A METHOD FOR MEASURING THE RESPIRATION OF ANIMAL CELLS *IN VITRO*, WITH SOME OBSERVATIONS ON THE MACROPHAGES OF THE RABBIT.

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CONVENTIONAL manometric respirometers are unsuitable for measurements on living animal cells *in vitro* for two main reasons. First, most of these cells survive for only very short periods at 37° in the buffered salt solutions commonly used in manometric respirometry. If serum, or some other biological fluid in which the cells do survive, is used as the medium, the respiration of the cells can only be measured indirectly, and with considerable error. Second, in order to insure that the uptake of oxygen by the cells is not limited by its rate of diffusion through the medium, continuous agitation of the respirometer flask is necessary. Many cells are killed by this treatment. In the present paper a new method for measuring cellular respiration is described. The method is thought to have a general application in respirometry, but it has been more particularly designed for studies on living animal cells. Some observations carried out by this method on the macrophages of the rabbit are reported.

EXPERIMENTAL.

The respiration of the cells was measured by a modification of the electro-chemical circuit described by Tödt, Teske, Windisch, Heumann and Goslich (1952). Electrodes were introduced into a coverslip chamber which contained the cells. The chamber was completely filled with medium, so that there was no gas space. The oxygen uptake of the cells was determined by measuring the fall in the O_2 tension of the medium.

The cells.

Macrophages were obtained from the peritoneal cavity of the rabbit by the technique originally described by Lucké, Strumia, Mudd, McCutcheon and Mudd (1933). Paraffin oil was injected into the peritoneal cavity and the resulting exudate removed some days later. After separation from the paraffin oil, the cell suspension obtained was centrifuged at 220 *g.* and the cells re-suspended at a suitable density in a medium containing 25 per cent of rabbit serum in Hanks' solution (Hanks, 1948). This suspension was introduced into a coverslip chamber.

The chamber.

The coverslip chamber was a modification of the one devised by Mackaness (1952). A plan of the chamber is shown in Fig. 1. The fluid in the chamber was stirred by a steel wire enclosed in a fine glass tube which rested on the Perspex ring attached to the bottom coverslip. This wire was rotated magnetically. After the chamber had been filled with the cell suspension, it was incubated at 37°. The cells settled out on the bottom coverslip and became adherent to it in about 15 min. (Fig. 3). The medium was withdrawn after the macrophages had adhered

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to the bottom coverslip, in order to remove any other cell types. Lymphocytes do not adhere to glass and polymorphonuclear leucocytes adhere much less tenaciously than macrophages ; the small number of these cells present in the exudate was thus removed by withdrawal of the medium.

The chamber was refilled completely with fresh medium. The O_2 tension and pH of this medium had been adjusted to the range required by gassing with either 5 per cent of CO_2 in oxygen, 5 per cent of CO_2 in air or 5 per cent of CO_2 in nitrogen. The number of cells adherent to the floor of the chamber was then counted directly under phase-contrast microscopy. The electrodes were introduced into the chamber through lateral drill-holes and the seals made air-tight with paraffin oil. The steel wire was rotated on the Perspex ring by placing the

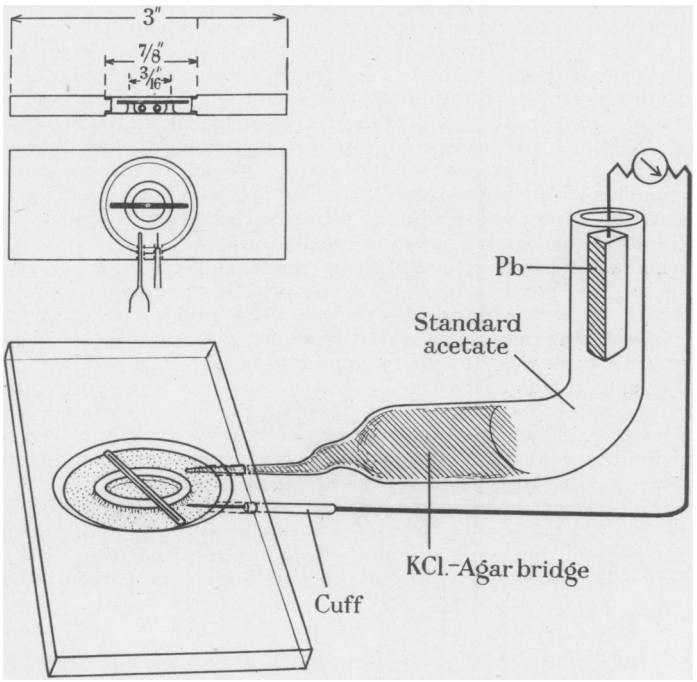

FIG. 1.—Plan of the electrodes and coverslip chamber.

chamber over a magnet which revolved at 30 to 40 r.p.m. This rate of stirring was found to give efficient mixing in the chamber, but it did not dislodge the cells from the bottom coverslip or injure them in any way. This was confirmed from time to time by direct microscopical observation. Since the stirrer itself was rotated on the top of the Perspex ring, it did not come into contact with the cells.

