CCXXXV. ACCURACY IN THE DETERMINATION OF BLOOD-UREA BY THE UREASE AERATION-TITRATION METHOD.

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In recently published studies of renal function [Dunn *et al.*, 1931; Kay and Sheehan, 1933] the percentage of urea in the renal arterial blood removed from the blood during its passage through the kidney was determined. This work involved the estimation of blood-urea contents with a degree of accuracy not usually demanded; for it was essential to be able to determine with the least possible error the difference between the urea contents of blood entering and leaving the kidney at the moment of the experiment. A range of error in estimations of the urea in each sample of blood of $\pm 0{\cdot}1$ mg. per 100 ml. was permissible at ordinary blood-urea levels and a correspondingly higher range at higher blood-urea levels. Results actually within the smaller range were obtained in two-thirds of the estimations, even though the blood-urea was frequently raised to several hundred mg. per 100 ml. In this paper is given an account of the method of estimation employed: sources of error are discussed and improvements in apparatus and technique to avoid them are described.

Of the methods available, only the urease aeration-titration method of Marshall [1913], Van Slyke and Cullen [1914, 1, 2] and Van Slyke and Zacharias [1914] appeared likely to give results with a high standard of accuracy compatible with reasonably quick work; it was therefore selected. Since the original publication the method does not appear to have undergone significant changes in technique; the most recent authoritative re-statement [Peters and Van Slyke, 1932] is little more than a transcription of the first descriptions. In the latter book, an accuracy of 1 % is claimed for the method. Actually this standard does not seem to be generally attained, but by improvements in apparatus and careful attention to technique it has been found possible in the present work to obtain results with an average relative error of only $\pm 0{\cdot}2$ mg. per 100 ml. at all levels of blood-urea. These improvements deal with technical detail rather than with principle. After they had been developed it was found that a number of the points had been recognised previously by Barnett [1917] in his studies on blood-ammonia, but the implications of his work do not appear to have been applied to the method of estimating blood-urea. The technique and apparatus as finally developed have proved not only to be reliable and accurate for research but also, in our hands and others', to be easy and convenient for routine clinical work. In clinical work blood-urea can be measured on blood samples of 0·2 ml. with an average error of less than 3 mg. per 100 ml.

For the purposes of discussion, errors and improvements are grouped according as they are related to accuracy in titrimetry, to the aeration process, to the enzymic reaction or to apparatus.

Titrimetry.

The degree of accuracy aimed at was 0·01 ml. of $N/100$ NaOH in titrating 25 ml. of $N/100$ H_2SO_4. This was attained by devoting attention to a number of details of technique in measurements and titration.

1. *General.* In washing apparatus, hands *etc.* scrupulous chemical cleanliness is essential. Measuring apparatus is kept clean and free from grease by frequent cleansing with chromic acid solution. Reaction- and acid-tubes are washed separately with separate brushes, and their stoppers are never interchanged, since those of the reaction-tubes appear to absorb alkali.

2. *Type of glass.* Resistance glass must be used for all parts which are to come into contact with standard alkali or acid for more than a few minutes; monax glass is suitable.

In different soda glass tubes, the titre of 25 ml. of $N/100$ H_2SO_4 was found to fall at varying rates, usually about 0·01 ml. per hour. In monax tubes, the titre was unchanged after 24 hours.

3. *Laboratory temperature.* During measurements, the temperature of the laboratory must be kept constant, since fluctuations of 1° introduce appreciable errors. In summer the variations of temperature observed were never more than 0·2 to 0·3° during critical periods, but in winter they were sometimes so great as to prevent work of very high accuracy under ordinary laboratory conditions.

4. *Burette and pipette deliveries.* In measuring from a pipette or burette the fluid at the point is "tipped off" against the inside of a clean dry beaker before beginning the delivery. Deliveries from a burette are finished by tipping off on the bubbling tube; from an automatic pipette, by tipping off against the inner wall of the receiver to give a standard "leave" in the tip of the pipette.

