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METABOLIC CHANGES IN EXCISED FRUIT TISSUE—I.

FACTORS AFFECTING THE DEVELOPMENT OF A MALATE DECARBOXYLATION SYSTEM DURING THE AGEING OF DISKS OF PRE-CLIMACTERIC APPLES

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Abstract—A system develops in peel from preclimacteric apples during ageing for periods up to 48 hr which rapidly decarboxylates added malate (the malate effect). This development is preceded by a peak of incorporation of ^{14}C-valine into the protein of the tissue. During the period of increased ^{14}C-valine incorporation the tissue develops the capacity to produce ethylene, and exogenous ethylene reduces the time taken to reach the full malate effect. Cycloheximide and other inhibitors of protein synthesis prevent the incorporation of ^{14}C-valine and the development of the malate effect. Changes in uptake of the various substrates do not appear to be involved in these phenomena. It is suggested that ethylene, RNA and protein synthesis are all involved in the development of the malate effect.

INTRODUCTION

THE OBJECT of the series of papers under the general title is to follow sequential changes in metabolic processes in disks of fruit tissue—in the first instance from apple peel—undergoing short-term incubation (up to 48 hr, at 25° termed "ageing") taken from fruit before the onset of respiration climacteric and through the development of the climacteric to the post-climacteric phase of the whole fruit. Since results will also be considered for disks taken immediately after excision from the fruit during the climacteric phase of the whole fruit, it is important to distinguish between the term "ageing" as solely used in the present context to represent the short-term incubation of excised disks of tissue and the use of the same term to describe effects associated with the climacteric and senescence of the whole fruit.

Dramatic metabolic changes rapidly ensue when thin slices excised from plant storage organs are incubated aerobically at physiological temperatures. During incubation of the tissue disks for up to 48 hr, the respiration rate increases 3–4 fold[1] and its sensitivity to respiratory inhibitors changes markedly.[2] Click and Hackett[3] showed that the development of enhanced respiration on ageing of potato disks is dependent upon renewed synthesis of both RNA and proteins. It is apparent from recent work that ageing may involve qualitative and quantitative changes in the pathways of respiratory metabolism,[4, 5] an increase in mitochondrial number,[6] the appearance of invertase and an increase in the activity of several

[1] G. G. LATIES, in *Control Mechanisms in Respiration and Fermentation* (edited by B. WRIGHT), p. 129, Ronald Press, New York (1963).
[2] K. V. THIMANN, C. S. YOCUM and D. P. HACKETT, *Arch. Biochem. Biophys.* **53**, 249 (1954).
[3] R. E. CLICK and D. P. HACKETT, *Proc. Nat. Acad. Sci. U.S.* **50**, 243 (1963).
[4] G. G. LATIES, *Plant Physiol.* **39**, 654 (1964).
[5] T. AP REES and H. BEEVERS, *Plant Physiol.* **36**, 839 (1961).
[6] S. G. LEE and R. M. CHASSON, *Physiol. Plantarum* **19**, 199 (1966).

other enzymes,[7] an increase in total protein content of the disks[8] and of the capacity to incorporate amino acids into protein,[3,9] an increase in total ribonucleic acid (RNA) content[10] and formation of polyribosomes from ribosomes,[11] increased fatty acid synthesis[9] and increased uptake of both salts and solutes.[5,12]

A recent survey[13] showed that slices of many plant tissues other than storage organs exhibit a similar increase in respiration on ageing. These changes are not, however, characteristic of all plant tissues and it has been shown that disks of apple peel and pulp do not show the characteristic increase in respiration on ageing.[14] The work on apples was carried out with fruit in the post-climacteric state and in view of the profound changes that occur during the respiration climacteric it was decided in the first instance to reinvestigate this problem using tissue from apples in the pre-climacteric state.

In a preliminary paper[15] we have shown that, although the increase in respiration on ageing of pre-climacteric apple disks is small, major metabolic changes do occur. In particular, a malate decarboxylation system, first described by Neal and Hulme,[16] markedly increases in activity during ageing. This decarboxylation of malate (termed the "malate effect") was thought to involve NADP malate dehydrogenase (E.C. 1.1.1.40) and pyruvate decarboxylase (E.C. 4.1.1.1).[17–19] The present paper shows that the development of the malate effect is dependent upon synthesis of RNA and protein and that ethylene is implicated in the process.

In the present study, uptakes of the metabolites have been measured and considered in relation to their utilization. Changes in uptake should give information on the permeability of the tissue[20] and its bearing on the metabolic changes we are investigating. The use of metabolic inhibitors such as cycloheximide (CH) can provide valuable data on the development of intracellular uptake and this approach has been employed in the present work.

RESULTS

Uptake of Valine and Uridine by Peel Disks During Ageing

The results for the rate of total uptake of valine and uridine by disks aged for various times up to 24 hr are shown in Fig. 1. During ageing there is a 2–3 fold increase in the capacity of the disks to take up both substrates and this increase is inhibited by the presence of cycloheximide in the medium in which the disks are aged. In the case of valine, the free-space uptake was also determined (see Fig. 1) and this was shown to be of a low order (5–10 per cent of rate of intracellular uptake) and to remain constant during the period of ageing. A similar situation also obtains for the rate of uptake of inorganic ^{32}P-phosphate during the ageing of disks in that a cycloheximide-sensitive increase in intracellular uptake (to a

