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THE RESPIRATION CLIMACTERIC IN THE APPLE.

PRODUCTION OF ETHYLENE AND FATTY ACIDS IN FRUIT ATTACHED TO AND DETACHED FROM THE TREE

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Abstract—Fruit from a single source was used to correlate the respiration rate, production of ethylene, lipoxidase activity and lipid constituents of the peel of the apple during the respiration climacteric. When ripening begins, the rise in lipoxidase activity precedes the evolution of ethylene, which in turn precedes the respiratory climacteric. The rise in respiratory activity is accompanied by rapid accumulation of free and esterified fatty acids. Subsequently free acids begin to disappear, followed later by a loss of esterified acids. The fatty acid reserves inside and outside the cuticle appear to fluctuate in the same manner without undue time lag. Metabolism of triterpenoid components of the skin shows no reaction to the respiratory changes of ripening. Ethylene production is higher in detached fruit than in fruit freshly picked from the tree, suggesting that an inhibitor of ethylene synthesis is supplied by the parent tree to the fruit during ripening on the tree.

INTRODUCTION

IN RECENT years the biochemical changes which occur during the respiration climacteric in apples have received detailed study,[1–3] but the part of the ripening mechanism which remains most obscure is the initial step in the process. The role of ethylene in ripening was in doubt for many years; the gas was difficult to analyse quantitatively. Some fruits were thought not to produce ethylene, and others appeared to evolve it only after the respiration climacteric had begun.[4] Following the application of gas chromatography to ethylene estimation[5] however, evidence has accumulated that ethylene is in fact the ripening hormone.[6,7]

Several reactions have been suggested as possible pathways for the biogenesis of ethylene. Among these is the route from linolenic acid, suggested by Lieberman and Mapson.[8] In the model chemical reaction the fatty acid is activated by lipoxidase and the production of ethylene is catalysed by cuprous ions. It has been pointed out[9] that in this respect the fat metabolism of the apple might well repay study.

Meigh and Hulme[10] showed that in the Cox's Orange Pippin apple the unsaturated fatty acids formed a substantial proportion of both the free and esterified acids of the peel. It was notable that the unsaturated C_{18} free acids increased rapidly in the peel during the climacteric, reaching a pronounced peak and then declining with comparable speed.

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3 J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *New Phytol.* **64**, 158 (1965).
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5 D. F. MEIGH, *J. Sci. Food Agr.* **11**, 381 (1960).
6 S. P. BURG and E. A. BURG, *Science* **148**, 1190 (1965).
7 S. P. BURG and E. A. BURG, *Plant Physiol.* **37**, 179 (1962).
8 M. LIEBERMAN and L. W. MAPSON, *Nature* **204**, 343 (1964).
9 L. S. C. WOOLTORTON, J. D. JONES and A. C. HULME, *Nature* **207**, 999 (1965).
10 D. F. MEIGH and A. C. HULME, *Phytochem.* **4**, 863 (1965).

The aim of the present work was to draw together data obtained from a single group of apples in a more detailed study of the relationships between respiration, ethylene production, lipoxidase activity and fatty acid production. One series of experiments concerned fruit detached from the tree and stored at 12°, the other concerned fruit freshly picked from the tree.

RESULTS

The development of the respiration climacteric followed the normal course in both the "on" and the "off" tree series of experiments. Figure 1 shows the correlation of changes in

