Microdetermination of Chloride in Blood Plasma and Cells, by Spectrophotometric Analysis Using Solid Silver Iodate

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As an extension of the silver iodate method of Sendroy, a convenient, reproducible, and sensitive method for analysis of chloride in 0.02-ml. samples of serum, whole blood, or red cells is described. The procedure involves (1) preparation of a protein-free filtrate of the sample, (2) AgI\textsubscript{3} reaction with the filtrate, and (3) spectrophotometric analysis of iodine as the quantitative measure of the amount of iodate exchanged for chloride. Conditions important for each of these steps have been investigated. A precision of ± 0.85 (S.D.) mM Cl per liter has been found for this method, the results of which are within 1% of those obtained by the macro nitric acid digestion method of Van Slyke.

The determination of chloride in solution, based on its reaction with solid silver iodate, was first described by Sendroy (2) with specific applications to a system of analysis of blood, plasma and urine (3–5).* In these methods, in which sample size ranged from 1.0 to 0.02 ml., iodate liberated from silver iodate by reaction with soluble chloride was analyzed by gasometric, titrimetric, and colorimetric proce-

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The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

*It is a pleasure for me to recollect and to note here that the methods cited were developed in Dr. Donald D. Van Slyke's laboratory at the Hospital of the Rockefeller Institute as a result of his suggestion that the principle of differential solubility might be utilized for chloride analysis.—J. S., Jr.
dures. Since the basic theoretical considerations were adequately presented in the foregoing papers, they will not be repeated here. It was found (3) that removal of protein from plasma (or serum) or urine was not necessary for the analysis of iodate by the gasometric or titrimetric procedure. However, colorimetric analysis required the deproteinization of such samples. Moreover, all analyses of whole blood, regardless of the method of iodate measurement, were likewise done on protein-free filtrates.

Work has been done to modify the method or to devise similar ones based on the silver iodate reaction (6–13). These modifications include the simultaneous precipitation of protein and reaction with AgIO₃, as well as changes in the titrimetric and colorimetric (or spectrophotometric) procedures for determination of the liberated iodate. The present study is an extension of Sendroy’s method to the use of spectrophotometry for the analysis of blood, plasma, serum, or packed red cells, on a micro scale. An effort has been made to utilize glassware and equipment normally present in a clinical chemistry laboratory. Some of the suggestions for improvement of the iodate procedure have been incorporated into the present easy, sensitive, and reproducible method.

Reagents

1. Standard 0.120 M chloride solution. Analytical grade KCl (8.946 gm./L.), dried at 110-120° is used. Working standards of 40 and 60 mM for the analysis of red cells, and of 75, 100, and 120 mM for the analysis of blood and serum are required. The first four solutions are made by the accurate addition of 10, 5, 3, and 1 ml. of water, respectively, to 5 ml. of the stock solution.

2. Phosphoric acid-sodium tungstate solution. This contains 0.30 M H₃PO₄ and 2.0% sodium tungstate. Twenty grams of Na₂WO₄·2H₂O (Baker’s reagent sodium tungstate “according to Dr. Folin”) are dissolved in about 800 ml. of water. Most samples of sodium tungstate require neutralization of an appreciable carbonate contamination. Syrupy phosphoric acid (reagent grade) is added dropwise until a drop of the tungstate solution is acid to an external indicator, such as phenol red solution (pH, 6-7) or an appropriate indicator paper. Then 20 ml. more of phosphoric acid is added, and dilution made to 1 L. This single stable protein precipitating solution is used for all analyses and for obtaining the standard chloride curve.
3. **Silver iodate** Merck's reagent "suitable for the determination of chloride" (14) is used.

4. **Reagent grade potassium iodide**

5. **0.020 N thiosulfate solution** Five grams of Na₂S₂O₃·5H₂O and 1.0 gm. of anhydrous Na₂CO₃ are dissolved in 1 L. of distilled water. After at least 24 hr., the thiosulfate concentration is tested and adjusted as follows. An accurately measured 5.0-ml. sample of 0.030 N KIO₃ solution (1.07 gm. of KIO₃ per L.) is mixed with 1 ml. of the phosphoric acid-sodium tungstate solution. One milliliter of 5% KI solution is added and the thiosulfate immediately run in from a 10-ml. buret until the solution is colorless. The volume of thiosulfate required should be 7.50 ml. If it differs from this by more than 0.20 ml., adjustment is made by the addition of water or thiosulfate to the stock solution until the titre is between 7.30 and 7.70 ml. The solution is sufficiently stable to be used for several months.

