On storage for several days in water the solvate slowly changed to fine, colorless needles, which analyzed correctly for cyclo-(triglycyl). Both the hydrated and the anhydrous forms were insoluble in acid and base and gave a negative ninhydrin test.[3]

Experimental[8]

Phthaloyltriglycine Ethyl Ester.—Phthaloylglycyl chloride[9] (25 g., 0.112 mole) dissolved in 100 ml. of methylene chloride was added during one-half hour to a rapidly stirred mixture of 23.2 g. (0.23 mole) of triethylamine and 21.9 g. (0.112 mole) of diglycine ethyl ester hydrochloride[10] in 300 ml. of methylene chloride. The temperature was maintained between −45 and −40° during the addition and for an additional one-half hour. The cooling bath was then removed and stirring continued 2 hours at room temperature. The colorless precipitate was removed by filtration, dried, pulverized and washed with 500 ml. of 0.5 *N* hydrochloric acid and then with water. The product weighed 34.2 g. (88%); m.p. 232–232.5°. Recrystallization from aqueous dimethylformamide yielded 32.6 g. (84%) of cotton-like colorless needles; m.p. 232.5–233° (reported[11] 228–230°).

Triglycine Ethyl Ester Hydrochloride.—A suspension of 17.5 g. (50.4 mmoles) of phthaloyltriglycine ethyl ester in 500 ml. of ethanol was heated to reflux and 5 g. (0.1 mole) of hydrazine hydrate (100%) was added. The resulting clear solution was stored 18 hours at 25°, then evaporated to dryness under reduced pressure. The residue was subjected to 0.01 mm. pressure for 16 hours to remove the last trace of hydrazine hydrate. Phthalhydrazide precipitated on addition of 150 ml. of 0.5 *N* hydrochloric acid. After cooling for 2 hours at 4° the solution was filtered to remove the precipitated phthalhydrazide and the filtrate was freeze-dried to a white powder. Crystallization from absolute ethanol yielded a total of 10.6 g. (84%) of colorless needles; m.p. 214–217° dec. (reported[12] m.p. 214–219° dec.).

Triglycine Hydrazide.—Triglycine ethyl ester hydrochloride (0.95 g., 3.75 mmoles) was suspended in 50 ml. of hot ethanol and 0.7 ml. (14 mmoles) of hydrazine hydrate (100%) was added. A clear solution resulted which, after storage three days at 25°, yielded a white precipitate. The mixture was evaporated to dryness under reduced pressure and stored *in vacuo* overnight. The colorless residue, dissolved in 25 ml. of water, was passed slowly through a 15 × 400 mm. column packed with Amberlite IRA-400 anion-exchange resin. The chloride-free effluent was evaporated to dryness under reduced pressure, and the residue was recrystallized from ethanol yielding 0.61 g. (80%) of hygroscopic, colorless needles which sintered around 100°, slowly decomposed, and completely charred by 205°. An analytical sample, recrystallized twice from ethanol, still decomposed over a similar wide temperature range.

Anal. Calcd. for $C_6H_{13}N_5O_3$: C, 35.46; H, 6.46; N, 34.47. Found: C, 35.23; H, 6.76; N, 34.26.

Cyclo-(triglycyl).—To a solution of 0.812 g. (4 mmoles) of triglycine hydrazide in 38.4 ml. (8 mmoles) of 0.2083 *N* hydrochloric acid, cooled to 0°, was added 0.276 g. (4 mmoles) of sodium nitrite in 2 ml. of water. After swirling gently for 15 minutes, the solution was poured into 2 l. of ice-water. Sodium bicarbonate (5 g.) was added and the solution stored 40 hours at 4°. Following adjustment to *p*H 5 with 2 *N* hydrochloric acid, the solution was concentrated to approximately 50 ml. Acetone (350 ml.) was added and the colorless precipitate was collected and triturated with 25 ml. of cold water. The water-insoluble material was separated by filtration, weight 0.542 g.

Some polymeric material was removed by boiling the product in 20 ml. of water and filtering while hot. Long colorless rods were recovered from the filtrate; weight 0.290 g. (42%). The product gave a negative ninhydrin test,[3] was insoluble in dilute acid or base, and charred slowly above 300°, becoming completely charred by 350° without melting. Recrystallization from hot water yielded a hemihydrate.

Anal. Calcd. for $C_6H_9N_3O_3 \cdot {}^1/_2H_2O$: C, 40.00; H, 5.60; N, 23.32. Found: C, 40.27; H, 5.96; N, 23.42.

Further recrystallization from hot water yielded long colorless rods which slowly changed to very fine needles on storing several days in water. This material gave a negative ninhydrin test[3] and charred without melting between 350 and 365°.

Anal. Calcd. for $C_6H_5N_3O_3$: C, 42.10; H, 5.30; N, 24.55. Found: C, 41.87; H, 5.23; N, 24.54.

