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# STUDIES ON THE TOXICITY OF COAL-TAR DYES. I. PHOTODECOMPOSED PRODUCTS OF FOUR XANTHENE DYES AND THEIR ACUTE TOXICITY TO FISH

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**Abstract**……The acute toxicity of photodecomposed products of Erythrosine, Eosine, Phloxine and Rose Bengale were studied, since it was found that toxicity of these dyes to fish increased after the dyes had been photoirradiated.

Photodecomposed products of the dyes were isolated and identified with UV, IR, NMR spectra and the acute toxicity of those compounds were determined by TLm test.

As results of these studies, it became clear that the toxicity of photodecomposed organic products (dehalogenated compounds of dyes) were lower than the mother compounds.

The increases in toxicity of the xanthene dyes by photo-irradiation were attributed to the liberated halogens by irradiation.

**Key words**: acute toxicity, photodecomposition, TLm test, halogen, fluorescein, xanthene dye, thin layer chromatography, high performance liquid chromatography

## INTRODUCTION

The authors in their previous papers (Tonogai et al., 1978) studied the effects of coal-tar dyes which were released into nature through industrial effluents discharged from dyestuff factories. For studying the decomposition pathways in the natural environment such as biodegradation, photolysis and chemical decomposition (Kitano et al., 1977), the authors carried out the microbial decomposition tests of dyes using activated sludge and TLm test using "Himedaka" (Oryzias latipes).

As its results it was clarified that xanthene dyes among the coal-tar dyes sho-

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wed smaller TLm values and the toxicity was found to incrcase further upon irradiation with light, which leads one to consider dyes' decomposed products has great influence on the lives in the environment.

Thus, to prevent environmental pollution due to coal-tar dyes in advance, it seems to be necessary to clarify their decomposition pathways under the natural conditions and to study the toxicities of the decomposed products.

The toxicity of xanthene dyes to fish is assumed to be caused by biological reactions of dyes and the effects of photolytic products to fish body. However in this paper, to trace the cause of fish poison under photoirradiation in view of the photolytic products of dyes, the major products of photolysis were separated to identify their structures, and their toxicity was investigated by the TLm test using "Himedaka".

As its results a rapid increase in xanthene dyes' toxicity under photoirradiation was clarified to be partly due to halogen yielded by photolysis of dyes.

## METHODS

1. Materials and equipment

1) Dye solution: The coal-tar dyes used were the reference standards of the National Institute of Hygienic Sciences. They were dissolved in water or methanol to make 2 mM.

2) Fluorescein compounds: 4, 5-Dibromofluorescein, 4, 5-diiodofluorescein and 12, 13, 14, 15-tetrachlorofluorescein supplied from Tokyo kasei kogyo Co., (G. R) were recrystallized from ethanol.

3) Photo-chemical reaction apparatus: Eiko HALOS-PIL 60 (inner irradiation type) equipped with a low pressure mercury lamp was used.

4) Spectrophotometer: Hitachi, Type 556, 2-wavelengths, self-registering.

5) Infrared spectrophotometer: Hitachi, Type EPI-G2.

6) Nuclear magnetic resonance absorber: Hitachi, Type R-2OB.

7) Gas chromatograph: Yanaco G-80, with flame ionization detection system.

8) High performance liquid chromatograph (HPLC): Shimadzu, Type E-336A.

2. Test methods

1) Conditions for photolysis of dyes: Into the reactor 500ml of 2 mM of dyes either in water or methanol were taken, and the light irradiation was performed at 80 V, 14 A passing through nitrogen into the reaction mixture.

2) Qualitative analysis by thin layer chromatography (TLC): As an adsorber 0.25mm layer of Kieselgel G and as a developer a mixture of ethyl acetate: methanol: 28% ammonia water (9 : 2 : 1.4) were used.

3) Qualitative analysis by gas chromatography (GC): The following two columns were used-5% DEGS+1% phosphoric acid/Chromosorb W (60-80) and 3% SE-30/Chromosorb W (60-80). Column temperature was programmed from 60 to 250°C.

Nitrogen flow rate was 20 ml/min.

4) Separation of photo-decomposed products: 500ml of 2 mM dyes in methanol solution was photoirradiated for 10 hours, then each 1,000ml of ether and petroleum ether were added and the mixture was allowed to stand. The yielded crystals were collected by filtration and dissolved into a small amount of methanol, and was subjected to the preparative separation on the TLC plate. The major band was scraped off, eluted with methanol and filtered. The recrystallization was performed and the photo-decomposed products of the dyes were obtained.