The volume of the chamber was determined gravimetrically for each experiment.

The electrodes.

The cathode consisted of platinum wire 0·018 in. (0·046 cm.) in diameter. This was coated with " Araldite "* resin except for a length of about $\frac{1}{8}$ in. (0·32 cm.) at one end. The uncoated end was immersed in the medium. A tapered polythene cuff was drawn over the resin-coated wire so that when the electrode was introduced into the chamber the cuff fitted tightly into the drill-hole. The anode was a piece of lead about 1 in. (2·54 cm.) long and $\frac{1}{8}$ in. (0·32 cm.) in diameter. This was immersed in standard acetate buffer contained in one end of a length of glass tubing about $\frac{3}{8}$ in. (0·95 cm.) in diameter. The other end of the tube was bent and drawn

* An adhesive resin supplied by Aero Research Ltd., Duxford, Cambridge.

out to a fine tip in which an isotonic KCl-agar bridge was incorporated. This tip could be inserted into the chamber through the second drill-hole (Fig. 1 and 2). Leads were soldered directly to the two metal electrodes and were connected to the terminals of a micro-ammeter.

Calibration of the electrodes.

When a noble metal cathode and a non-polarisable anode are immersed in an electrolyte solution and the circuit is completed, the current produced is proportional to the surface area of the cathode and the O_2 tension of the solution (Tödt *et al.*, 1952). Freier, Tödt and Wickert (1951) examined the relationship between the O_2 tension and the current produced in such a system and considered that it was not far from linear. In the above study the dissolved O_2 was measured by the toluidine-rhodamine method. Preliminary experiments with the electrodes described above led us to suspect that the relationship between O_2 tension and current was not linear, but logarithmic. A detailed calibration of the electrodes was therefore carried out, making use of Henry's law to vary the O_2 tension of the solution.

The electrodes were introduced into a 250-ml. vacuum flask containing some rabbit serum diluted 1/4 in Hanks' solution. The tips of the KCl-agar bridge and of the platinum cathode were immersed in the fluid. The leads from the electrodes were attached to brass screws which had been passed through the stopper of the vacuum flask. Leads were taken from these screws to the terminals of the micro-ammeter. The flask was connected through a mercury manometer to a suction pump, and the fluid in it agitated continuously by means of a magnetic stirrer. The calibration was carried out in an incubator at 37°.

To expel the gases initially dissolved in the medium the pressure in the vacuum flask was reduced until the solution boiled. Atmospheric air was then reintroduced into the flask, the fluid allowed to come into equilibrium with the gas phase, and a reading taken on the micro-ammeter. The pressure was then reduced in stages until zero was almost reached. After each change in pressure the fluid was allowed to come into equilibrium with the gas phase before the reading was taken. It was found that with continuous stirring equilibrium was attained under the present conditions in 15 to 20 min. ; the readings were therefore taken 30 min. after each change in pressure. The calibration was repeated using pure O_2 as the gas phase. The O_2 tension of the fluid was calculated from the level of the mercury manometer after a correction had been made for the pressure of water vapour in the flask.

The relationship between the current produced and the O_2 tension of the fluid is shown in Fig. 4. It will be seen that this relationship is not linear, approaching linearity only at high O_2 tensions. If, however, the current is plotted against the logarithm of the O_2 tension, a straight line relationship is obtained (Fig. 5). This relationship is given by the expression :

$$\text{Log } T = k_1 I + k_2$$

where T = the O_2 tension of the fluid, I = the current generated, and k_1 and k_2 are constants describing the characteristics of the platinum electrode. The values for these constants are obtained by empirical calibration of the electrode.

It will be noticed that a small current persists even when the pressure in the flask has been reduced virtually to zero. It is not known whether this current is due to the presence of traces of O_2 in the medium, or whether it is due to some intrinsic property of the electrode system. In any case, it is not possible to eliminate this residual current even if the medium is allowed to boil, or the O_2 in it is removed by chemical means.

Technique of taking the readings.

