The Determination of Uric Acid in Biological Materials

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Uric acid is commonly determined in biological fluids by measuring the blue colour produced by its reaction with reagents containing phosphotungstic or arsenotungstic acids under conditions which minimize interference by other reducing substances (Folin & Denis, 1912; Folin, 1933, 1934; Benedict, 1912, 1922; Brown, 1945; Bidmead, 1951). Recovery of uric acid added to blood has never been complete and values as low as 50% have been recorded. Substances other than uric acid which react with the reagents occur in blood, notably ergothioneine (Hunter & Eagles, 1925) which occurs mainly in the erythrocytes. Benedict & Behre (1931) found that ergothioneine is removed when silver chloride is precipitated in the solution. Treatment with the enzyme uricase has been used to distinguish uric acid from other reacting substances (Blauch & Koch, 1939; Bulger & Johns, 1941; Block & Geib, 1947; Johnstone, 1952). The present paper describes experiments which have led to a satisfactory method for the assay of uric acid with a phosphotungstic acid reagent.

MATERIALS AND METHODS

AnalaR reagents were used where available. Sodium tungstate was tested by the xanthate reaction for molybdate and purified where necessary. The phosphotungstic acid reagent was prepared by the method of Folin (1934). Batches vary slightly in chromogenic activity and so one batch was used for each set of comparative experiments. The reagent yielded reproducible results over several months. The sodium cyanide-urea reagent was prepared as one solution from assayed sodium cyanide and was stored at 2°. Standard uric acid solution (1 mg./ml.) was prepared by the method of Folin (1930) and diluted as required. The method of Folin (1933) was used as the basic procedure, with 1 ml. of uric acid solution, 0.5 ml. of phosphotungstic acid reagent and 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea. The reaction time was 20 min. at 20°. The optical density of the blue reaction mixture was measured at 690 m μ with a Unicam spectrophotometer and 1 cm. light path.

RESULTS

Concentration of reactants. Experiments were carried out to determine the optimum proportions of reactants and the time and temperature for

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development of the colour. Greater colour production was achieved when the concentration of phosphotungstate reagent was increased, and standard curves were linear up to 25 μ g. of uric acid in the reaction mixture with final concentrations of the reagent of 14 and 19% (v/v). At 9% concentration the curve was linear up to 10 μ g. of uric acid. Standard curves were linear for final concentrations of sodium cyanide between 1.1 and 3.4% (w/v). Maximum colour is produced with a final concentration of sodium cyanide between 2.3 and 3.1% (w/v); a further increase yields less colour (cf. Fig. 3, broken line). Beyond 4.5% (w/v) of sodium cyanide cloudy reaction mixtures are obtained. In all the experiments recorded 15% (w/v) urea was incorporated in the sodium cyanide solution, but the amount of urea does influence the colour. A steady increase of 11.7% in colour production was observed as the concentration of urea was increased from 7.5 to 22.5%.

Time and temperature. Fig. 1 indicates that at elevated temperatures colour production is more rapid and some colour destruction takes place. The time required to reach maximum optical density depends on the temperature and is more prolonged at lower temperatures. Blank values rise rapidly

Fig. 1. Optical density produced from 1 ml. of uric acid solution (5 μ g./ml.) when treated with 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea and 0.5 ml. of phosphotungstate reagent at given temperatures as a function of time. Blank values were obtained by substituting water for the uric acid solution. Curve M is given by the modified method described in the text.

Fig. 2. Optical density produced by treatment of a series of uric acid standards (1 ml.) with 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea and 0.5 ml. of phosphotungstate reagent at different time and temperature combinations. Curve *M* is given by the modified method described in the text.

with increase in temperature. To test if the rate of colour production was proportional to the concentration of uric acid throughout the reaction period standards were treated at the times and temperatures shown in Fig. 2. Curves similar to those of Fig. 1 were obtained with the methods of Brown (1945) and Bidmead (1951). Maximum optical densities were less than with the reagent of Folin (1934) for the same amount of uric acid, colour destruction was more rapid and standard curves were not linear above 10 $\mu\text{g.}$ of uric acid/ml. of test solution.

