Plant. Physiol. (1968) 43, 1154–1161

Metabolic Changes in Excised Fruit Tissue. IV. Changes Occurring in Discs of Apple Peel During the Development of the Respiration Climacteric

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Received March 13, 1968.

Abstract. It is shown that a sequential development of a series of enzyme systems occurs in the peel of the apple as the respiration climacteric develops in the whole fruit. The sequence of development of these systems, *i.e.* acetate incorporation into lipid, production of ethylene, incorporation of amino acid into protein and, finally, the decarboxylation of added malate (malate effect) is the same as that shown earlier for the short term (24 hr) aging of peel discs from pre-climacteric apples. As these systems appear in the initial discs from fruit passing through the climacteric they gradually cease to increase during the 24 hour aging period. Uptake studies show that none of the changes in these systems can be due solely to changes in the permeability of the tissue over the climacteric period. On the basis of these results it is tentatively suggested that the aging of discs from pre-climacteric tissue might provide a model system for a detailed study of the physiological and biochemical changes occurring during the climacteric of apple fruits.

It is almost 40 years since Kidd and West attempted to explain the climacteric rise in respiration which precedes visible ripening in apples in terms of a change in "organization resistance" or permeability of the tissue (11). This hypothesis, based on studies of changes in membrane permeability, has been revived in recent years and become 1 of the 2 main theories advanced to explain the series of biochemical changes which are now known to be associated with the climacteric in apples as well as various other fruits. Bain and Mercer (1) found that changes in the inner structure of the chloroplasts had already occurred by the time climacteric rise had begun and as the rise progressed there was also a disorganization of the ultrastructure of the protoplasts. Their results were consistent with an increase in the permeability of the cells especially in the early stages of the climacteric. Rhodes and Wooltorton (19) showed that considerable disorganization of chloroplast lamellae accompanied the loss of chlorophyll in the peel of the fruit during the climacteric period. Sacher (25) from his work on the banana concluded that the initiation of permeability changes may be construed as a cause of the onset of the climacteric. At the climacteric peak his tissue was "essentially 100 % free space to mannitol, sucrose, fructose, and chloride". In general agreement with this hypothesis, Young and Biale (26) from studies of ^{32}P-uptake by discs of the avocado pear, concluded that the respiratory climacteric is initiated by the failure of the cellular membranes to maintain their permeability characteristics.

The other major theory concerning the climacteric originated in the discovery of a net increase in protein (as nitrogenous material insoluble in 80 % ethanol) in apples during the climacteric, the increase not taking place under conditions which suppress the development of the climacteric (7). A similar increase in protein was found in avocados and tomatoes by Rowan *et al.* (24). More recently, Richmond and Biale (22) have shown that in avocados, while there appears to be a period of increased protein synthesis during the early stages of the climacteric, there is no direct relationship between protein synthesis and increased O_2-uptake throughout the climacteric period. This was implicit for apples in the earlier work of Hulme (7) in which an increase in the ratio of rate of respiration to protein content (the R/P ratio) was demonstrated and linked with the suggestion that a change in the pattern of protein to a more enzymatically active type might occur during the climacteric rise, a synthesis of the "enzymes of ripening". Indeed, during the past few years increases in the activity of several mitochondrial (6, 12, 13) and soluble enzyme systems (2, 8, 9, 14, 19, 27) have been reported. These changes do not appear to be due to the removal of enzymic inhibitors and are not associated with marked changes in the levels of pyridine nucleotides in the tissue (20). In the early stages of the respiration climacteric in avocados, Richmond and Biale (23) showed that there was a stimulation in the rate of synthesis of ribosomal and messenger RNA. Over the climacteric period as a whole in apples, Looney and Patterson (15) found an increase in total RNA.

Previous reports from this laboratory (4, 5, 21) have described various changes which occur when discs from pre-climacteric apples are aged by incubation in solution for time periods up to 24 hours.

