Changes in absorbance of monolayer of living cells induced by laser radiation at 633, 670, and 820 nm

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Changes in Absorbance of Monolayer of Living Cells Induced by Laser Radiation at 633, 670, and 820 nm

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Invited Paper

Abstract—Redox absorbance changes in living cells (monolayer of HeLa *cells*) under laser irradiation at 633, 670, and 820 nm have been studied by the method of multichannel recording in spectral range 530–890 nm. It has been found that the irradiation causes changes in the absorption spectrum of the cells in two regions, near 754–795 nm (maxima at 757, 775, and 795 nm) and near 812–873 nm (maxima at 819, 837, 858, and 873 nm). Changes occur in band parameters (peak positions, width, and integral intensity). Virtually no changes occur in the red spectral region and a few changes are recorded in the green region near 556–565 nm. The results obtained evidence that cytochrome c oxidase becomes more oxidized (which means that the oxidative metabolism is increased) due to irradiation at all wavelengths used. The results of present experiment support the suggestion (Karu, Lasers Life Sci., 2:53, 1988) that the mechanism of low-power laser therapy *at the* cellular level is based on the electronic excitation of chromophores in cytochrome c oxidase which modulates *a* redox status of the molecule and enhances its functional activity.

Index Terms—Cytochrome c oxidase, low power laser therapy, redox-absorbance changes in living cells.

I. INTRODUCTION

IT IS well known beginning with the work of Arwanitaki and Chalazonitis [1] that mitochondria of mammalian cells have photosensitivity. A large body of experimental data confirms this finding (reviews [2], [3]).

It was suggested in 1988 [4] that the mechanism of low-power laser therapy at the cellular level is based on the absorption of monochromatic visible and near IR radiation by components of the mitochondrial respiratory chain which causes changes in their redox properties and acceleration of electron transfer (primary reactions). Primary reactions are followed by a modulation of intracellular redox state (secondary reactions, cellular signaling) [4]. The question of the photoacceptor remained open until 1995 when the analysis of five action spectra allowed us

to suggest that the primary photoacceptor for red-near IR region is a mixed valence form of cytochrome c oxidase ([5], review [2]). The analysis in the work [5] was made by analogy with the metal-ligand system absorption spectra characteristic for this spectral range. It was suggested that the peaks at 620 and 820 nm in all action spectra are in most part connected with Cu_A in reduced and oxidized states respectively and the peaks at 680 and 760 nm belong to Cu_B respectively in oxidized and reduced states [2], [5].

One important step in identifying the photoacceptor molecule is a comparison of absorption and action spectra. As a rule, the action spectrum has the shape of the absorption spectrum of the photoacceptor molecule [6]. The absorption spectra of individual cells up to 650 nm were recorded years ago with the aim to identify respiratory chain enzymes. The absorption spectrum of whole cells in the visible region was found to be quantitatively similar to that of isolated mitochondria (review [7]). An extension of optical measurements from visible spectral range to far-red and near IR (650–1000 nm) was undertaken at the end of seventies for monitoring the redox behavior of cytochrome c oxidase *in vivo*[8]. These studies led to the discovery of near IR window into the body and to development of near IR spectroscopy for noninvasive monitoring of tissue oxygenation [9], [10].

