ANTHOCYANIN AND ANTHOCYANIN FORMATION IN SEEDLINGS OF FAGOPYRUM ESCULENTUM MOENCH

by

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(with Tab. II).

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INTRODUCTION.

The almost general distribution of the anthocyanin pigments (water-soluble red, violet or blue colouring matters) amongst the representatives of the Cormophyta (Von Wettstein 1935) gives the impression that the formation of these pigments in plants is connected with some very important metabolic process. About the nature of this process we hardly know anything. The number of theories never proved and never refuted in a very large number of publications of many investigators clearly shows our lack of knowledge in this matter. Where the last edition of Mrs Wheldale Onslow's monograph on "The Anthocyanin Pigments of Plants" appeared in 1925 it seemed desirable to give a short review of the literature concerning the formation of these pigments in plants.

According to the nature of the processes involved in anthocyanin-formation the theories are to be divided into two groups;

1. reduction-theories
2. oxidation-theories.

According to the nature of vegetable substances considered to be used in pigment-formation the more important theories are

a. the anthocyanins are derived from tannins

b. flavones are the source of these pigments.

In the case of tannins as mother-substances oxidation would play an important part whereas in the case of the flavones some investigators consider reduction as the process by which anthocyanin would be formed while several others are of the opinion that oxidation of flavones give rise to the appearance of anthocyanins.

a. Tannins as mother-substance.

In 1862, when Wigand proposed tannins as mother-substance in anthocyanin-formation, nothing was known of the constitution of all of the substances involved in this process. Until the end of the nineteenth century this theory was supported by many investigators. A great difficulty and one of the weak points in this theory was to find specific reactions to prove the transformation of the tannins present in the plant material into anthocyanins.

In recent years, after the constitution of the catechins had been elucidated by the work of Freudenberg and his co-workers

(Freudenberg 1933; see however Nierenstein 1934) the attention was redrawn to the tannins.

In 1929 Dekker states in a publication under the heading Verhalten in der Pflanze;

„Von den Pflanzenbestandteilen wurden schon früh Gerbstoffe und Zucker mit der Anthocyanbildung in Verbindung gebracht, und die Resultate der chemischen Forschung haben diese Schlussfolgerung der botanischen Untersuchungen bestätigt.

Wahrscheinlich ist es sogar, das nach der völligen Ausbildung der Anthocyane in den gefärbten Pflanzenorganen ein Gleichgewichtszustand eintritt, wobei fortwährend diese Substanzgruppen (i.e. Flavones, Anthocyanins and Catechins) in einander übergehen mittels Oxydase- und Reduktionswirkung!"

In 1920 Freudenberg in his book "Die Chemie der natürlichen Gerbstoffe" gives as his opinion that there exists a chemical relation between anthocyanins and a certain group of tannins. He is, however, very careful not to transfer this to a possible physiological relation between these two groups of vegetable substances.

In 1925, in a publication on the connection between tannins and vegetable colouring matters, Freudenberg hints at a possible transformation of these substances in plants. In his book "Tannin, Cellulose, Lignin (1933) (second edition of "Die Chemie der natürlichen Gerbstoffe (1920)) the author gives the same data as he did in the first edition.

b. Flavones as mother-substance.

Miss Wheldale suggested in 1909 that anthocyanins might be derived from the flavone or flavonol pigments. The flavones are a group of yellow natural colouring matters almost generally distributed in plants. The constitution became clear by the work of Von Kostanecki and his co-workers (1893-1904). Perkin and his collaborators (1895-1909) isolated a number from plants used for dying.

There exist two theories on the origin of anthocyanins from flavones. One we shall call in the following lines the oxidation-theory.

Miss Wheldale held the opinion that anthocyanins were formed in plants by oxidation of flavones.

We shall not discuss in detail the pro and contra of this hypothesis. The oxidation theory appeared before the constitution of the anthocyanin pigments was known. This theory was based on some genetical evidences, analogy with other processes and the fact that without oxygen no anthocyanins are formed. Later, investigations on the distribution of oxidases and anthocyanins in the same tissues of the plant gave some evidence.

The second theory, the so-called reduction theory, advances the idea that anthocyanin pigments are derived from flavones by reduction.

In 1914 Willstätter reduced a flavone solution and obtained a red coloured solution. Two red pigments proved to be present, one of which was a true anthocyanin. Notwithstanding the fact that only a very small quantity of this anthocyanin was present and Willstätter very positively declared that it would be dangerous to transfer this reaction in vitro to the processes which occur in the living plant the plant physiologists took possession of this fact. They reduced all sorts of plant extracts, often obtained red coloured solutions and concluded;

a. this red substance is an anthocyanin
b. it is formed by reduction of a flavone
c. in the plant the same thing occurs.

It seemed more reasonable to try to prove the occurrence, in the same plant, of flavones and anthocyanins with an analogous constitution. Only in a few cases the right combination was found, in the majority of cases, however, no such relation could be demonstrated.

In the last years Sando (1935, 1937) investigated the pigment of the apple and of cornhusks and established the occurrence of flavones and anthocyanins with an analogous constitution. At the other hand Miss Scott-Moncrieff (1930) investigated the pigments of the Snapdragon (Antirrhinum majus L.) and did not find the expected combination.

The older publications on this subject are not very convincing while in all the cases the pigments had not been isolated and reactions of doubtful value were used.

The methods described in the publications of Kozlowski (1921) and Jonesco (1930, 1931), both supporters of the theory of the oxidation of flavones to anthocyanins, diminish their value very much.

It is impossible and without any value to discuss the work of all those authors who support some theory without any experimental data.

It is very interesting to read the opinion of the Robinson's on this topic.

In 1935 Robinson gives his opinion in the following lines;

"I consider that the identification of the anthocyanin chromogen with the flavones was an unfortunate obsession of the plant physiologists, and that in a different form the oxidase hypothesis of Keeble and Armstrong will be revived".

The hypothesis of Keeble and Armstrong (1912*a* and *b*) is described in detail and criticized by Wheldale in her Monograph. These authors gave the following scheme;

glucoside + enzyme → sugar + chromogen
chromogen + enzyme + peroxide → pigment.

Keeble and Armstrong tried to prove their hypothesis in two ways

1. presence of an oxidizing enzyme
2. presence of some chromogen.

Point 1 is of interest because they could show that in different plant material the distribution of oxidizing enzymes and anthocyanins were about the same.

In 1935 the Robinsons again give their opinion;

"The discussions on the problem of the origin of anthocyanin in the plant have been much obscured in the past by arbitrary assumption that the chromogen (Palladin's respiratory prochromogens) are the flavones or their glycosides. This was a natural suggestion in 1911 (Wheldale) but it is not supported by any physiological evidence or by a statistical comparison of the constitutions of congeneric pyrones and pyrylium salts. Indeed, when the chemical relations of these groups became clear as a result of the work of Willstätter on the anthocyanins, the flavone-chromogen obsession was so potent that the acceptable hypothesis of anthocyanin formation by oxidation was discarded by many authors and in some quarters it was maintained that the pigments owe their formation to the reduction of γ-pyrones such as apigenin and quercetin or their glycosides.

Doubtless the details of the oxidase theory of Keeble and Armstrong require adjustment in the light of present knowledge, but in general terms it is surely in better harmony with the whole of the circumstances of anthocyanin formation than the view attributing a predominant rôle to reduction. Even the experiments in vitro cannot be logically interpreted in favour of the reduction hypothesis. The flavones can only be reduced to flavylium-salts by metals in strongly acidic media and the yields are very poor; thus no close analogy with the assumed reduction in vivo has been provided (Everest, Willstätter, Combes, Shibata et alii).

From the above it becomes clear that the present state of our knowledge is not very hopeful. Still there remains the possibility to obtain some information by physiological experiments rather than by the very difficult and sterile method of trying to find some chromogen.

STATEMENT OF THE PROBLEM.

In the course of time several groups of vegetable substances have been mentioned in connection with the production of anthocyanins. In the last few years Robinson and Robinson (1933, 1934, 1935 *a* and *b*, 1937) have redrawn the attention to a group of widely distributed substances, the so-called leuco-anthocyanins.

This name has been introduced by Rosenheim (1920) and indicates substances which are characterised by the property to give anthocyanins by heating with acids.

The Robinsons very carefully avoid to call the leuco-anthocyanins the precursors of anthocyanin pigments in plants. They only mention a few times the possibility of the process.

Jonesco (1930) however called these substances; "chromogènes générateurs d'anthocyanidines".

Many authors deny the occurrence of leuco-anthocyanins in plants. According to them these substances are tannins which also give red products on heating with acids.

It is a remarkable fact that quantitative work on anthocyanin-formation has so seldom been done.

As far as I know only one author (Kuilman 1930) estimated the amount of anthocyanin produced in experiments on the effect of light and temperature on anthocyanin-formation.

A second point of interest is the fact that physiological work on anthocyanin-formation neither seems to have been attractive to botanists. In the following lines we have endeavoured to obtain some data to make towards the elucidation of the problem of the formation of these pigments in plant.

For this purpose we intend to study with one object;

a. the chemical properties of some substances present in the material
b. the distribution of these substances in the material
c. the metabolic processes involved in the pigment-formation.

CHAPTER I.

ON CERTAIN SUBSTANCES PRESENT IN BUCKWHEAT-SEEDLINGS.

§ 1. *Material.*

As material we used the pale-yellow etiolated seedlings of Fagopyrum esculentum Moench. This material has been used by several authors; for the first time, as far as we know, by Batalin in 1879.

There are many advantages to use this material. The big seedlings grow very quickly, the material is available in every season and by the action of light an intense red colour rapidly appears. We obtained the Buckwheat-seed from the Firm Sluis and Groot, Enkhuizen (Holland). From 1937 onward we used seed received from the Firm Visser, Steenwijk (Holland).

Before using the seed was examined very closely, small and broken seeds were removed. After this treatment, which took very much time, good uniform seed had been obtained. The seeds from Sluis and Groot were much bigger than those from the other firm but the seed from Visser germinated far better. The percentage of germination of the Buckwheat seed obtained from Sluis and Groot was about 70 %, that from Visser more than 90 %. 10 Portions of 10 gms from the Sluis and Groot seed contained resp. 328, 337, 328, 334, 330, 335, 334, 339, 332 and 328 seeds while 10 portions of the same weight of the Visser seed contained resp. 450, 445, 442, 446, 452, 452, 452, 453, 450 and 458 seeds. The seed obtained from Visser agrees very well with the description given by Miège (1910), in his Thesis, of the seed of the variety "gris argenté" of Fagopyrum esculentum Moench. The mean-value for seed of this variety according to this author is 455 in 10 gms, while our mean-value is 450.

The seed from Visser has been used for most of the experiments, that of Sluis and Groot was only used for a few preliminary ones. To prevent growth of parasitical fungi the seeds were always sterilised by treatment with a solution of Calcium hypochlorite in tap-water.

At first portions of 10 gms of seed were laid out on wet filter-paper in crystallizing dishes with a diameter of 20 cm. covered with glass and placed in the dark in a constant temperature room at 25° C. Later on the seeds were laid out on well-washed paper pulp

containing a known amount of water. With this method well-grown seedlings were obtained in a much shorter time than ever was possible by means of the other method.

§ 2. *The Anthocyanin Pigment.*

a. General remarks.

When Buckwheat is cultivated in the open air the seedlings show an intense red coloration. This colour is due to some anthocyanin pigment.

As far as we know the constitution of this pigment is unknown. In connection with a possible physiological relation between a leucoanthocyanin and this anthocyanin pigment it has been thought desirable to investigate the nature of the pigment present in the red-coloured Buckwheat-seedlings.

In one respect the material used proved to be very unsatisfactory. The relatively small amount of colouring matter together with the presence of much slimy material made it practically impossible to obtain the anthocyanin pigment in a crystalline form.

By means of the methods described by Robinson and Robinson (1931, 1932, 1933, 1934) for anthocyanin solutions purified in a special way it has been possible to ascertain the constitution of the anthocyanin pigment of the red Buckwheat-seedling.

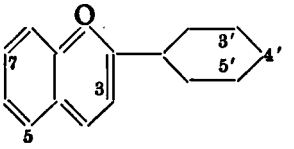
For obtaining some preliminary information as to the constitution of the pigment the extract of the methods of the Robinson's by Miss Scott Moncrieff (1936) proved to be very handy.

The chemistry of the colour reactions and some other properties of anthocyanin pigments found by Willstätter and his co-workers (1913-1917) has been elucidated by Robinson and his collaborators (1924-now) by means of their very extensive syntheses.

From the work of these and also of some of the older authors it became clear that the anthocyanin pigments are glycosides.

The general name for the sugar-containing pigment is anthocyanin, while the sugar-free pigments, obtained from the first one by hydrolysis are called anthocyanidin.

The general formule of the sugar-free nucleus of the anthocyanidin is given below:



Apart from a special group of nitrogen-containing anthocyanin-pigments, the so-called betanins, the anthocyanin molecules contain only C, H and O. Characteristic for the anthocyanin-molecule is the presence of a so-called oxonium-atom, a tetravalent oxygen-atom, by which salts of different acids may be formed. In this way Willstätter was able to obtain many anthocyanin-pigments from natural material in a beautiful crystalline form, mostly in the form of chlorides or picrates. A great number of different anthocyanins is possible, many of them have been found already to occur in nature. In natural material the following variations have been found.

1. at 3, 5 and 7 hydroxyl-groups are present; one or more may be methylated.
2. one, two or three hydroxyl-groups are present at 4′, 3′ and 4′, or 3′, 4′ and 5′; one or two of these groups may be methylated.
3. the special group of the so-called betanins are characterised by a nitrogen-atom at 4′. It is of great interest that this special group of anthocyanin-pigments is only present in a number of closely-related plant-families of the Centrospermae; Chenopodiaceae, Amarantaceae, Phytolaccaceae, Nyctaginaceae, Aizoaceae, Cactaceae and Portulaccaceae, while only one family of the Centrospermae; the Caryophyllaceae, as far as known, do not possess these special anthocyanins.
4. the place and nature of the sugar-residue is further of importance. In natural material the sugar-residue is attached at 3, or both at 3 and 5. In the first case one or two sugar molecules may be present, in the second case only one sugar molecule is present at 3 and at 5.
   Glucose is very common but also galactose and rhamnose or other pentoses may be present.
5. finally more complications occur by the presence of an organic acid combined with the sugar-molecules, to form the so-called complex-anthocyanins.

Specially Robinson and his co-workers were able to obtain a great number of anthocyanins (some of them as yet not found in nature) by means of synthesis.

The position of the sugar-residue, the number of OH-groups, methylation of OH-groups and several other points are the cause of specific colour reactions by which a great deal may be learned about the constitution of the pigment molecule.

The comparison of the synthetical and natural anthocyanin by Robinson and his co-workers led to the convinction of the great value of these colour-reactions for the determination of the

chemical constitution of anthocyanins present in solutions purified in a special way.

The methods Robinson and Robinson describe in their Survey of Anthocyanins I-IV (1931, 1932, 1933, 1934) are the result of those extensive investigations.

By these methods we obtained sufficient information to learn the principal points in the constitution of the pigment of the red Buckwheat-seedling.

As far as we know very little is known about the anthocyanin of Buckwheat. Robinson and Robinson do not mention this plant in their lists of several hundreds of plants they investigated (Survey I-IV).

Jonesco (1930) investigated the pigments of Fagopyrum, but his colour-reactions clearly show that his substances were not pure. In addition he does not suggest anything relative to the constitution of the pigments.

We could not confirm his statement of the presence of free anthocyanidin in this material.

Jonesco isolated three different substances which he looked upon as leuco-anthocyanins but his methods do not warrant any certainty on this point. In any case he does not mention anything on the constitution of the anthocyanidin derived from the leuco-anthocyanin by treatment with hydrochloric acid. His conclusion, however, is that the anthocyanidin obtained by hydrolysis of the natural pigment is identical with the one derived from the leuco-anthocyanin by heating these substances with hydrochloric acid.

b. Extraction and purification of the pigment.

About 400 gms of red coloured hypocotyledons of Fagopyrum were extracted by means of 350 cc of 0.5 % HCl aq. during two days. The extract was decanted and the material strained through cloth. The still fairly intensively coloured residue was treated again with 250 cc of hydrochloric acid of the same concentration and extracted for another two days. The second extract was obtained in the same way as the first.

Both extracts were joined and filtered, after which half the volume of 96 % ethyl alcohol was added. By this treatment a large quantity of mucilaginous material separated as a gelatinous mass.

Filtration proved to be impossible in every way but centrifuging gave a clear solution from which no more gelatinous material could be precipitated on addition of ethyl alcohol.

The extract obtained was saturated with sodium chloride and the pigment taken up in amyl alcohol. The amyl alcoholic extract was filtered, half the volume of benzene added and the anthocyanin taken up in 0.5 % HCl aq.

The filtered acid-aqueous solution was repeatedly washed with large volumes of ethyl acetate, until the ethyl acetate did no longer yield any yellow coloration on the addition of alkali.

After saturation with sodium chloride the acid solution was extracted with butyl alcohol. Half the volume of light petrol (b.p. 40—60° C) was added and the pigment taken up in the minimum quantity of 1 % HCl aq.

This extract is sufficiently pure to give the right colour reactions with alkali.

The following method however proved to be more satisfactory for this material.

40 gms of red coloured hypocotyledons were extracted with 100 cc 2 % hydrochloric methyl alcohol during 24 hours. The extract was decanted and the residue treated in the same way.

At the end of 24 hours the residue was pressed through cloth and the two extracts were put together.

After filtration the pigment was precipitated with a large excess of ether.

The dark-red sirup obtained in this way had a volume of about 25 cc and was mixed with 75 cc 0.5 % HCl aq. and the intensely red solution was saturated with sodium chloride. The pigment solution was further purified in the same way as described above.

c. Colour reactions of the glycosidal pigment.

To establish the constitution of the pigment its solution was compared with solutions of known anthocyanins. For this purpose purified extracts of certain flowers served (Robinson 1931, Scott Moncrieff 1936).

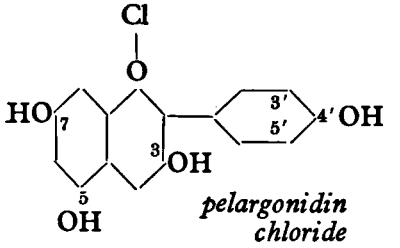
The petals of the flowers were macerated with 1 % HCl aq. The extracts obtained in this way were filtered and washed several times with a large excess of ethyl acetate until this solvent did not show any yellow coloration on the addition of alkali.

The solutions proved to be pure as might be seen by means of their typical colour reactions with alkali.

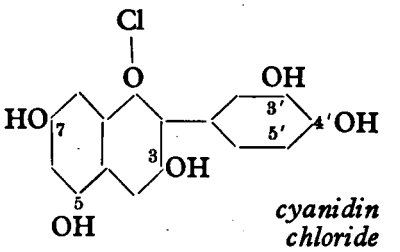
The material used is shown in table 1, with the addition of the class to which the anthocyanin belongs and the authors who studied the pigment.

See table 1.

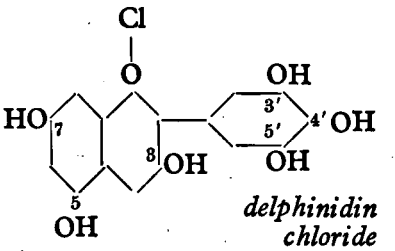
The formulae of the sugar free pigments as chlorides are given here;



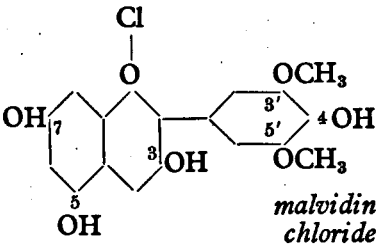
pelargonidin chloride



cyanidin chloride



delphinidin chloride



malvidin chloride

TABLE 1.

Name of the Plant	Part of the plant	Anthocyanin	Author
Phaseolus multiflorus Willd.	flower	pelargonidin 3-glycoside	Robinson and Robinson (1933)
Pelargonium zonale L'Hérit.	flower	pelargonidin 3-5-glycoside	Willstätter and Bolton (1915) Robinson and Todd (1932*a*)
Papaver Rhoeas L.	flower	cyanidin 3-glycoside	Willstätter and Weil (1917)
Antirrhinum majus L.	flower	cyanidin 3-glycoside	Scott-Moncrieff (1930)
Rosa spec.	flower	cyanidin 3-5-glycoside	Willstätter and Nolan (1915) Robinson and Todd (1932 *a*)
Delphinium spec.	flower	delphinidin 3-5-glycoside	Willstätter and Mieg (1915)
Vitis vinifera L.	skin of berries	malvidin 3-glycoside	Willstätter and Zollinger (1915, 1917) Levy, Posternack and Robinson (1931)
Clarkia pulchella Pursh	flower	malvidin 3-5-glycoside	Robinson and Robinson (1931) Robinson and Todd (1932*b*)

Lack of material was the cause that derivatives of peonidin (i.e. 3'-methylcyanidin), petunidin (i.e. 3'-methyl-delphinidin) and hirsutin (i.e. 3'-5'-7-trimethyl delphinidin) could not be tested. From notes of several authors it was clear that these pigments give per-

TABLE 2.

