APPLICATION OF GRADIENT DIVER MICRO-RESPIROMETRY TO PROTOZOAN STUDIES

A. J. COWLING

British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge CB3 0ET, UK

ABSTRACT. The techniques of gradient diver micro-respirometry, developed by Nexo, Hamburger and Zeuthen (1972), are evaluated for the purpose of their application to respiration studies of small Protozoa, particularly Antarctic testate amoebae. The method relies on measurements of the downward migration of ampulla divers in an aqueous linear density gradient. Basic techniques, together with modifications developed from experiments with *Tetrahymena pyriformis* (a ciliate) and *Corythion dubium* (a testacean), are detailed. Sufficiently small divers are required for adequate sensitivity, and a standardized method of culture contaminant removal is required when dealing with non-axenic protozoans.

INTRODUCTION

Numerous investigations have contributed to information on the respiratory physiology of Protozoa (see reviews of Danforth, 1967; Heal, 1971; Fenchel and Finlay, 1983) and the methods used to measure aerobic protozoan respiration have varied widely. Macro-respirometric studies (i.e. utilizing large numbers of protozoan cells in relatively concentrated suspensions) have included titration methods (Lund, 1918; Leichsenring, 1923) and gas analysis (Root, 1930), but have largely utilized standard manometric methods involving the use of Warburg, or differential (e.g. Barcroft) type respirometers (e.g. Leiner and others, 1968). Polarographic techniques for determining dissolved oxygen tensions (Clark, 1956) have also been applied to measurements of respiratory levels in a range of protozoans (e.g. Heinrich and Cook, 1967; Lloyd and others, 1978; Weik and John, 1977).

Micro-respirometric studies (i.e. in which relatively few or even single protozoans are used for measurements) have utilized capillary manometers (e.g. Cunningham and Kirk, 1942; Kalisz, 1973), but most commonly those methods in which flotation principles are exploited to enable measurement of very small gas exchanges. The versatility of the most widely used of these methods, the Cartesian diver, has been indicated by Zeuthen (1964 and references therein) and its application to ciliate energetics by Laybourn (1975; 1976*a*, *b*; Laybourn and Finlay, 1976). The subsequent development, from similar principles, of ampulla Cartesian divers (Zajiceck and Zeuthen, 1956) to manometric (Lovlie and Zeuthen, 1962) and non-manometric (Nexo and others, 1972) density gradient (non-Cartesian) diver methods has improved sensitivity and, in the latter case, practicability. The dispensation with manometry has permitted automated photographic recording of measurements (Hamburger and Zeuthen, 1974) and by such means, density gradient respirometers have been put to wide-ranging use, from determinations of relative glycolytic activity in clonal populations of the yeast *Schizosaccharomyces* (Hamburger and others, 1977) to the measurement of respiratory activity in Collembola (Petersen, 1981), for example. In addition, gradient diver methods have been employed in respiration studies of both small (Hamburger, 1975) and large (Chapman-Andersen and Hamburger, 1981) naked amoebae, and have been further developed, particularly for use in eco-physiological studies, by Klekowski and others (1980).

For the purpose of an energetics study of maritime Antarctic terrestrial Protozoa, gradient diver micro-respirometry was considered the most promising means available for measurements of respiration rates in selected species of small testate rhizopods (in particular *Corythion dubium* Taranek) that dominate the protozoan fauna of the Signy Island Reference Sites (Smith, 1973). The method appeared suitable both in terms of its potential sensitivity and amenability to modification. Such considerations were of importance because of the difficulty in obtaining large numbers of contaminant-free cultured amoebae for experimental purposes, with the resultant necessity for determinations to be made on single or few individuals.

This paper describes how modified gradient diver techniques, of particular application to testate amoebae studies, were developed from preliminary experiments with the ciliate *Tetrahymena pyriformis* (Ehrenberg) and from considerations of the sensitivity, accuracy and practicability of the proposed methods.

Gradient Diver Micro-Respirometry

General theory and apparatus

The principles of gradient diver microgasometry, as described by Nexo and others (1972), underlie the experimental methods summarized as follows. Respiring material is confined in a droplet of culture medium held in the neck of a small glass ampulla vessel (diver) and separated from alkali held in the narrow, open diver tail by an air space, the opening at the tip of the diver neck being sealed (Fig. 1A). When the diver is submerged in a vertical, aqueous linear density gradient, it sinks to a level in the gradient at which its compound density is approximately equal to the density of the surrounding fluid and there remains buoyant. As respiration depletes the volume of oxygen in the air space (evolved carbon dioxide being absorbed by alkali), gradient fluid is drawn up through the opening into the tail to replace the volume lost, with the result that the diver's compound density increases, so the buoyancy decreases and it sinks to a lower level in the density gradient. The diver therefore remains in a state of quasi-equilibrium with respect to its position in the gradient at any one time, such that the rate of its vertical descent is directly proportional to the rate at which oxygen is consumed by the respiring material. This vertical diver migration rate, if accurately measured, can be converted to the equivalent oxygen uptake rate by means of a constant (K_v), which is derived from the steepness of the gradient and various physical properties of the diver. Measurements of the migration rate of control divers (containing no respiring material) are also necessary to correct for the effects of temperature and pressure fluctuations where these are not held constant during the experiment. Under such conditions, experimental divers exhibit downward or upward migration independently of the respiration effect in response to temperature- and pressure-mediated gas volume changes. Even where both physical parameters are kept constant, a control diver will migrate downwards (control drift) in response to compound density changes caused by diffusion effects through the open tail so that controls are also essential in such thermoregulated, 'closed' (constant pressure) system experiments. The latter regime permits greater accuracy through better control over experimental conditions.

The apparatus (Fig. 1B) consists of a cylindrical glass gradient vessel (4 cm diameter, 450 cm length) fixed in a vertical position inside a perspex bath, its facing edge *c.* 5 cm from the bath front, and connected as shown to an outlet tube and compensation vessel. Constancy of pressure is effected by using a three-way tap, which regulates this connection, thus allowing equalization or closure of both vessels respectively when in an open or closed position and with the gas-tight gradient vessel