In this earlier work changes in 4 enzyme systems, namely increased incorporation of ^{14}C acetate into lipid, the development of the capacity of the discs to produce ethylene, the increased incorporation of labeled valine into protein and finally increased capacity of the discs to decarboxylate added malate [the 'malate effect' (3, 17)], were demonstrated.

In the present paper we will consider these same enzyme systems in relation to 2 further processes *i e.* A) changes occurring in the whole fruit during the climacteric rise in respiration as measured by the activity of discs of peel tissue taken from fruit at various stages of the climacteric and assayed immediately as 'initial' discs, and B) the further changes that occur when such discs from fruit in the climacteric phase are subsequently aged for periods up to 24 hours.

Materials and Methods

The fruit used was taken from a group of 32 Cox's Orange Pippin apple trees growing on Malling IX rootstocks in an orchard at Burlingham Horticultural Station, Norfolk, England. Samples of 20 apples of uniform size were stored in glass vessels at 12° (see table I). A stream of CO_2-free air was passed through the vessels at a constant rate and facilities were incorporated into the apparatus so that measurements of CO_2 and ethylene production by the fruit could be made at intervals (5, 9). During the period of storage of up to 35 days the respiration of the fruit was monitored daily and samples taken for analysis at suitable intervals as the respiration climacteric developed. Discs of peel were prepared from the apples after they had been surface sterilized by the methods previously described (21). Discs studied immediately after removal from the apple were called "initial discs" while "aged discs" were those incubated aerobically for periods up to 24 hours in 0.05 M KH_2PO_4 at pH 4.5 containing 50 μg/ml of chloramphenicol (termed CAP/phosphate medium).

The measurement of the basic respiration, the L-malate decarboxylating capacity of the discs (manometrically and radiochemically), the measurement of the uptake and incorporation of 1-^{14}C-acetate, U-^{14}C-valine and uptake of $^{32}PO_4$ were as previously described (4, 21). In the incorporation of U-^{14}C-valine into protein we have shown that at least 90 % of the measured radioactivity was due to peptide-bonded radioactive valine (21).

In the experiment in which the effect of cycloheximide on the uptake and incorporation of valine into protein was studied, discs from apples at the climacteric peak were aged for 6 hours in CAP/phosphate medium. Then batches of 20 discs were incubated for 1 hour in CAP/phosphate medium containing 1 μc of U-^{14}C-valine (6.9 mc mmole^{-1}) and various concentrations of cycloheximide (as indicated in fig 5) at 25°. At the end of this period the uptake of valine and the incorporation into protein were determined as previously described (21).

Chloramphenicol was used in the aging and incubation medium to minimize growth of microorganisms. We have already shown that it does not itself, at the concentrations used, inhibit the activity of the peel discs (21). Microbial contamination of the discs has been investigated, and the total contamination was found to be in the region of 10^4 organisms/g tissue; this did not increase significantly with aging.

Results

The Respiration of Discs and the Malate Effect. Figure 1 shows the course of CO_2-production over the period of the respiration climacteric at 12° for the whole fruit from which the peel discs were taken at various stages. Changes in the O_2-uptake and CO_2-output of the discs alone and the increments in O_2-uptake and CO_2-output after the addition of malate (the "malate effect") are also shown. It will be seen that the discs themselves are exhibiting a "climacteric" although the peak lags behind the climacteric in the whole fruit by about 10 days.

