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# Stimulation by *Erwinia carotovora* of the Synthesis of Ethylene in Cauliflower Tissue

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The synthesis of ethylene by cauliflower floret tissue was increased when the tissue was inoculated with the soft-rot bacterium *Erwinia carotovora*. This effect was clearly associated with the production of pectic enzymes by the micro-organism. These enzymes, acting together with the plant enzymes, stimulated the production of ethylene from methionine. The increased synthetic activity was due to the release and increased activity of a glucose oxidase enzyme apparently attached to plant cell-wall material and liberated by the action of pectic enzymes of the bacterium.

Ethylene is usually produced from fresh cauliflower florets at a relatively low but steady rate. However, if the florets were partially immersed in liquid, a similar slow production of ethylene was observed for the first few hours but this was followed by a greatly increased rate of ethylene production during incubation at 20°C. The maximum rate of ethylene production achieved was very variable in replicate samples. During this time growth of bacteria took place, the numbers and types of which were dependent on the varied contamination initially present on the floret tissue. Attempts to inhibit the growth of bacteria by pretreatment of the florets with sodium hypochlorite solution (1%, w/v) or by adding antibiotics (chloramphenicol, neomycin or streptomycin) to the liquid decreased the rate of growth of bacteria and also decreased the rate of ethylene production. Viable counts of bacteria on floret tissue that was rapidly producing ethylene showed that an increase in count of bacteria, including a high proportion of the pectolytic organism *Erwinia carotovora*, was correlated with a marked increase in rate of ethylene production by the plant tissue.

Twenty-two strains of bacteria, which represented the most numerous types present, were isolated from the viable count plates. Of these strains, 15 were *E. carotovora*; when cultures of these were inoculated back on to floret tissue a marked stimulation of ethylene production occurred. The remaining seven strains were not pectolytic and when a mixture of these was inoculated on to floret tissue no marked stimulation of ethylene production occurred.

The pectic enzymes of *E. carotovora* (Moran, Nasuno & Starr, 1968; Nasuno & Starr, 1966) are released extracellularly and probably play a major part in the degradation of plant tissues (Bateman & Millar, 1966). In the work reported here, cultures of this bacterium were grown on a pectate-containing medium and the culture fluid was separated from the cells by filtration through a bacteria-proof filter. This crude culture fluid stimulated ethylene production by cauliflower florets. The mechanism of this stimulation is the subject of the work reported here.

## EXPERIMENTAL

Sodium polypectate, polygalacturonic acid and pectin (U.S. National Formulary) were obtained from Sunkist Growers Inc., Ontario, Calif., U.S.A.

*Determination of ethylene.* This was measured by g.l.c. as described by Mapson & Wardale (1967).

*Ethylene production from floret tissue.* The ethylene production from floret tissue was determined in closed flasks (250ml) from small pieces of tissue (5–6cm across the head) in which the stalk was cut but not the floret tissue itself. The florets were partially immersed in 15ml of liquor containing sucrose (0.4M), EDTA (2mM), or in the same liquid with the addition of 0.1M-sodium–potassium phosphate buffer, pH6.5.

Homogenates of floret tissue were prepared by macerating florets in a mortar with 30ml of either 0.4M-sucrose–0.1M-sodium–potassium phosphate buffer (pH6.5)–2mM-EDTA, or 0.4M-sucrose–2mM-EDTA. In later experiments phosphate buffer alone was used; no difference was observed by using these different media for preparing homogenates. All determinations were carried out in oxygen (100%) at 25°C.

*Viable counts of bacteria on floret tissue.* A 5g sample of floret tissue was transferred to 100ml of sterile diluent [0.1% (w/v) Difco Bacto-peptone–0.5% (w/v) NaCl] containing glass beads, and mixed by shaking. A series of tenfold dilutions was prepared, and 0.025ml samples

were plated on to Heart Infusion Agar (Difco Laboratories, Detroit 1, Mich., U.S.A.) and on to a pectate medium (Paton, 1959). Colony counts were made after incubation at 25°C for 3 days, but where strongly pectolytic bacteria grew on the pectate plates, colony counts were recorded after 2 days at 25°C, longer incubation allowing liquefaction of the whole of the medium.

*Identification of* Erwinia carotovora. Colonies showing extensive liquefaction of pectate were transferred into a nutrient medium and purified by successive plating on to Heart Infusion Agar (Difco). The identification was based on some of the tests described by Graham (1964), and was kindly confirmed by Dr D. C. Graham by using slide agglutination and antisera to known strains of *Erwinia*.

*Bacteria-free culture liquid of* E. carotovora. The culture medium was prepared from two solutions that were each adjusted to pH 7, autoclaved separately and mixed before use. Solution 1 contained yeast extract (Difco) (5g), $Na_2HPO_4,2H_2O$ (7.2g), $KH_2PO_4$ (3.5g) and water to 500ml; solution 2 contained sodium polypectate (5g) and water to 500ml.

The bacteria were grown in 200ml of medium in 1 litre flasks on a rotary shaker for 18h at 25°C to the end of the exponential phase of growth, and harvested by centrifugation. The culture liquid was then freed from remaining viable *E. carotovora* by filtration through a 0.45$\mu$m Millipore filter (Millipore Filter Co., Bedford, Mass., U.S.A.), dialysed against water for 24h at 1°C, and stored at −25°C until used. This culture liquid is referred to below as 'bacterial enzyme preparation'.

*Enzyme assays.* The bacterial enzyme preparations were assayed for the following enzymes.

Pectate lyase (EC 4.2.99.3) was determined by measuring the production of unsaturated compounds with an absorption maximum at 235nm (Moran *et al.* 1968). Reaction mixtures contained 10% (w/v) enzyme preparation, 0.25% (w/v) sodium polygalacturonate, 0.25mM-$CaCl_2$, and 0.05M-tris–HCl buffer, pH 8.5, in a total volume of 4ml. The rate of reaction was measured for 1–2min after adding the enzyme. An increase of 0.26 extinction units at 235nm in a 2ml reaction mixture is equivalent to the release of 0.1$\mu$mol of aldehyde groups (Moran *et al.* 1968). A unit of pectate lyase activity was taken as the amount of enzyme that liberates 1$\mu$mol of aldehyde groups from polygalacturonic acid/min at pH 8.6 and 25°C.

Polygalacturonase (EC 3.2.1.15) was measured by using the iodometric method of Yemm (1935) to follow release of aldehyde groups during hydrolysis of the substrate. Flasks were set up containing 10% (v/v) enzyme solution, 0.5% (w/v) sodium polygalacturonate, 0.1M-NaCl and 0.05M-sodium acetate buffer, pH 5.2. After a suitable reaction time samples (5ml) were removed from the reaction mixture and added to 0.9ml of 1M-$Na_2CO_3$, followed by 5ml of 0.025M-$I_2$ solution. After 20min the reaction mixture was acidified with 2ml of 2M-$H_2SO_4$ and the liberated $I_2$ was titrated with 2.5mM-$Na_2S_2O_3$ with starch as indicator.

A unit of polygalacturonase activity was taken as the amount of enzyme that liberates 1$\mu$mol of aldehyde groups/min at 25°C under the above conditions.

Pectinesterase (EC 3.1.1.11) activity was assayed by measuring the carboxyl groups released by enzymic hydrolysis of pectin. The reaction mixture contained 0.5% (w/v) pectin NF, 0.1M-NaCl and 10% (v/v) enzyme preparation; liberated acid in the reaction mixture was titrated continuously at 25°C at pH 7.5 with 0.01M-NaOH. The rate of addition of alkali was measured for the first 10min after addition of the enzyme. A unit of pectinesterase activity was taken as the amount of enzyme that releases 1$\mu$mol of carboxyl groups/min under these conditions.

Glucose oxidase was determined either by measuring manometrically the increased oxygen uptake due to the addition of D-glucose or by measuring the ability of the sample to produce ethylene from 4-methylmercapto-2-oxobutyric acid in a test system (Mapson, March & Wardale, 1969) complete but for the presence of a glucose oxidase.

