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Fluorescence Characteristics of Benzidine and Its Application to Determination of Microgram Quantities of Nitrite

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1. Fluorescence characteristics of benzidine was examined. Benzidine had an emission maximum at 387 nm under excitation at 295 nm in 90% ethanol. The fluorescence intensity increased the most in 70% ethanol. The concentration of HCl in 70% ethanol also influenced the fluorescence intensity.

2. The nature of fluorescence of benzidine was changed by the treatment with nitrite. The wavelengths for both excitation and emission maxima shifted to 275 and 348 nm, respectively, and its fluorescence intensity decreased to about one-third that of benzidine.

3. For the determination of nitrite, a fluorescence measurement was carried out at 326 and 387 nm for excitation and emission, respectively. The best results was obtained from the following procedure. Benzidine is treated with nitrite at 98° for 1 min in 0.1 N HCl and the mixture is diluted 10-fold with ethanol, then the fluorescence intensity is measured. Under these conditions, 0.25 to 1.25×10^{-6} M nitrite was successfully determined by the use of 10^{-6} M benzidine.

4. Among cations tested, Cu^{2+}, Sn^{2+}, Fe^{2+}, and Al^{3+} interfered seriously with the determination of nitrite at a concentration of 5×10^{-4} M. Among the anions, citrate, malonate, thiocyanate, molybdate, and tungstate also interfered at a level of 10^{-4} M. Oxidants and reductants had also a serious effect on the determination of nitrite at a level of 5×10^{-5} M.

Not only are nitrites of importance as an indicator showing the presence of reducing agents, but nitrates may be quantitatively converted to nitrites for their determination. Moreover, the nitrite content of water is a factor in the prediction of its past history.

Numerous methods of nitrite determination have been reported.[2] The most sensitive and commonly employed methods are the colorimetric determination of a nitrite based on successive diazotization and coupling reaction. In acidic solution, primary aromatic amines react with a nitrite to form diazonium salts which will undergo coupling to form various azo dyes. Sulfanilic acid and α-naphthylamine have been the best-known reagents for this reaction, although many other reagents are used.

Benzidine can react with nitrite in an acidic solution of acetic acid to form a yellow compound which serves as an agent for the colorimetric determination of nitrite.[3] However, fluorometric method for nitrite determination has not been reported. Taking note of a violet fluorescence of benzidine in alcohols,[4] we attempted developing a method for fluorometric determination of nitrite.

This paper describes the fluorescence characteristics of benzidine and its application to fluorometric determination of a nitrite. The method presented is as sensitive as the colorimetric determination of nitrite by diazotization and coupling. Because this method is manipulatively simple and rapid, it is readily adaptable to the determination of a large number of samples.

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1) Location: *5-9-1, Shirokane, Minato-ku, Tokyo.*

2) E.A. Burns, "Chemical Analysis: The Analytical Chemistry of Nitrogen and Its Compounds," Vol. 28, Part 1, ed. by C.A. Streuli and P.R. Averell, Wiley-Interscience, New York, 1970, p. 117.

3) N.A. Tannaeff and A.M. Schapowalenko, *Z. Anal. Chem.*, **100**, 351 (1935).

4) K. Yagi, Z. Yoshida and T. Tabata, "Keiko (Fluorescence)," Nankodo, Tokyo, 1962, p. 106.

Experimental

Materials——All the reagents were analytical grade and were used without further purification. Stock solutions (0.1 M) of metal salts were prepared by dissolving each salt of nitrate, chloride, acetate, or sulfate in distilled water. Stock solutions (0.1 M) of anions were made by dissolving each sodium or potassium salt in distilled water. Solutions (10^{-2}M) of oxidatns and reductants were freshly prepared by dissolving each salt in distilled water before use.

Sodium Nitrite Stock Solution (1×10^{-3}M): Anhydrous nitrite (69.0 mg) was dissolved in distilled water and diluted to 1 liter. The solution added with 1 ml of chloroform was preserved in a refrigerator.

Standard Sodium Nitrite Solution (2×10^{-5}M): The stock solution (20 ml) was diluted to 1 liter with distilled water. This solution was freshly prepared before use.

