The Peroxidase-Catalysed Oxidation of a Chalcone and its Possible Physiological Significance

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1. Crystalline horseradish peroxidase catalysed the oxidation of 2',4,4'-trihydroxychalcone (isoliquiritigenin) in the presence of trace amounts of hydrogen peroxide under aerobic conditions. One atom of oxygen was consumed for each molecule of substrate. 2. The reaction course comprised a lag phase and a linear phase. The optimum pH for the linear phase of the reaction was about 7.5. The length of the lag phase decreased with increasing pH. It is suggested that the chalcone anion is the actual substrate for the reaction. 3. No evidence for the production of reducing free radicals or perhydroxyl radicals during the reaction could be found. 4. 4',7-Dihydroxyflavonol and 4',6-dihydroxyaurone were isolated from the reaction mixture. The immediate products of the reaction may have included 3,4',7-trihydroxyflavanone and 4',6-dihydroxy-2-(α -hydroxybenzyl)coumaranone, which can be readily converted non-enzymically into the flavonol and aurone respectively. 5. A similar reaction was catalysed by cell-free extracts of hypocotyls of *Phaseolus vulgaris*. 6. The physiological significance of the reaction is discussed in terms of a possible free-radical mechanism. An analogy may exist between flavonoid biosynthesis and lignin formation.

Wong & Grisebach (1969) have suggested that chalcones are the immediate precursors of several classes of flavonoid compound in plants. They have demonstrated the incorporation of radioactivity from labelled chalcones into other flavonoids in whole plants and in cell-free systems. We have observed that gel-filtered extracts of *Phaseolus vulgaris* hypocotyls rapidly metabolize 2',4,4'-trihydroxychalcone (isoliquiritigenin, I; Scheme 1) with the uptake of oxygen. We later observed the catalysis of a similar reaction by horseradish peroxidase in the presence of hydrogen peroxide. The present paper describes the reaction, and discusses its possible physiological significance. A preliminary account of this work has been published (Rathmell & Bendall, 1972); a brief report of a similar reaction has also been published by Wong & Wilson (1972).

Materials and Methods

Methods of assay

The activity of *Phaseolus vulgaris* hypocotyl extracts or solutions of crystalline horseradish peroxidase with 2',4,4'-trihydroxychalcone (isoliquiritigenin) as substrate was assayed either spectrophotometrically or by oxygen uptake measured with a Clark-type oxygen electrode. Crystalline chalcone was dissolved in 100 ml of 25 mM-sodium phosphate buffer, pH 7.5. For the spectrophotometric assay approx. 1 mg was dissolved and 2 ml of the resulting

solution was used in the assay. For the oxygen-electrode assay 10 mg was dissolved and the volume was 3 ml. For routine assays, 0.2 ml of hypocotyl extract or 1-1.5 ng of crystalline horseradish peroxidase [Reinheitszahl (R.Z.: E_{402}/E_{280}) = 2.71 (Seravac Ltd., Cape Town, South Africa)] was added. All assays were performed at room temperature.

Synthesis of flavonoids

Isoliquiritigenin. This compound was synthesized by the cold condensation method of Geissman & Clinton (1946), starting from *p*-hydroxybenzaldehyde and resacetophenone. The product was recrystallized three times from aq. ethanol, and had m.p. (uncorr.) 209°C.

4',7-Dihydroxyflavonol. This compound was synthesized by the method of Wong *et al.* (1965). It was recrystallized four times from aq. ethanol, and had m.p. (uncorr.) 303°C.

4',6-Dihydroxyaurone. This compound was obtained by the oxidation of isoliquiritigenin with alkaline permanganate as described by Wong (1966).

