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STUDIES OF FUNGAL AND PLANT LACCASES

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Abstract—The actions of an enzyme from *Polyporus versicolor* and of laccase from *Rhus vernicifera* on several phenolic substrates have been found to be identical, though the two enzymes have markedly different pH optima. The results obtained with laccase from *Rhus vernicifera* necessitate a revision of views expressed earlier that this enzyme does not calalyse oxidation of monophenols. The products from the action of these laccases on various phenols have been characterized and their significance for the biosynthesis of natural products is discussed. The basic reaction catalysed by laccase is the production of aryloxy-radicals from phenols.

YOSHIDA[1] in 1883 first detected laccase activity in the latex of *Rhus vernicifera.* The enzyme has recently been designated *p*-diphenol oxidase (1.10.3.2; *p*-diphenol:O_2 oxidoreductase) by the Commission on Enzymes of the I.U.B.[2] The substrate specificity of the enzyme has been investigated by several workers[3–6] but is, nevertheless, ill-defined.[7]

In a recent paper[8] we have described some of the reactions catalysed by an extracellular oxidase produced by the wood-rotting fungus, *Polyporus versicolor.*[9] This oxidase has been described in the literature both as a phenol oxidase and as a laccase.[10,11] We have decided to adhere to the use of the term catechol oxidase (1.10.3.1)[2] to cover those enzymes which catalyse the oxidation of monophenols and *o*-diphenols to the corresponding *o*-quinones, via the formation of a hydroxylated intermediate in the case of monophenols. The extracellular oxidase produced by *Polyporus versicolor* can be clearly differentiated from catechol oxidase by its action on *p*-cresol and by the fact that it does not catalyse the oxidation of tyrosine.[8] However, the literature contains such conflicting statements about the substrate specificity of enzymes referred to as laccases, obtained from the lacquer trees, *Rhus vernicifera* and *Rhus succedanea*,[3,4] and from the mushrooms, *Russula delica* and *Russula foetens*,[5,6] that we could not with certainty describe the enzyme from *Polyporus versicolor* as a laccase.

Graubard[5] reported the production of a white precipitate when laccase from *Russula delica* or *Russula foetens* catalysed the oxidation of *p*-cresol. Later, Keilin and Mann[4,12] reported that purified laccase from *Rhus succedanea* or *Rhus vernicifera* had no action on *p*-cresol. Their results were based on oxygen-uptake experiments during which the enzyme

[1] H. YOSHIDA, *J. Chem. Soc.* **43**, 472 (1883).
[2] *Report of the Commission on Enzymes of the I.U.B.* p. 81, Pergamon Press, Oxford (1961).
[3] G. BERTRAND, *Compt. rend.* **120**, 266 (1895); **122**, 1132 (1896).
[4] D. KEILIN and T. MANN, *Nature* **143**, 23 (1939).
[5] M. GRAUBARD, *Enzymologia*, **5**, 332 (1939).
[6] D. C. GREGG and W. H. MILLER, *J. Amer. Chem. Soc.* **62**, 1374 (1940).
[7] E. FRIEDEN in *Horizons in Biochemistry*, Ed. M. KASHA and B. PULLMAN, p. 474, Academic Press, New York (1962).
[8] B. R. BROWN and SHEILA M. BOCKS, "Some New Enzymic Reactions of Phenols" in *Enzyme Chemistry of Phenolic Compounds*, Ed. J. B. Pridham, Pergamon Press, Oxford (1963).
[9] G. MALMSTRÖM, G. FÅHRAEUS and R. MOSBACH, *Biochim. Biophys. Acta* **28**, 652 (1958).
[10] G. LINDEBERG and G. FÅHRAEUS, *Physiol. Plant.* **5**, 277 (1952).
[11] G. FÅHRAEUS, *Physiol. Plant.* **5**, 284 (1952).
[12] D. KEILIN and T. MANN, *Nature* **145**, 304 (1940).

was incubated for 90 min with this substrate. Dawson and Tarpley,[13] summarizing the results of earlier workers, state that laccase catalyses the oxidation of various polyhydric phenols but does not oxidize monophenols such as *p*-cresol. In addition they state that resorcinol is not oxidized by laccase.

