LXII. AN ABSORPTION APPARATUS FOR THE MICRO-DETERMINATION OF CERTAIN VOLATILE SUBSTANCES.

II. THE DETERMINATION OF UREA AND AMMONIA IN BODY FLUIDS.

By EDWARD JOSEPH CONWAY.

From the Department of Physiology and Biochemistry, University College, Dublin.

(*Received January 9th, 1933.*)

The urea of blood.

The determination of urea is an obvious application of the previous method of determining ammonia [Conway and Byrne, 1933]. By the action of urease an equivalent quantity of ammonia is formed and determined as previously described. The ferment action is very conveniently carried out in the outer chamber of the unit. The volume of 0·2 cc. has been selected for illustration since it represents an amount of blood easily obtained from the finger or ear lobe, and the error of the individual estimation on standards of 50 mg./100 cc. is only 0·6 mg. (expressed as a standard deviation), which accuracy is easily attained.

Method for 0·2 cc. blood. Into the inner chamber of a clean dry unit is run 1 cc. of 0·00667 *N* sulphuric acid (Solution I). Into the outer chamber 0·2 cc. of the blood for analysis is introduced from an Ostwald pipette. This volume of blood for convenience is kept in the one region rather than distributed over the surface. (The pipette should be standardised for its exact blood delivery without washing.) 0·5 cc. of the urease-phosphate (Solution II) is now added from a pipette—being dropped on the fluid already in the outer chamber. The lid is placed on the unit, the fluid in the outer chamber is further mixed by rotation, and the apparatus is then set aside for 15 minutes at room temperature or 10 minutes at 38°. The unit is then slightly tilted, the glass lid is a little displaced to introduce the tip of a pipette and 1 cc. of saturated potassium carbonate run into the outer chamber, the lid being quickly replaced in position. The fluids in the outer chamber are now well mixed by rotation and then left aside at room temperature for 90 minutes or incubated for 1 hour at 38°. At the end of this time the acid in the central chamber is titrated with carbon dioxide-free alkali (0·00267 *N*) (Solution III) from a 2 cc. standard burette (provided with a soda-lime trap) using some of the special indicator described in the previous communication.

Blank estimations are carried out with the reagents, the ammonia of which should be practically negligible and may in fact be reduced to zero by a suitable manner of preparation. A few such blanks with the fresh reagents with subsequent occasional estimations will suffice. A blank with the blood *plus* carbonate alone is advisable in certain diseased conditions or where the blood has been long standing. When the blood is first drawn its ammonia content, as is well known, is normally quite negligible compared with the urea.

Calculation. The volume of titrating alkali in cc. corresponding to the urea-ammonia (after deducting the blank value) is multiplied by 40 to give the mg. urea per 100 cc. in the blood. This may be subsequently corrected for pipette and alkali factors. Where the titrating alkali does not give exactly 2·50 cc. for 1 cc. of the acid a factor must be correspondingly introduced—the acid being taken as the standard of reference. The estimations may be suitably controlled by using the above procedure on 0·05 % urea. A description of the solutions used follows.

Solution I. 0·00667 *N* sulphuric acid. This is prepared by running 33·33 cc. of *N* sulphuric acid into a 500 cc. flask and making up to volume with carbon dioxide-free water. This solution is diluted 10 times. The weak solution is best kept in a paraffined or pyrex bottle with soda-lime trap and siphon—the tubing being of pyrex glass. The solution should then keep indefinitely.

Solution II. 0·00267 *N* carbon dioxide-free sodium hydroxide. This may be prepared from carbon dioxide-free alkali prepared as indicated in the previous communication. The carbon dioxide-free alkali should be stored with the same precautions as the weak acid. The diluted solution should not be used for longer than a week.

Solution III. The urease-phosphate solution. A stock glycerol extract of urease is prepared as follows in the manner described by Schmidt [1928] with but little variation. 22 g. of finely powdered permutite are washed with 2 % acetic acid, which is then decanted off, the permutite being subsequently washed twice with distilled water. The permutite is mixed with 45 g. of finely powdered jack bean meal and 75 cc. of distilled water. These are shaken for about half an hour, and then 225 cc. of pure glycerol are added and mixed. The whole is filtered and the filtrate collected. To prepare the dilute solution for use, one volume of the glycerol extract is diluted with the addition of one volume of the phosphate solution to ten volumes. Sufficient of this urease-phosphate solution should be freshly prepared for each set of estimations.

Any other method—such as the alcoholic extract described by Folin [1923]—for preparing a urease solution will suffice, but the urease should be tested with the 0·05 % urea control before use.

The phosphate solution is made by dissolving 69 g. of NaH_2PO_4 and 179 g. of crystallised Na_2HPO_4 in 100 cc. of distilled water. It is stored in the refrigerator. At room temperature it tends to become mouldy.

