apparently more resistant M_B (Tables 1 and 2). The reason for this discrepancy is not known. However, these figures show that about a tenth of the whole mycelium, including a part of each fraction analysed, is resistant to enzymic digestion or is not available to the enzyme. On the other hand, proteases, cellulases and chitinases are widespread in micro-organisms and invertebrates and their presence from these sources in the soil will in the absence of any stabilizing factors bring about the dispersion of the greater part of such fungal mycelium.

SUMMARY

1. Air-dried mycelium of *Penicillium griseofulvum* was subjected to autolysis. The suspended matter in the final product contained 16% of the original protein, 64% of the carbohydrate and 50% of the chitin. The solution contained dispersed protein and carbohydrate, but no α-amino acids or reducing sugar.

2. Air-dried mycelium after 2½–3 yr. storage was autolysed. The suspended material contained 39% of the original protein, 71% of the carbohydrate and 70% of the chitin. The solution contained amino acids, reducing sugar and *N*-acetylglucosamine.

3. The protease of the mycelium is stable to storage. Much polysaccharide-splitting and chitinase activity is lost on drying, but some of the residual activity remains after 3 yr. Such stored mycelium contains a very weak glucose oxidase, but no α-amino-acid oxidase, which was presumably very active in the fresh dry mycelium.

4. Little change takes place in the composition of the mycelium on storage except for a slight hydrolysis of protein.

5. 10–15% of the mycelium protein resists digestion with proteases; 10–25% of the carbohydrate and about 10% of the chitin resists digestion with juice from the alimentary tract of snails.

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REFERENCES

Bawden, F. C. & Pirie, N. W. (1946). *Brit. J. exp. Path.* **27**, 81.
Bender, A. E. & Krebs, H. A. (1950). *Biochem. J.* **46**, 210.
Benger, J., Johnson, M. J. & Peterson, W. H. (1937). *J. biol. Chem.* **117**, 429.
Bohonos, N., Woolley, D. W. & Peterson, W. H. (1942). *Arch. Biochem.* **1**, 319.
Dion, W. M. (1950). *Canad. J. Res.* C, **28**, 577.
Foster, J. W. (1949). *Chemical Activities of Fungi*. New York: Academic Press.
Holden, M. (1950). *Biochem. J.* **47**, 426.
Holden, M. & Tracey, M. V. (1950). *Biochem. J.* **47**, 421.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Pirie, N. W. (1936). *Brit. J. exp. Path.* **17**, 269.
Smithies, W. R. (1952). *Biochem. J.* **51**, 259.
Smithies, W. R. (1953). *Biochem. J.* **53**, xxix.
Tracey, M. V. (1948). *Biochem. J.* **42**, 281.
Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.
Woolley, D. W. & Peterson, W. H. (1937). *J. biol. Chem.* **121**, 507.

The Oxidation of Phenylacetaldehyde by Plant Saps

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Kenten & Mann (1951) found that the total oxygen uptake obtained when pea-seedling extracts catalysed the oxidation of β-phenylethylamine was in excess of that required for the oxidation of the amine to phenylacetaldehyde. This suggested the presence of an aldehyde oxidase in the extracts. In accordance with this view it was found that large increases in the oxygen uptake were produced when phenylacetaldehyde was added to pea-seedling extracts. The oxidation products were not determined, although preliminary tests suggested that the oxidation was accompanied with hydrogen peroxide formation. By analogy with the known aldehyde oxidases phenylacetic acid was presumed to be the oxidation product. Since Skoog (1937) and Gordon & Sanchez-Nieva (1949) had obtained evidence that tryptamine could function as a precursor of 3-indolylacetic acid in plants, Kenten & Mann (1951) suggested that the successive action of amine oxidase and the aldehyde oxidase on tryptamine was a possible mechanism for the formation of 3-indolylacetic acid.

The present work was undertaken to investigate the system in pea-seedling extracts responsible for the oxidation of phenylacetaldehyde and to determine the oxidation products.

MATERIALS AND METHODS

Plant sap. Most of the plants examined were glasshouse-grown in a John Innes potting compost. The rest of the plants were from gardens in the neighbourhood of Rothamsted. The plant material was washed free from soil and then either ground in a mortar with sand or passed through a triple roller mill (Bawden & Pirie, 1944). The pulp was squeezed by hand through madapollam and centrifuged for 20–30 min. at 8000 rev./min. Pea-seedling sap was normally stored at 0° overnight and then centrifuged again. This treatment reduced the O_2 uptake of the sap without significant loss of activity towards the phenylacetaldehyde.

Peroxidase. Peroxidase preparations were made from horseradish by Mann's (1953) method. The purpurogallinzahl (P.Z.) (Willstätter & Stoll, 1918) of the preparations was estimated by Keilin & Mann's (1937) method. In the text the peroxidase activity of some preparations is given as purpurogallin (P.G.) units. A P.G. unit is the number of mg. of purpurogallin formed by 1 ml. of the preparation under Keilin & Mann's conditions.

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 Katalasefähigkeit (Kat.f.) was 5000. Using the conversion factor of Chance & Herbert (1950) that Kat.f. $= 520\ k_1/M$, where M is the molecular weight of catalase (230000), $k_1 = 2{\cdot}2 \times 10^6 \text{M}^{-1}$ sec.$^{-1}$. For each experiment a sample of the preparation was suspended in 0·2M-orthophosphate at pH 7 (10 mg./ml.) and the insoluble material removed by centrifuging.

Substrates. Samples of phenylacetaldehyde from L. Light and Co. Ltd. and A. Boake Roberts and Co. Ltd. were redistilled twice *in vacuo*, the head and tail fractions being discarded. The middle fraction was stored at 0° to reduce the rate of polymerization; even at this temperature slow polymerization occurred and distillation *in vacuo* at about fortnightly intervals was necessary to keep the content of polymer low. 1-Naphthylacetaldehyde was prepared from 2-(1-naphthyl)-1:1-di(ethoxycarbonylamino)ethane by the method of Jensen & Christensen (1950).

Methods. Carbonyl compounds were estimated by the bisulphite method of Clift & Cook (1932) and formic acid was estimated by Pirie's (1946) method. Absorption spectra were determined using a Unicam Model S.P. 500 quartz spectrophotometer.

Manometric measurements. These were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. KOH was omitted from the centre cup unless otherwise stated. CO_2 formation was measured by Warburg's direct method (Dixon, 1943).

EXPERIMENTAL AND RESULTS

Manometric studies of the oxidation of phenylacetaldehyde by plant saps

The addition of phenylacetaldehyde to most of the plant saps used caused an increase in oxygen uptake (Table 1). The rate of oxygen uptake was considerably reduced by previous heating of the pea-seedling sap at 100° (Fig. 1). The effect of heat was somewhat variable, but in general, while 5 min. at 100° reduced the activity to about 20% of the original activity, 20 min. at 100° did not completely inactivate the extract.