Reduction of hydrogen acceptors

To test for the reduction of cytochrome *c* or Methylene Blue by a mixture of isoliquiritigenin and peroxidase in the absence of oxygen, a Thunberg tube with a spectrophotometer cuvette attached was

used. Sodium phosphate buffer (3ml; 25mM), containing isoliquiritigenin (100 μ g/ml), hydrogen peroxide (0.5mM) and cytochrome *c* (10 μ M) or Methylene Blue (300 μ M), was placed in the cuvette. Crystalline peroxidase (40ng) was placed in the side arm and the tube was evacuated. After the contents of cuvette and side arm had been mixed, the tube was placed in a spectrophotometer and the reduction of cytochrome *c* or Methylene Blue followed at 550 or 662nm respectively.

Anaerobic experiments. The Thunberg tube described above was also used for experiments on the metabolism of isoliquiritigenin by peroxidase and hydrogen peroxide in the absence of oxygen. The standard spectrophotometric assay conditions were used: the hydrogen peroxide (85 μ M) was added from the side arm.

Chromatographic separation of reaction products

Isoliquiritigenin (2mg) was dissolved in 50ml of 25mM-sodium phosphate buffer, pH 7.0. To this was added 0.25 μ g of crystalline horseradish peroxidase and 2 μ l of hydrogen peroxide (100vol.). When the yellow colour of the chalcone had been discharged, KCN (1mM final concn.) was added to prevent further reaction. The solution was extracted three times with 50ml of diethyl ether, and the ether layers were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of ethanol for chromatography. The chromatograms were run on thin (0.25mm) layers of cellulose, which were developed in 30% (v/v) acetic acid. The material was loaded on the plates in 3cm streaks, each chromatogram receiving a loading of 100 μ l of the ethanolic solution.

After development of the chromatograms, bands of material were detected by their fluorescence in u.v. light, or by spraying the chromatogram with a freshly prepared mixture of equal quantities of 0.1% solutions of ferric chloride and potassium ferricyanide (Barton *et al.*, 1952). The bands were eluted by scraping the area of cellulose containing them off the plate into aq. 85% (v/v) ethanol in a test tube. The tube was shaken on a Whirlimixer and the cellulose removed by centrifugation.

Conversion of reaction products into 4',6-dihydroxyaurone

Eluates of bands A and B (Table 1) in aq. 85% (v/v) ethanol were concentrated *in vacuo* to 1 ml. One drop of 2M-NaOH or 2M-HCl was added to 0.5 ml portions of the eluates from each of the two bands. The solutions were left at room temperature for 3–4h and then made approximately neutral, the neutralization being followed with indicator paper. The solutions were then chromatographed in the above chromatographic system. 4',6-Dihydroxyaurone was re-

cognized by its intense blue-green fluorescence (which became yellow-green in NH₃ vapour). Its identity was confirmed by elution and co-chromatography with the synthetic compound in three different systems.

Preparation and assay of cell-free extracts

Seedlings of *Phaseolus vulgaris* were grown as previously described (Rathmell & Bendall, 1971). Hypocotyls (60g) were excised from 8-day-old seedlings and homogenized in 40 ml of 25mM-sodium phosphate buffer, pH 7.5, for 20s in a blender. The homogenate was filtered through two layers of muslin, and 50ml of the filtrate was gel-filtered in a column (400ml bed vol.) of Sephadex G-25 (coarse grade). The effluent was collected, stored in ice and

Fig. 1. Effect of peroxidase and hydrogen peroxide on the absorption spectrum of isoliquiritigenin

Normal assay conditions were used with the components at the following concentrations: peroxidase, 0.5ng/ml; hydrogen peroxide, 85 μ M; isoliquiritigenin, 8 μ g/ml. The spectra were plotted immediately before and at 1min intervals after starting the reaction, and are numbered 1–4 in order of plotting.

used within 8h. All the above manipulations were performed with ice-cold solutions.