Lipoxygenase activity was measured as described by Surrey (1964).

Peroxidase was determined by measuring the rate of formation of purpurogallin from pyrogallol (Willstatter & Stoll, 1917).

D-Amino acid oxidase was assayed by the increase in oxygen uptake observed in the presence of catalase due to the addition of a D-amino acid (Negelein & Brömel, 1939).

Transaminase was tested for by determining the production of 4-methylmercapto-2-oxobutyric acid from D- or L-methionine in the presence of an amino acceptor (pyruvate) and pyridoxal phosphate. The formation of the oxo acid was subsequently determined by measuring the ethylene produced on addition of a peroxidase and other components of the ethylene-forming system (Mapson & Wardale, 1968).

*Gel electrophoresis of bacterial enzyme preparation.* The technique for electrophoresis in polyacrylamide gel with a discontinuous buffer system was described by Lund (1965); proteins were stained with Coomassie Blue solution (Weber & Osborn, 1969) and de-stained with 7.5% (v/v) acetic acid.

*Separation of components of bacterial enzyme preparation by continuous-flow electrophoresis.* The crude bacterial enzyme preparation (800ml) was treated with $(NH_4)_2SO_4$ to give 90% saturation. The precipitate was redissolved in 13ml 0.1M-tris–HCl buffer, pH 7.4, and dialysed for 5h against 750 vol. of water. The turbid solution was centrifuged at 25000*g* for 30min, the precipitate was discarded and the clear supernatant liquid containing pectate lyase and polygalacturonase activity was retained. The enzymically active material was again concentrated by addition of $(NH_4)_2SO_4$ to give 90% saturation. The precipitate was redissolved in buffer containing 0.1M-tris–0.05M-acetic acid at pH 8.3 and dialysed against the tris–acetate buffer. All manipulations were made at 1°C. The solution was fractionated in the continuous-electrophoresis apparatus of Hannig (1964) (Elphor model VaP-2; Bender und Holbein G.m.b.H, Munich, Germany). Cellophan membranes were used between the separation and electrode chambers. The electrode chambers were perfused with 0.2M-tris–acetate buffer, pH 8.3. Electrophoresis was carried out in 0.1M-tris–acetate buffer, pH 8.3, at 1900V (38V/cm), 170mA at 4°C, with a buffer flow rate of approx. 110ml/h.

The system was equilibrated for 30min and 1ml of sample was then introduced at a rate of approx. 1ml/h.

Fractions (vol. approx. 2ml) were collected and frozen at −20°C until required. Protein content of the material applied to the separator was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

*Radioactive-tracer experiments.* The incorporation of label from $^{14}$C-labelled methionine or 4-methylmercapto-2-oxobutyric acid into ethylene was determined on floret tissue and on floret tissue previously inoculated with the bacterial enzyme preparation. The technique used was described by Mapson, March, Rhodes & Wooltorton (1970).

*Cell-wall material.* The floret tissue was frozen by the application of solid $CO_2$ and ground in this condition in a mechanical mortar and pestle. The powdered tissue was thawed and diluted with an equal volume of 0.1M-sodium–potassium phosphate buffer, pH6.5, and passed through a French press (Milner, Lawrence & French, 1950). The resulting fluid was centrifuged at approx. 1000*g* to separate cell contents from cell-wall material. The cell-wall fraction was washed three times with ten times its volume of phosphate buffer by successive centrifugations. The washed cell-wall material was again diluted with buffer and homogenized in a Potter–Elvehjem homogenizer. The cell-wall fraction was finally exhaustively washed with buffer to remove all traces of cytoplasmic substrates or enzymes. Photomicrographs of this material after staining showed it to be free of cell nuclei and to contain only broken cells. Tests carried out on this material showed it to be devoid of both peroxidase and invertase, enzymes normally associated with cytoplasmic proteins.

## RESULTS

*Viable counts of bacteria on cauliflower florets during ethylene production.* Samples of cauliflower florets (15g) were placed in 250ml flasks with various amounts of a solution containing sucrose (0.4M) and EDTA (2mM). Viable counts were made on initial samples of the florets, and on 5g samples of florets removed from the flasks during incubation at 20°C. The results are shown in Fig. 1 and Table 1. The dry floret tissue, flasks 1 and 2, showed only a low ethylene production during incubation for 90h. In flasks with liquid present more ethylene was produced; flask 8, which showed much greater ethylene production than the replicate flasks 5 and 6, also showed a much higher viable count, the majority of the bacteria being strongly pectolytic. Tissue in flask 18 showed the most rapid production of ethylene, the most rapid increase in bacterial count, and a high proportion of pectolytic bacteria. From sectors of the pectate-count plates showing suitable numbers, colonies were picked in a systematic way to represent the main types of pectolytic or non-pectolytic bacteria present. Fifteen strains of pectolytic bacteria were isolated in this experiment. All were identified as *Erwinia carotovora* and a single strain was used for most of the subsequent experiments described.

*Effect of bacteria on ethylene production by floret tissue.* A series of 250ml flasks was set up, each containing 12g of florets and 20ml of a solution of sucrose (0.4M) and EDTA (2mM). To flasks 1–4 no further addition was made; to flasks 5–8 was added 1ml of a suspension of a pectolytic isolate; to flasks

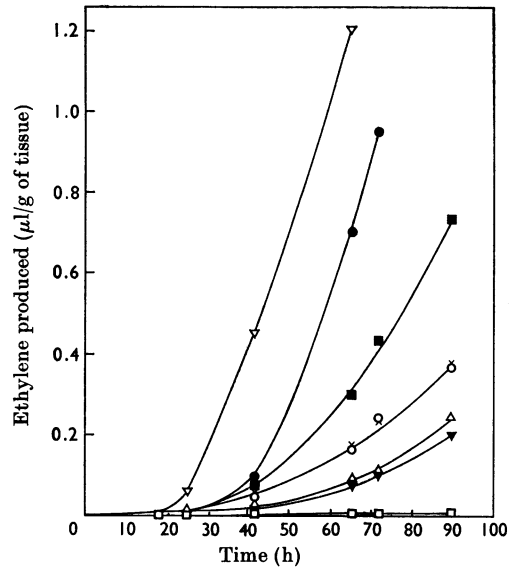



Fig. 1. Ethylene production by cauliflower florets. Samples (15g) of florets were placed in 250ml flasks. Flasks 1 and 2 (□) contained dry florets; flasks 5 (△), 6 (▼) and 8 (●) contained florets immersed in 0.4M-sucrose+2mM-EDTA solution for 30min then drained, leaving 3ml of liquid; flasks 15 (×) and 16 (○) contained 4ml of sucrose–EDTA solution added to dry florets; flasks 17 (■) and 18 (▽) contained 6ml of sucrose–EDTA solution added to dry florets. Flasks were flushed with oxygen and incubated on a shaker at 20°C.

