MICROMETHODS FOR DETERMINATION OF AMMONIA, UREA, TOTAL NITROGEN, URIC ACID, CREATININE (AND CREATINE), AND ALLANTOIN

By HENRY BORSOOK

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

(Received for publication, May 13, 1935)

The success with which Krebs and Henseleit (1) employed Warburg's method of surviving tissue slices in the problem of the formation of urea in the liver encouraged its use for a direct attack on other problems of nitrogen metabolism in animals.

For this purpose methods were developed (or adapted) for simultaneous multiple determinations (twenty to forty) of very low concentrations of ammonia, uric acid, creatinine, and allantoin in 1 to 2 cc. of solution. In addition to the convenience of saving of time in carrying out a large number of determinations simultaneously, these methods offer the advantages of improved specificity and accuracy. They have been in continuous use for more than a year by a number of different workers for literally thousands of determinations, and have stood the test of use by a class of undergraduate students. With only slight modifications they can be employed for the analysis of blood, urine, and tissue fluids and extracts. The procedure finally settled upon in every case ended in a colorimetric measurement. For this purpose a spectrophotometer is available here. The advantage of this instrument is that the most suitable band of approximately monochromatic light can be selected for measuring the intensity of a given color. The use of monochromatic light extends the region of linear relationship between depth of color and concentration of substance to be analyzed. With suitable color filters and an increased intensity of light it should be possible to obtain this advantage with a colorimeter, thus obviating the necessity for bracketing the unknown with standards of nearly the same concentration.

The volumes of solution analyzed and reagents were in each case so adjusted that the volume of the final colored solution was 3.0 to 3.5 cc. This is the lowest convenient volume for the absorption cell which we could use. This volume can, of course, be increased or decreased according to the needs of the colorimetric instrument employed.

Ammonia

The ammonia determination involved distillation by the method of Conway and Byrne (2), followed by assay of the ammonia by a modification of Van Slyke and Hiller's (3) procedure with the phenate-hypochlorite reagent.

In the experiments for which these analytical methods were primarily devised there were a large number of ammonia determinations. The usual ammonia micromethods designed for single measurements would have been too time-consuming. With the distillation method of Conway and Byrne it is possible to carry out conveniently a large number of distillations simultaneously. It was desirable therefore to obtain a method for detecting at least 0.0005 mg. of ammonia nitrogen per cc. of solution to be analyzed, with a reasonable accuracy, ± 1 per cent, which could be used for multiple determinations.

The technique described by Van Slyke and Hiller for the use of the phenate-hypochlorite reagent, heating for 3 minutes in a boiling water bath after addition of the phenate and hypochlorite and then cooling rapidly to room temperature, is not completely satisfactory for this purpose. The duration of heating in the water bath must be exactly the same or discordant results are obtained. Secondly, on cooling, the color changes in hue and attains its maximum intensity in about 7 minutes, after which it fades slowly, losing 10 to 15 per cent in 1 hour. These are not serious inconveniences where only a single determination is to be made, especially if the concentration of ammonia is approximately known. The standard and unknown can be handled together and will change together. Spectrophotometric measurements showed that with such a technique no significant error is incurred in single determinations. It is unsatisfactory when there are forty or more ammonia determinations in an experiment and the approximate concentrations in the individual samples are not known beforehand.

By studying the factors affecting the color development with the phenate-hypochlorite reagent, a procedure was worked out which gave a 10 per cent greater color intensity for a given concentration of ammonia than heating in a boiling water bath, and at the same time the color was stable (measured spectrophotometrically) for at least an hour. With this modification it was possible to obtain all the advantages of macromethods with a technique which permits the measurement of 0.0005 mg. of ammonia nitrogen per cc. of solution with an error not greater than ± 2 per cent. With higher concentrations the uncertainty is reduced to ± 1 per cent. By reducing the capacity of the vessels and the volumes it should be possible without any great difficulty to increase the sensitivity by a factor of 5. With this modification of the colorimetric procedure twenty to thirty solutions with unknown ammonia concentrations, varying between 0 and 1.5 mg. per cent, can be assayed with only four or five standards which may be developed simultaneously with the unknowns or separately. The time required for such a series of analyses is about 3 hours.

Reagents

Dilute hydrochloric acid, 0.01 N and 0.1 N. It is essential only that at the end of the distillation there be some excess of acid.

