

AN ABSTRACT OF THE THESIS OF

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Title: FACTORS INFLUENCING ENZYMATIC BROWNING OF
RIPENING BANANAS

Abstract approved: _____
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Activity of polyphenol oxidase, concentration of 3, 4-dihydroxyphenylethylamine (dopamine), and concentration of ascorbic acid in the pulp of bananas as they ripened were measured and relationships between these factors and both the extent of browning and the susceptibility to discoloration of the fruit during a 30-minute holding period were studied. Moisture and protein content were determined also. Bananas from three lots were analyzed as purchased and after either two or four days of additional ripening.

Initial browning of the filtrates of bananas and the increase in browning upon standing were greater as the fruit ripened. Protein content also increased with ripening. Moisture content of bananas increased significantly in those bananas held for four additional days. The specific activity of polyphenol oxidase (units of activity/mg protein) in bananas decreased with ripening, but when the activity was

calculated on a basis of dry weight of banana no significant change was observed. Both the dopamine and ascorbic acid contents decreased in individual bananas with ripening.

Decrease in the concentration of dopamine was the variable with the highest correlation to the increase in browning in ripening bananas. Both the increase in browning and the decrease in dopamine in ripening bananas were associated with a decrease in ascorbic acid content. Thus, the concentration of dopamine as influenced in part by concentration of ascorbic acid appears to be the limiting factor in the browning of bananas. The specific activity of polyphenol oxidase did not appear to be a limiting factor in the browning of bananas.

Dopamine was located histochemically in the vacuoles of the latex vessels and in a few isolated parenchyma cells of banana. Polyphenol oxidase appeared to occur throughout the pulp.

Factors Influencing Enzymatic Browning
of Ripening Bananas

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FACTORS INFLUENCING ENZYMATIC BROWNING OF RIPENING BANANAS

INTRODUCTION

Discoloration in bananas which involves the enzyme-catalyzed oxidation of phenolic compounds is of practical concern. This type of browning may occur in plants during the normal life cycle. Browning may also take place rapidly following mechanical injury as in the preparation of certain fruits for serving.

Early workers concerned with this type of discoloration attributed browning to a heterogeneous group of phenolic compounds known collectively as tannins. Griffiths (1959) identified 3, 4-dihydroxyphenylethylamine (dopamine) as the specific phenolic substrate for enzymatic browning in bananas. Palmer (1963) purified banana polyphenol oxidase and reported that dopamine was the major substrate.

Nandi (1972) attempted to relate the activity of polyphenol oxidase and the concentration of dopamine in ripening bananas to their susceptibility to discoloration. The study reported here is a continuation of that work. Analyses were made initially and after a period of ripening on two halves of the same fruit instead of on different fruit at different stages of ripeness, as did Nandi. Browning was measured on extracts of the banana instead of on the cut surface

of the fruit. A fluorimetric assay of dopamine was used instead of the colorimetric method using silver oxide. In addition, ascorbic acid, a naturally-occurring inhibitor to enzymatic browning, was measured to assess its influence on the susceptibility of bananas to discoloration. Enzyme and substrate in banana were located histochemically.

REVIEW OF LITERATURE

Polyphenol Oxidases

Properties and Localization

Polyphenol oxidases¹ catalyze the oxidation of phenolic compounds in the presence of molecular oxygen. Polyphenol oxidases have copper in the prosthetic group. The copper content of mushroom polyphenol oxidase, for example, is 0.2 percent and it appears in univalent form (Kertesz, 1966).

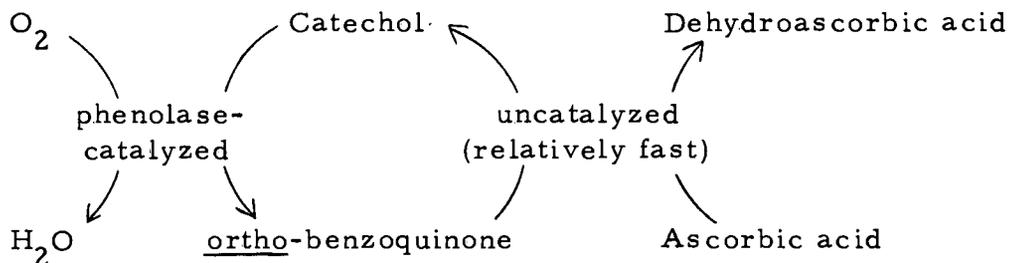
Polyphenol oxidases have multiple components (Constantinides and Bedford, 1967). These multiple forms may result from configurational changes in the protein molecule or from different combinations or degrees of polymerization of one or more subunits (Jolley and Mason, 1965). Mathew and Parpia (1971) suggested that each form may be bound to a specific site within a subcellular structure where it fulfills a specific role. Harel et al. (1964, 1965) found that apple polyphenol oxidase occurred in both the chloroplast and the mitochondrial fractions. Specificity of substrate and value of Michaelis constant differed for the two fractions. A soluble fraction of the enzyme was

¹The official name is (1. 10. 3. 1) ortho-diphenol:oxygen oxidoreductase as recommended by the Commission on Enzymes of the International Union of Biochemists (1961).

also identified. They found that the soluble fraction of apple polyphenol oxidase increased and the particulate fraction decreased with ripening. This suggested that the enzyme was released from the organelles during ripening. Walker and Hulme (1966) questioned the association of the enzyme with particulate fractions of the cell, suggesting that this was an artifact of the extraction procedure.

Assay

One way that activity of polyphenol oxidase can be assessed is by measuring oxygen consumption by employing polarographic or manometric techniques. Mayer et al. (1966) recommended the polarographic method in preference to manometric procedures. A second way to assess activity of polyphenol oxidase is to measure the products of reactions by colorimetry or titration. For example, the phenolase-catalyzed formation of ortho-benzoquinone from catechol in a coupled oxidation with ascorbic acid proceeds in the manner as shown by Dawson and Tarpley (1951):



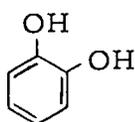
Joslyn (1970) criticized colorimetric methods because the rate and extent of pigmentation during the enzymatic reaction are not necessarily proportional to the rate of oxidation.

Enzymes must be freed of substances that may interfere with assessment of enzyme activity. Isolation of active enzymes in the presence of phenolic compounds is difficult because the two combine reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensation (Loomis and Battaile, 1966). Tannin-precipitating agents such as polyvinylpyrrolidone (Loomis and Battaile, 1966) and nonionic detergents (Goldstein and Swain, 1965; Walker and Hulme, 1966) have proved effective in extracting enzymes.

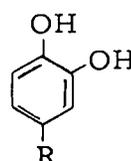
Substrates for Polyphenol Oxidase

Characteristics

In the browning of fruit tissue, polyphenol oxidases catalyze the oxidation of certain phenolic compounds related to catechol. The



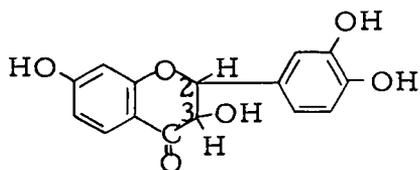
Catechol



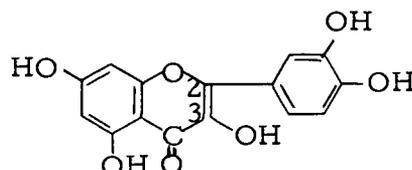
Catechol Derivative

nature of the R group gives rise to differences in reactivities of the substrates (Corse, 1964). Subtle differences may render one compound a substrate for polyphenol oxidase and a similar compound

unaffected. For example, Shannon and Pratt (1967) found that dihydroquercetin was a substrate for apple polyphenol oxidase but quercetin was not. The two molecules differ only in the bonding between carbons at the 2 and 3 positions. Quercetin is more planar



Dihydroquercetin



Quercetin

and less soluble than dihydroquercetin and contains a double bond conjugated to an aromatic ring which confers stability. Stability, decreased solubility, or perhaps steric effects may explain the difference in reactivity of the two compounds.

Localization

Reeve (1951) located compounds he referred to as tannins in pears in the sclerids, core parenchyma, the outside region of the fleshy tissue, and in scattered parenchyma cells in the intervening area of fleshy tissue. In apples, the skin area, phloem and associated parenchyma cells, and many of the storage parenchyma cells showed a positive reaction for tannins. Phenolics were frequently found in vacuoles, although they do not necessarily originate there. Methods used in the localization and estimation of phenolics may disrupt cells

and allow these substrates to appear in the vacuolar sap. Reeve (1951) suggested that these phenolic compounds may be involved in the formation of the vacuoles in meristematic tissues of many plants.

Changes with Ripening

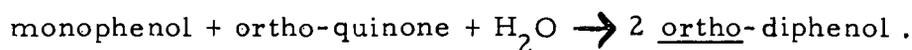
Phenolic content of fruits usually reaches a maximum during growth and declines during ripening. Increases in catechol derivatives have been associated with early maturation and with lignification of the pit and sclereids of the endocarp in peaches (Reeve, 1959; Craft, 1961). Upon ripening, the amount of catechol derivatives decreased in this fruit. Reeve suggested that they may be metabolized to form various sugars and acids or converted to other forms such as flavonoids and aromatics.

Assay

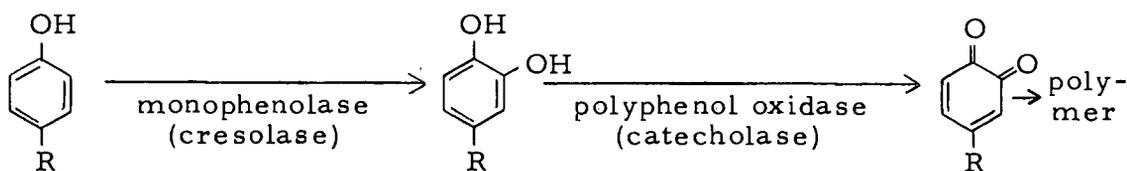
A problem in the assay of phenolic compounds in tissue may be that these compounds are absorbed onto cell walls, thus reducing their extractability. Once extracted, they can be measured by ultraviolet spectroscopy, nephelometry, and colorimetry. None of these methods are completely satisfactory, according to Schanderl (1970). Fluorimetry may be employed to analyze samples for dopamine (Carlsson and Waldeck, 1958).

Mechanism of Enzymatic Oxidation
of Phenolic Compounds

The fundamental step in enzymatic browning is the oxidation of phenolic compounds to ortho-quinones. Frequently, the catechol-related substrate is formed by hydroxylation of a monohydroxyphenol to the ortho-dihydroxyphenol. Mason (1955) proposed that this step is catalyzed by a monophenol oxidase. This action is called the cresolase activity. Kertesz and Zito (1962) suggested that the ortho-quinone produced by the oxidation of ortho-diphenol causes the hydroxylation of monophenols, thus:



The ortho-dihydroxyphenol so formed is oxidized subsequently to an ortho-quinone. The latter action is called the catecholase activity.

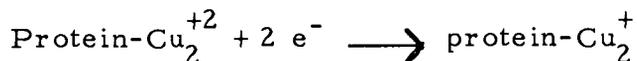
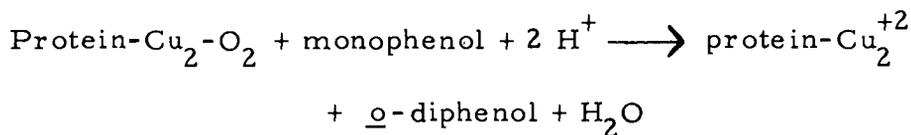
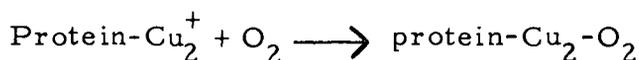


Catecholase activity is more important than cresolase action in foods because most of the phenolic substrates in foods are dihydroxyphenols (Mathew and Parpia, 1971). Ortho-quinones can undergo further autooxidation, condensation, and rearrangement to form brown or black polymers. Singleton (1972) characterized this polymerization as

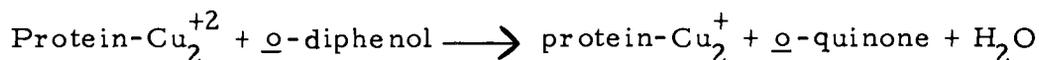
a random free-radical type process even though initiated by enzymatic oxidation. He theorized that polymerization is a stabilizing process whereby labile chromophores are incorporated into one molecule. Because these chromophores are of varying shapes, strains are produced and the polymers appear brown or black.

In the reactions outlined above, copper plays an important role. An aged polyphenol oxidase apoenzyme can be reactivated by addition of fresh cupric copper which is reduced to the active cuprous form (Kertesz, 1966). Mason (1956) suggested that the hydroxylation of monophenols occurs after phenolase with copper in the cuprous state combines with molecular oxygen. The monophenol then combines with this phenolase-oxygen complex to yield phenolase in the cupric state and diphenol.

Cresolase Reactions



Reduction of copper back to the cuprous state may occur during the oxidation of the ortho-diphenol to the ortho-quinone state.



Guadagni et al. (1949) reported a linear relationship between the amount of oxidizable phenols and change in optical density of the filtrate of frozen peaches. Less than half of the oxidizable phenols remained after the peach filtrate had been allowed to brown for 15 minutes, and browning no longer occurred at a rate proportional to the original activity of the enzyme. Guadagni et al. concluded that total browning at the end of 60 minutes was more dependent on the original phenolic content than on enzyme activity.

Weurman and Swain (1955) reported that browning in apples decreased 75 percent during the period of ripening from 37 days to 170 days from petal drop, but they concluded that activity of the enzyme was a more important factor than concentration of phenolic substances. Harel et al. (1966) contended that both enzyme and substrate are factors influencing extent of browning. Because the concentration of both substrate and enzyme changes as the fruit develops, their relative importance probably changes also.

Inhibitors of Browning

Evidence thus indicates that the browning of fruits by polyphenol oxidase may be delayed or stopped by various inhibitors to the reactants, to the enzyme, or to product formation. These inhibitors may occur naturally in fruits or they may be applied. Two practical means of preventing discoloration in fruits are blanching to inactivate

the polyphenol oxidases and removal of oxygen by preservation methods, such as vacuum packing or packing in a syrup. Enzymatic browning can also be retarded by chilling or by lowering the pH with acid.

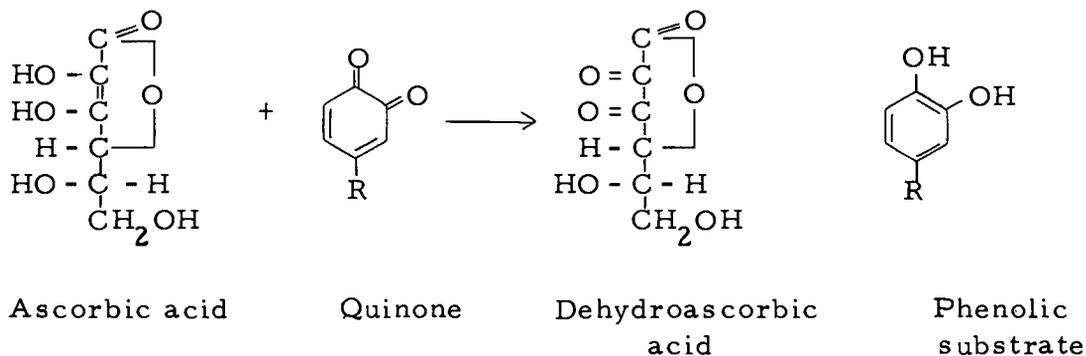
Compounds containing sulfur act as inhibitors to discoloration, although their mechanism of action is not fully understood. Sulfur dioxide may act by reducing the available oxygen, it may react with quinones or other intermediates of oxidation, or it may inhibit the enzyme because of its reducing capacity (Ponting, 1960). Sulfite (Embs and Markakis, 1965) and thiol compounds (Walker, 1964) are thought to act by combining with the ortho-quinones, thus stopping their condensation to melanins. Other chemicals act through competitive inhibition of polyphenols for polyphenol oxidase or through non-competitive inhibition by complexing the enzyme or by replacing the prosthetic group of the enzyme.

