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Substrate-Specific Reduction of Tetrazolium Salts by Isolated Mitochondria, Tissues, and Leukocytes

N. I. Fedotcheva[1]*, E. G. Litvinova[1], M. V. Zakharchenko[1], N. V. Khunderyakova[1], R. S. Fadeev[1], V. V. Teplova[1], T. A. Fedotcheva[2], N. V. Beloborodova[3], and M. N. Kondrashova[1]

[1]*Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; E-mail: nfedotcheva@mail.ru*
[2]*Pirogov Russian National Research Medical University, 117997 Moscow, Russia*
[3]*Negovskii Scientific Research Institute of General Reanimatology, 107031 Moscow, Russia*

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Abstract—Tetrazolium salts are commonly used in cytochemical and biochemical studies as indicators of metabolic activity of cells. Formazans, formed by reduction of tetrazolium salts, behave as pseudo-solutions during initial incubation, which allows monitoring their optical density throughout incubation. The criteria and conditions for measuring oxidative activity of mitochondria and dehydrogenase activity in reduction of nitroblue tetrazolium (NBT) and methyl thiazolyl tetrazolium (MTT) in suspensions of isolated mitochondria, tissue homogenates, and leukocytes were investigated in this work. We found that the reduction of these two acceptors depended on the oxidized substrate – NBT was reduced more readily during succinate oxidation, while MTT – during oxidation of NAD-dependent substrates. Reduction of both acceptors was more sensitive to dehydrogenase inhibitors that to respiratory chain inhibitors. The reduction of NBT in isolated mitochondria, in leukocytes in the presence of digitonin, and in liver and kidney homogenates was completely blocked by succinate dehydrogenase inhibitors – malonate and TTFA. Based on these criteria, activation of succinate oxidation was revealed from the increase in malonate-sensitive fraction of the reduced NBT under physiological stress. The effect of progesterone and its synthetic analogs on oxidation of NAD-dependent substrates by mitochondria was investigated using MTT. Both acceptors are also reduced by superoxide anion; the impact of this reaction is negligible or completely absent under physiological conditions, but can become detectable on generation of superoxide induced by inhibitors of individual enzyme complexes or in the case of mitochondrial dysfunction. The results indicate that the recording of optical density of reduced NBT and MTT is a highly sensitive method for evaluation of metabolic activity of mitochondria applicable for different incubation conditions, it offers certain advantages in comparison with other methods (simultaneous incubation of a large set of probes in spectral cuvettes or plates); moreover, it allows determination of activity of separate redox-dependent enzymes when selective inhibitors are available.

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Tetrazolium salts are used in cell biology for measuring metabolic activity, generation of superoxide, activity of oxidizing enzymes, quantitative assessment of cell proliferation, and survival of cells under the action of various factors [1-4]. All tetrazolium salts form colored products – formazans – during reduction, and application in histochemical and biochemical studies depends on the degree of their hydrophobicity. Another important difference between different tetrazolium salts involves the fact that intermediate electron carriers such as phenazine methosulfate and its derivatives are required for their reduction [1, 2].

* To whom correspondence should be addressed.

Nitroblue tetrazolium (NBT) and methyl thiazolyl tetrazolium (MTT) are reduced upon direct interaction with the reduced redox sites, forming diformazan and monoformazan in the process, respectively:

$$\text{ditetrazolium} + 2e^- \rightarrow \text{diformazan}, \quad (1)$$

$$\text{monotetrazolium} + e^- \rightarrow \text{monoformazan}. \quad (2)$$

Reaction (1) is characteristic for NBT reduction, which is accompanied by formation of insoluble granules of nitroblue tetrazolium formazan. NBT is most often used as a histochemical and cytochemical stain for determination of activity and localization of oxidases [1, 3].

Dehydrogenase activities were determined using NBT. It is generally recognized that NBT accepts electrons from flavins in the respiratory chain in the coenzyme Q site [5, 6]. In addition to determination of succinate dehydrogenase (SDH) activity, NBT is used for estimation of activities of NAD^+-dependent dehydrogenases from mitochondria, as well as of lactate dehydrogenase and other cytosolic enzymes [7]. NBT is also sensitive to superoxide formed in either biological or chemical systems. In clinical studies, NBT is effectively used for determination of activation of neutrophils and phagocytes in different pathologies such as immune disorders [8-11]. Superoxide production from data on NBT reduction was quantitatively evaluated in neurons under oxidative stress, in fibroblasts with electron transport chain deficiencies, and in other cells [12, 13].

Unlike NBT, reduction of MTT is accompanied by production of monoformazan (reaction (2)). This reaction is an indicator of metabolic activity, proliferation, and cell viability. Although the succinate-dependent reduction of MTT in mitochondria has been demonstrated, it is generally recognized that this acceptor is reduced predominantly with participation of NAD(P)H as a cofactor of mitochondrial dehydrogenases, cytochrome P-450, and other NAD(P)H-dependent enzymes [1, 14, 15].

It must be mentioned that data on substrate specificity of tetrazolium reduction reactions and their sensitivity to electron transport chain inhibitors are few and contradictory. It was shown that the succinate-dependent reduction of MTT was inhibited by antimycin and thenoyltrifluoroacetone (TTFA) and insensitive to azide, while the NAD(P)H-dependent reduction of the acceptor was not inhibited by the electron transport chain inhibitors [1, 15].

It is known that formazans formed on reduction of tetrazolium salts behave as pseudo-solutions during short-term incubation, which allows monitoring their optical density during incubation. This property was used in our study to elucidate substrate specificity of NBT and MTT reduction in isolated mitochondria, tissue homogenates, and leukocytes. The data show that monitoring of the reduction of these acceptors is a sensitive method for determination of metabolic activity of mitochondria and of separate redox-dependent enzymes if selective inhibitors are available. One of the significant advantages of NBT and MTT in comparison with other acceptors (dichlorophenolindophenol (DCPIP), N-tetramethyl-*p*-phenylenediamine (TMPD)) or with the polarographic method for evaluation of mitochondrial oxidative activity is the possibility of simultaneous analysis of many samples with various additives and different incubation times, which is of importance for investigation of the effect of therapeutic drugs and natural or synthetic physiologically active compounds on oxidation processes.

MATERIALS ANS METHODS

Isolation of mitochondria and preparation of homogenates from rat liver. Experiments were conducted with male rats of the Wistar line. Liver mitochondria were isolated using a standard technique of differential centrifugation in medium containing 300 mM sucrose, 1 mM EGTA, and 10 mM Tris-HCl buffer (pH 7.4). The mitochondria preparation was washed with isolation medium without EGTA, resuspended in the same medium, and stored on ice.

Concentrated liver homogenates were produced using a fast procedure involving cooling of tissue, crushing through a press, and homogenization. An aliquot of tissue was cooled in medium containing 125 mM KCl, 15 mM HEPES, pH 7.4, washed three times with the same medium, and crushed through a press. The resulting mass was placed in a glass homogenizer, isolation medium was added at ratio of 1 ml per 1 g of tissue, and the mixture was homogenized to the homogenous state. The homogenate was filtered through a layer of kapron and stored on ice. Physiological stress was induced by placing an animal in a tight perforated box for a short period (30 min). Next, the animal was placed in a chamber saturated with CO_2 for 20 sec followed by decapitation and preparation of liver homogenates using the fast procedure described above.

