EFFECT OF VISIBLE LASER RADIATION ON CULTURED CELLS

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I. INTRODUCTION

A developing therapeutic role for laser phototherapy in treating patients with skin diseases[1] has led to interest in the effects of visible light on cultured cells. The need to examine the action of different visible radiation wavelengths upon cellular cultures arises partially from the knowledge that disorders which respond to laser phototherapy, such as indolent wounds and trophic ulcers [2,3] may be associated with increased proliferation of cells surrounding the injuries [4].

To investigate the mechanism of wound healing by laser light, we looked for a model system which would more closely resemble tissue cell population (as compared with cultures of, e. g., microorganisms), while still permitting the simplicity of in vitro manipulation. Usually mammalian cellular cultures in an actively proliferating state (exponentially growing cultures) are used as models in such experiments.

In this type of culture a large number of cells is traversing the cell cycle. Actually, the cellular populations in vitro are heterogeneous, containing a number of subpopulations. In terms of the diversity of cellular subpopulations, cultured mammalian cell populations in the plateau phase of growth are close to cellular populations in vitro [5,6]. Plateau phase cellular cultures as model systems have been proved to be useful for studies of cellular effects of chemotherapeutic drugs and γ-radiation[7,8]. In our experiments we used both types of models (exponentially growing and plateau phase cells). The effects of laser irradiation on colony forming ability, DNA and RNA synthesis and progression through the cell cycle will be described.

Literature available to us contained no data about the action of low power laser light on the proliferative activity of plateau phase cells. In experiments with exponentially growing populations using radiation from various visible light lasers, the irradiation has been found to act in a stimulating, indifferent or even inhibiting manner on the proliferation of cellular cultures. These data are reviewed in detail in Reference 9.

Some experiments performed to explain the quantitative effects of monochromatic visible light on various microorganisms and cellular cultures, as well as the possible mechanisms have been discussed earlier in the In-

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ternational School of Quantum Electronics Course on "Laser Photobiology and Photomedicine" [10]. Some data may also be found in References 11 and 12.

II. CELLULAR CYCLE

Cell proliferation encompasses the overall dynamics of a cell in passing from its initial resting state through the entire division procedure, including its interaction with the remaining cell population[13]. The chain of events which a cell passes through while proceeding towards division has been termed the cell cycle. A typical cell cycle can be represented schematically as shown in Figure 1. It consists of a mitotic phase (M), a G_2 phase, a DNA synthetic phase (S), and a G_1 phase prior to next division. A cell can leave the cycle either during the G_1 or the G_2 period, entering into G_0 (resting or quiescent) state[13]. Replicative synthesis of DNA occurs only during the S period of cell cycle, reaching a maximum approximately midway through it [14], and is easily monitored by administering a radioactive precursor of its synthesis, thymidine. Synthesis of RNA occurs during G_1, S and G_2, but undergoes a two-fold increase in rate during the first half of the S phase [15,16] and can be monitored by means of a radioactive precursor of its synthesis, uridine (Figure 1).

In our experiments, He-La cells were cultivated as monolayers in scintillation vials with a bottom diameter of 24 mm, in 2 ml of nutrient medium (199 synthetic medium, supplemented with 10% calf serum and 100 units/ml kanamycine or lincomycine). Under these conditions the growth curve of the population resembles a typical curve of mammalian cells growth in vitro. The curve presents an initial phase in which cell number does not increase (lag phase), followed by an exponential region (log phase), and finally by a plateau phase. The laser irradiation experiments were performed when the number of cells in the vial was from 2.5 to 6± 0.2 x 10^5 (exponentially growing population) or 1.0 ± *0.3* x 10^6 cells (plateau-phase population). The labeling index (I_s) of the log-phase population was 19.1 ±3.0 %, and that of plateau-phase culture was 5.0 $^+$ 0.8 %. The mitotic indices (I_m) of the log-phase and plateau-phase populations were 1.1 $^+$ 0.1 % and 0.1%, respectively.

III. VIABILITY AND CLONIGENITY OF PLATEAU-PHASE HeLa CELLS AFTER IRRADIATION WITH He-Ne LASER

To investigate the viability and clonigenity of HeLa cells, the plateau-phase cultures were irradiated with a He-Ne laser and replated at various intervals after the irradiation[17]. Using this technique, not all subpopulations will be attached and start to divide after the replating. Figure 2 illustrates the diversity of subpopulations of a plateau phase cell culture [6], and shows the clonigenic cells starting to divide after trypsinization and replating.

In the first series of experiments we studied the growth curves and, in the second series, the clonigenity of cells by the Puck technique after the irradiation of plateau-phase HeLa cells with a He-Ne laser.

A He-Ne laser (model LG-52/1, λ = 632,9 nm) was used for irradiation (I1=5 W/m^2, t = 20 sec, D = 100 J/m^2). This dose has been found to be optimal to stimulate synthesis of DNA[17-20] : a short focus positive lens was used to expand a beam from a light source to a diameter of 24 mm, equal to that of the monolayer of cells. The cells were irradiated in darkness through the bottom of the flask. During irradiation the flask was attached to a special support. At various intervals (from 5 to 240 min) after the irradiation the monolayer was trypsinized and the cells were replated into fresh nutrient medium.

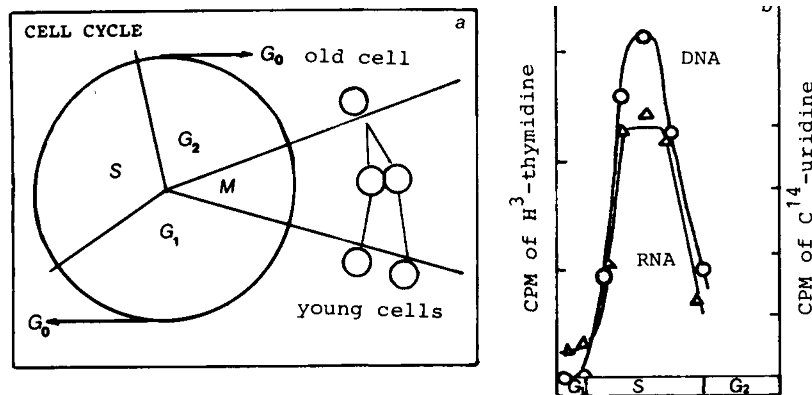


Fig. 1. Schematic diagram: (a) of the cell cycle of a mammalian cell, and (b) the synthesis of nucleic acids in the cell cycle.

The laser growth stimulation during the exponential phase of growth was observed to last 6-7 days when the interval between irradiation (λ= 632,8 nm, Δ = 100 J/m^2) and plating was 30 min and more (Figure 3). Worthy of notice is the difference in the shape of growth curves between the irradiated and non-irradiated cultures. In the control, beginning from the 8th day there appears the plateau-phase of growth and the number of cells in the flasks does not change till the 15th day. As distinct from the control, in the irradiated cultures where the interval between the

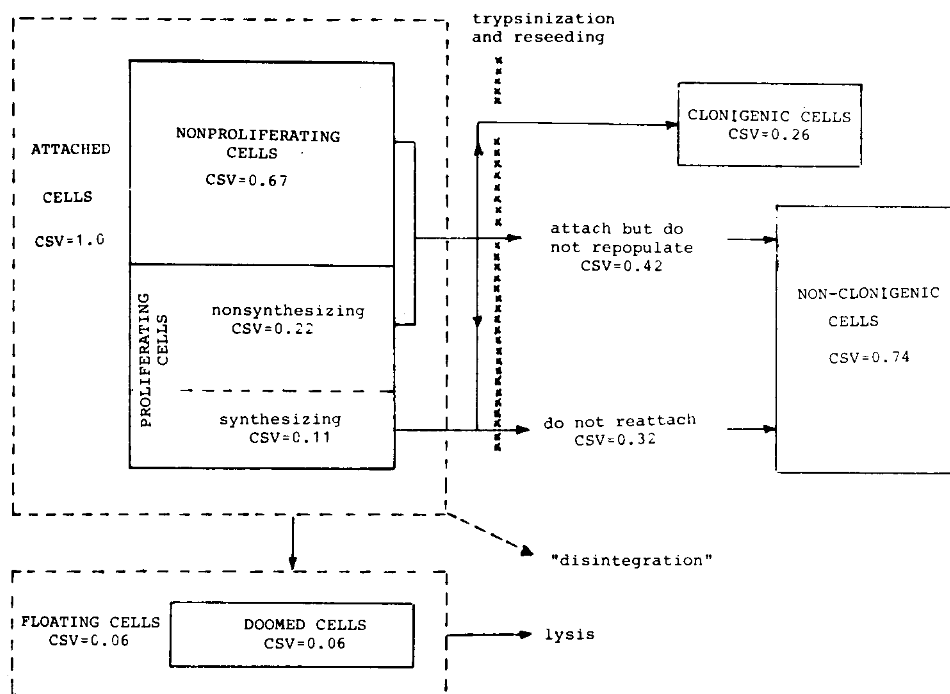


Fig. 2. Scheme of compartments of a plateau-phase population of Chinese hamster cells[6].