[7] J. EDELMAN and M.A. HALL, *Biochem. J.* **95**, 403 (1965).
[8] I. R. MACDONALD, J. S. D. BACON, D. VAUGHAN and R. J. ELLIS, *J. Exp. Botany* **17**, 822 (1966).
[9] C. WILLEMOT and P. K. STUMPF, *Plant Physiol.* **42**, 391 (1967).
[10] C. J. LEAVER and J. EDELMAN, *Biochem. J.* **97**, 27P (1965).
[11] C. J. LEAVER and J. L. KEY, *Proc. Nat. Acad. Sci. U.S.* **57**, 1338 (1967).
[12] R. L. BIELESKI and G. G. LATIES, *Plant Physiol.* **38**, 586 (1963).
[13] T. AP REES, *Australian J. Biol. Sci.* **19**, 981 (1966).
[14] F. M. V. HACKNEY, *Proc. Linn. Soc. N.S.W.* **70**, 333 (1945).
[15] M. J. C. RHODES, T. GALLIARD, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 405 (1968).
[16] G. E. NEAL and A. C. HULME, *J. Exp. Botany* **9**, 142 (1958).
[17] A. E. FLOOD, A. C. HULME and L. S. C. WOOLTORTON, *J. Exp. Botany* **11**, 136 (1960).
[18] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. Lond. Ser. B* **158**, 519 (1963).
[19] D. R. DILLEY, *Nature* **196**, 388 (1962).
[20] J. A. SACHER, *Plant Physiol.* **41**, 701 (1966).

maximum of 26 per cent of the applied phosphate per 2 hr incubation) occurs with no change in free-space uptake (3 per cent of the applied phosphate per 2 hr incubation).

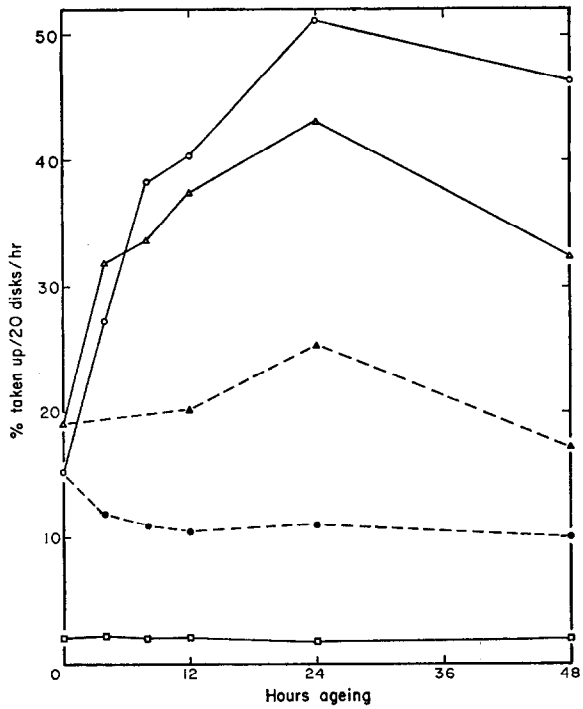

FIG. 1. TIME COURSE OF THE DEVELOPMENT OF THE CAPACITY OF APPLE PEEL DISKS TO TAKE UP ADDED METABOLITES DURING AGEING IN THE PRESENCE AND ABSENCE OF CYCLOHEXIMIDE.

●, ○: uptake of valine in the presence (discontinuous line) and absence (continuous line).
▲, △: uptake of uridine in the presence (discontinuous line) and absence (continuous line).
Also shown, the free-space uptake of valine (□).

Uptake of Organic Acids

We have already shown[21] that no change in uptake of acetate occurs during ageing of disks from pre-climacteric fruit. Figure 2 shows that there is no change in the uptake of malate and later results (Table 3) indicate a similar situation for pyruvate.

The Development of the Malate Decarboxylation System

Figure 2 shows the time course of development of the malate decarboxylating system (malate effect) during the ageing of peel disks assayed both manometrically and by radiochemical assay of the $^{14}CO_2$ produced by the disks from uniformly labelled ^{14}C-malate. Both assays show a similar time course of development with an initial short lag phase up to 6 hr, followed by a rapid phase in which the rate of decarboxylation rises 2–3 fold and, finally, a steady phase is reached after 24 hr of ageing. During a 24 hr period of ageing there is no significant increase in the rate of uptake of malate by the disks.

[21] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* 7., 1453 (1968).

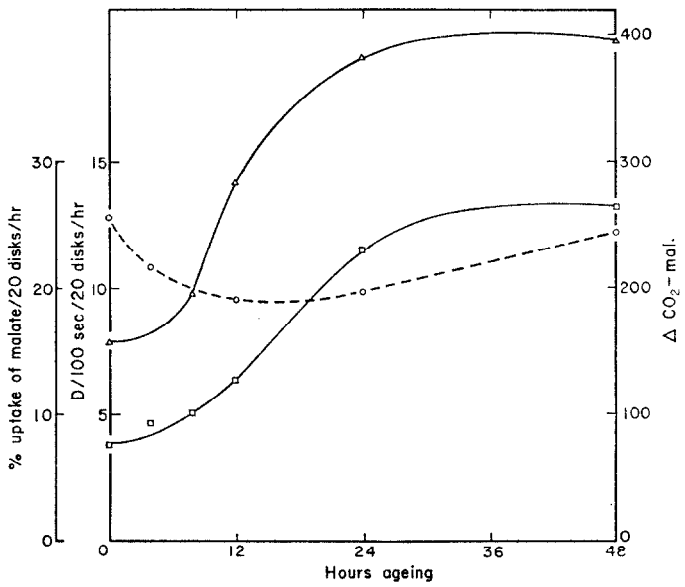

FIG. 2. TIME COURSE OF DEVELOPMENT OF MALATE DECARBOXYLATION SYSTEM DURING THE AGEING OF APPLE PEEL DISKS.