Table 1. *Viable counts of bacteria on cauliflower florets during ethylene production*

Viable counts were made on 5g samples from flasks 1, 2, 5, 6, 8 and 18 (Fig. 1) at the times indicated. T, Total viable count on Heart Infusion Agar; P, count of pectolytic bacteria.

| | | $10^{-5}$ × Viable count/g of floret tissue | | | |
|---|---|---|---|---|---|
| Time (h)... | T | T | P | T | P |
| Flask | 0 | 42 | 42 | 65 | 65 |
| 1 | | 8.2 | 1.8 | — | — |
| 2 | | — | — | 68 | 10 |
| 5 | 0.96 | 70 | 12 | — | — |
| 6 | | — | — | 580 | 580 |
| 8 | | — | — | 1200 | 1200 |
| 18 | | 7400 | 2800 | — | — |

9–12 was added 1 ml of a mixed suspension of seven non-pectolytic isolates. After 30 min the excess of liquid was drained from all the flasks, leaving about 1 ml. Flasks 4, 8 and 12 were immediately used for determination of bacterial counts and the remaining flasks were incubated at 20°C and ethylene production was recorded. After various incubation times one flask from each group was removed and bacterial counts were made on the floret tissue. The results are shown in Fig. 2 and Table 2. Florets not inoculated with bacteria (flasks 1–4) produced very little ethylene during incubation for 65 h at 20°C; during this time bacteria naturally present on the florets multiplied to give a 100-fold increase in count; some weakly pectolytic colonies were observed, but their proportion at the end of the experiment was too low to be estimated. Florets inoculated with the pectolytic bacteria (flasks 5–8) gave a rapid production of ethylene during a 20–65 h incubation; after 65 h the bacterial count had increased 1000-fold, and most of the organisms were the pectolytic isolate used as inoculum. Florets inoculated with the non-pectolytic isolates (flasks 9–12) produced very little ethylene; although the bacterial counts were similar in magnitude to those in flasks 5–8 the proportion of pectolytic bacteria (natural content) at the end of the experiment was too low to be estimated. This experiment indicated that growth of the pectolytic bacterium *E. carotovora* stimulated ethylene production by floret tissue, but the non-pectolytic bacteria isolated did not show this effect.

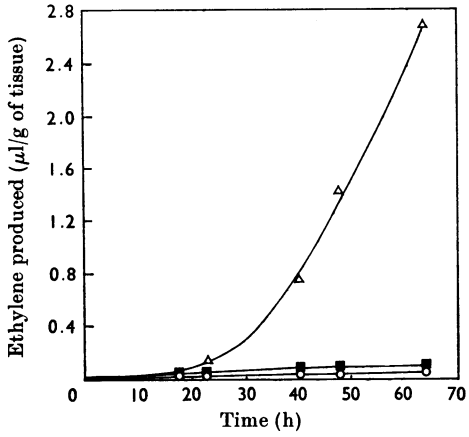



Fig. 2. Ethylene production by cauliflower florets inoculated with bacteria. Samples (12 g) of florets were placed in 250 ml flasks and to each flask was added 20 ml of sucrose (0.4 M)+EDTA (2 mM) solution and 1 ml of the inoculum indicated. After 30 min excess of liquid was drained from the florets; flasks were flushed with oxygen and incubated on a shaker at 20°C. Flask 1 (○), no added inoculum; flask 5 (△), inoculated with 1 ml of suspension of *E. carotovora*; flask 9 (■), inoculated with 1 ml of a mixed suspension of non-pectolytic isolates. Replicate flasks were set up for viable counts of bacteria (see Table 2).

*Test for ethylene production by* E. carotovora *in pure culture.* Closed flasks (250 ml) containing 50 ml of (*a*) pectate growth medium, or (*b*) heat-sterilized solution of cauliflower extract+DL-methionine (2 mM) were inoculated with *E. carotovora* and incubated on a rotary shaker at 25°C. After 16 h growth had reached the end of the exponential phase, giving (*a*) 1.7 mg and (*b*) 1.1 mg dry wt. of bacteria/ml. No ethylene production could be detected in the flasks containing these bacterial cultures.

*Effect of bacteria-free culture liquid (bacterial enzyme preparation) on ethylene production by floret tissue.* A culture of *E. carotovora* was grown in pectate medium and harvested at the end of the exponential growth phase (18 h at 25°C); 50 ml of culture was kept at 5°C, the remainder was centrifuged at 23 000 *g* for 30 min and the supernatant was removed and sterilized by passing through a Millipore filter, pore diameter 0.45 μm. The active culture and the bacteria-free culture liquid were compared for ability to stimulate ethylene produc-

Table 2. *Viable counts of bacteria on cauliflower florets inoculated with bacteria and showing ethylene production (Fig. 2)*

Viable counts were made on 5 g samples from replicate flasks at the times indicated. T, Total viable count on Heart Infusion Agar; P, count of pectolytic bacteria; *, weakly pectolytic bacteria; L, proportion of pectolytic colonies too low to count.

| | $10^{-5}$ × Viable count/g of floret tissue | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment of florets | T | P | T | P | T | P | T | P |
| Time (h) ... | 0 | 0 | 24 | 24 | 43 | 43 | 67 | 67 |
| No inoculum | 0.5 | — | 0.3 | 0.02 | 15 | 1.3* | 29 | L |
| Inoculated with *E. carotovora* | 34 | 31 | — | — | 820 | 820 | 40000 | 40000 |
| Inoculated with non-pectolytic isolates | 10 | — | 190 | 3.6 | 390 | — | 16000 | L |

Table 3. *Effect of bacteria-free culture liquid on ethylene production by floret tissue*

To 12g of cauliflower floret tissue in a 250ml flask was added 12ml of the bacterial culture preparations shown in the Table. Dilutions were prepared in solution containing sucrose (0.4M) and EDTA (2mM). Flasks were flushed with $O_2$, sealed and incubated on a shaker at 20°C. Results are the averages of duplicate flasks.

| Addition to flask | Total ethylene produced (μl/100g of tissue) | | Final appearance of floret tissue |
|---|---|---|---|
| Time (h) ... | 17.5 | 24 | |
| *E. carotovora*, culture (1) | 17.1 | 17.5 | Maceration, liquid turbid |
| *E. carotovora*, bacteria-free culture liquid (2) | 35.5 | 47 | Maceration, liquid not turbid |
| *E. carotovora*, boiled bacteria-free culture liquid | 3.0 | 5.6 | No maceration, liquid not turbid |
| 1:10 dilution of (1) | 48 | 71 | Maceration, liquid turbid |
| 1:50 dilution of (1) | 22 | 49 | Slight maceration, liquid turbid |
| 1:10 dilution of (2) | 24 | 40 | Slight maceration, liquid not turbid |
| 1:50 dilution of (2) | 3.6 | 8 | No maceration, liquid not turbid |

tion. The results (Table 3) show that (1) ethylene-inducing activity was as high in the cell-free culture liquid as in the bacterial culture, (2) the activity was heat-labile, (3) dilutions of the bacterial culture were more active than dilutions of the bacteria-free culture liquid; this would be expected because diluted cultures gave extensive growth of *E. carotovora*, eventually evidenced by the turbidity of the liquid in the flask.

*Properties of the bacterial enzyme preparation.* Several batches of this material were used in these experiments. The results of enzyme assays on a typical batch were: pectate lyase, 1.5 units/ml; polygalacturonase, 0.25 unit/ml; pectinesterase, 0.05 unit/ml; lipoxygenase, peroxidase, D-amino acid oxidase and D- or L-methionine transaminase activities were not detected.

The ability of this enzyme preparation to stimulate ethylene production by intact florets or by homogenates in the presence of 4-methylmercapto-2-oxobutyric acid (oxo acid) is shown in Figs. 4 and 5.

*Gel electrophoresis of bacterial enzyme preparation.* The enzyme preparation was concentrated by freeze-drying and re-dissolved in buffer containing 0.2M-tris and 0.1M-acetic acid (pH 8.3). Turbid material was removed by centrifugation, and the clear supernatant material (protein content approx. 19mg/ml) was examined by electrophoresis in polyacrylamide gel which was then stained for proteins. Five major protein bands were detected. In view of the marked differences in the electrophoretic mobility of these components, fractionation of the enzyme preparation was studied by continuous-flow electrophoresis.

*Investigation of components of bacterial enzyme preparation by continuous-flow electrophoresis.* Enzymes present in the bacteria-free culture liquid were concentrated by precipitation with ammonium sulphate to 90% saturation as described in the Experimental section and applied to the Elphor VaP-2 separator. Analyses of the fractions collected are shown in Fig. 3. Stimulation of ethylene production was tested with a homogenate of floret tissue and the precursor of ethylene in plant tissue, 4-methylmercapto-2-oxobutyric acid (see below). A positive result indicates a marked increase in ethylene production, compared with control flasks without added bacterial enzyme, during incubation for 8h at 25°C.

