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AN ABSTRACT OF THE THESIS OF

Mary Ellen Elizabeth Tracy for the M.S. in Chemistry
(Name) (Degree) (Major)

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Title: Pear Polyphenolase

Abstract approved: 
(Major Professor)

Polyphenolases (O-diphenol: O_2 oxidoreductase E.C. 1.10.3.1) have been isolated from a wide variety of plant and animal sources. This work deals with the isolation and characterization of polyphenolase from a previously unreported source, Pyrus communis, the common pear, horticultural variety D'Anjou.

The chronometric method of assay was used, in which the enzymic oxidation of the substrate, usually catechol, is coupled to the oxidation of ascorbic acid and the time required to oxidize a specific amount of substrate is noted as the time required to colorize an external starch-iodide indicator. Various methods of isolation and purification were attempted. After a suitable isolation procedure was established, the enzyme was characterized by its substrate specificity, and its sensitivity to temperature, pH and inhibitors.

Pear polyphenolase was characterized in particulate and soluble forms. The enzyme differs from other reported catechol oxidases in that it does not oxidize monophenols. A new spectrophotometric assay is described.

PEAR POLYPHENOLASE

by

MARY ELLEN ELIZABETH TRACY

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in

CHEMISTRY

Portland State University


1970

TO THE OFFICE OF GRADUATE STUDIES:

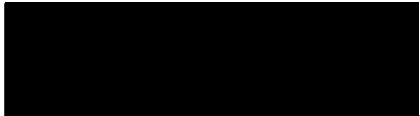
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May 18, 1970

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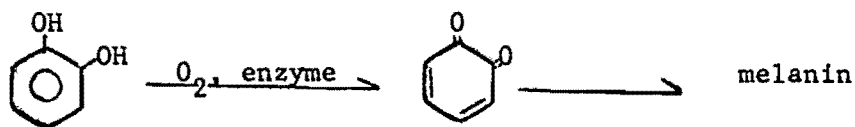
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I. INTRODUCTION

The enzyme polyphenolase (catechol oxidase, o-diphenol: O_2 oxidoreductase, E.C. 1.10.3.1) occurs in a wide variety of animals and plants. This work deals with the isolation and characterization of polyphenolase from pears, Pyrus communis, horticultural variety D'Anjou. Polyphenolase catalyzes the oxidation of catechol to O-benzoquinone; the ultimate product of the reaction is melanin.



The exact intermediates and method of action has as yet not been determined, though much work has been done in that area. Phenolase was first isolated from mushrooms by Bertrand in 1895 (1). An extensive review of work done in phenolase enzymes in plants to 1963 can be found in Enzyme Chemistry of Phenolic Compounds (2). In 1963 a phenolase was first crystallized from special high yielding strains of Neurospora crassa (3). However, despite this success, the most popular source of the enzyme remains mushrooms.

The phenolase enzymes come with a variety of common names: tyrosinase (the old classic name), cresolase, catecholase, and phenol oxidase, among them. Many of the enzymes isolated from various sources will accept a wide variety of substrates; that is, assorted mono and ortho diphenols from which their common names derive. It is customary, however, to distinguish these from the enzymes which will oxidize quinols, ortho-, meta-, and para- diphenols and occasionally monophenols, by putting these latter in the class called laccases. For many years

attempts were made to identify two distinct phenolase enzymes one for the oxidation of monophenols and one for the oxidation of diphenols. As yet there has been no success in separating the two activities.

Several methods of assay are used for phenolase enzymes: the measurement of oxygen uptake in the Warburg manometer; the spectrophotometric following of colored products; the measurement of disappearance of ascorbic acid due to coupled oxidation with the phenolic substrate, either spectrophotometrically or by titration; and the following of appearance of o-quinone by detection with an external indicator. Since there exist several methods of assay for the phenolase enzymes, there exist in the literature an equal number of ways for defining enzyme units. These various units are not, unfortunately, always interconvertible. The method of assay used in this work is that of Miller and Dawson (4) modified for smaller volumes. The unit employed is that recommended by the Enzyme Commission of the International Union of Biochemists, a unit being the amount of enzyme which catalyzes the transformation of one micromole of substrate per minute under defined conditions.

II. MATERIALS

D'Anjou pears (Hood River Valley Orchards); acetone (Mallinckrodt) spectrophotometric grade; pyrogallol (J. T. Baker) purified; catechol (Matheson, Coleman & Bell) practical; ammonium sulfate (J. T. Baker) enzyme grade; Sephadex (Pharmacia, Uppsalla, Sweden); Albumin Bovine, Fraction V Powder; amylase (Mann Research Laboratories) Bacterae; sodium deoxycholate (Nutritional Biochemical Corporation); L-tyrosine (Nutritional Biochemical Corporation); p-phenylenediamine (Matheson, Coleman & Bell) practical; 3,4 dihydroxyphenylacetic acid, cyclohexylamine salt (Calbiochem) A grade; D, L-3,4 dihydroxyphenylalamine (Aldrich); p-cresol (Aldrich); 4-methyl catechol (Aldrich); 3-isopropyl phenol (Aldrich); 3,4 dihydroxybenzoic acid (Aldrich); 3-isopropyl catechol (Aldrich); 3,4 dihydroxycinnamic acid (Aldrich); sodium diethyl dithiocarbamate (Amen Drug).

III. INSTRUMENTS

Oster Automatic Juice Extractor; Sorvall Omni Mixer Homogenizer; Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge; Beckman Spectrophotometer with Gilford 2000 Multiple Sample Absorbance Recorder; Beckman Model L Preparative Ultracentrifuge; Biosonik III, sonic oscillator, Browell Scientific Division of Will Scientific Inc.; French Pressure Cell. American Instrument Company; Cary 14 Recording Spectrophotometer, Varian.

IV. EXPERIMENTAL

Several methods of isolation of the enzyme were attempted. The first attempt employed the procedure used by Bouchilloux, McMahon and Mason (5), involving 30% acetone extraction of an acetone powder, followed by acetone precipitation. Assays of the fractions from the pear preparation showed small amounts of activity everywhere, but none great enough to consider the separation worthwhile. The next attempt followed the procedure of J. R. Walker (6), as used on apples. His procedure involved homogenizing in phosphate buffer, filtering, centrifuging, suspending in 1% KCl, acetone precipitation. His active precipitate at this point dissolved in sodium carbonate. No activity was found in any fraction from the pear preparation.

The third attempt was very simple and crude, but gave assayable material. The procedure consisted of homogenizing pears in a Waring Blendor, squeezing the pulp through a cloth by hand, centrifuging and saving the supernatant. Though the pulp appeared to have more activity than the juice because a few suspended particles of pulp would give activity to otherwise inactive juice, the supernatant was used because it was impossible, at this point, to remove the enzyme from the pulp and obtain a soluble material. Since small particles of pulp would greatly affect the activity of the supernatant and give spurious results, it was necessary to develop a method which would give complete separation of juice and pulp. A commercial apple press was used with excellent results. As this machine was unavailable for further use, a home-made press consisting of a perforated coffee can with wooden plunger was constructed.

However, this press gave no activity in the juice. Since the major difference between the two presses was that the home made press did not macerate the pears before pressing, it was concluded that some sort of grinding of the pears was necessary to release activity into the supernatant. Walker remarked in his work on apples that the enzyme in mature fruit was difficult to isolate because of the tedious separation from starch granules. Activity in the pears was noticed to increase in the juice as the pears ripened. It was thought that possibly the pear enzyme might also be bound to starch and as the pears ripened and the starch became converted to sugar, the enzyme was released. An attempt was made to remove the starch by breaking it down with amylase. However, the supernatant solution from the pear preparation inactivated the amylase. It was not determined what in the supernatant was affecting the amylase. However, there are reports in the literature (7) that certain phenolases can deactivate other enzymes by acting on their tyrosyl residues. However, the pear enzymes, when tested, did not use tyrosine itself as a substrate.

Since this attempt at releasing the enzyme from the pulp failed, it was decided to pursue the small amount of enzyme which appeared to be soluble in the juice. Initial work was done on Bartlett pears. When the season for Bartletts was over, work was switched to D'Anjou pears. The juice from both of these pears gave similar activities.

Purification of the enzyme was next attempted using the following method: Green pears were obtained from Hood River Valley orchards. These were ripened at room temperature. Activity of the enzyme in the

juice increased with ripening and seemed to coincide with an increase in pH from 3 to 5.

