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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Photoaggregation of Water-soluble Protein (Carboanhydrase) Induced by the Ultraviolet Radiation of Xe–Cl Laser

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Molecular mechanisms of the origin and development of certain forms of cataract, including photocataractogenesis, are based on the degradation and aggregation of the main water-soluble proteins of the eye lens (α-, β-, and γ-crystallines) [1–4]. The light-induced degradation of these proteins and the whole lens substantially simulates the processes associated with the development of senile cataract, one of the most widespread forms of cataract [5]. Detailed studies of the mechanisms of the UV-induced degradation and aggregation of crystallines in solution should be regarded as an appropriate model of the processes of lenticular opacity, primarily, the UV-induced lenticular opacity.

The goal of this work was to describe the results of experimental and theoretical analysis of the kinetics of the UV-induced aggregation of carboanhydrase, a model water-soluble protein. The kinetic curves of the dependence of the intensity of the scattering of a monitoring light beam on the dose of UV-radiation were measured during the exposure of a carboanhydrase solution to excimer laser radiation (308 nm).

Carboanhydrase is a monomer protein with a molecular weight of 29 kDa, which is very close to the molecular weight of γ-crystalline (19 kDa) and certain forms of β-crystallines (23–29 kDa). The behavior of the kinetic curve is similar to the behavior of similar dependences for γ- and β-crystallines [6, 7].

As far as we know from the literature, the effects of UV-radiation were studied at a constant energy density of light pulses (w) and constant pulse repetition frequency (F). The specific goal of this work was to study the effects of UV-radiation on protein within a broad range of variation of parameters w and F. This study provided information about the time course of the formation of high-molecular-weight complexes (cross-links) and revealed a strong dependence of the probability of their formation on parameters w and F. A physical model of the UV-induced protein aggregation was constructed on the basis of the results of this study. This model suggests that protein aggregation is caused by interaction between molecules modified by light and determines some characteristic parameters of this process.

A beam of a single-mode He–Ne laser ($\lambda = 633$ nm) was used as the monitoring light. The optical scheme of the experimental unit [8, 9] differed from the optical schemes used earlier. First, the intensity of monitoring-beam scattering was measured by the method of dark field [10], which allowed the absolute value of scattered power to be measured. Second, the energy of the excimer laser pulses passing through the cuvette was measured simultaneously. A large-aperture detector was placed immediately behind the cuvette to measure the protein solution absorption increase at 308 nm. The fraction of the energy of the laser pulses that passed through the cuvette was detected with an UV-sensitive photodiode (input aperture diameter, 1 mm), which was placed at a distance of 4 mm from the cuvette. The UV-sensitive photodiode detected the signal associated with both absorption and light scattering changes at 308 nm in the cuvette containing the protein solution. This scheme of signal detection allowed light scattering changes at 308 nm to be detected at a dose levels one order of magnitude lower than the value of D^* typical of the initial phase of the scattering of the He–Ne laser radiation. The optical transmission spectra and molecular weight distribution (MWD) of carboanhydrase were measured before and after the exposure to UV radiation at various doses. The extinction coefficient (α) of a carboanhydrase solution with a concentration

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Table 1. The results of theoretical analysis of kinetic curves at different values of w and F

Energy density of laser pulses, w, mJ/cm^2	Frequency of repetition of laser pulses, F, Hz	The dose of UV radiation above which scattering of He–Ne laser beam is observed, D^*, J/cm^2	Steepness of the kinetic curve, mW · cm^2/J
40	3	>800	0
	4	500	0.002
	6–16	350	0.006
84	1	600	0.002
	2	350	0.009
	4	300	0.009
300	0.15	300	0.0006
	0.25	275	0.005
	0.5	200	0.006
	1.0	200	0.014

of 0.5 mg/ml within the UV-radiation range w = (2–300) mJ/cm^2 was equal to 6×10^{-2} cm^{-1} and remained invariable within the whole range of changes in the value of w. This can be regarded as evidence for the absence of nonlinear (primarily, two-photon) absorption. At this value of α and maximum value of w used in our experiments (w = 300 mJ/cm^2), the temperature increase of the protein solution per pulse of laser radiation was $\Delta T_1 = 1.6 \times 10^{-3}$°C, whereas the temperature increase of the protein solution during the whole exposure time did not exceed ΔT = 2°C. Protein solution was exposed to UV-radiation at room temperature (23 ± 2)°C.

