A RAPID AND ACCURATE METHOD FOR THE DISTILLATION OF AMMONIA WITH APPLICATION TO THE DETERMINATION OF NITROGEN, AMMONIA, AND UREA IN BIOLOGICAL FLUIDS

by

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A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in the partial fulfillment
of the requirements for the degree of
Master of Science

September, 1944

[redacted]

[redacted]

Aug 22, 1944 [redacted]

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INTRODUCTION

The Determination of Total Nitrogen

The quantitative determination of the nitrogen content of organic substances has been one of the most extensively studied subjects in modern analytical chemistry. The Dumas[1] method, even though it is outstanding for its accuracy and general applicability, has been found to be too time consuming and difficult to be extensively applied to the many nitrogen analyses that are necessary in biological work. It has therefore in more recent years been used primarily for checking other procedures of nitrogen analysis and for nitrogen analysis on substances that are not amendable to the more simple procedures.

Kjeldahl[2] was the first worker to devise a method of quantitative analysis for nitrogen in biological substances that was universally acceptable and yet reasonably simple. This method consists of two well defined procedures. The first consists in the digestion of the substance to free nitrogen from its bonds with other atoms and form ammonium sulfate. The second procedure involves the quantitative determination of the ammonium sulfate nitrogen contained in the digestion mixture.

There have been many modifications of both of these steps to meet special analytical needs, to avoid complex manipulations, to save time, and to adapt the method to the determination of micro quantities of nitrogen. With respect to the digestion process the modifications are for the most part limited to various types of reagents and catalysts used to enhance the digestion process. It is not the purpose of this paper to discuss the various digestion mixtures and their application

since there are already many excellent discussions of the subject. It does seem worthwhile to mention some of the limitations of the Kjeldahl method that may be encountered in biological analysis. Dudley and Dakin[3] have pointed out that compounds of the pyridine, piperadine, quinoline, pyrazole, and piperazine types are all resistant to the ordinary Kjeldahl sulfuric acid process. According to these authors, these compounds routinely give low results unless special precautions are taken to insure complete digestion.

The determination of the nitrogen that is contained in the digestion mixture as ammonium sulfate may be accomplished by four different procedures. The nitrogen may be determined by making the digestion mixture alkaline and distilling the liberated ammonia into a receiving vessel containing standard acid. The nitrogen is then estimated by titrating the excess of standard acid or by nesslerizing the acid solution. A second method is to remove the ammonia from the alkalinized digestion mixture by aeration. In this method the ammonia is carried into an acid receiving solution by a stream of air bubbling through the two solutions thus eliminating the boiling process of distillation. The nitrogen in this instance is determined either by titrating or by nesslerizing the standard acid solution. The nitrogen contained in the digestion mixture may be determined by directly nesslerizing the digestion mixture, and in this way eliminate the procedure of removing the ammonia from the digestion mixture. The nitrogen content of the digestion mixture may also be determined by treating the mixture with sodium hypobromite and volumetrically determining the nitrogen gas formed.

The original Kjeldahl procedure directed that ammonia be removed

from the digestion mixture by making alkaline and then distilling into standard acid solution. The nitrogen was then determined by titrating the unneutralized acid with alkali. This method has proven itself, with only slight modification in apparatus, to be entirely reliable and accurate. Miller[4] demonstrated that this distillation procedure gave a negative error of as much as 1.25 per cent on samples containing 35 mg. of nitrogen. He claimed that the error is due to loss of ammonia at the start of distillation due to dilution of the ammonia gas with air and inefficient absorption by the receiving acid. He proposed a delivery tube with 8-10 perforations in the tip to give better diffusion of the ammonia in the acid receiving solution and thus eliminate the error. In general it is accepted that the distillation technique will give results with less than 0.25 per cent error on samples containing as little as 1 mg. of nitrogen.

The distillation procedure does have the following disadvantages. It is very time consuming. It requires cumbersome apparatus. It is prone to explosive type boiling with loss of the determination. Folin and Wright[5] modified the apparatus to avoid these difficulties. These workers used a simple glass "U" tube as distilling head to distill the ammonia into the acid solution. This procedure requires only 30 minutes for the determination of macro amounts of nitrogen in urine and gives results that are sufficiently accurate for clinical purposes.

Folin and Farmer[6] and Van Slyke and Cullen[7] adapted the aeration method to the removal of ammonia from the digestion mixture. In this method the ammonia is carried by a current of air from the digestion flask into the acid receiving solution and the nitrogen determined by either titration or nesslerization. Van Slyke and Cullen[8] later pointed out that much of the ammonia is lost if the air current is too rapid

at first or if the delivery tube is not placed deep into the acid solution. With these precautions the authors compared the aeration and distillation procedures and found no significant difference in the accuracy of the two methods. This aeration method did eliminate the difficulties of manipulation encountered in the distillation method but if anything, lengthened the time required and was not any better adapted for the use on micro quantities of nitrogen.

In general strong acids such as hydrochloric and sulfuric have been recommended for the absorption of ammonia in both the distillation and aeration methods. Stover and Sandin[9] proposed the use of boric acid for the absorption of ammonia in modified Kjeldahl methods. Ma and Zuazaga[10] again suggested the use of boric acid for the absorption agent in nitrogen determinations. These authors also recommended the use of a mixture of bromoresol green and methyl red as the indicator for the titration of boric acid solutions since it gives a more distinct end point. The method eliminates the use of a standard sodium hydroxide solution and requires only two minutes for the absorption of ammonia. Results showed an accuracy involving less than five per cent error on samples containing only 0.5 mg. of nitrogen. Sobel, Yuskn, and Cohen[11] proposed the use of boric acid as the receiving solution for ammonia in the aeration method. These workers recommended a mixture of methyl red and methylene blue as indicator for the acid titration and obtained nitrogen recovery with less than two per cent error on ammonium sulfate solutions containing 1 mg. of nitrogen.

The first widely used colorimetric procedure for the determination of nitrogen was proposed by Nessler[12]. In this method an alkaline aqueous solution of mercuric chloride and potassium iodide is used which in the presence of ammonium ions forms a complex mercury ammonium

iodide derivative. This mercury-ammonia complex gives a reddish-yellow color to the solution the intensity of which is proportional to the amount of ammonia present. This first method had many disadvantages. The solutions became turbid, other nitrogen compounds gave false colors, the mercury ammonium iodide complexes were not stable and frequently precipitated. Winkler[13] modified the reagent by using mercuric iodide instead of mercuric chloride in an effort to make Nessler's solution more stable. Folin and Denis[14] felt that excess of sulfate ion in the digestion solution causes turbidity when direct nesslerization is attempted. They suggested using a digestion mixture of three parts phosphoric acid to one part sulfuric acid to avoid this in urine analysis. These workers also found that increasing the ratio of potassium iodide to mercuric iodide, and adjusting the pH of the digestion mixture to approximate neutrality before nesslerizing gives clearer and more stable solutions. With these modifications Folin and Denis obtained results which agreed closely with those by the Kjeldahl distillation methods in the sample range of 0.7 to 1.5 mg. of nitrogen.

Folin and Farmer[6] found that if the Nessler-Winkler solution is diluted five times with water it gives less turbid results when added to the acid receiving solution of the aeration or distillation procedures. Folin and Denis[15] recommended that this same type of indirect nesslerization be applied to the determination of the nitrogen constituents of blood.

Peters[16], using the Folin and Denis procedure, obtained results on samples containing 0.1 mg. nitrogen that showed less than 5 per cent error. Bock and Benedict[17] compared the Folin and Farmer nesslerization method with Kjeldahl distillation and found an average variation of 10 per cent. These men felt that part of this discrepancy was due to a

1-2 per cent unavoidable error in colorimetry.

