A MODIFICATION OF ARNOLD-GUNNING'S PROCEDURE FOR THE DETERMINATION OF NITROGEN IN THE BIOLOGICAL FLUIDS

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Inasmuch as the conventional Kjeldahl methods which utilize copper salts or hydrogen peroxide as catalyst for digestion fail to convert such nitogenous substances as pyridine, proline and choline into the ammonium salts, more drastic oxydative procedure has been desired in our laboratory for the purpose of accurate determination of total and non-protein nitrogen of blood. Koch and Hanke (1948)[1] recommended a digestion at higher temperature employing mercury salts as catalyst, which was devised by Arnold and Gunning and enables the recovery of 98-105 per cent of nitrogen even for the substances which are extremely resistant to oxidation. The procedure to be presented in this paper is its modification which is transformed to suit the condition of our laboratory which is not equipped with draught-chamber. The digestion is run in ordinary test tubes placed on a digestion rack and the digest is directly nesslerized for colorimetry. This has been in routine use for two years in our laboratory with excellent efficiency.

METHOD

Reagents

(1) Digestion reagent: Dissolve 0.8 gm. of secondary mercury oxide HgO and 40 gm. of anhydrous potassium sulfate K_2SO_4 in about 40 ml. of distilled water contained in a beaker which has a marking at 250 ml. (these salts do not dissolve perfectly), and warm it to complete transparancy after adding 43.5 ml. of concentrated sulfuric acid (spec. gr. 1.84) drop by drop with constant agitation by means of glass rod. To this add at once approximately 200 ml. of distilled water and dilute to the volume of 250 ml. when it has cooled to room temperature.

(2) 10 mg./dl. N solution: Weigh accurately 0.4716 gm. of pure ammonium sulfate $(NH_4)_2SO_4$ crystals which has been placed overnight in a thermostat chamber regulated at 100°C, dissolve it in distilled water in a 1000 ml. volumetric flask and make to volume with distilled water after adding a few drops of concentrated sulfuric acid.

(3) Nessler's reagent: Prepare mercuric iodide solution according to Koch and McMeekin,[2] preserve in a brown bottle, and mix one volume of it to five volumes of 10 gm./dl. aqueous sodium hydroxide solution immediately before use.

(4) 10 gm./dl. aqueous trichloroacetic acid solution.

Digestion rack. An iron-wire rack with two grid planes a and b which are illustrated in Figure 1 is used as digestion rack. It allows nine digestion tubes (ordinary test tubes of the size 16 × 190 mm. and 1.5 mm. in the thickness of wall) to be inserted for oxidation simultaneously.

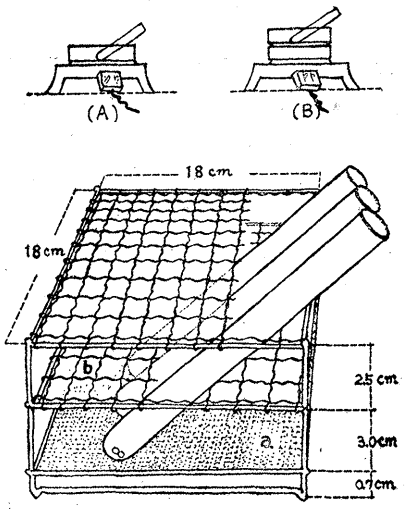

Fig. 1. Digestion rack

procedure

(1) Preparation of the materials. Dilute 0.10 ml. of serum to 20.0 ml. with distilled water (200 fold dilution); or to 49.0 ml. of distilled water add an aliquot of 1.0 ml. of the mixture composed of 1.0 ml. of urine and 4.0 ml. of distilled water (250 fold dilution). For the determination of non-protein nitrogen (NPN) add 1.2 ml. of 10 g./dl. trichloroacetic acid to 0.3 ml. of serum in a test tube drop by drop by shaking; allow to stand for more than three minutes; add 1.5 ml. of distilled water and filter with Tôyô filter paper No. 7 to get a clear protein-free fluid (tenfold dilution of the original serum) as soon as the mixture commences to separate into the supernatant and precipitate.

(2) Into the digestion tubes A, B, C and D introduce the following materials and glass beads (diameter: 1mm.), two for each, in the order given, left to right, mixing by lateral shaking after each addition.

	fluid prepared in (1)	10 mg./dl. N solution	digestion reagent	10 gm./dl. trichloroacetic acid*
A	1.0 ml.		0.7 ml.	
B		0.2 ml.	0.7 ml.	0.4 ml.*
C		0.4 ml.	0.7 ml.	0.4 ml.*
D		0.7 ml.	0.7 ml.	0.4 ml.*

* Trichloroacetic acid is not introduced when total N is determined, but added in case of NPN determination to eliminate the blank N content of trichloroacetic acid solution.

Insert the tubes in the digestion rack so that their bottom may touch on the plane a.

