Coulometric Determination of Protein Nitrogen
Application to Direct Titration of Kjeldahl Digests

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A coulometric method is described for the determination of ammonia resulting from micro-Kjeldahl digestion of protein samples. The method has been applied to the direct titration of the ammonia in the digests. Routine titration of the ammonia resulting from 10-μl serum samples is described; the method has been applied to 1-μl samples. Results are compared with standard macro- and micro-Kjeldahl procedures.

Coulometric titration methods offer several advantages over standard volumetric procedures (1). For example, no standard solutions are required and the only volumetric measurement that need be made is in the measuring of the sample. The authors have been investigating application of coulometric procedures to the determination of important biological substances (1-4).

Arcand and Swift (5) developed a coulometric method for the titration of ammonia with electrogenerated hypobromite. The authors recently described a direct amperometric end-point detection for the titration, which they applied to the determination of small amounts of ammonia (9). Automatic recording of the current and a pretitration procedure developed to eliminate reagent blank readings were described. A coulometric determination of urea nitrogen has been developed using this method based on titration of the ammonia resulting from urease hydrolysis (1).

Krivis et al. (6) also described a direct amperometric end-point detection, which they applied to the titration of sulfamic acid.

The micro-Kjeldahl method for determining protein nitrogen is recog-
nized as the standard on which other methods for protein determination are based. Since the ammonia formed is determined by an acid-base titration, it must be separated from the digest prior to determination. This is usually accomplished by distillation. Also, the method requires the preparation and storage of one or two different standard solutions with inherent volumetric errors and danger of atmospheric and other contamination.

These added manipulations combine to increase chance of technician error. A coulometric method would eliminate these. In addition, the method can be made very sensitive with little or no modification and without danger of large errors. Except for the coulometer, only standard laboratory equipment is required.

In the present investigation, a coulometric procedure was developed for the titration of ammonia formed from protein nitrogen following micro Kjeldahl digestion. A method is described for the direct titration of the ammonia in the digest. The method has been easily applied to the determination of protein nitrogen in as little as 1 μl of serum. Results are compared with standard macro- and micro-Kjeldahl procedures.

**Experimental**

Reagent-grade chemicals were used without further purification. The stock buffer solution used in coulometric titrations was prepared by dissolving 38.1 gm. of sodium tetraborate decahydrate in 1 L of water and adding 72% perchloric acid to adjust the pH to 8.6. The mercuric sulfate solution employed in digestion was prepared by dissolving 10 gm. of red mercuric oxide in a solution of 12 ml. concentrated sulfuric acid diluted to 100 ml. with water. A saturated solution of potassium hydroxide was prepared and then was boiled for 5 min. to remove ammonia.

The micro-Kjeldahl procedure employed was that described by Kabat and Mayer (7). The macro-Kjeldahl procedure was that given in *Standard Methods of Clinical Chemistry* (8).

All coulometric titrations were made with a Sargent coulometric current source, Model IV,* at a generating current of 4.825 or 9.650 ma. (0.05 or 0.1 μEq/sec., respectively). The generating anode and cathode were platinum foils of 2 sq. cm. and 0.8 sq. cm., respectively. The cathode was isolated from the test solution by placing it in a glass tube fitted with a sintered-glass frit end containing the stock buffer solution as catholyte. The indicating electrodes were two platinum foils (2 sq. cm.) with 150

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*E. H. Sargent & Co., Chicago, III.
my impressed between them. The impressed potential was supplied by a Polarograph, Model XV*. The amperometric measurements were made by recording the current on this instrument automatically, as previously described (3, 9).

During generation and current measurement, the solution was stirred with a magnetic stirring bar. The titration vessel was a 100-ml. or a 50-ml beaker.

All pH measurements were made with a Beckman pH meter (Model G1).

The Conway microdiffusion cells employed were described before (1). They were prepared and cleaned by the methods previously given. A 200-μl. pipet calibrated to contain was used to pipet serum samples; the volumetric tolerance of 0.2% conformed to ACS recommendations (10). Self-filling, 1- and 2-μl. pipets, with straight bore,‡ were calibrated to contain at a volumetric tolerance of 1.0% (10). The 10-μl. pipet employed was made of polyethylene by the methods given by Mattenheimer (11) and was calibrated coulometrically (12) with a precision of 0.2%. Its volume was determined to be 10.31 μl.

