Photosensitized Oxidation of Leuco-uranine and the Identification of the Photobleached Product of Uranine in Ethanol Solution

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According to Imamura and Koizumi[1,2], the reductive photobleaching of xanthene dyes such as eosine and uranine takes place when the degassed solutions of such dyes in ethanol are irradiated by visible light. They studied the primary processes of this reaction to some extent, but did not undertake the identification of the final product, suspecting that the dye structure might be completely destroyed since the dye is not restored by the introduction of air after the reaction. There remains, however, a possibility that the leuco dye itself is stable and exists as such in the presence of air, and even in case the above speculation be true, it seems at least possible that the decomposition would take place after the formation of leuco dye; the transient existence of a rather stable semiquinone has been established by the flash technique[3], and this makes more plausible the ultimate formation of leuco dye through disproportionation reaction.

With regard to leuco-uranine, Kul'berg and Matveev[4] had reported that this compound, being rather stable in the presence of air, can be conveniently utilized for the quantitative analysis of a minute quantity of oxidant. The present authors have now found that this leuco compound is easily converted photochemically to uranine by the use of some dyes and acridine as photosensitizers. Moreover, this reaction, contrary to our expectation, has proved to take place more rapidly under the deaerated condition. A similar reaction was found to occur with the photobleached product in question; this and some other facts have now unambiguously confirmed that most of the photobleached product exists as leuco dye.

Preparation of Leuco-uranine and Some of its Physico-chemical Properties

Preparation.—*Reagents.* — 1) Sodium amalgam: Prepared from Merck's sodium and distilled mercury.

2) Sodium hydroxide solution: Saturated solution was prepared by dissolving Wako Pure Chemical Industries' G. R. grade sodium hydroxide. To minimize the contamination of sodium carbonate, the supernatant liquid was used.

3) Fluorescein: For the preparation of leuco-uranine, Hikotaro Shudzui & Co. G. R. grade fluorescein was used without further purification. For chromatography, polarography and sensitized reaction, the above sample was neutralized and then twice recrystallized from ethanol by evaporating the solvent without heating. The uranine crystal thus obtained was desiccated in a high vacuum. This sample has an extinction coefficient of 8.2×10^4 at 490 mμ, the highest value ever obtained[5]; this assures its high purity.

Procedure. — Fluorescein was dissolved in water, neutralizing it with a small excess of sodium hydroxide, and then reduced by sodium amalgam. When the dye color became quite faint, the amalgam was removed from the solution and the precipitate of leuco-fluorescein was obtained by adding dilute hydrochloric acid. The precipitate had a tint of yellow at first but gradually turned brown. It is a sticky resin-like material but solidifies by slight cooling.

This substance, after being dissolved in 99% ethanol, was neutralized with an equimolar sodium hydroxide pellet; evaporation of the solvent under reduced pressure gave a crystalline substance.

Further purification was performed to remove the sodum carbonate and the small amount remaining of the oxidized form of uranine. The latter substance, being more soluble in ethanol than leuco dye, can be washed out by a small quantity of ethanol. Then the substance is dissolved in a large quantity of ethanol; sodium carbonate which is insoluble in ethanol can be eliminated by this procedure. The solvent was distilled off in a high vacuum, and the crystal deposited was treated further in the above way several times. The final evacuation was continued for more than ten hours to remove any trace of ethanol; this was continued until the ethanol vapor could not be detected by Geissler's tube.

It is, of course, necessary to perform all the above procedures under a safety lamp. Heating in the course of dissolution and precipitation is undesirable since it tends to promote the decomposition of the sample. Only at the last stage of desiccation was the sample heated up to 40°C.

The final substance obtained with a slight tint of brown is very deliquescent; hence, it was preserved in a sealed ampoule and was kept in the dark.

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[1] M. Imamura and M. Koizumi, This Bulletin, **28**, 117 (1955); ibid., **29**, 899 (1956); ibid., **29**, 913 (1956).

[2] M. Imamura, ibid., **30**, 249 (1957).