Pectate lyase and polygalacturonase showed different electrophoretic mobilities from the main u.v.-absorbing material, and the ability to stimulate ethylene production was correlated with the presence of these enzymes (Fig. 3). With material from a duplicate separation, samples of fractions 32+33 and 36+37 stimulated ethylene production but no pectinesterase activity could be detected, indicating that the presence of bacterial pectinesterase was not essential. Thus the stimulating effect of the bacterial enzyme preparation was associated with pectate lyase and polygalacturonase activity. Further work is necessary to separate these enzymes and to elucidate their relative importance.

*Pathway of ethylene synthesis.* Plant tissue in which the enzymes had been inactivated by heat treatments did not produce ethylene when infected either with the organisms or with the bacterial enzyme preparation. Conversely, inoculation of the plant tissue after heat-inactivation of the bacterial enzyme preparation did not induce any stimulation in the production of the hydrocarbon (Fig. 4). These results indicated that the stimulation of ethylene production observed was the result of the action of both bacterial and plant enzymes.

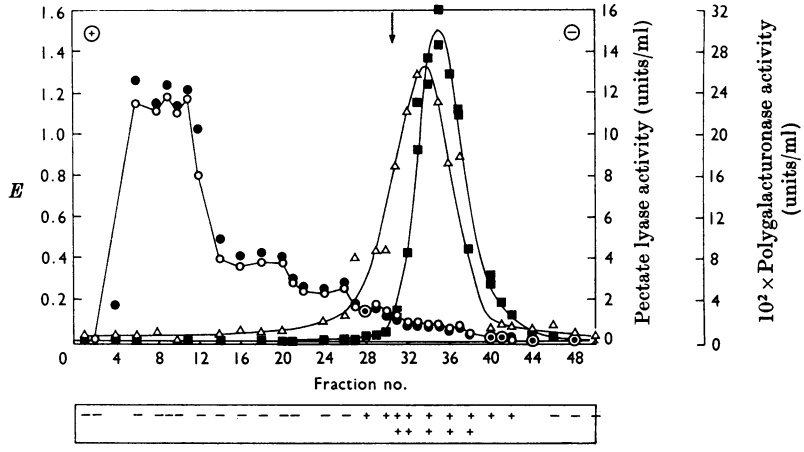



Fig. 3. Fractionation of components of bacterial enzyme preparation by continuous-flow electrophoresis. The buffer was 0.1 M-tris–acetate, pH 8.3; the voltage was 1900 V (38 V/cm), the current 170 mA and the temperature 4°C. Approx. 1 ml of sample containing 4.15 mg of protein/ml, 219 units of pectate lyase activity/ml and 16 units of polygalacturonase activity/ml was introduced opposite outlet no. 30 (↓). The volume of fractions was approx. 2 ml. The symbol + indicates a marked stimulatory effect of fraction on ethylene production. (●), $E_{260}$; (○), $E_{280}$; (△), polygalacturonase activity; (■), pectate lyase activity.

The fact that the plant enzymes were involved pointed to the possibility that the increased rate of synthesis was simply due to a stimulation of the normal synthetic system.

Recent work has shown that ethylene is formed from methionine in plants, and the biosynthetic route was described by Mapson & Wardale (1967, 1968). In floret tissue, the synthesis of ethylene depends on (1) a transaminase converting methionine into 4-methylmercapto-2-oxobutyric acid (oxo acid), (2) a glucose oxidase generating hydrogen peroxide from the oxidation of D-glucose, and (3) the presence of a peroxidase utilizing the peroxide so formed to catalyse the production of ethylene from the C-3 and C-4 carbon atoms of the oxo acid. In this latter reaction, two cofactors are essential, a phenolic compound such as *p*-hydroxybenzoate, and a sulphinic acid (methyl- or benzene-sulphinic acid). Mapson *et al.* (1969) showed that the addition of 4-methylmercapto-2-oxobutyric acid had a much greater stimulative effect on the synthesis than methionine, both with floret tissue and with homogenates prepared therefrom. They concluded on this and other evidence that the oxo acid was an intermediate in the formation of ethylene from methionine.

The conclusion that the increased production of ethylene resulting from the action of *E. carotovora* was due to an increased rate of production by the plant enzyme system described above required further proof. If, therefore, the bacterial enzymes create conditions that accelerate the normal method of ethylene production, the rate of formation of the hydrocarbon from the intermediate 4-methylmercapto-2-oxobutyric acid should be increased after the addition of these enzymes. The results (Fig. 5) show that with a homogenate prepared from floret tissue, the conversion of oxo acid into ethylene was greatly accelerated by the addition of the enzyme preparations from *E. carotovora*. This was subsequently confirmed in experiments with labelled methionine and its derivative 4-methylmercapto-2-oxobutyric acid. These experiments were designed to determine if the incorporation of label from 1,2,3,4-$^{14}$C-labelled methionine and similarly labelled oxo acid into ethylene in floret tissue was stimulated by the addition of the enzymes in the culture supernatant. The results (Table 4) showed that the incorporation of label from either methionine or oxo acid was increased under the influence of these enzymes although the quantitative increase (threefold) was somewhat lower than the increase (fivefold) in total ethylene; an observation that may perhaps partially be explained by the lower absorption of the amino acid and oxo acid in the tissue inoculated with the bacterial enzyme preparation. In view of these discrepancies the possibility exists that bacterial enzymes stimulate reactions other than those in which methionine is involved, but our other results make this explanation improbable.

*Role of hydrogen peroxide.* Mapson & Wardale

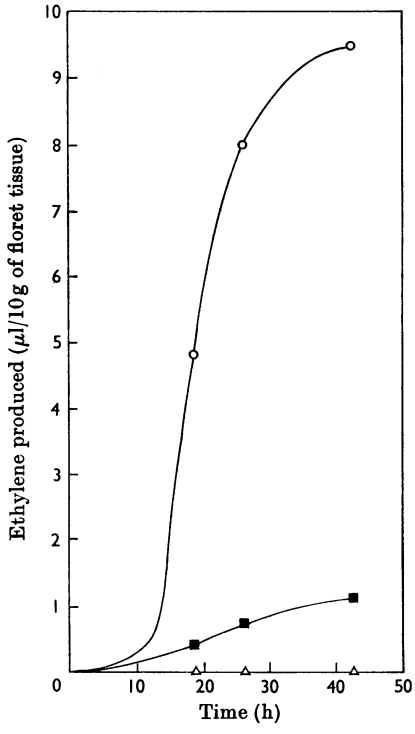



Fig. 4. Stimulation of ethylene production dependent on both plant and bacterial enzymes. Floret tissue (10g) was incubated in 8ml of phosphate buffer (0.1M), pH6.8, to which the following additions were made: ○, 2ml of bacterial enzyme preparation; ■, 2ml of autoclaved bacterial enzyme preparation; △, 2ml of bacterial enzyme preparation, but enzymes of floret tissue had been inactivated by heat before addition.

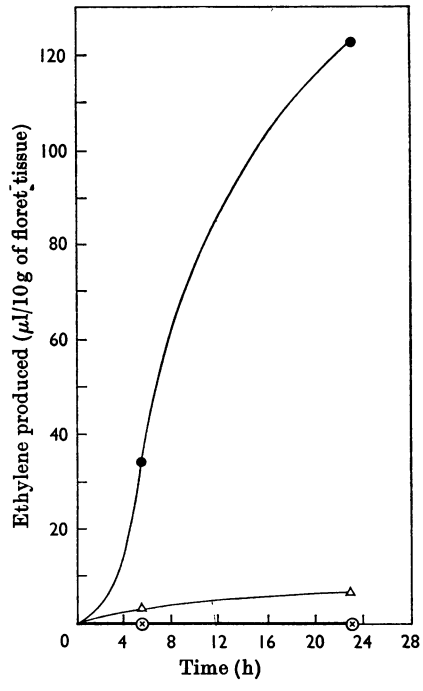



Fig. 5. Stimulation by bacterial enzyme preparation from *E. carotovora* of the synthesis of ethylene from 4-methylmercapto-2-oxobutyric acid by homogenate of cauliflower tissue. Homogenate was prepared from floret tissue (20g) with 15ml of a solution containing 0.4M-sucrose, 2mM-EDTA and 0.1M-phosphate buffer, pH6.5. Homogenate (5g) was placed in each flask with the following additions: ●, oxo acid (3mM) and bacterial enzyme preparation (0.5ml); △, oxo acid (3mM); ○, no addition; ×, bacterial enzyme preparation.

