A MICROMETHOD FOR THE DETERMINATION OF TOTAL NITROGEN (ACCURACY 0.005 μg N)

by

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1. INTRODUCTION

The problem of determining the quantity of total nitrogen in small amounts of organic material has occupied the members of our laboratory for a good many years. Ever since two of the authors in 1933[1] succeeded in developing a micromethod for the determination of ammonia (accuracy 0.005 μg N) it has been attempted — at intervals — to adapt this method to the estimation of total nitrogen without loss of sensitivity. In these attempts however we have run into severe difficulties, the origin of which has remained somewhat nebulous to the present day. This does not imply that none of the causes of our failure have been revealed, but only that a number of poor results, obtained with devices long ago discarded in despair, have had to be left unexplained. Everybody who has worked with micromethods of the sensitivity in question will know how capricious the results can be. During short intervals everything may look promising enough, but if the investigator is sufficiently persistent he is likely to run into periods where the results begin to spread and larger deviations appear (one out of 5, one out of 10 determinations) indicating that after all he is not master of the numerous sources of error involved in the method. This phenomenon — the periodic success — has made us extremely cautious and we have come to the conclusion that no method which has not been tested with satisfactory result during a long period and preferably by more than one investigator should be published.

The method described in the following fulfils these requirements in as much as hundreds of determinations, and have been carried out during almost a year by two investigators without any single one deviating for unknown reasons. The method is comparatively simple and has so many features in common with the ordinary KJELDAHL method that theoretically it ought to give correct results. The sources of error due to the reduced dimensions of the instruments employed (increase of surfaces towards air and towards the materials forming vessels, pipettes, etc.) are, we think, sufficiently well mapped out to ensure the inexperienced worker a reasonably uneventful period of practising the method.

We do not postulate that the method is the only or even the best approach to the problem in question, but we hope that some of our observations may be useful to others and that some of our devices may be adopted in other laboratories even if the sequence of operations as a whole may be subjected to individual alterations. In order to save workers, who want to remodel the method to their own convenience, from the multitude of mistakes we have made, a review will be given of some of the steps leading to the present method. This review together with a discussion of the literature will be found at the conclusion of this paper.

As mentioned above a number of workers have participated in the development of the method in the course of time. Members of the staff (Mr A. TORP, Mrs A. L. MADSEN-MYGDAL) as well as guests visiting the laboratory (Dr C. J. VIRDEN, Dr W. L. DOYLE, Dr A. B. HASTINGS, Dr G. PERLMANN, Dr H. LEVI) have been helpful in giving advice and taking part in the experimental work. We wish to thank these helpers most heartily for their assistance. Special thanks are due to Mrs K. M. GJERLOFF to whose patience and caution we owe much of the success in the final stage of the investigation.

2. THE METHOD

2. 1. *GENERAL PROCEDURE*

Before going into the details of the method its general principle will be outlined below.

The organic material to be investigated is introduced into the destruction tube, a long narrow tube, closed at one end, and an aqueous solution containing K_2SO_4, $CuSO_4$, and a small amount of H_2SO_4 is added. The water is removed in a vacuum desiccator over P_2O_5, and 1 µl of concentrated H_2SO_4 containing selenium is added. The tube is then heated for some 6 hours in a small sulphuric acid bath at 295°. After cooling, the digestion mixture is transferred with water to the bottom of a larger paraffin-coated distillation tube of the type previously described [1], NaOH is added in excess, a seal of water is introduced into the neck of the tube (see Fig. 9), dilute sulphuric acid is added to the seal, and the distillation tube is halfway immersed into a thermostat at 40°. After 1—2 hours the ammonia which has distilled from the bottom drop into the seal is titrated in the usual way [1, 2] by means of a suitable micro-burette.

2. 2. *DESTRUCTION*

2. 21 *The destruction tubes.*

The destruction tubes have the following dimensions: Length 40 mm, inner diameter 1.8 mm, outer diameter 2.4 mm. These dimensions need not be strictly maintained, but for reasons that will appear from the following they should not vary too much (especially the inner diameter). The tubes are easily manufactured by drawing them from a wide glass tube of a suitable wall thickness. We have made our tubes of Thüringer resistance glass but any other glass resistant to H_2SO_4 may be used. The bottom should be well rounded off, which makes the emptying of the tubes easier (see section 2.331).

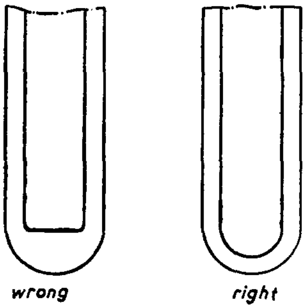

Fig. 1.

2. 22 *Rinsing of destruction tubes.*

Prior to use the tubes are cleaned by boiling them in sulphuric acid + chromic acid. After this treatment they are placed in a dish with cold distilled water and from there they are picked up one by one preferably by means of forceps (care being taken not to touch their rim with naked fingers) and rinsed inside by leading a rapid stream of cold distilled water through them. This is done by means of a capillary tube connected with a water bottle placed 1—2 meters above the sink. The capillary must be so thin that its tip can be led to the bottom of the destruction tubes. The water that remains in the tubes after rinsing is removed by suction, again using a thin capillary. Finally the tubes are dried in vacuo at 100° for 10 min. They are kept well protected in PETRI dishes on filter-paper (in a room where ammonia is banned and smoking prohibited) or in a desiccator.

2. 23 *Filling of destruction tubes.*

The organic material may be introduced into the tubes in several ways. If in liquid form, it is added by means of one of the micro-pipettes previously described[2]; if solid, the object in question may be introduced either by means of a fine glass needle or, in the case of living organisms, in the manner described by HOLTER[3] (p. 434).

After introducing the material, about 4 μl of the following solution (,,blue mixture") are added.

$$\left.\begin{array}{l} 1 \text{ g of } CuSO_4,\ 5\,H_2O \\ 10 \text{ g of } K_2SO_4 \\ 0.2 \text{ g of sucrose} \\ 5 \text{ ml conc. } H_2SO_4 \end{array}\right\} \text{diluted to 100 ml}$$

The composition and quantity of this solution are so adjusted that the amounts of $CuSO_4$ and K_2SO_4, etc. added, together with 1 μl of concentrated H_2SO_4 containing, 10 mg selenium per ml, will form a destruction mixture composed as follows:

1.2 μl conc. H_2SO_4
0.04 mg $CuSO_4$, $5H_2O$
0.4 mg K_2SO_4
10 μg selenium
8 μg sucrose

Fig. 2.

The sucrose is added in order to insure that reduction of nitrogenous impurities in the reagents takes place also in the blanks.

The pipetting of "blue mixture" is done by means of a LEVY semi-automatic pipette (Fig. 2), the destruction tube being held by a diver clamp as described by HOLTER[3] (p. 431 and following). Care should be taken that the solution is delivered at the bottom of the tubes and is not drawn up between the pipette and the inner wall. (For centering see HOLTER[3]). The pipette is connected with an air pump and a pressure regulator[2] (see p. 1137 or[4]) which is adjusted in such a way that the capillary forces at C and C' (Fig.2) just counterbalance the pressure exerted, when C', the tip of the pipette, is free, so that the pipette is discharged automatically as soon as C' touches a wall or penetrates a liquid surface. Sometimes this pressure adjustment requires a good deal of patience and for the present purpose (pipetting of "blue mixture") where the greatest accuracy is not needed, a coarser pressure adjustment may be used and the discharge of the pipette initiated by squeezing the rubber tubing connecting pipette and pressure regulator[2] (see p. 1138). In the case of more accurate pipetting, however, it pays to use the finer adjustment.

In fact, the time spent on this is mostly regained through the increased convenience and speed of pipetting.

The pipette should be rinsed with distilled water outside and inside before beginning a series of pipettings (see below).

After the addition of "blue mixture" the tubes are placed in a vacuum desiccator and the water is evaporated at room temperature. In order to avoid creeping, the main quantity of water is evaporated at 150 mm Hg, which generally takes about 24 hours. Then the pressure is lowered to 0.1 mm Hg and the drying continued for another 24 hours.

The tubes which contain the now blackened organic material at the bottom are next placed in a diver clamp and 1 µl of concentrated H_2SO_4 containing 10 mg of selenium per ml is introduced by means of a graduated pipette as shown in Fig. 3. This pipette, which is filled and blown out by mouth, is made as indicated by HOLTER [3] (p. 435), but the capillary has a wider lumen, 1 µl corresponding to a column length of about 10 mm. The scale is simply a strip of plotting paper glued to the capillary.

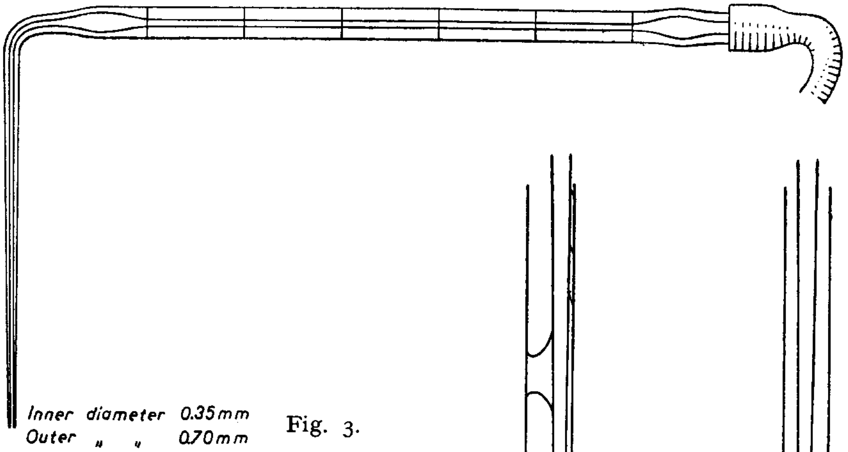

Fig. 3.

