

## The Action of Tyrosinase on Monophenols

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The many studies of tyrosinase have left unsolved the problem of the relationship between the so-called 'monophenolase' and 'catecholase' or 'polyphenolase' functions of this enzyme. It has seemed improbable that the same enzyme should effect two such dissimilar reactions as the introduction of a second hydroxyl group into the phenol ring and the dehydrogenation of an *o*-diphenol to give an *o*-quinone; yet the postulation of an independent specific monophenolase must remain under grave suspicion until fractions are obtained in which the relative monophenolase/catecholase activity is greater than in the crude mushroom juice. The alternative postulate, that the oxidation of monophenol is a secondary non-enzymic reaction, due to hydrogen peroxide or *o*-quinone formed from traces of *o*-diphenol which are presumed to be present, was first made by Onslow & Robinson (1928). It was criticized by Pugh (1929) and by Bordner & Nelson (1939), and a reconsideration of the evidence has led Nelson & Dawson (1944) to dismiss this possibility. The latter authors conclude that the monophenolase and catecholase functions are originally exercised by the same enzyme or enzyme complex, but that a centre in the enzyme which is essential for the monophenolase function, but not for the catecholase function, is (*a*) rather unstable towards the fractionation techniques which have been used, so that monophenolase activity is easily lost, and (*b*) inactive towards monophenol unless *o*-diphenol is being simultaneously oxidized by the enzyme, so that there is an induction period only relieved either by the slow build up in *o*-diphenol concentration initiated by spontaneous oxidation, or by the addition of *o*-diphenol to the system.

The first product of oxidation of monophenol is believed to be the corresponding *o*-diphenol, although the *o*-diphenol has only been isolated in the case of tyrosine  $\rightarrow$  dihydroxyphenylalanine (Raper, 1926). The *o*-diphenol so formed is then also available as substrate to the enzyme, and may be oxidized to the quinone and beyond in the case of the primary substrates most studied (tyrosine, phenol, *p*-cresol) to a stage involving a further uptake of oxygen of 1 atom/mol. or more. The composition of the reaction mixture thus becomes quite complex with several different reactions proceeding simultaneously, but, in the studies of 'monophenolase' activity so far made, the monophenolase activity has been assessed

in terms of the total oxygen uptake in this complex system. Keilin & Mann (1938) first reported the catalyzed oxidation of ascorbic acid in solutions containing tyrosinase and catechol, and Miller & Dawson (1941) have made use of this in their 'chrometric' method for measuring catecholase activity. The presence of ascorbic acid does not affect the catecholase activity of the enzyme, but *o*-quinone formed is then almost instantaneously reconverted to *o*-diphenol by the ascorbic acid, and is not detectable in the reaction mixture until all the ascorbic acid has been oxidized. This suggested that a study of the oxidation of monophenols by the enzyme in the presence of ascorbic acid might be of value. Under such conditions *o*-diphenol might be expected to accumulate, and the reaction mixture to remain uncomplicated by the accumulation of pigmented oxidation products of unknown constitution. The conditions would in fact be suitable for a study of the primary reaction, monophenol  $\rightarrow$  *o*-diphenol. The present paper describes methods for the estimation of *o*-diphenol in reaction mixtures containing initially monophenol, tyrosinase and ascorbic acid; and results obtained with these methods in studies of the conversion of monophenol to *o*-diphenol in the cases of tyrosine, phenol, *p*-cresol and 4:5-dimethylphenol.\* The experiments were carried out in the hope that the data obtained might throw more light on the vexed question of the mechanism of monophenol oxidation by tyrosinase.

### METHODS

*Enzyme preparation.* The enzyme was prepared from the common cultivated mushroom, *Psalliota campestris*, by the method of Keilin & Mann (1938). The second fractional adsorption on  $\text{Ca}_3(\text{PO}_4)_2$  in this procedure gave two fractions, the first of which, having  $Q_{O_2}$  (catechol)† and  $Q_{O_2}$  (*p*-cresol) values of c. 70,000 and 35,000, respectively, when freshly prepared, was used in all the experiments described in this paper. It was stored in the refrigerator under toluene, and had been so kept for 2 years when the work was begun. During this time its activity towards *p*-cresol had decreased by about one third, whilst the activity towards catechol appeared to have remained unchanged.

\* This compound is strictly named '3:4-dimethylphenol'. The alternative name '4:5-dimethylphenol' is used throughout this paper to emphasize the relationship of the phenol to the *o*-diphenol, 4:5-dimethylcatechol, formed from it.

† All  $Q_{O_2}$  values are in  $\mu\text{l. O}_2/\text{mg. dry wt./hr.}$

*Conditions in tyrosinase experiments.* The reaction mixtures in all cases contained 0.1 M-phosphate buffer (pH 7.0), and the temperature was 25°. The reactions were in general carried out in open vessels vigorously aerated by a stream of air for the provision of samples for *o*-diphenol and ascorbic acid estimation, and in the Warburg manometric apparatus with identical reaction mixtures, using a 2.00 ml. fluid phase and the standard technique, for the measurement of O<sub>2</sub> absorption. In the early experiments, with tyrosine as substrate, excellent agreement was obtained between the O<sub>2</sub> requirement calculated to correspond with the changes in ascorbic acid and *o*-diphenol concentration, and the O<sub>2</sub> uptake measured in parallel experiments. Later it was found in certain cases, viz. with phenol as substrate at the highest concentration used (0.1 M) and also with 4:5-dimethylphenol at quite low concentrations, that the oxidation of ascorbic acid and formation of *o*-diphenol was equivalent to a much smaller O<sub>2</sub> uptake than was observed in the parallel experiment. The difference could in neither case be attributed to a deficiency in aeration in the open vessel experiment, but, when this difference was observed, a rather persistent froth was always present above the surface of the reaction mixture in the open vessel, and the most likely explanation is that a major fraction of the enzyme was adsorbed in this froth and rendered ineffective by removal from the solution. To overcome this difficulty when it arose, manometric experiments only were carried out, in triplicate. Individual manometers were removed from the bath at different times after the beginning of the reaction, and their reaction mixtures immediately analyzed for ascorbic acid and *o*-diphenol. Composite curves were constructed from the data to show the changes in the reaction mixture with time.

#### *Estimation of o-diphenols*

It was desired to estimate ascorbic acid as well as *o*-diphenol in colourless reaction mixtures containing the corresponding monophenol, tyrosinase, ascorbic acid and 0.1 M-phosphate buffer. The reaction was always stopped at the appointed time by pipetting a measured sample into an equal volume of 4% metaphosphoric acid and the protein-free filtrate was used for both *o*-diphenol and ascorbic acid estimations. The following account of methods of *o*-diphenol estimation refers, therefore, only to estimations in this particular type of filtrate. The standard solutions of *o*-diphenols, used for the preparation of calibration curves, contained the same amounts of phosphate buffer and metaphosphoric acid as are present in such a filtrate. In no case was the presence of monophenol found to have any effect on the diphenol estimation. Ascorbic acid was only added to the standard solutions when it was found materially to affect the rate of development of the colour which was the basis of the method of estimation.

A. 3:4-Dihydroxyphenylalanine (DOPA). Schild (1933) and von Euler (1933) used the red colour developed on oxidation with I<sub>2</sub> for the colorimetric estimation of adrenaline. Evans & Raper (1937*a*) found that a similar method could be used for the estimation of DOPA. The method adopted in the present work was essentially that of Evans & Raper, improved by the use of the Spekker photoelectric absorptiometer instead of visual colour matching. A calibration curve was first prepared. The solution required for establishing a point on the curve was made by mixing in the order given: (a) 8.0 ml. of standard DOPA solution,

(b) 14.0 ml. of 0.5 M-phosphate buffer (pH 6.0), (c) 10.0 ml. of 0.192 N-NaOH and (d) 3.8 ml. of 0.1 N-I<sub>2</sub>. This mixture gave a final pH of 6.0. Ninety seconds after the I<sub>2</sub> addition (e) 4.2 ml. of 0.1 N-Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> were added, and the mixture immediately transferred to the 4 cm. absorptiometer cell. Absorptiometer readings were taken, with a blue filter (no. 6 of Hilger set H455), at timed intervals for a period of c. 5 min. The red colour remaining in the solution after the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> addition, due to the presence of DOPA, was found to diminish appreciably in intensity even within this time. The logarithms of the absorptiometer readings were plotted against time after Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> addition, and the linearity of the plot permitted extrapolation back to zero time. By following the same procedure with the enzyme reaction filtrate, using the latter instead of (a) above, a zero-time absorptiometer reading was obtained and the corresponding DOPA concentration read on the calibration curve. The latter covered DOPA concentrations up to 0.20 mg./ml.