(1968) showed that the rate of ethylene production from methionine or its derivatives could be influenced by an alteration in the rate of production of hydrogen peroxide by the tissue. This was increased by the direct addition of glucose oxidase, or decreased by the addition of catalase. In the first case ethylene synthesis was stimulated, and in the second case diminished, when the concentration of hydrogen peroxide was decreased by the action of catalase. The inhibitory effects of certain phenols, e.g. catechol, chlorogenic acid, on the production of ethylene, could similarly be attributed, in part at least, to the ease with which they utilized the peroxide in forming oxidation products, and this was the chief cause of the delay in the formation of ethylene from the oxo acid when this was added to a homogenate of floret tissue. Both the shortening of this latent phase and the subsequent rate of formation of ethylene are thus an index of the rate at which hydrogen peroxide is being generated in this tissue.

In the present work, we have shown that (1) a stimulation of the conversion of the oxo acid into ethylene, similar to that achieved by the addition of the bacterial enzyme preparation to a tissue homogenate, was obtained by the addition of small amounts of glucose oxidase, (2) the stimulatory effect of the bacterial enzyme preparation was inhibited or prevented by suitable additions of catalase and (3) the stimulatory effect of the bacterial enzyme preparation was decreased by the addition of a phenol such as chlorogenic acid.

The results (Fig. 6) obtained from experiments in which various amounts of glucose oxidase were added show that as the concentration of this enzyme was increased the latent period, before rapid production of ethylene was observed, was decreased. Calculations of the amount of additional hydrogen peroxide formed in the medium as a result of adding these various amounts of glucose oxidase show that the additional production of peroxide at

Table 4. *Effect of pectic enzymes on the incorporation of* $^{14}C$ *from labelled* L-*methionine or 4-methylmercapto-2-oxobutyric acid into ethylene in cauliflower florets*

Florets (3g) in 3ml of 0.1M-sodium–potassium phosphate buffer, pH6.5, and EDTA (2mM) to which either L-[1,2,3,4-$^{14}C_4$]methionine (0.63$\mu$Ci) or 4-methylmercapto-2-oxo[1,2,3,4-$^{14}C_4$]butyric acid (0.69$\mu$Ci) were added, were incubated at 25°C for 16h in oxygen. Bacterial enzyme preparation (0.6ml) was added as indicated.

| Addition | % absorption of substrate | Ethylene produced ($\mu$l) | Ethylene produced (d.p.m.) | $CO_2$ produced ($\mu$l) | $CO_2$ produced (d.p.m.) | $CO_2$/ethylene ratio total | $CO_2$/ethylene ratio labelled | % incorporation into ethylene |
|---|---|---|---|---|---|---|---|---|
| L-Methionine | 33 | 0.25 | 2550 | 22200 | 30050 | 89000 | 11.8 | 1.1 |
| | 34 | 0.25 | 2480 | 22300 | 29700 | 89000 | 12.0 | 1.0 |
| L-Methionine+ bacterial enzyme preparation | 25 | 1.56 | 5860 | 30100 | 26200 | 19300 | 4.5 | 3.4 |
| | 27 | 1.35 | 5910 | 28500 | 29800 | 21100 | 5.0 | 3.2 |
| Oxo acid | 26 | 0.26 | 1770 | 21400 | 32600 | 82000 | 18.4 | 0.91 |
| | 26 | 0.29 | 1980 | 24200 | 40300 | 83000 | 20.4 | 0.98 |
| Oxo acid+ bacterial enzyme preparation | 16 | 1.26 | 3980 | 25900 | 48800 | 21000 | 12.3 | 3.2 |
| | 24 | 1.32 | 4070 | 26200 | 60650 | 20000 | 14.9 | 2.3 |

| Sample | Mass of ethylene produced: Enzyme/control ratio | Incorporation into ethylene: Enzyme/control ratio |
|---|---|---|
| L-Methionine | 5.9 | 3.2 |
| Oxo acid | 4.7 | 2.9 |

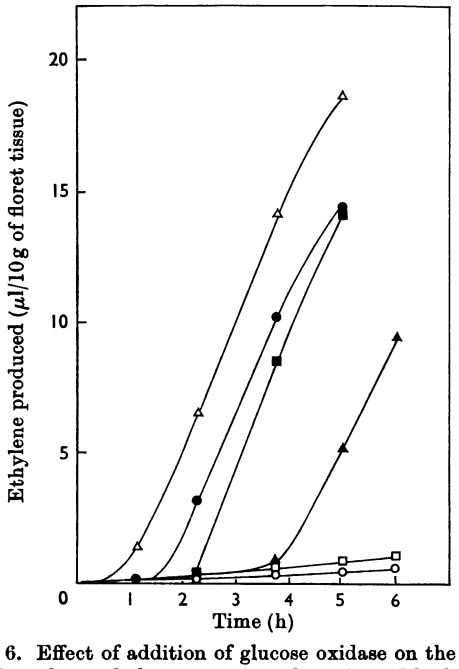



Fig. 6. Effect of addition of glucose oxidase on the conversion of 4-methylmercapto-2-oxobutyric acid by homogenates of floret tissue. Homogenate was prepared by maceration of floret tissue (20g) with 15ml of the sucrose–EDTA–phosphate solution, with oxo acid (1mM). Glucose oxidase at concentrations producing 0.22 (□), 1.1 (▲), 2.2 (■), 4.4 (●) or 11 (△) $\mu$mol of $H_2O_2$/h was added to homogenate (≡3g of floret tissue). ○, No glucose oxidase added.

a rate of approximately 1.1$\mu$mol/h was sufficient to duplicate the similar effect observed when the bacterial enzyme preparation alone was added to the same homogenate of floret tissue. An increase in the rate of peroxide formation above this value decreased the latent phase, but did not significantly affect the ensuing rate of ethylene production. The inhibitory effects of catalase and of a phenol such as chlorogenic acid on the stimulative action of the bacterial enzyme preparations are illustrated in Fig. 7.

These results are all consistent with the concept that the stimulative effect of the bacterial enzyme preparations is due to an ability to increase the rate of production of hydrogen peroxide. We obtained no evidence to suggest that the bacterial enzyme preparations themselves produced hydrogen peroxide or hydroperoxide. No glucose oxidase, lipoxygenase or D- or L-amino acid oxidases or transaminases were detected, nor could the bacterial enzyme preparation itself produce ethylene from the oxo acid in a test system in which the only component missing was hydrogen peroxide or enzymes capable of producing it. This was shown by using a solution containing the oxo acid, a peroxidase with its cofactors (benzenesulphinic acid and *p*-hydroxybenzoate) and a substrate for an oxidase. The only component missing was an oxidase capable of producing either hydrogen peroxide or a hydroperoxide, either of which could initiate the production of ethylene from the oxo acid (Mapson & Wardale, 1968). In the first