These experiments have not been repeated recently and we thought it necessary to make a comparative study of the action of laccase from *Rhus vernicifera* and of the *Polyporus versicolor* oxidase on a number of phenols of differing structure in order to establish clearly (*a*) whether these enzymes have the same chemical effects as each other; (*b*) how they differ from catechol oxidase (tyrosinase) in their chemical effects; and (*c*) the structures of the compounds produced when these laccases act catalytically on certain phenols, chosen either because they have for many years been used routinely as substrates for phenol oxidases (e.g. catechol, pyrogallol, and *p*-cresol) or because compounds of interest from a biosynthetic point of view could be expected as oxidation products (e.g. from 2,6-disubstituted phenols and from griseophenone A).

RESULTS AND DISCUSSION

Though phenol oxidases have not yet been obtained crystalline or pure, a great amount of work has been reported in which crude solutions have been investigated. The work described here has been carried out with solutions of laccase from *Rhus vernicifera* and of the oxidase from *Polyporus versicolor*. Since we are primarily interested in the chemical effects of the enzymes, we consider this to be a justifiable procedure, especially as a definition of the chemical effects of phenol oxidases can contribute to problems of assay, differentiation, and eventual purification of individual enzymes.

Evidence that the chemical effects which we describe are enzymic rests upon controls carried out with boiled enzyme preparations (see Experimental section) and upon comparative rate measurements made on the oxidation of 2,6-dimethoxyphenol with the fungal enzyme inhibited in various ways (Table 1).

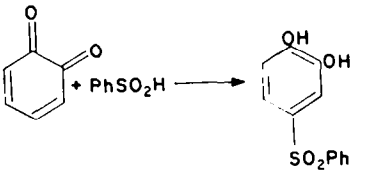
TABLE 1. COMPARATIVE RATES OF OXIDATION OF 2,6-DIMETHOXYPHENOL IN PRESENCE OF THE FUNGAL ENZYME AND INHIBITORS

System used	Relative rates*
Fungal enzyme	56·5
Fungal enzume + $3{\cdot}036.10^{-7}$ M sodium azide	43·1
Fungal enzyme + $1{\cdot}2144.10^{-6}$ M sodium azide	26·4
Fungal enzyme + $> 5.10^{-5}$ M sodium azide	0
Acetate buffer only	0
Boiled fungal enzyme	0
Fungal enzyme after dialysis against aqueous potassium cyanide	6·20
Fungal enzyme after dialysis against aqueous potassium cyanide + $2{\cdot}604.10^{-5}$ M copper sulphate	6·10
$2{\cdot}604.10^{-5}$ M Copper sulphate	0

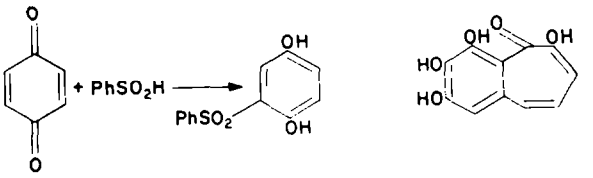
* Arbitrary units of increase in optical density at 468 mμ per min with the substrate $1{\cdot}718.10^{-5}$ M in acetate buffer of pH 4·0 at 29·8°.

[13] C. R. DAWSON and W. B. TARPLEY in *The Enzymes*, Ed. J. B. SUMNER and K. MYRBÄCK, 1st Ed., Vol. II, Part I, p. 484, Acad. Press. Inc., New York (1951).

Both enzymes catalyse the oxidation of catechol to *o*-benzoquinone (I), which then undergoes further reactions to yield a complex mixture of products, revealed by paper chromatography.[8] However, when sodium benzene sulphinate is present in the incubation mixture,[14] the phenylsulphone (II) of catechol is obtained, indicating that the *o*-quinone is the primary oxidation product. Similarly, both enzymes catalyse the oxidation of hydroquinone to *p*-benzoquinone (III), also identified as a phenylsulphone (IV), and of pyrogallol to purpurogallin (V).

