Direct Determination of Blood Ammonia by Berthelot's Coloration of Protein-free Supernatant Obtained by Folin-Wu Deproteinization

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An elevated blood ammonia level is encountered in some cases of severe hepatic disturbances, and it is said that increased concentration of ammonia in blood circulating the central nervous system is responsible for the neurologic symptoms typical of hepatic coma. So, determination of blood ammonia is now universally evaluated as a useful test for the diagnosis of terminal stage of hepatobiliary diseases.[1)2)]

Currently the most favored procedure for the estimation of blood ammonia is micro-gas diffusion analysis which was developed by Conway[3)] and improved by Seligson.[4)] Miller and Rice reported recently a new method which empolyed adsorption of ammonia on ion-exchange resin.[5)] In our experience their procedure was not satisfactory, because not only ammonia but also a significant amount of non-NH_3 nitrogenous substance is adsorbed to and, on elution, released from the resin, resulting in the interference with coloration specific to NH_3.

Shibata tried to scale down the volume of blood necessary for the determination by combined use of Seligson's micro-gas diffusion and hypobromite-phenosafranine coloration of ammonia.[6)] It enables us to perform ammonia determination with 1 ml of blood sample. However, this is not without fault. In the following the short-comings of Shibata's procedure are mentioned. Ammonia is formed rapidly in vitro after withdrawal of blood from the vessels, causing a significant rise of ammonia level unless the procedure of determination is started immediately. Accordingly Shibata's procedure demands a strict control of time. Furthermore, it requires a specially constructed assembly of apparatus which must be operated by skilled hands. This hinders the procedure from being used widely in the ordinary laboratory of clinical chemistry.

A few years ago Fujii and his associates devised a technique for the determination of blood ammonia which is new in principle without resorting to micro-gas diffusion.[7)] The blood is treated with a modified Folin-Wu reagent

and centrifuged to get a clear deproteinized supernatant receiving and containing the whole amount of ammonia in the original sample of blood. The supernatant is colorized by Berthelot's reaction which is specific to ammonia, and subjected to photoelectric colorimetry. In our hands this new method proved to be satisfactory with exception of minor disadvantages which concern with the concentration of buffer solution employed for coloration, composition of standard ammonia solution, and the procedure of collection of blood. The concentration of buffer solution was raised over the level specified in Fujii's procedure in order to improve the sensitivity of coloration for ammonia without being adversely affected by the coexistence of non-NH_3 nitrogenous substance. Augmentation of blood ammonia was prevented by immersing the blood container in the cakes of ice shortly after collection of blood.

METHODS

Principle.

The blood (arterial or venous) is deproteinized by addition of modified Folin-Wu reagent and filtration. To the filtrate is added a mixture of phosphate buffer solution (pH 11.2) and Berthelot's colorizing reagent which is composed of phenol, sodium nitroprusside and antiformin, to be incubated at 37°C for 30 minutes for full development of blue coloration of NH_3 contained in the filtrate. The optical density of the solution thus colored is measured at 620mμ in a photoelectric colorimeter.

Reagents.

1. Sodium tungstate solution (10 g/dl) : Dissolve 1 g of sodium tungstate $Na_2WO_4 \cdot 2H_2O$ (commercially available) in 10 ml of distilled water.
2. 1N H_2SO_4 solution : Add 3 ml of concentrated sulfuric acid (guaranteed reagent : GR) to 97 ml of distilled water. This should be prepared shortly before use lest it should be contaminated with ammonia absorbed from the air.
3. Phenol-nitroprusside solution : Dissolve 5 g of phenol (GR) and 25 mg of sodium nitroprusside in distilled water and make to volume of 500 ml. Store the solution in a refrigerator.
4. Antiformin solution (buffered : 0.2 M, pH 12.0) : Dissolve 4 g of sodium hydroxide NaOH (GR) and 35.8 g of disodium monohydrogenphosphate $Na_2HPO_4 \cdot 12H_2O$ (GR) in 400 ml of distilled water, followed by addition of 5 ml of antiformin (content of effective chloride 10 per cent).* Dilute the solution

* Check the effective chlorine content in the following way : — To one ml of 5-fold dilution of antiformin (commercially available) with water add 5 ml of water, 1 ml of potassium iodide olution, 0.5 ml of glacial acetic acid and a drop of 1 per cent starch solution, mix, and titrate with 0.1 N sodium thiosulfate $Na_2S_2O_3$ solution. A consumption of 4 ml of

to 500 ml.

