NEW METHODS FOR THE DETERMINATION OF TOTAL NON-PROTEIN NITROGEN, UREA AND AMMONIA IN BLOOD.

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I. Method for drawing blood ....................................... 527
II. Isolation of non-protein nitrogenous constituents ........ 528
III. Determination of the total non-protein nitrogen ......... 529
IV. Determination of the urea .................................... 531
V. Determination of the ammonia ............................... 532

The analytical technique described in the preceding three papers lends itself peculiarly well to the determination of total uncoagulable nitrogen, urea and ammonia in blood, milk, eggs and other liquids where we are dealing with minute amounts of these different constituents. In two earlier papers results obtained by adaptations of these methods to blood analysis were published.¹

The procedures by means of which these results were obtained are described in this paper.

I. METHOD FOR DRAWING BLOOD.

Before going into the details of the chemical work it would seem worth while to describe our method of drawing blood because so far as we have been able to learn it is somewhat different from the procedures employed by physiologists and because we believe it to be expeditious, neat and exact and therefore particularly suitable for quantitative work.

We use neither cannulae nor syringes but simply hypodermic needles and pipettes. The needles are about 1 mm. in diameter and about 25 mm. long. They are immersed in a dilute solution

¹ This Journal, xi, p. 87; Ibid, p. 161, 1912.
of vaseline in ether and then allowed to drain and dry on a clean paper for a few minutes before being used. (This does not apply of course to the drawing of human blood when the needles must be thoroughly sterilized.) An adequate supply of these needles is kept on hand so that we do not need to use any needle more than once in any one experiment. The needle is attached to the tip of a 2 or 5 cc. pipette by means of a short piece of narrow pure gum tubing. A small pinch of powdered potassium oxalate is introduced into the upper end of the pipette (which must be clean and perfectly dry) and is allowed to run down into the tip and the needle. The other end of the pipette is connected with a rubber tube which in turn connects with a mouth piece consisting of a short tapering glass tube. Close to the pipette the rubber tube carries a pinchcock.

To draw the blood one of us inserts the needle in the vein or artery and the other regulates the flow of the blood by means of the pinchcock and by suction. The exact quantity of blood desired is thus obtained without any waste and without clotting.

II. ISOLATION OF NON-PROTEIN NITROGENOUS CONSTITUENTS.

To separate the non-protein nitrogenous constituents from the protein materials we make use of pure (acetone-free) methyl alcohol and an alcoholic solution of zinc chloride. Ordinary methyl alcohol cannot be used because the impurities in it, particularly the acetone, combine with more or less of the urea so that it escapes decomposition in the subsequent treatment and is not quantitatively recovered. We have satisfied ourselves by means of determinations on pure urea solutions that the presence of acetone results in a loss of urea.

As soon as the blood is drawn it is transferred into measuring flasks half filled with methyl alcohol and the flasks are then filled up to the mark with methyl alcohol and vigorously shaken. Two cubic centimeters of blood we dilute to 25, while for 5 of blood we use 50 cc. flasks. At the end of two hours, or as soon after that as is convenient, the contents of the flasks are filtered through dry filters. To the filtrate are then added two or three drops of a saturated alcoholic solution of zinc chloride and after standing for a few minutes the mixture is again filtered through a dry
Otto Folin and W. Denis

paper. The zinc chloride brings down an appreciable precipitate and the last traces of coloring matters so that when the second filtration is made a perfectly colorless filtrate is obtained. Five cubic centimeters of these filtrates, corresponding to 0.4 or to 0.5 cc. of blood, depending on whether 2 or 5 cc. of blood were drawn, are taken for each determination. The precipitation procedure described above is the one which we ordinarily use. There are objections to it. We are not certain that traces of protein-like materials may not escape precipitation by this as by every other method and we do know that the filtrate does not contain all of the non-protein materials. When relatively large quantities (equivalent to 100 mgm. of nitrogen per 100 cc. of blood) of creatine, or asparagine are added to blood and treated as described above there is invariably an appreciable loss of material. To overcome this loss we have tried to triturate and wash the first alcoholic precipitate with methyl alcohol, and with some substances, as for example, with glycocoll, urea and acetamide, we are thus able to get practically quantitative results while with others, such as creatine, asparagine, and tyrosine, we still do not get quite all. Moreover, such trituration and washing does leach out a small amount of the coloring matters of the blood so that except for special experiments with less soluble substances we consider the simpler procedure rather more satisfactory.

