QUANTITATIVE DETERMINATION BY A SPECIAL "AMPULLA-DIVER" OF CHOLINESTERASE ACTIVITY IN INDIVIDUAL CELLS, WITH NOTES ON OTHER USES OF THE METHOD

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I. INTRODUCTION

The most sensitive method hitherto evolved for cholinesterase (ChE) determination is the Cartesian diver microgasometer of Linderstrøm-Lang (1937) developed by him and Holter (Holter, this volume). With this apparatus analyses were made of serum samples (Linderstrøm-Lang and Glick, 1938) and tissue fragments (Boell and Shen, 1950) with ChE activity corresponding to the liberation of about 0 · 01 to 0 · 1 μl CO₂ per hour.

The following paper describes a Cartesian diver method which permits quantitative determination of enzyme activity in single megakaryocytes of rat bone marrow. The method measures the evolution of 10⁻⁴ μl CO₂ per hour with a sensitivity of ± 5%. It is a modification of the ampulla-diver respirometer (Zeuthen, 1953) used in conjunction with the sensitive manometer for measuring the respiration of single protozoan or egg cells (Zeuthen, 1953, 1955).
The substrate found to meet the requirements for both stability and specificity was the thioanalogue of acetylcholine (ACh), acetylthiocholine (AThCh). This ester was first synthesized by Renshaw et al., (1938), who noted that its pharmacologic actions were similar to those of ACh, but were of briefer duration. Glick (1939) reported that AThCh underwent more rapid hydrolysis, both enzymatically and non-enzymatically, than ACh. This finding was confirmed by Koelle and Friedenwald, who also introduced the thiocholine esters as substrates for the histochemical demonstration of ChE and acetylcholinesterase (AChE) (1949). With a modified thiocholine method it was demonstrated histochemically that the esterase found in the megakaryocytes was AChE (Zajicek, et al., 1953). This enzyme displays optimum activity at AThCh concentrations ranging from $4 \times 10^{-3}$ to $6 \times 10^{-3}$ M (Koelle, 1950). We therefore decided to measure the esterase activity in megakaryocytes with $6 \times 10^{-3}$ M AThCh as the substrate (Acetylthiocholine iodide, F. Hoffmann-La Roche, Basle).

<table>
<thead>
<tr>
<th>Solution % (w/v)</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.8</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.28</td>
</tr>
<tr>
<td>CaCl₂·6H₂O</td>
<td>1.34</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.4</td>
</tr>
</tbody>
</table>

The quantity of AThCh dissolved to make the concentration $6 \times 10^{-3}$ M was 35.4 mg. The pH of the AThCh-bicarbonate buffer solution when saturated with a mixture of 5% CO₂ and 95% N₂ is about 7.4 (Umbreit et al., 1949).

B. Preparation of Bone-marrow Cell Suspension

Bone-marrow cells from the femur of an 8-week-old albino rat were suspended in a solution of 5% sodium citrate (1 part) and 0.8% sodium chloride (4 parts). As the anticoagulant citrate is an inhibitor of ChE, the cells were washed free of citrate by centrifugation and were placed in
0.9% sodium chloride. Centrifugation at 1500 r.p.m. for about 45 sec then produced a fraction rich in megakaryocytes. A small drop of this fraction was resuspended in 0.5 ml of AThCh-bicarbonate buffer solution.

C. ISOLATION OF INDIVIDUAL MEGAKARYOCYTES

A few drops of the bone-marrow cell suspension in AThCh-bicarbonate buffer were placed on a hollow slide. Megakaryocytes, like platelets, display a strong tendency to adhere to glass. This makes the handling of individual megakaryocytes very difficult, if not impossible. To overcome this difficulty we first tried, without success, coating the slides with paraffin or silicone. It was finally found that agar gave the desired effect. On an agar-coated hollow slide the megakaryocytes behave as

Fig. 1. Polymorphonuclear megakaryocyte isolated from rat bone marrow and placed in diver (from Zajicek, 1956b).
though freely suspended. The cell to be investigated can thus readily be sucked into a braking pipette and transferred to another agar-coated slide. When the accompanying erythropoietic or granulopoietic cells have been pipetted off, the megakaryocyte is sucked into the diver (Fig. 1), to the position indicated in Fig. 2, V. With a little practice all these operations can be performed under a standard dissection microscope, without the aid of a micromanipulator.

D. The Manometric Measurement of CO₂

The method for determining ChE is best described graphically. Figure 2 and its extended legend give an impression of how the method functions. The reader is therefore advised at this point to consult Fig. 2.
QUANTITATIVE DETERMINATION OF CHOLINESTERASE ACTIVITY

Fig. 2. (I) The braking pipette. A capillary (diameter 0.5 mm) is pulled from a thin-walled test tube and is fitted into a glass jacket, using De-Khotinsky cement. The pressure is regulated by mouth, through rubber tubing. Details inside the dotted frame are enlarged in II to V.

(II) The lower portion of the braking pipette forms an "ampulla" approximately 3 mm long with a "shaft" measuring about 20 mm and a "tip" of about 10 mm. The internal diameter of the shaft and the tip is 40 to 60 μ.