In order to avoid the continuous passage of current through the medium for many hours, the circuit was connected only while a reading was being taken. This made it necessary to standardise the technique of taking the readings. When the connection was made the initial reading was higher than the equilibrium level which could be correlated with the O_2 tension. Tödt (1950) has shown that for a given O_2 tension the height of this initial reading is a function of the time interval between readings. As the current flows, however, the reading rapidly falls

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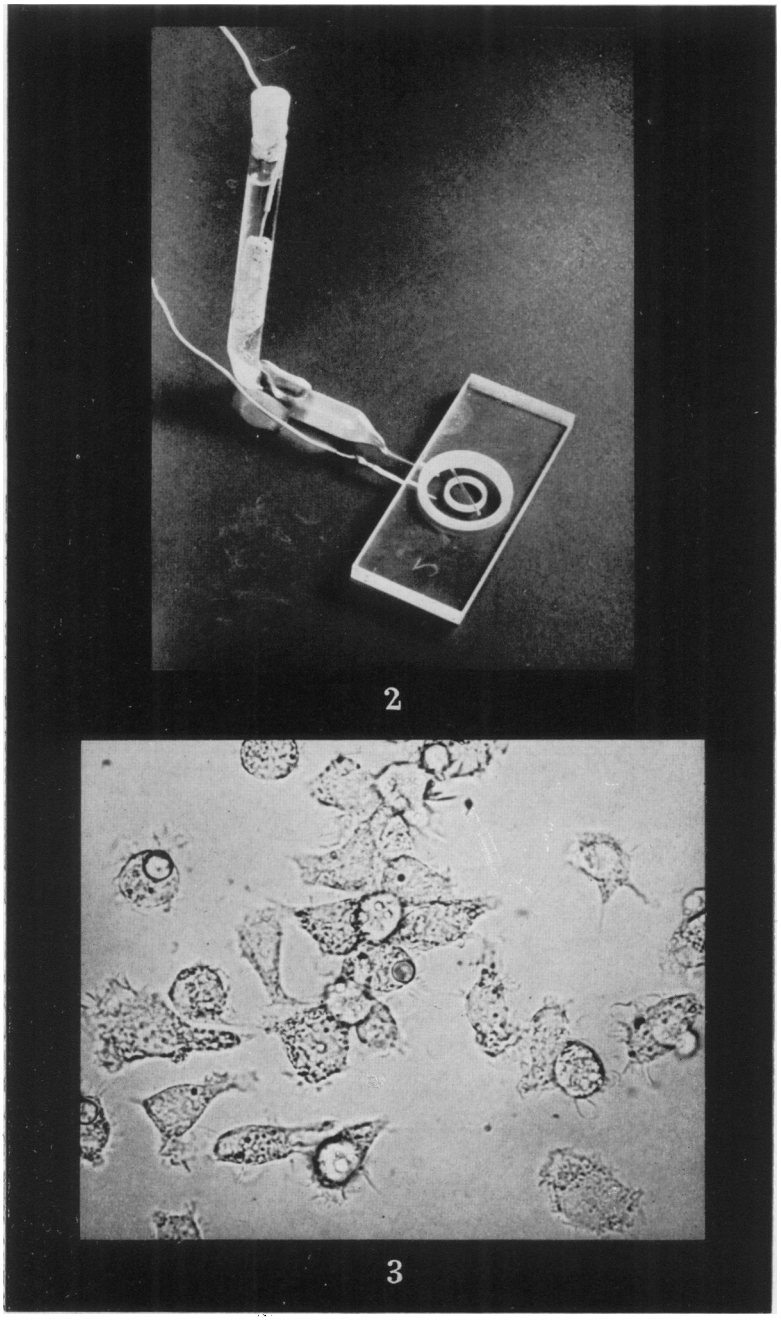
EXPLANATION OF PLATES.

FIG. 2.—The electrodes and chamber assembled.

FIG. 3.—Macrophages adherent to the floor of the chamber. ($\times$ 600).