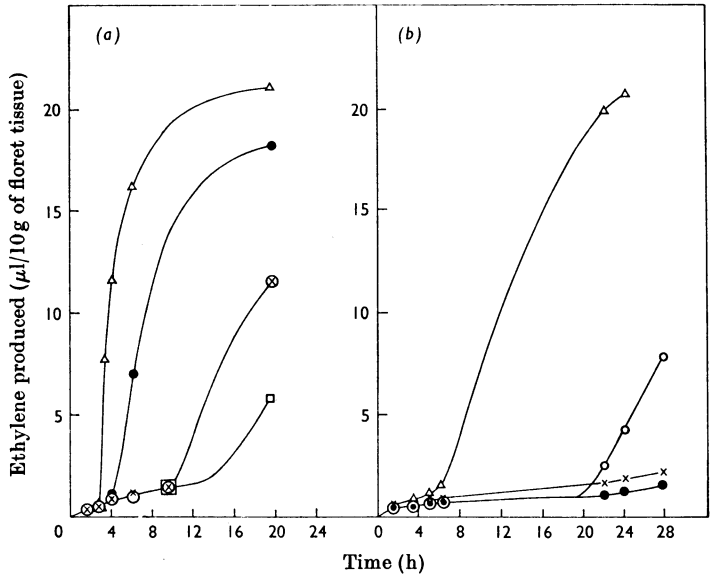



Fig. 7. Inhibitory effect of catalase and chlorogenic acid on the stimulatory action of a bacterial enzyme preparation on the conversion of 4-methylmercapto-2-oxobutyric acid into ethylene by a homogenate of floret tissue. Homogenate (5g) was prepared as described in Fig. 4 and the oxo acid was added in 1mM concentration. (*a*) ×, Homogenate alone; △, homogenate+bacterial enzyme preparation (0.5ml); ●, homogenate+bacterial enzyme preparation (0.5ml)+catalase (0.01ml) (≡$4\times10^3$ units); ○, +catalase (0.05ml) (≡$20\times10^3$ units); □, +catalase (0.1ml) (≡$40\times10^3$ units). (*b*) ×, Homogenate alone; △, homogenate+bacterial enzyme preparation; ●, homogenate+chlorogenic acid (1mM); ○, homogenate+bacterial enzyme preparation +chlorogenic acid (1mM).

Table 5. *Test for the presence of oxidase enzymes in the bacterial enzyme preparations*

The test system consisted of the following components in 10ml of 0.1M-sodium–potassium phosphate buffer, pH6.5; oxo acid (0.5mM), horseradish peroxidase (0.9 purpurogallin unit), benzenesulphinic acid (0.5mM), *p*-hydroxybenzoate (0.5mM) and either D-glucose (1%, w/v) in Expt. 1 or heated homogenate (5min at 100°C) of floret tissue (≡3g of floret tissue) in Expt. 2 to provide substrate for oxidases. In both experiments positive controls were included with glucose oxidase or with enzymes in the unheated homogenate.

| | Addition to test system | Duration of test (h) | Ethylene produced (μl) |
|---|---|---|---|
| Expt. 1 | Bacterial enzyme preparation (0.5ml)+D-glucose | 1 | 0 |
| | Bacterial enzyme preparation+D-glucose and glucose oxidase (1μg) | 1 | 3.5 |
| Expt. 2 | Bacterial enzyme preparation+heated homogenate of floret tissue | 3 | 0 |
| | | 18 | 0.10 |
| | Bacterial enzyme preparation+unheated homogenate of floret tissue | 3 | 1.7 |
| | | 18 | 8.90 |

experiment (Table 5), D-glucose was added as substrate, together with bacterial enzyme preparations. If, therefore, the latter contained a glucose oxidase, ethylene should be produced. In the second experiment, homogenates of floret tissue in which the enzymes had been destroyed by heat replaced the glucose to provide a substrate for any other oxidase present in the bacterial enzyme preparation. As the results show, no ethylene was produced in either test, indicating the absence of an oxidase in the bacterial enzyme preparation. Positive results were obtained only on the addition of glucose oxidase or in the presence of the plant enzymes in the unheated homogenate.

Stimulation of peroxide formation by the bacterial enzymes might also occur if these latter

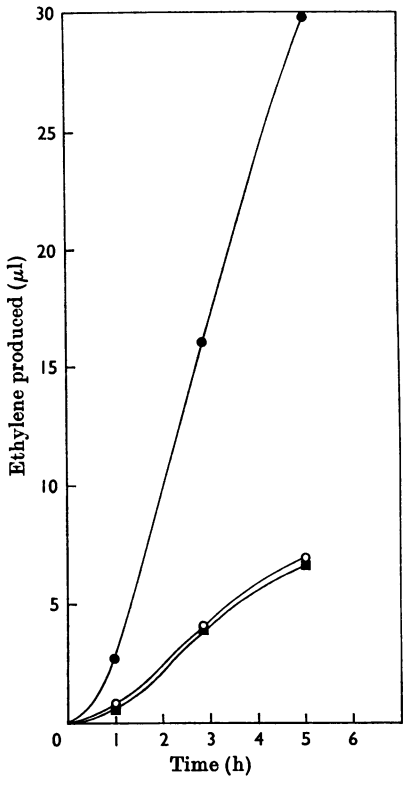


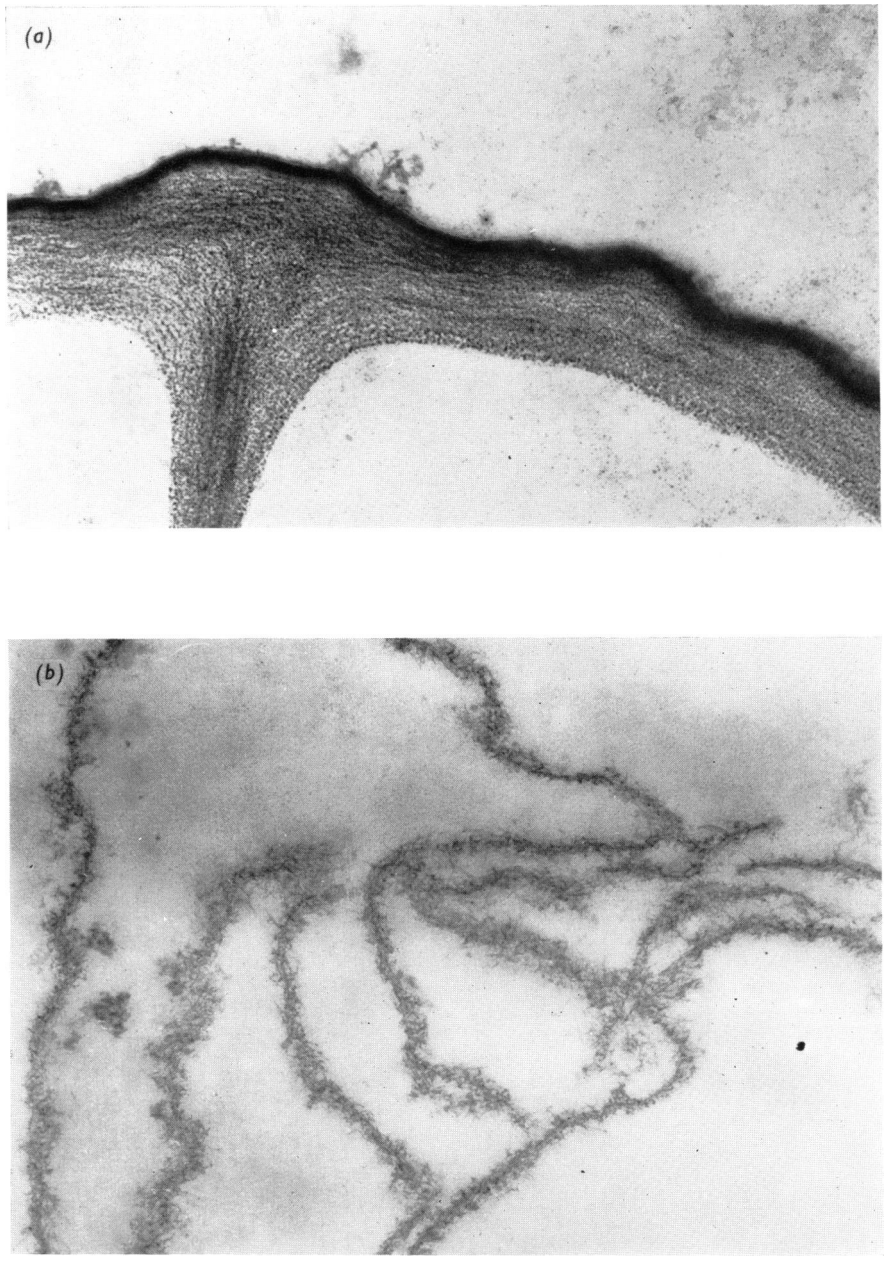

Fig. 8. Stimulation of ethylene production from 4-methylmercapto-2-oxobutyric acid by cell-wall material supplemented with cofactors after pretreatment with bacterial enzyme preparation. Cell-wall material (≡5g of floret tissue in 5ml of 0.1M-sodium–potassium phosphate buffer, pH6.5), was treated as follows: ○, not preincubated; ■, preincubated at 25°C for 2h; ●, preincubated at 25°C for 2h with bacterial enzyme preparation (0.5ml). After preincubation oxo acid (1mM), benzenesulphinic acid (0.5mM), *p*-hydroxybenzoate (0.5mM), D-glucose (0.5M) and horseradish peroxidase (0.9 purpurogallin unit) was added to each sample. The ethylene formed at 25°C was subsequently determined.