Specificity. Formaldehyde, acetaldehyde, glyoxal, *n*-butyraldehyde, *iso*valeraldehyde, cinnamaldehyde and phenylpropionaldehyde were tested with pea-seedling sap at a concentration of 10 mg./ml. under conditions similar to those used for Table 1. 1-Naphthylacetaldehyde was tested at a lower concentration (3·4 mg./ml.). In a period of 2 hr. only 1-naphthylacetaldehyde and *iso*butyraldehyde significantly increased the oxygen uptake, the effect being large with the former and small with the latter substrate.

Effect of dialysis and protein precipitants. Precipitates of pea-seedling sap, obtained by addition of ammonium sulphate to saturation, and dialysed sap (48 hr. at 0° against 200 vol. of water) catalysed the oxidation of phenylacetaldehyde by oxygen though at a much slower rate than an equivalent amount of untreated sap. Precipitates obtained by addition of

Table 1. *The oxidation of phenylacetaldehyde by plant saps*

(Reaction mixtures consisted of phenylacetaldehyde (10 mg. in 0·1 ml. ethanol) in 0·067M-orthophosphate at pH 7. Plant sap (0·2–0·5 ml.) was added from the side arm. Blank O_2 uptakes were obtained using mixtures of ethanol (0·1 ml.), orthophosphate and plant sap.)

Plant	Part	Increased O_2 uptake (μl./ml. sap in 1 hr.)	Blank O_2 uptake (μl./ml. in 1 hr.)
Dandelion (*Taraxacum officinale* Weber)	Root	40	35
Lupin (*Lupinus*, garden hybrids)	Root	70	15
Potato (*Solanum tuberosum* L.)	Tuber	170	33
Pea seedling (*Pisum sativum* L.)	Root	2100	22
	Stem	1300	16
	Leaf	670	20
Tobacco (*Nicotiana tabacum* L.)	Leaf	700	16
Red clover (*Trifolium pratense* L.)	Leaf	260	18

2 vol. of acetone or ethanol to sap at 0° were more active than dialysed extract, but dialysis (48 hr. at 0° against 200 vol. of water) of water suspensions of these precipitates caused a considerable loss of activity (Table 2). These results suggested that the system responsible for the oxidation consisted of a non-dialysable factor which was precipitated by the precipitants used, and a dialysable factor not precipitated by ammonium sulphate, but at least in part by acetone and ethanol. In agreement with this it was found that the addition of protein-free filtrates of pea-seedling sap to dialysed sap, dialysed ethanol or acetone precipitates, or ammonium sulphate precipitates caused a considerable increase in activity (Table 2).

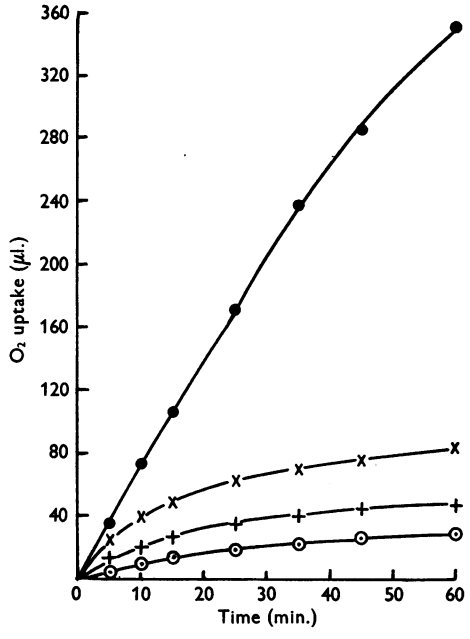

Fig. 1. The effect of previous heating of pea-seedling sap on the rate of oxidation of phenylacetaldehyde. Reaction mixtures as in Table 1; sap (0·2 ml.) previously heated at 100° for: ●—●, 0 min.; ×—×, 5 min.; +—+, 10 min.; ⊙—⊙, 20 min. The O_2 uptakes are corrected for the uptake in absence of phenylacetaldehyde.

Effect of ultrafiltration. Pea-seedling sap which had been kept at 0° overnight was centrifuged at 8000 rev./min. for 30 min. to remove chloroplast material and 30 ml. of the supernatant were filtered overnight at 0° in an ultrafiltration apparatus of the type described by Paterson, Pirie & Stableforth (1947). In this time nearly all the liquid passed through leaving a pasty residue inside the cellophan sac which was suspended in water (30 ml.). The oxygen uptake brought about by the addition of these fractions to phenylacetaldehyde is shown in Fig. 2. While the ultrafiltrate alone showed little

Table 2. *Effect of addition of ultrafiltrate on the oxidation of phenylacetaldehyde by fractions obtained from pea sap*

(Reaction mixtures as in Table 1; the various fractions (0·2 ml.) were added from the side arm alone or together with 0·2 ml. of ultrafiltrate. The O_2 uptake in the first hour was measured. Correction has been made for blank O_2 uptakes in mixtures from which phenylacetaldehyde, but not ethanol, was omitted. These were less than 10 µl. O_2 in 1 hr.)

Fraction	Uptake of O_2 (µl. in 1 hr.) Alone	Ultrafiltrate added
Pea sap	408	487
Dialysed pea sap	103	400
Ethanol precipitate	249	385
Dialysed ethanol precipitate	64	316
Acetone precipitate	327	418
Dialysed acetone precipitate	74	349
Saturated ammonium sulphate precipitate	134	359
Ultrafiltrate	12	—

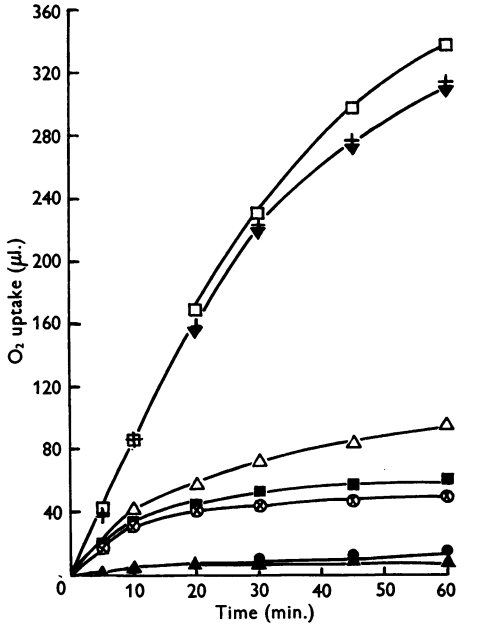

Fig. 2. The effect of ultrafiltration on the rate of oxidation of phenylacetaldehyde by pea-seedling sap. Reaction mixtures as in Table 1; after equilibration 0·2 ml. each of the following added from side arm: □—□, sap; ■—■, sap, heated; △—△, residue; ▲—▲, residue, heated; ●—●, ultrafiltrate; +—+, residue + ultrafiltrate; ⊗—⊗, residue, heated + ultrafiltrate; ▼—▼, residue + heated ultrafiltrate. Heat treatment was 15 min. at 100°.

activity and the residue alone only about one-third of the activity of the sap, together they gave an activity of the same order as sap. The effect of previous heating of the ultrafiltrate and the residue at 100° for 15 min. is also shown in Fig. 2. Whereas only slight activity remained in the residue after heating, the activating effect of the ultrafiltrate was not affected by heat treatment. Some ultrafiltrates had an activity of 30–60 μl. oxygen/0·2 ml./hr. with phenylacetaldehyde; this was presumably due to slight contamination by leakage during filtration since the activity was considerably reduced by heating for 15 min. at 100°.

