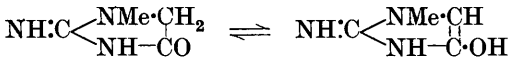
CLXI.—*The Constitution of the Red Isomeride of Creatinine Picrate responsible for Jaffé's Colour Reaction.*

By Winston Kennay Anslow and Harold King.

It was shown by Jaffé in 1886 (*Z. physiol. Chem.*, **10**, 399) that an intense red colour is produced when a solution of creatinine is treated with aqueous picric acid and then with caustic alkali. Although this reaction has been extensively used for the quantitative estimation of creatinine in biological fluids since Folin (*ibid.*, 1904, **41**, 223) made it the basis of a colorimetric method, it cannot be said that the chemistry underlying Jaffé's observation is at all understood. Greenwald (and Gross) have devoted considerable attention to this reaction and have discovered a number of facts of importance. They dismissed (*J. Biol. Chem.*, 1924, **59**, 601) the suggestion (Chapman, *Analyst*, 1909, **34**, 475) that the red colour was due to sodium salts of reduction products of picric acid (picramic acid, etc.) and produced evidence for the existence of a red "tautomeride" of

yellow creatinine picrate. The constitutional formula suggested by Greenwald (*J. Amer. Chem. Soc.*, 1925, **47**, 1440), however, is open to criticism.

By comparing the reaction of creatinine with that given by a number of its derivatives, Greenwald showed that the red colour was produced only by those derivatives in which a keto-enolic change was possible.



Furthermore, Weise and Trop (*Z. physiol. Chem.*, 1928, **178**, 125) conclude from a study of the reaction of numerous other substances with alkaline picric acid that Jaffé's reaction for creatinine is but a special case of a general reaction for active methylene or methine groups. Any explanation of the reaction must, therefore, take cognisance of the necessity for the presence of a mobile hydrogen atom of acidic character.

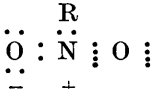
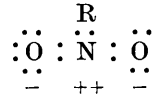
When the red alkaline solution of creatinine picrate was acidified, Greenwald and Gross, following on some earlier unpublished observations of S. R. Benedict and H. W. Banks, found that a red substance was precipitated which proved to be, on their interpretation, a " tautomeric " form of creatinine picrate. This could be changed by the application of heat into the yellow form of creatinine picrate. In a discussion of the conditions necessary for the production of the red colour, Greenwald (*loc. cit.*) assumed that creatinine or its substitutes function as bases, forming salts with picric acid. It is difficult to reconcile such an assumption with the observations that the red colour is only generated in alkaline solution and that it is given by non-basic substances such as malonic ester and ethyl acetoacetate.

The results of an investigation of Jaffé's reaction with which we have been engaged for some time confirm the existence of a red *isomeric* form of creatinine picrate and permit us to put forward a constitutional formula for this substance which is in agreement with the whole of the experimental evidence.

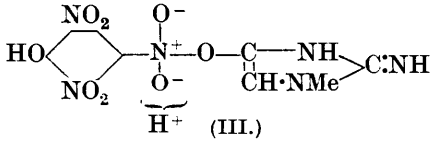
Red creatinine picrate may readily be prepared by the method to be described, a point of importance for obtaining a good yield being that acidification of the reaction mixture should be practically instantaneous. The product when freed from small amounts of uncombined picric acid by ether extraction is very sparingly soluble in water, readily soluble in sodium hydrogen carbonate solution, and is precipitated unchanged from the latter by mineral acids. It is not affected by contact with concentrated hydrochloric acid. It is, therefore, evident that the red alkaline solutions contain a red sodium salt of which red creatinine picrate is the free acid.

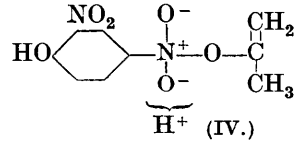
It is, indeed, possible to determine the number of points of acidity in the molecule by means of baryta. If red creatinine picrate be shaken with saturated baryta solution, in absence of carbon dioxide, it is converted into an insoluble scarlet barium salt. On analysis of the product the significant fact emerges that red creatinine picrate is a dibasic acid, and in presence of excess of baryta one acidic centre can form basic salts. Precisely similar results are obtained starting from a saturated solution of yellow creatinine picrate, containing yellow creatinine picrate in suspension. When treated with gradually increasing proportions of baryta, red barium salts of the same composition as those from red creatinine picrate are obtained.

