II. THE COLORIMETRIC ESTIMATION OF URIC ACID IN FOWL'S EXCRETA

By JOHN CHARLES DUNCAN HUTCHINSON

*Department of Poultry Nutrition, Animal Nutrition
Institute, School of Agriculture, Cambridge*

(Received 30 November 1940)

NUMEROUS methods have been elaborated for the estimation of uric acid in avian excreta. These methods and their defects have been admirably reviewed by Fritz [1935]. In this laboratory a modification of Woodman's [1924] method has been in use, in which the nitrogen in the precipitate obtained is estimated by the Kjeldahl method. It is found that this nitrogen is about 35 % less than would correspond to the weight of the precipitate if the latter were pure uric acid. It has been assumed that the impurities present in the precipitate contain no nitrogen and if this is the case the method may be accurate, but if nitrogenous impurities are present the results must be too high. Fritz [1935] has found that uric acid is liable to be destroyed by extraction with lithia, and Folin & Denis [1912] have called attention to the fact that uric acid is very rapidly destroyed on boiling in alkaline solution. The results may be too low on this account. In addition, the method is very laborious, owing to the filtration difficulties and the numerous processes involved. Consequently it is not very practical for metabolic work in which it is necessary to make a large number of estimations and to obtain the results quickly.

St John & Johnson [1931] have worked out a more refined isolation technique in which piperidine replaces lithia as the solvent for uric acid. This method appears to be reliable, but from the results published in their paper it seems that the quantity of nitrogen found in their precipitate averages 2.7 % less than corresponds with the weight of precipitate. There is thus still a small error caused by lack of specificity. If there is any nitrogenous impurity in the precipitate the error may be greater than 2.7 %. If on the other hand the uric acid content is calculated from a nitrogen estimation on the precipitate to eliminate the error, the method becomes nearly as cumbrous as Woodman's procedure, and in any case a more rapid method is desirable. Fritz [1935] also states that loss of uric acid is very liable to occur in piperidine solution when this method is employed.

The author has tried to separate uric acid added to human faeces by the methods of St John & Johnson [1931] and of Woodman [1924]. It was not possible to get a full recovery, because the filtration processes under these conditions are very lengthy and loss of uric acid occurred in alkaline solution, but it was found that by St John & Johnson's method the nitrogen obtained from the precipitate was 21 % less than corresponded to the weight of precipitate, while by Woodman's method it was 34 % less. There was no evidence of nitrogenous impurities in the precipitate obtained by St John & Johnson's method, but nitrogenous impurities were present in the precipitate obtained by Woodman's method.

The colorimetric method is much less laborious than the isolation methods, and it has been developed to a high degree of specificity for estimations in blood and urine, chiefly by Benedict and by Folin. Preliminary experiments were

made by the author, using the Benedict & Franke [1922] method for urine, on phosphate and lithia extracts of dried sheep's and human faeces to which 7 % of uric acid had been added. The uric acid recovered exceeded by 4.3 % the amount added to sheep's faeces and by 3.3 % in the case of human faeces. The extracts were not sufficiently dark coloured to affect the colorimeter readings. It was clear from these experiments that the colorimetric method was highly specific for uric acid in faecal extracts and that it would only be necessary to introduce one stage of purification to obtain an accurate method.

