

METHODS FOR THE DETERMINATION OF SUBMICRO QUANTITIES OF TOTAL NITROGEN, AMMONIA, AMINO NITROGEN, AMIDES, PEPTIDES, ADENYLIC ACID, AND NITRATES

BY HENRY BORSOOK AND JACOB W. DUBNOFF

*(From the William G. Kerckhoff Laboratories of the Biological Sciences,
California Institute of Technology, Pasadena)*

(Received for publication, August 9, 1939)

In 1935 one of us described micromethods for ammonia, urea, total nitrogen, uric acid, creatinine, and allantoin (1). These methods are adapted for the analysis of 1 to 2 ml. aliquots of dilute solutions, *e.g.* 0.1 mg. per cent of ammonia or urea, and for carrying out a large number of analyses simultaneously. These methods all end in a colorimetric measurement.

In the present communication methods are described for the determination of submicro quantities of nitrogen in the form of ammonia, amino groups, amides, urea, total nitrogen (and by difference, protein nitrogen), peptides, adenylic acid, and nitrate. The aliquots used are 0.1 to 0.2 ml.; the minimum dilution may be somewhat lower than in the methods we described previously.

Three innovations are introduced in these methods. One is a convenient glass electrode submicrotitration procedure. The problem here was to devise an electrical system which is rugged, quick recording, easy to operate, and from which electrical leaks are excluded. The Conway-Byrne ammonia distillation procedure (2) has been made more rapid. A Kjeldahl procedure has been devised which is simpler and more convenient than any of the preexisting methods for determining total nitrogen in submicro quantities. The same principle is also used in the determination of amides.

In all of these new methods the final procedure is an electro-metric titration with a glass electrode. This is more accurate, of wider application, quicker, and more convenient than the colori-

metric procedure employed formerly. The new methods are also better suited to the carrying out of a large number of analyses simultaneously.

These methods constitute a "system" of analysis of the common nitrogen compounds in animal and plant fluids. For any individual determination 0.1 ml. containing 0.0003 mg. of N suffices. For the fractionation of a mixture of compounds into total, protein, amino, ammonia, amide, peptide, adenylic acid, and nitrate nitrogen, about 2 ml. containing about 0.5 mg. of N will permit carrying out all the determinations in duplicate. The ordinary disagreement between duplicates and the absolute error of all except the Kjeldahl determinations are under 1 per cent. The difference between duplicates in the Kjeldahl determinations is about ± 2.0 per cent. The recovery of nitrogen in this Kjeldahl method is the same as in the macro-Kjeldahl procedure.

An estimate of the convenience and speed of these methods may be obtained from the fact that two of us have determined in duplicate all of the above fractions in six different solutions in 2 normal working days. All of the necessary equipment can be placed on one laboratory bench 8 feet long.

These methods have been used by different workers in this laboratory during the last 2 years in the study of the nitrogen metabolism of plant seedlings, isolated growing pea embryos, marine invertebrate embryos, and thin slices of mammalian tissues.

Micropipettes

The pipettes used are those described by Levy (3). We have found it convenient to construct a battery of different sizes from 0.1 to 0.3 ml. as shown in Fig. 1. The fluid to be measured is drawn by gentle suction a few mm. above the upper constriction, after which, with the point of the pipette still dipping into the solution below, the liquid in the pipette is allowed to fall by its own weight. Under these conditions it drops always to the same point in the upper constriction. The solution into which the point is dipping is withdrawn, and then the contents of the pipette are delivered into the desired container by nitrogen (or air) under a constant head of pressure. Fig. 1 shows the arrangement of the stop-cocks which permits the use of each pipette in the battery separately.

The pipettes are calibrated either by the weights of water or by titrating the volumes of a strong standardized acid solution which they deliver. The uniformity of the volumes delivered is the same as with ordinary macropipettes.

