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ENZYMES INVOLVED IN THE SYNTHESIS OF ETHYLENE FROM METHIONINE, OR ITS DERIVATIVES, IN TOMATOES

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Abstract—Three enzymes, a transaminase, an enzyme with lipoxygenase activity and a peroxidase, appear to be involved in the synthesis of ethylene from methionine in extracts from tomatoes. Fatty acid hydroperoxides formed by the action of soya bean lipoxygenase or by the tomato enzyme on linolenate can initiate the production of ethylene from its precursor 4-methylmercapto-2-oxobutyric acid in the presence of a peroxidase and its co-factors. Changes in the activities of these three enzymes during the ripening of tomatoes have been determined and a quantitative assessment of their activities has been attempted. The results indicate that they can account for the production of ethylene from either whole fruit or tissue slices.

INTRODUCTION

PREVIOUS work[1] with floret tissue from cauliflowers, has implicated three enzymes in the formation of ethylene from methionine. A transaminase enzyme converting the amino acid to the corresponding oxo acid, 4-methylmercapto-2-oxobutyric acid, a glucose oxidase producing hydrogen peroxide during the oxidation of β-D-glucose and a peroxidase utilising this peroxide to convert the C_3, C_4 moiety of the oxo acid to ethylene. The present work is a continuation of earlier work aimed at determining for a typical climacteric fruit whether the enzymes involved were identical with those in the floret tissue, and whether changes in the activities of these enzymes were reflected in changes in the synthesis of ethylene during the ripening of the fruit. Previous work[2,3] with ^{14}C labelled substrates has already shown that methionine and its derivatives are the precursors of ethylene in both tissues.

RESULTS

Transaminase. The presence of a transaminase enzyme catalysing the transfer of the amino group of methionine to other oxo acids has been identified in tomato tissue. Its presence was demonstrated by the formation of the oxo acid, 4-methylmercapto-2-oxobutyric acid, the latter being identified by its ability to produce ethylene on the addition of a peroxidase, glucose oxidase, glucose and the cofactors, a sulphinic acid and a phenol.[2] In contrast to the floret tissue, the enzyme was found in both particulate and nonparticulate fractions. A number of oxo acids can act as amino acceptors but the most efficient found were oxoheptanoic, oxovaleric or pyruvate. The transaminase activity from the fresh particulate fraction was not affected in the absence of pyridoxal phosphate, but the activity of preparations made from aged particulates was reduced in its absence. With the enzyme derived from the non-particulate fraction, its activity was reduced (50%) in the absence of

[1] L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **107**, 433 (1968).
[2] L. W. MAPSON, J. F. MARCH and D. A. WARDALE, *Biochem. J.* **115**, 653 (1969).
[3] L. W. MAPSON, J. F. MARCH, M. J. C. RHODES and L. S. C. WOOLTORTON, *Biochem. J.* (in press).

TABLE 1. ETHYLENE PRODUCTION AS AN INDEX OF THE FORMATION OF 4-METHYL-MERCAPTO-2-OXOBUTYRIC ACID BY A TRANSAMINASE ENZYME FROM TOMATOES

Omission from complete system	Enzyme source	Ethylene produced (μl)
None	Fresh particulate	2·94
Pyridoxal phosphate	Fresh particulate	2·8
Pyridoxal and pyruvate	Fresh particulate	1·7
None	Aged particulate	13·5
Pyridoxal phosphate	Aged particulate	10·8
Pyridoxal and pyruvate	Aged particulate	10·4
None	Fresh non-particulate	1·8
Pyridoxal phosphate	Fresh non-particulate	0·88
Pyridoxal and pyruvate	Fresh non-particulate	0·3
None	Aged non-particulate	0

The enzyme extract (≡ 10 g tissue from tomatoes in their climacteric phase) was incubated for 21 hr at 25° in 0·1M-sodium phosphate buffer, 6·8, with mannitol (0·4M), EDTA (2mM), L-methionine (5mM), pyridoxal phosphate (0·1mM) and pyruvate (1mM) as indicated. The concentration of oxo acid formed from methionine was measured by adding the peroxidase system and determining the ethylene produced. The cell fractions were aged by leaving at room temperature for 24 hr.

