XLIH. THE ESTIMATION OF AMMONIA AND UREA IN URINE AND OTHER FLUIDS.

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F. W. FOREMAN [1920] has used steam alcoholic distillation to separate volatile bases from amino acids, estimations being afterwards made by his well-known method of titrating in alcohol. He has since applied the method to mixtures of urea and ammonia, and finds that it is applicable to urine¹.

The fact that when a mixture of urea and ammonia is steam distilled in alcohol only the ammonia distils over during a certain stage of the distillation has been made use of in the Cambridge Biochemical Laboratory—at Mr Foreman's suggestion—to estimate ammonia and urea in solutions containing either or both. The method is so very rapid and easy to handle that it should be generally employed. Its use can only be attended with confidence however if the conditions for the distillation are carefully defined. It is the purpose of this paper to show that with due attention to conditions it can be made absolutely reliable. When it is borne in mind that a single determination occupies only a few minutes, the advantages of the method over the aeration and other methods at present in use will be sufficiently obvious.

METHOD.

The apparatus consists of a steam boiler, a large (1 litre) distilling flask and a condenser. The distilling flask, which is placed at 45° to the horizontal, is fitted with a rubber cork carrying a tap funnel containing an alcoholic solution of caustic potash coloured with phenolphthalein; and a glass tube which is bent so that it dips right down into the lowest part of the flask. This tube is connected by a movable piece of rubber tubing to the steam boiler. The side tube of the flask is connected to the condenser, which is arranged as nearly vertical in position as possible. A collecting flask containing standard HCl is placed so as to receive the distillate. A sample (5 to 10 cc.) of the fluid under investigation is taken and mixed with (90 cc. of) alcohol and placed in the distilling flask. The boiler is heated but it is not connected to the distilling flask until a vigorous jet of steam is issuing from the connecting tube. The coloured alcoholic potash solution is next run in from the tap funnel until the fluid in the distilling flask is just alkaline to phenolphthalein. If necessary more potash may be added in this way during

¹ Private communication.

the distillation. This method of rendering the fluid alkaline in order that ammonia may be distilled off has been found necessary in order to ensure that no leakage of ammonia occurs. The fluid soon boils and ammonia and other volatile bases are distilled over into the collecting flask, where they neutralise the acid placed there; and are subsequently estimated by titration with CO_2 -free soda, using alizarin-red as an indicator. After a few minutes a foam or froth appears on the fluid in the distilling flask. This indicates that the alcohol has nearly all distilled off from the fluid; frothing commences when the alcohol concentration is still as high as 3 %. At the first sign of frothing, therefore, the rubber stopper of the flask is turned round so that the bent end of the steam delivery tube is turned up out of the fluid. The steam then passes through the distilling flask and condenser and carries away any ammonia present there. This "washing" is allowed to continue for $1\frac{1}{2}$ minutes and the collecting flask is then removed and titrated as described above.

Practical Notes.

1. Frothing should not be confused with the splashing and bubbling that occurs in the first few minutes. It is a steady foam that gradually spreads over the surface of the fluid. Frothing may be made very obvious by the addition of a minute amount of fat or fatty acid, but it is easily detected after a few attempts at using the apparatus.

2. The condenser tube should be arranged nearly vertically to ensure complete drainage. Otherwise the condenser must be washed through with distilled water at the end of the distillation.

3. To avoid fluid in the distilling flask being splashed over, use a large flask (about one litre) and incline it as much as possible.

Note on corrections applied to the results.

The alcohol used was 96 %; wherever alcohol content is expressed as a percentage, it is this percentage of 96 % alcohol to which we refer. For example "80 % alcohol" should read "80 cc. of 96 % alcohol in 100 cc. of fluid."

The distilled water used was not neutral to the indicator (alizarin-red) used. The correction was applied where necessary:

25 cc. distilled water found equivalent to 0.10 cc. of $N/50$ HCl.

The blank correction for the apparatus was obtained by distilling a mixture of alcohol and water until frothing:

Blank correction = + 0.2 cc. $N/50$ HCl.

