THE DETERMINATION OF AMMONIA IN BLOOD AND OTHER BIOLOGICAL FLUIDS

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INTRODUCTION

In 1912 Folin and Denis (1) published a series of methods for the analysis of blood and urine, all based on the principle of Nesslerization, and among those methods was one for the determination of ammonia in blood. By the help of this method Folin and Denis proved the then important point that the relatively high ammonia content of portal blood is mostly putrefactive ammonia absorbed from the gut. After having further found by a general survey that nothing else of physiological or clinical significance could be elucidated by the method we lost interest in it, and we failed to grasp the fact that the blood ammonia values found by the new method might be too small to account for the very large quantities of ammonium salts occurring in urine. Several years later, in 1921, Nash and Benedict (2) seemingly proved by substantially the same method that the ammonia found in urine must be liberated in the kidneys. Bliss (3), at my suggestion, repeated the essential parts of the work of Nash and Benedict and confirmed their finding that the blood of the renal arteries contains less ammonia than the blood of the renal veins. Later, Bliss continued the work from a new point of view, and he has endeavored to prove that there is probably ammonia formation in all tissues, but that this ammonia, originally used to neutralize acids formed in the tissues, is carried in the blood in the form of amide nitrogen contained in the blood protein of the corpuscles from which it is liberated as ammonia in the kidneys. This paper has no bearing on the controversy over the validity of Bliss' findings and interpretations.
Parnas (4) and collaborators have determined the ammonia in blood and tissues by a somewhat different method. They remove the ammonia from blood by steam distillation in a partial vacuum and they claim that by this means they obtain more dependable values.

The ammonia in blood, according to all these different investigators, though always very small, is subject to considerable variations. Most of the figures for the ammonia in systemic blood run below 0.1 mg. per 100 cc. and many figures, particularly those reported by Nash and Benedict and by Parnas, are as low as from 0.03 to 0.05 mg. Even lower figures, 0.01 and 0.02 mg. per cent, have been obtained by Mann and Bollman, by the Nash-Benedict procedure.

For several years I have been aware that one somewhat serious error was made in the elaboration of the aeration-Nesslerization system of analysis which was published in 1912, and the effect of this error should be particularly noticeable in the determination of the minute amounts of ammonia present in blood. In connection with the methods of 1912, we abandoned the use of the special ammonia absorption tube which I had devised many years earlier for the aeration-titration method (5). We failed to realize that the quantitative extraction of very small amounts of ammonia by the help of a rapid air current is more difficult, instead of less difficult, than the recovery of large amounts (several mg.). In our check work we probably very seldom used less than 1 mg. of ammonia and probably never used less than several tenths of a mg. We thus failed to find the important fact that the losses of ammonia, due to incomplete absorption in the receiver, may be almost as large in absolute quantity when working with 0.05 mg. as when working with 0.5 mg. A technically important error was therefore made when we replaced the effective special ammonia absorption tube by one consisting only of a straight glass tube with a perforated bulb at the lower end.

Isolation of Ammonia by Air Currents

One of the first steps in the present research was to adapt the aeration-Nesslerization method for the determination of small amounts of ammonia to the use of the special absorption tube. The original tube, even when made entirely of glass, is much too
large for use with test-tubes, but a series of smaller ones was made, and no difficulty was encountered in getting them small enough for use with test-tubes having an internal diameter of 20
to 22 mm. These small ammonia absorption tubes, made of Pyrex glass, are just as effective as the original larger ones. They are made for us by the Macalaster Bicknell Company, Cambridge, Massachusetts. Fig. 1 is a diagram of the tube.

Having once made and perpetuated an error with regard to the absorption of ammonia from a rapid air current, I have now endeavored to determine just how much reliance can be placed on the new smaller absorption tube. For this purpose I made use of an excellent gas meter, borrowed from the Nutrition Laboratory, Carnegie Institution of Washington. This precaution seemed particularly necessary because our air supply (outside air) is delivered in practically unlimited quantities under a pressure of about 20 pounds to the sq. inch. The essential points found in the course of these tests can be briefly stated.

1. From 1 mg. to 10 mg. of ammonia nitrogen were quantitatively recovered from the fastest air current which it was practical to use—12 liters per minute.

"Quantitatively recovered" in this case does not mean absolutely quantitative. Slight losses must have occurred, but these were so small that they escaped detection in the determinations. The 1 mg. quantities (or less) were determined by Nesslerization, the large quantities by titration.

2. With 0.05 mg. nearly one-third fails to be absorbed by 10 cc. of water plus 1 cc. of 0.1 N acid when the speed of the air current is 14 to 15 liters per minute.