The phenate and hypochlorite solutions used are those described by Van Slyke and Hiller.

Sodium phenate. 25 gm. of phenol are dissolved in a small amount of ammonia-free water. 50 cc. of 40 per cent sodium hydroxide are then added, and the mixture diluted to 100 cc. It is then immediately transferred to a dark bottle and stoppered.

Javel water. Dissolve (or suspend) 50 gm. of calcium hypochlorite with 56.6 per cent of available Cl (or more) in about 500 cc. of hot water. This is mixed with a solution of 50 gm. of anhydrous potassium carbonate in 200 cc. of cold water. The resulting suspension is cooled and diluted to 1 liter. The clear filtrate must give no precipitate on addition of more K_2CO_3 solution. If necessary, add more of the alkali until all the calcium is precipitated. The solution used here contains 1.35 gm. of Cl per 100 cc. The prescription of Van Slyke and Hiller calls for 1 gm. of Cl per 100 cc. This reagent is best kept in an ice box in a number of small, dark bottles, so that the main solution is not opened each day.

The glass distillation vessels described by Conway and Byrne are not at present purchasable in this country. White glazed procelain vessels were made for us by a local potter which served quite well.

Procedure—The complete procedure is as follows: 1 cc. of dilute acid is pipetted into the central well of the distillation vessel. The outer rim of the vessel and the glass plate to cover it are then coated with vaseline. The vessel is tilted by resting the opposite edge on a glass plate. 1 cc. of the solution to be analyzed is then pipetted into one side of the outer well. The glass cover is now put on the vessel, leaving just sufficient room on the side opposite to that containing the solution to be analyzed to permit the insertion of a pipette point. 1 drop of phenol red is added, then 1 cc. of saturated potassium carbonate-potassium chloride solution. The cover is quickly slid over, covering the vessel, and made tight with a little pressure. By gentle rotation (about 10 times) the solutions in the outer well are thoroughly mixed, as shown by the uniform distribution of the phenol red. The covered vessel is set away in an air bath at 37° for 1 hour. At the end of this time the ammonia has distilled over into the central well. The contents of this well are transferred to a 50 cc. Erlenmeyer flask with a capillary syringe. The central well is then washed with five successive portions, 0.2 cc. each, of ammonia-free water into the Erlenmeyer flask.

To the 2 cc. of solution transferred as above, or directly into 50 cc. Erlenmeyer flasks, 1 cc. of the phenate solution is added. The flask is closed with a rubber stopper and cooled for 5 minutes on the draining board of a sink over which tap water is running. 0.5 cc. of Javel water is then added, after which the stoppered flask is set away in a water bath at 37° for 50 minutes. It is cooled again in running water for 5 minutes, after which the intensity of the color is read.

Exposure of the surface to air intensifies the color finally developed, hence the use of 50 cc. Erlenmeyer flasks. There is no increase or loss in color by incubating longer than 50 minutes.

The colored solution is transferred to an absorption vessel, in our case of the following dimensions (inside), 10 mm. deep, 5 mm. wide, and 30 mm. high. The absorption curve is a plateau with a maximum between 608 and 627$\mu\mu$. In this range of the spectrum

the absorption is a linear function of the concentration of ammonia from 0 to 1.5 mg. per cent, and only slightly curved between 1.5 and 2.0 mg. per cent.

When the color is developed by heating in a boiling water bath, it is at first green, changing to blue on cooling. The maximum absorption at first is at 627$\mu\mu$. On standing it shifts slowly toward 575$\mu\mu$.

The distillation vessels must be cleansed free of the last traces of vaseline. With the glazed porcelain vessels this is very difficult. The following procedure proved finally to be satisfactory. The vaseline is wiped off and the vessels cleaned with a hydrocarbon (Stoddard) solvent. The vessel is then scrubbed by hand with trisodium phosphate and soaked overnight in a 30 to 40 per cent solution of sodium silicate. It is then thoroughly rinsed successively with tap water, distilled water, and ammonia-free distilled water, and then dried in an air bath.

The above method for ammonia was used for the determination of urea and total nitrogen.

Urea

Urea was determined as ammonia after hydrolysis by urease.

Urease solutions were prepared from jack bean meal according to the method of Van Slyke and Cullen (4) or that of Krebs and Henseleit (1). Before being used the enzyme solution was shaken with purified permutit for 10 minutes after the pH had been adjusted to 6.0, to remove the ammonia which is continually formed.