Optical measurements of individual cells or cell monolayers in far-red and near IR spectral range are still limited due to extremely week absorption of cytochrome c oxidase in this region as well as due to limited sensitivity of commercial spectrophotometers. First measurements using a multichannel recording setup specially developed for irradiation experiments with cellular monolayer, showed a possibility to measure optical densities 0.005 and less ([11], review [2]). In the present work, we report the results of cell monolayer absorbance measurements in wide spectral range from 530 to 890 nm. Cells were grown in closed cuvettes. The cuvette was not open during the irradiation measurements to avoid changes in oxygen concentration and intracellular pH. The wavelengths used for the irradiation (632.8, 670, and 820 nm) were chosen in accordance with the maxima in the action spectra recorded earlier (reviews [2], [3]). As a result of present measurements we will demonstrate that the irradiation causes redox absorbance changes in living cells which can be interpreted as changes in functional activity of cytochrome c oxidase.
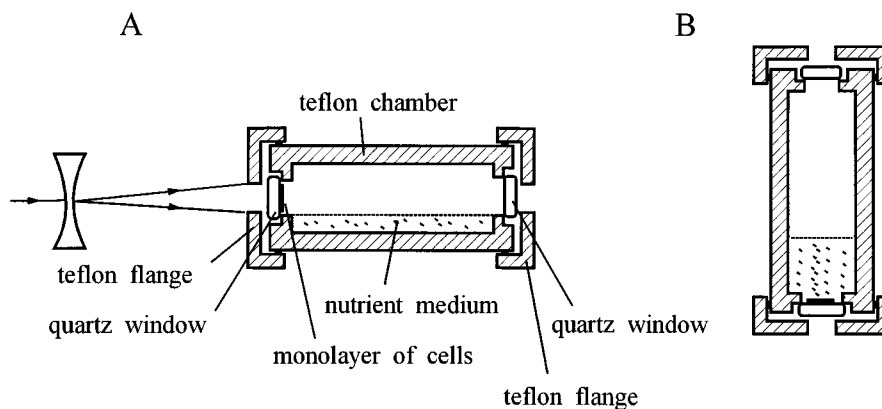
A

B

Fig. 1.   Cell culture chamber for irradiation of monolayers: (a) position during irradiation and (b) position during cultivation.
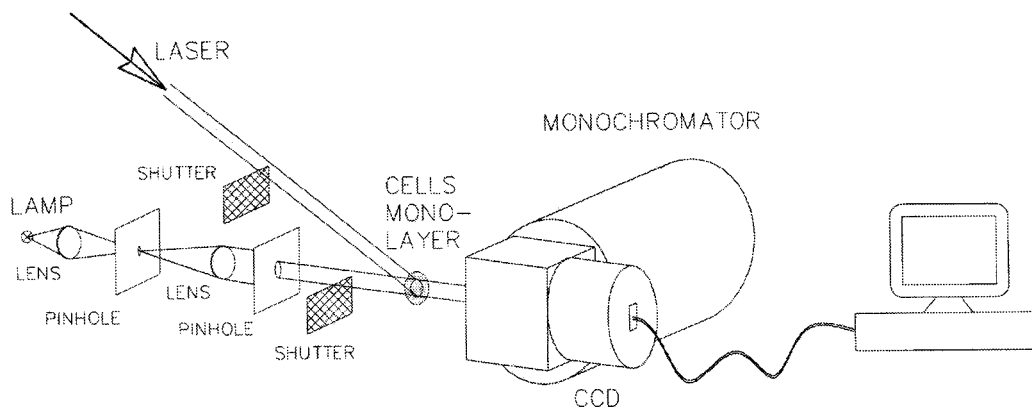
Fig. 2.   Schematic representation of experimental setup used for absorbance measurements.

II. MATERIAL AND METHODS

HeLa cells, initially obtained from the Institute of Virology, Moscow, Russia, were cultivated at 37°C and irradiated in special Teflon chambers with quartz windows allowing irradiation of the monolayer in the closed cuvette without removal of the nutrient medium (Fig. 1). A sample of 4×10^5 cells in 2 ml medium 199 supplemented with 10% bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin was inoculated into each cuvette. The cells were assayed 72 h after the inoculation, when a uniform monolayer covered the entire surface of the cuvette window.

The absorption spectra of the cell monolayer were measured using the following setup (Fig. 2). A parallel light beam from a small incandescent lamp with a radiant power of 20 μW was applied to a spectrophotometer with a linear dispersion of 120 Å/mm. Absorption spectra were detected with a multichannel optical analyzer equipped with a Toshiba (Japan) model TCD1301D charge-coupled linear photodetector. The total number of sensitive elements was 3648 per each 8×200 μm. The spectrophotometer and the optical analyzer were constructed by Dr. E. Silkis, Institute of Spectroscopy, Troitsk, Russia. The analyzer was connected to a computer, which implemented signal averaging and data processing. Spectral data were processed (transmission-to-absorption conversion, smoothing, baseline correction, deconvolution of spectra with Lorentzian fitting) using the ORIGIN (Microcal) software program.

