

VIII. A NEW TYPE OF MICRORESPIROMETER

BY N. G. HEATLEY, I. BERENBLUM¹ AND E. CHAIN

From the Sir William Dunn School of Pathology, University of Oxford

(Received 29 October 1938)

INTRODUCTION

MANOMETRIC methods are among the most useful and widely applied techniques in the field of biochemistry. The respirometers of Barcroft or Warburg are most commonly used, and the theory and practical manipulation of these and other types has been summarized by Dixon [1934].

These instruments are capable of measuring gas exchanges of not less than about 30 μ l./hr., so that 5–50 mg. of tissue are normally used. However, there is a need for methods of measuring very much smaller gas changes, and several instruments capable of measuring O₂ uptakes of 1 μ l./hr. or less have been described during the last decade. Stefanelli [1937] has described a miniature Barcroft-Warburg type of instrument. In others the respiration chamber is connected to a horizontal length of capillary tubing, the movements of a drop of fluid in the latter indicating the changes in volume [Fenn, 1927; 1928; Jackson *et al.* 1930; Schmitt, 1932; 1936; Gerard & Hartline, 1933; Victor, 1935; Waddington *et al.* 1936 and Duryee, 1936]. An entirely different method described by Linderstrøm-Lang [1937] and extended by Needham & Boell [1939], is based on the principle of the Cartesian Diver.

While each of these types possesses certain advantages, it appears that none of them in their present form can carry out on a micro-scale all the kinds of measurement which can be done on a larger scale in the Barcroft or Warburg apparatus.

The apparatus described below employs a principle which has so far not been used in a respirometer. As regards sensitivity, it is capable of measuring uptakes and outputs of about 1 μ l./hr. with an accuracy at least as great as that of the Warburg apparatus; it is therefore less sensitive than some of the instruments described above. However, unlike these others, it possesses all the potentialities of the Warburg apparatus. It can be assembled at one temperature and used at a widely different one; it can be filled with any gas mixture, and two or more separate fluids contained in it can be mixed at any desired time during an experiment. The fact that it can be used under sterile conditions is an additional advantage.

PRINCIPLE

The respiring material rests in a completely closed chamber, one wall of which consists of a thin sheet of mica. Two plane mirrors are attached to the latter, and when the volume of the gas in the chamber changes, the mica will bulge (either inwards or outwards) with the result that the mirrors will tilt in opposite directions. By applying a positive or negative pressure to the *outside* of the mica membrane, the latter may be brought back to its original position, a simple optical system being used to detect when the mirrors are in the same plane. Then, knowing the volume of the gas space and the change in pressure in it, the amount of gas absorbed or given out may be calculated.

¹ Beit Memorial Research Fellow.

CONSTRUCTION

The component parts of the apparatus may be described under five headings:

- (1) The chamber containing the tissue.
- (2) The metal case containing the chamber.
- (3) The optical system.
- (4) Thermostat, with heaters, thermoregulator etc.
- (5) The pressure-regulating mechanism and manometer, and arrangements for filling the chambers with any desired gas mixture.

(1) *The respiration chamber.* This consists of two plane slabs of glass 25 mm. square, one 3–4 mm. thick, and the other about half this thickness. The thicker of the two—which will be referred to in the rest of this paper as the “cup”—has a cavity of 40 to 80 μ l. capacity in one side, in which lies the material being studied. The inside of the cavity, which may be of any shape, is lined with wax, so that two or more separate drops of fluid may be placed in the different arms of the cavity without mixing. (Fig. 1a shows a “cup” capable of holding

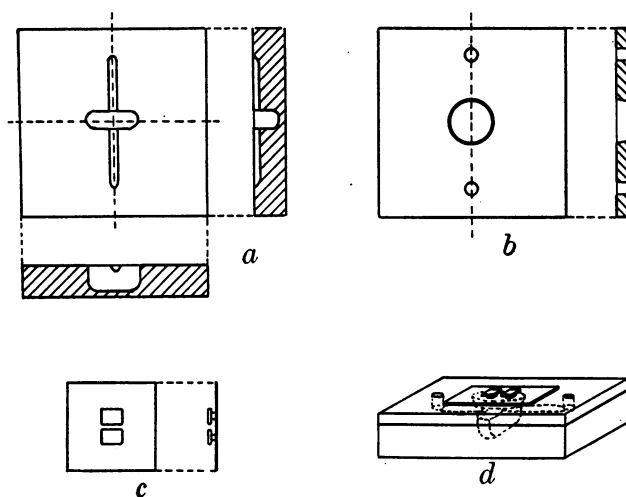


Fig. 1. Components of respiration chamber. *a*, “cup”. *b*, “plate”. *c*, mica membrane with mirrors attached. *d*, complete assembly.

two separate drops of fluid; Plate I A shows “cups” for two, three and four drops of fluid.) The “cup” also has two grooves cut in the plane surface, which lead to the cavity, as shown in Fig. 1a.

The thinner slab—referred to in this paper as the “plate”—has a central hole about 7 mm. in diameter, which is covered on one side by the mica membrane. The “plate” (Fig. 1b) also has two smaller holes, 18 mm. between centres, which coincide with the ends of the grooves in the “cup” when the two are in apposition. Fig. 1d shows a complete assembly of “cup”, “plate” and mica ready to be placed in the brass case.

The respiration chamber may be filled with any desired gas mixture by leading the latter through one hole in the “plate”. (The way in which this is done will be understood after reading the following section.) The gas enters the respiration chamber through one groove and leaves it by the other, and after a sufficient stream of gas has been passed, the chamber can be completely closed by rotating the “cup” relative to the “plate”.

The "cup" cavities can be made by drilling a number of holes in the glass slab with a china-riveter's diamond drill [Heatley, 1938], and then enlarging them and running them together by means of a suitably shaped carborundum dental burr; the holes in the "plates" are made with the same tools. Up to the present the "plates" have been made from microscope slides carefully selected for flatness of surface; optically worked glass has been used for the "cups",¹ but good quality plate glass can also be used.

