[Chem. Pharm. Bull. 23(5)1032—1038(1975)]

UDC 547.495.2.04.08 : 543.426

A New Fluorometric Analysis of Dulcin using Sodium Nitrite. IV.[1] Studies on the Reaction Mechanism

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(Received September 18, 1974)

The reaction mechanism of the fluorometric determination of dulcin with sodium nitrite was studied and estimated as follows: Dulcin produces 4-ethoxybenzenediazonium (4EBD) ion *via*. *p*-phenetidine by the action of nitrous acid. The ion itself decomposes in part to the substances possessing the active methylene neighbouring to a carbony group. Finally, 4EBD ion reacts with the active methylene in the presence of an alkaline solution containing nitrous oxide to form the essential fluorescent compound, 1,3-bis(4-ethoxyphenyl)-5-tetrazolone.

Recently, the present authors reported a new fluorometric determination of dulcin using sodium nitrite.[3] In the procedure, dulcin was treated with hydrochloric acid and sodium nitrite, and then with alkali to produce fluorescence.

The structure of the fluorescent compound, which should be the essential fluorescent product in the determination, was decided to be 1,3-bis(4-ethoxyphenyl)-5-tetrazolone ((compound A) by the single crystal X-ray diffraction method.[1] This paper describes the reaction mechanism of this fluorometric determination.

Experimental

Material——EtOH-1-^{14}C and EtOH-2-^{14}C were purchased from The Radiochemical Centre Amersham. 4-Ethoxybenzenediazonium (4EBD) fluoroborate was synthesized from *p*-phenetidine-HCl by the method reported.[4]

General Analytical Procedures——The fluorescence spectra were taken in chloroform solution on a Hitachi Fluorescence Spectrophotometer MPF-2A. The gas–liquid chromatography (GLC) was carried out with a Shimadzu Gas Chromatograph Model GC-3A equipped with a hydrogen flame ionization detector (FID). Radioactivity of ^{14}C-labeled compound was measured with an Aloka Liquid Scintillation Counter Model LSC-601. Radioactivity on a thin–layer plate was measured with an Aloka Thin–Layer Chromatogram Scanner Model TRM-1B.

Formation of Compound A from the Expective Intermediates——To each 1 ml solution of dulcin, *p*-phenetidine–HCl or 4EBD-fluoroborate (each concentration: 5.54×10^{-7} mole/ml) in a test tube with glass stopper were added 0.4 ml of 1 N HCl and 0.5 ml of 10% $NaNO_2$. The mixture was then allowed to react with HNO_2 for various intervals. The solution was made alkaline by addition of 0.5 ml of 6% NaOH, was allowed to stand for 40 min, and then was extracted with 10 ml of chloroform in the same manner of the determination. The chloroform extract was excited at 354 nm and the fluorescence intensity was measured at 446 nm (Fig. 1).

Source of 5-Position Carbon of the Tetrazolone Ring——a) Detection of *p*-Ethoxyphenol and *p*-Hydroquinone: To a mixture of dulcin (7 g), $NaNO_2$ (7 g) and distilled water (200 ml) in a flask was added 40 ml of 6 N HCl. The flask was sealed loosely with a stopper and allowed to stand for 3 hours. The reaction was stopped by addition of 20 g of urea. The reaction mixture was extracted with 200 ml portions of ether three times. The residual acid solution was made alkaline with conc. NaOH and then the alkaline solution was extracted with 200 ml portions of ether three times. The extracts were washed with small amounts of water and dried over anhyd. Na_2SO_4 and concentrated under reduced pressure, and then the two residues

1) Part III: Y. Iitaka, S. Uchiyama, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **20**, 1181 (1972).
2) Location: *a) 1–18–1, Kamiyoga, Setagaya-ku, Tokyo; b) Hongo, Bunkyo-ku, Tokyo.*
3) S. Uchiyama, T. Kondo, and I. Kawashiro, *Yakugaku Zasshi*, **89**, 828 (1969).
4) S. Uchiyama, H. Tanabe, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **20**, 357 (1972).