Biochemical modification of substrate is being explored as means to prevent discoloration of fruits. For example, catechase is being used to oxidize and split the benzene ring of phenolic substrates (Corse, 1964). Corse also reported some success with O-methyltransferase used to methylate chlorogenic acid and other catechols to their corresponding 3-methyl esters.

Ascorbic acid is an inexpensive, harmless chemical used by processors of frozen fruit and by homemakers to prevent browning.

Although Walker (1962) found no inverse relationship between the concentration of naturally-occurring ascorbic acid and browning of apples, Ponting and Joslyn (1948) found that no darkening occurred in this fruit until all of the ascorbic acid was oxidized. Ponting and Joslyn attributed both the oxidation of ascorbic acid and darkening to action of polyphenol oxidase. Baruah and Swain (1953) suggested that ascorbic acid affects the copper moiety of the polyphenol oxidase. They found that the inhibitory effect of ascorbic acid on potato polyphenol oxidase was directly proportional to the concentration of ascorbic acid. Weurman and Swain (1955) reported that ascorbic acid did not influence enzyme activity in apples. Pierpoint (1966) reported that ascorbic acid greatly decreased the initial rate at which tobacco-leaf ortho-diphenol oxidase oxidized chlorogenic acid, suggesting that ascorbic acid was acting on the enzyme. In addition, rate of oxygen uptake was increased by the addition of ascorbic acid. The latter observation suggested that ascorbic acid was being oxidized, thereby keeping the substrate reduced. Extra oxygen uptake and delay in browning were proportional to the amount of ascorbic acid added. The quinones, rapidly reduced by ascorbic acid, accumulated and condensed to brown products only after all the ascorbic acid had been oxidized. Krueger (1950) reported that addition of ascorbic acid increased oxygen uptake and acted as a reducing agent in the enzymatic oxidation of tyrosine and DOPA in the general reaction as given by

Corse (1964):



In plant tissue, ascorbic acid is associated with cell walls (Jenson and Kavaljuan, 1956). Accumulation of ascorbic acid may precede and condition cell expansion by influencing the absorption and retention of substances at the surface of cells (Reid, 1941).

Enzymatic Browning in Bananas

Bananas

The banana (*Musa paradisiaca sapientum* L.) belongs to the genus *Musa*, the family Musaceae, and the order Zingerberales. The *Eumusa* group of species is the most widespread and includes the major edible varieties. The banana is a berry fruit thought to be of Indian origin (Czyhrinciw, 1969).

The anatomy and development of the banana are discussed by von Loesecke (1949), Simmonds (1959), and Palmer (1971). The edible banana is parthenocarpic and is propagated from a rhizome.

The fruit consists of an inedible skin which encircles the edible pulp. The skin is approximately one-fifth the diameter of the pulp. The pulp-to-skin ratio increases throughout development. The skin has a sharply ridged outer margin and its boundary with the pulp is clearly marked by the color contrast with the white, starchy pulp tissue. The peel consists of an epidermis interrupted by stomata, parenchyma cells, and fibrovascular bundles parallel to the long axis. The parenchyma cells of the peel become more and more rounded nearer the center of the fruit and there are more intercellular spaces. A thin layer of protoplasm in which plastids are embedded lines the inner walls of these cells. In outer layers of tissue the plastids have pigments, but in deeper layers the plastids serve as centers for accumulation of starch. A vacuole is found in the center of each cell which accumulates sugars as the starch is hydrolyzed to sugars during ripening.

The pulp develops from the outer edges of the locules. The sterile ovules degenerate to brown flecks embedded in the edible pulp. The three arms of the inner locular walls extend radially from the center. Vascular strands are scattered abundantly in the skin and grouped in three pairs in the center of the pulp. Vascular bundles and associated latex vessels and parenchymatous cells adjoin the vascular strands. The latex system consists of the most conspicuous cells, large and barrel-shaped, joined end to end in a sausage-like fashion.

Latex tubes run at right angles to the long axis of the fruit and outline the margin of the individual locules.

Structure of the pulp other than gross observations are not available (Palmer, 1971). The parenchyma cells of green fruit are long and box-like in shape with thin walls. The cells contain a nucleus and cytoplasm. Cells of preclimacteric fruit, i. e., fruit which has not reached its peak in respiration, are closely packed with little or no intercellular spaces and are obscured by the numerous large starch grains present. During ripening, the cells become progressively depleted of starch and more details of the individual cells are revealed.

Banana Polyphenol Oxidase

Palmer (1963) found that banana polyphenol oxidase did not oxidize monophenols. The enzyme did catalyze the oxidation of a variety of ortho-dihydroxy phenols, including dopamine, DOPA, catechol, chlorogenic acid, and arterenol. Dopamine is the most reactive substrate for this enzyme. The Michaelis constant for this substrate is 6.3×10^{-4} M. The Michaelis constant for the substrate, L-DOPA, is 6.6×10^{-2} M. The enzyme was saturated by concentrations of dopamine higher than 3×10^{-3} M and inhibited by concentrations above 12×10^{-3} M. Banana polyphenol oxidase had an optimum pH of 7.0 when catalyzing the oxidation of dopamine. The pH of

green bananas, 5.0 to 5.7, decreases to 4.2 to 5.0 with ripening (von Loesecke, 1949; Nandi, 1972). Palmer (1963) reported evidence that banana polyphenol oxidase is adsorbed on or structurally associated with the cell wall.

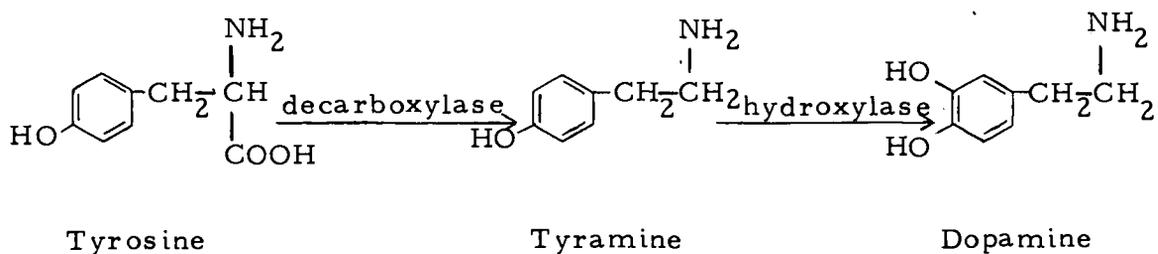
Dopamine

Griffiths (1959) identified dopamine (3,4-dihydroxyphenylethylamine) as the substrate for the polyphenol oxidase of banana. DOPA (3,4-dihydroxyphenylalanine) was not detected. Later (1961) Griffiths found that the concentration of dopamine in bananas is determined by the extent of the contribution of the acuminata genome to the genotype. Waalkes et al. (1958) measured the content of dopamine in bananas and found 700 $\mu\text{g/g}$ of peel and 7.9 $\mu\text{g/g}$ of pulp.

Barnell and Barnell (1945) located what they referred to as tannins, although not specifically dopamine, in the latex vessels of the skin and pulp and in scattered isolated parenchyma cells in three-fourths full, green bananas. After the fingers had sprung, i. e., the yellow began to show through the green of the skin, the latex in the pulp tended to dry out and the tannins gradually disappeared from the dried latex contents. Little change was observed between the eating-ripe and overripe stages. Buckley (1964) reported the dopamine content in peel of developing bananas. Ten to 15 percent accumulated

prior to emergence (0.7 mg/g fresh weight) and 85 to 90 percent (2.0 to 3.5 mg/g fresh weight) accumulated during the first month after emergence. Half of the dopamine was located in the outer millimeter of peel. Dopamine decreased 20 percent with ripening. However, the dopamine content of the inner 2 to 3 ml of peel increased 35 percent with ripening so that the content per entire peel seemed to remain constant to harvest. The peel enlarged during this period, giving a final concentration of 1.0 to 1.2 mg/g fresh weight. Dopamine concentration was least in the end attached to the stem and greatest in the floral end of the fruit.

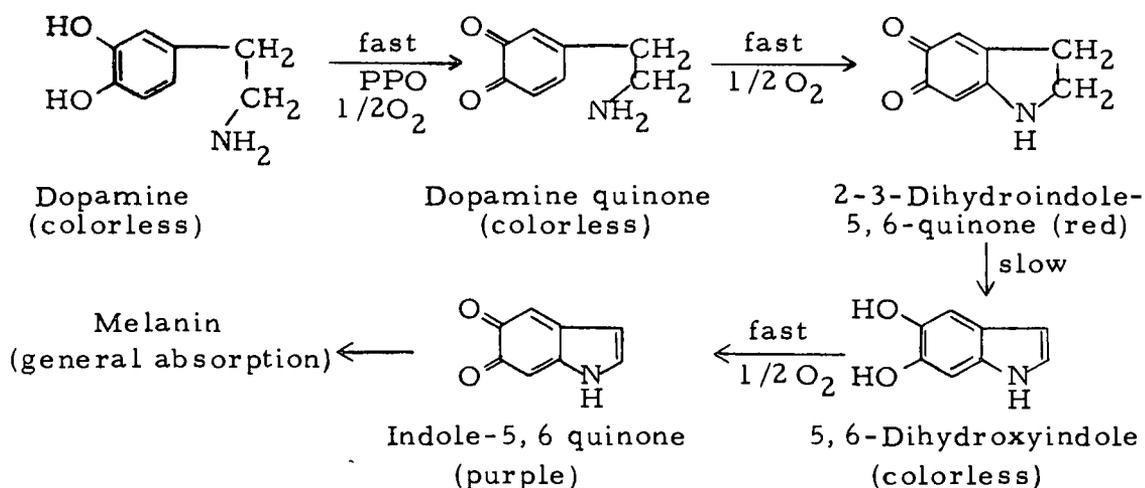
Dopamine is synthesized from tyrosine. In mammalian systems dopamine is formed by the decarboxylation of DOPA formed from tyrosine through the action of tyrosine hydroxylase. However, in bananas tyramine is the intermediate compound (Buckley, 1964). Deacon and Marsh (1971) isolated an enzyme from bananas that appeared to be the hydroxylase that converts tyramine to dopamine.



Waalkes et al. (1958) and Udenfriend et al. (1959) found a high content of other physiologically active amines in bananas. These do not act as substrates for polyphenol oxidase, however.

Mechanism of Oxidation of Dopamine

Based on spectrochemical evidence, Palmer (1963) proposed the following reaction mechanism for the oxidation of dopamine by banana polyphenol oxidase:



It is analogous to an earlier scheme proposed by Mason (1947, 1948) for the oxidation of DOPA. Not all intermediates are known. DeSwardt et al. (1967) reported that polymerization of phenolic compounds does occur in ripening bananas as shown by a decrease in extractable phenolic compounds. Palmer (1963) suggested that melanins may

also be produced in banana tissue from arterenol, a substrate for banana polyphenol oxidase. He confirmed the presence of dopamine β -oxidase in bananas which can convert dopamine to arterenol. This reaction was negligible in vitro, however, according to Palmer.

Ascorbic Acid

Ascorbic acid is a naturally-occurring inhibitor of the oxidation of dopamine. Harris and Poland (1939) found that ascorbic acid increased from an average of 5.3 mg/100 g pulp in green bananas to 11.0 mg/100 g pulp during ripening, but decreased to an average of 3.2 mg/100 g pulp as the fruit became overripe. The range of values of ascorbic acid was 1.0 to 14.3 mg/100 g pulp. The average value was 10 mg/100 g pulp.

Palmer (1964) studied the effects of various levels of ascorbic acid on delay of the enzymatic oxidation of dopamine and on the activity of banana polyphenol oxidase. He found a delay of 0.2 minute and 14 percent inhibition of polyphenol oxidase activity with 1.4×10^{-5} M ascorbic acid. The delay increased to 12.7 minutes, and inhibition of polyphenol oxidase activity increased to 78 percent with 1.7×10^{-4} M ascorbic acid.

Many questions remain unanswered about the importance in the metabolism of plant tissue of the oxidation of dopamine by polyphenol oxidase. Formation of melanin from dopamine is associated with root

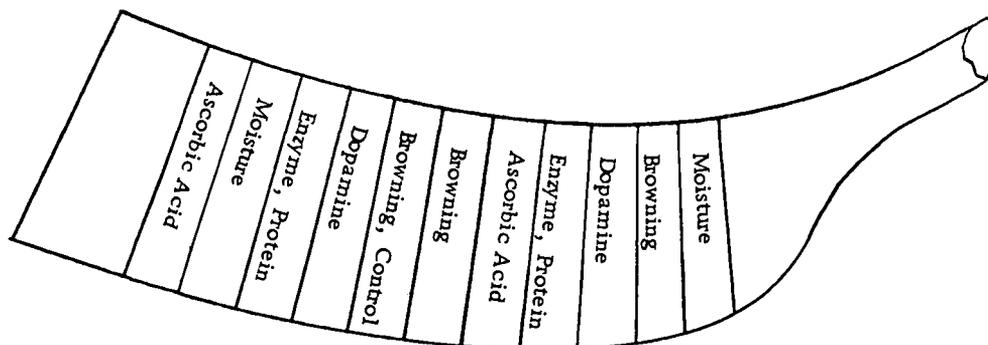
wounding and infection, and may play a significant role in resistance to infection. Sondheimer (1962) reported that plants susceptible to infection accumulate ascorbic acid, whereas resistant species accumulate polyphenols with an accompanying decrease in ascorbic acid. He says that oxidation products of ortho-dihydroxy compounds may be the antiviral substances. DeSwardt et al. (1967) suggested that low molecular weight tannins may control the activity of enzymes in preclimacteric fruit, thus influencing ripening. As tannins polymerize, they may be bound by hydrogen bonds to these proteins or to cell walls. Palmer (1963) suggested that further study of this unique relationship could help answer some of the problems concerning the synthesis, localization, and role of phenol oxidases in plant tissue.

MATERIALS AND METHODS

General Plan

Three hands of bananas ripened to the green-tip stage were obtained from a local grocer. Three bananas from each of two hands and four bananas from a third hand, making a total of ten bananas, were analyzed. One-half of each banana was analyzed as purchased (day zero) and the cut surface of the other half was wrapped to prevent evaporation. These halves were held at room temperature for additional ripening. Halves of two of the bananas of hand I were analyzed after two days and the half of the third banana was analyzed after four days of additional ripening. From hand II, the half of one banana was analyzed after two days and the halves of the other two bananas were analyzed after four days of additional ripening. From hand III, halves of two of the bananas were analyzed after two days and halves from the other two bananas were analyzed after four days of additional ripening.

Each of the halves was subsampled according to the following plan:



For the halves of bananas that were analyzed at day zero, the floral end and stock end were used alternately. Bananas were sectioned with a plastic knife and these subsamples were weighed on a Mettler balance to four decimal places except for the measurements of browning, the samples for which were weighed to two decimal places.

Browning

Banana slices were cut to weigh 10.00 g. The control slices were placed in 20 ml of 1 percent thiourea which stopped the enzymatic browning reaction. The other slices were placed in 20 ml of distilled water. The tissue was homogenized in a microblender and filtered in a Buchner funnel using Whatman No. 1 filter paper.

The extent and the susceptibility to browning were measured by the method of Gaudagni et al. (1949). One ml of filtrate was added to 5 ml of water in a cuvette and optical density was read at 475 nm with a spectrophotometer². Readings were taken periodically over a 30-minute period and curves were plotted as percent transmittance against time.

²Spectronic-20 Spectrophotometer, Bausch and Lomb Inc., Analytical Systems Division, Linden Ave., Rochester, New York 14625

Moisture

To calculate concentrations of constituents in banana on a dry weight basis, moisture was determined. Pieces of aluminum foil (13 cm square) were coded, dried in a vacuum oven, weighed, and stored in a desiccator. Samples of banana were mashed with a fork and transferred to the appropriate foil. After the banana and foil were weighed, the samples were frozen until they could be dried. The samples were dried in a vacuum oven, preheated to 70°C, at a pressure of 170 mm Hg for 12 hours. The samples were equilibrated in a desiccator before they were weighed to determine percentage of moisture in each sample.

