Respiration Rate of Rat-Liver Cells at Low Oxygen Concentrations

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Liver damage following chronic oxygen lack has been demonstrated experimentally in rabbits by Martin, Bunting & Lowenhart (1916) and in dogs by Goldschmidt, Ravdin & Lucke (1937). A similar type of damage has been found in the livers of airmen dying of acute anoxia (Kritzler, 1944). Recently, Bassi & Bernelli-Zazzera (1955) have made a quantitative study of the changes occurring in the livers of rats made acutely hypoxic. They found that after the animals had been kept for 2 hr. in an atmosphere containing 3% of oxygen the liver cells became vacuolated and their metabolic pattern altered. It is reasonable to suppose that this change was brought about by an impairment of the oxygen supply to the liver cells. For this reason it would be interesting to determine the effect of low oxygen concentrations on the respiration rate of the liver, as has been done with many other biological systems.

The selection of the best biological preparation of liver to use raises some difficulties. Hill (1948) discussed this problem in relation to frog muscles, whose respiration rate he measured as a function of oxygen concentration. The slices which Cruickshank & Trotter (1956) found satisfactory in their studies of skin respiration would not be suitable, for histological reasons. The preparation must be such that the suspending medium containing oxygen bears a similar spatial relation to the cells as blood plasma does in the intact organ. Elias (1955) has shown that the cells of rat liver form a muralium, or sponge-like structure of plates, one cell thick. Thus, in general, the rat-liver cell receives its oxygen supply directly from the plasma without any other parenchymal cell intervening. Because of this it would be reasonable to study the respiration of individual cells suspended in an oxygenated solution. Such suspensions would have to be free of extraneous respiratory particles; since the removal of mitochondria from cell suspensions is very difficult because of their stickiness, the method of preparation should be so gentle that no cells are disrupted. Anderson (1953) has described a method of obtaining individual cells by softening the cement substance with calcium-complexing agents, and Dulbecco & Vogt (1954) have used trypsin for the same purpose. Since these methods introduce substances foreign to the cell, a method was developed

which avoided this difficulty (Longmuir & ap Rees, 1956).

There are two general methods of measuring the respiration rate of a biological system as a function of oxygen concentration: (i) The manometric method used by Warburg & Kubowitz (1929) on bacteria and recently improved by Baender & Kiese (1955) working with mitochondria; by this method, however, each experiment yields only one point on the curve of respiration rate against oxygen concentration and, because of the time required, could not be expected to give a complete curve on one preparation. (ii) The polarographic methods used by Winzler (1941) on yeast, Hill (1948) on frog muscle and by Longmuir (1954) on bacteria; these methods produce a complete curve in a few minutes and so are suitable for application to preparations which may not retain their activity for long. Since the results are produced as a continuous curve rather than as discrete measurements the latter methods more readily demonstrate such critical points as the concentrations of oxygen at which the respiration rate just begins to fall and at which it reaches its half value. For these reasons the polarographic method was used.

In order to obtain results comparable with those of Baender & Kiese (1955), obtained with mitochondria, succinate was used as the substrate. The experiments of Baender & Kiese (1955) were repeated with the polarographic technique.

MATERIALS AND METHODS

Solutions. The cells were suspended in a mixture of equal parts of 0.1M-KCl and 0.1M phosphate buffer, pH 7.3, prepared as previously described (Longmuir, 1954). To this was added 0.4M sodium succinate, the quantity of which was determined by preliminary experiments described below.

Cells. These suspensions were prepared by the methods of Anderson (1953) (slightly simplified), of Dulbecco & Vogt (1954) and of Longmuir & ap Rees (1956). The reagents used were as far as possible Polaritan (Hopkins and Williams) and the water was de-ionized. The activity of the preparations is expressed as q_{O_2} (N) (μ l. of O_2 /mg. of total N/hr.), because the determination of the wet or dry weight of cells suspended in a relatively large amount of saline is not very accurate.

In practice it was found that all methods gave similar results except that the preparation of Dulbecco & Vogt was rather more quickly inactivated by the experimental

technique used. After the preliminary experiments using all three methods the method of Longmuir & ap Rees (1956) was used exclusively, as it was found to be most convenient.