Extracts were added to approx. 10 times their own volume of isoliquiritigenin soln. ($10\mu\text{g/ml}$), prepared as described above, for spectrophotometric or polarographic assay. For identification of reaction products, reaction mixtures were prepared for chromatography as described above, except that the reaction was stopped with trichloroacetic acid (5%, w/v, final concn.). After filtration through fibre-glass filter paper the solution was neutralized with NaOH. It was then extracted with ether as described above.

Results

Isoliquiritigenin has a major u.v.-absorption peak at 386nm in aqueous solution at pH7.5. When horseradish peroxidase and hydrogen peroxide were added the peak declined in intensity, and was replaced by another peak at 335nm. The two peaks had an isosbestic point at 349nm (Fig. 1). When the time-course of the decline in absorption at 386nm

was followed, the reaction was seen to comprise a lag phase followed by a linear phase (Fig. 2). During the reaction, oxygen was consumed, and the reaction course observed with an oxygen electrode also showed a lag phase and a linear phase. Under the standard assay conditions, the flavanone isomer of isoliquiritigenin (liquiritigenin) was not affected by the enzyme.

The reaction with isoliquiritigenin could be observed over the range pH5–10. Owing to the ionization of the substrate, however, the wavelengths of the peaks and of the isosbestic point were not constant over this range. Both the length of the lag phase and the rate of the linear phase of the reaction were affected by alterations of pH (Figs. 3 and 4). The pH optimum (7.5) for the linear phase of the reaction was markedly more alkaline than the optimum for the oxidation of guaiacol by the enzyme, which was found to be at pH6.5. Fig. 5 shows that isoliquiritigenin becomes appreciably ionized only at pH7.5. The ionization of the 4-hydroxyl group would be expected to have the lowest pK value; possibly the ionization of this group is necessary for the reaction to take place.

Fig. 2. Time-course of removal of isoliquiritigenin by peroxidase and hydrogen peroxide

A typical experimental recording of the decline in E_{386} is shown. Peroxidase was added at A, hydrogen peroxide at B; the experimental conditions were as described in Fig. 1.

Fig. 3. Rate of the linear phase of the reaction at different pH values

Isoliquiritigenin ($10\mu\text{g/ml}$) was dissolved in a buffer which was 25mM in respect of each of H_3BO_3 , NaH_2PO_4 and maleic acid. The solution was adjusted to various values in the range pH5–10 with NaOH. The linear rate of decline in extinction at the absorption maximum of the substrate after the addition of peroxidase (0.3ng/ml) and hydrogen peroxide ($85\mu\text{M}$) was measured, and plotted as substrate consumed $\text{min}^{-1} \cdot \text{ml}^{-1}$.

Fig. 4. Length of lag phase plotted as a function of pH

Experimental details were as described in Fig. 3.

Stoichiometry

When the concentration of hydrogen peroxide exceeded that of substrate, one atom of oxygen was consumed for every molecule of isoliquiritigenin metabolized (Fig. 6). In the absence of oxygen no reaction could be observed. When sufficient peroxidase was present to cause a rapid rate of reaction, only catalytic quantities of hydrogen peroxide were required. For example, when $0.1\text{ }\mu\text{g}$ of peroxidase was added to $1\text{ }\mu\text{mol}$ of isoliquiritigenin (dissolved in 3 ml of buffer), only 50 nmol of hydrogen peroxide was necessary for complete removal of the chalcone. Under these conditions, however, an increase in oxygen consumption was observed; approx. 30% more than the stoichiometric amount was taken up. In the complete absence of hydrogen peroxide no reaction took place.

Effects of inhibitors

Potassium cyanide (final concn. 0.1 mM) brought about 98% inhibition of the reaction as observed either spectrophotometrically or by the oxygen electrode. Catalase ($1\text{ }\mu\text{g/ml}$) completely inhibited the reaction. Between the time of addition of this enzyme and the time of complete abolition of oxygen uptake or change in E_{386} there was always an interval of 1–2 min. Carbon monoxide did not appear to inhibit the reaction. Provided that the amount of gas bubbled was not so great that more than 40% of the

Fig. 5. Effect of pH on the position of the u.v.-absorption peak of isoliquiritigenin

The buffer system used is described in Fig. 3. Wavelength values take the form of a titration curve.

dissolved oxygen was removed from solution (as measured with an oxygen electrode), no significant decrease in the rate of the reaction occurred. This experiment was conducted in the dark.