The mica for the membrane should not be thicker than $18\ \mu$, preliminary tests having shown that even very much thinner sheets are apparently quite impervious to gases. A freshly split mica sheet of the required thickness is divided into squares of 12 mm. side, by cutting it between two sheets of paper—preferably squared paper—with scissors. The mirrors are made from no. 3 cover-glasses, which are silvered, or better, aluminized, and cut up into rectangular pieces measuring 2×3 mm. These are stuck to small pieces of cover-glass 1 mm. square, which in turn are fixed to the mica (Fig. 1c); the insertion of the glass stalk makes the component less fragile and more sensitive. It is a considerable help when cutting the cover-glasses with a diamond chip, to lay them on a piece of oiled silk on plate glass. Bakelite varnish no. V-5209/2 has been used for attaching the mirrors to the mica, and though they easily become detached, a more suitable adhesive has not yet been found. (Cements which cannot be autoclaved, or which are affected by water, have not been tried.)

(2) *The metal case.* Considerable latitude is permissible in the design of this component, and the drawings which are appended are of the cases which have actually been used by the authors.

Fig. 2 is self-explanatory. The main body of the case is built of the thick brass disk *A*, to the back of which a thin disk *B*, having a central square hole 25.5×25.5 mm., is permanently fixed by two countersunk screws. A detachable disk *C*, having a central hole about 22 mm. in diameter, has three curved slots through which the three threaded rods *D* on the main body of the apparatus will pass. Projecting from one surface of *C* are eight pins enclosing an imaginary square of 25.5 mm. side. *A* has a central hole *E*, 7 mm. in diameter, which is closed on the outside by a glass window. The brass rod *J* acts as a handle, and by means of a hook soldered to it the case can be suspended from a horizontal bar fixed across the thermostat.

The "cup", "plate" and mica having been assembled into a unit (Fig. 1d), are placed mica downwards on to the main body of the case, which is held horizontally as in Plate IA, *b*, so that the mirrors lie in the central hole *E*, facing the glass window. The rotating back *C* is now placed on top of the glass parts with the eight pins surrounding the "cup", and the plain and spring washers are slipped on to the rods *D*, followed by the nuts *F*, which are tightened. It will now be seen that with the help of the two partially sunk rubber washers *I*, the two outer holes in the glass plate are making gastight contact with the tubes *G* and *G'* of the case, enabling gas to be passed through the respiration chamber. Fig. 3 shows the components of the whole assembly in an expanded diagrammatic form.

The slots in the disk *C* allow it to be rotated through an angle of 60° , and the pins projecting from it will force the cup to rotate with it. On the other hand the "plate" is partially sunk into the square hole in *B* and so will be held stationary. Thus the rotation of the "cup" relative to the "plate", which is required to close the respiration chamber, is carried out with ease

¹ Blanks of optically worked glass suitable for the "cups" may be obtained from Henry Hughes and Son, Ltd., Husun Works, New North Road, Barkingside, Essex.

and accuracy. Plate IA shows a photograph of some of the components already mentioned.

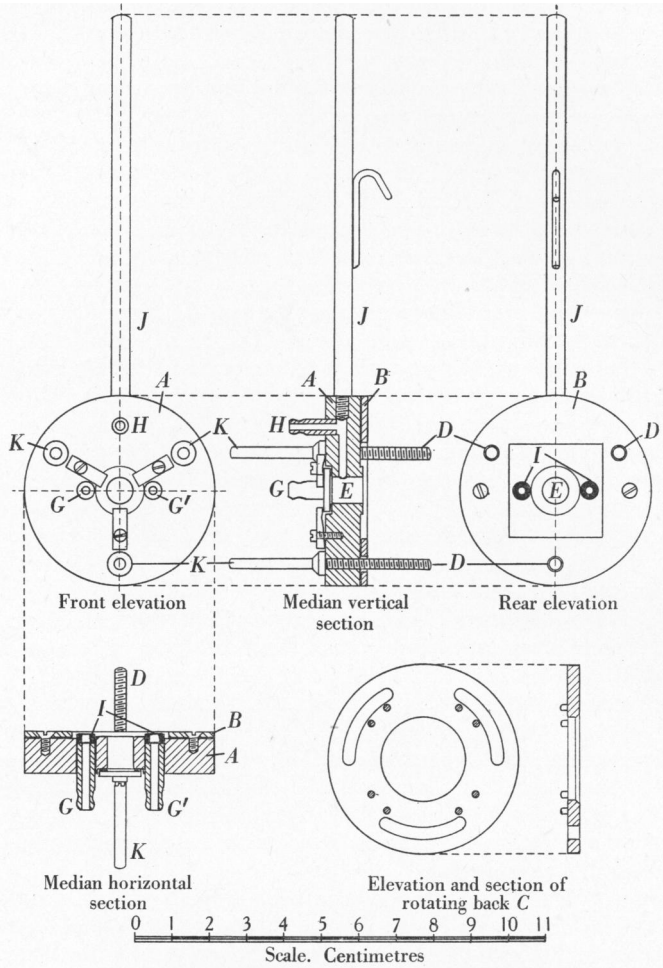


Fig. 2. Diagram showing construction of brass case.

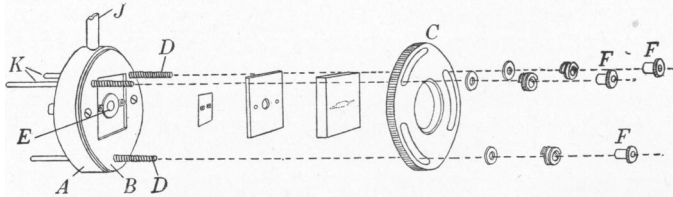


Fig. 3. Diagram showing disposition of components of brass case and respiration chamber.