Pears were cut into inch cubes, with removal of cores and seeds. These cubes were put into an Oster juicer. The resulting sludge was centrifuged for ten minutes at 12,000 x g at 4°C and filtered through cheese cloth to remove any floating pulp particles. The pulp was discarded. The supernatant solution was heated at 55 degrees centigrade for thirty minutes and centrifuged. The precipitate was discarded. The supernatant was brought to 50% saturation with solid ammonium sulfate and centrifuged. The precipitate contained no activity and was discarded. The supernatant was brought to 85% saturation with solid ammonium sulfate and centrifuged. The precipitate contained some activity and was saved. The supernatant was taken to 100% saturation and centrifuged. The precipitate contained most of the activity, though some remained still in solution. The precipitate was suspended in ten percent sucrose. The enzyme suspension was put on a Sephadex G-100 column and the column eluted with 0.1M Na_2HPO_4 . Resolution into two brown bands was observed. The faster moving band was eluted in the void volume and contained the activity. The second slow moving band showed no activity at all. The spectrum of 280 protein absorption taken as the fractions came off the column showed two peaks, corresponding to the two bands. The first peak was sharply peaked; the second was broad and jagged. In order to resolve the active band, the enzyme solution was put onto a column of Sephadex G-200. Again the solution resolved into 2 brown bands and the enzyme activity was eluted in the void volume.

Since this enzyme required 85 to 100% ammonium sulfate saturation to be salted out, one concluded that it had a relatively small molecular weight. However, this conclusion was contradicted by its activity on the Sephadex columns. One possible explanation for this behavior was that the enzyme was particulate; hence, since it was not really soluble, it would not salt out, and being particulate, it would be extremely large and would not be included in even the largest Sephadex. This explanation was tested by spinning the enzyme preparation in the Beckman, Model L preparative ultra centrifuge for 45 minutes at 125,000 x g. All of the activity was located in the pellet. Hence, the enzyme could not be called soluble. The problem remained to dislodge the enzyme from whatever its place of attachment. The preparation was examined under the microscope and found not to consist of whole cells. Sonic oscillation was employed using the Biosonic oscillator, but proved of no value. The enzyme suspension was brought to two percent in sodium deoxycholate and let stand overnight in the refrigerator. The detergent treatment released no activity into solution. At this point, it was decided to improve the particulate preparation and work with it.

The enzyme 'solution' which was used for the characterization work was prepared as follows. Very ripe pears were cut into cubes and put into the Oster Juicer. The resulting sludge was ground in the Omni Mixer at high speed for three minutes. The slurry was squeezed through cloth using a hydraulic press. The liquid was centrifuged at 800 x g. The supernatant solution was saved. The pellet was ground with one percent sucrose in a Potter Elvehjem Homogenizer; the resulting sludge was

centrifuged at 800 x g and the supernatant combined with the supernatant from the first centrifugation. This combined liquid was centrifuged in the Serval Model RC2-B for two hours at 48,500 x g or the Ultra Centrifuge Model L for forty-five minutes at 125,00 x g. Though the separation obtained from the Model L was superior to that from the Model RC2-B, the Model L head available could handle only one hundred thirty milliliters at a time, whereas the Model RC2-B could accommodate two liters, and hence was used for working up large batches. The pellet from this centrifugation was suspended in one percent sucrose by means of sonic oscillation. This preparation could be stored frozen and could be thawed and frozen again repeatedly with no loss of activity. It could also sit overnight at room temperature with no loss of activity.

Since the enzyme preparation turned a dark brown during the work up, which indicated the formation of product, and since some phenolases are reported to be inactivated during formation of product (8) and thought to polymerize product on their surfaces, a preparation was worked up under an inert atmosphere using a nitrogen filled glove bag and adding ascorbic acid to the slurry from the Oster juicer. The ascorbic acid would reduce any quinone formed and thus prevent polymerization of brown melanin. Since O_2 is consumed in the oxidation, removal of O_2 would prevent oxidation of the substrate. The enzyme solution was dialyzed after suspension in sucrose solution to remove the ascorbic acid which would interfere with the assay system used. The resulting solution was light tan and had a specific activity of thirty which was similar to the previous dark brown preparations. Several methods of estimating

protein concentrations were tried including the Kjeldahl (9), the Biuret (10), and 260-280 absorption (10) (11). The Biuret was used on the final suspensions. The only advantage at this point to the nitrogen-ascorbic acid variation on the preparation was in the lower blank readings for the Biuret protein estimations.

Because of the low specific activity of the pear preparations, the assay system of Miller and Dawson (4) was modified from the original two hundred fifty milliliters final volume to fifty milliliters final volume, in assays for the characterization of the enzyme. The assay system consisted of a three neck flask placed in a constant temperature water bath. Through one neck a tube entered through which air was bubbled. The bubbling air provided the necessary oxygen for the reaction and also mixed the reactants. Through another neck a thick walled capillary tube removed drops of reaction mixture to an external starch-iodide indicator. Through the third neck substrate was added to begin the reaction. The rate of flow of drops through the siphon tube could be regulated by pressure on the stopper in this neck. It was desirable that the reaction be complete within one minute, otherwise it became very difficult to determine the end point because of the self-darkening of the indicator. The reaction flask contained five milliliters of buffer of a pH lower than the desired pH, so that dilution to the final 50 milliliter volume would result in the desired pH. One milliliter of ascorbic acid solution containing one milligram (28 micromoles) of ascorbic acid was added. An amount of water was added which would bring the final volume to 50 milliliters, taking into consideration the amount of enzyme and

substrate which was to be added. Next the enzyme preparation was added - a typical run involved 0.2 milliliters. In characterization work, other than that to determine Michaelis-constants, a tenth of a millimole of substrate in ten milliliters distilled water was added at time zero. The time was recorded at which point the ascorbic acid had all been oxidized. At that time the siphoning drops turned the external starch-iodide blue. It is assumed that the rate determining step in the coupled reaction is the oxidation of the diphenol to quinone by the enzyme and that the ascorbic acid-o-benzoquinone oxidation-reduction takes place instantaneously. Ascorbic acid has been shown to have no effect on the rate of the enzymic reaction (12).

V. RESULTS

Influence of Temperature on Reaction Rate

The reaction flask was placed in a constant temperature water bath of the desired temperature. Water, buffer and ascorbic acid were added to the flask and permitted to come to the desired temperature. The temperature was again checked after addition of enzyme and substrate and no change was noted. Optimum activity of the pear enzyme was spread over a wide range from thirty degrees to fifty degrees centigrade, as can be seen from Figure 1. The pH was maintained at 5.0 as this was the pH used in the assay system as described by Miller and Dawson (4) in their original paper. A temperature of thirty five degrees centigrade was selected at which to run future assays, as this temperature was on the high activity plateau and also required no great length of time in equilibrating reagents to that temperature. Much difficulty was experienced in maintaining temperatures around fifty degrees and above because of the cooling effect of the air being bubbled through the system. This problem was reduced by heating the water above the desired temperature before addition to the flask and by wrapping the air hose around the water bath several times.

Influence of pH on Reaction Rate

Citrate-phosphate, phosphate-phosphate and tris buffers were used to maintain the desired pH. Optimum pH is 7.0 as obtained from Figure 2. Since activity of the enzyme dropped to zero at pH 8.5 and above the

