Biosynthesis of Ethylene

4-METHYLMERCAPTO-2-OXOBUTYRIC ACID: AN INTERMEDIATE IN THE FORMATION FROM METHIONINE

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The enzyme responsible for the conversion of methionine into a precursor of ethylene in cauliflower florets is a transaminase. The formation of 4-methylmercapto-2-oxobutyric acid by this enzyme has been shown. The oxo acid stimulates the synthesis of ethylene when added to floret tissue, and tracer experiments have shown that ^{14}C is incorporated into ethylene from the labelled oxo acid. The evidence is consistent with the view that the oxo acid is an intermediate in the formation of ethylene from methionine.

In previous work we have shown the synthesis of ethylene from methional (3-methylmercaptopropanaldehyde) by enzymes contained in the non-particulate fraction from the plant tissue of the cauliflower (Mapson & Wardale, 1967). These enzymes have since been identified as a glucose oxidase and a peroxidase (Mapson & Wardale, 1968). The peroxidase utilizes the hydrogen peroxide produced by the oxidase to convert the C-3 and C-4 atoms of methional into ethylene in the presence of two cofactors, a phenolic acid and a sulphinic acid (Mapson & Mead, 1968; Mapson, Self & Wardale, 1969). In the earlier work, we also showed the necessity for the synthesis from methionine of an enzyme present in the mitochondrial-microsomal fraction, and suggested that the function of this enzyme in the synthetic pathway was to produce a precursor similar to or identical with methional. In the present paper we show this enzyme to be a transaminase that converts methionine into the corresponding oxo acid 4-methylmercapto-2-oxobutyric acid, and it is this oxo acid that is converted into ethylene by the same enzymes that form the hydrocarbon from methional. The synthesis of ethylene from methionine in this tissue thus depends on the activities of a transaminase, a glucose oxidase and a peroxidase enzyme, together with their respective substrates and cofactors.

EXPERIMENTAL

Ethylene. This was measured by g.l.c., as described previously (Mapson & Wardale, 1967). Incorporation of ^{14}C label into ethylene was measured by a g.l.c. technique described by Galliard & Grey (1969), modified to allow measurement of both the mass and radioactivity of the gas.

The modification employed an effluent splitter whereby 10% of the ethylene was estimated directly by using a flame-ionization detector. The remaining 90% of the gas was passed through a low-background detector (Panax Equipment Ltd.) by using methods similar to those described by Simpson (1968).

Peroxidase system. The peroxidase system used for measuring the amount of ethylene formed from the oxo acid consisted of glucose oxidase (10 $\mu\text{g.}$), horseradish peroxidase (0.9 purpurogallin unit), *p*-hydroxybenzoate (5 μmoles), benzenesulphinic acid (5 μmoles) and glucose (1%, w/v) in 10 ml. of 0.1 M-sodium phosphate buffer, pH 6.8, containing EDTA (2 mM).

Preparation of the 2,4-dinitrophenylhydrazone of 4-methylmercapto-2-oxobutyric acid. The oxo acid was prepared by the enzymic oxidation of D-methionine with D-amino acid oxidase (Bender & Krebs, 1950). The oxo acid was extracted with ether after acidification with metaphosphoric acid. The ether was removed and the acid converted into its 2,4-dinitrophenylhydrazone. The hydrazone was purified by extraction into chloroform-ether (7:3, v/v), followed by extraction with 0.1 M- NaHCO_3 . After acidification it was extracted with ether. The hydrazone was crystallized (m.p. 149°) from the ethereal solution after drying over anhydrous Na_2SO_4 .

Determination of 4-methylmercapto-2-oxobutyric acid as the 2,4-dinitrophenylhydrazone. The oxo acid was determined by the method described by Kawano, Katsuki, Yoshida & Tanaka (1962). Particular care was found to be necessary in the separation and extraction of the hydrazone to avoid loss due to inefficient extraction. In our experiments, by adhering strictly to the following procedure, extraction efficiencies approximating to 100% were obtained. The hydrazone contained in 5 ml. of solution was extracted twice with benzene, 10 ml. and 5 ml. successively. Then 5 ml. of light petroleum (b.p. 40–60°) was added to the combined benzene extracts and the solution was extracted with 5 ml. of 0.1 M- NaHCO_3 . The organic phase was washed with 2 ml. of 0.1 M- NaHCO_3 . To the combined bicarbonate extracts, 1 ml. of M-HCl was added

and the acidified solution was extracted twice with benzene as described above. After the addition of the 5ml. of light petroleum to the combined benzene extracts, the solution was extracted with 0.1M-NaHCO₃ as before. The combined bicarbonate extracts were made up to a known volume with 0.1M-NaHCO₃. The extinction of the solution was then measured at 374nm.