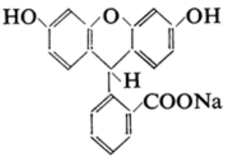
[3] S. Kato and M. Koizumi, *Nature*, **184**, 1620 (1959); S. Kato, T. Watanabe, S. Nagaki and M. Koizumi, This Bulletin, **33**, 262 (1960).

[4] L. Kul'berg and L. Matveev, *J. Gen. Chem.* (*U.S.S.R.*), **17**, 457 (1947); *Chem. Abstr.*, **42**, 478 (1948).

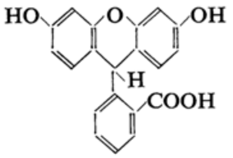
[5] M. Imamura, This Bulletin. **31**, 962 (1958).

The crystal does not melt up to 210°C.

Analysis of Leuco-uranine. — The sample is considered to be practically pure (I) from the results of acid and base titration.



I



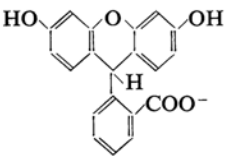
II

The sample was transformed into II by adding hydrochloric acid and, after washing with water and after desiccation through pumping in a high vacuum, was submitted to elementary analysis. The result is as follows:

Found: C, 68.06; H, 4.89. (a) Calcd. for II: C, 71.85; H, 4.22. (b) Calcd. for II with one mole of water (as crystalline water): C, 68.18; H, 4.58%.

The experimental value closely approximates the one for b.

Some Physico-chemical Properties of Leuco-uranine.—*Polarographic Studies.*—According to Delahay[6], who studied the polarogram of fluorescein in the pH range 2.00∼10.83, two-stage waves appear only in the alkaline region; this indicates a two-steps reduction involving the formation of stable semiquinone. He considered the ultimate form of leuco dye to be III.



III

To ascertain that the same III is obtained by treating our sample of fluorescein with sodium amalgam, our starting substance, fluorescein was re-examined polarographically in the alkaline region. The result gave two-stage waves, the half-wave potentials being −1.12 and −1.36 V. at pH 10.5, and −1.16 and −1.60 V. in a 1.2 N sodium hydroxide solution (referred to as S. C. E.). Delahay's value at pH 10.5 (interpolated value) is −1.18 V.

It is most plausible from the above results that the treatment of fluorescein with sodium amalgam in the alkaline solution gives I as a final product.

Absorption Spectra.—As shown in Fig. 1, the absorption spectra of leuco-uranine have two peaks, at 280 mμ $(\varepsilon\sim5\times10^3)$ and at 210 mμ. The extinction in the visible region is different from sample to sample, probably because of being contaminated with a different quantity of uranine, which has a large absorption maximum at 490 mμ. The second peak of leuco-uranine at 210 mμ is not so clear-cut, since it is superposed by the absorption of coexisting uranine.

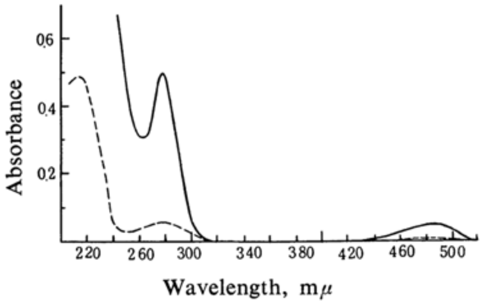


Fig. 1. Absorption spectra of leuco-uranine.
—— 1.0×10^{-4} M; ---- 1.0×10^{-5} M

Measurement of pK of Leuco-uranine. — For the measurement of pH, a Hitachi pH-meter E. H. P-1 was used. Acid and alkaline titration of leuco-uranine gave the two-step curves as shown in Fig. 2.

