Decolorization of Anthocyanins by Fungal Enzymes

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Several crude fungal enzyme preparations exert a significant decolorizing effect on extracts of pigments derived from berry fruits. This effect was studied in detail, within a range of pH 3.0 to 4.5, using a sample of a pure anthocyanin chloride isolated from blackberry as the principal substrate. The over-all decolorization process involves an enzymatic hydrolysis of the anthocyanin to anthocyanidin and sugar, and a spontaneous transformation of the aglucone into colorless derivatives.

THE POSSIBLE OCCURRENCE of an enzyme-catalyzed decolorization of anthocyanins was first brought to the author's attention by Meschter (*11*). He found that treatment of strawberry extracts with large doses of Pectinol, a commercial pectic enzyme preparation of fungal origin (Rohm & Haas Co., Philadelphia, Pa.), resulted in a significant loss of color, and that similar, but less pronounced, effects could be obtained with pigment extracts of other common berries.

The author has confirmed these observations. Results indicate that the decolorizing activity of Pectinol itself is such that, at the enzyme concentrations recommended, the amount of color loss which might be incurred during a normal clarifying operation (*26*) on a highly pigmented berry juice—e.g., grape or blackberry—would hardly be noticeable (*8*). However, in preliminary experiments using other fungal enzyme preparations, it was found that several derived from aspergilli, particularly one from a strain of *Aspergillus niger* and one from an unidentified aspergillus, contained a much higher order of anthocyanin-decolorizing activity. The availability of active preparations and the possibility that a specific anthocyanin-decolorizing enzyme might be involved prompted a detailed investigation of the nature and mechanism of the decolorization phenomenon.

Materials and Methods

The pigment extracts (kindly supplied by E. E. Meschter) of strawberry, red raspberry, black raspberry, cranberry, blackberry, and grape had been prepared by the procedure developed by Sondheimer and Kertesz (*23*) for the isolation of pelargonidin-3-β-monoglucoside from strawberries, and represented aqueous solutions of the material precipitated by the addition of ether to an ethyl alcohol solution of the original 1-butanol concentrate. A sample of anthocyanin chloride was obtained from the aqueous pigment extract of blackberry, *Rubus fructicosus*, by continuing the above isolation procedure to the chloride stage —picration of the aqueous extract, solution of the picrate in 5% hydrochloric acid in ethyl alcohol, and precipitation of the chloride with ether.

Table I. Decolorization of Chrysanthemin by Fungal Preparations

Preparation from Code No.	Organism	% Loss in Absorbance in 30 Min.
CN 558	*Aspergillus* (unidentified)	65
CN 424	*A. niger*	25
CN 72	*A. niger*	67
CN 36	*A. oryzae*	3
CN 595	*A. parasiticus*	4

To 1.03μM chrysanthemin chloride in 5 ml. 0.05M lactate buffer at pH 3.95 was added 1 ml. of a solution containing 1 mg. of the fungal preparation. A control tube was run to which 1 ml. of water was added. The tubes were incubated at 30° C. and readings of the absorbance at 510 mμ were made using lactate buffer as the blank. The initial absorbance in all the tubes was 0.68.

The crude fungal enzymes employed in this study were prepared from surface cultures of a series of aspergilli (Table I), and are identified by the code number of the organism from which the enzyme was derived. The typical growth medium consisted of 2 parts of wheat bran, 4 parts of middlings, and 7 parts of water. The medium was inoculated with a spore suspension of the organism grown on damp oat hulls, and then incubated at 30° C. for 70 to 80 hours until abundant mycelial growth was obtained. The whole culture was dried thoroughly in air, ground to a fine powder, and then extracted with twice its weight of water. The aqueous extract was cooled to 0° C. and sufficient ethyl alcohol at about 5° C. was added with stirring until the final ethyl alcohol concentration was approximately 87%. After standing for several hours, the precipitate was collected by centrifugation, and dried in air. The resultant dry solid containing active enzymes was stored in the refrigerator. For decolorization experiments the solid material was weighed out, dissolved in water, made up to the desired volume, and, if necessary, filtered before use.

The β-glucosidase activity of the emulsin (sample obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio) used was such that in a test system containing 10 mg. of salicin in 4 ml. of 0.08M acetate buffer and 1 ml. of enzyme solution at pH 5.0 and 37° C., each milligram of material liberated 75 γ of glucose in 10 minutes.