Samples were heated on a Thomas-Labconco high-temperature Kjeldahl digestion apparatus.§

Digestions

A 200-μl. serum sample was added to a 30 ml. Kjeldahl flask containing 1.0 gm. potassium sulfate. To this was added 1.0 ml. of the mercuric sulfate solution and 2.0 ml. of concentrated sulfuric acid. Two glass beads were added to prevent bumping. After the water was evaporated at low heat, the digestion was continued at high heat for 1½-4 hr. after the samples had cleared. Blanks were run in duplicate. Following digestion, all samples were transferred to a 25-ml. volumetric flask and diluted to volume with water.

Alternatively, a 10-μl. sample was added to a flask containing 0.200 gm. potassium sulfate. To this was added 0.200 ml. of the mercuric sulfate solution and 0.500 ml. of concentrated sulfuric acid. Two glass beads were added and the digestion was performed as above. Again, blanks were run in duplicate. Either the complete digested sample was taken for analysis or the sample was diluted to 25 ml. as above, and an aliquot was taken.

After 1- and 2-μl. samples were digested with 1.20 ml. of 4.5M sulfuric

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†Beckman Instruments, Inc., Fullerton, Calif.
‡Research Specialties Co., Richmond, Calif.
acid, 0.100 ml. mercuric sulfate solution, and 2.00 ml. 5% potassium sulfate solution, the complete digested sample was taken for analysis.

**Titration Procedures**

In addition to the micro- and macro-Kjeldahl procedures, a third method was employed with some samples to check the accuracy of the coulometric methods. A 1.000-ml aliquot of the digested sample was added to the outer chamber of a Conway microdiffusion cell. To the inner chamber was added 1.000 ml. of standard 0.015N sulfuric acid. The cell was covered and 1.0 ml. of 10% (w/v) sodium thiosulfate pentahydrate in 60% (w/v) potassium hydroxide was added to the outer chamber. The cells were rotated to mix the contents of the outer chamber and were allowed to stand at room temperature 3–3½ hr. At this time the contents of the inner chamber were transferred to a 25-ml Erlenmeyer flask with a Pasteur disposal pipet, as described previously (1), and then were titrated with standard 0.015N sodium hydroxide using methyl red indicator. A 2-ml microburet was employed.

In the direct coulometric titration of 200-μl samples, a 1.000-ml aliquot of the sample was added to a 100-ml beaker containing 10.0 ml. stock buffer solution, 6.00 ml. 5M sodium bromide solution, and 20 ml. water. The pH was adjusted to 8.5–8.6 with saturated potassium hydroxide, dropwise, while stirring with a magnetic bar. The sample was titrated immediately using a generating current of 9.650 ma. A correction for the blank was made.

In the separation procedure for 200-μl samples, a 1.000-ml aliquot was added to a microdiffusion cell. The ammonia was liberated as described above and was collected in 1.0 ml. of 0.05N sulfuric acid. The cells were allowed to incubate at room temperature for 2½–3 hr. The contents of the inner compartment were transferred to a 100-ml beaker containing the same amounts of reagents as described under the direct procedure. The sample was then titrated at 9.650 ma and a blank correction was applied.

In the direct titration of 10-μl samples, a 10.00-ml aliquot was added to the beaker containing the same reagents as above, but with 10 ml. water. The pH was adjusted and the sample was titrated at a generating current of 4.825 ma. A blank correction was applied. Alternatively, the complete sample in the digestion flask was transferred to the beaker and prepared in the same way. The titration was performed at 9.650 ma. generating current.

After 1- and 2-μl samples were transferred from the digestion flask to a 50-ml beaker, 5 ml. buffer and 3.00 ml. 5M sodium bromide were added. The pH was adjusted and the samples were titrated at 4.825 ma.
Results and Discussion

A number of catalysts can be used in Kjeldahl digests. Mercury is generally considered to give the best recoveries of protein nitrogen (13, 14). Therefore, in most of this investigation, a mercury catalyst was employed. An added advantage of this catalyst in the coulometric titrations is that ammonia can be titrated directly in the presence of the catalyst. If a selenium catalyst is used, the ammonia must be separated prior to titration, as the selenium is oxidized by hypobromite.

