THE DEGRADATION OF A CHERRY ANTHOCYANIN BY TYROSINASE

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This is to certify that the

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Pericles Markakis

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AN ABSTRACT

THE DEGRADATION OF A CHERRY ANTHOCYANIN BY TYROSINASE

By

Chung-Yen Peng

Anthocyanins are the chief pigments responsible for the red, blue, and violet colors of fruits and flowers. During the handling and preservation of the fruits, color changes may occur by destruction of the anthocyanin pigment or the development of browning. These changes can be of chemical or biochemical nature.

The effect of the tyrosinase (polyphenol oxidase) enzyme from an edible mushroom on the breakdown of the mecocyanin pigment of red tart cherry, Prunus cerasus, L. var. Montmorency was investigated under various assay conditions.

The mecocyanin pigment, cyanidin 3-gentiobioside, was extracted from cherries with ethanol and purified chromatographically and electrophoretically. The enzymatic degradation of the pigment was measured by optical density decrease at 520 mμ.

The reaction exhibited a pH optimum at 6.5 and a temperature optimum around 50° C.

The reaction was activated by catechol with maximum activation occurring at 0.01 M concentration of catechol.

When the concentration of the enzyme was varied the rate of the reaction increased rapidly with increasing enzyme concentration, and levelled-off after a certain level of enzyme concentration was reached under the assay conditions.

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INTRODUCTION

Color is a significant factor of food quality. It is used as a criterion of picking and shipping maturity of fruits and vegetables. Color evaluation and color control of fresh and processed foods are becoming more and more important. The U. S. Production and Marketing Administration has established color specifications as to appearance for all processed products.

In the red cherry industry one of the major problems during the handling and processing of the fruit is cherry "scald." Scald has been described as the appearance of a light colored area on the cherry skin. In severe scald these areas turn brown. Scald causes a downgrading of both raw and processed products. It was originally thought that the decrease in red color in the skin areas was primarily due to diffusion of the anthocyanin pigments into the flesh. However, Van Buren, et al. (22) showed that the pigment was converted to a colorless form by an enzyme present in cherry. Such an enzyme, specifically attacking anthocyanins, has not yet been defined. Anthocyanins, however, are polyphenols and polyphenolases are widely distributed in the plant kingdom without being very substrate-specific. The objective of the

present work has been to investigate the effect of a known plant polyphenolase on a pure anthocyanin. The choice of this enzyme was rather fortunate, since Bedford (1) has subsequently shown that there is polyphenolase activity in red tart cherries.

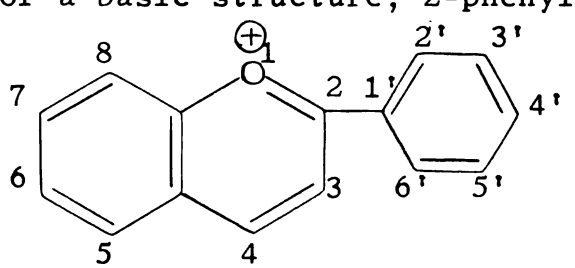
REVIEW OF LITERATURE

Occurrence of Anthocyanins

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

Structure of Anthocyanins

Willstätter in 1913 (23) studied the structure of anthocyanins and found them to be glycosides of anthocyanidins, the latter being oxonium salts of polyhydroxy (and methoxy) derivatives of a basic structure, 2-phenylbenzopyrylium.



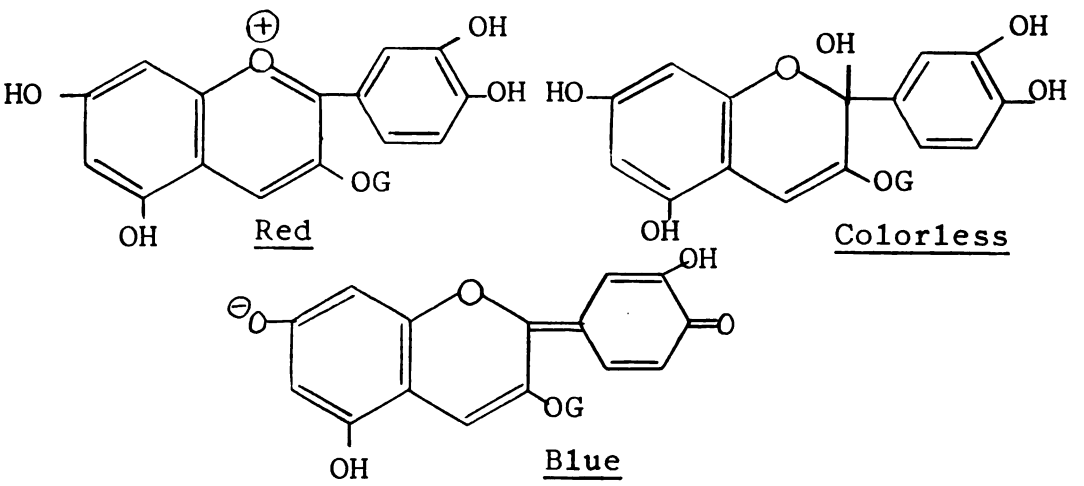
Upon hydrolysis anthocyanins give an anthocyanidin and one or more sugars (pentose, hexose) (7). Sometimes, a third

component can be found in association with the glycoside. This component can be an organic acid, a metal, or another flavonoid (14).

The position of the sugar residues in the anthocyanins is determined by complete methylation of the pigments, followed by hydrolysis of the glycosidic bonds. In mecocyanin there are two glucose units forming a gentiobiose attached to carbon 3 of the aglycone.

Properties of Anthocyanins

Anthocyanins are soluble in water and lower alcohols, but insoluble in ether, benzene, chloroform, or carbon bisulfide. Their color is due to the extensive conjugated double bond system, a strong chromophore (3). Anthocyanins are pH indicators, changing from red to colorless to blue as the pH increases. The following structures are associated with the color changes.



Anthocyanins also lose their color by reduction or oxidation (11, 12).