The urea used was twice recrystallised. A small amount of ammonium carbonate, etc. was however still present as impurity. Thus:

4 cc. of 25 % urea yielded ammonia equivalent to 1.15 cc. $N/50$ HCl.

The correct value of the ammonium carbonate solutions was obtained by distillation in aqueous solution and by the Van Slyke modification of Folin's aeration method.

The value of ammonia is expressed throughout in terms of its equivalent standard acid.

The amount of ammonia given off before frothing depends upon the time of frothing and the rate of boiling, but these are determined by the composition and bulk of the fluid used. Our first experiments, therefore, were intended to show the relationship between percentage of alcohol, bulk of alcohol and aqueous solution, and the amount of ammonia given off before frothing.

Exp. 1. Effect of varying the percentage of alcohol with constant bulk of fluid (100 cc.) and constant volume of ammonium carbonate solution (5 cc.).

Percentage alcohol	(1)			(2)	
	Ammonia present cc. N/50 HCl	Ammonia distilled cc. N/50 HCl	Time to frothing m. s.	Ammonia distilled cc. N/50 HCl	Time to frothing m. s.
10	21.30	8.20	2 10	7.40	2 5
30	21.30	15.40	3 0	13.15	3 0
50	21.30	18.80	3 30	18.00	3 30
70	21.30	21.00	4 50	21.10	4 40
80	21.30	21.25	5 0	21.30	5 0
90	21.30	21.30	5 30	21.30	5 40
95	21.30	21.30	5 40	21.30	5 40

This experiment shows that only a proportion of 21.3 cc. of ammonia (N/50 acid) is distilled over before frothing if the percentage of alcohol is 70 or under. It is also evident that with constant total volume of fluid, increasing the percentage of alcohol increases the time up to frothing.

Exp. 2. The effect of varying the volume of aqueous solution, with constant volume of alcohol (95 cc.) and constant volume of ammonium carbonate solution (5 cc.).

Volume alcohol cc.	Volume of aqueous solution cc.	Ammonia present cc. N/50 acid	Ammonia distilled cc. N/50 acid	Calculated percentage alcohol
95	5	127.0	127.00	95
95	25	127.0	126.60	80
95	40	127.0	126.15	70
95	55	127.0	126.00	63
95	70	127.0	124.40	58
95	95	127.0	121.10	50

This experiment shows that although the large volume of 95 cc. alcohol is present, it is also necessary to have the percentage alcohol above a critical value to ensure that all the ammonia has distilled over before frothing.

Exp. 3. Effect of varying the total volume of fluid, percentage of alcohol being kept constant.

Percentage alcohol	Total volume of solution cc.	Ammonia present cc. N/10 acid	Ammonia distilled cc. N/10 acid	Time to frothing m. s.
90	100	42.60	42.65	5 20
90	50	42.60	41.50	4 0
"		cc. N/50 acid	cc. N/50 acid	
90	100	42.35	42.15	5 20
90	50	42.35	42.30	4 0

This experiment shows that in addition to critical percentage, a critical volume is necessary to ensure that all the ammonia distills over before frothing. This critical volume varies with the amount of ammonia originally present in solution.

The first three experiments indicated that 90 % alcohol and 100 cc. total volume were values which ensured complete distillation of these quantities of ammonia before frothing; and our next step was to ascertain the range of ammonia concentration for which they held.

Exp. 4. Effect of varying the amount of ammonia, with constant percentage of alcohol (90 %) and constant total volume of fluid (100 cc.).