Polyphenol Oxidase

Extraction

Each slice of banana (approximately 4 g) was placed in a beaker containing 18 ml of 1 percent detergent³ buffered at pH 7.0 with 0.1 M phosphate buffer (Palmer, 1963). The banana and extractant were homogenized in a microblender⁴. The homogenate was centrifuged in a refrigerated centrifuge⁵ at 15,000 x g for 15 minutes. All operations were carried out at 0°C.

³ Igepal CO-630, phenylphenoxypoly (ethyleneoxy) ethanol, GAF Corporation, 140 West 51 Street, New York 10020.

⁴ Virtis Co., Route 209, Gardiner, New York 12525.

⁵ Model RC 2-B, Sorvall, Ivan Inc., Peck's Lane, Newtown, Connecticut 06470.

Assay

Activity of polyphenol oxidase was assayed by the method of Palmer (1963). The reagents used in the assay were prepared as follows:

1. Phosphate buffer (0.1 M, pH 7.0). $K_2HPO_4 \cdot 3 H_2O$ (15.22 g) and 4.54 g of KH_2PO_4 were dissolved in water and diluted to one liter. A 0.02 M solution was made by diluting one part with five parts water.
2. Dopamine solution. 3,4-dihydroxyphenylethylamine-HCl (284.4 mg) was dissolved in redistilled water and diluted to 100 ml.

The procedure used in the assay was as follows:

1. One ml of supernatant of banana extract was transferred to a 100 ml volumetric flask and made to volume with 0.02 M phosphate buffer.
2. To measure activity of the enzyme, 1.0 ml of dopamine solution (to give a final concentration of 5×10^{-3} M) and 1 ml of 0.1 M phosphate buffer were pipetted into the cuvette of a recording spectrophotometer⁶. After the cuvette was inserted into the spectrophotometer, 1.0 ml of the diluted extract containing

⁶ Cary 11 Recording Spectrophotometer, Varian Associates, Cary Instrument Division, 611-T Hansen Way, Palo Alto, California 94303.

banana polyphenol oxidase was added, and at once the master switch was turned on.

3. The increase in optical density at 470 nm was followed at 25°C.

Rate of enzyme activity was calculated as $\Delta OD/\Delta t$ from the initial slope of the curve. A change of 0.1 unit optical density represents the oxidation of 8 μ g of dopamine per ml of solution as determined by oxidation with Ag_2O using the spectrometric technique of Mason (1948). Specific activity of the enzyme was calculated as units of activity of banana polyphenol oxidase/mg of protein.

Protein

To calculate specific activity of the enzyme, protein content was determined using the biuret method (Layne, 1957). Substances containing two or more peptide bonds form a purple complex with copper salt in alkaline solution.

Reagents used in the assay were as follows:

1. Bovine serum albumin standard.⁷ Protein (100 mg) was dissolved in 10 ml of distilled water.
2. Biuret solution. $CuSO_4 \cdot 5 H_2O$ (1.50 g) and 6.0 g of $NaKC_4H_4O_6 \cdot 4 H_2O$ were dissolved in 500 ml of distilled water.

⁷ Albumin, Bovine Fraction V Powder, No. A-4503 Lot 23 C-1630, Sigma Chemical Co., Sigma International, Ltd., P. O. Box 14508, St. Louis, Missouri 63178.

Ten percent NaOH (300 ml) was added with constant swirling. The solution was diluted to one liter with distilled water and stored in a paraffin-lined bottle.

The procedure used in the assay was as follows:

1. One ml of bovine serum albumin standard or 1 ml of supernatant from the enzyme extraction was pipetted into a cuvette.
2. Biuret solution (4.0 ml) was added. The contents of the cuvette were mixed and allowed to stand for 30 minutes at room temperature (20° to 25°C). Optical density was recorded at 550 nm^8 . A reagent blank of 1.0 ml of distilled water and 4.0 ml of biuret solution was used and a tissue blank of 1.0 ml of supernatant from the enzyme extraction plus 4.0 ml of water was used.

Concentration of protein in a sample was obtained by reference to a standard curve made with bovine standard albumin at concentrations of 1.0, 2.5, 5.0, 7.5 and 10.0 mg/ml. The standard curve (Figure 1) was made by plotting optical density against the concentration of bovine serum albumin standard.

⁸Spectronic-20 Spectrophotometer.

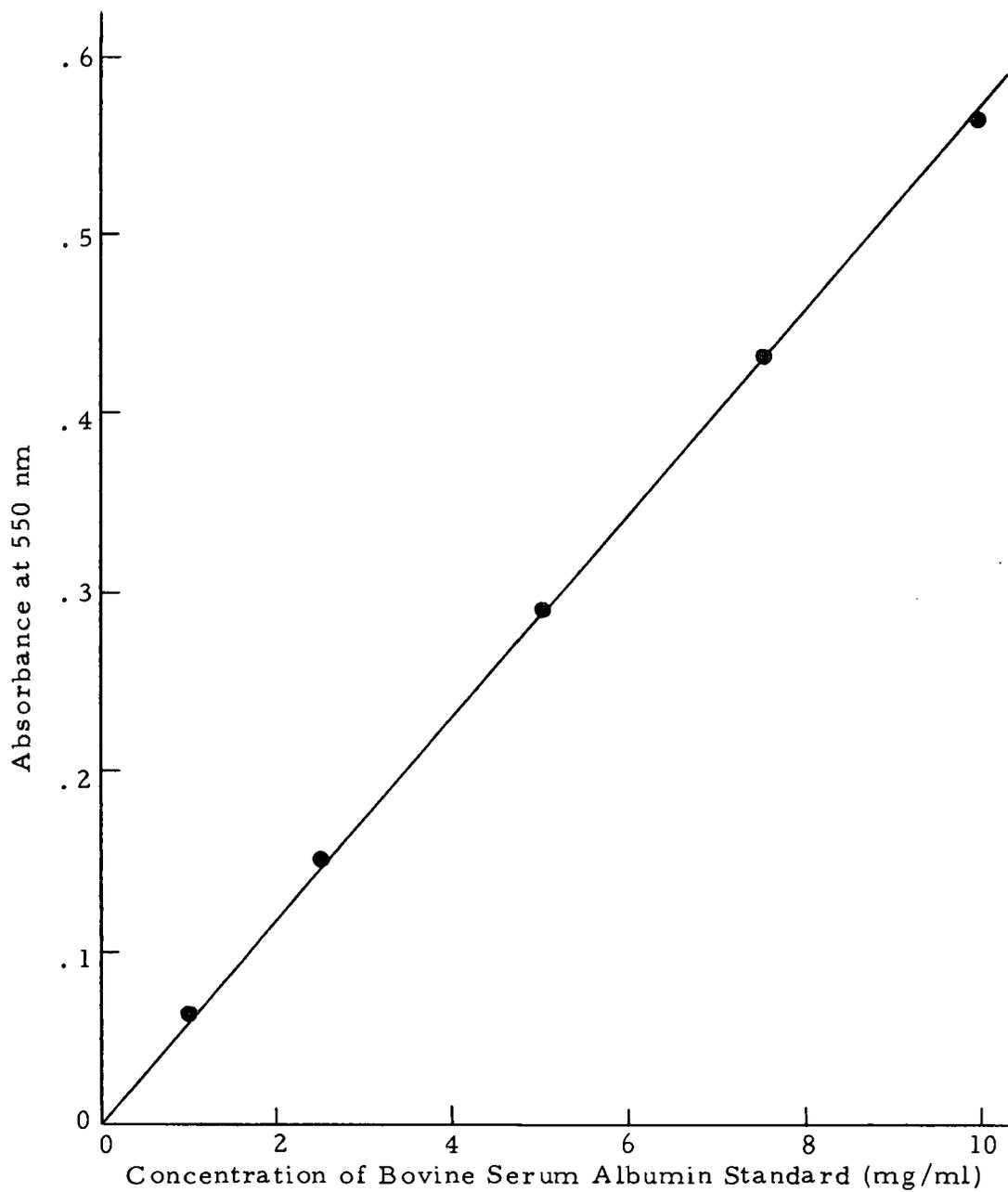


Figure 1. Standard Curve for Protein.

Absorbance measured on a Bausch and Lomb Spectrophotometer.

Dopamine

Extraction

Glass distilled water was used throughout the experiment. A slice of banana (approximately 2 g) was placed in a beaker containing 15 ml of 0.1 N HCl. After the tissue was homogenized in a microblender, the homogenate was frozen and held overnight. After it was thawed, the homogenate was centrifuged at 15,000 x g for 15 minutes at 0°C. The supernatant was adjusted to a pH of 4 with 5 N K₂CO₃, using one drop of 0.04 percent brom phenol blue in ethanol as an indicator. The volume was adjusted to 20 ml with water.

Purification

A Dowex 50 X-8 column was used to purify the dopamine (Bertler, Carlsson and Rosengren, 1958). The resin (200-400 mesh, dry weight 200 mg) was washed several times with 2 N HCl and packed in a glass column 5 mm in diameter. The column had a stopcock to aid in adjusting the rate of flow.

The column was charged by passing the following solutions through it:

- a) 20 ml 2 N HCl
- b) 5 ml water
- c) 10 ml 1 N sodium acetate-acetic acid buffer, pH 6.0
- d) 5 ml water

A 5 ml aliquot of the extract containing dopamine was then passed through the charged column and the column rinsed with 20 ml of water. Dopamine was eluted from the column at a flow rate of 0.25 ml per minute with 6 ml 1 N HCl followed by 6 ml 2 N HCl. Recovery of a known amount of dopamine off the column was 96 percent.

Assay

Concentration of dopamine in the eluate was assayed by a fluorimetric method (Carlsson and Waldeck, 1958). The reagents used in the assay were prepared as follows:

1. Phosphate buffer (0.1 M, pH 6.5). Na_2HPO_4 (4.25 g) and 9.52 g of KH_2PO_4 were dissolved in water and brought to a final volume of one liter.
2. Iodine solution (0.02 N). I_2 (0.252 g) and 5 g KI were dissolved in 5 ml water and diluted to 100 ml.
3. 5 N NaOH
4. Alkaline sulfite solution. Na_2SO_3 (2.52 g) was dissolved in 10 ml water and diluted to 100 ml with 5 N NaOH.
5. Acetic acid (5 N). Glacial acetic acid (28.5 ml) was diluted to 100 ml with water.

The following steps were performed in carrying out the assay:

1. The pH of the eluate was adjusted to approximately 6.5 with 5 N K_2CO_3 and made to 50 ml with glass-distilled water. (A sodium base cannot be used to adjust the pH!)

2. One to 3 ml of sample (0.2 to 2 μ g dopamine), 0.5 ml buffer, water to give a total volume of 3.8 ml, and 0.05 ml iodine solution were added to a test tube.
3. After 5 minutes, 0.5 ml alkaline sulfite solution was added.
4. After another 5 minutes, 0.6 ml 5 N acetic acid was added (pH drops to about 5.3).
5. The tubes were heated for 30 minutes at 45°C under standard laboratory lighting conditions (Udenfriend, 1962).
6. Fluorescence was measured with a spectrofluorometer⁹ using an activation wavelength of 345 nm and a fluorescence wavelength of 410 nm.

A standard and a reagent blank were run along with the sample. When tissue extracts were analyzed, an internal standard and a tissue blank were run. The internal standard was an aliquot of tissue extract treated as above, except that a known amount of dopamine was added to check for quenching substances that might be present in the banana extract. The tissue blank was an aliquot of tissue extract treated as above, except that 5 N NaOH replaced the alkaline sulfite solution.

Iodine caused the oxidation of dopamine to dopamine quinone. Rearrangement to 5,6-dihydroxyindole was effected by the alkaline sulfite solution. Fluorescence was increased by lowering the pH with

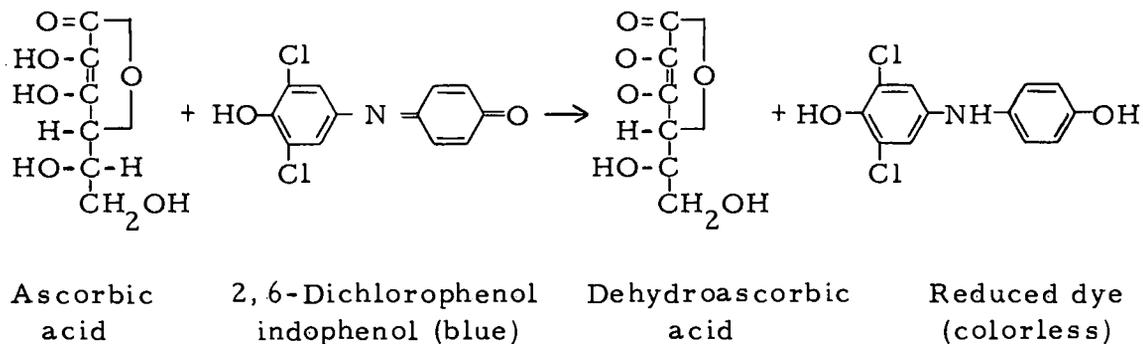
⁹Aminco-Bowman Spectrophotofluorometer, American Instrument Company, Inc., Silver Springs, Maryland 20910.

acetic acid. Concentration of dopamine in an extract of banana was determined by reference to a standard curve (Figure 2). The standard curve was prepared by plotting relative fluorescence against concentration of dopamine. Concentrations of dopamine of 0.05, 0.10, 0.15, 0.20, 0.30 and 0.40 μg per ml were used for the standard curve. A linear relationship was found between relative fluorescence and concentration of dopamine, up to 0.3 μg per ml. Recovery of a known amount of dopamine added to banana prior to extraction was 91 percent.

Ascorbic Acid

Extraction

Ascorbic acid was extracted from the bananas and measured by the method of Loeffler and Ponting (1942) This method was based upon measurement of the extent to which ascorbic acid decolorizes a solution of 2,6-dichlorophenol indophenol in the following reaction given by Bell (1968):



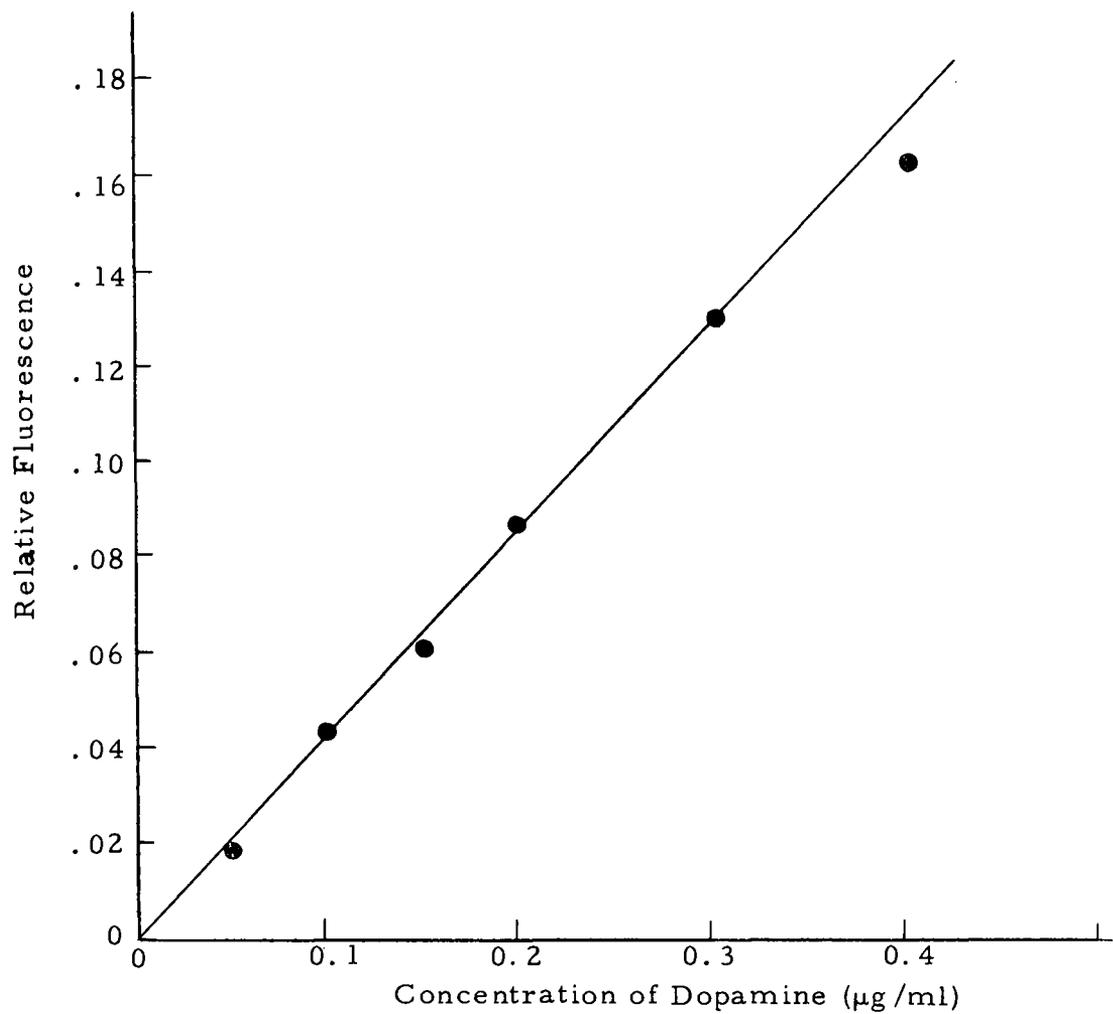


Figure 2. Standard Curve for Dopamine.

