ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 1, pp. 72-80. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © E. G. Salina, Yu. A. Zhogina, M. O. Shleeva, G. M. Sorokoumova, A. A. Selishcheva, A. S. Kaprelyants, 2010, published in Biokhimiya, 2010, Vol. 75, No. 1, pp. 88-98.

Biochemical and Morphological Changes in Dormant ("Nonculturable") *Mycobacterium smegmatis* Cells

E. G. Salina[1]*, Yu. A. Zhogina[2], M. O. Shleeva[1], G. M. Sorokoumova[2], A. A. Selishcheva[3], and A. S. Kaprelyants[1]

[1]*Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, 119071 Moscow, Russia; fax: (495) 954-2732; E-mail: elenasalina@mail.ru*
[2]*Lomonosov State Academy of Fine Chemical Technology, pr. Vernadskogo 86, 119571 Moscow, Russia; fax: (495) 434-8233; E-mail: Galinams@yandex.ru*
[3]*Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-4309*

Received December 12, 2008
Revision received March 18, 2009

Abstract—Biochemical and morphological changes have been studied during transition of *Mycobacterium smegmatis* cells into their dormant ("nonculturable") state. A significant fraction of the population of irreversibly "nonculturable" (NC) cells has a thicker cell wall, condensed cytoplasm, and a decreased number of ribosomes. The lipid contents in the NC cells are lower than in the metabolically active cells, with a relatively decreased amount of phospholipid and neutral lipid. Free mycolic acids, which are abundant in metabolically active cells, were not found in the NC cells. The NC forms are also characterized by decreased respiratory activity on endogenous substrates; however, the respiratory chain enzymes retain their activities in the isolated membranes. Activities of the Krebs cycle and glyoxylate cycle enzymes are markedly decreased. Despite a significant decrease in metabolic activity, NC cells possess membrane potential that seems to provide for reversibility of the NC state of mycobacteria, i.e. their capability of reactivating.

DOI: 10.1134/S0006297910010098

Key words: dormancy, "nonculturable" (NC) cells, latent tuberculosis infection, *Mycobacterium smegmatis*, *M. tuberculosis*

The ability of the non-sporulating bacterium *Mycobacterium* to generate dormant ("nonculturable" (NC)) forms attracts special interest because a latent tubercular infection in living organisms seems to be associated with transition of the agent cells, the *M. tuberculosis* bacteria, into a state of dormancy. It is virtually impossible to reveal dormant cells of *M. tuberculosis* in the organism by routine biochemical and microbiological methods because in this state the bacteria cannot be cultured on standard laboratory media and their metabolic activity is decreased, which makes them invulnerable to antibiotics. The latent state is especially dangerous because the agent cells are capable of sudden and rapid transition into a metabolically active state that results in development of acute disease. Factors initiating activation of the latent tubercular infection need to be studied, although it is known that in many cases this can be caused by weakness of the human immune system [1].

The ability for transition into dormancy has been shown *in vitro* for many non-sporulating bacteria [2-4] including mycobacteria [5-9]. Therefore, the onset of dormancy characterized by absence of cell division and significantly decreased metabolic activity can be considered a phase of the life cycle of the bacteria.

The *in vitro* model of reversible transition of *M. smegmatis*, which is a rapidly growing close relative of *M. tuberculosis*, into the nonculturable state was developed in our laboratory using a special potassium-free growth medium [8]. It is important that in this case the population of NC cells appears under aerobic conditions, as discriminated from the traditional approach, which includes creation of anaerobic conditions to obtain dormant mycobacteria [5, 7]. But it should not be ruled out that dormancy of cells under anaerobic conditions is a result of adaptation of obligate aerobic mycobacteria to unfavorable environmental conditions associated with oxygen

Abbreviations: CL, cardiolipin; DCPIP, 2,6-dichlorophenolindophenol; H-dB, Hartman's–de Bont medium; NC cells, "nonculturable" cells; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

* To whom correspondence should be addressed.

shortage. In any case, up to now there is no strong evidence that just hypoxia initiates the mycobacterial *in vivo* transition into dormancy. Moreover, in tissues of infected mice *M. tuberculosis* cells are not exposed to lack of oxygen [10].

However, our knowledge of the "nonculturability" of *M. smegmatis* under aerobic conditions was essentially only the assurance that such a phenomenon should exist [8]. Biochemical and morphological changes in the cells during their transition into the nonculturable state were not sufficiently studied. Also, features of NC cells were not characterized. Little was also known about factors contributing to the transition of *M. smegmatis* into the NC state.

The purpose of the present work was to study biochemical and morphological changes associated with the transition of *M. smegmatis* cells into the state of "nonculturability".

MATERIALS AND METHODS

Cultivation of bacteria. Cells of the *M. smegmatis* strain mc^2155 were initially grown for 20-48 h at 37°C on an orbital shaker (240 rpm) in 50-ml flasks containing 20 ml of Nutrient Broth medium (Himedia, India) supplemented with Tween 80 to 0.05% by volume. Then 1 ml of the resulting culture was inoculated into a 750-ml flask containing 150 ml of modified Hartman's–de Bont (H-dB) medium of the following composition (per liter): 17.9 g $Na_2HPO_4 \cdot 12H_2O$, 1.2 g citric acid, 20 g $(NH_4)_2SO_4$, 30 ml of glycerol, 10 ml of 5% Tween 80, and 10 ml of microelement solution. The microelement solution contained (per liter): 1 g EDTA, 10 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.04 g $CoCl_2 \cdot 6H_2O$, 0.12 g $MnCl_2 \cdot 2H_2O$, 0.02 g $Na_2MoO_4 \cdot 2H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.02 g $CuSO_4 \cdot 5H_2O$, and 0.5 g $FeSO_4 \cdot 7H_2O$. To obtain NC cells, the modified H-dB medium was supplemented with a sterile solution of BSA (Cohn Analog) from Sigma (USA) to the final concentration of 0.5%.

