THE COLORIMETRIC ESTIMATION OF SMALL AMOUNTS OF AMMONIA BY THE PHENOL-HYPOCHLORITE REACTION

By JANE A. RUSSELL

(From the Department of Physiological Chemistry, Yale University School of Medicine, New Haven)

(Received for publication, September 8, 1944)

The micro diffusion technique of Conway (1) is a satisfactory procedure for the isolation of small quantities of ammonia. The estimation of very small amounts of ammonia by the usual back titration offers some difficulties, since it requires the use of special ultramicro burettes, extensive precautions against contamination with carbon dioxide and other acids and bases, and considerable experience with the method on the part of the operator. A simple and sensitive colorimetric method for ammonia has been elaborated as an alternative procedure. The reaction, first used extensively by Van Slyke and Hiller (2) and by Borsook (3), is that which occurs between ammonia, phenol, and hypochlorite in alkaline solution to yield an intense blue product (believed to be indophenol or a closely related substance). Further study of the reaction has led to a several fold increase in its sensitivity. With a photoelectric colorimeter, it is possible to detect 0.1 γ per ml. and to determine with reasonable accuracy 0.5 γ per ml. or more of ammonia nitrogen.

Reagents—

1. Alkaline phenol reagent. 25 per cent phenol in 2.7 N sodium hydroxide. Mix 25 gm. of crystalline phenol with water; add with stirring 54 ml. of 5.0 N sodium hydroxide and make to 100 ml. Preserve in a brown bottle in the refrigerator.

2. Hypochlorite solution. Grind and sift 25 gm. of calcium hypochlorite (bleaching powder, U. S. P.) and dissolve it as far as possible in 300 ml. of hot water. Add with stirring 135 ml. of potassium carbonate solution (20 gm. of anhydrous salt in 100 ml. of solution, previously boiled to free it of ammonia). Mix thoroughly, heat briefly to about 90°, cool, and make to 500 ml. Filter a small portion of the mixture and test for calcium ion; if the test is positive, add more carbonate to the mixture until a negative test is obtained. Filter the mixture and store the filtrate in small brown bottles in the refrigerator. This solution should be water-clear and contain 1.30 to 1.40 gm. of free chlorine per 100 ml.

Test for Calcium Ion—To 1 ml. of the solution add a little of the potassium carbonate solution and heat in boiling water a few minutes. The solution should remain crystal-clear in the absence of calcium ion.
DETERMINATION OF AMMONIA

Test for Free Chlorine—To 2.00 ml. of the hypochlorite solution add 10 ml. of water, 2 ml. of 5 per cent potassium iodide, and 1 ml. of glacial acetic acid. Titrate with 0.100 N sodium thiosulfate (starch indicator); 7.5 to 8.0 ml. of thiosulfate should be required. Retest the strength of the solution occasionally.

3. Manganese salt. Manganous chloride or sulfate 0.003 M.

Color Development—Place in a calibrated test-tube or colorimeter tube 1.5 ml. of sample containing 0.5 to 6 γ of ammonia N, 1 drop (about 0.05 ml.) of 0.003 M manganous salt solution, 1 ml. of alkaline phenol reagent, and 0.5 ml. of hypochlorite solution. The two latter solutions should be cold when added to the sample; loss of ammonia from the alkaline solution may be further reduced by keeping the sample tubes in an ice bath during their preparation. Mix the contents of the tubes by gentle rotation, and place them immediately in a briskly boiling water bath for about 5 minutes. Cool. Make to a convenient volume (e.g., 6 or 10 ml.) and read in a photo-electric colorimeter with a filter having an absorption maximum near 625 μm.

The sample volume may be as much as 5 ml. if larger amounts of nitrogen are present. In this case the amounts of manganese and hypochlorite added should be increased approximately in proportion to the total volume of the solution during color development. Standards and blanks must of course be prepared to have the same composition. With the volumes given above and a volume at a reading of 6 ml., 0.1 γ of nitrogen gives a detectable color and 0.5 to 6 γ may be determined. With a sample volume of 5 ml., 1 ml. of phenol reagent and 1 ml. of hypochlorite solution and a final volume at a reading of 10 ml., 2 to 20 γ of nitrogen may be determined.

The ammonia sample should be neutral or in acid not more than 0.01 to 0.02 N in strength. When the method is used in conjunction with the Conway micro diffusion technique, the contents of the inner wells of the absorption units are conveniently rinsed into colorimeter tubes with a small bulb pipette. The contents of blank Conway units may serve as blanks in the colorimetric method if standards are run in Conway units simultaneously; this is the usual procedure when the interrupted absorption period is used, as in the determination of blood ammonia. When the full absorption period is allowed, it may be more convenient to prepare standards and blanks in the usual fashion and to correct the sample readings for the small and fairly constant value of the blank from the Conway unit.

