Phytochemistry, 1972, Vol. 11, pp. 1371 to 1387. Pergamon Press. Printed in England.

ROLE OF INDOLYL-3-ACETIC ACID IN THE FORMATION OF ETHYLENE FROM 4-METHYLMERCAPTO-2-OXO BUTYRIC ACID BY PEROXIDASE

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(*Received* 10 *September* 1971)

Abstract—Investigation of three systems producing ethylene from 4-methylmercapto-2-oxobutyric acid in the presence of peroxidase has shown that the addition of indolyl-3-acetic acid (IAA) to two of them increases the amount of ethylene produced and is essential for the third. The system investigated in the present paper produces ethylene from the oxo acid in the presence of peroxidase, *p*-hydroxybenzoate, IAA and sulphinic acid. Of these components only sulphinic acid inhibits the oxidation of IAA, but no ethylene is produced in its absence. Indolyl-3-acetic acid leads to the production of ethylene by reason of its ability to initiate the oxidation of the sulphinic acid which in turn causes the breakdown of the oxo acid.

INTRODUCTION

INDOLYL-3-acetic acid and closely related indole derivatives have been shown to stimulate the synthesis of ethylene when applied to several plant tissues.[1-3] Many of the physiological responses, which have been observed after addition of auxins to plant tissues, have also been explained on the basis that these are due to the ethylene produced as a result of the action of the hormone.[4,5] Reactions involved in the enzymic breakdown of methionine via 4-methylmercapto-2-oxobutyric acid to ethylene in plant tissue have already been fully described.[6,7] In one system a glucose oxidase and in the other a lipoxygenase enzyme is involved with their respective substrates reacting in conjunction with a peroxidase and its co-factors. The observation that the addition of indolyl-3-acetic acid to either system increased the rate of production of ethylene from the oxo acid and that ethylene production could also occur in the absence of peroxide or hydroperoxide provided IAA was present, prompted us to investigate the cause and make an attempt at determining the mechanism involved in the three systems.

RESULTS

Oxidation of Indolyl-3-acetic Acid and Production of Ethylene from 4-Methylmercapto-2-oxo Butyric Acid

Indolyl-3-acetic acid (IAA) was only slowly oxidised in the presence of low concentrations of peroxidase in phosphate buffer at pH 5·5. Oxidation of the indole was, however, greatly increased on the addition of a suitable phenol, in our experiments *p*-hydroxybenzoate

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or 2-4-dichlorophenol. Observations of the stimulating effect of phenolic compounds on oxidation of IAA by peroxidase enzymes are well authenticated.[8,9]

Our interest in these reactions began from the observation that IAA in the presence of peroxidase, *p*-hydroxybenzoate and a sulphinic acid will catalyse the production of ethylene from 4-methylmercapto-2-oxo butyric acid (oxo acid) or from methional (3-methylmercapto-propionaldehyde). Further investigation revealed that this stimulating effect on the indole compound was dependent on a number of factors which included the concentration of indolyl-3-acetic acid, of enzyme, of phenolic and sulphinic acid and pH and oxygen tension. The effect of each of these has been examined separately on, (1) the oxidation of indolyl-3-acetic acid by peroxidase, and (2) on the production of ethylene from 4-methylmercapto-2-oxo butyric acid or methional when a sulphinic acid was also present.

Concentration of Peroxidase and Phenolic Acid

Both the rate of oxidation of IAA and the production of ethylene from the oxo acid or methional in the presence of a sulphinic acid are linearly dependent on the concentration of the peroxidase. Whereas, however, the rate of oxidation of the indole was directly related to the concentration of enzyme, this direct relation was only observed in the rate of production of ethylene in the first few minutes of the reaction. Over longer periods relatively more ethylene was produced with higher concentrations of enzyme. This difference between the two reactions appears to be connected with the progress of the reactions, for whereas the rate of oxidation of IAA in the presence of *p*-hydroxybenzoate does not increase as the reaction proceeds, the graph relating ethylene production with time is sigmoid in shape (Fig. 1). A direct linear relationship between rate and enzyme concentration can therefore only be observed in the early stages, which in turn suggests that peroxidase initiates but does not control the subsequent reactions. This viewpoint receives support from the fact that the enzymic oxidation of IAA is always completed well before ethylene production ceases. Thus, in one experiment, (Fig. 1) with a concentration of enzyme of 3 units, *p*-hydroxybenzoate (0·5 mM) and sulphinic acid in a concentration of 0·1 mM, oxidation of the indole (0·1 mM) was completed within 12 min, whereas ethylene production from the oxo acid (1 mM) continued for a further 15 min.

There was a direct linear relationship between the concentration of the phenolic acid (*p*-hydroxybenzoate) and the rate of oxidation of IAA at least over a 0–1·0 mM concentration range. An increase in the rate of production of ethylene from the oxo acid in the presence of the sulphinic acid also occurred under these conditions. A further increase in the concentration of benzoate leads, however, to a decrease in the rate of both reactions (Fig. 2). An essentially similar state of affairs existed when 2,4-dichlorophenol (DCP) was substituted for the benzoate, except that the optimum rate obtained and inhibitory effects of the phenol on both reactions were apparent at a much lower concentration (Fig. 2). Dichlorophenol also appeared more efficient than *p*-hydroxybenzoate in both reactions. The close agreement between these effects of the phenols suggests that their stimulating and inhibitory influences were primarily due to their role in modifying the rate of oxidation of the indole.

In the absence of a phenol and with a high enzyme level (9 units) IAA was oxidized and ethylene produced from the oxo acid on addition of a sulphinic acid, but both reaction rates were slow. Thus, 3 μM indole was oxidized per min with 9 units peroxidase in 0·1 M phosphate buffer, pH 5·5, and 57 μM indole oxidized when 0·5 mM *p*-hydroxybenzoate was also

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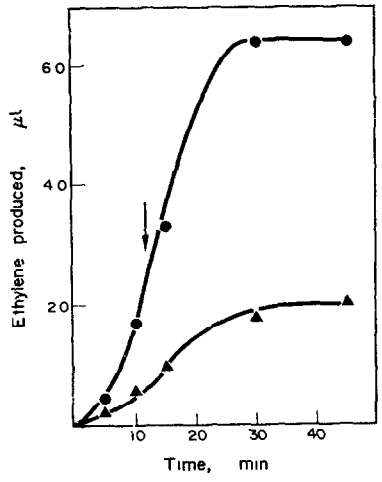


FIG. 1.

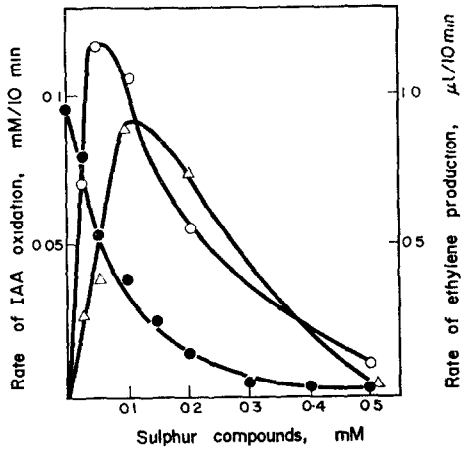


FIG. 3.

