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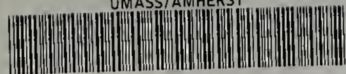
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THE STRAWBERRY ANTHOCYANINS AND THEIR DEGRADATION

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THE STRAWBERRY ANTHOCYANINS AND THEIR DEGRADATION

by

Pericles Markakis

Thesis Submitted in
Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

University of Massachusetts
Amherst, Massachusetts

June 1955

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INTRODUCTION

Color is an important organoleptic property of food. To obtain a "good", "attractive" color in a food product - in the language of previous experience and culinary tradition - presents a problem not only to the housemaker but to the industrial food processor as well.

The color problem is especially important in fruit preservation. Browning, fading, or discoloration in general, is a very common defect in preserved fruit products. The development of undesirable discoloration in a fruit product may start at several stages before the fruit reaches the processor. The food technologist, however, is generally concerned with the fate of the fruit after harvest and until it reaches the consumer. During that period, various characteristics of the raw product must be preserved, and color is one of them.

In the commercial handling of preserved fruit products, one may distinguish between two stages affecting the chromatic appearance of the fruit; i.e., the processing (dehydration, freezing, heat processing, etc.), and the storage after processing. During these stages many physicochemical factors may affect the color, and numerous chemical reactions may lead to discoloration. Temperature, light, pH, redox potential are examples of such factors; enzymatic changes, metallic salt formations, polymerizations, oxidations, reductions, and the Maillard condensations are examples of such reactions.

The color of a fruit product may be changed during

preservation by either or both of the following causes: destruction of the original pigment and/or formation of new colored products. The latter products are brown as a rule, hence the term "browning". Browning has been widely studied in recent years and its chemistry markedly clarified. The destruction of the original fruit pigment has been studied to a less extensive degree.

Anthocyanins are the main pigments of many fruits. In some products, such as strawberry spreads, the anthocyanin is destroyed at a more rapid rate than the brownish coloration appears, which has led to the statement that "the loss of red pigment is by far the more important" of the two causes of discoloration (Sondheimer and Kertesz, 1948b).

The present investigation is being undertaken as an attempt to stabilize the red color in strawberry products, and eventually in similar products of other small fruits. Strawberry preserves represent more than one fourth of all fruit preserves packed annually in the United States, and about one eighth of all fruit spreads are strawberry products. The color of these products turns from bright red to dull maroon-brown with progressive storage. It is known that both destruction of the anthocyanin and browning takes place in this discoloration process. The destruction of anthocyanin will be primarily studied in the course of this work; nonetheless, the possible connection with the browning will also be considered.

REVIEW OF LITERATURE

Anthocyanins have been extensively investigated from the chemical and botanical point of view. Their food technological aspects, however, have not been studied to any satisfactory degree. For this reason, the emphasis in this review will fall on the chemical literature pertaining to anthocyanins.

Occurrence of Anthocyanins

The term "antho-cyanin" comes from two Greek roots denoting "flower" and "blue" respectively. It was introduced by Marquart in 1835 (Onslow, 1925) to designate the blue pigments of the flowers. Later, it was realized that the innumerable shades of blue, purple, violet, mauve, and magenta and nearly all the reds which appear in flowers, fruits, leaves, and stems of plants are due to pigments similar chemically to Marquart's "flower-blues", the anthocyanins.

Although, taxonomically, anthocyanins are widely distributed over the plant kingdom (Onslow, 1925), histologically, they display the tendency of being localized in the epidermal and subepidermal tissues rather than in deeper seated ones; a notable exception to this is the red beetroot. In the cell they are present, as a rule, in the cell-sap occupying the vacuoles. De Vries (1871) showed that in living cells the protoplasm is impermeable to anthocyanins, but when the protoplasm is dead, semipermeability ceases and the anthocyanins diffuse out of the cell. This is important in extracting these pigments from plant tissues. If the concentration of the

pigment in the cell-sap becomes too high, the anthocyanin precipitates out in crystalline or amorphous form. Another histological possibility is for the anthocyanins to be adsorbed on the cell-walls of dying or lignifying tissues.

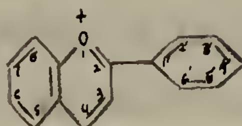
Isolation and Constitution of Anthocyanins

Isolation and analyses of anthocyanins were attempted long before Willstaetter's time. However, it was the pioneer work of Willstaetter and his students which laid the foundations of the chemistry of these plant pigments. According to Robinson (1936) "Willstaetter owed his triumph largely to recognition of the fact that the anthocyanins, although non-nitrogenous compounds, form salts with strong acids, and these salts can be purified by means of the technique appropriate to many ammonium salts, that is solution in a hydroxylic solvent and precipitation with a non-hydroxylic solvent".

To isolate the pigments, Willstaetter and his coworkers extracted the fresh or dried plant tissue by means of various solvents (glacial acetic acid, acidified water, methanol, ethanol, etc.), the pigment then being precipitated with ether, dissolved in water, and purified either through formation of lead salts or through picration. The crystalline anthocyanin chlorides were usually obtained from methanolic solutions containing an excess of hydrochloric acid.

Studies of their structures led Willstaetter to the conclusion that anthocyanins are all glycosides of anthocyanidins, the latter being oxonium salts of polyhydroxy (and methoxy)

derivatives of a basic structure, i.e., the 2-phenyl-benzo-pyrylium cation (flavylium):

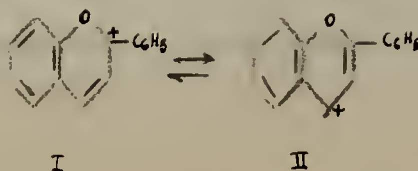


In addition to the preceding, several other formulae, utilizing the quadrivalent oxygen, were proposed, one of which has a centric configuration, with the positive charge regarded as being attached to the cation complex as a whole.

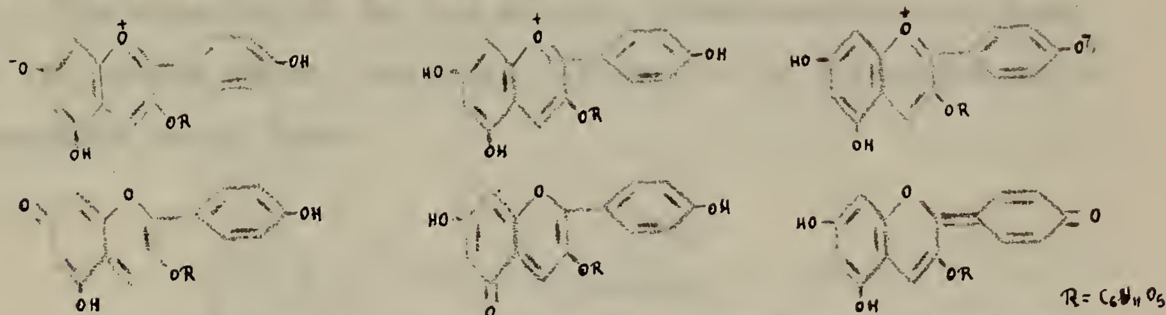
Dilthey and Quint (1931), and Quint and Dilthey (1931) challenged the oxonium configuration, and proposed a carbonium formula, instead. According to their theory the anion is linked to the pyrone ring at an "ionized coordinatively unsaturated carbon atom" (the heteropolar atom being indicated by a point). The carbon atom in the 2 position was considered as the heteropolar atom:

Hill (1935, 1936) showed that the 4-carbon atom may also act as a heteropolar atom.

Later, Shriner and Moffet (1939, 1940, 1941) presented more evidence against the oxonium theory. They suggested that the 2-, 3- and 4-carbon atoms constitute a mobile allylic system through which the flavylium salts may resonate between structures I and II:



According to Ingold (1935), an anthocyanin, such as the pelargonidin-3-glucoside, could exist in three tautomeric forms differing in the distribution of the phenolic protons, each tautomer being mesomeric between betainoid and quinoid valency structures as illustrated below:



The anthocyanins are grouped in the following categories according to the nature and position of the residues attached to the 3- or 3,5,-hydroxyl groups (Link, 1943):

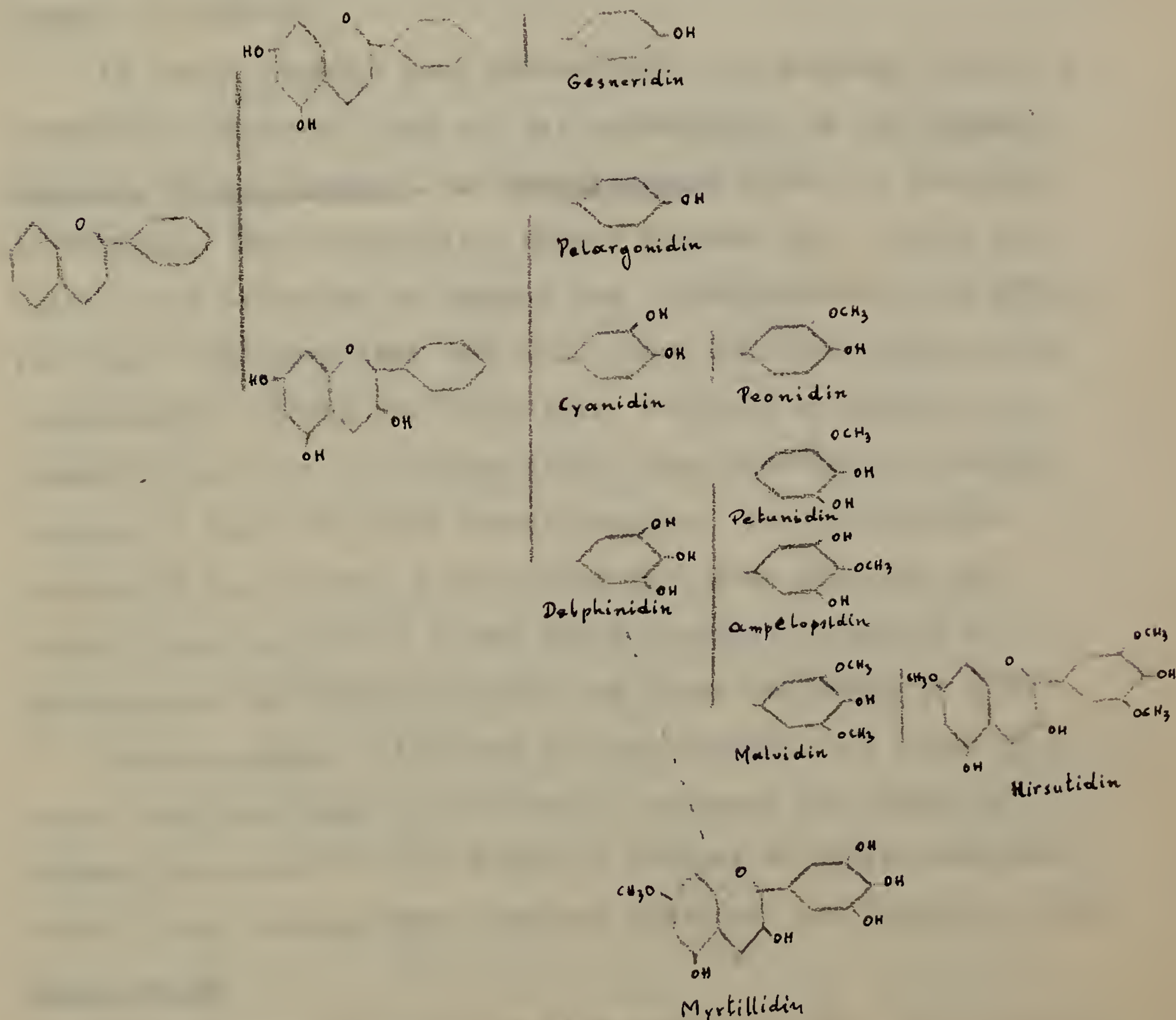
a) 3-monoglucosides, and 3-monogalactosides
b) 3-rhamnosides, and other 3-pentosides
c) 3-biosides
d) 3,5-diglucosides
e) acylated anthocyanins

Willstaetter (1914) was able to synthesize two natural anthocyanidins (pelargonidin, and cyanidin). Robinson and his coworkers (Robinson and Todd, 1932), using a different method, synthesized the same two and also four more anthocyanidins (delphinidin, peonidin, malvidin, and hirsutidin); he also succeeded in synthesizing five naturally occurring anthocyanins (chrysanthemin, oenin, pelargonin, cyanin, and malvin).

Ten natural anthocyanidins have been described in the literature (Mayer and Cook, 1943; McIlroy, 1950; Sannie and Sauvain, 1952). Many more anthocyanins are possible and have

been reported, since more than one sugar residue may be involved in glycosidation in more than one position, and organic acids also occur as a third component in some anthocyanins (Karrer, et al, 1927; Karrer and Widmer, 1927; Karrer and Meuron, 1932).

The formulae of the ten natural anthocyanidins are given in the scheme below, which also indicates the structural relationships among them:



Properties of Anthocyanins

Solubility

Anthocyanins are soluble in water. The anthocyanidins, which are obtained by hydrolysis of the anthocyanins, are far less soluble in water than the former, and in some cases quite insoluble (Wheldale and Basset, 1914).

Anthocyanins are insoluble in ether, benzene, carbon bisulfide, chloroform and similar solvents in which plastid pigments are soluble.

In lower alcohols most anthocyanins are soluble; there are exceptions, however, such as, the anthocyanins of the Amaranthaceae, Chenopodiaceae, and Phytolaccaceae which are insoluble in ethanol. The distribution number between amyl alcohol and dilute acid solutions is highest for anthocyanidins, much lower for their monoglycosides and still lower for the corresponding diglycosides. There are deviations, however; keracyanin and prunicyanin, which are diglucosides, have distribution numbers similar to those of their monoglucosides. The distribution numbers of the picrate salts render them more suitable for quantitative separation of the three classes, according to Willstaetter and Schudel (1918), and Grove and Robinson (1931).

Anthocyanidins, dissolved in amyl alcohol, are taken up in dilute acid solutions by addition of benzene; the amount of benzene necessary for the change of solvent is fairly characteristic of the anthocyanidin involved (Robinson and Robinson, 1931).

Color and pH

Anthocyanins change color with changes in pH. Willstaetter

attributed this property to the amphoteric character of these pigments, which are capable of forming salts with both acids and alkalies. For example, the pigments of the red rose and the blue cornflower are identical (Willstaetter and Everest, 1913; Willstaetter and Nolan, 1915). The rose contains salts of cyanin with acids, while in the cornflower salts of cyanin with metals are present. However, since the cornflower-sap is acidic, other factors besides pH should be taken into consideration to account for the discrepancy in this and many other cases. (see p.12).

Haas (1916) used buffer solutions of pH 1 to 13 to study the color change of anthocyanin extracts from several flowers, fruits and the red beet.

Attempts to use anthocyanins as pH indicators were made, among others, by Smith (1923) and Matula (1924). Smith found that a pigment from Ipomoea Learii was pink at pH 6.0 turning to full blue at pH 7.6, and Matula stated that an anthocyanin from the red cabbage is a good indicator, comparable to litmus and phenolphthalein.

Karrer and co-workers (1927) observed that, in alkaline solutions, peonidin was rapidly decolorized, while peonin retained its blue color after 24 hours.

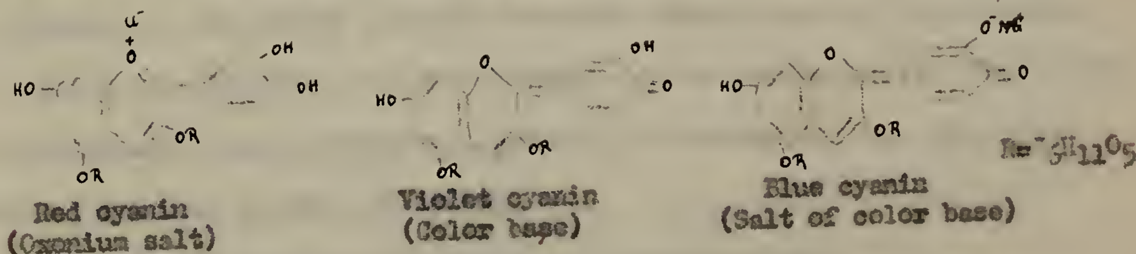
The importance of a definite pH of the solution in examining the color of anthocyanins was emphasized by Fear and Nierenstein (1928).

Buxton and Darbishire (1927, 19[illegible], 1929a), studying the effect of H+ concentration on the color of flower pigments,

recorded a definite trend of change from red to blue as the pH increased.

Robertson and Robinson (1929) found that anthocyanins can be characterized by means of their color reaction with alkalies. Using these reactions, and supplementary information from the distribution between immiscible solvents and from the ferric chloride reaction, Robinson and Robinson (1931, 1932, 1933, 1934) were able to make an extensive survey of the anthocyanins over the plant kingdom. These authors stated that their "methods throw little light on the nature of the carbohydrate group of anthocyanins, but in most cases they can give the position of attachment, as a result of comparison with pure natural or synthetic anthocyanins of known constitution".

Robinson (1933a) proposed the following structures for the red form of cyanin (at pH 3.0 or less), the violet form (around pH 8.5), and the blue form (at pH 11.0) of same.

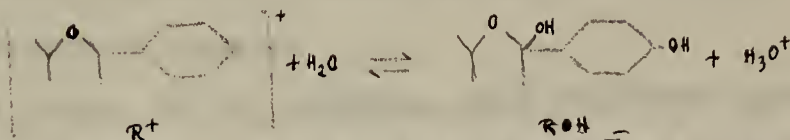


Red cyanin (Oxonium salt) | Violet cyanin (Color base) | Blue cyanin (Salt of color base)

These structures have not been definitely proven.

Sondheimer and Kertesz (1948) developed a method for determining the red anthocyanins in strawberries and strawberry products by measuring the light absorption at two different pH levels.

Later, Sondheimer (1952) presented evidence to support the hypothesis of an equilibrium reaction between hydronium ions, a red modification R^+, and a colorless form ROH of the anthocyanin:



Nebesky et al (1949) stated that adjustment of pH had little, if any, effect on the rate of deterioration of anthocyanin color in fruit juices, although lower pH values exerted a protective action on solutions of purified pigments. However, in the opinion of Thimann and Edmondson (1949), and Robinson (1951), a greater stability of anthocyanins is attained in more acidic solutions.

Li (1952), working with buffered solutions of purified cranberry pigment found that low pH levels resulted in better color retention.

Recently, Meschter (1953) reported that, in his experiments with buffered solutions of strawberry juice concentrate, the rate of anthocyanin degradation was greatly affected by the pH, high acidity favoring color retention.

Other factors affecting the color of anthocyanins.

In Willstaetter's laboratory it was observed, at an early date, that factors other than the pH of the cell-sap, were able to affect the anthocyanin colors of flowers and fruits. Willstaetter and Mallison (1915a) mentioned such factors as the concentration of an anthocyanin, the mixing of two or more

anthocyanins, and the background effect of yellow pigments. Furthermore, Willstaetter and Zollinger (1916) made the important observation that the red color of oenin chloride, in dilute HCl solution, turned to a blue-red and more intense color on the addition of tannin.

Haas (1916) found, to his surprise, that cranberry juice remained red even at pH 11, and Buxton and Darbishire (1919a) cited many cases in which crude anthocyanin extracts failed to turn blue on passing from acid to alkali.

In 1931 Robinson and Robinson used the term "copigmentation" to denote the synergistic effect of a substance in intensifying and modifying the color of an anthocyanin. Organic substances, and possibly metals, such as iron, may serve as copigments. The effect is more or less specific; gallotannin is a copigment for oenin and malvin, but not for cyanin. 2-hydroxyxanthone is a powerful copigment for cyanin but not for its isomeric mecocyanin. Copigmentation is considered to have little or nothing to do with salt formation, and occurs even in the presence of a large excess of mineral acids. It is the result of the formation of weak additive complexes, which are dissociated at an elevated temperature, or by the action of a solvent.

The copigment effect was also observed by Lawrence (1932), who studied the effect of ivory-colored flavones on crude anthocyanin extracts of widely different plants.

Robinson and Robinson (1932) observed that the acid extracts of flowers were almost always more blue-toned than the

solutions of pure anthocyanins, and they were able to reproduce most of the natural tones by means of the common copigments, tannins, and flavone and flavonol glycosides.

Robinson (1933), showed that aqueous extracts of the blue cornflower contained the cyanin pigment as a negatively charged colloid. This colloid was not precipitated by sodium chloride, a fact indicating the presence of a protective colloid. An artificial colloidal complex of cyanin chloride was prepared by the same author, using starch, xylan, or agar; the colloidal solution obtained was blue at pH around 7.5, whereas the molecular solution of this pigment is violet-red at the same pH; the corn-flower cyanin extract was blue, although the pH was below 5. Robinson further suggested that all blue flowers are colored by colloidal solutions of their respective anthocyanin pigments.

The alkaloids papaverine and nicotine were reported by Robinson and Robinson (1934) as having copigmentation effect on anthocyanins.

Excellent summaries on the modification of flower colors appear in Robinson's reports (1933, 1935).

The following relationships between chemical structure and color hue of anthocyanins have been observed by Willstaetter and Nolan (1915) and Gatewood and Robinson (1926). Methylation of the hydroxyl groups increases the redness; f.i. peonidin is redder than cyanidin and malvidin is redder than delphinidin. In the absence of copigments and other interfering substances, increased blueness follows the increase of hydroxylation at the

side phenyl ring; thus, in sodium acetate solution, the color of pelargonin is bright bluish-red, that of cyanin is violet and that of the delphinidin glycosides is blue.