5. Working standard solution of ammonium sulfate : Prepare mother solution of ammonium sulfate (50 mg/dl ammonia–N) by dissolving 2.35 g of ammonium sulfate $(NH_4)_2SO_4$ (GR) in distilled water in a 1000 ml volumetric flask and making to the volume. Dilute 1.0 ml of the mother solution with distilled water in a 100 ml volumetric flask to make 500γ/dl ammonia–N solution. Make aliquots of 4.0, 3.0, 2.0 and 1.0 ml of 500γ/dl ammonia–N solution to 5.0 ml with distilled water to get 400, 300, 200 and 100γ/dl ammonia–N solutions.

Procedure

1. Withdraw about 3 ml of blood from the vessel (antecubital vein or arteries), transfer in a vial containing about 6 mg of potassium oxalate crystals (anticoagulant) and mix to prevent coagulation. (Keep the vial cold in ice cubes).

2. Deproteinization : — Introduce an aliquot of 1.0 ml of blood sample in a centrifugetube. Add 0.5 ml of 10 g/dl sodium tungstate solution, mix gently by several times of inversion (with its mouth closed with a thumb covered with a parafilm sheet), mix with 0.5 ml of 1 N sulfuric acid solution, and allow to stand for 15 minutes. Centrifuge the tube (3000 rpm., for 5 minutes) to get clear supernatant without any floating flocculant.

3. Colorization : — Transfer 0.5 ml of the supernatant into test tube S. Introduce 1.0 ml aliquots of 0 (water), 100, 200, 300 and 400 γ/dl $(NH_4)_2SO_4$–N solutions to tubes B, C_1, C_2, C_3 and C_4, respectively. Add to these tubes, 10 g/dl sodium tungstate solution (0.5 ml) and 1 N sulfuric acid solution (0.5 ml) and mix. Transfer 0.5 ml aliquots of these mixtures individually into test tubes b, c_1, c_2, c_3 and c_4, respectively.

Add to all of these tubes 2.0 ml of phenol-nitroprusside solution and 2.0 ml of antiformin solution, mix and incubate in a 37°C water bath for 3 minutes.

4. Colorimetry : — Measure the optical densities of the colorized mixture in a photoelectric colorimeter with a filter of maximum transmittance at 620 mμ. (Set zero reading with colorized solution b).

Construct a calibration curve by collating the optical densities of colorized solutions c_1, c_2, c and c_4 with ammonia–N concentrations of 100, 200, 300 and 400 γ/dl. Read ammonia–N concentration correnspoding to the optical density of colorized solution of tube S from the calibration curve thus prepared. This is the ammonia–N concentration of the blood sample.

0.1 N sodium thiosulfate solution refers to 1.4 per cent effective chloride content.
If the effective chlorine is less than 10 per cent, use antiformin in excess of 5 ml to prepare the reagent so that adequate concentration of effective chlorine may be maintained.

RESULTS AND DISCUSSION

There are several points to which special attention should be paid for the successful performance of the procedure in this method.

Time necessary for maximal colorization and the stablity of coloration : — The coloration (production of indophenol blue) of ammonia with Berthelot's reagent is suppressed by the coexistence of the non-ammoniacal non-protein nitrogen. To eliminate this undesirable interference Fujii and his associates employed 10 g/dl sodium tungstate solution and 1 N sulfuric acid as deproteinizing reagent. Of course, colorization should be carried out at optimum pH.

Colorization was tested with various buffer solution (pH 11.2) of different concentrations to see the time necessary for attainment to maximum. To the blood sample and the standard ammonia solution (300γ NH_3–N ammonium sulfate solution) was added the deproteinizing reagent (composed of sodium tungstate and 1 N sulfuric acid), followed by addition of phenol-nitroprusside solution and antiformin solution in a way as specified in the procedure. The mixtures were incubated at 37°C for 10, 20, 30, 40 and 50 minutes. They were measured for optical density at 620 mμ in a ADS photoelectric colorimeter (adjusting 0 reading with distilled water). The results are shown in Figure 1. Stability of the color of colorized solution of deproteinized supernatant of a blood sample was traced for 2 hours. This is presented in Figure 2. It will be seen from these figures that the optical density attains the maximum at the end of 20 minutes and forms a plateau for the succeeding 2 hours.