Preparation of mononuclear leukocyte fraction from human blood. The leukocyte fraction of human blood (70% lymphocytes, 25% monocytes, 4% neutrophils) was obtained from healthy donors in the Scientific Clinical Center of Children's Hematology, Oncology, and Immunology. Mononuclear leukocytes were isolated by centrifugation at 900*g* in a Histopaque-1077 density gradient (Sigma-Aldrich, USA) for 25 min, room temperature, followed by washing in Hanks solution. Live cell content was 95 ± 4%, and cell concentration in the suspension was $1 \cdot 10^7$/ml.

Determination of dehydrogenase activity from reduction of NBT and MTT. Incubation medium (2 ml) containing 125 mM KCl, 20 mM HEPES, pH 7.4, 125 μM NBT, and the respective oxidation substrate was mixed with mitochondria (0.5 mg protein per ml) or homogenate (0.5 mg protein per ml) and incubated for 5, 10, and 20 min. The examined samples were placed simultaneously in a series of spectrophotometric cuvettes. The reaction of acceptor reduction was initiated by addition of mitochondria or homogenate. NBT reduction was accompanied by emerging of blue color. Mitochondria were lysed by Triton X-100 (20 μl of 10% solution) after the specified incubation time and optical density was recorded at 560 nm with an Ocean Optics USB4000 spectrophotometer.

Incubation of leukocytes with NBT and respective additives was conducted in a 96-well plate. Incubation medium (200 μl, containing 125 mM KCl, 15 mM

HEPES, 1.5 mM phosphate, pH 7.25) was supplemented with 250 μM NBT and 2-5 million cells. In addition to the specified components, digitonin was added to the sample at concentration of 50 μM to facilitate penetration of substrates and other compounds through cell membrane to determine activity of mitochondrial enzymes. The development of color occurred very slowly in the samples, which is why incubation was conducted for a longer time – from 3 to 5 h. Optical density of the reduced acceptor was recorded at 550 nm with an Infinite F200 plate spectrophotometer.

RESULTS AND DISCUSSION

Investigation of NBT substrate specificity. The data on optical density of reduced NBT (diformazan) in isolated mitochondria during oxidation of different substrates during incubation for 5-20 min are presented in Fig. 1. The highest rate of color development was observed in the first 5 min, and the rate decreased significantly with increase in incubation time. The NBT reduction was much faster in samples with succinate compared to samples containing NAD-dependent substrates – α-ketoglutarate (KGL) and glutamate (Fig. 1a). Optical density of the reduced NBT formed during oxidation of KGL and glutamate was 60 and 70%, respectively, of that observed during succinate oxidation for all incubation times. Endogenous substrates contribute significantly to the optical density of reduced NBT comprising a major part of the optical density values recorded in the presence of added substrates. Considering that their contribution decreased twofold in the presence of malonate, an inhibitor of SDH, it was suggested than a significant part of endogenous substrates was endogenous succinate produced in mitochondria. NBT reduction via oxidation of added succinate was completely inhibited by malonate (Fig. 1b). TTFA – an inhibitor of SDH affecting the ubiquinone-binding site of SDH [16] – was also effective, while the inhibitors of respiratory chain (antimycin A and cyanide) decrease the rate of NBT reduction by 65-70% (Fig. 1c). Rotenone did not affect the NBT reduction during succinate oxidation. NBT reduction was inhibited during KGL oxidation by rotenone and to a lesser degree by antimycin and cyanide. Partial inhibition of NBT reduction during KGL oxidation was caused by malonate (Fig. 1d). The results indicate that NBT in isolated mitochondria serves predominantly as an acceptor of electrons from SDH and site I of the respiratory chain. Furthermore, these data show that the activity of SDH in a mitochondrial suspension can be efficiently evaluated using malonate as an SDH inhibitor.

Measuring of SDH activity from NBT reduction in tissue homogenates. Taking these data into consideration, in the next experiments we measured the SDH activity in tissue homogenates and in human leukocytes. Use of these test objects avoiding the procedure of mitochondria isolation is of great practical importance for determination of metabolic activity in various pathologies and when an organism is exposed to different external factors. In these experiments, the animals were subjected to physiological stress followed by fast preparation of liver and kidney homogenates. Data on optical density of reduced NBT during succinate oxidation in these homogenates in control experiments and in experiments with the stressed animals are presented in Fig. 2. Even though the contribution of endogenous substrates to the reduction of NBT was higher in homogenates than in isolated mitochondria, it was greatly decreased (by more than 50%) with malonate, indicating the availability of endogenous succinate. The effect of stress on the SDH activity while measuring the reduced NBT after 5-20 min of incubation is presented in Fig. 2, c and d. The specificity of these measurements was controlled using parallel samples containing malonate in each time interval of incubation of the homogenate with succinate and NBT. The graphs present the malonate-sensitive fraction of the reduced NBT calculated from the difference between the NBT reduced in the presence of succinate and the NBT reduced in the presence of succinate and malonate. As can be seen from the graphs, stress activates succinate oxidation in both liver and in kidney, which is most pronounced at 10-min incubation. The degree of activation in liver is 28% and in kidney – 24%. These data are consistent with the data on 30% activation of liver mitochondria functions (respiration and maintenance of membrane potential) under stress [6].

Measurement of SDH activity from the reduction of NBT in human leukocytes. Use of NBT as an acceptor of electrons from SDH and malonate as enzyme inhibitor also allows evaluating the activity of succinate oxidation in leukocyte suspension. Evaluation of functions of mitochondria in leukocytes in norm and pathology is an important issue, because mitochondrial dysfunction in blood cells, which is characteristic of many chronic diseases, affects the phenotype of cells and efficacy of immune response [17, 18]. In our experiments, we investigated the fraction of mononuclear leukocytes from healthy donors. The changes in NBT optical density during incubation of the washed leukocytes in the control without additives, in the presence of digitonin facilitating permeability of cell membranes, and upon addition of succinate and inhibitor to the samples with digitonin are presented in Fig. 3. In contrast to mitochondria and tissue homogenates whose incubation with NBT was accompanied by rapid development of color, prolonged incubation for several hours was required for NBT reduction in leukocytes. As can be seen in the graph, the NBT reduction observed during incubation of intact leukocytes with NBT decreased sharply upon incubation with digitonin, and increased in the presence of digitonin and succinate. The observed inhibition by malonate indicates specific contribution of succinate oxidation to the NBT reduction. This result demonstrates the