In Fig. 2 will be seen a third tube, *H*, which leads directly into the space *E* between the glass window and the mica membrane; this tube is attached to the manometer and pressure-regulating apparatus, by which any bulging of the

mica can be corrected. (The bulging is detected by the optical system described in the next section.)

Some simple method for rapidly and accurately aligning the metal case with the optical system (when readings are to be taken) is required. This is done by placing the legs *K* of the case against a glass observation window let into the side of the thermostat, and preventing the case from rotating or sliding in a plane parallel with the window by a fixed V-shaped plate; all that is required now is some means for pressing the case against the window, and one simple mechanism for doing this is shown in plan and elevation in Fig. 4. The observation window is a small yacht decklight which is fixed to the thermostat wall by five bolts, three of which project inside and are used to secure the brass aligning-plate *L*. This has a V-shaped cutaway portion in which rest the legs *K* of the case. A stout pillar *M*, attached to this plate, is linked by the two freely pivoted strips *N* and *N'* to the rod *O*. An extension spring of stainless steel, anchored to suitable positions on the strips *N* and *N'*, ensures that *O* will press firmly against the back of the respirometer case. *O* is conveniently controlled by a knob (above the level of the water in the thermostat) which is rigidly connected to *N* (or *N'*) by the rod *P*. (In Fig. 4 the decklight, is shown in section in the plan and in dotted outline in the elevation.)

(3) *The optical system.* This is shown in its simplest form in Fig. 5*a*. An achromatic converging lens *V* is placed in front of an illuminated slit *S* so that an image of the latter would be formed, say, 70 cm. behind the lens. The mirrors *W* of the mica membrane are placed in the path of the convergent rays, some of which are reflected back to form an image on a ground glass screen *X*. When the mirrors are in parallel planes a single image of the slit will be seen, but as soon as the mica bulges one way or the other, the mirrors will tilt in opposite directions and the single image will split into two.

Even when special care is taken in the mounting of the mirrors on the mica, it will be found that only in a few cases are the planes of the mirrors approxi-

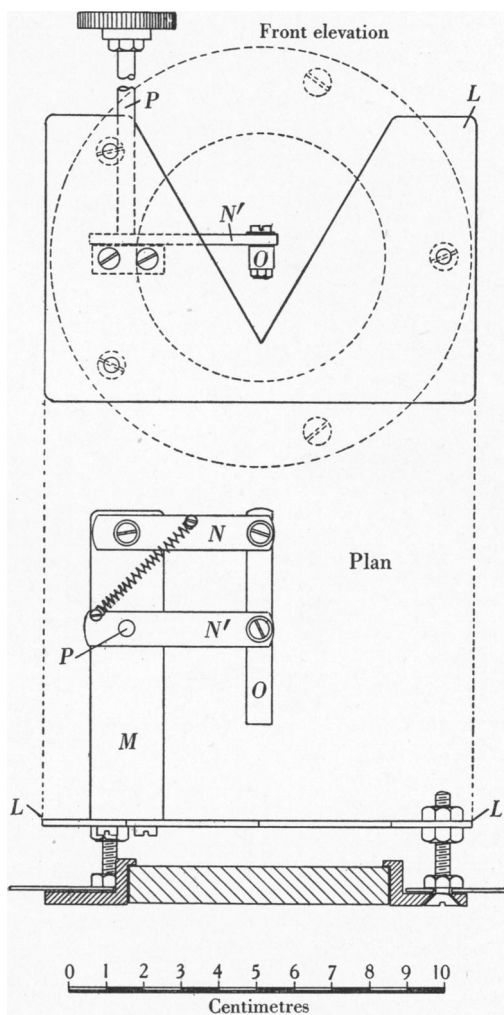


Fig. 4. Mechanism for holding brass case.

mately parallel; in the remaining cases the two images of the slit will be wide apart, and the zero position (i.e. when the two images fuse into one) can only be obtained when the mica is made to bulge. It is obvious that the membrane is most sensitive to deformation when it is flat, the sensitivity falling off rapidly as the bulging becomes greater. To avoid this loss of sensitivity a compensating arrangement of mirrors is provided by which the two images may be made to coincide, even when the pencils of rays from the two mica mirrors are widely

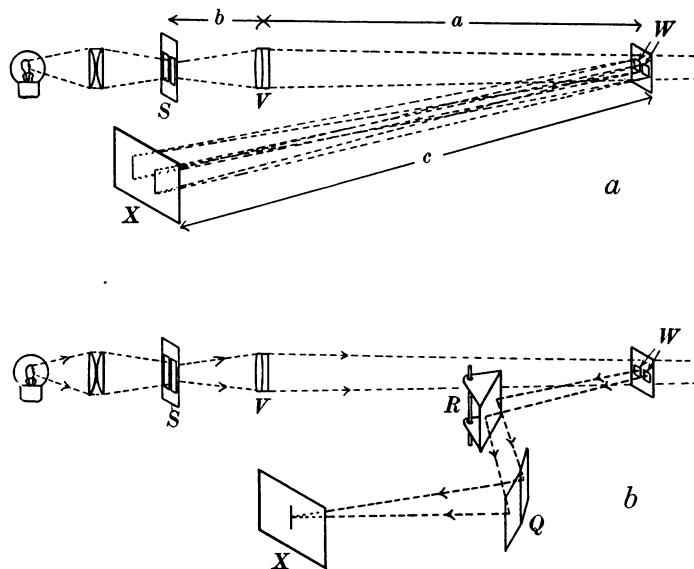


Fig. 5. Optical systems: *a*, in simplest form; *b*, with divided mirror.

divergent. Fig. 5*b* shows the principle of the method employed. Before falling on the ground glass screen, both pencils of rays are reflected from the vertically divided mirror *Q*, one half of which can be rotated about the dividing line. The tilt of the mirrors attached to the mica may be such that both pencils of reflecting rays, although diverging, may fall on the same half of the divided mirror *Q*. This contingency is met by interposing between the mica and *Q* another rotating mirror *R*, which can be so adjusted that one ray will fall on each half of *Q*.

