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INTERMEDIATES IN THE ENZYMIC OXIDATION OF CATECHOL

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INTRODUCTION

There is general agreement that the first step in the oxidation of catechol to "catechol-melanin" in the presence of polyphenol oxidase is to *o*-benzoquinone. This has been confirmed by chemical[1], spectrographic[2] and polarographic[3] studies. There has been considerable controversy about the subsequent reactions[2,4,5,6]. Much of the controversy has revolved around the quantitative oxygen uptake at different ratios of enzyme to substrate and different substrate concentrations. This has been re-investigated using paper chromatographic methods together with the manometric. Under some conditions phenolic spots other than catechol are detected on the paper. Some of these substances are also produced by inorganic oxidants and have been isolated by chromatography on cellulose powder columns.

MATERIALS

The catechol was sublimed immediately before use to obtain chromatographically pure material, m.p. 105° C. Mushroom polyphenol oxidase (purchased from the Treemond Company, New York) was used. As supplied, the material was in aqueous solution assaying 3,300 catecholase units per ml or 3,000 catecholase units per mg dry matter by MILLER AND DAWSON'S[7] chronometric method.

RESULTS AND DISCUSSION

A. Manometry and paper chromatography

The curves obtained for oxygen uptake at different ratios of polyphenol oxidase to catechol were very similar to those obtained by WRIGHT AND MASON[8], and fully con-

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firmed their results for the oxygen consumption by this system under acid conditions. They need not be reported in detail. During the oxidation of catechol by polyphenol oxidase, the enzyme is stoichometrically inactivated[6] but in all the experiments that follow sufficient enzyme was used so that this inactivation did not become a limiting factor. The total uptakes after 150 min over a range of catechol concentrations are shown in Fig. 1. The maximum uptake is 2.5 atoms/mole, obtained at catechol concentrations from $1 \cdot 10^{-3}M$ to $1 \cdot 10^{-4}M$. At concentrations greater than $1 \cdot 10^{-3}M$ catechol the uptake decreases, approaching 2 atoms/mole at $5 \cdot 10^{-3}M$. Many of the earlier investigations were done at this or even higher concentrations of substrate.

Fig. 2 shows the main components detected by paper chromatography of the enzymic reaction mixture at a concentration of $5 \cdot 10^{-3}M$ substrate. Three colourless phenols A, B, C, a bright purple-red component D, and a non-phenolic yellow component E, are always present. Other faint spots are frequently found. If the substrate concentration is increased or the enzyme is decreased A, B, C, and E increase and D decreases. Contrariwise as the substrate concentration is decreased, D increases while the others decrease, and at $5 \cdot 10^{-4}M$, D, the bright purple-red spot, is the only intermediate that can be detected. It should be noted that even then some catechol can be detected and presumably it is being continually reformed by dismutation[3].

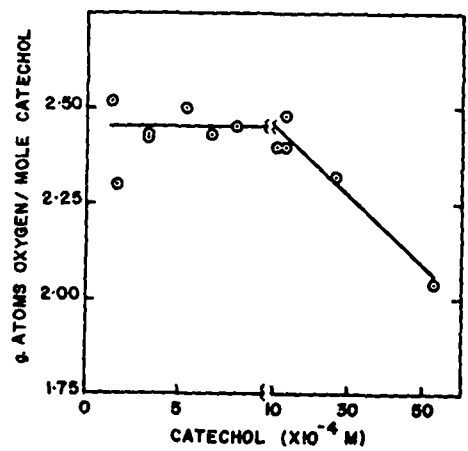

Fig. 1. Oxygen uptake over a range of substrate concentration. (44 catecholase units of polyphenol oxidase were used for each 3 ml total reaction volume except at $53 \cdot 10^{-4}M$ substrate when 88 units were used to ensure that the destruction of enzyme is not the limiting factor (see text)).

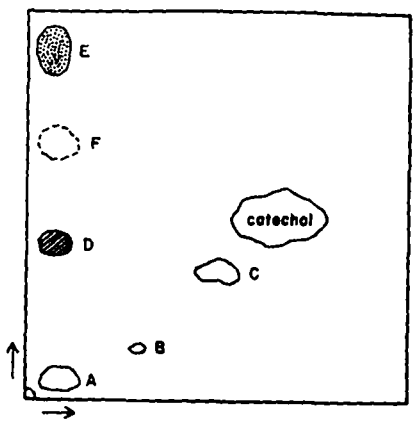

Fig. 2. Two-way paper chromatogram of intermediates in the oxidation of catechol ($5 \cdot 10^{-3}M$) with polyphenol oxidase. (First from left to right in water then upwards in the benzene-acetic acid solvent). Legend: A – 3,4,3′,4′-tetrahydroxydiphenyl; B – 2,3,3′,4-tetrahydroxydiphenyl; C – 2,3,2′,3′-tetrahydroxydiphenyl; D – purple-red pigment; E – yellow quinone; F – phenol produced by ascorbic acid reductionof E.