Name of the Plant	Constitution of the Pigment	Colour 1% HCl aq solution	Na_2CO_3	NaOH	Na-acetate	Ferric Chloride
Phaseolus multiflorus Willd. (flowers)	Pelargonidin 3-glycoside	orange-red thin layers same colour	violet-red	violet-red (rather stable)	very dull violet-red	brown-orange
Pelargonium zonale L'Hérit. (flowers)	Pelargonidin 3-5-glycoside (Pelargonin)	orange-red thin layers same colour	red-violet, with acetone pure blue	reddish-violet	violet-red	brown
Antirrhinum majus L. (flowers)	Cyanidin 3-glycoside (Antirrhinin)	red thin layers pink	blue-violet	pure blue [2]	red-violet	pure dark violet. in alcohol blue
Papaver Rhoeas L. (flowers)	Cyanidin 3-glycoside (Meco-cyanin)	red thin layers pink	blue-violet	pure blue [2]	red-violet	pure dark violet. in alcohol blue
Rosa spec. (flowers)	Cyanidin 3-5-glycoside (Cyanin)	red thin layers pink	pure blue [2]	pure blue (unstable)	violet	pure dark violet. in alcohol blue
Delphinium var. (flowers)	Delphinidin 3-5-glycoside	reddish-violet	pure blue [2]	pure blue [2]	blue	pure dark violet. in alcohol blue
Vitis vinifera L. (skins of fruits)	Malvidin 3-glycoside (Oenin)	violet-red	violet-blue	violet-blue	somewhat dull violet	orange-brown
Clarkia pulchella Pursh (flowers)	Malvidin 3-5-glycoside (Malvin)	bright violet-red. in thin layers reddish-violet	bright pure blue	pure blue (unstable) [2]	very bright violet	decoloration
Fagopyrum esculentum Moench (hypocotyledons)	?	red thin layers pink	blue-violet	pure blue [2]	violet-red	pure dark violet. in alcohol blue

[1] A violet reaction with ferric chloride is a so-called positive reaction.
[2] pure blue is somewhat greenish-blue, not violet tinge.

fectly different colour reactions than the Buckwheat anthocyanin. Table 2 shows the colour reactions of the anthocyanins used.

See table 2.

From these reactions it is clear that the anthocyanin isolated from red Buckwheat seedlings is probably a cyanidin 3-glycoside. The positive ferric chloride test (anthocyanins with 2 OH-groups in ortho-position in the phenylgroup give a very intense violet colour reaction with ferric chloride) excludes the possibility of the presence of all pelargonidin derivatives. The same is true for all those cyanidin and delphinidin derivatives which do not possess two free OH-groups in ortho-position in the phenylgroup e.g. peonidin-malvidin, and hirsutidin derivatives where one or two OH-groups are methylated.

The alkaline colour reaction with Na_2CO_3, NaOH and Na-acetate enables us to distinguish between cyanidin, delphinidin and petunidin glycosides.

d. The sugar-free pigment.

75 cc of the purified dark red anthocyanin solution were heated with 50 cc of 35 % hydrochloric acid during 3 minutes. At the end of this time the liquid was cooled, filtered and the pigment taken up in amyl alcohol.

The dark violet-red amyl alcoholic solution was washed several times first with distilled water and later with 1 % HCl aq. in order to remove possible traces of unhydrolysed anthocyanins.

A large excess (about 6 volumes) of benzene was added to the amyl alcoholic extract and the pigment taken up in 1 % HCl aq.

For comparison a purified cyanin (= cyanidin 3-5-diglucoside) solution extracted from the petals of a red rose was treated in the same way.

See table 3.

From Table 3 it may be seen at once that the anthocyanidin derived by hydrolysis from the anthocyanin of red Buckwheat seedlings is identical with cyanidin.

For the cyanidin reagent test [1] all precautions had been taken as Robinson and Robinson (1932) indicate. These precautions are to exclude ethyl alcohol and its derivatives in the preparation of the test-solution and to see that the concentration of the acid is 1 %.

e. The sugar-residue.

The blue-violet colour reaction of the anthocyanin with sodium

[1] The cyanidin reagent is a mixture of 1 vol. of cyclo-hexanol and 5 vols toluene (Robinson and Robinson 1931).

TABLE 3.

Name of the Plant	Colour 1% HCl aq solution	Colour acid amyl alcoh. solution	Na_2CO_3	NaOH	Na acetate in amyl alcoh.	Na amyl alcoh. solution $FeCl_3$	Cyanidin reagent	Delphinidin reagent	Oxydation test
Rosa spec. flowers	thick layers salmon-orange thin layers bluish-pink	thick layers red to violet-red thin layers violet-pink	via violet to pure blue [1]	pure blue [2]	reddish-violet (slightly more blue than next one)	bright blue	pink	not wholly extracted	fairly stable (somewhat less than foll.)
Fagopyrum esculentum Moench hypocotyledons	thick layers salmon-orange thin layers bluish-pink	thick layers red to violet-red thin layers violet-pink	via violet to pure blue [1]	pure blue [2]	reddish-violet	bright blue	pink	not wholly extracted	fairly stable

[1] pure blue is more greenish blue than violet-blue.
[2] excess of reagent causes a quick change over green to yellow.

carbonate and the pure blue colour produced with the same reagent on the sugar-free pigment indicate the 3-place for the carbohydrate attachment.

More difficult is to obtain some information as to the nature of the sugar-residue.

By means of the distribution number between amylalcohol and 1 % HCl aq., both well balanced with the other solvent, something may be learned about the carbohydrate-residue.

Robinson and Robinson (1932) devised simple tests to distinguish the three classes; diglycosides, pentose glycosides and monoglycosides. It was already known by the work of Willstätter that diglycosides (i.e. 3 biosides and 3-5 dimonosides) at the one hand and monoglycosides and pentose-glycosides at the other might be distinguished by the fact that the representatives of the first class in concentrated solutions have a low distribution in the alcoholic solvent while the numbers of the second class have a considerable higher distribution.

To make a distinction possible between pentose-glycosides and monoglycosides Robinson and Robinson describe the following tests;

On dilution of the acid-aqueous solution the distribution number of the pentose glycosides falls, that of the monoglycosides rises and that of the diglycosides remains low. This means that it is difficult to extract the pigment from a very dilute pentose-glycoside solution with amyl alcohol while monoglycosides pass easily into the amyl alcohol.

The difference of a very dilute acid solution of pentose glycosides and diglycosides lies in the fact that on addition of sodium chloride the distribution number of diglycosidic pigments is barely altered while the pentose-glycoside is extracted almost quantitatively by the amyl alcohol.

By testing purified solutions of Buckwheat anthocyanin with this method it seemed at first likely that the pigment is a pentose-glycoside.

In the second part of their Survey of Anthocyanins Robinson and Robinson (1932) give some chemical tests for a further distinction of normal pentose-glycosides and methyl pentose-glycosides.

A purified anthocyanin solution is mixed with an equal volume of concentrated hydrochloric acid and distilled. The presence of furfuraldehyde or methylfurfuraldehyde in the distillate is examined by the acetic acid-aniline test and by a modification of the naphtoresorcinol test (Tollens and Rorive 1908, Votoček 1897).

I. Tests with pure carbohydrates.

25 mgs of rhamnose, xylose and arabinose (Merck) were each distilled with 5 cc distilled water and 5 cc concentrated hydrochloric acid (pro anal.). 5 cc of the distillate were collected.

1. acetic acid-aniline test.

A mixture of 1 cc distillate, 5 cc acetic acid and 2 cc aniline gave in the case of rhamnose an orange-red colour while xylose and arabinose gave a very intense crimson, in thin layers bright pink.

2. naphto-resorcinol test modified according to Robinson and Robinson.

1 cc of the distillate was added to 10 cc of 5 % alcoholic hydrochloric acid containing 5 mg of naphtoresorcinol. Rhamnose gave a pale orange-red, slowly increasing in intensity. Xylose and arabinose gave an orange-yellow coloration quickly changing through brown, dark brown to very intense brown. After half an hour the rhamnose solution is still orange-red while the solutions from xylose and arabinose are of a very intense blue. After two hours and a half the solutions from xylose and arabinose are still very intense blue with an intense green fluorescence while the rhamnose-solution is only brownish orange-red.

At the end of seven hours the rhamnose solution is brownish-red with some green fluorescence while the xylose and arabinose solutions have still the same appearance.

To test the sensitivity of the reaction we diluted the distillate ten times. 1 cc of the dilute distillate with 2 cc of the reagent gave the following colorations;

Rhamnose changes from brownish pink to a stable yellow while xylose and arabinose changes from rather intense yellow through greenish yellow, green to blue green. After some time an intense green fluorescence appears.

II. Tests with a purified anthocyanin solution.

50 cc of a purified anthocyanin solution were distilled with 50 cc of concentrated hydrochloric acid. 5 cc of the distillate were collected.

1. acetic acid-aniline test.

1 cc of the distillate with 5 cc acetic acid and 2 cc aniline gives a pale pink colour, which quickly disappears. This coloration might indicate a weak positive pentose reaction. It is, however, possible that the coloration is due to the presence of some hexose in the anthocyanin molecule, for hexoses heated with hydrochloric acid give a small amount of furfurol.

2. naphto-resorcinol test.

1 cc of the distillate was mixed with the naphto-resorcinol reagent. A pale yellow colour appeared. After 5 minutes the colour was a pale reddish-orange. After one hour the colour was changed a little to a pale reddish-brown with an extremely intense yellowish-green fluorescence. After three hours the colour is rather pale orange-yellow with the same fluorescence which is still of the same intensity at the end of two days.

The result of this test clearly shows that pentose is not present in the Buckwheat anthocyanin. At the other hand the second colour reaction is sufficiently interesting to be worth some more attention.

III. Tests with different sugars and mixtures of sugars.

Where the result with the distillate of the Buckwheat anthocyanin was very striking some experiments have been done to try to throw some light on the rather strange reaction.

The following sugars and sugar-mixtures in equivalent quantities were distilled with 5 cc distilled water and 5 cc concentrated hydrochloric acid to which was added;

1. 36 mg glucose
2. 33 mg rhamnose
3. 30 mg xylose
4. 25 mg arabinose
5. 18 mg glucose + 15 mg xylose
6. 18 mg glucose + 16.5 mg rhamnose
7. 25 mg xylose + 25 mg rhamnose.

1 cc of each distillate was mixed with the required quantity of the naphtoresorcinol reagent and the colours observed for some time.

1. reddish-yellow, after 10 minutes brownish-yellow. After one hour greenish-yellow, while at the end of three hours the colour was of a greenish-yellow with a green fluorescence.
2. pinkish orange changing to reddish-orange and orange-scarlet, in thin layers eosin-red. At the end of 5 minutes no more change was observed. After one hour and 3 hours still the same. At this time no fluorescence could be observed.
3. dark yellow quickly darkening. After 5 minutes the colour was dark brown. After one hour a very intense pure blue without fluorescence could be observed. After three hours the blue colour was still the same but a dark green fluorescence had appeared.
4. perfectly the same as 3.
5. dark yellow, quickly darkening. After 5 minutes a dark brown colour had appeared. After one hour the colour was very intense pure blue with a weak green fluorescence. After three hours the blue colour had not changed. An intense green fluorescence was present, more intense than in No. 3.
6. salmon changing to brownish orange, in thin layers eosin-red. After 5 minutes the colour did not change any longer. After one hour the colour was brown-red, after three hours more reddish-brown without any fluorescence.
7. brown-yellow changing to brown orange. After 5 minutes a dark brown coloration had appeared. At the end of 45 minutes the colour had changed to violet-brown, no fluorescence was apparent. After two hours 30 minutes the colour was not pure blue but blue-violet with a green fluorescence.

From these tests it becomes clear that no pentose can possibly be present in the Buckwheat anthocyanin.

From a purely chemical point of view it might be desirable to investigate this matter further.

In connection with the results obtained with the pigment formed from the so-called leuco-anthocyanin by treatment with acids (see § 3) we thought it not worth while to continue our investigation on the sugar residue.

As a matter of fact the sugar-free pigment, the anthocyanidin, is, from a biochemical point of view, the more important part of the anthocyanin molecule.

Summarising the results it is clear that the anthocyanin pigment of the Buckwheat seedling is a derivative of cyanidin, the formula of which has been given above. Concerning the sugar-residue we have the certainty that pentose is not present, while the distribution number might be caused by the presence of some organic acid molecule in the sugar residue.

§ 3. *Leuco-anthocyanins or Tannins?*

a. Review of the literature.

A great number of authors mention the fact that plants or plant-extracts very often turn red on treatment with several acids.

A number of these investigators hold the view that the produced red substances are anthocyanins or anthocyanin-like substances. Others deny this and give as their opinion that those red substances are no anthocyanins but products derived from tannins. L a b o r d e (1908 *a*, *b* and *c*) prepared a bright violet-red anthocyanin-like pigment from the skins of unripe white and purple grapes by autoclaving them at 120° C in 2 % aqueous hydrochloric acid during 30 minutes. This author describes two substances which may be transformed into red pigments, one soluble in 96 % ethyl alcohol, the other insoluble in this solvent.

L a b o r d e concludes from his experiments that anthocyanins are derived from tannins.

The same is the case with M a l v e z i n (1908). This author heated unripe grapes with distilled water in a water-bath at 85° C. At the end of seventeen hours an intense yellow colour was developed which changed into a brilliant violet-red solution after heating for another seven hours.

It was found by the author that oxygen was necessary to produce the red colour. Heating in a sealed tube without oxygen only results

in a yellow solution. After acces of oxygen the well-known red colour appears.

Dezani (1910) also mentions the presence of a substance in white grapes giving an anthocyanin-like pigment on treatment with hydrochloric acid.

Willstätter and Nolan (1915) described a peculiar phenomenon. A methyl alcoholic hydrochloric acid extract of the petals of a dark-red rose becomes more and more intense in colour in the course of a few days till about twice the original concentration. In 1918 Noack published an important article on the formation of anthocyanins in plants. Amyl alcoholic extracts of leaves of Polygonum compactum Hook. and a variety of the Garden Peony heated with hydrochloric acid in a water-bath at 100° C turn red by the production of an anthocyanin-like pigment.

While Laborde, Malvezin and Dezani called the substances from which the red pigments are derived, tannins, Noack supposes that the anthocyanins in the plants, mentioned by him, are present in a colourless form, the so-called pseudobases (formula see p. 22). In 1922 a second publication of Noack appeared. He prepared amyl alcoholic extracts from red leaves of Ribes aureum Pursh and from leaves of several species of Anthurium. These extracts turned intensely red on heating with hydrochloric acid.

From the work of Hamorak (1915) we know that most species of Polygonum and several Anthurium-species contain large quantities of tannins in their leaves.

Shibata (1919) obtained a chromogenic substance from a number of plants which yielded a deep-red colour on heating with hydrochloric acid. Some colour reactions performed on this product give the impression that the resulting pigment is an anthocyanin.

Rosenheim (1920) again mentioned the fact that amyl alcoholic extracts from unripe purple and ripe white grapes contain a substance which he called "leuco-anthocyanin" producing a violet-red anthocyanin-like substance with the same properties as the natural pigment of the purple grape.

In the root of Beta vulgaris L. a similar substance has been described by Kozlowski (1921).

In a very interesting paper Nagai (1921) gives a long list of plants in which chromogenic substances occur which yield a red colour on heating with hydrochloric acid. This author discovered these substances in many different parts of plants. The number of species investigated is very large indeed. Nagai mentions in his list Fagopyrum esculentum Moench. which contains a large

quantity of these chromogenic substances in the unripe, colourless achene and in the leaves.

From the work of Fessler (1913) we know that in the achenes of Buckwheat a pyrocatechin tannin is present.

In a series of publications Jonesco (1921, 1922, 1930, 1931) describes the isolation and properties of a number of substances indicated as leuco-anthocyanins, following Rosenheim's nomenclature. Leuco-anthocyanins were isolated from leaves of Ampelopsis hederacea D C., Prunus Pissardi Carr. and Acer platanoides L., from flowers of Pelargonium and Papaver and from the seedlings of Fagopyrum esculentum Moench.

Kuilman (1930) briefly mentioned the fact that etiolated seedlings of Buckwheat turn red on heating with hydrochloric acid.

Very often the opinion is brought forward that these red products are phlobaphenes, formed by the action of acids on tannins (Combes 1922 *a* and *b*, 1923).

This author rather sharply criticized the work of Noack, Kozlowski, and Jonesco.

Kuilman (1930) states:

"Es ist heutzutage nicht mehr gestattet jede rote, anthocyan-artige Farbe als von einem Anthocyan herrührend zu betrachten".

No arguments, however, are mentioned to substantiate this view. It is very important to cite Robinson and Robinson (1935*a*) in relation to this problem. These authors (1933, 1934, 1935*a* and *b*) investigated the red products derived from leuco-anthocyanins found in almost every sort of plant material. They could prove that anthocyanidins are obtained by heating leuco-compounds with acids.

These authors state;

"The occurrence in flowers and fruits of colourless substances, affording anthocyanin-like pigments on treatment with acids has been frequently noticed, but the significance of these observations has not been fully recognised and the pigments have often been dismissed as "phlobaphenes" a term which in respect of its precision may be classed with "humic acids"."

In 1933 the authors published Number III of their "Survey of Anthocyanins". This publication contains notes on the distribution of leuco-anthocyanins in the Vegetable Kingdom. By means of the reactions published by the same authors in Number I and II of their Survey (1931, 1932) they could prove that the red pigments obtained from plant material were indeed anthocyanidins.

It is remarkable that so many leuco-anthocyanins were found by Robinson and Robinson in many kinds of wood and seeds. In the majority of cases cyanidin was obtained by the action of acids on the leuco-anthocyanin.

In 1934 Number IV of the Survey of Anthocyanins was published, completing the extensive list of the above-mentioned publication by several more examples.

The same authors published in 1935 (*a*) an article on the constitution of a special leuco-anthocyanin. Several more general remarks and literature on this topic are added.

Finally in 1937 Mrs. Robinson published an article on the formation of cyanidin chloride from the gum of Butea frondosa Roxb. With several methods anthocyanin-like substances were produced which proved to be known substances. Only in a special way cyanidin was obtained.

Störmer and Witsch (1937) mention the fact that the veins of the petals and the anthers of special Petunia-varieties turn violet or red on treating the still colourless buds with HCl vapors. The so-obtained pigment shows the same distribution as the anthocyanin does in the fully-developed and coloured flower.

From the review of the literature on this subject it may be seen that it is very probably that in many cases the red products are derivatives of tannins; in other cases we have no certainty whatever as to the nature of these substances. Only from a small number of publications it is perfectly certain that anthocyanidins are formed from leuco-anthocyanins by special treatment. The occurrence of the leuco-anthocyanins in parts of plants where anthocyanins will never appear does not give much hope for a direct transformation.

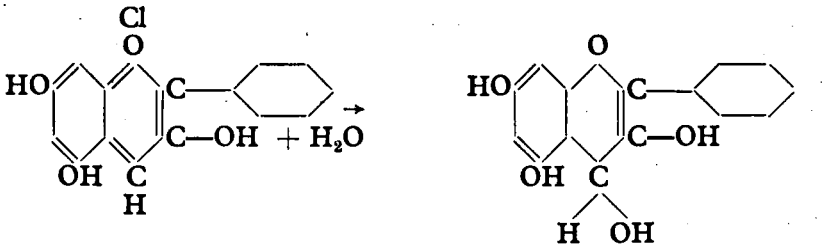
b. Leuco-anthocyanins or tannins in the seedlings?

In the review of the literature we mentioned a number of publications on the transformation of colourless substances into red anthocyanin-like substances by the action of hot hydrochloric acid.

Several authors (Noack 1918, 1922, Störmer and Von Witsch 1937) hold the view that leuco-anthocyanins are pseudo-bases of anthocyanidins. Pseudo-bases of anthocyanins were investigated by Willstätter (1914-1916). When a solution of some anthocyanin or anthocyanidin is diluted with water or alcohol the colour disappears by the formation of a pseudo-base. On addition of some acid the colour reappears at once in the case of the glycosidal pigment while in the case of anthocyanidins it is necessary to heat the solution with some acid to obtain again the pigment.

The change of the coloured phase into the colourless one is a process of isomerisation.

The coloured phase takes up one molecule of water in the following way;



Coloured oxonium salt *Colourless pseudobase*

One of the properties of the pseudo-bases is their solubility in ether. The leuco-anthocyanins, however, are not soluble in ether (Rosenheim (1920), Jonesco (1930).)

Jonesco (1930) describes in etiolated seedlings and in the fruits of Buckwheat the presence of substances which he called leuco-anthocyanins. By heating with hydrochloric acid a violet-red substance was formed. According to Jonesco this pigment is a true anthocyanidin with all the properties of the aglucon of the anthocyanidin obtained by hydrolysis from the natural anthocyanin of Buckwheat.

Kuilman (1930) mentions in short the fact that Buckwheat eedlings give a red coloration on heating with dilute hydrochloric acid.

To decide whether leuco-anthocyanins or tannins are present in the etiolated Buckwheat seedlings this matter had been investigated by us.

Etiolated Buckwheat seedlings were ground with sand and talcum into a homogeneous paste and extracted on a Buchner funnel with benzene and ether to remove carotenoids. After this treatment the residue is washed with ethyl alcoholic hydrochloric acid.

The intensely greenish-yellow extract obtained in this way develops a very dark coloured red anthocyanin-like colour on heating with hydrochloric acid.

In order to decide whether this colouring matter is an anthocyanin or a product derived from some tannin it has been tried first of all to purify the pigment in the same way as described above for the natural anthocyanidin.

At first the pigment seemed to be an anthocyanidin but very soon it has appeared that purification of the pigment in this way is impossible. Where anthocyanidins give still rather intensely coloured solutions in dilute aqueous hydrochloric acid in the case of this red pigment only very dilute, more or less salmon-pink, solutions

have been obtained. Another difference was the fact that, in contrast to anthocyanidin-solutions, solutions of this pigment proved to be very unstable. Within twenty-four hours the pink colour changed to a dull brownish-pink. Under these circumstances true anthocyanidin solutions keep their original bright colour.

Other points of difference were discovered by trying the usual reactions on anthocyanins.

With sodium carbonate a dull bluish-grey colour has been obtained, not the bright blue or greenish-blue colour anthocyanidin always give.

Another point of difference is the distribution between the aqueous solution and the cyanidin-reagent (see above). Cyanidin gives a pink colour to this reagent but this red colouring matter does not give off any colour to the reagent. By this reaction it was proved that the red pigment is certainly not cyanidin as would be the case according to Jonesco (1930).