5. *Automatic pipette for standard acid.* As it is difficult to maintain a high standard of accuracy with an ordinary pipette or burette, measurement of standard acid is done by an automatic 25 ml. pipette, filled from an overhead reservoir. This pipette is changed frequently to avoid errors from grease, although a moderate amount of grease in the pipette is not objectionable except in so far as it may hold air bubbles. Standard drainage time must be allowed; 2 minutes for emptying and draining were used in the present work.

Fifteen successive deliveries made from the automatic pipette used were weighed and the range of variation was found to be 0·0099 g. The deliveries were therefore constant to within 0·01 ml.

6. *The burette.* Accurate burette readings are made with a 50 ml. Schellbach blue line burette graduated in 0·1 ml. Associated conditions, *e.g.* speed of working and use of CO_2-free standard alkali solution, precluded the use of micro-burettes and weighing burettes. The burette must be kept scrupulously free from grease. All measurements are taken from the same initial point, and the portion of the burette used recalibrated by the weighing method.

Readings on such a burette can be made accurately to 0·01 ml. if certain precautions are observed. A reading tube is required to ensure that readings are made along a line perpendicular to the burette. A convenient tube is the barrel of a 5 ml. Record syringe with the entire needle mount removed; the cap mount gives a right-angled metal end, which is placed against the burette with the meniscus on the diameter. Three readings are then made with the eye at a constant distance from the burette, (*a*) looking down the centre of the tube, (*b*) looking along the inner surface of the top of the tube and (*c*) along the inner surface of the bottom of the tube. These positions are easily recognised by the

relationship of the circle of the burette end of the tube to the circle of the observer's end. In (*a*) the circles are concentric, in (*b*) the circles touch at the top, and in (*c*) they touch at the bottom. Reading (*a*) is made exactly at right angles to the burette. Readings (*b*) and (*c*) involve equal but opposite parallax, and the mean figure should be the same as that of reading (*a*). At the normal distance of reading, (*b*) is about 0·01 ml. above (*a*), and (*c*) is about 0·01 ml. below (*a*).

Measurements are most easily made by reading the short horizontal fine line of light which crosses the junction of the two points of the blue line in the meniscus, and which is enhanced by placing behind the meniscus the middle finger of the hand holding the reading tube.

Without any previous practice, it is easy to keep within an error of $\pm 0{\cdot}01$ ml. in reading the burette in this way, and the error is smaller as skill is acquired. Independent readings were made by both observers in all titrations in the present work and agreed exactly.

As a test of accuracy, eight deliveries of distilled water at 15° were made from a part of the burette which had been carefully recalibrated by weighing. Each measured volume involved two burette readings. The quantities delivered were then weighed and their volumes calculated from the known density of water. The results are given below; the greatest difference is 0·0076 ml. and the average is 0·0036 ml.

Volume by burette ml.	Volume by weighing ml.	Volume by burette ml.	Volume by weighing ml.
0·69	0·6951	0·75	0·7479
0·70	0·6966	0·76	0·7589
0·72	0·7232	0·78	0·7724
0·74	0·7460	0·81	0·8100

7. *Indicators.* The indicator usually employed for titrations was methyl red. The critical colour change selected as the end-point occurs after a gradual change from red to reddish yellow; there is then an abrupt change to greenish yellow accompanied by an increase of brightness due to discharge of the red shade. Using CO_2-free solutions this change is brought about in 40–50 ml. of fluid by less than 0·01 ml. $N/100$ NaOH in the absence of ammonium salts, and by no more than 0·01 ml. $N/100$ NaOH even when ammonium salts are present, the colour change being instantaneous and permanent so long as a CO_2-free atmosphere is preserved. In this volume of fluid the amounts of indicator required for satisfactory recognition of the colour change are 0·2 ml. of 0·02 % methyl red in 50 % alcohol, or 0·3 ml. of the B.D.H. stock solution. These amounts must not only be fixed but must also be accurately measured. If a micro-burette is used, the tip must be enclosed between measurements to prevent evaporation of alcohol and the formation of crusts of indicator. Accurate measurement is necessary because each 0·1 ml. of the indicator solution used requires 0·02 ml. of $N/100$ NaOH to produce the colour change.