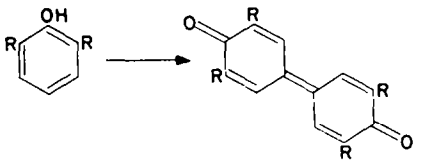


I II



III IV V

With 2,6-dimethoxyphenol (VI, R = OMe) as substrate, both enzymes were found to produce 3,5,3′,5′-tetramethoxydiphenoquinone (VII, R = OMe) and with 2,6-dimethylphenol (VI, R = Me), the fungal enzyme yielded 3,5,3′,5′-tetramethyldiphenoquinone (VII, R = Me). The possible relevance of these reactions in the biosynthetic schemes proposed[15] for naturally occurring extended quinones has already been pointed out[16] and such reactions have led to a new synthesis of perylenequinones by dimerization of naphthols.[17]



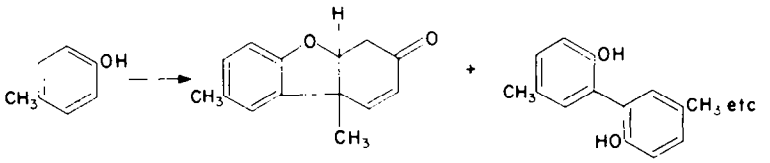
VI VII

Both enzymes were also found to catalyse the oxidation of resorcinol to a yellowish product (λ_{max} 485 mμ) with green fluorescence. After a few days' incubation with the enzyme, a pink solid accumulated in the solution. The structures of the products of this reaction have not yet been elucidated.

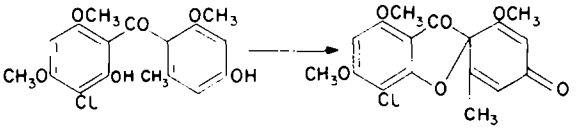
With *p*-cresol (VIII) as substrate, both enzymes produced a white solid precipitate which was resolved into Pummerer's ketone (IX) and alkali-soluble polymers (e.g. X). The realization that this reaction is the prototype of a large number of oxidations of phenols which have

[14] Cf. C. E. M. Pugh and H. S. Raper, *Biochem. J.* **21**, 1370 (1927).
[15] H. Brockmann and H. Eggers, *Angew. Chem.* 706 (1955); Bu'Lock and Allport, *Proc. Chem. Soc.* 264 (1957).
[16] Sheila M. Bocks, B. R. Brown and A. H. Todd, *Proc. Chem. Soc.* 117 (1962).
[17] B. R. Brown and A. H. Todd, *J. Chem. Soc.* in press.

been proposed for the biosynthesis of various natural products[18] led us to investigate this aspect. Griseophenone A (XI) has been suggested[18] to be the immediate precursor of (−)-dehydrogriseofulvin (XII) in the biosynthetic scheme for the production of (−)-griseofulvin by *Penicillium griseofulvum* or *P. patulum* and chemical oxidation of griseophenone A leads to (±)-dehydrogriseofulvin.[19] In the presence of the fungal enzyme, griseophenone A



VIII IX X



XI XII

(XI) is smoothly converted into (±)-dehydrogriseofulvin (XII). The production of a racemate is of importance and correlates with the fact that Pummerer's ketone (IX), produced by the enzymes from *p*-cresol, is also optically inactive. This result indicates either that griseophenone A is not the immediate precursor of dehydrogriseofulvin in the biosynthesis of griseofulvin by *P. griseofulvum* or *P. patulum*, i.e. that another mechanism involving enzymes of a different type is operative, or that a stereospecific enzyme of the laccase type is involved. Recent work of the Glaxo group[20] indicates that the former explanation is the more probable.

TABLE 2. ENZYMIC AND CHEMICAL OXIDATION PRODUCTS OF PHENOLS

Phenol	Product	Yield per cent isolated		
		Laccase	Fungal enzyme	Chemical oxidants
Catechol	*o*-Benzoquinone	33*	60*	Not stated[21]
Hydroquinone	*p*-Benzoquinone	22*	48*	83[22]
Pyrogallol	Purpurogallin	100	100	72[23]
2,6-Dimethoxyphenol	3,5,3′,5′-Tetramethoxydiphenoquinone	90	90	96[24]
2,6-Dimethylphenol	3,5,3′,5′-Tetramethyldiphenoquinone	—	30	50[24]
p-Cresol	Pummerer's ketone	18	28	22[24]
Griseophenone A	(±)-Dehydrogriseofulvin	—	37	60[19]

* Isolated as a phenylsulphone.

