Structure Elucidation of Chromogen Resulting from Jaffe's Reaction

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Summary: Jaffe's reaction is a reaction of creatinine with alkaline picric acid resulting in red chromogen. This color is being used widely for the estimation of creatinine in biological fluids without knowing the exact structure of the chromogen. The red species is isolated and its UV/Visible, ^{1}H- and ^{13}C- NMR studies are carried out. It is found that the red species is not a pure compound but a mixture of stereoisomers. Moreover, the red species in water reflects the same UV/Visible spectra as given by the reaction of creatinine with alkaline picrate in aqueous media. The red species is a 2:3 molar Meisenheimer σ-complex of creatinine-picric acid **(6)**.

Introduction

Determination of creatinine is an important marker in evaluation of renal function in the clinical laboratories. In the recent years, it is important due to the discovery of inherited defects of creatine biosynthesis [1, 2]. The most common methods for the determination of creatinine is based on the Jaffe's reaction in which creatinine and picric acid form a complex under alkaline conditions [3,4]. Despite of simplicity of method, the original Jaffe's procedure has been modified many times due to its lack of selectivity and interference of metabolites [5-9]. Although, a large number of modifications of the Jaffe's reaction were proposed, yet none has eliminated all interferences and serum samples remained a problem. No doubt, the reaction was adopted by many workers but the nature of the red species formed in the reaction has never been determined with certainty.

Firstly, the structure was suggested to be a tautomer of creatinine picrate but did not specify the exact structure of the tautomer [10]. It was suggested that one mol of the picric acid appeared to be required for each mol of creatinine, but the full chromogenic value of the creatinine develops in the presence of an excess of picric acid. They roughly estimated that 2.5 moles of picric acid were needed for each mole of creatinine. Similar views were also expressed by others [11-13]. Greenwald [14] isolated a red solid from the reaction mixture and postulated both 1:1 and 2:1 complexes of creatinine and picric acid. This red solid was quite different from the picrate, but he reasserted his previous views that formation of red colored tautomer of creatinine-picrate was the cause of color. Bollinger [15] obtained a red solid which was analyzed as an equimolar complex of picric acid, and creatinine (1:1) and two moles of sodium hydroxide. Seeling [16] from spectroscopic and chromatographic studies, showed that the red species was not picramic acid whereas Selling and Wust [17] proposed structure **(1)**. Same type of species was detected in the reaction of amines with polynitro compounds [11], but it had an exceedingly short life-time and it seemed unlikely that **(1)** is the correct structure. Butler [18] claimed that red species formed in the Jaffe's reaction was the result of attack by the creatinine anion on the un-substituted position of picrate to give 1:1 and 2:1 complexes. On the basis of spectrophotometric, kinetic and nuclear magnetic resonance studies, it was tried to prove that alkaline picrate and creatinine reacted to form a 1:1 adduct [19]. He proposed structure **(2)** in addition to **(1)**, but the spectroscopic evidences given in their support were inconsistent with **(2)** and **(1)**. Kohashi *et al.*, [13] described a mechanism of color reaction of active methylene compound with 1,3,5- trinitrobenzene derivatives and proposed structures **(3-5)**. The color observed in the Jaffe's reaction was the result of isomer **(3)**. The formation of **(4)** and the presence of equilibrium between the isomers of **(3)** and **(4)** were confirmed by H-NMR. The augmentation of the color intensity on neutralizing the alkaline reaction mixture of picric acid and creatinine might be explained in terms of shifts of the equivalence between the isomers of **(3)** and **(4)**. Three isomers of **(5)** were formed *via* the isomers of **(3)** and **(4)** in an alkaline mixture of picric acid and creatinine which were not found in excess

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amount of picric acid, as detected by HPLC. Butler [20] defended his work as described earlier and rejected the evidence illustrated by Kroll *et al.*, [21] against Janovsky complex formation. An artificial receptor for the detection of creatinine was also designed to elucidate the chromogenic mechanism by X-ray crystal structure of creatinine, the free receptor and the complex [22] but the structure of complex resulted from Jaffe's reaction is still mystery.

The main purpose of the present study is to deduce the structure of chromogen by spectroscopic techniques responsible for red color formation in Jaffe's reaction.

Results and Discussion

It is found that picric acid reacts with creatinine in alkaline media to afford a bright red substance **(6)** which does not melt at all due to an in-volatile salt. It does not show a molecular ion peak and we cannot deduce anything from its mass spectrum. However, on the basis of elemental analysis and spectroscopic evidence, we suggest a molecular formula of $C_{26}H_{12}N_{15}O_{23}Na_7$ for compound **(6)**. The percentage of hydrogen found is greater than the required amount because the compound is highly hygroscopic in nature and difficult to dry. However, after prolong drying under reduced pressure at 100 °C, it does not show any absorption above 3000 cm^{-1} which negates the presence of NH stretching and indicates uptake of moisture during sample handling. When it is kept open for five minutes at room temperature, it showed a very broad absorption band due to the hydroxyl group. Other portion of the spectrum (C=O and C=N group) remained consistent. The same problem is encountered during sodium analysis. In normal way, the percentage of sodium is found to be 13.6 units but using a dry box technique under nitrogen, the value has increased to 14.15% (required 15.1%). Despite all possible precautions, we could not avoid some moisture absorption, probably during the sample transfer to dry box. This explains the poor analysis of the compound **(6)**. Moreover, it is a mixture of stereo-isomers as has indicated by its ^{1}H- and ^{13}C-NMR studies. It is believed that nature of the mixture has also contributed in the poor analysis of compound **(6)**.