Reduction of hydrogen acceptors

In peroxidase-catalysed reactions in which oxygen is consumed, reducing free radicals may be formed. In the absence of oxygen these may reduce hydrogen acceptors such as Methylene Blue or cytochrome *c* (Yamazaki & Piette, 1963). To test for the production of reducing free radicals from isoliquiritigenin by peroxidase, we mixed cytochrome *c* or Methylene Blue with peroxidase, isoliquiritigenin and hydrogen peroxide under anaerobic conditions. Neither cytochrome *c* nor Methylene Blue was reduced under these conditions. When oxygen was dissolved in the reaction mixture, the chalcone was rapidly metabolized, but again no reduction of the hydrogen acceptors could be observed. It should be noted that Methylene Blue appeared to react with isoliquiritigenin to produce a green precipitate. However, sufficient of each compound remained in solution (as judged by their absorption spectra) for the above experiments.

Products of the reaction

The changes in the u.v.-absorption spectrum which occurred during the reaction (Fig. 1) suggested that either it was a simple reaction or, if several products were produced, they all had similar absorption characteristics. Chromatographic analysis of

Fig. 6. *Stoichiometry of oxygen uptake*

Solutions of isoliquiritigenin of various concentrations were treated with peroxidase (40 ng/ml) and hydrogen peroxide (0.6 mM) in the oxygen-electrode assay. The amount of oxygen consumed as the reaction went rapidly to completion was plotted against the original concentration of substrate, on a log-log graph. The points are the experimentally determined values; the line drawn shows the result that would be expected for an oxygen consumption of one atom for every molecule of substrate.

the reaction products by t.l.c. on cellulose, with 30% (v/v) acetic acid as developing solvent, produced four bands of u.v.-absorbing material. The characteristics of these bands (A–D) are described in Table 1. Two of them (A and B) had similar absorption spectra, and together they always represented over 70% of the u.v.-absorbing material on the chromatograms, although the actual amount was somewhat variable. Elution and rechromatography of either of these bands gave rise to the other two bands (C and D) as artifacts. Bands C and D had different absorption spectra from bands A and B (Table 1). These observations indicated that the contents of bands A and B were the actual reaction products, and that they were partially converted into the contents of bands C and D during the manipulations of chromatography.

The contents of band C were not identified. However, band D contained 4',7-dihydroxyflavonol (II), which was recognized by its u.v.-absorption spectrum and fluorescence properties. The identification was confirmed by co-chromatography with the synthetic compound in three solvent systems. The simplest

explanation for the ready formation of this compound from the contents of bands A and B is that these bands contain diastereoisomers of 3,4',7-trihydroxyflavonone (garbanzol; III). This is known to be readily oxidized to 4',7-dihydroxyflavonol as an artifact of chromatography (Wong, 1965). Garbanzol may therefore be an immediate product of the oxidation of isoliquiritigenin catalysed by peroxidase.

Treatment of either band A or B with dilute acid or alkali yielded 4',6-dihydroxyaurone (IV). This indicated that, as well as garbanzol, these two bands contained diastereoisomers of 4',6-dihydroxy-2-(α -hydroxybenzyl)coumaranone (V). This compound, which was first described by Wong (1967), readily dehydrates to form the aurone (IV) on treatment with acid or alkali. It appears therefore to be another product of the reaction.

Band B contained a small amount of the flavanone isomer of isoliquiritigenin (VI). This was detected with the borohydride spray reagent of Eigen *et al.* (1957). It was always present as a contaminant of chalcone solutions and was not produced enzymically.

