PREEXCITATION OF ACRIDINE YELLOW IN POLYMETHACRYLATE

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The effect of preexcitation on the spectral features of acridine yellow in polymethacrylate is investigated. Reversible changes of the intensity and degree of polarization of the fluorescence have been found to exist in irradiated samples. The magnitude of these changes is a function of concentration and the time of sample preexcitation. The lifetime of fluorescence and the shape of the absorption and emission spectra remain unchanged. Preexcitation generates an EPR signal which decays much more slowly than the changes in the intensity and degree of polarization of the fluorescence. Irradiation only causes the samples to become slightly dichroic.

An attempt is made to explain the observed changes as due to the superposition of two effects: the reorientation of excited molecules and the photoionization of dye molecules. Reorientation may take place because of a decrease in the medium's viscosity very close to an excited molecule which undergoes a so-called initial shock. Reorienting molecules are more apt to become, aggregated, after which they no longer partake in emission. Consequently, when preexcitation is accomplished with plane-polarized light there is observed a stronger decrease in the intensity of the fluorescence component polarized perpendicularly to the direction of polarization of the preexciting light than for the parallel component.

The photoionization of some of the dye molecules gives rise to an EPR signal.

1. Introduction

The preexcitation effect consists in a change of the properties of luminescent dye samples under the effect of irradiation with light. Reversible effects of preexcitation have been discovered for a large number of dyes [1–7]. As was found, these effects depend on dye concentration, the mutual orientation of the electric vectors of the preexciting light and exciting light, temperature, and so on. Preexcitation has been explained in many ways: by a change in the orientation of the illuminated molecules [8, 9], by reversible photoioniza-

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tion of the dye [6], or, finally, by the formation of unstable isomers [3, 10, 11]. Neporent [8] found that under the effect of irradiation dichroism appears in low-temperature viscous dye solutions. This dichroism vanished in the dark. The author links the observed effect with the "initial shock" of excited molecules, proposed by Jabłoński [12, 13, 8]. Some of the excitation energy is expended in raising the temperature in the molecule's vicinity, thus decreasing viscosity, what makes reorientation of the molecule easier. Neporent gave a phenomenological theory of excited molecule reorientation [9]. It does not, however, provide an explanation of all effects observed [8, 9].

Hoijtink [6, 7] found that illuminating glassy solutions of aromatic ring compounds in boracic acid causes reversible ionization. When the illuminating light is polarized, dichroims occurs.

The preexcitation of acridine yellow had been investigated earlier in gelatine phosphors [5, 10, 11]. The effects observed were explained as due to the formation of unstable isomers. Gelatine preparations are not homogeneous media. The dye at different regions of the sample may have the properties of adsorbed dye or the properties of dye in solution. Hence, the observed effects may be a superposition of contributions from these two groups of luminescence centers. On the other hand, the medium adopted in the present study, polymethacrylate, is stable, homogeneous and isotropic. This enables information on the mechanism of reversible changes induced by light in acridine dyes to be obtained.

An interesting point is the mechanism of the interaction between the acridine dyes with the medium. These dyes are used in research on the action of cancerigenic agents on nucleic acids [14, 15]. Ignorance of the interaction of acridine dyes with various kinds of polymers renders difficult the interpretation of the obtained results. The interaction of acridine dyes with polyanions was studied by Duuren [14] and Oster [15]. Duuren suggests that there are two types of aggregation of acridine orange: one for dye in solution and a different one for dye bound with the polymer. In the dye bound with the polyanion, the dye is aligned with monomial charges towards the polymer, so that parallelly arranged dye molecules interact with each other, whereas in aggregates in a solution the rings become aligned "antiparallelly". Duuren states that a change in the interaction between dye molecules is responsible for the changes in the spectrum of the dye after the polymer is added, whereas Oster declares that the changes are due to interaction between the dye and polyanion. The latter proposes a "sandwich" model of the aggregate of acridine dye with nucleic acids.

