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Fluorescence Reaction of Bisulfite with Indophenol Blue and Its Application to Determination of Bisulfite

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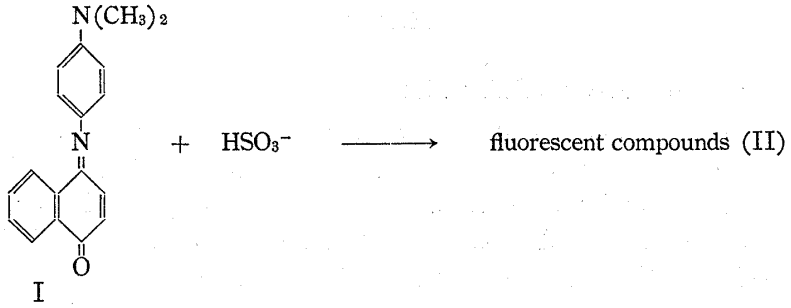
Bisulfite reacted with N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine(indophenol blue, I) to yield blue fluorescence in alkaline media. The reaction was dependent upon pH and temperature, being optimal at pH 3.5—4.0 and around 37°, and completed within 30 min. Based on these observations, a fluorometric method has been developed for the determination of bisulfite. A linear relationship was obtained in the range of 1.5×10^{-8}—1.5×10^{-7} mole of bisulfite. By column chromatography, thin-layer chromatography and paper electrophoresis, it was proved that at least two fluorescent compounds were formed in the reaction mixture and they were supposed to be bisulfite adducts of I containing one and two $-SO_3H$ groups.

Sulfur dioxide is one of main air pollutants and sulfite is biochemically important as an intermediate of biosynthesis and degradation of organic sulfur compounds. Consequently, the demand of accurate method for determination of sulfur dioxide or bisulfite is quite large. For that purpose, the methods using triphenylmethane dyes[2] have widely been used, though thiols, sulfide and thiosulfate ions also exhibit positive responses to them. There have been many other methods such as colorimetric methods using mercuric chloranilate,[3] 5,5′-dithiobis-(2-nitrobenzoic acid),[4] 2,4,6-tri(2-pyridyl)1,3,5-triazine[5] and 1,10-phenanthroline,[6] ultraviolet absorption methods,[4,7] and a quenching fluorescence method,[8] all of which are of less specificity.

In a previous communication,[9] we found that N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine(indophenol blue, I) reacts almost specifically with bisulfite among natural compounds to give fluorescent compounds (II) as shown below.



I

1) Location: *Hongo-7-3-1, Bunkyo-ku, Tokyo, 113, Japan.*
2) P.W. West and G.C. Gaeke, Jr., *Anal. Chem.*, **28**, 1816 (1956); P.W. West and F. Ordovea, *ibid.*, **34**, 1324 (1962); A.J. Steigmann, *J. Soc. Chem. Ind.*, **61**, 18 (1950); S. Atkin, *Anal. Chem.*, **22**, 947 (1950); P.F. Urone and W.E. Boggs, *ibid.*, **23**, 1517 (1951); R.V. Nauman, P.W. West, F. Tron, and G.C. Gaeke, *ibid.*, **32**, 1307 (1960); F.P. Scaringelli, B.E. Saltzman, and S.A. Frey, *ibid.*, **39**, 1709 (1967).
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9) H. Nakamura and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **22**, 1950 (1974).

The present paper deals with the method of determination of bisulfite utilizing the fluorescence reaction. The characteristics of II are also discussed.

Experimental

Reagents and Materials——Sodium sulfite-^{35}S($Na_2{}^{35}SO_3$, specific activity, 0.77 mCi/mg) was purchased from New England Nuclear. Sodium bisulfite ($NaHSO_3$, GR) and ethylenediaminetetraacetic acid disodium salt(EDTA, GR) were purchased from Kanto Chemical Co., Tokyo. N-(*p*-dimethylaminophenyl)-1,4-naphthoquinoneimine(indophenol blue, I), *p*-chloromercuribenzoic acid(PCMB, GR) were obtained from Tokyo Kasei Kogyo Co., Tokyo. Other reagents and solvents used were all reagent grade and obtained from commercial sources. Thin-layer chromatography was performed with commercially available silica gel chromatoplates (SPOTFILM S195, 5×20 cm, Tokyo Kasei Kogyo). Paper electrophoresis was performed with Toyo Filter Paper No. 514 (Toyo Roshi Kaisha, Ltd., Tokyo).

Apparatuses——Hitachi MPF-2A fluorescence spectrophotometer was used throughout this work. The pH meter used was Toa HM-5A(Toa Denpa Kogyo Co., Tokyo). The water bath incubator used was Yamato's BT-21 (Tokyo). For paper electrophoresis, SJ-1050 (A) was used (Mitsumi Scientific Co., Tokyo). Radioactivity measurements were carried out with a Beckman LS-230 liquid scintillation system.