3. With an air current of 8.5 liters per minute, a slight loss, about 5 per cent, may be encountered when one is working with 0.05 mg. of ammonia nitrogen.

4. 7 to 8 liters per minute represent, therefore, the maximum speed of air current which will permit the complete recovery of 0.05 mg. of ammonia nitrogen by the use of the new ammonia absorption tube.

For actual work it is best to use only a speed of from 6 to 7 liters per minute. A speed of only 4 liters per minute is somewhat too slow to be effective for the removal of ammonia from 10 cc. of blood according to the process described in this paper.

The ammonia absorption tube is sufficiently small so that it might be used with the micro-Kjeldahl digestion tubes as receivers. But though there is distinctly less spattering with the new absorp-
tion tube than with a simple plain bulb tube, the maximum speed permissible with the digestion tube as receiver is only about 5 liters per minute. It is therefore better to use special tubes as receivers; mine are about 260 mm. long and have an outside diameter of about 25 mm. They are graduated at 25 cc. and at 50 cc.

After having thus found an adequate remedy for the treacherous uncertainty in the aeration method for isolating very small amounts of ammonia, it seemed worth while to reexamine the determination of ammonia in blood, a determination which represents the most exacting application of the aeration-Nesslerization principle. There were one or two added reasons for making such a critical study, one of which was that I hoped to be able to introduce one important modification applicable to all kinds of biological fluids.

The Foaming Problem

No generally adequate procedure has been found for eliminating foaming in connection with the distillation or aeration of colloidal solutions and this difficulty has always tended to limit analytical procedures. My recently described antifoaming reagent, excellent as it is for micro distillations, has proved inadequate when used in connection with the rapid aeration of blood. Other well known antifoaming reagents are unsatisfactory, because they are themselves quickly removed by the air current and in part collect in the receiver. The foaming difficulty is particularly great with such prolonged and repeated aeration of blood as may be necessary for critical studies. The viscosity of blood might well serve to retard the complete removal of the last traces of ammonia and this interference may be further increased by the mechanical obstruction offered by the millions of corpuscles which are present. Finally, some of the ammonia might be within the blood cells, and one does not know how rapidly this part of the blood ammonia will come out under the experimental conditions employed. Nor does laking of the blood help much, since it must involve either a considerable dilution or a greatly increased viscosity. It would therefore seem important, at least for check work, that one should be able to continue the aeration process until one is obtaining only the ammonia which is due to decomposition. It has been practically impossible to do this because of the foaming.
These considerations have led me to try a new, or rather an old but almost forgotten, principle applicable to the determination of ammonia. In the Schlösing method for the determination of ammonia in urine, the latter was left with a suitable alkali in a desiccator and above the liquid was placed a small dish containing standard acid for the absorption of the gradually escaping ammonia. Shaffer (6) showed nearly 30 years ago that under really suitable conditions this primitive method could yield fairly good results in the course of 2 or 3 days. It is rather remarkable that no one ever proposed to improve that old method by passing a slow air current through the desiccator and into a receiver containing the acid.

There is only a slight resemblance of the process described here to the Schlösing method, but like the latter, it depends to a certain extent on a large surface area for the escape of the ammonia. Instead of violently agitating the blood in a test-tube, by means of a rapid air current, I now sweep it out by letting the air current play over the blood in an Erlenmeyer flask. This sweeping process is not more efficient than the agitating process; it is possibly a trifle less efficient; but the important point is gained that even with the most rapid air current scarcely a single air bubble and not a single drop of spattering are encountered. For 10 cc. of blood, I use 300 cc. Erlenmeyer flasks, the bottoms of which have a diameter of about 8 cm.

In order to secure the maximum effect of such an air current, the delivery tube is drawn out to a point 1 to 2 cm. long, the internal diameter of which is about 1.5 mm. This point never touches the blood, the tip is about 2 to 3 mm. above the surface, but the force of the current is so strong that it pushes the blood away from the center, thus exposing the bottom of the flask in a circle, the diameter of which depends on the speed of the current. Under the given conditions the size of this circle furnishes an adequate practical index of the speed of the air current.

A 4 liter current will produce a clear circle about 0.7 cm. in diameter, while a 7 liter current will produce a circle about 1.5 cm. in diameter, and 15 liters per minute will give a circle with a diameter of 4 cm.

By means of this modification it should be possible to isolate the ammonia from any kind of colloidal solution. Even soap solution,
probably the most effective known foam producer, can be aerated by this process. The method has one limitation. It is absolutely essential, for rapid aerations, that the depth of the fluid shall not be much greater than that obtained from 10 to 15 cc. in a 300 cc. Erlenmeyer flask.