In the case of muscle analysis, on the other hand, we thoroughly triturate and wash with the alcohol. Incidentally it should be said that, muscles as soon as cut out, while still twitching, are cut with a pair of sharp scissors and immediately immersed in methyl alcohol (about 50 cc. in an Erlenmeyer flask). After being allowed to stand for a few hours the coagulated muscle is thoroughly ground up and then extracted overnight with a fresh portion of alcohol. The various extracts and washings are then combined, filtered into a 100 cc. volumetric flask and after the addition of a few drops of alcoholic zinc chloride solution, made up to volume with methyl alcohol and again filtered. We invariably start with 5 grams of muscle and use 10 cc. of the filtrate for each determination of total nitrogen as well as of urea.

III. DETERMINATION OF THE TOTAL NON-PROTEIN NITROGEN.

To determine the total non-protein nitrogen of the blood 5 cc. of the alcoholic filtrate is transferred to a large Jena test tube of the same kind as is used in urine analysis (see p. 494). One drop
of sulphuric acid, one of kerosene and a pebble are added and the methyl alcohol is driven off by immersing the test tube in a beaker of boiling water for five to ten minutes. When the alcohol is removed 1 cc. of concentrated sulphuric acid, a gram of potassium sulphate, and a drop of copper sulphate solution are added and the mixture is boiled, cooled and diluted as in the analysis of urine (see p. 494).

From this digestion mixture the ammonia is removed in the usual manner. It is, however, not collected directly in a measuring flask (as in urine analysis) but in a second large test tube previously charged with 1 cc. of H₂SO₄ acid and 2 to 3 cc. of water. The reason for this variation is that 0.4 to 0.5 cc. of blood contains only 0.1 to 0.2 mgm. of non-protein nitrogen. The final Nesslerized solution cannot be diluted to 100 cc. and smaller volumetric flasks cannot be used as receivers during the air current treatment because of spattering. Large test tubes are therefore used as receivers and the ammonia is Nesslerized in these before the liquids are transferred to measuring flasks. Ordinarily the colored solutions obtained from cat's blood are transferred to 25 cc. flasks and are then found to have a depth of color which permits of a sure and accurate reading in the colorimeter. In some of our absorption experiments the total non-protein nitrogen runs up to very high figures and then the solutions are diluted to 50, sometimes even to 100 cc., before being read in the colorimeter.

Human blood contains scarcely more than one half as much non-protein nitrogen as cat's blood. In the case of human blood we therefore never draw less than 5 cc. and we take 10 cc. of the filtrate for each determination. In all other respects we use the same procedure for human blood as for cat's blood. In all ordinary cases 7 to 8 cc. of diluted Nessler's reagent (dilution 1:5) are added for the production of the color. If much ammonia is present so that the resulting colored solution must be diluted to 50 or 100 cc. correspondingly larger amounts of Nessler's reagent are added.

The calculation of the analytical results to milligrams of nitrogen per 100 cc. of blood is not difficult but the formulae given below may prove useful. In these formulae the standard solution contains 1 mgm. of nitrogen (as ammonium sulphate) Ness-
Otto Folin and W. Denis

lerized in a 100 cc. flask and the colorimeter prism of the standard is set at 20 millimeters. \[ \frac{50}{R} \times D \] in which \( R \) stands for the reading of the unknown and \( D \) represents the volume to which its ammonia has been diluted gives the desired figure. The reason for the figures is that we are here working with 0.4 cc. of blood.

When 5 cc. of blood is taken and it is diluted to 50 the formula becomes \[ \frac{40}{R} \times D. \]

When working with human blood and taking 10 cc. of the filtrate obtained from 5 cc. of blood diluted to 50 the formula is \[ \frac{20}{R} \times D. \]

It may be thought that we are using unnecessarily small amounts of blood in these analyses. We are, however, by no means sure that working with larger amounts would yield more accurate results and we have satisfied ourselves by scores of duplicate analyses that the method as outlined gives trustworthy figures. Further, the smaller the quantity of blood which can be made to give reliable results the greater becomes the usefulness of the method. The work which we have already done on cats could not have been done on such a small animal except by means of these microchemical methods. Finally, small amounts of blood must be used for the urea determinations because of the disturbing effects of the sugar present (see p. 520).

IV. DETERMINATION OF UREA.

Having described in some detail the preliminary treatment of the blood for the removal of the proteins and also the procedure for determining the total non-protein nitrogen, the urea determination in blood can be described very briefly.

