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An Improved Method for the Millon Reaction

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An improved method for the Millon reaction using cupric ion together with mercuric ion is presented for the determination of tyrosine. Blue-violet coloration in the present method is based on the mercuration and nitrosation of tyrosine, according to the conventional Millon reaction, followed by the chelate formation of nitrosotyrosine with cupric ion. This method is more sensitive than the conventional reaction, and the colored substance is more stable than that formed by the conventional method.

The colored substance was identified as bis(4-methyl-2-nitrosophenolato) copper(II) by isolating it from the reaction mixture of 2-chloromercuri-4-methylphenol (an intermediate of the conventional Millon reaction of *p*-cresol), hydrogen nitrite and cupric ion. Millon-positive substances in urine were successfully determined by the present method by pretreating the sample with mercuric acetate.

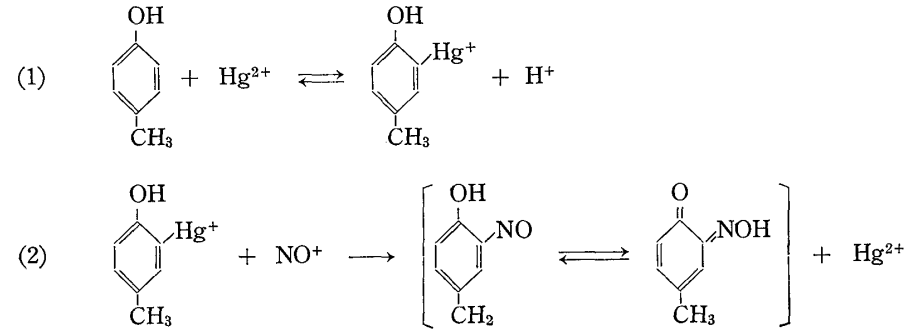
Keywords——Millon reaction; improved Millon reaction; tyrosine; *p*-cresol; bis(4-methyl-2-nitrosophenolato) mercury(II); bis(4-methyl-2-nitrosophenolato) copper(II); tyrosinosis

It was reported that the Millon reaction of p-cresol under the conditions used for quantitative analysis proceeded through the reaction steps (1), (2), and (3B) in Chart 1 to afford bis-(4-methyl-2-nitrosophenolato) mercury(II) as the main coloring substance.[2]

As the source of mercuric ion for the Millon reaction, mercuric sulfate or mercuric nitrate has been used because of its complete ionization. However, the high hydrogen ion concentration required to dissolve these salts in water prevents the chelate formation in step (3) in Chart 1, and thus decreases the sensitivity of this color reaction. Therefore, if reactions (1) and (2) are completed with a small amount of mercuric ion, and the reaction (3B) proceeds upon replacing mercuric ion with another metal ion having a higher solubility in water and good chelate-forming ability, the acidity of the reaction mixture can be decreased to result in an enhancement of the sensitivity of the Millon reaction.

When tyrosine was mercurated with a reduced concentration of mercuric sulfate followed by reaction with sodium nitrite in the presence of various heavy metal ions, only cupric ion gave a clear, blue-violet solution and other ions precipitated insoluble salts or colored substances.

In the present paper, an improved Millon reaction of tyrosine involving the use of mercuric ion together with cupric ion is described and a reaction mechanism through steps (1), (2), and (4) in Chart 1 is proposed for the case of *p*-cresol.