In the light of the above facts, it is now possible to formulate red creatinine picrate and its salts. Since an alkaline solution of picric acid becomes deep red on addition of creatinine, it is unlikely that the phenolic group of the picric acid molecule is involved. Combination of picric acid and creatinine therefore takes place through the nitro-groups. The nitro-group contains normally one semi-polar double bond (I) and in its reactive phase two semi-polar double bonds (II) (compare Bennett and Willis, this vol., p. 258).

(I.)  and  (II.)

The anions of substances capable of keto-enol tautomerism and of substances containing reactive methylene groups are predominant in alkaline solution and will be attracted by the positively charged nitrogen atom in (I). The approach of the anion induces a greater degree of polarity as shown by the reactive phase of the nitro-group (II), with which co-ordination can then take place with consequent re-arrangement of charges. Alternatively, one might postulate an exiguous concentration of the reactive phase (II) as being present in solution in equilibrium with the normal form. Combination of creatinine and picric acid in alkaline solution thus takes place through co-ordination of the nitro-group with the enolic group and development of a new centre of acidity. Red creatinine picrate would therefore be written as (III). The problem as to which nitro-group

 (III.)

 (IV.)

is involved with creatinine and which, if any, is present in quinonoid form (compare Hantzsch, *Ber.*, 1906, **39**, 1084) with caustic alkali

remains undecided. It is of interest that two sodium salts, one containing 1 molecule of creatinine, 1 of picric acid and 2 of sodium hydroxide, the other, 2 molecules of creatinine, 1 of picric acid and 3 of sodium hydroxide have been prepared by Greenwald (*J. Biol. Chem.*, 1928, **77**, 539; 1928, **80**, 104) and fit in with the above formulation of the red acid. The presence of three nitro-groups in picric acid would allow of the addition of at least two molecules of creatinine in the way formulated above, and the compound formed would require 3 atoms of sodium as found by Greenwald.

In support of our formulation we may adduce an important observation of Reissert (*Ber.*, 1904, **37**, 837) which has now been followed up. When a solution of sodium ethoxide is added to an acetone solution of 2 : 4-dinitrophenol, the solution becomes red and if sufficiently concentrated deposits a brick-red sodium salt. When an aqueous solution of this salt is acidified with acetic acid, an intense violet-red colour is produced which is stable for some minutes, but if acidified with hydrochloric acid only a transient colour change occurs. We have prepared this sodium salt and find that under the most favourable conditions it consists of a mixture of 15·5% of the sodium salt of dinitrophenol with 84·5% of the disodium salt of an addition compound of acetone with dinitrophenol. The formulation (IV) of the additive compound is exactly analogous to that of red creatinine picrate.

When the disodium salt is acidified, the intense colour produced is evidently that of the unstable dibasic acid liberated.

Greenwald and Gross comment on the fact that picric acid cannot be replaced in Jaffé's reaction by other nitrophenols. Contrary, however, to the observations of these authors, we find that addition of creatinine to alkaline solutions of 2 : 4-dinitrophenol and of 3-methylpicric acid gives orange-red solutions, although in contradistinction to the reaction with picric acid a few minutes are required for the colour to develop. Furthermore, when alkaline solutions of 2 : 4-dinitrophenol, 2 : 6-dinitrophenol, 3-methylpicric acid, and 2 : 4-dinitro-1-naphthol-7-sulphonic acid containing creatinine are gradually treated with *N*-hydrochloric acid, they each give a transient red colour which at neutrality pervades the whole fluid and is temporarily stable, but disappears on addition of excess of acid. The colour cannot be reproduced by approaching the reaction mixture from the acid side. Ionisation of the enolic form or ionisation of the reactive methylene compound is an essential preliminary to formation of the co-ordination complex, and this condition is most readily attained by salt formation in alkaline solution.

EXPERIMENTAL.