Enzymatic Degradation of Anthocyanins

The enzymatic degradation of anthocyanins has not been studied very extensively. In 1921 Nagai (15) found that the red anthocyanin pigments of scarlet Papaver were destroyed by a peroxidase prepared from soybean seedling hypocotyls and rootlets. He noticed that the color of anthocyanins disappeared rapidly when this peroxidase and peroxide were present. The enzyme was unstable, losing its activity with time.

Huang (7) observed that crude enzyme extracts from Aspergilli decolorized the anthocyanins of blackberries. The decolorization was rapid at 30° C. and pH ranging from 3.0 to 4.5. He also ascertained that the anthocyanins were hydrolyzed to anthocyanidin and sugar followed by a spontaneous transformation of the aglycone to colorless derivatives. This enzyme seems to be a glycosidase.

An enzyme preparation from the leaves of Coleus hybridus was obtained by Bayer and Wegmann (2) which could rapidly decolorize the anthocyanin of red roses at pH 7.0 to 7.5 in the presence of catechol. They called the enzyme cyaninoxidase and found that the presence of oxygen was necessary for this enzymic reaction.

Van Buren et al. (22) reported the presence of an oxidizing enzyme which destroyed the anthocyanin color in a number of fruits.

Scheiner (19) recently obtained a crude enzyme from sweet cherries which decolorized cherry anthocyanins.

Purified preparations were almost completely inactive unless catechol or some other o-dihydroxyphenol compound was present in the reaction mixture. Catechol was oxidized by all the preparations that decolorized anthocyanins, and catechol oxidase activity and anthocyanin decolorizing activity followed each other closely during the purification of the enzyme.

METHODS AND MATERIALS

Preparation of Anthocyanins

Fresh or frozen, pitted Montmorency cherries were placed in boiling 95 per cent (v/v) ethanol in such a proportion as to achieve a 70-75 per cent (v/v) final ethanol concentration. The mixture was boiled for five minutes and allowed to cool. This treatment extracted the anthocyanins, inactivated the enzymes, and precipitated the pectins.

The mixture was filtered through a milk filter and the filtrate was concentrated under reduced pressure in a rotatory film evaporator thermostated at 37° C.

The concentrated anthocyanin extract was applied to a 2 x 5 cm. column of Dowex 50W-X8 (100-200 mesh, H^+ form) resin which retained the pigments along with other basic components of the extract. The column was washed with 50 ml. of water and the pigments were eluted with 100-150 ml. of methanol 0.35 N in hydrochloric acid. The eluate was concentrated in vacuo and applied as a narrow band to Whatman 3MM paper. The paper was placed in a chromatography cabinet and irrigated ascendingly with 1 N acetic acid for 15-20 minutes. At the end of this time the two Montmorency cherry anthocyanins appeared as well-separated zones which were cut

off and eluted separately with methanol containing a trace of hydrochloric acid.

The eluate of the pigment with the higher R_f was concentrated *in vacuo* and further purified by zone electrophoresis. For this purpose a Reco Model E apparatus was used. Whatman cellulose powder, standard grade, was made into a paste with 0.5 N acetic acid solution and spread evenly on the plate of the apparatus. The electrode vessels contained 0.5 N acetic acid and were connected to the paste with filter paper. The pigment was applied as a narrow band on the paste at four places across the electric field and 700 volts of direct current, approximately 20 milliamperes, were applied for about five hours. At the end of this time the pigment had moved 2-3 cm. and the colored zones of the cellulose paste were removed and eluted with methanol. The eluate was concentrated *in vacuo* and the dry pigment was dissolved in water.

This pigment was found to be identical with the mecocyanin of Li and Wagenknecht (10) on the basis of its absorption spectrum, paper chromatographic behavior, and the sodium carbonate test. It has been defined as cyanidin 3-gentiobioside.

The Enzyme

Tyrosinase is a copper-containing enzyme which is widely distributed throughout the plant and animal kingdoms. The enzyme, as extracted from the edible mushroom, is characterized

by its ability to catalyze the aerobic oxidation of both monohydric and o-dihydric phenols. The monophenolic activity, commonly called cresolase activity, is less stable than the o-diphenolic activity, commonly called catecholase activity, during purification (4, 5, 17).

Its molecular weight is over 200,000 and its optimum pH is generally in the range of 5.5 to 7.0 (4, 5, 9, 17). Copper is essential for activity of the enzyme and in different preparations the enzyme activity is proportional to the copper content.

The enzyme preparation used in this study was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. Its activity was found to be 450 units per milligram. A unit of tyrosinase activity equals an increase in absorbance of 0.001 per minute under the specified conditions of the assay.

The Enzymic Reaction

The anthocyanin and the enzyme were allowed to react in a citrate-phosphate buffer and the progress of the reaction was followed colorimetrically. Four factors affecting the reaction were studied: pH, temperature, enzyme concentration, and catechol concentration.

Three buffer solutions were employed. One of these consisted of equal volumes of 0.1 M citric and 0.1 M phosphoric acids adjusted to the desired pH level with sodium hydroxide at the time of preparing the reaction mixture. The other

two buffers were 1.0 M citric acid solutions adjusted to pH 2.0 and 7.0, respectively, with sodium hydroxide and were used for differential color development as will be explained later. A 2×10^{-4} M solution of catechol was used in the preparation of the reaction mixture and a 0.5 M potassium cyanide solution was used for the preparation of the mixtures in which the color was measured. A tyrosinase stock solution was prepared containing 25 mg. of enzyme in 25 ml. of water.

The reaction was performed in a 30 ml. beaker. Three ml. of the citric-phosphoric acid mixture were transferred to the beaker followed by 1.0 ml. of anthocyanin solution and 1.0 ml. of catechol solution. The pH was adjusted to the desired level by adding 5 N sodium hydroxide solution from a graduated pipette and the volume of the base was noted. After addition of enough water to make a total volume of 6.8 ml., 0.5 ml. of the mixture was transferred to a tube containing 2.5 ml. of the citrate buffer of pH 2.0 and 0.1 ml. of 0.5 M potassium cyanide, and another 0.5 ml. of the mixture to a tube containing 2.5 ml. of the citrate buffer, pH 7.0, and 0.1 ml. of the cyanide solution. These tubes served for determining the zero time anthocyanin concentration. Immediately after the removal of the first ml. of reaction mixture, 0.2 ml. of the enzyme solution was added to the remaining mixture, a stop watch was started, and at intervals of time as short as 30 seconds, two aliquots, 0.5 ml. of each, were transferred to tubes containing citrate-cyanide solution

as it was done for the zero time determinations.