enzymes increased the activity of glucose oxidase, an enzyme known to be present in the plant tissue (Mapson & Wardale, 1968). This might be brought about by an increase in concentration of the enzyme, or activation of a latent enzyme; the result, however, would be the same, namely a stimulation of ethylene production from its precursor 4-methylmercapto-2-oxobutyric acid.

*Location of the action of the bacterial enzyme preparations.* Attempts were first made to locate the action of these bacterial enzymes on the organelles of the plant cells. Experiments in which cell-wall material was separated from other cell components showed that only when the bacterial enzyme preparation was added to the cell-wall fraction was an accelerated rate of synthesis subsequently observed on the addition of the oxo acid. No increase in the rate of synthesis was observed if the bacterial enzyme preparation was added to the cell contents. Cell-wall material, exhaustively washed to remove cell contents, did not produce ethylene from the oxo acid because it did not contain a peroxidase enzyme, its cofactors and glucose. When, however, these were added to the cell-wall preparations, production of ethylene was observed (Fig. 8). Pretreatment of these cell-wall preparations with the bacterial enzymes for 2h at 25°C produced a marked increase in the rate of synthesis of ethylene on addition of the other components (peroxidase, glucose and cofactors). In such experiments, glucose could not be replaced by other sugars, such as sucrose or other hexoses, results which confirm that the oxidase of the cell wall was a typical glucose oxidase specific for D-glucose (Table 6). The inability to utilize sucrose also indicated the absence of an invertase and thus indirectly the absence of cytoplasmic enzymes from our cell-wall preparations.

Similar experiments carried out with the cell contents showed that the rate of synthesis on addition of the oxo acid was not increased by pretreatment with the bacterial enzymes, nor could this rate of production be increased by the addition of peroxidase, glucose or cofactors. The action of the bacterial enzyme preparation in producing an accelerated rate of synthesis of ethylene was thus located in the cell-wall material and this appeared to be due to an increase in glucose oxidase activity. The component in the cell-wall material responsible for the accelerated rate of synthesis as a result of the action of the bacterial enzyme preparation was heat-labile and was inhibited by catalase. These observations are therefore all consistent with the view that the production or activation of a glucose oxidase is one result of the action of the bacterial enzyme preparation on the cell-wall material, the increase in oxidase resulting therefrom stimulating the formation of ethylene from the oxo acid. Electron micrographs of cell-wall material before and after treatment (45min at 25°C) with the bacterial enzyme preparation are shown in Plate 1. Solubilization of the pectin material of the wall has occurred, leaving a residual structure that appears to consist mainly of cellulose fibres.

*Glucose oxidase.* Direct evidence that the glucose oxidase activity of these cell-wall preparations increased as a result of the action of the bacterial enzyme preparation was obtained by determining manometrically the increase in oxygen uptake on addition of D-glucose. After incubation of the cell-wall preparation with the bacterial enzyme preparation for 2h at 25°C, the oxidase activity had




EXPLANATION OF PLATE 1

Electron micrographs of cell-wall material from florets (*a*) freshly prepared and (*b*) after treatment with the bacterial enzyme preparation for 45 min at 25°C (×48000). Material was fixed in glutaraldehyde (2%) at pH 7.2 and post-fixed with osmium tetroxide (1%) and stained with uranyl and lead acetate.

Table 6. *Specificity of the cell-wall oxidase for* D-*glucose*

The following components were added to the cell-wall material (obtained from 5g of floret tissue) in 0.1M-sodium–potassium phosphate buffer, pH 6.5, in a final volume of 10ml: horseradish peroxidase (0.9 purpurogallin unit), benzenesulphinic acid (0.5mM), *p*-hydroxybenzoate (0.5mM) and oxo acid (1.0mM).

| | | Sugar added (0.028M) | Ethylene produced (μl) 1h | 2h | 3h | 6h |
|---|---|---|---|---|---|---|
| Expt. 1 | Cell-wall material | None | 0.06 | 0.15 | 0.28 | 1.0 |
| | | D-Glucose | 0.95 | 1.9 | 3.10 | 6.0 |
| | | 2-Deoxy-D-glucose | 0.01 | 0.35 | 0.20 | 2.0 |
| | | D-Galactose | 0.01 | 0.25 | 0.60 | 1.8 |
| | | L-Rhamnose | 0.015 | 0.26 | 0.46 | 1.7 |
| | | D-Fructose | 0.02 | 0.35 | 0.86 | 2.5 |
| | | D-Mannose | 0.01 | 0.35 | 0.60 | 1.5 |
| | Cell-wall material treated with bacterial enzyme preparation for 2h at 25°C before test | None | 0.75 | 1.5 | 2.35 | 5.0 |
| | | D-Glucose | 3.0 | 5.8 | 8.0 | 15.4 |
| | | 2-Deoxy-D-glucose | 0.85 | 1.85 | 2.9 | 5.75 |
| Expt. 2 | Cell-wall material | None | 0.05 | 0.015 | 0.04 | 1.25 |
| | | Sucrose | 0.05 | 0.015 | 0.035 | 0.90 |
| | | D-Glucose | 0.50 | 1.35 | 2.25 | 4.75 |
| | Cell-wall material treated with bacterial enzyme preparation for 2h at 25°C before test | None | 0.40 | 1.0 | 1.6 | 3.5 |
| | | Sucrose | 0.40 | 1.0 | 1.5 | 3.4 |
| | | D-Glucose | 2.75 | 5.0 | 7.6 | 14.0 |

increased fourfold. This activity, expressed as μmol of hydrogen peroxide formed/h from that amount of cell-wall preparation equivalent to 1g of floret tissue, showed that 0.12μmol was formed by untreated tissue as compared with 0.48μmol formed after treatment with the bacterial enzyme preparation. No increase in glucose oxidase activity of the cell-wall preparation occurred when it was incubated alone for 2h at 25°C (cf. Fig. 8). The increased ethylene produced by a similar cell-wall preparation on addition of glucose, peroxidase and cofactors with various amounts of glucose oxidase was of a similar order of magnitude to that produced by the addition of the bacterial enzyme preparation. With added glucose oxidase the ethylene production was approximately doubled by increasing the rate of peroxide formation by 0.20μmol/h and nearly trebled with an increase in rate of 0.40μmol/h.

In the freshly prepared cell-wall material, all the glucose oxidase activity was found in the insoluble fraction. As digestion with the bacterial enzyme preparation proceeded, however, there was an increase in total activity of the oxidase and this increase occurred in both soluble (Fig. 9*a*) and insoluble (Fig. 9*b*) fractions, indicating that not only had the total amount of enzyme activity increased, but also that some degree of detachment from the cell-wall material with consequent solubilization of the enzyme had occurred.

