THE PERMEABILITY OF CELLS FOR OXYGEN AND ITS SIGNIFICANCE FOR THE THEORY OF STIMULATION.

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Although much research has been carried out on the permeability of cells to other substances, relatively little has been done on their permeability to oxygen and carbon dioxide. Yet it is obvious that permeability for these gases is of great theoretical significance in connection with theories of stimulation. Since it is quite certain that oxygen and CO_2 must enter and leave cells during respiration, the question is not one of penetrability, but rather of the rapidity with which they enter or leave the cell. Increased oxidation may result on stimulation of a cell. Is this increase to be explained by assuming increased permeability to oxygen or carbon dioxide? A comparison of the rate of penetration of living and dead cells by these gases will give an answer to the question.

The most significant work in this connection has been done by Jacobs (1920), who finds that carbon dioxide belongs to the group of acids which penetrate a cell with great rapidity. Among acids, CO_2 is comparable to NH_3 among alkalies (Harvey, 1911). Entrance into living cells is just as rapid as entrance into dead cells. Presumably then, we may say that passage of CO_2 from the cell is perfectly free at all times. Is the entrance of oxygen into the cell perfectly free at all times also? A number of lines of evidence bear on this problem and the larger problem whether stimulation brings about an increased permeability of the cell for oxygen.

It is quite possible to show directly by an indicator method, that many kinds of cells are freely permeable to oxygen. I have tried this experiment on epithelial cells of *Chætopterus*, on *Limulus* muscle, on ovarian epithelial cells and the pyloric cæca of the starfish, on the

tentacles of hydroids (*Eudendrium* and *Campanularia*), on gill cilia of *Mytilus*, on liver, muscle, and nerve of the frog. The following experiments will serve as examples of all.

If a frog's muscle is stained in methylene blue and then placed in an atmosphere devoid of oxygen, the blue color disappears. The tissue becomes colorless, an indication that the methylene blue has been reduced to its leuco base. If air is now readmitted to the muscle, the blue color returns *almost immediately* (within 15 seconds). The muscle is perfectly capable of contraction if stimulated electrically, even while the methylene blue is in the reduced or colorless condition. It is not injured by the methylene blue or the removal of the oxygen. The leucomethylene blue simply acts as an indicator of the entrance of oxygen to the cell and gives us unequivocal evidence that the cell is readily permeable for oxygen at all times, even in the resting condition.

If we stain two sartorius muscles in methylene blue, deprive them of oxygen until decolorized, and then kill one muscle with steam in the absence of oxygen, the two, when simultaneously exposed to air, become blue again at approximately the same rate. A dead muscle is, therefore, no more permeable to oxygen than a living muscle. Oxygen enters so readily that no difference is to be seen.

In this experiment, it is convenient to allow the muscle to use up the oxygen, as readily happens. It is sufficient to place a sartorius muscle (carefully removed from the frog, so as to avoid injury, and stained in methylene blue) in Ringer's solution on a slide and cover with a cover-slip. Under the cover, the blue color of the muscle fibers disappears in a short time, since they are denied access to oxygen, and the muscle remains blue only at the edge, where it comes in contact with dissolved oxygen. Oxygen is readmitted by lifting the cover-slip, when the muscle becomes blue throughout.

Use may be made of the avidity of muscles for oxygen to render small amounts of fluids practically oxygen-free. For this purpose, strips of frog muscle (or any muscle tissue) are arranged in the form of a ring on a slide, enclosing a space filled with Ringer's solution or sea water, and containing the tissue upon which the effects of lack of oxygen are to be observed. A cover-slip is now placed over the preparation, with careful exclusion of air bubbles, and we have the

tissue for observation enclosed in a "muscle pen" which quickly frees it of oxygen. This arrangement, which may be easily made, does away with the inconvenience of passing purified hydrogen or nitrogen gas through microscopic gas chambers to remove the oxygen. A control slide with muscle and tissue should be prepared and kept exposed to the air, in order to make sure that non-volatile products, such as lactic acid, diffusing from the muscle are not responsible for effects attributed to lack of oxygen. I have observed no effects which might be due to lactic acid, but it is quite possible that the production of CO_2 may be involved in causing any physiological changes observed. CO_2 accumulation does not affect the color change of the methylene blue, which is entirely dependent on lack of oxygen. The "muscle pen" method is well adapted for class experiments to demonstrate oxygen permeability, as the following example indicates.

Small pieces of the ciliated gill of the mussel, *Mytilus*, are removed and stained in methylene blue. A "muscle pen," using *Limulus* muscle, is then prepared on each of two slides, (A and B), and the *Mytilus* gill tissue placed within in sea water, and covered with a cover-slip. A third slide, (C), is prepared in a similar manner, but is not covered. The cilia in all three preparations are beating vigorously at first, but in the covered preparations (A and B) they gradually slow down and stop, standing straight out from the cells; this occurs in 15 to 30 minutes. The methylene blue color disappears at approximately the same time that the cilia stop beating.[1] In slide C, the blue color does not disappear, and the cilia continue beating vigorously.

