75. Microdiffusion Methods. Ammonia and Urea Using Buffered Absorbents (Revised Methods for Ranges Greater than 10 μg. N)

By **E. J. Conway** and **E. O'Malley**, *From the Biochemical Department, University College, Dublin*

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Microdiffusion methods for determinations of minute amounts of ammonia, urea, carbon dioxide, the halogens etc., are now established in many laboratories. A general account of apparatus and several applications have been given elsewhere [Conway, 1939]. Recent extensions include submicro-determinations of total N by Needham & Boell [1939], as well as a further very accurate submicro-total N method by Tompkins & Kirk [1942], also amide- and nitrate-N etc. by Borsook & Dubnoff [1939], quantitative acetone determination by Werch [1940] and an accurate acetone method using bisulphite absorption by Winnick [1941; 1942], who has also extended the technique to lactic acid and threonine estimations.

As shown elsewhere micro-NH_3 determinations by the microdiffusion technique can be brought to any desirable level of accuracy. Recoveries are better than with aeration methods, the apparatus required is simpler and large numbers of determinations can be carried out in one series.

Using the standard unit and 0·2 ml. of the NH_3-containing fluid the full absorption time at room temperature is 45 min., but with rocking this can be reduced to 10 min. Such times arise out of the principles discussed and the experimental results described. (Borsook & Dubnoff [1939] have erred in claiming an absorption time of 90 min. as an advance on the microdiffusion technique.)

The present study is directed especially to the removal of certain objections to the clinical use of the microdiffusion method for urea, but has also a more general application for concentrations of NH_3 beyond a certain level.

The blood urea method as originally described [Conway, 1933] had the disadvantage for clinical use, as commented on by Lee & Widdowson [1937], that the upper limit was 100 mg. urea/100 ml. Occasional blood ureas exceed this figure, though as pointed out [Conway, 1939], the use of 0·1 ml. as well as 0·2 ml. blood in a duplicate estimation raised the limit without any extra trouble to 200 mg./100 ml. However, this disadvantage is fully removed in the method described below, in which there is no upper limit. With this revised method also the amount of cleaning required need not exceed that given to ordinary analytical glassware, nor is any standardization of solutions required, apart from the original making up of $N/50$ HCl, which remains indefinitely stable. This removes the objection raised by Dukes [1939] who considered the microdiffusion method unsuitable for occasional blood urea determinations as distinct from large series.

In the new method for urine and blood urea, as also urine NH_3 or NH_3 in quantities greater than about 10 μg. NH_3-N, there is used the principle introduced into the microdiffusion technique by Abelin [1938], and further studied by Kawerau [1941], of absorbing the NH_3 into a solution such as dilute boric acid and titrating back with standard HCl. Improvements are included arising out of a further study of the conditions of the microdiffusion procedure, an account of which is given at the outset.

The boric acid procedure of Abelin [1938] was criticized by one of us [Conway, 1939] as giving a poor end point with the Tashiro indicator compared with the barium hydroxide titration, but it was noted that the principle was certainly a good one and that a change

in conditions might make it very suitable. This requisite change is described below; it includes the use of an indicator which is more suitable for the purpose, since it changes at an end point where the buffering is minimal and it is more sensitive than the Tashiro indicator when titrating in the alkaline to acid direction; also by a reduction in the strength of the boric acid absorbent (or the use of half-saturated $MgCl_2$) combined with potassium metaborate as alkali. The end point is now quite good and the accuracy of the new blood urea method is practically the same as that of the original, or even superior at the lower levels, while securing in addition some practical advantages.

Before a brief account of the methods, some studies in the microdiffusion conditions are described.

