3. The relation between the dye concentration and the apparent value of the Michaelis constant is not linear. It resembles the function entailed by the 'apparent competitive' inhibition mechanism of Segal *et al.* (1952), but on analysis is found to deviate significantly from it.

4. An inhibition mechanism is proposed which accounts for the experimental results, including the relation between the inhibitions of the hydrolyses of two different substrates.

5. Distribution experiments and freezing-point measurements show that rhodamine B is monomeric in the aqueous solutions of pH 7·4 used for the enzyme studies.

6. Measurements of the polarization of fluorescence demonstrate the reversible combination of rhodamine with large molecules in the enzyme preparation. The combination is accompanied by a change in absorption spectrum and an increase in fluorescent intensity.

7. Displacement of the dye from combination by ethyl acetate gives evidence that the binding is specifically related to the esterase inhibition.

8. The fluorescence data are analysed quantitatively, and their relation to the inhibition results is discussed.

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The Oxidation of Indolyl-3-Acetic Acid by Waxpod Bean Root Sap and Peroxidase Systems

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The presence in extracts of certain plant tissues of systems which oxidize indolyl-3-acetic acid (IAA) is well established (e.g. by Tang & Bonner, 1947, 1948; Wagenknecht & Burris, 1950; Gortner & Kent, 1953). The enzyme system present in extracts of etiolated pea epicotyls has been most studied, and it has been suggested that the system consists of a flavoprotein which gives rise to hydrogen peroxide and a peroxidase which catalyses the oxidation of IAA by hydrogen peroxide (cf. Galston, Bonner & Baker, 1953). The enzyme in extracts of waxpod bean roots has received little attention, although according to Wagenknecht & Burris (1950) such extracts show greater activity towards IAA than extracts of etiolated pea epicotyls. Wagenknecht & Burris (1950) have shown that the rate of oxidation of IAA by dialysed extracts of waxpod bean roots is increased in the presence of suitable amounts of Mn^{2+}. It has been shown by Galston *et al.* (1953) that horseradish peroxidase preparations catalyse the oxidation of IAA in the absence of added hydrogen peroxide, and Kenten (1953) has reported that the rate of this oxidation is stimulated in the presence of Mn^{2+}. These facts suggested that the system peroxidase–Mn^{2+} might be responsible for the activity of extracts of waxpod bean roots towards IAA, and the present work was undertaken to investigate this possibility.

MATERIALS AND METHODS

Plant sap. The waxpod beans (*Phaseolus vulgaris*, L., var. Sutton's Golden Waxpod) were glasshouse-grown in a John Innes potting compost. They were harvested 4–8 weeks after sowing. The rest of the plants were found growing wild or in gardens in the neighbourhood of Harpenden. The plant material was washed free from soil, and either ground in a mortar with sand or passed through a domestic meat mincer. The pulp was squeezed by hand through strong cotton cloth, the sap centrifuged for 15–20 min. at 6000 rev./min., the precipitate discarded and the supernatant stored at 0°.

Peroxidase. Peroxidase preparations were made from horseradish root by the method of Kenten & Mann (1954). The purpurogallin number (P.N.) of the preparations was estimated by the method of Keilin & Hartree (1951). The most highly purified preparation used had a P.N. of 1040 and a haemin content of 1·12%. In the text the peroxidase activity of some preparations is given as enzyme units (E.U.) (Keilin & Hartree, 1951). An amount of preparation containing 1 E.U. would form 1 g. of purpurogallin under Keilin & Hartree's conditions. With a dry preparation the number of E.U. = P.N. × wt. of the preparation in g.

Catalase. This was prepared from horse liver by a method based on that of Agner (1938) and the activity of the preparation was estimated as described previously (Kenten & Mann, 1952). The Katalasefahigkeit (Kat.f.) was 5000. Using the equation of Chance & Herbert (1950) that Kat.f. $= 520k_1'/M$, where M is the molecular weight of catalase (230000), the velocity constant of the preparation, $k_1' = 2{\cdot}2 \times 10^6 \text{M}^{-1}$ sec.$^{-1}$. For each experiment a sample of the preparation was suspended in 0·5% (w/v) NaCl (10 mg./ml.) and the insoluble material removed by centrifuging.

D-*Amino acid oxidase.* This was prepared from sheep kidney. The method of Negelein & Brömel (1939) was followed as far as the first precipitation with $(NH_4)_2SO_4$. This precipitate was dissolved in 0·003M pyrophosphate, pH 8·5, and reprecipitated by the addition of 30 g. $(NH_4)_2SO_4$/100 ml. The precipitate was separated by centrifuging and dissolved in 0·003M pyrophosphate at pH 8·3. 1 ml. of this preparation catalysed the uptake of approximately 100 μl. O_2/10 min. with DL-alanine as substrate under the conditions of Negelein & Brömel (1939).

IAA solutions. Indolyl-3-acetic acid solutions were adjusted to the appropriate pH (usually pH 6) with 5% (w/v) NH_3 and stored in the dark at 0°.

Manometric experiments. These were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated. The formation of CO_2 was measured by Warburg's direct method (Dixon, 1943). For all the experiments the apparatus was covered by a hood; this considerably reduced the amount of light reaching the reaction mixtures. While light was not completely excluded, the intensity of illumination at the surface of the bath was always less than 1 ft. candle.

EXPERIMENTAL AND RESULTS

Manometric studies of the oxidation of IAA by waxpod bean root sap

Wagenknecht & Burris (1950) showed that waxpod bean root sap lost about one-third of its activity towards IAA after dialysing for 24 hr. Using suspensions of ground pea epicotyls, Galston & Baker (1951) reported large increases in activity following dialysis, whereas Goldacre, Galston & Weintraub (1953) found a partial loss of activity, which was restored by the addition of dialysate. In the present work with waxpod bean root sap a loss of activity towards IAA has always been found following dialysis, and the results suggest that this decrease is due to the loss of a thermostable fraction. By ultrafiltration the IAA-oxidizing system in waxpod bean root sap can be separated into a thermolabile fraction and a thermostable fraction. The thermolabile fraction alone oxidizes IAA but the rate of oxidation is increased in the presence of the thermostable fraction. The thermolabile fraction can be replaced by horseradish peroxidase preparations.

The effect of ultrafiltration. Centrifuged waxpod bean root sap (15 ml.) was filtered for 24 hr. in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947). In this time nearly all the liquid passed through, leaving a small pasty residue inside the cellophan sac. The residue was suspended in 15 ml. of water. The O_2 uptake brought about by the addition of these fractions to IAA is shown in Fig. 1. While the ultrafiltrate alone showed negligible activity and the residue alone about half the activity of the sap, together they gave an activity only slightly less than the sap. The activating effect of the ultrafiltrate was not affected by previous heating at 100° for 15 min., whereas only slight activity remained in the residue after similar heating.

