[Chem. Pharm. Bull. 19(9)1842—1848(1971)]

UDC 547.457.08 : 546.562.04.09 : 543.426

Fluorimetric Determination of Hexose with 5-Hydroxy-1-tetralone using Cupric Sulfate as an Accelerator of the Reaction[1]

Yosuke Ohkura, Yoshihiko Watanabe and Tsutomu Momose

Faculty of Pharmaceutical Sciences, Kyushu University[2]

(Received January 25, 1971)

A revised fluorimetric method for the determination of hexose with 5-hydroxy-1-tetralone was established which uses cupric sulfate as an accelerator of the fluorescence reaction, on the basis of the study on the effects of usual inorganic ions on the fluorescence reaction and the fluorescing reaction mixture.

This method is very sensitive and is not affected by pentose and other organic compounds than hexose, and so may be useful for determining microamount of hexose in biological materials.

5-Hydroxy-1-tetralone was first found as a sensitive and selective fluorescence developing reagent for hexoses, and oligo- and polysaccharides containing hexose unit in their molecules,[3] and then applied to the determination of hexose,[4] blood sugar[5] and individual hexoses being contained in the hydrolyzates of glycopeptide.[6] The reaction mechanism was also discussed.[7]

In this paper, the effects of usual inorganic ions including transition metal and heavy atom ions were examined on the above fluorescence reaction and the fluorescing reaction mixture, and some ions were found to accelerate the reaction or sensitize the fluorescence. Of those ions, cupric ion was appropriate as an accelerator of the reaction to be utilized in the fluorimetric microdetermination of hexose.

Experimental

Reagents[8]——Reagents for the Study on the Effect of Ions: Glucose Solutions: Prepare 5 and 10 μg/ml solutions (2.87×10^{-5} and 5.74×10^{-5}M solutions, respectively), store in a refrigerator, and use within 3 days.

5-Hydroxy-1-tetralone Solution (1): Dissolve 125 mg of pure 5-hydroxy-1-tetralone[9] in 500 ml of conc.H_2SO_4 (d_4^{15}: 1.84), and store in a refrigerator. This solution is stable for about 1 month.

Cation Solutions: Prepare 5.74×10^{-5}, $\times10^{-4}$, $\times10^{-3}$ and $\times10^{-2}$M, and 2.87×10^{-5}, $\times10^{-4}$, $\times10^{-3}$ and $\times10^{-2}$M solutions of NH_4^+, Na^+, K^+, Al^{3+}, Ca^{2+}, Cr^{2+}, Cr^{3+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Cu^+, Cu^{2+}, Zn^{2+}, Ni^{2+}, Ag^+, Cd^{2+}, Sn^{2+} and Ce^{4+} by dissolving their sulfates in H_2O, Ba^{2+} by dissolving its acetate in H_2O, and Hg_2^{2+} and Hg^{2+} by dissolving their sulfates in 10% (by volume) H_2SO_4, respectively.

Anion Solutions: Prepare the same concentrated solutions as those of the cation solutions of NO_2^-, NO_3^-, F^-, Cl^-, Br^-, I^-, ClO_3^-, BrO_3^-, IO_3^-, $Mo_7O_{24}^{6-}$, MnO_4^-, $Cr_2O_7^{2-}$, $S_2O_8^{2-}$, $Co(NO_2)_6^{3-}$, S^{2-}, $S_2O_3^{2-}$, CH_3COO^-, $C_2O_4^{2-}$, PO_4^{3-}, SCN^-, SO_3^{2-}, $Fe(CN)_6^{4-}$, $Fe(CN)_6^{3-}$, AsO_4^{3-}, $Sb_2O_7^{4-}$ and WO_4^{2-} by dissolving their potassium, sodium or ammonium salts in H_2O, and AsO_2^- by dissolving As_2O_3 in 1% NaOH.

