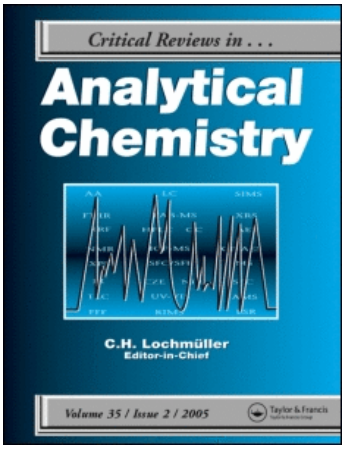

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THE DETERMINATION OF NITROGEN IN BIOLOGICAL MATERIALS

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I. INTRODUCTION

The determination of nitrogen in biological materials may be performed quantitatively by any of four methods, each being named after the author who devised the particular experimental procedure: Dumas,[1] Kjeldahl,[2] Ter Meulen,[3] and Ohashi.[4] The four original methods have been employed to determine quantitatively the nitrogen in organic compounds of biological interest; the methods have been improved and made more sensitive by the introduction of modern physicochemical techniques. Thermal conductivity and mass spectrometry measurements have improved the modifications of the original methods.

The Dumas method has been applied to the determination of nitrogren in a wide variety of solids down to the microscale of analysis. The original procedure has been modified by replacing the copper oxide with more efficient catalysts such as nickel oxide[5] or cobalto-cobaltic oxide[6,7] and by decreasing the time of combustion.[8-15] Other improvements in the method will be discussed in greater detail later.

The Kjeldahl method, which was originally described in 1883, has been greatly modified. Originally, sulfuric acid was used alone without a catalyst or salts to raise its boiling point. The decomposition of the organic substance under examination was hastened by the addition of potassium permanganate crystals as a finely ground powder. Mercuric oxide was introduced as a catalyst by Wilfarth[16] to reduce the decomposition time of the sample by concentrated sulfuric acid. The temperature of the boiling acid digestive mixture was raised when Gunning[17] began adding potassium sulfate. More recently, there have been attempts to standardize the digestive procedure[18-20] and investigate the effect of adding salts and catalysts to the concentrated sulfuric acid before the digestion of the sample.[18-25] The sealed-tube digestion method, with a catalyst, was described by White and Long.[26] This procedure raises the boiling point of the digestive mixture to a much higher temperature (e.g., from 328 to 470°C) and has been modified by several authors.[27-30] The application of more sensitive reagents and the use of spectrophotometric procedures of the determination of ammonia have led to more accurate measurements of the quantity of nitrogen in biological materials.[30-37]

The Ter Meulen method as originally described[3] has been used and improved by Smith and West[38] for the determination of nitrogen in grass, hay, albumen, yeast, meat, cheese, milk, and other biological materials,[39] as well as in hides and leather.[40] This method has not received the same attention as the two classical methods (the Dumas or Kjeldahl).

The Varrentrap and Will[41] method is not well known; however, it has been modified and improved by Shamrai et al.[42] and used for the analysis of a number of biological specimens. The modified method is rapid and has been employed for analysis of samples in the microrange; the authors have reported that the method is as accurate as the Kjeldahl procedure. A more recently developed method was described by Ohashi[4] and later modified by Takagi and Tusginori.[43] It has also been employed as a rapid method for the microanalysis of a variety of biological materials.

The foregoing methods have been applied to the determination of total nitrogen in particular biological specimens; however, it is sometimes necessary to determine the nitrogen in minor components of the specimens. Recently, there have been applications of ion-exchange resin chromatography to determine the individual α-amino acids in the protein hydrolysate as an alternative method of checking the total nitrogen content of a protein in a food diet.[30,44] The quantities of individual nitroso compounds present as minor toxic constituents in food products have been determined by gas-liquid chromatography, which will be discussed later in detail.[45,46] The determination of trace amounts of nitrate in biological materials has been made with an ammonia-gas-sensing electrode, following reduction of the nitrate with Devarda alloy.[47] A similar technique

has been used to determine nitrate in food using a nitrate-ion-selective electrode.[48] Recently, a noninvasive technique using muonic X-rays to determine the nitrogen present in tissue has been developed.[49] The X-ray spectra from several types of tissue were used to calculate the amounts of the elements, including nitrogen, in the tissue. An isotope dilution method has been used to determine the nitrogen in samples containing urea and sulfamate nitrogen as well as ammonia nitrogen or easily reducible amino groups.[50] Another radiometric method, proton activation, has been employed to determine ^{14}O produced by the irradiation of the protein content of grain by protons.[51]

II. THE DUMAS METHOD

A. The Effect of Temperature

The Dumas procedure followed by early workers and recommended by Pregl[52] depended on the combustion of the sample (at a temperature of 700 to 750°C) after it had been intimately mixed with copper oxide and copper. A device was designed by Clark and Stillson[53] to reduce the period of combustion and simulate manual burning of the sample; another automatic control of the rate of combustion was used by Fischer.[54] Similar rapid methods of combustion have been described.[55,56] The rapid method that required nickel oxide and nickel metal in the combustion tube instead of copper oxide and copper metal needed a higher temperature of 1050°C for more effective combustion.[5]

The Dumas method, as adapted by Pregl,[52] is known to produce inaccurate results with certain types of compounds such as purines, pyrimidines, and long-chain fatty acid amides.[9] Methane may be produced from the incomplete combustion of the sample, or nitrogen may be retained by carbonaceous residue deposited in the combustion tube: these errors cause loss of nitrogen. These difficulties may be avoided in some cases by recycling the gaseous combustion products.[8] Manganese dioxide, heated to 500 to 600°C and forming part of the combustion tube filling, has been used. More recently, Rezl and Kaplanova[57] have maintained different temperatures in separate portions of the combustion tube. They used both cupric oxide and cobaltic-cobaltous oxide in the oxidizing section heated to 850 to 950°C and copper metal in the reducing section at 500 to 650°C. Higher temperatures of combustion and the use of more effective catalysts are the primary improvements of the Dumas method in its application to the determination of nitrogen in many materials of biological interest.

B. The Influence of Various Catalysts at Different Temperatures

Copper oxide and copper metal in intimate mixture was used as a catalyst in the origial Dumas method; however, several workers subsequently found that this catalyst was unsatisfactory at the temperatures used at the time (700 to 750°C). Haas[58] showed that there was incomplete oxidation of methane on a macroscale of analysis and reported that this error could be prevented by mixing the sample with either cuprous chloride, cupric chloride, lead chloride, or lead chromate in place of the copper oxide in the combustion tube. Other catalysts used for the Dumas technique included potassium chlorate,[59] potassium dichromate,[60] mercuric acetate,[61] copper acetate,[62] or cobaltic oxide[6,7] in admixture with the sample.

The use of copper oxide in the Dumas procedure may produce two possible errors. Kirsten[63,64] showed that the copper oxide may yield significant quantities of oxygen at the temperatures he used and that in the presence of the oxygen the copper oxide may retain oxides of nitrogen. Kirsten replaced the copper oxide with nickel oxide and raised the temperature of combustion to 1050 to 1100°C.[13] Cobalto-cobaltic oxide was introduced as a catalyst in a later modification.[6,7] A recent improvement in the Dumas method has been described in which vanadium pentoxide was used as a catalyst for a

gas-solid chromatographic determination of nitrogen, carbon, and hydrogen by an automated procedure.[65] When the combustion temperature is raised and a more efficient catalysts is used, the quantitative determination of nitrogen is possible in samples of 0.5 to 2.5 mg.[57]

C. The Empty Tube Method

The Pregl-Dumas microcombustion procedure was considered by some observers to be very tedious, and several modifications of the method were developed to make possible more rapid analyses. Belcher and co-workers[66-68] demonstrated that the combustion of organic compounds on the macroscale can be effective with quite simple apparatus, without loss of accuracy. The sample was heated in a stream of oxygen which passed through an empty combustion maintained at a temperature of 800°C by a furnace of length 25 cm. The rate of flow of oxygen was controlled so that the total time for an analysis, including the time taken for the weighing operations, did not exceed 30 min. This procedure could be effective for the combustion of samples weighing 3 to 5 mg.[69]

Modifications of this empty tube method have been developed by Pfab[70] and Baba[71,72] in which the oxygen was flushed through the combustion tube at a rate of 8 to 10 mℓ/hr while the temperature was maintained at 850°C. The excess oxygen was carried by a stream of carbon dioxide and absorbed on copper heated to 500 to 600°C. The oxides of nitrogen were reduced to nitrogen, which accummulated in the nitrometer. This procedure has been applied to samples of 1.0 to 1.5 mg, and the results from eight compounds showed absolute errors of less than ± 0.43%.