5) Quantitative analysis by use of HPLC: Column-"Zipax" SAX. Elution-concentration gradient. Basic liquid-2 M boric acid and 0.02M sodium perchlorate dissolved in water and adjusted to pH 8.5 As additive-12.2 g of sodium perchlorate (0.1M) was dissolved to a mixture of 600ml acetonitrile and 400ml water. Flow rate was adjusted to 0.7 ml/min. and measurement was made at wavelength of 254 nm.

6) Quantitative analyiss of iodine by potentiometric titration: Using silver as indicator electrode and mercurous sulfate as reference electrode, iodine in the test solution (50ml) was titrated (Shimura et al., 1969).

7) TLm tests with fish: The same as stated in the previous papers (Tonogai et al., 1978), using 10 "HIMEDAKA" per trial. The TLm value was obtained from the 50 % survival rate of fish at 25°C after 48 hours. As for Eosine, Erythrosine, Phloxime, Rose Bengale and Acid Red, the TLm test was also carried out after irradiation for 10 hrs.

## RESULTS AND DISCUSSION

### 1. photo-decomposition of xanthene dyes

Some reports are available referring to the photochemical reactions of xanthene dyes in various solvents (for example, Immamura et al., 1956, 1958, 1970). However,

**Table.** 1. Comparison of TLm value about fluorescein related compounds

| Coal-tar dye | Before irradiation | After irradiation |
|---|---|---|
| Fluorescein sodium | 3,000 (mg/l) | — (mg/l) |
| Tetra-Cl-fluorescein Na* | 2,300 | — |
| Eosine | 1,800 | 1,200 |
| Erythrosine | 900 | 500 |
| Phloxine | 200 | 60 |
| Rose Bengale | 100 | 40 |
| Acid Red | >3,000 | >3,000 |

Ten fish of HIMEDAKA were used per trial and TLm values were derived from 50% survival of fish after 48 hrs. at 25°C. *12, 13, 14, 15-Tetrachlorofluorescein sodium

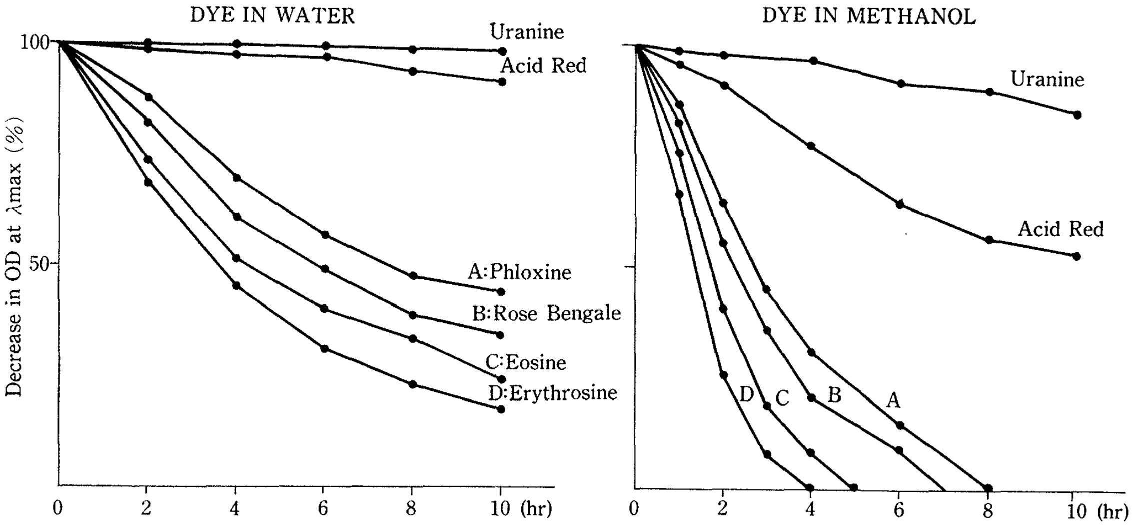


**Fig. 1.** Photodecomposition patterns of various xanthene dyes in water and methanol 2 mM of dye solution was irradiated by low pressure mercury lamp, and change in absorbance at λmax of each dye was determined.