Benzidine Solution (2×10^{-5}M): Benzidine (184.2 mg) was dissolved in 100 ml of ethanol and stored in a refrigerator. This solution (2.0 ml) was diluted to 1 liter with 0.2 N HCl before use unless otherwise stated.

Standard Assay Procedure: To 0.5 ml of the diluted benzidine solution, 0.5 ml of solution containing a nitrite was added and immersed in a boiling water bath for 1 min. After cooling in ice-water, 9.0 ml of ethanol was added to the mixture and the fluorescence intensity at 387 nm under an excitation at 326 nm was recorded.

Apparatus——Fluorescence measurement was made with an Aminco–Bowman spectrofluorometer using 10-mm rectangular cells.

Result and Discussion

Concentration of the reagents was expressed as the final concentration in a reaction mixture in which the fluorescence was measured.

Fluorescence Characteristics of Benzidine

The fluorescence excitation and emission spectra of benzidine in water and 90% ethanol are given in Fig. 1. The fluorescence spectra in both solvents were quite similar. The emission spectra changed slightly in water, in which the wavelength giving a maximum fluorescence intensity shifted to a slightly longer wavelength with a tailing in water than in 90% ethanol. The fluorescence intensity of benzidine in water decreased to about one-third of that in 90% ethanol, indicating that a solvent with low dielectric constant enhances the fluorescence quantum yield of benzidine under irradiation with ultraviolet ray. Wavelengths

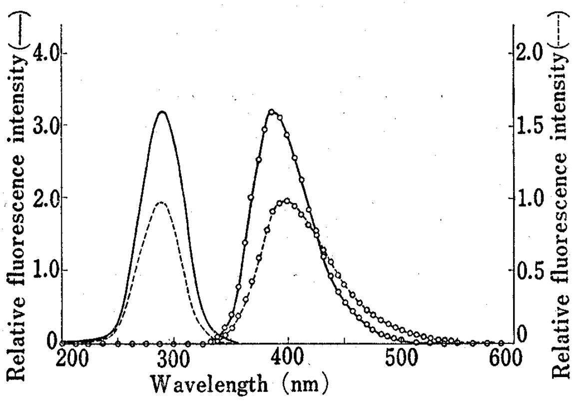

Fig. 1. Excitation and Emission Spectra of Benzidine in Water and 90% (v/v) Ethanol

Each cuvette contained 10^{-6}M benzidine. The excitation spectra were recorded at 387 nm for emission, and the emission spectra were taken under excitation at 295 nm.
——: Excitation spectrum in 90% ethanol,
---------: excitation spectrum in water,
—○—: emission spectrum in 90% ethanol
---○---: emission spectrum in water

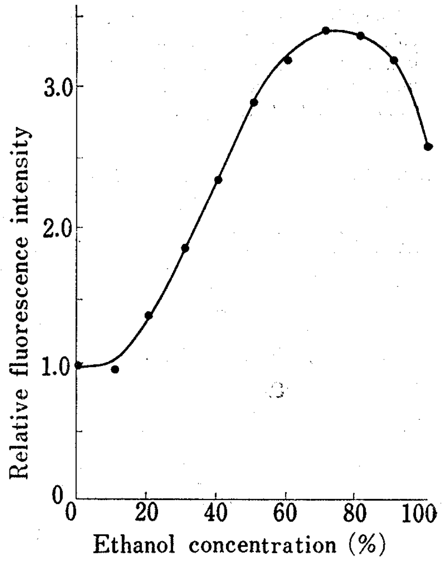

Fig. 2. Effect of Ethanol Concentration on Fluorescence Intensity of Benzidine

The mixture contained 10^{-6}M benzidine in ethanol of the indicated **final** concentration (% v/v). The fluorescence intensity was measured at 387 nm under excitation at 295 nm.

giving the maximum fluorescence excitation and emission were found to be 295 nm and 387 nm in 90% ethanol, respectively.