The results suggest that the oxidation of phenylacetaldehyde by the pea-seedling sap is brought about by a thermolabile factor which requires the addition of a thermostable factor for maximal activity.

The nature of the thermostable factor. The activating effect of the ultrafiltrate was largely destroyed by ashing. However, acid extracts of the ash, after neutralization, had a slight activating effect when added to the residue in small amounts, but inhibited in larger amounts (Table 3). The effect of

Table 3. *Effect of ultrafiltrate ash on the rate of oxidation of phenylacetaldehyde by pea-sap residue*

(Ultrafiltrate (15 ml.) was ashed and the ash extracted with N-HCl. The extract was neutralized and diluted to 15 ml. Mn content, determined by periodate oxidation, was 2·3 μg./ml. Reaction mixtures as in Table 1 with varying additions of ash solution or $MnSO_4$ (0·0002 M); pea-sap residue (0·2 ml.) was added from the side arm. The O_2 uptake in the first hr. was measured. Correction has been made for O_2 uptakes of blanks which were less than 10 μl. in 1 hr.)

Addition	Mn content (μg.)	Uptake of O_2 (μl. O_2 in 1 hr.)
None	—	118
Ash solution	0·23	144
	0·46	149
	0·92	125·5
	1·84	109·5
$MnSO_4$	0·55	167
	2·75	217
	22·0	291
Ultrafiltrate	0·47	443

adding Co^{2+}, Ni^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+} on the oxidation of phenylacetaldehyde by the residue was tested. At the concentrations used (0·001 M) only Mn^{2+} produced an increase in the rate of oxidation, while Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} reduced the rate, the effect being most marked with Cu^{2+} which caused a 50 % decrease. The activating effect of Mn^{2+} on the residue is also shown in Table 3. The rate of oxygen uptake increased with increasing amounts of Mn^{2+}, but the addition of 22 μg. Mn^{2+} did not increase the rate as much as the addition of ultrafiltrate containing 0·47 μg. Mn^{2+}. The fact that the addition of ultrafiltrate ash containing 1–2 μg. Mn^{2+} had little effect on the activity of the residue suggested that the ash contained an inhibiting factor. When ultrafiltrate was taken to dryness at 100° and then heated for 24–48 hr. at this temperature, 20–30 % of the activating effect was lost.

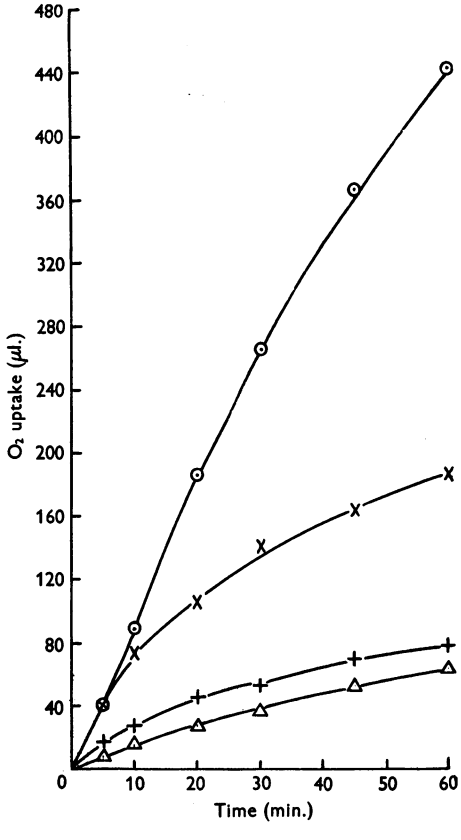

Fig. 3. The effect of catalase on the rate of oxidation of phenylacetaldehyde by pea-seedling sap. Reaction mixtures as in Table 1 with addition of catalase; pea-seedling sap (0·2 ml.) added after equilibration. Catalase added; ⊙—⊙, none; ×—×, 0·2 mg.; +—+, 1 mg.; △—△, 5 mg.

The effluent obtained when ultrafiltrate was passed three or four times through a column of the acid form of Zeo-Karb 215 resin had little effect on the activity of pea-sap residue. Over 90 % of the manganese contained in the ultrafiltrate was removed by the resin, but addition of Mn^{2+} to the effluent failed to restore its activity. If the resin used to deionize the ultrafiltrate was washed with N-hydrochloric acid, an eluate containing most of the manganese and 50–60 % of the original activity was obtained. Addition of effluent or Mn^{2+} to this eluate failed to increase its activity if allowance was made for the effect of Mn^{2+} alone.

The results suggest that at least part of the activating effect of ultrafiltrate is due to a factor(s) which is positively charged in weakly acid solution. If manganese is involved, it seems likely that either a cationic complex of manganese, or other inorganic or organic cations in addition to manganese, are responsible for the activating effect of ultrafiltrate on the residue.

Effect of catalase. The effect of catalase on the oxidation of phenylacetaldehyde by pea-seedling sap is shown in Fig. 3. The rate of oxygen uptake decreases with the increasing catalase addition, a concentration of 5 mg./3 ml. reducing the rate by about 85 %. The inhibiting effect of catalase suggested that the reaction studied depended on the production of hydrogen peroxide and was a peroxidatic oxidation, since with a simple flavoprotein enzyme system addition of catalase would, at most, halve the rate of uptake. It is known that manganese together with peroxidase forms a system capable of catalysing the oxidation of a number of substrates by oxygen (Kenten & Mann, 1953). The activating effect of manganese on the residue and the inhibition by catalase suggested that peroxidase might be the thermolabile factor in pea-seedling sap.

Manometric studies of the oxidation of phenylacetaldehyde by horseradish peroxidase and manganous ions

Horseradish-peroxidase preparations catalyse the oxidation of phenylacetaldehyde and the rate of oxidation is increased by the addition of ultrafiltrate or Mn^{2+}. This is shown by the results of Fig. 4. Control experiments with reaction mixtures from which phenylacetaldehyde but not ethanol was omitted gave negligible oxygen uptakes. The oxygen uptake with peroxidase alone was small and was only 30–35 % of that given by pea-sap residue of approximately the same peroxidase content. The greater activity of the residue is most likely due to the presence of small amounts of the natural activating factor. The rate of oxygen uptake increased markedly on addition of Mn^{2+} or ultrafiltrate. The increased uptake on addition of ultrafiltrate containing 0·27 μg. Mn^{2+} was considerably greater than that obtained by the addition of 2·75 μg. Mn^{2+}, showing that, as found with pea-sap residue, Mn^{2+} alone cannot be the natural activating factor in ultrafiltrate.

Effect of catalase. This was tested in reaction mixtures with varying additions of catalase. The resulting oxygen uptakes are shown in Fig. 5. The inhibiting effect of catalase is similar to that obtained when pea-seedling sap is used to bring about the oxidation (Fig. 3).