CAMBRIDGE 39, MASSACHUSETTS

(8) All melting points are corrected unless otherwise stated. We are indebted to Dr. S. M. Nagy and his associates for the microanalytical data.

(9) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949).

(10) E. Fischer and E. Fourneau, *Ber.*, **34**, 2868 (1901).

(11) K. Hofmann, A. Lindenmann, M. Z. Magee and N. H. Khan, THIS JOURNAL, **74**, 470 (1952).

(12) E. Fischer, *Ber.*, **36**, 2985 (1903).

[CONTRIBUTION FROM THE RESEARCH DEPARTMENT OF CIBA PHARMACEUTICAL PRODUCTS, INC.]

A Study of the Kinetics of Potato Phenoloxidase Inhibition

BY HANS HEYMANN, ZOSIA ROGACH AND R. L. MAYER

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The effect of 4-chlororesorcinol and of N-phenylthiourea on the rate of oxidation of catechol by crude potato phenoloxidase has been observed by means of Miller and Dawson's chronometric method. The data suggest that both inhibitors act by a largely competitive mechanism. Manometric measurements of the oxidation of *p*-cresol and its inhibition by phenylthiourea, 4-chlororesorcinol and *m*-hydroxybenzoic acid failed to demonstrate a difference between the response of the catecholase activity and that of the cresolase activity. 4-Chlororesorcinol progressively inactivates both catecholase and cresolase; this explains its great potency as an inhibitor of melanin formation. 2,4-Dihydroxyphenylalanine also acts as an inhibitor with progressive inactivating effect, as shown manometrically. The results are discussed.

The literature contains many reports on inhibitors of phenoloxidase; references are cited in reviews by Dawson and Tarpley,[1] Massart,[2] and Lerner.[3] The last named author presents a classification of compounds known to interfere with melanin formation according to their presumed mode of action, *e.g.*, compounds known to form complexes with copper, metals that may replace copper, substances resembling the substrate and thus probably acting competitively, and other classes. The large amount of work that has been done on the inhibition of phenoloxidase includes only a few papers in which velocity studies are reported that apply current theories of enzyme action to phenoloxidase systems. Baur[4] included potato phenoloxidase in a manometric study of reaction rates and their inhibition but did not evaluate his findings with refer-

(1) C. R. Dawson and W. B. Tarpley in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. II, Academic Press, Inc., New York, N. Y., 1951, p. 472.

(2) L. Massart, *ibid.*, Vol. I, 1950, p. 337.

(3) A. B. Lerner, *Advances in Enzymol.*, **14**, 74 (1953).

(4) E. Baur, *Helv. Chim. Acta*, **22**, 810 (1939).

ence to the Michaelis–Menten theory. Hackney[5] studied the inhibition of apple phenoloxidase by *inter alia*, resorcinol, using a manometric method; her results have been discussed critically by Warner.[6] Warner applied the chronometric method of Miller, *et al.*,[7] to determine that *m*-hydroxybenzoic acid acts as a competitive inhibitor in the enzymatic oxidation of catechol by potato and mushroom phenoloxidases.

Recently, a considerable number of compounds were examined in these Laboratories[8,9] as potential inhibitors of phenoloxidase by means of a simple and sensitive test in which drop-portions of increasingly more dilute solutions of the test substances are applied to filter paper which is then sprayed with a mixture of tyrosine and potato juice. A gray color of melanin develops on the test sheet and inhibition manifests itself in the form of lighter or uncolored spots at the sites where the test substances were deposited. In the spray test, the well-known inhibitor, phenylthiourea,[1-3] was found to be one of the most potent substances, equalled or surpassed only by 4-chlororesorcinol. On the other hand, *d,l*-2,4-dihydroxyphenylalanine,[10] another resorcinol derivative, proved to be only a feeble inhibitor. While this work was in progress, Lambooy[11] described the preparation of several isomers of dihydroxyphenylalanine including the 2,4-substituted product, and demonstrated that the latter inhibits melanin formation from tyrosine.

The present paper reports a study of the effect of varying substrate concentration on the velocity of the uninhibited and of the partially inhibited enzymatic oxidation of mono- and diphenols, especially of *p*-cresol and catechol. Our purpose was to gain information about the mode of action of the inhibitory compounds and to observe whether the cresolase and catecholase activities of phenoloxidase preparations are affected by one and the same, or by different inhibitory mechanisms. Although Gregg and Nelson[12] have shown that both types of activity are inhibited to an equal degree by 4-nitrocatechol or by sodium N,N-diethyldithiocarbamate, this finding does not necessarily rule out the possible existence of different inhibitory mechanisms. In the present investigation, the oxidation of cresols was observed by measuring the oxygen consumption of the reaction mixtures manometrically. For the study of catechol oxidation, the chronometric method[7] was employed, in which one measures the time required for a known amount of ascorbic acid to be oxidized by the *o*-benzoquinone produced enzymatically. No benzoquinone accumulates during the observation period and only the oxidation of catechol, but not of its reaction products, is measured.

