867. *Submicro-methods for the Analysis of Organic Compounds. Part I. The Determination of Nitrogen.*

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Nitrogen in organic compounds is determined by decomposing a sample, about 50 μg., in a sealed tube and titrating the ammonia thus formed with a standard solution of sodium hypochlorite.

The results are comparable in accuracy with those obtained on the micro-scale.

SEVERAL years ago we began investigations to devise a system of organic analysis for the determination of elements and functional groups requiring sample weights of about 0·05 mg. (50 μg.). This weight was chosen because it was considered to be the smallest that could be practically manipulated without use of magnifying lenses: *e.g.*, 0·05 mg. of a sucrose crystal is only just visible to the naked eye.

Several methods have now been perfected: those developed so far are based on classical principles and the only specialised apparatus required is the balance previously described [1] and a micrometer-syringe burette.

[1] Asbury, Belcher, and West, *Mikrochim. Acta*, 1956, 598.

Submicro-methods should have far-reaching applications, particularly in biochemistry, physiological chemistry, or wherever only minute amounts of material are available. They should also prove invaluable for use in conjunction with modern techniques such as vapour-phase chromatography where minute samples of high purity can be obtained. They are not intended to replace the conventional micro-methods preferable when sufficient material is available.

Determination of Nitrogen.—After consideration of modifications of Kjeldahl's method it was concluded that the best means of decomposition would be by digestion in a sealed tube.[2] This has several advantages over more conventional methods of decomposition; complete decomposition is more certain, catalysts and other additions (potassium sulphate) are unnecessary, the temperature can be controlled, and, most important in view of the scale, risk of atmospheric contamination is eliminated. For completing the determination, titration of the ammonia with standard hypochlorite was chosen.[3] This titrant had previously been used on the micro-scale [4] and offered the advantage that the digestion and the titration could be done in the same vessel.

Since this work was started Kirk and his co-workers have reported the successful determination of μg. amounts of nitrogen [5] and phosphorus.[6] Much of their analysis was on biological material, with relatively large samples. Kirk *et al.* checked the method for nitrogen by analysing some pure organic compounds. Satisfactory results were obtained on sample weights similar to those described below; sealed-tube digestion was also used for decomposition, but a diffusion method followed by an acid–base titration was used for determination of the ammonia.

Our general procedure was developed stepwise, starting from simple ammonium salts in pure solution, to eliminate errors associated with a particular stage. The most serious difficulty was to find a simple method for adjusting the pH of the digest (the hypochlorite titration is only satisfactory from pH 7·5 to 9·6). Organic indicators reacted with hypochlorite and yielded variable blanks, but satisfactory adjustment was obtained by the addition of a mercuric salt; [4] the precipitation of mercuric oxide indicated the approach of the neutralisation point and final adjustment was obtained by the use of a sodium hydrogen carbonate buffer. When the pH was too low a yellow colour developed owing to decomposition of hypochlorite; at too high a pH, the mercuric oxide precipitate did not dissolve when potassium bromide was added.

An electrically heated metal block drilled to accommodate the digestion tubes was used for decomposition. Satisfactory recoveries were obtained over the range 400—450°. A period of 30 min. was adequate; but a longer time (60 min.) showed no significant differences. Below temperatures of about 380°, decomposition was incomplete, and above 500° there were losses of ammonia. The temperature range 420—430° was always used subsequently. These findings agree substantially with those of Kirk *et al.*[5]

A series of compounds was analysed by this procedure; the results are included in the Table, Column A, and in most cases are the average of 10 determinations. Although the absolute errors were generally within 0·3%, nearly all were negative. These results were calculated on the basis of the hypochlorite solution standardised against thiosulphate which had been standardised in turn against potassium iodate. When the hypochlorite solution was standardised directly against a pure ammonium salt, the normality was always slightly higher. The results in Column B have been calculated from hypochlorite standardised in this way. This empirical standardisation of hypochlorite has been advocated by earlier investigators [7, 8] and in view of our own results is recommended.

[2] White and Long, *Analyt. Chem.*, 1951, **23**, 363.
[3] Kolthoff and Stenger, *ibid.*, 1935, **7**, 79.
[4] Belcher and Bhatty, *Mikrochim. Acta*, 1956, 1183.
[5] Grunbaum, Schaffer, and Kirk, *Analyt. Chem.*, 1952, **24**, 1487.
[6] Schaffer, Long, and Kirk, *ibid.*, 1953, **25**, 343.
[7] Wolfel, *Z. analyt. Chem.*, 1932, **90**, 170.
[8] Willard and Cake, *J. Amer. Chem. Soc.*, 1920, **42**, 2646.