---

1) This work was partly presented at "Symposium on Fluorimetric Technics" in the 7th International Congress of Clinical Chemistry (September 12, 1969, Geneva) and forms "Organic Analysis LXXVI." Part LXXV: Y. Ohkura, K. Matsumura, H. Hamada and T. Momose, *Bunseki Kagaku*, **20**, 480 (1971).
2) Location: *Katakasu, Fukuoka.*
3) T. Momose and Y. Ohkura, *Pharm. Bull.* (Japan), **4**, 209 (1956).
4) T. Momose and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), **7**, 31 (1959).
5) T. Momose and Y. Ohkura, *Talanta*, **3**, 191 (1959); B. Bourne, *Clin. Chem.*, **9**, 502 (1963); **10**, 1721 (1964).
6) P. Weber, I. Bornstein and R.J. Winzler, *Anal. Biochem.*, **14**, 100 (1966).
7) T. Momose and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), **6**, 412 (1958).
8) All reagents used are JIS Reagent Grade unless otherwise specified. The reagent solutions should be prepared and stored avoiding contamination of cotton dust.
9) Purchased from Ishizu Seiyaku Kabushiki-Kaisha, Osaka.

Reagents for the Determination of Hexose: 5-Hydroxy-1-tetralone Solution (2): Dissolve 150 mg of 5-hydroxy-1-tetralone in 500 ml of conc.H_2SO_4 and store in a refrigerator.

Cupric Sulfate Solution: Dissolve 200 mg of $CuSO_4 \cdot 5H_2O$ in 500 ml of H_2O.

Hexose Standard Solutions: Prepare first a stock solution by dissolving 100 mg of dried hexose (glucose, galactose, mannose, fructose, sucrose, maltose, lactose or raffinose) in sufficient 0.2% aqueous solution of benzoic acid to measure 1000 ml (100 μg/ml solution), and store in a refrigerator. This solution is stable for at least 3 months.

Prepare 0.5, 1,2,4,5 and 10 μg/ml solutions by diluting the stock solution with H_2O, store in a refrigerator and use within 2 days.

Instrument——Fluorescence intensity was measured by a Hitachi spectrofluorimeter, 203, equipped with Xenon lamp (Instrument 1), and fluorescence excitation and emission spectra by a Hitachi spectrofluorimeter, MPF-2A (Instrument 2), in a cell of 10×10 mm optical length.

Correction of excitation spectrum was performed by the Melhuish method[10] using rhodamine B solution (5 g in 1000 ml of ethylene glycol) placed in a triangle cell, and that of emission spectrum by the Lippert method[11] using 3-aminophthalimide.

Absorption spectrum was measured by a Shimadzu self-recording spectrophotometer, SV-50A, in a cell of 10 mm optical length.

Procedures[12] for the Study on Effects of Ions——Effect on the Fluorescence Reaction (Procedure(1)): To 0.25 ml of 5.74×10^{-5}M glucose solution placed in a glass-stoppered test-tube, and 0.25 ml of ion solution (5.74×10^{-5}—5.74×10^{-2}M) and 2.0 ml of 5-hydroxy-1-tetralone solution(1) under ice–water cooling, mix and then heat in a boiling water-bath at 100° for 30 min. After cooling in an ice–water bath, add 2.5 ml of H_2O. At the same time, prepare a reagent blank and a fluorescence standard solution by treating 0.5 ml of H_2O and 0.5 ml of 2.87×10^{-5}M glucose solution, respectively, in the same way as described above. Measure the fluorescence intensity at room temperature (about 25°) at an emission wave length of 530 mμ and an excitation one of 470 mμ, setting the intensities of reagent blank and fluorescence standard solution to zero and an arbitrary unit, respectively.

