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STUDIES ON THE DIPHENOL OXIDASE OF THE PHYTOPATHOGENIC FUNGUS *GLOMERELLA CINGULATA*: INHIBITION BY QUATERNARY AMMONIUM COMPOUNDS

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Abstract—The properties of the *p*-diphenol oxidase ("laccase") present in the mycelium of *Glomerella cingulata* have been investigated. The enzyme rapidly oxidised chlorogenic acid, *d*-catechin, catechol and *p*-phenylene diamine. Electrophoresis showed that only one enzyme was present. By contrast with *o*-diphenol oxidases from fruits and leaves the fungal *p*-diphenol oxidase was not inhibited by polyvinylpyrrolidone (PVP) but was non-competetively inhibited by cetyl-trimethyl ammonium bromide (CETAB) and other cationic detergents.

INTRODUCTION

MANY fungi that parasitize plants are known to produce enzymes capable of oxidizing phenolic compounds[1,2] and these enzymes may be classified as *o*-diphenol: O_2 oxidoreductases (E.C. No. 1.10.3.1), commonly referred to as "phenolases" or "tyrosinases", or as *p*-diphenol: O_2 oxidoreductases (E.C. No. 1.10.3.2) which are more usually known as "laccases". Both types of diphenol oxidase may occur in different parts of the same organism and, following investigations of the levels and distribution of *o*- and *p*-diphenol oxidase in the different tissues of the common mushroom (*P salliota bispora*), Lindeberg[3] suggested that the two enzymes may be interconvertible. The ability of certain wood-rotting fungi to give a positive Bavendamm[4] reaction on an agar medium containing gallic or tannic acid has been correlated with the production of extracellular *p*-diphenol oxidases by these organisms.[1] There is also a reasonable correlation between a positive Bavendamm reaction and the formation of a white rot,[5] whilst by contrast, brown rot fungi do not usually produce coloured zones in the Bavendamm test. Kuster[6] has also used a modified type of Bavendamm reaction to test for the presence of diphenol oxidases in *Streptomycetes* sp.

This paper reports investigations of the properties of the diphenol oxidase produced by *Glomerella cingulata*, the cause of "bitter rot" or "ripe rot" in apples and a common parasite in N.Z. apple orchards. Earlier studies by Sussman[7,8] and his co-workers investigated the effect of environmental conditions upon the development of *p*-diphenol oxidase in *G. cingulata* but these workers did not investigate fully the properties of the enzyme. In view

[1] V. W. COCHRANE, *Physiology of Fungi*. John Wiley, New York (1958).
[2] W. D. BONNER, *Ann. Rev. Plant Physiol.* **8**, 440 (1957).
[3] G. LINDEBERG, *Nature* **166**, 739 (1950).
[4] W. BAVENDAMM, *Z. Pflzkrankh. Pflzschutz.* **38**, 257 (1928).
[5] H. LYR, *Planta* **50**, 359 (1958).
[6] E. KUSTER, in *Enzyme Chemistry of Phenolic Compounds* (edited by J. B. PRIDHAM), p. 81. Pergamon Press, Oxford (1963).
[7] A. S. SUSSMAN and C. L. MARKERT, *Arch. Biochem. Biophys.* **45**, 31 (1953).
[8] A. S. SUSSMAN, P. COUGHEY and J. C. STRAIN, *Am. J. Botany* **42**, 810 (1955).

of the current upsurge of interest in the question of the host-parasite relationship in plants and also because of the local economic importance of this phytopathogen it was considered of importance to investigate its biochemistry in more detail.

RESULTS

Optimum pH

The optimum pH for *p*-diphenol oxidase activity with catechol as substrate was measured by determining the rate of O_2 absorption in reaction mixtures at different pH values. Maximum activity occurred around pH 6·5; therefore all subsequent experiments were carried out at this pH value.