[18] D. H. R. BARTON and T. COHEN, *Festschrift A. Stoll*, p. 144, Birkhäuser, Basel (1957).
[19] A. C. DAY, J. NABNEY and A. I. SCOTT, *Proc. Chem. Soc.* 284 (1960).
[20] A. RHODES, private communication (1962).
[21] R. WILLSTÄTTER and A. PFANNENSTIEL, *Ber.* 37, 4744 (1904).
[22] A. I. VOGEL, *A Textbook of Practical Organic Chemistry*, p. 709, Longmans, Green and Co., London (1948).
[23] J. A. BARLTROP and J. S. NICHOLSON, *J. Chem. Soc.* 116 (1948).
[24] C. G. HAYNES, A. H. TURNER and W. A. WATERS, *J. Chem. Soc.* 2823 (1956).

The yields of primary products obtained from the catalytic action of both enzymes on the phenols we have used are shown in Table 2 and compared with those produced by chemical oxidizing agents of the one-electron type. It is known[25] that the action of chemical oxidants of the one-electron type on phenols produces aryloxy-radicals (e.g. XIII) which undergo subsequent changes. Since, in the varied examples we have studied, laccase has always produced primary products identical with those from the action of chemical oxidants, we conclude that the chemical mechanism of the catalytic action of laccase is also to produce an aryloxy-radical (e.g. XIII) from a phenol. In doing so, cupric copper in laccase is reduced to the cuprous state which is then oxidized to the cupric state by molecular oxygen.[26] Thus, for example, the mechanism of the catalytic action of laccase on 2,6-dimethoxyphenol can be written as shown in Fig. 1. It should be noted that the state of the copper in laccase with respect to co-ordinated ligands is as yet unknown.

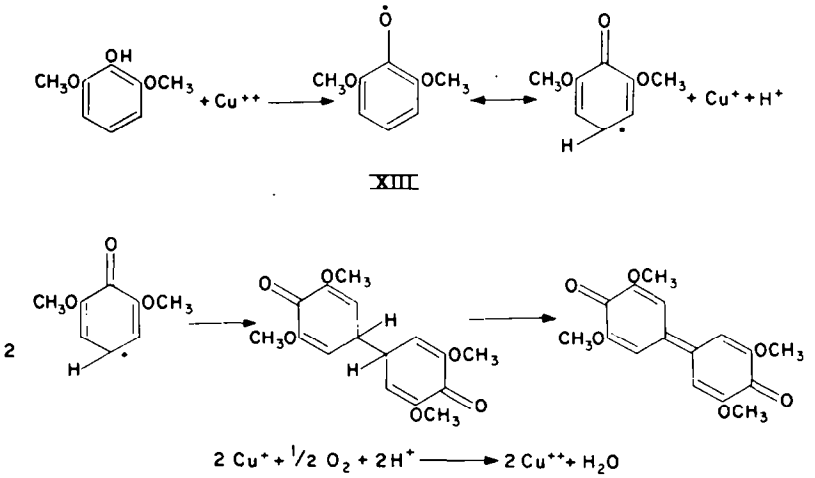


FIG. 1.

The main difference between the two enzymes is that the fungal enzyme was found to have its pH optima between pH 3·6 and 5·2, while laccase from *Rhus vernicifera* was found to have pH optima between 6·8 and 7·4 (Figs. 2 and 3). The low pH optimum of the fungal enzyme is to be expected since *Polyporus versicolor* is well adapted to grow under acidic conditions. The plant enzyme, being intra-cellular, may be expected to have its pH optimum nearer the physiological range. The differences in pH optima may point also to a difference in function. The fungal enzyme may be a mechanism for removing toxic phenols from the medium in which these fungi grow under natural conditions; the plant enzyme may be involved in synthetic processes, e.g. lignin formation.[27]

With regard to the substrate specificity of laccase from *Rhus vernicifera*, the results obtained from these studies do not agree with those reported by Keilin and Mann[4,12] in as much as

[25] J. K. BECCONSALL, S. CLOUGH and G. SCOTT, *Proc. Chem. Soc.* 308 (1959); A. FAIRBOURN and E. A. C. LUCKEN, *Proc. Chem. Soc.* 67 (1960); T. J. STONE and W. A. WATERS, *Proc. Chem. Soc.* 253 (1962).