Comparison of pea-sap residue and horseradish peroxidase. Pea-sap residue has high peroxidase activity; under the standard conditions (Keilin & Mann, 1937) with pyrogallol as substrate the P.Z. of different preparations varied from 5 to 30.

The activating effect of Mn^{2+} on a pea-sap residue of P.Z. 25 and a horseradish peroxidase of P.Z. 485 was compared in the following way. After equilibration, samples of residue and horseradish peroxidase equivalent to 7 P.G. units were added from the side arm to a mixture containing phenylacetaldehyde and varying amounts of Mn^{2+} in orthophosphate at pH 7. The oxygen uptakes in the first hour are recorded in Table 4. While both the horseradish peroxidase and the residue gave increased uptakes in the presence of Mn^{2+}, the horseradish peroxidase gave larger increases for a given Mn^{2+} addition than did the pea residue.

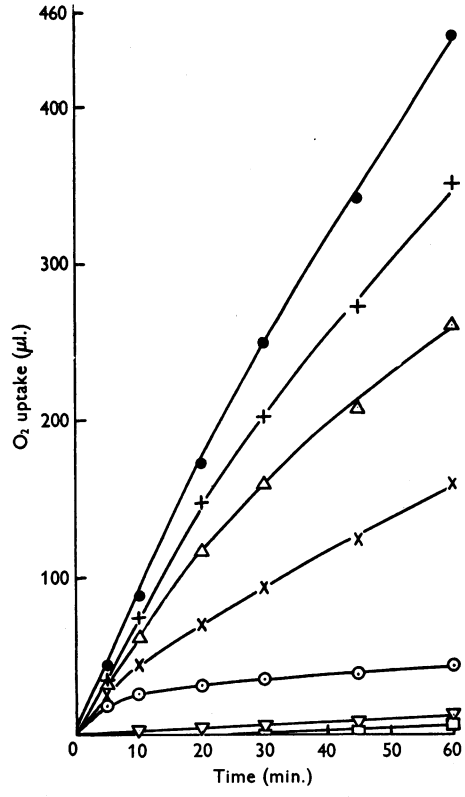

Fig. 4. The effect of Mn^{2+} and ultrafiltrate on the rate of oxidation of phenylacetaldehyde by horseradish peroxidase. Reaction mixtures as in Table 1 with varying additions of $MnSO_4$ or 0·2 ml. of ultrafiltrate; 0·2 ml. horseradish peroxidase of P.Z. 485 (65 μg./ml.) added from side arm. ⊙—⊙, ×—×, △—△, +—+, peroxidase with 0, 0·22, 0·55 and 2·75 μg. Mn^{2+}, respectively; ●—●, peroxidase + ultrafiltrate; ▽—▽, ultrafiltrate; □—□, 2·75 μg. Mn^{2+}.

Effect of pH. When horseradish peroxidase plus Mn^{2+} catalysed the oxidation of phenylacetaldehyde in phosphate-borate buffers the rate of oxygen uptake increased with increase in pH value from 6 to 9. At pH values above 9 freshly made solutions of phenylacetaldehyde went rapidly turbid, the change being more rapid the higher the pH. Aldehyde estimations showed that this change was accompanied by a decrease in the total aldehyde content of the solutions. This change (which was presumably due to polymerization of the aldehyde, possibly by an aldol condensation) was small at pH 7·5 where the total aldehyde content of 0·01 M-phenylacetaldehyde decreased less than 5% in 8 hr. Accordingly, although the activity of the system was less at pH 7–7·5 than at more alkaline pH, the experiments of the present work have been carried out at pH 7–7·5 to avoid the loss of substrate which takes place at higher pH values.

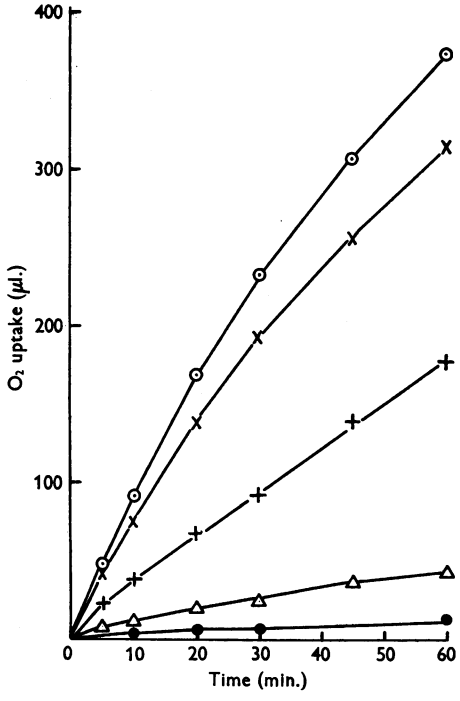

Fig. 5. The effect of catalase on the rate of oxidation of phenylacetaldehyde by horseradish peroxidase plus Mn^{2+}. Reaction mixtures as in Table 1 with addition of 2·75 μg. Mn^{2+} and varying amounts of catalase; 0·2 ml. horseradish peroxidase of P.Z. 485 (70 μg./ml.) added from side arm. Catalase added: ⊙—⊙, none; ×—×, 0·2 mg.; +—+, 1 mg.; △—△, 5 mg.; ●—●, 5 mg. catalase, peroxidase omitted.

Table 4. *Comparison of pea-sap residue and horseradish peroxidase*

(Reaction mixtures as in Table 1 with varying amounts of $MnSO_4$. After equilibration 0·2 ml. of pea-sap residue of P.Z. 25 (34·5 P.G. units/ml.), or 14·2 μg. horseradish peroxidase of P.Z. 485 in 0·2 ml. of water, was added from the side arm. Correction has been made for O_2 uptakes of blanks which were less than 10 μl. in 1 hr.)

Mn^{2+} added (μg.)	Uptake of O_2 (μl. in 1 hr.) Residue	Horseradish peroxidase
0	127	32
0·55	191	236
2·75	235	311
22·0	232	384

Effect of substrate concentrations. The effect of substrate concentration on the reaction velocity was tested with pea-seedling sap and horseradish peroxidase plus Mn^{2+} in 0·067 M orthophosphate at pH 7. The initial velocity increased markedly with increase in substrate concentration (Table 5).

Table 5. *The effect of substrate concentration*

(Reaction mixtures as in Table 1, with 0·5 ml. of 0·0001 M-$MnSO_4$ (2·75 μg. Mn^{2+}) where peroxidase was used. After equilibration 0·2 ml. of pea-seedling sap, or 20 μg. of horseradish peroxidase of P.Z. 485 in 0·2 ml. of water, was added from the side arm.)

Phenylacetaldehyde concentration (mg./3 ml.)	Increased O_2 uptake (μl. in 10 min.) Sap	Peroxidase and Mn^{2+}
10	75·5	72
5	67	64
1	15	20·5
0·5	5·5	11
0·1	1	4·5
0·05	0·5	2·5

Maximum velocity was not reached at a concentration of 5 mg./3 ml. where not all the phenylacetaldehyde was in solution. This suggested that the rate of oxidation was limited by the solubility of the phenylacetaldehyde; increase in the amount of phenylacetaldehyde by increasing the rate of solution would help to maintain saturation and therefore increase the velocity of the reaction.

