GASOMETRIC METHOD OF ESTIMATING OXIDASE ACTIVITY

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In the study of biological oxidations, two criteria are used as indications of oxidase activity. One of these is the oxidation-reduction potential of the system concerned, determined as described by Clark (3) and others. The other is the rate of oxidation measured by one of several methods. In our particular studies on the oxidase systems of various fruits, measurement of rate is much more useful than measurement of oxidation-reduction potential.

Rate measurements

Four important methods of measuring the rate of enzymic oxidation have been reported in the literature. These are the (1) colorimetric, (2) titrimetric, (3) electrometric, and (4) manometric methods.

If fruit oxidase is at all specific, it is very probable that the rate at which it oxidizes an added indicator is different from that at which it oxidizes the naturally occurring substrate. It is also possible that different catalysts are involved in oxidation of the natural and the added substrates. There are other objectionable features also, such as interference of turbidity of the juice, and difficulty in duplicating tints in permanent color standards.

Several of the more important colorimetric methods are those of Dye (6), Röhman and Spitzer (13), and Willstätter and Weber (17). Most colorimetric methods require the addition of H2O2. This addition alters natural conditions, and CrueSS and Fong (4) have shown that its concentration greatly affects the results obtained.

Guthrie (8) uses in a titrimetric technique a special substrate formed by the action of NaOH on glucose solution. HaeHN and Stern (9) have reported upon a method in which unchanged added tyrosin is determined at intervals.

In the electrometric method of Stearn and DAY (15), hydroquinone of the quinone-hydroquinone complex used with the quinhydrone electrode is oxidized by the oxidase, changing the ratio of quinone to hydroquinone and thus the E.M.F. of the quinhydrone electrode. The resulting drift in potential is taken as a measure of the rate of oxidation.

One of the earliest applications of the manometric method was made by Foa (7). The well known Barcroft manometric apparatus and its modified form, the Warburg (16) apparatus, are in very general use. Dixon and Elliott (5) have recently still further modified the Barcroft apparatus. The Bunzel (1, 2) apparatus also makes use of the manometric principle (see also Sennhauser 14).

Practical objections to the manometric apparatus are its high cost and
the rather large high differential pressures developed. The volume of liquid used is also small, usually less than 5 cc.

**Volumetric apparatus used**

In the apparatus devised by the writers, a number of the objections to the colorimetric, titrimetric, potentiometric, and manometric methods are obviated. It is volumetric instead of manometric; hence the internal pressure need not exceed 1 mm. of Hg compared with pressures occasionally exceeding 50 mm. of Hg in the manometric method. There is, therefore, little tendency for leakage of gas outward or inward. The volume of the reaction flask may be much larger than in the manometric method, and it is not necessary that its volume be known accurately, since change in volume at atmospheric pressure is measured. Ground glass connections are not necessary, and since relatively large volumes of O₂ are absorbed during the course of an experiment, standard gas burettes may be used for measurement of the volume of gas absorbed. Also, since the volume of the flask and burette is much larger in proportion to the volume of substrate than is the case in the usual manometric apparatus, there is less danger of retardation of reaction rate because of O₂ absorption and reduction of the partial pressure of O₂. In building the apparatus common and relatively inexpensive laboratory equipment may be used.

The constant temperature bath is 18 × 18 × 8 inches, and of galvanized sheet metal thermally insulated by asbestos. The water is brought to operating temperature by means of a 500-watt knife-type Cenco "lagless" heater, and is maintained at operating temperature by means of a similar 125-watt heater controlled by a mercury-filled electric regulator and small relay. The relay is operated by a 6-volt battery charged by a radio battery charger. It is possible to maintain the temperature within a range of 0.02° C.

Four 250-cc. Erlenmeyer flasks are held in position in the bath by clamps to two movable ½-inch rods suspended above the tank. The rods are moved back and forth horizontally at the rate of 90 times a minute by means of a ½-H. P. motor, geared down to the proper speed by wooden pulleys.

The four Erlenmeyer reaction flasks are fitted with rubber stoppers and connected to gas burettes outside the reaction chamber by means of heavy-walled, capillary glass tubing. The volume of the connecting capillary of each flask is less than 1 cc. A stop-cock connected to a small thistle tube fitted through the stopper of each flask is used to introduce added solutions and to equalize the pressure at the beginning of the run.

There is suspended inside each flask from the stopper a short wire holding a small glass cup in which is placed a small roll of filter paper, cut in
the form of a rosette at the top, and saturated with N/1 NaOH solution to absorb CO₂ liberated during the experiment. With fruit juices, the volume of CO₂ liberated is relatively large and must be removed in this manner in order to avoid serious error.