D. The Ohashi Method

This procedure was first described by Kiba et al.[73] for a volumetric analysis of elemental carbon by decomposition of the sample with iodic acid in the presence of phosphoric acid. Ohashi[4] then applied the method to the rapid analysis of nitrogen in organic compounds. Later, Tagaki and Tsuginori[43] used the technique to determine the nitrogen in samples of casein, fish meal, soybean powder, green tea, and wheat flour and obtained results which agreed well with the data obtained by the Kjeldahl method on composite samples. An analysis by this method could be completed in 20 min.

III. THE KJELDAHL METHOD

A. The Influence of Temperature on the Digestive Process

The original Kjeldahl method required only concentrated sulfuric acid to which was added a small quantity of potassium permanganate crystals to aid in the decomposition of the sample for analysis. The addition of oxidizing agent raised the boiling point of the digestive mixture only slightly above that of the concentrated sulfuric acid (330°C). Wilfarth[16] was the first observer to discover that mercuric oxide acts as a catalyst in the decomposition of the sample, and Gunning[17] introduced the idea of raising the boiling point of the sulfuric acid by means of potassium sulfate. Arnold[74] considered a mixture of copper and mercury to be more efficient than either metal alone, and in a later publication with Wedemeyer,[75] a mixture of copper, mercury, and potassium sulfate was used. All of these modifications of the original Kjeldahl method raise the boiling point of the digestive mixture; however, the use of one or more catalysts added to the concentrated sulfuric acid was considered essential. More recently, Belcher et al.[28] decomposed certain compounds in a sealed tube with concentrated sulfuric acid alone at a temperature of 420 to 430°C for 30 min. Using a quartz torsion balance, they were able to analyze submicroquantities of material (less than 50 μg).

White and Long[26] used the sealed tube technique at a temperature of 470°C and

added a catalyst to aid the decomposition of the sample. More recent work by Jacobs[20,30,31] shows that refractory substances such as nicotinic acid, 8-hydroxyquinoline, 5-methyl-2-*n*-propyl-4-hydroxypyrimidine, and 5-methyl-2-*n*-propyl-4-thiol pyrimidine may be effectively analyzed for nitrogen by this procedure. It has been shown[20] that the quantitative recovery of nitrogen from nicotinic acid is dependent upon the temperature and the presence of a catalyst in the digestive mixture is not critical, provided the period of digestion is adequate. The presence of a catalyst will expedite the decomposition of the sample and allow the quantitative recovery of the nitrogen at a slightly reduced temperature.

B. The Effect of a Catalyst on the Decomposition of the Sample

Mercuric oxide was used as a catalyst by Wilfath[16] and has been shown by more recent observers[20,76,77] to be more efficient than the other known catalysts employed with sulfuric acid for the decomposition of biological substances. Other catalysts used in studies on the digestion of nitrogenous substances by concentrated sulfuric acid include copper sulfate,[16,80] selenium,[81] selenium oxide,[18-24,80,82] sodium selenate,[83] selenium oxychloride,[84] and tellurium.[19,25] Several other catalysts have been employed[16,85,86] with concentrated sulfuric acid, but they were found to be less efficient than mercury, copper, and selenium.

Beet[87] was the first worker to use a combined mercury and selenium catalyst in the digestive mixture. Several other combinations of catalysts have been employed successfully.[30,37,88,89] Several observers have reported that mercuric oxide is more efficient than other catalysts; however, in their studies of the comparative efficiencies of the catalysts, no accounts of the temperatures or variations in the digestion periods were reported. Recently, a study by Jacobs[20] of the Kjeldahl digestion procedures at controlled temperatures in sealed tubes has shown that the presence of catalyst, whether mercuric oxide alone or mixed with copper sulfate and selenium, is not critical, provided the temperature is sufficiently high and the period of digestion is adequate.

C. Comparison between the Kjeldahl Open Tube and Sealed Tube Methods

The factors which may affect the digestion of the nitrogenous biological material by concentrated sulfuric acid are manifold. The effects of the variations in concentrations of the salts and the presence of catalysts in the acid digestive mixture have been discussed in the previous section. The rate of heating and the prevention of superheating in the digestive mixture are difficult to control in the open tube method. The main cause of the uncontrolled conditions is the source of heat. The change in concentration of salts during the digestive period, due to decomposition of the concentrated sulfuric acid by reaction with the sample, has been studied by some observers;[18,19,24] however, the different boiling points recorded for the same nominal digestive mixture by six observers[18,19,21-23,25] indicate either lack of control in heating or errors in measuring the boiling points.

The original Kjeldahl method[2] involved three stages: decomposition of the sample, separation by distillation of the ammonia after the digestive mixture had been made alkaline, and titration of the residue of a quantity of standard acid after the ammonia had been absorbed in and neutralized by part of a known excess of standard acid. During each of the three stages, loss of nitrogen may occur. Direct loss of ammonia may occur during the digestive procedure if the operation is prolonged[76-78,81,90] or an unsuitable catalyst [77,79,81,88-90] is employed for the given conditions. The loss of nitrogen as ammonia during the digestive procedure may result in the case of a sample containing a relatively low concentration of nitrogen but a high concentration of carbohydrate or other substance difficult to digest within a reasonable period, e.g., a food diet.[44] The distillation procedure also may involve a loss of ammonia if the addition

of alkali to the digested sample does not release all of the ammonia. This may occur when a mercury catalyst has been used and the mercury-ammonia complex is not completely decomposed.

The use of the sealed tube method eliminates the loss of ammonia during the digestion stage unless the temperature of the digestive mixture is too high.[27,30] The digestion of the sample may be completed in a sealed tube without the use of a mercury catalyst, in which case there will be no difficulty in recovering the ammonia quantitatively when the digestion mixture is made alkaline and the ammonia is separated by steam distillation. As a consequence, the sealed tube method has two advantages over the normal open tube Kjeldahl procedure. Table 1 indicates that the digestion in concentrated sulfuric acid of nicotinic acid (a substance known to be refractory) is complete when performed in a sealed tube without a catalyst, provided the temperature and period of digestion are adequate. [20]

The sealed tube method may be used advantageously when the sample requires pretreatment before the final digestion stage;[31] a further possibility of loss of nitrogen in the sample may be avoided. The ammonia in the digested sample may be determined quantitatively by a colorimetric procedure[30,31,37] without separating the ammonia by either steam distillation or diffusion. The ammonia is quantitatively determined as a colored complex when allowed to react with indanetrione hydrate at 100°C for 30 min. in a suitably buffered medium. The indanetrione hydrate method may be used on the ultramicroscale.[20,91] Recently, the sealed tube method has been used by Jacobs[20,44] to determine quantitatively the nitrogen in food diets; however, the food diet must first be hydrolyzed in a large volume of 6 *N* hydrochloric acid prior to analysis of a small aliquot of the hydrolysate by the Kjeldahl sealed tube technique. Table 2 shows results obtained when composite samples of food diets are analyzed by the Kjeldahl open tube method and sealed tube method to quantitatively determine nitrogen content. The quantitative determinations of nitrogen in proteins and mucopolysaccharides by the sealed tube method are listed in Table 3. The samples were hydrolyzed in 6 *N* hydrochloric acid at 105°C for 24 hr, and the solutions of hydrolyzates were dried by evaporation in a vacuum desiccator in the presence of sodium hydroxide pellets.[20] The resultant dry mixture of amino acids or amino acids plus amino sugars was dissolved in a sodium citrate buffer solution and analyzed by the sealed tube technique.[44] Aliquots of the hydrolyzates were examined by the Beckman Spinco® amino acid analyzer (Model 120C) using the ion-exchange chromatographic method described by Jacobs[91] and modified by using Zeokarb 225® ion-exchange resin spherical beads of a very fine grade with diameters mostly from 8 to 10 μm.[92] The right-hand column of Table 3 shows total nitrogen contents of the individual amino acids and amino sugars determined quantitatively by reaction with indanetrion hydrate after separation on the column of ion-exchange resin.[20]

A tracing from the recorder of the Bechman Spinco amino acid analyzer (Figure 1) indicates that only ammonia was present in the buffered digestive mixture, and the nitrogen recovery was quantitative. The decomposition of the normal horse serum protein in 50%(v/v) sulfuric acid at 470 ± 5°C was complete.