Effect of Ions on the Fluorescing Reaction Mixture (Procedure(2)): Treat 0.5 ml of 2.87×10^{-5}M glucose solution with 2.0 ml of 5-hydroxy-1-tetralone solution (1) to develop the fluorescence as described above. To the resulting reaction mixture, add 2.0 ml of H_2O and 0.5 ml of ion solution (2.87×10^{-5}—2.87×10^{-2}M) under ice–water cooling. Prepare a reagent blank by treating 0.5 ml of H_2O in the same manner and then diluting with 2.5 ml of H_2O, and prepare a fluorescence standard solution by diluting the reaction mixture with 2.5 ml of H_2O. Measure the fluorescence intensities within 20 min after adding ion solution, as in the procedure (1).

Method for the Determination of Hexose——Procedure (3): To 0.25 ml of test solution, add successively 0.25 ml of $CuSO_4$ solution and 2.0 ml of 5-hydroxy-1-tetralone solution (2) under ice–water cooling. Treat the mixture to develop the fluorescence and dilute with H_2O, as in the procedure (1). Prepare a reagent blank by treating 0.25 ml of H_2O in the same manner. Measure the fluorescence intensity within 3 hr, as in the procedure (1).

Calibration Curve: Treat three 0.25 ml aliquots of individual hexose standard solutions and of H_2O for blanks as in the procedure, and measure the fluorescence intensities setting that of the pooled blank to zero.

Result and Discussion

Effects of Ions on the Fluorescence Reaction and the Fluorescing Reaction Mixture

The effects were examined using glucose as a representative hexose. The fluorescence excitation and emission spectra of the reaction mixture obtained by treating glucose under the procedure (1) in the absence of ions tested are shown in Fig. 1. An apparent excitation maximum of 495 mμ (Fig. 1,c) gives the maximum intensity, but is so close to the apparent emission maximum, 530 mμ (Fig. 1,d), that the scatter of exciting light may slightly exert influence on the measured fluorescence intensity with the instrument used, and so another apparent excitation maximum, 470 mμ, was used in this study.

Effects on the Fluorescence Reaction——The fluorescence intensity increases with increasing concentration of ferric, cupric, cuprous and heptamolybdate ions in the reaction

10) W.H. Melhuish, *J. Opt. Soc, Amer.*, **52**, 1256 (1952).
11) F. Lippert, W. Naegele, I. Seibold-Blankenstein, U. Steigner and W. Vass, *Z. Anal. Chem.*, **170**, 1 (1959).
12) The reaction conditions used here were already proved to give the maximum fluorescence intensity in the absence of foreign ion, and the intensity had a linear correlation with the concentration of glucose below 6 μg/ml.

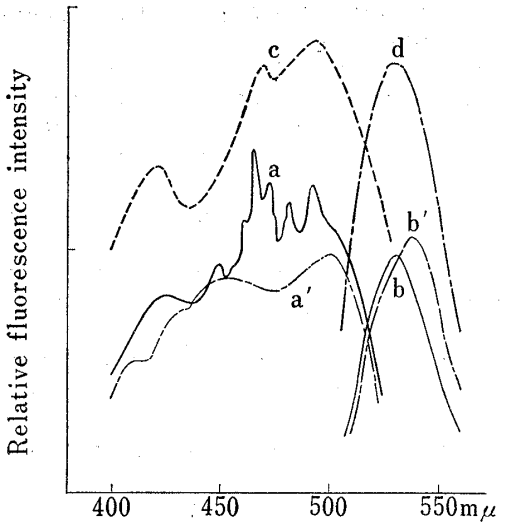

Fig. 1. Excitation and Emission Spectra of the Reaction Mixture

Glucose solutions (5.74×10^{-5}M) were treated by the procedure (1) without foreign ion. a and c, and b and d were the excitation and emission spectra, respectively. a′ and b′ were the corresponding corrected spectra. a and b, and c and d were measured by Instrument 2 and 1, respectively.

as shown in Fig. 2, without changing the shapes and maxima of the excitation and emission spectra. Those ions might be considered to behave as oxidizing agents to accelerate the reaction under the conditions of procedure, because an oxidation process might be required in the reaction to form a fluorescent compound, benzonaphthenedione.[7]

The above assumption was suggested from the following facts: A larger adsorption band of the fluorescent compound was observed in the chromatographic separation of the benzene extract of reaction mixture on alumina column[7] when the reaction was carried out in the presence of cupric sulfate. The fluorescence intensity of isolated fluorescent compound dissolved in 40% (by volume) sulfuric acid did not change on adding ferric, cupric or heptamolybdate ion.