Titration of acid which contains drops of octyl alcohol is exceedingly difficult, as some of the methyl red is dissolved in the alcohol giving an orange solution which does not take part in the colour change. This obscures the critical colour change. The present aeration method dispenses with the use of the anti-foam liquid in the acid and this complication does not arise.

Another satisfactory indicator is the "4·5" indicator of B.D.H. This has the advantage of having a colour change after the critical one used as the end-point; there is thus less danger of not recognising over-titration. Near the

end-point abrupt colour changes occur with each addition of 0·01 ml. $N/100$ NaOH to 50 ml. of solution being titrated, these are (*a*) grey to (*b*) dull green-blue to (*c*) a clear blue; the addition of a further 0·01 ml. $N/100$ NaOH causes a marked change to an intense bright blue which is distinctive.

The critical colour changes in these two indicators can only be recognised if the titration is completed by the addition of separate amounts of 0·01 ml. of the $N/100$ NaOH. It is advisable for the observer before using any indicator to carry out preliminary test titrations to familiarise himself with the critical colour change.

8. *The avoidance of CO_2.* The avoidance of CO_2 in the solutions involved in titration is one of the principal factors in securing accuracy; the effects of CO_2 in titrations involving $N/100$ solutions are generally underestimated [see also Acree and Brunell, 1906; Marshall, 1913]. A series of experiments was conducted to investigate this point quantitatively. 25 ml. of $N/100$ H_2SO_4 were titrated to near the end-point with $N/100$ NaOH. Small measured quantities of $N/100$ Na_2CO_3 were then added and the titration completed with the $N/100$ NaOH (to avoid removal of the added CO_2, the solution was not aerated during the final titration). Each addition of 0·2 ml. $N/100$ Na_2CO_3 in these titrations corresponds to 1 % Na_2CO_3 in the total solid alkali present. The results are shown in the table below. The essential points are that with increasing amounts of carbonate present:

(*a*) Steadily increasing quantities of total alkali are required.

(*b*) The end-point becomes spread more widely, *i.e.* the amount of alkali required to complete the critical colour change is increased.

(*c*) The colour change is harder to recognise owing to the bleaching of the indicator. This is marked when 0·4 ml. of $N/100$ Na_2CO_3 is present, and makes titration very difficult when 0·8 ml. is present.

(*d*) "Swing back," *i.e.* return to the previous colour, occurs owing to the buffering action of the carbonate so that more alkali must be used to produce permanence of the end-point.

$N/100$ Na_2CO_3 added ml.	Total alkali used in titration $N/100$ NaOH + $N/100$ Na_2CO_3 ml.	End-point "Spread" $N/100$ NaOH ml.	End-point Swing back $N/100$ NaOH ml.
0	25·71	0·01	0
0·1	25·75	0·02	0·01
0·2	25·76	0·04	0·02
0·3	25·78	0·04	0·03
0·4	25·81	0·06	0·04
0·5	25·83	0·07	0·05
0·8	25·94	0·08	0·05

In the titrations the most important source of CO_2 is the standard alkali. The avoidance of this CO_2 is most fundamental and important. The method of preparing CO_2-free standard NaOH solutions and the fitting of a burette for their use is described in a subsequent paper [Kay and Sheehan, 1934].

It is also very important that the air coming into contact with the standard acid during titrations should not contain CO_2. This is secured by passing a stream of air washed free from CO_2 and NH_3 through the acid during titration. A convenient source of clean air is obtained by passing the outflowing frothy water from a water suction-pump into a large Woulff's bottle. The water is siphoned away from the bottom and the liberated air is led from the upper part of the bottle through a stop tap and then through caustic soda and

sulphuric acid wash-bottles and finally through distilled water and an empty guard-tube to the acid.