Roe and Irish[18] suggested the use of calcium phosphate as an agent to prevent turbidity in nesslerized solutions. Looney[19] used gum ghatti as a protective colloid to stabilize nesslerized solutions. This agent had been previously suggested by Folin[20] for stabilizing the colloids in sugar determinations. However, gum ghatti gave erroneous results since it changed the color of the resulting solution. Wong[21] proposed the use of persulfate for digestion in direct nesslerization methods since he found that it prevented turbidity caused by the dissolved silica formed by the usual phosphoric acid digestion mixture. Since the directly nesslerized solutions of Folin were usually tinted green Marr[22] used a standard comparison solution containing iron, nickel and cobalt salts. This standard solution has a greenish tint corresponding to the nesslerized solutions and is read against the sample in a visual colorimeter.

Koch and McMeekin[23] changed the iodide-iodine ratio and alkalinity of the Nessler-Folin reagent, and found that this solution is slightly more sensitive and less likely to cause turbid solutions than the original Nessler-Folin solution. These authors also suggested the use of 30 per cent hydrogen peroxide in the digestion process. They claimed that it also helps prevent turbid solutions. Davenport[24] recommended 3 per cent in place of 30 per cent peroxide for the digestion process. This author also indicated the importance of correcting the nitrogen values to allow for the nitrogen contained in the acetenilid used to preserve the peroxide.

Wicks[25] found that the Koch and McMeekin Nessler reagent could be made from mercuric oxide instead of mercuric iodide with a considerable decrease in cost.

Barrett[26] felt that the turbidity of nesslerized solutions is due to the presence of reducing substances in the mixture, and added hypochlorite, an oxidizing agent, to the Nessler solution to counteract this effect. Gentzkow[27] also felt that turbidity is due to reducing substances and suggested using potassium persulfate as an oxidizing agent and either potassium gluconate or tartrate as reducing agent to partially counteract the effect of persulfate. This author also demonstrated that the color intensity of nesslerized solutions is proportional to the ammonia concentration only when a light of 490-520mμ wave length is used.

Hoffman and Osgood[28] found that the color intensity of nesslerized solutions varies with the time that they are allowed to stand, and with the amount of Nessler's solution used. These authors also suggested using blood filtrates made with trichloroacetic acid when nitrogen is determined by direct nesslerization with the Koch and McMeekin reagent. Dehn[29] pointed out the mechanical and optical errors of visual colorimetry and showed that unless care is taken to eliminate these an accumulated error of 10 per cent might occur. Allen and Davisson[30] compared the Folin nesslerization procedure with the titrametric method and came to the following conclusions. On quantities of nitrogen of 0.5 mg. or over the titrametric method gives results with less than 0.4 per cent error. The nesslerization procedure gives at least 2 per cent error on 0.5 mg. samples and this error increases as the size of nitrogen sample increases. For samples containing less than 0.5 mg. nitrogen nesslerization gives more accurate results than titration.

Even though the reaction of sodium hypobromite with ammonia to give nitrogen gas has been known for over fifty years, it was not until 1920 that Stehle[31] first adapted this reaction to gasometric nitrogen analysis. In his method he reacted the digestion mixture with

sodium hypobromite and measured the amount of nitrogen gas in the Van Slyke carbon dioxide apparatus. With this method he obtained results on 25 mg. samples with less than 0.2 per cent error. In a later publication Stehle[32] stated that copper sulfate in the digestion mixture and an excess of bromine in the sodium hypobromite solution liberate oxygen which gives high results. Van Slyke[33] demonstrated that this method could be used for micro analysis of nitrogen and obtained nitrogen recovery with less than one per cent error on ammonium sulfate samples containing 1 mg. of nitrogen. Gad-Andresen[34] applied the hypobromite reaction to the determination of blood ammonia. In this method he treated 1 cc. of blood with borax, aerated the ammonia into an acid receiving solution, and determined the ammonia nitrogen in this solution by the hypobromite method.

The gasometric method is one of the most accurate procedures for determining both macro and micro amounts of nitrogen; however it has not been of wide clinical application since the skill required to operate the apparatus cannot be readily mastered by the ordinary technician.

THE DETERMINATION OF UREA

Bunsen[35] first showed that urea decomposes into ammonia and carbon dioxide when it is heated. Benedict[36] made use of this fact for determining the urea concentration in urine when he autoclaved the urine with magnesium chloride at temperatures of 160-170 degrees centigrade, distilled the ammonia into an acid receiving solution, and determined the ammonia by titrating the excess acid. Benedict and Gephart[37] showed that magnesium chloride is not a good reagent for this method because it does not completely liberate the ammonia from the urine and usually contains ammonia as a contaminant. These authors used hydrochloric acid instead

of magnesium chloride to avoid these difficulties and obtained results with less than 1 per cent error on samples containing 50 mg. of urea. Kober[38] also demonstrated that it is extremely difficult to completely distill ammonia from solutions containing calcium and magnesium due to the formation of ammonium complexes with salts of these metals.

Folin[39] used potassium acetate for hydrolyzing the urea in urine in an effort to avoid decomposing nitrogenous substances other than urea. In this method the liberated ammonia is aerated into an acid receiving solution, and the nitrogen estimated by either titration or nesslerization. Folin and Dennis[15] used this method on blood filtrates for determining blood urea concentration. Folin and Wu[40] recommended hydrochloric acid for the autoclave procedure but felt that it gave high results due to the decomposition of other nitrogenous material. Clark and Collip[41] later showed that the hydrochloric acid method of Folin and Wu does not cause hydrolysis of non urea substances in blood filtrates. Lieboff and Kahn[42] used sulfuric acid to decompose urea in blood filtrates and determined the nitrogen in the resulting solution by direct nesslerization with the Koch and McMeekin modification of the Nessler solution. These authors obtained results that were about 5 per cent higher than those obtained by other methods.

Marshall[43] used urease in the quantitative determination of urea by estimating the amount of ammonia formed by the enzymatic hydrolysis of urea. In this method the ammonia is determined from the difference obtained by acid titration of the samples before and after hydrolysis. Marshall stated that this method gives less than 2 per cent error on both known urea solutions and urine. Marshall[44] later used Folin's aeration procedure for removing ammonia formed by the action of urease and determined it by titrating the acid receiving solution. On 5 mg.

samples this method gives a maximum error of five per cent. Folin and Svedburg[45] suggested using an anti-bumping rod and an anti-foam agent in the aeration method to avoid foaming.

Van Slyke and Cullen[46] demonstrated that both temperature and pH affect the rate of urease action and recommended the use of a phosphate buffer in the urease procedure. Behre[47] found that whole blood gives higher urea values than blood filtrates when the urease method is used. He attributed these high values on whole blood to the urease hydrolysis of non urea substances contained in the red blood cells and concluded that blood filtrates should be used for blood urea determinations. Addis[48] found that blood specimens that are heated before enzymatic hydrolysis give lower urea nitrogen levels than non heated specimens. He attributes this discrepancy to the enzyme, arginase, which becomes inactivated by heating. Howell[49] demonstrated the presence of two ammonia forming enzymes other than urease in jack bean meal. One of these enzymes is present only in fresh urease preparation, while the second can be demonstrated only after the preparation has become 48 hours old. This author feels that these two enzymes account for the factors discussed by Behre and Addis. Since urease preparations contain these two enzymes, Howell suggests using urease solutions that are not less than five and not over 20 hours old for quantitative urea estimations.