Development of Diphenol Oxidase in G. cingulata

The level of *p*-diphenol oxidase activity in stationary cultures of *Glomerella cingulata* was recorded at daily intervals for 5 days and the results are shown in Fig. 1. There was a regular increase in the weight of mycelial tissue but *p*-diphenol oxidase activity reached a maximum

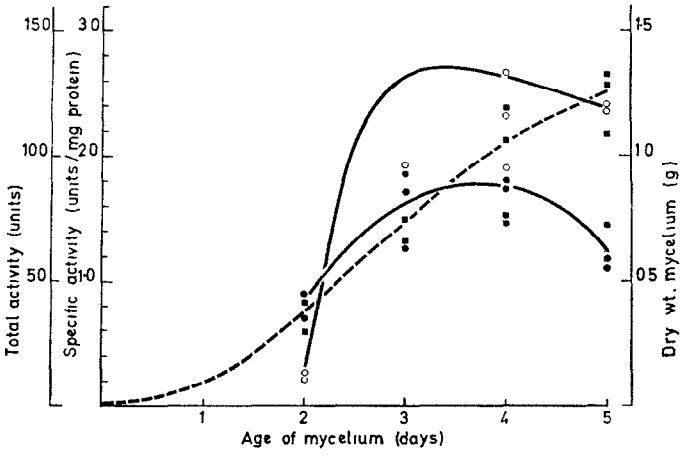

FIG. 1. GROWTH OF *G. cingulata* AND PRODUCTION OF *p*-DIPHENOL OXIDASE ACTIVITY IN THE MYCELIUM.
Dry weight of mycelium (—■—); total yield of enzyme, activity (μl O_2/min) × volume of extract (○); Specific activity, μl O_2/min/mg protein (●).

between the third and fourth days. Subsequent cultures for enzyme production were harvested after 4 days growth at which stage the mycelial mats were creamy white in appearance. After growth for 10 days, the mats became grey and showed little or no diphenol oxidase activity. By contrast shake culture of *G. cingulata* did not produce detectable amounts of *p*-diphenol oxidase activity, a finding which is in agreement with the observation by Bocks[9] that shake cultures of *Polyporus versicolor* did not produce *p*-diphenol oxidase whereas stationary cultures gave high yields of this enzyme.

Substrate Specificity

The substrate specificity of the *p*-diphenol oxidase from *G. cingulata* was investigated by incubating aliquots of the mycelial extract with 10 μmoles each of a number of suitable phenolic substrates and recording the rate of O_2 absorption. The results of this experiment

[9] S. M. BOCKS, *Phytochem.* 6, 777 (1967).

(Table 1) suggest that the enzyme is a *p*-diphenol oxidase ("laccase") since *p*-cresol was not oxidized whereas *p*-phenylene diamine, *p*-quinol and guaiacol were. The mycelial extracts used in these experiments showed negligible rates of endogenous respiration whilst other parallel experiments using an acetone-precipitated enzyme preparation gave a similar pattern of results. It was therefore concluded that the mycelial extracts were reasonably free of endogenous substrate(s) that might catalyse the oxidation of phenolic substrates such as *p*-quinol.

TABLE 1. OXIDATION OF PHENOLIC COMPOUNDS BY EXTRACTS OF *G. cingulata*

Substrate	Initial rate of O_2-absorption (μl/min)
Chlorogenic acid	5·6
(+)-Catechin	4·6
Catechol	3·2
Hydrocaffeic acid	2·1
Caffeic acid	2·0
4-Methylcatechol	1·4
L-β-(3,4-Dihydroxy-phenyl) alanine	1·1
3,4-Dihydroxybenzoic acid	1·1
3,4-Dihydroxyphenylacetic acid	1·0
p-Phenylene diamine	3·2
Guaiacol (*o*-methoxyphenol)	1·8
p-Quinol	1·5
p-Cresol	Nil
p-Cresol + 1% gelatin	Nil
p-Cresol + 1% PVP	Nil
p-Cresol + 0·1% Tween 80	Nil

Enzyme (0·5 ml) incubated with substrate (10 μmoles) in 0·1 M phosphate buffer pH 6·5 at 30° in atmosphere of air. Each Warburg flask contained 3·0 ml solution.

Michaelis constants (K_m values) were determined for the following substrates: chlorogenic acid $8{\cdot}5 \times 10^{-3}$ M, *d*-catechin $5{\cdot}7 \times 10^{-3}$ M, catechol $3{\cdot}8 \times 10^{-3}$ M, *p*-phenylene diamine $1{\cdot}1 \times 10^{-3}$ M. A number of other possible substrates, including tyrosine, *p*-cresol, *p*-coumaric acid, resorcinol, ascorbate and several quercetin-glycosides were also tested but were not oxidized by the *p*-diphenol oxidase from *G. cingulata*. Both chlorogenic acid and catechin, key substrates for the *o*-diphenol oxidase of the host apple, were readily oxidized by the mycelial *p*-diphenol oxidase.

