A COLORIMETRIC METHOD FOR THE DETERMINATION OF INORGANIC SULFATE IN SERUM AND URINE*

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(Received for publication, December 23, 1935)

The study of serum sulfate has been handicapped by uncertainty concerning the accuracy and dependability of available micromethods for determination of inorganic sulfate. While a variety of procedures have been described, the results obtained differ, depending on the method employed. Thus Hoffman and Cardon (5) have tabulated values found by the application of various methods to analysis of normal blood serum. The lack of agreement is impressive and Hoffman and Cardon justly conclude that practically all procedures that have been advocated give incorrect results. These authors propose a modified oxidimetric, benzidine precipitation technique that yields for most normal specimens of serum less than 1 mg. per cent of inorganic sulfur. We have for some time engaged in an investigation of serum inorganic sulfate and sulfate clearance with the aid of a revised colorimetric benzidine method. The average normal concentration that we have found is roughly one-third higher than the corresponding average published by Hoffman and Cardon. Careful examination of our procedure fails to reveal a source of error of this magnitude. Criteria of purity for the benzidine sulfate precipitate mentioned by Hoffman and Cardon appear to be fulfilled in our method. Solutions containing known concentrations of inorganic sulfate are correctly analyzed and sulfate added to serum can be recovered with reasonable accuracy. Furthermore, comparison of the colorimetric method with a standard macrogravimetric method showed close agreement. We are

* The technique was described and demonstrated at the May, 1934, meeting of the Physiological Society of Philadelphia.
obliged to conclude, as a consequence, that the method proposed by Hoffman and Cardon yields somewhat low results. It is our belief that the average normal serum inorganic sulfur concentration is slightly over 1 mg. per cent.

The improved colorimetric procedure for the determination of inorganic sulfate described in the present paper avoids objectionable features of several of the older methods that cause such methods to give incorrect results. The modified method is an outgrowth of unsuccessful attempts to use the colorimetric method of Hubbard (6), as adapted by Wakefield (10), described by Peters and Van Slyke (8). The principal difficulty encountered was the feebleness and instability of the color given by benzidine, when treated with ferric chloride and hydrogen peroxide. A search was made, consequently, for other reagents that might give a stronger and more stable color. Of the substances tested, sodium β-naphthoquinone-4-sulfonate was the most promising. It has been employed by Folin (4) for the determination of amino acids, and is known to give intense red colors with aniline and similar compounds. With benzidine in alkaline solution, sodium β-naphthoquinone-4-sulfonate develops a red-brown color which changes to red on addition of acetone. This color is stable and well adapted to colorimetry. Furthermore, the yellow color of the excess of reagent is almost entirely discharged by the acetone.

Another innovation, so far as sulfate determination is concerned, is the use of uranium acetate as a precipitant of protein. Inorganic phosphorus is removed by this reagent simultaneously with protein, hence error due to precipitation of benzidine by phosphate and the consequent need for removing phosphate as a separate step in the method are avoided.

Procedure

Reagents—Distilled water of known purity should be employed. Uranium acetate solution, 0.80 gm. of uranium acetate in 200 cc. of water.

Benzidine solution. 1 gm. of benzidine is dissolved in 100 cc. of acetone and filtered. The solution is stored in a refrigerator in a brown bottle and discarded when it becomes highly colored.

Glacial acetic acid.

Acetone, 99.5 per cent.
Standard benzidine hydrochloride solution. 0.1606 gm. of purified benzidine hydrochloride is transferred to a 200 cc. volumetric flask, dissolved in water previously warmed to about 50°, cooled, and diluted to volume. The solution should be stored in the cold. 10 cc. are equivalent to 1.0 mg. of sulfur. For a working standard, 10 cc. of this solution are diluted to 100 cc. with water. 1 cc. contains benzidine equivalent to 0.01 mg. of sulfur. The solution should be stored in the cold.

Sodium hydroxide-sodium borate solution. 1 gm. of powdered sodium borate is dissolved in 100 cc. of 0.1 N sodium hydroxide. Preserve in a Pyrex bottle.

Sodium β-naphthoquinone-4-sulfonate solution. 0.15 gm. of a pure preparation is dissolved in 100 cc. of distilled water. The solution will keep about 2 weeks in the cold. Each sample of this reagent should be tested by treating 2 cc. and 4 cc. of the working standard solution of benzidine hydrochloride with the color reagent, borate, water, and acetone as described below. Acceptable preparations do not deviate from the theoretical Beer's law relationship by more than 5 per cent.

