STUDIES OF TYROSINASE ACTIVATION

AND

ITS REVERSIBILITY

by

James O. Alben

A THESIS

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(Chairman, Graduate Council)

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INTRODUCTION AND REVIEW OF THE LITERATURE

The manner in which molecular oxygen is controlled by non-heme enzymes is only beginning to be understood. The enzymic mechanism of incorporation of molecular oxygen into a substrate is completely unknown. The copper enzyme (1-4), tyrosinase, is a convenient material for studying these mechanisms. Tyrosinase, which has a molecular weight in the order of 100,000 (5) and contains four atoms of copper per molecule (5), catalyzes two distinct types of reactions (6-8): the aerobic (9, 10) dehydrogenation of o-diphenols forming o-quinone (9-11), plus water, and the incorporation of one atom of molecular (12) oxygen into monophenols to form either the corresponding o-diphenol (13-15) or o-quinone (11). Hydrogen peroxide is not formed (10, 16, 17). These reactions have been referred to as the diphenolase and monophenolase activities of tyrosinase.

The enzymically catalyzed oxidation of monophenols is characterized by an initially low rate which increases to a maximum as the reaction progresses (18-20). This sub-maximal rate has been termed the induction period, and compounds which increase the initial rate are called activators. Several types of reducing agents (19) activate enzymically catalyzed monophenol oxidation. Among them are ortho-diphenols (1, 18), ascorbic acid (15, 20, 21), reduced diphosphopyridine nucleotide (20) and ferrocyanide (19). The reaction products of o-diphenol oxidation do not activate the monophenolase function (22). This activation has been

studied indirectly by measuring the rate of oxygen consumption in the Warburg respirometer (19) and directly by measuring the monophenol concentration during the reaction (15, 20, 23). It is not known whether reducing agents other than o-diphenols act directly on the enzyme molecule (20) or indirectly by favoring o-diphenol accumulation (19, 22, 24). The first possibility was suggested by the rapid initial rate of decrease in monophenol concentration in the presence of ascorbic acid or reduced diphosphopyridine nucleotide (20). Kubowitz (2) has demonstrated that tyrosinase from the potato will reversibly bind carbon monoxide in the presence, but not in the absence, of catechol with a stoichiometry of two atoms of copper per molecule of carbon monoxide. Correlating this data with the activation by reducing agents, several workers have suggested that activation of the monophenolase activity may involve a reduction of cupric enzyme to the cuprous form (2, 19, 25). However, the valence state of the metal has not been determined.

The autocatalytic nature of the monophenolase reaction has intrigued investigators for many years. Since o-quinones, which are produced in the reaction, undergo reductive polymerization (26), or condensation with nucleophilic groups (cf. 11), and in the presence of reducing agents are rapidly reduced to o-diphenols (13, 15, 27), it has been suggested that the increase from an initially low rate to a maximum (cf. 20, 21, 23) is caused by the accumulation of small amounts of o-diphenol in the reaction mixture (17, 19). Laccase (6, 19), or pyrocatechase (24), which remove o-diphenols from the reaction mixture, inhibit monophenol oxidation.

Added o-diphenol relieves the inhibition by pyrocatechase only as long as the diphenol is present in the reaction mixture. Reducing agents which are not oxidized by tyrosinase relieve the pyrochatechase inhibition only when diphenol is also added (24).

Thus, two general mechanisms of monophenolase activation have been suggested: 1. activation consists in the reduction of cupric-tyrosinase to the cuprous form (2, 19, 25); 2. o-diphenol must be oxidized simultaneously in order for monophenol to be oxidized (19, 22, 24).

Doskocil (17) has suggested that the first possibility represents the activation mechanism but that the cuprous-enzyme is highly unstable in the presence of oxygen and is rapidly reoxidized, requiring diphenol to be oxidized simultaneously with monophenol. Mason (25, 28) has suggested several possible reaction mechanisms which might explain the observed data. Tsou and Li (29) have observed that in the presence of high enzyme concentration, phenol, but not p-cresol, is aerobically oxidized more rapidly in the presence of 50% ethanol than in its absence. Thus a third possible mechanism of activation might involve the structure of the protein rather than the enzymic oxidation of o-diphenol. Kertesz (30) has recently still maintained his position that monophenols are non-enzymically oxidized by quinone and hydrogen peroxide in the presence of tyrosinase in spite of the large amount of evidence against this possibility (cf. 8, 10, 12, 16, 17).

For a more general discussion of tyrosinase, the reader is referred to several reviews (28, 32-39).

STATEMENT OF THE PROBLEM

Previous workers have demonstrated that during the monophenolase reaction, conditions which tend to decrease the concentration of o-diphenol also decrease the rate of monophenol oxidation. This has usually been interpreted in terms of the valence state of copper in the enzyme, copper being reduced to cuprous, thereby activating the monophenolase function; or with the removal of o-diphenol, the active cuprous enzyme being rapidly oxidized to an inactive cupric form by molecular oxygen. The data presented may be more generally interpreted, however, to mean that whatever the mode of monophenolase activation by o-diphenols, that activation is reversible in the presence of molecular oxygen. Thus, it was decided to determine whether the enzyme could be activated anaerobically, testing for activation by injecting the "anaerobically activated" enzyme into oxygenated monophenol and following the formation of quinone spectrophotometrically. The approach is similar to that used by Kearney (40) in her study of succinic dehydrogenase activation. Catechol, which may act as a substrate as well as an activator, was added to a solution of tyrosinase in the absence of a second required substrate, oxygen, equilibrated, then dialyzed to remove most of the activator and any products of the activation reaction. The enzyme was then tested for activation as indicated above. If activation were observed under these conditions, it would demonstrate that activation could occur prior to oxygenation of the enzyme. If an increased

rate of monophenol oxidation were not observed, it would indicate either that activation could not occur prior to oxygenation, or that the activation was reversible. By using catechol (a substrate) as the activator, it was anticipated that any oxygenated form of the enzyme which might still be present under anaerobic conditions would be converted to the completely deoxygenated form. In addition, other reducing agents which act as activators may not affect the enzyme molecule directly, but rather may act only by reducing o-quinone to o-diphenol.

In view of the finding of Tsou and Li (29) that under certain conditions tyrosinase may be activated by organic solvents, it was desirable to determine whether this activation also might be reversible.

METHODS AND MATERIALS

Tyrosinase Preparation. Thirty pounds of frozen mushrooms were ground into five gallons of acetone at minus 20-25°C and extracted for two hours. The precipitate was filtered and extracted overnight with 16 liters of 0.06 M sodium benzoate solution. The residue was removed by filtration. Solid ammonium sulfate was added to saturation and the suspension stirred overnight. The precipitate was filtered, packed dry into dialysis tubing, and dialyzed against running tap water until no precipitate was observed in the dialyzing water with barium acetate. The dialyzed enzyme was filtered and assayed (ammonium sulfate precipitate) (Fraction VIa).

The first lead acetate precipitation and first fractional adsorption on calcium phosphate gel were carried out according to the method of Keilin and Mann (3). Four milliliters of 1/10 saturated lead acetate solution was added to each 100 ml of VIa. The suspension was centrifuged and the supernatant fluid (Fraction VII) decanted and assayed. Two grams of calcium phosphate gel were stirred into fraction VII, permitted to stand overnight, and filtered. The filtrate was mixed with 9 grams of freshly precipitated calcium phosphate gel, permitted to stand for 30 minutes, and centrifuged. The gel was eluted with 0.5 M K_2HPO_4. Since the supernatant fluid from the phosphate gel treatment of the filtrate contained most of the activity and was lighter in color than the eluate, only the supernatant fluid was used for further

purification. The supernatant fluid was dialyzed against tap water for two days, against deionized water for an additional day (Fraction VIIIbd), and assayed.

Calcium phosphate gel was prepared by adding excess Na_3PO_4 to a solution of $CaCl_2$ and washing the precipitate several times with distilled water to remove the excess Na_3PO_4 as well as the largest and smallest particles of gel. Sufficient gel suspension (3.5 grams of gel in a volume of 93 ml) was added to 1.46 liters of fraction VIIIbd (which contained 1.13 grams of enzyme mixture) to adsorb 98% of the catecholase activity. The mixture was permitted to equilibrate for 90 minutes, and then centrifuged. The gel was resuspended in 83 ml of distilled water, mixed with an equal volume of Hi Flow Supercel suspension (11.7 grams) which had been previously washed several times with distilled water to remove the small particles, packed into a column and eluted under vacuum from a water pump. Deionized water (100 ml) was passed through the column to remove weakly adsorbed protein, and was followed by 381 ml of 0.001 M K_2HPO_4 which eluted most of the enzyme. Fractions with the highest specific activity were combined and dialyzed for two hours against 8 liters of continuously flowing deionized water (IXab-1). The purification procedures are summarized in Tables I and II. All procedures following filtration of the first acetone extract were conducted at 0° - 2° C. in a cold room. The protein concentrations of fractions listed in Table II were determined by drying aliquots to constant weight under vacuum over P_2O_5. Two of the purification procedures listed in

TABLE I

TYROSINASE PURIFICATION PROCEDURE

Thirty pounds of frozen mushrooms ground and extracted into 5 gallons of acetone at -20° to -25°C for 2 hours

↓ Filtered

Precipitate — Filtrate

Extracted overnight into 16 liters of 0.06 M sodium bemzoate at 0°-1°C

↓ Filtered

Precipitate — Filtrate = 21 liters Saturated with solid $(NH_4)_2SO_4$

↓ Filtered

Precipitate — Filtrate

Dialyzed against running tap water until sulfate-free

↓ Filtered

Precipitate — Filtrate (VIa), 1.425 liters, 2,780,000 units Plus 4 ml of 1/10 saturated $Pb(OAc)_2$/100 ml of VIa

↓ Centrifuged

Precipitate — Supernate (VII), 1.46 liters, 2,810,000 units Plus 2 grams Calcium Phosphate gel; equilibrated overnight

↓ Filtered

Precipitate 138,000 units — Filtrate, 1.50 liters, 4,390,000 units Plus 9 grams fresh Calcium Phosphate gel; equilibrated 30 minutes

↓ Centrifuged

Precipitate — Supernate (8b)

TABLE I (Continued)

Supernate (8b), 1.61 liters, 2,660,000 units
Dialyzed against running tap water for two days and against deionized water for one day.

Dialyzed Enzyme (VIIIbd), 1.46 liters, 1,570,000 units
Plus 3.5 grams fresh Calcium Phosphate gel; equilibrated 90 minutes

Centrifuged

Precipitate
Suspended in 83 ml of distilled water. Plus 11.7 grams High Flow Supercel suspended in 83 ml of water

Supernate, 1.61 liters, 30,000 units

Eluted under vacuum from water pump:
1) with 100 ml of deionized water
2) with 0.001 M K_2HPO_4

Eluate	ml	Total Units	Units/ml	O.D.$_{280}$/cm	$\frac{\text{Units/ml}}{\text{O.D.}_{280}/\text{cm}}$
1a	28	--	--	--	--
b	201	--	--	0.630	--
2a	70	210,000	3,000	2.18	1,380
b	30	389,400	13,000	6.24	2,080
c	153	757,000	4,950	4.62	1,071
d	128	70,300	570	1.26	452

Eluate Fractions 2a and 2b were combined and dialyzed against 8 liters of continuously flowing deionized water for two hours to yield Fraction IXab-1.

TABLE II

PURIFICATION OF TYROSINASE

Fraction	Procedure	Units*/ml	Total Units	mg/ml	Units/mg	Total Protein (mg)	Fold Purified Over VIa
VIa	Ammonium Sulfate Precipitate	1,950	2,780,000	9.48	206	13,400	1
VII	Lead Acetate Supernate	1,920	2,810,000	8.84	217	12,950	1.05
VIIIbd	1st Fractional Calcium Phosphate Supernate	1,074	1,570,000	0.777	1,382	1,136	6.7
IXab-1	2nd Fractional Calcium Phosphate Eluate	6,009	551,000	1.76	3,414	161	16.61
Xc**	Concentrate	32,200	493,000	9.44	3,414	144	---
Xw**	Washings From Concentrate	6,390	40,800	1.87	3,414	12	---

*Catecholase units were determined as described in the text.

**Activity and dry weight were estimated from IXab-1.

Table I produced an increase in apparent catecholase activity. However, the purification procedure was not thoroughly studied, and no explanation for the increase in activity can be given at this time.

The dialyzed enzyme was concentrated over $CaCl_2$ and KOH under vacuum from a water pump and then poured into a small, glass-stoppered cylinder (Fraction Xc). The sides of the concentrating-vessel were washed with deionized water which was collected separately to yield fraction Xw. The concentrated enzyme (Fractions Xc and Xw) were stored at 0° - 5° C. in a refrigerator until used. Free boundary micro-electrophoresis in the Antweiler apparatus (0.5 ml of Xc in 0.2 $\frac{\Gamma}{2}$ sodium barbital buffer, pH 8.6, electrophoresed 14 minutes at 74 volts and 1.8 milliamperes) indicated that two components were present in the concentrated enzyme solution. The copper content of fraction Xc was 10.5 micrograms/ml (estimated to be 0.11% of the dry weight of the enzyme preparation). After four months of storage in the cold, a small amount of brown precipitate was detected in fraction Xc, which was removed by filtration. The filtrate was designated Xf, and contained 12.0 micrograms of copper/ml. The spectral characteristics of Xf are described in Table III.

Assay of Catecholase Activity. Catecholase activity was determined with the Cary recording spectrophotometer essentially according to the method of El-Bayoumi and Frieden (41) by following the decrease in ascorbate concentration at 265 millimicrons. The method was

modified by bubbling oxygen through the reaction mixture for two minutes prior to initiating the reaction by adding enzyme. This procedure did not change the initially constant rate but rather prolonged the zero order portion of the reaction which was measured during the period between 12 and 30 seconds after adding the enzyme. According to the data of El-Bayoumi and Frieden, one catecholase unit (10 cmm oxygen consumed per minute at 37° C.) is equal to a change in absorbancy of 2.3 per minute at 25° C., using a 1cm cuvette. For enzyme assay, 0.04 to 0.18 catecholase units were added per ml of reaction mixture.

Copper was determined according to the method of Peterson and Bollier (42).

Substrates used were Distillation Products Industries Division of Eastman Kodak Company's white label grade of catechol and 3,4-dimethylphenol, which were used without further purification. The catechol melted at 103-104°C., and the 3,4-dimethylphenol at 64.0-64.7°C. The latter was recrystallized once from either 10% ethanol in water, or benzene, and dryed over P_2O_5 plus KOH in a dessicator, or in a current of nitrogen, respectively, giving melting points of 64.6 - 65.2°C., and 64.2 - 65.0°C. Thus no gross contamination was present in the 3,4-dimethylphenol. All reagents were dissolved in distilled, deionized water.

Anaerobic Dialysis Apparatus. Since it was essential for the purpose of this experiment to minimize the oxygen concentration during the "anaerobic activation", great precautions were taken toward this end. In general, the apparatus consisted of stores of deoxygenated nitrogen and deoxygenated deionized water which could be flowed past a cellophane dialysis sack containing enzyme. After flowing past the dialysis sack, the water was led through a cell containing a Clark (43) polyethylene membrane-covered electrode* which permitted measurement of oxygen tension. By this means, oxygen tensions, estimated to be less than 0.01% of one atmosphere, could be maintained.

Oxygen-free nitrogen was prepared by passing pre-purified nitrogen** through a series of three gas washing bottles each containing 25 grams of pyrogallol in 250 ml of 30% NaOH, followed by two gas washing bottles containing concentrated sulfuric acid, and a third partially filled with KOH pellets. The dried nitrogen was then passed through a series of two copper furnaces at approximately 450° C., the copper in which had been reduced with hydrogen immediately prior to use.

A store of deoxygenated water was prepared by filling four 5-gallon Pyrex carboys with distilled, deionized water, boiling the water under vacuum from a water pump, and replacing the vapor phase

*Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio. Loaned to the author through the courtesy of Dr. B.B. Ross, Department of Physiology, University of Oregon Medical School.