were chromatographed on a silica gel plates. The developing solvent system for the acid extract was benzene–ethyl acetate (35: 15) or chloroform–EtOH (50: 5) and that for the alkaline extract was benzene–ethyl acetate (1: 1) or chloroform–EtOH (50: 5). As the result, *p*-ethoxyphenol, *p*-hydroquinone and *p*-phenetidine were detected (Fig. 2.)

b) Detection of EtOH and Acetaldehyde: To a mixture of 100 ml of 20% $NaNO_2$ and *p*-phenetidine–HCl (5 g) in a flask was added 50 ml of conc. HCl. The fiask was sealed with a stopper loosely and was allowed to stand for an hour. After being neutralized with 20% NaOH, the reaction mixture was distilled twice to obtaine 1 ml of the final distillate. GLC of the distillate was performed with two kinds of column packings (Porapack Q and Chromosorb 101). As the result, EtOH and acetaldehyde were detected (Fig. 3).

F-Reaction for *p*-Ethoxyphenol, *p*-Hydroquinone, EtOH and Acetaldehyde——To 1 ml of each test solution (concentration: 10^{-8}—10^{-5} mole/ml) in a test tube with a stopper were added 0.4 ml of 1 N HCl and 0.5 ml of 10% $NaNO_2$. After the mixture was allowed to stand for 30 min at room temperature, 0.5 ml of 6% NaOH and 1 ml of 4EBD-fluoroborate solution (concentration: 4.24×10^{-7} mole/ml) were added and mixed well. After further standing for 40 min at room temperature, the mixture was extracted with 10 ml of chloroform.

The relationship between the fluorescence intensity and the concentration of each substance was then examined by the measurement of the fluorescence intensity in 10 ml of the chloroform extract from the reaction mixture (Fig. 4 and Fig. 5). In addition, the thin-layer chromatography (TLC) and the fluorescence spectra of the chloroform extracts showed that those fluorescent compounds obtained from the four substances were identical with compound A.

Fluorescence-Producing Compounds on F-Reaction——After 1 ml of each test solution (concentration: 10^{-8}—10^{-3} mole/ml) was treated by the same procedure as the above F-reaction, the reaction mixture was extracted with 10 ml of chloroform. The extracts were excited at 354 nm and the fluorescence intensities were measured at 446 nm. From the data of the measurements, the relative fluorescence intensity and the mole number which gave the highest fluorescence intensity for 4.24×10^{-7} mole of 4EBD-fluoroborate were examined. The relative fluorescence intensities were measured representing the difference between the highest fluorescence intensity of acetaldehyde and the blank value (blank: 6) to be 100 (Table I). In the substances which showed fluorescence, the fluorescence spectra and TLC of the chloroform extracts showed that those fluorescent products were identical with compound A.

Incorporation of EtOH-^{14}C into Compound A——To a mixture of 5 ml of *p*-phenetidine–HCl solution (concentration: 20 mg/ml) and 2 ml of 1 N HCl in a test tube with a stopper were added 2.5 ml of 10% $NaNO_2$ and 1 ml of 10% EtOH containing ^{14}C-labeled EtOH. The reaction mixture was allowed to stand for 30 min at room temperature. After being made alkaline by addition of 2.5 ml of 6% NaOH and allowed to stand for 40 min, the mixture was extracted with 2 ml of chloroform and then the extract was concentrated under reduced pressure, and the whole concentrated solution was chromatographed on a thin-layer plate. After the development, the radioactivity on a palte was measured by the radiochromatogram-scanner (Fig. 6). Radioactivities of ^{14}C-labeled EtOH used for the reaction were 4.53×10^6 dpm to EtOH-1-^{14}C and 2.86×10^6 dpm to EtOH-2-^{14}C.

In the reaction using EtOH-2-^{14}C, the silica gel adsorbent, which showed a fluorescence spot of compound A on a plate, was separated and extracted with EtOH. The combined extract from several plates (radioactivity: about 15000 dpm) was mixed with 97 mg of non-labeled compound A. The mixture was passed through a column of celite-charcoal (1: 1) and then was recrystallized from a mixture of chloroform–EtOH (2: 5). At the first place, 3.347 mg of ^{14}C-labeled compound A, which had been obtained by repeating recrystallization a few times, was oxidized by the Kametani's method[5] and then its specific radioactivity was measured with the liquid scintillation counter. Furthermore, the residual crystals of compound A was recrystallized and was then used as the second sample (Table II).