If the reaction is stopped with ascorbic acid to reduce any quinones present, then at a concentration of $5 \cdot 10^{-3}M$ substrate, A, B and C can be detected as before, confirming that they are formed during the actual reaction and not during the subsequent treatment. The coloured spots D and E which are presumably quinones have disappeared, but a new phenolic spot F can be detected. The catechol, as would be expected, is much increased in amount by reduction of *o*-benzoquinone, the

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primary intermediate, and, at $5 \cdot 10^{-4} M$ substrate concentration, catechol is in fact the only mobile spot detectable.

It would seem, therefore, that a direct path of oxidation under optimum conditions is via D but that, even at $5 \cdot 10^{-3} M$, the concentration of substrate causes appreciable side reaction leading to compounds A, B, C and E which, perhaps, by consuming less oxygen than does catechol, are responsible for the lower oxygen uptake (per mole of catechol) under these conditions.

B. Preparation and identification of intermediates

$FeCl_3$ oxidation produces A, B, and C (*cf.* the oxidation of methyl catechols[9,10] with $FeCl_3$) but $AgNO_3$ gives better results. When catechol is oxidised in acid acetate buffer with 1 mol. prop. $AgNO_3$ for 15 min, compounds A, B, C, and F are produced and have been shown by paper chromatography to be identical to the products of the enzyme reaction. They are isolated by extracting the reaction mixture with ethyl acetate, distilling off the solvent, and chromatographing the residue with water on cellulose powder columns, as described in the experimental section. The melting points of compound A (229° C), (Found: C, 65.9; H, 4.7. Calc. for $C_{12}H_{10}O_4$: C, 66.0; H, 4.6) and its tetramethoxy derivative (133.5° C), (Found: C, 69.9; H, 6.6; OCH_3, 45.2. Calc. for $C_{12}H_6(OCH_3)_4$: C, 70.0; H, 6.6; OCH_3, 45.3) are identical with those of 3,4,3′,4′-tetrahydroxydiphenyl[11]. Compound C, (m.p. 221° C) (Found: C, 65.7; H, 4.6. Calc. for $C_{12}H_{10}O_4$: C, 66.0; H, 4.6) is identical with an authentic sample of 2,3,2′,3′-tetrahydroxydiphenyl (supplied by Dr. O. GISVOLD)[12] and gives a tetramethoxy derivative (m.p. 96°–98° C) which cannot be distinguished from synthetic 2,3,2′,3′-tetramethoxydiphenyl prepared from vanillin via the 2,2′-dihydroxy-3,3′-dimethoxydiphenyl[13]. Compound B (m.p. 183° C) (Found: C, 65.9; H, 4.8. Calc. for $C_{12}H_{10}O_4$: C, 66.0; H, 4.6) also gives a tetramethoxy compound (m.p. 71.5° C) (Found: C, 69.5; H, 6.6; OCH_3, 43.8. Calc. for $C_{12}H_6(OCH_3)_4$: C, 70.0; H, 6.6; OCH_3, 45.3) and from its intermediate R_F values and properties is almost certainly the third possible isomer 2,3,3′,4′-tetrahydroxydiphenyl. The infra-red spectra of the tetramethoxy A, B, and C are consistent with this conclusion[14]. The structure of compound F (Found: C, 67.1; H, 3.9. Calc. for $C_{12}H_8O_4$: C, 66.7; H, 3.7) which forms a dimethoxy compound (m.p. 111.5°–113° C) (Found: C, 68.6; H, 4.9; OCH_3, 25.5. Calc. for $C_{12}H_6O_2(OCH_3)_2$: C, 68.8; H, 4.9; OCH_3, 25.4) is being investigated and will be reported elsewhere; it is probably a dihydroxydiphenylene dioxide. E is the corresponding quinone and can be obtained by enzymic or chemical (ceric sulphate) oxidation of isolated F. Unfortunately we have not yet found a method of oxidising catechol by non-enzymic methods to produce D in amounts sufficient for organic chemical studies.

C. Manometric studies with the isolated intermediates

The enzymic oxidation of A, B and C both alone and in the presence of catechol was followed manometrically with the results shown in Fig. 3.