Preparation of Red Creatinine Picrate.—A solution of picric acid (10 g.; 1 mol.) in boiling water (200 c.c.) was added to a solution of creatinine (5 g.; 1 mol.) in 50 c.c. of water. When cold, the solution, containing yellow creatinine picrate in suspension, was made alkaline with 50 c.c. of 2*N*-sodium hydroxide solution. The resultant deep red solution was cooled in ice and before crystallisation of sodium picrate had begun 50 c.c. of concentrated hydrochloric acid were added all at once with vigorous stirring. (Slow addition of acid gives a mixture of yellow and red precipitates which may finally be entirely yellow.) The red precipitate was collected and washed free from chlorides. When dry it weighed 8·6 g. and after removal of a small amount of picric acid by extraction in a Soxhlet extractor with ether, it weighed 7·1 g. When so prepared, "red" creatinine picrate is a carmine-coloured powder, and is definitely crystalline and anisotropic. When heated in a capillary tube, it turns yellow between 160° and 170° and then melts in the neighbourhood of 213°. Pure yellow creatinine picrate melts at 220—221° (Found: loss at 120°, 4·5; picric acid by nitron, 63·7. $C_4H_7ON_3,C_6H_3O_7N_3,H_2O$ requires H_2O, 5·0; picric acid, 63·6%).

The effect on the yield of varying the initial relative proportion of creatinine and picric acid is shown in the following table. The yield in each case is that obtained from 1 g. of creatinine.

Creatinine/picric acid	2 : 1	1 : 1	1 : 1·5	1 : 2	1 : 2·5
Yield of precipitate (g.).....	0·7	1·7	1·8	3·1	3·9
Yield after ether extraction (g.)	0·65	1·3	1·1	1·1	1·3
Loss at 120°, %.....	1·7	2·8	1·5	4·4	4·3
% Picric acid on anhydrous solid.....	66·3	66·2	66·6	66·6	66·7

($C_4H_7ON_3,C_6H_3O_7N_3$ requires picric acid, 66·9%).

From this table it is clear that the proportion recommended by Greenwald, 1 mol. of creatinine to 2½ mols. of picric acid shown in the last column, possesses no advantage over the proportion recommended by us as shown in the second column. The use of excess of picric acid results in an increased weight of precipitate, but most of this is free picric acid which has to be removed by prolonged ether extraction.

Conversion of Red into Yellow Creatinine Picrate.—Red creatinine picrate (0·53 g.) was boiled with water (17 c.c.) until a clear yellow solution was obtained. On cooling, pale yellow needles (0·44 g.) of yellow creatinine picrate were obtained as was shown by m. p., crystalline form and analysis (Found: picric acid, 66·7. Calc.: 66·9%). This property of forming a clear yellow solution on boiling with water was made use of for the estimation of the picric acid

content of red creatinine picrate. Once in solution, precipitation by nitron was carried out in the usual way.

Red Barium Creatinine Picrates.—The calculated amount of saturated, standard baryta solution (0·4 *N*) free from carbonate was added to very finely powdered red creatinine picrate in a stoppered vessel, and the mixture shaken for ½ hour. The product, a scarlet solid, was rapidly filtered off and then quickly transferred to porous plate without washing so as to avoid possible hydrolysis. It was then dried in a vacuum.

For analysis of these salts, the barium compound was dissolved in boiling water, dilute sulphuric acid added, and the barium sulphate collected and weighed. Picric acid was then determined in the filtrate by nitron. Nitrogen was determined by Kjeldahl's method after preliminary reduction of the nitro-groups with tin and hydrochloric acid.

The following table shows the results of analysis of the products obtained when red creatinine picrate was treated in the manner described with increasing amounts of baryta.

