V. THE MICRO-ESTIMATION OF CHLORINE IN WHOLE BLOOD SERUM OR CORPUSCLES.

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PREVIOUS METHODS.

CLAUDIUS [1924] described a method of micro-estimation of blood chloride in which he treated 0.2 cc. of blood with 20 cc. of $N/25$ silver nitrate and a few drops of nitric acid. After boiling this down to 0.25 cc. he completed the oxidation with potassium permanganate. Alcohol was added and the solution titrated with alcoholic ammonium thiocyanate. The use of alcohol was a considerable advance, but is open to the objection that it forms an explosive mixture with high concentrations of nitric acid. When titration is carried out in the presence of silver chloride, a large excess of nitric acid is necessary owing to the reversibility of the reaction

In addition, boiling down to small bulk is tedious and frequently causes loss by bumping.

Rehberg [1925] recently overcame these difficulties by maintaining a large excess of nitric acid, but keeping the reacting materials in small bulk. He utilised the special method of titration described by himself. The blood was digested with 100 volume hydrogen peroxide as introduced by Fowweather [1926] for blood-iron estimations. A single estimation of whole blood is completed in 2 hours, for the most part occupied by digestion in a water-bath.

The following method, using ammonium persulphate and nitric acid on 0.2 cc. of blood, gives a smaller margin of error for whole blood than Rehberg's method; it can be completed in 15 minutes for a single sample, or 12 minutes per sample over a series of estimations. It has the advantage that acetone intensifies the end-point more than alcohol and can be used in the presence of high concentrations of nitric acid.

PRINCIPLE OF THE METHOD.

The principle of this method is the destruction of the organic matter of the blood by ammonium persulphate and concentrated nitric acid, with the usual precipitation of the chlorine as silver chloride by excess of silver nitrate. The excess is titrated against alcoholic ammonium thiocyanate solution with concentrated ferric iron alum as indicator.

The titration is performed in the presence of acetone which intensifies the end-point by decreasing the dissociation of the ferric thiocyanate, and for a similar reason renders it more permanent. This enables a series of titrations to be brought to the same depth of red, which with the sharpened gradation of the end-point coloration permits accurate comparative observations to be made.

PROCEDURE.

Deliver 0.2 cc. of blood from a standardised capillary pipette to the bottom of a Jena or Pyrex test-tube. Wash the pipette carefully with 3 or 4 drops of distilled water contained in a small centrifuge tube and transfer the washings by means of the pipette to the Jena tube; *repeat* the washings twice. Add 0.5 cc. of aqueous silver nitrate (7.265 g. per litre; 1 cc. = 0.0025 g. NaCl), using a standardised Ostwald pipette. After mixing these, add very approximately 0.33 g. of pure powdered ammonium persulphate and wash down the adhering particles of persulphate and added liquids with 3.0 cc. of concentrated nitric acid (chlorine-free) from a pipette or a burette with ungreased tap.

Heat this mixture gently with moderate agitation and avoidance of frothing till the protein has dissolved. Clamp the test-tube in a retort stand at about 60° to the horizontal and bubble air through its contents by a fine capillary tube not necessarily of Jena glass (see Fig. 1).

Fig. 1. A. Capillary tube.
B. Short rubber tube carrying the capillary tube A in a slit.
C. Glass tube to air supply.

Bumping is entirely prevented by the stream of air bubbles, and the liquid is boiled freely for 15 or 20 seconds, using a small Bunsen burner or a larger size of microburner. The opalescence of the silver chloride precipitate is replaced by a clear greenish yellow solution containing small compact clumps or granules of silver chloride. At this stage the flame is removed and the capillary tube detached from the valvular slit in the rubber tube delivering air. The test-tube, carrying with it the capillary tube, is removed from the stand and allowed to cool.

All the samples of a series are brought to this stage and cooled thoroughly; after which air is again bubbled through by means of the capillary tube which is very readily attached and detached. 3.5 cc. of acetone are added to each

in turn from a small measuring cylinder, the air bubbles ensuring complete mixing. When more than 6 samples are in progress they should be treated with acetone and carried to the final stage in two groups; but if no more than 6, all may go through together.

When the acetone has been added to the last sample the heat of mixing will have passed off sufficiently in the first of the series, and, again connecting the air supply to its capillary tube, 0.3 cc. of concentrated ferric iron alum solution is added. A whitish precipitate usually forms due to the insolubility of the alum in acetone, and distilled water should be added drop by drop till it dissolves (usually 6 drops).

The Jena tubes are best ranged in an ordinary single row test-tube stand, with a white porcelain plate behind them and white paper beneath, using a strong front illumination from a shaded electric lamp about 6 inches away.

The first sample is then titrated against an alcoholic solution of ammonium thiocyanate from a 5 cc. microburette delivered directly into the solution by a fine capillary extension of the microburette attached by pressure tubing. The bubbles of air produce effective stirring, and the end-point is very sharp and sensitive in this illumination, especially in a darkened room. The observer's eyes should be completely protected from glare by a shade on the lamp. Titration is carried in the first sample to the earliest definite pink, and subsequent samples should be titrated by comparison to the depth of colour of the first sample.