The decolorization experiments were carried out at 30° C. and within a pH range of 3.0 to 4.5. The pigment solutions were made up in a sodium lactate buffer or in Sorensen's citrate buffers. In most experiments, 1 ml. of the enzyme solution was mixed well with 5 ml. of the substrate solution in a 12.5 × 125 mm. optically calibrated test tube. The tube was stoppered and the absorbance of the solution at 510 mμ was read immediately in a Coleman Junior spectrophotometer against an appropriate buffer blank. The tube was then incubated in a constant temperature bath maintained at 30° ± 0.05° C. At selected time intervals, the tube was removed from the bath and the absorbance of the reacting solution at 510 mμ was again measured. Although the aqueous pigment extracts employed were readily soluble in the buffers utilized, the solid samples of blackberry anthocyanin chloride were not. It was found desirable to prepare a concentrated stock solution of this pigment in 0.01% hydrochloric acid, from which suitable dilutions were made using the selected buffer solution.

When the stock solution was mixed with buffer, the intensity of the color was seen to decrease rapidly, until in 5 to 10 minutes a stable equilibrium intensity value was reached. Because such an operation involves not only a dilution

of the pigment solution, but also a change in the pH of the medium, which has a profound effect on the quality and intensity of the color of the anthocyanins in solution, this observation may be largely attributed to interaction between pigment and hydrogen ion (*22*). In all these experiments the buffered substrate solution was, therefore, permitted to stand at room temperature for 30 to 60 minutes before it was utilized.

Paper chromatography was performed in all cases on sheets of Whatman No. 1 paper using the descending one-dimensional technique at 23° to 28° C. Chromatograms of anthocyanin pigments were developed with the upper phase of a mixture of 1-butanol–acetic acid–water (4:1:5 by volume) according to the procedure of Bate-Smith (*2*). Phenolic compounds were also examined with the above solvent as described by Bate-Smith and Westall (*3*), and the spots were rendered visible with 0.2% ferric chloride or an ammoniacal silver nitrate reagent. Two solvent systems were used for the identification of sugars: the organic phase of a mixture of ethyl acetate–acetic acid–water (3:1:3 by volume) (*5*), and a mixture of 1-butanol–pyridine–water (6:4:3 by volume) (*4*). The chromatograms after development and drying were sprayed with ammoniacal silver nitrate (*17*), dinitrosalicylic acid (*4*), or *m*-phenylenediamine (*4*) in appropriate solvents and incubated in an oven as directed. The dinitrosalicylic acid reagent was particularly satisfactory for the routine identification of sugars.

Glucose was determined colorimetrically by a modification of Somogyi's micromethod (*27*), using the chromogenic reagent of Nelson (*14*).

Isolation of Chrysanthemin Chloride from Blackberry

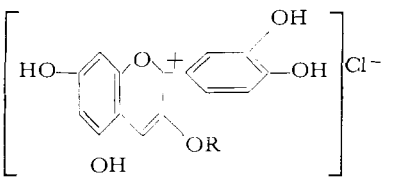
All the pigment extracts were analyzed by paper chromatography as solutions in 1% hydrochloric acid (*2*). Each extract contained at least two pigment components. Because blackberry extract was available in larger amounts than the other extracts, it was decided to isolate a pure anthocyanin from it for use as the principal substrate in later experiments. The pigment in the extract was converted to the picrate and subsequently to the chloride, as described by Sondheimer and Kertesz (*23*). The solid chloride obtained was similar in appearance and in chromatographic behavior to the sample of blackberry anthocyanin chloride. It was resolved into a major component (R_f = 0.29) and a minor component (R_f = 0.38) on the paper chromatogram. The material was further purified by repeating picration and chloride precipitation. The progress of purification was conveniently followed by paper chromatography, as with each operation the amount of minor component in the material was visibly decreased. After three cycles of the purification procedure, a solid amorphous pigment chloride was obtained which traveled as a single component on the paper chromatogram.

When a solution of the pigment in a 1 to 1 mixture of anhydrous methanol and concentrated hydrochloric acid was permitted to evaporate slowly at room temperature, clusters of tiny, violet-red, elongated, rhombic leaflets were obtained. These crystals, therefore, appear to bear a closer resemblance to those of chrysanthemin chloride from *Chrysanthemum indicum* L., as reproduced in a microphotograph by Willstätter and Bolten (*28*), than to those of the anthocyanin chloride isolated by Karrer and Pieper (*6*) from the wild and garden varieties of blackberry. An air-dried sample gave carbon, 50.3, hydrogen, 4.8, and loss of weight upon drying in vacuo at 80° C., 4.0% (calculated for $C_{21}H_{21}O_{11}Cl.H_2O$: C 50.2, H 4.6, and H_2O 3.6%).

