METHODS

AN ULTRAMICROMETHOD OF DETERMINING THE TOTAL NITROGEN

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The necessity of biochemical investigation of small quantities of biological material calls for the use of micro- and ultramicromethods, one of which is an ultramicromethod of determination of the total nitrogen.

Methods of determination of the total nitrogen in quantities of the order of micrograms which have so far been described [3, 4, 5, 7, 10] are complicated, require special equipment and are not always reproducible. We have devised a method which enables the total nitrogen to be determined in quantities of 0.5 to 10 μg in one histological section of tissue, based on the principle put forward by Hawes and Skavinski [6].

Reagents and Apparatus. An incineration mixture [5] is prepared by heating concentrated sulfuric acid, containing a 10% suspension of metallic Se, at a temperature of 290° until complete decolorization and translucency is obtained.

The following reagents are also required: a 25% solution of NaOH; a 0.1 M solution of KH_2PO_4; a 0.01 N solution of HCl; a 0.1% solution of bromcresol green in 20% ethyl alcohol (reagent A); a 0.05 m buffer of borax-succinic acid at pH = 4.5 (reagent B); a mixture of reagents A and B in a ratio of 1:9 and a mixture of water and reagent A in a ratio of 9:1.

All solutions are made up in double-distilled water; the KH_2PO_4, borax and succinic acid are twice recrystallized and made up to constant weight.

In order to measure accurately small volumes of solutions, we used a semiautomatic pipette of the Levy [8] type, calibrated gravimetrically. Otherwise we used ordinary 0.1 and 0.2 ml micropipettes.

Mineralization and subsequent diffusion were carried out in the same vessel, made from pyrex glass (Fig. 1). During diffusion and vessel was covered with a ground glass stopper.

Drops of the solution which absorbs the diffusing ammonia are held in a spiral coil, 1.5 mm in diameter, made of platinum wire, 0.2 mm in diameter. The distance between the turns and the length of the spiral are so adjusted that the volume of the drop is approximately 10 μl. The spiral is fixed to a capillary tube by means of paraffin wax.

When not in use, the platinum spirals are kept in double-distilled water, and immediately before the experiment they are cleaned by being dipped three times in boiling double-distilled water, and then flamed.

Mineralization was carried out on a sand bath, 1 cm wide, 30 cm long and 2 cm deep, heated by means of a nichrome coil buried in the sand. The heat generated by the coil was regulated by a rheostat.

In order to fix the vessels on the sand bath, clips are used, made of wire bent in the shape of round rings and attached to the sand bath, parallel to it and at a height of 1.5 cm from the surface of the sand.

Titration of the ammonia by the direct method [9] was carried out with the aid of an ultramicroburette (UMB) (Fig. 2).

From the number of ultramicroburettes described in the literature [2, 7] we selected the capillary UMB constructed by the method suggested by Korenman and Gronsberg [1, 2].

Solutions are drawn into and expelled from such a UMB by means of a pneumatic regulator. Estimation of the volume expelled is done by means of a scale, graduated in 0.5 mm. The UMB is calibrated gravimetrically. The total volume of the UMB is 17.8 μl, and one scale division corresponds to 0.024 μl. All the parts of the UMB are fixed to a panel of organic glass.

The solution to be titrated is poured into a glass dish with a diameter of 9 mm and a depth of 12 mm. By means of a glass rod, fused to it, mounted in a rubber stopper, the dish is fixed to a pedestal covered with opaque glass. The pedestal is supplied with a vertical screw transmission and a system of hinges which permit its movement in the horizontal and vertical directions.

Mixing of the fluid during titration is achieved by an electromagnetic mixer, consisting of a thin glass rod and a nonsparking electric bell AZ-127, from which the lid is removed as a preliminary measure. The glass rod is joined at one end to the handle of the striker of the bell, and the other end is dipped into the fluid to be titrated to the level of the tip of the capillary UMB.

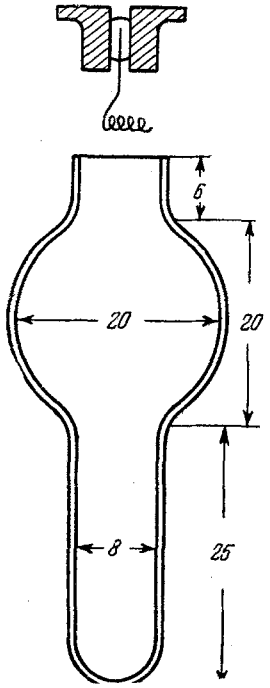

Fig. 1. Vessel for incineration of the tissue and diffusion (measurements given in mm).

The UMB, titration pedestal and electromagnetic mixer are mounted on a metal laboratory stand.

Procedure. A histological section of fresh tissue, 20-30 μ thick and 1-2 mm^2 in area, obtained with a freezing microtome, is placed at the bottom of the vessel illustrated in Fig. 1, and to it is added 18-20 ml of the incineration mixture from an ordinary 0.1 ml micropipette with a drawn out capillary delivery tube.