When the preformed ammonia in the solution to be analyzed is low, the urea nitrogen can be obtained by difference before and after treatment with urease. If it is high, it is better to remove it first by shaking with permutit at pH 6.0. A variable quantity, less than 0.1 mg. per cent, remains after the permutit treatment. Hence blank determinations are necessary.

Our usual procedure is to incubate 2 cc. of the solution to be analyzed with 0.5 cc. of the urease solution in a stoppered Erlenmeyer flask for 1 hour at 37°. 1 cc. aliquots are then assayed for ammonia.

The smallest quantity of urea nitrogen which can be measured by this method is 0.001 mg. in 1 cc. of solution.

Total Nitrogen

The digestion procedure is only a slight modification of that described by Van Slyke (5). The subsequent ammonia determination is as described above.

1 cc. of solution containing not more than 0.15 mg. of nitrogen is pipetted into a Pyrex ignition tube, 170 × 20 mm., and 1 drop of 0.5 per cent selenium oxide and 0.5 cc. of sulfuric-phosphoric (3:1) acid digestion mixture are added. A special antibumping tube is shaken to the bottom of the tube. This tube is made of glass tubing and is conveniently 70 mm. long and 6 mm. outside diameter, closed at both ends, with a dimple about 3 mm. deep in the end which rests in the solution. The combustion tubes are placed on a wire gauze shelf over microburners. The heating is continued for 3 minutes after the solution is quite clear.

The tops of the tubes are covered with small funnels which lead into a manifold connected with a water suction pump. The difficulty with this simple digestion rack is to prevent violent bumping when a drop of condensate runs down the tube into the hot melt. This can be prevented by playing a microburner flame over the upper part of the tube.

After the tubes have cooled, 0.5 cm. of potassium persulfate (Merck's "Reagent, for Dr. D. D. Van Slyke's micro-Kjeldahl determination of nitrogen") is added, and 2 drops of ammonia-free water. The heating is resumed and continued for 3 minutes after the strong fuming has stopped. Heating for a shorter period gives low results with proteins and amino acids. The tube is again allowed to cool at room temperature. 2 cc. of ammonia-free water are now added and the mixture is then carefully brought to the boil in a Bunsen flame. It is cooled in running tap water, 1 drop of phenol red solution is added, then 50 per cent sodium hydroxide drop by drop down the side of the tube, with frequent cooling under the tap, until the solution is colored a perfectly clear, bright yellow. The contents are then transferred with a capillary syringe to a 10 cc. volumetric flask. 1 cc. portions of water are used to wash the sides of the ignition tube and the antibumping tube. The washings are transferred to the volumetric flask and the final dilution is made up to the 10 cc. mark.

1 cc. portions of this final dilution are distilled and assayed for ammonia by the methods described above.

Direct colorimetric determination of the ammonia, without distillation, is unreliable because traces of potassium persulfate interfere with the color development by the phenate-Javel water reagent.

Uric Acid

In the following methods for uric acid, creatinine, and allantoin we found it convenient to use Pyrex test-tubes, 125 × 9 mm. inside dimensions, provided with solid ground glass stoppers.

The method for the determination of uric acid is a combination of features of a number of other methods, with a modification of the final color development and colorimetry.

The uric acid is precipitated with zinc (6). The precipitate is dissolved in dilute acid and water. Cyanide is added and then the arsenophosphotungstic acid reagent of Benedict (7). The procedure of Benedict and Behre (8) from this point on is heating in a boiling water bath for 3 minutes, followed by 2 minutes at room temperature, and then a quick reading in the colorimeter while the solution is still warm. This procedure gives solutions too turbid to be read in a spectrophotometer, and in our hands, duplicate or triplicate readings varied by as much as 20 per cent. These difficulties were overcome by carrying out the color development for 40 minutes at 37°, followed by 15 minutes in chopped ice. At the low temperature the salt responsible for the turbidity precipitates out. On centrifuging, a perfectly clear solution is obtained, whose color remains unchanged and perfectly clear for at least 24 hours. The depth of color at 540 to 630$\mu\mu$ is a linear function of the concentration of uric acid (with 1 cc. of solution) from 0 to 1 mg. per cent.

Reagents

2.5 per cent zinc chloride in water.

10 per cent sodium carbonate.