When this method of estimation was applied to samples taken at intervals from an aerated reaction mixture containing initially tyrosinase, ascorbic acid and DOPA (0.2 mg./ml.) but no tyrosine, the DOPA concentrations found 0.5, 5, 10, 15, 20, 30, 40, 50, 60 and 70 min. after the beginning of the enzyme reaction were respectively 0.207, 0.207, 0.206, 0.208, 0.206, 0.207, 0.206, 0.207, 0.204 and 0.208 mg./ml. These figures vouch for the reliability of the method, and for the absence of interference by ascorbic acid, the concentration of which fell from 4.0 to 0.2 mg./ml. during the experiment. They also illustrate the efficiency with which ascorbic acid reconverts DOPA quinone into DOPA.

B. *Catechol*. The molybdate method, used by Rae (1930) for adrenaline estimation and said to be applicable to all catechol derivatives, was considered. The statement of Evans & Raper (1937*a*) that ascorbic acid gives a green-blue coloration with the molybdate reagent was discouraging, but trial did not reveal any trace of such coloration, and the following procedure was found to be applicable even in the presence of ascorbic acid. For the determination of a point on a calibration curve a solution was made up by mixing (a) 4.0 ml. of catechol standard solution, (b) 7.0 ml. of 0.5 M-phosphate buffer (pH 6.0), (c) 5.0 ml. of 0.192 N-NaOH and (d) 2.0 ml. of 20% ammonium molybdate. An absorptiometer reading was taken 20 min. after the molybdate addition, using the 1 cm. cell and filter no. 7 of Hilger set H 455. The colour intensity continues to increase after its first almost instantaneous development, but not so rapidly (e.g. absorptiometer reading increasing from 0.246 at 20 min. to 0.261 at 150 min.) as to lead to error, provided that the interval between molybdate addition and absorptiometer reading is kept within the limits of 20–30 min. A calibration curve was constructed covering catechol concentrations up to 0.8 mg./ml. To increase the accuracy of the method at the lower concentrations a second curve was prepared using the 4 cm. absorptiometer cells with doubled volumes of solutions (a) to (d) above. This covered a range up to 0.3 mg./ml. For the estimation of catechol formed from phenol in enzymic reaction mixtures, the metaphosphoric acid filtrates of the latter were used in place of solution (a) in the procedure which has been described.

C. *Homocatechol* (4-methylcatechol). When a beginning was made with the preparation of a calibration curve for this estimation by the procedure used for catechol, it was noticed that the solutions prepared for absorptiometer examination,

left standing at room temperature for a day or two, increased in colour intensity by a factor of about 3. The possibility of increasing the sensitivity of the estimation by delaying the colour measurement until this increase had reached its maximum was obvious. Ascorbic acid was found not to affect significantly the colour intensity developing within 20 min. of the addition of molybdate, but it markedly delayed the subsequent increase in colour in such a way as to suggest that the onset of a phase of relatively rapid increase waited upon the exhaustion of the ascorbic acid by spontaneous oxidation. Independently of the amount of ascorbic acid present, the maximum colour finally reached was constant for a given homocatechol concentration and was stable for 2-3 days.

For the preparation of the calibration curves finally used, ascorbic acid was added to the standard homocatechol solutions in an amount equal to the maximum to be expected in the enzyme reaction mixtures (4 mg./ml.). The procedure was the same as in the estimation of catechol, but absorptiometer readings were taken 20 min. and 3 hr. after molybdate addition and each day until the maximum colour was attained (not more than 5 days). The filter used was Ilford spectrum violet no. 601 which was found to give readings c. 50% greater than the filter previously used. Two calibration curves were drawn, one from the 20 min. readings covering concentrations up to 1.5 mg./ml., the other from the maximum readings for concentrations up to 0.3 mg./ml. only. The latter curve was used unless homocatechol concentrations outside its range, giving maximum colours too intense for satisfactory measurement in the absorptiometer, were encountered. In the concentration range 0.2-0.3 mg./ml., in which a reliable comparison could be made between assessments based on the 20 min. and on the maximal colour readings, good agreement was found.

D. 4:5-Dimethylcatechol. This diphenol behaved very like catechol in the molybdate reaction under the conditions which have been described. For the estimation of 4:5-dimethylcatechol formed enzymically from 4:5-dimethylphenol the procedure was the same as for the homocatechol estimations.

#### Estimation of ascorbic acid

Samples of the metaphosphoric acid filtrates of the reaction mixtures, which had been prepared as already described for *o*-diphenol estimation, were titrated with a standardized solution of 2:6-dichlorophenolindophenol from a microburette.

## RESULTS

### Oxidation of tyrosine

*Course of oxidation in presence of ascorbic acid.* The results were of the type illustrated in Fig. 1. It will be seen that the observed oxygen uptake was in good agreement with that calculated for the oxidation of ascorbic acid and the formation of DOPA. The lag phase at the beginning of the oxygen-uptake curve corresponds with the lag in the initiation of ascorbic acid oxidation. Since the latter is a secondary oxidation effected by DOPA quinone, as the concentration of DOPA and, therefore, the rate of DOPA-quinone formation increases from zero, the rate of ascorbic acid oxidation also increases. In this experi-

ment it reaches a maximal value when the DOPA concentration has reached 0.0005M, and remains almost linear until all the ascorbic acid has been oxidized. The curve in Fig. 1, showing the changing concentration of DOPA during the experiment, provides no evidence of the occurrence of a lag phase in the oxidation of tyrosine to DOPA.

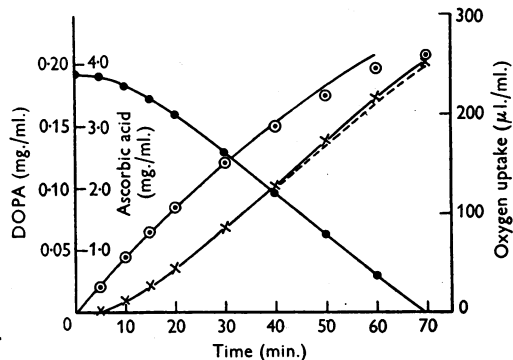


Fig. 1. Oxidation of tyrosine by tyrosinase in the presence of ascorbic acid. Solution saturated with tyrosine, excess solid present. Enzyme concentration, 0.07 mg. (dry wt.)/ml.  $\odot$ — $\odot$ , dihydroxyphenylalanine concentration, the curve representing the expression  $Q=0.77t/(160+t)$ , where  $Q$ =concentration and  $t$ =time;  $\bullet$ — $\bullet$ , ascorbic acid concentration;  $\times$ — $\times$ , oxygen uptake, the broken line showing the calculated oxygen requirement for ascorbic acid oxidation and DOPA formation.

*Decline in rate of formation of DOPA.* The rate of DOPA accumulation appears to be maximal at the start, and then progressively declines, although the concentration of DOPA continues to increase so long as ascorbic acid is present to prevent its oxidation beyond the reversible DOPA-quinone stage. Since the concentration of the substrate of the primary oxidation (tyrosine: saturated solution with solid phase present) remains constant throughout the experiment, the decline in rate of DOPA formation must be due either to inactivation of enzyme or to inhibition by reaction products, i.e. DOPA or dehydroascorbic acid. In the absence of any other indication of the second of these alternatives, the first seems the more probable, the susceptibility of tyrosinase to inactivation during its reactions being widely recognized.