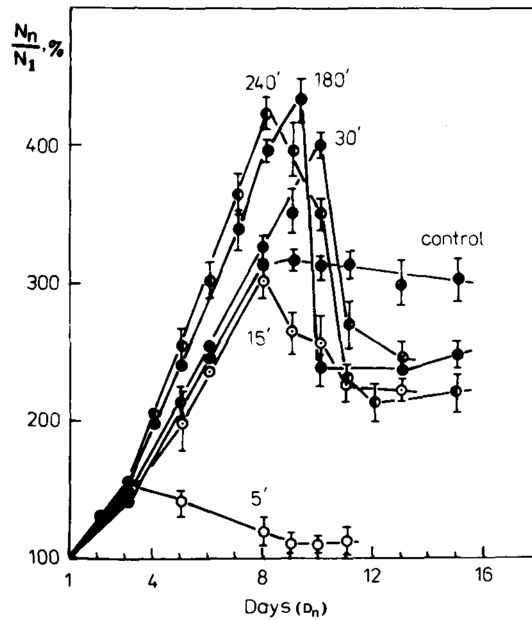


Fig. 3. Subcultivation growth curves of HeLa cells following irradiation of plateau-phase cultures with a He-Ne laser with 100 J/m^2 dose and inoculation of the irradiated cells into fresh nutrient medium 5, 15, 30, 180 or 240 min after irradiation. The ordinate gives the ratio of the number of cells on day n (N_n) to the number at the end of the first day (N_l), and the abscissa gives the days (D_n) after plating.

irradiation and plating ranged between 30 and 240 min, the number of cells decreases sharply at the end of the long phase of growth (8th-9th day), and the plateau occurs below that in the control and practically at one and the same level for all groups, except for the culture for which the interval between irradiation and plating was short (5 min). In the latter case, the culture stopped growing altogether in three days (Figure 3).

The differences in growth kinetics between the control and test groups of cells can apparently be explained as follows. As an effect of the radi ation, in the plateau-phase cells there occur changes which become mani fested upon subcultivation. On the one hand, these changes are due to the acceleration of proliferation with subsequent loss by some members of the population of the ability to enter the plateau phase of growth. Such a phenomenon was observed when irradiating plateau-phase HeLa cells with small doses of γ-radiation (0.1 Gy)[21]. On the other hand, the sharp cell growth retardation in the case of 5 min interval between irradiation and plating is evidently explained by the fact that the action of He-Ne laser radiation is in this case a sensitizing factor aggravating the damage to the cells due to subcultivation.

We also studied the influence of irradiation on the number of clonigenic cells as well as on the clone size distribution, by the Puck tech nique [22]. The plateau phase He-La cells were irradiated with a HeNe laser at a dose of 100 J/m^2, trypsinized 180 min after irradiation, resuspended in Hanks solution and counted with a hemocytometer [11]. Suitable delutions were made and the same number (100) of cells was inoculated into all vials, both control and test.

after 14 days. The percentage of clonigenic cells in the nonirradiated cul ture was 45.2± 0.4 %, and this number increased after irradiation, reaching 50.4±0.5, 58.3± 0.4 and 54.5±0.9 % at irradiation doses 10, 10^2 and 10^s J/m^2, respectively. This increase of the plating efficiency may be due to increased attachment of cells caused by irradiation. The histograms of the frequency distribution of clone sizes are presented in Figure 4. The clones were counted in four groups: clones with diameter 0.25 mm and less (abortive clones), from 0.25 to 0.35 mm (small clones), from 0.35 to 0.5 mm (middle clones), and from 0.5 to 0.8 mm (big clones).

As seen in Figure 4, the per cent of abortive clones practically does not change after irradiation as compared with the non-exposed control. The per cent of small clones decreased and the number of medium and large clones increased in a dose-dependent manner. The size of the large clones did not increase. Therefore the stimulative effect of He-Ne laser irradi ation is most noticeable on the proliferative activity of the slowly-growing subpopulations (the slowly dividing cells yield small and medium clones). After irradiation the clone-size distribution becomes more homogeneous. For example, when irradiating with a dose of 100 J/m^2, the percentage of small, medium and large clones is almost equal (near 30%). In the control experiment the distribution was 42:36:11 %.

From this group of experiments it is possible to conclude that irradi ation with a He-Ne laser stimulates the proliferation of He-La cells under

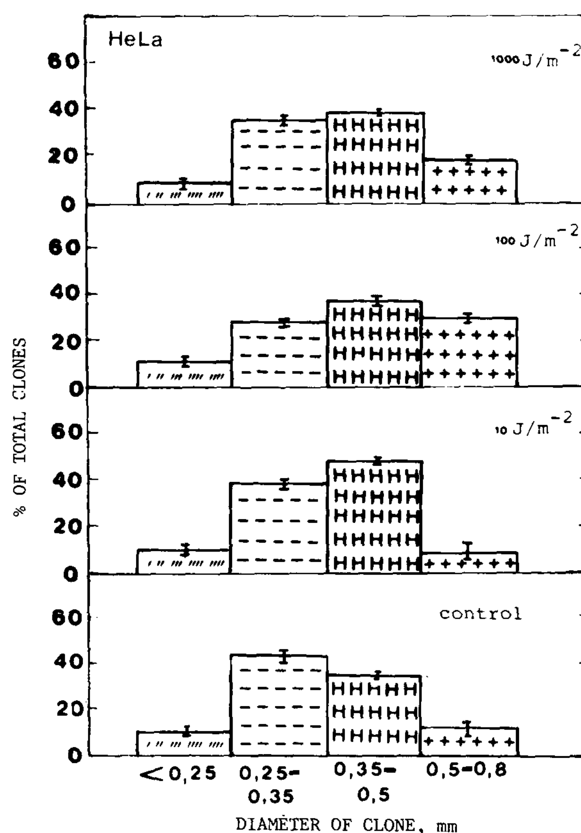


Fig. 4.Histograms of clone-size distribution of cell population at 14 days after the irradiation of He-La cells with a He-Ne laser (D = 10, 100 or 1000 J/m^2).

our experimental conditions (irradiating the plateau phase cells, with post-irradiational replating).

IV. CHANGES IN DNA AND RNA SYNTHESIS RATE AFTER THE IRRADIATION

Replicative DNA synthesis occurs in the S phase of the cellular cycle (Figure 1), and synthesis of RNA occurs through G_1, S and G_2 with a maximum in the S phase[15]. The synthesis rates can be monitored by means of radioactive precursors, ^{3}H-rhymidine and ^{14}C-uridine respectively. These two parameters were used to characterize the quantitative laws of laser action (dependence on the wavelength, dose, intensity and irradiation regime) [4, 17-20,22, 24].

Reparative DNA synthesis occurs during the entire cell cycle. DNA, RNA and proteins do not absorb visible light[25], and the energy of visible light photons is too small to cause damage to these molecules by molecular bonds (as in the case of γ-radiation, for example). For these reasons we believe that in our case we are dealing with replicative DNA synthesis.

IV.1. Exponentially growing He-La cells

Dose dependence. The dose dependence of the DNA synthesis stimulation effect in proliferating HeLa cells was determined at various wavelengths and it was found that the effect was different in the red and blue-ultra violet parts of the spectrum. In the red and far red regions the optimal dose was 100 J/m^2 (Figure 5) and it was relatively insensitive to the wave length. In the blue-ultraviolet region the dose causing maximal effect was lower by about an order of magnitude (see λ = 404 nm as an example in Figure 5). The dose dependence of the RNA synthesis stimulation effect in proliferating HeLa cells is shown in Figure 6.