Oxidation reactions.

Hydrogen peroxide. Willstaetter and Everest (1913) reported that oxidation of cyanidin by H_2O_2 yields a yellow crystalline product resembling a flavonol. Pratt and Robinson (1925) carried out numerous experiments to obtain the same transformation with no success; they obtained carboxylic acids and coumarin derivatives instead. Karrer and coworkers (1927, 1932, 1942, 1944a, 1945) studied the oxidation of anthocyanins and anthocyanidins extensively. They found that these pigments are generally readily oxidized by H_2O_2 at room temperature to off-color products, one of them being a flavonol.

According to Joslyn (1941), the addition of H_2O_2 to red wines causes rapid decrease of color, whereas the effect of oxygen is to intensify the color at the beginning, gradually causing it to fade, as the coloring matter is precipitated.

Tauber and Laufer (1943), studying the effect of H_2O_2 on crude preparations of anthocyanins in alkaline solutions, observed no appreciable difference in color between solutions containing H_2O_2 and blanks with no H_2O_2.

Sondheimer and Kertesz (1951) found that strawberry anthocyanin is readily oxidized by H_2O_2 and they studied the kinetics of this oxidation in solutions of pure pigment and in strawberry juice. Recently, the same authors (1953) proposed

an indirect ascorbic acid-induced destruction of strawberry anthocyanin, their contention being that the H_2O_2 formed during the aerobic oxidation of ascorbic acid, is responsible, partly at least, for the pigment destruction in strawberry products. However, the presence of H_2O_2 in strawberry products has not been demonstrated.

<u>Oxygen</u>

Tressler and Pederson (1936) reported that oxygen increases the deterioration of color in grape juice and strawberry juice. Nebesky and coworkers (1949) also stated that the oxygen content, along with the storage temperature, were the most specific agents in the deterioration of color in several fruit juices, or their purified pigments. However, Sastry and Tischer (1952, 1952a), showed that deterioration of the pigment in grape juice was just as rapid with nitrogen in the head space as with oxygen, and Li (1952) concluded that the action of oxygen on the pigment of cranberries is negligible.

<u>Ferric ions</u>

Ferric chloride bleaches anthocyanin solutions, the decoloration being faster with anthocyanidins than with their glycosides. Thus, Levy and Robinson (1931) observed that 3-glucosidyl peonidin exhibited greater stability than its aglucone in the presence of $FeCl_3$. Similarly, Leon et al (1931) found pelargonin, callistephin, and salvinin were resistant than the corresponding anthocyanidins to the action of $FeCl_3$.

In agreement with these findings is the general statement by Karrer and his coworkers (1927) that a free hydroxyl group

at carbon 3 renders the anthocyanidin molecule very vulnerable.

However, ferric chloride can, under suitable conditions, form additive compounds with the oxonium salts of flavylium derivatives (Everest and Hall, 1921; Pratt and Robinson, 1923).

Other oxidations.

Chromic acid (Rulof and Wegner, 1903), and permanganates (Anderson and Nabenhauer, 1926; Shriner and Anderson, 1928) have been successfully used to oxidize hydroxy-flavylium salts.

Reduction reactions.

On treating acid solutions of anthocyanins with zinc dust the color rapidly disappears, and the solution remains colorless if air is excluded. On exposure to air, if the reducing action is not too severe, the color returns with the surface layers of the solution becoming colored before the deeper ones. Keeble (1905) did not consider this reaction a reduction, because the color did not return on treatment with oxidizing enzymes. In this connection, Wheldale and Bassett (1914) remarked that the return of color on exposure to air is not equally great with all acids, which may indicate that the reaction is not a simple reversible reduction.

Willstaetter and Mallison (1915) decolorized idaein chloride in acid solution by means of zinc dust or hydrosulfite, and were able to regenerate the color by shaking with air or with H_2O_2.

Studying the corrosion in canned fruits, Kohman and Sanborn (1924) speculated on a possible reduction of the anthocyanins by the hydrogen generated from the reaction of organic

acids with the can, and reported that the color of red fruits can be frequently restored by the action of oxygen.

Freudenberg et al (1925) reduced cyanidin and its pentamethyl ether to D, L-epicatechol and pentamethyl-D, L-epicatechol, respectively, by hydrogenation over platinum. Freudenberg and Harder (1927) also prepared a catechol from luteolinidin tetramethyl ether. The catechols were colorless.

Karrer et al (1927) observed an important difference in catalytic reduction between 3-OH anthocyanidins and 3-substituted ones. Malvin, cyanin, peonin, and pelargonin could not be reduced in these experiments, whereas the sugar-free anthocyanidins could. The authors were unable, however, to explain why cyanidin pentamethyl ether, in which there is no free hydroxyl group in position 3, could also be easily reduced to epicatechin.

Kuhn and Winterstein (1932), using zinc dust in pyridine, reduced cyanidin into hydrocyanidin, a very labile compound, which reoxidized to cyanidin on exposure to air. Charlesworth, Chavan and Robinson (1933) repeated the decolorization of cyanin and cyanidin with zinc in acid solutions, and observed that, on prolonged action of the zinc, the color was not recoverable.

The reaction of sulfites and hydrosulfites with anthocyanin was studied by Koztowsky (1936). He reported that these agents decolorize anthocyanin solutions, and that the original color can be restored by adding tincture of iodine.

On the other hand, the same author observed that magnesium, in the presence of organic acids, decolorizes anthocyanins more or less irreversibly.

Reichel (1937) investigated a biochemical reduction of anthocyanidins. Solutions of several anthocyanidin chlorides were decolorized at 37°C., in evacuated flasks, in the presence of yeast or liver; a substrate, such as acetaldehyde, being necessary as a hydrogen donor. Introduction of air dehydrogenated the leuco-pigment back to the color form; removal of air was again followed by decolorization, and the cycle could be repeated. Reichel suggested that anthocyanins may play a role in the oxidation-reduction systems of living plant cells.

Beattie, Wheeler, and Pederson (1943) observed that ascorbic acid and red color disappeared at about the same rate in strawberry, raspberry, and currant juices; they advanced the hypothesis that ascorbic acid may be oxidized by reducing the pigment.

The same deleterious effect of ascorbic acid on anthocyanins was reported by Esselen, Powers, and Woodward (1945). L-ascorbic acid, D-isoascorbic acid added to grape juice reduced the intensity of the color of the latter.

Esselen, Powers, and Fellers (1946) also noted that addition of ascorbic acid to cranberry juice reduces the intensity of the red color, and Li (195[illegible]) reported on a very rapid decolorization of purified cranberry anthocyanin by ascorbic acid.

Nebesky et al (1949) noticed the bleaching effect of ascorbic acid on strawberry, blueberry, and grape juices.

Sondheimer and Kertesz's (1953) hypothesis of an indirect effect of ascorbic acid on strawberry pigment has already been mentioned. (p.14).

Meschter (1953) reported a logarithmic destruction of pigment with time when ascorbic or dehydroascorbic acids were added to strawberry juice or to purified extracts of strawberry pigment.

Reactions with metallic salts

Willstaetter and Mallison (1915) reported several reactions of idaein chloride with metallic salts. Ferric chloride, copper acetate, or zinc acetate, when added to alcoholic solution of the pigment, gave a blue coloration, while lead acetate gave a blue precipitate. In aqueous solution of the pigment, alum produced a very stable violet color, and bismuth nitrate a red-violet coloration.

Shibata *et al* (1919) studied the behavior of a number of natural anthocyanin extracts in the presence of salts of many metals. Salts of Na, K, Ca, Ba, Sr, Zn, Sn, Pb, Al, Mg, Co, Ni, Mn, Cu, Cr, and Hg always exerted a bathochromic effect, shifting the color of the extracts toward the violet end of the spectrum.

Everest and Hall (1921) found that small amounts of ferric chloride, when added to the oxonium salts of cyanin or violanin, produced an intense blue coloration, stable on standing, whereas salts of Na, K, Ca, or Mg caused a gradual decolorization of the pigments.

Bigelow (1922) noticed a more or less extensive bleaching of red fruits packed in ordinary tin cans.

Morse (1927) found that salts of iron, added to solutions of purified cranberry pigment, caused the formation of dark precipitates, while stannous chloride produced a purplish tint. Aluminum salts caused no noticeable change.

Culpepper and Caldwell (1927) stated that formation of purplish salts with tin is a general property of the red anthocyanin pigments. In the case of freshly cooked cranberry juice, no change of color was noticed when $SnCl_2$ or $AlCl_3$ was added. However, on raising the pH to near neutrality, either of those agents produced a faint purpling, followed by the deposition of a purple precipitate. Similarly, $FeCl_3$ produced little or no change of color in the same juice, but on partial neutralization, a brown-black colorization developed within a few minutes.

Li (1952) noticed a brown precipitate on the addition of $FeCl_3$ to fresh cranberry juice, and a dark purple precipitate on the addition of $SnCl_2$.

<u>Hydrolytic reactions</u>

Fading on treatment with a base or with a large amount of water is characteristic of all anthocyanins. Willstaetter and Everest (1913) were the first to show that this type of decolorization was not due to reduction, as earlier workers assumed, but to formation of a colorless pseudobase, often referred to as the chromenol or carbinol base. Addition of acid or evaporation of the solvent usually brought the color back.

Willstaetter and Mallison (1915) noticed the tendency of

idaein chloride to form a pseudobase in aqueous or alcoholic solutions.

Pratt and Robinson (1923) reported that 7-hydroxy, 3-methoxyflavilium chloride, on addition of sodium acetate, gave a colored quinonoid anhydrobase, which was completely decolorized to the pseudobase on further addition of an excess of water. However 7-hydroxy, 4-methoxyflavilium chloride which also formed a stable red anhydrobase on addition of sodium acetate, displayed no tendency to form a pseudobase with water, according to Pratt, Robinson and Williams (1924).

Pratt and Robinson (1924) also reported that pelargonidin chloride, dissolved in distilled water, decolorized on heating, owing to the formation of a pseudobase.

Irving and Robinson (1927), experimenting with simpler hydroxyflavylium salts, found that 4-hydroxyflavylium chloride yielded a colored quinoid anhydrobase, which had a marked tendency to pass to the colorless pseudobase form by hydration, while 3-hydroxyflavylium chloride showed such a stong inclination to form a pseudobase that the anhydroform could not be isolated in a pure state.

Chapman <u>et al</u> (1927) stated that the effect of a 3-hydroxy or 3-methoxy group in facilitating pseudobase formation is well established.

Hill and Melhuish (1935) treated 3-unsubstituted flavylium salts with a 10% NaOH solution at room temperature for a week, and obtained a mixture of a pseudobase, a chalcone, and a flavone.

Karrer and Trugenberger (1945) also reported the isolation of colorless pseudobases from the hydrolysis products of benzopyrylium salts.

Recently, Huang (1955) showed that fungal enzymes hydrolyze the glycosidic bond of many anthocyanins, the liberated anthocyanidins being subsequently decolorized "spontaneously".

<u>Stabilization of Anthocyanins in Fruit Products.</u>

Two problems arose when fruits were first packed in tinned containers: discoloration of the fruit pigment, and corrosion of the tin can. The introduction of enamels solved the first problem to some extent, but aggravated the second (Bibelow, 19[illegible]). The manner in which anthocyanins function as corrosion accelerators is not well understood. It has been suggested that pigments may act as depolarizers, by removing hydrogen from exposed iron, thereby increasing the rate of solution of the latter (Curl and Talburt, 1954). Hartwell (1951), however, states that, with better steels available today, the chief kind of container failure formerly attributed to anthocyanins (perforations) is no longer common, and the shelf-life of canned fruit products has been extended three-fold or more.

Koch (1931) suggested chromium plated copper or brass kettles for the cooking of red colored fruits; he also found that strong acidity could prevent discoloration by tin.

Tressler and Pederson (1936) recommended evacuation or replacement of air by steam in bottles of grape juice for better retention of color. Sastry and Tischer (1952), however, could not find a difference in the rate of pigment deterioration

between oxygen packed and nitrogen packed grape juice.

Kertesz and Sondheimer (1948, 1948a) stated that short times and low temperatures of pasteurization, prompt cooling below 65°C, and refrigeration during long storage are essential to the retention of desired color in strawberry products.

Hall (1949) obtained a patent for stabilizing the color of red fruits by adding alkaline phosphates before cooking. Angevin (1950), however, found that this method has an adverse effect on the color of cranberry juice.

Nebesky et al (1949) suggested low temperature storage and the removal of oxygen for better retention of color of fruit juices. They also indicated that increasing the sugar and/or the citric acid concentrations enhanced the color stability of strawberry fountain syrup.

Cohee and Nelson (1951) reported that addition of phytic acid or phytates is very effective in preserving the color of sour cherries packed in glass and stored at room temperature, but nearly as effective when packed in cans.

Li (1952) found that the color of cranberry juice could be stabilized by thiourea, or tannic acid; he attributed the preserving effect to antioxidative property of these agents.

Meschter (1953) reported that avoiding excessive heating in cooking, increasing the acidity, using low storage temperatures and smaller amounts of sugars favors the retention of color in strawberry preserves.

EXPERIMENTAL

The problem of color stability in strawberry products was approached in this work by studying the nature and stability of the red strawberry pigments in pure form. Strawberry juice was used when it was desirable to study the pigment degradation under conditions closer to those in actual products.

Analysis of Strawberry Juice

Available data on the composition of strawberries (U.S.D.A. Agric. Handbook No. 8, 1950; Bate-Smith and Morris, 1952), and of strawberry juice (Beattie *et al*, 1943) are not numerous and do not indicate good agreement among results reported by different authors. For this reason, and for the purpose of characterizing the materials used in these experiments, an analysis was carried out on juice obtained from fresh Sparkle strawberries.

The strawberries used for the analysis were grown on the farms of the University of Massachusetts, at Amherst, during the 1954 season. The juice was extracted by means of a wooden hand press. The acidity was measured by potentiometric titration against 0.1 N NaOH, using a Beckman Model G pH meter (1). The equivalence point of the titration was at pH 8.0. The anthocyanin content was determined by the method of Sondheimer and

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(1) Manufactured by Beckman Instruments, Inc., Pasadena, Cal.

Kertesz (1948), crystalline callistephin chloride [2] being used to prepare the reference curve. The ascorbic acid content was measured by the indophenol-xylene extraction method of Robinson and Stotz (1945). A Beckman Model G pH Meter [1] was used for obtaining the pH of the juice, and a Beckman Model H-2 pH Meter [1] for the oxidation-reduction potential. For the latter measurement, a simple cell was improvised. A three-neck, 200 ml. round-bottom flask was equipped with 3-inch calomel and platinum electrodes, which were introduced through the side apertures. A glass tube, drawn to a capillary tip, was brought in through the center hole. An outlet for gas escape was provided by an orifice next to the glass tube, and the inside of the flask, as well as the electrodes and the capillary tube, were made water-repellent by treatment with Beckman Desicote.[1] The flask was kept at constant temperature by immersion in a water bath at 25°C., and nitrogen gas, washed by means of alkaline pyrogallol (15% pyrogallol and 6% sodium hydroxide in distilled water), was bubbled through the capillary opening. The gas flow was maintained throughout the measurement period. Constant readings were obtained after about two hours. The sugar content was estimated refractometrically. The resulting data are summarized in Table 1.

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(2) Since the pelargonidin chloride 3-monoglucoside of strawberries, as described by Sondheimer and Kertesz (1948a), seems to be identical in every respect with the callistephin chloride of asters, as described by Willstaetter and Burdick (1917), the two names will be used interchangeably. The pigment for the reference curve used in this study was prepared from strawberries by the chromatographic method described on p. 31.

Table 1. Analysis of fresh Sparkle Strawberry Juice

Acidity (% citric acid)	0.77
pH (at 25°C.)	3.42
Redox potential (mv. at pH 3.4; 25°C.)	115.3
Ascorbic acid (mg.%)	59.5
Anthocyanin (mg.%)	30.2
Sugar (%)	6.8

Table 2. Comparative Efficiency of Solvents Used for Strawberry Pigment Extraction

Extractant	Light Absorbence at 500 mμ (%)	
	Extracted Pigment	Residual Pigment
n-Pentanol	18.5	98.0
Isobutanol	54.2	89.3
n-Butanol	75.5	78.5
Cyclohexanol	75.8	72.0

When mixed samples of juice, made from varieties of strawberries grown at the University of Massachusetts, were analyzed, the results did not deviate by more than 15% from the figures shown in Table 1, with the exception of the anthocyanin content, which showed a greater variation.

Selection of Anthocyanin Extractant

Several water-immiscible alcohols have been used for the extraction of anthocyanin pigments from aqueous solutions. n-Pentanol was used in some early studies in the field. Rosenheim (1920) used n-butanol as an extractant for anthocyanidins, as well as for anthocyanins, the same solvent being used more recently by Sondheimer and Kertesz (1948a) in their experiments with strawberry pigment. Nebesky et al (1949) used isobutanol.

A comparison of the afore-mentioned solvents and also cyclohexanol was made to determine the most suitable extractant for strawberry pigment. The cyclohexanol used (3) was at first found to cause a partial destruction of the pigment in preliminary experiments, only 84% of the pigment originally present in the juice being recovered as extracted plus residual pigment in such an experiment. The cyclohexanol was, therefore, subsequently redistilled just prior to use.

Two volumes of clear strawberry juice acidified to contain 1% HCl, were shaken with one volume of solvent. After separation of the two phases, the aqueous layer was diluted 30 times with

(3) Obtained from Distillation Products Industries, Rochester 4, N. Y.

water, the pH was adjusted to [illegible].00, and the light transmittance at 500 mμ was determined using a Beckman Model DU Spectrophotometer.(1) An aliquot of the alcohol extract was mixed with 1.5 volume of 0.5% HCl, and the mixture was shaken with 1.5 volumes of petroleum ether in order to effect a quantitative transfer of the pigment into the aqueous phase. The resultant solution was diluted 40 times with water, its pH adjusted to [illegible].0[illegible], and the light transmittance measured as above. A comparison of the efficiency of extraction of the four solvents used is presented in Table [illegible].

It is apparent that cyclohexanol is a somewhat more efficient solvent for the extraction of strawberry pigment. However, in view of the destruction of the pigment which may take place if the solvent is not properly purified and the fact that a slightly better separation of the liquid phases occurs when n-butanol is used, the latter solvent was used in subsequent experiments.

<u>Isolation and Identification of a Second Anthocyanin in Strawberries</u>

Robinson and Robinson (193[illegible]), working with plant tissue extracts, identified pelargonidin 3-monoglucoside in English cultivated strawberries (*Fragaria virginiana*). Sondheimer and Kertesz (1948a) obtained the same pigment in crystalline form from American cultivated strawberries (*Fragaria chiloensis*). Robinson (1934) found a pelargonidin 3-galactoside in wild strawberries (*Fragaria vesca*).

1. Extraction and Separation of Pigments.

Meschter (1953) isolated and purified crystalline pelargonidin 3-glucoside using the method described by Sondheimer and Kertesz (1948a). This method involves saturation of strawberry juice with NaCl, extraction of the pigment by means of n-butanol, concentration of the extract under nitrogen at low pressure, and precipitation of the pigment from the concentrated extract by ether. The precipitated pigment is dissolved in 0.01% HCl, reprecipitated as a picrate salt which is then converted back to the chloride salt. When Meschter chromatographed the purified anthocyanin chloride by ascending paper chromatography, using as solvent the organic phase of the mixture n-butanol:acetic acid:water (4:1:5 by volume) he observed only one red-orange spot. But when the pigment extract was chromatographed just prior to the picration step, a second, purple spot, thought to be an isomer of pelargonidin 3-glucoside, was also found.

Meschter's results were easily duplicated and confirmed in this investigation. It was also possible to follow the progress of purification in the preparation method of Sondheimer and Kertesz, (1948a) by chromatographing on paper the material obtained after each step in the procedure. Whatman No. 1 filter paper and the solvent used by Meschter (1953) were employed.

The strawberry juice as such was found incapable of being chromatographed by this method. When the pigments were extracted from the juice with n-butanol, and precipitated out, using petroleum ether into 1% HCl, two well colored bands

appeared on the paper after 24 hours of development at room temperature (Fig. 1). A faint yellow zone also appeared above the red pigments. This band turned bright yellow on exposure to ammonia vapors and gave a dark green color on streaking with $FeCl_3$ solution. These reactions characterize this band as a flavone (Sannié and Sauvain, 1952). It should be noted that petroleum ether was used in this experiment, rather than the ethyl ether suggested by previous authors, because a more complete precipitation of the pigments from the butanol phase is obtained with this solvent. The R_f values for the three pigments, thus isolated on Whatman No. 1 paper at room temperature, are given in Table 3.

In Fig. 2, chromatogram IV shows four bands corresponding to the minor, purple, pigment, the major, red, pigment, their picrates (in one band), and the excess free picric acid (yellow) present in solution after a first picration. Chromatogram III, obtained after hydrolysis of the first picration precipitate, shows the minor pigment, the major pigment and the free picric acid. Chromatogram II shows the major pigment and the free picric acid obtained after hydrolysis of the precipitate of a second picration. Chromatogram I shows the major pigment alone. Fig. 3 shows a chromatogram of the mother liquor of the first picrate precipitation after removal of the excess picric acid. The minor and major pigments can be seen along with their picrates band.

Upon exposure to hydrochloric acid fumes, the minor pigment turned from purple to reddish, denoting its anthocyanic

Fig. 1. Paper chromatogram of the strawberry anthocyanins.

