A COLORIMETRIC METHOD FOR THE MEASUREMENT OF POLYPHENOLOXIDASE ACTIVITY

A colorimetric method of measuring polyphenoloxidase activity by measuring the rate of formation of brown melanin-type pigment formed by this enzyme from a polyphenol substrate was described by Ponting and Joslyn (1948). These workers reported a straight-line relationship between optical density and time. However, Warner (1951) found that a straight-line relationship held only for the first 2·5 min, while Baruah and Swain (1953) found that under their experimental conditions the straight-line relationship held only at low enzyme concentrations.

Miller *et al.* (1944) showed that, in this reaction, o-benzoquinone is produced according to the equation

$$Q = at/(b+at),$$

where Q is the concentration of quinone, t is the time in seconds, and a and b are constants determined by the extent of enzyme inactivation during the initial stage of the reaction. This can be rewritten as

$$\frac{1}{Q} = \frac{b}{a}\frac{(1)}{t} + \frac{1}{a},$$

which represents a straight line.

Nelson and Dawson (1944) stated that the transformation of the initial oxidation product from polyphenoloxidase activity is independent of enzyme activity and virtually instantaneous. In view of this, Baruah and Swain (1953) suggested that the quinone produced may be related directly to absorbence, and they showed that a straight-line relationship does exist between the reciprocal of extinction and the reciprocal of time.

In an investigation at the Food Preservation Research Laboratory of a storage disorder of apples known as "superficial scald", the polyphenoloxidase activity of the apple skin was studied. Under no conditions of enzyme concentration or time was a straight-line relationship obtained when optical density was plotted against time. However, a straight-line relationship was obtained between the reciprocal of extinction and the reciprocal of time. This paper presents some observations on the reactions involved in the action of polyphenoloxidase.

The method of preparing the enzyme closely followed that of Ponting and Joslyn (1948), except that the final treatment with Amberlite IR-100 cation exchanger was omitted. Enzyme activity was measured by the method of Ponting and Joslyn (1948), using catechol as substrate, employing a citrate-sodium phosphate buffer (pH 4·85), and measuring the rate of colour formation with an Optica CF4 spectrophotometer.

Results and Discussion

When it was attempted to obtain a straight line, as described by Ponting and Joslyn (1948), Warner (1951) and Baruah and Swain (1953), for the plot of optical density against time, the graphs always resulted in curves. A

typical graph is shown in Figure 1. However, when the curve shown in Figure 1 is plotted as a reciprocal plot, the graph shown in Figure 2 is obtained. It follows then from the equation of Miller *et al.* (1944) that the o-benzoquinone produced in the reaction is being measured.

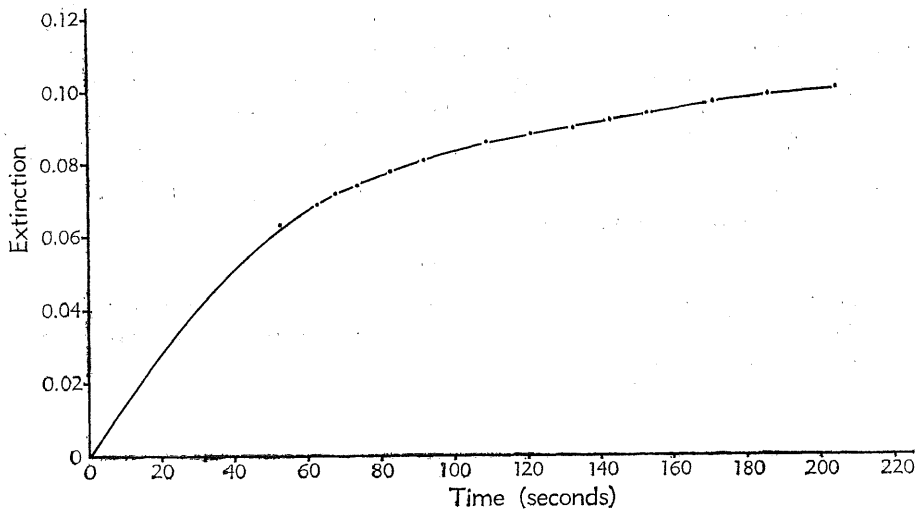

Fig. 1.—Increase of extinction with time in the catechol-polyphenoloxidase system, showing a typical curve obtained. Reaction mixture: enzyme 1·0 ml; catechol (0·25M) 1·0 ml; citrate-phosphate buffer 1·2 ml.

