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The Murexide Reaction of Caffeine with Hydrogen Peroxide and Hydrochloric Acid

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The murexide reaction was investigated to clarify the mechanism of the coloration, with caffeine as a model compound. From the reaction mixture of caffeine with hydrogen peroxide and hydrochloric acid, 1-hydroxy-5,7-dimethyl-2,4,6-trioxo-1H,5H,7H-oxazolo-[4,5-*d*]pyrimidine (yellow oil) (I) and 1,3,7-trimethyl-2,6,8-trioxo-9-hydroxy-1H,3H,7H-xanthine (red powder) (II) were isolated, and these two compounds were shown to be responsible for the murexide reaction of caffeine. Compound I was regarded as a key intermediate, since its purple coloration with dil. ammonia was similar to that of caffeine developed by the murexide reaction. The absorption maximum of II corresponds to that of the red-colored solution obtained from the reaction of caffeine with hydrogen peroxide and hydrochloric acid.

Keywords——murexide reaction; coloration; caffeine; amalic acid; murexoin; hydrogen peroxide; oxazolopyrimidine

Pront reported that treatment of uric acid with nitric acid followed by ammonia gave a purple coloration.[1] Wöhler and Liebig called this colored substance murexide[2] and Winslow studied the structure of murexide.[3] Wöhler *et al.* claimed that this coloration could be effectively used to detect uric acid and related purines.[2] This test for caffeine has been named the murexide reaction and used in pharmaceutical analysis and forensic chemistry. It is accepted as an authorized method in The Japanese Pharmacopoeia. Schreiber proposed a mechanism for the murexide reaction of uric acid with nitric acid, and pointed out that other oxidizing agents such as bromine were necessary for the coloration of caffeine.[4]

The mechanism of the murexide reaction for uric acid and caffeine has been assumed to be as shown in Chart 1.[5] By this pathway, caffeine is oxidized with partial hydrolysis and condensed to amalic aicd as an intermediate, which is converted to murexoin, giving rise to the purple coloration. We were interested in the role of amalic acid as an intermediate and in the effect of oxidizing agents on the murexide reaction of caffeine. A preliminary examination showed that hydrogen peroxide and hydrochloric acid brought about more intense colora-

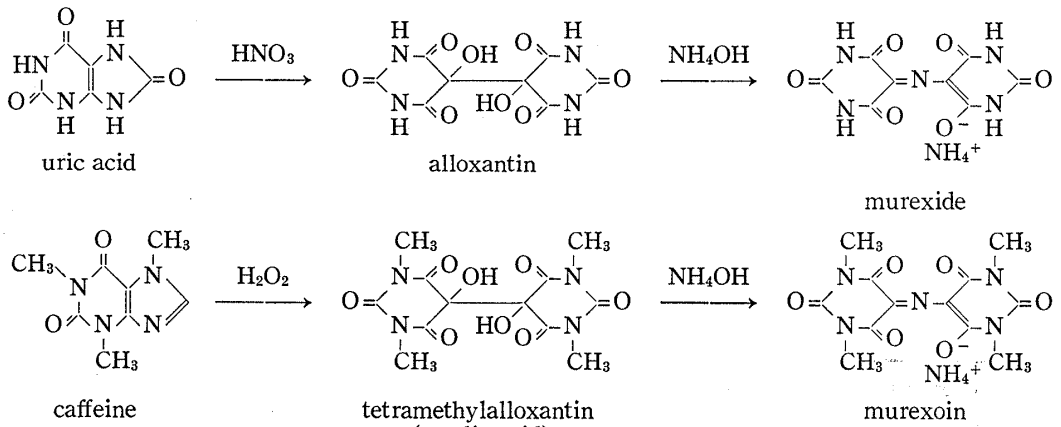


Chart 1

tion than bromine or nitric acid. However, our reexamination of this reaction of caffeine brought to light an unexpected intermediate and colored substance. This paper describes the elucidation of the mechanism of the murexide reaction of caffeine with hydrogen peroxide and hydrochloric acid.

