XVII. AN ULTRAMICRO-KJELDAHL TECHNIQUE

By JOSEPH NEEDHAM and E. J. BOELL

From the Biochemical Laboratory, Cambridge

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The problem of making Kjeldahl determinations of total nitrogen in amounts of the order of 1 μg. is not an easy one, and has not yet been satisfactorily solved. The present paper records a contribution to this end which has proved extremely useful in work described elsewhere on the metabolism of isolated regions of the amphibian gastrula [Needham *et al.* 1939; Boell & Needham, 1939; Boell *et al.* 1939]. As an indication of the delicacy of technique required, it may be recalled that the Pregl methods need amounts of from 100 to 300 μg. N [Pregl, 1937]. The amounts of total N which were to be estimated in the isolates from gastrulae varied from 1 to 20 μg.

Three previous papers have been devoted to this subject. Kirk [1934] worked out a method for Kjeldahl determinations on amounts of protein-N ranging from 3 to 15 μg. His method involved a rather undesirable transfer from the incineration vessel to a complicated steam distillation apparatus. He titrated some 300 μl. of distillate with 0·05 *N* NaOH. Levy [1936] abandoned transference and steam distillation; after dilution and neutralization of the digest, he estimated the nitrogen colorimetrically with the aid of Nessler solution and a Pulfrich photometer. Colorimetric estimations permitted him to measure amounts down to 0·4 μg., but most investigators would prefer to use a titration method, if possible. Lastly, Bentley & Kirk [1936] also abandoned steam distillation and had recourse to the diffusion principle, originally perfected by Conway & Byrne [1933] for ammonia determinations. They too, however, transferred the distillate from incineration vessel to diffusion vessel.

The essence of the problem may be said to lie in combining in one technique the Kjeldahl digestion, which logically demands a long narrow tube, and the Conway diffusion, which logically demands a maximal liquid-air interface, and hence a shallow vessel. At the same time it is desirable to neutralize the digest in a closed system so that none of the NH_3 first coming off shall escape.

The principle of our method lies in turning the Kjeldahl digestion vessel on its side after neutralization, and diffusing off the NH_3 from the resulting relatively large liquid-air interface into a hanging film of acid in a tube fitted into the mouth of the Kjeldahl vessel.

The vessel used by us[1] is shown in Fig. 1 (*a*). It consists of a digestion vessel with two bulbs blown in it (pyrex glass), some 7·5 cm. long. At the top there is an opening ground to receive a small tube of about the same dimensions as those used by Linderstrøm-Lang & Holter [1933] in their NH_3 estimations. Like their tubes also, it is coated on the inside with high melting-point paraffin wax. We refer to it as the receptor tube.

The digestion of the tissue proceeds as follows. The source of nitrogen is placed or pipetted into the bottom of the Kjeldahl vessel and 50–60 μl. of the digestion mixture added. The digestion mixture used by us was made up by

[1] Vessels of this kind can be obtained from Messrs C. Dixon, London, W.C.

adding 3 g. pure $CuSO_4$, 1 g. pure K_2SO_4 and 0·1 g. SeO_2 to 300 ml. pure conc. H_2SO_4. The tubes are then placed in an air oven at about 120° for *ca.* 3 hr. to drive off traces of water. At the end of that time a good deal of charring will be visible, and the digestion proper is carried out over pin flames constructed from old hypodermic needles (Fig. 1 (*b*)). Two glass beads are usually added, and we employ, as suggested by Levy, a mobile flame which the operator uses to heat the upper bulb when necessary. With the amounts of nitrogen used by us, digestion is complete in 3–5 min. If the tubes have been well heated in the air oven beforehand, and if they are constantly agitated during the heating by the use of the stem of the mobile flame, there is little danger of loss by sputtering, although as always where incinerations are concerned, care and practice cannot be dispensed with. When the liquid has become straw-coloured, the heating is continued for ½–1 hr. over a pin flame about 2 mm. high.

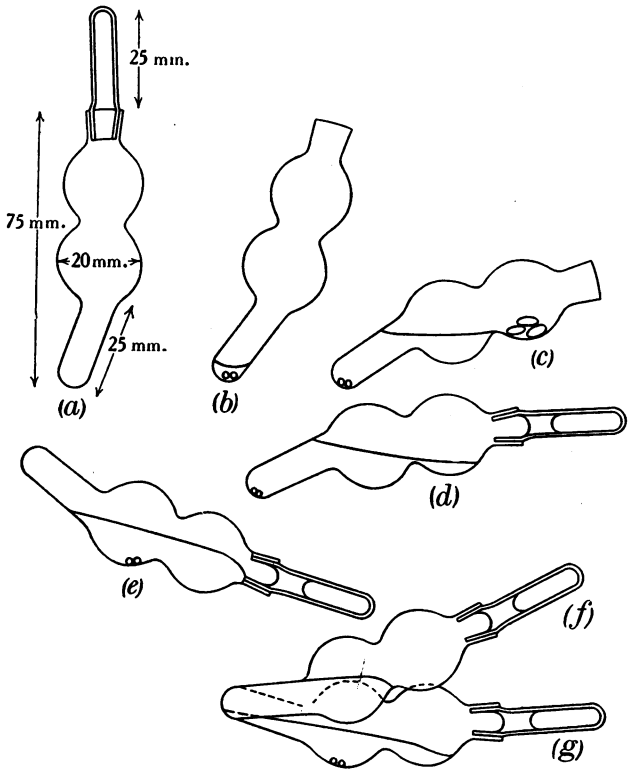

Fig. 1

The vessels are now removed from their holders and the digest diluted by the addition of 0·7 ml. NH_3-free distilled water. This is done with a pipette ending at an angle of 45° so as to facilitate the access of the water to all the interior surface of the vessel. The vessel is now tilted as shown in Fig. 1 (*c*) and three pellets of NaOH (corresponding to 300 mg. in all and giving 22 % NaOH) are placed in the upper bulb.