## DISCUSSION

Infection of plant tissue with fungi has been shown to be associated with increase in ethylene production by the infected tissue (Burg, 1962; Pratt & Goeschl, 1969). In some cases ethylene is produced by fungi *in vitro* (Ilag & Curtis, 1968). Stahmann, Clare & Woodbury (1966) found that strains of *Ceratocystis fimbriata* failed to produce ethylene when grown on potato dextrose agar, but when inoculated on to sweet-potato tissue these strains caused ethylene production. The hydrocarbon produced was thought to increase resistance of adjacent tissue to invasion by the fungus. Chalutz & De Vay (1969) reported that ethylene was produced by their isolates of *C. fimbriata* growing on potato dextrose agar. Carrot and sweet-potato roots inoculated with *C. fimbriata* produced more ethylene than uninoculated roots, and the concentrations of ethylene inside inoculated root cavities were 4–20 times greater than those above fungal cultures on potato dextrose agar. These workers concluded that the fungus stimulated ethylene production by the host, possibly due to 'the continuous physical injury caused by the growing fungus', but the relative participation of the host and of the pathogen in ethylene production was not determined.

To our knowledge, the only previous report that implicated bacteria in ethylene production by plant tissue was that of Freebairn & Buddenhagen (1964) who showed that banana fruit infected with *Pseudomonas solanacearum* produced more ethylene than uninfected fruit. Several strains of this bacterium produced ethylene when growing in peptone–glucose broth in shake culture at 30°C; rates of production were as high as 0.1μl/h per flask containing 20ml of bacterial suspension; one

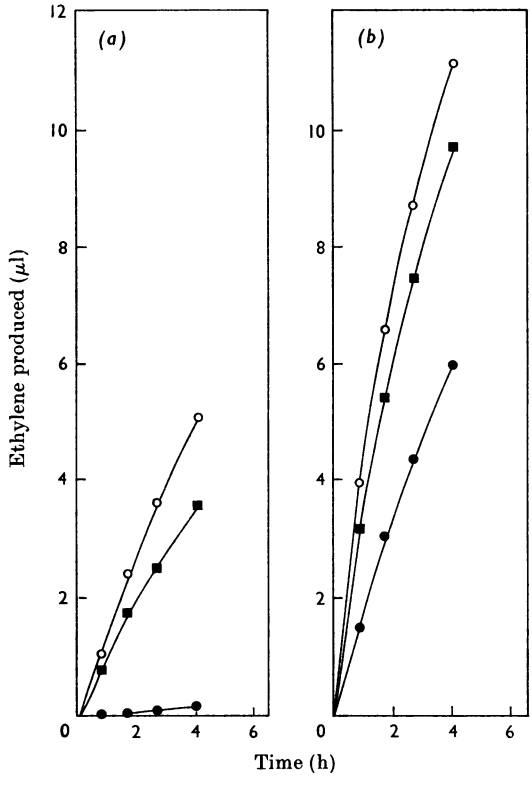



Fig. 9. Stimulation of ethylene production resulting from the increased activity of a glucose oxidase enzyme derived from cell-wall material after treatment with the bacterial enzyme preparation. Cell-wall material (≡5g of floret tissue) in 0.1M-sodium–potassium phosphate buffer, pH6.5, was incubated for 1 or 2h with bacterial enzyme preparation. The material was separated into soluble and insoluble fractions by centrifugation at 20000*g* for 10min. The residue was washed with 15ml of phosphate buffer. To soluble and insoluble fractions, benzenesulphinic acid, *p*-hydroxybenzoate, D-glucose and peroxidase were added with oxo acid (1mM) and the ethylene produced measured. (*a*) Soluble fraction; (*b*) insoluble fraction. ●, Cell-wall material incubated 2h; ■, cell-wall material incubated 1h with bacterial enzyme preparation; ○, cell-wall material incubated 2h with bacterial enzyme preparation.

banana strain of *Ps. solanacearum* failed to produce ethylene when grown in laboratory media. It was suggested that in infected fruit part of the ethylene could be produced by the infecting bacteria, but that triggering of ethylene production by the host cells might also occur.

The work reported here shows that the occurrence of a strongly pectolytic bacterium, *E. carotovora*, on cauliflower florets, resulted in stimulation of ethylene production by the plant tissue. We have also found that another pectolytic bacterium, *Xanthomonas campestris*, which gives rise to systemic infection and black rot of cauliflowers, stimulated ethylene production when inoculated on to floret tissue.

No ethylene production could be detected by pure cultures of *E. carotovora* growing in pectate medium, in sterilized solution of cauliflower extract or homogenate of cauliflower tissue, indicating that the stimulation of ethylene production by florets inoculated with this organism was due to the effect of the bacteria on the plant tissue. The effect could also be caused by the bacteria-free culture liquid of *E. carotovora*, and fractionation of the culture liquid showed a correlation between pectate-degrading activity of fractions and stimulation of ethylene production, but further work is required to separate the pectate lyase and polygalacturonase to determine whether one or both of these enzymes is involved. Pectinesterase, although present at low activity in the crude bacterial culture liquid, could not be detected in purified fractions that showed stimulation of ethylene production.

The ability of the bacterial enzyme preparations to stimulate ethylene production seems to result from the action of bacterial pectic enzymes on the plant cell-wall material. Any contamination of this cell-wall material with cytoplasmic membrane was very slight as judged by the absence of invertase activity in these preparations. No stimulation of ethylene production occurred when the pectic enzymes were added to the extracted cell contents. Such evidence suggests that the oxidase that was activated was normally attached to cell-wall material, although the possibility of adsorption of oxidase on to the cell wall during the preparation procedures cannot be completely excluded. If this was the case the assumption must also be made that in this process its activity was eliminated or severely curtailed. The complete absence of invertase from our cell-wall preparations argues against the adsorption of cytoplasmic enzymes.

The action of the bacterial pectic enzymes not only increased the activity of the oxidase attached to cell-wall material, but also caused a partial solubilization of the enzyme protein. The oxidase in the cell-wall material appears to be a glucose oxidase since it is specific for its substrate, D-glucose, a finding in agreement with our previous results on the oxidase present in the non-particulate fraction of the cytoplasm (Mapson & Wardale, 1968). The increased activity of the cell-wall oxidase leads to an increase in the production of hydrogen peroxide, which has been shown to be one limiting factor in the production of ethylene from methionine or its derivatives in cauliflower tissue (Mapson & Wardale, 1967). This concept is supported by our

present experiments, both with homogenates and with cell-wall preparations in which the stimulation of ethylene production from added oxo acid could also be achieved by the simple addition of a fungal glucose oxidase whereby an increased production of hydrogen peroxide was also obtained. Further, the fact that the stimulation of ethylene under these conditions, whether induced by the bacterial pectic enzymes or by the addition of glucose oxidase, was inhibited by catalase, indicates that in either case increased production of hydrogen peroxide is the cause of the stimulation observed.

We have also produced evidence from studies on the conversion of $^{14}C$-labelled methionine or oxo acid that the increased production of ethylene resulting from the action of these bacterial enzymes is due to a stimulation of the normal synthetic pathway. This fact taken in conjunction with our other findings does emphasize the role played by glucose oxidase in the production of ethylene by non-infected tissue.

Neither the micro-organisms themselves nor the pectic enzyme preparation derived therefrom stimulated the production of ethylene from freshly prepared homogenates of floret tissue, although both stimulated its production from florets. Supplementation of a homogenate with methionine did not alter the position, although as shown above, a marked stimulation of the conversion of 4-methylmercapto-2-oxobutyric acid into ethylene did occur. Disintegration of the tissue must therefore have so disorganized it that methionine was no longer actively converted into the oxo acid. This suggests that the transaminase enzyme that has been reported to be involved in this reaction in this tissue (Mapson *et al.* 1969) had been inhibited. Large amounts of acetaldehyde produced by such homogenates suggest decomposition of pyruvate, which is an amino-acceptor in the transaminase reaction. However, the addition of either pyruvate or oxovalerate did not lead, in the presence or absence of the bacterial pectic enzymes, to production of ethylene. The cause of this failure has not so far been found.

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