The reaction of caffeine with hydrogen peroxide and hydrochloric acid gives rise to a yellowish-red coloration, with an absorption maximum at 450 nm, as shown in Fig. 1.

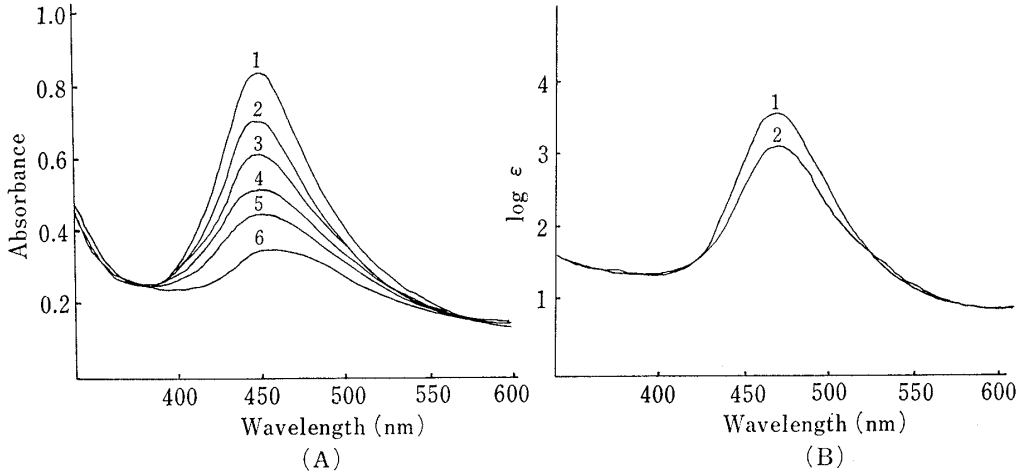


Fig. 1. Absorption Spectra of the Reaction Mixture of Caffeine with Hydrogen Peroxide and Hydrochloric Acid (A), and Absorption Spectra of II in Methanol (B)

(A) An oily residue was obtained by concentration of the reaction mixture of caffeine (0.5 m mol) with 3 % H_2O_2 (3 ml) and conc. HCl (1—2 drops). This residue was dissolved in 5 ml of methanol, and kept for various times (1—6) at room temperature (15°). The times (min) were as follows: 1, immediately; 2, 5; 3, 10; 4, 15; 5, 20; 6, 25.
(B) Spectrum 1 was recorded immediately, and spectrum 2 at 24 hr after dissolving II in methanol.

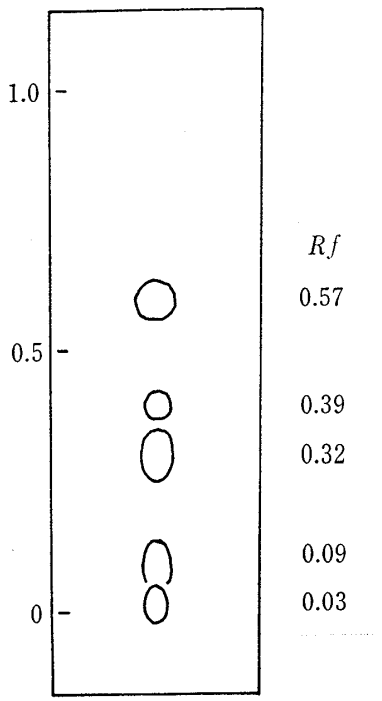


Fig. 2. TLC of the Reaction Mixture of Caffeine with Hydrogen Peroxide and Hydrochloric Acid

Solvent, benzene–methanol (20: 1, v/v).

A thin-layer chromatogram (TLC) of the reaction mixture (Fig. 2) showed five spots. From the above colored reaction mixture, the starting material (*Rf* 0.39) and four products, I (*Rf* 0.32), II (*Rf* 0.09), III (*Rf* 0.57) and IV (*Rf* 0.03), were isolated by column chromatographic fractionation. Among them, I showed a purple coloration with dil. ammonia, and II was a red-colored substance. The other compounds were uncolored and were not changeable to any colored product by addition of dil. ammonia. They were concluded not to be involved in this coloration.