(III) Substrate previously bubbled through with 5% CO₂ and 95% N₂ is sucked into the ampulla, shaft and adjacent parts of the pipette. This is followed by a bubble of the same gas mixture, taken from the slanted tube to the left (during interruption of the gas flow). Finally substrate is sucked into the tip and the lower part of the ampulla, leaving gas and fluids inside the diver in the positions indicated.

(IV) The cell is deposited in a droplet on an agar-coated hollow slide.

(V) The cell is sucked up to the position shown. The tip is sealed with a resin-wax mixture (1 part resin, 2 parts wax) heated to about 80°C. Breaking of the shaft at the site indicated separates the charged ampulla-diver from the pipette. The latter now has a sealed tip and what was the shaft becomes the "tail". The diver is transferred to a special vessel (VI) containing CO₂-N₂-bubbled substrate.

(VI) If the diver floats to the surface the stopper is inserted and the gas space may be reduced stepwise until buoyancy is attained at, or in the vicinity of, atmospheric pressure. This can be accomplished by suction (arrow), with return to atmospheric pressure whenever a small bubble is observed to escape through the tail of the diver. [By repeated suction, however, the liquid in the diver is depleted of gases and the time required for equilibration may thus be prolonged.

We therefore later worked with slightly overweight divers, i.e. diver with excessively long tails (2 cm) and regulated buoyancy by progressively shortening the tails]. From (VI) the diver is transferred to the flotation vessel of the "manometer" (VII), disconnecting and reassembling being carried out at (c).

The manometer is really a burette (d, e, f), by means of which accurately measured volumes of fluid can be withdrawn from, or injected into, a closed air space (b), thereby creating regulated pressure changes in the space. In our apparatus the approximate calibration of the burette is 1 μl/mm and that of the air space (b) with all ramifications 200,000 μl. A 1-mm shift of the meniscus (e) in the burette therefore corresponds to a pressure change of $5 \times 10^{-6}$ atm. or 0.05 mm H₂O. The diver floats in substrate in a pocket (a) which opens to (b). The assembled manometer is fixed at the edge of a special (±0.002°) water bath (supplied by O. Dich, Avedøre, Copenhagen), in which (a), (b), (c) and (d) are submerged. The manometer is perfused with 5% CO₂ and 95% N₂ through narrow polyethylene tubing, which is introduced deep into the pocket (a) (cf. insert B). The tubing passes the 3-way stopcocks g and d above the flotation vessel (insert A). The flow is continued for 20 min at an approximate rate of 50 ml/min. The gas escapes at (h), (i). Meanwhile the burette (d), (e) and the water manometer (k), (l) are cleared of air by pumping, using screws (f) and (l). The flow of gas is stopped by disconnecting the polyethylene tubing (insert A) and closing the stopcocks (g) and (h) to the atmosphere. With the water level (c) in a position suitable for the proposed measurements, the initial flotation pressure of the diver is adjusted and read with the aid of the manometer k, l. Finally (d) is closed to the atmosphere. During the actual experiments—which may now be begun—gas exchange in the diver is read as the pressure changes (movements of the meniscus (e)) necessary to keep the diver afloat at a predetermined level. If readings are discontinuous a suitable excess pressure must be applied between the measurements, so that the diver remains at rest on the bottom of the flotation vessel (from Zajicek and Zeuthen, 1956).
The method discussed is for a standard diver, gas volume 0·5 μl, liquid charge 0·6 μl. It is easy to charge divers with less than 0·6 μl of liquid, and indeed in most of our experiments the charge was only about 0·2 μl. A large liquid charge, however, causes increase of the initial period ($t_0$, cf. p. 140) before gas formation or gas uptake can be registered manometrically. The standard diver then provides less favourable experimental conditions. Since the absorption coefficient for CO$_2$ in water at 22°C is 0·83, our standard diver compares with a 1 μl-diver without liquid charge.

The diver floats only if made of glass so thin that it readily cracks during manipulation with fingers. In all the steps of making and filling, however, the diver remains attached to the braking pipette capillary from which it is pulled, and need not be touched by hand. Our divers were made from thin-walled test tubes (Jena glass) pulled in several operations to the dimensions indicated in Fig. 2. It is not essential that the cross-section of the capillary (diameter c. 0·5 mm) used for the braking pipette be circular. Most often, in fact, it is not. The “shaft” and “tip” of the “ampulla” (cf. legend, Fig. 2) are pulled in a microflame without rotation of the glass. The shaft becomes the “tail” of the diver. The glass is heated from one side when the shaft is pulled and from the other side when the tip is made. Thus the glass tapers excentrically into both tip and shaft, but the balance of the two is well adjusted and the diver will float upright (Fig. 2, VI). Personal experience is the only true guide in the choice of glass and in the method of pulling it to form a diver which can be made to float with a reasonable charge, i.e. one not essentially different from that illustrated in Fig. 2.

