d) Contacting with acid : When the all-*trans* compound was treated with *N* HCl, only a trace of *trans*→*cis* rearrangement was found on the chromatogram (cf. Table I), while this was almost entirely decomposed by contacting with conc. HCl.

The author is grateful to Professors Z. Horii and K. Tsukida for their encouragements and valuable discussions throughout this investigation. He is indebted to Mr. Nakamachi of Takeda Chemical Industries for the infrared spectra.

Summary

1) All-*trans* mutatochrome, $C_{40}H_{56}O$, undergoes rearrangement to give *cis* isomers which can be resolved by column chromatography.

2) Five *cis* compounds were isolated, of which three are mono-*cis* isomers, termed neo-U, -A, and -B.

3) Quantitative study of the stereoisomeric mutatochrome was made and all-*trans* mutatochrome was found to be more stable to thermal, photochemical, and catalytic treatments than all-*trans* luteochrome.

4) Some related spectroscopic phenomena were discussed and tentative configurational assignments of (II), (III), and (IV) were respectively proposed for neo-U, -A, and -B.

(Received June 29, 1960)

UDC 543.854.73

40. Tsutomu Momose and Akira Inaba : Organic Analysis. XXVIII.[1] Mechanism of the Color Reaction of 3,6-Dinitrophthalic Acid with Reducing Sugars.

(*Institute of Pharmaceutical Sciences, Faculty of Medicine, Kyushu University*[*1])

3,6-Dinitrophthalic acid gives a very sensitive color reaction with a reducing sugar and is successfully used in the microdetection,[2] approximate estimation,[3] and determination[1,4] of the sugar. When the reagent dissolved in aqueous alkaline solution is heated with a small amount of a reducing sugar, a deep wine-red color appears in a few minutes. This initial color is unstable but, if the reaction is carried out for a longer time (10 minutes) in the presence of sodium thiosulfate, a very stable orange-red color is produced. This paper presents the mechanism of these reactions, isolating the main coloring matters in a crystalline form.

Coloring Matter of the Initial Unstable Color Reaction

The initial wine-red color of the reagent produced with glucose in the absence of sodium thiosulfate fades gradually to a faint yellow from the upper part of the colored solution, where the coloring matter is in contact with the air. The absorption spectrum of the color could only be observed by adding sodium metaphosphate in the solution and such a spectrum is shown in Fig. 1-A.

*1 Katakasu, Fukuoka (百瀬 勉, 稲葉 頴).

1) Part XXVII : T. Momose, Y. Mukai : Yakugaku Zasshi, **81**, 227 (1961).
2) T. Momose, A. Inaba : This Bulletin, **7**, 541 (1959).
3) T. Momose, A. Inaba, Y. Mukai, T. Shinkai : *Ibid.*, **8**, 514 (1960).
4) T. Momose, Y. Mukai, M. Watanabe : Talanta, **5**, 275 (1961).

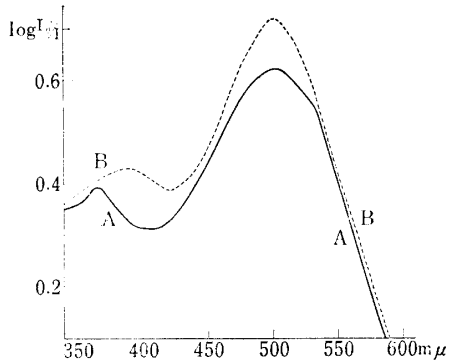


Fig. 1.

Absorption Spectra for Developed Color and 3-Hydroxyamino-6-nitrophthalic Acid

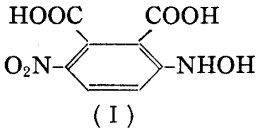
A : 0.1 cc. of glucose solution (0.5%) was heated with 5 cc. of 0.1% 3,6-dinitrophthalic acid dissolved in 5% Na_2CO_3 for 2 min., and diluted to 20 cc. with 5% Na_2CO_3 which contained 2% of $NaPO_3$.

B : 13.16 mg. of 3-hydroxyamino-6-nitrophthalic acid was dissolved in 500 cc. of 5% Na_2CO_3 which contained 2% of $NaPO_3$. λ_{max} : 500 mμ, log ε : 4.98.