Oxidation of Mn^{2+} *during the oxidation of phenylacetaldehyde by peroxidase.* Kenten & Mann (1949, 1950) have shown that the peroxidase-catalysed oxidation of Mn^{2+} by hydrogen peroxide depends on the presence of a phenolic substrate, such as *p*-cresol. In pyrophosphate at pH 7 it was shown that a pink manganipyrophosphate complex was formed. The manganipyrophosphate was estimated manometrically with hydrazine by measuring the evolution of nitrogen.

If the oxidation of phenylacetaldehyde by peroxidase plus Mn^{2+} is carried out in pyrophosphate at pH 7 the reaction mixture develops a pink colour suggesting the formation of manganipyrophosphate.

The composition of the reaction mixtures and results are given in Table 6. On adding the peroxidase from the side arm there was a short lag of about 2–3 min. before the oxygen uptake commenced both with the complete reaction mixture and where *p*-cresol was omitted. Whereas the presence of *p*-cresol with peroxidase in the reaction mixture from which Mn^{2+} had been omitted considerably reduced the rate of oxygen uptake, in the presence of Mn^{2+} the inhibiting effect of *p*-cresol was negligible.

Table 6. *The oxidation of manganese during the oxidation of phenylacetaldehyde by peroxidase*

(Reaction mixtures consisted of 0·1 ml. 0·2 M-phenylacetaldehyde, 0·2 ml. 0·1 M-$MnSO_4$ and 0·3 ml. 0·001 M-*p*-cresol in 0·33 M-pyrophosphate at pH 7. After equilibration 50 μg. horseradish peroxidase of P.Z. 485 in 0·2 ml. of water were added from the side arm. Two hr. after the peroxidase addition the manometers were gassed with N_2 and 0·2 ml. saturated hydrazine sulphate was added from the second side arm.)

Substance omitted from reaction mixture	Uptake of O_2 (μl.)	Output of N_2 with hydrazine (μl.)
None	346	28·5
p-Cresol	349	24
$MnSO_4$	75	6
Peroxidase	38	4
$MnSO_4$ and *p*-cresol	127·5	4
p-Cresol and peroxidase	49·5	6·5

Where Mn^{2+} was present in the absence of peroxidase a considerable oxygen uptake was obtained but no pink coloration was observed in the reaction mixtures. The Mn^{2+}, at the high concentration used, catalyses the autoxidation of the aldehyde but does not lead to the accumulation of manganipyrophosphate. With both the complete reaction mixture and that from which *p*-cresol was omitted a pink colour developed and appeared to increase in intensity as the reaction proceeded. After gassing with nitrogen the addition of hydrazine discharged this pink colour and the largest gas output (presumably nitrogen, Kenten & Mann, 1949) was obtained from the complete reaction mixture and the mixture from which *p*-cresol was omitted. These results suggest that oxidation of Mn^{2+} can take place during the reaction and leads to the accumulation of manganipyrophosphate. The accumulation of oxidized manganese was, however, small being equivalent to 0·28 and 0·24 mg. Mn^{3+} in the complete reaction mixture and in the mixture from which only *p*-cresol was omitted, respectively. Since phenylacetaldehyde slowly reduces manganipyrophosphate at pH 7 the amounts of manganipyrophosphate formed are probably somewhat greater than those found experimentally.

Specificity. The aldehydes previously tested with pea-seedling sap were tested at a concentration of 3 mg./ml. with horseradish peroxidase of P.Z. 485 and 2·75 μg. Mn^{2+} in orthophosphate at pH 7. In 2 hr. a small oxygen uptake was obtained with *iso*-butyraldehyde. Under the conditions used for Fig. 6, 1-naphthylacetaldehyde was oxidized by peroxidase and Mn^{2+}, but with this substrate there was a short lag period of about 5–10 min. before the oxygen uptake started and the rate of oxidation was less than with phenylacetaldehyde. Peroxidase in the absence of Mn^{2+} catalysed the oxidation though at a slower rate.

The course of the oxidation

A study has been made of the oxygen uptake and the oxidation products when pea-seedling sap and horseradish peroxidase plus Mn^{2+} oxidize phenylacetaldehyde. The results suggest that benzaldehyde and formic acid are two of the products while the formation of hydrogen peroxide as an obligatory intermediate is suggested by the inhibiting effect of catalase. Most of the results have been got using peroxidase plus Mn^{2+} as catalyst of the oxidation. With pea-seedling sap, particularly when large amounts of sap were used, aldehyde estimations by bisulphite titration were unsatisfactory owing to the large blank and indefinite end point.

Oxygen uptake and carbon dioxide output. With the amounts of pea-seedling sap and horseradish peroxidase used, the initial rate of oxygen uptake was rapid until a value approaching 1 mol. O_2/mol. phenylacetaldehyde was reached, when the rate of uptake fell off rapidly. Generally, 4–6 hr. was required for the total uptake to reach 0·9–1 mol. oxygen/mol. aldehyde. The oxygen uptake then continued at a slow rate varying between 4 and 10 μl. oxygen/hr. in different experiments. Increasing the amount of pea sap or peroxidase and Mn^{2+} present initially or adding second amounts from the side arm after the initial rapid uptake was complete gave increases of only 5–10 μl. oxygen in 6 hr. Typical curves showing the rate of oxygen uptake are shown in Fig. 6.

It would appear that the first stage of the reaction is enzyme catalysed and leads to the consumption of 1 mol. oxygen/mol. phenylacetaldehyde. The secondary slow oxygen uptake is presumably due to further oxidation of the primary reaction products and in view of the rate may not be enzyme catalysed.

Small amounts of carbon dioxide were apparently produced. Using Warburg's direct method the final respiratory quotients determined at oxygen uptakes between 0·9 and 1 mol. oxygen/mol. phenylacetaldehyde were 0·05–0·1 with pea-seedling sap and 0·02–0·04 with horseradish peroxidase and Mn^{2+}. Where potassium hydroxide was present in the inner cup slight yellowing of the filter-paper wick showed that some aldehyde had been

lost from the solution. Control experiments with phenylacetaldehyde in orthophosphate at pH 7·5 in the main vessel showed that this loss could amount to 5–10 % in 6 hr. Since the oxidation proceeds rapidly in the initial stages the actual loss during the enzyme reaction was probably less than this.