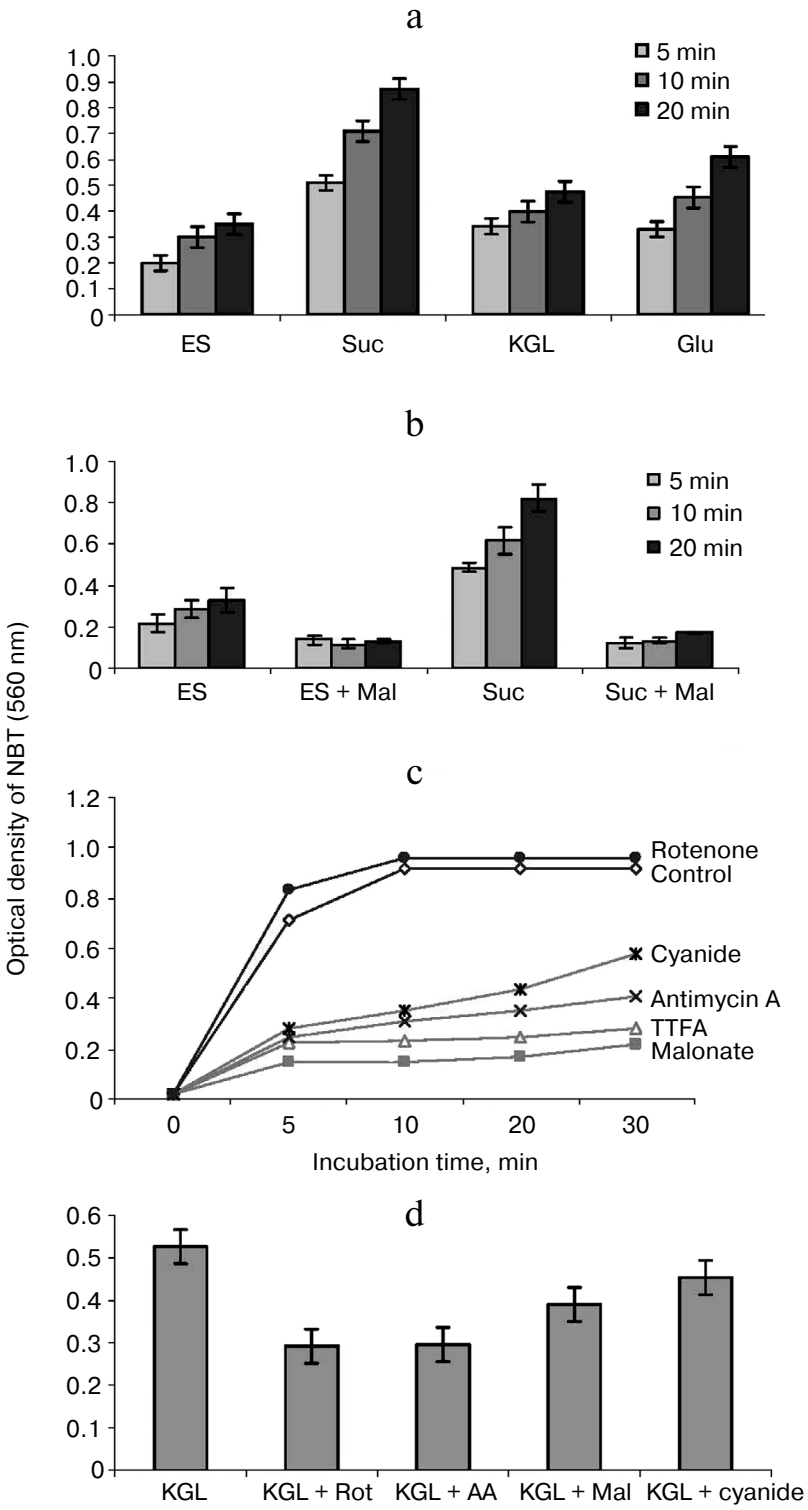

Fig. 1. Reduction of NBT during different durations of incubation of mitochondria with substrates and acceptor. Effect of inhibitors of dehydrogenases and respiratory chain. a) Optical density of reduced NBT after 5, 10, and 20 min incubation of isolated mitochondria in the presence of succinate (Suc), α-ketoglutarate (KGL), glutamate (Glu), and in the absence exogenous substrates (endogenous substrates, ES); b) effect of malonate (Mal) on NBT reduction during succinate oxidation; c) effect of inhibitors of succinate dehydrogenase and respiratory chain on NBT reduction during oxidation of succinate; d) effect of inhibitors on reduction of NBT during oxidation of α-ketoglutarate. Incubation medium: 125 mM KCl, 15 mM HEPES, 1.5 mM phosphate, pH 7.3. Additives: substrates – 5 mM each, NBT 100 μM, malonate 5 mM, TTFA 20 μM, cyanide 2 mM, antimycin A (AA) and rotenone (Rot) – 2 μM each. Mean values of ten independent experiments adjusted to 1 mg of protein and with subtracted background of uncolored medium are presented.