Role of light intensity. In next series of experiments, the influence of light intensity on the DNA synthesis stimulation effect was studied. In

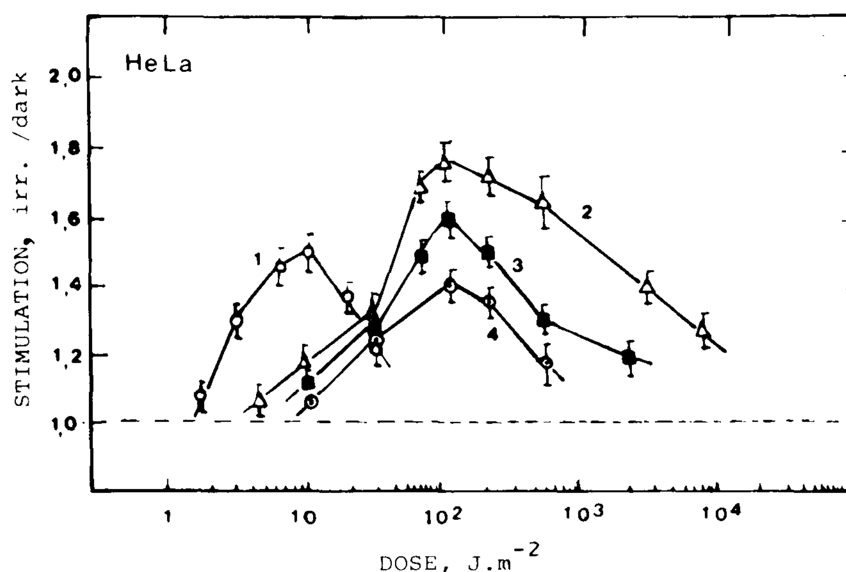


Fig. 5. The effect of cw light on stimulation of DNA synthesis inexponentially growing HeLa cells (1 - λ = 404 nm; 2 - λ = 760 nm; 3 - λ = 620 nm; 4 - λ = 680 nm) 1.5 h after irradiation.

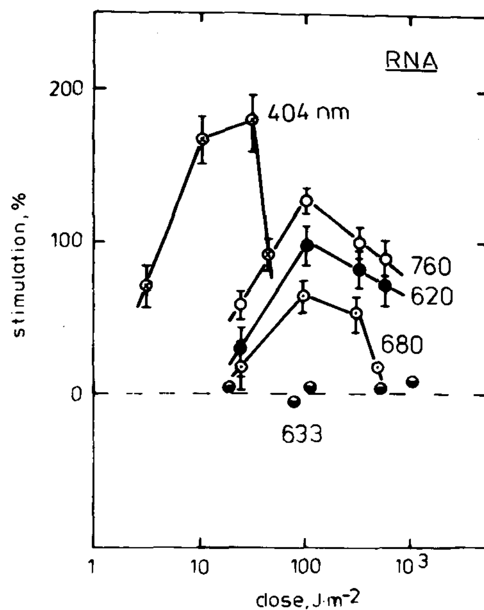


Fig. 6. The effect of cw light irradiation dose on stimulation of DNA synthesis in exponentially growing HeLa cells (☻ - λ= 404 nm; o- λ = 760 nm; • - λ = 620 nm; ⊙ - λ = 680 nm; **O** - λ= 630 nm) 1.5 h after irradiation.

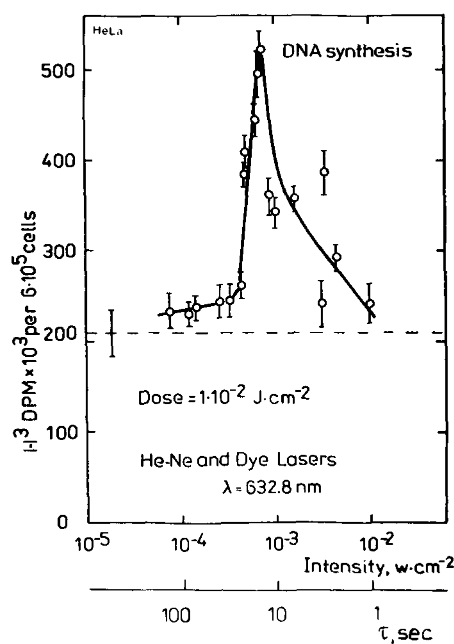


Fig. 7. The influence od cw light intensity at \ = 633 run (D = 10 J/m^2 = const.) on the rate of DNA synthesis in exponentially growing HeLa cells (H^3 - thymidine incorporation was measured 1.5 h after irradiation.

experiments with cw light at λ = 633 nm the radiation dose D = I x τ received by the cells was changed by varying the intensity of light I and the duration or irradiation T. A possible influence of the duration or of the radiation intensity on the stimulation effect in the case of a fixed dose is quite possible. This measurement was verified by using He-Ne laser ra diation (I_{max} = 10 W/m^2) as well as radiation from a cw dye laser pumped by an argon laser (λ_{out} = 633 nm, I_{max} = 80 W/m^2). The intensity of light was attenuated with calibrated neutral density filters. The radiation dose received by the cells was constant and amounted to 100 J/m^2, which was optimal for the rate of DNA synthesis in a culture of proliferating HeLa cells (Figure 5).

The results of measurements of the influence of the intensity of light on the rate of DNA synthesis are presented in Figure 7. It is clear from this figure that the DNA synthesis stimulation was very sensitive to the irradiation time and to the intensity of .light when the light intensity was 8-10 W/m^2 or when the irradiation time was 10-12 sec. It decreased very rapidly with an increase in the irradiation time or a reduction in the light intensity, being observed clearly during an irradiation time from 2 to 20 sec in our experimental conditions.

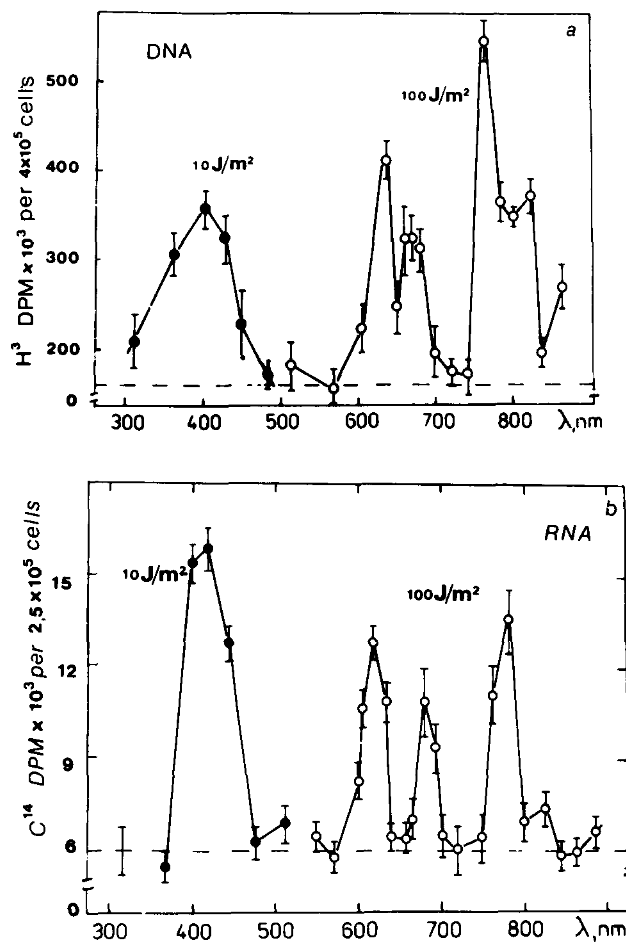


Fig. 8. Action spectra of visible monochromatic light on (a) DMA and, (b) RNA synthesis in exponentially growing HeLa cells, measured 1.5 h after irradiation.

Dependence on wavelength. Figure 8 shows the results obtained from measuring the action spectra of light, on the nucleic acid synthesis rate 1.5 h after the irradiation of proliferating HeLa cells. From these figures (Figures 8a and 8b) it follows that the synthesis rate of DNA and RNA increases in some spectral intervals. Stimulation of DNA synthesis can be observed in a wavelength range of about 320 to 450, 600 to 650, 660 to 720, 740 to 840 nm with maxima nearby 400, 630, 680 and 760 nm. In the infrared region there is a structure in the form of a peak near 820 nm. The action spectrum of RNA synthesis is very close to the corresponding action spectrum of light on DNA synthesis. The maxima in the stimulation of RNA synthesis correspond approximately to the wavelengths of 400, 615, 680, 780 and 820 nm. In the range from 450 to 580 nm, we have not observed statisstically valid changes in the DNA and RNA synthesis rate under these experimental conditions. Such an effect might be detectable when other para meters (irradiation time, intensity) are used. It is quite possible that intensity dependences are not similar for all spectral regions.

IV.2. Plateau-phase HeLa cells

As concerning the plateau phase HeLa cells, the irradiation causes changes in DNA and RNA synthesis beginning 2 to 2.5 h after the irradiation (Figure 9). The maximum effect is observed 4 to 6 h after irradiation. A comparison of results for DNA and RNA synthesis (Figures 9 and 10) gives grounds for assuming that the changes in the synthesis rate of DNA during the first 3 h after irradiation occur faster than for RNA. This conclu sion, however, needs further investigation. The results of the measurements show that the stimulation of DNA and RNA synthesis is maximum at irradiation doses 100 J/m^2, practically the same as for proliferating cells.

The action spectra of DNA and RNA synthesis stimulation in plateau-phase HeLa cells were measured for a dose 100 J/m^2 in the range of wavelengths from 580 to 880 nm (Figure 11). These action spectra obtained are very similar to the ones observed earlier for proliferating HeLa cells (Figure 8).