Cuprous ion is easily oxidized in hot sulfuric acid, and so it might act as cupric ion in the reaction.

From the above results, the intensifying effect of strong oxidative ions might be expected to occur on the reaction within a limited concentration. In fact, chlorate, bromate, iodate, nitrite, nitrate, dichromate, persulfate and cobaltinitrite ions intensify the fluorescence in a lower molar ratio of each ion to glucose as shown in Fig. 2, without changing the excitation and emission spectra. With more increased molar ratio of the ions, they decompose glucose, 5-hydroxy-1-tetralone and the fluorescent compound under the conditions of the procedure,[13] tint the reaction mixtures, giving the inner filter effect,[14] and so decrease the fluorescence considerably. In these cases, however, the excitation and emission spectra of the reaction mixtures were observed almost unchanged, in spite of the inner filter effect present, except those of the reaction mixture with cobaltinitrite ion. This phenomenon might be caused by the almost flat absorption spectra of reaction mixtures in the excitation and emission spectral regions (Fig. 3). In the reaction mixture with cobaltinitrite ion, the emission spectrum was observed to be almost identical with the original one, but the excitation spectrum became undiscernible in a shorter wave length region below about 450 mμ, due to the intense light absorption (Fig. 3).

Permanganate ion, a strong oxidizing agent in hot sulfuric acid, did not show the sensitizing effect even in a low concentration (Fig. 2).

Thiosulfate ion strongly hinders the reaction by liberating colloidal sulfur under the conditions employed (Fig. 2), which does not precipitate by centrifuging and might interfere with the reaction in the oxidation process,[15] and scatters the exciting light and fluorescence. Those effects might make the excitation and emission spectra undiscernible.

13) This might be clear from the following experimental facts: The fluorescence intensity was greatly reduced when the fluorescent compound[7] was heated in sulfuric acid in the presence of potassium chlorate. A decreased fluorescence intensity was observed when glucose solution was treated as in the procedure (1) with 5-hydroxy-1-tetralone solution which had already been heated in the presence of excessive potassium chlorate. A low fluorescence intensity was obtained with glucose which was preheated in 80% (by volume) sulfuric acid, and no fluorescence was observed with glucose preheated in the presence of potassium chlorate.

14) C.A. Parker and W.T. Rees, *Analyst*, **87**, 83 (1962).

15) A lower fluorescence intensity was also observed when the reaction was carried out by dispersing sulfur prepared from sodium thiosulfate and sulfuric acid.

Sulfide ion might be partly oxidized to colloidal sulfur under the reaction conditions to give a slightly turbid reaction mixture, and minimizes the fluorescence as in thiosulfate ion (Fig. 2).