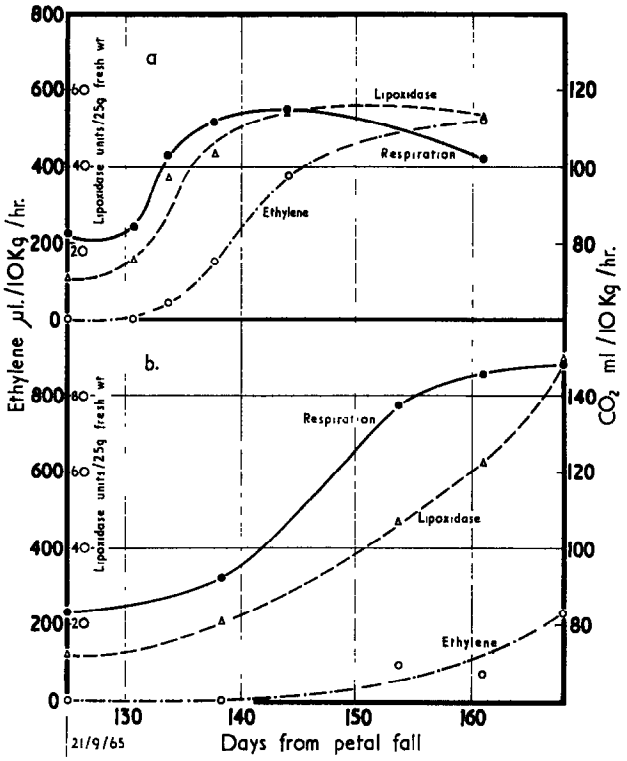

FIG. 1. COX'S ORANGE PIPPIN APPLES (*a*) STORED AT 12° AND (*b*) DEVELOPING ON THE TREE. RESPIRATION AND ETHYLENE PRODUCTION OF WHOLE FRUIT AND LIPOXIDASE ACTIVITY OF THE PEEL.
Respiration—●——●; ethylene production—○— · —○; lipoxidase activity—△——△.

respiration, lipoxidase activity and ethylene production. The curves showing the content of free and esterified C_{18} fatty acids in the fruit were strikingly similar to those determined in the previous year[10] and are not shown here.

Tables 1–3 show analytical figures for the C_{18} fatty acids, total figures for saturated and unsaturated fatty acids and for ursolic and oleanolic acids. The other constituent fatty acids were determined, but are not individually recorded here since they were similar in nature and quantity to those of the previous year.[10] The results of the ursolic and oleanolic acid analyses must be viewed with caution, in common with all those obtained from colour reactions the mechanism of which is imperfectly understood. Further complexity results from the minor

TABLE 1. FREE AND ESTERIFIED FATTY ACID AND TRITERPENOID ACID COMPOSITION OF COX'S ORANGE PIPPIN APPLE PEEL; FRUIT "OFF" THE TREE

Free fatty acid	Amount of acid (μg/g fresh wt.) after storage at 12° for various times (days) after picking on 21 September											
	0		6		9		13		20		37	
	Int*	Ext*	Int	Ext	Int	Ext	Int	Ext	Int	Ext	Int	Ext
$C_{18:0}$	2·2	0·2	1·6	Fraction lost	2·0	2·9	2·7	4·5	5·0	12·2	6·3	3·4
$C_{18:1}$	1·5	1·2	2·5		4·0	8·8	4·2	8·8	5·9	22·7	13·5	6·0
$C_{18:2}$	0·0	0·0	0·0		0·0	8·2	0·0	8·6	15·6	36·2	12·9	10·2
$C_{18:3}$	0·0	0·0	3·4		1·8	0·0	2·6	0·0	2·4	8·3	0·8	1·0
Total												
Saturated	92	237	453	—	43	83	56	75	75	138	44	50
Unsaturated	4	8	7	—	7	19	12	24	28	75	31	28
Esterified fatty acid												
$C_{18:0}$	15·6	32·7	24·3	14·4	48·3	25·8	60·9	18·2	64·8	37·2	69·8	17·3
$C_{18:1}$	23·5	123·5	20·4	60·1	36·8	59·8	59·2	68·7	70·9	98·4	101·7	70·3
$C_{18:2}$	52·6	142·0	58·2	56·8	215·2	75·2	243·3	88·3	294·2	152·8	391·0	151·3
$C_{18:3}$	26·6	11·8	39·4	11·0	163·0	10·8	156·6	11·7	138·1	26·1	128·1	18·2
Total												
Saturated	93	271	133	72	213	121	278	103	262	168	269	117
Unsaturated	116	290	119	135	429	178	481	195	573	365	733	400
Ursolic acid	—	377	4717	251	—	279	2701	248	3136	291	893	233
Oleanolic acid	—	355	2342	209	—	193	1596	259	1422	217	463	200

* "Int" = fraction extracted with chloroform–methanol from sample of peel which had been removed from apples, the surface of which had previously been washed with carbon tetrachloride to give the "Ext" fraction.