The basic difference in the reaction of cells of the plateau culture as compared with that of exponentially growing cells is that their stimu lation after irradiation occurs several hours later than in the case of proliferating cells. This result can be explained by the deceleration of many metabolic processes in the plateau-phase cells.

V. CHANGES IN CELL CYCLE PARAMETERS AFTER IRRADIATION

From experiments performed with radioactive precursors, it was not clear whether the increased incorporation of H^3thymidine, was connected with the enhancement of DNA synthesis in the S-phase cells, with changes in the proliferation kinetics of population, e.g. shortening of the duration of the G_1 phase of the cell cycle of cells of the proliferative pool, or with an increase in the proliferative pool. To answer these questions we conducted the following autoradiographical experiments'*.

To determine the changes in the number of S-phase cells and M-phase cells after irradiation, the cultures were pulse-labeled at various times after the irradiation and fixed. The results of these experiments with exponentially growing cultures indicate (Figure 12) that the number of DNA-synthesizing cells increases during first 3-4 h after irradiation and then diminishes to the control level. On the basis of this curve, one can sup pose that the increased number of S-phase cells originates from a part of the G_1 phase population that is ready to pass into the S-phase.

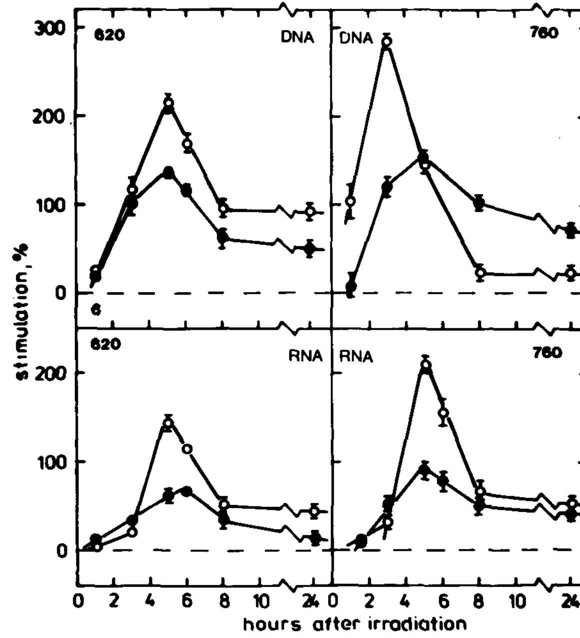


Fig. 9. The influence of irradiation with red (λ = 620 nm) and far red (λ = 760 nm) light on DNA or RNA synthesis in plateau-phase HeLa cells. Doses were (-o-o-o-) 100 J/m^2 or (-•-•-•-) 800 J/m^2 .

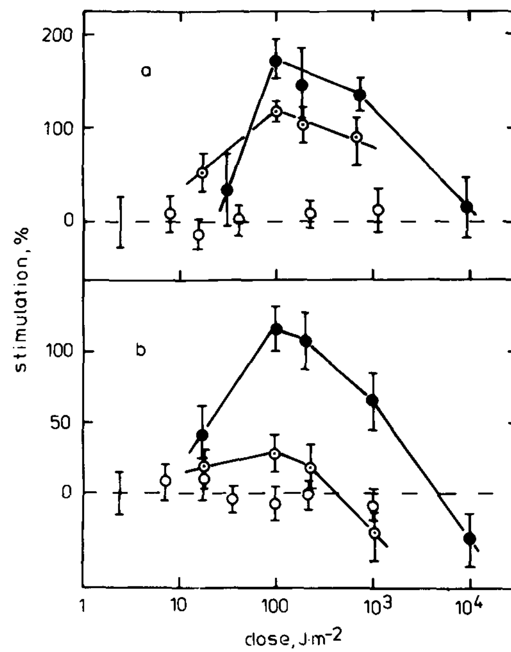


Fig. 10. The dependence of (a) DNA and, (b) RNA synthesis stimulation by an irr adiation dose of 100 J/m^2, for times 1.5 h (-o-o-o-), 3.0 h (-⊙-⊙-⊙-) and 6 h (-•-•-•-) after irradiation. Plateau-phase HeLa cells, irradiated with red light at λ = 620 nm.

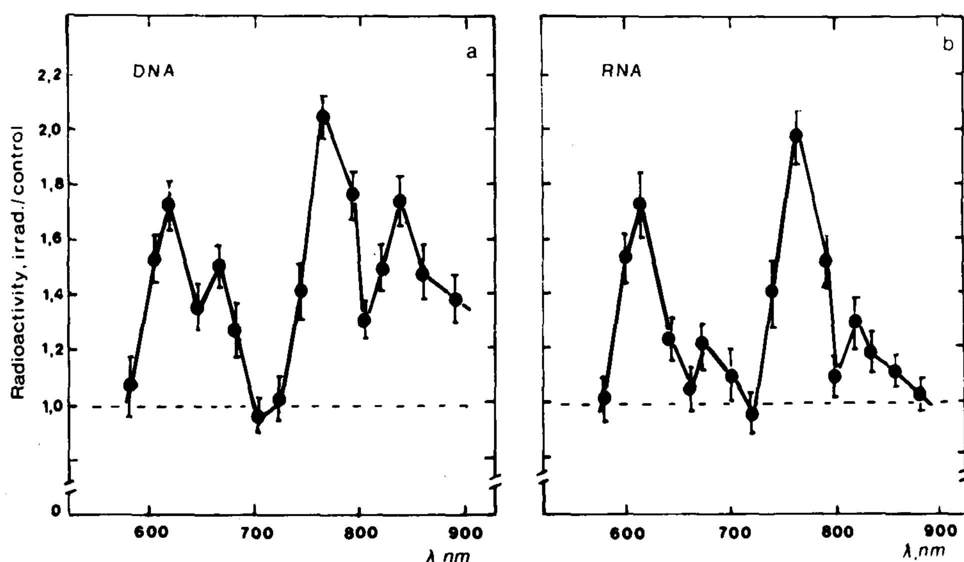


Fig.11.Action spectra of visible monochromatic light on (a) DNA and, (b) RNA synthesis in plateau-phase HeLa cells for an irradiation dose of 100 J/m ^{2}, measured 4.0 h after irradiation.

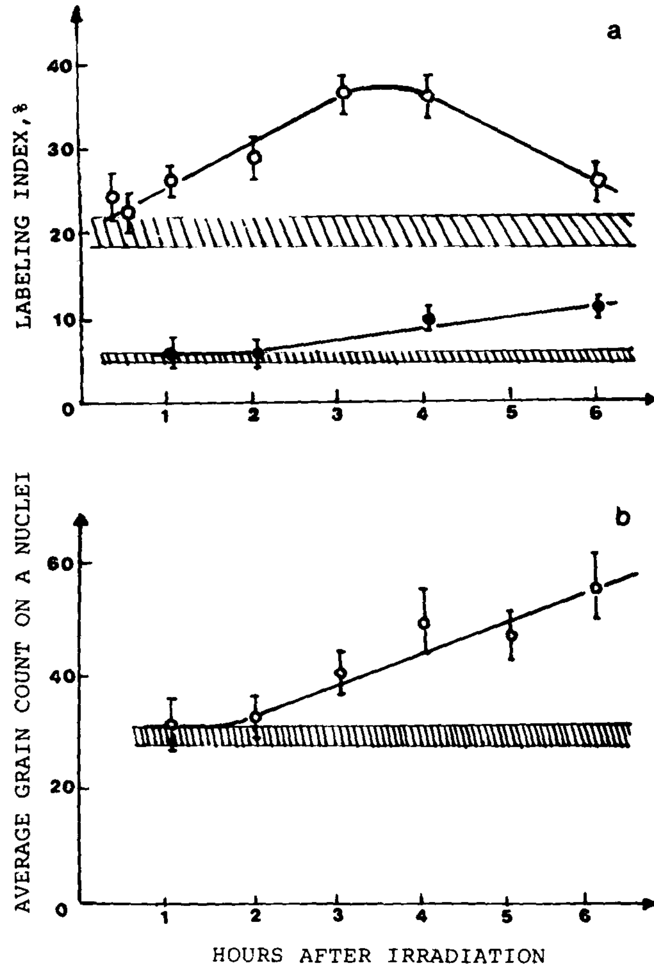
In case of plateau-phase cells (Figure 12), the increase of τ_s above the baseline level only starts 3-4 h after irrradiation, and is probably connected with $G_1 \rightarrow S$ transition of the cells of the proliferating fraction of the population, because 6 h is not sufficient time for a $G_o \rightarrow S$ transition."

The stimulative effect of the irradiation on the progression of G_1-phase cells into S-phase was confirmed in autoradiographic experiments with continuous labeling (Figure 13). When a cell population, immediately after the irradiation, is treated with H^3-thymidine and continuously incubated with it for hours, the fraction initially labeled represents the cells in S-phase at the moment of irradiation, while the subsequent increase in the Ig reflects the flow of the cells from G_1 into S during the interval studied. It is obvious from Figure 13 that the percent of labeled cells increases after the irradiation of both log-phase and plateau-phase populations.