For the irradiation, the Biotherapy 3ML semiconductor laser (Omega, London, U.K.) or He–Ne laser (LG-30, Lvov, Ukraine) were used. The parameters of the "Omega" device were 670 or 820 nm, 15 mW, 700 Hz (duty cycle 80%), the dose used was 6.3×10^3 J/m^2. The parameters of the He–Ne laser radiation were 632.8 nm, 15 mW CW, dose 6.3×10^3 J/m^2. The irradiation time was 10 s and the recording time of the spectrum (530–890 nm) was 600 ms. The irradiation and measurement procedures were performed nonstop after each other. The height of the monolayer segment exposed to the monitoring beam was equal to the height of the linear photodetector, and the width of the segment was equal to the spectrophotometer slit width (0.2×0.2 mm). The total area of the segment contained about 1×10^3 cells. The cuvette was fixed as shown in Fig. 2 during all experiment to irradiate (with all wavelengths) and measure after every irradiation exactly the same area of the monolayer. All preparation procedures, spectral measurements and laser treatment were performed at room temperature in the dark. During the measurements, the chamber with cells was at room temperature not more than 10 min.

III. RESULTS

Fig. 3 presents the absorption spectrum of the control cellular monolayer. This spectrum is typical for the cells grown in these particular conditions (cells were grown and the measurements were made without opening the cuvette to avoid changes in oxygen concentration and pH).
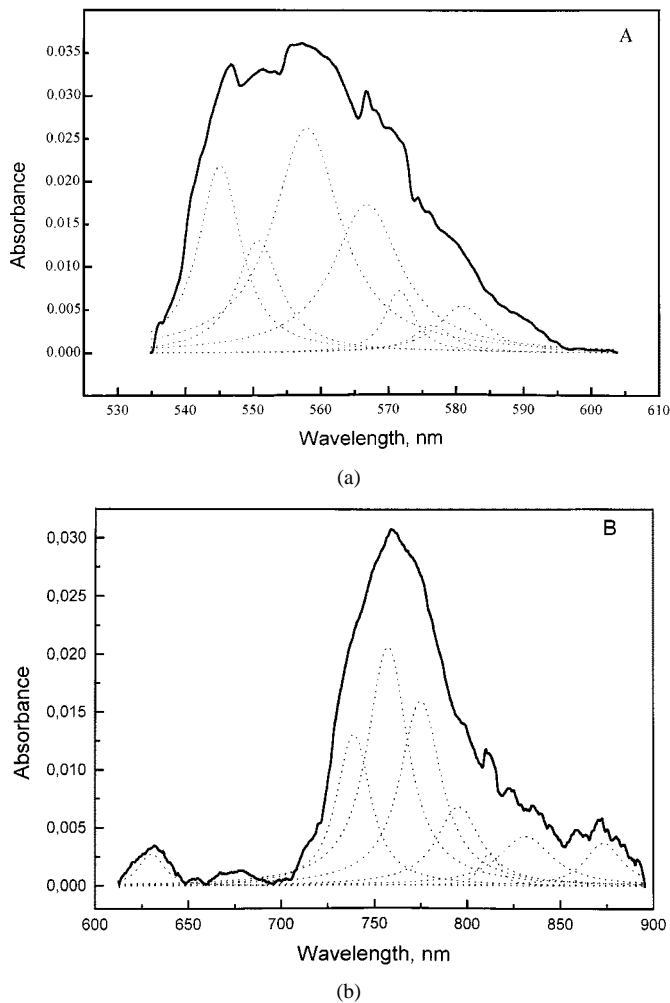
(a)

(b)

Fig. 3. (a) and (b) Absorption spectrum of non irradiated cell monolayer. The dashed lines mark the results of Lorentzian fit (data in Table I, spectrum 1).

The spectrum of the control cells is characterized by six peaks in the green region at 545, 551, 558, 567, 572, and 581 nm [Fig. 3(a)] and by four peaks in the red-near IR region (739, 757, 775, and 795 nm) [Fig. 3(b)]. There are also four peaks with low absorbance at 630, 812, 831, and 873 nm) in this spectrum. Table I presents the data of Lorentzian fitting of the spectra.