Sodium cyanide-urea reagent. When 2 ml. of a 5% solution of sodium cyanide is diluted to 10 ml. with water and titrated with $\text{N-H}_2\text{SO}_4$ the best grades show a titratable alkalinity of 1.85 ± 0.02 ml. Modification of the titratable alkalinity influences the colour yield with uric acid and the phosphotungstate reagent considerably, as illustrated in Fig. 3. Different batches of sodium cyanide gave widely different colour yields when tested with uric acid and the phosphotungstate reagent under identical conditions, as shown in Table 1, which also gives values for titratable alkalinity and assay of cyanide.

Fig. 3. Effect of modifying the alkalinity of the sodium cyanide-urea solution on the optical density of the final reaction mixture. Volumes of 5N-NaOH or 5N- H_2SO_4 were diluted to 0.5 ml. with water, mixed with 0.5 ml. of a uric acid standard containing 5 $\mu\text{g.}$ and treated with 2 ml. of sodium cyanide solution containing 15% (w/v) of urea and 0.5 ml. of phosphotungstate reagent at 35° for 30 min. The final concentration of sodium cyanide (w/v) in the reaction mixture is indicated on the curves. The broken line joins points where neither acid nor alkali was added. Curve *B* gives blank values obtained by substituting water for uric acid and with a final concentration of sodium cyanide of 3.43% (w/v).

Table 1. Optical density produced with different samples of sodium cyanide

The basic procedure was used, with 5 $\mu\text{g.}$ of uric acid.

Description	Sodium cyanide		<i>E</i> _{690mμ}
	NaCN content (%)	Titratable alkalinity (ml. of N-alcid/0.1 g.)	
Laboratory reagent			
1	78.1	1.61	0.492
2	93.3	1.85	0.540
3	92.4	1.85	0.335
4	93.1	1.86	0.321
Technical grade	61.8	1.33	0.083

Recovery of uric acid. Tungstic acid filtrates of serum prepared by the methods of Folin & Wu (1919) and of Folin (1930) with both slow and rapid addition of acid yielded identical uric acid assays. When uric acid was added to serum or to filtrates from the same serum recoveries were of the same

order. These findings indicate that loss of uric acid by co-precipitation with proteins is an improbable source of error in assays of uric acid in serum. The recovery of uric acid added to normal sera by three different techniques is given in Table 2.

Interfering substances. Investigation into the effects of interference in the colour reaction by a wide range of organic and inorganic compounds showed that the amino acids gave rise to a reduction in the colour produced by uric acid with the reagents when present in concentrations similar to those found in blood serum. Addition of glycine to serum tungstic acid filtrates in amounts estimated to double the amino acid nitrogen content gave an average reduction of 11.5% in the uric acid assays (three sera tested) as determined by the

Table 2. *Recovery of uric acid added to serum determined by three methods*

Uric acid added ranged from 4 to 12 mg./100 ml. of serum.

Method	No. of tests	Average recovery (%)	S.D.
Folin (1933)	19	76.8	4.85
Brown (1945)	11	74.3	5.88
Bidmead (1951)	8	76.5	4.85

Fig. 4. Optical density produced when different volumes of tungstic acid filtrate alone (Test) and containing added uric acid (5 μ g./ml.) (Recovery) are made up to 1 ml. and treated with 2 ml. of 5% (w/v) sodium cyanide containing 15% (w/v) of urea and 0.5 ml. of phosphotungstate reagent at 35° for 30 min. The broken curve is the algebraic sum of the test and standard (5 μ g./ml.) curves. Curve A is the calculated serum uric acid and curve B the calculated percentage recovery.

method of Folin (1933). A serum (J.L.) from a patient with severe gout and renal failure had a uric acid content of 11.2 mg./100 ml. and gave a recovery of added uric acid of 59.5% by this method. The serum had a markedly raised amino acid content.

Effect of diluting filtrate. Volumes of tungstic acid filtrate prepared from serum both with and without addition of uric acid were diluted with water to 1 ml. and treated with 5% sodium cyanide-15% urea solution (2 ml.) and the phosphotungstate reagent (0.5 ml.). A series of uric acid standards were similarly treated. Fig. 4 shows typical curves from a series of tests. Comparison of the 'standard' with the 'test' curve gives the uric acid concentration of the original serum for the appropriate filtrate dilution (curve A), and comparison with the 'recovery' curve gives the percentage recovery of added uric acid for each filtrate dilution (curve B). A similar pattern was obtained with urines, assays reaching a constant value when the urine dilution is greater than 1 in 400. These results indicate that recovery of uric acid is improved by using suitable dilutions of tungstic acid filtrate or urine; with serum less than 0.3 ml. of filtrate, and with urine less than 0.25 ml. of a 1 in 100 dilution should be present in 3.5 ml. of reaction mixture.