Folin and Youngburg[50] proposed a method for determining urea of urine in which the urease is purified by adsorption with permutit and then the sample is hydrolyzed with this purified urease and the ammonia is determined by direct nesslerization. Folin and Wu[40] found that the direct nesslerization of blood filtrates following enzyme hydrolysis gives erroneous colors due to the presence of amino acids and creatinine. Karr[19] determined the urea nitrogen of blood by urease action on Folin

and Wu filtrates followed by nesslerization. In this procedure he used a comparison standard made with cobalt, iron, and nickel salts since this solution more nearly matched the color of the nesslerized blood filtrates.

To avoid turbid solutions Gruskin[51] enzymatically hydrolyzed the urea of whole blood and then directly nesslerized a tungstic acid filtrate prepared from this blood. Taylor and Blair[52] recommended Folin and Youngburg's purified urease in the Gruskin procedure. Taylor, Hayes, and Wells[53] made tungstic acid filtrates of urine following enzyme hydrolysis and obtained clearer nesslerized solutions. Hoffman and Osgood[54] felt that Folin-Wu tungstic acid filtrates give turbid solutions when nesslerized and recommended filtrates prepared with zinc sulfate and sodium hydroxide. These authors obtained less than 2 per cent error when urea determinations were made on 0.2 cc. of whole blood. Using tungstic acid filtrates of blood, urease, and the Koch-McMeekin reagent for direct nesslerization, Sure and Wilder[55] obtained results with less than 5 per cent error in blood urea estimations. Hindmarsh and Priestley[56] combined the aeration method with nesslerization of the receiving solution and obtained less than 5 per cent error on 0.1 cc. blood samples. Fienblatt[57] ran a large series of determinations comparing the aeration-nesslerization method with direct nesslerization and concluded that there is no significant difference in the accuracy of the two methods.

Mirkin[58] was the first to make urea nitrogen estimations by measuring carbon dioxide liberated by the action of urease. Using this method he was able to obtain results that deviated no more than 2 per cent from those obtained by nesslerization. Van Slyke[59] adapted this method to his special carbon dioxide apparatus and obtained results

with less than 1 per cent error on 0.2 cc. samples of blood.

In the presence of sodium hydroxide urea reacts with sodium hypobromite to give nitrogen gas, sodium bromide, sodium carbonate, and water. Stehle[60] made quantitative urea determinations on urine by measuring the nitrogen gas formed by this reaction. The results with this method were 3-4 per cent higher than those obtained by the aeration method. Menaul[61] claimed that this reaction is not quantitative due to the formation of oxides of nitrogen. Stehle[62] later stated that he could not verify Menaul's work and that nitrogen oxides are not formed. Van Slyke[63] adapted the hypobromite-urea reaction to blood urea determinations and obtained results with less than 4 per cent error on 5 cc. of tungstic acid filtrate. Gad-Andresen[64] used sodium hypobromite to liberate nitrogen from the acid receiving solution in the digestion-aeration procedure. Using this method on blood samples of 0.05-0.1 cc. he obtained results that were not over 1 per cent in error.

Kiech and Luck[65] estimated the urea concentration of biological materials by precipitating the urea from tungstic acid filtrates with xanthydrol. This precipitate of dixanthydrol urea is dried and weighed on an analytical balance. The method is less than 1 per cent in error on samples containing 4-12 mg. of urea. Luck[66] later precipitated urea in the same manner but determined the amount of dixanthydrol urea by titrating with standard potassium permanganate solution. He used this method on samples containing 0.1 mg. of urea and obtained better than 95 per cent recovery. Beattie[67] precipitated dixanthydrol urea from tungstic acid filtrates, then redissolved the precipitate and estimated the urea concentration of the yellow solution by comparing it in a visual colorimeter with a urea standard treated in a similar fashion. Using this method samples containing 0.02 mg. of urea were analysed

with an error of less than 3 per cent.

Ormsby[68] treated urea with diacetyl monoxime in hydrochloric acid solution, intensified the orange color that was produced by adding potassium persulfate, and estimated the urea concentration of the solution colorimetrically. Using this method on 3 cc. of tungstic acid filtrate he obtained results that deviated less than 2 per cent from those done according to the aeration procedure of Van Slyke and Cullen. Even though this color reaction is not specific for urea he found that the other color producing substances such as methyl urea and citrulline give red colors and therefore do not interfere when a photoelectric colorimeter is used. Barker[69] modified the method by using sulfuric rather than hydrochloric acid and by adding potassium persulfate to the hot solution in order to develop the color more rapidly. With these modifications he was able to get good results using only 1 cc. of filtrate.

THE DETERMINATION OF AMMONIA

The determination of ammonia as well as urea and total nitrogen of urine has undergone many modifications.

Schloesing[70] determined urinary ammonia be placing the urine which had been made alkaline with calcium hydroxide under a container of hydrochloric acid in a sealed bell jar. After about twelve hours ammonia nitrogen was estimated by titrating the excess hydrochloric acid. Folin[71] estimated urine ammonia by adding magnesium hydroxide to the urine and distilling the liberated ammonia into an acid receiving solution and titrating. Shaffer[72] pointed out the inaccuracy of both these methods and later Kober[58] demonstrated the inadvisability of distilling ammonia in the presence of calcium and magnesium ions.

Folin[73] later described a method in which the urine is made

alkaline with sodium bicarbonate, the liberated ammonia aerated into standard acid, and the nitrogen estimated by titration. Shaffer[72] modified the Boussingault[74] vacuum distillation procedure for determining urine ammonia and obtained excellent results. Steel[75] showed that the Folin method using sodium bicarbonate did not completely liberate ammonia from magnesium ammonium phosphate present in the urine. In the place of bicarbonate he recommended sodium hydroxide in saturated sodium chloride. Folin[76] later pointed out that the saturated sodium chloride solution suggested by Steel did not completely prevent decomposition of other nitrogenous substances by sodium hydroxide. This method therefore gave erroneously high results and he suggested adding potassium oxalate to the sodium chloride reagent. Folin and MacCallum[77] then proposed a mixture of potassium oxalate and potassium carbonate for liberating urine ammonia. Folin and Denis[15] later recommended potassium oxalate and potassium carbonate mixture for blood ammonia determinations. Yee and Davis[78] designed a special vacuum aeration apparatus and recommended an alcoholic solution of sodium hydroxide for liberating urine ammonia.

Bandemer and Schaible[79] determined the ammonia in biological materials by placing hydrochloric acid in a container surrounded by the sample alkalinized with potassium carbonate. The dish is then covered, placed in an incubator at 38°C. for 1.5 hours, and the ammonia determined by titrating the acid. These authors obtained better than 95 per cent recovery on samples containing 0.02 mg. of ammonia nitrogen. Brown[80] proposed a simple method for estimating urine ammonia. In this method the urine is made neutral to phenolphthalein with sodium hydroxide, neutral formaldehyde is added and the urine titrated back to the end point with sodium hydroxide. The ammonia

nitrogen is estimated from the sodium hydroxide used in the second titration. The author suggests this method only for clinical use since it is not as accurate, but is more rapid than other methods.

Orr[81] proposed a colorimetric procedure for determining the ammonia concentration of urine. In this method phenol is added to urine in the presence of sodium hypochlorite and the resulting blue solution is compared with an ammonium sulfate standard treated similarly.

The colorimetric method of direct nesslerization has also been applied to the determination of ammonia in urine. Folin and Denis[14] used charcoal to adsorb ammonia from urine and then liberated it from the charcoal with sodium hydroxide and estimated the ammonia nitrogen by direct nesslerization. Folin and Bell[82] found that permutit, a crude aluminum silicate, was better adapted than charcoal to the adsorbtion of ammonia. Using this agent they made urine ammonia determinations with less than 1 per cent error. Sumner[83] estimated the ammonia nitrogen of urine by precipitating the non ammonia interfering substances in urine with alkaline copper sulfate, and directly nesslerizing the remaining solution.