To answer the question, does the irradiation influence the rate of DNA synthesis in S-phase cells , the number of grains was counted on the labeled nuclei. As seen in Figure 12b, the average grain count in the nuclei of cells increases after the irradiation, being detectable above the control level 3-6 h after the irradiation. The average grain count does not allow us to determine how the individual cells are influenced by irradiation. In Figure 14, we show the distributions of grain number per nucleus after the irradiation. The behavior of the cumulative curves indicates that the number of cells with higher grain counts increases following irradiation.

The percentage of cells in mitosis (I_m) does not change during the first few hours after irradiation (Table 1). The only significant difference from control level was noticed 6 h after the irradiation.

On the basis of these data it is possible to conclude that the enhanced incorporation of H^3-thymidine into DNA found earlier could be due to intensification of DNA synthesis in S-phase cells, as well as due to an



Pig. 12. Changes in: (a) labeling index and, (b) average grain count in (-o-o-o-) exponentially growing and (-•-•-•-) plateau-phase HeLa cells, pulse labeled with H^3-thymidine at various times after irradiation with a He-Ne laser at a dose of 100 J/m^2.

enhanced $G_1 \rightarrow S$ transition for a part of the population. Different kinetics of both these processes after irradiation makes it possible to suggest that in the first hours after the irradiation the increase of H^3-thymidine is caused mainly by an increased number of S-phase cells, but at longer times after irradiation (e.g. 6 h in our experiments) by enhanced DNA synthesis in S-phase cells.

Table 1. Changes in mitotic index of exponentially growing HeLa culture at various times after irradiation (D = 100 J/m^2, λ= 632.8 nm)

Time after irradiation, h	I_M, %
1.0	1.1 ± 0.1
2.0	1.3 ± 0.1
3.0	1 .2 ± 0.1
6.0	2.2 ± 0.1

VI. INFLUENCE OF DICHROMATIC IRRADIATION

The experiments described above showed that the effects were caused by relatively narrow-band radiation (±7 nm) . Therefore it is of interest to investigate the stimulation effect when the radiation spectrum is widened beyond the biologically active spectral intervals, going in the limit to white light.

A monolayer of cells at the bottom of a scintillation flask was ir radiated with stimulating red light (λ = 633 ± 7 nm) through the bottom of the flask and wide band radiation was directed to the cells through the open neck of the flask (arriving from the opposite side (Figure 15). A comparison was made of the effects of simultaneous irradiation of cells with red and wide band light with the effects of separate irradiations. The red light dose was optimal for DNA synthesis (100 J/m^2) and the dose of of wide band radiation was selected to be about 4 times greater. This ratio of doses corresponds approximately to the fraction of the red light (600-650 nm, 750-840 nm) in natural solar radiation to which the various biological objects have become adapted in the course of their evolution.

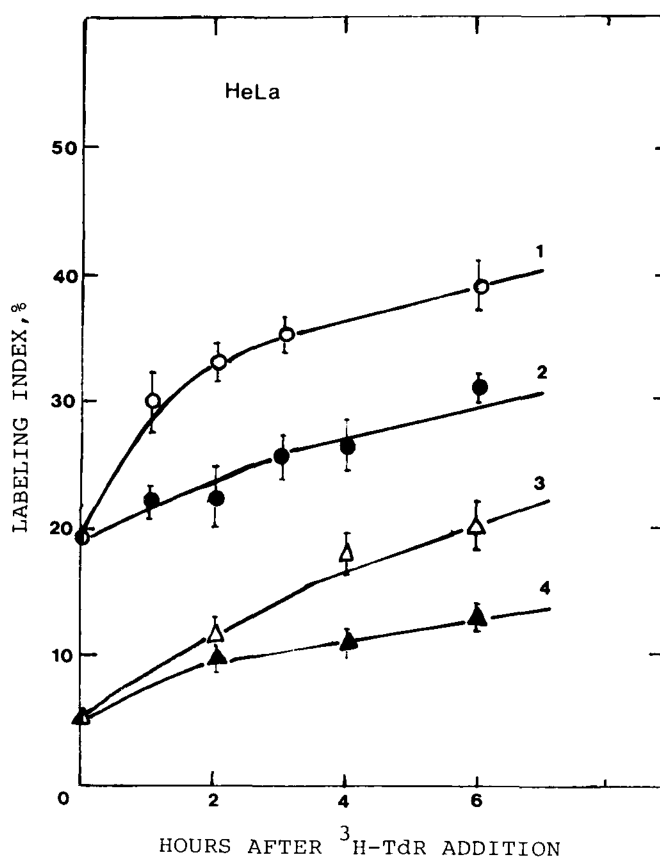


Fig. 13.The variation of the percentage of labeled cells during continuous labeling with H^3-thymidine in: (1, 2) the exponentially growing and, (3, 4) the plateau-phase. HeLa cells (2, 4), without irradiation or, (3, 4) after irradiation with He-Ne laser with a dose of 100 J/m^2.

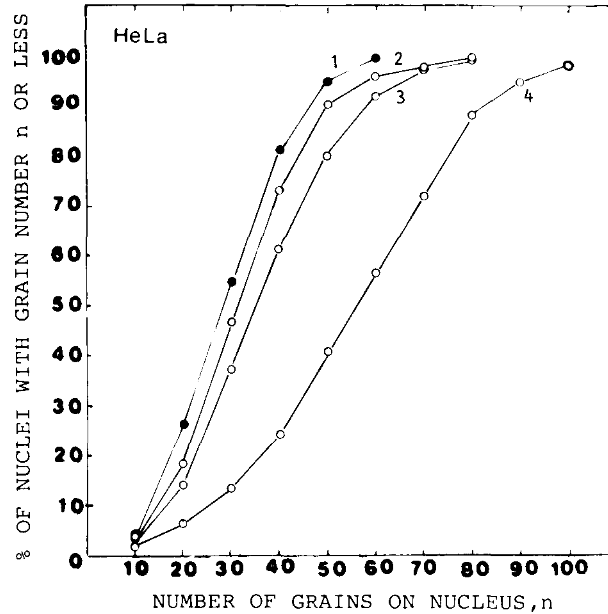


Fig. 14. Autoradiographically measured distribution of silver grains per nucleus of exponentially growing HeLa cells in: (1) control culture and, (2, 4) a culture irradiated with a He-Ne laser (100 J/m^2), for (2) 15 min, (3) 3 h, (4) 6 h after irradiation.

Figure 15 shows the results obtained. Irradiation of cells with just wide band light, including the red interval, in these experimental conditions resulted in practically no statistically significant variations in DNA synthesis as compared with the nonirradiated control.

The disappearance of the DNA stimulation effect (caused by irradiation with light 633 $^{+}$ 7 nm) when irradiating simultaneously with the narrow-band and the wide-band light from the blue-yellow region can be explained qualitatively as follows.

Light of the investigated range of wavelengths is not absorbed directly by DNA. Therefore, it is natural to assume that light is first absorbed by some other molecule or molecules M. The photoproducts formed as a result of reactions of an excited molecule M which acts as a light acceptor (dissociation and isomerization products, complexes with other molecules, etc.) should clearly influence the metabolic processes in cells.

We shall assume that light in the blue-yellow region is absorbed more effectively by the photoproducts of M than by the initial molecule M itself. Consequently, following the process $M \rightarrow M_{prod}$, as a result of the subsequent photoreactions, the concentration of the biologically active molecules M_{prod} decreases. The different efficiencies of the two processes in the spectral range under consideration may be due to differences between the absorption of the M and M prod molecules or due to the spectral dependences of the quantum efficiencies (yields) of the photoreactions involved.

We may assume that a reduction in the rate of DNA synthesis as a result of dichromatic irradiation is not due to the wide-band nature of light but due to the specification of those wavelengths which are within the investigated spectral range.