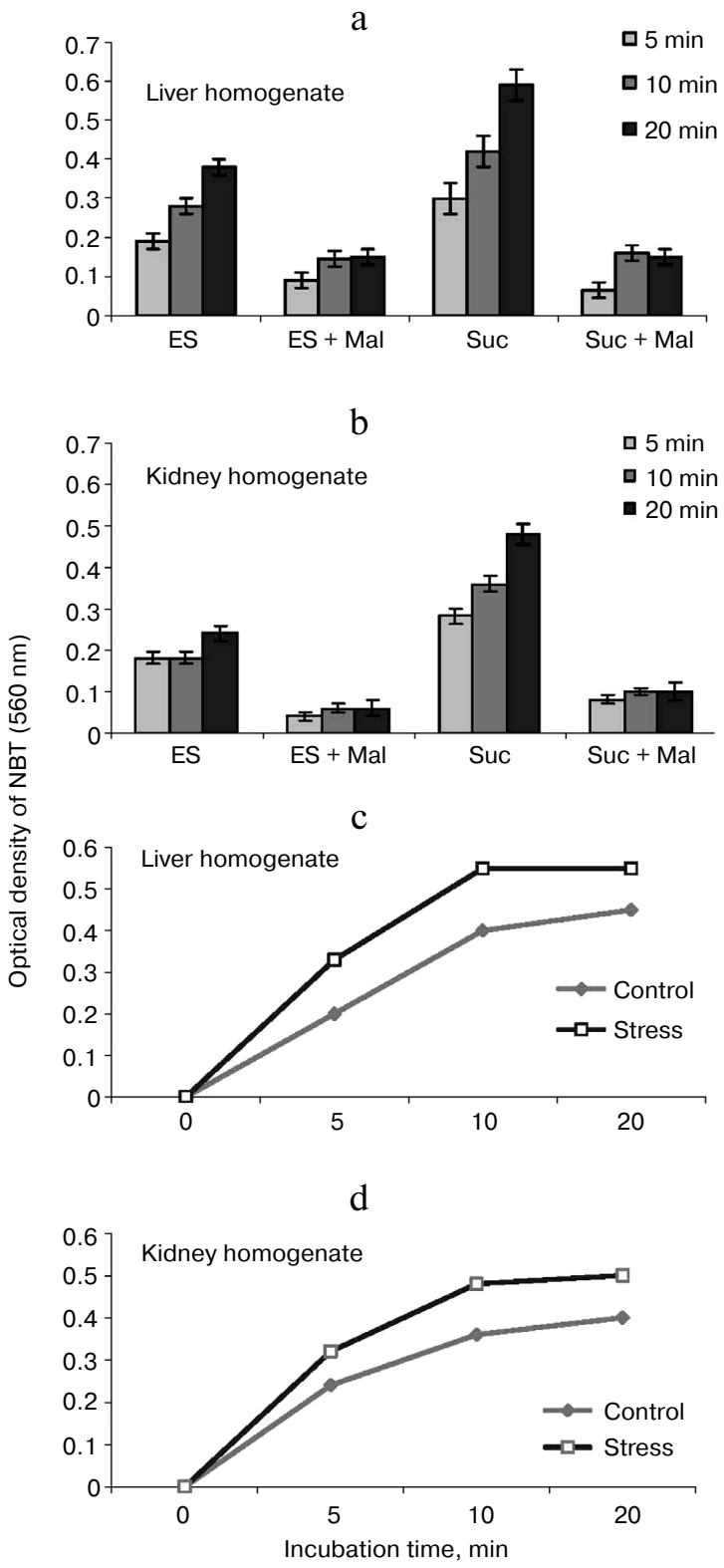

Fig. 2. Reduction of NBT during succinate oxidation and effect of stress on activity of SDH in liver and kidney homogenates. Optical density of reduced NBT formed after 5-, 10-, and 20-min incubation of homogenates with acceptor in the presence of endogenous substrates (ES) and in the presence of succinates (Suc) and malonate (Mal) in liver (a) and kidney (b) homogenates. The malonate-sensitive fraction of the reduced NBT in liver (c) and kidney (d) homogenates derived from stressed animals. Incubation medium: 125 mM KCl, 15 mM HEPES, pH 7.3, 1.5 mM phosphate, 5 mM succinate, 5 mM malonate, 100 μM NBT. Mean values of five independent experiments adjusted to 1 mg protein and with subtracted background of uncolored medium are presented.

possibility for application of NBT for evaluation of mitochondrial bioenergetic functions in leukocytes. The NBT reduction observed in the intact cells decreased during their incubation with superoxide dismutase (Fig. 3). Hence, the NBT reduction in these samples is due to generation of superoxide by the NADPH-oxidase present in the leukocyte cell membrane. Disruption of the cell membrane integrity in the samples with digitonin decreases activity of the NADPH-oxidase, which is accompanied by a decrease in superoxide generation and, hence, by the decrease in the degree of NBT reduction.

Investigation of substrate specificity of MTT. As can be seen in Fig. 4 from the values of optical density of the reduced acceptors at equal concentrations (100 μM) and incubation times of mitochondria with substrates and acceptors, the MTT reduction occurs more actively than the NBT reduction. The rate of MTT reduction is 2-3-fold higher during incubation with all substrates than the rate of NBT reduction under the same conditions. In addition to the differences in the rate of reduction, the acceptors differ in their substrate specificity. Unlike NBT, MTT is more sensitive to the oxidation of NAD-dependent substrates in comparison with succinate oxidation. The rate of MTT reduction by glutamate and KGL is by 20% higher, and the rate NBT reduction by these substrates is, on the contrary, by 20-30% lower than the rate of reduction by succinate. The reduction of these acceptors during incubation with pyruvate is significantly lower than during incubation with these substrates.

The differences between the NAD-dependent substrates in the reactions of MTT reduction could be related to the effect of glutamate and KGL on the oxidation of the fastest metabolizing substrate – succinate. KGL forms endogenous succinate during oxidation, and glutamate activates SDH by removing oxaloacetate, which inhibits the enzyme in the absence of added succinate. It can be suggested that oxidation of endogenous succinate contributes to a certain degree to the reduction of both acceptors by these substrates. However, further studies are needed to elucidate the causes of different effects of endogenous succinate on reduction of MTT and NBT.

The effect of inhibitors was estimated from the decrease in the level of reduced MTT during oxidation of glutamate, which was shown to be the most active substrate in this case (Fig. 4c). The same as in the case with NBT, rotenone decreased the MTT reduction to a greater degree than cyanide. N-ethylmaleimide (NEM) demonstrated the highest degree of inhibition. This effect can be explained by the simultaneous action of this inhibitor on thiol groups of site I of the respiratory chain and of glutamate dehydrogenase [19, 20]. The more efficient reduction of MTT during oxidation of NAD-dependent substrates compared to succinate oxidation was also observed in the liver homogenate, which was also clearly pronounced for KGL and glutamate. MTT reduction was also observed during incubation of the homogenate with lactate (Fig. 5). These data demonstrate that MTT is predominantly reduced by the NAD-dependent dehydrogenases. Taking this into consideration, MTT was used in the next experiments for evaluation of the effect of progesterone – a known inhibitor of site I of respiratory chain and NAD-dependent oxidation [21, 22], and of the synthetic progesterone analogs buterol (17α-acetoxy-3β-butanoyloxy-6-methyl-pregna-4,6-dien-20-on) and medr-

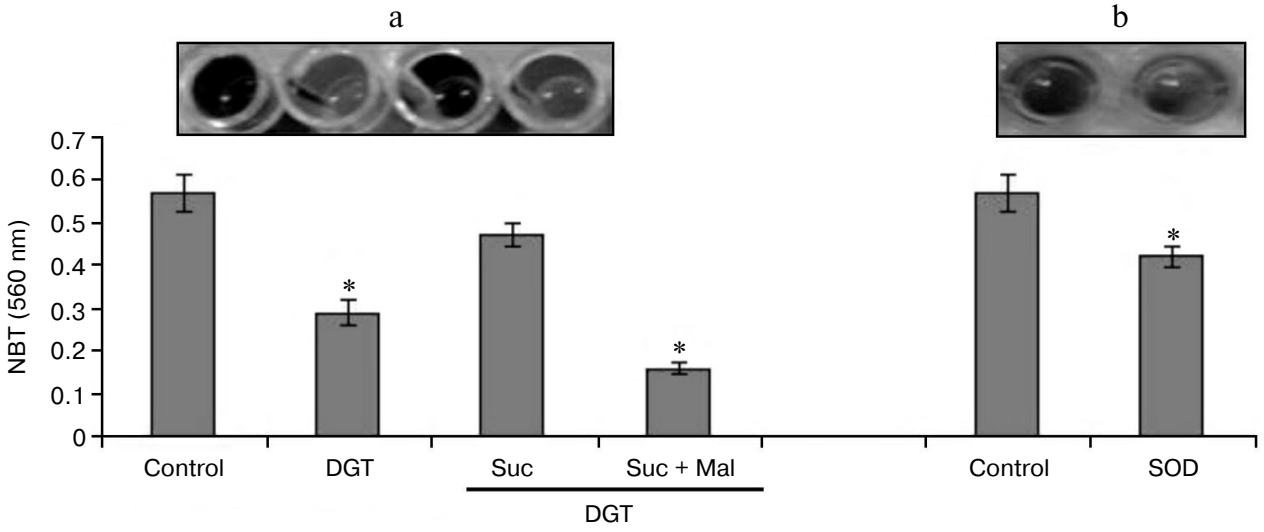

Fig. 3. Reduction of NBT in human leukocytes under different incubation conditions. a) Optical density of reduced NBT following incubation of leukocytes without additives (control), in the presence of 50 μM of digitonin (DGT), and following incubation with succinate (Suc) and malonate (Mal) in the presence of digitonin. b) Effect of antioxidants on the reduction of NBT during incubation of leukocytes in control and in the presence of superoxide dismutase (SOD, 1000 units). Images of the respective samples are presented above the graph in the same order as in the graph. Mean values with subtracted uncolored background for three independent experiments adjusted for 1 million cells are presented. Incubation medium: 125 mM KCl, 15 mM HEPES, pH 7.3, 1.5 mM phosphate, 250 μM NBT. * $p < 0.01$ in comparison with the respective control.