Pelargonidin 3-monoglucoside (upper band).
Minor anthocyanin (lower band).

Fig. 2. Paper chromatograms showing the progress of purification of the strawberry anthocyanins in the method of Sondheimer and Kertesz (1948a).

Identity of the bands:

I	II	III	IV
Major pigment	Picric acid Major pigment	Picric acid Major pigment Minor pigment	Picric acid Pigment picrates Major pigment Minor pigment

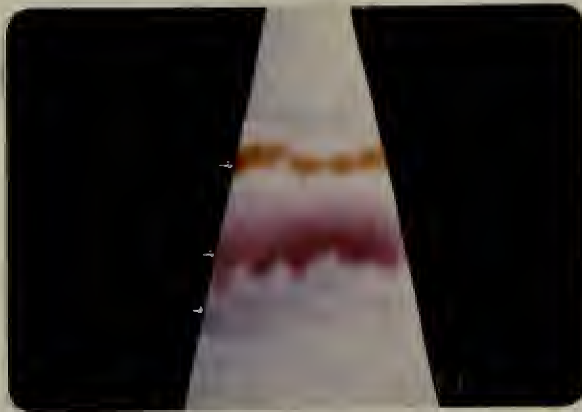

Fig. 3. Paper chromatogram of the mother liquor from the picrate precipitation of the strawberry anthocyanins.

Anthocyanin picrates (upper band)
Major anthocyanin (middle band)
Minor anthocyanin (lower band)

Table 3. Strawberry Pigments Separated by Paper Chromatography at Room Temperature. (Whatman No. 1)

Pigment	Color	R_f
Flavone	yellow	0.56
Pelargonidin 3-glucoside	red-orange	0.48
Unidentified	purple	0.38

Table 4. Paper Chromatography of Strawberry and Rose Anthocyanins and Anthocyanidins (Whatman No. 1).

Pigment	R_f
Cyanin	0.28
Unknown anthocyanin	0.38
Pelargonidin 3-glucoside	0.48
Cyanidin	0.56
Unknown anthocyanidin	0.56
Pelargonidin	0.85

nature.

2. Purification of Pigments.

After successfully completing the paper chromatographic separation of the minor pigment, an attempt was made to find a method for obtaining greater yields of the product, to allow for subsequent identification studies. Accordingly, the possibility of using column chromatography was investigated. Aluminum oxide, aluminum sulfate silicic acid with and without Dicalite filter aid, (4) and filter paper pulp were tried as adsorbents. 1% HCl, as well as the n-butanol: acetic acid: water mixture, were used as developers. Unfortunately, none of the separations were satisfactory. A chromatopile, and 0.1 inch Eaton-Dikeman filter paper were also tested but they displayed a poorer resolving power than the Whatman No. 1 or No. 4 papers.

As an alternative, triangular strips of standard filter paper were used instead of the conventional rectangular strips employed in paper partition chromatography. By this means, a relatively large volume of pigment can be applied to the base of the triangle as a stream, and a concentrating effect is achieved as the development progresses and the bands rise toward the apex. That the rate of flow of the solvent in this type of chromatogram is greater than it is in rectangular strips has been ascertained by Muller and Clegg (1951). Furthermore it may be presumed that a higher resolving power results from the fact that the faster fractions migrate still faster,

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(4) Obtained from The Dicalite Co. Chicago, Ill.

because of higher solvent flow rates at narrower sections of the paper.

Conical and circular shaped papers, with a solvent flow towards the apex of the cone or the center of the circle, were also tried using Whatman No. 1 paper, but the triangular paper, with a base as wide as the conventional chromatographic trough (8.5 inches) and a height of about 18 inches, was found to be the most practical.

The ascending technique was preferred over the descending; the advantage of being able to collect fractions directly from the apex of the triangle in the latter technique was overcome by the greater purity of the fractions in the former.

Rather than using the upper layer of the mixture n-butanol: acetic acid:water 4:1:5 (by volume) as a developer, a miscible solvent mixture, consisting of the same above reagents in a 40:10:15 ratio (by volume), was employed. This mixture contains all the water which a 4:1 n-butanol:acetic acid solution can dissolve.

In a typical preparative experiment, two liters of clear strawberry juice, obtained from strawberries of mixed varieties held frozen for two months, were acidified to contain 1% HCl. The solution was then extracted with about 800 ml. of n-butanol, using four separatory funnels in countercurrent manner. On treating the alcohol extract with about two liters of petroleum ether, the water dissolved in the n-butanol separated out. This aqueous phase contained all the red color matter present in the extract, and could be concentrated, under vacuum at

temperatures below 50°C., to half or one third of its original volume. The concentrate was streaked on triangular strips of Whatman No. 1 or 4 paper, about 3 cm. above the base of the triangle. No. 1 Whatman paper was found to be less rapid than No. 4. but yielded narrower bands on separation. Narrow streaking of the concentrate on the paper was achieved using pipettes drawn to a capillary tip. The bands were dried immediately after each streaking by means of a hot air blower, and many streaks were applied on the same band. After ascending development for 12-16 hours, the two anthocyanin zones were well separated. The chromatograms were dried, cut, and the colored bands, corresponding to each pigment, were collected separately. The bands were eluted by immersing one end of the strip in a beaker containing methanol with a trace of HCl and allowing this solvent to descend by capillarity into a lower beaker. The methanolic eluate was evaporated under vacuum, leaving an amorphous residue, which was redissolved in 1% HCl and chromatographed again as previously. The chromatograms of the second run showed that the purification obtained by the first run was not complete, each pigment fraction contained traces of the other pigment. The second methanolic eluate of each pigment was acidified to give a final concentration of 5% HCl and allowed to evaporate slowly in air at room temperature. The red-orange pigment formed purple-red leaflets. 0.1083 grams of pelargonidin 3-monoglucoside and 0.01[illegible]0 grams of minor pigment were thus obtained. Stored in a vacuum desiccator at room temperature for several days the minor pigment lost 8.5% of its weight.

3. Identification of the Minor Anthocyanin.

The tests of Robinson and Robinson (1931) and the development of paper chromatography of anthocyanins by Bate-Smith (1948) have greatly simplified the identification of anthocyanins. Both of these procedures were used in the present investigation.

The purified minor pigment was dissolved in 1% HCl and a small portion of the solution was extracted using n-pentanol. Only part of the pigment was taken up by this alcohol. This and the low R_f indicate that the pigment is not an anthocyanidin.

An equal volume of concentrated HCl was added to a larger portion of the pigment solution, and the mixture was heated and kept boiling for one minute. Upon cooling, the hydrolysate was shaken with n-pentanol, which now extracted the anthocyanidin present.

a. Identification of the Aglycone.

The pentanol extract was first washed with water and then with 1% HCl. Subsequently, 3 volumes of 0.5% HCl were added to the pentanol extract and the mixture was shaken with enough benzene to cause the pigment to be transferred quantitatively into the aqueous layer. Six volumes of benzene were required, indicating that the pigment might be cyanidin or malvidin (Robinson and Robinson, 1931).

The aqueous solution of the anthocyanidin was extracted again with n-pentanol and, using benzene, the pigment was transferred once more into 0.5% HCl. After removing all

traces of alcohol, by washing with benzene, the aqueous solution of the anthocyanidin was compared visually and spectrophotometrically with an aqueous solution of cyanidin, obtained by acid hydrolysis of the cyanin of roses. The latter was prepared from dried red rose petals by extraction with methanol, precipitation using petroleum ether, and the same paper chromatographic separation used in the case of strawberry pigment. The two anthocyanidins showed the same color and identical spectra (Fig. 4).

To a small portion of the anthocyanidin in n-pentanol were added a few drops of 5% sodium acetate solution. The color of the aqueous layer turned red-violet, and on further addition of a drop of 10% $FeCl_3$, the color turned bright blue. The same color reactions were given by the cyanidin of roses.

When an aqueous solution of the unknown anthocyanidin was shaken with an equal volume of toluene:cyclohexanol (5:1 by volume), a rose color was obtained in the organic phase. The same color resulted when the cyanidin was thus tested. This and the preceding test originate from the work of Robinson and Robinson (1931), and indicate the similarity of the two anthocyanidins.

Paper chromatography of the unknown anthocyanin and its anthocyanidin along with the cyanin and cyanidin from roses, and the strawberry pelargonidin and its glucoside was carried out on rectangular Whatman No. 1 paper using the usual solvent system. Development was allowed to proceed for six hours at 25°C., longer times causing excessive fading of the antho-

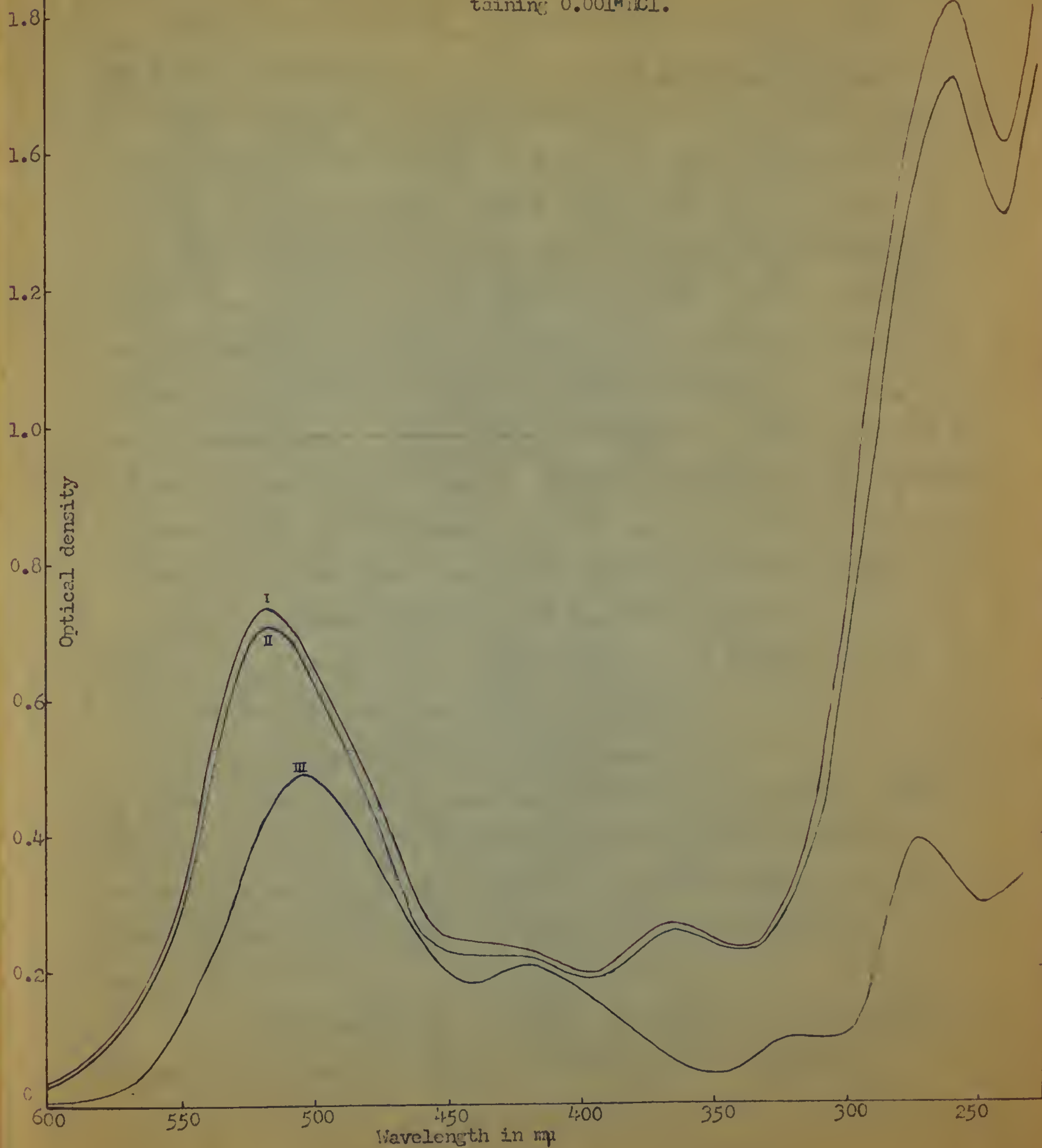

Fig. 4. Absorption spectra of cyanidin (I), pelargonidin (III) and the unknown anthocyanidin (II), in methanol containing 0.001% HCl.

cyanidins, especially of the unknown one and the cyanidin. The R_f values obtained are reported in Table 4.

In another experiment, the cyanidin of the idaein extracted from the European cranberry (<u>Vaccinium</u> <u>vitis</u> <u>idaea</u>) was chromatographed concurrently with the unknown anthocyanidin. The modified solvent system and an 18-hour development were applied. The two pigments showed the same R_f values (0.49).

Since no malvidin pigments were available for comparison, use was made of the observations of Parkinson (1954) and Bate-Smith (1948) that cyanidin glycosides have low (0.2 to 0.3) R_f values, while malvidin glycosides show much higher R_f values (0.7 to 0.8) when a m-cresol:acetic acid:water system (50:2:48 by volume) is used as a developer. Chromatographing idaein (cyanidin 3-galactoside) and the unknown anthocyanin in this solvent, for 24 hours, gave the same low (0.27) R_f for both these pigments.

Thus, it seems apparent that the minor pigment of strawberries first reported, but not identified by Meschter (1953) is an anthocyanin whose aglycone is cyanidin.

<u>b. Identification of the Sugar Moiety</u>.

The aqueous solution remaining after the extraction of the hydrolyzed anthocyanin with n-pentanol was divided into two parts: one for the identification of the sugar component, the other for the detection of any organic acid which might be present.

The aliquot meant for the sugar work was neutralized with concentrated ammonia solution, and evaporated to dryness at

100°C. The sugar was extracted from the NH_4Cl cake by using dry pyridine held at 100°C. for 10 minutes. The pyridine extract was cooled, filtered, and the solvent evaporated, under reduced pressure, at a temperature not exceeding 40°C., as recommended by Walpress and Morrison (1949). The residue was dissolved in 10% isopropanol and aliquots were chromatographed on Whatman No. 1 and No. 4 papers, using n-butanol:pyridine: water (3:1:1.5 by vol.) as solvent in descending run for 24 hours. After drying, the chromatograms were sprayed with 3% p-anisidine hydrochloride solution in n-butanol, and heated for 5 minutes at 100°C. The R_f values of the unknown sugar and of several known sugars were thus determined and are reported in Table 5. Glucose, galactose and rhamnose have been reported as being most commonly present in natural anthocyanins (Sannié and Sauvain, 1952; McIlroy, 1951). Table 5 also shows the distances of the sugar spots from the origin, after a 36-hour development, in which the solvent dripped from the lower edge of the paper. In an effort to simplify the separation of the sugar from the NH_4Cl cake, absolute ethanol was tried as an extractant for the sugar, and the ethanolic extract was applied on the paper and chromatographed as above. The R_f values and distances thus obtained also appear in Table 5. Fig. 5 illustrates one of the sugar chromatograms.

Ascending paper chromatography was also tried, using Whatman No. 1 paper and the solvent, spray, and time and temperature of development applied to the descending technique. The

Table 5. Descending Paper Chromatography of Unknown and Known Sugars.

Sugar	R_f	Distance from origin in cm.	
	Whatman No. 1	Whatman No. 1	Whatman No. 4
Glucose	0.23	10.8	23.6
Galactose	0.19	8.9	21.0
Rhamnose	0.46	21.8	44.5
Fructose	0.27	13.2	28.5
Unknown (pyridine extraction)	0.19	8.8	20.7
Unknown (ethanol extraction)	0.18	8.9	20.8

Table 6. Ascending Paper Chromatography of Unknown and Known Sugars (Whatman No. 1)

Sugar	R_f
Glucose	0.58
Galactose	0.53
Galactose from idaein	0.52
Unknown sugar	0.51

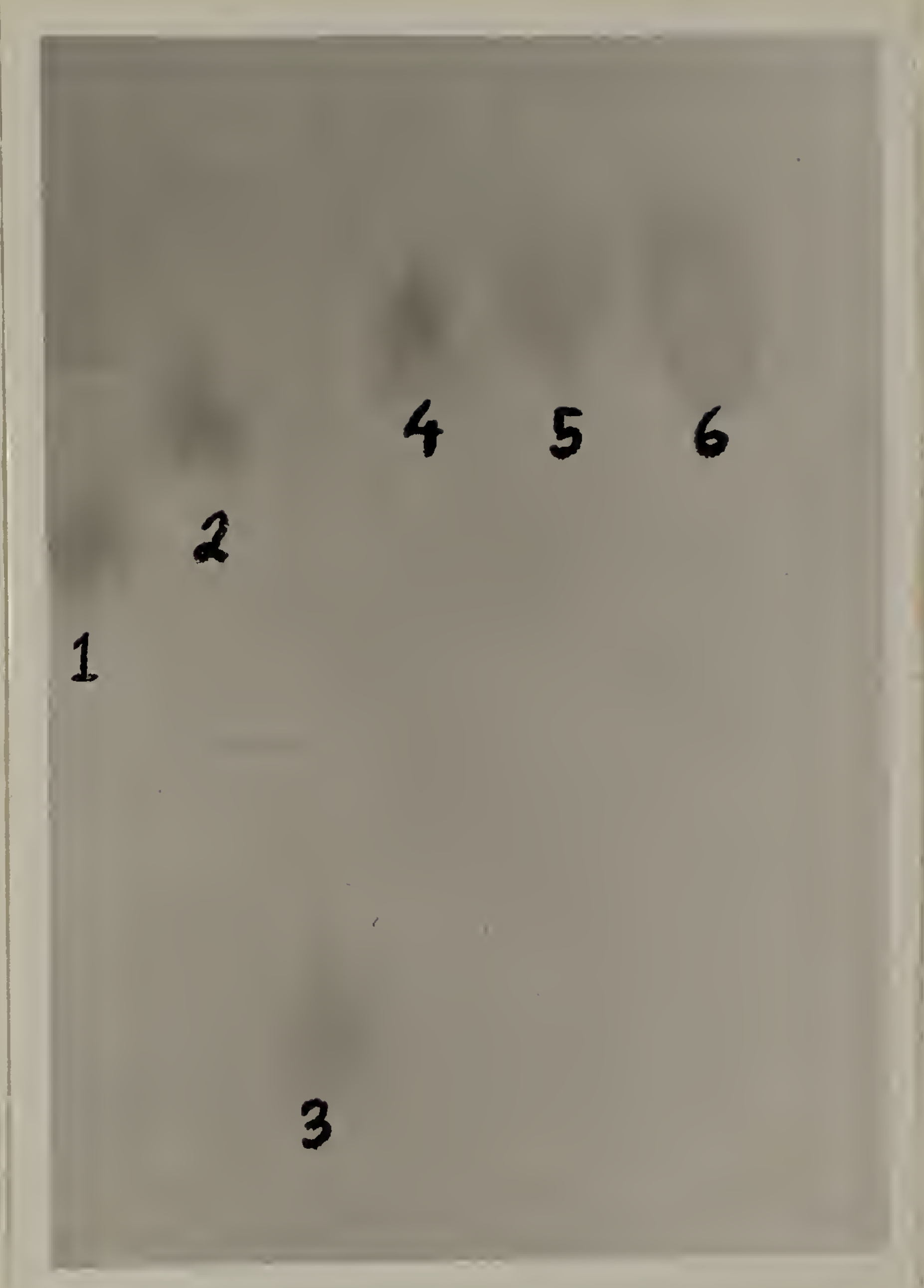

Fig. 5. Paper chromatogram of sugars.

1.Fructose 2.Glucose 3.Rhamnose 4.Galactose
5.Unknown sugar (pyridine method) 6.Unknown sugar (ethanol method)

R_f value of the unknown sugar, and that of known sugars, one of which was galactose obtained by acid hydrolysis of idaein chloride, appear in Table 6.

In order to liberate the sugar without subjecting the pigment to the severe conditions of the acid treatment, enzymatic hydrolysis of the glycosidic bond was attempted. Idaein, being a cyanidin 3-β-galactoside, appeared to be the anthocyanin most resembling the minor pigment of strawberries. It was speculated that β-glucosidase, being an enzyme specific for the steric glycosidic configuration, rather than the entire structure of the substrate, might hydrolyze the minor strawberry pigment. A system containing one mg. of the enzyme and 0.[illegible] mg. of the pigment in 5 ml. McIlvaine's citrate-phosphate buffer, pH 4.[illegible], was prepared and incubated at [illegible]2°C., overnight. After the incubation the system was deionized, using Amberlites IRC-50 and IRC-410,[5] and an aliquot was chromatographed, using Whatman No. 1 paper and the same conditions as in the descending chromatography of the acid hydrolysate. A very faint spot, showing the same R_f as galactose, was obtained.

A much clearer spot, also corresponding to galactose, was observed, when the hydrolysis was effected by the anthocyanase preparation, CN-7[illegible] of Rohm and Haas[5] (Huang, 1955), in 0.05M citrate buffer, pH 3.9. The reaction system contained two mg. of the enzyme preparation and 0.1 mg. of the pigment in 5 ml.

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(5) Obtained from Rohm and Haas, Inc., Philadelphia, Pa.

of the buffer. After an incubation at 12°C., for 1[illegible] hours, the system was deionized, using the pyridine extraction method (Malpress and Morrison, 1949), and the sugar extract was chromatographed, using Whatman No. 1 paper and the same conditions as in the descending chromatography of the acid hydrolysate. A control, consisting of the above reaction system, but containing no pigment, was subjected to the same treatment; no spot, attributable to the presence of sugar, was observed in the control.