The ^{1}H-NMR spectrum in D_2O at 400 MHz shows two singlets at δ 3.61 and 3.96 (-NCH_3), one doublet at 3.89 ($2H_c$) and two other doublets which are almost overlapping at 6.15 ($2H_d$). The singlet at 9.6 is assigned to aromatic protons (2H). The integrated peak areas match well with their respective number of protons. In the ^{13}C-NMR spectrum, two N-Me groups (C_1) absorb at δ 30.87 and 31.95, C_2 at 41.59, 43.13 and 43.35, respectively. In addition, two more peaks appear at 60.88 and 61.25 which we assign to C_3. After careful look at the spectra, it seems that C_3 shows three peaks and one of them is overlapped. In the off-resonance spectrum, C_1 splits into quartets, C_2 and C_3 into doublets. The aromatic carbon (C_5) resonates at 127.9 and also exhibits a doublet. A detailed study of spectral data suggests that the structure of compound **(6)** appears to be symmetrical and a small discrepancy in chemical shifts of C_1 (30.87, 31.95), C_2 (41.59, 43.13 and 43.35) and C_3 (60.88 and 61.25) may be the cause of very slight twist in molecule induced by the rotation of C_2-C_3 bond (rotational isomers). There are two chiral carbons (C_3) and at least four stereoisomers are possible of compound **(6)** and chemical shift discrepancy may be due to this reason. Similarly, C_7 displays two absorptions at δ 170.00 and 171.93, and a group of C_8 at 188.96, 189.35 and 189.68, respectively. The resonance at 119.38 is unique and is assigned to C_4. Its chemical shift agrees with literature value [23] and it remains as a singlet in the off- resonance spectrum which provides an additional proof for the symmetry of molecule **(6)**. Otherwise, the picture of the molecule would have been different. Hence, we assign two structures **(6)** and **(7)** to $C_{26}H_{12}N_{15}O_{23}Na_7$, and it is difficult to distinguish between two, in addition to their stereoisomer.

In compound **(6)**, sp^2-carbons ($C_{9\text{-}12}$) containing nitro groups do not appear in the spectrum. Probably, the nitrogen-14 nucleus possesses an electric quadrupole moment, and is, therefore, able to interact with both electric and magnetic field gradients, which cause the nucleus to tumble rapidly, so the spin-lattice relaxation, especially dipole-dipole relaxation, is greatly affected. Since the spin-lattice relaxation time is longer, signals for carbon bearing a nitrogen are either broad or small and sometimes do not appear in the spectrum. Similar effects have also been reported in the case of nitrobenzene [24], 1,3,4-trichlorobenzene and other quaternary carbons [25]. Despite this, the suppression of peak intensity is further aggravated due to the solvent used which adversely affects relaxation phenomena, in addition to changing chemical shift positions.

^{1}H-NMR data of picric acid has further endorsed our findings. As mentioned in the experimental section, ring protons in various solvents resonate at their respective place (small differences in chemical shifts are due to solvent). On addition of a few drops of D_2O, the peak intensity was reduced. Then the spectrum was recorded in pure D_2O and a very small peak appeared at 8.9 ppm.

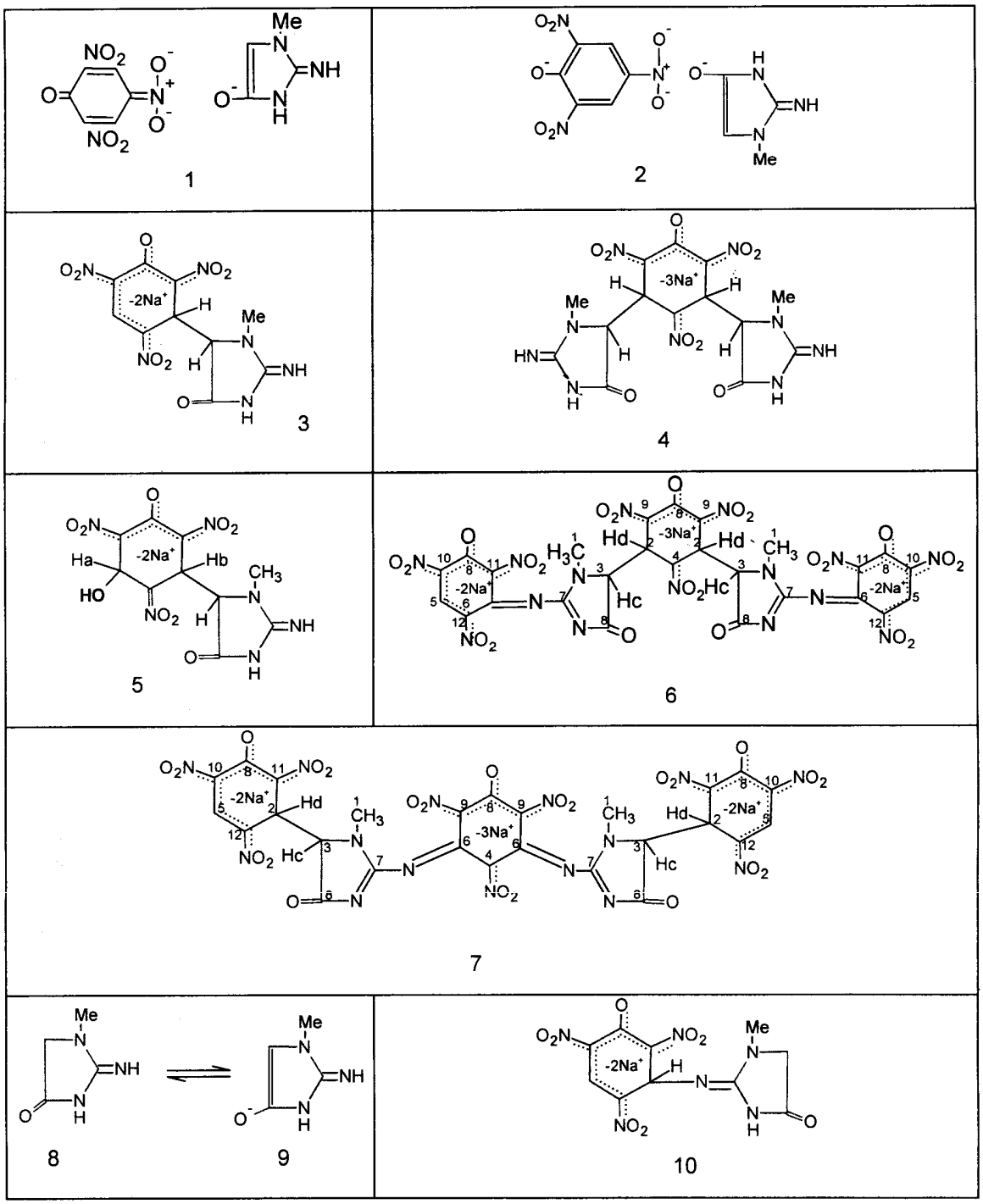
Creatinine behaved differently in neutral and alkaline media. Methyl and methylene protons appeared at 3.04 ppm and 4.03ppm in D_2O, respectively. The proton of the NH group is exchanged with deuterium. Kohashi [13] and Butler [18] reported that the carbanion of creatinine attacked the unsubstituted positions of picric acid to give **(3)** and **(4)** and Vasiliades [19] claimed that it was enolate anion of creatinine which involved in the formation of 1:1 complex. He suggested structures **(1)** and **(2)**. Likewise, Kroll *et al.*, [21] suggested that the enolate anions of ketones reacted with picric acid to yield Janovsky complex and hence, enolate anion of the creatinine follows the same path. Contrarily, Butler [20] reported that the formation of a C-C bond which appears to be the driving force in a Janovsky complex formation is due to carbanion and not enolate anion. However, our findings do not agree with any one of these structures. There is, of course, another possibility that imino (C=N-) nitrogen may attack the *m*-positions of picric acid resulting in **(10)** and **(11)**, and again our findings are inconsistent with structures **(10)** and **(11)**.