From the preceding discussion it is apparent that a rapid and accurate method for determining total nitrogen, urea, and ammonia in biological materials is important in both clinical and research work. The difficulties that are encountered in the methods used at present are of considerable significance. The following method eliminates most of these problems and in our laboratory has proven superior to other methods. This procedure is based upon a modification and adaptation of the apparatus described by Van Slyke, MacFadyen, and Hamilton[85] for the determination of carbon dioxide liberated from free amino acids by the reaction of ninhydrin.

EXPERIMENTAL*

Apparatus

The apparatus is pictured in Fig. 1. Two sizes will be described, the dimensions given having proven quite satisfactory for our type of work. It is to be understood that these dimensions may be varied to suit individual needs. However it should be remembered that a vertical piece which is too short will permit the sodium hydroxide solution to bubble over into the receiving vessel and ruin the determination.

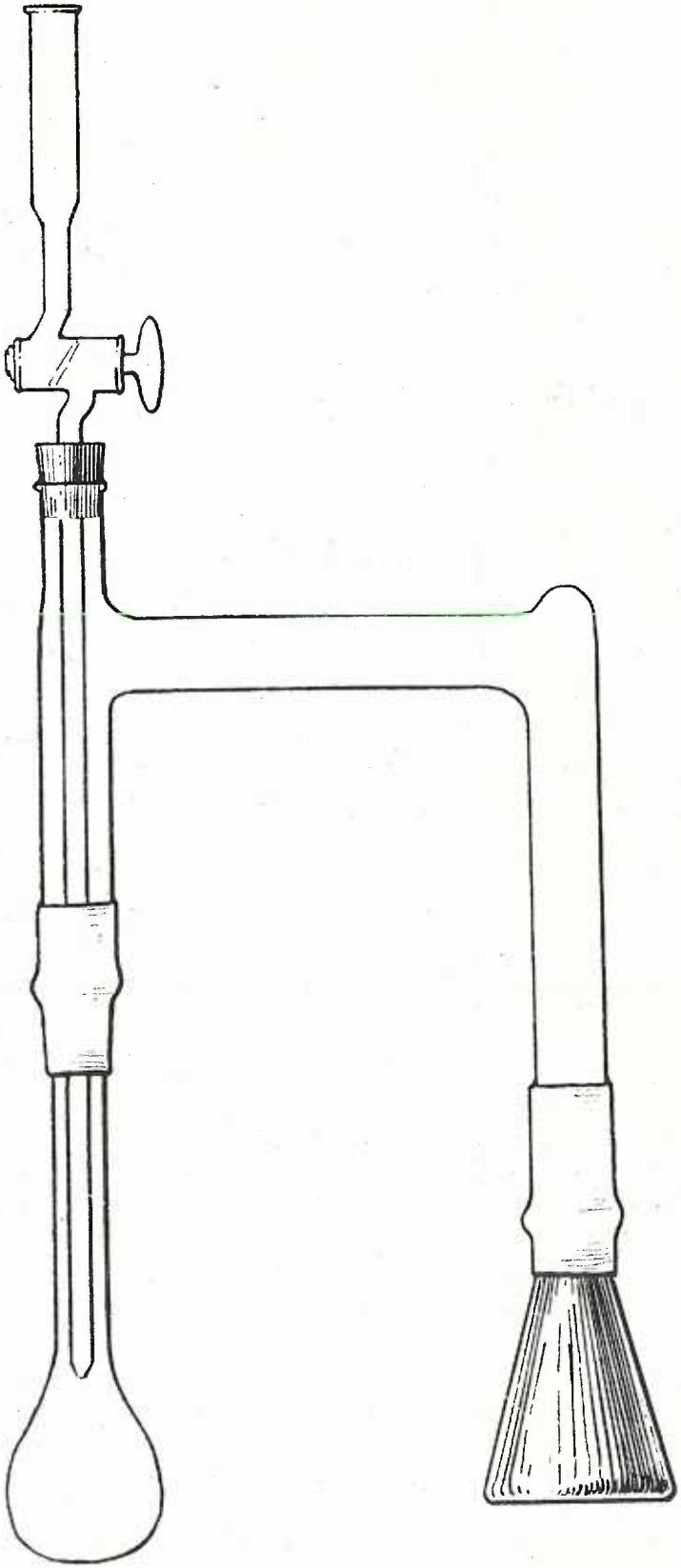
The larger apparatus is constructed from 1" pyrex tubing (discarded test tubes are quite satisfactory). The length of the receiving vertical section is 9", of the side distilling vertical section 5", and the connecting arm 5". A 2" length of 5/8" pyrex tubing is attached to the short arm for insertion of the dropping funnel. The distilling vessel is a regulation 100 ml. Kjeldahl flask. The receiving vessel is a narrow mouth 125 ml. Erlenmeyer flask with the flange removed. The dropping funnel is constructed from a two-way capillary stopcock, the lower end being attached to a 12" length of capillary tubing and the upper end to a cup designed to hold 10-15 ml. The use of capillary tubing at this point has the advantage of permitting the liquid to flow slowly and helps to prevent loss of the vacuum. However, it is very prone to become obstructed and in this manner it prevents adequate evacuation and low values result. If care is used in handling the apparatus an ordinary glass tube may be used. The dropping funnel assembly is attached to the apparatus by use of a one-hole rubber stopper.

*Rinehart, Grondahl, and West[94]

FIGURE I

Ammonia Distillation Apparatus

(Description in text)



The receiving and distilling vessels are attached by means of 2" lengths of heavy rubber tubing of 3/4 to 7/8" internal diameter. It is important that the glass to glass ends be smooth and square.

The smaller apparatus is constructed in the same manner with slightly different proportions, using $\frac{1}{2}$" pyrex tubing. The length of the receiving section is $2\frac{1}{2}$", of the distilling section 2", and the connecting arm 5". The short arm is continued upwards for 1" to provide an opening for the dropping funnel. The latter is the same as described above except that the stem is only 6-8" in length. For distillation a regulation 30 ml. Kjeldahl flask is used. A narrow mouth 50 ml. Erlenmeyer flask with the flange removed serves as receiving vessel, or it is replaced by a graduated Klett-Summerson colorimeter tube when the distillate is to be nesslerized. Connections are made with $1\frac{1}{2}$" lengths of $\frac{1}{2}$" rubber tubing.

Hot and cold water baths are necessary for the distillation and should be so placed that the distilling and receiving vessels may be immersed simultaneously and held in place by a clamp. The hot water bath should be boiling.

A Klett-Summerson photoelectric colorimeter is used for the nesslerized distillates.

REAGENTS

Sulfuric acid N/70, 2/3N and 2.5N.

Sodium hydroxide N/70, and concentrated (40 per cent).

Saturated potassium carbonate, about 90 per cent.

Phosphate buffer, 2.5 per cent potassium dihydrogen phosphate.

Urease, any good commercial preparation.

Indicators. Methyl red, 0.04 per cent in 95 per cent alcohol. Methylene blue, 0.02 per cent in water.

Caprylic alcohol.

Selenium digestion mixture. Gradually stir 250 ml. concentrated sulfuric acid into an equal volume of water. (CAUTION!) Cool the mixture and saturate it with nitrogen-free K_2SO_4 using about 40 g. Add 1.0 ml. of selenium oxychloride and mix.

