XL. SOME OBSERVATIONS ON THE ESTIMATION OF UREA.

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The chief aim of the following investigation was to find out a simpler method for the estimation of urea than those at present in use. The hypobromite method, although rapid and simple, has been shown to be unreliable [Mörner, 1903]. All the more accurate methods depend like that of Folin [1901, 1902] on the conversion of urea into an ammonium salt, and the estimation of the ammonia by distillation. These methods necessarily involve a preliminary estimation of the preformed ammonia of the urine. No useful purpose would be served by an enumeration of the chief modifications which the methods have undergone. Reference may be made to the exhaustive account given in the revised edition of Neubauer and Huppert's *Analyse des Harns* [1910].

The distillation of the preformed ammonia and that derived from the hydrolysis of urea occupies in each case about an hour. A considerable reduction of time and simplification of the method would be secured, if the distillation were replaced in both instances by titration in the presence of formaldehyde. The main objections to the latter method are that other amine derivatives, notably amino- and diamino-acids affect the result of the titration [Schiff, 1899, 1901, 1902], and that the best method for the determination of the neutral point, from which one starts, is still unsettled. Ronchese [1907] and Malfatti [1908] employ phenolphthalein throughout as indicator in their method for the estimation of ammonia in the urine by formol-titration, while Sörensen [1907, 1910] and Henriques [1909] use azolitmin paper as the indicator in neutralising the solution and phenolphthalein for the titration of the acids set free by the addition of neutral formol in their method for estimating amino-acids. De Jager [1909, 1; 1910, 1] follows the same procedure as Malfatti after adding excess of neutral potassium oxalate or of potassium sulphate. Folin [1903] had

previously made similar use of potassium oxalate in determining the acidity of the urine. The fact that solutions of chemically pure ammonium salts react acid to phenolphthalein has long been recognised. In consequence, the results of formol-titrations with this as the sole indicator are too low. One objection to the method of overcoming this difficulty adopted by Sørensen and Henriques is the inconvenience of the frequent testing with azolitmin paper required in the course of the titration. The addition of litmus to the solution renders the titration difficult and uncertain owing to the conflicting colour changes of the two indicators. In its place, I have used methyl-red [Rupp and Loose, 1908; Tizard, 1910; Howard and Pope, 1911] as the indicator of the neutral point and phenolphthalein for the determination of the amount of acid set free by the addition of neutral formol. Methyl-red has the advantage of being an exceedingly sensitive indicator for the titration of weak bases such as ammonia, while it is comparatively insensitive to weak acids. On the other hand, phenolphthalein is a good indicator for weak acids, but is not a reliable indicator for weak bases. The colour changes of the two indicators do not interfere with one another, and they may therefore be used together without any difficulty arising. Methyl-orange may be used instead of methyl-red, but it does not give such a sharp endpoint.

1. PRELIMINARY OBSERVATIONS ON THE TITRATION OF PURE AMMONIUM SALTS.

The following experiments were carried out in order to ascertain the degree of accuracy of the method as compared with formol-titrations of ammonium salts in the presence of phenolphthalein alone. Five drops of a 0.5% alcoholic solution of phenolphthalein were added to 10 cc. of a decinormal solution of pure ammonium chloride, which had been previously rendered faintly acid by the addition of dilute hydrochloric acid. The solution was then neutralised by the addition of sufficient decinormal caustic potash just to render the solution faintly red. Excess (about 5 cc.) of neutral formol (50 cc. 40% formaldehyde, 1 cc. of 0.5% alcoholic phenolphthalein, and sufficient decinormal caustic potash to render the solution faintly pink) was then added, and the addition of decinormal alkali continued until the solution just acquired a faint permanent red colour. The addition of 9.55 cc. of decinormal alkali was found to be necessary to reach this point instead of the true value 10 cc. Two to three drops of a 0.2% alcoholic solution of methyl-red were added to another 10 cc. of a decinormal solution of

