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Studies of the Transient Intermediates of a Thiopyronine Aqueous Solution under Flash Excitation

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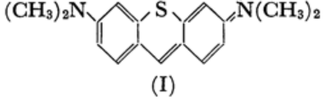
Flash photolytic studies of the transient intermediates produced in the flash excitation of an aqueous buffered solution of the photosensitizing dye "thiopyronine" were undertaken in order to obtain direct insight into the reaction mechanism of the photosensitizing action of the dye. As had been expected from the structural similarity to a well-known photosensitizer, methylene blue, previously reported on, the flash excitation of thiopyronine, in a deaerated buffered solution at pH 7.4 without any substrate, produced three transient intermediates: a triplet-state (D^t), a half-oxidized form (Oxd), and a half-reduced form or semiquinone (Red) of the dye. Red and Oxd were generated simultaneously through electron tansfer reactions, *i. e.*, $D^t + D^t \rightarrow \mathrm{Red} + \mathrm{Oxd}$ and $D^t + D \rightarrow \mathrm{Red} + \mathrm{Oxd}$, where D is the dye in the ground state. Thiopyronine solutions containing some reducing and oxidizing agents were studied in order to identify the intermediates. The absorption spectra of these intermediates were identified, and their reaction mechanism studied. The rate constants of some elementary processes obtained are:

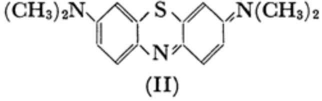
$$\begin{array}{rcl}
D^t & \longrightarrow & k_1 = 2.3_6 \times 10^3\ \mathrm{sec}^{-1} \\
D^t + D^t & \longrightarrow & k' = 7.6_9 \times 10^8\ \mathrm{M}^{-1}\mathrm{sec}^{-1} \\
D^t + D & \longrightarrow & k'' = 7.6_9 \times 10^8\ \mathrm{M}^{-1}\mathrm{sec}^{-1} \\
\mathrm{Red} + \mathrm{Red} & \longrightarrow & k_6 = (1\text{—}2) \times 10^9\ \mathrm{M}^{-1}\mathrm{sec}^{-1} \\
\mathrm{Oxd} & \longrightarrow & k_7 = (1\text{—}3) \times 10^3\ \mathrm{sec}^{-1} \\
D^t + \mathrm{EDTA} & \longrightarrow & k_8 = 1.6 \times 10^7\ \mathrm{M}^{-1}\mathrm{sec}^{-1} \\
D^t + \mathrm{ATU} & \longrightarrow & k_9 \approx \mathrm{small} \\
\mathrm{Oxd} + \mathrm{ATU} & \longrightarrow & k_{10} = (2\text{—}5) \times 10^6\ \mathrm{M}^{-1}\mathrm{sec}^{-1}.
\end{array}$$

The high efficiency of thiopyronine in its photodynamic effect was discussed in connection with the high quantum yield of triplet-state production and the high reversibility of the photochemical redox reaction of the dye.

Thiopyronine (I) is an efficient photosensitizing dye; it has a structure similar to that of the well-known photosensitizer, methylene blue (II).



(I)



(II)

Recently, investigations of the photodynamic effect of thiopyronine, as a photosensitizer, on various systems, such as bacterium, virus, nucleic acids, and their base components, have been reported by several authors, and the reaction mechanisms have been discussed.[1] However, all these investigations have been performed by steady-light illumination and were concerned only with the product analysis of the reaction or with physicochemical changes in the irradiated systems.

A more direct investigation of the intermediates is now necessary in order to elucidate the reaction mechanism.

Flash photolytic studies of the transient intermediates in the photoreduction of methylene blue

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1) K. Herzberg, K. Reuss and R. Dahn, *Naturwissenschaften,* **10**, 376 (1963); A. Wacker, G. Türck and A. Gerstenberger, *ibid.*, **10**, 377 (1963); A. Wacker, H. Dellweg, L. Träger, A. Kornhauser, E. Lodeman, G. Türck, R. Selzer, P. Chandra and M. Ishimoto, *Photochem. Photobiol.*, **3**, 369 (1964); J. S. Bellin and L. I. Grossman, *ibid.*, **4**, 45 (1965); H. Dellweg and W. Oprée, *Biophysik,* **3**, 241 (1966); E.-R. Lochmann, *ibid.*, **3**, 300 (1967); E.-R. Lochmann, *Z. Naturforschg.*, **22b**, 196 (1967).

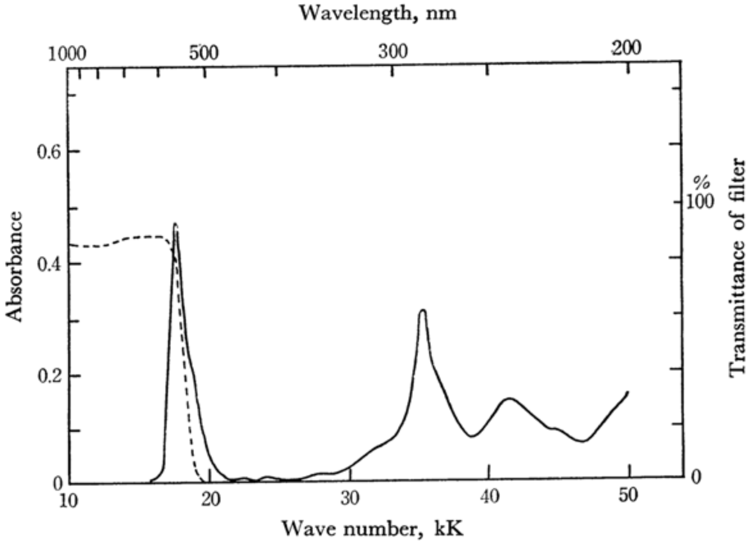

Fig. 1. Absorption spectrum of thiopyronine in buffered solution at pH 7.4; Dye concn. 6.4 μM. Optical path 1.0 cm. Broken line: Transmittance of the orange glass filter (Hoya Crystal Co., 0-54) used.

and thionine have been reported previously by the present authors.[2] That paper reported that the triplet-state of methylene blue produced by flash excitation interacts with the ground-state dye, producing semiquinone and a half-oxidized form.

This is an essential process in the photoreduction of methylene blue in a deaerated phosphate buffered solution in the absence of any reducing agent. A similar result obtained for thionine shows that the photochemical self redox reactions of these dyes take place readily.[3]

The photosensitizing efficiency of dyes has been discussed in connection with their photochemical redox reversibility.[4]

It will be interesting to investigate the correlation between the photosensitizing ability and the photochemical redox properties of these dyes from the point of view described above.

Also, it is expected that thiopyronine has a photochemical redox property similar to that of methylene blue, for the molecular structures are similar, and both have high photosensitizing powers. Thus, the flash photolytic investigation of thiopyronine was undertaken in order to clarify the reaction mechanism of the dye-sensitized photochemistry.