λmax Uranine : 456nm
Acid Red : 566nm
Phloxine : 538nm
Rose Bengale : 548nm
Eosine : 517nm
Erythrosine : 526nm

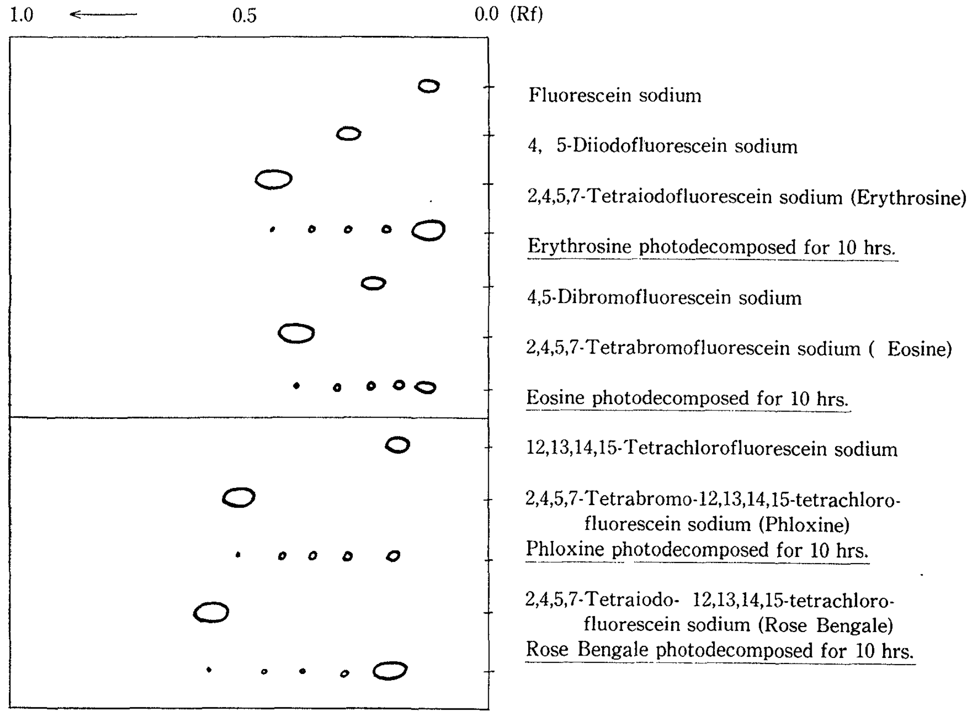


**Fig. 2.** Thin-layer chromatograms of fluorescein compounds and photodecomposed products of Erythrosine, Eosine, Phloxine and Rose Bengale
Adsorbent : Kieselgel G Solvent : ethyl acetate:methanol : 28% ammonia water (9 : 2 : 1. 4)

none of the papers deals with qualitative or quantitative analysis of photolytic products with the lapse of time, identification of structures of the photo-decomposed products as well as their toxicity.

In this paper several xanthene dyes, namely Erythrosine, Eosine, Phloxine, Rose Bengale, Acid Red and Uranine (fluorescein sodium), were dissolved separately in water or methanol to make 2 mM, and then photoirradiated for 10 hours. The results are indicated in Fig. 1.

It was found that little change was observed with Acid Red and Uranine in water solution, while the other 4 dyes indicated 50-80% decrease in OD after 10 hours.

On the other hand, in methanol solution Erythrosine, Eosine, Phloxine and Rose Bengale showed complete discoloration after 4-8 hours, while Acid Red and Uranine indicated decreases of about 50% and 20%, after 10 hours respectively.

All dyes were known to be decomposed rapidly in methanol than in water, which might be attributed to the formation of radical structure of dyes in methanol (Koizumi, 1969) being easily decomposed on exposure to light.