TABLE 2. SPECIFICITY OF THE TRANSAMINASE FOR L-METHIONINE

Substrate	Enzyme source	Ethylene produced (μl)
L-Methionine	Particulate fraction	3·72
D-Methionine	Particulate fraction	0·9
L-Methionine	Heat inactivated particulate fraction	0·46
L-Methionine	Non-particulate fraction	3·2
D-Methionine	Non-particulate fraction	0·9
L-Methionine	Heat inactivated non-particulate fraction	0·6

The oxo acid formed from methionine by the activity of the transaminase enzyme was determined by measuring the ethylene produced on addition of the peroxidase enzyme system. The conditions for assessing enzyme activity were as described in Table 1.

pyridoxal phosphate (Table 1). The omission of both pyruvate and pyridoxal reduced the activity of the enzyme derived from the non-particulate to virtually zero, but had much less effect on the activity of the enzyme associated with the particulate fractions. Both enzymes reacted readily with L-methionine and showed only very slight activity with the D-isomer (Table 2).

This result is in strong contrast with results obtained from floret tissue where the D-isomer was much more efficiently converted to the oxo acid than the L-isomer. Preliminary attempts to solubilize the particulate enzyme using either acetone or acetone followed by butanol treatments were unsuccessful. This failure may have been due to the extraction of the acetone powder with neutral rather than with alkaline buffer solutions, for as Frenkel,

Klein and Dilley[4] have shown, protein recovery from acetone powders of pome fruits was materially increased by buffer solutions at a pH of 10. Subsequent experiments using more alkaline buffers have yielded results indicating some solubilization of the enzyme.

Transaminase activity of the particulate fraction increased greatly (Table 1) if these cell fractions were "aged" by holding either at 1° or room temp. for 24–48 hr. Longer periods of "ageing" lead, however, to a loss of enzyme activity. The reason for this increase in activity, whether due to the removal of an inhibitor or to some other cause, was not investigated. Electron microphotographs gave no clear indication that the mitochondria were breaking down during these shorter periods of "ageing", nor could the enzyme be more easily solubilized from the "aged" compared with the fresh preparation. The pH for optimum activity of the transaminase was 6·5.

Peroxidase. This enzyme was identified as being present in the non-particulate fraction of the cell. It was found in all fruit examined, whether ripe or unripe. Estimates of its activity were determined either on extracts after removal of the mitochondrial/microsomal organelles of the cell, or after the enzyme had been removed from such extracts by precipitation with ammonium sulphate (0–80% saturation) followed by dialysis. The activity of the enzyme was always found to be lower when it was assayed by the second procedure, presumably due to some destruction during precipitation and dialysis.

Oxidase. The generation of ethylene from methionine or its derivatives, requires the participation of an oxidase producing either hydrogen peroxide or a hydroperoxide.[1] In a search for the oxidase operating in tomato fruit, enzymic proteins were precipitated with ammonium sulphate (80% saturation), excess ammonium salt being removed by dialysis. This procedure served both to concentrate the enzymic proteins and at the same time remove phenolic substances, especially dihydroxyphenols which have been shown to inhibit strongly the synthesis of ethylene in extracts of fruit and vegetable tissues.[1,5] To these enzyme extracts, substrates of known oxidases were added together with the peroxidase co-factors (sulphinic acid and a phenolic) and 4-methylmercapto-2-oxobutyric acid. The system then became a test system for the presence of any oxidase generating hydrogen peroxide or a hydroperoxide, the presence of which could be detected by the production of ethylene from the oxo acid. The substrates added included D-glucose, DL-alanine or DL-glutamic acid, glycollic acid or linolenic acid, denoting respectively the presence or absence of glucose oxidase, D-amino acid oxidase, glycollic acid oxidase or lipoxygenase type enzymes. As the results (Table 3) show, only with linolenic acid was there any appreciable amount of ethylene formed, and that this was dependent on the presence of a heat labile catalyst was evident from the absence of any ethylene production when the enzyme extract from the tomato fruit was held at 100° for 5–10 min before test. These results indicated that with this fruit, a fatty acid oxidase rather than a glucose oxidase, or other oxidase, is involved in the production of ethylene from methionine or its derivatives.

Blain, Patterson and Pearce[6] have also observed the presence of a similar enzyme in tomato fruit. They found that their fatty acid oxidase had properties resembling a haem protein rather than those of a typical lipoxygenase. Our findings confirm this in that the tomato enzyme is inhibited by cyanide (50% inhibition with 0·1mM) at a pH of 6·0 and catalyses the oxidation of linolenate in a heterogeneous system at pH 6·0, but not in a

[4] C. Frenkel, I. Klein and D. R. Dilley, *Phytochem.* **8**, 945 (1969).
[5] H. S. Ku, S. F. Yang and H. K. Pratt, *Phytochem.* **8**, 567 (1969).
[6] J. A. Blain, J. D. E. Patterson and M. Pearce, *J. Sci. Food Agri.* **19**, 713 (1968).