Nessler's reagent (Wicks[25]). Dissolve 51 g. of C.P. KI in 100-150 ml. of ammonia-free water. Heat this solution to 90-100°C. and add 16.2 g. of C.P. red mercuric oxide in small portions with stirring. Dilute 160 ml. of 50 per cent sodium hydroxide to about 500 ml., add the iodide solution, and dilute to one liter. One volume of solution is diluted with 5 volumes of water before use. The diluted solution keeps well but should be checked with standard $(NH_4)_2SO_4$ at weekly intervals.

Carbonate-oxalate reagent. Dissolve 100 g. of pure K_2CO_3 in 90 ml. of distilled water and boil for 5 minutes. Cool, add 10 ml. of saturated neutral potassium oxalate solution (30 per cent) and dilute to 140 ml.

Standard ammonium sulfate solution. Dissolve 0.472 g. of $(NH_4)_2SO_4$ in distilled water and dilute to 1000 ml. This contains 0.10 mg. of ammonia nitrogen per ml. Varying amounts and dilutions of this are used for preparing standards for use with the colorimeter.

GENERAL PROCEDURE

The Kjeldahl digestion flask containing the preformed ammonia is attached to the short arm of the apparatus, and the receiving flask containing the absorbing acid is attached to the longer arm. The dropping funnel is attached to the apparatus and its open end attached

to the water suction. The entire apparatus is evacuated to a pressure of 20-30 mm. of Hg (3 to 5 minutes), and the stopcock of the dropping funnel closed. The water pump is disconnected and the requisite amount of alkali placed in the dropping funnel. The alkali is cautiously run into the distilling flask, care being taken to exclude air. The vessels of the apparatus are immersed in the hot and cold water baths and the distillation allowed to proceed for three to five minutes. Observation of this upper limit of time is not essential in cases where no substances are present that may be converted to ammonia by prolonged heating with alkali.

When the distillation is complete air is allowed to enter the apparatus by opening the stopcock. The receiving flask is then disconnected and the ammonia determined by back-titration or nesslerization.

The following precautions should be taken to insure accurate results with this distillation procedure. Be sure that the system is adequately evacuated before the alkali is added. The common causes of error at this point are a water pump that does not produce adequate vacuum and an occlusion of the capillary tube. If the vacuum is sufficient to cause the N/70 acid solution to boil it will be satisfactory. The distillation apparatus should always be rinsed with distilled water between determinations to avoid introducing alkali into the receiving flask. To prevent the alkali from bumping over into the receiving flask anti-bump rods or boiling stones should always be used. In addition the volume of solutions should be held to a minimum.

RESULTS WITH AMMONIUM SULFATE SOLUTIONS

The results of a series of analyses with the macroapparatus are given in Table I. The results of a series of analyses using ammonium

sulfate solutions of known nitrogen content are given. The ammonia was liberated in these determinations by the addition of 10 ml. of 40 per cent sodium hydroxide. The values given are unselected. Variations of ± 1 per cent may be encountered. The amount of N/70 sulfuric acid may be varied to suit the expected recovery of ammonia. 25.00 ml. was used in these determinations. Samples containing as much as 20 mg. of nitrogen have been satisfactorily distilled in this apparatus as shown in Table I. 0.1N acid was placed in the absorbing vessel and 0.1N base was used for back titration in these analyses.

Table II gives the results of a series of analyses with the micro-apparatus. 5.00 ml. of N/70 sulfuric acid was used to absorb the ammonia and back titration was carried out with N/70 sodium hydroxide from a 5.00 ml. microburette. 5 ml. of 40 per cent sodium hydroxide was used to liberate the ammonia. Variations of 5 per cent may be expected when the amount of nitrogen determined is less than 0.1 mg. Larger amounts should give an accuracy of 2 per cent.

Table III gives results of a series of direct nesslerizations of varying amounts of standard ammonium sulfate solution. In these analyses the required volume of solution was pipetted into the colorimeter tube, diluted to the 5 ml. mark with water, and 2.0 ml. of Nessler's reagent added. After standing for fifteen minutes the tubes were read in the colorimeter. Blanks on water read zero. The factor, f, is obtained by dividing the average reading of several determinations by the milligrams of ammonia nitrogen in the sample divided by 0.01. C (the concentration of ammonia nitrogen in 0.01 mg. units) can be calculated from the equation:

$$C = \frac{R}{f}$$

in which R is the colorimeter reading.

TABLE I

Macroanalysis of Ammonium Sulfate Solutions

$(NH_4)_2SO_4$ 0.10 mg. N per ml.	Ammonia N	
	Found	Per cent of theory
ml.	mg.	
10.0	1.01	101.0
	1.00	100.0
	1.02	102.0
	1.00	100.0
15.0	1.51	100.7
	1.50	100.0
	1.50	100.0
20.0	1.98	99.0
	2.00	100.0
	2.00	100.0
25.0	2.50	100.0
	2.50	100.0
	2.51	100.2
	2.49	99.6
30.0	3.01	100.3
	3.00	100.0
	3.00	100.0
47.0	4.69	99.8
	4.71	100.2
	4.72	100.4
$(NH_4)_2SO_4$ 1.00 mg. N per ml.		
ml.		
10.0	10.05	100.5
	10.05	100.5
	10.08	100.8
20.0	20.21	101.0
	20.21	101.0
	20.27	101.3

TABLE II

Microanalysis of Ammonium Sulfate Solutions

$(NH_4)_2SO_4$ 0.05 mg. N per ml.	Ammonia N	
	Found	Per cent of theory
ml.	mg.	
1.0	0.048	96.0
	0.046	92.0
	0.052	104.0
2.0	0.098	98.0
	0.100	100.0
	0.100	100.0
	0.102	102.0
3.0	0.148	98.6
	0.154	102.6
	0.152	101.4
	0.150	100.0
4.0	0.198	99.0
	0.196	98.0
	0.200	100.0
	0.200	100.0
5.0	0.250	100.0
	0.248	99.2
	0.246	98.8
	0.250	100.0
6.0	0.296	98.7
	0.298	99.3
	0.302	100.7
	0.300	100.0
8.0	0.399	99.7
	0.398	99.5
	0.402	100.5

Table IV gives the results of a series of analyses in which the distillate was nesslerized. In these determinations the microapparatus was used and ammonia was absorbed in 1 drop of 2.5N sulfuric acid. The colorimeter tube served as receiving vessel. The ammonia was liberated with 5 ml. of 40 per cent sodium hydroxide and distilled for not less than five minutes. After admitting air to the apparatus the distillate was diluted to 5.0 ml., 2.0 ml. of Nessler's reagent added, the tube stoppered, and the solutions mixed by inversion. Readings were made after fifteen minutes.

KJELDAHL DIGESTS

The macro and micro procedures have been applied to the determination of total nitrogen of blood, blood filtrates, spinal fluid and filtrates, feces, wood, blood fertilizer, urine, casein glues, purified blood proteins, ascitic fluid, and leather. Excellent results have been obtained with all these substances when the selenium digestion mixture described above was used. Undoubtedly other digestion mixtures would be satisfactory. There are many cases where more rapid digestion could be accomplished, as in the case of fertilizer and leather, by using other digestion mixtures; but we found this to be an excellent all purpose reagent.

