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METABOLIC CHANGES IN EXCISED FRUIT TISSUE—III.

THE DEVELOPMENT OF ETHYLENE BIOSYNTHESIS DURING THE AGEING OF DISKS OF APPLE PEEL

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Abstract—The production of ethylene by disks of peel from pre-climacteric apples is induced by ageing the disks aerobically at 25°. The development of the ethylene-producing system is dependent upon protein synthesis. Ethylene evolution is stimulated by peroxidation products from linolenic acid, but not by methionine. The relationship between changes occurring during the ageing of pre-climacteric apple peel disks during the development of the climacteric in whole fruit is discussed. A rapid and sensitive method for the determination of ethylene is described.

INTRODUCTION

THE ROLE of ethylene as a ripening hormone in fruit was first recognized over 30 years ago[1, 2] but until recently little progress had been made in understanding either the mode of action or the biosynthesis of ethylene. Recent work has indicated that the stimulation by ethylene of both the abscission of leaves and the ripening of bananas is due to the synthesis of specific messenger RNA molecules.[3]

Synthesis of RNA and protein are initiated in plant storage organs when the tissues are sliced and "aged" by incubation in moist air or in suspension in aqueous solutions.[4] In the preceding two papers[5, 6] we have shown that, during the ageing of disks prepared from the peel of pre-climacteric apples, there is an increased synthesis of new protein leading to the development of several enzyme systems. The present paper extends this work to show the formation of an ethylene-producing system during the ageing of disks of peel from pre-climacteric apples.

RESULTS

The rate of ethylene production by disks of apple peel was determined after periods of ageing in various media at 25°. Figure 1 shows the change in rate during ageing of disks taken from peel from samples of pre-climacteric apples which themselves produce negligible amounts of ethylene even after 2 weeks at 12° from the time that the peel was prepared from them. With peel from immature apples there is a lag phase before the production of ethylene commences. Curve *B* in Fig. 1 illustrates this effect in disks prepared from apples from trees

[1] F. KIDD and C. WEST, *Rep. Food Invest. Bd., London*, p. 33 (1933).
[2] R. GANE, *J. Pomology* **13**, 351 (1935).
[3] R. E. HOLM and F. B. ABELES, *Plant Physiol.* **42**, 1094 (1967).
[4] R. E. CLICK and D. P. HACKETT, *Proc. Nat. Acad. Sci. U.S.* **50**, 243 (1963).
[5] M. J. C. RHODES, L. S. C. WOOLTORTON, T. GALLIARD and A. C. HULME, *Phytochem.* **7**, 1439 (1968).
[6] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 1453 (1968).

sprayed with the growth-retardant, N-dimethylamino succinamic acid; indications of a similar lag phase have been observed in normal apples taken at earlier pre-climacteric stages. No lag phase was observed in disks from fruit picked from the tree as the climacteric commenced (curve *A*) and maximum ethylene production occurs after 6–7 hr.

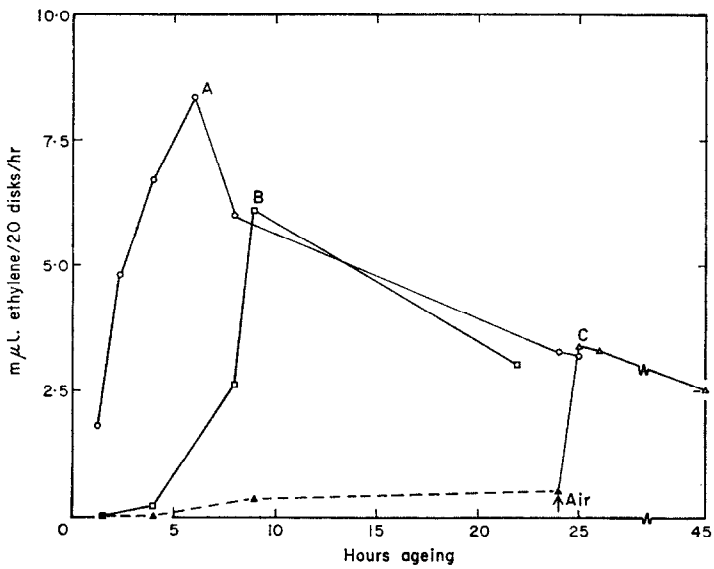

FIG. 1. DEVELOPMENT OF ETHYLENE PRODUCTION DURING AGEING OF APPLE PEEL DISKS.

