XXIX. THE ACCURATE MICRO-DETERMINATION OF CHLORINE AND IRON IN BLOOD AND OTHER LIQUIDS.

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INTRODUCTION.

Most micro-chemical analyses are carried out by colorimetric methods or by titration. In titration, the accuracy rests largely on the determination of an end-point. It is obvious that when the volume of fluid in which a reaction is carried out becomes doubled, at least twice the excess of standard reagent will have to be added to produce the same excess of end-point products; and if a coloured end-product has any tendency to dissociate into colourless ions, as is the case with ferric thiocyanate, then the excess required to produce an end-point of equal intensity will be more than doubled.

The success of a micro-titration method would appear, therefore, to depend on:

- (1) the maintenance of reacting materials in small bulk [Rehberg, 1926];
- (2) the minimisation of ionisation and reversible reactions in the end-products;
- (3) the selection of a distinct colour change for the end-point.

If these factors can be controlled in small bulk, there is no reason why the error from them should be larger than for a corresponding macro-method, so that the determination will depend for accuracy on the measurement of small quantities of material; and the elimination of such errors as those due to the exposure of reagents to a greater relative surface of glassware when one is dealing with minute amounts of liquid.

When equal amounts of ferric thiocyanate are diluted to a given volume with water, alcohol and acetone, it is found that the depths of colour are in ascending order. Now ferric thiocyanate is the end-point product in the chlorine determination previously described [Smirk, 1927, 1] and the substance used for colorimetric comparison in the iron determination [Smirk, 1927, 2]. If, therefore, titration is carried out in the presence of acetone, the end-point will be sharper where ferric thiocyanate is the determining factor.

If equal quantities of ammonium thiocyanate are treated with varying amounts of ferric iron alum and diluted to a definite volume, the depth of colour increases with the amount of iron alum added, owing to the reaction

being displaced to the right in the presence of excess of iron alum.

As these three conditions of bulk, non-reversibility and end-point are readily maintained, the reduction of the quantity of blood, etc. used, from 0.2 cc. to 0.015 cc. or less, has been readily accomplished with only a slight loss of accuracy in the method of chloride estimation previously described. The sole difficulty was the accurate measurement of the material, which was overcome by the use of a capillary pipette which may be made and graduated with ease and without special knowledge or equipment.

In the calibration of an ordinary micro-pipette graduated to contain 0.2 cc. the error is small but greater than in ordinary macro-pipettes, because in the micro-pipettes the stem is relatively wider, so that a small linear error in reading or graduating the pipette represents a greater percentage error in the volume of the contents. The width of the stem is necessary, however, in order that the 0.2 cc. graduation mark may lie within its length. If the stem is made very narrow compared with the bulb, only a small percentage of the pipettes would have a bulb whose volume content was so accurately gauged that the calibration mark would fall on the stem.

Fig. 1. Micro-pipette. To measure accurately 1/75 cc. or less.

It will, therefore, be seen that although it may be almost a technical impossibility to make micro-pipettes to contain accurately a fixed and stated volume of liquid, such as 0.05 or 0.01 cc., yet it will be possible, by having a graduation mark over a sufficiently narrow constriction or stem, to get a pipette which measures say 0.0135 cc. very accurately indeed. The obvious difficulty in the use of such pipettes is, however, that the proportion of the essential elements in a reaction mixture such as serum and silver nitrate, tends to be disturbed and large errors result. But if the strength of silver nitrate solution is so adjusted that equal quantities of serum and silver nitrate solution give the correct silver nitrate excess in the reaction mixture, then the same pipette may be used for the addition of both liquids.

It was, therefore, decided to make a capillary pipette using as graduation mark a fine hair capillary constriction such as holds the mercury column in a clinical thermometer.

TECHNICAL DETAILS.

A length of thick capillary tubing is drawn out so that the bore is about $\frac{3}{4}$ millimetre. It is then tapered abruptly so that the point will not readily be broken. A length of 2 or 4 cm. of this capillary tubing, containing approximately 0.02 to 0.01 cc., is almost sealed off from the main body by a micro-burner, so that a fine constriction results. The tube is allowed to cool and is cleaned with concentrated nitric acid, distilled water, alcohol, and ether; the last three being drawn through the pipette by a good suction pump.

The pipette is next filled with mercury to the middle of the capillary constriction, and this mercury is delivered into a small glass bottle and weighed to the fourth decimal place. The volume of the pipette is calculated by dividing the weight of mercury by the specific gravity of mercury at room temperature. Graduation of the pipette with mercury is extremely accurate but often, as in other micro-pipettes, tiresome owing to the difficulty of striking the middle of the arbitrary calibration mark. The best way is to fill the pipette just beyond the constriction and then to tap the tapered end of the pipette gently on a piece of filter paper held over the thumb. Tiny globules of mercury can in this way be squeezed out of the pipette and, with practice, calibration becomes easier than for the ordinary micro-pipettes of larger size, graduated to contain a definite volume of liquid.

Alternatively, the pipette has been calibrated with a strong standard solution of silver nitrate which is titrated against standard ammonium thiocyanate.