Distilled water used for preparing standard acid or alkali is boiled thoroughly in a flask before use and cooled out of contact with ordinary air by connecting up to a caustic soda wash-bottle.

9. *Technique of titration.* The method of titration is as follows.

After use, the burette is always left full of alkali. As this alters in strength on standing even for half an hour in an ordinary burette, it is changed completely before starting titrations.

After filling the burette and adjusting to zero, a drainage time of 1 minute is allowed before reading. Meanwhile the exact amount of indicator is added to the acid-tube. The stopper of the acid-tube is raised but not removed and the bubbling tube is then connected by rubber tubing to the stream of air freed from NH_3 and CO_2; this stirs the liquid efficiently during titration and preserves a neutral atmosphere.

Titration is then proceeded with, timed by stop-watch, until the end-point is approached and about 0·2 ml. of alkali has still to be added. The acid-tube is then removed from the burette, stoppered, disconnected from the air stream and shaken twice to wash down the walls. The acid remaining in the bubbling tube is blown out twice into the rest of the solution by temporary connections of the air stream tubing, and a proper connection is finally made again. All the acid in the tube is thus collected for the complete titration. To obtain an accurate end-point the titration is completed by "tipping off" portions of a drop of alkali (about 0·01 ml. each) from the burette tip on to the bubbling tube and transferring them to the acid.

The burette is read exactly 3 minutes after starting to run in the alkali.

As a test of the reliability of measurements and titration, five successive lots of $N/100$ H_2SO_4 were measured by the automatic pipette and titrated by the standard method. In each case the amount of standard alkali required was exactly 21·91 ml.

The aeration process.

1. *Rate of aeration.* The rapid short aeration (5 litres of air per minute) recommended by previous workers was not used in view of the alkaline spray which must inevitably have interfered with accuracy in their form of apparatus. The rate used was 1 litre of air per minute for 2 hours, standardised by the timed displacement of a measured volume of water. The total volume of air passed is roughly the same as that claimed by other workers to be enough; ammonia transport depends on the volume of air passed rather than on any factor of time or rate [Van Slyke and Cullen, 1916].

The quantitative recovery of ammonia by aeration claimed by other workers [Folin and Macallum, 1912; Kober and Graves, 1913; Van Slyke and Cullen, 1916; Folin and Wu, 1919] cannot be accepted; it is probably due to inadequate precautions to control alkaline spray which thus reaches the $N/100$ acid and is estimated as ammonia, so that sometimes more than 100 % of the actual ammonia is accounted for. The present work has been done with almost complete avoidance of alkaline spray, the performance of adequate blanks to account for such constant traces of spray as do pass and very accurate titrations. With four quantities of pure NH_4Cl varying from 0·669 to 10·698 mg. and the passage of 120 litres of air in 2 hours, only about 96 % transfer of NH_3 was found. Greater transfer can be obtained by more prolonged aeration at the same rate but complete transfer does not occur except possibly with very small

quantities of ammonia. This is shown from the figures below for two different amounts.

Duration of aeration hours	Percentage of NH_3 transported from	
	0·669 mg. NH_4Cl	10·698 mg. NH_4Cl
½	79	92·1
2	96	95·6
2½	97	96·1
4	97	96·3
12	98	97·2
23	100	97·8

Heating the reaction tubes to 50° appears to accelerate the transfer of NH_3, but the method was not applied to blood in view of the strong alkali present.

The addition of urease to the NH_4Cl has no obvious effect on the efficiency of aeration.

Though the 2 hours' aeration does not transport all the ammonia it was proved in many experiments that all tubes aerated together in series gave the same percentage transport when the amounts of NH_3 were not widely different from each other.