TABLE 2. FREE AND ESTERIFIED FATTY ACID AND TRITERPENOID ACID COMPOSITION OF COX'S ORANGE PIPPIN APPLE PEEL: FRUIT "ON" THE TREE

Free fatty acid	Amount of acid (μg/g fresh wt.) at various times (days) after initial picking*									
	0		14		29		36		43	
	Int†	Ext†	Int	Ext	Int	Ext	Int	Ext	Int	Ext
$C_{18:0}$	2·2	0·2	2·1	5·0	13·0	21·5	17·0	11·4	4·0	4·8
$C_{18:1}$	1·5	1·2	1·8	11·3	15·1	19·2	20·7	12·9	10·9	8·0
$C_{18:2}$	0·0	0·0	9·7	9·7	38·0	22·8	21·0	15·2	10·7	9·7
$C_{18:3}$	0·0	0·0	3·7	0·2	16·9	0·6	5·0	2·2	2·3	2·2
Total Saturated	92	237	27	71	285	259	130	88	49	47
Unsaturated	4	8	15	27	89	54	56	45	28	39
Esterified fatty acid										
$C_{18:0}$	15·6	32·7	45·8	17·5	47·1	55·4	94·5	41·6	49·4	45·7
$C_{18:1}$	23·5	123·5	56·2	76·4	65·8	145·2	107·2	112·7	66·6	89·6
$C_{18:2}$	52·6	142·0	191·1	79·5	225·5	199·8	409·4	145·6	217·6	108·6
$C_{18:3}$	26·6	11·8	164·6	13·7	124·9	12·6	173·5	32·1	94·7	22·1
Total Saturated	93	271	184	163	148	324	397	252	222	161
Unsaturated	116	290	424	185	469	472	766	369	1039	296
Ursolic acid	—	377	2362	336	1619	183	1023	153	2102	185
Oleanolic acid	—	355	834	294	963	171	585	139	846	222

* 21 September, 125 days after petal fall.
† See Table 1.

TABLE 3. COX'S ORANGE PIPPIN APPLES PICKED AT VARIOUS TIMES AND STORED AT 12°. VALUES AT TIME OF PICKING AND AFTER STORAGE, FOR RESPIRATION RATE (ml CO_2/10 kg/hr) AND ETHYLENE PRODUCTION (μl./10 kg/hr) (WHOLE FRUIT), LIPOXIDASE ACTIVITY (SUPERNATANT, UNITS/25 g FRESH WT.) AND AMOUNTS OF FREE AND ESTERIFIED FATTY ACIDS AND TRITERPENOID ACIDS (μg/g FRESH WT.)