2. Materials and methods

Reagent grade substances were used. Acridine yellow dissolves poorly in methyl methacrylate. The dye was dissolved in ethyl alcohol and then mixed with the methacrylate (1:9 volume ratio). Solutions with appropriate dye concentrations were sealed in glass ampoules. The alcohol did not bear any effect on the polymerization of the methyl methacrylate, which took 72 hours at a temperature of 72°C. Platelets were cut to size from the obtained transparent and uniformly coloured lumps of polymethacrylate; they were then polished with fine-grained polishing paste. Finished luminophors maintain their properties for many months if kept in the dark.

Extinction coefficient measurements were performed on VSU-1 (manuf. Zeiss) and Spectromom 202 spectrophotometers, supplemented if necessary with polarizers.

The arrangement of instruments used in measuring the fluorescence spectra of preexcited samples is shown in Fig. 1. The exciting and preexciting beams were not parallel, but made an angle of about 7 degrees. The sample was excited and preexcited by plane polarized light,

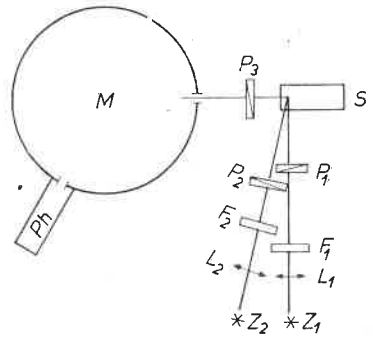

Fig. 1. Diagram of arrangement for measuring fluorescence spectra Z_1, Z_2 — sources of exciting and preexciting light, respectively, L_1, L_2 — lenses, F_1, F_2 — filters, P_1, P_2, P_3 — polaroids, M — monochromator, Ph — photomultiplier, S — sample

the electric vector of which was perpendicular to the plane of the drawing in Fig. 1. The polarized components of the fluorescence light was picked out with the polaroid P_3. The parallel component $I_{||}$ was obtained when the axes of all three polaroids were aligned parallelly, whereas the perpendicular component $I_{\perp}$ by a turn of P_3 by 90 degrees.

The dependence of the emission spectrum on wavelength of exciting or preexciting light was found by exchanging the interference filters (IF Zeiss) F_1 and F_2 in Fig. 1.

The dependence of the emission spectra on the intensity of the preexciting light was measured by attenuating the preexciting beam by means of nylon nets which were calibrated earlier.

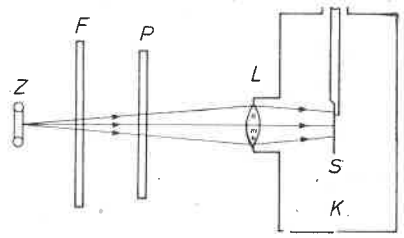

Fig. 2. Alignment of sample in EPR spectrum measurement. Z — quartz lamp, F — filter, P — polaroid, L — lens, S — sample, K — spectrophotometer chamber

In measurements of the dependence of the fluorescence spectra on preexcitation time the interval between the ending of preexcitation and spectrum measurements was some 30 seconds, while the dark periods between successive measurements of the same sample lasted from 12 to 24 hours.

The EPR signal was investigated by means of a JES-3BX reflex microwave spectrophotometer (manuf. JEOL). The scheme of the sample illumination during measurements is depicted in Fig. 2.

The degree of polarization of fluorescence was found in two ways:

1. by calculating it from separately measured intensities of the fluorescence components (from the results obtained by means of the arrangement shown in Fig. 2), and

2. by a direct measurement with the use of an instrumental arrangement of the Bauer and Rozwadowski type [18].

The first of these methods carries two errors:

1) the different losses in light intensity for the various polarizations in the monochromator M are not accounted for, and

2) the beam of exciting light is not parallel to the beam of preexciting light (Fig. 1).

These effects give rise to large quantitative discrepancies between results calculated from different methods. However, the essential result of investigations, namely, the appearance of the maximum of the degree of polarization of fluorescence in the 2 to 3 minute preexcitation range, is obtained with the use of both methods (Fig. 9).

Fluorescence lifetime measurements were carried out with the use of a Bauer-Rozwadowski fluorometer [15].