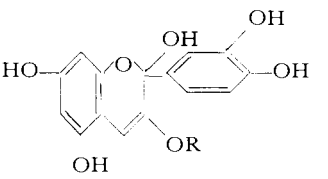
Both the noncrystalline and crystalline samples gave an R_f value of 0.29 when solutions in 1% hydrochloric acid were developed chromatographically on filter paper at 23° C. The R_f value given by Bate-Smith (*2*) for cyanidin-3-monoglucoside in the same system is 0.30. The purified anthocyanin chloride also showed all the color properties and reactions (*13*, *28*) attributed to chrysanthemin chloride.

Hydrolysis of the anthocyanin chloride in boiling 20% hydrochloric acid led to the isolation of a crystalline anthocyanidin chloride. When analyzed chromatographically on paper as a solution in 1% hydrochloric acid, the aglucone traveled as a single spot with a R_f value of 0.72 to 0.73. This substance exhibited all the characteristic color properties and reactions which are recommended for the identification of cyanidin in plant materials (*18*). The mother liquor from the hydrolysis was evaporated to dryness in vacuo. The residue was taken up in water and then examined by paper chromatography using two different solvent systems (*4*, *5*). Only one sugar was present in the aqueous solution and was shown to be identical with glucose.

These results confirm the observation of Karrer and Pieper (*6*) that the principal pigment of blackberry is a cyanidin-3-monoglucoside. It was, therefore, assumed in the present work that the material isolated is chrysanthemin chloride, which may be represented by expression I. The cation is probably a resonance hybrid of two principal structures: I, and a corresponding expression in which the positive charge is placed on position 4 (*9*).



I (R = β-glucosidyl)
III (R = H)



II (R = β-glucosidyl)
IV (R = H)

Absorption spectra of the anthocyanin in aqueous solution at pH < 1 and 3.95 are presented in Figure 1. Both curves exhibit a maximum at 510 mμ, which was accordingly chosen as the standard wave length for following the course of the decolorization reaction in subsequent experiments.

Preliminary Decolorization Data

The results of preliminary experiments on the decolorization of blackberry anthocyanin by a series of crude enzyme preparations derived from aspergilli are summarized in Table I. CN 558 and CN 72 are the most effective decolorizing agents. That such activity is not restricted to chrysanthemin as the specific substrate is suggested by Table II, in which the susceptibilities of a number of anthocyanin extracts from berry fruits toward decolorization by CN 558 are compared. These data indicate

Table II. Decolorization of Berry Fruit Anthocyanin Pigments by CN 558 at 30° C.

	Loss in Absorbancy, %					
	Strawberry	Red raspberry	Black raspberry	Cranberry	Blackberry	Grape
1 hour	23	21	8	13	82	20
19 hours	44	35	22	31	82	43

To 5 ml. of the pigment extract in 0.045*M* lactate buffer at pH 3.95 was added 1 ml. of a solution containing 1 mg. CN 558. Controls were run for each pigment using 1 ml. of water in place of the enzyme solution. Readings of absorbance at 510 mμ were made using lactate buffer as the blank. The pigment extracts were diluted with buffer until the absorbance in the color tubes at the start of the incubation period was within a suitable range for photometric measurements—i.e., from 0.60 to 0.80.

that all the pigment extracts listed in Table II are decolorized, although the degree of decolorization may vary considerably from one extract to another.

A very slight loss in the absorbance of the control tubes, amounting to about 1% of the initial value in 1 hour and not more than 5% in 19 hours, was noted for all the pigments studied. This spontaneous color loss is presumably a reflection of the inherent instability of the anthocyanin pigments at the pH level employed (*12*). In both series of experiments, the decrease in color density was not accompanied by any perceptible evidence of precipitate formation during the first few hours of incubation. When the incubation was continued for 19 hours—i.e., overnight—traces of pigmented sediment were visible in tubes containing extracts of black raspberry, cranberry, and grape. Thus, decolorization can occur independently of pigment precipitation, although precipitation of pigment may follow decolorization in certain cases, indicating that the two observations are interrelated.