	Time of picking and of removal from storage (days after petal fall)									
	125→162		139→177		154→182		161→189		168→191	
Respiration rate	83·7	102·5	92·0	104·4	137·6	118·2	144·6	122·6	149·6	122·0
Ethylene production	<0·04	525·0	1·7	599·6	99·5	660·2	77·5	503·7	232·4	625·0
Lipoxidase activity	10·9	53·0	20·0	83·7	47·0	90·5	62·0	120·7	90·0	110·2
Free fatty acids										
$C_{18:0}$ Int*	2·2	6·3	2·1	1·8	13·0	8·9	17·0	6·3	4·0	6·1
Ext*	0·2	3·4	5·0	1·7	21·5	4·3	11·4	3·2	4·8	9·7
$C_{18:1}$ Int	1·5	13·4	1·8	2·6	15·1	8·7	20·7	4·3	10·9	5·1
Ext	1·2	6·0	11·3	3·6	19·2	3·6	12·9	3·3	8·0	8·4
$C_{18:2}$ Int	0·0	12·9	9·7	4·5	38·0	7·5	21·0	5·8	10·7	6·7
Ext	0·0	10·2	9·7	5·9	22·8	5·4	15·2	4·4	9·7	13·0
$C_{18:3}$ Int	0·0	0·8	3·7	3·0	16·9	3·1	5·0	1·4	2·3	1·8
Ext	0·0	1·0	0·2	0·3	0·6	1·1	2·2	0·3	2·2	1·1
Total										
Saturated Int	92	44	27	15	285	77	130	26	49	35
Ext	237	50	71	22	259	50	88	35	47	77
Unsaturated Int	4	31	15	13	89	22	56	13	28	15
Ext	8	28	27	28	54	38	45	36	39	40
Esterified fatty acids										
$C_{18:0}$ Int	15·6	69·8	45·8	61·6	47·1	91·5	94·5	89·4	49·4	65·1
Ext	32·7	17·3	17·5	7·3	55·4	18·3	41·6	14·2	45·7	44·8
$C_{18:1}$ Int	23·5	101·7	56·2	94·5	65·8	115·8	107·2	117·5	66·6	89·6
Ext	123·5	70·3	76·4	21·1	145·2	38·5	112·7	18·8	89·6	78·4
$C_{18:2}$ Int	52·6	391·0	191·1	308·7	225·5	348·8	409·4	347·4	217·6	268·8
Ext	142·0	151·3	79·5	43·1	199·8	63·6	145·6	32·8	108·6	141·6
$C_{18·3}$ Int	26·6	128·1	164·6	97·1	124·9	118·6	173·5	83·7	94·7	85·4
Ext	11·8	18·2	13·7	6·8	12·6	12·3	32·1	8·1	22·1	32·3
Total										
Saturated Int	93	269	184	269	148	355	397	310	222	218
Ext	271	117	163	59	324	93	252	81	161	220
Unsaturated Int	116	733	424	121	469	1067	766	1236	1039	841
Ext	290	400	185	1067	472	223	369	212	296	610
Ursolic acid Int	—	893	2362	989	1619	2334	1023	2484	2102	1784
Ext	377	233	834	299	183	230	153	360	185	968
Oleanolic acid Int	—	463	834	402	963	1256	585	1256	846	258
Ext	355	220	294	328	171	266	139	321	222	271

* See Table 1.

components of the triterpenoid fraction; Brieskorn and Klinger[11] found four components in apple peel—ursolic acid, oleanolic acid and two unidentified triterpenes—in the ratio 35:11:24:8. Lawrie, McLean and Younes[12] have recently identified two triterpenes from apple skin as 20β-hydroxyursolic acid and 2ζ-acetoxy-20β-hydroxyursolic acid. These are perhaps the unidentified compounds of Brieskorn and Klinger; their response in the Liebermann–Burchard reaction is unknown.

[11] C. H. BRIESKORN and H. KLINGER, *Z. Lebensmitt-Untersuch.* **120**, 269 (1963).
[12] W. LAWRIE, J. MCLEAN and M. E. JOUNES, *Chem. Ind.* 1720 (1966).

Each analytical figure is divided into the contributions from the "internal" and "external" fractions, a classification which was not attempted by Meigh and Hulme.[10] "Internal" fractions comprised the lipids of the epidermal cells together with fatty material occluded in the cuticle and unaffected by washing the intact apple with carbon tetrachloride. "External" fractions consisted of the material extractable from the surface of the apple (cf. Richmond and Martin[13]).

"*Off*" *the Tree Series*

Lipoxidase activity (Fig. 1) was already rising some days before the respiration climacteric began, while ethylene production (Fig. 2) reached a detectable level about 24 hr beforehand. Subsequently lipoxidase activity and ethylene production continued to rise, closely paralleling respiration rate. Accumulation of C_{18} acids, free and esterified, began slightly later than the respiration rise. The free acids, after reaching a peak a few days later than the respiration,