The colour of the alkali reaction added to the unstable nature of the substance points to the possibility that the pigment is no anthocyanidin at all.

The possibility remains that the red colour obtained by heating the acid alcoholic extract of Buckwheat seedlings is caused by a so-called phlobaphene, red coloured substances derived from tannins by oxidation and polymerisation. It is a well-known fact that tannins belonging to a certain group are very easily transformed into red products by the action of mineral acids. The instability of the red product obtained from Buckwheat-seedlings giving a brown substance on further oxidation pointed in the direction of the presence of some tannin.

In the literature one encounters several times the statement that tannins are present in Buckwheat (Schmidt 1879, Woodcock 1914, Molisch 1923, Merkenschlager 1926) but on the chemical properties only one publication appeared as far as we know.

Fessler (1913) investigated the components of the ripe fruit-scales of Fagopyrum esculentum Moench. With several solvents dark brown extracts were obtained. They contained some phlobaphene as was proved by several reactions.

c. General remarks on tannins.

Tannins are complex amorphous substances with many phenolic hydroxyl-groups, which groups are the cause of several reactions, like the colour-reactions with metals, precipitation from their solution by gelatin, by several alkaloids, and by different salts.

Many of these reactions have already been known for a very long time (see in this connection Nierenstein 1934).

In the course of time authors tried to find a classification for this difficult group of plant substances in relation to their chemical properties.

One of the best-known is the Stenhouse-Procter classification (1842, 1894). The tannins, according to this classification, are divided into iron-bluing tannins, which contain a pyrogallol-nucleus and iron-greening tannins, the so-called catechol tannins, which contain a phloroglucinol nucleus and yield phlobaphenes (red or brown complicated oxidation-products).

This well-known classification is abolished now because there are many tannins which cannot be placed in one of the two classes as they appear to possess properties of both.

The classification that meets the case best is that of Freudenberg (1920). Freudenberg divides the tannins in two main groups;

1. those that can be hydrolysed by acids and enzymes
2. those that cannot be hydrolysed by these agents, the so-called "condensed tannins".

Group 1, the Hydrolysable Tannins, contain benzene-nuclei combined to a complex by means of oxygen-atoms.

This group may be divided into three parts;

a. esters of phenol-carboxylic acids and esters of phenol-carboxylic acids and other hydroxy-acids (so-called depsides)
b. esters of phenol-carboxylic acids and carbohydrates or polyhydric alcohols
c. glucosides.

Group 2, the Condensed Tannins, are characterised by the fact that the benzene-nuclei are kept together by carbon atoms. The tannins of this group are able to be transformed by condensation into so-called phlobaphenes. This group is to be divided into two parts;

a. tannins containing phloroglucinol
b. tannins without phloroglucinol in their molecule.

While the classification of Freudenberg seems the best one from a chemical point of view we know there actually exists no great difference between Freudenberg's classification and the one of Stenhouse-Procter. The group of the hydrolysable tannins is about the same as the group of the pyrogallol tannins of Stenhouse-Procter and the group of the condensed tannins covers more or less the catechol tannins.

In connection with the above we shall use some reactions which

differentiate between pyrogallol and catechin tannins as an indication to which group of Freudenberg a tannin belongs. Apart from the general reactions mentioned above there are some special reactions enabling us to carry out this differentiation.

All of the tannins which contain phloroglucinol yield a precipitate with bromine (Stenhouse 1842). Other reactions for a phloroglucinol-nucleus are the "Hlasiwitz's pine-wood test" (1867), the vanillin-hydrochloric acid test of Lindt (1885) and the Joachimovitz-test (1917).

To Stiasny we owe two tests to differentiate the two groups of tannins according to Stenhouse-Procter.

With formaldehyde-hydrochloric acid (1905) the catechol tannins are precipitated while the pyrogallol tannins remain dissolved.

In the test of Stiasny (1911) lead-acetate in acetic acid medium is used, which precipitates the pyrogallol tannins while, in this case, the catechol tannins remain dissolved.

By means of the above reactions it is possible to classify tannins.

d. The tannin present in the material.

As mentioned before, Fessler (1913) investigated the tannin of the scales of the ripe fruit of Buckwheat. With an aqueous solution of bromine a precipitate was obtained, while on heating with hydrochloric acid a red colour developed. These reactions indicate that the tannin belongs to group 2*a* of Freudenberg i.e. a condensed tannin containing a phloroglucinol nucleus.

In order to classify a possibly present tannin in the seedlings the following method has been tried.

About 200 gms of hypocotyledons from etiolated seedlings were extracted several times with boiling ethyl alcohol.

The extracts are mixed and evaporated under diminished pressure.

To test for catechins (the mother substances of group 2*a* of Freudenberg which are soluble in ether while tannins are insoluble in that solvent), the sticky residu is washed with ether. The ether extract has a reddish-brown colour. After evaporation of the ether a sticky and fatty mass remained behind which was washed with distilled water.

Only a very small part of the ether residu dissolved in the water.

With this aqueous extract some reactions on catechins were performed.

1. with bromine-water in the presence of acetic acid no precipitate has been obtained
2. with vanillin and concentrated hydrochloric acid no red colour developed
3. with a 1 % solution of ferric alum a green colour developed

4. on heating with hydrochloric acid no change in colour has been observed.

The result of these reactions makes it clear that no catechins are present in Buckwheat seedlings. The colour developed in reaction 3 might point in the direction of some flavone pigment.

The residue of the ethyl alcoholic extracts after being washed with ether was dissolved in distilled water. The extract is of a clear yellowish-brown colour.

This aqueous extract has been tested on the presence of tannins according to G n a m m (1933).

First of all the general reactions on a possible presence of tannins were carried out;

1. with gelatin-sodium chloride solution a heavy precipitate is formed
2. the addition of a 1 % caffein solution causes a rather heavy precipitate
3. a dense precipitate is formed on the addition of an ethyl alcoholic solution of potassium-acetate
4. with hexamethylene tetramin solution, prepared according to H o u g h (1931), a rather heavy precipitate results.

From the positive result of the general reactions on tannins the presence of tannins in Buckwheat seedlings is established. In order to obtain more information special reactions were carried out;

1. the neutral solution yields a dirty blackish decoloration on the addition of a 1 % ferric alum solution.
2. with bromine water a precipitate is formed
3. with vanillin and hydrochloric acid an intense red colour appears which is more violet-red in thin layers
4. with the para-dimethylaminobenzaldehyde-sulfuric acid reagent of J o a c h i m o v i t z (1917) a similar colour appears
5. a chip of pine-wood moistened with the extract and with concentrated hydrochloric acid developed a violet-red coloration.

Reactions 2-5 clearly show that some phloroglucinol-compound is present, while the first reaction does not give a clear answer whether catechol or pyrogallol tannins are present. To settle this question the reactions of S t i a s n y had to be tried.

1. the formaldehyde-hydrochloric acid test.

5 cc of the extract were mixed with 0.5 cc of concentrated hydrochloric acid and 1 cc of a 40 % formaldehyde solution and boiled for half an hour under a reflux condensor. At the moment that the different components were mixed a white precipitate was formed at once which turned into a red-brown on heating. The contents of the flask were filtered and to the clear yellowish-brown filtrate ferric alum and sodium acetate were added.

No violet coloration appeared.

From the nature of the precipitate it becomes clear that some catechol tannin is present while from the negative test with ferric alum and sodium acetate on the filtrate one might learn that pyrogallol tannins are not present.

2. the lead-acetate, acetic-acid test.

1 cc of the extract was mixed with 2 cc of 10 % acetic acid and 1 cc of 10 % lead-acetate added. A heavy white precipitate appears which, however, cannot be a precipitate of pyrogallol tannins because these are not present. The precipitate cannot possibly be caused by catechol tannins because these substances do not give a precipitate under these conditions. It is very probable that the precipitate is caused by the presence of some protein or sugar compound. After filtration a few drops of ferric alum were added to the clear filtrate. An olive green colour appears which becomes more intense on the addition of some sodium acetate. This colour-reaction indicates that some catechol tannin is present.

All things considered it is clear that a tannin is present in Buckwheat seedlings belonging, according to the Stenhouse-Procter classification to the catechol tannins while the place of the substance in the system of Freudenberg is No. *2a*; i.e. condensed tannins with a phloroglucinol nucleus.

The properties of the tannin present in the hypocotyledons of Fagopyrum agrees well with those of the tannin described by Fessler (1913) from the brown scales of the ripe fruits.

The relation of the tannin to the red colour obtained when an extract of the hypocotyledons is heated with hydrochloric acid has been studied a little more in detail.

Hypocotyledons of etiolated seedlings were rubbed with sand, talcum and acidified ethyl alcohol in the usual way. On extraction with acidified ethyl alcohol on a Buchner-funnel a clear greenish-yellow solution results. This extract shows a very intense red colour on heating with hydrochloric acid and a rather intense red colour on the addition of vanillin and hydrochloric acid. The extract was divided into two equal parts.

a. after acidification with acetic acid and warming a solution of lead-acetate was added. A heavy precipitate appears. After the precipitate was settled the solution was filtered. The white precipitate was washed several times with acetic acid ethyl alcohol. After washing the precipitate was heated with some concentrated hydrochloric acid. Only a trace of a pink colour appears.

The greenish-yellow filtrate heated with hydrochloric acid develops the well-known, very intensely red, colour.

Another part of the filtrate gives a red colour on the addition of vanillin and hydrochloric acid.

b. the other part of the original extract was treated with alkaline lead acetate.

A very voluminous yellow precipitate appeared. After filtration the pinkish brown filtrate was heated with hydrochloric acid. No red colour develops, only a yellowish-brown coloration appears. With vanillin and hydrochloric acid no red colour appears.

The yellow precipitate is soluble in dilute acetic acid. This solution heated with hydrochloric acid produces the well-known intense red coloration, while on the addition of vanillin and hydrochloric acid a red colour appears.

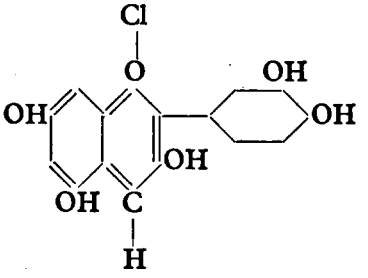
From the above it is obvious that a red colour only develops when the tannin is not precipitated and filtered off.

§ 4. *Flavones.*

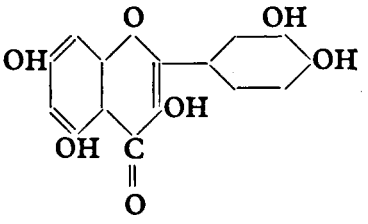
The narrow relation existing between the chemical constitutions of anthocyanins and flavones or flavonols [1] made it necessary to test the material on the presence of the latter pigments.

As stated above the chemical constitution of many of these yellow pigments was elucidated by the work of Von Kostanecki (1893-1904).

The comparison of the formulae given below of representatives of the groups of the flavone and anthocyanin pigments clearly show the relation between those two groups of widely distributed plant pigments.



cyanidin chloride
(*anthocyanin pigment*)



quercetin
(*flavone pigment*)

[1] Flavonol pigments contain an OH-group at place 3, while in the flavone pigments s.s. place 3 lacks the OH-group.

In the future however we shall use the name flavone as a general name both for flavone and flavonol pigments.

Several authors describe a flavone pigment in different parts of Buckwheat. This pigment is identical with the yellow substance Rutin, isolated from Ruta graveolens L. Rutin is a rhamnoglucoside of quercetin.

Wunderlich (1908) isolated this pigment from the dried flowers of Fagopyrum esculentum Moench. and proved the identity with the pigment from Ruta graveolens L. The pigment was present in the material up to more than two percent of the dry weight.

Brandl and Schärtel (1912) again investigated this matter and confirmed the work of Wunderlich. They give some data on the quantity of the pigment in different parts of the full-grown Buckwheat plant.

According to these authors the following quantities are present;

1. 1610 gms of fresh leaves contained 28 gms of Rutin = 1.78 %
2. 1820 gms of fresh flowers contained 13 gms of Rutin = 0.71 %
3. 5820 gms of fresh stems contained 5.5 gms of Rutin = 0.09 %

Nagai (1921) briefly mentions the presence of some flavone pigment in small quantity in the etiolated seedlings, the leaves and the unripe achenes of Fagopyrum.

Kuilman (1930) coult not find any flavone pigment in the etiolated Buckwheat seedlings.

To solve this question we investigated Buckwheat seedlings in different circumstances i.e. etiolated, irradiated but not yet red coloured, and red coloured individuals.

For the extraction we used the method described by Miss Steenhauer (1919) in her Doctors' Thesis.

Portions of seedlings were extracted several times with boiling 96 % ethyl alcohol. The extracts were mixed and evaporated to dryness under reduced pressure. The residue was treated several times with small quantities of boiling distilled water. These aqueous extracts were shaken some times with ether until no longer yellow coloured. In connection with the fact that flavones are easily extracted from the ether by alkaline solutions and take an intense yellow colour with these solvents the ether has been extracted with a 2 % solution of ammonium-carbonate.

In the case of the etiolated seedlings no yellow coloration of the ammonium-carbonate resulted. It is clear that in etiolated seedlings no flavones are present.

The possibility remains that light in necessary for the development of flavone pigment. For this reason we took seedlings in train to form anthocyanin pigment. Neither in this material we found a trace of some flavone pigment. We conclude that in the Buckwheat seedlings flavones are never present.

§ 5. *Discussion.*

The results of the data given above are unsatisfactory in many ways. It appears, however, that certain theories on anthocyanin formation need modification.

In the first place it is certain that the opinion of Jonesco (1930) that leuco-anthocyanins should be the precursors of anthocyanin pigments in the seedlings of Buckwheat is not justified by the simple reason that leuco-anthocyanins are not present in this material. Even if leuco-anthocyanins were present in the Buckwheat seedlings it would not be allowed to conclude from this simple fact that in nature the anthocyanins are derived from these substances. Without experimental work it is inadmissable to draw such sweeping conclusions.

The same thing is true for the opinion of the flavone-transformation. Many authors have, as described above, reduced plant extracts by means of nascent hydrogen and concluded from the resulting red coloration that the natural anthocyanin pigment was formed in the plant by transformation of flavone pigments. It seems to us unnecessary to discuss this matter more in detail.

The question as to the nature of the anthocyanin-precursor, however, has to be put again. It is as yet impossible to give an answer to this question, the only thing we may say is that in our material, some substances cannot be considered to be precursors of the anthocyanin pigments.

Considering the fact that some other substances like tannins and sugars were brought into connection with anthocyanin formation it might be of some value to study the distribution of these substances in the plant in relation to the presence of the anthocyanin pigment.

CHAPTER II

TOPOGRAPHICAL DISTRIBUTION OF SOME CONSTITUENTS.

§ 1. *Growth and Development of the Seedlings.*

As there exists a rather narrow relation between the development of the seedlings and anthocyanin formation as will be demonstrated later, it seemed justified to give a short account of the way the seeds germinate and give rise to the young plant.

In a publication of 1914 on the development and germination of het seed of Polygonaceae Woodcock gives some interesting

details on the morphology of the seed together with some observations on the germination and the early stages of growth of several Polygonaceae, amidst them, Fagopyrum esculentum M o e n c h. We could confirm W o o d c o c k's observations.

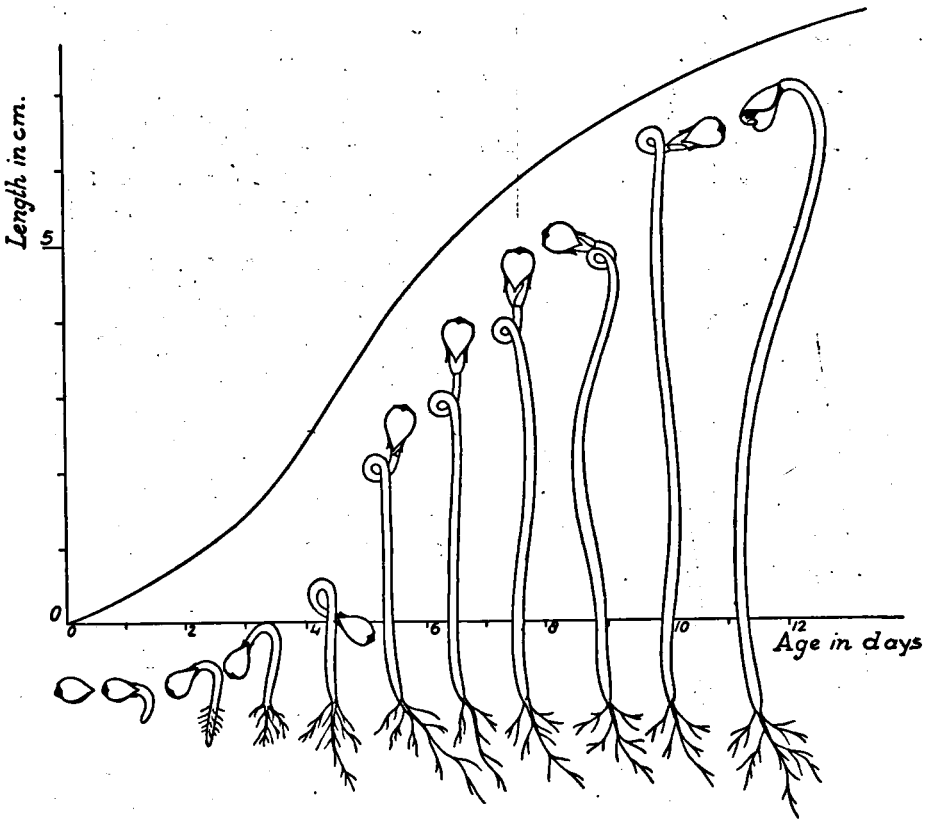


Fig. 1. Growth and growth-curve of etiolated Buckwheat seedlings.

Figure 1 depicts the way the young seedling grows. Within 24 hours after sowing the seed on moist paper pulp in a constant temperature room at 25° C the root appears. In another 24 hours the young hypocotyledon has developed by division and stretching of cells in certain zones between the base of the root and the base of the cotyledons.

The part of the hypocotyledonary axis where cell-division occurs is situated below the cotyledons while the zone where the cells

elongate very quickly is situated below the part where the division of the cells takes place.

The best way to study the growth of this material is to measure the epidermal cells. As may be seen from Figure 2*a* the epidermal cells in the division zone are more or less isodiametric, later the cells appear to be stretched parallel to the long-axis of the seedling, while their width remains the same (see Figure 2*b*). This is illustrated in Figure 3. The abscissa represents the top of a five days old seedling, divided into zones of 1 mm high, 0 represents the top of the seedling. It becomes clear from the figure that the division zone is between 0 and 3, while between 3 and 4 the stretching-zone is apparent. [1]

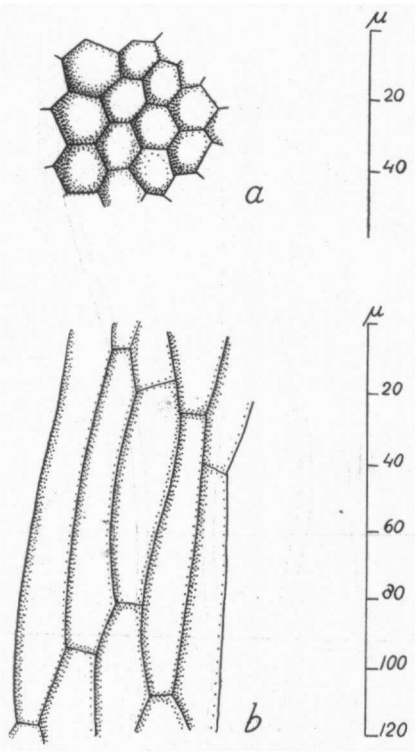


Fig. 2. Epidermal cells of the hypocotyledon.
a. Young epidermal cells.
b. Full-grown epidermal cells.

As the growth of the seedlings is very rapid under the circumstances described the stretching must also be rapid.

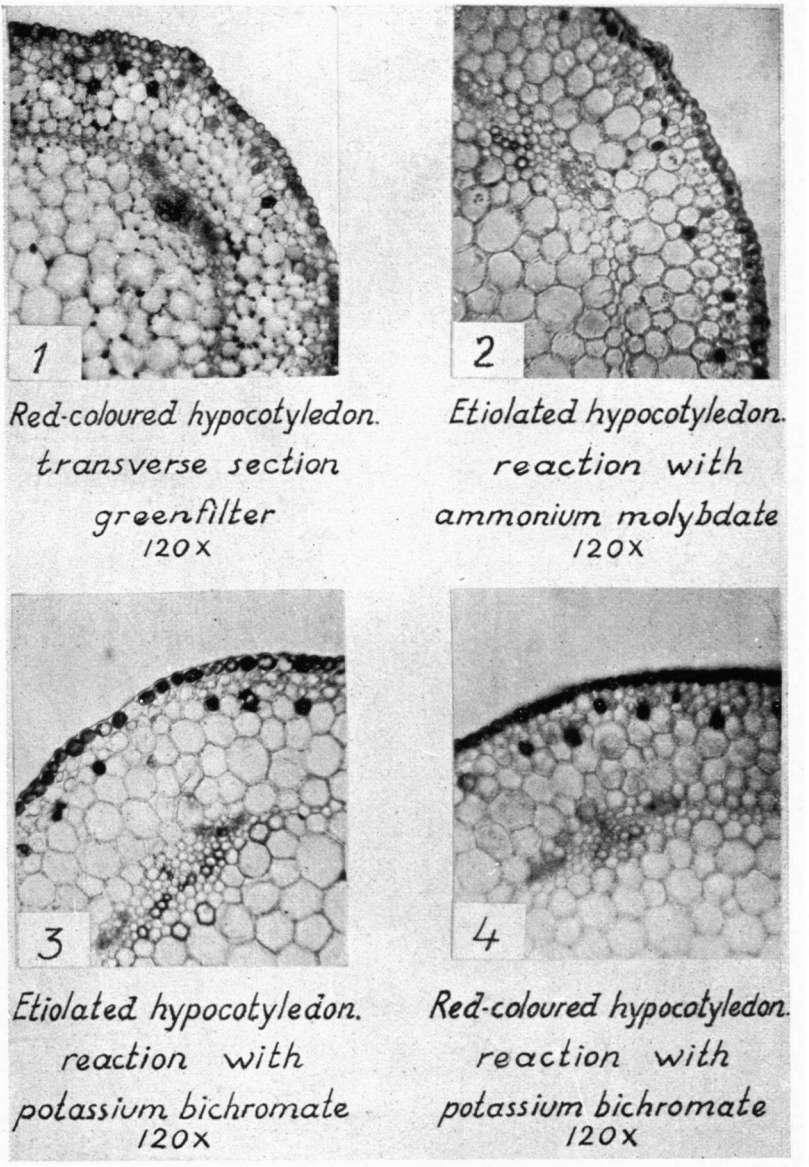
The growth of etiolated Buckwheat seedlings is closely related to the presence of reserve material.