Mitochondria. These were prepared by the method of Klemperer (1955).

Methods. The method used is essentially that previously described (Longmuir, 1954) but with some improvements (Fig. 1). A self-centring ball race (3) replaces the journal bearing between the rod (5) and the disk (2) and the rod is connected to the electrode by insertion directly into the glass tube (6); the metal and glass are separated by a layer of rubber (4). Both the motor and the cell are securely attached to brass plates bolted to one another and also to the

Fig. 1. Diagram of the apparatus. 1, Shaft of constant-speed motor; 2, disk; 3, self-centring bearing set into 2; 4, rubber tubing between eccentric shaft 5 and glass tube 6; 7, rubber disk; 8, gold electrode; 9, sintered-glass disk; 10, 2% agar in saturated KCl.

Fig. 2. Differentiating circuit. G_1 , Recorder of the Cambridge Polarograph; G_2 , galvanometer (125 scale divisions/ μA); R_1 , decade box 500–8000 Ω ; R_2 , potential divider of 10 000 Ω ; C , paper condenser of 10 μF .

side of a thermostat bath (accurate to 0.01°). The connexion to the anode is made through a constricted continuation of the cell (10) below the sintered-glass disk (9). The agar is placed in this part instead of in the cell proper. A wide-bore polyvinyl chloride tube filled with saturated KCl makes connexion with the anode. The anode, which is sufficiently far away from the bath to avoid temperature effects, consists of a mercury pool (60 cm.²); this is superior to a calomel electrode (Heyrowsky, 1957). The mechanical improvements simplify the technique and make the diffusion current more stable. Provided that prepolarization and ageing are duly carried out this assembly gives current $\times$ voltage curves which fulfil Heyrowsky's (1957) criterion of reproducibility. In order to facilitate direct measurements of respiration rate a differentiating circuit is incorporated in the polarograph (Fig. 2). The reading of the galvanometer is a measure of the respiration rate of the biological system being studied.

A different technique of re-aeration is used. Syringes (20 ml.) are attached to the inlet and outlet of the polarographic cell. These are each one-quarter full of a suspension of cells or mitochondria, and one-quarter full of air or oxygen. The polarographic cell contains only the suspension. When the oxygen in the suspension contained in the polarographic cell is exhausted, it can be renewed by drawing the liquid from one syringe to the other without introducing a gas phase into the cell.

RESULTS

Initial experiments showed that the activity of the liver-cell suspensions fell off while in the polarographic cell. The greatest rate of inactivation observed at 40° was 10%/hr. It was decided to perform all experiments quickly rather than devise a method of preventing inactivation.

The effect of succinate concentration on the q_{O_2} (N) of the liver-cell suspension was investigated. The reciprocal of the respiration rate was plotted against the reciprocal of the succinate concentration. The points lay on a straight line. The value of the maximum respiration rate and the concentration of succinate (K_m) producing half the maximum rate were derived from this line by the method of Lineweaver & Burk (1934). The value of K_m at 40° was found to be 1.03 mM. At lower temperatures it was lower. In all subsequent experiments a concentration of succinate (greater than 12 mM) sufficient to give the maximum respiration rate was used.

Fig. 3 shows the results of a typical experiment with an optimum concentration of succinate. Curve (a) is obtained from the recorder of the polarograph and is a plot of oxygen concentration against time. Curve (b) is a plot of respiration rate, the galvanometer reading, against time. In Fig. 4 the results are plotted in the more orthodox manner of respiration rate against oxygen concentration.

It will be seen that there is an appreciable scatter of the experimental points about the horizontal line in curve (b) of Fig. 3. It is not absolutely clear from this experiment alone that there is a discontinuity in

Fig. 3

Fig. 4

Fig. 3. Plots of the oxygen concentration of the cell suspension and respiration rate of the liver cells against time. Curve (a) is the trace obtained from the recorder of the polarograph and its ordinate is expressed as $\mu\text{moles of O}_2/\text{l.}$ Curve (b) is obtained by plotting the readings of the galvanometer (the respiration rate) expressed as percentages of the maximum rate.