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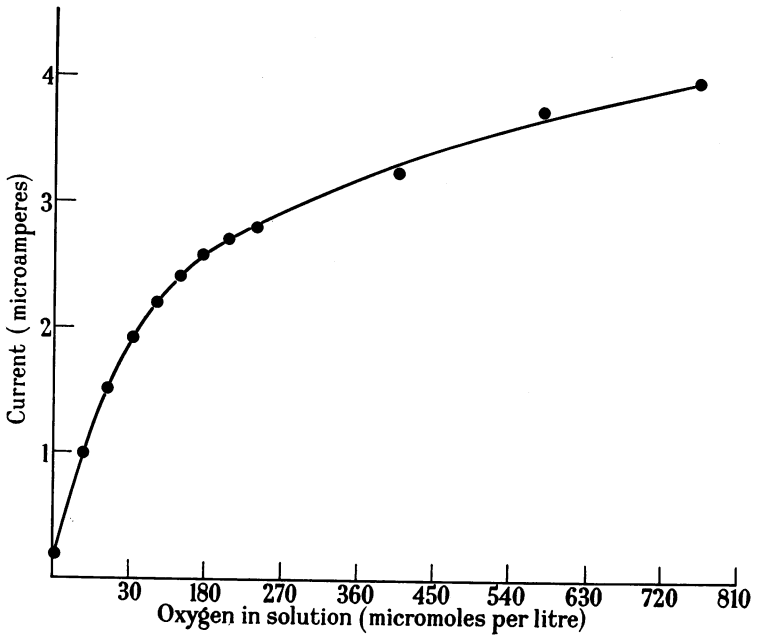

FIG. 4.—Calibration curve showing the relationship between O_2 tension and current.

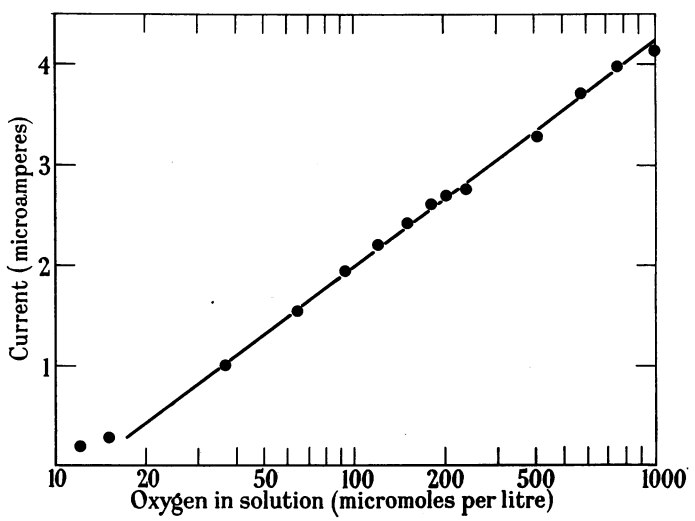

FIG. 5.—The relationship between the logarithm of the O_2 tension and the current.

to the equilibrium level. Under the present conditions, equilibrium was attained within a minute. All readings were therefore taken 1 min. after the connection had been made, and at intervals of exactly 30 min. With the technique of taking readings standardised in this way, there was no difficulty in obtaining an accurate and reproducible correlation between the current and the O_2 tension.

The effect of pH.

In order to determine whether the current produced was influenced by the changes in pH which might be expected to occur in the medium during cellular respiration, a series of Sørensen's buffers ranging from pH 5·9 to pH 8·1, and equilibrated with the same O_2 tension, were tested. Over this range the current was found to be independent of the pH.

Chemical methods.

The glucose concentration of the medium was determined by the method of Hagedorn and Jensen (1923); the lactic acid concentration by the method of Barker and Summerson (1941).

RESULTS.

The respiration of rabbit macrophages.

In Fig. 6 the results of a typical experiment are shown. The O_2 tension in the medium containing a given number of macrophages fell in a linear fashion until virtually all the O_2 was exhausted. The O_2 uptake of the cells was thus independent

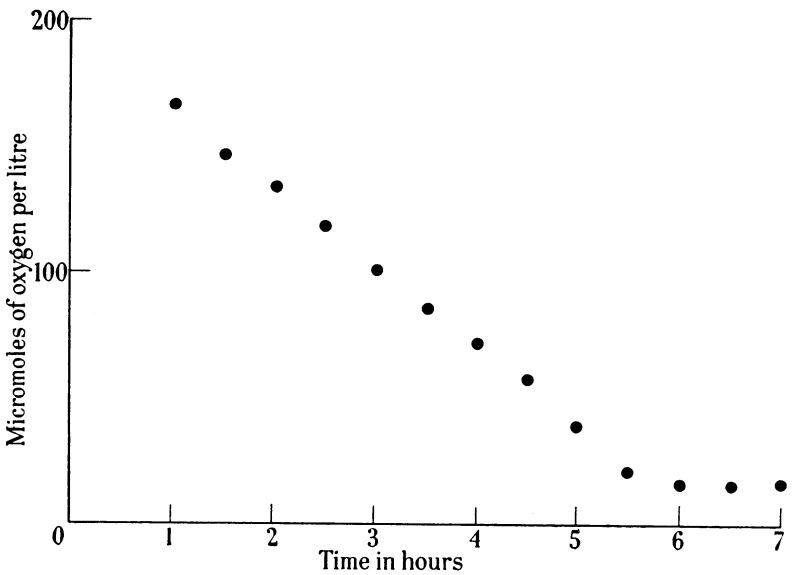

FIG. 6.—The fall in the O_2 tension of the medium in a chamber containing living macrophages. In this experiment the chamber contained 600,000 cells and 0·776 ml. of medium.