Determination of Sulfate in Serum—6 cc. of uranium acetate solution are measured into a 15 cc. centrifuge tube and 2 cc. of non-hemolyzed serum are added slowly. After mixing by inverting four times, the mixture is centrifuged for 10 minutes. The clear fluid is transferred to a test-tube by means of a medicine dropper.

4 cc. of the centrifugate are measured into a centrifuge tube (selected so that the tip will retain precipitates). 1 cc. of glacial acetic acid is added. This is followed by 9 cc. of benzidine solution. The tube is capped and placed in ice water for 30 minutes or longer, then centrifuged for 15 minutes at 3000 r.p.m. The

In the purification of benzidine hydrochloride 5 gm. of benzidine hydrochloride are dissolved in 200 cc. of 5 per cent hydrochloric acid by warming to about 50°. Any insoluble residue is filtered. 20 cc. of concentrated hydrochloric acid are added with continuous stirring. The solution is cooled in ice water for about 30 minutes, when the crystals that have formed are collected on a Buchner funnel. The material is washed with cold diluted hydrochloric acid (15 cc. of concentrated acid to 100 cc. of water). After removing the hydrochloric acid by a vacuum, the crystals are washed with two 25 cc. portions of cold ethyl alcohol and four portions of ether. After all traces of ether are removed, the dry crystals are transferred to a brown bottle.
supernatant fluid is decanted and discarded. The tube is permitted to drain in an inverted position for 3 minutes. 14 cc. of acetone are added. The precipitate is suspended in the acetone, then again centrifuged for 15 minutes at high speed. The acetone is decanted and the tube allowed to drain 5 minutes. After the mouth of the tube has been wiped, 1 cc. of the borate solution is added and the precipitate is dissolved by stirring. (The tube may be placed in warm water at 60° if solution is slow.) Finally 10 cc. of water and 1 cc. of the color reagent are added. The solutions are mixed and allowed to stand 5 minutes, then 2 cc. of acetone are added. At the same time, two standards are prepared by measuring 2 and 5 cc. of benzidine hydrochloride solution into two test-tubes. 1 cc. of borate solution is added to each, together with 8 cc. and 5 cc. of water respectively. 1 cc. of color reagent is added and the development of color carried out as described. The unknown solutions are compared with standards in the colorimeter. Calculation is made by the usual formula.

Determination of Sulfate in Urine—To 1 cc. of urine are added 4 cc. of 0.4 per cent uranium acetate solution. Depending on the volume of urine secreted, dilution with water is carried out as follows: for urine volumes less than 50 cc. per hour dilute to 20 volumes; between 50 and 100 cc. per hour, dilute to 10 volumes; between 100 and 200 cc. per hour, dilute to 5 volumes; between 200 and 300 cc. per hour, dilute to 2 volumes; over 300 cc. per hour, omit dilution. After mixing, the precipitate of phosphate and protein is removed by filtration. 1 cc. of the filtrate is analyzed as described for the protein-free centrifugate of plasma. Calculation is made as indicated by taking into account the dilution employed.

EXPERIMENTAL

Uranium acetate was found to be the most satisfactory of numerous agents tested for removal of proteins preliminary to sulfate precipitation. Phosphate is removed with the protein. While normal concentrations of phosphate in serum exert no appreciable influence on the results of inorganic sulfate determination by the technique described, phosphate in high concentration may be precipitated by benzidine and so cause high results.
Also in analysis of urine for inorganic sulfate, removal of phosphate is a necessary preliminary step. Uranium acetate is convenient and highly effective for this purpose. We have failed to find any effect of chloride in concentrations encountered in serum or urine. Hoffman and Cardon imply that chloride may cause high results. It is to be noted that these authors introduce appreciable amounts of chloride with their benzidine reagent.

The choice of a protein precipitant may definitely influence the results of sulfate determinations. Higher values are obtained when trichloroacetic acid is employed for removal of proteins, as compared with figures found when uranium acetate is used. Sixteen determinations of inorganic sulfate in samples of horse serum averaged 2.82 mg. per cent as sulfur when protein was precipitated by uranium acetate. The same samples yielded an average of 3.56 mg. per cent of sulfur when trichloroacetic acid (purified sulfate-free) was employed, as described by Hubbard for removal of protein. Deproteinization by heat in the presence of 1 per cent acetic acid gave even higher results, ranging 10 per cent above the trichloroacetic acid figures. On the other hand, sulfate analyses following removal of protein by the reagents employed by Hoffman and Cardon (ferric chloride-ammonium hydroxide-ammonium acetate) agree with the values found by the use of the uranium acetate precipitant.