The method described in this paper has been worked out for use with Benedict's [1922] arsenophosphotungstic acid reagent and Newton's [1937] lithium arsenotungstate reagent. A great deal of work has been published on methods of intensifying the blue colour. Christman & Ravwitch [1932] found no true proportionality of colour intensity to the concentration of uric acid when Benedict's [1922] 5 % cyanide solution was used. Moreover, it varied with different samples of cyanide and also was less reliable for diluted urine than for pure uric acid solutions. They found that they could correct the proportionality by adding 10 % of sodium carbonate to the cyanide solution and claimed that this solution gave a higher degree of specificity to the colour reaction. They considered that the variation in different samples of cyanide was due to their different contents of carbonate. In the author's experience however the proportionality is not the same with different samples of cyanide of similar origin used for making up Christman's solution, so that it seems that some other factor besides the carbonate content is involved. The author has compared the proportionality observed when Christman's solution was used with that observed when Folin's [1933] cyanide urea solution (which is free from carbonate) was employed. Both solutions were prepared from the same sample of sodium cyanide (B.D.H. pure). Uric acid standards containing 0.2, 0.3 and 0.4 mg. per 10 ml. were prepared by dissolving pure uric acid in warm lithium carbonate and adding acid lithium chloride, as described later, to prevent turbidity from developing during the colour reaction. 10 ml. of the standard solution, 5 ml. of cyanide and 2 ml. of undiluted Newton's reagent were used to develop the colour. On comparing the 0.2 and 0.4 mg. standards with the normal standard of 0.3 mg., the proportional colour intensity was found to increase about 5 % over this range as the concentration of uric acid was increased when Christman's solution was used. That is, the 0.4 mg. standard was 2.5 % too high and the 0.2 mg. standard was 2.5 % too low. When Folin's cyanide urea solution was employed the proportional colour intensity decreased 6 %. Thus the effect of adding the carbonate was to reverse the error in proportionality. It decreased the total intensity of the colour as Folin states. It seems that there should be an optimum quantity of carbonate which will give true proportionality with any given sample of cyanide, though this quantity varies with different samples. For example, a second sample of cyanide (also B.D.H. pure) gave true proportionality with Newton's reagent and Christman's cyanide. With a third sample (B.D.H. pure) the proportional colour intensity increased 5.5 %, practically the same amount as with the first sample.

Similar results were obtained with 2 ml. of the Benedict & Franke [1922] reagent. With the first sample of cyanide the proportional colour intensity increased 7 %, the second gave true proportionality, while the third gave an increase of 5 %.

A large number of experiments on proportionality were carried out with extracts of fowl's excreta both by the direct and indirect methods described later. In contrast to the experience of Christman & Ravwitch [1932] with diluted

human urine, the proportionality was found to be exactly the same with extracts of excreta as with lithium carbonate solutions of uric acid.

When an ammoniacal precipitating solution is used for the uric acid, the residual ammonia remaining in the precipitate can depress the colour reaction. This depressant effect is considerably greater for cyanide solutions with high driving power, such as Benedict's [1922] and Folin's [1933], than for Christman's solution. When the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitating solution' is used, as in the method described below, there appears to be an advantage in using Christman's solution for the colour development. Moreover, Christman & Ravwitch [1932] claim that their solution is more specific for uric acid than a 5 % solution of sodium cyanide without the addition of carbonate. Folin [1934], on the other hand, considers that increasing the driving power by removal of traces of carbonate increases the specificity and therefore uses cyanide solutions in which the carbonate is completely removed. For the estimation of uric acid in fowl's excreta no marked difference has been observed as regards the specificity of Folin's and Christman's solutions, but if ammonia can affect the action of Folin's solution more than that of Christman's solution, it may be that other extraneous factors can also affect it more than Christman's solution under practical conditions. For this work therefore Christman's solution has been used as the standard reagent. It has been confirmed that its driving power remains unimpaired for several months.

The preliminary experiments reported above on faecal extracts showed that to obtain the necessary specificity it was desirable to separate the uric acid from the extract prior to developing the colour. For the precipitation of uric acid the zinc hydroxide method of Morris & Macleod [1922] and the Salkowski reaction as employed by Benedict & Hitchcock [1915] have been tried. The zinc method appeared to bring down a considerable quantity of colouring matter as well as the uric acid. Moreover, the zinc was found to exert a depressant effect on the colour reaction, as Kern & Štransky [1937] had also observed, so that only 97 % recovery could be obtained from lithium carbonate solutions of uric acid. The results by this method, after correcting for the loss in the recovery, were practically the same as with the Benedict & Hitchcock [1915] method, in spite of the lack of specificity. Benedict and his co-workers consider that the Salkowski reaction provides the most specific method of precipitating uric acid, and it has therefore been adopted as the standard method. Here again difficulty was experienced in getting a full recovery of uric acid from lithium carbonate solution. This is partly because the reaction is not absolutely quantitative [Folin & Wu, 1919], and partly because the residual ammonia present in the precipitate can depress the colour reaction. The best results were obtained by using the proportions of precipitant recommended by Benedict & Hitchcock [1915], namely, 1 ml. precipitant to 5 ml. extract, and not 5 ml. precipitant to 5 ml. extract as used by Christman & Ravwitch [1932]. If the neck of the centrifuge tube is swabbed out carefully after draining and a current of air is passed over the precipitate, most of the residual ammonia can be eliminated so as not to interfere with the colour reaction. By this means a recovery of 98.5 % was obtained and the results were corrected accordingly. This correction may vary up to 1 % with individual workers and should be determined individually. The amount of ammonia remaining in the silver magnesium urate precipitate after passing the current of air should not exceed 1 ml. N/10.