**99.996% N_2 purchased from the Matheson Company, Newark, Calif.

by bubbling in deoxygenated nitrogen. The water was radiantly heated during the boiling operation with six external 300 watt reflector lamps. The sides of the carboys opposite the lamps were painted black and covered with a layer of aluminum foil to increase the absorption of the radiant heat. The carboys were arranged in parallel with respect to the gas phase, and in series with respect to water. Each water siphon between the carboys was connected to the vacuum line through a high vacuum stopcock, permitting removal of trapped gas or vapor bubbles each time after the water was boiled. Connections to each carboy were made through a large rubber stopper which was effectively sealed against the atmosphere with a thick covering of an epoxy resin (Weatherban*).

Water and nitrogen saturated with water were conducted from the carboys through a cooling coil or cooling coil and water trap, respectively, in a controlled-temperature water bath, to the dialysis vessel. The details of this apparatus are shown in Figures 1 and 2. The entire apparatus was constructed of Pyrex brand glass except for the rubber stoppers closing the carboys, sealed as indicated above, and the copper furnace tubes, which were of Vycor brand glass. The components were connected with 8 mm OD glass tubing fitted with size 12/5 ball and socket joints as indicated in Figure 1. All ground glass joints and stopcocks were lubricated with Heavy Celvacene** vacuum grease. The dialysis

*Generous samples were donated through the courtesy of the Minnesota Mining and Manufacturing Company, Detroit, Michigan.

**Purchased from Consolidated Electrodynamics Corp., Rochester, N.Y.

Figure 1. Schematic Diagram of Deoxygenated Nitrogen and Water Supply Systems.

I. Nitrogen enters at the mercury pressure trap (A), passes through pyrogallol gas washing bottles (B), sulfuric acid gas washing bottles (C), drying bottle partially filled with KOH pellets (D), and copper furnaces at 450°C. (E), and is bubbled through deionized water in 5-gallon carboys (F). Nitrogen is led from the carboys (F) past a mercury manometer, and through a cooling coil and water trap (G). The gas pressure over the water may be reduced by closing the appropriate stopcocks and connecting the line at "Vac 1" to a water pump. The water siphons may be cleared of bubbles with reduced pressure at "Vacuum 2." Water from the carboys is led through a cooling coil to point "H." Size 12/5 ball and socket joints are indicated by arcs, and stopcocks by circles.

II. Connections to Anaerobic Dialysis Apparatus:

Gas Phase: Nitrogen entering from "G" may be conducted directly through the Anaerobic Addition Chamber and Upper Chamber of the dialysis vessel (I) (see Figure 2), or in the reverse direction to increase the pressure in the Upper Chamber, or past the dialysis apparatus, as desired.

Water Phase: Water from "H" passes through the Lower Chamber (J) of one or both dialysis vessels, and out either past the Clark electrode (K) or to the side for sampling.

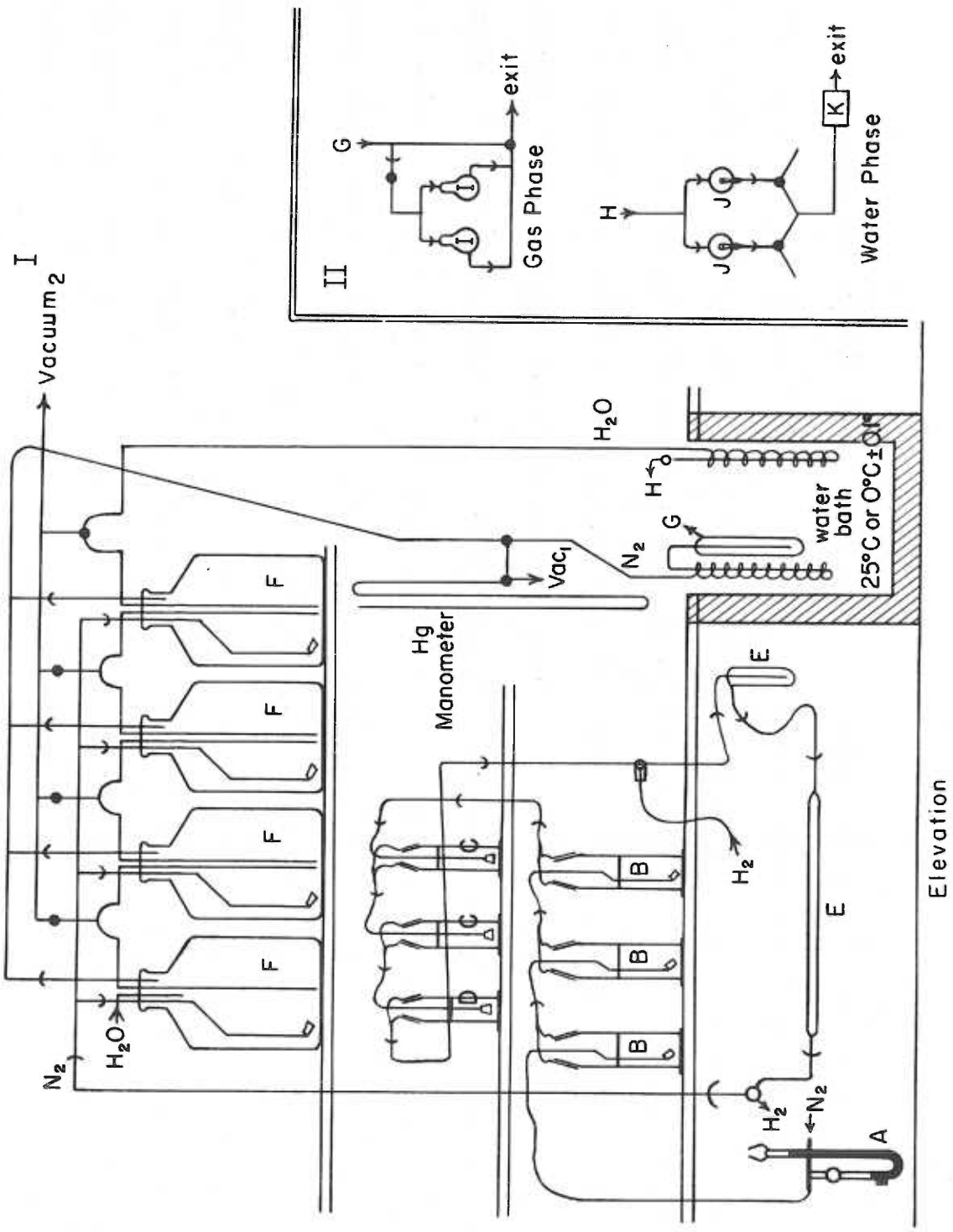
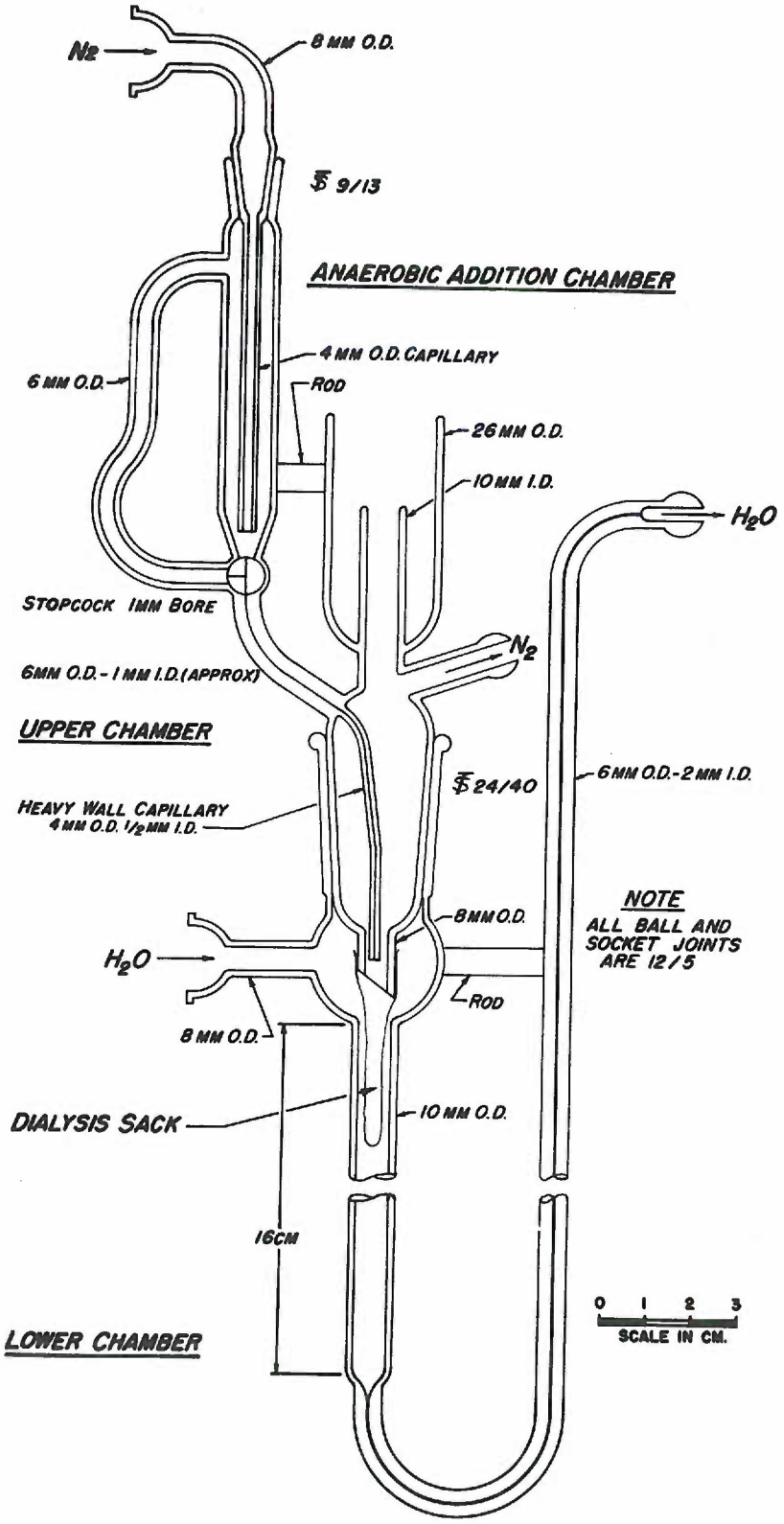

I
Vacuum 2
II
G
exit
Gas Phase
H
J
K
exit
Water Phase
H2O
H
G
water bath
25°C or 0°C ± 0.1°
N2
Vac1
Hg Manometer
F
F
F
F
E
E
B
B
B
C
C
D
H2
H2
N2
N2
H2O
A
Elevation

Figure 2. Anaerobic Dialysis Vessel. Constructed to the author's specifications by Mr. Joseph Griffith, Electro-Glass Laboratories.



UNIVERSITY OF OREGON MEDICAL SCHOOL

ANAEROBIC DIALYSIS VESSEL

DESIGNED BY J.O.A. OCT. 22, 1958 DRAWN BY T.H.R.

vessels (Fig. 2) were mounted in a water bath which was controlled at either 0-0.1° C. or 25° ± 0.1° C.

Anaerobic Sampling from the Dialysis Sack. The upper section of the dialysis vessel (Fig. 2) contained a vertical tube which could be fitted with a rubber stopper such as those used for injection bottles. The glass cup surrounding this tube was filled with water to about one inch above the injection stopper. A layer of toluene was placed over the water, which was bubbled with deoxygenated nitrogen. This procedure not only effectively sealed the gas phase of the dialysis vessel against the atmosphere, but also permitted the admission of an injection needle into the dialysis vessel for sampling of the enzyme. The sampling device consisted of a 28-gauge "Vim" style needle*, 5 inches long, the shaft of which was supported with a 22-gauge outer sheath to within 3/8 inch of the tip, and a 1 ml tuberculin syringe, graduated to 0.01 ml, containing a close-fitting plunger. The needle was sealed to the syringe with an epoxy adhesive** which prevented air from being drawn in as the plunger was pulled back. The plunger and barrel of the syringe were coated with a thick layer of Heavy Celvacene Vacuum Grease, which acted as an effective seal against the atmosphere. The sampling consisted in rinsing the syringe three times by drawing in deoxygenated

*#N700A, purchased from the C.H. Hamilton Company, Whittier, Calif.

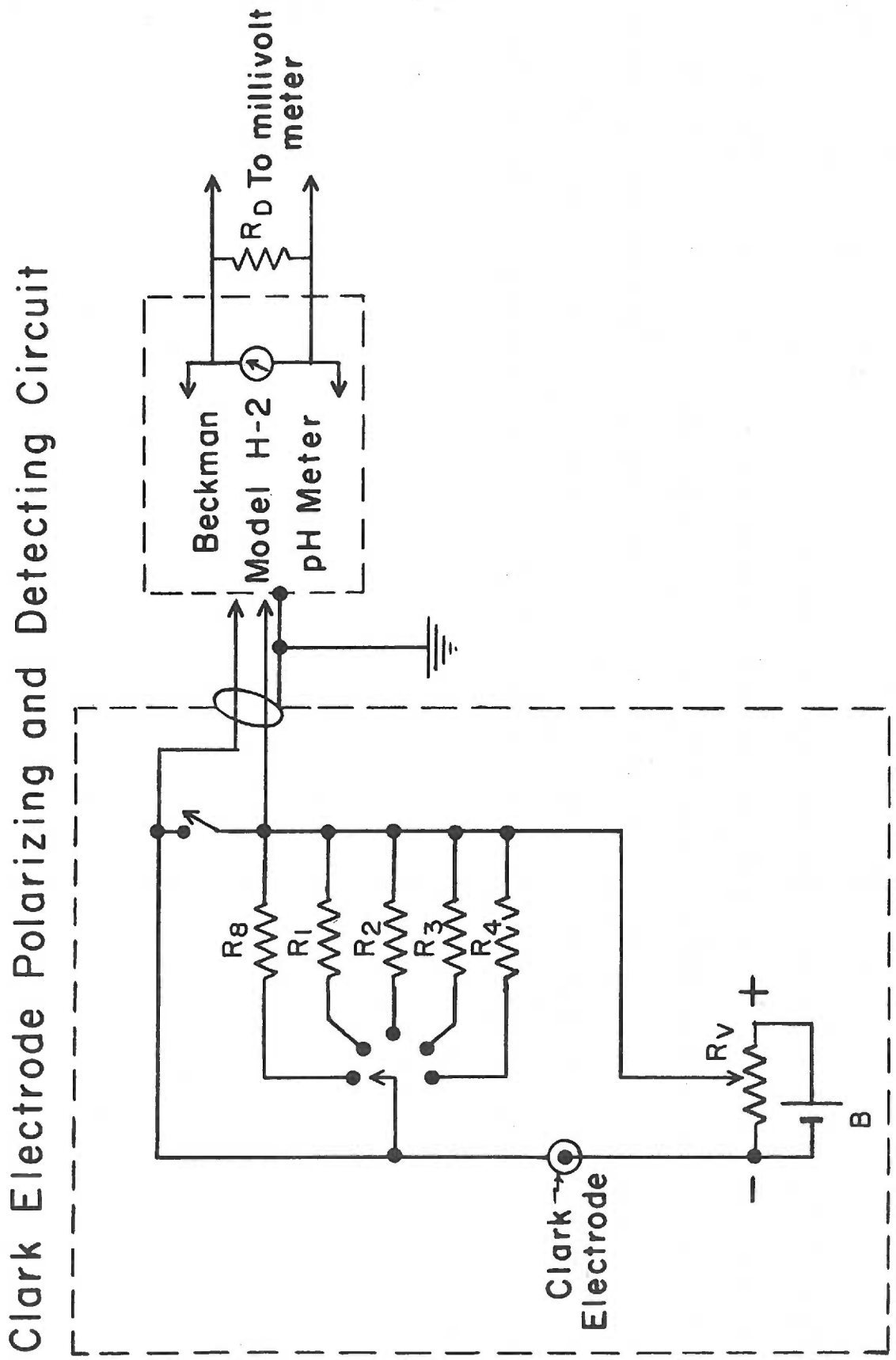
**EC-1838. Generous samples of this adhesive were donated by the Minnesota Mining and Manufacturing Company, Detroit, Michigan, for which the author wishes to express his appreciation.

water, carefully dislodging any air bubbles, and forcing the water out through the needle. Since the volume of rinse water was about ten times the dead space in the needle, the minimum theoretical oxygen tension of the water remaining in the needle which could be obtained by this procedure was about 0.02% of one atmosphere. The dialysis sack was collapsed by increasing the surrounding water pressure, which raised the enzyme solution into the upper chamber where it could be sampled. The needle was passed through the rubber stopper of the dialysis vessel, into the enzyme solution and the desired volume drawn into the syringe. The needle was withdrawn and the sample injected into the oxygenated monophenol reaction mixture approximately one minute later. The small cross section of the five-inch, 28-gauge needle separated most of the sample from the atmosphere during this interval.