Picrate / Baryta	% Loss at 120°.	% Ba.	% Picric acid.	% N.	Ba : Picric acid : N.
1 : 1	15·6	24·0	37·3	13·7	1·07 : 1 : 5·99
1 : 1·1	14·8	25·5	35·5	12·5	1·2 : 1 : 5·76
1 : 1·5	14·7	29·3	33·6	12·7	1·45 : 1 : 6·18
1 : 1·65	14·3	29·8	29·7	11·8	1·67 : 1 : 6·49
1 : 2·75	15·2	31·2	30·0	11·4	1·73 : 1 : 6·2

[Calc. for the normal salt, $(C_{10}H_8O_8N_6)_2Ba_2,14H_2O$: H_2O, 20·9; Ba, 22·8; N, 13·9; picric acid, 37·9%. Calc. for the basic salt, $(C_{10}H_8O_8N_6)_2,Ba_3(OH)_2,20H_2O$: H_2O, 24·2; Ba, 27·7; N, 11·3; picric acid, 30·8%.]

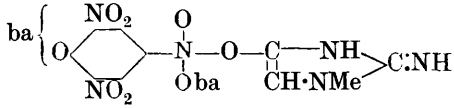
When yellow creatinine picrate was used in exactly the same way instead of red creatinine picrate, the results obtained were very similar and are shown in the following table :

Picrate / Baryta	% Loss at 120°.	% Ba.	% Picric acid.	% N.	Ba : Picric acid : N.
1 : 1	13·3	26·9	39·0	13·4	1·15 : 1 : 5·61
1 : 1·5	14·1	29·5	35·0	12·0	1·4 : 1 : 6·07
1 : 2	18·8	28·4	27·7	10·3	1·71 : 1 : 6·13
1 : 2·5	15·3	32·3	31·4	11·5	1·71 : 1 : 6·00

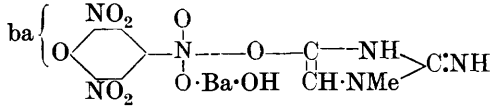
A study of these two tables, particularly the columns involving ratios, shows that red barium creatinine picrate is the barium salt of a dibasic acid. In the first row in both tables, where the baryta and creatinine picrate (red or yellow) are used in equimolecular proportion, the whole of the available barium is fixed and is equivalent to two acidic centres. Whatever excess of baryta is used, the barium fixed only rises very slightly above three equivalents. The high barium ratios found for the salts of highest barium content,

namely, 1·7 instead of 1·5, is explained by the difficulty of removing adhering baryta by the method of preparation employed. For this reason the analytical figures for barium are probably slightly high in every case.

The simplest interpretation of these results is that in the presence of two equivalents of baryta a normal barium salt is formed of the following formula, whereas in presence of excess of baryta a partly



basic salt is formed of the following formula :



When red barium creatinine picrate (1·0 g.) was ground with concentrated hydrochloric acid (5 c.c.), red creatinine picrate was liberated (yield, 0·25 g.). On heating in a capillary tube, it became yellow at about 150° and melted at about 209°. A mixture with red creatinine picrate melted at 211°.

Action of Sodium Ethoxide on 2 : 4-*Dinitrophenol in Presence of Acetone.*—2 : 4-Dinitrophenol (2 g.) was dissolved in a mixture of absolute alcohol (50 c.c.) and acetone (25 c.c.). The addition of sodium ethoxide solution (from 2·5 g. of sodium in 50 c.c. of alcohol) produced a deep red solution and in a few seconds this was followed by separation of a brick-red precipitate. This was collected, washed with absolute alcohol and ether, and dried in a vacuum (yield, 3·19 g.). The substance has no m. p. and explodes on heating. When dissolved in dilute sulphuric acid and extracted with ether, it gave a good yield of unchanged dinitrophenol, and when a small quantity was dissolved in dilute sulphuric acid and distilled, the first few drops of distillate had the odour of acetone and gave Legal's test.

On analysis the product proved to be mainly the disodium salt of acetone–dinitrophenol mixed with a small proportion of sodium dinitrophenoxide. Attempts to prepare a product uncontaminated with the latter salt were unsuccessful (Found : Na, 14·2; N, 8·8; acetone by Messinger's method, 15·4, whence ratio Na : N : acetone is 0·98 : 1 : 0·42. Calc. for a 84·5% mixture of $C_6H_3O_5N_2Na,C_3H_5ONa$ and 15·5% of $C_6H_3O_5N_2Na$, the whole containing 10% of moisture : Na, 13·8; N, 9·3; acetone, 15·4%).

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