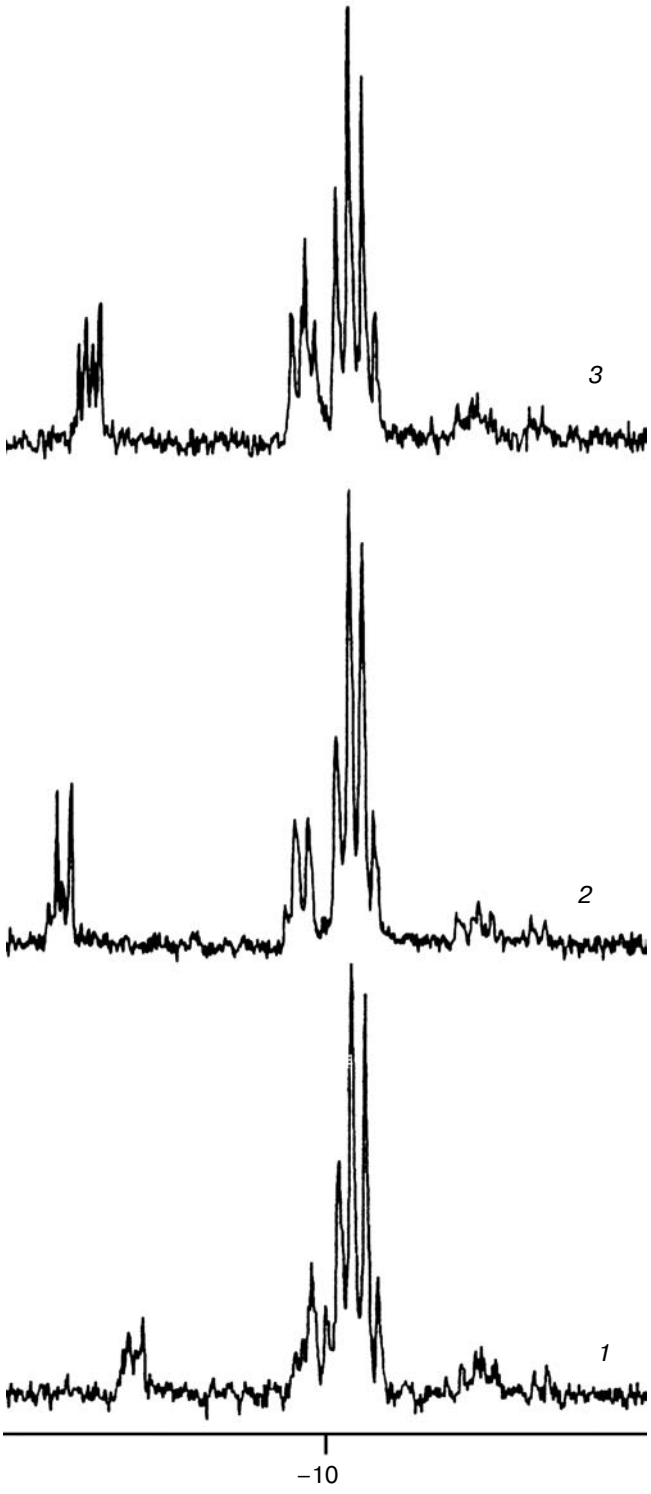
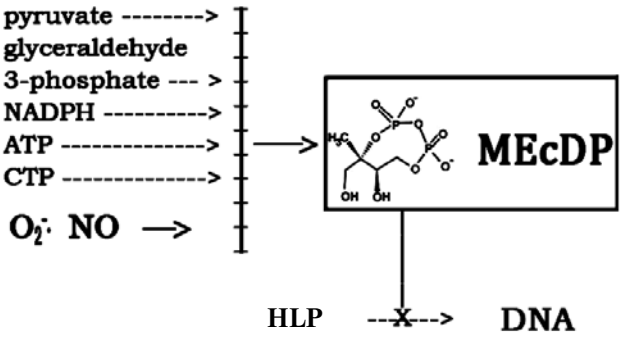


Fig. 5. Effect of NO upon *Myc. smegmatis*. ^{31}P-NMR spectra of the ethanol extract of control *Myc. smegmatis* cells (*1*) or cells grown on medium supplemented with DETA/NO (*2*) or supplemented with nitrosoglutathione (*3*). The region of diphosphate NMR signals on the chemical shifts scale is shown.

a signal at −7.5 ppm belongs to β-phosphate of isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP) (the products of IspH (Scheme 1)), then its stationary concentration should decrease on addition of inhibitory nitric oxide. Note that our suggestion on the nature of the signal at chemical shift −7.5 ppm correlates with the presented results (Fig. 2) on the influence of cadmium and oxidative stress on NMR spectra of *Myc. smegmatis*. We expect simultaneous increase in the concentration of the IspH substrate – HMBPP (signals at −6.8/−7.0 and −9.7 ppm), while the concentration of the product of the enzyme IspH affected by nitric oxide decreases. Activity of IspG is believed to be a limiting step of the MEP pathway [13], so even substantial inhibition of IspH may not lead to accumulation of MEcDP in amounts sufficient for NMR analysis. It is important to note that ^{31}P-NMR requires relatively large concentrations of the analyzed substances. That is why the registered increase in the signals in spectra *2* and *3* (Fig. 5) at chemical shift −6.8/−7.0 and −9.7 corresponds to substantial quantities of the organic diphosphates. Earlier [24] we calculated that in corynebacteria the concentration of MEcDP exceeds 50 mM when cells are grown under oxidative stress.