The development of the malate decarboxylation system is shown measured by the manometric method (△) and expressed as ΔCO_2-MAL and by the radiochemical method (□). The rate of uptake of malate at various stages of ageing is also shown (○---○).

TABLE 1. EFFECT OF VARIOUS INHIBITORS ON THE DEVELOPMENT OF THE MALATE EFFECT DURING AGEING OF APPLE PEEL DISKS AND ON THE MANOMETRIC ASSAY OF THE EFFECT

Conditions of ageing	Additions to Warburg flask during assay	μlCO_2/hr-ΔCO_2MAL Experiment number					
		1	2	3	4	5	6
Fresh disks (zero time)	—	214	213	155	166	190	180
Disks aged for 24 hr in:							
0·05 M phosphate, pH 4·5	—	423	—	—	—	—	—
0·05 M phosphate, pH 4·5, containing 50 μg/ml chloramphenicol (CAP/phosphate)	—	438	376	387	409	380	400
CAP/phosphate	Cycloheximide (5 μg/ml)	—	—	380	—	—	—
CAP/phosphate	*p*-Fluorophenylalanine (1·83 mg/ml)	—	340	—	—	—	—
CAP/phosphate	6-Azauracil (100 μg/ml)	437	—	—	—	—	—
CAP-phosphate	Actinomycin D (50 μg/ml)	—	—	—	386	—	—
Cycloheximide (0·1 μg/ml)	—	—	—	172	—	—	—
p-Flurophenylalanine (92 μg/ml)	—	—	210	—	—	—	—
6-Azauracil (10 μg/ml)	—	208	—	—	—	—	—
Actinomycin D (50 μg/ml)	—	—	—	—	175	—	—
Chloramphenicol (2 mg/ml)	—	—	—	—	—	16	—
5-Fluorouracil (100 μg/ml)	—	—	—	—	—	319	—
Puromycin (100 μg/ml)	—	—	—	—	—	90	—

The Effect of Inhibitors on the Malate Effect

Table 1 shows the effect of inhibitors (see also Fig. 3) on the development of the malate effect over a 24 hr period. The data show that the protein synthesis inhibitors, cycloheximide

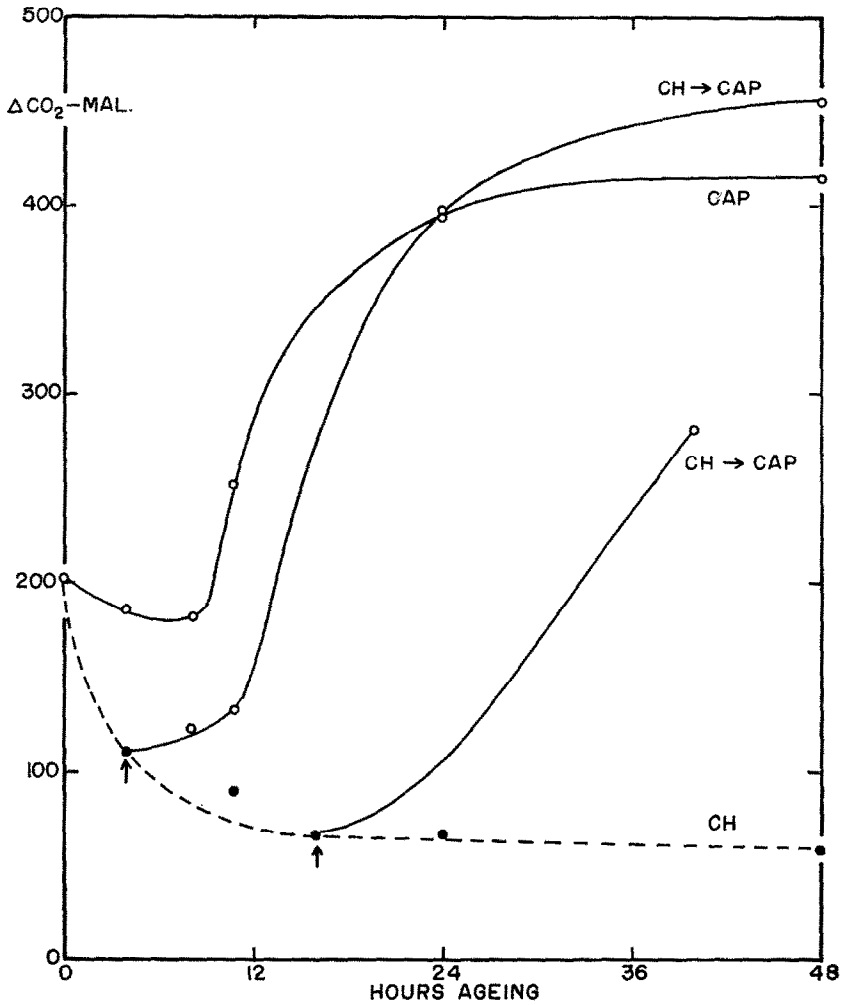

FIG. 3. REVERSIBILITY OF INHIBITION OF THE DEVELOPMENT OF THE MALATE EFFECT BY CYCLOHEXIMIDE.

The figure shows a normal time curve of development of the malate effect measured manometrically in CAP/PO_4 (○—○) and in the presence of CH (0·5 μg/ml) (●---●). At two intervals in time (shown by arrows) disks were transferred from the CH medium to CAP/PO_4, the recovery and full development of the malate effect in these disks is shown (○—○).