Ammonia

Vessels—The ammonia is distilled by the method of Conway and Byrne. The distillation vessels we now use are shown in

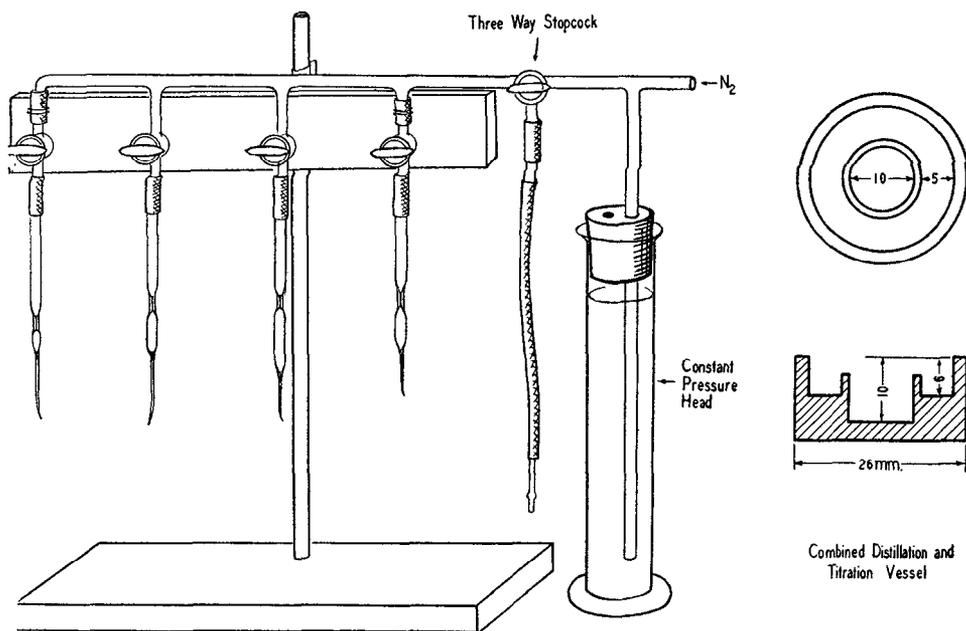


FIG. 1. Battery of pipettes and distillation vessel

Fig. 1. The time required for the ammonia to distil completely from the alkaline solution in the outer well to the standard acid in the inner well depends greatly on the dimensions of the distillation vessel. The dimensions shown in Fig. 1 are the best we have found for the volumes of solution employed.

The vessels we used until recently were made from Pyrex glass tubing. We now are using vessels cut on a lathe from clear lucite rod.¹ The lucite vessels offer the following advantages over

¹ Manufactured by E. I. du Pont de Nemours and Company, Inc.

glass, they are more quickly and easily made, unbreakable, more uniform, and the upper edge of the outer wall does not chip and is cut perfectly plane by the lathe.

Reagents—

A stock borate solution is prepared containing 12.4 gm. of H_3BO_3 and 100 ml. of *N* NaOH per liter, and brought to pH 10.3 with concentrated NaOH. Each day as required the final alkalizing solution is prepared by mixing 2 parts of this solution with 1 part of 95 per cent ethanol. The alcohol is used to promote the spreading of the solution in the distillation vessel.

Commercial glycerol with enough strong NaOH just to turn phenolphthalein pink. This alkaline glycerol is used for sealing the vessels. It is as efficient for this purpose as any vaseline, rubber, or grease mixture we have used. It is completely washed away by cold water and thus the main difficulty of washing the distillation vessels is avoided.

Standard H_2SO_4 solutions whose strength is varied according to the amount of ammonia to be distilled.

Standard NaOH solution between 0.02 and 0.04 *N* containing 1 per cent KCl to prevent the retraction of the mercury from the wall of the capillary of the burette.

*Procedure—*A quantity of standard acid is pipetted into the central well. This quantity must of course be more than sufficient to neutralize all the ammonia which distils over, and it must form a column in the central well so deep that the bulb of the glass electrode will be more than half immersed during the final titration. The strength of the standard acid need not be known precisely. It is "standardized" always by the blank titrations.

Either 0.1 or 0.2 ml. of the ammonia-containing solution is pipetted into the outer well. 0.1 ml. of the alcoholic borate solution is pipetted with a hand pipette into the opposite side of the outer well. If the solution containing the ammonia is too acid to be overneutralized by 0.1 ml. of the borate solution, it must be nearly neutralized beforehand.