2. *Alkaline spray.* As mentioned above, there is a great danger of carrying over alkaline spray during aeration from the reaction-tubes to the acid-tubes. Various workers have dealt with this problem by putting cotton-wool plugs in the outlet tubes of the reaction-tubes [*e.g.* Barnett, 1917]. Such plugs are relatively efficient; the chief objections to their use, apart from possible absorption of traces of NH_3, are the interference with smooth aeration in serially connected units and the necessity for strong suction to overcome their resistance.

Very satisfactory control of alkaline spray was obtained in the present work by using a zig-zag resistance glass connecting tube between the reaction- and acid-tubes. This is bent at 15 points; 11 of these are in the ascending limb as this arrangement was found most effective. During aeration, alkali is deposited up to the 11th bend where the tube finally becomes horizontal, but none can be shown in the long straight descending limb by subsequently running indicator along the tube. The minute amount of alkaline mist which passes through the tube to the acid is proportional to the rate as well as the time of aeration, but is quite constant for all tubes aerated in series and is therefore accounted for in the blank. At the standard rate of aeration it neutralises slightly less than 0·01 ml. of $N/100$ H_2SO_4 per hour, corresponding to about 0·0000086 ml. of the strong alkaline solution in the reaction-tube.

These glass connecting tubes are joined to the tubes from the reaction- and acid-tubes by short pieces of rubber tubing lubricated with a little distilled water, and the ends of the glass tubes are pushed together so that the air stream does not come into contact with the rubber tubing.

Barnett [1917] and Folin and Wu [1919] have drawn attention to the chemical defects of the long pieces of rubber tubing usually employed for connecting the reaction-tubes to the acid-tubes. They are certainly not satisfactory for accurate estimations; in the present work the average difference between 16 pairs of estimations of urea in a series using rubber tubing connections was over four times greater than in the series using the glass connections, the rest of the technique and apparatus being the same.

Soda glass connecting tubes are theoretically not quite so satisfactory as resistance glass, owing to the danger of moisture condensing in the downward limb and falling with alkali dissolved from the glass into the acid. This is not

however of great significance; the results were very nearly as good in 62 pairs of estimations of urea using soda glass connections as in the present larger series using resistance glass.

3. *The addition of potassium carbonate.* Many errors arise from the usual addition of solid potassium carbonate to liberate ammonia. The reaction-tube has to be opened; this involves possible loss of ammonia. As there is usually gross spilling, the amount of potassium carbonate added is never constant, and as the carbonate often contains traces of ammonia it is not possible to do a satisfactory blank. This is however not of such importance as the fine cloud of K_2CO_3 dust which remains for some time in the upper part of the reaction-tube and some of which may be carried by aeration into the $N/100$ acid. Only 0·000007 g. of solid K_2CO_3 is required to neutralise 0·01 ml. of $N/100$ acid: very small traces may therefore produce serious errors.

All these sources of inaccuracy are avoided by the use of saturated solution of K_2CO_3 as suggested by Fiske [1915]. The statement of Van Slyke and Cullen [1916] that the difference between the use of K_2CO_3 as a solid and in solution is "one of convenience rather than accuracy" cannot be accepted. The amount of saturated K_2CO_3 solution required for blood-urea estimations is that to produce two-thirds saturation in the final mixture. Greater amounts produce no improvement; half-saturation as recommended by Van Slyke and Cullen is almost but not quite as good. The measured 20 ml. of saturated K_2CO_3 solution required for the usual volume of fluid in the reaction tube are added by a tap-funnel, which is an integral fitting in the reaction-tube stopper. This is clean and accurate in use and avoids the danger of ammonia loss. The alkali should be added drop by drop during aeration, taking 3–5 minutes, in order to avoid too coarse precipitation of the blood-proteins. The last few drops must not be allowed to blow spray into the reaction-tube.

The presence of NH_3 in the K_2CO_3 solution must be tested for with Nessler's solution. If NH_3 is found in substantial amount it must be removed before using the solution by prolonged aeration with clean air; any traces remaining are dealt with by the blank.

