RELATIONSHIP OF SUGAR, ANTHOCYANIDIN, AND PHOSPHORUS LEVELS IN FLOWERS AND LEAVES OF HYDRANGEA MACROPHYLLA

by

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B. S., National Taiwan University,
Taiwan, China, 1960

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture
and
Landscape Architecture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1963

Approved by:

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INTRODUCTION

Numerous summaries of anthocyanidins in plants have been reported in the literature. These indicate that the formation of anthocyanins is associated with the accumulation of sugars in plant tissues. Molisch (79) assumed that anthocyanin formation is dependent on the accumulation of sugar in a leaf. Frey-Wyssling and Blank (36) stated that sugars are not directly involved in pigment synthesis, because they found no strict linear relationship between reducing sugars and pigment contents in plants, but their data showed a reasonably good correlation. Thimann (111) indicated that phosphate enables growth to take place, but has no influence on the formation of anthocyanin. MacGillivray (69) noted in the tomato that the absence of phosphorus greatly increases both reducing and non-reducing sugars in the plant. Thus, the increased pigment formation accompanying a deficiency of phosphorus is caused by increased amounts of available sugars through the inhibition of growth.

Because of such complicating factors as soil fertility and environment in the formation of anthocyanin, a more careful study of this relationship between sugar and anthocyanin was undertaken. The purpose of the present investigation was to study the relationship of sugar, anthocyanidin, and phosphorus levels in flowers and leaves of two hydrangea cultivars, Heite's Red and Kuhnert.

REVIEW OF LITERATURE

Anthocyanin Isolation and Identification

The word, anthocyanin, was first coined by Marquart (70) who used it for the red, violet, and blue pigment of flowers. Boyle (17), in 1664, gave an account of color changes which take place on adding acids and alkalies to extracts from flowers and other plant parts. Wheldale (118) made mention of the presence of solid anthocyanins in the flower petals of Solanum nigrum and Salvia splendens and the fruits of Coffea arabica. Wigand (121) was the first to mention that anthocyanins have no relation to chlorophyll, but arise from a chromogen, a tannin that gives anthocyanin on oxidation. Strasburger (109) published an account of the histological distribution of anthocyanins in various flower petals in 1884, whereas Kny (54), in 1889, studied the distribution of anthocyanins in leaves. In 1901, Goppelsroeder (43) described a method which could be adapted for the separation of mixtures of pigments in solution. Strips of specially prepared filter papers were allowed to dip slightly into the solutions, and various pigments rose to different heights. By cutting the zones of paper and repeating the process, he was successful in obtaining a certain amount of pure pigment.

Weigert (115), by qualitative tests, differentiated anthocyanin pigments into two groups. Grafe (44) reported the preparation and analysis of anthocyanin pigments in hollyhock (Althea rosea).

The fact that anthocyanins are present in plants as glycosides was brought forward by Willstatter and his collaborators (122,123,124). They said that these pigments belong to a group of glucosides, the sugar-free pigments or aglycones which are called anthocyanidins. Wheldale (113), in 1909, suggested that the anthocyanins were formed from chromogens, which are glucosides, possibly by the action of oxidase. Successive oxidative stages, according to Wheldale, gave rise to red, purplish red, and purple pigments. Wheldale (120), in a later work in 1913, gave a detailed account of the preparation and purification of anthocyanin pigments from several cultivars of Antirrhinum majus. Willstatter et al. (124) have pointed out that the various anthocyanins are relatives of -phenylbenzopyrilium, usually found in the form of its chloride, and as such, designated as the flavilium chloride. Robinson and Robinson (94) surveyed the work to 1931. They also published a detailed list of plants, indicating the anthocyanin pigments they contain. Schriner et al. (101) stated that anthocyanidins have been observed in plants only in rare cases. They further added that as a rule they occur in nature attached to one or more sugars as anthocyanins. Blank (15) is of the opinion that the anthocyanins appearing in nature are partly mono- and partly di-glycosides. Sugars like glucose, rhamnose, galactose, and gentiobiose have been isolated as sugar components; one of these sugar molecules is always attached at the 3-position. If a second sugar molecule is present, it is either coupled with the first or attached to the anthocyanidin in the 5-position.

Regarding the presence of more than one anthocyanin in plants, Blank (15) remarked that they are usually found as mixtures in plants. The components of these mixtures may be separated either by fractional crystallization of picrates or by the use of chromatographic absorption techniques. Robinson et al. (94) extracted anthocyanins from well-desiccated plant tissue by means of methyl alcohol containing 1 to 2 percent hydrochloric acid, and precipitated the anthocyanins by ether or lead salts.

Extraction of anthocyanidins from the tissue has been accomplished by grinding the tissue with water or polar organic solvents such as methyl alcohol or ethyl alcohol or a mixture of the two (94,95). Usually an acidic solvent is used. 2N hydrochloric acid has been used successfully for the extraction of anthocyanidins by several workers (9,10). Heating the plant tissue in 2N hydrochloric acid for 15 minutes to an hour or more over a steam bath has been reported (10,94,95). This duration of hydrolysis depends on the quantity of the pigment present, the nature of the pigment, and the type of plant tissue used. By heating the anthocyanin pigments in 2N hydrochloric acid for a short time, the pigments are converted to anthocyanidins, and the sugar moiety separates. Anthocyanidins are insoluble in water and hence can be separated from the hydrolyzed extract with isoamyl alcohol or n-butyl alcohol (9,10).

Robinson and Robinson (96) developed a number of qualitative tests based on the chemical behavior of anthocyanins and anthocyanidins prepared synthetically or isolated from natural sources.

Using these tests, they made a detailed survey on the occurrence of anthocynidins in the vegetable kingdom. Apart from the flowers, the other anthocyanin-containing organs of the plants were also investigated.

Specific work on the identification and isolation of anthocyanin and anthocyanidin pigments is fairly recent. The first specific work on the identification of anthocyanins of Pelargonium was done by Willstatter and Mallison (123). They isolated and analyzed the pigment in three varieties of Pelargonium and concluded that Pelargonium zonale cultivar 'Meteor' has pelargonin, and the bluish pink Pelargonium peltatum has the same pigment. Willstatter and Bolton (122) first identified the pigments from the petals of Tulipa gesneriana. They found that the scarlet red color of some varieties was due to a mixture of cyanidin diglucoside (Cyanin) and carotenoids. Robinson and Robinson (94) found that the garden tulip contained either a mixture of cyanidin and pelargonidin biosides or cyanidin biosides and delphinidin diglucoside. Further work revealed that the identification of anthocyanins of tulips was much more difficult than that of most other plants.

Robinson and Robinson (94) examined 34 varieties of tulips and separated them into two groups. They stated that there was one group in which pelargonidin and cyanidin occur as 3-biosides, and a second group containing delphinidin derivatives sometimes with cyanidin but free from pelargonidins.

Anthocyanins have been identified by precipitating them as lead salts (94), and with ether (95). In recent years chromatographic procedures have been extensively used. Paper chromatography was introduced by Consden et al. (27) in 1944, and since then has been extensively used in the identification and separation of plant pigments.

Chromatography is a simple procedure requiring only a simple apparatus. It has been successfully used at room temperatures and normal atmospheric pressures for the separation of similar compounds (81). Spaeth et al. (104) used columns of silicic acid for separating small amounts of mixtures of synthetic anthocyanins. Chandler et al. (21) employed a 50 x 4.4 cm. column of Whatman standard grade cellulose powder in identifying the anthocyanins from the black walnut. Lesins et al. (60) described the necessity of a rapid and distinct separation of the sap-soluble pigments. They used a 5 percent aqueous phosphoric acid solution with circular filter paper and found that it required four to six hours to make a chromatogram. Bate-Smith (9) and Nordstrom (85) have all used ascending chromatography in various studies of anthocyanins. Asen (5) and Halevy et al. (46) have used descending paper chromatography for the identification of anthocyanidins.

The possibility of applying filter paper chromatography to the study of sap-soluble plant pigments was discussed by Bate-Smith (10). He pointed out that the anthocyanidins and their mono- and di-glucosides form spots well differentiated in their Rf values and give characteristic color reactions with ammonia vapor. He further pointed out that anthocyanidins (aglycones of

anthocyanins) have to be run under standard conditions of temperature, composition of flowing solvent, and the substance which is applied to the paper. He stressed the importance of mineral acid in considerable concentration to prevent the decomposition of anthocyanins during the run. Bate-Smith (11) listed in great detail the factors which might affect the Rf values, and the precautions to be taken for getting the correct Rf. Bate-Smith (12), in another paper, has improved the technique of separation of pigments and applied it to the pigments of leaves and other tissues in numerous plant species. He concluded that except in Rosaceae and a few Leguminoseae (which appear to contain leucopeonidin), the leuco-anthocyanins appear to be restricted to leuco-cyanidins and leuco-delphinidins. He published a detailed list of many families in dicotyledons and a few families in monocots, Gymnosperms, and Pteridophyta, indicating the plants and the anthocyanins they contain.

Bate-Smith (12), while discussing the merits of other solvents, has stressed the superiority of Forestal solvent. He has listed the Rf values of various anthocyanidins and anthocyanins in three different solvents including the Forestal solvent. Bate-Smith and Westall (13) suggested maintaining a low Ph of solvent during chromatography to prevent the anthocyanidins from fading out. This was achieved by them by using the upper layer of the mixture of n-butyl alcohol: 2N-HCl(1:1 v/v). Asen (5) investigated the anthocyanidins and anthocyanins in Euphorbia pulcherrima in three different solvents.

Some varieties of Hydrangea macrophylla produce flowers with sepal color ranging from red to pink through mauve and magenta to blue. The pigments in sepals of hydrangeas have been examined by several investigators. Robinson and Robinson (34) showed that the red sepals of the cultivar Parcival and the blue sepals of cultivar Marechal both contained a delphinidin diglycoside. Further investigations revealed a delphinidin pentose-glycoside in the red sepals of the cultivar Marechal. Differences in the concentration of anthocyanin in red and blue sepals of hydrangeas were noted by Robinson (94) who found six to seven times as much delphinidin-3-monoglucoside in red sepals as in the blue. The red and blue sepals of Merville hydrangeas contained the same anthocyanin pigment. Asen et al. (7) found by chromatographic and spectrophotometric methods that the anthocyanins in red and blue sepals of Hydrangea macrophylla cv. 'Merville' were identical.