Ammonia present cc. N/10 acid	Ammonia distilled cc. N/10 acid	Ammonia distilled cc. N/10 acid
144.10	144.10	144.15
43.50	43.45	43.50
26.10	26.10	26.10
8.70	8.70	8.70
cc. N/50 acid	cc. N/50 acid	cc. N/50 acid
0.395	0.395	0.390

This experiment shows that with 90 % alcohol and 100 cc. total volume of fluid, any value of ammonia between 144.1 cc. (N/10 acid) and 0.395 cc. (N/50 acid) can be completely distilled over before frothing. We did not attempt to estimate still smaller quantities of ammonia, since the blank correction was greater than 50 % of this ammonia value. The highest value of ammonia estimated represents 5 cc. of saturated ammonium carbonate solution, *i.e.* the highest concentration possible. The values of 90 % alcohol and 100 cc. total fluid are therefore safe and accurate for this large range of ammonia values.

We then investigated whether the presence of urea in the solution would influence the proportion of ammonia given off before frothing.

Exp. 5. Effect of varying the percentage of alcohol, total volume of fluid (100 cc.) and the ammonia content being kept constant. The solution contains 4 cc. of 25 % urea.

Percentage alcohol	Ammonia present cc. N/10 acid	Ammonia distilled, cc. N/10 acid	
		(1)	(2)
90	144.10	144.10	144.10
80	144.10	144.05	144.05
70	144.10	143.40	143.50
60	144.10	141.60	141.65

The experiment shows that the presence of urea in solution does not apparently influence the distillation of the ammonia. With 90 % alcohol and total volume of 100 cc. the whole of the ammonia distills over before frothing (*cf.* *Exp. 1*).

Exp. 6. Effect of extreme ratios of urea and ammonia, with percentage of alcohol constant (90 %) and total volume of fluid constant (100 cc.).

Saturated urea solution	0.04 % urea solution	Ammonia present cc. N/50 acid	Ammonia present cc. N/10 acid	Ammonia distilled, cc.	
				(1)	(2)
5	—	0.395	—	0.395	0.400
5	—	—	144.10	144.05	144.15
—	5	0.395	—	0.390	0.395
—	5	—	144.10	144.20	144.10

This experiment shows that very small and very large amounts of ammonia can be estimated accurately in the presence of very large and very small concentrations of urea.

Time course of the Distillation.

It was necessary to ascertain for different percentages of alcohol the exact time relation of frothing to the end of ammonia distillation and the commencement of urea decomposition. This was done by finding the volume of ammonia given off at different periods after the commencement of distillation. The collecting flasks containing standard acid were changed at noted times. Volumes of ammonia (equivalents in $N/50$ acid) were plotted along the ordinates, and time along the abscissae, and the time curves thus obtained. The total volume of fluid in all cases was 100 cc.

Fig. 1. Curves showing time relations of the analysis.

Abscissae = time from commencement of analysis in minutes.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Total bulk of fluid 100 cc. Ammonia content the same in all the curves except the first.

See Exp. 6.

Arrows indicate commencement of frothing.

Practical Notes.

1. The ammonia in the condenser at the time of removing a receiving flask is inevitably collected in the next flask.

2. The slight loss of ammonia that may occur on changing collecting flasks does not affect the well-marked nature of the curves.

3. The time curve for urea alone shows that the ammonia from the impurity of ammonium carbonate is distilled over completely before frothing. The value of the ammonia impurity was confirmed by Van Slyke's method, thus permitting a correction for the remaining time curves.

Exp. 7. Data for time curves. The following tables illustrate the nature of the data upon which the curves are based.

The time of frothing and the value of ammonia distilled over by that time are denoted by black figures. In each case total volume of fluid is 100 cc.; urea content is constant (4 cc. of 25 % solution) and ammonia content is constant (21.40 cc. *N*/50).

(1) Urea alone in 100 cc. 90 % alcohol		(2) Urea and ammonia in 30 % alcohol		(3) Urea and ammonia in 90 % alcohol	
Time m. s.	Ammonia distilled cc. <i>N</i> /50	Time m. s.	Ammonia distilled cc. <i>N</i> /50	Time m. s.	Ammonia distilled cc. <i>N</i> /50
2 0	0.1	2 0	0.3	2 0	5.50
4 0	0.45	3 20	12.1	4 0	18.90
6 30	1.10	4 0	15.2	5 30	21.40
7 10	1.10	4 50	18.1	6 10	21.40
8 0	1.50	6 50	22.5	6 50	21.45
8 50	2.10	8 50	26.7	7 40	21.85
10 50	5.30	11 50	33.3	9 40	24.35
13 0	10.10	15 0	40.0	11 40	27.55
5 20	15.30			15 0	32.85

N.B. 1.10 cc. represents impurity (*vide supra*).