Buckwheat belongs to the group of plants that have a starchy endosperm. In the triangular fruit only one seed is present. The embryo consists of two cotyledons folded together in a peculiar way and a very short rootlet. All the corners between the folded cotyledons are filled up with the endosperm.

As soon as the seed starts to germinate the starch from the endosperm is hydrolysed by the enzymes secreted by the aleurone layer, the products are absorbed and replaced in the hypocotyle-

[1] At this place I should like to thank Mr J. J. t e r P e l k w ij k, B. A. for his keen interest and help in this part of the work.

TABLE II.



Micro-photographs of transverse sections through the hypocotyledon. Localisation of anthocyanin pigment and tannin.

donary axis in the form of starch. This starch is used later for further growth and development of the seedling.

As may be seen from the growth-curve in Figure 1, the period of dissolving the starch from the endosperm and rebuilding it in

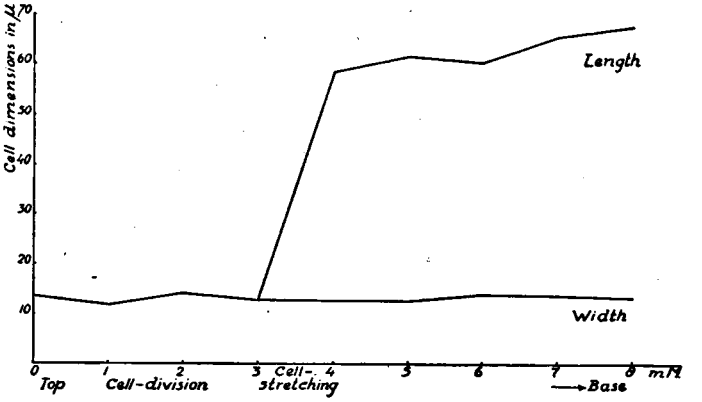


Fig. 3. Relation between length and width of the epidermal cells of the Buckwheat seedling.

the hypocotyledon is realised in the first few days. A second period is the rapid elongation for which, apparently, much starch is used. The end of the growth of the etiolated seedling, which can only live on the reserve-material from the endosperm, begins about nine days after sowing.

From the succession of these three periods in the life of the etiolated Buckwheat seedling the well-known S-curve results.

§ 2. *Anatomy of the Hypocotyledon.*

To understand the localisation of some constituents it might be desirable to describe in short the anatomy of the hypocotyledon of the Buckwheat seedling.

Buckwheat seedlings, especially etiolated ones, possess a long hypocotyledonary axis.

The anatomy of the growing Buckwheat plant is described in detail by Miège in his doctors thesis (1910). The data on the anatomy of the seedling, however, are rather scarce.

Some other authors; Schmidt (1879), Perdrigeat (1900), Woodcock (1914) and Steenhauer (1919) give details on the anatomy of different Polygonaceae, but no data on the anatomy of the seedling are given.

We only shall give those details which might be of use in relation to the description of the localisation of several substances.

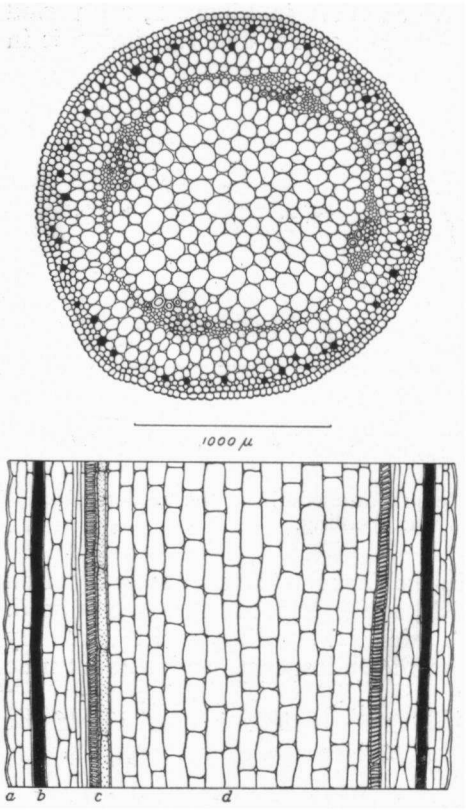


Fig. 4. Transverse and longitudinal section through the hypocotyledon.
a. epidermis. *c*. vascular bundle.
b. tannin sack. *d*. pith.

The transverse- and longitudinal sections, represented in Figure 4 show that the thin-walled, large-celled pith (*d*) takes up a great part of the hypocotyledonary axis.

Around the pith the other parts of the stele become evident.

Four xylem bundles are present, while the four phloem bundles of the stem are divided into eight bundles in the hypocotyledon.

The vessels in the xylem-parts are narrow and only give a rather pale coloration with phloroglucinol and hydrochloric acid: a fact hardly astonishing in relation to the age of the seedlings. Moreover, many of the vessels still contain their protoplasmatic contents.

The pericycle consists of small seized fibre-prosenchymatouslike cells. The inner cells of the cortex have the same character as the cells from the pith; they are rather large, thin-walled cells with many intercellular spaces (see Figure 4 and Tab. II, No. 1, where the intercellular spaces are filled with air).

The subepidermal cells of the cortex are much smaller than the cells of the inner cortex.

Very conspicuous in this part of the hypocotyledonary axis are the tannin sacks (*b*), unbranched, long, tubular elements amidst the cortex. These tannin sacks have a more or less opaque content and contain large quantities of tannin as will be described in § 5 of this chapter. These tannin sacs are formed in a very early stage as

described by Woodcock (1914) in the following words:

"In sections of the seed at the stage in which the radicle is just appearing outside the seed, the elongated tannin-sacs, mentioned by Solereder (1899) as occurring in species of Fagopyrum and Polygonum, are very evident. As further growth occurs these sacs increase markedly in length".

Schmidt (1879) in his thesis on the vegetative organs of the genus Polygonum and Fagopyrum, gives the following statement.

„Ausserdem bemerkt man schliesslich unmittelbar am Bast und im Mark, besonders im peripherischen Theile desselben, mit meist braunem Inhalt erfüllte Elemente: es sind Schläuche gleicher Art, wie die von Sambucus. Ich habe sie beobachtet bei Polygonum tinctorium Ait., P. Hydropiper L., P. virginianum L., P. Amphibium L., P. Persicaria L., P. lapathifolium L., P. orientale L. und sämtlichen Species von Fagopyrum. Die Lage der Schläuche ist auch bei den anderen Arten in ersten Linie dieselbe wie bei P. Amphibium L.; einerseits in peripherischen Teile des Markes, andererseits am Bast. Bei Fagopyrum sind sie freilich an der ersteren Stelle sehr selten".

Schmidt states that the tannin-sacs may reach a length of 7 cm.

Finally the epidermis consists of long-narrow cells (see fig. 2*b*) closely fitting together. Stomata are present in a rather large number. Their long-axis runs parallel with that of the hypocotyledon.

§ 3. *General Remarks on Localisation.*

In general we may say that work done on the localisation of special substances in plant tissues is often started with the ambition to find some direct relation or succession in the presence of certain constituents.

In connection with the problem of anthocyanin formation this method to detect relationships between anthocyanin and some precursor, between anthocyanin and enzymes has been used several times.

As early as 1837 Von Mohl describes the distribution of chlorophyll and anthocyanin in plant tissues. By his investigations he proved that the theory on anthocyanin formation by transformation of chlorophyll is erroneous. This theory had many supporters in the first decades of the nineteenth century.

In 1883 Pick tries to prove that anthocyanins are formed from tannin. Certain cells in the growing-points of a number of plants contain tannins. The same cells contain, in a more advanced stage, anthocyanin pigments. His methods to prove the presence of the different substances are, however, not reliable. Zopf (1887)

describes in a beautifully illustrated publication on "Gerbstoff- und Anthocyan-Behälter der Fumariaceen" the relation between tannins and anthocyanins in special cells in the tubers of plants of this family.

In 1899 Overton comes to the same conclusion as Pick. His methods, however, seem more reliable. Important is the work of Wissemann (1911) who describes in his thesis the localisation of anthocyanin and tannin in a great number of plants. He comes to the conclusion that in nearly all cases the distribution of the two classes of vegetable substances is not the same.

In his publication "Beiträge zur Mikrochemie des Spaltöffnungsapparates" Hamorak (1915) describes the localisation of several substances, amongst which tannins and anthocyanin pigments, in the epidermis of a number of plants. He shows himself to be a supporter of Wigand's theory on the formation of anthocyanins from tannins. His opinion on this subject is laid down in the following sentences:

„Was die von mir in dieser Arbeit aufgeworfene Frage anbetrifft, so hat es sich ganz unzweideutig herausgestellt, dass sich die beiden Stoffe in der Nähe von Spaltöffnungen vertreten können, ja es wurden sogar an manchen Objekten ganz deutliche Uebergänge von Gerbstoff zum Anthokyan aufgefunden".

The relation of flavones and anthocyanins in the stem of Fagopyrum esculentum Moench. has been described in short by Miège (1911). This author proved the presence in the cells of flavone pigments by treating sections with weakly alkaline solutions. According to him the cells which contain flavones in their youth later form anthocyanins. [1]

As already mentioned above Keeble and Armstrong (1912) find a close relationship between the presence of oxidizing enzymes and anthocyanins in petals of several flowers.

Atkins (1913, 1914, 1915) in a series of publications on the presence of anthocyanins and oxidases in Iris flowers could not confirm the view of Keeble and Armstrong.

Finally in 1922 Miranda, working with bulbs of white lilies investigated the distribution of oxidizing enzymes and anthocyanins and came to the conclusion that the localisation of the two groups of substances was the same.

In transverse sections we tried to obtain some information on the

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[1] We used the method of Klein (1922) to study the localisation of flavones with a negative result (see chapter I § 4).

localisation of oxidizing enzymes with the Nadi reagent. We got the impression that apart from several elements of the vascular bundles the tissues outside the stele gave a more intense coloration than the tissues of the stele. The fact, however, that the cell walls became coloured too, makes it undesirable to attach much value to this observation.

From the above mentioned examples it becomes clear how difficult it is to draw conclusions from the observational data. In some cases a totally different result has been obtained by different authors, often with other material. It seems to be possible that only a difference in distribution of the substances concerned should justify us to draw conclusions about their physiological relation while this is probably not allowed when there is no difference in their relative distributions.

§ 4. *Localisation of Anthocyanins.*

In this paragraph we intend to describe the distribution of the pigment in normal red parts of the seedling. As we shall see later in this chapter there is a relation between the age of a certain part of the hypocotyledon and pigmentation. Old seedlings only have some coloration at the top of the plant while the basal part is totally or almost unpigmented. In this series of experiments normal red coloured seedlings were used exclusively.

In a living transverse section which must not be too thin in order to keep large or long cells intact we see that the pigment is restricted to the tissues outside the stele.

The cell sap of each epidermal cell, except the guard cells of the stomata, contains anthocyanin. This is also true for the small-sized subepidermal cortical cells, while the larger-sized cortical cells only partly contain anthocyanin pigment. We have the impression that in very intense coloured parts no more cells are pigmented but that the cell sap of each cell contains more of the coloured substance.

The colour of the epidermal cells is not of the same intensity in all cells. By making surface sections the epidermal cells which are situated around the guard cells of the stomata prove to be more intensively pigmented. This seems to be often the case as may be seen from the work of Hamorak (1915).

§ 5. *Localisation of Tannins.*

Where so many authors consider tannins to be the precursors of anthocyanin we studied the distribution of these substances in the Buckwheat seedling. Another reason why the distribution of tannins has been examined was the possibility that the distribution of the

tannins might be perfectly different from that of the anthocyanin pigment. This would be another argument against the opinion of Jonescо (1930) that the transformation of leuco-anthocyanins (later proved to be tannin) gives rise to the presence of anthocyanin pigment. This point will be discussed in detail in the next paragraph.

To demonstrate the presence and distribution of tannins present in the material several microchemical reactions were carried out. Several of the reactions used are non-specific for tannins. For example a positive potassium-bichromate reaction of Sanio (1863) is given by tannins, alkaloids, several phenols. The reagent of Lindt (1885), containing vanillin and concentrated hydrochloric acid, gives a positive reaction with the tannins of the *2a* group of Freudenberg, and derivatives of phloroglucinol like Maclurin, Catechins, Cyanomaclurin and Phloretin.

The fact, however, that all the reactions tried, including specific reactions like precipitation with alkaloids, gave perfectly the same result clearly shows that real tannins were present.

The following reagents were used:

1. 10 % potassium-bichromate, brown precipitate, Sanio (1863).
2. a saturated solution of cupric acetate in water. After precipitation the precipitate is stained black with ferric-chloride. Moll (1884).
3. equal parts of a 5 % solution of ammonium molybdate and a 25 % solution of ammonium chloride made alkaline with some ammonia. A dark precipitate results. Möller (1888).
4. 1 gm of sodium wolframate and 2 gms of sodium acetate dissolved in 10 cc water gave a precipitate. Braemer (1889).
5. a 1 % solution of caffein gives a heavy white precipitate. Overton (1899).
6. a similar result is obtained with a 1 % solution of antipyrin. Overton (1899).
7. with the reagent of Lindt (1885), a solution of 0.05 gms of vanillin dissolved in a mixture of equal parts of ethyl alcohol and water and 3 gms of concentrated hydrochloric acid, the tannin-containing cells give a bright violet-red coloration.
8. the reagent of Joachimowitz (1917), a solution of 0.5 gms of dimethylaminobenzaldehyde in a mixture of 8.5 gms of concentrated sulfuric acid and 8.5 gms of water gives similar results as reaction Number 7.

Identical results were obtained with these reagents. Tannins proved to be present in the tannin-sacs and in the epidermal cells. Doubtful reactions were obtained in some elements of the vascular bundles.

§ 6. *Relations between Anthocyanins and Tannins.*

We described already in detail the different publications concerning the relation between the distribution of anthocyanins and tannins in plants. From the authors cited the conclusion may be drawn that in some cases a narrow relation exists in the topographical distribution but in the majority such a relation certainly does not exist.

By the reactions described above we studied the distribution of the tannin present in the hypocotyledon of the etiolated and red-coloured Buckwheat seedling and comparing the distribution of this substance with the localisation of the anthocyanin pigment we come to the conclusion that a relation does not exist. [1]

Anthocyanins are present in the cells of the subepidermal cortex, a locality where tannins are not present, while the tannin sacs are never coloured by anthocyanins. The only tissue where anthocyanins and tannins are both present is the epidermal layer. The fact, however, that the guard-cells of the stomata never contain anthocyanins and always give a positive reaction on tannins makes it very probable that also in this tissue no direct relation exists between these representatives of two, from the chemical point of view, related groups of vegetable substances.

Tab. II shows the localisation of anthocyanin pigments in a living transverse section of the hypocotyledon (1), the localisation of tannin in etiolated (3) and red-coloured (4) hypocotyledons by means of the potassium-bichromate reaction, and the localisation of tannins in etiolated seedlings as indicated by the ammonium molybdate reagent (2). A discussion of the photographs seems superfluous in view of the remarks given above.

Perhaps not so important in this case, but certainly of importance from a more general point of view is the quantitative study of tannin- and anthocyanin localisation.

For this reason the number of coloured cells in relation to the remaining colourless cells was determined in transverse sections. To make the counting easier a circular diaphragm of metal, from which a sector was removed, was placed in the eye-piece of the microscope. To count we used ten sectors of transverse sections from

a. red-coloured seedlings
b. red-coloured seedlings treated with potassium-bichromate
c. etiolated seedlings treated with potassium-bichromate
d. etiolated seedlings treated with ammonium-molybdate reagent.

The results are summarised in the following tables.

[1] I am very much indebted to Miss W. S c h u i l i n g B. A. whose accurate work materially contributed to the results described in these paragraphs.

TABLE 4. *a.* Red-coloured Seedlings.

Number of Colourless Cells	Number of Coloured Cells	Ratio
410	191	2.1
199	79	2.5
214	74	2.9
209	72	2.9
220	95	2.3
223	81	2.8
266	87	3.0
200	73	2.7
247	87	2.8
174	94	1.9
Total 2362	Total 933	Average 2.6 ± 1.1

TABLE 5. *b.* Red-coloured Seedlings, treated with potassium-bichromate.

Number of Colourless Cells	Number of Coloured Cells	Ratio
260	71	3.7
283	67	4.2
206	51	4.0
140	35	4.0
307	66	4.7
252	59	4.3
241	55	4.4
267	58	4.6
257	55	4.7
254	59	4.3
Total 2467	Total 576	Average 4.3 ± 0.9

TABLE 6. *c.* Etiolated Seedlings, treated with potassium-bichromate.

Number of Colourless Cells	Number of Coloured Cells	Ratio
324	75	4.3
401	88	4.6
282	60	4.7
253	68	3.7
240	61	4.0
215	61	3.5
328	70	4.7
444	95	4.8
231	53	4.4
357	86	4.2
Total 3075	Total 717	Average 4.3 ± 1.3

TABLE 7. *d.* Etiolated Seedlings, treated with ammonium-molybdate reagent.

Number of Colourless Cells	Numner of Coloured Cells	Ratio
245	58	4.2
264	57	4.6
337	70	4.8
322	71	4.5
298	60	5.0
308	66	4.7
247	52	4.8
234	50	4.7
320	66	4.8
322	73	4.4
Total 2897	Total 623	Average 4.7 ± 0.9

From Tables 4—7 we may conclude that;

a. the method gives reliable and comparable results

b. the distribution of the anthocyanin is perfectly different from that of the tannin.

c. the different reactions on tannins give the same results.

d. the distribution of tannins is identical in etiolated and red coloured seedlings.

§ 7. *Relations between Anthocyanins, Starch and Sugars.*

Until now we do not know from what precursor anthocyanin pigments arise. Only one thing we know with certainty; there is a close relation between the products of photosynthesis and the formation of these pigments. Of these products carbohydrates seem to be the most important.

Several authors succeeded to induce anthocyanin-formation or increase of the quantities of anthocyanin present in the material by sugar-feeding.

Overton (1899), Katić (1905) and Gertz (1912) studied the behaviour of a great number of plants on the influence of sugar-feeding. In the majority of cases a very important increase of the anthocyanin pigments resulted.

Although there are several objections to be made against these experiments it seemed to be worth while to study the localisation of the reserve food of the hypocotyledon, the sugars formed by hydrolysis of the starch and the anthocyanin pigment. For this purpose longitudinal sections of seedlings of different ages were made and tested by the following methods

a. anthocyanin

The distribution of the pigment was studied in not too thin longitudinal sections by the red or pink colour imparted to the cell sap.

b. starch

The distribution of this substance could be easily studied after coloration with JKJ solution.

c. sugars

To study the distribution of these substances we used the method described by S e n f t (1904).

Phenyl hydrazin and Na-acetate are separately dissolved in a ratio 1 : 10 in water-free glycerol. To test a section on the presence of sugars this section was placed in a freshly prepared mixture of equal parts of the two solutions. If treated in the cold within 48 hours bundles and sphaerotrichites of osazone-crystals appear if glucose or fructose are present. By heating on a water bath at 100° C for 10 minutes the osazones appear very quickly. By using heat more complex sugars are hydrolysed and more crystals appear.

We were not able to prove the presence of fructose by the method of G r a f e (1905) who proposed the use of methyl-hydrazin which gives only an osazone with fructose.

Where we were only interested to know if any sugar was present we only used S e n f t's hot reagent.

To study the distributions of the sugars in the different tissues we tried to make sections containing as far as possible only one special tissue.

No difficulties arose by taking the epidermis from the underlying cells. By making surface-sections we were able to obtain the cortex. With the aid of a small instrument made from two safety-blades it was possible to isolate the pith of the seedling.

Figure 5 shows the results of these tests.

The distribution of the three substances in seedlings of different ages show a remarkable close connection with the growth and development of the young plants.

Very young seedlings are literally stuffed with starch. When the seedling is about four days old hydrolysis of the starch begins. The hydrolysis goes very quickly in the period of the fastest growth that is between their 6th and 8th day. In the tip i.e. the zone of division and the stretching zone there is still much starch present but the lower half of the seedling except a very small quantity in the pith of the basal part of the plant seems entirely devoid of starch. Gradually the amount of starch in the tip of the seedling diminishes and growth runs parallel with this process.

The starch is present in the pith, the endodermis and the cortex.