After recording the initial absorption spectrum (Fig. 3(a) and (b), the same cells were irradiated at 820, 670, 632.8, and 670 nm (dose 6×10^3 J/m^2). The absorption spectrum was recorded after every irradiation procedure. The results of Lorentzian fitting of these spectra are presented in Table I. Irradiation with $\lambda = 820$ nm (first irradiation procedure) causes changes in the peak positions, as it can be seen by comparison the spectra 1 and 2 in Table I. The peaks at 630, 739, 775, and 795 nm disappear and a new peak at 858 nm appears. The peak at 831 shifts to 837 nm. Irradiation also causes alterations in the peak areas. Most significant increases in the peak area (integral intensity) occur at 757 nm (from 0.838 to 2.537, Table I) and at 812 nm (from 0.049 to 0.836, Table I). Virtually no changes can be detected in the green spectral region.

Irradiation of the cells at 670 nm (second irradiation procedure) causes appearance of a new peak at 556 nm which was not present neither in the control spectrum (Fig. 3(a)), nor in the

spectrum of cells irradiated at 820 nm (spectrum 2, Table I). At the same time, the peak at 558 nm changes its position to 561 nm and the peak at 757 nm shifts to 754 nm (Table I). New peaks appear at 799 and 820 nm (Table I). Areas (integral intensities) of the peaks at 754, 837, and 858 nm increase (spectrum 3, Table I) as compared to those in the previous spectra (1, 2, Table I). The area of the peak at 873 nm is the same as that in the spectrum of 820 nm-irradiated cells (spectra 2 and 3, Table I).

The third irradiation procedure was performed at 632.8 nm. Comparison of the spectrum of the control cells (Fig. 3) and that of the cells irradiated at 632.8 nm evidences disappearance of the peaks at 572, 739, 795, 812, 831, and 873 nm (Table I). The peaks at 572 and 873 nm were present in all previous spectra recorded so far (spectra 1–3, Table I). The peak at 739 was present only in the control spectrum (Table I). It was not detectable in the previous spectra 2 and 3 (Table I). A new peak, which was not present in the control spectrum 1, Table I), appears at 863 nm (spectrum 4, Table I). The spectrum 4 (Table I) is also characterized by a broad peak at 754 nm. The integral intensity (area) of the peak at 820 nm (which appeared only in the spectrum 3, Table I) is increased approximately eight times as compared with the previous spectrum (compare spectra 3 and 4 in Table I).

The last irradiation with $\lambda = 670$ nm causes two alterations in the green region. The peak at 580 nm disappears. Also, of new peaks at 715, 738, and 794 nm appear (spectrum 5, Table I). The peak at 715 nm is characteristic only for this particular spectrum, the peak at 795 nm is present in the control spectrum as well (spectrum 1, Table I). The integral intensity (area) of the peak at 819 nm is decreased and that of the peak at 863 nm is increased in comparison with the previous spectrum (spectra 4 and 5, Table I). Fig. 4 presents the absorption spectrum of the monolayer after the last (fourth) irradiation procedure (spectrum 5 in Table I).

IV. DISCUSSION

Optical measurements performed with cells, mitochondria, submitochondrial particles or purified enzymes of the respiratory chain are based on the circumstance that optical density as well as positions of the peaks in the absorption spectrum depend on the redox level of respiratory chain components. Previous experiments show that irradiation with different wavelengths can induce redox absorbance changes in mitochondria as well as in purified cytochrome c oxidase. Purified cytochrome c oxidase and the enzyme in isolated mitochondria was photoreduced by green light [12] as well as by UV radiation ($\lambda < 300$ nm) [13]. Pulsed laser light at 532 nm caused redox absorbance changes and electrogenetic events in partly reduced cytochrome c oxidase, indicating increased electron transfer from Cu$_B$ to Fe$_{a3}^{3+}$ [14]. In experiments [14] fully oxidized cytochrome c oxidase did not display any measurable absorbance changes. It has been found recently that He–Ne laser radiation causes *a* remarkable increase in electron transfer in purified cytochrome c oxidase as well as of that in isolated mitochondria. At the same time, activities of cytochrome c and cytochrome bc_1 complex were not affected by the irradiation [15]. Measurements of light-induced electronic events and absorbance indicate that irradiation can