Replacement of waxpod bean root residue with horseradish peroxidase preparations. The waxpod bean root residue has high peroxidase activity;

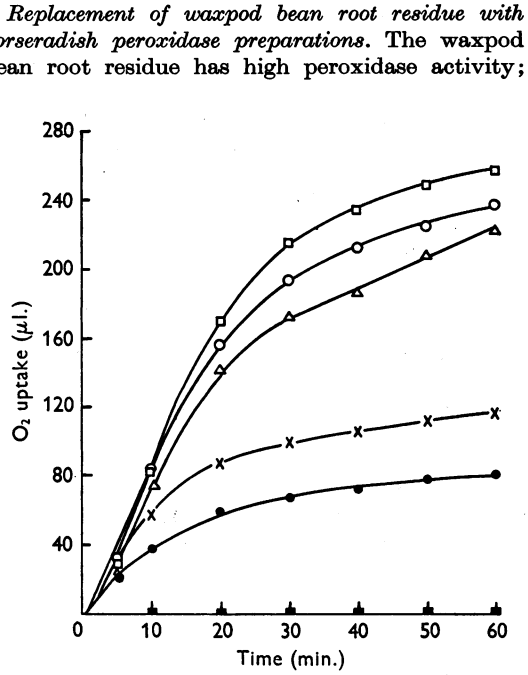

Fig. 1. Effect of ultrafiltration on the rate of oxidation of IAA by waxpod bean root sap. Reaction mixtures in 0·067M phosphate at pH 6 and 0·3 ml. 0·05M IAA added from the side arm at zero time, temp. 25°; gas phase, air. ○—○, 0·5 ml. sap.; ×—×, 0·5 ml. residue (0·17 E.U./ml.); ■—■, 0·5 ml. ultrafiltrate; △—△, 0·5 ml. residue +0·5 ml. ultrafiltrate; ●—●, 85 μg. horseradish peroxidase of P.N. 1040; □—□, 85 μg. horseradish peroxidase of P.N. 1040 + 0·5 ultrafiltrate. The O_2 uptakes are corrected for the uptake in absence of IAA.

under the standard conditions (Keilin & Hartree, 1951) with pyrogallol as substrate, the activity of different preparations varied from 0·045 to 0·19 E.U./ml. The effect of replacing residue with an amount of a horseradish peroxidase preparation was accordingly tested. The O_2 uptakes are shown in Fig. 1. In this experiment the amount of horseradish peroxidase used gave a lower rate of uptake than did waxpod bean root residue, but its activity was raised above that of sap by adding ultrafiltrate.

This experiment suggests that peroxidase together with some thermostable factor or factors of small molecular weight form a powerful IAA oxidase system. However, since Galston *et al.* (1953) have suggested that the IAA-oxidizing system in extracts of pea epicotyls consists of a flavoprotein–peroxidase system, it might be argued that the peroxidase preparation used was contaminated with flavoprotein or with the protein moiety of a flavoprotein, and that the function of the ultrafiltrate was to provide flavoprotein substrate or flavins respectively, and that in effect a flavoprotein–peroxidase system was being reconstituted. The evidence obtained by Kenten & Mann (1954) suggests that the peroxidase preparation of P.N. 1040 which was used is about 90% peroxidase. Furthermore, whereas riboflavin or flavin mononucleotide could be readily detected at a concentration of 0·1 μg./ml. by the appearance of a yellow fluorescence in ultraviolet light, when a solution of horseradish peroxidase of P.N. 1040 (1 mg./ml.) was heat denatured and centrifuged, no yellow fluorescence appeared when the supernatant was exposed to ultraviolet light. Galston *et al.* (1953) made use of the technique suggested by Warburg & Christian (1938) for the removal of the prosthetic group of flavoproteins. By treating an IAA oxidase preparation from pea epicotyls with $(NH_4)_2SO_4$ at pH 2·5 they obtained two fractions; a protein which precipitated at 0·22 saturation, and a soluble fraction containing peroxidase and flavins. Neither of these fractions was significantly active towards IAA, but when mixed they readily oxidized IAA. The peroxidase which was used to replace the non-filtrable residue in the experiment described above was accordingly treated in a similar fashion.

A solution of peroxidase, 10 ml., containing 1 mg. peroxidase of P.N. 1040/ml., was cooled in ice water to 0°, and saturated $(NH_4)_2SO_4$ in water at 0° and 0·1 N-H_2SO_4 added until the pH was 2·5, and the solution 0·25 saturated with respect to $(NH_4)_2SO_4$ at 0°. After 30 min. at 0° the solution was centrifuged but yielded only a trace of grey precipitate. The supernatant was then adjusted to pH 6 with N-NaOH after the addition of 1 ml. 0·2 M-phosphate, pH 6, and dialysed overnight against water. The dialysed solution was centrifuged and a trace of yellow precipitate was obtained and discarded. Activity measurements on the supernatant showed that 75% of the peroxidase activity had been recovered. The activity of this acid–$(NH_4)_2SO_4$–treated peroxidase was compared with the activity of the untreated peroxidase and the results are given in Table 1. The results obtained with a crude horseradish peroxidase of P.N. 130 are also included in this table. There is little difference between the activities of the three peroxidases in the presence of the ultrafiltrate but in the absence of the ultrafiltrate the acid-treated peroxidase was slightly less active than was the untreated peroxidases.

Under the conditions of Table 1 the presence of 5 or 50 μg. of either riboflavin or flavin mononucleotide did not affect the rate at which the acid–$(NH_4)_2SO_4$-treated peroxidase oxidized IAA. A preparation of flavin-adenine dinucleotide (FAD) was not available, but the addition of 1 ml. boiled D-amino acid oxidase preparation (the prosthetic group of this oxidase is FAD) was also without effect on the rate of oxidation of IAA by the treated peroxidase.

The results suggest that the activity of ultrafiltrate is not due to the presence of flavin compounds. Also, the fact that the $(NH_4)_2SO_4$–acid treatment has comparatively little effect on the activity of the purified peroxidase suggests that the observed activity is due to peroxidase and not to peroxidase plus flavoprotein.

Effect of varying the concentration of bean root residue, horseradish peroxidase and ultrafiltrate. The effect of varying the amounts of bean root residue, peroxidase and ultrafiltrate on the rate of O_2 uptake by solutions of IAA is shown in Table 2 and Fig. 2.

In the experiment given in Table 2 the activity of the bean root residue alone is less than that of an amount of highly purified peroxidase having the same peroxidase activity. This was not invariably the case; the activity of different batches of bean

Table 1. *Effect of acid–ammonium sulphate treatment on the activity of a highly purified horseradish peroxidase preparation*

Reaction mixtures consisted of: in the main vessel horseradish peroxidase preparation equivalent to 0·08 E.U., 0·067 M phosphate at pH 6, and, where present, 0·5 ml. waxpod bean root ultrafiltrate. After equilibration, 0·3 ml. of 0·05 M IAA was added from the side arm. The O_2 uptakes obtained when IAA or peroxidase was omitted were negligible.

Peroxidase	P.N.	Uptake of O_2 (μl. in 15 min.) Alone	Ultrafiltrate added
Horseradish	1040	62·5	155·5
Horseradish, acid–$(NH_4)_2SO_4$ treated	1040	45·5	139·5
Horseradish	130	57·5	140·5

Table 2. *Effect of varying the amount of peroxidase and waxpod bean root residue in the presence of a constant amount of ultrafiltrate*

Reaction mixtures consisted of varying amounts of horseradish peroxidase of P.N. 1040 and bean root residue, and where present, either 0·4 or 0·8 ml. ultrafiltrate in 0·067 M phosphate at pH 6. After equilibration, 0·3 ml. 0·05 M IAA was added from the side arm. All the O_2 uptakes recorded were obtained using the same batch of ultrafiltrate. The O_2 uptakes are corrected for the blank O_2 uptakes in mixtures from which IAA was omitted. These were less than 7 μl. in 30 min.