(CH) and *p*-fluorophenylalanine when supplied at relatively low concentrations, suppress the increase in the malate effect. These inhibitors have no effect on the *assay* of the malate decarboxylative capacity once the full value has developed in aged tissue, even when used at ten to twenty times the level required to inhibit the development of the effect. Table 1 also shows that the inhibitors of RNA synthesis, actinomycin D and 6-azauracil inhibit the

development of the malate effect but not the assay of the effect in disks aged in the absence of inhibitors. Low concentrations of chloramphenicol (CAP; 50 μg/ml), which inhibit bacterial protein synthesis completely, have no effect on the development of the malate effect in apple disks, but at much higher concentrations (2 mg/ml) complete inhibition was observed. 5-Fluorouracil, which inhibits ribosomal and soluble RNA synthesis in some plant tissues,[22] only marginally inhibits the increase in malate decarboxylation.

Table 2 shows the effect of a range of different concentrations of cycloheximide on the ageing process. At a concentration of 0·01 μg/ml there is a partial inhibition of the increase in the malate effect. Complete inhibition was obtained at concentrations of 0·1 μg/ml. At higher concentrations (0·25–0·5 μg/ml) both the basic respiration and the malate decarboxylation system are reduced below the initial to minimal levels which cease to be sensitive to further increase in the cycloheximide concentration.

TABLE 2. THE EFFECT OF A RANGE OF CONCENTRATIONS OF CYCLOHEXIMIDE ON THE BASIC RESPIRATION AND ON THE DEVELOPMENT OF THE MALATE EFFECT DURING AGEING OF PEEL DISKS

	Basic respiration (μl O_2/hr/20 disks)		Malate decarboxylation (μl CO_2/hr/20 disks (ΔCO_2-MAL))	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Fresh disks	119	126	155	214
Aged controls	141	126	406	376
Aged in presence of cycloheximide (μg/ml)				
0·01	125	—	330	—
0·05	118	—	209	—
0·10	113	—	172	—
0·25	97	—	90	—
0·5	91	74	52	110
1·0	—	75	—	98

Even relatively high concentrations of cycloheximide (*ca.* 0·5 μg/ml) have no irreversible effects either on the basic respiration of the disks or on the development of the malate effect, i.e., when disks in which the development of the malate effect had been inhibited by the presence of CH were returned to CAP/phosphate medium free of CH, the development of the malate effect was resumed and returned eventually to values comparable with those developed in untreated control disks. The reversibility of inhibition was demonstrated after periods of inhibition of 4 and 16 hr, as shown in Fig. 3.

Amino Acid Incorporation into Protein During Ageing

Figure 4 shows changes in the capacity of disks to incorporate radioactive valine into the protein fraction (see Experimental) during ageing of disks in the presence and absence of cycloheximide. During the first few hours of ageing in the absence of CH there is a stimulation of amino acid incorporation leading to a peak after 8 hr. The rate of incorporation then declines until it returns to the initial value after 24–48 hr. The presence of cycloheximide almost completely inhibits (*ca.* 95 per cent) protein synthesis in the disks at all stages of ageing.

[22] J. L. KEY and J. INGLE, *Proc. Nat. Acad. Sci. U.S.* **52**, 1382 (1964).