TABLE I
PARAMETERS OF THE PEAKS IN ABSORPTION SPECTRA OF CELL MONOLAYERS

Before the irradiation (spectrum 1)				After the irradiation at															
				820 nm (spectrum 2)				670 nm (spectrum 3)				632.8 nm (spectrum 4)				670 nm (spectrum 5)			
Position, nm	Area	Width, nm	Height	Position, nm	Area	Width, nm	Height	Position, nm	Area	Width, nm	Height	Position, nm	Area	Width, nm	Height	Position, nm	Area	Width, nm	Height
545	0.253	7.1	0.023	545	0.354	7.8	0.029	545	0.305	8.1	0.024	544	0.321	8.3	0.025	545	0.228	8.3	0.022
551	0.161	7.6	0.013	550	0.374	9.7	0.025	551	0.221	8.2	0.017	550	0.198	7.9	0.016	551	0.157	7.2	0.014
-	-	-	-	-	-	-	-	556	0.154	6.6	0.015	556	0.237	8.7	0.017	556	0.185	7.4	0.016
558	0.492	11.8	0.027	558	0.527	11.3	0.030	561	0.335	9.6	0.022	561	0.350	10.9	0.020	561	0.272	9.1	0.019
565	0.328	11.9	0.018	565	0.300	10.3	0.018	567	0.172	6.9	0.016	568	0.369	11.4	0.020	568	0.135	7.0	0.012
572	0.056	4.6	0.008	571	0.194	7.6	0.016	572	0.075	4.4	0.011	-	-	-	-	573	0.355	15.2	0.015
581	0.072	8.2	0.006	581	0.051	5.1	0.006	580	0.062	6.3	0.006	580	0.303	17.7	0.011	-	-	-	-
630	0.654	13.3	0.003	-	-	-	-	-	-	-	-	-	-	-	-	715	0.108	11.3	0.006
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	738	0.571	23.8	0.015
739	0.430	19.5	0.014	-	-	-	-	-	-	-	-	-	-	-	-				
757	0.838	24.7	0.022	757	2.537	43.8	0.037	754	2.665	44.7	0.038	754	2.589	55.8	0.030	759	1.040	32.0	0.021
775	0.654	24.9	0.017	-	-	-	-					-	-	-	-	-	-	-	-
795	0.299	27.5	0.007	-	-	-	-	799	0.941	47.2	0.013	-	-	-	-	794	0.808	38.9	0.013
812	0.049	8.3	0.004	811	0.836	49.2	0.010					-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	820	0.251	20.0	0.008	819	2.093	62.5	0.021	819	1.650	50.2	0.021
831	0.228	33.3	0.004	837	0.295	30.0	0.006	837	0.407	25.2	0.010	-	-	-	-	-	-	-	-
-	-	-	-	858	0.170	19.9	0.005	858	0.366	24.7	0.009	863	0.420	28.2	0.009	863	0.708	39.5	0.011
873	0.134	21.9	0.004	872	0.174	19.3	0.006	873	0.174	15.4	0.007	-	-	-	-	-	-	-	-

induce structural changes in cytochrome c oxidase ([12]–[15]). These experiments were performed with the purified enzyme. First experiments with irradiation of living cells evidence that light can induce redox absorbance changes is this case as well ([11], reviews [2], [3]).

However, respiratory chain enzymes, especially cytochrome c oxidase, react differently *in vivo* than *in vitro* experiments with isolated mitochondria [9], [10],[16]. Primary difference is the steady redox state of cytochrome c oxidase. It appeared to be more reduced *in vivo* at any state of metabolism [14]. For example, hemes a and a_3 are 40 or even 50% reduced and become more oxidized when oxidative metabolism is increased. This is the opposite of what observed in vitro experiments [9]. Also, the control of respiration *in vivo* by cytochrome c oxidase is much tighter than it was assumed on the basis of experiments carried out on isolated mitochondria [16]. One reason why the absorption spectra recorded *in vivo* and *in vitro* are different, is probably a disturbed (and damaged in purification process) molecular environment of copper atoms Cu_A and Cu_B [10].