From the data presented above, it is quite apparent that the sugar present in the minor pigment is galactose.

<u>c. Test for the Presence of Organic Acids.</u>

The aliquot from the acid hydrolysate of the minor anthocyanin, reserved for the detection of organic acids, was extracted twice with ether and the combined extracts were evaporated to dryness. No residue was left, indicating the absence of organic acids in the molecule of the anthocyanin.

<u>d. Identification of the Glycoside.</u>

The monoglycosidic nature of the minor pigment is suggested by its R_f value (0.38), which is intermediate between those of the diglycoside, cyanin (0.28), and the aglycone, cyanidin (0.55). Bate-Smith (1948, 1950) was the first to establish this relationship between R_f values and the extent of sugar substitution in anthocyanins.

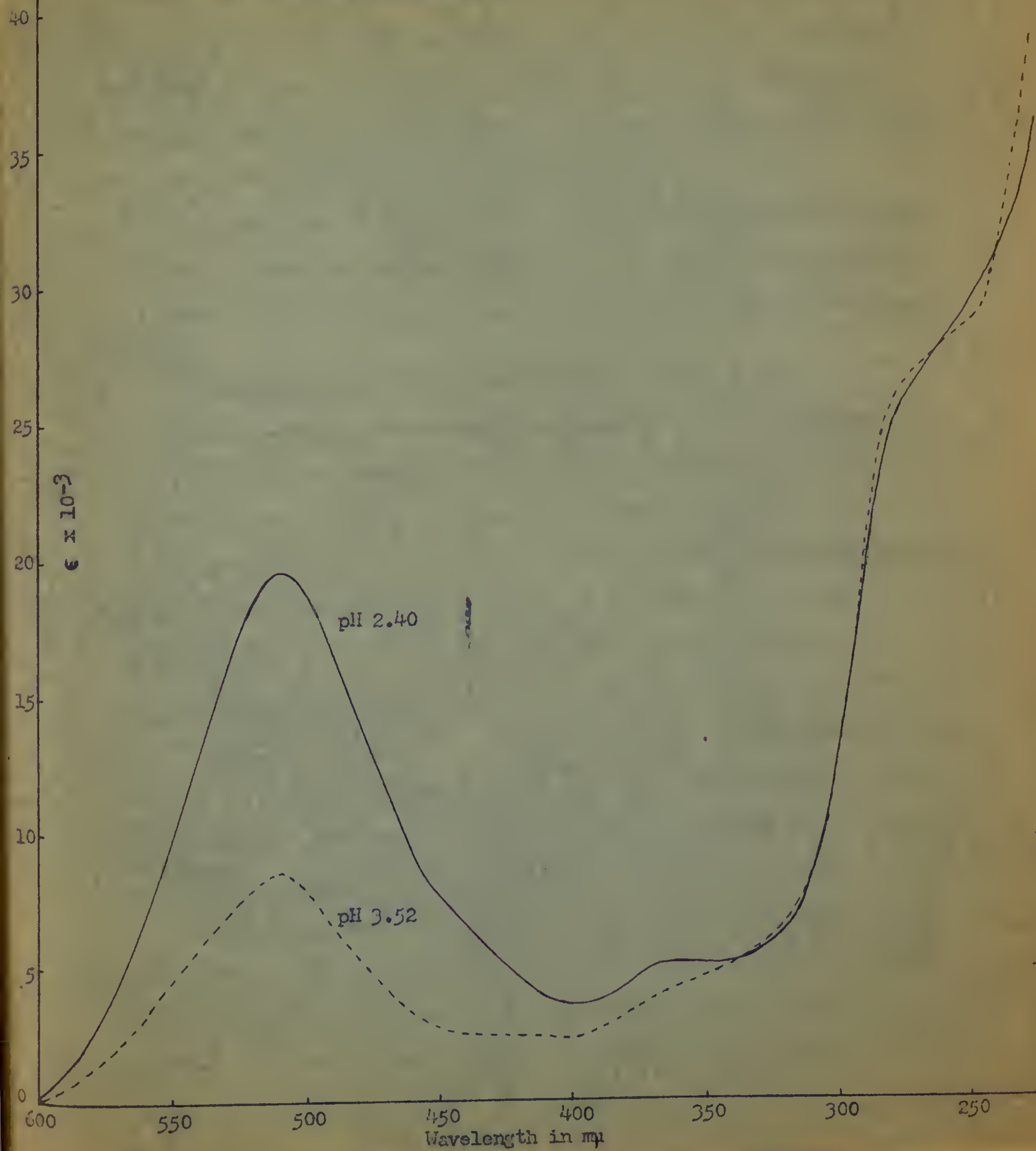
As a further test, the cyanidin galactosides from the leaves of the red beech tree (Robinson and Robinson, 193[illegible]), from the skin of Stayman Winesap apples (Duncan and Dustman, 1936; [illegible], 1937), and from the European cranberry (Willstaetter and

Mallison, 1915) were chromatographed concurrently with the strawberry minor anthocyanin. R_f values were obtained which did not vary more than [illegible] from each other.

The following characterizing reactions, based upon the work of Willstaetter and Mallison (1915), support the evidence that the minor pigment of strawberries is a cyanidin monogalactoside: Alkalinization of the pigment solution using NaOH caused a blue color, turning to green and then yellow within a few minutes. Addition of $FeCl_3$ to an ethanol solution of the pigment caused a blue color to appear. Addition of copper acetate to an ethanol solution of the pigment caused a blue color, while the same reagent, when added to an aqueous solution of the pigment, caused the formation of a red-violet precipitate.

The visible spectrum of the pigment, both in aqueous and methanolic solutions, is similar to that of the known cyanidin 3-galactosides. The maximum of absorption is at 510 mμ in aqueous acidic solution, and at 530 mμ in methanolic solution containing 0.1% HCl. In the ultraviolet region, however, the apple and cranberry galactosides exhibit an absorption maximum at 280 mμ and a minimum at about 255 mμ. This band is less marked in the red beech galactoside spectrum, while in the absorption spectrum of the minor strawberry pigment it is reduced to a mere inflection. Fig. 6 shows the molecular extinction curves of the minor strawberry pigment in aqueous solution at the pH of [illegible].40 and 3.52, adjusted with hydrochloric acid. The calculation was based on the formula $C_{21}H_{21}O_{11}Cl$ for the vacuum dried substance.

Fig. 6. Molar extinction curves of cyanidin galactoside from strawberries, at pH 2.40 and 3.52, in aqueous solution containing HCl.



The optical rotation of a methanolic solution containing 0.001 gram of pigment per ml. was -0.25° at $25^{\circ}C.$, for either the sodium or white light, using a 1 dm. tube. This corresponds to a specific rotation of -250°. Practically the same rotation was obtained for an aqueous solution of the pigment under similar conditions. The strong laevo-rotation is indicative of a β-glycosidic configuration.

Testing the stability of the minor strawberry pigment in aqueous solution against $FeCl_3$, it was found that this galactoside and its aglycone were decolorized at comparable rates.

Spectrophotometric and Chemical Study of the Degradation of Pelargonidin 3-glucoside (Callistephin)

Callistephin being the pigment primarily responsible for the red color of strawberries, its deterioration was investigated spectrophotometrically in the following experiment, using simple model systems. Reactions, characteristic of possible breakdown products of the pigment were also carried out.

Spectral Changes

Since the crystalline pigment, as well as the purified pigment on paper chromatograms, did not show any signs of deterioration when stored dry over a period of 18 months, stability in aqueous solution of the pigment was first considered.

Distilled water solutions of the crystalline pigment were prepared, (p.33) and the effect of pH adjustment was studied as a first variable. To avoid possible interference, no buffers were used. The pH was adjusted to three levels by means

of hydrochloric acid. Small shifts of the pH during the subsequent treatment would not seriously affect the results.

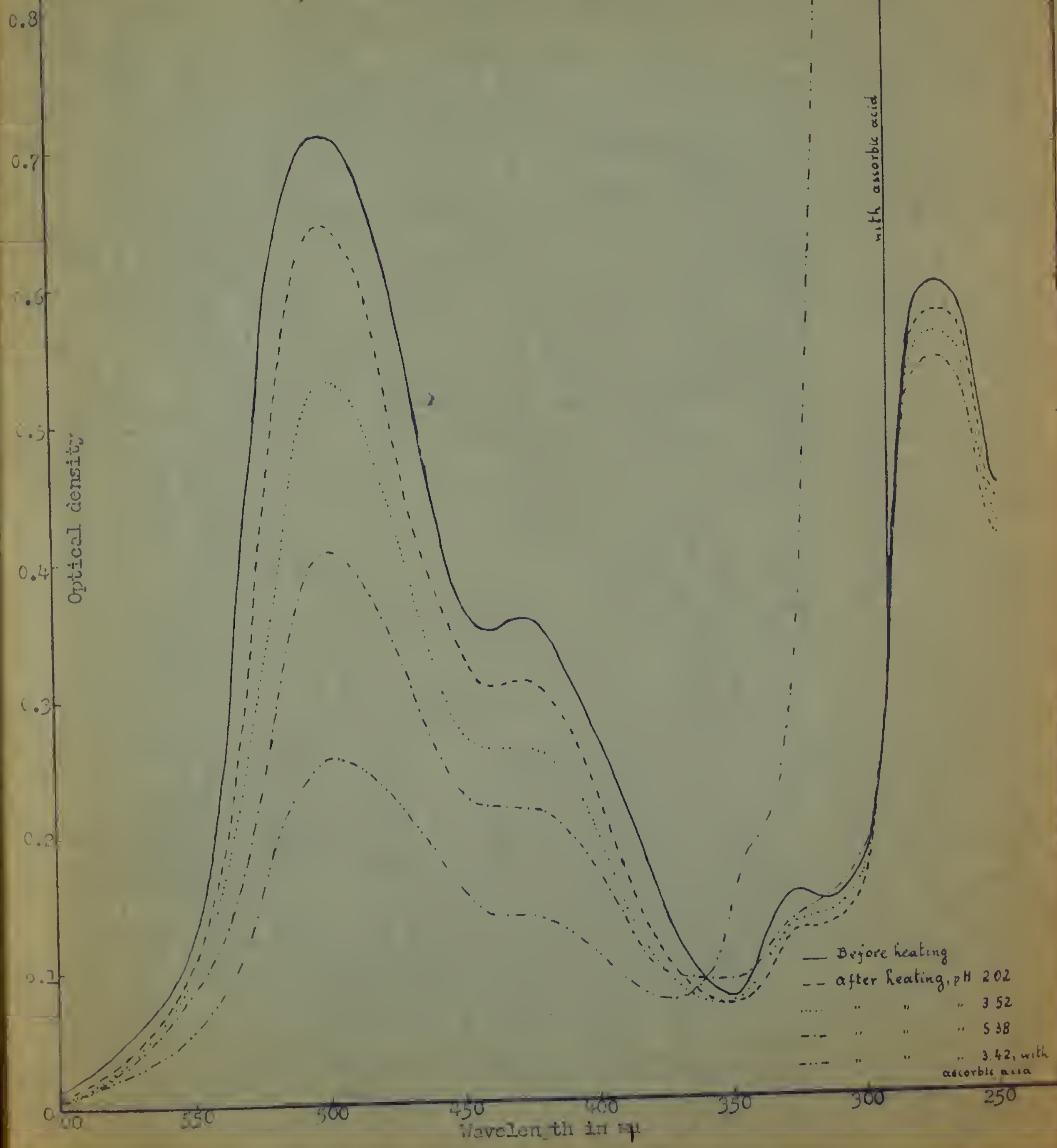
In view of the importance of ascorbic acid in the strawberry anthocyanin degradation (p.18), this vitamin was introduced into one of the above solutions in chemically pure form. Accordingly, the following four aqueous solutions were prepared:

1. 0.15 mM callistephin chloride, pH 2.02
2. " " " " , pH 3.52
3. " " " " , pH 5.38
4. " " " " , pH 3.42, 3 mM ascorbic acid.

The absorption spectra of these solutions were determined using a Beckman Model DU Spectrophotometer and 1 cm. silica cells. Two ml. of each solution were diluted to 10 ml. with citrate-hydrochloric acid buffer of pH 2.00, and the pH checked to insure it was 2.00. The spectral transmittance was measured after allowing the solutions to stand at room temperature for an hour.

Spectral measurements in the range of 220 mμ to 600 mμ were made immediately after preparation of the solutions, again after heating at 100°C. for 20 minutes 3 ml. aliquots in flame-sealed 15 x 125 mm. Pyrex test tubes, and a third time after storing the heated solutions at 35°C. for 7 days. The solution at pH 3.52 was examined a fourth time after it became visually colorless (about 5 weeks at 35°C.). Since a fine brown precipitate could be seen in this particular solution, the supernatant liquid only was examined. The spectra are shown in Fig. 7 and 8.

Fig. 7. Absorption spectra of callistephin chloride in aqueous solution, before and after heating (100°C, 20 min.), at various pH, with or without ascorbic acid. (All determinations were made at pH 2.00)



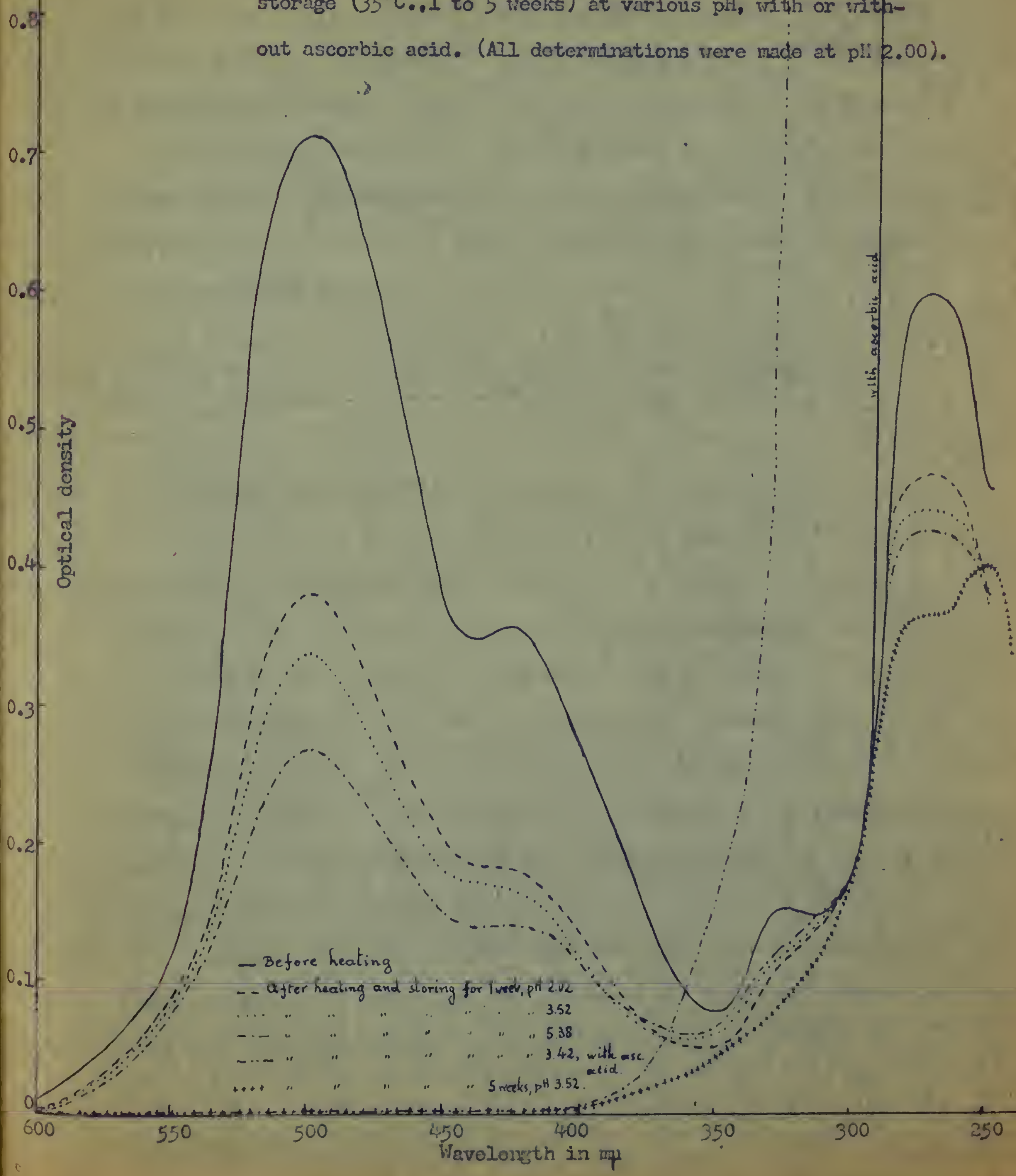
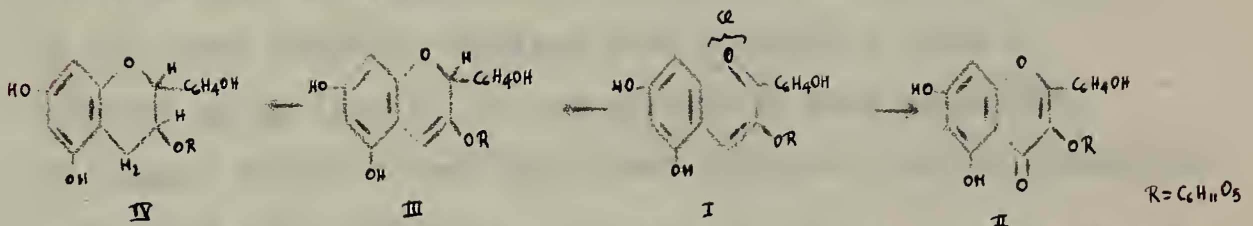


Fig. 8. Absorption spectra of callistephin chloride in aqueous solution, before and after heating (100°C., 20 min.) and storage (35°C., 1 to 5 weeks) at various pH, with or without ascorbic acid. (All determinations were made at pH 2.00).

The Degradation Reactions.

If the decolorization products of the anthocyanin were similar to the natural leucoanthocyanins, treatment with hot 20% HCl should return the color (Robinson and Robinson, 1933). This test was tried with solutions decolorized both in the absence and presence of ascorbic acid, with negative results.

If, on the other hand, the pigment (I) were oxidized to a flavonol (II), or reduced to a hydroanthocyanidin (III), and eventually to a catechin (IV), the following reactions should have been positive:



Pigment solution, decolorized in the presence or absence of ascorbic acid, was evaporated under vacuum at 50°C. and the residue was extracted with acetone. The extract was divided into two equal portions. To one portion was added a mixture of equal parts of acetone saturated with boric acid, and 10% citric acid in acetone, to the other portion, the control, was added a mixture of equal parts of acetone and 10% citric acid in acetone. No color was developed in the boric acid containing portion, as compared with the other portion, indicating absence of flavonol (Wilson, 1939).

Although the air had not been removed from the reaction tubes, additional aeration was produced by bubbling oxygen into the solution after decolorization, in an attempt to

restore the color, as in previously described reduction experiments (p.16). No color return was observed.

Catechins are known to turn yellow on treatment with NaOH in solution, and to become green-black with $FeCl_3$ (Encycl. Chem. Technology, 1954). These tests were also negative with the pigment degradation products.

The Brown Precipitate.

When any of the pure pigment solutions used in this study were allowed to stand long enough, a red-brown precipitate settled out. This precipitate displayed the characteristics of the brown sediment obtained from strawberry juice by Pederson et al (1947). It was soluble in NaOH solutions, yielding a yellow rather than brown solution, with an absorption maximum at about 420 mμ.

It was also attempted to dissolve the precipitate of the pure pigment solutions in ether, petroleum ether, acetone, conc. HCl, conc. H_2SO_4, sirupy phosphoric acid, and 5% $NaHCO_3$ solutions with no success. However, methanol dissolved part of the precipitate, the residue being soluble in 5% NaOH solution. The volumes of these two solutions were made equal and the absorption spectra determined (Fig. 9).

Hydrolysis of the Glycosidic Bond.

