The Detection and Estimation of Uric Acid by 2:6-Dichloroquinone-chloroimide

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When a very dilute (0.001–0.05 %) solution of uric acid is made slightly alkaline by addition of a pH 9–10 buffer, and treated with two to three drops of 0.4 % 2:6-dichloroquinone-chloroimide in ethanol, the mixture rapidly develops a bright yellow colour, which becomes red on addition of a few drops of 1 % silver nitrate. If the uric acid be in excess, the secondary colour change may be obscured by the formation of the dark brown silver reduction complex described by Schiff as a test for uric acid.

Although many biological compounds give colours with the chloroimide reagent in alkaline solution, cysteine and reduced glutathione were the only ones found to yield yellow pigments, and these differ from the uric acid pigment in that they are not turned red by Ag^+ . Less oxidized purines, such as hypoxanthine and xanthine, give no colour with the reagent. With 20 ml. of solution, the dilution limit of the test for uric acid is about 1 in 10^6 , and is of the same order of delicacy as the phosphotungstate reduction test developed by Folin (1922, 1934).

EXPERIMENTAL

Scope of the chloroimide reagent. The reaction with uric acid was observed while urine was being tested for free phenols by the method of Gibbs (1927 *a, b*), who has shown that the chloroimide reagent is a general test for phenols unsubstituted in the *para*-position, with which it forms blue or violet indophenol pigments. The reagent also has been used by Raybin (1938) for the detection of thiamine, and by Scudi (1941) for the estimation of pyridoxin.

Scudi reports that creatine, creatinine, and phenylhydrazine give a yellow, uric acid and carbon-bisulphide a yellow to pink, and furfuraldehyde a green colour with the buffered reagent. This has not been confirmed for a specimen of pure creatine, and the validity of the colour tests has been found to depend on several factors: the pH of the mixture, the form in which the reagent is added, the presence or absence of metallic catalysts, and the time allowed for reaction. For most purposes pH 9–10 is the best buffer range. At values below pH 8 colour development is slow, and the shade of the uric acid pigment is orange, although the test can be demonstrated by buffering the solution by addition of solid sodium acetate. In solutions above pH 10, the reagent

tends to undergo spontaneous decomposition, with production of a red-brown mixture of pigments. Gibbs has found that this decomposition is accelerated by exposure to light, as well as by increasing alkalinity. During the present work, it has been

Table 1. *Colour reactions of various substances with 2:6-dichloroquinone-chloroimide at pH 9.2*

Amino compounds:	
NH_3	No change
Methylamine	Slow purple, stable
Glycine	Slow violet, stable
Alanine	No change
Cystine	No change
Tyrosine	No change
Tryptophan	No change
Proline	Slow purple, turns brown
Arginine	No change
Histidine	Slow purple, turns brown
Ornithine	Slow purple, turns brown
Sulphur compounds:	
H_2S	Transient red
KSCN	No change
Thiourea	Slow violet, catalyzed by Cu^{++}
Formamidine disulphide	Rapid violet
Cysteine	Rapid yellow
Glutathione, reduced	Rapid yellow
Purines:	
Hypoxanthine	No change
Xanthine	No change
Guanine	No change
Uric acid	Rapid yellow, turned red by Ag^+
Caffeine	No change
Water-soluble vitamins:	
Thiamine	Slow orange-red, soluble in chloroform
Riboflavin	No change
Pyridoxin	Red-violet
Inositol	No change
Ascorbic acid	No change
Urinary solutes:	
Urea	No change
Creatine	No change
Creatinine	Slow red, turns brown
Hippuric acid	No change
Allantoin	No change
Miscellaneous:	
Adrenaline	Transient red
Indole	Slow violet
Phenols, substituted in <i>para</i> -position	No change
Phenols, unsubstituted	Blue or violet

found possible to repress the decomposition of the reagent by incorporating 10% of NaCl in the buffer mixture.