Respiratory chains of cells grown in closed vials with limited amount of oxygen (our experimental conditions) are evidently more reduced than the respiratory chains of isolated mitochondria. Also, the vicinity of copper atoms of cytochrome c oxidase is intact and not disturbed in case of whole cells. The absorption spectrum of an air-dried monolayer (in this case cytochrome c oxidase is fully oxidized) is presented in Fig. 5. This spectrum has bands with maxima at 620, 680 (with a shoulder at 665 nm), 810, and 870 nm and bands with week absorption near 715, 730, and 765 nm. The spectrum recorded in the closed vial has strong absorption peaks at 739, 757, and 775 nm and weak maxima at 795, 812, 831, 873 as well as at 630 nm (Fig. 3, Table I). Comparison of two spectra in Figs. 3 and 5 allows us to make a rough estimation that the peaks in red (620–680 nm) and near IR region (812–870 nm) of spectrum in Fig. 3 are characteristic to the absorption spectra of more oxidized cytochrome c oxidase and the peaks in the region 730–775 nm are characteristic to the spectra of more reduced cytochrome c oxidase.

With irradiation, the most remarkable changes in the absorption spectra occur with the peaks at 739–799 nm and with those at 812–873 nm (Table I). There are practically no changes in absorption in the red region (600–700 nm) and a few changes occur in the green region (peaks at 545–581 nm). Comparison of the spectra in Figs. 3–5 suggests that the spectrum in Fig. 5 belongs to cytochrome c oxidase in a more oxidized state than that in Fig. 3. In other words, cytochrome c is more oxidized after four irradiation procedures. When cytochrome c oxidase becomes more oxidized *in vivo*, it indicates that the oxidative metabolism is increased [9].

Electronic excitation of absorbing chromophores in cytochrome c oxidase alters their redox status [17]. Based on the earlier identification of peaks in the action spectra [5], we supposedly excite Cu_{Aoxid} by the irradiation at 820 nm (spectrum 2), then Cu_{Boxid} (670 nm, spectrum 3), then Cu_{Ared} (632.8 nm, spectrum 4), and finally once more Cu_{Boxid} (670 nm, spectrum 5). One has to take into account that the peaks in cytochrome c oxidase spectrum are caused by absorption not only by one chromophore. The exact contribution of different chromophores in absorption bands is not clear. For example, it is known that *in situ* only 85% of the absorption at 820–840 nm is due to Cu_{Aoxid} [18].