The rim of the distillation vessel, *i.e.* the upper edge of the outer wall, is moistened with the alkaline glycerol by rubbing over it a glass stirring rod coated with the glycerol. The distillation vessel is now closed with a square piece of thin plate glass the covering surface of which is thinly smeared with glycerol. This lid is kept

firmly on the vessel by a rubber band stretched over the cover and under the bottom of the vessel. The two solutions in the outer well are now mixed by tilting back and forward. It is important that the alkaline mixture spread and cover the whole of the bottom of the outer well. Otherwise the distillation is considerably retarded.

The vessel is now placed on a rocking platform driven at about forty-five oscillations per minute, 15° up and down from the horizontal. We have found that in a distillation vessel of these dimensions all the ammonia in a 0.1 ml. aliquot distils over in 2 hours with rocking at room temperature; 80 to 85 per cent in 1 hour. In an air bath at 40° distillation is complete, without rocking, within $1\frac{1}{2}$ hours.

The only limits to the number of distillations which can be carried out simultaneously are the number of distillation vessels available and the size of the rocking table when rocking is employed.

At the end of the distillation the cover of the distillation vessel is removed and the acid in the central well titrated with standard NaOH. The difference between this titer and the titration value of the blanks which have been run simultaneously gives the amount of ammonia.

The vessels are cleaned by soaking in dilute HCl strong enough to neutralize all the borate, and then washing with copious amounts of distilled water. The lucite vessels are dried in air; lucite does not withstand heating to 100° . Glass vessels may be dried in the oven at 100° or higher. They are washed by the same procedure as the lucite vessels.

Electrometric Titration

Apparatus Assembly.—The electrometric titration assembly is shown in Fig. 2. The pH is measured on the Beckman pH meter. The cable (*f*) from the glass electrode to the pH meter is a shielded cable and grounded to the metal stand to which the titration assembly is clamped and to the metal shield inside the pH meter. The belt for the motor-driven stirrer is a coiled brass spring wire.

The details of the electrode assembly are shown in Fig. 3. The glass electrode (*A*) is made of Corning glass No. 015. The whole of the soft glass from just above the bulb to the top is coated

with a film of De Khotinsky cement. It is filled with 0.1 N HCl to which some finely ground quinhydrone is added. The upper electrode (*B*) containing mercury from which a fused-in platinum wire dips into the 0.1 N HCl in electrode *A* below is also

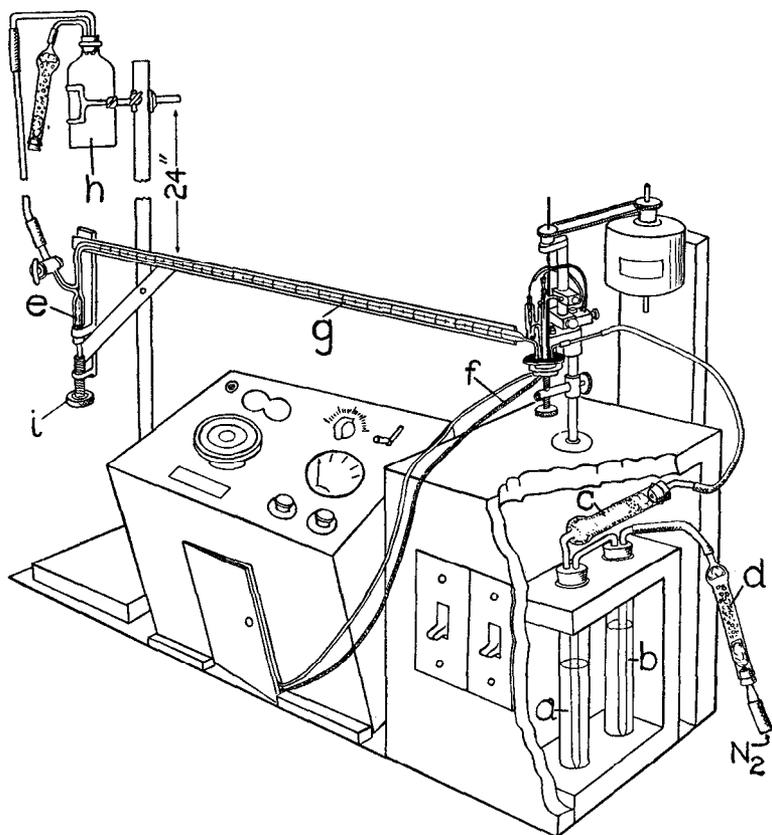


FIG. 2. Titration apparatus. *a*, water; *b*, NaOH; *c*, cotton; *d*, soda lime; *e*, mercury; *f*, shielded cable; *g*, microburette; *h*, standard NaOH; *i*, hand screw for propelling mercury, covered with rubber.

coated on the outside with a film of De Khotinsky cement. Electrode *B* may be of any glass. The films of De Khotinsky cement constitute electrical insulation and shielding. The two parts of the calomel electrode are sealed together above with De Khotinsky cement.