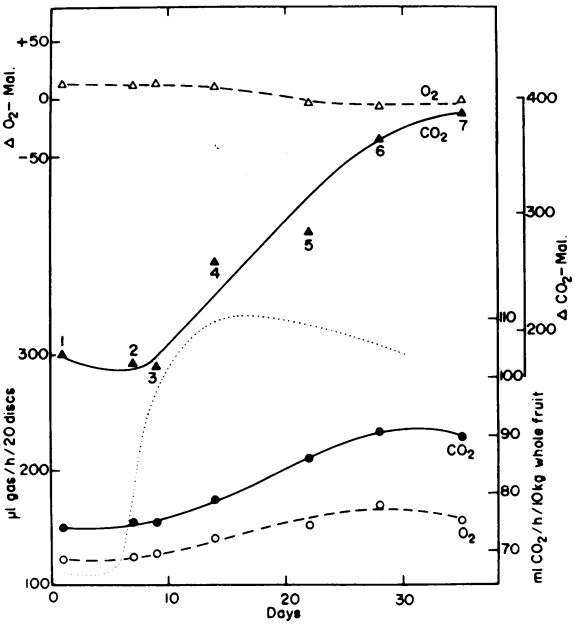

FIG. 1. Respiration of whole fruit and peel discs prepared therefrom, and the effect of added malate on the rates of CO_2 output and O_2 uptake by peel discs during the course of the respiration climacteric in the whole fruit. Dotted line - - CO_2-output of whole fruit; on addition of malate; △----△, change in O_2-uptake peel discs; ▲—▲, additional CO_2-output rate of discs on addition of malate; △----△, change in O_2-uptake rate of discs following addition of malate. The numbers represent stages in the development of the climacteric and refer to points on the curve of respiration rate of the whole fruit; for convenience they have been placed against the points on the curve of the extra CO_2 evolved from added malate.

Addition of L-malate to the discs brings about very little change in O_2-uptake (top curve, fig 1) but the increased CO_2-production is very marked. The increase in this malate decarboxylating system does not commence until the climacteric in the whole fruit has become well developed and continues past the peak. Figure 2 shows the yields of $^{14}CO_2$ from ^{14}C-malate supplied to discs from fruit during the climacteric. The correspondence with CO_2-production from added malate in the Warburg respirometer is close especially in the early stages of the climacteric (*cf.* figs 1 and 2). The rate of uptake of malate (fig 2, lower curve) changes very little over the climacteric period.

It has already been shown (21) that in initial discs from pre-climacteric fruit the effect of the addition of malate is relatively small but during a 24 hour period of aging the rate of CO_2-production from added malate rises 2 to 3 times to a value of approximately 400 μl hr^{-1} g^{-1}. As the climacteric develops in the whole fruit, the malate effect of initial discs gradually increases until, by the time it has reached its maximum value (also about 400 μl hr^{-1} g^{-1}), there is no further increase when the discs are aged for 24 hours. This is shown in figure 3 in which the excess CO_2-production on addition of malate to initial discs is depicted as open columns and the excess CO_2 from malate added to 24 hour aged discs appears as black columns for the 7 stages of the climacteric studied. The upper series of histograms in figure 3 illustrates the percent conversion of U-^{14}C-malate to $^{14}CO_2$ for both initial and aged discs taken from fruit at various stages of the climacteric. Here again decarboxylation reaches

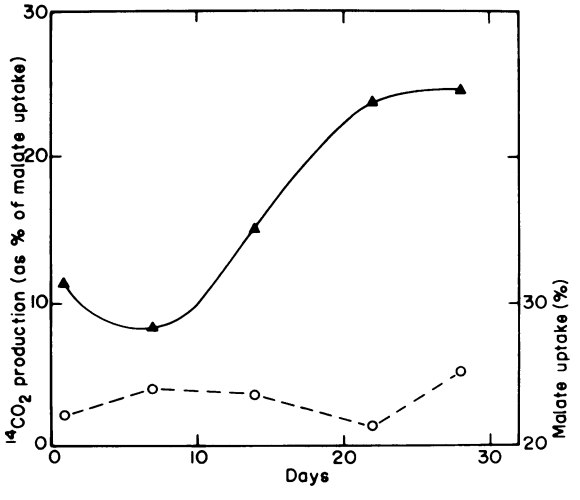

FIG. 2 Rates of production of $^{14}CO_2$ (▲——▲) from added U-^{14}C-malate and of malate uptake (○---○) of discs taken from apples passing through the respiration climacteric (Stages 1,2 4,5, 6 — see figure 1). Based on 20 discs (1 g) hour. During the incubation period of 1 hour the CO_2-production and malate uptake were linear. The U-^{14}C-L-maleate supplied was 0.5 μc (spec. activity, 34.7 mc $mmole^{-1}$)