3. Results of measurements

Absorption spectra

Absorption spectra of acridine yellow in ethyl alcohol and in polymethacrylate are shown in Fig. 3. A comparison of the positions of the acridine yellow absorption maxima in various solvents is given in Table I. It is seen that the short-wave absorption maximum for acridine yellow in polymethacrylate is shifted towards the longer wavelengths relative to the position of this maximum for the alcohol solution.

A change in solvent brings about a change in the ratio of the absorption in the long- and short-wave maxima. It is higher for polymethacrylate than alcohol.

For alcohol solutions with concentrations lower than 3×10^{-5} g/cm^3 the acridine yellow absorption coefficient is in practice independent of concentration. In polymethacrylate solutions of the same range of concentrations the absorption spectrum changes markedly and irregularly with concentration (Fig. 3). With increasing concentration the long-wave maximum grows relative to the short-wave maximum (Fig. 3).

A presupposition comes to mind that in polymethacrylate two processes having different effects on the shape of the absorption curve compete with each other. It may be that these are the association of the dye with the polymer and the aggregation of the acridine yellow molecules.

When comparing the spectra of polymethacrylate and methyl methacrylate it was found that polymerization bears no effect on the absorption spectrum of acridine yellow. This implies that there are interactions between the medium and dye which do not have the character of chemical bonds. The sitution in the case of the interactions between acridine

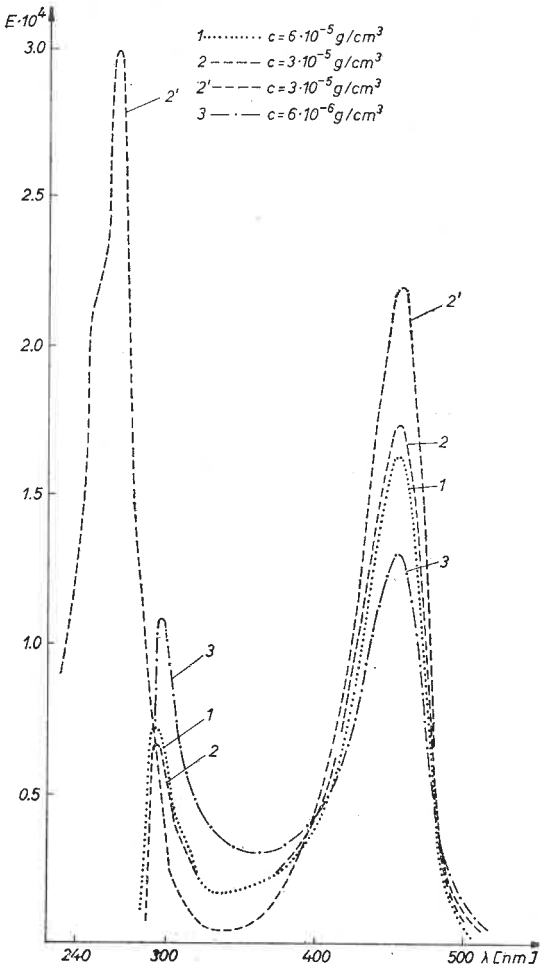

Fig. 3. Absorption spectrum of acridine yellow. *1*, *2*, *3* — in polymethacrylate, *2'* — in ethyl alcoho.

TABLE I

Solvent	Absorption maximum (nm)		Emission maximum (nm)
	short-wave	long-wave	
Water	266	437	508
Ethyl alcohol	266	457	502
Methyl methacrylate	294—296	457	508
Polymethacrylate	293—296	457	494

dyes and polyanions and DNA was found to be different [14]. Here, Duuren [14] revealed a strong dependence of the spectral properties of the dye on the length of the polymer, show ing that the dye occupies certain positions in the polymer.

Irradiation of a sample for 10 minutes with light causing large changes in emission does not give rise to any changes in absorption perceptible through a measurement of the entire spectrum on the VSU-1 spectrophotometer. There was success, however, in achieving measurable changes in absorption at the 450 nm peak (Fig. 4) by means of the Spektromom 202 spectrophotometer. The sample was preexcited by polarized light of wavelength 450 nm.