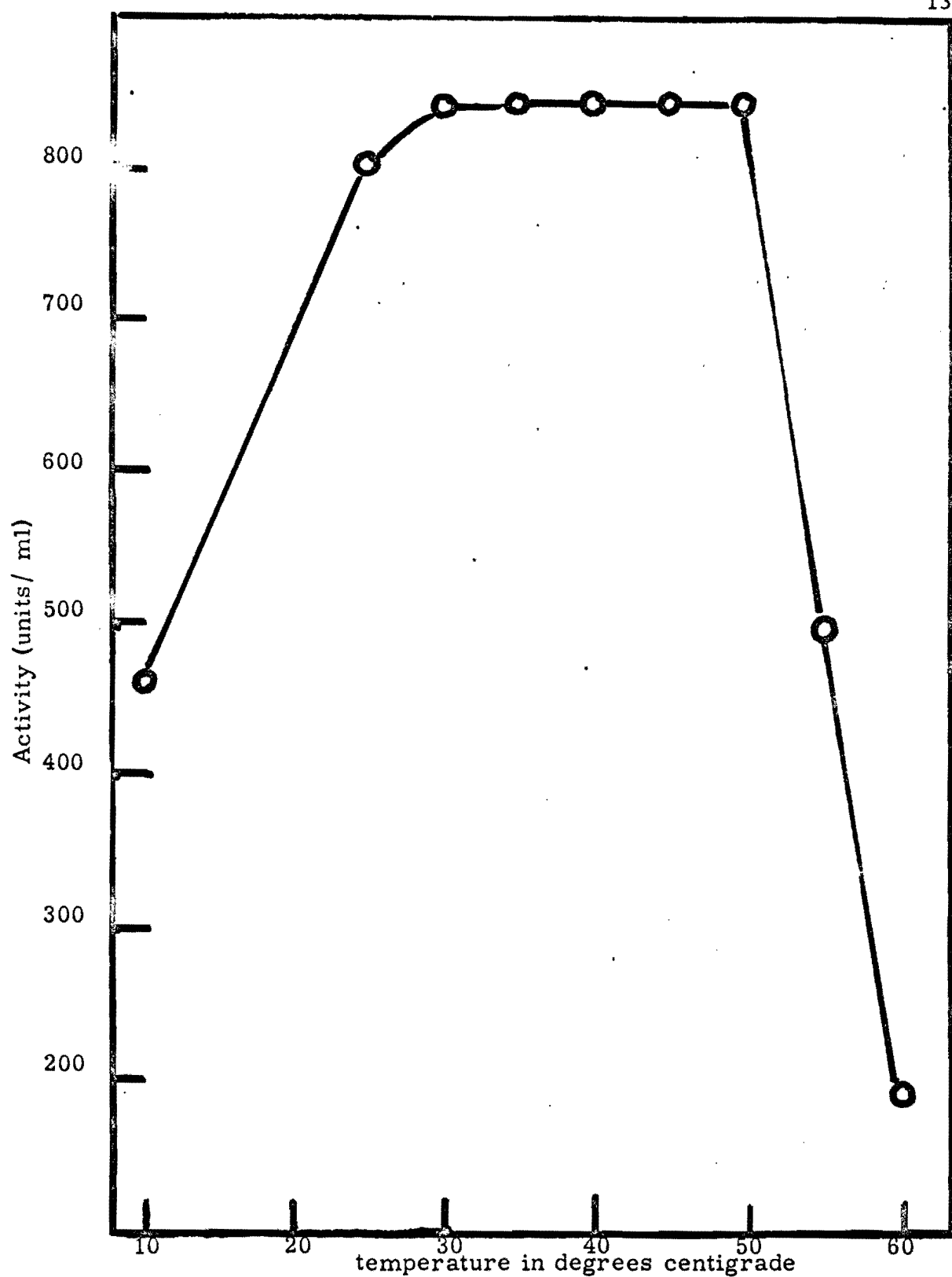


Figure 1. Influence of temperature on reaction rate

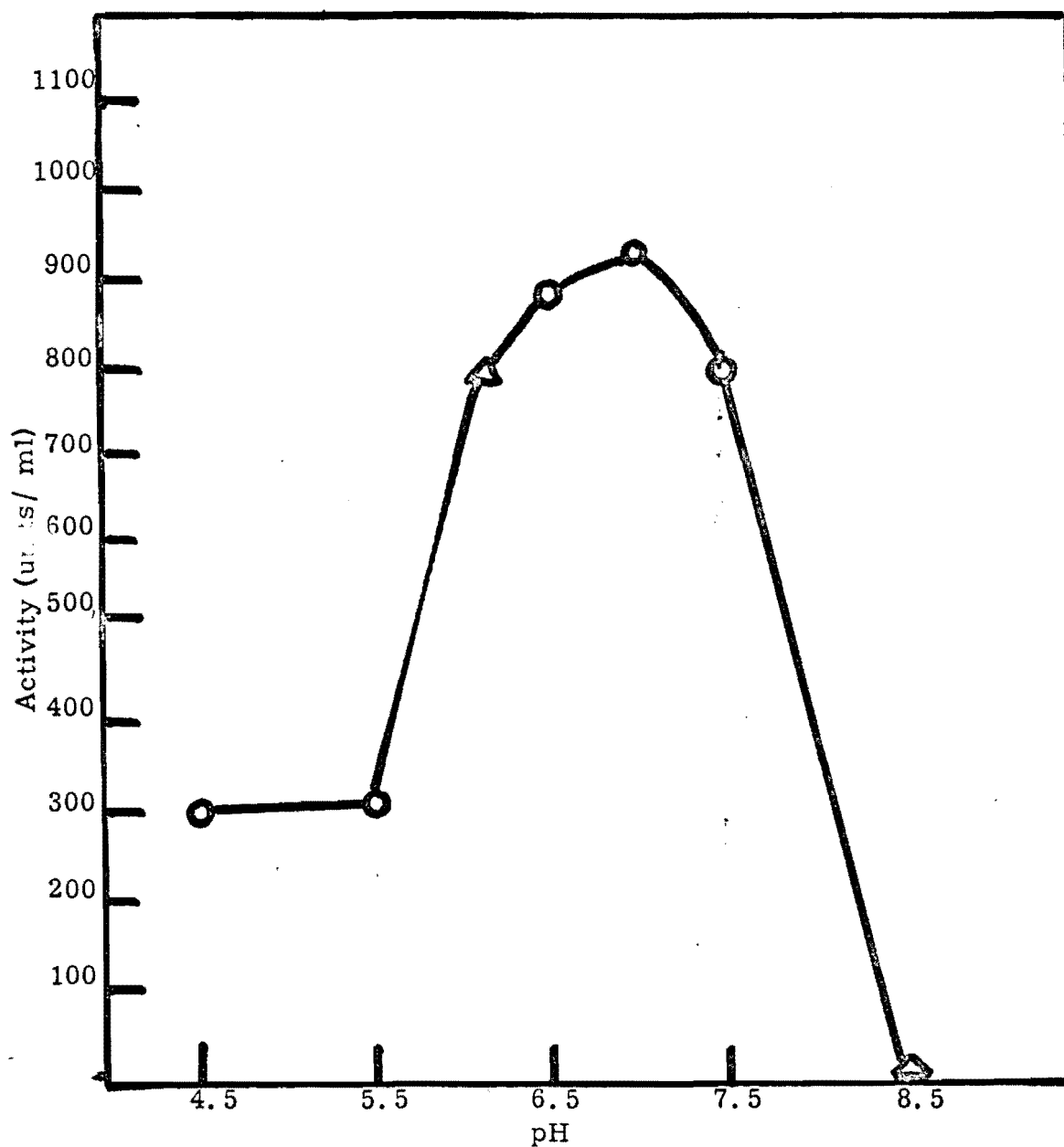


Figure 2. Influence of pH on reaction rate

● citrate-phosphate buffer
▲ phosphate buffer

points using the tris buffers are omitted from the graph, as these were used at pH 9 and 9.5. Since the activity drop to zero corresponded to a change in buffers from citrate-phosphate to phosphate-phosphate, the phosphate-phosphate buffers were checked at a lower pH. The drop in activity was shown not to be a function of the change in buffers. pH 7.0 was selected for future assays. A 0.1 M citrate-0.2M phosphate buffer of pH 6.6 which dilutes to pH 7.0 when 5.0 ml is brought to 50 ml total final volume was employed in all further assay systems. The first pK for catechol at twenty degrees is 9.85 (13). It appears that the enzyme will not act upon the substrate when it is ionized.

Substrate Specificity of the Particulate Enzyme

The particulate pear enzyme was very specific for orthodiphenols only. It did not oxidize mono phenols or meta or para diphenols. Table I summarizes the compounds tested. Cathechol, 4-methyl cathechol and 3-isopropyl catechol were purified by sublimination using a cold finger under vacuum. Recrystallization was used to purify 3,4-dihydroxycinamic acid and p-phenylenediamine. Purity was checked by melting point determinations using the Thiele tube. All substrates tested were at a concentration of 0.1 mM per flask ($2 \times 10^{-3} M$), solubility permitting. The most effective substrate tested was 4-methyl catechol.

Tyrosine was also tried with trace amounts of catechol present in the reaction flask, as other experimentors (14) had found the addition of ortho diphenols to be necessary to overcome a lag period experienced by certain phenolases in oxidizing the monophenols. The addition of

Table I

The Relative Rates of Oxidation of Phenols by the Particulate Enzyme

Compound	Relative Oxidation Rate
4-methyl catechol	100
catechol	59
3-isopropyl catechol	0
DL 3,4 dihydroxyphenylalanine	0
3,4-dihydroxycinamic acid	0
3,4-dihydroxybenzoic acid	0
3,4-dihydroxyphenylacetic acid	0
tyrosine	0
2-isopropyl phenol	0
p-cresol	0
pyrogallol	0
p-phenylene diamine	0

catechol was found to have no effect on inducing the pear enzyme to utilize tyrosine.