The sulphuric acid + selenium is prepared by boiling 1 ml H_2SO_4 with 10 mg of selenium until the solution is clear.

What is said about the pipetting of „blue mixture" is also true about the addition of H_2SO_4. Care should be taken that H_2SO_4 is not drawn up along the pipette to form seals in the upper part of the tubes (Fig. 4). A certain degree of drawing up of the viscous acid can hardly be avoided in all cases, but an experiment need only be discarded if the acid is drawn up as high as the rim of the tube where impurities are most likely to collect. If a seal is formed some distance from the bottom it can be brought down by swinging the tube.

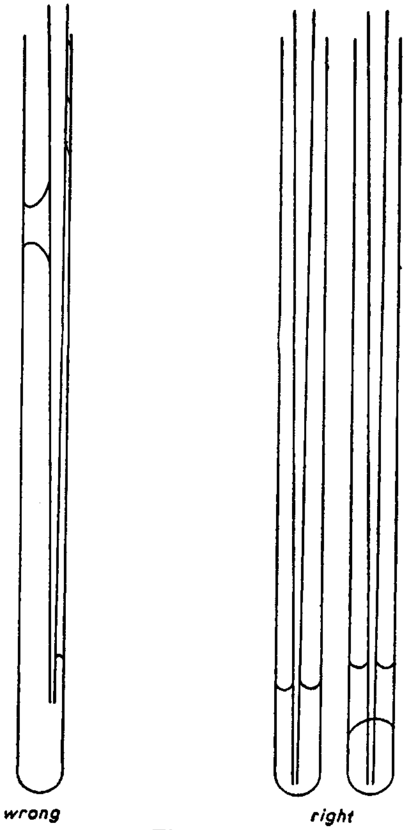

Fig. 4.

It is very important that every day before beginning a series of pipettings the sulphuric acid pipette is rinsed carefully, *outside as well as inside.* Already in the course of 24 hours detectable quantities of ammonia from the air are absorbed by the thin layers of sulphuric acid covering the surfaces of an unrinsed pipette. (See section 3.3).

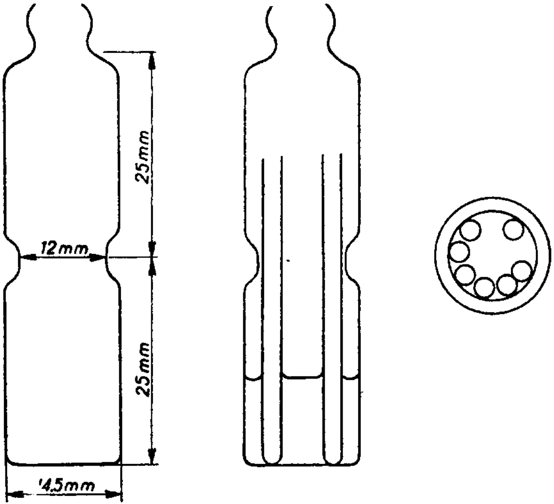

Fig. 5.

2. 24. *The process of destruction.*

The destruction is carried out in a small flask as shown in Fig. 5.

We have made these flasks from insulin ampullae by pulling a constriction approximately in the middle of them. The bottom of the flask is covered with 1 ml concentrated sulphuric acid containing 0.4 g K_2SO_4, *i.e.*, a solution of about the same composition as that in the destruction tubes. Before being used for the first time the solution is boiled in the flask to drive off water and gases. When not in use the flask is closed with a small glass ball (Fig. 6).

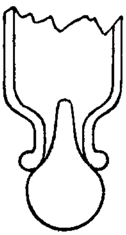

Fig. 6.

Preliminary to the destruction the flask (or flasks) is placed in a small electrically heated copper block[5, 6] with cylindrical holes fitting the flasks tightly and being about 14 mm deep. The temperature of the block is kept constant at 295° at which temperature the sulphuric acid (containing K_2SO_4) is clear, fumes readily, but does not boil. When inserting the destruction tubes the flask is removed from the block and permitted to cool for half a minute in the air; the tubes are picked up one by one by means of forceps and placed in one layer following the wall of the constriction (see Fig. 5) *. During the first minutes after replacing the flask in the copper block it is advisable to have the tubes under frequent observation, because in rare cases, especially when the quantity of organic material is large, the black destruction mixture in the tubes forms liquid seals which are pressed upwards by the gases evolved. If such liquid seals rise higher than about the middle of the tubes, the flask is removed from the copper block and allowed to cool for a minute or two, which causes the seals to move downwards and break. The phenomenon generally disappears after one such cooling process.

After this initial stage of gas evolution the process needs no further supervision. Under the conditions given sulphuric acid will condense all along the walls of the tubes up to the constriction of the flask where the cooling is most effective; above the constriction little or no condensation takes place. The condensed acid will form "islands" (see Fig. 7) that slide down the wall, but formation of seals has not been observed in any case.

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* The tubes must be marked in a suitable way.

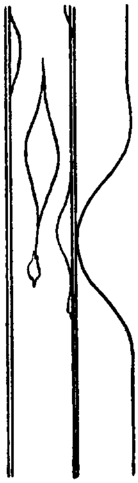

Fig. 7.

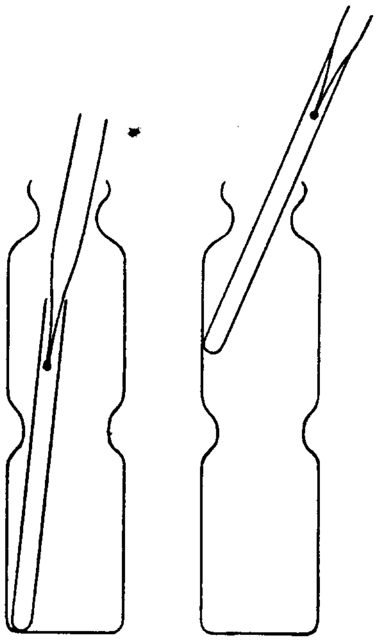

Fig. 8.

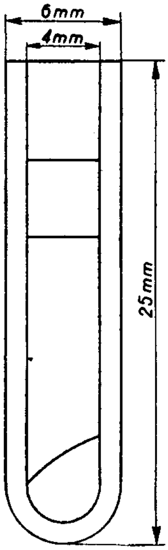

Fig. 9.

The time of destruction is about 5—6 hours (see section 3.4). After this time the tubes are removed from the slightly cooled digestion flask by means of a conical glass rod as shown in Fig. 8. They are rinsed on the outside with distilled water, dried with a clean towel and placed in a desiccator over P_2O_5 where they remain until distillation.

2. 3. *DISTILLATION.*

2. 31. *The distillation vessels.*

The vessels used for distillation are of the type previously described [1], *i.e.* with the dimensions given in Fig. 9. Their inside is coated with a layer of paraffin in the following way :

The clean, dry glass vessels are immersed in molten paraffin at 150—200°. The paraffin used was a German synthetic product probably made according to FISCHER and TROPSCH's method, (M.P. 82°). When the vessels have assumed the temperature of the paraffin bath they are taken up one by one with forceps, quickly knocked out and rotated in a clean towel between the fingers until the paraffin is setting. Care should be taken that a substantial layer of paraffin is formed at the bottom of the vessels and a reasonably thin and homogeneous one on the upper part of their inner wall. Any paraffin on the outside is removed with a dry cloth.

The paraffin bath may be used many times but should be kept well protected against dust and ammonia from the air.

2. 32. *Rinsing of the distillation vessels.*

After use the vessels are rinsed, first with water then with acetone, next with hot toluene, again with acetone, and finally they are boiled a couple of times with water[7]. Here as in the case of the destruction tubes the utmost cleanliness is recommended. The vessels (paraffined or unparaffined) are kept in a well closed glass container while not in use.

2. 33. *Transfer of the acid digest to the distillation vessels, and the distillation proper.*

2. 331. *Operation.* When transferring the acid digest from the destruction tube to the distillation vessel a drop (A) of distilled water (volume about 20 µl) is placed on a clean paraffin block by means of an ordinary hand pipette with or without constriction[8, 9]. Approximately one third of this drop is sucked up into another hand pipette (P) without constriction. This pipette is introduced by hand into the destruction tube and its tip is led to the center of the bottom of the tube (until it rests lightly against the deepest point of the concave bottom) (Fig. 10). The stem of the pipette is approximately centered too, the water is blown out (by mouth), immediately sucked up again together with the greater part of the acid digest, and finally blown out to form a new drop (B) on the paraffin block some distance from drop A (Fig. 11).