The ideal absorbent for NH_3, with subsequent acidic titration

If NH_3 is absorbed into a boric acid solution in the central chamber of a microdiffusion unit, the *p*H rises rapidly at first and then more slowly as the boric acid buffering comes more fully into play. If, at equilibrium, it rises to a *p*H of 9·4, the NH_3 would be half in the form of undissociated gas, and half as the ammonium cation. The gas would exert the same tension as that in the outer chamber, and if 2 ml. half-saturated potassium carbonate or metaborate were in the outer chamber and 2 ml. boric acid solution in the central, the gas would be shared in the proportion of 1 : 3·4, the relative solubilities being as 3·4 : 1. Hence the proportion of total NH_3 remaining in the outer chamber would be $1/(1+6{\cdot}8)$ or 0·13. If the equilibrium *p*H were less than 9·4 higher proportions would be absorbed and it may be shown that the proportion unabsorbed approximates to

$$U=\frac{bs}{a}\times\frac{10^{-9\cdot4}}{10^{-9\cdot4}+[\mathrm{H}]},$$

where a and b are the volumes in the inner and outer chambers [H] the equilibrium hydrogen ion content of the absorbent, and s the relative solubility (outer/inner) of the NH_3 gas.

Where b and a are equal and s approximately 0·3, only 0·01 is left (i.e. 99 % is absorbed) at a *p*H of 8·0. Reducing b relative to a and having a greater percentage saturation of potassium carbonate (or better—metaborate) the conditions of absorption are even more favourable. Considering the problem of the buffer strength required to prevent a central chamber rise of over *p*H 8·0, the maximum absorption of NH_3 likely to occur must be known. For blood urea determinations we may consider that 300 mg. urea/100 ml. is rarely exceeded, and if in any case a 99 % absorption is secured at this level, a 97 % efficiency will still be had with 600 mg. urea/100 ml. Using 0·2 ml. blood, 300 mg. urea/100 ml. are equivalent to 1 ml. of $N/50$ NH_3.

Concentrations of buffers with varying *p*K values may be worked out theoretically to give with 2 ml. volumes a *p*H value of 8·0 after absorbing 1 ml. of $N/50$ NH_3. These are given in Table 1, which includes the *p*H of minimal buffering on titrating with standard HCl solution, and the value of this buffering when $N/25$ HCl is used, since such a strength of acid would be suitable for the highest ranges.

It will be seen that theoretically a whole range of buffers almost equally suitable for the purpose could exist, with *p*K values ranging from 11·0 to 8·5, the end point *p*H and the minimal buffering being almost identical. In practice the ideal buffer should also have good keeping power, not affect the indicator used and have the required solubility. Of the many buffers tested only boric acid and $MgCl_2$ were found suitable, boric acid being used in 0·5 % (or in 1 %) strength and $MgCl_2$ as the half-saturated solution (see Table 2). There is little if anything to choose between these two when pure reagents for analysis are used. It may be noted that $MgCl_2$ possesses the added quality—besides the direct buffering power—of reducing the NH_3 tension for a given amount dissolved.

A buffering value of $9{\cdot}5\times10^{-5}$ at the end point when titrating with $N/25$ acid (as may be used for very high blood ureas—which are indicated by a special change in the mixed

Table 1

pK of buffer	Conc. of the 2 ml. buffer solution used for absorption	pH of end point giving minimal buffering (after absorption of 0·02 m.eq. NH_3-N)	Buffering (β) at end point $\beta=\frac{d\beta}{dp\text{H}}$ where β is equivalents of acid
11·0	5·0	4·93	$9{\cdot}4\times10^{-5}$
10·0	0·50	4·93	$9{\cdot}4\times10^{-5}$
9·5	0·16	4·93	$9{\cdot}4\times10^{-5}$
9·0	0·055	4·92	$9{\cdot}5\times10^{-5}$
8·5	0·021	4·89	$9{\cdot}8\times10^{-5}$
8·0	0·010	4·82	$10{\cdot}7\times10^{-5}$
7·5	0·0066	4·67	$13{\cdot}6\times10^{-5}$
7·0	0·0055	4·47	$20{\cdot}4\times10^{-5}$

C (conc. of buffer) $=0{\cdot}005\,(10^{-8{\cdot}0}/K+1)$

[H] (at ideal end point) $=\sqrt{0{\cdot}01\,(10^{-8{\cdot}0}+K)+V\times1{\cdot}4\times10^{-11{\cdot}0}}$

β (buffering at ideal end point) $=2{\cdot}3\left(\frac{0{\cdot}01\times10^{-8{\cdot}0}}{[\text{H}]}+\frac{1{\cdot}4\times V\times10^{-11{\cdot}0}}{[\text{H}]}+V\times[\text{H}]\right)$

In the equation for buffering at the ideal end point (or point of minimal buffering) account is taken of the CO_2 of the air and the buffering of the hydrogen ion concentration of the mixture as well as the special buffer used; the buffering of the indicator is not taken into account. End point volume (V) = 2·5 ml.