DISCUSSION

The reaction as used by Van Slyke and Hiller and by Borsook was carried out in strongly alkaline solution, the phenol reagent consisting of 25 per cent phenol dissolved in 20 per cent sodium hydroxide. In this
circumstance, when the reaction takes place at or near 100°, the color formed is not readily reproducible or suitable for photometric measurement. At lower temperatures, as at 37°, recommended by Borsook, the reaction takes some time to go to completion (1 to 2 hours) and the color is less than that produced at 100°. However, if the final alkali concentration is made equivalent to the molar strength of the phenol present (pH about 12), the reaction may be carried out at 100°, with the production of color about 3 times as intense as that produced by the more strongly alkaline reagent

![Graph](http://www.jbc.org/)

Fig. 1. The relationship of pH and of phenol concentration (0.1 to 8 per cent, Curves F to A) to color development in the estimation of ammonia by the phenol-hypochlorite reaction. All samples contained 10 γ of ammonia nitrogen and the volume of solution during color development was 10 ml. The concentration of hypochlorite and the measurement of pH are described in the text.

The colored substance produced in this way has a smooth reproducible absorption curve with a maximum at 625 mμ, as determined with a Beckman spectrophotometer at 10 mμ intervals.

The relation of the pH of the reaction and of the final phenol concentration to the density of color produced is shown in Fig. 1. The pH was maintained with appropriate buffer mixtures when the phenol concentration was low. The pH was measured with a glass electrode after color development, correction being made for the error due to the presence of sodium ion (4). In the case of the highest concentration of phenol shown in Fig. 1 (8 per cent), the pH could not be measured but was calculated from the
composition of the reagent. The optimum pH for concentrations of phenol from 1 to 10 per cent is between 11 and 12, and at this pH the maximum color is obtained with approximately 8 per cent phenol. Lower concentrations, down to 3 per cent, may be used, however, if the amounts of nitrogen present are such as to make a less sensitive reaction desirable. With concentrations of phenol less than 1 per cent, the optimum pH is lower; it is sharply at 10 for 0.25 per cent phenol and between 8 and 9 for 0.1 per cent. (The latter concentration of phenol was used by Hinsburg and Mucke (5), without control of the pH other than initial neutrality of the sample.) Below pH 7, little or no color is produced. It had been expected that at a pH lower than 9 the reaction might be more efficient because of better retention of ammonia, but since only low concentrations of phenol (about 0.1 per cent) could be used here, maximum colors comparable to those produced at higher pH with higher phenol concentrations were not obtained.

The concentration of hypochlorite found satisfactory is nearly that used by Borsook, and Borsook's reagent is recommended. About 2.0 (1.5 to 2.5) mg. of available chlorine per ml. are required during color development when the phenol concentration is over 1 per cent. With lower concentrations of phenol, the hypochlorite concentration must be carefully balanced with it, in about equimolar proportions with respect to phenol and free chlorine. Such concentrations were used in preparing the curves presented in Fig. 1.

The color production by this reaction is influenced by the presence of certain ions. Iron, chromium, and manganous ions catalyze the reaction; Mn⁺⁺ is the most effective in enhancing color development, increasing it about 25 per cent, and also increasing its reproducibility. Copper ions tend to inhibit color development.

The density of color produced by the procedure described above, when measured with an Evelyn colorimeter and Filter 620, is a linear function of the concentration of ammonia present. The color is reasonably reproducible but, as in other colorimetric methods, accurate work requires comparison with known standards prepared at the same time. The color is stable for at least an hour after development.

This method of estimating ammonia has not been applied directly to biological fluids, because certain amino acids react to a small extent. Urea does not participate in this reaction. It might therefore be possible under some circumstances to estimate urea by direct determination of ammonia before and after the action of urease. Direct application of the colorimetric method to micro-Kjeldahl digests is not recommended, since traces of peroxide interfere with the reaction, and the control of the final pH offers some difficulties. It would of course be possible to apply the method to
larger samples of ammonia obtained by distillation or aeration procedures other than the Conway technique.

SUMMARY

The conditions for maximum color development in the reaction of ammonia with phenol and hypochlorite have been investigated and the sensitivity of this test for ammonia has been considerably increased. A simple and reliable colorimetric procedure for estimating very small amounts of ammonia nitrogen has been described, which is suitable for use with the Conway micro diffusion method or other distillation or aeration procedures.

BIBLIOGRAPHY

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