The two p*K* values obtained were $\mathrm{p}K_1=3.8$ and $\mathrm{p}K_2=9.6$ at 30°C. The first of these surely corresponds to the dissociation of the carboxyl group, II $\rightleftarrows$ III, in view of the fact that the p*K* values of benzoic acid, 2-ethylbenzoic acid

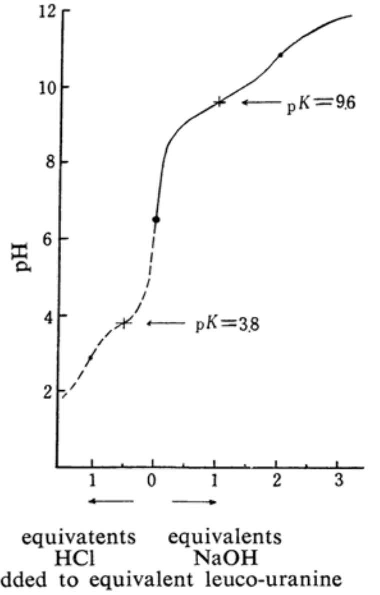


Fig. 2. An example of the titration curve of leuco-uranine.

Concn. of leuco-uranine: 2.76×10^{-2} M (25 ml.)
—— Alkali titration, NaOH 0.335 N
---- Acid titration, HCl 1.09 N

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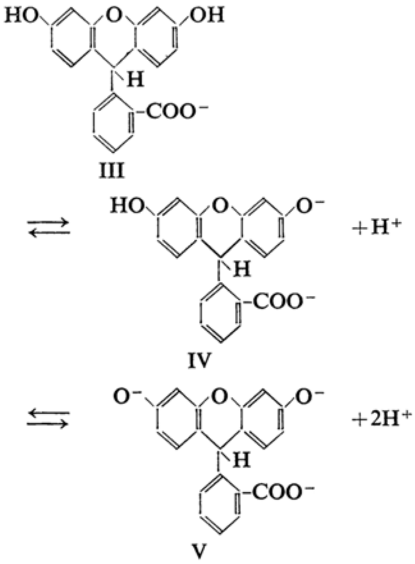
[6] P. Delahay, *Bull. soc. chim. France*, **15**, 348 (1948).

and 2-*tert*-butylbenzoic aid are respectively 4.2, 3.8[7] and 3.5[8].

The second value is most certainly an average of the two p*K* values corresponding to the following two dissociation processes:



Strong evidence for this is the fact that about two equivalents of sodium hydroxide are needed for neutralization. The value is reasonable in view of the p*K* value of phenol, 10.0. Table I gives a comparison of the above values with those of fluorescein. It seems worth while to note that leuco dye has somewhat different values.

TABLE I. p*K*-VALUES OF FLUORESCEIN AND ITS LEUCO COMPOUND

	Fluorescein		Leuco compound
	Zanker[9]	Lindqvist[10]	
Cation∼Neutral	1.95	2.2	
Neutral∼Univalent anion	5.05	4.4	3.8
Univalent∼Divalent	7.00	6.7	9.6
Divalent∼Tervalent			

Photosensitized Oxidation of Leuco-uranine

It was found that leuco-uranine in the aqueous solution is photochemically oxidized to uranine by adding some substances as sensitizers. Quite unexpectedly, the reaction was further found to take place more promptly in the deaerated condition. Acridine, xanthene dyes such as uranine, eosine, erythrosine and thiazine dyes such as methylene blue and thionine are more or less effective sensitizers. Some azo dyes and rhodamine were found to be not effective.

Detailed studies were made using acridine, uranine and methylene blue.

Materials.—*Acridine.*—Tokyo Kasei G. R. grade acridine was submitted to repeated crystallization using a water-ethanol mixture. The concentration of acridine employed as a sensitizer was usually 1.5×10^{-4} M.

Methylene Blue.—Grübler's sample was twice recrystallized from *n*-butanol.

Other Dyes. — Commercial reagents were used without further purification.

Water.—Twice distilled.

Ethanol.—Wako Pure Chemical Industries' G. R. grade ethanol was used without further purification.

The concentration of leuco-uranine was usually 1×10^{-5} M.

The solution for each experiment was prepared by diluting the stock solution of 1.0×10^{-4} M. The stock solution when kept in the dark scarcely showed any change.