After a 15-minute interval at room temperature the contents of the tubes were transferred to 1.0 cm. cuvettes for optical density measurements in a Beckman DU Spectrophotometer at 520 mμ. The concentration of anthocyanin was expressed in optical density difference between pH 2.0 and 7.0.

Sondheimer and Kertesz (20) showed that the difference in absorbance at two pH levels was a more accurate measure of the anthocyanin concentration in strawberry products than the absorbance at one pH, when interferring substances were present. In the system used in the present research, browning, chiefly due to catechol oxidation, was the source of interference. The absorbance at 520 mμ, however, of these brown products did not change with pH as is shown in Table 1 and cancelled out when the difference of absorbance at two pH levels was calculated for the reaction system. Similarly the slight absorbance of the enzyme at 520 mμ cancelled out in this differential colorimetry.

The results of each experiment were plotted as decrease in the difference of optical density between pH 2.0 and 7.0 at 520 mμ, $(OD_{pH\ 2.0} - OD_{pH\ 7.0})_{520}$, against time. The zero time optical density was multiplied by 0.966 before plotting to correct for the dilution occurring by the addition of the enzyme solution.

The reaction rates, $(OD_{pH\ 2.0} - OD_{pH\ 7.0})_{520}$ per minute, were generally linear during the first 60 seconds of the

reaction. Since, however, some deviation from linearity was observed at the end of 60 seconds in some cases, the 30 seconds readings were used for calculating the reaction rate in all cases (6, 16).

RESULTS AND DISCUSSION

The four factors investigated in the tyrosinase-mecocyanin reaction were pH, concentration of enzyme, temperature, and concentration of catechol.

The effect of pH (Table II) in the range of 2.5 to 7.5, on the rate of reaction is shown in Figure 1. The reaction took place at 23° C, with 3.4 mg. per cent of enzyme, and in the presence of 2.8×10^{-5} M catechol. The bell-shaped curve shows that the enzyme activity increased with increasing pH reaching a maximum at about pH 6.5, after which the rate fell off sharply.

The pH optimum is similar to that previously reported (4, 5, 9, 17). Dawson and Tarpley (1951) found that the pH optimum of tyrosinase varies with the degree of purity of the enzyme and the nature of the substrate, the general range being pH 5.5 to 7.0. This pH optimum is higher than the pH of the juice of most fruits, however, even at the more acidic pH of fruit juices (e.g., 3.5 for cherries) the activity of tyrosinase is considerable.

The following enzyme concentrations were used: 0.2, 0.5, 0.8, 1.7, 3.4, and 7.1 mg. of enzyme per 100 ml. (mg. per cent) of reaction mixture (Table III). The reaction was carried out at pH 6.5, 23° C, and in the presence of 2.8 x