With some practice it can be avoided that the liquid is drawn up between pipette and tube in this first part of the transfer. Should it happen however, one need not discard the determination for this reason alone, only the emptying of the tube is so much more rapid and easy if the liquid stays collected at the bottom, that it is strongly recommended to center the pipette well. The more time one uses in removing this first portion of diluted digest, the more the liquid is stirred and the greater is the danger of the crystallization of K_2SO_4, which, if it takes place in the pipette, can block it up completely and spoil the determination beyond help.

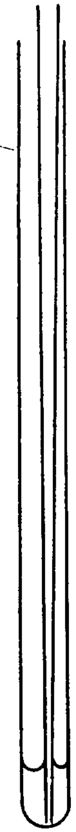

Fig. 10.

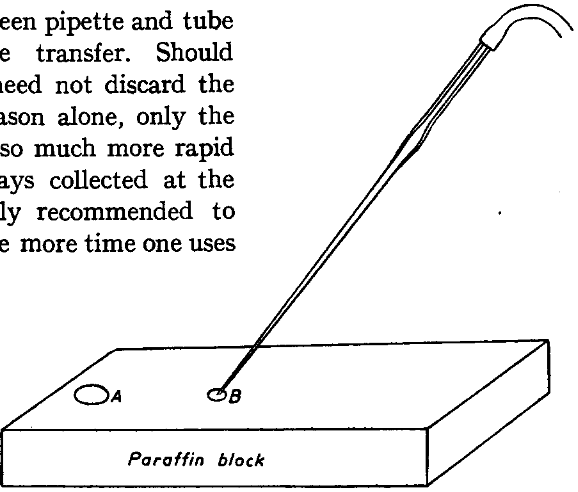

Fig. 11.

We should like to emphasize that crystallization is a very rare phenomenon even if no special precautions are taken. It is however recommended to heat the bottom of the destruction tube slightly in a flame immediately before the addition of water. Ever since this measure of precaution was introduced in our experiments mishaps due to crystallization stopped occurring.

The above described transfer being completed another third of drop A is sucked up into P and introduced into the destruction tube. This time however * the entire inner wall of the tube is moistened with water by leading the stem of the pipette along the inner wall some 5—10 times around. Here again it is relatively easy by centering

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* where there is no danger of crystallization.

the pipette once more in the hand to collect the liquid at the bottom of the tube and suck it up almost quantitatively. The last visible trace of liquid generally follows the tip of the pipette when this is pulled out along the inside wall of the tube and can be sucked up near the rim. The contents of the pipette are added to drop B and the rinsing process is repeated once more with the last third of drop A. Due to the hydrophobic properties of the paraffin surface the removal of drop A is complete. With a little training it is easy to avoid sucking air through the tip of the pipette when the last trace of A leaves the paraffin. (The tip should not be too open though ; see section 2.333).

Finally drop B is likewise sucked up completely in the pipette and transferred to the bottom of the distillation vessel. In order to exclude that the strongly acid solution comes into contact with the paraffined wall at undesired places (*viz.* where the receiving seal is going to be situated) the pipette is fastened in a vertical position in a holder of the type described by Holter[3] (p. 432), and the vessel is lifted towards the pipette by means of a pipetting stand with rack and pinion[2,4].

The pipette P need not be rinsed with water to collect the negligible quantity of diluted digest adhering to its inner wall. It may be used over and over again without intermediate rinsing, unless special conditions make a rinsing advisable (see section 3.2).

To the transferred drop of water + acid digest (21 µl) 9 µl of 18 N NaOH are added by means of a pipette similar to that applied when adding sulphuric acid. A pipetting stand is again used in order to exclude contact between the pipette and the upper part of the vessel. The NaOH is delivered directly into the drop of diluted digest. Quickly afterwards (see section 3.12) a seal of water (45 µl) is introduced into the neck of the vessel (see Fig. 9) and 7 µl of a 0.015 N H_2SO_4 are added to the seal using a semi-automatic pipette with pressure adjustment (see section 2.23). The vessel is covered with a rubber cap and placed halfway immersed in a water thermostat at 40° as shown in Fig. 12. The distillation requires some 1.5 hours, after which time the tubes are taken up and kept covered until the start of the titration.

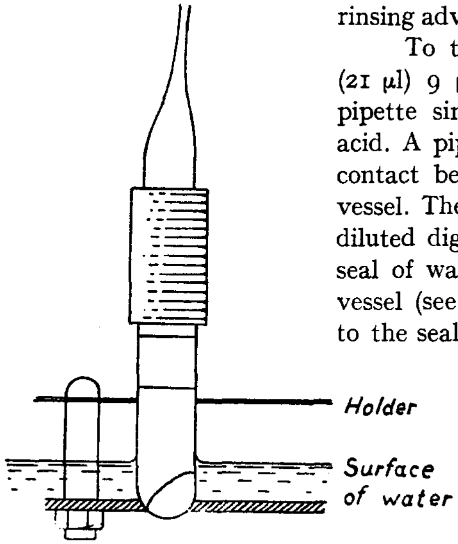

Fig. 12.

Before going into this part of the method a few details wil be given of the instruments employed in the transfer.

2. 332. *The paraffin block.* The paraffin block was cut from an ordinary "paravox" block (melting point 54°) and its surface was smoothed by scraping it with a knife. While not in use it was kept immersed in distilled water. At the beginning of each series of experiments it was taken up and dried with a piece of filter paper. The same block was used for all determinations reported in the present paper. Its surface was smoothed at intervals.

2. 333. *The pipette used in the transfer.* It is important that the pipette P (Fig. 13) has suitable dimensions with regard both to length and to thickness. The opening

at the tip must not be too narrow because if so, small crystals, dust particles or pieces of paraffin may block it up. Neither must it be too wide because then it is very difficult to blow out the pipette by mouth without blowing air through the tip, a thing that must be carefully avoided because it gives rise to spattering from the drops on the paraffin block or in the distillation vessel. The stem of the pipette should not be too thin because then it is liable to break when pulled along the wall of the digestion tube and it should not be too thick on account of the drawing up of liquid between pipette and tube.

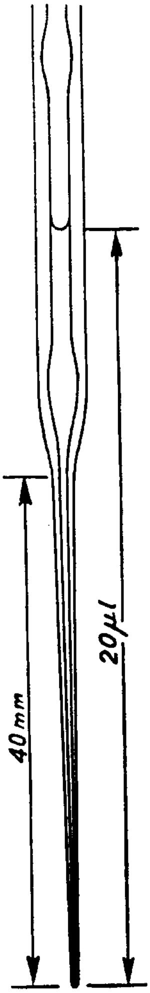

Fig. 13.

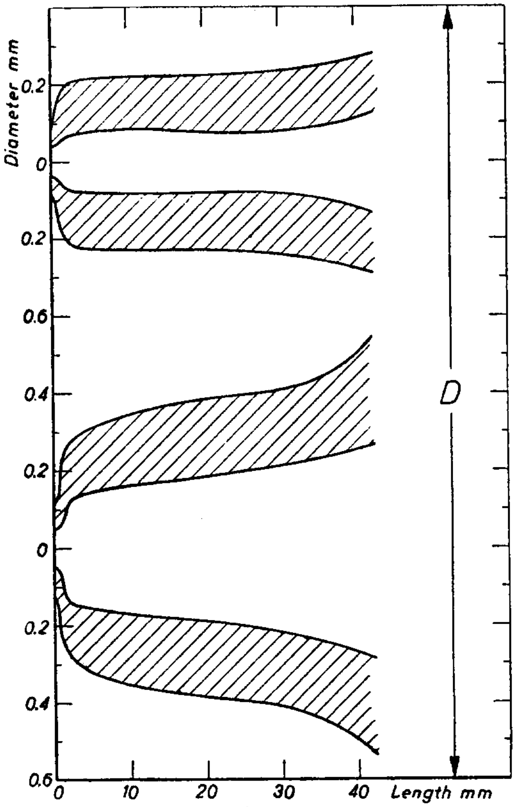

Fig. 14.

These conditions may look rather exacting but in practice it has proven very simple to make good pipettes of this type (using the principle given by Holter[3] (p. 435). In order to demonstrate the wide limits within which a pipette still may be called good two pipettes one rather thin and one rather thick have been measured out from the tip and 40 mm up the stem. The result is seen in Fig. 14. D is the inner diameter of the destruction tube.

2. 334. *Introduction of the water seal.* This procedure has been described before[8, 1, 2]. In the present case however, where speed of operation is required, it is recommended to use a pipette of the type shown in Fig. 15. This pipette is an ordinary hand pipette filled and blown out by mouth. Water is sucked up to the mark X and the entire amount of water is used in making the seal, which facilitates this process. Immediately afterwards the excess of water is removed by sucking up to the mark Y. A may be anything between 20 and 40 µl.

2. 335. *Pipetting of receiving acid.* In the pipetting of the receiving acid (7 µl 0.015 N H_2SO_4) where the highest accuracy is needed, the fine pressure adjustment mentioned in section 2.23 is recommended. In order to give an impression of the dimensions of

a suitable pipette one of our pipettes has been measured out*. The results are seen in Fig 16.