However, it was decided to examine the behavior of only creatinine in neutral and in alkaline media. The ^{13}C-NMR spectrum in D_2O exhibited four absorptions at δ 32.91, 59.09, 172.11 and 191.26 respectively. The first two absorptions were assigned to -NMe and methylene (-CH_2-) groups and they split into a quartet and a triplet in the off-resonance spectrum. The last two remained unchanged and were attributed to imino-carbon and carbonyl groups respectively. An aged solution did not show any deuterium exchange with methylene protons. However, on addition of a few drops of NaOH into aqueous creatinine solution, a series of lines emerged on the spectrum. After careful study of data, as mentioned in the experimental section, we found that, in addition to NH proton, sodium hydroxide had abstracted only one proton from methylene group and the resulting trianion **(12)** remained in equilibrium with the starting material **(8)**. That is why, two -NMe absorptions were observed at δ 31.12 (C_1) and 37.46 (C_2). A collection of five lines resulting from C_3 (doublet) and C_4 (triplet) was also seen in the off-resonance spectrum. Two signals appeared at δ 54.81 and 57.14ppm attributed to C_3 and C_4 respectively. Besides this, C_5, C_6, C_7 and C_8 in creatinine molecules **(12-14)** absorbed at δ 162.25, 173.68, 177.81 and 187.92 respectively, and they remained unsplit in the off-resonance spectrum. All the carbons pertaining to structure **(12)** moved upfield compared to the carbons of structure **(8)**. This study unambiguously indicates the maximum population of structures **(8)** and **(12)** in solution. Moreover, resonances belonging to C_9 and C_{10} were not seen which reflected that probably species **(13)** and **(14)** were in a very low concentration or did not exist, although theoretically, their existence is possible. Removal of both hydrogens from methylene group in presence of protic solvent seems to be unlikely. Despite this, when creatinine spectrum in D_2O + NaOD was taken, a broad hump resulting from attack of deuterium at methylene carbon (C_3) came up while other absorption peaks remained unaffected.

There is, of course, another possibility. Does creatinine skeleton remain intact or rupture in a basic media? We found that there was no change in creatinine structure when it was stirred in 5% sodium hydroxide solution at room temperature. The material was recovered unchanged and showed no depression in mixed melting point. Even during hydrolysis of compound **(15)** with concentrated sodium hydroxide, the creatinine skeleton remained intact and the resulting product **(16)** was identified by all means. Thus, the facts so far we have obtained, reflect that creatinine in a basic media behaves as a molecule with two nucleophilic centres, i. e., carbanion and imino nitrogen anion (C=N-) both are involved in the formation of complex **(6)**.