IV. THE DETERMINATION OF AMMONIA PRODUCED IN THE DIGESTIVE PROCESS

A. Separation of the Ammonia by Distillation or Diffusion

The original Kjeldahl procedure involved the separation by steam distillation of the ammonia from the digestive mixture after the latter had been rendered alkaline. The ammonia was absorbed and neutralized by a known excess quantity of a standard acid solution. The residue of the standard acid was determined by titration, and the quan-

TABLE I

Determination of Nitrogen in Nicotinic Acid: Effect of Temperature and Period of Digestion[20]

Temperature (°C)[a]	Period of digestion (min)	Nitrogen content[b] (%)
No Catalyst		
350	30	0.2
	60	0.3
	120	0.9
370	30	0.3
	60	0.3
	120	1.2
390	30	0.6
	60	3.2
	120	3.4
410	30	1.4
	60	3.3
	120	8.5
430	30	2.5
	60	8.0
	120	10.4
450	30	6.6
	60	9.2
	120	11.3
470	30	11.3
	60	11.3
	120	11.6
490	30	11.5
	60	11.1
	120	11.3
Mercury Catalyst		
350	30	0.3
	60	1.2
	120	3.6
370	30	0.4
	60	2.8
	120	9.5
390	30	0.8
	60	8.2
	120	9.8
410	30	1.1
	60	8.3
	120	10.7
430	30	7.4
	60	11.3
	120	11.6
450	30	9.3
	60	11.4
	120	11.3
470	30	11.4
	60	11.4
	120	11.2
490	30	11.4
	60	11.5
	120	11.3
Mercury-selenium-copper Catalyst		
350	30	0.6
	60	1.3
	120	3.8
370	30	0.6
	60	1.9
	120	5.1
390	30	1.4
	60	7.9
	120	9.5
410	30	3.4
	60	8.5
	120	10.6
430	30	6.9
	60	11.5
	120	11.5
450	30	9.4
	60	11.4
	120	11.3
470	30	11.3
	60	11.4
	120	11.4
490	30	11.3
	60	11.1
	120	11.3

[a] ± 5°C.

[b] Mean of triplicate determinations (theoretical value 11.39%).

From Jacobs, S., *Analyst* (London), 89, 489 (1964). With permission.

TABLE 2

Nitrogen Contents (% N) of Food Diets: Comparison of Kjeldahl Open Tube with Sealed Tube Method

Food diet (No.)	Kjeldahl open tube (whole diet)	Sealed tube (hydolyzate)
1	5.09	5.07
	4.67	5.12
	4.87	5.00
	4.96	4.97
2	3.22	3.32
	3.21	3.32
	3.17	3.29
3	4.37	4.26
	3.89	4.48
	4.45	4.34
4	3.08	3.11
	3.03	3.19
	3.04	3.15
5	6.06	6.11
	6.12	6.15
	6.05	6.19

From Jacobs, S., *Analyst* (London), 89, 489 (1964). With permission.

TABLE 3

Nitrogen Contents of Proteins and Mucopolysaccharides

Sample	Method: Sealed Tube	Method: Beckman Spinco® amino acid analyzer
Proteins (μg N)		
Human fetal globin		
Sample 1	361	360
Sample 2	362	372
Rabbit globin α-chains	232	225
Rabbit globin β-chains	237	229
Mucopolysacchardies (%N)		
Chondroitin-4-sulfate peptide		
Sample 1	3.15	3.15
	3.10	3.09
Sample 2	3.20	3.22
	3.14	3.20
	3.18	3.15
Heparin sulfate-peptide	3.24	3.20
	3.18	3.12
	3.17	—

From Jacobs, S., *Analyst* (London), 89, 489 (1964). With permission.

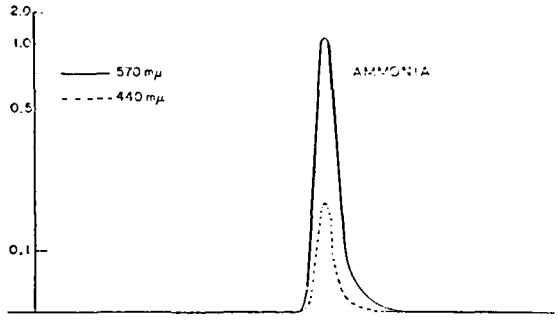

FIGURE 1. Diagram showing the relation between volume of eluate and absorbance of indanetrione hydrate-positive constituent in the sample of protein digested by sulfuric acid in the sealed tube Kjeldahl method.[187]

tity of standard acid required to neutralize the ammonia could then be calculated. A modified form of the Kjeldahl method allowed the ammonia to be absorbed into a 2% solution of boric acid after steam distillation from the digestive mixture (already alkaline). The ammonia then could be titrated directly against the standard acid with the use of a suitable mixed indicator.

The microdiffusion technique, first described by Durig,[93] relies upon a similar principle for the determination of very small quantities of ammonia. The method was improved by Neuberg;[94] however, the greatest development arose from the studies by Conway and Byrne,[95] who contolled the procedure, obtaining with great precision the quantitative determination of nitrogen as ammonia. The microdiffusion method has been developed by Byrne[96] for the analysis of other volatile substances, and other workers further modified this method for the special requirements of their protein studies.[97-102] The Cavett flask[103] was developed for the determination of ammonia by the microdiffusion technique and was applied by Milton and Duffield[104] to the analysis of clinical specimens. The ammonia separated by microdiffusion was quantitatively determined more conveniently by titration against dilute standard acid rather than by a colorimetric method.

Ammonia may be determined quantitatively by any one of several colorimetric procedures (after separation by steam distillation or thermal diffusion from sulfuric acid digests which have been rendered alakaline). The spectrophotometric procedures are usually more convenient when larger quantitites of nitrogen are to be determined (cf. the indanetrione hydrate method).[105] In such cases, the separated ammonia is absorbed by a suitable medium which may be diluted to a predetermined volume of solution from which a convenient aliquot may be taken for the colorimetric analysis.

B. Quantitative Determination of Ammonia without Prior Separation

1. The Nessler Method

The Kjeldahl procedure involving the sequence of digestion, distillation, and titration operations was considered by many observers to be tedious and time consuming. The Nessler technique was introduced for the rapid analysis of replicates of a particular material or serial analysis of specimens of a similar type. Other colorimetric methods have been used to determine directly the ammonia in aliquots of the acid digestive mixture after the latter had been neutralized or treated chemically in an appropriate manner.

The Nessler method is rapid and simple in operation; however, this technique has several disadvantages. The reagent is not stable on storage for any appreciable period

of time; therefore, it is necessary to examine standard samples containing known quantities of ammonia together with the unknown samples. The Nessler reagent only reacts with ammonia and will not detect organic amines or other nitrogen-containing compounds, such as amino acids, that may be present in the original Kjeldahl digestive mixture.[106] The factors which influence the reaction between ammonia and the Nessler reagent were studied by Yuen and Pollard.[107] They showed that the period of reaction, the amount of reagent, the temperature of the reaction during the color development, the presence of other cations or anions, and the pH of the reaction mixture were critical to the procedure. Their technique was used successfully to determine the nitrogen in agricultural materials; it was necessary to control rigidly all factors involved in operations. The same authors[108] modified the digestive procedures in a later study and used the Nessler method to examine soil extracts and plant tissue. A more stable Nessler reagent was used by Connerty et al.[109] to determine the nitrogen in samples of blood urea. There have been two other modified and improved forms of the Nessler reagent, and these have been described by Middleton[110] and Hill-Cottingham and Wagner.[111]

Although the Nessler reagent has been used frequently for many purposes,[106-113] great care is required to make a precise, quantitative determination of the nitrogen in samples containing 10 μg of the element.[114] Varieties of barley and amino acids of barley protein have been examined by Hecht and Fritz,[115] who used the Nessler reagent to determine nitrogen contents. They observed that, although the Nessler method gave useful results in the hands of trained personnel, the Kjeldahl procedures proved superior for the analyses of their biological materials. The Nessler procedure was used by Hutchinson and Labby,[116] combined with an ion-exchange chromatography technique, to determine the ammonia in blood samples.

2. The Phenol-Hypochlorite Method

The phenol-hypochlorite spectrophotometric method described by Russell[117] has been used extensively to determine nitrogen as ammonia that was allowed to react with sodium phenate and potassium hypochlorite solutions. The mixture was heated and the absorbance of the solution containing the blue complex was determined by a photometer. Both the temperature of the reaction mixture and the period of the reaction were shown by Riley[118] to be critical in controlling the procedures; however, he also discovered that the addition of Mn^{2+} ions had a critical effect upon the reaction. In later studies by Lubochinsky and Zalta,[119] it was shown that nitroprusside could act as a catalyst in the reaction between sodium phenate and hypochlorite and the Russell method could be controlled with greater precision. Exley[120] has applied the phenol-hypochlorite method as an ultramicrotechnique to the determination of nitrogen in bovine serum albumin, casein, egg albumin, human serum, and insulin. He has also used the method for the analysis of cat and human cerebrospinal fluids; however, special precautions were necessary because certain critical factors involved in the procedures rendered the method difficult to use.