Analysis of Radioactive Fluorescent Compounds——(a) Column Chromatography: The sample (0.1 ml) was charged onto a column of QAE-Sephadex A-25 (OH form, 0.8×12 cm) and a linear gradient elution was performed with 300 ml of 0—1 M NH_4HCO_3. The rate of flow was kept constant (150 ml/hr) by the aid of a peristallic pump (type SJ-1210, Mitsumi Scientific Ind.). An aliquot (50μl) of the collected fraction (5 ml) was measured for radioactivity in 10 ml of Bray's solution.[10]

(b) Paper Electrophoresis and Thin-Layer Chromatogrphy: The radioactive fractions obtained by the column chromatography were pooled and evaporated to dryness *in vacuo* below at 25°. Large amount of NH_4HCO_3 was removed by repeating evaporation with water. The residue was dissolved in a minimum volume of water and spotted to a chromatoplate or paper. Ascending thin-layer chromatography was performed with a solvent system of *n*-butanol–acetic acid–water (5:2:3, v/v). Paper electrophoresis was performed for 10 min at 36 v/cm in 1 M HCOOH(pH 1.75). The chromatogram or electrophoretogram was air-dried, cut into pieces of 10 mm or 5 mm width and measured for radioactivity in 10 ml of Bray's solution.[10] Detection of non-radioactive compounds was performed as follows. Lactose, used as a neutral marker, was sprayed with *n*-butanol solution saturated with ninhydrin, and successively with 2 N NaOH solution and heated for 5 min at 100°. Lactose was detected as a brown spot on a yellowish background. For the detection of the reaction products of I and bisulfite, the samples were sprayed with 2 N NaOH solution after observation of colored spots. Fluorescent compounds were visualized by the aid of a Pan ultraviolet (UV) lamp (PUV-1A type, Tokyo Kogaku Kikai). 1-Naphthol-3-sulfonate and 1-naphthol-3,6-disulfonate were detected by their blue and yellowish green fluorescence respectively under the UV lamp.

Result

Basal Data for Fluorometric Assay of Bisulfite

As shown in Fig. 1, formation of II from I and bisulfite was maximal at pH 3.5—4.0 (citrate) and substantially no fluorescence was observed when the reaction was performed under alkaline or strong acidic condition. The reaction depended on temperature, being optimal at around 37° (Fig. 2), and it was completed within 30 min at 37° (Fig. 3). Figure 4 shows that there exists optimal concentration of I according to the amounts of bisulfite.

As shown in Fig. 5, the fluorescence intensity of II was dependent on pH and it was maximal at above pH 11. The excitation maximum of II is at 340 nm and fluorescence at 435 nm at pH 13.5 (uncorrected).[9] The effect of organic solvents on the fluorescence intensity of II was summarized in Table I. *n*-Butanol markedly increased the intensity, but acetone abolished the fluorescence completely. The other solvents scarcely affected the fluorescence intensity.

As shown in Fig. 6, the fluorescence intensity was unstable in a dilute alkaline solution (0.1 N NaOH) probably because of neutralizing effect of CO_2 in the air, while in 0.5—2N NaOH the fluorescence was stable for the first 2 hr and the decrease in fluorescence intensity was only 8% even after 6 hr.

10) G.A. Bray, *Anal. Biochem.*, 1, 279 (1960).

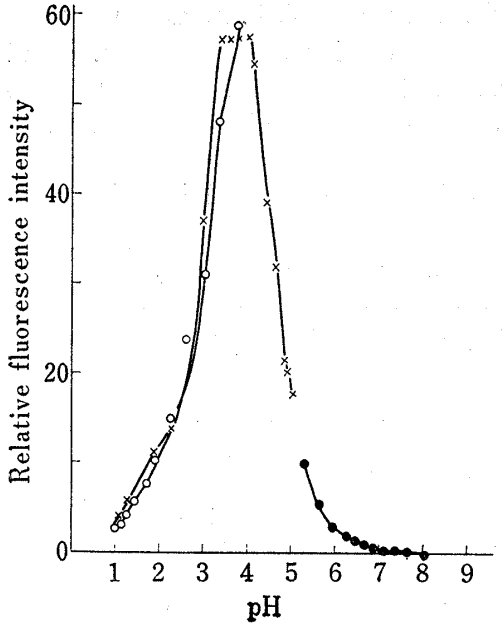

Fig. 1. The Dependence of Fluorescence Reaction upon pH

One ml of 200 μM $NaHSO_3$ solution was treated with the procedure described in the text by using the following buffers.
—○— : 1/10 M HCl–glycine–NaCl
—×— : 1/10 M HCl–citrate
—●— : 1/15 M phosphate

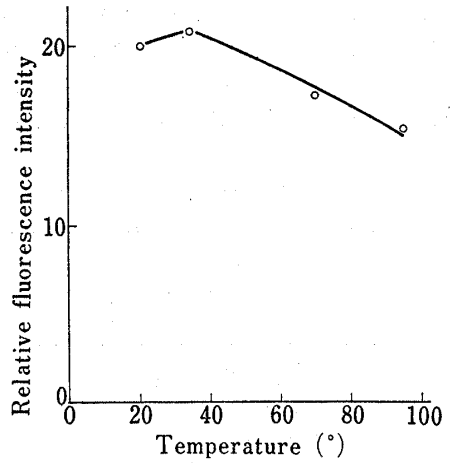

Fig. 2. Fluorescence Intensity in the Function of Reaction Temperature

One ml of 200 μM $NaHSO_3$ solution was treated at various temperatures with the procedure in the text. The values of blanks, similarly treated at the specified temperatures, were subtracted.