*Calculation of  $Q_{O_2}$  for oxidation of DOPA.* On the assumption that the decrease in rate of DOPA formation is due solely to enzyme inactivation this rate at any given time, i.e. the slope of the tangent to the DOPA concentration curve at that time, may be taken as a measure of the amount of active enzyme still present. By drawing tangents to the ascorbic acid concentration curve the rate of oxidation of ascorbic acid at any moment may be determined, and this is equal to the rate of oxidation of DOPA to

DOPA quinone. From this, by the use of the figure for the amount of residual active enzyme at the same moment obtained in the manner indicated above, one can calculate a  $Q_{0_2}$  value for the oxidation of DOPA by the enzyme preparation used in the experiment. The  $Q_{0_2}$  values so obtained for different times after the beginning of the reaction each relate to a different substrate (DOPA) concentration, the magnitude of which may be found from the DOPA-concentration curve.

Thus the data required for the construction of an activity-substrate concentration curve can be abstracted, the activity referred to being the catecholase (as distinct from the monophenolase) activity of the enzyme, with DOPA as substrate. This curve

$Q = 0.77t/(160 + t)$ , in which  $Q$  is the DOPA concentration in mg./ml. and  $t$  is the time in minutes after the start of oxidation. The values of  $Q$  calculated from this expression are compared with the observed values in columns 2 and 3 of Table 1. The fourth column of Table 1 gives the rates of DOPA formation at different times, i.e. the calculated values of  $dQ/dt = 123/(160 + t)^2$ , these being taken as a measure of the amount of enzyme still in the active condition. The fifth column shows this amount as a percentage of the amount initially present. The  $Q_{0_2}$  values for ascorbic acid oxidation, representing DOPA oxidation by the residual active enzyme, are in column 6, and the corresponding DOPA concentrations in molar terms in the last column of the table.

Table 1. Oxidation of tyrosine + ascorbic acid by tyrosinase

Time (min.)	DOPA concentration (mg./ml.)		Rate of DOPA formation (mg./ml./min.)†	Enzyme still active (%)	$Q_{0_2}$ of ascorbic acid oxidation	DOPA concentration ( $10^{-4}$ M)
	Found	Calc.*				
0	0.00	0.00	0.0048	100	—	—
5	0.021	0.023	0.0045	94	—	1.2
10	0.045	0.045	0.0043	90	2400	2.3
15	0.065	0.066	0.0040	83	3000	3.3
20	0.085	0.086	0.0038	79	3700	4.3
25	—	0.104	0.0036	75	4500	5.2
30	0.120	0.122	0.0034	71	5000	6.2
40	0.150	0.154	0.0031	66	5600	7.8
50	0.175	0.183	0.0028	58	6150	9.3
60	0.197	0.210	0.0025	52	6800	10.7

\* Calculated from  $Q = 0.77t/(160 + t)$ .

† Calculated from  $dQ/dt = 123/(160 + t)^2$ .

may then be compared with the corresponding curve determined in direct experiments with the enzyme and DOPA; if the two curves coincide, the above interpretation of events in the reacting system would be strongly supported.

*Rate of DOPA formation at zero time.* In attempting to treat the experimental data in the above way, a primary difficulty is in the satisfactory assessment of the rate of DOPA formation at zero time, before any enzyme inactivation has taken place. For the oxidation of catechol by tyrosinase in the presence of ascorbic acid Miller & Dawson (1941) have shown that the experimental data fit the relationship  $Q = at/(b + t)$ , where  $Q$  is the total substrate which has been oxidized in time  $t$ , and  $a$  and  $b$  are constants, the magnitude of  $b$  being determined by the rate of inactivation of the enzyme. The reciprocal of  $Q$  plotted against the reciprocal of  $t$  gives a straight line, and as the slope of this line is the reciprocal of the initial reaction velocity ( $dQ/dt$  for  $t = 0$ ) the latter may readily be determined with considerable accuracy.

Applying this device in the instance under discussion, it is found that the DOPA-concentration curve during the greater part of the experiment may be represented reasonably well by the expression

*Reaction velocity and DOPA concentration.* In a separate series of manometric experiments the relationship between reaction velocity and substrate

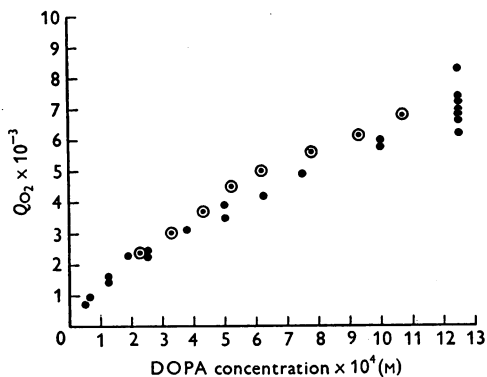


Fig. 2. Oxidation of DOPA by tyrosinase. Enzyme activity-substrate concentration curve: ●, direct determinations; ○, calculated from data of tyrosine oxidation experiment.

(DOPA) concentration was studied directly. DOPA itself in known concentration without tyrosine was used as substrate, and ascorbic acid and buffer concentrations were the same as in the experiments

already described. The oxygen uptake during the first 5 min. after the addition of the enzyme to the DOPA-ascorbic-buffer mixture was used for the purpose of calculating the  $Q_{O_2}$  value. The  $Q_{O_2}$  values obtained by this direct method are plotted against substrate concentration in Fig. 2, together with the values of Table 1 calculated from the data of the tyrosine oxidation experiment. The two sets of values are seen to be consistent with each other, and this lends support to the interpretation of the tyrosine oxidation experiment which has been put forward.

rate continued to increase until in saturated (c. 0.025M) DOPA solution  $Q_{O_2}$  values of about 25,000 were recorded.

$Q_{O_2}$  of true monophenolase and of the overall reaction. The initial rate of DOPA formation in the experiment represented in Fig. 1, calculated from the reciprocal plot of the DOPA-accumulation curve, was 0.0048 mg./ml./min., which corresponds to a  $Q_{O_2}$  ( $\mu$ l.  $O_2$ /mg. dry wt./hr.) of 230 for the enzyme preparation used. When tyrosine in saturated solution in 0.1M-phosphate buffer (pH 7.0) was oxidized in the absence of ascorbic acid, with the same enzyme

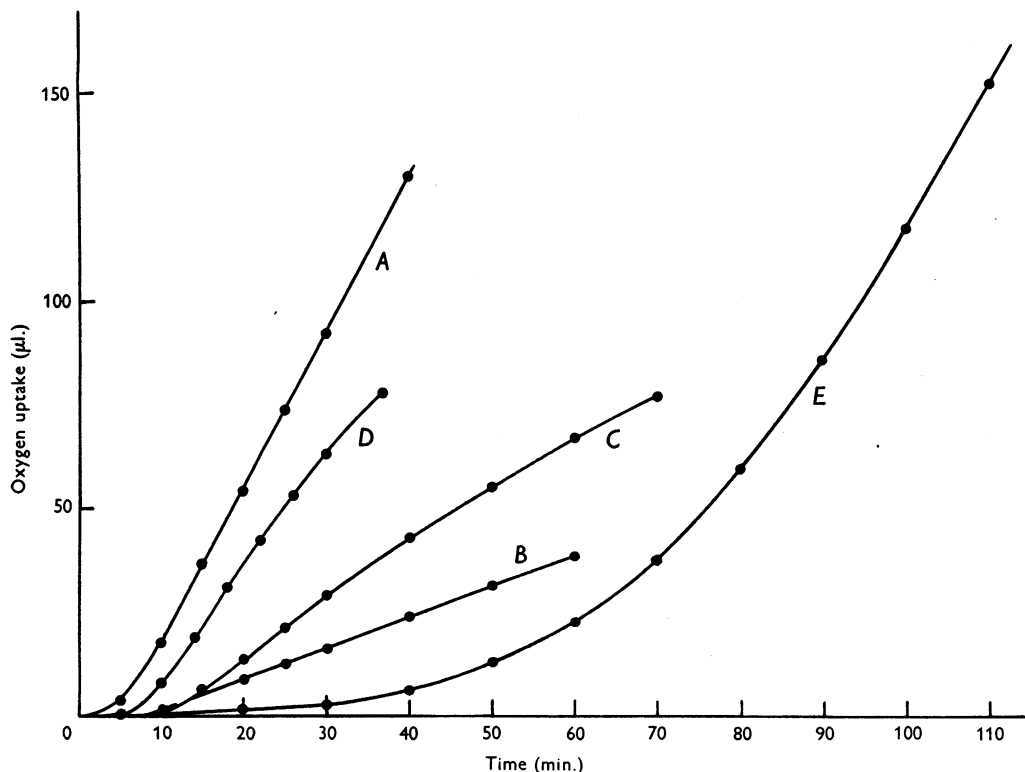


Fig. 3. Oxidation of monophenols with tyrosinase, in the absence of ascorbic acid. Curves A and B, tyrosine, saturated solution (c. 0.0025M); A, 0.35 mg. enzyme; B, 0.07 mg. enzyme. Curve C, 0.005M-phenol, 0.02 mg. enzyme. Curve D, 0.005M-*p*-cresol, 0.007 mg. enzyme. Curve E, 0.017M-4:5-dimethylphenol, 0.35 mg. enzyme.