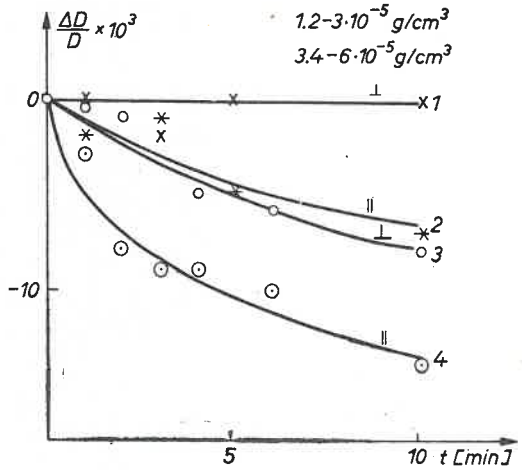

Fig. 4. Dependence of changes in optical density (ΔD) on preexcitation time. ΔD — change after illumination of appropriate components of the absorbed light of a vector parallel or perpendicular to the electric vector of the preexciting light, D — optical density at absorption maximum ($\lambda = 450$ nm)

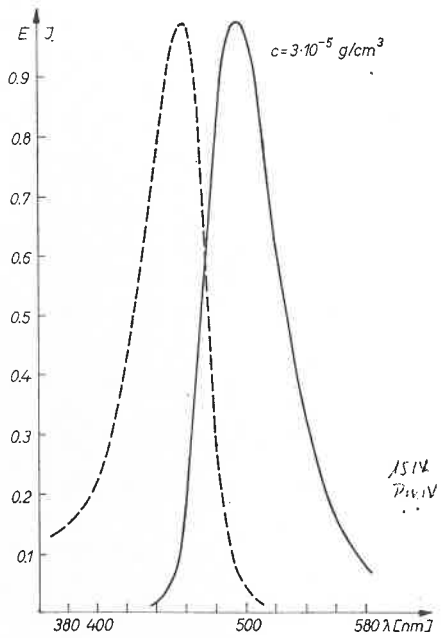

Fig. 5. Normalized spectra of absorption (E) and emission (I) of acridine yellow in polymethacrylate

The changes in the absorption of light plane-polarized in a direction parallel to the polarization of the preexciting light are greater than changes in the perpendicular component. Solutions of higher concentration exhibit larger changes (Fig. 4). The found changes in absorption do not exceed 2 per cent of the absorption in the non-preexcited state.

Emission spectra

The emission spectra exhibit mirror symmetry relative to the absorption spectra (Fig. 5). The curves intersect at a wavelength of 476 nm, what may be considered as approximate to the wavelength corresponding to a pure electronic transition.

The emission peak shifts only slightly with a change of preexciting light wavelength (Fig. 6). Hence, we have to do here with one fluorescing ionic species, for larger spectral changes than those in Fig. 6 usually correspond to different ionic forms of acridine dyes.