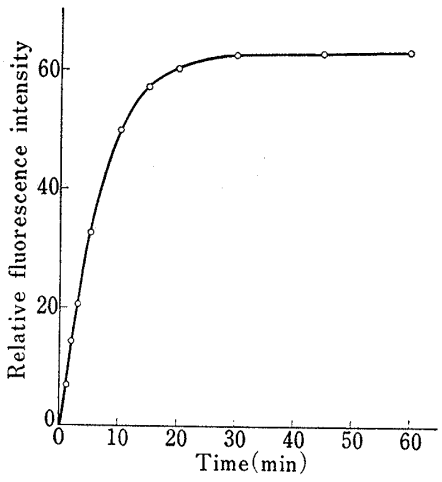

Fig. 3. Time Course of the Fluorescence Reaction

One ml of 200 μM $NaHSO_3$ solution was treated with the procedure described in the text, except for the reaction time.

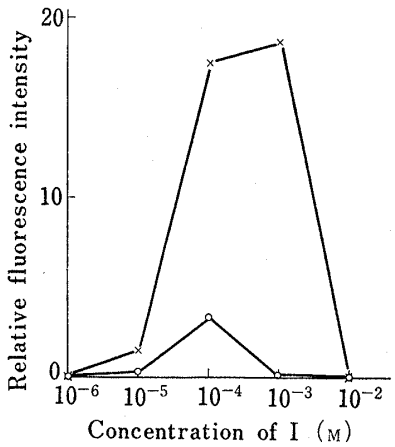

Fig. 4. Optimal Concentration of I

One ml of 50 μM (-○-) or 500 μM (-×-) of $NaHSO_3$ solution was mixed with one ml of water and one ml of appropriate concentration of I in N,N-dimethylformamide, incubated at 37° for 16 hr, and added with one ml of 2N NaOH.

Ethylenediaminetetraacetic acid (EDTA) was proved to suppress the autoxidation of bisulfite in solution.

Procedure for Determination of Bisulfite

Based on above observations, the procedure was established as follows.

The standard solution of bisulfite or the blank solution is made with an aqueous solution of 5 mM EDTA. One ml of the solution is added with 2.0 ml of the mixture of 0.1M HCl and 0.1M potassium citrate (pH 3.62, citrate buffer). One ml of the sample solution is added

with 2.0 ml of the citrate buffer supplemented with 2.5 mM EDTA. Add 1.0 ml of 0.1 mM I in ethanol to the resultant solution, mix, and allow to stand at 37° for 30 min. Add 1.0 ml of 2N NaOH, mix, excitate at 340 nm, and measure the fluorescence intensity at 435 nm. From calibration plots of fluorescence readings *vs.* the amount of bisulfite in standard solutions, the amount of bisulfite in the sample is calculated.

As shown in Table II, a good reproducibility was demonstrated in this procedure.

Table I. Effect of Organic Solvents upon Fluorescence Intensity

Solvent	Relative fluorescence intensity	
	1 ml	2 ml
H_2O (control)	100	100
MeOH	104	104
EtOH	100	102
n-BuOH	125	174
$CH_3COOC_2H_5$	102	117
Dioxane	104	106
DMF	104	105
DMSO	105	105
Acetone	0	0

The mixture of 1 ml of 200 μM $NaHSO_3$ solution, 2 ml of the citrate buffer (pH 3.62) and 1 ml of 0.1 mM I in EtOH was incubated at 37° for 30 min and added with 1 ml of 2N NaOH. To the resultant solution, 1 ml or 2 ml of an organic solvent was added and the fluorescence intensity was measured at $\lambda_{ex}340$ and $\lambda_{em}440$.

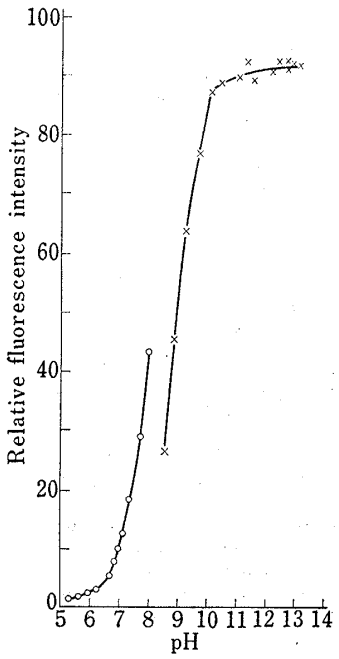

Fig. 5. The Dependence of Fluorescence Intensity of II upon pH

One ml of 200 μM $NaHSO_3$ solution was added with 2 ml of water and 1 ml of 0.1 mM I in ethanol. The mixture was incubated at 37° for 30 min and the fluorescence intensity was measured after addition of 2 ml of 1/15M phosphate buffer (-○-) or 1/10M Glycine-NaCl-NaOH buffer (-×-).