(5) F. M. V. Hackney, *Proc. Linn. Soc. N.W.W.*, **73**, 439 (1949).

(6) C. Warner, *Australian J. Sci.*, **B4**, 554 (1951).

(7) W. H. Miller, M. F. Mallette, L. J. Roth and C. R. Dawson, THIS JOURNAL, **66**, 514 (1944).

(8) R. L. Mayer, M. R. Grimm and F. C. Kull, *Proc. Soc. Exptl. Biol. Med.*, **81**, 163 (1952).

(9) F. C. Kull, M. R. Grimm and R. L. Mayer, *ibid.*, **86**, 330 (1954).

(10) We thank Dr. R. Mizzoni for the preparation of a sample of *d,l*-2,4-dihydroxyphenylalanine.

(11) J. P. Lambooy, THIS JOURNAL, **76**, 133 (1954).

(12) D. C. Gregg and J. M. Nelson, *ibid.*, **62**, 2500 (1940).

Experimental

Materials.—All of the salts and reagents employed to prepare buffers were of reagent grade.

Substrates.—Catechol and *p*-cresol were Eastman Kodak White Label products; *p*-cresol was distilled *in vacuo* and the white solid was kept in a closed vessel under refrigeration prior to use. L-Tyrosine and *d,l*-3,4-dihydroxyphenylalanine were products of Winthrop-Stearns, Inc.

Inhibitors.—4-Chlororesorcinol, *m*-hydroxybenzoic acid and N-phenylthiourea were Eastman Kodak White Label products. *d,l*-2,4-Dihydroxyphenylalanine was prepared by Dr. R. Mizzoni.[10]

Phenoloxidase Preparation.—Cold potatoes were sliced or grated under a blanket of nitrogen and ground in a Waring Blendor. The supernatant was recovered by centrifugation or, preferably, pressing through Curity diaper cloth. The liquid was kept cool at all times and was processed as rapidly as possible by alcohol precipitation and phosphate extraction of the solids, followed by dialysis in an Aminco agitating dialyzer. The preparation is equivalent to the "dialyzed potato juice" of Graubard and Nelson.[13] Centrifugation in the cold was found preferable to filtration through Super-cel of the alcohol precipitation mixture. Several batches of enzyme were prepared which remained active for weeks when stored in the cold. On the basis of the extrapolated values for V_{max}, the preparation contained (at 30°) 2–3 cresolase units per ml. and 5–8 catecholase units per ml. The batches were not pooled, but experiments to be compared with one another were carried out with one and the same batch.

Chronometric Measurements.—The procedure given in ref. 7 was followed with the exception that the reactions were carried out at 30° and that the 2 *N* sulfuric acid used in the indicator mixture contained 2% instead of 1% pyrogallol. With the use of 5.0 ml. of enzyme preparation, a suitable range of substrate concentrations was found to be from 2×10^{-3} to 10×10^{-3} *M*. At first, quantities of catechol (1.0, 0.80, 0.60, 0.40, 0.20×10^{-3} mole) were weighed out to ±0.2 mg., dissolved in 10 ml. of water kept at 30° and immediately added to the reaction mixture of enzyme, buffer and ascorbic acid to give a total volume of 100 ml. Stock solutions of catechol underwent air oxidation fairly rapidly as evidenced by the appearance of a yellow color within 1–2 hours; however, it was found expedient to prepare 100-ml. samples of catechol solutions (1.0, 0.80, etc., $\times 10^{-2}$ *M*) by dissolving the appropriate amount of substrate in boiled (*i.e.*, degassed) water immediately before initiation of a series of measurements. Such solutions showed no observable change during the time required for measurements, which did not exceed, and generally was less than, one-half hour. Phenylthiourea was incorporated in the reaction mixture to give a concentration of 1.5×10^{-6} *M*. For reasons to be discussed, 4-chlororesorcinol was added to the various catechol solutions to give a final concentration of 1.2×10^{-5} *M*; by this expedient, substrate and inhibitor were allowed simultaneously to interact with the enzyme. The final *p*H of the reaction mixture was 5.5; this value was selected because the originators of the chronometric method[7] state that at *p*H 5.5 the end-points are sharper than at *p*H 7.

Manometric Measurements.—The measurements were carried out at 30° and *p*H 7.0; all reagents were added as solutions in *M*/10 phosphate buffer with the exception of the phenoloxidase solution.

A suitable range of *p*-cresol concentrations was 1.0, 0.70, 0.50, 0.30 mg. per 3.0 ml. reaction mixture (30.8×10^{-4}–9.25×10^{-4} *M*), when 0.50 ml. of potato extract was used. Substrate and inhibitor or buffer were placed in the main chamber, and the enzyme solution was put into the sidearm; after equilibration the vessels were tipped and readings were taken at 5-minute intervals for 30 minutes.