Table 2

Solution in central chamber	Absorption using 0·2 ml. 0·1 N $(NH_4)_2SO_4$ %	Absorption using 0·4 ml. 0·1 N $(NH_4)_2SO_4$ %
2 ml. ½ sat. $MgCl_2$	98	96
1 ml. sat. $MgCl_2$ adding 1 ml. water before titrating	99	99
1 ml. 0·5% boric acid	93	90
2 ml. 0·5% boric acid	99	98
1 ml. 1·0% boric acid	99	96
1 ml. 2·0% boric acid	100	100

To the 0·2 ml. $(NH_4)_2SO_4$ in the outer chamber 1·0 ml. of saturated potassium metaborate was added to each unit, and the absorption allowed to continue for 90 min. 0·1 N $(NH_4)_2SO_4$ is equivalent to 300 mg. urea/100 ml.

indicator used) means $0{\cdot}2\times10^{-3}$ ml. to change the pH by 0·1, a change readily recognized. This corresponds to 0·2 of a small division on the horizontal burette, or to 0·14 mg. urea/100 ml. blood. Titrating with $N/75$ acid as in the usual routine method described, will give the same equivalent value of 0·14 mg. urea/100 ml., or slightly less, as the end point changes. Theoretically therefore the end point should be quite satisfactory, and this will be found so in practice with the indicator described, though when the titration is prolonged, as with very high blood ureas, appreciable CO_2 will be absorbed and the end point when reached will fade a little. On standing a few minutes after such titration a sharp end point will be obtained on adding a little more acid from the burette.

The alkali for use in the outer chamber. As already described [Conway, 1939], the salts of potassium have the most marked action in decreasing the solubility or in raising the tension of NH_3 gas in the outer chamber, and potassium carbonate, metaborate and fluoride were the three most effective salts. Further investigation has shown that though the metaborate and carbonate act almost equally when in half saturated strength in the outer chamber, as the percentage saturation is increased, the metaborate becomes more effective. Fig 1 shows the absorption rates with 1 ml. of saturated potassium metaborate and 1 ml. saturated potassium carbonate added to 0·2 ml. of $(NH_4)_2SO_4$ solution. It will be seen that the metaborate is more effective in equal percentage saturation at the higher levels. The metaborate is likewise more suitable when the solution to be introduced into the outer chamber is acidic, since then no CO_2 will be evolved.

The indicator. In the previous account of the microdiffusion method, Tashiro's indicator was used in the change from an alkaline to an acid direction, and with a maximum sensitivity around a *p*H of 5·4. Here, however, the change is in an alkaline to acid direction with the end point of minimal buffering at *p*H 4·9, hence a combination of bromocresol green and methyl red is more suitable. These are best used in a concentration of $0{\cdot}66 \times 10^{-3}$ % for methyl red and half that strength for bromocresol green. With these may be incorporated phenol red in similar strength to that of the methyl red. This addition, though by no means essential, is an advantage for indicating very high NH_3 values. Thus when NH_3 to the extent of 10^{-2} m.eq. is absorbed the colour of the indicator fades and becomes greyish; with 2×10^{-2} m.eq. absorption it changes to pink and with 4×10^{-2} to a cherry red.

The fixative. For room temperature work, good quality white vaseline has been found satisfactory. When the units are to be incubated the vaseline is hardened by melting with 3 parts of it 1 part of paraffin wax (M.P. 55°). Kawerau [1941] has found liquid paraffin serviceable and his method of applying it may be consulted. Borsook & Dubnoff [1939] used with success glycerol, rendered slightly alkaline, for small microdiffusion units made out of synthetic resin. We have found pure glycerol not as serviceable with the standard unit, as it tends to collect in drops which may fall on to the floor of the unit.

