# A NEW METHOD FOR THE DETERMINATION OF TOTAL NITROGEN IN URINE.

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No one analytical method has done so much to further metabolism investigations as Kjeldahl's method for the determination of total nitrogen. While applicable to all kinds of nitrogenous products of interest to the biochemist it has proved particularly serviceable in urine analysis. In its modern modifications it is one of the most rapid, convenient and accurate methods we have. At first sight it might therefore seem a thankless and superfluous task to attempt to find a substitute for such an admirable tool for research. Our original idea in attempting to find another method for the determination of total nitrogen in urine was, however, to fill a gap which Kjeldahl's method does not fill; it just falls short of being suitable for clinical work except in the very best hospital laboratories.

Our original purpose was to decompose an accurately measured minute quantity of urine by means of sulphuric acid and mercury. Then, making use of the mercury for the formation of Nessler's reagent, produce the color reaction directly in the digestion mixture. We should thus have had an ideal clinical method. Because of the difficulties encountered in trying to overcome the turbidity produced on Nesslerizing the ammonia in such mixtures we have temporarily at least abandoned that scheme.

In principle our new method may be described as a microchemical method based on the Kjeldahl-Gunning process for decomposing nitrogenous materials and on the methods of Nessler and of Folin for the determination of ammonia. Rapidity in every stage of the process is secured by reducing the amount of urine taken for an analysis. In the ordinary Kjeldahl determination from 30 to 100 mgms. of nitrogen is used while we work with only about 1 mgm. To many it may at first seem questionable whether a considerable element of error is not inevitably intro-

duced by reducing the amount of urine taken for a determination to the quantitative nitrogen level that is employed in water analysis. The accuracy of analytical results depends, however, far more on the nature of the chemical reactions employed than on the quantity of material actually weighed or measured. By means of suitable so-called Ostwald pipettes[1] 1 cc. can easily be measured to within an accuracy of about 0.1 per cent and so far as this one phase of the work is concerned nothing is gained by using 5 or 10 cc.[2] The only precaution called for in the use of these pipettes is to let them drain against the sides of the test tube for ten seconds and then blow them out clean so that nothing is left behind in the tip. One cubic centimeter of urine contains ordinarily from 5 to 20 mgms. of nitrogen. For colorimetric work with Nessler's reagent even 1 cc. of urine is therefore much more than can be advantageously used, although we have improved the Nesslerization process so that several milligrams of ammonia can be satisfactorily determined colorimetrically. We occasionally used 1 cc. of undiluted urine and titrate the ammonia, as in the Kjeldahl method (see p. 500). For the colorimetric determination, however, we invariably dilute the urine until 1 cc. contains from 0.75 to 1.5 mgms. of nitrogen.

The method, as we have now used it in this laboratory for nearly two years, is as follows:

Five cubic centimeters of urine is measured into a 50 cc.measuring flask if the specific gravity of the urine is over 1.018, or into a 25 cc. flask if the specific gravity is less than 1.018. The flask is filled to the mark with water and inverted a few times to secure thorough mixing. One cubic centimeter of the diluted urine is then measured into a large test tube made of Jena glass (size 20 to 25 mm. by 200 mm.). To the urine in the test tube add 1 cc. of concentrated sulphuric acid, 1 gram of potassium sulphate, 1 drop of 5 per cent copper sulphate solution and a small, clean quartz pebble (to prevent bumping). Boil over a micro-burner[3]

[1] Oswald-Luther: *Physiko-Chemische Messungen*, 2d ed., p. 135.

[2] From Eimer and Amend can now be obtained the kind of pipettes which we use in our work. The only difference between them and Ostwald's is that they are made of thicker glass tubing and the stems are longer.

[3] The microburner, No. 2587 Eimer and Amend, is very satisfactory. The flame must of course not be so high as to unduly heat the test tube above the liquid.

for about six minutes, *i.e.*, about two minutes after the mixture has become colorless. Allow to cool about three minutes until the digestion mixture is beginning to become viscous (it must not be allowed to solidify). Then add about 6 cc. of water, at first a few drops at a time, then more rapidly so as to prevent the mixture from solidifying. To the acid solution is then added an excess of sodium hydrate (3 cc. of saturated solution) and the ammonia is aspirated by means of a rapid air current into a measuring flask (volume 100 cc.) containing about 20 cc. of water and 2 cc. of $\frac{N}{10}$ hydrochloric acid. The air current used for driving off the ammonia may well be rather moderate for the first two minutes but thereafter for eight minutes should be as rapid as the apparatus can stand.