It follows from this result that the presence of tyrosine in saturated solution (c. 0.0025M) does not significantly inhibit the oxidation of DOPA by the enzyme. The apparently linear course of the oxidation of ascorbic acid seen in Fig. 1 after the maximal rate is reached must be ascribed to a fortuitous balance of two opposing factors, viz. the progressive inactivation of the enzyme which would of itself diminish, and the progressive increase in DOPA concentration which would of itself increase, the reaction rate. With continued increase in DOPA concentration above the range of Fig. 2 the reaction

preparation, the oxygen uptake followed the curves shown in Fig. 3. After the induction period the rate of uptake became nearly linear at values of 3.82 and 0.75  $\mu$ l./min. at the two enzyme concentrations employed, one of which was five times the other. The corresponding  $Q_{O_2}$  values are 650 and 640, and two other similar determinations gave values of 650.

These  $Q_{O_2}$  values of 230 for the true monophenolase activity and 650 for the overall reaction are in accordance with the relative speeds of the component steps in the oxidation of tyrosine suggested by Evans & Raper (1937b). According to the scheme put

forward by these authors the primary slow oxidation to DOPA, requiring one atom of oxygen/mol. of tyrosine, is followed by three successive relatively fast reactions, two of which, the conversion of DOPA to DOPA quinone, and, after ring closure, the oxidation of 5:6-dihydroxydihydroindole-1-carboxylic acid to its quinone, each require one atom of oxygen. If the secondary reactions are markedly faster than the first reaction, one would then expect the  $Q_{O_2}$  for the whole oxidation to be approximately three times the  $Q_{O_2}$  of the isolated primary reaction. The activity-substrate concentration curve of Fig. 2 shows that the  $Q_{O_2}$  for DOPA oxidation reaches a value of 230 at a DOPA concentration of c. 0.0002M. During the oxidation of tyrosine by tyrosinase in the absence of ascorbic acid one would, therefore, not expect DOPA to accumulate beyond this concentration, at which rate of removal would balance rate of formation; and the difficulty experienced by earlier workers (Raper, 1926) in attempting to demonstrate DOPA formation by isolation is consequently not surprising.

#### Oxidation of phenol

*Rate of formation of catechol in presence of ascorbic acid.* The results obtained with phenol were, except in one respect, qualitatively similar to the results of the tyrosine experiments. The course of catechol accumulation at the three phenol concentrations studied is shown in Fig. 4. The curves provide no evidence of an induction period in the primary oxidation of phenol to catechol. The catechol concentrations which had been built up when the experiments were terminated by the exhaustion of the ascorbic acid were 0.0053M from 0.1M-phenol, 0.0021M from 0.01M-phenol and 0.00095M from 0.001M-phenol, representing a conversion of 5, 21 and 95% respectively of the substrate initially present. Only in the case of the lowest phenol concentration is the fall in primary substrate concentration during the course of the experiment likely to have been a significant factor in bringing about the slowing down of catechol formation, and the form of the catechol accumulation curves at the higher phenol concentrations is attributed to the result of enzyme inactivation. These curves can be represented reasonably well by the equations  $Q = 2.0t/(122 + t)$  and  $Q = 0.53t/(38 + t)$ ; the lines drawn in Fig. 4 are the plots of these equations and show the closeness of fit.

The equations give initial  $Q_{O_2}$  values for the isolated primary oxidation of 2900 with 0.1M- and 2400 with 0.01M-phenol and in other similar experiments figures of 3100 and 2700, respectively, were obtained. In the experiment with 0.001M-phenol the initial  $Q_{O_2}$  relating to this concentration cannot be judged with confidence because of the rapid fall in substrate concentration. The use of the first two points only of

the catechol-accumulation curve by the reciprocal plot method gives a  $Q_{O_2}$  of 1900. When phenol was presented as substrate to the same enzyme preparation in the absence of ascorbic acid, the initial lag in oxygen absorption was much more pronounced and the uptake followed a course such as that shown in Fig. 3. The maximal rate of oxygen usage, reached after 20 min., corresponds to a  $Q_{O_2}$  of 4700, which is of the order of magnitude to be expected if the rate of the primary reaction determines the overall oxygen uptake rate.

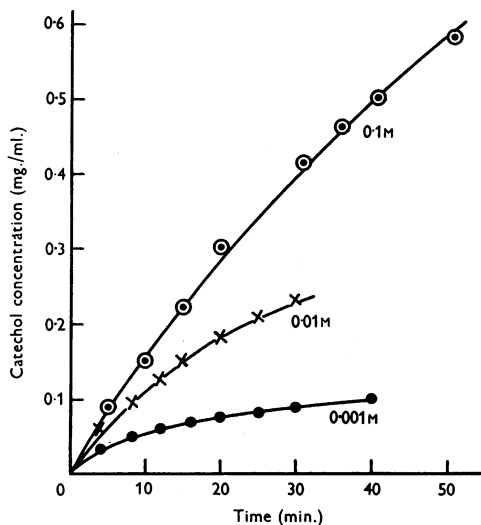


Fig. 4. Accumulation of catechol during the oxidation of phenol by tyrosinase in the presence of ascorbic acid. The initial phenol concentration is indicated alongside each curve. Enzyme concentration, 0.035 mg./ml. The curve drawn for 0.1M-phenol represents the expression  $Q = 2.0t/(122 + t)$ , and that for 0.01M-phenol the expression  $Q = 0.53t/(38 + t)$ ,  $Q$  being catechol concentration in mg./ml.

*Inhibition of catechol oxidation by phenol.* The results obtained in the experiments with phenol differed qualitatively from those given by tyrosine in one respect. Analysis of the results with tyrosine produced no evidence that tyrosine had any inhibitory effect on the oxidation of DOPA by the enzyme. The results of a similar analysis of the data of the phenol experiments, however, could only be satisfactorily interpreted if an inhibitory effect on catechol oxidation were attributed to phenol. It is not proposed to give this analysis in detail, since the same effect is seen in a more intense form during the oxidation of 4:5-dimethylphenol, to be described later. The inhibitory effect of phenol on catechol oxidation was demonstrated in direct experiments such as that illustrated in Fig. 5. The inhibition was competitive in type. When catechol and phenol were present in equimolar proportions the phenol was

without significant effect. But when the relative catechol concentration was reduced to 1/5 there was marked inhibition, and at 1/20 strong inhibition, of the initial oxygen-uptake rate. It must be borne in mind that in the phenol-containing reaction mixtures the concentration of catechol increases from the moment at which the enzyme is added, owing to the conversion of phenol to catechol by the enzyme. As a result of the increase in catechol concentration and the decrease in phenol concentration, the inhibition might be expected to pass off with time, and that it does so is evident in the curves.