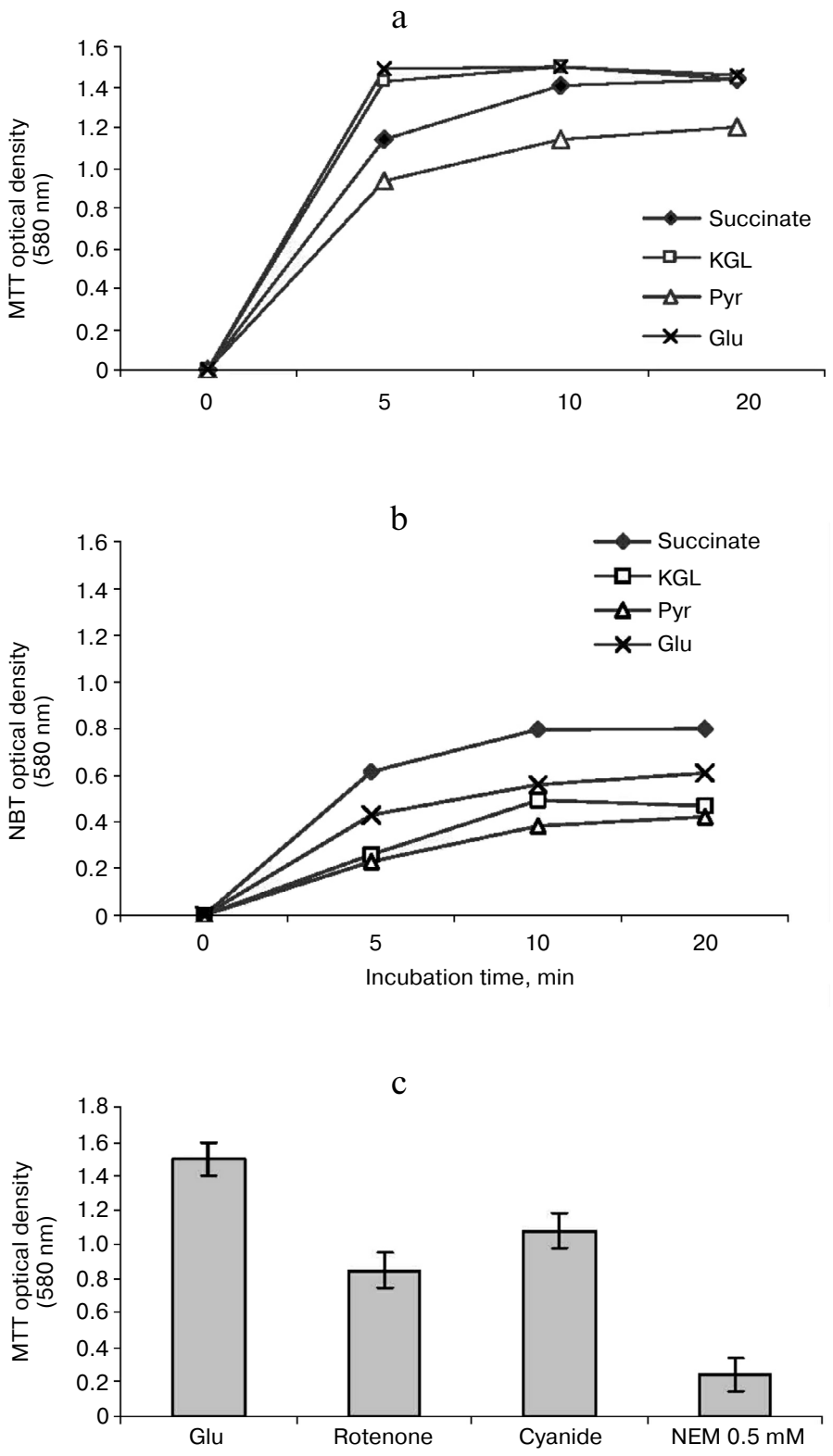

Fig. 4. Comparison of substrate specificity of reduction of NBT and MTT acceptors during oxidation of succinate and NAD-dependent substrates in isolated liver mitochondria. Effect of inhibitors. Optical density of the reduced acceptors after 5-, 10-, and 20-min incubation of isolated liver mitochondria in the presence of succinate (Suc), α-ketoglutarate (KGL), glutamate (Glu), and pyruvate (Pyr) with 100 μM MTT (a) and 100 μM NBT (b). Effect of inhibitors on the MTT reduction following incubation with glutamate for 5 min (c). Incubation medium: 125 mM KCl, 15 mM HEPES, 1.5 mM phosphate, pH 7.3, mitochondria (1 mg of protein); substrates 5 mM each, rotenone 2 μM, cyanide 2 mM, N-ethylmaleimide (NEM) 0.5 mM.

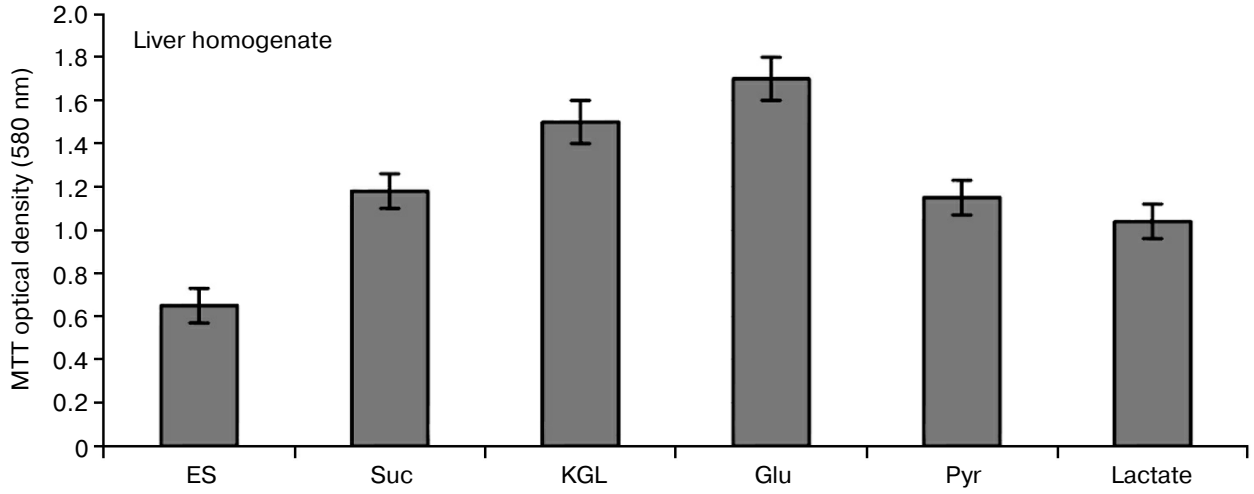

Fig. 5. Differences in optical density of reduced MTT during oxidation of succinate and NAD-dependent substrates in liver homogenate. Values of optical density following 10-min incubation of liver homogenate with MTT without substrate (endogenous substrates, ES) and in the presence of indicated substrates are presented. Incubation medium: 125 mM KCl, 15 mM HEPES, 1.5 mM phosphate, pH 7.3, MTT 100 μM, substrates – 5 μM each.