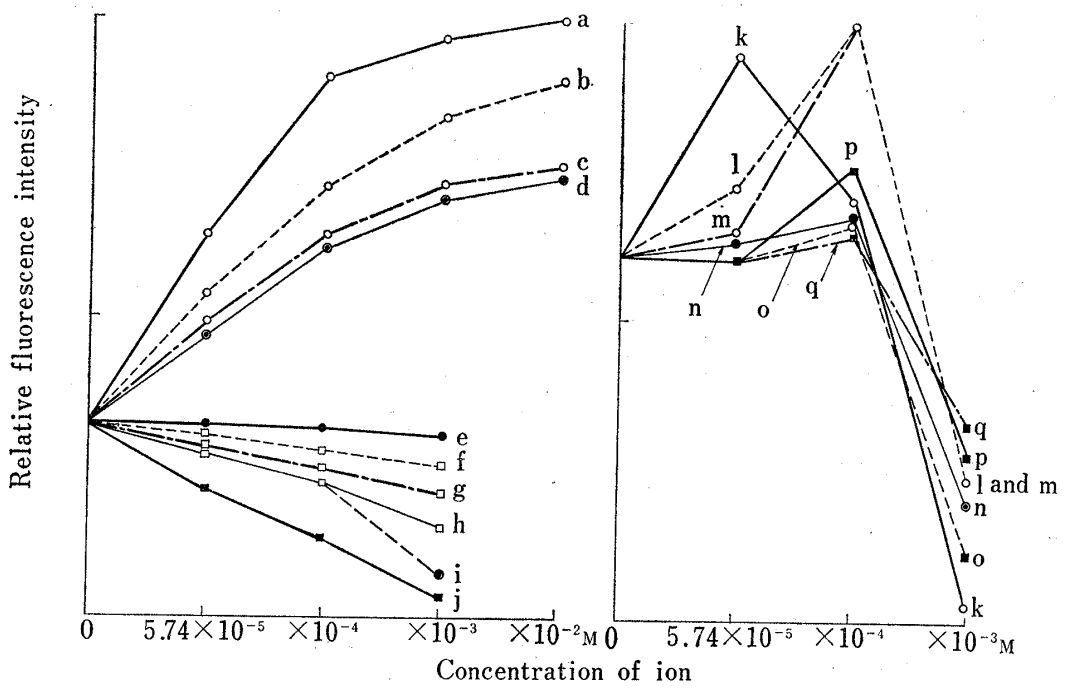

Fig. 2. Effect of Concentration of Ion on the Fluorescence Reaction

Glucose solutions (5.74×10^{-5}M) were treated as in the procedure (1) with the following ion solutions, a, Fe^{3+}; b, $Mo_7O_{24}^{6-}$; c, Cu^{2+}; d, Cu^{+}; e, I^{-}; f, Cl^{-}; g, Br^{-}; h, MnO_4^{-}; i, S^{2-}; j, $S_2O_3^{2-}$; k, $Co(NO_2)_6^{3-}$; l, NO_2^{-}; m, NO_3^{-}; n, $Cr_2O_7^{2-}$; o, ClO_3^{-} and BrO_3^{-}; p, IO_3^{-}; q, $S_2O_8^{2-}$. Each plot was the mean value of 4 determinations.

Chloride, bromide and iodide ions weakly repressed the fluorescence development (Fig. 2), and the excitation and emission spectra of reaction mixtures were identical with the original ones. This fact might be explained by the intramolecular heavy-atom effect of halogenated fluorescent compound and/or the external heavy-atom effect[16] of halogenated 5-hydroxy-1-tetralones which might be produced in the reaction.[17]

Other ions tested which are listed in the experimental part did not affect the fluorescence development even in the molar ratio of ion to glucose, 100: 1.

Effect on the Fluorescing Reaction Mixture——Cations tested did not affect the fluorescence even in the concentration of 2.87×10^{-3}M.

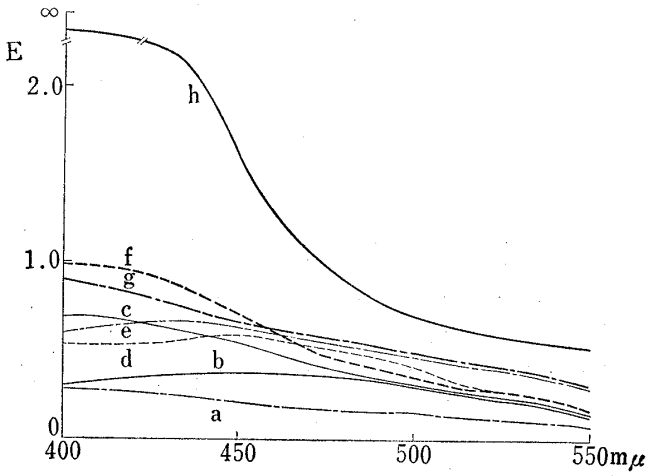

Fig. 3. Absorption Spectra of the Reaction Mixtures obtained by Treating Glucose with Ions

