cal than for the corresponding oxidized derivatives. This is in keeping with the known role of lipoyl dehydrogenase in keto acid oxidation, in which the function of the enzyme is to oxidize a reduced lipoic acid derivative at the expense of DPN reduction[14,15].

The demonstration of potent lipoyl dehydrogenase activity of diaphorase suggests that this may be the physiological function of the enzyme, rather than the function of a DPNH oxidase in the electron-transport mechanism that has previously been ascribed to it[16]. Thus the function of diaphorase in oxidative metabolism would appear to be to produce DPNH from DPN^+, rather than to oxidise DPNH to DPN^+. Further evidence for the validity of this idea has been presented[2,17], and will be detailed in the following paper.

REFERENCES

1 V. MASSEY, *Biochim. Biophys. Acta*, 30 (1958) 205.
2 V. MASSEY, *Biochim. Biophys. Acta*, 32 (1959) 286.
3 N. SAVAGE, *Biochem. J.*, 67 (1957) 146.
4 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 41 (1947) 500.
5 V. MASSEY, *Biochim. Biophys. Acta*, 37 (1960) 310.
6 S. M. SWINGLE AND A. TISELIUS, *Biochem. J.*, 48 (1951) 171.
7 F. B. STRAUB, *Biochem. J.*, 33 (1939) 787.
8 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
9 K. BURTON AND T. H. WILSON, *Biochem. J.*, 54 (1953) 86.
10 D. R. SANADI, M. LANGLEY AND R. L. SEARLS, *J. Biol. Chem.*, 234 (1959) 178.
11 D. S. GOLDMAN, *Biochim. Biophys. Acta*, 32 (1959) 80.
12 A. F. WAGNER AND L. J. REED, personal communications.
13 L. J. REED, M. KOIKE, M. E. LEVITCH AND F. R. LEACH, *J. Biol. Chem.*, 232 (1958) 143.
14 L. J. REED, *Advances in Enzymol.*, 18 (1957) 319.
15 I. C. GUNSALUS, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, Baltimore, 1954, p. 545.
16 E. C. SLATER, *Advances in Enzymol.*, 20 (1958) 147.
17 V. MASSEY, *Biochem. J.*, 72 (1959) 7P.

Biochim. Biophys. Acta, 37 (1960) 314–322

DIPHENYLENEDIOXIDE-2,3-QUINONE: AN INTERMEDIATE IN THE ENZYMIC OXIDATION OF CATECHOL

W. G. C. FORSYTH*, V. C. QUESNEL AND J. B. ROBERTS
Colonial Microbiological Research Institute, Port of Spain, Trinidad (West Indies)

(Received April 7th, 1959)

SUMMARY

A yellow quinone is formed from catechol by oxidation with polyphenol oxidase or iodates. It has been isolated in crystalline form and shown by degradation and synthesis to be diphenylenedioxide-2,3-quinone.

* Present address: Division of Tropical Research, Tela Railroad Company, La Lima, Honduras Central America.

INTRODUCTION

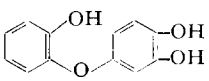
The oxidation of catechol in the presence of polyphenol oxidase has been shown to yield both a yellow and a purple quinone in addition to the three isomeric tetrahydroxydiphenyls (dicatechols)[1]. The yellow pigment (E) produced at a substrate concentration greater than $1 \cdot 10^{-3}$ *M* can be reduced to a phenol (F), $C_{12}H_8O_4$, with ascorbic acid and F can be re-oxidized to E with ceric sulphate or with the enzyme. F contains an *ortho* diphenol group which can be methylated to give the di-O-methyl derivative. It was therefore suggested in a previous paper[1] that F is probably a dihydroxydiphenylenedioxide. This has been confirmed and the structure of the phenol and quinone proved by degradation and synthesis.