2.4. TITRATION

Since it was found that dilute alkali reacted sufficiently rapidly with the glass in our micro-burettes to give rise to capricious errors in the titration we have decided to reverse the process, *i.e.* to add an excess of dilute alkali by means of a suitable pipette and titrate back with 0.01 N acid (HCl). The base used was secondary sodium phosphate, and the general process was as follows :

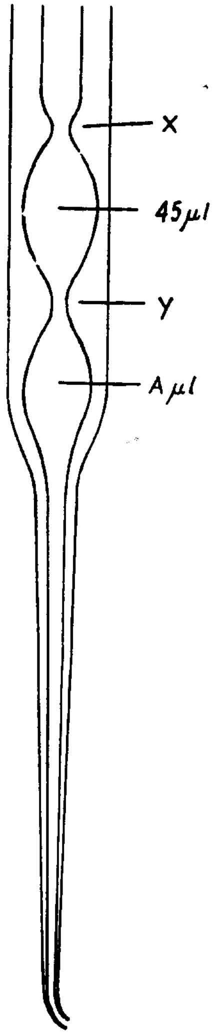

Fig. 15.

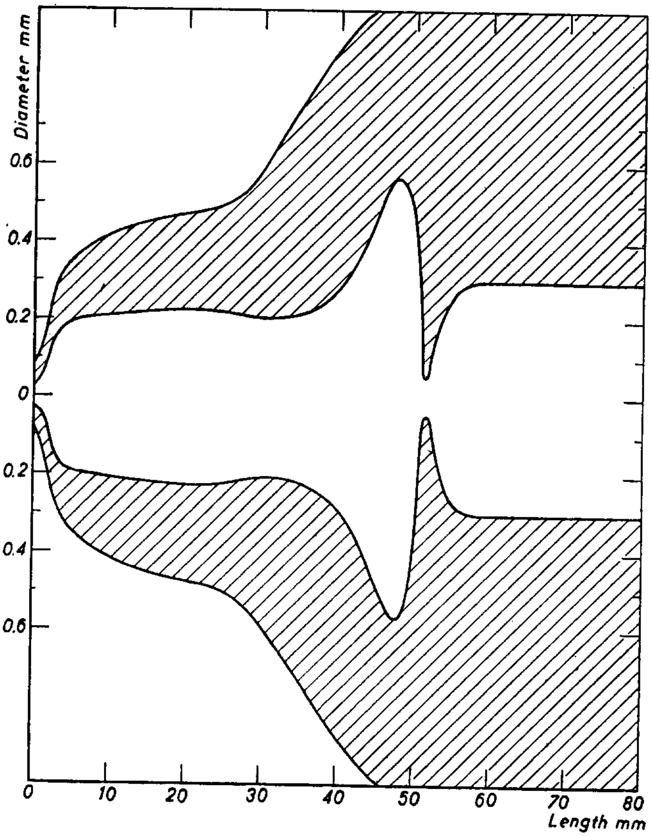

Fig. 16.

18 μl of the following solution are added to the receiving seal by means of a semi-automatic pipette (fine pressure adjustment recommended but not strictly necessary) :

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* The method used was very simple. The pipette was placed in benzene and the outer and inner diameter were measured under the microscope by means of an ocular micrometer. The values found for cylindrical parts of the pipette were controlled by the mercury drop method and found to be correct within 5 %.

0.5 ml m/15 Na_2HPO_4
2 ml bromo cresol green (aqueous solution 0.4 mg dye per ml, p_H 4.6)
2.5 ml boiled-out water.

This solution is made up fresh every day and is kept in a well closed vessel; the highest accuracy is not required in making it.

The acid used in the titration contains so much bromo cresol green (*viz.* 10 ml per 100 ml) that its indicator concentration is equal to that of the solution in the receiving seal. The endpoint of the titration is marked by a green blue colour corresponding to a p_H of about 4.6. A colour standard is made by titrating a blank (45 µl water + 7 µl 0.015 N H_2SO_4 + 18 µl phosphate sol. with indicator) until its colour matches that of a citrate buffer of p_H 4.6. We have the impression that the shade of the indicator is a little different in the citrate buffer and we therefore prefer the titrated blank as a colour standard in the titration.

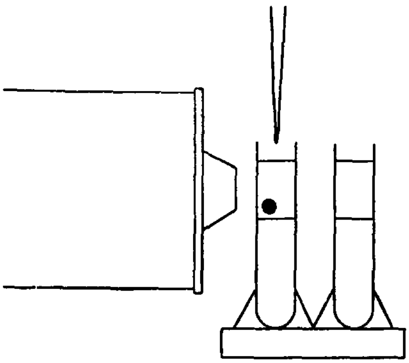

Fig. 17.

The titration apparatus is similar to that previously used [1] only the vessels have a vertical position and the burette is of the type B [8]. The stirrers used (see Fig. 17) must be very clean, otherwise they adhere to the surfaces of the liquid. The same stirrer was frequently used for a whole series of titrations. It was then picked up by fine tweezers and transferred from vessel to vessel via a clean piece of filter paper.

As regards the relative quantities of acid and base in a blank we find that

7 µl 0.015 N H_2SO_4	corresponds to	10.50 µl 0.01 N HCl
18 µl phosphate	corresponds to	12.00 µl 0.01 N HCl
Difference		1.50 µl 0.01 N HCl

Hence 1.50 µl 0.01 N HCl should be used to bring a blank to the endpoint. Naturally these quantities need not be strictly maintained. Inevitable deviations of the volumes of the pipettes from the values given (7 µl, 18 µl) may cause the value 1.5 µl to vary. The essential thing is that there is an excess of base and that this excess is the same throughout a series of titrations.

2.5. *RANGE AND ACCURACY OF THE METHOD.*

In its present form the method permits determination of $<$ 1.2 µg N ∽ 8—9 µl 0.01 N HCl. The accuracy is of the order of magnitude of 0.03 — 0.05 µl 0.01 N HCl (0.004 — 0.007 µg N). We should like to point out however that considerably greater quantities of N may be estimated if the amounts and concentrations of the reagents are changed accordingly. E. ZEUTHEN, working with the method in the field of comparative physiology (larvae of marine animals), determined quantities of N up to 5 µg using the same quantity of concentrated H_2SO_4 as here given, but 3 times as great strengths of receiving acid, phosphate and titration acid. In the case of larger quantities of organic material the amount of concentrated H_2SO_4 should be increased in proportion.

3. DISCUSSION OF THE METHOD

3.1. *TITRATION AND DISTILLATION PROPER.*

When starting the discussion of the present method it is more logical to take up the problem of distillation and titration first because these operations are used for testing the efficiency of the destruction and the transfer of the acid digest. In addition it might be expedient to compare these two operations in their present form with those of the original ammonia method.

3. 11. *Titration method.*

In the previous method [1] borate solution was used in the titration. We wrote at that time that "while N/100 borate gives completely reliable results under the conditions of experiment, we found it impossible to use N/100 NaOH under the same conditions since during one day's titrations its strength increased 1—1½ per cent, and again decreased after continued rinsing of the system of tubes". Now, when the method was taken up again in 1934 about a year after its publication, we found that borate too gave rise to difficulties of the type ascribed to NaOH, and after an endless series of unsatisfactory experiments we agreed upon the explanation that the continuous contact between the alkali and the inner surface of the capillary brought the latter into a state of swelling which facilitated the exchange of ions (Na^+, OH^-), between solution and glass. We consequently dropped the principle of using our microburettes for the titration with very dilute base and tried instead to develop a "reversed" titration as described section 2.4.

The advantage of this procedure is obvious. The inner diameter of a micropipette is much larger than that of a burette and hence the surface brought into contact with the alkali is correspondingly smaller. Furthermore the time in which the contact is effective is many times shorter than in the microburette. The danger of ion exchange is therefore strongly reduced.

The choice of secondary sodium phosphate as the base to be added in excess originated in an intermediary method the main principle of which is published in a paper by BOTTELIER, HOLTER, and LINDERSTRØM-LANG [10]. It was found here that ammonia can be titrated very accurately with acid in the presence of phosphate if the endpoint is around 4.6 where the buffer capacity of the phosphate ion is negligible. The shift in endpoint, from 5.9 (brom cresol purple, previous method [1]) to 4.6 (brom cresol green) is an advantage due to the reduced CO_2 effect, and since the p_H of a secondary phosphate solution is between 8 and 9 its CO_2, uptake and corrosive effect upon glass is negligible. We do not postulate, of course, that some other base may not be found which will serve the purpose equally well. But the present method has given so satisfactory results that we have not gone more fully into this question.

The change in the position of the titration vessels (from skew to vertical position) simplifies the titration and makes it possible to use ordinary titration stands. Since the titration fluid is an aqueous solution and its surface tension therefore sufficiently high, we prefer a burette of type B[8] for titration, as it avoids high hydrostatic pressures and the direct contact of the titration fluid with mercury. If type B is not available, any other suitable burette may serve as well.

Table I shows a series of titration values obtained in the following way:

In a great number of our experiments two sorts of blanks were run parallel with the determinations proper.

One sort of blank ("control blank", C.B.) was made with the double purpose of testing the titration and controlling the purity of the reagents (see below). The vessels in question were just charged with water seal, receiving acid and phosphate solution.

The other blanks (B) were charged and treated exactly as the determinations run parallel with them (they contained conc. H_2SO_4, strong NaOH, etc. (but naturally no ammonia).