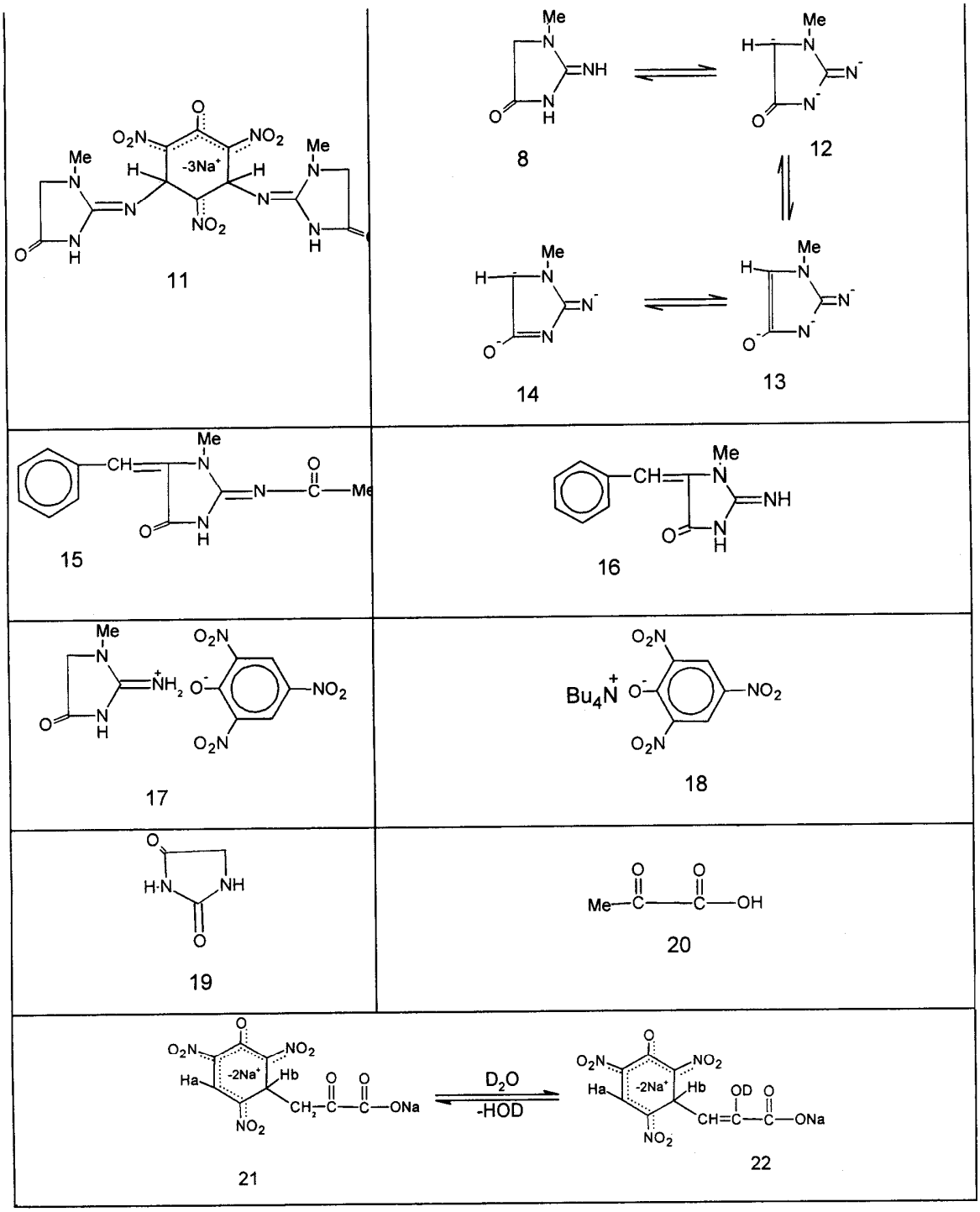
Complex **(6)** is soluble in water and on acidification (HCl) of aqueous solution of complex **(6)**, a red solid settled down **(17)**. Greenwald and Gross [10, 14] also reported similar chemical changes. This red solid changed its color to yellow when heated at 130-140°. This picrate **(17)** was further confirmed by comparison with authentic sample. From the filtrate, sodium chloride and picric acid were recovered and proved by physical and chemical means.

In another experiment, we tried to replace sodium cation by organic counterpart (phase-transfer catalyst). During vigorous shaking and multiple

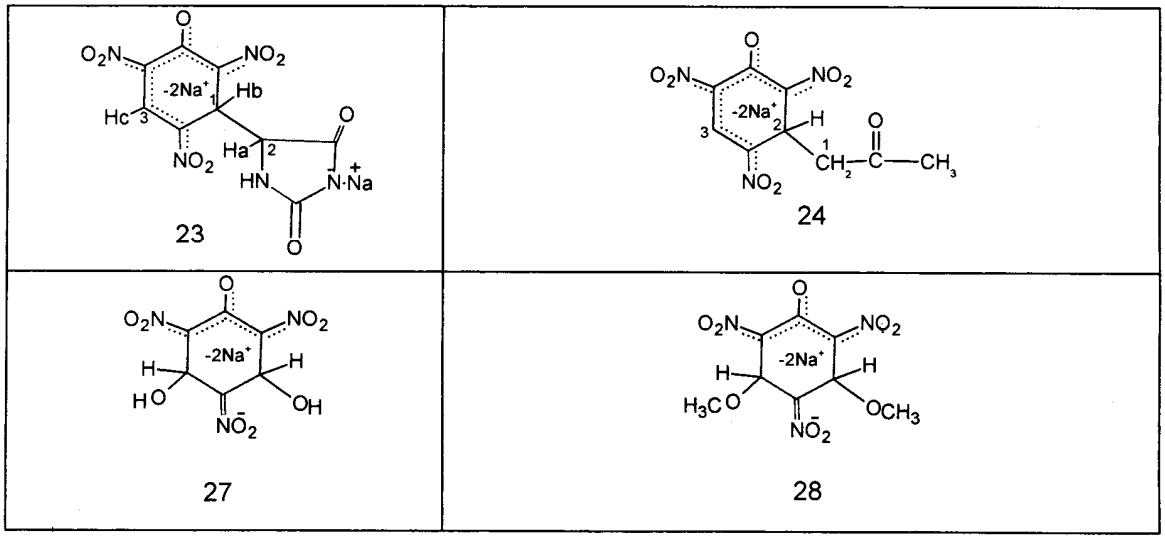


extractions, again we observed that compound **(6)** had fragmented and we could only isolate picrate of tetrabutylammonium **(18)**.

There are several functional groups on the creatinine molecule, any one of which may be involved in the Jaffe' reaction. We tried to methylate nitrogens of creatinine, especially the imino group (C=NH) but were unsuccessful. However, compounds like hydantoin **(19)** and pyruvic acid **(20)** were taken which had no imino group (C=NH) and acted as a molecule with single nucleophilic centre in



alkaline media. Several researchers have reported interference of pyruvate and other α-keto compounds [21, 26-30]; bilirubin [21, 27-33]; cephalothrin [34] hemoglobin and lipids [30] in the estimation of creatinine present in biological fluids. This piece of information also prompted us to look into the reaction of picric acid with alkaline solution of pyruvic acid and hydantoin. Compounds **(19)** and



(20), both gave bright red powder which did not melt, so they must be salts. Their mass spectra were not informative. The characteristics of both were akin to **(6)**. Elemental analysis and spectroscopic evidence suggested molecular formulae of $C_9H_4N_3O_{10}Na_3$ **(21)** and $C_9H_4N_5O_9Na_3$ **(23)**.