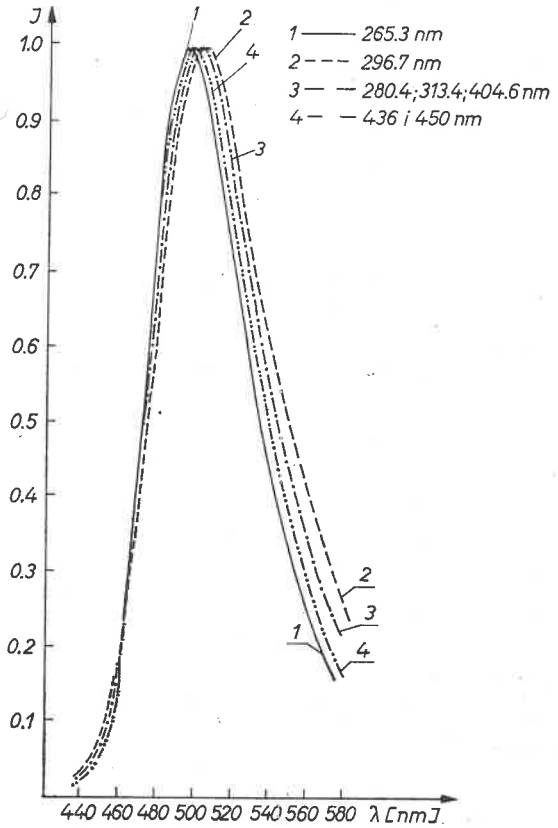

Fig. 6. Dependence of emission spectrum on wavelength of exciting light

The emission spectrum normalized per unit dye concentration is shown in Fig. 7. Strong concentrational extinction is observed. This may be associated with the poor solubility of acridine yellow in methyl methacrylate, what may cause the appearance of local concentrations higher than the average.

Concentrational changes in emission are much larger than the absorption changes in the same range of concentrations. Weill and Calvin [20] detected self-quenching of fluorescence of acridine dyes only for concentrations higher than 10^{-4}M, whereas Millich and Oster [21] had measurable results for concentrations as low as 10^{-6}M. The present results

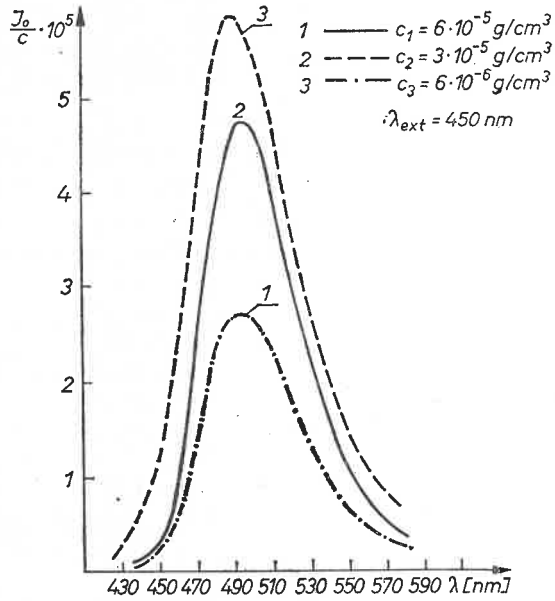

Fig. 7. Dependence of emission spectrum on concentration. I_0 — intensity of fluorescence of non-preexcited sample, c — concentration of acridine yellow

confirm the appearance of very strong concentrational extinction of fluorescence of acridine dyes in some media.

Fig. 8 presents the intensities of the two fluorescence components as a function of preexcitation time. The absolute intensity of the parallel component is obviously much higher than that of the perpendicular one, but in Fig. 8 the intensities are normalized relative to the corresponding intensities of non-preexcited components. As is seen in this figure, the perpendicular component varies much more than the parallel component. The curves exhibit distinct minima for preexcitation times of two to three minutes for all of the examined concentrations. The differences between the components are larger for the higher concentrations. At a concentration of 6×10^{-6} g cm^{-3} the shape of both components is the same within error limits. For the higher concentrations the divergence between the components pronouncedly exceeds experimental error.

A similar shape is also obtained for the total emission *versus* preexcitation time curve when the sample is preexcited with natural light.

Fig. 9 provides a comparison of the dependence of the degree of fluorescence polarization on preexcitation time obtained by the two methods. The degree of polarization is positive, as $I_{\parallel} > I_{\perp}$. In both of the methods a maximum degree of polarization is obtained in the range of preexcitation times from 2 to 3 minutes. The reasons why different values