Which Alkali Should Be Used for Liberating the Ammonia Present in Blood?

For the mere removal of ammonia from a solution by air currents one can use an alkali as weak as a mixture of 1 part of carbonate to 3 parts of bicarbonate. But to remove every trace of the ammonia in the course of an hour under such conditions one must apply air currents of greater speed than is permissible, if every trace of the ammonia is to be collected in a receiver. After a great many experiments with carbonate-bicarbonate mixtures I have come back to carbonates as the most suitable.

In the course of this work I have tried the alkaline borate solutions so highly recommended by Parnas. We have long used solutions of ordinary borax for the isolation of small amounts of ammonia by distillation in blood urea determinations and it is possible that alkaline borates can be used at the intermediate temperatures prevailing in Parnas' steam distillations. But for use in connection with the removal of ammonia by aeration the borate solution which I tried is less effective even than a mixture of 1 part of carbonate and 3 parts of bicarbonate. My borate solution contained 9.4 gm. of boric acid and 105 cc. of \( \times \) NaOH in 250 cc. This is a supersaturated solution, but the excess borate is easily brought back into solution by a very little warming.

For many purposes, potassium borates should prove more serviceable than sodium borates, because of their much greater solubility, but for the liberation of ammonia, even highly concentrated potassium borate solutions are inferior to the carbonates. The superiority of the carbonates may be due in part to the fact that CO\(_2\) as well as the NH\(_3\) is easily driven off by air currents.

The results obtained in two typical series of comparative experiments are given in Tables I and II. In these experiments the air current was adjusted at the beginning to the stated speeds per minute, and was not interrupted until all the determinations had been finished. The ammonia, from the standard ammonium sul-
fate solution, was contained in 10 cc. of solution, in a 300 cc. Erlenmeyer flask.

**Nesslerization**

The somewhat elaborate provisions described by Folin and Denis for the Nesslerization of the ammonia obtained from 10 cc.

### TABLE I

*Illustrating Recovery of 0.05 mg. of Ammonia N by Air Current of 7 Liters per Minute, and Relative Efficiency of Different Alkalies*

<table>
<thead>
<tr>
<th>Aeration time (min.)</th>
<th>Alkali added</th>
<th>Per cent recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.2 gm. Li₂CO₃</td>
<td>101</td>
</tr>
<tr>
<td>10</td>
<td>0.2 &quot; &quot;</td>
<td>84</td>
</tr>
<tr>
<td>15</td>
<td>0.2 &quot; &quot;</td>
<td>93</td>
</tr>
<tr>
<td>15</td>
<td>2 cc. carbonate-oxalate solution</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>2 &quot; borate solution</td>
<td>71</td>
</tr>
<tr>
<td>20</td>
<td>2 &quot; &quot;</td>
<td>80</td>
</tr>
</tbody>
</table>

### TABLE II

*Illustrating Rate at Which 0.05 Mg. Ammonia N Is Isolated by Air Currents of 4 Liters and 6 Liters per Minute When 2 Cc. of Carbonate-Oxalate Solution Are Added to 10 Cc. of Ammonia Solution*

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Speed of air current</th>
<th>4 liters</th>
<th>6 liters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>55</td>
<td>74</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>70</td>
<td>85</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>90*</td>
<td>98†</td>
</tr>
</tbody>
</table>

* Residue in Erlenmeyer flask was about 0.005 mg. of NH₃-N.
† Residue in Erlenmeyer flask was about 0.001 mg. of NH₃-N.

of blood represent an early stage in the development of quantitative Nesslerizations, and they have become quite superfluous especially since the Nessler's reagent used at that time has long since been replaced by the reagent of Folin and Wu. It has been pointed out repeatedly that there is no need for getting turbid or smoky solutions instead of crystal-clear ones when Nesslerizing, but beginners are still publishing modifications designed to facili-
tate the production of clear solutions (7). In these circumstances, it seems best to call attention once more to the remarkable power of gum ghatti solutions to prevent the formation of turbidities (8). 2 or 3 drops of a 2 per cent gum ghatti extract are all that I ever use, although I have recommended as much as 1 cc. These gum ghatti solutions have proved very useful in the check work on the quantitative isolation of minute amounts of ammonia. For example, in working with a possibly ineffective ammonia absorption tube, incomplete recovery of the ammonia might be due either to inadequate aeration or to incomplete absorption. By Nesslerizing the aerated solution one can tell at once whether any ammonia is left and if so, how much. But if the carbonate-oxalate mixture is used as an alkali, Nesslerization of the mother liquor will give a turbidity almost immediately except in the presence of a little gum ghatti, whereas with only 2 drops of the latter present the Nesslerized mother liquor will remain clear for hours. Our gum ghatti solutions are preserved with about 0.1 per cent of benzoic acid which is introduced in the form of a 20 per cent alcoholic solution (5 cc. per liter).