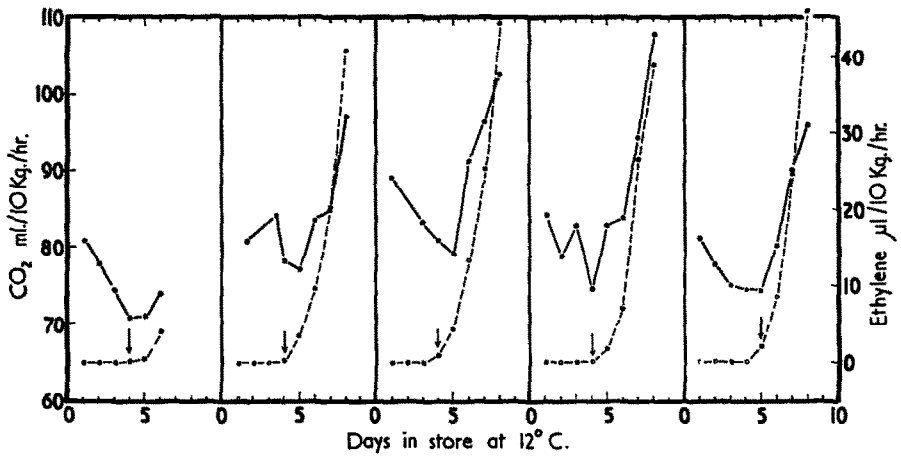

FIG. 2. COX'S ORANGE PIPPIN APPLES STORED AT 12°. RESPIRATION RATE AND ETHYLENE PRODUCTION OF FIVE SAMPLES OF WHOLE FRUIT, SHOWING THE START OF THE CLIMACTERIC.

Respiration—●——●; ethylene production—○——○.

began to break down, but the esterified acids, particularly linoleic, continued to accumulate throughout the period of the experiment.

More esters were extracted from "inside" the fruit than "outside" (Table 1) but the converse was true for free acids. The fatty acids in the two regions tended to change in the same manner during development except that the "external" esters decreased in the period before the climacteric minimum and the "internal" group were deficient in acids above C_{20}. The results for total saturated acids differed from the general pattern where an unexplained large contribution from branched C_{21} and C_{24} acids enlarged the "external" group of the first sample and from branched C_{17} and C_{23} acids enlarged the first and second samples respectively of the "internal" group. Ursolic and oleanolic acids showed no clear trends with time but overall the "external" extracts were considerably smaller than the "internal".

For the lipid determinations five individual samples of fruit were kept. The respiration and ethylene production of these were recorded and are plotted in Fig. 2 for the crucial initial

[13] D. V. RICHMOND and J. T. MARTIN, *Ann. Appl. Biol.* **47**, 583 (1959).

period where the climacteric is initiated. It will be seen that in every experiment ethylene production reached a detectable level about 24 hr before the respiration climacteric began.

"On" the Tree Series

Here both respiration rate and lipoxidase activity rose to levels some 50 per cent higher than in the "off" the tree series. In contrast, ethylene production reached less than half the level "off" the tree. The accumulation of free C_{18} acids followed a course similar to the "off" the tree series, but reached a peak slightly later. The esterified C_{18} acids were again similar but the experiment continued longer than the "off" the tree series and the final sample indicated the beginning of a breakdown of fatty reserves. The detailed figures (Table 2) show a less consistent difference in the quantities of "internal" and "external" fractions than in the "off" the tree series, but the "external" esters again lost weight in the period before the climacteric minimum and the "internal" esters were deficient in acids above C_{20}. Large contributions from branched C_{21} and C_{24} acids increased the first "external" sample and from a branched C_{17} acid increased the first "internal" sample. The amounts of ursolic and oleanolic acids found were comparable with those from the "off" the tree series.