The Meisenheimer complex of acetone-picrate **(24)** [35] is included for comparison. In this case, there is little deuterium exchange with methylene protons and all other protons absorbed at their right places. The chemical shifts and shape of peaks in ^{13}C and ^{1}H NMR of acetone-picrate well resemble with the Meisenheimer complexes of creatinine, pyruvate and hydantoin as reported herein. Spectral data of **(24)** are given in the experimental section.

Compound **(23)** is extremely hygroscopic and exhibited a very broad band at 3400-3100 cm^{-1} which was assigned to NH/OH groups. It did not lose water even after prolonged drying in vacuum at 100 °C. It seemed as the enolic form of compound **(23)** predominated. The remaining portion of the IR spectrum was consistent with C=O and NO_2 groups.

In the ^{1}H-NMR spectrum at 400 MHz, there were two doublets and a multiplet. The doublets at δ 4.3 (J_{ab}=4.0 Hz) and 8:75 (J_{cb}=5.0 Hz) were assigned to protons H_a and H_c. The multiplet at 5.36 was attributed to proton H_b. The resonance lines were too close to measure the exact coupling constant for H_b ($J_{ba,\ bc}$ =4.0 to 6.0 Hz). Here, the distinguishing feature that we observed in this spectrum was allylic couplings, but this type of allylic coupling was not observed in compounds **(21)** and **(24)**. Probably, restricted rotation of carbon-carbon bond (i.e., C_1 – C_2) due to size of hydantoin had fixed the proton H_b at such an angle where it could easily be coupled with proton H_c while free rotation in **(21)** and **(24)** did not permit allylic couplings. This observation suggests that structure **(6)** seems to be right. If structure **(7)** is assumed to be correct then it should exhibit allylic coupling like compound **(23)**, but nothing like this was observed. Hence, we propose structure **(6)**. Moreover, Kohashi et al.[13] have reported allylic coupling in picric acid – creatinine 1:1 σ–complex. But this kind of coupling is not found in our finding pertaining to structure **(6)**. A brief look at the structure of **(6)** indicates that it comprises two segments i.e., 1:1 and 1:2 picric acid – creatinine σ-complexes. So its proton NMR spectrum may resemble with a mixture of compounds **(3)** and **(4)**. Hence, it is doubtful to say weather compound **(6)** is pure compound or a mixture of compounds **(3)** and **(4)**. Contrarily, our findings indicate that presence of peak at δ 130 in ^{13}C-NMR spectrum negates the existence of compound **(4)** and absence of allylic coupling in ^{1}H-NMR spectrum rejects the formation of compound **(3)** and confirm that compound **(6)** is a pure compound with at least 2 dl pairs with several diastereo-isomers (rotational isomers). It is neither a mixture of σ 1:1-complex **(3)** and σ 1:2 –complex **(4)** nor a pure single compound of any one of two **(3)** and **(4)** with its diastereo-isomers.

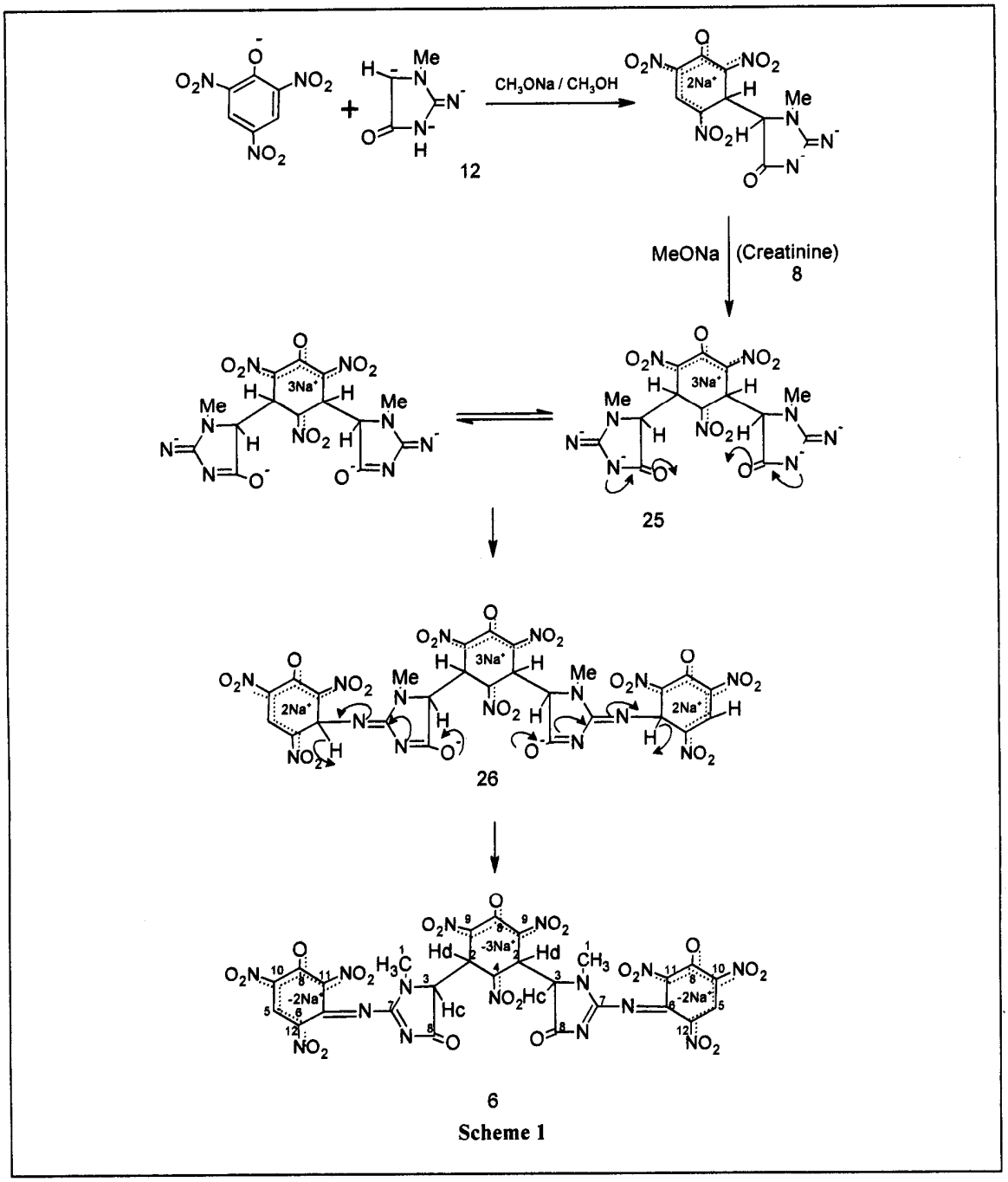
In the ^{13}C-NMR spectrum of compound **(23)**, C_1, C_2 and C_3 resonated at δ 43.32, 60.88 and