6. **KI-thiosulfate diluent for color development** This contains 0.5% KI and 4.0 × 10⁻⁴ N Na₂S₂O₃. It is prepared with 5 gm. of KI and 20.0 ml. of the stock 0.020 N Na₂S₂O₃ in 1 L. of solution.*

**Procedure**

The following procedure, described for the analysis of plasma or serum, is applied without change to whole blood analysis. Red cells are also analyzed by the same procedure when standard chloride and KI-Na₂S₂O₃ solutions of appropriate concentrations are used.

**Preparation of Protein-free Filtrate**

A 5-ml. centrifuge tube or a 13 × 100-mm. test tube is prepared for each standard, and in duplicate for each unknown. In each tube 0.50 ml. of water is placed. It is essential that this volume measurement be the same in all tubes, for which reason a single Lang-Levy constriction pipet (commercially available) should be used. Into each tube, by use of an accurate Sahli pipet, 0.020-ml. samples of standard chloride solutions (75, 100, and 120 mM) or plasma or serum are transferred. The volume is adjusted to the mark by repeated touching of the tip of the pipet to the surface of a sheet of bond paper.† The pipet is rinsed by

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*These dilute thiosulfate solutions gradually decrease in normality. Although they may ordinarily be used for a period of nearly a week, it is preferable that they be freshly prepared. The same thiosulfate solution must be used in the analysis of each standard and unknown.

†The common practice of using the surface of one's finger or hand for this purpose should be avoided.
repeated suction of the water into it and expulsion back into the tube. Only minimal quantities of solution are allowed to adhere to the pipet as it is removed from the tube, the contents of which are then mixed by gentle shaking. With the same precautions indicated for the addition of water, 0.50 ml. of the phosphoric acid-tungstate solution is added. The tubes are covered tightly (a small piece of Parafilm is very convenient) and shaken vigorously for about 1 min. The protein-containing tubes are centrifuged 3–5 min. at about 800g (2200 rpm in an International No. 2 centrifuge). The clear supernatant fluid is decanted into a 5-ml. centrifuge tube.

Reaction with Silver Iodate

To each tube containing chloride solution or protein-free filtrate, 6–10 mg. of AgIO₄ is added. The tubes are sealed with Parafilm, shaken vigorously 40–60 sec., and centrifuged 3–5 min. at approximately 800g. Contamination by solid silver salts in the subsequent transfer of supernatant solution from the centrifuge tubes is avoided as follows. When the desired speed is reached, centrifugation is stopped and the sealed tubes are gently tilted to wash off any solids adhering to the rims and walls of the tubes. The centrifugation is then resumed. The iodate in these solutions, whether they be immediately separated from the solid silver salts or left standing over them, is stable and has been analyzed without observable change over a period of 24 hr.

Color Development

A Folin-Ostwald pipet is used to transfer 0.5 ml. of the clear supernatant solution from the silver iodate reaction into 10.0 ml. of the KI-Na₂S₂O₃ diluent solution in a test tube.* The pipet is rinsed† with the solution by repeated suction of the diluent from, and expulsion back into, the tube. Finally, the tube is capped with Parafilm, inverted, and the contents mixed. The absorption is read at 400 mμ in 1-cm. cells in a Beckman (Model DU) or other suitable spectrophotometer. It is convenient to set the spectrophotometer at zero absorbance with the standard chloride solution of lowest concentration in the light path.

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*Should the analyst find it necessary or desirable to use the cotton plug filter originally recommended ($) for the transfer of the centrifuged solution, chloride contamination from moist fingers during the insertion of the plug should be avoided.

†Although Folin-Ostwald pipettes are calibrated to deliver by blowout, their use for washout, uniformly applied to the delivery of both standards and unknown, leaves unimpaired the accuracy of the analysis described. On the other hand, quantitative transfer of the sample to the diluent solution by blowout, is extremely difficult.
By this means, only the excess of concentration above that of this standard is measured in all of the other solutions.