The pattern of changes in the kinetic curves is shown in Fig. 1. It follows from Fig. 1 that, for each value of w, there exists a characteristic level of F^*. If the value of w goes below this level, this is accompanied by a substantial increase in the value of D^*, which can be regarded as evidence for an abrupt decrease in the cross-linking probability. In addition, the value of D^* depends on the value of w: parameter D^* declines upon increasing the value of w. The results of analysis of the kinetic curves are shown in the table. It should be noted that a similar dependence $D^*(w)$ was earlier observed in studies on the photoaggregation of pig eye lens exposed to the UV radiation with the wavelength of 308 nm [11]. On the other hand, the processes of photolysis were dominant under the conditions of eye lens exposure to the UV radiation with a wavelength of 266 nm; under these conditions, $D^*(w) \approx$ const.

In addition to kinetic curves, signals detected with the energy meter and UV-sensitive photodiode are also shown in Fig. 1 (curves *1* and *2*, respectively). At low exposure doses, these curves coincided with one another. However, at exposure doses above $D \sim 20$ J/cm^2, the signal detected with the UV-sensitive photodiode declines faster than the signal detected with the energy meter. This implied that, at low exposure doses, the experimentally measured value corresponded only to the increase in the optical absorption at 308 nm in the protein solution, whereas at higher exposure doses, the UV-beam scattering also contributed to the decrease in the signal detected with the UV-sensitive photodiode. The dose ratio at which the onset of scattering of the radiation of both He–Ne and Xe-Cl lasers was observed was close to (633 nm/308 nm)[4]. This suggested that, at least at the initial segment of the dose curve, the scattering of laser radiation with the wavelengths λ = 633 nm and λ = 308 nm in the protein solution obeyed the Rayleigh law. In other words, in the two cases, the following condition was observed: $d \ll \lambda$, where d is the size of scattering particles (the radius of carboanhydrase molecule is 2.36 nm [12]). The molecular weight distributions of the protein exposed to different doses of UV-radiation are shown in Fig. 2. The chromatogram of the initial sample contains peaks of the main fraction of the protein monomer and dimer forms. At $D = 0.1D^*$, the contribution of the dimer fraction increases, and oligomer forms emerge. At $D = D^*$, this process continues, and a fraction emerges in the region of the exclusion limit of the column (molecular weight, 2000 kDa and more). This fraction is represented by high-molecular-weight protein aggregates. The content of these aggregates, including forms with an apparent molecular weight of more than 2000 kDa, increases at an exposure dose of $1.5D^*$.

Analysis of the changes in the absorption spectra of the protein solution demonstrated that, within the wavelength range from 200 to 600 nm, a monotonic decrease in the transmittance value occurred with an increase in the dose of UV radiation (Fig. 3). The spectrum of induced absorption was found to be similar to that observed in [1] in the case of the water-soluble fraction of the bovine eye lens exposed to UV radiation.

To explain the results described above, let us assume that the process of aggregation is based on the interaction between two protein molecules, each of them being activated as a result of the absorption of a quantum of incident radiation. It should also be taken into consideration that the lifetime of the activated state of the molecule is limited. We considered only the formation of protein dimers, because the accumulation of protein dimers was assumed to determine the increase in the intensity of light scattering in the protein solution. Let these processes be described by the following equations:

$$\frac{dR}{dt} = \Re - kR^2 - \frac{R}{\tau}, \quad \frac{dN}{dt} = \beta k R^2, \qquad (1)$$

