MICRODIFFUSION ANALYSIS AND VOLUMETRIC ERROR

BY

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CHAPTER I

INTRODUCTORY

In the development of modern biological research large numbers of observations become increasingly necessary in determining the questions at issue. It becomes essential to have analytical methods of a micro kind in which, without sacrifice of accuracy with respect to macro procedure, the labour and time expended with the single analysis are reduced to a minimum.

In the search for such a method, primarily for ammonia and urea, the microdiffusion principle of analysis was elaborated here and a special diffusion apparatus and burette designed to give it effect. It was subsequently found applicable to a variety of micro determinations by the author and other workers.

The method in general would appear to be the simplest possible consistent with the maximum attainable accuracy in the handling of micro volumes. Distillation and aeration are eliminated, the passage of ammonia or other substance taking place by diffusion from one chamber, in which it exerts a certain tension, into an absorbing fluid in another chamber in which its tension is reduced to zero. With a knowledge of the diffusion conditions controlling this passage and used in the design of the standard microdiffusion apparatus or unit, full absorption times with many substances run from about half an hour to two hours.

With serial determinations the time expended on each determination need be only a few minutes; one of the special advantages of the method being the ease with which large numbers of accurate data can be assembled.

With the standard unit, the accuracy of ammonia and consequently, of urea and other determinations, is limited only by the accuracy of delivering and titrating fluid volumes of the order of 1 ml. With the development of the titration principles described in the text, this accuracy can be brought to any desirable level in practice, so that with comparative ease the percentage error need

not exceed that for ordinary macro titrations on the 25 ml. level.

The following is a list of the determinations developed so far in which the microdiffusion procedure has been found of value :

(1) Ammonia, including special application to normal blood ammonia determinations.
(2) Total nitrogen.
(3) Urea, with applications to renal function tests.
(4) Adenosinetriphosphoric acid.
(5) Adenylic acid.
(6) Adenosine.
(7) Amines.
(8) Amides.
(9) Glutamine.
(10) Histaminase.
(11) D-amino oxidase.
(12) Nitrate.
(13) Acetaldehyde.
(14) Threonine
(15) Lactic acid (revised).
(16) Formaldehyde.
(17) Formaldehydogenic steroids.
(18) Glycine.
(19) Ethanol.
(20) Urethane.
(21) Methanol.
(22) Isopropanol.
(23) Phenol.
(24) Cyanide.
(25) Azide.
(26) Sulphide.
(27) Chloride.
(28) Bromide.
(29) Iodide.
(30) Halogen mixtures.
(31) Halogenated hydrocarbons.
(32) Chloroform.
(33) Acetic acid and volatile fatty acids.
(34) Acetylcholine esterase.
(35) Carbon dioxide—with applications to determination of (*a*) blood bicarbonate or ' alkali reserve ', (*b*) oxidation rates of organic substance.
(36) Glucose.
(37) Carbon monoxide—with application to determination of oxygen capacity of blood.
(38) Separation of crystals and gums.
(39) Total molecular concentration in 3–4 milligrams of fluid.

Many of these methods are now in considerable use in other laboratories, and as mentioned in the preface several have been developed elsewhere.

With the standard ' unit ' a special burette was developed, the use of which may be extended to general micro titrations (e.g. its use for arsenic determinations in biological material by Levvy, 1943, and by Ramsay, 1944, in the determination of iron).

For the microdiffusion technique evolved it may now be claimed that it represents a new and special section of micro technique with a wide range of application and the purpose of the present volume is to give an account of the methods which have been tested in practice.

The general error of volumetric procedure. During the development of this technique the more general question of the error involved in volumetric procedure was studied firstly in order to compare the error of the new technique with those already established. Certain principles, however, were in this way discovered of practical help in assigning standards of attainable accuracies, and likely to prove useful in micro work.