The results in Table 1 are consistent with the evidence presented above that garbanzol and 4',6-dihydroxy-2-(α -hydroxybenzyl)coumaranone are products of the oxidation of isoliquiritigenin by peroxidase and hydrogen peroxide. Both compounds have similar absorption spectra with peaks at about 275 nm and 311–320 nm in organic solvents (Wong *et al.*, 1965; Wong, 1967). The spectra of bands A and B are similar to this (Table 1). In addition the R_F values of bands A and B are similar to those given by Wong (1967) for the diastereoisomers of the coumaranone derivative (V), and that of band B is similar to the value given by Wong *et al.* (1965) for garbanzol. Finally, the observed stoichiometry of oxygen and chalcone consumption is consistent with the formation of these two compounds in the reaction (Fig. 6). However, Wong & Wilson (1972) have presented evidence that other products are formed in the peroxidase-catalysed oxidation of chalcone.

Reaction of cell-free extracts of Phaseolus vulgaris with isoliquiritigenin

The reaction catalysed by gel-filtered cell-free extracts of *Phaseolus vulgaris* hypocotyls appeared similar to that catalysed by crystalline peroxidase. The reaction course measured by the spectrophotometer and the oxygen electrode was the same. The extracts contained sufficient endogenous hydrogen peroxide for reaction to be observed without any more being added. The reaction was inhibited by both catalase and potassium cyanide in the same way as the reaction with crystalline enzyme, and chromatography of the reaction products gave comparable chromatograms.

Table 1. *Characteristics of bands of u.v.-absorbing material on chromatograms*

The fluorescence characteristics are given as the appearance of the bands on the chromatogram observed by long-wavelength u.v. light. The changes in colour caused by exposing the chromatogram to NH_3 vapour are indicated by arrows (n.v. = not visible; b = bright; Y = yellow; G = green; R = red; Bn = brown; B = blue). The u.v.-absorption spectra were determined on eluates of the bands in aq. 85% (v/v) ethanol (sh = shoulder). There was no other significant amount of u.v.-absorbing material on the chromatograms.

Band	R_F	Fluorescence characteristics	U.v.-absorption maxima (nm)
A	0.80	n.v. $\rightarrow$ bYG	278, 318
B	0.69	n.v. $\rightarrow$ bYG	277, 317
C	0.47	RBn $\rightarrow$ B	324, 342 (sh)
D	0.25	bYG	261, 320, 357

Discussion

Wong (1965) has described the incorporation by cell-free extracts of *Cicer arietinum* of radioactively labelled isoliquiritigenin (I) into garbanzol (III), which may spontaneously oxidize to 4',7-dihydroxyflavonol (II). He has also shown the formation of diastereoisomers of 4',6-dihydroxy-2-(α -hydroxybenzyl)coumaranone (V) from isoliquiritigenin by cell-free extracts from *Soja hispida* (Wong, 1967). The coumaranone readily dehydrates in dilute acid or alkali to form 4',6-dihydroxyaurone (IV). In this paper we have presented chromatographic evidence that the conversion of the chalcone into garbanzol and the coumaranone can be catalysed by crystalline horseradish peroxidase in the presence of hydrogen peroxide. An identical reaction is catalysed by cell-free extracts of *Phaseolus vulgaris* hypocotyls. It seems probable therefore that the transformations observed by Wong (1965, 1967) and by Wong & Grisebach (1969) were catalysed by peroxidase in the cell-free extracts. Peroxidase thus has the ability to bring about the incorporation of oxygen into the chalcone molecule, a reaction that could be of physiological significance since the flavonol and aurone are compounds of natural occurrence.

Peroxidase is believed to catalyse the 1-equivalent oxidation of electron donors (Saunders *et al.*, 1964). The nature of the reaction products depends on the course of the subsequent free-radical reaction. When oxygen is consumed, it is thought that the first-formed free radical reacts with molecular oxygen and initiates a chain reaction (Yamazaki & Piette, 1963). Free-radical reactions often give rise to products that are complex mixtures of compounds. A mixture of different products appears to be produced in the reaction described here.