(3) Oxidation. Place the digestion rack upon an electric range (1 KW per hour) as shown in the Figure 1 (A), and holding an edge of the rack with a wooden forceps shake it incessantly to prevent the sudden outflow of the contents of tubes by ebullition. The contents will begin to boil within a few minutes, grows brown in color and is concentrated to form a caramel-like substance at the bottom of the tubes in about ten minutes. Rotate several times the test tubes in their position with wooden forceps or gloved fingers so that their bottoms may change the parts which are directly heated over the fire. The brown substances which adhere to the lower inner sutface of the tubes are washed down and oxidized completely by the flow of hot sulfuric acid condensed near the opening of the tubes. When the contents of the digestion tube A has turned colorless and transparent, remove all the tubes from the grid plane a and place them on the grid plane b or insert below the rack another one to leave a space between the bottom of the tubes and the fire (as in Figure 1 (B)) for the purpose of mild heating. Additional heating in this way, rotating the tubes to decolorize the brown substances, if any reappear, is required for the digestion of serum, while five minutes suffices for that of urine and NPN. Place again the tubes directly over the fire (on the grid plane a or in the position of Figure 1 (A)), heat them until they are filled with white fumes, switch off the electric range and allow the tubes to cool for three or four minutes.

(4) Nesslerization. To each of the digestion tubes introduce 5.0 ml. of distilled water while they are warm, plug their opening, invert several times to get a clear solution. Transfer 1.0 ml. aliquots of the resultant solutions into the test tubes a, b, c and d which corresponds to the digestion tubes A, B, C and D respectively, add 3.0 ml. of distilled water and 1.5 ml. of Nessler's solution to each tube, shake, and allow to stand for five to twenty mintes depending on the room temperature to produce a perfect yellowish color which is suitable for colorimetry.[3)]

(5) Colorimetry. Read them in the photoelectric coiorimeter, set to zero with distilled water and using a filter that has a maximum transmission at 470 mμ. To construct a calibration curve, plot N concentration as abscissa, assuming that

those of the tubes B(b), C(c) and D(d) are 20, 40 and 70 mg./dl. N respectively, against optical density as the ordinate on rectangular co-ordinate graph paper.[4] From this curve get s mg./dl. for the optical density of A(a). Then, NPN, total N for serum and N for urine are given as s mg., 20s mg. and 25s mg. per deciliter, depending upon the sample that has been treated in the tube A is the deproteinized serum filtrate, the whole serum or the urine, respectively.

Results and Discussion

To appraise the accuracy of our procedure, a comparison of the values obtained in duplicate by the hands of a technical assistant of our laboratory who was under exercise of this method is presented in Table I. This reveals that the discrepancy usually resides within two per cent, seldom exceeding five per cent of the smaller N estimation. Discordance over five per cent is, therefore, attributed

Tabele I.

Duplicate N determination

Protein N of serums (mg./dl.)	1131	1131
(total N–NPN)	1100	1110
	1280	1281
	1320	1305
	955	963
NPN of serum (mg./dl.)	34.6	34.0
	20.7	20.5
Total N of urine (mg./dl.)	1040	1038
	1047	1063
	1860	1875

to technical failure. When N values of highest accuracy are desired, averaging the two estimates which are ascertained to be in good accordance within the range of three per cent is recommended. Duplicate determination for a sample does not burden the operator, because the procedure is simple as described above.

Hitachi's protein meter (P.R.P-A type) is a kind of refractometer that allows the reading of serum protein concentration in terms of gm./dl. Inasmuch as an intimate correlationship between the refractive index and the concentration of protein solution is doubted by some workers,[5] the reliability of this instrument was checked by our N estimations with respect to the blood serums whose protein content varies over a wide range of concentration. The results are listed in Table II in which a fairly good agreement between these two methods assures the trustworthiness of the instrument. Incidentally, the protein concentration was computed from the N-content of serum (mg./dl.) by the equation

$$\text{protein concentration (gm/dl.)} = 0.00625 \times (\text{total N—NPN}) \text{ (mg./dl.)}$$

Although proline and choline which resists the digestion were not available for recovery test, it was ascertained in our hands that the nitrogen added in the

form of ammonium sulfate was recovered in 98-103 per cent.

TABELE II.

Protein-meter reading as compared with the values obtained by our procedure

Protein-meter (gm./dl.)	N estimation (gm./dl.)	Protein-meter (gm./dl.)	N estimation (gm./dl.)
4.1	3.81	7.2	7.39
4.3	4.29	7.2	7.44
5.9	5.40	7.3	7.33
6.0	6.01	7.5	7.56
6.7	6.79	7.5	7.60
7.0	6.96	8.0	8.01
7.0	7.09	8.1	8.09
7.1	7.13	8.1	8.26
7.2	7.20	5.8	8.66
7.2	7.21	9.8	9.23

SUMMARY

A modified Arnold-Gunning's procedure for the determination of nitrogen in biological fluids was presented. It enables the whole operation to be carried out in the ordinary laboratory room without draught equipment. The digestion of the materials to be assayed is run in the test tubes inserted in a simple metal-wire digestion rack, and the digest is directly colorized with Nessler's reagent for photoelectric colorimetry, thus obviating the tedious process of distillation. The reliability of Hitachi's protein meter (P.R.P-A type) as an instrument to measure the serum protein concentration was assessed and approved by this method of nitrogen determination.

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