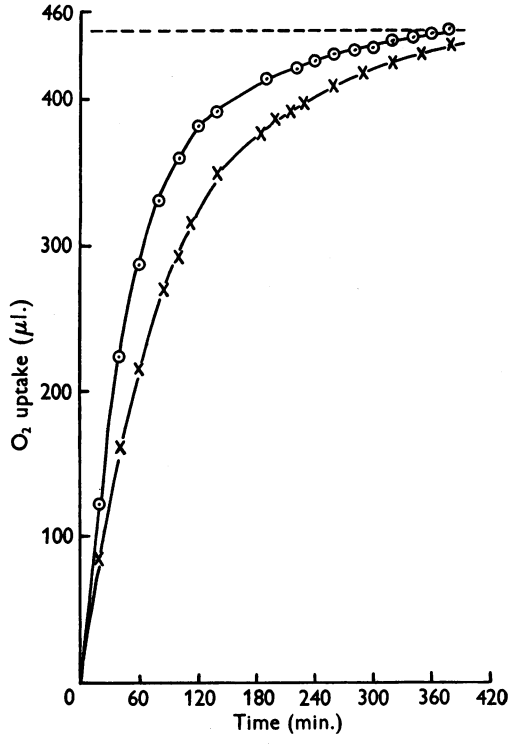

Fig. 6. The oxidation of phenylacetaldehyde by pea-seedling root sap and horseradish peroxidase plus Mn^{2+}. Reaction mixtures consisted of 0·1 ml. of 0·2 M-phenylacetaldehyde in ethanol in 0·067 M orthophosphate at pH 7·5 and 0·5 ml. of 0·0001 M-$MnSO_4$ where peroxidase was used. After equilibration 0·5 ml. of pea-seedling root sap or 100 μg. of horseradish peroxidase of P.Z. 485 in 0·1 ml. of water were added from the side arm. KOH was present in the inner cup. ⊙—⊙, Sap; × — ×, peroxidase plus Mn^{2+}. The dotted line shows the uptake for 1 mol. O_2/mol. aldehyde.

The formation of benzaldehyde. The oxidation of phenylacetaldehyde by pea-seedling sap and horseradish peroxidase plus Mn^{2+} is accompanied by the formation of a product giving an absorption maximum at 250 mμ. A typical set of curves obtained, using horseradish peroxidase plus Mn^{2+}, are shown in Fig. 7. In this experiment a reaction mixture identical to that used for Fig. 6 was shaken in air at 25°. Samples (0·4 ml.) were pipetted at intervals into 24·6 ml. of water at 0° and the ultraviolet-absorption curve of this solution was determined, using in the blank cell a suitably diluted mixture from which phenylacetaldehyde had been omitted.

In other experiments with a similar reaction mixture on a larger scale, the reaction was stopped when the O_2 uptake had reached a value of about 1 mol. O_2/mol. aldehyde, by cooling the mixture to 0°. Steam distillation of 0·5 ml. portions in the Markham (1942) still was carried out collecting exactly 50 ml. distillate. The ultraviolet-absorption curve was determined using a distillate from a suitable enzyme-phosphate mixture in the blank cell. The absorption curve of these distillates was very similar to that given by benzaldehyde in water, Fig. 8. That benzaldehyde is a product of the oxidation was confirmed by the isolation of benzaldehyde 2:4-dinitrophenylhydrazone from large-scale reaction mixtures (p. 358).

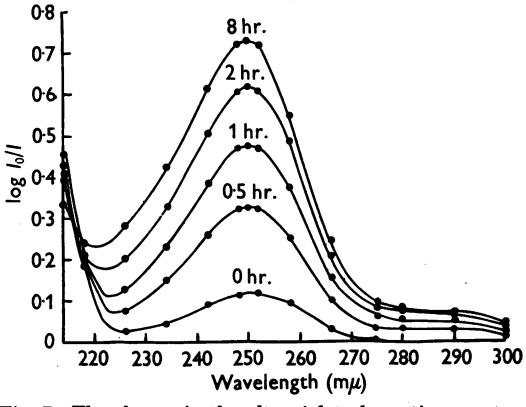

Fig. 7. The change in the ultraviolet-absorption spectrum when phenylacetaldehyde is oxidized by horseradish peroxidase plus Mn^{2+}. Reaction mixture as in Fig. 6.

Further proof that benzaldehyde was the product causing most of the absorption at 250 mμ. was shown by the change in the absorption curve to one closely resembling benzoic acid when the final reaction mixture was treated with Ag_2O. Many aldehydes are quantitatively oxidized to the corresponding acid by this reagent (Bailey & Knox, 1951; Smith & Mitchell, 1950).

One ml. of final reaction mixture and 1 ml. of a suspension of freshly precipitated Ag_2O (50 mg./ml.) were incubated at 37° overnight. After centrifuging, 1 ml. of the supernatant, together with 3 ml. 4 N-H_2SO_4, was steam distilled and 50 ml. distillate collected. The absorption curve determined against a suitable blank distillate was practically identical with that of a solution of benzoic acid in water (Fig. 8). It was found by measuring the absorption at 250 mμ. that benzaldehyde solutions in water obeyed Beer's Law over the range 1·5–6 μg./ml. and log ε was 4·10. Furthermore, at 226 mμ. log ε of benzoic acid in water was found to be 3·97 at a concentration of 5·6 μg./ml. These results permit the following calculations. Assuming the absorption at 250 mμ. is entirely due to benzaldehyde, then the concentration of benzaldehyde would be 0·37 mg./ml. in the final reaction mixture and 0·35 mg./ml. in the steam distillate. From the absorption at 226 mμ. after Ag_2O treatment and steam distillation, 0·46 mg. benzoic acid/ml. is present suggesting the presence of 0·40 mg. benzaldehyde/ml. in the final reaction mixture. If the contents of the still remaining after

the initial steam distillation of the neutral reaction mixtures are acidified with 3 ml. 4N-H_2SO_4 and distillation is continued until a further 50 ml. distillate is obtained the absorption curve of this solution (Fig. 8) suggests that 0·11 mg./ml. of benzoic acid may be present in the final reaction mixture. The value obtained above for benzoic acid formed by the Ag_2O treatment is, therefore, too high. The corrected value would be 0·35 mg. benzoic acid/ml., which is equivalent to 0·30 mg. benzaldehyde/ml.

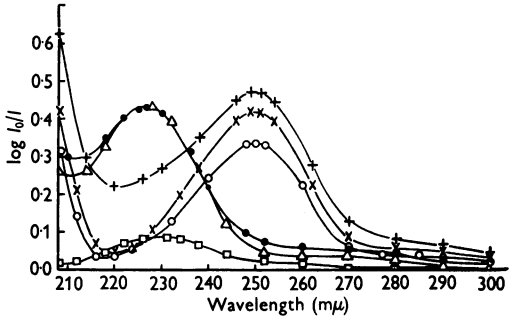

Fig. 8. Ultraviolet-absorption spectra of reaction mixture of horseradish peroxidase, Mn^{2+} and phenylacetaldehyde after steam distillation and treatment with Ag_2O. +—+, Reaction mixture; ×—×, steam distillate of reaction mixture; O—O, benzaldehyde 70 μg./25 ml.; ●—●, steam distillate of acidified Ag_2O-treated reaction mixture; △—△, benzoic acid 140 μg./25 ml.; □—□, steam distillate of acidified reaction mixture obtained subsequent to a preliminary distillation at neutral pH. Only a few of the experimental points are plotted for the sake of clarity.

