A COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NITRATES AND NITRITES IN BIOLOGIC FLUIDS.

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An accurate method for the determination of nitrates in blood and urine is necessary for complete studies of nitrogen metabolism in patients to whom nitrates are given as diuretics.

Various colorimetric methods for the determination of nitrates have been described. Caron (1) used diphenylamine, but the results were widely variable. Letts and Rea (2) replaced diphenylamine with diphenylbenzidine. The resulting blue color, however, fades rather rapidly and colorimetric determinations could not be made. In the technique to be described in this paper, the uses of diphenylbenzidine and of sulfuric acid were retained. Otherwise, the method is substantially new. By this method it was possible to get a uniform development of color under conditions that permitted the colorimetric comparison of standard and unknown. The method permits the determination of 0.0003 mg. of nitrate nitrogen for each cc. of material and so is suitable for biologic fluids, such as blood, urine, edema and ascitic fluids. The essential elements in control of the development of color are prevention of rapid fading of color, control of temperature, and removal of protein.

Method.

Solutions and Reagents.—The following solutions are needed: (1) Mercuric chloride, 5 per cent. (2) Sodium carbonate, 1 per cent. (3) Standard potassium nitrate. To make this standard potassium nitrate solution, 0.3608 gm. of pure potassium nitrate is dissolved in distilled water, diluted to 1 liter, and thoroughly
mixed. 1 cc. of this solution is diluted to 100 cc. 1 cc. of this
diluted solution equals 0.0005 mg. of nitrate nitrogen. (4) Concentrated sulfuric acid, specific gravity 1.84. The acid must be
free of nitrous and nitric acids. (5) Diphenylbenzidine. A good
quality of this reagent can be purchased from the Eastman Kodak
Company. 200 cc. of concentrated sulfuric acid are added to
50 mg. of diphenylbenzidine and 50 mg. of sodium chloride. A
fresh supply of the reagent should be made up every 3 days, since
such solutions slowly turn blue. After 3 days or so the reagent
gradually loses its ability to produce the blue color in the colori-
metric determination. The reagent is kept in the ice box.

Just before the reagent is used it is diluted with an equal
quantity of distilled water. The dilution is carried on in an ice
bath and care is taken not to add the reagent too rapidly. The
diluted reagent should be cooled to room temperature before it is
used.

Preparation of the Unknown Material.—The standard containing
0.0005 mg. of nitrate nitrogen for each cc. produces a depth of
color which gives a satisfactory reading when set at 20 mm. in the
colorimeter. It has been found more advisable to dilute the un-
known to match this standard than to compare a definite dilution
with a series of standards. Consequently several dilutions of the
unknown are necessary.

If the unknown material is urine, and if toluene has been used
as the preservative, it must be removed, for toluene prevents the
development of color on the addition of diphenylbenzidine. 5 cc.
of urine, made alkaline to litmus with sodium hydroxide, are
evaporated to dryness on the steam bath. When the residue is
dry it is transferred with as small a quantity of water as possible to
a 100 cc. volumetric flask and acidified with 3 N HCl. Since a
specimen of urine has occasionally been found which did not pro-
duce a blue color with diphenylbenzidine, even when considerable
nitrate was known to be present, the procedure of removing the
interfering substances with mercuric chloride has been adopted.
5 per cent mercuric chloride is added to the urine until a little,
added to sodium carbonate in a spot plate, gives a yellow color.
It is then made up to volume and filtered. If the solution is
allowed to stand for some time (about 15 minutes) a clear filtrate
is more readily obtained. From this filtrate a suitable dilution
is made. Normal urine requires a dilution of from 1:10 to 1:25. If the patient has been given nitrates, much higher dilutions must be made.

It is better not to use toluene as a preservative and thus avoid the preliminary treatment necessary for its removal. Much better precipitation occurs if mercuric chloride is added to urine directly. The procedure for toluene-free urines is as follows: To 1 cc. of urine 5 cc. of HgCl₂ are added and the solution is then diluted and filtered. From the filtrate, higher dilutions are made.