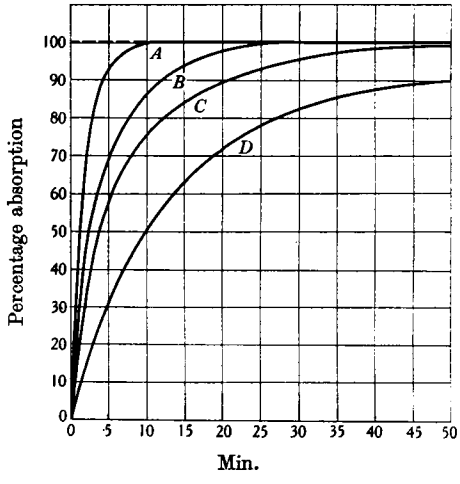

Fig. 1. The curves show the percentage absorption of NH_3 in the standard unit plotted against the time in minutes. *A*, outer chamber contained 0·2 ml. standard $(NH_4)_2SO_4$ and 1 ml. sat. potassium metaborate; rocking maintained at about 60 per min. *B*, same as *A*, but without rocking. *C*, same as *B*, but 1 ml. sat. potassium carbonate used instead of metaborate. *D*, same as *C*, but 1 ml. standard $(NH_4)_2SO_4$ instead of 0·2 ml.

We have found the following water-soluble fixative satisfactory over the period during which it has been examined.

Water-soluble fixative. To 10 parts by weight of powdered gum acacia are added 15 vol. water, 5 vol. glycerol and 5 vol. saturated K_2CO_3. In preparing this fixative, water is added slowly to the powder, the mixture being ground in a large mortar to hasten the solution of the gum. The glycerol and carbonate are then added and stirred in. The mixture may then be used or poured into a large separating funnel and allowed to settle overnight, when the lower fluid can be separated easily from a somewhat frothy upper layer and stored in a stoppered vessel.

For use a few drops are smeared over a glass lid, or, in the manner of Kawerau [1941], some drops are poured on one slide and another is placed on top and drawn across, so that two are prepared together. Results obtained with this fixative have been found to be the same as with vaseline. If traces of NH_3 are present, the mixture may be heated to 100° in a large beaker, with careful stirring throughout to avoid charring, when the NH_3 will be driven off. In the blood urea determinations we did not find any appreciable amount of NH_3 in the fixative.

An alternative and perhaps better water-soluble fixative may be prepared with gum tragacanth, of which much less is required. In preparing this 3 parts of the powdered gum are ground with water which is gradually added until 34 parts are finally included, as well as 15 parts of glycerol and 8 parts of saturated K_2CO_3 (all parts being added by volume except the gum, which is added by weight). The whole forms a soft jelly which may be easily spread on the lids.

Outline of revised NH_3 method for NH_3-N in amounts exceeding 10 μg.

The NH_3-containing fluid in any volume up to 2 ml. is introduced into the outer chamber, but the smaller the volume the more rapid will be the rate of absorption. Into the central chamber is introduced 1 ml. of the boric acid reagent containing indicator (2 ml. are suitable when the NH_3-N exceeds about 100 μg.); alternatively 1 or 2 ml. of half-saturated $MgCl_2$ with indicator may be used. The lid is smeared with the fixative, and after placing in position is slightly displaced in the usual manner to allow the introduction of the alkali (1 ml. of saturated potassium metaborate), then replaced; the outer chamber contents are mixed by rotation and the units left on the bench.

Suitable times for the absorption are as follows:

ml. of NH_3 fluid	No rocking min.	Rocking min.
0·2	45	10
0·5	75	20
1·0	120	60
2·0	240	—

At 38° these times will be reduced to 0·6–0·7 of the above values. At this higher temperature vaseline and hard paraffin 3 : 1 should be used as fixative.

The titration is carried out with standard HCl (N/200 or stronger) from the horizontal burette [Conway, 1939] of which an improved type is now available. A 2 ml. Bang burette may also be used, but it will not be found so convenient, especially for a series of titrations.

The end point is sharp, but when the NH_3 content of the analysed fluid is very high and a comparatively long time is taken with the titration, the end point when reached may fade a little. On standing a few minutes, a stable end point is reached by the addition of very little further acid. This fading results from the liberation of some CO_2 absorbed during the titration.