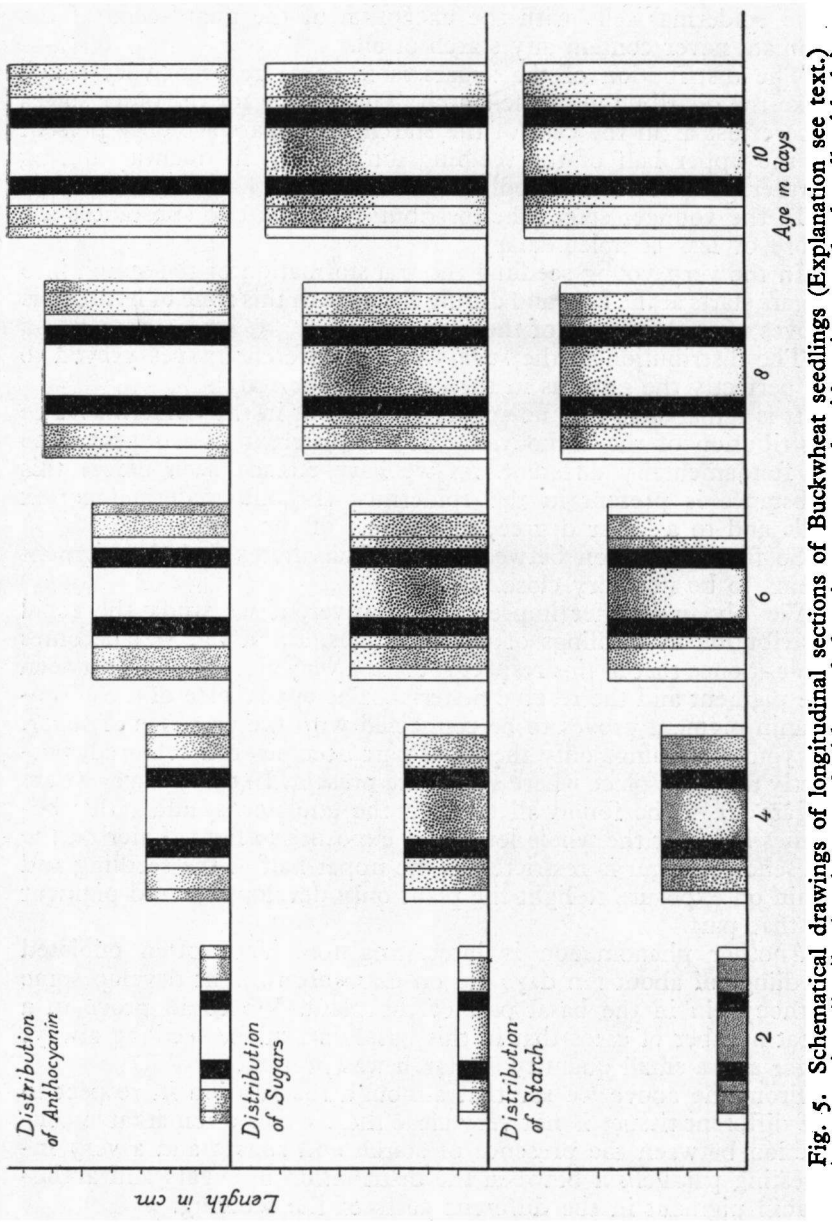

Fig. 5. Schematical drawings of longitudinal sections of Buckwheat seedlings (Explanation see text.) (representing distribution in central pith, endodermis, cortex and epidermis; vascular bundles black.)

The epidermal cells with the exception of the guard-cells of the stomata never contain any starch at all.

The distribution of the sugars is in some respects very much alike the distribution of the starch. This is true for the older stages where just as in the case of the starch the sugars are only present in the upper half of the seedling diminishing in quantity during further growth of the seedling.

In the younger stages the distribution of the two substances are more or less complementary.

In the very young seedling the transformation of the starch into sugars starts at the base and during the growth this zone of hydrolysis moves towards the tip of the plant.

The distribution of the sugars in the different tissues proved to be perfectly the same as in the case with the starch.

It is remarkable that no sugars are present in the epidermis. The distribution of the anthocyanin in the different tissues proved to be fundamentally different. As we have already seen before this substance is present in the epidermis, the sub-epidermal cortex cells and to a lesser degree, in the rest of the cortex.

So far the relation between the carbohydrates and the pigment seems to be not very close.

We obtain another impression, however, if we study the zonal distribution in seedlings of different ages. From fig. 5 it becomes clear at once that in this respect there is a very close relation between the pigment and the reserve material. The appearance of the anthocyanin pigment proves to be connected with the presence of sugar. In young seedlings only the basal part becomes red coloured, perfectly the same place where sugars are present. In those stages where sugars are to be found all through the etiolated seedling this becomes red over the whole length on exposure to light. Later on the presence of sugar is restricted to the upper half of the seedling and again on exposure to light the plant only develops the red pigment in that part.

Another phenomenon is interesting too. Very often etiolated seedlings of about ten days old on exposure to light develop some anthocyanin in the basal part of the plant. We could prove in a great number of cases that in this basal part of the seedling always sugar and a small quantity of starch was present.

From the above we see that although the relation in respect to the different tissues is not very close there exists a remarkable connection between the presence of starch and sugars and a very interesting parallelism between the distribution of sugars and anthocyanin pigment in the different parts of the seedling.

§ 8. *Age of the Seedling and Anthocyanin formation.*

From the foregoing paragraph we conclude that the seedling of Fagopyrum is very unfit material indeed to study anthocyanin formation. To study metabolic processes it is necessary that one has homogeneous material at one's the disposal. From the above it becomes clear that this is not the case with Buckwheat seedlings and with fast growing plant material in general. The different zones have perfectly different metabolic processes. While in the young stages starch is built up in the upper part this substance is already hydrolysed in the basal part and while in the tip no anthocyanin appears this pigment is produced in noticeable quantities at the base. At the other hand seedlings of about ten days old consist of a basal part where by lack of material hydrolysis of starch and formation of anthocyanins no longer take place and an upper part where these processes go on with undiminished intensity.

It becomes clear that in the seedlings at different distances from the tip the long chain of reactions shows different links. By studying the effect of the age of the seedling on the quantity of anthocyanin formed under standard conditions we obtained some more data which illustrate the opinion developed above.

Seedlings of different age were exposed to light during some time and after exposure the anthocyanin formed was extracted after

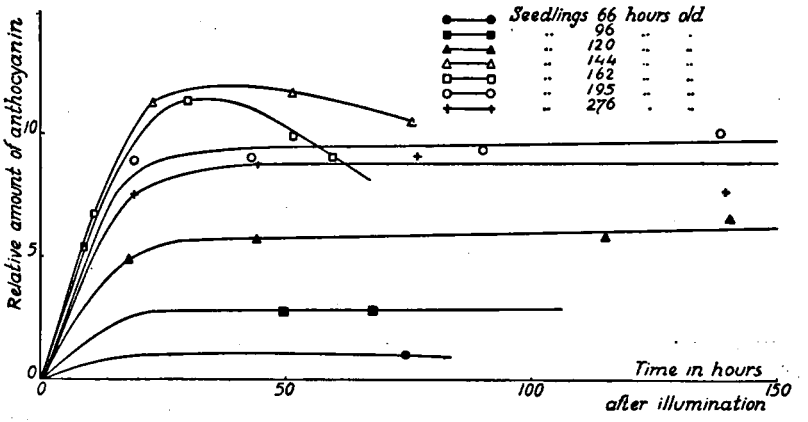

Fig. 6. Relation between age, duration of time after illumination, and anthocyanin content in hypocotyledons of Buckwheat.

different periods of time. The amount of the pigment present was determined by means of the large L e i t z colorimeter. Details of the methods used for this work will be described later on (Chapter

IV). The results of the series of experiments [1] is given in Table 8 while the same is expressed in Figure 6.

TABLE 8.

Age in Days	Time in Hours after Exposure to Light	Relative Amounts of Anthocyanin formed
2.75	74	1.0
4	49	2.8
	67	2.8
5	18	4.9
	43.5	5.7
	115	5.8
	140	6.5
6	9	5.4
	22.75	11.3
	51.30	11.7
	75.25	10.5
6.75	9	5.4
	11	6.7
	30	11.4
	51.5	9.9
	59.1	9.1
8.13	19.2	8.9
	42.7	9.0
	90	9.4
	138.5	10.1
11.50	19	7.5
	44.25	8.8
	76.50	9.1
	139.75	7.7

From Figure 6 we may conclude several things. First of all it is obvious that in a seedling of a certain age a certain amount of available material is present from which a certain quantity of anthocyanin pigment may be formed. As soon as the material is exhausted no more anthocyanin is formed. At the other hand it seems to be certain that the anthocyanin formed is not resorbed, or changed into colourless

[1] We are very much obliged to Mr E. Boeke B.A. to whom we owe the data used in this paragraph.

products, in any case not in the course of the first six days after the exposure to light. Exceptions, however, occur in the case of six or seven days-old seedlings in which, after a certain height is obtained, the amount of anthocyanin diminishes rather rapidly.

The curves given by K u i l m a n in his thesis show a striking similarity to those we obtained with six of seven days-old seedlings. It has to be remarked that this author always uses Buckwheat seedlings of about six days old, grown in the same circumstances as our seedlings.

It is possible, of course, that this is only a mere coincidence but the possibility remains that this exceptional behaviour is characteristic for seedlings of this special age. [1]

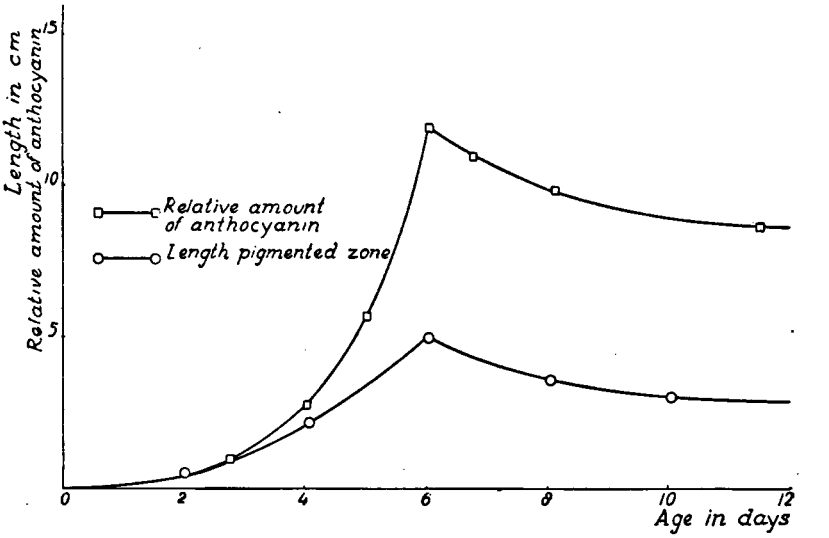

Fig. 7. Relation between age, length of pigmented zone of the seedling, and anthocyanin content.

We may learn something more from the set of curves given in Figure 6. By plotting the maximum quantity of anthocyanins formed, a point reached at the end of 40 hours after the exposure to light, against the age of the seedling the curve of Figure 7 results. The significance of this line seems to us the following.

[1] In several cases we obtained for seedlings of six or seven days old curves like those described for younger or older seedlings. This fact, however, does not change any fundamental point in our argument.

By comparing this figure with the distribution of anthocyanins in seedlings of different age as given in Figure 5 we see that there appears to be some relation between these two figures.

In the first six days the pigmented part of the seedling increases while later the pigmented zone slowly decreases.

By comparing the quantity of anthocyanins formed in seedlings of different age and the length of the coloured zone we observe that there is a very close relation between the two curves.

The results of this series of experiments shows once more that the formation of anthocyanin pigments totally depends upon the carbohydrates available for this process both in their distribution and in their quantity.

§ 9. *Discussion.*

In the foregoing pages we came to the following results;

a. a formation of anthocyanin from tannin is impossible

b. there exists a close relation between age and development of the seedling, on one hand, and the distribution of starch, sugars and anthocyanin pigment in the material on the other.

CHAPTER III.

OXIDATIVE PROCESSES AND ANTHOCYANIN FORMATION.

§ 1. *Review of the Literature.*

We have already mentioned several times that there exist several theories according to which oxidation should play a part in the formation of anthocyanin pigments.

In the last chapter we quoted several authors who studied the relation between the localisation of oxidizing enzymes and the distribution of anthocyanin.

In the introduction we had the opportunity to mention some investigators who developed theories on the formation of anthocyanin pigments from some chromogen. The number of authors however who studied a possible connection between oxidative processes and anthocyanin formation by means of physiological methods is very small.

In 1876 M e r observed that leaves of Cissus do not redden under water. The same was described by E m e r y (1889) for the flowers of the tulip.

More data are obtained from the thesis of K a t i ć (1905) who

studied the effect of sugar-feeding on anthocyanin formation and in addition describes some other experiments. Leaves, enclosed in vessels containing air devoid of oxygen by treatment with pyrogallol in caustic soda, do not form any pigment.

He further describes that submerged leaves of Canna indica L., Veronica Chamaedrys L., and Rosa spec. remain free from anthocyanin. The leaves of Saxifraga cordifolia H a w., however, develop anthocyanin under the same circumstances.

N a g a i (1921) made the observation that etiolated Buckwheat seedlings which develop a red pigment on exposure to light did not form the pigment in an atmosphere of hydrogen even when exposed to light. He describes also some experiments with seedlings of certain varieties of the Soy Bean, Glycine Soja S i e b. & Z u c c.

Seedlings in a hydrogen atmosphere never developed any trace of anthocyanin pigments while the control in air became deep purple. Seedlings after staying in hydrogen for four days did not form anthocyanin when the hydrogen was replaced by normal air.

As far as we know no more literature appeared on this subject.

§ 2. *The Effect of Nitrogen on Anthocyanin Formation.*

To obtain some more information about the connection between oxygen and anthocyanin formation some experiments have been performed in order to establish the dependance of the formation of the pigment upon the presence of oxygen.

Furthermore the experiments of N a g a i do not yield any information about the state of the seedlings after a sojourn in the hydrogen atmosphere. The fact that the soy bean seedlings did not give the pigment after staying for four days in the hydrogen might be accounted for in two ways.

a. all the material used ordinarily for the formation of the pigment is consumed for other purposes.

b. the material is in such a bad state after the hydrogen treatment that one can hardly speak of living seedlings.

The experiments with nitrogen were arranged in the following way. Four groups were made each containing two samples of about eight days old seedlings.

a. seedlings exposed to light in a nitrogen atmosphere. After the treatment the nitrogen has been replaced by common air (No. I and II)

b. seedlings exposed to light in common air. After the exposure to light the air has been replaced by nitrogen (No. III and IV)

c. seedlings exposed to light in a nitrogen atmosphere. After this treatment the nitrogen has not been replaced by air (No. V and VI)

d. seedlings exposed to light in common air. After treatment the air has not been replaced by nitrogen (No. VII and VIII)

The nitrogen from a bomb was purified as follows;

First of all the gas was bubbled through an alkaline pyrogallol solution and washed in a washing-bottle with dilute sulfuric acid to retain possible traces of alkali. Next the gas was led through a quartz tube containing three close-fitting rolls of copper gauze, heated to redness by three wing burners to absorb every trace of oxygen. Finally the nitrogen was cooled in a glass spiral before passing through the vessels containing the seedlings.

The air in the vessels with the seedlings was replaced by the purified nitrogen by flushing them with four times their volume After this procedure the vessels were closed and together with the control vessels, were exposed to the light of a mercury-arc lamp for five hours. At the end of this time the different numbers were examined on the presence of the first trace of anthocyanin.

After the examination the nitrogen in Nos. I and II was replaced by air, Nos. III and IV were filled with nitrogen and Nos. V, VI, VII and VIII left undisturbed.

After sixteen hours in the dark the seedlings were examined again. After the examination all the vessels were filled with air, and exposed to light again. After four hours of illumination the seedlings were examined again and placed in the dark for about eighteen hours. After this the seedlings were examined for the last time.

While the first exposure to light was performed to get some information on the influence of nitrogen on the formation of colouring matter the reason for the second exposure was to see whether the seedlings were still alive and able to form anthocyanin.

The results are given in Table 9.

TABLE 9.

No.	At the end of 5 hours exposure	16 hours later	After second exposure of 4 hours	18 hours later
I	—	—	±	+
II	—	—	±	+
III	±	±	++	+++
IV	±	±	+	+++
V	—	—	±	+
VI	—	—	±	+
VII	±	++++	++++	++++
VIII	±	++++	++++	++++

— uncoloured
± very pale coloured
\+ pale coloured
++ fairly coloured
+++ intensely coloured
++++ very intensely coloured.

From the results we may draw several conclusions. In the first place it is obvious that exposure to light in a nitrogen-atmosphere never results in formation of anthocyanin in the seedlings whether or not the nitrogen be replaced by air.

It seems in the second place to be apparent that prolonged sojourn in a nitrogen atmosphere in the dark after exposure to light in air stops the anthocyanin formation at once that had already begun during the exposure.

From the results obtained we may conclude that there are two processes in anthocyanin formation for which oxygen is necessary i.e.
a. during the exposure to light
b. during the period in the dark after the exposure to light.

The exposure to light in air gives some more data.

First of all the fact ought to be mentioned that all seedlings form anthocyanin pigment to a greater or lesser degree.

In the second place it is clear that during the first exposure to light those samples which have been irradiated in a nitrogen atmosphere develop much less anthocyanin after the second light-treatment. This might be explained in this way that during the first exposure to light nearly all the material that otherwise is transformed into the anthocyanin-precursors in the period after the exposure to light is used for some other purpose. This is not the case in samples III and IV which after being exposed to light in a normal atmosphere were brought into nitrogen after the light treatment. These two samples are still able to form appreciable amounts of anthocyanin pigments after the second treatment with light.

Finally the fact that all of the seedlings are still able to form anthocyanin means that we are justified to regard the lack of formation of anthocyanin pigments in seedlings in a nitrogen atmosphere as a physiological phenomenon.

§ 3. *Oxidative Systems present in Buckwheat Seedlings.*

As now we have the certainty that anthocyanin formation in Buckwheat seedlings is a chain of oxidative processes it might be desirable to investigate the nature of the oxidative systems present in our material.

By means of the usual reactions described in the handbooks (see f.i. Von Euler 1934) the presence of several oxidizing enzymes could be shown.

To eliminate the presence of disturbing substances like soluble carbohydrates and different salts the seedlings were ground with pure sand in a mortar and washed several times to remove the above mentioned substances. As polyphenol-oxidase is water-soluble we

used in the tests for this enzyme unwashed tissue. To be sure that possible positive reactions were really caused by the presence of enzymes always a control-experiment has been performed with material previously heated to 100° C for some time. We tested the material on the presence of the following enzymes; oxidases, peroxidases and katalase.

For the oxidizing enzymes we used the following tests; guaiac, benzidin, α-naphtol, Nadi-reagent, pyrogallol, guaiacol with and without hydrogen-peroxide.

The presence of katalase is shown by the formation of oxygen from hydrogen-peroxide.

The results are shown in Table 10.

TABLE 10.

	$- H_2O_2$	$+ H_2O_2$
Nadi-reagent	+	+
benzidin	—	+
α-naphtol	—	+
guaiac	—	+
guaiacol	—	+
pyrogallol	—	+

The positive reaction with the Nadi-reagent in the absence of hydrogen-peroxide is an indication that cytochrome oxidase is present.

We tried to obtain more information on the presence of cytochrome oxidase and for cytochrome itself.

Keilin (1929) describes the enzyme as thermolabile, being irreversibly destroyed by boiling or heating to 70° C. On treatment in this way the tissue suspension hardly gave any colour on the addition of Nadi-reagent.

Like all oxidizing enzymes the cytochrome oxidase is very sensitive to HCN and H_2S. In the presence of HCN and H_2S in a total concentration of 0.005 mol. the reaction with Nadi-reagent proved to be perfectly negative.

The next thing to try was to detect cytochrome itself in the material. Where cytochrome has a characteristic absorption spectrum (Keilin 1925, 1929, 1930) we hoped to be able to see the absorption bands described by this author. While we had not the opportunity to use the same instruments as described by Keilin we first investigated the presence of cytochrome in yeast by means of a Hoffmann-spectroscope. We were able to detect the

absorption bands *b* and *c* of 5645 and 5490 Å. The fact that the bands, with our instrument, were very vague did not give us much hope as to the abundance of cytochrome, which appears to be much less than e.g. in yeast. This proved to be the case and addition of reducing substances or pyridin did not help to give the sharper absorption bands of cytochrome derivatives. Still we are fairly certain that cytochrome is present.

The fact that polyphenol oxidase was not present is interesting in connection with the opinion of Von Euler (1934) who states:

„Die in den Blüten vorkommenden Oxydasen sind namentlich wegen des zu erwartenden Zusammenhang mit der Farbstoffbildung des öfteren untersucht worden. Ueber den Chemismus der Oxydasewirkung ist wenig bekannt, doch zeigt ein Blick auf die beiden Formeln des Quercetins und des Cyanidins als zweier typischen Vertreter der grossen Farbstoffgruppen der Flavonole und der Anthocyanidine, dass hier prinzipiell ein Betätigungsfeld der Polyphenolase vorliegt".

Peroxidases are present in large quantities. We did not find any difference in the peroxidase content of etiolated and red coloured seedlings. Perhaps a point of interest is that embryo's picked out from seeds of Buckwheat do not contain a trace of oxidising enzymes. As soon as the primary root appears the reactions on these enzymes become positive. The presence of HCN and H_2S in a concentration of 0.005 mol. prevents the appearance of a coloration with the above-mentioned reagents.

Finally katalase proved to be always present. The presence of the so-called "yellow respiratory enzyme" could not be established.

§ 4. *Discussion.*

The experiments described in the second paragraph of this chapter strengthen the assumption that anthocyanin formation in Buckwheat seedlings is connected with oxidative processes. We could prove that apart from the photochemical reaction two oxidative processes occur. Evidence for the presence of several oxidizing enzymes could be obtained.

CHAPTER IV.

INFLUENCE OF HCN ON ANTHOCYANIN FORMATION.

§ 1. *Introduction.*

The evidence adduced for the oxidative anthocyanin formation is sufficient to warrant a further study in this direction.

In the last chapter we obtained some qualitative data and it is evident that quantitative data might throw more light upon the phenomena narrowly connected with anthocyanin formation.

§ 2. *Material.*

For quantitative work the quality of the material used is of the greatest importance. We therefore worked out the conditions necessary to obtain reliable material.