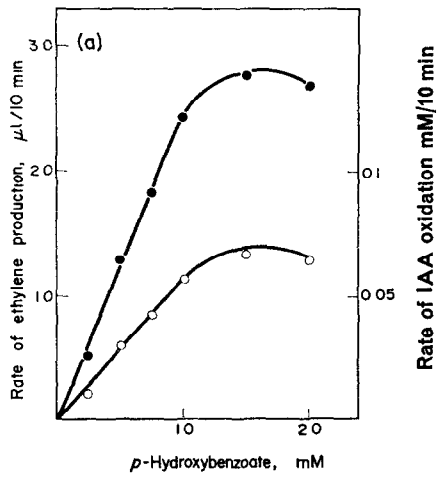
FIG. 1. THE RELATION BETWEEN CONCENTRATION OF PEROXIDASE AND THE RATE OF ETHYLENE PRODUCTION.

Flasks contained IAA (0·1 mM) *p*-hydroxybenzoate (0·5 mM) benzene sulphinic acid (0·1 mM) oxo acid (1 mM) and peroxidase in a total vol. of 10 ml 0·1 M-sodium phosphate buffer, pH 5·5 containing EDTA (2 mM). ●—peroxidase 3 units; ▲—peroxidase 1·5 units. ↓ denotes completion of IAA oxidation with sulphinic acid present.

FIG. 3. THE RELATION BETWEEN CONCENTRATION OF SULPHINIC ACID OR SULPHITE AND THE RATE OF ETHYLENE PRODUCTION AND THE RATE OF OXIDATION OF IAA.

Flasks contained IAA (0·1 mM), *p*-hydroxy benzoate (0·5 mM), benzene sulphinic acid or sulphite, oxo acid (1 mM) and peroxidase (1·5 units). ○—production of ethylene with sulphinic acid; △—with sulphite; ●—oxidation of IAA with sulphinic acid.

present. Similarly, the rate of ethylene production from the oxo acid was increased ten-fold by the addition of the benzoate. This rate, however, continued for only a short period, whereas the slow rate from the reaction lacking the phenol kept on for over 1 hr.



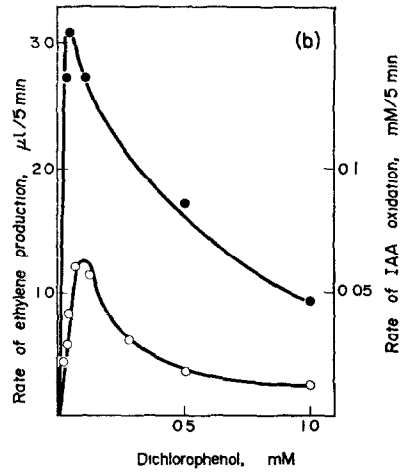


FIG. 2. THE RELATION BETWEEN CONCENTRATION OF PHENOLIC COMPONENT AND THE RATE OF OXIDATION OF IAA AND THE RATE OF ETHYLENE PRODUCTION.

○—oxidation of IAA with IAA (0·1 mM), phenolic, benzene sulphinic acid (0·1 mM) and peroxidase (1·5 units); ●—production of ethylene witth oxo acid (1 mM).

Concentration of Indolyl-3-acetic Acid

The stimulating effect of the indole on the production of ethylene from the oxo acid by peroxidase and co-factors was observed only over a narrow range of concentration. With *p*-hydroxybenzoate (1 mM) and benzene sulphinic acid (0·1 mM) optimum stimulation was observed with the indole in a concentration of 0·05–0·1 mM, decreasing as the concentration of the indole was raised or lowered (Table 1). No ethylene was produced at all in concentrations of the indole of 0·5 mM or higher. An increase in the concentration of enzyme merely shifts the optimum level to higher values, but does not otherwise alter qualitatively the general response obtained.

Various possibilities exist to explain the inhibitory effect of the indole at these higher concentrations. The most obvious, that the oxidation products of the indole, such as 3-hydroxy methyloxindole and methyloxindol, were inactivating the peroxidase enzyme, was disproved on showing that the rate of production of ethylene from the oxo acid was unaffected in their presence. This was achieved by forming the oxidation products by the action of the peroxidase on IAA in the presence of the phenolic, then inactivating any peroxidase by short heat treatment and showing that the residual solution had no inhibitory effect by comparing the rate of ethylene produced by it and a similar solution devoid of oxidation products.

Some clue as to the nature of the inhibition by higher concentrations of IAA was obtained by the observation that the inhibition may be offset by increasing the concentration of the *p*-hydroxybenzoate. Thus, with concentrations of IAA in the range 0·05–0·25 mM, the same rate of ethylene production may be achieved throughout the range if the ratio of the concentration of phenolic to that of the indole is increased exponentially (Table 1). Higher concentrations of the indole than 0·25 mM could not be tested because of the limit imposed by the solubility of the phenol. The significance of the results is alluded to later in this paper.

Tests carried out to determine if a similar competitive element existed between the sulphinic acid and IAA have yielded negative results. In these experiments the concentration of the phenol was held constant and both IAA and sulphinic acid varied (Table 1).

Specificity of Reaction

When peroxidase acts as an oxygenase, as in these reactions, the specificity appears to be absolute for indolyl-3-acetic acid. Not even the closely related indolyl-3-propionic and indolyl-3-butyric acids are capable of initiating ethylene and correspondingly no oxidation occurs on the addition of the indoles to the peroxidase enzyme. Similarly, the unnatural auxins, 1- and 2-naphthyl acetic acids and 2,4- and 2,4,5-trichlorophenoxyacetic acids are also not oxidized.

Influence of Sulphinic Acid

The presence of a sulphinic acid or a sulphite is an absolute requirement for the production of ethylene from either the oxo acid or methional in all the enzymic systems so far described in which peroxidase participates.[10,11,7] The production of ethylene by the

[9] R. H. KENTEN, *Biochem. J.* **59**, 110 (1955).
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TABLE 1. THE EFFECT OF INCREASING *p*-HYDROXYBENZOATE OR BENZENE SULPHINIC ACID TO OFFSET THE INHIBITION ON THE PRODUCTION OF ETHYLENE AT HIGHER INDOLYL-3-ACETIC ACID CONCENTRATIONS

	IAA (mM)	*p*-Hydroxybenzoate (mM)	Benzene sulphinic acid (mM)	Ethylene produced (μl/10 min)
Expt. 1a				
	0·025	1·0	0·1	0·065
	0·05	1·0	0·1	1·02
	0·1	1·0	0·1	0·88
	0·17	1·0	0·1	0·56
	0·25	1·0	0·1	0·32
	0·5	1·0	0·1	0·04
Expt. 1b				
	0·05	0·5	0·1	0·7
	0·1	1·0	0·1	1·4
	0·25	2·5	0·1	1·0
	0·5	5·0	0·1	0·38
Expt. 1c				
	0·05	0·6	0·1	1·26
	0·1	1·0	0·1	1·4
	0·17	2·14	0·1	1·24
	0·25	5·0	0·1	1·3
Expt. 2a				
	0·1	0·5	0·1	1·1
	0·1	0·5	0·2	0·2
	0·1	0·5	0·5	0·08
Expt. 2b				
	0·5	0·5	0·1	0·06
	0·5	0·5	0·2	0·07
	0·5	0·5	0·5	0·07