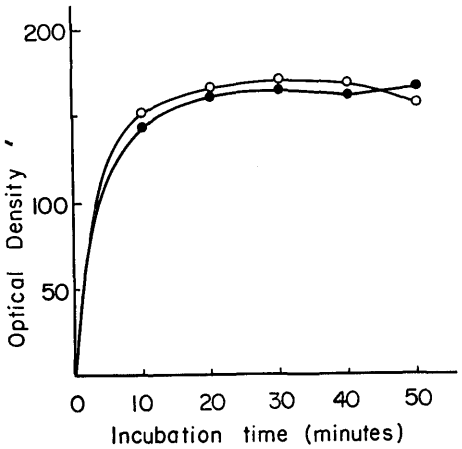

Fig. 1. Time necessary for maximal coloration
o——o : The present method.
•——• : Fujii's original method.
"Optical density" taken on the ordinate represents the reading of dial in S-ADS colorimeter.

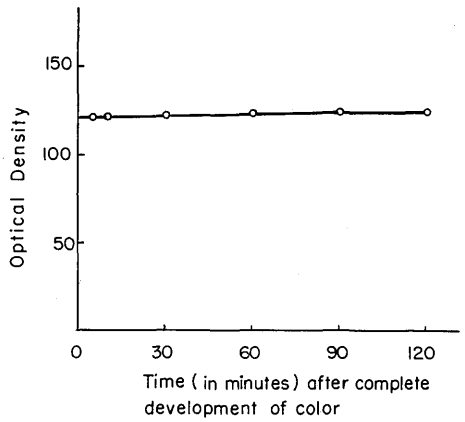

Fig. 2. Stability of the completely developed coloration.
"Optical density" taken on the ordinate represents the reading of dial in S-ADS colorimeter.

Absorption spectrum of the colored solution: — The absorption curves of the colored solutions (deproteinized blood sample and 200 γ NH_3-N/dl ammonium sulfate solution) have their peaks at 625 mμ. (Figure 3). Accordingly, maximum sensitivity of reading is expected to be obtained if an interference filter possessing maximum transmittance at 620 mμ is employed for the measurement of their optical density.

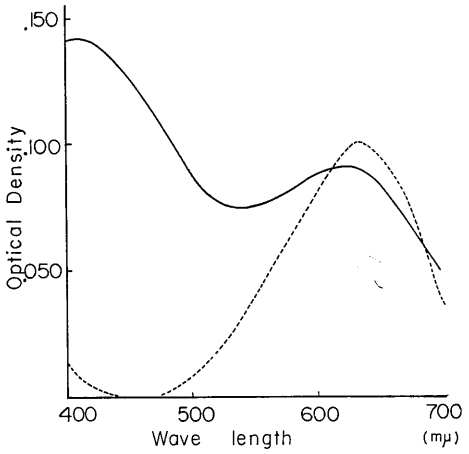

Fig. 3. The absorption curve of the coloration of a standard solution.
——: The present method.
……: Fujii's original method.

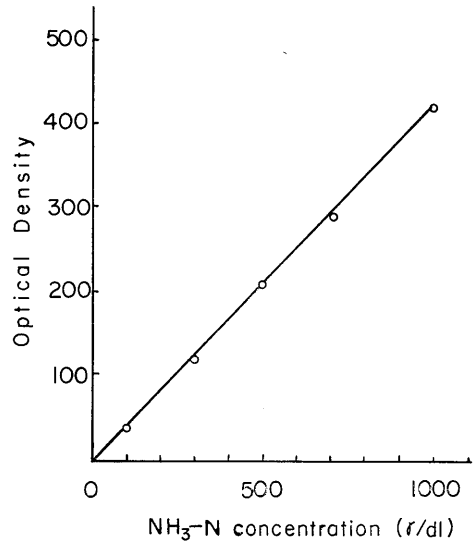

Fig. 4. Calibration curve.
"Optical density" taken on the ordinate represents the reading of dial in S-ADS colorimeter.

