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Mechanism of the Color Reaction of Active Methylene Compounds with 1,3,5-Trinitrobenzene Derivatives. XI.[1] The Jaffé Reaction[2]

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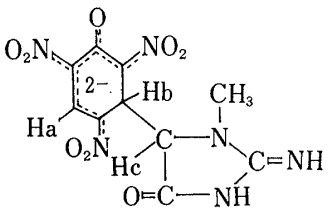
The color observed in the Jaffé reaction is due to the formation of the isomers of the 1:1 σ-complex (I) of creatinine and picric acid. The formation of hydroxylated anions of I (I′) and the presence of equilibria between the isomers of I and I′ were confirmed by nuclear magnetic resonance studies. The phenomenon of the augmentation of the color intensity on neutralizing the alkaline reaction mixture of picric acid and creatinine can be explained in terms of shifts of the equilibria between the isomers of I and I′. Three isomers of the 1:2 σ-complex are formed *via* the isomers of I and I′ in an alkaline mixture of picric acid and creatinine, but not in the presence of a large excess of picric acid, as determined by high performance liquid chromatography.

Keywords——Jaffé reaction; creatinine; picric acid; Zimmermann complex; Janovsky complex; 1:1 σ-complex of picric acid and creatinine; 1:2 σ-complex of picric acid and creatinine

Picric acid and active methylene compounds react in an alkaline medium to give an orange or red color.[4] The color increases in intensity when the alkaline reaction mixture is neutralized with sodium dihydrogen phosphate solution.[5] The phenomenon was also observed in the Jaffé reaction and successfully applied for the microdetermination of creatinine in serum.[6] However, the mechanism of this augmentation in color intensity is not known.

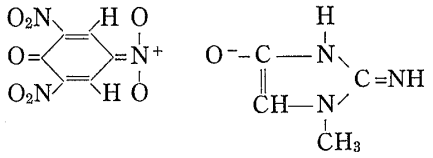
The color observed in the Jaffé reaction[7] has been thought to be due to the formation of a 1:1 complex of picric acid and creatinine. Three types of 1:1 complexes, *i.e.*, 1:1 σ-complex (I) (called Janovsky complex[7a,b]) (Chart 1), 1:1 ionic complex (II) (Chart 2) and the Zimmermann complex (III)[7a,b] (Chart 3), have been considered as the main coloring matter of the Jaffé reaction. The Janovsky complex (I), described first by Kimura,[8] was confirmed to be involved by proton nuclear magnetic resonance (NMR) and kinetic studies of the Jaffé reaction, though the complex was not isolated in a pure state.[8b,c,d] The 1:1 ionic structure (II), formerly described by Anslow and King[9a] and Seelig and Wüst,[9b] was

1) Part X: K. Kohashi, Y. Tsuruta, and Y. Ohkura, *Chem. Pharm. Bull.* (Tokyo), **26**, 2914 (1978).
2) A part of this paper was presented at the 94th Meeting of the Kyushu Branch (Fukuoka, Oct., 1975) and at the 96th and 97th Annual Meetings (Nagoya, Apr., 1976 and Tokyo, Apr., 1977) of the Pharmaceutical Society of Japan.
3) Location: *Maidashi, Higashi-ku, Fukuoka, 812 Japan.*
4) W. Weise and C. Trop, *Z. Physiol. Chem.*, **178**, 125 (1928).
5) T. Momose, Y. Ohkura, and K. Kohashi, *Chem. Pharm. Bull.* (Tokyo), **11**, 301 (1963).
6) T. Momose and Y. Mukai, *Rinshokensa*, **5**, 529 (1961).
7) M. Jaffé, *Z. Physiol. Chem.*, **10**, 391 (1886). For reviews, see *a*) T. Nambara, *Bunseki Kagaku*, **13**, 184 (1964); *b*) K. Kohashi and Y. Ohkura, *ibid.*, **23**, 91 (1974).
8) *a*) M. Kimura, *Pharm. Bull.* (Tokyo), **3**, 81 (1955); *b*) A.R. Butler, *Clin. Chim. Acta*, **59**, 227 (1975); *c*) A.R. Butler, *J. Chem. Soc. Perkin Trans.*, **2**, 853 (1975); *d*) A. Ellinger, R. Seidel, and K.-A. Kovar, *Arch. Pharm.*, **309**, 603 (1976).
9) *a*) W.K. Anslow and H. King, *J. Chem. Soc.*, 1210 (1929); *b*) H.P. Seelig and H. Wüst, *Arztl. Lab.*, **15**, 34 (1969); *c*) J. Vasiliades, *Clin. Chem.*, **22**, 1664 (1976).