Oxygen Detection. Water which had flowed past the dialysis sack was passed through a coil maintained at constant temperature with flowing tap water (9°C.) immediately prior to entering the Clark electrode chamber which is similar to that described by Sproule and coworkers (44). Some of the characteristics of membrane-covered electrodes have been recently described (45). The polarizing and detecting circuits are shown in Figure 3. The platinum electrode of the Clark cell was covered with a polyethylene membrane 1.5 mils (0.038 mm) thick and was polarized with -0.6V versus a silver-silver chloride anode.

Figure 3. Clark Electrode Polarizing and Detecting Circuit.

The Clark electrode and polarizing circuit are housed in an aluminum box which is grounded as indicated. The polarizing voltage source is a mercury battery (B) connected across a 1,000 ohm variable resistor (Rv). Current through the Clark electrode is determined by measuring the decrease in potential across one of the following resistors: R_4 = 4,700 ohms, R_3 = 47,000 ohms, R_2 = 100,000 ohms, R_1 = 470,000 ohms, and R_8 = 1.5 megohms. R_D is a dampening resistor = 180 ohms.


Clark Electrode Polarizing and Detecting Circuit
Beckman
Model H-2
pH Meter
R_D To millivolt meter
R_8
R_1
R_2
R_3
R_4
R_V
B
Clark Electrode

The supporting electrolyte was saturated KCl. Current was measured by determining the drop in potential across a suitable resistor. In order for the measured decrease in potential to be an accurate measure of the current passing through a resistor, it is necessary for the current drawn by the measuring instrument to be small relative to the total current. Therefore, a Beckman Model H-2 pH meter, which has an input grid current of 10^{-12} amperes, was used as a power amplifier. A millivolt meter (either the Brown Recorder from a Sargent Model XX Polarograph or a Hewlett-Packard Model 425A micro voltammeter) was connected across the meter terminals of the Beckman instrument. By this means it was possible to measure currents as low as 50-100 micro-microamperes accurately within a few per cent. The precision of measurement was limited to $\pm$ 0.10 millivolts due to electrical noise in the pH meter. When measuring across the 1.5 megohm resistor in the polarizing circuit, this was equivalent to about 66 micromicroamperes. The Beckman pH meter was specially stabilized for use as a high impedance input power amplifier by replacing the #932 electrometer tube with a #2532 tube* which was specially selected for high emission and low grid current, and by replacing a noisy slidewire resistor with a suitable potentiometer. The pH meter was stabilized by Mr. John Dahnke of the Scientific Instrument Section of the University of Oregon Medical School.

*These tubes are identical except for the ground connection. The first is numbered for use with the Beckman Model H-2 pH meter, while the second is numbered for use with the Model DU spectrophotometer.

The Clark electrode was calibrated with water saturated with oxygen, air and purified nitrogen at room temperature. Comparison with water flowing through the cooling coil at 9° C. indicated that current flow through the electrode was reduced by approximately one-half at this temperature. Low oxygen tensions were estimated by extrapolation, assuming zero residual current, and a constant ratio of oxygen tension to current passed by the electrode.

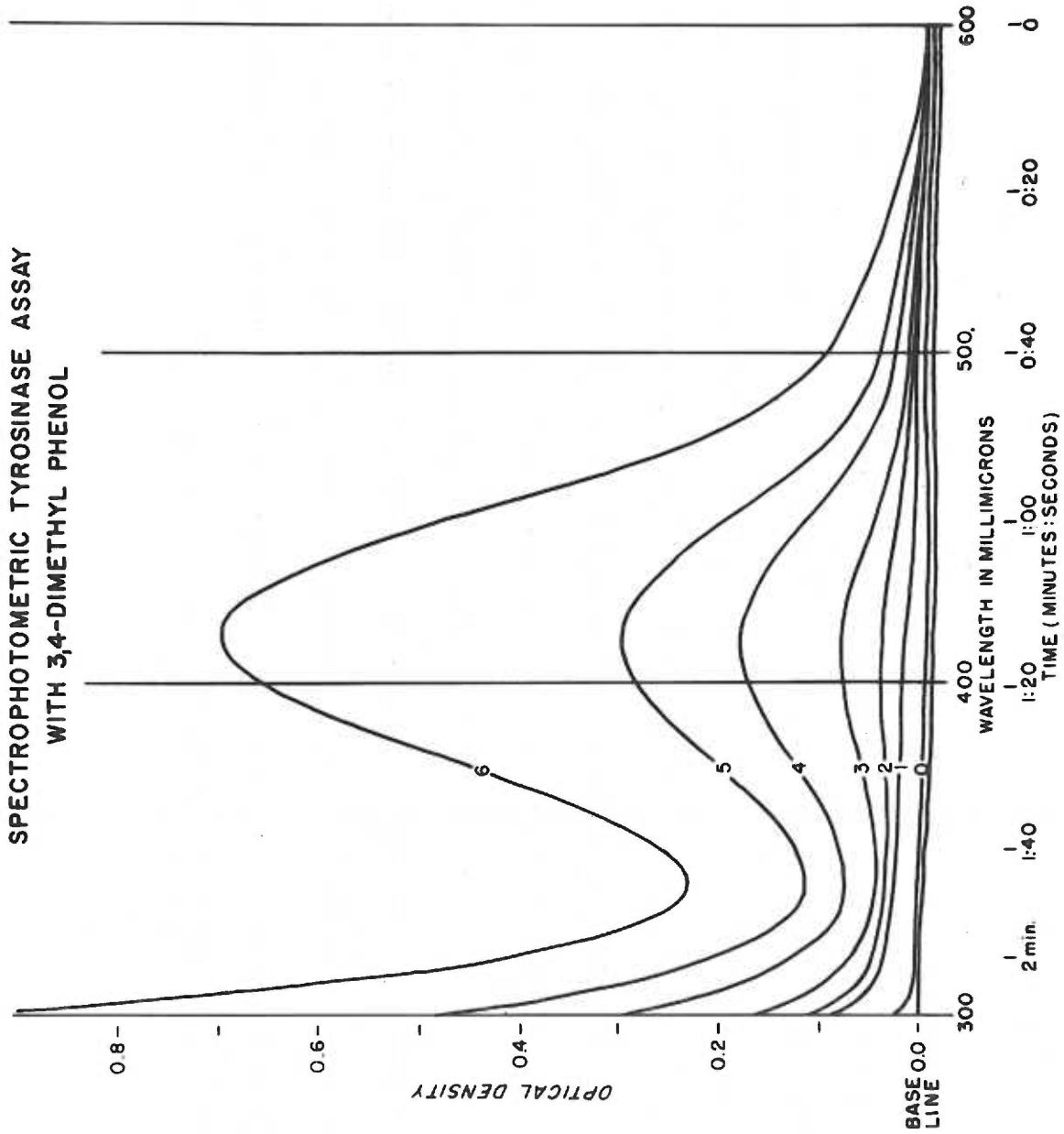
EXPERIMENTAL

In order to study the activation of monophenol oxidation, it was desirable to work with conditions such that the "induction period" was relatively long and the quinone formed from the monophenol was relatively stable. Using 3,4-dimethylphenol as substrate satisfied both these conditions. A Cary Model 14 spectrophotometer was used to record the reaction continuously. The quinone concentration was followed at 412 millimicrons (cf. Fig. 4). The maintained symmetry of the 412 millimicron peak during the reaction suggested that polymerization or condensation products of 4,5-dimethyl-1,2-quinone contributed little to the absorbancy, and were thus present to only a small extent in contrast to o-benzoquinone which rapidly polymerizes (cf. 26). The molar absorbancy coefficient (a_M) of 4,5-dimethyl-1,2-quinone was estimated by oxidizing 0.25 mg of 3,4-dimethylphenol in the presence of a large amount of enzyme (containing 2.4 micrograms Cu) and following the changes in absorbancy. The reaction was complete in 12.5 minutes. Following the decrease in absorbancy after completion of the reaction (0.0007 $minute^{-1}$)* permitted extrapolation of the maximum absorbancy to zero time. The molar absorbancy coefficient (a_M) was estimated to be between 1,070 and 1,050 $l mole^{-1} cm^{-1}$. The molar absorbancy coefficient for o-benzoquinone from enzymically oxidized

*Because of the low rate of decrease in absorbancy when one of the substrates is exhausted, the phrase "quinone formation" will be used interchangeably with "increase in absorbancy at 412 millimicrons".

Figure 4. Spectrophotometric Tyrosinase Assay With 3,4-Dimethylphenol.

The spectra of the reaction mixture were determined before and after adding enzyme. The absorption spectra were scanned from right to left. The time scale indicates the time elapsed after beginning each scan. Starting times were as follows: curve 0, before the enzyme was added; 1, 30 seconds; 2, 5 minutes; 3, 10 minutes; 4, 16 minutes; 5, 20 minutes; 6, 28 minutes. The absorption spectra were determined using a Cary Model 14 Recording Spectrophotometer, with one-centimeter cuvettes and deionized water in the reference cell. The base line was determined with deionized water in both reference and sample cuvettes. Oxygen was bubbled through the reaction mixture for two minutes before adding the enzyme. The temperature was maintained at 25°C. The reaction mixture consisted of 0.20 ml of 3,4-dimethylphenol (5.00 mg/ml), 0.20 ml of 0.201 M potassium phosphate buffer, pH 7.2, 2.50 ml of deionized water, and 0.10 ml of enzyme (Xw, Table I). All reagents were dissolved in distilled, deionized water.


SPECTROPHOTOMETRIC TYROSINASE ASSAY
WITH 3,4-DIMETHYL PHENOL
OPTICAL DENSITY
0.8
0.6
0.4
0.2
BASE LINE 0.0
6
5
4
3
2
1
0
300
400
500
600
WAVELENGTH IN MILLIMICRONS
2 min.
1:40
1:20
1:00
0:40
0:20
0
TIME (MINUTES:SECONDS)

catechol was estimated to be 1,120 ℓmole^{-1} cm^{-1} at 412 millimicrons and 1,440 ℓmole^{-1} cm^{-1} at the maximum at 386 millimicrons.

Before proceeding with the "anaerobic activation" experiments, it was necessary to determine the effects of concentration of the reactants on the rate of quinone formation. Thus, the effects of various concentrations of 3, 4-dimethylphenol, oxygen, and catechol were investigated. Figure 5 shows the rate of quinone formation from four different initial concentrations of 3, 4-dimethylphenol. The reaction mixture was saturated with oxygen prior to adding enzyme. The reaction conditions are described in the caption. Oxygen became limiting at an absorbancy of about 1.2. Bubbling oxygen into the reaction mixture produced a rapid increase in absorbancy, demonstrating that oxygen was the limiting factor. The very sharp decrease in the rate of quinone formation as oxygen is depleted indicates that the Michaelis (45) constant for oxygen under these conditions is very low. Ingraham (47) has determined the equilibrium constant for oxygen and tyrosinase in the absence of substrate to be approximately 1.5% of one atmosphere of oxygen. In order that oxygen not be rate-limiting during the portion of the reaction studied, it was necessary to saturate the reaction mixture before starting the reaction with enzyme. Bubbling the reaction mixture continuously with oxygen during the reaction produced a much greater rate of quinone formation than bubbling for two or three minutes prior to adding the enzyme, probably due to non-enzymic formation of diphenol from the monophenol.

Figure 5. Monophenolase Reaction: Varying Monophenol Concentrations.

The formation of o-quinone from the enzymically catalyzed oxidation of 3,4-dimethylphenol was followed at 412 millimicrons with the Cary Recording Spectrophotometer. The initial absorbancy (0.045) is that of the added enzyme. The reaction mixtures were similar to those described in Figure 4, except that the reaction was started with 0.20 ml of enzyme (Xw, Table II), and various monophenol concentrations were used as follows: curve A, 2.60 ml of 3,4-dimethylphenol (5.00 mg/ml); B, 1.00 ml; C, 0.40 ml; and D, 0.20 ml. The total volume in all instances was 3.00 ml. The temperature was maintained at 24°C. The dotted line indicates the initial portion of the reaction which was studied in Figure 7. Each curve represents a single experiment.

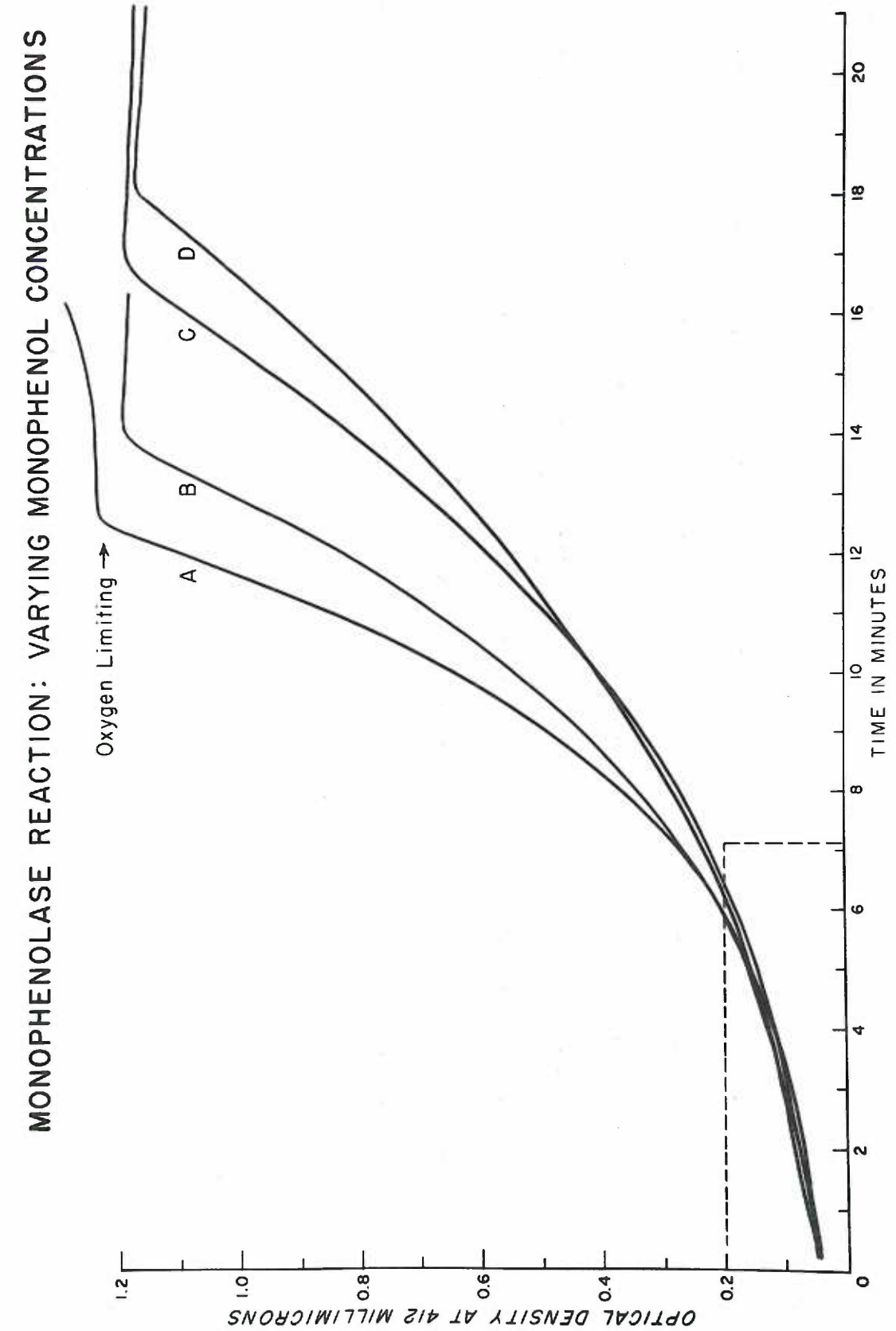

MONOPHENOLASE REACTION: VARYING MONOPHENOL CONCENTRATIONS
Oxygen Limiting →
A
B
C
D
OPTICAL DENSITY AT 412 MILLIMICRONS
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
16
18
20
TIME IN MINUTES

Figure 5 demonstrates that after about the first eight minutes, both the total amount of quinone formed and the rate of quinone formation vary in the same direction as the monophenol concentration. The latter is better seen in Figure 6 in which the rate of change of absorbancy (ΔO.D./minute) is plotted against time. The slopes of these curves are the acceleration of quinone formation, and thus the rate of activation. With the lower monophenol concentrations, the acceleration appears to be relatively constant, whereas it increases with time with higher initial concentrations. After the initial period, both the rate and acceleration vary in the same direction as the monophenol concentration. The dotted lines connect points of equal change in absorbancy, indicating that the differences in rate and acceleration are not due to either product concentration or the amount of enzymic turnover.