Compound I was a yellow oily substance, and the molecular formula was established as $C_7H_7N_3O_5$ by high resolution mass spectrometry. The infrared (IR) spectrum of I (Fig. 3) showed C=O bands at 1840, 1800 and 1750 cm^{-1} and O-H bands at *ca.* 3200 and 1020 cm^{-1}. The absorption band at 1840 cm^{-1} indicated the presence of a lactone ring. The nuclear magnetic resonance (NMR) spectrum of I showed signals at 2.82 (singlet, 3H) and 3.08 ppm (singlet, 3H), which were assigned as N–CH_3 protons. An O–H proton signal was observed

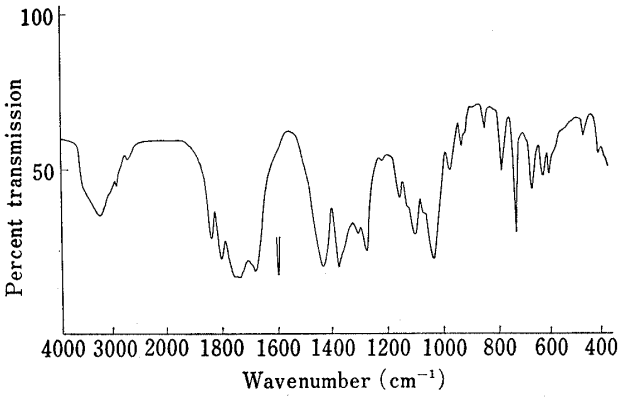


Fig. 3. IR Spectrum of I (in KBr)

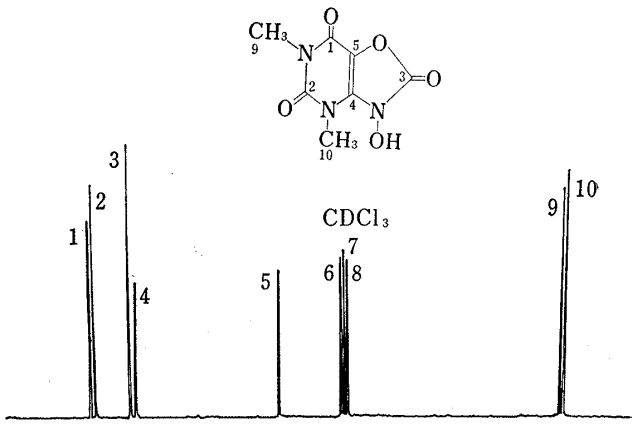
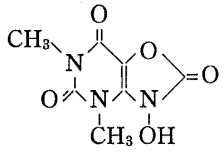


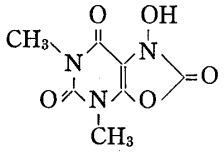
Fig. 4. ^{13}C-NMR Spectrum of I in $CDCl_3$

No.	ppm	No.	ppm
1.	164.37	6.	78.39
2.	164.00	7.	77.11
3.	154.77	8.	75.90
4.	152.47	9.	27.06
5.	91.13	10.	25.42



4, 5-*d*

Ia



5, 4-*d*

Ib

Chart 2

at 9.40 ppm (broad, 1H). Furthermore, ^{13}C-nuclear magnetic resonance (^{13}C-NMR) of I (Fig. 4) showed signals at 25.42 and 27.06 ppm, which were assigned as N–CH_3 carbons. The signals of C=O carbons were observed at 154.77, 164.00 and 164.37 ppm. The signals at 91.13 and 152.47 ppm were assigned as C=C bridgehead carbons. From the above spectral data, the oxazolo[4,5-*d*]pyrimidine (Ia) and oxazolo[5,4-*d*]pyrimidine (Ib) structures could be considered for compound I (Chart 2). However, the structure Ia was favored by the mass spectral fragmentation sequences (Chart 3, Table I). The loss of a CO_2 moiety was observed at the first stage, followed by the successive losses of CCO, N-CH_3 and N–OH moieties. In the case of Ib, however, the sequential losses of CO_2 and N–OH moieties would be expected prior to the loss of the CCO moiety (Chart 3). Therefore, I may be assigned as 1-hydroxy-5,7-dimethyl-2,4,6-trioxo-1H,5H,7H-oxazolo[4,5-*d*]pyrimidine (Ia).