In the experiments with tyrosine, 2-ml. aliquots of substrate solution were added containing 1.75, 1.50, 1.25 and 1.0 mg. of tyrosine (the stock solution contained 35 mg. of tyrosine in 40.0 ml. of warm buffer; the measuring error introduced by the use of a hot solution is negligible; the final concentration of tyrosine in the flasks did not exceed the solubility limit). The enzyme (1.0 ml.) was added directly to the main chamber and 20% potassium hydroxide

(13) M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **111**, 757 (1935).

was placed in the center well fitted with the customary filter paper strip. Buffer or inhibitor solution brought the final volume to 3.50 ml. Readings were taken for six hours at hourly intervals.

Treatment of Data.—For the construction of the double reciprocal plots shown in Figs. 1, 3, 5 and 6 the data were treated as a regression of $1/v$ on $1/S$ to obtain the line minimizing the deviation of the $1/v$ component (method of least squares). Although this method substitutes a certain arbitrariness for the personal bias in line fitting by eye and gives undue weight, in a reciprocal plot, to the least reliable points, it must still be regarded as the best method available to avoid personal bias. The values for the slopes and intercepts recorded in Table I are computed from the regression equations.

The manometric rate data from six estimations of the velocity–substrate relation show an average scatter of ± 0.19 μl./min. On the assumption that this value is a measure of the precision attained, the probable uncertainty of the experimental points has been calculated and is indicated by markers on the figures. Absence of markers means that the uncertainty is equal to or smaller than the limits encompassed by the circle indicating an experimental point.

The individual measurements obtained in chronometric velocity estimations of 5 different substrate concentrations in which the rate was linear with time (case of phenylthiourea) were compared to the rates as determined by passing a straight line through the experimental points. The average scatter of the 20 individual points was $\pm 0.05 \times 10^{-2}$ mg./sec. of ascorbic acid. This value was taken as a measure of the precision attained and treated as in the preceding paragraph.

Results[14]

Rate comparisons in studies of enzyme activity are meaningful only under certain conditions, *e.g.*, in a region where the amount of reactants consumed is proportional to time, *i.e.*, where the enzyme activity remains constant and the change in substrate concentration is negligible, or where the velocity changes obey a simple rate law that allows extrapolation to zero time. By its nature, the chronometric method[7] is well suited to such a study of catecholase activity. We found that at the highest substrate concentrations employed, the initial velocity could be extrapolated by the graphic method given in reference 7, and that at the lower catechol concentrations, a plot of mg. of ascorbic acid used *versus* time gave a straight line through the origin.

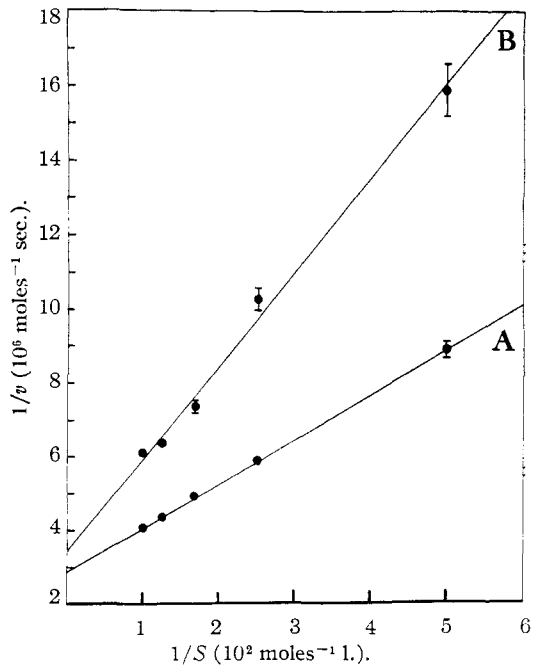

Fig. 1.—Oxidation of catechol with phenoloxidase preparation 427: line A, no inhibitor; line B, in presence of 1.5×10^{-6} M N-phenylthiourea.

Application of the treatment of Lineweaver and Burk[15,16] yielded lines A of the $1/v$ *versus* $1/S$ plots presented in Figs. 1 and 3. Pertinent data as computed from the regression lines are assembled in Table I. The slope/intercept ratios of lines A in Figs. 1 and 3 correspond to values of K_S = 4.2, 4.6×10^{-3} mole l.$^{-1}$, in agreement with Warner's observation of 5×10^{-3} mole l.$^{-1}$.