To anyone with the laboratory experience it will seem that there are few more practical questions of micro technique than the comparative accuracies in the use of glass ware in volumetric procedure and titration. The constant error of general titrimetric procedure has no doubt been expounded by Bjerrum (1914) and others, and the question of exact calibration long considered. The variable error, however, has not hitherto been equally appreciated, and for the biological worker with his comparative series it is the more important ; not only this, but a correct understanding of the variable error affects in turn the question of calibration, and forms much of the rationale of micro-volumetric procedure. This latter turns on an equation representing the addition of the variable glass and the chemical errors, which serves also as a means whereby, for given quantities, we can choose ideal volumes.

In the study of the variable error the exact influence of certain factors on the constant error was also discovered, which may be of interest to laboratory workers in general, applying a volumetric technique.

Note on the description of micro-concentrations. As is well known the description of the concentrations of standard solutions is generally made in terms of normality or molarity (with the symbols N or M associated with the descriptive number). The immediate results of analysis, however, are more usually stated in terms of the absolute units of mass and volume, such as grams per ml., per 100 ml. or per litre.

Macro-concentrations may in this way be considered as those most conveniently expressed as grams per 100 ml. or per cent., and such description may be retained no matter how low the quantity analysed provided the relation of quantity to volume remains on the same level, though for the actual designation of the minute quantities or volumes themselves we have the milli-

gram (mg.), and the microgram (μg) also termed the 'gamma' (γ or 0·001 mg.), and the lambda (λ or 0·001 ml.—introduced by Kirk).

Micro-concentrations may be expressed as milli-equivalents or as millimols per litre, but analytical results are more immediately given as milligrams per litre (or per cent.), parts per million, or μg (or γ) per ml.

These three units of concentration are alike and the term 'gammil' as the equivalent of the three was suggested by the author in the previous edition as a convenience in the laboratory. In relation to more accepted conventions this might be better termed 'microgrammil' shortened conveniently to 'micril'.

The distinction between the micro-quantity (or volume) and the micro-concentration is in any case one worth drawing, because of its significance in relation to the theory of titration and the variable chemical error (as expounded in Part III of the present volume). From a consideration of the latter the analytical practice with small quantities should be to avoid or raise the micro-concentration by scaling down on the micro-volume. There is a 'micril' level, however, above which there is little or no practical value in increasing the concentration (taking certain standard precautions of acidimetric micro-titration), and this for ammonia is approximately 10 'micrils', though at 1 'micril' the error need not exceed 2%.

For colorimetry or spectrophotometry of the ordinary kind 0·3 'micril' is the order of limit for accurate working (within 2%). This concentration of ammonia nitrogen after Nesslerisation and viewed through a stratum depth of 50 mm. gives an extinction value (E) of approximately 0·3, with filter *S* 43 of the Pulfrich photometer. An extinction of 0·3 is towards the lower end of the extinction scale for accurate observation.

In comparing such 'micril' limits, the relation of the molecular weights must of course be borne in mind when these diverge greatly.

Scale and accuracy of the microdiffusion methods described. Using the standard unit or absorption apparatus, accuracies of the order of 0·5% are easily obtainable for ammonia, urea, chloride, bromide and carbon dioxide at the 20, 50, 250, 500 and

500 ' micril ' level respectively. Accuracies of the order of 1–2% can be obtained with one-tenth these amounts, and of 5% with as low as 0·4 ' micril ' of ammonia and a few ' micrils ' of chloride and bromide.

With 1 ml. volumes used in the standard unit, the ' micril ' represents the ' microgram ' as the quantity analysed. By scaling down the dimensions of the apparatus and the volumes used much lower quantities could be dealt with having the same concentrations and with the same accuracies. It is in short the ' micril ' level (i.e. the concentration) that sets the possible accuracy and not the quantity or volume considered separately.

While such suggestions as to terminology are made to draw attention to the significance of *concentrations* as distinct from *quantities* in micro or ultra micro work, what is here termed the ' micril ' will be represented in the usual symbols of μg/ml.

The present volume is divided into three parts. Part I deals with the apparatus and principles used in microdiffusion analysis. Part II gives an account of the methods for laboratory usage. In Part III is expounded a new treatment of volumetric error with particular reference to micro-quantities and the rationale of micro-volumetric technique.