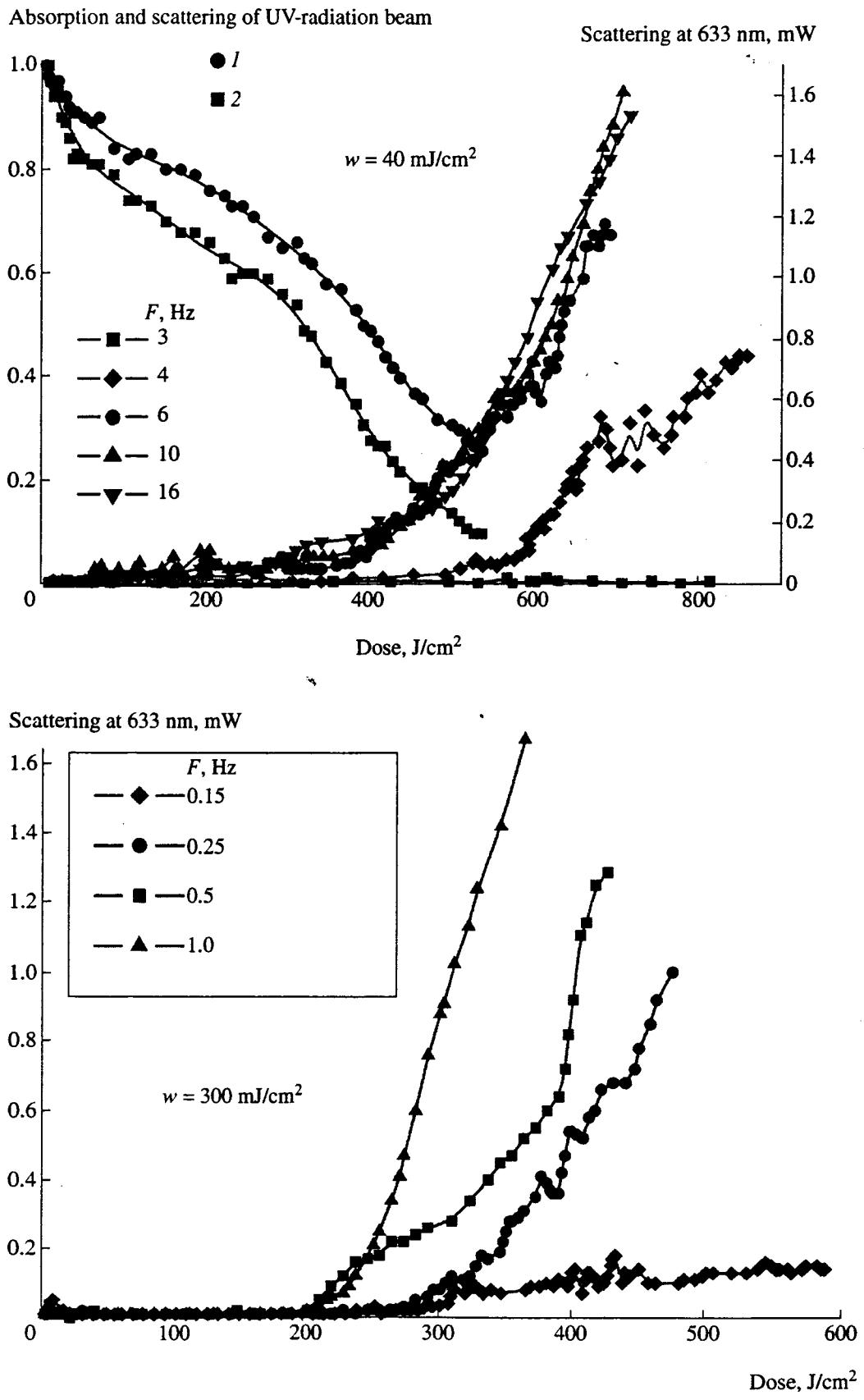

Fig. 1. Kinetic curves at different levels of energy density of laser pulses and frequency of repetition of laser pulses and characteristics of UV-beam absorption and scattering: (*1*) signal of energy meter; (*2*) signal of UV-sensitive photodiode.