Experimental conditions are described in the text. The effects are shown for disks from apples at the commencement of the climacteric (curve *A*), from pre-climacteric apples (curve *B*), and from pre-climacteric apples when the disks were aged in 3 per cent O_2–97 per cent N_2 for 24 hr before transferring to aerobic conditions (curve *C*).

Low concentrations (1 μg/ml) of cycloheximide, an inhibitor of protein synthesis, completely inhibited the production of ethylene by disks during ageing (Table 1). Control experiments in which disks were aged normally in the absence of inhibitor and then assayed for ethylene production in the presence of cycloheximide (1 μg/ml) gave the same amount of ethylene as the controls assayed without inhibitor. Thus only the *development* of an ethylene-producing system and not the *production* of ethylene is sensitive to inhibition by cycloheximide.

TABLE 1. EFFECT OF AGEING FOR 24 hr ON THE RATE OF ETHYLENE PRODUCTION BY DISKS OF PRE-CLIMACTERIC APPLE PEEL

Conditions of ageing*	Ethylene production (mμl/hr/20 disks) Experiment number: 1	2	3
Fresh disks (no ageing)	<0·5	<0·5	<0·5
Aged controls	7·2	10·4	12·7
Aged in presence of cycloheximide (1 μg/ml)	<0·5	<0·5	<0·5

* For conditions of ageing and incubation—see Experimental.

Ageing of disks of apple peel in an atmosphere of 3 per cent O_2 and 97 per cent N_2 ("3 per cent O_2") inhibits the development of a malate decarboxylating system[5] but does not inhibit the onset of lipid synthesis.[6] Curve *C*, Fig. 1, shows that disks aged and incubated in 3 per cent O_2 have a very low rate of ethylene production. When these disks are then transferred to incubate in air, a rapid evolution of the gas ensues. The rate of ethylene production on transferring from 3 per cent O_2 to air was found to be variable, in some experiments reaching a level up to five times that of controls maintained throughout under aerobic conditions.

The results of a study of various conditions of incubation on ethylene production are shown in Table 2. Malate (0·1 M) inhibited ethylene production; in these experiments both

TABLE 2. EFFECTS OF INCUBATION CONDITIONS OF ETHYLENE PRODUCTION BY FRESH AND AGED DISKS ON APPLE PEEL

	Experiment number				
	1	2	3	4	5†
	Ageing period (hr)				
Conditions of incubation*	24	24	15	15	0
	Ethylene production (mμl/hr/20 disks)				
Fresh disks—no additions	<0·5	<0·5			11·6
Aged disks—no additions	7·2	10·4	6·0	15·0	
+malate	1·7	<0·5			
+methionine		8·4			
anaerobic				0·7	<0·5
+linolenic acid		9·4	6·7		
+lipoxidase			6·7		
+lipoxidase+linolenic acid			52·6	60·3	53·0
+lipoxidase+linolenic acid+methionine				47·0	
+lipoxidase+linolenic acid (anaerobic)					<0·5
+lipoxidase+linolenic acid (disks boiled)				0·7	
+lipoxidase+linoleic acid				10·3	
+lipoxidase+oleic acid				13·3	

* For ageing and control incubation conditions—see Experimental. Additions to incubation as indicated were: potassium malate, 0·1 M; methionine, 1 mM; oleic, linoleic and linolenic acids (as ammonium salts) 1 mM; lipoxidase, 0·3 mg (17,000 units) per ml.