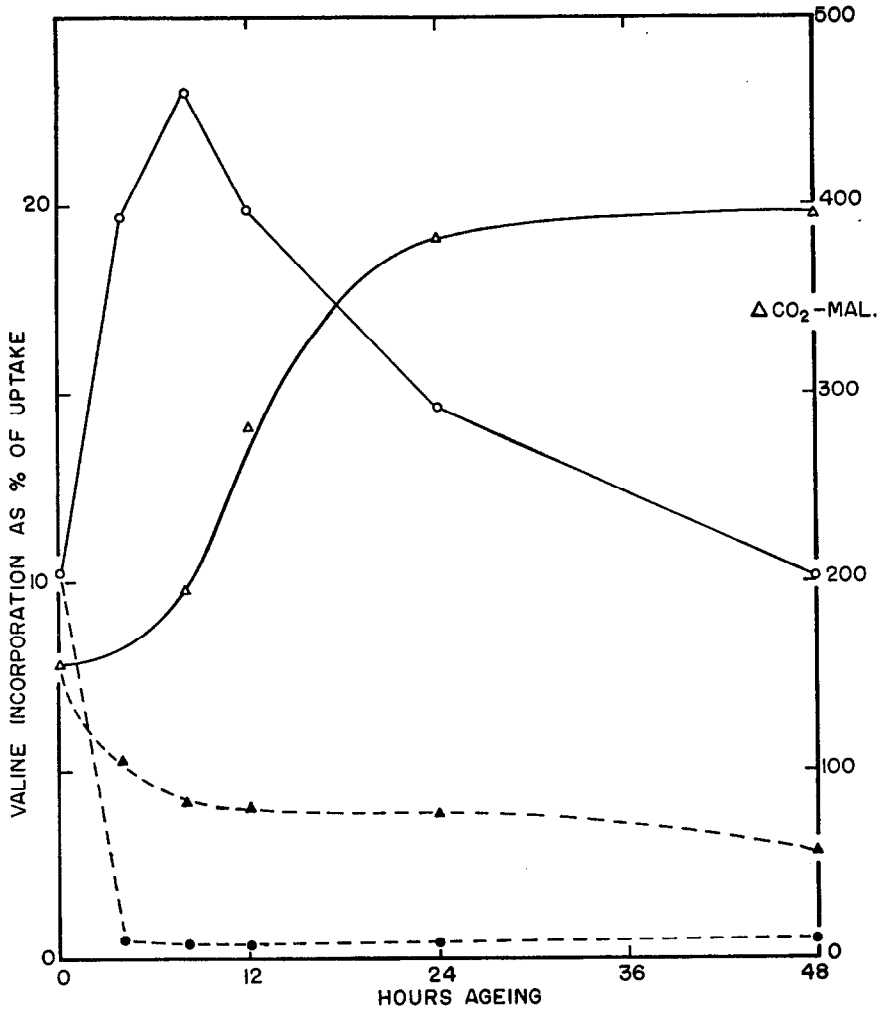

FIG. 4. TIME CURVES OF THE DEVELOPMENT OF THE MALATE EFFECT AND OF AMINO ACID INCORPORATION DURING THE AGEING OF APPLE PEEL DISKS, IN THE PRESENCE AND ABSENCE OF CYCLOHEXIMIDE.

The development of the malate effect (△) is shown in the presence (▲---▲) and absence (△—△) of CH. The ability of disks to incorporate valine into protein was measured at various times of ageing in the presence (●---●) and absence (○—○) of CH. All assays were carried out in the absence of CH.

Ethylene in Relation to Ageing

Figure 5 shows a time course of ethylene production by disks of pre-climacteric apples in relation to the onset of the malate effect. Ethylene production rises to a miximum after 6 hr of ageing.

The effect of addition of ethylene to the atmosphere above ageing disks on the time course of development of the malate effect is shown in Fig. 6. The presence of ethylene almost completely eliminates the lag phase, so that full development of the effect is attained in 12 hr rather than in 24 hr as in the control disks. Ethylene, however, has no effect on the level of the malate effect finally attained.

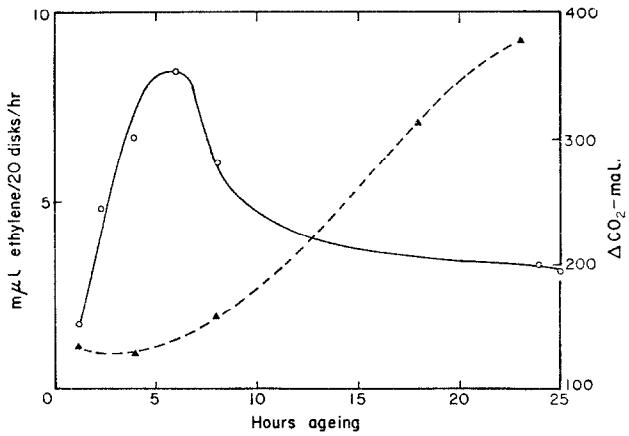

FIG. 5. TIME COURSE OF ENDOGENEOUS ETHYLENE PRODUCTION IN RELATION TO THE DEVELOPMENT OF THE MALATE EFFECT IN APPLE PEEL DISKS.

The rate of ethylene production (○—○) is shown together with the capacity of disks to decarboxylate malate (▲---▲) for disks at various stages of ageing.

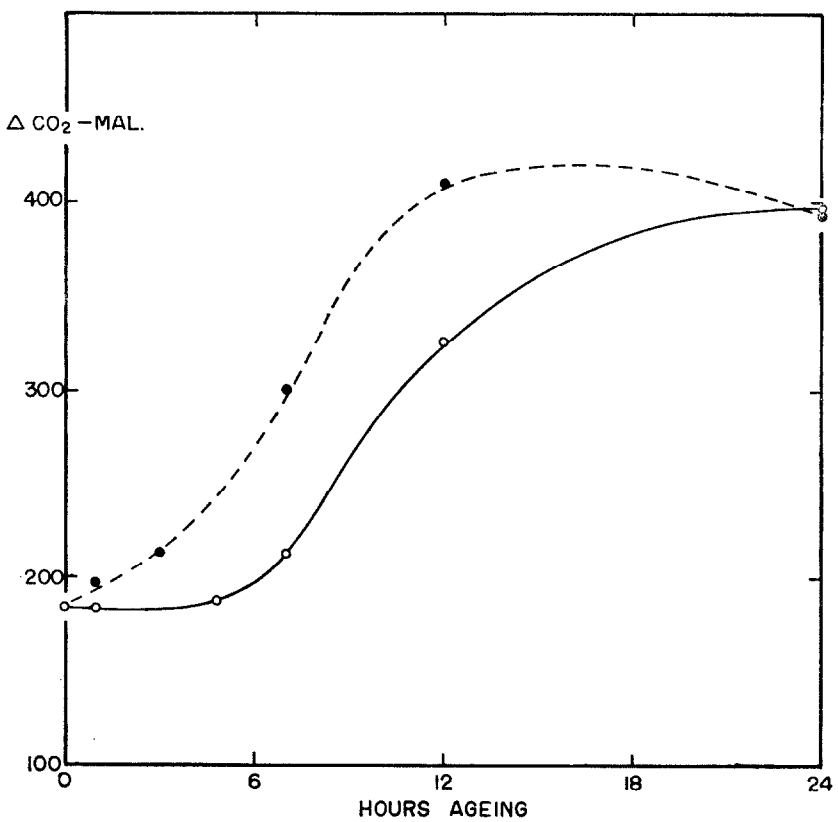

FIG. 6. TIME COURSE OF THE DEVELOPMENT OF THE MALATE EFFECT IN THE PRESENCE AND ABSENCE OF EXOGENOUSLY ADDED ETHYLENE.