Effect of ethanol concentration on the fluorescence intensity of benzidine is shown in Fig. 2. When the intensity of benzidine was measured at 387 nm under an excitation at 295 nm, a maximum intensity was obtained in a solution of 70% ethanol. The ethanol concentration giving a maximum intensity of benzidine changed to 90% in the presence of 5×10^{-3}N HCl, though the data are not presented here. HCl concentration also affected the fluorescence intensity of benzidine. The intensity in 70% ethanol increased in a concentration below 2×10^{-3}N HCl and decreased in that above 5×10^{-3}N HCl. The maximum intensity of benzidine in 70% ethanol was obtained with 2×10^{-4}N HCl. If 10^{-3}M or 10^{-7}M benzidine was used, the maximum intensity was found with 10^{-2}N and 10^{-4}N HCl, respectively.

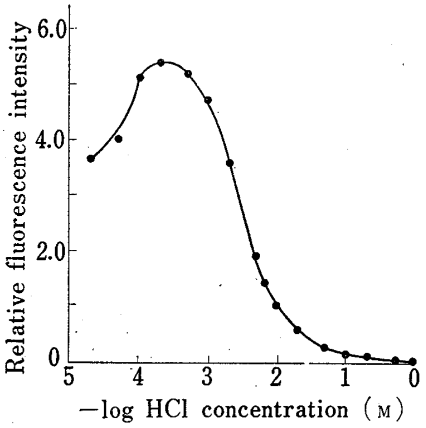

Fig. 3. Effect of HCl Concentration on Fluorescence Intensity of Benzidine

The mixture consisted of 10^{-6}M benzidine in 70% (v/v) ethanol containing HCl of the indicated final concentration. Fluorescence was measured under the same conditions as described in the footnote to Fig. 2.

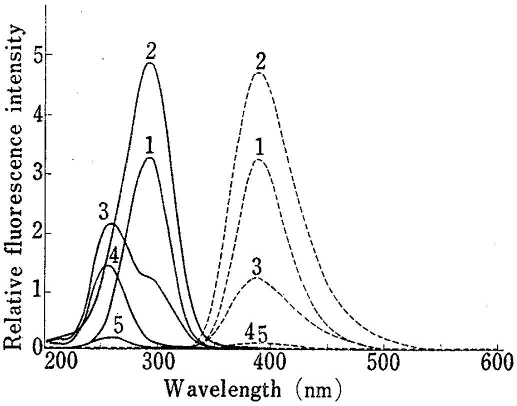

Fig. 4. Excitation and Emission Spectra of Benzidine in HCl of Different Concentrations

Each cuvette contained 10^{-6}M benzidine in 70% (v/v) ethanol containing HCl of the indicated concentration. The excitation and emission spectra were recorded under the same conditions as described in the footnote to Fig. 1. 1: none, 2: 10^{-3}N HCl, 3: 10^{-2}N HCl, 4: 10^{-1}N HCl, 5: 1.0N HCl

——: excitation spectra
------: emission spectra

Fluorescence excitation and emission spectra of benzidine at various concentrations of HCl are presented in Fig. 4. The emission spectra of benzidine obtained were similar to each other, though the intensity varied with HCl concentration. On the other hand, the excitation spectra of benzidine in 70% ethanol changed markedly with HCl concentration. The excitation spectrum in 70% ethanol with 10^{-3}N HCl was similar to that of free benzidine in 70% ethanol, except for a change in its fluorescence intensity. The excitation spectrum with 10^{-2}N HCl had a new peak at 262 nm with decrease in the intensity at 295 nm. The spectrum with 0.1N HCl showed only a single peak at 258 nm for the excitation of benzidine, accompanied with a marked decrease in the fluorescence intensity. As can also be seen in Fig. 4, the intensity under an excitation at 258 nm was very small in 70% ethanol with 1N HCl. The peak giving a maximum fluorescence intensity completely shifted to 260 nm in water with 10^{-2}N HCl, though the data are not presented here. This observation indicates that ethanol partially protects the shift of wavelength exciting benzidine from 295 nm to 258 nm with HCl. These findings suggest that the wavelength for activating benzidine varies from 295 to 258 nm according to the progress in dissociation of amino groups with decrease in the fluorescence intensity.