Glucose solutions (5.74×10^{-5}M) were treated as in the procedure (1) with the following ion solutions, a, $S_2O_3^{2-}$; b, $Cr_2O_7^{2-}$; c, IO_3^{-}; d, ClO_3^{-}; e, BrO_3^{-}; f, NO_2^{-}; g, NO_3^{-}; h, $Co(NO_2)_6^{3-}$.

16) E.L. Wehry and L.B. Rogers, "Fluorescence and Phosphorescence Analysis," Chapter III ed. by D.M. Hercules, Interscience Publishers, New York, London and Sydney, 1966.
17) This may be clarified by measuring the fluorescence and/or phosphorescence of the compound produced. The study on this subject is now going on.

Chlorate, bromate, iodate, persulfate and cobaltinitrite ions quench the fluorescence with increasing concentrations of the ions (Fig. 4). The emission spectra of the resulted solutions with chlorate, iodate and cobaltinitrite ions are identical to the original one in their shapes and maxima. However, their excitation spectra slightly change in a shorter wave length region below about 470 mμ to have lower intensities when compared with the original one. This phenomenon was more distinctly observed especially in the case of cobaltinitrite ion. Therefore, the quenching effect of those ions might partly rise in the inner filter effect due to the light absorptions of the resulted solutions mainly in the excitation spectral region (Fig. 5). Bromate and persulfate ions give only weak absorbance to the reaction mixture. Therefore, the quenching actions might be caused by unknown factors other than the inner filter effect.

On the other hand, dichromate ion showed unexpectedly the sensitizing effect on the fluorescence without changing the excitation and emission spectra, and the maximum effect was observed at the concentration of 2.87×10^{-4}M. The fluorescence intensity decreased on standing the resulted solution for a long time (Fig. 4), probably due to the decomposition of the fluorescent compound. The more concentrated ion decreased the fluorescence, partly owing to the intense light absorption (Fig. 5).

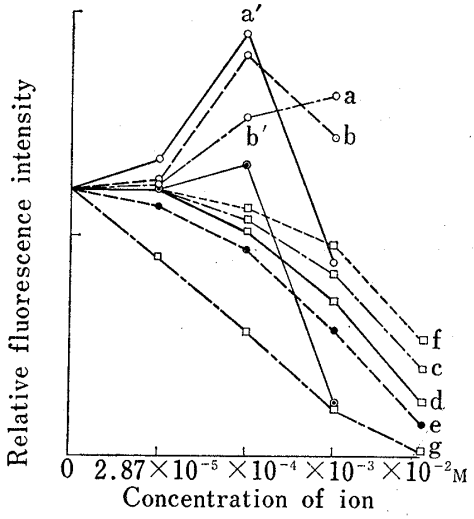

Fig. 4. Effect of the Concentration of Ion on the Fluorescing Reaction Mixture

The reaction mixture obtained by treating glucose solution (2.87×10^{-5}M) was diluted as in the procedure (2) with water and the following ion solutions, a, NO_3^-; b, $Cr_2O_7^{2-}$; c, ClO_3^-; d, BrO_3^-; e, IO_3^-; f, $S_2O_8^{2-}$; g, $Co(NO_2)_6^{3-}$. a′ and b′ were the results obtained by standing the mixtures diluted with a and b, respectively, at 25° for 19 hr. Each plot was the mean value of 4 determinations.

