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A METHOD FOR DETERMINING TOTAL CARBON DIOXIDE IN SMALL VOLUMES OF LIQUID

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INTRODUCTION

The method of Conway (1962) for determining total dissolved carbon dioxide by microdiffusion is frequently used to estimate bicarbonate concentrations in body fluids. However, the method as described requires 0·1 ml. of fluid, and in work on small invertebrates this volume is frequently not available. The present paper describes modifications of the microdiffusion method which enable measurements of total carbon dioxide to be made using volumes of liquid of the order of 1 μl.

PRINCIPLE OF THE METHOD

If a substance present in solution can be volatilized and the volatile form can then be trapped in such a way as to alter the measurable properties of the absorbing liquid, the quantity of the original substance can be estimated, usually without interference from other substances present in the original solution. In Conway's (1962) methods for estimating a variety of substances, the sample is placed in one part of a microdiffusion dish, while the absorbant is placed in a separate part. The substance is volatilized by addition of some reagent, the dish is sealed and the volatile substance diffuses from the original solution to the absorbant. Change in composition of the absorbant is then estimated by colorimetric titration.

This method is an excellent one for use with volumes of the order of 0·1–1·0 ml., but if attempts are made to reduce the volume of liquid while retaining a similar procedure, the problems of evaporation of the sample and of the inaccuracy of colorimetric titrations become more important. In the design of a similar method applicable to very small volumes, the microdiffusion process must be carried out in a cavity which is never open to the air, and a different method of determining the change in composition of the absorbant must be devised.

In our method for the determination of dissolved carbon dioxide the first problem has been solved by performing the microdiffusion process under liquid paraffin (mineral oil) in conjunction with the use of mercury, and by the use of micropipettes filled with petroleum ether. The second problem was approached by considering the reaction involved in the absorption of carbon dioxide in barium hydroxide after its volatilization by the addition of acid. This can be represented:

$$CO_2 + Ba(OH)_2 \rightarrow BaCO_3 + H_2O.$$

The original absorbing solution of barium hydroxide is converted to the insoluble

carbonate and water. This means that the freezing point of the absorbing solution changes, the change depending on the quantity of carbon dioxide absorbed. We have therefore used the micro-method of Ramsay & Brown (1955) to measure the change in freezing point of the barium hydroxide, which varies in proportion to the amount of dissolved carbon dioxide in the original sample.

METHOD

(1) *Preparation of microdiffusion tubes, micropipettes and solutions*

Tubes

The tubing used is Pyrex, internal bore approximately 1·0 mm., wall thickness 0·3 mm. (obtainable from Townson & Mercer Ltd.). This is broken into pieces approximately 13 mm. long. These pieces are made water-repellent by dipping in silicone 'Repelcote' (Hopkin & Williams Ltd.). After drying, they are soaked in distilled water, washed in acetone and allowed to dry in air.

One end of each tube is closed by passing the tube through a wire loop carrying molten paraffin wax, the wax being deposited as a disk attached to the end of the tube. This prevents samples from moving along the tube after they have been injected at a particular place. Nitrogen is blown into the open end of the tube through a fine jet for approximately 10 sec., and the tube is immediately plunged into a dish of liquid paraffin (mineral oil).

Pipettes

Samples, barium hydroxide solutions and sulphuric acid solutions are all manipulated in mouth-operated constriction pipettes. Pyrex tubing (2 mm. internal bore, wall thickness 1 mm.) is drawn out to a diameter of 0·5–1·0 mm., and then drawn to a fine

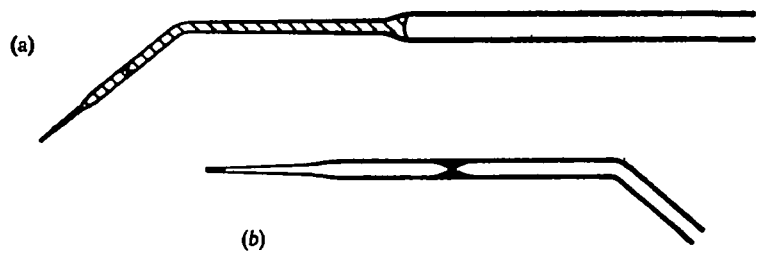

Fig. 1. Micropipettes. (*a*) shows the whole pipette, with shading indicating the level to which petrol is drawn up; (*b*) shows an enlarged view of the tip and constriction.