Determination of 4-methylmercapto-2-oxobutyric acid in the presence of pyruvic acid. The oxo acid formed from methionine in the transaminase reaction was separated from unchanged pyruvic acid by a paper-chromatography technique by using the 2,4-dinitrophenylhydrazone derivatives. The hydrazones, dissolved in ethyl acetate, were chromatographed on Whatman no. 1 paper buffered to pH 6.5 with 0.2M-sodium phosphate buffer, with 2-methylbutan-2-ol-water (5:1, v/v) at room temperature. The chromatogram was developed for 16-17hr. The band due to the oxo acid (R_F 0.72) separated in the solvent from the two bands due to the pyruvic acid (R_F 0.36 and 0.60). The concentration of the oxo acid was then determined after elution of the band (R_F 0.72) with 0.1M-NaHCO₃.

Methyl esters. The methyl esters of the oxo acid formed from methionine by the transaminase and from 4-methylmercapto-2-oxobutyric acid were prepared with diazomethane. The esters were examined by g.l.c. on a 5-ft. glass column loaded with 5% diethylene glycol succinate on acid-washed dichlorodimethylsilane-treated Chromosorb G. The column was used isothermally at 110° with argon as carrier gas; the flow rate was 40ml./min. The system employed a flame ionization detector.

Transaminase. This enzyme was extracted from a particulate fraction and was prepared as follows. Cauliflower florets (400g.) were extracted with 400ml. of a chilled sucrose-sodium phosphate solution (0.4M-sucrose and 0.1M-sodium phosphate buffer, pH 7.4) by maceration in a Waring Blendor. Cell debris was removed by filtration through muslin, and the solution centrifuged at 40000g for 30min. The particulate fraction so obtained was suspended in 20ml. of 0.01M-sodium phosphate buffer, pH 7.4, and 200ml. of cold acetone added. The precipitate formed was collected by centrifugation, washed with cold acetone (-20°) and centrifuged again: the powder was dried *in vacuo* and suspended in 0.01M-sodium phosphate buffer, pH 7.4. The enzyme extract was further centrifuged at 20000g before dialysis (2hr.) against the phosphate buffer. Transaminase activity was measured by determination of the oxo acid formed from methionine by incubating the enzyme at 25° with D- or L-methionine (5mM) in 0.1M-sodium phosphate buffer, pH 7.4, containing EDTA (2mM), pyruvate (1mM) and pyridoxal phosphate (0.1mM), for periods of 16-20hr. The oxo acid was determined either directly as described above, or indirectly by its conversion into ethylene by the peroxidase system. Determinations of the oxo acid formed by the transaminase by the first procedure agreed well with the ethylene produced from it by the peroxidase system on the basis of a 25% conversion (see below) by the latter system.

RESULTS

Transaminase. Mitochondrial preparations from flocet tissue when incubated with DL-methionine in phosphate buffer, pH 6.8, do not produce any ethylene. If, however, to these preparations the

enzymes glucose oxidase and peroxidase and the cofactors *p*-hydroxybenzoate and a sulphinic acid (benzenesulphinic acid) together with D-glucose are added, small amounts of ethylene are produced. The formation of a precursor by the action of the mitochondrial enzymes may be simply demonstrated by allowing the mitochondria to act on methionine, inactivating the enzymes by heating, and subsequently producing ethylene by the addition of the enzymes and cofactors listed above. The formation of the precursor was increased, first, by the addition of certain oxo acids, notably pyruvic acid, and, secondly, by extraction of the enzyme from the particulate fraction.