Either the 2:6-dibromo- or the 2:6-dichloroquinone can be used as the reagent, the latter being chosen on account of its greater stability in the buffer-chloride mixture. Owing to the very low solubility of both reagents in water they were applied as 0.4% solutions in aldehyde-free 98% ethanol. This is a higher concentration than that used by Scudi, as it was observed that excess of ethanol added with the reagent promoted decomposition during the test.

To ascertain the scope of the reaction, the following tests were carried out by mixing 2 ml. of a pH 9.2 borate buffer with 2 ml. of a 0.01–0.1% solution of the compound examined. Two drops of the 0.4% reagent were added, and 5 min. allowed for colour development. The results are shown in Table 1. The reactions with methylamine, glycine, thiourea and indole were accelerated by shaking and aeration, though this promoted the decomposition of the reagent in the tests with proline, histidine and ornithine. On acidification with glacial acetic acid the phenolic colours were changed to bright red, the colours given by methylamine and glycine were changed to a stable violet, and the other pigments were more or less bleached, depending on the degree to which the reagent had undergone spontaneous decomposition. No colour reactions were given by the

mask the original colour reaction. When this is suppressed by the use of a buffer-chloride mixture, the original colours are much more stable and characteristic; that given by uric acid persists for at least an hour unchanged, as shown by absorptiometer measurements.

Comparison of the formulae suggests that the test for uric acid requires the presence of a labile H in position 7, adjacent to the OH at 8, and the double bond at 4–5. This, as Biltz (1936) has shown, constitutes a reacting system altogether unlike that in the less oxidized purines. Under appropriate conditions condensation appears to involve only one molecule of each of the reactants, and HCl is liberated in the process. It is independent of oxidation, although, once the pigment has formed, it can be reversibly bleached by reducing agents.

If the yellow pigment is formed by condensation between the chloroimide grouping in the quinone and the NH— at position 7 in the purine ring, the subsequent change to red on addition of Ag^+ can be explained as an example of the silver-binding NH group effect resembling that shown by the substituted rhodanines (Feigl, 1940). This condensation can be formulated in accordance with the reaction scheme worked out for the phenols by Gibbs (1927*b*), and adopted by Scudi (1941):

common sugars, aliphatic, hydroxy- or keto-acids, or by higher proteins with the exception of samples of dried egg albumin, which were found to be contaminated with uric acid.

Mechanism of the test. Three types of colour reaction are recognizable: (1) rapid coupling, as occurs with the phenols, uric acid and some thiols; (2) slow interaction, probably accompanied by oxidation, as occurs with amino and imino compounds, and yields red or purple pigments closely alike in shade; and (3) spontaneous decomposition of the chloroimide with formation of orange-brown pigments that may

Attempts at isolation of the pigment were unsuccessful, partly owing to the very low solubility of both reactants. The best yields were got when a finely divided and well-mixed suspension of equimolecular parts of uric acid (170 mg.) and dichloroquinone-chloroimide (210 mg.) was suspended in 20 ml. of glass-distilled water, and slowly titrated with $\text{N}/10\text{-NaOH}$. The uric acid acted as a buffer, and the mixture was kept in the region of pH 8–8.5 by continual stirring and slow addition of the alkali. At such pH the colour of the pigment is a deep orange, changing to yellow when a drop of the filtrate is added to 5 ml. of pH 10 buffer. Although it was possible to obtain very concentrated solutions of the pigment by this method, all underwent further changes during attempts at crystallization.

ESTIMATION OF URIC ACID IN URINE

Owing to the excess of chloride in the buffer mixture employed, the red pigment cannot be satisfactorily developed by addition of Ag^+ , but the primary yellow pigment is sufficiently intense and stable to be used for the colorimetric estimation of uric acid in concentrations down to 1 in 100,000.

Reagents

pH 10 buffer-chloride mixture. Add 43.9 ml. of $\text{N}/5\text{-NaOH}$ to 50 ml. of 2M-boric acid in 0.2M-KCl, and make up to 200 ml. with 20% NaCl, using copper-free distilled water and A.R. reagents.