As an illustration of the results obtained by the method Table I shows the first six analyses of a set of 19. Table II shows the results of some experiments on 0·2 cc. of different blood samples prepared from the one stock by adding 5 cc. of water or of a urea solution to 20 cc. of the blood and demonstrates the agreement of the "recovered" quantities with those added. The volume of acid used in the above estimations suffices for 100 mg. urea per 100 cc. If there is any reason to expect a greater urea content than this in the blood half the quantity of blood may be analysed.

Table I.

Number in series	Blank estimation *minus* titration figure in cc. of 0·00267 *N* alkali. (Factor of alkali = 1·012)	Estimated conc. of urea in the 0·2 cc. sample, corrected for alkali and pipette factors
1	1·225	50·1
2	1·215	49·7
3	1·200	49·1
4	1·215	49·7
5	1·225	50·3
6	1·230	50·3

The above 6 experiments are the first of a series of 19. In each 0·2 cc. of a standard urea solution (50 mg./100 cc.) was analysed. Factor of pipette = 0·990. Example of the calculation taking the first result: 1·225 cc. of alkali corrected for factor becomes 1·240. This multiplied by 40 gives 49·60 mg./100 cc. which corrected for the pipette factor gives 50·1 mg./100 cc.

Table II.

Blood sample	Urea conc. found mg./100 cc.	Urea added as mg./100 cc. of mixture	Urea "recovered" mg./100 cc.
I	26·8	00·0	—
II	46·4	20·0	19·6
III	58·6	30·0	31·8
IV	66·0	40·0	39·2
V	76·4	50·0	49·6

To make each blood sample 20 cc. of blood were taken from the same stock, and 5 cc. of water or of a standard urea solution were added, 0·2 cc. of each mixture being taken for analysis.

Use of Nessler's reagent for determination of the ammonia. The advantage of this modification is that it eliminates the use of weak acid and alkali solutions, as well as the use of standard 2 cc. burettes. Into the central chamber is run 1 cc. of $N/10$ or $N/100$ sulphuric acid, the remaining procedure up to the end of the absorption being the same as in the previous method.

The lid is now removed and the contents of the inner chamber are removed by a simple glass pipette with rubber teat and transferred to a 25 cc. volumetric flask, subsequently washing and transferring the washings in a similar manner. 5 cc. of Nessler's solution prepared according to the directions of Folin and Wu [1919] are added to the flask, and the mixture is shaken and made up to the 25 cc. mark. A standard is prepared for colorimetric comparison by making up 16·6 cc. of 0·1N ammonium sulphate to a litre and taking 2 cc. of this with the addition of Nessler as before. Calculation of the result is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 50 = \text{mg. urea/100 cc. in blood.}$$

A blank is also carried out and the ammonia in it calculated in terms of urea percentage as in the above formula. This is subtracted from the first result.

Method for 1 cc. of a protein-free filtrate. In this section we may consider a Folin-Wu tungstate filtrate. The procedure is similar to the previous analysis except that 1 cc. of the filtrate from an Ostwald pipette is substituted for the 0·2 cc. of blood. The acid and alkali however should be half the strength of the reagents used in the first titration method with 0·2 cc. of blood. The time of the absorption after the addition of the carbonate may be taken as 2 hours at room temperature or 1¼ hours at 38°. The Nessler modification may be used as before, the standard being half the strength.

The method may be also extended to the analysis of 1 cc. of plasma with the present apparatus. The proteins present have no appreciable influence on the ammonia tension. With 1 cc. of whole blood the absorption is somewhat delayed by the formation of a scum after some time, due to the saturated carbonate addition.

In using 1 cc. of plasma the acid and alkali should be five times stronger than that previously described.

The urea of urine.

Method for 0·2 cc. of diluted urine. Seeing that in the analysis of 0·2 cc. of urea solutions of 50 mg./100 cc. the standard deviation is only 0·6 mg./100 cc., if no further accuracy is desired the urine may be diluted 50 times and estimated in the same way as blood. The dilution has the advantage that only one set o reagents need be used for urine and blood. With the diluted urine an additiona

blank estimation is carried out to determine the small correction to be made for the ammonia in the urine. In this blank, if the ammonia content itself is not accurately required, the blank need only consist of the diluted urine and the potassium carbonate in the same volumes as above. Having determined the quantity of ammonia due to the urea alone (expressed as cc. of the titrating alkali) the calculation is the same as before—the result being finally multiplied by 50 for the dilution.

The analysis of 40 duplicate estimations of urine by this method showed an average difference in the duplicates of 0·5 mg./100 cc., that is the same average difference as shown in 40 duplicate blood analyses carried out in the same way.

As with blood the method may be extended to the analyses of larger volumes.

The ammonia of urine.

Method for 0·2 cc. of diluted urine. The urine is diluted four times and 0·2 cc. of the diluted urine analysed as in the preceding 0·2 cc. methods except that the urease is omitted. The absorption rate will here be faster owing to the omission of the urease. An hour at room temperature will suffice or about 40 minutes at 38°. A blank estimation may be carried out on the alkali and acid alone. In the calculation the result may be first expressed as though urea were estimated in the same way as in 0·2 cc. of blood. The calculated figure is multiplied by $\frac{17}{30}$ to reduce it to ammonia. The result is then multiplied by the dilution. The method as before can be extended to larger volumes of diluted or undiluted urine, using suitable strengths of absorbing acid and titrating alkali.

