Mechanism of the Millon Reaction. I. Isolation of Coloring Substances

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(Received January 23, 1968)

The mechanism of the Millon reaction of p-cresol using the Hopkins–Cole reagent was investigated and three kinds of pigments as the final coloring substances were isolated. Namely, Pigment 1, 2 and 3 were obtained from the reaction mixture under various qualitative conditions using a 10% solution of mercuric sulfate. Pigment 1 was found to consist of 2-mercuri-4-methylphenol (MMP) and 2-nitroso-4-methylphenol (NMP), Pigment 2 of NMP and mercuric ion, and Pigment 3 was a polymer consisting of NMP and a compound assumed to be a mercurated nitrosocresol.

On the other hand, the main pigment produced under the quantitative condition was demonstrated to be Pigment 2 by its isolation.

MMP which is assumed to be an intermediate of these pigments, produces Pigment 1 on treatment with sodium nitrite in acidic medium. Consequently, Millon reaction of p-cresol proceeds through the mercuration at the o-position of hydroxyl group, which is followed by the electrophilic substitution of mercury atom with nitrosoylcation generated from sodium nitrite in acidic medium to give nitrosocresol. This resulted nitrosocresol, finally, coordinates to either organic or inorganic mercury to develop the characteristic Millon color.

The color reaction of protein using a solution of mercury dissolved in fuming nitric acid (the Millon reagent) was firstly reported by Millon in 1849. Afterward, it was found that the coloration was due to tyrosine residues in protein, and the reaction was also positive for tryptophan. Folin and Ciocalteu reported the improved Millon reaction for estimation of tyrosine in protein homogenate in which tyrosine was determined by coloration with sodium nitrite after previous elimination of tryptophan using a solution of mercuric sulfate in sulfuric acid (the Hopkins–Cole reagent). This modified method has been widely applied for determination of tyrosines in the field of protein.

Although a few works on the mechanism of the Millon reaction have been reported, they do not explain the mechanism satisfactorily. Namely, Meyer pointed out that a small amount of nitrous acid contained in the Millon reagent would be essential, and this consideration was also reported in the modified method by Folin, et al.

1) This work was reported at the 19th and 21st Annual Meeting of the Pharmaceutical Society of Japan.
2) Location: Hongo-7-chome, Bunkyo-ku, Tokyo.
3) E. Millon, Compt. Rend., 27, 40 (1849); Chem. Zentr., 29, 185 (1849).
4) R. Hofmann, Ann., 87, 123 (1853).
intermediates,5,8,10 and that nitroso-phenols or o-benzoquinone monoximes participated in coloration.6,11 Recently, Sakakibara, et al.19 and Katsumata19 presumed that the main coloring substance should have a structure as shown below on the basis of the fact that 2,6- and 3,5-disubstituted phenols were negative in this reaction.14 However, any intermediate or colored substance could not be isolated.

The present investigation was undertaken to isolate the colored substances in the modified Millon reaction of p-cresol which is a compound having a functional group concerning with the Millon reaction, and also reveal its mechanism.

The Pigments produced by the Qualitative Millon Reaction

In the qualitative Millon reaction of p-cresol using a 10% solution of mercuric sulfate in 10% sulfuric acid and a 5% solution of sodium nitrite, three kinds of pigments, Pigment 1,2 and 3, are variably produced depending on the amount of mercuric sulfate used.

When a solution of p-cresol was heated with a 10% solution of mercuric sulfate in 10% sulfuric acid, the white precipitate which was considered to be mercurated p-cresol was afforded and, moreover, this precipitate colored blood red, a typical Millon color, on treatment with a solution of sodium nitrite in sulfuric acid. It was, therefore, concluded that the first step of this color reaction should be the mercuration of p-cresol. Then, the assumed intermediate, 2-mercuri-4-methylphenol (MMP) was synthesized as the chloride form to make clear this assumption.