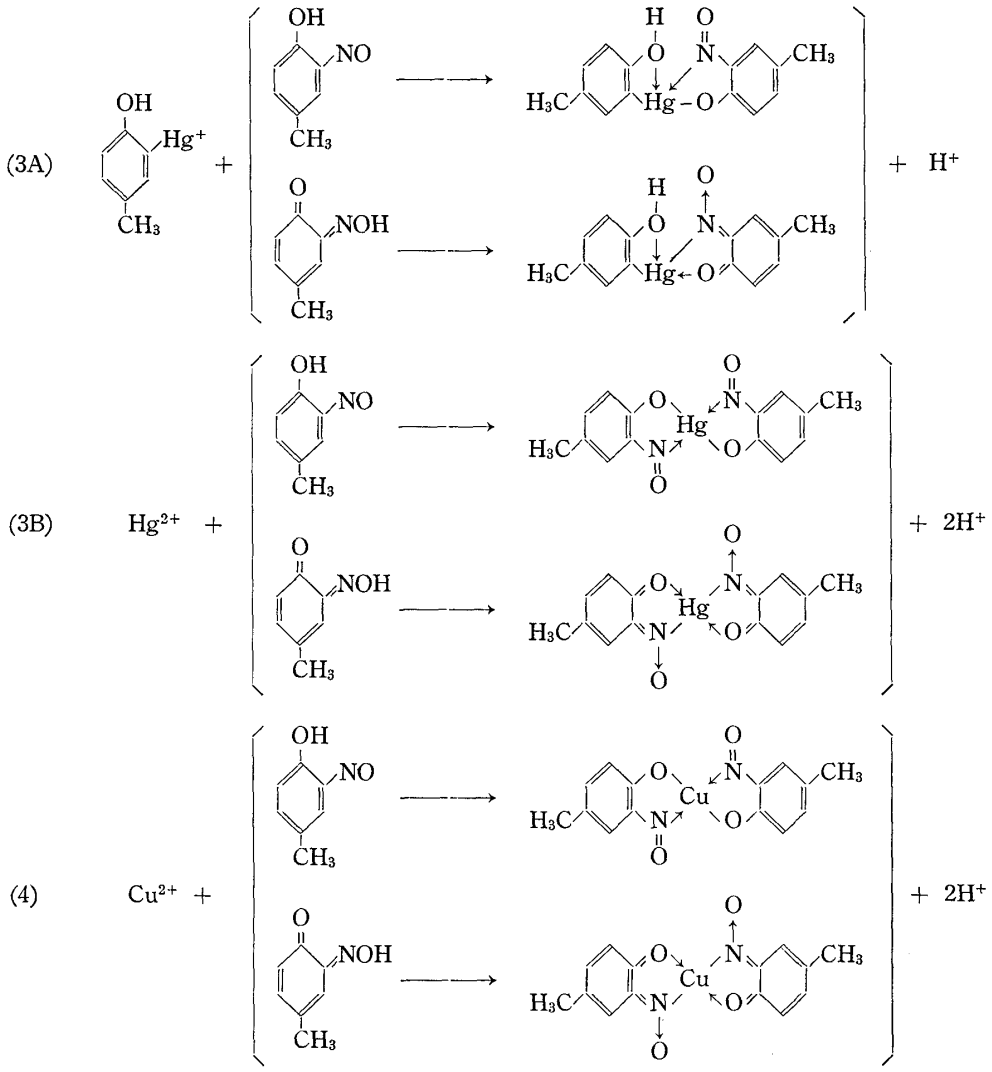


Chart 1

Results and Discussion

Conditions of Mercuration

Among the three steps of the modified Millon reaction in Chart 1, steps (2) and (4) proceed rapidly. Step (1) was completed in about 40 min in a solution of 1×10^{-2} M mercuric sulfate in 2.25 N sulfuric acid (Fig. 1). The optimal concentration of mercuric sulfate at the mercuration stage was higher than 1×10^{-2} M.

As shown in Fig. 2, the absorbance decreased with increase in concentration of sulfuric acid and with decrease in concentration of mercuric sulfate at the mercuration stage, and even when the color development was carried out at a final, constant pH of 4, the absorbance decreased with increasing acid concentration at the mercuration stages (Fig. 3).

The above observations indicate that step (1) in Chart 1 is an equilibrium reaction and is the rate-determining step. Therefore, mercuration should be carried out at as high a concentration of mercuric sulfate and as low a concentration of sulfuric acid as possible. Practically, the concentration of sulfuric acid used in the optimal concentration of mercuric sulfate is the limiting concentration required to dissolve the slightly soluble mercurated intermediate of tyrosine.

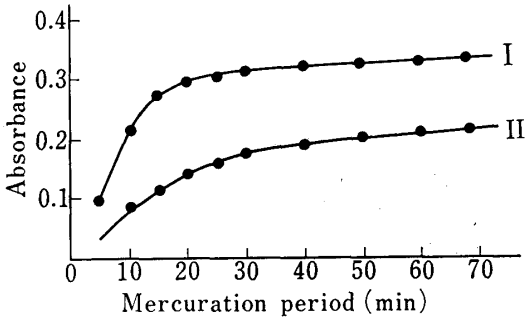


Fig. 1. Relation between the Mercuration Period and the Absorbance at 524 nm

Tyrosine (final): I, II, 8×10^{-5} M.
Mercuric sulfate (at the mercuration stage): I, 1×10^{-2}; II, 2×10^{-3} M.
Sulfuric acid (at the mercuration stage): I, II, 0.25 N.
Cupric sulfate: I, II, 2×10^{-1} M.
Sodium nitrite: I, II, 2×10^{-2} M.