The initial rates, as indicated in Figure 6, appeared to be inversely related to monophenol concentration. By increasing both the sensitivity and the chart speed of the Cary recording spectrophotometer, it was possible to investigate the initial portion of the reaction more closely (Fig. 7). It is seen that during the interval between 20 and 40 seconds, both the slope and total amount of quinone formed are inversely related to monophenol concentration. In Figure 8, the uncertainty of determining the initial rate is clearly demonstrated. However, both the overall pattern and the individual curves are fairly reproducible.

In spite of the complexity of the curves in Figure 8, several characteristics may be discerned. With several of the plotted curves,

Figure 6. Monophenolase Reaction: Rate of Quinone Formation vs. Time.

The data from Figure 5 have been analyzed by plotting the rate of change of absorbancy (Δ O. D. /minute) against time. The rates were obtained from tangents drawn with the aid of a mirror. The curves are identified by letters corresponding to those in Figure 5. The dotted lines connect points of identical change of absorbancy of 0.20, 0.35, 0.55, and 0.75, respectively.

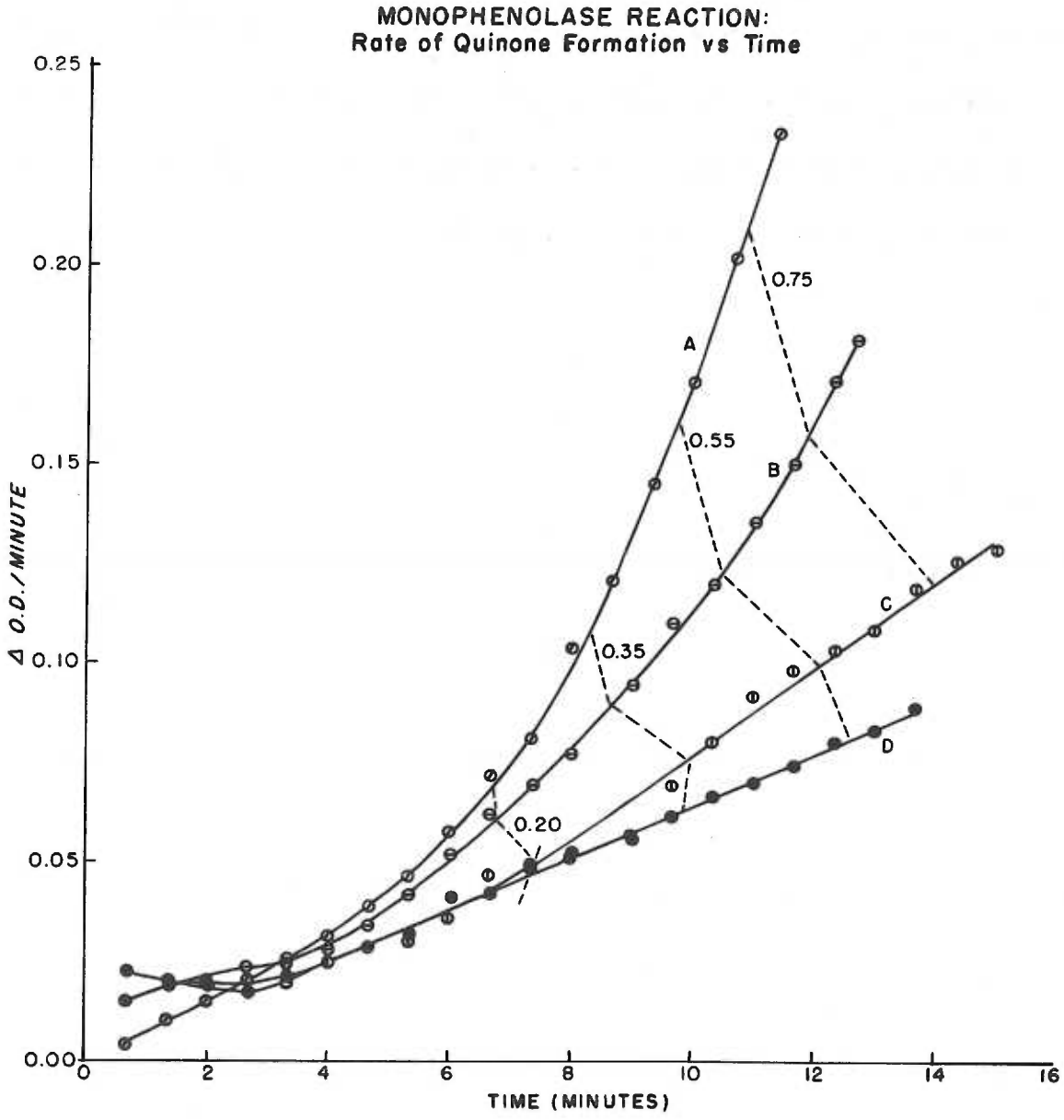

MONOPHENOLASE REACTION:
Rate of Quinone Formation vs Time
Δ O.D./MINUTE
TIME (MINUTES)
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
14
16
A
B
C
D
0.75
0.55
0.35
0.20

Figure 7. Monophenolase Reaction: Varying Monophenol Concentrations.

The first few minutes of the monophenolase reaction were followed in the Cary Recording Spectrophotometer at 412 millimicrons by increasing the sensitivity tenfold (with a special slidewire) and increasing the chart speed to five inches per minute. The reaction conditions were similar to those described in Figure 5. The reaction mixtures for Curves a, b, c, and d were similar to the corresponding mixtures in Figure 5. Curves e and f were similar except that they each contained 0.05 ml of 3, 4-dimethylphenol (5.00 mg/ml). The total volume in all cases was 3.00 ml. The temperature was maintained at 24 - 25°C. Each curve represents a single experiment.

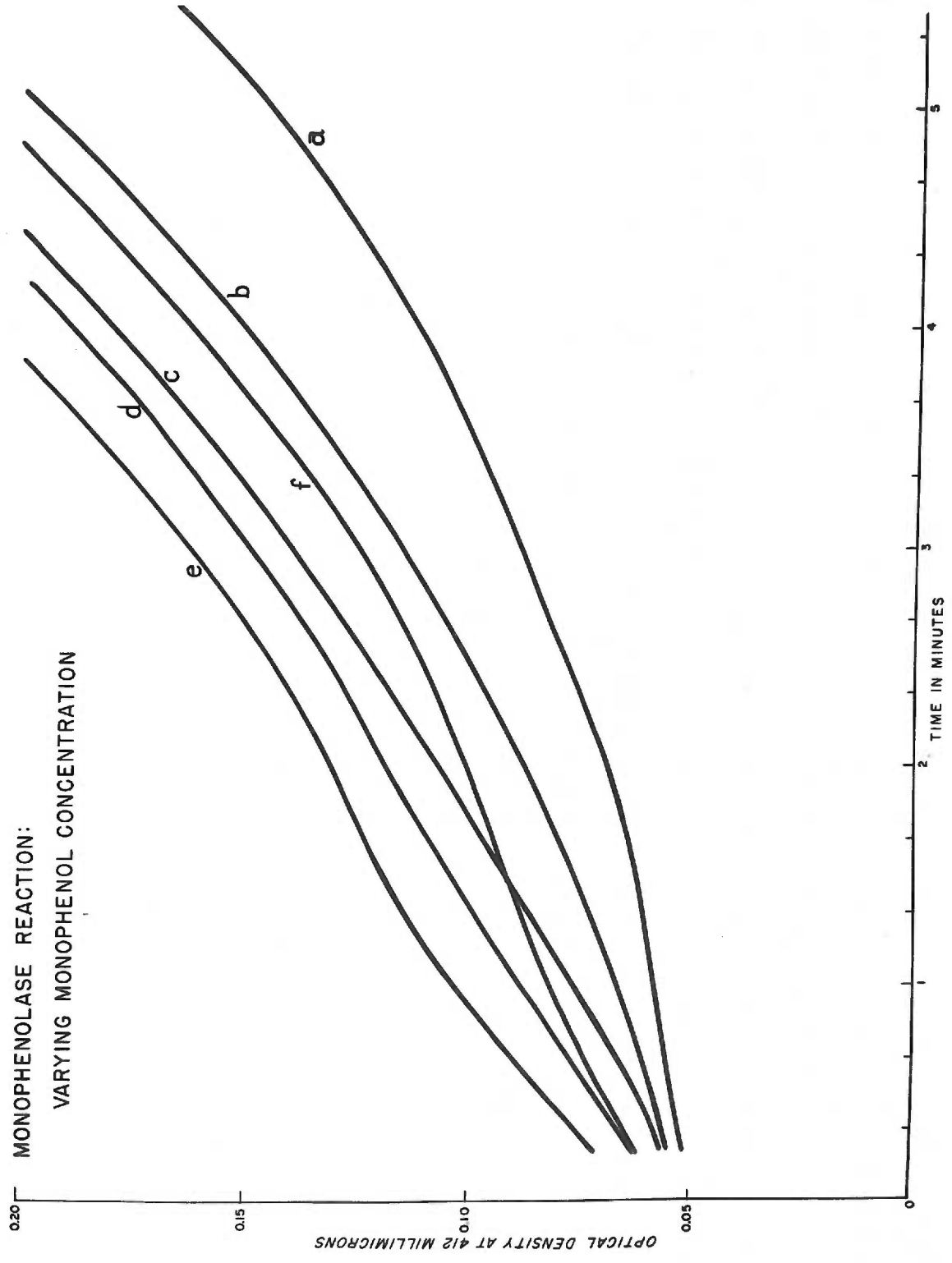
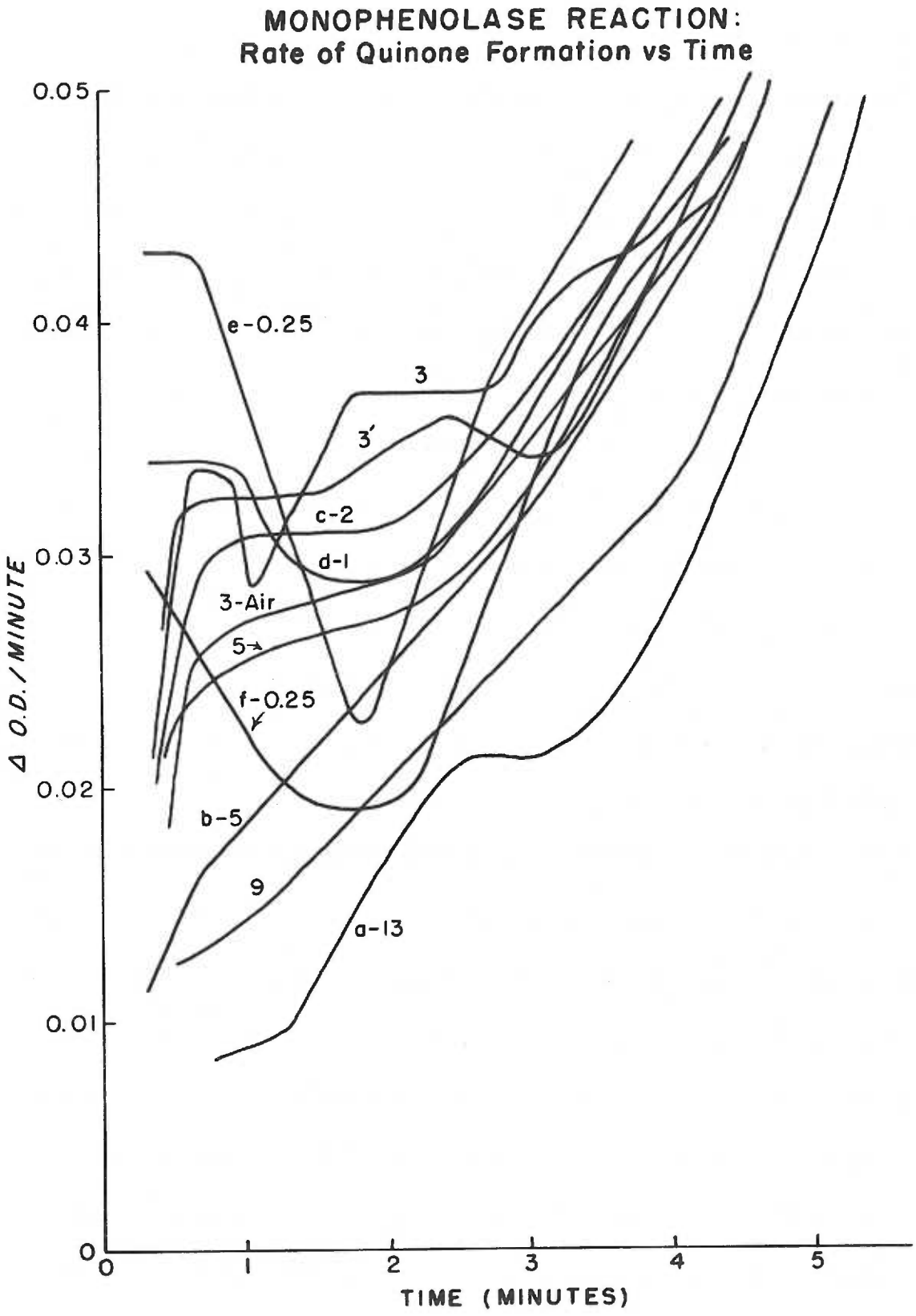

MONOPHENOLASE REACTION:
VARYING MONOPHENOL CONCENTRATION
a
b
c
d
e
f
0.20
0.15
0.10
0.05
OPTICAL DENSITY AT 412 MILLIMICRONS
0
1
2
3
4
5
TIME IN MINUTES

Figure 8. Monophenolase Reaction: Rate of Quinone Formation vs. Time.

The rates of change of absorbancy were determined as described for Figure 6. Curves a- through f- are derived from similarly designated curves in Figure 7. Numbers on the curves indicate the milligrams of 3, 4-dimethylphenol in a reaction mixture with a total volume of 3.00 ml. The reaction mixtures for the unlettered curves were similar to those in Figure 7 except that they were bubbled with oxygen for three minutes instead of two prior to starting the reaction with enzyme (0.06 ml Xf, Methods). The reaction mixture for curve 3-Air was similar to those for curves 3 and 3' except that air instead of oxygen was bubbled through the reaction mixture before starting the reaction. Curves 5 and 9 each represent duplicate experiments in which the maximum deviation of the rates from the respective curves is $\pm$ 0.002. The other curves (for which the maximum deviation of the rates from the respective curves is $\pm$0.001) were derived from single experiments, curves 3 and 3', and e-0.25 and f-0.25 being duplicates. For all curves, tangents were taken at a frequency of 6 - 8/minute. Control reaction mixtures similar to those for curve 9 except that the enzyme was replaced with an equal volume of boiled enzyme or water, gave rates of change of absorbancy equal to zero, with maximum deviations of $\pm$ 0.002 and $\pm$ 0.0002, respectively. The temperature for all reactions was 24 - 25° C.


