CXX. NOTE ON THE ESTIMATION OF URIC ACID BY THE HOPKINS-FOLIN METHOD.

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IN metabolic experiments where uric acid in the urine is estimated the Hopkins-Folin method is the one most commonly used. In spite of this popularity difficulty is almost universally found in the determination of the end point of the titration with permanganate, and, in addition, disconcerting variations not infrequently occur in a series of parallel estimations.

As long ago as 1876 a method of estimating uric acid in urine was given by Sutton [1876], and, from what is said there, permanganate had evidently been used for estimation of uric acid before that. There it is advised to precipitate uric acid from 300 to 500 cc. of urine by means of 5 to 8 cc. of pure HCl. The precipitation is complete in 24 to 30 hours. The precipitate is then washed once with cold distilled water, dissolved in a solution of pure KOH, and, after acidifying with H_2SO_4 , titrated with decinormal permanganate.

In 1887 Blarez and Denigès [1887] drew attention to two factors modifying this titration with permanganate. They found (a) that the amount of $N/10$ permanganate required to reach the end point increased as the amount of 20 % H_2SO_4 used in acidifying before titration increased, (b) that the degree of dilution of uric acid markedly affected the amount of permanganate required to reach the end point. When the dilution of uric acid was above 1:8000 (from 1:8000 to 1:160,000) there was no effect on the end point, but below 1:8000 (1:8000 to 1:2000) the amount of permanganate required increased as the concentration of uric acid increased. (c) That temperature did not affect the end point.

The next stage in the evolution of this method was when Hopkins [1891, 1892, 1893] showed that uric acid could be quantitatively precipitated from urine by saturation with ammonium chloride in two hours. The final estimation was by weighing or, preferably, by titration with permanganate after acidifying with 20 cc. of pure strong sulphuric acid which produced a temperature in the neighbourhood of 60° . This temperature was considered the optimum for titration.

Shortly afterwards Folin [1898, 1901] investigated the method and the instructions given in his second paper are practically those followed at the present day. It was in the second paper that the uranium solution was introduced to precipitate the mucoid bodies in the urine in order to facilitate filtration.

This uranium solution contains 500 g. ammonium sulphate, 5 g. uranium acetate and 60 cc. 10 % acetic acid in 650 cc. water. The resulting volume is about 1 litre. When 25 cc. of this solution is added to 100 cc. of urine the concentration of ammonium sulphate in the mixture is about 10 %.

The method employed in this laboratory is as follows:

100 cc. urine is measured into a tall narrow cylinder and 25 cc. of the uranium solution is added. The mixture is allowed to stand without stirring for about half an hour. The uranium precipitate has then settled and the clear supernatant fluid may be removed by decantation. 100 cc. of this fluid is measured into a beaker, 5 cc. of strong ammonia added and the mixture allowed to stand overnight. The precipitate is then filtered off and washed with 10 % ammonium sulphate solution until chloride-free. The filter paper is removed from the funnel, unfolded, and the precipitate washed back into the beaker with water, the filter paper and water to make about 100 cc. added. The precipitate is now dissolved by the addition of 15 cc. concentrated H_2SO_4 and, at once, whilst warm, titrated with $N/20$ potassium permanganate. The end point is the first appearance of a permanent pink colour. A correction of 2.4 mg. due to the solubility of ammonium urate is added to the result.

Folin [1901] states that uric acid exposed to a solution of ammonia is destroyed, but in the presence of 10 % ammonium sulphate no destruction occurs after 48 hours' exposure. The addition of the filter paper to the 100 cc. water before adding the strong sulphuric acid is not advised by Folin as he considers the strong acid may destroy the paper liberating substances capable of reducing the permanganate solution. The optimum temperature [1898] for the titration is given as lying between 55° and 65° . Below 50° the end point is not sharp and above 70° the colour disappears too rapidly. The correction factor of 2.4 mg. of uric acid for 80 cc. of urine is obtained from Folin's calculation that the solubility of this acid in urine is about 3 mg. per 100 cc. urine. Also it is stated by Folin that traces of chloride do not disturb the titration.

In the following estimations a standard solution was made by dissolving the uric acid by the aid of Li_2CO_3 in water. The addition of a trace of thymol prevented destruction for several weeks.

An obvious source of error is the presence of reducing substances either in the water or in the concentrated sulphuric acid used.