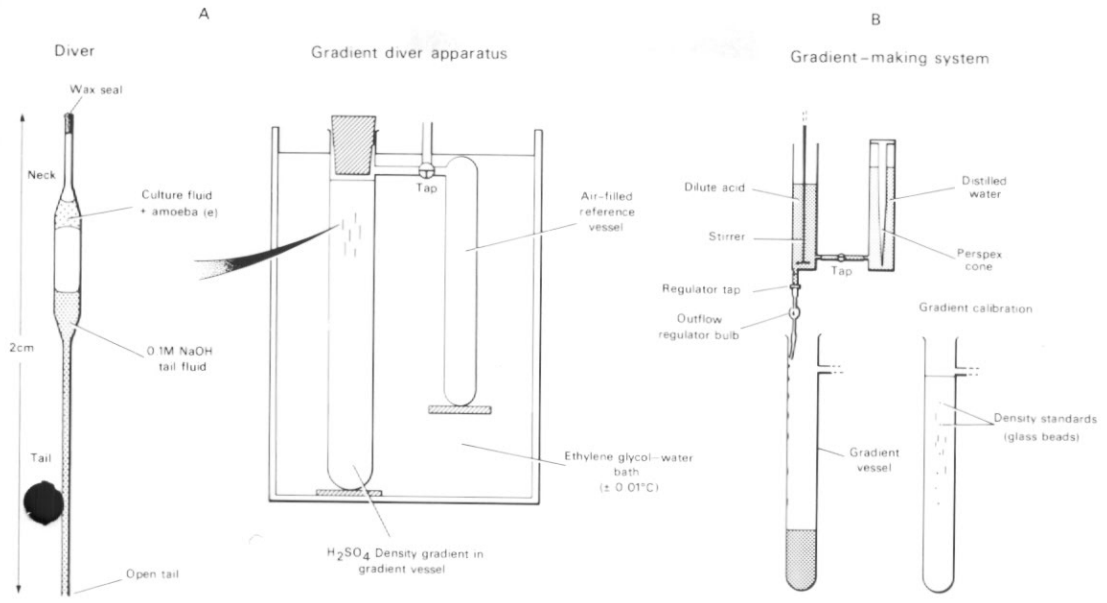

Fig. 1. Gradient diver micro-respirometer (A) and gradient-making apparatus (B).

stopper in place. The bath liquid (an ethylene glycol–water mixture) is cooled continuously (Hetofrig CA3) and its temperature controlled to ± 0.001 deg. by an ultrathermostat heater (Heto, Denmark), regulated by a mercury contact thermometer. A cathetometer (Precision Tool and Instrument Co.) mounted at a fixed distance from the gradient vessel outside the bath is used to measure diver migrations to an accuracy of 0.01 mm over a vertical distance of *c.* 300 mm. The apparatus is housed in a constant-temperature room in which all experimental procedures are carried out at an ambient temperature equal to that of the bath liquid.

Calculations

For each experiment, sequential readings of each diver's position in the gradient are plotted against time to indicate its cumulative migration (mm) over the time period t_0 to t_n (h). A line of best fit is calculated by least-squares regression, the slope giving the uncorrected diver migration rate in mm h^{-1}. This is converted by subtracting the mean control diver migration rate, calculated similarly from the cumulative migrations of control divers.

The hourly oxygen uptake rate for each experimental diver (ΔV, mm^3) is a product of the corrected diver migration rate (ΔH_E, mm^{-1}) and the 'volume diver constant' (K_v, mm^2), where:

$$K_v = \frac{273}{10300\ T} \times \frac{U\ P_o}{\phi_{op}} \cdot \frac{d\phi}{dH} - (\phi_{op} \cdot V_o)$$

and

$$P_o = P_{top} - \pi + (\phi_{op} + \phi_w)\ H_{op}$$

and

$$U = \frac{mgl}{\phi_{gl}} + \frac{mf - mgl}{\phi_w}$$

and

$$V_o = mgl \frac{1}{\phi_{mp}} - \frac{1}{\phi_{gl}}.$$

The values and dimensions of the various constants and variables are given in Table I.

Table I. Constants and variables used in calculation of oxygen uptake rates per diver. *i.e. mg mm^{-3} mm^{-1}; †approximated to values from published tables of water vapour pressure at T.

T	K	Experimental temperature
$d\phi/dH$	mg mm^{-4}	Gradient steepness*
P_{top}	mm H_2O	Pressure on the top of the gradient (equal to the barometric pressure at closure).
P_o	mm H_2O	Pressure of gases in the diver at t_o
V_o	mm^3	Volume of gas in the diver at t_o
π	mm H_2O	Vapour pressure inside the diver†
H_{op}	mm	Height of the diver opening at t_o
U	mm^3	Total volume of the diver
ϕgl	mg mm^{-3}	Density of diver glass (= 2.229)
ϕ_w	mg mm^{-3}	Density of water (= 1.00)
ϕ_{op}	mg mm^{-3}	Density of the gradient at the initial (t_o) position of the diver opening
ϕ_{mp}	mg mm^{-3}	Density of the gradient at the initial (t_o) position of the diver mid-point
mgl	mg	Weight of the glass of the diver
mf	mg	Weight of the diver filled with water

Density gradients

Linear gradients of *c.* 430 mm height and density range of 1.00 to 1.04 g ml^{-1} (surface to base) are prepared using the apparatus shown diagrammatically in Fig 1B, which is permanently fixed above the respirometer bath. Equal volumes of water and 4–5% (v/v) sulphuric acid (density 1.04 g ml^{-1}) are placed in the right- and left-hand vessels respectively, and the tap opened to allow mixing while the stirrer operates continuously at a slow speed (*c.* 2 rev s^{-1}). The gradient is slowly built up over 1–2 h by adjusting the flow from the outlet below the left-hand vessel to a rate of *c.* 2–3 drops per second by means of the regulator tap. The need for additional apparatus for removal of divers without disturbance of the gradient (Klekowski and others, 1980) is obviated by preparing fresh gradients before each experiment.

Sulphuric acid is used as a heavier fluid in preference to sodium sulphate solution, which is usually employed (Zeuthen and Hamburger, 1977), because reduced solubility of the latter at lower temperatures can cause problems in gradient making. An additional advantage is that the gradual penetration of acid into the diver's tail through the opening can be monitored if the alkaline tail fluid is stained with phenolphthalein. This indicates the point in time at which ions from the gradient fl[illegible] eventually reach the tail fluid–air interface, after which no further rate measurement should be made, since the diver then responds to effects caused by the resultant liberation of previously bound CO_2 and water vapour tension decrease (Lints and others, 1967).

The density gradient is calibrated upon completion of migration rate measurements and before divers are removed. Between 5 and 8 density standards (hollow glass beads) are placed in the gradient and their positions recorded with the cathetometer after an equilibration period of 30–60 min (Fig. 1B). Details of the manufacture and calibration of glass bead standards are in Møller and Ottolenghi (1964) and Klekowski and others (1980).