Meanwhile the receptor tubes have been prepared. 70 μl. of distilled water are placed as a hanging film at the top of the waxed interior and 7 μl. of 0·294 *N* HCl

delivered into the film from a constriction pipette [*vide* Linderstrøm-Lang & Holter, 1931]. The acid contains bromocresol purple in double the strength used in the Linderstrøm-Lang NH_3 method. It is important to put enough water in the receptor tube, as owing to the osmotic pressure difference, the film loses water as it gains in ammonia, eventually falling to perhaps 30–40 μl. The receptor tube is well sealed into the Kjeldahl vessel with a grease composed of one part high melting-point paraffin wax and two parts white vaseline. The NaOH and the diluted digest are carefully mixed, care being taken to see that the heat evolved does not blow out the receptor tube, see Fig. 1 (*d*). The whole is then cooled in water.

Now comes the most novel and delicate part of the technique. If traces of strong acid are left between the alkaline digest and the film of acid in the receptor tube, gross errors will result, for the ammonia will never reach its proper destination. The vessel is therefore held as in Fig. 1 (*e*) and rotated so that the alkaline digest runs right round the opening of the receptor tube. The wax coating of the interior of the receptor tube, and the rim of wax grease around the ground joint alike prevent the entry of the slightest trace of strongly alkaline digest into the receptor tube. It is essential to cool the vessel well before carrying out this part of the technique, for otherwise the wax grease and the wax coating may melt, with unfortunate results.

It is now simply a question of time before the acid in the receptor tube is ready for titration. We place the vessels, however, in a horizontal position in a rocker at 37° overnight and titrate the following day. The rocker was easily made from an electric gramophone motor connected to an eccentric so as to give the motion seen in Fig. 1 (*f*, *g*). At each slow rise and fall the alkaline digest spills into the upper bulb and then falls back again into the bottom of the vessel.

At the end of the period of diffusion desired, the receptor tubes are removed, the ends carefully wiped, and the films titrated against 0·102 *N* NaOH from a Rehberg-Heatley micro-burette of 50 μl. capacity [*v.* Heatley, 1939]. Agitation of the film is accomplished magnetically as described by Linderstrøm-Lang & Holter [1931].

Using a solution of NH_4Cl we first determined the time taken under our conditions for 100 % of the NH_3 to diffuse at 37° from the alkaline digest into the acid film in the receptor tube. As is shown by Fig. 2, 95·2 % recovery was reached

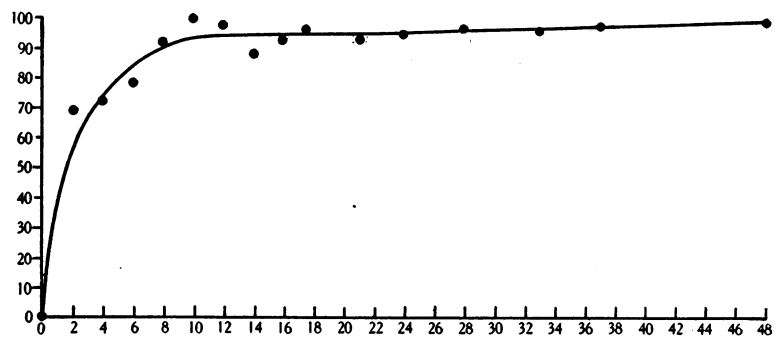

Fig. 2. Average recovery after 10 hours' distillation = 95·2 %.

in 10 hr. so that our usual practice of letting the rocking proceed for 18 hr. appears to be well on the safe side. The fact that the time required is a good deal longer than that in the NH_3 methods of Conway & Byrne [1933] and Conway

[1935] must certainly be due to the fact that the Conway vessel has a large donator surface and a large receptor surface; under our conditions only a large donator surface is possible.

Table I shows the percentage recoveries from solutions of casein (Hammarsten).

Table I

Exp.	Casein (μg.)	Recovery (μg.)	Percentage
II	0·58	0·525	90
	1·17	1·11	95
III	1·13	1·19	105
	1·13	1·07	95
	1·14	1·12	98
I	2·06	2·36	115
	3·09	3·09	100
	3·09	3·11	101
	4·12	4·11	100
	6·57	5·83	89
	6·57	6·00	91
	6·57	6·56	100
	6·57	5·84	89
	6·57	5·85	89
	6·57	6·52	99
	6·57	6·25	95
	6·57	6·38	97
	6·57	6·62	101
	6·57	6·40	97
		Av.	97·5

We see no reason why this method should not be applied to the estimation of quantities of total N much less than 1 μg., but we believe that many further precautions would be necessary, especially as regards the purity of the reagents and the contamination of the atmosphere, as by smoking etc.

Summary

An ultramicro-Kjeldahl technique, suitable for amounts of total N from 1 to 20 μg. is described. It includes a new type of Kjeldahl vessel, which, when turned on its side, acts as a Conway dish with a relatively large donator surface. The NH_3 diffuses into an acid film hanging in a modified Linderstrøm-Lang wax-coated receptor tube, which blocks up the mouth of the Kjeldahl vessel. Tests of the method, with a working description, are given in the text.

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