A rapid method for the determination of urea in serum has been developed by Searey and Gough,[121] who converted the urea to ammonia by means of urease and measured the ammonia with the phenol-hypochlorite technique. The Russell method has also been applied by Konitzer and Voigt[122] to the analysis of blood samples, whereas a simple ion-exchange technique, used in conjunction with the phenol-hypochlorite procedure, was employed by Miller and Rice[123] to determine the ammonia nitrogen in blood plasma.

3. The Indanetrione Hydrate Method

The reagent indanetrione hydrate was first described by Ruhemann[124] as triketohydrindene hydrate. Later, the reagent became commonly known as ninhydrin. The re-

action between indanetrione hydrate and α-amino nitrogen groups was first described by Herzfeld.[125] The use of the reagent for the colorimetric determination of α-amino nitrogen was confirmed by Harding and McClean.[126] Harding and Warneford[127] reported the use of indanetrione hydrate for the determination of ammonium compounds. Harding and McClean.[128] also employed the reagent for the detection of amines and amides. Moore and Stein[129] employed the indanetrione hydrate reagent for the analysis of amino acids in protein hydrolysates after separation of the individual amino acids by a chromatographic technique using columns of starch.[13] This latter procedure was useful in separating the ammonia from the individual amino acids present in the sample of protein hydrolysate. Moore and Stein modified the reagent by dissolving the indanetrione hydrate in a suitable strong buffer solution so that the eluates of varied pH produced a reaction mixture with a pH confined close to 5.0. In a later publication, Moore and Stein[131] further modified the reaction mixture to facilitate the analysis of the eluates containing amino acids or ammonia obtained from columns of ion-exchange resin.

The Ruhemann's purple complex, which results from the sensitive reaction between ammonia and indanetrione hydrate, has been used by Jacobs[20,30,37,105] to determine the total nitrogen in proteins. The mechanism of the reaction between the indanetrione hydrate and α-amino acids has been studied by Moore and Stein[129] and Boissonas and Haselbach.[132] The absorption spectrum for the purple complex obtained from the reaction between indanetrione hydrate and the α-amino acids appears to be identical with the complex formed during the reaction between indanetrione hydrate and ammonia.[105,129,133] Other workers have employed the reagent to determine the nitrogen in proteins; Baudet and Cherbuliez[137] used indanetrione hydrate to determine the nitrogen of polypeptides and amino acids isolated from paper chromatograms and ionophoretograms after digesting the eluate by heating it with 18 *N* sulfuric acid to 450°C in a sealed capillary tube. The ammonia nitrogen and amide nitrogen in several native proteins have been determined by Chibnall et al.[138]

The nitrogen in many heterocyclic organic compounds has been determined by means of the sealed tube digestion in concentrated sulfuric acid. The resultant ammonia has been quantitatively determined by means of indanetrione hydrate in compounds such as nicotinic acid,[30] 8-hydrixyquinoline,[20] streptomycin,[30] viomycin,[133] and collistin.[139] Certain pyrimidines containing the $=N-CH_3$ group which were found difficult to analyze by the Dumas method could be digested satisfactorily by concentrated sulfuric acid in a sealed tube heated to 460 to 480°C, and the ammonia was determined quantitatively by indanetrione hydrate.[31]

The great advantage in using indanetrione hydrate for the spectrophotometric determination of ammonia is the lack of interference by cations[30] and anions[30,105] in the reaction mixture, provided the pH of the reaction mixture is suitably adjusted.[30] The excess indanetrione hydrate reagent contributes no absorbance at the wavelength of light (570 nm) at which the Ruhemann purple complex is measured (Figure 2).

The molar extinction value of the complex is 20,125 when measured in a cell path length of 10 mm. When a sample aliquot of 2.0 mℓ volume from the neutralized and buffered digestion mixture is allowed to react at 100°C for 30 min. with 2.0 mℓ of a 2% buffered solution of indanetrione hydrate and the cooled reaction mixture diluted with 6.0 mℓ of a 50% (v/v) aqueous solution of ethanol, a 0.4 μmol of ammonia will produce a sufficient quantity of the Ruhemann's purple in the 10-mm cell to give an absorbance of 0.805 ± 0.01 at 570 nm wavelength.[30] If microcells of path length 10 mm and small volume (≈ 0.5 mℓ) are used, a 0.2-mℓ sample containing 0.04 μ*M* ammonia and processed in a similar manner to yield a final volume of 1,0 mℓ will produce a sufficient quantity of the colored complex to give an absorbance of 0.805 as above.[105]

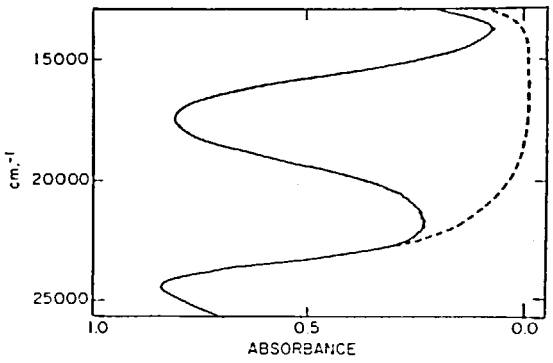

FIGURE 2. Diagram showing the absorbance spectra of indanetrione hydrate (broken line) and the ammonia-indanetrione hydrate complex (continuous line) between 25,000 cm^{-1} (400 nm) and 13,000 cm^{-1} (780 nm).[187]

4. Other Colorimetric Methods

The colorimetric determination of α-amino nitrogen has been made by several methods (cf. the Van Slyke method). Pope and Stevens[140] described a photometric copper phosphate method that has been used extensively to determine the α-amino nitrogen in individual amino acids, tryptic digests of fibrin, and the acid hydrolysates of zein, edistin, and gelatin. Sobel et al.[141] used the photometric copper phosphate method to determine the α-amino nitrogen in samples of urine, and the authors compared that method with the naphthoquinone method and the gasometric indanetrione hydrate method. They isolated the amino acids on a column of ion-exchange resin (Dowex® 50W × 8 200 to 400 mesh analytical grade H form) in a preliminary procedure and concluded that the specificity of the copper phosphate and the naphthoquinone method was improved by the isolation procedure. Kaniuga and Toczko[142] also used the photometric method together with a modified ion-exchange chromatography technique to examine urine samples for α-amino nitrogen content. Modifications of the photometric copper complex method have been described by other workers for samples of urine[143] and blood plasma.[144]

The microdetermination of ammonia by a novel colorimetric procedure was described by Stone,[145] who applied the method to the analysis of glutamine, blood, and brain tissue. The small samples of ammonia (0.05 to 0.5 μmol) were oxidized by the addition of hypobromite; the excess of hypobromite was determined by its ability to decolorize phenosafronin.

The nitrogen contents of samples of wheat and barley have been determined by Jennings,[146] who used a very simple procedure. The sample (500 ± 5 mg) was treated with a small volume (2 mℓ) of carbon tetrachloride and 50 mℓ of a reagent mixture containing potassium sodium tartrate, potassium hydroxide, and copper sulfate. The final mixture of reagent and sample was shaken for 1 hr, and aliquots were centrifuged before being examined spectrophotometrically.

The chloramine-T photometric method for the determination of the nitrogen content of plants was used by Pochinok.[147] The same photometric method was employed by Fric[148] to determine the nitrogen in biological and soil samples. He used a normal Kjeldahl open tube method of digestion and directly determined the ammonia in the digestive mixture without separating the ammonia by distillation. Both of the latter authors reported that the method is accurate and more convenient and rapid than the normal Kjeldahl distillation and titration procedure.

C. A Comparison between the Titration and Spectrophotometric Methods

Each of the Kjeldahl titration and modern spectrophotometric methods used to determine ammonia quantitatively may be adapted to the measurement of micro- and ultramicroquantities of the compound. The relative merits of the two methods may be determined in terms of the apparatus and materials available, the purity of the reagents commercially available, and the availability of techniques that may be required to remove ammonia contamination from the reagents.