RESULTS AND DISCUSSION

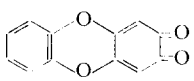
The quinone (E) has been isolated from an enzymic oxidation of catechol but better yields are obtained by oxidation with sodium iodate. It is a typical *o*-quinone, easily reduced to an *o*-diphenol (F) and condenses with *o*-phenylenediamine to give a brilliant blue phenazine. The infra-red spectrum of di-O-methyl F shows that only ether-linked oxygen atoms are present[2]. When F is refluxed with a mixture of acetic and hydrobromic acids, catechol can be recovered from the reaction mixture showing that the two aromatic rings are joined by oxygen and not carbon linkages. Cleavage of F with aniline hydrochloride yields a compound not separable on chromatography in three different solvent mixtures from 2,3',4'-trihydroxy-diphenyl ether (I). 2,3',4'-Trimethoxy-diphenyl ether was synthesized by condensation of 4-iodoveratrole with potassium guaiacol and demethylated to yield (I) by heating with pyridine hydrochloride.

The quinone (E) is much more labile than F and can be hydrolysed with acids and alkalis and most conveniently with perchloric acid to yield catechol and 2,5-dihydroxy-benzo-1,4-quinone, characterized as 2,5-diethoxy-benzo-1,4-quinone. Methanolysis of E yields catechol and what is probably 4,5-dimethoxy-benzo-1,2-quinone. The isolation of the 1,2,4,5-tetrahydroxy derivatives shows that the oxidative formation of the diphenylenedioxide ring system has occurred through *para* and not *ortho* condensation of the catechol.

Oxidation of 2,3',4'-trihydroxydiphenyl ether (I) with aqueous silver nitrate yields the diphenylenedioxide-2,3-quinone (E).



I



E

This work shows that during the formation of "catechol melanin" by enzymic oxidation of catechol both carbon–oxygen and carbon–carbon linkage occurs.

EXPERIMENTAL

Melting points recorded in this paper represent uncorrected values. *U.v. spectra* were all determined with a Unicam S.P. 500 spectrophotometer.

Preparation of quinone (E)

(1) *By enzymic oxidation.* Catechol (0.025 mole) in 250 ml. McIlvaine's buffer pH 5.1 was stirred with 250 ml of polyphenol oxidase solution, containing 50,000 catecholase units[1] for 10 min at room temperature. The yellow-brown solution was extracted with 2 × 250 ml lots of chloroform. The chloroform layers were combined and washed with 0.25 *M* sodium acetate (100 ml) and dried (Na_2SO_4). The chloroform solution was passed through a column of silica gel (15 × 2.5 cm) which removed phenolic contaminants. The solution could then be shown by paper chromatography to contain only E. The chloroform was removed under reduced pressure and the quinone recrystallized from acetone as orange-red prisms, m.p. 260–261° with decomposition. Yield: 35 mg, 1.3 % (found: C, 66.8; H, 2.9; O, 30.1; calc. for $C_{12}H_6O_4$: C, 67.2; H, 2.8; O, 29.9 %); ultraviolet max. at 282, 410 and min. at 248, 350 mμ (log ε, 4.13, 2.74, and 3.45, 2.57 respectively).

(2) *By iodate oxidation.* Superior yields of E can be obtained by oxidation of catechol with iodates. Catechol (0.2 mole) was dissolved with stirring in chloroform (2 l) and a solution of sodium iodate (0.2 mole) in water (400 ml) added with continuous stirring. After 10 min "Celite 545" (Johns-Mannville, 50 g) was added to the thick black emulsion and the mixture filtered on a sinter glass funnel. The chloroform layer was removed and the aqueous layer and celite washed with fresh chloroform (150 ml). The combined extracts and washings were shaken with 0.1 *M* sodium thiosulphate (400 ml) to remove iodine, and then with 0.25 *M* sodium acetate (400 ml). The chloroform solution was dried ($CaCl_2$), concentrated under reduced pressure to about 200 ml, and run through a silica gel (B.D.H. "for chromatographic adsorption") column (30 × 3.5 cm). E was eluted with chloroform, concentrated under reduced pressure, precipitated by dilution with light petroleum B.P. 60–80°, and recrystallized from acetone. Yield 1.88 g, 8.7 %, m.p. 260–261° unchanged on mixing with the quinone from the enzymic oxidation.