In an Appendix to the volume an account is given of the best as well as the easiest method of applying blood and urine urea determinations to obtain a measure of renal function. This is expressed as a urea *normality ratio* which is very similar to but not quite the same as the ' clearance test ' of Van Slyke.

The student desiring a rapid account of the essential microdiffusion technique will find this in Chapters II, V and the first part of Chapter VI, describing the horizontal burette ; he will find in Part II a detailed practical account of the various methods.

PART I

APPARATUS AND PRINCIPLES USED IN MICRODIFFUSION ANALYSIS

CHAPTER II

A STANDARD MICRODIFFUSION APPARATUS OR UNIT

THE standard microdiffusion apparatus or 'unit' consists of a pyrex glass container resembling a small Petri dish with thick glass walls, within which a second chamber is formed by a circular wall of glass arising from the floor. The wall of the inner chamber is about half the height of the outer.

The apparatus is covered during determinations by a square glass lid on which is smeared a small amount of fixative, such as vaselin or a mixture of vaselin and paraffin wax, and in this way makes an air-tight compartment.

Figs. 1 and 1*a* illustrate the standard pyrex unit (No. 1) at present available from Messrs. A. Gallenkamp, Finsbury Square, London.

Principle of use. The general principle of the microdiffusion methods consists in the absorption by simple gaseous diffusion of the volatile substance from the outer chamber where it exerts a certain tension into the inner chamber where its tension is zero on the surface of the absorbing fluid. Though this on a first consideration would seem a very slow process, yet from the manner of construction of the 'unit' the rate is comparatively fast, and determinations may be carried out in about 10 minutes to two hours for a number of different substances. Where large numbers are being carried out the procedure can be so arranged that the average working time for the single determination is only a few minutes. Ammonia analyses by simple gaseous diffusion were carried out by Schlösing and by Shaffer (as given in Neuberg's *Der Harn*,

1911) some fifty years ago. Separate vessels were used, both being covered by a large bell jar, and upwards of 48 hours with the Schlösing method and 3 to 4 days with the Shaffer modification were required for the absorption, which even then gave somewhat

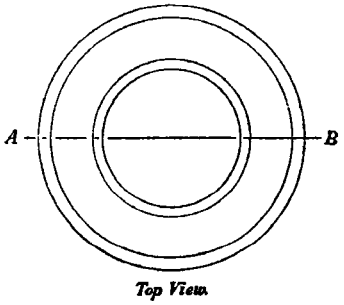

FIG. 1. Plan of standard unit, Conway and Byrne.
(From *Biochemical Journal*, **27**, 420, 1933.)

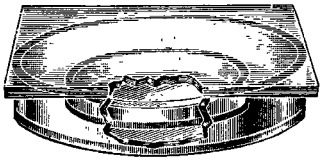

FIG. 1*a*. Standard unit (as supplied by Messrs. A. Gallenkamp & Co., Finsbury Square, London).

low results. Diffusion in this form was of little analytical value, the controlling factors were not understood and the method was abandoned. The recognition that the process could rapidly reach completion as a microdiffusion led to the introduction of this as a new principle of analysis the general application to volatile substances being rationally elaborated. Full absorptions could be obtained in as short a time as 10 minutes with the standard

' unit ' using 0·2 ml. ammonium sulphate solution and shorter times may be obtained with small drops on enclosed surfaces—of paraffin or other material.

Procedure as exemplified by ammonia determinations. 1 ml. of standard acid is introduced into the central chamber. This may be introduced either from an Ostwald or from a straight tube pipette, with a delivery time not less than about five seconds, or what is often convenient for routine work, from a 2 c.c. standard burette of the Bang type (1920) (*vide* Fig. 10) with comparatively large reservoir. Into the outer chamber is run a measured volume of the fluid to be investigated—usually ranging from about 0·1 to 2·0 c.c. For this also an Ostwald pipette or straight tube pipette may be used. The lid is suitably smeared with the fixative (vaselin, vaselin plus paraffin or a mixture of paraffins) and placed in position on the ' unit '. The apparatus is then slightly tilted—most conveniently by resting one edge on a spare lid. The tilting collects the fluid in the outer chamber away from where the alkali is introduced. The lid is horizontally displaced so that a small opening appears into the outer chamber sufficient to allow the introduction of the tip of a pipette containing a measured quantity of reagent liberating the volatile substance, which for ammonia determinations is usually one ml. of saturated potassium carbonate.