Horseradish peroxidase (E.U. $\times 10^{-3}$ present)	Waxpod bean root residue (E.U. $\times 10^{-3}$ present)	O_2 uptake (μl./30 min.)			Increase due to presence of ultrafiltrate (ml.)	
		No ultrafiltrate	Ultrafiltrate added (ml.) 0·4	0·8	0·4	0·8
0·5	—	0	23·5	45·5	23·5	45·5
2·0	—	0	61	88	61	88
8·0	—	3	79·5	132	76·5	129
16	—	9·5	113·5	174	104	164·5
32	—	29·5	128·5	199	99	169·5
64	—	57·5	160	228·5	102·5	171
—	2·0	1·5	4·5	12·5	3	11
—	8·0	0	18	33·5	18	33·5
—	16	0	37·5	58·5	37·5	58·5
—	32	7	61	92	54	85
—	64	20	85	138	65	118

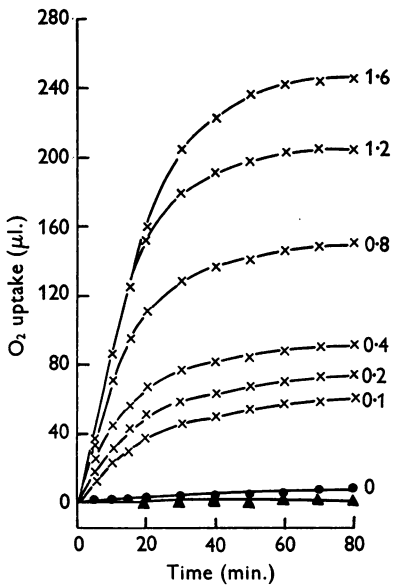

Fig. 2. Effect of variation in the concentration of ultrafiltrate on the rate of oxidation of IAA by a horseradish peroxidase preparation of P.N. 1040. Reaction mixtures consisted of 8 μg. horseradish peroxidase of P.N. 1040, 0·067 M phosphate at pH 6 and varying amounts of waxpod bean root ultrafiltrate (ml. added denoted by the number to the right of each curve). After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. ●—●, peroxidase; ▲—▲, 1·6 ml. ultrafiltrate; ×—×, peroxidase + ultrafiltrate.

root residue varied considerably, and sometimes had a greater activity than an equivalent amount of horseradish peroxidase. One of the reasons for this variation may have been variation in the degree of contamination of the bean root residue by the natural activating factor(s).

The ultrafiltrate alone was always inactive (< 10 μl. O_2/ml./hr. with IAA), and while it invariably increased the activity of bean root residue and horseradish peroxidase towards IAA, its capacity in this respect varied widely. With small amounts of bean root residue alone or peroxidase alone the activity is negligible, but if suitable amounts of ultrafiltrate are also present the oxidation of IAA proceeds readily (Fig. 2). With any one preparation of ultrafiltrate, and using an amount of horseradish peroxidase equivalent to the peroxidase activity of bean root residue, the system, residue + ultrafiltrate, was less active than horseradish peroxidase + ultrafiltrate. This suggests that waxpod bean root peroxidase catalyses the oxidation of IAA less readily than does horseradish peroxidase.

In the presence of a constant amount of ultrafiltrate (0·4 or 0·8 ml.), increasing the amount of horseradish peroxidase from 0–16 μg. increases the activity of the system, but with amounts of peroxidase greater than 16 μg. the further increase in activity can be accounted for by the activity of peroxidase alone (Table 2). Increasing the amount of ultrafiltrate in the presence of a constant amount of horseradish peroxidase increases the activity of the system (Fig. 2), although under the conditions of the experiment there is little difference in the initial rate of oxidation in those cases where 1·2 and 1·6 ml. of ultrafiltrate are present. It can be seen that the effect of the ultrafiltrate is largely restricted to the initial 30–40 min. of the reaction period, after which the reaction practically ceases, although with the smaller amounts of ultrafiltrate less than half the added IAA has been oxidized. The effect of

making a second addition of ultrafiltrate or peroxidase after the initial rapid phase of the reaction was complete is shown in Table 3. It is clear that while a second addition of ultrafiltrate has only a comparatively small effect, a second addition of peroxidase causes a further O_2 uptake only slightly less than that obtained initially. This suggests that the peroxidase, not the ultrafiltrate factors, is inactivated during the oxidation of IAA. Incubation of peroxidase with IAA for 1 hr., under conditions where little oxidation of IAA takes place, does lead to small loss of peroxidase activity, since on adding ultrafiltrate the reaction proceeds at a slightly slower rate than that found with mixtures in which the peroxidase has not been incubated with IAA. Peroxidase estimations on initial and final reaction mixtures of similar composition to those used in the experiments given in Table 3, showed that where peroxidase had been incubated with IAA there was only a small loss of peroxidase activity (about 30 %) in the solution, but where bean root ultrafiltrate was also present and a large amount of IAA had been oxidized there was a large loss of peroxidase activity (about 90–95 %). Control reaction mixtures showed that 8 μg. peroxidase incubated at 25° with phosphate at pH 6 either in the presence or the absence of the ultrafiltrate lost about 15 % of its activity in 1 hr. It would appear, therefore, that the activity of the ultrafiltrate is not a protection of the peroxidase from inactivation during the oxidation of IAA. The loss of peroxidase activity would appear to be a consequence of the oxidation of IAA and could be due to the formation of toxic or inhibiting oxidation products.

Although the results with bean root residue and horseradish peroxidase differ quantitatively, they are essentially similar, and suggest that the activity of the waxpod bean root sap towards IAA could be due to peroxidase plus thermostable factors.

Table 3. *Loss of peroxidase activity during the oxidation of IAA*

Reaction mixtures consisted of: in the main vessel 0·3 ml. 0·05 M IAA in 0·067 M phosphate at pH 6 and where present 0·3 ml. waxpod bean root ultrafiltrate. After equilibration 8 μg. horseradish peroxidase of P.N. 1040 in 0·1 ml. were added from the side arm. After 1 hr. a further addition of peroxidase or ultrafiltrate was made from the second side arm as shown below. Blank O_2 uptakes with IAA omitted or IAA + ultrafiltrate were less than 5 μl. in 2 hr.