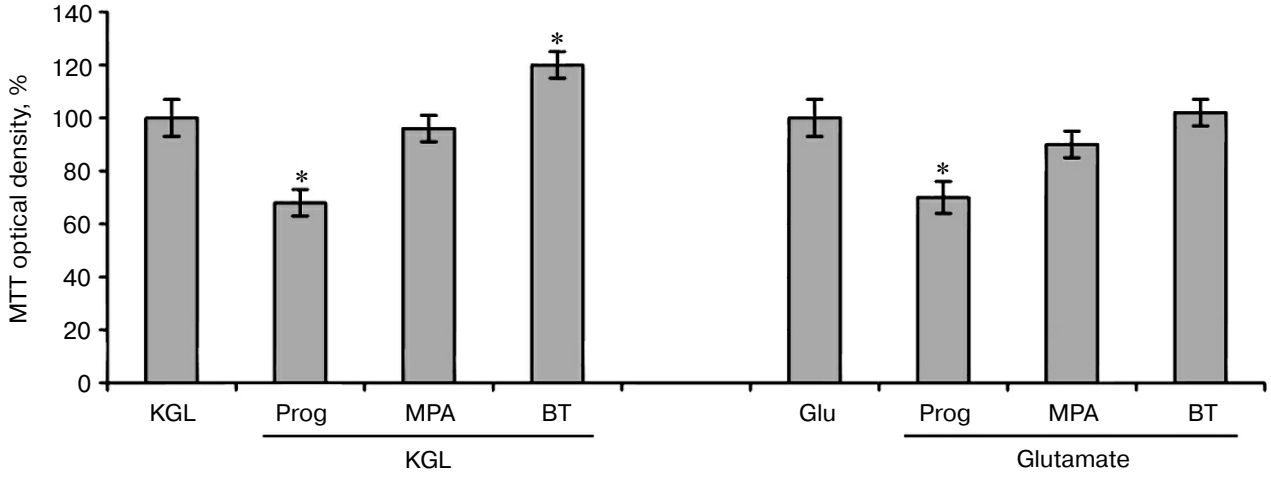

Fig. 6. Effect of progesterone and its synthetic analogs on MTT reduction in mitochondria during oxidation of NAD-dependent substrates. Values of optical density of reduced MTT in percentage of control following 5 min incubation of liver mitochondria with α-ketoglutarate (KGL, control), glutamate (Glu, control), progesterone (Prog), medroxyprogesterone acetate (MPA), and buterol (BT) are presented. Incubation medium: 125 mM KCl, 15 mM HEPES, 1.5 mM phosphate, pH 7.3, mitochondria (1 mg of protein), MTT 100 μM, substrates 5 mM, hormones 50 μM.

oxyprogesterone acetate (methylacetooxyprogesterone) used in hormone replacement therapy [23, 24] on oxidation of KGL and glutamate in isolated liver mitochondria. As can be seen in Fig. 6, progesterone inhibits oxidation of both substrates, decreasing MTT reduction by almost 30%, while medroxyprogesterone acetate does not change the level of MTT reduction in comparison with control, and buterol activates KGL oxidation. These results are in good agreement with the data on opposite directions of the action of progesterone and its analogs on such parameters as respiration rate, mitochondrial pore induction, and other factors [22, 25]. As shown, progesterone inhibited NAD-dependent respiration and stimulated pore opening, medroxyprogesterone acetate did not affect these functions, while buterol exhibited protective effect on pore induction while not affecting respiration. Unlike the measurements requiring addition of activating agents to mitochondria (ADP, calcium, oxidants), the samples with MTT revealed differences in the action of these compounds on mitochondria in the state of rest due to higher sensitivity. This can be clearly seen from activation of the KGL oxidation by buterol, which has not been observed

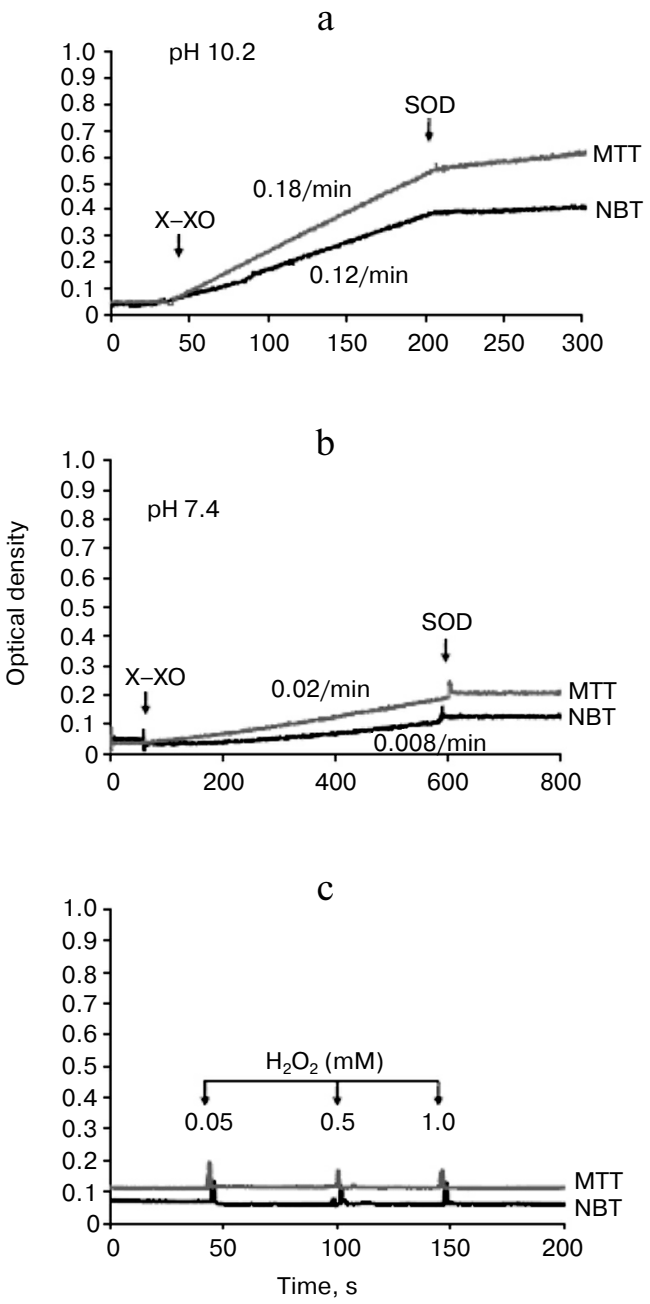

Fig. 7. Reactions of NBT and MTT with reactive oxygen species. Changes in optical densities of NBT and MTT during generation of superoxide by xanthine–xanthine oxidase complex at pH 10.2 in carbonate buffer (a), at pH 7.4 in KCl-HEPES buffer (b), and lack of effect of hydrogen peroxide on their reduction (c). Buffers contained 100 μM NBT or MTT, which was then supplemented with xanthine (0.5 mM), xanthine oxidase (0.3 unit/ml), and SOD (500 units/ml).

by other methods. At the same time, this effect of buterol can explain its role in protection of mitochondria.