Some results have already been reported in a short form.[5] The aim of the present paper is to report in detail the spectroscopic and kinetic behavior of the transient intermediates of thiopyronine in an aqueous solution with or without substrate.

Experimental

Chemicals. The thiopyronine was synthesized in the way described by Biehringer and Topaloff.[6]

The results of an elementary analysis of the synthesized dye as a zinc double salt were in good agreement with the calculated values. (Found: C, 51.02; H, 4.97; N, 7.00%. Calcd for $C_{17}H_{19}N_2S\cdot\frac{1}{2}ZnCl_2$: C, 52.70; H, 4.95; N, 7.24%.)

After the zinc had been removed by adding ammonia, the dye was recrystallized, in the form of golden-green needles, from concentrated hydrochloric acid as the dichloride ($C_{17}H_{19}N_2SCl\cdot HCl$) which was then recrystallized three times from concentrated hydrochloric acid and desiccated over calcium oxide and concentrated sulfuric acid. The purity was checked by paper chromatography. The solvent used was a mixture of butanol (4), acetic acid (1), and water (4). The chromatogram showed only one distinct single R_f value, 0.61_4. The absorption spectrum of thiopyronine in a buffered solution at pH 7.4 is shown in Fig. 1. The molar extinction coefficient is $7.4\times10^4\text{M}^{-1}\text{cm}^{-1}$ at 564 nm.

The *p*-benzoquinone (Wako Junyaku Co., G.R.-grade reagent) was recrystallized from ethanol. The hydroquinone (Wako Junyaku Co., G.R.-grade reagent),

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[2] S. Kato, M. Morita and M. Koizumi, This Bulletin, **37**, 117 (1964).

[3] M. Morita and S. Kato, The 18th Annual Meeting of Chem. Soc. Japan, Osaka (1965).

[4] L. Lindqvist, *Arkiu Kemi*, **16**, 79 (1960); V. Kasche and L. Lindqvist, *Photochem. Photobiol.*, **4**, 923 (1965); L. Lindqvist, *Acta Chem. Scand.*, **20**, 2067 (1966).

[5] M. Morita and S. Kato, *Z. Naturforschg.*, **Ser. b,** in press.

[6] J. Biehringer and W. Topaloff, *J. Prakt. Chem.*, **65/66**, 499 (1902).

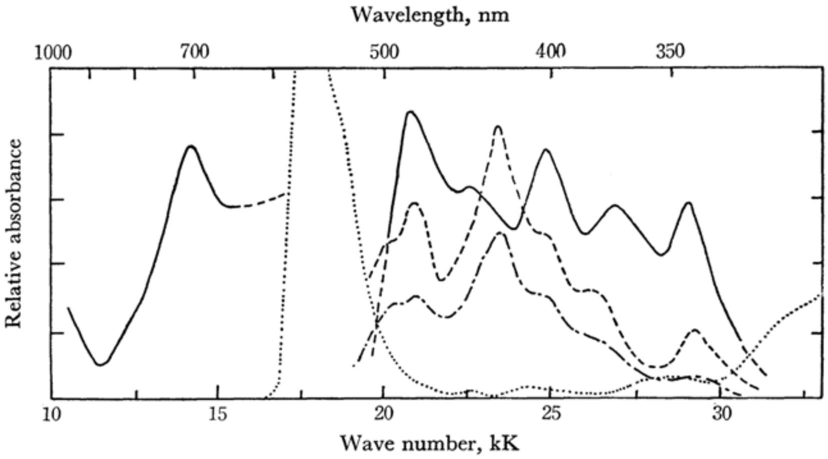

Fig. 2. Transient absorption spectra of thiopyronine in phosphate buffered solution at pH 7.4; Dye concn. 6.5 μM.
—— 22 μsec; - - - 130 μsec; —-— 200 μsec after flashing. The absorption spectrum in near-infrared region was constructed by an extrapolation of the decay curves measured at a series of wavelengths to zero time flashing. The absorption spectrum of thiopyronine is shown for the sake of comparison …….

allylthiourea (ATU) (Tokyo Kasei Co., G.R.-grade), and potassium dihydrogen phosphate (Wako Junyaku Co., G.R.-grade) were recrystallized three times from redistilled water. The sodium hydroxide solutions were prepared from a saturated solution of Wako Junyaku Co., G.R.-grade reagent. The disodium ethylene diamine tetraacetate (EDTA) and potassium persulfate, both Wako Junyaku Co., G.R.-grade reagent, were used without further treatment.

Preparation of Sample Solutions. The sample solutions were prepared from a stock thiopyronine solution of 5×10^{-4} M; the pH values were controlled with a 1/25 M KH_2PO_4-NaOH buffered solution and measured with a Hitachi-Horiba M-5 pH meter.

The solution was deaerated by more than five cycles of freezing-pumping-thawing. The absorption spectra before and after flashing were measured by means of a Shimadzu QB50 spectrophotometer or a Shimadzu multipurpose, auto-recording spectrophotometer (MPS-50).

Every measurement of flash photolysis was made with a fresh sample, even when the permanent change resulted from a single flash was negligibly small.

Apparatus. For photolysis, two quartz flash lamps filled with xenon (Ushio Electric Co., UF-693) were powered by a 4-μF capacitor charged up to 10 kV, if not otherwise mentioned. The duration time of photolysis lamps was 15 μsec.

A capillary flash lamp filled with argon was employed as a light source for the spectrophotography; it was worked at 3 kV with a 10-μF capacitor. The transient spectra were taken on Fuji Neopan plates, Fuji Neopan SS films, or Sakura Infrared 750 films by means of a Nalumi RM-23 grating spectrograph, which could also be used as a monochromator by turning a reflecting mirror. A triggering circuit controlled the time interval between the photolysis and the spectro flash from 10 μsec to 800 msec. Some measurements of the transient spectra were photographed by a double-beam flash apparatus described elswhere.[7]

The decays of the transient absorption spectra were measured with a xenon short-arc lamp (Ushio Electric Co., UXL-150D), photomultipliers (RCA 1P28 for the UV and visible region and 7102, Hamamatsu TV Co., for the red to near-infrared region), the monochromator described above, and a Hitachi V-116 synchroscope working through a cathode follower. The photolysis cells were glass tubes 10 cm long and 8 mm in inner diameter, to both ends of which were cemented quartz plane plates.

Hoya 0-54 orange glass cut-off filters were inserted between the photolysis flash lamps and the photolysis cell so as to exclude any light shorter than 540 nm, as is shown in Fig. 1, where the absorption spectrum of the dye is also depicted.

Results and Discussion

A. Transient Intermediates in the System Without Any Substrate.

A deaerated, plain, aqueous solution and a buffered solution of the dye were almost completely decolorized transiently just after irradiation by a 200 J flash. The permanent bleaching by a single flash was, however, less than 2%.

