AN ABSTRACT OF THE THESIS OF

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Title: Polyphenol Oxidase, Dopamine Content, and Discoloration in Ripening Bananas

Abstract approved: Helen G. Charley

This study attempted to relate the activity of polyphenol oxidase and concentration of the substrate, 3,4-dihydroxyphenylethylamine (dopamine), in bananas as they ripened to the susceptibility to discoloration of cut slices of the fruit. Bananas from three lots were analyzed as purchased and after two and five days of ripening. Ripeness was assessed by measuring the rate of respiration.

Individual fruits were respiring at different rates initially, and the respiratory activity differed as they ripened. The entire fruit lost weight as it ripened, but the edible pulp gained weight. The pH of the pulp decreased from an average of 5.6 to near 5.0 at the end of five days of ripening. Dopamine content of the bananas varied from 12.5 μg/g to 77.5 μg/g fresh weight and was lowest in those bananas in Lot I. While the dopamine content in fruit in Lot I was higher after two days of ripening than it was either initially or after five days of

ripening, the dopamine content decreased in fruits in Lots II and III as they ripened. In some instances, however, differences in dopamine content of individual bananas from the same hand ripened for the same period of time exceeded the difference in average values due to ripening. In all three lots of bananas, the activity of the enzyme decreased as the fruit ripened, the first two days resulting in a greater decrease than did the last three. Although the activity of polyphenol oxidase was highest in the least ripe bananas, fruit at this stage was not most susceptible to browning. In fact, bananas in Lot III were most susceptible to discoloration after five days of ripening when both the average dopamine content and the activity of the enzyme was lowest.

POLYPHENOL OXIDASE, DOPAMINE CONTENT, AND DISCOLORATION IN RIPENING BANANAS

by

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INTRODUCTION

Fruits undergo rapid, undesirable color changes following mechanical or physiological injury during harvesting, transportation, storage, and preparation for serving. Phenolic compounds of fruits and vegetables are oxidized to quinones which undergo polymerization and further oxidation, imparting characteristic brown or black coloration (Aylward and Haisman, 1969). According to these authors, phenols may be oxidized catalytically by phenol oxidases, by peroxidases or even nonenzymatically, resulting in darkening in color, deterioration in flavor and loss of ascorbic acid.

Bananas, like many other fruits, are susceptible to enzymic browning. Bananas were chosen for this study because world-wide they are a major fruit crop, with more than 11.5 billion pounds entering international trade in 1968 (U.S.D.A., 1970). They are readily available the year around and widely used not only in this country but also in India. Griffiths (1959) reported that browning of bananas results from the enzymic oxidation of 3,4-dihydroxyphenylethylamine (dopamine) by polyphenol oxidase. Since his work was reported, Palmer (1963) extracted and identified polyphenol oxidase from banana and reported that dopamine was the major substrate.

While several workers have attempted to relate enzymic browning with enzyme activity and substrate concentration in a number of fruits and vegetables (Weurman and Swain, 1955; Harel, Mayer and Shain, 1966; Walker, 1962), no such work has been done with bananas. This was attempted in the present study. Bananas were brought to different stages of ripeness, assessed by respiratory activity. Fruits at each stage of ripeness were analyzed for 3,4-dihydroxyphenylethylamine (dopamine) and for activity of polyphenol oxidase enzyme towards this substrate. As a measure of the rate and extent of browning, diffuse reflectance from the cut surfaces of the banana slices was determined.

REVIEW OF LITERATURE

BANANAS

A banana (Musa sapientum, L.) is a seedless tropical fruit which belongs to the family Musaceae (Von Loesecke, 1950; Czyhrincin, 1969). Bananas grow in regions where the temperature does not fall below 10°C and seldom goes higher than 40°C and where rainfall is frequent and scattered throughout the year. The plant is propagated vegetatively by division of the main stem (rhizome) or by planting suckers which arise by the outgrowth of the buds on the rhizome (Simmonds, 1966). In India, the banana is one of the most popular fruits, with hundreds of varieties grown on a large scale in most parts of the country. K. S. Venkataramani (cited in Von Loesecke, 1950) reported about 400 varieties in Madras province alone. Famous varieties include Cresole, Saint Pierre, Black, Horn and Eyeless.

Structurally, banana fruit consists of inedible peel and edible pulp. The outer part of the peel is made up of hexagonal epidermal cells, interrupted by stomata and coated with a thin film of protective cutin. Underlying the epidermis is the parenchyma tissue whose rectangular shaped cells make up the bulk of the peel. Fibrovascular bundles are scattered throughout the parenchyma tissue (Von Loesecke, 1950).

The edible pulp consists of large cells with thin

walls. These cells are closely packed, with little intercellular space. Latex vessels run longitudinally through the center of the pulp with branches running horizontally (Barnell and Barnell, 1945).

CHANGES IN BANANA DURING RIPENING

Bananas are harvested while they are green. In tropical countries the entire bunch is hung in a warm, shady place to ripen. In the United States and other cold countries the fruit is ripened commercially under controlled conditions of temperature, humidity, and ventilation. Volatile accelerators of respiration such as ethylene are used to hasten ripening and to get uniform ripeness (Von Loesecke, 1950; Biale and Young, 1959; Spencer, 1965). Hultin and Proctor (1961) reported that the actual mechanism by which ethylene hastens the ripening of the banana is unknown.

Changes in Color. The unripe banana is dark green in color. Visible signs of ripening involve changes in the color of the skin from dark green to pale greenish yellow and then to bright yellow, at which point all trace of green disappears except at the tip and in the stalk. The change in color is due to a decrease in the chlorophyll content of the fruit. The yellow pigments, xanthophylls and carotenes, remain constant. The green tip disappears slowly and the yellow color deepens. Finally, the skin

begins to get brown flecks which soon spread over the entire surface when the fruit reaches the overripe stage. Respiration rate is considered a more accurate criterion of ripeness in banana (DeSwardt and co-workers, 1967) than is skin color because ripening starts from the center of the fruit (Simmonds, 1966) and the skin may remain green when the fruit is well advanced in the ripening process (Maxie and Sommer, 1968).

Respiration. During ripening of the harvested fruit under conditions of controlled temperature and humidity, chemical changes take place. Starch, embedded in the cytoplasm of the parenchyma cells of the green banana, undergoes hydrolysis during ripening. Reducing sugars are formed in much greater quantities than sucrose. In the overripe banana sugars are probably used as respiratory substrates (Biale, 1960). Even when the fruit is detached from the plant, the tissue is alive and respiring, utilizing oxygen and evolving carbon dioxide along with ethylene and probably small amounts of volatile esters.

Bananas, like many other fruits, have a characteristic respiration curve (carbon dioxide evolution and oxygen consumption with time) as they ripen. At the green stage, carbon dioxide production is low and steady, but it rises sharply to a peak at or just prior to the first visible signs of ripening. At the peak, the respiration rate is about two to five times as great as the initial steady

level. The actual magnitude of the rate depends upon temperature and many other factors of the environment, including the composition of the air in which the fruit is kept. The peak is referred to as "climacteric," the green fruit as "preclimacteric" and ripe fruit as "postclimacteric" (Simmonds, 1966). The climacteric stage is a transition phase when fruit tissues are passing from a low metabolic activity to a higher one (Von Loesecke, 1950).

A number of factors can alter the normal respiratory activity of the fruit. Mapson and Robinson (1966) reported that the ability of unripe bananas to synthesize ethylene is dependent on the oxygen tension of the atmosphere surrounding the fruit. According to Quazi and Freebairn (1970) low oxygen concentration and high carbon dioxide concentration inhibit ethylene synthesis and ripening in uninitiated fruit (unexposed to ethylene) and low oxygen concentration greatly reduces the total respiration necessary for a given degree of ripening. However, the initiated fruit under similar conditions shows more or less normal respiration pattern. Young, Romani and Biale (1962) reported that treatment with carbon dioxide delayed the beginning of ripening of both avocado and bananas, although the rate of respiration at the climacteric peak for the latter was unaffected. Ethylene accelerates the onset of the climacteric if applied before the rise (Biale and co-workers, 1954). Burg and associates (1965)

reported that the ethylene content of bananas (Musa sapientum L., var. Silk fig) remains constant throughout their growth and development. But when ripening commences, an abrupt increase in ethylene synthesis precedes the onset of the climacteric rise in respiration.

Intensity of respiration in fruits responds within limits to temperature in a manner characteristic of chemical reactions. At low temperature the rise in respiration is approximately exponential, whereas at high temperature the rate declines with time (Biale, 1960). "The climacteric rise in respiration is earlier in incidence, shorter in duration and correspondingly more sharply defined, the higher the temperature" (Simmonds, 1966).

Maintenance of proper relative humidity in the storage atmosphere is an important factor for ripening of banana fruit. Relative humidity is generally maintained at 90-95% for green fruit and 75-85% for the ripened fruit. Bananas stored at below 80% relative humidity did not undergo respiratory climacteric and did not show visual characteristics of normal ripening. Also, they did not develop characteristic aroma of ripened banana fruit and were astringent to taste. Abnormalities were found in many respects similar to those observed for chilled fruit (Haard and Hultin, 1969).