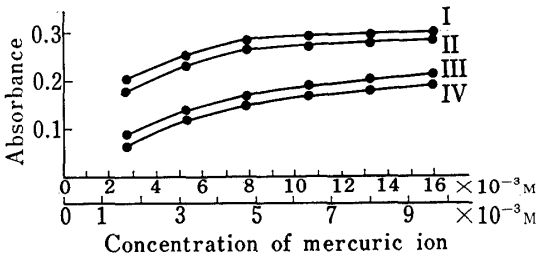


Fig. 2. Effect of Acid Concentration on the Absorbance

Upper scale: concentration at the mercuration stage.
Lower scale: final concentration.
Concentration (N) of H_2SO_4 at the mercuration stage: I, 0.29; II, 0.43; III, 1.30; IV, 1.73.
Concentration (N) of H_2SO_4 in the final reaction mixture: I, 0.17; II, 0.26; III, 0.78; IV, 1.04.

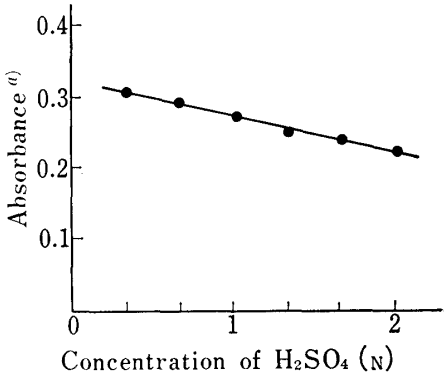


Fig. 3. Effect of Acid Concentration at the Mercuration Stage on the Absorbance

a) Absorbance is the optical density at pH 4.05 ± 0.05 of the final reaction mixture.

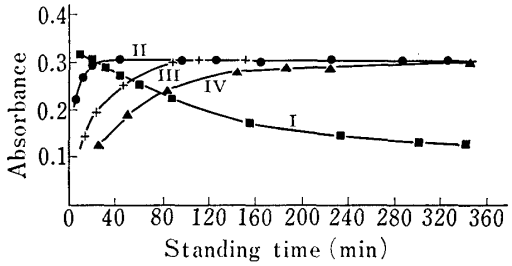


Fig. 4. Effect of Sodium Nitrite Concentration on the Coloration

Final concentration of $NaNO_2$: I, 4.0×10^{-2} M; II, 4.0×10^{-3} M; III, 8.0×10^{-4} M; IV, 4.0×10^{-4} M.

Optimal Concentration of Cupric Sulfate and Relation between pH and Sensitivity at the Color Development

Almost constant absorbance was obtained at cupric ion concentrations of more than 2×10^{-1} M. Even if the pH of the final reaction mixture was increased to 4.5 with triethanolamine, the absorbance was not much increased at such a high concentration of cupric sulfate.

Optimal Concentration of Sodium Nitrite and Stability of Coloring Substance

The higher the concentration of sodium nitrite, the faster the intensity reached the maximum, although the shorter the duration of color. The optimal concentration of nitrite was found to be 4×10^{-3} M, and the maximum intensity was constant for at least six hours, as shown in Fig. 4.

Sensitivity and Working Curve

The most appropriate conditions for determination of tyrosine established above were also applicable to *p*-cresol. As shown in Table I, the present method has higher sensitivity than Snell's method.[3]

TABLE I. Comparison of the Present Method with Snell's Method (Reaction Conditions and Sensitivity)

	Concentration of Hg^{2+}(M)	Concentration of H_2SO_4(N)	Concentration of Cu^{2+}(M)	Concentration of NO_2^-(M)	Mercuration time (min)	Standing time (min)	ε
Improved method	1.4×10^{-2} a)	3.6×10^{-1} a)	2.0×10^{-1}	4.0×10^{-3}	30	40	*p*-Cresol 3710
	8.0×10^{-3} b)	2.0×10^{-1} b)					Tyrosine 4860
Snell's method	1.5×10^{-1} a)	2.3 a)	—	4.1×10^{-3}	10	10	*p*-Cresol 2470
	1.4×10^{-1} b)	1.9 b)					Tyrosine 2770

a) Concentration at the mercuration stage.
b) Final concentration.
Final concentrations of the samples were as follows.
Improved method: *p*-cresol, 8×10^{-2} mM; tyrosine, 6.5×10^{-2} mM.
Snell's method: *p*-cresol, 12.2×10^{-2} mM; tyrosine, 17.6×10^{-2} mM.