The absorption spectra of free benzidine in water or 90% ethanol possessed a peak at 288 nm, which shifted to 249 nm in 10^{-2}N HCl or 90% ethanol containing 10^{-2}N HCl, with decrease in its extinction coefficient (our unpublished observation). It seems that benzidine has a close similarity between variabilities in the absorption and fluorescence-excitation spectra with HCl.

Aniline, a fluorescent substance, becomes non-fluorescent by its dissociation to an anilinium ion.[5] The fluorescence of benzidine practically disappeared in the presence of 1 N HCl (see Fig. 4). This fact indicates that benzidine molecule loses its fluorescence with dissociation of its two amino groups like the anilinium ion.

Determination of Nitrite

Benzidine was treated with nitrite in acidic solution. The product had no visible fluorescence under irradiation with ultraviolet ray, but it had a fluorescence in the ultraviolet region. The excitation and emission spectra of the product are given in Fig. 5. The maximum fluorescence intensity of the product was found at 348 nm under an excitation at 279 nm. However, the fluorescence intensity under the conditions used decreased to about one-third of that of benzidine in 90% ethanol with 10^{-2}N HCl. When fluorescence emission was taken at 387 nm, the product had a considerable intensity under irradiation at 295 nm, at which benzidine was subjected to a maximum activation. However, it had hardly any measurable fluorescence intensity under an excitation at 326 nm, though the fluorescence intensity of benzidine decreased to about one-half of that activated at 295 nm.

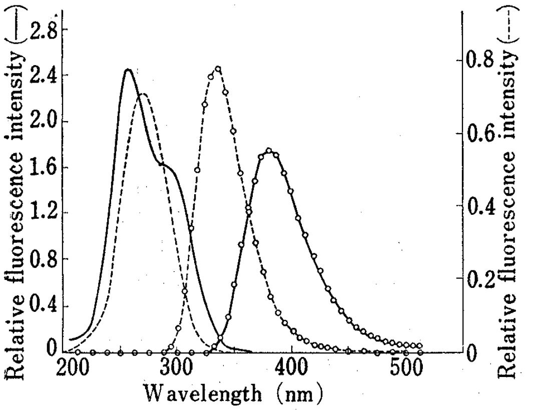

Fig. 5. Excitation and Emission Spectra of the Product Obtained by Benzidine Treatment of Nitrite

The reaction mixture contained 10^{-4}M benzidine and 5×10^{-3}M $NaNO_2$ in 1.0 ml of 0.1 N HCl. After standing for 1 hr at 37°, 9.0 ml of ethanol was added to the mixture. The emission spectrum of the product was recorded under excitation at 275 nm and the excitation spectrum taken at 348 nm for emission. The spectra of control were obtained under the conditions described in the footnote to Fig. 1. ———: excitation spectrum of control, ---------: excitation spectrum of product, —○—: emission spectrum of control, ---○---: emission spectrum of product

Table I. Effect of HCl Concentration on Fluorescence Intensity of Benzidine Treated with Nitrite

HCl concn. (N)	Decrease in intensity (%)
2×10^{-1}	82[a)]
1×10^{-1}	87[a)]
5×10^{-2}	75[a)]
2×10^{-2}	84.8
1×10^{-2}	71.5
5×10^{-3}	64.1
2×10^{-3}	68.5
1×10^{-3}	83.6
5×10^{-4}	98.5
2×10^{-4}	104.0
1×10^{-4}	108.6
5×10^{-5}	109.0
2×10^{-5}	109.7
0	116.5

The mixture consisted of 10^{-5}M benzidine, 7.5×10^{-6}M nitritc, and HCl of the indicated concentration in 1.0 ml. After heating at 98° for 1 min, 7.0 ml of ethanol and 2.0 ml of water were added to the mixture. Fluorescence intensiy was measured at 387 nm under excitation at 326 nm.

a) Incorrect values due to low fluorescence intensity.