Slide B is now steamed for 2 minutes to kill the cilia, and, when cool, the cover-glasses of slides A and B are lifted simultaneously, thus exposing both tissues to the air. The blue color returns in 15 seconds, and at the same rate in both slides. The cilia in the unsteamed slide begin beating again within a minute or two after the blue color has returned. Thus, oxygen enters the cells with practically no resistance.[2]

[1] The relation between stoppage of the cilia and color change of methylene blue depends somewhat on the depth of blue color in the tissue.

[2] This experiment can be performed with practically the same result, if the ciliated cells, stained in methylene blue, are mounted in an Englemann micro-

If kept without oxygen in a "muscle pen" for 6 to 7 hours, the blue color again reappears in 15 seconds after air is admitted, but the cilia do not begin beating so quickly as when the exposure to lack of oxygen is less prolonged. It should be remarked in passing that cilia which have stopped beating from lack of oxygen, will not beat if stimulated with interrupted induced shocks which are sufficiently intense readily to stimulate muscle tissue. It is well known that cilia are remarkably independent of stimulation and in this respect the contractile tissue of cilia behaves quite differently from the contractile tissue of muscle.

Lack of oxygen may be studied with larger whole animals, as follows:

An entire *Chætopterus* is stained in methylene blue and then placed in a glass tube of just the size to fit it. The tube is filled completely with a small amount of sea water, and a rubber stopper carrying a platinum wire (for stimulation) is inserted in each end. The worm gradually uses up the oxygen in the tube. The effect of this on the normal movements of the fan segments, the contraction of muscles and appearance of luminescence on stimulation, and the reduction of methylene blue may be observed.

The first function to disappear is luminescence. The worm may be stimulated to luminescence for 3 to 4 minutes after being placed in the tube, but not after this time. Movement of the fan segments is very slow and weak at the end of 30 minutes, but the methylene blue has not yet been reduced. After $2\frac{1}{2}$ hours, the worms (not observed in the meantime) were colorless, and no spontaneous movement occurred, but contraction of the muscles could be produced on stimulation. If now removed from the glass tube to aerated sea water, the blue color returns in 15 to 20 seconds, and the rhythm of the fan segments is likewise regained. The power of luminescence returns very slowly, however. No luminescence resulted from stimulation $1\frac{1}{2}$ hours after removal to the fresh sea water, but the luminescence had returned in 20 hours.

scopic gas chamber, and nitrogen or (electrolytic) hydrogen gas is passed through the chamber to remove the oxygen. CO_2 passed through the chamber will cause the cilia to stop beating very quickly, but does not decolorize the methylene blue until all the oxygen is driven out.

It may be readily shown by the "muscle pen" method that the living epithelial cells of *Chætopterus* allow oxygen to enter as readily as the dead cells.

I believe we are therefore justified in making the general statement that cells are freely permeable to oxygen at all times. Any theory of stimulation which supposes a sudden increase in permeability to oxygen as a result of stimulation has no basis in fact.[3]

A somewhat similar problem to that of stimulation has to do with the changes occurring in a cell after death. The change occurring on stimulation may be similar in some respects to that resulting from the death of the cell. Thus, a muscle goes into a permanent contracted condition, *rigor mortis*, "death rigor." A luminous cell produces a continual, steady luminescence, the "death glow." The change on stimulation is temporary, that on death is permanent.

On the death of any cell, the chemical reactions run riot. This is especially well seen in some plants, as the potato, false indigo (*Baptisia tinctoria*), or Indian-pipe (*Monotropa uniflora*), which become black on death. Blackening is dependent on oxidation, and may be used as an indication that too rapid oxidation is proceeding within the cells. Why does not this blackening proceed during the life of the cell?

Blackening is due to oxidation of a chromogen to black melanin in the presence of an enzyme, an oxidase. Does this reaction proceed on death of the cell because oxygen can now readily gain entrance, or for some other reason? Perhaps the chromogen and enzyme are kept apart by some mechanism or phase boundary in the living cell which breaks down on the death of the cell.

I have endeavored to gain some light on this problem by subjecting the leaves of the false indigo plant, *Baptisia tinctoria*, to pressures of 1,000 to 1,500 lbs. per square inch of pure oxygen for varying periods of time. This has been accomplished in a small, specially constructed,

[3] This statement must be applied to luminous animals with caution. *Campanularia flexuosa*, a luminous hydroid, possesses cells as freely permeable to oxygen as non-luminous hydroids (*Eudendrium*), but it is impossible to say whether the luminous cells are those which take up the methylene blue. The same statement applies to *Chætopterus*. The photogenic cells of the firefly are large and too easily injured for clear results.

brass chamber connected with a high pressure oxygen tank and gauge.[4] When the pressure chamber was properly connected with lead washers, there was practically no leakage over long periods of time.