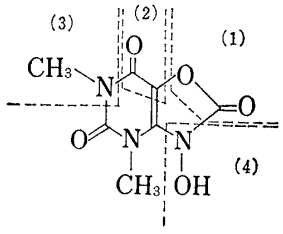


Chart 3

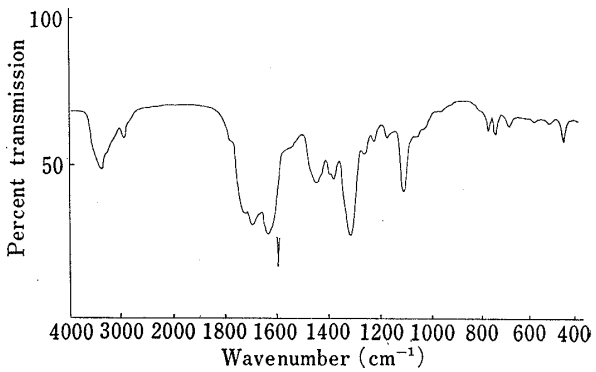


Fig. 5. IR Spectrum of II (in KBr)

Table I. Mass Spectral Data for I

m/e		Calcd	Found	Formula
213	(M^+)	213.039	213.038	$C_7H_7N_3O_5$
169	($213-CO_2$)	169.052	169.048	$C_6H_7N_3O_3$
129	($169-CCO$)	129.055	124.053	$C_4H_7N_3O_2$
100	($129-NCH_3$)	100.028	100.027	$C_3H_4N_2O_2$
69	($100-NOH$)	69.021	69.021	C_3H_3NO

Compound II was a red-colored substance, and the molecular formula was established as $C_8H_{10}N_4O_4$ from the high resolution mass spectral data. The IR spectrum of II (Fig. 5) showed C=O absorption bands at 1730, 1700 and 1640 cm^{-1}, and an O–H band at *ca.* 3400 cm^{-1}. The NMR of II showed signals at 3.04 (singlet, 3H), 3.16 (singlet, 3H) and 3.32 ppm (singlet, 3H), which were assigned as N–CH_3 protons. The signal at 8.20 ppm (broad, 1H) was assigned as an O–H proton. From these data, II was assumed to be 1,3,7-trimethyl-2,6,8-trioxo-9-hydroxy-1H,3H,7H-xanthine.

Compound III, corresponding to the spot at *Rf* 0.57, was assigned as 1,3,7-trimethyl-2,6-dioxo-8-chloro-1H,3H,7H-xanthine from the spectral data; this compound was main product of the reaction in terms of yield. The spot at *Rf* 0.39 was caffeine. Compound IV, which corresponded to the spot at *Rf* 0.03, was identified as 1,3,7-trimethyl-2,6-dioxo-8-hydroxy-1H,3H,7H-xanthine.