Thus, the fluorescence intensity in the following experiments was measured at 387 nm under excitation at 326 nm. When HCl concentration was varied, an increase in the fluorescence intensity was observed below 2×10^{-4}N HCl in 70% ethanol. This indicates that the reaction of benzidine with nitrite does not occur at such a low concentration of HCl. Correct values of the fluorescence intensity were not obtained above 5×10^{-2}N HCl due to its very low intensity under the conditions used. A good result was obtained with 5×10^{-3}N

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[5] S. Udenfriend, "Fluorescence Assay in Biology and Medicine," ed. by N.O. Kaplan and H.A. Scheraga, Academic Press Inc., New York and London, 1962, p. 28.

HCl (Table I). When ethanol concentration was varied, a large decrease in the fluorescence intensity was found in 70% ethanol with 5×10^{-3}N HCl, 80% ethanol with 5×10^{-3}N HCl, and 90% ethanol with 10^{-2}N HCl. Among them, a maximum fluorescence decrease was found in 90% ethanol with 10^{-2}N HCl (Table II).

TABLE II. Effect of Ethanol Concentration on Fluorescence Intensity of Benzidine Treated with Nitrite

Final concn. of HCl (N)	Final concn. of ethanol (%)	Relative intensity −Nitrite	Relative intensity +Nitrite	Decrease in intrnsity (%)
2×10^{-3}	70	1.53	1.11	73.0
2×10^{-3}	80	2.03	1.32	65.0
2×10^{-3}	90	2.45	1.61	65.7
5×10^{-3}	70	0.72	0.46	63.9
5×10^{-3}	80	0.93	0.58	62.4
5×10^{-3}	90	1.17	0.71	60.6
1×10^{-2}	70	0.37	0.25	67.6
1×10^{-2}	80	0.50	0.33	66.0
1×10^{-2}	90	0.57	0.34	59.7
2×10^{-2}	70	0.19	0.16	84.2
2×10^{-2}	80	0.23	0.17	74.0
2×10^{-2}	90	0.28	0.17	60.7

The mixture consisted of 10^{-5}M benzidine, 7.5×10^{-6}M nitrite, and HCl of the indicated concentration in 1.0 ml. After treatment under the condition described in the footnote to Table I, ethanol of the desired final volume was added to the mixture and was made up to 10 ml with distilled water.

In the experiments shown in Tables I and II, ethanol was added to the system before fluorescence measurement after treatment of benzidine with nitrite in aqueous solution. When 10^{-6}M benzidine was treated with 1.4×10^{-5}M nitrite in 90% ethanol containing 10^{-2}N

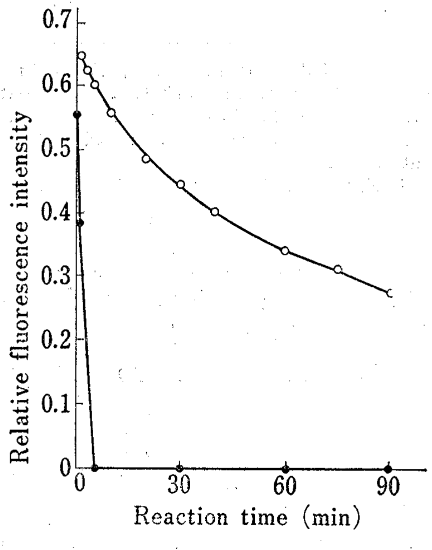

Fig. 6. Comparison of Reaction Rates in Water and 90% (v/v) Ethanol

The reaction mixture contained 10^{-5}M benzidine and 1.4×10^{-4}M $NaNO_2$ in 10 ml of 0.1 N HCl, with or without 90% ethanol. After standing for the indicated period at 22°, 0.5 ml aliquot was diluted 10-fold with 90% ethanol or ethanol, respectively. Fluorescence intensity at 387 nm under excitation at 326 nm was recorded. —●—: in water, —○—: in 90% ethanol