The agreement between these values suggests that measurement of the absorption of reaction mixtures at 250 mμ. can be used to estimate (probably to ± 20 %) the amount of benzaldehyde present. The values given for the rate of benzaldehyde formation in Table 7 have been calculated using the absorption at 250 mμ. and are corrected for the absorption of the phenylacetaldehyde present, assuming that solutions of phenylacetaldehyde obey Beer's Law and that a molecule of phenylacetaldehyde is lost per molecule of benzaldehyde formed.

The formation of formic acid. The oxidation of phenylacetaldehyde to benzaldehyde requires the formation of an equivalent amount of a compound containing one carbon atom. The evidence suggests that this compound is formic acid.

The results for formic acid and steam-volatile acid formation given in Table 7 were obtained in the following way. A reaction mixture of the same composition as that used for Fig. 6 was shaken in air at 25°, and 10 ml. samples were pipetted at intervals into 5 ml. of 4N-H_2SO_4 to stop the reaction. Portions (7 ml.) of these acidified samples were distilled in the Markham (1942) still collecting two successive 50 ml. of distillate. The distillates were titrated to pH 7·5 with 0·01N-$Ba(OH)_2$ to measure the total steam-volatile acid present. The first 50 ml. of distillate, which contained 80–85 % of the total acid, was concentrated and used for the estimation of formic acid. By distilling control mixtures of formic acid, orthophosphate and H_2SO_4 it was found that an average of 85 % of the formic acid was recovered in 50 ml. distillate when distillation was carried out from a volume of 7 ml. The values for formic acid have accordingly been corrected on this basis. The total steam-volatile acid finally present was some 20–30 % greater than the amount of formic acid present, suggesting that small amounts of other acids, possibly phenylacetic or benzoic acid, are formed during the reaction. Distillation of control mixtures of benzoic or phenylacetic acid with orthophosphate and H_2SO_4 gave recoveries 95 and 62 % respectively in 100 ml. of distillate.

Loss of aldehyde. The total amount of carbonyl compounds present in reaction mixtures containing phenylacetaldehyde, horseradish peroxidase and Mn^{2+} decreased as the oxygen uptake proceeded. In general, under the conditions given in Table 7, 25–35 % of the total carbonyl content was lost in 6–8 hr.

Isolation of benzaldehyde 2:4-dinitrophenylhydrazone. A reaction mixture containing 220 mg. phenylacetaldehyde, 4 ml. 0·001M-$MnSO_4$, 5 mg. horseradish peroxidase of P.Z. 485 and 100 ml. 0·2M-orthophosphate at pH 7·5, in a total volume of 300 ml. was shaken in air at room temperature for 8 hr. The mixture was left at 0° overnight and then filtered. Samples were removed at intervals and the benzaldehyde

Table 7. *Oxygen uptake and the formation of benzaldehyde, formic acid and steam-volatile acid during the oxidation of phenylacetaldehyde by peroxidase plus* Mn^{2+}

(The O_2 uptake was followed manometrically in reaction mixtures of 3 ml. consisting of 0·1 ml. 0·2M phenylacetaldehyde in ethanol, 0·5 ml. 0·0001M-$MnSO_4$ and 100 μg. horseradish peroxidase of P.Z. 485 in 0·067M orthophosphate, pH 7·5. Reaction mixtures of the same composition but on a larger scale were shaken in air at 25° and samples withdrawn at intervals for the estimation of benzaldehyde, formic acid, steam-volatile acid and total carbonyl compounds.)

Time (hr.)	Uptake of O_2 (μmoles)	Benzaldehyde (μmoles)	Formic acid (μmoles)	Steam-volatile acid (μmoles)	Total carbonyl compounds (μmoles)
0	0	0	0·3	0·9	20·2
1	9·6	5·8	4·7	5·5	18·2
2	14·4	8·1	6·5	8·4	16·9
4	17·9	9·2	8·4	10·4	15·6
8	19·0	9·9	8·8	12·5	13·4

content estimated spectrophotometrically; such estimations showed a total of 67 mg. of benzaldehyde in the residual filtrate (263 ml.). To this filtrate 100 ml. of a saturated solution of 2:4-dinitrophenylhydrazone in 2N-HCl were added, and the mixture was allowed to stand for 15 min. The orange precipitate was centrifuged off, washed with water on the centrifuge, and then transferred with hot ethanol to a tared beaker. Removal of the ethanol and drying *in vacuo* gave 261 mg. of orange-brown product. This was suspended in boiling benzene and freed from an insoluble brown material by filtration. Concentration of the filtrate yielded 49·5 mg. of orange crystals, m.p. 229–230°. Mixed m.p. with an authentic sample of benzaldehyde 2:4-dinitrophenylhydrazone (m.p. 233–234°) was 231–232°. (All m.p.'s uncorr.) The yield based on benzaldehyde formed was 27%. A second crop of 22 mg. orange crystals, m.p. 203–208°, was obtained from the mother liquor.

DISCUSSION

The results of the present work show that many plant saps catalyse the oxidation of phenylacetaldehyde by oxygen. The system responsible for this oxidation consists of, in pea-seedling sap, a thermolabile and a thermostable factor. The evidence suggests that the thermolabile factor is a peroxidase. It can be replaced by horseradish-peroxidase preparations, and in agreement with the thermolabile properties of peroxidase reported by Gallagher (1924) and Herrlinger & Kiermeier (1944) prolonged heating of the natural system at 100° is necessary for inactivation. Furthermore, the inhibiting effect of catalase suggests that the reaction is peroxidatic. The thermostable factor can be partially replaced by Mn^{2+}, and using horseradish peroxidase plus Mn^{2+} it is possible to construct a system with similar properties to that in pea-seedling sap. The thermostable factor in pea sap, however, is not identical with Mn^{2+} since addition of this factor to peroxidase gives a system of much greater activity than addition of Mn^{2+} at the concentration contained in the thermostable factor. Although the thermostable factor in the natural system has not been identified, the experiments with ion-exchange resin suggest that the activity of this factor is partially dependent on a substance or substances which are positively charged in weakly acid solution, and it is possible that the activity is due to a cationic complex of manganese which is more effective than Mn^{2+}.

The mechanism of the reaction is obscure, but the formation of hydrogen peroxide as an obligatory intermediate is suggested by the inhibiting effect of catalase. Hydrogen peroxide accumulation during the reaction has not been demonstrated. Chance (1952) has suggested that a Mn^{2+}-activated peroxidase-peroxide complex is the catalyst for the oxidase reaction with dihydroxymaleic acid, peroxidase and Mn^{2+}, and it is possible that a similar complex catalyses the oxidation of phenylacetaldehyde. The results of Kenten & Mann (1953) suggested that the oxidation of certain dicarboxylic acids by peroxidase and Mn^{2+} was dependent on manganese oxidation. While manganese oxidation can be demonstrated when phenylacetaldehyde is oxidized by peroxidase in pyrophosphate at high Mn^{2+} concentrations, it may be that oxidation of manganese is a side reaction. Neither hydrated manganese dioxide nor manganipyrophosphate appear to oxidize phenylacetaldehyde rapidly at neutral pH and, if manganese oxidation plays a part in the reaction, the oxidized manganese must react with an intermediate product. In contrast to the peroxidase systems studied by Kenten & Mann (1950, 1952) manganese oxidation takes place in the absence of a phenolic compound such as *p*-cresol suggesting the formation either of a manganocomplex which is directly oxidized by peroxidase, or of phenolic compounds during the reaction. Manganese catalyses the autoxidation of phenylacetaldehyde though at a very slow rate unless present in high concentration. Such autoxidation if it leads to the production of hydrogen peroxide may accelerate the reaction.