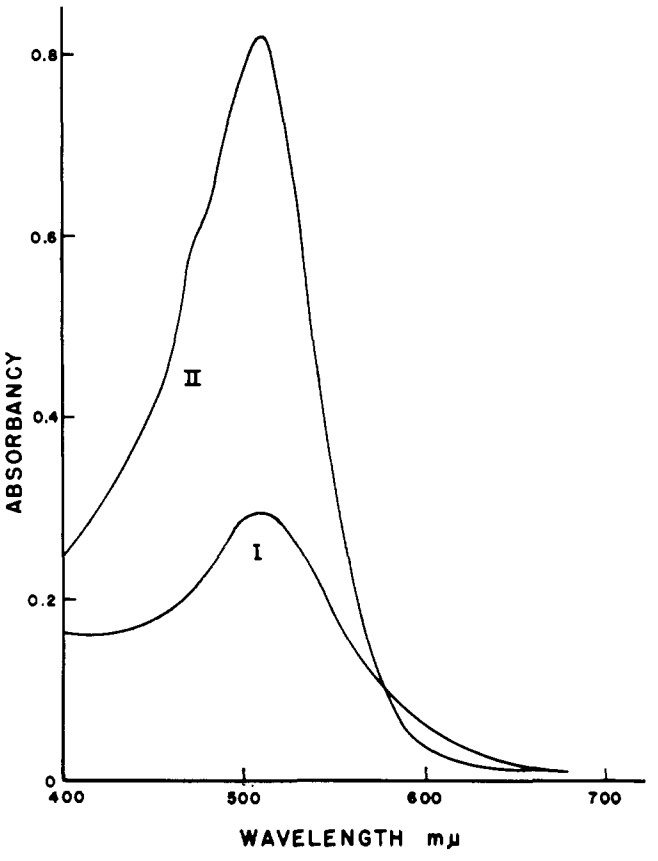


Figure 1. Absorption spectra of anthocyanin isolated from blackberry

I. pH 3.95
II. pH <1

Pigment concentrations. I, 7.44 × 10^{-5}M chrysanthemin chloride in 0.045M sodium lactate buffer; II, 3.72 × 10^{-5}M chrysanthemin chloride in 1 to 1 mixture of buffer and 1.6N hydrochloric acid. Absorbances read in 12.5 × 125 mm. optically calibrated test tubes in Coleman Junior spectrophotometer.

Nature of Decolorizing Activity

Further experiments were carried out using CN 558 as the decolorizing agent and the chrysanthemin chloride isolated from blackberry as the substrate. To ascertain the nature of the decolorizing activity, the effect of various factors which might influence this activity was studied. Data on the effect of pH on the extent of decolorization are summarized in Table III.

Although the anthocyanin concentrations at all four pH levels are identical, the initial absorbance value decreases as the pH is increased. This observation is consistent with the view that the anthocyanin in solution exists as an equilibrium mixture of a colored cation, I, and a colorless "pseudobase" form, II (*22*). The point of immediate concern, however, is that the data in Table III are adequate to demonstrate that the decolorizing activity is definitely pH-dependent. When the absolute loss of absorbance is considered, the activity is highest at pH 3.50, while on the basis of a percentage loss in absorbance, the reaction is most rapid at pH 3.95. The decolorizing activity is operative over a pH range of 3.0 to 4.5, within which the pH values of most natural fruit juices are found (*20*).

The course of a typical decolorization reaction is illustrated by a plot of absorbancy versus time in the lowest curve (solid circle) in Figure 2. When a residual absorbance value, amounting to approximately 9% of the original, is reached, no further decolorization occurs. The same curve could apply to data in experiments in which the CN 558 solution had been dialyzed at 10° C. for 16 hours in running tap water prior to use. However, when the solution was held at 80° C. for 15 minutes the decolorizing activity was completely lost. Less severe heat treatment caused correspondingly less destruction of activity. Thus, the activity of the catalytic agent responsible for the decolorization process is pH-dependent, nondialyzable, and heat-labile, and is consequently enzymic in nature. Furthermore, the results of dialysis experiments indicate that no activators or inhibitors are present in the crude preparation.

Using the conditions pertaining to the lowest curve, Figure 2, no gas exchange was observed during decolorization in 1 to 2 hours, when the experiments were performed in the Warburg constant-volume respirometer (*24*) at 30° C. However, upon prolonged incubation subsequent to complete decolorization, a very slow uptake of oxygen was evident. Although the rate of oxygen utilization was too slow to be measured conveniently the amount of oxygen consumed in 24 hours was suggestive of extensive oxidation of the anthocyanin molecule. Shaking of the reaction tube in air during decolorization had no effect on the course of color loss, as shown by the lowest curve in Figure 2. These data indicate that the actual decolorization process does not involve oxygen, although the end product of this process may be more susceptible to attack by molecular oxygen than the original anthocyanin.

Data on the effect of enzyme concentration on decolorization rate are presented in Figure 2. The curves show that the extent of decolorization in a given time interval of reasonable length increases with increasing enzyme concentration. The actual relationship between enzyme concentration and the rate of decolorization, however, cannot be formulated until the kinetics of the decolorization process have been satisfactorily interpreted. These aspects of the problem are under investigation. Nevertheless, the shape of the decolorization curves in Figure 2 shows that for concentrations of CN 558 greater than 0.1 mg. per tube, the loss in absorbance for 0 to 10 minutes is significantly less than that for 10 to 20 minutes. Similar curves were obtained when the decolorization data from experiments with CN-72 and CN 424 were treated in the same way. Apparently, the rate of decolorization increases from an initial value to a maximum, and then falls off gradually to zero. This suggests that the decolorization of anthocyanin occurs via an intermediate, the concentration of which is the immediate rate-determining factor in the over-all decolorization process. The concentration of this colored intermediate may be expected to increase from zero to a maximum steady state value before it gradually declines.