Michaelis Constants for the two effective substrates were obtained from double reciprocal plots of activity versus substrate concentration using the method of Lineweaver and Burk (15). See Figures 3 and 4. The K_m for catechol is 1.6×10^{-4} ; that for 4-methyl catechol is 7.2×10^{-3} .

Influence of Oxygen on Reaction Rate

Since oxygen is necessary for the enzymic reaction, the effect of varying the oxygen concentration was studied. The Michaelis Constants mentioned in the last section were obtained from a system using air, which would give a twenty percent oxygen concentration. Another Lineweaver-Burk plot was done using the same experimental methods and reaction flask concentrations as in the twenty percent work, but bubbling one hundred percent oxygen through the system. The Michaelis Constant obtained for catechol at one hundred percent oxygen concentration is 4.6×10^{-3} . See Figure 5.

Inhibitors of Pear Polyphenolase

The inhibitors were added to the reaction flask containing the enzyme and incubated for fifteen minutes before addition of the substrate, catechol. Results are summarized in Table II. Phenolase enzymes contain copper. Inhibition of the pear enzyme by sodium diethyldithiocarbamate suggests that a heavy metal ion, most probably copper in some form, is necessary for this enzyme's activity. Lack of inhibition by iodoacetamide suggests that the activity of pear polyphenolase is not