The first paragraph of the second chapter contains some data on the growth and development of the seedlings. It is obvious that the age of the seedlings used for physiological purposes is important. We have seen that the age of the seedling in which the maximum anthocyanin is formed is about six days. In the following experiments seedlings of this age have been used.

A second point to consider is the water supply. The presence of much water causes rapid growth. In the first chapter we mentioned the fact that we found a very suitable medium in paper pulp, a substance containing no nutrient substances and which is easily soaked with a certain amount of water.

The good seeds were selected, the broken and shrivelled seeds discarded. After sterilising with a dilute chlorine solution the seeds were sown on paper pulp, which pulp was washed twice to remove possible toxic substances. The paper pulp used finally contained 160 gms of tapwater, and 90 gms of dry substance. The moisture content proved to be sufficient for about twelve days.

Portions of 100 sterilized seeds were sown in P e t r i-dishes with a diameter of 10 cm containing 62.5 gms of wet paperpulp. The P e t r i-dishes were placed in crystallization dishes with a diameter of about 20 cm, 8 cm high and covered with a glass-plate. After sowing the seeds were allowed to germinate in a constant temperature room in the dark at 25° C. At the end of six days the seedlings were used for the experiments.

As we had not the disposal of a uniform variety of Buckwheat the rate of growth was not very uniform as might be seen in Figure 8.

The average length of samples of the same age, however, proved to be practically the same.

§ 3. *Determination of Anthocyanin.*

The amount of anthocyanin pigment present in the sample was determined colorimetrically. For the colorimetric determination of the concentration of a coloured solution this solution has to meet certain requirements. These requirements are that the coloured solution follows Beer and Lambert's law.

If a solution of a light-absorbing substance dissolved in a non-

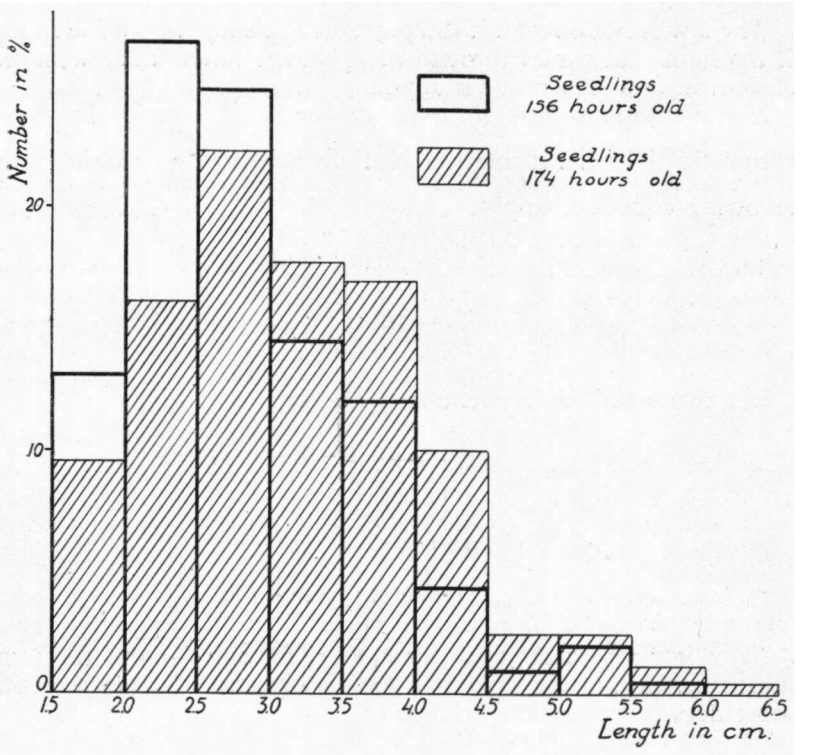

Fig. 8. Graph illustrating the difference of length of seedlings in two samples of different age.

absorbing solvent follows Beer and Lambert's law this means that the following relation exists;

$$I = I_0 . e^{-k . l . c.}$$

in which I_0 = Light intensity before passing the solution.
I = Light intensity after the passage.
e = Natural logarithm.
k = Light absorption constant.
l = Thickness of layer.
c = Concentration of the substance.

Expressed in Briggian logarithms;

$I = I_0 . 10^{-0.4343\ k . l . c}$ or
$I = I_0 . 10^{-\varepsilon . l . c}$ in which ε (extinction coefficient) = 0.4343 k.

The law is not obeyed if the pigment is changed by dilution and if the light that passes through the pigment solution is not monochromatic (see Karsten 1934).

If two solutions show the same colour on observation in monochromatic light i.e. if the two solutions have the same ratio $\frac{I_0}{I}$, the following equations apply;

$$I = I_0 . 10^{-\epsilon} . l . c.$$

$$\frac{I_0}{I} = 10^{\epsilon} . l . c.$$

For two solutions as mentioned above;

$$\frac{I_{01}}{I_1} = \frac{I_{02}}{I_2} \text{ or}$$

$$10^{\epsilon_1} . l_1 . c_1 = 10^{\epsilon_2} . l_2 . c_2$$

$$\varepsilon_1 . l_1 . c_1 = \varepsilon_2 . l_2 . c_2$$

As ε is a molecular quantity $\varepsilon_1 = \varepsilon_2$ for solutions of the same substance. In the case where we compare two solutions of the same substance we obtain the relation;

$$l_1 . c_1 = l_2 . c_2$$

It is clear that in this way unknown concentrations may be compared with a standard solution of the same substance.

As we had not the disposal of crystalline anthocyanin we were obliged to use some other pigment-solution with known optical properties.

For this purpose a Greyfilter-solution is made available by the Firm of Leitz.

The ε of this solution is not given. For the Greyfilter another quantity is given i.e. E, the extinction, $\log \frac{I_0}{I}$.

The value of $E = \frac{1}{2}$. for $l = 1$ cm.

It is clear that $E = \varepsilon . l.c.$

In the case of the Grey-filter solution; $E = \varepsilon . c. = \frac{1}{2}$ or, for 1 cm of the solution compared with the filter solution;

$$E_1 . l_1 = E_2 . l_2$$
$$E_1 . l_1 = \tfrac{1}{2} . l_2$$
$$E_1 = \frac{l_2}{2 . l_1}$$

In this equation the concentration c_1 is not used. For calculating ε_1 the following formula may be used;

$$\varepsilon_1 = \frac{l_2}{2 . l_1 . c_1}$$

in which ε_1 = Extinction coefficient of the investigated solution
l_1 = Thickness of layer of this solution
c_1 = Concentration of this solution
l_2 = Thickness of layer of the Greyfilter solution.

As we did not know the concentration of the anthocyanin pigment in mol./L we could not calculate ε_1. If we divide the different values of E_1 by the respective relative concentrations we certainly must obtain a constant value only different from ε_1 by some factor.

We further used a Leitz Colorimeter. To be able to work with monochromatic light of different wave-length, this instrument is provided with a set of colour-filters. To obtain a greater accuracy it is desirable to use a filter with the complementary colour of the pigment solution.

By means of a Keuffel and Essen spectrophotometer [1] the absorption of different wave-lengths of a purified solution of Buckwheat anthocyanin in 0.5 % HCl aq. has been determined. The maximum absorption proved to be situated at about 5200 Å. As the absorptionband of anthocyanins is a broad one it did not matter that the filter used possessed a transmission-maximum of a slightly different value. We had to use a filter with a maximum transmission at 5300 Å.

As far as we know nothing is known on the validity of Beer and Lambert's law for anthocyanin solutions. We investigated this matter to be certain that a colorimetric determination is feasible.

As we had not the disposal of the crystalline anthocyanin pigment from Buckwheat seedlings we used a purified solution in 0.5 % HCl aq. in a range of dilutions we expected to obtain in our experiments. The results are shown in Table 11.

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[1] For the use of which instrument I am indebted to Dr H. P. Wolvekamp of the Zoological Department of the Leyden University.

TABLE II.

Concentration	Anthocyanin (mm.)	Greyfilter [1]) (mm.)	E calculated	$\frac{E}{c}$
8	5	2.1	0.2100	0.0263
	10	4.2	0.2100	0.0263
	20	8.4	0.2100	0.0263
	40	17.1	0.2138	0.0267
6	5	1.6	0.1600	0.0267
	10	3.2	0.1600	0.0267
	20	6.4	0.1600	0.0267
	40	12.6	0.1575	0.0263
4	10	2.1	0.1050	0.0263
	20	4.2	0.1050	0.0263
	40	8.5	0.1063	0.0266
2	20	2.1	0.0525	0.0263
	40	4.3	0.0538	0.0269
1	40	2.2	0.0275	0.0275

The constant value obtained for $\frac{E}{c}$ in a range of dilutions gives the certainty that the anthocyanin solution follows Beer and Lambert's law.

The anthocyanin solutions used for colorimetric determination were prepared in the following way.

The hypocotyledons of the seedlings were ground with sand and talcum and a small quantity of acidified ethyl alcohol. The paste was sucked dry on a Buchner funnel and washed several times with acidified alcohol in order to extract all of the pigment. The total ethyl alcoholic extract, amounting to about 50 cc, was mixed with 20 cc of amyl alcohol and 20 cc of ether; 40 cc of benzene was added and the mixture washed several times with small portions of distilled water in order to extract the pigment. A large quantity of yellow impurities remained in the amyl alcohol-ether-benzene mixture.

The aqueous anthocyanin solution was finally made up to a certain volume and the amount of anthocyanin present determined by the colorimetric method as described above.

§ 4. *Determination of HCN.*

In all experiments on the influence of HCN on physiological processes the objects were submersed in the HCN solutions (Warburg 1928, v. d. Paauw 1935, Van Raalte 1937).

[1] average of at least 6 determinations.

Only in certain cases where HCN has been used in forcing experiments, the substance was present in the gaseous phase (Gassner 1927).

The small HCN content of solutions is easily controlled by making use of dilution of concentrated standard solutions. In the experiments with gaseous HCN it is difficult to control the quantity of HCN present. The only way to obtain safely a HCN- containing atmosphere is to have a cyanide solution and acid brought together in a closed vessel in which the air may be mixed thoroughly with the developing HCN. Another difficulty is to measure the volume of the vessel with sufficient accuracy. As it was of interest to know how much hydrocyanic acid the seedlings contained after remaining for some time in a special air-HCN mixture a micro-method was worked out to suit our special case. We based our method on some preliminary results of Klaassen (1931) with a micro-modification of the bromometric method of Schulek (1923).

This method is based on the quantitative formation of bromocyanide by the action of bromine to hydrogen cyanide in a weakly acid medium. The quantity of bromocyanide formed may be determined by the addition of potassium iodide. According to the equation $BrCN + 2\,HJ = HCN + HBr + 2\,J$ an equivalent quantity of iodine is set free and may be titrated with standard sodium thiosulfate. As we wanted to estimate the amount of prussic acid in seedlings which had been in contact with an atmosphere containing this substance for a certain time, it was, in addition, necessary to have the disposal of a method to isolate the hydrogen cyanide from the seedlings.

A suitable method was to distil the HCN from the ground seedlings from an acid medium into 1 N NaOH. At the end of the distillation the amount of sodium cyanide was determined by means of the above-mentioned method. We used a small Jena-glass distillation-apparatus. First of all the duration of the distillation and the sensitivity of the method had to be investigated.

a. duration of the distillation.

2 cc 0.0097 mol. KCN [1]) containing 0.524 mgr HCN, 30 cc of distilled water and 2 cc of concentrated phosphoric acid were heated to boiling and the distillate received in 5 cc 1 N NaOH. At the end of the distillation the amount of HCN was determined as follows.

The alkaline solution is acidified with some drops of concentrated hydrochloric acid, a trace of methyl orange added, and bromine-water added drop by drop until a permanent pale-yellow colour is obtained, after a few minutes

[1] pro analysi KCN of Kahlbaum was used for the experiments.

some drops of liquid phenol are added and the flask shaken. At the end of 5 minutes, necessary to neutralise the excess of bromine a few small crystals of potassium iodide (pro anal. K a h l b a u m) are added and the stoppered flask placed in the dark for 15 minutes after which the iodine liberated is titrated with 0.01 N sodium thiosulfate with starch as indicator.

Some blanks were prepared in the same way as described above, only without hydrogen cyanide. These blanks remained perfectly colourless and did not yield any coloration with starch. We distilled the above-mentioned quantity of HCN i.e. 0.524 mgr during different times. The results are given in Table 12. To illustrate the reliability of the method we give the data in full.

TABLE 12.

Duration of the Distillation	mgr HCN added	mgr HCN recovered	HCN recovered in % HCN added
10 min.	0.524	0.512	97.71
	„	0.499	95.23
	„	0.521	99.43
	Average	Average	Average
	0.524	0.511	97.46
15 min.	0.524	0.515	98.28
	„	0.518	98.85
	„	0.517	98.66
	„	0.519	99.05
	Average	Average	Average
	0.524	0.518	98.71
20 min.	0.524	0.519	99.05
	„	0.520	99.23
	„	0.526	100.38
	„	0.524	100.00
	Average	Average	Average
	0.524	0.522	99.67

The results of Table 12 show that the method is reliable and gives accurate results. If not mentioned otherwise the duration of the distillation will be always 20 minutes in the following experiments.

To obtain an idea on the sensitivity of the method different quantities of HCN have been distilled. The results are shown in Table 13, each giving the average of four determinations.

The method described seems to be suitable and allows to determine very small quantities of HCN with proper accuracy. The loss of 0.003 mgr. will be used as a correction.

There remained the possibility that the presence of plant material

TABLE 13.

mgr HCN added	mgr HCN recovered	mgr HGN lost	HCN recovered in % HCN added
0.524	0.522	0.002	99.67
0.262	0.259	0.003	98.87
0.131	0.128	0.003	97.86
0.0655	0.063	0.003	96.22

might influence the quantity of prussic acid recovered. To investigate this point different quantities of HCN have been distilled in the presence of plant material of different age.

The experiments were executed as follows.

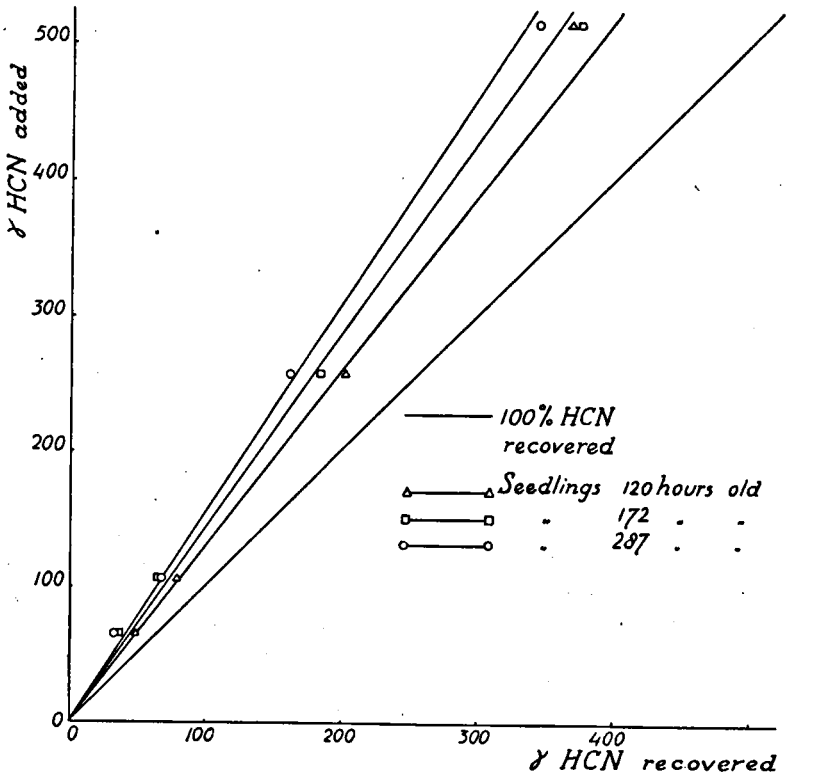


Fig. 9. Influence of plant material on the quantity of HCN recovered.

The hypocotyledons of 40 seedlings to which were added 5 drops of 1 N NaOH, the HCN containing solution and 3 cc of distilled water were finely ground in a mortar and washed into the distillationflask with 35 cc of distilled water; 2 cc of phosphoric acid were added and the mixture heated to boiling in order to distil the amount of HCN present.

The results are shown in Table 14 and Figure 9.
The values given are the average of two or three determinations.

TABLE 14.

Age in Hours	mgr HCN added	mgr HCN recovered	mgr HCN not recovered	HCN recovered in % HCN added
120	0.514	0,369	0.145	71.70
	0.257	0.203	0.054	78.79
	0.129	0.099	0.030	76.36
	0.064	0.050	0.014	78.13
172	0.514	0.375	0.139	73.05
	0.257	0.182	0.075	70.62
	0.129	0.082	0.047	63.88
	0.064	0.038	0.026	59.37
287	0.514	0.345	0.169	67.12
	0.257	0.165	0.092	64.20
	0.129	0.084	0.045	65.12
	0.064	0.036	0.028	56.25

The results of Table 14 make it possible to correct for the loss of HCN caused by the presence of plant material. The loss of prussic acid seems to be caused by the presence of some substance in the seedlings because there is a relation between the age of the seedlings used and the loss of hydrogen cyanide. After these preliminary experiments it is possible to determine the quantity of HCN present with a fair amount of accuracy.

§ 5. *Experiments.*

To study the influence of different quantities of HCN on the anthocyanin formation in Buckwheat seedlings a simple apparatus has been designed. A small stand is placed in a large crystallization dish and covered with a glass-jar on short feet. On the stand two half P e t r i-dishes are placed, the highest containing paper-pulp and seedlings, the lower one contains the KCN-containing solution and a small tube placed in a slanting position and which contains 1 N hydrochloric acid. A bent glass tube connects the interior of the glass-jar with the atmosphere outside. The crystallization dish

contains paraffine oil up to a certain mark to separate the interior of the glass-jar from the outside. The bent glass tube is closed by means of a rubber tube and a pinch-cock. The small tube containing hydrochloric acid is made to slip into the KCN-containing solution and the HCN developed by the action of the acid divided through the whole volume of the glass-jar i.e. 2 Liters by pumping with a rubber-balloon.

To study the influence of HCN on the anthocyanin formation always a series of four glass-jars were used.

One glass-jar was always used as a control while the three remaining jars contained different concentrations of HCN.

We only studied the effect of HCN on the second oxidative process and in addition obtained some data on the influence of the presence of HCN on the activity of the peroxidase present in the material.

It is very difficult to obtain some evidence whether a poison like HCN only acts upon one process and does not interfere with the whole metabolism of the organism. We had the same difficulty in studying the effect of HCN on the anthocyanin formation. If fairly strong concentrations of HCN are administered to Buckwheat seedlings, for example 5 mgr HCN/1L air during seven hours they prove to have a completely lethal effect. By trying several concentrations we finally arrived at the following combination of intensity and length of the action. Samples of seedlings of about six days old were exposed to the light of a low-pressure mercury-arc lamp during 5 hours and a half and treated for half an hour in a series of concentrations of HCN ranging from 1 to 5 mgr HCN/L atmosphere. After this treatment the P e t r i-dishes with the seedlings were placed in the dark at about 20° C and the length of the seedlings and the anthocyanin-content determined after 40 hours. We used the length of the seedlings for comparison with the anthocyanin-content but later it proved not to be justified in all respects. The difficulty to find some other easily determined property as a function of the vitality of the seedlings pressed us more or less to use the length of the young plants as a measure of the condition of the seedling.

As a matter of fact it has been shown that HCN checks the elongation of the cells but does not interfere with the processes of cell-division. As the elongation-zone is situated, as we have seen before, just underneath the top of the hypocotyledon and etiolated seedlings of about six days old turn red over their whole surface on exposure to light we need not be afraid that checking one of the processes of growth directly influences the quantity of anthocyanin formed.

We therefore give the data on the length of the seedlings after treatment with different quantities of hydrogen cyanide chiefly in order to obtain some impression of the condition of the plants. The quantity of anthocyanins present in the material has been determined according to the methods described in § 3 of this chapter while we determined the quantity of HCN in the plants after a treatment of half an hour, as described above. The results of the determination of the amount of HCN present in the seedlings after remaining for half an hour in a HCN atmosphere are given in Table 15.

TABLE 15.

Age of Seedlings in Hours	HCN recovered in γ/40 Seedlings	HCN recovered in γ / 40 Seedlings corr.	HCN recovered in γ / 1 seedling. corr.	HCN recovered in γ / cc plant material. corr.	HCN added in γ / cc atmosphere
170	64	92	2.3	28.8	9.9
	59	84	2.1	26.3	
	59	84	2.1	26.3	
	59	84	2.1	26.3	
156	62	85	2.1	26.3	
			Average	Average	
			2.1	26.8	
170	30	45	1.1	14.1	4.9
	37	53	1.3	16.6	
	39	55	1.3	16.6	
156	33	45	1.1	14.1	
	35	48	1.2	15.0	
			Average	Average	
			1.2	15.3	
156	13	18	0.5	5.6	2.5
	12	17	0.4	5.3	
	10	14	0.4	4.4	
	17	26	0.7	8.1	
			Average	Average	
			0.5	5.9	

As mentioned above all the publications on the influence of some substance or another always give the concentration of the substance in the surrounding liquid or atmosphere. That this procedure (excluding the concentration within the tissue) may be considered to be inaccurate shows Figure 10 very clearly. In this graph the amounts of HCN/cc plant material and of HCN/cc atmosphere have been plotted. There seems to be a rectilinear relation between these two quantities. More important is the fact that the plant seems to ac-

cumulate the HCN as one cc plant material contains three times the quantity that is originally present in the atmosphere around the treated seedlings.