Calibration curve (Figure 4) : — The standard solutions of ammonium sulfate used for the construction of calibration curve should be colorized with color reagents in exactly the same way as the serum samples. Standard ammonium sulfate solutions to which water is simply added so that the finally colored solution may have the specified volume give optical densities lower than those of the standard solutions colored according to the specified procedure by treating with deproteinizing solution. In Fujii's original method, standard solutions and samples are colored in different ways: To the former solutions is added water, while to the latters the deproteinizing reagent. However, in our opinion, it is necessary to treat samples and standard solution in exactly the same way if one wants to read true ammonia concentration from the calibration curve.

Duplicate determinations and recovery test: — The discrepancies between the duplicate determinations were 4.4 per cent on an average when 28 samples of blood collected from healthy subjects and patients with hepatobiliary disorders

were estimated for ammonia by this method. Tests with 40 samples gave a recovery rate of 95 ± 5.6 (SD) per cent.

Comparison with Seligson's microdiffusion method (Figure 5) :— A comparative study of this method with Seligson's microdiffusion method with 30 indiscriminately collected blood samples disclosed a satisfactory correlationship as shown in Figure 5 (coefficient of correlation between these two procedures was equal to $+0.82$).

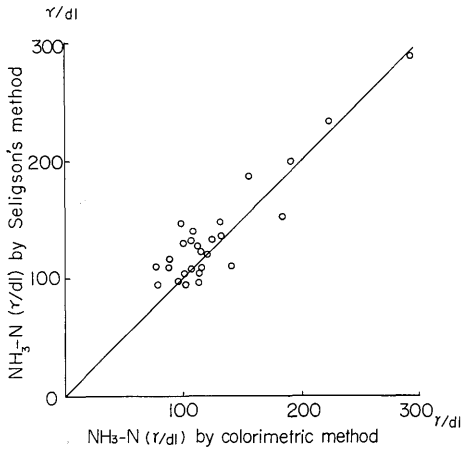

Fig. 5. Comparison of the present method (colori-metric method) with Seligsons' procedure.

Intensity of coloration in relation to the pH and the composition of buffer solution: — It has been well known that coloration of ammonium salt with Berthelot's reagent attains its maximum intensity in the range of pH between 11 and 12. In fact there is an increase of as much as 20 per cent in coloration when pH of the medium is elevated to 11.2 from 10.1. (The latter pH level was specified by Fujii for the buffer solution of his original method.) The intensity of color reaches its plateau at pH 11.2 to 11.4 without affecting the correct reading of ammonia concentration (Figure 6). Since the blood of a normal subject contains a very minute amount of ammonia, the procedure of coloration ought to be improved to display its maximum potentiality so that it may become as sensitive to ammonia as possible. With this in mind, several buffer solutions which are different in pH and composition (NaOH to Na_2HPO_4 $12H_2O$ ratio) were examined (Figure 7). The largest inclination of the calibration curve which assures the largest sensitivity was obtained with the buffer solution containing 4 to 5 g of sodium hydroxide and 35.8 g of Na_2HPO_4 $12H_2O$ in 500 ml of distilled water.

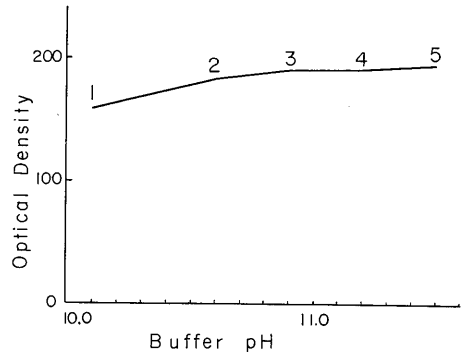

Fig. 6. Intensity of coloration in relation to pH. The numerals 1-5 represent the results obtained by employing the buffer solutions (1)-(5) described in Figure 7, which are different in pH and composition.
"Optical density" taken on the ordinate represents the reading of dial in S-ADS colorimeter.