Dilute hydrochloric acid (0.1 N).

Cyanide solution (Benedict and Behre). 5 per cent sodium cyanide, containing 2 cc. of concentrated ammonia per liter, made fresh every 6 to 7 weeks.

Benedict's arsenophosphotungstate reagent, prepared as follows: 100 gm. of pure sodium tungstate, dissolved in 600 cc. of water in a liter flask. 50 gm. of pure arsenic pentoxide are now added,

followed by 25 cc. of 85 per cent phosphoric acid, and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter.

Folin's stock standard uric acid solution was employed (9). 1 gm. of uric acid weighed to within 1 mg. is transferred to a 1 liter volumetric flask. To 0.6 gm. of lithium carbonate in a 250 cc. flask add 150 cc. of water and shake until the salt is dissolved. Filter. Heat the filtered solution to 60° and warm the flask containing the uric acid in running hot water. Pour the warm lithium carbonate solution into the volumetric flask containing the uric acid, washing down crystals adhering to the neck. Shake the warm mixture until all the uric acid has dissolved, about 5 minutes. Cool under the tap. Add 20 cc. of formalin (40 per cent solution of formaldehyde) and half fill the flask with water. Add a few drops of methyl orange and then introduce slowly and with shaking, by means of a pipette, 25 cc. of N sulfuric acid. The solution should turn pink while 2 to 3 cc. are still in the pipette. Dilute to 1 liter. Mix. Transfer to a tightly stoppered bottle, and store in the dark. The diluted standards are made up from this stock solution. They will keep for several days, and should not be used sooner than 1 hour after they are made.

In the experiments for which this method was primarily intended the solutions to be analyzed contained varying amounts of amino acids. Accordingly all the solutions, including the standards, were so diluted that the concentration of amino acids was nearly the same in all. Without this additional precaution the method has been used on Folin-Wu blood filtrates and has given normal values.

Procedure—The detailed procedure is as follows: 1 cc. of solution (or 2 cc. if the uric acid content is low), 1 cc. of water or special diluting fluid, and 0.05 cc. of 2.5 per cent zinc chloride are pipetted into one of the test-tubes described above. The contents are mixed by inversion several times. Then 0.4 cc. of 10 per cent sodium carbonate is added and the contents are again mixed by inversion. The test-tube is centrifuged, the supernatant liquid is poured off, and the last drop taken up with filter paper. 0.5 cc. of N/14 HCl, 1.5 cc. of water, and 1 cc. of the cyanide solution are added next. The stoppered test-tube is shaken vigorously until all the precipitate has dissolved, giving a crystal-clear,

colorless solution. 0.2 cc. of the arsenophosphotungstate solution is then added. The contents are again mixed thoroughly by inverting the test-tube a number of times. The stoppered test-tube is set away for 40 minutes in a water bath at 37°, after which it is immersed in a beaker of ice water for 15 minutes and then centrifuged. The supernatant solution is poured off into the absorption cell (or colorimeter cup) and the color read at 610$\mu\mu$. The absorption increases toward the red end of the spectrum, and any convenient color filter in this region will serve.

The standards are treated simultaneously with the unknowns in exactly the same manner, diluted, precipitated with zinc, etc. Triplicates agree within the personal differences of the spectrophotometer readings, $\pm 0.1°$. A large number, twenty to forty, of such determinations can be carried out simultaneously with one set of four standards (1 cc.) to cover the range from 0 to 1.0 mg. per cent of uric acid. In the absorption cell employed here, the absorption is too great to be read conveniently with concentrations above 1.0 mg. per cent.

Creatine and Creatinine

Two changes in the classical alkaline picrate method are introduced here. The creatinine is adsorbed on Lloyd's reagent, and in this state washed with acid free of impurities which are not adsorbed. The creatinine is then removed from the Lloyd's reagent by the same alkaline picrate in which the color is developed. The color is measured with light whose wave-length is approximately 525$\mu\mu$.

The reagents required are two concentrations of HCl, 0.1 N and 0.01 N, Lloyd's reagent (Eli Lilly and Company), and freshly made alkaline picrate solution consisting of 10 parts of saturated aqueous picric acid and 1 part of 10 per cent sodium hydroxide.

In the details of the method the procedure employed for converting creatine to creatinine prefaces the description of the actual determination of the creatinine.