Microscopy. Mycobacteria were observed with an Eclipse E4000 light microscope (Nikon, Japan) equipped with apparatuses for phase contrast and fluorescent microscopy and with a Camedia C-4040 digital camera (Olympus, Japan) for recording the results. The magnification was ×1000. To assess cell membrane damage, the cells were stained with fluorescent dye propidium iodide (10 μM), which stained cells with a disturbed permeability due to penetration inside and interaction with nucleic acids. The membrane potential was detected with fluorescent probe rhodamine 123 (0.2 μM) incubated with the cells for 15 min at 30°C. Respiratory activity of the bacterial cells was assessed using dye 5-cyano-2,3-ditolyl tetrazolium chloride, which gives a fluorescent product formazan upon reduction by the respiratory chain. This was obtained on incubation of the cells in the presence of 5 mM 5-cyano-2,3-ditolyl tetrazolium chloride for 30 min at 37°C [11].

For studies by electron microscopy, mycobacterial cells were precipitated by centrifugation and fixed as described in [12]. Then the preparation was additionally fixed with 1% solution of osmium tetroxide in 0.05 M cacodylate buffer (pH 7.0) and 1% aqueous solution of uranyl acetate, dehydrated in alcohols with increasing concentration (from 50 to 100%), and mounted into methacrylate LR White as described in [13]. Ultrathin sections (of 200-300 Å in thickness) were prepared using an LKB 3 ultratome and placed onto nickel grids with a formvar support. The ultrathin sections were stained with lead citrate by Reynolds' method [14] and analyzed with a JEM-100B electron microscope.

Determination of enzyme activities. Activity of isocitrate lyase was determined fluorometrically by changes in fluorescence of NADH on its oxidation to NAD^+ at the excitation and emission wavelengths of 340 and 455 nm, respectively. Glyoxylate produced from citrate under the influence of isocitrate lyase is reduced into glycolate with involvement of lactate dehydrogenase and concomitant oxidation of NADH. The reaction mixture (0.5 ml) contained 10 U of L-lactate dehydrogenase (Sigma), 0.15 mM NADH (DiaM, Russia), 1.5 mM DL-isocitrate (Sigma), and the cell extract (0.1-0.5 mg protein) in 50 mM buffer Mops-NaOH (Sigma). The enzyme activity was expressed in μmol NADH oxidized per minute. The specific activity of the enzyme was calculated per mg protein.

The isocitrate dehydrogenase activity was determined fluorometrically at the same excitation and emission wavelengths by changes in the fluorescence of $NADP^+$ on its reduction to NADPH, which accompanies the conversion of isocitrate into α-ketoglutarate. The reaction mixture (0.5 ml) contained 0.2 mM $NADP^+$, 2 mM DL-isocitrate, 5 mM $MgCl_2$, 1 mM EDTA, and the cell extract (0.1-0.5 mg protein) in 50 mM Mops-NaOH (Sigma). The enzyme activity was expressed in μmol NADPH reduced per minute. The specific activity of the enzyme was calculated per mg protein.

Activities of NADH and malate dehydrogenases were determined spectrophotometrically by changes in optical absorption at 600 nm of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) in the presence of menadione at 25°C with an SF-2000 spectrophotometer (Spektr, Russia). The reaction mixture (1 ml) contained 0.5 mM DCPIP, 0.145 mM menadione, and the membrane preparation (0.5-1.5 μg protein) in 0.1 M phosphate buffer (pH 7.0). The reaction was initiated by adding 5 μmol substrate. The activity that decreased optical absorption of the solution by 0.001 per minute was taken as the enzyme activity unit. The specific activity of the enzyme was calculated per μg protein.

Activities of endogenous DCPIP reductases in the cells were determined under the same conditions but

without adding the substrate. The activity that decreased optical absorption of the solution by 0.001 per minute was taken as the enzyme activity unit. The specific activity was calculated per mg of cells dried at 70°C to constant weight.

The NADH oxidase activity was determined by decrease in the fluorescence intensity of NADH on its oxidation to NAD^+ at the above-mentioned wavelengths of excitation and emission. The reaction mixture (0.5 ml) contained 0.1 mM NADH and the membrane preparation (1-5 μg protein) in 0.1 M phosphate buffer (pH 7.0). As the enzyme activity unit, the amount of the enzyme that oxidized 1 μmol NADH per minute was taken. The specific activity of the enzyme was calculated per μg protein.

Determination of ATP concentration in cells. Biomass of *M. smegmatis* cells (10^8 cells) was destroyed using a BeadBeater disintegrator (BioSpec Products, USA) with zirconium beads in dimethyl sulfoxide (Sigma). The beads and undestroyed cells were separated by centrifugation for 2 min at 4000*g*. ATP was determined in the cell extract by bioluminescent ATP-metry with a luciferin/luciferase system on a LUM-1 luminometer (Lumtek, Russia) using a commercial ATP reagent kit (Lumtek) according to the producer's recommendations.

Preparation of lipid and hydrophobic protein fractions. The biomass was dried and flooded with methanol–chloroform–water mixture (2 : 1 : 0.8 v/v) at the ratio of 1 liter per 15 g of dry cells. The resulting mixture was stirred for 2 h and then centrifuged at 12,000*g* for 15 min. The supernatant was separated, and the cells were re-extracted with chloroform–methanol mixture (2 : 1) and centrifuged under the same conditions. The supernatant was combined with the previous supernatant and evaporated to dryness on a rotary evaporator (Heidolph, Germany). The resulting film was supplemented with 40 ml of methanol–chloroform–water mixture (10 : 5 : 3 v/v), shaken until the film was fully dissolved, decanted into a separating funnel, and left for stratification. Then the upper protein-containing aqueous–methanol layer was separated from the lower lipid-containing chloroform layer.

Adsorption column chromatography of the lipid fraction was performed using Silica gel 60, 70-230 (Merck, Germany) on a 70 × 5 mm column. Neutral lipids were eluted with chloroform (25 ml), glycolipids and glycopeptidolipids were eluted with acetone (10 ml), and phospholipids were eluted with methanol (20 ml); 3-ml fractions were collected. The fractions were combined for each class of lipids (neutral lipids, glycolipids and glycopeptidolipids, phospholipids), dried, and weighed.