Aminco-Bowman Spectrophotofluorometer

Activation wavelength, 345 nm

Fluorescent wavelength, 410 nm

Each slice of banana (approximately 2 g) was placed in a beaker containing 14 ml of 0.04 percent oxalic acid. The mixture was homogenized in a microblender and filtered with suction through Whatman No. 1 filter paper using a Buchner funnel.

Assay

Glass-distilled water was used throughout. The reagents used were prepared in the following manner:

1. Oxalic acid (0.04 percent)
2. Dye solution. 2,6-dichlorophenol indophenol (20 mg) was dissolved in water and diluted to one liter. New solution was made weekly. The dye was stored in a dark bottle under refrigeration and diluted 1:10 before use.
3. Standard ascorbic acid solution. Ascorbic acid (40 mg) was dissolved and diluted to 100 ml with 0.04 percent oxalic acid. The ascorbic acid solution (1 ml = 0.4 g) was used immediately.

The procedure used in the assay was as follows:

1. Determination of K value (mg of ascorbic acid per 100 g of banana tissue represented by each unit of the scale of the Klett colorimeter).
 - a) Three ml of stock ascorbic acid solution were added to each of three 100-ml volumetric flasks. The aliquots were diluted to mark with 0.4 percent oxalic acid to give a

concentration of 1.20 mg per 100 ml. These solutions were the working standards.

- b) The instrument¹⁰ was zeroed with a tube of redistilled water. A green filter was used.
- c) In another tube¹¹, 1 ml of 0.4 percent oxalic acid was pipetted. Seven ml of dye were added. The tube was inverted to mix, and the color was read after 30 seconds (G_1).
- d) One ml of each working standard was pipetted into a tube. To each tube, 7 ml of dye were added. The tubes were inverted and read after 30 seconds (G_2).
- e) The K value was then calculated:

$$K = \frac{\text{mg ascorbic acid per 100 ml}}{G_1 - G_2}$$

2. Colorimetric determination of ascorbic acid in the extract from banana tissue.

- a) The instrument was zeroed with a turbidity blank (1 ml of filtrate and 7 ml water).
- b) One ml of filtrate was pipetted into a tube. Seven ml of dye were added. The contents of the tube were mixed and the

¹⁰ Klett-Summerson Colorimeter, Klett Mfg. Co., 179 E 87th Street, New York 10028.

¹¹ Matched tubes were used.

color was read after 15 and 30 seconds. Ascorbic acid reduces the dye almost instantaneously, and interfering substances presumably reduce the dye slowly. To correct for the presence of the latter, the decrease in intensity of the color between 15 and 30 seconds ($G_{15} - G_{30}$) can be added to the reading after 15 seconds to give a reading (G_3) for the sample which excludes any reduction by interfering substances as represented by the formula:

$$G_3 = G_{15} + (G_{15} - G_{30}) .$$

- c) Calculations of ascorbic acid in the bananas were made as follows:

$$K(G_1 - G_3) \left(\frac{\text{weight of acid} + \% \text{ moisture} \times \text{weight sample}}{\text{weight of sample}} \right) .$$

Histochemical Localization of Dopamine

The technique used was that of Reeve (1959) which involved the following steps:

1. Slices of banana tissue were quartered and fixed in 10 percent formalin containing 2 percent ferrous sulfate. The tissue was aspirated and held 48 hours.
2. The tissue was dehydrated by placing it in successively higher concentrations of tertiary butyl alcohol.

3. Infiltration with paraffin, embedding, and sectioning followed.
4. The sections were affixed to slides with Haupt's reagent with the aid of a drop of 4 percent formalin solution.
5. Paraffin was removed with xylene and the tissue was counter-stained with fast green. The cover slip was mounted with Canada balsam dissolved in xylene.

A blue-black precipitate indicates the presence of tannins. As this test is not specific for dopamine, Reeve's test (1951) for catechol-related tannins was performed for confirmation of results. This test is based upon the fact that phenols react with nitrous acid to form a cherry-red color. Fresh frozen sections were passed in succession through equal volumes of:

- a) 10 percent sodium nitrite
- b) 20 percent urea, and
- c) 10 percent acetic acid, followed, after three minutes, by two volumes of
- d) 2 N NaOH.

Histochemical Localization of Polyphenol Oxidase

Pearse's method (1953) for dopa-oxidase was adapted to localize polyphenol oxidase. Blackish-brown granules indicate the presence of dopamine oxidase. Reagents used were prepared as follows:

1. Phosphate buffer (0.1 M, pH 7.4). $K_2HPO_4 \cdot 3 H_2O$ (1.522 g)

and 0.454 g KH_2PO_4 were dissolved in redistilled water and diluted to 100 ml.

2. Dopamine solution (0.0056 M). 3,4-dihydroxyphenylethylamine (106 mg) was dissolved in buffer and diluted to 100 ml.

The following technique was employed:

1. Slices of banana were quartered and fixed in 10 percent formalin for one hour at 22°C .
2. This tissue was then washed in running water for three minutes.
3. The tissue was incubated in the solution of dopamine for one hour at 37°C . This solution was replaced by fresh solution and the incubation was continued for 12 to 15 hours.
4. The solution was washed from the tissue with running water.
5. The tissue was fixed in FAA (formalin, acetic acid and alcohol).
6. Dehydration was achieved by passing the tissue through a series of tertiary butyl alcohol.
7. After the tissue was embedded in paraffin, sections were cut.
8. The tissue was affixed to slides, the paraffin was cleared with xylene, and the coverslips were mounted with Canada balsam in xylene.

In addition to localizing the enzymes in fixed tissue, Gurr's method (1960) which utilizes fresh tissue was used so that development of melanin granules could be observed under the microscope. The development of blackish-brown granules was observed in fresh tissue

to rule out the possibility of granules in the fixed tissue being artifacts.

RESULTS AND DISCUSSION

Browning

The initial percent transmittance of filtrates of each banana as purchased and after either two or four days of additional ripening is given in Table 1. The filtrates were browner, i. e., the percent transmittance was less, in those extracts from bananas which had ripened for an additional two days as compared to those analyzed as purchased and an even greater increase in browning was found for those bananas analyzed after an additional four days of ripening. The increase in browning associated with ripening was highly significant, as shown by a two-way analysis of variance (Table 2). Measurement of the percent transmittance in filtrates of banana treated with thiourea to block any browning that might occur during maceration of the tissue verified the fact that the increase in browning with ripening occurred in the intact tissue.

The decrease in percent transmittance, i. e., the increase in browning, upon standing for 30 minutes for the filtrates of bananas analyzed as purchased and after either two or four days of additional ripening is given in Table 3. This is a measure of the susceptibility of the fruit to discoloration as a result of ripening. Although bananas increased in susceptibility to discoloration with ripening, the difference between bananas ripened for four days and those ripened for two days was not significant (Table 2).

Table 1. Percent Transmittance of Filtrates of Ripening Bananas.

Hand	Banana	Subsample	Days of Ripening		
			None	Two	Four
I	1	1	95.0	88.0	
		2	94.0	89.0	
	2	1	94.0	92.0	
		2	96.5	91.5	
II	4	1	74.5	72.0	
		2	75.0	69.0	
III	7	1	74.5	73.0	
		2	78.0	70.0	
	8	1	73.5	70.0	
		2	78.0	64.0	
I	3	1	99.0		72.5
		2	94.0		74.0
II	5	1	71.0		53.0
		2	64.0		50.0
	6	1	65.0		53.0
		2	65.0		50.0
III	9	1	72.0		62.0
		2	72.5		62.0
	10	1	76.0		57.0
		2	76.0		59.0

Table 2. Analysis of Variance of Browning, Moisture, Protein, Activity of Polyphenol Oxidase, Dopamine, and Ascorbic Acid in Ripening Bananas.

Variable	Source	Degrees of Freedom	F-test*
Initial percent transmittance	Hands	2	1.09
	Ripening	1	37.22
Change in percent transmittance over 30 minutes	Hands	2	0.83
	Ripening	1	1.03
Moisture	Hands	2	2.21
	Ripening	1	0.48
Protein (mg/g dry weight banana)	Hands	2	3.05
	Ripening	1	14.43
Polyphenol Oxidase (units of activity/ mg protein)	Hands	2	8.55
	Ripening	1	8.02
Polyphenol Oxidase (units of activity/ g dry weight banana)	Hands	2	4.65
	Ripening	1	1.06
Dopamine (μ g/g dry weight banana)	Hands	2	2.66
	Ripening	1	7.23
Ascorbic Acid (mg/100 g dry weight banana)	Hands	2	0.11
	Ripening	1	4.08

*	Degrees of Freedom	Random Error	F Values
95%	2	16	> 3.63
	1	16	> 4.49
99%	2	16	> 8.53
	1	16	> 6.23

Table 3. Change in Percent Transmittance over 30 Minutes in Filtrates of Ripening Bananas.

Hand	Banana	Subsample	Days of Ripening		
			None	Two	Four
I	1	1	1.5	2.5	
		2	2.0	2.0	
	2	1	1.5	1.5	
		2	3.0	2.0	
II	4	1	1.0	3.0	
		2	2.0	5.0	
III	7	1	3.5	3.0	
		2	4.5	4.5	
	8	1	2.5	5.0	
		2	2.0	5.0	
Average				3.4	
I	3	1	1.0		6.0
		2	1.5		2.5
II	5	1	2.0		4.0
		2	1.5		3.5
	6	1	1.5		4.0
		2	2.0		6.0
III	9	1	0.5		3.0
		2	5.0		5.0
	10	1	1.5		3.0
		2	2.0		3.0
Average			2.1		4.0

The effect of ripening on initial percent transmittance as well as on the change in percent transmittance upon standing for 30 minutes is shown graphically in Appendix Figures 4 and 5.

Moisture

Moisture content of the bananas is given in Table 4. The average percent moisture in bananas analyzed as purchased was 75.2, for bananas analyzed after two days of ripening 75.6, and for those after four days of ripening 76.3. The difference in moisture content between bananas analyzed after two days and after four days of additional ripening was not significant according to the analysis of variance (Table 2). The change in moisture in bananas held for two days varied widely, the average increase being 0.8 percent which was not significant (Table 5). However, the increase in moisture content of bananas held for four days of additional ripening averaged 2.0 percent which was significant at the 1 percent level (Table 5). Nandi (1972) and von Loesecke (1949) also reported an increase in moisture in bananas as they ripened. Von Loesecke attributed this increase to a withdrawal of water from the peel.

Protein

Protein content of the bananas is given in Table 6. The protein content averaged 8.4, 9.7, and 12.0 mg/g banana on a fresh weight

Table 4. Percentage of Moisture in Ripening Bananas.^a

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
I	1	73.9	73.3		-0.9
	2	74.0	74.1		+0.2
II	4	76.7	78.4		+2.2
III	7	74.4	76.8		+3.2
	8	75.8	75.4		-0.6
Average			75.6		+0.8
I	3	73.5		74.0	+0.7
II	5	76.5		78.5	+2.6
	6	77.1		78.6	+1.9
III	9	74.6		77.5	+3.9
	10	75.5		76.0	+0.7
Average		75.2		76.3	+2.0

^aAverage of two subsamples.

Table 5. t-test for Percent Moisture in Ripening Bananas.

Days of Ripening	Degrees of Freedom	t-value*
2	9	-1.11
4	9	-5.28

* t-value must be > 2.26 or < -2.26 at 95% level and > 3.25 or < -3.25 at 99% level.

Table 6. Protein Content of Ripening Bananas.^a

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
<u>mg/g Banana (fresh weight)</u>					
I	1	11.6	13.5		+16.4
	2	10.1	14.5		+43.6
II	4	9.0	7.8		-13.4
III	7	6.1	7.3		+19.0
	8	5.1	5.7		+12.9
Average			9.7		+15.7
I	3	12.1		17.4	+43.8
II	5	10.2		11.0	+7.8
	6	8.9		12.4	+39.8
III	9	4.9		9.2	+89.1
	10	6.0		9.9	+65.9
Average		8.4		12.0	+49.3
<u>mg/g Banana (dry weight)</u>					
I	1	44.5	50.5		+13.5
	2	38.7	56.2		+45.2
II	4	38.4	35.9		-6.5
III	7	23.9	31.3		+31.0
	8	20.9	23.1		+10.5
Average			39.4		+18.7
I	3	45.6		66.7	+46.3
II	5	43.4		51.2	+18.0
	6	38.8		57.8	+49.0
III	9	19.2		41.0	+113.5
	10	24.3		41.2	+69.5
Average		33.8		51.6	+59.2

^aAverage of two readings on each of two subsamples.

basis for bananas analyzed as purchased and after two and four days of additional ripening, respectively. On a dry weight basis, the corresponding figures are 33.8, 39.4, and 51.6 mg/g. The protein in bananas held for two days increased by an average of 15.7 percent on a fresh weight basis of banana or 18.9 percent on a dry weight basis. The protein in bananas held for four days increased by an average of 49.3 percent on a fresh weight basis of banana or 59.2 percent on a dry weight basis. The increase in protein in bananas ripened for four days as compared to those ripened for two days was significant at the 1 percent level (Table 2).

Polyphenol Oxidase

Activity of polyphenol oxidase in bananas as expressed three ways, units of activity per mg protein, per g fresh weight of banana, and per g dry weight of banana, is given in Table 7. The specific activity of the enzyme (units of activity/mg protein) averaged 6.4, 5.2, and 4.1 for bananas analyzed as purchased, after two days of additional ripening, and after four days of additional ripening, respectively. Specific activity of the polyphenol oxidase decreased with ripening in each banana, but it must be kept in mind that protein increased with ripening. The decrease in specific activity in those bananas analyzed two days apart averaged 15.6 percent and for those analyzed four days apart averaged 31.0 percent. The difference in the

Table 7. Activity of Polyphenol Oxidase in Ripening Bananas. ^a

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
<u>Units of activity^b /mg protein</u>					
I	1	4.1	3.7		- 9.5
	2	4.2	3.6		- 1.4
II	4	5.9	4.9		-17.7
III	7	9.3	5.3		-43.3
	8	9.0	8.4		- 6.2
Average			5.2		-15.6
I	3	3.5		3.0	-15.3
II	5	3.9		3.2	-17.5
	6	5.5		2.6	-51.8
III	9	10.3		6.2	-39.7
	10	7.8		5.4	-30.8
Average		6.4		4.1	-31.0
<u>Units of activity/g banana (fresh weight)</u>					
I	1	47.7	49.6		+ 4.0
	2	42.4	52.9		+24.8
II	4	53.7	37.6		-30.0
III	7	56.4	38.3		-32.1
	8	44.8	46.4		+ 3.4
Average			45.0		- 6.0
I	3	42.5		51.2	+20.5
II	5	39.6		35.1	-11.4
	6	44.9		32.5	-27.6
III	9	49.7		57.0	+14.7
	10	46.5		50.7	+ 9.0
Average		46.8		45.3	+ 1.1

(Continued on next page)

Table 7. (Continued)

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
<u>Units of activity/g banana (dry weight)</u>					
I	1	183	186		+ 1.6
	2	163	204		+25.2
II	4	230	174		+24.3
III	7	220	165		-25.0
	8	185	188		+ 1.6
Average			183		- 4.2
I	3	160		197	+23.1
II	5	169		163	- 3.6
	6	196		152	-22.4
III	9	196		254	+29.6
	10	190		211	+11.1
Average		189		195	+ 7.6

^a Average of at least two recordings on each of two subsamples.