The analysis of the action spectra performed earlier ([5], review [2]) allowed us to suggest that the peak at 760 nm is characteristics to Cu_B in a reduced state. How does irradiation effect this peak? The peak at 757 nm in the absorption spectrum is increased by all parameters (height, width, area) after the first irradiation (spectrum 2 in Table I). After the second irradiation, two peaks appear at 754 and 799 nm instead of one (spectrum 3, Table I). After the third irradiation, only one large peak at 754 nm is recorded (spectrum 4, Table I). After the fourth irradiation (spectrum 5, Table I) two peaks (759 794 nm) are recorded again similar to the result of the previous irradiation at the same wavelength (670 nm, compare spectra 3 and 5 in Table I). In spectrum 5 (Table I), the peaks at 759 nm and 819 nm have almost equal optical density.
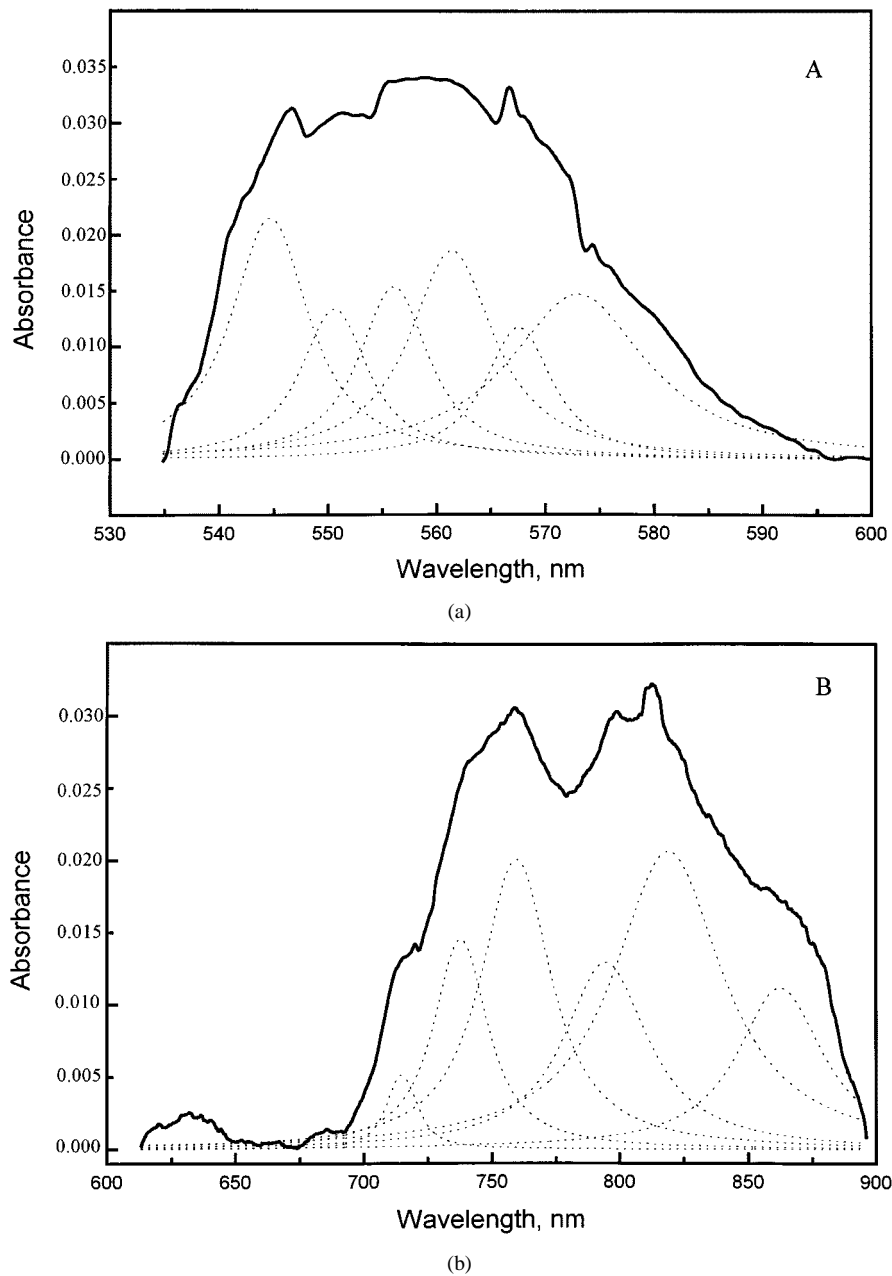
Fig. 4. (a) and (b) Absorption spectrum of the same cell monolayer as in Fig. 3 after the last (fourth) irradiation procedure. The dashed lines mark the results Lorentzian fit (spectrum 5 in Table I).
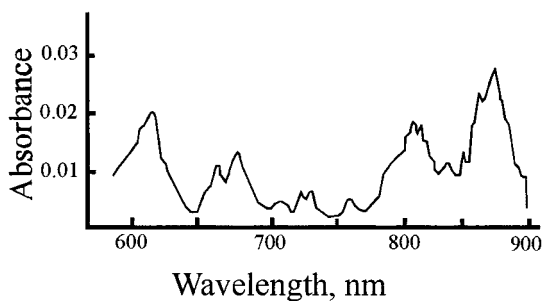

Fig. 5. Absorption spectrum of air-dried cell monolayer.

The following occurs with the peaks at 812–873 nm: the peak at 819 nm is not present in the initial spectrum 1 (Table I). It

appears only after the first irradiation at 670 nm (spectrum 3, Table I) and grows significantly after irradiation at 632.8 nm (spectrum 4, Table I). Another peak (858–863 nm) in this spectral region is not present in the initial spectrum (spectrum 1, Table I) but appears and grows after irradiation (spectra 2–5, Table I).