The four gas burettes are attached by clamps to the desk in front of the constant temperature bath. The top of each burette is slightly above the surface of the bath, in order that the connection to the reaction flask shall be as short as possible. The connecting capillary glass tubing is joined to the burette and to the flask by flexible rubber connections. The connecting capillary glass tubing is cut above the flask stopper, and the ends held together tightly by a flexible rubber connection; it is similarly connected to the gas burette. The rubber connections permit movement by the shaker mechanism. Each burette is surrounded by a Pyrex glass jacket such as that used on Liebig condensers. The jackets are connected in parallel to the constant temperature water bath and to a small rotary pump from a discarded automobile engine. The pump is operated by the same ½-H. P. motor that operates the reaction-flask shaker device, the pump and motor shafts being directly connected.

The burettes are filled with distilled water and the lower ends connected by rubber tubing to a single leveling bottle. Pinch-cocks on each tube permit leveling of each burette individually by the one bottle.

Mercury is of such high density that a small difference in height in the leveling bottle and burette represents a relatively large volume of gas. Clear petroleum oil of low density (nujol) gave a poor meniscus because of distillation of moisture from the reaction flask. Distilled water previously allowed to stand in air several days was found to be very satisfactory.

In order to prevent growth of algae in the bath and in the water jackets of the burettes, a small amount of formaldehyde (about 1:1000) was added to the water in the bath.

Substrate

While it would have been desirable to use the volumetric apparatus without addition of an oxidizable substance, it was found that oxygen absorption was too small and inconsistent in the absence of such addition. Thus, 50 cc. of the freshly expressed apple juice absorbed less than 1 cc. of O₂ in 130 minutes. Peaches gave a similar result.

Bunzel (1, 2), in his tests with the oxidase of potatoes and beet leaves, used pyrogallol as the substrate in his manometric apparatus. However, we found that the rate of absorption of O₂ by the pyrogallol was much greater at pH 5.7 in the absence of fruit juice than at pH 6.0 in juice. Evidently the fruit juice inhibited rather than catalyzed the reaction. At pH 10.3, non-enzymic oxidation was extremely rapid. Various tests gave additional evidence that pyrogallol, while probably satisfactory for potato
juice, is not very satisfactory for fruit juice. However, it proved useful with asparagus, spinach, string bean, and pea juices.\(^1\)

Several of the commonly used colorimetric oxidase indicators, among them benzidine, hydroquinone, and guaiacol, were found to cause no significant additional \(O_2\) absorption. Tannins and fruit coloring matter from several sources also were found of no value as substrates.

Since catechol tannins occur naturally in many fruits, and because Onslow (10, 11, 12) has reported that plant materials that give a positive test with guaiacum contain substances with a catechol grouping, this phenol was used in a number of trials with apple, apricot, avocado, olive, prune, peach, and pear juices. It was found to be a very satisfactory substrate. There was also only slight absorption of \(O_2\) in water and in the boiled juice in the presence of the catechol, and absorption in these media ceased or became very slow after 15 minutes.

The \(CO_2\) evolved by respiration of fresh fruit tissues and freshly expressed juice under the conditions of our tests was found to be appreciable, and it was evident that some means of absorbing this gas (such as that previously described) is necessary. The initial rate of absorption increased with increase in ratio of enzyme to juice but not in a strictly proportional manner.

Although the \(O_2\) absorbed at the close of an experiment varied with the quantity of catechol initially present, the variation was not strictly proportional to the concentration of catechol. Thus when the quantity of catechol in one flask was 20 times that in another, the \(O_2\) absorbed was less than twice that in the second. Possibly the reaction product inhibits enzymic action at higher concentrations of catechol. Two cc. of 5 per cent. catechol to 50 cc. of sample was found satisfactory.

Portions of apple juice were brought to various pH values ranging from pH 2.25 to 6.75 by addition of NaOH or of N/1 acid. The pH of the untreated juice was 4.0. The rates of gas absorption were determined at 25°C.

Reducing the pH value from 4.0 to 3.7 very greatly retarded \(O_2\) absorption, and at 2.25–3.0 it practically ceased, being approximately the same as in boiled juice. Increasing the pH to 5.4 and 6.75 greatly increased the absorption of \(O_2\).

Alkalinity naturally greatly favored \(O_2\) absorption. The rate of absorption and the total amount of \(O_2\) absorbed were much greater at pH 9, 10.1, and 10.8 than at pH 4.5; but much of this absorption is undoubtedly due to non-enzymic oxidation, as absorption is nearly as great in boiled as in unheated juices.

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\(^1\) In tests made by H. M. Pancoast.
LITERATURE CITED