Early in this investigation a selenium catalyst was employed to digest weighed samples of bovine serum albumin. It appears that satisfactory results can be obtained if the ammonia in a 1-ml. aliquot of the digested sample (ca. 14 mg. protein; final volume, 25 ml.) is separated by microdiffusion (15). The liberating agent was 1.0 ml. of 40% (w/v) potassium hydroxide, and the ammonia was collected in 1.0 ml. of 0.05N sulfuric acid.

A series of human serum samples were digested using a mercury catalyst as described under Titration Procedures and the ammonia was then titrated coulometrically. The results were compared with those obtained on a separate sample, using both the macro- and micro-Kjeldahl procedure. In the former procedure, a 1.5- to 2.5-ml. sample was taken for analysis and, in the micro-procedure, a 200-μl. sample was taken. The microprocedure employed a selenium catalyst while the macroprocedure used a mercury catalyst. A third comparison was made by titrating, with standard acid, the ammonia from a 1-ml. aliquot of the same sample taken for coulometric analysis; the ammonia was separated by microdiffusion (see Titration Procedures). Comparison of results is summarized in Table 1.

The micro-Kjeldahl procedure described in Standard Methods of Clinical Chemistry (8), employing a mercury catalyst, recommends continuing digestion for 30 min. after the mixture has cleared. Initially, the 200-μl. samples were heated 1 1/2–2 hr. following clearing. The results are given in Column 6 of the table. As may be seen, this is not sufficient time for complete destruction of protein using the described procedure. When the samples were titrated directly, results were high by several per cent. In a few instances they were close to the Kjeldahl value. These high results are easily explained. It was noted earlier that traces of protein gave high titration values with coulometrically generated hypobromite (1). It is evident from the data of Column 6 that a small amount of protein was not always completely oxidized in 1 1/2–2 hr. of digestive time. Results by the microdiffusion procedure after 2 hr. digestion were about 0.5% less than those shown in Column 4. The diges-
Table 1. COULOMETRIC AND KJELDAHL RESULTS* FOR HUMAN SERUM PROTEIN NITROGEN

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<th>Micro-Kjeldahl</th>
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<th>Coulometric Conway†</th>
<th>Coulometric direct†</th>
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*In grams of nitrogen per 100 ml. sample.
†Average of two titrations.
‡A 200-μl. sample, digested 4 hr.
§A 200-μl. sample, digested 2 hr.
¶A 10-μl. sample, digested 4 hr.
∥A 10-μl. sample, digested 2 hr.

The results are shown in Columns 4 and 5. It appears here that 4 hr. of digestion time following clearing of the mixture nearly completely oxidizes the organic matter. In the microdiffusion separation procedure (Column 4) the results agree with those obtained by the macro-Kjeldahl procedure, with an average of + 0.95%, while the direct titration procedure agrees within + 1.95%. Compared to the micro-Kjeldahl procedure, the microdiffusion-coulometric method agrees within an average of + 1.88%, and the direct titration differs by an average of + 2.53%. The direct coulometric titration procedure gives results which are an average of + 0.88% different from those obtained by the microdiffusion-coulometric method.

Samples can be titrated directly because the bromide ions in the generating solution complex the mercury as HgBr₄²⁻, thereby releasing the ammonia from the mercury. It is important that the bromide be added to the sample before adjusting the pH to 8.5–8.6. If it is not, the mercury will react with the alkali to form mercuric oxide. Then, when the bromide is added, hydroxyl ions will be released, causing an increase in pH. Also, the blank reading will be increased because of added impurities from the base.

In the microdiffusion separation, the mercury is precipitated as mercuric sulfide by the liberating agent, preventing mercury-ammonia com-
plexes. This is a procedure described by Weil-Malherbe and Green (16). These authors did not list incubation times. They collected the ammonia in boric acid solution. We employed 2½–3 hr. diffusion time, finding this to be satisfactory when the acid in the central compartment was 0.05N sulfuric acid. When the ammonia was collected in 0.015N sulfuric acid, 3–3½ hr. diffusion time was used.