Components present with IAA	Uptake of O_2 in first hr. (μl.)	After 1 hr. add	Uptake of O_2 in second hr. (μl.)
Peroxidase	3	—	3·5
	2·5	Peroxidase	4·5
	4·5	Ultrafiltrate	94
Peroxidase + ultrafiltrate	109	—	7
	104·5	Ultrafiltrate	18
	111	Peroxidase	74

The nature of the thermostable factors. Studies of the effect of Mn^{2+} on the peroxidase-catalysed oxidation of IAA by O_2, which will be described later in this paper, show that while Mn^{2+}, under suitable conditions, increases the rate of oxidation, this effect is small compared with that of waxpod bean root ultrafiltrate. The activity of ultrafiltrate cannot, therefore, be due to the presence of Mn^{2+} alone. Goldacre *et al.* (1953) have shown that certain phenols, when present in small amounts, bring about large increases in the activity of pea epicotyl brei towards IAA. These authors, while not excluding the possibility that the active phenols were operating in a catalytic manner by a reversible oxidation–reduction, suggested that they might serve as cofactors. They found that a large number of monophenols, particularly chloromonophenols, were active, but with dihydric phenols no activity could be demonstrated. In this connexion it is of interest that Kenten & Mann (1950) found that monohydric phenols but not di- or tri-hydric phenols (with the exception of resorcinol) were active as carriers in the peroxidase-catalysed oxidation of Mn^{2+} by H_2O_2. The results of Kenten & Mann (1950) suggested that it was the intermediate and not the final oxidation products of the phenols which were active, since prior incubation with peroxidase and H_2O_2 destroyed the activity of the phenols. If the active factor in the ultrafiltrate is acting catalytically, being oxidized by peroxidase and reduced by IAA, then in the absence of IAA it might be oxidized to an irreversible oxidation product.

This was tested in reaction mixtures consisting of: ultrafiltrate, 2·5 ml.; 100 μg. horseradish peroxidase of P.N. 1040; 0·05 M-H_2O_2, 0·4 ml.; and water to a final volume of 4 ml. Control mixtures were made up from which either peroxidase, or H_2O_2, or both peroxidase and H_2O_2 were omitted. After 3 hr. at room temperature 20 μg. catalase in 0·1 ml. were added to decompose the residual H_2O_2 and 10 min. later the solutions were heated at 100° for 20 min. to inactivate the peroxidase and catalase. After cooling, portions of the solutions were tested manometrically under the conditions given in Table 4.

The results show that while the treatment of the ultrafiltrate with H_2O_2 alone or peroxidase alone has little effect on its activity, the treatment with both peroxidase and H_2O_2 present has reduced the O_2 uptake obtained in the first 30 min. by about 60 %. That this effect was not due to the formation of inhibiting substances is shown by the O_2 uptake obtained when solutions from treatments 1 and 2 were both present (Table 4). Increasing the time of the treatment with peroxidase and H_2O_2 or in-

Table 4. *Loss of activity of ultrafiltrate, 2:4:6-trichlorophenol and p-chlorophenol by treatment with peroxidase plus* H_2O_2

Treatments with peroxidase and H_2O_2 as described in the text. Manometric reaction mixtures consisted of: in the main vessel 1 ml. of the treated ultrafiltrate solutions or 0·8 ml. treated chlorophenol solutions or 0·6 or 0·06 ml. untreated ultrafiltrate, 16 μg. horseradish peroxidase of P.N. 1040 and phosphate at pH 6, 0·067 M (ultrafiltrate experiment) and 0·035 M (chlorophenol experiment). After equilibration, 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptake due to peroxidase in the absence of the chlorophenols or ultrafiltrate which was 16 μl.

Treatment or addition	Uptake of O_2 (μl. in 30 min.)		
	Ultrafiltrate	2:4:6-Trichlorophenol	*p*-Chlorophenol
None	123·5	65	98
Peroxidase + H_2O_2	49	6·5	18
H_2O_2 alone	134·5	61·5	76·5
Peroxidase alone	123	72·5	97·5
Equal volumes of solutions from treatments 1 and 2 mixed and 2 ml. (ultrafiltrate experiment) or 1·6 ml. (chlorophenol experiment) tested	161	77	109
Ultrafiltrate 0·6 ml.	124·5	—	—
Ultrafiltrate 0·06 ml.	35	—	—

Table 5. *Activity of phenols, aromatic amines and maleic hydrazide*

Reaction mixtures consisted of: in the main vessel 16 μg. horseradish peroxidase of P.N. 1040 and the substances tested in 0·067 M phosphate at pH 6. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptake due to the action of peroxidase alone on IAA. These blank uptakes varied between 10 and 16 μl. O_2. Blank uptakes when IAA was omitted were negligible.

Concentration (M) ...	Increased uptake in 30 min. (μl. O_2)			
	5×10^{-3}	10^{-3}	10^{-4}	10^{-5}
Substance				
Phenol	38	82·5	42·5	14
p-Chlorophenol	14	62·5	84	44
4-Methylumbelliferone	—	109·5	58	26
Resorcinol	4·5	32·5	79·5	20·5
Aniline	25	45·5	37	15·5
Maleic hydrazide	52·5	8	0	0

Catechol, hydroquinone, pyrogallol and *p*-phenylenediamine inhibited when present in concentrations of 5×10^{-3}–10^{-5} M.

creasing the amounts of peroxidase and H_2O_2 did not result in a significantly larger decrease in the activity of ultrafiltrate. With other batches of ultrafiltrate the loss of activity following treatment with peroxidase plus H_2O_2 varied between 65 and 50 %, based on the O_2 uptake. However, the increase in O_2 uptake is not directly proportional to the amount of ultrafiltrate present. The amount of ultrafiltrate present in the portions of treated ultrafiltrate solutions tested manometrically is equivalent to 0·6 ml., and it is shown in Table 4 that reducing the amount of ultrafiltrate from 0·6 to 0·06 ml. reduces the O_2 uptake by only 70 %. Thus, the results suggest that a large part of the activity of the ultrafiltrate is due to the presence of peroxidase substrates which give rise to inactive products when oxidized by peroxidase and H_2O_2. It is of course possible that the activity remaining in the ultrafiltrate after treatment with peroxidase and H_2O_2 is also due to peroxidase substrates which are not irreversibly oxidized under the conditions used.

The effect of peroxidase substrates on the oxidation of IAA by horseradish peroxidase preparations. In agreement with the suggestion that peroxidase substrates are partially responsible for the activity of bean root ultrafiltrate, it was found that certain monophenols, and also aniline, increased the rate of oxidation of IAA by peroxidase. In addition to a number of known peroxidase substrates, maleic hydrazide, 4-methylumbelliferone and *p*-chlorophenol were also tested, since these compounds are known to increase the activity of pea epicotyl preparations towards IAA (Goldacre *et al.* 1953; Andreae & Andreae, 1953). At the concentration used, resorcinol, aniline, maleic hydrazide and the monohydric phenols increased the activity of the system, while the dihydric and trihydric phenols and *p*-phenylenediamine decreased the activity (Table 5). The results suggest that with *p*-chlorophenol, resorcinol and aniline there is an optimum concentration, and that if these compounds are present in too high a concentration little or no increase in activity is observed. With the exception of maleic hydrazide, 4-methylumbelliferone and *p*-chlorophenol, all of the compounds tested in the experiments recorded in Table 5 are already known to be peroxidase substrates. Colorimetric evidence was obtained that both 4-methylumbelliferone and *p*-chlorophenol are peroxidase substrates, since when peroxidase was added to solutions of these compounds in dilute H_2O_2 at pH 6, the umbelliferone solution went rapidly turbid and finally a white insoluble oxidation product separated, while with *p*-chlorophenol coloured oxidation products

were formed. Also, as will be shown later, the activating capacity of *p*-chlorophenol on the peroxidase-catalysed oxidation of IAA by O_2 is largely destroyed by prior incubation with peroxidase plus H_2O_2. With maleic hydrazide, however, no visual evidence of oxidation by peroxidase plus H_2O_2 at pH 6 was obtained, and its activating capacity was not affected by this treatment.