Preparation of phenazine derivative

E (140 mg) in acetic acid (10 ml) was mixed with *o*-phenylenediamine (400 mg) in the same solvent (10 ml). Very dark blue crystalline material was deposited almost immediately and was recrystallized from acetic acid. M.p. > 300°, yield 36 mg. (Found: N, 10.6; calc. for $C_{18}H_{10}N_2O_2$: N, 9.8 %. Visible absorption spectrum shows max. at 540, 578, 622 and min. at 550, 596 mμ (log ε, 4.05, 4.92, 5.13 and 4.04, 4.21 respectively).

Reduction of E

(1) E (350 mg) was dissolved in chloroform (150 ml) and the solution saturated with sulphur dioxide. The chloroform was removed under reduced pressure and the residue crystallized from 20 % aqueous alcohol containing SO_2. Colourless needles, m.p. 197° decomp. (rapid heating) undepressed by admixture with a sample of compound F[1]. Yield 246 mg 70 %.

(2) E (90 mg) was dissolved in 50 ml 90 % ethanol containing ascorbic acid (1 g). After decolorisation had occurred, water (4 volumes) was added and the solution extracted with ethyl acetate (2 × 100 ml). The ethyl acetate was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was recrystallized from aqueous ethanol to give F. Yield 67 mg, 74 %.

Cleavage of F

Compound F (100 mg) was refluxed with glacial acetic acid (10 ml) and 48% aqueous hydrobromic acid (10 ml) for 10 h. The solution was brought to pH 4 by addition of sodium hydroxide (4.5 g) in water (80 ml) and extracted with ethyl acetate (3 × 25 ml). The combined extracts were taken to small volume and mixed with a small quantity of cellulose powder and allowed to dry. The dry powder was packed on top of a cellulose powder column (2.6 × 2.8 cm) and the column eluted with water. The first 15 ml of eluate was collected and extracted with four successive 0.5 volume of ethyl acetate and the combined extract taken to small volume. Toluene was added and the mixture again concentrated. On standing crystals of catechol formed, m.p. and mixed m.p. 103°, not separable on chromatography from an authentic specimen.

Compound F (10 mg) and aniline hydrochloride (50 mg) were heated in a sealed tube at 210° for 1 h. The black residue was dissolved in water, acidified, and extracted with ethyl acetate. The extract contained only a single phenolic substance which could not be separated from 2,3',4'-trihydroxydiphenyl ether (I) on paper chromatography in water; n-butanol–acetic acid–water (4:1:5); or chloroform–acetic acid (5:3) saturated with water.

Hydrolysis of E

E is hydrolysed by acids and alkalis. E (100 mg) was dissolved in boiling glacial acetic acid (15 ml) and 60% perchloric acid (5 ml) added to the hot solution. The colour immediately changed from a clear orange to a brown yellow. The solution was cooled after 2 min and solid potassium carbonate added to remove the perchloric acid. The solution was diluted with water and extracted 3 times with ethyl acetate (0.5 volume). Paper chromatography indicated the presence of catechol and 2,5-dihydroxy-benzo-1,4-quinone (by co-chromatography with the commercially available compound). A saturated solution of calcium acetate in boiling methanol was added until there was no further precipitation. The purplish precipitate was removed by centrifugation and suspended in water. Concentrated phosphoric acid was added dropwise until the precipitate had dissolved. The solution was extracted 3 times with ethyl acetate (0.5 volume), the extracts dried (Na_2SO_4) and the ethyl acetate removed under reduced pressure. The residue was dissolved in ethanol (10 volumes), saturated with HCl gas, and allowed to stand overnight at 0°. The yellow crystals were filtered off and washed with a few drops of ethanol. Yield 17.5 mg (20%). M.p. 183° undepressed by admixture with an authentic sample of 2,5-diethoxy-benzo-1,4-quinone. (Found: C, 62.3; H, 6.3; calc. for $C_{10}H_{12}O_4$; C, 61.3; H, 6.1%.) Under the same conditions of hydrolysis catechol remains unchanged.