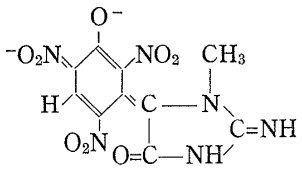
I

Chart 1



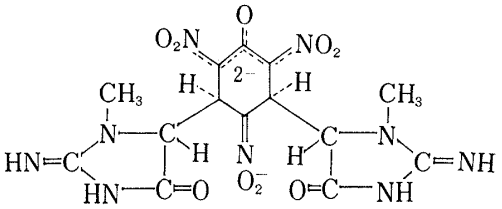
II

Chart 2



III

Chart 3



IV

Chart 4

again proposed by Vasiliades[9c] on the basis of proton and carbon-13 NMR studies. However, the significance of the ionic complex has been questioned.[10] The Zimmermann complex (III) was postulated by analogy with the mechanism of the Zimmermann reaction with m-dinitrobenzene, though the oxidation of I to III by excess picric acid has not been established.[7a]

In the previous paper of this series,[1] we isolated three isomers of a 1:2 σ-complex of picric acid and creatinine (IV) (Chart 4) in crystalline form from the alkaline reaction mixture of picric acid and creatinine. This paper describes the color reaction of creatinine with excess picric acid (the Zimmermann reaction[7a,b]) and that of picric acid with excess creatinine (the Janovsky reaction[7a,b]), and discusses the mechanism of the Jaffé reaction.

Results and Discussion

The absorption spectra of alkaline reaction mixtures of creatinine and excess picric acid (Zimmermann reaction) are shown in Fig. 1 (a). The neutralized mixtures (Fig. 1 (b)) show higher absorption intensity than the alkaline reaction mixtures in the visible region. The resulting orange color did not change for at least 2 hr at 1—3°, but gradually faded at 37°.

On addition of methanol to the alkaline reaction mixture, the species responsible for the orange color was obtained as a red precipitate which was hygroscopic and soluble in water, but insoluble in usual organic solvents.[12] The absorption spectrum of the red precipitate in water showed three maxima at 232, 406 and 480 nm with a shoulder around 335 nm (Fig. 2 (1)), which changed gradually (Fig. 2) when the solution was allowed to stand for 60 min at 25°. At 1—3°, the spectrum did not change for at least 2 hr. When the solution was adjusted to pH 6 or less by addition of some acids,[13] changes of the spectrum, as shown in Fig. 2, occurred more rapidly. The resulting band (Fig. 2 (4)) did not change further and is similar to the absorption spectrum of creatinine picrate in water, with two maxima at 232 and 352 nm, and a shoulder around 400 nm. On the other hand, when 10% sodium hydroxide

10) A.R. Butler, *Clin. Chem.*, **23**, 613 (1977).
11) T. Momose, Y. Ohkura, K. Kohashi, T. Tanaka, Y. Yano, and N. Itakura, *Rinshokensa*, **10**, 747 (1966).
12) Methanol, ethanol, dimethylformamide, dimethylsulfoxide, acetone, ethyl acetate, benzene, ether and acetic acid were examined.
13) Acetic acid, hydrochloric acid, phosphoric acid and sodium dihydrogen phosphate solutions were examined.