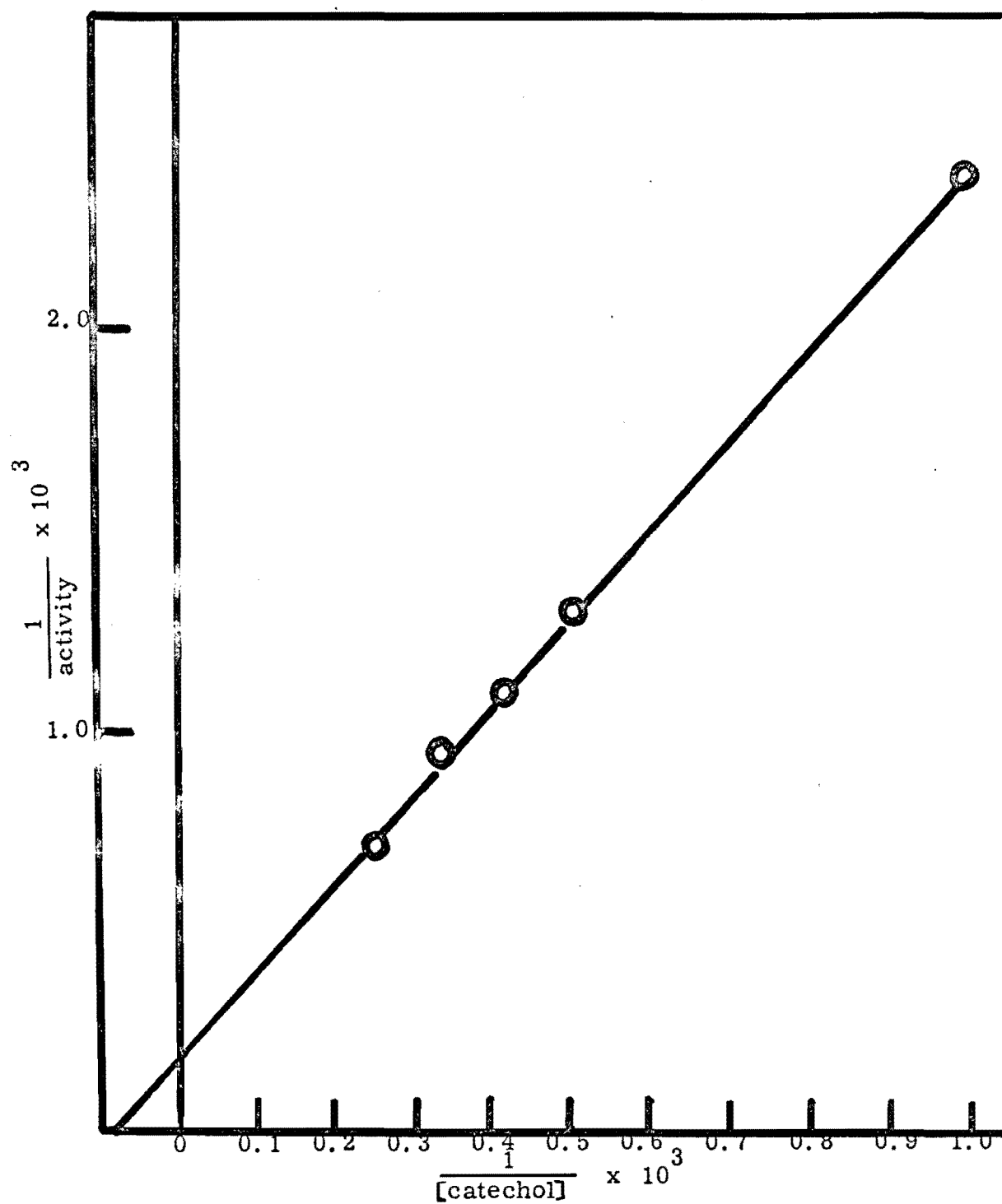


Figure 3: Lineweaver-Burk plot: catechol with 20% oxygen

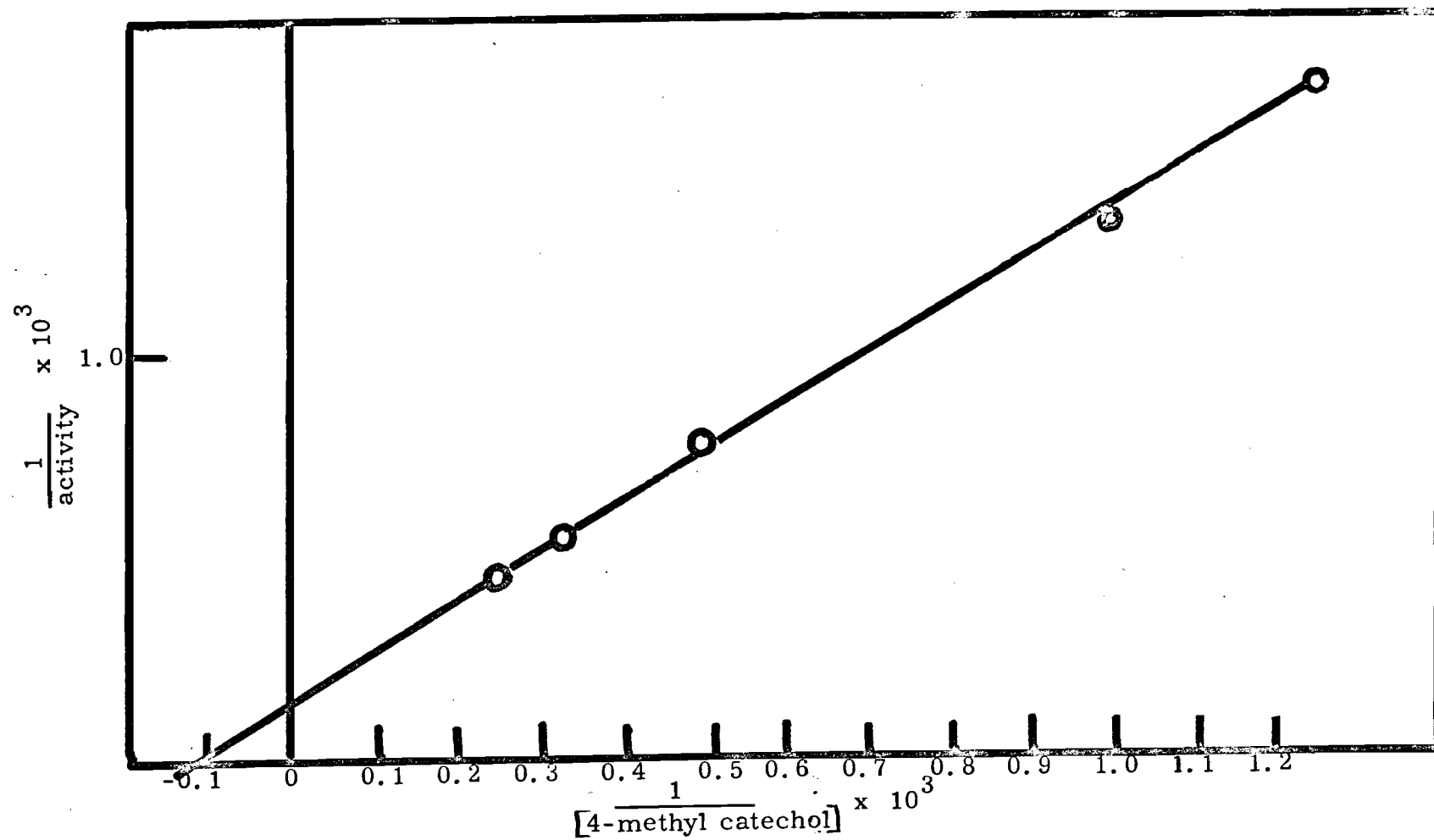


Figure 4: Lineweaver-Burk plot: 4-methyl catechol with 20% oxygen

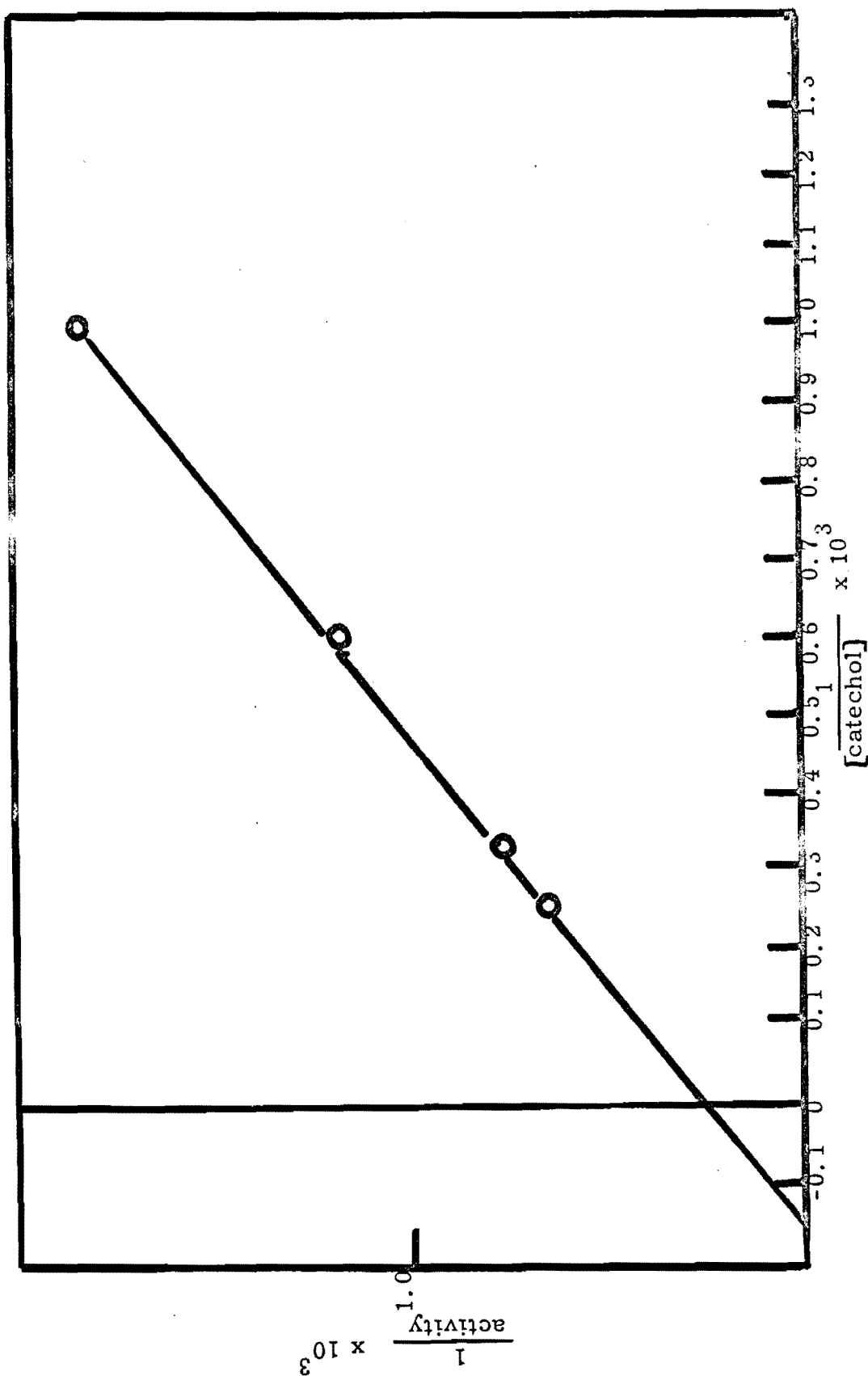


Figure 5. Lineweaver-Burk plot: catechol with 100% oxygen

Table II

Inhibitors of Pear Polyphenolase

Compound	Concentration		% Inhibition
sodium diethldithiocarbamate	1×10^{-3}	1×10^{-3}	100
	1×10^{-4}	1×10^{-4}	100
	0.5×10^{-4}	$.5 \times 10^{-4}$	66
	1×10^{-5}	1×10^{-5}	12
iodoacetamide	1×10^{-3}	1×10^{-3}	0
3-isopropyl catechol	2×10^{-3}	2×10^{-3}	0

dependent on sulfhydryl groups. This is typical of phenolases from many sources.

Two phenolic compounds which do not act as substrates were checked as inhibitors. The compound 3-isopropyl catechol is not utilized by the enzyme as a substrate and does not interfere with the enzyme's activity toward catechol. The compound 3,4-dihydroxyphenylalanine is also not oxidized by the pear enzyme. However, when added to the reaction flask with catechol, the dopa was oxidized to dopachrome, as was evidenced by a red color in the reaction flask. This was investigated further and will be discussed in a later section.

Partial Purification

A particulate enzyme preparation was worked up as described in the Methods section using the nitrogen-ascorbic acid modification. Assay using 0.2 milliliters of the enzyme preparation, 28 micromoles of ascorbic acid and 0.1 millimole of catechol in a total volume of 50 ml, gave an activity of 619 units per milliliter. Twenty milliliters (12,380 total units) of the preparation was put into a French Pressure Cell. (American Instrument Company, Inc.) The cell was placed in a hydraulic press. Pressure was applied to seven thousand pounds per square inch. The cell valve was released gently and the solution run into a fifty milliliter erlenmeyer flask. The solution was warm. Assay of this solution gave an activity of 467 units per milliliter, for a loss of twenty four percent. Thirteen milliliters of the twenty were spun for one hour in the Beckman Model L Preparative Ultracentrifuge at 125,000 x g. Assay of the clear supernatant solution gave an activity of 132 units per

milliliter for a release of twenty one percent into solution. Protein estimation on the supernatant using the 260-280 spectrophotometric method (10) (11) gave a protein concentration of 3.7 mg/ml, which gives the supernatant a specific activity of 36. The pellet from the centrifugation was resuspended in thirteen milliliters of one percent sucrose. Assay on this suspension gave an activity of 330 units per milliliter.

Substrate Specificity of Soluble Enzyme

Selected phenolic compounds were tested as substrates using the modified chronometric method of assay as previously described. The system contained 5.0 ml 0.1M citrate - 0.2M phosphate buffer pH 6.6, 0.5 ml ascorbic acid (14 μ m) 0.5 ml soluble enzyme preparation specific activity 34, 1.0 ml (0.1 mm) substrate, with the total volume brought to 50 ml with distilled water. A summary of the tested compounds is contained in Table III.

Again, as with the particulate enzyme, 4-methyl catechol was the most effective substrate. The relative rate of oxidation of catechol was increased to 73.5%. Also, as in the case of the particulate enzyme, tyrosine was not utilized as a substrate. The case with dopa was uncertain. No end point was noted; however, the reaction solution did turn faint pink at around 200 seconds, which would indicate the possible formation of dopachrome, and gave a relative oxidation rate of approximately 7%. In order to check this, a spectrophotometric assay was run using the Cary 14. The 3 ml cuvette contained 0.1 ml enzyme solution, 0.5 ml catechol (1 μ m) and 2.5 ml 0.1M citrate-0.2M phosphate buffer

Table III

Relative Oxidation of Phenols by Soluble Enzyme

Compound	Relative Oxidation Rate
4-methyl catechol	100
catechol	73.5
tyrosine	0
DL 3,4-dihydroxyphenylalanine	0

pH 7.0. The blank contained buffer only. The reaction was scanned from 250 mμ through 500 mμ. No peaks were observed at 390 or 475 mμ where the dopaquinone and dopachrome would be observed. Curve A, Figure 6 shows dopa itself. Curve B shows dopa after the addition of enzyme. The cause of the pink coloration in the chronometric method was not further investigated.