Reduction of NBT and MTT by reactive oxygen species. Role of these reactions in determination of dehydrogenase activities in mitochondria. Considering that reduction of some tetrazolium salts can occur in reactions with reactive oxygen species, in the next experiments we examined the sensitivity of NBT and MTT to superoxide anion and hydrogen peroxide. While the reaction of NBT with superoxide anion is well known, and it is used in the "NBT-test" for determination of phagocytic activity [8, 9], data on the effect of reactive oxygen species (ROS) on MTT reduction and on the role of ROS in reduction of both acceptors in mitochondria are lacking.

The change in optical density of both acceptors during their interaction with superoxide anion produced by the xanthine–xanthine oxidase (X–XO) enzyme–substrate complex as well on addition of hydrogen peroxide are presented in Fig. 7. As seen in Fig. 7a, both acceptors are reduced with high rate under standard conditions (carbonate buffer, pH 10.2) optimal for production and detection of superoxide [26]. The rate of MTT reduction is 50% higher than the rate of NBT reduction. Both reactions are completely inhibited by addition of SOD, which confirms their specificity towards superoxide. The rates of reduction of both acceptors decrease by more than one order of magnitude at physiological pH 7.4 (Fig. 7b). Neither NBT nor MTT react to addition of hydrogen peroxide even at concentrations as high as 1 mM, as seen in Fig. 7c.

These data indicate that both acceptors are reduced by superoxide anion and are not sensitive to hydrogen peroxide. In the next experiments, participation of superoxide in the reactions of NBT and MTT reduction under conditions used for determination of activity of dehydrogenases in mitochondria was examined. For this purpose, mitochondria were incubated with substrates and acceptors in the presence of SOD. As seen in Fig. 8a, the level of MTT reduction following 5-min incubation with substrates does not change in the presence of SOD. As expected, SOD affected the reduction of acceptors in the presence of the respiratory chain inhibitors, i.e. under conditions stimulating superoxide generation. Under these conditions, SOD decreased the acceptor reduction both during succinate oxidation in the presence of antimycin and cyanide (Fig. 8b), and during oxidation of NAD-dependent substrates in the presence of rotenone and cyanide (Fig. 8c). Furthermore, the decrease in optical density of acceptors in the presence of SOD did not exceed 10-15% of the control values recorded at this time of incubation with inhibitors but without SOD.

Hence, the contribution of ROS to the reduction of both acceptors during oxidation of the dehydrogenase substrates is insignificant or completely absent under physiological incubation conditions and pH 7.4, but it can emerge due to generation of superoxide induced by the inhibitors of certain enzyme complexes. To compare NBT and MTT with other known acceptors used for determination of dehydrogenase activities, we conducted experiments with dichlorophenolindophenol (DCPIP) commonly used in a standard method for determination of activity of succinate dehydrogenase [27, 28]. In partic-

ular, it is still unknown whether DCPIP is sensitive to ROS. It was found that in addition to reduction by succinate dehydrogenase during the substrate oxidation, DCPIP is also reduced by superoxide produced by the X–XO system in carbonate buffer at pH 10.2 (Fig. 9). The rate of DCPIP reduction is comparable with the rate of NBT reduction if the conditions of superoxide generation are the same, i.e. the same rate of superoxide production by XO. The dependence of DCPIP reduction on superoxide is also corroborated by the inhibition of reduction on addition of SOD (Fig. 9). The reduction of DCPIP also was observed under physiological conditions in the pres-