The transient absorption spectrum has rather complex feature, as is shown in Fig. 2. It has many peaks which behave in different ways.

The absorption around 350 nm and those in the near-infrared region decay rapidly, while the peak at 425 nm increases after flashing and the

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[7] S. Kato, "Zikken Kagaku Koza, Zoku 11, Denshisupekutoru (Handbook of Experimental Chemistry, Continued Vol. 11)," ed. by S. Nagakura, Maruzen Inc., Tokyo (1965).

peak at 475—500 nm decays slowly.

From this kinetical difference, it is clear that the overall spectrum consists of several bands of different natures, overlapping with each other.

The absorption decays measured at 340 and 690 nm were analyzed according to the first-order rate formura, as is shown in Fig. 3; the same rate constant was obtained for both wavelengths.

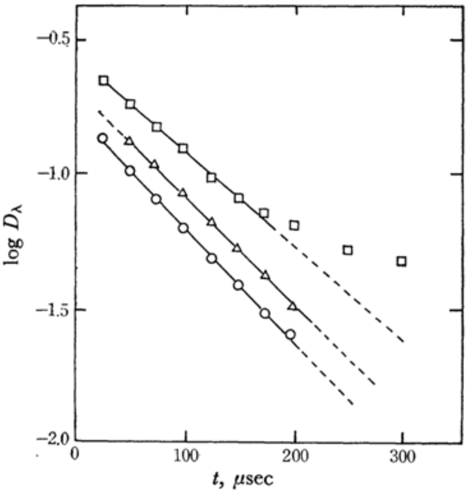

Fig. 3. First-order decay of the triplet state of the dye.
Dye concn. 8.8 μM. pH 7.4.
Wavelengths: ○ 340 nm, □ 375 nm, △ 690 nm

The plot for the first order at 375 nm is also nearly parallel, in the first part, to the other lines.

The coincidence of the rate constants make it possible to attribute these peaks to a single species, which may be assigned to the triplet-state of the dye. This assignment is strongly supported by the good correlation of the peaks with the energies for the triplet-triplet transition caluculated by means of the LCAO-MO-SCF method based on a variable *beta* approximation.[8]

Every single run for the decay at 340 or 690 nm can be well described as a first-order process, but the observed rate constant increases linearly with the initial dye concentration, as is shown in Fig. 4, which gives the following relation:

$$k_{\mathrm{obs}} = k_1 + k(\mathrm{D}).$$

On the other hand, the rate constant are quite independent of the flash intensity, whether it is high enough to make a complete transient decoloralization or is so low as to make a 20% conversion (see Fig. 5).

These results can be explained only by a postulating that the triplet-state and the ground-state of the dye make the same contribution to the decay of the triplet-state:

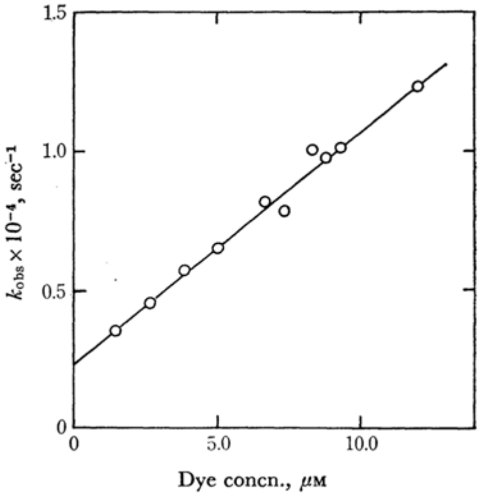

Fig. 4. Concentration dependence of the decay rate of the thiopyronine triplet in a phosphate buffered soultion at pH 7.4 measured at 340 nm.

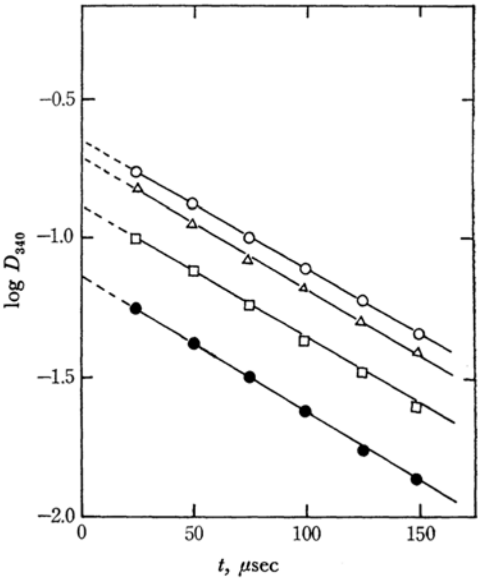

Fig. 5. The effect of the flash-light intensity (as a function of the input electric energy) on the decay rate of the triplet state measured at 340 nm.
Dye concn. 8.4 Mμ. pH 7.4.
○ 200J, △ 180J, □ 72J, ● 32J

$$\mathrm{D}^t \longrightarrow k_1$$
$$\mathrm{D}^t + \mathrm{D}^t \longrightarrow k'$$
$$\mathrm{D}^t + \mathrm{D} \longrightarrow k''.$$

The peaks at 425 and 475—500 nm have distinctly longer lives than the triplet state, but the kinetical behavior is rather too complex for quantitative analysis. This means that at least two species have absorption bands, greatly overlapping, in

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[8] M. Morita, S. Kato, K. Nishimoto and L. S. Forster, to be published.

this spectral region. Qualitatively speaking, there exist at least two species, one of which decays in the first-order with a lifetime of less than 100 μsec, and while the other decays in the second order.

From the experimental evidence presented above, and also analogously with the results for methylene blue,[2)] it is conceivable to attribute these long-living peaks to the half-oxidized form (475—500 nm) and the half-reduced form or semiquinone (425 nm) of the dye. This assignment was confirmed by the experiments to be described later.

The following reaction mechanism may, then, be proposed for the primary processes of thiopyronine in a buffered solution (pH 7.4) without any substrate:

$$\begin{array}{rcllr}
D + h\nu & \longrightarrow & D^* & & \\
D^* & \longrightarrow & D^t & & \\
D^t & \longrightarrow & D & k_1 & (1) \\
D^t + D & \longrightarrow & 2D & k_2 & (2) \\
D^t + D^t & \longrightarrow & D^t + D & k_3 & (3) \\
D^t + D & \longrightarrow & \text{Red} + \text{Oxd} & k_4 & (4) \\
D^t + D^t & \longrightarrow & \text{Red} + \text{Oxd} & k_5 & (5)
\end{array}$$

where D, D*, D^t, Red, and Oxd are the ground state, the first excited singlet state, the triplet-state, the half-reduced form, and the half-oxidized form of the dye respectively.