^b Units of activity were calculated as $\Delta OD/\Delta t$ from the initial slope of the reaction curve for the enzymatic oxidation of dopamine.

mean changes between bananas analyzed two days apart and those analyzed four days apart was significant at the 1 percent level (Table 2). The difference in specific activity due to hand was also significant at the 1 percent level (Table 2).

While it is customary to report the activity of an enzyme in terms of specific activity, the protein content of bananas increased with ripening. Therefore, activity of polyphenol oxidase was also calculated and reported as units of activity/g of both fresh and dry weight of banana. The activity of the enzyme averaged 46.8, 45.0, and 45.3 units of activity/g of banana on a fresh weight basis for bananas analyzed as purchased and after two and four days of additional ripening, respectively. On a dry weight basis, the corresponding figures are 189, 183, and 195 units of activity/g. On this basis, the decrease in activity of polyphenol oxidase with ripening was not significant, but the difference in activity due to hand was significant at the 5 percent level (Table 2). Nandi (1972) reported a decrease in enzyme activity with ripening of bananas. However, Palmer (1963) recommended that a final dopamine concentration of 5×10^{-3} M be used in the assay of polyphenol oxidase. This would require a stock solution of dopamine of 2.84 mg/ml. Nandi used a solution with a concentration of 0.2 mg/ml which may have caused the substrate to be a limiting factor in her assay.

Dopamine

Dopamine content of the bananas is given in Table 8. The dopamine content averaged 58.1, 43.5, and 24.5 $\mu\text{g/g}$ banana on a fresh weight basis for bananas analyzed as purchased and after two and four days of additional ripening, respectively. On a dry weight basis, the corresponding figures are 236, 175, and 106 $\mu\text{g/g}$. The decrease in dopamine content averaged 2.6 and 61.7 percent for bananas ripened two days and four days, respectively, calculated on a fresh weight basis. Corresponding decreases on a dry weight basis were less than 1 percent and 52.1 percent. The decrease in the dopamine content after four days of ripening was significantly different (1 percent level) from the decrease after two days of ripening (Table 2). Reeve (1959) and Craft (1961) previously reported a decrease in catechol derivatives in apples upon ripening and Barnell and Barnell (1945) reported a decrease in what they referred to as tannins which they observed histologically in ripening bananas.

Ascorbic Acid

Concentration of ascorbic acid in the bananas is given in Table 9. The content of ascorbic acid averaged 5.0, 2.7, and 3.2 mg/100 g banana on a fresh weight basis for bananas analyzed as purchased and after two and four days of additional ripening, respectively.

Table 8. Dopamine (3,4-dihydroxyphenylethylamine) Content of Ripening Bananas. ^a

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
<u>µg/g Banana (fresh weight)</u>					
I	1	60.2	63.1		+ 4.8
	2	31.9	61.8		+93.7
II	4	49.3	37.7		-23.5
III	7	38.2	29.6		-22.5
	8	73.1	25.1		-65.7
Average			43.5		- 2.6
I	3	66.9		28.2	-57.8
II	5	75.8		17.5	-76.9
	6	69.2		18.9	-72.7
III	9	63.4		33.1	-47.8
	10	52.7		24.6	-53.3
Average		58.1		24.5	-61.7
<u>µg/g Banana (dry weight)</u>					
I	1	231	232		+ 0.4
	2	122	239		+95.9
II	4	212	175		-17.5
III	7	149	128		-14.1
	8	302	102		-66.2
Average			175		- 0.3
I	3	253		108	-57.3
II	5	322		82	-74.7
	6	302		88	-70.9
III	9	250		147	-41.2
	10	215		103	-52.1
Average		236		106	-52.1

^aAverage of two readings on each of two subsamples.

Table 9. Ascorbic Acid Content of Ripening Bananas. ^a

Hand	Banana	Days of Ripening			% Change with Ripening
		None	Two	Four	
<u>mg/100 g Banana (fresh weight)</u>					
I	1	4.1	1.7		-58.9
	2	6.8	5.5		-18.8
II	4	6.1	3.4		-44.7
III	7	4.7	1.5		-69.3
	8	5.1	1.3		-74.7
Average			2.7		-53.3
I	3	8.2		4.8	-41.2
II	5	2.4		2.0	-15.5
	6	4.5		1.7	-62.3
III	9	5.0		5.0	- 0.4
	10	3.3		2.8	-15.0
Average		5.0		3.2	-26.9
<u>mg/100 g Banana (dry weight)</u>					
I	1	15.9	6.4		-59.9
	2	26.2	21.4		-18.3
II	4	26.1	15.6		-40.2
III	7	18.4	6.2		-66.1
	8	21.1	5.3		-75.1
Average			11.0		-51.9
I	3	30.8		18.4	-40.2
II	5	9.5		9.4	- 1.4
	6	19.6		7.9	-59.9
III	9	19.7		22.1	+14.5
	10	13.3		11.6	-12.8
Average		20.1		13.9	-20.0

^aAverage of two readings on each of two subsamples.

Corresponding figures on a dry weight basis are 20.1, 11.0, and 13.9 mg/100 g. The decrease due to two days of additional ripening averaged 53.3 percent on a fresh weight basis (52.9 percent dry weight). After four days of additional ripening, the decrease in ascorbic acid was only 26.9 percent on a fresh weight basis (20.0 percent dry weight). This decrease was significantly less (5 percent level) than that observed in bananas held to ripen for an additional two days (Table 2). It must be kept in mind that analyses were done on different bananas and biological variability may have resulted in slower metabolism of ascorbic acid in those particular bananas that were held for four days of additional ripening compared to those held for two days. An unlikely alternative is that ascorbic acid may decrease and later increase in ripening bananas. Harris and Poland (1939) reported a decrease in ascorbic acid in bananas as they matured from ripe to overripe.

The data on ripening bananas presented thus far show the extent of browning and the quantitative changes in the factors which might be involved. To determine whether interrelationships between the variables studied were significant, the Student-Fischer's t-test for zero correlation was employed (Table 10). The strongest relationship found was between dopamine content and susceptibility of the bananas to discoloration, i. e., the change in percent transmittance over 30 minutes. The correlation between dopamine content and

Table 10. Relationships between Polyphenol Oxidase, Dopamine, Ascorbic Acid, and Browning.*

	Dopamine ($\mu\text{g/g}$ dry wt. banana)	Ascorbic Acid ($\text{mg}/100$ g dry wt. banana)	Initial % Transmittance	Change in % Transmittance over 30 min.
Polyphenol Oxidase (units of activity/ mg protein)	+0.16	+0.09	-0.08	+0.04
Polyphenol Oxidase (units of activity/ g dry wt. banana)	+0.01	+0.26	-0.07	+0.02
Dopamine ($\mu\text{g}/\text{g}$ dry wt. banana)		+0.30	+0.36	-0.59
Ascorbic Acid ($\text{mg}/100$ g dry wt. banana)			+0.48	-0.41
Initial % Transmittance				-0.50

* Student-Fischer's t-test for zero correlation. Significance at 5 percent level, $r > 0.23$ or < -0.23 .

extent of browning, i. e. , initial percent transmittance of filtrates, was also significant at the 5 percent level. As the content of dopamine decreased with ripening, the initial percent transmittance decreased and the change in percent transmittance over 30 minutes increased. The decrease in dopamine as bananas ripened may have been due to oxidation by polyphenol oxidase, according to the mechanism proposed by Palmer (see p. 19). One intermediate in this oxidation is the red compound, 2,3-dihydroindole-5,6-quinone. Measurements of percent transmittance were made at 475 nm which would have reflected the presence of this compound or the presence of melanin which has a general absorption. If some of the dopamine had been converted to either the red intermediate or to melanin, this could account for both the lower dopamine content and the lower initial percent transmittance with ripening. Moreover, if some of the dopamine had been converted to intermediates closer to the formation of the red quinone or to melanin, this could account for the increase in susceptibility to discoloration over 30 minutes, as more of these compounds could have been formed during this time. The latter appears to have been more predominant because the change in percent transmittance over 30 minutes was more positively correlated to the disappearance of dopamine than was the initial percent transmittance. If much of the dopamine had been converted to melanin during ripening, the rate of browning would have been limited as compared to the initial percent

transmittance. This was the case as shown in Table 2. The increase in browning in the intact fruit with ripening was much greater than was the increase in susceptibility to browning.

Weurman and Swain (1955) claimed that browning was not related to phenolic content in apples. They based their argument on the fact that the content of substrate per apple increased with ripening, and this did not parallel the decrease in extent of browning of the apple filtrate. However, the apples gained weight with ripening, giving a decrease in phenolic content per gram of apple which did parallel the decrease in extent of browning.

DeSwardt et al. (1967) suggested that unpolymerized tannins may inhibit enzymes, thus influencing ripening. In this study, no significant correlation was found between the concentration of dopamine and the activity of polyphenol oxidase (Table 10).

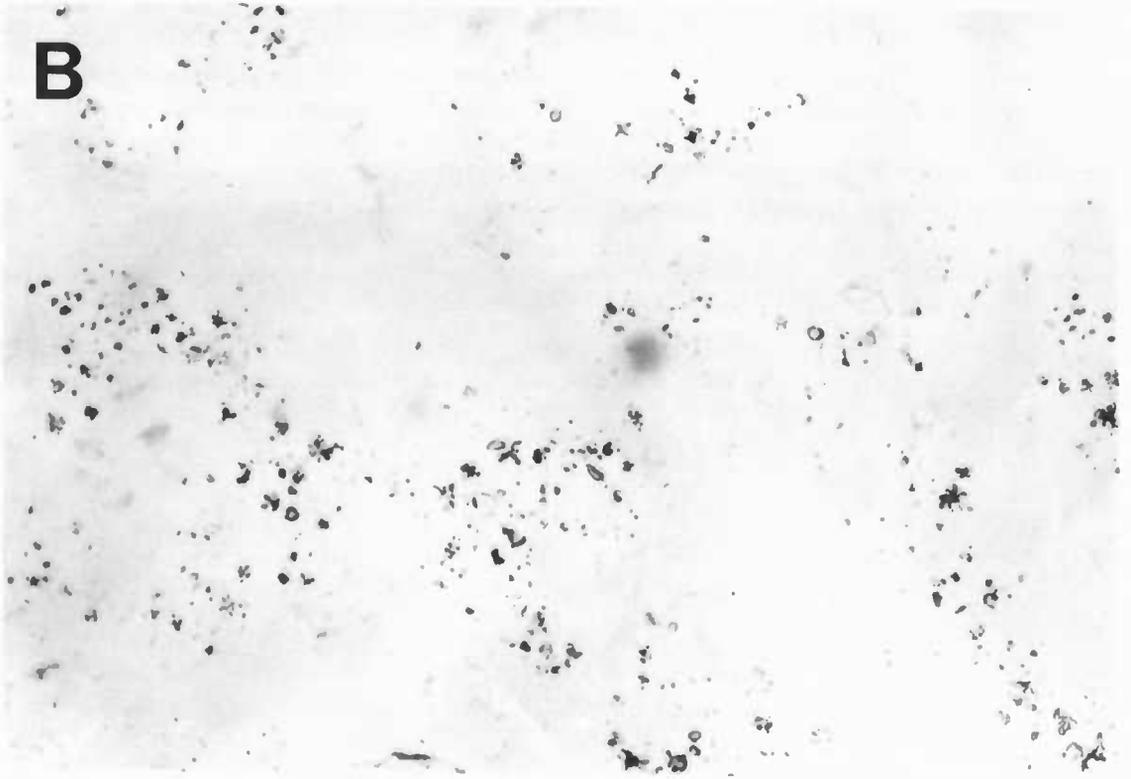
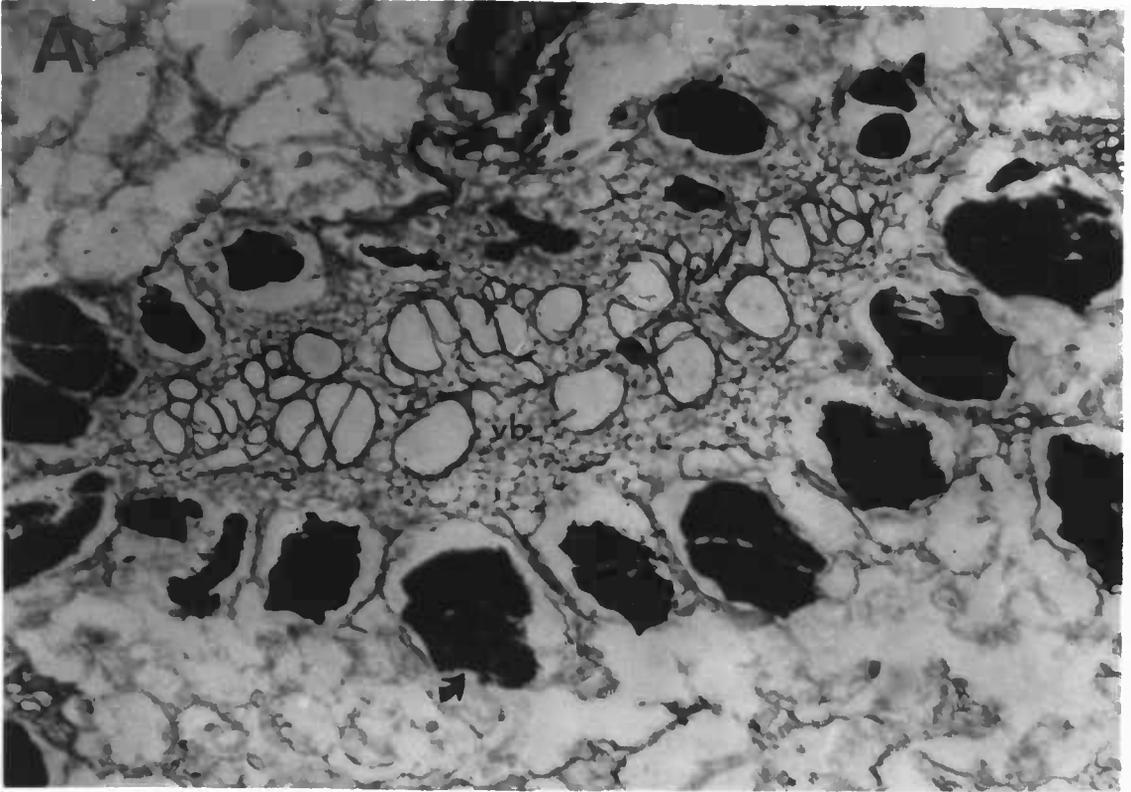
Significant relationships (5 percent level) were found between the concentration of ascorbic acid and both the extent of browning and the susceptibility to discoloration (Table 10). As ascorbic acid decreased with ripening in individual bananas, the rate and extent of browning increased. Palmer (1964) reported that ascorbic acid delayed the enzymatic oxidation of dopamine, the delay being less with decreasing levels of ascorbic acid. The inhibitory effect of ascorbic acid on browning may be due to its reducing capacity, as suggested by Kreuger (1950) who studied its effects on the oxidation of tyrosine and

DOPA. Baruah and Swain (1953), who studied the effects of ascorbic acid on potato polyphenol oxidase, suggested that the effects of ascorbic acid may be due to a direct inhibition of the enzyme. Pierpoint (1966) suggested that ascorbic acid acted both as a reducing agent and as an inhibitor of the enzyme in the oxidation of chlorogenic acid in tobacco leaves. In the present study, the concentration of ascorbic acid was more significantly correlated with the concentration of dopamine than with the activity of the enzyme (Table 10). The specific activity of banana polyphenol oxidase was not significantly related to the concentration of ascorbic acid or to either the extent of browning or the susceptibility to discoloration. This suggests that polyphenol oxidase was not the limiting factor. However, a significant correlation between the concentration of ascorbic acid and the concentration of dopamine was found. As the level of ascorbic acid diminished, so did the dopamine. If the ascorbic acid were oxidized, quinones would accumulate instead of being reduced to dopamine. Because the strongest relationship is between the concentration of dopamine and the susceptibility of ripening bananas to browning and because the concentration of dopamine is significantly related to the concentration of ascorbic acid, the concentration of dopamine as influenced in part by the concentration of reduced ascorbic acid appears to be the limiting factor in the browning of bananas.

