CCXV. A SIMPLE METHOD OF VOLUMETRIC ANALYSIS FOR SMALL QUANTITIES OF FLUID: ESTIMATION OF CHLORIDE IN 0.3 μ l. OF TISSUE FLUID

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IN recent years many quantitative methods of analysis for small amounts of fluid have been devised; notably by Richards and his colleagues for studies on the glomerular filtrate in the kidney [Westfall *et al.* 1934] and by Linderstrøm-Lang & Holter [1931; 1935] for enzyme studies on single cells. The only advantage of the method here described lies in its simplicity.

In the colorimetric estimation of *pH* in the body fluids of insects [Wigglesworth, 1927] use was made of capillaries lined with paraffin. This technique has now been extended to titration. The method was developed for the purpose of estimating the chloride in the blood of single mosquito larvae [Wigglesworth, 1937], and it was this requirement which determined the volume of fluid used for analysis. But much smaller volumes could be used if necessary; and the principle may be applied to many other titrimetric procedures.

Principle

By using capillary tubes lined with paraffin, and so reducing surface tension, it is possible to manipulate, measure and transfer quantitatively, very minute quantities of fluid. To measure concentration it is not necessary to know the absolute volumes employed. A measured volume of tissue fluid is treated with excess of nitric acid and an arbitrary volume of standard silver nitrate of suitable concentration, filtered and the filtrate titrated in arbitrary units with sodium thiocyanate in the presence of ferric ammonium sulphate. The equivalent value of the units of thiocyanate in terms of chloride is obtained by titrating the standard volume of silver nitrate alone and after the addition of sodium chloride of known concentration measured from the same pipette as the tissue fluid.

Preparation of the pipettes

The capillaries are made by drawing out capillary tubing of 2 mm. external and 0.6–0.7 mm. internal diameter. 12 cm. is a convenient length. The lumen varies and the pipettes are chosen later according to the purpose required. They are lined with paraffin of high melting-point (B.D.H. m.p. 65–71°), the method being as follows. The paraffin is melted in a long test-tube held obliquely in a clamp. Heating should be continued until the wax begins to smoke freely: the lining is apt to peel off if it is applied at too low a temperature. The capillary pipette with a rubber tube attached is then immersed in the melted paraffin up to the neck. When the paraffin has risen to this level, with the pipette still immersed, it is blown out; and as soon as bubbles of air flow from the tip the pipette is withdrawn, air being blown through all the time. The paraffin hardens

instantly and forms a perfectly smooth coat. It is an advantage to leave the paraffin on the outside of the capillary also, since it facilitates observation of the meniscus. With very fine pipettes it may not be possible to blow out the paraffin by the mouth. It is easily forced out if the rubber tube is folded over near the top of the pipette and squeezed between the finger and thumb.

The capillary is now cut through just where it widens out, inserted into a sheath consisting of a length of the tubing from which it was drawn, and secured at both ends with paraffin (Fig. 1 *A*). The capillary projects a few mm. at the lower end. A rubber tube is attached at the upper end, and it is advisable to insert a small roll of filter paper in this tubing to prevent condensed vapour from the breath entering the pipette.

For marking graduations a strip of transparent adhesive paper (music mending paper) is applied to the glass sheath and the marks made with a finely pointed pencil. The "burette" for thiocyanate is graduated by drawing in a column of water of convenient length (say 5 mm.), marking its upper limit, drawing it along until its lower limit coincides with the mark, and repeating this process until the whole burette is divided into a series of segments of varying length but equal capacity. To prepare and graduate such a burette requires about half an hour.

A convenient type of pipette for the rapid measurement of small volumes where great accuracy is not required (e.g. for the nitric acid or for distilled water) is shown in Fig. 1 *B*. The capillary is inserted into a tube as shown. Its tip is

Fig. 1. *A*, standard type of capillary pipette or burette; *B*, pipette for rapid measurement of approximately constant volumes; *C*, method of titration on paraffin coated slide, stirring with jet of air; *D*, method of filtration. In the sectional drawings glass parts are shown clear, paraffin black, rubber tubing stippled.

lined and coated with paraffin (so that it can be discharged without spluttering) but above this the glass is clean. As soon as fluid is drawn past the paraffin coated tip, a pipette of this type will fill itself automatically to the end of the capillary, and will therefore always deliver a constant volume.

The capacity of the pipette used for tissue fluid (estimated by filling with mercury and measuring with the microscope the diameter of the droplet after discharge) was about 0.3 mm.³ This formed a column from 6 to 9 cm. in length in different pipettes so that the mean diameter of the lumen was 65–75 μ . The units in the thiocyanate burette (each about 5 mm. in length) were rather less than 0.04 mm.³

Care of the pipettes. Each pipette is used only for one solution. When these solutions contain no organic material the pipette is left completely dry and never requires cleaning. The pipettes should be kept dry. Organic fluids adhere slightly to the walls, so that the pipette must be washed out repeatedly with 1 % sodium chloride followed by distilled water. Even then a film of organic material may accumulate in course of time; this is eliminated by filling the pipette with 50 % nitric acid and leaving for an hour at room temperature. It is surprising that the pipettes will withstand cleaning repeatedly in this way. They can even be cleaned several times with pure nitric acid before the film of paraffin breaks down. The pipette for measuring blood usually requires replacement after 100 or 150 titrations; the other pipettes can apparently be used indefinitely.