According to the concentration of creatine (or creatinine) present 1 to 5 cc. of solution are pipetted into one of the test-tubes described above. One-quarter the volume of 0.1 N HCl is added, and the solutions mixed by inversion. A piece of thread is inserted in the neck of the test-tube containing the ground glass

stopper. The otherwise stoppered test-tube is autoclaved for 20 minutes at 30 pounds pressure (130°). After it has cooled, the thread is removed, 30 to 40 mg. of Lloyd's reagent are added, and the now tightly stoppered test-tube is continuously shaken to and fro by inversion for 10 minutes. It is then centrifuged. The supernatant liquid is poured off, the last drop taken off the lip with filter paper. 1 cc. of 0.01 N HCl is added, the precipitate resuspended, and then another 1 cc. of the acid is used to wash the precipitate down the stopper and the sides of the test-tube. It is centrifuged again, the supernatant fluid again discarded, and the last drop again taken up with filter paper. 3 cc. of sodium picrate solution are then added. The creatinine is removed from the Lloyd's reagent, and at the same time the color is developed by continuous gentle rotatory inversion or shaking for 10 minutes. The tube is centrifuged again. The supernatant solution is poured into the absorption cell, and the absorption read at 525$\mu\mu$. At this wave-length there is a linear relationship between absorption and the concentration of creatinine from 0 to 2.0 mg. per cent (2 cc. of solution analyzed). The standards are adsorbed with Lloyd's reagent and washed, etc., with the unknowns.

We have employed the usual creatinine-zinc chloride standard (10) in N/14 HCl. This standard solution does not keep indefinitely. We found it necessary to renew it at least once a month.

With 3 cc. of picrate solution, the smallest quantity which can be measured in our absorption cell is 0.001 mg. of creatinine, with an error of $\pm$0.0001 mg. The absolute error is the same with the highest concentrations of creatinine. The sensitivity of the method can be increased considerably by using a smaller volume of picrate solution, and a suitable change in the absorption vessel to increase the depth of solution through which the light passes.

Allantoin

The allantoin method is based upon that of Fosse and his collaborators (11). In the presence of cyanide to prevent the conversion of uric acid to allantoin, allantoin is converted to allantoic acid by an enzyme prepared from soy bean meal. The allantoic acid is then hydrolyzed by acid to glyoxylic acid and urea. The glyoxylic acid is measured colorimetrically. The technique described by Fosse and his collaborators in our hands gave solu-

tions too turbid to be read in the spectrophotometer; the color once developed with pure glyoxylic acid faded rapidly, and duplicates differed by as much as 40 to 50 per cent.

After a great deal of study a technique was worked out which is tolerably satisfactory. Differences between duplicates are still as high as ±5 per cent. The method is not time-consuming, but it is laborious. Its chief advantage is its sensitivity. With 2 cc. of solution the lowest limit is 0.05 mg. per cent, with an error of ±5 per cent.

Reagents

Enzyme powder. This is prepared from soy bean meal by Van Slyke and Cullen's method for the preparation of urease (4).

Ammonium carbonate-sodium cyanide. 1.153 gm. of NH_4HCO_3, 0.891 gm. of $(NH_4)_2CO_3$, and 0.46 gm. of NaCN are dissolved together in water and made up to 200 cc.

10 per cent aqueous trichloroacetic acid.

2 per cent sodium tungstate.

N/15 sulfuric acid.

0.5 per cent phenylhydrazine hydrochloride in N/14 HCl. Commercial phenylhydrazine hydrochloride is dissolved in water and decolorized by boiling with decolorizing charcoal. The hot solution is filtered, and after being cooled in an ice-salt bath the phenylhydrazine hydrochloride is precipitated by the addition of concentrated hydrochloric acid, or by dry HCl gas. The precipitate is filtered with suction, washed once quickly with very cold HCl, and then set away in a desiccator over calcium oxide in the dark. The solution is made up freshly just before it is to be used.

1.25 per cent potassium ferricyanide in water, also made just before it is to be used.