TABLE I

DATA PERTAINING TO FIGURES 1,3, 5, 6

Fig. and line	Slope 10^4 sec. l.$^{-1}$	Intercept 10^6 moles^{-1} sec.	Ratio Slope B/A	Ratio Slope C/A	Ratio Inter-cept B/A	Ratio Inter-cept C/A
1 A	1.21	2.90				
1 B	2.52	3.46	2.10		1.23	
3 A	1.20	2.63				
3 B	1.81	3.02	1.51		1.15	
	10^3 min. l.$^{-1}$	10^6 mole^{-1} min.				
5 A	0.281	1.61				
5 B	0.756	2.25	2.69		1.41	
5 C	1.148	3.17		4.09		1.97
6 A	0.232	1.96				
6 B	0.366	2.60	1.58		1.33	
6 C	0.504	2.88		2.17		1.47

In order to learn whether the inhibitor actions were reversible and constant with time, we varied the period of enzyme–inhibitor interaction that takes place before addition of substrate. The results are given in Fig. 2; clearly no loss in enzyme activity occurs when the diluted phenoloxidase solution is allowed to stand for periods up to 4 minutes in the presence or absence of phenylthiourea, whereas an appreciable loss is noted in the case of inhibition by 4-chlororesorcinol. To minimize this effect during rate measurements, 4-chlororesorcinol was incorporated in the various substrate solutions and the chronometric measurements were made between 20 and 45 seconds. As Fig. 2 indicates, the inactivation error is likely to be small during the first 45 seconds. The $1/v$ *versus* $1/S$ plot of the chlororesorcinol reactions is shown as line B in Fig. 3; the parameters as computed from the regression line appear in the table. The ratio V/V_i as given by the intercepts is 1.15, whereas the corresponding ratio of the two slopes is 1.51.

Line B in Fig. 1 gives the results of chronometric rate measurements in the presence of N-phenylthiourea and the regression parameters appear in the table. The ratio V/V_i is 1.23 and the ratio of the slopes is 2.10.

In both cases the velocity of the inhibited reac-

(14) Symbols employed in accord with common usage are: S, substrate concentration; K_s, dissociation constant of the enzyme–substrate compound; K_I, dissociation constant of the enzyme–inhibitor compound; V, maximum velocity of the uninhibited reaction; V_i, maximum velocity of the inhibited reaction; v, reaction velocity.

(15) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(16) *Cf.* P. W. Wilson in H. A. Lardy, "Respiratory Enzymes," Burgess Publishing Co., Minneapolis, Minn., 1949.

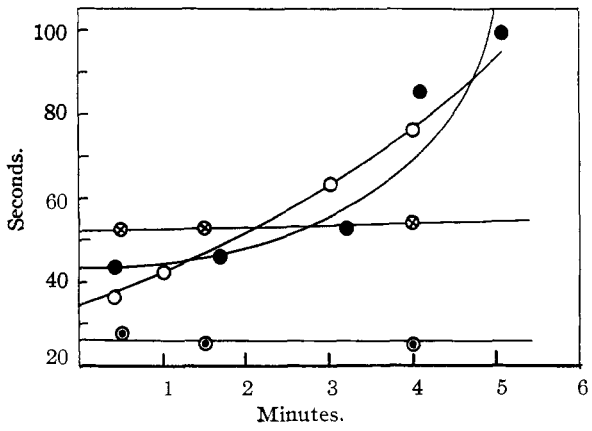

Fig. 2.—Oxidation of catechol with phenoloxidase preparation 427: abscissa, time elapsed between addition of inhibitor to enzyme and addition of substrate; ordinate, time required for oxidation of specified amount of ascorbic acid: ⊙, no inhibitor, 1.0 mg. of ascorbic acid; ⊗, 1.5×10^{-6} *M* phenylthiourea, 1.5 mg. of ascorbic acid; O●, 1.2×10^{-5} *M* 4-chlororesorcinol, 0.5 mg. of ascorbic acid, and 1.5 mg. of ascorbic acid, respectively; ⊙⊗●, 10×10^{-3} *M* catechol; O, 2×10^{-3} *M* catechol.

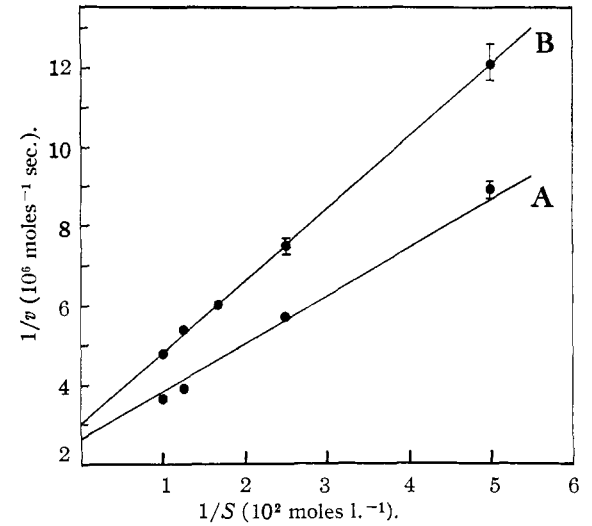

Fig. 3.—Oxidation of catechol with phenoloxidase preparation 423: line A, no inhibitor, line B, 1.2×10^{-5} *M* 4-chlororesorcinol.

tion approximates that of the normal reaction upon extrapolation to infinite substrate concentration. Although the effect of the inhibitor is not completely abolished in either case, the effect of the inhibitors upon the slopes of lines B in both cases is appreciably greater than the effect upon the intercepts. Thus, while neither of the two simple conditions of the Lineweaver–Burk interpretation is fulfilled, the data do permit the conclusion that both inhibitors act largely by a competitive mechanism. Values of K_I computed on that assumption are 1.3×10^{-6} mole $l.^{-1}$ for phenylthiourea and 2.4×10^{-5} mole $l.^{-1}$ for 4-chlororesorcinol.