The gradient steepness, $d\phi/dH$ (mg mm^{-4}) is given by the gradient of the regression line calculated from a plot of bead density against bead position (depth) in the

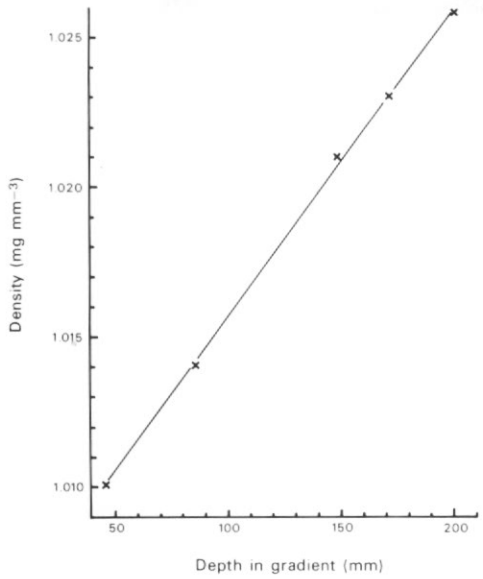

Fig. 2. Typical example of linear density gradient calibration using glass bead density standards. Density = $(1.017626 \times 10^{-4})$ depth + 1.005444.

gradient, a typical calibration being shown in Fig. 2. Divers should only be operated in gradients whose steepness (in mg mm^{-4}) is above a critical value, given by:

$$\frac{\phi_{op}{}^2 V_o}{U.P_o}$$

for the equations upon which gas volume changes are based to be valid (see Nexo and others, 1982).

Construction of ampulla divers

A natural gas and oxygen flame is used to pull thin capillaries of *c.* 1 m length from Pyrex glass tubing (10 mm diameter, 1 mm wall thickness; density of glass 2.229 mg mm^{-3}). The narrow capillaries should be of uniform wall thickness ($\leqslant 10\%$ their diameter), circular in cross-section, and of a precise dimension where divers of $\leqslant 1$ mm^3 total volume (U) are required.

From such capillaries, ampulla diver 'units' (Fig. 3A) are constructed using a microburner flame of *c.* 2 mm length (Fig. 3B–3E). At this stage, it is useful to sort the diver units into approximate size categories by visual comparison with previously constructed divers (already used successfully in preliminary experiments), which are of known volume and of the correct dimensions and shape for the purposes required. This ensures, firstly, that time is not wasted subsequently on unsuitable divers, which may be particularly difficult or impossible to charge, balance or weigh, and, secondly, that control divers are as closely similar as possible to those used as experimental ones.

After sorting, diver units are boiled for 10 min in water to remove any grease, dried and then placed in screw-capped bottles to be heat-sterilized (140°C, 2.5 h) before being left to cool overnight at the experimental temperature.

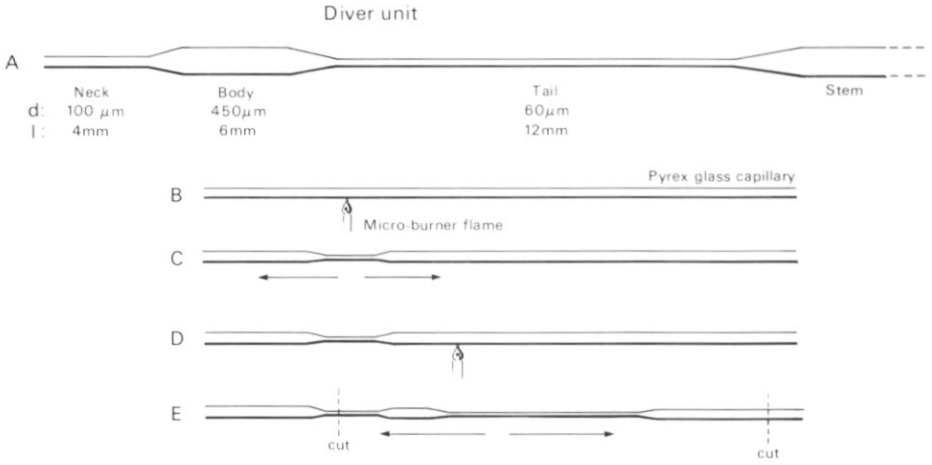

Fig. 3. Ampulla diver construction. Dimensions given are typical for a diver of *c.* 1 mm³ total volume. d, diameter; l, length. (Modified after Klekowski and others (1980).)

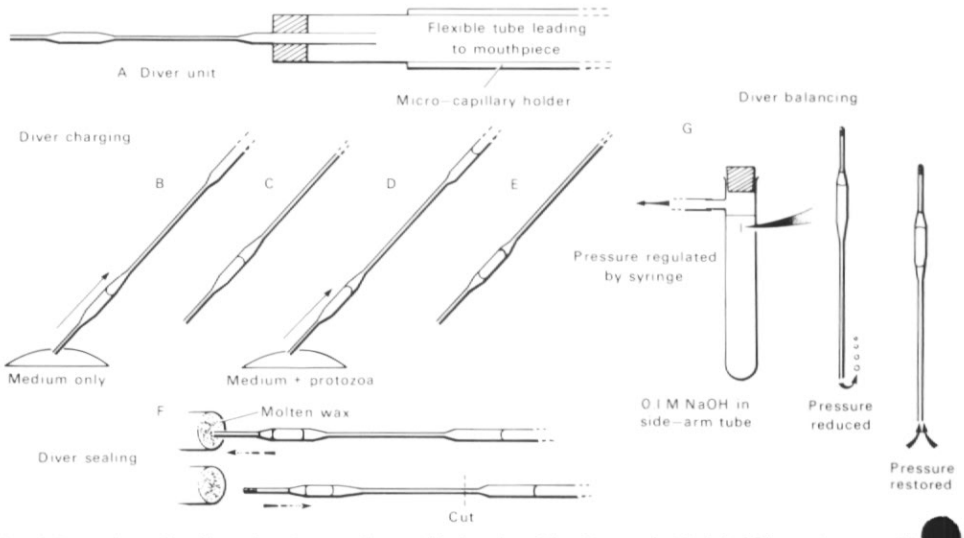

Fig. 4. Procedures for diver charging, sealing and balancing. The diver unit (A) is held in a micro-capillary holder and used throughout as a micropipette. After rinsing the diver with sterile culture medium (B) a small amount is retained in the tail (C) before medium and protozans are introduced (D, E). The diver neck opening is then sealed with molten wax held in an electrically heated wire loop (F). During balancing (G), successive applications of pressure reduction are used to replace medium and air in the diver tail with NaOH (stained with phenolphthalein) until the diver's density becomes just slightly greater than that of the surrounding alkali. (Modified after Klekowski and others (1980).)

Diver charging, sealing and balancing

Details of the serial sterile-wash method, which precedes charging of divers with testate amoebae, are given below. Experimental divers are 'charged' – i.e. Protozoa introduced into the diver neck – as illustrated in Fig. 4A–E. It is important to ensure that some fluid initially extends into the diver body (C) because menisci move toward

the tail as the protozoans are introduced (D). Also, the length of gap between the droplet and tail fluid meniscus must, at this stage, be approximately correct (E) since this air volume dictates the subsequent buoyancy of the diver.