Glasgow (Loch Katrine) water, which was used throughout, contains 0.80 parts per 100,000 of organic matter and absorbs 0.16 part of oxygen per 100,000 from permanganate when exposed for 4 hours at 80°F . Nevertheless it was found that one drop of $N/20$ KMnO_4 gave a lasting pink colour to 100 cc. water at all temperatures.

With the "pure" sulphuric acid it was quite otherwise. Varying quantities of concentrated sulphuric acid were added to 100 cc. water and titrated at 40° with $N/20$ permanganate. The end point was taken when the pink coloration persisted just beyond 10 seconds. The following results are each the average of at least three readings (Table I).

Table I.

cc. concentrated H_2SO_4	1	5	10	15
cc. $N/20$ KMnO_4 used A	0.08	0.15	0.235	0.32
" " B	0.045	0.07	0.085	0.115

Many other samples of "pure" sulphuric acid were obtained and all showed similar variations.

Sample *B* was then used in estimating uric acid, e.g. as in Table II.

Table II.

cc. concentrated H_2SO_4	1	5	10	15
cc. $N/20$ KMnO_4 used in titration	1.355	1.38	1.385	1.42
Corrected figures	1.31	1.31	1.30	1.305

This, then, is a possible explanation of the error observed by Blarez and Denigès, and also of the discrepancies in a series of estimations if no blank titration is performed as a routine.

By varying at one time the solvent and at another the solute, the effect of the degree of dilution of uric acid on the permanganate was investigated (Table III). The conditions of titration were as above, the temperature being raised or lowered to 40° and a correction factor for H_2SO_4 used.

Table III.

Variation in solvent.

Dilution	1 : 20,000	1 : 15,000	1 : 10,000	1 : 8,000	1 : 6,000	1 : 4,000	1 : 2,500
cc. $N/20$ KMnO_4	2.615	2.595	2.575	2.575	2.575	2.615	2.625

Variation in solute.

Dilution	1 : 20,000	1 : 10,000	1 : 6 666	1 : 5 000	1 : 2,000
cc. $N/20$ KMnO_4	2.60	2.575	2.591	2.583	2.588

Many other series gave similar results. This does not substantiate the findings of Blarez and Denigès that concentration of uric acid plays an important rôle. In this connection it is interesting to note that, from his figures, Folin must have been working at times with a dilution of only 1:1000. In ordinary metabolic work also, the dilution of uric acid from normal urine in the mixture on titration can but rarely be above 1:8000.

The effect of temperature on the end point of the titration is as follows. Under conditions similar to the above a mixture of 15 cc. H_2SO_4 and 100 cc. water was titrated against $N/100$ KMnO_4 . The temperature was varied by heating or cooling the mixture after the addition of the sulphuric acid (Table IV).

Table IV.

Degrees centigrade	30	40	50	60	70	80
cc $N/100$ KMnO_4	0.46	0.50	0.53	0.53	0.56	0.56

Following on this 1 cc. $N/100$ KMnO_4 was added to such a mixture of water and sulphuric acid at different temperatures and the time taken for the colour to disappear noted.

Table V.

Degrees centigrade	30	40	50	60	70	80
Time in seconds	420	150	60	44	32	25

This effect when estimating uric acid is shown below (Table VI):

Table VI.

Degrees centigrade	25	35	45	55	65	75	85
cc. <i>N</i> /20 KMnO_4 used	2.74	2.74	2.76	2.77	2.78	2.79	2.82
Corrected	2.64	2.62	2.60	2.63	2.61	2.62	2.64

Two points are shown here. First, the titrations should always be at the same temperature. The simplest way to ensure this regularity is to make the fluid suspending the ammonium urate exactly up to 100 cc. and to add exactly 15 cc. strong sulphuric acid. This has the advantage of giving consistently a temperature in the neighbourhood of 60° , the optimum for the titration. Other temperatures, however, are quite possible, especially if the second point is attended to. The essential factor is that the temperature should not vary throughout a series of titrations.

Second, in determining the end point, permanganate should be added drop by drop until the final addition gives a pink diffuse colour persisting just beyond a fixed time. Such a suitable time is 10 seconds. Beginners then have not the unsatisfactory experience of deciding for themselves what is meant by a "persistent pink colour."