Halevy and Asen (46) stated that the isolation and purification of antyocyanins were accomplished by column chromatography in their research with tulips. They found that the variety Pride of Haarlem contained derivatives of delphinidin, cyanidin, and pelargonidin. Asen (5) identified the anthocyanins and anthocyanidins in the bracts from Euphorbia pulcherrima plants in three cultivars by paper chromatographic and spectrophotometric methods. He concluded that anthocyanins in the bracts from the Poinsettia cultivars examined were identical. Halevy and Asen (46) identified the anthocyanins from the tulip varieties Smiling Queen and Pride of Haarlem. The Rf values of anthocyanidins from

these varieties were listed in three different solvents and compared with the Rf values of authentic anthocyanidins for the purpose of identification.

Bate-Smith (9) has tabulated Rf values for 22 anthocyanins and anthocyanidins. Geisman (38) has recorded Rf values for over 100 polyphenols in four solvents.

As early as 1870, Schonn (99) mentioned the possibility of spectroscopic examination of plant pigments. Anthocyanins and anthocyanidins have been found to absorb strongly in the investigated range of 200 to 600 mu. (15). The Beckman model DU spectrophotometer has been used to determine the absorption spectra of several plant pigments. An absorption maximum is present in the visible range of the spectrum. The absorption spectrum has been used as a reliable guide for the identification of anthocyanins and anthocyanidins. Halevy et al. (46) identified the flower pigments of Tulipa gesneriana by this method. Schou (100) stated that the anthocyanins and anthocyanidins have approximately the same absorption spectra. Bate-Smith (12), Asen (5), and Halevy and Asen (46) have all used the absorption spectra as a guide for the identification of anthocyanidins and anthocyanins. The absorption maximum does not differ very much for a given anthocyanin and its anthocyanidin. The absorption maxima, as obtained by the above workers, are as follows for the anthocyanidins: Pelargonidin, 530; Cyanidin and Peonidin, 545; and Delphinidin and Malvidin, 555. Asen (5) and Bate-Smith (12) have given the maximum for Petunidin as 555 mu., but Halevy and Asen (46) got a higher value for the same anthocyanidin. They

gave 557 mu. as the value for Petunidin. The absorption data were obtained by means of a Beckman DU spectrophotometer.

Relationship of Sugar, Anthocyanin, and Phosphate

Although the chemistry of anthocyanin pigments has been studied extensively, very little is known of its mode of formation in plants. The genetic capacity for anthocyanin synthesis differs considerably with the kind of plant. Synthesis of anthocyanins will not occur in plants, even if the necessary genes are present, without favorable environmental conditions. The formation of anthocyanins seems to be commonly associated with the accumulation of sugars in plant tissues. Any environmental factor favorable for an increase in the sugar content of a given plant tissue such as high light intensity, low temperature, drought, or low nitrogen supply often favors synthesis of anthocyanin in that tissue. Likewise, environmental factors which check the formation or accumulation of sugar often have a similar effect on anthocyanin synthesis (73).

The earliest investigation of anthocyanin formation using artificial nourishment by means of various kinds of sugars was carried out by Overton (87). The culture of Hydrocharis Morsus-ranae in sugar solutions developed larger quantities of anthocyanin in their leaves. Further experiments showed the phenomenon to be constant for quite a number of species when isolated leaves and twigs were fed on solutions of cane sugar, dextrose, levulose, and maltose. Repetition of experiments along

these lines by Katic (51), Gertz (40), and others confirmed Overton's results. Overton concluded that, in the normal plant, reddening of leaves, etc. are correlated with excesses of sugar in the plant tissue. Further tests upon red autumnal leaves revealed more sugar in red than in green leaves. He concluded that the appearance of red cell sap was in close relation to the sugar content of the cell sap. This assumption has found many adherents even today, though it has never been proved by exacting tests. More elaborate and conclusive work was commenced by Combes in 1909. He (24,25) had observed that decortication in some plants brought about considerable development of anthocyanin in the leaves above the point of decortication. Analyses showed that red leaves contain greater quantities of sugars and glucosides than green ones from the same plant. It may be safely inferred that the accumulation of synthetic products in the leaves leads to the production of anthocyanin. For example, we frequently find abnormal reddening of a single leaf on a plant otherwise in full vigor, and investigation almost invariably shows the reddening to be accompanied by injury. The injury, whether it be due to mechanical cutting or breaking, or to the attacks of insects, will be found to affect those tissues which conduct away the synthetic products of the leaf (86). Some investigators assume a close relationship between anthocyanin formation and the quantity of assimilates, i.e., sugar. Gleisberg (42), on the other hand, obtained no clear results in his experiments with cane sugar as a nutritive solution. Griffin (45) also was unable to find a pronounced dependence of pigment formation on sugar content.

Exact research studies on the connection between sugar metabolism and anthocyanin formations in seedlings of red cabbage have shown no such intimate relation (36) as that assumed by Overton and others. In seedlings, more anthocyanin was formed as the sugar content increased, but a comparison of individual results was unfavorable for the "Sugar Theory." The lack of regularity in the relationship between anthocyanin and sugar content in the individually investigated organs and in the whole seedling, renders a quantitative relation between sugar and anthocyanin content highly improbable.

Lippmaa (64,65) succeeded, by means of artificial feeding with sugar, in increasing the formation in different plants not only of anthocyanins but of carotenoids as well. In these experiments chloroplasts were changed into chromoplasts, giving the leaves of the plant in question a significantly darker appearance. According to Lippmaa, sugar is of importance as a precipitating factor in the formation of anthocyanin. He sharply rejected the idea of a connection between sugar metabolism and anthocyanin formation.

Noack (84) reported that the formation of antocyanins, after sugar addition, can be traced to a destructive effect on the chloroplasts or on the assimilation of accumulated sugar in the tissue. He is of the opinion that other factors which promote the formation of anthocyanins (temperature, lack of mineral substances, etc.) can be explained in this way as well.

It may be pointed out in this connection that the content of other plant aromatic compounds can be increased by the use of a

nutritive solution rich in sugar. Lang (59) has made such observations with naphochinones and tannins. Danner (28) was able to increase significantly the arbutin content of his experimental plants (Saxifraga) by artificial sugar feeding.

Stanescu (106) believes that autumnal formation of anthocyanin takes place as a result of reduced starch reserves. Molisch (79) assumed, on the basis of his observations in Japan, that anthocyanin formation is dependent on the accumulation of sugar in a leaf.

Frey-Wyssling and Blank (36) concluded that sugars are not directly involved in pigment synthesis, because they found no strict linear relation between reducing sugar and pigment content in plants. Their data did, however, show a reasonably good correlation. Thimann, Edmondson, and Radner (111) reported that sugar-pigment relationship, in Spirodela at least, is not linear. Until now no direct conversion of sugar to anthocyanin has been proved.

Thimann, Edmondson, and Radner (111) found that the anthocyanin formation in growing cultures of Spirodela is promoted by sucrose but not by glucose; conversely, growth is promoted by glucose but not by sucrose. Fructose is intermediate in both respects. In non-growing cultures, however, all three sugars are equally effective in promoting anthocyanin formation. A number of treatments which increase or decrease the anthocyanin content have parallel effects on the reducing sugar content. A plot of anthocyanin content against reducing sugar content shows a smooth relationship. Variations in the sucrose content are smaller and

show no parallelism with pigmentation. It is deduced that anthocyanin may be formed independently from any of three sugars, but that glucose is preferentially consumed for growth.

Phosphorus is important in protein formation, since in its absence, sugars increase in amount and coagulable proteins decrease (74).

It was observed by Reed (91), in 1907, that the transformation of starch into water-soluble carbohydrates was seriously impaired in the absence of phosphorus. Hartwell (47), in 1917, noted that simultaneously with the increase in the absorption of phosphorus by the turnip root, which previously had been deprived of it, the leucoplasts containing the starch grains shrank in size as the grains were corroded and dissolved until finally all the starch had disappeared from the root tissue. When phosphorus was again withdrawn from the nutrient solution, the starch reappeared. MacGillivray (69), in 1926, noted that the tomato in the absence of phosphorus greatly increased both reducing and nonreducing sugars in the plant. This increase of sugars in the absence of phosphorus has also been noticed by Eckerson (32) in 1929 and Kraybill (56) in 1930. Eidelman (33), in 1939, stated that for average lengths of day and with relatively high temperatures, photosynthetic activity tended to show a positive correlation with the content of phosphorus. The maxima, however, do not coincide under the conditions of a shortened day and low temperatures.

Plants which have a foodstuff deficiency often show increased anthocyanin formation. Gassner and Straib (37) investigated the formation of anthocyanin in young barley plants with a deficiency of phosphorus, potassium, and nitrogen. They were of the opinion that the increased pigment formation was explained by the amount of available carbohydrates. The tomato (72) is very sensitive to a deficiency of phosphorus. When this nutritive element is deficient, the lower side of cotyledons and foliage leaves show an especially high content in anthocyanin.

Thimann et al. (111) found that the conversion of sugar into anthocyanin does not proceed via the usual glycolytic breakdown system since (a) none of the intermediates tested, from hexose phosphate to pyruvate, was active in forming anthocyanins, and (b) phosphate exerts apparently little if any influence. The action of phosphate in growing cultures is best appreciated by a comparison with other agents. When growth is reduced by a copper deficiency, the plant's anthocyanin formation is reduced. But when growth is reduced by a lack of phosphate, the anthocyanin concentration is increased and the total yield per culture is essentially unchanged. The pigment-forming mechanism thus runs independently of the phosphate concentration, and this is in agreement with the behavior of the non-nutrient cultures.

Phosphate apparently does not participate in the formation of anthocyanin, and if the process does take place directly from sugars it probably does not proceed via the usual glycolytic pathway, since none of a number of glycolytic intermediates gives rise to any anthocyanin.

Several quantitative methods for reducing sugars have been based on the reduction of ferricyanide to ferrocyanide. The reaction between the ferricyanide and reducing sugars was first suggested by Gentele (39). In Strepkov's method (110) for the microdetermination of carbohydrates in plant materials, the excess ferricyanide was determined by an iodometric titration. Hassid (48) determined quantitatively the ferrocyanide formed by titration with a standard ceric sulfate solution. In a procedure for the determination of glucose in blood and urine, Hoffman (50) made use of the fact that ferricyanide solutions are yellow whereas ferrocyanide solutions are colorless. Glucose was thus estimated by measuring in a photoelectric colorimeter the diminution in yellow color of an excess of ferricyanide. Forsee (35) adopted this method in the determination of reducing sugar in plant materials. The extract or plant juice must be clarified so as to be free of all coloring matter and must be waterclear. The method, as outlined by Hassid (49), has been found entirely satisfactory by Forsee. This photocolorimetric method is rapid and accurate, the procedure is simple, and only one standard solution is necessary.