The decay of the triplet-state can now be represented as:

$$-\mathrm{d}(D^t)/\mathrm{d}t = k_1(D^t) + k_2(D^t)(D) + k_3(D^t)^2 + k_4(D^t)(D) + 2k_5(D^t)^2.$$

When (k_2+k_4) is assumed to be equal to (k_3+2k_5), the above equation turns into the following:

$$-\mathrm{d}(D^t)/\mathrm{d}t = k_1(D^t) + (k_3 + 2k_5)(D_0)(D^t),$$

where $(D_0) = (D^t) + (D)$,

and where (D_0) is the initial dye concentration. The integration of the simplified equation yields:

$$-\mathrm{d}\ln(D^t)/\mathrm{d}t = k_{\text{obs}} = k_1 + (k_3 + 2k_5)(D_0).$$

This also holds in the case of eosine.[9)]

Using the values obtained from Fig. 4, one obtains:

$$k_1 = 2.3_6\times10^3\ \text{sec}^{-1}$$

$$k_3 + 2k_5 = 7.6_9\times10^8\ \text{M}^{-1}\text{sec}^{-1}$$

$$k_2 + k_4 = 7.6_9\times10^8\ \text{M}^{-1}\text{sec}^{-1}.$$

B. Transient Intermediates in the Systems Containing Oxidants. *Potassium Persulfate* ($K_2S_2O_8$). In the presence of $K_2S_2O_8$ added as an oxidant, a partial fading of the dye gradually took place in a deaerated solution, even in the dark. When the solution containing 5×10^{-4} M $K_2S_2O_8$ at pH 6.9 was flashed, there appeared only one absorption band, between 450 and 510 nm, as is shown in Fig. 6, and a permanent bleaching of the dye was observed.

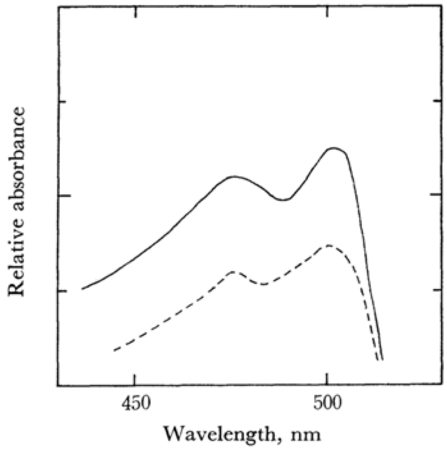

Fig. 6. Absorption spectra of transient species in a buffered solution at pH 6.9 containing 5×10^{-4} M $K_2S_2O_8$.
Dye concn. 3 μM
—— 40 μsec; - - - 105 μsec after flashing.

The absorption spectrum of the product has no distinct band, except for some rise in the shorter wavelength region.

p-Benzoquinone. In the case of the addition of *p*-benzoquinone as an oxidizing agent, two different transient absorption spectra were observed by flash excitation. Figure 7 shows the transient absorption spectra in this system.

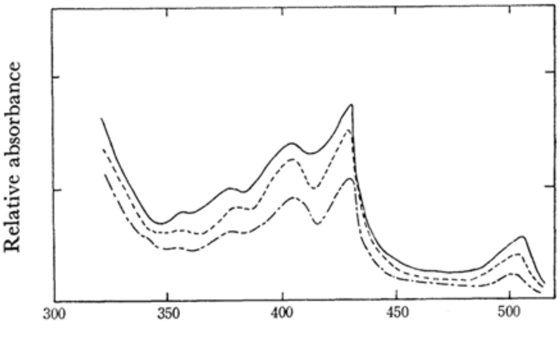

Fig. 7. Transient absorption spectra of thiopyronine phosphate buffered solution containing 5×10^{-4} M *p*-benzoquinone at pH 7.4.
Dye concn. 8 μM
—— 85 μsec; - - - 200 μsec; —·— 440 μsec after flashing.

One has four peaks, at 355, 375, 405, and 432 nm, and the other has one peak, at 500—505 nm. The former agrees with the absorption spectrum of the *p*-benzosemiquinone ion reported by several authors.[10)]

[9)] T. Ohno, S. Kato and M. Koizumi, This Bulletin, **39**, 232 (1966).

[10)] H. Diebler, M. Eigen and P. Matthies, *Z. Elektrochem.*, **65**, 634 (1961); G. E. Adams and B. D. Michael, *Trans. Faraday Soc.*, **63**, 1171 (1967).

Also, some kinetic studies undertaken at 430 and 370 nm show that these transient species decay according to the second-order rate formula, as is shown in Fig. 8; the slopes of the decay curves give the rate constant of $1.7_3\times10^8$ M^{-1} sec^{-1} at 430 nm and of $1.8_2\times10^8$ M^{-1} sec^{-1} at 370 nm, using $\varepsilon_{430}=7.3\times10^3$ M^{-1} cm^{-1} and $\varepsilon_{370}=4.6\times10^3$ M$^{-1}\cdot$cm^{-1} as the extinction coefficients of the *p*-benzosemiquinone ion at 430 and 370 nm respectively.

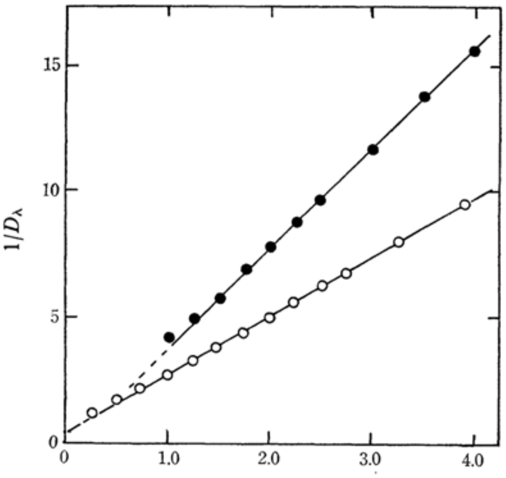

Fig. 8. Second-order decay of the *p*-benzosemiquinone ion.
Dye concn. 8.0 μM. *p*-Benzoquinone concn. 5× 10^{-4}M. pH 7.4. Wavelengths: ● 370 nm, ○ 430 mn

These rate constants agree with the value for the dismutation process of the *p*-benzosemiquinone ion previously reported by Diebler, Eigen, and Matthies if one takes into account the temperature difference in the measurements.[10)]

Thus, it is very likely that the *p*-benzosemiquinone ion decays through its own dismutation process, without interaction with a dye or any other intermediates.

The transient species, the absorption spectrum of which appeared in the region from 480 to 510 nm, with the peak at 500—505 nm may be identified as the half-oxidized form of the dye on the basis of a comparison with the case of $K_2S_2O_8$. There is no doubt that this intermediate is produced by the following reaction:

$$D^t + Q \longrightarrow Oxd + Q^-$$

where Q and Q$^-$ indicate *p*-benzoquinone and its semiquinone ion respectively. The transient decay kinetics at 490 nm gave the first-order decay rate constant of 1×10^3 sec^{-1}. This evidence confirms conclusively that the transient species with an absorption around 500 nm is not due to the semiquinnoe ion of *p*-benzoquinone, but to the half-oxidized form of the dye.