Thus, all the above results illustrate that both oxidative and nitrosative stress inhibit Fe–S enzymes of the MEP pathway of isoprenoid biosynthesis in mycobacteria and corynebacteria. However, bacterial growth *in vitro* is not completely suppressed, so what might be the significant sense for the host immune system in the struggle against bacterial infections to block the MEP pathway by oxygen radicals and by nitric oxide? Because of the specific requirements for NMR as a detector of the phosphate-containing intermediates, we used high-density bacterial cultures (>$5\cdot10^8$ cells/ml) actively growing on a rich medium. Also, we used low concentration of compounds generating small quantities of the nitric oxide and active oxygen that would not decrease substantially the growth rate in order to obtain good biomass (much higher concentration of these agents are usually used to stop the growth of *Myc. tuberculosis* [5]). It is clear that under our experimental conditions the sensitivity to the inhibitors might be decreased, and also the cells may continue to grow due to accumulated substrates and by cryptic growth when metabolic pathways are blocked. For example, in our experiments 0.15 mM $CdCl_2$ only slightly reduced the growth rate of *Myc. smegmatis* culture (Fig. 1, curve *3*), while 0.04 mM $CdCl_2$ completely inhibit the growth when added together with inoculum (not shown). That is why a certain role of sensitivity of non-mevalonate pathway of isoprenoid biosynthesis in the struggle of the immune system against bacterial infections cannot be excluded. Of course, experiments *in vivo* and with macrophages are required to make this statement definite. However, our experiments with cadmium ions incline us to think that substantial bactericidal effect can be expressed by induction of oxidative and nitrosative stress concomitant with inhibition of resynthesis and/or reparation of IspH and IspG enzymes. That is why the system of resynthesis and reparation of Fe–S centers (and possibly the unique SUF system for mobilization of sulfur in mycobacteria [25]) might be a good target in the search for new antibacterial drugs. Quite recent data on activation of genes responsible for reparation of Fe–S centers in enzymes [5] in the case of oxidative and nitrosative stress and activation of transport of iron ions are in accord with our suggestion. Of special interest is the fact that activation is observed at low concentration of the inhibitors (lower than bacteriostatic) and these concentrations correspond to that used in our experiments.

Another aspect of the problem is the possibility of other functions of MEcDP beyond being an intermediate of the MEP pathway. The unusual cyclodiphosphate structure, stability, and the ability to accumulate in large quantities [15] suggest some yet unknown function for this compound. These speculations are interesting in the light of a recent publication on the MEP pathway in the bacterium *Listeria innocua* [26], which has a complete mevalonate pathway and truncated MEP pathway terminated by MEcDP-synthase – IspF. Transformation of this organism with the *Bacillus subtilis IspG*-gene-bearing plasmid demonstrated that the truncated MEP pathway is functionally very active as it can produce extremely high concentrations of HMBPP [26]. Retention in evolution of an enzyme chain leading to "useless" compound MEcDP seems unreasonable (the genes *IspG* and *IspH* are not found in the genome of this strain). So what may be an additional metabolically important function for MEcDP in this case? To answer this, we should understand how the synthesis of MEcDP is regulated in the bacterial cell. Our data illustrating that most intensive accumulation of MEcDP is often observed only in growing cultures of bacteria when many important substrates are sufficiently supplied suggest that the activity of the non-mevalonate pathway (in particular, the stationary



Components necessary for induction of accumulation of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate and a possible locus of its action on the genome of the producing bacterium

Scheme 2

level of its unusual intermediate – MEcDP) might serve as a sensor of energetic wellbeing. In fact, high levels of important metabolites (ATP, CTP, NADPH, pyruvate, phosphoglycerate) are required at the same time for the successful MEcDP biosynthesis (Schemes 1 and 2). On the other hand, the level of MEcDP clearly depends on the level of oxygen radicals and NO, and thus MEcDP could well be a sensor of oxidative and nitrosative stress. The exact mechanism of this is still obscure, but it is clear that high negative charge and expressed stability of this cyclic compound is somehow involved. One recent observation in our laboratory on the specific properties of MEcDP (A. Anuchin et al., unpublished) may lead the way. The *in vitro* interaction of the plasmid DNA with the recombinant histone-like protein HLP of *Myc. smegmatis* [27] is followed by a change in circular dichroism (CD), but on addition of MEcDP (isolated from *C. ammoniagenes*) [28] these CD spectral changes were reversed. It is very tempting to suggest that highly negatively charged MEcDP may directly control some positively charged histone-like activators and suppressors of DNA and thus be involved in regulation of gene activity and sensory functions (Scheme 2). Moreover, we suggested earlier that MEcDP can act as an endogenous stabilizing agent for bacterial cells subjected to oxidative stress. When added to a solution of DNA in the presence of Fenton reagent, MEcDP prevented the decomposition of the DNA by complexation of ferrous ions, which attenuated their ability to catalyze the formation of hydroxyl radicals from hydrogen peroxide [29].

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