The complete system contained benzene sulphinic acid, oxo acid (1 mM), *p*-hydroxybenzoate, IAA and horse radish peroxidase (1·5 units) in 10 ml of 0·1 M sodium phosphate buffer, pH 5·5 containing EDTA (2 mM).

peroxidase–IAA system is no exception. Whilst, however, these sulphur compounds are essential, too high a concentration of either compound inhibits the production of the hydrocarbon. The results of experiments in which the production of ethylene was followed when sulphinate or sulphite was present in varying concentrations are shown in Fig. 3. The data indicate a stimulation of the reaction with low concentrations of sulphinic or sulphite and a maximum production of ethylene with the acid in a concentration of 0·05 mM and sulphite at 0·1 mM, when peroxidase was present in a concentration of 1·5 units, IAA 0·1 mM and *p*-hydroxybenzoate 0·5 mM. At higher concentrations both sulphur compounds inhibited strongly. The efficiency of the sulphinic acid for the production of ethylene was higher than that of the sulphite. Similar results were obtained when methional replaced the oxo acid as the source of ethylene, although the efficiency of the sulphite for the production of ethylene was markedly reduced.

The rate of oxidation of IAA by peroxidase in the presence of *p*-hydroxybenzoate was also affected by the sulphinic acid. In this case, however, the inhibition increased progressively with an increase in concentration of the sulphinic acid and between 0·3 and 0·5 mM the rate of oxidation was almost reduced to zero. These were the same concentrations of sulphinic acid which reduced the production of ethylene from the oxo acid to negligible amounts. In no case did we observe any stimulation of the oxidation of IAA with low concentrations of sulphinic or sulphite.

There is thus further evidence of the dependence of this ethylene producing system on the ability of peroxidase to oxodize IAA. The fact that low concentrations of sulphinic acid stimulate the former but reduce the rate of the latter seems to indicate that at this level the sulphinic is present in a sufficient concentration to exert its catalytic influence on the production of ethylene, whilst not completely inhibiting the oxidation of IAA. At higher concentrations the oxidation is increasingly inhibited and this then limits the production of ethylene. These observations suggest that the sulphinic acid influences these reactions in two distinct ways.

Higher amounts of sulphinic acid can be tolerated by the ethylene producing system provided that the concentration of *p*-hydroxybenzoate, IAA and peroxidase are also increased. Thus, in one experiment 0·46 μl ethylene were produced from the oxo acid (1 mM) in 1 hr with 0·5 mM *p*-hydroxybenzoate, 0·5 mM sulphinic acid, 0·1 mM IAA and 1·5 units peroxidase. Increasing the benzoate to 5 mM and IAA to 0·25 mM whilst keeping the peroxidase at the same level produced 0·9 μl ethylene and by increasing the peroxidase to 9 units with the higher indole and benzoate, 7·3 μl ethylene were produced from the oxo acid.

Oxygen Tension

The dependence of ethylene production from the oxo acid on the oxidation of IAA by peroxidase is also shown by the effect of altering the partial pressure of oxygen on the two reactions. In nitrogen, no oxidation of IAA occurred in the presence of enzyme and phenolic and no ethylene was produced on addition of the sulphinic and oxo acids. Increasing the oxygen from 2 to 20% had very little effect on the rate of oxidation of IAA by peroxidase in the presence of the benzoate, although a decreased rate occurred in pure oxygen. When, however, a sulphinic acid was also present, there was first an increase in the rate of oxidation of the indole as the oxygen tension was increased from 2 to 5% and that was followed by a decrease in the rate, as the oxygen tension was further increased to 100%. The rate of production of ethylene was likewise affected. The relation between rate of oxidation of IAA in the presence of a fixed concentration of sulphinic acid and rate of ethylene production from the oxo acid was linear in relation to varying oxygen tension. This linear relation between oxygen tension and the rate of both reactions is further confirmation of the dependence of ethylene production in this system on the oxidation of the indole.

Further examination showed that increasing oxygen tension from 5 to 100% had merely reduced the concentration of sulphinic acid which was required to yield the maximum amount of ethylene. The relation between total production of ethylene, sulphinic acid concentration and oxygen tension is illustrated in Fig. 4; in 5% oxygen, maximum production of ethylene occurred with sulphinic at 0·1 mM, in air, at almost half this concentration and in pure oxygen, at 0·025 mM. Only in pure oxygen was the total production of ethylene at the optimum, below that at lower oxygen tensions, and the cause of this appears to be due to the much more rapid destruction of peroxidase in pure oxygen (48% loss in pure oxygen as against 8–16% loss in air, or 5% oxygen in the first 10 min of the reaction).

Effect of pH

The effect of pH on the oxidation of IAA by peroxidase in the presence of *p*-hydroxybenzoate and on the generation of ethylene from the oxo acid when sulphinic acid is present is shown in Fig. 5. Both reactions proceed at their maximum rate at approximately pH 5·7–6·0. On the acid side both fall in rate as the pH decreases from 5·7 to 4·5 and on the

alkaline.side there is a very sharp fall of ethylene production between 6·0 and 6·25; at pH 6·5 the ethylene production is negligible. Oxidation of IAA also declines over this pH range and so there is a fairly close correspondence between the effect of pH and activity of the two systems.

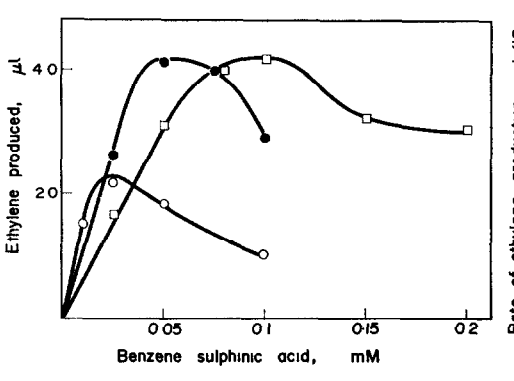


FIG. 4.

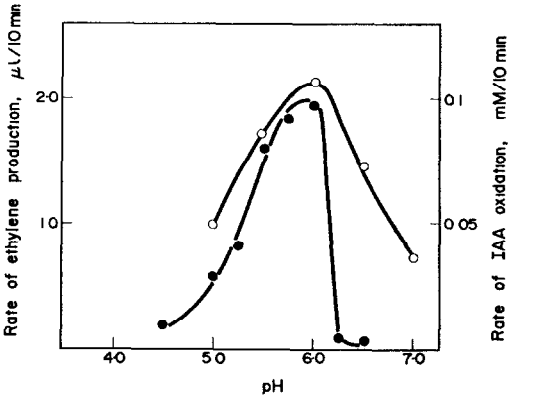


FIG. 5.