To decompose the precipitate and remove the silver, which may interfere with the colour reaction, 7 % lithium chloride in 0.125 N HCl was used in place of Folin's acid NaCl procedure. The advantage in the use of lithium chloride is that

it prevents the development of turbidity during the colour reaction. It was found that if lithium chloride was not present the urea in the cyanide solution was barely sufficient to prevent turbidity when Benedict's reagent was used, and definitely insufficient with Newton's reagent. Benedict & Behre [1931] have also used an acid lithium chloride solution for this reason. The decomposition of the precipitate by acid chloride not only removes the silver, but has the advantage of bringing down most of the colouring matter which may be present, so that a colourless solution of uric acid is obtained even from the most deeply coloured extracts of excreta. It is found that no uric acid is lost by this procedure, even though the concentration of uric acid approximates to, or in some cases even exceeds, its solubility in acid solution.

The author, like Christman, has not found Benedict & Hitchcock's [1915] phosphate standard uric acid solution to be reliable. Folin's [1930] most recent formalin standard has proved to have excellent keeping qualities. On dilution it was found to be exactly equivalent in colour-producing property to a lithium carbonate solution of uric acid of the same strength. A diluted solution of this standard containing 0.3 mg. uric acid in 10 ml. is prepared for use and this will keep for 14 days. A quantity of acid lithium chloride solution equal to that present in the unknown must be added to the standard, because chloride intensifies the colour reaction, at all events when Benedict's arsenophosphotungstic acid reagent is employed.

Fritz [1935] found that loss of uric acid is very liable to occur when it is extracted from the excreta by lithia or piperidine. The author has found that piperidine depresses the colour reaction. Phosphate is not suitable because it interferes with the precipitation of uric acid, causing turbidity in the subsequent colour reaction, though it gives only a lightly coloured extract. Dilute lithium carbonate has been successfully employed by Yamaguchi [1930] and by Fürth & Edel [1933] for the extraction of uric acid from tissues. It has proved very successful for extracting the uric acid from fowl's excreta in the method described below.

Woodman [1924] considered it desirable to convert any ammonium urate present in the excreta into uric acid with HCl before extracting with lithia, since unless this were done the extraction of uric acid was apparently not complete. St John & Johnson [1931] also adopt this procedure before their piperidine extraction. Some experiments were made to find out whether this preliminary treatment was necessary in the case of fowl's excreta. Samples were estimated by the standard colorimetric technique described below, with and without pre-treatment with HCl. 0.8 g. of excreta was covered with 7 ml. of distilled water and 7 ml. 0.3 *N* HCl were added. The mixture was brought to boiling point and held there for 4 min. In the first two experiments enough lithium carbonate was added to neutralize the HCl in addition to the 12 ml. normally used for the extraction. In the other two experiments the mixture was left for 24 hr. after boiling with HCl and then filtered and washed once with distilled water. The residual HCl was neutralized with lithium carbonate and extracted in the same way. The results of these experiments were as follows (Table 1).

Table 1

No. of exp.	% uric acid on air-dried wt.	
	With HCl pretreatment	Without HCl pretreatment
1	8.49	8.43
2	8.57	8.60
3	10.13	10.32
4	9.25	9.10

It is apparent that preliminary treatment with HCl is unnecessary.

The following standard procedure has been adopted for the estimation of uric acid in avian excreta. Since eight estimations can be performed in one day, there is a considerable saving of labour as compared with the older methods.

Method

1. Enough air-dried excreta to give 50–100 mg. uric acid is weighed and washed into a 250 ml. volumetric flask with 20 ml. of distilled water.

2. 12 ml. 0.5 % lithium carbonate solution or, if more than 0.8 g. excreta were taken, 15 ml., are added. The flask is heated on a water bath for 15 min., cooled immediately and made up to volume.