Aerated Buffered Solution. When an aerated solution of the dye was subjected to flash excitation, only a weak transient absorption spectrum appeared during irradiation; it was in the region from 450 to 510 nm, with two peaks, at 475 and 505 nm. The other transient absorption had completely vanished.

This shows that the transient intermediates which appear in a deaerated solution are due to oxygen sensitive species, *i. e.*, the triplet-state of the dye and the intermediates with longer lifetimes produced from the triplet state. This finding confirms the assignment presented above.

The intermediate with the weak absorption around 450—510 nm may be produced by the reaction between the triplet state of the dye and oxygen. However, a large portion of the triplet state of the dye is likely to be quenched physically with oxygen, thus regenerating the ground-state dye. It seems that only a small portion of the triplet-state dye reacts to give the transient species *via* the following process:

$$D^t + O_2 \longrightarrow Oxd + O_2^-.$$

Thus, the transient intermediate with an absorption around 450—510 nm may be assigned to the half-oxidized form of the dye.

C. Transient Intermediates in the Systems Containing Reductants. *Hydroquinone.* In the presence of 5×10^{-4} M hydroquinone added as a reducing agent, upon flash excitation, there appeared a very intense transient absorption spectrum, with 343, 370, 405, and 428 nm peaks as is shown in Fig. 9.

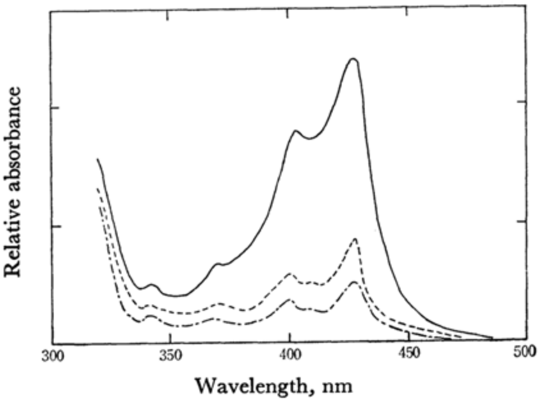

Fig. 9. Transient absorption spectra of a thiopyronine phosphate buffered solution at pH 7.4 containing 5×10^{-4} M benzohydroquinone.
Dye concn. 7.2 μM
—— 55 μsec; - - - 250 μsec; -·- 550 μsec after flashing.

The spectra obtained at 250 μsec and at 550 μsec have almost the same structure as that shown in Fig. 7 in the region from 350 to 450 nm. There is no doubt that these spectra are due to the *p*-

benzosemiquinone ion. On the other hand, the spectrum at 55 μsec is similar to the others in its peak positions, but different in the relative intensities. These results lead to the conclusion that an absorption band of another transient species is superposed in this spectral region; this species is certainly a semiquinone of the dye and has a shorter lifetime. In the case of methylene blue, the superposition of the transient absorptions due to the corresponding semiquinone in this wavelength region has been reported previously.[11] It may be noted that the transient absorption band from 470 to 510 nm which appeared in the dye-*p*-benzoquinone system completely vanishes in this system. Thus, there is scarcely any doubt that the electron transfer or hydrogen transfer occurs from the hydroquinone to the triplet state of the dye. Decay kinetics were undertaken at 430 and 370 nm. As is shown in Fig. 10, decay curves plotted on the second-order rate formula give the rate constants of $1.8_7 \times 10^8$ M^{-1} sec^{-1} at 430 nm and of $1.6_2 \times 10^8$ M^{-1} sec^{-1} at 370 nm, using the values of extinction coefficient for each wavelength presented above.

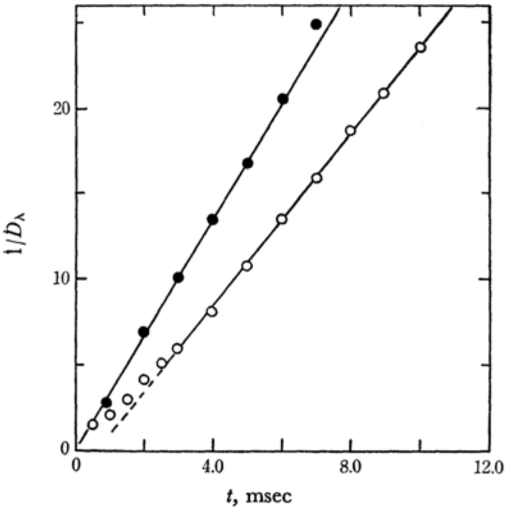

Fig. 10. Second-order decay of the *p*-benzosemiquinone ion.
Dye concn. 7.2 μM
Hydroquinone concn. 5×10^{-4} M. pH 7.4.
Wavelengths: ● 370 nm, ○ 430 nm

These values are in very good agreement with that of the dismutation rate constant of the *p*-benzoquinone ion obtained in the dye-*p*-benzoquinone system. This finding leads to the conclusion that the *p*-benzosemiquinone ion mainly disappears through its own dismutation process in any system examined. Meanwhile, the dye-hydroquinone solution became almost completely transparent at 205 nm, which is within the absorption region of the original dye, immediately after flashing, and then it turned back to become perfectly opaque in 200 μsec. The ground-state dye recovers according to the first-order rate formula. As regards the recovery process of the ground-state dye, such reactions as follows may be supposed:

$$\text{Red} + \text{Red} \longrightarrow \text{D} + \text{Leuco}$$

or: $$\text{Red} + \text{Q}^- \longrightarrow \text{D} + \text{QH}_2$$

where Q^- and QH_2 are the *p*-benzosemiquinone ion and hydroquinone. If such is the case, the recovery of the ground-state dye should be analyzed according to the second-order rate formula. Thus, this rapid recovery of the ground-state dye is likely to take place directly from an intermediate with a shorter lifetime, an intermediate which is certainly the triplet-state.

Disodium Ethylene Diamine Tetraacetate (*EDTA*). EDTA has been used as a reducing agent for the photochemical reduction of various classes of dyes.[12] When a deaerated thiopyronine solution containing 5×10^{-3} M EDTA was flashed, more than 50% of the dye was permanently bleached by a single flash.