URINE TOTAL NITROGEN

To a sample of urine containing 2-4 mg. of nitrogen in a 100 ml. Kjeldahl flask add 3 ml. of the selenium digestion mixture. Boil until the concentrated mixture has become colorless and continue the heating for 10 minutes longer. Cool the flask, add 10 ml. of water and attach to the apparatus. Attach the receiving flask, containing 25.00 ml. of

TABLE III

Direct Nesslerization of Standard Ammonium Sulfate Solutions

$(NH_4)_2SO_4$ 0.02 mg. per ml. ml.	R Colorimeter readings	R_a Average of readings	$F = \frac{R_a}{\text{mg. N/0.01}}$
1.0	63,62,63 63,64,63	63	31.5
2.0	126,126,125 127,126,126	126	31.5
4.0	250,252,251 253,253,252	252	31.5

TABLE IV

Recovery of Ammonia Nitrogen from Known Solutions

$(NH_4)_2SO_4$ 0.01 mg. per ml.	Colorimeter readings	Ammonia N Found	Per cent of theory
ml.		mg.	
1.0	31, 30, 30 29, 31, 31	0.0099	99
2.0	63, 62, 63 64, 65, 63	0.02	100
4.0	125, 126, 125 127, 127, 126	0.04	100
5.0	158, 157, 157 158, 158, 157	0.05	100
10.0	316, 315, 315 314, 315, 316	0.10	100

N/70 sulfuric acid and three drops each of methyl red and methylene blue, to the apparatus. Evacuate at the pump, detach the suction, add 10 ml. of 40 per cent sodium hydroxide, and distill for 3-5 minutes. Admit air to the apparatus, detach the receiving flask, and titrate the excess sulfuric acid with N/70 sodium hydroxide. Typical results on aliquots of urine are given in Table V.

TABLE V

Total Nitrogen of Urine by Kjeldahl Digestion

Vacuum distillation grams per 100 ml.	Regular distillation grams per 100 ml.
1.051	1.030
1.051	1.035
1.050	1.054

The advantage of this procedure over the standard type of Kjeldahl procedure is that it requires only 3-5 minutes to accomplish the distillation while in the previous methods as much as 15 minutes may be required, and in our experience this method has given slightly more consistent results than the ordinary Kjeldahl distillation procedure.

URINE AMMONIA

Excellent results have been obtained with this determination. The short time required to complete an analysis is the main advantage of the modification.

Determination of Urine Ammonia. Into a clean 100 ml. Kjeldahl flask measure exactly 5.0 ml. of urine, which must be acid to congo red. It is recommended that a few drops of 2.5N sulfuric be introduced into the urine sample as a routine procedure since this eliminates any possibility

of ammonia escaping during the evacuating process. The flask is connected to the apparatus and the remainder of the determination carried out exactly as described for the analysis of Kjeldahl digest, except that 10 ml. of potassium carbonate reagent is used in place of sodium hydroxide. Both sodium hydroxide and potassium oxalate-carbonate reagent were found to give high values for ammonia nitrogen regardless of how the time of distillation was controlled. Potassium carbonate gives very satisfactory checks against the aeration procedure. Distillation must not proceed for more than five minutes if the formation of ammonia from other nitrogenous substances is to be avoided. Table VI shows the agreement between this method and the aeration procedure. Table VII shows the importance of limiting the distillation time to 2-3 minutes, since this time is adequate for complete distillation yet does not cause errors due to alkaline decomposition of non-ammonia nitrogenous compounds.

UREA NITROGEN

Recovery from Urea Solutions. About 50 g. of Mallinkrodt A.R. urea was recrystallized from 200 ml. of hot alcohol. The air dried crystals were further dried in a vacuum desiccator over concentrated sulfuric acid for 24 hours. Kjeldahl analysis of these purified crystals, using vacuum distillation, gave 46.9 per cent nitrogen; theoretical 46.6. 0.2140 g. of this urea was made up to 1000 ml. (1.0 mg. N in 10 ml.). 25.00 ml. samples were used for analysis. Results are given in Table VIII. The procedure was the same as described for blood urea.

Blood Urea Nitrogen, Macro. Into a clean 100 ml. Kjeldahl flask place 4.0 ml. of whole blood, 1.5 ml. of phosphate buffer, and a pinch

TABLE VI

Determination of Urine Ammonia

Vacuum distillation mg. per 100 ml.	Aeration mg. per 100 ml.
36.2	37.6
37.4	36.8
37.6	
37.3	
64.2	63.4
63.3	64.2
63.6	65.5

TABLE VII

Effect of Prolonged Distillation on Recovery of Urinary Ammonia

Time in minutes	Ammonia N mg. per 100 ml.
2	34.8, 34.6
4	34.8, 35.2
6	50.6, 56.8

TABLE VIII

Determination of Urea Nitrogen

Solution containing 0.10 mg. urea N per ml.	Urea nitrogen	
	Found	Per cent of theory
ml.	mg.	
25.00	2.51	100.4
	2.50	100.0
	2.51	100.4
	2.51	100.4

(10-20 mg.) of urease powder. Incubate at 50-55°C. for thirty minutes. Place methylene blue and methyl red in the receiving flask. Attach the flasks to the apparatus and evacuate at the pump. Disconnect the apparatus from the suction and place 6 ml. of carbonate-oxalate reagent in the funnel. Allow this to enter the distilling flask and follow with about 0.5 ml. of caprylic alcohol, care being taken to close the stopcock before air is admitted to the system. Distill for 3-5 minutes and determine the ammonia liberated by back titration with N/70 sodium hydroxide. Distillation time must be limited to 5 minutes as a maximum to avoid decomposition of other nitrogen containing substances.

Blood Urea Nitrogen, Micro. This method may be used when only 0.1 to 1.0 ml. of blood is available. The accuracy depends almost entirely on the accuracy of measurement and dilution of the sample. Several determinations may be done on the larger sample. The procedure is based on the action of urease on diluted whole blood with subsequent deproteinization as described by Gentzkow[27]. The ammonia is then distilled from the alkalinized filtrate and determined by nesslerization. Folin and Svedberg[45] described a method for the direct distillation of hydrolyzed filtrates to which the urease has been added after deproteinization, a technique that may give rise to excessive foaming.

One volume of blood (0.1 to 1.0 ml.) is mixed with seven volumes of water in a 15 ml. centrifuge tube and 5-10 mg. of urease powder are added with shaking. The tube is stoppered and incubated for thirty minutes at 50-55°C. in a water bath. One volume of 10 per cent sodium tungstate solution is next added and mixed, followed by one volume of 2/3N sulfuric acid. The tube is stoppered, shaken well, allowed to stand for 10 minutes in water at room temperature, and then centri-

fuged for 30 minutes. 0.5-2.0 ml. of the supernatant fluid is transferred to a 30 ml. Kjeldahl flask which is then connected to the apparatus. For the nesslerization procedure it is best to choose a volume of filtrate containing 0.01 to 0.10 mg. of nitrogen. For the titration procedure the sample should contain 0.10 to 0.50 mg. of nitrogen.

For nesslerization the Kjeldahl flask is connected to the distilling side of the apparatus and a Klett-Summerson photoelectric colorimeter tube graduated at 5.0 ml. and containing one drop of 2.5 N sulfuric acid is attached to the receiving side. The dropping funnel is attached and the apparatus evacuated as described above. Two ml. of carbonate-oxalate reagent are added through the dropping funnel and the apparatus immersed in the water baths for from 3-5 minutes, during which time distillation is complete. The colorimeter tube is detached from the apparatus and its contents diluted to 5.0 ml. 2.0 ml. of Nessler's reagent are then added and the contents of the tube mixed by inverting. The color is allowed to develop for 15 minutes, after which it is quite stable for at least two hours. If good checks on aliquot samples are desired it is essential to use calibrated pipettes and colorimeter tubes throughout.

The procedure for titration is exactly as described above except that a sample containing 0.10 to 0.50 mg. of nitrogen is used and the distillate is collected in a 50 ml. Erlenmeyer flask containing 5.00 ml. of N/70 sulfuric acid. The ammonia is determined by back titration with N/70 sodium hydroxide from a 5.00 ml. burette. The indicator is the same as described above.