When initially divergent rays are made to form a single image by the use of the divided mirror *Q*, there will be two different pressures which can give a zero reading, according to whether the rays are crossed before impinging on *Q* or not. The correct reading is easily selected, but this point may cause confusion at first. (At the correct reading a small change in pressure will cause the images to move a considerable distance relative to each other—at the other null point the same pressure change will cause only a small displacement of the images.)

The illuminated slit was found to be more efficient than a straight filament lamp.

The relation of the components of the optical system must be a matter of compromise. The distance *c* (Fig. 5*a*) must be great in order to obtain an efficient optical lever; but if it is made too big relative to *a* the area of the mirrors *W* will be small at that position in relation to the cross-sectional area of the converging beam, and much light will be lost. If, however, *b* is large, the lens will collect only a small amount of light from the slit; but if *b* is too small in

relation to $a+c$, the magnification of the slit will be excessive and loss of definition and brilliancy of the ground glass image will be the result.

Some light will be reflected from the window of the brass case, causing a secondary image, and another image may be formed in the same way from the thermostat window. The first unwanted image may be eliminated by having the small window in the case bedded down on a washer of uneven thickness, so that it is no longer perpendicular to the optical axis. The second may be eliminated by inclining the case at, say, 5° to the vertical, either by slightly lengthening the bottom leg K , or preferably by a suitable packing, such as a microscope slide, between it and the thermostat window. The optical system must be tilted a corresponding amount so that its optical axis is still perpendicular to the two mica mirrors W . The thermostat window will now no longer be perpendicular to the optical axis, and any reflexions from it will not interfere.

The exact arrangement of the optical system will depend on the characteristics of the individual components, but Fig. 6 shows a horizontal section through the optical box of the instrument at present in use. An image of the filament of the 36 W. 12 V. car headlamp bulb U is focussed on the vertical slit S by means of the condenser T . The achromatic lens V has an approximate focal length of 9 cm., and throws a beam of light from the slit on to the mirror R . This rotates about a vertical axis and is controlled by the screw Y , backlash being prevented by a spring. The beam of light passes through the thermostat window (shown in section in Fig. 6) and falls on to the two mirrors mounted on the mica membrane, from which two pencils of light are reflected back on to R and thence to the divided mirror Q . When the pencils of rays are initially divergent, Y may be adjusted so that one pencil of rays falls on to each half of the mirror Q ; then by tilting one half of the latter by rotating the graduated drum Z (which is fixed to a threaded rod) the two separate images of the slit may be made to superimpose: Mirror X is merely for the sake of convenience, as it allows the ground glass screen to be at the front, instead of at the back of the apparatus; the screen is situated above the drum Z , and therefore is not shown in Fig. 6. In Plate I B the hood which screens it from stray light while it is being viewed can be seen. A ball bearing hinge such as that described by Baker [1938] is used for the divided mirror Q .

(4) *Thermostat, etc.* The tank at present in use is made of copper and measures 65 cm. long by 28 cm. broad by 25 cm. high. It is heated electrically by three immersion heaters of 250 W. each, and one kettle unit taking either 100 or 700 W. The thermoregulator is of the ordinary toluene-mercury type, the toluene-containing part being thin-walled glass tubing about 1 cm. in diameter. Part of the heating current is controlled by the thermoregulator, and part is fed continuously; the advantage of this arrangement—which is well known, but not used as often as it might be—is that heat is continuously supplied to the bath at a rate which would allow it to cool only very slowly; hence the intermittent heating current need only be small, and fluctuations in temperature caused by thermoregulator lag, etc., will be correspondingly reduced. The thermostat can be warmed from room temperature to 37° in about an hour by employing the 700 W. heater and one of the 250 W. ones. When the bath has warmed up, the heaters are connected as follows:

Intermittent (controlled by relay): 700 W. heater in series with one 250 W. heater.

Continuous: two 250 W. heaters in series.

In a very warm or very cold room another of the many possible heater combinations may be more suitable.

The thermostat is stirred by a three-bladed vane driven by an electric gramophone motor situated under the tank. The stirring is efficient, noiseless and trouble-free, and by having the motor under the tank (private communication from Prof. R. A. Peters) more room is left at the top, and troublesome pulleys are avoided.

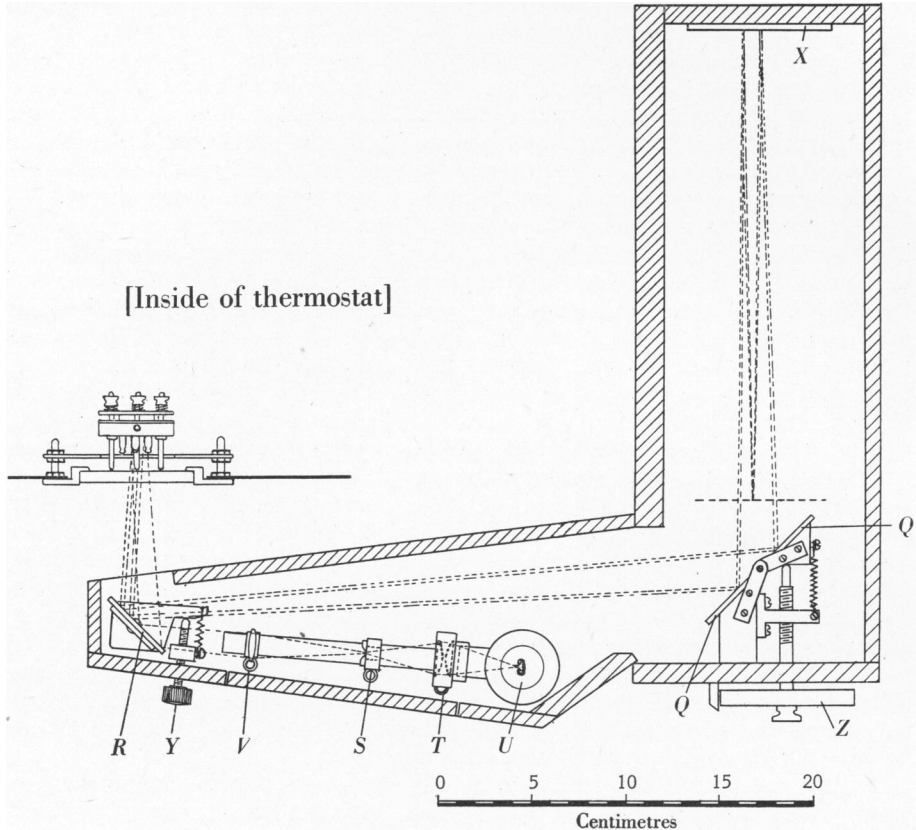


Fig. 6. Horizontal section through optical box.