Other Chemical Changes. Acidity in the banana pulp increases with ripening, the pH varying from 5.02 to 5.6

in the green banana to 4.2 to 4.75 in the ripe fruit (Gane, 1936; Von Loesecke, 1950). The insoluble pectins (protopectins) disappear gradually during ripening, while the soluble pectin content increases (Biale, 1960). The concentration of tannins falls during ripening, resulting in disappearance of astringency of the fruit (Barnell and Barnell, 1945; Spencer, 1965). Amino acid and protein contents of bananas remain more or less constant during ripening (Simmonds, 1966). Free space increases in banana tissue during ripening, and the proportion of cells which are permeable to solutes increases (Sacher, 1966). Rate of ion leakage increases as the fruit ripens (Baur and Workman, 1964). Ripe bananas have a characteristic aroma which is due to increase in volatile constituents as ripening progresses (Wick and associates, 1966). McCarthy et al. (1963) reported that "banana like" aroma is due to amyl esters of acetic, propionic, and butyric acids, while the characteristic "fruity" and estery tones are due to butyl acetate, butyl butyrate, hexylacetate and amyl butyrate.

During ripening of the fruit, the pulp increases in weight due to an increase in water content, some of which is drawn from the peel and stalk by osmotic withdrawal and the remainder produced by respiration. Consequently, the peel loses weight, causing a change in the pulp to peel ratio, as the fruit ripens. This ratio is called the coefficient of ripeness and is sometimes used as index of

maturity (Von Loesecke, 1950).

POLYPHENOL OXIDASE

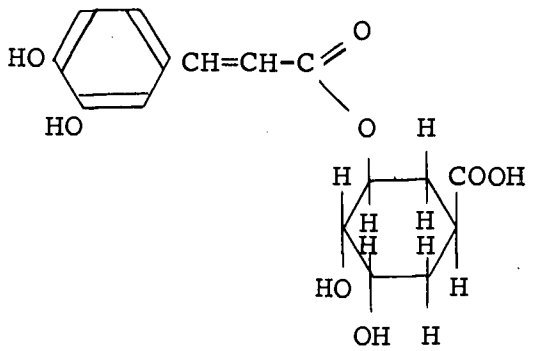
For rapid browning of fruit to occur, a substrate, oxygen and an oxidative enzyme must be present. Oxidative enzymes known as oxidases include polyphenol oxidases and peroxidases. The latter oxidizes substrates in the presence of hydrogen peroxide as an oxidizing agent (Karlson, 1968). Polyphenol oxidases, on the other hand, require oxygen as an oxidizing agent. These enzymes are copper-proteins, copper being in the prosthetic group. Polyphenol oxidases have been found in potatoes, mushrooms, fungi and many other plants, and they display activity toward a great range of substrates. Thus they are known by various trivial names such as catecholase, tyrosinase, cresolase, polyphenol oxidase, and phenolase (Aylward and Haisman, 1969). The name recommended for such enzymes by the commission on enzymes of the International Union of Biochemists (1961) is (1.10.3.1) o-diphenol : oxygen oxidoreductase. Constantinides and Bedford (1967) reported that the phenol oxidase system in the tissue of mushrooms, potatoes and apples has multiple forms, each of which has substrate specificity. Polyphenol oxidases from Solanum tuberosum L-1 were separated into seven bands by polyacrylamide gel electrophoresis, one band of which was capable of oxidizing L-dihydroxyphenylalanine (Dopa), catechol, chlorogenic acid,

and pyrogallol.

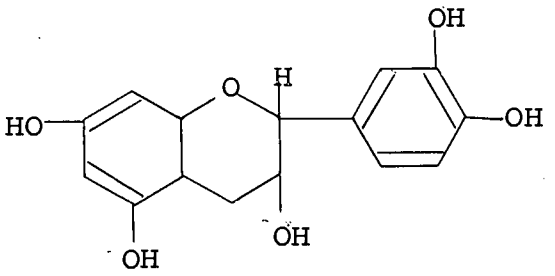
SUBSTRATES FOR POLYPHENOL OXIDASE

Several compounds can be oxidized by polyphenol oxidase, only a few of which are important in the browning of fruit tissue. Phenol oxidase from Bartlett pears was found active only toward o-dihydric phenols (Tate et al., 1964). Using catechol as substrate, Cushing (1948) found that the introduction of ortho-directing groups ($-CH_3$ and $-Cl$) into the fourth position of the ring produced a substrate for tyrosinase whose oxygen consumption was two atoms per mole, while meta-directing groups such as $-CHO$, $-COOH$, $-COCH_3$, $-COCH_2Cl$ and $-SO_3Na$ introduced at the fourth position of the catechol ring produced substrates with oxygen consumption of one atom per mole.

Chlorogenic acid is a substrate for polyphenol oxidase which is found in many fruits, including apples (Hulme, 1958; Siegelman, 1955), apricots (Sayed and Luh, 1965), egg plant (Knapp, 1965), peaches (Craft, 1961), and pears (Hulme, 1958). The principal browning substrate in the extracts of skin from Grimes Golden and Golden Delicious apples and Bartlett pears was l-epicatechin. Another browning substrate reported in Bartlett pears was d-catechin (Siegelman, 1955).

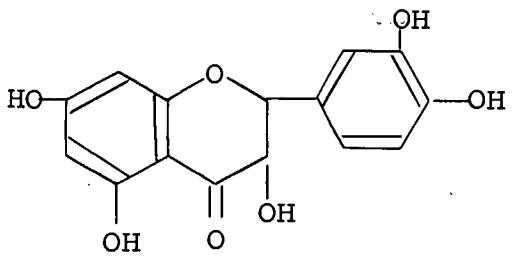


Chlorogenic acid

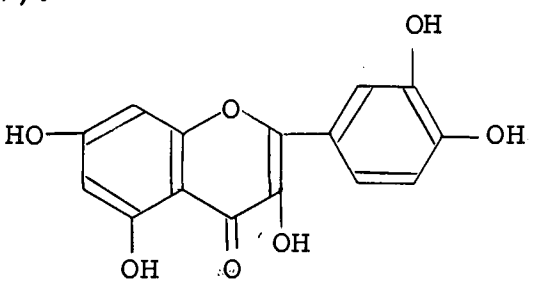


Catechin (and epicatechin)

Apple polyphenol oxidase showed substrate specificity toward dihydroquercetin but not to quercetin which is structurally quite similar except for a double bond in the latter (Shannon and Pratt, 1967).

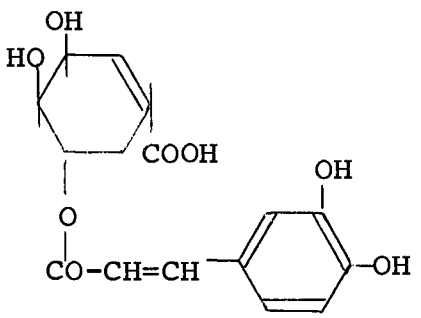


Dihydroquercetin



Quercetin

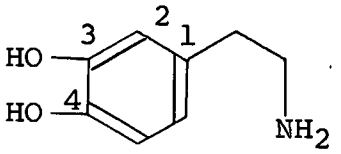
Caffeoylshikimic acid, a cinnamic acid derivative also known as dactylifric acid is the main substrate for polyphenol oxidase in dates (Mayer and Metzler, 1965).



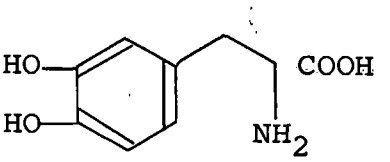
Caffeoylshikimic acid (dactylifric acid)

Polyphenol oxidase enzyme from avocado showed more affinity for nordihydroguaiaretic acid than for catechol or catechin (Knapp, 1965), while another polyphenol oxidase from egg plant was found active toward anthocyanin (Sakamura et al., 1966). Leucocyanidins in bananas do not contribute to enzymic browning (Griffiths, 1959).

According to Barnell and Barnell (1945) the browning substrate in bananas, which they referred to as "tannins," is present in the latex vessels of the fruit. Griffith (1959) identified the major browning substrate of the banana as 3,4-dihydroxyphenylethylamine (dopamine). Dopamine is abundant in the skin of the fruit but relatively sparse in the pulp. Dopamine differs chemically from L-Dopa by the presence of a carboxyl group on the side chain of the latter. While the two compounds are quite similar structurally, the K_m values for the two differ by a factor of 100 (K_m 6.3 x 10^{-4} M for dopamine; K_m 4.4 x 10^{-2} M for dopa) (Palmer, 1963).



