STUDIES OF ONION ROOT RESPIRATION

III. AMPEREOMETRIC TITRATION AS A METHOD FOR THE MEASUREMENT OF RESPIRATORY OVERSHOOT*

by

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The respiratory rebound, which has been most clearly defined by BURTON[1] can be measured by means of ampereometric titration. The method developed was adapted especially for onion root segments and it has made possible kinetic study of ordinary respiration rates in addition to overshoot rates produced after an anaerobic period at any temperature of experimental interest.

In contrast to ordinary polarographic analysis, where the impressed voltage across a dropping mercury electrode and an electrode consisting of a quiescent pool of mercury is increased continually, in the ampereometric technique the applied voltage is kept constant at a magnitude where the diffusion current is linearly proportional to the concentration of the material under investigation — in the case of oxygen, −0.5 V. For use in respiratory studies, the decrease in the concentration of oxygen of a solution surrounding the respiring tissue can be related directly to the oxygen consumption of the tissue; this change is recorded in units of microamperes of "diffusion" current. Use of this method for determining oxygen consumption by a living tissue was first suggested by MÜLLER[2] for yeast suspensions. Applications to studies of photosynthetic activity have been made by PETERING AND DANIELS[3] and DUTTON AND MANNING[4]. BAUMBERGER[5] also employed it for measurement of respiration in yeast, in order to relate the "redox" potential to the rate of oxygen consumption. Special application to root respiration has been made by DUBUY AND OLSON[6] and by WANNER[7] who worked with *Avena coleoptile* and with *Triticum sativum* and *Allium cepa* roots, respectively.

In designing a method that could be employed for the investigation of rebound phenomena in respiration, several factors had to be taken into consideration. First the tissue (in this study, onion root segments) must be placed in a vessel permitting ampereometric titration in such a fashion that there is no physical contact either with the dropping mercury or with the quiescent mercury pool, not only because the mercury might be toxic to the living tissue, but also because there must be no mechanical hindrance on the mercury drop as it forms, ripens and falls, if reproducibility is to be at-

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tained. Second, the vessel must be designed in such a fashion that rapid changes in liquid environment (from initial aerobiosis to anaerobiosis back to aerobiosis) are possible without loss in number of roots, without physical damage to the tissue, and with minimum liquid turbulence. Third, the vessel has to be constructed in such a fashion that the volume occupied by the tissue and the solution is a constant one. This means that there must be some device for the removal of excess mercury as it accumulates. Fourth, and finally, it must be possible to place the entire system in a water bath in order that it be kept at constant temperature.

The cell and inset designed to meet these requirements is shown in Figs 1 and 2. The cell is a modification of that used first by DUBUY AND OLSEN[6] and later by WANNER[7]. Fig. 2 shows an isometric representation of the Plexi-Glass* support employed inside the cell. The inset was machined in such a fashion that the top disc could be easily removed to permit admission of the root segments but would not come off as the liquid

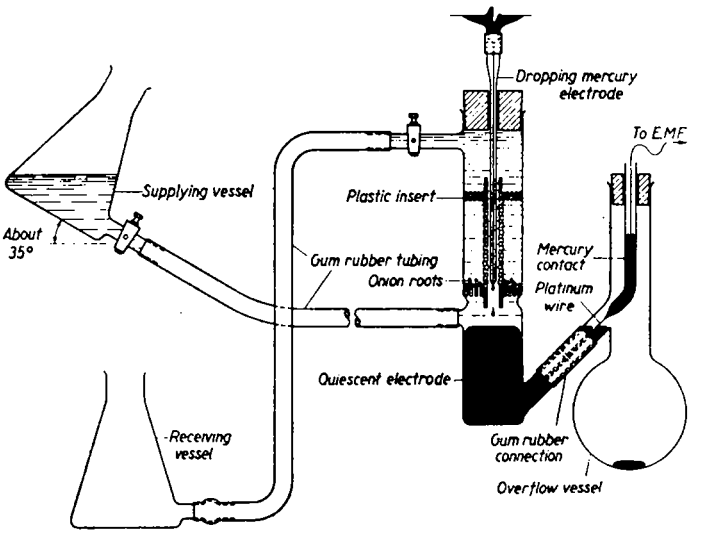

Fig. 1. Cell with inset for the polarographic determination of oxygen consumption before and after an anaerobic period