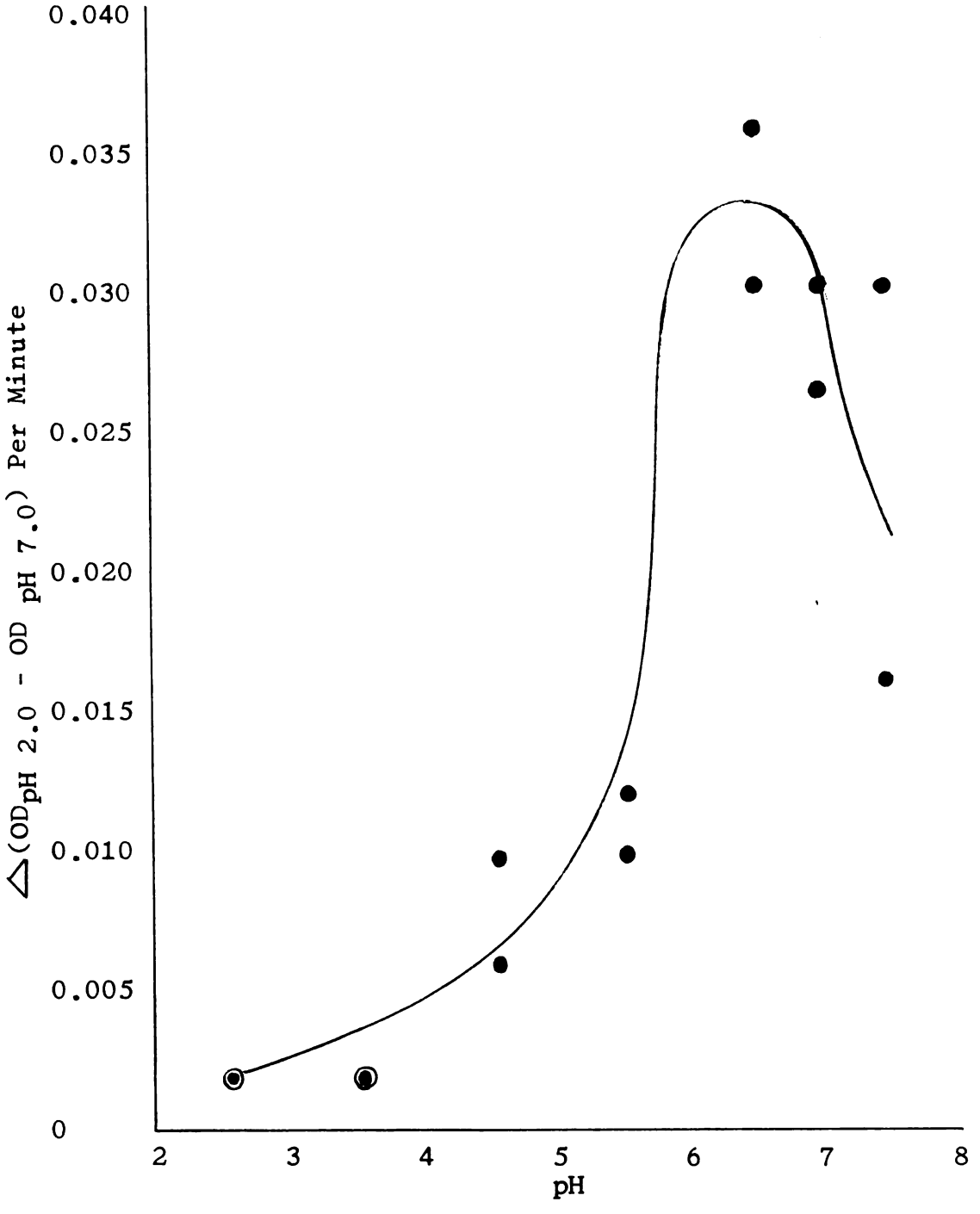


Figure 1. Influence of pH on Tyrosinase Degradation of Cherry Mecocyanin with 3.4 mg. per cent Enzyme, 2.8×10^{-5} M Catechol, at 23° C.

10^{-5} M catechol. The rate of the anthocyanin destruction was proportional to the enzyme concentration in the range of 0.2 to 2.0 mg. per cent, reached a maximal value at about 3.0 mg. per cent and then it levelled off (Figure 2). Such a relationship between enzyme concentration and reaction rate is typical of many enzymes.

The effect of temperature (Table IV) on the reaction rate was studied in the range of 5° to 60° C. The reactions were performed at pH 6.5, 3.4 mg. per cent of enzyme, and 2.8×10^{-5} M of catechol. As can be seen in Figure 3, the rate of reaction increased with temperature and reached a maximum value at about 50° C. At still higher temperature the rate of reaction dropped quickly.

The effect of temperature was also typical of enzyme reactions and it is explained by a combination of two phenomena: (1) the general increase in chemical reaction rates with temperature, and (b) the destruction of the enzyme at higher temperatures.

The determination of the effect of catechol (Table V) on the rate of reaction at eight different catechol concentrations ranging from 0 mM to 20 mM was carried out at pH 6.5, 23° C, and 3.4 mg. per cent of enzyme. The data are presented graphically in Figure 4. In the absence of catechol the enzymic degradation of the anthocyanin was very slow. As the catechol concentration increased, however, the

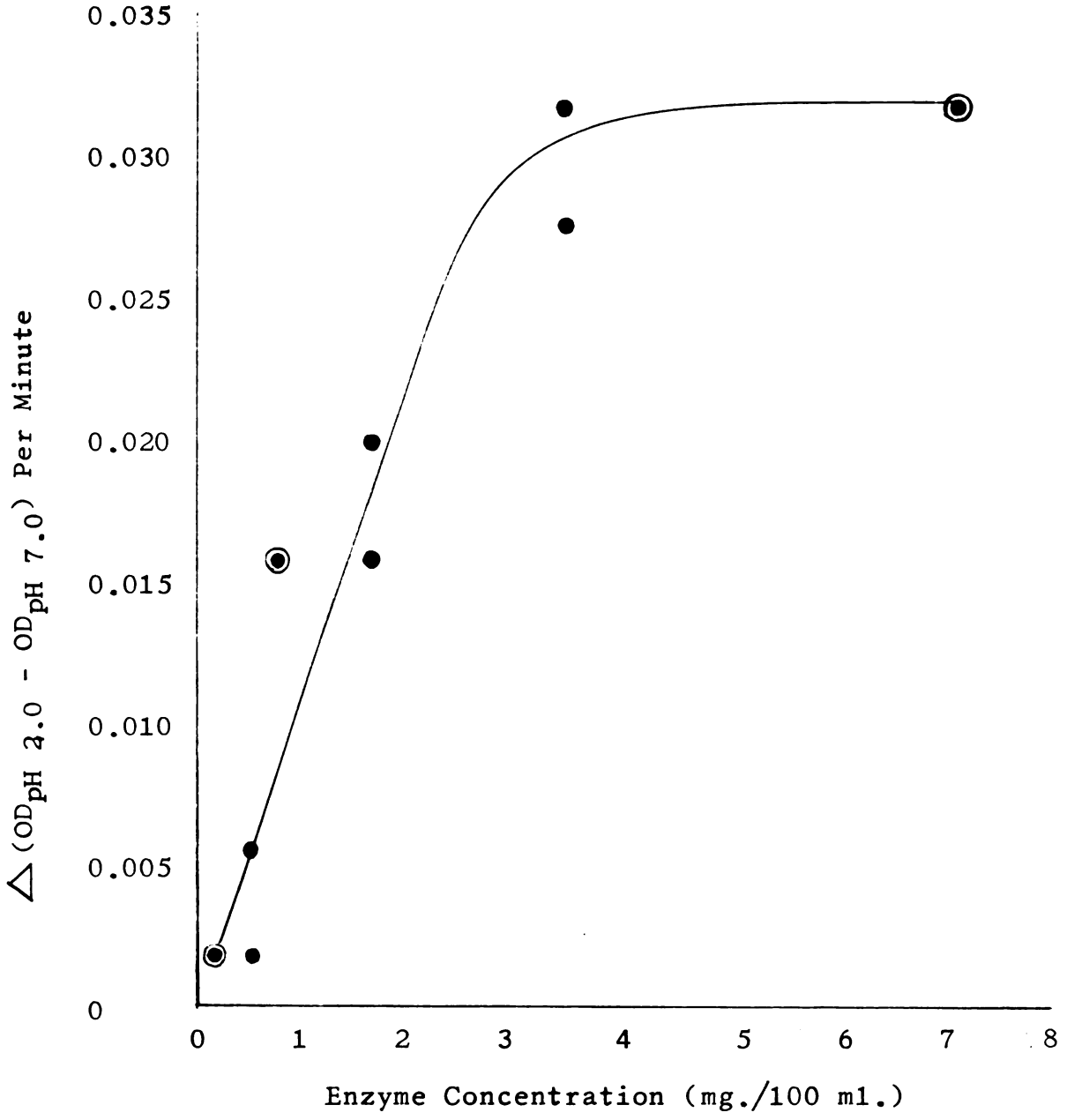


Figure 2. Influence of Tyrosinase Concentration on the Degradation of Cherry Mecocyanin with 2.8 x 10^{-5} M Catechol at pH 6.5 and 23° C.