[26] T. NAKAMURA, *Biochim. Biophys. Acta* **30**, 538, 640 (1958); **42**, 499 (1960); B. G. MALMSTRÖM, R. MOSBACH and T. VÄNNGÅRD, *Nature* **183**, 321 (1959).

[27] K. FREUDENBERG, *J. Polymer Science* **48**, 371 (1960).

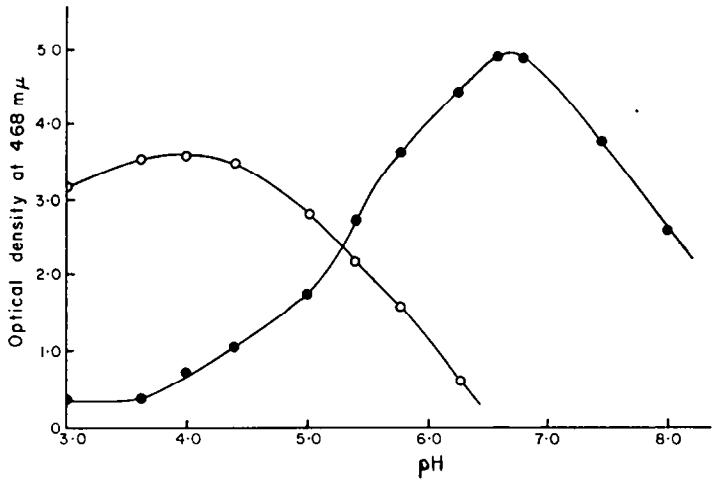


FIG. 2. EFFECT OF pH ON THE OXIDATION OF 2,6-DIMETHOXYPHENOL BY *Polyporus versicolor* OXIDASE (—○—○—○—) IN 0·01 M SODIUM ACETATE BUFFER AND *Rhus vernicifera* LACCASE (—●—●—●—) IN 0·01 M POTASSIUM PHOSPHATE BUFFER AT 25°.

The phenol was 0·0065 M and optical density was measured after 10 min.

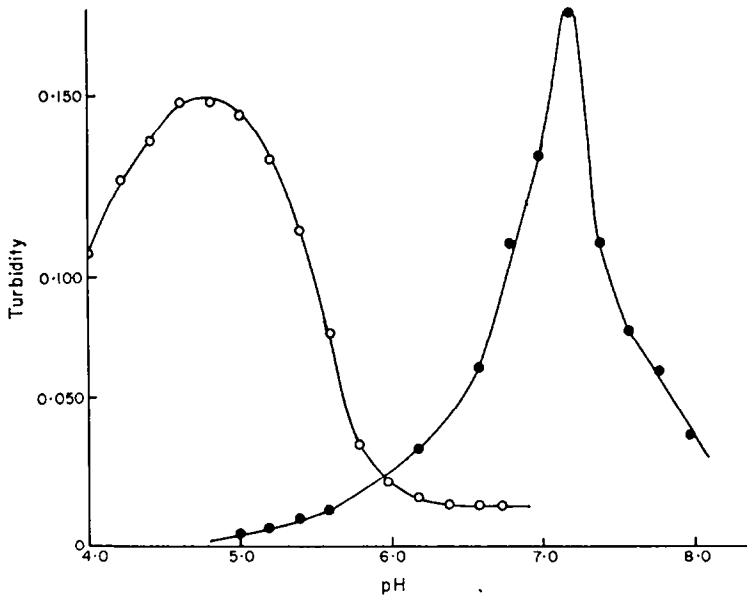


FIG. 3. EFFECT OF pH ON THE OXIDATION OF *p*-CRESOL BY *Polyporus versicolor* OXIDASE (—○—○—○—) IN 0·2 M DISODIUM HYDROGEN PHOSPHATE–CITRIC ACID BUFFER AND *Rhus vernicifera* LACCASE (—●—●—●—) IN 0·2 M DISODIUM HYDROGEN PHOSPHATE–CITRIC ACID BUFFER AT 25°.