128.01 and each carbon was split into a doublet in the off-resonance spectrum. The carbonyl groups absorbed at δ 163.36 and 170.22 and also remained unchanged in the off-resonance spectrum. Hence, the studies of spectral data, physical and chemical properties of **(21, 23),** and **(24)** gave substantial support to structure **(6)** and confirmed that creatinine acted as a molecule with two nucleophilic centres in alkaline media.

It was further authenticated by ^{1}H- and ^{13}C-NMR study. In a broadband proton decoupling ^{13}C spectra, four lines of -NCH_3 carbon (C_1) appeared at δ 32.75, 32.82, 33.83 and 33.95 respectively. Similarly, a group of four lines due to C_2 appeared in the range of δ 43.50 to 45.30. Four peaks of C_3 emerged at d 68.86, 68.89, 69.16 and 69.32 respectively. C_4 absorbed at δ 121.19, 121.28, 121.39 and 121.47. C_5 with a very high intensity peak appeared at δ 130.06 while quaternary carbons (carbons with nitro groups) appeared little bit broader as compared to non-quaternary carbons. They have absorbed at δ 130.90 131.40 (C_9), 135.97 (C_{10}), 136.71 (C_{11}), 144.05 (C_{12}), 165.46, 170.16, 170.31, 171.88, 172.02, 173.78, 173.91 (C_7) and 178.55, 179.19 (C_6), respectively. A group of carbonyl carbons appeared at 190.94, 191.34, 191.44, 191.54, 191.74 (C_8). On spectral expansion all the peaks become quite discernable and recognized with respect to their chemical shift values. Kohashi *et al.*, [13] have also reported that 1:1 σ-complex is thought to have eight diastereo-isomers because of steric hindrance among the bulky creatinine residue and two nitro groups. Moreover, the shape and the intensity of the peaks reflect that some peaks have overlapped each other and do not give their own individual values. In DEPT spectra no methylene group has appeared and CH_3/CH groups have emerged at their right places. It further indicates that there is no decomposition of compound **(6)** during spectral recording and no contamination of un-reacted materials. All quaternary carbons (C_4, $C_{6\text{-}12}$) have disappeared suggesting the structure of compound is **(6)**. Moreover, this compound also rotates the plane polarized light to +1.5° when its 0.1% solution was used on polarimeter, model ATAGO Polax-2L, Japan. Above this concentration the color of the solution is too dense to measure the optical activity.

^{1}H-NMR in D_2O at 400 MHz further clarified the situation of compound **(6)**. Ten independent lines appeared in the range of δ 2.59 to 3.88 and were assigned to –NCH_3 groups. Five well defined doublets centered at δ 4.07, 4.11, 4.36, 4.46 and 4.51 were designated to H_c proton which in turn H_d proton coupled with H_c and gave well resolved four doublets (δ 5.12, 5.15, 5.21 and 5.25), the shape and integration of the peaks indicated that one more doublet in addition to four above-said had overlapped. Their coupling constant (J_{cd}) ranged from 2.4 to 3.6 Hz depending upon dihedral angle. Three lines of equal intensity and their proton integration ratios emerged at δ 8.63, 8.72 and 8.02 and we assigned them meta protons of the rings. The ^{1}H-NMR spectral studies reflected that at least five diastereo-isomers are present which have different chemical shifts in addition to enantiotropic protons which have the same chemical shifts. No allylic coupling is seen in any part of the spectrum and hence supports the structure **(6)**.

The reaction mechanism of creatinine-picrate is shown in Scheme 1. The reactive part of the creatinine molecule is the methylene group, activated by the neighbouring carbonyl group, and the other functional group in the molecule is C=NH. In the presence of base, both carbanion and imino anions are formed. As the carbanion is a better nucleophile than nitrogen anion, so carbanion first attacks the unsubstituted positions of picrate to give 2:1 complex **(25)**. Kohashi *et al.*, [13] and Butler [18] also proposed the same complex on the basis of kinetic evidence and ^{1}H-NMR spectroscopy. Greenwald and Gross [10], Butler [18] and Vasiliads [19] reported that the full chromogenic value of the creatinine develops in the presence of excess picric acid. However, here the nitrogen of imino group attacks the meta position of picrate ion to afford **(26)** which rearranges to complex **(6)** with removal of hydride ion. Although imino anion is a poorer nucleophile than carbon, yet there are precedents where aliphatic as well as aromatic amines and other related amino compounds form Meisenheimer complexes with polynitro aromatic compounds [35-39]. The removal of hydride ion in complex **(26)** is difficult to explain. However, there is evidence of removal of hydride ion from the reaction of nitrobenzene with KOH and trinitrobenzene with phosphorus (V) compounds. In this case, the presence of air or other oxidising agents encourage the elimination of hydride ion. Some conversion does occur in the absence of any added oxidising agent because nitrobenzene can act as an oxidising agent [40-41]. So, there is no reason why



Scheme 1

picric acid, which belongs to the same class of compounds as nitro derivatives of benzene, shall not facilitate the elimination of hydride anion and, to destroy it as it is formed. Moreover, the presence of methanol as a solvent in this reaction further helps in this connection. Makosza and Winiarski [42] reported the migration of hydride anion from the aromatic carbon to the electron deficient carbon of the nucleophile in the reaction of nitro compounds with α- halosulphones, N, N-dialkyl-1-haloalkane sulphonamides and acetonitrile derivatives. Similarly, the evidence for the migration of the hydride ion was obtained with the use of 3-deuterio-2, 4-dinitroanisole. The final product contained deuterium

at the two position. The deuterium exchange in the system 1,3,5-tricyanobenzene-$NaBD_4$ ($LiAlD_4$) also occur via the formation of a corresponding hydride σ-complex [43-44]. In complex **(6)**, we could not locate the hydrogen resulting from the hydride migration, but suggests probably, this hydride anion has migrated to unreacted picric acid (intermolecular rearrangement) which has been destroyed or washed away during washing of Meisenheimer complex **(6)** with methanol.