The difference B.C.B. gave information about the impurities in the reagents (H_2SO_4, NaOH, etc.) or the possible uptake of ammonia from the outside during the operations leading to B.

TABLE I

TITRATION OF "CONTROL-BLANKS" (C.B.)

n = Number of titrations ; δ = Largest deviation from mean.

Date 1944	n	mean values µl 0.01 N HCl	δ	Date 1944	n	mean values µl 0.01 N HCl	δ
8.8	4	2.41	0.03	14.9	4	1.63	0.07
9.8	3	2.24	0.00	27.9	4	2.11	0.03
10.8	4	1.83	0.05	29.9	4	1.67	0.05
12.8	4	1.89	0.03	2.10	4	1.62	0.02
23.8	4	1.73	0.05	5.10	4	2.35	0.03
24.8	4	1.98	0.02	6.10	4	2.43	0.03
25.8	4	2.00	0.04	16.10	4	2.30	0.04
28.8	3	2.00	0.00	18.10	4	2.40	0.02
29.8	4	2.17	0.01	19.10	4	2.24	0.02
31.8	4	2.01	0.01	20.10	4	2.24	0.04
1.9	4	2.07	0.03	24.10	3	2.19	0.03
2.9	3	2.10	0.02	26.10	3	2.19	0.03
5.9	4	2.09	0.01	31.10	2	2.23	0.03
6.9	4	2.02	0.02	3.11	2	2.28	0.02
7.9	4	2.05	0.03	6.11	3	2.19	0.01
8.9	4	2.02	0.02	10.11	2	2.34	0.00
11.9	4	2.02	0.02	13.11	3	2.34	0.04
12.9	4	1.94	0.04	14.11	2	2.31	0.01
13.9	3	1.67	0.03	18.11	3	2.43	0.03

Standard deviation for the whole material s = 0.022

Generally 2—4 C.B.'s were run in each experiment and the results obtained during the period 8/8—18/11 1944, are reported in Table I. It is observed that the C.B. values vary considerably from day to day which is due to the fact that the phosphate solution was not made up very accurately. The figures show however in a sufficient number of cases the total error involved in the pipetting of receiving acid and phosphate, and in the titration itself. Apart from one experiment (14/9) where the largest deviation from the mean is too high (due to one single "outsider") the agreement between simultaneous titrations is satisfactory. The standard deviation is calculated

from the formula
$$s = \sqrt{\frac{S_1 + S_2 + \ldots\ldots}{n_1 + n_2 + \ldots\ldots}}$$
where S_1, S_2 ..... are the sums of the squares of the deviations from the mean for each day, and n_1, n_2 ..... are the corresponding numbers of titrations. The value $s = 0.022$ is of the same magnitude as that which can be derived from the experiments in the previous paper ($s = 0.025$, from[1] Table I, page 7,12 blanks).

3. 12. *The distillation proper.*

The deviations from our previous method are here a change of paraffin, a change in the mode of adding NaOH, and a change in the time of distillation.

The change of paraffin is hardly significant and was mainly due to the circumstance that we were unable to procure the same paraffin (melting point 72°) as was used before. We do not doubt that any high melting paraffin may do for the coating of the distillation vessels provided it is free from acid impurities and does not crack on cooling.

All our attempts to introduce new materials ("Plexiglas" or Lucite) were unsuccessful, but we quite realize that some time in the future (or may be already now) a "plastic" may be brought on the market which will meet the necessary requirements, being at the same time transparent, hydrophobic, and resistant to strong alkali and acid. If such a substance is found the tedious process of coating the glass vessels could be avoided, which would be a great advantage.

The change in the mode of adding NaOH was necessitated by the considerable rise in the concentration of the base and the correspondingly increased danger of contaminating the receiving seal with NaOH by touching the paraffin wall with the tip of the pipette. The loss of ammonia during the time elapsing between the addition of soda and the insertion of the water seal (10—15 sec.) is perfectly negligible, one of the reasons being that, owing to its higher density, the soda sinks to the bottom of the drop of acid digest, and is covered by this.

As regards the time of distillation, the decrease from 5 to 1.5 hours[1] is mainly due to the higher concentration of soda (and salt) in the bottom drop (4—5 N against 0.6 N in the previous method)[11]. It is possible however that also a better position of the vessels relative to the water surface in the water bath (see Fig. 12) may have something to do with it. In Tables II and III the results of several distillation experiments are reported.

The notations are as follows:

n_B	Number of blanks
B.	Blank (mean value, µl 0.01 N HCl)
$\triangle B$	Standard deviation calculated from the formula $\triangle B = \sqrt{\frac{\Sigma x^2}{n_B - 1}}$
dB	Largest deviation from mean
n_E	Number of determinations
E	Determination (mean value, µl 0.01 N HCl)
$\triangle E$	Standard deviation (as $\triangle B$)
δE	as δB
D	Difference between determination and blank
$\triangle D$	Standard deviation of D calculated from the expression

$$\Delta D = \sqrt{(\Delta B)^2 + (\Delta E)^2}$$

εD, Error of mean calculated from the expression

$$\varepsilon D = \sqrt{\frac{(\Delta B)^2}{n_B} + \frac{(\Delta E)^2}{n_E}}$$

D_{cal}, Quantity of nitrogen (μl 0.01 N HCl) calculated from macro-analysis.

From Table II it appears that half an hour's distillation is insufficient which is shown partly by the too low value found and in part also by the greater spreading of the results *.

TABLE II

DISTILLATION EXPERIMENT

Ammonium chloride solution directly pipetted down at the bottom of the distillation vessel, H_2SO_4 and NaOH added and distillation started. μl 0.01N HCl.

Date	n_B	B	ΔB	δB	n_E	E	ΔE	δE	D	ΔD	D cal.	Dist. time
14.11 1944	2	2.55	0.03	0.05	4	10.43	0.09	0.09	7.88	0.09	7.98	30 min
	2	2.58			4	10.54	0.03	0.04	7.96	0.04	7.98	60 ,,
	2	2.54			4	10.52	0.01	0.02	7.98	0.03	7.98	90 ,,
	2	2.61			4	10.59	0.03	0.03	7.98	0.04	7.98	120 ,,

TABLE III

DISTILLATION EXPERIMENTS

Ammonium chloride solution directly pipetted down at the bottom of the distillation vessel, H_2SO_4 and NaOH added, and distillation started. Distillation time 90 min. μl 0.01 N HCl.

Date	n_B	B	ΔB	δB	n_E	E	ΔE	δE	D	ΔD	εD	D cal.
1.4	5	3.23	0.02	0.03	7	7.19	0.03	0.05	3.96	0.04	0.02	3.99
19.4	6	3.03	0.01	0.03	8	7.00	0.03	0.04	3.97	0.04	0.01	3.99
20.4	6	3.29	0.02	0.03	8	7.27	0.02	0.03	3.98	0.03	0.01	3.99
10.6	6	2.24	0.03	0.04	6	6.23	0.04	0.07	3.99	0.05	0.02	3.99
24.8	4	2.10	0.03	0.04	8	6.15	0.02	0.03	4.05	0.04	0.02	4.07
22.9.43	4	2.99	0.03	0.03	8	10.98	0.04	0.06	7.99	0.05	0.02	7.98
10.6.44	6	2.24	0.03	0.04	5	9.97	0.05	0.07	7.73	0.06	0.03	7.74
14.6	4	1.95	0.04	0.05	3	9.91	0.01	0.03	7.96	0.04	0.02	7.98
24.8	4	2.12	0.03	0.04	8	10.10	0.03	0.06	7.98	0.04	0.02	7.98

One hour's distillation seems to be enough, but for the sake of security we have chosen 1.5 hour.

From Table III it appears that the spreading of the values is higher than that

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* It is true that ΔB is not a very accurate measure of the standard deviation when only 4 values enter into the mean. For the present purpose however this rough estimate may suffice.

of the C.B. values in Table I, especially in case of the E values; but the difference is not very marked. We have considered the result satisfactory in view of the increased number of operations involved. As regards blanks see section 3.3.

3.2. *THE TRANSFER OF ACID DIGEST.*

When transferring aqueous solutions from one vessel to another in quantitative macro-analysis the inner wall of the vessel being emptied is meticulously rinsed with water to remove remnants of the transferred solutions, adhering to the glass surface. Offhand, one would imagine that in micro-analysis still greater care was needed in the rinsing process because of the much greater relative extension of the surfaces in question. However the reverse seems to be the case. In several instances we have been unable to demonstrate any significant difference between the weight of the content of a full micropipette and that of the blown out liquid.

For one of our transferring pipettes (P) we found:

Weight of dry pipette 2.09113 g
,, of water-filled pipette 2.11057 g
,, of emptied pipette 2.09119 g

Hence of 19.44 mg water only 0.06 mg remains in the pipette after emptying! In a 20 ml standardized pipette with very slow delivery 88 mg were retained. In a fast delivering pipette as much as 322 mg retention was observed.