Solutions

Boric acid, 0·5% in 20% alcohol. To make 1 l. of the reagent, 5 g. of the purest boric acid are introduced into a 1 l. flask, 200 ml. alcohol are added, then about 700 ml. distilled water. The boric acid is brought into solution and 10 ml. of the mixed indicator (bromocresol green and methyl red) are added. On mixing, the whole is brought to the desired end point colour of faint reddish, which usually requires the addition of a little alkali, and the mixture made up to the mark.

A further refinement may be secured by adding, after the 10 ml. of mixed indicator, 10 ml. of a phenol red solution (0·066% in alcohol), which serves to indicate very high NH_3 or urea values, when a stronger acid in titrating will be a convenience.

Mixed indicator. This contains bromocresol green, 0·033% and methyl red, 0·066% in alcohol. It keeps indefinitely.

Magnesium chloride. Exactly as for boric acid above, except that 28 g. purest $MgCl_2$ are used instead of the 5 g. boric acid.

Saturated potassium metaborate. 69 g. of the metaborate/100 ml. water are necessary to form a saturated solution at room temperature.

Saturated potassium carbonate. This may also be used instead of saturated metaborate. To prepare a saturated solution 110 g. may be allowed for every 100 ml. water.

Revised method for blood urea

2 hr. method at room temperature. 0·2 ml. blood is delivered into the outer chamber, and 0·5 ml. of urease-phosphate solution is dropped on to it and mixed with it. Into the central chamber are pipetted 2 ml. of the 0·5% boric acid reagent or the $MgCl_2$ solution (this need be only roughly delivered). After 15 min., 1 ml. of saturated metaborate is

added and the outer contents are mixed by rotation. After a further 1¾ hr. the lid is detached and the contents of the central chamber are titrated from the horizontal burette with $N/50$ HCl. (For general research purposes where there is no likelihood of very high values $N/100$ may be used.) When very high blood ureas are likely to be encountered it is also a convenience to have a second burette filled with $N/10$ HCl, to be used when the colour in the central chamber is greyish or reddish, if phenol red is included in the absorbing reagent.

If any tendency is experienced to have spills from the central chamber 1·0 ml. of a 1% boric acid reagent may be substituted for the 2 ml.

If a 2 or 3 ml. Bang burette is used, $N/250$ HCl would be a suitable strength, with an alternative burette with $N/100$ for the very high levels. The volume of acid used in ml. is multiplied by 60 to give mg. urea/100 ml. or by 150 when using the $N/100$. Also in using the Bang burette 1 ml. of a 1% boric acid reagent is advisable.

Calculation. The result is obtained by multiplying the number of large divisions (each division = 0·01 ml.) on the burette reading by 3, which gives mg./100 ml. urea in the blood.

A blank may be run at the same time if there is any NH_3 in the reagents, and the burette reading is then subtracted from the previous one before multiplying by 3.

Accuracy of the determination. The end point here is sensitive to about half a small division, or 0·15 mg. urea/100 ml. blood, and when titrating with the horizontal burette the movement of the meniscus should be under perfect control (which is readily secured in the new instrument as supplied by Messrs Gallenkamp). The other errors may be controlled to any desirable level as described elsewhere [Conway, 1939]. From a series of 51 routine duplicate determinations the standard deviation of the individual estimate was calculated as 0·34 mg. urea/100 ml.

Solutions. The boric acid and $MgCl_2$ absorbents are prepared as above, as are also the solutions of metaborate or K_2CO_3.

