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

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SUMMARY

1. A microdiffusion method is described by which total carbon dioxide can be determined in 2 nl samples of liquid. The standard deviation for 0–30 mM/l HCO_3^- is approximately 1 mM/l.

2. The volume of sample can be reduced to 0·8 nl, but the S.D. then rises to 1·6 mM/l. With volumes of 0·4 nl, the S.D. is 2·7 mM/l. It is not possible, without modification, to use volumes smaller than about 0·4 nl.

3. In samples where the HCO_3^- concentration is low, accuracy can be increased by using larger volumes: 10 mM/l HCO_3^- can be determined in 6 nl samples with a standard deviation of 0·5 mM/l.

INTRODUCTION

The method of Conway (1962) for determining total dissolved carbon dioxide by microdiffusion was adapted by Little & Ruston (1970) for use with volumes of liquid of the order of 1 μl. Because of problems arising from diffusion of water vapour, the sample volume could not be further reduced without reducing the accuracy of the method. The present paper describes a modification of the microdiffusion method whereby total carbon dioxide can be determined using volumes of liquid of the order of 1 nl (10^{-3} μl).

PRINCIPLE OF THE METHOD

In the method of Conway (1962) the carbon dioxide is volatilized from the sample by addition of acid, allowed to diffuse through an air space, and finally trapped in barium hydroxide. Consequent change in the concentration of the barium hydroxide is determined by titration. In the method of Little & Ruston (1970) the same basic principles are followed, but the diffusion occurs through nitrogen and the change in concentration of the barium hydroxide is determined by measuring the change in freezing point. In the present method the carbon dioxide diffuses through liquid paraffin so as to prevent significant diffusion of water vapour. Reduction in the scale of operations allows the barium hydroxide to be transferred directly to the freezing-point apparatus of Ramsay & Brown (1955), without separate sampling. The reduction of scale and the lack of any gas space in the system also enable the liquids to be handled in a single syringe, and ensure that the microdiffusion process is completed in a relatively short time.

With this method small volumes of mercury, barium hydroxide, the sample

(containing HCO_3^-), sulphuric acid and again mercury are drawn successively into a fine tapering microdiffusion chamber: each of these liquids being separated by liquid paraffin (see Fig. 3). Once the series is complete, it is drawn further into the chamber, where the increase in diameter causes the oil barrier between sample and acid to break. The carbon dioxide liberated, being unable to escape through the mercury, is eventually trapped in the nearest aliquot of barium hydroxide solution. The freezing point of the latter is then compared with that of an unchanged aliquot, using the apparatus of Ramsay & Brown (1955).

APPARATUS

(1) *The general design*

Fluid volumes are measured by coupling a micrometer-driven syringe to a small microdiffusion chamber and drawing liquids into this chamber under liquid paraffin (mineral oil). This method requires: a rigid mechanical bench, a bath of liquid paraffin in which to manipulate liquids, fine microdiffusion chambers, and a seal between these chambers and the syringe which can be easily connected and disconnected. These items are described in turn.

(2) *The mechanical bench*

The various items are mounted on a steel plate (60 × 10 × 1·2 cm) and viewed with a stereo microscope (Wild M 5) with substage illumination (Fig. 1). One of the legs of the steel plate is on an extended arm and bears a counter-weight.

The Perspex bath is attached via a pump to the reservoir. Beneath the Perspex bath is a rectangular hole through which light is transmitted to the stereo microscope. Smaller holes are necessary to accommodate two micromanipulators.

(3) *The manipulating bath*

The Perspex bath contains liquid paraffin ('light', S.G. 0·840–0·860) under which the manipulation of liquids is carried out. The level of the liquid paraffin can be raised or lowered by a peristaltic pump which is connected by a Polythene tube to the bath (Fig. 1). The various liquids used, including the samples, are placed in horizontal 1·0 mm diameter Pyrex tubes held in a Perspex rack whose position can be adjusted by a micromanipulator. The microdiffusion chambers (see below) are held in a stainless-steel tube inserted through the end wall of the bath at a height of 3·2 cm from the bottom. Below this tube a small 'drip cup' is attached to the outside of the bath.