A Michaelic Constant for catechol was obtained from double reciprocal plots of activity versus substrate concentration using the method of Lineweaver and Burk. See Figure 7. The K_m for catechol is 5.5×10^{-3} for the soluble enzyme as compared with 1.6×10^{-4} for the particulate enzyme preparation.

Spectrum of Soluble Enzyme

The soluble enzyme preparation was diluted from 0.2 milliliters to 3.0 milliliters with 0.1M citrate-0.2M phosphate buffer pH 7.0. The soluble enzyme dilution was then scanned using the Cary 14 from 1900 to 7000 Angstroms. The spectrum was free of any peaks other than that for protein from 260 to 280 millimicrons. See Figure 8.

Spectrophotometric Assay

The spectrophotometric course of the oxidation of catechol at pH 7.0 was followed using the Cary 14. The 3.0 ml cuvette contained 2.5 ml of 0.1M citrate-0.2M phosphate buffer pH 7.0, 0.5 ml of catechol ($1\mu\text{M}$) and 0.1 enzyme solution added to initiate the reaction for a total of 3.0 ml. Figure 9 shows the spectrophotometric course of the reaction.

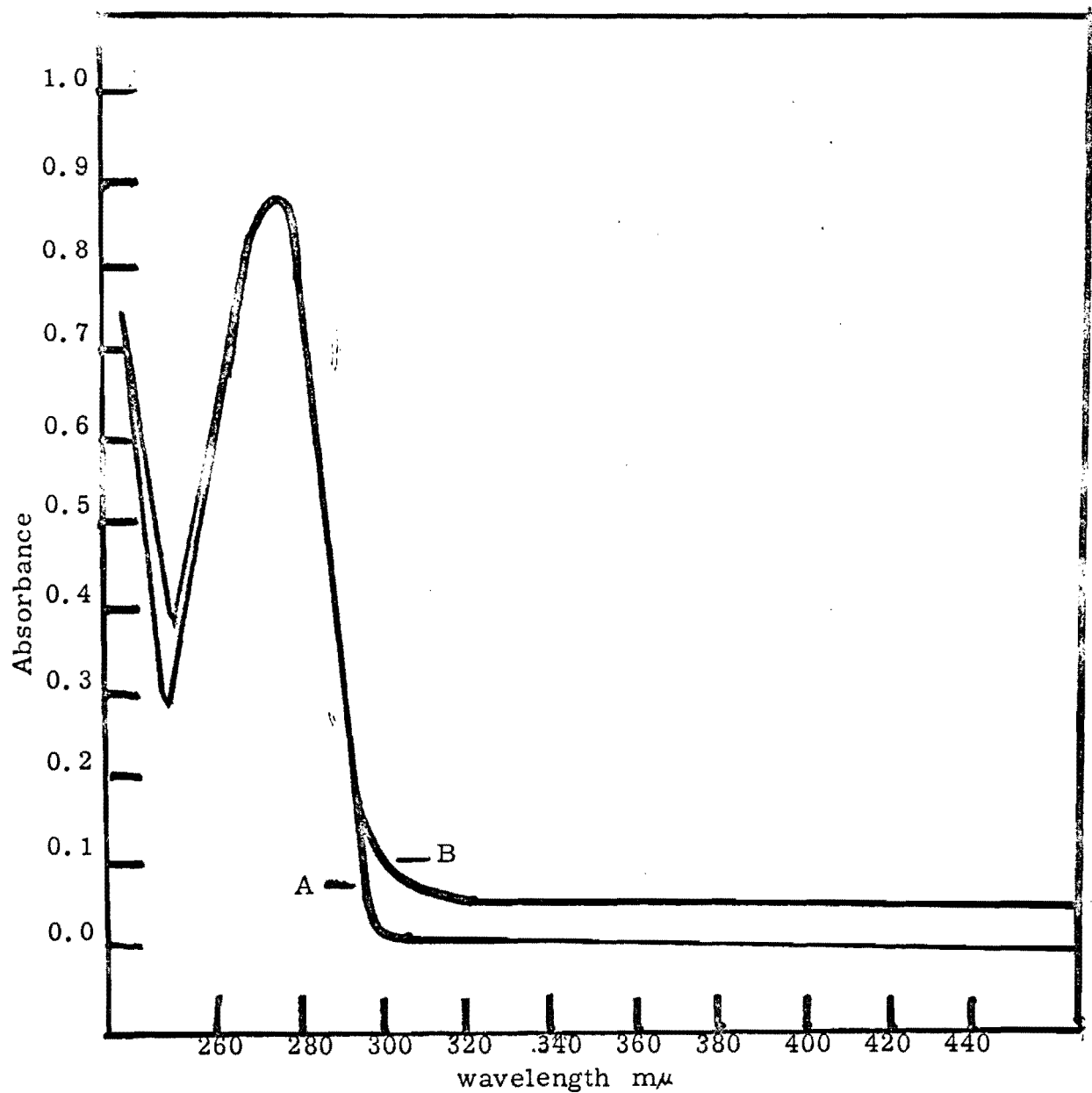


Fig 6 : Spectrophotometric course of enzymic oxidation of dopa. Curve A represents the absorption spectrum before addition of enzyme, Curve B, five minutes after addition of enzyme.