Fig. 4. Variation of the respiration rate of liver cells in $\mu\text{moles of O}_2/\text{l./sec.}$ (ordinate) with oxygen concentration in $\mu\text{moles of O}_2/\text{l.}$ (abscissa).

the plot of respiration rate against oxygen tension, the respiration rate beginning suddenly to fall at this value. However, it is possible to establish that this is so. The scatter is due to noise coming from an extraneous source and it is independent of the signal strength. Thus the error in this region can be reduced by increasing the amount of rat-liver cells placed in the polarographic cell. In experiments in which this was done it was found that the galvanometer reading remained constant and then fell abruptly, i.e. that the respiration rate does fall abruptly from the maximum. In these experiments the rate changes too rapidly to permit the accurate reading of points in the second part of the curve.

Fig. 5 shows plots of $\log v/(v_{\max} - v)$ against $\log$ (oxygen concentration) for experiments at two different temperatures, where v is respiration rate; the significance of this plot is discussed below. The results obtained with mitochondria gave similarly shaped curves.

Experiments were conducted at various temperatures between 1.40° and 40.00° . The logarithm of the respiration rate was plotted against the reciprocal of the absolute temperature, and gave a straight line. From the slope of this line the energy of activation of the whole system was found to be 16 000 cal. By a similar treatment, the logarithm of the concentration of oxygen at which the cytochrome oxidase is half saturated [calculated from the equation of Baender & Kiese (1955)]

Fig. 5. A plot of $\log v/(v_{\max} - v)$ (ordinate) against $\log$ (oxygen concentration) (abscissa), showing the deviation from a straight line at higher respiration rates. Curve (a) at 10° , curve (b) at 40° .

being used, the value of the heat of the reaction was found to be 13 500 cal.

In all the experiments in which the respiration rate was varied by altering either the concentration of succinate or the temperature it was found that the concentration of oxygen (K) at which the cells respired at one-half of their maximum rate was linearly related to the maximum respiration rate within the limits of experimental error (average coefficient of variation 4%). The relationship obtained by least squares is $K = \alpha q_{O_2}(N) + \beta$, where $\alpha = 1.2 \times 10^{-9}$ and $\beta = 4.26 \times 10^{-8}$.

DISCUSSION

It has been held for some time (Tang, 1933) that the relationship between the respiration rate (v) of various biological systems and the partial pressure of oxygen (p_{O_2}) is

$$v = \frac{p_{O_2}}{K_1 + K_2 \times p_{O_2}}, \quad (1)$$

where K_1 and K_2 are constants. Baender & Kiese (1955) have recently shown that for mitochondria, for a bacterium and for a yeast, the relationship is

$$v = \frac{v_{\max.} \times p_{O_2}^{1.4}}{K + p_{O_2}^{1.4}}, \quad (2)$$

where $v_{\max.}$ is the maximum respiration rate. They obtained this by plotting $\log v/(v_{\max.} - v)$ against $\log p_{O_2}$, when they found that between 10 and 90% of the maximum rate, their full experimental range, the points lay on a straight line of slope 1.4. In Fig. 5 the results obtained with liver cells at two different temperatures are plotted in this way. In the straight region the slope is 1.4 but the upper ends rise more steeply. This means that the respiration rate approaches the maximum at a lower oxygen concentration than predicted by either of the equations. Baender & Kiese (1955) reported that at three times the concentration of oxygen at which the respiration rate was half maximal the respiration rate had just begun to fall, and called this the 'critical oxygen concentration'; but later in their paper they assume that their equation is valid at higher concentrations, i.e. that the respiration rate approaches the maximum rate asymptotically. There is thus no theoretical 'critical oxygen concentration'.

The curves shown in Figs. 3 and 4 show that with liver cells there is a true 'critical oxygen concentration' which is between four and five times the value of the oxygen concentration at which the rate is one-half of the maximum. According to Eqns. (1) or (2) the respiration rate at this critical value should be only 80 or 90% of the maximum respectively. The instrumental error in the determination of the

respiration rate is about 3%, and it is less in experiments designed to clarify this region.