(4) *The microdiffusion chambers*

These are made from micro-electrode tubing (internal bore 1·0–1·25 mm and external diameter 2·0 mm, obtainable from Jencons Ltd), using an electrode puller, so as to produce a thin 'whisker' (approximately 1·0 cm long) attached to a 5 cm length of original tubing. The 'whisker' is broken off where it has an internal diameter of approximately 30 μm (external diameter 40 μm) and is coated internally with silicone 'Repelcote' (Hopkin & Williams Ltd). The internal diameter must never be smaller than 20 μm because it is not then possible to draw mercury into the capillary (see page 675).

The chambers are held firmly in the stainless-steel tube by melting paraffin wax between the walls of the steel tube and the chamber, using a small electrically heated wire placed near the outer end of the steel tube.

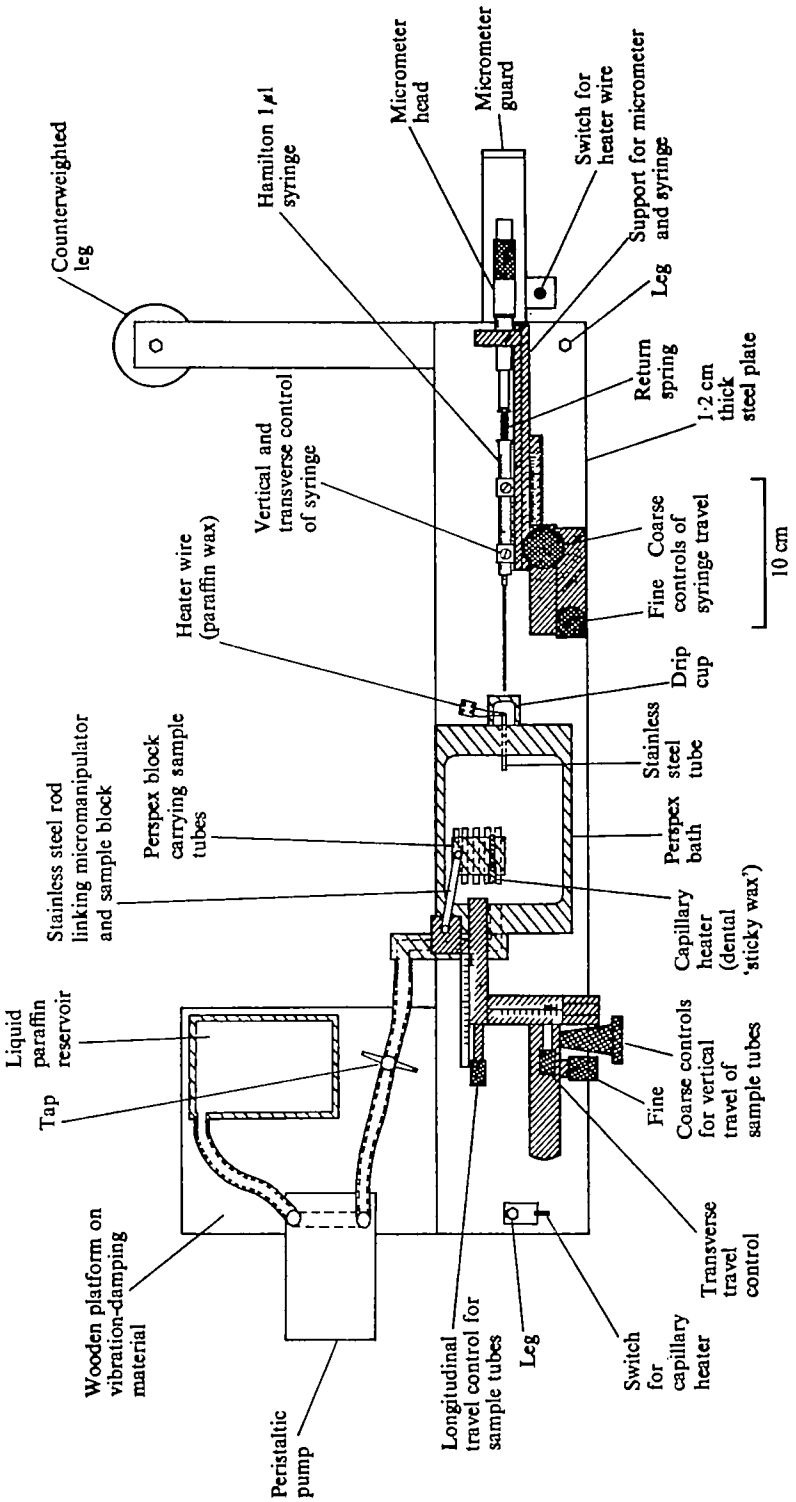

Fig. 1. A plan view of the apparatus to show the positioning of the various items on the mechanical bench.

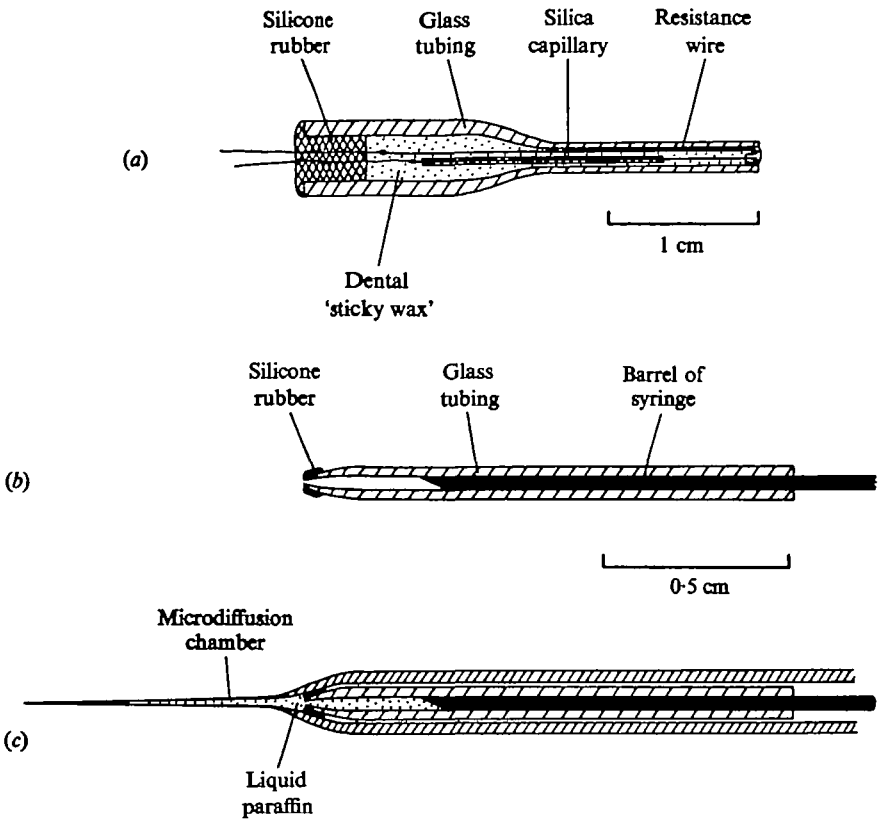

Fig. 2. Some details of the apparatus. (*a*) The heater containing dental 'sticky wax' used to seal the tip of the microdiffusion chambers. (*b*) The tip of the syringe needle, showing the attached glass tubing and the position of the silicone rubber seal. (*c*) The syringe and its attached glass in place inside the microdiffusion chamber.