The ability of *p*-diphenol oxidase preparations to oxidize monophenols, such as *p*-cresol, appears to depend upon the source of the enzyme. This topic has been investigated in detail by Fahraeus and Ljunggren[10] who showed that *p*-diphenol oxidase preparations from *P. versicolor* were able to oxidize *p*-cresol rapidly when stimulated by catalytic quantities of catechol or when gelatine or Tween 80 (poly-oxyethylene sorbitan mono-oleate) were added to the reaction medium. Similar experiments with the enzyme preparation from *G. cingulata* were negative and *p*-cresol was not oxidized.

A number of workers[11, 12] have reported that extracellular *p*-diphenol oxidases secreted by a number of fungi were able to oxidize indole-3-acetic acid. The ability of *G. cingulata* to

[10] G. FAHRAEUS and H. LJUNGGREN, *Biochem. Biophys. Acta* **46**, 22 (1961).
[11] N. E. TONHAZY and M. J. PELCZAR, *Science* **120**, 141 (1954).
[12] G. FAHRAEUS, *Physiol. Plantarum* **14**, 171 (1961).

oxidize this type of compound was checked by incubating both mycelial extracts and the extracellular enzyme with 10 μmoles each of indole-3-acetic acid, indole-3-butyric acid, indole-3-propionic acid and 1-naphthyl-acetic acid. None of these compounds were oxidized. Thus there appear to be several points of difference between the *p*-diphenol oxidase from *G. cingulata* and the corresponding enzyme from basidomycetes and *P. versicolor*.

Electrophoresis Experiments

In view of the observation that mycelial extracts of *G. cingulata* oxidized both *ortho-* and *para-* diphenols it was necessary to find out if these extracts contained distinct *o-* and *p*-diphenol oxidases. The enzyme preparation was therefore subjected to electrophoresis on starch and polyacrylamide gels and the pherograms developed with a range of different phenolic substrates. Electrophoresis was carried out on starch gels at pH 6·5 (the optimum pH for activity) and also at pH 5·0 and 8·6. "Disc" electrophoresis on polyacrylamide gel was carried out at pH 6·5. In all experiments only one coloured zone appeared when the gels were treated with a phenolic substrate and these all showed the same electrophoretic mobility. Thus it was concluded that *G. cingulata* contained only one *p*-diphenol oxidase.

Effect of Inhibitors

Both types of diphenol oxidase contain copper as a prosthetic group and are inhibited by Cu- complexing reagents such as sodium diethyldithiocarbamate (DIECA) or 8-hydroxyquinoline. Carbon monoxide has been shown to be an inhibitor of *o*-diphenol oxidases[13] but to have little effect on *p*-diphenol oxidases and Lindeberg[3] took advantage of this difference in his studies of the location of diphenol oxidases in the different tissues of basidiomycetes. The effect of carbon monoxide upon the oxidation of phenolic compounds by *G. cingulata p*-diphenol oxidase was investigated by using an atmosphere of $CO+O_2$ (80:20) in the Warburg flasks. The results of these experiments are summarized in Table 2 where it will be seen that DIECA, 2:3 dimercapto-propanol (BAL), sodium azide and 8-hydroxyquinoline all inhibited *G. cingulata p*-diphenol oxidase whereas carbon monoxide was without effect. This latter observation supports the hypothesis that the enzyme from *G. cingulata* is a *p*-diphenol oxidase ("laccase").

Experiments with PVP and High Molecular Weight Carbohydrates

Polyvinylpyrrolidone (PVP) has been shown to act as a competitive inhibitor of the *o*-diphenol oxidases extracted from fruits,[14] leaves[15] and potato tubers.[16] However when this compound was included in reaction mixtures containing *p*-diphenol oxidase and chlorogenic acid, catechol or *p*-phenylene diamine it was devoid of any inhibitory effect (see also Fig. 2).

A number of high molecular weight carbohydrate substances, such as might occur in the natural host tissues, were similarly tested for any possible stimulatory or inhibitory effect upon the oxidation of phenolic compounds by *G. cingulata* extracts. None of the compounds tested (sodium polypectate, polygalacturonic acid, pectin, methyl cellulose) affected the rate of oxidation of the phenolic substrates.