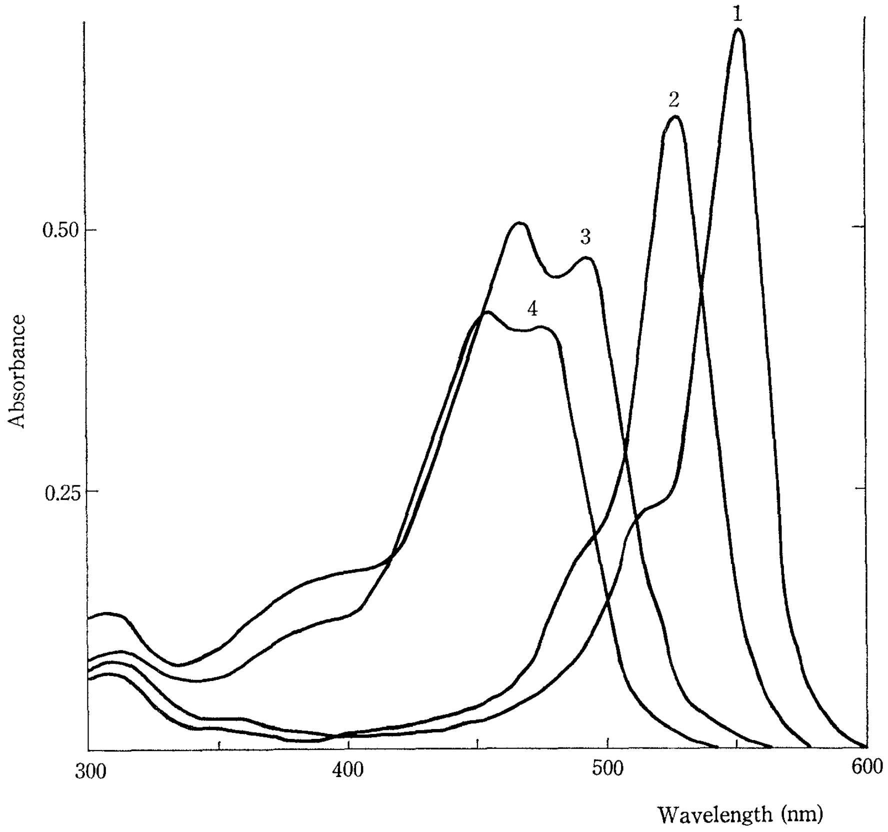


Fig. 3. Ultraviolet and visible absorption spectra of Rose Bengale, Erythrosine and their photodecomposed products
1 : Standard Rose Bengale 2 : Standard Erythrosine
3 : Major product of Rose Bengale
4 : Major product of Erythrosine