ammonium chloride, and then sufficient decinormal caustic potash just to convert the red colour of the solution to a yellow. On the addition of neutral formol, the solution again acquired a red colour owing to the hydrochloric acid, set free by the interaction of the formaldehyde with the ammonium chloride, acting upon the methyl-red. On the further gradual addition of a measured quantity of decinormal caustic potash, the red colour fades and is replaced by a yellow. The addition of alkali was continued until a faint permanent red colour, due to the action of the alkali on the phenolphthalein, just appeared. 9.95–10 cc. were required to reach this point. Repeated control estimations of the same character with solutions of pure ammonium sulphate as well as of ammonium chloride gave similar results. It will be noted that, when the two indicators are used, the solution during titration passes through three changes of colour. It is first red owing to the action of free mineral acid on the methyl-red, then orange to yellow when the solution contains only free organic acid, and finally red when just alkaline owing to the action of slight excess of free alkali on the phenolphthalein. The organic acid is formic acid present in the formaldehyde solution and resulting from the oxidation of part of the formaldehyde. Thus, prior to the addition of the neutral formol, the solution contains only ammonium chloride; while after its addition the fluid contains hexamethylenetetramine, free hydrochloric and formic acids, the latter two being in varying proportions according to the amount of formic acid present in the original solution of formaldehyde. In the presence of traces of free formic acid, methyl-red has nearly the same tint as in neutral solution, and, therefore, when all the free mineral acid has been neutralised, the colour of the methyl-red changes to faint orange and later to yellow, although the solution is found still to be distinctly acid to phenolphthalein.

2. APPLICATION OF THE FOREGOING RESULTS TO THE ESTIMATION OF PURE UREA IN AQUEOUS SOLUTION.

Folin's method of hydrolysis [1901, 1902] is not applicable in this case, since the presence of a large amount of magnesium chloride interferes with the subsequent titration. A slight modification of Benedict and Gephart's method of hydrolysis [1909] was consequently adopted. The modification consisted merely in using more dilute acid for the hydrolysis of the urea. When a considerable excess of acid is used, the large amount of neutral salt formed in the course of neutralisation interferes with the accuracy of the determination of the endpoint [Michaelis and Rona, 1909; Andersen, 1911].

The use of a smaller amount of acid also lessens the risk of the decomposition of other nitrogenous substances, when the method is applied to urine. After using this method of hydrolysis for some time, I found that it had been previously employed by Henriques and Gammeltoft [1911].

At first the hydrolysis was carried out in sealed glass tubes heated in a glycerol bath to 160° for four hours. Two cc. of a 2.006% solution of pure urea and 2 cc. of normal sulphuric acid were placed in a glass tube, which was then sealed and heated in a glycerol bath. After being allowed to cool, the sealed tube was opened and the contents carefully transferred to a flask, the broken tube being thoroughly washed with distilled water. The fluid was then neutralised in the presence of methyl-red as indicator, and the ammonia determined by means of formol-titration using phenolphthalein as the indicator. The following are the results given in tabular form. One cc. of decinormal alkali is equivalent to 0.003 g. of urea.

Cc. of 2.006% urea	Cc. of N. acid for hydrolysis	Cc. of N/10 alkali (formol titr.)	G. of urea found	Theoretical amount of urea
2	2 H ₂ SO ₄	13.3-13.35	0.0399-0.04	0.0401
2	2.5 HCl	13.4	0.0402	0.0401

Since the use of sealed tubes is somewhat inconvenient, Benedict and Gephart's method [1909] of heating the solution in test-tubes covered with leadfoil and placed in an autoclave kept at a temperature of 155° for 1.5 hours was afterwards employed. A large number of estimations can readily be carried out simultaneously by means of this method. The temperature in the autoclave was varied in the first experiments in order to find the most suitable temperature for hydrolysis.

2.3101 g. of pure urea were placed in a 200 cc. flask, dissolved in water, 100 cc. of normal sulphuric acid were added, and the whole made up to 200 cc.; 5 cc. portions of the acid solution were then hydrolysed. The following table gives the results.

Temperature	Cc. of N/10 alkali for formol-titr.	G. of urea found	Theoretical g. of urea
144-145° (70 minutes)	18.8	0.0564	0.0577
152-153° (30 minutes)	(1) 19.15	0.05745	"
	(2) 19.2	0.0576	"
longer)	(3) 19.15	0.05745	"

Since the application of the foregoing results to the estimation of the urea in the urine involves a preliminary formol-titration of preformed ammonia and other amine derivatives, it was essential to test the method by applying it to the titration of amino-acids as well as to mixtures of amino-acids with ammonium salts.

3. TITRATION OF AMINO-ACIDS ALONE AND WITH AMMONIUM SALTS.

Glycine being easily prepared in a pure state, was selected as a type for the tests. Since one has to deal with a mineral acid solution in the final formol titration, it was obviously essential to carry out the estimation under similar conditions, and also to compare the results with those obtained when phenolphthalein was used as the sole indicator. The following are the results given in tabular form. The glycine solution was 0.71 %.