Finally the mean values of length of seedlings and the quantity

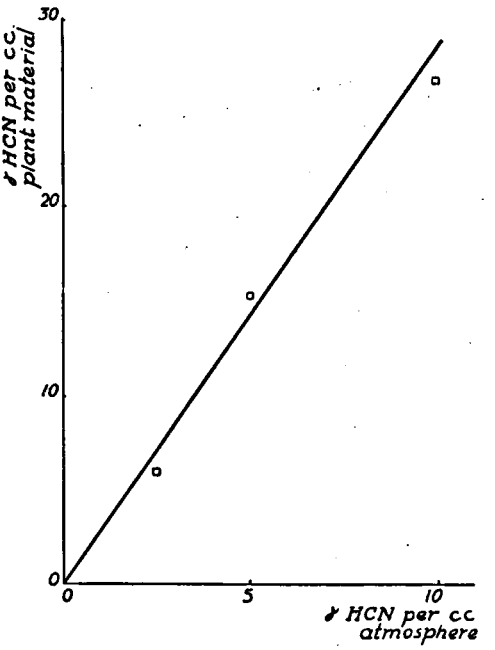


Fig. 10. Relation between the quantity of HCN present in the plant and in the atmosphere /cc.

of anthocyanin present, expressed in % of the value = 100 of the control, are given in Table 16 and Figure 11.

TABLE 16.

HCN in γ/cc plant material . . .	0	5.9	15.3	26.8
Length of Seedlings	100	95.5	92.0	85.5
Quantity of Anthocyanin	100	87.5	76.5	57.0

It is obvious that an influence of HCN upon anthocyanin formation exists; a direct conclusion is that there exists a connection between oxidative processes and the pigment formation.

In addition we investigated the influence of a similar range of HCN-concentrations on the activity of the peroxidase present in the Buckwheat seedling according to the purpurogallin method of Willstätter (1918).

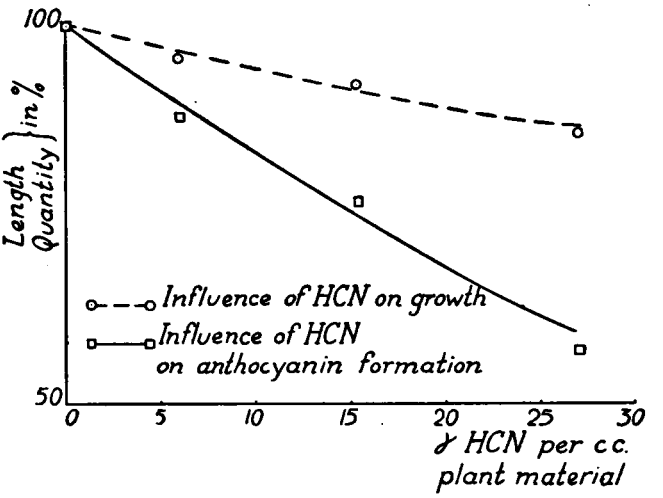


Fig. 11. Influence of HCN on anthocyanin formation and length of the seedlings.

To this purpose 380 hypocotyledons of six or seven days old were ground in a mortar and washed several times on a Buchner-funnel with distilled water. The tissue has been collected and suspended in a mixture of 76 cc of a phosphate buffer with pH 7.0 and 76 cc of distilled water. In this suspension 2 cc contained the material of 5 seedlings. In test-tubes 5 cc distilled water, 1 cc 5 % pyrogallol, 1 cc 1 % hydrogen peroxide, 2 cc of the tissue-suspension and 1 cc of a KCN solution of different concentrations were mixed. At the end of 10 minutes the reaction was checked by the addition of 2 drops of concentrated hydrochloric acid. The amount of purpurogallin formed has been estimated colorimetrically by shaking out with ether. At pH 7.0 a slight auto-oxidation of pyrogallol takes place. Blanks with the same HCN-content have been taken into consideration.

The results are shown in Figure 12 in addition with the data on the influence of HCN on anthocyanin formation. In Table 17 the results of four series are given.

TABLE 17.

HCN in γ / cc	0	1.8	3.7	7.4	14.7	29.4	58.9
Relative Quantities of Purpurogallin	100	47	41	26	12	11	8
	100	43	39	16	16	19	12
	100	64	37	28	16	13	14
	100	53	45	36	29	—	16
Average;	100	52	41	27	18	14	13

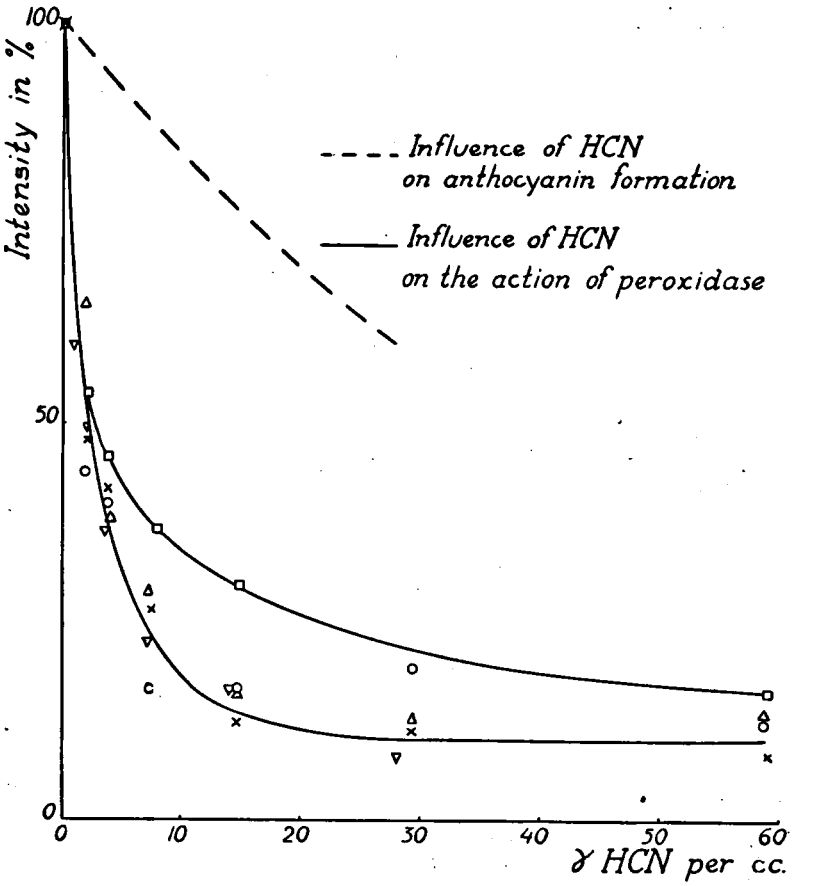


Fig. 12. Influence of HCN on the activity of peroxidase.

From Table 17 we may, moreover, conclude that HCN, even in higher concentration, does not inhibit the peroxidase entirely. The curves, shown in Figure 12 show an asymptotic relation at HCN values higher than $\pm$ 30 γ/cc.

It may be, therefore, that besides cytochrome or other metal-containing enzymative substances a small quantity of other oxidative enzymes are present.

It is clear that the influence of HCN already in low concentrations is very great, while anthocyanin formation is much less sensitive to the action of HCN.

§ 6. *Discussion.*

In this chapter we tried to obtain more information on the relation between anthocyanin-formation and oxidative processes. While we obtained qualitative data in the last chapter, in this chapter we affirmed the results of Chapter III by means of quantitative methods.

We established the connection of anthocyanin formation with oxidative processes by studying the effect of HCN, one of the well-known poisons for respiratory processes, on the pigment formation.

In one respect our data show a discrepancy with those of other authors who studied the influence of hydrogen cyanide on the respiration of several organisms. From their publications results that the quantity HCN/cc solution was in a range of dilutions about one tenth of the quantity HCN/cc atmosphere used by us. These authors, however, studied the influence of HCN on the organisms by placing those organisms into very dilute prussic acid solutions. The difference between the results might be explained in the following way;

a. we determined the amount of HCN present within the material
b. the rate of penetration in an aqueous milieu is larger than in air
c. a certain amount of HCN may be present in the intercellular spaces.

Finally the results obtained with experiments on the influence of prussic acid on the activity of the peroxidase present in the Buckwheat seedlings show that there seems to be no direct relation between peroxidase-activity and anthocyanin-formation. There remains, however, the possibility that the properties of the oxidizing enzyme might be totally different in the tissue-suspension than in the living plant.

In the next chapter the influence of a second substance, toxic to respiratory processes on anthocyanin formation, will be investigated.

CHAPTER V.

INFLUENCE OF H_2S ON ANTHOCYANIN FORMATION.

§ 1. *Introduction.*

In the last chapter we made it probable that anthocyanin formation is connected with some oxidative process as this formation is checked to a different degree by means of different concentrations of prussic acid.

In order to make certain that the influence was not characteristic for hydrogen cyanide we repeated the experiments with another poison i.e. hydrogen sulphide.

The influence of H_2S on respiration of plant material has been studied by various authors. For literature see Pop (1936).

§ 2. *Determination of H_2S.*

In the determination of small amounts of hydrogen sulphide one meets with several difficulties of which the most outstanding is the easy autoxidation of H_2S.

As we had to determine the H_2S-content in plant material we were obliged to find a method to drive out the substance from the ground plant material. For this reason the colorimetric method of Ter Meulen as described by Pop (1936) could not be applied.

We, therefore, had to use a micro-modification on the usual method (Treadwell 1930) together with all the troubles connected with this method. The fact, after all, that we did not obtain the desired results, makes us hesitate to describe the method here.

Nitrogen was purified as described in chapter III § 2, and bubbled through a small vessel (contents $\pm$ 25 cc) containing the material to be analysed. By means of a dropping-funnel acid may be added to the vessel without exposing this to the air. The nitrogen drives out the H_2S and is led through a capillary tube into a glass-tube placed in a slanting position and which tube contains a known amount of standard iodine solution. The small gas bubbles proceed relatively slowly through the iodine in order to facilitate the passing of the hydrogen sulphide from the bubble into the iodine. Back titration of the iodine-solution gives the amount of H_2S combined with the iodine.

Points to be considered are;

a. the nitrogen must be perfectly pure
Small amounts of oxygen already cause partial oxidation of the H_2S, accompanied with separation of sulphur.

b. nitrogen must be led through the H_2S containing material for a very long time in order to remove the last traces of hydrogen sulphide.

c. bubbling nitrogen through an iodine solution diminishes the concentration of the iodine.

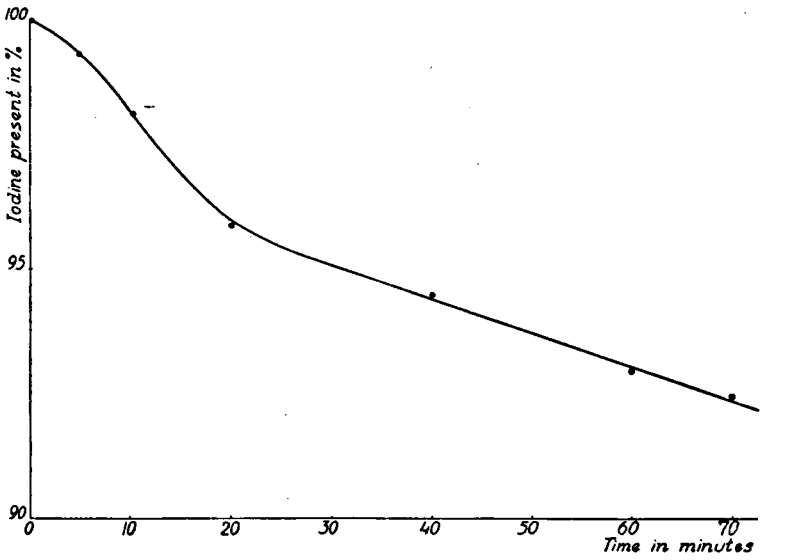


Fig. 13. Explanation see text.

In connection with the points considered above the method has to be standardised.

First of all blank experiments have to be performed on the changes in the iodine concentration caused by bubbling nitrogen at a constant rate through its solution for different periods.

The results are given in Figure 13; for our apparatus by bubbling nitrogen at a constant rate we used 5 cc of 0.0087 N iodine throughout.

In the second place the duration of washing the H_2S containing material with nitrogen to remove the last traces had to be estimated. For this purpose certain amounts of hydrogen-suphide solutions are placed in the small vessel, dilute sulphuric acid added and nitrogen passed through for a certain time. From Figure 14 we may conclude that washing nitrogen through the sulphide solution for 70 minutes is sufficient to remove all of the H_2S present. Next to

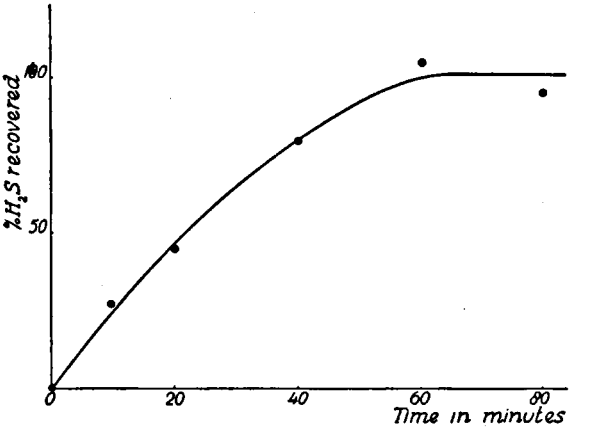


Fig. 14. Explanation see text.

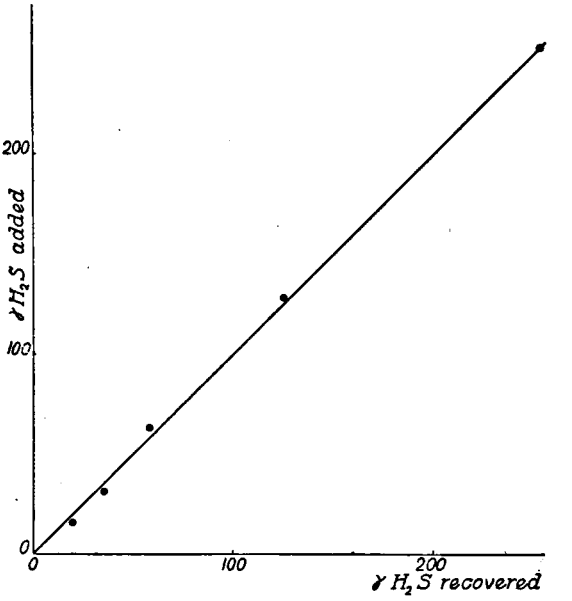


Fig. 15. Explanation see text.

this the sensitivity of the method had to be tested. Different volumes of sulphide solutions containing 127 γ H_2S/cc have been used.

The results are shown in Table 18 and Figure 15.

TABLE 18.

γ H_2S added	cc 0.0087 N iodine used	γ H_2S recovered	% H_2S recovered
254.1	1.718	254.1	100.0
127.2	0.855	126.5	99.5
63.6	0.385	56.9	89.5
31.8	0.239	35.3	111.0
15.9	0.126	18.6	117.0

It is clear that the method is only reliable in cases where more than 60 γ H_2S are present.

Finally some blanks and control determinations in the presence of plant material have been executed.

No substances were driven out by bubbling nitrogen through the plant material which combine with iodine. In the case where plant material was used in the presence of a certain amount of H_2S a great difference has been found between H_2S added and recovered (see Figure 16). It is remarkable that the angle between the theoretical and the empirical line is perfectly the same as the one in Figure 9 where HCN and plant material were distilled together. There seems to be the same substance present which combines equally well with HCN as with H_2S and which combination is very stable even in the presence of acid.

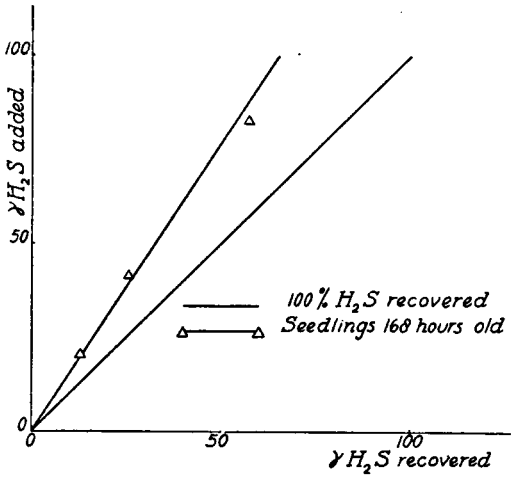


Fig. 16. Influence of plant material on the quantity of H_2S recovered.

Last of all we endeavoured to determine the amount of H_2S in seedlings which had remained for some time in a H_2S-atmosphere.

Seedlings of about 170 hours old were placed in an atmosphere containing different amounts of H_2S. To obtain a H_2S atmosphere the same procedure has been followed as described for the HCN.

The same concentration as used for the experiments of the influence of H_2S on anthocyanin formation were given to the seedlings

here. The concentrations are 4.38, 2.5 and 1.25 γ H_2S/cc atmosphere.

The concentrations were lower than in the experiments with HCN. The reason was that only very wet Na_2S was available by means of which the same concentrations have not been obtained. The time used in these experiments was 3 hours i.e. 6 × the time of exposure to HCN.

40 seedlings after exposure to H_2S were ground in a mortar with 5 drops of 1 N NaOH and a few cc of freshly boiled water. The ground material has been washed into the small vessel by means of about 10 cc of freshly boiled and cooled distilled water, 5 cc of 1 N sulphuric acid added and the H_2S driven from the acidified solution by means of a stream of purified nitrogen into 5 cc of standard iodine solution.

The result proved to be in some way disappointing. As a matter of fact practically no iodine had been used. The amount of H_2S present in the material after the described treatment seems to be too small to be determined by our method.

Still some deduction seems justified from these negative data. It means in any case that in the highest concentration originally less than 30 γ H_2S was present in the plant material. Accepting the same rate of penetration into the seedlings for HCN and H_2S we are able to verify these results.

As there exists a rectilinear relation between diffusion and the square root of time during which the diffusion has taken place we have the following relation.

In Table 19 the corresponding values are given for H_2S and HCN in the comparable concentrations used.

TABLE 19.

	γ / cc atmosphere	γ / cc plant material	ratio	time of exposure	$\sqrt{\text{time of exposure}}$
HCN	2.5	5.9	2.36	30	5.48
H_2S	2.5	y	x	180	13.42

As the ratio is the result of the diffusion we may say

$$5.48 : 13.42 = 2.36 : x \quad \text{or } x = 5.8.$$

As the ratio between the quantity of the substance in the unit volume atmosphere and that of plant material remains constant in the range of concentrations used we are allowed to multiply the quantity of H_2S in one cc of the atmosphere used by the factor 5.8. The result is for the H_2S concentrations used (4.38, 2.5 and 1.25/cc

atm. resp.) that $y = 25.4$, 14.5 and 7.3 γ/cc plant material resp.; results which very well agree with our experimental data.

§ 3. *Experiments.*

The experiments on the influence of H_2S on anthocyanin formation are executed perfectly in the same way as given in § 5 of the last chapter for the influence of HCN. Here again in a range of

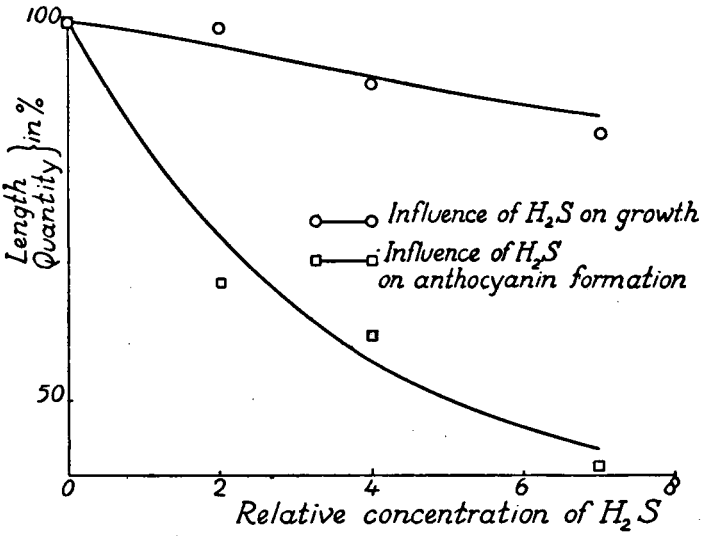


Fig. 17. Influence of H_2S on anthocyanin formation and length of the seedlings.

concentrations seedlings have been treated with H_2S and the length of the hypocotyledons and the quantity of anthocyanin determined.

For a couple of series the results are shown in Table 20 and Figure 17.

TABLE 20.

Relative concentration of H_2S	0	2	4	7
Length of Seedlings	100	99	91.8	85.2
Quantity of Anthocyanin	100	65.5	58.5	41

§ 4. *Discussion.*

Comparison of Figures 11 and 17 shows clearly that the figures represent, in general, a similar relation.

The influence of H_2S appears to be qualitatively the same as that of HCN as might be expected.

However, quantitatively, the influence of H_2S on anthocyanin formation seems to be more pronounced; the influence of H_2S on the length of the seedlings is nearly the same as that of HCN.

These data give us the certainty that anthocyanin formation has something to do with oxidative processes, particularly with those oxidative processes where heavy metals are involved.

CHAPTER VI.

THEORETICAL CONSIDERATIONS.

§ 1. *Literature.*

In the introduction we had the opportunity to discuss some of the theories brought forward on the formation of anthocyanin in plants.

In all the cases mentioned a so-called chromogen is postulated from which the pigment should be formed. These chromogens are in general substances with a related chemical structure f.i. flavones and catechins. The change from chromogen to pigment is only small and is often conceived as a simple oxidation or reduction at a certain place of the molecule.

Supposing these narrowly related substances to be the precursors of the anthocyanin pigments this supposition only shifts the difficulty.

New theories on anthocyanin formation in publications of the last years may be divided into three groups;

a. theories founded on the chemical structure of the pigment.

b. theories based on physiological experiments.

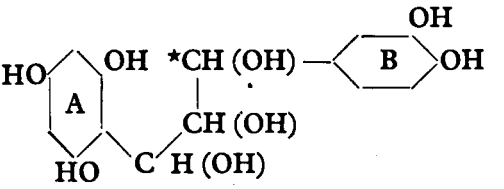
c. theories based on genetical evidence.