(5) *The microsyringe and its connexion to the microdiffusion chamber*

Appropriate volumes of liquids are drawn into the microdiffusion chamber by the microsyringe (Fig. 1). A 1 μl syringe (Hamilton) is used, with the plunger operated by a micrometer drive. The micrometer employed advances 0·5 mm for each turn of the micrometer head, which has 50 divisions: a rotation of the micrometer head by 5 divisions thus corresponds to the movement of 1 nl of liquid at the tip of the microsyringe. To avoid relative movement of the microsyringe and the micrometer, both are mounted on a steel plate (thickness 0·6 cm), held on a micromanipulator, to facilitate connexion and disconnexion with the microdiffusion chambers. This micromanipulator is mounted on the steel platform so that the barrel of the microsyringe is in line with the steel tube in the wall of the Perspex bath, which bears the microdiffusion chamber. Provision is made in the mounting of the syringe on the steel plate for small vertical and horizontal adjustments. A small Polythene bearing is attached to the steel platform to provide support for the steel plate over the whole travel of the micromanipulator.

To provide a leakproof connexion with the microdiffusion chamber a small Pyrex tube, with a tapering profile towards the free end (Fig. 2*b*), is glued to the outside

the steel barrel of the microsyringe. The tip of this taper is surrounded by a thin layer of silicone rubber sealant (either opaque (e.g. Dow Corning) or transparent (e.g. Trophix)) so as to form an effective seal when the glass tip is pressed into the wide end of the microdiffusion chamber.

PROCEDURE

(1) *Solutions and their manipulation*

A number of liquids are placed into the sample tubes held in the sample rack, after these have been filled with liquid paraffin and lowered beneath the general level of liquid in the bath. Mercury is placed in the tube farthest from the operator and 0·5 N-H_2SO_4 in the adjacent one, the next two tubes being siliconed internally and containing either samples or standard solutions. Samples are placed near the mouth of the tubes with micropipettes. Standard solutions consist of appropriate bicarbonate solutions, normally 10, 20 and 30 mM/l $NaHCO_3$. In the last tube is placed 75–80 mM/l $Ba(OH)_2$ (see Little & Ruston, (1970)).

(2) *Preparation of the microdiffusion chambers for use*

A siliconed microdiffusion chamber is inserted through the steel tube in the end wall of the paraffin bath and sealed there with paraffin wax as described above. The level of liquid paraffin in the bath is then raised to cover the microdiffusion chamber. The barrel of the microsyringe (filled with 'light' paraffin) is then directed into the wide end of the microdiffusion chamber. Using the coarse control of the micromanipulator the tip is driven along the microdiffusion chamber until the silicone rubber sealant just touches the walls of the chamber where they begin to narrow. With the fine adjustment of the micromanipulator the tip is moved forward until it can be seen that the silicone rubber sealant has made a complete seal. Liquid paraffin is then driven in from the syringe until the microdiffusion chamber is nearly filled, leaving an air space at the tip. This makes the tip easily visible, and allows the sample tube containing mercury to be accurately positioned in line with the tip of the microdiffusion chamber. The apparatus is now ready for use.

(3) *The microdiffusion process*

Two nl (2×10^{-3} μl) samples have been used routinely, although smaller volumes can be analysed, as will be described later.

When the tube containing mercury has been aligned with the microdiffusion chamber, the residual air is driven out. Since there may be some backlash in the micrometer-syringe system, it is best to draw some liquid paraffin into the microdiffusion chamber initially to ensure that all 'slack' is taken up. The tube containing mercury is then moved until the tip of the microdiffusion chamber protrudes into the mercury. The micrometer head is turned approximately 10 divisions so that 2 nl of mercury is drawn into the microdiffusion chamber. On withdrawal from the mercury, a little paraffin runs into the chamber. Three samples of $Ba(OH)_2$ are then drawn in, each of 5 divisions (1 nl), and each separated from the next by 5 divisions of liquid paraffin. Another 5 divisions of paraffin is taken in, followed by 10 divisions (2 nl) of sample or standard; 0·5 divisions of liquid paraffin separates this from 10 divisions of 0·5 N-I_2SO_4. Lastly, 5 divisions of mercury is drawn in (see Fig. 3*a*). After this sequence,