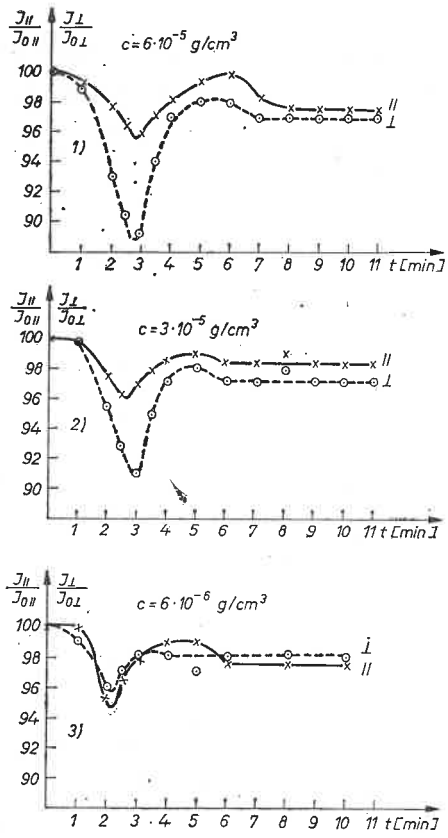

Fig. 8

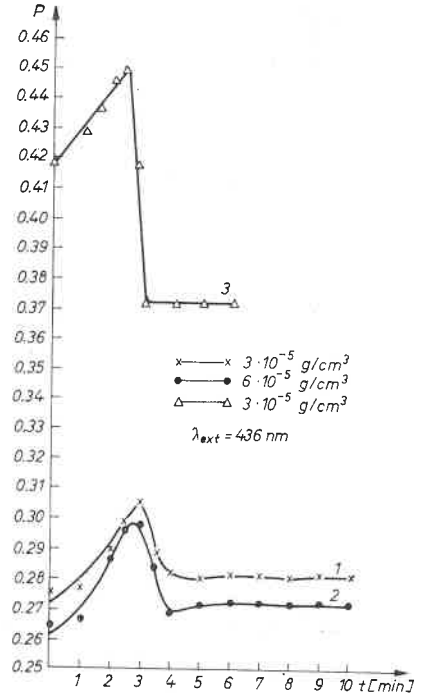

Fig. 9

Fig. 8. Dependence of fluorescence components on preexcitation time. $I_{||}$, $I_{\perp}$ — corresponding intensities of fluorescence components, $I_{0||}$, $I_{0\perp}$ — intensities of fluorescence components of non-preexcited sample
Fig. 9. Dependence of polarization degree of fluorescence of acridine yellow in polymethacrylate on preexcitation time. *1*, *2* — calculated from intensities of separate fluorescence components, *3* — determined with Bauer-Rozwadowski polarimeter

of degree of polarization are found with the use of different methods had been given in the preceding section.

Table II corroborates the results obtained from emission spectrum measurements, namely, that in the examined solutions there is very strong interaction between dye molecules. Hence the strong concentrational depolarization.

TABLE II

Concentration (gcm^{-3})	Degree of polarization of fluorescence (%)
6×10^{-6}	29.25
3×10^{-5}	26.75
6×10^{-5}	25.25

Fig. 10 presents the results of EPR measurements. No signal was observed for undyed samples and non-preexcited dyed samples. In the dyed samples the signal arises during irradiation. After irradiation is terminated the signal diminishes very slowly; the signal became

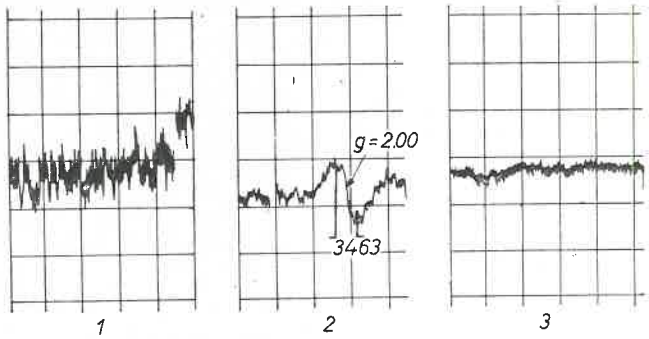

Fig. 10. EPR spectrum of acridine yellow in polymethacrylate. *1* — non-preexcited sample (measurement sensitivity 80 dB), 2 — preexcited sample (sensitivity 70 dB, other parameters identical as in *1*, *3* — sample 48 hrs after preexcitation (parameters as in *2*)

immeasurable only after 48 hours (Fig. 10, *3*). In accord with literature data [22] the signal should be ascribed to unpaired electrons in acridine yellow.