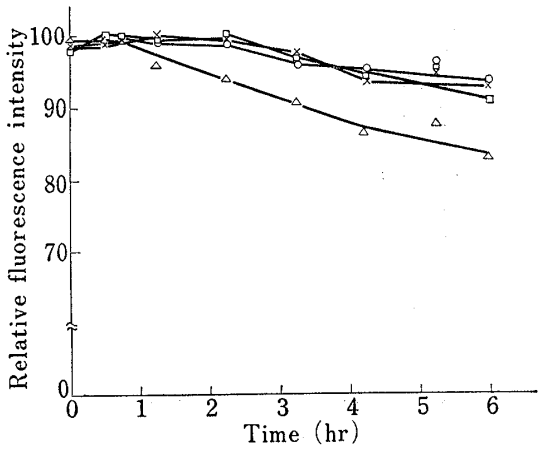

Fig. 6. Stability of the Fluorescence in Alkaline Media

The fluorescent compounds were prepared as described in Fig. 5. After addition of 2 ml of various alkaline solutions, the fluorescence intensity was followed.
—○— : 2.0 N NaOH
—×— : 1.0 N NaOH
—□— : 0.5 N NaOH
—△— : 0.1 N NaOH

Interfering Substances

The effect of 26 cations preexisted in the reaction system on the fluorescence intensity was summarized in Table III.

As shown in Table IV, pyrosulfite($S_2O_5^{2-}$) and sulfite gave approximately the same degree of fluorescence intensities as that of bisulfite. Hyposulfite($S_2O_4^{2-}$) and sulfide(S^{2-}) ions

TABLE II. Reproducibility of the Present Method

Sample No.	$NaHSO_3$ added (10^{-8} mole)	
	5.00	10.0
1	4.84	9.83
2	5.03	10.07
3	5.02	10.18
4	5.01	9.91
5	5.03	10.10
6	5.08	10.09
7	5.09	9.93
8	4.95	10.03
9	4.49	9.89
10	5.00	9.91
Mean	5.00	9.99
σ	0.07	0.10

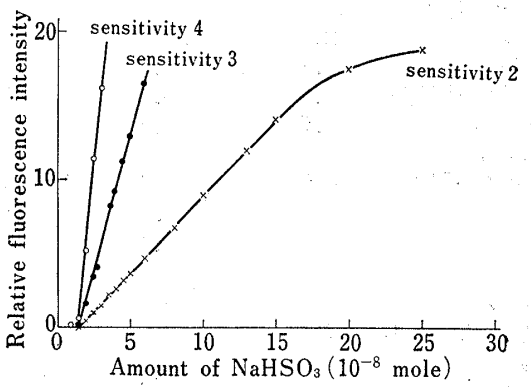

Fig. 7. Working Curves for Bisulfite

TABLE III. Effect of Foreign Cations on Fluorescence Intensity

Cation	Source	Inhibition (%)		
		5×10^{-7} mole	5×10^{-6} mole	5×10^{-5} mole
None		0	0	0
Fe^{3+}	$Fe(NO_3)_3\cdot9H_2O$	3.5	86.6	100
Cr^{3+}	$Cr(NO_3)_3\cdot9H_2O$	0	0	69
Cu^{2+}	$Cu(NO_3)_2\cdot3H_2O$	0	0	86
Mn^{2+}	$Mn(NO_3)_2\cdot5H_2O$	27	88	96
Pb^{2+}	$Pb(NO_3)_2$	0	0	70
Ag^{+}	$AgNO_3$	6.2	27	44
Sn^{4+}	$SnCl_4\cdot5H_2O$	0.8	73	
Sn^{2+}	$SnCl_2\cdot2H_2O$	2.4	94	
Hg^{+}	$HgNO_3\cdot H_2O$	6.4	7.0	
Bi^{3+}	$Bi(NO_3)_3\cdot5H_2O$	3.9	0	
Cd^{2+}	$Cd(NO_3)_2\cdot4H_2O$	0	0	0
Ni^{2+}	$Ni(NO_3)_2\cdot6H_2O$	0	0	47
Hg^{2+}	$Hg(NO_3)_2\cdot xH_2O$	5.1	77	
Co^{2+}	$Co(NO_3)_2\cdot6H_2O$	0	5.6	91
Sb^{3+}	$SbCl_3$	5.8	73	
Zn^{2+}	$Zn(NO_3)_2\cdot6H_2O$	0	0	35
As^{3+}	As_2O_3	0.4	73	
As^{5+}	As_2O_5	0	37	
Ba^{2+}	$Ba(NO_3)_2$	0	0	5.5
Ca^{2+}	$Ca(NO_3)_2\cdot4H_2O$	0	0	13
Al^{3+}	$Al(NO_3)_3\cdot9H_2O$	0	4.0	88
Sr^{2+}	$Sr(NO_3)_2$	0	0	10
Mg^{2+}	$Mg(NO_3)_2\cdot6H_2O$	0	0	13
K^{+}	KNO_3	0	0	1.6
Na^{+}	$NaNO_3$	0	0	0.6
NH_4^{+}	NH_4NO_3	0	0	5.0

The conditions used were same as in the procedure in the text except for the additional presence of 1 ml of a 5×10^{-4}M, 5×10^{-3}M or 5×10^{-2}M foreign cation solution.