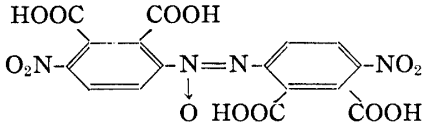
The coloring matter was so unstable in its solution, that the acidified reaction mixture was extracted with ether in which a small amount of sulfurous acid was added. The product of orange prisms, m.p. 178°, formed an unstable deep red color in aqueous solution of sodium carbonate, which gradually turned yellow in the air. The absorption spectrum (Fig. 1-B) of the color measured in the presence of sodium metaphosphate was the same as that of the developed color in the maximum (500 mμ) and shape of the curve. Therefore, this substance might be the main coloring matter of the initial reaction.

The dye had the formula $C_8H_6O_7N_2$ and consumed 4 moles of hydrogen on catalytic hydrogenation over palladium-carbon. The aqueous solution of the compound reduced an ammoniacal solution of silver nitrate. These data indicated that the compound might be 3-hydroxyamino-6-nitrophthalic acid (I).

This assumption was comfirmed by the fact that the compound formed 3,3'-azoxybis-(6-nitrophthalic acid) (II) as almost colorless needles, m.p. 220°, by air oxidation in alkaline medium. The latter compound (II) and its dimethyl ester, yellow needles, m.p. 180~181°, were separately prepared by the oxidation of 3,3'-azobis(6-nitrophthalic acid), and identified by the mixed melting point test and infrared spectra.



(I)



(II)

Coloring Matter of the Stable Color Reaction

The absorption spectrum of the stable orange-red color of the reagent produced by glucose in the presence of sodium thiosulfate is shown in Fig. 2-A. The main coloring matter of this reaction was isolated in a crystalline form, as yellow prisms, m.p. 222°, by extracting the acidified reaction mixture with ether. An alkaline solution of the

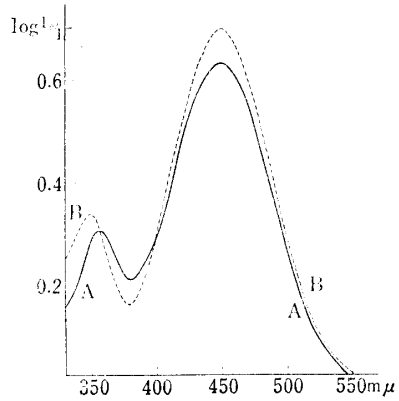


Fig. 2.

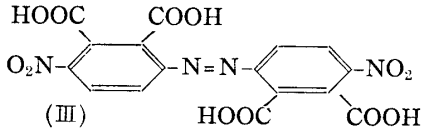
Absorption Spectra for Developed Color and 3,3'-Azobis(6-nitrophthalic acid)

A : 2 cc. of glucose solution (80 γ/cc.) was heated with 2 cc. of 0.1% 3,6-dinitrophthalic acid dissolved in 10% Na_2CO_3 which contained 2.5% of $Na_2S_2O_3 \cdot 5H_2O$ for 10 min. and diluted to 20 cc.

B : 8.96 mg. of 3,3'-azobis(6-nitrophthalic acid) was dissolved in 20 cc. of 5% Na_2CO_3, and diluted to 800 cc. λ_{max} : 450 mμ, log ε : 4.45.

compound had a deep orange-red color and its absorption spectrum (Fig. 2-B) was identical with that of the developed color in the maximum (450 mμ) and shape of the curve.

This compound had the formula $C_{16}H_8O_{12}N_4$ and consumed 8 moles of hydrogen in catalytic hydrogenation. Its dimethyl ester had the molecular weight of 522. These data indicated that the compound is 3,3′-azobis(6-nitrophthalic acid) (Ⅲ).



(Ⅲ)

The other reduction products of 3,6-dinitrophthalic acid were also synthesized to compare their absorption curves with those of the above dyes. 3-Amino-6-nitrophthalic acid, yellow needles, m.p. 242°, was prepared by carrying out the color reaction for a much longer time (30 minutes). An alkaline solution of the compound showed a yellow color and its absorption spectrum is shown in Fig. 3. 3,6-Diaminophthalic acid was prepared by catalytic hydrogenation of the dinitro compound over palladium-carbon and isolated as its hydrochloride of colorless needles. This compound was so unstable in alkaline or neutral solution, that the absorption spectrum could not be observed.