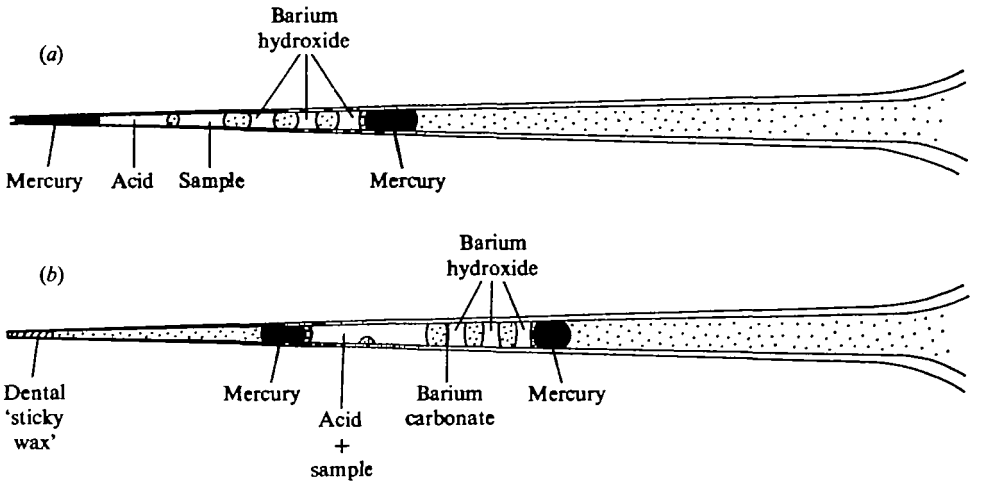

Fig. 3. Liquids in the microdiffusion chamber. Stippled areas indicate liquid paraffin. (*a*) The liquids when they have just been drawn into the chamber. (*b*) The final position of the liquids. The microdiffusion has taken place, and a crust of $BaCO_3$ has formed at the left-hand meniscus of the $Ba(OH)_2$ aliquot nearest to the sample.

the liquids are drawn further up the diffusion chamber until the meniscus of the liquid paraffin separating the acid and the sample is about to break. At this stage, a small quantity of molten dental 'sticky wax' is drawn in. Acid and sample mix, and as the sticky wax cools, it blocks the tip of the diffusion chamber (see Fig. 3*b*). Molten sticky wax is produced from a tube 1·0 mm in diameter, placed above the sample rack, and containing sticky wax (Ash Model Cement: Amalgamated Dental Trade Distributors Ltd) and a heater wire (see Fig. 2*a*).

As the acid and sample mix, carbon dioxide is evolved from the sample, and since the mercury barrier prevents it from diffusing in that direction, it diffuses through the liquid paraffin and is trapped in the nearest aliquot of $Ba(OH)_2$ as $BaCO_3$. The aliquot of $Ba(OH)_2$ which was first drawn in has cleaned the tube, and the middle one remains unaltered and uncontaminated, so that it indicates the freezing point with no addition of carbon dioxide.

The microdiffusion chamber can now be detached from the syringe in one of two ways. Normally, the paraffin wax holding the chamber in the stainless-steel tube is melted, using the heater wire, so as to detach the chamber from the tip of the syringe. The syringe can be withdrawn using the coarse control of the micromanipulator. Alternatively, if more control is needed in the breaking of the seal (when smaller volumes are used), the syringe tip can be withdrawn slowly using the fine control of the micromanipulator, while at the same time liquid paraffin is injected into the chamber using the micrometer. In this way pressure change within the chamber can be kept to a minimum. When the seal is completely broken, the syringe is removed as before.

When the seal has been broken, the level of liquid paraffin in the bath is lowered, and the chamber can be removed from the stainless steel tube by operating the heater wire and sliding the chamber out into the bath above the liquid paraffin. This whole sequence of operations, although lengthy to describe, takes only about 5 min to perform.