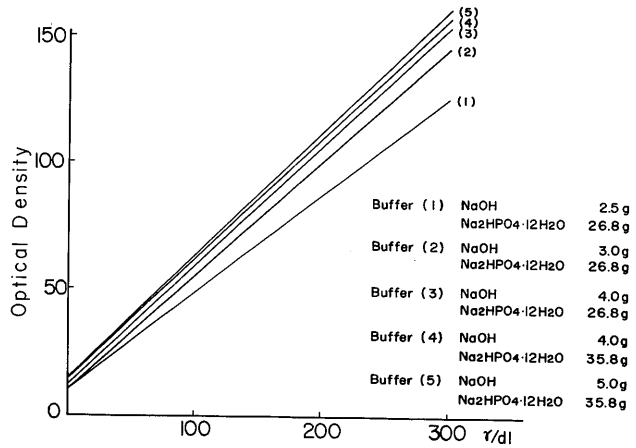

Fig. 7. Calibration curve constructed by employing various buffer solutions, (1)-(5), which are different in pH and composition.
"Optical density" taken on the ordinate represents the reading of dial in S-ADS colorimeter.

Effect of non-NH_3 nitrogen substances upon the coloration: — Some of the non-NH_3 nitrogen substances coexisting with ammonia intensify or inhibit the Berthelot coloration. This effect or the interference by the non-NH_3 nitrogen substances varies according as the buffer solutions employed for coloration are different. Figure 8 presents an example which was examined by adding glycine to the buffer solution as an interfering substance. It is apparent from the scrutiny of this figure that the buffer solution with 4.0 g of NaOH and 35.8 g

of Na_2HPO_4 $12H_2O$ in 500 ml of H_2O is again among the best one which is useful for exact coloration without being affected by interference. This was named buffer solution #4.

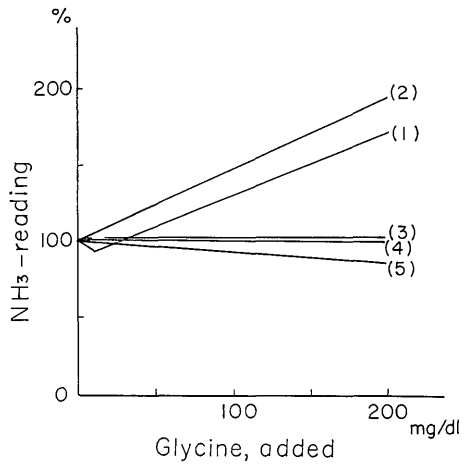

Fig. 8. Influence of non-protein nitrogen (glycine) upon the Berthelot coloration observed by employing various buffer solutions, (1)-(5), whose composition are given in Figure 7. NH_3-reading refers to the apparent NH_3 concentrations expressed in terms of the per-centage of the NH_3 concentration of a test sample to which glycine is not added.

Non-protein nitrogen and the Berthelot coloration: — The effect of non-protein nitrogen on the Berthelot coloration was studied in detail with buffer solution #4, because this seemed to be the best by the examinations described above. None of the non-protein nitrogen substances examined in this experiment disturbed the Berthelot coloration significantly : Urea caused only 1 per cent increase in optical density at the concentration of 100 mg/dl, while creatinine resulted in only 2 per cent suppression of optical density at 10 mg/dl. The coloration was not interefered with by glycine even at its concentration of 200 mg/dl, but leucine exerted an effect toward increase in coloration by 2 per cent and 15 per cent at 10 mg/dl and 50 mg/dl, respectively.

Since the normal ranges of the concentrations of urea, creatinine, and amino acids are far below these levels, it is expected that buffer solution #4 will assure an exact Berthelot coloration which is exempted from the interference by coexisting non-NH_3 nitrogen substances.

Collection and transportation of blood samples: — Collection and transportation of blood samples make important preliminary steps exerting decisive effect on the determination of ammonia. Ammonia is produced incessantly anew by hydrolysis of non-NH_3 nitrogen substances after collection, if the blood sample

is allowed to stand at room temperature. Anticoagulants possessing NH_4–salts can not be used for the prevention of blood from clotting, because it obviously causes falsely high blood ammonia value as a result of contamination. Accordingly, Fujii recommended direct pipetting of blood sample (from its container) into the deproteinizing reagent immediately after its withdrawal from the vein with a syringe. However, this is not always an easy task on account of occasional addident of accelerated blood coagulation. Quantitation of blood which has been kept fluid by addition of anticoagulant free from ammonium salts is easier and more convenient. In our experience potassium oxalate proved to be the anticoagulant of choice. Release of ammonia from the non–NH_3 nitrogen substances was successfully suppressed when the test tube containing potassium oxalate-added blood was cooled with ice cubes. This enabled us to have sufficient time for (1) the collection of blood sample from the patients on the ward, (2) its transportation to the laboratory and (3) the arrangements necessary for the determination of ammonia.