Result

Under the similar reaction conditions previously reported,[3] equimolar dulcin, *p*-phenetidine and 4EBD ion produced the same quantities of compound A, while the reaction rate was quite different from each other as shown in Fig. 1. The results indicated that the fluorescent reaction proceeded in the following pathway (Chart 1).

As the probable sources of 5-position carbon of the tetrazolone ring in compound A, *p*-ethoxyphenol and *p*-hydroquinone were produced by the reaction of nitrous acid from dulcin (Fig. 2), and also ethanol and acetaldehyde from *p*-phenetidine (Fig. 3).

5) K. Kametani, Y. Inoue, and K. Maruyama, *Radioisotopes.*, **16**, 41 (1967).

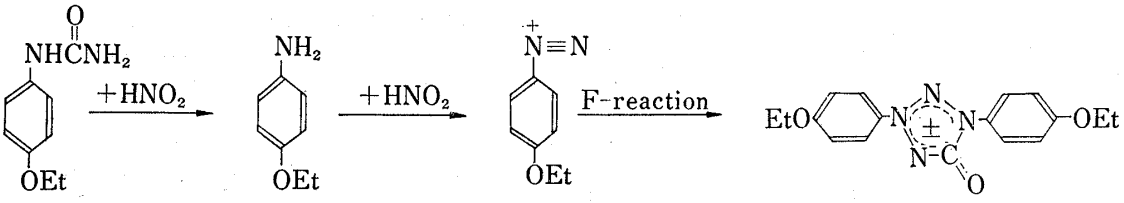


Chart 1

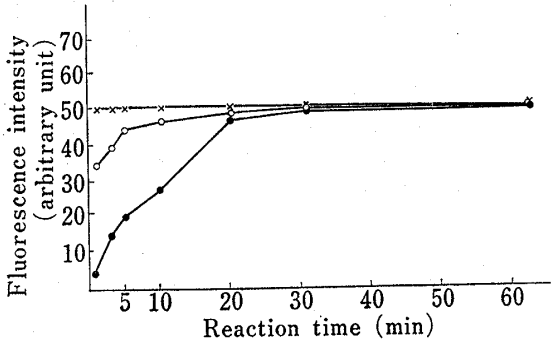


Fig. 1. Relationship between Fluorescence Intensity and Reaction Time with Nitrous Acid in the Fluorescent Reaction

—×— : 4EBD-fluoroborate, —○— : *p*-phenetidine-HCl, —●— : dulcin
The fluorescence intensity is measured at 354 nm (excitation) and 446 nm (emission).
Amount of each compound is 5.54×10^{-7} mole.

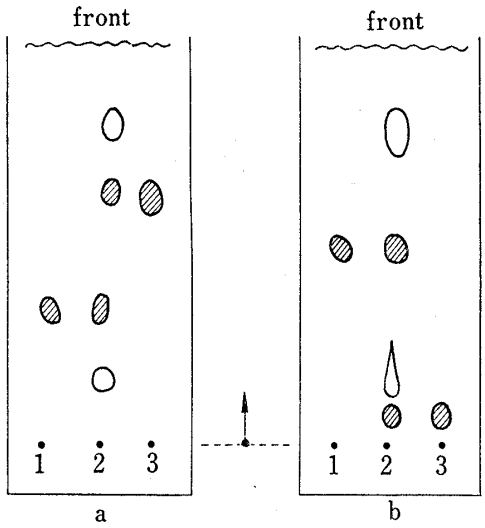


Fig. 2. Thin-Layer Chromatograms of Ether Extracts from the Reaction Mixture of Dulcin with Nitrous Acid

a): ether extract from the acid mixture
1) *p*-hydroquinone, 2) ether extract, 3) *p*-ethoxyphenol plate: Silica gel G (250 μ, activated at 110° for 1 hr), developing solvent : benzene-ethyl acetate (35: 15), color-producing reagent: 0.5% 2, 6-dichloroquinone chloroimide ethanolic solution
b): ether extract from the alkaline mixture
1) *p*-phenetidine, 2) ether extract, 3) dulcin, plate: Silica gel G (250 μ, activated at 110° for 1 hr), developing solvent: benzene-ethyl acetate (1: 1), color-producing reagent: 1% *p*-dimethylaminobenzaldehyde HCl-ethanolic solution