In the first group the following authors must be mentioned; Robinson (1921, 1936), Freudenberg (1925), Mrs Wheldale Onslow (1931, 1932) and Frey-Wyssling (1938).

In 1921 Robinson held a lecture for the British Association on the genesis of plant pigments and related substances. Only a short review appeared in the Reports of this Association. In 1936 an article of the same author appeared in Nature. His opinion in

general did not change during the period 1921—1936. Robinson considers the flavones, flavonols, anthocyanins and related substances as C_{15} systems built up as C_6-C_3-C_6. The first aromatic nucleus (A) is normally present as a derivative of trihydroxybenzene, the normal condition of the second (B) is represented by a dihydroxybenzene. The main feature of the hypothesis is that each nucleus is derived from a hexose and that these nuclei are connected by means of a triose (glycerose) by aldole condensations.

In this way a hypothetical intermediate substance is formed the formula of which is given below.



Oxidation of the -CH(OH)- (marked with an asterisk) to CO and dehydration results in the formation of cyanidin derivatives. According to Robinson the photosynthetic process would be the condensation of C_6 (B) with C_3 followed by one of $C_6(B)C_3$ with C_6 (A).

The author lays stress on the fact that many C_6—C_3 substances (in which C_6 is benzenoid) are known among natural products f.i. coniferyl alcohol.

Freudenberg (1925) gives some data on the chemical relation of catechins, flavones and anthocyanins. All these substances might be considered as derivatives of α, γ-diphenylpropane;

$$C_6H_5\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}C_6H_5.$$

From the co-existence of representatives of the three classes in the same material and the chemical constitution of these substances he draws the conclusion;

„Wo neben einander in einer Pflanze verschiedene Qxydationsstufen, also Varianten in der Kette der drei verbindenden Kohlenstoffatome vorkommen, so stimmen diese Naturstoffe in der Anordnung der Phenolhydroxyle überein".

It is not possible to obtain the certainty from Freudenberg's paper whether the three classes change from one into the other by oxidative processes or that the representatives originate independently from some common source.

In 1931 and 1932 Mrs Wheldale Onslow proposes a

very interesting hypothesis on the formation of certain pigments which will be best understood by verbal citation;

"Anthocyanin pigments are found, on the whole, in flowers, fruits, young leaves, autumnal leaves, and leaves subjected to drought and injuring .... Two fundamental conditions are characteristic, in general of organs producing these pigments as contrasted with those from which the pigments are usually absent. First, in flowers, fruits, young leaves, and autumnal leaves, photosynthesis is less than in the normal leaf, or may have ceased alltogether. This, as recent work on nitrogen metabolism has demonstrated, leads to hydrolysis of proteins and subsequent de-amination, with production of the residues of amino-acids, both aliphatic and aromatic. Secondly, in some of the organs quoted above, there is relatively little protection against loss of water, and, moreover, in the case of petals, fruits and autumnal leaves, approaching separation from the parent plant and other causes render supply of water increasingly difficult .... The deduction drawn from many observations connected with the two conditions mentioned above is that anthocyanin pigments are produced from the residues of aromatic amino-acids after de-amination. Under conditions of relative desiccation, condensation among these residues takes place with formation of anthocyanin pigments ....". .... There are three classes of aromatic compounds which may be synthesised from such residues, the flavone pigments, the anthocyanin pigments, are universally distributed in the tissues under all conditions. Hence it is likely that they are products of de-amination and condensation under the usual conditions of growth and development. Anthocyanin pigments are only formed under special conditions. Finally, the tannins only arise in a certain type of plant.

Upon the precise reactions of condensation leading to the formation of anthocyanin pigments it is idle to speculate. The phenyl ring, we would assume, has its origin from tyrosine or phenyl-alanine. The phloroglucinol ring, possibly, from short-chained residues of aliphatic amino-acids; or; possibly, from hexose. By oxidation, either by enzymes or chemical agents, hydroxyl groups are readily introduced into the phenyl ring in a position ortho to that of an existing group".

From the above two points are worth to emphasise i.e.;

1. the de-amination residues of amino acids are used in the plant for the synthesis of flavones, anthocyanins and tannins
2. each of these products originate directly from these residues.

It seems certain that Frey-Wyssling (1938) has not seen the publications of Mrs Wheldale Onslow; in any case he does not mention her work in a short article on the origin of secundary plant-substances. The hypothesis of this author is perfectly the same as the hypothesis of the de-aminated amino acids of Mrs Wheldale Onslow. The only difference is that Frey-Wyssling considers the de-amination residues as the source for all sorts of plant substances.

Frey-Wyssling states in this article;

„Es wäre unstatthaft eine so weitgehende chemische Hypothese auf-zustellen, wenn nicht gewichtige physiologische Gründe für die hervorragende

Rolle sprechen würden, welche die desaminierten Aminosäuren im pflanzlichen Stoffwechsel spielen müssen".

By this statement it is difficult to classify Frey-Wyssling's hypothesis. As, in relation to the anthocyanin pigments, the theory is more chemically than physiologically founded we discuss his work here.

Frey-Wyssling states further;

„Nicht nur aus den sich differenzierenden Meristemen, sondern auch aus den Blütenblättern wandert der Stickstoff aus, während der Kohlenstoff zurück bleibt. Die Eiweisspaltung setzt vielfach schon mit dem Aufblühen ein. Es ist deshalb eine naheliegende Hypothese, die Riech- und Farbstoffe der Blüten als die umgewandelten Desaminierungsprodukte der Aminosäuren zu betrachten deren Kohlenstoffgerüst wir in diesen Blütenstoffen finden".

In connection with the formation of flower pigments the author points to the results of Schumacher's work (1932) on the breakdown of the proteins that often starts during the opening of flowers.

From the data of Schumacher we come to different conclusions. In the first place the number of flowers examined where a decrease of the protein-content starts during the opening, is small in relation to the cases where flowers have been used in a full-grown condition. In addition the opening flowers used in these experiments are flowers of Cactaceae which are known for their short life.

Apart from this point it is clear that we are not allowed to draw any conclusions from the work of Schumacher in connection with pigment-formation as anthocyanin pigments are in the majority of cases already formed in the bud. As Schumacher did not analyse growing buds during pigment formation it is clear that it is dangerous to draw conclusions from his work in favour of Wheldale Onslow's and Frey-Wyssling's hypothesis. Investigations of this matter with a view to pigment formation might give, meanwhile, interesting results.

b. based on purely physiological work are the considerations of Kuilman (1930) on the processes involved in anthocyanin formation.

In the second part of his thesis Kuilman describes the influence of some factors like temperature and light on the pigment formation in Buckwheat seedlings.

From his experiments the following points are to be considered;

1. at low temperature (5° C) after exposure to light the seedlings develop much anthocyanin but slowly; after some time the amount of pigment remains constant.

2. at higher temperature (20° C, 35° C) the anthocyanin pigment appears more quickly, the quantity is less than by lower temperature, and after course of time the amount of anthocyanin decreases.

Together with the fact that anthocyanin is developed in the seedlings in the dark after exposure to light the above mentioned phenomena are explained by Kuilman as follows.

Apart from the photochemical reaction two reactions take place in the anthocyanin formation.

During the illumination, apart from the photochemical reaction, some reaction takes place. Kuilman observed that by illumination at different temperatures different amounts of anthocyanins are formed.

In the second reaction some chromogen is transformed into anthocyanin. Beside this reaction a third reaction takes place, in which the chromogen is transformed into a substance different from anthocyanin.

Summarizing we come to the conclusion that after the photochemical reaction two chemical reactions take place in the formation of the anthocyanin pigments of Fagopyrum seedlings. Finally a third reaction takes place, particularly at higher temperature by which the quantity of chromogen available for anthocyanin formation decreases.

c. In 1935 a publication of Lawrence and Scott-Moncrieff appeared on the genetics and chemistry of the flower colour in Dahlia. In this article the authors investigated the flower pigments of the Dahlia and arrived at the following opinion;

"In Dahlia the subtle balance in the formation of both flavones and anthocyanins points to these pigments all being produced through some common fundamental chemical reaction, or from some limited common source from which all four pigments (i.e. two flavone and two anthocyanin pigments) are at least in part derived, and for which they all compete. The supply of this hypothetical source must be so limited that the quantity of each pigment derived from it can only increase at the expense of the others, and depends upon the number, nature and balance of all the dominant pigment factors present, and upon the relative claims to existence made by the pigment formed, as demonstrated by their specific interaction effects".

From the opinion of Lawrence and Scott-Moncrieff on the relation of flavones and anthocyanins it is apparent that the one class is not formed by transformation of representatives of the other, but both sorts of pigments are derived from one mother substance.

§ 2. *General considerations on anthocyanin formation.*

In general we may be certain that biochemical reactions take place in such a way that first of all the substances to be transformed become fixed on the surface of some colloidal substance after which by the action of enzymes the chemical transformation take place. As the enzymes themselves are often colloidal substances these substances may act at the same time as substrate for the reactions. In connection with this point of view one may expect that the principal reactions take place in the protoplasm of the plant cell or at the boundary-layer between protoplasm and cell-sap. It is hardly possible that important chemical reactions take place in the cell-sap. The cell-sap which in many cases is a homogeneous system the number of different molecules and ions is so great that a reaction in a special direction is hardly feasible.

As a matter of fact it is conspicuous that many important biochemical reactions take place in more solid centra of the protoplasmic layer the so-called plastids. For the formation of anthocyanins it was originally thought that special plastids were able to form anthocyanins (Politis 1923). Guilliermond (1913, 1931, 1933) was of this opinion but later, influenced by the work of Dangeard (1922) he changed his views. At present it seems to be certain that anthocyanin when formed in very young cells develops in the so-called vacuome, part of the protoplast, from which, by enlargement, the vacuoles arise. In any case it seems to be sure that anthocyanin pigments are formed in some heterogeneous system. Further information is to be obtained from physiological work on anthocyanin formation.

First of all we have to consider the photochemical reaction involved in the pigment formation.

It is a well-known fact that light has a great influence on anthocyanin formation. In the majority of cases light is necessary for the appearance of the colouring matter. There are, however, many exceptions. It is remarkable that in the case of these exceptions, always very large quantities of reserve food are present from which some substance necessary for pigment formation may arise.

Experiments on the influence of light of different wave-length on anthocyanin formation gives some evidence according to the photo-chemical reaction. The first photochemical law, the Grotthus-Draper law states that only light which is absorbed by the reacting system can produce chemical change. From the work of Magness (1928), Pearce and Streeter (1931), and Arthur (1932) on the effect of light of different wave-length on the formation of the red pigment of apples we may learn something.

From the experiments of these authors who only gave qualitative data it becomes clear that wave-lengths larger than 7000 Å do not have any effect, that visible light up to 5000 Å is of very little value for the formation of anthocyanins, finally that blue, violet and ultra-violet light up to 2900 Å (the solar limit) produce considerable quantities of the pigment. The best effect gave ultra-violet rays close to the solar limit. Light of shorter wave-length than 2900 Å proved to be very injurious. From these results we may conclude that a substance absorbing light in the blue, violet and ultra-violet range

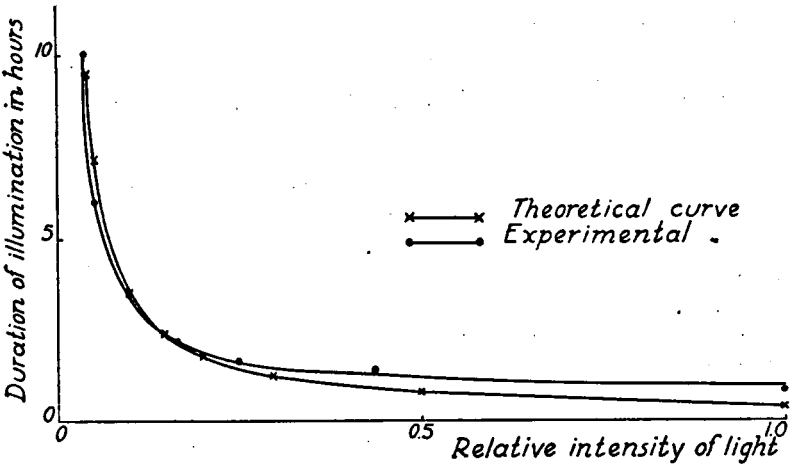


Fig. 18. Relation between intensity of light and duration of illumination necessary for the formation of a certain amount of anthocyanin pigment.

is connected with anthocyanin formation. This substance is probably yellowish or colourless.

As to the second law which demands that a photochemical reaction is proportional to the amount of light energy absorbed very little experimental work has been done.

From the data of L i n s b a u e r (1908) who studied the influence of the combination of duration and intensity of the illumination on the first appearance of anthocyanin pigment in etiolated Buckwheat seedlings we are able to calculate that the product of duration and intensity of the illumination is constant, which is shown by the hyperbolic shape of the curve obtained.

In Figure 18 the data of L i n s b a u e r are given in comparison

with the theoretical hyperbola. The difference is very small indeed and proves that a photo-chemical process really exists. More data are, in the mean time, badly needed on this point. As to the processes which follow the photo-chemical reaction data are available leading to the same conclusion and obtained by perfectly different methods given by Kuilman (1930) and those described in the second paragraph of chapter III.

Kuilman concluded by his experiments on the influence of the temperature on anthocyanin formation that two processes must be necessary present.

1. a chemical reaction during illumination
2. a chemical reaction after the illumination.

We came to the same conclusion by exposing seedlings to light in nitrogen or in ordinary air and by changing the nitrogen or air in different ways for the period after illumination.

In addition we proved that the two processes which occur during the illumination and the dark reaction are oxidative processes.

We are able to give still more details on the processes of anthocyanin formation.

In the eighth paragraph of chapter II we gave some data on the development of anthocyanin pigment in Buckwheat seedlings in the dark after illumination for a certain time. A glance at Figure 6 is sufficient to obtain the impression that these curves represent the progress of a monomolecular reaction.

A monomolecular reaction is a reaction during which one sort of molecules is transformed into other molecules f.i.

$$A = B + C.$$

For this sort of reactions the following equation holds;

$$K = \frac{1}{t} \, l \, \frac{a}{a - x}$$

in which K = constant
t = time
a = number of molecules of A originally present
x = number of molecules of A transformed at time t.

In our case a and x are not known but we know that at the end of the reaction b molecules of substance B have been formed. At time t a number of y molecules of B is present.

Suppose that our reaction is monomolecular.

In the second place we suppose that the number of molecules of substance A (= a) before the reaction starts is equal to the number

of molecules of substance B (= b) formed at the end of the reaction; follows that a = b.

At the time t a number (a — x) of A is not yet transformed and a number (b — y) of B is not formed while at the other hand x of A has been transformed and y of B has been formed.

It is clear that x = y, and a — x = b — y. This discussion may be illustrated by Figures 19 and 20.

It is obvious that the curve, obtained by plotting the number of molecules of B formed against the time, is the reverse of the curve

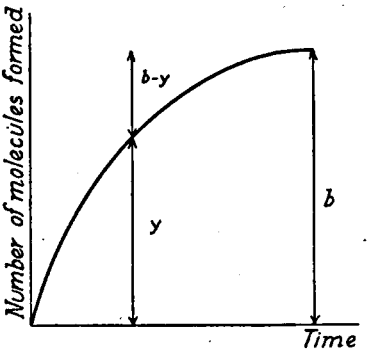


Fig. 19. Explanation see text.

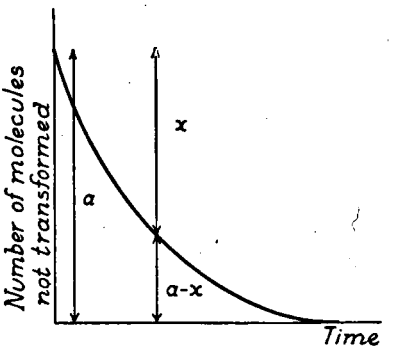


Fig. 20. Explanation see text.

to be obtained by plotting the number of molecules of substance A to be transformed against the time.

To prove that the bundle of curves of Figure 6 represents the progress of monomolecular reactions we must plot the time against $l \frac{b}{b-y}$. In the case of monomolecular reactions straight lines should result.

In plotting our data in this way we really obtain a straight line which proves that a monomolecular reaction plays a part somewhere in the process of anthocyanin formation in illuminated Buckwheat seedlings (see Figure 21).

A possibility for a monomolecular reaction in the process of anthocyanin formation gives the following consideration. In a great number of cases biochemical pigments are fixed on a substrate from which the pigment may be dissolved by suitable solvents. As anthocyanin pigments are soluble in dilute acids the possibility exists

that at the end of the second oxidative process by which a colourless chromogen is changed into the coloured pigment (by rearrangement

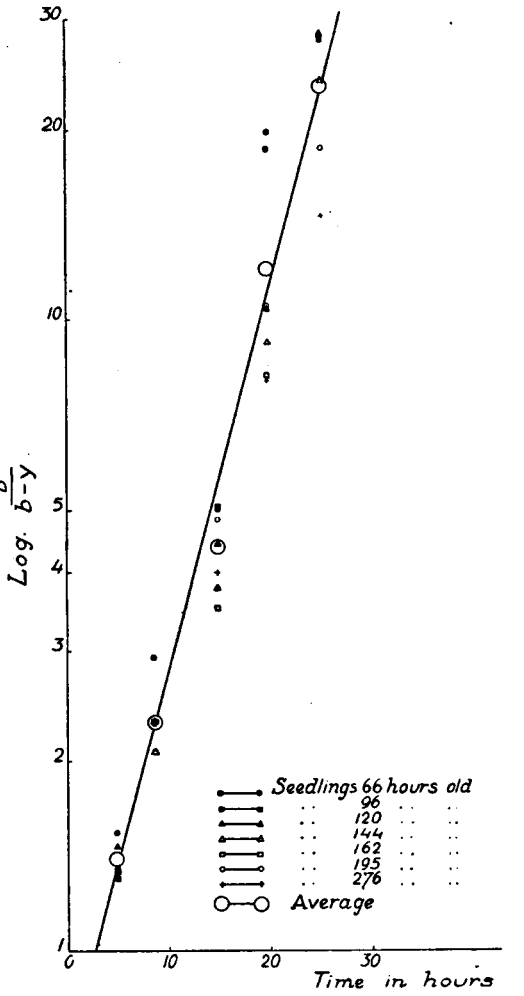


Fig. 21. Explanation see text.

of double bonds) this pigment is pushed off from the substrate and dissolved in the cell-sap. This hypothesis is in accordance with the

cytological processes as described above. This process is clearly a monomolecular reaction [1].

This investigation was carried out 1934—1938 at the Botanical Institute of the Government University of Leyden, Holland, Director Prof. Dr. L. G. M. Baas Becking.

At the end of this period I want to express my most sincere thanks to Professor Baas Becking for all he has done for me.

[1] At this place I should like to thank Mr. J. E. Bevelander for the way he executed most of the drawings.

SUMMARY.

In the foregoing lines we have tried to elucidate some points in the chaotic topic of anthocyanin formation.

We, therefore, studied the processes in anthocyanin formation in different directions in one object. We choose the etiolated seedlings of Fagopyrum esculentum M o e n c h, a material readily available in every season.

The disadvantages as well as the advantages of the material used are described in full in chapter I, § 1 and in chapter II, § 8.

The chemical composition of the anthocyanin present has been partly elucidated. The same is true for the tannin, present in large quantities.

We could not confirm the statement of J o n e s c o (1930) on the presence of leuco-anthocyanins in Buckwheat seedlings. As a matter of fact we are sure that the so-called anthocyanidin derived from the leuco-anthocyanin is a coloured product obtained from the tannin by the action of mineral acids (chapter I, § 3).

The results of the chemical investigation added to microchemical observations lead to the opinion that there certainly does not exist any physiological relation between anthocyanins and tannins (chapter II, § 9). Of other substances looked upon as possible chromogens of anthocyanin pigments, we investigated the flavones. In the material used flavones could never be demonstrated (chapter I, § 4).

The localisation of certain substances present in the material and studied by means of microchemical methods proved to be interesting. There seems to exist a very narrow relation between the presence of reserve food and anthocyanin formation in the hypocotyledons of Buckwheat seedlings. A narrow relation exists between development of the seedling and starch and sugars present. At the same time a similar relation exists between the presence of sugars and anthocyanin. The result is that there exist a relation between the age of the seedling and the quantity of anthocyanin to be formed (chapter II).

By physiological methods we could obtain some information as to the processes involved in anthocyanin formation. Apart from the photo-chemical reaction we could prove that two oxidative processes are present during the pigment formation, the first one of which follows the photo-chemical reaction very closely, the second takes

place in the dark after the exposure to light. We added more data to the knowledge obtained by studying the influence of characteristic respiratory poisons i.e. HCN and H_2S. (Chapter III, IV and V).

These poisons proved to have a marked influence on the formation of the anthocyanin pigment.

Micro-methods to determine the quantities of hydrocyanic acid and hydrogen sulphide present in the plant material after staying in an atmosphere containing these substances were worked out (chapters IV and V). A number of oxidizing enzymes proved to be present in the material. The influence of HCN on the activity of one of these enzymes has been studied quantitatively (chapter IV).

In chapter VI the literature, appeared in the last years, has been discussed and some general considerations on the reactions involved in anthocyanin formation added.

We came to the following conclusions;

a. a photo chemical reaction starts the chain of reactions involved in anthocyanin formation.

b. favourable conditions for this photo-chemical reaction are, for instance, the presence of carbohydrates.

c. the substance used in the photo chemical process has either a yellow colour or will be colourless.

d. two oxidative processes are necessary.

e. the reactions take place while the substances being transformed are fixed on a substrate.

f. the pigment formation may involve a monomolecular reaction.

g. this monomolecular reaction takes place during the loosening of the pigment from the substrate.

h. the pigment after loosening from the substrate dissolves in the acid cell-sap.

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