3,4-dihydroxyphehylethylamine
(Dopamine)

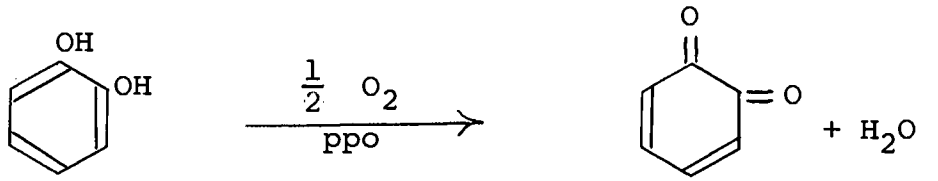


3,4-dihydroxyphenylalanine
(Dopa)

OXIDATION OF PHENOLIC COMPOUNDS

Discoloration in fruit occurs when the cells are altered mechanically by cutting, bruising, or freezing. Probable reason for this is that enzyme and substrate are separated from each other in the intact fruit and come in contact only when the cells are ruptured. In apple, polyphenol oxidase is localized in both the mitochondria and the chloroplasts (Harel et al., 1965).

Polyphenol oxidase is highly specific for polyphenols such as catechol and pyrogallol. The first step in the action of polyphenol oxidase is to convert ortho- and para-polyhydroxy phenols to the corresponding quinones as follows (Mason, 1948). Ortho-quinones further undergo autooxidation.



Catechol

o-benzo quinone

The o-diphenolic activity of PPO has been referred to as 'catecholase', since catechol is frequently used as the substrate, while the monophenolic activity has been termed 'cresolase' activity since cresol, a monophenol, is used as the substrate in this case. Cresolase activity is easily lost during purification of phenol oxidase, as its center is more readily inactivated than is that of catecholase. The first step in the oxidation of a monophenol is its conversion to the corresponding o-diphenol (Mason, 1959).

$$\text{Monophenol} + 2\bar{e} + O_2 \xrightarrow{\text{Enzyme}} \text{o-diphenol} + O^{=}$$

Dawson and Tarpley (1951) observed an initial induction period for oxidation of monophenols by oxidase (cresolase activity). The length of this period was influenced by the source and purity of the enzyme. Once the o-diphenol is formed, it is oxidized to quinones as shown for catechol (Mason, 1959). When oxidation of catechol is proceeding simultaneously with oxidation of cresol, the former facilitates the latter reaction, probably due to the fact that the cresolase activity requires electrons and the oxidation of catechol provides for this (Mason, 1959).

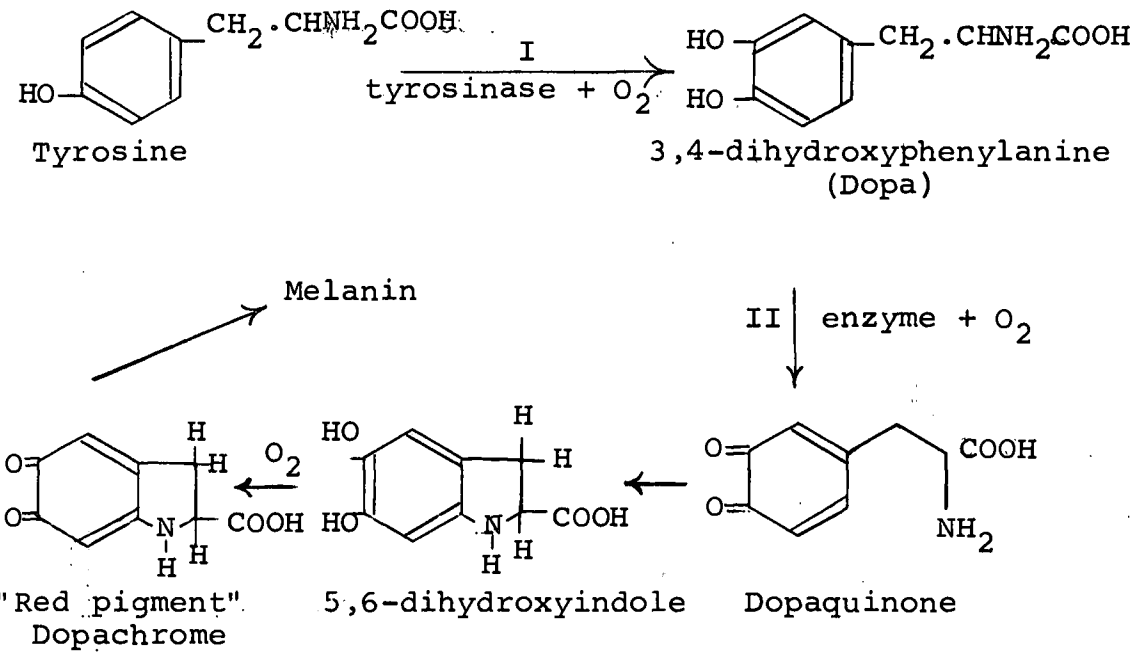
Walker (1964) reported that not only is the o-dihydroxy phenol structure essential for a compound to be readily oxidized by polyphenol oxidase but other structural features also play an important role. He found that the

rate of oxidation by apple polyphenol oxidase of the following phenolic acids decreased in this order: hydrocaffeic acid, chlorogenic acid, isochlorogenic acid, caffeic acid, 3,4 dihydroxyphenylacetic acid, and 3,4 dihydroxybenzoic acid.

The polyphenol oxidases in mitochondria and in chloroplasts of apple differ in their activities. That in chloroplasts has a slightly greater catecholase than cresolase activity, while polyphenol oxidase in mitochondria has greater cresolase than catecholase activity (Harel et al., 1964). The polyphenol oxidase in chloroplasts is in tightly bound form and is probably associated with the lipid material in the plant cell (Walker and Hulme, 1966). In studies with apricots (Samisch and Cruess, 1934) and with pears (Scott et al., 1960), evenly intense browning was found in all parts of the fruit, suggesting that, although the enzyme is segregated in specific structures, these are more or less evenly distributed in all parts of the fruit.

When a monohydroxy phenol, like tyrosine, is acted upon by tyrosinase, the first step consists in the addition of a hydroxyl group ortho to the one already present, yielding 3,4-dihydroxyphenylalanine (Dopa). This first step undergoes quinone formation and then rearrangement to 5,6-dihydroxyindole. This indole is then oxidized to a 5,6-quinone which is a red pigment called dopachrome.

This red pigment is further oxidized and polymerized to form the high molecular weight black pigment, melanin (Mason, 1948).



Only steps I and II are mediated by the polyphenol oxidase enzymes (Makower and Schwimmer, 1957). Palmer (1964) suggested that Dopamine is oxidized by a mechanism similar to that proposed by Mason (1948) for Dopa.

Both monophenolic and o-diphenolic oxidation were inhibited by the same copper complexing agents, suggesting that both functions of the enzyme complex probably had the same copper atoms (Dawson and Tarpley, 1951). In regard to the role of copper in polyphenol oxidase, Kertesz (1957) proposed that copper is in the cuprous state in the enzyme and remains so throughout the reaction. Bendall and Gregory (1963) presented evidence that copper is

normally in the cupric state (confirmed by electron spin study by Malmstrome et al., 1959), but that it changes to cuprous during oxidation of the substrates. They propose that copper acts as a built-in electron carrier which can undergo a reversible oxidation-reduction reaction.

ENZYMIC BROWNING

Effect of Enzyme and Substrate Concentration. When the concentration of the enzyme increases, the reaction rate increases as in any other enzymic reaction, but only to the point where concentration of the substrate becomes limiting. When concentration of the substrate increases, reaction rate increases to the point where enzyme concentration becomes limiting. Mapson and co-workers (1963) reported that the concentration of tyrosine was the major factor in enzymic browning in potatoes. Harel, Mayer and Shain (1966) reported similar results with apples, in which they found a good correlation between browning and o-diphenol content.

Effect of pH. Polyphenol oxidase, like any enzyme, has a pH optimum at which its activity is greatest. Above and below this optimum pH, the activity is decreased until the enzyme is finally inactivated. At pH 4.1, browning was only about 30% of that at pH 6.2 for pear polyphenol oxidase. At a pH of 2.5-2.7, the enzymic activity was completely lost and even when the pH was returned to the

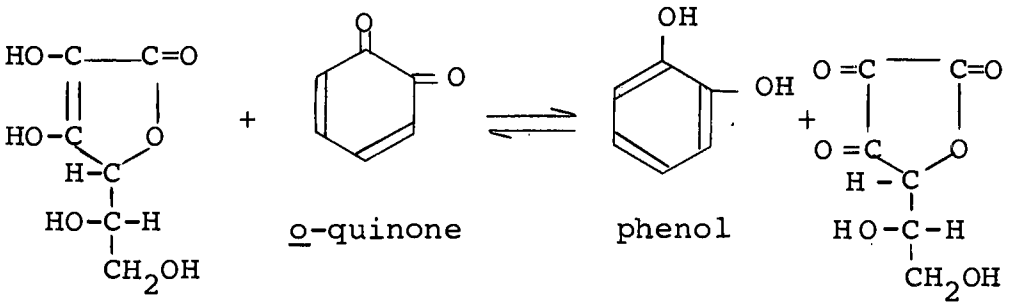
original value, the enzyme activity could not be returned (Ponting, 1951). When the pH is lowered by adding acid to the system, enzymic browning is controlled, as acid reduces the activity of the enzyme. Citric acid is often used for this purpose. The optimum pH for polyphenol oxidase for the substrate dopamine (in banana) is 7 (Palmer, 1963), for catechol (in pears) is 5.8 to 6.4 (Tate et al., 1964), and for chlorogenic acid (in apples) it ranges from 4 to 8 (Sondheimer, 1962). Joslyn and Ponting (1951) reported an optimum pH of 7 for polyphenol oxidase from apricots and prunes with catechol as substrate.