(●---●) with exogenously added ethylene to a final concentration of 1%. (○—○) in the absence of added ethylene (see Experimental section for details).

Figure 7 shows that the ageing of disks in an atmosphere of 3 per cent O_2 completely inhibits the development of the malate effect. When such disks are returned to air and the ageing continued the development of the malate effect starts immediately with the normal lag phase eliminated.

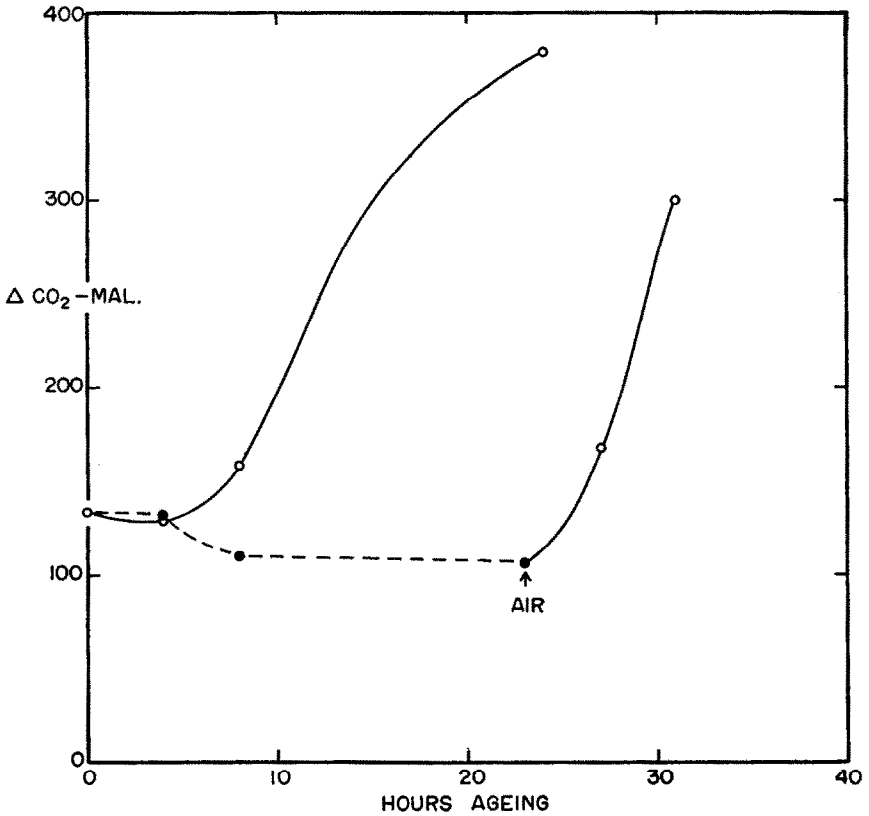

FIG. 7. INHIBITION OF THE DEVELOPMENT OF THE MALATE EFFECT IN DISKS AGED IN 3% O_2–97% N_2 AND THE REVERSAL OF INHIBITION ON RETURN TO AIR.
Disks were aged in air (○—○) or in 3% O_2–97% N_2 (●---●) and then transferred to air.

Pyruvate Decarboxylation During Ageing

Table 3 shows that no changes corresponding to the malate effect occur when pyruvate is added to disks during ageing.

TABLE 3. THE CAPACITY TO DECARBOXYLATE PYRUVATE OF FRESH DISKS AND OF DISKS AGED IN THE PRESENCE AND ABSENCE OF CYCLOHEXIMIDE

	Increase in CO_2 production on addition of pyruvate	$^{14}CO_2$ formation from pyruvate-1-^{14}C	
		% Uptake of counts applied	% Conversion of counts taken up to CO_2
Fresh disks	132	23	60
Disks aged for 24 hr in CAP/phosphate	138	25	58
Disks aged for 24 hr in presence of 1 μg/ml CH	137	24	62

DISCUSSION

The results for uptake of acetate[21] and malate show that whatever the mechanism of uptake may be, uptake cannot be the overriding factor in the utilization of the added metabolites during the ageing of peel disks.

With valine, uptake increases with ageing and this increase is prevented by inhibitors of protein synthesis (CH). We have no data on active uptake compared with free-space uptake for uridine but there is no reason to suppose that free-space uptake is greater than for valine.

It should be noted that the tissue of "peel" has characteristic differences from "pulp" or cortical tissue and the cortical tissue of bananas[20] and the parenchyma cells of many storage organs which could well result in considerable differences in permeability. Peel disks have only one major cut surface, the cells are relatively small and have few intercellular spaces.

The sensitivity of the development of the malate decarboxylation system on ageing of disks from pre-climacteric apples to inhibitors of nucleic acid and protein synthesis is consistent with the view that the development depends upon nucleic acid and protein synthesis. The involvement of RNA synthesis in the effect is shown by the inhibitory action of actinomycin D and 6-azauracil. Actinomycin D inhibits DNA-dependent RNA synthesis by combining with the DNA and so preventing the attachment of the DNA to the enzyme RNA polymerase,[23] while azauracil acts as a pyrimidine base analogue.[24] Cycloheximide and puromycin, which inhibit protein synthesis by preventing the formation of the polypeptide on the ribosomes,[25] both inhibit the development of the malate effect. Cycloheximide is a potent inhibitor of the effect even at a very low concentration (0·1 μg/ml) and it has been demonstrated that the inhibitory effects are completely reversible. The capacity of disks to incorporate amino acids into proteins rises to a peak after 8 hr of ageing and then falls off steadily. This peak in synthetic capacity is, therefore, reached considerably before the full development of the malate decarboxylation system is attained (see Fig.3).