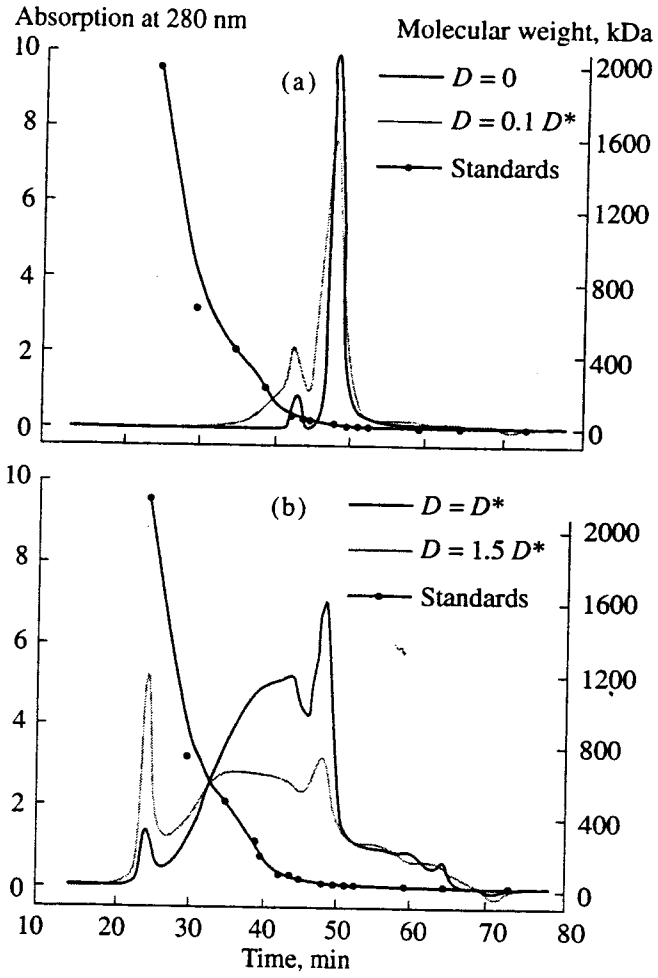

Fig. 2. The calibration curve and curves of molecular weight distribution measured before and after the exposure to UV radiation at different doses.

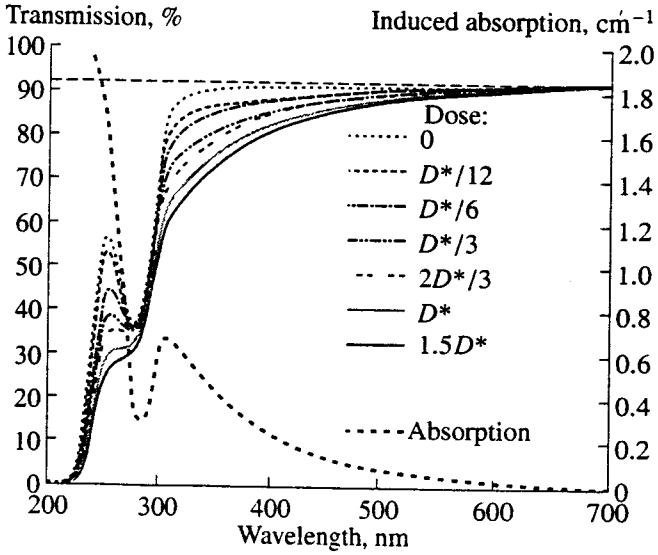

Fig. 3. Spectra of optical transmission and induced absorption measured after the exposure to UV radiation (D = $1.5D^*$).

where R is the concentration of activated molecules; N is the concentration of dimers; k is the rate constant of the decrease in the concentration of activated molecules in the process of interaction in pairs; β is the probability of dimer formation; τ is the characteristic lifetime of the molecule in the activated state; $\Re$ is the source of activated molecules.

In the case of exposure to continuous UV radiation, the set of simultaneous equations (1) can be recast as follows:

$$\frac{dR}{dt} = \eta\sigma C_0 \frac{I}{\hbar\omega} - kR^2 - \frac{R}{\tau}, \quad \frac{dN}{dt} = \beta k R^2, \qquad (2)$$

where C_0 is the concentration of monomer molecules; η is the quantum yield of the formation of activated molecules; σ is the effective absorption cross-section; I is the radiation intensity; $\hbar\omega$ is the energy of a radiation quantum.

In the case of a steady-state concentration of activated molecules ($dR/dt = 0$),

$$N(t) = N_0 + t \cdot \beta k \frac{\left(\sqrt{1 + 4k\tau A} - 1\right)^2}{(8k\tau)^2},$$

where $A = \eta\sigma C_0 \frac{I}{\hbar\omega}\tau$.