As shown in Fig. 3, the tip of the burette, the calomel and glass electrodes, the end of the stirrer, and the tip of the tube delivering the nitrogen pass through holes cut in a bakelite plate. It is essential that this plate be thoroughly dry during a titration.

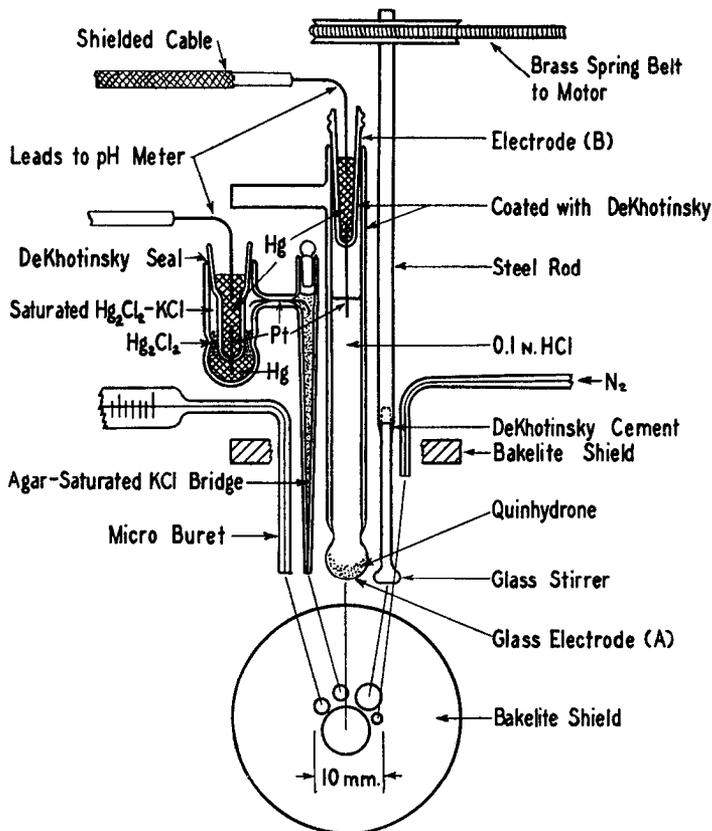


FIG. 3. Electrode assembly details of titration apparatus

The burette delivers about 100 c.mm. of alkali over a length of about 2 feet. It is drawn out to a point so that there is a negligible amount of back diffusion from the solution which is being titrated, and no alkali drops out except when it is pushed out by the mercury. The hand screw (*i*) which drives the mercury is covered with rubber. The bottle of standard alkali is clamped a suffi-

cient distance above the burette so that the hydrostatic pressure is sufficient to flush out the burette when the stop-cock connecting it with the burette is opened, and to balance the pressure of the short column of mercury. Otherwise the mercury creeps along the burette forcing out NaOH. The burette is filled by screwing the mercury back until the alkali in the burette is in contact with the alkali in the side tube. The stop-cock is then opened. After a drop of alkali has been forced off the point of the burette, the stop-cock is closed. The mercury is screwed up until it is near the zero mark on the burette. The tip of the burette is washed with water, and the point dried with filter paper. The mercury is then screwed to the zero mark, and the hanging drop of alkali taken up with filter paper.

Details of the construction and use of this type of burette are given by Linderstrøm-Lang and Holter (4).

Procedure—The central well containing the standard acid is placed on an adjustable stand as shown in Fig. 2. It is now screwed up until it touches the bakelite plate. The heights of the glass and calomel electrodes are previously adjusted so that their ends will dip into the acid but not touch the bottom when the top of the distillation vessel touches the bakelite plate. The bulb of the glass electrode is about half immersed. The tips of the burette and of the glass stirrer are similarly adjusted so that they also will dip into the solution. The tip of the tube which delivers the washing nitrogen does not dip into the solution; it is just below the bakelite plate. The nitrogen gas is run through only during a formol titration, which is described below.