The enzyme was solubilized and extracted from the mitochondrial preparation by treatment with cold acetone (-20°) followed by extraction of the protein precipitate in sodium phosphate buffer, pH 7.4. This was then dialysed against phosphate buffer to remove acetone. The resulting extract was capable of catalysing the transfer of the amino group from methionine to a suitable oxo acid. Several oxo acids were capable of acting as acceptor molecules for the amino group. The oxo acids tested included oxaloacetic acid, pyruvic acid, phenylpyruvic acid, 2-oxobutyric acid, 2-oxovaleric acid, 2-oxoheptanoic acid, oxoglutaric acid and glyoxylic acid. The most effective were pyruvic acid and oxaloacetic acid; the least effective was oxoglutaric acid.

The oxo acid formed was provisionally identified as 4-methylmercapto-2-oxobutyric acid by the isolation of a 2,4-dinitrophenylhydrazone, and the similar chromatographic behaviour of this to that of the authentic 2,4-dinitrophenylhydrazone of 4-methylmercapto-2-oxobutyric acid (m.p. 149°). The identities of the two hydrazones were established by their similar behaviour on chromatography in two systems: (1) 2-methylbutan-2-ol-water (6:1, v/v) developed on paper buffered with 0.2M-sodium phosphate buffer, pH 6.5 (R_F 0.71); (2) butan-1-ol-ethanol-0.5M-ammonia (7:1:2, by vol.) (R_F 0.75). Further identification of the oxo acid formed by the transaminase enzyme from methionine was provided by the preparation of the methyl ester. This ester and one prepared from authentic 4-methylmercapto-2-oxobutyric acid gave identical responses when examined by g.l.c.

The mitochondrial enzyme may be replaced in the synthesis of ethylene from methionine by either D-amino acid oxidase and D-methionine or L-amino acid oxidase and L-methionine, the product of whose action is the same 4-methylmercapto-2-oxobutyric acid. The possibility that the production of the oxo acid from methionine was due to these latter enzymes, rather than to a transaminase, was dismissed on finding no evidence of any increased oxygen consumption on the addition

to the mitochondrial preparations of either D- or L-methionine with or without added FAD.

The concentration of the transaminase in the floret is low, necessitating relatively long periods of enzyme activity (16–24 hr.) to demonstrate formation of appreciable amounts of the oxo acid. The results of an experiment illustrating the progress of the reaction is shown in Fig. 1. In this experiment, the product of the action of the transaminase on D-methionine was converted into ethylene after

Fig. 1. Formation of a precursor of ethylene from D-methionine by transaminase. Enzyme from 100g. (●) or 25g. (▲) of floret tissue was incubated in 0.1M-sodium phosphate buffer, pH 6.8, containing EDTA (2mM), D-methionine (5mM), oxalosuccinate (1mM) and pyridoxal 5-phosphate (0.05mM). After the times indicated the amount of precursor formed was determined by its conversion into ethylene by using the peroxidase system.

addition of the peroxidase system. This low concentration of the enzyme agrees with the small amounts of ethylene normally produced by the florets, which can vary between 0.20 and 1.5 μl./hr./100g. at 25° depending on age, variety and season. Estimations of the amount of ethylene to be expected from the rate of formation of the oxo acid by the extracted transaminase from floret tissue gave values of 0.16–0.30 μl./hr./100g. and the comparable amounts of ethylene produced from the same florets gave values of 0.15–0.50 μl./hr./100g. The activity of the extracted transaminase could therefore account for the actual production of ethylene observed, assuming that *in vivo* the enzyme was fully saturated with its substrate methionine and acceptors for the amino group and that some loss of enzyme during extraction from particulate preparations is almost inevitable.

Table 2. Activity of D- or L-methionine as substrates for the transaminase

The enzyme extract was incubated for 18 hr. at 25° in 0.1M-sodium phosphate buffer, pH 6.8, with EDTA (2mM) and D- or L-methionine (5mM) and pyruvate (1mM) as indicated. All solutions contained chlorobutanol (5mg./ml.). The concentration of oxo acid formed from methionine was measured by adding the peroxidase system and determining the ethylene produced.