The results are summerized in Table IX.

TABLE IX

Blood Urea Nitrogen, mg. per 100 ml.

Sample No.	Aeration	Macro-titration	Micro-titration	Micro-Nesslerization	Nitrogen determined mg.
1	22.3 25.0	25.4 25.1 24.5			1.0
2		20.6 20.6 21.2			0.82
3		7.0 6.9	6.8 6.9 6.8		0.07
4		20.5 20.4	20.5 20.1 20.2		0.1
5		29.6 29.3		28.4 29.1 28.6	0.029
6		63.4 63.0		63.2 63.0 62.5	0.063
7		9.5 9.1		8.9 9.0 8.6	0.018

SUMMARY

A new method for determining total nitrogen, urea, and ammonia in biological fluids has been described. This method consists in liberating ammonia from ammonium sulfate by adding alkali and distilling it under reduced pressure into an acid receiving solution. In our laboratory this procedure has given results that are as accurate as those obtained by either the aeration or Kjeldahl distillation methods. This procedure does not present any of the apparatus difficulties that are frequently encountered in the Kjeldahl distillation method and eliminates the possible source of error due to ammonia loss that may occur when the aeration method is used. The vacuum distillation method is a more rapid means for quantitatively determining ammonia since it requires only 5 minutes per determination compared to the 15 to 30 minutes necessary for aeration or distillation methods.

By nesslerizing the receiving solution, samples containing 0.02 mg. of nitrogen can be analysed. This micro modification makes the method adaptable to nitrogen analysis on the small quantities of blood that are available when laboratory animals are used for research work.

BIBLIOGRAPHY

1. Pregl, Fritz, and Fyleman, E. "Quantitative Organic Microanalysis", P. Blakiston's Son and Co., Inc., Philadelphia, Pa., 1930. pp. 81-108.

2. Kjeldahl, J. Neue Methode zur Bestimmung der Stickstoffs in Organischen Korpen. Z. anal. Chem. 22:366 (1883).

3. Dakin, H. D. and Dudley, H. W. Some Limitations of the Kjeldahl Method. J. Biol. Chem. 17:275-280 (1914).

4. Miller, H. S. A Source of Loss of Ammonia in Kjeldahl Distillations. Ind. and Eng. Chem. (Anal. Ed.) 8:50-51 (1935).

5. Folin, O. and Wright, L. E. A Simple Macro-Kjeldahl Method for Urine. J. Biol. Chem. 38:461-464 (1919).

6. Folin, O. and Farmer, C. J. A New Method for Determining Nitrogen in Urine. J. Biol. Chem. 11:493-501 (1912).

7. Van Slyke, D. D. and Cullen, G. E. A Permanent Preparation of Urease and its Use in the Determination of Urea. J. Biol. Chem. 19:211-228 (1914).

8. Van Slyke, D. D. and Cullen, G. E. The Determination of Urea by the Urease Method. J. Biol. Chem. 24:117-122 (1916).

9. Stover, N. M. and Sandin, R. B. Use of Boric Acid in Micro-Kjeldahl Determinations of Nitrogen. Ind. and Eng. Chem. (Anal. Ed.) 5:240-242 (1933).

10. Ma, T. S. and Zuazaga, G. Micro-Kjeldahl Determination of Nitrogen. Ind. and Eng. Chem. (Anal. Ed.) 14:280-282 (1942).

11. Sobel, A., Yuskn, H., and Cohen, J. A Convenient Method of Determining Small Amounts of Ammonia and Other Bases by the use of Boric Acid. J. Biol. Chem. 118:443-446 (1937).

12. Nessler, J. Ueber das Verhalten des Jodquecksilber und der Quecksilberverbindungen ueberhaupt su Ammoniak und uber eine neue Reaction auf Ammoniak. Freiburg i. Br. (1856).

13. Winkler, L. W. Z. Untors. Nahr. Genussm. 49:164 (1925).

14. Folin, Otto and Denis, W. The Determination of Nitrogen by Direct Nesslerization. J. Biol. Chem. 26:473-489 (1916).

15. Folin, Otto and Denis, W. New Methods for the Determination of Total Nitrogen, Urea, and Ammonia. J. Biol. Chem. 11:527-536 (1912).

16. Peter, Amos W. The Micro-determination of Nitrogen by Direct Nesslerization. J. Biol. Chem. 39:285-298 (1919).

17. Bock, Joseph C. and Benedict, S. R. An Examination of the Folin-Farmer Method for Colorimetric Estimation of Nitrogen. J. Biol. Chem. 20:27-59 (1915).

18. Roe, Joseph H. and Irish, Oliver J. An Accurate Method for the Estimation of Urea in Blood and Urine by Direct Nesslerization. J. Lab. Clin. Med. 11:1087-1090 (1926).

19. Looney, Joseph M. The Determination of Blood Urea Nitrogen by Direct Nesslerization. J. Biol. Chem. 88:189-195 (1930).

20. Folin, Otto. A New Blood Sugar Method. J. Biol. Chem. 77:421-430 (1928).

21. Wong, San Yin. Persulfate in Direct Nesslerization. J. Biol. Chem. 55:431-435 (1923).

22. Karr, Walter G. A Method for the Determination of Blood Urea Nitrogen. J. Lab. Clin. Med. 9:329-333 (1923).

23. Koch, F. C., and McMeekin, T. L. A New Direct Nesslerization Micro-Kjeldahl Method and a Modification of the Nessler-Folin Reagent for Ammonia. J. Am. Chem. Soc. 46:2066-2069 (1924).

24. Davenport, H. A. A Note on the Koch and McMeekin Method for the Determination of Nitrogen. J. Lab. Clin. Med. 12:286-288 (1926).

25. Wicks, L. F. A Cheaper Nessler's Reagent by the Use of Mercuric Oxide. J. Lab. Clin. Med. 27:118-121 (1941).

26. Barrett, John Frederick. A Modified Nessler's Reagent for Micro-determinations of Urea in Tungstic Acid Filtrates. Biochem. J. 29:2442-2445 (1935).

27. Gentzkow, Cleon J. An Accurate Method for the Determination of Blood Urea Nitrogen by Direct Nesslerization. J. Biol. Chem. 143:531-544 (1942).

28. Hoffman, W. S. and Osgood, Bess. The Photoelectric Micro-determination of Nitrogenous Constituents of Blood and Urine by Direct Nesslerization. J. Lab. Clin. Med. 25:856-862 (1939).

29. Dehn, William H. Fallacies in Colorimetry. J. Am. Chem. Soc. 39:1392-1404 (1917).

30. Allen, E. R. and Davisson, B. S. On the Relative Accuracy of Colorimetric and Titrametric Procedures for the Determination of Nitrogen as Ammonia. J. Biol. Chem. 40:183-197 (1919).

31. Stehle, R. L. Gasometric Determination of Nitrogen and its Application to the Estimation of N. P. N. of Blood. J. Biol. Chem. 45:223-228 (1920).

32. Stehle, R. L. Note on the Gasometric Determination of Nitrogen. J. Biol. Chem. 47:11-16 (1921).

33. Van Slyke, D. D. The Gasometric Micro-Kjeldahl Determination of Nitrogen. J. Biol. Chem. 71:235-248 (1927).

34. Gad-Andresen, K. L. A Micro Method for the Estimation of Ammonia in Blood. J. Biol. Chem. 51:367-372 (1922).

35. Bunsen, R. Ueber quantitative Bestimmung des Harnstoffs. Ann. der Chem. 65:375 (1848).

36. Benedict, Stanley R. The Estimation of Urea. J. Biol. Chem. 8:405-421 (1910).

37. Benedict, Stanley R. and Gephart, Frank. The Estimation of Urea in Urine. J. Am. Chem. Soc. 30:1760-1764 (1908).

38. Keber, Philip A. Ammonia Distillation in the Presence of Calcium and Magnesium Salts. J. Am. Chem. Soc. 30:1279-1281 (1908).