These results indicate that, when the two indicators are used, the estimations of amino-acids and ammonium salts are more accurate than when phenolphthalein is the sole indicator. The fact that formol-titration yields too low results in the case of mixtures of amino-acids and ammonium salts was first noted by de Jager [1909, 2]. Henriques and Sørensen [1910] have suggested an explanation of this anomaly. More recently de Jager [1910, 2] has shown that the addition of urea enables one to obtain correct results. The latter fact is illustrated by the results given in (4) of the table.

Solutions used	Cc. N/10 alkali (for neutralisation)	Formol-titr. (found)	Theoretical result
(1) 10 cc. glycine 2.55 cc. N/10 HCl	2.55 (indicator methyl-red)	9.4	9.46
(2) 10 cc. glycine 2.75 cc. N/10 HCl	2.85-2.9 (indicator phenol- phthalein)	9.25	„
(3) 10 cc. glycine 10 cc. N/10 NH ₄ Cl 2 cc. N/10 HCl	2 cc. (methyl-red)	18.65	19.46
(4) 10 cc. glycine 10 cc. N/10 NH ₄ Cl 2 cc. N/10 HCl 0.5 g. urea	2 cc. (methyl-red)	19.4	„

4. ESTIMATION OF UREA IN URINE.

(1) *Without a preliminary purification of the urea.*

Since the presence of phosphates renders the neutral point indefinite for both indicators, and since the neutral points for the two indicators are markedly different in solutions of phosphates, it was necessary to precipitate the phosphates with baryta mixture. The addition of an unduly large excess of barium chloride is to be avoided, as its presence interferes slightly with the accuracy of the titration; but a small excess is immaterial. When sulphuric acid is used for the hydrolysis of the urea the excess of barium present in the filtrate is precipitated as barium sulphate, and it

was mainly for this reason that normal sulphuric acid rather than hydrochloric acid was used for the majority of the hydrolyses. The results of the method of formol-titration were controlled by comparison with the values for ammonia obtained by distillation with magnesia and with barium hydrate.

100 cc. of normal urine were mixed with 10 cc. of a binormal solution of barium chloride and sufficient barium hydrate to make the mixture distinctly alkaline, and finally made up with water to 200 cc. After the fluid had been thoroughly shaken, it was allowed to stand either for a few minutes or preferably in the presence of toluene for twelve hours. Henriques' method [1909] of freeing the urine from phosphates, sulphates and carbonates may be used as an alternative to the foregoing. The solution was then filtered. 25 cc. of the filtrate required 18.5 cc. of decinormal hydrochloric acid to render it neutral to methyl-red. The endpoint is not quite sharp. Excess of neutral formol (about 5 cc.) was added, and the titration with decinormal alkali completed. The average of two titrations was 5.2 cc. of decinormal alkali. This result gives the approximate amount of nitrogen in the form of preformed ammonia and amino-acids in 12.5 cc. of urine.

A number of 10 cc. portions of the filtrate were then mixed with 7-8 cc. of normal sulphuric acid, and placed in test-tubes in the autoclave, which was kept at a temperature of 155° for 1.5 hours. Although the fluid had darkened considerably in colour as a result of the heating with acid, the colour on dilution was not so deep as to interfere appreciably with the subsequent formol-titration. The ammonia in some portions was then set free by the addition of magnesia or barium hydrate, distilled off, and absorbed by excess of decinormal sulphuric acid (50-100 cc.) placed in the receiver. In others the ammonia and amino-acids were determined by formol-titration. Prior to carrying out the formol-titration it is advisable although not essential to filter off the barium sulphate. A considerable amount of pigment adheres to this and is removed with it. The test-tube and precipitate were then thoroughly washed, and the collected filtrate and washings treated by formol-titration. The following table (I) gives the results obtained. The deduction referred to in the table corresponds with the amount of substances in the original urine capable of titration by the formol method, consisting mainly of preformed ammonia and amino-acids.

An examination of the table indicates that the results of the formol-titration are invariably higher than those obtained by distillation; but agree well with one another. The difference is to be explained by the presence of non-volatile amino-groups set free by the hydrolysis with acid,

which raise the results obtained by formol-titration; but obviously cannot affect the values for ammonia obtained by distillation. The hydrolysis with normal acid splits up hippuric acid and any polypeptides which may be present, and thus renders the results of formol-titration higher than those obtained by distillation. The preliminary formol-titration, on which the deduction is based, gives the amount of nitrogen in the form of preformed ammonia and amino-acids: while, after hydrolysis, the final formol-titration gives the preformed ammonia and amino-acids, together with the amino-acids set free by hydrolysis, and the ammonia resulting from the decomposition of the urea. The difference between these two values represents the ammonia derived from urea plus the nitrogen of any amino-acids derived from hydrolysis of polypeptides and of hippuric acid. On the other hand, the method of distillation gives only the preformed ammonia and that formed by hydrolysis of urea. In calculating the results given in the table, the same deduction was made from the values obtained by distillation as from those by formol-titration. Strictly speaking, a smaller deduction should have been made from the latter. An independent determination of the preformed ammonia would be required to ascertain the true value of this correction. This point will be dealt with in a later part of this paper.