System	Ethylene formed (μl.)
Enzyme extract + pyruvate + L-methionine	1.78
Enzyme extract + L-methionine	1.5
Enzyme extract (heat-inactivated) + pyruvate + L-methionine	0.23
Enzyme extract + pyruvate + D-methionine	4.0
Enzyme extract + D-methionine	1.1
Enzyme extract (heat-inactivated) + pyruvate + D-methionine	0.24

Table 1. Production of ethylene from the oxo acid formed by the transaminase and 4-methylmercapto-2-oxobutyric acid

The oxo acid formed by the enzyme was prepared by incubating the transaminase for 18 hr. at 25° with D-methionine (5mM), pyruvate (1mM) and EDTA (2mM) in 0.1M-sodium phosphate buffer, pH 6.8.

Oxo acid formed by transaminase (μmoles)	Pure oxo acid added (μmoles)	Total ethylene formed on addition of glucose oxidase-peroxidase system (μmole)	Conversion (%)
0.66	—	0.134	20
1.96	—	0.50	25
—	5.0	1.0	20
—	2.5	0.55	22

Direct determinations of the oxo acid formed by the transaminase and of the amount of ethylene produced from it on addition of the peroxidase system have also been made, and compared with the amount of ethylene produced from authentic 4-methylmercapto-2-oxobutyric acid in comparable concentrations and tested under the same conditions. The results (Table 1) show that the enzymically produced oxo acid behaves in a manner similar to that of the pure oxo acid.

Specificity. The transaminase from the florets reacted more readily with D- than with L-methionine (Table 2). The rate of conversion of the L-isomer was only about one-third that of the D-isomer. The results obtained may have been due either to the presence of two different transaminases or of one enzyme with racemase activity, or to the presence of a distinct racemase enzyme. A fourth possibility, that the results were due to the development of micro-organisms during the incubation period (20 hr.), was disproved when the experiment was carried out under sterile conditions. In these experiments all reaction components were sterilized by filtration through Millipore filters (0.22 μ m. pore size) and aseptically distributed into sterile flasks.

Unlike L-amino acid transaminases, participation of pyridoxal phosphate in the reaction could not be shown. Dialysis of the extracts did not decrease their activity and the subsequent addition of pyridoxal phosphate or pyridoxamine did not enhance the reaction, a result which may mean that the pyridoxal group, if present, is tightly bound to the enzyme. In this respect, the properties of the enzyme resembled those reported by Thorne & Molnar (1955) for the D-amino acid transaminase from *Bacillus anthracis*. The poor response shown by oxoglutaric acid as compared with pyruvic acid and other oxo acids as acceptor molecules is another point of resemblance between the two enzymes.

The transaminase from the florets was as effective with DL-ethionine as with methionine, and the oxo acid derived from the former was also readily converted into ethylene by the peroxidase system.

Methional as intermediate. The possibility that methional may be formed as an intermediate between the oxo acid and ethylene has been examined. The presence of this aldehyde could not, however, be detected at any time during the decomposition of the oxo acid into ethylene by the peroxidase enzyme system. The enzymic reaction was arrested by acidification of the solution with metaphosphoric acid, and the solution was extracted with diethyl ether to remove any methional present. The ethereal solution was then examined by g.l.c. for the presence of the aldehyde by using the technique described for methyl esters. Only trace amounts of methional were detected, and appeared to be due to slight decomposition of the

oxo acid, since no increase occurred during the enzymic reaction.

Influence of pH. The effect of pH on the activity of the transaminase enzyme has been measured by forming the oxo acid at different pH values and determining its concentration by conversion into ethylene by the peroxidase system. The optimum pH appears to be about 7.5 (Fig. 2), falling sharply on either side of this value.

Efficiency of conversion. The complete glucose oxidase-peroxidase system that has been found to be necessary for the production of ethylene from methional (Mapson & Wardale, 1968) was also necessary for the production of the hydrocarbon from the oxo acid (Table 3). It is of note that whereas Mn^{2+} can replace the glucose-glucose oxidase peroxide-generating system when sulphite is used as cofactor for the peroxidase system (Yang, 1967) it cannot replace the glucose oxidase when sulphonic acids replace sulphite. This is true whether methional or oxo acid is used as substrate.