When sealing the diver (Fig. 4F), some wax is pulled into the diver neck before solidifying to form an effective plug. Care must be taken to ensure that the neck is completely sealed and that the wax tip is rounded at its extremity, so that air bubbles cannot form when the diver is immersed in the gradient fluid. Dentists' modelling wax (Klekowski and others, 1980) is ideal for sealing purposes.

The diver, once charged, sealed and cut from its stem is then removed to a beaker of distilled water in order to determine its approximate buoyancy. Divers that remain at the surface with their tails submerged are relatively easy to balance, while those that sink directly to the bottom are too heavy, although this can sometimes be remedied by shortening the tail to reduce diver weight.

After balancing individual divers (Fig. 4G), each is then ready for introducing into the density gradient. Control divers are charged (with culture medium only) and balanced in the same way as experimental divers.

Experimental procedure

Each diver is loaded separately into the density gradient by using special forceps to release it gently, from a submerged position just below the gradient surface. All divers should reach equilibrium positions away from other divers and from the gradient vessel walls, and below the upper 50 mm of the gradient (which part tends to be non-linear). Similarly, the positions of experimental divers should be within the range of the controls, or as close as possible to them.

Divers are left to equilibrate for *c.* 35 min at atmospheric pressure, after which time the system is closed by means of the tap (Fig. 1), while the gradient vessel stopper is in place, and atmospheric pressure recorded from a barometer. The positions (heights) of the tip of each diver are measured with the cathetometer, and thereafter readings are repeated at regular 30 or 60 min intervals. The height of the surface of the density gradient is recorded, and also the position of each diver's tail opening, the latter to enable later calculation of diver lengths.

The use of lamps to reflect light on to the floating divers ensures maximum contrast between background and divers and enables accurate cathetometer readings.

Each diver is carefully scrutinized during experiments to check that no air bubble forms on its surface, or at the tail opening. Divers having external air bubbles usually exhibit erratic migrations, and when they are present the results should be discarded.

Experiments are terminated immediately after the final reading (5–6 h after closure) by opening the tap before removal of the gradient vessel stopper (Fig. 1). The density standards are then introduced separately into the gradient, beginning with the heaviest, and their positions recorded after equilibration. Experimental divers are carefully removed from the gradient by means of a long pipette and placed in numbered sample tubes containing distilled water.

Diver examination and weighing

The contents of experimental divers are examined *in situ* before removal of the wax plug in order to record the number and viability of protozoan cells confined to the droplet. For experiments with highly motile ciliates, such as *Tetrahymena*, it is sufficient to record cell viability and numbers per diver by using low-power magnification of a stereo microscope to focus on the diver inside its stoppered sample tube.

For testate amoebae, however, it is essential to locate and examine each protozoan closely enough to establish its viability. This is carried out by placing the diver into a drop of water on a microscope slide and using 10–40 × phase objectives with both phase contrast and dark field illumination. Removal of the wax plug (without breaking the diver) would otherwise allow extraction of Protozoa intact and unharmed, but none of several methods tried was totally successful.

After examination, diver contents and wax plug are removed by a modification of the method of J. R. Caldwell (pers. comm.). The neck of the diver is inserted into a hypodermic needle of suitable diameter attached to a 10 ml syringe, and suction pressure applied to loosen the plug with the diver and needle tip immersed in a beaker of hot (> 62°C) water. After rinsing to flush out the contents, the diver is removed to a numbered sample tube and dried in an oven at 60°C overnight.

Each diver is then weighed, both empty and filled with distilled water, to 0.5 μg on a microbalance. Filling is carried out by carefully inserting the diver neck into a modified micro-capillary holder and sucking up water into the diver through its tail. If the diver tail becomes blocked with detritus or wax residues, it may be cleared by passing over the hot air current from an electrically heated wire coil, taking care not to melt the glass.

Experiments with *Tetrahymena pyriformis*

The aims of these experiments were, firstly, to test the reproducibility of results obtained, secondly, to determine the suitability of the materials and methods to be used subsequently with testate amoebae and thirdly, to provide data for comparison with the literature. Because axenically cultured ciliates were used, there was little possibility of bacterial contamination. Similarly, by avoiding the necessity of a lengthy sterile-wash procedure, a larger number of replicate divers could be prepared for each experiment.

Methods

Tetrahymena pyriformis (CCAP LI630/1f) was maintained axenically at 20°C in proteose peptone (Oxoid L46) 20 g l^{-1}; yeast extract (Oxoid L21) 2.5 g l^{-1} and used in a series of four separate experiments at 20°C over a 9-day period. The ciliates were in logarithmic growth phase. Between one and four individuals were randomly selected from a drop of cell suspension and introduced into each diver. The prepared diver units were unsorted, so that a wide size range of divers were used. Between eight and eleven experimental and three control divers were charged for each experiment and diver migrations recorded over 3 to 5 h. The equilibration period was between 36 and 60 min in three of the experiments, but was 104 min in a fourth. Diver contents were examined carefully before each was balanced and loaded into the gradient and again immediately after termination of the experiment.

A 3-day-old culture was used to determine the mean cell volume from measurements of 30 individuals immobilized in Lugol's iodine. Individual cell volumes were derived from length measurements of major (a) and minor (b) cell axes and by approximation to a prolate spheroid:

$$volume = 4/3\ \pi\ a\ b^2.$$

A calibrated eyepiece graticule and × 40 objective were used to measure groups of five freshly immobilized ciliates at a time. In this way, any possible distortion of cells caused by shrinkage or swelling in response to the stain was avoided.

Results

Table II summarizes the results obtained from 33 experimental divers used in the four experiments and shows that, even with the large size range used, consistent mean rates of oxygen uptake per *Tetrahymena* cell were determined. These were not significantly different from each other. The slowest diver migration rates recorded were at least 60% greater than the corresponding mean control diver migration rate and were for divers of relatively large total volume charged with a single *Tetrahymena*. Fig. 5 shows diver movements during the fourth experimental run as an example.

Tetrahymena mean cell volume and 95% confidence limits was $13000 \pm 1406\ \mu m^3$.

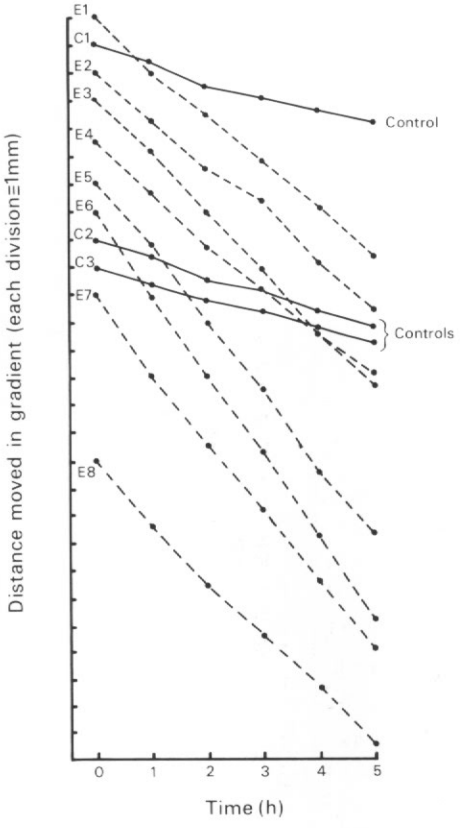

Fig. 5. Diver migrations recorded in Experiment 4 with *Tetrahymena pyriformis*. Curves are separated for clarity and do not represent relative distances between divers.