Urease-phosphate mixture. The glycerol extract of jack bean [Conway, 1939] has been found very satisfactory; it retains its activity for many months in the refrigerator. In the usual routine this was diluted ten times with an equal volume of phosphate solution before use. Kawerau [1941] found that incorporating the phosphate in the original preparation produced an extract which has very good keeping powers and filters quickly. The preparation is made as follows. To 10 g. of jack bean meal 20 ml. of water are added and the mixture is agitated vigorously for about 15 min. (a mechanical stirrer or shaker may be conveniently employed). 100 ml. phosphate buffer at *p*H 7·4 (3 g. anhydrous Na_2HPO_4 and 2 g. anhydrous KH_2PO_4 in 100 ml. water) and 20 ml. glycerol are added. This mixture is shaken well, allowed to stand for a short time and the fluid is decanted on to a large folded filter paper. The mixture filters comparatively quickly and may then be cleared of traces of NH_3 or stored directly. To clear from NH_3 5 g. finely powdered permutit are washed with 2% acetic acid and allowed to settle; the acid is decanted and the permutit washed twice with distilled water. To the permutit is added the urease-phosphate preparation; the mixture is well shaken for some time and allowed to settle, when the clear fluid is decanted off and stored in the refrigerator. A small volume of this is diluted three times when required for a series of determinations.

1 hr. method. 0·5 ml. blood is mixed well with 0·5 ml. of the urease-phosphate mixture (diluted twice) in a small clean dry tube (about 2 ml. capacity) and allowed to stand for 10 min. 0·2 ml. volumes are transferred to the outer chamber of a prepared unit with 2 ml. absorbent in the central chamber, and the unit is closed. 1 ml. of saturated potassium metaborate is then introduced into the outer chamber and the contents are well mixed. After 45 min. the contents of the central chamber are titrated with $N/100$ HCl. The calculation is made as before, the number of large units on the burette being multiplied by 3·0. The accuracy is not appreciably less than in the previous method.

It is obvious that by the use of rocking and increased temperatures the time could be further reduced.

Urea in urine. This is carried out by pipetting 0·2 ml. urine suitably diluted to resemble blood strength [Conway, 1939; p. 279] into the outer chamber of a unit containing 1 ml. boric acid reagent and adding 1 ml. saturated potassium metaborate in the usual manner. Titrate with $N/100$ acid.

NH_3 in urine. 0·2 ml. undiluted urine is transferred to the outer chamber of a unit and 1 ml. boric acid reagent into the central chamber. The NH_3 is liberated by 1 ml. saturated potassium metaborate in the usual manner. After 45 min. the titration may be carried out with the $N/50$ acid. Each large division corresponds to 1·4 mg. NH_3-N/100 ml. urine (about 28 large divisions will be the average for the 24 hr. urine).

Discussion

In the blood urea method described, it will be seen that grounds for some previous objections against the microdiffusion method for clinical use are removed. There is, practically speaking, no upper limit, the titrating fluid is perfectly stable, as is also the absorbing fluid, and the cleaning of the units requires no more attention than that given to analytical glassware, but naturally some fixed routine [Conway, 1939] is still advisable. Defective cleaning of the central chamber in an acid-alkali sense reveals itself at once when the absorbing fluid with indicator is introduced. Since, if present, it will be almost always due to the presence of alkali, it may be corrected by a small addition from the burette used to titrate the units, or alternatively, such a unit may be discarded. Apart from the acid used in titration, the only accurate delivery required is that of the 0·2 ml. blood. Errors of the order of 10% in the introduction of the absorbent, the metaborate (or carbonate) or the urease-phosphate solution (provided that the urease preparation is reasonably free from NH_3), are without significance to the determination. The calculation of the result is very simple.

The essential changes in the revised method for ranges $> 10\,\mu g.$ N have proceeded from Abelin's [1938] use of boric acid as absorbent (also studied by Kawerau [1941]). The reduction in concentration of the boric acid in the absorbent, or the substitution of half-saturated $MgCl_2$, with the use of a new indicator more suitable for this particular titration from the alkaline to the acid side, make the end point much more satisfactory. Also the use of potassium metaborate increases somewhat the speed of the absorption, and it is to be noted that full NH_3 absorption can be secured at room temperature in 45 min. using 0·2 ml. of fluid and 1 ml. of the saturated potassium metaborate, and that—at least using standard $(NH_4)_2SO_4$ solutions—complete absorption, if necessary, can be secured in 10 min. by rocking.

Summary

1. A revised microdiffusion method (using the standard unit) is described for estimating quantities of NH_3 greater than 10 $\mu g.$ NH_3-N, and for determining blood urea. Some minor disadvantages of the older method for clinical use have been removed.

2. Further studies of microdiffusion conditions are described.

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