The efficiency of conversion of the oxo acid into ethylene has been determined by using the glucose oxidase-peroxidase system. Both the ethylene produced and the decrease in concentration of oxo acid were measured over periods in which the rate

Fig. 2. Effect of pH on the activity of the transaminase. Enzyme from 80g. of floret tissue was incubated in 0.1 M-sodium phosphate buffers at the pH indicated, containing EDTA (1 mM), D-methionine (5 mM) and pyruvate (1 mM). After 20 hr. the pH was adjusted to 6.8 and the amount of ethylene formed from the oxo acid was determined on addition of the peroxidase system.

Table 3. *Enzymes and cofactors required for the conversion of 4-methylmercapto-2-oxobutyric acid into ethylene*

The complete system contained benzenesulphinic acid (5 μ moles), *p*-hydroxybenzoate (5 μ moles), glucose (1%, w/v), glucose oxidase (10 μ g.), oxo acid (10 μ moles) and horseradish peroxidase (0.9 purpurogallin unit) in 10 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, containing EDTA (2 mm).

Omission from complete system	Ethylene produced (μ l.)		
	15 min.	30 min.	1 hr.
None	8.8	17.6	28.4
<i>p</i> -Hydroxybenzoate	0.34	0.66	1.26
Sulphinic acid	0.08	0.16	0.28
Peroxidase	0.01	0.02	0.04
Glucose oxidase	0.01	0.02	0.04
Glucose oxidase*	0.02	0.09	0.25

* 0.5 μ mole of MnSO_4 was added to the system.

of production of ethylene with time was linear. In these experiments the rate of the reaction was varied by controlling the rate of production of peroxide by the addition of different amounts of glucose oxidase. Within the limits of peroxide production tested, which led to the formation of ethylene at a rate of 0.4–1.5 μ l./min., no significant effect on the efficiency of conversion of the oxo acid into ethylene was observed. The values obtained ranged from 24 to 30%. This low conversion by the enzymic system was almost certainly due to the decomposition of the oxo acid into products other than ethylene. This was confirmed by the observation that about 7 μ moles of carbon dioxide were produced/ μ mole of ethylene formed. The corresponding ratio for methional was 3 μ moles/ μ mole of ethylene produced.

Tracer experiments. The incorporation of ^{14}C from L-methionine labelled at C-1, C-2, C-3 and C-4 and of the oxo acid similarly labelled, into ethylene by floret tissue has been determined. In other experiments, the incorporation of label into ethylene on supplying the labelled oxo acid and unlabelled methionine has also been studied as well as the reverse procedure of supplying labelled methionine and unlabelled oxo acid.

The results (Table 4) show that, whereas incorporation of label into ethylene occurs with both methionine and oxo acid, the extent to which incorporation occurs is greater with the oxo acid (1–2%) than with methionine (0.3–0.6%). These estimates are based on the assumption that in each case only C-3 and C-4 are converted into ethylene (Lieberman, Kunishi, Mapson & Wardale, 1966; Burg & Claggett, 1967). The poor incorporation of tracer into ethylene from labelled methionine, compared with the incorporation from oxo acid, cannot be ascribed to defective absorption of methionine. In Expt. 2, at the end of the incubation

period, 90% of the labelled methionine had been absorbed by the floret tissue compared with 80% for the oxo acid under the same conditions in other experiments.

When unlabelled methionine was supplied in a concentration equal to that of the labelled oxo acid, the specific radioactivity and incorporation of tracer into ethylene was not decreased. Even when the unlabelled methionine was increased to a concentration 270 times that of the labelled oxo acid, the incorporation of label decreased only from 1.1 to 0.4%. On the other hand when unlabelled oxo acid was supplied in addition to labelled methionine, the specific radioactivity and percentage incorporation of tracer into ethylene were, in most experiments, decreased. This decrease was especially marked when the concentration of oxo acid supplied was increased (Expt. 4, Table 4). Table 4 also shows the disproportionate amount of labelled carbon dioxide in relation to labelled ethylene produced on addition of 1,2,3,4- ^{14}C -labelled oxo acid or methionine. On the basis of C-3 and C-4 being converted into ethylene, and on a mole-to-mole conversion, the labelling of carbon dioxide should not be greater than that incorporated into ethylene. The fact that the carbon dioxide produced was labelled to an extent 10–20-fold that of ethylene indicates the extent to which conversion into products other than ethylene occurred.