† In experiment 5 the disks used were prepared from climacteric apples which were themselves producing ethylene.

ethylene and acetaldehyde production were measured and the production of acetaldehyde was eight times greater in disks aged for 24 hr than in fresh disks. Cycloheximide abolished the increased formation of acetaldehyde in aged disks. Methionine, which has been shown to be a direct precursor of ethylene in several plant systems,[7–10] including senescent apples,[11] did not stimulate ethylene production in aged disks from pre-climacteric apples (Table 2). Linolenic acid has also been suggested as a precursor of ethylene in plants[12] and it has been

[7] M. LIEBERMAN, A. T. KUNISHI, L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **97**, 449 (1965).
[8] S. P. BURG and C. O. CLAGETT, *Biochem. Biophys. Res. Commun.* **27**, 125 (1967).
[9] L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **107**, 433 (1968).
[10] H. S. KU, S. F. YANG and H. K. PRATT, *Arch. Biochem. Biophys.* **118**, 756 (1967).
[11] M. LIEBERMAN, A. T. KUNISHI, L. W. MAPSON and D. A. WARDALE, *Plant Physiol.* **41**, 376 (1966).
[12] M. LIEBERMAN and L. W. MAPSON, *Nature* **204**, 343 (1964).

further suggested that the hydroperoxidation of long-chain unsaturated fatty acids mediated by lipoxidase is involved in the production of ethylene in ripening apples.[13] Addition of linolenic acid or lipoxidase separately to aged disks of apple peel caused no stimulation of ethylene production but, when added together, caused a 4–8 fold increase. If incubations were performed anaerobically, or with boiled disks aerobically, no ethylene was produced with added linolenic acid and lipoxidase. Thus the formation of ethylene in the presence of linolenic acid and lipoxidase is an aerobic, enzymic process. The specificity for linolenic acid as substrate is shown by the fact that linoleic acid (an alternative substrate for lipoxidase) caused no stimulation of ethylene production in the presence of lipoxidase. Furthermore, we have been unable to stimulate ethylene formation by disks with other peroxide-generating systems, e.g. hydrogen peroxide, glucose with glucose oxidase or with glycollic acid. Although no ethylene is produced by fresh disks from pre-climacteric apples, addition of lipoxidase with linolenic acid to fresh disks causes ethylene production at a rate similar to that observed with aged disks in the presence of linolenic acid and lipoxidase.

No attempt was made in the present experiments to determine the uptake of linolenic acid by the disks, but studies on utilization of linoleic acid-1-^{14}C described in the previous paper in this series[6] showed an appreciable uptake of this acid even though it was added as the ammonium salt, in the absence of detergent, to the incubation mixture at pH 4·5. Evidence against the contribution of microbial contamination to the results obtained in these experiments with disks of apple peel has been presented in a previous paper.[5]

DISCUSSION

The production of ethylene during ripening is characteristic of many fruits.[14] The present paper demonstrates that disks prepared from the peel of apples in the pre-climacteric state and not themselves producing ethylene can be induced to produce ethylene simply by ageing. The development of the system producing ethylene is initiated by excision of the peel, and the fact that the inhibitor of protein synthesis, cycloheximide, prevents the development of the system during ageing suggests that synthesis of new protein is involved in the process.

In common with the development of a malate decarboxylating system during ageing,[5] the formation of ethylene in disks of apple peel is dependent on O_2 tension. However, when once transferred from a low O_2 tension to air the disks immediately produce ethylene at a rapid rate. A similar effect was found by Burg and Thimann[15] when whole ripe apples and plugs of tissue prepared from them were transferred from an atmosphere low in O_2 to air. It appears possible that part of the enzyme system involved in ethylene production is synthesized during ageing in low O_2 (that part inhibited by cycloheximide) but that the final conversion of precursors to ethylene involves a step requiring a much higher tension of O_2. The fact that ethylene production on transfer of disks from low to normal levels of O_2 is often higher than in the controls maintained in air throughout, may be due to an accumulation of a precursor during the period in low O_2.