tip. A constriction is formed near the tip, so as to enclose a known volume between the constriction and the tip, by holding the pipette vertically in a horizontal microflame. The pipette is then bent at 45° just behind the constriction (see Fig. 1). The extreme tip is sealed and the external surface only is coated with 'Repelcote', after which the seal at the tip is broken off. Pipettes are required for approximately 1·5, 0·60 and 0·25 μl., and are partially filled with petrol (petroleum ether). We have positioned them by hand, but they may be positioned using a micromanipulator. A simple micropipette with no constriction is also required for the injection of mercury.

Solutions

The barium hydroxide is approximately 80 mM/l. Initially a saturated solution is made up, and this is centrifuged under liquid paraffin to remove the insoluble barium carbonate which forms in air. A saturated solution at 22° C. contains 184 mM/l. The concentration is then adjusted by dilution with distilled water (always keeping the solution under liquid paraffin) to 80 mM/l., and the depression of freezing point is then approximately 0·370° C. This must not be greater than 0·395° C., since at −0·400° C. barium hydroxide is precipitated from solutions which should theoretically have a lower freezing point. Because of the rapid absorption of carbon dioxide from the air it is not convenient to make up a solution of 80 mM/l. $Ba(OH)_2$ directly.

The acid used to liberate CO_2 from the sample is 0·5 N-H_2SO_4.

Standard solutions of bicarbonate are 10, 20, and 30 mM/l. $NaHCO_3$, and are kept under liquid paraffin.

(2) *Procedure*

A series of microdiffusion tubes is placed under liquid paraffin (S.G. 0·865–0·890) as described above. The following operations are carried out under a low-power

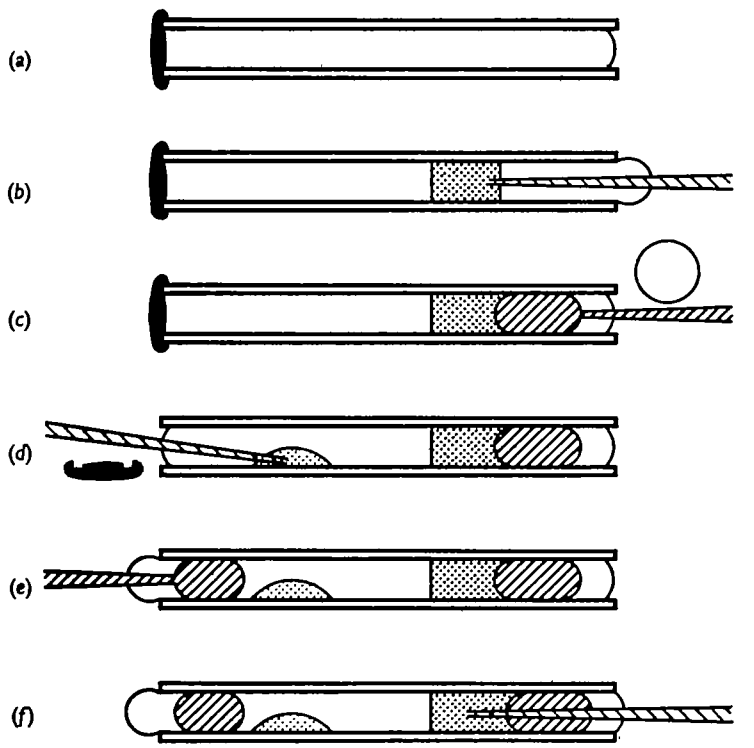

Fig. 2. Details of procedure. (*a*) Nitrogen-filled tube, under liquid paraffin, with wax disk at one end. (*b*) Injection of sample. (*c*) Injection of first mercury barrier. (*d*) Removal of wax disk and injection of barium hydroxide. (*e*) Injection of second mercury barrier. (*f*) Final injection of acid into sample solution. The tube is shown in black outline, with the wax disk in black. Fine stipple shows barium hydroxide; heavy stiple shows the sample solution. Heavy diagonal shading (right-hand) shows mercury, while light diagnonal shading (left-hand) shows petroleum ether. The nitrogen space is left clear.

binocular microscope using transmitted light. Any dish such as a Petri dish, which can be accommodated on the microscope stage, is suitable.