While presence of both substrate and enzyme is essential in the susceptibility of a fruit to discoloration, of equal importance may be the location of each and the ability of the enzyme to come in contact with the substrate. Dopamine was located histochemically in a few isolated parenchyma cells and in the vacuoles of the latex vessels, as indicated by an arrow in Figure 3A. This photomicrograph confirms the location of dopamine in banana tissue which Barnell and Barnell (1945) previously illustrated by drawings. Melanin granules produced by polyphenol oxidase appeared to occur in the pulp ubiquitously, as illustrated in Figure 3B. Buckley (1964) reported that polyphenol oxidase is of "general distribution" in the cells of banana roots. Because the melanin granules produced by the action of the enzyme on application of dopamine were so large, it was impossible to determine whether the enzyme was associated with the vacuole, the cytoplasm, or with the cell walls, as Palmer has suggested (1963). Harel et al. (1964, 1965) suggested that polyphenol oxidases in apples are associated with organelles in green fruit but are released to the cytoplasm during ripening. Polyphenol oxidases may also occur in the latex vessels, but at least most of the enzyme and substrate are compartmentalized.

Ripening may be the result of a progressive increase in cell permeability leading to an increased contact between enzymes and substrates already present in tissue, and thereby to an enhanced

- Figure 3. A. Localization of Dopamine (615 X).
 vb = vascular bundle
- B. Localization of Polyphenol Oxidase (2,050 X).



metabolism. Alternately, ripening may be a differentiation process under genetic control involving the de novo synthesis of proteins. Two pertinent studies have been done on bananas. Sacher (1966, 1967) studied permeability in ripening bananas and found that an increase in permeability occurred two days prior to and rose exponentially during the respiratory climacteric of bananas. At the respiratory peak, he found the tissue was 100 percent permeable to mannitol, sucrose, fructose, and chloride. Support for this theory is given by Palmer and McGlasson (1969) who found an increase in respiration after cutting banana tissue. A breakdown of compartmentalization may have caused the increase in respiration. No change in levels of protein or amino acids during the climacteric was reported by Sacher. On the other hand, Brady et al. (1970) reported an increase in the incorporation of amino acids into proteins and a change in the pattern of proteins in bananas undergoing a climacteric rise in respiration as observed by gel electrophoresis.

If Sacher (1966, 1967) is correct in assuming that ripening results from an increase in permeability, banana polyphenol oxidase and dopamine may be allowed to come into contact and, thereby, cause an increase in discoloration in ripening bananas. On the other hand, an increase in protein was observed with ripening in this study which supports the differentiation hypothesis of Brady et al. (1970). Perhaps both of these processes are involved in the ripening of the banana fruit.

SUMMARY

Bananas were analyzed as purchased and after either two or four days of additional ripening for the extent of browning and for their susceptibility to discoloration during a 30-minute holding period. Dopamine (3, 4-dihydroxyphenylethylamine) content, activity of polyphenol oxidase, and ascorbic acid content were measured. Moisture and protein content were determined also. In addition, dopamine and polyphenol oxidase were localized histochemically. The results were as follows:

1. Browning in the filtrates of bananas that had ripened for four days was greater than in the filtrates of bananas that had ripened for two days and the difference was highly significant.
2. Susceptibility to discoloration of bananas upon standing for 30 minutes increased with ripening, but the difference between bananas ripened for two additional days and bananas ripened for four additional days was not significant.
3. Moisture content of bananas analyzed as purchased averaged 75.2 percent. No significant change in moisture was observed in bananas ripened for an additional two days, but a highly significant increase was found in bananas analyzed after four days of additional ripening.
4. The protein content averaged 33.8, 39.4, and 51.6 mg/g banana

- on a dry weight basis for bananas analyzed as purchased, and after either two or four days of additional ripening, respectively and the increase was highly significant.
5. The specific activity of polyphenol oxidase in bananas decreased with ripening and the difference in the change in specific activity between bananas ripened for four additional days and those ripened for two additional days was highly significant. When the change in activity of polyphenol oxidase was calculated on a basis of dry weight of banana, the difference was not significant.
 6. The dopamine content averaged 236, 175, and 106 $\mu\text{g/g}$ banana on a dry weight basis in bananas analyzed as purchased and after two and four days of ripening, respectively, and the decrease was highly significant.
 7. The content of ascorbic acid averaged 20.1, 11.0, and 13.9 mg/100 g banana on a dry weight basis for bananas analyzed as purchased and after two and four days of ripening, respectively. The decrease in ascorbic acid associated with four days of additional ripening was significantly less (5 percent level) than that observed in bananas held to ripen for an additional two days.
 8. As the concentration of dopamine decreased with ripening, the extent of browning and susceptibility to discoloration increased. Dopamine may have been converted to melanin or to the red intermediate, 2,3-dihydroindole-5,6-quinone, or to intermediates

closer to these compounds, thereby resulting in an increase in brownness of the intact fruit and an increase in susceptibility to discoloration upon standing.

9. The increase in brownness and the increase in susceptibility to discoloration with ripening were associated with a decrease of ascorbic acid in the fruit. The decrease in dopamine was also associated with a decrease in ascorbic acid.
10. The specific activity of the polyphenol oxidase did not appear to be the limiting factor for either the brownness of the tissue or for its susceptibility to browning.
11. Dopamine was located histochemically in the vacuoles of the latex vessels and in a few isolated parenchyma cells of bananas.
12. Polyphenol oxidase appeared to occur throughout the pulp.
13. The concentration of dopamine as influenced in part by concentration of ascorbic acid appears to be the limiting factor in the browning of bananas.

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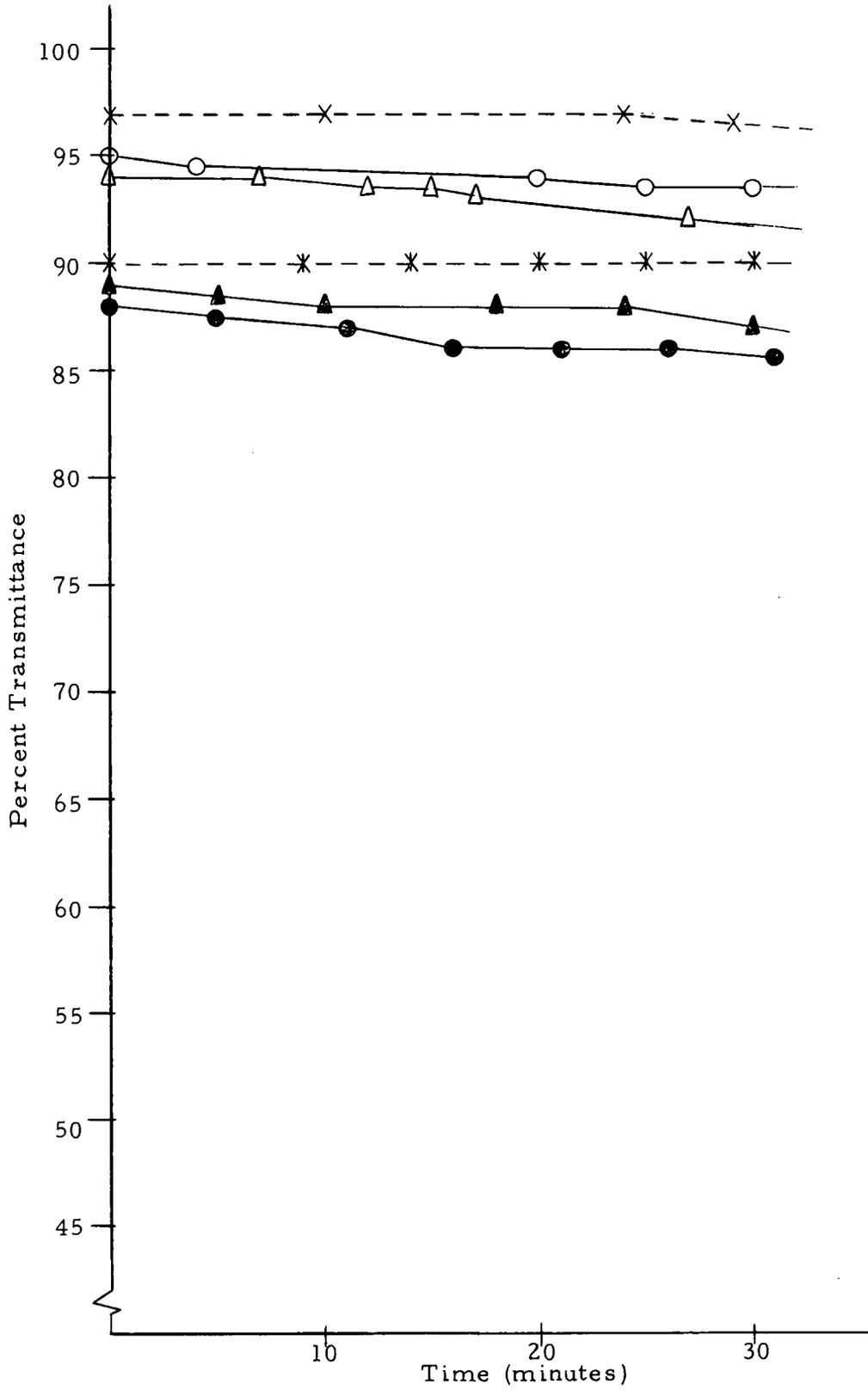
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APPENDIX

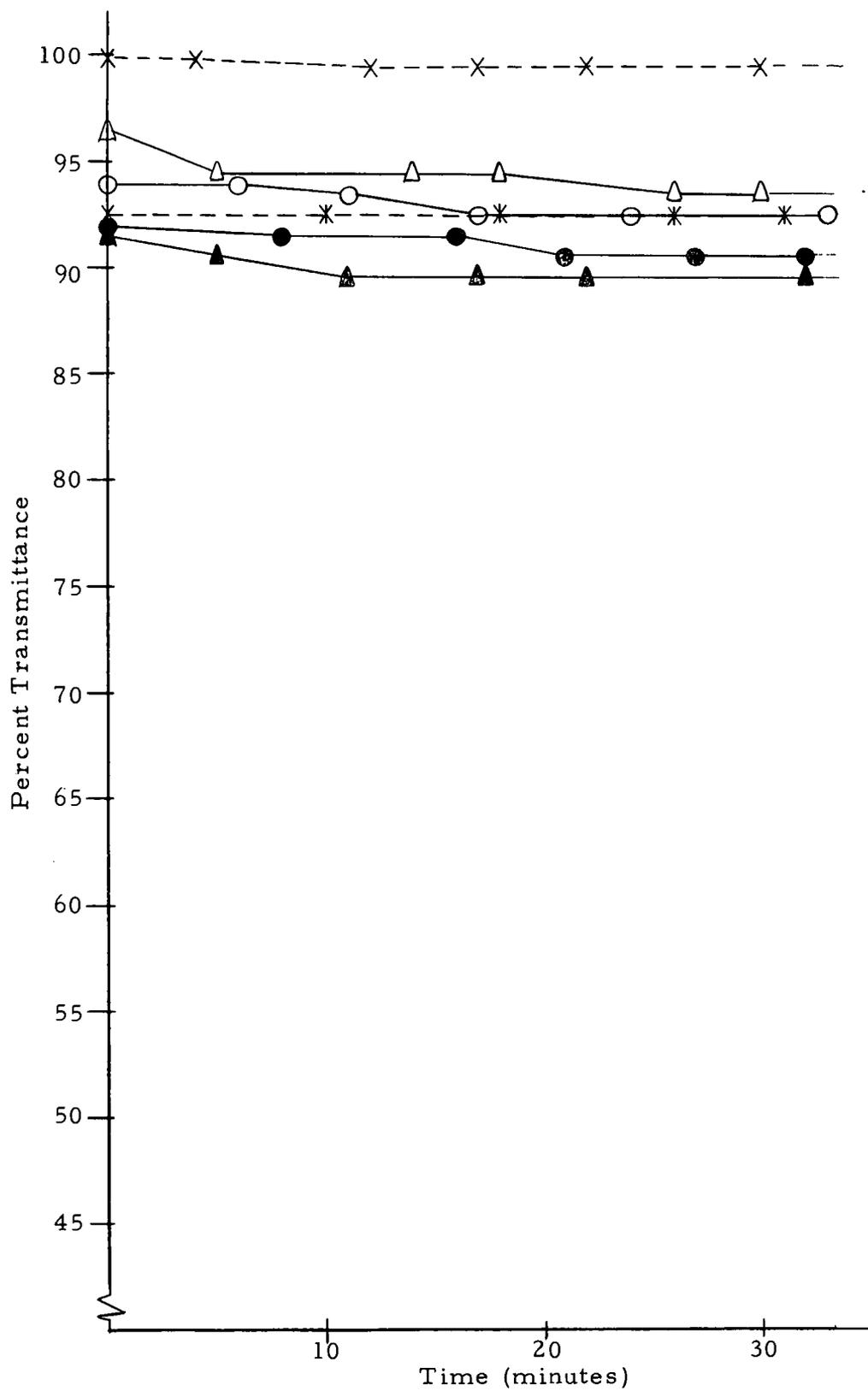
Figure 4A-E. Browning of the Filtrates from Banana Tissue Analyzed as Purchased and after Two Days of Additional Ripening.