Folin's objection to the filter paper does not seem to hold good, at least when using a Green's 1 F paper 9 cm. in diameter. Such a paper was cut into segments ranging from $\frac{1}{16}$ to a $\frac{1}{2}$ of the total area, which were then added to the uric acid solution before the sulphuric acid was added. In numerous titrations the paper did not seem to affect the end point. Nevertheless, it can do no harm to add a filter paper, such as is used in filtering off the ammonium urate, to the blank titration.

The direction to remove all chlorides from the precipitate of ammonium urate by washing with 10 % ammonium sulphate is vague. Apart from the almost practical impossibility of removing the last traces of chloride, and Folin's statement that traces of chloride do not affect the end point, there would seem to be a risk of dissolving an appreciable quantity of the ammonium urate on the filter paper by washing with unknown, possibly large, volumes of ammonium sulphate solution in a vain attempt to remove the last traces of chloride. That sodium chloride in quantities of 15 mg. seems to have no effect is shown in the following table. 15 mg. sodium chloride was found to give a marked precipitate with silver nitrate solution, much more marked than any precipitate obtained in the sulphate washings. Varying quantities of sodium chloride were added to uric acid solution before titration. The results are as follows:

Table VII.

mg. NaCl	0	1	5	10	15
cc. <i>N</i> /20 KMnO_4	2.53	2.55	2.60	2.60	2.58

The effect of washing with ammonium sulphate solution was another matter. The ammonium urate precipitate was obtained by adding a known volume of standard uric acid solution to 10 % ammonium sulphate solution

made alkaline with strong ammonia solution, S.G. 0.880. After standing overnight the precipitate was collected on a filter paper and washed with varying amounts of sulphate solution. The uric acid in the precipitate and also in the mother liquor plus wash water was then estimated. Corrections by blank titrations were used in all cases (see Table VIII).

Table VIII.

mg. uric acid taken	...	...	...	29	29	29	29
cc. $(\text{NH}_4)_2\text{SO}_4$ used in washing	...	...	...	—	50	100	200
mg. uric acid in precipitate	...	...	...	27.11	26.96	26.85	26.25
mg. uric acid in mother liquor plus wash water	...	...	...	1.58	1.73	1.95	2.25
Total uric acid recovered	...	...	...	28.69	28.69	28.80	28.50

Other series gave similar results. The sulphate solution then has quite a definite solvent action on the ammonium urate.

The question whether strong ammonia solution could rapidly destroy uric acid in water whilst having no effect in the presence of 10 % ammonium sulphate seemed worth testing. In the first series the amount of ammonia solution used in precipitating the uric acid from the sulphate solution varied and the time of exposure to the alkali remained the same, viz. 48 hours, while in the second series the amount of alkali was fixed, 5 cc. as recommended in the method, but the time of exposure varied (see Table IX).

Table IX.

mg. uric acid taken	...	19.39	19.39	19.39	19.39
cc. ammonia solution added	...	0.5	2.0	5.0	10.0
Uric acid recovered	...	19.31	19.22	18.82	18.64
mg. uric acid taken	...	19.39	19.39	19.39	19.39
Time of exposure in hours	...	21	47	96	96
Uric acid recovered	...	18.88	18.84	18.66	18.66

Destruction, although present, is slight.

CONCLUSIONS.

(1) In this method blank titrations are essential, the blank containing 100 cc. water, 15 cc. concentrated sulphuric acid and a filter paper similar to the one used in collecting the ammonium urate precipitate. This blank should be repeated daily with the materials to be used in the estimation that day.

(2) The degree of dilution of uric acid does not affect the titration.

(3) In gauging the end point a time limit, such as 10 seconds, should be used as suggested above.

(4) The temperature should be the same day by day, the optimum being about 60°.

(5) Traces of chloride do not upset the titration with permanganate.

(6) The amount of 10 % ammonium sulphate solution used in washing the precipitate of urate should be not more than 100 cc. and the worker should continue to use the same amount all through his series.

(7) Strong ammonia solution, even in the presence of ammonium sulphate solution, attacks the uric acid so that the amount of ammonia used and the

time the uric acid is allowed to stand in contact with it should not vary from day to day.

These conclusions show that, in spite of the accuracy of the method, comparable results can be obtained only by observing the same technique day after day. This is all the more important as, in metabolic work, the amount of uric acid in the urine per diem is obtained by multiplying the results of such experiments by at least ten, so that a small alteration in technique may give a result which will appear as a noteworthy variation in total output of uric acid.

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