Calculations

Since unknown and standard solutions all have identical treatment at every stage of the analysis, the chloride concentration of the unknown is read directly from the curve of absorption vs. concentration of the standards. It is important to use standard solutions of concentrations both lower and higher than those of the unknown solutions.

The following is an example of the calculation for the analysis of a sample of human serum. When the absorbance of the 75 mM standard was set at zero, the absorbancies obtained for the 100-mM and 120-mM KCl solutions, and the serum, were 0.345, 0.621, and 0.410, respectively. From the absorbance change of 0.276/20 = 0.0138 U./mM Cl\(^-\) for the standards, the concentration of chloride in the serum sample was calculated to be: 75 + (0.410/0.0138) = 104.7 mM.

Analysis of Whole Blood and Red Cells

Whole blood may be analyzed by the procedure described without change. The analysis of red cells requires standard KCl solutions of 40 and 60 mM and a color development solution containing 0.5% KI and \(2.0 \times 10^{-4}\) N Na\(_2\)S\(_2\)O\(_3\). No other change is needed.

Experimental Results

The Species of Iodine in Solution

In the silver iodate method carried out by titrimetric and colorimetric or spectrophotometric technics, the iodate liberated is measured as the iodine formed on reaction with iodide ion in acid solution. In the original colorimetric method (5), the blue color of the starch-iodine complex was used. This procedure was later converted to a photoelectric technic in which the yellow-brown color of iodine in potassium iodide solution was measured. Subsequently (9), a wave length of 420 m\(\mu\) was used for convenience, although maximum absorption, attributed to the formation of the addition compound, KI\(_3\), was found to be at 355 m\(\mu\) (8, 9). In fact, Jones and Kaplan (15) had shown that there was a reversible equilibrium between molecular iodine and iodide ion with the formation of the univalent triiodide ion. They found the dissociation constant of the triiodide ion, \(K = (I_2)(I^-)/(I^-_3)\), to be 1.40 \(\times\) \(10^{-8}\) at 25\(^\circ\).
Absorption Curves of Iodine and Triiodide Ion

In order to determine the optical properties of the forms of iodine (molecular I₂ and I⁻₃ ion), the absorption spectra of iodine in various solutions similar to those used by Awtrey and Connick (16) have been determined in the visible and near ultraviolet. The absorption spectra in Fig. 1 show that a solution containing only molecular iodine, I₂, and essentially no iodide ion (Curve A) has an absorption maximum at 460 mμ, but no absorption near 350 mμ. On the other hand, when essentially all of the iodine is in the form of triiodide, I⁻₃ (Curve B), there are intense absorption maxima at 287 and 353 mμ, but no maximum in the region of 460 mμ. Pure resublimed iodine dissolved in redistilled water (Curve C) produces absorption maxima at 460, 353, and 287 mμ, indicating, as a result of hydrolysis, the presence of both I₂ and I⁻₃. The molar absorption index, aₘμ, of the triiodide ion at 350 mμ was observed to be 25,500, whereas the value for I₂ at 460 mμ was found to be 748.* Indeed, the molar absorption index of I⁻₃ is always greater than that of I₂ even at the absorption maximum of the latter (16).

Thus, it is apparent that over the span of wave length 350–460 mμ, the species of iodine which contributes most to the absorption is the triiodide ion. It is, therefore, important to determine the concentration of iodide required to give maximal absorption with a given amount of iodine. Figure 2 shows that maximal absorption at 350 mμ is reached with 5 × 10⁻² M iodide, about 2000 times the molar concentration of iodine. The lower concentration of 3.0 × 10⁻² M (0.5% KI)

*These values are in excellent agreement with those reported by Awtrey and Connick (16) and those recalculated from the data of Custer and Natelson (17).
has been chosen for the present method. Under the conditions of analysis, this concentration of iodide keeps about 95% of the iodine in the strongly absorbing, nonvolatile triiodide form. The spontaneous formation of iodine by air oxidation of acidified iodide solution, is also minimized (Curve B, Fig. 2).

