COMPARATIVE STUDIES ON RESPIRATION.

I. Introduction.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication September 6, 1918.)

In the course of studies on antagonism the writer made experiments on the action of antagonistic substances on respiration. As a result of his experiments he became dissatisfied with the existing means of studying respiration in plants and undertook to devise more satisfactory methods. At his suggestion Dr. Haas developed an indicator method[1] which proved to be very satisfactory for aquatic organisms. However, it had serious limitations which were subsequently removed by a method devised by the writer. The new method is accurate, rapid, and convenient, and can be used for organisms of all kinds.[2]

A series of studies has been made in the writer's laboratory by the use of these methods, the first of which are here brought together. The work of Mr. Gustafson deals with higher fungi, that of Mrs. Brooks with bacteria, that of Miss Thomas with flowering plants, and that of Miss Irwin with a variety of animal material. The work of Haas[3] on the marine alga *Laminaria* was carried out under the same conditions, and may therefore be compared with the results described in this series.

In the first investigations the technique described by Haas[1] was employed in most cases. Phenolsulfonephthalein was added to the liquid containing the organisms, and respiration was allowed to proceed until a definite change in acidity had occurred. Usually the organism was then placed in a fresh sample of the solution and the process was repeated until the normal rate of respiration was ascer-

[1] Haas, A. R., *Science*, 1916, xliv, 105.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1918, i, 17.

[3] Haas, *Science*, 1917, xlvi, 462; *Proc. Nat. Acad. Sc.*, 1917, iii, 688.

tained;[4] the reagent was then added and new measurements of respiration were made in the same manner as before.

The rate of respiration after the addition of the reagent is expressed in each case as per cent of the normal rate. The normal rate is usually determined by taking the average of successive determinations. The normal rate may be different in each experiment (on account of differences in the quantity or kind of material used), but it is in all cases taken as 100 per cent.

The relative rate was ascertained in all cases by comparing the times required to produce a definite change of acidity and not by a comparison of the changes in acidity produced in equal times. The former method compares the reaction velocities directly, while the latter may not.[5]

This method of comparison has the further advantage of making it unnecessary to know what change in pH value is produced by adding a definite amount of CO_2. For if we always start the measurement from the same pH value and carry it to the same end-point, we can be certain that the same amount of CO_2 has been produced in each case, although we may not know what this amount is. It is, in fact, quite unnecessary to know it if we are comparing the times required to do equal amounts of work.

An additional advantage of this method is that an error in the buffer solutions does not affect the results, providing we use the same buffer solutions throughout. For this purpose they are made up in large quantities.[6] Two solutions are chosen, the pH value of one being taken as the starting point (to which the experimental solution is always brought when starting a measurement) and that of the other as the end-point (to which the experimental solution comes as the result of respiration).

Certain precautions which are essential to accurate work may be briefly mentioned, in addition to those described by Haas.[3] It is necessary to use the purest reagents and in particular to see that they are neutral (or practically so); stock solutions may be kept in Pyrex flasks or tubes to avoid alkalinity due to the glass. Slight departure

[4] Unless the normal rate was fairly constant the experiment was rejected.

[5] Osterhout, *Science*, 1918, xlviii, 172.

[6] Buffer solutions of phosphates or borates will keep for a long time.

from neutrality may be compensated by adding CO_2 to the tap water or removing CO_2 (by means of a current of air free from CO_2 or by a current of hydrogen). In this way the solution may be brought to the most convenient pH value; all experiments are then started at this value. It is desirable to choose a value in the region where the indicator is sensitive.

In case alkali is added to bring the solution to a given pH value it must be done before the normal respiration is measured, so that the buffer effect of the alkali will be the same in the measurements of normal and abnormal respiration.

The buffer effect of all reagents must be carefully measured. This may be done by means of an apparatus recently described.[7] In the case of the reagents employed in these studies there is practically no buffer effect (unless the contrary is expressly stated). The result of buffer action is to make the amount of respiration appear smaller than it really is.