In recent years the accurate ash analysis of specified plant tissue has been used to ascertain the nutritional status of the plant (112).

Wolf (125) used the rapid photometric method to determine the phosphorus quantitatively. The plant material is rapidly ashed by means of sulfuric acid and hydrogen peroxide. A test for the phosphorus is run on an aliquot of the ash extract by

means of a photoelectric colorimeter. This rapid method provides for a very rapid determination of phosphorus with sufficient accuracy for many routine purposes.

Effects of Fertilizer Elements and Environmental Condition

Temperature. Observations have indicated that an increase of anthocyanin is correlated with lowering of the temperature. Starch synthesis from sugar is a process which is retarded by low temperature. Thus Muller-Thurgau (82) has shown that at temperatures below 5° C., quite a considerable portion of the starch contents of the potato is changed to sugar, and with a rise in temperature the greater portion of starch is again regenerated. According to Lidforss (61), evergreen leaves in winter are also completely starch-free but contain very considerable quantities of glucose, which again, to a large extent, changed back to starch if the leaves are artificially warmed. Overton (87) examined the sugar content of autumnal leaves and found considerable quantities present, appreciably more than in the same species at midsummer. It has been reported that low temperature may greatly affect the sugar content of the tissues, and hence may in this way cause the reddening, apart from any more direct effect (86).

Low temperatures favor pigment formation; this is demonstrated by autumnal coloration and the winter reddening of leaves of Hedera, Ligustrum, Mahonia, and other evergreens (86). Conversely, Overton (87) found in Hydrocharis, the higher the

temperature, the less anthocyanin. Klebs (53) also noted that flowers of Campanula trachelium and Primula sinensis may be almost white in a greenhouse, but the same plant kept in the cold will bear colored flowers. Klebs is of the opinion that the color changes induced by changes of temperature are not directly due to the effect of temperature on pigment formation, but indirectly to the effect of temperature on metabolism. At high temperatures, growth is so rapid that the substances used in pigment formation are not present in sufficient quantity.

Several investigators (98,128) drew from their studies the conclusion that low temperatures have a favorable influence on the formation of anthocyanins in general. Weisse (116), working with Pelargonium and Geranium species, observed the opposite effect. The investigations of Harder and co-workers, which will be dealt with in another connection, also often showed an increase in anthocyanin formation at a higher temperature.

Frey-Wyssling and Blank (36) have followed the formation of anthocyanin quantitatively in seedlings of red cabbage in the dark at temperatures of 10°, 20°, and 30° C. At 20° and 30° C. the anthocyanin content was much higher than at 10° C. and 30° C.; however, a noticeable decrease in the pigment content of the germinating seed started to set in. The optimum temperature for anthocyanin formation lay, in this case, probably between 10° and 30° C.

Light. The relationship between pigment formation and light constitutes a problem to which there is no very satisfactory solution.

As early as 1799, Senebier (103) noted that the crocus and the tulip develop colored flowers in the dark. The same observations were recorded by Marquart (70) in 1835 for Crocus sativus. Later, Sachs (97), Askenasy (8), and others tried the obvious methods of growing plants in the dark with controls in the light, of darkening leaves while leaving inflorescences uncovered, and so forth. The outcome of these researches, as well as of several others, has been to show that in many cases, for example, in flowers of Tulipa, Hyacinthus, Iris, and Crocus, anthocyanin develops equally well in the dark; in other cases, such as Pulmonaria, Antirrhinum, and Prunella, the development is feeble or absent. Numerous cases may be quoted in which light appears necessary for the formation of the pigment. Reddening of seedlings is entirely absent in the dark in Polygonum tartaricum, Celosi, and Beta (117). The most casual observation will also afford instances of cases where anthocyanin is developed on the sides of stems, twigs, and petioles which are exposed to the sun, the opposite side remaining green. Such phenomena are especially mentioned in stems of Cornus sanguinea, C. sibirica, species of Tilia, Rosa, and Rubus (41). The development of autumnal coloration often takes place only in the parts of leaves and stems exposed to light, as was noted long ago in Viburnum lantana (Voigt, 113).

Linsbauer (62), in 1908, found more precise relationships between light and the formation of anthocyanin. He used seedlings of Fagopyrum esculentum which had been grown in the dark, and were quite etiolated. Such seedlings were then exposed to

artificial light of different intensities and for varying lengths of time. From his results, Linsbauer concluded that the photochemical process of anthocyanin production in light is a typical stimulus reaction, and is dependent upon both the intensity and duration of light.

Mirande (75,76) made some interesting observations on the effect of light on the development of anthocyanin in the detached scales from the bulbs of Lilium candidum. At whatever the altitude the experiment was carried out, the pigment is never produced in direct light; it is produced only in diffuse light, the amount required varying with the altitude. Only the rays of the luminous part of the spectrum are effective, and of these, the blue and the indigo are most active, the red less so; the green are inactive.

Favorable influence of strong illumination in promoting formation of anthocyanins has been observed in the chrysanthemum and Abutilon (55), in Geraniaceae (128) and Coleus (34), and in Diervilla (52).

Chi-Yuen-Chia (23) was able to attain a significant decrease of anthocyanin content in Amaranthus odoratus by decreasing illumination of the pigment in his experiments.

Pearce and Streeter (88) showed that the region from 3,600 Å to 4,500 Å of the solar spectrum is most influential in coloring apples. Allen (2) was able to accelerate the formation of anthocyanin in plums by means of illumination. However, anthocyanin is also found when sunlight is excluded. On the other hand, apples, apricots, pears, and peaches all require sunlight for the

formation of their anthocyanin. For this reason peaches do not take on a red color in storage. Bunning (19) also was able to observe formation of anthocyanin in the dark. Seedlings of red cabbage take on color by means of anthocyanin formation likewise without any illumination whatsoever; they become reddish-violet (36).

Nutrition. The production of red pigment through the oxidation of a chromogen was the hypothesis brought forward by Wigand (121) as early as 1862. That the process is controlled by a specific oxidase has been postulated by Buscalioni and Pollacci (20), Mirande (77), and Wheldale (118,119). The actual dependence of the process on the presence of oxygen is illustrated by the experiments of Mer (71), who mentions the fact that leaves of Cissus do not redden under water. According to Combes' experiments (26), he concluded that the appearance of anthocyanin is accompanied by an accumulation of oxygen in the tissues; the disappearance of the pigment is, on the contrary, accompanied by a considerable loss of oxygen.

Molisch (80) found that leaves of Peireskia aculeata, Tradescantia, Panicum variegatum, and Fuchsia reddened strongly if watered only a little. Eberhardt (31) also found an increase of anthocyanin in leaves of Coleus blumei and Achyranthes angustifolia when grown in a very dry atmosphere. According to Warming (114), plants such as Tillaea aquatica, Peplis portula, and Elatine are green when growing in water, though individuals on land may be strongly red.

Plants which have a foodstuff deficiency often show increased anthocyanin formation. Steinecke (107) found a large quantity of anthocyanin in Lathyrus and Viola species growing on sand dunes particularly poor in foodstuffs. Sugar beets, as revealed in extensive research material (57), often show increased formation of red or violet pigments during deficiency conditions. Lettuce shows the same tendency (126). Calcium deficiency can also be the cause of an increase in pigment formation (68). Berthold (14), together with Boysen Jensen (18), stated that maize reacts to foodstuff deprival by a stronger formation of anthocyanins. Red coloration is also promoted by the addition of potassium to the diet of red cabbage, whereas nitrogen and phosphorus addition decreases the pigment content (92).

Sprengel (105), in 1817, reported that iron salts mixed with the soil in which hydrangeas were growing produced blue and violet flowers. According to Schubler's (102) experiments, the effectiveness of soil was due to its greater carbon and humus content which absorbed the oxygen in the soil, and under this condition little oxygen was supplied through the root, causing a certain deoxidation which changes the pink color to blue. Donald (29) indicated that hydrangea plants treated with aluminum produced blue flowers. Molisch (78) found that aluminum, aluminum sulphate, and ferrous sulphate were able to produce the change in color, and the soils in which hydrangeas produce blue flowers were acid. He concluded that this was due to the greater solubility of Al and Fe in an acid medium.

Allen (3), Cheney (22), and Stock (108) showed that aluminum was essential for the blue color of hydrangea macrophylla sepals. This blue color was due to a complex formation of the aluminum with delphinidin.

Pierre and Stuart (90) and Wright (127) have shown that large applications of available P precipitate Al not only from the soil solution but also within the plant. Based on the results of the Asen, Stuart, and Specht (6) experiments, they indicated that increased concentration of P available to plants of Merville and Todi had no effect on the amount of delphinidin-3-glucoside, but decreased the amount of Al in these tissues. The redder sepals of Merville and Todi hydrangeas were supplied with high concentration of P in these tissues, causing less Al to be available for complexing with delphinidin-3-glucoside. Thus, total Al in sepals of hydrangeas may not always be indicative of their color.

Effect of Infection and Injury. Many authors describe an increase in anthocyanin formation in plants attacked by parasites, in infected plants, and those which have suffered some sort of injury. Stienecke (107) noticed the formation of anthocyanin in leaves, caused by aphids. Kuster (58) found anthocyanin in the supporting tissue of galls and in infected plants. Bodmer (16) observed how species of thrips stimulated anthocyanin formation in the pollen of Lythrum salicaria. Lippmaa (63) also reported an increase in anthocyanin formation after mold infection. Longley (66) described the distribution of anthocyanin in tulips after they had been infected with mosaic disease.

Injured corn plants have manifested increased formation of pigment (67). Increased formation of anthocyanin also was observed when apples were sprinkled with thiocyanates (30). Although, in general, formation of red and violet pigments in infected and injured plants may be attributed to anthocyanins, there are two investigations in which the pigments formed upon infection did not turn out to be identical with anthocyanins. Nierenstein (83) found in a chemical examination of the pigment from the red pea gall on Quercus pedunculata that it had no relation to the anthocyanins. Petrie (89) also could find no trace of anthocyanins in the leaves of Eucalyptus stricta which had been attacked by Eriophyes and subsequently showed strong red coloration. The red and violet pigments in diseased plants obviously are not identical with anthocyanins in every case.

