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THE REDUCTION OF ALAMAR BLUE BY PERIPHERAL BLOOD LYMPHOCYTES AND ISOLATED MITOCHONDRIA

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Alamar Blue – нетоксичний індикатор проліферативної активності клітин, здатний швидко проникати крізь біологічні мембрани і легко відновлюватись внутрішньоклітинними ферментами. Накопичення відновленої флуоресцентної форми Alamar Blue за умови короткострокового культивування лімфоцитів периферичної крові людини може бути використано як тест на життєздатність, оскільки оброблення клітин дигітонином, що викликає руйнування плазматичної мембрани, практично повністю пригнічує відновлення індикатору. Інгібування відновлення Alamar Blue азидом натрія свідчить, що його метаболізм пов'язаний із мітохондріальною активністю. Порівняльний аналіз відновлення Alamar Blue та споживання кисню ізольованими мітохондріями печінки щурів показав, що відновлення Alamar Blue здійснюється не якимось певним ферментом дихального ланцюга, а може бути розглянуте як інтегральний показник активності його окислювально-відновлювальних компонентів.

К л ю ч о в і с л о в а : Alamar Blue, лімфоцити, мітохондрії, інгібіторний аналіз, ферменти дихального ланцюга, споживання кисню, флуоресценція.

The successful search of informative and sensitive method for determination of the viability and proliferative activity of different cell types plays an important role in the efficiency of biomedical and cytotoxic studies. Nowadays, test based on the reduction of resazurin dye, which is commercially known as Alamar Blue (AB), takes a great interest in the investigators all over the world (Fig. 1) [1].

The oxidation-reduction (REDOX) potential of AB is +380 mV (pH =7.2, 25 °C degree). It penetrates quickly through the biological membranes and can be easily reduced by intracellular enzymes. After the reduction, AB changes from nonfluorescent "blue" form with the maximal absorption of 600 nm to the "red" fluorescent form with maximal absorption of 570 nm. This event makes it possible to determine the reduced form of AB by measuring both absorption and fluorescence. The accumulation of the reduced form of AB is proportional to the activity of REDOX enzymes, i.e. it has a capacity to reflex the metabolic state of cells [2]. AB has several benefits compared to MTT (3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or XTT (sodium 3′-[1-phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) tetrazolium salts. It is non-toxic to the cells and it is soluble in culture media in oxidative form as well as in the reduced one. So, it is possible to make a long-term monitoring of cells in culture without any negative effects. Some studies on long-term culture of plant cells [3], fibroblasts [4], osteoblasts [5], epithelium cells [6], lymphocytes [7] and transformed cell lines [8, 9] show that the reduction of AB correlates with the viability and proliferative activity of cells. However, studies that show the possibility of using AB assay for determining cell viability under short-term culture conditions were not reported yet. At the same time, a question of enzymatic systems, which are responsible for AB reduction is still open. Different authors consider that mitochondria take the main part in AB reduction. This view is based on the high activity of mitochondrial respiratory chain REDOX enzymes as well as on the ability of cytochrome oxidase (1.9.3.1.) inhibitor sodium azide to inhibit the AB reduction [1, 2]. Another study, which was carried out on rats medulla synaptosomes showed that treatment of synaptosomes with mitochondria inhibitors such as antimicyn A or malonate inhibited the AB reduction [10]. At the same time, taking into account the high sensitivity of AB assay (measurements were carried out on samples, contained

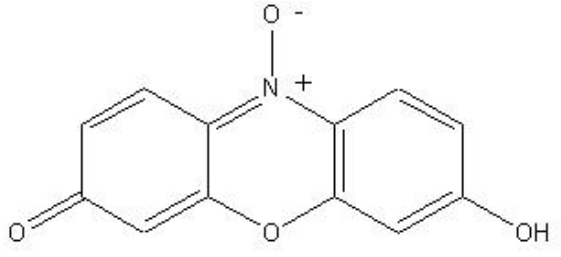

Fig. 1. Chemical formula of resazurin.

only 25 μg of protein), authors consider it to be the easiest method for determination of functional activity of intracellular mitochondria. However, studies on isolated mitochondria have not been carried out yet.