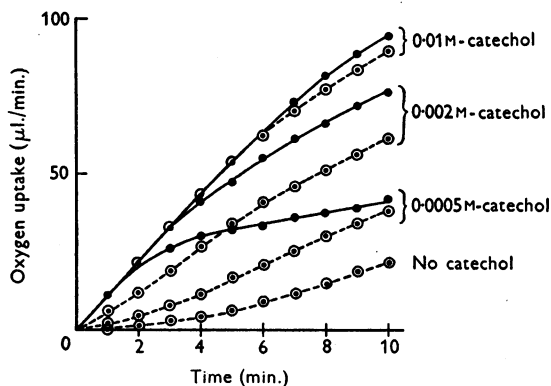


Fig. 5. Effect of phenol on oxidation in the ascorbic acid + catechol + tyrosinase system. ●—●—●, without phenol; ○—○—○, with 0.01 M-phenol.

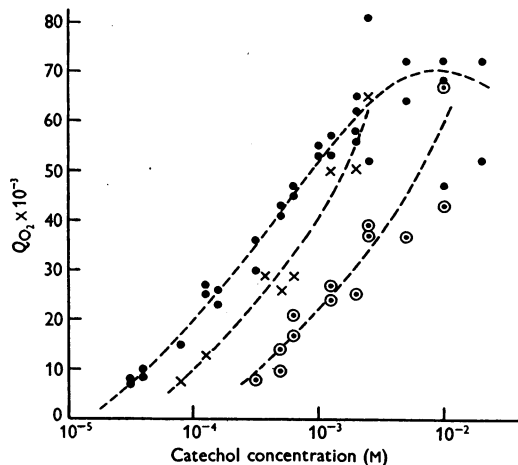


Fig. 6. Oxidation of catechol by tyrosinase. Effect of phenol on the enzyme activity-substrate concentration curve. Dots, no phenol present; crosses, with 0.001 M-phenol; circles, with 0.01 M-phenol.

$Q_{O_2}$  of catechol oxidation. The  $Q_{O_2}$  values obtained in a series of determinations similar to those shown in Fig. 5 and using reaction mixtures with various catechol concentrations and (a) no phenol, (b) 0.001 M-

phenol and (c) 0.01 M-phenol are plotted against catechol concentration in Fig. 6. The  $Q_{O_2}$  values are based on the oxygen uptake in the first 2 min. of the reaction and are admittedly subject to a rather large possibility of experimental error, and this is evident in the scatter of points in Fig. 6. But the nature of the relationship between enzyme activity and catechol concentration, and the competitive inhibitory effect of phenol, are clearly discernible. One half the maximal activity is reached at c. 0.0003 M-catechol in the absence of phenol and at c. 0.0007 and 0.002 M-catechol in the presence of 0.001 and 0.01 M-phenol respectively.

#### Oxidation of *p*-cresol

Course of homocatechol formation in presence of ascorbic acid. The course of homocatechol accumulation in a series of experiments at different *p*-cresol

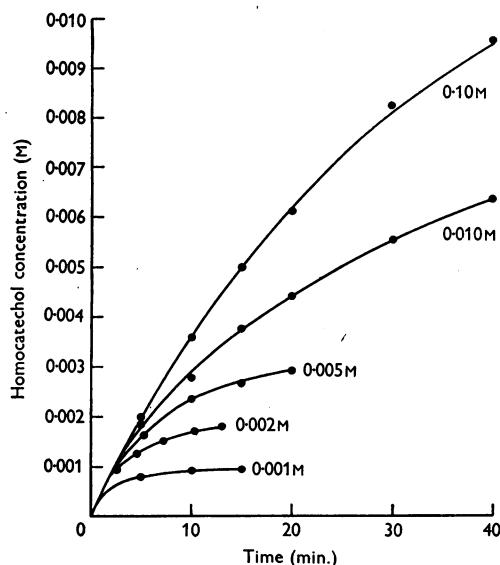


Fig. 7. Accumulation of homocatechol during oxidation of *p*-cresol with tyrosinase in the presence of ascorbic acid; enzyme concentration, 0.035 mg. (dry wt.)/ml. The initial *p*-cresol concentrations in the various experiments are recorded at the ends of the curves. The uppermost curve represents the expression  $Q = 2.5t/(45.6 + t)$ .

concentrations is shown in Fig. 7. The enzyme concentration was the same as in the experiments with phenol, but the formation of *o*-diphenol was much faster. With the smaller *p*-cresol concentrations, the asymptotic approach to complete conversion to homocatechol is apparent and even with 0.01 M-*p*-cresol 64% conversion had taken place in 40 min. Only at the highest *p*-cresol concentration (0.1 M) is the resultant decrease in *p*-cresol sufficiently small relative to the initial concentration to make it

unlikely that the rate of the primary oxidation is affected during the course of the experiment by the fall in substrate concentration.

The uppermost curve in Fig. 7 is the curve of the expression  $Q = 2.5t/(45.6 + t)$ , where  $Q$  is the homocatechol concentration in mg./ml. This expression fits the points for the experiment with 0.10M-*p*-cresol very well. The initial reaction velocity ( $t = 0$ ) is 0.55 mg./ml./min. corresponding to a  $Q_{O_2}$  of 8500. Using the homocatechol formation in the first 5 min. for the calculation of  $Q_{O_2}$  values to represent the monophenolase activity of the enzyme in each case, the results shown in Table 2 were obtained. The *p*-cresol concentrations are the means of the values at the beginning and at the end of the 5 min. period. The figures suggest that half the maximal reaction velocity is reached at a substrate concentration of about 0.001M.

Table 2. Oxidation of *p*-cresol + ascorbic acid by tyrosinase: monophenolase activity at various substrate concentrations

<i>p</i> -Cresol concentration (M)	Monophenolase $Q_{O_2}$
0.098	7880
0.0096	7200
0.0046	6200
0.0013	5180
0.0006	3100

$Q_{O_2}$  of overall oxidation. When *p*-cresol in the absence of ascorbic acid is oxidized by excess of tyrosinase, the oxygen uptake approaches three atoms/molecule of *p*-cresol. If the overall rate of the reaction is determined by the rate of the primary oxidation of *p*-cresol to homocatechol, one would expect it, in terms of oxygen-uptake rate, to be about three times that of the primary oxidation. The course of oxygen uptake when the enzyme preparation used in this series of experiments oxidized *p*-cresol in the absence of ascorbic acid is shown in Fig. 3. The maximal rate, reached after 10 min., corresponds to a  $Q_{O_2}$  of 25,000, which is of the order of magnitude expected in relation to the figure of about 8500 found for the isolated primary reaction.

$Q_{O_2}$  of homocatechol oxidation. Inhibition by monophenol. Using the rate of ascorbic acid oxidation in these experiments as an index of the rate of homocatechol oxidation,  $Q_{O_2}$  values were calculated in the manner already given in the description of the tyrosine experiments. They were found to be lower than the values obtained in direct determinations with homocatechol as substrate at equivalent concentrations. As in the case of phenol-catechol, this suggested the operation of an inhibitory effect of the monophenol on the oxidation of the corresponding *o*-diphenol, which was verified by direct experiment (Fig. 8).

#### Oxidation of 4:5-dimethylphenol + ascorbic acid

Rate of accumulation of 4:5-dimethylcatechol. This was found by the reciprocal plot method to conform well with the relationships  $Q = 0.76t/(49 + t)$  and  $Q = 1.0t/(115 + t)$  for initial monol concentrations of

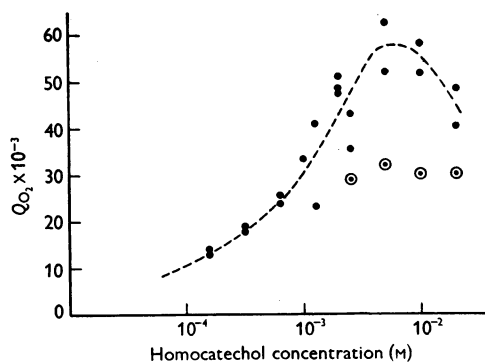


Fig. 8. Oxidation of homocatechol by tyrosinase. Enzyme activity-substrate concentration curve.  $\odot \odot$ ,  $Q_{O_2}$  values determined in the presence of 0.01M-*p*-cresol.