![Graph](image)

**Fig. 2.** Effect of iodide concentration (Curve A) on the absorption at 350 μm, of 2.38 × 10⁻⁴ M iodine solutions in 0.2 M H₃PO₄. Curve B is for blank solutions from which the iodine was omitted. Length of light path, 10 mm.

**Sensitivity of Absorption Measurements**

The high molar absorption index of I⁻ at 350 μm would make it necessary to dilute a 100-mM chloride solution such as serum approximately 15,000 times in order to measure all of the chloride and to obtain absorbance below 0.7 at this wave length. Furthermore, the sensitivity, i.e., Δ A/Δ Cl⁻, is only approximately 0.004 absorbance unit for a change in chloride concentration of 1 mM. At lower dilution, approximately the same sensitivity may be obtained from the absorption at 400 μm. The sensitivity is increased 10-fold or more when a constant amount of the iodine is made to react with thiosulfate in the manner suggested by Stiff (11). In the present procedure, with dilution of about 1000 and measurement at 400 μm, the sensitivity is approximately 0.016 absorbance unit per millimole change in chloride. Typical standard curves, measured with two instruments, are shown in Fig. 3.*

*A linear relationship was observed between absorption and chloride concentration when measurements were made with the Beckman spectrophotometer within the concentration range shown. When the Coleman Jr. instrument was used, the standard curve was not linear, probably owing to the greater spread of wave length of incident light of this instrument.
Fig. 3. Change of absorption with chloride concentration of standard solutions. For the Beckman spectrophotometer, 10-mm. cell: Curve A, reagent blank (KI-Na$_2$S$_2$O$_3$) set at zero, 400 mM; Curve B, reagent blank (KI-Na$_2$S$_2$O$_3$) set at zero, 385 mM; and Curve D, 80 mM Cl standard set at zero, 400 mM. For the Coleman Jr. spectrophotometer, 12 × 75 mm. cuvet: Curve C, reagent blank (KI-Na$_2$S$_2$O$_3$) set at zero, 400 mM.

Results of Analyses of Serum and Whole Blood by Present Method

Several samples of pooled human serum were analyzed as prescribed in the foregoing and by the Van Slyke nitric acid digestion method (18). Table 1 shows that mean values for the present method average about 1% higher than results obtained by digestion. This mean increase is more than 3 times the standard error of the mean as calculated for small samples (19) and is, therefore, considered statistically significant (P = 0.01). This difference confirms earlier work in which

Table 1. Comparison of Chloride Analyses of Human Serum by Two Methods

<table>
<thead>
<tr>
<th>Sample</th>
<th>Digestion (mM/L)</th>
<th>Spectrophotometric (mM/L)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>102.6</td>
<td>103.9</td>
<td>1.013</td>
</tr>
<tr>
<td>2</td>
<td>104.9</td>
<td>105.3</td>
<td>1.004</td>
</tr>
<tr>
<td>3</td>
<td>105.0</td>
<td>105.1</td>
<td>1.001</td>
</tr>
<tr>
<td>4</td>
<td>104.2</td>
<td>104.8</td>
<td>1.006</td>
</tr>
<tr>
<td>5</td>
<td>102.9</td>
<td>104.6</td>
<td>1.017</td>
</tr>
<tr>
<td>6</td>
<td>105.2</td>
<td>107.7</td>
<td>1.024</td>
</tr>
<tr>
<td>7</td>
<td>104.8</td>
<td>106.0</td>
<td>1.011</td>
</tr>
<tr>
<td>8</td>
<td>104.3</td>
<td>104.0</td>
<td>0.997</td>
</tr>
<tr>
<td>9</td>
<td>104.3</td>
<td>105.4</td>
<td>1.011</td>
</tr>
</tbody>
</table>

Av. 1.009

* Spectrophotometric divided by digestion values.
the iodate of tungstic acid filtrates was measured by gasometric and titrimetric means (3, 4, 10). As suggested previously (3), it seems unlikely that the observed difference lies in the extent of serum dilution; essentially the same value has been found by all three methods over a range of dilution from 10- to 50-fold. Table 2 shows similar comparative values for analyses of human whole blood. A difference of 1% is likewise apparent but this is not statistically significant. Furthermore, none was previously observed for tungstic acid filtrates (3).