Chloroimide reagent. 0.4% 2:6-dichloroquinone-chloroimide (British Drug Houses Ltd.) in aldehyde-free absolute ethanol. Kept in brown glass bottles, and stored in the dark, the reagent is stable for at least 3 months. Exposed to daylight, deterioration begins within 12 hr., and the solution darkens on being mixed with the buffer. Similar results were obtained with a purified sample of the reagent, synthesized by Prof. T. J. Nolan.

Uric acid standard. The stock solution, containing 1 g./l., is prepared according to Folin's method (1922), which is in general use. The stock solution, when compared with freshly prepared standards, was found to have retained its original value after storage for a year in the dark. Uric acid standards prepared by other methods deteriorated within a month. The colorimetric standard, containing 1 mg. uric acid/100 ml., is prepared freshly as required, by diluting 1 ml. of stock to 100 ml., with glass-distilled water. It does not keep for more than a day or two.

Method

For the concentration range of 10–80 mg. uric acid/100 ml., which includes all values normally found in human urine, the urine is diluted 1 in 50 with distilled water. Five ml. of the diluted urine are transferred to a 10 ml. measuring flask, and mixed with 1 ml. of pH 10 buffer-chloride solution and 0.05 ml. of the 0.4% chloroimide reagent. Within a minute the mixture is diluted to 10 ml. with distilled water, and, within 5–10 min., compared with a standard prepared in the same way, 5 ml. of a solution containing 1 mg. uric acid/100 ml. being used. Readings are taken in the ordinary type of colorimeter, the standard being set at 20 mm. Alternatively, readings can be taken by means of the Hilger Spekker absorptiometer, with the violet glass filter no. 7.

Calculation. $X = 50 \times S/U$,

where X is uric acid in mg./100 ml. in the original undiluted urine, S is the reading of the standard ($S=20$), U is the reading of the unknown solution.

Urines very rich in uric acid may require to be diluted 1 in 100.

RESULTS

Recovery. Recovery values, obtained by adding various amounts of the stock solution to urine, showed that the method gives consistent results within the concentration range of uric acid found in ordinary urines (Table 2).

Table 2. *Recovery values for uric acid in urine*

Uric acid added (mg./100 ml.)	Uric acid recovered (mg./100 ml.)	Recovery (%)
10	9.99	99.9
20	19.92	99.6
30	29.89	99.6
40	39.78	99.4
50	49.90	99.8

Interfering substances. Urinary solutes capable of interfering with the reaction are: (1) amino compounds, including creatinine and free amino-acids; (2) thiols, including cysteine and reduced glutathione; (3) free phenols unsubstituted in the *para*-position. The concentrations of all these, with the exception of creatinine, and, occasionally, histidine and phenols, are so low that their effects are completely suppressed by diluting the urine 1 in 50; while at this dilution, creatinine reacts too slowly to interfere, if the estimation is completed within 10 min. Although early morning samples of pregnancy urine may contain 30–100 mg. of histidine/100 ml. (O'Brien & Quelch, 1943), specimens of pregnancy urine rich in histidine gave uric acid colours that could be matched without difficulty, and addition of 100 mg. of histidine to 100 ml. of normal urine did not obviously affect the uric acid value. This, again, is probably due to the relative slowness of the amino-acid reaction. Interference due to phenols is shown by the development of a green colour during the test. The phenolic reaction is very delicate, and can only be eliminated by extraction of the acidified urine with ether before carrying out the estimation. This is rarely necessary, as *p*-cresol, the commonest urinary phenol, does not give a colour with the reagent, and the other phenols are almost entirely in esterified non-reacting form in fresh urine. It appears doubtful if free phenols occur in significant amounts in ordinary urine, unless hydrolysis has taken place on keeping. The commoner pathological constituents: proteins, reducing sugars, acetone and acetic acid, do not interfere with the estimation. Pile pigments, as might be expected, obscure the reaction.