Compound	N, calc. (%)	A		B	
		N, found (%)	Δ *	N, found (%)	Δ *
Acetanilide	10·36	10·16	0·20	10·37	0·20
Phenacetin	7·82	7·69	0·16	7·85	0·15
Hippuric acid	7·82	7·78	0·21	7·93	0·16
4-Benzylideneaminodiphenyl	5·45	5·40	0·25	5·51	0·24
Diphenylacetonitrile	7·25	7·08	0·12	7·23	0·15
s-Diphenylurea	13·21	13·04	0·20	13·30	0·20
α-Benzoin oxime	6·16	6·05	0·20	6·17	0·20
s-Diphenylguanidine	19·89	19·79	0·20	20·18	0·19
Benzamide	11·57	11·41	0·19	11·64	0·19
o-Tolidine	13·20	12·86	0·20	13·12	0·20

Column A. Hypochlorite standardised against standard thiosulphate.
Column B. Hypochlorite standardised against ammonium sulphate.
* Standard deviation.

Experimental

A. *Reagents.—Sulphuric acid.* Microanalytical reagent (M.A.R.).

Mercuric sulphate. 2% Solution, prepared by adding a few drops of M.A.R. sulphuric acid to 2 g. of M.A.R. mercuric sulphate, followed by about 20 ml. of distilled water. The mixture was thoroughly stirred, and more distilled water added, with continued stirring, to dissolve the last traces of solid. The solution was then diluted to 100 ml.

Sodium hydroxide. 2N-Solution prepared by dilution of the standard commercial solution from ampoules.

Sodium hydrogen carbonate. 5% Solution, prepared from " AnalaR " solid.

Potassium bromide. 30% Solution, prepared from " AnalaR " solid.

Sodium hypochlorite. 0·04N-Solution, prepared by diluting the appropriate volume of 10—14% (available chlorine) solution with distilled water. B.D.H. bromine-free reagent was used.

Potassium iodide. 30% Solution, prepared from " AnalaR " solid. The solution was made freshly each day in order to limit the blank caused by decomposition.

Sulphuric acid. 4N-Solution, prepared by dilution of M.A.R. acid.

Sodium thiosulphate. 0·04N-Solution, prepared from " AnalaR " salt.

Indicator. " Thyodene," which could be added as solid and was therefore more convenient than a starch solution.

Ammonium sulphate. " AnalaR " salt was very finely ground with a micro-pestle and mortar and then dried (P_2O_5) under vacuum.

B. *Apparatus.—Ultramicrobalance.* The balance and its operation were as described by Asbury, Belcher, and West.[1]

Digestion tubes. These were of borosilicate glass tubing (13 mm. external diam., 11 mm. internal diam., and 7 cm. length) with a hemispherical seal at one end. They were cleaned by soaking either overnight in chromic acid or for a few minutes in a 5% mixture of hydrofluoric acid (about 40% solution) with Teepol, followed by thorough washing with tap- and then distilled-water. The clean tubes were dried at 120°, and stored over phosphoric oxide. It was important for the glass to be perfectly dry and free from grease, etc., so that none of the small crystals of an ultramicro-sample would stick to the sides of a tube during transfer.

Heating block. This consisted of an electrically heated Dural block drilled with 8 holes to accommodate the digestion tubes.

Centrifuge. An electric semimicro-centrifuge capable of 4000 r.p.m.

Titration equipment. " Agla " micrometer syringes (Burroughs Wellcome & Co.) were employed throughout. Each had a graduated total capacity of 0·5 ml., the individual graduations corresponding to a volume of 0·0002 ml. (*i.e.*, 0·2 μl.). It was possible to estimate to 0·1 μl. without difficulty. In place of the metal needle normally provided, a glass capillary bent at a right angle was sealed on the nozzle of the syringe. It could be used in this form as either a pipette or a burette. A coiled metal spring was always kept over the syringe plunger in practice to prevent the plunger's slipping into the barrel of the syringe without pressure being exerted on it from the micrometer screw-gauge.

Rotating magnetic stirring was employed. Each stirrer consisted of a short thin steel rod sealed into a glass capillary (1 cm. long), a very thin glass capillary (0·5 cm.) being sealed at right angles to the centre of the latter to give a T-shape.