The changes in the intensity of fluorescence which are caused by preexcitation decay already within 15 minutes (Fig. 11). The various points of the curve in Fig. 12 were obtained by preexciting a sample kept in the dark for 24 hours, the intensity being measured at times

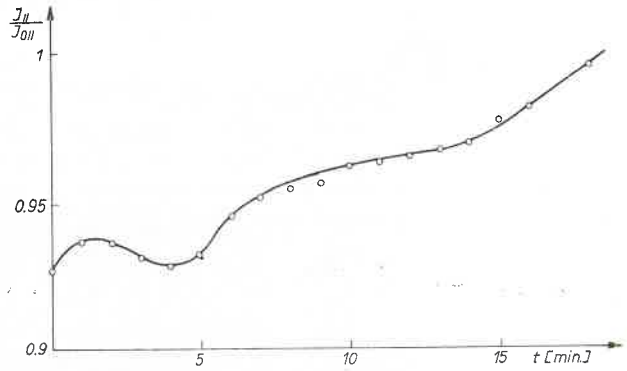

Fig. 11. Decay of preexcitation effect in time. $I_{||}$ — intensity of parallel component of fluorescence after preexcitation, $I_{0||}$ — ditto for non-preexcited sample, t — lapse of time between termination of preexcitation and measurement

after preexcitation as given in the figure. The whole curve was obtained over a long period of time by preexciting the same sample many times over.

It is true that the decay time of the changes in emission polarization, absorption and intensity depends to some extent on the "age" of the sample (that is, the time between the preparation of the sample and the measurement), but not so much as to be able to explain

on this basis the discordance between the decay time of the EPR signal and the decay times of other spectral changes. Therefore, different mechanisms should be ascribed to these effects.

Tentative measurements of the preexcitation effect on wavelength of the preexciting light were made. The results obtained were not recalculated per unit energy of preexciting light and unit absorption of the dye in a given region. Even from these approximate results it is seen, however, that there are changes in intensity and degree of polarization of fluorescence when preexcitation is in the short-wave region of the long-wave absorption band. It decreases abruptly in the vicinity of the 0-0 transition (470 nm) and does not appear when preexcitation is with longer waves.

The lifetime in the excited state as a function of preexcitation time is given numerically in Table III. As is seen, within the limits of measurement error the fluorescence lifetime does not depend on preexcitation time.

TABLE III

Preexcitation time (min)	Lifetime (nsec)
0	4.35
1	4.35
2	4.35
3	4.35 ± 0.05
4	4.35
20	4.35

The preexcitation effect appears most strongly near the sample surface. When preexcitation and excitation includes the deep inner layers of the glassy solution the effect is weaker.

There was no long-lived luminescence from the examined preparations. This was checked by means of a phosphoroscope with a photomultiplier at room temperature.

4. Interpretation of results

It is not easy to give an explanation of the set of data presented above, especially as regards the dependence of the degree of polarization of fluorescence on the time of sample preexcitation. Above all, therefore, it is necessary to look at the possible secondary effects stemming from the experimental conditions.

The first possibility which should be considered is the change in concentrational depolarization and concentrational quenching which may be caused by the elimination of some of the molecules from the emission process due to a reversible photoreaction (for instance, reversible photo-oxidation). Molecules altered by light may cease to participate in concentrational quenching, what gives rise to increased intensity. On the other hand, the decrease in the number of luminescence centers weakens the intensity of fluorescence.

These two overlapping effects could give an observable and rather complex dependence of fluorescence intensity on preexcitation time. Preexcitation with plane-polarized light would give rise to a certain anisotropy of emission owing to the exclusion from the emission process of the molecules with directions of absorption oscillators close to the direction of the preexciting light's electric vector.

Should this possibility be the true one, the following would be observed:

1. changes in the parallel component of the fluorescent light larger than those in the perpendicular component, hence, exactly the opposite than observed in Fig. 8, and

2. changes in absorption which would explain the magnitude of the changes observed in the emission.