The polyphenol oxidase activity is dependent on the available oxygen, an increase in oxygen increasing the rate of reaction when pH and other conditions are not unfavorable. The activity of polyphenol oxidase from Bartlett pears was greatly decreased when the oxygen concentration in the reaction mixture was lowered (Tate et al., 1964).

Effect of Temperature. As with any other enzyme, polyphenol oxidase activity increases with temperature, until that temperature is reached which inactivates the enzyme. "The point of balance between the accelerating effect of temperature upon the enzyme-catalyzed reaction and the effect of destruction of the enzyme by heat is known as the temperature optimum" (Joslyn and Ponting, 1951). Reyes and Luh (1960) reported that freezing does

not inactivate the oxidative enzymes from Elberta Freestone peaches.

Effect of Inhibitors. Many compounds containing sulfur have been widely used to prevent discoloration in fruits. These include sulfurous acid and its salts (bisulfites, sulfites and meta bisulfites), cysteine and glutathione. According to Embs and Markakis (1965), sulfite prevents browning by combining with the enzymatically produced o-quinones and thus stopping their condensation to melanin. Muneta and Walradt (1968) reported that the initial site of cysteine inhibition of enzymic browning caused by oxidation of tyrosine occurs at the conversion of tyrosine to 3,4-dihydroxyphenylalanine (Dopa). Sulphur compounds present in plant tissue may inhibit enzymic discoloration. For example, pineapple juice because of its high content of sulfhydryl compounds prevents discoloration of fruit tissue. Ascorbic acid is another widely used inhibitor of enzymic browning which is also present in many plants. Ascorbic acid reduces quinones to the original phenolic compounds by donating two hydrogens and is itself thus oxidized into dehydroascorbic acid (Ponting and Joslyn, 1949).



L-Ascorbic acid

L-Dehydroascorbic acid

Biale and Young (1963) reported that ascorbic acid content in bananas at picking stage was 5.3 mg / 100 g fresh weight and increased to 11.1 mg / 100 g after storage.

METHOD

PREPARATION OF BANANAS FOR ANALYSIS

Three lots of bananas were obtained from a local wholesaler at one week intervals. Each lot consisted of six bananas (fingers) from a single hand. The bananas were uninitiated, i.e., not treated with ethylene gas. The six bananas from each hand were divided into three groups of two bananas each for analysis at different stages of ripeness. Two of the fingers from each hand were analyzed at the stage of ripeness as purchased (Stage I). Each of the remaining four bananas was put into separate respiration jars to ripen. The bananas were held at 70°F, and air at a constant flow rate of 12,000 ml/hour was maintained in the respiration chambers. The air that entered the chamber was made carbon dioxide free by passing through a sodium hydroxide solution. Two of the bananas from each hand were analyzed at the end of the second day of ripening (Stage II) and the remaining two were analyzed at the end of the fifth day (Stage III). Bananas were analyzed individually. The weight of each banana as purchased and upon removal from the respiration chamber was recorded.

ASSESSING RIPENESS OF THE BANANAS

Two methods were used to assess the ripeness of each banana at the time of analysis. The carbon dioxide output,

a measure of the respiration rate, was determined with an infrared analyzer.[1] Readings were taken twice daily and carbon dioxide output was read from a calibration curve[2] which indicated the concentration of carbon dioxide in ppm in the air around the banana and from which ml CO_2/100 g banana were calculated. The values for the bananas analyzed as purchased were based on the CO_2 output on that day, of the four bananas that were placed in the respiration chambers. The respiratory rates for bananas analyzed after two days of ripening were based on CO_2 output of the same bananas at the end of two days. Values for bananas at the end of the fifth day of ripening were based on CO_2 output on the fifth day of the remaining two bananas.

A second method of assessing the ripeness of each banana was to match the color of the skin with a commercial color chart devised to show six stages of ripeness (Von Loesecke, 1950). The stages are numbered from 2 to 7. At stage 2 the skin of the banana is green with faint yellow undertones, at stage 3 the yellow is more pronounced, and at stage 4 most of the green has disappeared. Skin of bananas at stages 5 and 6 is bright yellow, with fruit at stage 5 retaining green tips. At stage 7 brown flecks have begun to appear on the deep yellow skin.

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[1] Beckman Instruments, Model 215A.

[2] Calibration curve provided by Prof. Elmer Hansen, Dept. of Horticulture.

DETERMINATION OF 3,4-DIHYDROXYPHENYLETHYLAMINE IN BANANAS

Extraction. The 3,4-dihydroxyphenylethylamine was ex-extracted from the bananas by the method of Griffiths (1959). Banana pulp (20 grams) was cut into small pieces with a stainless steel knife and transferred to a microblender containing 20 milliliters of ice-cold 1% methanolic hydrochloric acid. The tissue was homogenized for ten minutes at medium speed. An additional 20 milliliters of the extractant was added and homogenizing continued for ten minutes. The homogenate was transferred to centrifuge tubes and centrifuged at 10,000 rpm for 20 minutes. The supernatant was transferred to a volumetric flask and was made to 100 milliliters with 0.04 M phosphate buffer (pH 5.6). The white cell debris left in the centrifuge tube, when dispersed in 0.1 M phosphate buffer (pH 6.8) and treated with polyphenol oxidase, remained colorless, thus indicating that no substrate was left in the marc.

Measurement. The initial oxidative step in the conversion of dopamine to colored compounds can be catalyzed either by Ag_2O or by polyphenol oxidase (Mason, 1948; Palmer, 1963). In this work, Ag_2O was used as the catalyst because of its stability. For the measurement (conversion), a ten milliliter aliquot of each extract was transferred to a test tube and to this 50 milligrams of Ag_2O was added. The contents of the tube were agitated by means of a mixer

for one and one-half minutes, after which they were then filtered through Whatman No. 42 filter paper, using a slight suction. The absorbance of the filtrate was read at the end of three minutes with a spectrophotometer[1] set at a wavelength of 475 mμ. The concentration of 3,4-dihydroxyphenylethylamine was read from a standard curve, data for which were obtained in the following way.

For the standard curve a stock solution was prepared by dissolving 100 milligrams of 3,4-dihydroxyphenylethylamine in 0.04 M phosphate buffer (pH 5.6) and diluting it to 100 milliliters. From this stock solution, five working standards at concentrations of 5, 10, 20, 50 and 80 micrograms per milliliter were prepared, using the same phosphate buffer. A ten milliliter aliquot of each working standard was then oxidized with Ag_2O in the manner outlined above and the optical density of the filtrate was read at 475 mμ. The standard curve (Figure 1) was made by plotting optical density against the concentration of 3,4-dihydroxyphenylethylamine.

POLYPHENOL OXIDASE IN BANANAS

Extraction. To release the enzyme, banana pulp (two grams) was homogenized for 20 minutes in a microblender

[1] B L Spectronic-20 Spectrophotometer.