This is quickly run in *without blowing* and—as its delivery does not require to be very accurate—it is most conveniently run in from a simple pipette made from a piece of glass tubing somewhat drawn out and with a mark at the required volume. The lid is at once replaced and the mixture in the outer chamber rotated about 10–20 times. This is easily done by gripping the unit between thumb and fingers and tilting slightly from side to side. It will be found that—at least with very little experience—there is no danger of spilling the contents of the chambers, when the volumes described in the determinations are used.

After a suitable time for the absorption the lid is detached and the contents of the inner chamber titrated. For other determinations it may be necessary to remove it for colorimetric investigation.

Variations in the above procedure occur in the different determinations described in text, particularly in the manner of intro-

ducing the reagents into the outer chamber. Instead of one such, several may be required, the last reagent liberating the volatile substance.

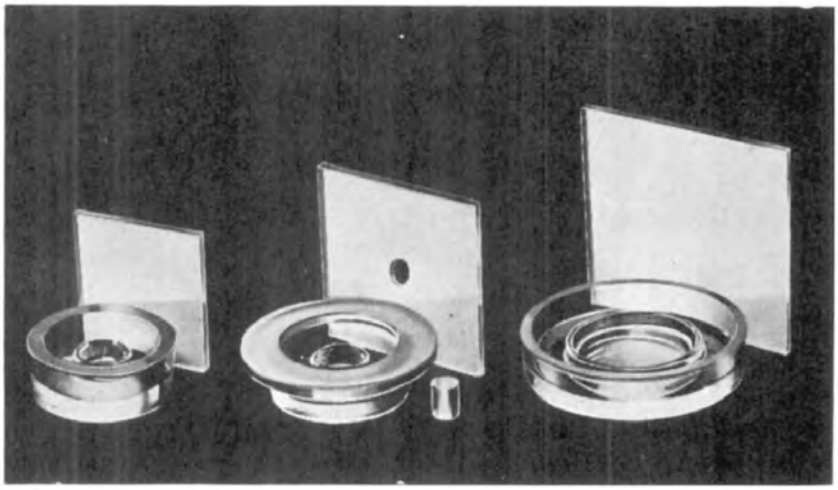

FIG. 2. Standard ' unit ' with ' units ' No. 2A and 2 (as supplied by Messrs. A. Gallenkamp, Finsbury Square, London).

In developing the method for blood glucose as later described, a smaller ' unit ' was designed than the No. 1. This ' unit '—No. 2—could be more easily secured from any leakage inwards of atmospheric CO_2, and much lower ranges of CO_2 could likewise be dealt with. A modification of this No. 2 ' unit ' was devised later—No. 2A—which had a much wider rim and a drilled hole through the lid, made air-tight by a small cylinder of glass treated with fixative. This ' unit ' may be preferred for fine carbon dioxide work, and is very efficient, when determining, say, the carbon dioxide content of 0·1 ml. blood, which may be done with a high degree of accuracy. These modifications of the original ' unit ' are shown in Fig. 2 and 2*a*. The No. 2 and 2A types have the same dimensions, except for the wide rim in 2A.

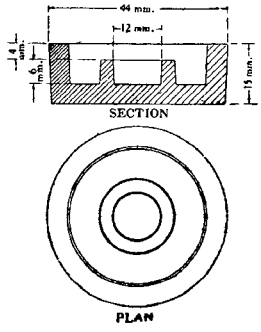

FIG. 2*a*. Plan of ' unit ' No. 2 (from the *Biochemical Journal*, **36**, 655).