When reacted with nitrous acid, made alkaline and added with 4EBD-fluoroborate, these compounds produced compound A in a good yield. The procedure was named F-reaction, since it was thought to be the critical part of the whole fluorescent reaction.

p-Hydroquinone gave higher fluorescence intensity at lower concentrations than *p*-ethoxyphenol (Fig. 4). It could be, therefore, presumed that 4EBD ion decomposed in part to *p*-hydroquinone *via* *p*-ethoxyphenol to become the source of the 5-position carbon (Chart 2). The figure also shows that the excess of these compounds inhibits the formation of compound A.

On the other hand, acetaldehyde gave higher fluorescence intensity at lower concentrations than ethanol (Fig. 5). This fact suggested that acetaldehyde was formed *via* ethanol from the ethoxyl group of 4EBD ion or *p*-ethoxyphenol in part for the source of the 5-position carbon (Chart 3). Thus, it was found that 4EBD ion and the above four compounds were associated with the formation of compound A.

In order to deal with the F-reaction in details, other substances were investigated as possible sources of the 5-position carbon (Table I).

The result indicates that not only the substances such as acetaldehyde and oxalacetic acid, which possess an active methylene neighbouring to a carbonyl group, but also those substances such as ethanol, which form active methylenes by oxidation, will be excellent sources of the 5-position carbon.

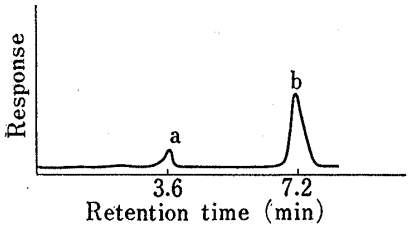


Fig. 3. Gas–Liquid Chromatogram of the Distillate obtained from the Reaction Mixture of *p*-Phenetidine with Nitrous Acid

column: Porapack Q, 3 mm×1 m; column temperature: 100°; detector: FID; peak a: acetaldehyde, peak b: ethanol

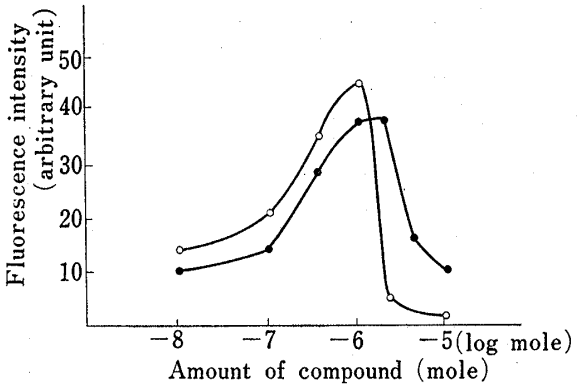


Fig. 4. Relationship between Fluorescence Intensity and Amounts of *p*-Hydroquinone and *p*-Ethoxyphenol in F-Reaction

—○— : *p*-hydroquinone, —●— : *p*-ethoxyphenol
The fluorescence intensity is measured at 354 nm (excitation) and 446 nm (emission).