The precision of the present procedure was tested with 14 separate analyses of a sample of human serum (Sample 9, Table 1). A mean chloride value of 105.4 with a standard deviation of ±0.85 mM was found. Replicate analyses of whole blood and of red cells have shown that the precision of the method is nearly the same for these materials, also. The major factor determining the variation appears to be the accuracy with which the small 0.02-ml. samples can be measured.

**Table 2. Comparison of Chloride Analyses of Human Blood by Two Methods**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Digestion (mM/L.)</th>
<th>Spectrophotometric (mM/L.)</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>79.1</td>
<td>82.3</td>
<td>1.040</td>
</tr>
<tr>
<td>2</td>
<td>83.2</td>
<td>84.3</td>
<td>1.013</td>
</tr>
<tr>
<td>3</td>
<td>71.4</td>
<td>70.7</td>
<td>0.990</td>
</tr>
<tr>
<td>4</td>
<td>81.4</td>
<td>82.5</td>
<td>1.014</td>
</tr>
<tr>
<td>5</td>
<td>83.0</td>
<td>84.8</td>
<td>1.022</td>
</tr>
<tr>
<td>6</td>
<td>87.9</td>
<td>87.4</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ave. 1.012</td>
</tr>
</tbody>
</table>

* Spectrophotometric divided by digestion values.

**Effect of Proteins on the Silver Iodate-Chloride Reaction**

The silver iodate method was primarily developed by Sendroy (3) as a means of direct determination of chloride in plasma, serum, and urine, without recourse to deproteinization, although technics were also outlined for the analysis of protein-free filtrates of plasma and whole blood.

Van Slyke and Hiller's modification (10), which was subsequently used by other workers (11-13), was to carry out in plasma samples, the simultaneous precipitation of protein and the silver iodate reaction. It seemed that such a procedure might be advantageous in its extension to the analysis of blood and red cells, particularly by the spectro-
photometric technic presently developed. To test this possibility a fresh sample of heparinized dog blood was used. The whole blood, and the plasma and red cells obtained therefrom, were each analyzed in triplicate (1-ml. samples) by the Van Slyke (18) digestion method as modified by Eisenman (20). Each sample was also analyzed by triplicate spectrophotometric measurement of the phosphoric-tungstic acid supernatant fluid obtained in two ways: in Method A, the precipitated protein was separated from the fluid prior to the addition of AgIO₃; in Method B, the AgIO₃ was added in the presence of the precipitated proteins.

The results, in Table 3, show that chloride values for blood and its two components, obtained by treating the protein-free filtrate with AgIO₃ (Method A), correspond closely to those found by the digestion method. By contrast, values obtained when the protein precipitate is present during the reaction with AgIO₃ (Method B) are increased roughly in proportion to the protein (cell) concentration. In comparison with the digestion method, the chloride values obtained for plasma, whole blood, and cells of this particular dog blood sample, treated with AgIO₃ in the presence of the precipitated protein, were respectively 4, 18, and 47% too high. Similar comparative analyses were carried out to ascertain the effect of the precipitated protein in samples from other species. Measurements were made by titration or spectrophotometric determination.

### Table 3. Comparative Chloride Analyses of Dog Blood by Two Methods

<table>
<thead>
<tr>
<th>Sample</th>
<th>Digestion (mM/L.)</th>
<th>Method A (mM/L.)</th>
<th>Method B (mM/L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood No. 1 (Het. = 0.435)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood</td>
<td>87.8</td>
<td>89.0</td>
<td>103.3</td>
</tr>
<tr>
<td>Plasma</td>
<td>114.7</td>
<td>115.8</td>
<td>119.2</td>
</tr>
<tr>
<td>Red cells</td>
<td>52.5</td>
<td>52.2</td>
<td>77.3</td>
</tr>
<tr>
<td>Red cells (calculated)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood No. 2 (Het. = 0.373)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood</td>
<td>82.1</td>
<td>82.1</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>104.8</td>
<td>106.0</td>
<td></td>
</tr>
<tr>
<td>Red cells</td>
<td>44.0</td>
<td>44.7</td>
<td></td>
</tr>
<tr>
<td>Red cells (calculated)†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>44.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Method A: according to the procedure described for the present method. AgIO₃ was added after the precipitated proteins were removed. Method B: AgIO₃ was added in the presence of the precipitating proteins.