Figure 4 shows the reaction curves observed during a typical manometric experiment in which the oxidation of *p*-cresol with phenoloxidase was inhibited by 4-chlororesorcinol. The progressive inactivation of the enzyme by the inhibitor is evident and an evaluation of the initial reaction velocities appears to be impossible without a detailed study of the rate of inactivation, which was not undertaken. Zero time in Fig. 4 is the moment at which the enzyme was added to the mixture; in other words, in the presence of 4-chlororesorcinol an induction period was not observed. Although Fig. 4 fails to show an induction period for the uninhibited reaction, this is exceptional and normally such a period was observed.

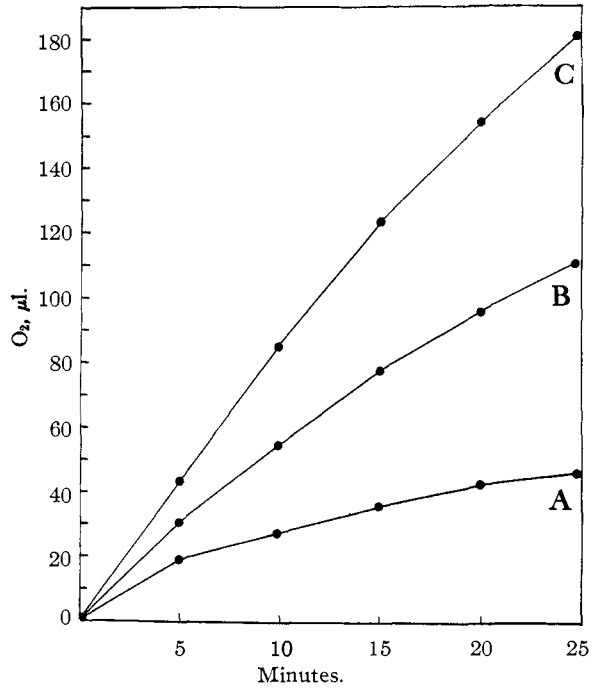

Fig. 4.—Oxidation of *p*-cresol with phenoloxidase preparation 312; $S = 30.8 \times 10^{-4}$ *M*; line C, no inhibitor; line B, 4.2×10^{-5} *M* 4-chlororesorcinol; line A, 7.5×10^{-5} *M* 4-chlororesorcinol. Zero time is the time of addition of enzyme to substrate.

In the presence of N-phenylthiourea, on the other hand, the velocity of *p*-cresol oxidation remained constant for at least 30–40 minutes. The initial rates were estimated from the linear, or nearly linear, portions of the reaction curves that obtained, after passage of an induction period, and a plot of $1/v$ *versus* $1/S$ was constructed (Fig. 5); the parameters of the regression lines appear in the table.

Line A refers to the uninhibited reaction and lines B and C are plots of the velocity data obtained in the presence of 5×10^{-7} and 10^{-6} *M* phenylthiourea. Even though here, again, neither the condition for strictly competitive nor non-competitive inhibition is fulfilled, the slopes are increased by about twice the increase of the intercepts, which allows one to conjecture that a competitive element is present in the inhibitory action.

Since Warner[6] had shown *m*-hydroxybenzoic acid to be a competitive inhibitor for catecholase oxidation, it was of interest to examine the effect of this substance on the oxidation of *p*-cresol. The initial rates were readily evaluated from the linear portion

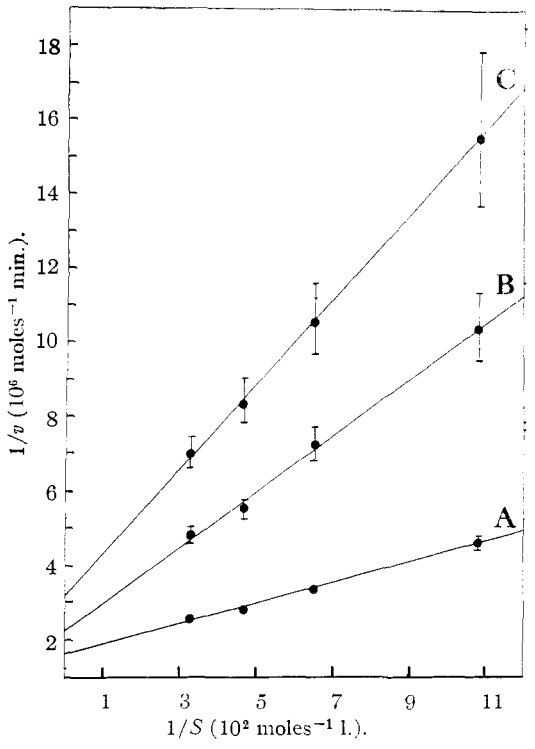

Fig. 5.—Oxidation of *p*-cresol with phenoloxidase preparation 323: line A, no inhibitor; line B, 5×10^{-7} *M* phenylthiourea; line C, 10^{-6} *M* phenylthiourea.