Deductions from Time Curves.

The curves for percentages of alcohol of 70 and under (Figs. 1 and 2) show that there is no appreciable time interval between the end of ammonia distillation and the commencement of urea decomposition. The ammonia from the urea decomposition distils over before the whole of the ammonia of the ammonium carbonate has been distilled. With these low percentages it is therefore impossible to stop distillation at any definite time and obtain a correct result. This explains the discrepant results obtained through not using a sufficiently high percentage of alcohol. Frothing in these cases is of no value as a time mark. The 80 % alcohol curve shows a minimum period of zero distillation but it is not absolutely flat and must therefore be classified as unreliable for accurate work. The 90 % alcohol curve, however, shows a well-marked flat zero period, which extends over an appreciable time; and during which no ammonia distils over. Frothing (indicated by a vertical arrow) is seen to fall at the commencement of this flat zero period, and marks in this case the completion of ammonia distillation. It therefore serves as a valuable signal

for the cessation of distillation. The flat zero period represents the safety interval during which no ammonia distils over, and this is followed by urea decomposition. 90 % alcohol and a total volume of 100 cc. are therefore the conditions necessary for accurate ammonia distillations in the presence of urea.

Fig. 2. Curves showing time relations of the analysis.

Abscissae = time from commencement of analysis in minutes.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Arrows indicate commencement of frothing.

The same bulk of fluid was used for each curve. The ammonia content was the same for each curve.

If working with unknown solutions a time curve for the conditions of experiment will show immediately whether the results obtained are reliable. If the curve resembles that for 90 % alcohol, the conditions are correct. If the zero period is absent or very short it is due to too low a percentage of alcohol or too small a total volume of fluid. This can be remedied accordingly. Thus the great value of an initial time curve for any accurate work is evident.

THE ESTIMATION OF UREA.

This is done by converting the urea into ammonium carbonate by the action of a strong solution of urease. At 40° C. complete conversion was

invariably obtained in 20 minutes. The urease solution was buffered to p_H 7.02 by means of a solution consisting of suitable proportions of 3 molar caustic potash and molar acid potassium phosphate solutions.

The effect of such a buffered urease solution on the analysis was first investigated.

Exp. 8. Analysis of 100 cc. fluid containing 1 cc. ammonia and 9 cc. strong urease solution buffered to p_H 7.02.

Cc. alcohol	Ammonia present cc. <i>N</i> /50	Ammonia found cc. <i>N</i> /50
90	44.10	44.15
90	44.10	44.10
90	44.10	44.10

This experiment shows that urease has no effect upon the amount of ammonia distilled over up to frothing from 100 cc. of a 90 % alcohol solution of ammonia.

Solutions containing 25 % and 0.04 % of urea were next analysed and the results compared with those given by the aeration method.

Exp. 9. Analysis of 1 cc. of 25 % urea solution.

Method	Correct result 80.10 cc. <i>N</i> /10. Results in cc. <i>N</i> /10.			Mean
	1st exp.	2nd exp.	3rd exp.	
Steam-alcohol distillation	80.05	80.10	80.10	80.08
Van Slyke aeration	80.10	80.15	80.15	80.13

Exp. 10. Analysis of 10 cc. of 0.04 % urea solution in 100 cc. 90 % alcohol.

Method	Results in cc. <i>N</i> /50.			
	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	6.35	6.37	6.37	6.37
Van Slyke aeration	6.34	6.33	6.36	6.34

The method is therefore quite accurate for the estimation of urea in either strong or weak solutions.

APPLICATION TO URINE.

We then successfully attempted to apply the method to the analysis of urine.