MATERIALS AND METHODS

The plant materials used in these experiments were the leaves and flowers of Hydrangea cultivars Heite's Red and Kuhnert, which were obtained from the Heite Wholesale Greenhouse, Wichita, Kansas.

Fifty dormant hydrangea plants of cultivars Kuhnert and Heite's Red that had been in a 40° F. storage since early October, 1961 were donated for this study by the Heite Wholesale Greenhouse of Wichita, Kansas. The two-year-old plants were under normal cultural practices before receipt. After removal from storage the plants were kept in a 55° F. greenhouse for two weeks and then transplanted to 6" diameter clay pots. The soil mixture

used contained 1:2 peat moss and a silty loam soil. The plants were grown in a 60° F. temperature greenhouse until flowering.

The hydrangeas of cultivar Heite's Red were divided into three equal groups after removal from storage. One group received six applications of 25-0-25 fertilizer solution at a concentration of 1 ounce (oz.) of fertilizer dissolved in 2 gallons (gal.) of water at 10-day intervals. The plants were fertilized from the third week after removing from storage until coloration of the sepals began. A second group received the same treatment with an additional three applications of 1/2 oz. of ammonium phosphate dissolved in 1 gal. of water applied at 20-day intervals beginning two weeks after removal of the plants from storage. The remaining group received six applications of 1/2 oz. of ammonium phosphate in 1 gal. of water at 10-day intervals in conjunction with the applications of the 25-0-25 fertilizer.

Hydrangeas of cultivar Kuhnert were divided into three groups after removal from storage. One group was fertilized with a liquid fertilizer solution containing 1 oz. of 25-0-25 fertilizer per 2 gal. of water every 10 days until sepal color appeared. The second group received an application of 1 oz. of a 25-4-10 fertilizer every 10 days. The remaining hydrangeas received 1 oz. of a 20-10-6 fertilizer dissolved in 2 gal. of water at 10-day intervals. Half of each group was treated with aluminum sulfate at the concentration of 1 pound (lb.) per 7 gal. of water on the following dates: February 1st, 10th, and 20th; March 2nd, 12th, and 22nd; and April 1st, 12th, and 22nd. The plants treated with aluminum sulfate received six applications,

the dates of application depending upon the date of removal from storage.

Ten plants of each of the two cultivars were removed from the 40° F. storage on these dates: January 1st, 12nd, and 20th; February 23rd; and March 1st, in order to obtain a continuous supply of flowers. Healthy fresh leaves and flowers of these cultivars were used for the identification, estimation of anthocyanidin, and quantitatively determination of sugars and phosphorus in the leaves and flowers from the nine different treatments at these three stages of sepal development:

1. The green stage. When most sepals of a flower head just separated from one another. The flower head remained green in color.
2. The white stage. A normal flower head development having two-thirds of the sepals half expanded with little pigment on the parts of sepals.
3. The colored stage. A flower head that has opened fully and maximum sepal color developed over the whole flower head.

Leaf samples were taken from the same stem below the flower head at the three different stages and from the nine different treatments. All samples of flowers were taken at random from the flower head. In the study made of the effect of aluminum sulfate on plants, only sepals of hydrangea of cultivar Kuhnert at the colored stage were used as samples. Sufficient care was taken to use the samples immediately after picking the flowers and leaves from the plants in order to prevent drying out.

Identification and Quantitative Estimation of Pigment

One-gram samples of leaves and flowers were removed at the green, white, and colored stages of sepal development and weighed on a chainomatic balance. The samples were placed in test tubes and a 5-milliliter (ml.) aliquot of acidic methyl alcohol was added to each tube. The contents of the tubes were homogenized by an electric homogenizer for two to four minutes until the sepals or leaf tissue became crushed. The homogenizer rod and the test tube were washed with the acidic methyl alcohol so as to avoid any loss of the pigment. The test tubes containing the extracts were then placed in a steam bath for concentration of each extract to about 3 ml. Vigil was necessary at this stage to insure that the contents of the tube did not boil over or become completely dry. Next, 15 ml. of 2N hydrochloric acid were added to each tube for hydrolysis. It was found that one hour was sufficient for the complete hydrolysis of all pigments. The tubes were then taken out of the steam bath and cooled by running water over them. This procedure was basically the same for both the identification and the estimation of pigments, but the following steps are different.

Identification. Three to four milliliters of n-butyl alcohol were added to each tube and shaken well. This separated the aglycone and the sugar moiety into the supernatant layer and hypo layer, respectively.

Following the isolation of aglycone, the colored solution was chromatographed on Whatman No. 1 filter paper. The Rf values

were calculated from the spotted chromatograms; whereas the streaked chromatograms were used for the elution of the pigment for the purpose of reading with the Beckman spectrophotometer. The chromatographed sheets were prepared by streaking the solution in a band 1 centimeter (cm.) wide across the broad width of the paper on the starting line 5 cm. from the base of the paper. A pipette of 1-ml. capacity was used for applying the solution. A micropipette of 100-microliter capacity was used to apply the concentrate in spots 5 cm. apart.

The spotted or streaked chromatographic paper was rolled, clipped, and placed in a presaturated glass chamber 24 in. high and 12 in. in diameter. The chamber had become saturated with the vapors of Forestal Solvent (acetic acid, hydrochloric acid, and water in the proportion of 30:3:10 v/v) over night before placing the paper in the chamber. A glass pie plate 10 in. in diameter was placed in the chamber containing about 150-200 ml. of solvent to be used. The chamber was covered on the top with a glass plate, which was sealed with modeling clay, and then covered with brown paper to keep out the light.

Ascending chromatography was carried out at room temperature. After 24 hours, the chromatogram was taken out of the chamber and air dried at a room temperature of 80° F. Rf values were calculated by measuring the distance the solute moved on the chromatogram and dividing this by the distance the solvent moved. Rf values for the isolated anthocyanidins were tentatively identified by comparison with those in the literature. Further identification was made by spectrophotometric examinations.

The chromatograms were air dried at room temperatures, and the pigment streaks cut from the moist but not wet chromatograms with scissors. The chromatographic strips were then placed in test tubes, were stoppered after the addition of 5-10 ml. of acidic methyl alcohol, and left in a dark cabinet for about an hour. During this period the tubes were shaken once or twice. The pigments from the paper were eluted almost completely after an hour. Each eluent was transferred to another tube, and the peaks of maximum absorption of anthocyanidins were determined with a Beckman model DU spectrophotometer. Both anthocyanins and anthocyanidins have definite peaks of maximum absorption in the visible spectrum. These peaks have been worked out for the authentic anthocyanidins, and their values described in the literature (5,13,46). The identification of the unknown anthocyanidins was determined by comparing their peaks of maximum absorption with the absorption maxima of the authentic anthocyanidins described in the literature.

Quantitative Estimation. Each sample, after cooling, was carefully transferred to a 125-ml. separatory funnel. The tube first was washed with about 10 ml. of distilled water and then with 5-10 ml. of n-butyl alcohol. The contents of each funnel was shaken vigorously after stoppering. The funnel was allowed to stand for two to three minutes until two phases had formed. The hypo phase was drained to another separatory funnel; 10 ml. of n-butyl alcohol were added, and the process repeated several times to insure that the extraction was complete.

Each funnel was washed with about 3 ml. of n-butyl alcohol; this insured that no pigment was left in the separatory funnel. All aliquots containing anthocyanidin were combined and transferred to 50-ml. volumetric flasks. Each solution was read at 545 millimicrons (mu.) in a Beckman DU spectrophotometer. The quantity of the pigment in the solution was determined by comparison with a standard curve for cyanidin concentration (Plate I) established by Ahuja (1).

Determination of Sugars

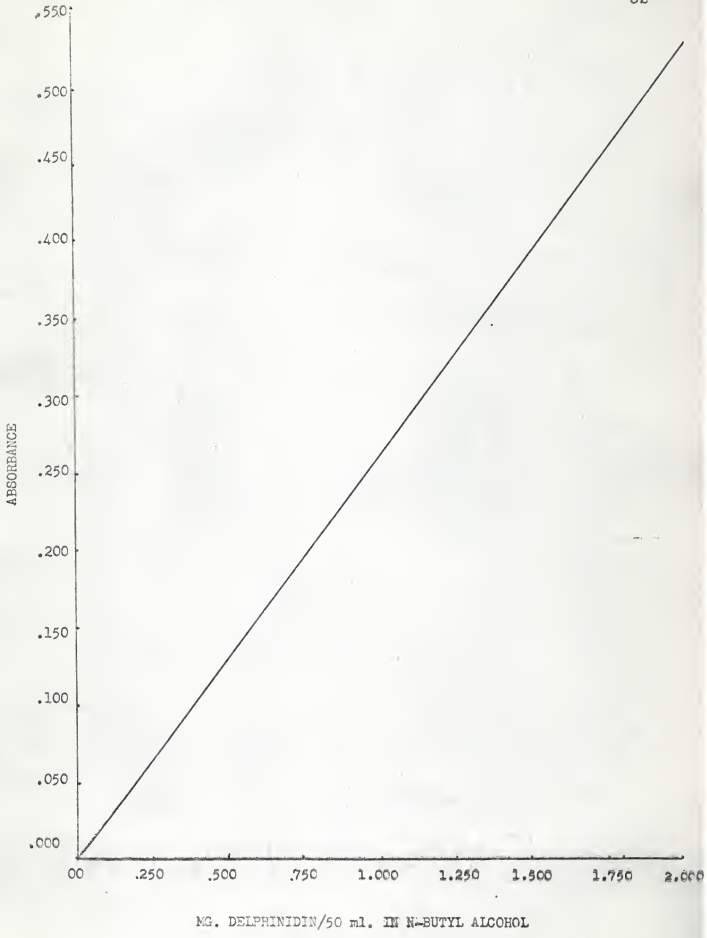
Two-gram samples of fresh plant materials were weighed and embedded in cotton in glass thimbles. The samples were pressed slightly and placed in the Goldfisch extractor. To each of the Goldfisch extractor cups, 30 ml. of 80 percent ethanol was added before the extractor cups were connected to the condensers. Extraction was done for six to eight hours. After extraction, the cups with the alcoholic extracts were placed on a steam bath and evaporated to about 10 ml. Water was added and the cups reheated for 10 more minutes to be sure all ethanol had been evaporated. After cooling to room temperature, water was added and the samples were transferred to 100-ml. graduated cylinders. Solutions were purified with 2 ml. of saturated neutral lead acetate, diluted with water to make the volume 100 ml. mixed, and left for 15 minutes. The solutions were filtered through E & D No. 615 filter paper directly into 250-ml. beakers. In order to remove the excess lead, 2 grams of potassium oxalate

EXPLANATION OF PLATE I

X-axis represents milligrams of delphinidin per 50 milliliters in n-butyl alcohol.