Now disconnect, dilute the contents in the flask to about 60 cc., and dilute similarly 1 mgm. of nitrogen, in the form of ammonium sulphate (see p. 496), to about the same volume in a second measuring flask. Nesslerize both solutions as nearly as possible at the same time with 5 cc. of Nessler's reagent diluted immediately beforehand with about 25 cc. of water. (Five cubic centimeters of Nessler's reagent gives the maximum color with 1 to 2 mgms. of ammonia and when diluted as indicated turbidity is avoided.) The color produced does not reach the maximum till the end of about half an hour but the increase is small and is immaterial to the result when the reagent is added as described, *i.e.*, practically simultaneously to the standard and to the unknown ammonium salt solution. The two flasks are therefore at once filled to the mark with distilled water, mixed, and the relative intensity of the colors is determined by means of a colorimeter.

In making colorimeter readings it is important to adjust the unknown to that of the standard both from above and from below the level of the latter. If the color is adjusted only from above one is apt to consider the two fields equal when the unknown is still too dark and if from below the reverse is the case. This is true for any kind of comparison of colors or of light intensity.

In all of our work we have used the Duboscq colorimeter. A much cheaper instrument designed by Professor White of Harvard University primarily for use in the iron and steel industry we have also found fairly serviceable.

The calculation of the result is simple. The reading of the standard divided by the reading of the unknown gives the nitrogen in milligrams in the volume of urine taken.

It has taken us a long time to devise the above simple procedure for the determination of nitrogen in urine.

1. At first we were unable to secure satisfactory results because our standard ammonium sulphate solutions were not trustworthy; notwithstanding the fact that they gave practically theoretical results when their ammonia was determined by distillation and titration. Other salts of ammonia were even worse than the sulphate. Because of this fact our results were too high and we were led to suspect the presence of ammonia or other nitrogenous products in our reagents. The error was due to pyridine bases present in all ammonium salts. These bases titrate like ammonia but do not give the reaction with Nessler's reagent.

Pure ammonium sulphate can be made by decomposing a high grade ammonium salt with caustic soda and passing the ammonia gas into pure sulphuric acid by means of the air current. The salt so obtained is precipitated by the addition of alcohol, is redissolved in water and again precipitated with alcohol and finally dried in a desiccator over sulphuric acid.[4]

2. Another difficulty which we had to overcome was the frequency with which the Nessler reagent produced turbidity instead of clear solutions. In water analysis the amounts of ammonia are very small though even in water analysis failures due to this cause are not uncommon. Winkler's modification of Nessler's solution consisting in substituting mercuric iodide for the chloride in the preparation in the reagent represents an effort to prevent turbidity. The remedy which we finally discovered consists, as indicated in the above description, in diluting the reagent with about five volumes of water. When so diluted the reagent can literally be dumped into the ammonia solution even when as much as 2 mgms. is present, and the result is a deep wine color but no turbidity. If turbidity does occur it is because the Nessler solution is not sufficiently diluted with water before being added to the ammonium salt solution. To secure the maximum color, the reagent is, however, best added about one third at a time. The diluted Nessler-Winkler solution does not keep for more than a few minutes, a brick red precipitate settling out, hence the dilution should not take place until just before it it needed. When

[4] Dr. R. L. Emerson, Boston, now prepares our ammonium sulphate for us in the manner described.

once added to the ammonium salt solution, even though the amount of ammonia present be very small, the decomposition of the reagent is checked.

3. As in nearly all other quantitative colorimetric comparisons it is here necessary for accurate work that the amount of color produced in the unknown should be reasonably near that of the standard (see, however, p. 534). Using 1 mgm. of nitrogen as a standard, the unknown should contain between 0.75 and 1.5 mgms. If much more or considerably less nitrogen is present the colorimetric readings become less accurate. The standard can be set at any desired depth, but 20 mm. represents the standards we ordinarily use (with the Duboscq colorimeter).

The color is extremely easy to read quantitatively. Diffused daylight is by far the best but it is possible to get fairly reliable readings with an electric light by interposing a sheet of smooth white paper between the source of light and the colorimeter; careful adjustment of the instrument so as to secure equal illumination in both fields is, however, imperatively necessary when artificial light is used.

4. In order to remove the ammonia from the digestion mixture in the shortest possible time the volume of the solution should be kept at a minimum. There is danger of loss of ammonia, however, if this attempt to keep down the volume is carried too far, for when sodium or potassium sulphate settles out, as it will do immediately on adding the alkali if the volume of water previously added is too small, it carries down more or less ammonium sulphate. The sulphates must therefore not begin to come down until the air current has already removed the greater part of the ammonia, *i.e.*, until it has been going a couple of minutes. After this time and when the solution is getting cold, more or less sulphate invariably settles out but this does no harm. It is of course perfectly feasible to dilute the digestion mixture with more than the 6 cc. of water prescribed above and thus entirely avoid the formation of any precipitate but the conditions described are the most advantageous and when followed, every trace of ammonia present in the digestion mixture will be removed by a strong air current in eight to ten minutes.