Having completed the whole series, the samples are then transferred to tubes of colourless glass and of exactly equal bore. The solutions are carefully poured out, leaving the last few drops containing the silver chloride in the Jena tubes, and titration is then carried accurately to the colour of the first sample. Deeper solutions, if any, are corrected by titrating a solution of the same depth as the first sample to the colour of the darker solution, and subsequently subtracting the excess added from the titre of that solution. Corrections usually amount to about 0.02 cc. of alcoholic thiocyanate.

Although there are several operations, it is found in practice that they are much easier to perform than to describe and there is very little to go wrong.

Certain supplementary points in technique, which must be carefully followed.

(1) Pipettes used must be carefully re-standardised; the 0.2 cc. with mercury, as their contents are to be washed out, and the 0.5 cc. Ostwald pipettes with water as they are to be blown out after 15 seconds' drainage. The Ostwald pipettes should deliver their contents slowly, about 30 seconds as a minimum time, or wetting of the glass causes appreciable error. Iron alum is added grossly in excess of that required to combine with the thiocyanate.

(2) Dissociation is also less in acetone; therefore titration is performed, in the presence of acetone, with the minimum of water for solution of the iron alum. The standard ammonium thiocyanate cannot therefore be used in acetone solution because the alum would be precipitated by the acetone added

in the course of the titration. Dissociation is much less in alcohol than in water, though greater in alcohol than in acetone. Therefore alcoholic ammonium thiocyanate is the best practicable substitute for a solution in acetone.

(3) Ferric thiocyanate dissociates into ferric and thiocyanate ions which are colourless. The minimum dissociation takes place when there is a marked excess of either thiocyanate or iron. Therefore, to obtain the maximum coloration with the minimum of thiocyanate excess, a large quantity of iron must be added.

(4) Very occasionally a sample turns pink 0.1 or 0.2 cc. before the true end-point—especially if the thiocyanate standard has been run in too quickly. These samples always fade completely before the final accurate colour comparison is made and no gross error results when the final correction is added.

(5) Brownish colours in the mixture before titration are due to incomplete cooling before or after addition of acetone. If samples are allowed to stand this colour disappears in half-an-hour.

Results of 9 consecutive observations on whole blood.

Total chloride per 100 cc. g.	Known chloride added per 100 cc. g.	Chloride of original blood sample g.
0.429	0.000	0.429*
0.446	0.025	0.421
0.424	0.000	0.424
0.468	0.044	0.424
0.519	0.094	0.425
0.423	0.000	0.423
0.452	0.031	0.421
0.423	0.000	0.423
0.517	0.094	0.423

Results of 9 consecutive observations on serum.

Total chloride per 100 cc. g.	Known chloride added per 100 cc. g.	Chloride of original serum sample g.
0.553	0.000	0.553
0.581	0.025	0.556
0.553	0.000	0.553
0.602	0.044	0.558
0.557	0.000	0.557
0.653	0.094	0.559
0.558	0.000	0.558
0.574	0.025	0.549*
0.596	0.038	0.558

* No known cause for this deviation. Rehberg claims errors of only 2 or 3 mg. in his estimations of serum chloride, using 0.1 cc. of serum. This amounts to an error of $\frac{1}{4}$ or $\frac{1}{2}$ %. Such results have frequently been obtained on a short series of estimations. Errors in the second figure are very rare. The published series contain the error due to pipette calibration since a different pipette was used for each sample. A closer approximation of results can be obtained by using the same pipette throughout.

SUMMARY.

- (1) A simple and rapid method of estimating blood chlorides on 0.2 cc. has been described.
- (2) Using whole blood the outside error is usually 4 milligrams of NaCl per 100 cc. of blood, with a similar percentage error for serum.
- (3) Larger errors occur for unknown reasons in about one-tenth of the samples, but remain in the third place of decimals.

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REFERENCES.

- Claudius (1924). *Acta Medica Scand.* 61, 3.
Fowweather (1926). *Biochem. J.* 20, 93.
Rehberg (1925). *Biochem. J.* 19, 270.

[Note added February 4th, 1927]. Although the method of titration already described, followed by correction in comparator tubes, is the better for occasional use and for those workers whose colour sense is not acute, a modification has been introduced since going to press, which yields better results in the hands of practised workers whose colour perception is good. The acetone is added to the cooled digested samples, taking not more than two or three of a series at once. The mixture is then cooled for about 30 seconds and titrated directly with ammonium thiocyanate. The thiocyanate is run in at a moderate rate and a *brisk* stream of air bubbles is passed to ensure *rapid* mixing. Titration is complete at the first distinct uniform red flush which appears throughout the solution.

Results in duplicate by this method compare very closely. The error is usually about 1 to 3 milligrams, occasionally 6 or 8 milligrams, in the serum and whole blood and about 6 or 8 milligrams in the corpuscles. Larger errors than 8 milligrams are rare in the corpuscles provided that the corpuscular mass has been thoroughly mixed.

The time of estimation is reduced by this modification to an average of 9 or 10 minutes per sample over a series, and 13 minutes for a single estimation.