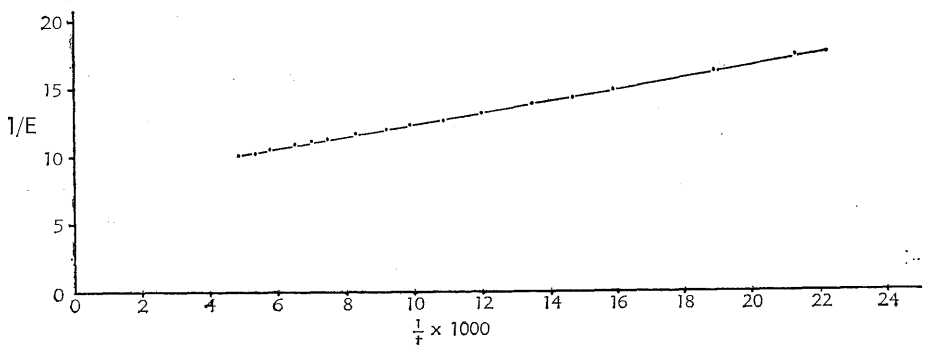

Fig. 2.—Results of Fig. 1 shown as a reciprocal plot.

Baruah and Swain (1953) pointed out that in using extinction as a measure of o-benzoquinone produced, they assumed that the same coloured condensation product is produced regardless of conditions. They stated that in other work (unpublished) they found that crude enzyme preparations from different sources gave easily observed differences in colour with catechol as substrate, but with the enzyme used in the work under discussion, the absorption maximum of the coloured product was always at 480 mμ. They concluded

therefore that their assumption was justified. Warner (1951) pointed out that in his system the colour changed from a red-brown to a bright yellow with increasing substrate concentration. He suggested that since he was working with relatively crude enzyme preparations the colour was probably due in part to secondary reactions between the oxidation products of catechol and other compounds present in his system. In the present work it was found that, using large differences in substrate concentration (approximately 0·02M to 0·5M), a colour change in the product from red-brown to yellow was observed. However, when using concentrations of the order of 10–20 mM, no visual differences in colour of products could be observed.

Using catechol at concentrations of 2, 10, 15 and 20 mM, absorption curves of the product were drawn after a constant reaction time. The absorption curves showed two peaks, one at 425 mμ and one at 500 mμ, the former being the greater. By comparing the ratio height of peak at 425 mμ: height of peak at 500 mμ, at the different catechol concentrations, it was found that this ratio decreased as catechol concentration increased (Table 1). A possible interpretation of these results is that at least two products are being formed, either concurrently or consecutively, and that they are affected differently by the different catechol concentrations.

TABLE 1

RATIO OF PEAK HEIGHTS AT VARIOUS CONCENTRATIONS OF CATECHOL

Catechol Concentration (mM)	Ratio $\frac{\text{Peak height at 425 m}\mu}{\text{Peak height at 500 m}\mu}$
2	1·56 : 1
10	1·46 : 1
15	1·37 : 1
20	1·16 : 1

Using a 0·25M catechol solution, absorption curves were drawn at different time intervals during a reaction. Again, by determining the above ratio, it can be see that the products are not being formed at the same rates (Table 2). The rate of the reaction leading to the peak at 500 mμ does not decrease as fast as that leading to the peak at 425 mμ.

TABLE 2

RATIO OF PEAK HEIGHTS AT VARIOUS TIME INTERVALS

Time Intervals (min)	Ratio $\frac{\text{Peak Height at 425 m}\mu}{\text{Peak Height at 500 m}\mu}$
0–1	1·74 : 1
1–2	1·69 : 1
2–3·5	1·66 : 1
3·5–5	1·61 : 1

Since the absorption peaks of the products formed in this polyphenoloxidase reaction were found to be at 425 mμ and 500 mμ, a reaction mixture was followed at these two wavelengths. Graphs of extinction (E) against time (t) at these wavelengths are shown in Figure 3. When these results were plotted in the form 1/E against 1/t (Figure 4), a straight line was obtained at 425 mμ, but at 500 mμ a curve resulted. In the case of readings at 425 mμ, since a straight line resulted, it is assumed that the absorbance at 425 mμ is directly related to the o-benzoquinone used in the reaction leading to the peak at this wavelength. This o-benzoquinone may or may not be the total amount of quinone produced by the polyphenoloxidase reaction. Since a curve was obtained at 500 mμ, it is assumed that at this wavelength the benzoquinone formed by the enzyme is not being measured. It is considered that the optical density changes at this wavelength are related to the product of either a reaction between catechol and an impurity in the enzyme solution, or a further reaction of the substance having the peak at 425 mμ and being formed from benzoquinone.