Control variability and absolute accuracy

The major limitation to the accuracy of the method is the precision with which the movement of control divers can be used to correct the experimental diver migration rates. This can be determined by the product of the diver constant (K_v) and the rate by which control divers move relative to each other (Nexo and others, 1972). This accuracy is different for each experiment, being dependent on the consistency of the

Table II. Measured oxygen uptake rates of *Tetrahymena pyriformis* at 20°C.

Experiment number	*Number of controls*	*Mean control diver migration rate* (ΔH_c) ($mm\ h^{-1}$)	*Range of experimental diver migration rates* ($mm\ h^{-1}$)	*Number of experimental divers*	*Range of diver volumes* (U) (mm^3)	*Oxygen uptake rate per individual* ($nl\ h^{-1}$)	
						$\bar{x} \pm 95\%$ *CL*	*Range*
1	3	0.585	0.900–2.744	10	1.35–2.50	0.06501 ±0.0115	0.04648–0.09664
2	3	0.936	1.231–2.500	9	1.26–2.39	0.06499 ±0.0113	0.04542–0.09075
3	3	0.561	0.926–1.950	6	1.40–2.20	0.05809 ±0.0133	0.03955–0.07310
4	3	0.580	1.678–2.581	8	1.14–2.47	0.06675 ±0.0172	0.04202–0.09130
Totals			0.900–2.581	33	1.14–2.50	0.06417 ±0.0057	0.03955–0.09664

control rates measured. The largest discrepancy between control diver rates occurred in the second experiment, in which a maximum difference of 0.844 mm h^{-1} resulted in an accuracy of *c.* $\pm$0.05 nl h^{-1}. However, Experiment 4 gave an absolute accuracy of $\pm$0.005 nl h^{-1} as there was better agreement between the controls, the maximum rate difference being only 0.077 mm h^{-1}. The close agreement between the mean oxygen uptake rates derived from these two experiments (Table II) suggests that the lower accuracy inherent in Experiment 2 was compensated for by an adequate number of replicates. It was concluded that consistent mean oxygen uptake rates could be determined even where variability between control migration rates was high. It was confirmed also that reliable measurements from single *Tetrahymena* could be obtained even where relatively large ($U \geqslant 2.00$ mm^3) ampulla divers were used. This suggested that, if smaller divers of a narrower size range were used later, control migration rate consistency would improve, enabling the measurement of smaller oxygen uptake rates. Similarly, such divers would have lower K_v values so that even if control rates remained as variable, absolute accuracy would be nevertheless improved.

Comparisons with published data

Many authors have reported respiration rate values for *Tetrahymena pyriformis* from studies in which Warburg, Cartesian diver and polarographic methods have, in decreasing order of frequency, been variously utilized. The total range of rates is very large and, although in part this may be attributable to differing respirometric methods used, it is probably also a result of differences in ciliate strains, cell volumes and the physiological state of the cultured Protozoa. In the case of exogenously respiring *Tetrahymena* (i.e. conditions comparable with the present study), Baldock and others (1982) reported a rate of 0.022 nl h^{-1} of oxygen per individual at 20°C using Cartesian diver methods, while a figure of 0.439 nl h^{-1} at 25°C was given by Pace and Lyman (1947), who utilized Warburg respirometers. These data, however, represent the extreme range of published estimates, the majority of reported values being between values of 0.038 (James and Read, 1957) and 0.185 nl h^{-1} (Khlebovich, 1974) at 20°C. A fuller comparison of the published data for *Tetrahymena* is made in Cowling (1983), and comparative analyses of respiration data for Protozoa in general have been the subject of several recent studies (Klekowski, 1981; Baldock and others, 1982; Fenchel and Finlay, 1983). The mean oxygen uptake rate obtained in the present study (0.0642 nl h^{-1} per individual at 20°C) lies at the lower end of the range from the literature, although it is slightly higher than those from recent comparable micro-technique studies.

A reliable estimate of *Tetrahymena* respiration rate could thus be obtained using the gradient diver, and it was shown that measurements on single protozoans were possible. The experimental method used, however, was uncomplicated by any preparative culture or sterile washing procedure, and the ciliates themselves, being highly active and easily maintained, were relatively amenable to manipulation with ampulla divers. Nevertheless, for its intended application to measurements of respiratory rates of similar sized testate amoebae, the technique showed much promise, given that further improvements of both sensitivity and accuracy were possible, and that a suitable serial washing technique could be developed.

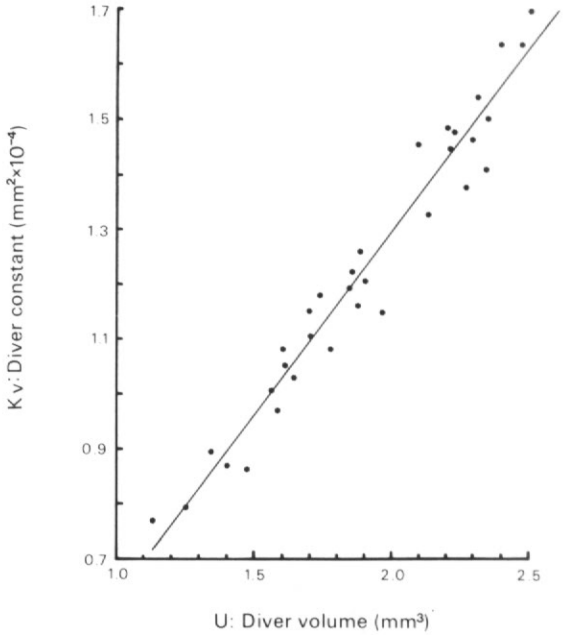

Fig. 6. Total diver volume (U) *v.* volume diver constant (K_v). $K_v \times 10^{-4} = 0.67\ U - 0.046$ ($r = 0.98$, $n = 33$). Data are from *Tetrahymena* experiments.