Thin-layer chromatography was performed on HPTLC Fergiplatten Kieselgel $60F_{254}$ plates (Merck) in the chloroform–methanol–water (65 : 25 : 4 v/v) system for phospholipids (system A) and in the hexane–diethyl ether (1 : 1 v/v) system for neutral lipids (system B). Commercial standards prepared from different natural sources were used: phosphatidylinositol (PI) from soybeans, phosphatidylethanolamine (PE) from *Escherichia coli* cells, cardiolipin (CL) from bovine heart, phosphatidylserine (PS) from bovine brain, and also synthetic mono-, di-, and triacylglycerides. Chromatograms were developed using Molybdenum Blue reagent for phospholipids, and using 10% sulfuric acid in ethanol with subsequent heating of the plate to 100°C for neutral lipids.

Preparative isolation of substances by TLC. Upon TLC for neutral lipids in system B, the spot of substance Y was cleaned from the plate together with Silica gel and flooded with chloroform–methanol mixture (1 : 1 v/v). The precipitate (silica gel) was separated using a water jet pump and Buchner funnel, and the resulting extract was dried.

Mass spectrometry. Mass spectra were obtained by electron scattering on a Finnigan LCQ Advantage tandem mass spectrometer (Thermo Electron Corp., USA) with an octapole ion trap. The range m/z = 100-2000 (200-1000 for the secondary fragmentation) was scanned in the negative ion-detecting regime (at specimen temperature 120-170°C, flow rate 50 μl/min, voltage 4.5 kV).

NMR spectroscopy. Substance Y was dissolved in $CDCl_3$, and NMR spectra were obtained using an Avance-3 (600 MHz) NMR spectrometer (Bruker, Germany).

RESULTS

Morphological changes of *M. smegmatis* cells in their NC state. Electron microscopy of ultrathin sections of metabolically active *M. smegmatis* cells and their NC cells revealed changes in size and shape of the NC cells, some of which appeared nearly spherical (Fig. 1). A significant part of the NC cell population was characterized by fragmentary and subtotal condensation of the cytoplasm and by a slightly thickened cell wall. In the majority of NC cells, the cytoplasm had rarefied regions. Sometimes DNA strands could be observed in these zones. Some cells had an increased number of vacuoles with electron-transparent contents and a sharply decreased number of ribosomes. The cells with a markedly rarefied cytoplasm seemed to be nonviable. Fluorescent microscopy with propidium iodide penetrating the cells with a damaged membrane confirmed the presence of such cells, but their number was no more than 30% (Fig. 2, a and b), whereas the population of NC cells mainly consisted of cells that retained viability.

Biochemical changes of *M. smegmatis* cells in their NC state. As shown by light microscopy, the resulting NC cells of *M. smegmatis* were unable to reduce 5-cyano-2,3-ditolyl tetrazolium chloride with generation of the fluorescent product formazan. The respiratory chain of actively growing bacteria could easily reduce this dye (not shown). The absence of 5-cyano-2,3-ditolyl tetrazolium

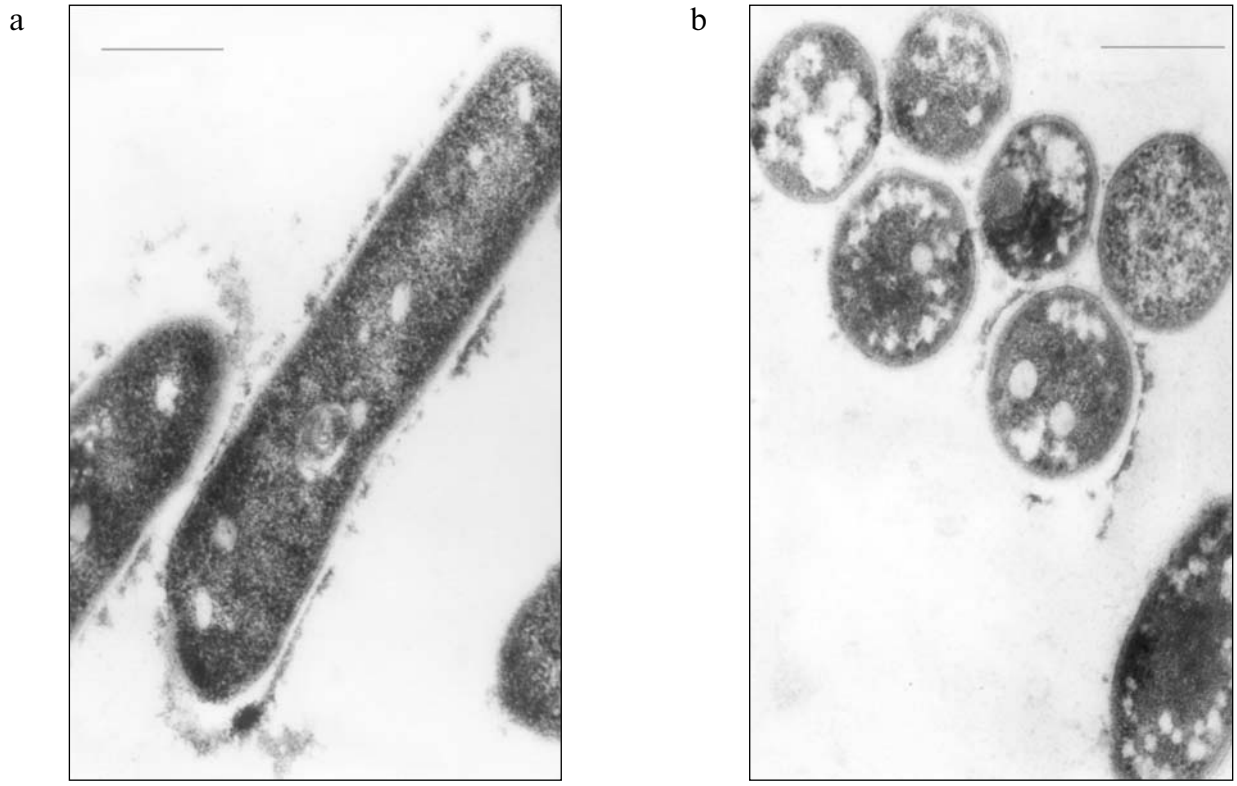

Fig. 1. Electron microscopy of ultrathin sections of *M. smegmatis* cells. a) Metabolically active cells; b) NC cells. The scale label corresponds to 0.5 μm.

chloride reduction indicated a decreased endogenous respiratory activity of the *M. smegmatis* NC cells, i.e. a decrease in their total metabolic activity.