The enzymic conversion of methionine and methional to ethylene has recently been shown to occur with partially purified enzyme preparations from plant tissues[9] in which a peroxide-generating enzyme (glucose oxidase) and a methional-splitting enzyme are involved. However, although Lieberman *et al.*[11] were able to show that methionine was a precursor of ethylene in apples which had been stored for prolonged periods, they could not demonstrate

[13] L. S. C. WOOLTORTON, J. D. JONES and A. C. HULME, *Nature* **207**, 999 (1965).
[14] S. P. BURG, *Ann. Rev. Plant Physiol.* **13**, 265 (1962).
[15] S. P. BURG and K. V. THIMANN, *Proc. Nat. Acad. Sci. U.S.* **45**, 335 (1959).

this for less mature apples. Burg and Clagett[8] have also demonstrated the conversion of methionine to ethylene in apple tissue but did not describe the stage of maturity of the apples used. We were unable to stimulate ethylene production by addition of methionine to fresh or aged disks of peel from pre-climacteric apples.

The increased capacity to decarboxylate added malate to acetaldehyde via pyruvate which develops during ageing of disks of apple peel[5] may well explain the inhibitory effect which malate has on ethylene production during ageing. Acetaldehyde is related structurally both to ethylene and to ethylene oxide and Lieberman and Mapson[16] have shown that acetaldehyde and ethylene oxide inhibit ethylene production in ripening whole apples.

Wooltorton *et al.*[13] have shown that the onset of the respiration climacteric in apples is accompanied by an increase in both ethylene production and lipoxidase activity; these authors suggest a relationship between lipid metabolism, lipoxidase activity and ethylene production during the ripening process. The previous paper in the present series[6] showed that ageing of peel disks brought about increased lipid synthesis; the present work has demonstrated the presence of an enzymic ethylene-producing system which is stimulated by linolenic acid and lipoxidase. The fact that fresh disks will evolve ethylene in the presence of added linolenic acid and lipoxidase may mean that, in fresh disks, either available substrate or lipoxidase is not present but is produced during ageing by a mechanism involving protein synthesis. Whilst this paper was in preparation Lieberman and Kunishi[17] published results showing that propanaldehyde, a major product in the peroxidation of linolenic acid, was a precursor of ethylene in model systems and stimulated the production of ethylene in slices of green tomatoes. Studies with disks of storage tissue[4] have shown that the development of increased respiration during ageing is due to m-RNA synthesis initiated by slicing. In a previous paper[5] we have shown that addition of ethylene eliminates the lag phase in the development of the malate decarboxylating system during ageing of peel disks and that the incorporation of ^{14}C-labelled uridine into the RNA of the disks is probably highest at the initial stages (1–2 hr) of ageing.

In the present series of papers we have described how the ageing of disks of peel from pre-climacteric apples results in the development of several systems each reaching a maximal level after different periods of ageing. The first to rise to a maximum is lipid synthesis (2–4 hr) followed by protein synthesis (8 hr) and ethylene evolution (6–8 hr) and, lastly, malate decarboxylation (16–24 hr). On the basis of present knowledge we tentatively propose the following sequence of events to explain these phenomena: removal of peel and preparation of disks initiates a de-repression of DNA, possibly by loss of a repressor; m-RNA is formed and causes the synthesis of some enzymes including those (possibly lipoxidase and fatty acid producing systems) concerned with ethylene biosynthesis; ethylene is produced and acts on a further portion of DNA leading to the formation of m-RNA associated with other enzyme systems such as malic enzyme. Subsequent papers will examine the relationship between these events occurring during the ageing of pre-climacteric tissue and those taking place in the whole fruits during the development of the climacteric.