[13] D. KEILIN and T. MANN, *Nature* **145**, 304 (1940).
[14] J. R. L. WALKER and A. C. HULME, *Phytochem.* **4**, 677 (1965).
[15] W D LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[16] D. A. ABOUKHARMA and H. W. WOOLHOUSE, *New Phytologist* **65**, 477 (1966).

TABLE 2. EFFECT OF INHIBITORS UPON THE *p*-DIPHENOL OXIDASE OF *G. cingulata*

(a) Cu-complexing compounds Inhibitor	Concentration (M)	Rate of O_2-absorption (μl/min)	Inhibition (%)
None (control)	—	7·7	0
Sodium azide	10^{-4}	5·2	32
	10^{-3}	1·1	86
	10^{-2}	0	100
Sodium diethyldithiocarbamate (DIECA)	10^{-4}	7·6	1
	10^{-3}	5·2	32
	10^{-2}	0	100
2,3-Dimercaptopropanol (BAL)	10^{-4}	1·08	86
	10^{-3}	0·23	97
8-Hydroxy-quinoline	10^{-4}	7·6	1
	10^{-3}	5·1	34

(b) Effect of Carbon monoxide Substrate	Rate of O_2-absorption (μl/min) Gas atmosphere:	
	Air	CO:O_2 mixture
Chlorogenic acid	8·1	8·0
(+)-Catechin	8·0	8·0
Catechol	6·0	6·1
p-Phenylene diamine	6·2	7·0
p-Quinol	3·0	3·0

Experiments were conducted at 30° in Warburg flasks or O_2-electrode cell containing enzyme (0·5 ml), 0·1 M-phosphate buffer pH 6·5 and substrate (10 μmoles) in total volume of 3 ml.

Effect of Surface Active Agents

A number of workers[14, 17, 18] have reported that anionic detergents, such as sodium dodecyl sulphate or sodium dioctyl sulphosuccinate ("Manoxol OT" Hardman and Holden Ltd., Gt. Britain), exert a stimulatory effect upon the oxidation of phenolic compounds by *o*-diphenol oxidases whilst non-ionic or cationic detergents had little or no effect. By contrast when similar experiments were carried out with the *p*-diphenol oxidase from *G. cingulata* anionic detergents (10^{-2} M-sodium dodecyl sulphate or sodium di-octyl succinate) and non-ionic detergents (10^{-2}M-Lissapol N or Triton X-100) failed to stimulate the rate of O_2-absorption whereas cationic detergents brought about a marked inhibition of the rate of oxidation (Table 3).

This observation that fungal *p*-diphenol oxidase was inhibited by cetyl-trimethyl ammonium bromide (CETAB) was surprising and therefore was investigated in more detail. These inhibitory effects were investigated for a number of phenolic substrates and for a range of quaternary ammonium compounds and acyl-substituted pyridinium-based cationic detergents (Table 3). Preliminary experiments suggested that a non-competitive type of inhibition was taking place and this finding was confirmed by a Lineweaver and Burk plot of the reciprocal of the initial rate of oxidation ($1/v$) against the reciprocal of the substrate concentration ($1/s$) for experiments carried out in the presence and absence of inhibitor. The interpretation

[17] R. H. KENTEN, *Biochem. J.* **68**, 224 (1958).
[18] T. SWAIN, L. W. MAPSON and D. A. ROBB, *Phytochem.* **5**, 469 (1966).

TABLE 3. EFFECT OF CATIONIC DETERGENTS UPON THE OXIDATION OF CATECHOL BY EXTRACTS OF *G. cingulata*

Detergent	Concentration (M)	Diphenol oxidase activity (O_2 uptake μl^2/min)	Inhibition (%)
None (control)	—	3·7	—
Cetyl-trimethyl ammonium bromide (CETAB)	10^{-4}	3 6	3
	5×10^{-4}	0 5	86
	10^{-3}	0 0	100
Cetyl-pyridinium bromide	10^{-4}	3·7	0
	10^{-3}	1·2	67
Vantoc N (Dodecyl-trimethyl ammonium bromide)	10^{-4}	3·5	5
	10^{-3}	0·7	81
Vantoc CL (Dodecyl-dimethyl-benzyl ammonium bromide)	10^{-4}	3·6	3
	10^{-3}	0 6	84

Warburg flasks contained 0·5 ml enzyme, 0·1 M-phosphate buffer pH 6·5 at 30°, 10 μmoles catechol added from side-arm. Total volume 3 ml.