The synthesized MMP colored blood red immediately by treating with sodium nitrite in diluted sulfuric acid, and the produced colored substance was separated by extracting with chloroform as brownish prisms, mp 172° (decomp.), C13H12O3NHg (Pigment 1).

Pigment 1 was easily decomposed with mineral acid to two components. One of them was extractable with chloroform from the reaction mixture decomposed with hydrochloric acid, and purified by recrystallization from diluted ethanol to give white needles which was identical with MMP by mixed melting point determination and also infrared spectroscopy (Fig. 1).

![Fig. 1. Infrared Spectra of 2-Chloromericuri-4-methylphenol (Cl-NMR) (in KBr)
--- synthesized from p-cresol, mercuric acetate and sodium chloride
--- isolated from the decomposed product of Pigment 1](image1)

![Fig. 2. Infrared Spectra of Potassium 2-Nitroso-4-methylphenolate (NMP-K) (in KBr)
--- prepared from copper chelate of 2-nitroso-4-methylphenol (NMP-Cu)
--- isolated from the decomposed product of Pigment 1](image2)

The other was also isolated as a potassium salt on treatment of chloroform layer with a solution of potassium hydroxide, which was confirmed to be the potassium salt of 2-nitroso-4-methylphenol (NMP-K) (Fig. 2).

Pigment 1 was also prepared by only mixing an aqueous solution of NMP–K and an alcoholic solution of MMP. From the determination of NMP content, Pigment 1 was considered as a chelate compound consisting of the equimolar amounts of MMP and NMP.

On the other hand, phenylmercuric acetate (PMA) colored violet red with NMP–K and its absorption spectrum showed $\lambda_{\text{max}}$: 492 μm similar to that of Pigment 1, and moreover, the pigment (reddish brown needles, mp 161°(decomp.)) produced from PMA and NMP–K contained NMP in the equimol to PMA. Therefore, the phenolic hydroxyl group of mercurated $p$-cresol in Pigment 1 should not perform important role in the chelate formation with NMP.

On the basis of above results, the mechanism of this reaction is proposed as follows (Chart 1):

![Chart 1](image)

The first step is the mercuration of $p$-cresol at the $o$–position of phenolic hydroxyl group. Then, a part of the mercurated $p$-cresol is attacked with nitrosyl cation to yield NMP, a tautomer of 4-methyl-1,2-benzoquinone-2-oxime which coordinates to MMP to produce Pigment 1.

The infrared spectrum of Pigment 1 (Fig. 3) shows a band attributable to $v_{o-H}$ at 3260 cm$^{-1}$ suggesting that, at least, one of the phenolic hydroxyl group is independent from the chelation. The band at 750 cm$^{-1}$ is attributable to $v_{c-N}$ and disappearances when the crystals are sucked for about 15 hours at 50° under reduced pressure. As will be shown in the forthcoming paper,

Pigment 1 contains chloroform in its crystal structure and \( r_{C-C} \) absorption band is due to the chloroform.

Pigment 1 could also be isolated from the reaction mixture colored with less equimolar amount of mercuric sulfate toward \( \beta \)-cresol.

As the amount of a 10% solution of mercuric sulfate to \( \beta \)-cresol increased, the yield of Pigment 1 decreased gradually, and at the molar ratio of \( \beta \)-cresol to mercuric ion 1:2, Pigment 1 could not be isolated at all, and a blood red clear solution together with reddish brown precipitate was obtained. From the former, Pigment 2 as dark, reddish violet prisms was isolated and its infrared spectrum was identical with that of the mercury chelate compound of NMP (dark, reddish violet prisms, mp 175°C (decomp.) \( \text{C}_{14}\text{H}_{12}\text{O}_4\text{N}_2\text{Hg} \)) synthesized from NMP–K and mercuric sulfate. The latter was difficult to crystallize, though being soluble in chloroform, dichloroethane, pyridine or dioxane. It was purified by reprecipitating with ethanol from its chloroform solution to give a reddish brown powder (Pigment 3). Pigment 3 in chloroform showed about 5000 in molecular weight measured by a \( \beta \)-Pressure Osmometer, and its mercury content was 52.5%. Pigment 3 was easily decomposed with sulfuric acid to afford two fractions; the soluble fraction in chloroform (yellowish brown solution) and insoluble one (brownish powder). From the former NMP–K was isolated. Since the latter was insoluble in most organic solvents, it was purified by reprecipitating from its alkaline solution with sulfuric acid to give brownish powder. This compound contains mercury, and its alkaline solution produced reddish precipitate with cupric ion and violet one with mercuric ion. These facts indicated that this substance was a mercurated nitroso phenol polymer having a ability to form chelate.