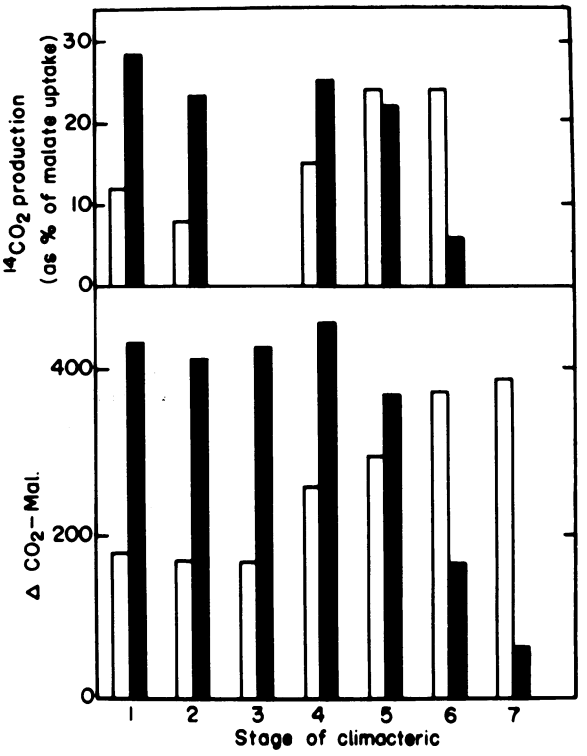

FIG. 3. Rates of increased CO_2 production on addition of malate to initial (white columns) and 24 hour-aged (black columns) discs of apple peel taken from various stages (see figure 1) of the climacteric. Lower histograms show CO_2 production in the Warburg respirometer; upper histograms show $^{14}CO_2$ absorbed in hyamine hydroxide. Results are calculated from rates per 20 discs (1 g) per hour.

a maximum in initial discs with no further increase during the 24 hour aging of discs taken from fruit in the immediate post-climacteric state. Presumably the fall off in the malate effect in aged discs taken after the climacteric peak (stages 6 and 7) in the whole fruit is due to rapid senescence as the discs age.

Uptake and Utilization of Acetate by Peel Discs. The rate of uptake of 1-^{14}C-acetate changes very little either during aging or the climacteric period (table 1). The percentage incorporation into lipid of the acetate taken up is also shown in table 1 in relation to the respiration of the whole fruit. The apples of the first 4 experiments in table 1 were in the pre-climacteric state (respiration about 60–65 ml CO_2 hr^{-1} 10 kg^{-1}), experiments 4 to 6 relate to the series of samples taken over the climacteric in store at 12° (see fig 1). In fruit in the pre-climacteric state (expts 1–4) the incorporation of acetate into lipid in initial discs is low and constant (it rises rapidly on subsequent aging—see ref 4). As the climacteric develops in detached fruits (expts 4–6) the rate of incorporation by initial discs rises until by the time the respiration of the whole fruit has risen to 80 μl hr^{-1} g^{-1} a maximum value is reached

Table I. *Uptake and Incorporation Into Lipid of Acetate*[2] *by Apple Peel Discs in Relation to Storage Conditions and Maturity (Respiration Rate) of the Whole Fruits*

Expt	Date of picking	Respiration of whole fruit	Stage of climacteric[1]	Treatment: Days at 12°	Treatment: Aging	Uptake: −cycloheximide	Uptake: +cycloheximide	Incorporation into lipid: −cycloheximide	Incorporation into lipid: +cycloheximide
		ml CO_2 hr^{-1} 10 kg^{-1}			*Hrs*	*as % of counts added*		*as % of counts taken up*	
1	August 21	65	Pre-	6	0	66	...	25	...
2	September 4	59	Pre-	5	0	80	...	20	...
3	September 20	60	Pre-	1	0	70	...	21	...
4	September 26	65	Pre-(Stage 1)	1	0	74	...	27	...
					2	81	71	66	23
					4	81	69	68	21
					6	82	70	65	35
					24	75	72	61	44
5	October 2	80	2	7	0	82	...	70	...
					3	86	80	77	70
					24	83	87	50	48
6	October 9	110	4	14	0	84	...	61	...
					3	88	85	64	66
					24	80	77	47	36