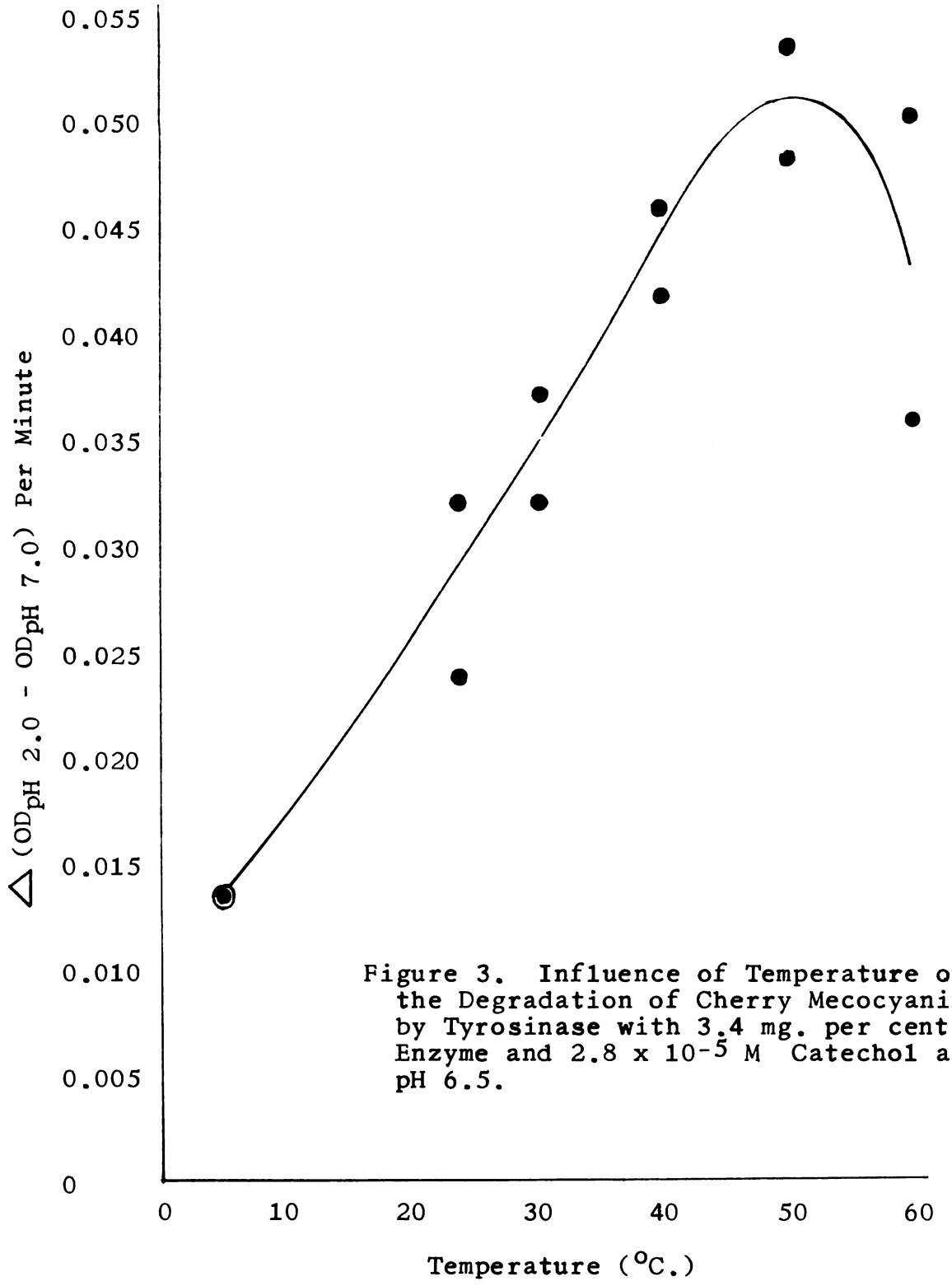


Figure 3. Influence of Temperature on the Degradation of Cherry Mecocyanin by Tyrosinase with 3.4 mg. per cent Enzyme and 2.8×10^{-5} M Catechol at pH 6.5.