The opened digestion tube, containing the centrifuged digest, was clamped on the surface of

a white tile resting on top of the case containing the electrically operated rotating magnet. A stirrer was added, and the syringe burette was clamped horizontally so that its capillary tip dipped below the surface of the liquid.

To provide standard illumination, a Daylight Blue Bulb (230 v, 60 w, Mazda B.T.H. Single Coil) was enclosed in a dark shade and clamped about one foot above the titration tube at a slight angle to the vertical. This produced an intense white light and enabled the progress of a titration to be watched from above the open end of the tube. The disappearance of the blue starch–iodine colour could be seen readily against the illuminated white tile background.

C. *Recommended Procedures.—Sealed-tube determination of organic nitrogen.* (i) Weighing. The contents of a weighing boat were transferred to an empty digestion tube by holding the boat in tweezers over the mouth of the tube, inverting, and tapping the tweezers supporting the boat on the edges of the tube mouth. The boat was then reinverted, and restored to the scale-pan of the ultramicro-balance. The sides of the digestion tube were well tapped to ensure that all the sample crystals fell to the bottom. During transfer, the tube had to be clamped upright.

For a series of determinations, 8 clean dry digestion tubes (4 for nitrogen and 4 for blank determinations) were placed in a metal-block holder in a desiccator containing phosphoric oxide. The total time to complete these 8 determinations was usually slightly more than 8 hr.

(ii) Digestion. Concentrated sulphuric acid (10 μl.) was added to each digestion tube from a syringe burette, the drop being removed from its tip by touching the latter lightly against the sides near the bottom of the tube. The closed end of the tube was then inserted about 2 cm. into a tight holder consisting of a B19 Quickfit socket lined with asbestos paper, and an oxygen–gas flame applied at a point some 2 cm. from the open end. When the glass was quite molten, the open end was pulled out with clean tweezers and a seal made as near to the main tube as possible.

The sealed tube was placed in a cavity of the block with tweezers and left for 30 min. at 420—430°. After digestion, the sealed tubes were removed from the heating block with tweezers and allowed to cool for a few minutes. They were then centrifuged thoroughly (about 5 min.) and returned to the holder in the phosphoric oxide desiccator.

Although the glass was thin where the tube was sealed, the pressure developed during digestion was normally never sufficient to cause an explosion. When a cooled digestion tube was opened, there appeared to be very little, if any, excess of pressure.

(iii) Titration. A scratch was made with a sharp glass knife some 1·5 cm. from the point of sealing and the tube opened by applying a piece of molten soft-glass rod lightly to the moistened scratch. The two portions of the tube were then placed in an oven at 90° for 5 min. to remove any sulphur dioxide from solution in the digest (this would interfere in the titration). After being removed from the oven, the two portions of the digestion tube were allowed to cool for about 1 min. in a metal-block holder in an otherwise empty desiccator.

Distilled water (1 ml.) was taken into a 2 ml. hypodermic syringe (Everett Syringes, metal nozzle) and 0·5 ml. washed down the sides of the main portion of the opened digestion tube. The tip of the digestion tube was washed out with two 0·25 ml. portions of distilled water from the hypodermic syringe, these being swilled over the inside surface of the tip several times before transfer to the main tube by means of a capillary dropper containing a 1 ml. reservoir bulb. The final contents of the main tube were gently swilled over its inside by tilting and rotating it carefully a few times.

A T-piece stirrer and 2% mercuric sulphate solution (1 drop) were added to the main tube (now a titration tube), the light was switched on, and the stirrer started. 2N-Sodium hydroxide was run in fairly quickly from a syringe burette until the first sign of a white turbidity, and then more slowly until the capillary tip of the syringe burette and stirrer were obscured from view by a yellowish-white cloudy precipitate. The alkali was then added very slowly. When crystalline material became visible against the cloudy background of colloidal material, further additions of sodium hydroxide were made only a little at a time. As soon as the background cloudiness cleared, no more alkali was added. It was important to avoid the addition of excess of alkali at this stage. 5% Sodium hydrogen carbonate (1 drop) and 30% potassium iodide (2 drops) were added at once, so that the solid material could not adsorb much alkali and be rendered insoluble. If some solid remained undissolved after being stirred for a few minutes, further additions of bromide and bicarbonate were made.