The *p*-cresol was 0·058 M.

this enzyme has now been found to catalyse the oxidation of monophenols, such as 2,6-dimethoxyphenol and *p*-cresol. It is possible that their method of isolation and purification of laccase from lacquer trees may have resulted in considerable denaturation of the enzyme protein. Preliminary experiments have shown that the *Polyporus versicolor* oxidase is considerably inactivated by pre-incubation with acetone. The purification procedure adopted for the preparation of laccase[4,12] involved treatment with acetone. It is also possible that the techniques used to measure the activity of these enzymes were unsuitable for the detection of very low activity with these monophenolic substrates.

EXPERIMENTAL

Preparation of the Fungal Enzyme

Polyporus versicolor was grown on a sterilized 3% aqueous malt medium at 22°. The enzyme was found to accumulate in the medium and maximum activity was reached approximately 72 hr after inoculation. Enzyme activity was also found in extracts of the mycelium obtained by grinding the mycelium with 0·01 M acetate buffer (pH 5·0) and fine glass beads. The medium and the extracts of the mycelium were dialysed against 0·001 M acetate buffer (pH 5·0) in the cold, with three changes of buffer, for 48 hr. The dialysed enzyme solutions so obtained were cooled to 0° and the enzyme was precipitated by addition of ammonium sulphate. The enzyme protein was separated by centrifugation at 10° and taken up in the minimum amount of 0·01 M acetate buffer (pH 5·0). The concentrated enzyme solution was dialysed in the cold against 0·001 M acetate buffer (pH 5·0) for 24 hr and stored at 0° until required for use.

Enzyme activity was assayed using 2,6-dimethoxyphenol as substrate in 0·01 M acetate buffer (10 mg/ml) at pH 4·0. Aliquots were removed from the incubation mixture at intervals and added to chloroform and dilute sulphuric acid. Optical density of the chloroform extracts was read at 468 mμ (λ_{max} of 3,5,3′,5′-tetramethoxydiphenoquinone).

Preparation of Plant Laccase

A freeze-dried extract of *Rhus vernicifera* (kindly supplied by Dr. T. Omura of Osaka University) was dissolved in distilled water and saturated with ammonium sulphate at 0°. The precipitate containing laccase was collected by centrifugation, redissolved in water and made 67 per cent saturated with respect to ammonium sulphate. The precipitate formed was discarded and the supernatant saturated with ammonium sulphate. The laccase-containing precipitate was collected by centrifugation, dissolved in 0·01 M phosphate buffer (pH 6·5), dialysed for 24 hr at 0° against distilled water and kept at 0° until required for use. This procedure was adapted from that of Omura,[28] except that acetone treatments were omitted. Assay of enzyme activity was carried out as for the fungal enzyme.

Oxidation of Catechol

(*a*) *Catalysed by the fungal enzyme.* A solution of catechol (200 mg) and sodium benzene sulphinate (300 mg) in 0·2 M acetate buffer (pH 5·2; 100 ml) was added to *Polyporus versicolor* enzyme solution (20 ml) and left for 2 days at 25°. The crystals which separated (140 mg) were recrystallized from water to yield a hydrate of the phenylsulphone, m.p. 150–153°. A further

[28] T. OMURA, *J. Biochem. Japan* **50**, 264 (1961).

quantity (80 mg) was obtained from the incubation mixture by extraction with ether. Recrystallization from anhydrous benzene gave colourless prisms, m.p. 160–162°. Comparison of the infrared spectra (Nujol) of the two forms of the phenyesulphone with those of the two forms of authentic material[29] established identity. An aqueous solution of all samples gave an intense blue–green colour with aqueous ferric chloride, which became deep red on addition of aqueous sodium carbonate.

(*b*) *Catalysed by laccase.* A similar procedure to that above, using catechol (100 mg), sodium benzene sulphinate (200 mg), 0·1 M sodium phosphate buffer (pH 7·0; 100 ml) and laccase solution (4 ml) after 24 hr yielded crystals (75 mg) which after recrystallization from benzene had m.p. 157–159° and were identical in all respects (infrared spectrum and colour reactions) with the authentic phenylsulphone.