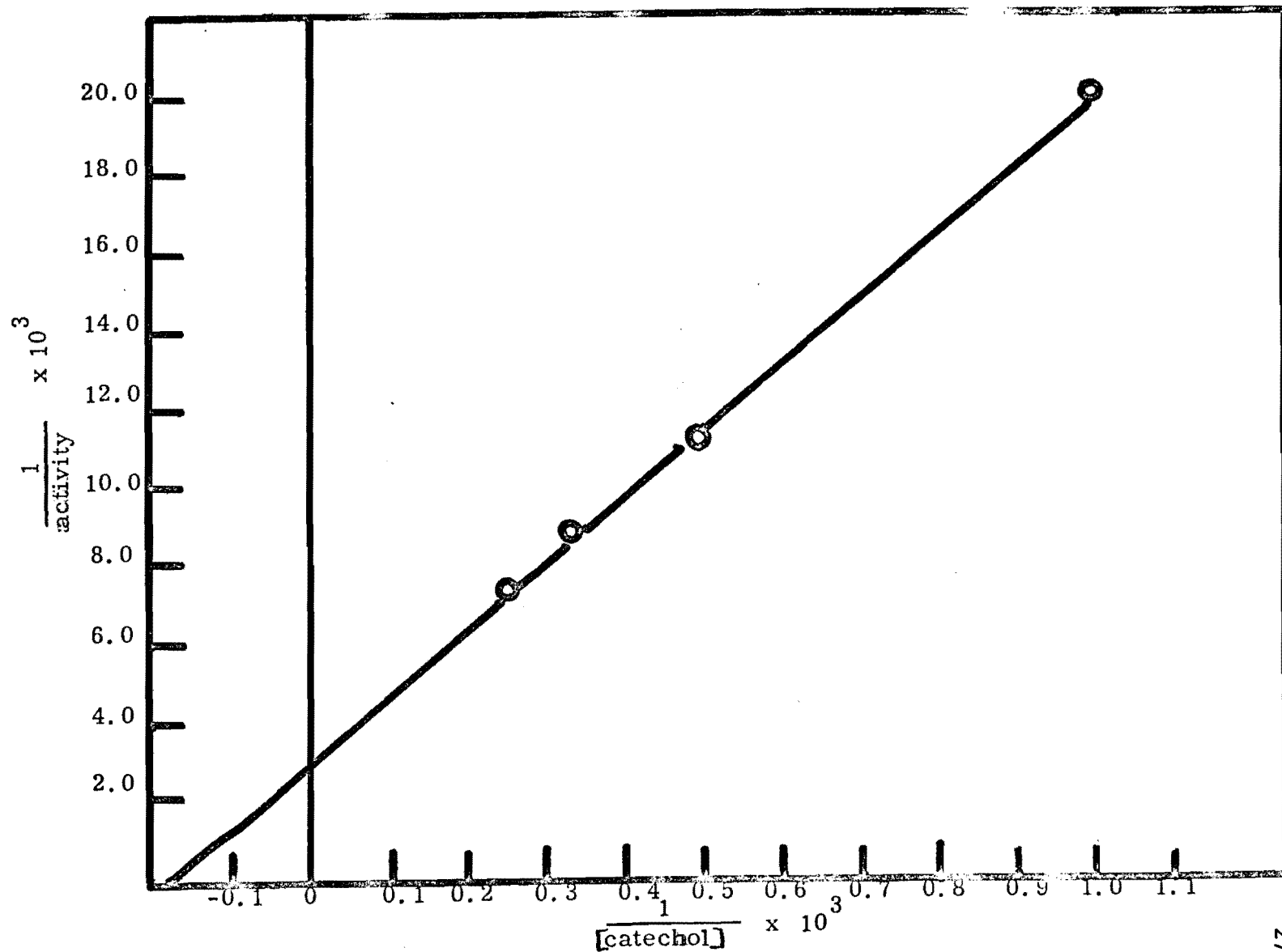


Figure 7: Lineweaver-Burk plot: catechol with 20% oxygen, soluble enzyme

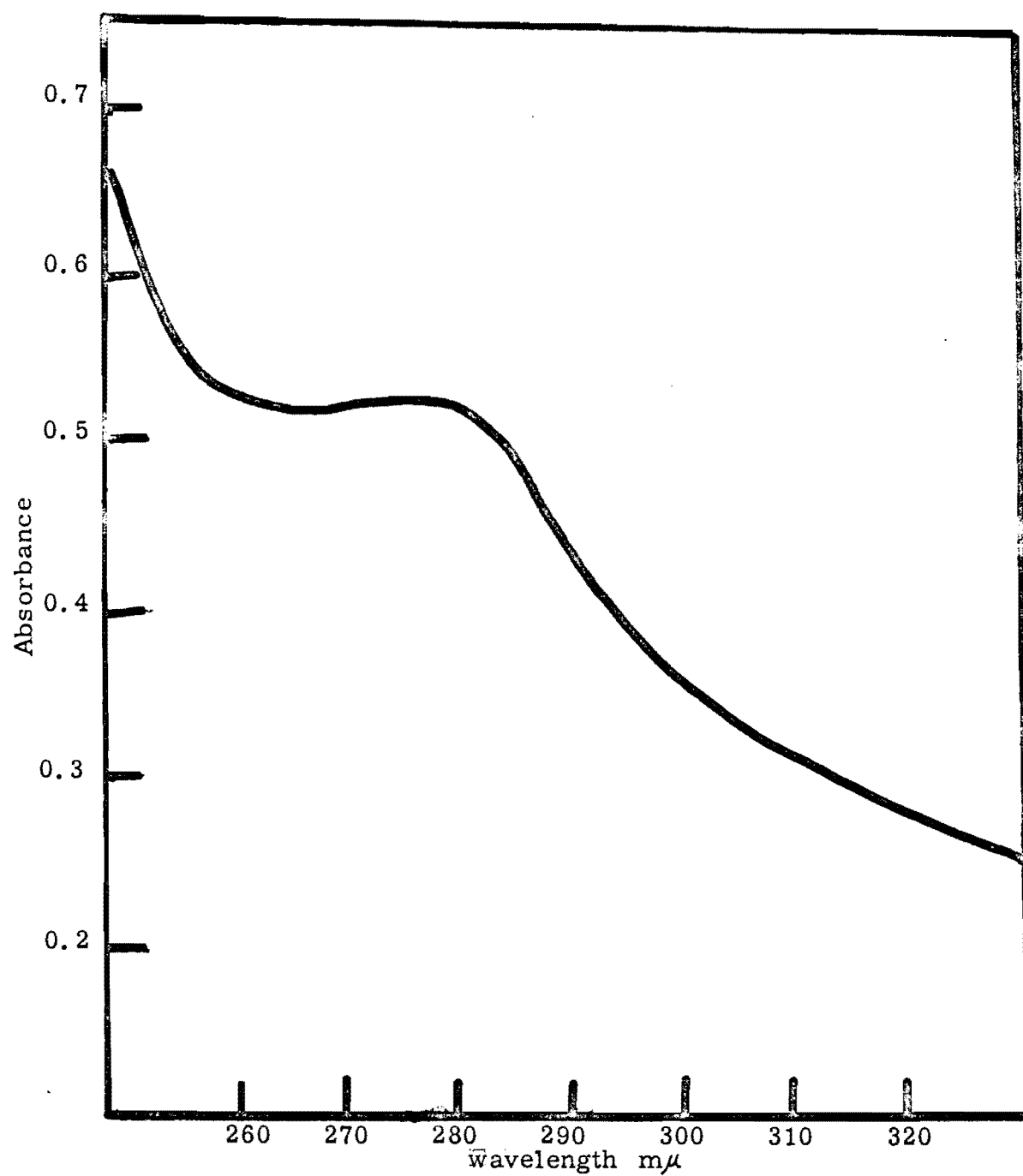


Figure 8: Spectrum of pear catechol oxidase

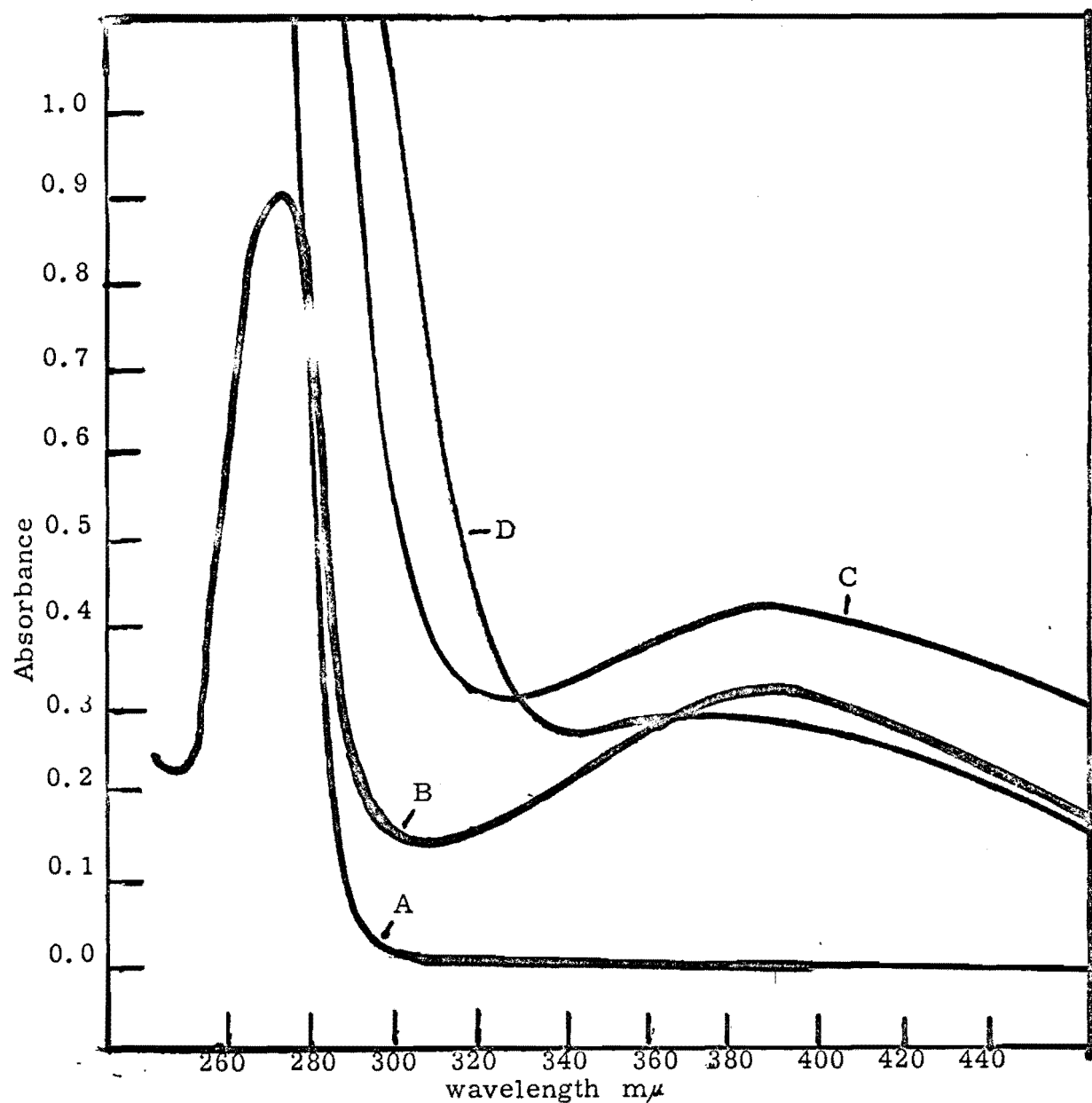


Fig. 9 : Spectrophotometric course of the enzymic oxidation of catechol. Curve A represents the absorption spectrum before addition of enzyme, Curve B, immediately after addition of enzyme, Curve C, after 5 minutes, Curve D, after 15 minutes.