Ammonia is ubiquitous and may be found in all reagents as a contaminant. The amount of the ammonia contaminant present in a reagent will depend on the particular batch of reagent or the source of the manufacture. Special precautions may be necessary to purify those reagents which either form addition complexes with ammonia or in which ammonia is soluble. It is necessary to perform control experiments in determining ammonia by either the titration or the spectrophotometric methods. The microanalytical reagent grade of chemicals in general is best suited for use in the determination of micro- and ultramicroquantities of ammonia.

The titration technique may be used to quantitatively determine extremely small quantities of ammonia,[28,95,149-151] provided suitable precautions are employed. The microanalytical grade reagents required for the titration operations are few in number and are generally of good quality. The control solutions may be expected to contain only very small quantities of ammonia as a contaminant. However, some of the modern spectrophotometric methods, including the indanetrione hydrate method, require the use of reagents which cannot easily be freed from the ammonia. In such cases, the control solutions may contain relatively large quantities of ammonia, and consequently, the methods will not be suitable for the determination of ultramicroquantities of nitrogen, unless special precautions are taken to purify the reagents.

A particular case is that of indanetrione hydrate reagent which is prepared as a 2% solution in a solvent mixture containing methyl cellosolve (2-methoxy ethanol) and a sodium acetate buffer solution. The methyl cellosolve usually contains peroxides (which interfere by reducing the development of the Ruhemann's purple) and ammonia. Both of these substances are removed in one operation so that cheapest technical grade of the methyl cellosolve may be used efficiently. A solution containing 50 g $FeSO_4 \cdot 7H_2O$ in 100 m*l* 2*M* sulfuric acid is prepared with conductivity water.[152] A small volume (10 m*l*) of the ferrous sulfate solution is added to each liter of the methyl cellosolve before the latter is distilled. The peroxides are removed from the methyl solvent when they convert ferrous sulfate to ferric sulfate, and the latter combines with the ammonia present to form ferric ammonium sulfate. It is necessary to store the distilled methyl cellosolve in a well-sealed glass container as a precaution. The indanetrione hydrate of technical grade is purified by a very simple procedure without any loss which would otherwise be incurred if it were recrystallized from water or 1.0 *M* hydrochloric acid. The indanetrione hydrate is dissolved as a 4% solution in methyl cellosolve purified as previously described, and a small quantity of cation-exchange resin beads in the hydrogen form (approximately 50 m*l*) is added to 1*l* or less of the 4% stock solution of indanetrione hydrate. The latter may be Zeokarb 225, Dowex 50, or any other suitable resin bead and the water regain value or cross-linkage value is unimportant.[153] The sodium acetate required for use in the indanetrione hydrate reagent mixture is easily recrystallized by adding ethanol to the almost saturated sodium acetate solution and cooling the mixture to 0°C. The crystals of sodium acetate are filtered on a sintered glass funnel and washed with ethanol which has been cooled to 0°C. Excess ethanol may be removed by suction from a water pump and the sodium acetate crystals are dried in a vacuum desiccator over silica gel.[154] The ethanol may be filtered through a shallow layer of suitable cation-exchange resin (similar to that used to purify the 4% indantrione hydrate reagent in methyl cellosolve) as a precautionary

measure to remove ammonia from the ethanol (in which ammonia is very soluble).

The spectrophotometric methods, which measure the absorbance of the complexes formed between ammonia and the more sensitive reagents may be used to determine ultramicroquantities of the ammonia with great precision[20,31,35,105] However, it is absolutely essential to apply great caution in order to avoid errors due to artifacts.

V. THE TER MEULEN METHOD

The Ter Meulen method has received little attention since it was first described by Ter Meulen[3] in 1924. The Kjeldahl method was known to produce low values for the determination of the nitrogen content of heterocyclic compounds such as substituted pyridines, quinolines, pyrroles, and other ring-type nitrogen compounds. The Dumas technique also may be unsatisfactory in dealing with certain pyrimidines, purines, and polymeric substances.[9] The original Ter Meulen procedure consisted of pyrolysis of the organic compound in a stream of hydrogen, and the products were passed over a nickel catalyst at 280 to 300°C. The nitrogen was converted quantitatively into ammonia. The catalyst was prepared by calcinating asbestos which had been impregnated with nickel nitrate. The resultant nickel oxide was then reduced at 300 to 380°C to the metal, and the latter could only be used on two or three occasions before it needed to be regenerated by a further calcination. The catalyst was susceptible to poisoning by sulfur. Opotzkii[155] modified the original Ter Meulen method by heating the sample with five times its weight of sodium in an iron tube through which hydrogen and water vapor passed. The ammonia formed was absorbed in a known excess of standard acid, and the residual excess of standard acid was determined by titration. Ter Meulen[39] modified the hydrogenation process and used the new procedure to quantitatively determine the total nitrogen in fresh and boiled meat, oxo® products, dried white of egg, horse blood, edestin (hemp seed), gluten, yeast, fresh boiled and skimmed milk, glue, cheese, malt, urine, and feces. Opotzkii and Mirlis[40] compared the Ter Meulen method with the Kjeldahl method on composite samples of skins and leather at different stages of their production. They concluded that when the determinations of total nitrogen were made by both methods, the values of total nitrogen determined by the Ter Meulen method were 2.2 to 2.8% higher than those values of total nitrogen determined by the Kjeldahl method on composite samples. Holowchak et al.[156] used the Ter Meulen method, modified to include a nickel-magnesium catalyst for the pyrolysis operation, to the analysis of hides, leather, albumen, and yeast. The authors claimed that they could quantitatively determine the nitrogen with an accuracy of 1% of the nitrogen present.

VI. THE WILL AND VARRENTRAP METHOD

In 1841, Will and Varrentrap[41] published a method for the quantitative determination of nitrogen in organic compounds. Although the method has received little attention in recent years, until the publication of the Kjeldahl method in 1883, it was used by many chemists as a reasonable alternative to the Dumas method. It relies on a simple procedure by which the sample was ignited in admixture with freshly ignited soda lime. The nitrogenous sample was completely decomposed into ammonia and other gaseous products which were absorbed in a known volume of a standard solution of acid. The residual quantity of standard acid solution, not neutralized by the ammonia, was titrated against a standard alkali solution. The original method was known to yield low values for the nitrogen content of the sample when the pyrolysis conditions caused part of the ammonia to decompose before the gaseous combustion products were absorbed by the standard acid.

More recently, Shamrai et al.[42] adapted the original method by igniting the sample with a standard mixture of freshly ignited calcium oxide (50 wt %) and sodium hydroxide (50 wt %). In addition to samples of biological tissues, they used the modified method to determine the nitrogen in homocyclic and heterocyclic compounds. The analyses were completed in 20 to 25 min, and the authors reported that the method is not less accurate than the Kjeldahl procedure.

VII. THE VAN SLYKE METHOD

The α-amino nitrogen in biological materials has been determined by several colorimetric procedures described in Section IV.B.4. These methods were based on the reactions of the α-amino group with a reagent such as indanetrione hydrate,[126] copper phosphate,[140,141] or β-naphthoquinone.[141] Van Slyke[158-161] has developed a manometric method in which the nitrogen evolved during the reaction between the α-amino group with nitrous acid is a quantitative measure of the α-amino nitrogen present in the samples. Subsequently, Van Slyke devised a manometric apparatus to determine the amount of nitrogen formed by measuring the pressure of the gas at constant volume; this apparatus has been extremely useful for several other analytical procedures.[162]

VIII. MODERN PHYSICAL METHODS OF DETERMINING NITROGEN

In the fields of chemistry and biochemistry, the major advances in research during the last 4 decades have been directly due to the development of physical methods. The advanced electronic equipment and fully automated procedures have been applied to the determination of nitrogen by the Dumas technique, as well as the Kjeldahl method. Buckee and Hickman[163] have reviewed methods of determining the total nitrogen, protein nitrogen, α-amino nitrogen, and amino acids in hops by automated and semiautomated methods of in-line analysis for other properties of the hops.

Mass spectrometric analysis of urinary nitrogen from growth-hormone-deficient children administered ^{15}N-labeled glycine have been performed by De Jongh and Hills.[164] Methods of determining ^{15}N by emission and mass spectrometry in biochemical analysis have been reviewed by Fiedler and Proksch.[165]

The combined gas-chromatographic and mass-spectrometric techniques have been employed by Mitsuo and Yoshimura[166] to investigate foods. Baker[167] also used these combined techniques to analyze permethylated nucleosides and nucleotides.