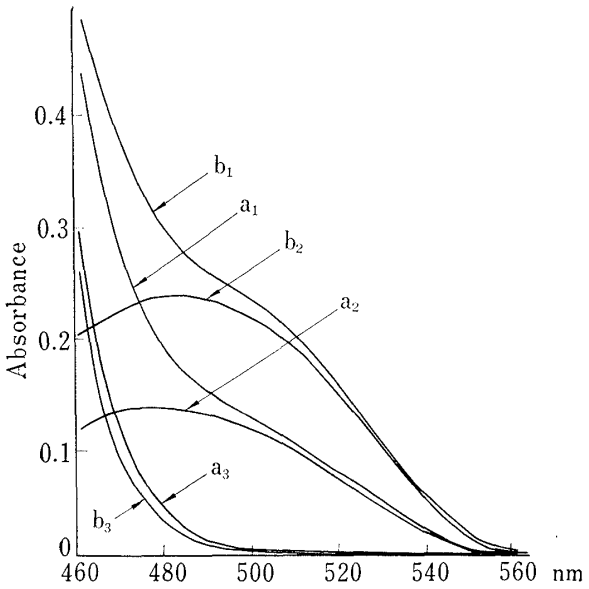


Fig. 1. Absorption Spectra of Color Reaction Mixtures

Picric acid (0.2%, 0.2 ml) and NaOH (20%, 0.4 ml) were added to 2.0 ml of a solution of creatinine (20 μg/ml). After standing for 20 min at 25° the mixture was either diluted with 3.40 ml of H_2O and measured against 1) H_2O and 2) the reagent blank (a) or neutralized with 3.40 ml of $NaH_2PO_4 \cdot 2H_2O$ (5%) and measured against 1) H_2O and 2) the reagent blank (b). a_3 and b_3 are the reagent blanks measured against H_2O, respectively.

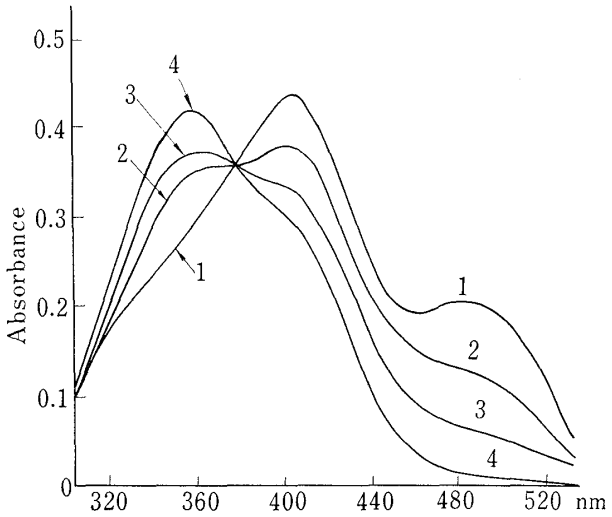


Fig. 2. Absorption Spectral Changes of the Red Precipitate in Water

The spectrum of the solution (1 mg/dl) was measured (1; immediately after dissolution, 2; after 10 min, 3; 30 min, 4; 60 min) against H_2O.

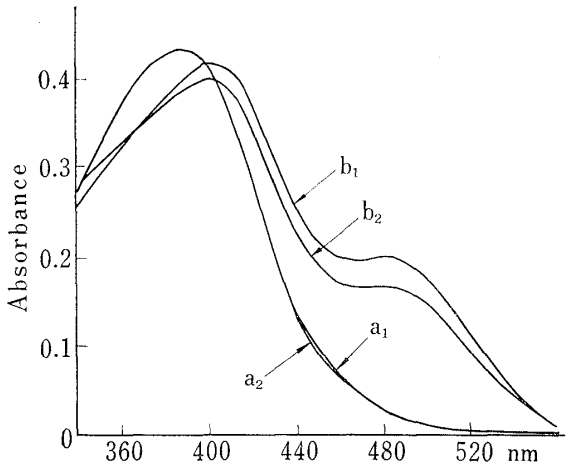


Fig. 3. Absorption Spectral Changes of the Red Precipitate in Alkaline Media

To 1.0 ml of an aq. solution of the red precipitate (4 mg/dl), 2.0 ml of H_2O and 1.0 ml of NaOH (a; 10%, b; 2%) were added successively. The spectra were measured 1) after 5 min, 2) after 60 min against H_2O.