Table IV. Relative Fluorescence Intensity of Compounds in the Reaction with I

Compound	RFI			Compound	RFI		
	10^{-7} mole	10^{-6} mole	10^{-5} mole		10^{-7} mole	10^{-6} mole	10^{-5} mole
$NaHSO_3$	100	222	267	Dithiodiglycolic acid	0		
Na_2SO_3	104	232	265	DL-α-Lipoic acid	0		
$Na_2S_2O_5$	130	222	244	Dimethyl sulfite	0	0.2	81
$Na_2S_2O_4$	40	33	27	Di-*n*-propyl sulfite	0	0	98
$Na_2S \cdot 9H_2O$	17	49	16	$HOCH_2SO_3Na \cdot H_2O$	0	0.7	54
NaSH	2.6	25	33	1,3-Dimethyl thiourea	0		
$K_2S_2O_7$	0.7	0.6	0.7	Thiosemicarbazide	0		
$Na_2S_2O_3 \cdot 5H_2O$	0.6	0.6	1.0	C_2H_5OCSSK	0		
Na_2SO_4	0.5	1.8	1.1	$CH_3CH_2CH_2CH_2$-OCSSK	0		
$K_2S_2O_8$	0	0	0	L-Cysteic acid $\cdot H_2O$	0		
$Na_2S_4O_6 \cdot H_2O$	0.5	0.7	0.9	DL-Homocysteic acid	0		
KCN	0.5	0.7	3.7	Formamidine sulfinic acid	0		
KSCN	0.4	0.4	0.4	Ammonium sulfamate	0		
L-Ascorbic acid	0	0	0	Sodium cyclohexyl sulfamate	0		
$FeSO_4 \cdot 7H_2O$	0	0	0	C_2H_5NCS	0		
$Ce(NO_3)_3 \cdot 6H_2O$	0	0	0	$C_6H_{11}NCS$	0		
Cu_2O	0	0	0	CH_3SCN	0		
L-Cysteine	0.7	1.0	2.8	C_2H_5SCN	0		
Homocysteine	1.6	1.6	2.9	DL-Lanthionine	0		
Cysteamine $\cdot$ HCl	0.7	0.9	1.0	Djenkolic acid	0		
Glutathione	7.7	16	19	*n*-Heptyl sulfide	0		
Dithiothreitol	2.8	4.3	4.6	D-Glucose-6-sulfate	0		
2-Mercaptoethanol	1.0	1.3	1.6	DL-Methionine sulfone	0		
L-Cysteine-S-sulfate	0			DL-Methionine sulfoxide	0		
Pantetheine-S-sulfate	0			$(CH_3)_2NCSSNa$	0		
Cysteamine-S-sulfate	0			$(C_2H_5)_2NCSSNa$	0		
Cystamine $\cdot 2H_2O$	0			Methanesulfonic acid ethyl ester	0		
Tetraethylthiuram disulfide	0						
Dipentamethylene thiuram disulfide	0						
Glutathione oxidized	0						

One ml of aqueous solution of a 1×10^{-4}M, 1×10^{-3}M or 1×10^{-2}M compound was mixed with 1 ml of 0.1 mM I in ethanol and 2 ml of the citrate buffer (pH 3.62), and incubated at 37° for 30 min.

also yielded fluorescence, the intensities being 40 and 17% respectively on a molar basis. Other inorganic sulfur compounds gave little or no fluorescence. Among usual organic sulfur compounds, only a few thios showed positive responses, and the maximum response of them was exhibited by glutathione whose relative fluorescence intensity to that of bisulfite was 7%.

Analysis of Bisulfite in The Presence of Thiols and Sulfide Ion

When large amounts of thiols and sulfide ions were present in the sample, they were able to be removed by treating with *p*-chloromercuribenzoic acid (PCMB) as follows.

Mix 1.0 ml of the sample, standard, or blank solution and 1.5 ml of 10 mM PCMB in 0.05M Tris–HCl buffer (pH 10.3). The buffer solution is preliminarily added with 3.33 mM EDTA when the sample solution is analyzed. Incubate the mixture at 37° for 10 min, and centrifuge at 4000 rpm for 5 min. Take 3.0 ml of the resultant supernatant, add 1.0 ml of 0.1 mM I in ethanol, and incubate at 37° for 30 min. Add 1.0 ml of 2N NaOH, mix, and measure the fluorescence intensity as described above.

With the procedure, the presence of 1.5×10^{-6} mole of sulfide ion gave no effect on the values of bisulfite. As shown in Fig. 7, the working curve for bisulfite was linear in the range from 1.5×10^{-8} to 1.5×10^{-7} mole.

Characteristics of Fluorescent Compounds (II)

Although the fluorescence spectrum showed one peak as reported in the previous communication,[9] two fluorescent compounds (IIa, blue fluorescence; IIb, violet fluorescence) with different mobilities toward the anode (IIa, 1.6 cm; IIb, 2.8 cm) were found in the reaction mixture of I and bisulfite by paper electrophoresis (1M HCOOH, pH 1.75, 36 v/cm, 10 min). In the early stage of the reaction, IIa was the major fluorescent spot together with the minor one, IIb, and the amount of IIb gradually increased to surpass that of IIa as the reaction proceeded. The fact that IIa and IIb moved toward the anode at pH 1.75 indicated the introduction of at least one $-SO_3H$ group into I. Therefore, by reacting radioactive $Na_2{}^{35}SO_3$ with I, the chemical structures of IIa and IIb were further estimated in the following manner.