TABLE 3. THE INITIATION OF ETHYLENE PRODUCTION FROM 4-METHYLMERCAPTO-2-OXOBUTYRIC ACID BY LIPOXYGENASE OF TOMATO FRUITS

Substrate of oxidase	Ethylene produced (μl)		
	0·5 hr	1 hr	2 hr
D-Glucose 0·1%	0·1	0·14	0·28
DL-Glutamic acid 0·1%	0·02	0·06	0·08
DL-Alanine			
Glycollic acid 0·1%	0·08	0·1	0·12
Linolenate 1mM	0·22	0·73	1·22
Linolenate* 1mM	0·03	0·07	0·1

* With heat denatured enzyme + horseradish peroxidase (0·9 units).

The non-particulate fraction from tomato fruit at the onset of their climacteric was used and the enzymes contained therein precipitated with 0–80%-saturated $(NH_4)_2SO_4$ and dialysed. The ethylene produced by these enzyme proteins in 0·1M-sodium phosphate buffer, pH 5·5, together with benzene sulphinic acid (5 μmoles), *p*-hydroxybenzoate (5 μmoles), oxo acid (10 μmoles) and EDTA (2mM) in a volume of 10 ml with substrates of different oxidases, was determined.

homogeneous system at pH 9·0.[7] Whether it is a haem protein remains to be determined. It does not appear to be identical with peroxidase, the most likely haem protein constituent of the non-particulate fraction of the cytoplasm, for a typical peroxidase cannot replace soya bean lipoxygenase in the enzymic production of ethylene from the oxo acid (cf. Table 4) and the changes in the activity of the tomato peroxidase and of the fatty acid oxidase that occur during ripening do not run parallel as would be expected if the activity observed were due to a single enzyme (cf. Fig. 2). Finally, fractionation of tomato extracts with ammonium sulphate has partially separated lipoxygenase (0–40% saturation) from peroxidase activity (40–70% saturation).

Synthesis initiated by hydroperoxide. We have repeated some of our earlier observations[1] on the conditions necessary for the synthesis of ethylene by a lipid hydroperoxide from either

TABLE 4. THE FORMATION OF ETHYLENE FROM THE OXO ACID OR METHIONAL WITH LIPOXYGENASE AS THE OXIDASE AND SULPHINIC ACID AS CO-FACTOR

Substrate	Omission from complete system	Ethylene produced (μl) 0·5 hr	1 hr	2 hr
4-Methylmercapto-2-oxobutyric acid (10 μmoles)	None	1·28	1·78	2·28
	Peroxidase	0·09	0·09	0·11
	Peroxidase and *p*-hydroxybenzoate	0·09	0·1	0·1
	Sulphinic acid	0·14	0·2	0·3
	Lipoxygenase	0·06	0·12	0·2
Methional (10 μmoles)	None	0·84	1·02	1·28
	Peroxidase	0·04	0·04	0·05
	Peroxidase and *p*-hydroxybenzoate	0·06	0·07	0·08
	Sulphinic acid	0·02	0·02	0·03
	Lipoxygenase	0·12	0·16	0·2

The complete system contained benzene sulphinic acid (5 μmoles), *p*-hydroxybenzoate (5 μmoles), linolenate (10 μmoles), lipoxygenase (soya bean) (4100 units), horseradish peroxidase (1·5 units) and substrate (10 μmoles) in 10 ml of 0·1M-sodium phosphate buffer, pH 6·0 containing EDTA (2mM).

[7] A. L. TAPPEL, *Food Res.* **18**, 104 (1953).

the oxo acid or its derivative methional (3-methylmercapto-propionaldehyde). The results (Table 4) show that a soybean lipoxygenase enzyme and its substrate linolenic acid will initiate the production of ethylene from both these derivatives of methionine. The requirements for a peroxidase, a sulphinic acid, and for a suitable phenolic are similar to those already reported for the glucose oxidase system; the lipoxygenase and its substrate thus substitute for glucose oxidase and its substrate.