When \( \beta \)-cresol was allowed to react with the higher molar ratio of mercuric sulfate under the same reaction condition, the pigment corresponding to Pigment 3 was getting insolvency in chloroform, and the soluble pigment disappeared.

The Pigment produced by the Quantitative Millon Reaction

Firstly, variation of absorption spectra by changing each concentrations of \( \beta \)-cresol, mercuric sulfate and sodium nitrite was examined. With a \( \beta \)-cresol solution of fixed concentration, \( 3.0 \times 10^{-4} \text{ M} \), an absorption peak at 490 \( \text{m}\mu \) appeared in the case of low concentration of mercuric ion, whereas, it was disappeared gradually by the increased concentration of mercuric ion producing a new peak at 490 \( \text{m}\mu \) (Fig. 4) to give a characteristic spectrum of the Millon reaction and the intensity of this peak became constant at the concentration of mercuric sulfate more than \( 2 \times 10^{-8} \text{ M} \) in about 1/5N sulfuric acid.

![Absorbance vs. Wavelength for Pigment 1](image1)

![Absorbance vs. Wavelength for Pigment 2](image2)

Fig. 4. Absorption Spectra in the Solution of Varying Mercuric Sulfate Concentration

\( \beta \)-cresol: \( 3.0 \times 10^{-4} \text{ M} \); mercuric sulfate: I, \( 0.2 \times 10^{-3} \text{ M} \); II, \( 0.4 \times 10^{-3} \text{ M} \); III, \( 4.0 \times 10^{-3} \text{ M} \); IV, \( 0.0 \times 10^{-3} \text{ M} \); V, \( 0.8 \times 10^{-3} \text{ M} \); VI, \( 0.0 \times 10^{-3} \text{ M} \), sodium nitrite: \( 4.0 \times 10^{-4} \text{ M} \), sulfuric acid: 0.16 M.

Fig. 5. Absorption Spectra in the Solution of Varying \( \beta \)-Cresol Concentration

\( \beta \)-cresol: I, \( 1.0 \times 10^{-4} \text{ M} \); II, \( 2.0 \times 10^{-4} \text{ M} \); III, \( 3.0 \times 10^{-4} \text{ M} \); IV, \( 4.0 \times 10^{-4} \text{ M} \); V, \( 5.0 \times 10^{-4} \text{ M} \); VI, \( 6.0 \times 10^{-4} \text{ M} \), mercuric sulfate: \( 4.0 \times 10^{-8} \text{ M} \), sodium nitrite: \( 4.0 \times 10^{-4} \text{ M} \), sulfuric acid: 0.16 M.

Variation of the concentration of \( \beta \)-cresol from \( 1.0 \times 10^{-4} \) to \( 6.0 \times 10^{-4} \text{ M} \) in the fixed concentration of mercuric ion, \( 4.0 \times 10^{-8} \text{ M} \), did not change its spectral shape, and its intensity
increased proportionally to the concentration of \( p \)-cresol (Fig. 5). Therefore, the factor which affected the spectral shape should be the concentration of mercuric ion.

When the concentration of added sodium nitrite was varied from \( 1.0 \times 10^{-4} \) to \( 8.6 \times 10^{-4} \) M, the intensity at 490 m\( \mu \) reached maximum near the concentration of \( 4.3 \times 10^{-4} \) M and tended to decrease gradually by the higher concentration. These observations suggested that the colored substances were decomposed with the higher concentration of nitrite ion (Fig. 6).