If the unknown material is blood, mercuric chloride is used as the precipitant of protein. Considerable difficulty was experienced in trying to find a protein precipitant for plasma and whole blood. All the precipitants (tungstic acid, trichloroacetic acid, metaphosphoric acid, phosphotungstic acid, lead acetate, alcohol, colloidal iron, and aluminum cream) which were tried, with the exception of mercuric chloride, gave results which were too low.

To 1 cc. of plasma, serum, or ascitic fluid, in a 10 cc. volumetric flask, water is added to about 6 cc. Then 1 cc. of 5 per cent mercuric chloride is added. The mixture is made up to volume and shaken. If whole blood is to be used, it is necessary to add 1 cc. of 1 per cent sodium carbonate before the mixture is made up to volume. Suitable dilutions may be made from the filtrate.

Procedure.—For these determinations it is convenient to use Pyrex test-tubes, 180 by 20 mm., with ground glass stoppers. The test-tubes, each of which contains 1 cc. of the diluted filtrate of urine or blood, or 1 cc. of standard solution, are placed in an ice and salt freezing mixture. Then 10 cc. of the diluted diphenylbenzidine reagent are added by allowing it to run slowly down the side of the tube. The acid and nitrate solutions are mixed gently with a stirring rod. Then 10 cc. of concentrated sulfuric acid are added under the solution of nitrate and reagent. The tip of the pipette should be kept beneath the surface until all the acid has been added. The solutions are then mixed gently. It is important that the solutions be mixed with little or no rise in temperature. When the solutions have been thoroughly mixed and cooled, the tubes are transferred to a water bath that is at a temperature of about 50° and are allowed to remain there for 5 minutes, with occasional stirring, or until the solutions are
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at about room temperature. They are then removed from
the water bath and allowed to stand for 1 hour and 30 minutes.
The results are read in the colorimeter (with the standard set
at 20 mm.).

Calculation.—The following equation is employed.

\[
\frac{20}{\text{Reading of unknown}} \times 0.0005 \times \text{dilution of unknown} \times 100 = \text{mg. nitrate N for each 100 cc. of material}
\]

Precautions.—1. Because of the prevalence of nitrates in tap
water, extra precautions should be taken in the cleaning of glass-
ware. It is advisable to clean all glassware in cleaning acid, and
to rinse thoroughly with distilled water.

2. Special care should be taken in mixing the reagent and ni-
trate solution, and in packing the tubes. It is convenient to use a
freezing mixture of salt and ice. The tubes may be inserted into
this with little danger of contamination and the outer surfaces of
the tubes are evenly exposed to the cooling medium. If nitrites
are present, even more care in mixing is necessary, for nitrites are
distinctly unstable. The presence of nitrites can be detected by
the immediate appearance of a blue color on the addition of the
reagent.

3. A colorimeter with clear glass cups, the bottoms of which
are fused on, should be used. This is advisable since such a con-
centrated solution of sulfuric acid is used.

DISCUSSION.

Fig. 1 shows the rate at which the color developed in the solu-
tion. There was a fairly uniform development which reached its
maximum in about 1 hour and 20 minutes. The readings were
taken with a spectrophotometer at intervals of a minute up to 83
minutes after the addition of the reagent. Readings taken at
100 and 130 minutes were the same as those taken at 83 minutes.
Letting the solution stand for 90 minutes insured complete de-
velopment of color, and the fact that the color did not begin to
fade for at least 40 minutes after that gave ample time for reading.

When standards of different strengths were read against each
other, the range of variation between the theoretic reading and the
actual reading in thirteen determinations was from 0 to 1.2 mm. and the average 0.26 mm.

Table I shows the results of addition of potassium nitrate to urine, serum, plasma, whole blood, and ascitic fluid. The average error of fourteen determinations on urine, of which seven are given in Table I, was about 2 per cent. The average error of thirty-eight determinations, of which twenty-one are recorded in Table I, and which were performed variously on serum, plasma, whole blood, and ascitic fluid was less than 2 per cent. In the work with serum, plasma, whole blood, and ascitic fluid, because of the preliminary addition of potassium nitrate, dilutions of 1:50 to 1:1000 were necessary.