It was found that no alkali or acid (other than carbonic) was given off by the organisms studied. This was determined by placing the organisms in tap water free from CO_2 and allowing them to respire for some time so as to change the color of the added indicator; the CO_2 was then driven off (by a current of air free from CO_2) and it was found that the indicator returned to its original color.

It was found by control experiments (in which the indicator was added after respiration took place) that the indicator itself (at the concentrations employed) had no effect upon respiration.

The accuracy of the measurements depends on developing skill in matching standard colors under uniform conditions. The standard colors are prepared by making a series of buffer mixtures[8] containing the same concentration of indicator as the experimental solutions and contained in Pyrex tubes of the same size as those in which the organisms are respiring. Various devices may be employed to make the matching more accurate. A background of white or light gray paper is used by many, while others prefer opal or ground glass. In some cases it is advisable to screen off the lower part of the tube or

[7] Osterhout, *J. Biol. Chem.*, 1918, xxxv, 237.

[8] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xlvii, 420, footnote 7. Frequent renewal of the buffer solutions is advisable.

to place the tube in a hole bored in a block of wood and to view it through another hole bored at right angles to the first. The last method is very useful when there is a color (due to small suspended organisms or to the giving off of coloring matter by the organism) which is superimposed on that of the indicator. This color is contained in the tube which is put into the wooden block in line with the tube containing the buffer solution, so that the light passes through both tubes before reaching the eye. In this way the color of the indicator can be varied independently of the disturbing color, which is due to the organism.

Uniform conditions for comparison of colors were secured by the use of the "Daylight" lamp.[9] This gives a uniform source of light under which colors can be matched with sufficient accuracy, so that the investigator is free from disturbances due to fluctuations of daylight.

The best test of the accuracy of the work is to make repeated measurements on the same material in its normal environment at constant temperature. Tests made with favorable material show that the variation is very small, the probable error (in the time required to produce a standard change in acidity) being less than 1 per cent. Hence if greater variations are found, they must be due to the variability of the material, to the personal equation of the observer, or to unfavorable conditions (*e.g.* of illumination) in comparing colors, rather than to the method. A probable error of less than 1 per cent must be regarded as highly satisfactory for biological measurements.

The results obtained by the use of the new apparatus[2] are quite as accurate as those obtained by the original method.[1]

Attention may be called to two things in respect to the curves: (1) After each measurement there is an interval during which the solution is changed; since the organism is exposed to the action of the reagent during this interval the time should be included in the total time of exposure (as shown in Fig. 1). (2) The rate is obtained by taking the reciprocal of the time required to produce the standard

[9] Cf. Luckiesh, *Science*, 1915, xlii, 764. The form used is known as "north light."

change in acidity; hence it represents the average rate for that period. If the rate changes during the period, the average rate is probably the actual one somewhere near the middle of the period. Hence in Fig. 1, each ordinate representing the average rate is placed in the middle of the period. In these papers all curves in which rate is

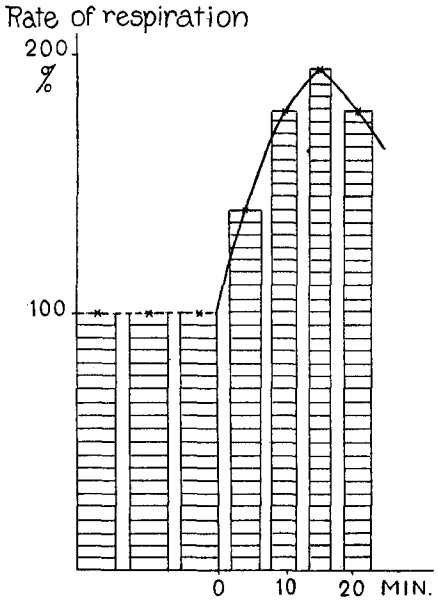

FIG. 1. Curve showing the rate of respiration under normal conditions (dotted line), and under the influence of a reagent (unbroken line). The periods during which measurements are made are indicated by horizontal shading; the intervals during which no measurements are made are left blank. Each measurement gives an average rate for the period; as this average rate is probably the actual rate near the middle of the period, the ordinate expressing the average rate is placed in the middle of the period. For convenience in comparison, time is reckoned from the beginning of exposure to the reagent.