E. Calculation of CO$_2$ Evolution

The formula reported in earlier papers (Zeuthen, 1953, 1955) was used for calculating exchange of gases with low solubility, such as O$_2$. When measuring the evolution of CO$_2$ the amount of this gas dissolved in the liquid contents of the diver must be taken into consideration. Some uncertainty remains concerning the extent to which the liquid in the narrow channels in both ends of the diver—far removed from the gas space—serves as a solvent for the CO$_2$, formed during the experiments. This liquid volume, however is very small. In the experiments presented in Fig. 4 it comprised less than 5% of the liquid in the diver and less than 3% of the total volume of the diver. The formula employed in this study for calculating CO$_2$ evolution was

$$\frac{CO_2}{t} = x \times \left( \frac{v \times V_G \times (B + h - e) \times 273}{V \times 10300 \times (273 + f)} + \frac{v \times V_F \times \alpha CO_2 \times (B + h - e)}{V \times 10300} \right)$$
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in which \( x \) is the change in scale reading (mm per accepted time unit), \( v \) is the volume (\( \mu l \)) per mm burette and \( V \) is the volume of the gas phase of the "manometer" (\( \mu l \)); in our apparatus \( v/V \) was almost 1/200,000. \( V_G \) is the gas volume of the floating diver. \( B \) is the barometric pressure in mm H\(_2\)O (760 mm Hg; 10,300 mm H\(_2\)O), \( h \) is the initial equilibrium pressure of the diver (mm H\(_2\)O) read as the difference in height of the menisci on the double-branched manometer \( (k) \) (cf. Fig. 2, in which the initial pressure \( h \) is positive), \( \varepsilon \) is the vapour tension of water at \( t^\circ \) (mm H\(_2\)O), \( V_F \) is the volume of water in the diver (\( \mu l \)) and \( \alpha \)CO\(_2\) is the absorption coefficient for CO\(_2\) at the temperature during the experiment.

F. DETERMINATION OF \( V_F \) AND \( V_G \)

The volume of the liquid charge \( (V_F) \) is computed by subtracting the weight of the diver after oven-drying from the weight of the diver as soon as the actual experiment ends—after washing in distilled water, removal of external liquid with a strip of filter paper and brief exposure to the air. The value thus arrived at is modified by the correction \( V_G(\varepsilon/B) \), to be added if the diver floats at negative pressure. The volume of gas \( (V_G) \) in the floating diver is determined as follows. There is a constant ratio \( (f) \) between the weight of the glass used for the diver and the volume of air required to make the aggregate of glass and air buoyant in the medium. \( f \) is determined independently for the type of glass used. \( V_G \) is the dry weight of the diver multiplied by \( f \).

III. RESULTS

A. CONTROL EXPERIMENTS

We are aware that our filling procedure (cf. stages III to V, Fig. 2) does not ensure initial saturation of the medium in the diver with 5\% CO\(_2\) and 95\% N\(_2\). It is possible for the pretreated medium to lose to the atmosphere some of its CO\(_2\) and N\(_2\) while being poured into the dish (Fig. 2, III) or on to the hollow slide (Fig. 2, IV) and to take up small amounts of O\(_2\). When the diver has been filled, sealed and balanced to buoyancy, therefore, the gases will diffuse to equilibrium inside the diver. This process may last long when the diffusion distances in the system are considerable. With the same concentration gradient the amount of any one gas diffusing must vary directly with the absorption coefficient. Reference is made to note 3.

Because of its high \( \alpha \) the diffusion of CO\(_2\) can be expected to dominate over that of other gases. It should be possible to define filling procedure and diver dimensions so as to reduce to an acceptable minimum the time necessary for initial equilibration of the control diver. Our search for
such conditions, however, led us to conclude that other sources of error play a dominant role. Glass, when suddenly exposed to CO$_2$-bicarbonate, seems to absorb CO$_2$ and to continue to do so for more than 24 hr. Figure 3 shows the series of control experiments from which this inference was drawn. Curve I in this figure shows typical scale readings for a freshly-pulled control diver charged with 5% CO$_2$ and 95% N$_2$, and with bubbled bicarbonate buffer medium as described on p. 135, but without AThCh. The flotation medium was the same as that in the diver charge. The manometer was perfused with gas as shown in Fig. 2. Curve I indicates that the diver became heavier with high initial rates. The system did not approach equilibrium for about 24 hr. This is much longer than we anticipated, judging from calculations based on formulas published by Linderstrom-Lang (1943, 1946).

With a slight variation in procedure we obtained quite different results. We interrupted the diver fillings at stage III (Fig. 2) for 24 hr, during which time the filled ampulla was kept in air. We then blew the ampulla empty and refilled it, but this time concluding the procedure and setting the diver afloat as described above. Curve II, Fig. 3, shows a typical result. Pre-conditioning of the glass by prolonged internal contact with bicarbonate (curve II) or with AThCh-bicarbonate solution (curve IV) clearly eliminated the initial phase in which the divers rapidly become heavier (from Zajicek and Zeuthen, 1956).