Influence of Chloride.—Sodium chloride added to the diphenylbenzidine reagent intensified and stabilized the color developed, but the presence of an additional amount of chloride had no effect on the color. Addition of 1 cc. of 0.01 N sodium chloride, or 1 cc. of 0.1 N sodium chloride to standard solutions made no difference in color as read in the colorimeter.
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*Determination of Nitrite.*—There has been some question as to the possibility of a portion of the ingested nitrate being excreted in the urine as nitrite. It probably is not produced in any considerable amount.

**TABLE I.**


<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Nitrate N added per 100 cc.</th>
<th>Nitrate N recovered per 100 cc.</th>
<th>Difference.</th>
<th>Fluid to which nitrate was added.*</th>
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<td>52.0</td>
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* The proteins were precipitated by mercuric chloride.
The use of potassium permanganate for the purpose of oxidizing nitrite to nitrate and thus of determining the nitrite content of fluids by the difference between nitrate determined before and after oxidation has been suggested by Letts and Rea (2) and others. In my experience, the addition of potassium permanganate to urine results in the oxidation of other substances besides nitrites. Nitrogenous substances, such as ammonia, urea, and amino acids, perhaps are oxidized to nitrates by being heated with potassium permanganate in the presence of sulfuric acid. In favor of this hypothesis is the fact that normal solutions of ammonium chloride and of urea, which were negative for nitrates, gave a definitely positive reaction with diphenylbenzidine after being heated with potassium permanganate in acid solution.

Addition of the diphenylbenzidine reagent to a solution of pure silver nitrite gave the characteristic blue color. In order to determine whether the reaction was quantitative, the procedure for nitrates was followed through on a solution of silver nitrite containing 0.000455 mg. of nitrite nitrogen for each cc. Duplicate determinations gave the following results, 0.000453 and 0.00045 mg., with an accuracy of 99.3 per cent and 98.9 per cent, respectively. Titrations of this silver nitrite solution with 0.01 N potassium permanganate solution gave 49.7 and 50 mg. of silver nitrite. The theoretic value was 50 mg. of silver nitrite for each 100 cc. The method, therefore, is quantitative for nitrates and nitrites.

Mitchell, Shonle, and Grindley (3) reported some determinations of nitrates in the urine after the ingestion of potassium nitrate. The method they used was that of Schulze in which the nitrates are reduced to nitric oxide (NO) by ferrous chloride and hydrochloric acid; the nitric oxide evolved is collected over concentrated alkali and its volume measured. They claimed an accuracy of 95 to 100 per cent for pure nitrate solutions and of 93 to 94 per cent for urine. Since Schulze's method for nitrate determinations has been accepted as standard, it was thought advisable to check the diphenylbenzidine colorimetric determination against it. I tried to follow the technique of Mitchell, Shonle, and Grindley, but found that several modifications were necessary in the method of removing protein and extracting the
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Nitrates from urine before, in my hands at least, accurate results could be obtained.

A lead acetate filtrate, from which excess lead was removed with solid potassium carbonate, was used and found to yield theoretic results on potassium nitrate solutions, and on urine to which potassium nitrate had been added. Table II gives the results of comparison of the two methods.

Since good duplicate determinations on a solution at different dilutions constitute as excellent a check on a method as good recovery of added material, multiple determinations were made on 25 per cent potassium nitrate and sodium nitrate solutions and on urine, with at least two dilutions on each sample.

### TABLE II.
Comparison of the Diphenylbenzidine Method with Schulze's Method.

<table>
<thead>
<tr>
<th>Nitrate N, mg. per 100 cc. urine.</th>
<th>Ferrous chloride and hydrochloric acid method (Schulze's).</th>
<th>Difference.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg.</td>
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<tr>
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</table>

In eleven cases, determinations were made in duplicate, in one case in triplicate, and in one case in quadruplicate. In each of these cases but one, the results of the readings with different dilutions corresponded to the third decimal place. In the one case in which correspondence was not so close, the quantities of nitrate and the consequent dilutions were nearly 40 times as large as in the general run of the determinations. Even in this case the error was less than 3 per cent.

**CONCLUSIONS.**

An accurate method for the determination of nitrates and nitrites, based on the development of a blue color by diphenylbenzidine, has been described. It has been applied to blood,
urine, ascitic fluid, pleural fluid, edema fluid, and saliva with an average error of ±2 per cent.

BIBLIOGRAPHY.

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