39. Folin, Otto. The Determination of Urea in Urine. J. Biol. Chem. 11:507-522 (1912).

40. Folin, Otto and Wu, Hsien. A System of Blood Analysis. J. Biol. Chem. 38:81-110 (1919).

41. Clark, E. P. and Collip, J. B. A Procedure for the Determination of Urea in Folin-Wu Filtrates by the Autoclave Method. J. Biol. Chem. 67:621-627 (1926).

42. Leiboff, S. L. and Kahn, Bernard. A Rapid and Accurate Method for the Determination of Urea in Blood. J. Biol. Chem. 83:347-352 (1929).

43. Marshall, E. K. A Rapid Clinical Method for Determining Urea in Urine. J. Biol. Chem. 14:283-290 (1913).

44. Marshall, E. K. A New Method for Determining Urea in Blood. J. Biol. Chem. 15:487-494 (1913).

45. Folin, Otto and Svedberg, Andrea. Improved Distillation Method for the Determination of Urea in Blood. J. Biol. Chem. 88: 77-83 (1930).

46. Van Slyke, D. D. and Cullen, G. E. The Mode of Action of Urease. J. Biol. Chem. 19:141-180 (1914).

47. Behre, J. A. Observations on the Determination of Blood Urea. J. Biol. Chem. 56:395-404 (1928).

48. Addis, T. An Error in the Urease Method for the Determination of Urea. Proc. Soc. Exp. Biol. and Med. 25:365-367 (1928).

49. Howell, S. F. The Determination of Blood Urea. J. Biol. Chem. 129:641-660 (1939).

50. Folin, Otto and Youngburg, G. E. Note on the Determination of Urea in Urine by Direct Nesslerization. J. Biol. Chem. 38:111-112 (1919).

51. Gruskin, B. A New Shorter Method for the Determination of Urea and Sugar in Blood. J. Lab. Clin. Med. 10:233-236 (1924).

52. Taylor, W. F. and Blair, W. M. An Improved Method for the Determination of Blood Urea Nitrogen by Direct Nesslerization. J. Lab. Clin. Med. 17:1256-1263 (1932).

53. Taylor, W. F., Hayes, E. R., and Wells, B. B. An Improved Method for the Determination of Urine Urea Nitrogen by Direct Nesslerization. J. Lab. Clin. Med. 23:188-191 (1937).

54. Hoffman, W. S. and Osgood, Bess. A Micro-photoelectric Method for Urea Determinations in Blood and Urine by Direct Nesslerization. J. Lab. Clin. Med. 25:862-866 (1939).

55. Sure, Barnett and Wilder, Violet M. Simplified Photoelectric Procedures for Micro-determination of Nonprotein Nitrogen, Urea, and Creatin. J. Lab. Clin. Med. 26:874-878 (1940).

56. Hindmarsh, E. M. and Priestley, Henry. A Method for the Estimation of Urea in 0.1 cc. of Blood. Biochem. J. 18:252-254 (1924).

57. Feinblatt, Henry. Determination of Urea Nitrogen in Blood Without Aeration. J. Lab. Clin. Med. 9:66 (1923).

58. Mirkin, A. A Gasometric Method for Determining Nitrogen of Urea in Blood. J. Lab. Clin. Med. 8:50-52 (1922).

59. Van Slyke, D. D. Determination of Urea by Gasometric Measurement of the Carbon Dioxide formed by the Action of Urease. J. Biol. Chem. 73:695-723 (1927).

60. Stehle, R. L. Gasometric Determination of Urea in Urine. J. Biol. Chem. 47:13-17 (1921).

61. Menaul, Paul. The Hypobromite Reaction. J. Biol. Chem. 51:87-88 (1922).

62. Stehle, R. L. Note on Gasometric Urea Determination. J. Biol. Chem. 51:89-92 (1922).

63. Van Slyke, D. D. The Manometric Determination of Urea in Blood and Urine by the Hypobromite Reaction. J. Biol. Chem. 83:449-461 (1929).

64. Gad-Andresen, K. L. A Micro Urease Method for the Estimation of Urea in Blood. J. Biol. Chem. 51:373-376 (1922).

65. Kiech, V. C. and Luck, J. M. The Estimation of Urea and Amino Acids in Animal Tissues. J. Biol. Chem. 77:753-781 (1928).

66. Luck, J. M. The Oxidation of Dixanthydryl Urea, A Micro Method for Determining Urea. J. Biol. Chem. 79:211-219 (1929).

67. Beattie, Florence. A Micro-method for the Colorimetric Determination of Urea in Blood. Biochem. J. 22:711-712 (1928).

68. Ormsby, A. A. A Direct Colorimetric Method for the Determination of Urea in Blood and Urine. J. Biol. Chem. 146:595-604 (1942).

69. Barker, S. B. The Direct Colorimetric Determination of Urea in Blood and Urine. J. Biol. Chem. 152:453-463 (1943).

70. Schloesng. Annales de chimie et de physique. 31:153 (1851).

71. Folin, Otto. Zeitschr. f. Physiol. Chem. 32:504 (1901).

72. Shaffer, Philip. On the Quantitative Determination of Ammonia in Urine. Am. J. Physiol. 8:330-354 (1903).

73. Folin, Otto. Eine neue Methode zur Bestimmung des Ammoniaks im Harne. Zeit. Physiol. Chem. 37:161 (1902).

74. Boussingault. Annales de chimie et de physique. 29:472 (1850).

75. Steel, Mathew. An Improvement of the Folin Method for the Determination of Urinary Ammonia Nitrogen. J. Biol. Chem. 8:365-379 (1910).

76. Folin, Otto. Note on the Determination of Ammonia in Urine. J. Biol. Chem. 9:497-498 (1910).

77. Folin, Otto and MacCallum, A. B. On the Determination of Ammonia in Urine. J. Biol. Chem. 11:523-526 (1912).

78. Yee, J. Y. and Davis, R. E. Determination of Ammoniacal and Urea Nitrogen. Ind. and Eng. Chem. (Anal. Ed.) 7:259-261 (1935).

79. Sandemer, S. L. and Schaible, Philip. Micro-determinations of Ammonia Nitrogen in Eggs. Ind. and Eng. Chem. (Anal. Ed.) 8:201-202 (1936).

80. Brown, W. E. The Clinical Estimation of Ammonia in Urine by the Formalin Method. J. Am. Med. Assn. 53:2071-2073 (1909).

81. Orr, A. P. A Colorimetric Method for Direct Estimation of Ammonia in Urine. Biochem. J. 18:806-808 (1924).

82. Folin, Otto and Bell, R. C. Application of a New Reagent for the Separation of Ammonia. J. Biol. Chem. 29:329-335 (1917).

83. Sumner, J. B. A New Method for Direct Nesslerization of Ammonia in Urine. J. Biol. Chem. 34:37-41 (1918).

84. Hinshart, Robert, Grondahl, R. D., West, E. S. A Rapid and Accurate Method for the Distillation of Ammonia Application to the Determination of Nitrogen, Ammonia, and Urea in Biological Fluids. Arch. Biochem. 2:163-174 (1943).

85. Van Slyke, D. D., MacFadyen, D. H., and Hamilton, P. H. J. Biol. Chem. 141:671-680 (1941).

Typed by Jean Wiedemann