A free-radical reaction of the type postulated by Yamazaki & Piette (1963) requires that the substrate be 'redogenic', i.e. capable of oxidation by two 1-equivalent steps. Redogenic substrates are believed to be converted by peroxidase into reducing free

radicals. These are able to reduce molecular oxygen to form perhydroxyl radicals. In the absence of oxygen, the substrate free radicals may instead reduce other hydrogen acceptors such as cytochrome c or Methylene Blue. In the present reaction we were unable to observe the reduction of either of these acceptors under anaerobic conditions. We conclude therefore that isoliquiritigenin is not redogenic. Further, no reduction of the acceptors was observed when the experiments were performed under aerobic conditions. This provides evidence that perhydroxyl radicals are not produced in the reaction, since it has been shown by McCord & Fridovich (1969) that they are capable of reducing cytochrome c.

It has been suggested that in the oxidation of indolylacetic acid by peroxidase the first-formed free radical may react directly with molecular oxygen (Hinman & Lang, 1965). If this is true in the present reaction, two types of complex between free radical and oxygen may be formed to account for the two different products that have been recognized. The subsequent reaction must in any case be complicated since, to fit the observed stoichiometry, the complex between free radical and oxygen must decompose in such a way as to yield the product and generate one half-molecule of hydrogen peroxide.

Alternative mechanisms, which do not involve free-radical chain reactions, have been postulated for peroxidase-catalysed reactions in which oxygen is consumed (Nicholls, 1962). For example, Lemberg & Legge (1949) suggested that substrates could be oxidized by direct transfer of molecular oxygen from an oxygenated ferropoxidase. Oxyferropoxidase may be formed during the present reaction, as it is in other peroxidase-catalysed reactions in which oxygen is consumed (Yamazaki & Piette, 1963). Our failure to observe inhibition by carbon monoxide suggests, however, that if it is formed, it is not an important part of the mechanism.

The oxidation of chalcones and flavanones by alkaline permanganate and Fenton's reagent may

Scheme 1. Possible relations of compounds discussed in the text

Unbroken arrows indicate reactions catalysed by peroxidase; broken arrows indicate non-enzymic transformations.

resemble the reaction described here, since free-radical reactions are involved. Wong (1966) obtained 4',6-dihydroxyaurone (IV) from isoliquiritigenin by oxidation with alkaline permanganate. However, Pelter *et al.* (1971) obtained 4',7-dihydroxyflavone from both isoliquiritigenin and liquiritigenin (VI) by using this oxidizing agent. Mahesh & Seshadri (1955) found that Fenton's reagent oxidized the flavanone naringenin to the corresponding 3-hydroxyflavanone and a 3,3-linked flavanone dimer. Thus in some cases these oxidizing agents yield products similar to those obtained in the present work.

Hence, although the details are obscure, the oxidation of isoliquiritigenin catalysed by peroxidase in the presence of hydrogen peroxide is likely to be a free-radical reaction. Peroxidase-catalysed free-radical reactions often yield a diversity of compounds as products (Saunders *et al.*, 1964). This is true for the present reaction. However, a characteristic of flavonoid metabolism is that a diversity of structural forms exists. Pelter *et al.* (1971) have suggested free-radical mechanisms for several important flavonoid transformations. Therefore it seems reasonable to

postulate, not only that the present reaction is of significance in the intact plant, but also that many of the oxidative transformations of flavonoid and isoflavonoid compounds that occur in plants are catalysed by an enzyme of low specificity, namely peroxidase.