The completely photobleached solution has a rather diffuse absorption spectrum in the UV region, as is shown in Fig. 11, in contrast to those of methylene blue and thionine.[13]

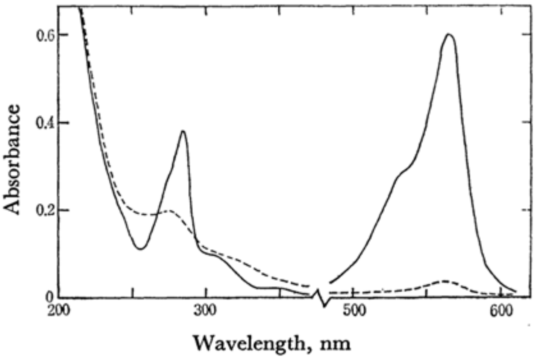

Fig. 11. Absorption spectrum of the photoproduct of thiopyronine containing 5×10^{-3} M EDTA as a reductant.
Dye concn. 8.1 μM
—— before flashing; - - - after flashing (photoproduct)

With the help of light, the photobleached solutions recover their color up to 60% or more of the original

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[11] S. Matsumoto, This Bulletin, **37**, 491 (1964).

[12] W. J. Nickerson and J. R. Merkel, *Biochem. Biophys. Acta*, **14**, 303 (1954); G. Oster and N. Wotherspoon, *J. Am. Chem. Soc.*, **79**, 4836 (1957); B. Holmström, *Photochem. Photobiol.*, **3**, 97 (1964).

[13] Y. Usui, H. Obata and M. Koizumi, This Bulletin, **34**, 1049 (1961); H. Obata, This Bulletin, **34**, 1057 (1961).

color upon the introduction of air. This shows that the major part of the photoproduct is the leuco dye. However, color recovery does not take place in dark, unlike the case of methylene blue and thionine.[14] When a buffered solution of thiopyronine at pH 7.4 containing 5×10^{-3} M EDTA was flashed, the absorption of the dye disappeared and a transient absorption spectrum with a peak at 426 nm appeared, as is shown in Fig. 12.

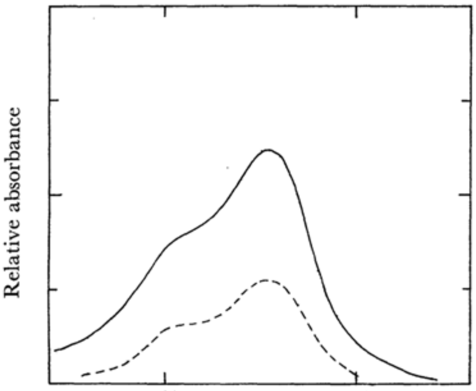

Fig. 12. Transient absorption spectrum of thiopyronine containing 5×10^{-3} M EDTA. pH 7.4. Dye concn. 8.2 μM
—— 60 μsec; - - - 260 μsec after flashing.

On the other hand, at lower concentrations of EDTA, transient spectra similar to those in the case of the plain aqueous solution and a buffered solution appeared at the initial stage, and then the 426 nm peak increased and the other peaks simultaneously decreased. At 200 μsec after flashing, the 426 nm peak alone survived. The general features of the photobleaching described above and the following kinetic investigations strongly suggest that this intermediate is due to the half-reduced form or the semiquinone of the dye.

The decay of the triplet state of the dye was accelerated by the addition of EDTA, as has been stated above. From the dependence of the decay-rate constant on the EDTA concentration, one can obtain the bimolecular rate constants for the following processes:

$$D^t + \text{EDTA} \longrightarrow \text{Red} + \text{EDTA}_{\text{oxd}} \quad k_r$$
$$\searrow \quad D + \text{EDTA} \quad k_d.$$

From the slope of the plot of the decay rate constant *vs.* the concentration of EDTA, the quenching constant k_q was obtained:

$$k_q = k_r + k_d = 1.6\times10^7\ \text{M}^{-1}\text{sec}^{-1}.$$

This value is comparable with that of methylene blue-EDTA, $k_r = k_d = 1.2\times10^7\ \text{M}^{-1}\ \text{sec}^{-1}$.[15]

The decay of the semiquinone, measured at 430 nm, is second order, as is shown in Fig. 13.

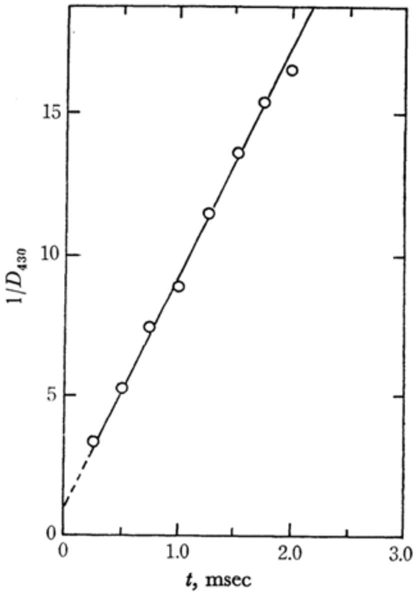

Fig. 13. Second-order decay of the semiquinone of thiopyronine.
Dye concn. 8.0 μM. EDTA concn. 5×10^{-3} M. pH 7.4
Monitored wavelengths: 430 nm

The slope of the line gives $k/\varepsilon_{430}d$, where k, ε_{430}, and d are the second-order rate constant, the extinction coefficient of the semiquinone at 430 nm, and the optical depth of the reaction cell respectively. It is not easy to determine the absolute value of k since ε_{430} is not certain. However, if it is assumed that the permanent photobleaching, or the production of leuco dye, takes place only through the dismutation process of the semiquinone:

$$\text{Red} + \text{Red} \longrightarrow \text{D} + \text{Leuco},$$

and if no other absorption band overlaps at this wavelength, (this is a reasonable assumption at a high EDTA concentration because it may effectively

TABLE 1. THE SECOND-ORDER RATE CONSTANT OF THE SEMIQUINONE OF THIOPYRONINE
Dye concn. 5.0μM. pH 7.4.

EDTA concn. M	k $\text{M}^{-1}\text{sec}^{-1}$
1.0×10^{-4}	$0.9_8\times10^9$
2.0×10^{-4}	$0.9_5\times10^9$
4.0×10^{-4}	$1.1_6\times10^9$
5.0×10^{-4}	$1.0_0\times10^9$
8.0×10^{-4}	$1.1_3\times10^9$
5.0×10^{-3}	$0.8_0\times10^9$
Average	$1.0_0\times10^9$

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[14] Y. Usui, This Bulletin, **38**, 206 (1965) and Ref. 13.

[15] N. Kosui, K. Uchida and M. Koizumi, *ibid.*, **38**, 1958 (1965).

consume the triplet-state, a state of which otherwise interferes with the measurement at this wavelength), ε_{430} can be estimated to be 1.0×10^4 M^{-1}cm^{-1} from the amount of the permanent bleaching and the transient increase in the absorbance at 430 nm.

The rate constants for the similar processes of rhodamine B, thionine, and methylene blue are illustrated in Table 2 for the sake of comparison, though the experimental conditions are not alike. The values for thiopyronine and methylene blue are as large as can be expected for a diffusion-controlled process.