A transformer giving an output of 3 amp. at 14 V. is housed behind the control panel (Plate IB). This feeds the 36 W. car headlamp bulb used in the optical system, and also a low tension metal rectifier which delivers 2 amp. at 9 V.; a three-way switch enables either of these circuits, but never both, to be connected, whilst the third position of the switch is "off". The current from the rectifier activates the electromagnet by which two drops in the respiration chamber may be mixed (for details of this see below). An intermediate transformer tapping at 4 V. supplies current for working the thermoregulator relay, which is also situated behind the control panel.

(5) *The manometer and pressure-regulating apparatus, etc.* The principle of this is shown in diagrammatic form in Fig. 7. In the actual instrument the two reservoirs, consisting of 3 oz. bottles suspended horizontally, are attached to opposite sides of an endless chain passing round a system of pulleys and sprockets. A control wheel fixed to one of the latter enables the pressure in the system to

be varied (see Plate I B and Fig. 7). Those outlet tubes which are not connected to tube *H* of the brass cases (see Fig. 2), are closed by rubber caps. It is convenient to read the level of fluid in both arms of the manometer, thus avoiding errors through drainage; the manometer scale should be at least 60 cm. long if the instrument is ever to be used for respiratory quotient determinations (to be described in a subsequent paper).

For filling the cups with any desired gas mixture, a main tube having six side tubes is connected to the gas source; a separate bubbler, containing water and immersed in the thermostat, is interposed between this manifold and each respiration vessel, partly to moisten the gas, and partly to enable the rate of passage of gas in each individual case to be checked. It is important that the gas should be as nearly saturated as possible before entering the vessel, as otherwise evaporation of the droplets may occur, with alteration in the volume of the fluid and thus of the vessel constants. It was found that when dry O_2 was passed through the respiration chambers via the bubblers, for 10 min. at 37° at the rate of 5–10 ml./min., only about $0.5 \mu\text{l.}$ of fluid was lost from a drop of $12 \mu\text{l.}$ of distilled water. However, though this would cause a negligible error in the vessel constants, it might have a deleterious effect on delicate cells by making the medium hypertonic. The evaporation can be reduced still further by inserting a plug of damp cotton wool in the rubber tube connecting the case to the bubbler.

A thermobarometer is required, as in the Warburg technique, to compensate for any changes in thermostat temperature or barometric pressure. Since the volume of the vessel used as thermobarometer is immaterial, a simple bulb connected to a manometer of capillary tubing serves the purpose. Apparently no error is introduced by the difference in shape or size of this vessel compared with the respiration chambers, though should temperature regulation not be good, errors would undoubtedly arise.

CALIBRATION AND PROCEDURE

Grease. The respiration chamber is made airtight by films of grease between the component parts. Many different kinds of grease were examined and found to be unsuitable on account of their physical or chemical properties (consistency, absorption of gases etc.). For working at 37° B.D.H. winter grade rubber grease was found to be entirely satisfactory. The exact method of applying the grease is important, and too much or too little may spoil the experiment. It is most suitably applied with the ball of the finger, being patted on, rather than spread or smeared. Of the surface to which the mica is to be fixed, only a small central area need be greased, and before the mica is placed in position the edge of the central hole should be wiped free from excess grease, and this is most easily done with the ball of the clean little finger; if this is omitted, a ridge of grease may be squeezed into the cavity which will prevent the mica from moving perfectly freely.

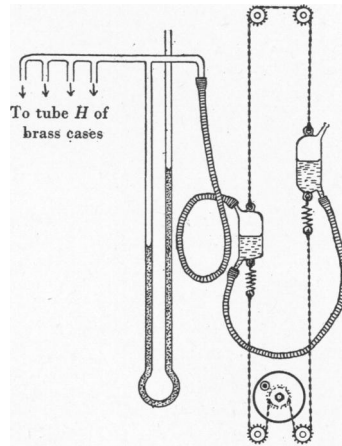


Fig. 7. Diagram showing principle of manometer and pressure regulator.

Effect of concentration of alkali, etc. In all microrespirometers where the gas volume is small, and the surface/volume ratio of the fluids is great, isothermal distillation from one drop of fluid to another may occur; if the different drops have different initial osmotic pressures, they will tend during the course of the experiment, to attain the same osmotic pressure, with resulting changes in the concentration of the solutes in each drop. This can easily be demonstrated by actual measurement of the volumes of the drops before and after the experiment, and at 37° changes of this kind may occur in a relatively short time.

An important example of this effect is seen when strong alkali is used to absorb CO₂. This is illustrated by the results of an experiment in which the O₂ uptake of a suspension of lymphocytes was measured in the presence of (a) 1% and (b) 10% NaOH. The actual values obtained were

(a) In 1% NaOH: 0.25, 0.21, 0.23 μ l. O₂/hr.

(b) In 10% NaOH: 0.14, 0.13, 0.19 μ l. O₂/hr.

It will be seen that the rate of O₂ uptake is markedly lower in the latter case. It seems that lymphocytes are considerably more sensitive to osmotic changes than other tissues, but in any experiment where concentration of medium is to be avoided, strongly hypertonic solutions of alkali, etc., should not be used.