When a clear titration medium had been obtained, a known amount (about 150% of theoretical) f standard 0·04N-sodium hypochlorite solution was run in from a syringe burette

with stirring throughout. The titration tube was then placed in a metal-block holder in the empty desiccator for 5 min. while oxidation of the ammonia was completed. After being removed from the desiccator and again clamped above the stirrer motor, the latter was switched on, freshly prepared 30% potassium iodide (1 drop) added, the solution acidified with 4N-sulphuric acid (1 drop), and the liberated iodine titrated at a *slow* steady rate with standard 0·04N-sodium thiosulphate solution until the yellow colour had nearly disappeared. A little solid Thyodene was added from the end of a micro-spatula, the light was switched on, and the titration completed with further 0·04N-thiosulphate solution. The disappearance of the blue starch–iodine colour was best seen from above the open end of the titration tube against the illuminated white tile. It was essential not to titrate very quickly.

The blank determination was carried out as described above, except that as there was no nitrogenous organic compound present no crystalline material was formed. After the appearance of the dense yellowish-white cloudy precipitate, alkali was added very slowly until the first sign of clearance was noted. Further addition of sodium hydroxide was made a little at a time. In the pauses between these additions, time was allowed for the alkali to mix into the whole system; the addition of alkali was stopped when the cloudiness disappeared. The nitrogen and blank titrations were done alternately, the sodium hydroxide, sodium hypochlorite, and sodium thiosulphate syringe burettes being refilled after each blank titration. Blanks varying between 2 and 5 μl. of 0·04N-hypochlorite have been found; they should not exceed 6 μl. The time between the opening of a sealed tube and reaching the iodometric end-point was about 30 min. Hence, the contents of the syringe burettes were only exposed to the laboratory conditions of light, etc., for about 1 hr. before being replaced.

1 μl. of 0·04N-NaOBr (*i.e.*, NaOCl) = 0·1868 μg. of nitrogen. Hence,

$$\text{nitrogen in sample} = (V - V') \times f \times 18{\cdot}68/W\%$$

where W = weight of sample taken (in μg.), f = normality factor of the 0·04N-sodium hypochlorite solution, V and V' = unconsumed 0·04N-hypochlorite in the blank and in the nitrogen determination, respectively (μl.).

Standardisation of Sodium Hypochlorite.—A weighed sample (about 50 μg.) of ammonium sulphate was transferred to a titration-type tube and digestion acid (10 μl.) run in from a syringe burette, the drop being taken from the burette tip by touching it lightly against the sides of a titration tube. Distilled water (1 ml.) was then added from a graduated hypodermic syringe, a T-piece stirrer introduced, and the acid neutralised as before. The oxidimetric titration of the ammonia was also done as outlined above.

The blank titrations were carried out in exactly the same way, except that no ammonium sulphate was present.

The nitrogen content of ammonium sulphate being taken as 21·19%, then:

Normality of NaOCl (*i.e.*, NaOBr) = $21{\cdot}19W \times 0{\cdot}04/[f(V - V') \times 18{\cdot}68]$N where W = weight of ammonium sulphate taken (in μg.), V and V' are analogous to V and V' in the hypochlorite titrations (above), and f = ratio of concentration of hypochlorite to that of thiosulphate (see below).

A series of 5 standardisations, with 5 blanks, was usually carried out in this way and the average value taken for the normality of the sodium hypochlorite solution. The normality of the sodium thiosulphate solution was then calculated from the known ratio of the concentrations of the two titrants (*i.e.*, f). The standard deviation of a series of standardisations was 0·009. In one typical series the average blank was 2·8 μl., the largest deviation from this mean value being 0·4 μl. (*i.e.*, 0·07 μg. of nitrogen).

Determination of the Ratio of the Concentrations of the Hypochlorite and Thiosulphate Solutions.—0·04N-Sodium hypochlorite (40 μl.) was run into a titration-type tube containing distilled water (1 ml.) and a T-piece stirrer inserted. The stirrer motor was switched on, freshly prepared 30% potassium iodide (1 drop) added, the solution acidified with 4N-sulphuric acid (1 drop), and the liberated iodine titrated with 0·04N-sodium thiosulphate, Thyodene being used as indicator in the manner described above.

Hence:

$$f = \frac{\text{Concn. of NaOCl solution}}{\text{Concn. of } Na_2S_2O_3 \text{ solution}} = \frac{40}{V}$$

where V = volume (μl.) of 0·04N-thiosulphate required to titrate the liberated iodine.

A series of 5 titrations was always carried out, and the average sodium thiosulphate titre taken for V.

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