The actual titration consists in screwing out alkali into the solution which is being titrated with continuous stirring until the pH is near to 4.0. Small amounts are then added, with stirring after each addition, up to pH 6.0 which is the end-point.

Formol Titration

Reagents—

Commercial formaldehyde diluted 1:1 with water and brought to pH 5.0 with strong NaOH. The solution is stored in a dark brown bottle.

Standard NaOH solution between 0.02 and 0.04 N containing 1 per cent KCl.

Nitrogen gas washed with alkali and water. It is necessary to maintain a positive pressure of CO₂-free nitrogen in the central well throughout the titration in order to exclude the CO₂ of the air. Without this precaution we have been unable to obtain satisfactory end-points.

Procedure—0.25 ml. is pipetted into the central well of a distillation vessel. After the vessel is mounted in place and screwed up to the bakelite plate, the nitrogen is streamed through the overlying space and continued throughout the titration.

The first stage of the titration consists in titrating to pH 7.0. The pH chosen for the end-point of the first stage is a compromise when the solution contains ammonia, a variety of organic acids, and amino acids. When one is titrating solutions of known amino acids, the end-point selected for the first stage will depend on the ionization constants of the compound.

At the end of the first stage 0.25 ml. of the formaldehyde solution is added to the central well. A hand micropipette is used for this purpose, similar to those shown in Fig. 1, with suction and pressure applied by mouth through rubber tubing. After the formaldehyde is added, the vessel is again screwed up to the bakelite plate and the titration resumed to an end-point of pH 8.0. This end-point also is a compromise, and may be modified in special cases. We have found by trial that ammonia is titrated by this procedure and gives a value 110 per cent of the theoretical. The amino nitrogen value is the titer in the second stage minus 110 per cent of the ammonia value which has been obtained independently by the method described above. A blank titration must be carried out with formaldehyde and water alone, and this blank value subtracted from the total titer.

Total Nitrogen

Apparatus—The digestion tubes are shown in Fig. 4. These are calibrated to a mark made around the constriction. Their volume is 0.45 to 0.50 ml.

The digestion rack is shown in Fig. 4. This may be made any desired length. The heating of the tubes all the way round and some distance above the melt prevents bumping. If the flames are not turned up too high (see below), there is no loss of nitrogen.

Reagents—

The digestion mixture consists of a 1:1 dilution of concentrated sulfuric acid with water containing 1 gm. of SeO_2 and 1 gm. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per 100 ml. of diluted acid. After many trials the use of phosphoric acid in the digestion mixture was abandoned because it etched the digestion tubes excessively.

A saturated solution of potassium persulfate. The salt used is Merck's Reagent variety "for Dr. D. D. Van Slyke's micro-Kjeldahl determination of nitrogen."

25 per cent NaOH.

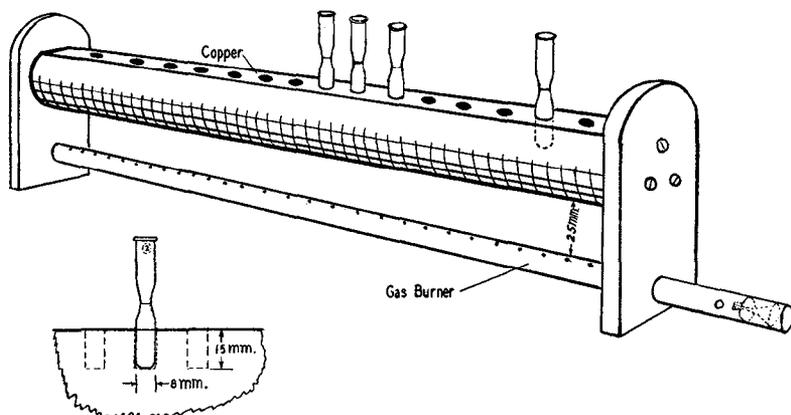


FIG. 4. Digestion rack and Kjeldahl tubes

Procedure—0.1 ml. or 0.2 ml. aliquots of the solution followed by 0.1 ml. of the digestion mixture are pipetted into a digestion tube. All the tubes so set up are placed in an air oven at about 105° until most of the water has evaporated off and the solution has become dark brown. Our practice is to leave the digestion tubes in the oven overnight. To prevent particles of dirt dropping in, the tubes are covered with a sheet of tin-foil.