Properties of Decolorized Material

In weakly acid or neutral media anthocyanins and anthocyanidins are present, to a greater or lesser extent, in colorless modifications known as pseudobases (*27, 29*) which are believed to have the carbinol structures exemplified by III and IV. These pseudobases are reconverted into the highly colored cations,

Table III. Effect of pH on Decolorization of Chrysanthemin Chloride by CN 558

pH ± 0.02	3.00	3.50	3.95	4.50
Absorbance at 510 mμ				
0 min.	1.57	1.32	0.85	0.47
60 min.	1.19	0.28	0.11	0.08
Loss in absorbance in 60 min.	0.38	1.03	0.74	0.39
% loss in absorbance in 60 min.	24	79	87	84

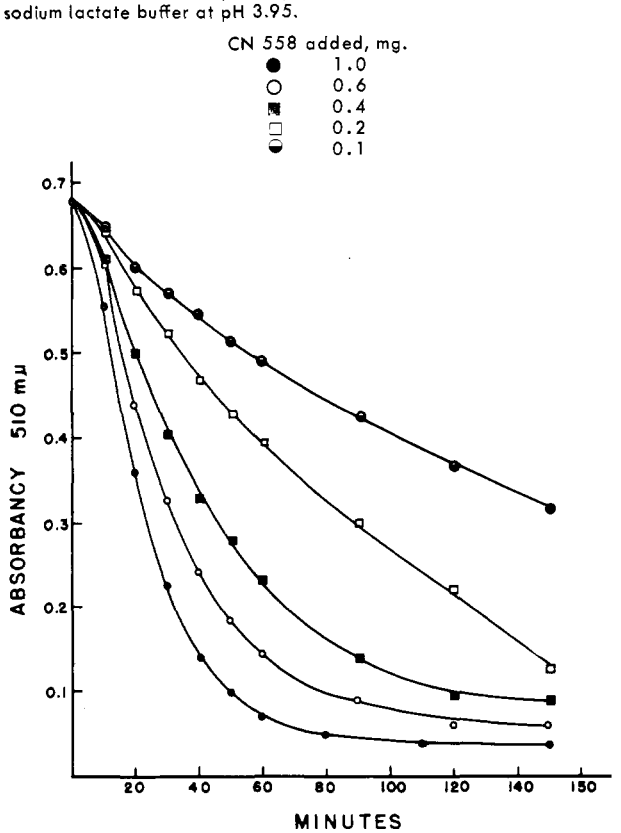
The test system contains 5 ml. of a solution of crystalline chrysanthemin chloride at a concentration of 0.207μM per ml. in Sorensen's citrate buffer, 0.045M with respect to citrate, and 1 mg. of CN 558 in 1 ml. of water. Controls were run for each pH level using 1 ml. of water.

I and II, under strongly acid conditions. Since the observed decolorization reaction does not immediately involve oxidative degradation, it would be informative to know whether a colored form can be regenerated from the decolorized material when the pH of the medium is lowered. Accordingly, in a number of experiments the decolorized solution was acidified to pH < 1 with hydrochloric acid. A slow regeneration of the original color occurred. Qualitatively the absorption spectrum of the restored color was the same as those recorded in Figure 1. The rate of color regeneration could, therefore, be studied conveniently by measuring the absorbance of the solution at 510 mμ. Typical results are summarized in Figure 3.

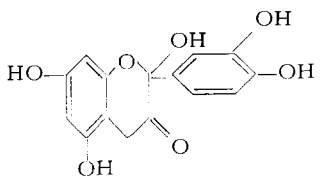
The data are noteworthy in two particular aspects. In the first place, as the length of incubation time before acidification was increased, the maximum intensity of the regenerated color decreased, and eventually dropped to zero. Concurrently, the barely perceptible residual color in the reacting solution turned gradually from pink to yellow, although the absorbance at 510 mμ remained practically unchanged. In a similar series of experiments carried out in an atmosphere of nitrogen, the capacity for color regeneration was not diminished and no change in the quality of the residual color was detectable even after 72 hours. Secondly, Figure 3 shows that the regenerated color is unstable in an environment of high acidity, in contrast to the color exhibited by the original anthocyanin in a medium of identical composition. The latter was found to maintain a constant intensity for more than 3 weeks at room temperature. From these considerations it may be inferred that the initial product of decolorization, which is convertible into pigment when the pH of the medium is lowered, undergoes further reaction when oxygen is present to give a product (or products) which is no longer so convertible, and that the regenerated pigment is similar to but not identical with the original anthocyanin.