Compound II showed an absorption maximum at 480 nm, as shown in Fig. 1, while no purple coloration was observed on dissolving II in dil. ammonia. On the other hand, Compound I turned purple on treatment with dil. ammonia. Its absorption maximum ($\lambda_{max}^{H_2O}$ 525 nm) and IR spectrum coincided with those of the purple-colored substance obtained by the murexide reaction of caffeine. Therefore, I was found to be an intermediate in the formation of the purple-colored substance produced by the murexide reaction of caffeine. Though III and IV appeared to be by-products, as described above, the murexide reactions of III and IV gave a purple coloration ($\lambda_{max}^{H_2O}$ 525 nm), and a spot corresponding to I was observed on TLC

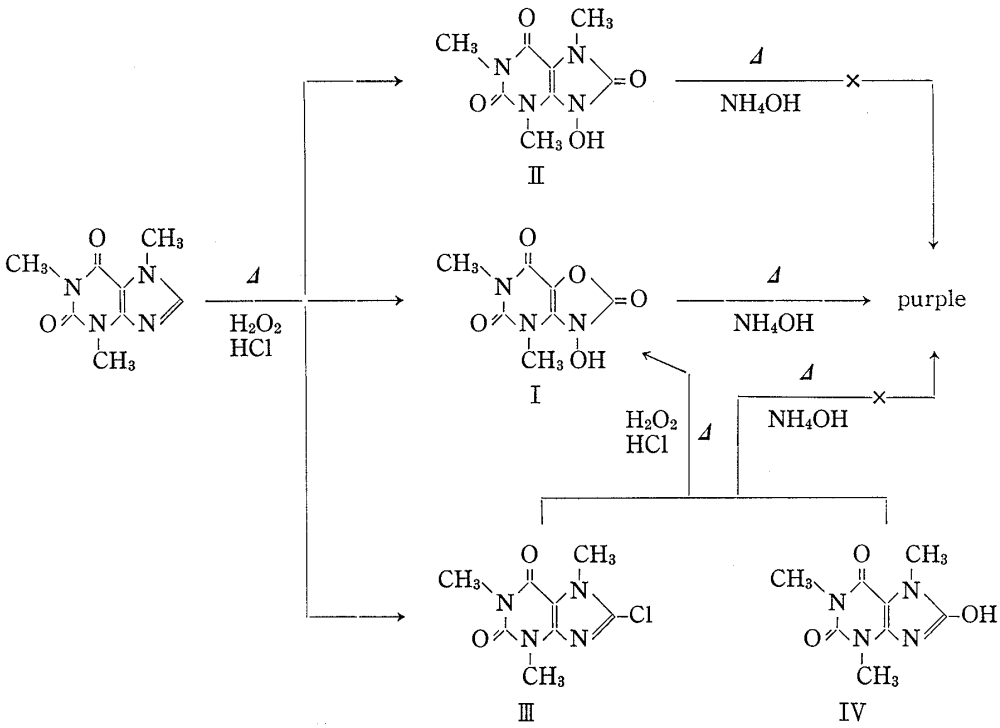


Chart 4

of the reaction mixtures of III and IV with hydrogen peroxide and hydrochloric acid. Consequently, it appeared that III and IV were also converted to I followed by the purple-colored substance. Further studies are required to determine whether III and IV are the initial intermediate of the coloration. The above results may be summarized as shown in Chart 4.

In conclusion, it was found that the reaction of caffeine with hydrogen peroxide and hydrochloric acid gave a yellow compound, 1-hydroxy-5,7-dimethyl-2,4,6-trioxo-1H,5H,7H-oxazolo[4,5-*d*]pyrimidine, and a red-colored compound, 1,3,7-trimethyl-2,6,8-trioxo-9-hydroxy-1H,3H,7H-xanthine. The red coloration of caffeine with hydrogen peroxide and hydrochloric acid was ascribed to the formation of II. Compound I further reacted with dil. ammonia to exhibit a purple coloration, and hence this compound is the key intermediate in the murexide reaction of caffeine. Amalic acid (see Chart 1) can be ruled out as an intermediate on the basis of our experimental results.

Experimental[6]

Reaction of Caffeine with Hydrogen Peroxide and Hydrochloric Acid——One g (0.005 mol) of caffeine was placed in a crucible, and 40 ml of 3% H_2O_2 and 1—2 drops of conc. HCl were added. This mixture was heated on the water bath and evaporated to dryness. A yellowish red oil was obtained. This oily product was subjected to column chromatography on silica gel, with benzene–methanol (20: 1, v/v) as an eluent.