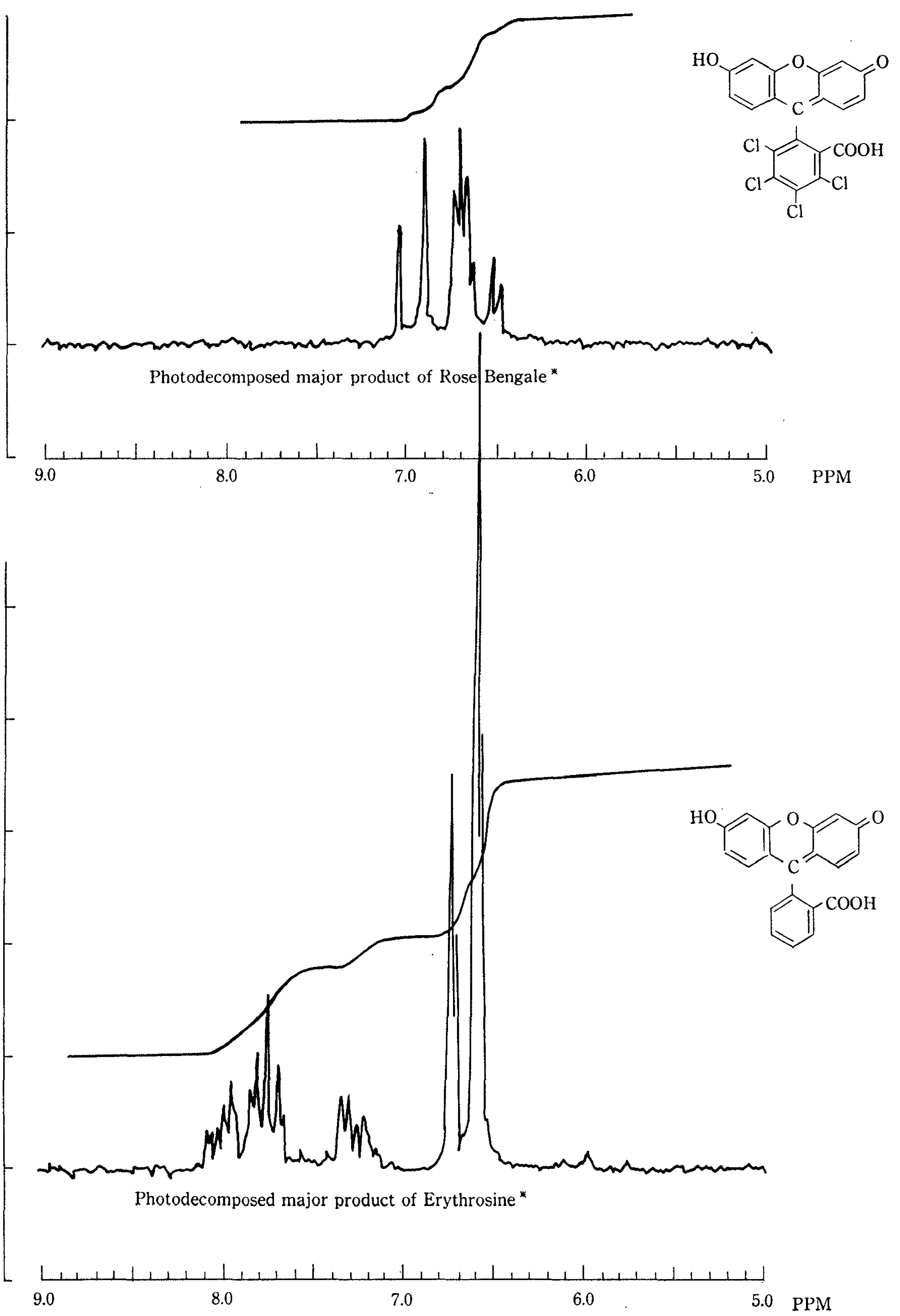


**Fig. 4.** Nuclear magnetic resonance spectra of photodecomposed major products of Erythrosine and Rose Bengale

So far as the decomposition patterns of dyes in the two solutions were concerned, no difference was observed between the photo-decomposed products in them; accordingly, pursuit of photodecomposition products of dyes will be carried out in methanol solution hereafter.

2. Qualitative analysis of photo-decomposed products of dyes

Aliquots of Erythrosine, Eosine, Phloxine and Rose Bengale solutions that had been irradiated for 10 hrs were spotted on silicagel thin-layer, and the developed spots were compared with fluorescein-related compounds.

As is known from Fig. 2, it became clear that 4 spots of photo-decomposed products were detected besides the original and that the major decomposition products of Erythrosine and Eosine showed the same Rf value as that of fluorescein (Rf 0.13), suggesting that 4 iodine or bromine atoms had been released from the original dyes; hence the intermediate spots were supposed to be partially dehalogened fluoresceins. Contrary to this, the major photo-decomposed products of Phloxine and Rose Bengale were supposed to be tetrachlorofluorescein having Rf at 0.19.

3. Identification of major photo-decomposed products

Ito et al. (1971) isolated photo-decomposed products of Phloxine and Rose Bengale in water solutions through cellulose column, and discussed their inhibitive effects on trypsin activity, but they did not succeed in elucidating the structure.

Crystals obtained were subjected to the measurements of absorbance spectra of visible, infrared and nuclear magnetic resonance (NMR), the results being indicated in Fig. 3, 4.

Referring to the UV and visible absorption spectra, Rose Bengale indicated a maximum absorbance at the wavelength of 550 nm while major phoho-decmposed products did both at 465 nm and 495 nm. Erythrosine indicated a maximum absorb-