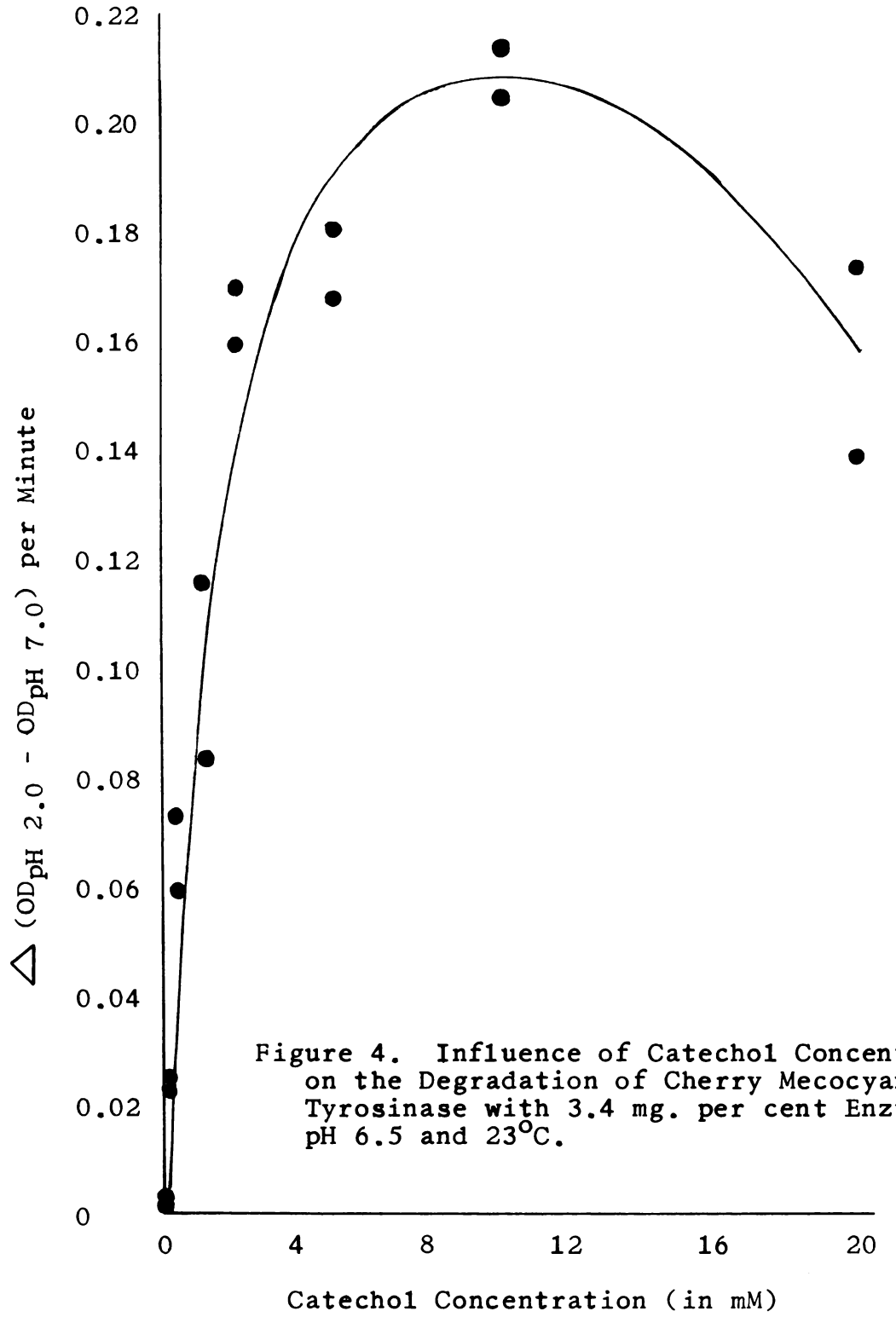
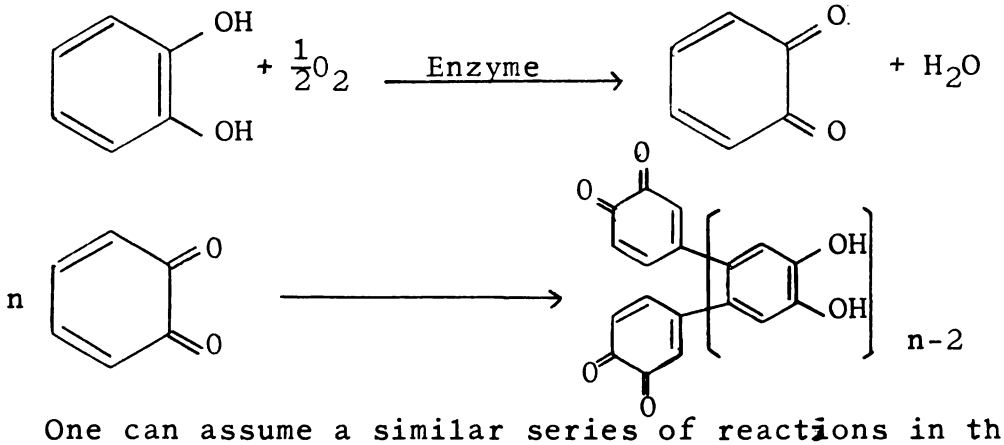


Figure 4. Influence of Catechol Concentration on the Degradation of Cherry Mecocyanin by Tyrosinase with 3.4 mg. per cent Enzyme at pH 6.5 and 23°C.

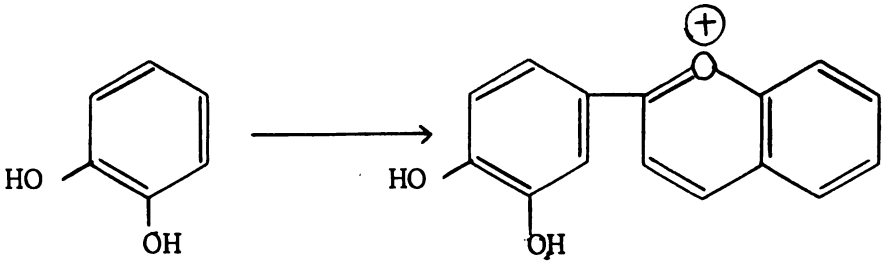
rate increased rapidly and reached a maximum at the concentration of 10 mM.

Mechanism of the Reaction

When catechol is oxidized by tyrosinase an o-benzoquinone is formed (8, 13, 21, 24) which is later polymerized to dark-colored compounds (Scheme 1):

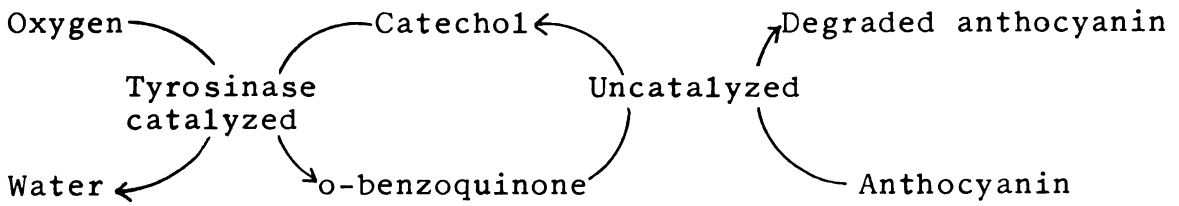


One can assume a similar series of reactions in the oxidation of mecocyanin by tyrosinase in the absence of catechol. In this analogy mecocyanin could be considered a derivative of catechol with a benzopyrylium in para position.



Obviously, this derivative is not as good a substrate for tyrosinase as catechol. If catechol is present in the system, however, the first step can still be the oxidation

of catechol to o-benzoquinone; this quinone could then oxidize the anthocyanin with concomittant loss of color as in Scheme 2:



A similar scheme has been proposed (5) for the effect of ascorbic acid in preventing the browning reaction of tyrosinase. Whether anthocyanin behaves as equally good an antioxidant in this case as ascorbic acid has not been established. In almost all the reaction systems tried in this work some browning occurred when catechol was present. This may indicate that a part of the o-benzoquinone formed from catechol probably follows Scheme 1.