![Fig. 3. The scale readings for freshly-pulled control divers charged with bicarbonate buffer solution (curve I) and with acetylthiocholine (AThCh)-bicarbonate solution (curve III). The preconditioning of the divers by prolonged internal contact with bicarbonate (curve II) or with AThCh-bicarbonate solution (curve IV) clearly eliminated the initial phase in which the divers rapidly become heavier.](image-url)
opinion the improvement as compared with curve I resulted from auto-
hydrolysis of AThCh, with subsequent liberation of CO₂ wherever the
CO₂ tension inside the diver was below that corresponding to 5% (in gas
of atmospheric pressure), and the pH thus higher than the stability limit
of the substrate. This implies that the non-conditioned diver filled with
CO₂-bicarbonate and in contact with this medium becomes progressively
heavier because CO₂ is lost from the diver's gas phase. Since this loss
does not occur by diffusion (cf. curves I and II), we suggest that the glass
continues to adsorb or absorb CO₂ for 24 hr or more after it is brought
into contact with CO₂-bicarbonate.

With the diver preconditioned by 12 hr internal exposure to CO₂-bi-
carbonate with added AThCh, and then refilled as described above with
AThCh-bicarbonate solution, curve IV is obtained. In five experiments it
was established that after an initial period of about 30 min such divers
showed no further significant change in equilibrium pressure. Over 8 hr
the scale readings were constant within ±10 mm, or within a pressure
range of less than 1 mm H₂O. During the 30-min initial period, however,
the equilibrium pressure in some divers showed variations up to ±20 mm.
See also the following Section B.

B. CHOLINESTERASE EXPERIMENTS

In the ChE experiments the divers were first filled with AThCh-bi-
carbonate solution plus gas, and the tips were sealed with wax. After

![Graph](image)

**Fig. 4.** Cholinesterase activity of a megakaryocyte (upper curve) and of a
megakaryoblast (lower curve) (from Zajicek and Zeuthen, 1956).

about 12 hr the seals were broken off and the solution present in the tips
was expelled. Fresh substrate-bicarbonate solution was sucked into the
divers along with a cell, and stages V to VII of Fig. 2 were carried out.
ChE activity curves of megakaryocyte (diameter 48 μ) and of a megakaryoblast (diameter 24 μ)—both from rat—are shown in Fig. 4. The experiments were conducted at 25° C. The megakaryocyte (upper curve) liberated from the bicarbonate buffer $100 \times 10^{-5} \mu l \text{CO}_2$ per hour, and the megakaryoblast (lower curve) $19.2 \times 10^{-5} \mu l \text{CO}_2$ per hour. These values correspond to the hydrolysis of about $0.013$ and $0.0024 \mu g$, respectively, of AThCh per hour.

The constants for the two divers were: $V_G = 0.8 \mu l$, $V_F = 0.44 \mu l$ (upper curve), and $V_G = 0.54 \mu l$, $V_F = 0.24 \mu l$ (lower curve).

After the completion of the experiments presented above, it was found that the observed absorption of carbon dioxide by the divers pulled from Jena glass did not take place in divers made from Phoenix glass. The use of Phoenix glass, therefore, eliminates the time-consuming pre-conditioning of the wall of the diver, and makes it possible to introduce the cells immediately after the divers have been charged with the gas bubble and the substrate. Phoenix glass has not recently been easily available. It may be replaced by Pyrex or other heat-resistant glass. The user of the method is advised to perform proper control runs with the type of glass selected for use. The substrate used is $5 \times 10^{-3} \text{M acetylcholine chloride}$.

The non-enzymatic hydrolysis of $0.1 \mu l$ of acetylcholine-bicarbonate solution (pH 7.4) was below the sensitivity of the present diver method.

IV. COMMENTS

A. THE INITIAL PERIOD

When the control diver, filled and treated as described for the typical experiment of Fig. 3, curve IV, is set afloat, we have frequently observed considerable (± 20 mm scale) changes of its equilibrium pressure. These initial changes, which usually involve less than the first half-hour of the experiment, probably reflect the diffusion to equilibrium of the gases inside the diver. It is essential to point out that the time ($t$) required for diffusion to equilibrium of a finite amount of CO$_2$, introduced at time 0 into a defined location in the diver, is expected to be much longer than the time ($t_e$) which must elapse before constant rate of gas formation or gas uptake is reflected in linear changes in equilibrium pressure with time (Linderstrom-Lang, 1943). In experiments with a biologic object, $t_e$ and not $t$ is of interest. The purpose of the following evaluation is to give some idea of the dependence of $t_e$ on the dimensions of the diver, a question of considerable practical importance.