Synthesis in floret tissue. With the demonstration of the presence of a transaminase in the mitochondria of floret tissue, all the enzymes and cofactors necessary for the conversion of methionine into ethylene have been shown to be present. Our failure in previous work to demonstrate an increased rate of synthesis when methional was added to intact floret tissue or slices has been confirmed and contrasts with the ease with which the same substance was converted by the enzymes *in vitro*.

Table 4. *Incorporation of ^{14}C into ethylene from labelled L-methionine or 4-methylmercapto-2-oxobutyric acid*

Cauliflower florets (3.0g.) were immersed in 3.0 ml. of 0.1 M-sodium phosphate buffer, pH 6.5, containing either labelled 4-methylmercapto-2-oxobutyric acid or labelled L-methionine. In some cases unlabelled oxo acid or L-methionine was also added as indicated. The reaction period was 16 hr. at 25°, in an atmosphere of oxygen.

	$10^{-3} \times$ Radio- activity in ethylene (d.p.m.)	Amount of ethylene ($\mu\text{l.}$)	$10^{-3} \times$ Radio- activity in CO_2 (d.p.m.)	$10^{-3} \times$ Sp. radioactivity of ethylene (d.p.m./ μmole)	Incorporation of labelled material into ethylene (%)
Expt. 1					
[1,2,3,4- $^{14}\text{C}_4$]Methylmercapto-2-oxobutyric acid (1.33 μmoles , 1.0 μC)	10.5	0.44	195	525	0.96
Labelled oxo acid + unlabelled L-methionine (1.33 μmoles)	16.3	0.51	213	710	1.48
L-[1,2,3,4- $^{14}\text{C}_4$]Methionine (1.33 μmoles , 1.0 μC)	1.9	0.39	119	112	0.17
Labelled L-methionine + unlabelled oxo acid (1.33 μmoles)	0.65	0.39	137	38	0.06
Expt. 2					
L-[1,2,3,4- $^{14}\text{C}_4$]Methionine (1.33 μmoles , 1.0 μC)	3.3	0.28	85	254	0.3
Labelled L-methionine + unlabelled oxo acid (1.33 μmoles)	1.2	0.73	41	36	0.1
Expt. 3					
Labelled oxo acid (1.33 μmoles , 0.5 μC)	8.7	1.12	173	174	1.58
Labelled oxo acid + unlabelled L-methionine (1.33 μmoles)	10.4	1.35	173	174	1.89
L-[1,2,3,4- $^{14}\text{C}_4$]Methionine (1.33 μmoles , 0.5 μC)	3.0	0.76	57	88	0.55
Labelled L-methionine + unlabelled oxo acid (1.33 μmoles)	3.2	0.93	79	78	0.58
Expt. 4					
Labelled oxo acid (0.11 μmole , 0.1 μC)	1.2	0.46	15.1	57	1.1
Labelled oxo acid + unlabelled L-methionine (30 μmoles)	0.41	0.48	13.8	20	0.37
L-[1,2,3,4- $^{14}\text{C}_4$]Methionine (0.11 μmole , 0.1 μC)	0.49	0.45	9.1	25	0.45
Labelled L-methionine + unlabelled oxo acid (30 μmoles)	Nil	2.83	7.8	Nil	Nil

This observation inevitably raised the question as to the validity of our observations in relation to the synthesis *in vivo*. For this reason we examined the effect on the production of ethylene from intact tissue of substituting 4-methylmercapto-2-oxobutyric acid for methional. In these experiments the oxo acid was added in concentrations in the range 0.4–6 mM to the external fluid surrounding the floret tissue. The results (Fig. 3) show that the stimulation of ethylene production was related directly to the concentration of oxo acid. The increased rate of production continued for 20–24 hr. before declining. Analysis at this stage of the concentration of the oxo acid in the external fluid indicated that less than 10% of the acid originally added remained; the fall off in rate of synthesis thus

corresponded to the disappearance of the oxo acid. Estimates of the conversion of the oxo acid under such conditions gave values of about 1–2%.