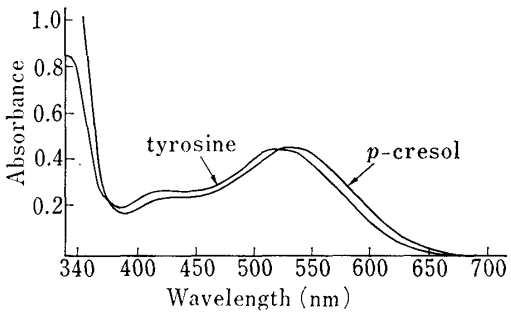


Fig. 5. Absorption Spectra of the Improved Millon Reaction Mixture

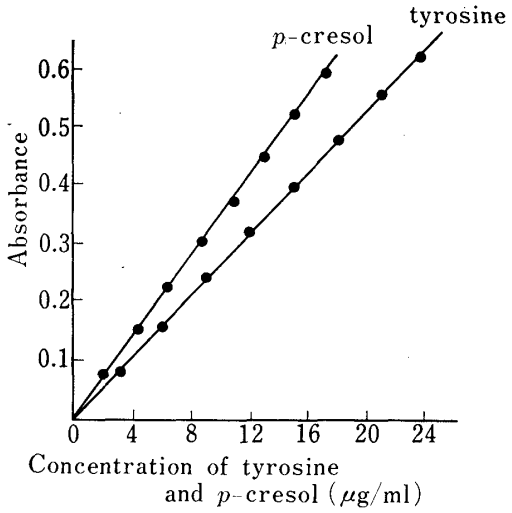


Fig. 6. Working Curves for Tyrosine and *p*-Cresol Obtained by the Present Method

The absorption spectra of the reaction mixtures of tyrosine and *p*-cresol (Fig. 5) show absorption maxima at 524 nm and 530 nm, respectively.

The working curves for tyrosine and *p*-cresol show good linearity. The regression equations are $Y=0.026X+0.003$ and $Y=0.034X-0.004$, respectively, as illustrated in Fig. 6.

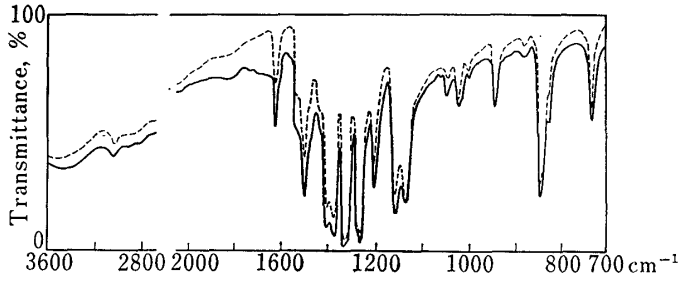


Fig. 7. Infrared Spectra of Bis (4-methyl-2-nitroso-phenolato) Copper(II)

——: synthesized from 2-chloromercuri-4-methylphenol, cupirc sulfate and sodium nitrite.
----: synthesized from p-cresol, cupric sulfate and sodium nitrite by Cronheim's method.

Reaction Mechanism

Since the initial procedure of the present method is the same as that of the conventional Millon reaction using Hopkins-Cole reagent,[4] the reaction should proceed through the mercuration of phenols.

2-Mercuri-4-methylphenol cation, the reaction product of *p*-cresol with mercuric sulfate, reacted with hydrogen nitrite in the presence of cupric ion to afford dark blue needles, which were shown to be identical with bis(4-methyl-2-nitrosophenolato) copper(II) (MNP-Cu) synthesized by Cronheim's method[5] by infrared spectroscopy (Fig. 7). Furthermore, the absorption spectrum of the color reaction mixture was identical with that of an acidic solution of MNP-Cu. Consequently, the coloring substance in this improved Millon reaction is MNP-Cu produced through the reactions (1), (2), and (4) in Chart 1.