0.02 and 0.005M respectively,  $Q$  being the 4:5-dimethylcatechol concentration in mg./ml./min. after time  $t$ . The initial rates of diol formation in the two cases ( $dQ/dt$ ,  $t = 0$ ) correspond to  $Q_{O_2}$  values of 330 and 180. No attempt was made to treat the data obtained at lower initial monol concentrations in this way; the method of estimation of the diol is not sufficiently reliable at lower concentrations, and there is the additional complication of a relatively rapidly changing monol concentration. At 0.001M, the latter had been reduced to half in about 20 min.

$Q_{O_2}$  for diphenol oxidation in presence and absence of the monophenol. In the experiments at the lower 4:5-dimethylphenol concentrations, the lag in the initiation of ascorbic acid oxidation and oxygen absorption was much more prolonged, and the subsequent acceleration much more dramatic, than in any of the experiments with the other substrates. The general nature of the difference may be appreciated if Fig. 9, showing the results of an experiment with 0.001M-4:5-dimethylphenol, is compared with Fig. 1. The results of an analysis of the data of Fig. 9 are recorded in Table 3. The rates of ascorbic acid oxidation given in the table were obtained from the slopes of tangents drawn to the ascorbic acid concentration curve at the appropriate points. They are taken to be equivalent to the rates of oxidation of 4:5-dimethylcatechol and the  $Q_{O_2}$  (catecholase) values given in the third column of the table are based on them. The dimethylcatechol concentrations in column 4 are taken from the accumulative curve of Fig. 9.



The rate of oxidation of ascorbic acid during the first 20 min. of this experiment was so small as to be within the range of spontaneous oxidation under these conditions in controls without enzyme, so that even the very small  $Q_{O_2}$  values recorded in this period must have been seriously overestimated. The later  $Q_{O_2}$  values, on the other hand, would be subject to a progressively greater magnification if it were possible to relate them to the amount of residual

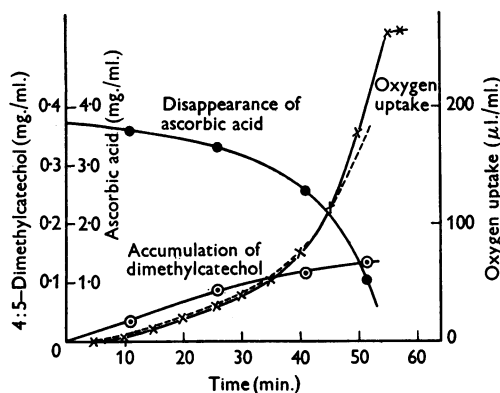


Fig. 9. Oxidation of 0.001 M 4:5-dimethylphenol by tyrosinase in the presence of ascorbic acid. Enzyme concentration, 0.115 mg. (dry wt./ml). The broken line shows the calculated oxygen requirement for ascorbic acid oxidation and dimethylcatechol formation.

active enzyme instead of to the amount of enzyme initially present. These considerations together indicate that the real increase in  $Q_{O_2}$  (catecholase) must have had a more sudden onset than the figures in Table 3 suggest. Since, in the absence of 4:5-dimethylphenol,  $Q_{O_2}$  values for the oxidation of 4:5-dimethylcatechol were found to be of the order of 40,000 at a dimethylcatechol concentration such as had been reached within less than 10 min. of the beginning of the experiment under discussion (Fig. 10), the delay in the attainment of a high  $Q_{O_2}$  must be due to the inhibitory effect of the dimethylphenol present.

The acceleration of ascorbic acid (=dimethylcatechol) oxidation is attributed to (a) the accumulation of the substrate 4:5-dimethylcatechol and

(b) the removal of the inhibitory effect of the monol on the oxidation of the diol, as the relative concentration of these changes. It is most marked when the conversion of monol to diol is approaching completion and the monol/diol ratio changing rapidly to

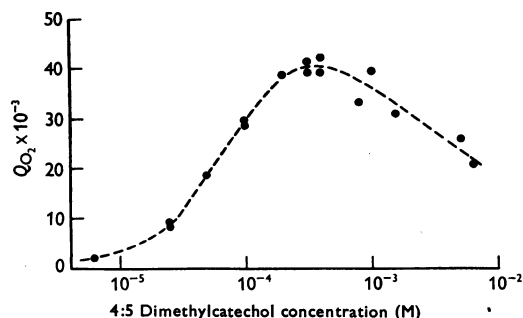


Fig. 10. Oxidation of 4:5-dimethylcatechol by tyrosinase; enzyme activity-substrate concentration curve.

very small values. The change in this ratio is shown in the last column of table 3. At higher initial dimethylphenol concentrations, e.g. 0.02 M, the molar ratio monol/diol remained greater than unity and the inhibitory effect of monol on the diol oxidation was maintained throughout the experiment.

The course of oxygen uptake in manometric experiments in which smaller concentrations of 4:5-dimethylphenol were used is seen in Fig. 11. In those curves which relate to primary substrate concentrations of less than 0.002 M the very slow initial uptake and subsequent rapid acceleration are strikingly evident. The initial rate is scarcely measurable, and of an order of magnitude accountable in terms of the monophenolase activity of the enzyme and the autoxidation of ascorbic acid. For a given enzyme concentration, the lower the concentration of dimethylphenol the earlier and the more sudden is the onset of the rapid phase of oxygen absorption; maximal rates with 0.0004, 0.0001 and 0.000025 M-dimethylphenol are reached respectively in 80, 22 and 8 min. (Fig. 11b). On the other hand, at a given substrate concentration the rapid phase appears earlier, the greater the enzyme concentration; with 0.0004 M-dimethylphenol, at 10–15 min.

Table 3. Oxidation of 4:5-dimethylphenol + ascorbic acid by tyrosinase

(Dimethylphenol concentration initially 0.001 M.)

Time (min.)	Rate of ascorbic acid oxidation ( $\mu\text{l. O}_2/\text{ml./min.}$ )	$Q_{O_2}$ * catecholase	Dimethylcatechol concentration ( $10^{-3}\text{M}$ )	Dimethylphenol concentration ( $10^{-3}\text{M}$ )	Molar ratio: monol/diol
10	0.8	610	0.26	0.74	2.9
20	1.3	670	0.49	0.51	1.0
30	2.3	1200	0.69	0.31	0.45
40	5.1	2600	0.84	0.16	0.19
50	12.4	6400	0.94	0.06	0.06

\* Not corrected for inactivation.

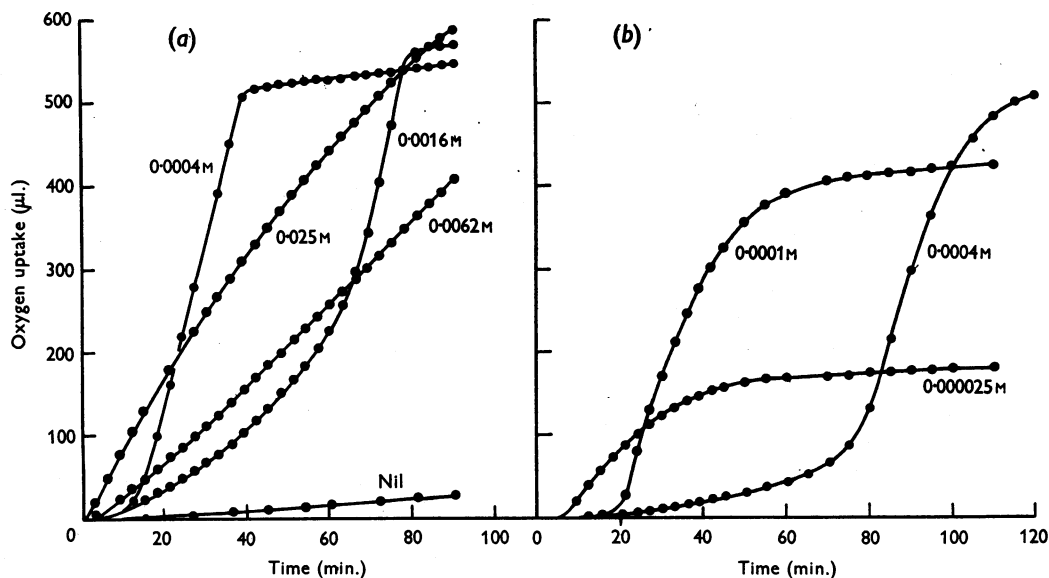


Fig. 11. Oxidation of 4:5-dimethylphenol + ascorbic acid by tyrosinase. Reaction mixture vol. 2.0 ml., containing 8 mg. ascorbic acid ( $\cong 508 \mu\text{l. O}_2$ ). Initial dimethylphenol concentrations as stated against the curves; (a) 0.23 mg., (b) 0.058 mg., tyrosinase (dry wt.).