In accordance with the recommendations made earlier in this paper our first experiment was to obtain a "time-ammonia" curve for the apparatus when 5 cc. normal urine, 90 cc. alcohol, and the equivalent in 5 cc. of ammonium carbonate solution of about 45 cc. of *N*/50 acid were present in the distilling flask. The curve obtained is shown in Fig. 3 c. The actual values are given below, Exp. 16.

The curve is of the type given by the 90 % alcohol solutions shown in Fig. 2. All the ammonia has therefore been distilled over before frothing commences. The conditions were therefore assumed to be correct and the following experiments were made.

A series of analyses of normal urine were made by this method and compared with the results given by the Van Slyke modification of the Folin aeration method.

Exp. 11. Analysis of 5 cc. urine in 100 cc. fluid containing 90 % alcohol.

Method	Results in cc. N/50.				
	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	7.25	7.20	7.24	7.25	7.235
Van Slyke aeration	7.22	7.24	7.25	7.25	7.240

Exp. 12. Analysis of 5 cc. urine in 100 cc. 90 % alcohol containing added ammonium carbonate.

Method	Results in cc. N/10.			
	1st exp.	2nd exp.	3rd exp.	Mean
Steam-alcohol distillation	44.12	44.10	44.15	44.120
Van Slyke aeration	44.10	44.15	44.10	44.120

This is as large a *quantity* of ammonia as is ever present in the quantities of urine which we recommend using, either before or after the urea has been converted into ammonium carbonate. The time curve (not shown) of this experiment was quite satisfactory.

These experiments show that the method is quite accurate for the estimation of ammonia in normal urine.

The urea was next estimated. 1 cc. of urine was acted on by 9 cc. of standard urease solution at 40° for 20 minutes. All the urea was found to be converted into ammonium carbonate by this procedure.

Exp. 13. Analysis of 1 cc. urine after treatment with urease to convert the urea into ammonium carbonate. 100 cc. of 90 % alcohol were used.

Method	Results in cc. N/50.				
	1st exp.	2nd exp.	3rd exp.	4th exp.	Mean
Steam-alcohol distillation	35.40	35.45	35.45	35.45	35.44
Van Slyke aeration	35.40	35.45	35.45	35.45	35.44

Again there is complete agreement with the Van Slyke-Folin method.

The method is therefore unaffected by urease in the presence of urine and it is accurate for the estimation of ammonia and urea in normal urine.

We then ascertained whether the method was equally accurate for the estimation of ammonia and urea in pathological urines. To do this we analysed samples of urine containing various foreign substances both with and without urease.

Exp. 14. Analysis of 5 cc. urine in 100 cc. 90 % alcohol in the presence of foreign substances.

Substance introduced	Results in cc. N/50.		Substance introduced	Results in cc. N/50.	
	Ammonia present	Ammonia found		Ammonia present	Ammonia found
1 g. glucose	7.00	7.00	1 cc. butyric acid	7.00	6.95
"	7.00	7.00	1 cc. conc. butyric acid	7.00	7.00
1 g. serum albumin	7.00	6.95	1 cc. 4 % aceto-acetic acid	7.00	7.00
"	7.00	6.95	" "	7.00	7.00
1 cc. conc. acetone	7.00	6.98			
"	7.00	6.95			

The results found in the presence of urease were exactly similar.

We conclude from these experiments that the method is applicable with great accuracy to the estimation of ammonia and urea in all urines whether normal or pathological.

Although the alcohol used may be easily recovered from the distillate after titration it is desirable that as little as possible be used when a large number of estimations are being made. We therefore investigated the effect of halving the volume of alcohol used, so that the total volume of fluid was only 50 cc. instead of 100 cc. We first repeated Exp. 11 using this smaller bulk.

Exp. 15. Analysis of urine in 50 cc. of fluid.