Determination of the Millon-Positive Substances in Human Urine

The present method showed poor sensitivity for tyrosine in urine, like the conventional Millon reaction. The reason was presumed to be reduction of the mercuric ion concentration either by binding and precipitating with urine components or by conversion to the scarcely dissociating mercuric chloride by the large amount of chloride ion present in urine. Actually, mercuric sulfate produced a large quantity of yellowish precipitates when added to a urine sample, and sodium chloride in a tyrosine solution also inhibited color development remarkably (Fig. 8).

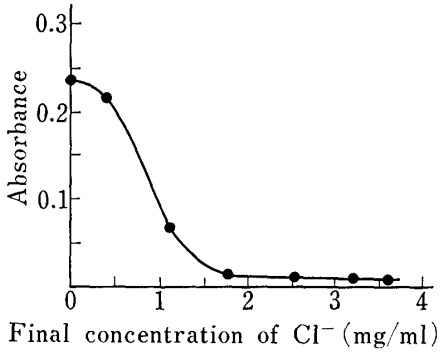


Fig. 8. Effect of Chloride Ion on the Absorbance

Final conceetration of mercuric sulfate: 2×10^{-2}M

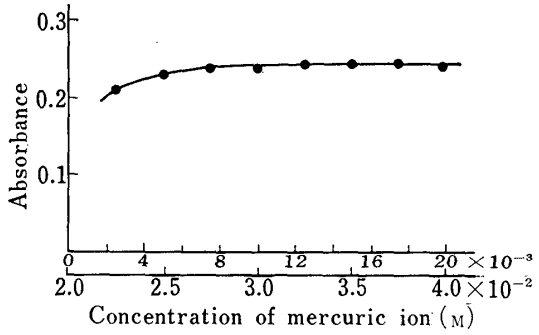


Fig. 9. Effect of Mercuric Acetate added to Tyrosine Solution Containing Sodium Chloride

Upper scale: final concentration of mercuric acetate.
Lower scale: concentration of total mercuric ion.

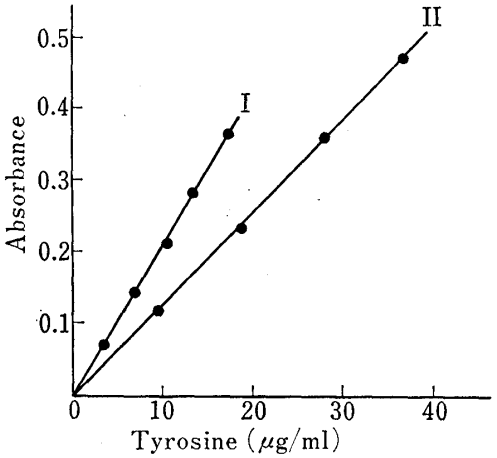


Fig. 10. Working Curves for Tyrosine in Urine

I: Present method.
II: Conventional method.

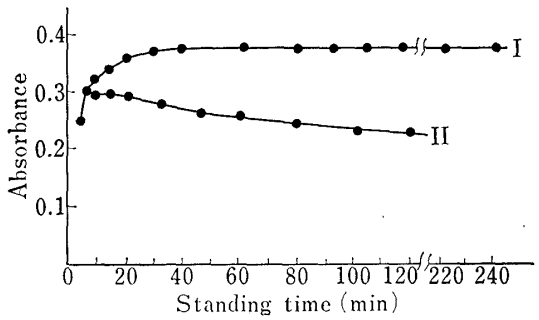


Fig. 11. Effect of Standing Time on the Absorbance with Urine Samples

I: Present method.
II: Conventional method.

Table II. Analytical Results obtained by the Present Method for Millon-Positive Substances in Human Urine and Recovery of Tyrosine

Sample No.	Determined Millon-positive substances (μg as tyrosine/ml urine)	Tyrosine (μg) Added	Tyrosine (μg) Recovered	Recovery (%)
1	239	183	154	84.2
2	71	183	156	85.2
3	434	183	137	74.9
4	162	147	118	80.2
5	150	147	113	76.9
6	241	147	130	88.4
7	79	147	129	87.8
8	119	147	128	87.1
9	165	147	131	89.1
10	166	147	127	86.4
			Mean	84.0

Pretreatment of the sample containing chloride ion with a sufficient amount of mercuric acetate gave satisfactory color development in the present Millon reaction (Fig. 9), with a sensitivity twice that of the screening test of tyrosinosis[6] (Fig. 10).

Furthermore, the color development was rather stable compared to that of the conventional method[6] (Fig. 11).