Late Picked Series

The detailed trends in respiration rate and ethylene production of this series are shown in Fig. 3. Only the earlier picked samples showed a rise to a peak of respiration. The later ones

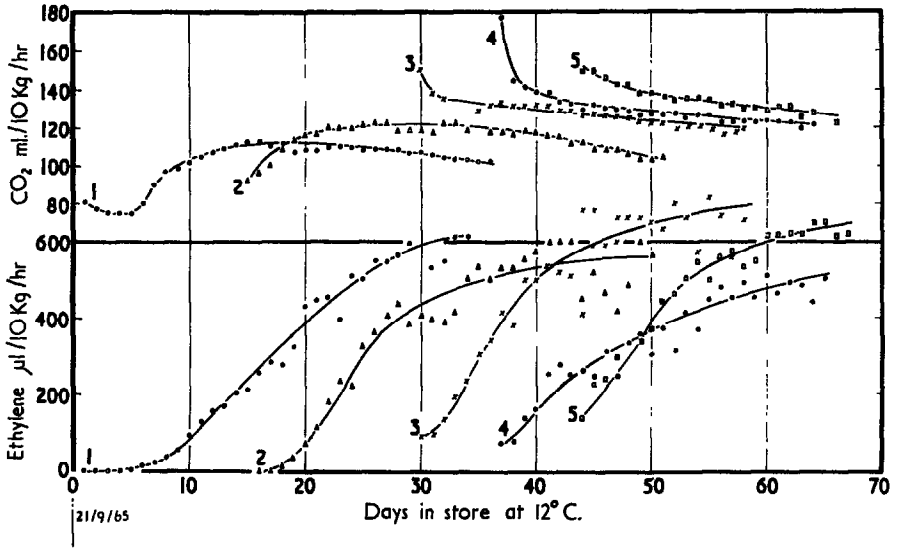

FIG. 3. COX'S ORANGE PIPPIN APPLES PICKED AT VARIOUS TIMES AND STORED AT 12°. RESPIRATION RATE AND ETHYLENE PRODUCTION OF WHOLE FRUIT.

started at an increasingly high level which subsequently fell. It can be expected that the riper the fruit is picked, the faster it will senesce subsequently. In every sample ethylene production increased markedly during storage. In Table 3 the inital and final points of these curves are summarized together with the corresponding analytical figures for lipoxidase activity, C_{18} fatty acids, total saturated and unsaturated fatty acids and ursolic and oleanolic acids.

DISCUSSION

Data from Ripening Apples

The pattern of the ripening process emerging from a study of apples detached from the tree shows that, in the period before the start of the climacteric, respiration rate is declining as is also the reserve of esterified fatty acids on the surface of the apple (Fig. 1, Table 1). Lipoxidase activity is however already rising. A few hours before the respiration rate begins to increase, the start of the climacteric is preceded by the first detectable evolution of ethylene (Fig. 3). This sequence of events is comparable with that in the cantaloupe, studied by Lyons, McGlasson and Pratt.[14] The rise in production of CO_2, ethylene, free and esterified acids and lipoxidase activity then proceeds at a steady rate for some 10 days before respiration and lipoxidase activity begin to level off. At this stage the production of free acids not only declines but a rapid breakdown also ensues (cf. Meigh and Hulme[10]). As a result the reserve of free acids is reduced almost to its preclimacteric level about 50 days after ripening began. It is notable, however, that reserves of the more abundant esterified acids are not lost at this stage.

If the fruit is allowed to remain on the tree the climacteric is similar in pattern but the detailed differences are interesting (Fig. 1, Table 2). Respiration rate and lipoxidase activity reach a higher level, perhaps because reserves of the tree are available to the apple. The free and esterified fatty acids of the skin follow a very similar course, attachment to the tree having little effect. But ethylene production on the tree is considerably depressed, perhaps by some inhibitor from the parent tree.

Fruits that are picked at successive dates behave, when stored, in a manner consistent with the "on" and "off" tree samples (Table 3). The respiration rates of the different picks tend on storage to converge to a common value, the earlier ones rising and the later ones dropping. Lipoxidase activity continues, during storage, to rise at about the same rate, fatty acid reserves tend to fall to a constant level and the rate of ethylene production in the picked fruit accelerates to reach the rate of the earliest stored sample. The respiration and ethylene trends compare well with previous results obtained with Cox's Orange Pippin.[3, 15]

It is worth noting that the rise and fall of fatty acid reserves in the "internal" and "external" extracts keep well in step. This is particularly noticeable in the "on" tree free fatty acids. It must be concluded that the movement of newly synthesized material from the epidermal cells, through the cuticle and on to the surface of the skin is comparatively rapid. Further subdivisions of these extracts would be desirable. The "internal" extract comprises the lipids of the living cells together with a large reserve of fatty material embedded within the cuticle. It is possible to obtain this reserve, without macerating the cuticle, by a thorough Soxhlet extraction[13] but in order to isolate the undegraded lipids of the epidermal cells it would be necessary to separate them from the cuticle.