Thereby, the aim of this study was to investigate the possibility of using AB assay for determining the viability of human peripheral blood (PB) lymphocytes under short-term culture conditions as well as for measuring the respiratory chain activity of isolated rat liver mitochondria.

Materials and Methods

Lymphocyte isolation. Human PB lymphocytes were separated in Ficoll-Paque (p = 1.077) density gradient [11]. For this purpose 4 ml of stabilized blood, diluted 1 : 1 by Hank's balanced salt solution («Sigma-Aldrich», USA) were layered on 2 ml of Ficoll-Paque («Pharmacia», Sweden) and centrifuged at 400 g for 15 min. After collection cells were washed twice by centrifugation at 200 g in Hank's solution. The cell suspension viability was determined by trypan blue («Sigma-Aldrich», USA) staining method. Cells were counted, using haemocytometer (USA).

Short-term culture of PB lymphocytes and Alamar Blue viability assay. Freshly isolated PB lymphocytes were resuspended in DMEM/F12 culture medium, supplemented with 10% of fetal calf serum (FCS), penicillin (50 units/ml) and streptomycin (50 mg/ml). Cell suspensions with densities of 10^4, 10^5 and 10^6 cells per ml were seeded into 96-well plate by 200 μl per well. 20 μl of AB («Serotec Ltd», USA) were added to each well and cell suspensions were cultured in 5% CO_2 / 95% humidity at 37 °C for 48 hrs. In parallel experiments digitonin («Sigma-Aldrich», USA) in concentration of 100 μg per 10^6 of cells [12,13] which causes the destruction of plasma membrane, or the cytochrome oxidase inhibitor sodium azide («Sigma-Aldrich», USA) in final concentration of 5 mM have been added. The reduced form of AB was determined by fluorescence measurements at excitation wavelength 550 nm and emission of 590 nm. Measurements were carried out using Tecan Genios microplate reader («Tecan inc.», Australia). Data analysis was made using software supplied with the Tecan reader. Results were demonstrated as the relative fluorescent units (RFU) which were calculated by this formula:

RFU = fluorescence measurement of cells − fluorescence measurement of background.

As a background the fluorescence of AB in the medium without cells was used.

Mitochondria isolation and investigation. Mitochondria were isolated from livers of white female rats with the body weight of about 200–300 g by differential centrifugation method [14, 15]. All manipulations with animals were carried out under the ether narcosis according to "European convention for defense from application of vertebrates with experimental and scientific aims". Mitochondria were incubated at +26 °C in the medium supplemented with 200 mM of mannitol, 50 mM of sucrose, 10 mM of KH_2PO_4, 0.5 mM EDTA and 30 mM of Tris-HCl (pH = 7.4). The oxygen consumption was determined using Clark's closed electrode on Rank Brothers polarographic analyzer, model 20 («Rank Brothers», UK).

In parallel, using the same conditions, the reduction of AB was measured as described above.

Glutamate and malate (5 mM) or succinate (8 mM) or ascorbate (5 mM) with TMPD (1 mM) were used as substrates [14, 15]. An uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP, 100 μM) and inhibitors mersalyl (10 mM), rotenone (1 μM), antimicyn A (2 μg), sodium azide (5 mM) and cyanide (5 mM) were used [14, 15]. All substrates were produced by «Sigma-Aldrich» (USA).

Protein content was measured by the method of Lowry [16].

Statistical analysis. Analyses were done using Student's *t* test. Means ± SEM are listed.

Results and Discussion

Alamar Blue reduction in peripheral blood lymphocytes culture. The viability of freshly isolated lymphocyte suspension assessed by trypan blue staining was 95 ± 2% and did not reduce during 24 hrs of culture. Then, the viability slowly decreased and was about 60% at 48 hrs of culture. The fluorescence intensity was studied immediately after AB addition into cell culture, and then every 4–12 hrs of culture. Fig. 2 demonstrates the dynamics of AB reduced form accumulation during PB lymphocytes culture in fi-