At several time intervals during the decolorization process, the pure pigment solutions were extracted with ethyl acetate to detect the presence of aglycone liberated as a result of hydrolysis of the glycosidic bond. No color was taken up by the ethyl acetate, indicating that no such

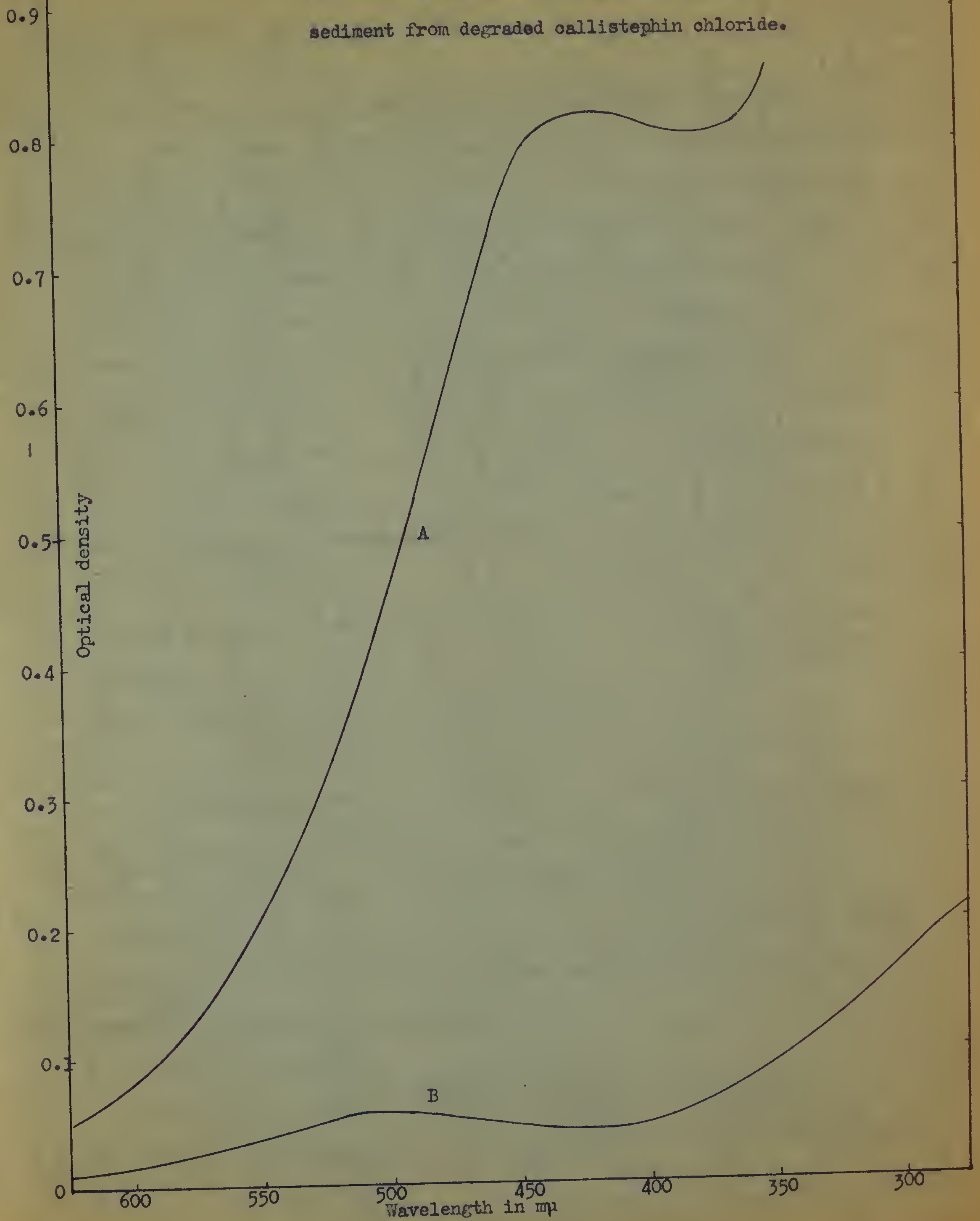


Fig. 9. Absorption spectra of the alkali-soluble (A) and methanol-soluble (B) fractions of the sediment from degraded callistephin chloride.

hydrolysis had occurred.

Kinetics of the Degradation of Callistephin Chloride.

Work on the kinetics of the strawberry pigment degradation has been reported by Sondheimer and Kertesz (1952, 1953), and by Meschter (1953). The former authors (1952) studied the kinetics of the reaction callistephin chloride-hydrogen peroxide and found it to be of second order, when the two substances were in nearly equimolecular amounts, and of "pseudofirst" order, when the hydrogen peroxide was in excess. In a later paper (1953), the same authors presented evidence, based on rate studies, that the destruction of pelargonidin 3-glucoside is an indirect effect of the air oxidizing the ascorbic acid in model systems and strawberry juice with production of H_2O_2, which in turn oxidizes the pigment. In the absence of air, however, they had to assume that "the pigment destruction in strawberry juice proceeds primarily by yet unknown mechanisms not involving ascorbic acid". Meschter (1953), working with strawberry juice, strawberry preserves, and a partially purified strawberry anthocyanin preparation, found first order reaction rates for the decolorization of the pigment at various temperatures, pH values, salt concentrations, ascorbic and dehydroascorbic acids concentrations, and sugar degradation products concentrations.

The Reaction Rates.

In this experiment the rate of degradation of pure callistephin chloride was studied in citrate-hydrochloric acid

buffer solutions, 0.05 M in disodium citrate, at two pH values and five temperatures. The pigment was prepared by the chromatographic method described on p. 31. Concentrations of pigment of the order of 0.1 mM were used. The change in pigment concentration was followed colorimetrically, using the reference curves of Fig. 10 and a Beckman model DU Spectrophotometer set at a slit width of 0.05 mm. The method of Sondheimer and Kertesz (1948) for determination of the pigment, in which the difference of optical densities at two pH values is related to pigment concentration, was found to give the same results as the relationship of optical density at one pH to pigment concentration. Therefore, all optical density measurements were made at the pH of the original solution.

The two pH values used for the original solutions were 2.00 and 3.40, and the five treatment temperatures were 110°, 100°, 80°, 60° and 45°C. About 3 ml. of pigment solution was placed in each of a number of Pyrex tubes 15 x 125 mm.; the tubes were sealed in an oxygen flame, and immediately immersed into a constant temperature oil bath; an air incubator was used for the 45°C. treatment. At suitable intervals of time two tubes were cooled in ice water, their content diluted with the buffer, and the optical density of an aliquot measured. The lag time correction for heating in the oil bath was calculated (Ball, 1928) and found to be four minutes.

When the per cent retention of pigment was plotted against time on a semilogarithmic paper, curved lines were obtained,

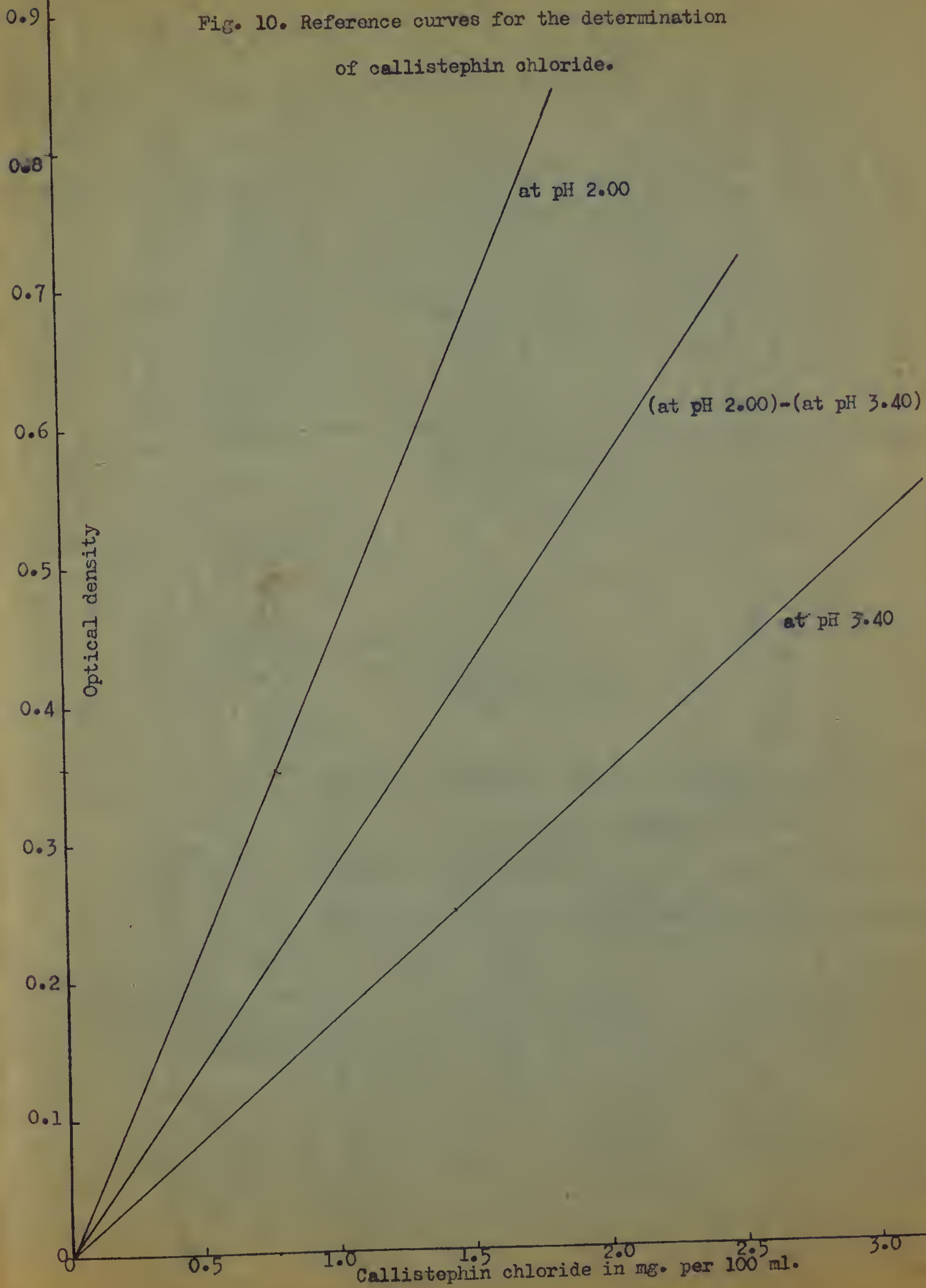


Fig. 10. Reference curves for the determination of callistephin chloride.

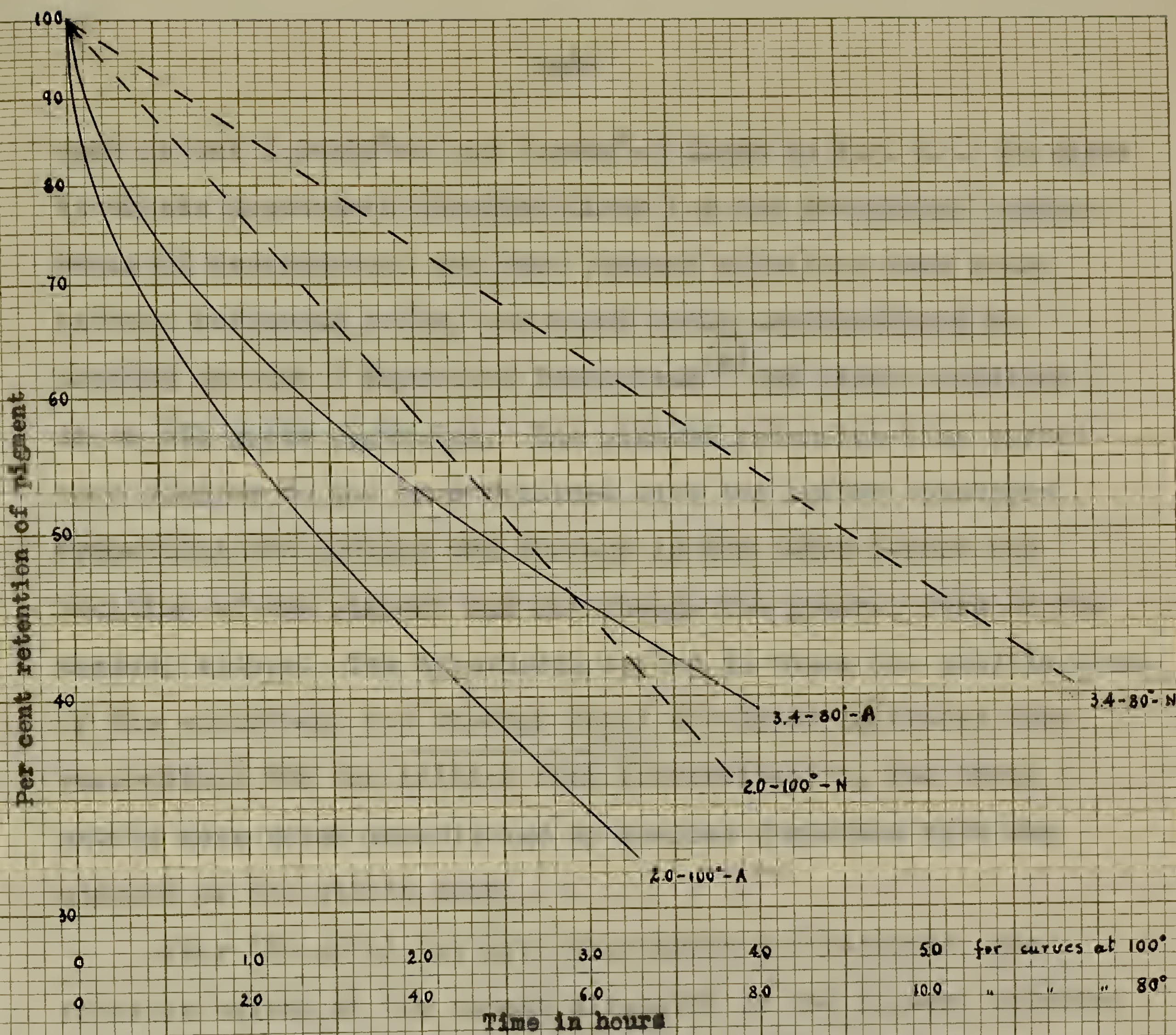


Fig. 11. Destruction rates of callistephin chloride in citrate buffer solutions.

(The first number indicates pH, the second, temperature in °C. A = air present in solution, N = air replaced by nitrogen.)

such as the $2.0-100^{\circ}$-A and $3.4-80^{\circ}$-A lines in Fig. 11. In order to obtain Meschter's straight lines for the strawberry pigment rates of destruction, new pure pigment solutions were made, without buffering salts, and using water demineralized by passing through a Crystalab Deeminizer[6] and twice distilled in an all glass apparatus. The pigment retention-time curves were similar to the ones obtained with the buffer solutions. Preheating and cooling the buffers in the tubes before the addition of the pigment did not change the general form of the curves, either. The hypothesis tested in these two modifications of the experiment was whether metal catalysts in traces were responsible for the initial fast decolorization, and these metals were later inactivated by complex formation with the pigment or the citric ions.

After the metal catalyst hypothesis was rejected, the possible effect of the oxygen dissolved in the pigment solution was tested. Tubes of pigment in citrate buffer solution were drawn to an open capillary end and placed almost horizontal in a vacuum desiccator. The pressure was reduced to 3 cm. Hg, and nitrogen, bubbled through alkaline pyrogallol (.5% pyrogallol, 6% NaOH), was introduced into the desiccator. The evacuation and refill with nitrogen was repeated five times. Vilece (1953) could thus reduce the oxygen content in the head-space of test tubes to 0%. After sealing the tubes in the

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(6) Obtained from Crystal Research Laboratories, Inc., Hartford, Conn.

flame, and exposing them to the constant temperature treatments, pigment destruction rates were obtained which yielded the straight lines in Figs. 12 and 13.

When the time required for 50% pigment destruction was plotted against temperature, the thermal destruction-time (TDT) curves A and B of Fig. 14 were obtained.

The straightness of the rate curves is indicative of a first order reaction. The specific rates (k) of such a reaction can be calculated from the half life (t1/2) of the compound by means of the formula

$$k = \frac{0.693}{t_{1/2}} ,$$

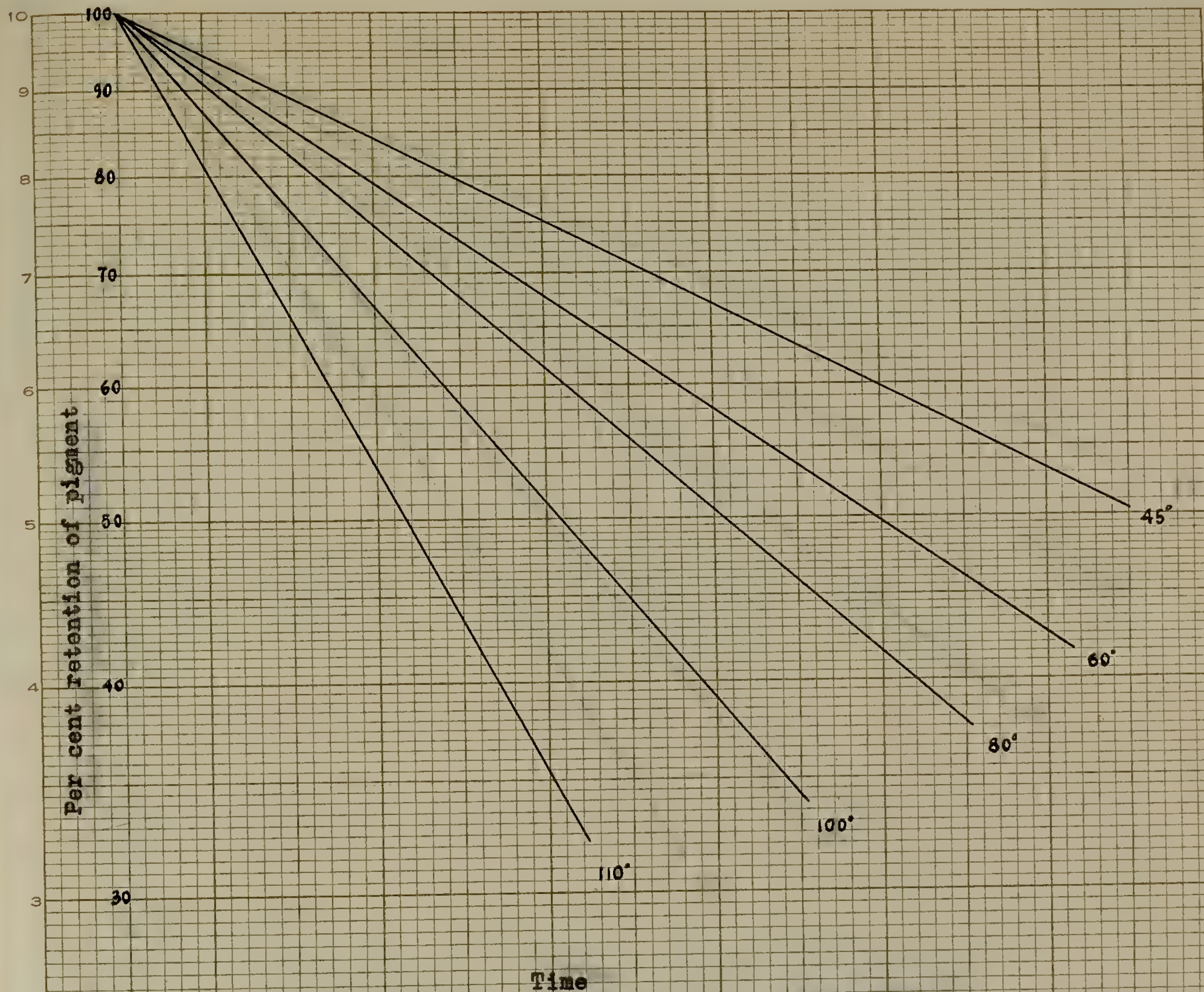
which is a corollary of the first order reaction equation,

$$\frac{dx}{dt} = k(a-x) ,$$

where a is the initial concentration of the reacting species, and x is the decrease of that concentration after time t. Table 7 gives the specific reaction rates at two pH values and three temperatures.

<u>The Temperature Coefficient of the Reaction.</u>

Since the TDT lines A and B of Fig. 14 are practically parallel, the temperature coefficient of the decolorization reaction must be the same at the two pH values used. Expressed as Q_{10} (ratio of the specific rates at two temperatures differing by 10°C.) this coefficient is about 2.85, while expressed as z (degrees of temperature covered by the TDT line traversing one logarithmic cycle) it is equal to about 22°C.



Time scales:

One division	equals	12	hours	at	45°
"	"	3.3	"	"	60°
"	"	0.5	"	"	80°
"	"	0.1	"	"	100°
"	"	0.05	"	"	110°

Fig. 12. Destruction rates of callistephin chloride in citrate buffer solution, pH 2.00, at various temperatures (°C). The air in solution was replaced by nitrogen.