Hence the quantity of liquid remaining in a micropipette and the quantity of liquid required for the rinsing of a micropipette (or a microtube) is generally smaller than anticipated. We have therefore simplified the transfer and reduced the final volume of diluted digest by omitting that extra rinsing of P with water which would serve to pick up the last trace of ammonia retained in the pipette. The error committed naturally depends upon the sequence of the determinations. If these are arranged after increasing N-content beginning with the blanks, the relative error will never exceed 0.3 per cent (relative quantity retained in pipette). If however solutions with low N-content succeed others with high N-content in the transferring the relative error may be quite high. But since the highest N-content estimated by the method in the present form is abt. 1 µg corresponding to 8—9 µl 0.01 N HCl the absolute error will only be around 0.02—0.03 µl which is within the limit of the experimental error anyhow. In fact, the deviation of blanks transferred directly after determinations did not exceed the standard deviation of blanks succeeding other blanks. In special cases however, P may be rinsed in water between the pipettings.

The transfer via the paraffin block has several advantages. It is rapid and safe and requires but little training. The alternative principle of separately adding the consecutive portions of diluted digest to the distillation vessel is time-consuming and dangerous (crystallization, touching of the wall). We should also like to point to the advantage of having the entire amount of rinsing water in one drop which is used up quantitatively. Under these circumstances the same pipette can be used for adding water and for removing the diluted digest without fear of loosing nitrogen.

3.3. *EXTRINSIC ERRORS IN THE DESTRUCTION PROCESS,* INCLUDING ERRORS IN TRANSFERRING THE DIGEST.*

In the ordinary macro and micro KJELDAHL (ANDERSEN and NORMAN JENSEN [7],

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* As extrinsic errors we consider all errors that do not originate in an incomplete transformation of organic N into ammonia.

PARNAS and WAGNER [12]) the ratio between organic nitrogen and H_2SO_4 is around 1 mg N/1 ml H_2SO_4 under optimum conditions. The same ratio was therefore maintained in the present method (*viz.* 1 µg N/1 µl H_2SO_4) so that errors due to impurities in the reagents should not be more dominant than in the macromethods.

An idea of the magnitude of these errors may be obtained by comparing the values of the "control-blanks" with those of simultaneously titrated blanks (see section 3.11). This has been done in 59 cases (comprising 4—10 C.B.s and 4—10 B.s each) with the following result:

1. No destruction (distillation experiments, see Table 2 and 3), H_2SO_4 directly introduced into distillation vessel).

B.—C.B. = 0.12 µl 0.01 N HCl, standard deviation 0.05 (15 cases) error of mean $0.05/\sqrt{15} = 0.01$.

2. Destruction (main experiments). H_2SO_4 treated with "blue mixture" for 6 hours and transferred to distillation tube in the normal way.

B.—C.B. = 0.25 µl 0.01 N HCl, standard deviation 0.09 (44 cases) error of mean $0.09/\sqrt{44} = 0.01$.

The spreading of these values is rather large due to differences in purity of the reagents used at different times. As to their absolute magnitude it is somewhat higher than anticipated (in ANDERSEN AND NORMAN JENSEN'S method (20 ml H_2SO_4) the quantity of ammonia found in blanks is about 0.2—0.3 mg or recalculated to 1 µl H_2SO_4 0.01—0.015 µg corresponding to 0.07—0.10 µl 0.01 HCl), but since systematic errors of this type are satisfactorily eliminated in the difference between determinations and blanks (see Tables II—VI) we have not studied this question in great detail.

We emphasize however, that uptake of ammonia from the air during some of the processes involved plays a part here, and point to the necessity of always running a suitable number of B blanks, treated in exactly the same way as the determinations proper.

The condition that this principle leads to satisfactory results is however that the error is small and relatively constant, and in this connection we shall turn to the question of how to reduce the ammonia uptake to a minimum.

First of all it must be made clear that not only ammonia but also ammonium salts or such substances as may be transformed into ammonia in the KJELDAHL process are a potential danger to the analysis if they are present in the atmosphere as dust particles. It is therefore important, as frequently mentioned in the foregoing, that the utmost cleanliness is observed in handling and storing the glass instruments employed in the analysis. It is however equally important that the atmosphere of the room in which the analysis — and especially the destruction process — is carried out is as clean as possible. For this reason tobacco smoking should not be permitted in the working room, and this should preferably be well separated from the rest of the laboratory. The necessity of the latter precaution naturally depends upon the daily work of the laboratory. If ammonia is not much used this measure need not be taken.

However, only the most radical measures of precaution (air conditioning with dust — and NH_3 — free air) can totally exclude ammonia from the air of the working room. According to the rather varying data of the literature (see THORPES Dictionary of Applied Chemistry or GMELIN KRAUT'S Handbuch der anorganischen Chemie) the atmosphere normally contains about 1 mg ammonia per cubic meter, and if to this we add the unknown but presumably strongly varying quantities of ammonia originating from human secretions (decomposition of urea) we may reach considerably higher values in a closed room.

For comparison, the smoke of one Danish cigarette gives about 2 mg of ammonia in the KJELDAHL analysis. Free ammonia (ammonium carbaminate) seems not to be present. Hence if the smoke were evenly distributed in the room (of say 100 m^3) the effect of tobacco smoking would be negligible.

The presence of such quantities of ammonia in the air naturally puts a limit to the possibility of increasing the accuracy of the method. 1 mg of NH_3 per m^3 corresponds to 0.005 µg (accuracy of present method) in 5 ml and it would seem rather astonishing that the method works at all under these conditions. The uptake of ammonia (or nitrogenous matter) seems however to be relatively slow. A sulphuric acid pipette (see section 2.23) covered with a thin layer of H_2SO_4 on the outside was exposed to the air of the working room for a week and the nitrogenous matter absorbed (ammonia, dust etc.) was determined in the Kjeldahl analysis. The uptake found — 0.15 µg N and 0.26 µg N in two cases — gives an idea of the error involved and indicates that air cannot contain free ammonia in the assumed concentration (1 mg per m^3). As shown by a simple calculation diffusion alone would be able in this case to transport much more molecular ammonia to the pipette than actually found.

Though not fatal in the present case, the absorption found is large enough to warn against exposing acid solutions to the air for longer periods than strictly necessary. In the destruction process where the exposure necessarily is longlasting the destruction tubes are protected to a certain extent by the H_2SO_4 atmosphere in which they are placed. The nitrogenous matter absorbed is dissolved in the entire volume of acid in the destruction flask and diluted in this way.

However, the destruction flask was not introduced for this reason alone. The reduction of the quantity of concentrated sulphuric acid to 1 µl made it necessary to minimize the escape of H_2SO_4 during the destruction process. The sulphuric acid bath serves this purpose in part by ensuring a gentle and uniform heating and a suitable cooling, in part by producing an atmosphere nearly saturated with sulphuric acid vapour.

Table IV shows the results of a series of experiments in which ammonium chloride was treated as "organic material". It will be observed that the spreading is not greater than in the distillation experiments reported in Table II and III. There is however

TABLE IV

NH_4Cl µl 0.01 N HCl

Date	n_B	B	ΔB	δB	n_E	E	ΔE	δE	D	ΔD	εD	D cal.
10.8.44	2	2.32	0	0	6	6.27	0.05	0.07	3.95	0.05	0.02	3.99
28.8	3	2.24	0.02	0.02	7	6.21	0.03	0.07	3.97	0.04	0.02	3.99
31.8	4	2.26	0.03	0.04	8	6.25	0.04	0.06	3.99	0.05	0.02	3.99
27.6	4	2.26	0.03	0.04	7	9.96	0.04	0.05	7.70	0.05	0.02	7.74
29.6	3	2.29	0.02	0.03	7	10.03	0.04	0.06	7.74	0.04	0.02	7.74
29.8	4	2.46	0.03	0.04	7	10.15	0.03	0.05	7.69	0.04	0.02	7.74
1.9	4	2.35	0.02	0.03	8	10.05	0.05	0.07	7.70	0.05	0.02	7.74

a slight tendency for the values to come out too low as is seen from the quantities εD (see section 3.12 ; the D values should not deviate more than 2 εD from D_{cal}). This may be due to incomplete transfer of acid digest, but since the effect is just at the limit of the experimental error we have considered the results satisfactory.

3. 4. *INTRINSIC ERRORS IN THE DESTRUCTION PROCESS.*

The problem of determining the best conditions for transforming the nitrogen of organic substances into ammonia is a very difficult one or, more correctly, one that requires elaborate work for its solution. During the period elapsed since the appearance of KJELDAHL'S original work an overwhelming number of modifications have been proposed, some being definite improvements, some being less valuable. It is not within the scope of the present paper to discuss this question in detail, but we should like to point out that among the modifications presented in the literature that of ANDERSEN and NORMAN JENSEN[7] seems to be the most well-founded one. Unfortunately this method includes mercury as a catalyst and since our micro-distillation has to take place at relatively low temperature (40°) the compounds of Hg and NH_3 formed cannot be completely decomposed even with Na_2S. We have therefore been forced to use selenium as a catalyst[9, 13, 14]. Although to our knowledge the application of selenium has not been quite so thoroughly tested as that of mercury there is sufficient evidence in the literature[14] that it leads to satisfactory results. However the quantity of catalyst varies considerably from author to author as is seen from the adjoining table.