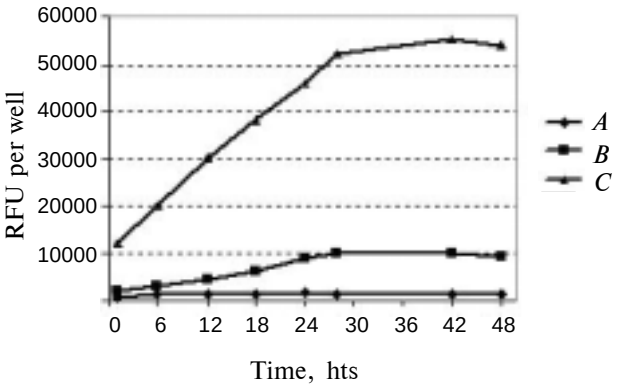

Fig. 2. The reduction of AB during PB lymphocytes culture. A − cell suspension in final concentration 10^4 cells per ml; B − cell suspension in final concentration 10^5 cells per ml; C − cell suspension in final concentration 10^6 cells per ml; (n = 4 in triplicate).

nal concentrations of 10^4, 10^5 and 10^6 cells per ml. It is shown that during 48 hrs of culture there was no significant difference in fluorescence between cell suspension in final concentration of 10^4 and a background (Fig. 2). At the same time, suspensions in final concentrations 10^5 and 10^6 showed significantly higher fluorescence levels compared to the background. The accumulation of AB reduced form which was accompanied with the enhancement of fluorescence was proportional to the cell concentration in culture. The fluorescence intensity increased linearly for cell suspensions in final concentrations of 10^5 and 10^6 cells per ml during 28 hrs of culture. The accumulation of AB reduced form increased 1.4 ± 0.1 times for every 6 hrs of culture in the both cell concentrations. Then, the rate of AB reduction decreased and after 42 hrs the accumulation of AB reduced form was not observed. It could be a result of formation of the final reaction product – colorless and nonfluorescent substance hydrorezoruphin [2]. The effect of additives on the accumulation of AB reduced form was investigated after 24 hrs of culture because the fluorescence growth was linear during this time.

The experiments with digitonin, which destroys the integrity of the plasma membrane, were carried out to study the capacity of using AB assay for determining cell viability. The addition of digitonin to culture medium caused the staining of all cells by trypan blue dye. Fig. 3 shows that the addition of digitonin simultaneously with lymphocytes almost completely inhibited the AB reduction after 24 hrs of culture in both cell concentrations.

Sodium azide – the inhibitor of mitochondrial cytochrome oxidase, as an additive to lymphocyte culture did not lead to significant cell staining, but did reduce the AB reduction for about 4 times (Fig. 3). These results show that AB assay may be used for viability determination and that its reduction is associated with mitochondrial enzyme activity. The conclusion does not contradict the opinion that digitonin does not destroy the mitochondrial membrane because of cholesterol absence. Earlier we have shown on isolated hepatocytes that the addition of digitonin quickly inhibits the respiratory activity of intracellular mitochondria [17]. The fact that the inhibitor of mitochondrial respiration did not completely reduce AB fluorescence and its effect was less than that of digitonin needs more detailed studying on isolated mitochondria.

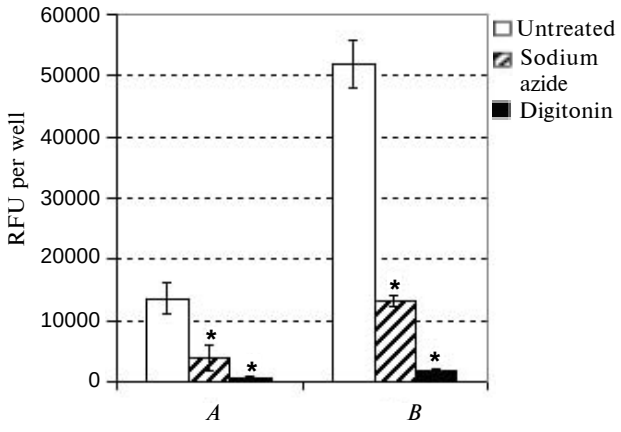

*Fig. 3. The effect of digitonin and sodium azide on AB reduction during 24 hrs of PB lymphocytes culture. A – cell suspension in final concentration 10^5 cells per ml; B – cell suspension in final concentration 10^6 cells per ml; (n = 5 in triplicate); * data is significantly different in comparison with untreated samples ($p < 0.05$).*