Procedure—The details of the procedure are as follows: 10 mg. of the dry enzyme powder are transferred to the special test-tubes. 0.5 cc. of the ammonium carbonate-cyanide solution, 2 cc. of the solution to be analyzed, and a drop of chloroform are then added. The stoppered test-tube is then set away at 37° overnight (12 hours). From time to time it is shaken to facilitate solution of the enzyme. 2 cc. of the solution with the enzyme powder suspended in it are then transferred to another test-tube. 0.2 cc. of 10 per cent trichloroacetic acid and 0.1 cc. of 2 per cent sodium tungstate

are added. The solution is mixed by inverting several times. Then 0.1 cc. of N/15 H_2SO_4 is added and the solution again mixed by inversion. These reagents precipitate the protein, and at the same time provide the acidity necessary for the hydrolysis of the allantoic acid. The stoppered test-tubes are placed in a large beaker of water at room temperature, which is then heated quickly to 90° for 5 minutes, and then cooled quickly in ice water for 2 minutes. 0.3 cc. of 0.5 per cent phenylhydrazine in N/14 HCl is now added, and the stoppered test-tube shaken vigorously and set in a water bath at 60° for 5 minutes, after which it is again quickly cooled in ice water. The tube is then centrifuged. Although the bulk of the coagulum is thrown to the bottom of the tube, the surface usually contains a number of coagulated particles. As a routine procedure a drop of 95 per cent ethyl alcohol is floated carefully on the surface after the first centrifuging, and the tube is centrifuged a second time. 2 cc. of the clear supernatant liquid are now carefully pipetted out and transferred to another dry test-tube. This is now placed in a beaker containing alcohol and solid CO_2. A bottle of concentrated HCl has previously been placed in this bath. This alcohol-CO_2 bath is surrounded by another ice-salt bath maintained at $-15°$ to $-20°$. A large saucepan will serve for the ice-salt bath, with a beaker in the middle for the alcohol-CO_2. After about 10 minutes in the alcohol-solid CO_2 bath 1.5 cc. of concentrated HCl are pipetted into the test-tube. This is then stoppered and continually inverted up and down in the air until the cake of ice melts. Just before the last of the ice disappears 0.2 cc. of 1.25 per cent potassium ferricyanide is added and quickly mixed by inverting several times. It is then set away in the ice-salt bath for 5 minutes, after which it is transferred to a water bath (a cup or beaker) at room temperature. After 10 minutes in this bath the color is read as quickly as possible afterwards, as it slowly fades and becomes turbid. The turbidity is associated with some constituent of the enzyme preparation which we have so far been unable to eliminate. It is not obtained when the color is developed with glyoxylic acid alone. The best region of the spectrum for the colorimetry is at 535$\mu\mu$. There is a linear relation at this wave-length between the absorption and concentrations of allantoin (2 cc. used) from 0 to 1.5 mg. per cent.

The standards are treated in the same manner as the unknowns

and at the same time. We have used aqueous solutions of allantoin made from a stock solution containing 1 mg. per cc. This stock solution must be freshly made each week. It deteriorates even when kept at 0–2°.

When a large number of determinations are to be made, two workers are required for this method. With two practised workers it is possible to carry out the completion of twenty to thirty determinations, *i.e.* after the overnight incubation with the enzyme, in about 3 hours.

SUMMARY

1. Improvements are described in the micromethods for the determination of ammonia, uric acid, creatinine, and allantoin. These improvements lead to a greater stability of the final color, increased sensitivity, and specificity, and, by use of a suitable approximately monochromatic light in the colorimetry, a great extension of the region of linear relationship between intensity of color and concentration.

2. These improvements make it possible to carry out a large number of determinations (twenty to forty) at the same time, with the use of only a few standards.

BIBLIOGRAPHY

1. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
2. Conway, E. J., and Byrne, A., *Biochem. J.*, **27**, 419 (1933).
3. Van Slyke, D. D., and Hiller, A., *J. Biol. Chem.*, **102**, 499 (1933).
4. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore, 545 (1932).
5. Van Slyke, D. D., *J. Biol. Chem.*, **71**, 235 (1926–27).
6. Morris, L. J., and Macleod, A. G., *J. Biol. Chem.*, **50**, 55 (1922).
7. Benedict, S. R., *J. Biol. Chem.*, **51**, 187 (1922).
8. Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, **92**, 161 (1931).
9. Folin, O., *J. Biol. Chem.*, **86**, 179 (1930).
10. Hunter, A., Creatine and creatinine, Monographs on biochemistry, London and New York, 70 (1928).
11. Fosse, R., Brunel, H., and Thomas, P., *Compt. rend. Acad.*, **192**, 1615 (1931); **193**, 7 (1931).