According to Linderstrom-Lang (1943), $t_e$ varies with $b^2$, $b$ representing the length of the diffusion path in the liquid medium in the diver. The $t_e$ value for CO$_2$ is little different from that for O$_2$. Linderstrom-Lang
(1946) applied calculations to a diver system resembling the sketch A in Fig. 5. The diver is charged with two columns of liquid, 3 and 2 mm long (total volume 16 μl), separated by an air bubble (volume 2 μl). The respiring layer is situated in the smaller water volume, either adjacent to the air bubble or close to the impermeable membrane which forms the floor of the diver chamber. When the respiring sheet is adjacent to the air bubble the value of \( b \) which determines \( t_s \) was accepted to be close to the average length of the two columns (Linderstrøm-Lang, 1946, p. 269). Our diver system closely resembles the system discussed by Linderstrøm-Lang. The actual shape of the charged diver may be any of those shown in Fig. 5. Lines have been drawn across the divers at distances of 0.5, 1.0 or 1.5 mm from the gas bubble, and the calculated values for \( t_s \) are reproduced. They are derived from the calculation that \( t_s = 100 \text{ min} \) for \( b = 2 \text{ mm} \) (Linderstrøm-Lang, 1946, p. 247, Table 4), accepting that \( t_s \) varies with \( b^2 \). The fact that the diver tapers off at both ends probably exaggerates the values given for \( t_s \).

The situation, however, is somewhat complicated by the fact that the diver—in addition to the liquid between the lines in Fig. 5—carries small volumes of liquid in the long tail and tip. If the volume in these channels is not allowed to exceed 5% of the total volume (air plus fluid) of the diver, its influence on the measurements can be disregarded, provided we do not claim more than about 5% accuracy for the method. In any case it is probably difficult, for other reasons, to improve on this.

In our experiments the initial period required about 30 min, which is the time necessary for performing stages V, VI and VII of Fig. 2. The readings, however, were not commenced until 30 min after closing the stopcock \( d \) to the atmosphere (stage VII, Fig. 2), when the control divers were shown to have attained equilibrium.
B. LEAKAGE OF CO\textsubscript{2} FROM THE DIVER

The equilibrium pressure of the control diver remained constant within 1 mm H\textsubscript{2}O (20 mm scale) for about 8 hr. This did not necessarily indicate that the diver was impermeable to CO\textsubscript{2}. It may merely have reflected the establishment of complete equilibrium between the gases inside and around the diver. Introduction of the cell entailed a CO\textsubscript{2} concentration difference which increased as CO\textsubscript{2} formed in the diver in the course of the experiment.

With our apparatus the capacity for pressure adjustment, without resetting of the instrument, is exhausted when the CO\textsubscript{2} tension in the diver exceeds the tension in the flotation medium by 35 mm H\textsubscript{2}O (initial equilibrium is assumed; length of burette—700 mm—corresponds to 35 mm water pressure). It is therefore of interest to calculate the maximum diffusion loss of CO\textsubscript{2}, i.e. when the gradient is 35 mm H\textsubscript{2}O. The formula employed is:

\[
\frac{\Delta \text{CO}_2}{h} = \frac{\delta \text{CO}_2 \times A \times (p_1 - p_2)}{1 \times B}
\]

\(\delta \text{CO}_2\) is the "standard rate of passage" of CO\textsubscript{2} in water. It is defined as \(\mu l\) passing through a column of 1 mm\(^2\), 1 mm long, in 1 hr, when the gradient is 1 atmosphere. Using Hufner’s values (Krogh, 1919, p. 407) we find that \(\delta \text{CO}_2 = 5 \cdot 4\); \((p_1 - p_2)\) is 35 (mm H\textsubscript{2}O); for \(B\) is introduced 10,300 (mm H\textsubscript{2}O). The cross-sectional area \((A)\) of the tail of the diver, through which pressure adjustment takes place, varies somewhat, but can easily be kept at less than 0·003 mm\(^2\) (diameter 60 \(\mu\)). As a rule the tail is longer than 15 mm, but not at its narrowest part. Hence we have inserted \((10 \text{ mm})\) for 1 in the above formula. For \(A = 0\cdot003\) mm\(^2\) we find \(\Delta \text{CO}_2/h = 5 \times 10^{-6}\mu l\).

In our standard diver a pressure change of 35 mm H\textsubscript{2}O corresponds to the formation or consumption of \(3 \cdot 5 \times 10^{-3}\mu l\) CO\textsubscript{2}. If this amount of CO\textsubscript{2} were linearly produced over 1 hr \((p_1 - p_2)\) would average 17·5 mm, and the CO\textsubscript{2} loss by diffusion through the diver tail would amount to 0·7\% of the simultaneous production in a diver with a tail 60 \(\mu\) in diameter. But we seldom measure enzyme activities high enough to liberate CO\textsubscript{2} at that rate. With the production of CO\textsubscript{2} constant at, say, \(3 \cdot 5 \times 10^{-4}\mu l\) per hour, the experiment can be run for 10 hr before the CO\textsubscript{2} tension in the diver reaches the accepted maximum of 35 mm H\textsubscript{2}O. The loss of CO\textsubscript{2}—in percentage of the simultaneous CO\textsubscript{2} production—evidently increases linearly with time, and attains a maximum by the end of the experiment. In the last hour it will be 1·4\% of the simultaneous CO\textsubscript{2} production in the diver in question. It should also be pointed out that
for identical divers and for any fixed hour of the experiment the leakage of CO₂ represents a constant percentage error, irrespective of the rate of production.