of the reaction curves that resulted after passage of an induction period. The double reciprocal plot (Fig. 6) for the two inhibitor concentrations employed (2.5×10^{-3} and 10^{-3} *M*) shows slope increases by factors of 2.17 and 1.47, respectively, as compared to increases of intercepts of 1.58 and 1.33. These data likewise indicate that some inhibitor action persists even at infinite substrate concentration.

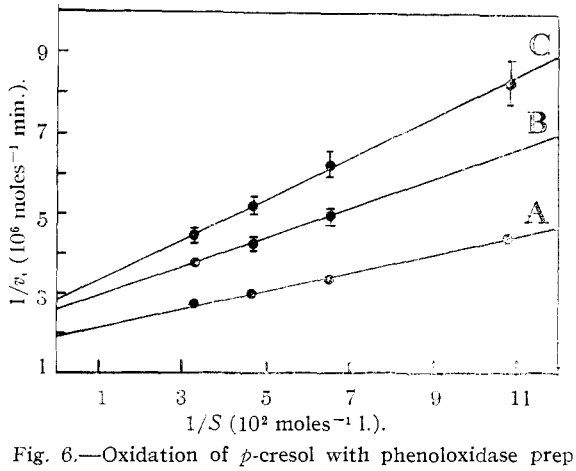

Fig. 6.—Oxidation of *p*-cresol with phenoloxidase preparation 329: line A, no inhibitor; line B, 10^{-3} *M* *m*-hydroxybenzoate; line C, 2.5×10^{-3} *M* *m*-hydroxybenzoate.

Experiments with *d,l*-2,4-dihydroxyphenylalanine were limited to manometric studies owing to the scarcity of the material. The inhibitor showed only feeble action in experiments with *p*-cresol. Under the conditions specified, the oxygen consumption during a 30-minute period of a 15.4×10^{-4} *M* solution of *p*-cresol was reduced to 70% by the addition of inhibitor to give a concentration of 38×10^{-3} *M*, a 25-fold molar excess. When the ratio was 30.8×10^{-4} *M* *p*-cresol:20.3×10^{-3} *M* inhibitor, no effect on oxygen consumption was noticeable. The oxidation of L-tyrosine, however, was significantly inhibited by approximately equimolar concentrations of *d,l*-2,4-dihydroxyphenylalanine, and an approximately tenfold molar excess of inhibitor completely arrested the reaction. As in the case of 4-chlororesorcinol, the rates of the inhibited reactions showed a marked decay with time (*cf.* Fig. 7) and were not suitable for a reliable estimate of initial velocities. No statement can be made about the presence or absence of an induction period because, as detailed in the experimental section, substrate and enzyme had to be mixed prior to equilibration; zero time on Fig. 7 is thus an arbitrary point in time.

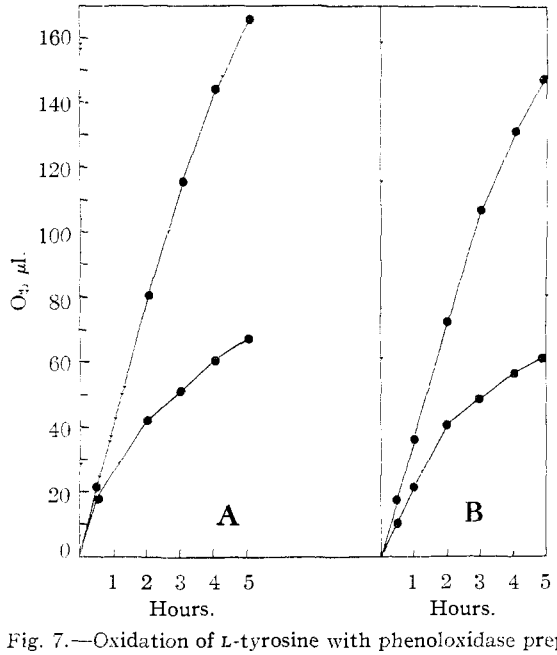

Fig. 7.—Oxidation of L-tyrosine with phenoloxidase preparation 329: section A, 3.2×10^{-3} *M* tyrosine in presence and absence of 4.3×10^{-3} *M* *d,l*-2,4-dihydroxyphenylalanine; section B, 2.8×10^{-3} *M* L-tyrosine in presence and absence of 4.3×10^{-3} *M* *d,l*-2,4-dihydroxyphenylalanine.