Y-axis represents absorbance with a Beckman DU spectrophotometer.

Any reading up to 0.550 with the Beckman DU spectrophotometer could be read directly to give quantity of delphinidin in milligrams in 50 milliliters of the extract in n-butyl alcohol. This 50 milliliters of the extract was prepared from a weighed quantity of flowers and leaves. Thus, the quantity of delphinidin per gram weight of fresh flowers and leaves was determined.


.550
.500
.450
.400
.350
.300
.250
.200
.150
.100
.050
.000
ABSORBANCE
00
.250
.500
.750
1.000
1.250
1.500
1.750
2.000
MG. DELPHINIDIN/50 ml. IN N-BUTYL ALCOHOL

were added to each solution and then mixed. This was again filtered through E & D No. 615 filter papers.

Aliquots of 50 ml. were placed in 250-ml. beakers. Five milliliters of concentrated HCl were added and left overnight. The next day, 2 drops of methyl red was added to each of the cups, and neutralized with 25 percent NaOH. The solutions were transferred to the 100-ml. volumetric flasks, and the beakers were rinsed with distilled water several times. The distilled water was then poured back to the same volumetric flask. Solutions were diluted to the mark and mixed.

Two milliliters of each of the above solution were placed in 15-ml. calibrated centrifuge tubes, and exactly 3 ml. of ferricyanide solution were added to each tube. The materials were mixed and then immersed in a boiling water bath for five minutes. Each tube was then removed, cooled, and diluted to the mark. After mixing the contents of the tubes, the color intensities were determined at 420 mu. with a Beckman spectrophotometer. If the 3 ml. of ferricyanide was insufficient to completely oxidize the reducing sugars, then the test was repeated using a 1-ml. sample of reducing sugars. This need for repeating the test could visibly be detected by the disappearance of the yellow color of the ferricyanide, the solution becoming colorless after being heated on the steam bath.

After the photoelectric colorimeter readings were obtained, the weight in milligrams of sugar in the aliquot could be determined by locating the readings on a standard curve. This standard curve was prepared by subjecting known amounts of

glucose to the reduction procedure (Plate II).

Determination of Phosphorus

Each 1-gram sample of fresh plant material was put in a 30-ml. micro-Kjeldahl flask; in certain cases the samples had to be divided into small bites to fit in the flask. Two milliliters of concentrated sulfuric acid were added to digest the samples. The flasks were rotated, mixing the plant material with the acid, and allowed to stand for a few minutes. Next, 0.5 ml. of 30 percent hydrogen peroxide were carefully added to the flasks, then heated on the hot plate of a micro-Kjeldahl digestion apparatus in which fumes from the micro-Kjeldahl flasks were removed through a suction tube after heating. If the material was still dark, the flask was cooled, rotated, and a few drops more of hydrogen peroxide were slowly added to the sides of the flask and reheated. Slow addition in this manner avoided spattering. Charred material adhering to the flask was washed down into the sample.

This process of adding a few drops of hydrogen peroxide and reheating was repeated several times if necessary to obtain a colorless solution. Then, solutions were heated slowly to expel excess hydrogen peroxide. After the bubbling ceased, the samples were cooled, and water was added.

The extraction solutions were transferred to 100-ml. volumetric flasks. Sufficient care was taken to avoid any loss of the phosphorus. Solutions were diluted to make the volume 100 ml. and mixed thoroughly. Forty-milliliter samples of the

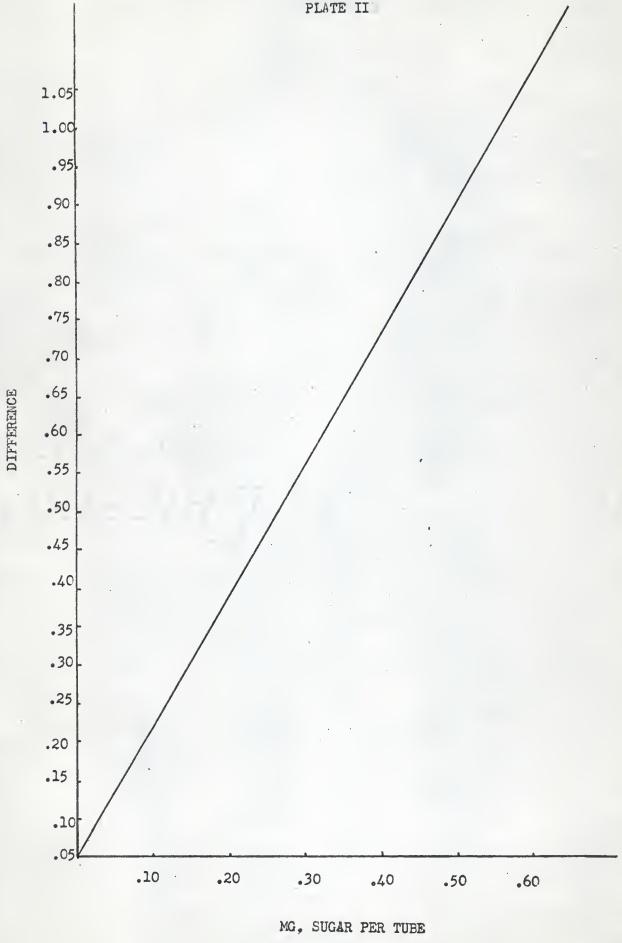
EXPLANATION OF PLATE II

X-axis represents milligrams of sugar per tube.

Y-axis represents the difference between the readings of the ferricyanide solution and the sugar solution plus the ferricyanide solution.

Any reading up to 1.05 with the Beckman DU spectrophotometer could be read directly to give quantity of sugar in milligrams in 15 milliliters of the extract. This 15 milliliters of the extract was prepared from a weighed quantity of flowers and leaves. Thus, the quantity of sugar per gram weight of fresh flowers and leaves was determined.

PLATE II


1.05
1.00
.95
.90
.85
.80
.75
.70
.65
.60
.55
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
DIFFERENCE
.10
.20
.30
.40
.50
.60
MG, SUGAR PER TUBE

solutions were transferred to other 100-ml. volumetric flasks with a pipette. Water was added to make the volumes from 50 to 75 ml.

Next, 4-ml. aliquots of molybdate solution were added to each of the flasks, and swirled. Two milliliters of aminonaphtholsulfonic acid solution were added to each flask and again mixed. The solutions were diluted with water to make the volume 100 ml., mixed thoroughly, and allowed to stand for 15 minutes in order to obtain the maximum development of blue color. Each solution was transferred to a photometer absorption cell, and readings were taken with a Beckman model DU spectrophotometer at 820 mμ. Readings were compared with a cell containing distilled water.

The concentration of phosphate per 100-ml. volume was determined by locating the photometer readings on a standard curve. The standard curve had been previously prepared by subjecting known amounts of phosphate to this test (Plate III).

RESULTS

Anthocyanidins

Anthocyanidins were isolated from Hydrangea Heite's Red and Kuhnert, and identified by ascending chromatography in Forestal solvent. The anthocyanidin Rf values were calculated; these are presented in Table 1. The Rf values for the authentic anthocyanidins from different sources described in the literature have been presented in Table 2. The Rf values in the literature for delphinidin ranged between 0.30 and 0.38. In the present study

PLATE III

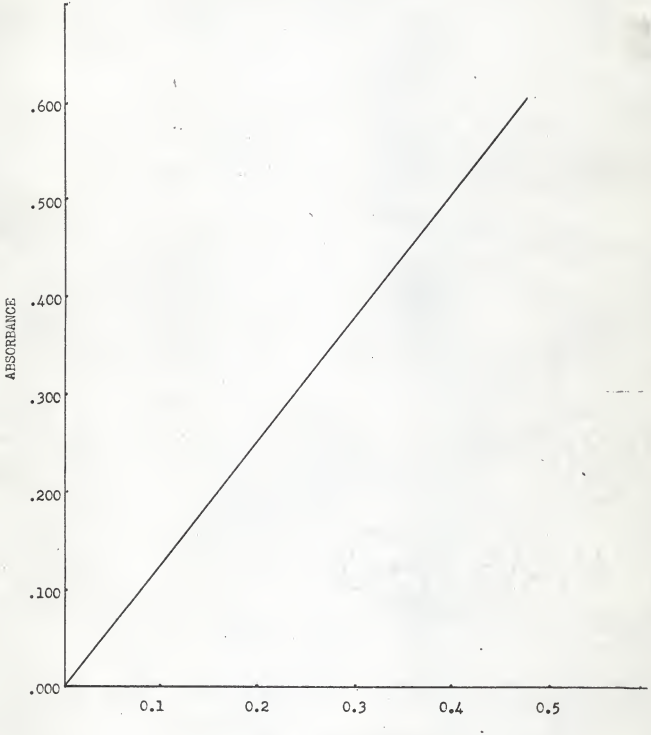

.600
.500
.400
.300
.200
.100
.000
ABSORBANCE
0.1
0.2
0.3
0.4
0.5
MG. PHOSPHORUS PER 100 ml.

Table 1. Rf values of anthocyanidins extracted with Forestal solvent from the sepals of hydrangeas 'Heite's Red' and 'Kuhnert.'

Source	Rf values	Maximum absorption in acidic methanol
Heite's Red	0.35	555
Kuhnert	0.31	545-555

Table 2. Rf values and absorption maxima of the authentic anthocyanidins.

Anthocyanidin	Absorption maxima mu.	Rf value in Forestal solvent	Author
Pelargonidin	530	0.68	Bate-Smith (12)
	530	0.76	Halevy and Asen (46)
	530	0.74	Asen (5)
Cyanidin	545	0.50	Bate-Smith (12)
	545	0.56	Halevy and Asen (46)
	545	0.60	Asen (5)
Peonidin	545	0.63	Bate-Smith (12)
Delphinidin	555	0.30	Bate-Smith (12)
	555	0.37	Halevy and Asen (46)
	555	0.38	Asen (5)
Petunidin	555	0.45	Bate-Smith (12)
	557	0.53	Halevy and Asen (46)
Malvidin	555	0.60	Bate-Smith (5)

the average Rf values were 0.35 for cultivar Heite's Red and 0.31 for cultivar Kuhnert. A comparison of Rf values indicated that the anthocyanidins in the sepals of these cultivars were delpninidin.