5. The most convenient method for adding 3 cc. of saturated sodium hydroxide solution to the warm digestion mixture is to

suck it up into the glass tube which goes to the bottom of the test tube and through which the air is forced through the alkaline mixture.[5] By means of a short rubber tube and a pinch cock the tube is temporarily used as a pipette for the transference of the alkali.

6. In Folin's air current method the ammonia was made to pass through a filter consisting of a calcium chloride tube filled with cotton wool. In this case no such filter is needed and is less desirable because of the small amounts of ammonia involved. A cheap (unmarked) 5 cc. pipette is used instead as shown in the drawings.

It is, however, highly desirable, if not necessary, to prevent the concentrated alkaline sulphate solution from splashing up into the tube (made from the pipette) for if much gets there a little will creep up along the sides of the tube and get into the receiver. Since the air current is to be a rapid one this is likely to happen if nothing is done to prevent it. A simple yet very effective trap is shown in the drawings below. It consists of a circular piece of rubber cut out of a two-holed rubber stopper or rubber matting about a quarter of an inch thick and is slipped on to the glass tube which reaches to the bottom of the test tube. It should be small enough to easily get into the test tube yet large enough to prevent the splash from striking the opening of the exit tube. One or two notches are cut into the edges so that the liquid which does get above the trap can easily flow back again without obstructing the air currents.

7. In most modern laboratories compressed air is available and where that is the case the air (and ammonia) is pushed through the apparatus. This is the most convenient method for isolating the ammonia since it is to be collected in a measuring flask the neck of which is not wide enough for a two-hole rubber stopper. The necessary air current can, however, be obtained without much trouble from a good suction pump. The air should be washed free from any traces of ammonia it may contain by passing it through a bottle of dilute sulphuric acid. When suction is

[5] It is important that the glass tubes passing through the rubber stopper should not be too large for the holes in the stopper. If the latter remains perfectly round the test tube is most easily closed perfectly tight without using undue pressure.

employed the ammonia is not absorbed directly in the measuring flask for the reason stated above. It is collected in a second large tube in 2 cc. of $\frac{N}{10}$ acid and about 5 cc. of water. The ammonium salt solution is then rinsed into the measuring flask with 40 to 50 cc. of water and is then Nesslerized as described.

The drawings below illustrate how the apparatus is set up for use (a) with compressed air or a force pump, (b) with a vacuum pump. When the short rubber tube carrying the pinch cock is withdrawn the alkali gets into the digestion mixture. Connection with the air current is then made and the aspiration is begun.

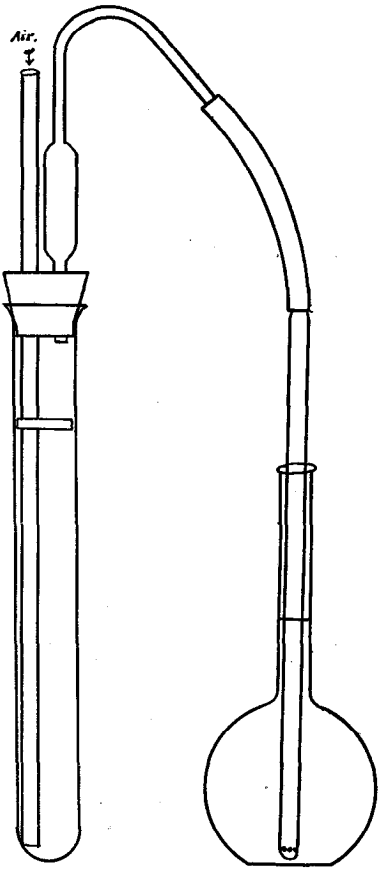


FIG. 1. APPARATUS FOR USE WITH COMPRESSED AIR.

8. The acid in the volumetric flask used as a receiver should be small in amount for with a large excess present the color develops rather more slowly. Two cubic centimeters of tenth normal acid is enough for the retention of 2 mgms. of ammonia nitrogen.

In order to secure perfect absorption of the ammonia a glass tube sealed at one end but containing three or four little holes drilled into the tube by means of a hot platinum wire is used. Such a tube can be made in a few minutes and is adequate as a substitute for the special absorption tube used by Folin for the absorption of larger amounts of ammonia.[6]

9. The microchemical method for the determination of nitrogen

[6] Many seem to have trouble about making holes by means of the hot platinum wire. By having the glass only moderately hot (not hot enough to be soft) and keeping the wire at a white heat all difficulties are avoided.

has been described above exclusively on the basis of colorimetric comparisons with standard ammonium salt solutions. The colorimetric principle is, however, not indispensable. One cubic centimeter of urine previously diluted with an equal volume of water can be decomposed as described above and the ammonia