was changed. As many holes as possible of the size shown in Fig. 2 (about 0.75 mm) were drilled in both shoulders and in the center cylinder to permit diffusion to the drops issuing from the capillary, shown in operating position in Fig. 1. The DUBUY-OLSON cell was further modified by the introduction of two side-arms, one placed with its lowest edge flush with the top of the mercury meniscus of the quiescent electrode, the other, above the top of the uppermost shoulder of the plastic inset. To the top side arm there was attached a micro stop-cock. The side arms were subsequently connected by means of gum rubber tubing to two Erlenmeyer flasks of 50 ml capacity. At the bottom of each of these there was affixed a piece of glass tubing in order that connection might be made with the electrolysis cell; in addition, the flask to be attached to the lower of the cell side arms was also equipped with a micro stop-cock. The tubing length between the receiving vessel and the electrolysis cell was 18.5 cm, that between the supplying vessel and the electrolysis cell was 30.0 cm. Before use the tubing was extracted con-

* Plexi-Glass supplied by Dr C. E. ANDERSON of the Rohm and Haas Co., Philadelphia, Penna.

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tinuously for 18 hours with boiling acetone in order that any free sulphur might be removed. The entire cell, with the exception of the two Erlenmeyer flasks was placed in a Plexi-Glass clamp (not shown in the figure) so constructed that the vessel containing the roots and the vessel for mercury overflow could be kept at a fixed distance from one another. The clamp fastened by means of a brass set screw to a brass rod of such length that it could be placed in the water bath. The end of this rod rested on a rubber pad which served as a damp for vibration in the bottom of the bath.

The receiving Erlenmeyer flask was placed in the water bath, the supplying flask above it, approximately in the position indicated in Fig. 1. These positions, particularly the angle of the supplying vessel, and the length of the connective tubing were important in keeping turbulence at a minimum while changing the solutions. Adherence to the specifications given above permitted flushing through of 30 ml of solutions (about 7 ml more than the actual volume of the electrolysis cell) in 23 seconds. The effect of this manipulation on the characteristics of the respiratory record will be discussed later.

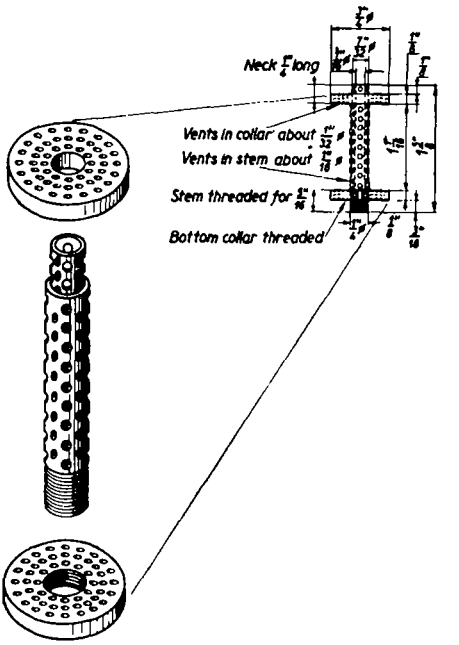

Fig. 2. Isomeric representation of Plexi-Glass inset

The dropping mercury electrode and the device for controlling the amount of pressure on the drop were constructed according to KOLTHOFF AND LINGANE[8]. In addition the mercury reservoir and the capillary were supported on a ring affixed to a stativ so that centering and raising or lowering of the capillary was possible to within very close dimensions.

The reproducibility obtainable from the dropping mercury electrode depends to a large extent on the purity of the mercury and solutions and the cleanliness of the apparatus employed. In all of the analyses the mercury used had been previously distilled four times to insure chemical purity. Previous to distillation it was aerated in dilute nitric acid until examination showed that the surface tension of the mercury was such that contaminants had been oxidized. Following the aeration the mercury was filtered and dried with alcohol and ether and then placed in the distillation apparatus.