This is a point which has sometimes been overlooked in previous work with microrespirometers, and the results obtained may therefore, in some cases, be erroneous.

Method of mixing drops. One or more of the droplets inside the respiratory chamber may be mixed by dragging a small iron ball from one to the other by means of an electromagnet. Mr Leemans has invented a method of applying a thin coating of glass to small balls of a special magnetic alloy ("platinersatzdraht")¹ but an alternative method of protecting the mixing ball from corrosion is as follows. Steel bearing balls, $\frac{1}{16}$ in. in diameter, are given several coats of Bakelite varnish no. V-5209/2, each coat being polymerized by stoving before applying the next. The balls are then heated to 100° in paraffin wax (m.p. 50°) for some minutes, the surplus wax being removed by rolling the balls on hot filter paper; by this means any flaws in the Bakelite coating are blocked with wax. They are then rolled in the palm of a warm, but clean and dry hand with some well washed kaolin; this is necessary because the paraffin-coated balls are otherwise not readily wetted, and hence are inefficient mixers. As Bakelite is attacked by certain reagents (e.g. KMnO₄), balls prepared by this second method cannot be used in the presence of such substances. Other ways of protecting steel balls from corrosion, such as gold or nickel plating, have been tested and found unreliable.

Cleaning and preparation of the apparatus. At the conclusion of an experiment the mica membrane is dragged off the "plate" and placed on a piece of clean filter paper. The adherent grease is removed from the upper surface by means of a blunt scalpel, and from the under side by moving the mica over the surface of the filter paper. The "plate" and "cup" are then cleaned in hot chromic acid, or better, in sulphuric-nitric acid mixture. They are rinsed under the tap, then in distilled water, and dried in the oven. The "cup" cavities are then lined with paraffin wax (m.p. 70°) by carefully heating them over a small flame and touching minute shavings of wax on to the hot surface; before the "cup" has cooled completely it is rubbed face downwards on a piece of clean silk in order to remove any wax which may have spread from the cavity to the plane surface.

¹ Mr Leemans, who is glassblower at the Clarendon Physics Laboratory, University of Oxford, is prepared to supply these balls.

In preparing for an experiment the "plates" are greased in the manner described above, and the mica squares carrying the mirrors are placed in position on them. The greased "plates" (with mica membrane attached) are best kept on a special stand protected from dust until they are required. As soon as each cup is charged with the material being studied, it is covered with a plate. The flat raised rim round the central hole E (Fig. 2) in a brass case is carefully wiped and greased, the inner edge being wiped free from excess grease by the ball of the little finger. The "cup"- "plate"-mica assembly is placed in position, the rotating back is added, followed by the plain and spring washers and the knurled nuts which are screwed up evenly all round. At this stage the numbers of the "cup" and "plate" are noted; to reduce the possibility of mistakes, the "cups" are numbered in Roman, and the "plates" in Arabic, characters. Each knurled nut is unscrewed one half turn to allow the back of the case to be rotated more easily, and the whole is placed in the thermostat and connected to the manifold leading to the manometer, and also to one of the bubblers. At some time during the passage of the gas, the case is clamped against the thermostat window and the zero point is examined. If the two images of the slit on the ground glass screen can be made to coincide by a pressure of not more than, say, ± 30 mm. of water, nothing further is done; but if a bigger pressure is required (i.e. if the mirrors on the mica are far from parallel), the pressure is kept at zero and the two images are made to coincide by adjusting the mirror Q by means of the calibrated drum Z . The drum reading is noted, and whenever a reading is taken of that particular respiration chamber, the drum is always set to the same reading.

When all the zero readings have been taken, the gas stream is stopped and the vessels are closed by rotating the back of each case backwards and forwards five or six times; after 2 or 3 min. temperature equilibration is attained and readings may be commenced. Each case has three rubber tubes leading from it, and when several cases are being used it is convenient to keep these tubes out of the way by suspending them in hooks attached to elastic cords which dangle from a permanent framework. The latter and the elastic and hooks can be seen in Plate I B.

Determination of constants. The constants of the respiration chambers are determined as for the Warburg vessels [*vide* Dixon, 1934]. The constant, K , is therefore given by the formula

$$K = \frac{v_G \frac{273}{t} + v_F \alpha}{P_0},$$

where v_G = volume of gas space,

v_F = volume of fluid,

α = solubility coefficient of the gas in the liquid concerned,

t = the absolute temperature,

P_0 = the normal pressure (760 mm. Hg) in mm. of manometric fluid. If

D is the density of the latter, then $P_0 = \frac{760 \times 13.6}{D}$.

Then, where h is the manometric reading observed, the amount of gas, x , evolved or absorbed = hK .

The volume of fluid, v_F , can be measured by a pipette, or by determining the increase in weight of the tared "cup" after adding the fluid.

The total volumes of the "cups" and "plates" are determined initially by weighing the amount of mercury required to fill completely the central space. In the case of a "cup" this is done by filling with mercury, laying a plane slab of glass on top, and squeezing out the excess of mercury. Adhering globules

of the latter are brushed off, and the mercury is transferred to a tared watch-glass, which is reweighed. The volume of the central cavity of a "plate" is found in the same way, except that the "plate" is squeezed between two slabs of plane glass. The volume of the gas space, v_G , is obtained by subtracting the volume of fluid, v_F , plus the volume of the mixing ball (if used) from the total volume.

A number of factors suspected of altering the effective volume of the whole assembly were investigated, since any alteration in the volume of the vessel will introduce corresponding errors in the constant. The first of these factors was the thickness of the film of grease between the components. The error from this cause is negligible, since bright interference colours can always be seen as soon as air enters between the faces of the "cup" and "plate" when these are wrung apart after an experiment. The second and third possible sources of error are (a) the volume of the wax used to line the cavity of the "cup", and (b) the volume of grease which will be squeezed into the respiration chamber when it is screwed up tightly in its metal case. No allowance is made for either of these factors, but quantitative investigations showed that from these two causes the results may be anything up to 3% too high.