In fact, in addition to the decrease in the respiratory activity, the *M. smegmatis* NC cells also manifested a pronounced decrease in the activity of DCPIP reductase on endogenous substrates. By the moment of complete loss of culturability on solid media (incubation for 72 h on potassium-free medium), the DCPIP reductase activity was decreased to zero (Fig. 3). Such a decrease in the DCPIP reductase and respiratory activities suggested an inhibition of the respiratory chain functioning, which, in turn, could be caused by inhibition of the respiratory chain enzymes in the cells in the NC state. Therefore, activities of some membrane enzymes of *M. smegmatis* were measured in preparations of isolated membranes. The activities of the membrane NADH dehydrogenase, malate dehydrogenase, and NADH oxidase in the NC cells were virtually the same as their activities in the metabolically active cells (table).

Thus, the low endogenous respiratory activity and a pronounced decrease in the endogenous DCPIP reduc-

Specific activities of membrane enzymes (calculated per μg protein of the membrane fraction) and contents of different lipid classes in the lipid fractions from the metabolically active and NC cells

Cells	NADH dehydrogenase, U/μg	Malate dehydrogenase, U/μg	NADH oxidase, U/μg	Fraction, %		
				phospholipids	glycolipids and glycopeptidolipids	neutral lipids
Metabolically active	61 ± 2*	8.9 ± 0.4	1.4 ± 0.1	36.9 ± 0.2*	15.1 ± 0.2*	48.0 ± 0.3*
NC	50 ± 2*	7.9 ± 0.4	1.5 ± 0.1	31.6 ± 0.3*	25.1 ± 0.1*	43.3 ± 0.2*

* Differences are significant at $P > 0.01$.

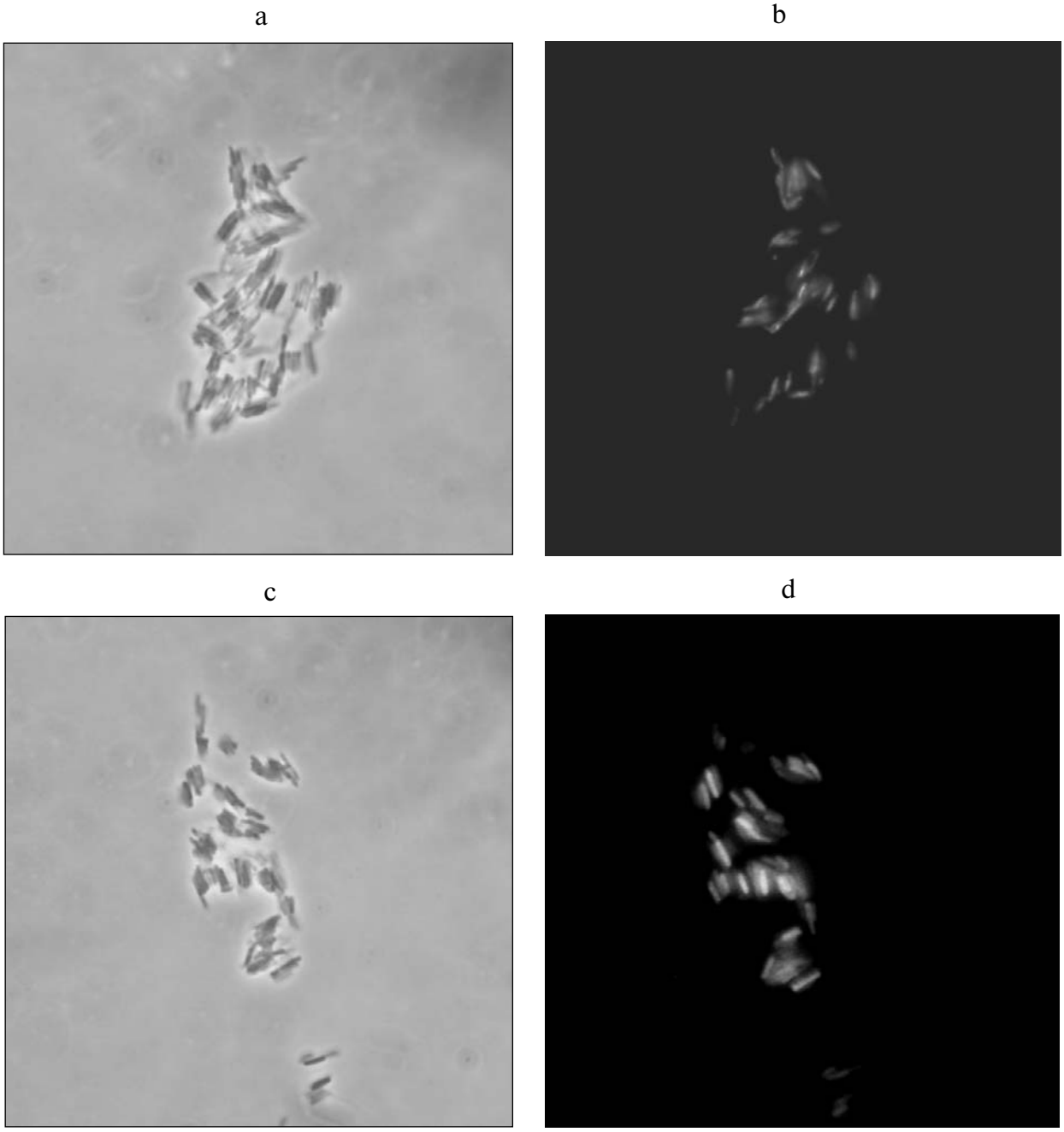

Fig. 2. Light microscopy of *M. smegmatis* NC cells. a, c) Phase-contrast microscopy; b) fluorescence microscopy of the preparation (a) stained with propidium iodide; d) fluorescence microscopy of the preparation (c) stained with rhodamine 123. Magnification ×1000.