Discussion

The inhibitory action of the two resorcinol derivatives as detailed above is complex. However, the progressive and irreversible deactivation of both cresolase and catecholase activity provides an explanation for the great potency exhibited by 4-chlororesorcinol in the spray test. The end-point in this test, it will be recalled, is that dilution of inhibitor which causes a distinct decrease in melanin formation as compared to the uninhibited reaction which is allowed to proceed for one-half to one hour. 4-Chlororesorcinol is effective in high dilution because of its ability progressively to inactivate the enzyme as time elapses. The competitive nature of the initial inhibition of catecholase by 4-chlororesorcinol is plausible in view of the phenolic nature

of the inhibitor, if the additional assumption is made that a *m*-dihydroxybenzene is unsuitable for enzymatic *o*-hydroxylation. The acid-strengthening effect of the chlorine atom may contribute to the affinity of the inhibitor for the enzyme.

A further complexity consists in the fact that in the presence of either of the resorcinol inhibitors, oxidation mixtures of *p*-cresol develop a bright orange-red color soon after the onset of the reaction. The color is not produced when any one of the constituents, oxygen, phenoloxidase, substrate or resorcinol derivative is omitted, nor does it develop in the presence of very high concentrations of chlororesorcinol which inhibit completely the oxidation of the substrate. In enzymatic oxidations of catechol in the presence of 4-chlororesorcinol, the red color is also seen to develop about 10–15 minutes after the presence of *o*-benzoquinone becomes apparent from the yellow color of the solution. Thus, it appears plausible that the color is due to a reaction between the resorcinol and an oxidation product of the substrates, conceivably the *o*-quinone. The literature contains examples of facile reactions of this type; Nietzki[17] states that *p*-benzoquinone and resorcinol condense in benzene solution to give a deeply colored body $C_{12}H_{10}C_4$, and acid catalyzed condensations yielding (*m*-hydroxyphenoxy)-hydroquinones are described by Blumenfeld and Friedländer.[18] Since no *o*-quinone is present during chronometric measurements, the red product cannot be responsible for the velocity decay noted, but the effects, if any, of its production during the oxidation of cresol upon gas consumption and/or velocity of oxidation present a question that cannot be answered at this time.

The disappearance of the induction period in the presence of 4-chlororesorcinol is interesting. During the induction period a small amount of dihydric phenol is generated and according to Bordner and Nelson,[19] this substance activates the enzyme for the hydroxylation of monophenols. Since the suggestion was voiced above that chlororesorcinol competitively replaces catechol in catechol oxidation, it is further possible that it also possesses the ability to exert a similar activating influence. Thus 4-chlororesorcinol would allow the oxidation of *p*-cresol to proceed at once by virtue of its ability to prime the enzyme; as time passes the irreversible inactivating effect comes into play and the reaction velocity drops off.

(17) R. Nietzki, *Ann.*, **215**, 136 (1882).
(18) S. Blumenfeld and P. Friedländer, *Ber.*, **30**, 2563 (1897).
(19) C. A. Bordner and J. M. Nelson, THIS JOURNAL, **61**, 1507 (1939).

If the interpretation of Fig. 1 as suggested above is accepted, then the competitive action of phenylthiourea is unexpected from the point of view that a competitive inhibitor is generally regarded as a substance that structurally resembles the substrate without being able to undergo its reactions. However, there is evidence that phenylthiourea is effective because of its affinity to form chelates with cupric ions, and that addition of cupric salts reverses the inhibition of catecholase by phenylthiourea.[20] It is thus entirely conceivable that phenylthiourea competes with the substrate catechol for the same coördination valences of the copper–protein enzyme. Conversely, the present observation might be cited as evidence that the active positions of catecholase are those where the cupric ions are located and that activation of catechol is initiated or mediated by chelate formation with the cupric ions.

The manometric data have been plotted in the fashion customary for reactions in which only a single rate process occurs, or is being measured. Attempts at interpretation of the manometric rate data require great caution. The observed velocities are based on total gas consumption, which involves the oxidation of both cresol and catechol, and such further oxidative changes as have been recognized.[1] The velocity of oxygen consumption is not identical with the rate of disappearance of *p*-cresol, the primary substrate, and may not even be proportional to that rate. Moreover, the chronometric measurements were performed at *p*H 5.5, whereas the manometric runs took place at *p*H 7.0, which casts some uncertainty upon a comparison of the data. Without a much more detailed analysis of the individual rate processes, a meaningful interpretation of the double reciprocal plot is impossible.

Such differences as appear to exist in the response of the cresolase and catecholase activities to the inhibitors used are small and, in view of the very crude nature of the enzyme preparation employed, probably not significant. More striking is the observation that both activities are subject to progressive inactivation by 4-chlororesorcinol. Thus the results of the present study have not brought forth a differentiation between the two activities of the enzyme preparation, but rather underline the close relationship between them.[21]

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(20) L. E. Tenenbaum and H. Jensen, *J. Biol. Chem.*, **147**, 27 (1943).
(21) *Cf.* reference 1, p. 460.