Author	g K_2SO_4 per ml H_2SO_4	g $CuSO_4 \cdot 5H_2O$ per ml H_2SO_4	g selenium per ml H_2SO_4	g $HgSO_4$ per ml H_2SO_4
Andersen and Norman Jensen[7]	0.5	0.05	0	0.037
Lauro[13]	0.4	0	0.004—0.008	
Levi[9]	0.625	0	0.075	
Chibnall et al.[14] . .	0.2	0.05	0.00035	
Boell[15]	0.02	0.02	0.02	
Present method . . .	0.33	0.033	0.008	

From this we conclude that the catalyst concentration is no important factor in the destruction process. As regards the addition of the potassium sulphate we wish to emphasize that in the present method where the temperature is maintained artificially at 295° it serves the sole purpose of depressing the vapour tension of H_2SO_4.

For the rest, only experiments can decide whether the destruction is complete under the conditions chosen. Tables V and VI show a series of determinations of the nitrogen content of casein and egg albumin, two proteins which may be regarded as representatives of easily and slowly digestible substances respectively. The figures demonstrate that 6—7 hours destruction suffices to give almost complete destruction. Longer destruction times are not advisable for practical reasons (times between the length of a working day and that of a night are generally inconvenient, and the destruction cannot very well be left over night) and have only led to more spreading results. It will be observed that casein is somewhat easier to decompose than egg albumin and that the spreading is a little greater in case of the latter substance even after 6.5 hours. There is also a tendency for the values to lie a little too low but the systematic error ($D_{cal} - D - 2\ \varepsilon D$) is always below 1 per cent.

In Tables V and VI as well as in the preceding tables the number of simultaneous determinations and blanks varies somewhat from case to case (from day to day). We wish to emphasize that this is not due to mishaps (so that on days with 7 simultaneous determinations one out of 8 was lost and so on). The number was mainly determined by the space available in the destruction flasks. It is true that now and then mishaps occurred, but we should say that they were not much more frequent than in macro-analysis.

TABLE V

CASEINOGEN. µl 0.01 N HCl

Date	n_B	B	ΔB	δB	n_E	E	ΔE	δE	D	ΔD	εD	D cal.	Destruction time hours
8.9.44	4	2.22	0.03	0.04	8	5.92	0.09	0.14	3.70	0.09	0.04	3.81	2.5
11.9	4	2.21	0.02	0.03	8	5.91	0.09	0.13	3.70	0.09	0.03	3.81	4.5
5.9	6	2.39	0.04	0.07	8	6.16	0.04	0.06	3.77	0.06	0.03	3.81	6.5
12.9	4	2.12	0.03	0.05	8	5.90	0.03	0.06	3.78	0.04	0.02	3.81	6.5
18.11	4	2.65	0.01	0.01	8	6.77	0.02	0.03	4.12	0.02	0.01	4.13	6.5
16.10	5	2.62	0.05	0.06	7	10.71	0.40	0.25	8.09	0.40	0.15	8.26	2
16.10	5	2.62	0.05	0.06	8	10.83	0.05	0.07	8.21	0.07	0.03	8.26	4
24.10	3	2.50	0.03	0.04	7	10.75	0.05	0.07	8.25	0.06	0.03	8.26	4
24.10	3	2.50	0.03	0.04	7	10.78	0.04	0.08	8.28	0.05	0.02	8.26	6
12.8	2	2.39	0.01	0.01	6	9.97	0.04	0.06	7.58	0.04	0.02	7.60	6
6.9	4	2.31	0.03	0.05	7	9.91	0.03	0.03	7.60	0.04	0.02	7.60	6
7.9	4	2.40	0.04	0.04	7	9.95	0.03	0.05	7.55	0.05	0.02	7.60	6
22.6.45	4	3.17	0.04	0.06	10	12.47	0.03	0.05	9.30	0.05	0.02	9.35	6.5

TABLE VI

EGG ALBUMIN. µl 0.01 N HCl

Date	n_B	B	ΔB	δB	n_E	E	ΔE	δE	D	ΔD	εD	D_{cal}	Destruction time hours.
6.11.44	4	2.43	0.03	0.03	8	5.83	0.11	0.17	3.40	0.11	0.04	3.53	2
6.11	4	2.48	0.03	0.04	8	5.94	0.07	0.12	3.46	0.08	0.03	3.53	4
13.11	4	2.56	0.01	0.02	8	5.95	0.09	0.15	3.39	0.09	0.03	3.53	4
13.11	4	2.58	0.02	0.02	7	6.08	0.03	0.06	3.50	0.04	0.02	3.53	$6^1/_2$
20.10	3	2.61	0.01	0.01	7	6.09	0.05	0.09	3.48	0.05	0.02	3.53	$6^1/_2$
31.10	3	2.50	0.02	0.02	6	6.02	0.04	0.06	3.52	0.05	0.02	3.53	$6^1/_2$
10.11	4	2.46	0.04	0.06	6	5.99	0.05	0.07	3.53	0.07	0.03	3.53	$6^1/_2$
26.10	4	2.44	0.02	0.02	8	9.09	0.20	0.29	6.65	0.20	0.07	7.06	2
26.10	4	2.50	0.02	0.02	8	9.30	0.08	0.12	6.80	0.08	0.03	7.06	4
3.11	4	2.56	0.03	0.04	7	9.27	0.10	0.19	6.71	0.11	0.04	7.06	4
3.11	3	2.53	0.02	0.03	6	9.49	0.03	0.05	6.96	0.04	0.02	7.06	$6^1/_2$
18.10	4	2.70	0.03	0.04	8	9.71	0.05	0.09	7.01	0.06	0.02	7.06	$6^1/_2$
19.10	4	2.57	0.03	0.05	7	9.56	0.04	0.06	6.99	0.05	0.02	7.06	$6^1/_2$
31.10	4	2.49	0.02	0.03	7	9.49	0.04	0.07	7.00	0.04	0.02	7.06	$6^1/_2$

An investigation of the substance pyridinium chloride, zinc chloride ($(C_5H_5N.HCl)_2\ ZnCl_2$), which is very difficult to decompose, gave definitely too low values (70 per cent of theory) but so did also a macro-determination in which the normal procedure of ANDERSEN and NORMAN-JENSEN was used (2.5 hours destruction, 60 per cent of theory). This is in agreement with the findings of these authors. For complete decomposition in the macro-analysis the pyridine compound requires boiling for 5—7 hours or a higher temperature (1 g K_2SO_4 per ml H_2SO_4).

We therefore believe the present method to be almost equivalent to the normal ANDERSEN, NORMAN-JENSEN procedure, which has been used with excellent results in our laboratory for almost 20 years. (Compare C. OLSEN[16]).

4. CONCLUSION

As mentioned in the introduction the present method has grown out of the principles laid down in a previous paper by two of the authors. These principles again originated in the range of ideas connected with the micro-enzymatic work begun some 15 years ago. For reasons that were explained at that time a reduction of all liquid volumes was unavoidable in this work and a system of instruments was devised serving to handle, chemically and mechanically, small volumes of liquid. However, when determining an inorganic substance like ammonia the same principles are not thrust so forcibly upon the investigator, because ammonia can be determined with great accuracy even in high dilution (see CONWAY and BYRNE[11, 17], LEVY[9]), and hence it seems evident that the ammonia formed in the destruction process can be determined in several other ways than by micro-titration in a small volume using a µl-burette. When notwithstanding this fact we have decided upon the method described it is for three reasons. In the first place the apparatus was at hand, a factor which is not without significance. In the second place the accuracy of the original ammonia method has not — to our knowledge — been reached by other methods developed since 1933. In the third place the limiting factor for the accuracy of the KJELDAHL method is not found in the ultimate determination of the ammonia liberated, but must be seeked somewhere else in the destruction and distillation processes.

We therefore consider the development of a suitable destruction technique as the main result of the present investigation and leave it an open question in what way the ammonia formed should be determined. It is possible that a more rapid and easy method can be found instead of that presented here. The use of colorimetry is favoured by many authors. We just want to point out that the method chosen need not be more accurate than the present one.

A lengthy discussion of the micro methods for total nitrogen determination already at hand is hardly necessary. To our knowledge M. LEVY[9] was the first to develop a method for the determination of nitrogen amounts of the magnitude in question. He used 10 µl of conc. H_2SO_4 with selenium and K_2SO_4 and determined the ammonia directly in the acid digest by means of NESSLER's reagent. The standard deviations of these estimations was about 0.03 µg N. Almost simultaneously BENTLEY and KIRK[18] published a method in which as much as 100 µl of conc. H_2SO_4 was used with copper and superoxol as catalysts. The standard deviation of their estimations was of the order of magnitude of 0.05—0.1 µg N. A capillary burette was used in the titration of the ammonia (0.025 N NaOH).

NEEDHAM and BOELL[19] in 1939 published a technique which, apart from some minor modifications, was similar to a method previously tried out and abandoned in our laboratory (see below). The standard deviation found from their control experiments is however 0.3 μg, an unnecessary high error even for this type of method. We believe that the large amount of destruction mixture (50—60 μl) and the long distillation time (18 hours) are the causes of the trouble. In the same year BORSOOK and DUBNOFF[20] described a method for quantities of nitrogen between 5 and 10 μg the extreme difference between duplicates being given as ± 2 per cent. The standard deviation may thus be judged to be about 0.05 μg. In their destruction procedure 50 μl of conc. sulphuric acid were used with selenium, copper and persulfate as catalyst. The CONWAY distillation unit was used in the estimation of the ammonia.