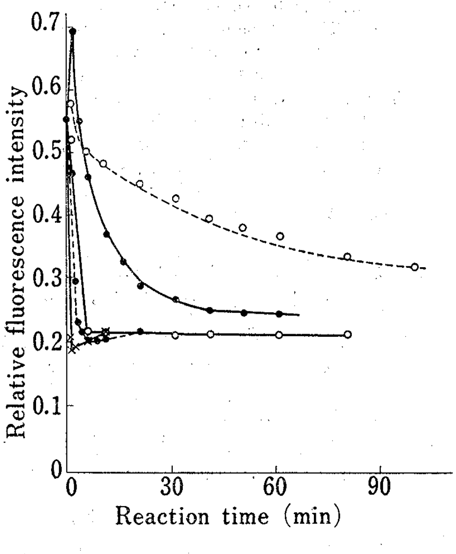

Fig. 7. Effect of Temperature on the Benzidine Treatment of Nitrite

The reaction mixture contained 10^{-5}M benzidine and 10^{-6}M $NaNO_2$ in 1.0 ml of 0.1 N HCl. After incubation at the indicated temperature, the mixture was diluted 10-fold with ethanol. The intensity was recorded under the conditions described in the footnote to Fig. 6. —○— :2°, —●—: 22°, ---○---: 37°, ---●---:56°, —×—: 98°

HCl, the decrease in fluorescence intensity was slower than by treatment in aqueous solution containing HCl before adding ethanol. As shown in Fig. 6, the fluorescence of benzidine disappeared after 5 min in the aqueous solution, while its fluorescence treated in 90% ethanol remained in about 40% of benzidine used initially after 90 min. This fact indicates that the reaction of nitrite with benzidine proceeds very slowly in 90% ethanol. Thus, in the following experiments, the treatment of nitrite with benzidine was carried out in aqueous solution containing HCl and the resulting reaction mixture was diluted 10-fold with ethanol before fluorescence measurement.

Effect of temperature on the treatment of nitrite with benzidine is shown in Fig. 7. The decrease in fluorescence intensity of benzidine treated with nitrite was slow at 2° and 22°, while it was rapid at temperatures above 37° and reached almost a constant level after the treatment for 5 min. A maximum decrease in the fluorescence intensity was obtained at 98° for 1 min. The fluorescence intensity of the remaining benzidine was stable in 90% ethanol containing 10^{-2} N HCl at room temperature and its significant change was not observed for a few hours. However, the fluorescence intensity of benzidine decreased with increasing temperature during fluorescence measurement. When the temperature was hard to control, it was necessary to measure the standards prepared simultaneously.

From the results described above, the following conditions for nitrite determination were selected. Nitrite is treated with benzidine in 10^{-1} N HCl aqueous solution at 98° for 1 min and the mixture is added with ethanol to bring its final concentration to 90%. The fluorescence intensity is measured at 387 nm under excitation at 326 nm. As can be seen in Fig. 8, there was a good linearity between the decrease in fluorescence intensity and the concentration of nitrite from 0.25 to 1.25×10^{-6} M in the presence of 10^{-6} M benzidine by the method presented here. When the conventional formula of quenching [$F = F_0/(1+kc)$] was applied to the nitrite determination, the plots obtained in Fig. 8 fitted well below 1×10^{-6} M nitrite. However, the plots above 1.25×10^{-6} M nitrite were considerably out of rule and, as shown in Fig. 5, the excitation and emission spectra of the product treated with nitrite differed from those of benzidine. Thus, the formula could not be directly applied to the nitrite

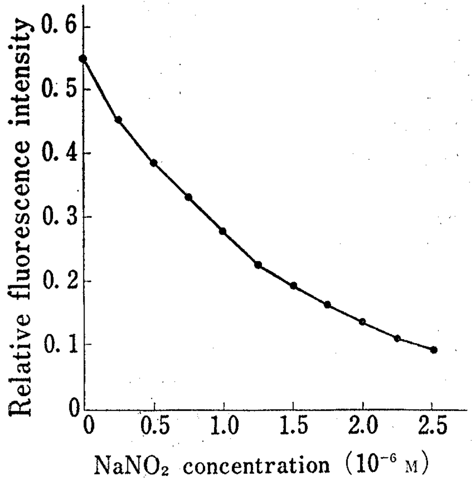

Fig. 8. Standard Curve for Nitrite Determination with Benzidine

The reaction mixture contained 10^{-5} M benzidine and nitrite of the indicated concentration in 1.0 ml of 0.1 N HCl. After heating at 98° for 1 min and then cooling in an ice-water bath, 9.0 ml of ethanol was added to the mixture. The fluorescence intensity was measured under the conditions described in the footnote to Fig. 6.