MONOPHENOLASE REACTION:
Rate of Quinone Formation vs Time
0.05
0.04
0.03
0.02
0.01
0
Δ O.D. / MINUTE
e-0.25
3
3'
c-2
d-1
3-Air
5
f-0.25
b-5
9
a-13
0
1
2
3
4
5
TIME (MINUTES)

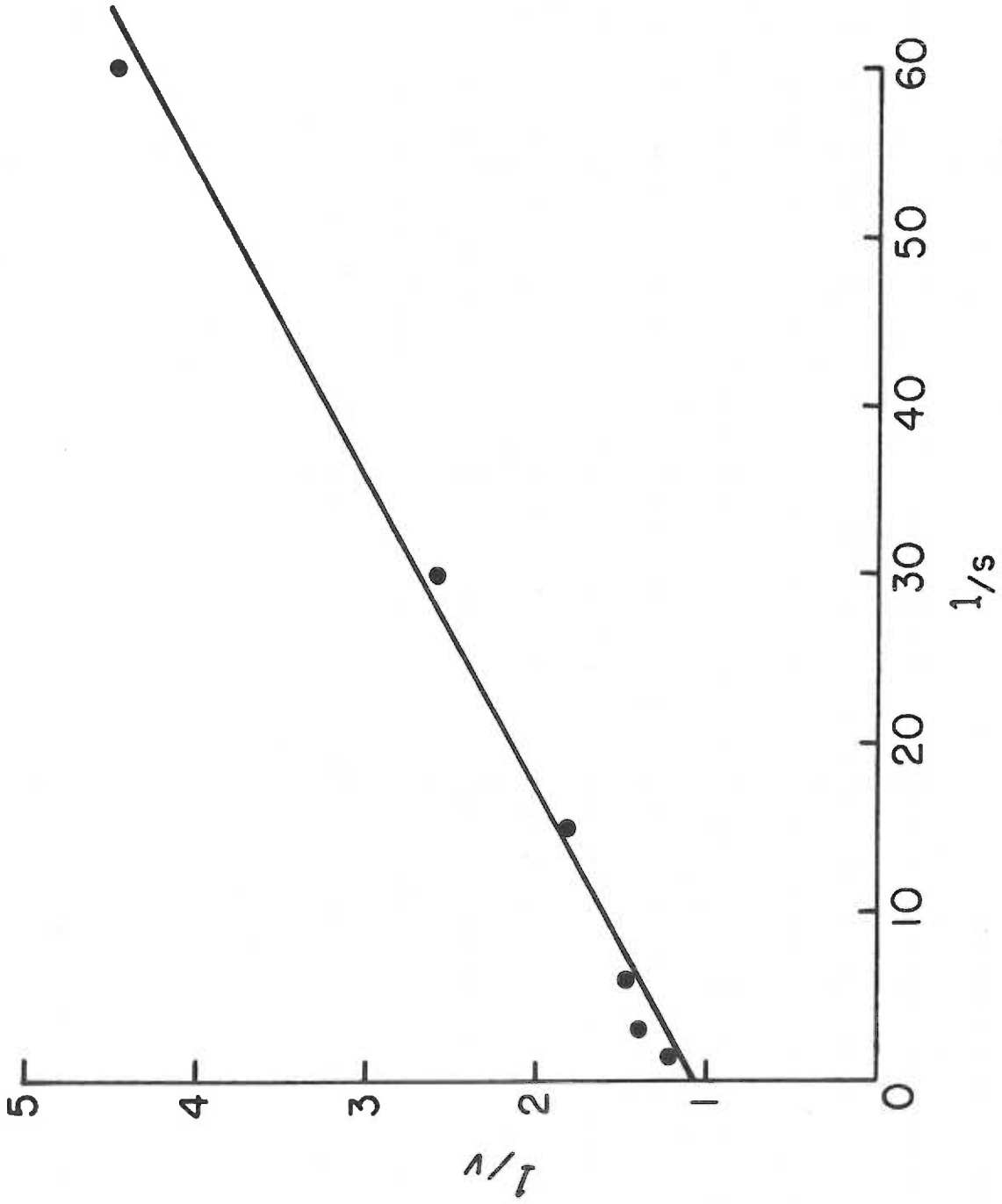
the rate of quinone formation appears to increase rapidly from an indeterminate low value during the first twenty or fourty seconds. After this initial rapid rise in rate, the rate of quinone formation is generally inversely related to monophenol concentration. A comparison of curve 3-Air with curves 3 and 3' or curve b-5 with curve 5 suggests that with these concentrations, at least, the rate between one and two minutes is directly related to the amount of oxygen which was bubbled through the monophenol reaction mixture before the enzyme was added to start the reaction. It would appear likely that the inverse relation between rate and monophenol concentration which was observed during the first few minutes of the reaction may be due to the presence of small amounts of 4, 5-dimethylcatechol either as an impurity in the 3, 4-dimethylphenol or derived from the monophenol by non-enzymic aerobic oxidation. The latter possibility would appear to be consistent with the data of Kendal (15) and others (6) who have demonstrated inhibition of diphenol oxidation by the corresponding monophenol. Under the present experimental conditions, it was not possible to distinguish between quinone formed from monophenol or diphenol. Since the reaction mixture containing 3 mg of 3, 4-dimethylphenol/ml (Curve 9, Fig. 8) appeared to produce a somewhat simpler rate form than lower concentrations, it was decided to use this monophenol concentration for activation studies.

In contrast to monophenol oxidation, the enzymic oxidation of diphenols follows straightforward two-substrate kinetics (47). The Michaelis constant for catechol has been determined from the Lineweaver-Burk (48) plot (Fig. 9) to be 5.2×10^{-4} M. El-Bayoumi and Frieden (41) have summarized several values for the Michaelis constant for catechol from their work and from the literature, which range from 1.7×10^{-4} M to 5×10^{-4} M. Tsou and Li (29) reported a value of 7.2×10^{-5} M which was obtained with enzyme before treatment with 50% ethanol, and a value of 4.9×10^{-4} M with the same enzyme but with reaction mixtures which were 50% (v/v) in ethanol. These values have been obtained with a variety of conditions and methods, so that they may not be directly compared. However, the work of Tsou and Li (29) suggests that some of the variation may be due to changes in the protein during the purification procedures. No thorough study of the effects of various factors on the Michaelis constant for catechol has been made.

The rate-limiting velocity constant for the overall reaction at maximum velocity may be calculated from the data of Figure 12, using the relation, $V_{Max} = k_f E_o$, where E_o is the final enzyme concentration. The maximum velocity is 0.926 ΔO.D.$_{390}$/minute, or 6.44×10^{-4}(moles catechol)(liter)$^{-1}$(minute)$^{-1}$, calculated using $a_M = 1,440$. The final enzyme-copper concentration in the reaction mixtures was 8.88 micromolar. Assuming four atoms of copper per mole of enzyme (cf. 4), the

Figure 9. Lineweaver-Burk Plot for Catechol.

The reciprocal of the change of absorbancy (at 390 millimicrons) per minute is plotted against the reciprocal of initial catechol concentration (milligrams/milliliter of reaction mixture). The rate was determined for the interval between 14 and 20 seconds after adding enzyme. The reaction mixture contained the indicated amount of catechol, 0.20 ml of 0.201 M potassium phosphate buffer, pH 7.2, and water to a total volume of 2.90 ml. Oxygen (99.8%) was bubbled through the reaction mixture for two minutes, and the reaction was started by adding 0.10 ml of a 1:625 dilution of enzyme fraction Xc (Table II). Control reaction mixtures containing 0.667 and 0.167 milligrams catechol/ml (the highest and third highest concentrations used) and water instead of enzyme, gave initial rates of 0.0005 and 0.0001/minute, respectively, which were less than 0.1% of the rates in the presence of enzyme. The rates were not corrected for autoxidation. The temperature was 24 - 25° C.


1/v
1/s
0
10
20
30
40
50
60
1
2
3
4
5

final enzyme concentration was 2.22 micromolar. The value obtained for k_f is therefore 290 minute^{-1}.

Monophenolase activation by catechol is more complex. Figure 10 shows some of the rate versus time curves obtained when small amounts of catechol are added to a reaction mixture similar to that used for Curve 9, Figure 8. Several of these curves suggest the presence of a rapid acceleration during the first 20-30 seconds. The decreases in acceleration following this first rapid acceleration, which are quite prominent with the lower monophenol concentrations in Figure 8, are suggested at only one catechol concentration (0.0498 micrograms/ml) in Figure 10. This intermediate region appears to be greatly shortened with increasing catechol concentration, and apparently disappears when the catechol is increased to 0.083 micrograms/ml (0.755 micromolar). Figure 11 illustrates the effect of higher catechol concentrations on the rate of quinone formation. The acceleration of quinone formation increases rapidly after the first one or two minutes, especially with the highest catechol concentration used in this study.

The rate of quinone formation at one minute has been plotted against the catechol concentration (Fig. 12). This time was chosen because of the uncertainty in the initial rate. It is apparent that the velocity is not a linear function of catechol concentration, but rather the curve is concave relative to the abscissa. Since the catechol concentrations in these experiments were low relative to the enzyme concentration, rather than the reverse, Michaelis-Menten (46) type of

Figure 10. Monophenolase Activation by Catechol: Rate of Quinone Formation vs. Time.

Small amounts of catechol were added to reaction mixtures similar to those used to obtain curve 9, Figure 8. Rates were determined as described for Figure 6. Final catechol concentrations in the reaction mixtures were as follows: Curves 8, 0.083 micrograms/ml; Curves 5, 0.0498 micrograms/ml; Curves 3, 0.0312, 0.0332, and 0.0332 micrograms/ml, respectively. Control reaction mixtures similar to those for Curves 5 and 3, except that boiled enzyme replaced Xf, gave rates equal to zero with maximum deviations of $\pm$ 0.002 and $\pm$ 0.0004/minute, respectively. The temperature was maintained at 25.2° C. $\pm$ 0.2° C. The maximum deviation of the rates from the respective curves is $\pm$ 0.0015. Tangents to the absorbancy curves were taken at a frequency of 7 - 10/minute.

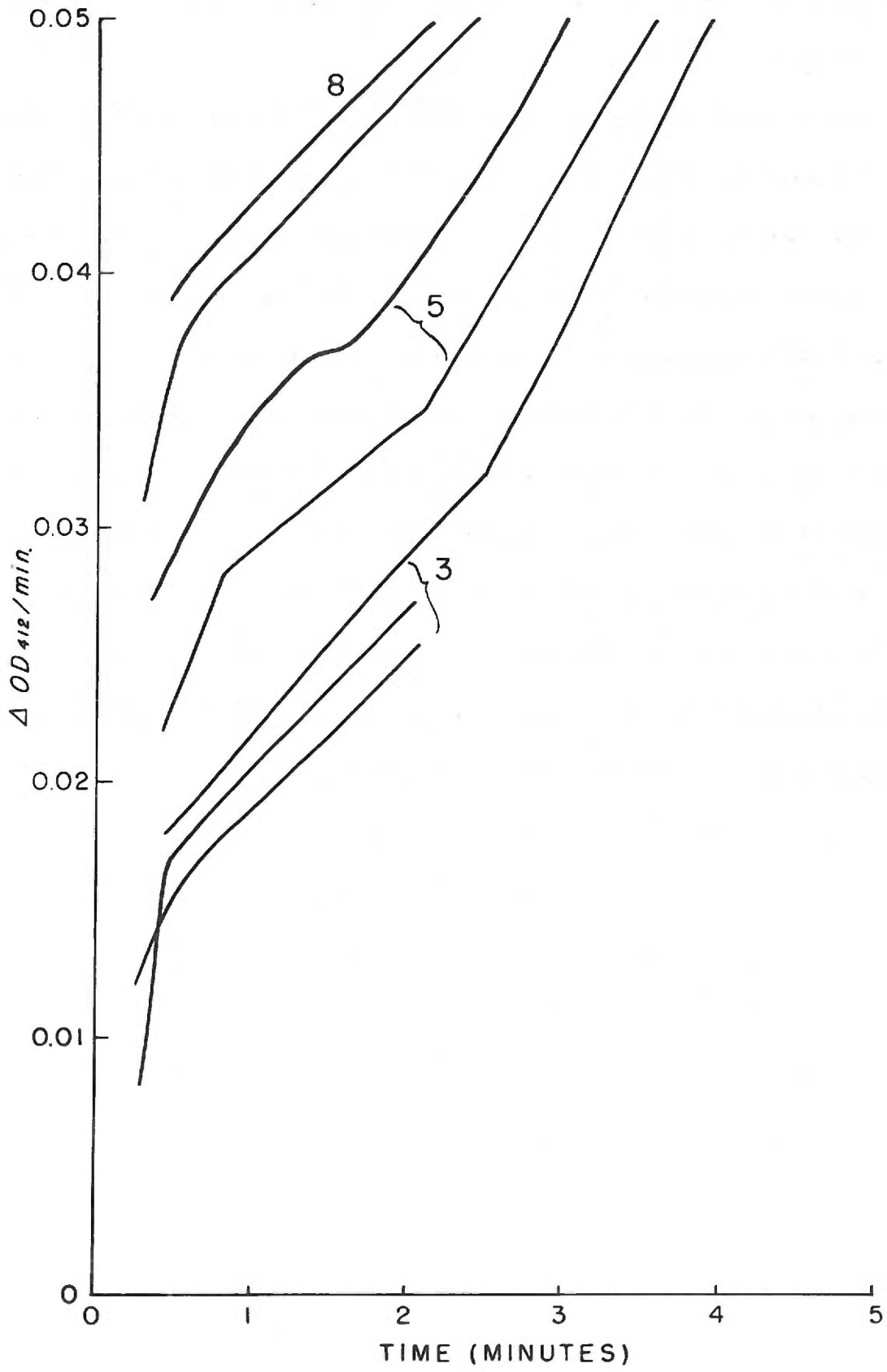

0.05
0.04
0.03
0.02
0.01
0
Δ OD 412 / min.
8
5
3
0
1
2
3
4
5
TIME (MINUTES)

Figure 11. Monophenolase Activation by Catechol: Rate of Quinone Formation vs. Time.

Curves 3, 5, and 8 are replotted from Figure 10. The reaction mixtures for Curves 16 and 31 are similar except that they contained 0.166 and 0.312 micrograms of catechol/ml, respectively. Curves 16 and 31 each represent duplicate experiments for the first two and three minutes, respectively. The dotted lines connect points of equal absorbancy. An initial absorbancy of approximately 0.05, due to enzyme, should be subtracted from the values 0.20, 0.30, 0.40, 0.50 and 0.55 to obtain change of absorbancy due to quinone formation. A control reaction mixture similar to that for Curve 31, except that enzyme was replaced with boiled enzyme, produced a rate of change of absorbancy equal to zero, with a maximum deviation of $\pm$ 0.002/minute. The temperature was maintained at 25.2° C. $\pm$ 0.2°C. Tangents to the absorbancy curves were taken at a frequency of 5/minute to obtain curve 31 and all except the first minute of curve 16, for which the frequency of tangents was greater than 10/minute. The maximum deviation of the rates from Curve 31 is $\pm$ 0.008/minute, and that from Curve 16 is $\pm$ 0.002/minute.