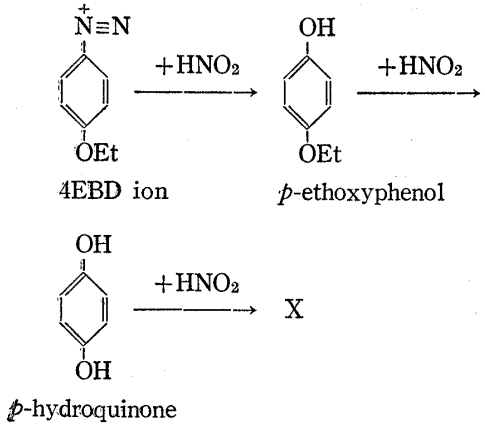


Chart 2

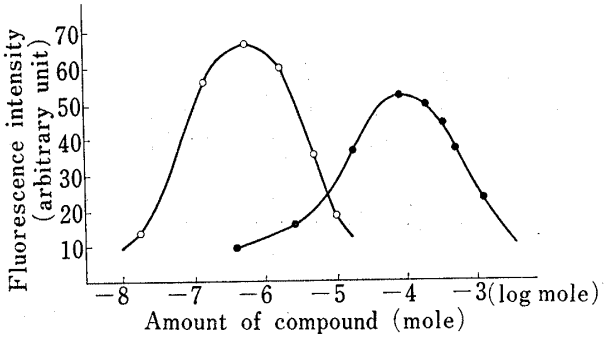


Fig. 5. Relationship between Fluorescence Intensity and Amounts of Acetaldehyde and Ethanol in F-Reaction

—○— : acetaldehyde, —●— : ethanol
The fluorescence intensity is measured at 354 nm (excitation) and 446 nm (emission).

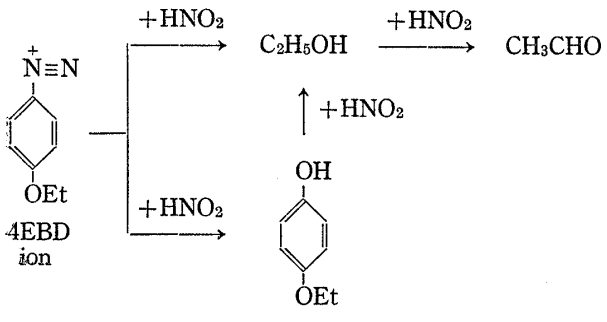


Chart 3

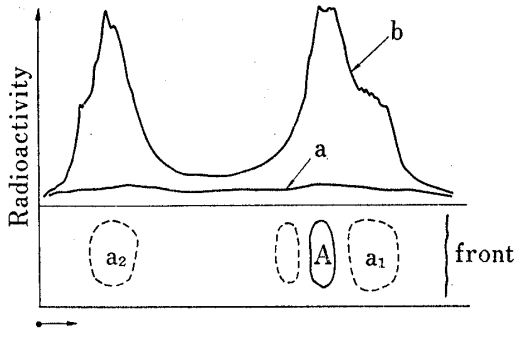


Fig. 6. Radioscannogram and Thin-Layer Chromatogram of Chloroform Extract from the Reaction Mixture of *p*-Phenetidine with ^{14}C-Labeled Ethanols in the Fluorescent Reaction

a: ethanol-1-^{14}C, b: ethanol-2-^{14}C,
a_1: compound a_1, a_2: compound a_2, A: compound A
conditions of TLC: silica gel G, 250 μ, $CHCl_3$–EtOH (50: 3)
○ : fluorescent band

Table I. Investigation of Fluorescence-Producing Compounds in F-Reaction with 4EBD-Fluoroborate

Compound	Amount[a] (mole)	Fluorescence[b] intensity	Compound	Amount[a] (mole)	Fluorescence[b] intensity
Ethanol	8.7×10^{-5}	82.0	*n*-Butylalcohol		0
Isopropyl alcohol	5.0×10^{-5}	100.0	*tert*-Butyl alcohol		0
Acetaldehyde	5.0×10^{-7}	100.0	Formaldehyde		0
Acetone	9.0×10^{-7}	100.0	Glyoxal		0
Pyruvic acid	9.5×10^{-7}	100.0	Glyoxylic acid		0
Pyruvaldehyde	5.0×10^{-6}	100.0	Formic acid		0
Oxalacetic acid	3.0×10^{-6}	100.0	Acetic acid		0
Malonic acid	1.0×10^{-5}	18.0	Succinic acid		0
p-Ethoxyphenol	2.5×10^{-6}	28.0	Fumaric acid		0
p-Hydroquinone	1.0×10^{-6}	34.0	Malic acid		0
			α-Ketobutyric acid		0
Methanol		0	Nitromethane		0
n-Propyl alcohol		0			

a) Amount of compound which give the highest fluorescence intensity for 4.24×10^{-7} mole of 4EBD-fluoroborate
b) Each fluorescence intensity, which is measured at 354 nm (excitation) and 446 nm (emission), is reduced by that of a blank and described as 100 in the case of acetaldehyde. Intensity of blank is 6.