Sulphite can replace a sulphinic acid in the formation of ethylene from either the oxo acid or methional,[8] and accordingly it was of interest to determine if sulphite could so act when the lipoxygenase enzyme replaced glucose oxidase. The results (Table 5) show that

TABLE 5. THE FORMATION OF ETHYLENE FROM THE OXO ACID OR METHIONAL WITH SULPHITE AS CO-FACTOR AND EITHER SOYA BEAN LIPOXYGENASE OR GLUCOSE OXIDASE AS OXIDASE

Substrate	Oxidase	Omission from complete system	Ethylene produced (μl) 0·25 hr	0·5 hr	1 hr	2 hr
4-Methylmercapto-2-oxobutyric acid (10 μmoles)	Lipoxygenase (41,000 units)	None	6·7	10·6	13·8	13·8
		Peroxidase	6·7	10·6	13·5	13·8
		Peroxidase and *p*-hydroxybenzoate	5·8	9·7	13·3	15·4
		Sulphite	0·1	0·17	0·3	0·4
		Lipoxygenase	2·8	4·5	8·0	10·6
		Lipoxygenase, peroxidase and *p*-hydroxybenzoate	1·8	3·8	7·1	11·4
Methional (10 μmoles)	Lipoxygenase (41,000 units)	None	0·75	1·5	2·2	3·3
		Peroxidase	0·52	1·2	1·8	3·0
		Peroxidase and *p*-hydroxybenzoate	0·48	1·3	2·2	3·4
		Sulphite	0·02	0·04	0·05	0·08
		Lipoxygenase	0·25	0·4	0·8	1·4
		Lipoxygenase, peroxidase and *p*-hydroxybenzoate	0·17	0·32	0·68	1·3
4-Methylmercapto-2-oxobutyric acid (10 μmoles)	Glucose oxidase (0·29 units)	None	16·1	16·2	16·3	—
		Peroxidase and *p*-hydroxybenzoate	0·04	0·06	0·06	—
		None*	10·2	17·8	27	—

* Sulphite replaced by benzene sulphinic acid (5 μmoles).

The complete system contained sulphite (5 μmoles), *p*-hydroxybenzoate (5 μmoles), linolenate (10 μmoles), horseradish peroxidase (1·2 units), oxidase and substrate in 10 ml of 0·1M-sodium phosphate buffer, pH 6·0, containing EDTA (2mM).

with the lipoxygenase–linolenate system as oxidase, there was no requirement for either a peroxidase enzyme or a phenolic with either oxo acid or methional as substrate, a finding which suggests that peroxidative activity is self-generated in this system. Indeed in such a system it was possible to dispense with a lipoxygenase; in the presence of sulphite and non-peroxidized linolenate, appreciable amounts of ethylene were found from either oxo acid or methional in the complete absence of enzymes. With glucose oxidase as oxidase and

[8] L. W. MAPSON, R. SELF and D. A. WARDALE, *Biochem. J.* **111**, 413 (1969).

sulphite as co-factor, the participation of peroxidase and attendant phenol is necessary. These are thus further differences[2] between a model system containing sulphite and an enzymic system in which sulphinic acid acts as an essential co-factor for the action of the peroxidase enzyme.

Effect of pH. The relation between pH and ethylene production by enzymes isolated from tomato tissue when the synthesis is initiated by the addition of linolenate is shown in Fig. 1. In these experiments, the tomato enzymes were supplemented with the fatty acid

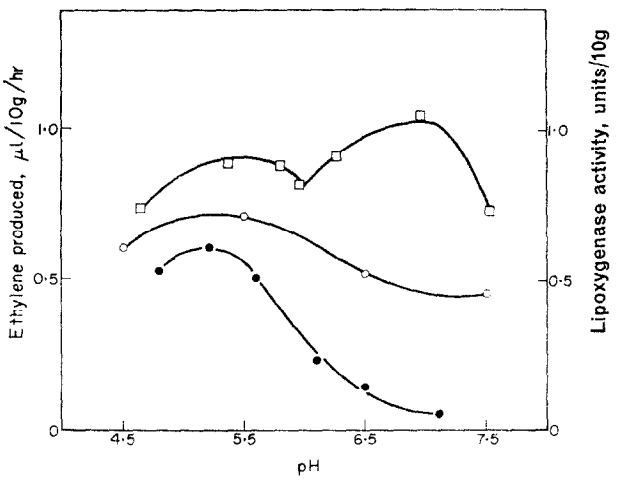

FIG. 1. THE RELATION BETWEEN pH AND (a) ETHYLENE PRODUCTION WHEN THE LATTER IS INITIATED BY TOMATO LIPOXYGENASE, (b) ETHYLENE PRODUCTION BY TISSUE PLUGS, AND (c) LIPOXYGENASE ACTIVITY.