It is clear from the results that certain peroxidase substrates, when present in catalytic amounts, are capable of increasing the rate of oxidation of IAA by peroxidase. Furthermore, those peroxidase substrates which are active in this respect, namely, the monohydric phenols, aniline, and resorcinol, are also active as carriers in the peroxidase-catalysed oxidation of Mn^{2+} by H_2O_2 (Kenten & Mann, 1950; Kenten & Mann, unpublished observations), while catechol, hydroquinone, pyrogallol and *p*-phenylenediamine, which inhibit the oxidation of IAA, are inactive in the oxidation of Mn^{2+}.

Loss of activity of 2:4:6-*trichlorophenol and* p-*chlorophenol following treatment with peroxidase plus* H_2O_2. Goldacre *et al.* (1953) found that 2:4:6-trichlorophenol activated the oxidation of IAA by dialysed pea epicotyl brei. They considered that this phenol was unlikely to be functioning in an oxidation–reduction system, since it appeared to be incapable of reversible oxidation via an *o*- or *p*-quinone. It was, therefore, considered of interest to test whether prior incubation with H_2O_2 and peroxidase would destroy the activating capacity of this phenol. In addition *p*-chlorophenol was also tested.

The experimental procedure was similar to that previously described for the ultrafiltrate, except that the reaction was allowed to proceed overnight (approximately 18 hr.). The complete reaction mixture consisted of: 0·002 M 2:4:6-trichlorophenol or *p*-chlorophenol, 1 ml.; 100 μg. horseradish peroxidase of P.N. 1040 and 0·05 M-H_2O_2, 0·5 ml., in a total volume of 5 ml., 0·04 M with respect to phosphate at pH 6.

The results (Table 4) show that the treatment, with both peroxidase and H_2O_2 present, has largely destroyed the activity of the phenols. Using *p*-chlorophenol and varying the amount of peroxidase from 50 to 200 μg. and the H_2O_2 from 1 to 0·2 ml., 0·05 M, did not result in a significantly larger decrease in activity than that given in Table 4. The small residual activating capacity of the phenol solutions after treatment with peroxidase plus H_2O_2 is of interest in connexion with the failure to completely inactivate ultrafiltrate by such treatment.

The inhibiting effect of certain peroxidase substrates and the reversal of such inhibition by the addition of H_2O_2. Small amounts of catechol, hydroquinone, pyrogallol, *p*-phenylenediamine and ascorbic acid strongly inhibit the oxidation of IAA by peroxidase plus ultrafiltrate or *p*-chlorophenol. It is shown in Table 6 that the addition of a small amount of H_2O_2 reverses this inhibition. In order to cause complete inhibition in the experiment with the ultrafiltrate it was necessary to use a higher concentration of pyrogallol ($3·3 \times 10^{-5}$ M) than in the experiment with *p*-chlorophenol, where 10^{-5} M was sufficient. Even so, with the ultrafiltrate, pyrogallol only inhibited initially. At the end of 1 hr. an O_2 uptake started and then proceeded with increasing velocity. With *p*-chlorophenol and the lower concentration of pyrogallol no O_2 uptake was observed after 1·5 hr. It is possible that the ultrafiltrate contains a factor which accelerates the autoxidation of pyrogallol or that the ultrafiltrate-containing system produces peroxide more readily than does the *p*-chlorophenol system. Using 10^{-4} M ascorbic acid as an inhibiting peroxidase substrate in place of pyrogallol, and working in citrate at pH 6 to reduce loss of ascorbic acid by autoxidation, essentially similar results were obtained, except that with the ultrafiltrate-containing system at this concentration of ascorbic acid, in the absence of added H_2O_2, the reaction had not started after a 2 hr. period.

Experiments with horseradish root sap. Horseradish root sap, although rich in peroxidase, does not catalyse the oxidation of IAA by O_2. However, if the sap is treated with a suitable amount of H_2O_2

Table 6. *Reversal of pyrogallol inhibition by addition of* H_2O_2

A. The complete reaction mixture consisted of: in the main vessel 16 μg. horseradish peroxidase of P.N. 1040, 0·001 M *p*-chlorophenol, 0·3 ml., and 0·0001 M pyrogallol, 0·3 ml. in 0·067 M phosphate at pH 6. After equilibration, 0·05 M IAA, 0·3 ml., and 0·001 M-H_2O_2, 0·2 ml., were added from the side arm.

B. The complete reaction mixture consisted of the same quantities of reagents as in *A* except that 0·6 ml. ultrafiltrate was used in place of *p*-chlorophenol solution, the amount of pyrogallol was 1 ml. 0·0001 M- and 0·2 ml. 0·002 M-H_2O_2 was present in the side arm with the IAA. The composition of the control reaction mixtures is shown below.

Components present with IAA	Uptake in 30 min. (μl. O_2)
A. With *p*-chlorophenol	
Peroxidase	16
Peroxidase + H_2O_2	15·5
Peroxidase + *p*-chlorophenol	104
Peroxidase + *p*-chlorophenol + H_2O_2	100·5
Peroxidase + *p*-chlorophenol + pyrogallol	1·5
Peroxidase + *p*-chlorophenol + pyrogallol + H_2O_2	96·5
Peroxidase + pyrogallol + H_2O_2	7
B. With ultrafiltrate	
Peroxidase	9
Peroxidase + ultrafiltrate	143
Peroxidase + ultrafiltrate + H_2O_2	114
Peroxidase + ultrafiltrate + pyrogallol	1·5
Peroxidase + ultrafiltrate + pyrogallol + H_2O_2	123
Ultrafiltrate + H_2O_2	2·5

before testing, then it readily oxidizes IAA. This is shown in Table 7. The results suggest that peroxidase substrates are present in the sap which inhibit in a way similar to that of pyrogallol.

Sap obtained from spinach beet (*Beta vulgaris* L.) leaves and from waxpod bean leaves had peroxidase activity but did not oxidize added IAA, and attempts to demonstrate IAA oxidation after treating the saps with H_2O_2 were unsuccessful. Unlike the sap of horseradish root, which has a very low catalatic activity, these leaf saps decompose H_2O_2 very rapidly, and it may be that the added H_2O_2 was decomposed too rapidly for the oxidation of inhibiting substances to take place.

Table 7. *Oxidation of IAA by horseradish root sap treated with* H_2O_2

Mixtures containing 1·5 ml. horseradish root sap adjusted to pH 6 with N-NaOH, varying amounts (0–0·5 ml.) of 0·05 M-H_2O_2, and water to a volume of 3 ml. were stood at room temperature for 1·5 hr. Then 20 μg. catalase in 0·2 ml. were added to decompose residual H_2O_2 and 30 min. later portions of the mixtures were tested manometrically with IAA. A mixture of 1·5 ml. sap at pH 6 with 1·5 ml. water which had been shaken for 4 hr. in air at room temperature was also tested. Manometric reaction mixtures consisted of: in the main vessel 1·2 ml. portions of the treated sap in 0·033 M phosphate at pH 6. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the uptakes obtained when IAA was omitted.