One ml of 1 mM I (1×10^{-6} mole) in N,N-dimethylformamide was added with 2 ml of the citrate buffer (pH 3.62) described above and 0.1 ml of $Na_2{}^{35}SO_3$ solution (1×10^{-6} mole), incubated at 37° and at appropriate intervals aliquots (0.5 ml) were withdrawn.

As shown in Fig. 8, analysis of the reaction products by paper electrophoresis revealed two main radioactive peaks together with one minor peak around the origin, the mobilities of the former two were identical to those of IIa and IIb, and the third to the fluorescent spot occasionally detected. As the reaction time was prolonged, the radioactive peak identical to IIa decreased, whereas the peak identical to IIb increased.

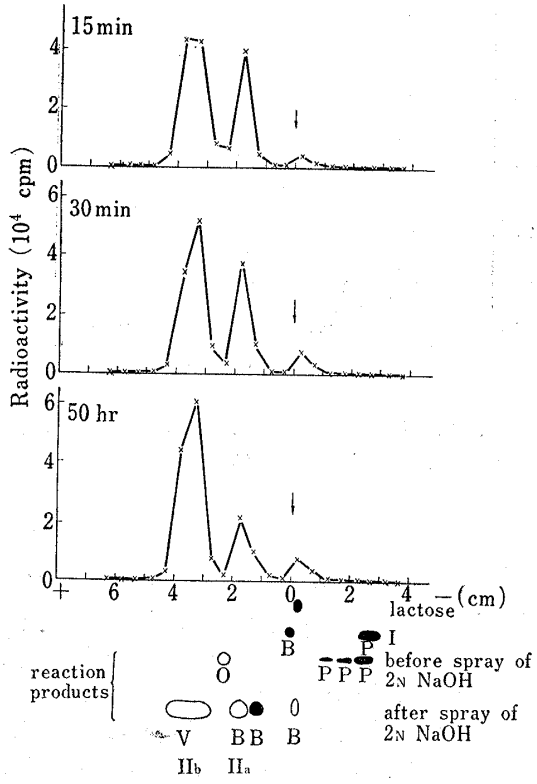

Fig. 8. Analysis of Radioactive Products by Paper Electrophoresis

An aliquot (10 μl) of the reaction mixture of I and $Na_2{}^{35}SO_3$ was analyzed as described in the text. The shadowed spots indicate the coloured ones and the others indicate the fluorescent ones. The arrows indicate the spotted points.
B=blue, P=pink, O=orange, V=violet

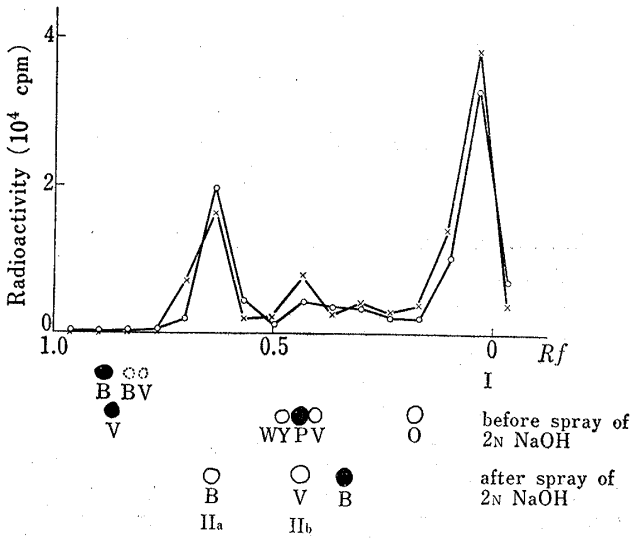

Fig. 9. Analysis of Radioactive Products by Thin-Layer Chromatography

An aliquot (5 μl) of the reaction mixture of I and $Na_2{}^{35}SO_3$ was analyzed by TLC on SPOTFILM (silica gel) with *n*-BuOH–AcOH–H_2O (5: 2: 3). The abbreviations used are same as in Fig. 8 except for WY which means white yellow.
—×— : 15 min
—○— : 30 min