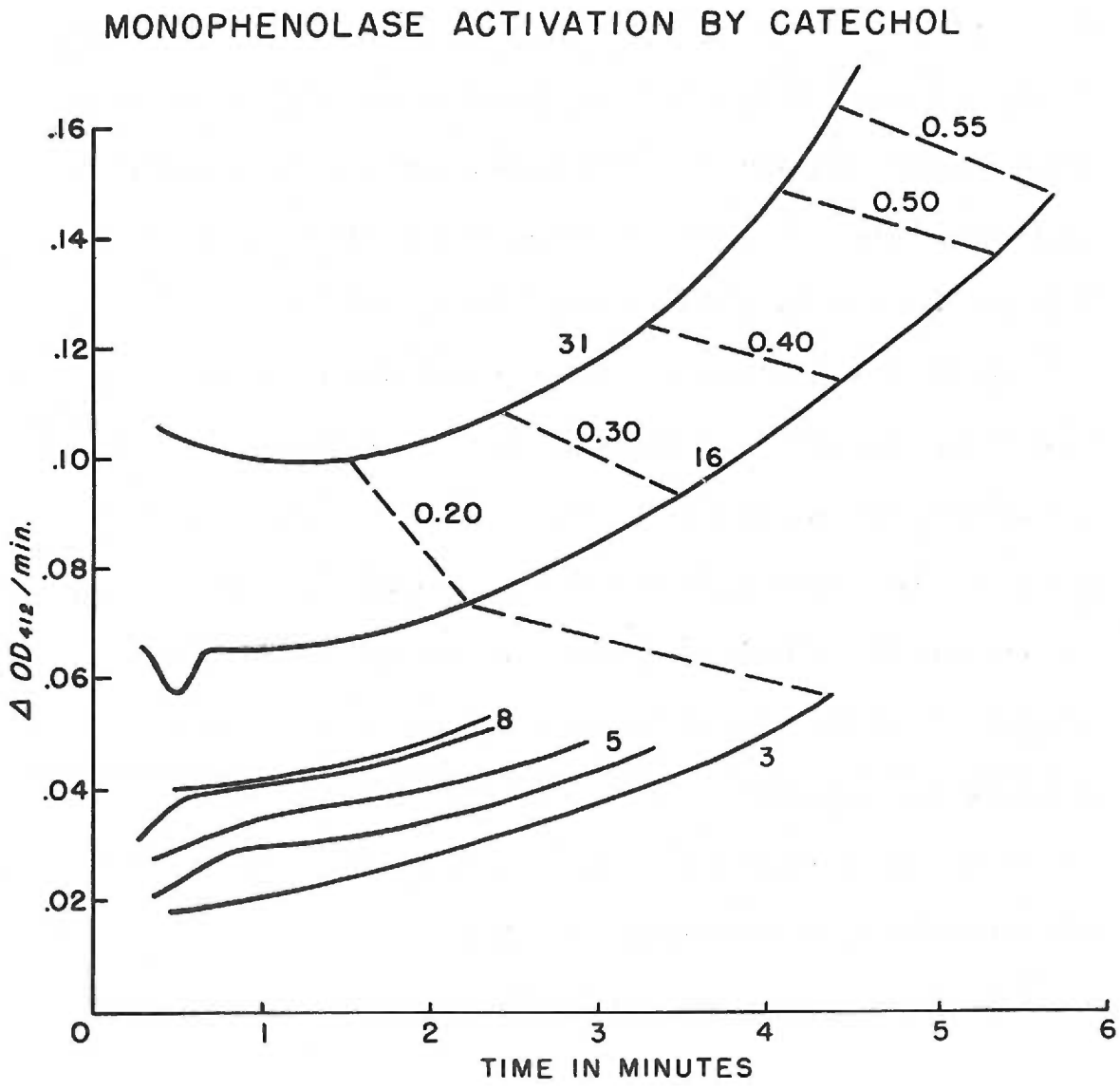
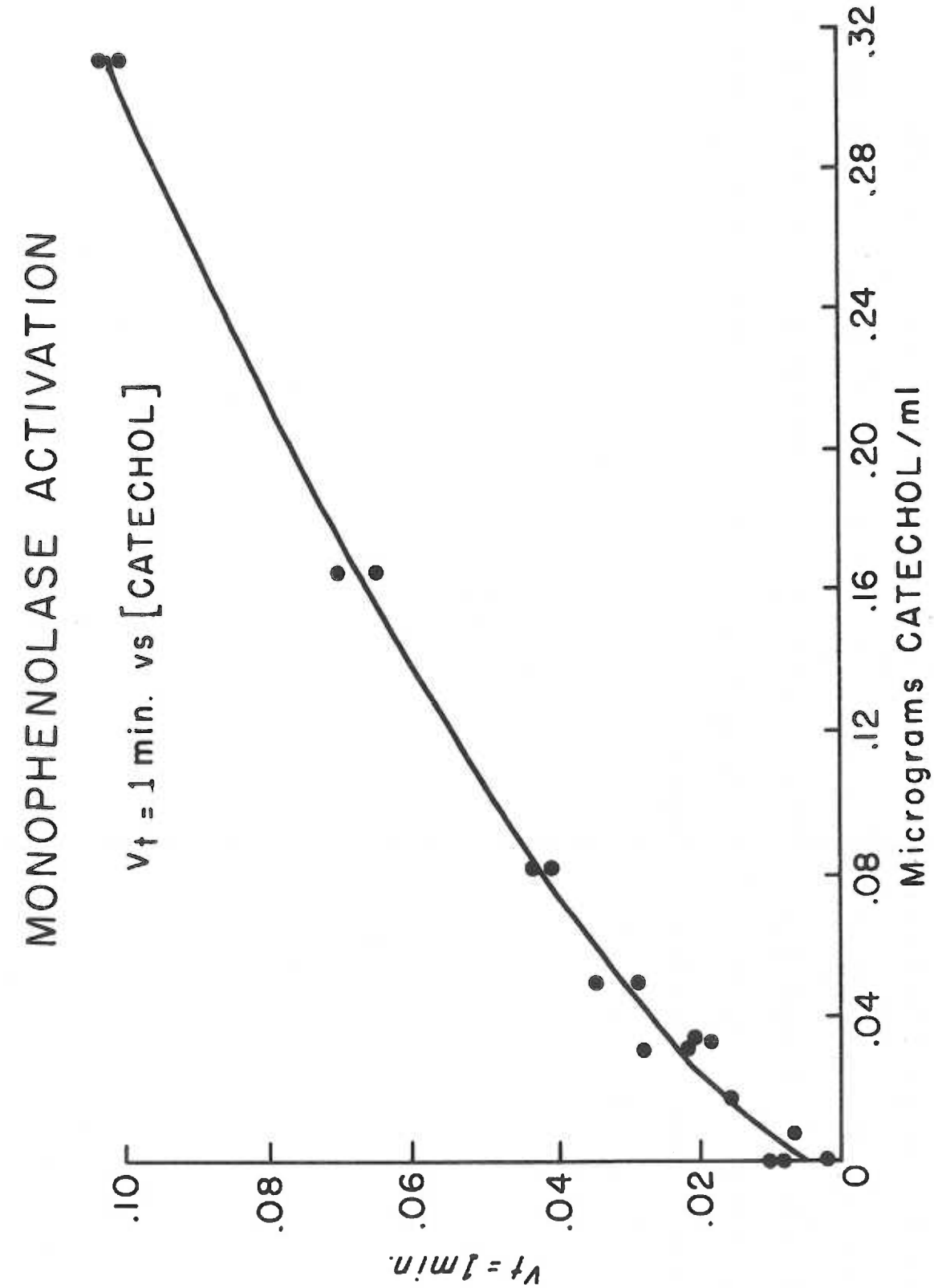

MONOPHENOLASE ACTIVATION BY CATECHOL
.16
.14
.12
.10
.08
.06
.04
.02
Δ OD412/min.
0.55
0.50
31
0.40
0.30
16
0.20
8
5
3
0
1
2
3
4
5
6
TIME IN MINUTES

Figure 12. Monophenolase Activation: Rate of Quinone Formation vs. Catechol Concentration.

Summary of monophenolase activation by catechol. The velocity is the rate of change in absorbancy at one minute after starting the reaction by adding enzyme (0.06 ml of Xf, Methods and Table IV). Some of the data are taken from Figures 10, 11 and 13. All reaction mixtures are similar to those for Curve 9, Figure 8, except for catechol concentration.


MONOPHENOLASE ACTIVATION
v_t = 1 min. vs [CATECHOL]
V_t = 1 min.
.10
.08
.06
.04
.02
0
.04
.08
.12
.16
.20
.24
.28
.32
Micrograms CATECHOL/ml

treatments do not apply. A mathematical approach to this problem will be treated in the discussion.

Anaerobic Activation. Having established suitable conditions for determining monophenolase activation by catechol, it was possible to test whether tyrosinase could be irreversibly activated in the relative absence of oxygen. The procedure followed, in general, was to anaerobically incubate tyrosinase with catechol, dialyze to remove the excess catechol and any reaction products, and test for activation by following the rate of quinone formation when the "anaerobically activated" enzyme was injected into a fully oxygenated monophenol reaction mixture. A preliminary anaerobic dialysis experiment indicated that neither a large activation or inactivation could be detected under these conditions. The following experiment was conducted to establish this point. Deoxygenated water and nitrogen were prepared as described in the "Methods". Enzyme (1.00 ml of Xf) and catechol (224 micrograms in 1.00 ml of deionized water) were pipetted into the dialysis sack and addition chamber, respectively, of the anaerobic dialysis vessel (Fig. 2). The water bath (Fig. 1) was maintained at 0° C. Deoxygenated nitrogen was passed through the catechol solution and over the surface of the enzyme, and deoxygenated water was permitted to flow continuously past the dialysis sack at about 10 ml/minute for 24 hours. Thus, oxygen was removed from the enzyme solution by diffusion only. The estimated oxygen tension in the water which flowed past the dialysis sack was reduced to less than 0.006%

of one atmosphere (which was equivalent to the electrical noise level of the oxygen-detecting instrument) during the first ten hours of anaerobic dialysis and remained below that level for the duration of the experiment. After deoxygenation of the enzyme and catechol, the temperature of the water bath was raised to 25° C., the dialysis sack was collapsed by increasing the surrounding water pressure, raising the enzyme into the upper chamber and minimizing dialysis, and the catechol solution was added, mixed with the enzyme, and permitted to equilibrate for 10 minutes before continuing the dialysis and lowering the bath temperature to 0° C. The water and nitrogen flow were stopped periodically and the enzyme was sampled as described in the section on Methods. The results are described in Figure 13 and Table III.

During the anaerobic dialysis, an attempt was made to sample that volume of enzyme which would give an initial absorbancy due to enzyme which was similar to that obtained with earlier aerobic activation studies. This approach was considered valid only when lower reaction rates were obtained so that the error in estimating the initial absorbancy would be small. Figure 13 indicates that the initial absorbancies obtained from Samples III, IV, and VII were similar to those obtained from the aerobic activation studies. In Table III, the third column lists the total time during which deoxygenated water was flowing past the enzyme contained in the dialysis sack after anaerobic addition of the catechol. It does not include the sampling time during which the dialysis sack was collapsed and the enzyme raised into the upper

Figure 13. Monophenolase Activation by Catechol.

Absorbancy at 412 millimicrons is plotted as a function of time from data obtained with the Cary Model 14 Recording Spectrophotometer. The initial absorbancy is that of the enzyme added to start the reactions. Reaction mixtures for all curves were similar to those from which Curve 9, Figure 8, was derived except for added catechol, and for Curves II, III, IV, VI, VII and VIII, pretreatment of the enzyme. Reaction mixtures for Curves 0, 5, 8, and 16 were as follows: 1.80 ml of 3, 4-dimethylphenol (5.00 mg/ml), 0.20 ml of 0.201 M potassium phosphate buffer, pH 7.2, catechol in a final concentration of zero, 0.0498, 0.083, and 0.166 micrograms/ml, respectively, and water to 2.94 ml. The reaction mixtures were bubbled with 99.8% oxygen for three minutes before completing the reaction mixture with 0.06 ml of enzyme fraction Xf (Methods and Tables II, III and IV). Reaction mixtures for Curves II, III, IV, VI and VII were similar except that no catechol was added separately from the enzyme, which was sampled from the anaerobic dialysis vessel as described in Table III and in the text. The final volume of all reaction mixtures was 3.00 ml. The reaction mixture for Curve VIII was similar to those for Curves II - VII except that it contained a final catechol concentration of 1.22 micrograms/ml. The temperature for all reactions was $25° \pm 1°$ C.

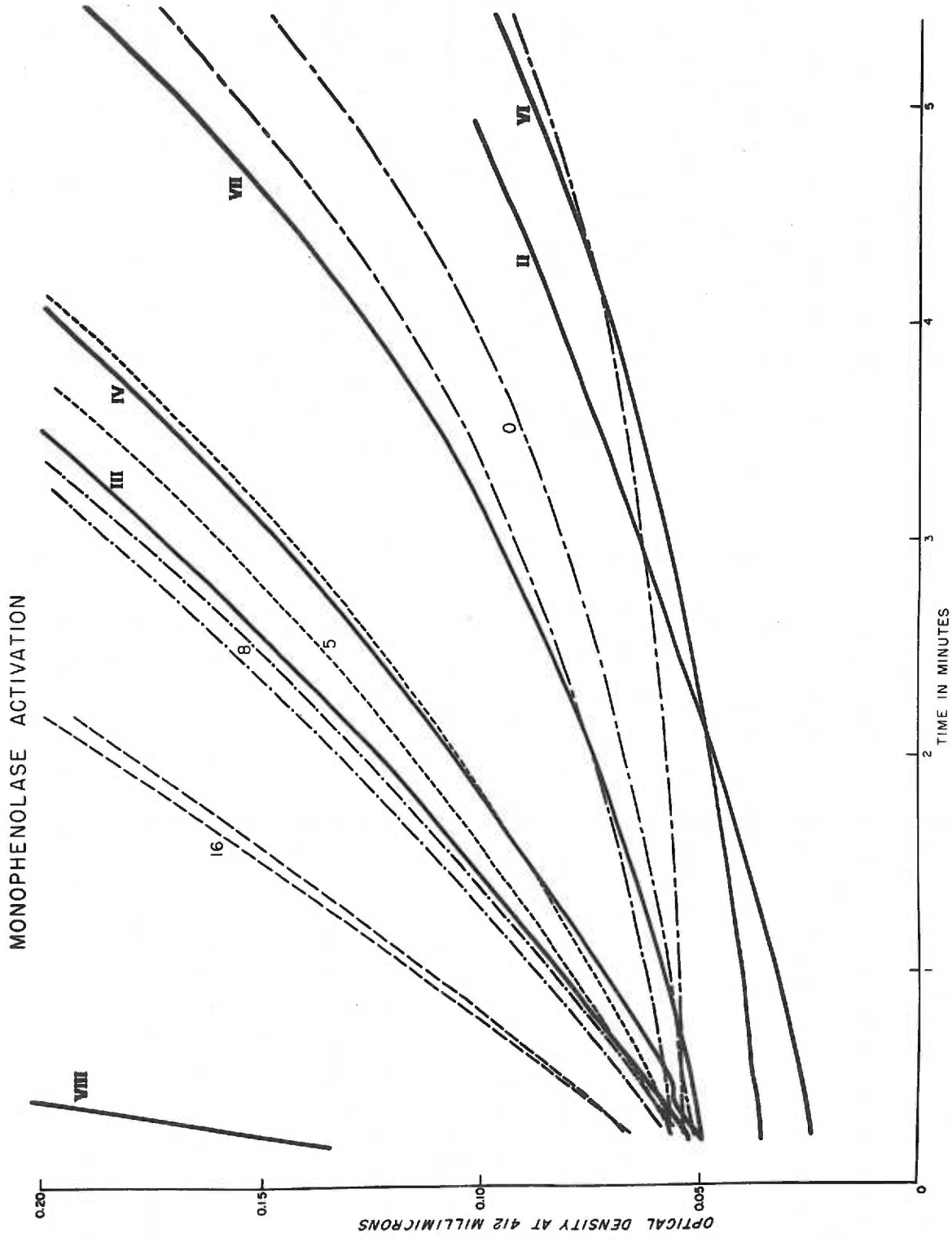

MONOPHENOLASE ACTIVATION
OPTICAL DENSITY AT 412 MILLIMICRONS
TIME IN MINUTES
0.20
0.15
0.10
0.05
0
1
2
3
4
5
VIII
VII
VI
IV
III
II
16
8
5
0

TABLE III

ANAEROBIC DIALYSIS EXPERIMENT

Sample No.	Volume	Time Dialyzed After Catechol Addition (hours:minutes)	Rate of Quinone Formation at One Minute (ΔO. D. per minute)	Rate: ΔO. D. per minute per ml. of Sample	Micrograms of Copper per Sample	Rate: ΔO. D. per minute per microgram of Copper
I	0.20 ml	0:57	0.420	2.10	---	---
II	0.06	1:52	0.0136	0.226	---	---
III	0.14	4:22	0.0400	0.286	---	---
IV	0.14	4:22	0.0328	0.234	---	---
V	0.15	27:52	0.0187	0.125	---	---
VI	0.12	38:47	0.0065	0.060	0.66	0.0109
VII	0.17	"	0.0136	0.080	0.93	0.0145
VIII*	0.17	"	0.252	1.48	0.93	0.269

*Reaction mixture contained 1.22 micrograms of added catechol per milliliter.

10-I-A	0.06 ml Xf	0.0025	0.041	0.72	0.0034
10-II-A	"	0.0104	0.173	"	0.0144
10-II-B	"	0.0086	0.143	"	0.0119

chamber. The fourth column indicates the observed rate obtained one minute after adding the anaerobic enzyme to the oxygenated monophenol reaction mixture, and the fifth column contains the calculated rate per milliliter of sampled enzyme. It is seen that the latter rate decreased sharply during the first two hours of dialysis and then much more slowly. It appears that the decrease in rate per milliliter of sampled enzyme may follow the decrease in catechol concentration during dialysis, although the latter concentration is not known; and it has not been established that the rate at one minute is a linear function of enzyme concentration, other factors being constant.