solution was added to a freshly prepared aqueous solution of the red precipitate, the spectrum changed rapidly, giving a band with a maximum at 398 nm, which was stable for 1 hr at ambient temperature (Fig. 3 (a)). In the case of 2% sodium hydroxide solution, the absorption intensity above 400 nm decreased gradually, as shown in Fig. 3 (b). When these alkaline solutions ((a) and (b) in Fig. 3) were neutralized, the mixtures showed the same absorption bands as a freshly prepared aqueous solution of the red precipitate (*cf.* Fig. 2 (1)). The regenerated bands changed gradually in the same way as those of the aqueous solution of the red precipitate shown in Fig. 2. Similar changes were observed even in the presence of excess picric acid. When an alkaline mixture of the red precipitate and excess picric acid was neutralized, the absorption intensity above 450 nm increased in the same way as that of the color reaction mixture shown in Fig. 1. These observations suggest that the red precipitate is the main coloring matter of the Jaffé reaction.

The proton NMR spectrum of the red precipitate in deuterium oxide (D_2O) changes with time, as shown in Fig. 4. The resulting spectrum (Fig. 4 (3)) was stable for more than a week and the signals were readily assigned to sodium picrate (δ 8.94) and three isomers of the 1 : 2 σ-complex (δ 3.10 and 2.75) since these compounds were isolated from the resulting

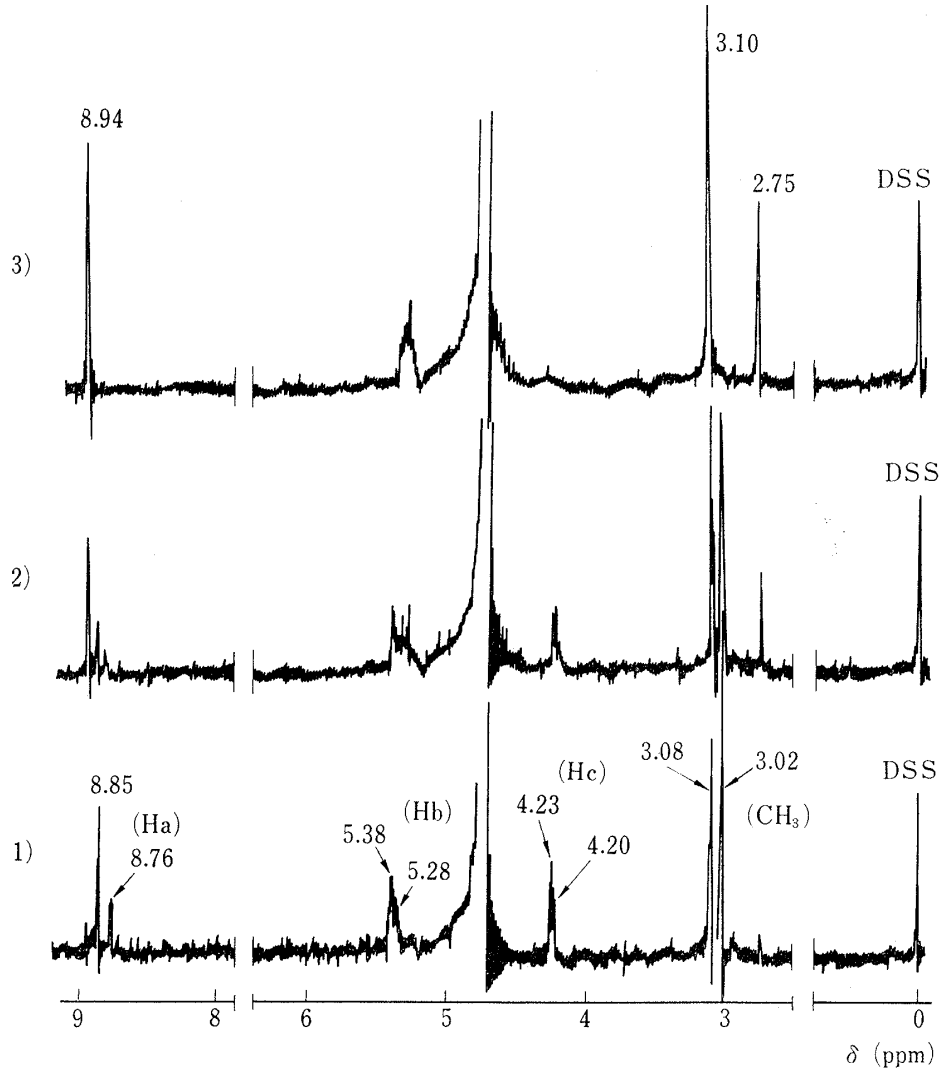
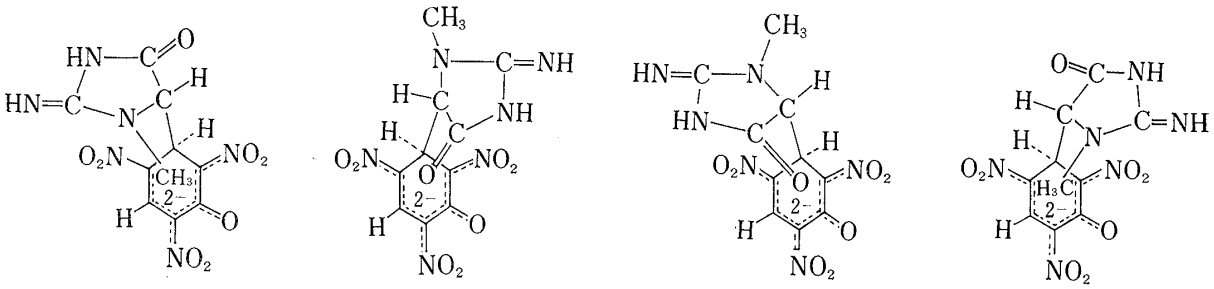