Practical Considerations

Although this research has been planned as a basic approach to the problem of the enzymatic degradation of anthocyanin pigments, some points of practical significance may be raised.

A. The finding that tyrosinase destroys the cherry anthocyanin, in conjunction with Bedford's recent evidence (1) that there is tyrosinase activity in cherries, points to one direction of preventing this reaction. It is known

that browning caused by tyrosinase can be checked by the addition of ascorbic acid or similar antioxidants. In this case ascorbic acid serves as the hydrogen donor to the o-benzoquinone of reaction. If ascorbic acid proves to be a better hydrogen donor than the anthocyanin pigment, then browning and anthocyanin destruction due to tyrosinase can be prevented in one act.

There are a number of substances that inhibit tyrosinase, but most of them are too toxic to be used in food additives: potassium cyanide, hydrogen sulfide, carbon monoxide, various thiouracils and thioureas, etc. There are a few tyrosinase inhibitors, however, which could be added to foods in small concentrations, such as p-aminobenzoic acid, cysteine, and glutathione. Their effect is the tyrosinase-anthocyanin reaction should be studied both in model systems and in actual fruit preparations. Ethylene-diaminotetraacetic acid, a metal chelating agent, is also worthy of testing in this connection.

B. The tyrosinase-anthocyanin reaction is oxygen dependent. The well-known fact that exclusion of air will minimize the color destruction of frozen cherries finds at least a partial explanation in this reaction.

C. The decrease of enzymatic activity at lower temperature is general knowledge and has found wide application in food preservation. The present data simply confirm it. It could be emphasized, however, that if tyrosinase

plays any important role in the destruction of cherry mecocyanin, the temperature zone from 40° to 55° C. should be traversed quickly when pasteurizing or sterilizing temperatures are to be used.

SUMMARY AND CONCLUSIONS

1. In this investigation the effect of a mushroom tyrosinase on a pure anthocyanin, cyanidin 3-gentiobioside, was studied under various conditions of pH, temperature, concentration of enzyme, and concentration of an activator, catechol.
2. The cyanidin derivative, mecocyanin, was extracted from red tart cherries with ethanol and separated and purified chromatographically and electrophoretically.
3. The progress of the tyrosinase-mecocyanin reaction was followed photometrically by measuring the optical density decrease of the reaction mixture at the wavelength of maximum absorption of the anthocyanin, 520 mμ. The interference of the concurrent browning was eliminated by measuring the optical density at two pH levels, after the reaction was stopped.
4. In studying the pH effect on the rate of reaction, it was found that at pH 6.5 the enzyme had its maximum activity.
5. By varying the enzyme concentration from 0.2 mg. to 7.1 mg. of enzyme per 100 ml. of reaction mixture, it was found that the rate increased as the enzyme

concentration went up to 3.4 mg. per cent, after which it levelled off.

6. The optimum temperature for the reaction was around 50° C.

7. Catechol was used to speed up the enzymic reaction. When the catechol concentration was varied from 0 mM to 20 mM the rate increased with increasing concentration of catechol up to 10 mM.

8. From the mechanistic viewpoint, the rapid destruction of mecocyanin in the presence of catechol seems to be a non-enzymic oxidation of the anthocyanin by the o-benzoquinone formed from the enzymic oxidation of catechol. In the absence of catechol the enzyme attack must be direct.

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APPENDIX

TABLE I

CATECHOL TEST WITHOUT ANTHOCYANIN

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)
0	0	0.001
1/2	0	0.001
1	0	0.001
2	0.001	0.002

Reaction mixture:
3.0 ml. 0.1 M citric-phosphoric acid
1.0 ml. 0.2 mM catechol
0.2 ml. enzyme
2.8 ml. water

TABLE II

EFFECT OF pH ON THE RATE OF REACTION

1. pH 2.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.168	0.012	0.156	0.168	0.008	0.160
1/2	0.168	0.013	0.155	0.167	0.008	0.159
1	0.167	0.013	0.154	0.165	0.008	0.157
2	0.165	0.014	0.151	0.159	0.008	0.151

Final pH - 2.5

2. pH 3.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.161	0.010	0.151	0.160	0.008	0.152
1/2	0.160	0.010	0.150	0.161	0.010	0.151
1	0.160	0.011	0.149	0.161	0.011	0.150
2	0.156	0.011	0.145	0.154	0.012	0.142

Final pH - 3.5

3. pH 4.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.165	0.016	0.149	0.167	0.017	0.150
1/2	0.165	0.019	0.146	0.164	0.019	0.145
1	0.163	0.019	0.144	0.163	0.019	0.144
2	0.160	0.019	0.141	0.159	0.019	0.140

Final pH - 4.6

4. pH 5.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.152	0.016	0.136	0.153	0.016	0.137
1/2	0.148	0.017	0.131	0.148	0.017	0.131
1	0.142	0.018	0.124	0.145	0.017	0.128
2	0.135	0.019	0.116	0.133	0.017	0.116

Final pH - 5.6

5. pH 6.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.143	0.032	0.111	0.144	0.032	0.112
1/2	0.126	0.033	0.093	0.130	0.033	0.097
1	0.120	0.033	0.087	0.120	0.033	0.087
2	0.109	0.033	0.076	0.113	0.035	0.078

Final pH - 6.6

6. pH 7.0

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.137	0.032	0.105	0.145	0.035	0.110
1/2	0.123	0.033	0.090	0.135	0.038	0.097
1	0.118	0.033	0.085	0.125	0.038	0.087
2	0.113	0.033	0.080	0.121	0.038	0.083

Final pH - 7.0

7. pH 7.5

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.133	0.042	0.091	0.135	0.049	0.086
1/2	0.119	0.043	0.076	0.130	0.052	0.078
1	0.110	0.043	0.067	0.113	0.052	0.061
2	0.102	0.043	0.059	0.103	0.052	0.051