When the experiment was repeated with florets that had first been subjected to heat to inactivate enzymes, the production of ethylene after the addition of the oxo acid was again observed. However, there were indications that the ethylene production was controlled by different factors in these two cases. With the untreated tissue the production of ethylene was linear with time during 24 hr. (Fig. 4), whereas with the heated tissue ethylene was produced rapidly initially but production ceased after a short time. Further, the effect of added ascorbate was very different. With the boiled tissue addition of ascorbate increased very

Fig. 3. Formation of ethylene from 4-methylmercapto-2-oxobutyric acid by floret tissue. The oxo acid in the amounts indicated was added to the floret tissue (3g.) in 3ml. of 0.1M-sodium phosphate buffer, pH 6.5, containing EDTA (2mm); the atmosphere was oxygen. Concentration of oxo acid: ●, none; Δ, 0.45 mm; □, 2.25 mm; ○, 4.5 mm; ▼, 6.25 mm.

markedly the initial burst of ethylene; with the untreated tissue ascorbate did not affect the production.

These observations are explicable on the basis of the operation of two distinct catalytic systems, the first enzymic and the second a model system involving ascorbate. With the untreated floret tissue the enzymic system alone appears to be operating, but this gives place after heat denaturation of protein to a model system that is dependent on the presence of ascorbate. The operation of these two systems may be shown as follows. If the floret tissue is homogenized in phosphate buffer, pH 6.8, the ascorbate is rapidly oxidized by the ascorbate oxidase present. This oxidation may be followed by titration against indophenol dye after acidification and removal of protein. After completion of the oxidation, ethylene may be produced on the addition of the oxo acid and production continues for some hours (Expt. 1, Table 5). The elimination of ascorbate has thus had no adverse effect on the catalytic activity. This activity can, however, be destroyed by heat and is therefore heat-labile (Expt. 1, Table 5). Further, the addition of ascorbate does not enhance the catalytic activity (Expt. 2, Table 5).

If, however, the enzymes of the homogenate are inactivated by heat after all the endogenous ascorbate has been oxidized, the addition of the oxo acid alone produced little or no ethylene (Expt. 2, Table 5). Only when ascorbate and oxo

Fig. 4. Production of ethylene from the oxo acid by either fresh floret tissue or tissue in which the enzymes had been inactivated by heat. Tissue (3g.) was incubated in 4ml. of 0.1M-sodium phosphate buffer, pH 6.5, containing EDTA (2mm) and oxo acid (4mm); the atmosphere was oxygen. ●, Heated tissue; □, heated tissue and ascorbate (4mm); Δ, fresh tissue; ○, fresh tissue and ascorbate (4mm).

acid are added together is there an appreciable production of ethylene and at a rate considerably in excess of that observed with unheated tissue. The production of ethylene in these circumstances is therefore dependent on the presence of ascorbate. Such observations differentiate an enzymic heat-labile system from a model system that operates only when the tissue proteins are denatured by heat. Presumably in the latter case metal ions, notably Cu^{2+} , normally present as prosthetic groups of enzymes, are released or altered and in the presence of ascorbate are capable of forming a catalytically active model system (cf. Lieberman & Mapson, 1964).

Both catalytic systems, the enzymic and non-enzymic, were effective in producing ethylene from methional when the latter compound was added to homogenized tissue. Therefore the inability of methional to stimulate ethylene production when added to floret tissue, in contrast with its ability to do this when added to homogenized floret tissue, suggests either a failure to penetrate cell membranes or a very rapid metabolism of the aldehyde by the intact tissue along other pathways (Expt. 3, Table 5).

Table 5. *Conversion of 4-methylmercapto-2-oxobutyric acid or methional into ethylene by homogenates of floret tissue*

Cauliflower florets (3g.) were homogenized with 4ml. of 0.1M-sodium phosphate buffer, pH 6.8, containing EDTA (2mm). The homogenate was left for 15–30 min. with aeration to oxidize ascorbate.