Accuracy of the constants. This was investigated by measuring the volume of O_2 liberated from a known amount of H_2O_2 by means of catalase, control experiments being carried out in the Warburg apparatus. The results are shown in Table I, the calculated values being obtained from titrations with $KMnO_4$. The

Table I. *Test experiments with H_2O_2*

In each case approximately 1 μ l. of gas was evolved in the microrespirometer
Vol. O_2 /ml. H_2O_2 solution

Exp.	Vol. O_2 /ml. H_2O_2 solution		
	Microrespirometer	Warburg	Calculated
1	204	—	202
	208		
2	198	—	216
	200		
3	78	78	—
	77		
	77		
	77		
	80		
4	81	77	84.5
	74	77	
	75	78	
	79	79	
	79	80	
5	79	77	82.5
	85	80	
	79	81	
	82		

occasional discrepancies are almost certainly due to the fact that the weak H_2O_2 solution decomposes slowly during the equilibration period. Another series of experiments was carried out in which the volume of CO_2 , liberated from bicarbonate solution in an atmosphere containing 5% CO_2 by a known amount of acid, was measured. The results are shown in Table II. The scatter in these experiments is due to the fact that it is difficult to get the "cup", and more particularly the mixing ball, completely free from traces of acid or base. We are convinced that neither of the two sets of figures given is a true indication of the accuracy of the instrument when used for measuring O_2 uptakes.

Table II. *Measurement of volume of CO₂ displaced from bicarbonate by a known amount of acid*

Calculated value, 0.90 μ l.		
Exp.	Vol. CO ₂ evolved μ l.	% error
1	0.83	-8
2	0.85	-5.5
3	0.90	0
4	0.90	0
5	0.82	-9
6	0.87	-3.5
7	0.84	-6.5
8	0.87	-3.5
9	0.85	-5.5
10	0.90	0
11	0.96	+6.5
12	0.87	-3.5
13	0.89	-1
14	0.88	-2
15	0.89	-1
16	0.92	+2

The role of diffusion. In most of the previously mentioned respirometers dealing with uptakes of the order of 1 μ l. O₂/hr., shaking is omitted on the assumption that diffusion of the gas in the droplet will be adequate. This point was investigated by comparing the rate of O₂ uptake in the microrespirometer with that in the Warburg apparatus as control. An inorganic system such as a spontaneously oxidizable titanous salt is unsuitable for this purpose, because the rate of O₂ uptake will be proportional to the partial pressure of O₂; as this will decrease towards the centre of the droplet, the rate of uptake of the inside of the drop will be less than that of the outside layer. What is required is a catalytic system in which the catalyst has a high affinity for oxygen, and experiments showed that kidney amino-acid oxidase with alanine as substrate [Krebs, 1935] was satisfactory in this respect.

A large number of experiments was carried out with this system in an atmosphere of O₂, with droplets between 4.0 and 16.0 μ l. in volume, with total uptakes ranging from 0.5 to 2.7 μ l. O₂/hr. Of the 59 microrespirometer experiments which were done, the standard deviation of the percentage error was 4.8, a similar scatter being obtained with the Warburg values. The agreement, in any particular series, between the two instruments was in most cases within $\pm 10\%$, and though occasionally even worse results were obtained, it is quite clear that in an atmosphere of O₂ there is no effect attributable to diffusion.

Comparative experiments in air, however, showed a definite effect of diffusion, for considerably lower values for the rate of O₂ uptake were obtained with the microrespirometer than with the Warburg apparatus, with drops down to 4.0 μ l. in volume having an uptake of 0.4 μ l./hr. Even when the drop was suspended in a platinum loop inside the vessel, so that maximum surface was exposed to the gas mixture, the values were low by as much as 20%.

In view of the results obtained in an atmosphere of air, it was thought desirable to test rates of uptake in O₂ under more stringent conditions. Tyramine oxidase has been shown by Kohn [1937] to be highly dependent on the O₂ pressure, and some comparative experiments with this system were carried out in the microrespirometer, with the Warburg apparatus as control, with the results shown in Table III. The activity of the extract fell off rapidly, so that accurate comparison of the results in the two instruments was not possible. The

Table III. *Experiments with tyramine oxidase*

In all experiments the volume of extract taken in the microrespirometer was 10.3 μl ., but this quantity was sometimes divided into two or three separate droplets in the respiration chamber.

Exp.	O ₂ uptake/hr./ μl . extract		Number of drops of fluid in microrespirometer
	Warburg	Microrespirometer	
1	285	306	2
	285	291	1
	303	264	2
		260	1
2	310	291	3
	320	276	3
	280	310	1

table shows clearly, however, that even when oxidative systems are used which are dependent on O₂ pressure, the rates of O₂ uptake of these systems can safely be measured in this instrument. Since correct values are obtained in O₂ with relatively large drops and rapid rates of uptake, it might have been expected that normal values would also be obtained in air, provided that the drop of fluid were sufficiently small. This is not the case and therefore factors other than simple diffusion must be operating to account for this phenomenon. However, a discussion of these other factors is beyond the scope of the present communication.

Comparative experiments, with the Warburg apparatus as control, have also been done on the rate of liberation of CO₂ from urea by the action of urease; entirely satisfactory agreement between the two instruments was obtained.

The apparatus described in this communication is suitable for the measurement of rates of O₂ uptake and other metabolic activities of small pieces of surviving tissues. As, however, the case of small pieces of tissue involves a number of special considerations and the application of special methods, this question is treated in detail in the following paper by Berenblum *et al.* [1939].

Details for the measurement of respiratory quotients and aerobic glycolysis in this instrument will be given in a subsequent paper.

SUMMARY

1. A new type of microrespirometer is described which will measure O₂ uptakes of the order of 1 μl ./hr., with a standard error similar to that normally obtained with the Warburg apparatus.