plotted against time are made in this manner. For purposes of comparison the time expressed on the abscissæ is usually reckoned from the beginning of exposure to the anesthetic. The preceding part of the experiment, during which respiration was going on under normal conditions, is also represented on the curve but in this part the

figures expressing time are omitted from the abscissæ in order to avoid confusion.

In averaging curves the following procedure was adopted. A curve was drawn for each experiment (plotting rate of respiration as ordinates and time as abscissæ). These curves were averaged graphically, making use of interpolated points. It may be stated that this procedure is perfectly sound since the error of interpolation is usually less than the experimental error.

The rate of respiration is stated in all cases as per cent of the normal rate (which is always taken as 100 per cent). The rate is taken as the reciprocal of the time required to produce the standard change in acidity. The time required to produce this change under normal circumstances is usually stated, and from this the actual time required to produce the same change under the influence of the reagent may be easily calculated. It is not necessary to find the reciprocals of the time under normal and abnormal conditions and then to divide the latter by the former in order to get the relative rate; for the same thing is accomplished more quickly by dividing the time required to produce the standard change under normal circumstances by the time required to produce the same change under the influence of the reagent.

Of particular interest is the fact that these investigations were made by a method which enables us to ascertain the time curves of respiration by determinations made at very brief intervals (in some cases 1 minute or less). When the intervals are long (as in practically all previous investigations) it may happen that a rise in the rate of respiration which is quickly followed by a fall cannot be detected; and in general the form of the curve cannot be determined with sufficient accuracy to enable us to study the dynamics of the process.

Another point of importance is that so wide a range of material was studied that it is possible to judge whether the results obtained are of general validity. The plants included bacteria, the higher fungi, algæ, and flowering plants, while the animals included insects, frog eggs and tadpoles, and fish embryos. Precisely similar experiments were made on all these forms and as these experiments were all carried out under the same conditions the results are comparable in all respects.

All of these papers deal primarily with the effect of anesthetics on respiration. The special interest of this problem is too well known to require extensive discussion. Of late it has centered largely about the theory of Verworn, which states that anesthesia is a kind of asphyxia, due to a checking of respiration by the anesthetic.

Although this theory has been widely accepted there are excellent reasons for regarding it as invalid. Among these may be mentioned the observations of Warburg,[10] who found that phenylurethane inhibited cell division in the sea urchin egg without altering the consumption of oxygen, and those of Winterstein[11] who found that anaerobic animals are easily narcotized, which is difficult to understand if narcosis depends on interference with oxygen consumption. Loeb and Wasteneys[12] showed that to produce complete narcosis in *Fundulus* embryos by lowering the rate of respiration (by means of KCN) it was necessary to diminish respiration to one-fourteenth of the normal; but the same degree of narcosis could be produced by chloroform with a lowering of respiration amounting to only 5 per cent (or even less). In this case it would appear that anesthesia is not due to the checking of respiration by the anesthetic but to some other mode of action. This conclusion was confirmed by a variety of experiments made with other anesthetics and upon other organisms. Winterstein[13] in a later paper has shown that in the spinal cord of the frog, anesthetized with alcohol, the rate of respiration is above the normal.

The experiments upon plants made by various observers[14] are not in agreement. This is doubtless due to differences in the method of experimentation. One point of great importance which has been brought out in recent studies, particularly by those in this series,

[10] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

[11] Winterstein, H., *Biochem, Z.*, 1913, li, 143.