Percent transmittance at 475 nm, BL spectrophotometer

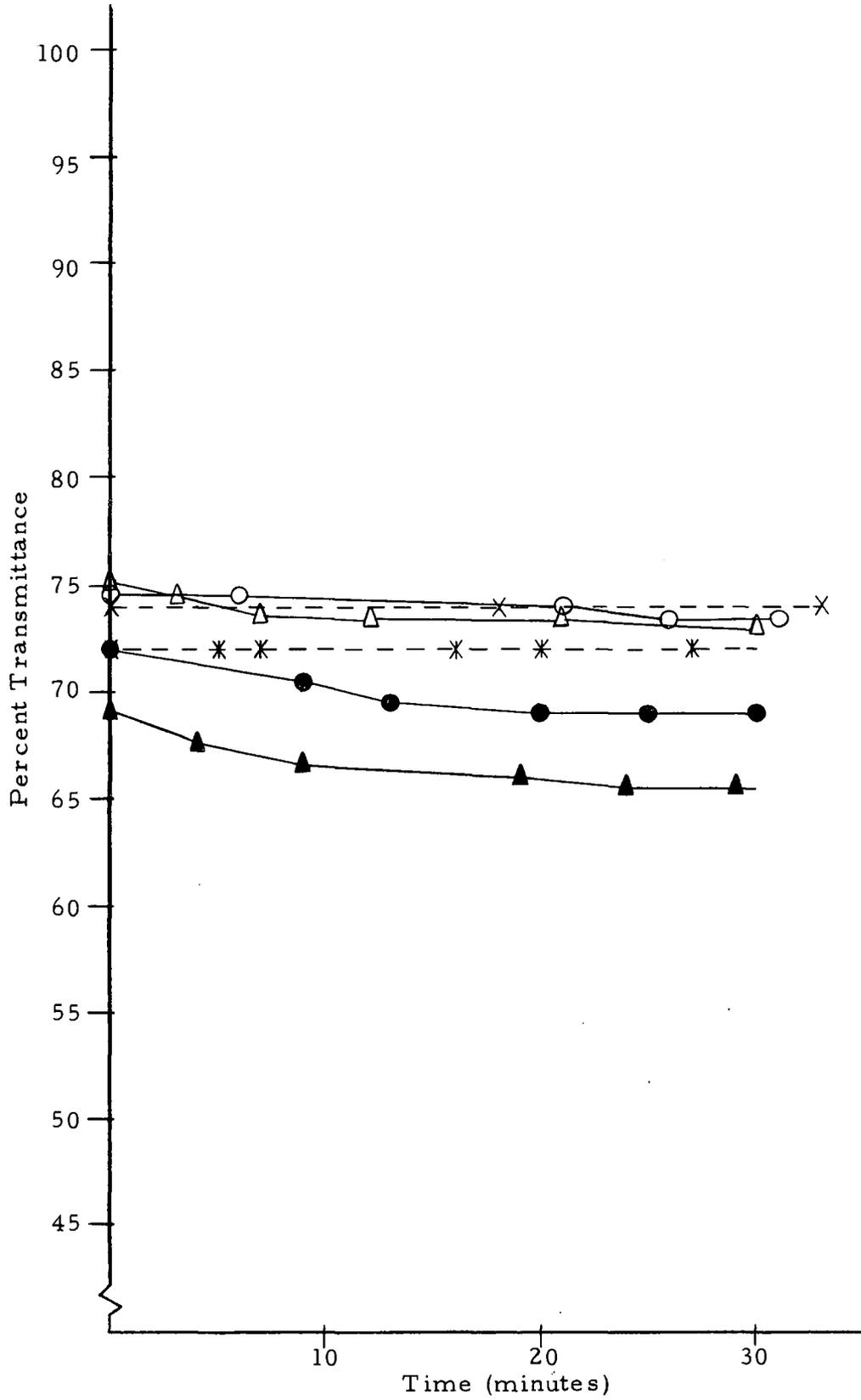
x---x	Control at day 0
---	Control at day 2
o---o	Subsample 1 at day 0
Δ---Δ	Subsample 2 at day 0
●---●	Subsample 1 at day 2
▲---▲	Subsample 2 at day 2



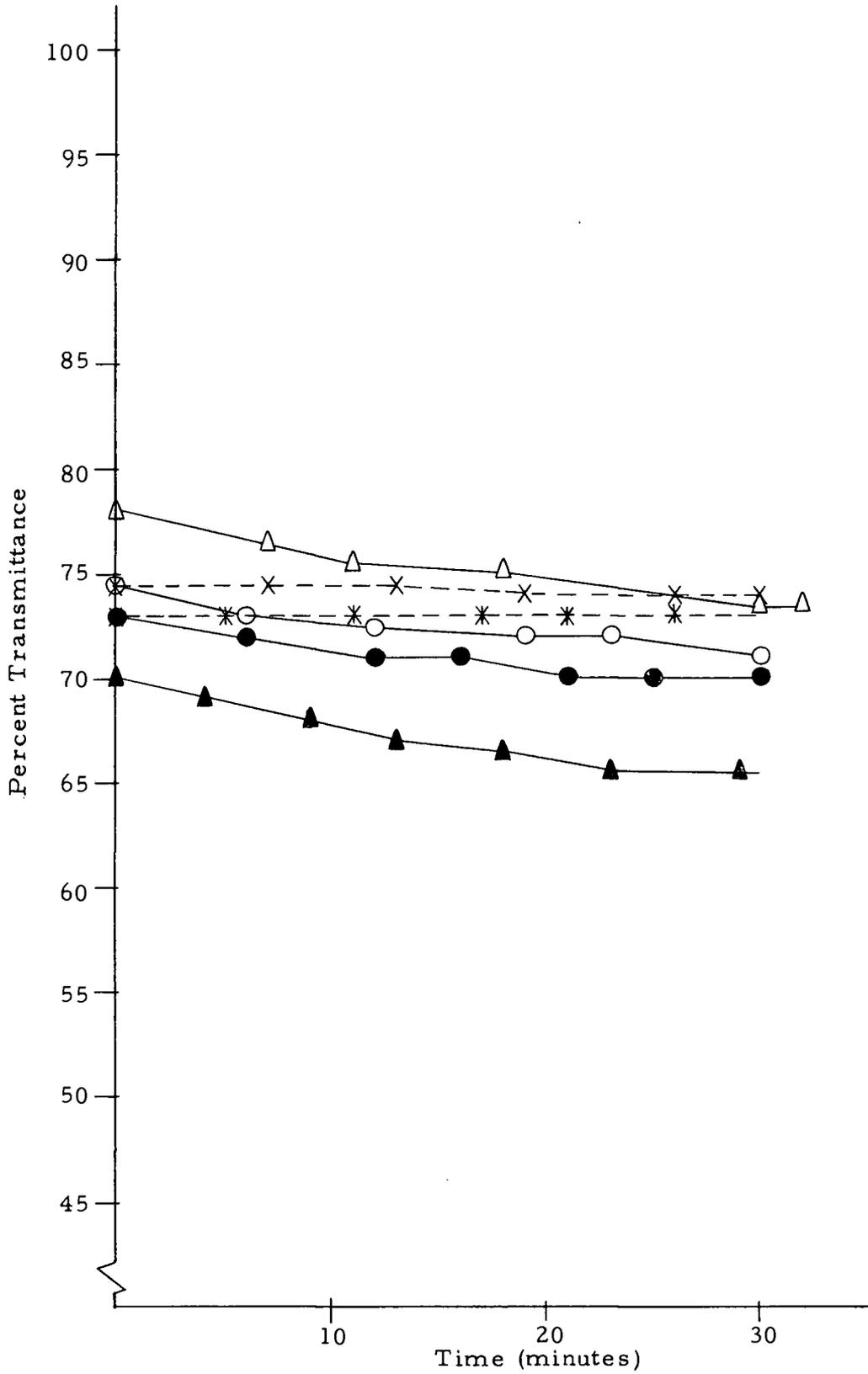
A. Banana 1



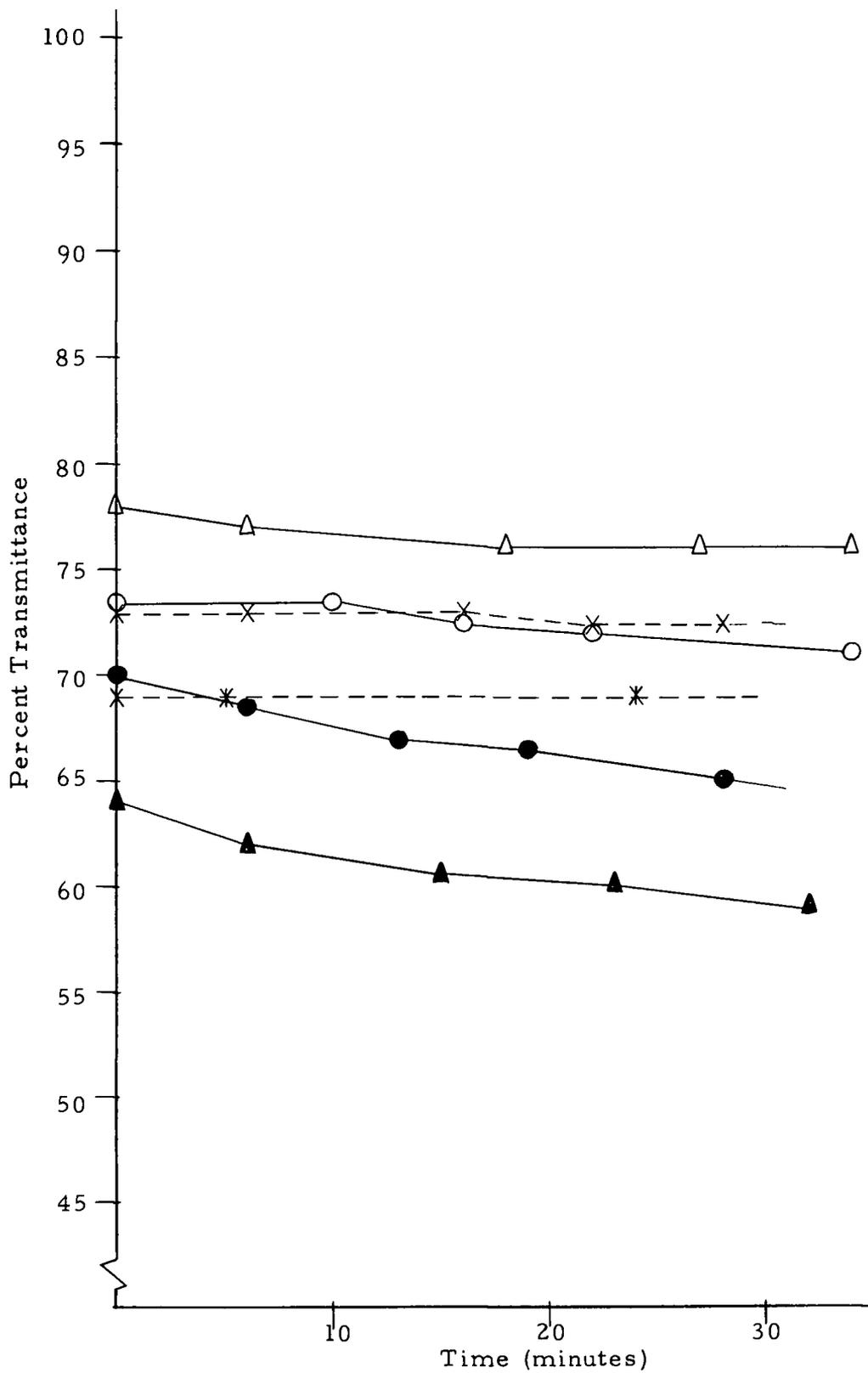
B. Banana 2



C. Banana 4



D. Banana 7

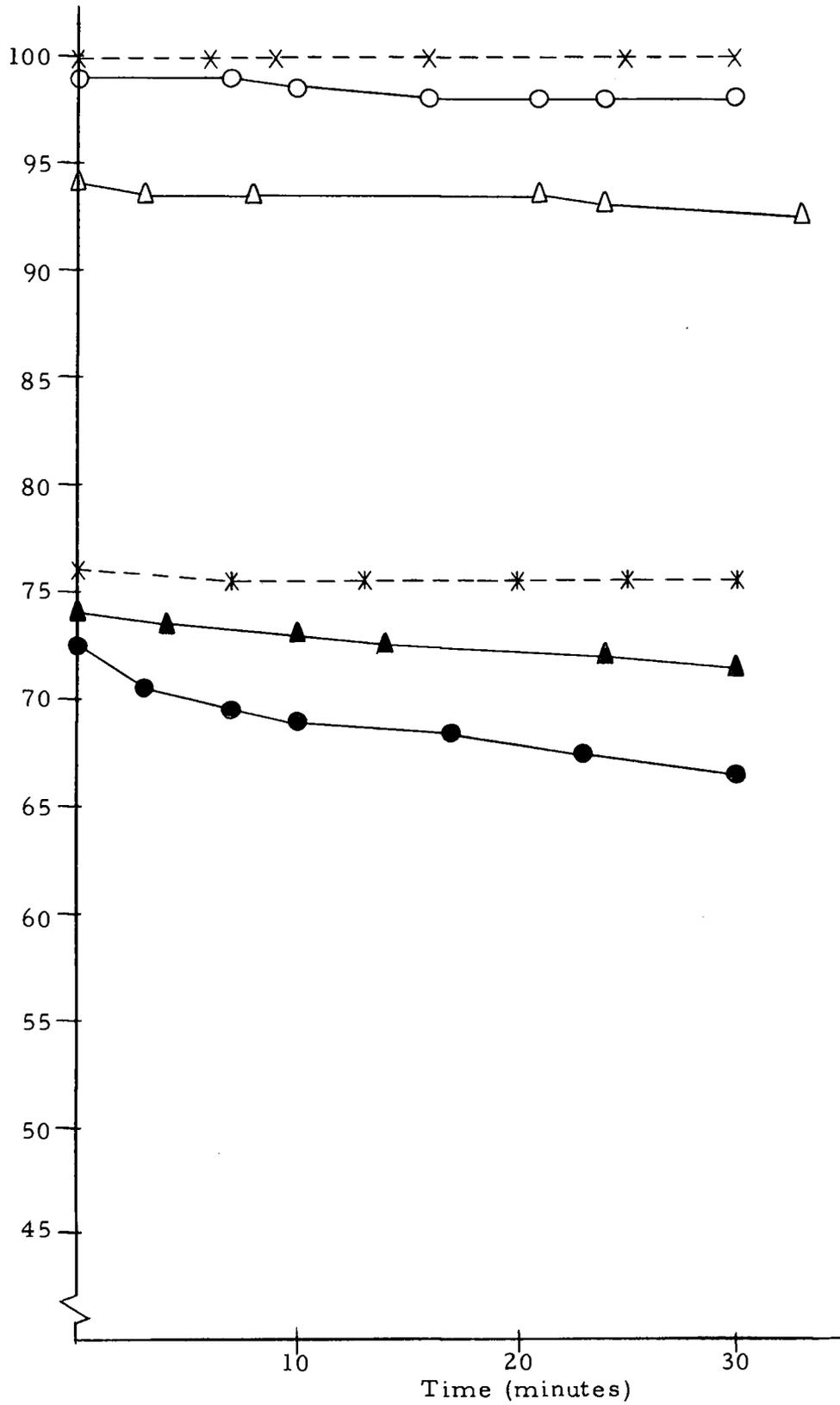


E. Banana 8

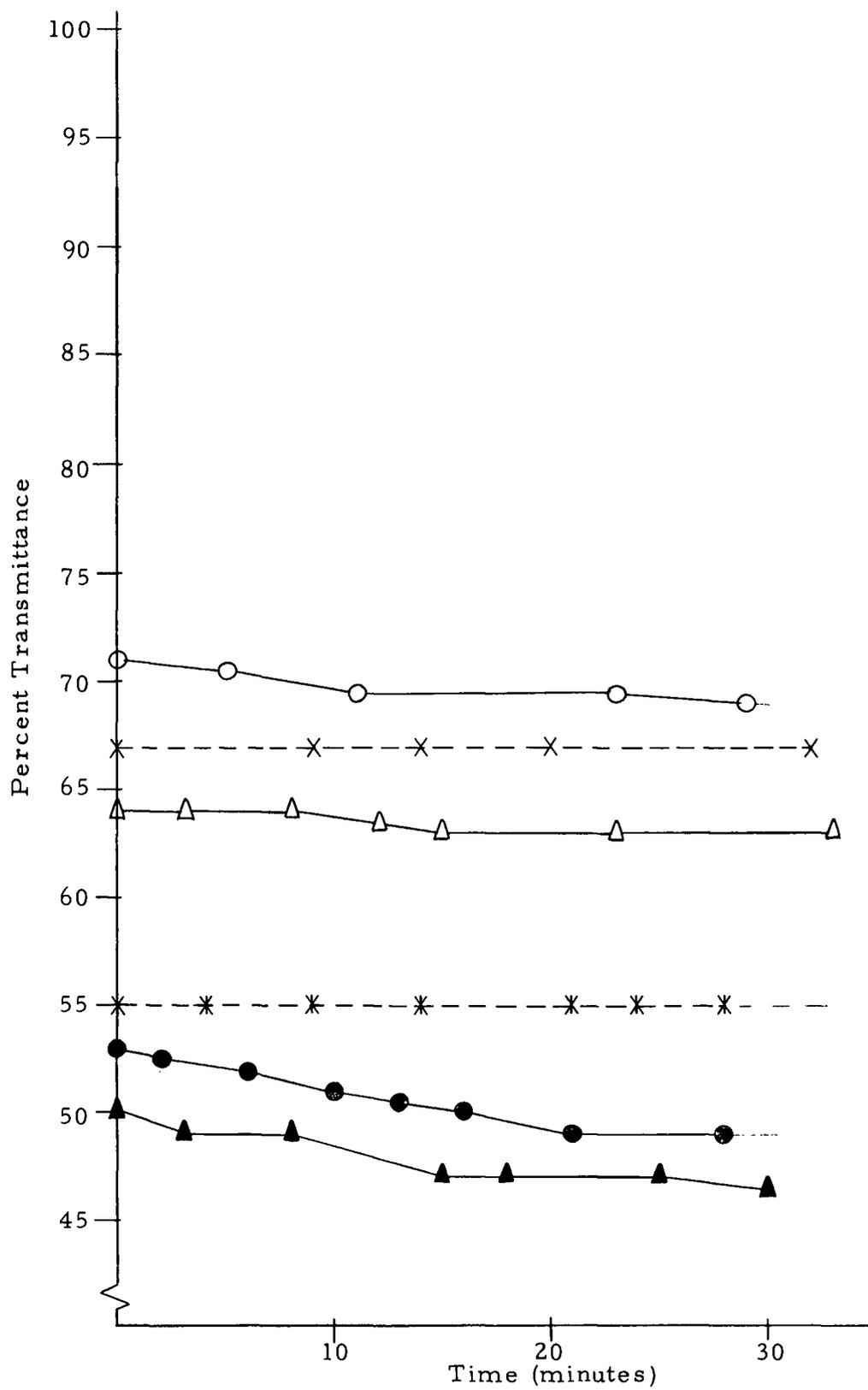
Figure 5A-E. Browning of the Filtrates from Banana Tissue Analyzed as Purchased and after Four Days of Additional Ripening.