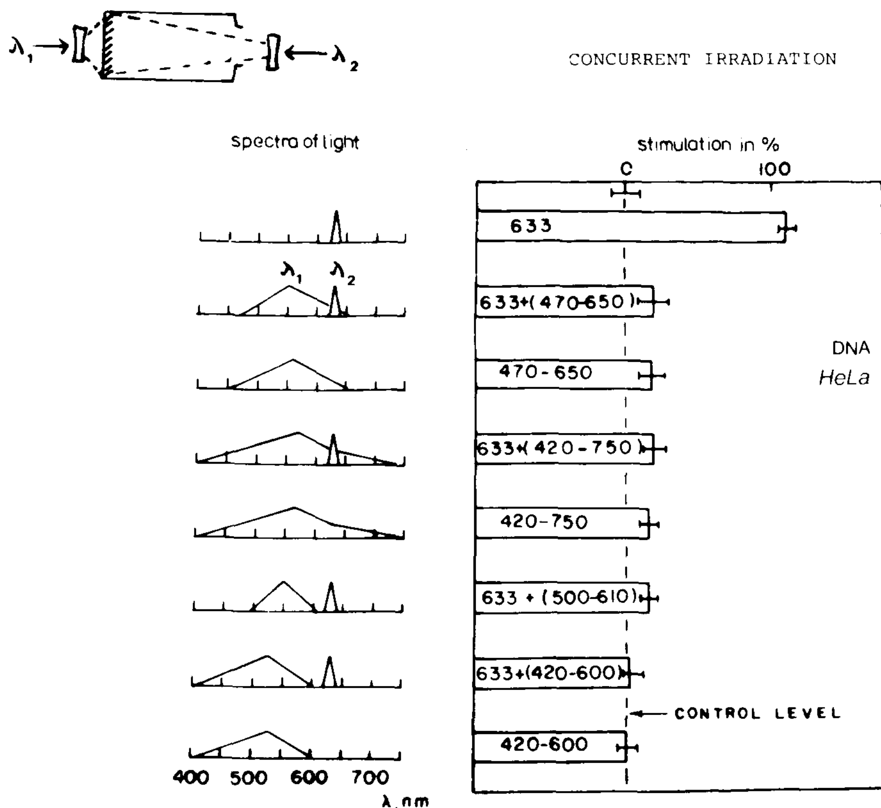


Fig. 15. Effects of broadening the spectrum of the irradiating light in simultaneous dichromatic irradiation with red (λ_2) and wideband (λ_1) light, on DNA synthesis in exponentially growing HeLa cells. Simplified spectra of the wide-band visible light (λ_1) and of the narrow-band stimulating red light (λ_2) sre shown on the left. The corresponding DNA synthesis rates are shown on the right. The dashed line is the control level, taken as 0 %.

Thus, it may be stated that low-intensity visible laser radiation causes clearly observable changes in the biochemical processes in a cell involving biomolecules which do not directly absorb laser radiation. Monochromatic light is needed but the spectral band can be 50-150 nm. This bandwidth is more or less 10^5 times wider than the spectral bandwidth of laser radiation (e.g.$\delta\ \lambda$ of He-Ne laser 10^{-3} nm). That is why the correlation between the stimulative effect of light and its monochromaticity in the range $\Delta\lambda >> \delta\lambda$ laser is not negligible.

Since there are several maxima in the action spectra of light-nucleic acids synthesis response (Figures 8 and 11) in our next experiments with dichromatic irradiation, we used those wavelengths that had been found to give maximal responses. Dichromatic irradiation experiments can answer the question of whether the hypothesized photoacceptor molecules are photoreversible pigments. In this way also, it is possible to determine those radiation wavelengths in the visible light spectrum which made the stimulative effect of the red component of the spectrum disappear (Figure 15).

HeLa cells in the exponential growth phase were cultivated and irradi-

ated [17],[19^20]. The rate of DNA and RNA synthesis was estimated 1.5 h after irradiation by a radiometric technique[24].

In the first series of experiments[24], HeLa cells in the form of a monolayer culture on the bottom of a scintillation vial were simultaneously irradiated with red light (λ = 632.8 nm) through the open neck of the flask and a variable wavelength monochromatic light (λ_{add}) through the bottom of the flask from the opposite side (Figure 16). The red light irradiation dose was always kept fixed at 100 J/m^2. The irradiation dose for the variable wavelength light was taken at 100 J/m^2 in the range (600-800) nm and 10 or 25 J/m^2 in the range (400-570) nm. Figure 16 presents the action spectra of dichromatic irradiation for the synthesis of DNA and RNA. In the red and far red regions of the spectrum (λ_{add} = 600-820 nm), the maximum at 620 nm remained the same as in the case of monochromatic irradiation (Figure 8) whereas that at 760 nm disappeared. With the irradiation dose for the variable .wavelength light λ_{add} set at 10 J/m^2 the maximum in the blue region of the spectrum, which was observed at 400 nm in Figure 8, now occurred at 450 nm, i.e. it shifted into a region of longer wavelengths. With the irradiation dose for λ_{add} increased to 25 J/m^2, the maximum in the blue region vanished and a new maximum appeared in the green region (λ = 550-570 nm). The synthesis of both DNA and RNA was observed to be inhibited in the green region when the irradiation dose for λ_{add} was set at 10 J/m ^{2}.

When the cells were subjected to consecutive dichromatic irradiation in the sequence λ_{add} + 633 nro with a 60 min interval between the successive irradiation events (Figure 17), these effects vanished and the respective action spectra for the synthesis of DNA and RNA became similar to those obtained earlier (Figure 8) for the wavelengths equal to λ_{add} in this spectral region. This means that only the first irradiation event proved effective, and, by the onset of the second irradiation event with λ = 633 nm, the system had changed into another state from which no further change was possible.

VI.1. Action of red and far red light upon HeLa cells

It is clear from results of the above experiments that the time inter-

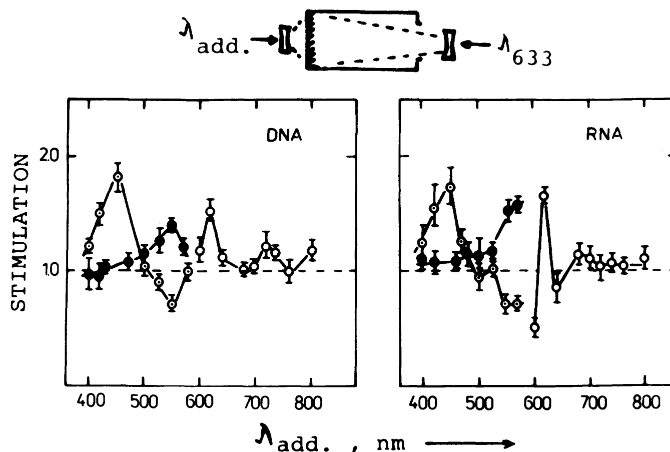


Fig. 16. Action spectra of concurrent dichromatic irradiation with λ = 632.8 nm and λ_{add} on the synthesis of DNA and RNA in exponentially growing HeLa cells, measured 1.5 h after irradiation. D632.8 = 100 J/m ^{2}; Dλ_{add} = 100 J/m^2 (-o-o-o-); 25 J/m^2 (-•-•-•-); 10 J/m^2 (-e-e-e-).

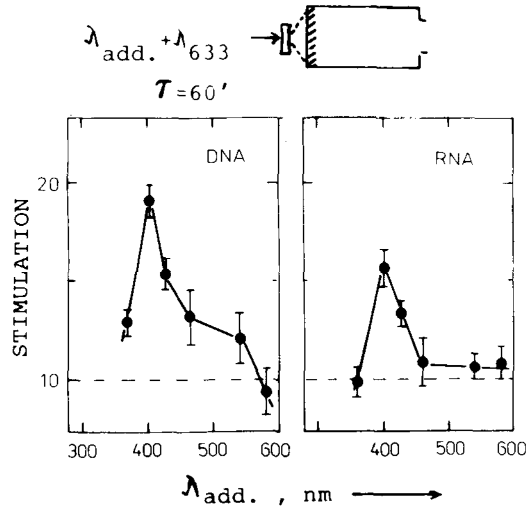


Fig. 17. Action spectra of consecutive dichromatic irradiation, in the sequence λ_{add} + 632.8 nm, on the synthesis of DNA and RNA in exponentially growing HeLa cells, 1.5 h after the last irradiation event. $D_{632.8} = 100\ J/m^2$; $D\ \lambda_{add} = 10\ J/m^2$.

val between the successive irradiation events in dichromatic irradiation plays an important part. We performed consecutive dichromatic irradiation with λ = 760 nm and 633 run, varying the time interval between the irradiation events over a broad range (from 1 sec to 2 h) (Figure 18). The irradiation doses were always taken at 100 J/m^2, which corresponded to the maximum stimulative effects for these wavelengths (Figure 5). Recall that the concurrent dichromatic irradiation with λ = 633 nm and 760 nm caused no changes in the rate of synthesis of DNA and RNA (Figure 16).

Figure 18 shows the rate of synthesis of DNA and RNA as a function of the time interval between the successive irradiation events. With short (110 sec) time intervals, the rate of DNA synthesis does not deviate from the control level. As the interval grows longer, the rate of DNA synthesis changes, the sense of the effect depending on the sequence of dichromatic irradiation wavelengths. Irradiation first with the far red light (λ = 760 nm) and then with the red light (λ = 633 nm) stimulated DNA synthesis, whereas that in the reverse order (633 + 760 nm) inhibited it. These effects reach their maxima when the time interval between the successive ir radiation events becomes 1 to 3 min and becomes progressively less pro nounced with further increases in the interval. It should be noted that the effects in their maxima are not equal in magnitude: stimulation amounts to 60%, while inhibition is only 20%.