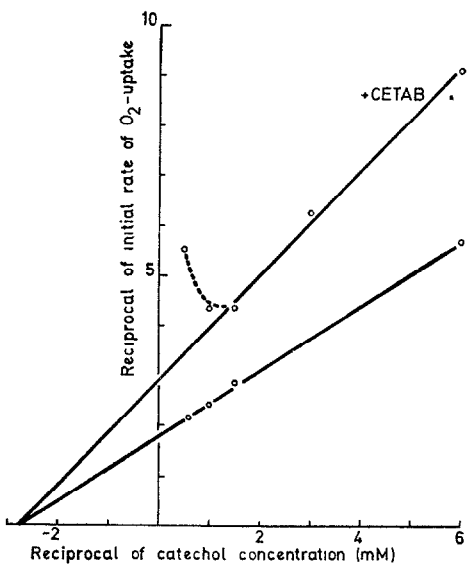

FIG. 2. INHIBITION OF THE *p*-diphenol OXIDASE FROM *G. cingulata* BY 5×10^{-4}M CETYL-TRIMETHYL AMMONIUM BROMIDE (CETAB): graphical determination of inhibitor constant (K_i) and type of inhibition.

of this type of plot has been comprehensively discussed by Dixon and Webb[19] and the results shown in Fig. 2 confirm the suggestion that CETAB acts as a non-competitive inhibitor for the fungal *p*-diphenol oxidase. A value of $1{\cdot}3 \times 10^{-3}$ M for the inhibitor constant (K_i) was calculated for this system. The upturning at the lower end of the plot for the inhibited system is due to the lower rate of oxidation of catechol at high concentrations in the presence of the

[19] M. DIXON and E. C. WEBB, *Enzymes*. Longmans, Green, London (1958).

inhibitor. This may be due to a lesser availability of "active sites" an effect which would become more marked with increasing concentration of substrate. The possibility also exists that CETAB may affect the tertiary structure of the enzyme in such a manner as to lessen its catalytic activity. By way of analogy it has been suggested that the stimulatory effect of anionic detergents upon *o*-diphenol oxidases[14, 17] may be due to their action upon the tertiary structure of the enzyme.[18, 20]

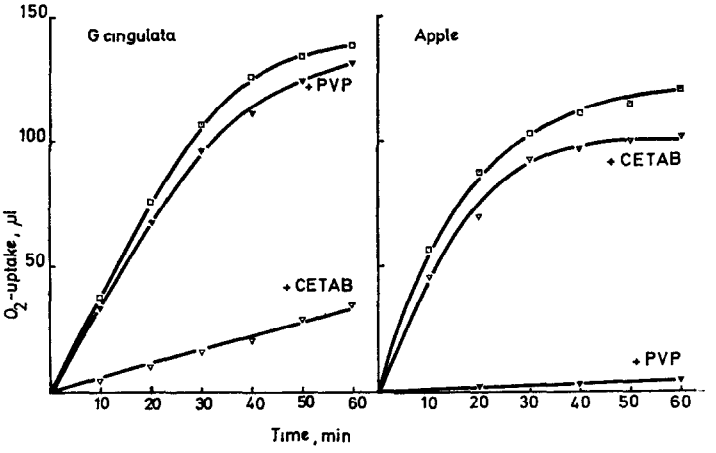

FIG. 3. COMPARISON OF EFFECT OF PVP AND CETAB UPON THE OXIDATION OF CHLOROGENIC ACID BY THE *p*-DIPHENOL OXIDASE FROM *G. cingulata* AND THE *o*-DIPHENOL OXIDASE FROM APPLE PEEL. Warburg flasks contained 1 % PVP (▼) or 0·0005 M CETAB (▽) is indicated; 10µ moles chlorogenic acid was added from the side arm. Open squares (□) denote control flasks without inhibitor.