Cc. alcohol	Cc. urine	Ammonia present cc. N/50	Ammonia found cc. N/50
45	5	42.35	42.30
45	5	42.35	42.35
40	1 + 9 cc. urease	39.12	39.15
40	1 + 9 cc. „	39.12	39.10

For these values therefore the correct result is obtained with the smaller bulk.

From Exp. 3 it will be remembered, however, that 42.6 cc. N/10 ammonia (the maximum amount which can possibly be present in our urine estimations) is not quite all distilled over before frothing commences when a total bulk of 50 cc. of 90 % alcohol is used. The same holds good for urine and is an objection to the use of a smaller quantity of alcohol than 90 cc.

The time curves were then investigated and compared with those of equal percentage alcohol content but of double the volume of fluid.

The curves are shown in Fig. 3.

Exp. 16. Time curves for urine. See Fig. 3. The following table illustrates the nature of the data from which the curves were constructed. Frothing occurred at the time denoted by black figures.

Analysis of 5 cc. urine A in 100 cc. and B in 50 cc. 90 % alcohol. Ammonia content = 42.35 cc. N/50.

Time from commencement		Cc. N/50 ammonia distilled over	
A	B	A	B
m. s.	m. s.		
2 0	2 0	16.0	18.2
5 0	4 20	42.0	41.7
5 20	4 50	42.3	42.3
6 0	5 20	42.3	42.3
6 40	6 0	42.3	42.8
7 30	8 0	42.4	43.3
9 0	10 0	43.1	44.6
12 0	13 30	45.1	46.6
15 0	—	49.7	—

From this experiment it is clear that in the smaller bulk the zero period is smaller than in the larger bulk of equal alcohol percentage; and, bearing this in mind, there are two objections to using the smaller bulk.

(1) The zero period is shorter in the smaller bulk than in the larger bulk of equal alcohol concentration and therefore if the distillation is not stopped exactly at the commencement of frothing an error may be introduced.

(2) If the ammonia present is greater than 42.35 cc. $N/50$ all the ammonia may not have distilled over before frothing commences.

Fig. 3. Time curves for urines. See Exp. 16.

Ordinates = cc. $N/50$ HCl neutralised by the ammonia distilled over.

Abscissae = time in minutes from commencement of analysis.

Arrows indicate commencement of frothing.

The ammonia content was the same in all cases.

This error may be avoided, of course, by using smaller quantities of urine.

For general work therefore we recommend conducting the analysis in 100 cc. of 90 % alcohol which we have shown to give accurate results in all cases. For analysing *quantities* of ammonia less than 42.35 cc. $N/50$, however, a total volume of 50 cc. may be used with perfect accuracy.

SUMMARY AND CONCLUSION.

1. It is shown that the estimation of ammonia and urea by means of a steam-alcohol distillation is accurate to a high degree for all quantities of ammonia investigated, from 0.395 cc. *N*/50 to 144.1 cc. *N*/10 ammonium carbonate, which represents the analysis of a saturated solution of this salt. The small differences shown in some results are within the limits of error of the titration.

2. The conditions governing the steam alcohol distillation of ammonia were thoroughly investigated and it was found that when the analysis was conducted in 100 cc. of 90 % alcohol all the ammonia was distilled over before frothing commenced. This gave accurate results for all concentrations of ammonia up to saturation.

3. It is shown that the accuracy of the method in any particular case may be tested by obtaining a time analysis curve, the form of which indicates whether correct results are being obtained; although the ammonia content may not be otherwise known.

4. The method is proved to be accurate for the investigation of the action of urease on urea.

5. The method is rapid. A complete determination of ammonia and urea may be made with one apparatus in 30 minutes, including the time taken for the conversion of urea into ammonium carbonate. A determination of ammonia requires seven minutes only.

6. The method is applied successfully to the estimation of ammonia and urea in urines and is shown to hold good for pathological as well as for normal urines.

In our opinion the accuracy, rapidity, and facility of manipulation which the method possesses render it more desirable as a means of estimating ammonia and urea than any of the methods at present in use.

REFERENCE.

Foreman (1920). *Biochem. J.* 14, 451.