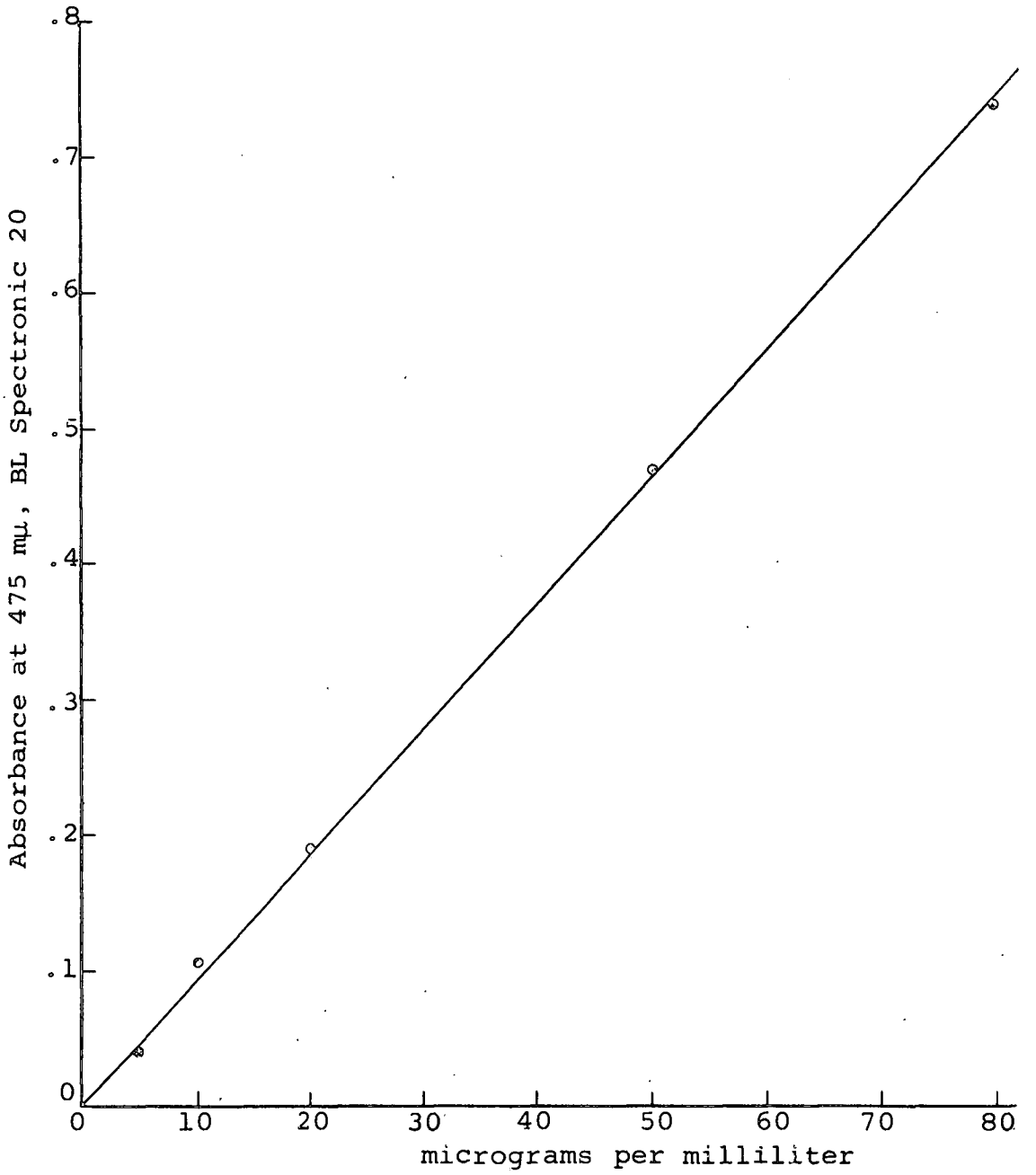

Figure 1. Standard curve for dopamine.
(in 0.04 M phosphate buffer, pH 5.6)

with 18 milliliters of 1% detergent (Igpal CO-630)[1] buffered at pH 6.8 with 0.1 M phosphate buffer (Palmer, 1963). The homogenate was centrifuged at 15,000 x g for 15 minutes and the activity of the enzyme in the supernatant was determined. All operations were carried out at 0°C.

Determination of Activity of the Enzyme. To measure the activity of the enzyme, one milliliter of a solution of 3,4-dihydroxyphenylethylamine-HCl (0.2 milligram per milliliter) and one milliliter of 0.1 M phosphate buffer (pH 6.8) were pipetted into the cuvette of a recording spectrophotometer.[2] After the cuvette was inserted into the instrument, one milliliter of the extract containing banana polyphenol oxidase was added, and at once the switch which activated the recording mechanism was turned on. The optical density versus time was recorded on the moving graph. On the curve which resulted, a tangent was drawn along the linear part near the origin, and from the slope, $\Delta OD/\Delta t$ was obtained, as shown in Figure 2.

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[1] Igpal CO-630, phenylphenoxypoly (ethyleneoxy) ethanol, GAF Corporation, 140 West 51 Street, New York, 10020.

[2] Cary Recording Spectrophotometer, model 11, series 311.

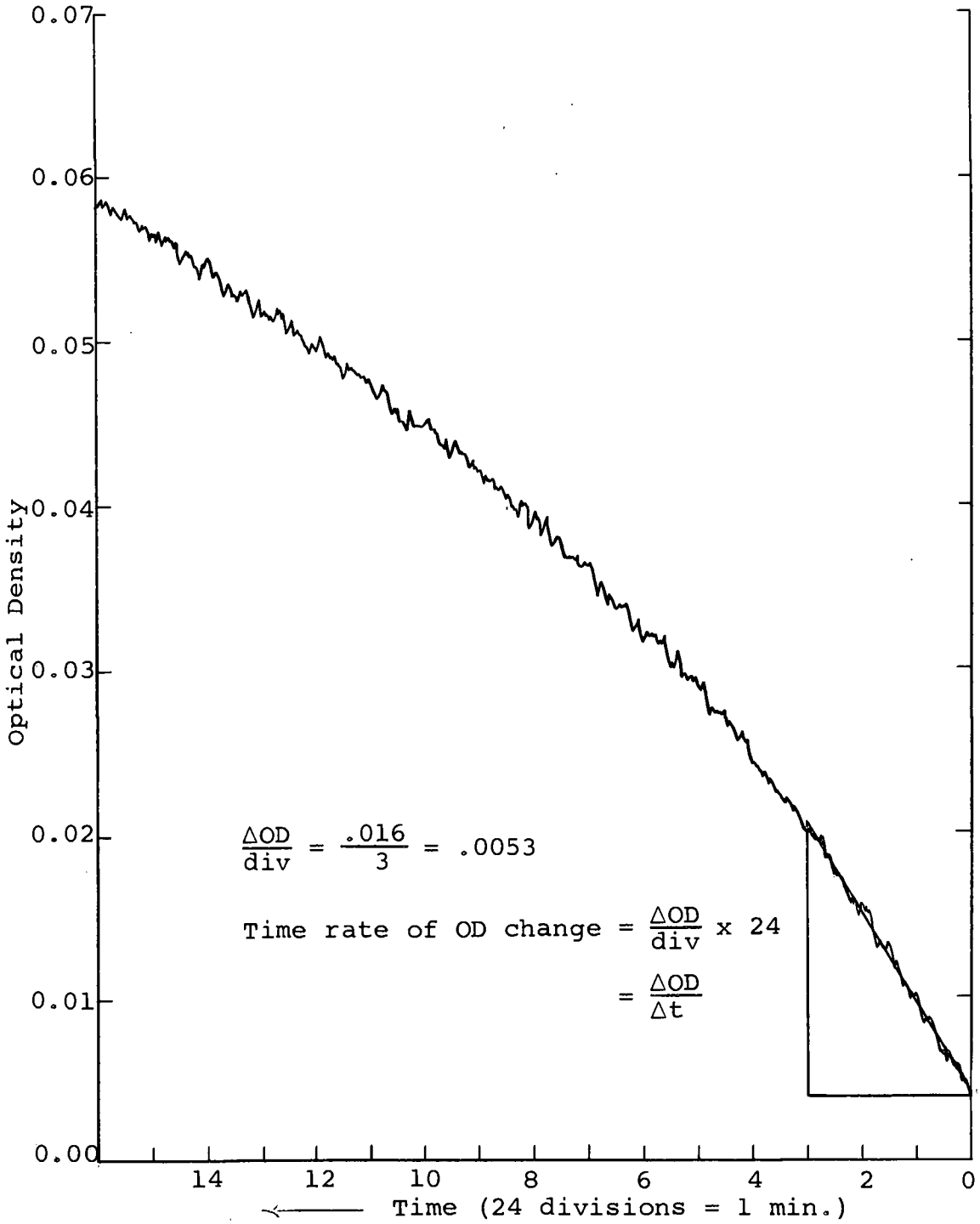

Figure 2. O.D. change due to dopaminechrome formation.

DISCOLORATION OF CUT SURFACE OF BANANA

From each banana, two half-inch thick slices were cut perpendicular to the long axis. Surface color was measured immediately after the slice was cut and at five minute intervals until 60 minutes had elapsed. For this purpose a reflection meter[1] with green tristimulus filter in the search unit was used. The instrument was calibrated against an enameled standard. To measure the discoloration of the banana, the cut surface was placed over the opening of the search unit and the diffuse reflectance read from the dial. Between readings the slices were covered with petri dishes to minimize evaporation. The extent of browning at each five minute interval was calculated by subtracting the diffuse reflectance at each interval from the diffuse reflectance of the sample when it was first cut.

pH DETERMINATION

Banana tissue was first ground, using a mortar and pestle, and then was homogenized in a microblender. The pH of this slurry was measured in duplicate, using a pH meter.[2]

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[1] Photovolt Reflection Meter, Model 670.

[2] Beckman Model G.

MOISTURE DETERMINATION

Aliquots of the homogenate prepared for pH determination were used to measure the moisture content of the bananas. The percentage of moisture in the bananas was determined in duplicates using an Ohaus Moisture Determination Balance.[1]

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[1]Model 6000, Ohaus Scale Corporation, N. J.

RESULTS AND DISCUSSION

RESPIRATION OF BANANAS

The outputs of carbon dioxide in ppm/100 g of banana at a constant rate of air flow (12,000 ml/hr) are shown in Table 1. The values are also given in ml of CO_2/100 g of banana. The latter values for each lot of bananas are plotted in Figure 3.