tase activity in the *M. smegmatis* NC cells were not caused by inhibition of the respiratory chain enzymes. In this case, the low level of the cell respiration could be caused by shortage of intracellular substrates, probably as a result of deceleration of the main metabolic pathways and a decrease in the total metabolic activity. To test this hypothesis, the activity of the Krebs cycle enzyme isocitrate dehydrogenase was determined. The isocitrate dehydrogenase activity in the NC cells was found to be no more than 30% of its activity at the point corresponding to the beginning of the stationary phase. A slight decrease in the isocitrate dehydrogenase activity was also recorded for metabolically active cells in the stationary phase (Fig. 4, a and b). The activity of isocitrate lyase, an enzyme of the glyoxylate shunt (a metabolic pathway alternative to the Krebs cycle), sharply decreased on transition into the stationary phase in both metabolically active cells and the cells with the growth associated with generation of NC

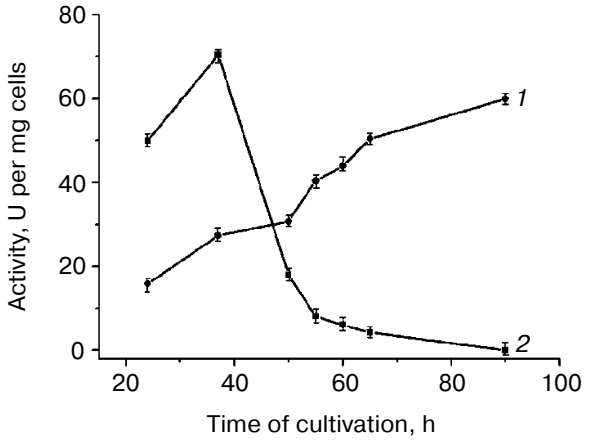

Fig. 3. DCPIP reductase activity in *M. smegmatis* cells. *1*) Metabolically active culture; *2*) the culture producing NC cells.

forms (Fig. 4, c and d). The ATP content in the NC cells was also very low (in the cell extract prepared from 10^8 cells it was 2.5 nM, whereas in the same quantity of metabolically active cells it was 508 nM).

It was also found that 50-80% of cells in the nonculturability phase were able to accumulate the fluorescent probe rhodamine 123 (Fig. 2, c and d). The accumulation was abolished under the influence of the uncoupler of oxidative phosphorylation carbonyl cyanide *m*-chlorophenylhydrazone, which indicated the retention of a certain level of membrane potential by the cells. Note that such an energy-dependent accumulation of rhodamine 123 was not recorded in the metabolically active *M. smegmatis* cells.

Mycobacterium smegmatis NC cells were earlier shown to have a higher value of floating density in a sucrose gradient [15], which suggested changes in the protein/lipids ratio. In fact, quantitative analysis revealed that the total amount of lipid in the NC cells was approximately twofold lower than in the metabolically active cells (15 and 7.2%, respectively, calculated per dry biomass). However, contents of hydrophobic proteins increased from 0.26% of dry biomass for metabolically active cells to 1.1% for the NC cells.

Qualitative analysis of the lipid fractions revealed the prevalence of neutral lipids in both the metabolically active and NC cells (table); the glycolipid and glycopep-