Treatment (ml. of H_2O_2 added)	Uptake of O_2 (μl. in 1 hr.)
0	5·5
0·1	12
0·2	113
0·4	69
0·5	48·5
Shaken 4 hr. in air	1
Equal volumes from treatments 1 and 3 mixed and 2·4 ml. tested	2

Table 8. *Effect of* Mn^{2+} *on the oxidation of IAA by different peroxidase preparations*

Reaction mixtures consisted of: peroxidase preparation equivalent to 0·05 E.U., 0·067 M phosphate at pH 6 and where present 0·1 ml. 0·001 M-$MnSO_4$. After equilibration 0·4 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes when peroxidase or IAA were omitted were negligible.

	P.N.	Uptake of O_2 (μl. in 90 min.): No addition	Mn^{2+} added	Increase due to Mn^{2+}
Horseradish	60	123·5	185·5	62
Horseradish	500	117	211	94
Horseradish	940	85	115	30
Horseradish	1040	89	120	31
Turnip root	70	100·5	204·5	104

Effect of catalase. The effect of catalase on the oxidation of IAA by horseradish peroxidase plus phenol was tested manometrically using 16 μg. peroxidase of P.N. 1040, 2×10^{-4} M phenol, 0·005 M IAA and 0·067 M phosphate buffer. The inhibiting effect of catalase varied with the pH, and it was necessary to use large amounts to obtain an inhibition. When 5 mg. catalase preparation were present in the reaction mixture at pH 6·5 or 7, no oxidation of IAA was observed in the experimental period of 1 hr.; but at pH 6 the presence of 5 mg. catalase caused only a short lag period (approximately 10 min.), after which the oxidation proceeded with increasing velocity and finally reached a velocity slightly greater than that of the control from which catalase was omitted. The inhibiting effect of catalase at pH 6·5 and 7, which is around the optimum for catalase action, suggests that the reaction depends on the production of H_2O_2.

Effect of manganese on the oxidation of IAA by horseradish peroxidase preparations. The effect of Mn^{2+} on the oxidation of IAA by horseradish peroxidase has been found to depend on the pH, the amount of Mn^{2+} present, the purity of the peroxidase preparation and the composition of the buffer solution.

Effect of pH. Under the conditions used (0·067 M phosphate–0·005 M IAA) the activating effect of small amounts of Mn^{2+} is greatest in the neighbourhood of pH 6, at which pH horseradish peroxidase alone catalyses the oxidation of IAA most readily.

Effect of purity of the peroxidase preparation. When a number of peroxidase preparations of varying degrees of purity were tested with Mn^{2+}, it was found that the activating effect of Mn^{2+} was least with the two most highly purified preparations used (Table 8). This suggests that the cruder preparations contain a factor which increases the effect of Mn^{2+}.

Effect of varying the concentration of Mn^{2+}. The effect of varying the concentration of Mn^{2+} on the oxidation of IAA by the peroxidase preparation of P.N. 500 in phosphate at pH 6 is shown in Fig. 3. With increasing amounts of Mn^{2+} up to about 10^{-5} M, the initial rate of O_2 uptake increases, but with further increase in Mn^{2+} there is a lag period before the oxidation begins. The length of the lag period varied in different experiments, but in general increased with increasing amounts of Mn^{2+}. Similar results were obtained when the highly purified peroxidase of P.N. 1040 was used, but here the activating effect of Mn^{2+} was less marked. It can be seen (Fig. 3) that the presence of an optimum amount of Mn^{2+} increased the rate of oxidation of IAA by 40 μg. peroxidase of P.N. 500 about 2–3 times; the effect of Mn^{2+} was no greater with small amounts of peroxidase (e.g. 8 μg. of P.N. 1040).

Since it has been shown (Fig. 2) that the rate of oxidation of IAA by 8 μg. peroxidase of P.N. 1040 is increased some 20–30 times in the presence of suitable amounts of waxpod bean root ultrafiltrate, it is clear that Mn^{2+} is much less active than is the ultrafiltrate.

Effect of the composition of the buffer solution. At pH 6 in the absence of Mn^{2+} the rate of oxidation of IAA by peroxidase was greater in pyrophosphate and phosphate–citrate media than the rate in phosphate. Under the conditions used this difference was greatest in the first 10–20 min. of the reaction where in pyrophosphate the rate of oxidation was nearly twice as fast as in phosphate, but since the rate of oxidation fell off more rapidly in pyrophosphate than in phosphate, the difference was less marked in the later stages of the reaction. However, in pyrophosphate or phosphate–citrate buffer at pH 6 the activating effect of low amounts of Mn^{2+} is almost entirely suppressed if the concentration of pyrophosphate and citrate is sufficiently high. This is shown in Table 9, where the conditions used were the same as for Fig. 3, except that pyrophosphate or phosphate–citrate buffers were employed in place of phosphate. The presence of Mn^{2+} at concentrations above 10^{-5} M caused a lag period in both phosphate–citrate and pyrophosphate buffers before the O_2 uptake started; the lag period with a given amount of Mn^{2+} was greater in the latter two buffers than when phosphate was used. The effect of citrate and pyrophosphate is presumably due to their capacity of forming complex ions with Mn^{2+}. It is not clear why the presence of pyrophosphate and citrate in sufficiently high concentration not only suppresses the activating effect of Mn^{2+} but also increases the lag period.

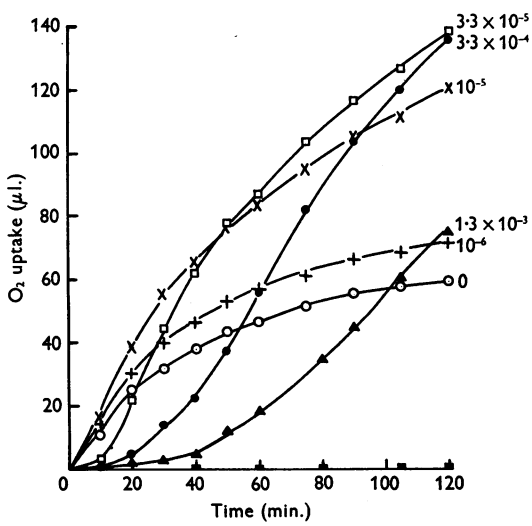

Fig. 3. Effect of variation in Mn^{2+} concentration on the oxidation of IAA by the horseradish peroxidase preparation of P.N. 500. Reaction mixtures consisted of 40 μg. horseradish peroxidase of P.N. 500, 0·067 M phosphate at pH 6 and varying amounts of Mn^{2+}. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. O—O, peroxidase; +—+, ×—×, □—□, ●—●, ▲—▲, peroxidase + Mn^{2+}; ■—■, $1·3 \times 10^{-3}$ or 10^{-5} M-Mn^{2+}, peroxidase omitted. The number to the right of each curve gives the molarity of Mn^{2+} used.