Lignin is believed to be synthesized from phenolic precursors by the activity of an enzyme that catalyses a 1-equivalent oxidation, and peroxidase has been suggested to be the enzyme involved (Seigel, 1968). The oxidative transformations of flavonoids and the production of lignin may therefore be catalysed by the same enzyme. In this connexion it should be noted that there is a high correlation between the occurrence of lignin and the occurrence of flavonoids in the plant kingdom (Bate-Smith, 1963).

The hypothesis that peroxidase is involved in the oxidative transformations of flavonoids could help to explain their sporadic occurrence throughout the plant kingdom. Certain classes of flavonoid that are of limited taxonomic distribution (for example aurones) are found in completely unrelated families. Although the enzymic potential for the synthesis of

certain classes of flavonoid exists in all plants in the form of peroxidase, the control of the expression of that potential would then be dependent on factors such as the interaction of the enzyme with other cellular components, and the availability of appropriate substrates. Novel control systems would also be required to explain the observation that some flavonoids may apparently be synthesized independently of others in the same plant. For example, we have shown that the synthesis of the isoflavonoid derivatives phaseollin and coumestrol in *Phaseolus vulgaris* can take place without appreciable influence on the production of other flavonoids (Rathmell & Bendall, 1971).

A further difficulty of the hypothesis is the observation that some flavonoids of natural occurrence (for example flavanones) show optical activity, although some of the most frequently occurring types (e.g. flavonols) do not have an asymmetric carbon atom. A free-radical reaction that took place in solution would not be expected to give rise to optically active products. However, if the reaction took place at the enzyme surface or if the products of the reaction became the substrates of other enzyme-catalysed reactions, optical activity could result.

We therefore suggest that the observations reported here provide a reasonable basis for the hypothesis that peroxidase, or a peroxidase-like enzyme, may be of importance in the oxidative anabolism of flavonoids.

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References

- Barton, G. M., Evans, R. S. & Gardner, J. A. F. (1952) *Nature (London)* **170**, 249
 Bate-Smith, E. C. (1963) in *Chemical Plant Taxonomy* (Swain, T., ed.), p. 127, Academic Press, London
 Eigen, E., Blitz, M. & Gunsberg, E. (1957) *Arch. Biochem. Biophys.* **68**, 501
 Geissman, T. A. & Clinton, R. O. (1946) *J. Amer. Chem. Soc.* **68**, 697
 Hinman, R. L. & Lang, J. (1965) *Biochemistry* **4**, 144
 Lemberg, R. & Legge, J. W. (1949) *Hematin Compounds and Bile Pigments*, p. 419, Interscience, New York
 Mahesh, V. B. & Seshadri, T. R. (1955) *J. Chem. Soc. London* 2503
 McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049
 Nicholls, P. (1962) in *Oxygenases* (Hayaishi, O., ed.), p. 273, Academic Press, London
 Pelter, A., Bradshaw, J. & Warren, R. F. (1971) *Phytochemistry* **10**, 835
 Rathmell, W. G. & Bendall, D. S. (1971) *Physiol. Plant Pathol.* **1**, 351
 Rathmell, W. G. & Bendall, D. S. (1972) *Phytochemistry* **11**, 873
 Saunders, B. C., Holmes-Siedle, A. G. & Stark, B. P. (1964) *Peroxidase*, Butterworths, London
 Seigel, S. M. (1968) *Compr. Biochem.* **26A**, 1
 Wong, E. (1965) *Biochim. Biophys. Acta* **111**, 358
 Wong, E. (1966) *Phytochemistry* **5**, 463
 Wong, E. (1967) *Phytochemistry* **6**, 1227
 Wong, E. & Grisebach, H. (1969) *Phytochemistry* **8**, 1419
 Wong, E. & Wilson, J. M. (1972) *Phytochemistry* **11**, 875
 Wong, E., Mortimer, P. I. & Geissman, T. A. (1965) *Phytochemistry* **4**, 89
 Yamazaki, I. & Piette, L. H. (1963) *Biochim. Biophys. Acta* **77**, 47