The enzymes were prepared from fruit at the onset of their climacteric by precipitation of the non-particulate fraction with 0–80%-saturated $(NH_4)_2SO_4$ and dialysis for 2 hr against 0·01M-sodium phosphate buffer, pH 6·0. (a) The flasks contained the enzyme fraction (≡ 10 g tomato tissue), EDTA (2mM), benzene sulphinic acid (5 μmoles), *p*-hydroxybenzoate (5 μmoles), linolenate (10 μmoles) and oxo acid (10 μmoles) in 0·1M-sodium phosphate buffer, at the appropriate pH, ●. (b) Twelve tomato plugs (6 g) were immersed in 10 ml 0·2M-sodium phosphate buffer, at the appropriate pH containing mannitol (0·2M) and EDTA (2mM) and left for a 5-hr period with shaking in oxygen at 25°, ○. (c) Lipoxygenase activity was measured spectrophotometrically on the enzyme fraction, at the appropriate pH, □.

and the other co-factors and the rate of formation of ethylene on addition of the oxo acid determined. In a second experiment, the concentration of the peroxidase present was increased by the addition of horseradish peroxidase to ensure that the enzymic activity being measured was due to the lipoxygenase and was not limited by peroxidase activity. In both experiments, the effect of pH on the ability to form ethylene from the oxo acid showed that maximum activity occurred within the pH range 5·2–5·5, being especially sharply curtailed on the alkaline side. It is interesting to compare these results with the activity of the tomato lipoxygenase as determined by the procedure of Surrey[9] and with the effect of pH on ethylene production by tomato plug tissue (Fig. 1). With the enzyme over the pH range 4·5–7·5, there were two peaks of maximum activity, one at ~pH 5·4 and one

[9] K. SURREY, *Plant Physiol.* **39**, 65 (1964).

at ~7·0. The effect of immersing tomato plugs in 0·2M phosphate buffers over the range pH 4·5–7·5 on their endogenous ethylene production resembles that observed with the extracted enzymes, with again a maximum at pH 5·4, but falling less sharply on the alkaline side.

Changes in enzyme activity during ripening. We have shown the presence in extracts of tomato of three enzymes, a transaminase, an enzyme with lipoxygenase activity and a peroxidase, which in combination with their substrates and co-factors are theoretically capable of forming ethylene from methionine or its derivatives. It remains to assess the evidence for the operation of these particular enzymes in the synthetic process *in vivo* in this fruit.

The activities of these separate enzymes during four stages in the ripening of the fruit, i.e. preclimacteric, onset of climacteric, climacteric peak and postclimacteric phase, have been studied. Extracts were made from the fruit in these different phases, the enzyme activities determined and compared with the ethylene production from similar fruit. Results (Fig. 2) shown as a percentage increase or decrease in activity of the various enzymes during the ripening syndrome, indicate that the activities of all three enzymes change. Peroxidase activity shows a steady decline throughout ripening, some increase in lipoxygenase activity occurs early before the onset of the climacteric phase, followed by a decline in activity before the peak of the climacteric is reached. Similar findings to these have been reported by Pratt *et al.*,[10] who also observed that lipoxygenase activity is high in the early

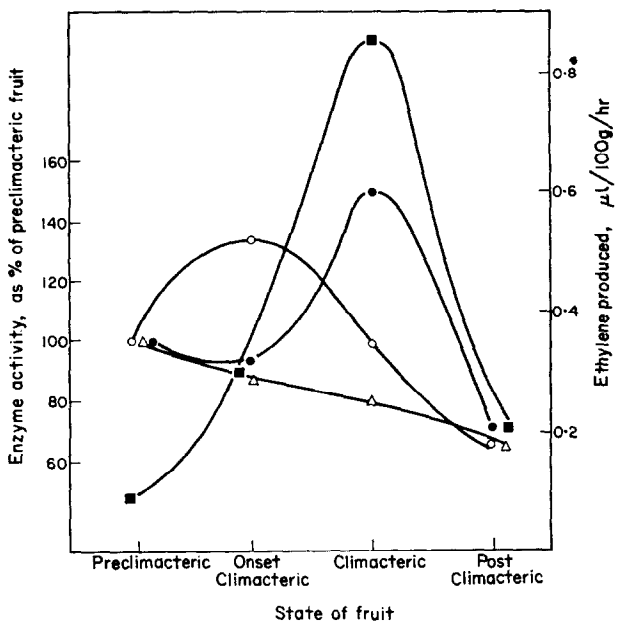

FIG. 2. CHANGES IN THE ACTIVITIES OF ENZYMES INVOLVED IN THE SYNTHESIS OF ETHYLENE DURING RIPENING OF TOMATOES.