Rate of respiration of the banana at the beginning of the ripening period was highest in those in Lot I and lowest in those in Lot III. At the end of the first day of ripening, respiration rose sharply in bananas in Lot I, only slightly in those in Lot III and remained essentially the same in those in Lot II. At the end of the second day of ripening (Stage II), respiration was at the climacteric for bananas in Lot I and Lot II, but respiration in bananas from Lot III did not attain maximum rate until the end of the fourth day of ripening. Between the third and the fifth day of ripening, the rate of respiration in bananas from Lots I and II declined somewhat while that for bananas in Lot III remained essentially the same. The values ranged from 1.94 to 4.04 ml CO_2/hr/100 g banana for bananas in Lot I, from 1.56 to 4.38 ml CO_2/hr/100 g banana for bananas in Lot II and from 1.20 to 3.00 ml CO_2/hr/100 g banana for bananas in Lot III. These results are in line with those reported for bananas by Biale and Young (1962)

TABLE 1. RESPIRATION RATES[1] OF RIPENING BANANAS

Days of Ripening	Skin[2] Color	CO_2/100g ppm/hr	CO_2/100g ml/hr	Days of Ripening	Skin[2] Color	CO_2/100g ppm/hr	CO_2/100g ml/hr	Days of Ripening	Skin[2] Color	CO_2/100g ppm/hr	CO_2/100g ml/hr
		Lot I				Lot II				Lot III	
0	2	162	1.94	0	2	130	1.56	0	2	100	1.20
1	3	261	3.13	1	2	137	1.64	1	2	125	1.50
2	4	360	4.32	2	3+	394	4.68	2	2+	207	2.48
3	4+	358	4.23	3	4	395	4.73	3	3	250	3.00
4	5	355	4.26	4	5	381	4.57	4	5	250	3.00
5	5+	337	4.04	5	5	365	4.38	5	5	250	3.00

1. Respiration rates were determined at constant air flow rate (12,000 ml/hr) at constant temperature (70°F).

2. A commercial color chart showing six stages of ripeness (Loesecke, 1950).

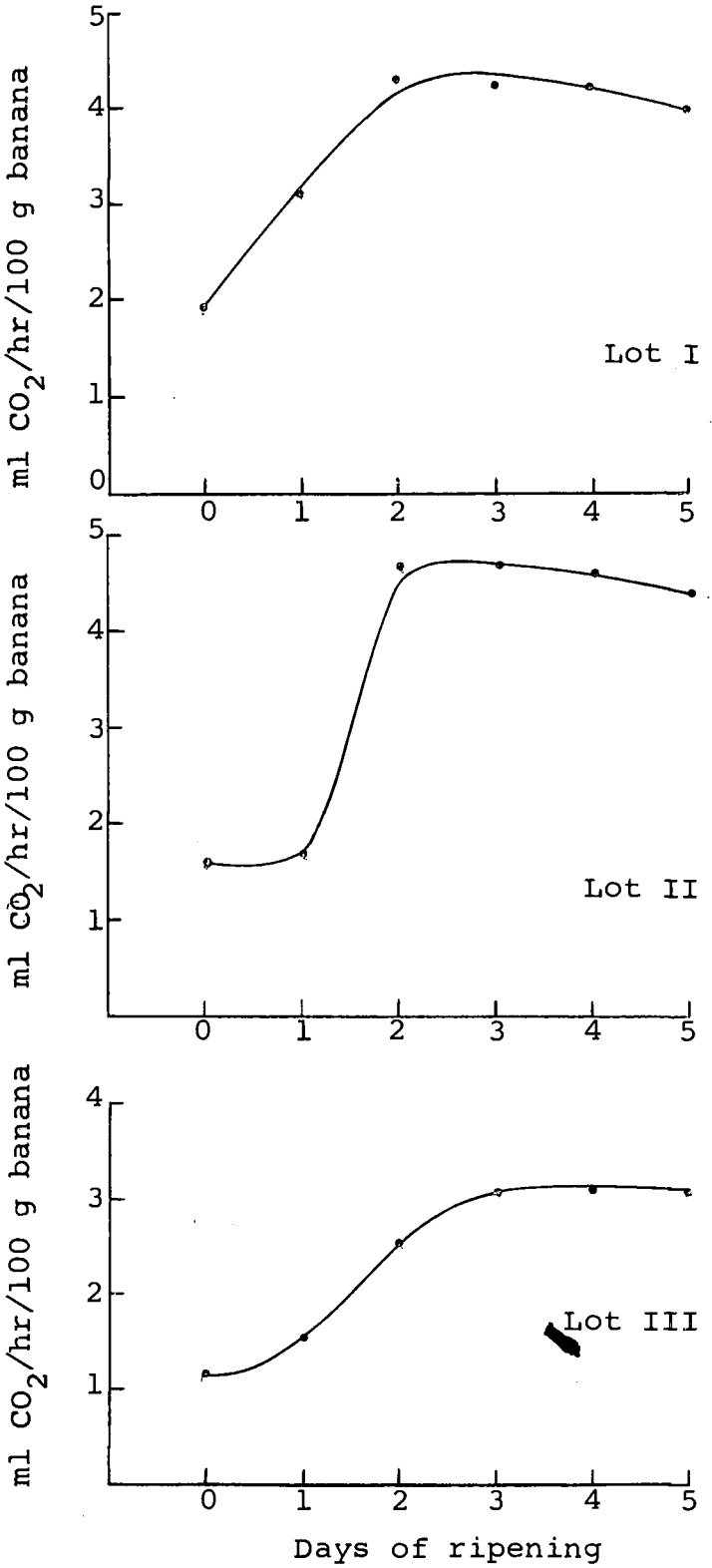

Figure 3. Respiration rates of ripening bananas.

who gave a range from 2.0 to 6.0 ml CO_2/hr/100 g. As shown in Table 1 and in Figure 3, the bananas as purchased were not only respiring at different rates but under the same ripening condition they did not show the same changes in respiration rate.

The color of the skin of the bananas when purchased was judged to match that of skin color 2 of the commercial color chart that shows six colors of ripeness. At the end of the second day of ripening, bananas from Lot I had changed to skin color 4 and those from Lot II to between 3 and 4, while those in Lot III had changed only slightly. At the end of the fifth day of ripening, bananas in Lots II and III were at skin color 5 while those in Lot I were between skin color 5 and 6.

pH OF BANANAS

The pH values of bananas at different stages of ripeness are given in Table 2. The pH of the bananas at the stage when they were first analyzed was 5.6 - 5.7. The pH decreased with ripening to near 5.0 at Stage III. Von Loesecke (1950) and Gane (1936) reported a similar decline in pH with ripening.

TABLE 2. pH OF RIPENING BANANAS (average of two sub-samples)

LOT	BANANA	Days of Ripening		
		NONE	TWO	FIVE
I	1	5.70	5.10	5.10
	2	5.60	5.30	5.10
	av.	5.65	5.20	5.10
II	1	5.70	5.00	4.95
	2	5.60	5.00	4.80
	av.	5.65	5.00	4.88
III	1	5.60	5.30	5.10
	2	5.60	5.40	5.10
	av.	5.60	5.35	5.10

MOISTURE CONTENT OF BANANAS

The moisture contents of bananas when analyzed are given in Table 3. As the figures show, the moisture in the

TABLE 3. PERCENTAGE OF MOISTURE IN RIPENING BANANAS (average of two sub-samples)

LOT	BANANA	Days of Ripening		
		NONE	TWO	FIVE
I	1	71.3	73.3	74.4
	2	70.1	74.5	74.8
	av.	70.7	73.9	74.6
II	1	69.1	71.2	72.3
	2	69.0	71.1	72.4
	av.	69.0	71.2	72.4
III	1	70.1	72.05	73.9
	2	71.8	71.65	71.5
	av.	71.0	71.8	72.7

pulp increased as the bananas ripened, the weight of bananas in Lot I increasing most. An increase in the weight of

the pulp occurred with ripening despite the fact that total weight decreased with ripening (Table 4). Total weight

TABLE 4. PERCENTAGE WEIGHT LOSS OF RIPENING BANANAS

LOT	BANANA	Days of Ripening TWO	FIVE
I	1	2.1	5.5
	2	2.5	5.0
	av.	2.3	5.2
II	1	2.1	4.8
	2	2.3	3.9
	av.	2.2	4.4
III	1	2.4	5.2
	2	2.3	5.6
	av.	2.4	5.4

losses of the bananas during the five days of ripening ranged from 3.9% to 5.6%. The loss is mainly due to evaporation. The increase in the moisture content of the pulp is due to withdrawal of water from the skin and stem (Von Loesecke, 1950; Simmonds, 1966).

DOPAMINE CONTENT OF BANANAS

Dopamine content of the bananas (calculated as 3,4-dihydroxyphenylethylamine-HCl) is given in Table 5. As the figures show, there was a great variation in dopamine content of bananas. Values ranged from 9 μg/g to 77.5 μg/g banana. Udenfriend, Lovenberg and Sjoerdsma (1959), reported an average value of 8 μg dopamine/g banana pulp. A recovery of a known amount of dopamine added to bananas

TABLE 5. DOPAMINE (3,4-dihydroxyphenylethylamine) CONTENT OF RIPENING BANANAS (μg/g fresh weight)*

LOT	BANANA	Days of Ripening		
		NONE	TWO	FIVE
I	1	12.5	20.0	9.0
	2	15.0	25.0	16.0
	av.	13.8	22.5	12.5
II	1	62.5	40.0	32.5
	2	45.0	37.0	23.0
	av.	53.8	38.5	27.8
III	1	77.5	70.0	40.0
	2	60.0	60.0	55.0
	av.	68.8	65.0	47.5

*Individual readings are based on at least three determinations on each of two sub-samples.

prior to extraction averaged 73.4% (based on four determinations). The higher values in the present study were not due to leucoanthocyanin or serotonin (both compounds are present in bananas in large quantities), because neither compound gave any color when tested with silver oxide.