Apparatus.—In the case of acridine being employed as a sensitizer, the irradiating light was 365 mμ from a high pressure mercury lamp, passed through a ultraviolet filter (D-2). When the sensitizer was uranine or methylene blue, a 75 V.-500 W tungsten lamp was used at 45.0 V., the light <360 mμ being cut off in the former case with a ultraviolet-cut off-filter, and the light <600 mμ in the latter case with a Walz R-2 filter. The degree of coloration was measured by a Hitachi spectrophotometer EPU-2A, using a $1\times1\times4$ cm. Terex cell which was designed for evacuation. Degassing of the sample was done by use of a mercury diffusion pump, melting and freezing (in a dry-ice bath) being repeated five or six times.

The aqueous solution of acridine has an absorption maximum at 355 mμ, at which there exists no absorption of leuco-uranine or uranine; hence, 365 mμ of mercury lamp is very convenient for studying this system. This is the reason that acridine was chiefly used as a sensitizer.

Results

When the aerated solution containing leuco-uranine and acridine was irradiated with 365 mμ light, the coloration due to the restoration of uranine took place, but it did not go over 40%; further irradiation after evacuation never induced any more coloration. Examination of this material by paper chromatography proved the absence of leuco-uranine. Thus, it seems plausible that the decomposition of the dye took place, perhaps photochemically, following the formation of uranine.

The absorption spectra of the irradiated solution completely coincide with those of uranine in the visible region, with a maximum at 490 mμ and a shoulder near 460 mμ.

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[7] "International Critical Table", VI, McGraw-Hill, New York (1929), p. 291.

[8] J. B. Shoesmith and A. Mackie, *J. Chem. Soc.*, **1936**, 300.

[9] V. Zanker and W. Peter, *Chem, Ber.*, **91**, 572 (1958).

[10] L. Lindqvist, *Arkiv. Kemi.*, **16**, 79 (1960).

Scarcely any change was perceived in the spectra of acridine (in the ultraviolet region).

The unexpected coloration which takes place in the highly degassed system proceeds with a much greater speed, and, moreover, it is almost quantitative.

In Fig. 3 the absorption spectra before and after the irradiation are shown. It is remarkable that there is no change in the absorption spectra of acridine, which precludes the hydrogenation of acridine taking place. There is no evolution of hydrogen*, but the exact nature of this reaction is quite unknown at the present time.

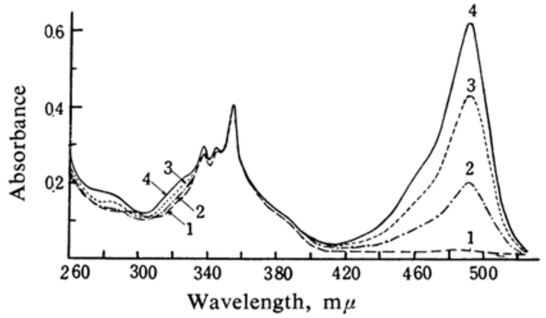

Fig. 3. Absorption spectra of leuco-uranine +acridine (sensitizer) at various irradiation times.

1 ——— Before irradiation
2 —·—· 1 min. irradiation
3 ------ 4 min. irradiation
4 ——— 30 min. irradiation

In the case of uranine as a sensitizer (with light >360 mμ), the reaction was also accelerated, so to speak in this case, autocatalytically. In this case also, degassing promotes the coloration and makes it more complete. The absorption spectra of the irradiated solution, as shown in Fig. 4, are quite similar to those of uranine. In the aerated solution, the coloration did not exceed 50%, whence the optical density at 490 mμ gradually declined, perhaps due to the irreversible photoöxidation.