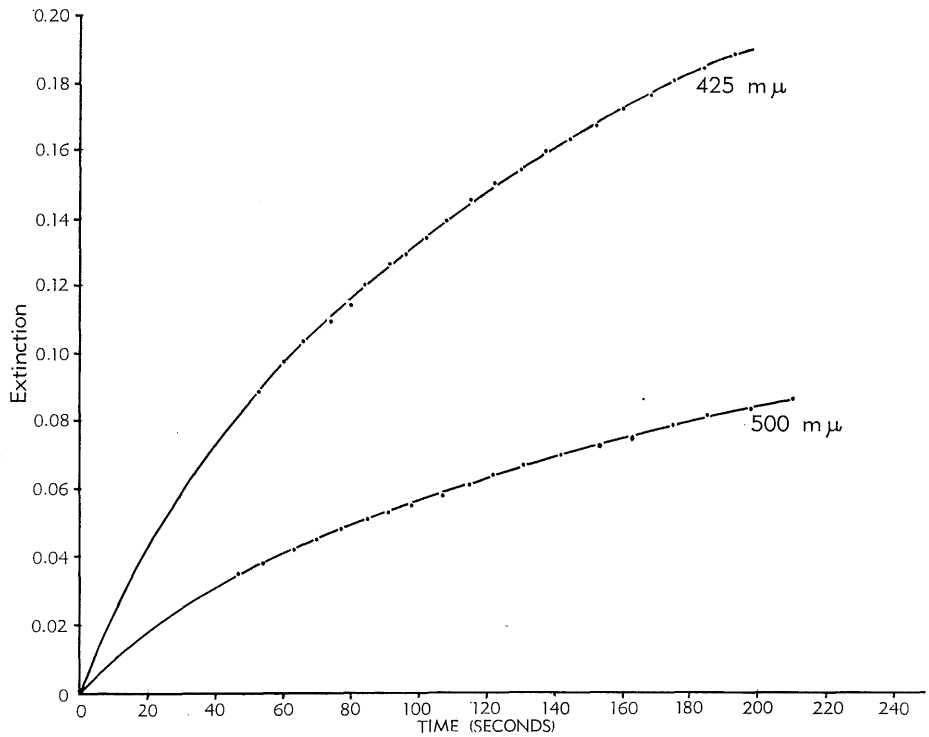

Fig. 3.—Increase of extinction, measured at two wavelengths, with time, in a catechol-polyphenoloxidase system. Reaction mixture: enzyme 1·0 ml; catechol (0·25M) 1·0 ml; citrate-phosphate buffer 1·2 ml.

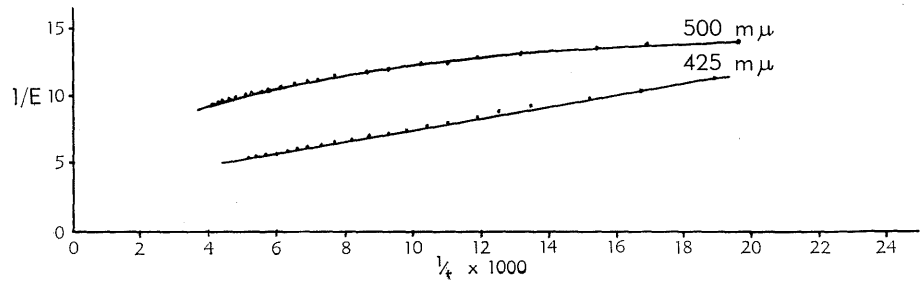

Fig. 4.—Results of Fig. 3 shown as a reciprocal plot.

Measuring the reaction at 425 mμ gives an absolute measure of the amount of polyphenoloxidase present only if all the benzoquinone being formed is used in the reaction leading to the peak at this wavelength. However, measuring polyphenoloxidase by the rate of formation of colour at 425 mμ probably can be used for such work as inhibitor studies on the enzyme, provided the inhibitor does not interfere with this colour reaction. This method could be used for comparing amounts of enzyme present in samples of the same material. It could not be used for samples from different types of materials, since reactions leading to peaks at wavelengths other than 425 mμ and 500 mμ may occur, or the amount of benzoquinone used in the 425 mμ reaction may not be a constant percentage of that formed by polyphenoloxidase.

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