The increase of 25 to 35 per cent in inorganic sulfur caused by the use of acid precipitating agents apparently represents liberation of sulfate from a constituent of serum. An analogous increase in inorganic phosphate when serum stands in the presence of trichloroacetic acid has been described by Kay (7), who attributes the change to decomposition of phospholipid. However the rise in inorganic sulfate differs in that it appears to occur at once on addition of acid. The readiness with which sulfate is liberated suggests that the sulfate so obtained is associated or combined with protein, since there is no similar change on acidification of uranium filtrates. The presence of phosphate in the filtrates when acid precipitants are used apparently does not explain this discrepancy. Phosphate added to uranium acetate filtrates in concentrations equal to or exceeding those of the serum employed fails to influence the results appreciably. It is evident that the...
use of trichloroacetic acid under these circumstances explains the higher values found by many authors. Liberation of sulfate from protein by bromine or by fuming nitric acid has been described by Blumenthal and Clarke (1).

Quantitative precipitation of sulfate by benzidine requires an acid reaction, although acidity must not be excessive. Acetic acid, added as directed, fulfills requirements satisfactorily. The precipitate is white and crystalline and free from discoloration. On standing in ice water, the crystals become aggregated into clumps. Unless aggregation occurs, loss of finely divided crystalline material with the supernatant fluid becomes a factor. For washing the precipitated benzidine sulfate, undiluted acetone is employed. Hoffman and Cardon found it necessary in their technique to use 90 per cent acetone for complete removal of benzidine hydrochloride. Benzidine sulfate is far from insoluble in this concentration of acetone. We have found that a single washing of the precipitated benzidine sulfate with 90 per cent acetone resulted in losses of 20 to 50 per cent, depending on concentration of sulfate present, even when contact between solvent and precipitate was minimal. Although Hoffman and Cardon by the use of special tubes attempt to avoid loss due to solubility, it is doubtful whether this measure is wholly effective, and we believe that the lower values obtained by these authors are explained in part at least by solution of benzidine sulfate during this operation. The fact that these authors obtain satisfactory recovery of added sulfate cannot be accepted as proof that such loss is avoided or that the results of determinations are correct. Quantities of benzidine sulfate removed in the wash solution would be quite constant and independent of the amount of precipitate under fixed conditions; consequently, recoveries might be quantitative. Hoffman and Cardon do not provide a comparison of their procedure with a standard method, although data are presented showing close agreement with what we conclude is a macromodification of the method they describe. Obviously, errors of the type in question would not be detected by such a comparison.

The color reaction between benzidine and β-naphthoquinone-4-sulfonate is well adapted to colorimetry. However, pure preparations of the color reagent are essential. Several samples that
have been purchased were not suitable for use. It is therefore necessary to test each preparation as described. When pure preparations are employed, the intensity of the color formed deviates only slightly from the expected color intensity for any given concentration over a wide range of concentrations. Such deviations are negligible when concentrations vary within 50 per cent of the concentration of the standard. When the difference in concentration approaches 100 per cent, results are low by about 5 per cent. Color contributed by the necessary excess of reagent is discharged by addition of acetone preceding color-

### TABLE I

Recovery of Sulfate Added to Horse Serum

Potassium sulfate containing 0.032 mg. of sulfur was mixed with horse serum containing 0.0282 mg. of inorganic sulfate sulfur and analyzed at once.

<table>
<thead>
<tr>
<th>S found (mg.)</th>
<th>Added S recovered (mg.)</th>
<th>per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0590</td>
<td>0.0308</td>
<td>97</td>
</tr>
<tr>
<td>0.0563</td>
<td>0.0286</td>
<td>90</td>
</tr>
<tr>
<td>0.0574</td>
<td>0.0292</td>
<td>91</td>
</tr>
<tr>
<td>0.0544</td>
<td>0.0262</td>
<td>82</td>
</tr>
<tr>
<td>0.0557</td>
<td>0.0272</td>
<td>86</td>
</tr>
<tr>
<td>0.0594</td>
<td>0.0312</td>
<td>96</td>
</tr>
<tr>
<td>0.0577</td>
<td>0.0295</td>
<td>92</td>
</tr>
<tr>
<td>0.0575</td>
<td>0.0293</td>
<td>92</td>
</tr>
<tr>
<td>0.0591</td>
<td>0.0309</td>
<td>96</td>
</tr>
</tbody>
</table>