Prior to taking enzyme sample number VI, the water flow past the dialysis sack was stopped and stationary dialysis continued for 110 minutes. The water which had been in contact with the dialysis sack was sampled and its absorbancy at 275 millimicrons (0.016) determined. If it is assumed that all of this absorbancy was due to catechol, then by comparison with standard catechol solutions of similar absorbancy, the concentration outside the dialysis sack would be estimated to be 0.45 micrograms/ml. If this concentration is taken as the catechol concentration in the enzyme solution, then the estimated catechol concentration after adding Sample VII to the monophenol reaction mixture would be 0.025 micrograms per milliliter of reaction mixture. Although this estimate is subject to very gross errors, the observed rate with Sample VII is consistent with that expected from the estimated catechol

concentration and enzyme which had not been activated before addition to the reaction mixture.

After sampling the dialyzing water, the flow was stopped and the dialysis sack maintained collapsed for the remainder of the experiment. At the conclusion of the experiment, the remaining enzyme (Anaerobic Test Enzyme) was removed from the dialysis vessel, and its absorption spectrum and copper content determined and compared with those of enzyme (Xf) which had not been subjected to anaerobic dialysis. The results are contained in Table IV. The characteristic wavelengths of the absorption spectra are a valley at 252 millimicrons, the usual peak at 280, and a shoulder at 290 millimicrons. The absorbancies at 412 millimicrons are included for comparison with the initial absorbancies due to enzyme in the monophenol reaction mixtures. Comparison of the ratios of absorbancies at the four wavelengths for the two enzyme fractions indicates that the spectral characteristics were not greatly changed by the anaerobic dialysis or catechol addition and removal. The ratios suggest that the absorbancy of the Anaerobic Test Enzyme may be about 6% lower than that of Xf at 412 millimicrons. No reason can be suggested for this at present. The copper content of the Anaerobic Test Enzyme appears to be 2 - 4% higher than that of Xf, but this difference is within the range of experimental error. However, the copper content per absorbancy at 412 millimicrons of the Anaerobic Test Enzyme is about 10% higher than that of Xf. At least one-half of this difference appears

TABLE IV

SPECTRAL ANALYSIS OF ENZYME FRACTION Xf and ANAEROBIC TEST ENZYME (ATE)

Enzyme Fraction	Wavelength (millimicrons) 252	280	290	412	Micrograms Copper/ml
	Optical Density per Centimeter				
Xf	15.6	20.4	16.8	2.88	12.0
ATE	6.88	8.90	7.32	1.20	5.5

	Reference Wave Length (millimicrons)	Optical Density Ratios				Micrograms of Copper per ml / O.D. per cm.
Xf	252	---	1.31	1.08	0.185	0.77
ATE	"	---	1.29	1.06	0.174	0.80
Xf	280	---	---	0.823	---	0.59
ATE	"	---	---	0.825	---	0.62
Xf	290	---	1.21	---	---	0.71
ATE	"	---	1.22	---	---	0.75
Xf	412	5.41	7.08	5.84	---	4.17
ATE	"	5.74	7.42	6.11	---	4.58

to be due to the decreased absorbancy of the Anaerobic Test Enzyme at 412 millimicrons.

The last two columns in Table III list the copper content and rate of quinone formation at one minute per microgram of copper for the last three samples of Anaerobic Test Enzyme. The lower section of the table lists comparable data for three replicate reactions (Curves "O", Fig. 13) using untreated enzyme (Xf), and containing no added catechol in the reaction mixture. It is seen that the rates per microgram copper obtained with Samples VI and VII are in the same range as those obtained with untreated enzyme without added catechol. Thus the data in Figure 13 and Table III clearly demonstrate that if activation of the anaerobically dialyzed enzyme occurred, it must have been reversible. The data from Sample VIII demonstrate that the enzyme could still be activated, after anaerobic dialysis, by the presence of catechol in the reaction mixture. If the final volume of enzyme-catechol mixture immediately after the anaerobic addition of the catechol is taken to be approximately 2.5 milliliters, then the catechol concentration in the mixture was about 90 micrograms/ml or about 73 times the catechol concentration in the monophenol reaction mixture used with Sample VIII.

Monophenolase Activation by Ethanol. Tsou and Li (29) have recently reported some effects of several organic solvents on the enzymic oxidation of catechol, and the effect of 50% ethanol on various o-diphenolic derivatives, and phenol and p-cresol. Of the catechol derivatives tested,

only catechol and pyrogallol showed an increased rate of oxidation in the presence of 50% ethanol, oxidation of the other derivatives being inhibited. The rate of catechol oxidation was also increased in the presence of 50% methanol, iso-propanol, ter-butanol, and acetone, the last being the most effective. Enzymic oxidation of catechol was inhibited by ethyleneglycol, glycerol, and dioxane. The effect of 50% ethanol present in a reaction mixture containing phenol or p-cresol was somewhat different. In the presence or absence of ascorbate, p-cresol oxidation was inhibited by 50% ethanol at all enzyme concentrations tested. With p-cresol, in the presence of a high ascorbate concentration, and 50% ethanol, the rate of oxygen consumption was an approximately linear function of enzyme concentration. Under the same conditions, except that phenol was substituted for p-cresol, the rate of oxygen consumption was a non-linear function of enzyme concentration, inhibition occurring with low amounts of enzyme and apparent activation with high amounts. It is apparent from the work of Tsou and Li (29) that organic solvents affect substrate oxidation in a rather complex manner. Whether this occurs by means of changing the folding of the protein molecule or binding constants for substrate or both is not known.

It was of interest to test the effect of a reaction mixture which was 50% (v/v) in ethanol, and contained 3, 4-dimethylphenol. Preliminary experiments indicated that 50% ethanol activates the enzymic oxidation of 3, 4-dimethylphenol only when the ethanol is present in the reaction

mixture. The following experiment was conducted to test this point: 0.50 ml of enzyme fraction Xc (cf. Table II) was incubated with an equal volume of ethanol* at 25° C. The mixture was sampled at intervals and the rates of change in absorbancy at 412 millimicrons in the presence of 3, 4-dimethylphenol were compared when the reaction mixture was zero or 50% (v/v) in ethanol (Fig. 14). The data demonstrate that under these conditions, previous incubation in 50% ethanol does not affect the initial monophenolase activity prior to denaturation and precipitation of the enzyme. The observed activation in the presence of 50% ethanol in the reaction mixture corresponds to that which might be expected from the nonprecipitated enzyme. However, this last point was not quantitated.

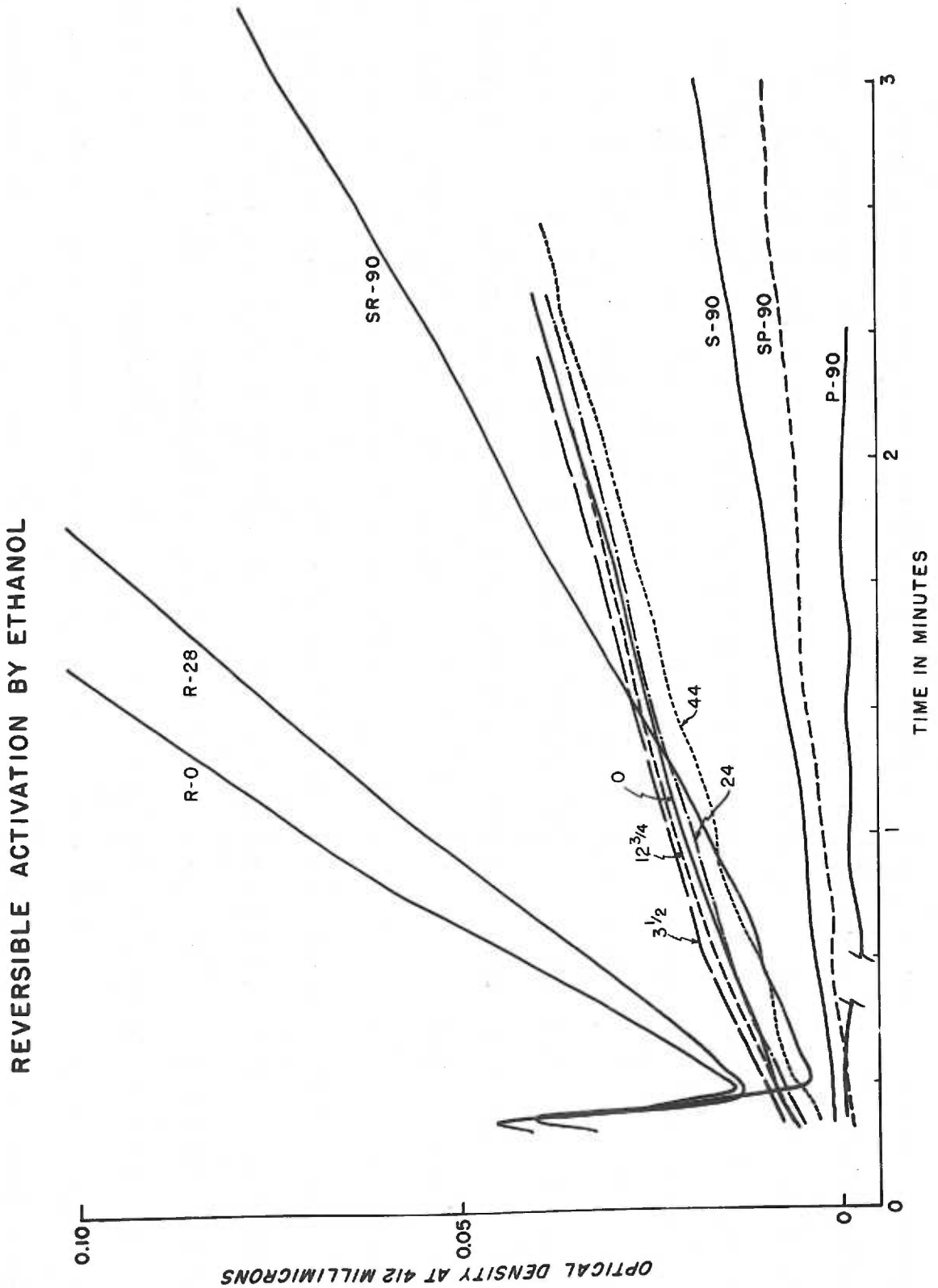
The data in Figure 14 demonstrate that the rate of the enzymically catalyzed oxidation of 3, 4-dimethylphenol is increased more than threefold (cf. Curves O, and R-O) when the reaction mixture is 50% in ethanol, but is not affected by preincubation of the enzyme in an equal volume of ethanol until denaturation occurs. Thus, if the change which is involved with this activation occurs in the absence of 3, 4-dimethylphenol, that change must be reversible. Tsou and Li (29) suggested but did not specifically demonstrate the reversibility of the activation of catechol oxidation by acetone.

*Rossville Gold Shield Alcohol, 200 proof, purchased from the Commercial Solvents Corporation, Terre Haute, Indiana.

Figure 14. Ethanol Activation of the Tyrosinase-Catalyzed Oxidation of 3,4-dimethylphenol. Absorbancy at 412 millimicrons is plotted as a function of time. Reactions in the presence or absence of 50% ethanol, which were catalyzed by enzyme (Xc) previously incubated in 50% ethanol at 25° C. for various lengths of time, are compared. Numbers on the curves indicate the time in minutes that the enzyme was preincubated before a sample was added to the reaction mixture. Reaction mixtures for curves marked R- were 50% (v/v) in ethanol. Precipitated protein was first observed in the ethanol-enzyme preincubation mixture after 20 minutes. After 70 minutes, the preincubation mixture was centrifuged, and the supernatant fluid (S) decanted. The pellet (P) was suspended in an approximately equivalent volume of distilled water. The reaction mixtures contained 0.20 ml of 0.201 M potassium phosphate buffer, pH 7.2; enzyme (0.05 ml of Fraction Xc for curves marked "0"; 0.10 ml of the preincubation mixture for curves marked 3-1/2, 12-3/4, 24, R-28, or 44; 0.10 ml of supernatant fluid for curves marked S-90 or SR-90; 0.10 ml of suspended pellet for curve SP-90); water; and ethanol for curves marked R- as indicated above. The reaction was started by adding 0.05 ml of 3,4-dimethylphenol solution containing 5.00 mg/ml. The volume of the completed reaction mixture was 3.00 ml in all instances. The reaction mixture was not bubbled with oxygen. The temperature was maintained at 25° C. The initial absorbancy at 412 millimicrons due to added 3,4-dimethylphenol is small and considered negligible.

Figure 14. (Continued)

The absorbancy obtained with Curve P-90 decreased below the recordable level, necessitating an adjustment of the "Balance Control" of the spectrophotometer. Therefore, after the break in Curve P-90, only the change in absorbancy, and not the relative absorbancy, was obtained. An initially high absorbancy was obtained with the reaction mixtures (R-0, R-28, and SR-90) which were 50% in ethanol. The reason for this is not known, but is presumed to be a mixing artifact from the addition of aqueous 3,4-dimethyiphenol solution to the 50% ethanol reaction mixture.


REVERSIBLE ACTIVATION BY ETHANOL
R-0
R-28
SR-90
S-90
SP-90
P-90
0
3½
12¾
24
44
0.10
0.05
0
0
1
2
3
OPTICAL DENSITY AT 412 MILLIMICRONS
TIME IN MINUTES

DISCUSSION

The central question in this dissertation is whether the activation of the monophenolase function of tyrosinase by catechol is reversible, and if so, whether the deactivation is mediated by oxygen. In order to obtain information regarding the activation of tyrosinase, several techniques have been applied to this field of study for the first time.

The question of whether the activation of the monophenolase function of tyrosinase by catechol is reversible is difficult to answer. In the present experiments, no activation was found when catechol was incubated with tyrosinase and removed by dialysis in the presence of undetectable oxygen tensions. Three possible explanations of this observation appear to be pertinent: 1. the enzyme was activated by catechol, but was deactivated by oxygen (cf. Doskocil's suggestion (17) on page 3 of the Introduction); 2. the enzyme was activated by catechol, but the activation was reversible independently of oxygen tension; 3. the enzyme was not activated by catechol in the absence of oxygen and monophenol. The work reported in this dissertation does not distinguish between the second and third possibilities, but tends to make the first possibility unlikely, not only because oxygen was not detected during the anaerobic dialysis experiment, but also because the

extreme precautions which were taken to remove oxygen might be expected to have lowered the oxygen tension to considerably below the detectable limit.

In the past, the autocatalytic form of the oxidation of monophenols catalyzed by tyrosinase has been difficult, if not impossible, to analyze from a kinetic standpoint. The description of the autocatalytic form has been broadened considerably by plotting the rate of quinone formation during the enzymic oxidation of 3,4-dimethylphenol, against time, since the rate of product formation is a function of activated enzyme concentration, as well as of substrate concentrations. The reactions which must be considered in any discussion of enzymic oxidation of monophenols are the following: 1. the oxidation of o-diphenol, independent from monophenol oxidation; 2. the activation of the monophenolase function by o-diphenol; 3. the oxidation of monophenol; 4. inhibition of o-diphenol oxidation by monophenol (demonstrated by Kendal (15)); and 5. accumulation of o-diphenol in the reaction mixture. One further relation which is suggested by the data of Figure 8, is an inhibition by high concentrations of 3,4-dimethylphenol, of monophenolase activation. By utilizing these six relations, it is possible to suggest meaningful interpretations of the rate-forms in Figure 8.