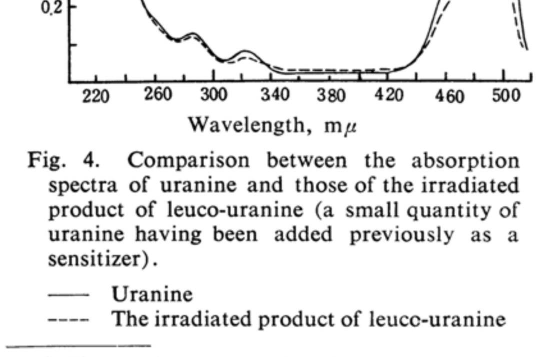

Fig. 4. Comparison between the absorption spectra of uranine and those of the irradiated product of leuco-uranine (a small quantity of uranine having been added previously as a sensitizer).

—— Uranine
---- The irradiated product of leuco-uranine

When methylene blue was used as a sensitizer under the irradiation with light >600 mμ, the absorption spectra of uranine with a maximum at 490 mμ also grew with a moderate speed. In the presence of air, no appreciable change was observed in the absorption spectra of methylene blue, with only a slight decrease of optical dentity, probable due to photoöxidation[11].

In case of the evacuated solution, methylene blue is photoreduced to leuco, dye accompanied by the change of leuco-uranine to uranine. Its rate is very large, as is seen from Fig. 5A, which shows the absorption spectra of the solution at various irradiation times. The maximum of methylene blue at 665 mμ gradually declines even when the solution is kept in the dark, but the decline is very rapid when the solution is irradiated, and it is restored up to about 96% when air is introduced after the reaction. The maximum at 490 mμ, which is due to uranine, grows gradually when the solution is kept in the dark. For example, the decrease of methylene blue and the increase of uranine when the solution is kept in the dark for four hours after evacuation, are respectively 0.33×10^{-8} mol. and 0.17×10^{-8} mol. The excess of the former over the latter is perhaps due to the adsorption of methylene blue to the cell wall. Irradiation accelerates the rate very much. The introduction of air causes a slight increase of absorbance at 490 mμ, which is probably due to the absorption of methylene blue restored having a small extinction in this region.

In the ultraviolet region, the absorption spectra of methylene blue, leuco methylene blue, uranine and leuco-uranine overlap each other, complicating the change of the entire absorption spectra. Subtracting the absorption spectra due to uranine and leuco-uranine from the entire spectra, one gets the resultant spectra shown in Fig. 5B.

Although some degree of error is unavoidable in this treatment, it is clear that the absorption maxima at 293 and 248 mμ of methylene blue existent prior to irradiation disappear and that in their place a new maximum appears at 256 mμ in the irradiated solution.

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* The experiment concerning this will be published shortly.

11) H. Obata, K. Kogasaka and M. Koizumi, This Bulletin, 32, 125 (1959).

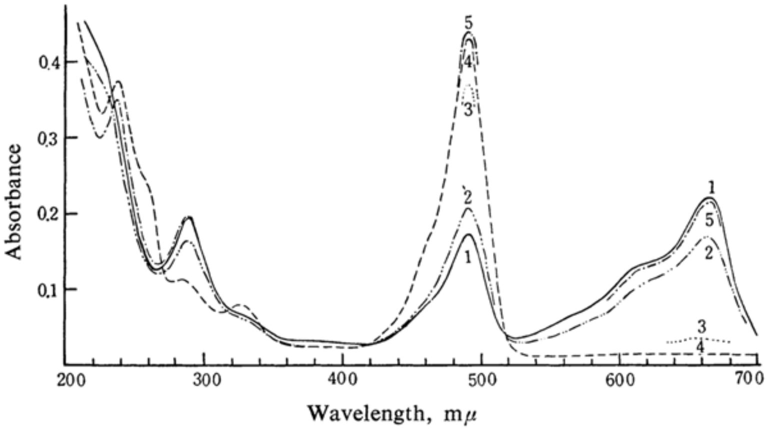

Fig. 5A. Absorption spectra of the solution containing leuco-uranine and methylene blue (sensitizer).