Moreover, in some types of extinction one should expect changes in the lifetime of the fluorescence. In the case of preexcitation the lifetime is invariable (Table III).

The second effect requires closer scrutiny. The largest observed change of absorption for 450 nm (Fig. 4, curve 4) is about 1.4 per cent. The degree of polarization for the same sample under similar preexcitation changes from 25 to 29 per cent (when calculated from the fluorescence components) or from 38 to 45 per cent (from direct measurements). Such large changes in degree of polarization should be accompanied by changes in concentration of an order of magnitude as long as depolarization is of a concentrational character only. This is seen in Table II. Such large changes in absorption are not observed (Fig. 4).

The found changes in fluorescence intensities (some 10 per cent for the perpendicular complonent and 4 per cent for the parallel one) are also much larger than the corresponding changes in absorption.

As is seen, the observed effects cannot be explained by a reversible photo-oxidation of the dye, but one cannot exclude there being a certain influence of reversible photoreactions on the obtained results.

A plausible hypothesis may be put forth to explain the results. It is based on the assumption that excited molecules which undergo initial shock become reoriented. The proposed explanation complies with the results obtained up to now, but several assumptions yet uncorroborated by experiments have to be made.

When molecules, during excitation, undergo a strong initial shock [12, 13] the viscosity of the medium immediately surrounding them decreases. Molecules located in an affected volume have for a short time a somewhat larger freedom of movenent. At high local concentrations there may be one or more unexcited molecules in the near vicinity of an excited molecule. A molecule, when it rotates, may form an aggregate (for instance, a dimer), which is non-fluorescent, with another molecule in its neighbourhood. In this way both molecules stop taking part in the emission. Consequently, the perpendicular component of the fluorescence of preexcited samples, to which the "rotated" molecules contribute above all, has a lower intensity than the parallel components, to which molecules unaffected by preexcitation primarily contribute energy.

As fluorescence extinction occurs due to the formation of non-fluorescing dimers, the fluorescence lifetime remains unchanged, whereas the changes in intensities become enhanced with increasing dye concentration.

The decrease of both components may also be in part associated with photoreactions

which illuminated molecules of the dye go into. Illumination produces few radicals, however, as the EPR signal is weak. Recombination takes place very slowly. The largest changes in emission intensity appear at definite preexcitation times for a fixed intensity and preexciting light wavelength. It may be that multiple excitations dissociate the formed agregates.

The slight dichroism of the preexcited samples can be explained by analogy with the results of Feofilov [23] obtained for acridone, another acridine dye. In the long wavelength region acridone has relatively small negative dichroism and high positive polarization. It may be that acridine yellow has low dichroism in the region of 450 nm. This assumption should be checked by making a measurement of the dichroism of acridine yellow in an anisotropic medium, what has hitherto not been done.

That the observed effects are related with the reorientation of molecules is corroborated by their appearance only when preexcitation occurs in the short-wave region of the absorption band. In this region molecules being excited get a greater reserve of vibrational energy than elsewhere.

The low degree of polarization of fluorescence, considering that the solution is rigid, is explained by the strong overlap of two electronic transitions of mutually perpendicular polarization directions (L_a and L_b) in the absorption region. The participation of each of these transitions in the preexcitation effect may be various, however.

The surface nature of the preexcitation effect may be associated with the participation of oxygen in the formation of non-fluorescing aggregates. Oxygen diffuses easier into the layers near the surface.

The examined preparations did not exhibit any long-lived luminescence like that discussed in the studies by Frąckowiak and others [5, 10, 11]. Hence, the described effects concern singlet states of the dye.

5. Conclusions

1. It was shown that for the examined sample the preexcitation effect cannot be explained by reversible photoionization nor by the formation of unstable isomeric forms. An explanation based on the effect of the initial shock on molecule orientation is proposed.

2. No effect of polymerization of the methyl methacrylate on the spectral properties of acridine yellow was observed, hence, in the examined case there is no occupation of free sites in the polymer by dye molecules, like that observed in polyanions.

3. There is high depolarization and concentrational extinction of the fluorescence of acridine yellow in polymethacrylate.

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