Finally BOELL[15] in 1945 described a method which reminds somewhat of BENTLEY-KIRK'S but in which the NEEDHAM-BOELL destruction mixture was used. The standard deviation is about 0.1 μg N.

Although in CONWAY'S paper from 1935 there is no mention of the use of his unit for the estimation of total nitrogen we should like to refer to this admirable piece of work. The accuracy obtained in his distillation and titration experiments is 0.02 μg, to our knowledge the best result hitherto obtained in "ml-scale" analysis. Next to it comes that obtained by TEORELL[21].

In modern science it is not frequent that detailed descriptions are given of the abortive experiments carried out during the development of the final method, and it is not our intention to use much space in exposing all our mistakes. There are however some elements of our discarded methods that either might be used in other connections or might serve to prevent workers in this field from running into similar difficulties.

When our experiments started in 1934 we worked with a slight modification of the original ammonia method. The distillation principle was described in a paper by LINDERSTRØM-LANG, WEIL, and HOLTER[22] and will appear from Fig. 18. The destruction was carried out over a small gas burner with 10 μl of H_2SO_4 containing K_2SO_4 and selenium as the only catalysts. The history of this method is typical for work of this type. At the start it gave surprisingly good results but in the detailed investigation it soon became capricious, both too high and too low values occurring so frequently that the method had to be abandoned. The main errors are :

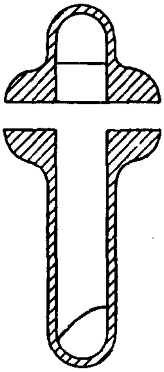


Fig. 18.

1. A too slow diffusion of ammonia from bottom drop to receiving drop, the distance being too great and the areas too small (see CONWAY and BYRNE[11], [17]). The long distillation times required (18—24 hours) promotes the influence of

2. A creeping of sodium hydroxide between paraffin and glass in the receiving cup. The necessity of moistening the whole inner wall of the destruction tube with soda (to exclude that acid, ammonia-absorbing areas remain) brings the alkali to the rim of the receiving cup.

3. The violent neutralization of 10 μl concentrated acid with soda gives rise to a formation of extremely fine alcaline droplets (fog) that are carried to the receiving seal with convection currents during the initial part of the distillation.

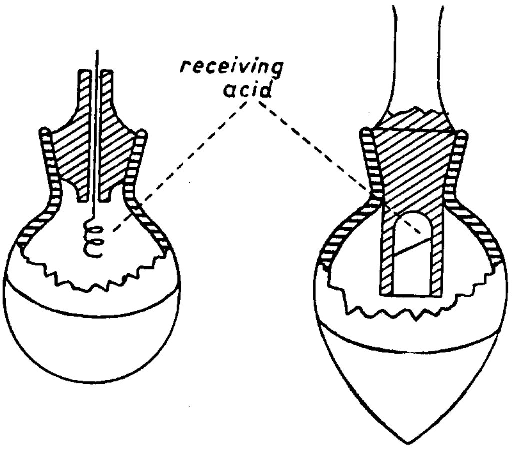
4. Errors due to impurities in H_2SO_4 and NaOH and to absorption of ammonia during the destruction process.

None of these errors is serious when the distillation method is used for the determination of ammonia under circumstances where the quantities of acid and alkali are low [22]. The consistent step to take was therefore to reduce these quantities, which per unit of nitrogen were 10 times those applied in macro-analysis, or to use a principle of ammonia determination in which either the distillation was totally omitted or in which at least contamination with acid and alkali did not appear as a disturbing factor. The beautiful principle of direct ammonia determination in the acid digest developed by TEORELL [23] was not applicable due to the catalysts chosen and the relatively large quantity of H_2SO_4 used. In addition hydrogen peroxide as the only catalyst [23] is known to fail in some cases. The idea of using TEORELL's bromate method (which is relatively independent of acidity) for the determination of ammonia after distillation was abandoned because of the paraffin coating of the receiving cup. Later on at a stage where paraffin was abandoned this method was tried out (see below) but did not give consistently good results (which probably was our fault).

To make a long story short the failure of our first method led to the above mentioned direct, colorimetric method of LEVY and to a series of attempts to reduce the quantity of sulphuric acid. In these endeavours we were led astray for a long time by an idea which (again) at the beginning looked promising, *viz.* the destruction with 1 µl H_2SO_4 (+ catalysts ; Cu, Se, K_2SO_4) in *closed* tubes or ampullae immersed in a H_2SO_4 — K_2SO_4 bath. The ratio gas-liquid in the tubes was about 50—1. The reason for adopting this principle was fear of the troubles connected with the direct heating in open tubes of such small quantities of acid. In addition the possibility of totally excluding the access of air during the destruction was very tempting (prevention of NH_3 uptake).

We should like to point out that this method never failed completely. In periods it gave quite good results, in fact so good that we ventured to use it in several biological investigations (see BOTTELIER, HOLTER, and LINDERSTRØM-LANG [10]) but the number of unexplained outsiders was too large and the spreading of the rest of the values was of the order of magnitude of 0.015 µg. Furthermore the nitrogen content of a protein like egg albumin was always found too low (5—10 per cent) especially if the quantity of protein destructed was large ($\backsim$1 µg N). A thorough investigation carried out during the war failed to reveal any special source of error except one. The presence of halides is decomposed to the determination and all attempts to drive off the halogens with H_2SO_4 in vacuo at 100° over P_2O_5 before closing the ampullae were in vain. But even in the absence of halides no consistently good results were obtained, and we believe that the influence of the decomposition products (SO_2, etc.), trapped within the ampullae, is behind most of the troubles. The main visible difference between the destruction in closed and in open tubes is that in the closed tubes free selenium is formed by reduction and precipitates in a red amorphous form upon dilution of the digest. The same does not occur after destruction in open tubes, which tends to show that the milieu is quite different in the two cases. The significance of the water formed and trapped in the closed ampullae has not been cleared up. Addition of SO_3 seems to have a very capricious effect probably depending upon the amount of water formed in the destruction.

The ampulla technique was tested with the distillation method described in the present paper so that the erroneous results were not due to deficiency of that part of the KJELDAHL method. (The acid digest was transferred from the ampullae to the distillation vessel in much the same way as described in section 2. 331). However it was not until rather late that we found out how little water was needed for this transfer and a series of distillation methods were therefore tested out in which the volume of diluted digest was large (50—100 µl). One of these methods was described in [10]. Here the ampulla is broken directly in dilute alkali in the distillation vessel. In other methods the contents of the ampulla were rinsed out into 100 µl water in the distillation vessel and alkali added afterwards. Common for all these methods was that in spite of all possible shapes of the distillation vessels and all possible stirring devices (rocking, rotating, with or without one-sided heating) the distillation time had to be made very long (24 hours) whereby many errors increased in significance and the number of mishaps grew (see Fig. 19). None of these methods was therefore satisfactory, and they are not to be recommended.



Main error:
Acid dropped off spiral.

Main error:
If receiving cup paraffined: acid slid off. If receiving cup unparaffined: alkali from glass.

Fig. 19.

The titration method was also modified several times [10]. For two shorter periods TEORELL'S bromate method was tried.

SUMMARY

The method described is based on the KJELDAHL principle. The sample is digested by sulphuric acid, with selenium and copper sulphate as catalysts. The digestion mixture is transferred by a special arrangement to a paraffin-coated distillation tube, and after addition of NaOH the ammonia is distilled at 40° into a liquid seal of known acid content. Finally the excess of acid is titrated.

In the present form the method permits the determination of 0.1—1 µg N with an accuracy of 0.005 µg N. Larger amounts of nitrogen may be determined by suitably increasing the concentrations of the reagents.

RÉSUMÉ

La méthode décrite repose sur le principe de KJELDAHL. L'échantillon est digéré par l'acide sulfurique avec du sélénium et du sulfate de cuivre comme catalyseurs. Lemélange digéré est amené par un dispositif spécial dans un tube de distillation revêtu de paraffine. Après addition de NaOH l'ammoniaque est transportée par distillation à 40° en un *liquid seal*, dont la teneur en acide est connue. L'excédent d'acide est ensuite titré.

Sous sa forme actuelle, la méthode permet la détermination de 0.1—1 µg N à une approximation de 0.005 µg N. Il est possible de déterminer de plus grandes teneurs en azote en augmentant convenablement les concentrations des réactifs.

ZUSAMMENFASSUNG

Die beschriebene Methode beruht auf dem Prinzip von KJELDAHL. Die Probe wird digeriert durch Schwefelsäure mit Selen und Kupfersulfat als Katalysatoren. Die digerierte Mischung wird durch eine Spezialvorrichtung in eine mit Paraffin überzogene Destillationsröhre geführt, und nach Zufügung von NaOH wird der Ammoniak durch Destillation bei 40° in ein *liquid seal* übergeführt, dessen Säureinhalt bekannt ist. Schließlich wird der Säureüberschuss titriert.

In ihrer jetzigen Form ermöglicht die Methode die Bestimmung von 0.1—1 µg N mit einer Annäherung von 0.005 µg N. Grössere Stickstoffmengen können durch eine passende Erhöhung der Konzentrationen der Reagentia bestimmt werden.

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Received July 4th, 1946