All platinum contacts and all glass apparatus were cleaned in the following solutions: concentrated nitric acid, dilute nitric acid, tap water, distilled water and the specially distilled water whose preparation will be described below. The plastic inset shown in Fig. 2 was aged in distilled water which contained a non-ionic wetting agent. This precaution was felt to be necessary in view of the fact that it has been found that plastics frequently discharge "Plastic-ions" into a solution if they have not been suitably aged*. Rubber stoppers were boiled in distilled water to remove any possible contaminants.

* Personal communication from L. G. VAN DE BOGART.

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In order that the tank nitrogen might be oxygen free it was bubbled through seven gas-washing tubes packed with glass beads and containing the following solutions: a) a 50% (vol) solution of ammonium hydroxide and distilled water saturated with ammonium chloride, to which there had been added pieces of bright copper foil, (b, c, d) a 10% sulphuric acid solution, (e, f, g) distilled water containing a drop of methyl red. Gassing of the solutions was always carried out at the temperature of the experiment.

The specially distilled water was prepared as follows: distilled water containing sulphuric acid and potassium permanganate was redistilled and condensed in block tin. Previous to distillation it was kept at 80–90° C while purified nitrogen gas was bubbled through it. The distillation *per se* was likewise carried out under nitrogen; the condensate was stored under nitrogen.

The supporting electrolyte was a phosphate buffer of the desired p_H containing a drop of 0.1% methyl red solution for the suppression of maxima. It was found that the methyl red was best added after the solutions had been gassed, just prior to being placed in the electrolysis cell. The buffer mixtures were made of M/15 Na_2HPO_4 and KH_2P_4.

Onion bulbs from the Barteldes Seed Company, Lawrence, Kansas, were sprouted in constantly aerated 25% HOAGLAND's solution[9]. The bulbs were placed on holes drilled through plastic racks so designed that the basal section of the bulb was just bathed by the nutrient, with a free circulation of the aerated solution around the bulb. The aquaria were kept in a dark room at an essentially constant temperature (25° C $\pm$ 2°). It was necessary to operate under these conditions to insure a constant growth environment and the absence of any possible growth anomalies[10]. Cutting of the roots was carried out under ruby light using the technique that has been described in detail by BERRY AND BROCK[11]. All experiments were made with 50 2 day old root segments, 5 mm in length, cut either 5 mm above the tip or at 10 and 15 mm above the tip. This meant that one of the segments had two cuts. BERRY has shown that respiration is not measurably influenced by this factor[12]. After cutting, the roots were placed by means of a spatula in the electrolysis cell containing about 17 ml of the oxygenated buffer solution. This was made possible by removing the upper flange of the plastic support shown in Fig. 2. After the roots had been added, the flange replaced, and the cell stoppered, the capillary was slowly lowered into such a position that the tip of the capillary was in approximately the same plane as the upper surface of the bottom flange, as the diagram shows. The cell was then completely filled with oxygenated buffer by means of the supplying vessel. Care was taken to obtain a continuum of fluid from the stop cock on the supplying vessel to the stop cock on the upper arm of the cell. This was insured by employing an excess volume of buffer.

At each temperature of measurement it was necessary to make two calibrations: the variation in drop time with applied pressure, and the variation in limiting current with oxygen concentration. The first of these was accomplished by the use of the apparatus described by LINGANE AND KOLTHOFF[13] for the collection of mercury drops at a constantly applied voltage. Because ILKOVIC[14], followed by MACGILLAVRY AND RIDEAL[15] have shown that the diffusion current is a linear function of the quantity $m^{2/3} t^{1/6}$, where m is drop mass and t is drop time, this quantity was calculated from the data obtained. The plot of these values against of the values applied pressure is given in Fig. 3.