The effects of varying the initial monophenol concentration

on the first few minutes of the rate of enzymically catalyzed quinone formation has been described in Figure 8. It was suggested in the Experimental section (page 24) that a small amount of diphenol was produced in the reaction mixtures during oxygenation. Thus, it is suggested that with moderate concentrations of 3,4-dimethylphenol (curves 3-5, Figure 8), the rapidly increasing rate during the first 30 seconds represents a rapid activation of a small portion of the enzyme by diphenol which was initially present. The diphenol which was available for activation is consumed as the slopes of the rate curves decrease, and further activation must await accumulation of additional diphenol, which produces the second phase of activation (second increase in the slopes of the rate curves). With lower initial concentrations of 3,4-dimethylphenol (curves e- and f-0.25 and d-1 of Figure 8), the oxidation (without activation) of the small amount of diphenol which is presumed to have been initially present, is inhibited to a lesser extent by monophenol. The initial rates for these curves therefore represent the sum of independent diphenol oxidation and monophenol oxidation, and this sum decreases as diphenol is consumed until further independent oxidation of diphenol is inhibited by monophenol. The monophenolase reaction then becomes the major contributor to the rate of quinone formation, and the rate again increases due to accumulation of diphenol and subsequent

activation of additional enzyme. In the case of the higher monophenol concentrations (curves b-5, 9, and a-13, Figure 8), it is suggested that the independent oxidation of diphenol is fully inhibited and activation by diphenol is partially inhibited. It is clear from Figure 8 that the rapid acceleration during the first 30 seconds which was observed with moderate monophenol concentrations was not observed with the higher monophenol concentrations. If this rapid acceleration is correctly interpreted as being a rapid activation of the monophenolase function of tyrosinase, then this activation is inhibited by monophenol. Whether this represents a competitive inhibition of activation by diphenol is not known.

The form of the rate curves is much simpler after the first five minutes of the monophenolase reaction (cf. Figure 6). The amount of quinone which has formed after a given time, the rate, acceleration, and increase in acceleration, all vary in the same direction as the initial monophenol concentration. It is suggested that these observations may be most simply interpreted by the following considerations: 1. diphenol accumulates as a function of quinone concentration; 2. the concentration of enzyme which is activated varies as the diphenol concentration (cf. Figure 12); and 3. monophenol exerts a mass action effect on the activated enzyme. However, in order to analyze the data of Figure 6, and test whether these considerations are sufficient, it would be necessary to know the mathematical functions for each of them. This infor-

mation has not yet been obtained.

The rate relations which are described in Figures 10 and 11 for activation of the monophenolase function by catechol are quite complex. However, it is suggested that the six reactions which have been described on page 49 may be both necessary and sufficient for a complete kinetic analysis.

There are two advantages to the formulation of model reaction mechanisms at this stage in the study of tyrosinase activation. The simplifying assumptions which must be made in setting up the model may be put to test in the future, and the results of assuming a given model may be determined by mathematical analysis. The simplest model could be obtained by assuming an irreversible activation mechanism.

1. $\underset{(x-y)}{E_i} + \underset{(c-y)}{C} \xrightarrow{k} \underset{y}{E_a}$

If the reaction were started with only inactive enzyme (E_i) and activator (C) present initially, then at any time the inactive enzyme concentration would be (x-y), the activator concentration, (c-y), and the total activated enzyme concentration, y, where the initial concentrations of inactive enzyme, activator, and activated enzyme are x, c, and zero, respectively. Since the activation reaction is defined as being irreversible for this case, further reactions of E_a would not affect the activation reaction, and the velocity (v) of quinone formation from monophenol would be a function ($G_{(A,B)}$)

of monophenol (B) and oxygen (A) concentrations times the total activated enzyme concentration (y) as follows:

$$v = yG_{(A,B)} \qquad 1.$$

The function $G_{(A,B)}$, would be constant at constant monophenol and oxygen concentrations. For the purpose of derivation, it is further assumed that E_i and C take part in no other reactions than Reaction 1., and that Equation 1. is a valid linear function of activated enzyme concentration. The rate equation for Reaction 1. is

$$\frac{dy}{dt} = k(x-y)(c-y) \qquad 2.$$

where k is the second order rate constant. Rearrangement gives the form,

$$\frac{dy}{(x-y)(c-y)} = kdt$$

which is a linear differential equation in y and t, and can be easily integrated. When x is not equal to c, the following form* may be used for integration,

$$\int \frac{dx}{(a+bx)(a'+b'x)} = \frac{1}{ab' - a'b} \cdot \ln \frac{a' + b'x}{a + bx}$$

giving,

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*Integral number 47., "Table of Integrals," Handbook of Chemistry and Physics, Thirty-seventh Edition, C. D. Hodgman, R. C. Weast, and S. M. Selby, Editors, Chemical Rubber Publishing Co., Cleveland, 1955., p. 250.

$$\frac{1}{c - x} \cdot \ln \frac{c - y}{x - y} = kt + K$$

The integration constant, K, is evaluated at t = 0, and y = 0:

$$K = \frac{1}{c - x} \cdot \ln \frac{c}{x}$$

Incorporation of the integration constant gives the form,

$$\frac{1}{c - x} \cdot \ln \frac{x(c - y)}{c(x - y)} = kt \qquad 3.$$

Rewriting Equation 3. in exponential form and solving for y gives Equation 4.:

$$y = \frac{cx(e^{kt(c - x)} - 1)}{ce^{kt(c - x)} - x} \qquad 4.$$

When t = 0, the numerator is zero, and y = 0; and as t approaches infinity, the exponential term becomes large if c is greater than x, so that y approaches x; or if c is less than x, the exponential term approaches zero, so that y approaches c. At intermediate values of the exponential term, y is a non-linear function of c and x when t is held constant. Reference to Equation 1. demonstrates that the velocity is a linear function of activator concentration only if the activation reaction is complete.

Under the condition that c = x, Equation 4. is indeterminate, since both numerator and denomenator are then equal to zero. Therefore, integration of Equation 2. must be carried out using the form*,

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*Integral number 30., ibid., p. 249.

$$\int \frac{dx}{(a + bx)^2} = \frac{-1}{b(a + bx)}$$

giving,

$$kt = \frac{1}{c - y} + K$$

At $t = 0$, $y = 0$, and the integration constant, $K = -1/c$. Incorporating this value of the integrating constant and solving for y, the following equation is obtained,

$$y = \frac{c^2kt}{ckt + 1}$$

which reduces to $y = c$ as t tends toward infinity. Thus if, and only if, all of the assumptions for the irreversible activation model which were listed on pages are correct, and the activation reaction is essentially complete at the time of velocity measurement, then y, and therefore the velocity, v, (cf. Equation 1.) will be a linear function of the activator concentration, c, for all values of c where c is less than x and x is constant. This would not be in agreement with the data of Figure 12. Inspection of Figure 11 indicates that while the requirements of the irreversible activation model for a linear relation between velocity and activator concentration may be roughly approximated at $t = 1$ minute, they are not strictly met by the experimental data. Therefore the irreversible activation model cannot be ruled out by comparison with the data of Figure 12.

Let us now consider the model system in which the activation

mechanism is assumed to be reversible.

$$2. \quad E_i + C \underset{k_i}{\overset{k_a}{\rightleftharpoons}} E_a$$

If we start Reaction 2. with only inactive enzyme (E_i), and activator (C), and assume for the first case of reversible activation that all of the enzyme which has been activated comes into equilibrium with E_i and C, then the concentration of E_i at any time is $(E_o - E_a)$, and that of C is $(c - E_a)$, where E_o is the total enzyme concentration, and c is the initial activator concentration. The equation for the dissociation constant (K_{dis}) for E_a is thus,

$$K_{dis} = \frac{(E_o - E_a)(c - E_a)}{E_a} \qquad 6.$$

Multiplying both sides of Equation 6. by E_a gives

$$K_{dis}E_a = (E_o - E_a)(c - E_a)$$

and expanding the binomial and collecting terms gives Equation 7:

$$E_a^2 - (E_o + c + K_{dis})E_a + E_oc = 0 \qquad 7.$$

Equation 7. may be written in terms of velocity of quinone formation from monophenol (v) according to Equation 1., from which

$$E_a = y = \frac{v}{G_{(A,B)}}$$

Substituting this value for E_a into Equation 7. and multiplying through by $(G_{(A,B)})^2$ gives a quadratic in terms of velocity of quinone formation,

$$v^2 - (E_o + c + K_{dis})(G_{(A,B)})v + E_o c(G_{(A,B)})^2 = 0 \qquad 8.$$

which appears to be consistent with the data of Figure 12.

Consideration of the model for activation in which only one form (E_a) of the total enzyme which has been activated(E_A) is in equilibrium with inactive enzyme (E_i) and activator (C) leads to a different form of Equation 6.:

$$K_{dis} = \frac{(E_o - E_A)(c - E_A)}{E_a} \qquad 9.$$

In this case, the inactive enzyme concentration from Reaction 2. is ($E_o - E_A$), the activator concentration at any time is ($c - E_A$), and the concentration of that activated enzyme which is directly in equilibrium with inactive enzyme and activator is E_a. Multiplication of both sides of Equation 9. by E_a and expansion of the binomial gives Equation 10.

$$K_{dis}E_a = E_A^2 - (E_o + c)E_A + E_o c \qquad 10.$$

The total activated enzyme, E_A, may be evaluated in terms of the usual kinetic constants as follows. Alberty (49) has derived a relation for two-substrate enzyme mechanisms which may include one or more sequential ternary enzyme-substrate complexes, (50), or may include only binary enzyme-substrate complexes (51), such as occur in the Theorell-Chance mechanism (52) for liver alcohol dehydrogenase. (It should be noted, however, that this equation is applicable only to mechanisms for which valid Michaelis constants can be derived for both substrates.) As modified by Frieden (50), Alberty's equation is:

$$\frac{V_A}{v} = 1 + \frac{K_A}{A} + \frac{K_B}{B} + \frac{k_2 K_B}{k_1 AB} = J_{(A,B)} \qquad 11.$$

where V_A is the maximum velocity of that enzyme which is activated (E_A), K_A is the Michaelis constant for the first substrate, A, and K_B is the Michaelis constant for the second substrate, B, and v is the overall velocity in the forward direction. The maximum velocity and Michaelis constants for A and B are all determined at maximum concentrations of both substrates. The function $J_{(A,B)}$ is defined in the above equation, and differs from the reciprocal of the function $G_{(A,B)}$ only by the rate constant k_f for the breakdown of the rate-limiting complex into enzyme and products. Thus it is possible to write the following relations:

$$V_A = k_f E_A = v J_{(A,B)} \quad \text{and} \quad E_A = \frac{v}{k_f} \cdot J_{(A,B)} \qquad 12.$$

The kinetic form for the concentration of free activated enzyme (E_a) has been derived for two types of two-substrate enzyme mechanisms, one of which requires a ternary enzyme-substrate complex, and has been previously described by Segal, Kachmar, and Boyer (53), and Ingraham and Makower (54); and the second, which requires only binary enzyme-substrate complexes, is the Theorell-Chance mechanism (52) which was proposed to explain the reactions of liver alcohol dehydrogenase. For the derivations it is assumed that the active enzyme concentration is low relative to substrate and product concentrations, the concentrations of the enzymic intermediates do not change (steady state

assumption), and the concentrations of the products are sufficiently low that the reverse reaction is negligible (initial velocity assumption). The mechanism involving a ternary enzyme-substrate complex is as follows:

3. $\underset{x}{E_a} + \underset{A}{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{y}{EA}$

4. $\underset{y}{EA} + \underset{B}{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \underset{z}{EAB}$

5. $\underset{z}{EAB} \xrightarrow{k_f} \underset{p}{P} + \underset{x}{E_a}$

The substrate concentrations are A and B, and the concentrations of the enzymic intermediates are x, y, and z. Independent equations describing these reactions are:

$$\frac{dy}{dt} = 0 = k_1Ax - k_2y - k_3By + k_4z \qquad x = \frac{y(k_2 + k_3B) - k_4z}{k_1A}$$

$$\frac{dz}{dt} = 0 = k_3By - z(k_4 + k_f) \qquad y = \frac{(k_4 + k_f)z}{k_3B}$$

$$\frac{dp}{dt} = v = k_fz \qquad z = \frac{v}{k_f}$$

By successively substituting the values for y and z into the equation for x, and simplifying, the following relation is obtained:

$$x = \frac{k_2(k_4 + k_f) + k_3k_fB}{k_1k_3AB} \cdot \frac{v}{k_f}$$

Ingraham (46) derived the following relations for the Michaelis

constants for A, and B, at high concentrations of both substrates:

$$K_A = \frac{k_f}{k_1} \quad \text{and} \quad K_B = \frac{k_4 + k_f}{k_3}$$

Substitution of these values into the relation for x, gives,

$$x = \left[\frac{k_2 K_B}{k_1 AB} + \frac{K_A}{A}\right]\frac{v}{k_f} = E_a \qquad 13.$$

which is the kinetic form of E_a. An identical relation for the free-enzyme concentration has been derived by the author for the Theorell-Chance mechanism (53), which follows:

6. $E_a + A \rightleftharpoons EA$

7. $EA + B \rightleftharpoons EA' + P_1$

8. $EA' \rightleftharpoons E_a + P_2$

By substituting the relations for E_A and E_a (Equations 12 and 13) into Equation 10, and rearranging, the following equation is obtained:

$$v^2 - k_f\left[\frac{(E_o + c)}{J_{(A,B)}} + \frac{K_{dis}}{(J_{(A,B)})^2}\left(\frac{k_2 K_B}{k_1 AB} + \frac{K_A}{A}\right)\right]v + \frac{E_o c k_f^2}{(J_{(A,B)})^2} = 0 \qquad 14.$$

The quadratic form of the velocity in Equation 14. is a result of the assumptions that the activation is reversible and that the magnitudes of the concentrations of the enzyme and activator are similar. This latter assumption is required since the concentration of the enzyme-copper in the reaction mixtures used for Figure 12 was 3.8 micromolar, while that of the highest amount

of catechol used was 2.84 micromolar. It is not possible to directly compare Equation 14. with the data of Figure 12, but the forms appear to be consistent.

In the past, most investigators have emphasized the importance of the valence state of copper or the state of oxygenation in the activation of the monophenolase function of tyrosinase. However, no direct evidence has been obtained to establish whether copper exists in more than one valence state in tyrosinase, or whether more than one state of oxygenation exists. Indeed, such data are difficult to obtain. The work of Tsou and Li (29) indicates that under certain conditions, an increased rate of either diphenol or monophenol oxidation can occur in the presence of 50% ethanol. It has been demonstrated in the present dissertation that under at least one set of conditions, the activation by 50% ethanol of enzymically catalyzed 3,4-dimethylphenol oxidation is reversible, if it occurs in the absence of monophenol. This work, with that of Tsou and Li (29), suggests that the degree of folding of the enzyme may contribute to both the specificity and the degree of activation of tyrosinase. It would be premature, however, to suggest that the mechanisms of activation by 50% ethanol and catechol are the same. A much more thorough investigation of both the kinetic or equilibrium or stoichiometric aspects of tyrosinase function must be conducted before the mechanisms of activation will be elucidated.

SUMMARY AND CONCLUSIONS

The possible activation of the monophenolase function of mushroom tyrosinase by catechol in the absence of detectable oxygen has been studied. A solution of the enzyme was anaerobically incubated with catechol and then dialyzed against deionized, deoxygenated water until virtually all of the catechol had been removed. A comparison of the initial monophenolase activity of this anaerobically incubated enzyme with that of equivalent amounts of untreated enzyme indicated that no differences could be observed. It is concluded that if activation of tyrosinase by catechol occurs in the absence of oxygen, that activation must be reversible. Although the possibility that the reversal of activation is mediated by oxygen cannot be entirely excluded, it appears improbable not only because oxygen was not detected, but also because of the extreme precautions which were taken to exclude it during the anaerobic dialysis procedure.

Ethanol, at a concentration of 50% (v/v) in the reaction mixture, produced greater than a three-fold increase in the initial rate of quinone formation from 3,4-dimethylphenol over that obtained in its absence. Preincubation of tyrosinase with an equal volume of ethanol produced no observed change in the initial monophenolase activity prior to denaturation of the enzyme. It is concluded that if this activation by ethanol occurs in the

absence of 3,4-dimethylphenol, it is reversible.

Changes in the rates of quinone formation during the enzymic oxidation of 3,4-dimethylphenol have been studied in the presence of various initial 3,4-dimethylphenol and catechol concentrations. Rate- and acceleration-determining reactions have been suggested for the several portions of the rate versus time curves.

Model mechanisms for reversible and irreversible activation have been described which might approximate conditions which occur during the enzymic oxidation of monophenols, and their respective rate laws derived. On the basis of the kinetic analyses, it would be possible to reject the irreversible activation mechanisms only if certain restrictive assumptions were valid. Insufficient data are presently available to decide this point.

The possibilities of various types of activation have been discussed.

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