It should be noted that the m.p. of the phenylsulphone is variable over the range 150–163°, depending upon its degree of hydration. For this reason identity was established by infrared spectra of samples which had been treated in an identical fashion.

Oxidation of Hydroquinone

(*a*) *Catalysed by the fungal enzyme.* Hydroquinone (100 mg) and sodium benzene sulphinate (250 mg) in 0·2 M acetate buffer (pH 4·8; 50 ml) and *Polyporus versicolor* enzyme solution (10 ml) were kept at 25° for 2 days. Colourless prisms (110 mg) of the phenylsulphone were formed and after recrystallization from aqueous ethanol had m.p. and mixed m.p. with authentic material, 200°. The two samples had identical infrared spectra (Nujol).

(*b*) *Catalysed by laccase.* Hydroquinone (40 mg) and sodium benzene sulphinate (60 mg) in 0·1 M sodium phosphate buffer (pH 7·0; 20 ml) and laccase solution (1 ml) were kept at 25° for 1 day. After recrystallization, the phenylsulphone (20 mg) had m.p. and mixed m.p. 199° and its infrared spectrum (Nujol) was identical with that of authentic material.

(*c*) *Uncatalysed.* When the quantities of substrates used in (*a*) above, omitting the enzyme solution, were kept at 25° the solution was still clear after 2 weeks and appeared unchanged in all respects. After 4 weeks, crystals (50 mg), m.p. and mixed m.p. 200°, presumably formed by aerial oxidation, were obtained.

Oxidation of Pyrogallol

(*a*) *Catalysed by the fungal enzyme.* Pyrogallol (23 mg) in 0·01 M acetate buffer (pH 4·0) was incubated at 20° with enzyme solution. Purpurogallin was formed and was estimated spectroscopically on ether extracts (λ_{max} 311 mμ). After 27 hr, formation of purpurogallin reached a maximum; thereafter dark-coloured products resulted. In order to obtain the maximum yield (20·4 mg), the purpurogallin was separated at intervals and fresh enzyme solution was added to the incubation mixture. The recrystallized purpurogallin was identified by comparison (m.p., mixed m.p., ultraviolet and infrared spectra) with authentic material.

The pH optimum for conversion of pyrogallol to purpurogallin was found to lie in the pH range 3·6–4·0.

(*b*) *Catalysed by laccase.* As first reported by Bertrand[3] and later confirmed by Suminokura,[30] laccase was found to catalyse the oxidation of pyrogallol to purpurogallin (theoretical yields).

[29] O. HINSBERG and A. HIMMELSCHEIN, *Ber.* **29**, 2023 (1896).
[30] K. SUMINOKURA, *Biochem. Z.* **224**, 292 (1930).

Oxidation of 2,6-Dimethoxyphenol

(*a*) *Catalysed by the fungal enzyme.* 2,6-Dimethoxyphenol (50 mg) in 0·01 M acetate buffer (pH 4·0; 40 ml) was incubated at 25° with enzyme solution (10 ml) for 24 hr. 3,5,3',5'-Tetramethoxydiphenoquinone (90%) separated as steel-blue needles, m.p. 289–293°, identical in ultraviolet ($CHCl_3$) and infrared (Nujol) spectra with authentic material.

The pH optimum for this substrate in 0·01 M acetate buffer was determined using the spectroscopic method described above for the assay of the enzyme (see Fig. 2).

(*b*) *Catalysed by laccase.* 2,6-Dimethoxyphenol (10 mg) in 0·01 M potassium phosphate buffer (pH 7·0) was incubated at 30° with a solution of laccase. After 6 hr 3,5,3',5'-tetramethoxyphenoquinone (90%) separated as steel-blue needles.

The pH optimum was determined using the spectroscopic method (Fig. 2).

Oxidation of 2,6-Dimethylphenol by the Fungal Enzyme

2,6-Dimethylphenol (50 mg) in 0·01 M acetate buffer (pH 5·4; 40 ml) was incubated with enzyme solution (10 ml) for 24 hr, after which further enzyme solution (50 ml) was added and the mixture incubated for 24 hr. Recrystallization of the resulting red precipitate from acetic acid gave 3,5,3',5'-tetramethyldiphenoquinone (30%) as deep red prisms, m.p. and mixed m.p. 217–219° (decomp.), identical in ultraviolet ($CHCl_3$) and infrared (Nujol) spectra with authentic material.