1 ——— Directly after deaeration
2 —··— After 4 hr. in the dark
3 ------ 10 sec. irradiation
4 ——— 90 sec. irradiation
5 —·—· After introduction of air

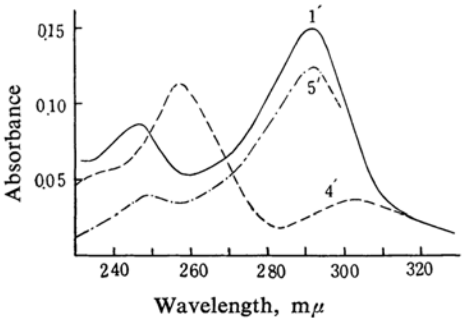

Fig. 5B. The resultant absorption spectra (240 ~320 mμ) when the contributions of leuco-uranine or uranine are subtracted from the observed spectra (Fig. 5A.).

1′ ——— Before irradiation
4′ ------ After irradiation
5′ —·—· After introduction of air

This is exactly equal to that of leuco methylene blue[12]. When the air is introduced after the reaction, the latter disappears and the two maxima due to methylene blue again come to exist.

The oxidation of leuco-uranine proceeds quite rapidly as long as methylene blue exists, but it becomes very slow when almost all the methylene blue turns into leuco form.

The quantity of methylene blue and leuco-uranine having reacted can be evaluated separately by the use of the extinction coefficients of methylene blue (6.0×10^4 at 665 mμ) and of uranine (8.2×10^4 at 490 mμ). Calculation showed that during the irradiation for 1.5 min., 1.4×10^{-8} mol. of methylene blue has disappeared and 1.2×10^{-8} mol. of uranine has been produced. The two values coincide well within the range of experimental error.

The reactions with the use of methylene blue are quite reasonable, since the oxidation-reduction potential of methylene blue and uranine are respectively ca. 0 and <-1 V.

Identification of the Photobleached Product of Imamura and Koizumi with Leuco-uranine

As to the photobleached product when the ethanol solution of uranine or eosine is irradiated by a visible light, Imamura and Koizumi suggested that the structure of the dye might be decomposed[1,2]. Now it is evident, however, from the results described above that the leuco dye is not so reactive, and hence there is ample possibility for the final product to be nothing but leuco dye. Therefore, the comparison between the chemically prepared leuco dye and the photobleached compound was undertaken spectroscopically and especially in respect to the photosensitized oxidation, which is highly specific.

Besides, paper-chromatographic comparison between them was tried.

Photosensitized Oxidation of the Photobleached Product.—The absorption spectra of the photobleached product, as shown in Fig. 6, resemble those of leuco-uranine, with maxima at 285 mμ and <220 mμ.

The photobleached product was prepared by irradiating the ethanol solution of uranine in the usual way. It was then separated from ethanol by distilling the latter in vacuo; to its alkaline solution was added acridine, and then the solution was irradiated by a 365 mμ

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[12] Y. Usui, H. Obata and M. Koizumi, ibid., **34**, 1049 (1961).

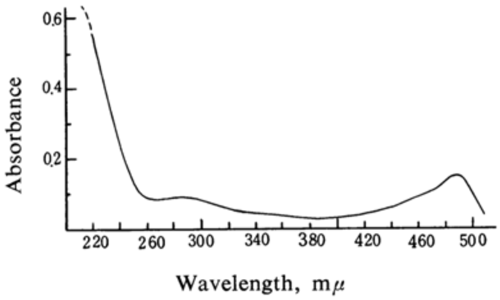

Fig. 6. Absorption spectra of the photobleached product of uranine as dissolved in a weak alkaline aqueous solution.

light. The formation of uranine was observed up to about 25% of the original quantity. In contrast to this result obtained for the aerated solution, degassing of the system accelerated the reaction, and the recovery attained about 60%. This indicates that at least more than 60% of the photobleached product exists as leuco dye.

It is to be added, however, that the restoration of color is slower than that of the chemically prepared leuco dye, the rate in the aerated and deaerated solution being respectively about half and one-fifth of that of the latter. This might be due to the inhibiting action of a certain substance produced during the photobleaching.

An analogous comparison was made with eosine.