Figure 2. Decolorization of chrysanthemin by CN 558

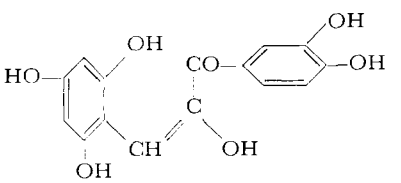
Each tube contained 1.03μM chrysanthemin chloride in 5 ml. of 0.045M sodium lactate buffer at pH 3.95.



The above conclusions strongly suggest that the regenerated pigment is the aglucone—i.e., cyanidin, II—corresponding to the substrate anthocyanin—i.e., chrysanthemin. If so, the decolorization reaction may be regarded as a two-step process involving a hydrolysis of the anthocyanidin, and a decolorization of the liberated anthocyanidin, II, by transformation into colorless pseudobase modifications such as the 2-carbinol, IV, and its tautomers, the ketone, V, and, perhaps, the chalcone, VI. These pseudobase forms can be reconverted to the color base, II, when the medium is acidified, but they are easily susceptible to attack by molecular oxygen.



V



VI

The author's results indicate that the first step—hydrolysis of a β-glucoside—must be enzyme-catalyzed. To examine the nature of the second step—i.e., decolorization of the aglucone—the properties of cyanidin chloride in aqueous solution were carefully scrutinized. It was found that a solution of the anthocyanidin in 1N hydrochloric acid lost color slowly upon standing at room temperature. When a stock solution of the aglucone containing more than 0.2 mg. per ml. in 0.01% hydrochloric acid was diluted 1 to 10 with 0.05M lactate buffer at pH 3.95, a precipitate of the anthocyanidin quickly separated from the solution. The dilute pigment solution in the above medium obtained by filtration decolorized within 5 to 10 minutes. Furthermore, the spontaneously decolorized solution upon acidification provided data which were essentially the same as those obtained for the enzyme-decolorized anthocyanin solution as summarized in Figure 3. These observations are qualitatively similar to those recorded by the Robinsons (*19*), who found that cyanidin was rapidly transformed into its pseudobase at pH levels within the range encountered in these experiments. The color base could be restored by acidification of the decolorized solution, and the facility with which the color was restored decreased with time. Accordingly, the decolorization of the aglucone produced by the enzymatic hydrolysis of chrysanthemin can be satisfactorily regarded as a spontaneous process.

Hydrolysis during Decolorization

A demonstration that glucose is liberated during the course of the enzyme-catalyzed decolorization of chrysanthemin chloride would provide a

simple and unequivocal verification of the above reaction scheme. Unbuffered chrysanthemin solutions at pH 3.95 were, therefore, decolorized with CN 558 and then treated consecutively with Amberlite IR-100 and ethyl acetate to remove unreacted anthocyanin and products derived from the decolorized aglucone. The aqueous phase was analyzed by paper chromatography to detect the presence of glucose and its reducing activity was determined by chemical analyses. The results of these experiments are summarized in Table IV.

Under the conditions of the experiment the maximum loss in absorbance was attained in less than 1.5 hours. The values for apparent glucose content found in samples from flasks 3 to 6, extracted with ethyl acetate, are in agreement with the theoretical value of 36.3 γ per ml. based on complete hydrolysis of the glucoside. The aqueous solution from flask 3 was partly concentrated and analyzed by paper chromatography. The presence of a sugar in the concentrate could be readily demonstrated. The sample spot when mixed with glucose traveled as a single component when irrigated with either of the two solvent systems employed (*4*, *5*). Attempts to detect the presence of phenolic substances in the aqueous concentrate failed to yield any positive results, indicating that the extraction by ethyl acetate was effective in removing all the anthocyanidin from the aqueous phase. Thus, the apparent glucose value quoted for flasks 3 to 6 must represent the true glucose concentration in these samples. These results, therefore, offer convincing evidence of the essential correctness of the previously proposed mechanism of decolorization.

Paper chromatography of the ethyl acetate extracts from samples 5 and 6 using the procedure for the separation of phenolic compounds (*3*) revealed the presence of two principal components. The first component corresponds to a fast-traveling yellow spot of R_f = 0.95 and may be tentatively regarded as the chalcone, VI, as chalcones are known to give high R_f values under the conditions employed (*3*). The second component (R_f = 0.80) was identical with protocatechuic acid in chromatographic behavior and in color response to ferric chloride and ammoniacal silver nitrate (*3*).