The technique of optical emission spectroscopy has been used by Stein et al.[168] in ^{15}N-tracer studies on humans. Urine was collected from a subject who had received an infusion of ^{15}N-glycine during a period of 8 hr. Ammonia was isolated from 10 m*l* of sample adjusted to pH 10 by freeze-drying for 1 hr at 50 mtorr and collected in 0.1 *M* H_2SO_4. Urea and amino acids were separated by passing the urine sample (pH 1.0) through a column (5.0 × 1.0 cm in diameter) of Dowex 50W × 8 resin, collecting the eluate (containing the urea), and then eluting the amino acids with 0.5 *M* NaOH. The urea and amino acids were converted to ammonium sulfate, and molecular nitrogen was liberated by BrO^-; the determination of nitrogen was performed by means of an optical emission spectroscopy technique.

McKenzie and Young[47] have described the use of an ammonia-gas-sensing electrode to determine ammonia, nitrate, and organic nitrogen in water and waste waters. The Orion® model 95-10 was used to determine ammonia after the addition of a quantity of sodium hydroxide and ethylene diamine tetraacetic acid to prevent the formation of insoluble hydroxides. The nitrate ion was determined as ammonia after the former had been reduced with Devarda alloy under acid conditions in the presence of 1 ppm fluoride ion, for a 24-hr reduction period. The method showed a recovery of 95%,

with a coefficient of variation of 6.8%. The organic nitrogen was determined by Kjeldahl digestion with sulfuric acid containing a mixture of potassium sulfate and mercuric sulfate. The digestive mixture was treated with a mixture of sodium hydroxide and sodium iodide, and the ammonia was determined by the ammonia-gas-sensing electrode.

Another technique, using a selective electrode, was developed by Lietke and Meloan[48] to determine nitrate in baby food by a rapid screening method. The sample (20 g) was mixed with Dowex 50W × 8 ion-exchange resin (≈ 3 g Al^{3+} form or 2 g Ag^{+} form) and stirred with water (20, 60, or 80 mℓ) to enable a reading to be made on the expanded scale (mV) of a pH meter linked to an Orion model 92-07 nitrate-selective electrode. The method is rapid (15 to 20 min) but yields results which are high by a few parts per million for samples having a nitrate content higher than 10 ppm. Fifty-three baby food samples were examined, and five samples contained more than 20 ppm nitrate ion.

Dohan et al.[51] have developed an entirely novel method of determining nitrogen in grain by proton activation. A cyclotron was used to produce a proton beam with particles that had an energy of 16 MeV. The proton beam was employed to irradiate the nitrogen atoms in the sample, converting the nitrogen atoms to oxygen-14 atoms with a simultaneous emission of 2.3-MeV photons. The nitrogen is determined off-line from the number of 2.3-MeV photons to the total number of protons striking the sample.

The whole or ground grain is placed in a cylindrical container covered at one end with a thin aluminium foil, and the sample is irradiated for 1 min through the foil and parallel to the axis of the cylinder. The cylinder is held in an insulated metal Faraday cup connected to earth through a current integrator to measure the total charge collected. The sample is then removed from the cylinder, and emitted X-rays are measured with two NaI (Tl) scintillation counters for 3 min with computer integration of the counts. The ^{14}N content of the sample is proportional to the ratio of the number of X-ray counts under the 2.3-MeV peak to the total charge that is collected in the Faraday cup. The method is nondestructive, and the sample does not need to be weighed, since its effective thickness (≈ 0.23 g cm^{-2}) is determined by the proton range. The results obtained for whole or ground grain (wheat, durum, barley, rye, triticale, and oats), rapeseed, and high-protein flour agreed well with results obtained with the Kjeldahl method. The new procedure for the determination of nitrogen in biological samples is capable of analyzing approximately ten samples per minute.

An isotope dilution method has been developed by Faust et al.[50] to determine nitrogen by an automated procedure. The automated nitrogen-15 analyzer NA-5A (Isonitromate 5200®, VEB Statron, Fuerstenwalde, East Germany) deals with samples in which the nitrogen is present as ammonia or easily reducible amino groups. The isotope dilution technique has been used to study conditions under which reliable results could be obtained for urea and sulfamate nitrogen, as well as for ammonia nitrogen. Solutions containing these compounds in concentrations corresponding to those present in human urine were examined after various amounts of nitrogen-15 in different forms had been added. A scheme was described, involving the additions of the tracer isotope and microdiffusion of the ammonia, for the determination of ammonium nitrogen, urea nitrogen, and total nitrogen in urine.

Another noninvasive technique has been described by Hutson et al.[49] for the chemical analysis of tissue to determine the relative amounts of elemental carbon, nitrogen, and oxygen present in the samples. A stoppered muon channel was used as a source of muons for X-ray analysis. The X-ray spectra from several types of tissue were used to quantitatively determine the elemental carbon, nitrogen, and oxygen, and the results of these analyses agreed well with data obtained from conventional chemical analyses. Muonic X-rays offer a noninvasive technique for the determination of the more abundant elements in selected regions of the body.

IX. THE DETERMINATION OF SPECIFIC NITROGEN COMPOUNDS

The determination of nitrogen in a biological sample usually involved the total nitrogen content, as in the case of proteins, mucopolysaccharides, or other compounds of biochemical interest, or the major portion of the nitrogen, which may occur in the form of α-amino nitrogen. However, it may occasionally be necessary to determine a quantity of a nitrogenous compound present in only a trace amount. It is under such conditions that a modern physical technique proves to be so valuable. In food analysis, it is very important to establish the quantity of N-nitrosamines in the cured meat or related product. Gough and Sugden[45] have developed a dual-column gas-chromatographic system for use in the mass-spectroscopic determinations of N-nitrosamines. They described an application of their system to the determinations of N-nitrosodimethylamine and N-nitrosopycrolidine in cured meats and related products. Two stainless-steel columns are connected in series and operated at 140°C with helium (4 mℓ/min) as carrier gas. One column of dimensions 1.6 m × 1.8 mm is packed with 15% Carbowax® 20M on AW-DMCS Chromosorb W® (80 to 100 mesh), and the other column, of dimensions 30 m × 0.5 mm, is a support-coated open tubular column containing Carbowax 20M. Two valves of low dead volume were placed in position between the columns to enable the solvent to be vented and N-nitrosamines of long retention time to be separated by the second column. Two flame ionization detectors are used; one is connected to a silicone membrane separator to monitor material entering the mass spectrometer, and the other monitors vent material and prevent toxic substances from entering the laboratory atmosphere. The limit of detection possible with this mass spectrometer system is 1 μg/mℓ.

Fine et al[46] described a thermal energy analyzer for the trace determination of volatile and nonvolatile N-nitroso compounds. They employed the equipment to investigate its sensitivity to different N-nitroso compounds present in biological materials and reported that it was possible to detect these compounds below 1 μg/kg in foodstuffs. This apparatus could be used as a gas-liquid chromatograph (GLC) detector. In a later paper, Fine and Rounbehler[169] reported on the trace analysis of volatile N-nitroso compounds by a procedure which combined gas chromatography and thermal energy analysis. They devised an apparatus incorporating a stainless steel column (0.5 × 2 mm) packed with 15% FFAP® on AW-DMCS Chromosorb W (80 to 100 mesh) and operated at 185°C with argon (10 to 30 mℓ/min) as the carrier gas and detection by thermal energy analysis.[170] The authors reported that with an injected sample of 200 $\mu\ell$ the N-nitroso compounds can be detected down to 1 ng/mℓ.

Another gas-chromatographic technique for the determination of N-nitrosamines in food has been developed by Kawabata.[171] The author described a newly devised alkali-flame ionization detector, incorporating a single crystal of KBr in the GLC of nitrosamines on glass columns (2.5 m × 3 mm) packed with Chromosorb W (60 to 80 mesh) supporting 25% PEG 6000 or 20% Versamid® 900 and operated at 140°C with nitrogen (60 mℓ/min) as the carrier gas. The injection port and detector connections were glass to ensure the best sensitivity (approximately 0.1 ng for 1 $\mu\ell$ of solution injected). Isobutylamine was used as a standard, and calibration graphs were shown for nitrosodimethylamine and nitrosoethylamine. A method for the determination of secondary amines in food, involving the extraction of the amines, nitrosation, and GLC technique, was described. The author also presented data on nonvolatile nitroso compounds such as N-nitrososarcosine, N-nitrosoproline, and N-nitrosopipecolic acid. There appeared to be little interference from primary or tertiary amines at levels normal in fish products, and the recoveries were 97%. The nitrosamines were methylated by diazomethane, and the esters were analyzed by GLC at 180°C on a column of 15% DEGS® on Celite® 545. The calibration graphs covered the range 0 to 50 ng for each compound tested.

