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Clinical Analysis of Urea Nitrogen. Chemical Structure of the Colored Reaction Product of Urea with Diacetylmonoxime and Thiosemicarbazide

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The chemical structure of the color reaction product, 2-amino-5,6-dimethyldihydro-3*H*-1,2,4-triazine-3-one (**3a**), obtained from the reaction of urea with diacetylmonoxime (DAM) and thiosemicarbazide (TSC) in 10% sulfuric acid in methanol was elucidated. The ethyl and *p*-tolyl derivatives (**3b** and **3c**) of **3a** were also obtained from the reactions of ethyl- and *p*-tolylurea with DAM and TSC, along with 1,1′-disubstituted-5,4′-dimethly-2,2′-dioxo-4,5′-diimidazolylmethanes. **3a** and its derivatives, **3b** and **3c**, develop a red color immediately in acidic media and are thought to be the main products of the color reaction of urea, ethylurea and *p*-tolylurea, respectively, with DAM in the presence of TSC. A reaction mechanism is proposed.

Keywords——urea; diacetylmonoxime; thiosemicarbazide; 2-amino-5,6-dimethyldihydro-3*H*-1,2,4-triazine-3-one; structural determination; diacetylmonoximethiosemicarbazone; urea derivatives

Methods for the determination of blood urea nitrogen can be classified into chemical and enzymatic methods. The chemical methods, which involve the reaction with α-diketone or diacetylmonoxime (DAM), are widely used in clinical analysis, including automated procedures, though their reaction mechanism and the chemical structures of the reaction products are still unkown. On the other hand, the reaction mechanisms of the enzymatic methods which use urease and the chemical methods which use α-isonitrosopropiophenone, xanthydrol, and Ehrlich's reagent have already been clarified.[1]

Fearon reported that diacetyl, DAM, or diacetyldioxime reacted with urea in acidic media in the presence of oxidizing agents to form red or red-brown colored substances.[2] In these reactions, urea or alkylated urea derivatives gave coloration while acylated urea derivatives did not. The reducing action of hydroxylamine, one of the reaction products, was reported to decrease the color intensity and the stability of the colored substance, so it had to be removed by addition of an oxidizing agent.[3] However, it was found that the color was unstable in the presence of oxidizing agents.[4] Thus, Ueda *et al.* used D-glucuronolactone as a sensitizer of the color reaction,[5] and they isolated imidazolone (**1**) as one of the main products in the reaction of DAM with butyl- or *p*-tolylurea in acidic media.[6] The compound **1** developed a red color immediately when dissolved in an acidic medium, giving an absorption maximum at 490 nm.

Recently, the color reaction using thiosemicarbazide (TSC) as a color-stabilizing agent has been widely used for the determination of blood urea nitrogen.[7] Although it has been shown that the absorption maximum of the reaction products in the TSC method (530 nm) differs from that obtained by the D-glucuronolactone method (490 nm), the chemical structure of the reaction product obtained by the TSC method still remains unclarified.

The present paper deals with the structural determination of the reaction product between DAM and urea in the presence of TSC in acidic media.

Results and Discussion

Urea was heated in a boiling water bath with DAM and TSC in aqueous phosphoric acid

for 1 hr. The reaction conditions were identical with those used routinely in clinical laboratories for the determination of blood urea nitrogen (this will be referred to as the "routine method" hereafter)[8]; DAM: TSC: urea=900: 50: 1 (molar ratio). The absorption spectrum of the colored solution showed a maximum at 530 nm as well as shoulder bands at 490 mn and 560 nm (Fig. 1). When the reaction mixture was neutralized with saturated potassium carbonate solution and then the decolorized solution was analyzed by thin-layer chromatography (TLC), 12 components could be detected under ultraviolet (UV) irradiation (Fig. 2). Of these spots, only three components turned red under acidic conditions, as shown in Fig. 2A (black spots). Both TLC and spectral analyses indicated the presence of a number of color reaction products. In order to avoid the formation of numerous by-products in the routine method, a simple procedure for the isolation of colored substances was utilized, in the following manner.