Table 2. The second-order rate constants for the decay of semiquinones of cationic dyes

Dyes	Conditions	k M^{-1}sec^{-1}	Ref.
Rhodamine B	EtOH/H_2O	6.8×10^6	16)
	i-PrOH/H_2O (pH 6.2)	2.3×10^6	16)
Thionine	0.1N H_2SO_4	2.4×10^9	17)
Methylene blue	0.1N H_2SO_4	1.5×10^9	18)

Allylthiourea (ATU). ATU has also been used as a reducing agent for the photoreductions of several dyes;[19] however, it is milder in reducing power than EDTA.

Thiopyronine solutions containing ATU were photobleached by steady-light irradiation as well as by flash excitation, but the rate of the bleaching is slower than for EDTA by the factor of ten. For a thiopyronine solution containing 5×10^{-3} M ATU, only about 5% of the original dye was photobleached by a single flash. The absorption spectrum of the photoproduct has no distinct band, as in the case of the EDTA system. After almost complete bleaching with steady illumination, the introduction of air into the reaction cell and the irradiation of UV light recovered about 20% of the original dye, without any shift of the absorption peak at 564 nm. This definite recovery of the original dye confirms that a considerable fraction of the photoproduct is leuco dye.

The transient spectrum of this system, as is shown in Fig. 14, consists of all the characteristic bands of the triplet state (340, 370, and 400 nm), the half-oxidized form (475 nm), and the semiquinone (430 nm) of the dye, which appeared in the plain aqueous solution and the buffered solution.

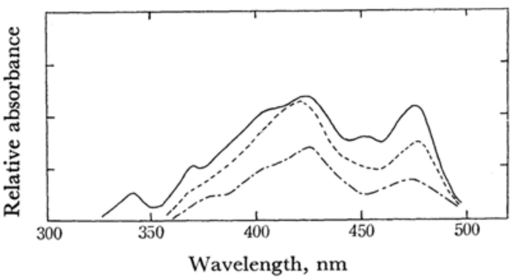

Fig. 14. Transient absorption spectrum of a thiopyronine buffered solution containing 5.0×10^{-3} M ATU. pH 7.6.
Dye concn. 10 μM
—— 45 μsec; - - - 110 μsec; —-— 255 μsec after flashing.

Kinetic investigations were undertaken at 430 nm for Red and at 480—500 nm for Oxd. Red decays according to the second-order rate formula, as is shown in Fig. 15.

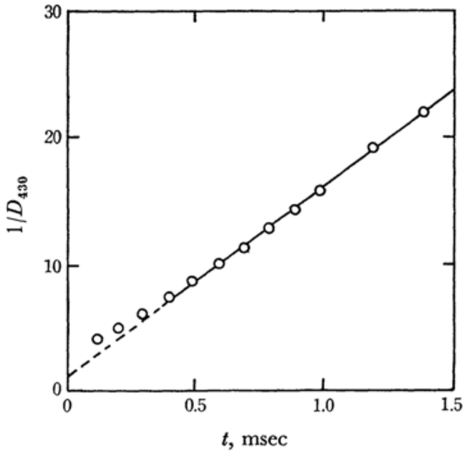

Fig. 15. Second-order decay of semiquinone of the thiopyronine.
Dye concn. 7.2 μM. ATU concn. 5×10^{-3} M. pH 7.6.
Monitored wavelength: 430 nm

The slope of this line gives 1.5×10^9 M^{-1}sec^{-1} for the second-order rate constant, using the value, $\varepsilon_{430}=1.0\times10^4$ M^{-1}cm^{-1}, estimated above. This rate constant is independent of the concentration of ATU added to the solution.

On the other hand, the half-oxidized form of the dye follows a pseudo first-order decay kinetics, the rate constant of which depends linearly on the concentration of the added ATU. From this relation, one obtains the rate constant for the following reaction:

$$\text{Oxd} + \text{ATU} \longrightarrow$$

to be (2—5)$\times10^6$ M^{-1}sec^{-1}.

Photochemically, ATU is not such a strong reducing agent as EDTA to the triplet-state of the

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16) B. Stevens, R. P. Sharpe and W. S. W. Bingham, *Photochem. Photobiol.*, **6**, 83 (1967).

17) C. G. Hatchard and C. A. Parker, *Trans. Faraday Soc.*, **57**, 1093 (1961).

18) C. A. Parker, *J. Phys. Chem.*, **63**, 26 (1959).

19) M. Pestemer, *Z. Elektrochem.*, **58**, 121 (1954); G. Oster and A. H. Adelman, *J. Am. Chem. Soc.*, **78**, 913 (1956); F. Millich and G. Oster, *ibid.*, **81**, 1357 (1959); K. Kikuchi and M. Koizumi, This Bulletin, **40**, 736 (1967).

dye. Because of this characteristics, the electron transfer between a dye molecule in the triplet-state and another in the ground-state or in the triplet-state predominates over the reaction between a dye molecule in the triplet-state and ATU.

Thus, the formation of the semiquinone and the half-oxidized form of the dye through dye-dye interaction is much more important than the direct attack of ATU on the triplet-state. Accordingly, ATU acts as a reductant mainly in the reaction with the half-oxidized form of the dye:

$$\mathrm{Oxd} + \mathrm{ATU} \longrightarrow \mathrm{D} + \mathrm{ATU}_{\mathrm{oxd}}$$

where $\mathrm{ATU}_{\mathrm{oxd}}$ is a one electron-oxidized form of ATU. This process takes place in the photoreduction of thiazine dyes, methylene blue and thionine. Koizumi and his co-workers have discussed this mechanism.[20]

If the photoreduction proceeds through this mechanism, the rate formula of the photoreduction by steady-illumination can be expected to be analyzed approximately by the second order with regard to the dye, as Koizumi *et al.* suggested. From this point of view, the steady-light photoreduction of thiopyronine was studied in our laboratory; this relation was confirmed in the case of ATU.

In general, two kinds of reaction

$$\mathrm{D}^{t} + \mathrm{D} \text{ or } \mathrm{D}^{t} \longrightarrow$$

and $$\mathrm{D}^{t} + \text{Reductant or Oxidant} \longrightarrow$$

compete with each other; the fraction of each path depends both on the reactivity of the added reagent and on its concentration relative to that of the dye.

This has been clearly demonstrated in the steady-light photoreduction of methylene blue reported by Kosui, Uchida, and Koizumi.[15]

The general features of the photochemical reactions of thiopyronine are very much like those of thiazine dyes.