Calibration of applied current variation with respect to oxygen tension was carried

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out by means of a modified Winkler technique. A sample of oxygenated buffer that did not contain any tissue was polarized in the electrolysis cell in the presence of the plastic inset. A portion of the solution was then removed and analysed according to a method recommended by the AMERICAN PUBLIC HEALTH ASSOCIATION[16] with the exception that the amount of iodine was estimated colorimetrically in a Coleman spectrophotometer at a wave length of 580 Å.

The Leeds and Northrup polarograph employed in these measurements was equipped with a galvanometer of the ballistic current detector type providing under the operating conditions described above a sensitivity equivalent to an oxygen concentration of 10^{-6} mol/l. This factor was of great importance at the time of the switch from aerobiosis to anaerobiosis and particularly in the switch from anaerobiosis to aerobiosis, for it was found that the electrical circuit experienced its own rebound, not as a result of the change in oxygen concentration, but as a result of the fact that diffusion relationships to the forming mercury drops were upset. For this reason, special measures were taken, first, to reduce the electrical rebound to a minimum for reasons of sensitivity, and second, to guarantee that the instrumental overshoot would be limited to a known period from measurement to measurement. Satisfaction of these specifications requires a fixed position of the supplying and receiving vessels, a constant length of rubber tubing from the vessels to the cell and the use of small stop cocks rather than pinch clamps for flow control. With maintenance of the mechanical conditions previously described it was found that the root environment could be changed completely within 23 seconds. The duration of the instrumental rebound under these conditions was such that when 150 seconds had elapsed, including the 23 seconds required for emptying and refilling the vessel, the recording and amplifying circuits were in all cases again indicating the oxygen concentration of the solutions. This is shown for a change in oxygen tension in both directions at 25° C in Fig. 4. This curve is typical of numerous controls run at all temperatures at which rebound measurements were made.

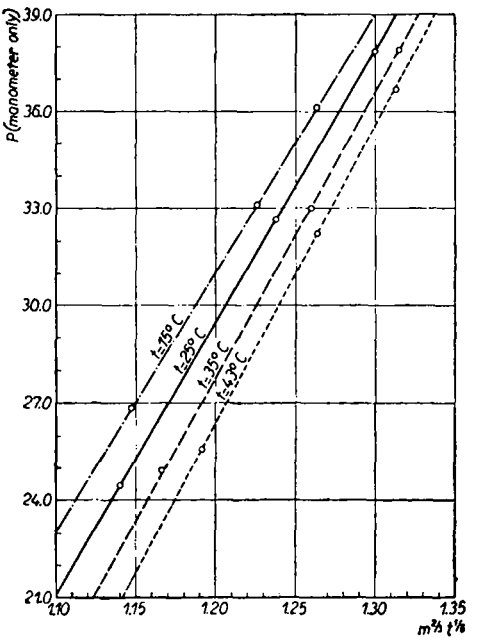

Fig. 3. Variation of $m^{2/3}$ $t^{1/6}$ with applied pressure in centimeters. m = mass of drop, t = drop time

In the course of the early experimental work it was soon noted that the use of the plastic inset reduced the recorded diffusion current to a value lower than that which would have been obtained in its absence. This is probably due to the fact that the support provides mechanical hindrance to diffusion to the surface of the drop. For this reason a group of experiments were made in which the center rib and the top flange of the inset were removed. Loss of roots was prevented by the introduction of a small platinum screen at the exit stop-cock; a small rubber cone whose largest diameter just slightly exceeded that of the opening of the lower flange was placed about 1 cm above