It is important that when the pipette or burette is discharged the fluid should stand out as a small sphere and show no tendency to creep around the tip. This is ensured by dipping the tip from time to time in melted paraffin, air being blown through the pipette meanwhile.

Procedure

The whole procedure is carried out on a microscope slide which has been dipped in melted paraffin. This rests on a white card to provide a suitable background. Droplets of fluid placed separately on such a paraffin surface can be transferred quantitatively from place to place, and mixed together when required, by blowing them with a jet of air.

A small drop of distilled water (say 1.5 mm. in diameter) is measured out. The tissue fluid is measured out beside it and the water used for washing out the pipette twice, each droplet being discharged separately. A volume of nitric acid of about four times the volume of the tissue fluid is next added and mixed with the fluid and washings from the pipette. The droplet of standard silver nitrate (0.5 %) is measured; a minute droplet of saturated ferric ammonium sulphate is added to the water; and all the droplets are brought together and mixed.

Filtration. The silver chloride is filtered off by a modification of Behren's method [Chamot, 1921]. A thick-walled capillary tube (3 mm. external, 0.75 mm. internal diameter), drawn out and ground to a point at one end, is lined with paraffin and the outside of the tip also well coated with paraffin. A rubber tube is attached to the other end. A piece of hardened filter-paper 1 mm. square is placed on the paraffin slide close to the drop to be filtered and the tip of the capillary pressed firmly down vertically upon it. The drop is then driven up against the filter paper, and a negative pressure is created within the capillary by suction and closure between the finger and thumb. Then the downward pressure upon the capillary is slowly relaxed until the fluid begins to rise into the lumen. When all the fluid has been drawn up, the filter paper is washed twice by drawing up in the same way two droplets of distilled water of suitable size previously put in readiness (Fig. 1 D). When the pipette is lifted up the silver chloride adheres to the filter paper and a perfectly clear filtrate can be discharged quantitatively upon a clean part of the paraffin surface.

Titration is carried out with 0.01 N sodium thiocyanate. If the tip of the thiocyanate burette is kept freshly coated with paraffin, the solution forms a minute sphere as it is gently blown out. This can be introduced into the droplet to be titrated by just touching its margin. During titration the droplet is stirred by directing a jet of air to one side of it: this causes it to spin rapidly round (Fig. 1 C).

Sources of error. Contamination with sodium chloride from the air is guarded against by coating the slide with a fresh layer of paraffin immediately before each titration, and by passing the air used for mixing and stirring through water.

With these precautions there have been no irregularities due to this cause. Interference by proteins and stability of the end-point are discussed in the next section.

Results

The following are values obtained in the calibration of a set of pipettes:

1 vol. AgNO_3 required 51, 51, 51 units NaSCN .

2 vol. AgNO_3 required 102, 103 units NaSCN .

Mean: 1 vol. $\text{AgNO}_3 = 51$ units.

1 vol. 0.50 % $\text{NaCl} + 2$ vol. AgNO_3 required 39, 40, 41 units NaSCN .

Mean: 1 vol. 0.5 % $\text{NaCl} = 102 - 40 = 62$ units.

It has been shown by Patterson [1928] that if an excess of concentrated nitric acid is added to plasma before the addition of the silver nitrate, the proteins do not interfere with the Volhard titration, which can be carried out directly without previous destruction of the proteins. This point was tested by estimating the chloride in a diluted blood serum by the present method and by Rehberg's [1926] method as used in this laboratory, in which the proteins are oxidized with nitric acid and perhydrol.

(i) 1 vol. serum + 1 vol. AgNO_3 required 11 units, $51 - 11 = 40 \times \frac{0.5}{62} = 0.32$ % NaCl .

(ii) 1 vol. serum + 1 vol. AgNO_3 required 12 units, $= 0.31$ % NaCl .

(iii) 1 vol. serum + 1 vol. AgNO_3 required 10 units, $= 0.33$ % NaCl .

Estimation by Rehberg's method (kindly carried out for me by Mr E. Lange) gave 0.33 % NaCl . Clearly there is no interference by proteins.

In working with these small volumes of fluid it is impossible to prevent the decomposition of the AgCl by having an excess of acetone present or by using an alcoholic solution of thiocyanate. It was for this reason that removal of the AgCl by filtration was adopted. The end-point is then very sharp and stable. It is of course possible to omit the filtration and so to simplify the procedure greatly. Although the end-point soon fades it is not difficult, with practice, to obtain consistent results. Three estimations on the same diluted serum as above, without filtration, gave values 0.31, 0.31, 0.32 %.

The accuracy of the method, as employed in my experiments, with or without filtration, is not greater than ± 0.02 % NaCl ; but there is little doubt that by the introduction of refinements a higher degree of accuracy could be obtained.

SUMMARY

If capillary pipettes coated with paraffin are used it is possible to carry out volumetric analyses on very small quantities of fluid. A Volhard titration, with or without removal of the silver chloride by filtration, is described. It is applicable to 0.3 mm.³ of tissue fluid, or less if required.

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