That all the aglucone was extracted by ethyl acetate is supported by the data on color regeneration presented in Table IV. Of the decolorized solutions, only samples 1 and 2, which had not been extracted with ethyl acetate, exhibited the ability to regenerate color upon acidification. Sample 1 also gave rise to a value of apparent glucose content almost twice as much as the maximum theoretical value of 36.3. Thus, the aglucone components, like the original anthocyanin, are capable of reducing alkaline copper under the conditions of the sugar determination.

Discussion

Of the few reports available on the enzymatic hydrolysis of anthocyanins (*7*, *15*, *16*), the work of Noack (*15*) provides the only satisfactory demonstration of such a reaction under in vitro conditions. He described the hydrolysis of several anthocyanins by a "tannase" of aspergillus. It is conceivable that the "anthocyanase" (*16*) reaction observed by Noack and by the author is adequately accounted for in terms of the present knowledge of the specificity of well established glycosidase systems (*25*). If the anthocyanin isolated from blackberry in this study is indeed chrysanthemin, which has been shown by chemical synthesis to be a β-glucoside (*13*), it would be reasonable to assume that the enzyme responsible for the reaction is a β-glucosidase. It might be inferred that the classical β-glucosidase of almond emulsin would be an effective decolorizing agent. However, emulsin is practically devoid of decolorizing activity toward chrysanthemin. This apparent discrepancy is now being investigated and results will be reported in a subsequent communication. Preliminary data, as summarized in Table II, suggest that these preparations can also hydrolyze a β-galactoside, since idaein—i.e., cyanidin-3-β-monogalactoside—is the principal coloring matter of cranberry (*10*). A separate β-galactosidase may be involved. Nevertheless, the specificity with respect to sugar groups of β-glucosidases from various sources (*25*) is not absolute; hence these enzymes may retain some activity toward β-galactosides. The reaction is, of course, by no means restricted to cyanidin glycosides. Thus, CN 558 hydrolyzes pelargonidin-3-β-monoglucoside or callestephin from strawberries (*23*) and malvidin 3-β-monoglucoside or oenin from grape (*30*). Because glucosides and galactosides are mong the most widely occurring types of anthocyanin pigments, and glycosidases are usually relatively nonspecific in their structural requirements on the aglucone portion of the substrate molecule, the decolorizing action of these preparations may operate on a large number of anthocyanins from a variety of sources.

The characteristic red color of chrysanthemin in acid media is due to the presence of the cation (I). Within the range of pH employed in these experiments, chrysanthemin may be assumed to exist in solution as an equilibrium mixture of I and colorless pseudobase structures, of which II is an example. The absorption at 510 mμ, and hence, the proportion of I increases as the pH is decreased. A similar situation may hold for the corresponding aglucone, cyanidin. In this case, however, at pH > 3, the equilibrium position is now so much in favor of the colorless forms that when III is formed from I, it is immediately converted to forms exemplified by IV. Moreover, while the pseudobase of the anthocyanin—e.g., II—is instantaneously converted into the corresponding colored cation, I, upon acidification, the transformation of the pseudobase of the anthocyanidin into its colored cation, III, is a very slow process (Figure 3). These observations point to a qualitative difference between the colorless forms of chrysanthemin and those of cyanidin in acid solution. Al-

Figure 3. Effect of acidification on decolorized solution

Each tube contained initially 0.692μM chrysanthemin chloride in 5 ml. of 0.045M sodium lactate buffer at pH 3.95 and 1 mg. of CN 558 in 1 ml. of water. At time indicated by arrow 4 ml. of 2.0N hydrochloric acid was added to each tube.