4. *The prevention of frothing; size of reaction-tubes.* The reaction-tubes (preferably of monax glass) should be at least 250 mm. long × 30 mm. diameter to allow satisfactory aeration[1]; this is much larger than the tubes which are usually used. To prevent frothing in the alkaline blood-containing liquid, technical *iso*butyl alcohol was preferred to octyl alcohol as some samples of the latter are not efficient for the purpose. In addition, the portion of the *iso*butyl alcohol that volatilises and is thus transported to the acid is completely dissolved in it, whereas octyl alcohol tends to form droplets which interfere with titration.

2 ml. of *iso*butyl alcohol were added through the tap-funnel of each reaction-tube after incubation; the tubes must be cooled for 5 minutes in cold water in the incubating bath before this addition in order to avoid distillation of the alcohol into the glass connecting tubes.

5. *The acid-tubes.* The acid-tube is the same size as the reaction-tube and is sufficiently big not to allow frothing to any dangerous height; it is therefore not necessary to use anti-foam liquid in the acid. The importance of this in the subsequent titration has already been pointed out, but the great advantage in the present connection is that a layer of bubbles about 1–2 cm. deep can be maintained on the surface of the acid throughout the aeration, thus prolonging the contact of air with acid and increasing the area of contact. This and the relatively slow rate of aeration give complete absorption of NH_3 by the acid;

[1] Recently, reaction- and acid-tubes 300 × 34 mm. have been used.

in tests, a second acid-tube after the first remained unaffected in titre. It is thus permissible to connect up estimating units in series; the actual results of such serial aerations never gave any indication of passage of ammonia from one unit to the next.

6. *Purification of air for aeration.* The air used for aeration is passed first through 20 % NaOH, then through 25 % H_2SO_4 and distilled water. The acid-tube must not precede the alkali-tube because any spray carried out of the acid-tube is liable to contain arrested ammonia. The efficiency of the NH_3 trap is such that, in a test, 6 hours' aeration through $N/100$ H_2SO_4 did not produce any measurable effect on the titre of the acid.

Hydrolysis of urea.

1. *The urease solution.* The urease solution was made by extracting freshly ground soya bean meal in 10 times its weight of distilled water for 1 hour. To obtain unvarying blank values the solutions must be quite homogeneous; this is attained by thorough centrifuging after straining through gauze. The ammonia which occurs in this urease solution is simply and exactly accounted for by the "blank" estimation done with every batch of estimations. It is possible to remove this NH_3 [Folin and Youngburg, 1919; Van Slyke and Cullen, 1914, 2], but it does not appear necessary. 5 ml. of the urease solution are used with 5 ml. of 0·6 % KH_2PO_4 buffer and incubation conducted for 30 minutes at 50°.

2. *The efficiency of hydrolysis.* In view of the incompleteness of aeration transfer of NH_3, 95–96 % estimation of urea is to be considered as indicating practically complete hydrolysis. Though hydrolysis of pure urea is not always complete, especially with small amounts, the proportion of any given quantity is always the same in solutions incubated and aerated under the same conditions. Thus, for example, eight paired estimations of pure urea solutions gave an average titration difference between pairs of 0·01 ml. $N/100$ NaOH, which is within the error of measurement.

Working with blood, the hydrolysis appears to be very constant and complete for varying amounts of urea and of blood. The following figures were obtained by paired estimations of different quantities of a sample of blood.

Volume of blood ml.	mg. urea per 100 ml. blood	
3	32·1	32·0
2	32·2	32·1
1	32·2	32·0
0·2	32·0	33·0

Hydrolysis of urea added to blood appears to be complete. Various quantities of urea were added to 2 ml. of blood whose urea content had been found to be 0·848 mg. The estimations gave results as follows, the slightly higher figures with small amounts of urea being accounted for by the relatively greater efficiency of aeration with small amounts of NH_3:

Amount of urea added (mg.)	Percentage of total urea estimated
0·625	96·0
1·250	95·6
5·000	95·0

With aqueous solutions of pure urea on the other hand, hydrolysis is incomplete when small quantities of urea are used though it is complete when large quantities are used. This is shown below in a series of estimations.