[12] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 517; *Biochem. Z.*, 1913, lvi, 295.

[13] Winterstein, *Biochem. Z.*, 1914, lxi, 81.

[14] For a review of the literature see Czapek, F., Biochemie der Pflanzen, Jena, 2nd edition, 1913, i, 195 ff. See also Ewart, A. J., *Ann. Bot.*, 1898, xii, 415, and Appleman, C. O., *Am. J. Bot.*, 1916, iii, 223. For a general review of the literature on animals, see Winterstein, H., *Biochem. Z.*, 1913, li, 143; also Höber, H., Physikalische Chemie der Zelle und der Gewebe, Jena, 4th edition, 1914, 460 ff.

is that the result depends in most cases on the length of the experiment. This is due to the fact that under the influence of the anesthetic the rate of respiration constantly changes. In the older experiments this was overlooked and the discrepancies in the results are doubtless due in large measure to this circumstance. It is important to be able to determine the rate of respiration at intervals of 5 or 10 minutes, or even less, as is possible by the method employed in these studies.

All of the plants studied in the writer's laboratory (including representatives of bacteria, higher fungi, algæ, and flowering plants) agree in their behavior toward anesthetics. While lower concentrations produce no effect on respiration, higher concentrations cause a rise followed by a fall. In general the rise of respiration appears to be associated with reversible anesthesia while the fall below the normal rate indicates irreversible toxic effects, at least if it goes much below the normal.

It is evident that these results are directly opposed to the theory of Verworn.

The results obtained by Miss Irwin upon animals are likewise not in agreement with Verworn's doctrines, for although they show that anesthetics may produce a temporary decrease of respiration, which cannot be wholly explained on the ground of cessation of movement, this decrease is much too small to produce anesthesia.

On the other hand Miss Irwin's observations are interesting as showing an apparent difference between animals and plants in their behavior toward anesthetics, in that the temporary decrease followed by an increase which is found in animals does not occur in plants. If this should prove to be generally true, it is significant.

The facts developed in these studies are of considerable interest but it seems wiser to defer their interpretation until more information is available. They offer suggestions for further attacks upon the problem and have an important bearing on the theory of anesthesia. These investigations are being continued and it is expected that additional results will be forthcoming in the near future.

It may be added that throughout these articles the term respiration is used as meaning oxidative processes which furnish energy, beginning with the taking up of O_2 and ending with the production of

CO_2 and H_2O. The consumption of O_2 and the production of CO_2 furnish the most convenient means of measuring respiration and it is always desirable to study both together. If we find that O_2 is consumed but no CO_2 is produced, as in some of Warburg's experiments,[15] we may speak of respiration. If on the other hand CO_2 is produced but no free O_2 is consumed, as is the case in anaerobic respiration, we may regard this as respiration also. Ordinarily a rise in the rate of CO_2 output is interpreted as a rise in the rate of respiration, but it might happen that the sudden production of an acid (*e.g.* lactic acid) might set free CO_2 from carbonates already present in the tissue. When there is reason to suspect that this is the case special precautions must be taken to ascertain how the CO_2 is produced.

Some writers endeavor to make a distinction between oxidation in the living cell and that which occurs after death. In the opinion of the writer there is no valid ground for such a distinction, and the term respiration is here used to include processes which occur immediately after death.

SUMMARY.

A series of investigations on respiration with improved quantitative methods has been commenced. The first of these are here described. They show that when anesthetics are employed in sufficient concentration to produce any result, plants show a rise in the rate of respiration which is followed by a fall. In the animals studied, the rise (found in higher concentrations only) was preceded by a temporary fall which is not entirely due to lowering of muscular activity or tonus. In lower concentrations the effect on animals was merely a decrease of respiration.

The results of all the investigations are opposed to the theory of Verworn.

[15] Warburg, *Ergebn. Physiol.*, 1914, xiv, 319.