Let N^* be the concentration of dimers, at which the intensity of scattered radiation increases. Then, the value of D^* can be described by the following expression:

$$D^* = D_0 \frac{I/I_0}{\left(\sqrt{1 + I/I_0} - 1\right)^2}, \qquad (3)$$

where $I_0 = \frac{\hbar\omega}{4k\tau^2\eta\sigma C_0}$; $D_0 = 16\frac{N^*\hbar\omega}{\beta\eta\sigma C_0}$.

In the limiting cases, (1) $\frac{I}{I_0} \ll 1$, $D^* = 4D_0\frac{I_0}{I}$, (2) $\frac{I}{I_0} \gg 1$, $D^* = D_0 = \text{const}$.

At low values of I, D^* is a decreasing function of radiation intensity ($D^*(I)$ is a decreasing curve), whereas at high radiation intensity, the value of D^* tends to a constant level (D_0). The value I_0 in this case can be regarded as a borderline between the area of sharp increase in D^* and the area within which the value of D^* tends to an asymptotic level.

In the case of exposure to pulsed radiation, the source of activated molecules is a periodic function of time: $\Re = \Re(t - nT)$, where n is integer; $T = F^{-1}$. Let the solution of the set of simultaneous equations (1) be sought for the experimental conditions described above. At a concentration of 10^{16} cm^{-3} (0.5 mg/ml), the time interval between collisions of protein molecules is about $\sim 10^{-5}$ s, which is significantly longer than the laser pulse duration (3×10^{-8} s). Therefore, the dimers have no enough time to be formed during the laser