Anthocyanidins have maximum absorption in the visible spectrum. The values of this peak remain constant for a particular

anthocyanidin. The values for authentic anthocyanidins are presented in Table 2. Chromatographs in this investigation were eluted with methyl alcohol containing 0.01N hydrochloric acid. The wavelengths of maximum absorption are presented in Table 1 for the anthocyanidins from the sepals of cultivars Kuhnert and Heite's Red. This spectrophotometric method revealed an absorption maximum at 545-555 mu. for cultivar Kuhnert and 555 mu. for cultivar Heite's Red. These readings indicated that the compound was delphinidin.

The quantities of anthocyanidin in milligrams per gram fresh weight of flowers and leaves were estimated for both cultivars at the green, white, and colored sepal stages at three different rates of phosphate application to the soil.

Sepals at the colored stage have maximum anthocyanidin development, while flowers at the green and white stages have no measurable quantities of anthocyanidin. This is true for both cultivars of Heite's Red (Table 3 and Plate IVa) and Kuhnert (Table 4 and Plate Va). The anthocyanidin contents at the colored stage in the flowers of Heite's Red hydrangea showed a decreasing trend with an increase in the frequency of phosphorus application to the soil. No differences in anthocyanidin content were found in the flowers of Kuhnert at the three phosphorus levels.

There were no measurable amounts of anthocyanidin in the leaves of cultivars Heite's Red and Kuhnert at any stage of sepal development and at any level of phosphorus.

Table 3. Anthocyanidin, sugar, and phosphorus content from fresh flowers of Heite's Red at three stages of sepal development and at three levels of phosphorus (expressed in mg/gm fresh weight).

Phosphorus level	Sepal development stages: Green	White	Colored	Mean	LSD 5%	F
Anthocyanidin						
0	0	0	1.376	.258	0.129	7.051**
3	0	0	1.211	.403		
6	0	0	.816	.272		
Mean	0	0	1.139			
LSD 5%	0.129					
F	330.162**					
P.S.[a]	**					
Sugar						
0	51	44	156	83.6	18.70	ns
3	31	51	126	69.3		
6	53	55	98	68.6		
Mean	45	50	126.6			
LSD 5%	18.70					
F	91.77**					
P.S.[a]	*					
Phosphorus						
0	.983	.708	.332	.674	.051	132.5**
3	.495	.280	.243	.339		
6	.578	.502	.417	.499		
Mean	.685	.496	.331			
LSD 5%	.051					
F	149.8**					
P.S.[a]	**					

* 5% level of significance

** 1% level of significance

ns Non-significant results

[a] Interaction between phosphorus and stages

EXPLANATION OF PLATE IV

a. Contents of anthocyanidin in the flowers of cultivar Heite's Red hydrangea at different stages of sepal development and phosphorus levels.

b. Contents of sugar in the flowers of cultivar Heite's Red hydrangea at different stages of sepal development and phosphorus levels.

c. Contents of phosphorus in the flowers of cultivar Heite's Red hydrangea at different stages of sepal development and phosphorus levels.

PLATE IV

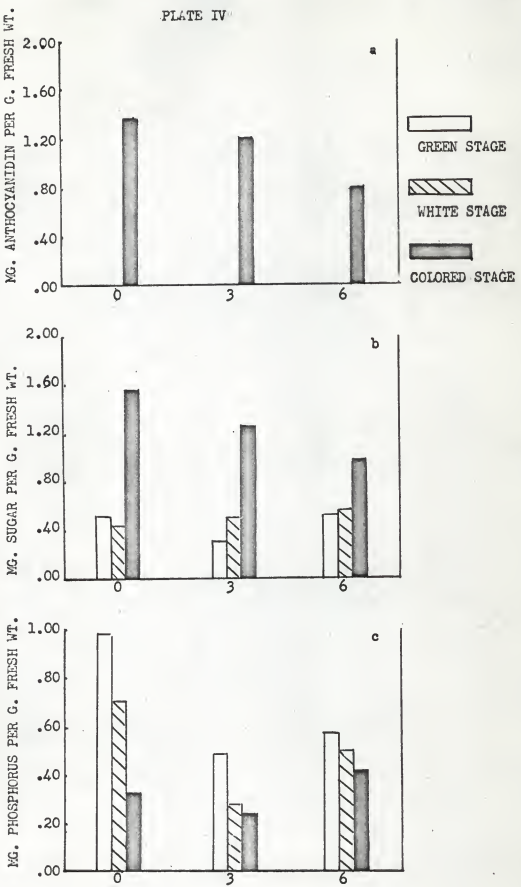

MG. ANTHOCYANIDIN PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
0
3
6
a
GREEN STAGE
WHITE STAGE
COLORED STAGE
MG. SUGAR PER G. FRESH WT.
b
MG. PHOSPHORUS PER G. FRESH WT.
1.00
.80
.60
.40
.20
c
APPLICATION OF PHOSPHORUS
TO THE SOIL

Table 4. Anthocyanidin, sugar, and phosphorus content from fresh flowers of the Kuhnert hydrangea at three stages of sepal development and at three levels of phosphorus (expressed as mg/gm fresh weight).

Phosphorus level	Sepal development stages								
	Green	White	Colored	Green	White	Colored	Green	White	Colored
	Anthocyanidin			Sugar			Phosphorus		
0	0	0	.586	49	46	82	.593	.279	.241
3	0	0	.544	71	83	86	.642	.330	.215
6	0	0	.558	60	78	128	.666	.512	.260

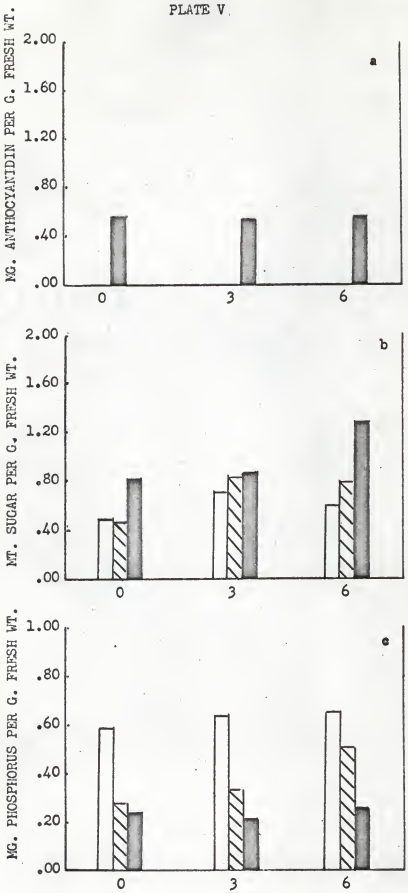
EXPLANATION OF PLATE V

a. Contents of anthocyanidin in the flowers of cultivar Kuhnert hydrangea at different stages of sepal development and phosphorus levels.

b. Contents of sugar in the flowers of cultivar Kuhnert hydrangea at different stages of sepal development and phosphorus levels.

c. Contents of phosphorus in the flowers of cultivar Kuhnert hydrangea at different stages of sepal development and phosphorus levels.

PLATE V


MG. ANTHOCYANIDIN PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
a
0
3
6
MT. SUGAR PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
b
0
3
6
MG. PHOSPHORUS PER G. FRESH WT.
1.00
.80
.60
.40
.20
.00
c
0
3
6
APPLICATION OF PHOSPHORUS
TO THE SOIL

Sugar

Large differences in the sugar content were found in the flowers of Heite's Red hydrangea at the different stages of sepal development. The sugar content increased rapidly with color appearance. The only exception was a slight decrease in the sugar content in the flowers of Heite's Red hydrangea at the colored stage, with an increase in the frequency of phosphorus application. With this exception there didn't appear to be an effect of phosphorus application on the sugar content in the flowers of Heite's Red hydrangea (Table 3 and Plate IVb). Analyses of flower samples from cultivar Kuhnert were made from plants treated with or without aluminum sulfate. Great differences in sugar content from the flowers of Kuhnert were found at different stages of sepal development and frequency of phosphorus application (Table 4 and Plate Vb). Differences in sugar content at the 5 percent level of statistical analysis were found in the leaves of Heite's Red at different stages of sepal development and at the various frequencies of phosphorus application (Table 5 and Plate VIa). The leaves of the Kuhnert hydrangea used as samples were taken from plants treated with or without aluminum sulfate. No consistent differences in the sugar content of leaves from the Kuhnert were found either at different stages of sepal development or phosphorus application (Table 6 and Plate VIIa).

Table 5. Sugar and phosphorus content from fresh leaves of Heite's Red at three stages of sepal development and at three levels of phosphorus (expressed in mg/gm fresh weight).

Phosphorus level	Sepal development stages: Green	White	Colored	Mean	LSD 5%	F
			Sugar			
0	34.0	38.0	48	40.0	14.62	8.97*
3	34.5	40.5	50	41.6		
6	54.0	47.0	79	60		
Mean	40.8	41.8	59			
LSD 5%	14.62					
F	7.6*					
P.S.[a]	ns					
			Phosphorus			
0	.365	.333	.341	.346	.194	6.936*
3	.333	.213	.289	.278		
6	.430	.695	.539	.555		
Mean	.376	.413	.390			
LSD 5%	.194					
F	ns					
P.S.[a]	ns					

* 5% level of significance

ns Non-significant results

[a] Interaction between phosphorus and stages

Phosphorus

The quantities of phosphorus in the flowers of Heite's Red hydrangea decreased greatly from the green sepal stage to the colored sepal stage. Although significant differences in phosphorus content in the flowers of Heite's Red hydrangea occurred at different frequencies of phosphorus application, the phosphorus content was not proportional to the rate of phosphorus

EXPLANATION OF PLATE VI

a. Contents of sugar in the leaves of cultivar Heite's Red hydrangea at different stages of sepal development and phosphorus levels.

b. Contents of phosphorus in the leaves of cultivar Heite's Red hydrangea at different stages of sepal development and phosphorus levels.