Curve A represents the spectrum before addition of the enzyme. This shows the absorption of catechol which comes about 276 m μ . Curve B represents the reaction immediately after addition of the enzyme. One observed the appearance of a peak at 390 m μ which is characteristic of o-benzoquinone (16). Curve C represents the reaction at five minutes, during which time the o-benzoquinone is still building up. Curve D represents the reaction after 15 minutes. It shows a diminishing of the o-benzoquinone peak and an increase in general absorption.

In the section on inhibitors, it was mentioned that the catechol with dopa oxidation gave unexpected results. Since dopa was shown not to be a substrate, and since the absorption of dopachrome is very easy to follow spectrophotometrically, an attempt was made to couple the enzymic oxidation of catechol to the oxidation of dopa to dopachrome for use as an assay method. Figure 10 shows the spectrum of the reaction immediately after addition of the enzyme. Here one observes the immediate formation of dopachrome as shown by the peak at 475 m μ . No peak at all is present at 390 m μ where the quinones would appear. It would appear from this that the oxidation of dopa to dopachrome (probably by the o-benzoquinone) takes place instantaneously. This would make the coupled reaction a possible spectrophotometric assay system for pear polyphenolase. Naturally, further study must be done to determine the equivalency of catechols to dopachrome and to insure that the presence of dopa does not increase the rate of the enzymatic oxidation of catechol.

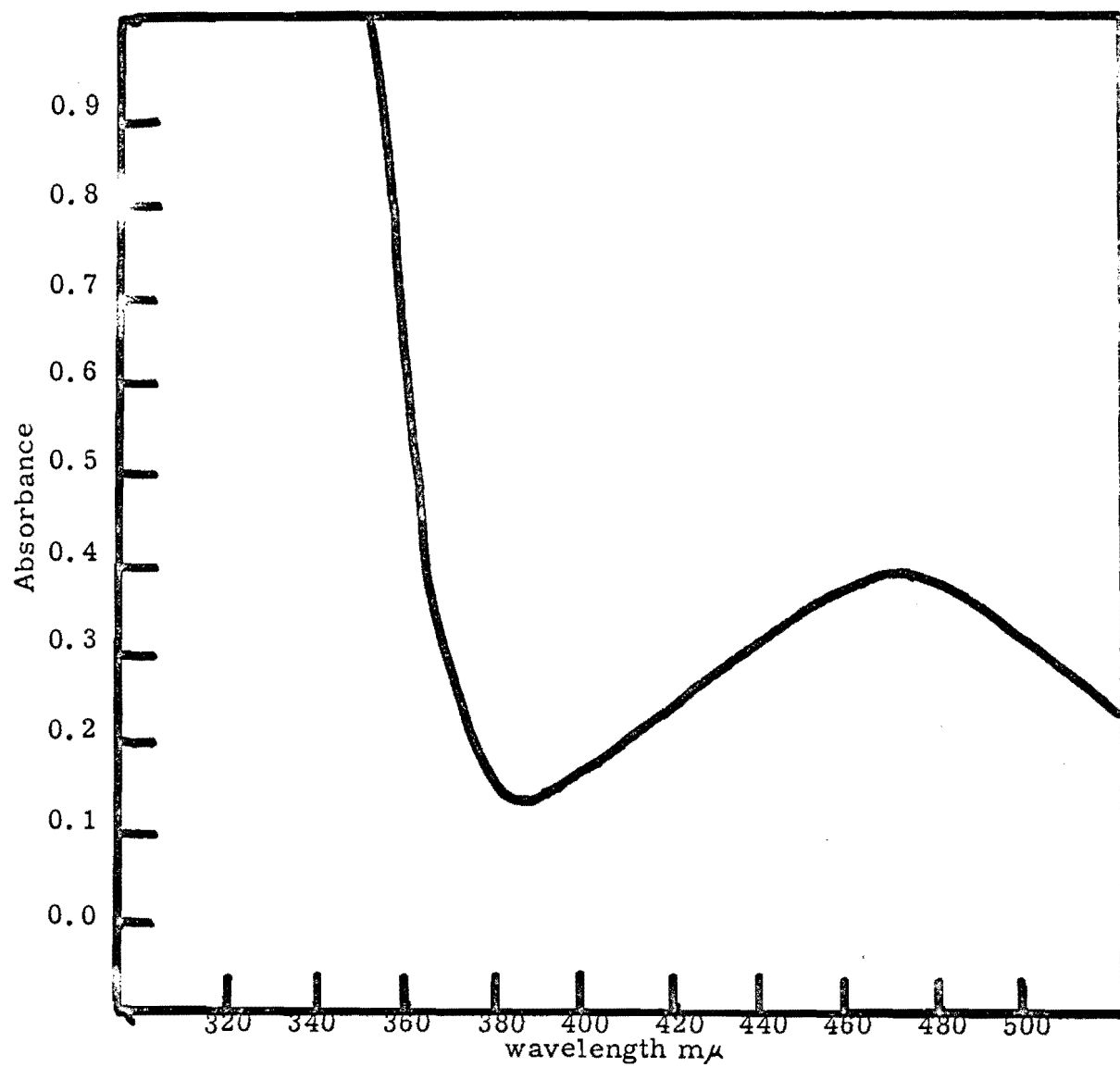


Figure 10: Absorption spectrum of the enzymic oxidation of catechol coupled to the oxidation of dopa.

VI. DISCUSSION

In dealing with enzymes which oxidize phenols, it is necessary to distinguish between the catechol oxidases and the laccases. Generally the catechol oxidases (often called tyrosinases for historical reasons) will use ortho- diphenols and monophenols as substrates. The laccases oxidize ortho-, para- and occasionally meta-diphenols. Some laccases also oxidize monophenols but yield a different immediate oxidation product than the tyrosinases (2). Another distinguishing substrate is p-phenylenediamine which is a substrate for laccase but not for catechol oxidase. Although the pear enzyme did not utilize any monophenols tested, it is classified as a catechol oxidase because it also did not oxidize meta-phenols or p-phenylenediamine.

It is interesting to note that 3-isopropyl catechol is not used by the enzyme, whereas the 4-methyl is. One is led to speculate that perhaps substitution in the three position results in steric interference. Other compounds such as the 3-methyl and 4-isopropyl catechols would shed light on steric effects, but unfortunately, these compounds were not readily available. The compound 1-3picatechin which is considered to be the principle substrate for polyphenolase in apple and pear skins (17) is a catechol derivative substituted in the four position.

The studies with enzyme inhibitors whose results typical of other polyphenolases. The enzyme does not depend on sulfhydryl groups for activity as shown by its insensitivity to iodoacetamide. Inhibition by sodium diethyldithiocarbamate is evidence that a heavy metal, most likely copper, is required for activity.

The exact nature of the particle binding the enzyme was not determined. From observation of the particulate preparation under a microscope, one concludes that all whole cells have been reaptured. The work with emylase is inconclusive as to starch binding. The attempt at solubilization with detergents would suggest that lipid binding is not involved. Another possible site of binding could be cellulose. This could be investigated by attempting to break down the cellulose with cellulase and hence release the enzyme into solution.

The particulate enzyme shows much greater stability than the soluble enzyme in that the particulate enzyme can be stored frozen for weeks with no loss of activity whereas the soluble preparation lost about one third of its activity on being frozen for three days.

VII. CONCLUSION

The pear enzyme is somewhat unique among catechol oxidases in that it does not exhibit both catechol and cresol oxidation activity. As such, it should be of interest to those investigators who have been trying to separate the two activities. Often the cresolase activity of polyphenolases is lost or diminished on purification, but the pear enzyme in even its crudest forms shows no oxidation of monophenols.

The catechol-dopa coupled oxidation needs further investigation before employment as an assay system, but shows promise in that it requires a very small amount of enzyme, is not dependent on human color judgement and can give a permanent record of the reaction.

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