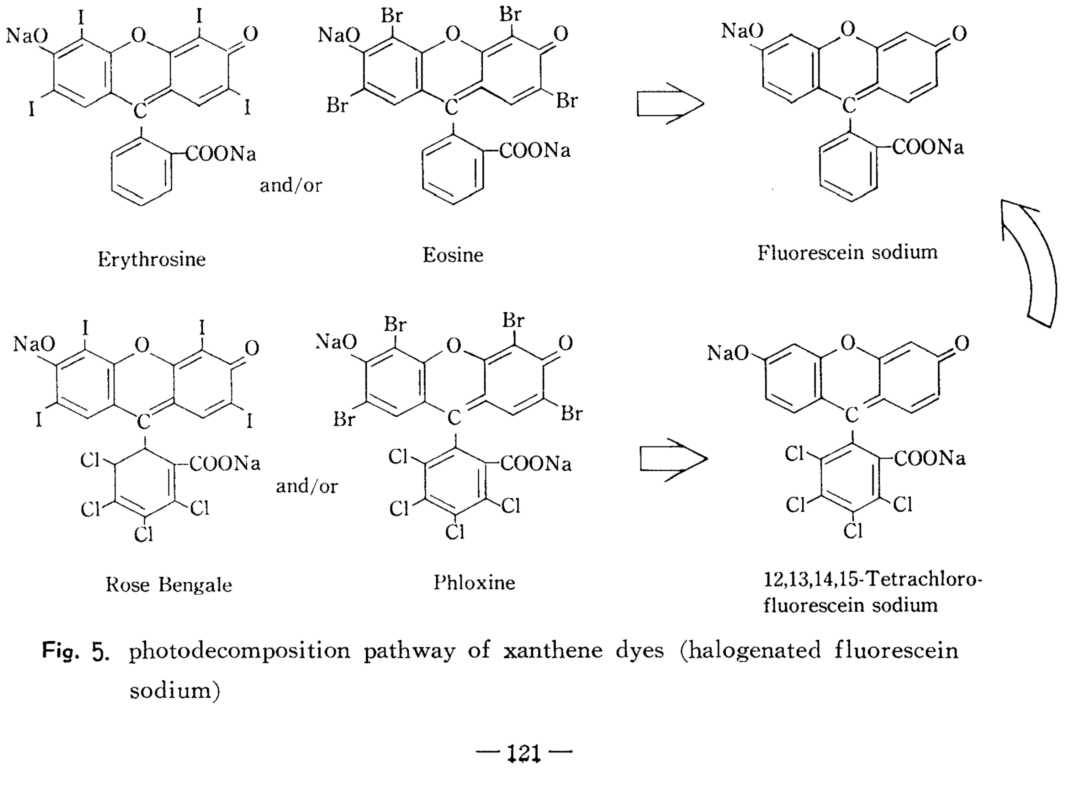


Fig. 5. photodecomposition pathway of xanthene dyes (halogenated fluorescein sodium)

ance at the wavelength of 525 nm while its major photo-decomposed products did at both 450 nm and 455 nm, in both cased the maximum absorbances of photo-decomposed products being shifted to lower wavelength.

From the Rf value, color tone, fluorescence on TLC and maximum absorbance in UV, the former was assumed to be 12, 13, 14, 15-tetrachloro-fluorescein while the latter to be fluorescein. The results of the NMR spectra were effective for the confirmation of the photo-decomposed products as well as those of IR spectra. Both were known to be present in free form.

### 4. Photo-decomposition pathways of xanthene dyes

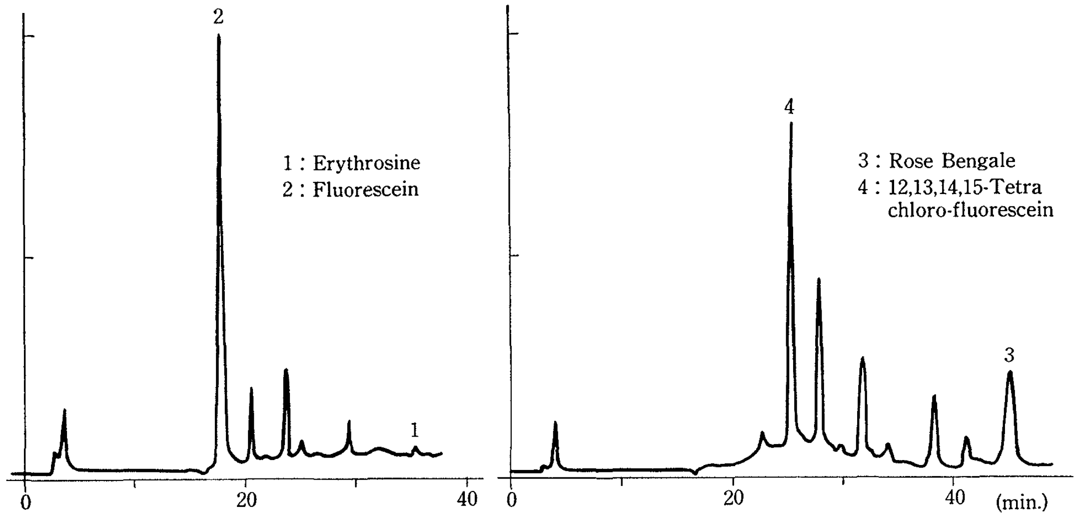
The photo-decomposition pathways of Erythrosine and Rose Bengale estimated from the above-mentioned results are demonstrated in Fig. 5.

Since Ishibashi (1965) reported that, under intensive photo-irradiation, xanthene skeleton of fluorescein was decomposed to resorcinol, phthalic acid, etc., the existence of volatie micro-components yielded by the photodecomposition was attempted by authors using gas chromatography.

However, none of the peaks corresponding to these photolytic products were detected, indicating that no fission of xanthene skeleton had taken place.