Oxidation of p-Cresol

(*a*) *Catalysed by the fungal enzyme.* *p*-Cresol (1·025 g) in 0·004 M acetate buffer (pH 4·8; 1 l.) and enzyme solution (25 ml) were kept for 4 days at 23°. A white suspension formed. Further enzyme solution (25 ml) was added and the mixture was kept at 23° for a further 12 days. Ether extraction yielded a residue (1·00 g) which was boiled under reflux for 4 hr in ethanolic acetic acid (9:1 v/v; 25 ml) with Girard's T reagent (1·964 g), after which the solution was poured into water (100 ml) containing sodium carbonate (2·04 g). The solution was extracted with ether, water added to the non-ketonic ether extract, the ether removed by distillation and the residue distilled in steam to give a distillate containing *p*-cresol and leaving polymeric material. On ether extraction of the distillate, *p*-cresol (0·166 g) was obtained and polymeric material (0·364 g) was recovered from the distillation residue. The aqueous ketonic solution was acidified to pH 2 with concentrated hydrochloric acid and after 12 hr the ketonic material was extracted into ether. This extract, after being washed with sodium carbonate solution, was dried and the ether removed leaving crystalline ketonic material (0·215 g; 28 per cent of the reacted *p*-cresol). Recrystallization from aqueous acetone yielded colourless plates, m.p. 123–124°, which had m.p., mixed m.p., infrared, and ultraviolet spectra identical with authentic Pummerer's ketone. The sample had zero optical rotation in chloroform solution.

The pH optimum was determined over the range 4·0–6·6 using 0·2 M disodium hydrogen phosphate–citric acid buffer solutions and 0·058 M *p*-cresol. After incubation for 1·5 hr at 23°, the mixtures were turbid but none showed visible precipitation or formation of crystal clusters which appear on longer incubation. The turbidity of the mixtures was measured with a Unican SP 600 Visible Spectrophotometer. A plot of turbidity against pH is shown in Fig. 3.

(*b*) *Catalysed by laccase.* *p*-Cresol (1·12 g) in 0·01 M disodium hydrogen phosphate–citric acid buffer (pH 7·2; 1 l.) and laccase solution (10 ml) were kept at 23° for 9 days. A

white precipitate was formed. Ether extraction was hindered by precipitation of the laccase protein which necessitated centrifugation at each ether extraction and reduced the total recovery (0·971 g). Thence the procedure was as above and resulted in isolation of *p*-cresol (0·333 g), polymers (0·28 g) and ketonic material (0·141 g; 18 per cent of the reacted *p*-cresol). Recrystallized ketonic material was identical with Pummerer's ketone and showed no optical rotation in chloroform solution.

The pH optimum in 0·2 M disodium hydrogen phosphate–citric acid buffer solutions was determined as described above. The turbidity was measured after 19 hr at 23° (Fig. 3).

Oxidation of Griseophenone A by Fungal Enzyme

Griseophenone A (100 mg) dissolved in acetone (10 ml) was slowly poured into 0·01 M acetate buffer (pH 5·4; 3·5 l.). The mixture was boiled and then cooled to room temperature. To the resulting clear solution was added enzyme solution (35 ml). The mixture was kept at 30° for 2 days with further addition of enzyme solution (total of 85 ml) at intervals. The resulting turbid liquid was extracted with chloroform and the product isolated by evaporation of the dried extract. A solution of the product in acetone (4 ml) had zero optical rotation. Crystallization of the product from acetone and then from acetone–ether yielded (±)-dehydrogriseofulvin (36·7 mg) as aggregates of prisms, m.p. 284°. The infrared spectrum ($CHCl_3$) and the behaviour on paper chromatography were identical with those of (−)-dehydrogriseofulvin.

Controls

In all cases control experiments were carried out in which the substrates in buffer solution were incubated (*a*) with boiled enzyme solution and (*b*) with no enzyme solution. In the time taken for the formation of products in the enzyme-catalysed reactions, no products could be detected in the control experiments.

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