△, Peroxidase activity; ●, transaminase activity; ○, lipoxygenase activity; ■, ethylene production.

[10] H. K. PRATT, A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Rep. Ditton Covent Garden Lab.* 44 (1964–65).

stages of the climacteric, falling rapidly as the fruit ripens. The activity of the transaminase likewise rises during the climacteric and falls in the postclimacteric phase. Hulme *et al.*[11] also observed that transaminase activity increased (10-fold) during the development of the climacteric in apple peel tissue. Our results show that the rise and fall of this enzyme coincides more closely in time with the rise and fall in ethylene production during ripening of the whole fruit. On their face value, the results suggest that the transaminase enzyme may be limiting the rate of conversion of methionine to ethylene and our previous results[3] do indicate that the incorporation of ^{14}C labelled methionine into ethylene by tomato discs follows a similar pattern.

If indeed the activity of the transaminase enzyme is limiting the overall rate of synthesis from methionine, the rate should be increased if the action of the enzyme was bypassed by supplying the product 4-methylmercapto-2-oxobutyric acid to the tissue. It was found that stimulation of ethylene production only occurred on the addition of the oxo acid to tissue plugs from fruit in the early climacteric and climacteric phases (Table 6). Little or no stimulation occurred with tissue from fruit in the pre- or postclimacteric state, suggesting that whilst the activity of the transaminase may have been limiting over the climacteric phase, other factors were controlling the synthesis in the pre- and postclimacteric stages.

TABLE 6. INCREASE IN ETHYLENE PRODUCTION OF TOMATO PLUGS BY 4-METHYLMERCAPTO-2-OXOBUTYRIC ACID

	pH Of external medium	Condition of fruit	Ethylene produced (μl/10 g/hr)		
			Control	With oxo acid (1mM)	Increase
Expt. 1	5·5	Preclimacteric	0·14	0·19	0·05
		Early climacteric	0·46	0·60	0·14
		Climacteric	0·68	0·9	0·22
		Postclimacteric	0·14	0·15	0·01
Expt. 2	7·4	Preclimacteric	0·06	0·09	0·03
		Early climacteric	0·27	0·43	0·16
		Climacteric	0·31	0·44	0·13
		Postclimacteric	0·03	0·07	0·04

The ethylene production from 12 tomato plugs (6 g) immersed in 10 ml 0·2M sodium phosphate buffer containing mannitol (0·2M) and EDTA (2mM) at the stated pH was measured over a 5-hr period in oxygen at 25°.

Quantitative assessment. Whilst the changes observed in the activities of these enzymes during ripening provide no proof that they are involved in the synthesis *in vivo*, they are not inconsistent with this concept. An attempt has been made to determine if the activities of these enzymes as assayed in extracts are sufficient to quantitatively account for the observed production of ethylene from the fruit. To test this for lipoxygenase activity the enzyme was isolated from fruit in the climacteric phase in a protein fraction extracted with 0·2M-Tris containing 0·02M $CaCl_2$, pH 8·7 and isolated by precipitation with ammonium sulphate (80% saturation). The enzyme proteins were taken up in 0·1M-sodium phosphate buffer, pH 5·5 and their activity in forming ethylene from the oxo acid (mM) on addition of linolenate, peroxidase and the co-factors (benzene sulphinic acid and *p*-hydroxybenzoate)

[11] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1343 (1967).

determined. The rate of production of ethylene from the same tissue in the form of tomato plugs, with and without the addition of oxo acid, was determined concurrently. The results showed that at pH 5·5, 0·55 μl C_2H_4/10 g/hr were produced from the isolated enzyme system as compared with 0·46 μl/10 g/hr from the same tissue in the form of plugs, and this latter was increased to 0·59 μl/10 g/hr when supplemented with oxo acid (mM). These rates are therefore comparable and indicate that the activity of the fatty acid oxidase was sufficient to account for the observed production of ethylene from the tissue slices, which are known to be considerably higher than the rate of ethylene production from intact fruit.[12,13]

The activity of the peroxidase enzyme in all stages of ripening was also more than sufficient to account for the rate of ethylene production. The lowest estimation of peroxidase activity after extraction from the fruit and partial purification by ammonium sulphate treatment was equivalent to 3·4 purpurogallin units/10 g fruit. Tests with model enzyme systems using horseradish peroxidase at half this concentration were sufficient to produce a rate of synthesis some 30-fold greater than that observed from tomato plugs with or without supplementation with the oxo acid.