FIG. 4. THE RELATION BETWEEN CONCENTRATION OF SULPHINIC ACID AND THE RATE OF ETHYLENE PRODUCTION AT VARIOUS OXYGEN TENSIONS.
Flask contents were as in Fig. 1 with 1·5 units of peroxidase. □—5% oxygen tension, ●—20%; ○—100%.

FIG. 5. EFFECT OF pH ON THE RATE OF OXIDATION OF IAA AND ON THE RATE OF ETHYLENE PRODUCTION.
Flask contents as for Fig. 4 with varying buffer pH. ○—IAA oxidation; ●—ethylene production.

Hydrogen Peroxide

We obtained no evidence to suggest that in air hydrogen peroxide was formed either in the oxidation of IAA by peroxidase in the presence of a phenol, or in the reactions in which ethylene was produced. In neither case did the addition of catalase influence the reactions. With the ethylene producing system in 100% oxygen, however, we found that the addition of catalase did reduce the rate of the reaction, suggesting that some peroxide may have been produced.

Oxygen Uptake Studies and Oxidation of Sulphinic Acid

Analysis of the rate of oxidation of IAA, the time course of oxygen uptake and of ethylene production has been made with different initial amounts of sulphinic acid added to enzyme, phenolic (5 mM) and IAA (0·25 mM). IAA was used in this concentration in order to obtain sufficient and more reproducible oxygen consumption data. The peroxidase concentration was also increased seven-fold. This was to ensure that active enzymes remained throughout the reaction period. With weak concentrations of enzyme, inactivation of enzymes that occurs during the oxidation of IAA prevents the reaction going to completion. When sulphinic acid was added to such a solution, the increased oxygen uptake over and above that due to IAA showed that the sulphur acid was completely oxidized with the uptake of 1 atom of oxygen/mol sulphinic acid (Table 2). Subsequent experiments in which the trimethyl silyl derivatives were prepared showed that the sulphinic acid was converted to the sulphonate under these conditions. The sulphur acid thus behaves similarly to results of experiments in which oxidation of sulphite to sulphate has been studied.[10]

TABLE 2. OXYGEN UPTAKE DUE TO THE OXIDATION OF THE BENZENE SULPHINIC ACID IN THE INDOLYL-3-ACETIC ACID, PEROXIDASE SYSTEM

Sulphinic acid added (μmol)	Total O_2 uptake (μl)	O_2 uptake of sulphinic (μl)	Sulphinic acid oxidised (μmol)
0	10·5	—	—
0·3	14	3·5	0·31
0·75	19	8·5	0·76
1·5	28	17·5	1·56
3·0	46·5	36	3·2

The complete system contained IAA (0·25 mM), *p*-hydroxybenzoate (5 mM), peroxidase 2·7 units and benzene sulphinic acid in a total vol. of 3 ml of 0·1 M sodium phosphate buffer, pH 5·5. The Warburg flasks were shaken at 25° and oxygen uptake determined every 5 min until reaction complete.

The time course of the oxygen uptake and ethylene production is shown in Fig. 6. The graphs indicate that ethylene continues to be produced until all the sulphinic acid has been oxidized and, with the two highest concentrations of sulphinic acids used, there was a noticeable lag phase during which no sulphinic acid was oxidized or ethylene produced. The rate of oxidation of the indole under these conditions also showed a lag phase with the two highest concentrations of acid. The results indicate the inter-relation of these two reactions and correlate the oxidation of the sulphinic acid with the production of ethylene, a conclusion similar to that reported by Yang,[10] in which sulphite oxidation in a manganese–peroxidase model system was correlated with the production of ethylene. As already noted,

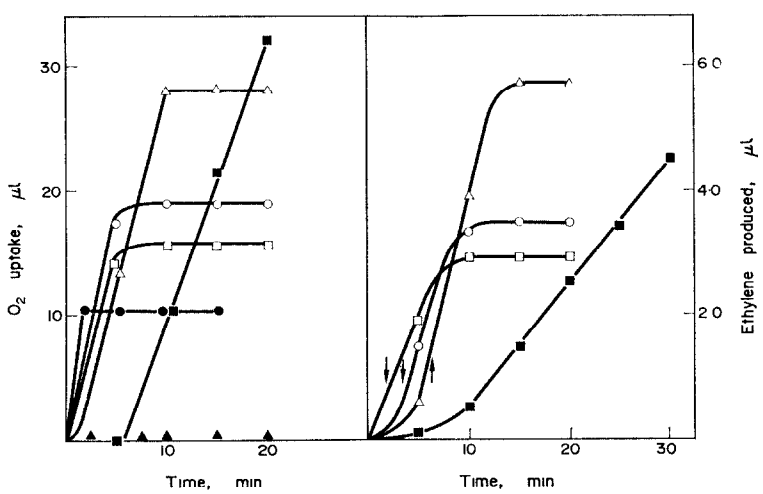


FIG. 6. THE RELATION BETWEEN O_2 UPTAKE AND THE PRODUCTION OF ETHYLENE BY INCREASING THE CONCENTRATION OF BENZENE SULPHINIC ACID.

Warburg flasks contained IAA (0·25 mM), *p*-hydroxy benzoate (5 mM), benzene sulphinic acid and peroxidase (2·7 units) in a total vol. of 3 ml buffer. Ethylene determinations were carried out with a vol. of 10 ml containing equivalent strength reagents and oxo acid (1 mM). Sulphinic acid ●—nil; □—0·1 mM; ○—0·25 mM; △—0·5 mM; ■—1·0 mM; ▲—0·5 mM and no IAA. ↓ denotes completion of IAA oxidation.

the oxidation of IAA in all cases stops well before either oxygen uptake or ethylene production ceases, which in the present context indicates that oxidation of IAA only initiates production of ethylene by reason of its ability to initiate the oxidation of the sulphinic acid.

Effect of Peroxide

The stimulation by indolyl-3-acetic acid of the reactions so far described are obtained when peroxidase is acting as an oxygenase. We have accordingly examined whether the indole so acts when peroxidase is acting as a peroxidase, i.e. in the presence of systems generating peroxide or hydroperoxide. Such an investigation is perhaps more pertinent to the events which may occur *in vivo* because the generation of ethylene in plant tissues appears to depend on the presence of peroxide producing systems.[6,7] In the experiments with lipoxygenase we have determined the production of ethylene from the oxo acid with IAA and lipoxygenase separately and then compared the summation of these results with that obtained from the complete system.

Lipoxygenase. The rate of oxidation of the indole that occurred in the presence of a phenol and peroxidase was increased by the addition of linolenate and lipoxygenase at pH 5·5, the rate of oxidation being proportional to the concentration of the lipoxygenase enzyme. If the peroxidase was omitted from the system, only a very slight oxidation occurred. The production of ethylene from the oxo acid was also increased by the addition of lipoxygenase and substrate over and above the summation of the two independent systems. This increased ethylene production (Fig. 7) was only apparent for up to 15 min and the rate was

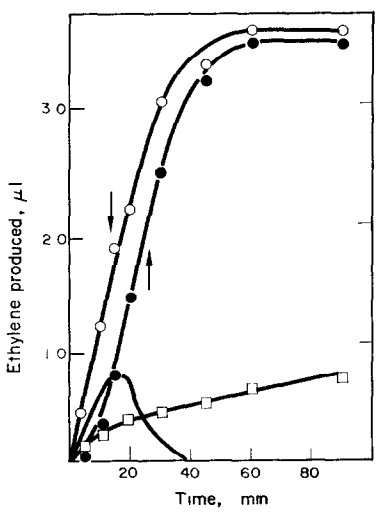


FIG. 7.