### 5. Quantitative analysis of photo-decomposed products from Erythrosine and Rose Bengale

Each two mM of Erythrosine and Rose Bengale in methanol was photoirradiated for 5 hours, then the photo-decomposed products were pursued by use of HPLC. The results are shown in Fig. 6



(photodecomposed sol. of Erythrosine) (photodecomposed sol. of Rose Bengale)

**Fig. 6.** High-performance liquid chromatography of photodecomposed products of Erythrosine and Rose Bengale
Column : "Zipax" SAX (0. 5m x 2)
Solvent : pri-2. 00 $MH_3BO_3+0.02M\ NaClO_4$ in$H_2O$ (pH8. 5)
sec-$CH_3CN$ 600ml+12. 2 g$NaCl_4$ in $H_2O$ 400ml
Detection : Absorbance at wavelength of 254nm

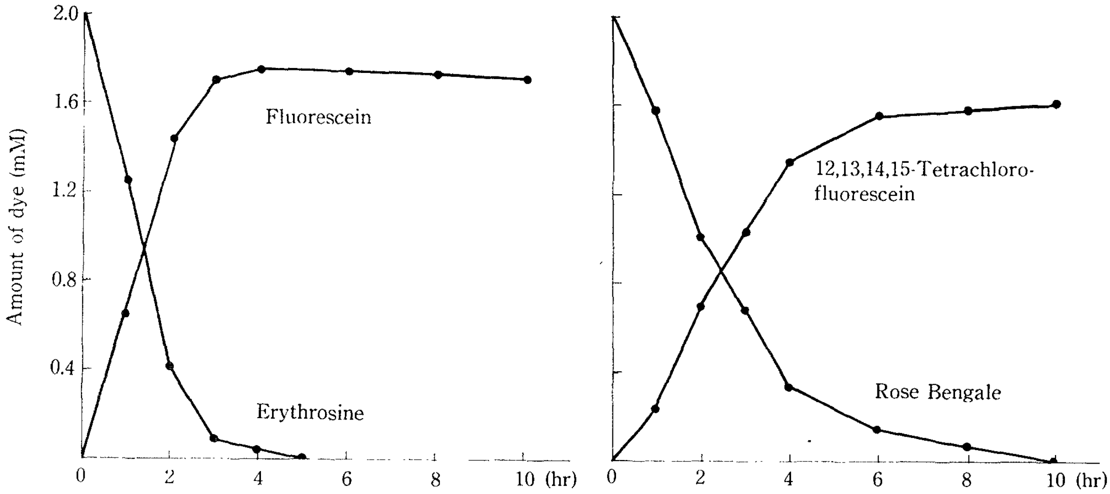


Fig. 7. photodecomposition of Erythrosine, Rose Bengale and formation of fluorescein, 12, 13, 14, 15-tetrachloro-fluorescein determined by high-performance liquid chromatography

Column : "Zipax" SAX (0. 5m x 2)

Solvent : pri-2. 00M$H_3BO_3$+0. 02M$NaClO_4$ in $H_2O$ (pH8. 5)

sec-$CH_3CN$ 600ml+12. 2g$NaClO_4$ in $H_2O$ 400ml

Detection : Absorbance at wavelength of 254nm

As for Erythrosine, the peak of main photo-decomposed product appeared at $t_R$ 18, while as for Rose Bengale, main photo-decomposed product appeared at $t_R$ 26. Relation between decrease of original dyes and increase of the main photo-decomposed products are shown in Fig. 7

Assuming that each 1 mole of main decomposition product yielded from 1 mole of dye, the conversion ratio was 90% for Erythrosine after 5 hrs and 80% for Rose Bengale after 10 hrs.

From quantitative analyses of halogen yielded by photoirradiation with Test Method 2-6), it was observed that the yields of iodine from Erythrosine and Rose Bengale was in proportion to those of fluorescein and 12, 13, 14, 15-tetrachlorofluorescein, respectively.

6. TLm test of dyes and photo-decomposition products

First, when the TLm values of the xanthene dyes before photo-irradiation were compared, no sudden death of fish occurred even for 48 hrs at 3, 000 mg/l with Acid Red, contrary to this, the TLm values of the other 4 dyes and their photolytic products increased in the order of Rose Bengale, Phloxine, Erythrosine, Eosine, 12, 13 14, 15-tetrachlorofluorescein and fluorescein sodium.