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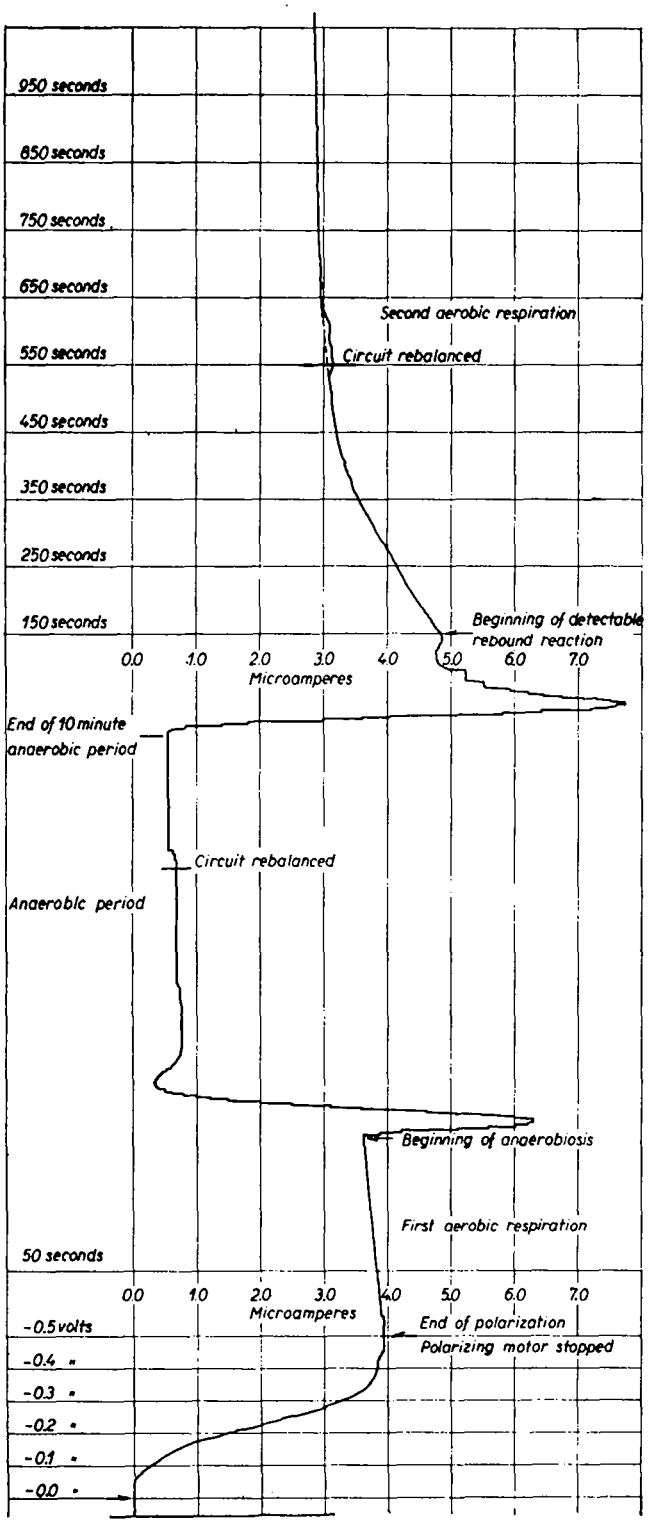

the tip of the capillary. At the time of the first change in solution the capillary was lowered by means of the stativ to which it was fixed until the lower opening was sealed. It was raised again after 50 seconds of the second aerobic period (the overshoot period) had elapsed. Under these conditions the instrumental rebound persisted for about 450 seconds. In addition the recorded diffusion current appeared to fluctuate sinusoidally for an additional 150 seconds. For these reasons use of this method was abandoned and the complete inset was employed.

In making an actual measurement use was made of the following procedure: the phosphate buffers were gassed at the correct temperature and the tissue placed in the cell according to the method previously described. After dropping was started, polarization was begun. The solution was polarized from 0 applied volts to −0.5 V. When this point was reached the polarization was stopped and a constant voltage of −0.5 V was applied. The respiration was permitted to proceed normally for 500–800 seconds and then the solution around the roots was changed to one free of oxygen. In making this

Fig. 4. Typical "ampereogram" showing electrical rebound and respiratory rebound. 50 onion root segments, 0–5 mm portion of root. t = 25° C. 10 minute anaerobic period. pH of buffer = 6.489