3. The extract is filtered, the first few ml. being rejected if there is any turbidity.

4. 5 ml. filtered extract are pipetted into a 25 ml. centrifuge tube and 1 ml. of the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitant' is added. The liquid is stirred very carefully with a fine glass rod so that it does not splash up the sides of the tube and is then centrifuged for 5 min.

5. The supernatant liquid is poured off and the tube is drained for 2 min. Its neck is then swabbed with a filter paper. The tube is placed in a beaker of hot water (70–80°) and a current of air is passed for 1 min. over the precipitate to remove ammonia. The precipitate is dislodged from the bottom of the tube by a smart tap, the tube is replaced in the beaker of hot water and the current of air is passed for another minute.

6. 20 ml. 7 % lithium chloride solution in 0.125 *N* HCl are added and the contents of the tube are stirred very thoroughly with a fine glass rod until all the precipitate is decomposed. The tube is then centrifuged for 2–3 min.

7. The supernatant liquid is poured into a 50 ml. volumetric flask, 5 ml. of the acid lithium chloride solution are added to the centrifuge tube and 10 ml. distilled water. The contents are stirred and centrifuged again for 2–3 min.

8. The supernatant liquid is poured off into the 50 ml. flask and the liquid is made up to volume with distilled water.

9. The standard and unknown are prepared simultaneously for the colour development. 10 ml. of the solution in the 50 ml. flask and of the diluted standard are pipetted each into 50 ml. volumetric flasks. 5 ml. of the Christman's cyanide solution are added and either 2 ml. arsenophosphotungstic acid reagent or 1.5 ml. Newton's lithium arsenotungstate reagent undiluted; the flasks are shaken gently. The colour is allowed to develop for 5 min. with Benedict's reagent or 8 min. with Newton's reagent, and the mixtures are made up to volume with distilled water.

10. The colours are compared in a colorimeter immediately. Readings between 15 and 30 mm. are satisfactory provided that the reagents are adjusted to give true proportionality as described above. The results are corrected for the empirical loss in the recovery of uric acid after precipitation.

Recovery of uric acid from fowl's excreta

The following experiment was performed to test the recovery of different quantities of uric acid from fowl's excreta. A sample of excreta was separated mechanically as far as possible from uric acid and dried. Different quantities of uric acid were added to this, and the uric acid was estimated by the standard colorimetric method described above, using Benedict's colorimetric reagent

The results are given in Fig. 1. The amounts of uric acid added were recovered quantitatively over the whole range of conditions likely to be encountered in practice, from the low uric acid content of excreta from birds on a nitrogen-free ration to the high content which is obtained when they are on a nitrogen-rich ration. It appears that the amount of protein which is present in the excreta is

Fig. 1. Recovery of uric acid added to 'nearly uric acid-free' fowl's excreta.

not sufficient to affect the precipitation at low concentrations of uric acid. The graph (Fig. 1) does not run through the origin because there was a small quantity of residual uric acid in the excreta after the mechanical separation.

Specificity

To test the specificity of the method it was necessary to add the uric acid to sheep's and human faeces because it was impossible to obtain fowl's excreta completely free from uric acid. A lithium carbonate extract was prepared from dried sheep's faeces, and a uric acid solution in lithium carbonate was added to this to provide the equivalent of 7.5% of uric acid reckoned on the air-dried weight of the faeces. Thus, any error in the result could not be caused by failure of the extraction process, but must be due to lack of specificity. The uric acid content of this mixture was then estimated by the standard method, using Benedict's colorimetric reagent. The results were as follows (Table 2):

Table 2

No. of exp.	% uric acid added	% uric acid recovered
1	7.50	7.55
2	7.50	7.58
3	7.50	7.58
4	7.50	7.53
Mean	7.50	7.56

Further experiments were performed on sheep's and human faeces. Ammonium urate instead of uric acid was added before extraction and Newton's colorimetric reagent was used. The results are given in Table 3.