Moreover, from such a diluted solution of \( p \)-cresol and the reagents, the insoluble pigment did not be isolated even when \( p \)-cresol was allowed to react with the higher molar ratio of mercuric sulfate. On the basis of informations of the quantitative reaction conditions mentioned above, the pigment produced in diluted solution was extracted perfectly with chloroform to give the dark, reddish violet prisms which was confirmed to be Pigment 2 by infrared spectroscopy (Fig. 7).

**Discussion**

The spectra of these three kinds of pigments isolated are very similar each other in chloroform solution (Fig. 8), suggesting that each of these colorations may be due to the chelate compounds of nitrosocresol. Furthermore, the spectra of Pigment 1 and 2 in the Hopkins–Cole reagent are also identical with that of typical colored mixture of the Millon reaction of \( p \)-cresol in their visible region. It is, consequently, concluded that these pigments are the coloring substances in the Millon reaction. Practically, they are produced under different coloring conditions, and under the qualitative condition using 10% solution of mercuric sulfate in 10% sulfuric acid, one of Pigment 1,2,3 or their mixture is produced while under the quantitative condition using the diluted solution of reactants, Pigment 2 is dominant.

The attempts to separate the coloring substances by column, paper and thin layer chromatographies were unsuccessful, since the pigments were decolorized during development. There are no means to obtain the detailed informations about the colored by–products.

It is assumed that mercuric ion participates in the two reaction steps. The first step is the mercuration of the phenol at the \( o \)-position to hydroxyl group, and the mercurated phenol contributes to formation of nitrosophenol by the electrophillic substitution of mercury atom.
with nitrosyl cation. The second is formation of the chelate compound of inorganic or organic mercury with nitrosophenol. These assumptions were supported by the facts that an application of cupric ion instead of mercuric ion in the Millon reaction causes no coloration and addition of nitrous acid in the presence of cupric ion after the mercurication with insufficient amount of mercuric sulfate produces the color of copper chelate of nitrosophenol.16)

As will be shown in the forthcoming paper, Pigment 2 consists of NMP and mercuric ion in the molar ratio of 2:1. So, stoichiometrical point of view, the Millon reaction can be achieved in the molar ratio of the phenol to mercuric ion 2:1, although, in the diluted solution of reactants, the concentration of mercuric ion is the problem rather than the ratio of phenol and mercuric ion. This means that the produced pigment tends to dissociate due to the strong acidity of reaction medium, the acidity is required to dissolve mercuric sulfate, and, therefore, the higher concentration of mercuric ion necessary in order to form the mercury chelate compound quantitatively.

In the present investigation, the pigment having the structure assumed by Sakakibara, et al. was not obtained. Since all the pigments obtained contain NMP as a part of their structure, it is elucidated why 2,6-disubstituted phenols are negative in this reaction. However, a question why 3,5-disubstituted phenols are negative, is still remaining unsolved and this problem will be discussed in the forthcoming paper.

Experimental17)

2-Chloromercuri-4-methylphenol (CI-MMP)——Mercuric acetate (150 g) was added to the p-cresol (46.2 g), previously heated at 150—180° in a 300 ml flask with small portions, and the mixture was heated at 140—150° for 30 min. The reaction mixture was immediately poured into 2 liter of boiling water, and the mixture was heated at the boiling temperature for 5 min. After the hot mixture was filtered into a 30% solution of NaCl (100 ml), the filtrate was cooled to room temperature, the product precipitated was collected and recrystallized from 40% EtOH to give white needles (35 g), mp 160°. Anal. Calcd. for C_{10}H_{7}O_{2}ClHg: C, 24.50; H, 2.06. Found: C, 24.43; H, 2.10.