There are two possible explanations for this limitation of the respiration rate. A reduction of the concentration of oxygen in the suspending medium eventually results in anoxia of those mitochondria furthest from the surface of the cell. This concept has been treated mathematically by Gerard (1931) and Rashewsky (1933) for a somewhat simplified system; the respiration rate, however, seems to fall off rather more sharply than these equations require. An alternative explanation is that above 80% saturation of the respiratory enzyme another component of the succinic oxidase system becomes limiting. Winzler (1941) found this to be true in yeast. He changed the limiting dehydrogenase by altering the substrate and found indirectly that the percentage saturation of cytochrome oxidase at the maximum respiration rate was changed. By direct spectroscopic examination Chance (1952) has shown that in the steady state, when the respiration rate is maximal, the cytochrome a_3 of heart-muscle preparation is only 87% oxidized. If this explanation is correct, the respiration rate of mitochondria above 80% of the maximum should rise with increasing oxygen concentration more rapidly than predicted by Baender & Kiese (1955). Experimentally, with the technique described in this paper, it was found that the respiration rate of mitochondria did increase in this way. These results do not contradict those of Baender & Kiese (1955); the deviation from the straight line occurs in a region of respiration rate which they did not investigate fully.

The values obtained for the energy of activation and the heat of reaction are the same, within the limits of experimental error, as those found by Baender & Kiese for mitochondria. This suggests that fundamentally the same process is being studied in both cells and mitochondria and that diffusion through the cell cytoplasm is not a limiting factor, as it may be in large bacteria (Longmuir, 1954). Direct comparison of the oxygen concentrations at which mitochondria and cells respire at one-half of their maximum rate is difficult owing to the marked variation of these oxygen concentrations with q_{O_2} , but a comparison with the figures of Baender & Kiese suggests that the value for cells with a comparable respiration rate is slightly higher.

SUMMARY

1. A method of determining simultaneously the respiration rate of a biological system and the concentration of oxygen in the suspending medium is described.

2. This method has been used to measure the relation between the respiration rate of rat-liver cells and oxygen concentration.

3. The significance of the relationship is discussed.

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The Structure of the Naturally Occurring Phosphoglycerides

3. ACTION OF MOCCASIN-VENOM PHOSPHOLIPASE A ON OVOLECITHIN AND RELATED SUBSTANCES*

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The enzyme phospholipase A, which is present in the venom of several species of snake, removes a single fatty acid radical from the naturally occurring lecithins to give the corresponding lysolecithins. Lecithin, when isolated from biological materials by solvent-fractionation procedures, usually contains saturated and unsaturated fatty acid radicals in approximately equimolar proportions; on treatment with phospholipase A, the unsaturated fatty acid radicals are found to be preferentially removed (Levene & Rolf, 1923; Chargaff & Cohen, 1939; Fairbairn, 1945). However, the enzyme does not act specifically on unsaturated fatty acid ester linkages, since Zeller (1952) and Hanahan, Rodbell & Turner (1954) have demonstrated the removal by phospholipase A of a single fatty acid radical from synthetic dimyristoyl-L- α -lecithin and yeast dipalmitoyllecithin respectively. In the first case, the lecithin contains two saturated fatty acid radicals; in the second, both are unsaturated. It has been shown by Hanahan (1954) and by ourselves

(Long & Penny, 1954) that it is the fatty acid radical attached to the α' position in the lecithin molecule which is selectively detached by phospholipase A.

In the present work, the phospholipase A of cottonmouth-moccasin venom has been studied with regard to its action on ovolectithin and other naturally occurring and synthetic phosphoglycerides. The enzyme has been found to require Ca^{2+} ions and to be specific for the L- α form of synthetic lecithins; phosphatidylethanolamine and probably certain other naturally occurring phosphoglycerides have also been found to act as substrates. Some of this work has been published in preliminary form (Davidson, Long & Penny, 1955).

EXPERIMENTAL

Materials

Source of phospholipase A. Samples of the dried venom of the cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), rattlesnake (*Crotalus adamanteus*) and cobra (*Naja naia*) were purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. Aqueous solutions (0.1%) were used either immediately or after storage for up to 24 hr. at 2°. Six samples of moccasin venom were examined; all showed a significantly higher phospholipase A activity than did the rattlesnake or cobra venom, and were therefore used in the experiments to be described.

* Part 2: Long & Maguire (1954).

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