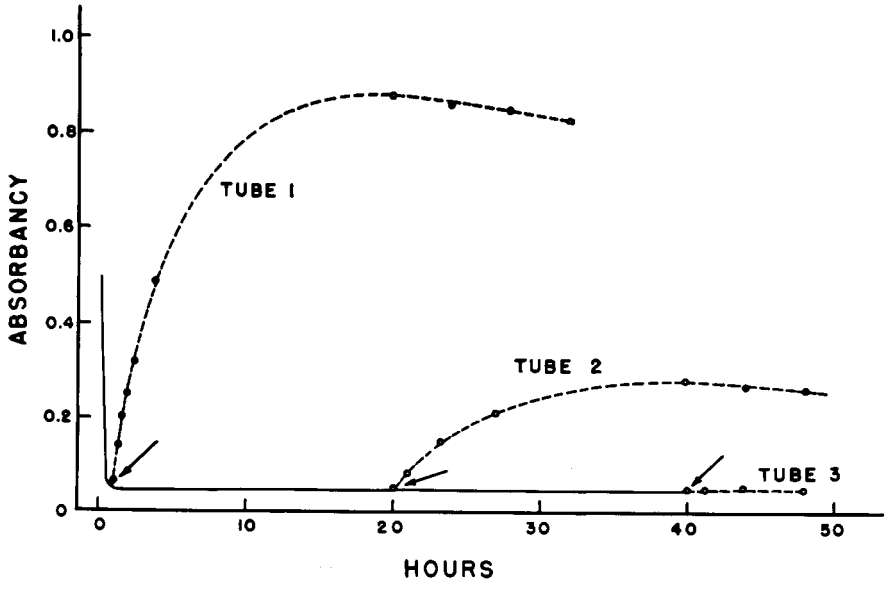


Table IV. Liberation of Glucose during Decolorization of Chrysanthemin Chloride

Flask No.	Enzyme[a] Addition	Hours[b] Incubation	Extraction: Amberlite[c] IR-100	Extraction: Ethyl[d] acetate	Apparent[e] Glucose Found, γ/Ml.	Slow Regeneration[f] of Color after Acidification
1	+	1.5			70	+
2	+	1.5	+		45	+
3	+	1.5	+	+	34	−
4	+	20	+	+	36	−
5	+	2.5		+	37	−
6	+	20		+	38	−
7	−	2.5			34	
8	−	2.5	+		None	

[a] 5 mg. of CN 558 in 0.5 ml. of water at pH 3.95 was added to 20-ml. solution of 4.13 μ*M* chrysanthemin chloride in water. In flasks 7 and 8, 0.5 ml. of water was used, and no decolorization was observed.

[b] Reacting solution was incubated at 30° C., pH 3.95 in 50-ml. conical flasks.

[c] Solution was shaken mechanically with 2.5 grams of Amberlite IR-100, analytical grade, in the hydrogen form, for 10 minutes. After treatment pH of solution was 2.85.

[d] Solution was extracted three times with an equal volume of reagent grade ethyl acetate and once with ether.

[e] Aqueous phase was analyzed for reducing sugar by the microcolorimetric method of Somogyi (*21*), and the final absorbance values were corrected to a glucose standard curve. Based on chrysanthemin content, the amount of glucose liberated after complete hydrolysis should be 36.3 γ per ml.

[f] 2 ml. of 2*N* hydrochloric acid added to 3 ml. of aqueous solution.

though the shape of the decolorization curves (Figure 1) intimates that the actual decolorization process involves a colored intermediate, which can be derived only from I, it does not necessarily imply that the colored cation, I, is the only form of chrysanthemin in solution which is hydrolyzed by the enzyme. The carbinol, II, is structurally no less satisfactory as a substrate for the enzyme than I. However, the hydrolysis of II would lead directly to the colorless carbinol, IV. The effect of this reaction on the actual decolorization picture is dependent on the rates of conversion between I and II as well as those involved in the hydrolysis of I and of II.

The proposed mechanism provides a simple explanation for the observed formation of pigment deposits during the enzymatic decolorization of some of the pigment extracts and also of pure chrysanthemin when it is present in concentrations substantially greater than those utilized in the experiments reported. It is apparent that in these cases, the concentration of the aglucone pigment is increased to a level considerably in excess of the saturation point. The anthocyanidin then precipitates from solution.

Since anthocyanidins are, in general, sparingly soluble in aqueous media, the major effect of an anthocyanase action on a concentrated anthocyanin solution would then be the removal of the pigment from solution in the form of an aglucone sediment.

It is possible that the anthocyanase activity in these fungal preparations may be of practical interest in situations wherein the hydrolysis of anthocyanin is actually desirable. For instance, excessive amounts of anthocyanin present in blackberry wines cause the formation of deposits around the walls of the bottles during storage (*31*). These might be removed by the judicious application of anthocyanase during the extraction of fruit juice or directly on the fermented wine. The aftersedimentation often associated with grape juice may conceivably be prevented in a similar manner. Moreover, in certain grape-growing areas, white varieties of grapes are difficult to cultivate and the wine maker finds it necessary to use the free-run juice of red varieties of grapes for the production of white wines (*1*). The grapes are then picked young, so that the pigment content of the free-run juice will be negligible. The time of picking is of considerable importance, as grapes which are too young will not have the desired flavor qualities and those which are mature will impart too much color to the free run juice. With the aid of the action of anthocyanase, it may be feasible to use the free-run juice of mature red grapes for the production of white wines.

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