The digestion tubes are then placed in the digestion rack. The flames are kept low at first until the acid begins to reflux. Then the flames are gradually raised but not at any time to such a height that the acid distils more than a few mm. above the constriction. The digestion is continued until the solution is colorless.

The tube is then cooled, 1 drop of the saturated persulfate solu-

tion is added, and the digestion continued for 15 minutes after the digestion mixture is water-clear.

This digestion may be carried out without a hood. There is practically no loss of acid and hence the digestion may be carried on for hours.

When the digestion is completed, the tubes are cooled and then water is added to the mark around the constriction. The water is added in two portions, with mixing by rotation and cooling between additions. After being made up to the mark the contents are mixed by vigorous shaking and inversion.

Duplicate or triplicate 0.1 ml. aliquots are pipetted into distillation vessels. Standard acid is pipetted into the central well. 0.1 ml. of 25 per cent NaOH is pipetted into the outer well some distance from the drop of solution containing the ammonia.

The subsequent procedure and titration are exactly the same as described above in the determination of ammonia. The total nitrogen is computed from the titer, the size of the aliquots taken for digestion and distillation, and the volume of the digestion tube used.

Blank determinations are carried through the same procedure simultaneously with the experimental determinations.

It is possible by this method to determine as little as 0.0003 mg. of N in an aliquot. The convenient amounts of nitrogen are between 0.01 and 0.005 mg. in each aliquot. In the course of a great many analyses the extreme difference between duplicates or triplicates has been approximately ± 2 per cent. When the difference occasionally was larger, the analysis was repeated. The error has been nearly always in the distillation. When we were using glass distillation vessels, this error was the result of microscopic leaks between the central and outer wells or of a film of acid creeping up the side of the vessel and remaining unneutralized. These mishaps have been eliminated by the use of lucite vessels.

Amide Nitrogen

Reagents—

3 N H_2SO_4 .

2.9 N NaOH.

Alcoholic borate solution used in the determination of ammonia above.

Procedure—A 0.2 ml. aliquot is pipetted into one of the Kjeldahl digestion tubes described above. 0.1 ml. of 3 N H_2SO_4 is added. The two solutions are mixed. The tube is covered with a cap of tin-foil and set away in a boiling water bath for 3 hours. A wire gauze rack was made to hold these tubes in the water bath.

After 3 hours digestion the tube is cooled; 0.1 ml. of 2.9 N NaOH is added and quickly mixed in. Water is then added to the mark.

The subsequent procedure is as described above for the determination of ammonia. The total ammonia present after acid hydrolysis is computed as in the determination of the total nitrogen, from the titer, the aliquots used for digestion and distillation, and the volume of the digestion tube used. The difference between this value and the preformed ammonia nitrogen is the amide nitrogen.

Nitrate Nitrogen

Reagents—

Finely powdered Devarda's alloy.

25 per cent NaOH.

Procedure—The nitrate determination is carried out on the solution which remains after the amide and ammonia nitrogen has been distilled off, as described above.

The acid in the central well of the distillation vessel at the end of the amide nitrogen determination is pipetted out, and replaced by another quantity of the standard acid.

4 mg. of finely powdered Devarda's alloy are now added to the outer well. The vessel is tilted a little and the metal powder placed on the side opposite to the fluid in the outer well. Then 0.1 ml. of 25 per cent NaOH is added. The strong alkali is required for the reduction of the NO_3 . From this point on the procedure is the same as in the determination of ammonia. At room temperature in 1 to 2 hours all of the nitrate is reduced and the resulting ammonia distilled over.

It is necessary to carry through two sets of blanks, one, in which the strong NaOH is added but no Devarda's alloy to control the effect of the alkali alone, and a second with Devarda's alloy added to the blanks of the amide determination, *i.e.* in which only reagents are present. The sum of both blank values must be subtracted from the titer of the experimental solution.