Final pH - 7.5

TABLE III

EFFECT OF ENZYME CONCENTRATION ON THE RATE OF REACTION

1. 7.1 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.079	0.015	0.064	0.070	0.006	0.064
1/2	0.065	0.017	0.048	0.060	0.012	0.048
1	0.058	0.019	0.039	0.056	0.012	0.044
2	0.048	0.019	0.029	0.044	0.012	0.032

Final pH - 6.5

2. 3.4 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.112	0.025	0.087	0.113	0.022	0.091
1/2	0.097	0.026	0.071	0.100	0.023	0.077
1	0.088	0.026	0.062	0.091	0.023	0.068
2	0.076	0.026	0.050	0.083	0.023	0.060

Final pH - 6.5

3. 1.7 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.116	0.027	0.089	0.114	0.023	0.091
1/2	0.109	0.028	0.081	0.105	0.024	0.081
1	0.098	0.028	0.070	0.099	0.024	0.075
2	0.085	0.028	0.057	0.085	0.024	0.061

Final pH - 6.5

4. 0.8 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.113	0.027	0.086	0.113	0.022	0.091
1/2	0.106	0.028	0.078	0.106	0.023	0.083
1	0.098	0.028	0.070	0.098	0.025	0.073
2	0.087	0.028	0.059	0.087	0.025	0.062

Final pH - 6.5

5. 0.5 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.106	0.020	0.086	0.113	0.021	0.092
1/2	0.106	0.021	0.085	0.111	0.022	0.089
1	0.105	0.021	0.084	0.106	0.022	0.084
2	0.097	0.021	0.076	0.098	0.022	0.076

Final pH - 6.5

6. 0.2 mg. per cent

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.116	0.025	0.091	0.119	0.030	0.089
1/2	0.116	0.026	0.090	0.119	0.031	0.088
1	0.111	0.026	0.085	0.116	0.031	0.085
2	0.105	0.026	0.079	0.111	0.031	0.080

Final pH - 6.5

TABLE IV

EFFECT OF TEMPERATURE ON THE RATE OF REACTION

1. 5° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.116	0.021	0.095	0.113	0.015	0.098
1/2	0.113	0.025	0.088	0.110	0.019	0.091
1	0.109	0.027	0.082	0.106	0.019	0.087
2	0.097	0.027	0.070	0.097	0.019	0.078

Final pH - 6.5

2. 23° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.109	0.018	0.091	0.112	0.021	0.091
1/2	0.098	0.019	0.079	0.097	0.022	0.075
1	0.088	0.019	0.069	0.088	0.022	0.066
2	0.082	0.019	0.063	0.076	0.022	0.054

Final pH - 6.5

3. 30° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.112	0.030	0.082	0.112	0.026	0.086
1/2	0.097	0.031	0.066	0.094	0.027	0.067
1	0.088	0.031	0.057	0.087	0.027	0.060
2	0.078	0.031	0.047	0.078	0.027	0.051

Final pH - 6.5

4. 40° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.111	0.029	0.082	0.111	0.026	0.085
1/2	0.091	0.030	0.061	0.089	0.027	0.062
1	0.085	0.030	0.055	0.081	0.027	0.054
2	0.072	0.030	0.042	0.072	0.027	0.045

Final pH - 6.5

5. 50° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.113	0.029	0.084	0.104	0.020	0.084
1/2	0.090	0.030	0.060	0.082	0.025	0.057
1	0.079	0.030	0.049	0.072	0.025	0.047
2	0.072	0.030	0.042	0.070	0.025	0.045

Final pH - 6.5

6. 60° C.

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.104	0.027	0.077	0.109	0.026	0.083
1/2	0.087	0.028	0.059	0.088	0.030	0.058
1	0.077	0.028	0.049	0.079	0.030	0.049
2	0.074	0.028	0.046	0.072	0.030	0.042

Final pH - 6.5

TABLE V

EFFECT OF CATECHOL CONCENTRATION ON THE RATE OF REACTION

1. 20 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.206	0.108	0.098	0.175	0.082	0.093
1/2	0.125	0.113	0.012	0.135	0.112	0.023
1	0.123	0.129	--	0.116	0.125	--
2	0.132	0.131	--	0.123	0.135	--

Final pH - 6.5

2. 10 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.210	0.065	0.145	0.206	0.055	0.151
1/2	0.135	0.093	0.042	0.135	0.091	0.044
1	0.119	0.108	0.011	0.119	0.103	0.016
2	0.117	0.120	--	0.116	0.116	0

Final pH - 6.5

3. 5 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.128	0.042	0.086	0.131	0.037	0.094
1/2	0.063	0.061	0.002	0.067	0.063	0.004
1	0.068	0.066	--	0.065	0.070	--

Final pH - 6.5

4. 2 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.126	0.040	0.086	0.124	0.029	0.095
1/2	0.062	0.056	0.006	0.057	0.047	0.010
1	0.053	0.059	--	0.059	0.055	--

Final pH - 6.5

5. 1 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.142	0.027	0.115	0.158	0.031	0.127
1	0.071	0.039	0.032	0.051	0.043	0.008
2	0.053	0.048	0.005	0.049	0.048	0.001
4	0.053	0.053	0	0.059	0.052	--

Final pH - 6.5

6. 0.5 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.150	0.027	0.123	0.137	0.025	0.112
1	0.095	0.032	0.063	0.070	0.032	0.038
2	0.069	0.035	0.034	0.056	0.033	0.023
4	0.056	0.035	0.021	0.054	0.038	0.016

Final pH - 6.4

7. 0.2 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.141	0.026	0.115	0.142	0.032	0.110
2	0.096	0.030	0.066	0.101	0.036	0.065
4	0.087	0.034	0.053	0.092	0.034	0.058
8	0.078	0.030	0.048	0.082	0.032	0.050

Final pH - 6.6

8. 0 mM

Time (min.)	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference	O.D. (pH 2.0)	O.D. (pH 7.0)	Difference
0	0.141	0.026	0.115	0.136	0.020	0.116
3	0.138	0.027	0.111	0.130	0.022	0.108
6	0.129	0.030	0.099	0.123	0.023	0.100
12	0.122	0.027	0.095	0.122	0.027	0.095

Final pH - 6.5