Butler [18] reported that in the clinical determination of creatinine both the picric acid and creatinine are at fairly low concentration and the red species formed under these conditions may not be the same as that precipitated from solution. We do not agree with this view because UV / Visible spectra of compound **(6)** when recorded on SP8-400 spectrophotometer are exactly identical to that given by Butler [18] and Vasiliades [19] in their publications. Again, Butler [18] pointed out that a study of the spectral changes occurring during Jaffe' reaction shows that addition of creatinine to alkaline picrate does not result in the formation of a new peak but broadening of that already present (470-550 nm). If the hydroxide ion present at high concentration the addition complex **(27)** is formed and it is this species which has such an intense absorption. However, the formation of **(27)** is likely, but could not find any evidence of complex **(28)** which is analogous to **(27)**. On the other hand, it is found that the absorption at 470-550 nm goes on increasing by increasing the amount of **(27)** in solution and the resulting spectra exactly coincide with that taken by Butler [18] at various concentrations. It may be possible that complex **(28)** acts as an intermediate and consumes during the formation of **(6)**. However, the broadening of peak (470-550 nm) is not associated with formation of complexes **(27)** or **(28)**. Moreover, the UV/Visible spectra of compound **(6)** in aqueous media was recorded on spectrophotometer Model U-2000. It showed the independent absorption peaks at 500.5, 505, 509.5, 518, 520, 531.5, 545, 550 and 560 nm in addition to the absorption peaks of creatinine and picric acid. These peaks move up and down with variation of concentration of the compound **(6)** and any one of them can be utilized for the estimation of creatinine in the biological fluids. An identical spectrum is obtained when creatinine is made to react with alkaline picrate in aqueous media. It means the compound **(6)** and the species resulting from the reaction of creatinine-alkaline picrate in aqueous media are the same. Hence, after working on every respect, we are certain that compound **(6)** is responsible for red coloration in Jaffe' reaction, but not compounds **(1-4)** and **(17)** as described previously by various workers.

Experimental

i) Reaction of Sodium Picrate with Creatinie

Freshly cut sodium metal (0.6 g) was dissolved in dry methanol (50 mL) and creatinine (1.13 g, 10 mmol) was added. On slightly warming and shaking, the solution became clear. A solution of picric acid (2.29 g, 10 mmol) in dry methanol was added drop-wise with constant stirring, at room temperature, during 3 h. A bright red solid settled out. It was washed with dry methanol and dried which gave Meisenheimer complex of creatinine-picric acid **(6)**.

$[\alpha]_D^{25^\circ}$ +1.5. IR (Nujol) ν_{max} cm^{-1}· 1640-1610 (C=O) and 1580-1560 (C=N); ^{1}H-NMR (D_2O, 400 MHz): δ 2.59, 2.60, 2.86, 2.88, 2.92, 2.94, 2.95, 3.20, 3.76 and 3.88 (–NCH_3), 4.07, 4.11, 4.36, 4.46 and 4.51 (d, J_{cd} 2.4-3.6 Hz, H_c), 5.12, 5.15, 5.21 and 5.25 (d, J_{cd} 2.4-3.6 Hz, H_d), 8.63, 8.72 and 8.02 (s, 1H); ^{13}C-NMR (D_2O, 100 MHz): δ 32.75, 32.82, 33.83 and 33.95 (-NCH_3, C_1), 43.50, 43.55, 45.07 and 45.30 (C_2), 68.86, 68.89, 69.16 and 69.32 (C_3), 121.19, 121.28, 121.39 and 121.47 (C_4), 130.06 (C_5), 130.90, 131.40 (C_9), 135.97 (C_{10}), 136.71 (C_{11}), 144.05 (C_{12}), 165.46, 170.16, 170.31, 171.88, 172.02, 173.78 and 173.91 (C_7), 178.55, 179.19 (C_6), 190.94, 191.34, 191.44, 191.54, 191.74 (C_8). (Found: C, 29.25; H, 1.46; N, 20.38; Na, 14.15 $C_{26}H_{12}N_{15}O_{23}Na_7$ requires C, 29.36; H, 1.13; N, 19.76; Na, 15.13%).

ii) Acidification of Complex (6) with HCl

Complex **(6)** (2.0 g, 1.88 mmol) was dissolved in water (20 ml) and slightly acidified with hydrochloric acid. On standing, a red solid settled out. It was washed with water, ether and then dried, m.p. 213° **(17)**. The filtrate was yellow in colour, extracted with ether which gave pure picric acid and from aqueous solution sodium chloride was recovered. Picrate of creatinine **(17)** (2 g, 6.9 mmol) in a dish was kept in an oven at 130- 140°. The red color changed into a yellow one, m. p. 213°, and there was no depression in mixed m. p.

m/z 229 and 113 (M^+); IR (Nujol) ν_{max} cm^{-1}): 3320-3300 (NH), 1795 (C=O), 1700- 1696 (C=N),

1635 and 1600 (C=C), 1560 and 1540 (NO_2). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 3.21 (s, 3H, -NCH_3), 4.34 (s, 2H) and 8.72 (s, 2H, Aryl. H); ^{13}C-NMR (DMSO-d_6): δ 31.17 (q), 54.02 (t), 125.21 (d), 141.78 (s), 157.46 (s), 160.85 (s) and 171.18 (s). (Found: C, 35.05; H, 2.74; N, 24.72 $C_{10}H_{10}N_6O_8$ requires C, 35.09, H, 2.94; N, 24.55%)

iii) Reaction of Sodium Picric Acid with Sodium Pyruviate

A methanolic solution of picric acid (2.29 g, 10 mmol) was added drop-wise to a basic solution of pyruvic acid (0.88 g, 10 mmol) (dissolved in a mixture of CH_3ONa + CH_3OH) with constant stirring at room temperature for 24 h. A red product was filtered off and washed thoroughly with anhydrous methanol which gave the Meisenheimer complex of pyruvic acid **(21)**.