In micro-colorimetric methods the accurate measurement of small bulks of material plays a similar part, but conditions for micro-analysis are simpler, since the main factor is the dilution of the colour to the optimal concentration for colorimetric work with a diluent which minimises ionisation or reversible reactions of the coloured substances. The conditions are fulfilled in the thiocyanate method for iron determination by the use of nitric acid and excess of thiocyanate, which renders the reaction more complete, and of acetone which has the same effect by diminishing the dissociation of ferric thiocyanate.

THE ACCURATE ESTIMATION OF CHLORINE ON 0.02 TO 0.01 cc. OF SERUM,
WHOLE BLOOD, CORPUSCLES, URINE OR SWEAT.

In an earlier paper [Smirk, 1927, 1] a method was described for the accurate estimation of chlorides on 0.2 cc. of blood in about 10 minutes. For combined investigations on the serum, whole blood, and corpuscular chlorides of capillary blood samples, it is necessary to work on much smaller quantities of material, since 0.2 or 0.3 cc. is all that can be obtained from a single skin puncture, without the use of procedures altering the composition of the blood, such as heating or congesting the fingers from which the samples are taken.

The rôle of the skin in chloride excretion which appears to be of great importance in nephritis, especially as regards treatment with vapour baths, has also been incompletely investigated because of the difficulty experienced in obtaining sufficient quantities of sweat for analysis. The error due to evaporation tends to be very great in the collection of the amount required for analysis by existing methods.

Procedure.

A well mixed sample of the liquid to be analysed is taken up in the pipette and accurately adjusted to the level of the constriction, soaking up the excess with pieces of thin filter paper. The contents of the pipette are delivered to

the bottom of a 9×1 centimetre test-tube, taking care that as much adherent fluid is removed from the outside of the pipette as possible. The pipette is next filled with distilled water to just above the graduation mark and the washings delivered $\frac{1}{2}$ " above the bottom and on one side of the test-tube, which has been marked with a glass pencil or a piece of gummed paper. This washing is repeated twice and the washings added to the contents of the test-tube with similar precautions. The distilled water should be taken from a drop hanging from a burette, so that any material from the outside of the tip of the pipette, contaminating the residue of the drop, will be delivered to the test-tube in the subsequent washings.

The pipette is next washed with distilled water, alcohol and ether and is filled to the graduation mark with silver nitrate solution. The silver nitrate is of the following strength:

For sweat and urinary chlorides 43.592 g. per litre (1 cc. = 0.015 g. NaCl).
For serum chlorides 21.796 g. per litre (1 cc. = 0.0075 g. NaCl).
For whole blood chlorides 17.437 g. per litre (1 cc. = 0.0060 g. NaCl).
For corpuscular blood chlorides 13.078 g. per litre (1 cc. = 0.0045 g. NaCl).

The silver nitrate of appropriate strength is delivered to the tube using the precaution already outlined, and the pipette is washed out four or five times with concentrated nitric acid also taken from the hanging drop of a burette.

About as much powdered ammonium persulphate as would go on a 2 mm. square is then added, and the reaction mixture heated with moderate agitation over a micro-burner until all protein is dissolved. Air is then bubbled through the liquid by a very fine capillary tube and heating is continued by the micro-burner until the silver chloride forms small discrete masses at the bottom of the tube and the solution above is clear.

The reaction mixture is then cooled and diluted with approximately an equal quantity of acetone, which is delivered down the sides of the test-tube so that it carries the particles of fluid spurts to the bottom of the test-tube. The mixture is again cooled, one large drop of saturated iron alum solution is added, and it is then ready for titration. This is carried out as described [Smirk, 1927, 1] by delivering the alcoholic ammonium thiocyanate (20 cc. = 1 cc. of AgNO_3 = 0.005 g. of NaCl) directly into the liquid by means of a fine capillary extension of the micro-burette, attached to it by pressure tubing of small bore, and stirring the titration mixture with a rapid stream of fine bubbles from a capillary tube.

The amount of thiocyanate added should be about 0.15 to 0.25 cc. in order that the extent of dilution should not be greatly altered. The end-point is changed distinctly by 0.003 cc. of the ammonium thiocyanate. It is, therefore, desirable that a capillary burette graduated to 0.001 cc. should be used. Excellent results can be obtained with a burette graduated in 0.01 cc. if the divisions are read with the aid of a lens, and the tenths of a division estimated. Delivery from the burette should be constant and slow, since the slightest error due to incomplete drainage will show in the results. The end-point is to

be taken at the earliest reddish flush through the solution. 0.003 cc. added after this should change the colour of the solution to a distinct red.

The accuracy of the method depends on attention to detail.

(1) Avoidance of contamination of the pipette after washing, by contact with the original contents.

(2) The very earliest perceptible change should be noted, and then the titration carried a shade beyond to confirm the observation.

(3) The use of a strong white front illumination from a shaded lamp and of a white porcelain or paper background in actual contact with the test-tube is absolutely essential for accurate titration.

(4) All the ammonium persulphate must have been destroyed by nitric acid before the acetone is added or the end-point is ruined by the oxidation of the ferric thiocyanate with consequent decoloration.