The Effect of CETAB on Diphenol Oxidases from High Plants

In order to provide further support for the hypothesis that CETAB and similar quaternary ammonium compounds act as specific inhibitors for fungal *p*-diphenol oxidase, the effect of CETAB and PVP upon the oxidation of phenolic compounds by mycelial extracts and by *o*-diphenol oxidase preparations from apple peel was investigated. The fungal *p*-diphenol oxidase was strongly inhibited by 5×10^{-4}M-CETAB but this concentration of CETAB had little effect on the plant *o*-diphenol oxidase whereas 1 % PVP inhibited the plant enzyme but did not affect the fungal *p*-diphenol oxidase. Similar results were obtained using chloroplast grana from tobacco leaves.

Studies on the Extracellular p-*Diphenol Oxidase of* G. cingulata

A number of workers[1, 2, 4, 9, 10] have shown that "white-rot" and other fungi secrete extra-cellular *p*-diphenol oxidases and it was found that older (7 days) cultures of *G. cingulata* similarly secreted an extracellular *p*-diphenol oxidase into the growth medium. Some characteristic properties (optimum pH, substrate specificity, electrophoresis) of this enzyme were investigated and found to be identical with those of the enzyme extracted from the mycelium.

[20] D. A. ROBB, L. W. MAPSON and T. SWAIN, *Nature* **207**, 503 (1964).

The effect of CETAB on the Growth of G. cingulata

Quaternary ammonium compounds are widely used as disinfectants and for the control of dermatophytes and other pathogenic fungi; they have also been shown to be active against powdery mildews.[21,22] The effect of CETAB on the growth of *G. cingulata* was investigated by adding different concentrations of CETAB to flasks containing the yeast malt broth[23] normally used for the growth of *G. cingulata*. The inoculated flasks were incubated in the dark and unshaken at 25°. After 4 days growth, the mycelial mats were harvested, washed and dried in vacuum at 60° prior to weighing. The results of this experiment are recorded in Table 4 and it can be seen that concentrations of CETAB greater than 10^{-4} M completely suppressed the growth of *G. cingulata*. The specific activity of the *p*-diphenol oxidase of the mycelium grown in the presence of 10^{-5} M CETAB was found to be the same as that of the control (no CETAB).

TABLE 4. EFFECT OF CETAB UPON THE GROWTH OF *G. cingulata*

Concentration of CETAB (M)	Dry wt mycelium mean value (mg)*
None (control)	862
10^{-5}	626
10^{-4}	32·7
10^{-3}	0

* Four experiments.

DISCUSSION

The results in this paper provide further confirmation of the commonly observed low specificity exhibited by *p*-diphenol oxidases, although the enzyme from *Glomerella cingulata* is considerably more specific in its choice of substrates than is the extracellular enzyme secreted by *Polyporus versicolor*. For example the latter organism is able to oxidize the monophenols, *p*-cresol[10] and 2:6-dimethoxyphenol,[9] and also the auxin indole-3-acetic acid[12] whereas the *G. cingulata* enzyme did not oxidize these compounds. *G. cingulata* also differs from *P. versicolor* in as much as the addition of tyrosine[10] to the growth medium did not lead to increased yields of *p*-diphenol oxidase.

Many fungi produce diphenol oxidases but it is not always easy to distinguish the *ortho*- from the *para*-enzyme. In general *o*-diphenol oxidases show some oxidative activity towards monophenols (but not in all cases) whereas *p*-diphenol oxidases are able to oxidize compounds such as *p*-quinol and *p*-phenylene diamine. Both enzymes contain copper and their prosthetic group and therefore are inhibited by copper-complexing reagents but only *o*-diphenol oxidases are appreciably inhibited by carbon monoxide.[13] Thus the ability of the enzyme from *G. cingulata* to oxidize *p*-phenylene diamine and *p*-quinol, the failure of carbon monoxide to inhibit these oxidations and the single electrophoresis band was taken as indicative that only a *p*-diphenol oxidase ("laccase") was present.

[21] A. H. M. KIRBY and E. L. FRICK, *Ann. Appl. Biol.* **52**, 45 (1963).
[22] E. SOMERS, R. J. PRING and R. J. W. BYRDE, *J. Sci. Food Agri.* **18**, 153 (1967).
[23] C. L. MARKERT, *Genetics* **35**, 60 (1950).