Fig. 4. NMR Spectral Changes of the Red Precipitate in D_2O

1) Immediately after dissolution, 2) after 30 min, 3) after 40 hr.

mixture as described previously.[1] The signals other than those of picrate and the three isomers of the 1:2 σ-complex, which are observed as main signals in Fig. 4 (1), could be divided into two groups according to their signal patterns. The signals of the two groups were assigned to the protons of the Janovsky complex (I) in the following manner. In one group of signals, the singlet at δ 8.85 (1H) is assigned to the proton (Ha) at the sp^2 ring carbon atom of I. The doublet at δ 5.38 (J=2.0 Hz, 1H) is assigned to the proton (Hb) at the sp^3 ring carbon atom and another doublet at δ 4.23 (J=2.0 Hz, 1H) to the methine proton (Hc) of creatinine. The singlet at δ 3.02 (3H) is assigned to the methyl protons of the creatinine residue. In the other group, the doublet at δ 8.76 (J=1.0 Hz, 1H) is assigned to the proton (Ha), which couples with the proton appearing as a doublet of doublets centered at δ 5.28 (J=1.0 and 2.0 Hz, 1H). The doublet of doublets and a doublet at δ 4.20 (J=2.0 Hz, 1H) are assigned to Hb and Hc, respectively. The singlet at δ 3.08 (3H) is assigned to the methyl protons of the creatinine residue. The chemical shifts of the latter group were similar to those of the corresponding protons of I assigned by Elinger *et al.*,[8d] who investigated the reaction mixture of equimolar amounts of sodium picrate, creatinine and deuterated sodium methoxide in deuterated dimethylsulfoxide.

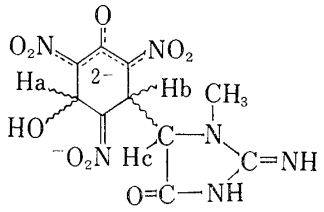
The 1:1 σ-complex (I) is thought to have eight diastereoisomers because of steric hindrance among the bulky creatinine residue and two nitro groups. Four of them are shown schematically by Ia, Ib, Ic and Id in Chart 5 and the others are their enantiomers,



Ia Ib Ic Id

Chart 5

Ia′, Ib′, Ic′ and Id′, respectively. Ia (Ia′) and Ic (Ic′) are rotational isomers of Ib (Ib′) and Id (Id′), respectively. These isomers can be classified into two groups, A (Ia, Ia′, Id and Id′) and B (Ib, Ib′, Ic and Ic′), on the basis of the following NMR spectral considerations. The NMR spectra of the four diastereoisomers in each group must be indistinguishable from each other. The methyl groups of the isomers in group A are over the trinitrooxocyclohexadienide ring, but those in group B are interposed by two nitro groups, which may cause some differences in the chemical shifts of corresponding protons between the isomers of groups A and B. Methyl protons of the isomers in group A are expected to appear at higher field than those in group B. Thus, signals of the first group are assigned to the protons of the isomers of group A and those of the second group to the isomers of group B.