We have not considered diffusion through the tip of the diver, assuming the solid wax to be impermeable to gases. Although there is some justification for this assumption (Zeuthen, 1943, Sect. XII, p. 3), we may be mistaken. In that case CO₂ leaks from the diver about twice as fast as the calculated rate. In other respects, however, we have assumed more adverse conditions, i.e. favourable for diffusion. We feel justified in stating that the diver used can be made impermeable to CO₂ in the sense that leakage is negligible as compared with other sources of error in the measurements. The tip and tail of the standard diver should not be wider than 60 μ and the length of the narrow parts should exceed 10 mm.

C. Sensitivity and Accuracy of the Method

The sensitivity of the method for absolute measurements is limited by the instability of the control diver. As reported in the foregoing, five runs with control divers showed that after an initial period of about 30 min the equilibrium pressure was stable within 1 mm H₂O for 8 hr. Stability is not substantially better over 1 hr than over many, as the scale readings vary rapidly within the 0·5 mm H₂O limit, probably reflecting the offs and ons of the heater in the water bath. A pressure change of 10 mm H₂O (200 mm scale) accumulated in an experiment can thus be measured with 5% accuracy. Accordingly, if 8 hr is the time stipulated to be the maximum duration of an experiment and the standard diver is used, half of the CO₂ being dissolved in the liquid, 10⁻⁴ μl CO₂ per hour will be measured with ± 5% accuracy. It is advisable, however, either to select the biologic object or to reduce the dimensions of the diver below those of the standard diver, so as to obtain more pronounced pressure changes. This will probably enhance the accuracy of the method.

The accuracy thus claimed for our diver technique of ChE determination is theoretical. We therefore checked the method by charging three divers with equal amounts of purified human plasma ChE. The error in charging the divers with this soluble plasma ChE was about 5%. For diver 1: \( V_G = 0.54 \ \mu l, \ V_F = 0.24 \ \mu l \); for diver 2: \( V_G = 0.38 \ \mu l, \ V_F = 0.15 \ \mu l \); and for diver 3: \( V_G = 0.4 \ \mu l, \ V_F = 0.12 \ \mu l \). The evolution of CO₂ in μl per hour was 22·1 \times 10⁻⁵ in diver 1, 21·0 \times 10⁻⁵ in diver 2 and 20·2 \times 10⁻⁵ in diver 3. The experimental evidence thus supports the accuracy claimed for the method on the basis of theoretical considerations.
V. SUMMARY

A Cartesian diver method is described for the determination of cholinesterase activity in single somatic cells (megakaryoblast, megakaryocyte). A megakaryoblast 24 μ in diameter was found to hydrolyse about 0.0024 μg, and a megakaryocyte measuring 48 μ, 0.013 μg of acetylthiocholine per hour. The accuracy of the method is discussed.

VI. NOTES

1. The bath (± 0.002°C) and the double-branched water manometer is that shown in Fig. 2 of Holter’s account in this volume. The sensitive manometer (Fig. 2, this paper) is hung over the edge of the bath in much the same way as a Warburg manometer. The air space b is submerged to between the stopcocks d and g, the burette and scale is fastened on the front side of the bath: Up to four manometers may be run simultaneously. The photograph Fig. 6 shows the general set-up.

Fig. 6. General set-up for work with the ampulla diver.

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2. The original method (Fig. 7, with text) for respiration (Zeuthen, 1953) is simpler than the one here described for CO₂. For respiration measurements the diver is charged with air, and the manometers are not gassed. Stopcock \( g \) in Fig. 2 may be omitted, and the water manometer is hooked directly to the free vertical glass tubing above \( d \). In the equation, p. 136, the second member in the parenthesis drops out. This method has been used also for determining succino-oxidase, succinic dehydrogenase and cytochrome oxidase in individual Deiter’s nerve cells and in lumps of glia cells, together representing the same mass as a nerve cell (Hydén, et al., 1958) and Hydén and Pigoń (unpublished, cf. Hydén 1958, 1959).

Substrates for cytochrome oxidase (Slater, 1949a; Potter, 1949):

(a) With sodium ascorbate as reducing agent: Phosphate buffer, pH 7.4, 0.0375 M; cytochrome c (Sigma) \( 10^{-4} \) M; Na-ascorbate 0.0125 M; \( \text{AlCl}_3 \) \( 5 \times 10^{-4} \) M.

(b) With \( p \)-phenylenediamine as reducing agent: Phosphate buffer, pH 7.4, 0.043 M; cytochrome c \( 1.2 \times 10^{-4} \) M; \( p \)-phenylenediamine 0.043 M; \( \text{AlCl}_3 \) \( 5.7 \times 10^{-4} \) M.

Substrate for succinic dehydrogenase (Slater, 1949b):

Phosphate buffer, pH 7.4, 0.075 M; Na-succinate 0.025 M; methylene blue 0.009 M; KCN, neutralized, 0.009 M.

The flotation media used for the divers were of the same composition as the respective substrates, except that cytochrome \( C \) and methylene blue were omitted, and sodium ascorbate and \( p \)-phenylenediamine were replaced by isotonic concentrations of sodium chloride. The fact that the flotation medium is, or may be, continuous with the substrate solution in the diver, makes the determination of enzymes by \( O_2 \)-uptake in certain respects simpler than respiration measurements in which the air bubble must constitute a safe separation between the alkaline flotation medium and the biologic reaction mixture (Zeuthen, 1953).