			Ethylene production (μ l./100 g.)		
	Treatment of aerated homogenate	Addition	Concn. (mm)	1.5 hr.	17 hr.
Expt. 1	—	—	—	0.6	3
	—	Oxo acid	3	6.3	280
	Heated at 100° for 3–5 min.	Oxo acid	3	0.6	3
Expt. 2	Heated at 100°	Oxo acid	3	6 hr.	23 hr.
	Heated at 100°	Oxo acid	3	8.3	10
		+ ascorbate	10	1300	1640
	—	Oxo acid	3	22	80
	—	Oxo acid	3	24	55
		+ ascorbate	10		
	Expt. 3	—	—	—	4 hr.
—		Oxo acid	2	0.9	5
—		Methional	2	9	54
			2	10	88

DISCUSSION

The work described in this paper has indicated the intermediate role of 4-methylmercapto-2-oxobutyric acid in the pathway of synthesis of ethylene from methionine by enzymes in the florets of the cauliflower. Ku, Yang & Pratt (1969) have also described experiments implicating this oxo acid as a precursor of ethylene when added to extracts of tomato fruit, provided that certain inhibitors of a phenolic nature were first removed. The efficiency with which the oxo acid is converted into ethylene has been shown in this work to be dependent on the enzymic system. With a purified glucose oxidase-peroxidase system in which side reactions are decreased to a minimum 30% of the oxo acid added was converted into ethylene, but under conditions in which greater possibilities exist for the decomposition of the oxo acid into products other than ethylene the conversion is much lower. Thus with tissue slices or homogenates the highest conversion on a mole-to-mole basis was about 2%.

The relatively low conversion with the purified enzymic system is almost certainly due to the conversion of the oxo acid into other products. Evidence of this is seen by the greater amount of carbon dioxide produced (over threefold) over and above that which would be expected from a mole-to-mole conversion of C-3 and C-4 into ethylene. The same is true to an even greater degree when tissue slices are used in place of the more active enzymic system. Then, the ratio of carbon dioxide

produced to ethylene formed is about 10–20:1 (Table 4), and the overall conversion of the oxo acid into ethylene is decreased accordingly. Even so, it has to be remembered that in the fresh floret tissue only 1 mole of ethylene is produced for approx. 20 000 moles of carbon dioxide, so that compared with this the conversion of the oxo acid is high.

One further conclusion appears possible from the work recorded here. The addition of increasing amounts of oxo acid to tissue slices resulted in proportionate increases in the production of ethylene. Such results indicate that the enzymes and cofactors necessary for the conversion of the oxo acid into ethylene are present in concentrations in the tissue sufficient to catalyse the production of the hydrocarbon in amounts far in excess of those normally observed. The addition of methionine, on the other hand, leads at best to only a small increase in the rate of synthesis of ethylene. This observation points to the conclusion that as a substrate the concentration of methionine is not limiting; the chief factor controlling the formation of the oxo acid would thus appear to be the rate of formation of the oxo acid, i.e. the activity of the transaminase. We have also shown that the activity of this enzyme as extracted is more closely related to the synthetic activity of the fresh florets than are the other enzymes involved. The results of experiments with ^{14}C -labelled methionine or oxo acid are consistent with the view that the oxo acid is an intermediate between methionine and ethy-

lene. The greater rate of incorporation of label from oxo acid as compared with methionine, and the failure of unlabelled methionine when present in high concentrations to eliminate the incorporation of label into ethylene from labelled oxo acid, and conversely the prevention of incorporation of label from labelled methionine in the presence of a high concentration of unlabelled oxo acid, also point to the same conclusion.

Methional, which could not be detected in the enzymic formation of ethylene from the oxo acid, cannot be excluded as a possible intermediate until more detailed information is available about the chemical mechanism involved. If decarboxylation of the oxo acid occurred simultaneously or almost simultaneously with the splitting off of C-3 and C-4 as ethylene, methional as an intermediate would have only a transitory existence.

Work with the floret tissue has underlined the necessity for caution in interpreting results, especially when oxo acid is used. The ease with which this compound decomposes into ethylene under the catalytic influence of either enzymic or model systems has been shown. In the experiments recorded here, the formation of an extremely active ascorbate-dependent model system, after elimination of the enzymic system by heat, renders it necessary to examine closely the origin of ethylene in other tissues, especially in those cases in which it has been shown that ascorbate is necessary, e.g.

the formation of ethylene from linolenic acid in apple tissue (Galliard, Hulme, Rhodes & Woollorton, 1968).

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