Diurnal variation. To see if colour development was modified in any way by variations in the composition of urine during the day, an analysis was made of each 2-hourly specimen over a period of 24 hr. No difficulty was found in matching the colours in the ordinary colorimeter, although, in the early morning specimens, where the uric acid output was very low, development of the full colour was slightly delayed (Table 3).

State of uric acid in urine. Since the chloroimide method is very rapid, and involves no drastic treatment of the urine, it should reveal if the uric acid is present as a simple solute, or is, in part at least, esterified or otherwise bound. This was investigated

Table 3. *Two-hourly output of uric acid in urine*

Time	Vol. (ml.)	Sp. gr.	pH	Uric acid	
				(mg./ 100 ml.)	(mg.)
12 noon	350	1005	6.4	14.8	51.8
2 p.m.	74	1018	7.4	41.7	31.3
4 p.m.	263	1006	5.8	20.0	52.6
6 p.m.	153	1010	6.0	32.7	49.1
8 p.m.	130	1012	5.8	34.5	45.0
10 p.m.	158	1012	6.6	33.0	39.5
12 p.m.	130	1011	6.6	37.9	50.5
2 a.m.	86	1015	5.4	33.0	26.4
4 a.m.	54	1018	5.2	35.4	17.8
6 a.m.	43	1020	5.2	45.4	19.5
8 a.m.	40	1020	5.4	55.5	22.2
10 a.m.	44	1018	5.4	53.5	23.1
Total, 10 a.m. to 10 a.m.					428.8

by comparing the uric acid values of fresh samples with samples kept for 24 hr. in presence of dilute acid and alkali. As the results were almost identical, it appears that uric acid in urine occurs as a simple solute (Table 4).

Table 4. *Uric acid values of urine after treatment with dilute acid or alkali at 18°*

Urine	Uric acid (mg./100 ml.)	
Fresh, pH 5.8	30.5	30.3
After 24 hr., undiluted	30.1	30.3
After 24 hr., diluted with an equal vol. of water	30.3	30.6
After 24 hr., diluted with an equal vol. of N/10-HCl	30.12	29.94
After 24 hr., diluted with an equal vol. of N/10-NaOH	30.5	30.3

DISCUSSION

Validity of the method. In general, the values obtained for urinary uric acid by the chloroimide

method are of the same order, but slightly lower than values obtained by the phosphotungstate reduction methods. Variations, however, often occurred in the results obtained by different clinical workers using different preparations of the phosphotungstate and cyanide reagents, and it was concluded that unless the entire group of methods was checked by the actual isolation and direct estimation of the uric acid present in the samples, conclusions could not be safely established. This procedure was adopted by Myers & Wardell (1928), but present circumstances did not allow of it being attempted with prospect of the same degree of accuracy as that postulated by the American workers. The exhaustive survey by Bröchner-Mortensen (1937) shows that any comparison of values for urinary uric acid depending on colorimetry can be misleading, as the same set of errors may reappear in different methods, notably those involving tungstate reduction, which, according to Folin (1934), 'represents probably the most complex reaction we have in the whole field of practical colorimetry'.

SUMMARY

1. In buffer solutions at pH 9–10, uric acid reacts with 2:6-dichloroquinone-chloroimide, forming a yellow pigment that turns red on addition of Ag⁺.
2. The test can be used for the estimation of uric acid in concentrations down to 1 in 100,000, and has been adapted for urine analysis.

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Some Hemicellulose Extraction Methods

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Hemicellulose preparations made by methods involving extraction of woody tissues with aqueous sodium hydroxide are mixtures, containing materials resistant to chlorination and others which are

rendered soluble in sodium sulphite solution by this treatment. The two types have tentatively been designated cellulosan and encrusting hemicelluloses (Norman, 1937; Preece, 1941), and their proportions