It is more difficult to assess quantitatively whether the transaminase activity of the fruit can account for the observed production of ethylene in the different stages of ripening owing to some unknown variables. Despite this, an attempt was made to assess the possibility by estimating the transaminase enzyme activity after extraction in both aged particulate and non-particulate fractions from preclimacteric, climacteric and postclimacteric tomato tissue. At the same time, the ethylene production of the whole fruit and tissue plugs was also determined. The results (Table 7) show the rate of production of ethylene from whole

TABLE 7. ESTIMATION OF TRANSAMINASE ACTIVITY BY FORMING ETHYLENE IN RELATION TO THAT PRODUCED BY WHOLE FRUIT OR TOMATO PLUGS

		Ethylene produced μl/10 g/hr
Whole fruit	Preclimacteric	0·008
	Climacteric	0·14
	Postclimacteric	0·02
Tissue plugs	Preclimacteric	0·14
	Climacteric	0·68
	Postclimacteric	0·14
Transaminase*	Preclimacteric	0·27
	Climacteric	0·41
	Postclimacteric	0·23

*Values in table are sum of enzyme activities in particulate and non-particulate fractions.

The production of ethylene was determined on single whole fruits in oxygen through the preclimacteric to postclimacteric phase. Ethylene production was also measured from 12 tomato plugs (6 g), immersed in 10 ml 0·2M-sodium phosphate buffer, pH 5·5, containing mannitol (0·2M) and EDTA (2mM), over a 5-hr period in oxygen at 25°. The ethylene produced from methionine as a result of transaminase action was determined on the same batch of fruit, as described in Table 1.

[12] M. S. SPENCER, *Can. J. Biochem. Physiol.* **34**, 1261 (1956).
[13] D. F. MEIGH, K. H. NORRIS, C. C. CRAFT and M. LIEBERMAN, *Nature* **186**, 902 (1960).

fruit and fruit tissue, in comparison with that formed by the action on methionine of the transaminase extracted from the same fruit, the resulting oxo acid then being converted to ethylene on addition of the peroxidase enzyme system. It seems clear that on such a basis, the activity of the transaminase enzyme could account for the ethylene production by whole fruit and for that produced by plugs from preclimacteric and postclimacteric tissue, but not quite for plugs from climacteric tissue. However, one variable which is not being assessed in such calculations is the efficiency of conversion of methionine or its precursors into ethylene. In the production of ethylene from the oxo acid by the peroxidase test system, only about 15% of the oxo acid present is converted under the conditions of the test. There is evidence to indicate that the efficiency with which exogenous oxo acid or methionine are converted to ethylene increases as the fruit passes from the preclimacteric to the climacteric state, decreasing again in the postclimacteric phase.[3] In the climacteric state, the efficiency of conversion of exogenous labelled oxo acid to ethylene was also of the order of 15%. If, as seems likely, the efficiency with which endogenous methionine or oxo acid is converted is higher than this, then the transaminase activity of the fruit might be more than sufficient to account for the ethylene production in plug tissue even at the height of the climacteric.

DISCUSSION

We have shown in this paper that tomatoes contain three enzymes, a transaminase, an enzyme with lipoxygenase activity, and a peroxidase, which, acting in concert, are capable of forming ethylene from methionine or its derivatives.

The possibility of the participation of lipoxygenase type enzymes has been raised by a number of workers. Wooltorton, Jones and Hulme[14] observed an increase in the concentration of the enzyme in apple peel tissue and observed that this increase coincided with a phase of rapid production of ethylene. Meigh, Jones and Hulme[15] made similar observations and Galliard[16] observed an increased breakdown of linolenate during ripening of pome fruits. In a later work, Galliard *et al.*[17] observed the stimulatory effect on ethylene production of adding lipoxygenase and linolenate or linolenate alone to preclimacteric apple peel discs.

We have been unable to stimulate ethylene production by preclimacteric tomato plugs by the simple addition of linolenate. Linolenate in a concentration (1mM) caused some inhibition in the production of ethylene although a marked loss of chlorophyll occurred, suggestive of the increased activity of a lipoxygenase. This could mean that in contrast to the apple experiment, lipoxygenase activity in tomato tissue is not a limiting factor. From the standpoint of the synthesis of ethylene it appears to be immaterial whether the fatty acid hydroperoxide is produced by a typical lipoxygenase or by the catalytic activity of a haem protein. Fatty acid hydroperoxide is capable of initiating the formation of ethylene from its precursor 4-methylmercapto-2-oxobutyric acid in the presence of a peroxidase and co-factors. In this respect, it is able to substitute for the similar system identified in cauliflower florets in which hydrogen peroxide appears to be involved.[1]

[14] L. S. C. WOOLTORTON, J. D. JONES and A. C. HULME, *Nature* **207**, 999 (1965).
[15] D. F. MEIGH, J. D. JONES and A. C. HULME, *Phytochem.* **6**, 1507 (1967).
[16] T. GALLIARD, *Phytochem.* **7**, 1915 (1968).
[17] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 1465 (1968).