Five cubic centimeters of the alcoholic filtrate from cat’s blood (or 10 cc. from human blood) are taken for each determination. This amount is measured into one of the large Jena test tubes in which the decomposition is to be made. A drop of dilute acetic acid and two or three of kerosene are added and the test tube is then closed by a two-hole rubber stopper. Through one of the holes in the stopper passes a glass tube drawn out to a capillary
several inches long. The capillary end reaches nearly to the bottom of the test tube. Through the other hole passes a short bent glass tube which is connected with a good water pump (see p. 523). The test tube is placed in warm water and the vacuum pump is started. In ten to thirty minutes the combined action of the gentle heat, the air current (through the capillary) and the vacuum removes all the alcohol. The rubber stopper is then removed and the capillary tube is broken off by bending it against the sides of the test tube and is left there. Two cubic centimeters of 25 per cent acetic acid, a temperature indicator, a pebble and 7 grams of dry potassium acetate are added and the decomposition of the urea is accomplished by heating it to 153 to 158°C. for about eight to ten minutes exactly as in the urea determination described for urine (see p. 515).

The ammonia set free by the subsequent air current treatment is collected in a large test tube, there Nesslerized (usually with only 3 cc. of the diluted reagent), is made up to volume in a 10 cc. volumetric flask and the color comparison is made as in the case of the total non-protein nitrogen against the same standard solution of ammonium sulphate. We usually Nesslerize the total nitrogen, and the urea, and the standard, all at the same time. Since only 10 cc. is available of the solution corresponding to the urea, all of it must be poured into the Duboscq colorimeter cylinder for the making of the color comparison. Dry cylinders must therefore be used. If only one cylinder is available the urea should be read first. We find it extremely convenient, however, to have several extra cylinders for the colorimeter and are thus able to read a series of urea determinations without stopping to rinse and wipe the inside of the cylinder for each determination.

V. DETERMINATION OF THE AMMONIA.

The accurate determination of the ammonia in blood is beset with far greater difficulties than any of the earlier inventors of methods for its estimation have realized. The blood decomposes spontaneously (and particularly in the presence of alkalies capable of setting free the ammonia) at all temperatures even when kept on ice. The ammonia thus produced by decomposition in the course of a few hours is much greater than the preformed ammonia
present in the strictly fresh blood and when distillation methods are applied, whether in the vacuum or otherwise, the determination becomes little else than a measure of the decomposition.

The decomposition in tissues such as the liver is even greater than in the blood and for this reason (among others) we are of the opinion that there is not a single experiment on record proving that macerated liver tissue splits off by hydrolysis the NH₃ groups from ordinary amino-acids when the latter are added to such tissue.

In view of the instability of blood or of certain components of blood the determination of its ammonia can be accomplished with a reasonable degree of accuracy only by the speediest kind of a process. Having once thoroughly realized this fact the problem of determining this ammonia became with us a problem of learning to work with the smallest possible amount of material—a serious problem in view of the minute quantities of ammonia present in normal blood.

The Nesslerization process lends itself as does none other to the quantitative estimation of small amounts of ammonia but instead of working with milligrams, as in urine, or with tenths of a milligram, as with blood in the estimation of total nitrogen and urea, it became a question of working with hundreths of a milligram. The quality of the color produced by Nessler's reagent with ammonium salt depends greatly on the amount of ammonia present, the tint is yellow or yellowish green when the amount of ammonia is very small (see p. 496) and such faintly colored solutions can not be read in a Duboscq colorimeter as ordinarily used. It would of course have been possible to fall back on the procedure as it is used and has been used for a long time in water analysis, but we felt sure that this old process is not as reliable as the ammonia determinations we made by the help of a high grade colorimeter.

By means of two important modifications of the Duboscq colorimeter we have succeeded in meeting all the necessary conditions. The chief reason why a dilute Nesslerized solution cannot be read against a much stronger one is that the light is absorbed in passing through a deep layer of the solution. Two such fields cannot therefore be made to look alike. After having unsuccessfully tried various kinds of screens for reducing the amount of
light passing through the thin layers of concentrated solutions we finally attained the desired result by the help of an iris diaphragm attached to one side of the colorimeter. By means of this diaphragm we are able to make use of 0.5 mgm. of nitrogen as a standard and against it read a solution containing only a few hundredths of a milligram of ammonia nitrogen.

The second modification consists in the use of a 100 mm. polariscope tube as container for our unknown ammonia solutions instead of the cylinders which go with the Duboscq colorimeter. These cylinders are so large in diameter that the solutions would have to be made impractically dilute in order to furnish a reasonably high column. Ten cubic centimeters, for example, will reach to a height of only about 30 mm. in the Duboscq cylinders yet these are about the smallest colorimeter cylinders in the market. With 10 cc. we can, however, comfortably fill a 100 mm. polariscope tube and, as it happens, such tubes just fit the Duboscq colorimeter when the solid movable glass prism is removed.