Actual urea mg.	Percentage estimated	Actual urea mg.	Percentage estimated
0·382	81·0	1·528	94·2
0·625	88·0	3·057	94·8
0·764	87·0	5·000	94·9
1·250	94·1	6·113	95·1

The use of soya bean extract obtained by extracting 20 g. of meal with 100 ml. of water gives more complete hydrolysis of small amounts of urea in water than those shown above. Thus with 0·382 mg. urea 96 % was estimated, and with 6·113 mg. 96·2 %. The use of this strong extract is not however necessary for estimations on blood.

More prolonged incubation also gives more complete hydrolysis; after incubation of 0·382 mg. urea for 2 hours with 10 % soya bean extract, 94 % was estimated. This long incubation is without effect on the soya bean extract blank but is to be avoided in work with blood as it allows the possibility of urea formation from interaction of blood-arginase with the arginine in the soya bean extract [Behre, 1923; Addis, 1928].

The conditions governing the complete hydrolysis of urea have been further investigated and an account is given in a subsequent paper [Kay and Reid, 1934]. The results of that work indicate that urea in blood undergoes practically complete hydrolysis and explain the relative inefficiency of hydrolysis in watery solutions of pure urea.

Apparatus.

1. *Blood pipettes.* Blood pipettes are calibrated to contain an exact volume. Errors up to ±2 % often occur in makers' graduations. Recalibration by the mercury weighing method can be made to an accuracy of ±0·1 %. To allow easy washing out of blood in delivery the bore should be about 1·0 to 1·5 mm., slightly narrower at the tip, the bulb fusiform, the top funnel-shaped to take the tip of the phosphate buffer pipette, and the tip ground to 60° so as to have as little glass at the point as possible.

2. *The blood-urea apparatus*[1]. The principal changes have already been indicated. Full details can be seen in Fig. 1. The apparatus is designed not to require opening from the first connecting-up until the end of the aeration, in order to avoid loss of NH_3. First the acid-tubes are charged and stoppered; next the reaction-tubes are charged with blood, buffer and urease, and stoppered. The reaction- and acid-tubes of each unit are connected by the zig-zag glass connecting tube previously described and the units are connected serially by rubber tubing. The battery of units is supported rigidly in a metal stand which is saddle-shaped (see Fig. 2), so that the reaction-tubes assembled on one side can be placed in a water-bath and the acid-tubes on the other side left outside. After assembling, the battery in its stand is an entity which can be incubated and subsequently lifted out on to the bench without disturbing the units. After the cooling, the *iso*butyl alcohol and K_2CO_3 solution are added through the tap-funnels during aeration.

In assembling the apparatus distilled water in minimum quantity is used as lubricant for rubber-to-glass joints. The first pair of tubes to receive incoming air during aeration must be that for the blank estimation. This avoids a slight error which may arise from blood being forced up the bubbling tube of the first unit during incubation and so partly escaping heating, and later, alkali. During incubation the reaction mixture is stirred from time to time by gently

[1] The improved blood-urea apparatus is supplied to the specification of this paper by Messrs Griffin and Tatlock, Ltd., 34 Great Ducie St, Manchester.

drawing a few bubbles of air through it. This allows for expansion of air and equalises the pressure inside the apparatus, thus preventing blood from being forced up the bubbling tubes.

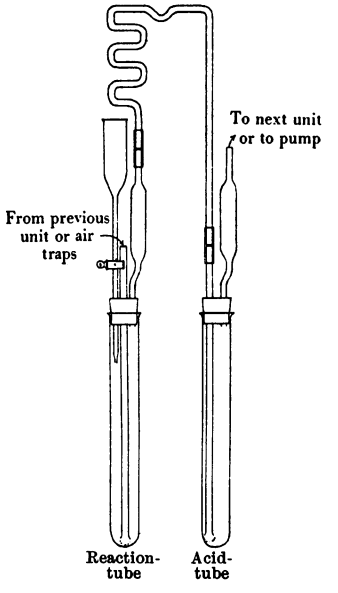

Fig. 1. Blood-urea apparatus. ($\times \frac{1}{3}$.)