Dopamine content was highest in bananas of Lot III, ranging from 77.5 μg/g in the first stage to 40 μg/g in the third stage of ripeness. Lot II was second highest in dopamine content, ranging from 62.5 μg/g in the first stage to 23 μg/g in the third stage. Bananas in Lot I, however, showed highest dopamine content at the second stage of ripeness, first and third stages being more or less the same. The average dopamine content of bananas in Lots II and III decreased with ripening. However,

variations in the dopamine content of individual bananas at the same stage of ripeness (even those in the same lot and adjacent each other on the same hand) in some instances were greater than the difference between bananas at different stages of ripeness. This was particularly true of bananas which were analyzed without being held for further ripening. So inherent differences in the dopamine content are reflected in the values obtained for the bananas held for additional ripening. From the data above, it is impossible to say what effect, if any, ripening has on the dopamine content of bananas. Analyzing parts of the same banana at different stages of ripeness would have eliminated the effects of inherent differences among individual bananas. In this study it was impossible to do so because other tests were performed on the same banana.

ACTIVITY OF POLYPHENOL OXIDASE FROM BANANA

In Table 6, initial rates of OD change due to dopaminechrome (2,3-dihydroindole-5,6-quinone) formation as a result of banana polyphenol oxidase activity on pure dopamine are given. The activity of the enzyme, as measured by the rate of change in optical density, decreased as the fruit was held to ripen. The first two days of ripening caused a greater decrease in the activity of the enzyme than did the last three. The bananas in Lot III at Stage I of ripeness had the highest

TABLE 6. INITIAL RATE OF OD CHANGE DUE TO DOPAMINECHROME FORMATION BY POLYPHENOL OXIDASE EXTRACTED FROM RIPENING BANANAS* (ΔOD/div)**

LOT	BANANA	Days of Ripening		
		NONE	TWO	FIVE
I	1	.0070	.0059	.0046
	2	.0075	.0049	.0055
	av.	.0072	.0054	.0050
II	1	.0065	.0059	.0038
	2	.0085	.0050	.0046
	av.	.0075	.0054	.0042
III	1	.0160	.0050	.0044
	2	.0120	.0055	.0050
	av.	.0140	.0052	.0047

*Average of two determinations.

**Time rate of OD change=(ΔOD/div) x 24; 24 div = 1 min.

enzyme activity (essentially twice that of bananas in Lots I and II). However, after only two days of ripening, the activity of the enzyme from all three lots of bananas was essentially the same, as was the case at the end of five days of ripening.

Polymerization of phenolic compounds in bananas and other fruits occurs upon ripening (Barnell and Barnell, 1945; Goldstein and Swain, 1963), and should this complex involve polyphenolase this could account for the lower activity of polyphenolase from the well ripened fruit. However, in this study a non-ionic detergent was used in the extractant to free the enzyme (Palmer, 1963; Goldstein and Swain, 1965). Phosphate buffer (pH 6.8) which was used in this experiment to extract the enzyme should have

prevented inactivation of the enzyme by the more acidic cell sap of the riper bananas. The decrease in enzyme activity with ripening of the bananas could reflect an actual decrease in enzyme concentration or a change in the enzyme which affects its activity. For example, Pirie (1959) pointed out that the percentage of protein in leaf tissue decreases as the leaf matures due to a decrease in protein synthesis. Pierpont (1959) reported greater activity of enzymes from leaves of younger plants and from younger leaves of older plants. Pirie (1959) suggested that difference in amino acid composition of young and old leaves could account for difference in enzyme activity.

DISCOLORATION OF THE CUT SURFACE OF THE BANANAS

Rates of browning of the cut surface of the bananas after three periods of ripening are plotted in Figure 4. The data (Table 7, Appendix) used to construct the graphs, are differences between the diffuse reflectance at each five minute interval and the diffuse reflectance of the sample when first cut.

The rate of browning was highest in bananas in Lot I at the end of the second day of ripening and in bananas in Lot III at the end of the fifth day of ripening. The rate of browning was lowest in bananas in Lot I when analyzed on the day they were purchased and in Lots II and III at the end of the second day of ripening.

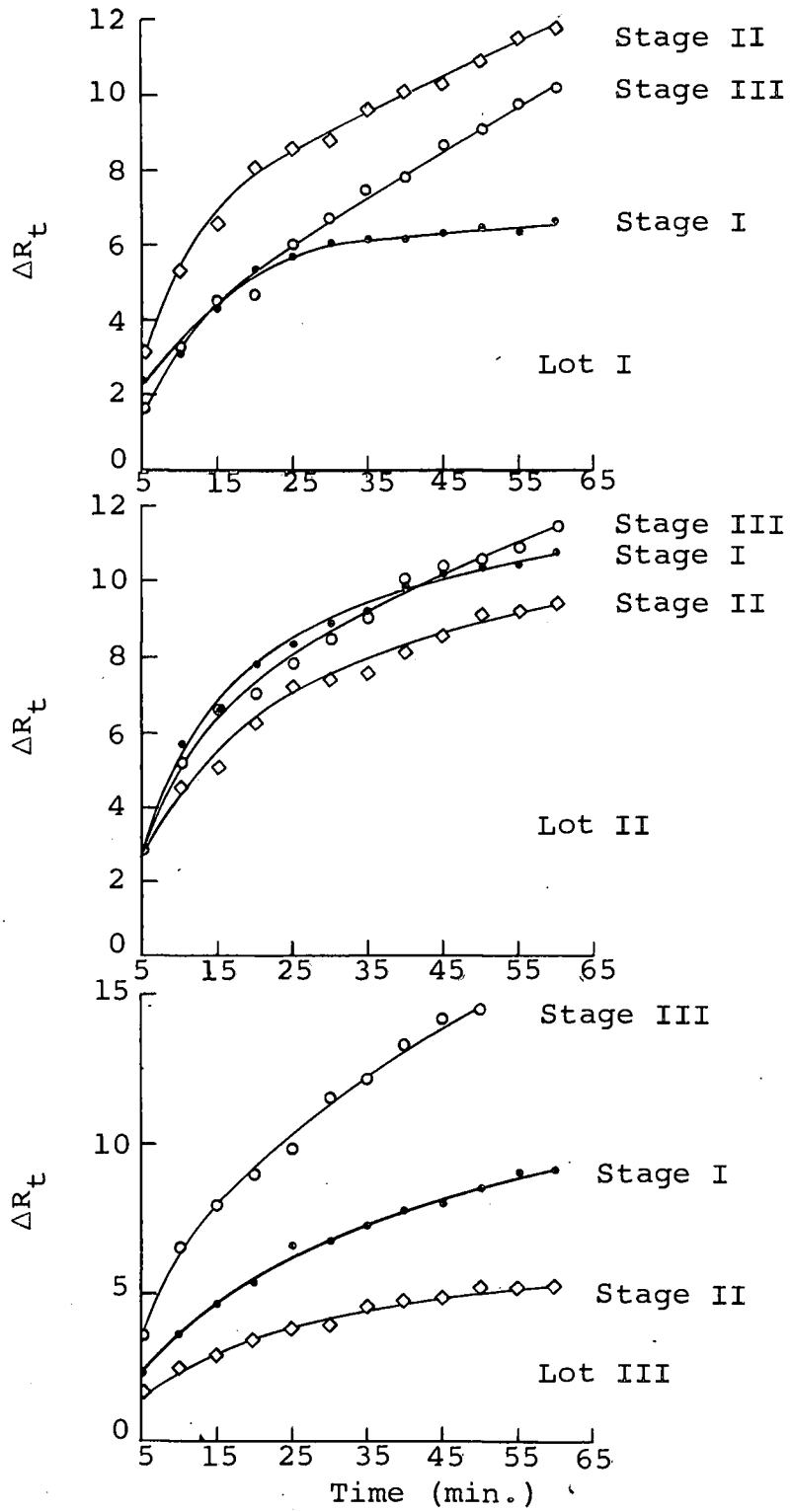

Figure 4. Enzymic browning[1] of ripening bananas.
1. Define $\Delta R_t = (R_o - R_t)$
where R_o = Initial reflectance (at t = 0)
R_t = Reflectance at time t

Ripeness made more difference in the rate of browning for bananas in Lot III and least for bananas in Lot II, where the stage of ripeness made little difference in the browning. Thus the rate of browning in these bananas was not directly related to ripening. Enzyme activity was greatest in bananas at Stage I of ripeness in all three lots, yet these bananas did not brown most. In fact, in Lot I these bananas browned least and in Lot III browning was much less in bananas with the most active enzyme, and the browning rate was greatest in bananas with the least active enzyme. Also, the rate of browning did not show any clear-cut relationship with the substrate concentration of the bananas.