The reduction of Alamar Blue by isolated rat liver mitochondria. The addition of mitochondria into incubation medium caused quick reduction of AB. The accumulation of fluorescent form was proportional to protein contents in suspension and to the protein concentration of 0.3–0.4 mg per ml and had a linear enhancement of fluorescence during incubation for at least 15 minutes. Fig. 4 shows the effect of mitochondrial substrates and inhibitors on AB fluorescence (*A*) and oxygen consumption rate (*B*) of mitochondria. For this aim mitochondria were pre-incubated with different substrates and inhibitors for 5 min, then AB was added and measurement was carried out after 5 min. Our results indicate that the addition of NAD-dependent substrates (glutamate + malate) or succinate did not sharply change the fluorescence of AB. At the same time the substrate of cytochrome oxidase TMPD + ascorbate increased the AB fluorescence 2.5 times compared to endogenous substrates values. In this case the fluorescence intensity in the absence of exogenous substrates was 33900 ± 8200 RFU per mg of protein. After oxidation of substrates of I, II and IV complexes of the respiratory chain the fluorescence indexes were 36700 ± 1900, 39200 ± 600, and 83300 ± 6800 RFU per mg of protein, respectively. The uncoupler DNP stimulated AB fluorescence index more than 2 times in the presence of NAD-dependent substrates and slightly affected these meanings in the presence of succinate or ascorbate + TMPD.

According to the AB designers' opinion [3], it has the capacity to be reduced by cytochrome oxidase – the terminal enzyme of the respiratory chain. In this case the AB fluorescence index must correlate with the activity of respiratory chain, which can be determined by the oxygen consumption rate. However, such correlation was not observed in this study. Fig. 4, *B* shows that the mitochondria respiratory rate during oxidation of malate + glutamate, succinate or TMPD significantly exceeded the endogenous respiratory rate and was 7.7 ± 1.3; 12.1 ±

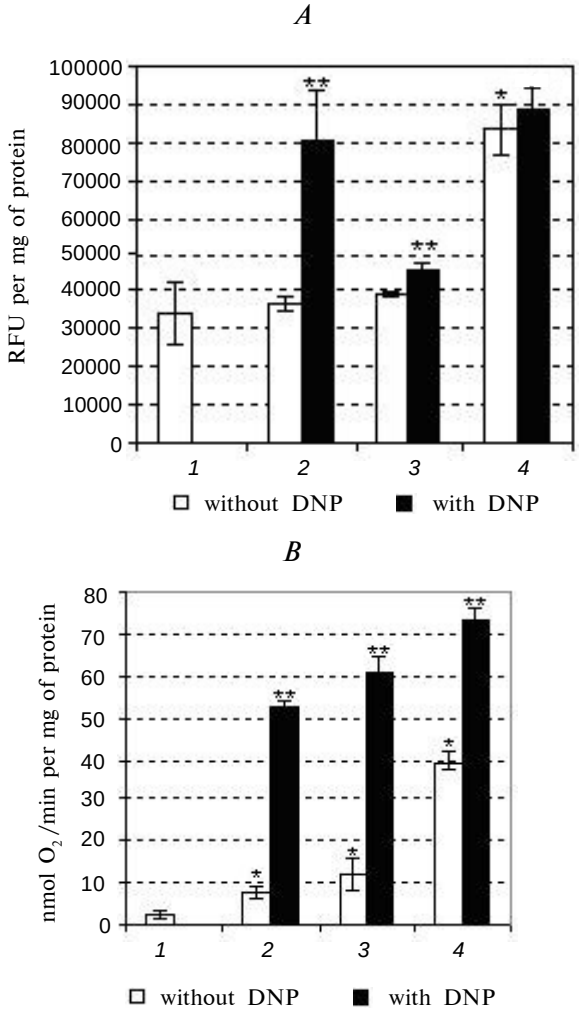

*Fig. 4. The effect of different substrates on AB fluorescence (A) and oxygen consumption rate (B) in mitochondria in the absence (white columns) or presence (dark columns) of DNP. 1 – no exogenous substrates; 2 – glutamate+malate; 3 – succinate; 4 – TMPD+ascorbate. (n = 5 in triplicate); * data is significantly different in comparison with the samples without exogenous substrates (p<0.05); ** data is significantly different in comparison with the samples without DNP (p < 0.05).*