with 0.232 mg. enzyme (Fig. 11a), but at 70–80 min. with 0.058 mg. enzyme (Fig. 11b). This is in agreement with the interpretation already put forward, that the catecholase function of the enzyme is strongly inhibited by the monophenol, the inhibition passing off swiftly when the ratio monophenol/diphenol falls rapidly as the conversion of the one to the other approaches completion. At the higher dimethylphenol concentrations, 0.025 and 0.0062M (Fig. 11a), the rapid phase of oxygen uptake was not reached during the course of the experiment; the earlier experiments in which dimethylcatechol concentrations were estimated indicated that this is because the concentration of dimethylphenol remained sufficiently high in these cases to exert a strong inhibitory effect throughout the experiments. At the lowest dimethylphenol concentrations (less than 0.0004M) the rapid phase of oxygen uptake finishes before all the ascorbic acid has been oxidized (which requires 508  $\mu\text{l. O}_2$ ); it thus appears that in this concentration range the rate of enzyme inactivation becomes greater with diminishing substrate concentration.

*Inhibition of diphenol oxidation by monophenol.* The inhibitory effect of 4:5-dimethylphenol on the oxidation of 4:5-dimethylcatechol was demonstrated directly in systems containing ascorbic acid, enzyme and known concentrations of the two substrates. The results of a typical experiment are shown in Fig. 12, where the course of oxygen uptake in the first few minutes in systems containing  $10^{-3}\text{M}$ -dimethylcatechol and various concentrations of di-

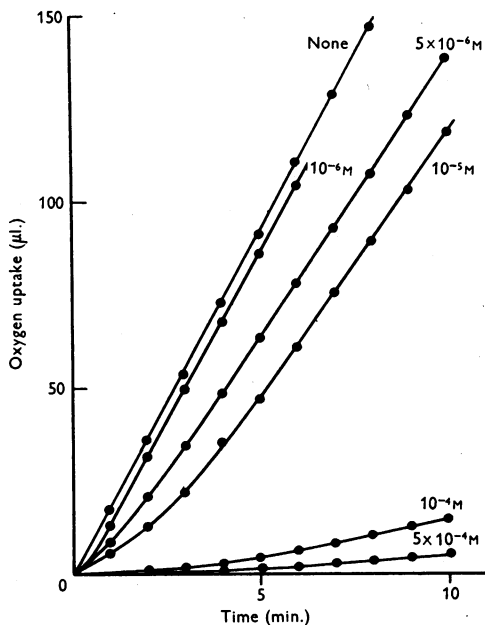


Fig. 12. Effect of 4:5-dimethylphenol on oxidation in the 4:5-dimethylcatechol + ascorbic acid + tyrosinase system; 0.029 mg. enzyme (dry wt.); 4:5-dimethylcatechol concentration,  $10^{-3}\text{M}$ ; 4:5-dimethylphenol concentrations as stated against each curve.

methylphenol are plotted. There is a marked depression of the initial uptake rate with  $5 \times 10^{-6}\text{M}$ -monol, i.e. when the ratio of monol to diol is only

1:200, and the inhibition is practically complete when this ratio is 1:10. At the lower monol concentrations the inhibition quickly passes off, as may be expected owing to the conversion of monol to diol by the enzyme. A rough quantitative estimate of the extent of the inhibition in this and another similar experiment is given in Table 4. The estimate is based on the oxygen uptake in the first minute; but it should be remembered that with the very low monol concentrations the inhibition may already be passing off even within this short time, on account of diminishing monol concentration.

Table 4. *Inhibition of oxidation of 4:5-dimethylcatechol by 4:5-dimethylphenol*

Initial concentration ( $10^{-3}$ M)		Ratio: monol/diol	Initial $O_2$ uptake rate ( $\mu\text{l./min.}$ )	Inhibition (%)
Diol	Monol			
1.0	Nil	—	18.0	—
	0.001	0.001	13.5	25
	0.005	0.005	9	50
	0.01	0.01	6.5	64
	0.1	0.1	1	94
	0.5	0.5	0.5	100
0.3	Nil	—	20	—
	0.0012	0.004	8.5	57
	0.005	0.016	5	75
	0.02	0.07	1.5	93

*Oxidation of 4:5-dimethylphenol in absence of ascorbic acid.* In this case, the induction period before maximal oxygen-uptake rate was attained was much longer than in the cases of the other monophenolic substrates under the same conditions. The oxygen uptake in such an experiment is included in Fig. 3. The maximal rate corresponded to a  $Q_{O_2}$  of 620, reached after the reaction had proceeded 100 min. This may be compared with the value of 330 found for the isolated primary reaction in the presence of ascorbic acid at a similar dimethylphenol concentration.

#### DISCUSSION

The behaviour of the reacting system containing monophenol, tyrosinase and ascorbic acid can in each of the cases studied be adequately explained in terms of: (a) specific monophenolase activity of the enzyme preparation, maximally effective, in the presence of ascorbic acid, at the moment of addition of enzyme to substrate; (b) specific catecholase activity of the preparation; (c) progressive inactivation of the enzyme during the reaction; and (d) competitive inhibition of catecholase activity by monophenols. The relative magnitude of these factors determines the course of oxidation in each particular case. Comparing the oxidation of tyrosine + ascorbic acid with that of 4:5-dimethylphenol + ascorbic acid, for example, we find that the  $Q_{O_2}$  values for the isolated primary oxidations were

not very different (230 for 0.0025 M-tyrosine, 180 for 0.005 M-dimethylphenol). The oxygen uptake reached its maximal rate much more rapidly with tyrosine than with dimethylphenol, because dimethylphenol strongly inhibits the oxidation of the dimethylcatechol formed from it in the primary reaction, whereas tyrosine has no such inhibitory effect on DOPA oxidation. Again, the effect of this inhibition in accentuating and prolonging the induction phase of the oxygen-uptake curves is much more striking in the case of dimethylphenol than with phenol or *p*-cresol not only because the inhibition for a given monol/diol concentration ratio is more powerful, but also because the rate of diphenol formation is much slower, the relative monophenolase activities for the substrates phenol/*p*-cresol/dimethylphenol being 1.0/2.6/0.1, whereas the relative catecholase activities with catechol/homocatechol/dimethylcatechol were 1.00/0.83/0.59.