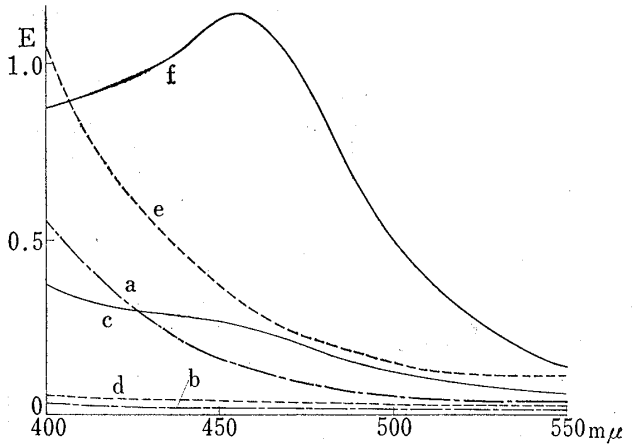

Fig. 5. Absorption Spectra of the Reaction Mixtures in the Presence of Ions

The reaction mixture obtained by treating glucose solution (2.87×10^{-5}M) was diluted as in the procedure (2) with water and the following ion solutions, a, ClO_3^-; b, BrO_3^-; c, IO_3^-; d, $S_2O_8^{2-}$; e, $Co(NO_2)_6^{3-}$; f, $Cr_2O_7^{2-}$. ion concentrations: a—e, 2.87×10^{-2}M; f, 2.87×10^{-3}M

It is of interest to note that nitrite ion sensitizes the fluorescence (Fig. 4) and the intensity increases reversibly with increasing temperature even in a range of 20 to 30°. The measured excitation and emission spectra were identical with those of the original reaction mixture. These behaviors were the reverse to those observed in common fluorescent compounds.[18] Furthermore, the mode of sensitizing effect changed on standing the resulted solution as shown in Fig. 4, suggesting that a part of benzonaphthenedione was converted to a

18) D.W. Ellis, "Fluorescence and Phosphorescence Analysis," Chapter II, ed. by D.M. Hercules, Interscience Publishers, New York, London and Sydney, 1965; E.J. Bowen and S. Sahu, *J. Phys. Chem.*, **63**, 4 (1959).

more strongly fluorescent compound.[17] The cause for the sensitizing effect of nitrite and dichromate ions remained unsolved.

Thiosulfate ion liberates colloidal sulfur which coheres and easily precipitates by centrifuging. Therefore, it does not give any effect on the fluorescence.

Other anions tested did not influence the fluorescence in the concentrations examined.

Determination of Hexose

In order to increase the fluorescence intensity in the reaction of hexose with 5-hydroxy-1-tetralone, cupric, ferric and heptamolybdate ions are now available as described before. Ferric and heptamolybdate ions are superior to cupric ion in the sensitizing effect on the reaction, but their salts, ferric sulfate, ferric ammonium sulfate and ammonium heptamolybdate are hardly supplied as pure reagents of constant quality for the use in the present purpose. Thus, cupric sulfate pentahydrate was selected as the optimum reagent. The reaction conditions for the determination of hexose were investigated by using glucose.

The concentration of 5-hydroxy-1-tetralone in the reaction mixture affects the fluorescence development, giving a constant intensity at a concentration higher than 0.025%, and the prescribed concentration, 0.03%, was selected for convenience. The concentration of sulfuric acid, which is used for dissolving 5-hydroxy-1-tetralone, also affects the fluorescence development. Concentrated sulfuric acid(96%) was employed to give the maximum intensity and became about 77% (by volume) in the reaction.

Cupric sulfate is sparingly soluble in the sulfuric acid, therefore, it could not be added to 5-hydroxy-1-tetralone solution for convenience in the procedure. The fluorescence intensity does not change over the concentration range of cupric sulfate 0.04—0.08% (as pentahydrate) with glucose concentration below 6 μg/ml, and 0.04% was selected for the optimum (Fig. 6). The fluorescence increases by about 2.2 times as much in this concentration of cupric sulfate.