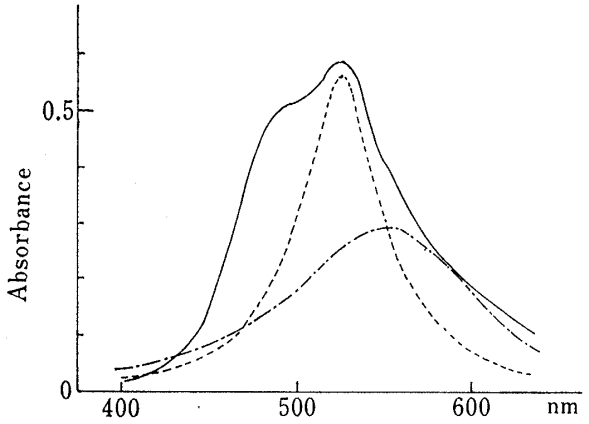

Fig. 1. Absorption Spectra of the Color Reaction Mixture and the Isolated Products

——: reaction mixture, ----: isolated **3a** (25 μM) on dilution with 6 N H_2SO_4, —·—: **3a** at 3 days after dissolution.

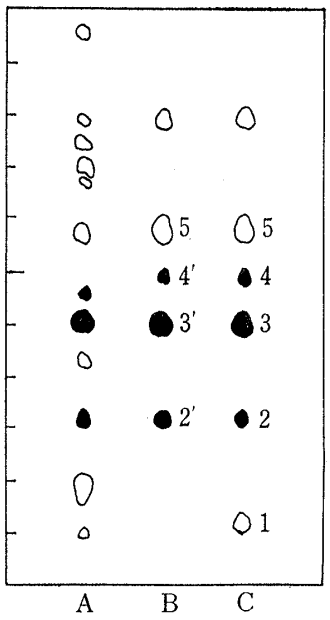

Fig. 2. Thin-Layer Chromatograms of the Color Reaction Products (Silica gel; 3% Methanol in Chloroform)

A: routine method. B: reaction of urea with DTZ in 10% sulfuric acid in methanol. C: reaction of *p*-tolylurea with DTZ in 10% sulfuric acid in methanol. Black spots represent reddish components that appeared after treatment with conc. H_2SO_4. 1: compound **5b**. 2: compound **1b**. 3': compound **3c**. 3': compound **3a**. 2', 4', and 4: unknown. 5: DTZ.

When an aqueous solution of equimolar amounts of DAM and TSC was stirred at room temperature, needle-shaped crystals of diacetylmonoximethiosemicarbazone (DTZ, **2**) separated out in a short time. DTZ, **2**, was also present in trace amounts in the DAM–TSC reagent which was used in the routine method as checked by TLC analysis. Equimolar amounts of urea and DTZ in 6 N sulfuric acid were heated in a boiling water bath for 1 hr. The reaction mixture became reddish by the end of heating. The absorption spectrum of this acidic solution coincided well with that of the solution obtained by the routine method. However, three color reaction products along with unreacted starting materials could be detected by TLC analysis (Fig. 2B). In addition, it appeared that the sensitivity of the color reaction was increased when the reaction was carried out in 10% sulfuric acid in methanol.

In order to isolate the colored substance, urea and DTZ were refluxed in 10% sulfuric acid in methanol. The reaction mixture was neutralized with saturated barium hydroxide solution, and separated by preparative TLC (silica gel) to give pale yellow amorphous **3a**. The

compound **3a** developed a red color when dissolved in an acidic medium and gave an absorption maximum at 530 nm, which was identical with the major absorption band of the reaction mixture. This sharp absorption maximum shifted bathochromically to 560 nm when the solution was allowed to stand for three days at room temperature, and the color of the acidic solution of **3** faded within one week (Fig. 1).