The oxidation of phenylacetaldehyde by pea-seedling sap or horseradish peroxidase and Mn^{2+} proceeds with the formation of benzaldehyde and formic acid as two of the products. Under the experimental conditions used, the oxygen uptake is rapid until about 0·9 mol. oxygen/mol. aldehyde is consumed when the oxygen uptake falls to a low rate. When the oxygen uptake has reached a value of 0·9–1·0 mol. oxygen/mol. phenylacetaldehyde, approximately 0·5 mol. benzaldehyde has been formed per mol. phenylacetaldehyde initially present. Some phenylacetic acid may be formed since the values for steam-volatile acid are somewhat greater than can be accounted for as formic acid, but the amount is not sufficient to resolve the discrepancy of about 0·5 mol. between the phenylacetaldehyde initially present and the benzaldehyde formed. The loss of aldehyde which occurs as the reaction proceeds shows that the reaction does not consist only of the conversion of phenylacetaldehyde to benzaldehyde and formic acid.

Evidence has been obtained that 1-naphthylacetaldehyde is converted to 1-naphthylacetic acid by *Avena*-coleoptile juice (Larsen, 1951) and by *Artemisia*-seedling-root juice (Ashby, 1951). Larsen (1951) found 1 mol. acid was formed per 2 mol. aldehyde added and suggested that the conversion process might be a dismutation in which equivalent amounts of alcohol and acid were formed. Ashby (1951), however, found considerably less acid formation than 1 mol. per 2 mol. aldehyde and he concluded that the root juice was able to change 1-naphthylacetaldehyde in some manner other than by oxidation or a dismutation to 1-naphthylacetic

acid. The results of Ashby (1951) are in better agreement with the results of the present work, if it is assumed that the oxidation of phenylacetaldehyde and 1-naphthylacetaldehyde proceed in the same way. It has been shown in the present work that 1-naphthylacetaldehyde is oxidized by both pea sap and horseradish peroxidase plus Mn^{2+}. By analogy with phenylacetaldehyde it seems likely that 1-naphthylacetaldehyde and also 3-indolylacetaldehyde would be oxidized with the formation of 1-naphthaldehyde and 3-indolealdehyde, respectively.

There is indirect evidence that partial conversion of 3-indolylacetaldehyde into 3-indolylacetic acid takes place in certain intact plants and with certain plant extracts (Larsen, 1949; Gordon & Sanchez-Nieva, 1949; Ashby, 1951; Brown, Henbest & Jones, 1952). Since most plant extracts and crude plant-enzyme preparations contain peroxidase and Mn^{2+} the results of the present work suggest that *in vitro* studies of the metabolism of 1-naphthylacetaldehyde and 3-indolylacetaldehyde with such preparations may lead to the demonstration of partial conversion of these compounds to 1-naphthaldehyde and 3-indolealdehyde respectively.

It is not yet known how far the system responsible for the oxidation of phenylacetaldehyde is of significance *in vivo*. Phenylacetaldehyde is not a known plant constituent, but β-phenylethylamine is, and by the action of plant amine oxidase could form phenylacetaldehyde. The oxidation proceeds readily with both pea-seedling sap and at physiological concentrations of peroxidase and Mn^{2+} with relatively high substrate concentration, but only with difficulty at low substrate concentration. The reaction is of interest since, like the previously reported oxidations of certain dicarboxylic acids (Swedin & Theorell, 1940; Kenten & Mann, 1953), it shows that peroxidase may bring about oxidations independently of external sources of hydrogen peroxide. Peroxidase, together with the natural activating factor, may act as a direct oxidase system in the intact plant, and peroxidase action *in vivo* may, therefore, proceed in the absence of flavoprotein enzyme systems. As yet few compounds have been tested, and no well-known plant metabolite has been shown to be oxidized by peroxidase and Mn^{2+} under physiological conditions. However, preliminary results suggest that horseradish-peroxidase preparations alone will catalyse the oxidation of 3-indolylacetic acid by oxygen, while peroxidase plus Mn^{2+} is a more efficient catalyst of the reaction.

SUMMARY

1. Many plant saps oxidize phenylacetaldehyde. The system in pea-seedling sap consists of a thermolabile factor which appears to be a peroxidase and a thermostable factor which can be partially replaced by manganous ions. A system with similar properties to that in pea-seedling sap can be constructed using horseradish-peroxidase preparations and manganous ions.

2. The oxidation when catalysed by either pea-seedling sap or horseradish peroxidase and manganous ions proceeds with the formation of benzaldehyde and formic acid.

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REFERENCES

Agner, K. (1938). *Biochem. J.* **32**, 1702.
Ashby, W. C. (1951). *Bot. Gaz.* **112**, 237.
Bailey, H. C. & Knox, J. H. (1951). *J. chem. Soc.* p. 2741.
Bawden, F. C. & Pirie, N. W. (1944). *Brit. J. exp. Path.* **25**, 68.
Brown, J. B., Henbest, H. B. & Jones, E. R. H. (1952). *Nature, Lond.*, **169**, 335.
Chance, B. (1952). *J. biol. Chem.* **197**, 577.
Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.
Dixon, M. (1943). *Manometric Methods*, 2nd ed. p. 64. Cambridge University Press.
Gallagher, P. H. (1924). *Biochem. J.* **18**, 39.
Gordon, S. A. & Sanchez-Nieva, F. (1949). *Arch. Biochem.* **20**, 367.
Herrlinger, F. & Kiermeier, F. (1944). *Biochem. Z.* **317**, 1.
Jensen, K. A. & Christensen, S. A. K. (1950). *Acta chem. scand.* **4**, 703.
Keilin, D. & Mann, T. (1937). *Proc. Roy. Soc.* B. **122**, 119.
Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
Kenten, R. H. & Mann, P. J. G. (1951). *Biochem. J.* **50**, 360.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **52**, 125.
Kenten, R. H. & Mann, P. J. G. (1953). *Biochem. J.* **53**, 498.
Larsen, P. (1949). *Amer. J. Bot.* **36**, 32.
Larsen, P. (1951). *Plant Physiol.* **26**, 697.
Mann, P. J. G. (1953). Private communication.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Paterson, J. S., Pirie, N. W. & Stableforth, A. W. (1947). *Brit. J. exp. Path.* **28**, 223.
Pirie, N. W. (1946). *Biochem. J.* **40**, 100.
Skoog, F. (1937). *J. gen. Physiol.* **20**, 311.
Smith, J. & Mitchell, D. M. (1950). *Analyt. Chem.* **22**, 746.
Swedin, B. & Theorell, H. (1940). *Nature, Lond.*, **169**, 335.
Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.