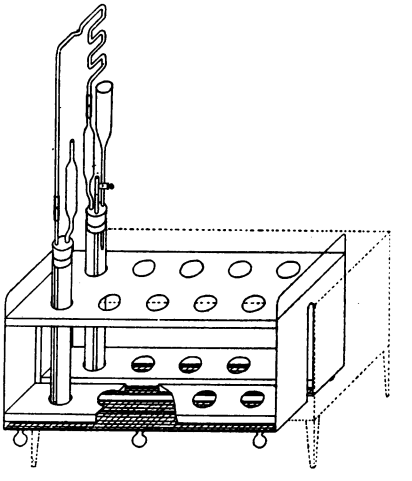

Fig. 2. Saddle-shaped metal stand for blood-urea apparatus. ($\times 1\frac{1}{2}$.)

Note on potassium oxalate. Some samples of potassium oxalate (used to prevent clotting of blood) contain NH_3. They must be tested with Nessler's solution; only those ammonia-free should be used.

Accuracy of the method.

Errors of measurement and titration are reduced to very fine limits, the urease hydrolysis in blood appears to be almost quantitative, but the aeration transfer of NH_3 is not complete. As a result the method gives with blood an absolute accuracy of only about 96 %, but the relative accuracy is limited only by that of the measurements. The high standard of relative accuracy is satisfactory from the point of view of the renal function studies for which the method was developed.

The test of relative accuracy is to examine the differences in terms of ml. of standard alkali (*N*/75 NaOH) between the titres of duplicate estimations on the same blood carried out simultaneously under identical conditions. Ninety consecutive pairs of estimations of blood-urea by the present method are available, mostly on 2 ml., the rest on 1 ml. The bloods had urea contents ranging from 14 to 600 mg. per 100 ml., the average being 93 mg. Magnitudes of differences are independent of blood-urea level. The distribution of differences is as follows:

Difference between pairs ml. of *N*/75 NaOH	Number of pairs		Difference between pairs ml. of *N*/75 NaOH	Number of pairs
0·00	17	80 %	0·05	1
0·01	42		0·06	3
0·02	13		0·07	1
0·03	4		0·09	1
0·04	7		(0·21!)	1

In any pair of estimations at normal blood-urea levels the range of possible error due to measurements is as follows:

	ml. $N/75$ NaOH
Blood pipette measurements	0·003
Automatic pipette measurements	0·010
Burette measurements	0·015

The total possible error of measurement is thus 0·028 ml. $N/75$ NaOH, though at high blood-urea levels the error due to blood pipette measurements is proportionately higher. Actually the mean of the differences in the 90 pairs of estimations is 0·02 ml. $N/75$ NaOH, and this average includes one quite anomalous pair where some gross accidental error must have occurred. 80 % of the pairs have a difference of 0·02 ml. or less; *i.e.* within the average.

Using 2 ml. of blood for each estimation this difference of 0·02 ml. $N/75$ NaOH corresponds to 0·4 mg. urea per 100 ml. blood, on an average blood-urea in the present experiments of 93 mg. per 100 ml.

Summary.

1. Sources of error in the urease aeration-titration method of estimating blood-urea are discussed, and improvements in technique and apparatus to overcome them are described.

2. The aeration method only removes about 96 % of ammonia under the conditions described so that the method only estimates this proportion of urea present.

3. Nevertheless the modified method gives a very high standard of relative accuracy for all levels of blood-urea. In estimations of blood-urea ranging from 14 to 600 mg. per 100 ml. the average difference between pairs corresponded to 0·02 ml. of $N/75$ alkali, or 0·4 mg. urea per 100 ml. of blood.

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