Average.............................. 91.6

imetry. A compensating colorimeter may be used but is not essential if the color reagent is of good quality. A borate buffer for use in conjunction with \( \beta \)-naphthoquinone-4-sulfonate as suggested by Danielson (2) has been adopted. Blank determinations without sulfate have been negative without exception.

Inorganic solutions containing known amounts of sulfate can be analyzed with acceptable precision. Seventeen separate examinations of the concentration of inorganic sulfate in a sample of horse serum showed an average deviation from the mean of 2.2 per cent. The greatest single differences were +5.7 per cent and -2.4 per cent.
Determination of Inorganic Sulfate

Sulfate added to serum can be recovered satisfactorily provided analysis is started at once after mixing the added sulfate with the serum (Table I). When this was done, the average recovery by the method described was 91.6 per cent in nine experiments. However, if analysis was delayed 5 to 10 minutes, recoveries averaged only 77 per cent, while longer delay led to larger losses of 40 to 50 per cent. This effect was observed regardless of the protein precipitant used and similar results were obtained when trichloroacetic acid, alcohol, or heat was employed for removal of protein. These findings suggest that sulfate added to serum becomes firmly bound, and is thus rendered non-precipitable by benzidine. One may surmise that a similarly bound fraction preexists in serum. However, the liberation of sulfate by acid previously described may not be and probably is not related to the disappearance of added sulfate, since the latter occurs also when acid precipitants are employed.2

Power, Keith, and Wakefield (9) have reported that adminis-

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**Table II**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Inorganic S excreted during 24 hrs.</th>
<th>Colorimetric analysis</th>
<th>Gravimetric analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm.</td>
<td>gm.</td>
<td>gm.</td>
</tr>
<tr>
<td>1</td>
<td>0.63</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.62</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.60</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.60</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.95</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.10</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.46</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.39</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.38</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.47</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.46</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>0.59</strong></td>
<td><strong>0.59</strong></td>
<td></td>
</tr>
</tbody>
</table>

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2 Conversion of sulfate added to serum to a non-precipitable form has not been mentioned by previous writers on the subject. The experiments presented in Table I were made with horse serum preserved with phenol; however, normal human serum without preservative behaved in a like manner.
tration of acacia intravenously markedly influences the results of serum sulfate determinations by their oxidative method. The results of our colorimetric method are not affected by the presence of acacia, although acacia is precipitated by acetone simultaneously with benzidine sulfate. It is not unlikely that other oxidizable material at times may escape precipitation with protein and yet be thrown down by acetone, thus introducing error if determinations are carried out by an oxidimetric technique.

In Table II are shown the results of determinations of inorganic sulfate of urine by the colorimetric method and also by the Folin (3) gravimetric method. The figures agree, a fact that further supports the accuracy of values found by use of our method. Sulfate added to urine can be recovered quantitatively irrespective of the time of analysis. The procedure described has proved to be convenient for the determination of sulfate clearance.

Serum inorganic sulfate of twenty-three normal individuals has been determined by the method described. The ages of the subjects ranged between 20 and 40 years. The figures so obtained fall within the comparatively narrow limits of 0.95 mg. and 1.16 mg. per cent as sulfur. It is believed that the average, 1.04 (±0.049) mg. per cent, represents the normal inorganic sulfur of serum more accurately than the higher or lower average levels that have been reported in the literature.

SUMMARY

A convenient colorimetric procedure for the determination of sulfate in serum and urine, based on the color-producing reaction between benzidine sulfate and sodium β-naphthoquinone-4-sulfonate, is described. Phosphate and proteins are removed preliminarily to analysis by the use of uranium acetate solution.

The inorganic sulfur of normal human serum, determined by this method, averages 1.04 mg. per 100 cc. The lowest value was 0.95 mg., the highest, 1.16 mg. per 100 cc. Additional sulfate is liberated when serum is treated with trichloroacetic acid for removal of proteins. Sulfate added to serum is rendered partially non-precipitable by benzidine.

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Determination of Inorganic Sulfate

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