Application of Techniques to Testate Amoebae Studies

Diver size

As diver sizes (or volumes) and sensitivities are, in general, inversely related (Nexo and others, 1972), it was necessary to quantify this relationship in order to determine the extent to which sensitivity is improved by reducing diver size. Data from the experiments with *Tetrahymena* provided a means by which the two factors could be equated, since the volume (U) of each diver used could be plotted against its calculated diver constant (K_v), the latter being, in effect, a measure of the diver's sensitivity under experimental conditions. The resulting graph (Fig. 6) confirmed that diver volume was a main determinant of the magnitude of the constant, the variation reflecting the other parameters used in the calculation of K_v. From the equation of the fitted regression line, approximate values of K_v could be predicted for prepared divers whose volumes would be within the range 0.5–1.0 mm³ (as determined by the diver sorting procedure) and which were intended for use in experiments. The sensitivity, or more strictly the limits of detection, of such divers could then be further anticipated in terms of the expected migration rate (ΔH) for a given oxygen uptake (ΔV), from the linear equation

$$\Delta V = K_v \cdot \Delta H.$$

This is illustrated graphically in Fig. 7 where representative plots are given for large ($U = 2.0$ mm³), small ($U = 1.0$ mm³) and very small ($U = 0.5$ mm³) divers – the gradient of each line being their respective K_v values as approximated by the above method. Given that a reasonable limit to a measurable diver migration rate is of the order of 0.5 mm h^{-1}, the corresponding sensitivity of such divers would be *c.* 0.065,

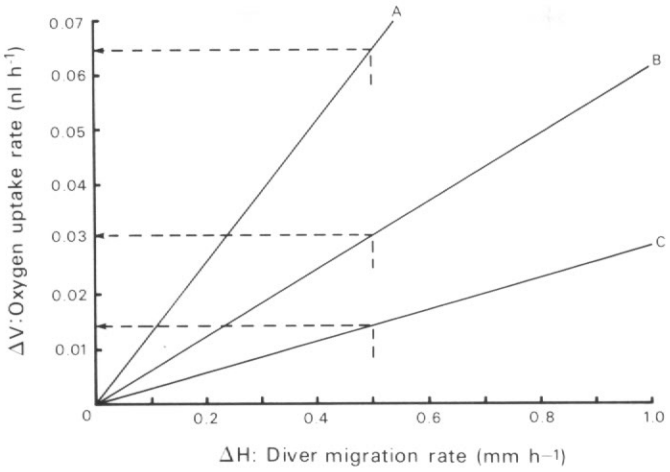

Fig. 7. Relationship between diver migration rate and equivalent oxygen uptake rate for divers of different sizes. A, large diver ($U = 2.0$ mm^3, $K_v = 1.29$); B, small diver ($U = 1.0$ mm^{-3}, $K_v = 0.62$); C, very small diver ($U = 0.5$ mm^3, $k_v = 0.29$). Dashed lines indicate expected uptake rates for a given migration rate of 0.5 mm h^{-1}.

0.030 and 0.015 nl h^{-1} respectively (dashed lines, Fig. 7). Construction of divers of $U < 0.5$ mm^3 had been found impracticable, while those of $\leqslant 1.0$ mm^3 can, with experience, be made consistently and in the numbers required for experiments. With such divers, therefore, detection of oxygen uptake rates from single testate amoebae (size *c.* 50μm) at 20°C is feasible, with the assumption that the magnitude of their respiration lies within the range reported for similar sized naked amoebae (Laybourn-Parry and others, 1980; Baldock and others, 1982). It was anticipated, however, that for experiments at lower temperatures, a greater number of amoebae per diver would be required in order to produce measurable diver migration rates and thus allow determination of the expected lower oxygen uptake rates per individual.

Development of serial washing and charging techniques

In order to eliminate microbial contaminants (originating in the cultures) from experimental material, the technique of repeated washing of amoebae through several changes of fresh, sterile culture medium was employed. This process is made difficult by the small size of the euglyphid testate amoebae and their ability to adhere strongly to the inside surfaces of both micropipettes and diver units. It was considered essential, therefore, to develop specific techniques to overcome this problem. A satisfactory method was only developed after much trial and error, and the techniques finally adopted are illustrated schematically in Fig. 8. Diver charging itself (H) must be precisely and rapidly executed in order to avoid the following.

(i) The introduction of too large a liquid droplet into the diver.

(ii) The attachment of amoebae to the inner walls of the diver. The first situation can be remedied (I), provided that the amoebae remain in a coherent group near (but not attached to) the inner meniscus of the droplet whilst the excess medium is expelled from the diver. The irreversible consequence of the second situation is that some amoebae become isolated outside the menisci as the droplet itself is subsequently positioned. In practice, therefore, the most expedient course is to charge the diver in a single, swift movement.

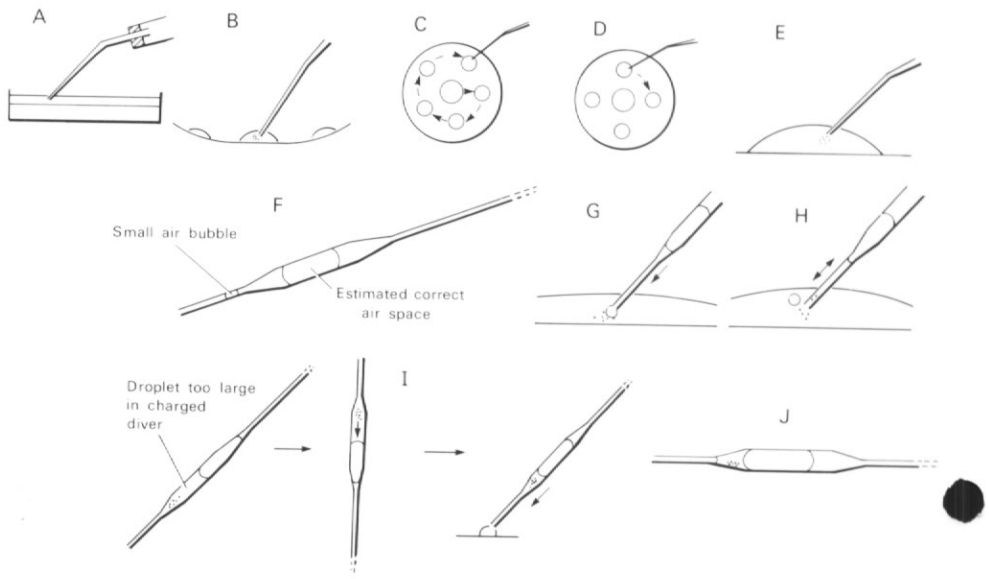

Fig. 8. Serial washing and diver charging procedure used in experiments with testate amoebae. Individual amoebae are transferred through a minimum of six separate drops of sterile culture medium (A–D) before being ejected, as closely together as possible, into a fresh drop (E). The prepared diver unit (F) is used to quickly draw amoebae into the diver using, if necessary, the air bubble as shown (G, H). These techniques ensure that the air space (shown in F) does not alter during any of the manipulations, so that the final charged diver (J) will have an approximately correct buoyancy.