The study of the triterpenoid acids of the skin has contributed little to the delineation of the climacteric since the results do not indicate any marked change in reserves of these compounds during the climacteric period, whether "on" or "off" the tree. This is in agreement with the work of Huelin and Gallop.[16] It has been suggested that the triterpenes and α-farnesene present in the skin are derived from a common farnesyl or nerolidyl intermediate.[17] It appears that the biosynthesis is completed at a preclimacteric stage.

[14] J. M. Lyons, W. B. McGlasson and H. K. Pratt, *Plant Physiol.* **37**, 31 (1962).
[15] B. G. Wilkinson, *Nature* **199**, 715 (1963).
[16] F. E. Huelin and R. A. Gallop, *Australian J. Sci. Res.* **4B**, 526 (1951).
[17] F. E. Huelin and K. E. Murray, *Nature* **210**, 1260 (1966).

Theories of the Climacteric Mechanism

If, from the results of these experiments, a theory of the early phases of ripening had to be outlined, the sequence of events might possibly begin with the production or activation of lipoxidase. This could have an influence on the membranes of the cell, increasing their permeability and permitting easier penetration of the substrates to enzyme systems.

The action of lipoxidase on unsaturated systems, possibly the unsaturated fatty acids in the membrane structures, would result in the formation of lipid peroxides. These might initiate the release of ethylene, for Mapson and Wardale[18] have shown that the presence of hydrogen peroxide is required in the production of ethylene from methionine by subcellular fractions from cauliflower florets. One of the reactions in this pathway appears to be inhibited by a product derived from the parent tree. When detached from the tree the inhibitor disappears and the rate of ethylene production rises to the level of fruit detached at an earlier stage. It is possible that ethylene itself may react with the inhibitor and the fact that artificial treatment with ethylene induces the climacteric in a preclimacteric fruit might support this theory. It is interesting to note that the progress of the climacteric in fruit attached to the tree is also slow by comparison with that of detached fruit. Whether this is a direct consequence of the inhibition of ethylene production remains to be studied.

When ethylene formation begins, ripening enters a new stage, for the presence of ethylene can be expected to cause further increases in membrane permeability (Lyons and Pratt[19]), and possibly other interactions with lipid metabolism. Reports that added ethylene alters the composition of the lipids of stored peas[20] and causes changes in the lipoproteins of roots, stems and leaves[21] give added weight to this possibility.

The present work raises two interesting questions for future investigation. Firstly, what is the mechanism which partially inhibits the production of ethylene in fruit attached to the tree, and secondly, how and at what point in the preclimacteric phase is lipoaidase activity initiated?

EXPERIMENTAL

Fruit

Fruit was taken from twenty-nine Cox's Orange Pippin trees on Malling IX rootstocks, at East Malling Research Station. Petal fall (the date at which about 90 per cent of the flowers had shed their petals) was 19 May 1965. The main pick was made on 21 September. Twelve samples were taken, two for immediate analysis and ten for storage at 12° in glass desiccators. At intervals of a few days two samples were selected from the stored ones as a pair matched for similar physiological state in the respiration climacteric. These samples comprised the "off" the tree series 1–6. Further samples were picked at intervals of one or two weeks for immediate analysis. These, together with the main pick, comprised the "on" the tree series 1–5, the initial member of each series being the same sample. Further samples from the second and subsequent picks were stored at 12° for some 25 days before analysis. These comprised the "late picked" series in which the first member is the same as the last member of the "off" the tree series.

Measurement of Respiration

Air freed of CO_2 was drawn over the apples at a rate of 10 l./hr. The effluent air was drawn through standard NaOH solution for periods of 24 hr and the absorbed CO_2 determined by titration (Hulme[22]).