On the other hand, when the fluorescent reaction of *p*-phenetidine was carried out by the addition of ^{14}C-labeled ethanols, the fluorescent band of compound A on a thin-layer plate showed radioactivity only when ethanol-2-^{14}C was used (Fig. 6).

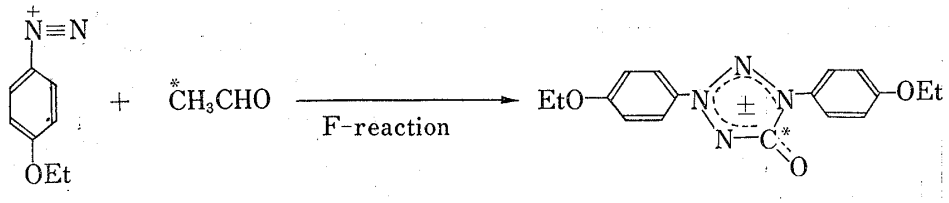
In addition, the specific radioactivity for a mixture of the extract from the radioactive fluorescent band and non-labeled compound A was constant on every recrystallization (Table II).

Table II. Results of the Isotope Dilution Analysis of Compound A

No.[a]	Weight (mg)	dpm	dpm/mg	Recovery (%)
1	3.347	465.1	138.96	100
2	4.495	628.2	139.75	100.59
3	4.801	675.4	140.67	101.23

a) recrystallization number

These facts showed that the 2-position carbon of ethanol and consequently the active methylene carbon was incorporated into compound A (Chart 4).



4EBD ion compound A

Chart 4

Discussion

In the quantitative procedure, the authors heated the test tube containing a mixture of dulcin and hydrochloric acid on account for avoiding the errors in the reaction. However,

the heating was not necessary when these reactants were mixed enough by shaking the test tube.

It was concluded that the fluorescent reaction was ultimately performed on the action of 4EBD ion and the active methylene neighbouring to a carbonyl group (represented as F-reaction). However, *p*-ethoxyphenol and *p*-hydroquinone, which were fluorescence-producing compounds in F-reaction, gave lower fluorescence intensities than the substances possessing the active methylene like acetaldehyde as shown in table I. It is therefore suggested that *p*-hydroquinone does not react directly with 4EBD ion, but it becomes once to be compound X possessing the active methylene by the action of nitrous acid to give compound A.

From the experiment for the incorporation of ethanol-^{14}C into compound A, it was clear that the active methylene carbon was incorporated into the 5-position carbon of the tetrazolne ring in compound A. However, the problem how the substances containing the active methylene groups are incorporated into compound A by F-reaction still remains.

On the thin-layer plate shown in Fig. 6, there were a dark brown band(compound a_1) and a yellowish brown band (compound a_2), both of them showed radioactivity in the case