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change the nitrogen saturated solution was placed in the supplying vessel maintained in the position shown in Fig. 1. The stop cock connected to it was first opened and immediately thereafter, the stop cock connected to the cell was opened. When the supplying vessel was nearly drained, the latter was closed, and then the stop cock connected to the supplying vessel was closed. It was found that the sequence of these operations was of great importance in order that a minimum electrical instrumental rebound would be obtained. This is probably due to the fact changes in hydrostatic pressure above the surface of the quiescent mercury pool produced changes in its level. The roots were kept anaerobic for the desired length of time. The completeness of anaerobiosis was easily checked by observing the current delivered. In the absence of oxygen the current should exactly equal the residual current for the phosphate buffer at an applied voltage of −0.5 V. When this was not the case the run was discarded. As previously stated the polarograph, under these conditions, could detect a concentration of oxygen equal to approximately 0.5 μl in the 23 ml of buffer. At 25° C, 50 root segments 5 mm long at air saturation consume 1.5 μl of oxygen per minute[12]. On this basis it would seem that nearly complete anaerobiosis was achieved. The change back to an environment of high oxygen tension was performed in an identical fashion. The analysis was continued until the rate of oxygen consumption returned to the initial level. No evidence of mercury poisoning was ever obtained during the time of the individual experiments since the post rebound rate of oxygen consumption was never less than that obtained in the initial aerobic period. Results of this type of measurement and an analysis of the data so obtained for different root segments, for varying periods of anaerobiosis, at different temperatures and at various concentration of hydrogen ion of phosphate buffer will be reported in subsequent papers.

Advantages of this type of rebound measurement may be most clearly seen by a comparison with other methods of measurement. A review of overshoot phenomena in nerves and muscles may be found in BURTON[1]; the general problem of recovery from anaerobiosis in invertebrates has been discussed by VON BRAND[17]; NORRIS[18] has carefully reviewed the existing literature on respiratory rebound and its possible relationship to rebound in electrical potential measurements.

Most of the reports in the literature have several features in common. In nearly all of the experimental work, the rebound has been treated as a static, rather than a dynamic phenomenon of the living organism. This arises, in part, from the fact that the techniques of measurement yield data which are not precisely amenable to a kinetic, dynamic treatment. Methods that have been employed include the WINKLER titration[19, 20]; an evaluation of the respiratory quotient, which is, in the final analysis, a static quantity[21, 22], at set times in the post-anaerobic period; in the case of plant tissue, use has been made of the FENN microrespirometer[23], and of the PETTENKOFER titration for carbon dioxide[24] and of the speed of reduction of methylene blue[18]. No attempt was made in any of these cases to describe the system kinetically, although in many instances, "rates" formed the basis of the discussion.

Secondly, reports are frequently characterized by lack of temperature control of the system being measured. This is perhaps the most serious indictment of a study that concerns "rates" and "rate measurements". It has been pointed out by PEASE[25] that if a reaction rate is doubled by a ten-degree rise in temperature (roughly true for most known chemical reactions) there may be approximately a 7% change in the reaction rate per degree, or a change of 7 parts in a 1000 for a one-tenth degree change. This

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omission is even more serious when one considers the fact that theoretical considerations demand a temperature-caused rebound. However, it must be remembered that in many cases control of temperature presents serious difficulties in the designing of apparatus.

Another feature characteristic of many measurements likewise stems in most cases from the type of measurement employed for the rebound reaction. Frequently the experimental conditions are such that data are lost in the post-anaerobic period for as long a time as 5 [23] to 90 minutes[24]. This should correspond to the portion of the reaction where numerical results should be of greatest interest. NORRIS's data (*loc. cit.*) have the advantage of permitting evaluation of rate from the inception of the second aerobic period.

Finally, there are examples reported in the literature for which there are no quantitative data whatsoever on the increased rate of oxygen consumption after anaerobiosis, caused, usually, by the experimental difficulties involved. Such a case is that reported by KITCHING[26] on the post- anaerobic response of the protozoan, *Cothutria kellicottiana.*

Use of the polarographic method permits the attainment of data 150 seconds after the inception of aerobiosis; it is capable of being carried out under controlled temperatures; metabolic waste products accumulated in the surrounding milieu during anaerobiosis are removed in the return to aerobiosis; checking of current delivered during anaerobiosis provides a means of ascertaining the completeness of the anaerobiosis, and the continuously recorded curves are amanable to kinetic analysis.