Chloramphenicol inhibits the ageing effect only at relatively high concentrations. This is in agreement with other workers[26] who have suggested that in higher organisms it acts by selectively inhibiting the action of the particulate protein synthetic sites which involve the 70S ribosomes. The significance of this inhibition in the present case is not clear, since the malate decarboxylation system appears to be a soluble one.[18]

We have shown elsewhere[15, 27] that disks prepared from pre-climacteric apples develop the capacity to produce ethylene during a short period of ageing, even when the whole apples from which they were prepared would have remained in the pre-climacteric state without producing ethylene for several weeks. It has also been shown that the production of ethylene is sensitive to protein synthesis inhibitors.[27] A peak in ethylene production is reached after 6 hr ageing and thus preceeds the peak in protein synthesis and the full development of the malate effect. It is difficult to prove an absolute requirement for ethylene in the processes leading to the changes on ageing because of the endogenous production of ethylene. However, ageing disks in an atmosphere of 3 per cent O_2/97 per cent N_2, which inhibits ethylene production in apples[27] and bananas,[28] also inhibits the development of the malate effect.

[23] J. M. KIRK, *Biochim. Biophys. Acta* **42**, 167 (1960).
[24] C. W. ROSS, *Biochim. Biophys. Acta* **87**, 564 (1964).
[25] N. R. COHEN, *Biol. Rev.* **41**, 503 (1966).
[26] M. HUANG, D. R. BRIGGS, G. D. CLARK-WALKER and A. W. LINANE, *Biochim. Biophys. Acta* **114**, 434 (1966).
[27] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 1465 (1968).
[28] L. W. MAPSON and J. E. ROBINSON, *J. Fd Technol.* **1**, 215 (1966).

This, together with the fact that the lag phase in the development of the malate effect is eliminated both when disks are aged in the presence of added ethylene and when disks aged initially in 3 per cent O_2 are returned to air (a procedure known to stimulate ethylene production[27]), suggest that ethylene as well as RNA and protein synthesis is involved.

The work of Holm and Abeles[29] on the mechanism of ethylene induced abscission of bean ex-plants and ripening of bananas suggest that ethylene acts by regulation of RNA synthesis. Ethylene in particular stimulates the synthesis of ribosomal and messenger RNA. Preliminary experiments (not shown here) on the incorporation of ^{14}C-labelled uridine into the RNA of apple peel disks suggest that although the incorporation is small it is highest at the initial stages (1–2 hr) of ageing.

In earlier work[16–18] it was suggested that the enzymic nature of the malate decarboxylation system in apples consists of the combined action of malic enzymic and pyruvate decarboxylase and it was shown[16] that the experimentally determined ratio of malate utilization; CO_2 production; acetaldehyde formation; closely fitted the theoretical value of 1:2:1. We have found that during ageing there is no increase in uptake or in decarboxylation of added pyruvate corresponding to the increase in the malate effect; this suggests that the malate effect is not limited by the level of pyruvate decarboxylase in the tissue. The almost complete absence of increased O_2 uptake and the consequent high respiratory quotient of the malate effect[16, 15] poses a problem in relation to the action of malic enzyme, but this can be explained if there is coupling with another system at the pyridine nucleotide level. Although it seems probable that malic enzyme and pyruvate decarboxylase form the basis of the malate decarboxylation system[18] more work is needed to elucidate the details of their combined action in the ageing of disks from pre-climacteric fruit.

EXPERIMENTAL

Pre-climacteric apples were picked from Cox's Orange Pippin trees growing on Malling IX rootstocks in an orchard at the Burlingham Horticultural Station, Norfolk, over a 6-week period from 9 August to 20 September 1967. They were either used immediately or were stored for short periods at 12° until required for use.

The preparation of disks of 1 cm dia. from uniform, thin strips of peel tissue and their subsequent ageing were carried out by the methods previously described.[15] When the effect of inhibitors on ageing was studied, the inhibitor was introduced at a suitable concentration into the medium (0·05 M potassium phosphate, pH 4·5, containing 50 μg/ml chloramphenicol, termed "CAP/phosphate") in which the disks were aged. In the experiments in which the reversibility of the effect of CH was demonstrated, the disks were initially aged for 4 or 16 hr in the presence of CH (0·5 μg CH/ml of CAP/phosphate) and then returned to CAP/phosphate.

The conditions under which the preparations and ageing of the peel disks was carried out were such as to minimize contamination by micro-organisms. The tissues were initially surface sterilized[15] and the washing and ageing carried out in the presence of low concentrations of chloramphenical (50 μg/ml) which prevents the growth of many bacteria and other micro-organisms.[30] The surface microbiological contamination of both fresh (initial) and aged disks was studied using standard plating techniques. The main contaminants found were yeasts with some bacteria (Pseudomonads). However, the total contamination by micro-organisms was relatively low (10^4 organisms/g plant tissue) and did not significantly increase on ageing. During the present series of papers various effects developing on ageing will be discussed which show vastly different time course characteristics and this could clearly not be due to the growth of a single species of micro-organism. Also it will be shown that these effects do not develop on ageing of disks of ripe apples in which a high degree of contamination (particularly by yeasts) is to be expected.