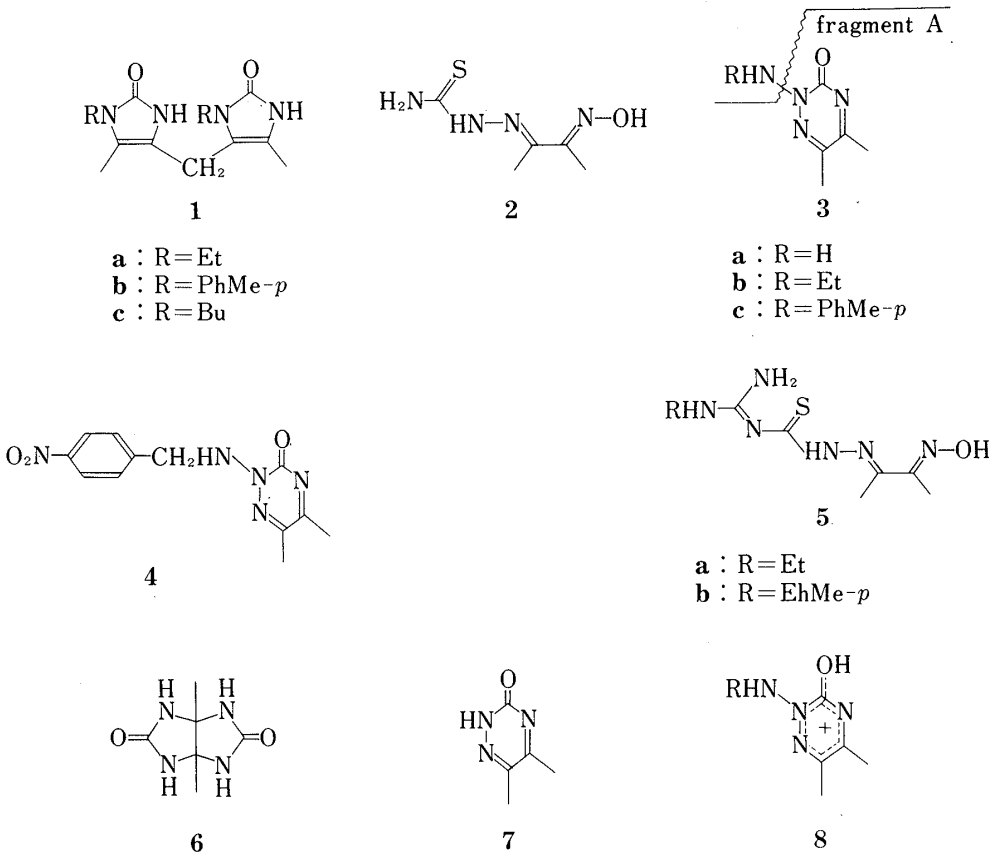

Fig. 3

In contrast to the formation of an amorphous product in the reaction of urea with DTZ, the reaction of DTZ with a urea derivative such as ethyl- or *p*-tolylurea provided a crystalline colored product. When ethyl- or *p*-tolylurea was heated with DTZ under the conditions used in the case of urea, the ethyl or *p*-tolyl derivative of **3**, **3b** or **3c**, was obtained along with a condensate, **5a** or **5b**, and an imidazolone derivative, **1a** or **1b**. A thin-layer chromatogram of the reaction mixture is shown in Fig. 2C. The major products, **3b** and **3c**, showed an absorption maximum at 530 nm and at 535 nm, respectively, in an acidic medium. The imidazolone derivative, **1**, was found to be the same product as that isolated after the D-glucuronolactone-sensitized reaction.[6] The absorption maximum of **1a** or **1b** in an acidic medium was observed near 490 nm, which corresponded to the minor peak (shoulder band) of the reaction mixture (Fig. 1). The structure of **1b** was established spectroscopically by comparing various data with the reported values.[6]

It was reported[9] that tetrahydroimidazoimidazolone-2,5-dione, **6**, was obtained in the reaction of urea with DAM in acidic media in the absence of oxidizing or sensitizing agents. However, **6** was not isolated at all during the color reactions in the presence of TSC.