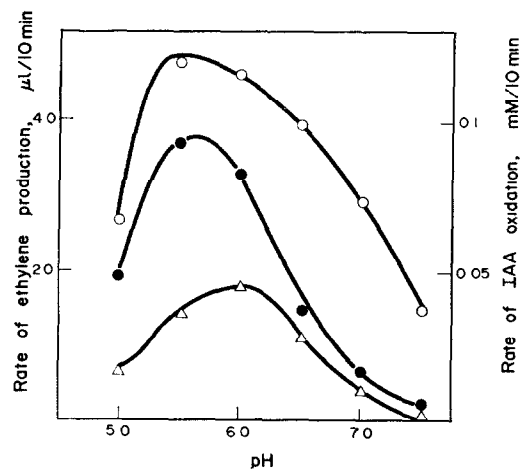


FIG. 8.

FIG. 7. EFFECT OF LIPOXYGENASE ON THE PRODUCTION OF ETHYLENE IN THE PRESENCE OF IAA. Flasks contained IAA (0·1 mM), *p*-hydroxybenzoate (0·5 mM), benzene sulphinic acid (0·1 mM), peroxidase, (1·5 units), oxo acid (1 mM), linolenate (1 mM) and lipoxygenase (13 400 units). ○—complete system; ●—no lipoxygenase; □—no IAA; —, estimated increase in ethylene in complete system over and above that formed by a summation of the individual systems. ↓ denotes completion of IAA oxidation.

FIG. 8. EFFECT OF pH AND LIPOXYGENASE ON THE RATE OF ETHYLENE PRODUCTION AND ON THE RATE OF OXIDATION OF IAA.

Flask contents for the complete system as for Fig. 7. Ethylene was also determined on samples without IAA and without lipoxygenase and the summation of these rates subtracted from the rate for the complete system. ●—complete system; △—estimated increase in ethylene; For the oxidation of IAA flasks did not contain oxo acid, ○—complete system.

proportional to enzyme concentration during this period. Over a longer period more ethylene was produced from the reaction containing IAA alone. The reason for this change is apparent when the two oxidation rates of IAA are compared. With the complete system, oxidation of IAA is faster and finished by 15 min, whereas the system without lipoxygenase takes a further 10 min before the indole is completely oxidized. No production of ethylene from the oxo acid could be observed from a system containing IAA, linolenate, lipoxygenase and the co-factors but lacking peroxidase.

The rate of oxidation of IAA by peroxidase, *p*-hydroxybenzoate and lipoxygenase was inhibited by the sulphinic acid, the inhibition increasing as the concentration of the acid was raised. However, the presence of lipoxygenase and substrate allowed a slow rate of IAA oxidation and the production of ethylene from the oxo acid to take place even with sulphinic at 0·5 mM, a concentration which would have almost completely inhibited both reactions in the simple system. The increased rate of ethylene production due to the presence of the indole and lipoxygenase went at a slower rate, but for a longer period, as the concentration of sulphinic was raised.

The presence of lipoxygenase made no difference to the inhibition of the production of ethylene that occurred with higher concentrations of the indole. Optimum stimulation was again observed with IAA at a concentration of 0·05–0·1 mM decreasing as the concentration was raised or lowered.

The effect of pH on the oxidation of IAA and the production of ethylene from the oxo acid by the addition of linolenate and lipoxygenase is shown in Fig. 8. Both reactions are at their maximum rates around pH 5·5, slightly more acidic than that found with IAA alone, but the sharp fall in the rate of ethylene production that was observed on the alkaline side in the simple system is much less pronounced when lipoxygenase is present. An increased rate of ethylene production from the complete system, over and above that found by the summation of the ethylene from the two independent systems, is also observed between pH 6·0–7·0 and this increased rate is sustained for a much longer period than at a lower pH.

We have already stated that only indolyl-3-acetic acid initiates the production of ethylene when peroxidase acts as an oxygenase. With the addition of lipoxygenase, however, the two closely related acids, indolyl-3-propionic and indolyl-3-butyric, are both capable of initiating ethylene from the oxo acid whilst indolyl-3-carboxylic acid is incapable (Table 3). Increasing the concentration of the indolyl-3-propionic from 0·05 mM to 0·1 mM considerably diminished the production of ethylene. The unnatural auxins, 1 and 2 naphthyl-acetic acids and the 2,4- and 2,4,5-trichlorophenoxyacetic acids were inactive.

Glucose oxidase. With glucose oxidase as the peroxide producing enzyme the stimulation of ethylene production by indolyl-3-acetic acid is observed at a pH of 6·8, under which conditions the enzyme must be acting wholly as a peroxidase in the oxidation of IAA, for, as we have already shown, it does not produce ethylene at this pH when acting as an oxygenase.

In the presence of low glucose oxidase concentrations (0·125 μg) IAA stimulated the production of ethylene from the oxo acid; with 1·5 μg oxidase, IAA stimulated only during the initial period of the reaction and with high concentrations (10 μg), ethylene production was inhibited by IAA (Fig. 9). The rate of oxidation of IAA in the presence of a peroxidase and phenol was also increased by the addition of glucose oxidase and glucose. With 10 μg glucose oxidase the indole was oxidized in 5 min, the same period during which a stimulation of ethylene production took place, whereas with 1·5 μg oxidase IAA was not completely oxidized until 20 min and correspondingly there was an increase in ethylene production

TABLE 3. THE PRODUCTION OF ETHYLENE FROM 4-METHYLMERCAPTO-2-OXO BUTYRIC ACID BY, (a) LIPOXYGENASE, (b) DERIVATIVES OF INDOLE, AND (c) BOTH TOGETHER

	Additions	Ethylene produced (μl) min 10	20	30	60
(a)	lipoxygenase and linolenate	0·5	0·58	0·62	0·84
(b)	indolyl-3-acetic acid	0·64	1·76	2·52	3·3
(c)	both	2·42	4·04	4·04	4·04
	increase in (c) over (a) + (b)	1·28	1·7	0·9	
(b)	indolyl-3-carboxylic acid	0	0	0	0·02
(c)	both	0·54	0·57	0·6	0·76
	increase in (c) over (a) + (b)	nil	nil	nil	
(b)	indolyl-3-propionic acid	0	0	0·02	0·02
(c)	both	0·88	1·00	1·10	1·42
	increase in (c) over (a) + (b)	0·38	0·42	0·46	0·56
(b)	indolyl-3-butyric acid	0	0	0	0·02
(c)	both	1·04	1·15	1·16	1·38
	increase in (c) over (a) + (b)	0·54	0·57	0·52	0·52

Flasks contained *p*-hydroxy benzoate (0·5 mM), benzene sulphinic acid (0·1 mM), oxo acid (1 mM) and peroxidase (1·5 units) in 10 ml 0·1 M sodium phosphate buffer, pH 5·5, containing EDTA (2 mM). The additions were; linolenate (1 mM), lipoxygenase (26 000 units) and indole derivatives (0·05 mM).

above the control for a longer period. There is thus evidence of the dependence of the ethylene producing system, at a pH of 6·8, on the ability of peroxidase, phenolic, glucose oxidase and glucose to oxidize IAA. No ethylene was produced from the oxo acid by peroxidase, phenolic, sulphinic and IAA in the absence of glucose oxidase at pH 6·8, although some oxidation of IAA does occur under the same conditions.