[1] See figure 1.
[2] 1 μc 1-^{14}C acetate (specific activity 44.4 mc mmole^{-1}) per 20 discs in a 1 hour incubation period.

and there is no significant increase on aging. This maximum value is reached early in the climacteric rise, well before the climacteric peak (stage 4 see fig 1). Increased incorporation of acetate during aging occurs only in discs from pre-climacteric fruit and is lost when once the maximum rate of incorporation is reached in initial discs during the development of the climacteric. It should be noted that as during the aging of "pre-climacteric discs" (4) the maximum rate of acetate incorporation is reached early in the aging process (*i.e.* at approx 2 hr), so does maximum incorporation in initial discs occur early in the development of the climacteric. Furthermore the extent of the maximum development of this acetate incorporation attained by initial discs taken from fruit during the climacteric rise is the same as that in aged pre-climacteric discs. The uptake of acetate is scarcely affected either by aging or by the presence of cycloheximide. Cycloheximide, however, completely inhibits increased incorporation of acetate into lipid over short periods of aging and only partially loses its effect after 24 hour aging (see also ref 4). It has already been shown that the assay of acetate incorporation into lipid is unaffected by the presence of cycloheximide (4).

Production of Ethylene by Peel Discs. We have no systematic data for ethylene production by peel discs over the complete climacteric period, but the average value for discs from pre-climacteric fruit was 3 mμl hr^{-1} g^{-1} (the corresponding rate of respiration of the whole fruit was 63 ml CO_2 hr^{-1} 10 kg^{-1}); discs from detached fruit at the climacteric peak evolved ethylene at the rate of 80 mμl hr^{-1} g^{-1} (respiration rate of the whole fruit was 112 ml CO_2 hr^{-1} 10 kg^{-1}). Thus there is a sharp rise in ethylene production by initial peel discs during the climacteric period just as there is in the whole fruit (18).

Uptake and Incorporation of Valine by Peel Discs. The lower half of figure 4 shows the

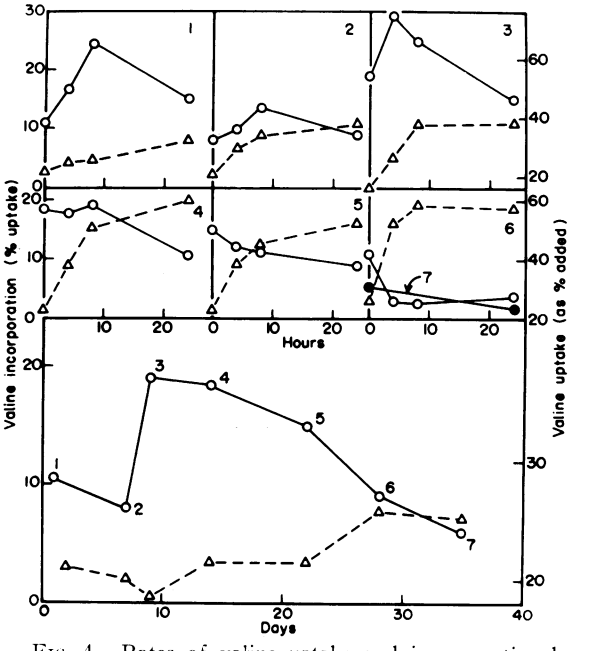

FIG. 4. Rates of valine uptake and incorporation by initial discs (lower graph) and during aging of discs (upper series of graphs) from apples at the 7 stages of the climacteric (see figure 1). O, (● stage 7 only) incorporation, and △---△ uptake. The rate of incorporation of valine into protein is expressed as a percentage of the radioactivity from 1 μc of U-^{14}C-valine (spec. activity 6.9 mc mmole^{-1}) taken up by 20 discs in an hour.