The sample, which is kept on a siliconed surface under liquid paraffin, is drawn up into the 1·5 μl. pipette, previously filled with petrol, until the petrol-sample meniscus reaches the centre of the constriction. The pipette is inserted into the open end of the microdiffusion tube, with the tip 2–3 mm. from the end, and the sample is blown out (Fig. 2*b*). Using a similar pipette without a constriction, mercury is then injected against the exposed surface of the sample (Fig. 2*c*). This will displace some or all of the nitrogen external to the sample. The wax cover at the opposite end of the tube is then removed with fine forceps, and 0·60 μl. of $Ba(OH)_2$ is injected on to the wall of the tube (Fig. 2*d*). Accidental injection of a small amount of petrol will not affect the determination. A second mercury barrier is formed external to the $Ba(OH)_2$ (Fig. 2*e*). Finally, 0·25 μl. of sulphuric acid is injected into the sample through the first mercury barrier (Fig. 2*f*). This first mercury barrier prevents the CO_2 which is liberated from the sample from diffusing into the paraffin; and the second barrier prevents CO_2 present in the paraffin from diffusing into the $Ba(OH)_2$.

When a sample or a volume of $Ba(OH)_2$ has been delivered, the petrol is blown out, and the pipette is rinsed twice in acetone up to the 45° angle. Fresh petrol is then drawn up and the pipette is again ready for use. This procedure is not necessary with the sulphuric acid pipette. After some time the pipettes become dirty and the meniscus does not travel freely along them. This may be cured by drawing up 1–2 washings of 50% HNO_3 followed by very thorough washing with distilled water and final washing with acetone.

It is convenient to prepare a series of tubes with samples and the first mercury barrier, and then to add the $Ba(OH)_2$ and the acid at specific times for each tube. One precaution must be observed during these operations: the micropipettes must be positioned near the axis of the tube when injecting liquids, as liquid paraffin tends to creep along them when they are in contact with the sides of the tube.

After 1 hr., and not later than 70 min. after adding the acid (see later notes on time course and interference), a sample of $Ba(OH)_2$ is removed under liquid paraffin in a silica capillary and its freezing-point is determined by the method of Ramsay & Brown (1955). We have found it convenient to take five samples in each capillary, to disregard the first one, and to average numbers 2, 3 and 4. Once these samples have been mounted ready for freezing-point determination, they may be left indefinitely before actually performing this determination.

The calibration curve is a straight line and can be prepared from two points: the initial freezing-point of the $Ba(OH)_2$ representing the reading for 0 mM/l. HCO_3^-; and the freezing-point given with 30 mM/l. HCO_3^- representing the maximum reading. If this latter freezing-point is higher than approximately −0·050° C., the reading of freezing-point becomes slightly inaccurate. Such a situation can be met by using a slightly smaller sample pipette, or a slightly larger $Ba(OH)_2$ pipette.

An experienced operator can set up at least 10 tubes for microdiffusion in 1 hr. By this time the initial tubes will be ready for determination of freezing-point. Samples can be taken and prepared for measurement of freezing-point in 2–3 min., and the final determination of freezing-point takes 5–10 min. (Ramsay & Brown, 1955).

Accuracy of the method and interference

The overall accuracy of the method depends upon several factors, some of which are now considered.

(*a*) Accuracy of constriction pipettes. The accuracy with which the liquid is drawn up into the pipettes depends upon the geometry of the constriction. If the constriction is one tenth of the diameter of the pipette, i.e. 0·05 mm. in a pipette of 0·5 mm.