Compound A is oxidised to the same extent as catechol. The oxidation of compounds B and C is very slow. However, in mixed oxidations with catechol, the oxygen uptake is the same as for solutions of equivalent molar concentrations of catechol alone. Therefore if all the reactions went via the dicatechols, 1.75 atoms $(1 + 2.5)/2$ would be consumed per aromatic ring. It is likely that a considerable proportion of the oxidation is taking this route at $5 \cdot 10^{-3} M$ but not at $5 \cdot 10^{-4} M$ catechol. That

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the three possible dicatechols are produced by enzymic oxidation of catechol, presumably via the mesomeric semi-quinones, is not surprising, but it has not hitherto been realised that this is a major route even at substrate concentrations of $5 \cdot 10^{-3} M$ and is probably the chief reason for the many low oxygen uptake values hitherto reported.

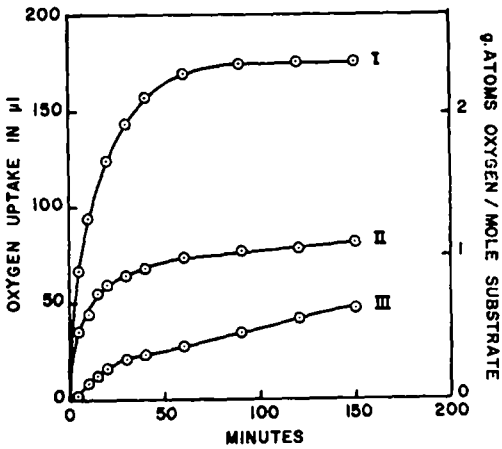

Fig. 3. Enzymic oxidation of tetrahydroxydiphenyls. 41 catecholase units were used for each 3 ml total reaction volume. Curve I. All the following give almost identical uptakes illustrated by this curve: (a) $2.21 \cdot 10^{-3} M$ catechol; (b) $2.21 \cdot 10^{-3} M$ 3,4,3',4'-tetrahydroxydiphenyl (A); (c) $1.77 \cdot 10^{-3} M$ catechol plus $0.44 M$ 3,4,3',4'-tetrahydroxydiphenyl; (d) $1.77 \cdot 10^{-3} M$ catechol plus $0.44 M$ 2,3,3',4'-tetrahydroxydiphenyl; (e) $1.77 \cdot 10^{-3} M$ catechol plus $0.44 M$ 2,3,2',3'-tetrahydroxydiphenyl. Curve II. $2.21 \cdot 10^{-3} M$ 2,3,3',4'-tetrahydroxydiphenyl (B). Curve III. $2.21 \cdot 10^{-3} M$ 2,3,2',3'-tetrahydroxydiphenyl (C).

EXPERIMENTAL

Manometry

Oxygen consumption was measured at 30° C in a conventional Warburg apparatus with manometers of about 18 ml gas space. Each flask contained 3.0 ml of the reaction mixture and 0.2 ml of 10% KOH or buffer in the centre well. Buffer was used after it had been confirmed that no CO_2 was evolved during the reaction. Suitably diluted enzyme was added from the side arm to the substrate in McIlvaine buffer at pH 5.1 and the oxygen uptake followed for at least 150 min. The rate of shaking was 120 oscillations per min.

Chromatography

To study the reaction by paper chromatography, air was slowly bubbled through a solution of catechol in McIlvaine's buffer pH 5.1 and suitably diluted enzyme added at zero time. The total reaction volume was 25 ml. After a fixed time, 2.5 ml $0.5 N$ HCl was added to stop the reaction and the aqueous solution shaken with 3 ml of ethyl acetate. The small (about 1 ml) layer of ethyl acetate was centrifuged to clarify it, and spotted on paper squares. At $5 \cdot 10^{-3} M$ catechol concentration, 24 µl ethyl acetate extract was sufficient to give good chromatograms. Proportionate amounts were used at other dilutions. In a duplicate run, the solutions were transferred to 5% ascorbic acid (2.5 ml) instead of HCl to reduce any quinones present in addition to stopping the reaction.

The two solvents routinely used were water and the top layer of a benzene; acetic acid; water (2; 4; 1) mixture. In general the paper was chromatographed by downward elution in water then by upward elution with the benzene–acetic acid solvent. Only small squares (effective dimensions 10 × 10 cm) were used. The phenolic

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spots were detected by the $FeCl_3$–$K_3Fe(CN)_6$ reagent[15]. The reaction was followed by paper chromatography both at optimum substrate concentration ($5 \cdot 10^{-4} M$ catechol) and at a greater concentration of substrate ($5 \cdot 10^{-3} M$ catechol). Running chromatograms at different times showed that by 10 min reaction time the detectable mobile intermediates had already reached a maximum. Longer times only led to a decrease in the detectable catechol and increase in complex material at the starting point.