Isolation of Pigment 1 from the Qualitative Millon Reaction Mixture—A mixture of 10% p-cresol (13 ml) and 10% H_{2}SO_{4} in 10% H_{2}SO_{4} (13 ml) was heated in a boiling water bath for 15 min. After cooling in running water, 5% NaNO_{2} (3 ml) was added to the reaction mixture. The pigment produced was extracted with CHCl_{3} (50 ml), and the extract was washed with H_{2}O (300 ml) five times. The needles separated were collected, and recrystallized from CHCl_{3} to afford brownish prisms (0.4 g) (Pigment 1), mp 172° (decomp.). Anal. Calcd. for C_{14}H_{10}Cl_{2}O_{2}N_{2}Hg: C, 37.98; H, 2.74; N, 3.16. Found: C, 37.82; H, 2.81; N, 3.20.

Pigment 1 from CI-MMP and Sodium Nitrite in Sulfuric Acid—A mixture of CI-MMP (4 g) in MeOH (200 ml), 40% NaNO_{2} (10 ml) and H_{2}SO_{4} (0.5 ml) was allowed to stand for four days at room temperature, and filtered. The filtrate was concentrated under a reduced pressure to about one-quarter volume. The resulting precipitate by adding H_{2}O (200 ml) was collected by filtration and purified by recrystallizing from its MeOH solution with H_{2}O. The reddish brown precipitate was recrystallized from CHCl_{3} to give brownish prisms (0.89 g, mp 172° (decomp.)), which was identical with Pigment 1 by infrared spectroscopy.

Copper Complex of 2-Nitroso-4-methylphenol (NMP-Cu) and Potassium 2-Nitroso-4-methylphenolate (NMP-K)——A mixture of CuSO_{4}·5H_{2}O (77 g) and NaNO_{2} (28 g) in H_{2}O (2 liter) was added to a solution of p-cresol (70 g) in a mixture of AcOH (150 ml) and H_{2}O (500 ml) adjusted to pH 4.2 with NaOAc, and the mixture was allowed to stand at room temperature for ten days. The precipitate separated (69 g) was collected, and washed with H_{2}O. Recrystallization from EtOH gave dark, blue-violet needles. Anal. Calcd. for C_{14}H_{12}O_{4}N_{2}Cu: C, 50.07; H, 3.61; N, 8.34. Found: C, 50.40; H, 3.88; N, 8.24.

NMP-Cu (1.2 g) in CHCl_{3} (50 ml) was decomposed by shaking with 10% H_{2}SO_{4} (20 ml) twice. The CHCl_{3} layer was extracted with 10% KOH (10 ml) twice, and the aqueous layer was mixed with an ice-cold, saturated KOH solution. The resulting precipitate was collected by filtration, dried, and recrystallized from EtOH to give brownish plates (0.15 g). Anal. Calcd. for C_{14}H_{16}O_{4}NK: C, 47.97; H, 3.45; N, 7.99. Found: C, 48.14; H, 3.47; N, 8.28.

16) The data will be reported by the forthcoming paper.
17) All melting points are uncorrected. Infrared and visible spectra were measured on a Koken Model DS-301 infrared spectrophotometer, and a Hitachi Model EPS-2 and EPS-3T recording spectrophotometer.
Pigment 1 from Cl-MMP and NMP-K—To a solution of NMP-K (130 mg) in H₂O (20 ml), was added a solution of Cl-MMP (195 mg) in EtOH (10 ml). The precipitate separated (260 mg) was collected, and recrystallized from CHCl₃ to give brownish prisms, mp 172° (decomp.). The infrared spectrum of this substance was identical with that of Pigment 1 obtained from the color reaction mixture (Fig. 3).

The Pigment Produced from Phenylmercuric Acetate (PMA) and NMP-K—To a solution of PMA (203 mg) in CHCl₃ (5 ml), was added NMP-K (154 mg) in H₂O (20 ml), and the CHCl₃ layer washed with H₂O was concentrated to afford precipitate (104 mg). The precipitate was recrystallized from EtOH to give dark, reddish brown needles (65 mg), mp 161° (decomp.). Anal. Calcd. for Cl₃H₃O₂N₂H₃: C, 37.73; H, 2.08. Found: C, 37.68; H, 2.81.