These smaller ' units ' may be used, of course, for any of the

other microdiffusion determinations as well as No. 1, the volumes being suitably scaled down, and their usage allows of determinations at lower micro levels. For the less soluble gases such as carbon dioxide, the halogens, etc., the times with the small 'units' are scarcely altered. For the highly soluble gases or vapours such as ammonia, amines, etc., the times will be very dependent on the fluid volumes in the outer chamber and much influenced also by the reduced emission and absorbing surfaces. The diagram for the times required for ammonia absorptions is given later. Throughout the present volume when the microdiffusion 'unit' is mentioned, the No. 1 type is to be understood unless the No. 2 and No. 2A 'units' are definitely stated.

Cleaning the 'units'. The success of the methods—particularly for the minute ammonia determinations—very largely depends on a satisfactory cleaning. This cleaning is quite easy, but requires the carrying out of a definite routine.

The following routine of cleaning is found suitable for all determinations.

1. The 'units' after use are well washed out under the cold-water and then under the hot-water tap.

2. They are then cleaned of any grease with a test-tube brush or pledget of cotton wool, using a little detergent, and subsequently well rinsed under the tap.

3. After this they are filled with dilute sulphuric acid ($N/200$ roughly) containing indicator. This acid may be made up in bulk in a large distilled water bottle and filled into each 'unit' from the outlet of the bottle. They are allowed to soak in the acid for fifteen minutes.

4. They are now well rinsed under the cold-water tap and finally with distilled water. The drops are shaken out (the interior of the apparatus intended for immediate use should never be dried with a cloth) and the 'units' are left to dry. For quick drying it will often be found very convenient to range them along the top of a radiator, resting upside down along the ridges. They also dry comparatively quickly after shaking out the drops well by mere exposure to the air.

Important points for correct usage in the general procedure. (*a*) The 'units' when used for determinations where the final

measurement is acidimetric should be perfectly clean in an acid-alkali sense and a routine similar to that given must be followed in cleaning.

(*b*) The surface of the inner chamber must be fully covered. With the use of a detergent in the cleaning process, as mentioned above, 1 ml. fluid will very easily cover the surface. The