Percent transmittance at 475 nm, BL spectrophotometer

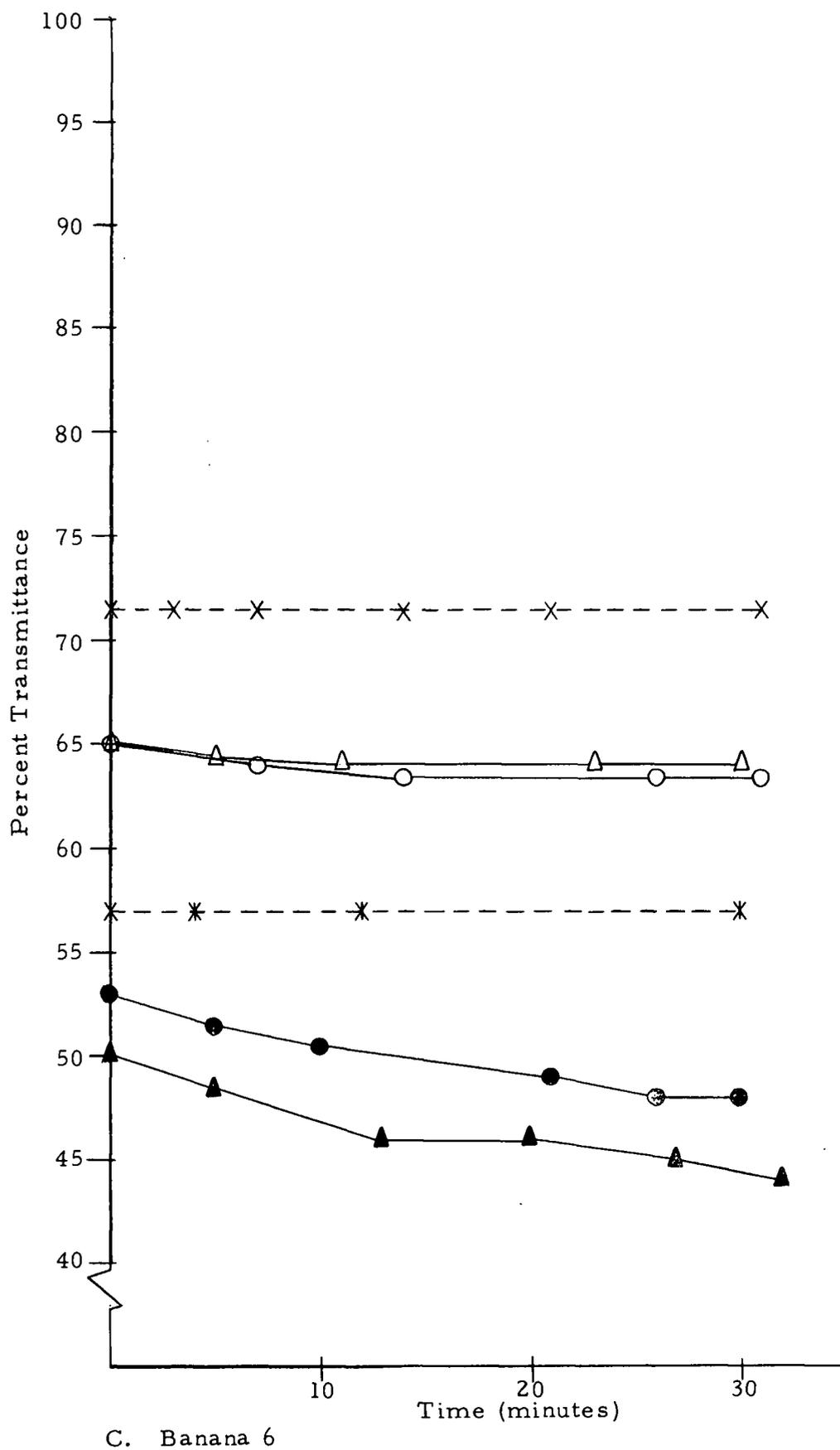
x---x Control at day 0
--- Control at day 4
o---o Subsample 1 at day 0
Δ---Δ Subsample 2 at day 0
●---● Subsample 1 at day 4
▲---▲ Subsample 2 at day 4

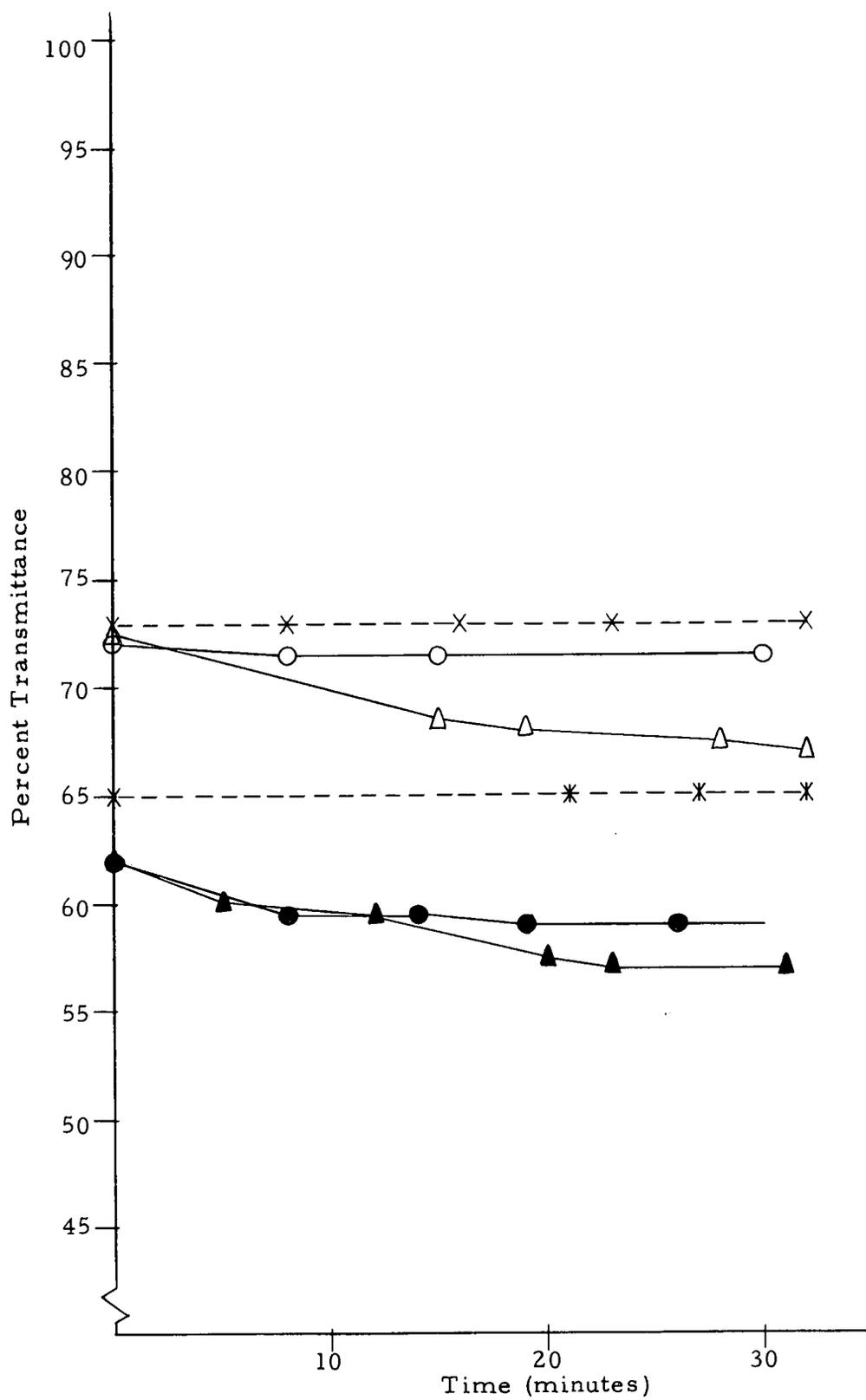


A. Banana 3

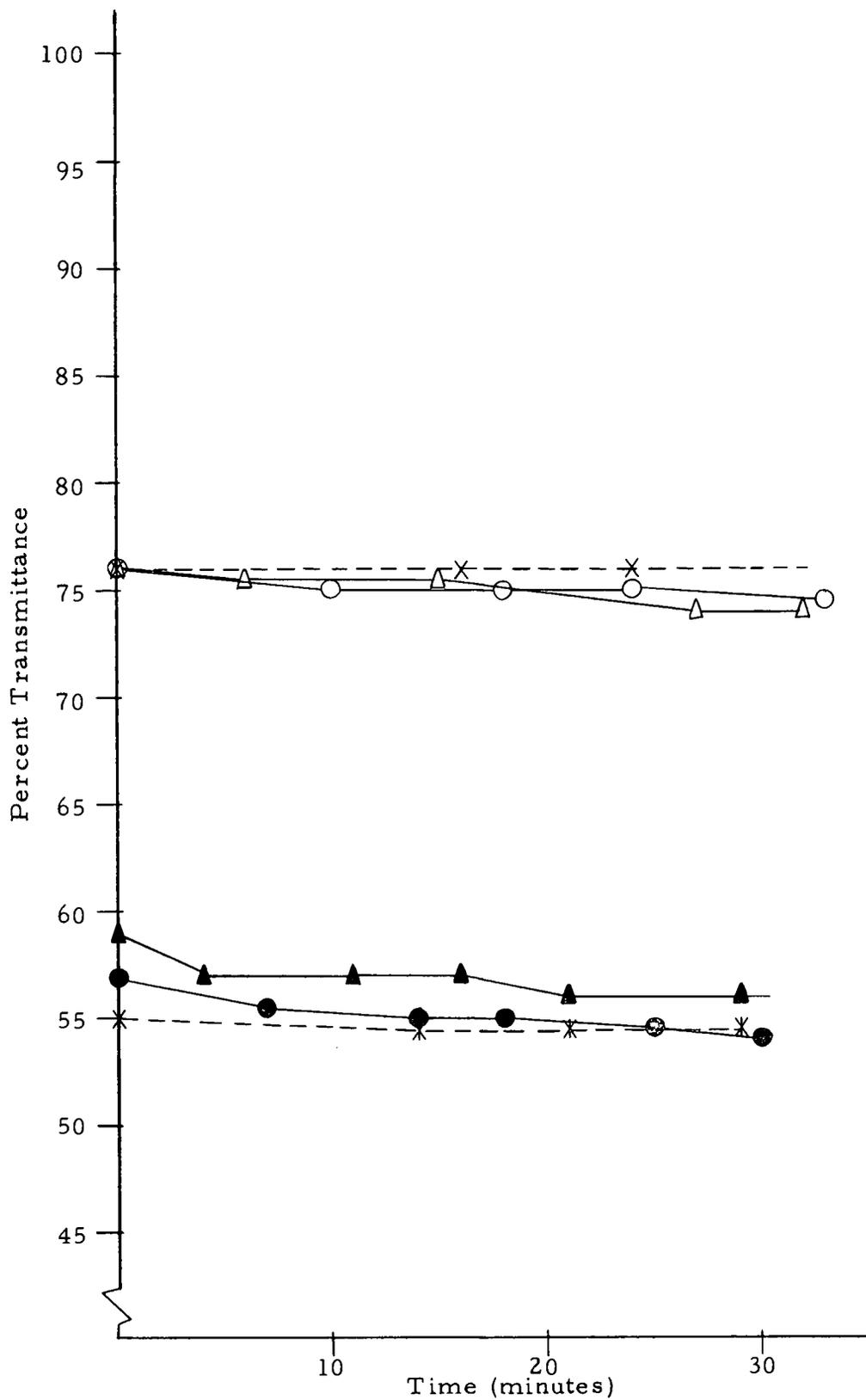


B. Banana 5





D. Banana 9



E. Banana 10

Table 11. Raw Data Used in Statistical Analyses.

Banana	Hand	Day	% Moisture	Protein (mg/g banana dry weight)	Polyphenol oxidase (units activity/ mg protein)	Polyphenol oxidase (units activity/g dry weight banana)	Dopamine (μ g/g dry weight banana)	Ascorbic acid (mg/100 g dry weight banana)	Initial Percent Transmittance	Change in Percent Transmittance over 30 minutes	
1	I	0	74.44	44.5	4.50	201	170	10.7	95.0	1.5	
			73.40	44.5	3.72	165	292	21.0	94.0	2.0	
			2	74.44	45.6	4.18	191	175	7.3	88.0	2.5
				72.13	55.4	3.26	181	298	5.4	89.0	2.0
				75.01	37.8	4.37	166	102	26.6	94.0	1.5
				72.92	39.5	4.06	160	143	25.8	96.5	3.0
2	I	2	73.24	53.6	3.42	183	195	20.9	92.0	1.5	
			75.00	58.8	3.84	226	283	21.8	91.5	2.0	
			77.67	40.5	5.59	227	173	26.8	74.5	1.0	
4	II	0	75.74	36.3	6.29	234	250	25.5	75.0	2.0	
			78.80	38.9	4.25	166	187	16.4	72.0	3.0	
			78.04	32.9	5.54	182	163	14.8	69.0	5.0	
	7	III	0	76.02	22.8	10.35	236	195	18.9	74.5	3.5
				72.82	24.9	8.20	204	103	18.0	78.0	4.5
				77.10	31.8	5.12	163	103	4.2	73.0	3.0
8	III	0	76.48	30.8	5.42	167	152	8.2	70.0	4.5	
			76.51	22.5	7.41	167	329	18.6	73.5	2.5	
			75.16	19.2	10.55	203	279	23.6	78.0	2.0	
			2	76.92	26.7	6.64	177	98	4.8	70.0	5.0
				73.77	19.5	10.21	199	106	5.7	64.0	5.0
				74.60	40.9	3.81	156	228	31.6	99.0	1.0
3	I	0	72.37	50.4	3.26	165	277	30.0	94.0	1.5	
			75.44	58.6	3.32	195	120	16.1	72.5	6.0	
			72.55	74.8	2.66	199	96	20.8	74.0	2.5	
	5	II	0	76.71	44.3	4.05	180	319	10.1	71.0	2.0
				76.27	42.4	3.71	158	326	8.9	64.0	1.5
				79.45	56.5	2.97	168	119	8.8	53.0	4.0
6	II	0	77.51	45.9	3.44	158	44	9.9	50.0	3.5	
			77.75	34.9	5.12	179	275	18.8	65.0	1.5	
			76.49	42.6	5.83	213	330	20.3	65.0	2.0	
			4	79.81	63.1	2.42	153	85	8.2	53.0	4.0
				77.41	52.4	2.87	151	92	7.6	50.0	6.0
				75.69	15.9	11.07	176	302	20.1	72.0	0.5
9	III	0	75.59	22.6	9.55	216	197	19.3	72.5	5.0	
			77.53	45.3	5.99	271	130	22.6	62.0	3.0	
			77.52	36.7	6.43	236	165	21.6	62.0	5.0	
			4	75.59	25.3	7.99	202	224	9.7	76.0	1.5
				74.67	23.4	7.60	178	206	17.0	76.0	2.0
				77.07	32.9	6.70	220	97	12.6	57.0	3.0
10	III	0	74.99	49.5	4.09	203	108	10.5	59.0	3.0	