A rapid method for determining gases in small blood samples by a chromatographic technique has been described by Schachinger.[172] The apparatus employed had a simple extraction chamber for removing oxygen, carbon dioxide, and nitrous oxide anaerobically from 50-$\mu\ell$ samples of blood. The N_2O was determined separately by gas-solid chromatography. The time required for an analysis was 6 min compared with 10 to 12 min by a manometric method.

The determination of nitrate in meat products has been reported by Selmeci et al.,[173] who used a colorimetric method. Aqueous extracts of meat were treated with $Na_4B_2O_7 \cdot 10\ H_2O$ to remove proteins and pigments; the nitrate ions were subsequently converted to nitrosalicylate by treatment with salicylic acid in the presence of sulfuric acid. The nitrosalicylate colored compound was determined by spectrophotometry at a wavelength of 415 nm. The sensitivity was 1 ppm, and the error was less than ± 5.4% for 1-g samples.

Postel[174] described another colorimetric method for the determination of nitrate in beer and brewing materials. The sample was clarified, and the NO_3^- ions were reduced with cadmium sponge (prepared by treating zinc dust with cadmium acetate solution). The NO_2^- ions were allowed to react with Griess reagents 1 and 11 (Sulfanilic acid and 1-naphthylamine) and determined spectrophotometrically at a wavelength of 530 nm. The calibration graph was rectilineal (for quantities less than 30 $\mu g/m\ell$ nitrate ions) and passed through the origin. Hamilton[175] used a similar colorimetric method to determine the NO_3^- in cheese. The aqueous slurry of the sample was clarified with aqueous zinc hydroxide, and the excess of Zn^{2+} ions was removed by 2% sodium hydroxide solution. The NO_3^- ions were reduced to NO_2^- ions by passage through a column of cadmium sponge. The NO_2^- ions were used to diazotize sulfanilic acid before being coupled with 1-naphthylamine.

X. DISCUSSION

The more senstitive and accurate determinations of nitrogen in biological materials by the Dumas and Kjeldahl methods depend on modern physical apparatus. The Dumas method has been improved considerably by the introduction of more efficient catalysts, such as nickel oxide,[5] cobalto-cobaltic oxide,[6,7] vanadium pentoxide,[65] and silver manganate.[57] However, the major advances have been due to the use of higher temperatures for the combustion process and the application of thermal conductivity techniques. Several commercial instruments are available that will perform elemental microanalysis, including nitrogen by automated equipment. The apparatus described by Rezl and Kaplanova[57] employed silver manganate as catalyst under dynamic conditions, either with the addition of oxygen or in an inert atmosphere of helium. The combustion tube contained copper oxide and cobalto-cobaltic oxide in the oxidizing section (heated to 850 to 950°C) and copper in the reducing section (500 to 650°C). The combustion products were diluted with helium and allowed to reach diffusion equilibrium before being subjected to gas-solid chromatography on Porapak® Q by the frontal technique with katharometer detection. This automated procedure requires a sample of 0.5 to 2.5 mg, and the analysis takes about 10 min. The authors claim satisfactory precision, with an absolute precision of 0.2, 0.2, and 0.1%, respectively, for carbon, nitrogen, and hydrogen.

Stoffel and Gade[65] have used another type of automated commercially available analyzer for the elementary microanalysis of drugs. They used vanadium pentoxide as a catalyst for a gas-solid chromatographic determination (in 10 min) of the atomic ratio of carbon, hydrogen, and nitrogen in samples weighing 0.7 to 1.3 mg. The results obtained for seven drugs were compared with the theoretical values; the standard deviations were ± 0.21% for carbon and ± 0.50% for hydrogen. The higher deviation

for hydrogen was due to the lower percentages of that element in the individual compound. The determination of the carbon/hydrogen/nitrogen ratio of a single substance was performed without weighing the sample; a desktop computer was used for calculating the atomic ratios of carbon and hydrogen relative to nitrogen (taken as unity). In a later paper,[176] the authors used the same equipment to calculate the carbon/hydrogen/nitrogen atomic ratios for the determination of the components of a mixture of two substances without separation. The substances analyzed included nicotinamide, antipyrine, hexamine, barbitone, caffeine, and benzocaine. Each analysis was performed in about 8 min. The method was recommended for powders and tablets having at least one nitrogen-containing component. Harrington and Bramstedt[177] have described an on-line data acquisition system for the Perken Elmer® 240 carbon, hydrogen, and nitrogen analyzer. They have reported data from an on-line remote PDP®-15/76 computer for the monitoring of an electrobalance (for weighing the samples) and the three thermal conductivity detectors used with this analyzer. The final report of the analysis is printed by means of a Silent® 700 teletype (Texas Instruments). Since Vecerea[178] first reported the thermal conductivity measurements on the gaseous products from the Dumas combustion process, there have been several advances in this technique. The performances of the catalysts have been improved, and more rapid analyses are possible with the employment of electronic equipment such as computers and teleprinters. The determination of nitrogen in a biological substance requires a sample weighing only 0.7 to 1.3 mg.

The Kjeldahl procedure is convenient for the analysis of either solid samples or solutions, and the method has been adapted to the analysis of ultramicroquantities of nitrogen. The open tube Kjeldahl digestion technique imposes the restriction that subsequent boiling point of the sulfuric acid digestive mixture cannot be raised to a level sufficiently high to decompose a refractory substance such as nicotinic acid. The sealed tube method may be used successfully without a catalyst on a wide variety of products and refractory substances,[20] provided the temperature and period of digestion are adequate. The temperature of the sulfuric acid digestive mixture should not exceed 500°C; otherwise, some of the ammonia may be converted to nitrogen.[27,30] The presence of a catalyst will expedite the decomposition of the sample by the sulfuric acid digestive mixture whether the digestion is performed in an open tube at lower temperature or in a sealed tube at a higher temperature.

There have been many catalysts used in the numerous modifications of the Kjeldahl digestion procedure since the original method was devised. Particular studies of the digestive process have been made in order to produce some convenient methods of standarization.[18,19] More recently, an interesting modification of Kjeldahl digestive procedure has been described by Stirrup and Hartley,[179] who used a mixture of titanium dioxide and copper sulfate as a catalyst. They determined the nitrogen to estimate the protein content of English wheat, skim milk powder, ground-nut meal, and whitefish meal. The authors used the mixture of titanium dioxide and copper sulfate instead of mercuric oxide catalyst, because it was cheaper and effluent problems could be avoided. The modified method produced marginally higher recoveries of nitrogen on a limited number of materials examined. Another modification of the Kjeldahl method has been described recently by Swaminathan and Sud.[180] They used chromium trioxide as a catalyst with potassium sulfate to raise the boiling point of the sulfuric acid digestive mixture in an open digestion flask. Examination of a large number of biological materials, including fruits, vegetables, meat, powdered milk, flour, and yogurt, produced results which compared favorably with those obtained by the standard Kjeldahl method.

An automated total nitrogen analysis of biological samples has been described by Hambraeus et al.[181] They used an aluminium block digestor with a mixture of sulfuric

acid and phosphoric acid in the presence of hydrogen peroxide and selenium at 370°C. The diluted digestion mixtures were treated with the phenol-hypochlorite reagents and examined by means of an Autoanalyser® (Technicon Co.); the samples tested included tissues, biological fluids, foods, protein concentrates, and casein.

Moll et al.[182] have described an automated method of determining total nitrogen in wort and beer. The samples were digested for 15 min at 320°C in sulfuric acid, and selenium and hydrogen peroxide were used to hasten the digestion of the samples. The resultant ammonium sulfate was allowed to react with phenol in an alkaline solution containing sodium hypochlorite. The extinction measurements were made at 630 nm and the reproducible results agreed well with those obtained by the standard Kjeldahl method. The procedure was also used for the determination of protein in wort and barley.

A rapid method for the determination of nitrogen in grain meal, by an automated procedure, has been reported by Kaul and Sharma.[183] They examined rice, rat-diet, and barley, containing 7 to 36% nitrogen, by a conventional microscale Kjeldahl method and also by the indophenol color reaction. The Kjeldahl method (using titration) correlated well with each of the spectrophotometric methods used. Their comparison of the methods included a cost evaluation scheme; the authors recommended the manual spectrophotometric method for nonautomated, direct screening at low cost.

Glebko et al.[184] have also made a comparative study of the phenol-hypochlorite reaction produced by different methods. Six variations of the method were compared, and the authors concluded that the best of these techniques was that in which phenol and $Na_2Fe(CN)_5NO$ reacted together. The lowest concentration of nitrogen was 0.1 $\mu g/m\ell$, with a standard deviation of 0.003 μg. The increased number of phenols acceptable for use in the method has not afforded any appreciable enhancement of activity. They believed that further improvement in the method of determining nitrogen by the phenol-hypochlorite reaction should be possible if side reactions are avoided.