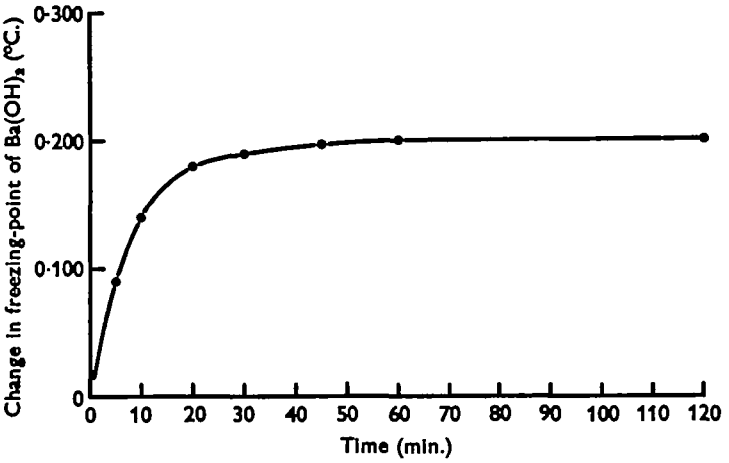

Fig. 3. The time course of a single determination of 20 mM/l. HCO_3.

Table 1. *Measurements on bicarbonate solutions*

Concentration (mM/l.)	Mean observed change in freezing-point of $(BaOH)_2$ (°C.)	S.D. of freezing-point determinations (mM/l.)	No. of observations
0·00	0·0000	±0·32	10
5·00	0·0510	±0·30	10
10·00	0·1000	±0·32	10
20·00	0·2020	±0·30	10
30·00	0·3000	±0·39	10
20·00 (with 600 mM/l. NaCl.)	0·2008	±0·36	10

diameter and 7·5 mm. length, which has a volume of approximately 1·5 μl., a movement of the meniscus 0·5 mm. along the constriction is equivalent to a change in total volume of only 1 %. Positioning of the meniscus in the constriction is thus not likely to be a significant source of error.

(*b*) Degree to which the reaction has proceeded and errors arising from diffusion of water vapour. A typical time course is shown in Fig. 3. The reaction appears to be completed after 1 hr. Further readings of freezing-point do not change measurably for 12 hr., when pure solutions of bicarbonate are used, but after 24 hr. a change of 0·005° C. may be detectable. This change is presumably due to diffusion of water vapour from the sample into the $Ba(OH)_2$. When the samples have a higher osmotic pressure than the $Ba(OH)_2$, i.e. a depression of freezing-point of more than 0·370° C.,

water vapour moves from the $Ba(OH)_2$ into the sample. When the sample contains 600 mM/l. NaCl, the change in freezing-point of the $Ba(OH)_2$ amounts to 0·003° C./hr., which is exactly the standard deviation quoted by Ramsay & Brown (1955) for the freezing-point determinations. Such a change after 1 hr. is not detectable (see Table 1), but it will become so after 1·5–2 hr. Errors arising from diffusion of water vapour into, say, sea water, are not appreciable when the method is used as described above; but such errors become unacceptable if attempts are made to reduce the scale of the method (see section *d*). If the vapour pressure of the sample solutions is very low, compensation for this could always be made, provided it is fairly accurately known.

(*c*) Accuracy of the method. The figures obtained from measurements using standards are given in Table 1. These show that 0–30 mM/l. HCO_3^- can be determined with a standard deviation of ± 0·3–0·4 mM/l.

(*d*) The possibility of reducing fluid volumes. Preliminary attempts to reduce the scale of the method have shown that the reaction can be followed using nanolitre samples; but under these conditions the errors arising from diffusion of water vapour become very large relative to the changes due to CO_2 movement, and the problem of compensating for these changes renders the method so tedious as to be impracticable.

SUMMARY

1. A microdiffusion method for estimating total dissolved carbon dioxide in volumes of approximately 1·5 µl. is described. The principle of the method is to displace the CO_2 with acid, to absorb it in barium hydroxide solution and to measure the change in the freezing-point depression of this solution.

2. The range of measurement is 0–30 mM/l. HCO_3^- with a standard deviation of ± 0·3–0·4 mM/l.

3. In its present form the method cannot be used with smaller volumes without correcting for errors arising from the diffusion of water vapour between the sample and the barium hydroxide solution.

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