In general, the smaller the size of the divers used, the greater are the problems described above, because of the strong capillary forces exerted through the narrow necks and tails of such divers. Consequently it is difficult to control the movement of the droplet and tail fluid, particularly in poorly made or very small divers.

Sterilized micropipettes (constructed from Pasteur pipettes) are used for serial washing. They are sorted by a procedure similar to that used for diver units, since only those with a sufficiently small tip diameter (*c.* 60–100 μm) are suitable, both for manipulating individual amoebae, and for transferring them to each drop with the minimum carry-over of residual medium from the previous wash.

Effects of serial washing

The efficacy of serial washing was assessed by comparing the apparent 20°C respiratory rates determined in a series of experiments using *Corythion dubium* individuals, which had either been taken straight from cultures (unwashed) or washed through 1, 4–5 or > 5 drops of sterile medium before charging. Amoebae were cultured at 20°C in Cerophyl–Prescott (CP) agar with acidified CP liquid and indigenous maritime Antarctic bacterial isolates (*Pseudomonas* spp.) as food. Experimental divers were charged with 1–4 *Corythion*. Control divers were charged with sterile medium in which amoebae had received five or less washes; above this number the final wash medium was used.

Table III gives details of the total of nine experiments carried out. Displayed graphically (Fig. 9), the results show that there was a reduction in apparent respiration rate with the extent of serial washing which clearly reflected the greater efficiency of

Table III. Details of respirometric experiments with *Corythion dubium* at 20°C to determine the effects of serial washing on apparent respiration rates per individual.

Number of serial washes	*Number of amoebae per diver*	*Experiment numbers*	*Total number of experimental divers (n)*	*Apparent oxygen uptake per individual nl h^{-1}*	
				Mean	*Range*
Unwashed	1–2	9, 10, 12, 13	7	0.1529	0.1154–0.2217
1	1–2	9, 13, 17	11	0.0725	0.0411–0.1101
4–5	1–3	9, 10, 13, 15, 17, 18	11	0.0379	0.0096–0.0793
> 5	1–4	20, 22	6	0.0271	0.0126–0.0427

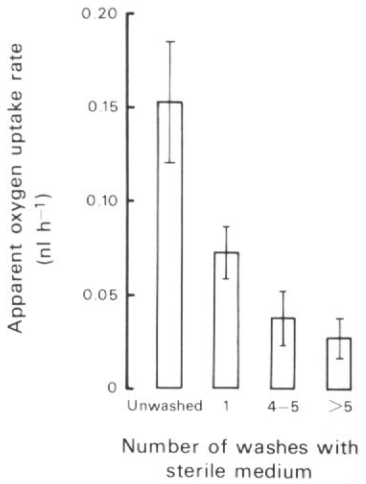

Fig. 9. Efficiency of serial washing method; oxygen uptake per individual from experiments with *Corythion dubium* at 20°C. Vertical bars represent 95% confidence limits.

microbial contaminant removal. The large reduction resulting from the use of a single wash only is attributable to the initial considerable dilution of the dense bacterial suspension which is otherwise introduced into divers, and which contributes the largest to the measured rates. Thereafter, Fig. 9 indicates that no further significant decrease in apparent respiration rate resulted from employing any more than five serial washes. Maximal elimination of contaminants could therefore be achieved using this number of washes as a minimum, such that the resultant measured rate was attributable to protozoan respiration alone. The restriction of serial washing to between five and ten transfers is proposed as a practical compromise to ensure both adequate contaminant removal and the avoidance of excessive manipulation of amoebae, which might influence amoebal respiration rate.

High-power microscopic examination confirmed the absence, both of microbial contaminants in the final wash medium and of larger aggregates of bacterial cells and/or culture debris, which occasionally adhered to some amoebae prior to washing. The possibility remains, however, that smaller numbers of bacteria may have persisted through serial washes by remaining closely attached to the surface of the amoeba test, or within the test chamber itself. Although it was difficult to observe the extent to which this occurred with washed amoebae, it is doubtful whether such bacteria would

have been present in sufficiently large numbers to have contributed significantly to the oxygen uptake rates measured.

There are no comparable data in the literature on respiration of testate amoebae with the exception of Zeuthen's (1943) Cartesian diver study, in which values from 0.39 to 1.75 nl h^{-1} of oxygen per individual were given for a species of *Difflugia* whose cell volume (estimated from the dimensions given) is *c.* 70 times that of *Corythion*. Figures for naked amoebae of similar size to *Corythion* are lacking, and published data are available for only a relatively small number of Gymnamoebia species. Oxygen uptake rates reported by different authors for one such better studied genus, *Acanthamoeba* (*c.* $\frac{1}{3}$ the cell volume of *Corythion*), are in reasonable agreement, being in the range 0.007 to 0.025 nl h^{-1} per individual at 27–30°C (see Byers, 1979), although distinct mean values of 0.0043 and 0.03161 nl h^{-1} per individual at 20°C were determined for a single species (*A. castellani*) by Baldock and others (1982) as a result of employing two different methods of measurement. Making allowances for such factors (including differing cell volumes, experimental temperatures, etc.) the presently determined oxygen uptake rate for *C. dubium* at 20°C of 0.03408 ± 0.0095 nl h^{-1} per individual ($n = 17$, mean $\pm$ 95% confidence limits) indicates a respiration intensity of at least the same order of magnitude as that found for naked amoebae in general. A fuller consideration of the respiration data arising from the continuation of this study (which included measurements at a range of temperatures) is intended for future publications dealing with the respiration and energetics of Antarctic Protozoa.

Acknowledgements

This research was undertaken during the tenure of an NERC CASE Research Studentship. I wish to thank J. R. Caldwell for his instruction in gradient diver techniques, Drs W. Block and H. G. Smith for their advice throughout and the British Antarctic Survey for laboratory facilities.

Received 27 April 1984; accepted 13 July 1984

References

Baldock, B. M., Rogerson, A. *and* Berger, J. 1982. Further studies on respiratory rates of freshwater amoebae (Rhizopoda, Gymnamoebia). *Microbial Ecology*, **8**, 55–60.

Byers, T. J. 1979. Growth, reproduction and differentiation in *Acanthamoeba*. *International Review of Cytology*, **61**, 283–338.

Chapman-Andresen, C *and* Hamburger, K. 1981. Respiratory studies on the giant amoeba *Pelomyxa palustris*. *Journal of Protozoology*, **28**, 433–40.

Clark, L. 1956. Monitor and control of blood and tissue oxygen tensions. *Transactions of the American Society for Artificial Internal Organs*, **2**, 41–8.

Cowling, A. J. 1983. Respiration and growth of terrestrial Protozoa from the maritime Antarctic. Ph.D. thesis (unpublished).

Cunningham, B. *and* Kirk, P. L. 1942. The oxygen consumption of single cells of *Paramecium caudatum* as measured by a capillary respirometer. *Journal of Cellular and Comparative Physiology*, **20**, 119–34.