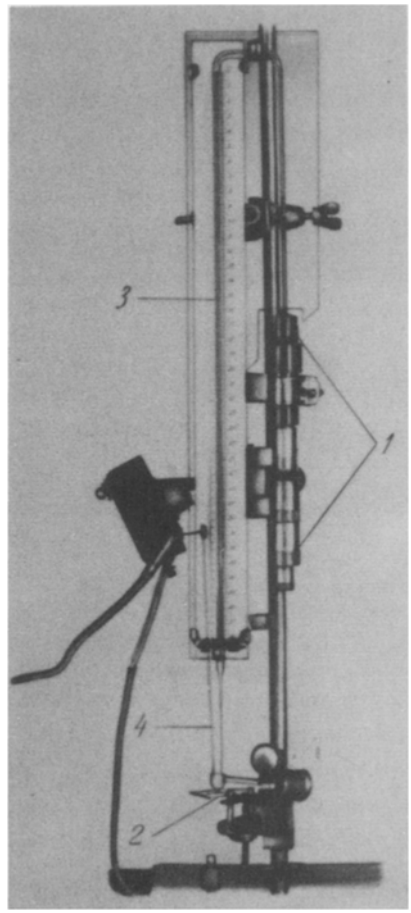

Fig. 2. Apparatus for titration. 1) Pneumatic regulator; 2) pedestal for titration with the dish fixed to it; 3) capillary tube of the ultramicroburette; 4) rod of the mixer.

The vessel is transferred to one of the wire rings so that its base is lightly buried in the sand, and inclined at an angle of about 60°.

Mineralization is continued for 4-5 hours at 255-260°, after which the vessel is allowed to cool. This time is used for preparation of the spiral coil.

The stopper of the tube is grasped with forceps and passed a few times over the flame of an alcohol lamp, in which it is heated until the paraffin wax in the capillary tube begins to melt. With another pair of forceps the platinum wire is carefully inserted into the capillary tube, the paraffin wax is allowed to coll, and the ground surface of the stopper is then smeared with a thin layer of vaseline. The stopper is turned with the spiral uppermost and left for subsequent manipulation.

After the spiral has been prepared, 0.2 ml of water is added to the cooled vessel and mixed, and the vessel is placed at an angle of 15-20° so that all the contents of the vessel are transferred to its narrow part.

Determination of the Total Nitrogen in a Standard (0.4 mg/ml) Urea Solution

Sample number	Total nitrogen content of sample (in μg)	Value determined		Error of the method (in %)
		(in μg)	(in %)	
1	1.287	1.368	106.2	+6.2
2	1.287	1.306	101.4	+1.4
3	1.287	1.336	103.8	+3.8
4	1.287	1.297	100.7	+0.7
5	1.287	1.270	98.6	−1.4
6	1.287	1.293	100.5	+0.5
7	1.287	1.269	98.6	−1.4
8	1.287	1.234	95.8	−4.2
9	1.287	1.289	100.1	+0.1

Into the spherical part of the vessel is carefully introduced 0.2 ml of 25% NaOH, the previously prepared stopper is taken and the attached spiral is immersed in a 0.1 M solution of KH_2PO_4 which, according to Hawes and Skavinski [6], possesses a higher absorbtive power than the boric acid usually used for this purpose [4, 9]. Since the absorbed ammonia is then estimated by direct titration [9], there is no necessity to measure accurately the volume of the drop, and it is sufficient to immerse the spiral once in the absorbent solution.

After withdrawl of the spiral from the solution, the vessel is quickly covered with the stopper, the fluid contained in it is thoroughly mixed by agitation, and the vessel is left in the horizontal position for 16 hours to allow diffusion and absorption of the ammonia.

After 16 hours the stopper with the spiral is removed from the vessel the spiral is carefully separated from the stopper with forceps, and placed in one piece in the titration dish, in which is 150 μl of a mixture of water and indicator solution.

Titration is performed from an ultramicroburette with a 0.01 N solution of HCl to pH = 4.5, with constant agitation of the titrated solution with the electromagnetic mixer, and with observer control (150 μl of a mixture of 0.05 M buffer of borax and succinic acid and indicator solution, in proportion of 9:1). The color change of the indicator was observed from above, against the opaque glass of the pedestal.

Each experiment must consist of a minimum of three parallel experimental and three control tests.

In order to test the completeness of mineralization, subsequent diffusion and absorption of ammonia, the total nitrogen content of a standard urea solution (0.4 mg/ml) was determined. In each sample 6.9 μl of urea solution was taken, which was equivalent to 2.76 μg urea or 1.287 μg nitrogen. In the control samples and urea solution was replaced by an equal volume of water.

Analysis of the samples of standard solution (see table) gave perfectly satisfactory results.

SUMMARY

The author describes the ultramicromethod in total nitrogen determination which makes it possible to analyze histological tissue sections (20 to 30 μ thick and with the area of 1-2 mm^2) to within the accuracy limits of ± 5-6% with the total nitrogen content in them comprising 0.5-10 ± 5-6%.

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