[18] L. W. MAPSON and D. A. WARDALE, *Biochem. J.* **102**, 574 (1967).
[19] J. M. LYONS and H. K. PRATT, *Arch. Biochem. Biophys.* **104**, 318 (1964).
[20] F. A. LEE, *Nature* **184**, 462 (1959).
[21] D. S. VAN FLEET, *Nature* **200**, 889 (1963).
[22] A. C. HULME, *Dept. Sci. Ind. Res. Food. Invest. Bd. Rep.*, 1937, p. 133. H.M.S.O., London (1938).

Measurement of Ethylene Production

The outgoing tube from each respiration chamber was fitted with a glass T-piece. The branch tube was closed with two layers of silicone rubber and a short capillary tube held in place with PVC tubing. This formed a sampling port for a hypodermic needle. Air samples were collected in 1 ml glass syringes and analysed with a gas chromatograph capable of detecting 0·001 ppm ethylene in a 1 ml air sample.[23] The chromatograph was calibrated with ethylene (C. P., Matheson Gas Co.) All measurements were made in duplicate and repeated if not in agreement.

Measurement of Lipoxidase Activity

This was determined as described by Meigh and Hulme[10] using the assay method of Surrey.[24] Both the mitochondrial and supernatant fractions, prepared as described by Hulme *et al.*,[1] were analysed. The results are expressed in lipoxidase units, where one unit is the activity which will produce an optical density of 1·000 at a wave-length of 234 nm in 1 min in a total volume of 10 ml of 60% aqueous ethanol.

Extraction of Fatty Acids

A sample consisted of three apples. The weight and circumference of each was measured. Each intact apple, held on a spike, was rotated for three 30-sec periods in CCl_4 (100 ml) in a beaker, and finally washed with fresh solvent (10 ml). The resulting solution yielded the "external" fatty acid fraction. Peel was then removed from the washed apples with a stainless-steel household peeler. A representative 5 g sample was extracted with $CHCl_3$–methanol mixture by the method described by Meigh and Hulme.[10] The $CHCl_3$ extract, isolated after overnight contact with water, formed the "internal" fatty acid fraction.

The "internal" and "external" fractions were each evaporated to dryness under vacuum at room temperature and dissolved in ether:methanol:water, 89:10:1. At this stage some insoluble residue was isolated and later combined with the ursolic acid fraction. The "internal" and "external" fractions were then fractionated on Sephadex by the method of Zinkel and Rowe[25] to obtain a neutral fraction, washed through the column with the same solvent, and an acidic fraction, eluted with ether:methanol, 90:10, saturated with CO_2. The acidic fraction was further divided into a free fatty acid fraction, soluble in light petroleum, and an ursolic acid fraction insoluble in light petroleum.

The neutral fraction was saponified and the liberated fatty acids isolated as previously described.[10] This formed the esterified fatty acid fraction.

Separation and Estimation of the Fatty Acids

An aliquot of each sample was esterified with freshly distilled CH_2N_2 in ethereal solution at 0° and the esters analysed by gas chromatography.[26] The identity of the acids was checked with a graph of log retention time against carbon chain length of the acids and comparison with a further separation after hydrogenation of the sample with Adams platinum oxide catalyst in ethanolic solution. For quantitative estimation, standard solutions of authentic methyl esters of the C_{12}, C_{14}, C_{16} and C_{18} normal fatty acids were run under the same conditions. Recorded peak areas were estimated by triangulation.

Estimation of Ursolic and Oleanolic Acids

The method of Brieskorn and Hofmann[27] which employs the colorimetric Liebermann–Burchard reaction at 40° in a heated cuvette was modified to enable it to be followed at 20° in a constant temperature room. At 20° the 528 nm absorption band was found to reach a maximum after 15 min reaction and the 610 nm band after 30 min. The original calibration with ursolic and oleanolic acids was repeated under the new conditions at concentrations of 0·05, 0·25 and 0·5 mg/ml. These were used to estimate the apple solutions.

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