(5) The titration must be carried out in the cold. It is not wise to have a hot electric lamp close to the test-tube except during the critical phase of the titration.

RESULTS.

The following represent consecutive series of results on samples of serum, whole blood and corpuscles (g. NaCl per 100 cc. of serum, whole blood and corpuscles respectively).

	<i>Sample A</i>	<i>Sample B</i>
Serum chloride	0.637 0.632	0.627 0.623 0.628
Whole blood chloride	0.484 0.486 0.490	0.492 0.491 0.497
Corpuscular chloride	0.256 0.256	0.485 0.327 0.333
	<i>Sample C</i>	
Serum chloride	0.620	
Corpuscular chloride	0.327	
Haemocrite reading: 46 % corpuscles, 54 % serum.		
Whole blood chloride by calculation = $0.620 \times 0.54 + 0.327 \times 0.46 = 0.487$.		
Whole blood chloride by estimation = 0.472.		

THE ACCURATE ESTIMATION OF IRON IN 0.02 TO 0.01 cc.

OF WHOLE BLOOD OR CORPUSCLES.

The principle of this method is practically that of a scale reduction of the original method on 0.2 cc. already quoted. It is, however, more rapid and if carefully performed more accurate. This increase in accuracy is probably due to the rapidity of the process which leaves little time for alteration in the colour of the solutions to be matched.

Procedure.

Using a micro-pipette as described for the estimation of chlorides on 0.02 to 0.01 cc., a small measured quantity of blood is delivered to the bottom of a Jena glass test-tube; approximately 2×12 cm. The pipette is washed out

(as described p. 204) three or more times, using in all 0.1 cc. of distilled water. 0.1 cc. of concentrated nitric acid is then added.

As much ammonium persulphate as will go on a 2 mm. square is then added with care, so that all of it reaches the bottom of the Jena tube and none remains adherent to the walls above the reaction mixture. In the absence of this precaution the ferric thiocyanate colour would fade slightly during the estimation owing to the oxidising action of the ammonium persulphate. The mixture is heated gently over a micro-burner and in about 20 seconds solution of the protein is complete. The clear solution is shaken round the lower walls of the test-tube to dissolve any small particles of protein which may have escaped from the main mass.

The lower part of the test-tube is then placed in an ordinary Bunsen burner for a few seconds with gentle agitation to keep the inner glass surface of the tube covered with liquid. As soon as vapours rise half or three-quarters of the way up the test-tube, it is removed from the Bunsen flame and cooled rapidly under the tap.

The tube now contains: x (say 0.01725 cc.) of blood, 0.1 cc. of water and 0.1 cc. of concentrated nitric acid.

Neglecting the slight change in volume, which will follow the addition and solution of the ammonium persulphate, the total volume is $(0.2 + x)$ cc.

Add to the test-tube 2 cc. of distilled water from an accurate pipette and then 2 cc. of acetone, mix thoroughly and cool for 20 seconds in running water. Now add 1 cc. of concentrated ammonium thiocyanate solution (24 g. per 100 cc.) to the solution in the test-tube, mix thoroughly and cool in running water.

The ferric thiocyanate colour is compared colorimetrically with the artificial standard already described in the earlier paper.

Calculation. Let the unknown blood and the iron standard be set at 15 mm. in the left-hand cup of the colorimeter and let the corresponding readings of the artificial standard be "B" and "S."

Then as the dilutions are similar, $\frac{15}{B}$ cc. of blood have the same iron content as $\frac{15}{S}$ cc. of iron standard.

Therefore the iron content of the blood is $\frac{50B}{S}$, where 50 mg. of ferric iron per 100 cc. is the adopted strength of the standard.

Notes and observations on the method.

(1) Each analysis, including the cleaning of the pipette and taking of the sample, occupies about 10 minutes.

(2) The colorimeter is very carefully cleaned with distilled water, alcohol and ether before the first colorimetric comparison is made. Afterwards, the cups are merely emptied, rapidly drained and filled at once with the next of a series of solutions. This changing of solutions must be carried out rapidly, with the

minimum delay and without any attempt at washing out the cups, in order that errors due to evaporation of the water-acetone mixture may be avoided.

(3) Great care must be taken with the quantities of the washings since 0.1 cc. more or less represents a 2 % error in 5 cc. of solution.

(4) For description of the preparation of an iron standard, see Fowweather [1926].

RESULTS.

Consecutive estimations of whole blood iron: 48.9, 48.0, 48.7, 48.0, 47.2 mg.

These results, as will be seen from the above account of the method, were obtained by comparison with an iron standard treated in a similar fashion. There is no essential difference from the method previously described for 0.2 cc. of blood.

SUMMARY.

(1) Some general principles of micro-analysis have been discussed.

(2) An accurate method for the estimation of chlorine on 0.02 cc. of serum, whole blood, corpuscles, urine or sweat has been described.

(3) An accurate and rapid method for the estimation of iron on 0.02 to 0.01 cc. of whole blood or corpuscles has been described.

REFERENCES.

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