Effect of pyrophosphate on the activity of the ultrafiltrate. It has been shown that the presence of pyrophosphate strongly inhibits the activating capacity of Mn^{2+} on the peroxidase-catalysed oxidation of IAA by O_2 (Table 9). Therefore, if Mn^{2+} is responsible for a major part of the activity of waxpod bean root ultrafiltrate on the peroxidase-catalysed oxidation of IAA by O_2, then the activity of the ultrafiltrate should be less in pyrophosphate than in phosphate.

Table 9. *Effect of pyrophosphate and citrate on the oxidation of IAA by horseradish peroxidase in the presence of* Mn^{2+}

Reaction mixtures as in Fig. 3 except that 0·067 M pyrophosphate, or 0·067 M phosphate plus 0·067 M citrate, were used in place of phosphate at pH 6.

Concentration of Mn^{2+} (M)	O_2 uptake (μl.) Pyrophosphate 60 min.	Pyrophosphate 120 min.	Phosphate–citrate 60 min.	Phosphate–citrate 120 min.
0	70	76	65	71·5
10^{-6}	64	69·5	66	71
10^{-5}	75	79·5	68	79
$3·3 \times 10^{-5}$	73·5	82·5	68	87·5
10^{-4}	32·5	71	5	54·5

Table 10. *Effect of pyrophosphate on the activity of ultrafiltrate*

Reaction mixtures consisted of: peroxidase preparation equivalent to 0·02 E.U. 0·067 M pyrophosphate or 0·067 M phosphate at pH 6 and where present ultrafiltrate. After equilibration 0·3 ml. 0·05 M IAA was added from the side arm. The O_2 uptakes are corrected for the blank O_2 uptakes in mixtures from which peroxidase was omitted. Two separate batches of ultrafiltrate were examined (*A* and *B*).

	Uptake of O_2 (μl. in 90 min.) Phosphate	Pyrophosphate
Horseradish (P.N. 1040)	40	48
Horseradish (P.N. 1040) + 0·4 ml. ultrafiltrate *A*	127·5	123
Horseradish (P.N. 1040) + 0·8 ml. ultrafiltrate *A*	199·5	176·5
Horseradish (P.N. 500)	62·5	68·5
Horseradish (P.N. 500) + 0·4 ml. ultrafiltrate *B*	175·5	161

Table 11. *Oxygen uptake and carbon dioxide formation during the oxidation of IAA by peroxidase*

Reaction mixtures consisted of: horseradish peroxidase of P.N. 1040 and where present 1 ml. waxpod bean root ultrafiltrate in 0·067 M phosphate at pH 6. IAA was added from the side arm. The duration of the experiments was 4 hr. The values given are corrected for the blank O_2 uptakes and CO_2 outputs with the systems IAA alone, IAA + ultrafiltrate, and ultrafiltrate + peroxidase. These blanks were very small, the largest being 3 μl. O_2 uptake with the system 10 μmoles IAA + ultrafiltrate.

		IAA (μmoles)					
		10		5		2·5	
Peroxidase (μg.)	Ultrafiltrate	O_2	CO_2	O_2	CO_2	O_2	CO_2
		μmoles					
100	—	4·8	4·7	3·3	3·7	2·3	2·7
300	—	5·5	5·5	4·2	4·1	2·4	2·5
100	1 ml.	7·2	7·7	4·1	4·1	2·3	2·0

The results with two different batches of ultrafiltrate are shown in Table 10. The O_2 uptakes obtained in the presence of pyrophosphate are only slightly less than those obtained in phosphate, which suggests that Mn^{2+} is only responsible for a small part of the activity of the ultrafiltrate. However, there is some doubt concerning the validity of this suggestion since, when amounts of ultrafiltrate in excess of about 0·8 ml. were tested they often caused a lag period in the presence of pyrophosphate and the subsequent rate of O_2 uptake was sometimes less than with smaller amounts of ultrafiltrate.

The course of the oxidation. A study has been made of the O_2 uptake and CO_2 formation when horseradish peroxidase and horseradish peroxidase plus waxpod bean root ultrafiltrate oxidize IAA. The results (Table 11) suggest that, under the conditions used, the oxidation of IAA proceeds more readily to completion with low concentrations of IAA. Thus, with 100 μg. peroxidase the final O_2 uptakes were 0·92 mole O_2/mole IAA when 2·5 μmoles IAA were present, but only 0·48 mole O_2/mole IAA with 10 μmoles IAA. Increasing the amount of peroxidase, or adding ultrafiltrate, increased the final O_2 uptake with the larger amounts of IAA, but was without effect where 2·5 μmoles IAA were present. Using the reagent of Tang & Bonner (1947), approximate colorimetric estimations of IAA in the final reaction mixtures showed that where the O_2 uptake exceeded 0·9 mole O_2/mole IAA, over 90 % of the IAA had been oxidized. The total amount of CO_2 produced was measured by Warburg's direct method. The values obtained were all in the neighbourhood of 1 mole CO_2/mole O_2 consumed.

DISCUSSION

The results of the present work show that highly purified horseradish peroxidase preparations catalyse the oxidation of IAA by O_2 and that in the presence of certain thermostable factors such as monophenols, aniline, resorcinol, manganese and maleic hydrazide the rate of oxidation is increased. The system in waxpod bean root sap responsible for the oxidation of IAA consists of a thermolabile fraction and a thermostable fraction. The evidence suggests that the thermolabile fraction is a peroxidase, since it can be replaced by highly purified horseradish peroxidase preparations. The thermostable fraction in the bean root system has not been identified but the results suggest that a large part of its activity is due to the presence of certain peroxidase substrates. It is considered that Mn^{2+} alone can be responsible for only a small part of the activity of the thermostable fraction; although under certain conditions low concentrations of Mn^{2+} increase the rate of oxidation of IAA by peroxidase, this effect of Mn^{2+} is small compared with that of the ultrafiltrate, particularly when the concentration of peroxidase is low. Furthermore, the activating effect of Mn^{2+} is strongly inhibited by pyrophosphate, while the activity of the thermostable fraction, when present in small amounts, is only slightly affected. However, anomalous results were obtained using large amounts of the thermostable fraction, since under these conditions an inhibiting effect of pyrophosphate was found. Evidence has been obtained that the oxidation of IAA by O_2 catalysed by the peroxidase systems studied is not dependent on the presence of flavoprotein, and this raises the question of the significance of the flavoprotein present in preparations of IAA oxidase from pea epicotyl (e.g. Galston *et al.* 1953). In contrast to the present work, where it was found that the oxidation of IAA by peroxidase proceeded without a lag period or with only a brief one, Galston *et al.* (1953) found that in darkness a lag period of about 2 hr. was necessary before the oxidation commenced. This lag period could be overcome by exposure of the reaction mixtures to white light. Also, Galston *et al.* (1953) found that the inhibiting effect of catalase on the peroxidase-catalysed oxidation of IAA by O_2 was partially reversed by light. These facts suggest that the effect of light in solutions containing peroxidase and IAA

is to increase the rate of production of peroxide. In preliminary experiments it has been found that the rate of oxidation of IAA by highly purified horseradish peroxidase is increased in the presence of strong daylight. This suggests that the stimulating effect of light on the oxidation of IAA by pea epicotyl preparations may not be entirely due to an effect on flavoprotein components, as has been previously supposed (Galston & Baker, 1949, 1951; Galston *et al.* 1953). It is not likely that light had much influence on the results obtained in the present work, since the reactions were carried out in light of very low intensity (< 1 f.c.) except for a brief exposure to daylight during the addition of materials from the side arm of the reaction flasks. However, this brief exposure to light and the different experimental conditions used in the present work may each be partly responsible for the absence of a long lag period before the oxidation of IAA by peroxidase commenced.