3. We have later (Brzin and Zeuthen, 1961) introduced a funnel (Fig. 8, VII), in which the operations III, IV and V shown in Fig. 2 may be performed. 5% CO₂, 95% N₂ flows continuously through the funnel. This ascertains that the substrate solution is saturated with the gas mixture with which first the diver and later the manometer is charged. The “tip” of the diver is sealed with a wax-resin mixture (2:1) which is deposited on a wire to be electrically heated. When, upon heating, the mixture just melts, the diver is sealed (wax-resin to water) in a quick operation: touch—suck—withdraw. To simplify the illustration only the sealing is shown to take place in the funnel. Steps V and VI are or may also be performed in the flow of \( N_2, CO_2 \).
Fig. 7. (I) The tip of a glass capillary is pulled in a microflame to form what is to be the diver respirometer. The pointed tip should be about 5–7 mm long, the wider piece about 1 cm, and the narrow channel about 1 cm. Inner dimensions of the order 50μ, 500μ, and 50μ, respectively. To select the right capillary from which to make the diver one should aim at a tube which will float in N/10 NaOH when filled with air to about half its length. After transfer to the vacuum bottle shown below in the figure the diver is made buoyant. Suction is applied so that first the nutrient medium filling the narrow channel, then air, is sucked out of the diver. When normal pressure is re-established, air has become replaced by alkali. This is repeated in steps so that buoyancy is approximated.

(II) The diver floats in N/10 NaOH in (a), thus freely exposed to the pressure prevailing in the air-space (b) which extends through (c) and (d) to the water surface (e). At the beginning of the experiment the pressure in (b) is adjusted by mouth through (n), (m), (l), with (d) in the position shown in the figure. With (m) closed, the manometer (k) is useful for the fine regulation of the initial flotation pressure in (b), (n) is a CO₂-trap; (l) is an air-brake. During actual measurements (d) is closed to (l), (m), (n) and to (k), but open to (e).

The volume of the "burette" from 0 cm to 70 cm is less than 2% of the volume of (b), extending to (e). Therefore linear movements of the indicator bubble (g) in the (very uniform) "burette" sets up proportional (within 2%) pressure changes in (b). The system can be reset when (g) has been moved across the scale. The gas exchange per unit time \( \Delta O_2 / \Delta t \) is calculated from the formula

\[
\frac{\Delta O_2}{\Delta t} = \frac{x \times v \times g_D \times f(B + h - e) \times 273}{V \times 10300 \times (273 + f)}
\]

in which \( x \) is the movement (in mm per unit time) of the bubble (g) in the "burette", \( v \) is the volume (μl) per mm "burette", \( V \) is the volume of (b) extending to (c) (c. 100,000 μl; \( v/V = 2 \times 10^{-5} \)), \( g_D \) is the dry weight (mg) of the diver determined after
Fig. 8. Technique for refilling divers. The hole in the stopper is pierced with a hypodermic needle which is then withdrawn leaving the diver's tail inserted in the stopper (from Brzin and Zeuthen, 1961).

4. Refilling of divers. Adding reactants and inhibitors in the course of the experiment. The specificity of AChE is best defined by its reaction to a number of inhibitors. In view of the fact that the activity for AChE varied considerably from one cell to another, Zajicek (1957) developed techniques which permitted a diver to be reused after full or partial refilling. A single cell could then be studied first in the absence, then in the presence of the inhibitor. In this procedure (Fig. 9) the diver is broken off the experiment. $f$ is a factor—determined independently for the glass from which the diver is made—indicating the ratio between volume air ($\mu l$) and dry weight (mg) of glass when the system is buoyant. $* B$ is the barometric pressure, mm Hg (760 mm Hg ~ 10300 mm H$_2$O). $h$ is the initial equilibrium pressure of diver read on (k) as the difference in height of the menisci (mm). $e$ is the vapour tension of water (or of N/10 NaOH and of 2% proteose-peptone) at $r'$ (mm H$_2$O).

In the present studies $h$ in the equation has been small compared to $B$. It should be noticed, however, as an interesting and promising feature of the present method, that $x$ varies inversely with $(B + h - e)$. Therefore the sensitivity of the system should increase greatly at low pressures and should approach infinity when $h$ approaches negative values equal to $B - e$ (from Zeuthen, 1953).

* $g_D \times f$ thus equals $V_0$ in the equation p. 136.
from the tail (a) before it is charged and sealed. The tail is excessively long. A small amount of water is introduced via the tip by briefly touching a water surface. The diver is then quickly submerged so that the air in it is locked between two menisci, one in the tip and one in the tail. The diver is tested for buoyancy. At this stage the diver should be too heavy because of the long tail. Buoyancy is obtained by chopping small bits off the tail. Divers which are buoyant with a minimum of liquid charge are used (Fig. 9 (c)).