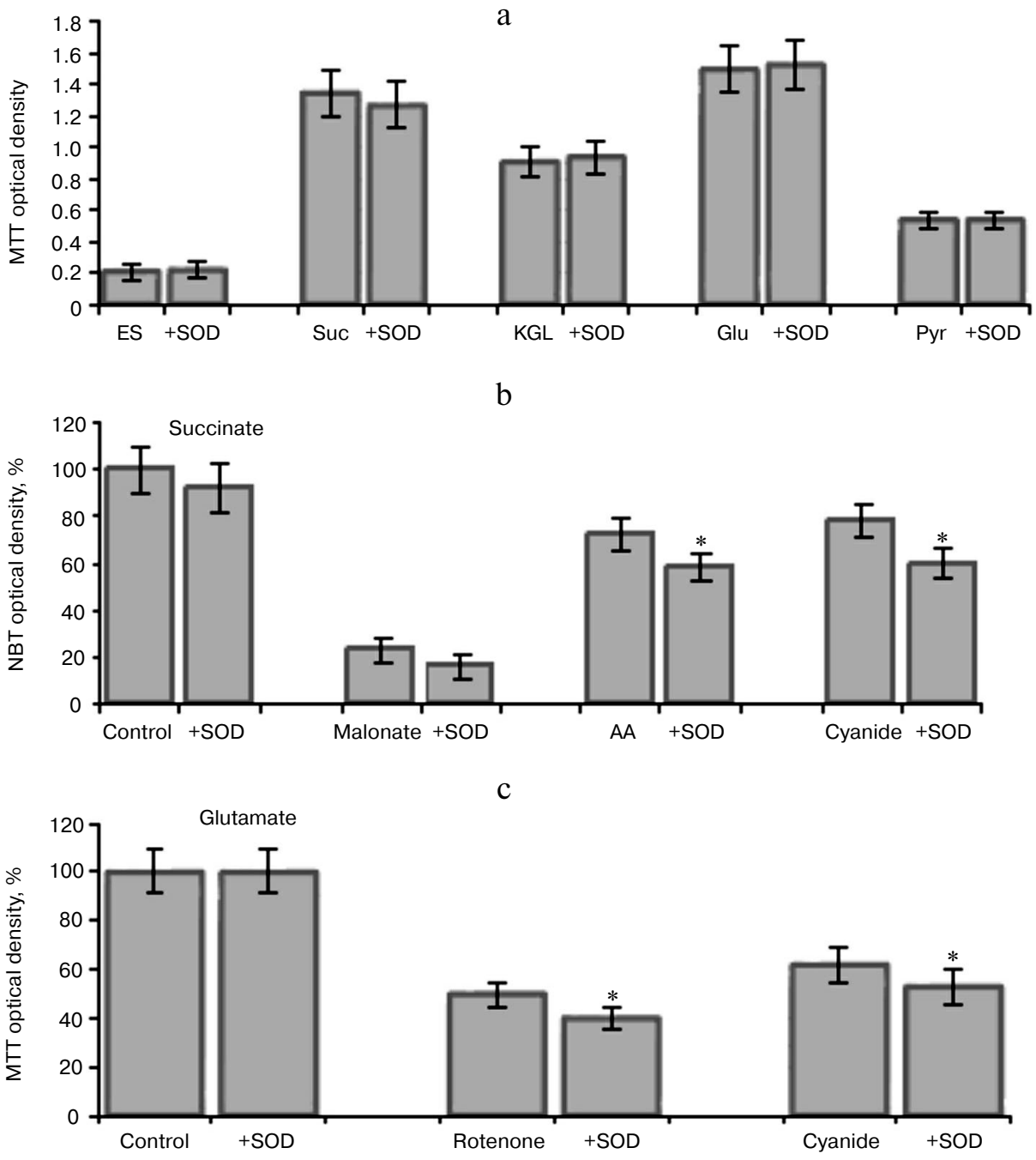

Fig. 8. Effect of superoxide anion on the change in activity of dehydrogenases in mitochondria. Effect of SOD (+SOD, 500 units/ml) on reduction of MTT during oxidation of indicated substrates (a), on reduction of NBT during oxidation of succinate in the presence of inhibitors (b), and on the reduction of MTT during oxidation of glutamate in the presence of inhibitors (c) during incubation of mitochondria (0.5 mg/ml) in KCl-HEPES buffer, pH 7.4. Optical density of the reduced acceptor during oxidation of the respective substrate (5 mM) in the absence of SOD and inhibitors is taken as 100%. Additives: malonate – 5 mM, rotenone – 2 μM, antimycin A (AA) – 2 μM, cyanide – 1 mM. * $p < 0.01$ in comparison with the respective control.

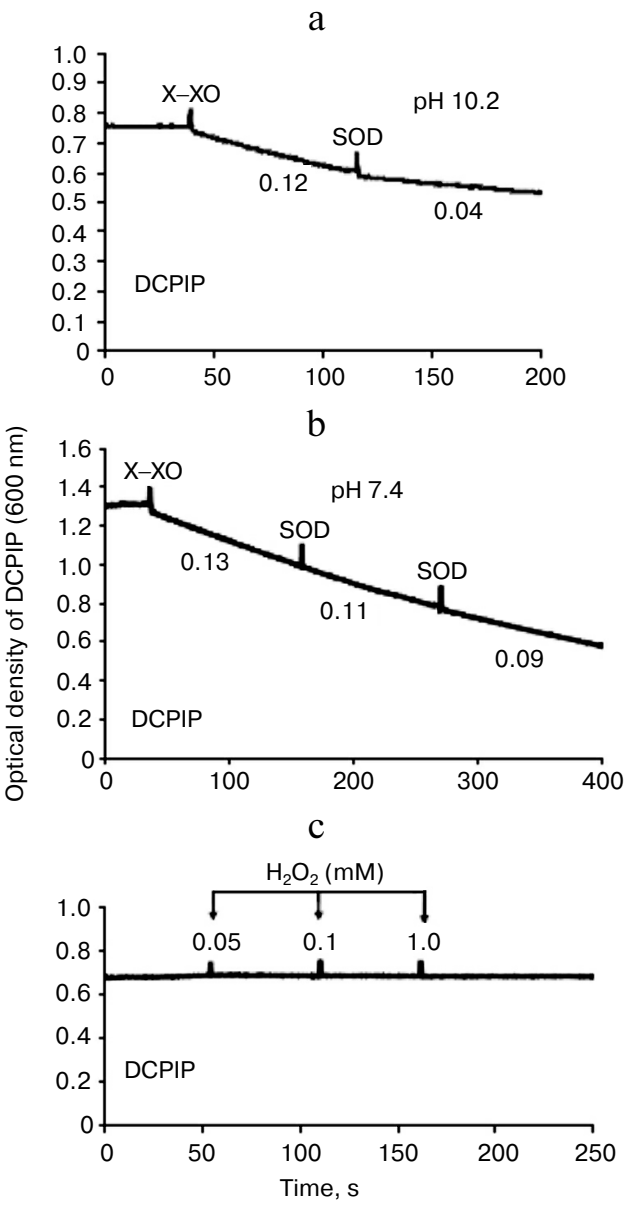

Fig. 9. Reactions of DCPIP with reactive oxygen species. Change in optical density of DCPIP during generation of superoxide by xanthine–xanthine oxidase complex at pH 10.2 in carbonate buffer (a), at pH 7.4 in KCl-HEPES buffer (b), and absence of effect of hydrogen peroxide on DCPIP reduction (c). Buffers contained 100 μM of DCPIP, followed by xanthine 0.5 mM, xanthine oxidase (0.3 unit/ml), and SOD (500 units/ml).

ence of X–XO at pH 7.4, but it was not inhibited by SOD. It is likely that the acceptor reduction under these conditions occurs directly by XO during oxidation of its substrate, xanthine, which is not observed in the samples with NBT and MTT. As in the case of NBT and MTT, DCPIP is insensitive to hydrogen peroxide – addition of hydrogen peroxide over a wide range of concentrations did not cause any changes in optical density of DCPIP; neither oxidation nor reduction of the acceptor occurred.

Hence, our data demonstrate that the tetrazolium salts NBT and MTT are effective for determination of mitochondrial oxidative activity and for evaluation of the activity of separate redox-dependent enzymes if selective inhibitors are available, as in the case of SDH and malonate. It must be mentioned that only short-term (5-10 min) incubation with mitochondria and homogenates is required for application of NBT and MTT for these purposes, because complete reduction of the acceptors with leveling of optical density in different samples occurs with increase in incubation time independent of oxidation substrate. This could be the reason for contradictions observed in different studies with prolonged incubation times. Moreover, considering that both acceptors are also reduced by superoxide anion, it is worthwhile considering that superoxide contribution to the process is possible under certain experimental conditions. As mentioned before, this property of NBT is used for evaluation of the NADPH oxidase activity of monocytes and macrophages in norm and pathology [8]. The contribution of superoxide to determination of activity of dehydrogenases in mitochondria is most probable in pathologies related to mutations in enzyme complexes [29-31].

As follows from the data, substrate specificity is characteristic for these acceptors. NBT is more specific for determination of SDH activity than activities of NAD-dependent dehydrogenases, while MTT exhibits higher specificity to NAD-dependent dehydrogenases. As follows from the results on inhibition of MTT reduction by rotenone and NEM, as well as from MTT reduction during lactate oxidation, MTT accepts electrons both from site I of the respiratory chain and from the NAD-dependent dehydrogenases. Moreover, the possibility of simultaneous incubation of many samples with different substrates and inhibitors (in spectral cuvettes or microplates) provides additional benefits to this method in comparison with those that involve sequential sampling and, thus, decrease in mitochondrial activity with time. Most clearly pronounced are the advantages of this method over the polarographic technique for measuring respiration, which is known for being totally dependent on oxygen concentration in the cell, not sufficiently sensitive for measuring low respiration rates, and involves prolonged times of mitochondria storage, and, thus, variability of the isolated mitochondria in time. Simultaneous measuring in a set of cuvettes or in a microplate with using NBT and MTT allows analyzing 20 samples within 20 min, while the sequential measuring of these samples using the polarographic technique could take as least 200 min. Such prolonged storage represents a well-known problem, because it is accompanied by shifts of all indicators of mitochondrial activity, thus increasing the differences between the state of mitochondria in an organism and in the test sample, which introduces more scatter to the data. These advantages are clearly demonstrated by the measuring of SDH activity from the quantitative

change of malonate-sensitive fraction of the reduced NBT in liver and kidney homogenates under stress in comparison with norm, as well as by the effect of progesterone and its analogs on the level of MTT reduction with participation of NAD-dependent enzymes.

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