uptake and incorporation (as percent of uptake) of U-^{14}C-valine by initial discs taken from fruit during the climacteric. The upper half of the figure gives similar data for discs taken at corresponding stages of the climacteric and aged for periods of up to 24 hours at 25°. It will be seen that uptake of valine by initial discs is reasonably constant until well after the climacteric peak (fig 4, lower half) has been reached. On subsequent aging of discs taken at various stages of the climacteric, increased uptake occurs (fig 4, upper half, interrupted lines) and is pronounced in the region of the climacteric and beyond. Incorporation of valine into protein rises sharply in the initial discs taken in the early stages of the climacteric and reaches a peak at stage 3, *i.e.* 4 or 5 days before the peak in respiration of the whole fruit (fig 4, lower half). Incorporation also increases rapidly during the first 8 to 10 hours of aging in discs taken before the climacteric peak is reached (fig 4, upper half, stage 3), but near the peak (stage 4) and beyond it either does not increase during aging or falls.

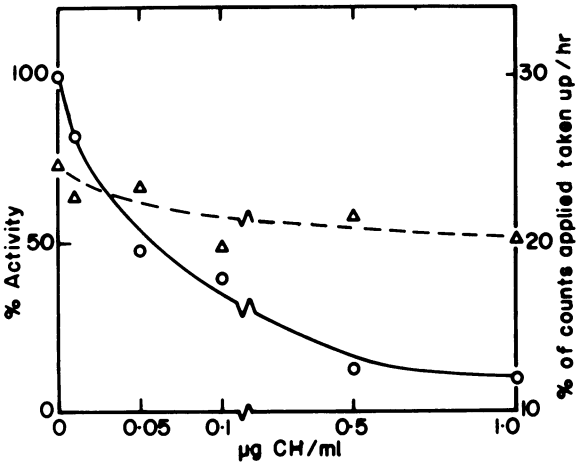

FIG. 5. The effect of cycloheximide on the assay of the rates of uptake (△---△) and incorporation (○—○) of U-^{14}C-valine by apple discs. The discs were taken at the point along the climacteric (of the whole fruit) of maximum valine incorporation and aged for 6 hours and then assayed in presence of U-^{14}C-valine and various concentrations of cycloheximide. The rates of incorporation are expressed as a percentage of the activity of the control discs incubated in the absence of cycloheximide.

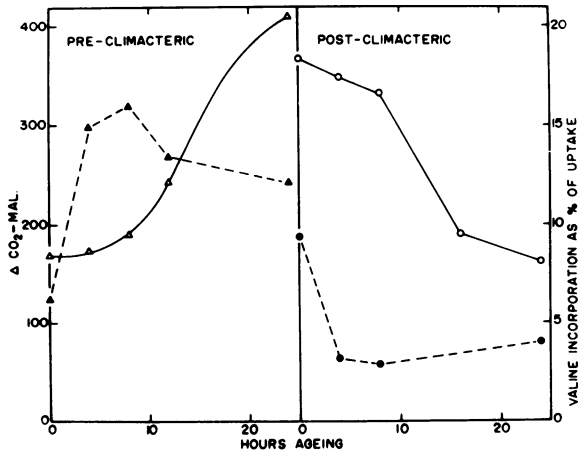

FIG. 6. The relationship between the development of the malate effect and of valine incorporation into protein in peel discs taken from fruit in the pre- and post climacteric state. △ and ○ represent the rate of excess CO_2 production from added malate; ▲ and ● represent the rate of valine incorporation as a percent of uptake. Results based on 20 discs incubated for 1 hour at each point.