To charge the diver with a cell, the tail is now inserted into a fine rubber tubing (inner diameter about 200 μ) which is fitted over a braking pipette containing the biological medium used (Fig. 9 (b)). By compressing the lumen of the rubber with a screw, the diver is held firmly in position. The filling procedure may now be continued according to the type of experiment to be performed. The final sealing is with hot wax. After the enzymatic hydrolysis of the substrate has been determined, the diver is removed from the flotation vessel and fastened by the tail to the braking pipette as before. After removal, by cutting with a diamond, of the waxed part of the tip (unsignificant in terms of weight) the diver is refilled with the same cell, but this time with a substrate containing the inhibitor. This method may not work with all cells. Whether or not the cell adheres to the glass is important; so is the degree of mechanical stability of the cell and of chemical stability of the enzyme. A further development is illustrated in Fig. 9, I-V (from Brzin and Zeuthen, 1961).

In the second filling the amount of inhibitor introduced may be controlled as follows: a small but well-defined volume of the inhibitor-substrate solution is placed under paraffin oil on a slide or in a capillary.
Without blowing anything out of the diver, this volume is quantitatively taken into the diver, and suction is continued until the oil-water interphase reaches the close vicinity of the megakaryocyte which always attaches itself somewhere to the glass. Mixing is accomplished by pushing the interphase back and forth a couple of times. The diver is then resealed and set afloat. The inhibitor concentration is defined if also the volume in which the cell was first introduced has been defined analogously to what has just been described. An illustration of a slight modification of Zajicek's procedure is shown in Fig. 10 (from Giacobini, 1959b).

Figure 11 (Giacobini, 1957) shows four curves which, listed from below, represent: a control, a 110-μ-long piece of the neurite from a sympathetic neurone from the frog, a sample of purified ChE, and the cell body (25 μ) of the sympathetic neurone mentioned. The interruptions of the three latter curves reflect the introduction of $5 \times 10^{-6}$ M mipafox, which inhibits unspecific esterases.

5. The methods described have been applied without further changes in a number of haematological and neurochemical studies in which it was desirable that single cells be studied. Zajicek (1956a) followed the increasing contents in AChE of rat megakaryoblasts (24 μ) maturing to megakaryocytes (45–55 μ). The measurements were in the range
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1 \times 10^{-4} to 15 \times 10^{-4} \mu l \text{ CO}_2 \text{ per hour. He \ (1956b, 1957) confirmed his histochemical results \ (1954) on the inverse distribution of } \text{AChE} \text{ between erythrocytes on the one side and platelets on the other. The hypothesis was advanced that the megakaryocytic and the erythropoietic cells have a common origin in the body. It is at the level of this origin that the enzyme is partitioned, differently in different species of animals, between the two lines. Clearly, the platelets are in the megakaryocytic line.}

![Graph of AChE activity curve](image)

**Fig. 11.** AChE activity curve of a cell body (25 \mu) isolated from a sympathetic ganglion of the rat (〇), that of 110 \mu long part of its neurite (□), of a sample of purified ChE (●), and of a control buffer-substrate solution (×) (from Giacobini, 1959b).

The diver was used for single motor end-plates by Brzin and Zajicek (1958), by Giacobini (1959c) and by Brzin and Zeuthen (1961). In the latter case it was accepted as the standard against which a gravimetric method for AChE was checked (cf. the chapter on the diver balance in this volume).

Giacobini and Zajicek (1956) initiated a series of studies by Giacobini (1957, 1959a, b, c (c is a review)), and by Giacobini and Holmstedt (1958) in which the AChE in single neurones and their constituent parts was determined. In single sympathetic ganglion cells from the frog and from the rat, the AChE varied from 2 to 30 \times 10^{-4} \mu l \text{ CO}_2 \text{ per hour. No classification into uniform groups was possible (Giacobini, 1957). The spinal ganglion cells of the rat showed about the same content of AChE, and the same variation per unit volume. Two separate groups of cells, one
with a high level of enzyme and one with a low level, could be distinguished. The AChE is several times higher in the axon than in the cell body. AChE is much lower in the motor horn cell than in the spinal ganglion cell (Giacobini, 1959a, c).

Using his micro-edition (0.005 μl) of this diver, Giacobini (1959b) sampled the neurone at various levels. The nucleus or μl samples of cytoplasm, of nucleoplasm or of nucleoli were removed from the cell. The equilibrium pressure of the controls was constant within 3.5–7.5 mm H2O over 12 hr. For a 0.005 μl diver this would correspond to a stability of 10^-7 μl CO2 per hour. The isolated nucleus of a large anterior horn cell showed an activity of 5 × 10^-6 μl CO2 per hour. A sample of cytoplasmic material exhibited the same activity, and a sample of nuclear sap evolved 4 × 10^-7 μl CO2 per hour. (For more data, see Table III in Giacobini, 1959c.) It would have been useful had the proper controls been published. The experiments demonstrate at least the possibilities of this diver method for the study of the intracellular distribution of enzymes. It is the inherent beauty of the method that the diver itself is a pipette before it becomes a diver-gasometer. It may therefore be used for the removal of samples from inside individual living cells.

REFERENCES