TABLE I.

(The quantity of urine was 5 cc. for each estimation.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 32.15	2.1 cc. N/10 alkali	1.803	0.8414
„	(2) 32.1	2.1	1.800	0.840
„	(3) 32.4	2.1	1.818	0.848
Distillation with barium hydrate	(1) 32.05	2.1	1.797	0.8386
„	(2) 32	2.1	1.794	0.837
„	(3) 31.9	2.1	1.788	0.834
Distillation with MgO	(1) 31.7	2.1	1.776	0.829
„ „	(2) 31.75	2.1	1.779	0.830

The difference between the result obtained by distillation and that by formol-titration gives an approximate value for the non-volatile amino-derivatives. A more accurate estimate might be made by taking advantage of the fact ascertained by de Jager [1910, 2] that the addition of urea is necessary in order to secure the true result for the sum of amino-acids and ammonia. This addition is unnecessary in the first formol-titration, since sufficient urea is present in the urine; but after hydrolysis the addition

of pure urea to the neutralised solution would be necessary before carrying out the second formol-titration. I have not considered the correction of sufficient importance in the case of the normal urines examined to warrant its introduction into the table; but in the event of a marked difference being found between the values obtained by distillation and formol-titration indicating excess of amino-acids, it would be advisable to make the correction.

The next table (II) gives the results of a similar series of estimations in a sample of another urine. Normal hydrochloric acid was used in this instance instead of sulphuric.

The same features will be noted as in the previous table. When barium hydrate is used as the fixed alkali, the values for ammonia are usually higher than when magnesia is employed. In order to obtain constant results, the distillation has to be more prolonged in the case of magnesia than with barium hydrate. The distillation usually occupied 1-2 hours in both cases. The differences between the results of formol-titration and of distillation is greater than in the other urine.

TABLE II.

(The quantity of urine used for each estimation was 5 cc.)

Method	Cc. of N/10 alkali	Deduction	% of urea	% of urea-N.
Formol-titration	(1) 36.65	2.5	2.049	0.9562
„	(2) 36.6	„	2.046	0.9548
„	(3) 36.6	„	2.046	0.9548
Distillation with barium hydrate	(1) 35.45	2.5	1.977	0.9226
„	(2) 35.65	„	1.989	0.9282
„	(3) 35.75	„	1.995	0.931
Distillation with MgO	(1) 35	2.5	1.95	0.910
„ „	(2) 35.5	„	1.98	0.924

(2) *Estimation of urea in urine after a preliminary extraction of the urea with a mixture of alcohol and ether.*

Folin's [1901] and Benedict and Gephart's methods [1909], as well as all other methods depending on the conversion of urea into the ammonium salt of the acid used for hydrolysis, give inaccurate results when carbohydrates are present in the urine. Mörner [1903] has shown that this is due to the fact that the humin-substances formed by the action of the acid on the sugar contain firmly bound nitrogen, when ammonium salts are present or formed during the hydrolysis.

Mörner adopted the following method in order to obtain a solution of urea free from carbohydrates. The phosphates were precipitated from 5 cc. of urine by the addition of 1·5–2 g. finely powdered barium hydrate, and the fluid was then extracted with 100 cc. of a mixture of two volumes of alcohol and one of ether. After being thoroughly mixed, the solution was allowed to stand for 24 hours, then filtered, and the precipitate washed with the mixture of alcohol and ether, the filtrate and washings being collected in the flask afterwards to be employed in the distillation. The alcohol and ether were then distilled off, and the residue after being freed from ammonia by concentration at 50° after the addition of magnesia, was hydrolysed by Folin's method [1901]. I carried out the first stages of the method in the same way; but instead of freeing the aqueous solution from ammonia by the addition of magnesia, I determined the amount of amino-nitrogen by means of formol-titration. The method of hydrolysis adopted was the modification of Benedict and Gephart's method [1909] previously described. In some cases normal hydrochloric acid was used for the hydrolysis, in others normal sulphuric acid. The following table summarises the results.

TABLE III.