Figure 5 illustrates the effect of varying the cycloheximide concentration in the assay system for the determination of valine uptake and incorporation in discs from, in this example, post climacteric apples. This shows that, although cycloheximide strongly inhibits valine incorporation, the direct effect on valine uptake is small. Thus the effect of cycloheximide in inhibiting changes in uptake during aging (ref 21, fig 1) is probably due to a direct effect on the development of an enzymic transport system.

Table II. *Uptake of Phosphate by Apple Peel Discs*

Stage of climacteric		Aging	Counts of ^{32}P as percent of counts ^{32}P added[2] — Cycloheximide			+ Cycloheximide		
			Internal	Free space[1]	Total	Internal	Free space	Total
		Hr						
1	September 26	0	...	...	9 0	...	...	...
		2	...	...	8.6	...	...	7.9
		4	...	...	9.7	...	...	5.5
		6	...	...	16.5	...	...	10.5
		24	...	...	20 9	...	...	13.6
4	October 9	0	3.8	1.0	4.8	...	...	...
		24	22	1.0	23	16	2.0	18
6	October 23	0	2 3	1.0	3.3	...	...	...
		5	5.7	1.5	7 2	3 1	2.2	5.3
		24	18	1.5	19.5	5.7	3.0	8.7

[1] For determination of internal "and free space" uptake see Rhodes *et al.* (1968)
[2] Incubation mixture contains 1 μc ^{32}P- orthophosphate (spec. activity 33 mcm mole^{-1}).

The interdependence of protein synthesis (in terms of valine incorporation) and the development of the malate effect is emphasized by an examination of the time courses of the extra CO_2 produced from added malate and of valine incorporation during aging of discs taken both from pre- and post-climacteric apples (fig 6).

Uptake of Phosphate by Peel Discs. As an example of a metabolically active anion, the uptake of ^{32}P-phosphate was studied during aging of discs taken from fruit at the immediate pre-climacteric, climacteric peak and post-climacteric stages. The results are shown in table II. Uptake does not increase in initial discs at the 3 stages, but total uptake increases during aging, the increase being confined almost entirely to "internal" uptake. Cycloheximide (1 μg/ml) partially prevents the development of increased uptake during aging. It is clear, however, that in apple peel tissue (unlike the avocado, see 26) there is no increase in the rate of phosphate uptake over the climacteric.

Discussion

In previous communications (4, 5, 21) we have shown that during the aging of peel from pre-climacteric apples there is the sequential development of 4 enzyme systems dependant upon the synthesis of specific RNA and protein moieties. The first system to develop is one giving increased incorporation of acetate into lipid which reaches a maximum value after 2 to 3 hours of aging; the second system is the enzymic production of ethylene by the discs (6–8 hr of aging), and while these 2 systems are developing a third system namely the capacity of the tissue to incorporate labeled valine into protein is rising and reaches a maximum after 8 hours of aging. Finally there is the development of the fourth system which rapidly decarboxylates added malate (the malate effect) which reaches its final level after 16 to 24 hours of aging. The development of all these systems is prevented by the presence of inhibitors of RNA and protein synthesis during aging (21). Although during aging there are changes in the uptake of some of the metabolites involved, the pattern of development of these systems cannot be explained in terms of changes in uptake. Of the metabolites studied, some (*i.e.* malate, acetate, pyruvate) show no significant changes in uptake during aging and inhibitors of protein synthesis have no effect on the observed uptake. Others (*i.e.* valine, uridine, phosphate), however, show an increase in uptake during aging which is inhibited by cycloheximide and thus probably enzyme mediated (21).

In the present work we have shown that there is a similar sequential development of these 4 systems in apples during the climacteric as measured in initial discs prepared from fruit during the rise in respiration. The incorporation of acetate into lipid rises to a maximum value within 1 to 2 days of the onset of the climacteric, the incorporation of valine into protein rises to a peak about 5 days after the onset of the climacteric, while the maximum of malate decarboxylation is not reached until well beyond the climacteric peak. The increases in these systems as measured in initial discs during the climacteric rise are quantitatively the same as those developing during the aging of discs from pre-climacteric apples. As these systems develop during the climacteric so the ability of the discs to increase in activity on aging is lost. Further, the sequence of disappearance of increased activity of acetate incorporation into lipid, incorporation of valine into protein and of the malate effect is in the same order as the attainment of the maximal operation of the systems during the aging of pre-climacteric tissue. We have no detailed data for the production of ethylene by discs between the pre- and post-climacteric stages but many other studies have shown (27) that increased ethylene production by whole fruit commences early in the climacteric rise and follows the same general course as the CO_2-production. During the development of the climacteric there are no significant differences in the uptake by initial discs of all the metabolites we have studied.