Variations in the rate of RNA synthesis caused by consecutive dichrom atic irradiation do not follow the pattern described above for DNA. RNA synthesis is observed to be stimulated with either of the two irradiation wavelength sequences (760 + 633 nm or 633 + 760 nm), but the maximum of the stimulative effect for the sequence 633 + 760 nm is observed to practically vanish (Figure 16). This fact provides every reason to search for some possible connection between these two wavelengths.

In the case of consecutive dichromatic irradiation with λ = 760 nm and 633 nm, variations in the rate of DNA synthesis (stimulation or inhibition of the synthesis) depend on both the irradiation wavelength sequence and the time interval between the successive irradiation events (Figure 18).

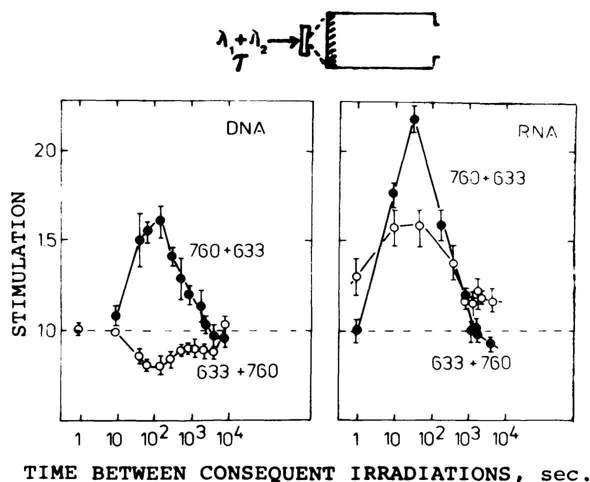


Fig. 18. Rate of DNA and RNA synthesis (1.5 h after last irradiation event) of exponentially growing HeLa cells, irradiated (D = 100 J/m^2) consecutively with λ = 760 nm and λ = 632.8 nm, as a function of the irradiation wavelength sequence and the time interval between the successive irradiation events.

These data point to the possibility that the photoacceptor may exist in two interconvertible forms having their absorption maxima located in the red and far red regions of the spectrum.

A well known example of such a photochromatic photoreversible pigment is phytochrome[26]. There are no published experimental data that would con firm the existence of a similar system in mammal cells. There are only data on the antagonistic action of red and far red light on the process of oxidizing phosphorylation in mitochondria [27], and on chromosome aberrations in mammalian cells [8]. In experiments described in Reference 27, the photoreversal of stimulation and inhibition of ATP synthesis in isolated rat live mitochondria was realized for two cycles.

VI.2. Action of blue and red light upon HeLa cells

The next series of experiments follows a method similar to the preced ing one, the only difference being that instead of far red light we used blue light with a wavelength of 404 nm (D = 10 J/m^2), which is close to one of the maxima in the action spectrum (Figure 8). Recall that concurrent irradiation with λ = 404 nm and 633 nm practically does not change the rate of synthesis of DNA and RNA as compared with the control level (Figure 19). In the case of consecutive dichromatic irradiation (Figure 19) with short (1-10 sec) time intervals between the irradiation events, the rate of DNA synthesis remains at the control level no matter what irradiation wavelength sequences are used. Increasing the time interval between the irra diation events in the case of the sequence (404 + 633 nm) enhances the rate of DNA synthesis, whereas the reverse irradiation wavelength sequence has for all cases no effect on the synthesis. RNA synthesis is observed to be stimulated with both irradiation wavelength sequences, the maximum of the stimulative effect being higher in the case of the sequence (404 + 633) nm. This effect already appears at short (1-10 sec) time intervals between the irradiation events. As the interval is increased (up to 2 h in our experi ments) the effect reaches its maximum and then remains practically unchanged .

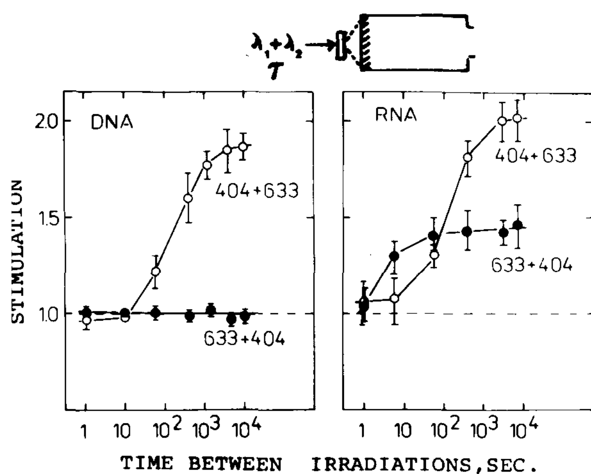


Fig. 19. Stimulation of DNA and RNA synthesis (1.5 h after last irradi ation event) of exponentially growing HeLa cells, irradiated consecutively with λ = 404 nm and λ = 633 nm (D_{633} = 100 J/m^2, D 404 =10 J/m^2), as a function of the irradiation wavelength sequence and the time interval between the successive irradi ation events.

In our experiments, consecutive dichromatic irradiation with red and blue light with different time intervals between the successive irradi ation events and in different wavelength sequences has different effects on the synthesis of macromolecules. Irradiation in the sequence 633 + 404 nm has either no effect at all (in the case of DNA) or a considerably weaker effect (in the of RNA, Figure 19) as compared with that in the sequence 404 + 633 nm. Irradiation in this latter sequence (404 + 633 nm) is observed to stimulate the synthesis of both DNA and RNA, irrespective of the time interval between the irradiation events. Our data agree with the data on the consecutive dichromatic irradiation of a culture of human embryonic skin-muscular fibroblasts [29]. No changes have been noticed in the mitotic activity of the cells in the case of irradiation in the sequence 633 +441 nm, whereas, in the case of irradiation in the sequence 441 + 633 nm, the mitotic index was observed to increase by 40 to 60 % within a 5 min interval between the successive irradiation events. Non-additive stimulation was also noticed in cases in which one of the two irradiation components has a inhibitive effect when used individually.

In our experimental conditions, we failed to observe in the action spectra for the rate of synthesis of DNA and RNA any statistically signif icant deviations from the control level in the green region of the spectrum (Figure 8). A blue maximum was found at around 404 nm, which was approximately 10 times as sensitive as the red and far red maxima (maximum effects at irradiation doses 10 and 100 J/m^2, respectively - see Figure 5). Concurrent dichromatic irradiation was found to affect the action spectra: the blue maximum shifted from 404 nm to 450 nm and changes were observed in the green region of the spectrum (Figure 16). These changes depend on the irradiation dose of the blue or green light (the red-light irradiation dose was kept fixed at 100 J/m^2). Thus, the light of these wavelengths is "biologically active". For these effects to occur, it is necessary that the irradiation doses should be low and the irradiation times short.

Here we are dealing with some photoreceptor(s) which can initiate changes in the final photoresponse. One may assume that concurrent dichro matic irradiation (Figure 16) shifts the equilibrium of the electron trans-

fer chain (respiratory chain) in such a way that the action spectrum reflects the other redox state of a chromophore absorbing at λ = 450 nm as well as of that chromophore absorbing in the green region (probably cytochromes). At 450 nm, the oxidized form of flavin features an absorption maximum[31]. The one electron reduction of oxidized flavins leads to the formation of either an anionic form of semiquinone having its absorption maxima located at 380, 400 and 490 nm (with a continuation up to 650 nm) or neutral semiquinone which absorbs light within almost the whole of the visible light region (up to 750 nm). The two-electron reduction of flavin molecule results in almost complete disappearance of the absorption band at 450 nm. It is quite possible that some flavoprotein is the primary photo-acceptor for which we search. Flavoproteins play a key part in redox regulation of mitochondrial activity. Due to their ability to form a stable intermediate semiquinone form, they serve as connecting links between one and two electron reduction-oxidation systems in the respiratory chain.

The picture here is complicated by the fact that hemoproteins to which the cytochromes belong also absorb light in the blue (λ = 400 nm) and green regions of the spectrum[25]. Therefore changes in the final macroeffect may occur, as in fact is shown by the sensitivity of nucleic acid synthesis to the irradiation dose of λ_{add} (Figures 16 and 17). It can be expected that for other ratios between the irradiation doses of the red light and λ, the action spectra will be different.