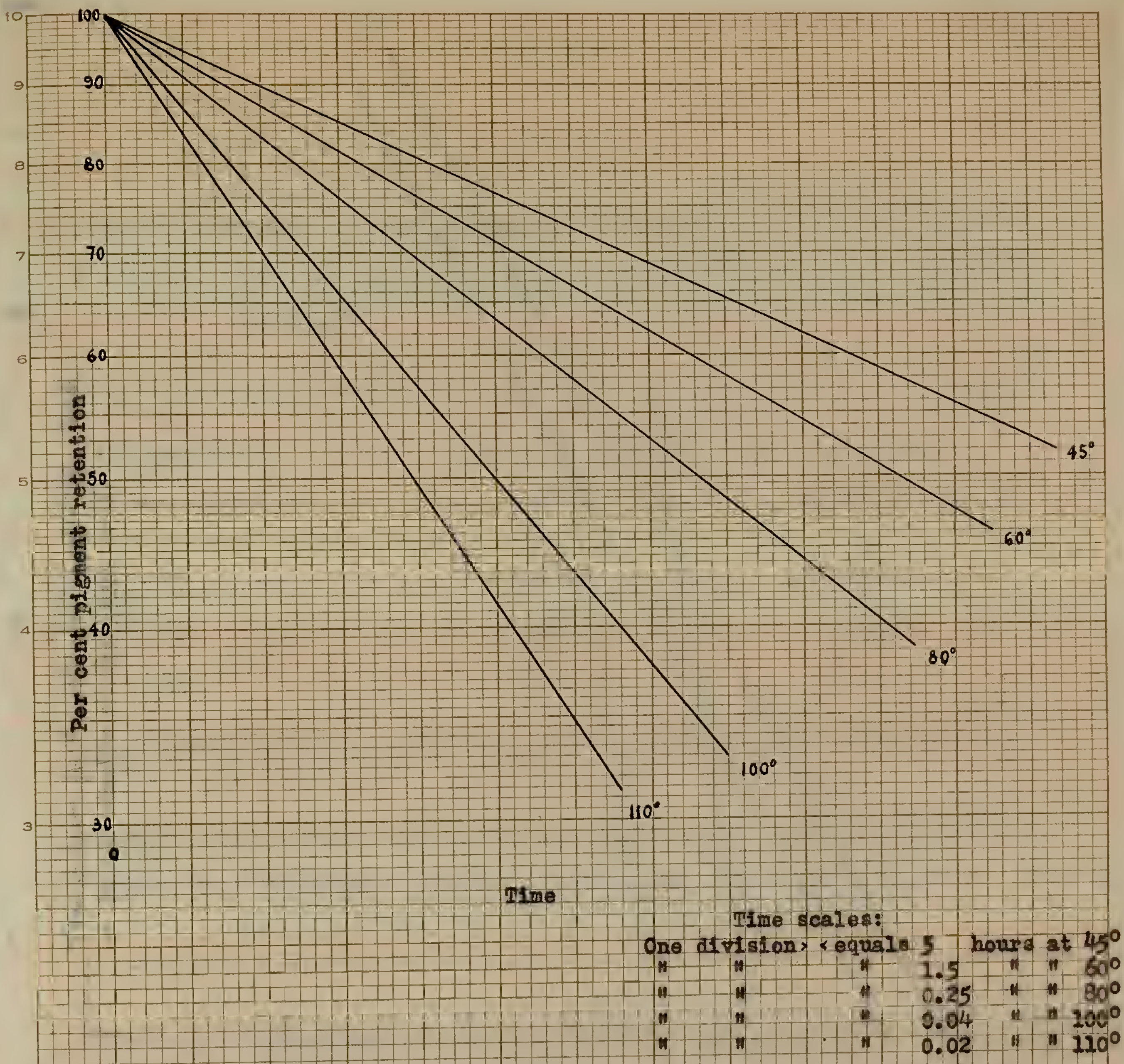


Fig. 13. Destruction rates of callistephin chloride in citrate buffer solution, pH 3.40, at various temperatures (°C). The air in solution was replaced by nitrogen.

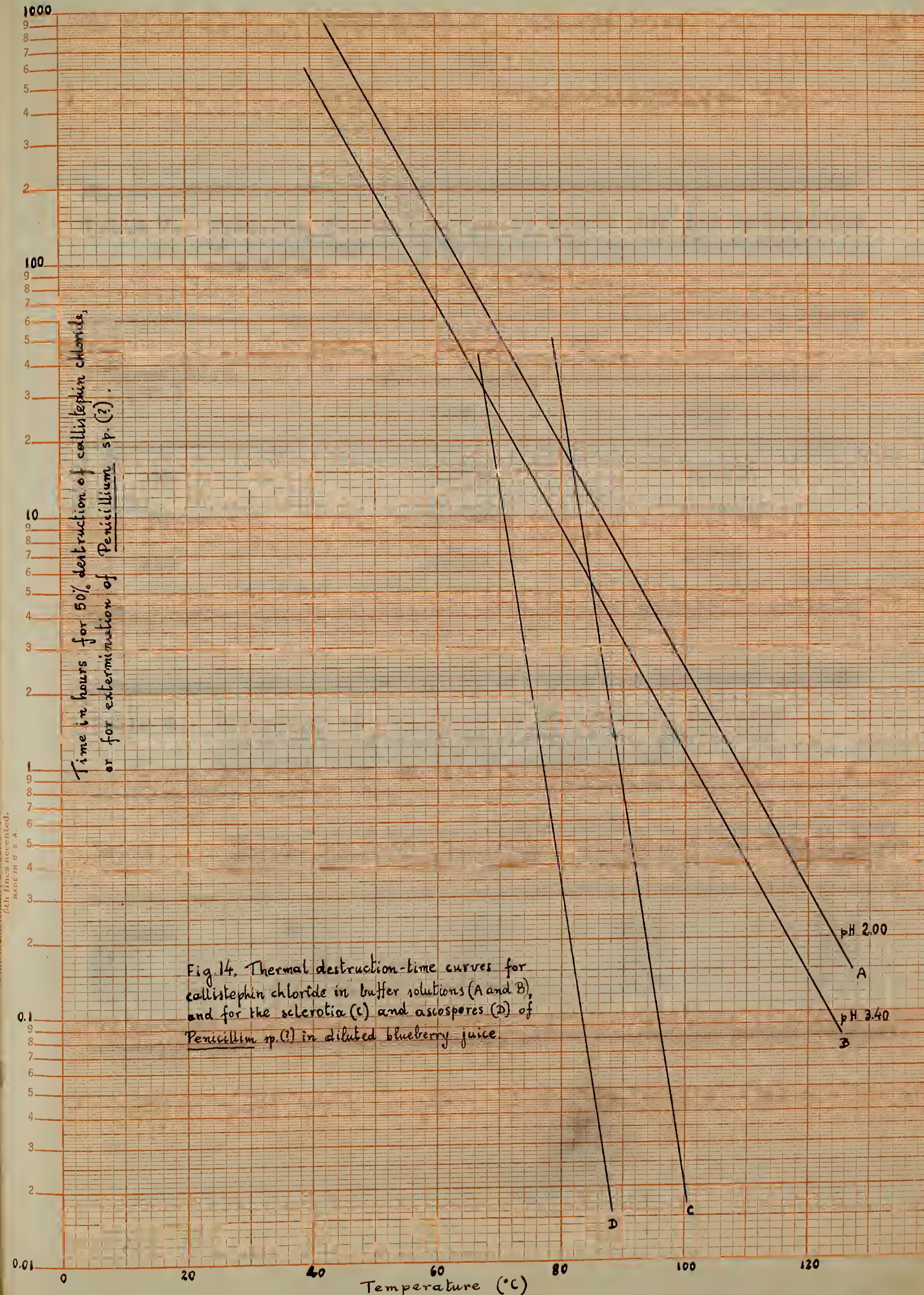


Fig. 14. Thermal destruction-time curves for callistephin chloride in buffer solutions (A and B), and for the sclerotia (C) and ascospores (D) of *Penicillium* sp.(?) in diluted blueberry juice.

Table 7. Specific reaction rates in hour^{-1} for the destruction of callistephin chloride in buffer solution under nitrogen.

Temperature (°C.)	pH	
	2.00	3.40
50	0.0016	0.0034
80	0.0364	0.0769
110	0.815	1.728

Table 8. Retention of callistephin chloride in buffered (citrate) and unbuffered solutions containing metallic ions at 0.[illegible] mM concentration.

Metal	% Retention			
	Heated for 1 hr. at 100°C.		Heated for 240 hrs. at 45°C.	
	Unbuffered	Buffered	Unbuffered	Buffered
Control	39.5	40.0	41.5	48.0
Fe^{++}	10.0	33.0	29.5	47.0
Fe^{+++}	5.8	27.0	23.6	48.0
Cu^{++}	4.3	15.6	18.8	47.5

The pH Coefficient of Reaction.

The degradation of the major strawberry pigment under the conditions of this experiment appears to have a "pH coefficient of reaction", which should be a measure of the change in specific rate with pH at a certain temperature. The limited available data do not permit the calculation of an exact value for this coefficient; they only indicate that the specific rate increases by a factor slightly higher than [illegible] when the pH increases from [illegible].00 to 3.40, independently of the temperature used.

The Energy of Activation.

The energy of activation of the decolorization reaction, as calculated by means of the Arrhenius equation

$$\frac{d \ln k}{dT} = \frac{E}{RT},$$

is 27,000 calories per mole of pigment.

The Effect of Oxygen on the Absorption Spectrum.

The difference in rate of reaction between solutions with air present, and air replaced by nitrogen, suggested a comparison of the absorption spectra under these two different conditions of decolorization. The spectra of the solutions with air replaced by nitrogen, determined at various stages of decolorization, as for the solutions with air present (p.40), did not show any significant differences between the two.

Similarly no differences could be observed between the absorption spectra of the brown sediments, obtained under "aerobic" and "anaerobic" conditions.

The Effect of Metallic Ions on the Pigment, and their Chelation by Citric Acid.

The effect of Cu^{++}, Fe^{++}, and Fe^{+++}, at the concentration of 0.2 mM, on the decolorization rate of pure callistephin chloride solutions, 0.1 mM in pigment, was studied at a pH of 3.40, in the presence and absence of 0.05M citrate buffer. The chlorides of Cu^{++} and Fe^{+++}, and the sulfate of Fe^{++} were used, all C.P. grade. Blanks, with no salts added, were also employed.

The solutions were transferred in 15 x 125 mm. Pyrex test tubes, 3 ml. in each, the tubes were sealed in the flame without replacement of the air by nitrogen, and subsequently, they were either heated for 1 hour at 100°C., or stored for 240 hours at 45°C. The pigment was determined colorimetrically at one pH, as described in p. 45. It was eventually necessary to centrifuge the solutions before taking their optical density, because of the dark precipitates which were formed with time, especially in the absence of citrate buffer. The results are presented in Table 8.

The Effect of 5-Hydroxymethyl-2-furfural on the Pigment.

The formation of 5-hydroxymethyl-2-furfural (HMF) upon heating with acids in solution is a reaction characteristic of hexoses. Since the latter are present in strawberries, and added, or formed from sucrose in the preparation of strawberry spreads, the effect of this very reactive aldehyde on the pigment was investigated.

Meschter (1953) found that addition of HMF, or of furfural,

which is the corresponding degradation product of pentoses, to buffered solutions of a crude preparation of strawberry pigment resulted in faster color loss than in similar solutions with no aldehydes added.

In the experiment described herein, crystalline HMF[7] was added at the concentration of [illegible]0 mM to 0.05M citrate buffer, pH 3.40, containing 0.1 mM pure callistephin chloride. The pigment was prepared by the chromatographic method described in p.31. A blank, with no HMF added, was also used.

Three ml. samples were transferred into Pyrex tubes 15 x 1[illegible]5 mm., which were then sealed in the flame, heated for 5 minutes at 100°C., and stored at 40°C. Pigment determinations were made before the tubes were sealed, and after [illegible], 4, 8, 16 and [illegible]5 days of storage, using the method of Sondheimer and Kertesz.(1948).

The resulting data are graphically presented in Fig. 15.

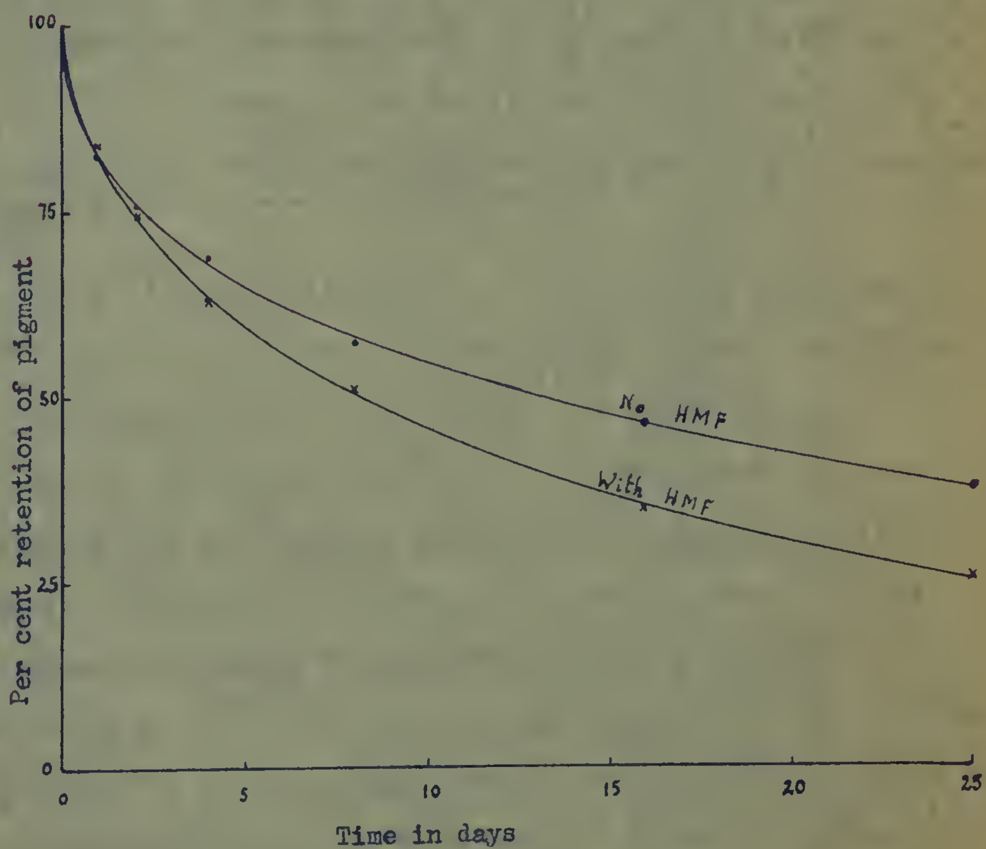
Stabilization Experiments.

Additives.

As a practical approach to the problem of fixing the natural red color of strawberry products, various chemical substances were added to strawberry juice, and to 0.05 M citrate buffer solutions, 0.1 mM in pure callistephin chloride. The additives used were selected mainly because of their high or

(7) Synthesized by M. A. Steinberg, University of Massachusetts, Amherst, using the method of Haworth, W. N. and Jones, W.G.M., J. Am. Chem. Soc. 66, 667 (1953).

Fig. 15. The effect of 5-hydroxymethyl-2-furfural on callistephin chloride in citrate buffer, pH 3.40, during storage at 40°C.



low oxidation-reduction potential, or of their metal-chelating properties.

The juice and the buffer solutions had the same pH of 3.42. Three ml. samples were placed into 15 x 125 mm. test tubes, which were then cotton-plugged and heated for 5 minutes at 105°C. Melted paraffin was added on top of the plugs, to prevent evaporation, and the tubes were stored at 35°C. Before heating, and after 12 days of storage, the samples were suitably diluted with citrate buffer of pH 3.42, and the optical density of the dilutions was determined at 500 mμ using a Beckman Model DU Spectrophotometer. Assuming that Beer's law was obeyed in all cases, the degree of color retention was as presented in Table 9.

In a later experiment, the color-stabilizing effect of phytic acid and of $AlCl_3$ were investigated. Phytic acid has been reported to prevent fading of the red color of sour cherries (Cohee and Nelson, 1952). Al was tried because of the possibility of forming, by means of its electronic sextet, an addition product with the oxygen of the pyrylium ring, thereby stabilizing the pigment molecule (Ketelaar, 1953).

Strawberry juice was used, and the phytic acid[8] was added at the levels of 0.5 and 1.0 per cent. The method of Sondheimer and Kertesz (1948) was used for the determination of the pigment.

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(8) Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. It contained 60% solids.

Table 9. Effect of additives on the retention of color of citrate buffer solutions of major strawberry pigment and strawberry juice, after heating at 105°C. for 5 min. and storing at 35°C. for 12 days.

Additive	Concentration (%)	% Retention (at 500 mμ) Buffer	Juice
Control	-	75.5	68.0
D,L-alanine	0.1	72.5	65.0
L-ascorbic acid	0.2	4.2	39.2
Calcium pantothenate	0.002	75.0	70.0
D,L-cystine	0.002	73.0	80.2
Gallic acid	0.2	75.0	82.0
Kojic acid	0.2	71.5	63.5
Phosphates after Hall (1949)	0.67	69.8	69.5
Propyl gallate	0.2	80.0	73.0
Quercetin	0.1	73.0	72.0
Riboflavin	0.001	59.[illegible]	69.0
Rutin	0.1	74.2	72.0
Versene (disodium salt)	0.002	75.6	65.0
Stannous chloride	0.002	75.5	65.0
Stannic chloride	0.002	75.[illegible]	64.5
Tannic acid	0.2	71.5	8[illegible].0
Thiamine	0.001	75.3	66.0
Thiourea	0.2	86.5	132.0

Heating the samples in sealed tubes for 5 minutes at 100°C. and storing them at 35°C. for three weeks did not reveal any appreciable protective or deleterious effect of the additives on the pigment of the juice.

Ascorbic Acid Oxidase (Ascorbase).

Strawberries contain considerable amounts of ascorbic acid, which, however is deleterious to the pigment (p. 18; also Table 9), once the living cells are ruptured. An attempt was made, therefore, to destroy the ascorbic acid in order to reduce the color loss. Ascorbase oxidizes ascorbic acid to dehydroascorbic acid without formation of H_2O_2, this peroxide being very destructive to the pigment (Sondheimer and Kertesz, 1951). Dehydroascorbic acid has also a detrimental effect on the pigment; its rate of reaction with the pigment, however, is slower than that of the ascorbic acid (Meschter, 1953).

100 ml. of strawberry juice, extracted from fruit kept frozen for 5 months, were used in one experiment. The juice contained 40.1 mg. of ascorbic acid, as determined by the method of Robinson and Stotz (1945), and 19.5 mg. of pigment, according to the analysis by the Sondheimer and Kertesz (1948) method. It had a pH of 3.45. Using a 20% NaOH solution, the pH was raised to 5.30, which is in the optimum pH-range for the activity of this enzyme, and 250 units of ascorbase[9] were added to the juice. Within 1 hour, at 25°C., the ascorbic

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(9) Obtained from Nutritional Biochemicals Corp., Cleveland 28 Ohio.

acid content of the juice was reduced to [illegible].3 mg%, after which the pH was restored to its original level by means of a 30% citric acid solution. In a preliminary experiment, restoration of the pH by using citric acid resulted in higher pigment retention than by using hydrochloric or phytic acids.

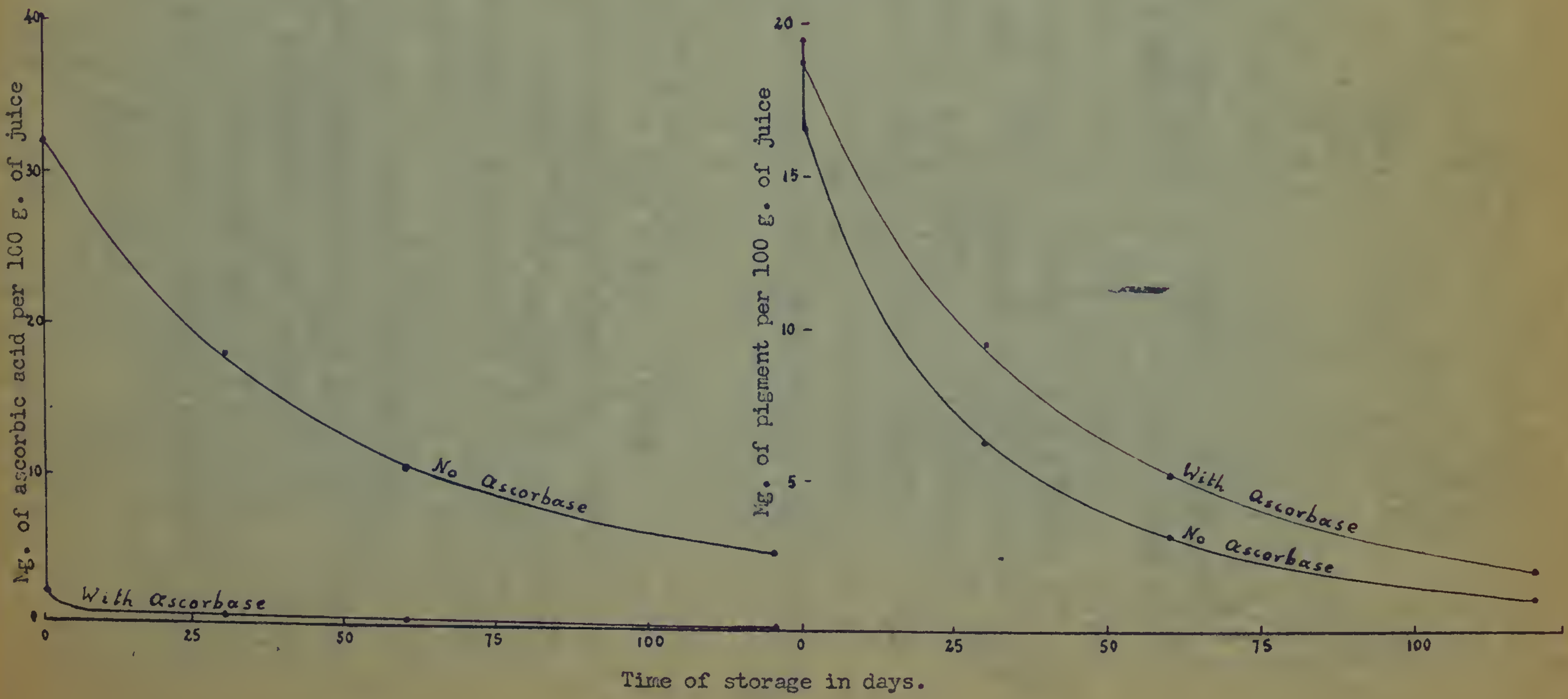
The enzyme-treated and the untreated juices, the latter after adjustment to the volume of the former by means of 1N citrate buffer of pH 3.45, were transferred to 15 x 125 mm. Pyrex tubes which were then sealed in the flame, heated for 6 minutes at 100°C., and stored at [illegible]2 ± 2°C. Determinations of the pigment and ascorbic acid contents were made before the enzyme treatment, after the enzyme and heat treatments, and at the 30th, 60th, and 120th day of storage, using the methods mentioned in the above paragraph. The results are presented in Fig. 16.

Oxygen and Ascorbic Acid.