EXPERIMENTAL

Materials

The source of apples used was described previously.[18] Other materials used were: chloramphenicol, B.P. (Boots Pure Drug Co.); cycloheximide (Sigma); all *cis*-oleic, linoleic and α-linolenic acids (Fluka A.G.);

[16] M. LIEBERMAN and L. W. MAPSON, *Nature* **196**, 660 (1962).
[17] M. LIEBERMAN and A. T. KUNISHI, *Science* **158**, 938 (1967).
[18] M. J. C. RHODES, T. GALLIARD, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 405 (1968).

fungal glucose oxidase (β-D-glucose:oxygen oxidoreductase, E.C. 1.1.3.4) 13 units/mg (Boehringer Corp.); lipoxidase (linoleate:oxygen oxidoreductase, E.C. 1.13.1.13) 50,000 units/mg (Koch-Light Laboratories). Gas mixtures were obtained from the British Oxygen Company and standard ethylene mixtures were prepared by mixing known volumes of ethylene and air.

Methods for Ageing and Incubating Disks

The method of preparation and ageing disks were described previously.[18] Peel disks used immediately after preparation (fresh disks) or after periods of ageing (aged disks) were washed well with water, lightly blotted and twenty disks placed in small conical flasks containing 3 ml solution of 0·05 M potassium phosphate, pH 4·5, chloramphenicol (50 μg/ml) and other additions as indicated in the text. The flask was closed with a rubber stopper with a narrow glass tube in the centre connected to a short piece of silicone-rubber fitted with a screw clamp. The gaseous volume of the flasks containing twenty disks and 3 ml solution was approximately 20 ml. Gas mixtures were introduced by evacuation and replacement of the gas phase through a three-way tap. Assays were performed by shaking the closed flask and contents at 25° for 1 hr.

Determination of Ethylene and Acetaldehyde

Following incubation, samples of the gas phase were taken with a gas-tight syringe connected to the flask with the silicone-rubber tubing and injected into a concentric tube-trap[19] modified by having an increased gap (8 mm) between the bottom of the inner tube and the base of the outer tube. In order to condense ethylene in the trap, the outer tube was previously immersed in liquid O_2 while the inner inlet tube was maintained at about 50° by ohmic heating. The trap containing the condensed sample was then connected into the carrier gas line of the gas chromatograph. The carrier gas was allowed to flow for a few seconds to establish a steady base line on the recorder before the condensed sample was evaporated onto the column by removing the trap from the liquid O_2 bath and allowing it to heat rapidly by ohmic heating.

For routine ethylene estimations, a gas chromatograph (W. G. Pye & Co. Ltd., Series 104) fitted with a flame-ionization detector was operated isothermally at ambient temperature. A stainless-steel column (5 ft × 0·125 in. i.d.) was packed with 10 per cent w/w Triton X-305 (Rohm & Haas Co.) on NAW Chromosorb G (Johns Manville), 80–100 mesh. Argon (20 ml/min) was the carrier gas and H_2 (20 ml/min) and air (300 ml/min) were used in the detector. A stainless-steel pre-column (17 cm × 4 mm i.d.) packed with 20 per cent w/w diglycerol on celite and fitted with a back-flushing device[20] was used to prevent polar volatile compounds from entering the analytical chromatograph column. By back-flushing the pre-column system immediately after the ethylene peak emerged from the chromatograph, the time taken to effect an analysis was greatly reduced and the interference by large amounts of other volatile compounds was eliminated. In experiments to determine the acetaldehyde production of disks the pre-column was omitted. Samples from a standard mixture of ethylene in air were analysed with each series of experiments to determine retention volume and response values for ethylene. The lower limit of detection of ethylene by this method was 0·03 ppm in the gas phase above the assay mixture representing a rate of ethylene production by the disks of 5×10^{-10} l/g/hr.

Confirmatory identification of ethylene as the gas produced in the experiments described in this paper was performed by the method of Meigh *et al.*[21]

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[21] D. F. MEIGH, K. H. NORRIS, C. CRAFT and M. LIEBERMAN, *Nature* **186**, 902 (1960).