Table 3

Source of faeces	Weight of air-dried faeces g.	Ammonium urate added g.	Ammonium urate recovered g.
Sheep	0.700	0.0805	0.0810
"	0.748	0.0825	0.0800
"	1.331	0.0835	0.0801
"	1.400	0.0815	0.0829
"	1.403	0.0830	0.0840
Human	0.754	0.0825	0.0833
"	1.503	0.0910	0.0946

These tests show that the method is highly specific and gives good results under conditions which caused a failure of the methods of Woodman [1924] and of St John & Johnson [1931] (see p. 81).

Experiments were also carried out to test the specificity of the Benedict and Newton colour reagents in the presence of certain other purine bodies which are also precipitated by the ammoniacal silver magnesium precipitant. Solutions or suspensions were made up in lithium carbonate of guanine, adenine, hypoxanthine and xanthine containing 0.75 mg. of the purine bodies and 0.3 mg. of uric acid per 10 ml. of solution. The colours produced by 10 ml. of these solutions were compared with that produced by 10 ml. of a standard uric acid solution (0.3 mg. per 10 ml.). Adenine and hypoxanthine gave no colour with either reagent. Xanthine gave 1/40th of the colour which would be given by the same quantity of uric acid when Newton's reagent was used for the colour reaction, and 1/30th of the colour with Benedict's reagent. Guanine gave 1/70th of the colour with Newton's reagent and 1/25th of the colour with Benedict's reagent. Thus the small quantity of the other purine bodies precipitated with uric acid could not affect the results. The Newton reagent appears to be rather more specific than the Benedict reagent, as Newton states. Fürth & Edel [1933], using Folin's colorimetric reagent, have also found that the decomposition products of the nucleic acids are either not precipitated or give no colour.

The possibility of using a direct method for the estimation of uric acid in avian excreta has been examined. Estimations made directly on the lithium carbonate extracts of the excreta have been compared with those made on the same extracts by the standard indirect method described above. Both Newton's and Benedict's colorimetric reagents were used in these determinations, and some experiments were made using Folin's carbonate-free cyanide urea solution. For the direct estimations with Christman's cyanide solution the lithium carbonate extracts were diluted 5 times to give a convenient concentration of uric acid, and the same amount of acid lithium chloride was added as was contained in the standard. For the direct estimations with Folin's cyanide-urea solution a standard was prepared containing 0.06 mg. uric acid per 10 ml. and the extracts of the excreta were diluted 25 times. To develop the colour when Folin's cyanide solution was used, 10 ml. diluted extract were taken, 5 ml. cyanide-urea solution and 1 ml. undiluted Newton's reagent were added. The volume was made up to 20 ml. after 10 min. The results of these experiments are given in Table 4.

The specificity of Newton's reagent combined with Christman's cyanide solution is high when used directly on the lithium carbonate extract. The results

obtained by this method were on the average 1.8% higher than those obtained by the indirect method. The Benedict reagent was not quite so specific, yielding results which averaged 3.9% above those obtained by the indirect method. The experiments in which the Newton reagent was combined with Folin's cyanide-urea solution gave values which were intermediate between those of the other two direct methods, being 2.6% above those of the indirect method. Samples 5, 6, 7 and 8 were used for comparing the Newton and the Benedict reagents in the indirect method in conjunction with Christman's solution. The results for these samples are not the same for the two series, because they were part of a series of estimations carried out at the extremes of the range of uric acid concentrations to test the proportionality of the colour to the uric acid concentration.

Table 4

No. of sample	Direct method		Indirect method	
	% uric acid on air-dried wt.	Colorimetric technique	% uric acid on air-dried wt.	Colorimetric technique
1	12.68	N C	12.51	N C
2	11.21	N C	11.11	N C
3	8.88	N C	8.55	N C
4	10.30	N C	9.96	N C
5	10.77	N C	10.76	Mean of B C & N C
6	9.37	N C	9.43	Mean of B C & N C
7	8.04	N C	7.67	Mean of B C & N C
8	10.02	N C	9.96	Mean of B C & N C
13	3.16	N C	3.01	Mean of B C & N C
5	11.10	B C	10.60	Mean of B C & N C
6	9.21	B C	9.04	Mean of B C & N C
7	8.84	B C	8.53	Mean of B C & N C
8	11.15	B C	10.41	Mean of B C & N C
13	3.11	B C	3.04	Mean of B C & N C
9	10.02	N F	9.72	B C
10	7.11	N F	6.86	B C
11	6.75	N F	6.42	B C
12	8.30	N F	8.24	B C
13	3.04	N F	3.03	Mean of B C & N C

N = Newton's colorimetric reagent.