2. The respiration chamber can be filled with any desired gas mixture, and two or more separate fluids in it may be mixed at any time during the experiment. The apparatus may be charged at one temperature and used at a widely different one, and it can be sterilized.

3. The estimation of substances which can be determined manometrically in the Warburg apparatus can also be carried out in the microrespirometer, the amounts which can be estimated being approximately 200 times smaller.

4. No special skill is required in its manipulation, and the setting up of an experiment takes only slightly longer than in the case of the Barcroft or Warburg apparatus. During the course of an actual experiment six readings can be taken in 5 min., with a little practice.

We wish to thank Dr H. G. Kuhn and Dr P. E. Lindahl for helpful suggestions. Our thanks are due to the Medical Research Council for a personal grant and a grant for apparatus to one of us (N. G. H.), and to the British Empire

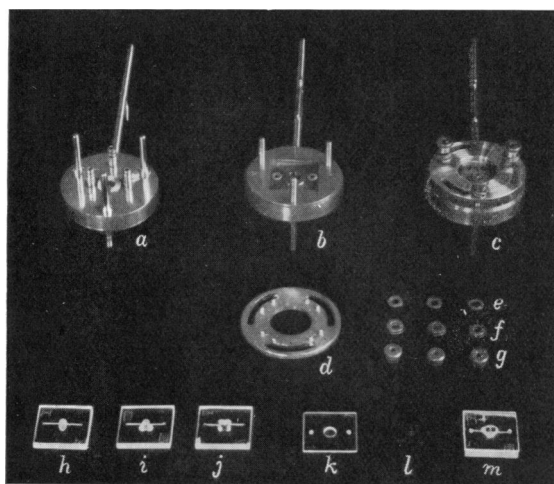


Plate I a

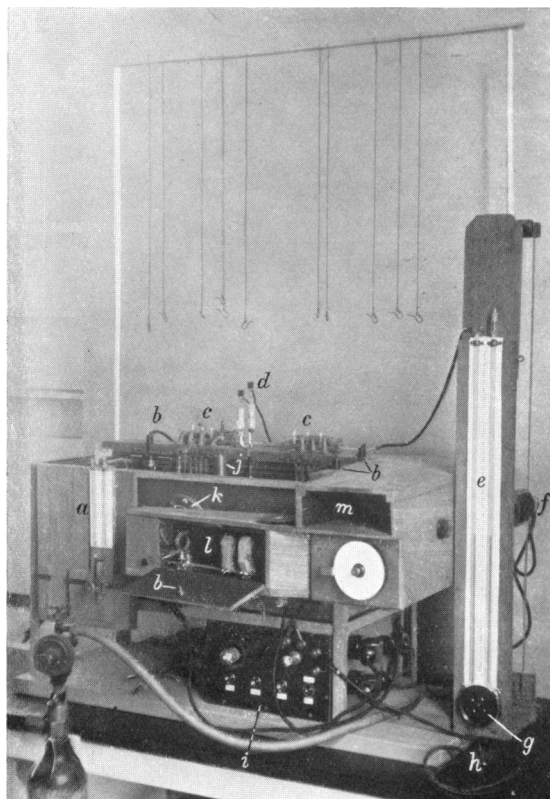


Plate I b

Cancer Campaign for personal grants to two of us (I. B. and E. C.). Preliminary experiments were carried out by one of us (N. G. H.) at the Biochemical Laboratory, Cambridge, England, during his tenure of the Benn W. Levy studentship.

REFERENCES

- Baker (1938). *J. sci. Instrum.* **15**, 304.
 Berenblum, Chain & Heatley (1939). *Biochem. J.* **33**, 68.
 Dixon (1934). *Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes*. Camb. University Press.
 Duryee (1936). *Z. vergl. Physiol.* **23**, 208.
 Fenn (1927). *Amer. J. Physiol.* **80**, 327.
 — (1928). *Amer. J. Physiol.* **84**, 110.
 Gerard & Hartline (1933). *J. cell. comp. Physiol.* **4**, 141.
 Heatley (1938). *J. sci. Instrum.* **15**, 340.
 Jackson, Parker & Glover (1930). *J. exp. Med.* **52**, 547.
 Kohn (1937). *Biochem. J.* **31**, 1693.
 Krebs (1935). *Biochem. J.* **29**, 1620.
 Linderstrøm-Lang (1937). *Nature, Lond.*, **140**, 108.
 Needham & Boell (1939). *Proc. roy. Soc. B* (in the Press).
 Schmitt (1932). *Amer. J. Physiol.* **104**, 303.
 — (1936). *Cold Spr. Harb. Symp.* **4**, 188.
 Stefanelli (1937). *J. exp. Biol.* **14**, 171.
 Victor (1935). *Amer. J. Physiol.* **111**, 477.
 Waddington, Needham & Brachet (1936). *Proc. roy. Soc. B*, **120**, 173.

EXPLANATION OF PLATE I

PLATE I A

Components of the respiration chamber and brass case. *a*, brass case, face upwards. *b*, brass case, face downwards. *c*, complete assembly ready for immersion in thermostat. *d*, rotating back of case, showing pins. *e*, plain washers. *f*, spring washers. *g*, knurled nuts. *h*, *i* and *j*, "cups" with cavities for two, three and four separate drops of fluid respectively. *k*, "plate". *l*, mica, with mirrors attached. *m*, complete respiration chamber ready for insertion in brass case.

PLATE I B

General view of complete apparatus. *a*, thermobarometer. *b*, immersion heaters. *c*, bubblers and manifold leading to manometer, etc. *d*, thermoregulator. *e*, manometer. *f*, one of the reservoirs of the pressure-regulating mechanism. *g*, crank actuating pressure-regulating mechanism. *h*, electromagnet—for mixing drops. *i*, control panel. *j*, top of thermostat stirrer. *k*, window in side of thermostat. *l*, part of optical system (lens, slit and condenser) seen through opened panel of optical box. *m*, hood protecting ground glass screen from stray light.

Note. The bar from which the brass cases are suspended is not shown in this photograph.