The photosensitized coloration of the chemically prepared leuco-eosine in the deaerated alkaline solution was only about 70% of the total quantity. For the photobleached product it was about 35%. From these results it can be said that about half of the photobleached product exists as leuco dye.

In the aerated solution the result is not so clear-cut, the coloration in both the photobleached and chemically prepared samples being only 15∼20%.

It is to be added that, in these cases, the shift of the maximum is accompanied by the oxidation of the leuco dye. Thus, the absorption peak of the recovered dye is 506∼505 mμ compared with 514 mμ of eosine in the alkaline solution. This is due to the debromination reaction occurring in the alkaline solution[13]; this reaction would take place during the photosensitized oxidation and, moreover, might occur in the course of the preparation of leuco dye, which was done in a 1.3 N alkaline solution to make easy its reduction by sodium amalgam. According to Oster[14], dibromofluorescein has a peak at 506 mμ, which is exactly the same as the above value.

Analysis by Means of Paper Chromatography.—Taylor's method[15] was employed except that the composition of the developer was somewhat modified. Our composition was 28% ammonia water 10 vol.%, ethanol 15 vol.%, water 75 vol.%. Since the concentration of both uranine and leuco-uranine was low, the position after the development was decided by the fluorescence emitted by ultraviolet irradiation.

The ascending method was employed, and the filter papers used were Toyo Roshi (filter paper) No. 50. All the procedures were done in the dark room.

The results obtained for uranine are shown in Table II. It is clear that the result for the photobleached product is quite the same as that of leuco-uranine except for the existence of a certain substance with $R_f=0.935$. This unknown substance emits fluorescence with a white tint. 0.79 of leuco-uranine and the photobleached product, without doubt, is due to contaminated uranine. The R_f of leuco compound (0.86) is easily ascertained, since it is oxidized to uranine when irradiated (after development) and emits the fluorescence characteristic to uranine. Samples c and d usually give only $R_f=0.78$, but in addition they sometimes give $R_f=0.86$ of leuco-uranine, though its quantity is always very small.

TABLE II. THE RESULTS OF PAPER CHROMATOGRAPHY FOR URANINE AND RELATED COMPOUNDS

	R_f		
a) Uranine	0.78		
b) Leuco-uranine	0.79	0.86	
c) The material obtained from leuco dye by oxidation in air	0.78		
d) The material obtained from leuco dye by oxidation in vacuo	0.79	0.86 (very little)	
e) The photobleached product from uranine	0.79	0.85	0.935(?)

In case of eosine the results are more complicated and the reproducibility of the R_f-values is not so good. It can be said, however, from the results of more than ten tests, that the photobleached product contains an appreciable quantity of leuco-eosine. The material obtained by the photosensitized oxidation of leuco-eosine gives spots other than those of eosine, which may perhaps be attributed to dibromofluorescein.

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[13] M. Imamura and M. Koizumi, ibid., **29**, 913 (1956). Eosine, when, irradiated in the alkaline water-ethanol mixture, undergoes a rapid debromination.

[14] A. H. Adelman and G. Oster, *J. Am. Chem. Soc.*, **78**, 3977 (1956).

[15] K. B. Taylor, *Nature*, **185**, 243 (1960).

Summary

Leuco-uranine was prepared by reducing uranine with sodium amalgam, and some of its properties were investigated. The aqueous solution of leuco-uranine is stable in the presence of air irrespective of its low oxidation-reduction potential.

It was found that leuco-uranine is photooxidized to uranine by the use of acridine and some dyes as sensitizers. The reaction may be attributed to the dissolved oxygen and is very reasonable. A quite unexpected result was, however, that a similar reaction takes place more rapidly and more quantitatively in the degassed solution than in the aerated solution. The uranine and acridine employed as sensitizers in this case were ascertained not to sustain any change. The nature of the reaction is not known at the present time.

It was further established that the photobleached product obtained by irradiating the deaerated ethanol solution of xanthene dyes, consists mainly of leuco dye. This was ascertained by comparing their absorption spectra, by the photosensitized coloration, acridine being used as a sensitizer, and, in addition, by means of paper chromatography.

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