When Nesslerizing the minute quantities of ammonia which may be obtained from blood, and especially when Nesslerizing the almost imperceptible traces which may be obtained from second and third aerations, there is more or less danger of getting turbidities unless the gum ghatti solution is used. In the presence of the protective colloid the solutions always remain perfectly clear, even when left overnight.

The Determination

There is really little to be said about the form of blood ammonia determination as I now use it which has not already been mentioned.

Transfer 10 cc. of blood to a clean, dry Erlenmeyer flask. Add 2 cc. of potassium oxalate-carbonate solution (10 per cent potassium carbonate (K₂CO₃) plus 15 per cent potassium oxalate). Insert a clean 2-hole rubber stopper carrying two glass tubes. The tube reaching to within about 1 cm. of the bottom of the flask is drawn out to a point having an internal diameter of about 1.5 mm. It connects with a large bottle containing 5 volumes per cent of sulfuric acid, where the air is washed free from am-
Ammonia Determination

Ammonia by passing through an ammonia absorption tube. The other glass tube connects with the ammonia absorption tube in the receiver. The receiver, a test-tube 260 mm. by 25 mm., is graduated at 25 cc. and contains 1 cc. of 0.1 N acid, together with water enough to reach the upper openings in the absorption tube. Start the air current and regulate it to a speed of about 6 liters per minute (5.5 to 6.5 liters). The bottom of the Erlenmeyer flask is exposed by a circle having a diameter of 1 to 1.2 cm. Keep the air current running at this speed for 40 to 45 minutes.

To each of two test-tubes, graduated at 25 cc., add 0.01 mg. and 0.007 mg. respectively of ammonia nitrogen, and to each add 1 cc. of 0.1 N acid, together with water enough to give a volume of about 22 cc.

Rinse the ammonia absorption tube with about 9 cc. of water, contained in a 10 cc. volumetric pipette, on the outside and on the inside through the opening in the top and through the large openings in the outside jacket of the tube. Add 2 drops of gum ghatti solution to each of the three tubes and then add 2 cc. of Folin-Wu Nessler's reagent to each of the three tubes. Make up to volume, mix, let stand for about 15 minutes (or longer if desired), and make the color comparison in the usual manner if the 0.01 mg. standard can be used. For values less than 0.07 mg. per cent, straight test-tube comparisons are fully as reliable as those made by help of the colorimeter, but they will require additional standards.

In check work involving more than one aeration period the quantities of ammonia obtained after the first aeration will be too small for the ordinary colorimetric comparisons against the 0.01 mg. standard. For these comparisons it is best to use test-tubes of equal size graduated at 25 cc. Pour the Nesslerized unknown into one test-tube and compare with the color obtained from 0.002 to 0.006 mg. of ammonia nitrogen in other similar tubes. This comparison should, of course, be made by looking down through the full length of the test-tubes. With a little experience, one can easily determine in this way the ammonia content of the unknown to the last 0.001 mg. corresponding to 10 times as much per 100 cc. of blood. It is particularly important to let these solutions stand for at least 15 minutes before making the final comparison, and here it is also important to use gum ghatti solution to prevent the formation of any turbidity.
In connection with the determination of 0.01 mg. of ammonia nitrogen, which is the amount, in round figures, to be expected from 10 cc. of blood, it is of course essential that no ammonia be introduced with the chemicals. The most important source of error in this respect is the potassium oxalate, used partly to prevent clotting of the blood, and even more important in the preparation of the alkaline oxalate-carbonate solution. The preparation of potassium oxalate absolutely free from ammonia is a simple matter, if one has a continuously available supply of compressed air.

Transfer 100 gm. of neutral potassium oxalate and 500 cc. of distilled water to a 1500 cc. Florence flask, add 20 cc. of 10 per cent solution of potassium hydroxide, and pass an air current through it, at 4 to 6 liters per minute, for 24 to 36 hours; i.e., until 5 cc. of the solution fail to give a trace of color with Nessler's reagent. Transfer the solution to a 2 liter beaker, add with stirring 1000 to 1200 cc. of alcohol, cool for a couple of hours, and filter with suction on a Buchner funnel. Wash three or four times with alcohol and two or three times with ether. Dry in a protected place. Yield, 85 to 90 gm.