Peptide and Adenylic Acid Nitrogen

Peptide nitrogen is determined by enzymatic hydrolysis of the peptides present. A satisfactory enzyme preparation was found in that described by Orcutt and Wilson (5), which we have modified slightly in order to prepare a more concentrated preparation and to free it of ammonia.

Reagents—

The preparation is as follows: *Aspergillus wentii* is grown by seeding skim milk containing 5 per cent dextrose with spores and incubating 6 to 8 days at 30°. The mold is cultured in 1 liter Erlenmeyer flasks with enough milk to give a depth of about 2 cm. At the end of 6 to 8 days, if satisfactory growth has been obtained, the heavy pad of mold has begun to crack and pull away from the wall of the flask. The pads are removed at this time, weighed, and frozen for several hours to inactivate any arginase which may be present. The pads are then thoroughly ground in a mortar with sand and finally enough water is added to give a suspension which contains about 10 per cent dry matter. (The moisture content of the pad is determined on a small quantity.) The pH is now adjusted to 7 to 7.5. The suspension is covered with toluene and allowed to stand at room temperature for 4 to 6 hours. At the end of this time it is filtered through sail-cloth under a vacuum. The proteolytic enzymes are in the filtrate. To the filtrate an equal volume of acetone is added. This precipitate is dissolved in one-fourth the original volume of water and centrifuged free of the insoluble material. This solution is shaken with permutit to free it of ammonia and then constitutes the final enzyme solution. Kept under toluene in a refrigerator it retains its potency unimpaired for several months. The solution darkens on standing; but this does not impair its potency.

The activity of this solution has been tested against Witte's peptone. It gives in 12 hours at 38° nearly the same increase in free amino nitrogen as does hydrolysis with strong acid.

When Witte's peptone or plant extracts are incubated with this enzyme preparation, in addition to an increase in free amino nitrogen, there is an increase in ammonia. We have tested a large number of amino acids, purines, amides, urea, creatine, and creatinine, and have found that with the exceptions of arginine and histidine only the amino group in the 6 position of adenine is hy-

drolyzed, when this purine is in the form of adenylic or nucleic acid. Free adenine is not attacked (6). A small amount of ammonia is slowly liberated from arginine and histidine. Direct determination of the adenylic acid in plant extracts by the method of Kerr and Blish (7) accounted for only half the increase in ammonia. We have not identified the source of the remaining ammonia in plant extracts, apart from the possibility of its being arginine and histidine.

Procedure—An equal volume of the protein-free extract (trichloroacetic acid filtrate) brought to pH 6.0 and the enzyme solution are incubated overnight under toluene. The hydrolysis is nearly complete in 6 hours. It is convenient, however, to allow the digestion to continue overnight.

Formol titrations and ammonia estimations are then carried out as described above. The ammonia found multiplied by 1.1 is subtracted from the formol titration value at the second stage. The peptide amino nitrogen is the difference between this value minus the sum of the formol titer of the enzyme solution and the free amino nitrogen before the enzymatic hydrolysis.

This enzyme preparation can be used as a convenient micro-method for the determination of adenylic acid, if other sources of ammonia can be excluded or controlled by one direct determination of the adenylic acid in the material to be analyzed.

SUMMARY

Methods are described for the determination of submicro quantities of total nitrogen, and nitrogen as ammonia, amino groups, amides, peptides, adenylic acid, and nitrates.

These methods are adapted for carrying out conveniently large numbers of determinations simultaneously.

The end-stage of all the methods described is an electrometric titration.

BIBLIOGRAPHY

1. Borsook, H., *J. Biol. Chem.*, **110**, 481 (1935).
2. Conway, E. J., and Byrne, A., *Biochem. J.*, **27**, 319 (1933).
3. Levy, M., *Z. physiol. Chem.*, **240**, 33 (1936).
4. Linderstrøm-Lang, K., and Holter, H., *Compt.-rend. trav. Lab. Carlsberg*, **19**, 1 (1931).
5. Orcutt, F. S., and Wilson, P. W., *Plant Physiol.*, **11**, 713 (1936).
6. Borsook, H., and Dubnoff, J. W., *Enzymologia*, in press.
7. Kerr, S. E., and Blish, M. E., *J. Biol. Chem.*, **98**, 193 (1932).