EXPERIMENTAL

Enzymes and chemicals. Fungal glucose oxidase (14·7 Sigma units/mg), soybean lipoxidase (8200 Sigma units/mg) and horseradish peroxidase (295 purpurogallin unit/mg) were purchased from the Sigma Chemical Co., St. Louis, Mo., USA. The oxo acid, 4-methylmercapto-2-oxobutyric acid was prepared as described previously.[2] Linolenate was used as the ammonium salt in enzyme experiments.

Ethylene. This was measured by GLC as described previously.[18]

Peroxidase system. The peroxidase system used for measuring the amount of ethylene formed from the oxo acid, consisted of glucose oxidase (20 μg), horseradish peroxidase (0·9 purpurogallin units), *p*-hydroxybenzoate (5 μmoles), benzene sulphinic acid (5 μmoles) and glucose (1%, w/v) in 10 ml of 0·1M-sodium phosphate buffer, pH 6·8, containing EDTA (2mM).

Transaminase. This enzyme was located mainly in the particulate fraction and was prepared as follows. Half-ripe tomatoes (180 g) were extracted with 230 ml of a chilled solution (0·1M Tris, 0·4M mannitol and 0·02M $CaCl_2$, pH 9·1) by maceration in a Waring blender. Cell debris was removed by filtration through muslin followed by a low speed centrifugation. If necessary, the solution was adjusted to pH 7·4, and recentrifuged at 40,000 *g* for 30 min. The particulate fraction so obtained was washed with 0·1M-sodium phosphate buffer, pH 7·2 containing mannitol (0·4 M) and finally suspended in 30 ml of the same buffer.

The non-particulate fraction obtained after centrifuging at 40,000 *g* was treated with $(NH_4)_2SO_4$ to 80 per cent saturation (56 g $(NH_4)_2SO_4$/100 ml solution). The precipitate was collected, dissolved in 30 ml of 0·01M sodium phosphate buffer, pH 6·8, and dialysed for 2 hr against 1 l of the same buffer. The solution was then centrifuged to remove debris and dialysed a further 2 hr against fresh 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 the phosphate-mannitol buffer, pH 6·8, containing EDTA (2mM) pyruvate (1mM) and pyridoxal phosphate (0·1mM), for periods of 20–24 hr. The oxo acid was determined by its conversion into ethylene by the peroxidase system.

Peroxidase and lipoxygenase. These enzymes were located in the non-particulate fraction and were prepared as follows. Tomatoes (200 g) at the onset of climacteric were extracted with an equal volume of a chilled solution (0·2M Tris and 0·02M $CaCl_2$, pH 8·7) by maceration in a Waring blender. It was found to be important to maintain the pH during extraction at ~8·2 in order to obtain maximum enzyme yield. Cell debris was removed by filtration through muslin and the solution centrifuged at 40,000 *g* for 30 min. The non-particulate fraction so obtained was either tested directly or treated with $(NH_4)_2SO_4$ to 80% saturation (56 g $(NH_4)_2SO_4$/100 ml solution) and the precipitate dissolved in 30 ml 0·1M-sodium phosphate buffer, pH 7·0 and dialysed for 2 hr against 1 l of the same buffer.

Peroxidase activity was measured by the formation of purpurogallin from pyrogallol. One "purpurogallin unit" caused the formation of 1 mg of purpurogallin in 20 sec at a pH of 6·0, with an initial concentration of H_2O_2 of 0·147M.

Lipoxygenase activity was measured by the method of Surrey.[9] One enzyme unit produces an O.D. change of 1·0 at 234 mμ in 1 min in a total vol. of 10 ml 60% ethanol at a pH of 6·0.

Tomatoes. The varieties used were Kingley Cross and Suttons Alicante. Plugs of tissue, 10 mm in dia. were cut from the flesh of the tomato walls with the skin attached to one face of the plug. Twelve discs (6 g) were immersed in 10 ml of 0·2M-sodium phosphate buffer, at various pHs, containing mannitol (0·2M) and EDTA (2mM) in a 50-ml flask with oxygen and shaken at 25°.

[18] L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **102**, 574 (1967).