Table 1. *Measurements on bicarbonate solutions (2 nl), with a sample: $Ba(OH)_2$ ratio of 2 : 1*

Concentration (mM/l)	Mean observed change in freezing-point of $Ba(OH)_2$ (°C)	Mean concentrations as determined (mM/l) ± S.D.	No. of observations
0·00	0·000	0·0 ± 0·3	10
5·00	0·041	5·0 ± 0·4	10
10·00	0·080	9·8 ± 0·8	10
20·00	0·168	20·4 ± 1·2	10
30·00	0·240	29·2 ± 0·7	10
20·00 with 600 mM/l NaCl	0·173	21·1 ± 1·0	10

The calibration curve from which changes in freezing point have been converted to mM/l HCO_3^- is the straight line of best fit passing through the origin.

The $Ba(OH)_2$ is now ready for freezing-point determination. The fine portion of the diffusion chamber is protected by a glass tube approximately 1 mm in diameter containing 'heavy' liquid paraffin (S.G. 0·870–0·890) which is sealed in place using dental sticky wax. Most of the wide portion of the chamber is then broken off, and the remainder is frozen in dry ice and alcohol, and placed in the freezing-point apparatus of Ramsay & Brown (1955). The freezing point of the middle aliquot of $Ba(OH)_2$ is determined first, and then that of the aliquot which has received the carbon dioxide.

The calibration curve is a straight line and can be prepared from the initial freezing point of unaltered $Ba(OH)_2$ and from the freezing point of $Ba(OH)_2$ exposed to 30 mM/l HCO_3^-. Any freezing points which are higher than approx. −0·050 °C should be regarded with suspicion, as the reading of freezing point becomes difficult and somewhat inaccurate above this temperature. Readings below −0·395 °C indicate that the $Ba(OH)_2$ is too concentrated, since the temperature at the eutectic point is −0·400 °C (see Little & Ruston, 1970).

ACCURACY OF THE METHOD

(1) *The degree to which the reaction has proceeded, and contamination from the glass*

Because of the small volumes and distances involved, the reaction proceeds much faster than in the method of Little & Ruston (1970). It has not been possible to plot a time-course for the present method because the reaction is always over by the time that the freezing point is measured, i.e. approx. 15 min. Measurements of the freezing point taken over 24 h show that it does not change measurably, but significant changes have been found in samples kept for 3 days.

(2) *The measurement of volumes and contamination of one liquid with another*

The measurement of volumes is accurate to better than 5% but even this could account for a final error of 1·5 mM/l in 30 mM/l HCO_3^-. In fact the standard deviation found during determinations on 30 mM/l HCO_3^- is less than 5% (Table 1).

Another possible source of error during the process of drawing up liquids into the microdiffusion chamber could be provided by any liquid which remains attached to the

Table 2. *Measurements on bicarbonate solutions*

Concentration (mM/l)	Volume (nl)	Sample: $Ba(OH)_2$ ratio	Mean observed change in freezing point of $Ba(OH)_2$ (°C)	S.D. of observations (mM/l)	No. of observations
10·00	6·0	6:1	0·199	±0·5	10
30·00	0·8	2:1	0·266	±1·6	10
30·00	0·4	2:1	0·240	±2·7	10

wall of the chamber after it has supposedly been drawn farther up. Such liquid might then contaminate subsequent liquids passing up the chamber. Siliconing eliminates this problem completely.

(3) *Diffusion of water vapour*

In the method of Little & Ruston (1970), water vapour could diffuse between the sample and the $Ba(OH)_2$, and this set a limit to the minimum volume of the sample. In the present method, the liquid paraffin effectively prevents significant diffusion of water vapour as is shown in the results in Table 1. The readings obtained for a solution of 20 mM/l $NaHCO_3$ and for a similar solution containing 600 mM/l NaCl were not significantly different.