Working with apples, Weurman and Swain (1955) reported that the intensity of browning was determined to a large extent by the activity of the enzyme present in the fruit. On the other hand, several researchers have reported that, for a number of fruits and vegetables, enzymic browning is directly related to substrate concentration (Guadagni, Sorber and Wilbur, 1949; Walker, 1962; Mapson, Swain, and Tomalin, 1963). Evidence for this came from the observation that browning of tissue increased after addition of substrate. However, Weurman and Swain (1955) did not accept the view that browning depended upon the concentration of substrate. From their work on apples at different stages of maturity, Weurman and Swain found that browning

and substrate concentration were not related. For example, from first to last picking, total phenols per apple increased roughly 40% while total browning decreased more than 75%. Harel, Mayer, and Shain (1966), who, like Weurman and Swain (1955) worked with developing apples, presented evidence that both substrate concentration and enzyme activity influence rate of browning.

The conflicting evidence cited above seems to indicate that possibly factors other than substrate concentration and enzyme activity are involved in enzymic browning. L-ascorbic acid is frequently added to prevent enzymic browning. This suggests that there might be an inverse relationship between ascorbic acid content of fruit and browning, but Walker (1962) found no such inverse relationship with the apples which he analyzed. Ascorbic acid content of the individual bananas in the present study was not determined. However, Biale and Young (1962) reported that bananas increased in ascorbic acid content as they ripened. Oxidation products of phenols may react with polyphenol oxidase and thus interfere with browning (Palmer, 1964), certain phenols having stronger inhibiting effect than others. On the other hand, browning of potatoes due to oxidation of the substrate, tyrosine, was greatly increased by the addition of chlorogenic acid (0 to 200 μg/ml), and in its absence the rate of browning was slow. In addition, L-ascorbic acid (100 μg/ml) had no effect on

the oxidation of tyrosine *in vitro* in the presence of sub-optimal amounts of chlorogenic acid (Mapson, Swain, and Tomalin, 1963).

To sum up, the enzymic browning of fruit is a complex problem. A statement made by Weurman and Swain in 1955 is still pertinent:

> A study of the separate phenols in the fruit, their individual contribution to the browning, and the inhibitory effect of their oxidation products on the activity of the enzyme will be of value in the efforts to solve the problems still outstanding in connection with the enzymic browning of injured fruit. In fact we think that no progress will be made until this is done.

SUMMARY

Bananas at different stages of ripeness were analyzed for 3,4-dihydroxyphenylethylamine (dopamine) content, for moisture and for pH. In addition, activity of polyphenol oxidase, in terms of rate of dopaminechrome formation, was determined and the rate of discoloration of the cut surface of the fruit was measured. Three lots of bananas, each consisting of six fruits from the same hand, were used. Two bananas from each lot representing the first stage of ripeness were analyzed the day they were purchased. The remaining four bananas in each lot were held in respiration jars for ripening at 68-70°F at constant rate of air flow. At the end of two days of ripening two of the bananas of each lot were analyzed (second stage of ripeness) and at the end of the fifth day the two fruits remaining were analyzed (third stage of ripeness). Respiration rate was determined to assess the stage of ripeness when each fruit was analyzed.

While the entire fruit lost weight (average 5%) during five days of ripening, the moisture in the edible pulp increased. The pH of the pulp decreased from an average of 5.6 at the first stage of ripeness to near 5.0 at the end of five days of ripening.

Dopamine content was highest in bananas of Lot III, ranging from 77.5 μg/g fresh weight at the first stage of

ripeness to 40 μg/g at the third stage of ripeness. Fruit in Lot II was second highest in dopamine content, ranging from 62.5 μg/g fresh weight in the first stage of ripeness to 23 μg/g in the third stage of ripeness. Bananas in Lot I, however, had the highest dopamine content (average 22.5 μg/g fresh weight) at the second stage of ripeness, first and third stages being more or less the same (14.0 to 12.5 μg/g fresh weight, respectively). While the average dopamine content of fruits from Lots II and III decreased as the fruit ripened and was lowest in the ripest fruit from Lot I, the great variation in the dopamine content of individual bananas at the same stage of ripeness precludes any unqualified statement about the decrease in dopamine content as bananas ripen.

The activity of the enzyme, as measured by the rate of dopaminechrome formation, decreased with the ripeness of the fruit in all three lots of bananas. From Stage I to Stage III, the average rate of pigment formation dropped from 0.1728 $\Delta OD/\Delta t$ to 0.1200 $\Delta OD/\Delta t$ in Lot I, from 0.1776 $\Delta OD/\Delta t$ to 0.1008 $\Delta OD/\Delta t$ in Lot II and 0.3360 $\Delta OD/\Delta t$ to 0.1128 $\Delta OD/\Delta t$ in Lot III. The first two days of ripening caused a greater decrease in the activity of the enzyme than did the last three.

In none of the three lots did the rate of browning show any definite pattern. The rate of browning was highest in the bananas in Lot I at the end of the second

day of ripening and in bananas in Lot III at the end of the fifth day of ripening. The rate of browning was lowest in bananas in Lot I when analyzed on the day they were purchased and in Lots II and III at the end of the second day of ripening. Results of this study did not show any relationship between either substrate concentration and browning or enzymic activity and browning. While substrate and enzyme are essential for browning of injured banana tissue other factors obviously are involved.

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APPENDIX

TABLE 7. ENZYMIC BROWNING OF RIPENING BANANAS[1] (ΔR_t)[2]

TIME (min.)	Days of Ripening								
	NONE BANANA			TWO BANANA			FIVE BANANA		
Lot I	1	2	av.	1	2	av.	1	2	av.
05	2.00	2.75	2.37	1.45	5.00	3.22	1.40	2.10	1.75
10	3.00	3.25	3.12	4.00	6.50	5.25	2.80	3.45	3.12
15	3.50	5.25	4.37	7.00	6.20	6.60	4.00	5.10	4.55
20	4.75	6.00	5.37	9.25	6.50	7.87	4.05	5.35	4.70
25	5.00	6.50	5.75	9.50	8.00	8.75	4.90	7.20	6.05
30	5.50	6.75	6.12	9.60	8.25	8.92	5.70	7.70	6.70
35	5.50	6.65	6.07	10.50	8.65	9.57	6.40	8.60	7.50
40	5.50	6.70	6.10	11.00	9.25	10.12	6.55	8.85	7.70
45	5.50	7.10	6.30	11.10	9.75	10.42	7.30	10.10	8.70
50	5.75	7.20	6.47	11.50	10.35	10.92	7.85	10.45	9.15
55	5.70	7.25	6.47	12.00	11.35	11.67	8.50	11.10	9.80
60	6.25	7.15	6.70	12.25	11.35	11.80	9.15	11.35	10.25
Lot II									
05	3.35	2.45	2.90	2.35	3.25	2.80	3.05	2.85	2.95
10	5.80	5.40	5.60	3.36	5.35	4.35	5.15	5.10	5.12
15	6.50	6.75	6.62	4.40	5.75	5.07	6.80	6.50	6.65
20	8.05	7.70	7.87	5.50	6.95	6.22	7.15	6.95	7.05
25	8.80	7.85	8.32	6.30	7.50	6.90	7.90	7.65	7.78
30	8.80	8.85	8.82	6.50	8.25	7.37	8.30	8.65	8.48
35	9.20	9.25	9.22	6.60	8.35	7.48	9.10	8.95	9.02
40	10.00	9.75	9.87	7.20	9.00	8.10	10.00	10.00	10.00
45	10.15	10.20	10.17	8.20	9.60	8.90	10.45	10.15	10.30
50	10.35	10.25	10.30	8.20	9.95	9.08	10.60	10.20	10.40
55	10.40	10.40	10.40	8.20	10.10	9.15	11.15	10.45	10.80
60	10.75	10.75	10.75	8.50	10.45	9.48	11.95	10.80	11.38
Lot III									
05	1.90	2.65	2.27	2.35	0.90	1.62	3.50	3.50	3.50
10	3.25	3.85	3.55	2.65	2.55	2.60	6.15	7.30	6.72
15	5.25	4.20	4.72	3.00	2.70	2.85	7.00	9.00	8.00
20	6.10	4.80	5.45	3.45	3.40	3.42	8.00	9.75	8.88
25	7.05	6.20	6.62	3.45	3.70	3.57	8.00	9.75	8.88
30	7.25	6.35	6.80	3.80	4.05	3.92	11.00	12.00	11.50
35	7.50	7.10	7.30	4.60	4.40	4.50	11.35	13.10	12.23
40	8.00	7.75	7.87	4.90	4.55	4.72	13.00	13.85	13.42
45	8.15	8.00	8.07	5.00	4.65	4.82	13.50	14.65	14.07
50	8.45	8.85	8.65	5.30	4.90	5.10	13.75	14.65	14.20
55	8.75	9.35	9.05	5.30	4.90	5.10	14.00	15.00	14.50
60	9.15	9.35	9.25	5.50	4.95	5.22	14.25	15.50	14.87

1. Average of two determinations.

2. $\Delta R_t = (R_o - R_t)$
where R_o = initial reflectance (at t=o)
R_t = reflectance at time t