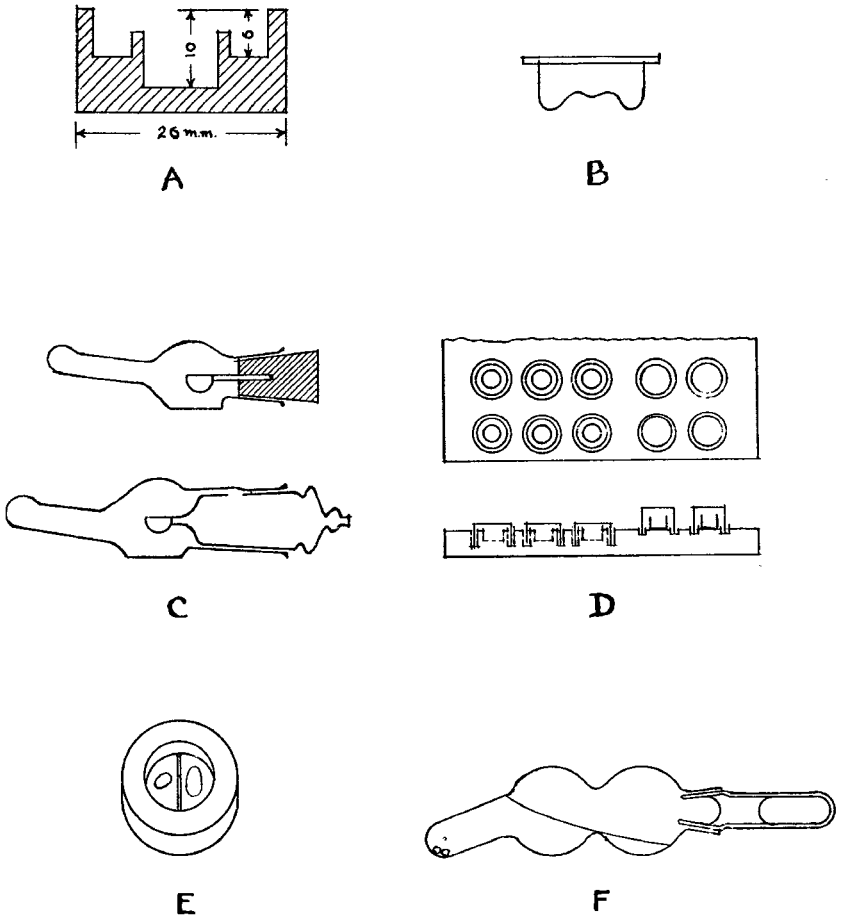

FIG. 3. Other forms of microdiffusion apparatus, for minute amounts of ammonia determination.
A—from Borsook and Dubnoff (1939), see text, page 140.
B—from Gibbs and Kirk (1934); 32 mm. outside diam.
C—from Tompkins and Kirk (1942), see text, page 144.
D—from Kinsey and Robison (1946), upper, top view; lower, side view.
E—from Conway and O'Malley (1943); see text, page 68.
F—from Needham and Boell (1939), see text, page 143.

introduction of soap means at the same time the introduction of alkali, and hence the need for soaking in acid for some time.

(*c*) The fluid volume in the outer chamber should not exceed that described in the various determinations. (The fluid volume in this chamber, as shown in the next chapter, has in general a very marked effect on the absorption rate, the time for a 99·5 clearance being proportional to this volume.)

Other forms of microdiffusion apparatus. Since the description of the standard unit (Fig. 1) was published, various forms of microdiffusion apparatus, applying the new principle have been elaborated and are shown in Figs. 3 and 3a. Recently Öbrink has

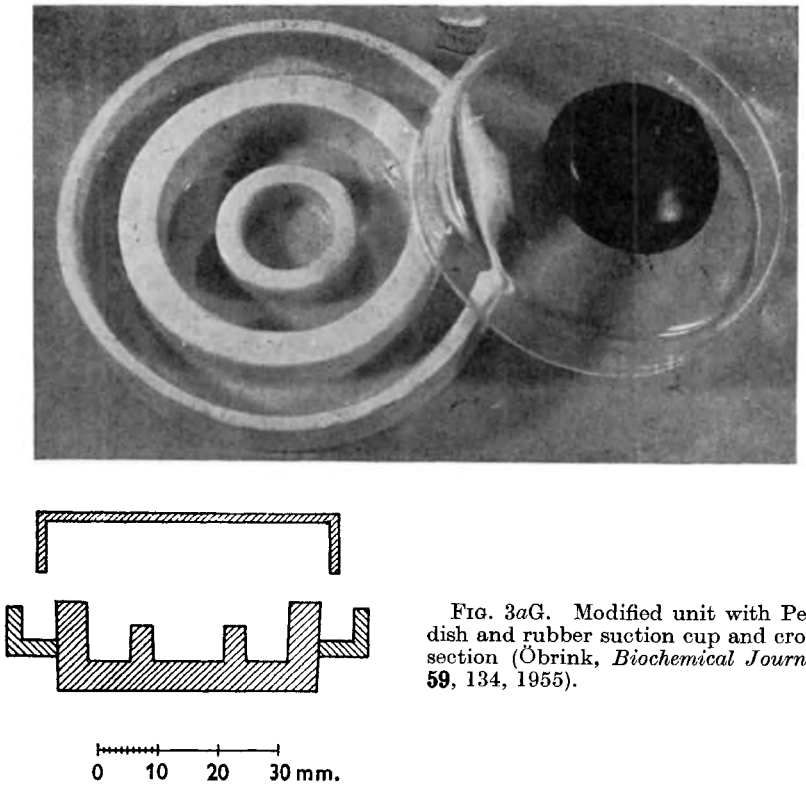

FIG. 3*a*G. Modified unit with Petri dish and rubber suction cup and cross-section (Öbrink, *Biochemical Journal*, **59**, 134, 1955).

introduced a modification of the No. 2 unit, as represented in Fig. 3*a*G. In this no fixative is required as the lid fits into a circular groove containing some of the fluid used to liberate the volatile substance.

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