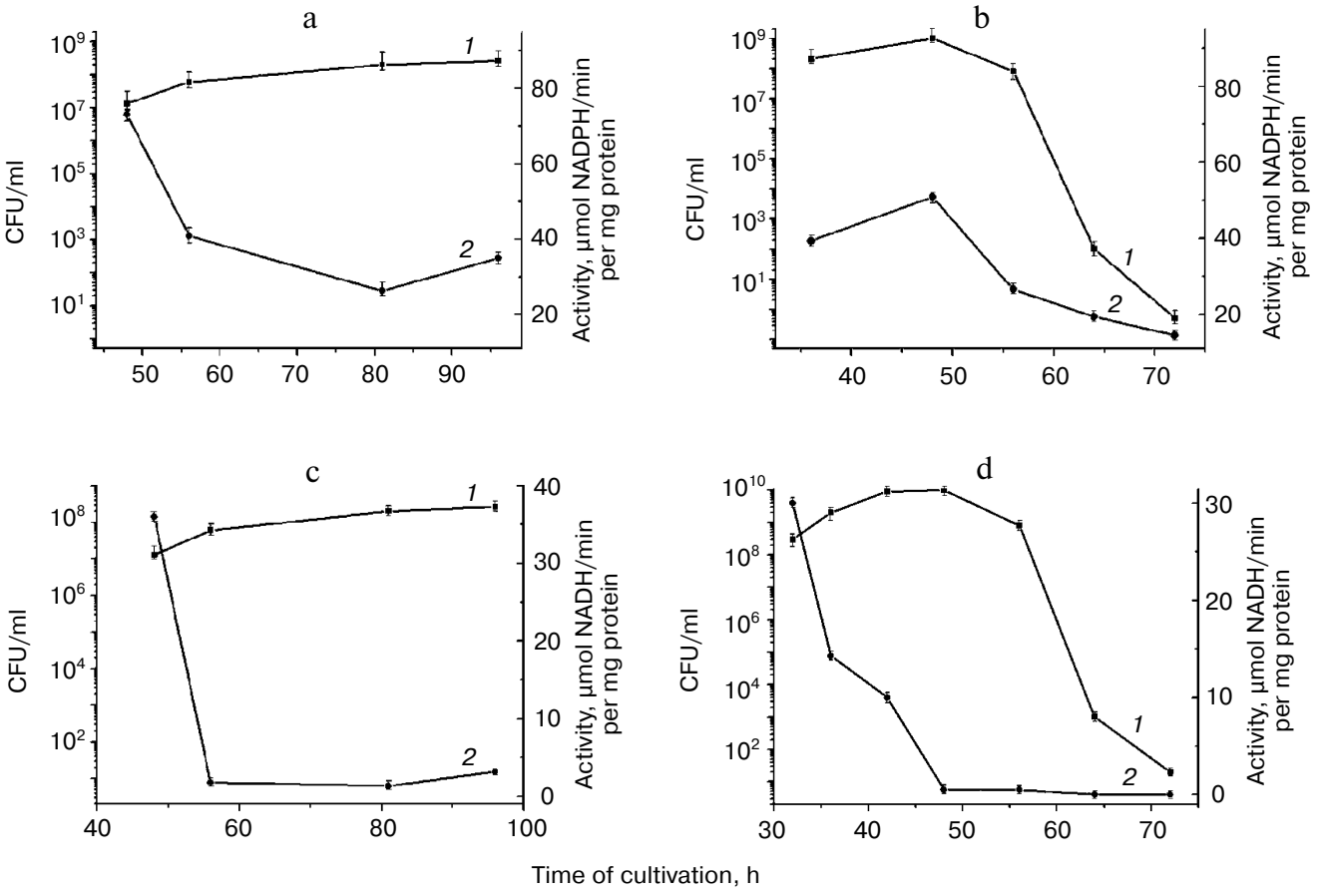

Fig. 4. Activities of isocitrate dehydrogenase (a, b) and isocitrate lyase (c, d) in *M. smegmatis* cells. a, c) Metabolically active cells; b, d) cells during transition into the NC state. *1*) The value of CFU/ml; *2*) enzymatic activity.

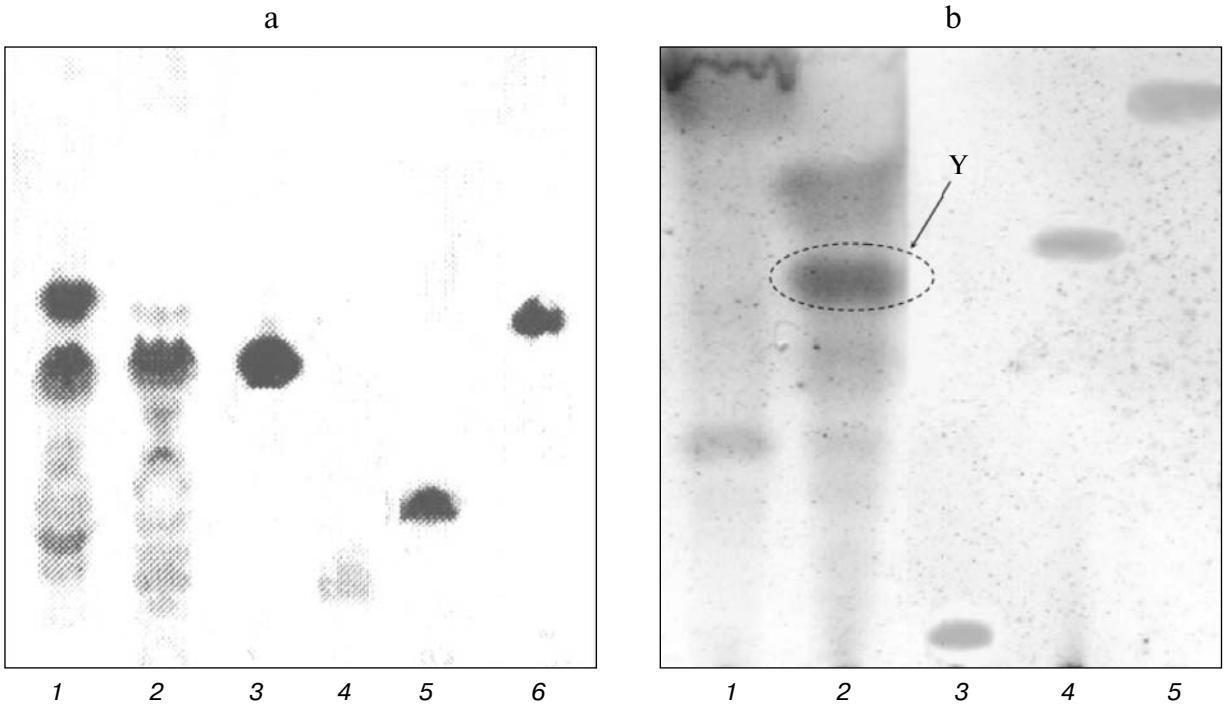

Fig. 5. a) TLC of phospholipids in system A with subsequent development with Molybdenum Blue reagent. *1*) Phospholipids of metabolically active cells; *2*) phospholipids of NC cells; *3*) PE standard; *4*) PS standard; *5*) PI standard; *6*) CL standard. b) TLC of neutral lipid in system B followed by treatment with 10% sulfuric acid in ethanol and heating the plate at 100°C. *1*) Neutral lipids of NC cells; *2*) neutral lipids of metabolically active cells; *3-5*) standards of mono-, di-, and triacylglycerides, respectively.

tidolipid fraction was minimal, but their contents were markedly higher in the NC cells. Contents of neutral lipids and phospholipids were also slightly decreased in the NC cells, similarly to the earlier observation for dormant *M. luteus* cells [16].