IR (Nnujol) ν_{max} cm^{-1}: 1640-1590 (C=O). ^{1}H-NMR (D_2O, 400 MHz): δ 4.93 ($1H_b$, s) and 9.14 ($1H_a$, s), 2.5 (d, 2H, -CH_2-); ^{13}C-NMR: δ 27.34 (t), 49.55 (d), 127.97 (d), 141.94 (s) and 163.35 (s); (Found: C, 28.15; H, 1.02; N, 10.81; Na, 17.95 $C_9H_4N_3O_{10}Na_3$ requires C; 28.21; H, 1.05; N, 10.96; Na, 18.0 %).

iv) Reaction of Hydantion with Picric Acid

Reaction of hydantoin (1.0 g, 10 mmol) with picric acid (2.29 g, 10 mmol) using similar conditions as described above gave the Meisenheimer complex of hydantoin-picric acid **(23)**.

IR (Nujol) ν_{max} cm^{-1}: 3100-3400 (NH/OH) and 1715 (C=O); ^{1}H-NMR (D_2O): δ 4.3 (d, J_{ab} =4Hz, $1H_a$), 5.36 [m (dd), $J_{ba,\ bc}$ =4-6 Hz, $1H_b$], 8.75 (d, J_{cb} =5.0 Hz, $1H_c$); ^{13}C-NMR: δ 43.32 (d), 60.88 (d), 128.01 (d), 163.36 (s) and 170.22 (s); (Found: C, 24.54; H, 2.43; N, 15.86; Na, 15.66. $C_9H_4N_5O_9Na_3.3H_2O$ requires C, 24.06; H, 2.24; N, 15.59; Na, 15.36%).

v) Meisenheimer complex of acetone-picric acid **(24)** was prepared by known method [39].

IR (Nujol) ν_{max} cm^{-1}: 1670-1690 (C=O); ^{1}H NMR (DMSO-d_6) δ 2.10 (s, -CH_3), 2.50 (d, J_{ab} =3.5Hz, 2H), 4.82 (t, J=3.5Hz, 1H) and 8.56 (s, Aryl. H), ^{13}C-NMR (DMSO-d_6): δ 29.37 (q), 36.76 (t), 47.18 (d), 118.74 (s), 122.79 (s), 129.0 (s), 132.02 (d), 159.99 (s), and 170.64 (s).

vi) NMR of Picric Acid in Various Solvents

IR (Nujol) ν_{max} cm^{-1}: 3100 (OH) 1630 and 1610 (C=C); ^{1}H-NMR (acetone-d_6): δ 9.06 (s, 2H), 8.12 (s, 2H) and 10.4 (s, 1H). ^{1}H-NMR (DMSO-d_6): δ 8.62 (s, 2H); ^{13}C-NMR: 126.56 (d), 138.71 (s), 153.21 (s) and 182.71 (s), ^{13}C-NMR (DMSO-d_6 + 2 drops NaOD): δ 126.48 (d), 128.15 (s), 142.14 (s) and 162.52 (s), ^{13}C-NMR (DMSO-d_6 + D_2O 1-2 drops): δ 127.02 (d), 130.79 (s), 141.61 (s) and 160.08 (s), ^{13}C-NMR (D_2O + NaOD or $NaHCO_3$): δ 130.01 (d).

vii) NMR of Creatinine

^{1}H-NMR (D_2O): δ 3.04 (s, -NCH_3), and 4.03 (s, 2H), ^{13}C-NMR (D_2O): δ 32.91 (q), 59.09 (t), 172.11 (s) and 191.26 (s). ^{13}C-NMR (D_2O + NaOH): δ 31.12 (q), 37.46 (q), 54.81 (d), 57.19 (t), 162.25 (s), 173.68 (s), 177.81 (s), and 187.92 (s), ^{13}C-NMR (D_2O + NaOD): δ 31.0 (q), 37.28 (q), 161.49 (s), 172.95 (s) 177.40 (s) and 188.59 (s).

viii) Creatinine (2.0 g) in 5% sodium hydroxide was stirred at room temperature for 1 h. The resulting solution was neutralized with hydrochloric acid and then poured into excess acetone. White solid was settled down, washed with cold water, acetone and finally with ether. When dried, it had m.p. 260°, mixed m.p. 260°. The filtrate was reduced to dryness; nothing was obtained except a few crystals of sodium chloride.

ix) When above experiment was repeated with compound **(15)**, another compound **(16)** was obtained but creatinine ring remained intact.

m.p. 338° (dec.), m/z 201 (M^+); IR (Nujol) ν_{max} cm^{-1}: 3290-3120 (NH), 1710 (C=O), 1665 (C=N), and 1630 (C=C), ^{13}C-NMR (DMSO-d_6): δ 27.77 (q), 113.24 (d), 127.63 (d), 130.04 (d), 133.81 (s), 135.04 (s), 166.31 (s) and 174.46 (s) (Found: C, 65.40; H, 5.45; N, 20.92 $C_{11}H_{11}N_3O$ requires C, 65.65; H, 5.50; N, 20.88%).

Conclusion

It is found that the red species is not a pure compound but a mixture of stereoisomers. The red species is not a 1:1, 2:1 Meisenheimer complex of creatinine-picric acid but is 2:3 Meisenheimer σ–complex. It is also an optically active.

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