The spectral data of the major reaction products, **3a**, **3b**, and **3c** are summarized in Table I. The proton magnetic resonance (PMR) spectrum of **3a** showed only three signals, two signals of methyl protons (3H×2) and one signal of active protons (2H) which disappeared upon addition

of D_2O. The infrared (IR) spectrum of **3a** showed a broad peak in the region of 3400 cm^{-1} (Table I). Therefore, it was concluded that the active protons of 3**a** were attributable to a primary amino group. The amorphous product, **3a**, was converted to a crystalline derivative, **4**, by reaction with *p*-nitrobenzyl bromide in the presence of sodium carbonate. The molecular formula of **4** was established to be $C_{12}H_{13}N_5O_3$ by elemental analysis and mass spectroscopy. The PMR signal of the benzyl protons of **4** at 4.72 ppm split into a doublet (J=7.2 Hz) due to coupling with the proton of the secondary amino group. Two singlet methyl signals were observed in common for **3a**, **3b**, and **3c** at near 2.2 and 2.4 ppm in DMSO-d_6. These signals were assumed to be associated with the dimethyltriazolone skeleton, since it was reported that **7** gave methyl signals at 2.20 and 2.40 ppm in the same solvent.[10] The mass spectra of **3a**, **3b**, and **3c** showed parent peaks at *m/e* 140, 168, and 230, respectively, and a fragment peak at 125 which is attributable to the molecular ion peak of the dimethyltriazolone moiety (fragment A). Therefore, it was assumed that the structure of **3** might be 2-(*N*-substituted-amino)-5,6-dimethyldihydro-3*H*-1,2,4-triazine-3-one.

TABLE I. Spectroscopic Data for the Main Reaction Products

	3a	**3b**	**3c**
λ_{max} (nm, 6 N H_2SO_4)	535(4.02)[a]	530(4.07)[a]	535(4.49)[a]
IR (ν_{max}^{KBr}, cm^{-1})	3400[b]	3320	3320
	1680	1675	1675
	1645	1640	1640
PMR (δ^{DMSO}, ppm)	2.20(s, 3H)	1.21(t[c], 3H)	2.28(s, 3H)
	2.45(s, 3H)	2.22(s, 3H)	2.40(s, 3H)
	5.2 (b, 2H)	2.47(s, 3H)	3.38(s, 3H)
		3.98(m, 2H)	4.7 (b, 1H)
		4.5 (b, 1H)	7.2—7.6(m, 4H)
MS (*m/e*)	140(M^+)	168(M^+)	230(M^+)
	125	139	215
	116	125[d]	125[d]
	60[d]	60	60
	44	44	44

a) log ε. *b*) Neat. *c*) J=7.5 Hz. *d*) Base peak.

The color reaction of urea derivatives with DAM is known to be a condensation reaction involving dehydroxylamination. One of the color reaction products **1** was obtained in the presence of both D-glucuronolactone and TSC. Therefore, these compounds should have contributed only to the elimination of hydroxylamine and the stabilization of the color of **1**. However, the skeletone of the main reaction product, **3**, contains three nitrogen atoms, two of which may be derived from TSC. These results indicated that the formation of **3** should involve an intramolecular rearrangement of an unstable cationic intermediate. It has been demonstrated that symmetrical tetrazole[11] and certain triazoles[12] become colored red in acidic media. Thus, we assumed that the color of the pigment **3** in an acidic medium was due to an aromatized cationic species such as **8**.

It was concluded that TSC condensed with DAM to form **2**, which then reacted with urea to yield the red-colored **8**. This reaction process definitely differs from the original Fearon reaction, which does not use TSC. A further investigation of the color reaction mechanisms regarding the rearrangement of cationic intermediates is in progress.