(4) *Stoichiometry*

The freezing point of 79 mM/l $Ba(OH)_2$ was found to be −0·365 °C. From this figure one might expect to be able to calculate the change in freezing point produced by reaction with a known quantity of CO_2. Thus the change for 30 mM/l HCO_3^- in Table 1, and all the changes in Table 2, ought to be 0·277 °C. There are significant differences between the means of some of the observed changes, and all these means are smaller than the calculated change. No reason for these differences has been established, but it follows that calibrations must be carried out using the same conditions as are used for making measurements.

(5) *The overall accuracy*

Table 1 shows the figures obtained using standard solutions of bicarbonate. 0–30 mM/l HCO_3^- can be determined with a standard deviation of approximately 1 mM/l.

LIMITATIONS AND POSSIBLE EXTENSIONS OF THE METHOD

(1) *Measurement of concentrations of HCO_3^- greater than 30 mM/l*

These must be determined by reducing the volume of the sample relative to that of the $Ba(OH)_2$. If, for instance, the sample volume is made the same as that of the $Ba(OH)_2$, the range of measurement is increased to 0–60 mM/l HCO_3^-. This, however, will increase the standard deviation of the measurements.

(2) *Increased accuracy in samples containing less than 30 mM/l HCO_3^-*

When the samples contain considerably less than 30 mM/l HCO_3^-, the accuracy of measurement can be increased by increasing the sample:$Ba(OH)_2$ ratio, and by

standardizing against, say, 10 mM/l HCO_3^-. A number of replicates were made on 10 mM/l HCO_3^- using 6 nl of sample and 1 nl of $Ba(OH)_2$, and the results are given in Table 2.

(3) *The minimum size of samples*

An attempt to reduce the size of samples has been made using a more sensitive micrometer (barrel diameter 3·8 cm, obtainable from Moore & Wright Ltd.). The increasing variation in the results obtained with volumes of less than 1 nl (Table 2) is probably due both to the inaccurate reading of the micrometer, and to variations in the shape of the meniscus at the tip of the microdiffusion chamber: since this is kept to a diameter of about 30 μm, the columns of liquid are much shorter and wider than in larger samples. It is not possible to reduce the diameter of this tip below about 20 μm because below this a pressure of 1 atm is not sufficient to force mercury into the capillary. Unless some liquid which is impermeable to carbon dioxide, and which has a surface tension nearer to that of water, can be found to replace mercury, this seems to place a limit on the minimum size of samples.

PRECAUTIONS

(1) The system for measuring volume ratios is very sensitive to sudden changes of temperature. Under normal conditions temperature effects are insignificant, but the apparatus must not be used in direct sunlight.

(2) After a time it may be found that the menisci tend to break when samples are drawn up into the microdiffusion chamber. This indicates that the liquid paraffin is dirty and must be changed.

(3) The syringe must not contain any air bubbles. If air bubbles are allowed to enter, or if air is trapped in the silicone rubber seal, it will probably not be possible to draw liquids into the microdiffusion chamber.

(4) When liquids are drawn into the microdiffusion chamber, they may continue to move when the micrometer is no longer being moved. This indicates either that the seal is not complete, or that the chamber is not held firmly in place by the paraffin wax.

I wish especially to thank Gary Ruston for his help and advice; without his assistance the method would probably not have been conceived, and would certainly not have been completed. I am also indebted to Mr E. Cox and Mr N. Ablett for much technical assistance. This account of the method has been much improved following advice from Dr G. M. Jarman and Dr A. E. Dorey, to whom I am most grateful.

REFERENCES

CONWAY, E. J. (1962). *Microdiffusion Analysis and Volumetric Error*. London: Crosby and Lockwood.

LITTLE, C. & RUSTON, G. (1970). A method for determining total carbon dioxide in small volumes of liquid. *J. exp. Biol.* **52**, 395–400.

RAMSAY, J. A. & BROWN, R. H. J. (1955). Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. scient. Instrum.* **32**, 372–5.