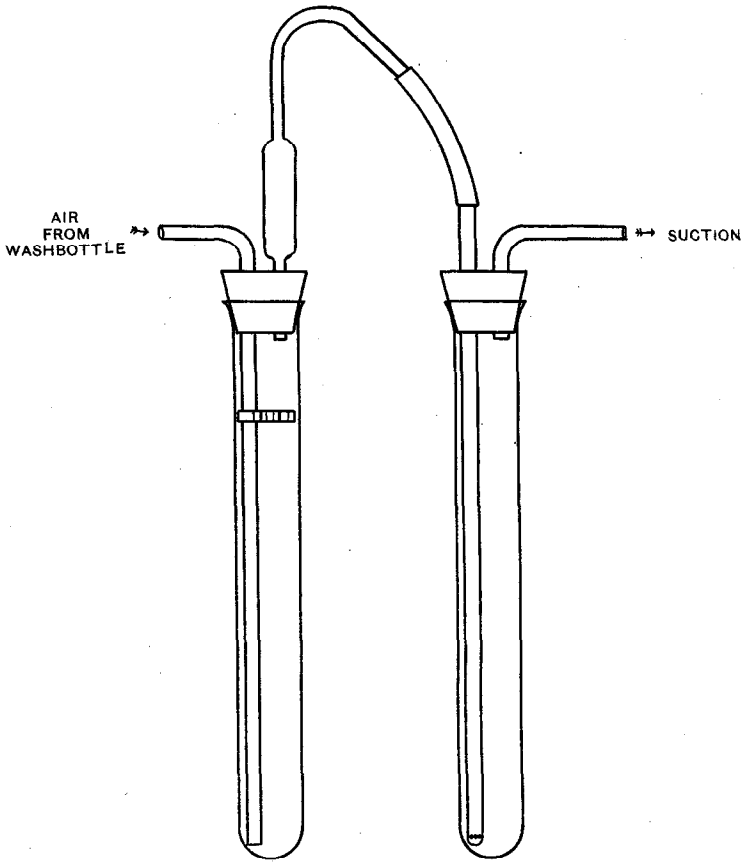


FIG. 2. APPARATUS FOR USE WITH SUCTION.

obtained is enough to titrate with a very fair degree of accuracy by the help of $\frac{N}{10}$ acid and $\frac{N}{50}$ alkali using alizarin red as indicator. The process is in every way similar to the method described on the preceding pages, except that the ammonia is collected in an ordinary small Florence flask (instead of in a measuring flask or

test tube) containing 10 cc. of $\frac{N}{10}$ acid and about 40 cc. of water. The solution is titrated in the ordinary manner, and the end point is sufficiently sharp to give very satisfactory results. Results obtained in this way are recorded below. Those who are color blind as well as those whose ability to match colors is rather poor can use the above miniature Kjeldahl process to good advantage.

Had the problem been purely a problem of total nitrogen determinations it is doubtful whether it would have been worth all the time that it has cost to develop the colorimetric procedure after it once had become clear that the color reaction seemingly could not be applied directly to the digestion mixture (see p. 493). As will be seen from the other analytical methods now published (see pp. 507-536) the total nitrogen determination was only one part of a general colorimetric scheme of analysis.

The determinations recorded below are cited to show the accuracy of our new method for the determination of nitrogen in urine. The middle column represents figures obtained by titrating the ammonia as described above. The figures represent grams of nitrogen per liter of urine.

| | NEW METHOD | | KJELDAHL'S METHOD | |
|---|---|---|---|---|
| 1 | 7.9 | 8.1 | 8.0 | |
| 2 | 10.0 | 10.2 | 9.9 | |
| 3 | 3.7 | 4.1 | 3.7 | |
| 4 | 10.5 | 10.0 | 10.2 | |
| 5 | 3.8 | 4.1 | 3.9 | |
| 6 | 9.4 | 9.3 | 9.2 | |
| 7 | 7.5 | 7.3 | 7.3 | |
| 8 | 9.2 | 9.3 | 9.2 | |
| 9 | 9.0 | 9.1 | 9.0 | |
| 10 | 9.3 | 9.1 | 9.2 | |
| 11 | 8.5 | | 8.3 | |
| 12 | 9.1 | | 9.3 | |
| 13 | 9.1 | | 9.4 | |
| 14 | 5.2 | | 5.3 | |
| 15 | 3.7 | | 3.7 | |
| 16 | 7.5 | | 7.7 | Diabetic urine. |
| 17 | 7.5 | | 7.6 | |
| 18 | 8.4 | | 8.4 | Diabetic urine. |
| 19 | 13.1 | | 13.1 | Nephritic urine. |
| 20 | 10.0 | | 10.2 | Nephritic urine. |