The principal limitations of the present method seem to be a) the interference with free diffusion of oxygen in the electrolysis cell imposed by the plastic inset. This apparently has little influence upon reproducibility (as shown by Tables I and II) but makes impossible the calculation of oxygen concentration in true molar units; b) the application of the technique to unicellular organisms, which offer certain advantages for kinetic studies, is impossible without modifications that pose serious technical difficulties (c) no evaluation of carbon dioxide was made along with the determination of oxygen consumption. This would provide valuable additional information.

TABLE I

REPRODUCIBILITY OF OVERSHOOT MEASUREMENT
0–5 mm ROOT SEGMENT

20 Minute Anaerobic Period; t = 25° C

Time (Seconds)	Test 1 % Increase*	Test 2 % Increase*	Test 3 % Increase*
150	1082	1069	1097
200	835	800	806
250	623	596	600
300	588	590	578
350	370	367	575
400	323	296	300
450	305	280	291
500	252	267	256
550	217	212	200
600	164	171	173
650	141	133	153
700	94	83	76
750	85	52	33

* % Increase of oxygen consumption after the anaerobic period

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TABLE II

REPRODUCIBILITY OF OVERSHOOT MEASUREMENT
10–15 mm ROOT SEGMENT

20 Minute Anaerobic Period; t = 25° C

Time (Seconds)	Test 1 % Increase*	Test 2 % Increase*	Test 3 % Increase*
150	465	442	402
200	408	395	340
250	377	367	389
300	229	259	238
350	200	217	189
400	170	163	159
450	164	152	157
500	108	108	100
550	92	65	87
600	—	44	13

* % Increase in oxygen consumption after the anaerobic period

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SUMMARY

The method of amperometric titration for oxygen, using the dropping mercury electrode, has been adapted for measuring the respiratory overshoot in segments of onion roots. The change in concentration of oxygen with time is followed continuously in the solution around the roots when an aerobic medium replaces one that is anaerobic. The period of electrical instability is limited to 150 sec, including the time required for changing the solutions. Data so obtained are amenable to kinetic treatment and reproducibility from experiment to experiment is good. The advantages and limitations of this method over those previously used are discussed.

RÉSUMÉ

La méthode du titrage ampérométrique pour l'oxygène, en utilisant l'électrode à goutte de mercure, est adaptée pour la mesure de l'excès respiratoire dans des segments de racines d'oignon. Les changements de la concentration de l'oxygène en fonction du temps ont été observés continuellement dans la solution entourant les racines, quand un milieu anaérobique est remplacé par un milieu aérobique. La période de l'instabilité électrique est limitée 150 secondes, le temps nécessaire pour le changement des solutions compris. Les données ainsi obtenues sont soumises au traitement cinétique, et sont reproductibles d'une expérience à l'autre. Les avantages et les limites de cette méthode, comparées à ceux des méthodes précédentes, sont discutés.

ZUSAMMENFASSUNG

Die amperometrische Titrationsmethode von Sauerstoff mit Hilfe der Quecksilbertropfelektrode wurde für die Messung des Atmungsüberschusses in Segmenten von Zwiebelwurzeln angepasst. Die Änderungen der Sauerstoffkonzentration mit der Zeit wurden fortlaufend in der die Wurzeln umgebenden Lösung verfolgt, wenn ein aerobes durch ein aneorobes Milieu ersetzt wird. Die Dauer der elektrischen Schwankungen ist auf 150 Sekunden beschränkt, wobei die zum Wechsel der Lösungen benötigte Zeit mit eingerechnet ist.

Die so erhaltenen Resultate können kinetisch ausgedrückt werden und sind gut reproduzierbar. Die Vorteile und die Grenzen dieser Methode im Vergleich zu früher gebräuchlichen Arbeitsweisen werden erörtert.

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