of the O_2 tension of the medium. It will be noted that there was a small residual current, as in the calibration curve (Fig. 5). If this current represents a trace of O_2 persisting in the medium, this O_2 is apparently unavailable to the cells.

The mean O_2 uptake of macrophages from 6 different exudates withdrawn from the peritoneal cavity on the fifth or sixth day after the injection of paraffin oil was 0·040 micromoles per 10^6 cells per hour (S.D. 0·006). Cells withdrawn from

the peritoneal cavity on the fourth day after injection had a somewhat higher O_2 uptake. The highest value recorded in an exudate withdrawn on the fourth day after injection was 0·091 micromoles per 10^6 cells per hour.

The behaviour of macrophages under anaerobic conditions.

The complete exhaustion of the available O_2 in the medium produced no observable effect on the behaviour of the macrophages. They remained motile and free from any visible degenerative change until the end products of their metabolism depressed the pH of the medium to levels below 6·8. It was not possible to distinguish microscopically between macrophages in an anaerobic environment and the same cells in the presence of O_2. Macrophages could be kept alive under anaerobic conditions for many days by periodic changes of medium. Before the fresh medium was introduced into the chamber, the O_2 in it was expelled by prolonged gassing with nitrogen, and the pH was adjusted with CO_2 and nitrogen. Medium treated in this way still contained a small amount of O_2, but this was consumed by the cells within an hour. Thus, in an experiment lasting several days, with daily changes of medium, the cells were in a completely anaerobic environment for at least 23 hr. out of 24.

The glucose consumption and lactic acid production of macrophages under aerobic and anaerobic conditions.

Since it was clear from the foregoing experiments that the macrophage was a facultative anaerobe, it was thought worth while to see whether a " Pasteur effect" could be demonstrated. The glucose consumption and lactic acid production of the cells under aerobic and anaerobic conditions were therefore compared. The results obtained from three representative experiments are given in the Table.

TABLE.—*Comparison of the Glucose Consumption and Lactic Acid Production of Macrophages under Aerobic and Anaerobic Conditions.*

Age of exudate. Number of days after injection of paraffin oil.		Oxygen uptake.	Consumption of glucose.	Production of lactic acid.
		Micromoles per 10^6 cells per hour.		
Aerobic	4	0·090	0·17	0·33
Anaerobic		—	0·27	0·60
Aerobic	8	0·037	0·18	0·24
Anaerobic		—	0·27	0·58
Aerobic	4	0·091	0·20	0·44
Anaerobic		—	0·34	0·79

Under anaerobic conditions there was a marked increase in the amount of glucose consumed and the amount of lactic acid produced. This " Pasteur effect " could always be demonstrated, although its magnitude varied somewhat from exudate to exudate, as did the O_2 uptake and the consumption of glucose. In most experiments about twice as much lactic acid was produced under anaerobic conditions. Moreover, it will be noticed that even if all the O_2 taken up by the cells was used in the combustion of glucose, it would account for only a small part of the total amount of glucose consumed. It thus appears that even in the presence of O_2 most of the glucose metabolised is converted to lactic acid. Under anaerobic

conditions the energy requirement of the cells is apparently met by an increase in this glycolysis.

SUMMARY.

A new method for measuring cellular respiration is described. This method is particularly suitable for the study of animal cells *in vitro*, as measurement of respiration can be carried out under conditions which permit direct microscopical observation of the cells. The electro-chemical circuit used in this method is able, without amplification, to record changes in oxygen tension of 1–2 micromoles per litre. Some observations carried out with this technique on the macrophages of the rabbit show that these cells are facultative anaerobes. Under aerobic conditions they have a constant oxygen uptake which is independent of the oxygen tension of the medium. However, even in the presence of oxygen, most of the glucose consumed is converted to lactic acid. Under anaerobic conditions the energy requirement of the cells is met by increased glycolysis.

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