Reference was made in the introduction to the conclusion of other workers that the oxidation of monophenols by tyrosinase is dependent in some way upon the simultaneous oxidation of *o*-diphenol by the enzyme, though not a result of secondary oxidation of monophenol by products of oxidation of diphenol. In the case of each monophenol studied in the present work, however, the rate of the primary oxidation to *o*-diphenol appears to be maximal at the very beginning of the enzyme reaction, and, when an induction period is evident before the maximal oxygen-uptake rate is reached, it is due in the main to the inhibiting effect of monophenol on diphenol oxidation. This leads inevitably to the conclusion that the presence of *o*-diphenol is not in fact necessary for monophenolase activity to be displayed. How then is the induction period observed when monophenol is oxidized in the absence of ascorbic acid to be explained? With 4:5-dimethylphenol as substrate the  $Q_{O_2}$  of the primary reaction in the presence of ascorbic acid was found to be of the order of 300 for the enzyme used in this work. In the absence of ascorbic acid, with the same substrate, the total oxygen-uptake rate reached a value corresponding to  $Q_{O_2} = 620$ , but only after a long induction period during the earlier part of which the oxygen uptake rate was almost zero (Fig. 3). This maximal value of 620 does not conflict with a  $Q_{O_2}$  (monophenolase) of about 300, but the fact that the initial oxygen-uptake rate was very much smaller than that appropriate to a  $Q_{O_2}$  of 300 points to the conclusion that in the absence of ascorbic acid the monophenolase function of the enzyme was at first relatively quiescent, and that it was activated by *o*-diphenol as the latter slowly accumulated. Ascorbic acid appears to bring about an almost instantaneous activation of monophenolase function and its effect in this respect is not, as other workers have supposed (Nelson & Dawson, 1944), dependent on its favouring *o*-diphenol accu-

mulation. Indeed from the moment of completion of the reaction mixture by addition of enzyme onwards, monophenolase activity diminishes on account of enzyme inactivation, in spite of the progressive increase of *o*-diphenol concentration and oxidation. Ascorbic acid and *o*-diphenols have in common rather strong reducing power and similar reducing groups, and it seems possible that the effect which they have on monophenolase function may be dependent on this property. At any rate the new evidence is against the existence of any specific role for *o*-diphenols in relation to monophenol oxidation.

It is regrettable that the methods available for *o*-diphenol estimation did not permit the studies of *o*-diphenol formation to be carried out over a sufficient range of monophenol concentration to provide reliable activity-substrate concentration curves for the isolated primary oxidation. With each monophenol substrate, monophenolase activity was highest at the highest substrate concentration employed, even when this was as great as 0.1 M, and there was no sign of inhibition such as is observed with the *o*-diphenolic substrates at such high concentrations. The strong competitive inhibitory effect of quite low concentrations of 4:5-dimethylphenol on the catecholase function of the enzyme suggests a much greater affinity of the specific 'catecholase centre' in the enzyme for this monophenol than for the *o*-diphenols or other monophenols used. Were the same centre responsible for monophenol oxidation, one would expect the substrate concentration necessary to saturate the enzyme to be particularly low in the case of dimethylphenol. The few facts available are not in accordance with this expectation. In the case of dimethylphenol the  $Q_{O_2}$  (monophenolase) was 330 at 0.02 M-substrate, but only 180 at 0.005 M-substrate, whilst with *p*-cresol a value of 7200 at 0.01 M had fallen only to 5000 at 0.001 M, suggesting that the affinity for dimethylphenol was appreciably less than for *p*-cresol. Thus it appears probable that the activities of the enzyme or enzyme complex in respect of monophenol and *o*-diphenol are located at different centres.

The optimal concentrations of the *o*-diphenolic substrates were 0.004 M for catechol, 0.006 M for homocatechol and 0.00035 M for 4:5-dimethylcatechol. Two of these values agree reasonably well with the figures given by Nelson & Dawson (1944), 0.002 and 0.0007 M for catechol and dimethylcatechol respectively. For homocatechol these authors give 0.0006 M as the optimal concentration and there appears to be no obvious explanation of the discrepancy. It would be unwise to assume that these differences in optimal concentration reflect differences in enzyme-substrate affinities, in the absence of knowledge of other factors which determine the shapes of the activity-substrate concentration curves, and in particular of the reason for

the falling off in activity at high substrate concentrations, which is especially marked in the case of dimethylcatechol. The relative activity towards the three *o*-diphenolic substrates, at the optimal concentration of each, of 1.00:0.83:0.59, does not differ greatly from the ratio of 1.00:0.88:0.74 quoted by Nelson & Dawson (1944).

So far as the experiments which have been described show, the monophenolase and catecholase activities of tyrosinase appear to be functionally independent, except as regards the inactivation of the enzyme during the reaction. In the treatment of the data obtained in the monophenol oxidation experiments, the rate of formation of *o*-diphenol was taken as a measure of the amount of enzyme still in the active condition, in those experiments in which it was considered that the decline in this rate could not be attributed to the diminution in monophenol concentration. Catecholase activity, assessed in terms of oxidation rate of ascorbic acid, was then related to the amount of residual active enzyme determined in this way. The  $Q_{O_2}$  (DOPA) values thus calculated from the data of the tyrosine oxidation experiment were found to agree very well with those obtained by direct manometric studies of the oxidation of DOPA itself (Fig. 2), and this appears to justify the calculation; but in the calculation the amount of residual active enzyme, to which the DOPA oxidation was attributed, was judged by the extent of residual monophenolase activity. This suggests that the process of inactivation affects equally both the catecholase and monophenolase functions, and, therefore, that these are properties of one and the same enzyme complex. The extent to which the data relating to the oxidation of the other monophenols conform with this view is not readily judged, because of the additional complicating factor of inhibition of *o*-diphenol oxidation by the monophenol present.

Nearly all previous experimental work on the oxidation of monophenols by tyrosinase has been carried out with the initially simpler system containing only monophenol, enzyme and buffer. The quantitative study of *o*-diphenol formation in such systems presents obvious difficulties on account of the pigment developed, but it is hoped that such studies may soon be made. It is by no means certain, however, that the behaviour of the enzyme in the absence of ascorbic acid is of greater biological significance than its behaviour in the presence of this substance. Of the two sources of tyrosinase which have been chiefly used, the mushroom is stated to contain no ascorbic acid (Medical Research Council, 1945), but the potato may contain up to 30 mg./100 g. Several authors have suggested that the *o*-diphenol-tyrosinase system may play a part in the economy of plant tissues, by forming the terminal link in a respiratory chain (Boswell & Whiting, 1938;

Baker & Nelson, 1943). According to their view the *o*-quinone formed in the presence of oxygen by the enzyme abstracts hydrogen from some unspecified hydrogen donator and is consequently reconverted to *o*-diphenol. In the potato one would expect ascorbic acid to be a powerful competitor for this quinone, unless it is in some way separated spatially from the enzyme in the tissue cells. The monophenolase function appears to have been regarded by the proponents of this theory merely as a mechanism for providing the *o*-diphenol necessary for such a respiratory function. Robinson & Nelson (1944) formed the opinion that in the presence of tyrosinase only a trace of DOPA sufficient for the above purpose accumulated, and suggested, to explain this, that the oxidation of tyrosine was strongly inhibited by DOPA. No direct experimental evidence in support of these speculations was given, however, and the evidence now presented has shown that considerably more than traces of DOPA may accumulate. There was, it is true, a falling off in the rate of DOPA formation during the enzyme reaction, although the tyrosine concentration remained constant, but this can be adequately accounted for by enzyme inactivation. The possibility that the monophenolase function of tyrosinase may also be important for syntheses of substances other than the substrate for the catecholase function ought still to be borne in mind.

#### SUMMARY

1. Colorimetric methods for the estimation of *o*-diphenol in enzymic reaction mixtures containing

ascorbic acid and the corresponding monophenol are described.

2. The results of studies of the course of oxygen uptake, *o*-diphenol formation and disappearance of ascorbic acid during the oxidation by tyrosinase, in the presence of ascorbic acid, of tyrosine, phenol, *p*-cresol and 4:5-dimethylphenol (3:4-dimethylphenol) are reported.

3. The primary oxidation of monophenol to *o*-diphenol by tyrosinase in the presence of ascorbic acid was found to proceed at maximal rate at the moment of addition of enzyme to substrate. There was no induction period such as is observed in the absence of ascorbic acid. This leads to the conclusion that *o*-diphenol plays no specific part in facilitating the oxidation of monophenols by tyrosinase.

4. The behaviour of the reacting system containing initially monophenol, tyrosinase and ascorbic acid can be adequately explained in terms of (a) specific monophenolase activity of the enzyme preparation; (b) specific catecholase activity of the enzyme preparation; (c) progressive inactivation of the enzyme during the reaction; (d) competitive inhibition of catecholase activity by monophenols.

5. The bearing of the results on current views of the nature and action of tyrosinase is discussed. It is suggested that they support the view that tyrosinase is a single enzyme or enzyme complex having independent centres associated specifically with its monophenolase and with its catecholase functions.

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