B = Benedict's colorimetric reagent.

C = Christman & Ravvitch's cyanide carbonate urea solution.

F = Folin's carbonate-free cyanide urea solution.

While under the conditions employed the proportionality was not exact, it was practically the same for the two colorimetric reagents. When Newton's reagent combined with either Christman's or Folin's cyanide solution is used directly on the extracts the method is at least as specific as that of St John & Johnson [1931]. It seems that the rather heroic efforts of Folin and Benedict and their co-workers to prepare a direct uric acid colorimetric reagent, if not altogether successful for blood estimations, have at least produced a technique of practical value for direct determinations on avian excreta. On the other hand, the indirect method is more reliable and should be employed as the standard procedure. Where, however, saving of time is important, and it is known that the direct and indirect methods agree in the estimation of uric acid in excreta obtained from birds on a ration of the same type as that under investigation, the direct method may be of considerable practical value. Close agreement between direct and indirect methods has been obtained also by Shannon [1938], working on fowl's urine which has been separated from the faeces.

Experiments have been carried out to test whether there is any difference between the results obtained in the indirect method when either Benedict's or Newton's reagent is combined with Christman's cyanide solution. The results are given in Table 5.

Table 5

No. of sample	% uric acid on air-dried wt.	
	Benedict's reagent	Newton's reagent
5	10.74	10.63
6	9.22	9.26
7	8.00	8.21
8	10.29	10.08

There was no significant difference between the results obtained by the two methods. In general it was found that Newton's reagent with Christman's cyanide solution did not give such close agreement between duplicates as the Benedict and Franke reagent.

Finally, a number of samples of fowl's excreta have been analysed by the author and other workers in this laboratory by the modification of Woodman's method used here hitherto; the results were compared with those obtained by the standard indirect colorimetric procedure and are given in Table 6.

Table 6

No. of sample	% uric acid on air-dried wt.	
	Colorimetric method	Woodman's method
18	9.61	8.98
19	8.46	9.04
20	9.92	8.66
21	7.35	6.48
22	8.20	7.90
23	8.19	7.17
24	9.78	7.18
25	6.84	6.04
26	6.51	6.03

The results by Woodman's method are in most instances considerably lower than those obtained by the colorimetric method. This is presumably due to the fact that the destruction of uric acid brought about by boiling with lithia more than compensates for any increase in the apparent value caused by nitrogenous impurities which may be present in the uric acid precipitate.

SUMMARY

A colorimetric method is described for the determination of uric acid in fowl's excreta; it is both more specific and less laborious than the methods at present in use.

The author is indebted to Mr E. T. Halnan and Mr F. W. Foreman for able criticism during the course of the work.

REFERENCES

- Benedict (1922). *J. biol. Chem.* **51**, 187.
—— & Behre (1931). *J. biol. Chem.* **92**, 161.
—— & Franke (1922). *J. biol. Chem.* **52**, 387.
—— & Hitchcock (1915). *J. biol. Chem.* **20**, 619.
Christman & Ravwitch (1932). *J. biol. Chem.* **95**, 115.
Folin (1930). *J. biol. Chem.* **86**, 179.
—— (1933). *J. biol. Chem.* **101**, 114.
—— (1934). *J. biol. Chem.* **106**, 311.
—— & Denis (1912). *J. biol. Chem.* **13**, 469.
—— & Wu (1919). *J. biol. Chem.* **38**, 81.
Fritz (1935). *J. industr. Engng Chem.* **7**, 123.
Fürth & Edel (1933). *Biochem. Z.* **263**, 9.
Kern & Stransky (1937). *Biochem. Z.* **290**, 419.
Morris & Macleod (1922). *J. biol. Chem.* **50**, 55.
Newton (1937). *J. biol. Chem.* **120**, 315.
St John & Johnson (1931). *J. biol. Chem.* **92**, 41.
Shannon (1938). *J. cell. comp. Physiol.* **11**, 135.
Woodman (1924). *J. agric. Sci.* **14**, 413.
Yamaguchi (1930). *Jap. J. med. Sci.* **iv**, Pharmacology, **5**, 72.