The determination of the ammonia in the digested sample, without separation by distillation or microdiffusion procedures, may be performed by titration with standard hypochlorite using the method of Kolthoff and Stenger[185] as modified by Belcher et al.[28] or by titration with sodium hypobromite[186] without control of pH or temperature. These procedures may be performed on digested material made alkaline before titration, and little or no ammonia is absorbed from the atmosphere during the operations. The use of Nessler reagent in alkaline solution may also avoid atmospheric contamination by ammonia; however, the color produced by the excess Nessler reagent contributes a considerable background to the total absorbance measured. It is essential that standard solutions be used even when the absorbance of the control solutions is maintained at a reasonable level. The method is not as sensitive as either the phenol-hypochlorite or the indanetrione hydrate method. Both of the latter two colorimetric methods may be used directly to determine the nitrogen, as ammonia, in digested samples without prior separation by distillation or diffusion of ammonia. The indanetrione hydrate method, combined with the sealed-tube high-temperature digestive procedure, has been shown to be satisfactory for the analysis of a wide range of biological materials.[20,30,31,44,91,105]

The sensitivity of the indanetrione hydrate colorimetric method may be compared with that of the titration method using indicator dyes. The modern spectrophotometer will detect precisely a difference of 0.01 in absorbance measurements at 570 nm, using a tungsten filament lamp as the light source. The molar extinction value of the ammonia-indanetrione complex, determined in a cell of path length 10 mm at a wavelength of 570 nm, is in excess of 20,100. A 0.4-μmol quantity of nitrogen (5.6 μg N) as ammonia, allowed to react with excess indanetrione hydrate under specified conditions, will produce in 10 $m\ell$ of solution an absorbance of 0.805 ± 0.008 at 570 nm in

a cell of path length 10 mm. Hence, a quantity equal to 1/80 of 5.6 μg of nitrogen may be detected, i.e., 0.07 μg nitrogen. The use of microcells of the same path length, but of a capacity of 0.5 mℓ, and a sample containing 0.04 μ*M* ammonia diluted to 1.0 mℓ will enable the observer to detect 0.007 μg nitrogen.

The titration of ammonia against 0.01 *M* hydrochloric acid is limited in its sensitivity to the end point change of the indicator. In practice, 0.02 mℓ of the 0.01 *M* acid is required under ideal conditions to change the color from purple through colorless or grey to green of an indicator such as the methyl red-methylene blue mixed indicator (pH range 4.5 to 5.5). This sensitivity is equivalent to 2.8 μg of nitrogen as ammonia. The precision of the colorimetric method is much greater than that of the titration procedure. When using the sensitive indanetrione hydrate reagent, it is necessary to purify the water and other reagents used in the reaction with ammonia.[20,30] In recent years, several fully automated amino acid analyzers have become available commercially. These have employed indanetrione hydrate reagent, which has been used to react with α-amino acids, and ammonia, which is present in the hydrolysates of proteins,[154,187] and mucopolysaccharides.[188,189]

A study has been made in the author's laboratory on the rate of reaction between indanetrione hydrate and each of the 17 common α-amino acids which occur in protein hydrolysates.[92] It was shown that the reaction between indanetrione hydrate and ammonia developed at a slower rate than that which occurred between the reagent and any of the 17 α-amino acids studied. A special Teflon® reaction coil 300-ft long was wound around a stainless steel former and used with the Beckman Spinco amino acid analyzer (Model C). It should be possible to automate the method of determining nitrogen by means of indanetrione reagent in place of phenol-hypochlorite reagent in the automated method already described.[181] The Ruhemann's purple complex,[124] formed by reaction between indanetrione hydrate and ammonia, has a molecular extinction coefficient greater than that of the indophenol complex formed by the reaction between ammonia and pheno-hypochlorite reagent.[190]

Tables 4 and 5 indicate the characteristics of the complexes formed by the reactions between ammonia and indanetrione hydrate and ammonia with the phenol-hypochlorite reagent (i.e., indophenol complex). The absorption spectrum of the complex formed by the reaction between ammonia and indanetrione hydrate is shown in Figure 3, and the absorption spectrum of the complex formed by the reaction of ammonia with the pheno-hypochlorite reagent is illustrated in Figure 4. When suitable precautions are taken to remove ammonia from the water and the solutions of the reagents, the sealed tube digestion procedure combined with a sensitive colorimetric method should be capable of precisely determining the nitrogen in biological samples.

XI. SUMMARY

This review of methods used to determine nitrogen in biological materials includes a survey of those procedures which have been applied to the analysis of organic compounds by the older classical methods (Dumas, Kjeldahl, and Ter Meulen) in addition to those developed more recently (e.g., the Ohashi method). A comparison between the respective methods has been described, and particular reference has been made to the improvements in the older classical chemical methods by the introduction of modern physicochemical techniques. The older procedures of volumetric determinations of nitrogen in the Dumas and Van Slyke methods have been replaced and improved by the use of thermal conductivity measurements, mass spectrometry measurements, and radiometric techniques using isotope dilution methods or muonic X-ray analysis. The more sensitive spectrophotometric methods for the determination of ammonia, including the indanetrione hydrate, indophenol, and other colorimetric procedures, have

TABLE 4

Comparative Absorbance Values of Ammonia Complexes

Complex	Ammonia (μmol)	Vol (mℓ)	Cell path (mm)	Absorbance value*
Indantrione hydrate	0	10	10	0.055
	0.4	10	10	0.860
Indophenol	0	50	40	0.032
	1.2	50	40	0.662

* Absorbance values were the mean of triplicate readings measured vs. ethanol/water (= 1:1 v/v) diluent in the case of indanetrione hydrate complex or water as diluent for the indophenol complex.

From Jacobs, S., *Proc. 12th Colloq. Spectroscopicum Int.*, Hilger Watts, London, 1965, 242. With permission.

TABLE 5

Characteristics of Ammonia Complexes

Complex	Absorbance maximum (nm)	Molar extinction (10 mm cell) × 10^3
Indanetrione Hydrate	570	20.1
Indophenol	630	6.6

From Jacobs, S., *Proc. 12th Colloq. Spectroscopicum Int.*, Hilger Watts, London, 1965, 242. With permission.

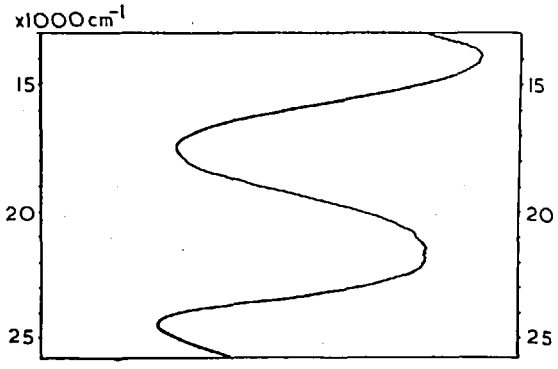

FIGURE 3. Absorption spectrum of ammonia-indanetrione hydrate complex.[190]

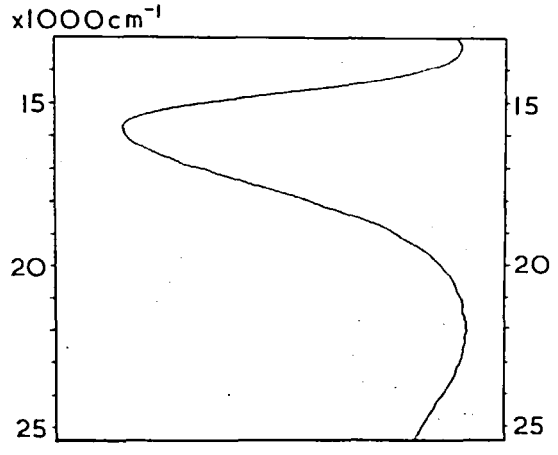

FIGURE 4. Absorption spectrum of ammonia-phenol-hypochlorite complex.[190]

been compared with the titration methods to determine the end product from the digestion of nitrogenous substances by sulfuric acid.

Biological samples in the form of tissues or tissue fluids have been examined by noninvasive techniques, using isotope dilution or X-ray spectra analysis. These modern physical methods may prove to have advantages over the older chemical methods.

The determination of nitrogen in organic compounds of biological interest, such as proteins, mucopolysaccharides, antibiotics, pyrimidines, and nicotinic acid, have been discussed. The review also includes data related to the nitrogen analysis of processed biological materials such as leather and food products from animal and vegetable sources.

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