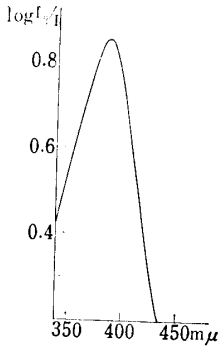


Fig. 3.

Absorption Spectrum of 3-Amino-6-nitrophthalic Acid

22.61 mg. of 3-amino-6-nitrophthalic acid was dissolved in 1000 cc. of Na_2CO_3, and diluted to 1500 cc. λ_{max} : 385 mμ, log ε : 4.10.

Discussion

Now it is clear that the initial wine-red color is caused by the monohydroxyamino compound. The same result had also been obtained in the color reaction of 3,4-dinitrobenzoic acid with a reducing sugar.[5] It is of interest to note that the stable orange-red color is produced by the azo compound, while the other nitro compounds which are used for the same purpose are reduced to their monoamino derivatives. These are picric acid[6] and 3,5-dinitrosalicylic acid.[7] In the present reaction, sodium thiosulfate plays an important rôle in stabilizing the developed color, protecting the hydroxylamino compound from air oxidation.

Experimental

3-Hydroxyamino-6-nitrophthalic Acid (I)——A solution of 1 g. of 3,6-dinitrophthalic acid, 1.4 g. of glucose, and 1.5 g. of Na_2CO_3 succesively dissolved in 50 cc. of H_2O was heated in a boiling water bath for 2 min. The mixture was rapidly cooled in ice water, acidified with H_3PO_4, and extracted with Et_2O which contained a small amount of SO_2. The solvent was evaporated from the extract, AcOEt was added to the residue, and separated orange prisms, m.p. 178°, were washed with AcOEt. Yield, 0.1 g. This substance was unstable in solution and could not be recrystallized.

5) E. Borel, H. Deuel : Helv. Chim. Acta, **36**, 801 (1953).
6) J. J. Willaman, R. F. Davison : J. Agr. Res., **28**, 479 (1926).
7) F. Hostettler, E. Borel, H. Deuel : Helv. Chim. Acta, **34**, 2132 (1951).

Anal. Calcd. for $C_8H_6O_7N_2$: C, 39.68; H, 2.49; N, 11.56. Found : C, 40.24; H, 2.64; N, 11.58. It consumed 4.90 cc. (in H_2O) of H_2 by catalytic hydrogenation (Calcd. for 12.15 mg. of the sample (24°): 4.88 cc.).

3,3'-Azoxybis(6-nitrophthalic acid) (II)——Air was passed through the solution of 1 g. of (I) dissolved in 100 cc. of 5% Na_2CO_3 for 20 hr., the mixture was acidified with HCl, extracted with Et_2O, and Et_2O was evaporated. The residue was recrystallized from 10% HCl to almost colorless needles, m.p. 222°. Yield, 0.4 g. *Anal.* Calcd. for $C_{16}H_8O_{13}N_4 \cdot 2H_2O$: C, 38.41; H, 2.41; N, 10.94; H_2O, 7.20. Found : C, 38.48; H, 2.68; N, 11.00; H_2O, 7.34.

It consumed 7.95 cc. (in H_2O) of H_2 by catalytic hydrogenation (Calcd. for 17.95 mg. of the sample (17°) : 7.68 cc.).

Dimethyl Ester : Prepared by methylation of (II) with CH_2N_2 and recrystallized from MeOH to yellow needles, m.p. 180~181°. *Anal.* Calcd. for $C_{20}H_{16}O_{17}N_4$: C, 46.20; H, 3.09; N, 10.75; mol. wt., 520.38. Found : C, 46.38; H, 3.01; N, 10.93; mol. wt. (Rast), 522.

3,3'-Azobis(6-nitrophthalic acid) (III)——A solution of 1 g. of 3,6-dinitrophthalic acid, 1.7 g. of glucose, 3 g. of Na_2CO_3, and 5 g. of $Na_2S_2O_3 \cdot 5H_2O$ successively dissolved in 50 cc. of H_2O was heated for 10 min. in a boiling water bath. The mixture was cooled, acidified with HCl, and extracted with Et_2O. The Et_2O solution was extracted with 5% Na_2CO_3 to separate the acid from sulfur. The extract was acidified and extracted with Et_2O. After evaporation of Et_2O, the residue was recrystallized from AcOEt and EtOH to yellow prisms, m.p. 222°. Yield, 0.2 g. *Anal.* Calcd. for $C_{16}H_8O_{12}N_4$: C, 42.86; H, 1.79; N, 12.49. Found : C, 42.87; H, 1.92; N, 12.36.