We have shown in previous work[11] that the peroxide producing system at pH 6·8 is capable of utilizing a high concentration of benzene sulphinic acid during the production of ethylene and that the reaction stops due to the oxidation of the sulphinic acid. The reaction, initiated by IAA, with 10 μg glucose oxidase stops before the control (Fig. 9) but can be reinitiated by the addition of sulphinic acid. By increasing the sulphinic acid concentration from 0·1 to 0·5 mM at the start of the reaction there is again an initial stimulation of ethylene production in the presence of IAA with 1·5 μg glucose oxidase over and above that produced from the oxidase alone. This continues until IAA is completely oxidized after which the ethylene production of the two reactions proceeds approximately at the same rate.

High concentrations of IAA were inhibitory. In an experiment where 0·1 mM IAA stimulated the production of ethylene from the oxo acid in the presence of *p*-hydroxybenzoate (0·5 mM), sulphinic acid (0·5 mM), glucose (1%, w/v), glucose oxidase (1·5 μg) and peroxidase (1·5 units); 0·5 mM IAA inhibited the production of ethylene by 75%.

Indolyl-3-propionic acid and indolyl-3-butyric acid are both capable of stimulating ethylene from the oxo acid when glucose oxidase and glucose are present but indolyl-3-carboxylic and indole are not. In a system containing 9 units of peroxidase, *p*-hydroxybenzoate (0·5 mM), benzene sulphinic acid (0·5 mM), glucose (1%, w/v), glucose oxidase (1·5 μg), indolyl-3-propionic acid (0·05 mM) and oxo acid (1 mM) in 0·1 M sodium phosphate buffer, the rate of ethylene production during 15 min over and above a control system without IPA was 0·94 μl at a pH of 5·5, 0·7 μl at both 6·0 and 6·5, 0·4 μl at 7·0 and 0·26 μl at a pH of 7·5. Under the same conditions but without oxo acid, the rate of oxidation

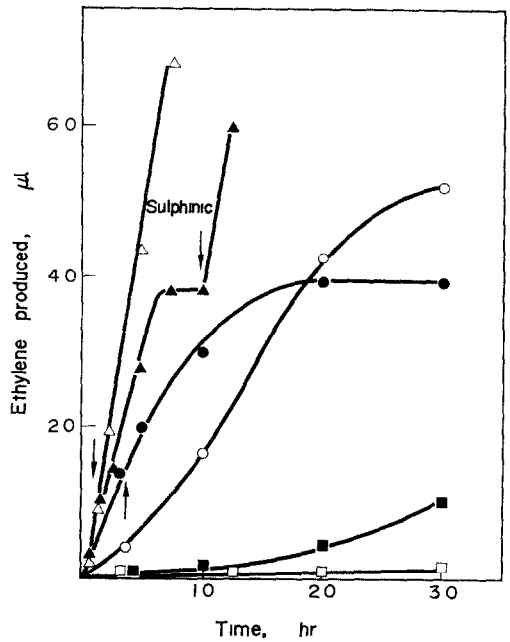


FIG. 9. EFFECT OF GLUCOSE OXIDASE ON THE RATE OF ETHYLENE PRODUCTION IN THE PRESENCE OF IAA. Flasks contained *p*-hydroxybenzoate (0·5 mM), benzene sulphinic acid (0·1 mM), glucose (1%, w/v), oxo acid (1 mM), peroxidase (1·5 units) and glucose oxidase in 0·1 M sodium phosphate buffer, pH 6·8. □—glucose oxidase 0·125 μg; ■—+IAA; ○—glucose oxidase 1·5 μg; ●—+IAA; △—glucose oxidase 10 μg; ▲—+IAA. ↓ denotes completion of IAA oxidation.

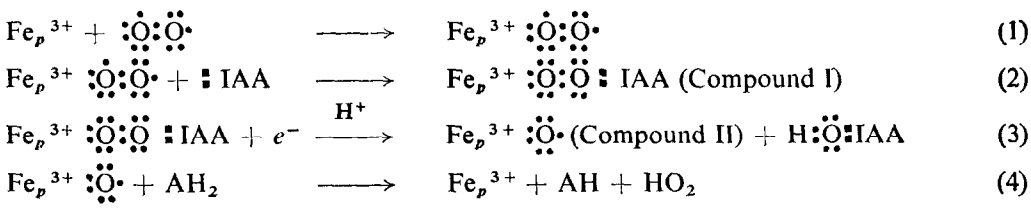
of IPA over a 10-min period was 0·017 mM at pH 5·5, 0·015 mM at 6·0, 0·01 mM at 6·5 and 0·006 mM at pH 7·5. It was also observed that peroxidase was lost at a faster rate when the pH was low; at a pH of 5·5 no peroxidase could be detected in either the control or IPA sample after 90 min reaction time, whereas 90% of the enzyme was still present in both samples when the pH was between 7·0 and 7·5. A comparison between the rate of ethylene production stimulated by the three active auxins showed that IPA and IBA were around 60% of the rate found with IAA. With the unnatural auxins a change was apparent in the role of sulphinic acid during their oxidation. With IAA, sulphinic acid always inhibited the oxidation and this was also true when glucose oxidase was present. However, the rate of oxidation of both IPA and IBA was increased when the sulphinic acid concentration was raised from 0·1 to 0·5 mM; higher concentrations (1 mM) slightly inhibited. We could detect no stimulation of the rate of ethylene production from 1-naphthylacetic acid, 2-naphthylacetic acid or 2,4-dichlorophenoxy acetic acid in the presence of glucose oxidase at a pH of 5·5 or 6·5 and with various concentrations of sulphinic acid.

Possible Mechanism of Reaction

A mechanism for the oxidation of IAA by peroxidase has been proposed by Fox *et al.*[12] It involves the formation of an oxygenated peroxidase by reaction with biradical oxygen and a reaction between this form of the enzyme and IAA to form an intermediate com-

[12] L. R. FOX, W. K. PURVES and H. I. NAKADA, *Biochem.* **4**, 2754 (1965).

pound. On reduction, this enzyme–IAA complex breaks down to give Compound II and 3-hydroxyindolenine-3-acetic acid. Compound II of peroxidase, which is convertible to the free enzyme be the addition of one electron,[14] could then react with other reducing substances to yield the free enzyme and generate a free radical component. The reactions visualized are as follows:



The Compounds I and II in the scheme are electronically and spectrally identical to those proposed by Theorell *et al.*[13] for those formed by the enzyme and H_2O_2.