I′

Chart 6

The visible absorption spectrum of the red precipitate[14] in concentrated sodium hydroxide solution, shown in Fig. 3 (a), is very similar to the spectra of three isomers of the 1:2 σ-complex dissolved in the same medium.[1] This similarity suggests that the species present in the alkaline medium are the hydroxylated anions of the isomers of I (I′ in Chart 6), having the same chromophore as the three isomers of the 1:2 σ-complex. The hydroxylated anion (I′) has a number of isomers and the stereochemistry of the species present in the alkaline medium was difficult to determine. When a few drops of 20% sodium deuterium oxide (NaOD) solution were added and mixed, the signals in the NMR spectrum of the red precipitate (*cf.* Fig. 4 (1)) at δ 8.85 and 8.76 due to the isomers of I disappeared and two singlets appeared at δ 6.24 and 6.12, which indicated the formation of the isomers of I′. In the NMR spectrum of the red precipitate in 10% NaOD solution, shown in Fig. 5, the two singlets at δ 6.24 and 6.12 are assigned to the proton (Ha) at the sp^3 ring carbon atom of the isomers of I′. The protons (Hb) at another sp^3 ring carbon atom of the isomers of I′ appear at δ 5.20 and 5.14 as singlets, because of the exchange of the methine proton of the creatinine residue (Hc) with deuterium in the medium.[1] Two singlets at δ 2.96 and 2.54 are assigned to the methyl protons of creatinine residues of the isomers of I′. In the case of 2% NaOD solution, mixed signals due to the isomers of I (*cf.* Fig. 4 (1)) and I′ (*cf.* Fig. 5) were observed. This supports the view that equilibria between the isomers of I and I′ are present in a dilute alkaline medium. The absorption spectrum of the red precipitate in dilute sodium hydroxide solution shown in

14) None of the isomers of I could be separated in a pure state from the red precipitate because they were unstable in water and changed rapidly into other species, such as sodium picrate and the three isomers of the 1:2 σ-complex during purification. The red precipitate obtained here is a mixture of large amounts of the isomers of I and very small amounts of the picrate and the isomers of the 1:2 σ-complex (*cf.* Fig. 4(1)).

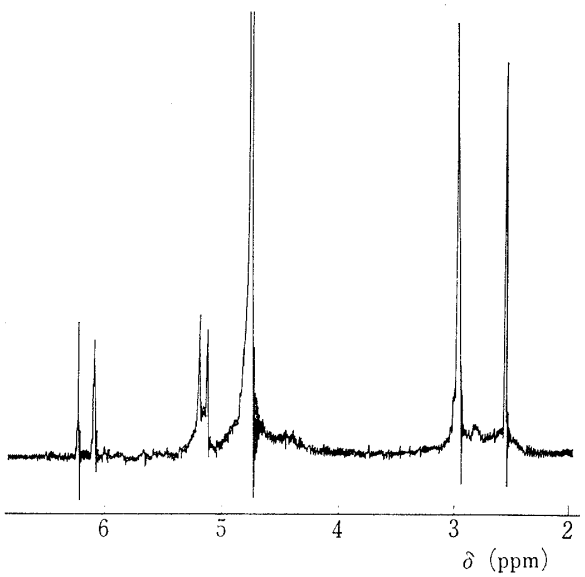


Fig. 5. NMR Spectrum of the Red Precipitate in Alkaline Medium

The red precipitate (50 mg) was dissolved in 10% NaOD solution (0.5 ml). The spectrum was measured immediately after dissolution.