Method	Results in cc. of N/10 NH ₃	Deduction	% urea	% urea-N.
1. Direct formol-titration	(a) 29·55	0·85	1·722	0·8036
	(b) 29·7	„	1·731	0·8078
	(c) 29·65	„	1·728	0·8064
2. Distillation with Ba(OH) ₂	(a) 29·1	0·85	1·695	0·791
	(b) 29·15	„	1·698	0·7924
3. Distillation with MgO	(a) 28·45	0·85	1·656	0·773
	(b) 28·5	„	1·659	0·774
	(c) 28·1	„	1·635	0·763

The following are the chief features revealed by an examination of the table. The comparatively low value of the deduction is to be explained by the fact that the method of preparing the aqueous solution of urea involves the loss of the greater part of the preformed ammonia. The values for urea obtained by the formol method are, as in the previous estimations, higher than those obtained by distillation. Amino-derivatives evidently pass into the alcohol and ether mixture. Hippuric acid is probably the most important of these. The glycine derived from its hydrolysis must raise the results of the formol-titration, while it obviously cannot affect the values obtained by distillation. The results obtained by distillation with magnesia are considerably lower than those with barium hydrate.

5. FINAL CONTROL OF THE METHOD BY AN INDEPENDENT DETERMINATION OF THE PREFORMED AMMONIA.

The estimations hitherto given suffer from the defect of not having been controlled by an independent determination of the preformed ammonia. Reference was previously made to this fact. In a final series of experiments, I have estimated the total nitrogen by Kjeldahl's method, the nitrogen of the preformed ammonia by the method of Krüger, Reich, and Schittenhelm [1903], the ammonia and urea nitrogen after hydrolysis by distillation, and the nitrogen of the amino-acids by Henriques' method [1909]. The results for 100 cc. of urine are stated in Table IV in terms of decinormal alkali and in Table V as percentages.

TABLE IV.

Results for 100 cc. urine expressed in terms of decinormal nitrogen.

Total N.	Urea and NH ₃ -N. (distillation)	Urea + NH ₃ + amino- acid nitrogen (formol-titration)	NH ₃ + amino-N. (in original urine)	Ammonia-N. (distillation)
587	529	550	33.6 (Sørensen)	23.9
585	528	544	35.2 (methyl-red)	24.2
583	524	546	—	—
585 (mean)	527 (mean)	547 (mean)	—	24.05 (mean)

TABLE V.

Total N.	Urea-N.	Urea-N. (formol-titr.)	Amino-N.	Ammonia-N.
585	503	513 (Sørensen)	9.6 (Sørensen)	24.05
—	—	511.4 (methyl-red)	11.2 (methyl-red)	—
0.819 %	0.704 %	0.718 % (S.) 0.716 % (M.)	0.013 % (S.) 0.0157 % (M.)	0.0337 %

The value given in column 2 of Table V is arrived at by deducting the amount of preformed ammonia as determined by the method of Krüger, Reich, and Schittenhelm from the total amount of ammonia obtained by distillation after complete hydrolysis of the urea. This may be regarded as the correct value for urea arrived at by a standard method. The values for the nitrogen of urea given in the third column of Table V are obtained by deducting the results of column 4, Table IV, from those of column 3, Table IV, using in the first instance the results of Henriques and Sørensen's method and in the second case those obtained by the use of methyl-red and phenolphthalein as the two indicators. The values for the nitrogen of the amino-acids given in column 4 of Table V are arrived at by subtracting the

results of the method of Krüger, Reich and Schittenhelm given in column 5 of Table IV from those of column 4, Table IV, using in the one case Henriques' method, and in the second formol-titration with methyl-red as the first indicator.

Sörensen [1907, 1910] used fifth-normal alkali for the titration of the amino-acids. Notwithstanding the fact that this strength is to be preferred for reasons stated by that author, I was obliged to continue the use of decinormal alkali as it had been used throughout the foregoing investigation. These final results indicate that the error in the method of estimating urea in this urine by formol-titration is a positive one amounting to approximately 1.7 %.

The chief advantages of the formol-method of estimating urea are that it is not only more rapidly carried out than the methods involving distillation, but also entails less arrangement of apparatus. The fact that the method gives at the same time an approximate estimate of the preformed ammonia and amino-acids is also an advantage, which compensates to some extent for the less degree of accuracy attainable. These facts suggest that it might find useful application for the examination of urines in clinical laboratories as a substitute for the hypobromite method. Another possible application of the method might be to the study of the hydrolysis of urea by enzymes and micro-organisms.

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