Methanolysis of E

E (200 mg) was dissolved in boiling methanol (100 ml) and HCl gas passed through the solution. The solution was allowed to cool in the refrigerator and the yellow crystals filtered off after 3 days. Yield 108 mg. M.p. 294–297° dec. (Found: C, 56.9; H, 5.0; calc. for $C_8H_8O_4$; C, 57.1; H, 4.7%.)

Synthesis of 2,3',4'-trihydroxydiphenyl ether (I)

Guaiacol (7.5 g) and potassium hydroxide (2.2 g) were mixed and heated *in vacuo* for 2 h at 150° to remove water. Freshly precipitated copper (0.4 g) was successively

washed with acetone and ether and heated with guaiacol (2 ml) at 150° for 5 min. The catalyst and 10.6 g of 4-iodoveratrole (prepared by the method of BRUCE AND SUTCLIFFE[3]) were added to the dry potassium salt of guaiacol and the mixture stirred and heated under reflux at 180° for 4 h. The reaction mixture was then cooled, broken up and extracted alternately with water and ether (300 ml each total). The extracts were combined, shaken, and the ether layer removed. The aqueous layer was re-extracted with ether and the combined ether extracts filtered to remove the catalyst and extracted with dilute sodium hydroxide solution to remove guaiacol. The ether was then dried (Na_2SO_4) and removed *in vacuo*. The residue, a brown gum, was steam-distilled to recover unchanged 4-iodoveratrole (3 g). The semi-solid residue was crystallized from 50% aqueous alcohol and after repeated crystallization gave 2.1 g (28% yield) of 2,3',4'-trimethoxydiphenyl ether, m.p. 72–73°. (Found: C, 68.5; H, 6.1; OCH_3, 35.5; calc. for $C_{12}H_7O(OCH_3)_3$: C, 69.3; H, 6.1; OCH_3, 35.7%.) The trimethoxy diphenyl ether (1.5 g) was mixed with freshly prepared pyridine hydrochloride (7 g) and heated at 210° for 2 h. The reaction mixture was cooled, dissolved in water, and extracted with 6 × 50 ml portions of ether. The combined ether extract was dried (Na_2SO_4) and taken to dryness *in vacuo*. The residual gum crystallized on rubbing with a glass rod and was re-crystallized from toluene giving clusters of needles of the trihydroxydiphenyl ether (930 mg 74% yield). M.p. 138°. (Found: C, 65.8; H, 4.7; calc. for $C_{12}H_{10}O_4$: C, 66.0; H, 4.6.)

2,3-Dihydroxydiphenylenedioxide (F)

Trihydroxydiphenyl ether (400 mg) was dissolved in water (75 ml) containing potassium acetate (1 g) and mixed with a solution of silver nitrate (1.24 g: 4 mol. prop.) in water (15 ml). After stirring for 1 h at 25° the black precipitate was filtered off. The filtrate was extracted with ether and the precipitate washed with ethanol until all the yellow colour was extracted. The ether and alcohol extracts, which were shown by paper chromatography to contain E and F, were combined, saturated with sulphur dioxide, and concentrated to small volume. Addition of water and cooling caused the separation of crystals of 2,3-dihydroxydiphenylenedioxide (270 mg, 68%). Recrystallization from 20% aqueous alcohol containing SO_2 yielded colourless felted needles, m.p. 197° decomp. (rapid heating) undepressed by admixture with a sample of compound F. The dihydroxydiphenylenedioxide was methylated with diazomethane giving a quantitative yield of the dimethyl ether m.p. 110°. The m.p. is undepressed by admixture with a sample of the dimethyl ether of compound F.

REFERENCES

1 W. G. C. FORSYTH AND V. C. QUESNEL, *Biochim. Biophys. Acta*, 25 (1957) 155.
2 V. C. FARMER, personal communication.
3 J. M. BRUCE AND F. K. SUTCLIFFE, *J. Chem. Soc.*, (1955) 4435.