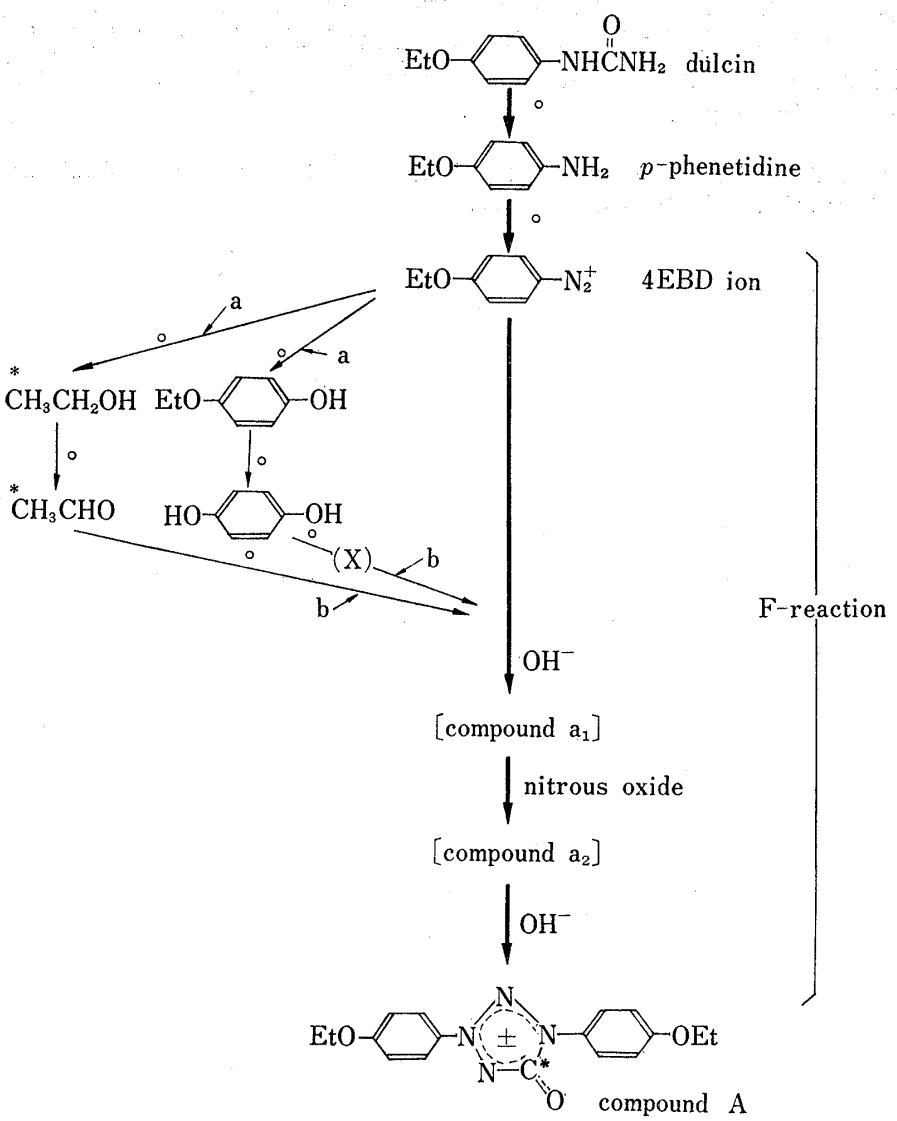


Fig. 7. Schema of the Fluorescent Reaction Mechanism

a: partial decomposition of 4EBD ion
b: incorporation to 5-position carbon of tetrazolone ring
x: the compound possessing the active methylene neighbouring to carbonyl group
o: the action of HCl and $NaNO_2$

of ethanol-2-^{14}C. Compound a_1, which was extracted from the adsorbent of silica gel with chloroform, produced compound a_2 easily by exposure to nitrous oxide. Compound a_2, which was extracted from the adsorbent of silica gel with methanol, formed easily compound A by the action of an alkaline solution of sodium hydroxide. At this point, the structural determination of these compounds might be the key to dissolve this F-reaction mechanism. These compounds decomposed, however, so quickly in the air that the structures of these compounds could not be elucidated.

Another important substance to form compound A may be nitrous oxide which presents in the alkaline solution (in the test tube), because compound A is not formed in the alkaline solution containing only nitrite ion (NO_2^-).

It is therefore assumed that the first step of F-reaction is the action of 4EBD ion and the active methylene in the presence of an alkali to form compound a_1. In this step, the 1-position and the 2-position carbons of acetaldehyde may be cleft like Japp-Klingemann reaction,[6] since compound a_1 is formed without nitrous acid or nitrous oxide in the system. The second step may be oxidation by nitrous acid or nitrous oxide to form compound a_2 from compound a_1, and then the third is the action with an alkali to form compound A from compound a_2. The result obtained in this experiment is summarized in Fig. 7.

The next paper will deal with the improved method using acetaldehyde as the source of the 5-position carbon for enhancing fluorescence intensity and also with the application to the other aromatic amines.

Acknowledgement The authors wish to thank members of Department of Radiochemistry, National Institute of Hygienic sciences for their kind advice.

6) R.R. Phillips, "Organic Reaction," Vol. 10, ed. by A.H. Blatt, John Wiley, and Sons, Inc., New York, 1959, p. 143.