†Calculated from values for hematocrit, and digestion analyses of whole blood and plasma.
Table 4. COMPARISON OF CHLORIDE ANALYSES SHOWING RESULTS OF AgIO₃ REACTION IN THE PRESENCE OF PRECIPITATED PROTEIN

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method of iodate analysis</th>
<th>Digestion (mM/L.)</th>
<th>AgIO₃ reaction* Method A (mM/L.)</th>
<th>Method B (mM/L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human blood</td>
<td>Titrimetric</td>
<td>83.0</td>
<td>84.7</td>
<td>89.1</td>
</tr>
<tr>
<td>Human plasma</td>
<td>Titrimetric</td>
<td>100.0</td>
<td>102.7</td>
<td></td>
</tr>
<tr>
<td>Ox serum</td>
<td>Titrimetric</td>
<td>101.8</td>
<td>104.8</td>
<td></td>
</tr>
<tr>
<td>Ox blood</td>
<td>Titrimetric</td>
<td>84.0</td>
<td>98.3</td>
<td></td>
</tr>
<tr>
<td>Ox red cells</td>
<td>Titrimetric</td>
<td>58.3</td>
<td>76.7</td>
<td></td>
</tr>
<tr>
<td>Ox serum</td>
<td>Spectrophotometric</td>
<td>100.8</td>
<td>101.0</td>
<td>102.3</td>
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<tr>
<td>Ox serum</td>
<td>Spectrophotometric</td>
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<td>102.9</td>
<td>106.4</td>
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<td>99.1</td>
<td>101.3</td>
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<tr>
<td>Ox blood</td>
<td>Spectrophotometric</td>
<td>84.3</td>
<td></td>
<td>95.9</td>
</tr>
<tr>
<td>Ox blood</td>
<td>Spectrophotometric</td>
<td>82.4</td>
<td></td>
<td>90.3</td>
</tr>
</tbody>
</table>

*Method A: According to the procedure described for the present method. AgIO₃ was added after the precipitated proteins were removed. Method B: AgIO₃ was added in the presence of the precipitating proteins.

photometry. Table 4 likewise shows that in all cases values obtained in the presence of the precipitated protein were higher than those from the corresponding protein-free filtrates, which in turn, were in substantial agreement with digestion values. The results for plasma and serum in Tables 3 and 4 indicate the effect of the presence of the precipitated proteins in the mean increase in values of 2.83 mM/L by Method B as compared with Method A. This is more than 12 times the standard error of the mean for this group of analyses, and must be regarded as being most highly significant (19). The differences for whole blood and cells are obviously valid. The effect noted apparently varies with the species, being least for human blood, and increasing with the red cell concentration. On the basis of the evidence given, the present method requires that the protein precipitate be removed before the addition of AgIO₃. This step is mandatory for the analysis of whole blood or red cells.

Discussion

Previous applications of the silver iodate method, with the exception of that to urine analysis (3, 21), have all been carried out in solutions of at least 3 mM chloride, to eliminate a correction for AgIO₃ solubility (3). Such a restriction is not important for the present procedure because the amount of AgIO₃ in solution is controlled by the analysis of
standard chloride solutions of approximately the same concentration as the unknown. When all standard and unknown solutions are treated alike throughout the procedure, factors such as the extent of dilution, temperature, time of mixing or standing, chloride contamination in reagents, and the solubility of AgIO₃, do not affect the analysis.

In the authors' experience, the suggested simplification of the iodate method (10) by simultaneous precipitation of plasma protein and reaction with AgIO₃ always yields higher values than those obtained by nitric acid digestion of the sample. Van Slyke and Hiller's data (10) indicate such results to be 1.7% too high for dog plasma whereas our results for the same (and the plasma of other species) are about 4% high. The extension of their procedure to the analysis of samples containing a high concentration of protein, such as blood, red cells, or other tissues, is evidently not feasible (Tables 3 and 4). Although the exact reason for this effect of precipitated protein has not been determined, it is at least partially a result of the binding of silver ions by the proteins, and, to a much lesser extent, of a reduction of iodate to iodide. Recent experimental evidence to this effect has been obtained as follows. The proteins were precipitated from samples of human blood according to the present method, then washed free of chlorides with successive portions of the precipitating solution. Upon the addition of AgIO₃, iodate was liberated in amount approximating the excess found for chloride in human blood when the steps of deproteinization and AgIO₃ treatment were combined (Table 4). On the other hand, as shown above, chloride values found for phosphoric-tungstic acid filtrates of plasma, whole blood, or red cells, from which the protein was separated and removed prior to treatment with AgIO₃, are in substantial agreement with the values obtained by digestion.