The peak at 873 nm which was weak in the control spectrum (spectrum 1, Table I), enlarges after the irradiation both at 820 nm (spectrum 2 in Table I) and 670 nm (spectrum 3 in Table I) but disappears completely following irradiation at 632.8 nm (spectrum 4, Table I) and 670 nm (spectrum 5, Table I). The peak near 870 nm was also recorded *in vivo* alongside with the maximum at 820 nm [10]. The identity of the components giving rise to two bands is not yet determined but it has been hypothesized [10] that these two bands in the near IR region (near 820

and 870 nm) are contributed separately by the Cu_A and Cu_B and that they are in some redox communication outside of the heme a $\rightarrow$ heme a_3 redox pathway. Many attempts with isolated mitochondria were unsuccessful in eliminating one or the other of the two bands or in reducing or oxidizing them differentially with inhibitors [10].

Our data is not ambiguous for exact identification of absorption peaks in the spectra recorded. Remarkable redox absorbance changes near 750–760 and 820–870 nm suggest that the irradiation induces structural (and respectively functional [19]) changes near Cu_A and Cu_B chromophores. The alteration of peak parameters (width, height, area) after the irradiation (peaks at 750–760 nm, Table I) indicates that the structure of a_3-Cu_B site (probably ligand–metal interactions) changes. Changes in absorption at 754–759 nm are accompanied with changes in circular dichroism spectra in this spectral region [22]. This finding confirms that conformational changes occur as well due to the irradiation. It is known that even small structural changes in the binuclear site may control both rates in the dioxygen reduction and rates of internal electron and proton transfer reactions [19]. The illumination of partially reduced cytochrome c oxidase at $\lambda = 532$ nm resulted in an increase of electron transfer from Cu_{B+} to Fe_{a3}^{3+} as a response to structural changes [19]. It was also shown in the work [19] that the light-induced events in one-electron-reduced cytochrome c oxidase reveal structural changes in the a_3-Cu_B site with characteristic time constants of catalytic events in dioxygen reduction by reduced cytochrome c oxidase.

The green spectral region is specific for absorption peaks of cytochromes b and c [17]. Irradiation in our experimental conditions causes little changes in this region (Table I). However, some changes can be seen in spectra 3, 4, 5 (Table I): appearance of new peaks at 556 and 561 nm, disappearance of that at 558 nm and a shift of the peak at 565 nm to 567–568 nm. Maxima at 565 and 558 nm are considered to be specific for cytochrome b_T, *and* that at 561 nm is characteristic for cytochrome b_K [20]. Cytochrome b_T and b_K are two forms of cytochrome b which differ functionally. They also respond quite differently to the energy state of mitochondria [20]. The peaks characteristic for cytochrome b_T and b_K were recorded also *in vivo* [21]. One can speculate that the radiation of red-near IR region which is expected to be absorbed by copper chromophores of cytochrome c oxidase and activates the enzyme functionally, causes changes in other components of the respiratory chain as well. This suggestion, however, needs further experimental proof.

V. CONCLUSION

Our experimental results demonstrate that irradiation with different wavelengths (632.8, 670, and 820 nm) causes changes in the absorption spectrum of HeLa cells in two regions near 754–759 nm and 819–873 nm. These changes can be interpreted as an increase of functional activity of cytochrome c oxidase. The changes in the absorption spectrum depend on the wavelength used for the irradiation but the tendency that cytochrome c oxidase became more oxidized can be seen throughout the whole experiment. This "nonspecifity" of wavelengths clearly needs more detailed investigation. Also, when the same cells are irradiated with different wavelengths, a cumulative effect can appear. Our results support the suggestion made earlier [4] that the mechanism of low power laser therapy on cellular level is based on increase of oxidative metabolism in mitochondria caused by electronic excitation of components of the respiratory chain. Our results also evidence that various wavelengths (670, 632.8, 820 nm) can be used for an increase of oxidative metabolism in mitochondria. Recall that the peaks at 620, 680, 760, and 820 nm were recorded earlier in various action spectra for HeLa cells in red-near IR spectral region (reviews [2], [3]). The wavelengths used in the present study for the irradiation were chosen in accordance of these previous results on the one side and in accordance of the most popular wavelengths used in the laser therapy (He–Ne laser at 632,8 nm and semiconductor laser or LED at 820 nm) on the other.

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