It consumed 9.70 cc. of H_2 by catalytic hydrogenation (Calcd. for 22.5 mg. of the sample (18°): 9.60 cc.).

Dimethyl Ester : Prepared by the methylation of (III) with CH_2N_2 and recrystallized from MeOH to yellow needles, m.p. 132°. *Anal.* Calcd. for $C_{20}H_{16}O_{12}N_4$: C, 47.62; H, 3.19; N, 11.10; mol. wt., 504.38. Found : C, 47.74; H, 3.15; N, 11.11; mol. wt. (Rast), 491.

3,3'-Azoxybis(6-nitrophthalic acid)——Prepared by the oxidation of (III) with H_2O_2 in AcOH by heating on a boiling water bath for about 5 hr. The reaction mixture was concentrated in a reduced pressure and separated crystals were recrystallized from 10% HCl to almost colorless needles, m.p. 222°. This compound and its dimethyl ester, m.p. 180~181°, prepared by methylation with CH_2N_2, were identified with the compounds mentioned above by the m.p. on admixture and infrared spectra.

3-Amino-6-nitrophthalic Acid——A solution of 1 g. of 3,6-dinitrophthalic acid, 2 g. of glucose, and 1 g. of Na_2CO_3 successively dissolved in 20 cc. of H_2O was heated for 30 min. in a boiling water bath. The mixture was cooled, acidified with HCl, and extracted with Et_2O. After evaporation of Et_2O, the residue was recrystallized from AcOEt to yellow prisms, m.p. 242°. Yield, 0.3 g. *Anal.* Calcd. for $C_8H_6O_6N_2$: C, 42.48; H, 2.67; N, 12.48. Found : C, 42.49; H, 3.00; N, 12.26. It consumed 6.30 cc. of H_2 by catalytic hydrogenation (Calcd. for 20.0 mg. of the sample (18°) : 6.30 cc.).

Dimethyl Ester : Prepared by methylation of the compound with CH_2N_2 and recrystallized from MeOH to yellow needles, m.p. 146°. *Anal.* Calcd. for $C_{10}H_{10}O_6N_2$: C, 47.24; H, 3.96; N, 11.02; mol. wt., 254.19. Found : C, 47.24; H, 3.91; N, 11.13; mol. wt. (Rast), 251.

3,6-Diaminophthalic Acid——A mixture of 2 g. of 3,6-dinitrophthalic acid, 10 cc. of 1% $PdCl_2$, 0.2 g. of activated carbon, and 100 cc. of H_2O was shaken for 6 hr. in H_2 atmosphere. At the end of the reaction, 5 cc. of HCl was added to dissolve the separated white crystals. After removal of the carbon by filtration, AcONa was added to the mixture and separated crystals were recrystallized from 10% HCl. Yield, 1 g. of the dihydrochloride as colorless needles, m.p. above 300°. The free amine was so unstable in the air that it could not successfully be purified. *Anal.* Calcd. for $C_8H_8O_4N_2 \cdot 2HCl$: C, 35.71; H, 3.75; N, 10.41; Cl, 26.35. Found : C, 35.83; H, 4.03; N, 10.25; Cl, 26.06.

Absorption Spectra——Measured in 10-mm. thickness by a Beckman DK-2 Ratio-recording Spectrophotometer.

The authors express their gratitude to Miss S. Indo and Mr. M. Shido for the microanalyses, and to Messrs. H. Matsui, H. Yano, and K. Hikita for the spectral analyses.

Summary

The mechanism of the color reaction between 3,6-dinitrophthalic acid and a reducing sugar was clarified. The initial wine-red color is produced by 3-hydroxyamino-6-nitrophthalic acid and the stable orange-red color by 3,3'-azobis(6-nitrophthalic acid). Some reduction products of the reagent were also prepared.

(Received July 1, 1960)