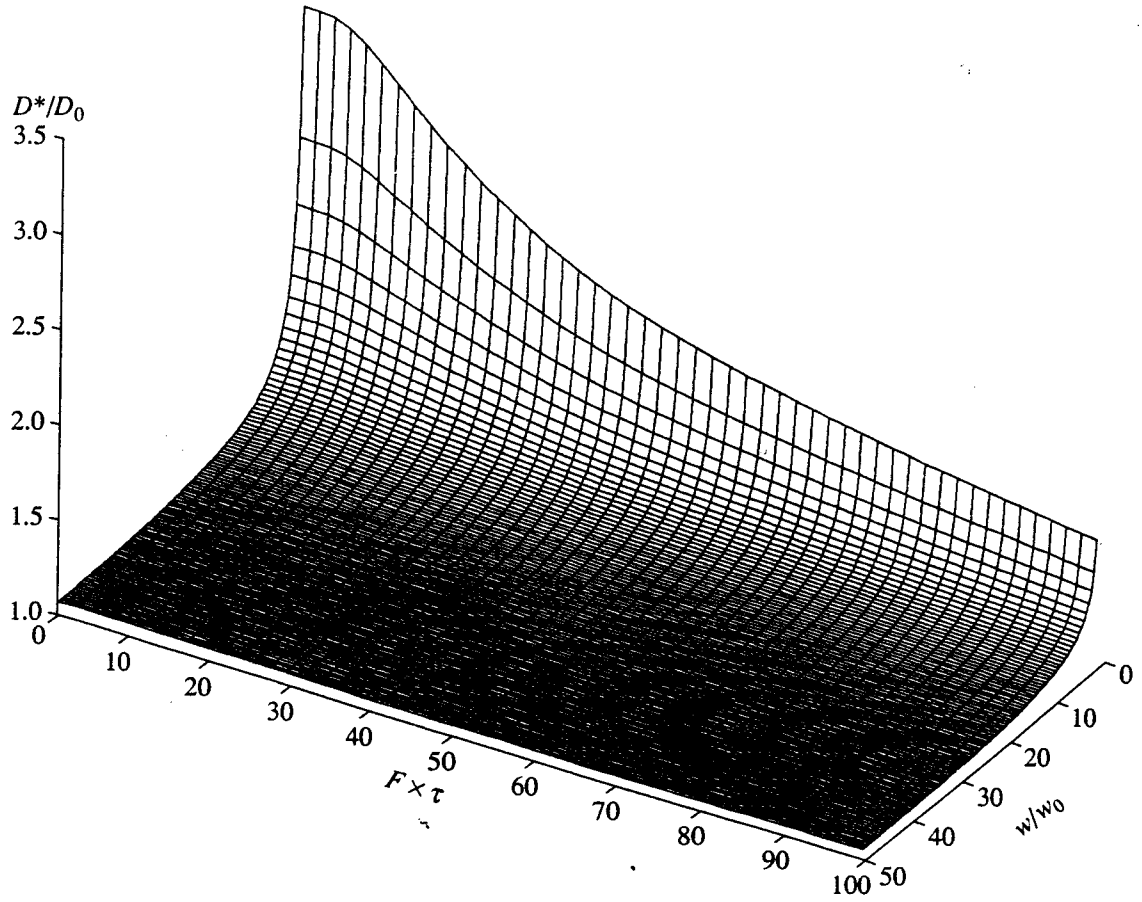

Fig. 4. Dependence of the UV radiation dose at which the intensity of scattered radiation of a monitoring beam (633 nm) begins to increase on the energy density of light pulses (w) and pulse repetition frequency (F).

pulse, and the current concentration of activated molecules (R_{0i}) can be taken as the source of activated molecules in Eqs. (1). Therefore, the set of simultaneous equations (1) can be recast as:

$$\frac{dR}{dt} = -kR^2 - \frac{R}{\tau}, \quad \frac{dN}{dt} = \beta kR^2. \qquad (4)$$

In the steady-state regime, the concentration R is a saw-toothed curve, and, immediately after a radiation pulse, it is equal to $(R_p + R_s)$, where $R_p = \eta\sigma C_0 \frac{w}{\hbar\omega}$. By the moment of the beginning of each succeeding pulse, this curve declines to a certain constant level R_s. The increment of the concentration of dimers in this case is

$$\Delta N(T) = \frac{\beta}{k\tau}\left[k\tau R_p + \ln\left(\frac{1 + k\tau R_s}{1 + k\tau(R_p + R_s)}\right)\right],$$

where $R_s = \frac{R_p}{2}\left(\sqrt{1 + \frac{4}{k\tau R_p(\exp(T/\tau) - 1)}} - 1\right)$.

The steady-state value of R_s was calculated assuming that, by the moment of beginning of each pulse, the concentration is the same as before the preceding pulse. Assuming that light scattering is observed only if the concentration of dimers goes above a certain level N^*, the following equation can be written:

$$D^*(w, F) = D_0 \frac{w/w_0}{\left(w/w_0 - \ln\left(1 + \frac{2w/w_0}{1 - w/w_0 + (w/w_0 + 1)\cdot Z}\right)\right)}, \qquad (5)$$

where

$$w_0 = \frac{\hbar\omega}{k\tau\eta\sigma C_0}, \quad D_0 = \frac{N^*\hbar\omega}{\beta\eta\sigma C_0}; \quad \text{and}$$

$$Z = \sqrt{1 + \frac{4w/w_0}{(w/w_0 + 1)^2\left(\exp\left(\frac{1}{F\tau}\right) - 1\right)}}.$$

The three-dimensional pattern described by Eq. (5) is shown in Fig. 4. It follows from Fig. 4 that, with an increase of the level of w, the value of D^* declines, tending to a constant level D_0, whereas a decrease in w is accompanied by an unlimited increase in the value of D^*. This behavior is similar to the pattern of D^* dependence on the intensity of continuous radiation. Indeed, with increasing F, the value of D^* declines, being lim-

ited from below by the same value D_0, which is typical of the curve $D^*(w)$; whereas a decrease in F was accompanied by an increase in the value of D^*. The parameters of Eq. (5) were adjusted to provide the best fit of the theoretical curves to the experimental data. As a result of this adjustment, the following estimates of model parameters were obtained: $D_0 \sim 2 \times 10^2$ J/cm^2, $\tau = (10–100)$ s, $\eta \sim 3 \times 10^{-8}$, and $N^*/\beta \sim 10^{12}$ cm^{-3}. For the sake of simplicity, only the processes of formation of dimers were included into the physical model of photoaggregation. This simple model allowed the experimental results obtained in this work to be interpreted at a qualitative level.

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