Thus, proceeding from the data on dichromatic irradiation, flavoproteins can be thought of as a photosensitive regulatory system in which the chromophores are $flavin_{educed} \rightarrow flavin_{seniquinone} \rightarrow flavin_{oxidied}$. There is information in the literature showing that irradiation with visible light at λ = 400 nm alters the reduction-oxidation state of flavins. It has also been demonstrated that there is a functional relationship between the degree of oxidation of flavin coenzymes and the ATP synthesis activity of mitochondria in vivo . Regulation is thought to occur through changes in the spatial conformation of flavoproteins, which is determined by the reduction-oxidation state of the chromophore[33]. Problems connected with possible primary photoacceptors and light signal transduction chains in cells (e.g. from respiratory chain to DNA) are discussed in detail in the References 12 and 30.

VII. STIMULATION AND INHIBITION

In experiments described above, the positive (stimulating) effects of irradiation were described. However, there also exists a tremendous amount of data describing inhibitory, and even lethal, effects of light (especially blue and fluorescent light) on various types of cells. Table 2 shows some light-growth responses of mammalian cell cultures[9]. An analysis of this data suggests that the dose and the intensity of the light used determine the sense stimulating versus inhibitory of the end macroeffect. The stimulative doses are 3-4 orders lower than inhibitory ones.

To verify the importance of light dose on the sense of the final effect, two series of experiments with HeLa cells were performed.

In the first series of experiments, we studied the dose-response dependences for lethal effect of different visible light wavelengths. HeLa cells were cultivated as usual and taken for the experiment 72 h after plating (during the log-phase culture). The culture was trypsinized, and a suspension of $2x10^5$ cells in 1 ml of Hank's solution was prepared for irradiation. The irradiation was performed in a special quartz cuvette with volume 90 µl or 250 µl ($S_{irrad} = 6.38 \times 10^{-2}$ and $2.12x10^{-1}$ cm^2, respectively). For irradiation, a Rhodamine S dye laser pumped with cw Ar laser (λ = 578

Table 2. Action of various bands of light on proliferation activity of mammalian cellular cultures.

Wavelength n	Culture	Stimulation	Inhibition
		Dose, J/m^2	Dose, J/m^2
400	HU-274 WI-38	10^4	10^5-10^6
441.6 632.8 694.3 741	Human embryonic skin fibroblasts	10^3-10^4	10^5-10^6
Cool white fluorescent lamps	Human embryonic diploid lung fibroblasts	Dose not shown. Exposition every day during 150 days for 2 h: cells came through 70 divisions; 40 h:60 div.; 6 h:53 div.; dark control: 53 div	Dose not shown. The cells exposed constantly, died within 2-3 days
405	Human lympho-blastoid cells	-	10^5
630-633	Chinese hamster fibroblasts	10^3	-
632.8	L	7.5	-
632.8	Human embryonic foreskin fibroblasts	10^2	-
632.8	HeLa	10^2	-
546-579	HeLa	Dose not shown, dose rate 10-50 W/cm^2	dose not shown, dose rate 100-300 W/cm^2
694.3	Human skin fibroblasts	$(1-4)x10^3$	-
694.3	Human melanoma in culture	$(2.4-48)x10^6$	-
694.3	Mouse fibroblasts L929	10^5	-
694.3	Human epidermal and mouse lung cells in culture		No damage till 10 J/m^2

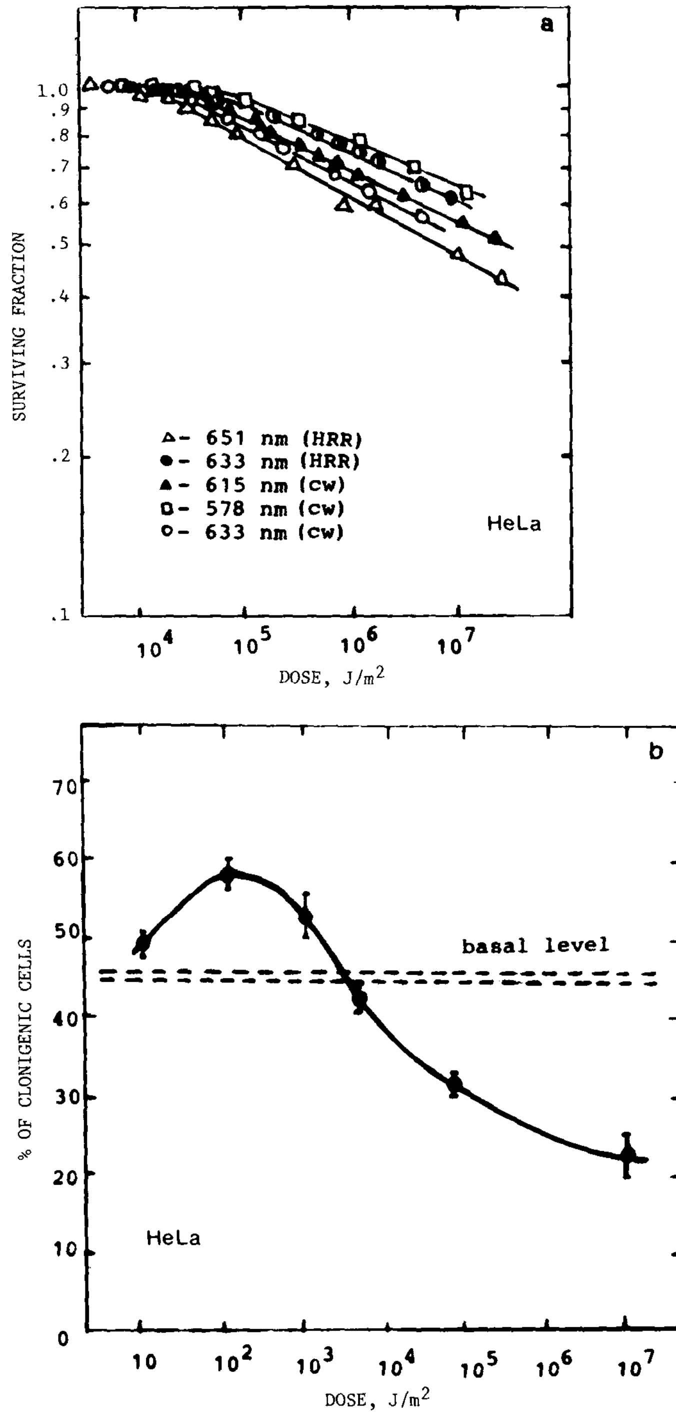


Fig. 20. (a) Survival of proliferating HeLa cells irradiated with a dye laser at different wavelengths and with different irradiation modes (cw or repetitively pulsed light) and, (b) change of number of clonigenic cells as a function of HeNe laser ra diation dose (plateau-phase HeLa cells were irradiated as a monolayer, and their clonigenic ability was evaluated by the Puck technique after replating).

or 615 nm), and Oxazin 170 dye laser pumped by copper vapor laser (λ = 633 651 and 670 nm, high repetition rate nanosecond pulses) were used. Just after the irradiation, cell viability was evaluated by trypan blue excluusive test. The viability of cells in nonexposed samples was 89 ± 3 %.

As seen in Figure 20a, the dose-effect dependences are very similar for all wavelengths used in our experiments. There are no lethal effects till approximately 10^4 J/m^2, beyond which increasing doses give an increasing lethal effect.

In the type of test just described it is impossible to investigate the positive and negative effects in one experiment. For that reason we performed the next series of experiments using the type of experiment described at the beginning of this chapter. Plateau-phase HeLa cells were irradiated with a He-Ne laser in different doses, trypsinized 3 h after irradiation and replated. The number of viable cells was determined by counting the number of clones.

Figure 20b shows the results obtained. As seen in Figure 20b, there is a rather abrupt switch from a positive to a negative effect upon increasing the light dose. A similar type of curve was obtained earlier when investigating the influence of near IR light at λ = 890 nm on the growth of E. coli[34], and at λ = 904 nm on ATP production in Saccharomyces calbergensis [35].

On the other hand, the negative effect seems not to increase monotonically with increasing irradiation dose. Irradiation of Saccharomycodes ludwigii with He-Ne laser did not cause growth inhibition more than 20%, even when the dose was increased by 2 orders of magnitude [36,37].

In explaining the positive (stimulating) effects of visible light, the respiratory chain components were proposed to be the primary photoaccep-tors [12,19,30]. Also, in the case of negative effects (which inhibit cellular metabolism or are lethal), the respiratory chain components have been shown to be primary photoacceptors [38,44]. For example, it has been demonstrated[43] that cytochromes c and c_1 failed to act as hydrogen acceptors following 10 pulses of 1 mW/cm^2 of green laser light at 530 nm, and cytochrome a/a_3 showed a similar response when irradiated at λ = 609.6 or 601.3 nm.

These data illustrate the principle that laser wavelengths which are appropriately matched to the absorption characteristics of target molecules can not only stimulate but also selectively inhibit specific molecular components in cells. The lack of wavelength specificity in our experiments can be probably explained by absorption and resulting changes in various molecules in the respiratory chain, the final results being overall inhibition of the electron transfer chain.

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