To summarizing the results obtained in the present investigation, the elementary reactions of thiopyronine are as follows:

$$\begin{array}{rcll}
\mathrm{D} + h\nu & \longrightarrow & \mathrm{D}^{*} & \\
\mathrm{D}^{*} & \longrightarrow & \mathrm{D} + \mathrm{h}\nu_{\mathrm{f}} & \\
\mathrm{D}^{*} & \longrightarrow & \mathrm{D} + \text{heat} & \\
\mathrm{D}^{*} & \longrightarrow & \mathrm{D}^{t} & \\
\mathrm{D}^{t} & \longrightarrow & \mathrm{D} \qquad k_1 = 2.3_6 \times 10^3\,\mathrm{sec}^{-1} & (1)\\
\mathrm{D}^{t} + \mathrm{D} & \longrightarrow & 2\mathrm{D} \qquad k_2 & (2)\\
\mathrm{D}^{t} + \mathrm{D}^{t} & \longrightarrow & \mathrm{D}^{t} + \mathrm{D}\ k_3 & (3)\\
 & \searrow & 2\mathrm{D} & \\
\mathrm{D}^{t} + \mathrm{D} & \longrightarrow & \mathrm{Red} + \mathrm{Oxd}\ k_4 & (4)\\
\mathrm{D}^{t} + \mathrm{D}^{t} & \longrightarrow & \mathrm{Red} + \mathrm{Oxd}\ k_5 & (5)
\end{array}$$

$$k' = k_3 + 2k_5 = 7.6_9 \times 10^8\,\mathrm{M}^{-1}\mathrm{sec}^{-1}$$

$$k'' = k_2 + k_4 = 7.6_9 \times 10^8\,\mathrm{M}^{-1}\mathrm{sec}^{-1}$$

$$\begin{array}{rcll}
\mathrm{Red} + \mathrm{Red} & \longrightarrow & \mathrm{D} + \mathrm{Leuco} & (6)\\
 & & k_6 = (1-2) \times 10^9\,\mathrm{M}^{-1}\mathrm{sec}^{-1} & \\
\mathrm{Oxd} & \longrightarrow & \mathrm{X} & (7)\\
 & & k_7 = (1-3) \times 10^3\,\mathrm{sec}^{-1} & \\
\mathrm{D}^{t} + \mathrm{EDTA} & \longrightarrow & k_8 = 1.6 \times 10^7\,\mathrm{M}^{-1}\mathrm{sec}^{-1} & (8)\\
\mathrm{D}^{t} + \mathrm{ATU} & \longrightarrow & k_9 \approx \text{small} & (9)\\
\mathrm{Oxd} + \mathrm{ATU} & \longrightarrow & k_{10} = (2-5) \times 10^6\,\mathrm{M}^{-1}\mathrm{sec}^{-1}. & (10)
\end{array}$$

Lindqvist gave weight to the reversible photochemical redox property of dye in the photosensitizing activity in his extensive investigations of the flash photolysis of xanthene dyes.[4] The efficient photosensitizing power of thiopyronine as well as of methylene blue can be well understood in the light of this photochemical redox reversibility.

By the way, pyronine G, with a structure similar to that of thiopyronine, was studied by the flash method, but no transient absorption appeared under our experimental conditions.

In order to examine the concentration change in the dye in the ground-state, further studies were undertaken by the fluorescence method, which makes it possible to evaluate the amount of the transient bleaching of the dye by measuring the change in fluorescence intensity during flash excitation.[21]

These results showed that the transient bleaching did not take place in the phosphate buffered solution of pyronine G at pH 7.4.

On the other hand, in the case of thiopyronine, it was found that 90% or more of the ground state of the dye was converted to the triplet-state or to intermediate species with long lives during 200 J flash excitation. In the case of rhodamine B, with a structure similar to that of pyronine G, it has been reported that no transient absorption was observed in an aqueous solution.[16]

At any rate, these results are in good agreement with the fact that pyronine G has little photodynamic effect.

The failure to find any intermediates for pyronine G might be due either to their short lifetimes under our experimental conditions, or to the low extinction coefficient of the transient species in the spectral region available. However, this latter seems not to be the case, because our theoretical studies of these photosensitizing dyes predict that they will have very similar electronic structures.[8] Therefore, thiopyronine and pyronine G may be expected to exhibit similar spectroscopic behavior.

An alternative interpretation of this different situation between thiopyronine and pyronine G may be made in terms of the heteroatom effect.

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20) M. Koizumi, H. Obata and S. Hayashi, This Bulletin, **37**, 108 (1964).

21) A. Kira and S. Kato, *Sci. Rep. Tohoku Univ., Ser. 1*, **158**, 142 (1965).

It is well known that the introduction of a hetero-atom into an aromatic system facilitates inter-system crossing.[22)]

Nurmukhametov and Gobov have studied the phosphorescence of a series of compounds, *i. e.*, fluorenone, diphenylene oxide, diphenylene sulfide, and carbazole.[23)]

They have found that the presence of a nitrogen atom or, more especially, a sulfur atom in an aromatic system appreciably increases the probability of the S*–T intersystem crossing. This was observed as an increase of the phosphorescence yield.

Since the intersystem crossing competes successfully with fluorescence, a decreased fluorescence quantum yield should result. This was confirmed experimentally in the series of compounds mentioned above.[24)]

As to pyronine G, there seem no such experimental results. However, comparing the fluorescence quantum yield of pyronine G with that of rhodamine B, a high quantum yield of fluorescence of pyronine G may be anticipated from the structural similarity of these dyes.[25)]

On the other hand, the fluorescence quantum yield, Φ_f, and the quantum yield of the triplet-state production, Φ_{S^*-T}, of thiopyronine were determined experimentally in our laboratory to be $\Phi_f=0.20$ and $\Phi_{S^*-T}=0.67$.[26)]

Thus, the difference in photochemical properties between thiopyronine and pyronine G can be well understood in terms of the difference in the quantum yield of the triplet-state production of the dyes.

Conclusion

The flash excitation of a deaerated buffered solution of thiopyronine without any substrate leads to three transient intermediates: the triplet-state (D^t), the half-oxidized form (Oxd), and the half-reduced or the semiquinone (Red) of the dye.

Red and Oxd were generated simultaneously through the electron-transfer reactions between D^t and D^t and between D^t and D.

It has been established that the high efficiency of thiopyronine in the photodynamic effect is due to the high quantum yield of the triplet-state production and to the high photochemical redox reversibility of the dye.

Thanks are due to Professor H. Tsubomura of the Faculty of Engineering Science, Osaka University, for his permission to use the microphotodensitometer. The authors wish also to thank the Ministry of Education for its Scientific Research Grant-in-Aid.

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22) M. Kasha and S. P. McGlynn, *Ann. Rev. Phys. Chem.*, **7**, 403 (1956).

23) R. N. Nurmukhametov and G. V. Gobov, *Optics and Spectry.*, **18**, 126 (1965) (*Optika i Spektroskopiya*, **18**, 227 (1965)).

24) D. W. Ellis and B. S. Solomon, *J. Chem. Phys.*, **46**, 3497 (1967).

25) W. H. Melhuish, *J. Opt. Soc. Am.*, **52**, 1256 (1962); H. V. Prushel, A. L. Sommers and R. C. Cox, *Anal. Chem.*, **35**, 2166 (1963).

26) Unpublished results obtained in our laboratory.