Analytical results for normal human urines and recovery of tyrosine added to the urine samples are listed in Table II.

Experimental[7]

Determination of the Mercuration Period——Aliquots of the reaction mixture (about 11 ml) of 4×10^{-4} M tyrosine (100.0 ml) and 2×10^{-2} M or 4×10^{-3} M $HgSO_4$ in 0.5 N H_2SO_4 (100.0 ml) were taken every 5 or 10 min during heat treatment in a boiling water bath and the samples obtained were cooled immediately in running water for 5 min. To the samples (10.0 ml) obtained above were added 0.5 M $CuSO_4$ (10.0 ml) and 0.1 M $NaNO_2$ (5.0 ml). The mixtures were allowed to stand for 60 min at room temperature and then their optical densities were determined at 524 nm against a blank test solution prepared similarly, except that H_2O was substituted for the tyrosine solution (Fig. 1).

Effect of Mercuric Sulfate and Sulfuric Acid Concentrations at the Mercuration Stage on the Optical Density——Mixtures of 1×10^{-3} M tyrosine (2.0 ml) and $HgSO_4$ solutions in H_2SO_4 (13.0 ml) at various concentrations were heated in a boiling water bath for 40 min then cooled immediately in running water. Next, 1 M $CuSO_4$ (5.0 ml) and 2×10^{-2} M $NaNO_2$ (5.0 ml) were added to each reaction mixture, and the mixtures were allowed to stand for 60 min at room temperature. Then, their optical densities were measured at 524 nm against the reagent solution (Fig. 2).

On the other hand, mixtures of 1×10^{-3} M tyrosine (5.0 ml) and 2×10^{-2} M $HgSO_4$ in various concentrations of H_2SO_4 (10.0 ml) were heated in a boiling water bath for 40 min and cooled in running water. Next, 1 M $CuSO_4$ (10.0 ml), 2×10^{-2} M $NaNO_2$ (10.0 ml) and 1 M triethanolamine (5.0 ml) were added to the mixtures. The pH of each solution was adjusted to 4.05 ± 0.05 with 10% NaOH solution, then H_2O was added up to 50.0 ml and the solution was processed by the procedure described above (Fig. 3).

Effect of the Sodium Nitrite Concentration on the Coloration——A mixture of 5×10^{-4} M tyrosine (20.0 ml) and 2×10^{-2} M $HgSO_4$ (50.0 ml) was heated in a boiling water bath for 40 min and cooled in running water. Next, 0.5 M $CuSO_4$ (50.0 ml) and $NaNO_2$ solution of various concentrations (5.0 ml) were added to the above reaction mixture, and the optical density of the colored solution at 524 nm was measured every 5 or 10 min during the standing time against the reagent solution (Fig. 4).

Bis(4-methyl-2-nitrosophenolato) Copper(II) (MNP-Cu) from 2-Chloromercuri-4-methylphenol (MMP)——A solution of $CuSO_4\cdot5H_2O$ (0.8 g) and $NaNO_2$ (1.4 g) in H_2O (10 ml) and 0.2 N H_2SO_4 (10 ml) was added to a solution of MMP (2.3 g) in EtOH (50 ml), and the mixture was stirred for about 5 h at room temperature. The precipitate was collected, washed with H_2O and dried to afford a dark blue powder (1.2 g). Recrystallization from EtOH gave dark blue-violet needles, which were identical (by infrared spectroscopy) with MNP-Cu synthesized by Cronheim's method[5] (Fig. 7).

Effect of Pretreatment of Tyrosine Solution Containing NaCl with $HgAc_2$ on the Optical Desity——Mixtures of 8×10^{-4} M tyrosine (2.0 ml), 2.5 M NaCl (0.2 ml), aqueous solution of $HgAc_2$ of various concentrations (1.8 ml) and 5×10^{-2} M $HgSO_4$ in 1 N H_2SO_4 (10.0 ml) were heated in a boiling water bath for 40 min, then cooled in running water. Next, 0.5 M $CuSO_4$ (10.0 ml) and 0.1 M $NaNO_2$ (1.0 ml) were added to each reaction mixture. The mixtures were allowed to stand for 60 min at room temperature and the optical densities were measured at 524 nm against the reagent blank solution prepared similarly (Fig. 9).