An advantage of the methods here described for the determination of urea in blood and urine and ammonia in urine on 0·2 cc. volumes is that the same acid and alkali may be used throughout.

Discussion.

In the analysis of 0·2 cc. of a standard urea solution—50 mg./100 cc.—by the method here described the error obtained expressed as a standard deviation was 0·6 mg. urea per 100 cc. or a coefficient of variation of 1·2. Similar results were obtained for the recovery of urea added to blood. These results are very easily reproducible. We may compare them with the Collip-Clark [1926] method for analysing the urea content of 5 cc. of blood-filtrate corresponding with 0·5 cc. of blood. This—by comparison—is a very elaborate method and was specially selected for the highest accuracy obtainable. After autoclaving to convert the urea to ammonia the latter is estimated by a Parnas-Pregl technique. With this technique the urea-ammonia will be present finally in about 10 cc. fluid.

Using methyl red as indicator, the error of titration as given by Pregl [1930] is $\pm$ 0·01 cc. of 0·01 N alkali. If we take as an example a blood-urea concentration of 50 mg./100 cc. the amount of 0·01 N alkali corresponding to the urea-ammonia analysed is 0·83 cc. The error therefore expressed as a percentage of the mean is 1·2. This arises from the titration alone without reckoning any other source. If we consider Pregl to have stated the titration error as a standard deviation it corresponds to the total error arising in the estimate of 0·2 cc. of blood by the method described in this communication. If however we take Pregl's figure as some practical statement of the range within which the observations fall, this range may be taken as $\pm$ 2 $\times$ the standard deviation. (It is scarcely necessary to point out that all such statements of error should be given definitely as standard deviations or coefficients of variation.) With the latter

assumption the standard deviation of titration of the individual estimation by the Collip-Clark method expressed as a coefficient of variation is ± 0·6 (where the standard analysed is 50 mg. urea per 100 cc.). The additional errors arising from the measurement of volumes, distillation, autoclaving *etc.*, must bring the final result to within ± 1·0 or what is practically equivalent to the error of the method here described. The latter however is carried out on two-fifths of the quantity of blood and has the advantage of an extreme simplicity of procedure and apparatus.

The present micro-method for urea in 0·2 cc. of blood compares very favourably with the comparatively macro-method of Van Slyke and Cullen [1914] in which 3 cc. of blood are used and seems to give but the same percentage error. As an illustration we may take the results of Addis and Watanabe [1916] who carried out a large number of estimations by the Van Slyke and Cullen method. They say that in 90 duplicate estimations of blood-urea the "average error" was 1·5 mg./100 cc. In a footnote to their paper however they state that this average error of the duplicates was subsequently reduced to 0·5 mg. which we may suppose to be the least error obtainable. In 7 duplicate blood estimations carried out personally by the author using 0·2 cc. of blood, with the present method the average error was 0·8 mg. urea per 100 cc. In 40 duplicate estimates carried out by a skilled attendant the "average error" of the duplicates was 0·52 mg. urea per 100 cc. The results therefore show the same degree of accuracy using 0·2 cc. of blood as in the Van Slyke and Cullen method using 3 cc., with the advantage of a much greater simplicity of technique and the possibility of carrying out larger numbers of estimates at the one time.

SUMMARY.

Methods have been described for the estimation of urea in blood and urine and for the estimation of the ammonia in urine. The analyses were conducted on 0·2 cc. volumes, but larger volumes may be used. The error of estimation of the urea in 0·2 cc. of blood expressed as a standard deviation is 0·6 mg./100 cc. The technique is an obvious extension of that used in the previous communication for the estimation of ammonia, the urea being converted into equivalent quantities of ammonia by the action of urease obtained from the jack bean as a glycerol extract. Large numbers of estimations may be carried out at the one time and the percentage error of the urea estimation on 0·2 cc. blood does not exceed that found in the Van Slyke and Cullen method using fifteen times the quantity of blood nor, apparently, that found in the Collip-Clark method using two and a half times the volume.

REFERENCES.

Addis and Watanabe (1916). *J. Biol. Chem.* **24**, 203.
Collip and Clark (1926). *J. Biol. Chem.* **67**, 679.
Conway and Byrne (1933). *Biochem. J.* **27**, 419.
Folin (1923). Laboratory manual of biological chemistry, 3rd edition. (New York and London.)
—— and Wu (1919). *J. Biol. Chem.* **38**, 81.
Pregl (1930). Quantitative organic microanalysis, 2nd English edit., 116. (London.)
Schmidt (1928). *J. Biol. Chem.* **78**, 54.
Van Slyke and Cullen (1914). *J. Biol. Chem.* **19**, 211.