Annino (22) has found that the native proteins increase the chloride values for serum when measured by the mercurimetric method of Schales and Schales (23). Such results indicate that mercuric ion reacts not only with chloride, but to some degree with serum proteins. Where there is evidence of significant error in chloride methods involving the use of mercuric or silver ions in the presence of protein, the latter should be not only precipitated, but removed prior to the continuation of the analysis.

King and Bain (13) were unable to measure chloride in standard NaCl solutions with silver iodate in the presence of sodium tungstate. No such difficulty has been encountered in the present work. The acid concentration used is higher than necessary to remove protein, but was
so chosen in order to provide sufficient acid for the subsequent color development.

The iodate may be measured by the gasometric procedure for which no standard or solution of known composition is required. When iodate is measured by reaction with excess KI in acid solution, the iodine released may be titrated with thiosulfate or measured spectrophotometrically. A combination of the latter two analytical steps has been used, as advocated by Stiff (11), who pointed out the advantages of the increased sensitivity obtained when most of the iodine was first reduced with thiosulfate, with resultant lower dilution of the sample. In his method, a dilute thiosulfate solution of exactly known concentration was used for partial reaction with the iodine. Such solutions are rather unstable and require constant preparation or restandardization. In the present method, a constant volume of a similar solution, whose thiosulfate concentration needs to be set only within certain limits, is used for all standard and unknown solutions. In effect, the dilution with a known amount of thiosulfate has so been chosen that the physical dilution is about 1000, but the "chemical dilution" caused by reaction of iodine with thiosulfate accounts for about 70% of the total chloride. Furthermore, potassium iodide has been added to the thiosulfate solution in order to simplify the color development. The amount of excess iodine observed by this procedure is the same as that found when all of the iodine is liberated before the addition of thiosulfate.*

The method as described is an attempt to provide adequate sensitivity over the range of concentration of chloride encountered in the analysis of blood, plasma, or cells. For special purposes, it is possible to alter the sensitivity of the measurements greatly by changing the total dilution of the sample, by altering the amount of iodine reacting with thiosulfate, and by reading the absorption at other wave lengths. The conditions prescribed are a compromise, since increasing the sensitivity further makes the technic considerably more demanding.†

Measurement of the absorption at 400 mμ has been chosen for routine analysis. The sensitivity of the method is doubled by reading absorbance at 385 mμ, and is about 5 times as great if measurements are made at 350 mμ. However, readings at this wave length will seldom be

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*Thiosulfate does react with iodate in the absence of iodide ion, but this reaction is very slow in comparison with the rate of reaction of iodide with iodate in acid solution.

†Although the molar absorbancy index of I2 is higher at 287 mμ, this wave length can not be conveniently used because quartz optics are required and because iodide and tungstate ions, present in excess in these solutions, seriously interfere with absorbancy at this wave length.
practical since a much more critical adjustment of the thiosulfate concentration is then required.

We have found the molar absorption index of $I^-_2$ at 350 $\mu$m to be approximately 2/3 that of the blue starch-iodine complex at 600 $\mu$m. The starch-iodine complex has not been used in this work, however, because increased sensitivity was not needed and because reproducible color development of the starch-iodine complex is much more difficult (7). Although the method is described for 0.02-ml. samples, it is obvious that larger samples may be used if desired. It is also possible to change the dilution, both in the original protein-free filtrate preparation and in the color development. When such modifications are made, care must be taken to use AgI$\textsubscript{2}$ sufficiently in excess, and to use a color development solution which leaves a small amount of excess iodine in the most dilute standard and unknown solution.

References

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5. Sendroy, J., Jr., J. Biol. Chem. 120, 419 (1937).