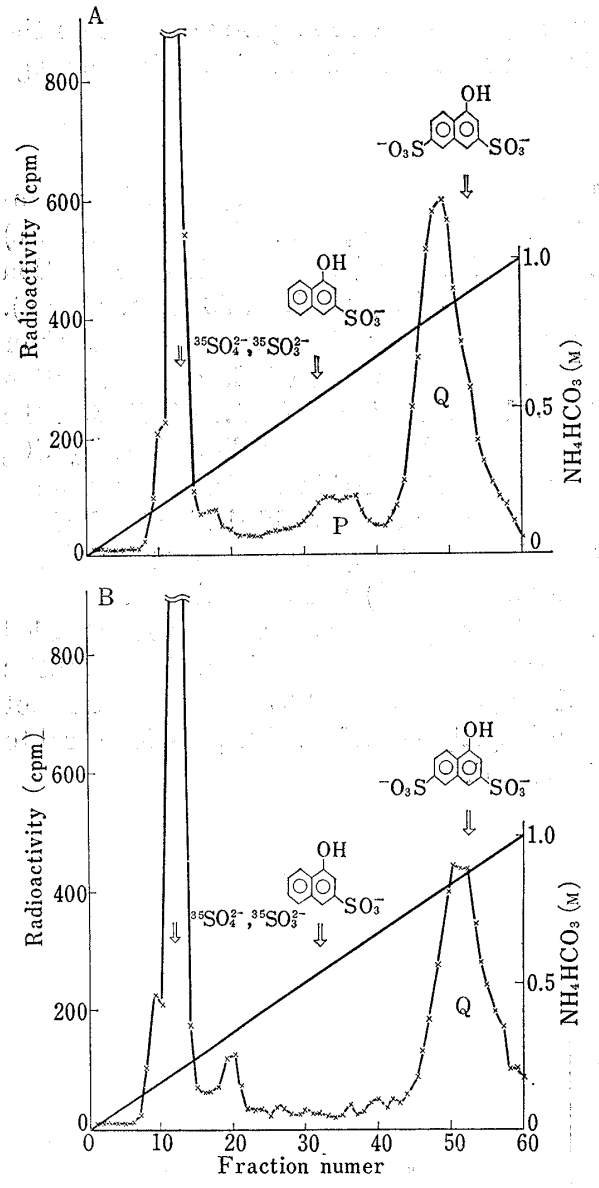

Fig. 10. Column Chromatograms of Reaction Products

The arrows indicate the elution positions of authentic compounds.
A: 15 min
B: 50 hr

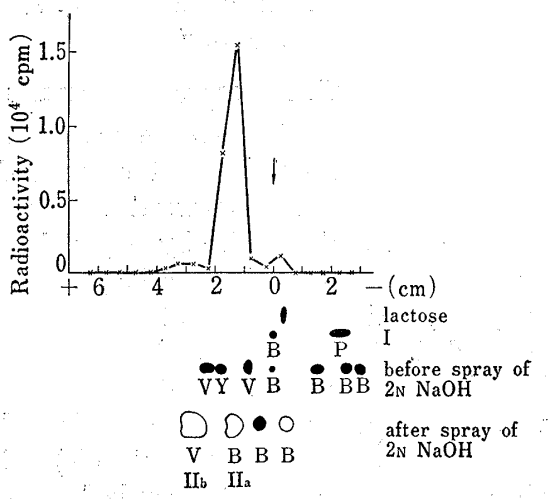

Fig. 11. Analysis of Peak P by Paper Electrophoresis

The arrow indicate the spotted point. The abbreviations used are same as in Fig. 8 except for Y which means yellow.

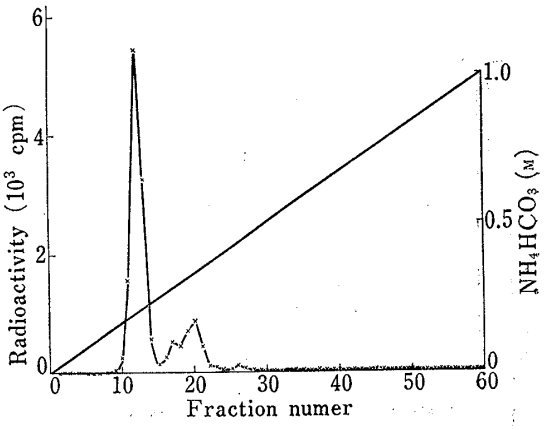

Fig. 12. Re-chromatography of Peak P on QAE-Sephadex A-25 Column

Three radioactive peaks were observed by thin-layer chromatography of the reaction products, the *Rf* values of two of which again coincided with those of IIa and IIb (Fig. 9). The largest radioactive peak located at *Rf* 0.03 seemed to be $^{35}SO_4^{2-}$ but not $^{35}SO_3^{2-}$ because sulfite was easily removed from the thin-layer with the acidic developing solvent.[11]

Another data were obtained by column chromatography. As representatives, chromatograms of 15 min- and 50 hr-reaction products are shown in Fig. 10, together with the elution positions of authentic $^{35}SO_4^{2-}$, $^{35}SO_3^{2-}$, 1-naphthol-3-sulfonate and 1-naphthol-3,6-disulfonate. Most remarkable characteristic common to all the elution patterns of samples taken at various times was a tendency of peak P to decrease with time, which coincided with the decrease in IIa with time as already shown in Fig. 8. The radioactive peak centered at fraction 12 to 13

11) H. Nakamura and Z. Tamura, *J. Chromatog.*, **104**, 389 (1975).