Some differences between active and dormant cells were found by TLC in the qualitative composition of phospholipids, in particular, several compounds were present in the region of phosphatidylinositol mannosides (R_f = 0.10-0.15) in the dormant cells and absent in the active cells (Fig. 5a). By contrast to dormant *M. luteus* cells, the cardiolipin contents in the NC cells were insignificant as compared to the metabolically active cells [16]. In the class of glycolipids and glycopeptidolipids ~20 compounds were found, without differences in them between the active and dormant cells (not shown). The metabolically active and NC cells were significantly different in the class of neutral lipids. In the metabolically active cells, two unknown compounds were present that were not detected in the NC cells (Fig. 5b). One of these compounds (compound Y) was isolated by preparative TLC and analyzed by mass spectrometry. According to analysis of the mass spectrum and literature data [17], compound Y seemed to be a mixture of free mycolic acids C_{86}-C_{92} with 24 carbon atoms in the α-carbon chain (Fig. 6). Thus, in the mass spectrum there are mass ions with values 1339 (C_{92}), 1311 (C_{90}), 1283 (C_{88}), and 1253 (C_{86}) corresponding to the above-mentioned mycolic acids. Peaks were also detected which corresponded to fragments produced on detachment of a hydroxyl group from fragment A: 821 (C_{92}), 849 (C_{90}), 877 (C_{88}), and 904 (C_{86}). Data of NMR confirmed the presence of mycolic acids in the preparation. The presence of a free carboxyl group in compound Y was also confirmed on chromatographic plates using bromocresol green (not shown).

DISCUSSION

Biochemical and morphological characteristics of NC cells of *M. smegmatis*, a rapidly growing nonpathogenic relative of *M. tuberculosis*, are presented for the first time. In the NC state, the cells change their size and shape and display a tendency for thickening of the cell wall, which is specific for dormant bacteria [16, 18]. A significant part of the NC cell population (no less than 70%) represented by cells with condensed cytoplasm are suggested to be truly nonculturable but retaining viability and capability of reactivating. The structure of the ribonucleoprotein complex in these changed cells seems to indicate a decrease in protein synthesis that can result in a reversible decrease in metabolic activity of the cells

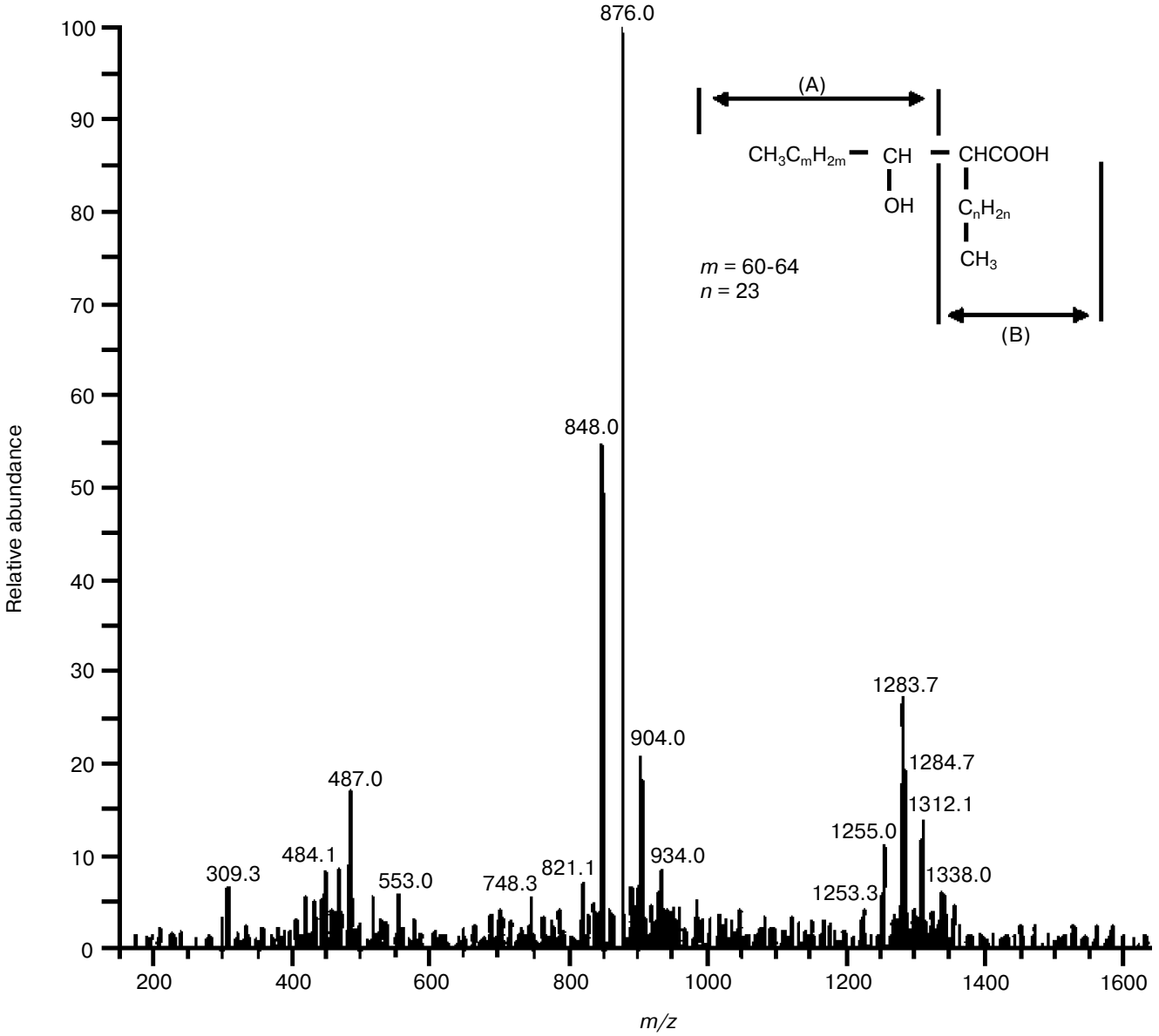

Fig. 6. Mass spectrometry of compound Y by electron scattering, m/z = 100-2000 (200-1000 for secondary fragmentation) in negative ion detection regimen. The supposed structure and fragmentation scheme of compound Y on ionization are shown.

and loss of ability for division and growth, i.e. endurance under unfavorable environmental conditions.