± 3.4 and 39.5 ± 6.4 nmol O_2/min per mg of protein respectively. Oxygen consumption rates during oxidation of glutamate + malate, succinate and TMPD + ascorbate in the presence of DNP were 52.8 ± 3.23, 60.9 ± 4.9 and 73.4 ± 6.5 nmol O_2/min per mg of protein correspondingly (Fig. 4, *B*). The stimulation of substrate oxidation rate by the uncoupler (respiration control index) was 7.56 ± ± 0.25 for NAD-dependent substrates, 4.86 ± 0.36 – for succinate and 1.92 ± 0.07 – for TMPD + ascorbate. These meanings were adequate to common values [14, 15].

A comparative study of AB reduction and oxygen consumption rate in isolated mitochondria showed that AB fluorescence did not correlate with the electron transport in the presence of the uncoupler and different substrates using the same conditions of measurement. The slight stimulation of succinate or TMPD + ascorbate oxidation by the uncoupler might be explained by the uncoupling effect of AB. Special experiments showed that at high concentrations (over 15%) AB really has the uncoupling effect on oxidative phosphorylation, however, in the chosen concentration (10%) this effect was not observed. The fact that DNP equally stimulated both fluorescence and oxygen consumption rate in the oxidation of NAD-dependent substrates also indicates that AB does not have an uncoupling effect on oxidative phosphorylation.

To determine components of respiratory chain, which take part in AB reduction, the inhibitory analysis was carried out (Fig. 5). Fig. 5, *A* shows that AB fluorescence intensity during succinate oxidation in the presence of DNP was 45900 ± 110 RFU per mg of protein. After the addition of inhibitor of bc_1-complex of respiratory chain antimicyn A or the inhibitor of cytochrome oxidase sodium azide, the fluorescence intensity was reduced but remained at the 50% point compared to the initial one (in the absence of inhibitors). At the same time, the inhibitors almost completely stopped the oxidation of succinate in the respiratory chain. During the oxidation of TMPD + ascorbate in the presence of DNP inhibitors of cytochrome oxidase (cyanide or azide) completely inhibited oxygen consumption, but the AB fluorescence intensity decreased by these inhibitors less than 2 times (Fig. 5, *B*). Inhibition of AB fluorescence by cyanide or azide indicates that cytochrome oxidase takes part in AB reduction. At the same time, the incomplete inhibition of fluorescence by cytochrome oxidase inhibitors allows to assume that the AB reduction may be caused by direct interaction of AB with TMPD. Indeed, in the absence of mitochondria TMPD reduced AB.

The AB fluorescence intensity during oxidation of NAD-dependent substrates in the presence of the uncoupler did not decrease after the addition of rotenone or antimicyn A (Fig. 5, *C*). These inhibitors completely prevented the oxygen consumption during oxidation of NAD-dependant substrates. Inhibition of substrate transport into mitochondria by mersalyl resulted in the decrease of AB fluorescence intensity, which was stimulated by the uncoupler, down to 32800 ± 7100 RFU per mg of protein. This value was equal to the fluorescence level without exogenous substrates. In the aggregate, these results allow to assume that NADH-dehydrogenase (1.6.5.3.) has the capacity to reduce AB.