Decomposition of Pigment 1 with Mineral Acid—A solution of Pigment 1 (200 mg) in CHCl₃ (20 ml) was shaken with 10% HCl (10 ml) twice, and the resulting yellowish CHCl₃ layer was evaporated to almost dryness. The residue was dissolved in EtOH (5 ml), and decolorized with a small amount of charcoal. The white needles (41 mg), mp 160° were obtained by adding of H₂O (5 ml) to the decolorized alcoholic solution, followed by recrystallization from diluted EtOH. This product was identical with Cl-MMP by mixed melting point determination and also by infrared spectroscopy (Fig. 1).

A solution of Pigment 1 (500 mg) in CHCl₃ (30 ml) was also decomposed by shaking with 10% H₂SO₄ (10 ml) four times. The resulting brownish CHCl₃ layer was extracted with 10% KOH solution (3 ml) twice. To this extract was added a saturated aqueous solution of KOH (10 ml) under ice cooling, and brownish crystals (310 mg) were produced. Recrystallization from EtOH gave brownish plates, of which infrared spectrum was identical with that of the NMP-K (Fig. 2).

Determination of NMP in Pigment 1—To each solution containing 62 μg and 124 μg of Pigment 1 in CHCl₃ (10 ml) was added 1 x H₂SO₄ (2 ml) and H₂O (6 ml). The mixture was shaken vigorously, and allowed to stand. The aqueous layer was removed carefully and the CHCl₃ layer was transferred to a cuvette by introducing a pipet directly through the center of CHCl₃ surface. Then, optical density of CHCl₃ layer was determined at 500 μm. The amount of NMP calculated from the calibration curve obtained from NMP-K by the same procedure for the sample was 24.03%. The theoretical value for CH₃Cl₂O₂N₂H₃·CHCl₃ is 24.34%.

Determination of NMP in the Pigment produced from PMA and NMP-K—To each solution containing 84 μg and 96 μg of the pigment in CHCl₃ (10 ml), were added 0.2 x H₂SO₄ (10 ml), and after the mixture was shaken vigorously, processed by the same procedure as the determination of NMP in Pigment 1. The amount of NMP was 34.61% to the theoretical value, 33.14%, for CH₃Cl₂O₂N₂H₃.

Isolation of Pigment 2 and Pigment 3 from the Qualitative Millon Reaction Mixture—A mixture of p-cresol (1.0 g) in H₂O (150 ml) and 10% HgSO₄ in 10% H₂SO₄ (55 ml) was heated in a boiling water bath for 30 min. After the reaction mixture was cooled in running water and poured into H₂O (700 ml), a 5% solution of NaNO₃ (13 ml) was added. The mixture was allowed to stand for about 1 min, and then filtered. The separated reddish brown precipitate was washed with H₂O, dried, and washed with EtOH.

The EtOH insoluble residue was extracted with CHCl₃ by a Soxlet’s apparatus, and the extract was concentrated to about 10 ml under a reduced pressure. The separated precipitate by adding EtOH (100 ml) to the concentrated solution was collected and purified by reprecipitating with EtOH from its CHCl₃ solution three times. The reddish brown powder (3.2 g) (Pigment 3) was obtained.

The filtrate of the reaction mixture was extracted with CHCl₃ (10 ml) four times. The combined extracts were concentrated under a reduced pressure, and the separated precipitate was recrystallized from EtOH to give dark, reddish violet prisms, Pigment 2 (320 mg).

Determination of Hg in Pigment 3—To Pigment 3 (15.9 mg) was added HNO₃ (4 ml) and 30% H₂O₂ (5 ml) under heating in a boiling water bath and the reaction mixture was refluxed for 1 hour. To the mixture, was added 30% H₂O₂ (5 ml) and the mixture was refluxed 1 hour moreover. After addition of H₂O (40 ml), Hg was determined by the chelate titration method. The Hg content was 52.59%. The Hg content in PMA determined by the same method as mentioned above was 101.47% comparing to theoretical value.