The results suggest that the oxidation of IAA by horseradish peroxidase in the absence and in the presence of the thermostable fraction of waxpod bean root proceeds with the consumption of 1 mole O_2/mole IAA oxidized, and the formation of 1 mole CO_2/mole O_2 consumed. These values are in agreement with the findings of Tang & Bonner (1947) and Wagenknecht & Burris (1950) for the IAA oxidizing systems present in extracts of pea epicotyls and waxpod bean roots.

The mechanism of the oxidation is not clear, but at pH 6·5 or 7, which is close to the optimum for catalase action, catalase inhibits strongly, suggesting that the reaction is accompanied by, and depends on, the formation of H_2O_2. The mechanism of the stimulation of the oxidation by Mn^{2+} or maleic hydrazide is obscure, but it is suggested that the activity of the peroxidase substrates such as monophenols, resorcinol, and aniline, depends on their capacity for reversible oxidation–reduction. Such a mechanism would account for the inhibiting effect of polyphenols, since in general these are more readily oxidized by peroxidase than are monophenols, and they would thus successfully compete for the H_2O_2 formed. It presupposes that polyphenols cannot act as redox carriers in the oxidation of IAA, but a similar situation is known in the case of Mn^{2+} oxidation by peroxidase and H_2O_2 where monophenols but not polyphenols are active as carriers (Kenten & Mann, 1950). The fact that those peroxidase substrates (e.g. *p*-chlorophenol) which increase the activity of peroxidase towards IAA are much less effective at high than at low concentration could be due to inhibition of peroxidase by the high phenol concentration. On the other hand, it is possible that these phenols are less easily oxidized by peroxidase and H_2O_2 than is IAA, and that when such substrates are present in high concentration their oxidation is favoured and they compete with IAA for the available H_2O_2. If this latter possibility is correct, then the activity of substrates such as *p*-chlorophenol at low concentration is due to some function other than that of a redox carrier.

The reaction bears a marked resemblance to the known direct oxidase reactions of peroxidase in which the oxidation of dioxymaleic acid (Swedin & Theorell, 1940), certain other dicarboxylic acids (Kenten & Mann, 1953), and phenylacetaldehyde (Kenten, 1953), by O_2 is catalysed by peroxidase. With the dioxymaleic acid reaction it has been previously supposed that H_2O_2 was produced by an initial autoxidation of the dioxymaleic acid, but Hartree (1953) has demonstrated that 'by rigorous purification of the dioxymaleic acid and buffer solution it is possible to abolish autoxidation virtually completely without affecting the rate of the enzyme-catalysed reaction'. Since neither phenylacetaldehyde nor the dicarboxylic acids studied by Kenten & Mann (1953) undergo significant autoxidation under the conditions in which they are oxidized by peroxidase, it is clear that peroxide must arise from some mechanism other than direct autoxidation of the substrate. It is possible, however, that extremely slow autoxidation of these substrates provides traces of H_2O_2 whereby the reaction is initiated, and further H_2O_2 could arise if the oxidation product, produced by the action of peroxidase, autoxidizes readily with the production of H_2O_2.

It is of interest that those compounds which have been demonstrated to increase the activity of IAA oxidase preparations of pea epicotyl, and pineapple leaf and stem tissue, also increase the rate of oxidation of IAA by highly purified horseradish peroxidase. Thus a variety of monophenols, particularly chlorophenols, 4-methylumbelliferone and maleic hydrazide have been shown to activate the pea epicotyl IAA oxidase (Goldacre *et al.* 1953; Andreae & Andreae, 1953), while Mn^{2+} has a large effect on the pineapple IAA oxidase (Gortner & Kent, 1953) and a comparatively small effect with dialysed pea epicotyl extracts (Wagenknecht & Burris, 1950; Goldacre *et al.* 1953). There is ample evidence suggesting the presence of a thermostable inhibitor of IAA oxidase in plant tissues (Tang & Bonner, 1948; Gortner & Kent, 1953) and with pineapple tissue the results of Gortner & Kent (1953) suggest that this may be a polyphenol. In this connexion the inhibiting effect of pyrogallol on the peroxidase catalysed oxidation of IAA by O_2 suggests that peroxidase may be concerned in the oxidation of IAA by pineapple-tissue extracts. The presence, in extracts of horseradish root, of peroxidase substrates which inhibit the reaction is suggested by the fact that, although such extracts are rich in peroxidase, they will not oxidize IAA unless they are first

treated with a suitable amount of H_2O_2. If too large an amount of H_2O_2 is used the activity of the treated extract is considerably less than that of an extract treated with the optimum amount of H_2O_2. This suggests that either the peroxidase in the extract is partially destroyed by exposure to the high concentration of peroxide (Theorell, 1951) or that active peroxidase substrates (e.g. monophenols) are irreversibly oxidized under these conditions.

Further work is necessary before the physiological significance of the peroxidase-catalysed oxidation of IAA by O_2 can be evaluated. Hill & Hartree (1953) have pointed out some of the difficulties in applying the results obtained with distintegrated tissues to a discussion of the metabolism of intact plants. It is known that peroxidase *in vitro* can catalyse the oxidation of a great many different substances by H_2O_2, and several direct oxidase reactions of peroxidase are known. Also, the range of peroxidase action *in vitro* has been further extended by the demonstration that substances which are not directly oxidized by peroxidase and H_2O_2 can be oxidized when certain peroxidase substrates are present and act as carriers. Peroxidase is widely distributed among the higher plants and presumably plays some part in metabolism, but in spite of our knowledge of the *in vitro* activities of peroxidase the relative significance of these activities *in vivo* is not yet known.

SUMMARY

1. The oxidation of indolyl-3-acetic acid (IAA) by oxygen is catalysed by highly purified horseradish peroxidase preparations. The rate of oxidation is increased in the presence of certain thermostable factors such as monophenols, aniline, resorcinol, manganese and maleic hydrazide. With the exception of manganese and maleic hydrazide, all the active factors are known, or have been shown to be, peroxidase substrates. Other peroxidase substrates, e.g. catechol, hydroquinone, pyrogallol and *p*-phenylenediamine (10^{-4}–10^{-5} M) strongly inhibit the peroxidase-catalysed oxidation of IAA.

2. The IAA oxidase of waxpod bean root sap consists of a thermolabile fraction, which appears to be a peroxidase and which can be replaced by horseradish peroxidase, and a thermostable fraction, the activity of which largely depends on the presence of peroxidase substrates.

3. The oxidation of IAA when catalysed by horseradish peroxidase in the absence or presence of waxpod bean root thermostable fraction proceeds with the consumption of 1 mole oxygen/mole IAA oxidized and the formation of 1 mole carbon dioxide/mole oxygen consumed.

4. Possible mechanisms of the reaction are discussed, and evidence is presented suggesting that the oxidation of IAA by the peroxidase systems studied is not dependent on the presence of flavoprotein.

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