Fox *et al.*[12] showed that on adding IAA to peroxidase under aerobic conditions that the free enzyme disappeared (decrease in A. 395 nm), that Compound II (increase in A 427 nm) was formed and that some inactivation of the enzyme occurred during these reactions. They, moreover, observed that in the presence of reducing substances, e.g. 2,4-dichlorophenol, no formation of Compound I or II could be observed, but that DCP added to Compound II did regenerate the free enzyme at a rate some 300 times greater than that observed in its absence and that no enzyme inactivation occurred in the process. This effect they ascribed to the known fact[15,16] that reducing compounds accelerate the transition between the transitional forms of peroxidase. We have confirmed their observation that Compound II of peroxidase is formed on addition of IAA and we have further found that in the presence of *p*-hydroxybenzoate no formation of Compound I or II can be observed but that the regeneration of free enzyme is accelerated by the addition of *p*-hydroxybenzoate to Compound II. Sulphinic acid or sulphite behave similarly, in that they accelerate the disappearance of Compound II and appearance of free enzyme, when they are added after the formation of Compound II.

The pH of the solution has a marked effect on the rate of formation of Compound II. In our experiments this was measured by determining both the rate of disappearance of the free enzyme (decrease in A at 395 nm) and the appearance of Compound II (increase in A. at 427 nm). The results of the addition of IAA to peroxidase at pH between 5·5 and 7·5 (Table 4) show that the rates of Compound II formation, calculated on its extinction coefficient,[17] decreased over this pH range and this is in agreement with those of Fox and Purves.[18] They afford some explanation of why the oxidation of IAA and ethylene production declines on the alkaline side of pH 6·0. It does not, however, account for the decreased rate of these reactions on the acid side of pH 6·0, which, in the case of the oxidation of IAA, must presumably be due to the effect of pH on the rate of reaction 4.

[13] H. Theorell, A. Ehrenberg and B. Chance, *Arch. Biochem. Biophys.* **37**, 237 (1952).
[14] P. George, *Nature, Lond.* **169**, 612 (1952).
[15] B. Chance, *Arch. Biochem. Biophys.* **37**, 235 (1952).
[16] P. George, *J. Biol. Chem.* **201**, 413 (1953).
[17] B. Chance, *Arch. Biochem. Biophys.* **41**, 404 (1952).
[18] L. R. Fox and W. K. Purves, in *Plant Growth Substances* (edited by F. Wightman and G. Setterfield), p. 301, Runge Press, Ottawa, Canada (1968).

TABLE 4. THE EFFECT OF pH ON THE RATE OF COMPOUND II FORMATION IN THE PRESENCE OF IAA OR H_2O_2 FROM PEROXIDASE

Substrate	pH	Rate of Compound II formation (μM/min)
IAA	5·5	11·5
	6·5	4·6
	7·0	1·3
	7·5	0
H_2O_2	5·5	1·87
	6·5	1·68
	7·5	2·7
Both	7·5	3·4

Compound II formation determined spectrally at 427 nm. Reagent concentration: IAA (30 μM), horse radish peroxidase (4 μM) and H_2O_2 (50 μM) in a total vol. of 3 ml 0·1 M sodium phosphate buffer.

DISCUSSION

In all the cases so far described in which ethylene is produced from either the oxo acid or methional, there is direct or indirect evidence of the formation of free radicals. In the non-enzymic system in which Cu^{2+} ions and ascorbate are the catalysts for ethylene production,[19] electron paramagnetic studies have shown the presence of the free radical, mono-dehydro-ascorbate.[20] The same applies to studies in which flavin compounds are the catalysts of ethylene synthesis.[21] In the enzymic systems in which peroxidase is a necessary participant, the production of free radicals has also been observed[22] and in those producing ethylene, their presence has been implicated on indirect evidence. The presence of free radicals has also been implicated by Fridovich and Handler,[23] in the lipoxygenase-linolenate system whose activity in generating ethylene from the oxo acid has recently been established.[7] It is also true that substances capable of trapping free radicals, e.g. catechol, chlorogenic acid and other dihydroxyphenols, are all inhibitors of the generation of ethylene by these systems.

Formulation of a free radical mechanism whereby ethylene is produced from methional or oxo acid has been proposed by Yang.[10] In the sequence of reactions the oxidation of a phenol to a phenoxy free radical is catalysed by peroxidase in the presence of H_2O_2 or systems which make peroxide. The phenoxy compound is believed to react with sulphite generating a sulphite free radical and the reaction of this with oxygen produces free radicals of the type $HO_2\cdot$, $O\cdot_2^-$ and $OH\cdot$, which finally react with methional or oxo acid to give ethylene. The reactions are postulated as follows:

[19] M. LIEBERMAN A. T. KUNISHI, L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **97**, 449 (1965).
[20] I. YAMAZAKI, H. S. MASON and L. H. PIETTE, *J. Biol. Chem.* **235**, 2444 (1960).
[21] S. F. YANG, H. S. KU and H. K. PRATT, *J. Biol. Chem.* **242**, 5274 (1967).
[22] I. YAMAZAKI and L. H. PIETTE, *Biochem. Biophys. Acta* **50**, 62 (1961).
[23] I.FRIDOVICH and P. HANDLER, *J. Biol. Chem.* **236**, 1836 (1961).

$$ROH + H_2O_2 \xrightarrow{\text{peroxidase}} RO^{\bullet} \quad (5)$$

$$RO^{\bullet} + SO_3^{2-} + H^+ \longrightarrow ROH + SO_3^{-\bullet} \quad (6)$$

$$SO_3^{-\bullet} + O_2 \longrightarrow SO_3 + O_2^{-\bullet} \quad (7)$$

$$O_2^{-\bullet} + H^+ \longrightarrow HO_2^{\bullet} \quad (8)$$

$$CH_3SCH_2CH_2CHO + HO_2^{\bullet} \longrightarrow CH_3S^{+\bullet}CH_2CH_2CHO + HO_2^- \quad (9)$$

$$CH_3S^{+\bullet}CH_2CH_2CHO + OH^- \longrightarrow CH_2{=}CH_2 + \text{— Products} \quad (10)$$

In our present system involving peroxidase and IAA, the production of free radicals could arise from the reaction of Compound II of peroxidase with either *p*-hydroxybenzoate or sulphinate (equation 4).

It is also probable that *p*-hydroxybenzoate, acting like other reducing agents, stimulates the oxidation of IAA by reacting with Compound I by the donation of an electron, thereby accelerating the production of Compound II and is itself converted to a phenoxy free radical (equation 3).

If the phenolic component reacted in both reactions 3 and 4 in this manner, the rate of production of phenoxy free radicals would be increased considerably and such an hypothesis might explain the relation already noted of the increase in the rate of oxidation of IAA with increasing benzoate and the increase in the production of ethylene. On this theory, the role of the phenolic component in both the oxidation of IAA and the generation of ethylene is to accelerate the formation of Compound II from Compound I.