PLATE VI

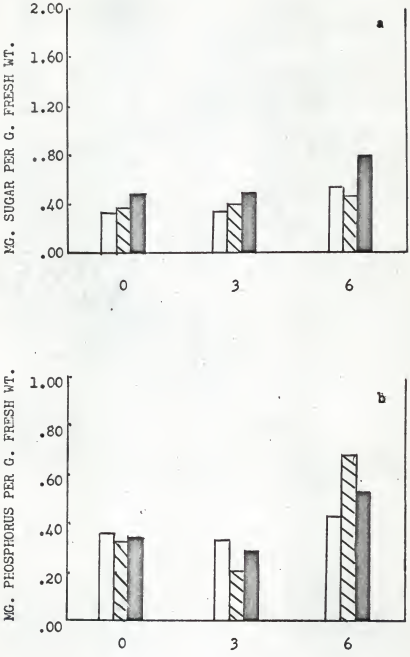

a
MG. SUGAR PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
0
3
6
b
MG. PHOSPHORUS PER G. FRESH WT.
1.00
.80
.60
.40
.20
.00
0
3
6
APPLICATION OF PHOSPHORUS
TO THE SOIL

Table 6. Sugar and phosphorus content from fresh leaves of the Kuhnert hydrangea at three stages of sepal development and at three levels of phosphorus (expressed as mg/gm fresh weight).

Phosphorus level	Sepal development stages					
	Green	White	Colored	Green	White	Colored
	Sugar			Phosphorus		
0	45	38	52	.251	.167	.224
3	41	38	44	.263	.188	.210
6	68	53	45	.363	.292	.230

application (Table 3 and Plate IVc). Although variability of phosphorus content in the leaves of Heite's Red hydrangea was large, non-significant differences in phosphorus content of the leaves were found at the different stages of sepal development. Significant differences in phosphorus content of the leaves occurred at different rates of phosphorus application, but the quantities of phosphorus in the leaves were not proportional to the rates of phosphorus application (Table 5 and Plate VIa).

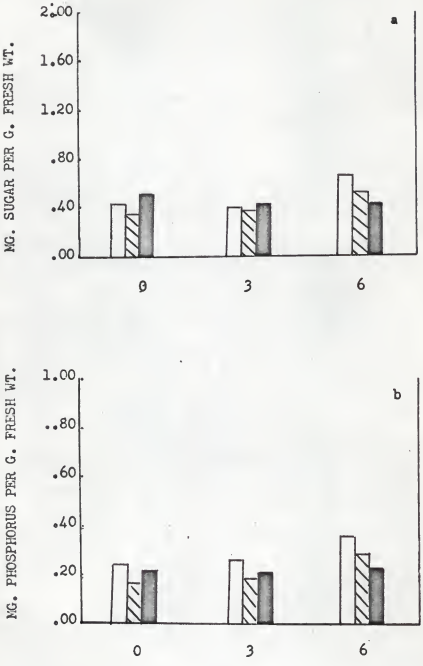
Samples of flowers and leaves from Kuhnert hydrangeas were taken both from plants treated with aluminum sulfate and plants not treated with aluminum sulfate. The quantities of phosphorus in the flowers of the Kuhnert hydrangea decreased from the green stage to the colored stage, and a slight increase in the phosphorus conetnt was associated with an increase in the phosphorus application (Table 4 and Plate Vc). No consistent differences were found in the amount of phosphorus in the leaves of the Kuhnert hydrangea at different stages of sepal development and phosphorus levels (Table 6 and Plate VIIb).

EXPLANATION OF PLATE VII

a. Contents of sugar in the leaves of cultivar Kuhnert hydrangea at different stages of sepal development and phosphorus levels.

b. Contents of phosphorus in the leaves of cultivar Kuhnert hydrangea at different stages of sepal development and phosphorus levels.

PLATE VII



APPLICATION OF PHOSPHORUS

TO THE SOIL

Effects of Aluminum Sulfate on the Contents of Anthocyanin, Sugar, and Phosphorus in the Sepals of the Kuhnert Hydrangea

The sepals of plants of cultivar Kuhnert not treated with aluminum sulfate appeared pink in color. The soil phosphorus level appeared to have no effect on the amount of delphinidin in the sepal (Table 7 and Plate VIIIa). Plants treated with aluminum sulfate produced flowers with sepal color ranging from pink to blue through mauve. Most of the sepals from plants supplied with high levels of phosphorus showed a desirable pink color, while plants supplied with low levels of phosphorus were blue in color. The quantities of anthocyanidin in the blue sepals were higher than the quantities in pink sepals, although the anthocyanidins were identical in both the pink and the blue sepals.

The applications of aluminum sulfate significantly decreased the sugar content at the colored sepal stage (Table 7 and Plate VIIIb). Although there was a slight decrease in the sugar content with the increase in the rates of phosphorus doses, there were no significant differences in sugar content in the sepals of plants not treated with aluminum sulfate at the three levels of phosphorus. The rates of phosphorus application significantly increased the sugar content in the sepals of the Kuhnert hydrangeas treated with aluminum sulfate.

The applications of aluminum sulfate decreased the phosphorus content in the sepals of the Kuhnert hydrangea treated with higher levels of phosphorus (Table 7 and Plate VIIIc).

Table 7. Anthocyanidin, sugar, and phosphorus content from fresh sepals of the Kuhnert hydrangea at the colored stage of sepal development at three phosphorus levels with or without applications of aluminum sulfate (expressed in mg/gm fresh weight).

Phosphorus level	Anthocyanidin		Sugar		Phosphorus	
	Treatment					
	No $Al_2(SO_4)_3$	$Al_2(SO_4)_3$	No $Al_2(SO_4)_3$	$Al_2(SO_4)_3$	No $Al_2(SO_4)_3$	$Al_2(SO_4)_3$
0	.480	.476	134	77.2	.258	.283
3	.504	.494	120	105.6	.187	.176
6	.479	.421	113	90.0	.265	.247
LSD 5%	.105	.130	23.0	5.2	.023	.024
F	ns	38.72**	ns	187.9**	120**	92.4**

A two-way analysis of variance of above data is presented here:

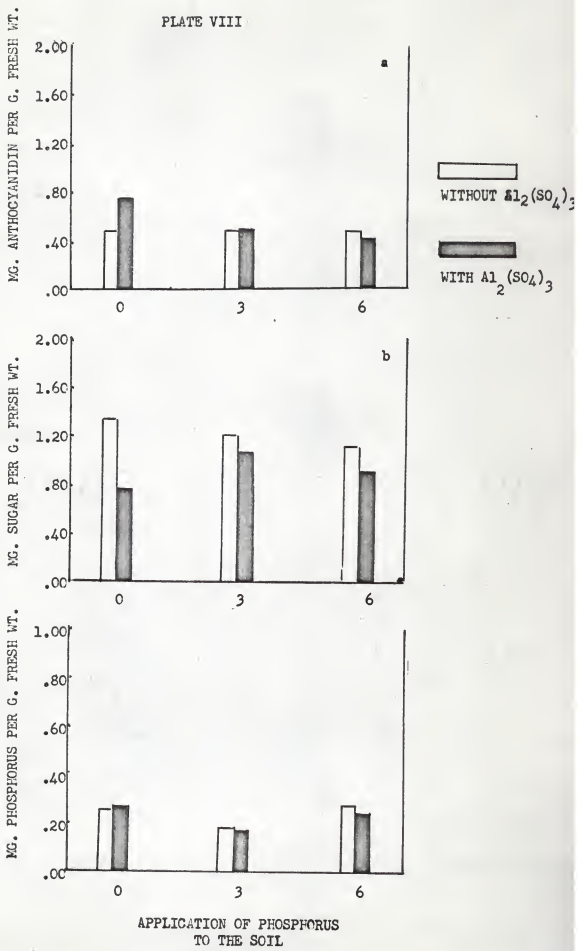
Source	df	Anthocyanidin	Sugar	Phosphorus
			Variance	
Phosphorus level	2	.0436**	198.16*	.0135**
Al_2SO_4 application	1	.0238**	4480.88**	.0010**
P x A	2	.0417**	758.68**	.0007**
Within	12	.0023	42.95	.000042
LSD 5% for phosphorus		.063	10.07	.0098
LSD 5% for $Al_2(SO_4)_3$		.048	6.71	.0068

* 5% level of significance

** 1% level of significance

ns Non-significant results

PLATE VIII


MG. ANTHOCYANIDIN PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
a
0
3
6
WITHOUT $Al_2(SO_4)_3$
WITH $Al_2(SO_4)_3$
MG. SUGAR PER G. FRESH WT.
2.00
1.60
1.20
.80
.40
.00
b
0
3
6
MG. PHOSPHORUS PER G. FRESH WT.
1.00
.80
.60
.40
.20
.00
0
3
6
APPLICATION OF PHOSPHORUS TO THE SOIL

DISCUSSION

The chromatographic technique used in the present study was similar to the one outlined by Bate-Smith (12). Identification of anthocyanidins was made by comparing the calculated Rf value with the values described in the literature for authentic anthocyanidins. Absorption spectra for the anthocyanidins under study were determined with a Beckman DU Spectrophotometer. The absorption spectra were compared with the absorption spectra of authentic anthocyanidins described in the literature. Final identification was made both on the basis of Rf value and absorption spectra.

The two cultivars under study had only one band. The Rf value of the pigment was within the range of Rf values for delphinidin as described in the literature. The absorption spectra for the pigments also were quite similar to the absorption spectra for delphinidin. A little difference in the values might be due to the difference in purity of the material or some experimental error. However, since both the Rf value as well as the absorption spectra are quite close to the Rf value and absorption spectra for the authentic delphinidin, it seems reasonable to conclude that the pigment in the two cultivars was delphinidin.

There was no anthocyanidin in the flowers at the white and green stages for both cultivars Heite's Red and Kuhnert hydrangea. It is possible that anthocyanin may be present in minute amounts which could not be detected in this experiment.

The amount of anthocyanidin in flowers of Heite's Red hydrangea at the colored stage decreased with the increase in the phosphorus level. This was probably due to the sugar content in the flowers. A slight decrease in the sugar content was associated with an increase in the phosphorus application (Table 3 and Plate IV). Thimann (111) stated that the pigment-forming mechanism is independent of the phosphorus concentration, and MacGillivray (69) noted in the tomato that the absence of phosphorus greatly increased both reducing and non-reducing sugar. Therefore, a decrease in the phosphorus level of the hydrangea increased the production of anthocyanidins indirectly. But, the phosphorus level seemed to have no effect on the anthocyanin content in the flowers of the Kuhnert hydrangea. Asen, Stuart, and Specht (6) observed that increased concentrations of phosphorus available to plants of Merville and Todi had no effect on the quantity of delphinidin-3-glucoside. It seems a reasonable explanation that the increased phosphorus levels have no effect on the amount of delphinidin in the flowers of the Kuhnert hydrangea. This possibly could be explained by varietal differences.