I——From the first fraction, a crude product (I) was obtained (200 mg). Repurification by column chromatography on silica gel gave a yellow oil (40 mg). MS *m/e*: 213 (M^+), $C_7H_7N_3O_5$ (Calcd 213.039, Found: 213.038). ^{1}H-NMR ($CDCl_3$) ppm: 2.82 (3H, s, N–CH_3), 3.08 (3H, s, N–CH_3), 9.40 (1H, brs, O–H). ^{13}C-NMR ($CDCl_3$) ppm: 25.42 (1C, N–CH_3), 27.06 (1C, N–CH_3), 154.77 (1C, C=O), 164.00 (1C, C=O), 164.37 (1C, C=O), 91.13 (1C, C=C), 152.47 (1C, C=C). IR ν_{max}^{KBr} cm^{-1}: 1840 (lactone), 1800 (C=O), 1750 (C=O), *ca.* 3200 (O–H), 1020 (O–H).

II——Successive elution from the column with the same solvent system gave a red product (II) (80 mg). Repurification from column chromatography gave a red powder (10 mg), mp 138—140°. MS *m/e*: 226 (M^+) $C_8H_{10}N_4O_4$ (Calcd 226.072, Found: 226.070). ^{1}H-NMR (DMSO-d_6) ppm: 3.04 (3H, s, N–CH_3), 3.16 (3H, s, N–CH_3), 3.32 (3H, s, N–CH_3), 8.20 (1H, brs, O–H). VIS λ_{max}^{MeOH} nm (log ε): 480 (3.24). IR ν_{max}^{KBr} cm^{-1}: 1730 (C=O), 1700 (C=O), *ca.* 3400 (O–H).

III——Successive elution from the column with the same solvent system afforded a colorless product (III) (400 mg). Recrystallization from methanol gave a colorless powder (170 mg), mp 185—186°. MS *m/e*: 228 (M^+), 230 (M+2). ^{1}H-NMR ($CDCl_3$) ppm: 3.36 (3H, s, N–CH_3), 3.52 (3H, s, N–CH_3), 3.92 (3H, s, N–CH_3). *Anal.* Calcd for $C_8H_9ClN_4O_2$: C, 42.02; H, 3.97; N, 24.51. Found: C, 42.16; H, 3.90; N, 24.60. IR ν_{max}^{KBr} cm^{-1}: 1700 (C=O), 1660 (C=O).

IV——Successive elution from the column with the same solvent system provided a colorless product (IV) (30 mg). Recrystallization from methanol gave a colorless powder (5 mg), mp>300°. MS *m/e* 210 (M^+) $C_8H_{10}N_4O_3$ (Calcd 210.078, Found: 210.075). ^{1}H-NMR (DMSO-d_6) ppm: 3.16 (3H, s, N–CH_3), 3.28 (3H, s, N–CH_3), 3.32 (3H, s, N–CH_3), 12.0 (1H, brs, O–H). IR ν_{max}^{KBr} cm^{-1}: 1670 (C=O), *ca.* 3400 (O–H), 1030 (O–H).

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References and Notes

1) Pront, *Phil. Trans. Roy. Soc., London*, 420 (1818).
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3) N.M. Winslow, *J. Am. Chem. Soc.*, **61**, 2089 (1938).
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5) "Interpretation of the Japanese Pharmacopoeia," ed. VIII Vol. 1, Hirokawa Publishing, Co, Tokyo, 1971, p. C-555.
6) Absorption spectra were measured with a Hitachi 124 spectrophotometer in a cell of 10 mm optical length, IR spectra with a JASCO IR-G spectrophotometer, NMR spectra with a JEOL EC100 spectrometer at 100 MHz with TMS as an internal standard, mass spectra (MS) with a JMS-D100 mass spectrometer, and high resolution mass spectra with a JMS-01S spectrometer. Elemental analysis was carried out with a Perkin-Elmer 240B elemental analyzer.