Increase in ammonia reading with lapse of time after deproteinization of blood sample: — In Fujii's original procedure there was an gradual increase in the reading of ammonia concentration with the passage of time when blood was mixed with deproteinizing reagent, and allowed to stand at room temperature so that the deproteinized supernatants of blood sample might be subjected to the Berthelot coloration at various time intervals. A significant and a remarkable increase were observed at 3 and 8.5 hours after mixing (Figure 9). In contrast,

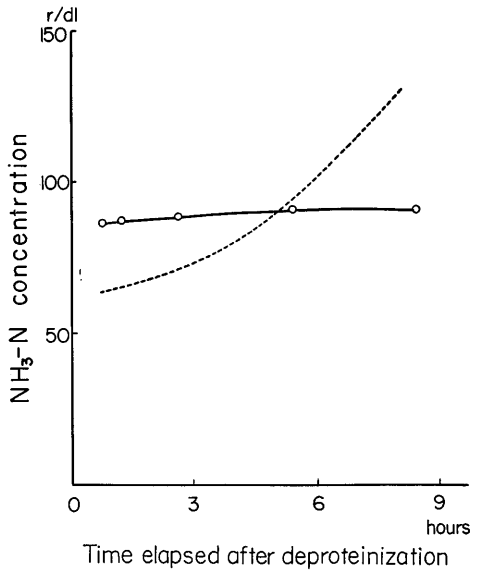

Fig. 9. Duration of allowing to stand the deproteinized supernatant of the sample with its precipitated protein in reference to the development of the Berthelot coloration (NH_3-reading).
o——o : The present procedure. ····· : Fujii's original method.

such increase was not seen in our procedure. The reading remained approximately constant for 9 hours with our deproteinizing reagent (Figure 9).

SUMMARY AND CONCLUSION

A direct determination of blood ammonia circumventing the use of micro-diffusion apparatus which was devised by Fujii consists in (1) deproteinization of blood by a modified Folin-Wu's procedure,(2) coloration of thus deproteinized supernatant of blood by the Berthelot reaction and (3) colorimetry. This is a simple and convenient method, but our experience disclosed several drawbacks in it. A series of experiments were carried out in our laboratory in the hope that Fujii's method might be improved. The following is the outline of our improvement.

1°. Collection of blood sample. Blood (about 3 ml) is withdrawn with a syringe by venous puncture, transferred into a test tube containing the crystals of potassium oxalate, stirred gently to prevent coagulation, and immediately cooled by inserting into a pile of ice cubes so that release of ammonia from non-NH_3 nitrogen substances may be stopped completely.

2°. Deproteinization. To the blood (1.0 ml) thus collected are added 10 g/dl sodium tungstate solution (0.5 ml) and 1 N sulfuric acid (0.5 ml), allowed to stand (for 15 minutes), and centrifuged to get clear supernatant. (This procedure of deproteinization insures the elimination of danger for the spontaneous increase in ammonia reading which may arise while the mixture of blood with deproteinzing reagent is allowed to stand at room temperature).

3°. Coloration. The deproteinzed supernatant of blood (0.5 ml) is mixed with phenol-nitroprusside solution (2.0 ml) and antiformin solution (2.0 ml), mixed and incubated at 37°C (for 3 minutes). (A stable blue coloration which is not disturbed by the co-existing non-NH_3 nitrogen substances is obtained in this way.)

4°. The standard solutions of ammonium sulfate (1.0 ml aliquots; 100, 400 mg N/dl) are treated with deproteinizing reagents and color reagents in the same way as described above (3°) in order to construct a calibration curve.

5°. Colorimetry at 630 mμ.

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