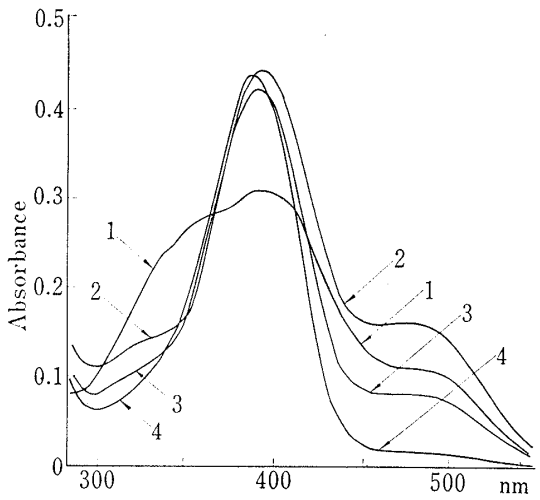


Fig. 6. Absorption Spectra of Reaction Mixtures of Picric Acid and Excess Creatinine

NaOH (10%, 1 ml) was added to 2.0 ml of aq. solution containing creatinine (0.14 mg) and picric acid (0.09 mg). The mixture was neutralized with 2.0 ml of 9% $NaH_2PO_4 \cdot 2H_2O$ after various times (1, 2, 3 and 4 were taken after 1, 30, 60 and 180 min, respectively). The spectra were measured against H_2O.

Fig. 3 (b) must be due to the isomers of I and I′. The spectral behavior of the red precipitate shown in Fig. 3 and that of the reaction mixture shown in Fig. 1 can thus be explained successfully in terms of shifts of the equilibria between the isomers of I and I′ in the media.

An aqueous sodium hydroxide (or NaOD) solution of equimolar amounts of creatinine and picric acid showed the same NMR spectra as the red precipitate described above, except for the signals due to unreacted creatinine (δ 3.03 and 4.04) and picrate (δ 9.84).[15] Although the alkaline mixture of creatinine and excess picric acid showed complicated NMR signals which appeared and disappeared with time, all of the signals could be assigned to the isomers of I and I′ and the three isomers of the 1:2 σ-complex,[15] and no signals ascribable to species such as II and III were observed. The absorption spectral behavior of the color reaction mixture shown in Fig. 1 could not be reasonably explained in terms of a reaction pathway including the species II[9] and/or III.[7a] These findings indicate that II and III are not the main coloring matter of the Jaffé reaction under the conditions described above.

The absorption spectra of reaction mixtures of picric acid and excess creatinine (Janovsky reaction) are shown in Fig. 6. The absorption at around 330 nm in the early stage of the reaction is due to the picrate (Fig. 6 (1)). The absorption intensity due to the isomers of I at around 500 nm increases and then decreases with time (Fig. 6 (1—3)). Finally, the mixture shows a band with a maximum at around 394 nm due to the isomers of the 1:2 σ-complex[1] (Fig. 6 (4)). The formation of the isomers of the 1:2 σ-complex could be followed by high performance liquid chromatography (HPLC).[1,16] In the early stage of the reaction, the mixture giving the absorption spectrum of Fig. 6 (1) did not show any peaks due to the three isomers of the 1:2 σ-complex, but after a reaction time of 60 min, the mixture giving the absorption spectrum of Fig. 6 (4) showed three peaks due to the three isomers of the 1:2 σ-complex.[1] Similar chromatographic changes were also observed in a reaction mixture of

15) Under the conditions of NMR spectral measurements, the formation of three isomers of the 1:2 σ-complex occurred even in the early stage of the reaction.

16) Unreacted picric acid and the isomers of I were adsorbed on the column paching under the conditions of HPLC.

equimolar amounts of picric acid and creatinine.[1] However, the color reaction mixtures with excess picric acid, as shown in Fig. 1, did not give any peaks due to the three isomers of the 1:2 σ-complex even after 60 min.[1]