In the study of the kinetics of the callistephin chloride degradation, it became apparent that replacing the air by nitrogen in the tube containing the solution resulted in higher retention of the pigment. This was attributed to a detrimental effect of the oxygen on the pigment. In the present experiment, the role of oxygen was investigated in combination with the effect of ascorbic acid on the pigment.

Six model systems, coded as shown below, were prepared and followed for content changes in pigment (P) and in ascorbic acid (A), in the presence (+) and in the absence (-) of each

Fig. 16. Retention of pigment and ascorbic acid in strawberry juice treated or non-treated with ascorbase, heated for 6 min. at 100°C., and stored at 22°C.



other and of oxygen (O).

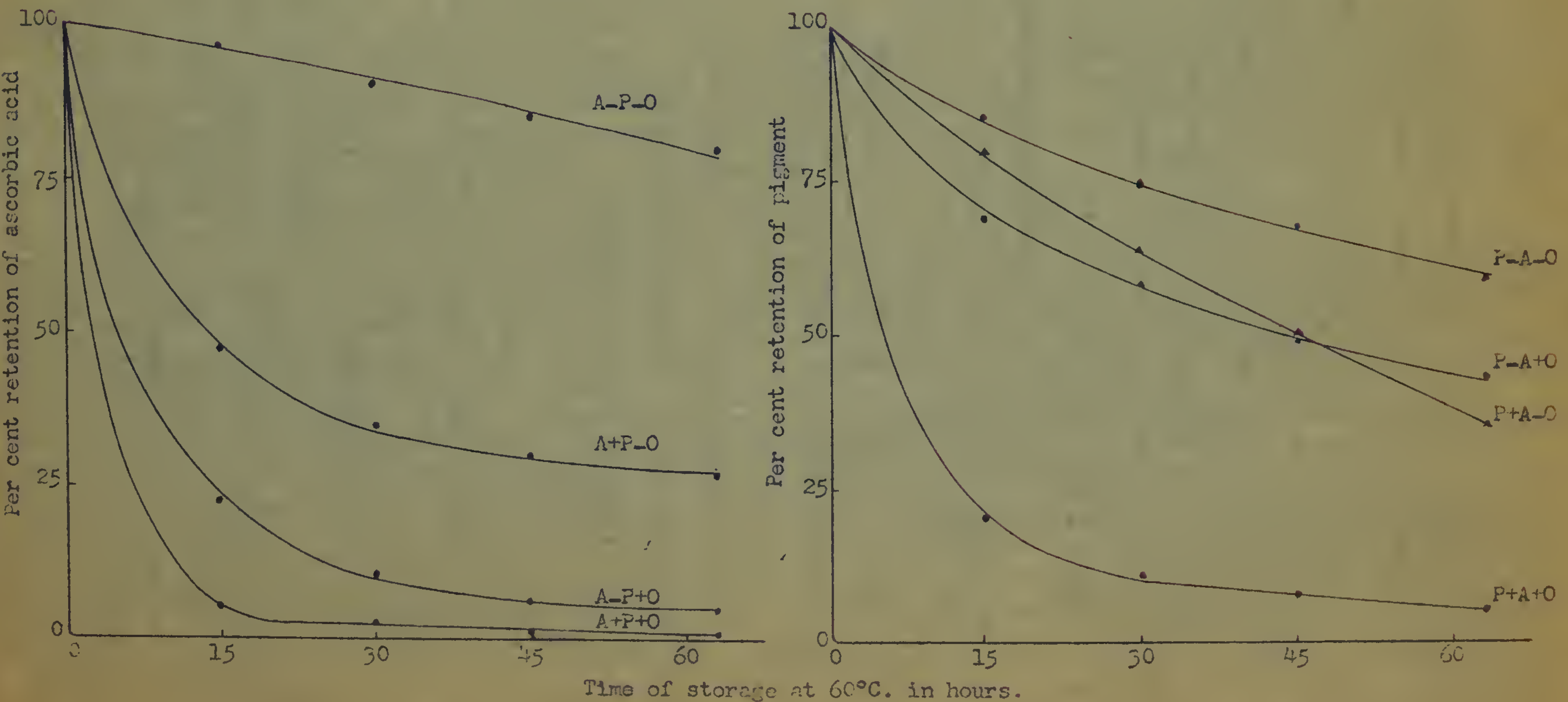
P+A+O
P+A-O
P-A+O
P-A-O
A-P+O
A-P-O

The systems contained the pigment in 0.15 mM, and the vitamin in 5 mM concentrations, in 0.05 M citrate buffer, pH 3.40. The air was replaced by nitrogen using the technique described on p.45. The methods of Sondheimer and Kertesz (1948) and Robinson and Stotz (1945) were used for the determination of the pigment and vitamin respectively. After sealing, the tubes (15 x 125 mm) containing the solutions were stored in an oil bath at 60°C., and determinations were performed after 0, 15, 30, 45, and 63 hours of storage. The results are presented in Fig. 17.

The Oxygen in Strawberry Juice.

In this experiment the retention of pigment and ascorbic acid was studied in strawberry juice stored with the air present in it or replaced by nitrogen. The juice was obtained from strawberries held frozen for 7 months, and it contained 18 mg% of pigment and 37 mg% of ascorbic acid. After its ascorbic acid content was raised to 60mg%, by adding vitamin of C.P. grade, the juice was distributed into 1[illegible] x 150 mm Pyrex tubes, 5 ml. in each. Half of the tubes were sealed with the air in, while the air in the other half was replaced by nitrogen before sealing. An aliquot of 10 ml. of juice was transferred into

Fig. 17. Retention of callistephin chloride (P) and ascorbic acid (A) in citrate buffer, pH 3.40, in the presence (+) or absence (-) of each other and of oxygen (O).



a 50 ml. cylinder, and used for bubbling oxygen through it. The tubes and the cylinder were incubated in an oil bath at 50°C. Oxygen was bubbled into the juice of the cylinder throughout the incubation the water lost through evaporation being frequently replaced. After 40, 70 and 160 hours of storage samples were taken from all three groups of juice and analyzed for pigment and ascorbic acid, using the methods of the preceding experiment. The results are presented in Fig. 18.

Ascorbic Acid Oxidase and Oxygen.

In this last stabilization experiment, the retention of pigment was studied in strawberry juice which did or did not receive the ascorbase treatment, and was subsequently stored under "aerobic" or "anaerobic" conditions, as in the preceding experiment.

The juice had the same composition as the one in the preceding experiment, its ascorbic acid content was raised to 60 mg% similarly, and the part of it which did not receive the enzymic treatment was tubed and sealed with the air left in or replaced by nitrogen as in that experiment. The part of the juice reserved for the enzymic treatment had its ascorbic acid content reduced to 3 mg% by the procedure used in the ascorbic acid oxidase experiment (p.52). It was then tubed and sealed in the same way as the control juice.

The storage temperature used was 60°C. Determinations of the pigment were made after 20, 40 and 60 hours of storage, using the Sondheimer and Kertesz (1948) method. The results are graphically shown in Fig. 19.

Fig. 18. Retention of pigment and ascorbic acid in strawberry juice with the air present (O), or replaced by nitrogen (N), or with oxygen bubbled (OB) through it, during storage at 50°C.

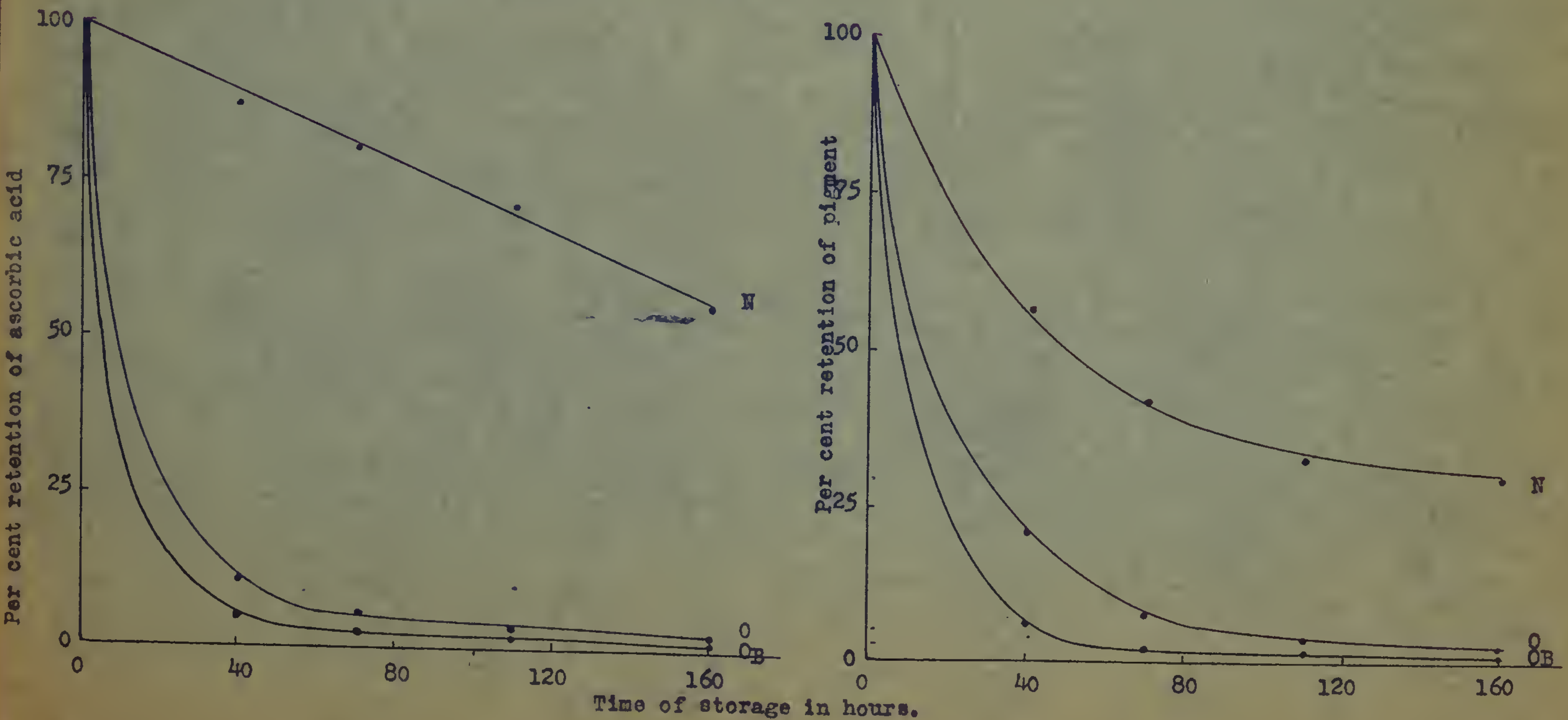
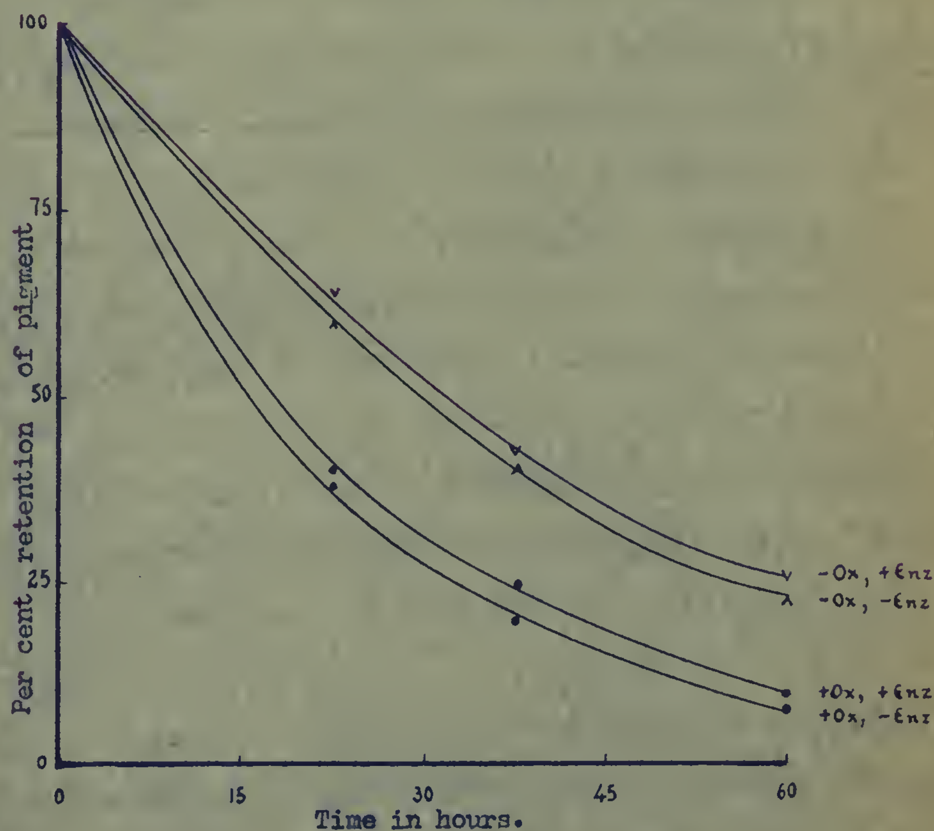


Fig. 19. Retention of pigment in strawberry juice treated (+Enz) or non-treated (-Enz) with ascorbase, and with the air replaced (-Ox) or non-replaced (+Ox) by nitrogen, during storage at 60°C.



Pelargonidin 3-monoglucoside-C^{14}

To facilitate the study of pigment degradation reactions in strawberry products, and specifically to provide a means of locating and isolating the degradation products, it was deemed desirable to obtain tagged anthocyanin pigment. Experiments were conducted which led to the biosynthesis and isolation of pelargonidin 3-monoglucoside-C^{14}.

Phytosynthesis of Radioactive Anthocyanin.

Five methods for introducing C^{14} into green strawberries on the plant were explored. They were: (1) injection of glucose-C^{14} solution into the stem, (2) injection of glucose-C^{14} solution into the berries, (3) absorption of glucose-C^{14} solution into the berries through their immersion in such solution, (4) application of small amounts of crystalline glucose-C^{14} to freshly made longitudinal cuts in the stems, the sugar being dissolved by the plant juices, and (5) exposure of the entire plant to $C^{14}O_2$ in a glass bell jar closing the plant.

The first two methods were unsuccessful. No measurable uptake of glucose was noted when berries were immersed in the sugar solution. The last two methods, however, were both successful.

40 g. of ripe strawberries were obtained from plants to which about 2 microcuries (30 milligrams) of glucose-C^{14} were administered. The berries were frozen, thawed, and their major pigment was isolated from the hand-expressed juice, using the paper chromatographic technique described on p.31.

About 8 mg. of crystalline pelargonidin chloride 3-monoglucoside-C^{14} were obtained.

In the tagged CO_2 experiment, one plant was covered with a glass bell jar, into which $C^{14}O_2$, generated from $Na_2C^{14}O_3$ and HCl solution in an attached small flask, was introduced. An atmosphere of 0.8% CO_2 was created into the jar, and 2 μc of $C^{14}O_2$ was administered to the plant in two doses within three weeks. Between the two doses, the plant was left uncovered for four days, because a few necrotic spots had appeared on its leaves. 14 g. of ripe berries were obtained from this experiment, and were processed for the isolation of the major anthocyanin as in the tagged glucose experiment. About 3 mg. of radioactive major anthocyanin were obtained from the CO_2 experiment.

Radioactivity Measurements.

While the berries were still on the plant, frequent measurements of their radioactivity were made, using an end-window Geiger-Muller tube and a Nuclear[10] count-rate meter. Increase in the radioactivity of the berries was observed.

The radioactivity of the pure pigment obtained from the two experiments was measured as follows: [illegible].5 mg. of pigment was dissolved in 1 ml. of water, and the solution uniformly spread on a circle, 4.[illegible]5 cm. in diameter, of Whatman No. 1 paper. After drying, the activity was measured using an end-

(10) Manufactured by Nuclear Instrument and Chemicals Corp., Chicago, Ill.

window G-M counter held against the paper, and a Tracerlab[11] scaler. The difference between the background count, for which a filter paper circle with no pigment was used, and the sample plus background count was 18.2 c/m and 6.9 c/m for the pigment of the carbon dioxide and glucose experiments, respectively. The differences were significant at the 1% statistical level.

Although the pigment used for the radioactivity measurements was purified chromatographically twice and finally crystallized, it was thought that the possibility of its contamination with radioactive glucose should be investigated. About 4 mg. of the same tagged glucose which was used for the phytosynthesis experiment was added to 10 ml. of inactive strawberry juice, and the major pigment was isolated as in that experiment. There was no difference between background and background plus sample counts.

In order to obtain an estimate of the absolute activity of the pigment samples, an effort was made to standardize the measurements by means of a calibrated 0.047M solution of $Na_2C^{14}O_3$, undergoing 1067 ± [illegible] disintegrations per second.[11] Impregnation of dilutions of the carbonate solution in filter paper circles, as in the measurements of the pigment solutions, resulted in counts decreasing rapidly with time.

It was, therefore, decided to use the deposit-in-planchet technique. The radiopigment samples were eluted with methanol

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(11) Manufactured by Tracerlab Inc., Boston, Mass.

from the paper circles, and the eluate was concentrated and dried in stainless steel planchets, 2.5 cm. in diameter and 0.7 cm. in height. The measurements were made with the end-window of the G-M tube touching the rim of the planchet. For a total count time of 100 minutes in each case, the background showed 2603 counts, the pigment of the carbon dioxide experiment 7613 , and the pigment of the glucose experiment 4520 counts. This corresponds to 951 19 and 379 7 disintegrations per minute per milligram of pigment from the carbon dioxide and glucose experiments respectively. The calculations for one of the two cases is shown below.

1. Sample weight: swt = 2.5 mg
2. " area: sar = 4.9 cm^2
3. " thickness: $t = \frac{2.5}{4.9} = 0.51\ mg/cm^2$
4. Self absorption factor: $saf = \frac{1-e^{-5t}}{5t} = 0.361$
5. Background corrected count: $bc = \frac{1}{100}(7613-2603 \pm \sqrt{7613+2603}) = 50.1 \pm 1.0\ c/m$
6. Resolving time correction = insignificant (Fig. 7.1 in "The Measurement of Radioisotopes" by D. Taylor, Methuen LTD, London, 1950)
7. Self absorption corr'd count: $sbc = \frac{bc}{saf} = 135.5\ 2.8$
8. Geometry factor: $\eta = \frac{1}{2}(1-\cos\beta) = 0.256$
9. Window thickness: 3.5 mg/cm^2
   Air " 0.9 " (1 mm of air corresponds to 0.13 mg/cm^2)
   Total T = 4.4 "

   Thickness to reduce activity to half for C^{14}, $d_{1/2} = 2.8\ mg/cm^2$
10. Effective thickness/actual thickness factor for geometry, f = 1.35 (Fig. 6.2 <u>ibid</u>.)

11. Absorption correction factor: $2^a = 4.42$

$$a = \frac{T \times f}{d_{1/2}}$$

12. Activity of sample: $\frac{abc \times 2^a}{\eta} = 2380 \pm 48$ disintegrations per min.

13. Activity per mg of sample: 951 ± 19 dis/min

14. Activity per mg of carbon, assuming the formula: $C_{21}H_{21}O_{10}Cl \cdot 2.5H_2O$ for the pigment, 1934 ± 39 dis/min

When the same measuring technique and calculation was applied to the calibrated carbonate solution the difference between expected and found activity was 5%.

Both the pigment of the $C^{14}O_2$ and the glucose-C^{14} experiments were acid-hydrolyzed, and the aglucone was extracted with amyl alcohol. The alcoholic extract was washed with 1% HCl, and the two moieties of the pigment were measured for radioactivity using the planchet technique. The counts above background were [illegible] and 11.[illegible] for the sugar and the aglucone respectively in the $C^{14}O_2$ experiment, and 5.6 and 6.1 in the glucose-C^{14} experiment, all values being significant at the 1% statistical level.

DISCUSSION OF RESULTS

<u>Ascorbic acid, redox potential, and reduction of the pigment.</u>

Ascorbic acid is a reducing compound, exhibiting a low redox potential. It is possible, therefore, that the low redox potential of strawberry juice is due, partly at least, to the high ascorbic acid content of the juice. Or, vice-versa, the accumulation of ascorbic acid in strawberries may be possible because of the low redox potential of the cell-sap.

The redox potential of strawberry juice was investigated in this study because anthocyanins are easily reducible (Link, 1943), and in a reducing system, in which the organization of the living cell does not exist, they might be degraded by a reductive process. The findings that strawberry juice has a low redox potential (115 mv., as against 350 mv. of the grape juice according to Hentschler and Tanner, 1952, and 27[illegible] mv. of the cranberry juice, according to Li, 1952) and that pigment and ascorbic acid disappear simultaneously, even under exclusion of air, lead to the speculation that pigment may be reduced at the expense of ascorbic acid in a direct oxidation-reduction reaction. However, the failure to detect the expected reduction products of the pigment is evidence against at least the hypothesis of a reduction of anthocyanin to hydroanthocyanin to catechin.