Sepal color changes from pink to blue for the Kuhnert hydrangea occurred when plants were treated with aluminum sulfate. It may be concluded that the change in color was a result of the absorption of aluminum, probably as ions from the soil solution followed by the formation within the flower tissue of a blue-colored aluminum-delphinidin complex. Increased quantities of phosphorus decrease the amount of aluminum in these tissues. Plants receiving higher levels of phosphorus had desirable pink-

colored sepals. The Rf values and absorption spectra of pigments from both blue and pink sepals of the Kuhnert hydrangea were quite similar to the Rf values and absorption for the authentic delphinidin. Therefore, the anthocyanidin in both pink and blue sepals of the Kuhnert hydrangea were identical, but the amount of anthocyanidin in blue sepals is greater than that in pink ones.

No anthocyanidins were found in measurable quantities in leaves of either cultivar. This possibly was due to a lower sugar content in the leaves. According to the literature (87), tests upon red autumnal leaves indicated more sugar in red than in green leaves; reddening of leaves is correlated with an excess of sugar in the plant tissues.

The sugar content in the flowers of both Heite's Red and Kuhnert hydrangeas increased from the green to the colored stages. Carbohydrates are required for the development of flower and fruit color. There was a direct correlation between the sugar content of the flower and the appearance of color, especially in the case of Heite's Red hydrangea. No large differences in sugar content in flowers of cultivar Kuhnert occurred at different stages of sepal development as compared with Heite's Red. Possibly this was due to the interference of aluminum sulfate or was simply varietal. Flower samples of the Kuhnert hydrangea were taken from both plants untreated and treated with aluminum sulfate. Results showed (Table 7 and Plate VIII) that the sepals of the Kuhnert hydrangea not treated with aluminum sulfate contained much more sugar than sepals from plants treated with aluminum sulfate. The rate of phosphorus application had no

effect on the sugar content of flowers of Heite's Red hydrangea, but the phosphorus level did influence sugar content of flowers of the Kuhnert hydrangea. This may have been caused by interaction between aluminum and phosphorus. The results presented have shown that there was an important effect of phosphorus application on the sugar content in the sepals of the Kuhnert hydrangea treated with aluminum sulfate, but there was no effect on the sugar content in the sepals of the Kuhnert hydrangea not treated with aluminum sulfate. The increase in sepal sugar content of plants treated with aluminum sulfate was not proportional to the rate of phosphorus dosage. This might also be explained by this elemental interrelationship.

Carbohydrates synthesized in the leaves are transferred to flowers, roots, and other organs; therefore, no great accumulation of sugars occurs in the leaves at any stage of sepal development regardless of phosphorus level. Anthocyanidin synthesis in the leaves of both cultivars was inhibited by the low concentration of sugars.

The quantities of phosphorus in the hydrangea flowers of both cultivars decreased greatly from the green to the colored stage at any phosphorus level. This result was the reverse of that for the sugar content in the flowers for both cultivars at different stages. It appeared that a correlation existed between sugar and phosphorus contents in the flowers at any particular stage. No differences in phosphorus content of leaves occurred at different stages of sepal development for either cultivar.

There is an effect of the rate of phosphorus application on the phosphorus content in the flowers and leaves of Heite's Red hydrangea, but increasing the phosphorus level is not proportional to the content in these tissues. No explanation can be offered for this phenomenon.

These results indicate a decreasing trend in the sugar content as the phosphorus content increases. These results were consistent when comparing the sugar content and phosphorus content at three stages of development at the same phosphorus level. The amount of anthocyanidin produced in the flowers of Heite's Red hydrangea increased with the sugar content in the flowers and with a decrease in the phosphorus level. There seemed to be a relationship between the anthocyanidin, sugar, and phosphorus levels in the flowers of Heite's Red hydrangea, but no such relationship was observed for cultivar Kuhnert.

Further research is needed with different cultivars to determine how aluminum sulfate influences the content of phosphorus and the production of anthocyanidin and sugar in the plant tissues.

SUMMARY

Fifty dormant hydrangea plants of cultivars Heite's Red and Kuhnert that had been in a 40° F. storage were used in this study. After removal from storage, the plants were kept in a 55° F. greenhouse for two weeks and then transplanted to 6" diameter clay pots. The plants were grown in a 60° F. greenhouse until flowering.

Three equal groups of 16 plants of each cultivar were treated at three different rates of application of phosphorus fertilizer. Half of each group of Kuhnert hydrangeas were treated with aluminum sulfate. Plants were fertilized from the third week after removing from storage until initial coloration of the sepals.

Weighed samples of flowers, leaves, and sepals from the plants were used for the identification and estimation of anthocyanidin and quantitative determinations of sugars and phosphorus at these three stages of sepal development: green, white, and colored.

Chromatographic and spectrophotometric methods were used to identify the anthocyanidins. The anthocyanidin, sugar, and phosphorus were estimated quantitatively by locating the photometer readings on a standard curve prepared for this purpose. The anthocyanidin in the two cultivars was identified as delphinidin. Sepal color changes from pink to blue with the Kuhnert hydrangea occurred when plants were treated with aluminum sulfate. The anthocyanidins in both pink and blue sepals of the Kuhnert hydrangea were identical, but the amount of anthocyanidins in blue sepals was higher than that in pink ones. It also was found that there was no delphinidin in leaves at any stage of sepal development and in the flowers at the white and green stages, but large quantities of delphinidin were isolated from flowers of both cultivars at the colored stage.

The sugar content of the flowers from both cultivars increased significantly from the green to the colored stages, but

the phosphorus content in the flowers decreased. The sugar content in the hydrangea sepals of Heite's Red increased rapidly with the appearance of color. The quantities of delphinidin in the flowers at the colored stage increased in the same manner as the sugar content when the phosphorus level was lowered. The amount of delphinidin in the flowers of the Kuhnert hydrangea treated with aluminum sulfate decreased with the increase of phosphorus applications. Plants receiving higher levels of phosphorus had a desirable pink color. Aluminum sulfate also inhibited the accumulation of sugar. Because of the interference of aluminum sulfate, no relationship between delphinidin, sugar, and phosphorus levels was observed in the Kuhnert hydrangea.

Further research is needed with different cultivars to determine how the aluminum sulfate influences the content of phosphorus and the production of sugar and anthocyanidin in plant tissues.

ACKNOWLEDGMENTS

The author is indebted to Dr. William J. Carpenter, major adviser, for his very valuable guidance and aid in conducting this research. The author is also indebted to Dr. Howard L. Mitchell, Head of the Department of Biochemistry for his technical guidance and valuable advice from time to time and for allowing the use of a Biochemistry laboratory for conducting this research. Thanks are also due Dr. Robert P. Ealy, Head of the Department of Horticulture, for giving permission to carry out this research.

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RELATIONSHIP OF SUGAR, ANTHOCYANIDIN, AND PHOSPHORUS LEVELS IN FLOWERS AND LEAVES OF HYDRANGEA MACROPHYLLA

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Horticulture
and
Landscape Architecture

KANSAS STATE UNIVERSITY
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1963

Fifty dormant hydrangea plants of cultivars Kuhnert and Heite's Red that had been in a 40° F. storage were donated for this study by the Heite Wholesale Greenhouse of Wichita, Kansas. After removal from storage, the plants were kept in a 55° F. greenhouse for two weeks and then transplanted to 6" diameter clay pots. The plants were grown in a 60° F. greenhouse until flowering.

Three equal groups of Heite's Red and Kuhnert hydrangeas received three different dosages of phosphorus. Half of each group of Kuhnert hydrangeas was treated with an additional application of aluminum sulfate. Plants received soil applications of fertilizers from the third week after removal from storage until initial coloration of the sepals.

Weighed samples of sepals, flowers, and leaves were used for the identification and estimation of anthocyanidins and quantitative determinations of sugar and phosphorus at three stages of sepal development: green, white, and colored stages, and from different treatments under study.

The chromatographic and spectrophotometric methods were used to identify the anthocyanidins. The anthocyanidin, sugar, and phosphorus were estimated quantitatively by locating the photometer readings on a standard curve prepared for this purpose. All measurements were expressed in milligrams per gram fresh weight. The Rf values and the maximum absorption values for isolated anthocyanidins were compared with the authentic values given in the literature. The anthocyanidins from the two cultivars were identified as delphinidin. Sepal color changes

from pink to blue for the Kuhnert hydrangea occurred when plants were treated with aluminum sulfate. The anthocyanins in both pink and blue sepals of the Kuhnert hydrangea were identical, but the quantities of anthocyanidins in blue sepals were higher than those in pink sepals. It was also found that there were no measurable quantities of delphinidin found in the leaves of these hydrangeas at any stage of sepal development and in the flowers at the white and green stages. Flowers at the colored stage of sepal development had measurable amounts of delphinidin for both cultivars.

The sugar content in the flowers of both cultivars increased greatly from the green to the colored stages, and significant differences in sugar content of the leaves of Heite's Red occurred at the different stages of sepal development. The quantities of phosphorus in the hydrangea flowers of both cultivars decreased greatly from the green to the colored stages at any of the three soil phosphosur levels, but no differences in the phosphorus content of leaves were found at any stage of sepal development.

The sugar content of sepals of Heite's Red hydrangea increased rapidly with the appearance of color. The quantities of delphinidin produced in the flowers at the colored stage increased as the sugar content increased at the lower rates of phosphorus application. There was no such relationship in the leaves of Heite's Red hydrangea. The amount of delphinidin in the flowers of the Kuhnert hydrangea treated with aluminum sulfate decreased with an increase in the frequency of phosphorus

application. Plants receiving higher levels of phosphorus had a desirable pink color. Aluminum sulfate also inhibited the accumulation of sugar, but because of the interference of aluminum sulfate, no relationship between delphinidin, sugar, and phosphorus level was observed in the Kuhnert hydrangea.

Further research is needed with different cultivars to determine how the aluminum sulfate influences the content of phosphorus and the production of sugar and anthocyanidin in plant tissues.