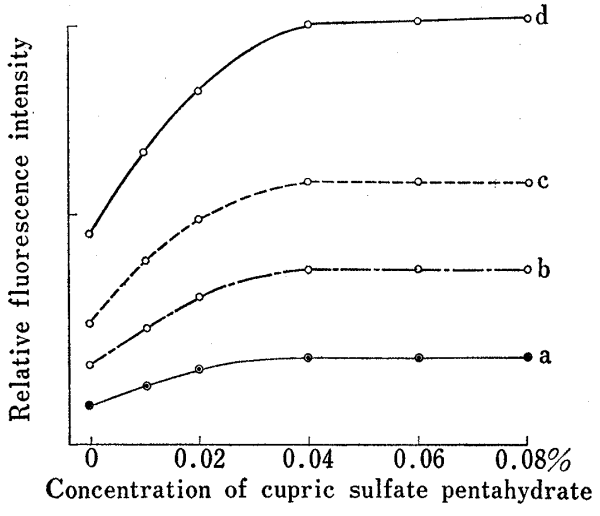

Fig. 6. Effect of the Concentration of Cupric Sulfate on the Fluorescence Development

Cupric sulfate solutions in various concentrations were treated by the procedure (3) with glucose solutions, a, 2; b, 4; c, 6; d, 10 μg/ml. Each plot was the mean value of 4 determinations.

Table I. Fluorescence Intensities of Hexoses obtained by the Present Method[a]

	Hexose	Fluorescence intensity
Monosaccharide	glucose	100
	fructose	99
	galactose	77
	mannose	69
Diasaccharide	sucrose	100
	maltose	99
	lactose	91
Trisacchride	raffinose	89

a) 0.25 ml of hexose solutions (2 μg/ml) were treated by the prescribed procedure(3), and the fluorescence intensity of glucose was taken as 100.

The reaction time influences the fluorescence development. The intensity reaches the maximum after heating the reaction mixture at 100° for 30 min, and then decreases when the heating is continued for longer time.

The concentration of sulfuric acid in the final reaction mixture affects the measured value of fluorescence and 38% (by volume) gives the maximum.

The fluorescence developed under the prescribed conditions is stable for at least 3 hr in daylight and 2 days in the dark.

Other hexoses give the fluorescence intensities different from that of glucose under the conditions of the present method as shown in Table I. The value of fluorescence intensity of di- or trisaccharide almost coincides with the additive values of the individual sugars which compose the di- or trisaccharide.

The calibration curve for each individual hexose is a straight line in a concentration of sugar below 5 μg/ml (actual amount of hexose up to 1.25 μg).

Pentoses such as xylose, ribose, arabinose and rhamnose give neither fluorescence nor interference with the fluorescence produced by 2 μg/ml solution of glucose, even at a concentration of 20 μg/ml.

Glucuronolactone, galacturonic acid and ascorbic acid give no interference at a concentration of 40 μg/ml. Cellulose fluoresces, and therefore, a contamination of cotton dust may cause a large error and a high value of blank. The fluorescence intensity of blank was usually measured in our laboratory as 15—20% of that produced by 4 μg/ml solution of glucose.

The precision of the method was studied with respect to repeatability, which was obtained by performing 40 analyses separately on 2.0 and 4.0 μg/ml solutions of glucose at the same time. The standard deviations were 0.044 and 0.064 μg/ml (coefficient of variation, 2.2 and 1.9%), respectively.

In the measurement of fluorescence intensty, a mercury line of 436 mμ is available for the excitation when a mercury lamp is equipped with the fluorimeter.

The improved reaction is selective and sensitive for hexose, and the excitation and emission maxima in the spectra are in a long wave length region, where usual organic substances hardly fluoresce. This method may be useful for the microdetermination of hexose in biological samples.

Acknowledgement This work is partially supported by a Grant-in-Aid for Scientific Research provided by the Ministry of Education to which we are indebted. We wish to thank Mr. K. Zaitsu for his technical assistance, Miss Y. Sodea for absorption spectral measurements, and the staff of Application Laboratory, Naka Works, Hitachi, Ltd. for the help of fluorescence spectral measurements.