<u>The minor pigment of strawberries. The chromatographic method.</u>

There is enough evidence to characterize the minor strawberry pigment as a cyanidin monogalactoside. The divergency

in the ultraviolet spectrum, however, does not permit the identification of the new anthocyanin with any one of the cyanidin galactosides used for comparison in this study. Actually, the lack of an absorption maximum at 270-280 mμ in the spectrum of this pigment appears as a unique characteristic, since all natural anthocyanins studied exhibit this maximum, according to the survey by Sannié and Sauvain (1952). On the other hand, all of the natural anthocyanins, in which the point of attachment of the sugar(s) to the aglycone has been ascertained, are 3-, or 3,5-glycosides. Therefore, the rare possibility emerges that the new pigment might be a 5-monogalactoside. Although no evidence can be offered for this hypothesis, the relative lability of the pigment to decolorization by $FeCl_3$ suggests a free 3-hydroxyl group, in view of the statements of Karrer and Helfenstein (1932). Unfortunately, not enough pigment was available to carry on further degradation studies, or use Karrer's complete methylation test for detecting substitution at 3-OH.

The paper chromatographic technique, used in this study for the isolation and purification of the pigments of strawberries, is a convenient method when small amounts of pigment are to be obtained in pure state. Furthermore, this method made possible the isolation of the otherwise elusive minor pigment. The conventional picration method allows this pigment to be lost. It is conceivable that the impurity which followed the major pigment even after two picrations in Sondheimer and Kertesz's (1948a) work, was indeed the minor

pigment. This appears probable since the purity test used by those authors was the $FeCl_3$ reaction, which is negative for the major but positive for the minor pigment of strawberries.

Using the chromatographic technique, also, improved the yield in pigment. Sondheimer and Kertesz obtained about 5.5% of the pelargonidin 3-glucoside that they estimated as present in the strawberry juice they used. According to their method of estimation, the two lt. of juice used in the present study contained 420 mg. of major pigment, out of which 208.3 mg. were obtained as air-dried crystalline material. Allowing 8.5% for the water of crystallization, the yield was 45.5%.

Assuming the same yield for the minor pigment, there should have been 1[illegible] mg. of cyanidin galactoside per kg. of juice used, and the ratio of the contents in major and minor pigment, should have been 11:1 approximately.

Incidentally, the method of Sondheimer and Kertesz (1948) for determining the anthocyanin pigment in strawberry products, becomes less accurate after the establishment of the existence of a second strawberry anthocyanin, having different optical characteristics and, probably, stability. The optical density at 500 mμ of the major pigment is about twice that of the minor pigment for the same weight, and, by changing the pH from [illegible].40 to 3.52, the optical density at 500 mμ of the major pigment is decreased by a factor of 3.4, while the same factor for the minor pigment is [illegible].4.

<u>The spectral changes, the kinetics, and the mechanism of the major pigment degradation.</u>

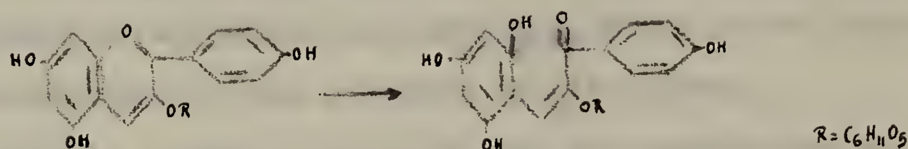
The spectra shown in Figures 7 and 8 indicate that:

1. The major anthocyanin pigment of strawberries suffers loss of optical density upon heating, or heating and storing in aqueous solutions containing traces of HCl.
2. Decrease of pH decelerates the loss of optical density in the above solutions.
3. Ascorbic acid accelerates the decrease in optical density, when added to an aqueous solution of the pigment.
4. The application of heat increases the rate of color loss in aqueous pigment solutions.
5. The decrease in optical density is much greater in the visible than in ultraviolet region of the spectrum. In the presence of ascorbic acid, the transmittance in the ultraviolet is dominated by the vitamin and its degradation products.
6. No shift of the 500 mμ absorption maximum is noticeable up to complete decolorization. The minimum at 350 mμ moves slightly toward larger wavelengths, while the maximum at 270 mμ gradually broadens. At an advanced stage of degradation of the pigment a new maximum emerges at [illegible]50 mμ.

It is apparent from the foregoing that the pigment primarily responsible for the red color of strawberries is a labile substance, being easily decolorized when water is present. The chemical changes involved in the above decolorizations can not be the pyrylium-chromenol reaction (Sondheimer, 195[illegible]), because acidification does not restore the color.

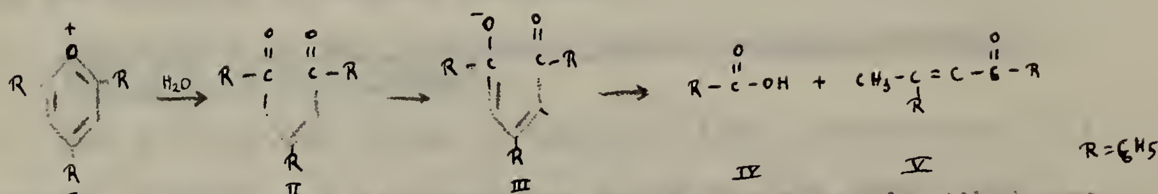
Hydrolysis of the glycosidic bond does not seem to take place either (p.43); such a hydrolysis would liberate a colored, but very unstable, aglycone (Huang, 1955).

The kinetics of the pigment degradation, on the other hand, suggest a first order reaction, when oxygen is absent in the system. Another type of hydrolysis was, therefore, considered possible, involving opening of the pyrylium ring at position 1-2, with formation of a ketone (a substituted chalcone):



Further degradation of this ketone would eventually lead to the brown precipitate.

Berson (195[illegible]) showed that crystalline triphenyl pyrylium pseudobases, obtained from the corresponding oxonium salts (I), occur in an open diketone form only (II). These pseudobases have a pink color (III) in alkaline solutions, which on standing or warming fade with concomitant formation of benzoic acid (IV) and a neutral oil (V):



The maximum of absorption at [illegible]27 mμ of Berson's diketones, which is very close to the [illegible]50 mμ absorption maximum appearing in the strawberry anthocyanin degradation, and also the greater lability of the anthocyanin at higher pH, suggest a similarity in the degradation mechanism of the two pyrylium classes of

compounds.

The solubility characteristics of the brown precipitate, along with its origin, are indicative of its polyphenolic nature. It is noteworthy that the surmise of Pederson et al (1947) that the brown sediment in stored strawberry juice may arise directly from the anthocyanin, was correct. When this insoluble brown anthocyanin degradation material forms in a jellied strawberry product the particles are held in suspension by the gel structure. This eventually results in intensification of the brown discoloration, which occurs during storage independently of the presence of anthocyanin.

The effect of oxygen in accelerating the degradation of the anthocyanin in aqueous solutions may be oxidative or catalytic. The failure to observe any difference in the spectra obtained under "aerobic" and "anaerobic" conditions of degradation does not favor the oxidation hypothesis. In the absence of quantitative gasometric data, however, it is difficult to exclude any oxidation at all. The catalytic effect of the oxygen, on the other hand, may be exerted through the formation of transient peroxides.

The effect of metallic ions, and of 5-hydroxymethyl-2-furfural on the pigment.

The data on the effect of metallic ions indicate that (1) ferrous, ferric, and cupric ions, in increasing order, are destructive to the pigment, (2) their toxicity to the pigment is attenuated by citric ions, and (3) high temperatures increase the toxic effect in both citrate buffered and unbuffered

solutions. It is not clear whether the metallic ions studied accelerate the degradation of the pigment catalytically, or by formation of pigment salts, or by both these processes. Their inactivation by citric ions, however, is most probably due to metal-citric complex formation.

The results of the HMF experiment corroborate Meschter's (1953) findings that the products formed on heating acidic sugar solutions are deleterious to the strawberry pigment.

<u>The effect of the additives.</u>

From the 19 different additives tried in this study as stabilizers of the red color in strawberry juice and in pure pigment solutions, thiourea showed a marked stabilizing effect in both of these media. Steinberg (1954), however, showed that heating thiourea with citric acid and sugar in solution produces brown color and "browning" precursors. Formation of such products absorbing at 500 mμ must be the reason for the increase of optical density above the original value, on heating and storing strawberry juice with thiourea added. Thiourea has a strong anti-peroxidase activity in the human metabolism (Best and Taylor, 1950), and it seems unlikely that it would ever find use as a food additive; it could, however, serve as a prototype for the search of other stabilizers.

Propyl gallate and quercetin showed a slight protective effect on the color of both the juice and the pigment solution, and they might have a commercial importance as fruit color stabilizers.

On the other hand, the detrimental effect of ascorbic

acid on the pigment was strikingly shown in this experiment.

<u>The effect of oxygen and ascorbic acid</u>.

The results of the study on the effect of ascorbic acid and oxygen on the pigment in model systems indicate the following:

1. In the absence of oxygen, the major strawberry pigment alone, or the ascorbic acid alone, are relatively stable, the pigment being less so than the vitamin.
2. In the presence of oxygen, the pigment alone, or the ascorbic acid alone, are relatively unstable, the pigment being less so than the vitamin.
3. In the absence of oxygen, the simultaneous presence of pigment and ascorbic acid leads to faster degradation of both of them than when they are alone. Stoichiometric relationships however, do not suggest a simple reaction between pigment and ascorbic acid. After 15 hours at 60°C., 1 molecule of pigment was destroyed for every 50 molecules of ascorbic acid.
4. In the simultaneous presence of oxygen and ascorbic acid, the destruction of pigment is greater than the addition of the single effects of oxygen and ascorbic acid on the pigment can account for.

This indicates a "positive interaction" of these two factors in regard to pigment. Isolating "the effect of single factors and their interactions", in the way in which it is commonly done in statistical analysis, it appears that out of the 70% destruction of pigment, occuring after 30 hours, i.e., under

the conditions of this experiment (Fig. 17), 35% may be ascribed to the effect of water, 11% to the effect of oxygen, 17.5% to the effect of ascorbic acid, and the rest 36.5% of destruction to the interaction of ascorbic acid and oxygen. These percentages and their ratios change with time of storage in the experiment, the percentage due to interaction becoming smaller. However, ascorbic acid reacts rapidly with oxygen, according to the present data, and, if hydrogen peroxide is formed from this reaction (Sondheimer and Kertesz, 1953), the destruction of pigment attributed to the interaction may be higher than the above analysis suggests.

The results of this experiment do not allow the establishment of the exact mechanism of the reaction callistephin chloride-ascorbic acid-oxygen, in citrate buffer of pH 3.40, at 60°C.; they make it clear, nonetheless, that there is more than one path for the degradation of the pigment to proceed in such a system.

The findings of the experiment with model systems are corroborated by the data on the retention of pigment and ascorbic acid in strawberry juice stored under "aerotic" and "anaerotic" conditions. Considerable amounts of pigment and ascorbic acid could be retained in the juice by replacing the air in it by nitrogen. Ten times as much pigment was found in strawberry juice stored at 50°C. for 160 hours under exclusion of oxygen, than in the same juice stored at the same temperature and time under its natural atmosphere.

The effect of the ascorbic acid oxidase treatment.

It was possible to increase the pigment retention in strawberry juice by destroying the ascorbic acid present using ascorbase. However when the comparison was made of the effect of oxygen with the combined effect of oxygen and ascorbase, it was found that little is to be gained in pigment retention by adding the ascorbase treatment to the removal of oxygen in strawberry juice under storage.

Food Processing Considerations.

The present study of strawberry pigment stability suggests that the following factors should be considered in the manufacture of preserved strawberry products:

1. Use of the lowest possible temperatures at any stage of the handling of the product.
2. Removal of the oxygen from the product.
3. Selection of strawberry varieties of high pigment content and low ascorbic acid content.
4. Decrease of the pH, within acceptable limits, preferably by using citric acid.
5. Avoidance of contamination with metallic ions.

Since heat is still the only means of preserving fruit spreads, is it advantageous to the strawberry pigment retention to use low temperature-long time heat treatments, or "high-short" ones?

This problem is handled, in the following discussion, as if heat were necessary only to prevent microbial spoilage, and as if no heat effects other than the pigment retention entered

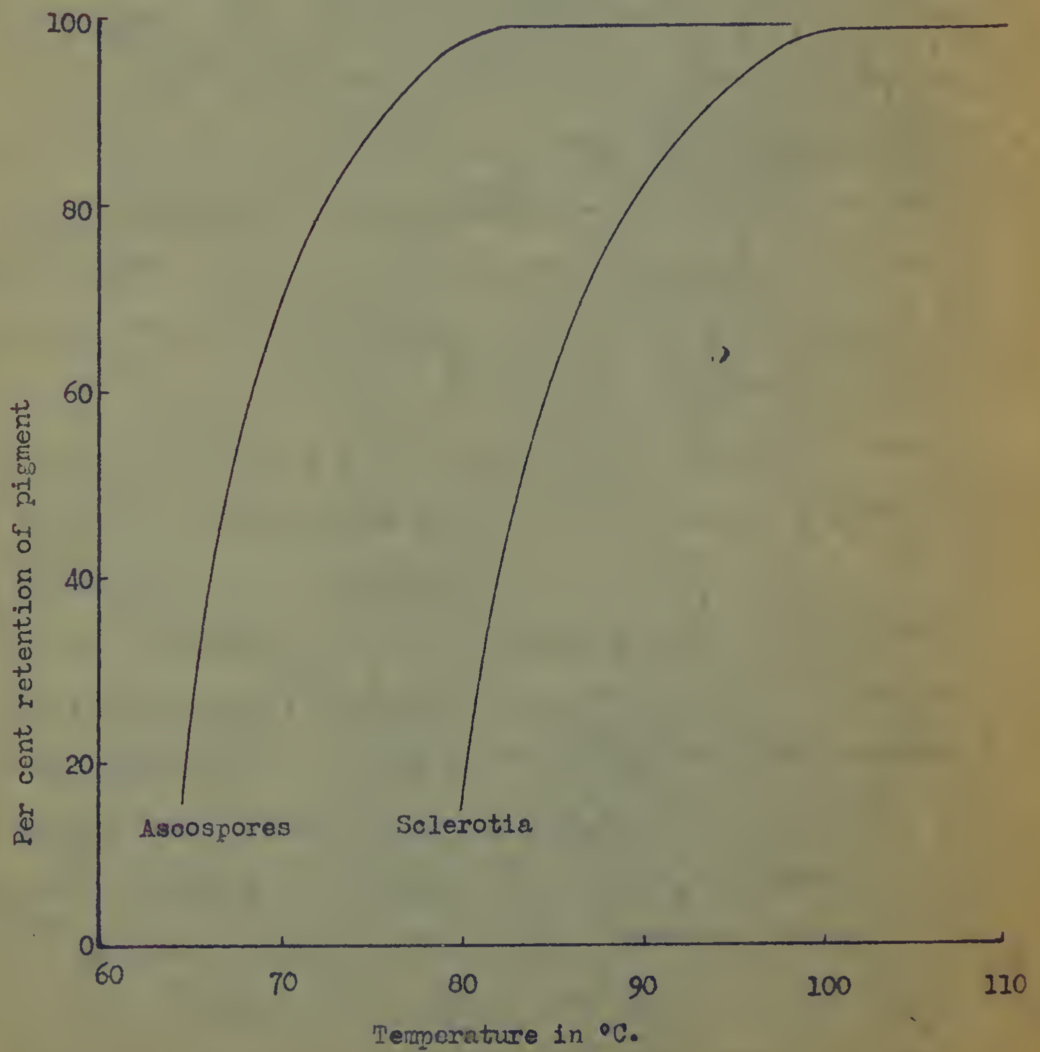
the picture.

In evaluating heat processes of food, the most heat resistant microorganism, likely to be a health hazard or to cause food spoilage, is used as a heat processing test organism. The unidentified species of Penicillium, which was isolated from canned blueberries by Williams, Cameron and Williams (1941) is very heat resistant in acid media, is capable of growth under high vacuum, and its thermal death characteristics have been studied in diluted blueberry juice and reported by the authors. These features make its selection as a test organism appropriate for the present discussion, bearing in mind the difference in pH and solids content between diluted blueberry juice and strawberry spreads.

Fig. 14 shows, along with the thermal destruction-time curves A and B of the major strawberry anthocyanin, the thermal death-time curves C and D for the sclerotia and ascospores of the test Penicillium, respectively. A simple inspection of the curves indicates that high-short heat treatments will cause less pigment destruction, while exterminating the mold, than low-long ones, since the organism is more sensitive than the pigment to increases in temperature.

A quantitative interpretation of the interplay of the four factors: time-temperature-death of the organism-destruction of the pigment is illustrated in Fig. 15, in which the retention of pigment is plotted against the temperature of microbially equivalent heat treatments. The two curves of Fig. 15 were obtained by taking from the lines C or D of Fig. 14 the

Fig. 20. Retention of pigment vs. temperature of heat treatments equivalent in exterminating the sclerotia or ascospores of Penicillium sp. described by Williams, Cameron and Williams (1941).



temperature required to exterminate the sclerotia or ascospores at various times, and securing the per cent destruction of pigment for each such time-temperature pair at the buffer solution of pH 3.40 (which is close to the pH at which the mold death curves were obtained), using the rate curves of Fig. 13. Extensions of the graphs were eventually used, and rates not appearing in Fig. 13 were drawn by taking the half-life of the pigment from line a, Fig. 14. It is apparent from Fig. 20 that a heat treatment at 100°C., at which a minute or two would exterminate the sclerotia, would leave the pigment practically undamaged, whereas heating at lower temperatures would necessitate times inflicting high pigment destruction. Similarly, if the ascospores were to be exterminated only, heating at 80°C. for 20 minutes would kill all these cells and cause very little damage to the pigment, while at lower temperatures, times of exposure would be necessary which would cause considerable loss of pigment.

It is not intended that the figures given above be taken as heat processing specifications for good pigment retention in strawberry products. For such a purpose, the destruction of the pigment and of the most heat resistant undesirable microorganism should be studied in the very product to be processed. Meschter (1953) gives a heat destruction-time curve for the pigment in strawberry preserves with a z value slightly higher than the one obtained with the model systems in this study. This further emphasizes the value of high-short heat treatment in the commercial practice, provided

heat is used for preservation only and against organisms of small z value.

The radioactive pigment.

The radioactive pelargonidin 3-glucoside, produced by phytosynthesis, was not obtained in quantities great enough to be used for degradation studies. It was shown, however, that (1) such a synthesis is possible, (2) carbon from CO_2 can enter into both the sugar and the aglucone moieties of the pigment molecule when the berries are green and 0.5-1.0 cm long, and (3) glucose can not only attach to the aglucone, but also serve as a precursor to it, at this early stage of berry development.

SUMMARY AND CONCLUSIONS

1. An analysis of strawberry juice was made. The low redox potential and the high ascorbic acid of the juice suggested a reductive degradation of the pigment; however, a hypothetical reduction scheme could not be verified.
2. In a comparison of several anthocyanin extractants, n-butanol and cyclohexanol exhibited the highest efficiency in extracting the color of strawberry juice.
3. A second anthocyanin pigment was isolated from strawberry juice, and was identified as a cyanidin monogalactoside; its glycosidic bond remains to be located. There is about one molecule of the newly identified pigment for every eleven molecules of the previously described anthocyanin.
4. A chromatographic technique was developed, employing triangular sheets of filter paper, and permitting the preparation of pure anthocyanins in small quantities, with high yields.
5. In testing the stability of the major strawberry anthocyanin in pure form, it was found that mere standing of the pigment in aqueous solution results in decolorization. This decolorization is accelerated by raising the temperature and/or the pH of the solution.
6. A brown precipitate was obtained as an end product of the degradation of the major anthocyanin in aqueous solution. This precipitate showed the characters of the brown sediment obtained early in the storage of strawberry juice.

7. The kinetics of the major anthocyanin degradation were studied in buffer solutions, at pH 2.00 and 3.40, under exclusion of oxygen, over the temperature range from 45°C. (113°F.) to 110°C. (230°F.). First order reaction rates, and straight thermal destruction-time lines were obtained.

8. A tentative scheme for the major anthocyanin degradation was proposed. This scheme involves hydrolytic opening of the pyrylium ring, with formation of a substituted chalcone, further degrading to an insoluble polyphenolic compound.

9. The effect of cupric, ferrous, and ferric ions was studied on pure major anthocyanin in solution. It was found that these ions accelerate the pigment destruction, but they are inactivated to a great extent by citric acid.

10. The effect of 5-hydroxymethyl-2-furfural was studied on pure major anthocyanin in solution. It was found that the pigment destruction is accelerated by this sugar degradation product.

11. Out of 17 different additives tested, thiourea, propyl gallate, and quercetin showed some protective effect on the color of model systems and of strawberry juice.

12. In studying the interaction of oxygen, ascorbic acid and major strawberry anthocyanin in model systems, it was found that oxygen or ascorbic acid are detrimental to the pigment. These two agents combined caused more destruction of pigment than the sum of their single effects. Also,

the pigment retention in strawberry juice stored under nitrogen were several-fold higher than in similar juice stored under air.

13. Destroying the ascorbic acid of strawberry juice by means of ascorbic acid oxidase resulted in greater pigment retention. However, when the enzyme-treatment was combined with the removal of the oxygen in the juice, the resultant pigment retention was only slightly higher than the one obtained through oxygen removal alone.

14. Assuming that heat treatment is necessary to prevent microbial spoilage only, and that, in evaluating such a treatment, a test organism is used having thermal death-time characteristics similar to those of the Penicillium described by Williams *et al* (1941), high-short "sterilization" is advisable for maximum pigment retention in strawberry products.

15. It was possible to biosynthesize pelargonidin 3-monoglucoside-C^{14} by administering $C^{14}O_2$, or glucose-C^{14} to strawberry plants at an early stage of the development of the berries.

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Approved by:

Gideon E. Livingston

Walter E. Conrad

James E. Fuller

D. J. Hankinson

Carl R. Fellers

Thesis committee

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