Working Curves for Tyrosine by the Present Method and the Conventional One——1) Present Method: To an aqueous solution of tyrosine of various concentrations (3.0 ml) was added 0.2 M $HgAc_2$ (1.0 ml), and the mixture was allowed to stand for 15 min at room temperature. Each mixture (2.0 ml) was heated in a boiling water bath for 40 min with 5×10^{-2} M $HgSO_4$ (10.0 ml) in 1 N H_2SO_4 and the resulting reaction mixture was cooled in running water. Next, 0.5 M $CuSO_4$ (10.0 ml) and 0.1 M $NaNO_2$ (1.0 ml) were added to each mixture (10.0 ml) and the whole was allowed to stand for 60 min at room temperature. The optical densities were measured at 524 nm against the reagent blank solution prepared similarly (Fig. 10).

2) Conventional Method: To an aqueous solution of tyrosine of various concentrations (2.5 ml) was added 15% $HgSO_4$ in 6 N H_2SO_4 (2.0 ml), and the whole was allowed to stand for 60 min at room temperature. After addition of 2 N H_2SO_4 (10.0 ml) to each mixture, the mixtures were heated in a boiling water bath for 15 min and cooled in running water. By adding 2 N H_2SO_4 (10.0 ml), 2% $NaNO_2$ (1.0 ml) and H_2O to each reaction mixture, the volume was made up to 50.0 ml. The colored solution was allowed to stand for 30 min at room temperature, and the optical density of each solution was measured at 480 nm against H_2O (Fig. 10).

Effect of Standing Time on the Optical Density in the Case of Urine Samples——1) Present Method: A mixture of human urine (8.0 ml), 8×10^{-4} M tyrosine (8.0 ml), 0.1 M $HgAc_2$ (8.0 ml) was allowed to stand for 15 min at room temperature, then centrifuged. To the resulting supernatant was added 5×10^{-2} M $HgSO_4$ in 1 N H_2SO_4 (40.0 ml), and the mixture was heated in a boiling water bath for 40 min, cooled immediately in running water and centrifuged to separate the precipitate. Next, 1 M $CuSO_4$ (20.0 ml) and 0.1 M $NaNO_2$ (4.0 ml) were added to the supernatant (60.0 ml), and the optical density of the reaction mixture at 524 nm was measured at intervals against a blank test solution prepared similarly, except that H_2O was substituted for urine and tyrosine solution (Fig. 11).

2) Conventional Method: A mixture of human urine (1.5 ml), 5×10^{-3} M tyrosine (1.0 ml) and 15% $HgSO_4$ in 6 N H_2SO_4 (2.0 ml) was allowed to stand for 60 min at room temperature and centrifuged to separate the precipitate. The supernatant and washing of the precipitate with H_2O (5.0 ml) were combined and transferred to a 50.0 ml flask, 2 N H_2SO_4 (10.0 ml) was added and the mixture was heated in a boiling water bath for 15 min. The reaction mixture was cooled in running water, and additional 2 N H_2SO_4 (10.0 ml), 2% $NaNO_2$ (1.0 ml) and H_2O were added to make 50.0 ml. The solution was filtered through filter paper. The optical density of the filtrate at 480 nm was measured against H_2O at intervals (Fig. 11).

Determination of Millon-Positive Substances in Urine and Recovery of Tyrosine added to Human Urine——Mixtures of normal human urine obtained in the morning (2.0 ml) and H_2O (1.0 ml) or 1×10^{-3} M or 8×10^{-4} M tyrosine (1.0 ml) and 0.2 M $HgAc_2$ (1.0 ml) were allowed to stand for 15 min at room temperature. The resulting reaction mixtures were centrifuged to separate the supernatant (2.0 ml), to which was added 5×10^{-2} M $HgSO_4$ in 1 N H_2SO_4 (10.0 ml). The mixtures were heated in a boiling water bath for 40 min and cooled immediately in running water, then 0.5 M $CuSO_4$ (10.0 ml) and 0.1 M $NaNO_2$ (1.0 ml) were added to each reaction mixture. The colored solutions were allowed to stand for 60 min at room temperature and the optical densities were measured at 524 nm against a solution prepared similarly except that H_2O was substituted for urine and the tyrosine solution (Table II).

References and Notes

1) Present address: *National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan.*
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7) Infrared and visible spectra and optical density were measured on a Koken DS-301 infrared spectrophotometer, a Hitachi EPS-3T recording spectrophotometer and a Hitachi EPU-2A spectrophotometer, respectively.