Modified method. On the basis of the results obtained in the experiments described above, the method of Folin for the determination of uric acid in serum or urine has been modified as follows:

Tungstic acid filtrate (0.2 ml.), 4% (w/v) sodium cyanide containing 15% (w/v) of urea (3 ml.) and Folin's reagent (0.5 ml.) are mixed and heated at 35° for 30 min. Standard solutions should be run alongside the tests. One containing 0.5 mg. of uric acid/100 ml. is usually satisfactory. The tubes are finally cooled and the optical densities of the solutions are read at 690-700 $m\mu$ or with a red filter in a photometer either directly or after adjusting to a convenient volume with water. Duplicate tests agree to within 0.12 mg./100 ml. of serum and recoveries are 96.8% (s.d. 6.3) in 25 tests on sera with a range of uric acid contents of 3.3-9.4 mg./100 ml. and added uric acid of from 4 to 10 mg./100 ml. The method can be applied to urine, 0.2 ml. of a 1 in 100 dilution being used for the reaction.

DISCUSSION

Published methods for the determination of uric acid in serum vary in the proportions of the reactants. The experiments described indicate that these proportions may materially affect the results of assays, particularly when the latter are high. The temperature and duration of the reaction have no influence on assays and conditions should be chosen to yield a high $E_{\text{test}}:E_{\text{blank}}$ ratio.

Sodium cyanide samples which gave different colour yields with a given amount of uric acid were assayed for total cyanide and titratable alkalinity, but little consistent agreement was obtained. One commercial grade giving a low colour yield had a low assay and revealed traces of iron on analysis, the latter in the ferric form considerably reduces the colour yield of uric acid. Changes in alkalinity of the reaction mixture influence considerably the maximum optical density produced by a fixed quantity of uric acid.

Several substances modify the colour reaction; the most significant in the assay of serum are the amino acids. Dilution of the test solutions has been shown to reduce this effect and to yield higher recoveries and subsequent higher assays.

SUMMARY

1. In the reaction between uric acid and phosphotungstic acid in the presence of sodium cyanide, the effects of time, temperature, concentration of reactants and alkalinity of the reaction mixture have been investigated.

2. A method is described which is suitable for the determination of uric acid in as little as 0.05 ml. of serum containing up to 25 mg. of uric acid/100 ml.

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Further Studies on the Cysteine-Cystine Content of the Foetal-Human Haemoglobin

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In a previous paper (Hommès, Santema-Drinkwaard & Huisman, 1956) we described analyses of the total half-cystine content and the number of free sulphydryl groups in foetal-human haemoglobin. With a chromatographic procedure (Schram, Moore & Bigwood, 1954; Kimmel, Thompson & Smith, 1955), a total number of six half-cystine residues was found, whereas with an amperometric-titration method with silver nitrate only four free -SH groups could be demonstrated. These data suggest the existence of one disulphide bridge in the foetal haemoglobin.

In this paper the total number of free -SH groups in this protein are given, as established by two other techniques. In addition, an attempt was made to divide the foetal haemoglobin into two different parts by reduction of the disulphide bridge which should be present. It should thus be possible to get indications if the disulphide linkage is present in one polypeptide chain or between two

polypeptide chains. In this study the results obtained with foetal haemoglobin are compared with those for the adult component.

METHODS

Normal adult haemoglobin, obtained from healthy adults (laboratory workers) and pure foetal haemoglobin were prepared as described earlier (van der Schaaf & Huisman, 1955). The carboxyhaemoglobins were stored at 0° for no longer than 1 week. No experimental difference was found between this preparation of adult haemoglobin and that obtained by starch electrophoresis according to Kunkel & Wallenius (1955). Amperometric titrations with AgNO_3 and 0.05M- NH_4NO_3 -aq. 0.1M- NH_3 soln. as supporting electrolyte [Hommès *et al.* (1956) described in detail in the Addendum] followed the method of Benesch, Lardy & Benesch (1955). In this amperometric method a titration mixture of 0.133M-2-amino-2-hydroxymethylpropane-1:3-diol, 0.1133M- HNO_3 and 0.01M-KCl was used in all experiments. The titrimetric experiments were carried out at room temperature. No change was found on lowering the