The larger the number of halogen in one molecule, the stronger was the toxicity of the dye. When the TLm values of decomposition products after 10 hrs photoirradiation were compared, a marked decrease in TLm value was observed especially

**Table.** 2. Median tolerance limit test of halide compounds

| Halide compound | TLm (mg/l) | Halide compound | TLm (mg/l) |
|---|---|---|---|
| Chlorine | <20 | Sodium chlorate | 600 |
| Bromine | <20 | Sodium bromate | 800 |
| Iodine | <20 | Sodium iodate | 700 |
| Potassium chloride | 500 | Sodium chloride | 1, 500 |
| Potassium bromide | 700 | Sodium bromide | 1, 300 |
| Potassium iodide | 800 | Sodium iodide | 1, 200 |

Ten fish of HIMEDAKA were used per trial and TLm values were derived from 50% survival of fish after 48 hrs. at 25°C.
>20 means that the TLm was below the calculation limit.

on the 4 dyes. The degree of toxic intensity among the dyes before irradiation, however, did not change by photoirradiation indicating that the principal source of fish poison is attributed to halogens liberated.

TLm values of halogens and halogen compounds are shown for refernce in Table 2.

TLm values of potassium and sodium salts of halogens were within the range of 500-800 and 1, 200-1, 500 mg/l, respectively, while those of their oxides showed 600-800 mg/l.

The toxicity of free halogens were very strong, being less than 20 mg/l in TLm value. National Institute of Occupational Safty and Health (1973) reports that the toxic concentration of chlorine gas in air to human being is 5 ppm, the LD50 of bromine to rats upon intraperitoneal injection is 85. 2 mg/l and the LD50 of iodine to mammals upon oral administration is 30 mg/kg.

From those reports and our experimental results, it was found that the effect of halogen on the vital bodies was so strong, that such a rapid increase in fish poison was caused by photoirradiation of xanthene dyes in water due to halogen yielded by photolysis of dyes.

## CONCLUSION

1. Four xanthene dyes, Erythrosine, Eosine, Phloxine and Rose Bengale, were subjected to photoirradiation by low pressure mercury lamp and the toxicity of the decomposition products were pursued by use of "Himedaka".
   The degradation was more rapid in methanol than in water.
2. From the results of UV, IR and NMR analysis the main degradation products of Erythrosine and Rose Bengale were identified to be fluorescein and 12, 13, 14 15-tetrachlorofluorescein, respectively.
3. Although 4 dyes indicated marked reduction of TLm values after photoirradiat-

ion, the toxicities of the dehalogenated major photolytic products were known to be smaller than those of the original dyes.

The increases in toxicity to fish by photoirradiation were attributed to the liberation of free halogens from the xanthene dyes.

## REFERENCES

Imamura, M. and Koizumi M. (1956) : Irreversible photobleaching of the solution of fluorescent dyes. Ⅲ. Photoreaction of Eosine in the aqueous alcoholic solutions under evacuated state. Bull. Chem. Soc. Japan, **29**, 913-918.

Imamura M. (1958) : Irreversible photobleaching of the solution of fluorescent dyes. V. Photobleaching of Erythrosin. ibid, **31**, 62-66.

Imamura, M. Kimura, K. and Miwa T. (1970) : The radiolysis and photolysis of methanolic solutions of Eosine. I. The radiolysis of neutral and alkaline solutions. ibid., **43**, 1329-1336.

Ishibashi T. (1965) : Hygienic studies on the fading and the decomposed products of coal-tar dyes used for food. Japanese Journal of Public Health. **12**, 613-627.

Ito, T., Ikezawa H. and Tejima S. (1971) : Food additives and digestive enzyme. Ⅲ. Food dyes and tryptic activity, (2), J. Hyg. Chem. **17**, 245-250.

Kitano, M. Takagi H. and Anan T. (1977) : Biodegradation and bioaccumulation of chemical substances, and methods of their estimation. BUNSEKI, **7**, 420-426.

Koizumi M. (1969) : Studies on the photobleaching first step of xanthene and thiazic dyes. J. Jap. Chem. Soc, **90**, 117-133.

Shimura, H. Chikamori R. and Terashima T. (1969) : Determination of chloride and sulfate in food coal-tar dyes. J. Hyg. Chem. **15**, 346-350.

Tonogai, Y. Iwaida, M. Tati, M. Ose Y. and Sato T. (1978) : Biochemical decomposition of coal-tar dyes. Acute toxicity of coal-tar dyes and their decomposed products. J. Toxicol. Sciences. **3**, 205-214.

National Institute of Occupational Safty and Health (1973) : "List of toxic substances", The international Technical Information Institute.