Table III. Effect of Metal Ions on Nitrite Determination

Metal	Decrease in fluorescence intensity (%)
None	49.2
Li^{1+}	49.7
Be^{2+}	47.9
Mg^{2+}	48.0
Ca^{2+}	48.3
Sr^{2+}	47.9
Ba^{2+}	49.3
Mn^{2+}	50.6
Fe^{3+}	100
Co^{2+}	49.2
Ni^{2+}	50.5
Cu^{2+}	61.6
Zn^{2+}	49.7
Cd^{2+}	48.5
Hg^{2+}	48.4
Al^{3+}	45.4
Sn^{2+}	4.3
Pb^{2+}	48.4[a]

The mixture consisted of 10^{-5} M benzidine, 10^{-6} M nitrite, 0.1 N HCl, and 5×10^{-3} M metal salt in 1.0 ml. After treatment at 98° for 1 min. 9.0 ml of ethanol was added to the mixture.

a) determined in the supernatant

determination throughout the nitrite concentration used. The detectable limit with this method, 0.012 to 0.057 μg of nitrite/ml, was approximately equal to that of the colorimetric determination using the diazo-coupling reaction.

There was no change in detection limit of the system when the order of addition of reagents except ethanol was altered. However, better reproducibility was obtained when a nitrite was added to the mixture of benzidine and HCl.

The effect of metal ions on the determination of 10^{-6}м nitrite is given in Table III. A few metal ions, *e.g.*, Cu^{2+}, Sn^{2+}, Fe^{3+}, and Al^{3+}, interfered seriously at a level of 5×10^{-4}м. Lead (II) at a concentration of 5×10^{-4}м formed a precipitate in this system, but did not interfere after the precipitate was separated by centrifugation at 3000 rpm for 5 min. As shown in Table IV, in which the effect of various anions is presented, citrate, molonate, thiocyanate, molybdate, and tungstate interfered with this reaction at a concentration of 10^{-4}м. As can be seen in Table V, oxidants and reductants had a serious effect on the determination of nitrite at a level of 5×10^{-5}м. The reductants inhibited the decrease in fluorescence intensity of benzidine with nitrite, while the oxidants promoted it.

Table IV. Effect of Anions on Nitrite Determination

Anion	Recovery (%)	Anion	Recovery (%)
None	100.0	Veronal	101.9
Fluoride	101.6	Nitrate	98.2
Chloride	102.6	Borate	100.9
Bromide	103.0	Cyanide	99.3
Iodide	98.3	Phosphate	101.0
Acetate	99.3	Pyrophosphate	101.7
Lactate	98.1	Sulfate	101.4
Citrate	93.7	Pyrosulfate	101.4
Malonate	95.4	Laurylsulfate	100.4
Benzoate	99.4	Arsenate	101.0
Phthalate	97.1	Thiocyanate	107.6
Oxalate	100.2	Molybdate	111.5
Tartrate	99.8	Tungstate	112.6

The conditions used were identical to those described in the footnote to Table III, except for the use of 10^{-3}м anions.

Table V. Effect of Oxidants and Reductants on Nitrite Determination

Agent	Relative fluorescence intesnity	Agent	Relative fluorescence intensity
Control	0.32	Persulfate	0.05
Sulfite	0.49	Iodate	0.04
Ferrocyanide	0.57	Periodate	0.03
Hydrosulfite	0.67	Ferricyanide	0.07

The reaction mixture consisted of 10^{-5}м benzidine, 10^{-5}м nitrite, 0.1ɴ HCl, and 5×10^{-4}м reagent in 1.0 ml. Fluorescence intensity was measured on 10-fold dilution with ethanol.