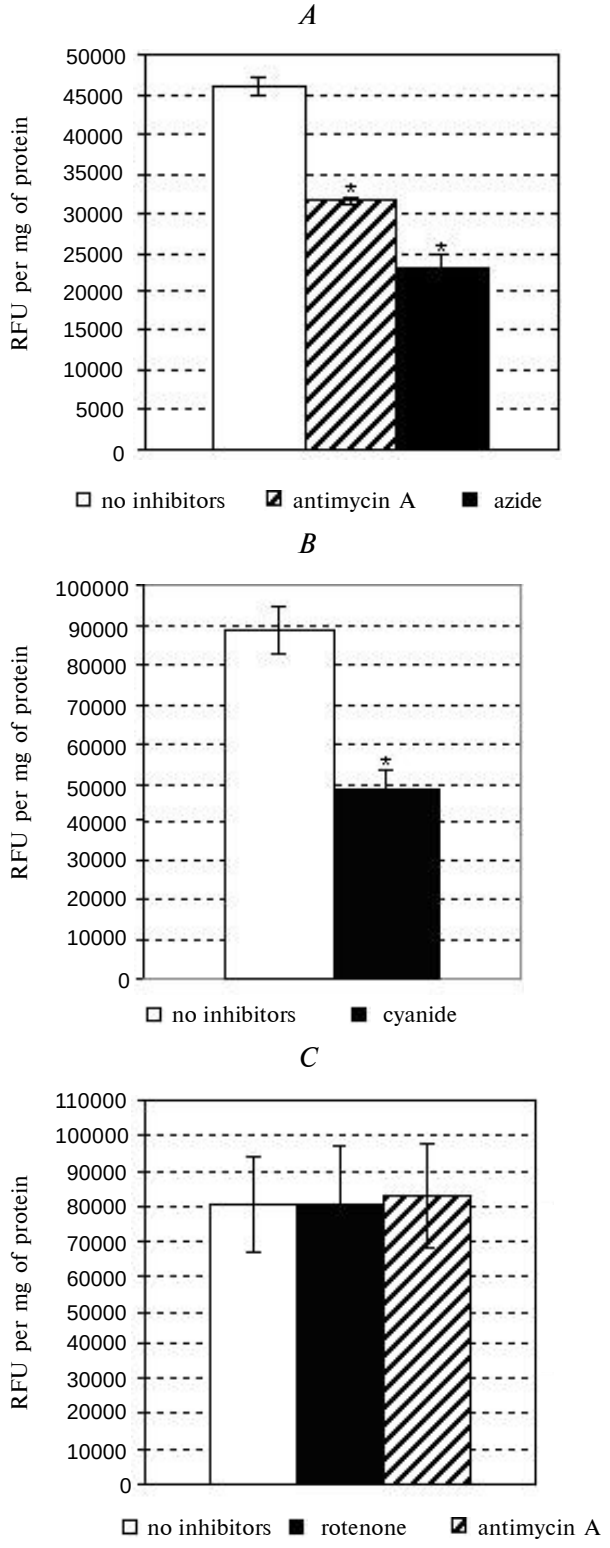

*Fig. 5. The effect of mitochondrial inhibitors on AB reduction during oxidation of different substrates in the presence of uncoupler DNP. Substrates: A — succinate; B — TMPD + ascorbate; C — glutamate + malate. (n = 5 in triplicate); * data is significantly different in comparison with the samples without inhibitors (p < 0.05).*

Thus, results obtained on isolated mitochondria did not allow to determine a single part of respiratory chain which is responsible for AB reduction. Being based on the fact that AB has a high oxidation-reduction potential (+380 mV) we can suppose that it has the capacity to act as an electrons acceptor for different components of respiratory chain, such as NADH-dehydrogenase, succinatedehydrogenase (1.3.5.1.), $FADH_2$, FMN, ubiquinone, cytochrome *c* etc.

Summarizing aforesaid, the data obtained on isolated cells and mitochondria show that AB assay can be used as a viability test, which is based on the determination of metabolic activity of mitochondria. Furthermore, the AB can be reduced not by a specific enzyme of the respiratory chain. It seems to be the integral indicator of oxidation-reduction activity of the respiratory chain components.

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S u m m a r y

Alamar Blue is a widely used nontoxic indicator of cell proliferative activity, which penetrates quickly through the biological membranes and can be easily reduced by intracellular enzymes. Accumulation of reduced fluorescent form of Alamar Blue during short-term culture of human peripheral blood lymphocytes may be used as a cell viability test since it was prevented by disruption of plasma membrane by digitonin. The inhibition of Alamar Blue reduction by NaN_3 indicates that its metabolism is associated with mitochondrial activity. A compaative study of Alamar Blue reduction and oxygen consumption on isolated rat liver mitochondria shows, that the Alamar Blue reduction is not associated with the activity of specific complex of respiratory chain and it seems to be an integral indicator of oxidation-reduction activity of respiratory chain components.

K e y w o r d s: Alamar Blue, lymphocytes, mitochondria, inhibitory analysis, respiratory chain enzymes, oxygen consumption, fluorescence.

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