Thus, the NC cells are found to have a decrease in the intensity of metabolism, which is first of all manifested by an extremely low endogenous respiratory activity. A decrease in the respiratory activity was earlier observed in dormant *M. luteus* cells [2, 19]. Note that unlike the metabolically active cells the NC cells are capable of accumulating rhodamine 123 and, thus, possess a certain membrane potential. The presence of membrane potential in the NC cells seems to indicate the retention of a certain metabolic activity in the nonculturability state that can be important for reversibility of this state. The inability of the *M. smegmatis* metabolically active cells to transport rhodamine 123 across the cytoplasmic membrane seems to be associated with its impermeability for this probe because of hydrophobicity of the cell wall. The cell wall in the NC cells, despite its thickening, allows rhodamine 123 to penetrate across it. In fact, in the present work the cells in the NC state displayed a change in the protein/lipids ratio towards a relative decrease in the lipid content, similarly to that reported for dormant *M. luteus* cells [16]. In particular, in the NC state there is a noticeable decrease in the contents of free mycolic acids, which are an important component of the cell wall and due to their hydrophobicity form a layer with low perme-

ability for external molecules. A decreased respiratory activity of the NC cells and the presence of membrane potential are formally contradicting each other because the membrane potential in aerobic bacteria is generated by the respiratory chain. However, even an extremely low activity of the respiratory chain is sufficient to support the membrane potential in bacteria [20].

It is interesting that in the case of *M. luteus* inhibition of the respiratory activity of dormant cells was associated with inhibition of the respiratory chain enzymes: activity of the membrane NADH oxidase rapidly fell to non-detectable values on transition of the *M. luteus* cells into dormancy, and activities of the membrane NADH- and malate dehydrogenases also decreased, although not so dramatically [16]. However, in the present work no decrease in the activities of NADH- and malate dehydrogenases and also of NADH oxidase from isolated membranes was recorded on transition of the *M. smegmatis* into the NC state. Consequently, the low endogenous activity was not caused by inhibition of the respiratory chain enzymes. The decrease in the metabolic activity of the *M. smegmatis* NC cells relative to that of the metabolically active cells seemed to be caused by the decrease in activities of at least a number of intracellular enzymes. As to the rather high enzymatic activity of membrane proteins in the NC state, their immobilization within the membranes is supposed to be an additional stabilizing factor preventing their rapid inactivation and providing for the possibility of cell reactivation from the NC state.

Thus, the present study has revealed pronounced morphological changes in the cells in the state of "non-culturability". Such cells are characterized by significantly decreased metabolic activity, but they retain a membrane potential that seems to represent their readiness for reactivation and be a basis for reversibility of the NC state in *M. smegmatis*.

This work was supported by the Russian Foundation for Basic Research (project No. 06-04-49201), FP6 project "New Medicines for Tuberculosis" (contract #LSHP-CT-2005-018923), and by the program on Molecular and Cell Biology of the Presidium of the Russian Academy of Sciences.

REFERENCES

1. Mehta, J., and Dutt, A. (1995) *Infect. Med.*, **12**, 40-46.
2. Kaprelyants, A. S., and Kell, D. B. (1993) *Appl. Environ. Microbiol.*, **59**, 3187-3196.
3. Mulyukin, A. L., Sorokin, V. V., Loiko, N. G., Suzina, N. E., Duda, V. I., Vorob'eva, E. A., and El-Registan, G. I. (1996) *Mikrobiologiya*, **65**, 782-789.
4. Demkina, E. V., Soina, V. S., and El-Registan, G. I. (2000) *Mikrobiologiya*, **69**, 383-388.
5. Wayne, L. G. (1994) *Eur. J. Clin. Microbiol. Infect. Dis.*, **13**, 908-914.
6. Wayne, L. G., and Hayes, L. G. (1996) *Infect. Immun.*, **64**, 2062-2069.
7. Shleeva, M. O., Bagramyan, K., Telkov, M. V., Mukamolova, G. V., Young, M., Kell, D. B., and Kaprelyants, A. S. (2002) *Microbiology*, **148**, 1581-1591.
8. Shleeva, M. O., Mukamolova, G. V., Young, M., Williams, H. D., and Kaprelyants, A. S. (2004) *Microbiology*, **150**, 1687-1697.
9. Mukamolova, G. V., Salina, E. G., and Kaprelyants, A. C. (2008) in *National Institute of Allergy and Infectious Diseases, NIH* (Georgiev, V., ed.) Vol. 1, Humana Press, pp. 83-90.
10. Tsai, M. G., Chakravaty, S., Zhu, G., Xu, J., Tanaka, K., Koch, C., Tufariello, J., Flynn, J., and Chan, J. (2006) *Cell Microbiol.*, **8**, 218-232.
11. Kaprelyants, A. S., and Kell, D. B. (1993) *J. Microbiol. Meth.*, **17**, 115-122.
12. Ito, S., and Karnovsky, M. (1969) *J. Cell Biol.*, **39**, 168-169.
13. Newman, G. R., Jasani, B., and Williams, E. D. (1983) *Histochem. J.*, **15**, 543-555.
14. Reynolds, E. S. (1963) *J. Cell Biol.*, **17**, 208-212.
15. Salina, E. G., Shleeva, M. O., Vostroknutova, G. N., and Kaprelyants, A. S. (2005) *Clin. Microbiol. Infect.*, **11**, 645.
16. Mukamolova, G. V., Yanopolskaya, N. D., Votyakova, T. V., Popov, V. I., Kaprelyants, A. S., and Kell, D. B. (1995) *Arch. Microbiol.*, **163**, 373-379.
17. Kaneda, K., Naito, S., Imaizumi, S., Yano, I., Mizuno, S., Tomiyasu, I., Baba, T., Kusunose, E., and Kusunose, M. (1986) *J. Clin. Microbiol.*, **24**, 1060-1070.
18. Suzina, N. E., Mulyukin, A. L., Kozlova, A. N., Shorokhova, A. P., Dmitriev, V. V., Barinova, E. S., Mokhova, O. N., El-Registan, G. I., and Duda, V. I. (2004) *Mikrobiologiya*, **73**, 516-529.
19. Mukamolova, G. V., Kormer, S. S., Yanopol'skaya, N. D., and Kaprelyants, A. S. (1995) *Mikrobiologiya*, **63**, 341-346.
20. Artsatbanov, V. Yu., Tikhonova, G. V., and Ostrovskii, D. N. (1983) *Biokhimiya*, **48**, 1568-1579.