Serum samples of 10 μl. were digested for 2 hr. after clearing and then were titrated directly by taking a 10-ml. aliquot, as described under Titration Procedures. The results, given in Column 8 of Table 1, are generally slightly higher than those in Column 4. The serum samples were approximately 2 weeks old when the 10-μl. samples were digested, which may explain the higher results. The samples were refrigerated but not frozen during this time. Subsequent analyses have given close agreement between 200-μl. and 10-μl. samples (Table 2). The protein in 10-μl. samples appears to be almost completely digested in 2 hr. because nearly the same results were obtained when the samples were digested for 4 hr. (Column 7). However, the authors prefer to use 3–4 hr. to insure complete digestion. An aliquot of these 10-μl. samples was taken for analysis only so titrations could be made in duplicate. The end point of the sample plus blank occurred at about 260 sec. with a blank of about 30 sec. using 4.825 ma. generating current. Most of the blank was due to impurities in the sodium bromide (3) and in the potassium hydroxide used to neutralize the acid digest.

It is preferable to transfer the complete digested sample to the titration vessel and to titrate this at a generating current of 9.650 ma. In this way, the only volumetric measurement made is in pipetting of serum samples. This is the method employed routinely in our laboratory in the titration of 10-μl. samples.

Extremely small samples can be taken for direct analysis if the complete digested sample is titrated. Using a generating current of 4.825 ma. and the procedure given, the authors have easily determined the ammonia in serum samples as small as 1 μl. Results for 1- and 2-μl. samples are compared in Table 2 with those obtained using 200-μl. and 10-μl. volumes of the same samples. All results given in Table 2 were obtained from

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*In grams of nitrogen per 100 ml. All values represent the average of duplicate runs.
samples digested 4 hr. The complete sample was titrated except for the 200-μl samples. Sample 1 is whole blood and Sample 3 is plasma taken from a rat which had been subjected to shock and mannitol infusion, resulting in low protein levels. It appears that 4 hr. of digestion time is required to digest 1- and 2-μl samples completely by the given procedure. When working with small amounts, it is necessary to measure reagents carefully in order to reproduce blank readings.

Because of the long digestion times involved, there is danger of loss of ammonia. Recoveries of 100.00 μg. of ammonia (as ammonium sulfate) added to 10 μl. of serum were determined after 4 hr. of digestion time. When duplicate samples were run, an average of 99.6 μg. of ammonia was recovered; when "digested" in the absence of protein, 99.8 μg. of ammonia were recovered. It therefore appears that with the given conditions, losses of ammonia are not appreciable. This is supported by the close agreement of coulometric results with standard Kjeldahl procedures and by agreement among coulometric procedures with different size samples.

It is important in direct titrations that care be taken to insure complete destruction of the sample lest high results be obtained. Under the conditions described, relatively long digestion times are required for complete oxidation. Undoubtedly, digestion times can be shortened by using larger amounts of digesting reagents than are employed here. The one disadvantage to this is that the blank would be increased because of the need of more alkali to neutralize the sulfuric acid. This would be a serious problem in the direct titration of very small samples. However, it would not seriously alter results for 200-μl. serum samples, because the blank in this case is small compared to that of the sample. If the ammonia is separated by microdiffusion or distillation, increased digesting acid would have little or no effect on blank readings, most of the blank being attributed to the generating reagents in these cases. Larger amounts of catalyst and potassium sulfate may be employed without markedly increasing the blank in a direct titration. This might be useful to shorten digestion times with both large and small samples. More precise results should be obtained with very small samples (1 or 2 μl.) if the ammonia is separated prior to titration and a pretitration procedure is employed (3). In this way the blank would be nearly eliminated.

With modifications (less reagents, smaller digestion tubes, smaller titration cell, lower generating current, and faster recorder) samples can be titrated directly that are smaller than have been employed here. For example, it should not be difficult to titrate the ammonia from a 0.1-μl. serum sample. If the ammonia is separated and if the generating reagent
blank is pretitrated, it would not be necessary to minimize the amounts of reagents used.

Ammonia in Kjeldahl distillates can of course be titrated coulometrically.

References