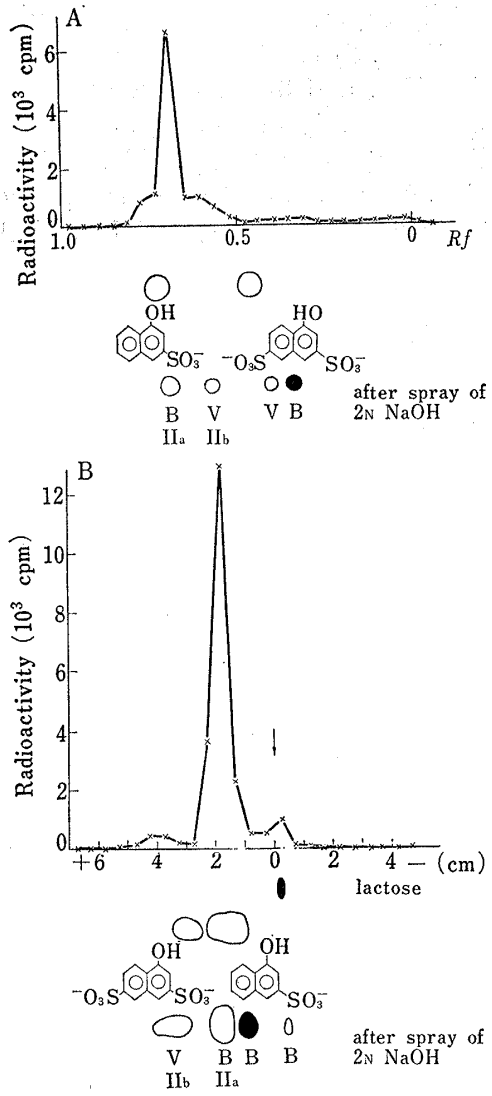

Fig. 13. Analysis of Peak Q by Thin-Layer Chromatography (A) and Paper Electrophoresis (B)

The abbreviations used are same as in Fig. 8. The arrow in (B) indicates the spotted point.

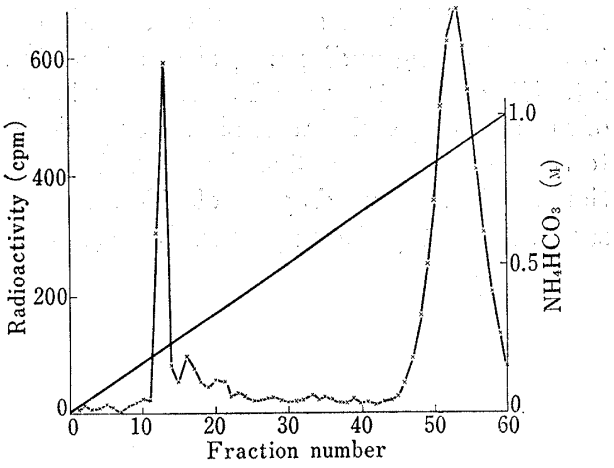

Fig. 14. Re-chromatography of Peak Q on QAE-Sephadex A-25 Column

was identified as $^{35}SO_4^{2-}$ and/or $^{35}SO_3^{2-}$ by paper electrophoresis and thin-layer chromatography. As the electrophoretogram in Fig. 11 shows, peak P was proved to contain IIa. Re-chromatography of peak P yielded the peak of $^{35}SO_4^{2-}$ and/or $^{35}SO_3^{2-}$ and minor compounds, but not peak P (Fig. 12).

With peak Q, both thin-layer chromatographic (Fig. 13A) and electrophoretic (Fig. 13B). results indicated the predominant presence of IIa, however, the presence of trace radioactive peak corresponding to IIb and its elution position from QAE-Sephadex A-25 column suggested the presence of IIb in peak Q and also the decomposition of IIb into IIa during the procedures. After re-chromatography of peak Q on the QAE-Sephadex column, the peak of $^{35}SO_4^{2-}$ and/or $^{35}SO_3^{2-}$ was emerged together with the main peak Q, though without the peak P (Fig. 14).

Discussion

Among the compounds tested as the fluorescence reagents for bisulfite, only I gave intense fluorescence. *p*-Benzoquinone reacted with bisulfite to yield fluorescence, but even 5 μg of $NaHSO_3$ was not detected. Methylene blue gave faint blue fluorescence. The following compounds did not yield fluorescence at any pH by the reaction-with bisulfite; sodium indophenol, indo-oxine sodium salt, gallocyanine, sufranine T, bromocresol green, orange G, phthalein complexon, aniline violet, sudan III, phenol red, fuchsine (basic), and malachite green.

The present method is the first one for the determination of bisulfite based on a fluorescence reaction of bisulfite. In practical point of view, the present method would permit selective determination of bisulfite in biological materials, since highly interfering compounds, pyrosulfite and hyposulfite, are usually not found in nature. Inorganic sulfide and thiols could be eliminated by treating with PCMB prior to addition of I to the sample, in this case, use of $HgCl_2$ as a masking agent for sulfide and thiols is undesirable because of its inhibition of formation of II formed.

IIa and IIb are supposed to contain one and two $-SO_3H$ groups respectively according to the following findings: 1) electrophoretic behaviors of them, 2) the transformation of IIa into IIb during the period of reaction between I and bisulfite, and 3) the elution positions of IIa and IIb, which were shown as peak P and peak Q respectively in Fig. 10, were similar to those of 1-naphthol-3-sulfonate and 1-naphthol-3,6-disulfonate respectively. The assumption may be supported by the clarified structures of bisulfite adducts of unsaturated compounds such as 2-methyl-1,4-naphthoquinone(vitamin K_3),[12] flavin,[13] folate[14] and nicotinamide.[15]

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