Experimental

General——The mass spectra were obtained with a Hitachi RMS-4 spectrometer; the IR spectra with a Hitachi 215 spectrometer; the PMR spectra with a JEOL PS-100 spectrometer (with Me_4Si as the internal standard), and absorption spectra with a Shimadzu UV-210A spectrometer.

DAM, TSC, urea, ethylurea, and *p*-tolylurea were obtained from commercial sources.

Isolation of 3a——A mixture of 1.5 g (8 mmol) of DTZ, 0.5 g (8 mmol) of urea, 40 ml of methanol, and 3 ml of conc. sulfuric acid was heated in a boiling water bath for 1 to 4 hr. The solution was cooled with running water, and the precipitate was removed by filtration. The filtrate was neutralized with saturated barium hydroxide solution. The solvent was evaporated off *in vacuo*, and the crude oily material obtained was purified by preparative TLC (silica gel, 10% methanol in chloroform) to give **3a** as a sole product. The UV, IR, mass (MS) and PMR spectral data of **3a** are listed in Table I.

***p*-Nitrobenzyl Derivative of 3a**——A mixture of 1.06 g (10 mmol) of sodium carbonate, 0.65 g (5 mmol) of **3a**, and 30 ml of water was treated with 2.16 g (10 mmol) of *p*-nitrobenzyl bromide in 60 ml of ethanol. The solution was refluxed for 1.5 hr, and then inorganic material was removed by filtration while the mixture was hot. The precipitate obtained by cooling the filtrate in an ice-salt bath was collected by filtration and recrystallized from water–methanol to give **4** as pale yellow crystals. **4**: yield, 584 mg (56%), mp, 250° (dec.); IR (KBr), 3120, 1680, 1635, and 1240 cm^{-1}; PMR (DMSO-d_6), δ 2.20 (s, 3H), 2.42 (s, 3H), 4.72 (d, $J=7.2$ Hz, 2H), 5.1 (b, 1H), and 7.3—7.7 (m, 4H). *Anal.* Calcd for $C_{12}H_{13}N_5O_3$: C, 52.36; H, 4.76; N, 25.45. Found: C, 52.98; H, 4.57; N, 25.27.

Reaction of Ethyl- or *p*-Tolylurea with DTZ——A mixture of 0.8 g (10 mmol) of ethylurea or 1.5 g (10 mmol) of *p*-tolylurea, 1.74 g (10 mmol) of DTZ, 50 ml of methanol, and 5 ml of conc. sulfuric acid was heated in a boiling water bath for 4 hr. The solution was cooled with running water, and the precipitate (A) was collected by filtration. The acidic filtrate was diluted with excess water and extracted with chloroform. The viscous residue obtained after removing the solvent *in vacuo* was purified by preparative TLC (silica gel, 5% methanol in chloroform) to give **3b** or **3c**. **3b**: mp, 224—226° (microcrystals). *Anal.* Calcd for $C_7H_{12}N_4O$; C, 49.98; H, 7.19; N, 33.31. Found: C, 49.81; H, 7.00; N, 33.49. **3c**: mp, 262—264° (microcrystals). *Anal.* Calcd for $C_{12}H_{14}N_4O$; C, 62.59; H, 6.13; N, 24.33. Found: C, 62.76; H, 6.42; N, 23.98.

In addition, the precipitate (A) was extracted with chloroform, and the extract was dried over anhy. sodium sulfate, then concentrated *in vacuo*. The crude residue obtained was further purified by preparative TLC (silica gel, 10% methanol in chloroform). **1a** or **1b** was recovered from the fast-moving band ($Rf=0.7$) and it was confirmed that the IR and PMR data of these compounds were identical with the reported values.[6] A condensate, **5a** or **5b**, was obtained from the slow-moving band ($Rf=0.15$). The IR and PMR spectra of **5a** and **5b** were identical with those of corresponding authentic samples.

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References and Notes

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