These striking similarities between the sequence of development and the final activities attained for both aging of discs from pre-climacteric tissue and over the climacteric period suggest that in some major respects the aging system reflects changes undergone during the climacteric. How then do these 2 phenomena differ? For instance, the capacity for uptake of valine and phosphate develops during aging of both pre- and post-climacteric tissue discs. This increase in uptake capacity on aging seems to be a general characteristic of excised plant tissue; it is not related to the climacteric since it is not apparent in initial discs taken from fruit as the climacteric develops. Another difference between aging and the climacteric is that the rise in respiration on aging of discs from fruit in the pre-climacteric stage is small (approximately 30 %) compared with the rise in respiration of the whole fruit during the climacteric (about 100 %). However, the rise in the respiration of initial discs over the climacteric is also relatively small (about 60 %). Compared with the aging of discs from storage organs such as the potato where the respiration increases 3- to 4-fold, the rise with apple discs is always very small. The respiration of initial discs of apple peel is at least twice that of initial potato discs (unpublished data) and the quantitatively greater increase in respiration on aging of potato discs may be associated with the proportionately greater energy demands made on the system during the rejuvenescence of the dormant tissue. Thus an increase in respiration is not a basic attribute of the aging process it merely provides mobilized energy where this is necessary (*e.g.* in storage organs).

This rationalization of the apparent discrepancies between the processes of aging and the climacteric leaves us with the view that, in apples at least, they are basically similar phenomena. Further evidence in this direction is the fact that treatments such as low oxygen storage and spraying of the trees at the appropriate time with *N*-dimethylaminosuccinamic acid which delay the onset of the respiration climacteric also inhibit the development of the malate effect during aging of peel discs (10).

The suggestion (25) that the respiration climacteric is purely a phenomenon centered around changes in permeability appears to be no longer tenable, at least in relation to the apple, since we have shown that the comparable changes undergone during aging and during the climacteric are not due to any general changes in permeability (uptake). Changes in permeability measured in terms of uptake appear to be a selective process characteristic of substrates or groups of substrates and may be reduced or prevented by the presence of inhibitors of protein synthesis. However, our results do not rule out the possibility that small, selective changes in permeability (especially of the tonoplast) mediated by enzyme carriers may be involved in the changes that lead to the increased enzyme synthesis during the climacteric.

The development of the various enzyme systems during the aging of discs of peel from pre-climacteric apples is dependent upon renewed RNA and protein synthesis but independent of general changes in the permeability of the tissue (21). The comparison of the development of the systems during aging of pre-climacteric tissue and during the respiration climacteric leads to the view that the sequence of events during the climacteric is also due to renewed RNA and protein synthesis and that it is essentially independent of general changes in permeability.

The production of ethylene by the tissue has usually been considered as the first step in the sequence of events leading to the onset of the climacteric. The present results suggest that an enhancement of turnover of lipid precedes the increase in the production of ethylene. It has been suggested that the combined action of lipoxidase [which increases in activity rapidly during the climacteric (16)] and long chain unsaturated fatty acids might be involved in ethylene biosynthesis (27). It might well be, therefore, that the climacteric in apples is initiated by a turnover of lipids leading to an increased production of ethylene.

Acknowledgments

Messrs. Harkett and Pryke and Mrs. E. Lowe and Miss S. Haylock assisted with the experimental work. We are indebted to Norfolk County Council for providing the fruit used from their Burlingham Horticultural Station.

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