Oxidation of catechol with aqueous silver nitrate

Catechol (0.25 mole in 0.5 l acetate buffer pH 5.1) was mixed with $AgNO_3$ solution (0.25 mole in 2.5 l acetate buffer pH 5.1) with stirring for 15 min at room temperature (25° C). The mixture was then passed through a Sharples centrifuge to remove the precipitated silver salts and "melanoid" brown pigments. The clarified solution was shown by paper chromatography to contain the components A, B, C, and F, identical with the intermediates of the enzymic reaction, together with some unchanged catechol. The proportions of the intermediates vary somewhat from run to run. The following is a typical experiment.

The solution was extracted with three successive lots of ethyl acetate (1500 ml, 750 ml, 375 ml) and the extracts combined and dried (Na_2SO_4) overnight. The ethyl acetate was removed *in vacuo* and the residue re-dissolved in water (250 ml) and added to a short wide column (6 × 10 cm) of washed cellulose powder in a sinter glass funnel. The column was eluted with water to give two fractions: I (1025 ml) containing catechol and components C and B; II (575 ml) containing only B. After all the B had been eluted the column was sucked dry and then washed with benzene. This yielded fraction III (425 ml) containing component F. The benzene was removed *in vacuo* and F crystallized from 20% aqueous alcohol containing SO_2 to maintain reducing conditions. The column after removal of fraction III was again sucked dry and eluted with water saturated with ethyl acetate to yield fraction IV (3550 ml) containing component A. The eluate was extracted with two successive lots of ethyl acetate (1 l, 0.5 l) and the extracts combined, concentrated, and diluted with toluene (2 vol.). The ethyl acetate remaining was then removed *in vacuo* and the volume reduced to about 250 ml. On standing at 0°, component A crystallized out.

Fraction I was extracted three times with ethyl acetate (0.5 vol) and the extracts combined and the ethyl acetate removed *in vacuo*. The unchanged catechol was recovered by vacuum distillation at 150° C (0.1 mm Hg). The residue was taken up in acetone and mixed into a paste with cellulose powder. This was packed, after allowing the acetone to evaporate off, on to the top of a long cellulose column (20 × 8 cm). The column was then eluted with water giving: fraction Ia (200 ml) containing catechol; Ib (200 ml) containing catechol + component C; Ic (300 ml) containing component C; and Id (4 l) containing component B.

Fractions Id and II were combined and B recovered by ethyl acetate extraction and crystallization from toluene in a similar manner to A. Component C was recovered from fraction Ic in the same way. Fraction Ib was concentrated to 20 ml frozen, thawed and filtered. The crystalline ppt. was recrystallized from water to yield additional C. The catechol was also recovered from fraction Ia in the usual manner. The following yields were obtained: A, 900 mg; B, 188 mg; C, 332 mg; F, 152 mg; together with 13.3 g of unchanged catechol.

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Component A but not the other substances may be conveniently prepared in greater yield by taking advantage of its ability to form an insoluble complex with cupric ions. A typical preparation follows: Catechol (20 g) in water (250 ml) was added with stirring to a solution of $AgNO_3$ (90 g) $Cu(NO_3)_2$ (60 g) and sodium acetate (100 g) in water (3750 ml). After 10 min the reaction mixture was filtered on a sinter glass funnel and the black precipitate washed with water. The precipitate was susl pended in 5% aqueous oxalic acid (800 ml) and extracted three times with 250 m-ethyl acetate. The ethyl acetate extract was concentrated and the recovered A was recrystallized as in the previous method from hot toluene (yield, 3 g).

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SUMMARY

The oxidation of catechol in the presence of polyphenol oxidase has been investigated using paper chromatographic methods together with the manometric.

Under conditions of very low substrate concentration and optimum oxygen uptake the only intermediate which can be detected is a purple-red pigment.

At higher substrate concentrations the three isomeric tetrahydroxydiphenyls are formed, together with another compound whose structure is being investigated. These compounds can also be prepared from catechol with inorganic oxidants.

The tetrahydroxydiphenyls only consume half the oxygen per aromatic ring compared to catechol and their production in varying amounts at different concentrations of catechol may explain the divergence in reported oxygen uptakes.

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