With the phenoxy free radicals so produced, the subsequent reaction with sulphinate or sulphite ions might proceed as visualized by Yang. At least his hypothesis explains the absolute need for these sulphur acids for the production of ethylene from methional or oxo acid. The possibility that IAA can also react with Compound II in reaction 4 as visualized by Fox *et al.*[12] would render intelligible our observations of the competitive element existing between IAA and the phenolic component in so far as the production of ethylene was concerned and the inhibition which occurs when the concentration of IAA is increased, with a fixed phenolic concentration. Only the phenoxy free radical and not the IAA free radical is therefore capable of generating other free radicals from sulphinate or sulphite.

Even at those concentrations at which sulphinic acid or sulphite stimulates the production of ethylene, their effect on the oxidation of IAA in the presence of peroxidase and *p*-hydroxybenzoate is to inhibit the reaction. This fact indicates that their inhibitory action at higher concentrations on the synthesis of ethylene resides in their influence on the oxidation of IAA. One possibility is that the sulphur compounds can react with the oxygenated form of the enzyme such that at higher concentrations they totally prevent the formation of Compounds I and II. This, however, appears unlikely because if they so reacted, their oxidation should be accomplished by enzyme and oxygen alone, whereas no oxidation of the sulphinic or sulphite occurs unless IAA is also present. It also appears unlikely that either sulphinic acid or sulphite can accelerate the formation of Compound II from Compound I because they both progressively inhibit the oxidation of IAA as their concentration is increased. Our evidence from the spectrophotometric assays rather indicates that like *p*-hydroxybenzoate they can react directly with Compound II, and in so doing generate a sulphinyl or sulphite free radical which, in turn can initiate ethylene production. That this can occur is seen from the fact that ethylene may be produced from the oxo acid by a high

level of peroxidase, IAA and sulphinic acid aerobically without the addition of *p*-hydroxybenzoate. Under these conditions the electron for the conversion of Compound I to II is provided by the reducing group on the enzyme[17] and no phenoxy free radicals are formed.

The inhibitory action of the sulphinic acid or sulphite at high concentrations is probably to be sought in its ability to act as a free radical inhibitor in a similar manner as metabisulphite, reported by Fox and Purves,[18] which donates electrons to free radicals. Increasing the velocity of the reactions 1–4 with higher concentrations of IAA, *p*-hydroxybenzoate and peroxidase raises the level at which sulphinic acid becomes inhibitory.

On the theories advanced here to explain the mechanism of the formation of ethylene, the step-wise reduction of oxygen by the sulphinyl or sulphite free radicals is the last reaction in the chain. It is not surprising therefore that oxygen tension may play some part in controlling the overall production of the hydrocarbon. In its absence, no IAA is oxidized or ethylene produced and as the oxygen tension is raised there is first an increase in the rate of production of ethylene followed by a decline. This effect of oxygen tension does not appear to be due to any major action on the reactions associated with the oxidation of IAA since, apart from some inhibition in pure oxygen (due to enzyme inactivation), these rates are not altered over the range 5–20% oxygen. Increase in oxygen tension over this range appears to increase the efficiency with which sulphinic acid initiates ethylene production, maximum production being obtained at lower concentrations of the sulphur acid as the oxygen tension rises. There was no evidence to implicate hydrogen peroxide formation as the cause of these observations. A more probable explanation is that with an increase in oxygen tension the formation of free radicals of the type $HO_2^{-\bullet}$, $O_2^{-\bullet}$ and $OH^\bullet$ may be facilitated at lower concentrations of the sulphur acids. The very poor absolute response in 100% oxygen is almost certainly due to the marked increase in rate of enzyme inactivation.

The mechanism underlying the reaction we have described here is based on the assumption of the production of a number of free radical forms. It is therefore only fair to point out at this stage that we have been unable to detect any free radicals by electron-spin resonance studies, during either the oxidation of IAA by peroxidase in the presence or absence of a phenolic, nor when the complete system forming ethylene was investigated. As far as we are aware, there is no direct evidence of the formation of free radicals during the oxidation of IAA by peroxidase, although their presence has been claimed on indirect evidence.[12,20] Although we feel that a free radical interpretation of our results is the most probable, it is conceivable that other explanations may be forthcoming.

A further question arises as to whether these results we have observed in *in vitro* studies have any relevance to the action of indolyl-3-acetic acid *in vivo*. We have shown that only the closely related acids, indolyl-3-propionic or indolyl-3-butyric, are capable of replacing indolyl-3-acetic when hydroperoxide or peroxide is present, and even this slight modification of the molecule renders them less effective. All the other indole derivatives tested were inactive as were the unnatural auxins, 1- and 2-naphthylacetic acids and 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxy acid. The fact that 1-naphthylacetic acid has been shown to stimulate ethylene formation when applied to plant tissues[2] would suggest that the reactions here being studied may not be those in which auxins participate *in vivo*. Preliminary experiments with tomato slices have shown that the increased ethylene production as a result of supplying indolyl-3-acetic acid takes 3–5 hr to develop, and moreover, is inhibited by certain protein inhibitors, notably cycloheximide, results which have been earlier observed by other workers. This latter compound does, however, not prevent or inhibit the formation of ethylene in our *in vitro* system. A final assessment of

whether the results reported have therefore any physiological significance must await further experimentation.

EXPERIMENTAL

Enzymes and chemicals. Fungal glucose oxidase (14·7 Sigma units/mg), soybean lipoxygenase (134 000 Sigma units/mg), horseradish peroxidase (300 purpurogallin units/mg) and indolyl-3-acetic acid were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. The oxo acid, 4-methylmercapto-2-oxo butyric acid was prepared as described previously.[6] Linolenate was used as the ammonium salt in enzyme experiments.

Ethylene. This was measured as described previously[24] by GLC.

Peroxidase system. The peroxidase system used for measuring the amount of ethylene formed from the oxo acid (mM) consisted of *p*-hydroxybenzoate (0·5 mM), benzene sulphinic acid (0·1 mM), indolyl-3-acetic acid (0·1 mM) and horseradish peroxidase (1·5 units) in 10 ml of 0·1 M sodium phosphate buffer, pH 5·5, containing EDTA (2 mM).

Indolyl-3-acetic acid oxidation. The oxidation of IAA was followed by the method of Bellando and Fiussello[25] modified by the addition of the sulphuric acid in an anaerobic atmosphere. The reaction was started by the addition of peroxidase and at timed intervals 1 ml samples were added to stoppered tubes previously flushed with N_2. The contents of each tube were immediately bubbled with N_2 for 20 sec, 3 ml H_2SO_4 added, reflushed with N_2 and the tubes stoppered for the 30 min. during which maximum colour formation occurred. Manometric measurements were made with a Warburg apparatus at 25° by adding peroxidase from the side arm.

[24] L. W. Mapson and D. A. Wardale, *Biochem. J.* **102,** 574 (1967).
[25] M. Bellando and N. Fiussello, *Z. Naturf.* **23B,** 398 (1968).

Key Word Index—Ethylene; biosynthesis; indolyl-3-acetic acid; peroxidase; 4-methylmercapto-2-oxo-butyric acid.