Decomposition of Pigment 3 with Mineral Acid—To a solution of Pigment 3 (1 g) in CHCl₃ (300 ml) was added 10% H₂SO₄ (250 ml) and the precipitate separated (170 mg) was collected by filtration. The brownish powder was obtained by reprecipitating repeatedly with 10% H₂SO₄ from its solution in 5% NaOH.

The decomposed matter of this powder with H₂SO₄·HNO₃ showed to be positive for the test of Hg with CuI. An alkaline solution of the brownish powder produced the violet precipitate with mercuric ion and the reddish one with cupric ion.

On the other hand, the CHCl₃ layer was extracted with 15% KOH (30 ml) and an ice-cold, saturated KOH solution was added to its aqueous layer to separate brownish precipitate which was recrystallized from EtOH twice to afford brownish crystals (70 mg) which was identical with NMP-K by infrared spectroscopy. Anal. Calcd. for Cl₃H₃O₂N₂K: C, 47.97; H, 3.46; N, 7.99. Found: C, 47.70; H, 3.38; N, 7.92.

Pigment 2 from NMP-K and Mercuric Sulfate—To a solution of NMP-K (400 mg) in H₂O (5 ml), was added 10% HgSO₄ in 3.3% H₂SO₄ (15 ml). The separated dark, reddish brown, muddy precipitate (480 mg) was collected, washed with H₂O, and dried. Recrystallization from EtOH gave dark, reddish violet prisms (220 mg), mp 175° (decomp.). Anal. Calcd. for C₁₄H₁₈O₅N₂Hg: C, 35.56; H, 2.56; N, 5.98. Found: C, 35.61; H, 2.61; N, 6.12.

Absorption Spectra in Various Conditions—Each mixture of 1/200μ p-cresol (1.5 ml) and HgSO₄ in 1/5μ H₂SO₄ (22.5 ml) in various concentrations was heated in a boiling water bath for 20 min, and cooled in running water. 1/100μ NaNO₂ (1.0 ml) was added to each reaction mixture, and the mixture was allowed to stand at room temperature for 1 hour. Then, their absorption spectra were measured.

Each mixture of p-cresol solution (14.0 ml) in various concentration and 1/100μ HgSO₄ (10.0 ml) in fixed concentration was heated in a boiling water bath for 20 min. After processed by the same procedure as mentioned above, their absorption spectra were measured.

1/100μ p-cresol (0.5 ml) and 1/50μ HgSO₄ (5.0 ml) were mixed, the mixture was heated in a boiling water bath for 20 min, and cooled in running water. After adding the NaNO₂ solution in various concentrations to each mixture, the mixtures were allowed to stand for 30 min at room temperature, and their absorption spectra were measured.

Isolation of Pigment 2 from the Quantitative Millon Reaction Mixture—1/100μ p-cresol (100 ml) was added to 1/100μ HgSO₄ in 1/5μ H₂SO₄ (1.4 liter) previously heated at 70°, and mixture was heated at 70—80° for 20 min. After cooling in running water, 1/100μ NaNO₂ (100 ml) was added, and the mixture was allowed to stand at room temperature for 90 min. The colored substance produced was extracted with CHCl₃ (10 ml) three times, and the combined extracts were washed with H₂O. After adding of EtOH (20 ml) into the combined extracts, the mixture was concentrated to a few ml under a reduced pressure. The precipitate separated was collected, and recrystallized from EtOH to give dark, reddish violet prisms (17 mg), mp 175° (decomp.), of which infrared spectrum was identical with that of Pigment 2 (Fig. 7).

Acknowledgement The authors wish to thank members of the Microanalytical Laboratories of this Faculty for elemental analyses and for the measurements of infrared and ultraviolet spectra. Elemental analyses were also carried out by members of the Sankyo Research Laboratory, Sankyo & Co., Ltd., to whom the authors thanks are due. The molecular weight was determined by Mr. A. Nara of Tokyo Laboratory of Kowa Co., Ltd., to whom the authors are very much indebted.