The NMR spectrum of the red precipitate in 10% NaOD solution, which showed signals due to the isomers of I′ (Fig. 5), changed when excess creatinine was added. The signals at δ 6.24 and 6.12 due to the isomers of I′ disappeared and signals due to the three isomers of the 1:2 σ-complex appeared at δ 2.96, 2.60 and 2.58[1] (*cf.* Fig. 4 (3)). This indicates that the isomers of the 1:2 σ-complex are formed in the reaction between the isomers of I′ and excess creatinine in a strongly alkaline medium. The NMR spectral changes of the red precipitate in D_2O (shown in Fig. 4) indicate that the isomers of the 1:2 σ-complex are also formed from the isomers of I with the release of sodium picrate in an alkaline medium.[17]

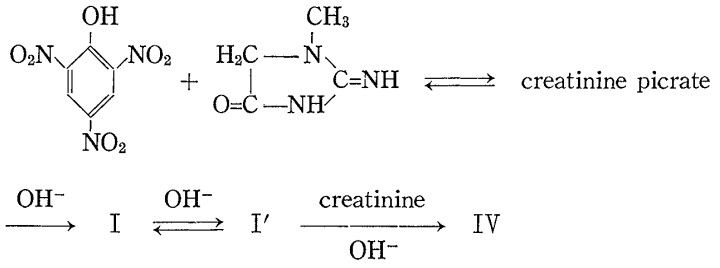


Chart 7

On the basis of the above results, the reactions between picric acid and creatinine can be summarized as shown in Chart 7 and explained as follows; when excess picric acid is added to an aqueous solution containing creatinine, creatinine picrate is formed. The picrate changes gradually into the isomers of I on addition of sodium hydroxide solution. The isomers of I change partly into the isomers of I′ in the presence of excess sodium picrate and sodium hydroxide. Since the isomers of I′ do not show intense absorption above 450 nm (*cf.* Fig. 3 (a_1)), the colors observed during the reaction are due to the isomers of I and excess sodium picrate (*cf.* Fig. 1 (a_1)). Under conditions of higher alkali concentration, formation of the isomers of I proceeds rapidly, but the color intensity due to the isomers of I will be lower because the equilibria between the isomers of I and I′ favor the isomers of I′. When the mixture is neutralized, the equilibria favor the isomers of I. This is the reason why the color intensity of the neutralized mixture becomes higher than that of the alkaline mixture (*cf.* Fig. 1 (a) and (b)). In the case of excess creatinine (Janovsky reaction), three isomers of the 1:2 σ-complex (IV) are formed via the isomers of I and I′ in the alkaline media. The colors observed during the reaction are due to the isomers of I initially and the isomers of IV finally.

Since the color intensity in the Jaffé reaction depends on the alkali concentration,[8c,9c] neutralizing the alkaline mixture of creatinine and excess picric acid can be recommended provided that the absorbance at around 490—510 nm due to the isomers of I is employed for the determination of creatinine.

Experimental

Red Precipitate——Powdered NaOH (1 g) was added to a solution of creatinine (0.5 g) and picric acid (2.3 g) in H_2O (100 ml). The mixture was stirred for 15 min at 25° and filtered, then MeOH (300 ml) was added. The resulting red precipitate was filtered off, washed several times with MeOH and dried *in vacuo*. Yield, 2.5 g.

Three isomers of the 1:2 σ-complex were prepared according to the procedure described previously.[1] NMR spectra were measured with a JEOL 100H NMR spectrometer using sodium dimethylsilapentane-

17) An aqueous solution of the red precipitate (10%) showed pH 11.

sulfonate (DSS) as an internal standard. To 2.0 ml of picric acid (50 mmol/l) in H_2O (or D_2O), 0.5 ml each of creatinine (6.25, 12.5 and 25.0 mmol/l) in H_2O (or D_2O) and NaOH in H_2O (or NaOD in D_2O) (2 and 10%) were added successively. The mixtures (0.5 ml) were measured at various times.

UV spectra were measured with a Shimadzu UV 200S double-beam spectrometer in a cell of 10 mm optical path length.

HPLC was performed with a Hitachi 635 liquid chromatograph, equipped with a Hitachi wavelength-tunable effluent monitor and a Hitachi 056 recorder. A stainless steel column (250×4 mm i.d.) packed with Hitachi gel #3011-N was used. The HPLC conditions were described previously.[1)]

pH was measured with a Hitachi-Horiba M-7 pH meter. The samples were dissolved in distilled water free from carbon dioxide.

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