The inhibitory effect of quaternary ammonium compounds upon the *p*-diphenol oxidase of *G. cingulata* is of considerable interest because these findings suggest that quaternary ammonium compounds may act as specific inhibitors for *p*-diphenol oxidase without effect on *o*-diphenol oxidase and this could provide a useful additional test for differentiating between these two closely related enzymes. From the practical point of view the use of CETAB as a selective inhibitor of *p*-diphenol oxidase is far less troublesome and less hazardous than the use of carbon monoxide/oxygen gas mixtures in the Warburg respirometer. However these preliminary observations need further confirmation using *p*-diphenol oxidases from other sources and this is the subject of current investigations. The observation that CETAB inhibits the growth of *G. cingulata* in liquid media may also have useful applications in horticultural practice and it may be beneficial to consider the use of quaternary ammonium compounds as wetting agents or dispersing agents in the formulation of fungicidal spray mixtures.

The finding that PVP did not inhibit the *p*-diphenol oxidase of *G. cingulata* is also relevant to the above discussion since this compound has been shown to be a competitive inhibitor of *o*-diphenol oxidase[14, 16] whereas CETAB had little effect upon this enzyme. Thus tests with CETAB and PVP might provide a useful means of differentiating *ortho-* and *para*-diphenol oxidases. Furthermore in those cases where it has been established that a phytopathogenic fungus secretes a *p*-diphenol oxidase the selective use of these two inhibitors could provide a means of identifying the origin of the enzymic browning that is a common feature in diseased plant tissue.

EXPERIMENTAL

The organism was isolated from "bitter rot" lesions on locally grown "Sturmer Pippin" apples, a variety susceptible to "bitter rot", and its identity confirmed by Mrs. Susan Davison (Plant Diseases Division, D.S.I.R., Auckland). Cultures were maintained on slopes of potato glucose agar whilst mycelial mats for biochemical experiments were grown on 150 ml yeast malt broth[23] in 750 ml Erlenmeyer flasks at 25°. After 4 days growth the mycelial mats were washed with distilled water and finally harvested on a Buchner funnel.

Preparation of Enzyme

The washed mycelial mats were disintegrated in 20 ml 0·01 M-phosphate buffer pH 6·5 by means of an "Ultra-Turrax" homogenizer (Janke and Kunkel K.G., Stauffen, Germany). The resultant slurry was centrifuged for 20 min at 10,000 × *g* in a refrigerated centrifuge and the clear supernatant was used as a source of *p*-diphenol oxidase for all experiments. This preparation was stored for up to 3 months at −15° without loss of activity.

Electrophoresis Experiments

Electrophoresis was carried out on 10 per cent (w/v) starch gels made up in pH 6·5 0·5 M-phosphate buffer, unless otherwise stated, on standard 3 × 1 in. microscope slides and run at a potential gradient of 15 V/cm for 18 hr. Polyacrylamide gels for disc electrophoresis were prepared in small glass tubes (5 × 65 mm) and operated at a potential gradient of 20 V/cm. All electrophoresis runs were conducted in a cold room at 4°. The separate enzyme bands were located by incubating the gels with dilute solutions (0·001 M) of the various substrates made up in pH 6·5 0·05 M phosphate buffer.

Experimental Techniques

Rates of O_2-absorption were measured by conventional Warburg techniques or by means of an O_2-electrode (Rank Bros, Bottisham, Cambridge, England) connected to a 1 mV recording potentiometer; all measurements were carried out at 30°. The Warburg flasks or the O_2-electrode cell normally contained pH 6·5 0·1 M phosphate buffer, 0·5 ml enzyme extract and 10 μmoles substrate in a total volume of 3 ml. The protein content of the mycelial extracts were estimated by the method of Lowry *et al.*[24]

[24] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

Analytical reagent grade chemicals were used where available whilst catechol, *p*-quinol, *p*-cresol and *p*-phenylene diamine HCl were purified by recrystallization. Chlorogenic acid and caffeic acid were obtained from Fluka A.G., Buchs, Switzerland; polygalacturonic acid from L. Light & Co. Ltd., England; sodium polypectate from Sigma Chemical Co. U.S.A. and pectin, (apple 250 grade) from British Drug Houses Ltd. The "Vantoc" series of cationic detergents were a generous gift from Imperial Chemical Industries (N.Z.) Ltd.

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