In the determination of the traces of ammonia here under discussion two precautions, not needed in any of the other methods described in the preceding three papers, are necessary. The first is that too much Nessler reagent must be avoided. The greenish tint observed in very dilute ammonia solutions when Nesslerized is almost wholly due to an excess of the reagent (see p. 497). The second precaution is the necessity of using only water that is strictly free from ammonia for diluting the unknown. The amount of ammonia in ordinary distilled water is sufficient to introduce a considerable error in this determination, while in those previously described it does not matter, partly because the ammonia is so small as to be negligible in view of the fact that the standard and the unknown are diluted to about the same extent with the same water. In this case where we read through 100 mm. of the unknown solution against about 10 mm. of the standard the case is different and the ammonia of the water must be eliminated.²

² Ammonia-free water is easily obtained from ordinary distilled water by the addition of a little saturated bromine water and a few drops of concentrated caustic soda. See Claessen's Text-book, ii, p. 116. Such water containing hypobromite and alkali cannot of course be used for the absorption of the ammonia but only for diluting the reagent and for the final dilution to a definite volume.
We do not use such ammonia-free water for the small amount, 2 to 3 cc., employed for the absorption of the ammonia, but only for the water subsequently added in Nesslerizing and making up to a volume.

The method for the determination of the ammonia is as follows:

Ten cubic centimeters of systemic blood or 5 cc. of portal or mesenteric blood are drawn in the usual manner (described above) by means of a pipette and transferred directly to one of the large Jena test tubes so extensively used in this work. To it are added 2 to 3 cc. of the oxalate-carbonate solution described on p. 524 (15 per cent potassium oxalate and 10 per cent sodium carbonate) and about 5 cc. of toluol. The air current is then started and is run as fast as the apparatus can stand for 20 to 30 minutes. The liberated ammonia is collected, as previously described, in another large test tube charged with 5 to 6 drops of tenth-normal acid and 1 cc. of water.

On account of the strong air current available in this laboratory, and also because of the relatively long period during which the process is carried out, we have found it desirable to cover the top of the test tube receiver with a small funnel from which the stem has been removed, thus obviating any loss which might be caused by spattering. At the end of the time indicated the contents of the receiver is Nesslerized in the usual manner but more cautiously, adding in all not over 1 cc. of the previously diluted reagent (dilution 1:5). The solution is then carefully transferred to a 10 cc. volumetric flask, diluted to the 10 cc. mark, mixed, and with this solution the 100 mm. polariscope tube is filled and closed as for ordinary polariscope work.

Two standard solutions, one containing 0.5 mgm. the other 1 mgm. of nitrogen, are Nesslerized simultaneously with the unknown solution made up to volume (100 cc.) and one or the other is used as a standard.

In this case, of course, the unknown remains stationary and the standard solution must be adjusted until the two colors match.

In making this comparison it is necessary to keep moving both the diaphragm and the colorimeter prism in the standard solution until the right position of each is secured.

The colorimeter, as thus used, represents, we believe, a new departure in colorimetry and we are taking steps to secure the
making of such instruments. So far we have used an ordinary diaphragm taken from a microscope and have fastened it by means of two screw clamps on top of the colorimeter platform on which stands the cylinder. A new zero point has of course to be established to allow for the altered position of the cylinder. Now we are compelled to use one instrument exclusively for such ammonia determinations but we hope later to see such instruments properly made by some manufacturer.

In view of the fact that we have already published a number of ammonia determinations, made as described above, it seems unnecessary to insert more figures here. We do not assert that even those figures may not ultimately be found to be too high but we do believe that they represent the nearest approach to the true values that have yet been published.

We believe that the methods described in this paper will be found more serviceable than any hitherto available for the study of many important problems which can be solved only on the basis of blood and tissue analysis. We have so far published two papers (loc. cit.) and shall soon publish another more extensive one on the fate of the amino-acids absorbed from the digestive tract (and the gradual formation of urea). We hereby expressly revoke our earlier reservation (loc. cit.) of the field of research referred to in those papers by means of these methods. We would like to reserve for a while, however, the use of the methods for clinical investigations. We wish particularly to investigate nephritic cases and fevers, and for this purpose are now gathering data as to the variations in the composition of normal blood. The retention of 3 to 4 grams of non-protein nitrogen in a person of average size should be easily demonstrable by means of these methods unless the normal variations are greater than we have yet found them.

3 This Journal, xi, p. 161, 1912.
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