The potassium oxalate so prepared has one especial merit besides that of being completely free from ammonia. It is very light and fluffy instead of compact and heavy like ordinary powdered oxalate and therefore goes into solution more quickly. This is helpful in the prevention of coagulation of the blood, at least when the minimum safe quantity is used (20 mg. for each 10 cc. of blood). It is neutral in reaction as the added KOH is washed away with the alcohol.

For the preparation of the carbonate-oxalate solution it is not worth while to use the ammonia-free potassium oxalate, because the potassium carbonate is also likely to yield ammonia. A fairly good reagent is obtained by boiling down to 80 cc. a solution of 15 gm. of potassium oxalate and 10 gm. of potassium carbonate in 150 cc. of water and diluting it to 100 cc. Solutions so prepared give invariably a slowly developing color with Nessler’s reagent, due to some impurity seemingly present in all brands of potassium carbonate, and it is neither removed by boiling nor destroyed by gentle ignition.

A reagent which gives absolutely no color with Nessler’s reagent,
even after several hours, can be made by the following almost equally simple process.

Dissolve 10 gm. of anhydrous potassium carbonate and 15 gm. of potassium oxalate in about 100 cc. of water in a 300 cc. Erlenmeyer flask. Add to this solution 0.1 to 0.2 cc. of bromine and shake until all of the bromine globules have dissolved. If any precipitate is formed, as may happen with some samples of bromine, filter on a quantitative filter paper into another 300 cc. flask. Dilute the straw-yellow filtrate (or solution) to about 150 cc., and boil down to a volume of 80 or 90 cc. Cool and dilute to 100 cc.

By this treatment, the ammonia in both the oxalate and the carbonate, as well as the unknown disturbing impurity of the carbonate, is completely destroyed and the surplus hypobromite is destroyed during the boiling.

Test—Transfer 2 drops of 2 per cent gum ghatti solution and about 10 cc. of the carbonate-oxalate solution to a clean test-tube. Add 2 cc. of Nessler's reagent. No trace of color should develop in the course of an hour.

It will be noted that, in the revised method described in this paper, the aeration is continued for 40 to 45 minutes, although 0.05 mg. in 10 cc. of water can be quantitatively recovered in 20 minutes. While I have made similar recovery experiments with ammonia added to blood, I think that the significance of such recoveries is not quite conclusive. Added ammonia seems to be recovered more easily than a part of the ammonia already present, hence it has seemed a little better to find the correct aeration period by studying the rate at which ammonia continues to escape from the blood during more prolonged aeration periods. These experiments have shown that the escape of ammonia never comes to a sharp and definite end. The ammonia due to decomposition in fresh blood is small; it usually amounts to no more than 0.01 or 0.02 mg. per 100 cc. of blood, in 30 minutes, and it may be even less than that during the first 30 minute period. The second 30 minute period, on the other hand, will usually yield more than 0.02 mg. per cent.

It is on the basis of such observations that I have finally come to adopt 40 to 45 minutes as the most dependable single aeration period.
Table III gives a few figures showing the amounts of blood ammonia obtained in successive 30 minute periods.

A large number of blood ammonia determinations have been made during the past season by the method described in the preceding pages. The results obtained differ from those recorded in the literature in only one respect. The conspicuously low values, less than 0.05 mg. per cent, have disappeared. In the light of this experience, I must frankly express my skepticism as to the accuracy and validity of many of the blood ammonia values reported in the literature, and I hope that Parnas will not take it amiss, if I specifically mention one peculiar set of values of his as illustrating the point. I refer to the paper in which he proves that the ammonia content of the blood in the arm is demonstrably and unmistakably increased by exercising the hand (9). Here all of the fourteen ammonia nitrogen values obtained with the subjects at rest fell between 0.02 and 0.04 mg. per cent.

**TABLE III**

*Showing That Last Trace of Preformed Blood Ammonia Is Not Always Obtained in First 30 Minute Aeration Period and Illustrating Occurrence of Some Ammoniacal Decomposition*

<table>
<thead>
<tr>
<th>Kind of blood</th>
<th>NH₃-N, mg. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period 1</td>
</tr>
<tr>
<td>Dog 1</td>
<td>0.075</td>
</tr>
<tr>
<td>&quot; 2, renal vein&quot;</td>
<td>0.26</td>
</tr>
<tr>
<td>&quot; 3</td>
<td>0.09</td>
</tr>
<tr>
<td>Human</td>
<td>0.116</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.084</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.092</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.11</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.075</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.06</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.095</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.08</td>
</tr>
</tbody>
</table>

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