Danforth, W. F. 1967. Respiratory metabolism. (*In* Chen, T. T. *ed. Research in Protozoology*, Oxford, Pergamon Press, 201–306.)

Fenchel, T. *and* Finlay, B. J. 1983. Respiration rates in heterotrophic, free-living Protozoa. *Microbial Ecology*, **9**, 99–122.

Hamburger, K. 1975. Respiratory rate through the growth-division cycle of *Acanthamoeba* sp. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **40**, 175–85.

Hamburger, K., Kramhoft, B., Nissen, S. B. *and* Zeuthen, E. 1977. Linear increase in glycolytic activity through the cell cycle of *Schizosaccharomyces pombe*. *Journal of Cell Science*, **24**, 69–79.

Hamburger, K. *and* Zeuthen, E. 1974. Recording mitotic cycles in single cleaving frog eggs. Gasometric studies with the gradient diver. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **39**, 415–32.

HEAL, O. W. 1971. Protozoa. (*In* PHILLIPSON, J. *ed. Methods of study in quantitative soil ecology, IBP Handbook No.* 18, Oxford, Blackwell, 51–71).

HEINRICH, B. *and* COOK, J. R. 1967. Studies on the respiratory physiology of *Euglena gracilis* cultured on acetate or glucose. *Journal of Protozoology*, **14**, 548–53.

JAMES, T. W. *and* READ, C. P. 1957. Effect of temperature on cell size of *Tetrahymena pyriformis*. *Experimental Cell Research*, **13**, 510–16.

KALISZ, B. 1973. Stimulation of respiration in *Amoeba proteus* by inducers of pinocytosis. *Folia Biologica* (*Krakow*), **21**, 169–72.

KHLEBOVICH, T. V. 1974. Respiration intensity in ciliates of various body size. *Tsitologiya*, **16**, 103–6. [In Russian.]

KLEKOWSKI, R. Z. 1981. Ecology of aquatic organisms. 3. Animals; size dependence of metabolism on protozoans. *Verhandlungen für Internationale Vereinigung der theoretische und angewandte Limnologie*, **21**, 1498–502.

KLEKOWSKI, R. Z. SCHIEMER, F. *and* DUNCAN, A. 1980. Ampulla gradient diver microrespirometry. *Ekologia Polska*, **28**, 675–83.

LAYBOURN, J. 1975. Respiratory energy losses in *Stentor coeruleus* Ehrenberg (Ciliophora). *Oecologia*, **21**, 273–8.

LAYBOURN, J. E. M. 1976*a*. Energy consumption and growth in the suctorian *Podophyra fixa* (Protozoa: Suctoria). *Journal of Zoology*, **180**, 85–91.

LAYBOURN, J. 1976*b*. Respiratory energy losses in *Podophyra fixa* Muller in relation to temperature and nutritional status. *Journal of General Microbiology*, **96**, 203–8.

LAYBOURN, J. *and* FINLAY, B. J. 1976. Respiratory energy losses related to cell weight and temperature in ciliated Protozoa. *Oecologia*, **24**, 349–55.

LAYBOURN-PARRY, J., BALDOCK, B. *and* KINGMILL-ROBINSON, C. 1980. Respiratory studies on two small freshwater amoebae. *Microbial Ecology*, **6**, 209–16.

LEICHSENRING, J. M. 1925. Factors influencing the rate of oxygen uptake in single cells. *American Journal of Physiology*, **75**, 84–92.

LEINER, M., SCHWEIKHARDT, F., KONIG, K., BLASCHKE, G. *and* FISCHER, M. 1968. Die Garung und Atmung von *Tetrahymena pyriformis* furgason. *Biologisches Zentralblatt*, **87**, 593–608.

LINTS, C. V., LINTS, F. A. *and* ZEUTHEN, E. 1967. Respiration in *Drosophila*. 1. Oxygen consumption during development of the egg in genotypes of *Drosophila melanogaster* with contributions to the gradient diver technique. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **36**, 35–66.

LLOYD, D., PHILLIPS, C. A. *and* STATHAM, M. 1978. Oscillations of respiration, adenine nucleotide levels and heat evolution in synchronous cultures of *Tetrahymena pyriformis* ST prepared by continuous-flow selection. *Journal of General Microbiology*, **106**, 19–26.

LOVLIE, A. *and* ZEUTHEN, E. 1962. The gradient diver – a recording instrument for gasometric micro-analysis. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **32**, 513–34.

LUND, E. J. 1918. Quantitative studies on intracellular respiration. III. Relation of the state of nutrition of *Paramecium* to the rate of intracellular oxidation. *American Journal of Physiology*, **47**, 167–77.

MØLLER, K. M. *and* OTTOLENGHI, P. 1964. The manufacture of small standardized glass floats for calibrating density gradients. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **34**, 169–85.

NEXØ, B. A. HAMBURGER, K. *and* ZEUTHEN, E. 1972. Simplified microgasometry with gradient divers. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **39**, 33–63.

PACE, D. M. *and* LYMAN, E. D. 1947. Oxygen consumption and carbon dioxide elimination in *Tetrahymena geleii* Furgason. *Biological Bulletin*, **92**, 210–16.

PETERSEN, H. 1981. Open gradient diver respirometry modified for terrestrial microarthropods. *Oikos*, **37**, 265–72.

ROOT, W. S. 1930. The influence of carbon dioxide tension upon the oxygen consumption of *Paramecium* and the egg of *Arbacia*. *Biological Bulletin*, **59**, 48–69.

SMITH, H. G. 1973. The Signy Island terrestrial reference sites: II. The Protozoa. British Antarctic Survey Bulletin, Nos. 33 and 34: 83–7.

WEIK, R. R. *and* JOHN, D. T. 1977. Cell size, macromolecular composition, and O_2 consumption during agitated cultivation of *Naegleria gruberi*. *Journal of Protozoology*, **24**, 196–200.

ZAJICEK, J. *and* ZEUTHEN, E. 1956. Quantitative determination of cholinesterase activity in individual cells. *Experimental Cell Research*, **11**, 568–79.

ZEUTHEN, E. 1943. A Cartesian diver respirometer with a gas volume of 0.1 μl. *Comptes rendus des travaux du Laboratoire de Carlsberg*, **24**, 479–518.

ZEUTHEN, E. 1964. Microgasometric methods. Cartesian divers. (*In: Proceedings of the 2nd International Congress of Histo- and Cytochemistry, Frankfurt, August 1964*, 70–80.)

ZEUTHEN, E. *and* HAMBURGER, K. 1977. Microgasometry with single cells using ampulla divers operated in density gradients. (*In* GLICK, D. *and* ROSENBAUM, R. M. *eds. Techniques of Biochemical and Biophysical Morphology*, Volume 3, New York, Wiley Interscience, 59–80.)