It was found that leaves of *Baptisia* would turn black in air saturated with chloroform[5] or ether vapor in less than 5 minutes. Killing the cells in any other way that leaves the enzyme unharmed, gives the same rapid blackening.

Exposure to 1,000 lbs. of pure oxygen for 3 hours does not affect the leaves to the slightest degree. They are green when removed from the chamber and remain green. As 1,000 lbs. of oxygen is about 330 times the pressure of oxygen in the air, we should expect many times more oxygen to dissolve in the cell membranes, although not 330 times as much, for Henry's law cannot be assumed to hold at these high pressures.

As the cells in the leaf which turn black contain chlorophyll (spongy and palisade mesophyll), they must allow oxygen to pass out during photosynthesis,[6] and if blackening depended merely on increased

[4] Caution should be exercised in subjecting dry organic matter to high pressures of oxygen. Explosions have been known to occur as a result of this procedure.

[5] Chloroform was used to kill the cells merely because of its convenience. The chloroform (or ether) has no specific effect in causing the cells to blacken, as blackening results from crushing or freezing the cells (but not from merely keeping the cells at 0°C. for 48 hours). For the same reason, we cannot necessarily suppose that the blackening is due to preponderance of oxidative over reductive processes, as the result of inhibition or destruction of the reducing enzymes by the chloroform. While it is probably true that chloroform destroys the reducing enzymes, but not the oxidizing enzymes, it is well known that plant extracts have reducing power in the absence of oxygen (see Bach, A., *Biochem. Z.*, 1913, lii, 412), and consequently crushing the cell to make an extract does not destroy the reducing enzymes. Nevertheless, crushing a *Baptisia* cell does cause it to blacken.

[6] One might suppose that oxygen never comes in contact with the mesophyll cells, because the stomata might close when the leaf is subjected to high oxygen pressures. While I have never been able to examine the stomata during actual exposure to high oxygen pressures, I have noted that the stomata were open on leaves before and after subjection to high oxygen pressures for times not sufficient to cause leaves to blacken; and also that a leaf cut across and then subjected to high oxygen pressure does not blacken at the cut edge, except in cells actually injured by the cutting. Oxygen under pressure cannot turn the cells black, even when it can come in direct contact with them through the intercellular spaces in the mesophyll leading from the cut edge.

rate of entrance of oxygen, blackening should be rapid at high oxygen pressures. Nevertheless, at 1,000 lbs. pressure, oxygen does not cause blackening as long as the cells are living.

Longer exposure to 1,000 lbs. of oxygen will cause blackening. In 4 hours, the older leaves are slightly darkened and in 7 hours they are completely black when removed from the chamber. This is apparently due to an injury of the cell by the high oxygen pressure, for a leaf removed from the oxygen pressure chamber when slightly blackened along the edges, will turn completely black in the air in the course of some hours. The previous exposure to high oxygen pressure has a progressive injurious effect.

The injurious effect is due to the oxygen, and not to the high pressure, as may be very readily shown by exposing leaves to high hydrogen pressure. Leaves in a pressure chamber filled with hydrogen at 1,800 lbs. of pressure (falling to 1,300 lbs. of pressure during the period of 24 hours) were still green when removed from the chamber, and *remained green for 6 hours afterwards*, when observation was discontinued. They were then subjected to chloroform vapor, to see if the hydrogen had in any way prevented blackening, even of killed cells. It was found that they turned black readily in chloroform vapor in air. Certainly, then, it is not the pressure itself, but the oxygen, which has the injurious action in the high concentration. It would seem that the oxygen under pressure must enter the cell in much higher concentration, but cannot react with the chromogen until some phase boundary or film of separation of chromogen and oxidase is broken down. This film is readily changed on death of the cell.

Finally, I might record the fact that a slice of potato subjected to 2,900 lbs. of air (= 580 lbs. of oxygen), or 1,700 lbs of oxygen pressure for 18 hours was not blackened in the slightest degree, whereas the control in chloroform vapor in air at atmospheric pressure was completely blackened. Again I would not interpret this result as showing that oxygen cannot be forced into living potato cells, but as indicating that it cannot react with the chromogen after it gets in. The boundaries separating chromogen and oxidase appear to be more resistant in the potato than in the false indigo plant.

SUMMARY.

It can be demonstrated by an indicator method that living cells are as freely permeable to oxygen as dead cells, and that sudden admission of oxygen to the cell cannot account for increased oxidation as a result of stimulation. Oxygen penetrates as readily as carbon dioxide among the acids and ammonia among the alkalies.

Exposure of *living* plant cells to *high oxygen pressures* does not initiate certain oxidations (except after some hours), which proceed readily in *dead* plant cells in the *air*. In the light of the preceding statement, about the permeability of cells for oxygen, this is interpreted to mean that more oxygen enters the cell at high pressure, but that the reacting substances (chromogen and oxidase) are kept apart by some phase boundary as long as the cell is alive. Increased oxygen concentration eventually produces injury to the cell.

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