Determination of Malate and Pyruvate Decarboxylation

The capacity of disks to decarboxylate malate was assayed using both manometric and radiochemical techniques. Using the manometric method of Neal and Hulme,[13] twenty disks (equivalent to about 1 g fresh

[29] R. E. Holm and F. B. Abeles, *Plant Physiol.* **42**, 1094 (1967).
[30] G. J. Leaver and J. Edelman, *Nature* **207**, 1000 (1965).

weight) were bathed in 2·8 ml 0·05 M potassium phosphate, pH 4·5, in the Warburg flask and the standard direct method was used for the determination of the basic respiration of the disks over a period of 30–60 min. Potassium malate, pH 4·5, was then added from the side arm to the main compartment of the Warburg flask to give a final concentration of 0·1 M. The increase in CO_2 output over the basic CO_2 output was measured in the period between 30 and 90 min after addition of the malate and was used as a measure of the malate decarboxylating capacity of the disks (expressed as: ΔCO_2-MAL). When the effect of inhibitors on the Warburg assay was studied, the inhibitor, at a concentration similar or in excess of that normally used to inhibit the development of the malate effect on ageing, was dissolved in the 0·05 M potassium phosphate medium.

The capacity of the disks to decarboxylate malate was also measured using uniformly labelled ^{14}C-malate. Twenty disks were incubated in 2·5 ml of 0·05 M potassium phosphate containing 0·5 μc of labelled malate (34·7 $\mu c/\mu$mole) at 25° for 1 hr in a 25 ml conical flask fitted with an ignition tube suspended by wire and sealed with a serum cap. After 1 hr incubation 0·2 ml of 1 M methanolic hydroxide of Hyamine 10-X (Rohm and Haas) was injected from a syringe into the suspended tube through the serum cap, and 0·4 ml of 3 N H_2SO_4 was injected into the bathing medium in order to kill the disks and to release dissolved CO_2 from the medium. A further 30 min period was allowed for complete absorption by the hyamine hydroxide of the CO_2 produced during the incubation. The suspended tube was removed from the conical flask and the hyamine hydroxide solution washed into a glass vial with 5 ml of a blended toluene phosphor containing 30 per cent ethanol, 4 g 2,5-diphenyloxasole (PPO) and 0·1 g 1:4 di-2-(5 phenyloxazolyl)–benzene (POPOP) per litre. The radioactivity was then determined by standard liquid scintillation techniques. Pyruvate decarboxylation was measured by similar manometric and radiochemical techniques to those used for the assay of the malate effect. In the radiochemical assay, 0·5 μc of sodium pyruvate-1-^{14}C (11·8 $\mu c/\mu$mole) was used in place of uniformly labelled malate.

Incorporation of Radioactive Valine into Protein

Twenty disks were incubated in 2·5 ml 0·05 M potassium phosphate containing 1 μc of uniformly labelled ^{14}C-valine (6·9 $\mu c/\mu$mole) at 25° for 1 hr. At the end of the incubation the disks were filtered, washed and then frozen in liquid N_2. The combined washings were used for the estimation of uptake by the disks. To obtain the protein-containing fraction, the frozen disks were ground to a fine powder in liquid N_2 and the powder was transferred to a polypropylene centrifuge tube and then sequentially extracted as follows:

Twenty ml of 5 per cent trichloracetic acid (TCA) containing 0·01 M ^{12}C DL-valine at 0°, 20 ml of 5 per cent TCA heated to 70° for 20 min, twice with 20 ml of 5 per cent TCA at 0°, twice with 20 ml of acetone, 20 ml of acetone and ether (1:1 v/v) and finally with 20 ml of ether. The extracted powder was dried *in vacuo* and then transferred from the polypropylene tube into a glass vial. 250 mg thixotropic gel powder (Cab-O-Sil, Packard) followed by 5 ml toluene phosphor (4 g PPO and 0·1 g POPOP dissolved in 1 l. toluene) were added and the powder counted suspended in the gel so formed. A suitable correction for quenching due to the presence of the powder was made after addition of an internal standard.

Chemical hydrolysis of the extracted powder with 6 N HCl at 110° for 14 hr, separation of the constituent amino acids by two-dimensional thin-layer chromatography using chloroform–methanol–17 per cent NH_3 (2:2:1 v/v/v) and phenol–water (75:25 v/v) as the solvents and finally autoradiography of the developed thin-layer plate, showed that all the radioactivity present was in the form of valine. Enzymic hydrolysis of the extracted powder with papain (57 enzyme units) and pronase (90 enzyme units) released more than 87 per cent of the radioactivity into a buffer soluble fraction showing that nearly 90 per cent of the radioactivity as assayed is present in the form of peptide bound valine.

Determination of Total, Intracellular and Free Space Uptake by Disks During Ageing

For total uptake, samples of 20 disks were taken during ageing and incubated in 25-ml conical flasks at 25° for 1 hr in 2·5 ml 0·05 M potassium phosphate, pH 4·5, containing the labelled metabolite (0·5 μC for malate and pyruvate and 1·0 μc for valine, uridine, acetate and inorganic phosphate). At the end of the incubation the disks were decanted from the labelled medium and then washed rapidly with ice-cold distilled water. The remaining medium and the washings were combined and made to 25 ml. 1 ml aliquots were taken for determination of the radioactivity remaining at the end of the incubation and counted in 9-ml toluene-Triton X-100 scintillation mixture[31] using standard liquid scintillation techniques. An aliquot of the original labelled medium was counted in a similar way and the total uptake of radioactivity by the disks was estimated by the difference (between the radioactivity added originally and that remaining at the end of the incubation).

Also for valine, the disks after the total uptake has been determined were washed in 0·01 M ^{12}C valine in 0·05 M phosphate at 1° for 20 min to exchange labelled valine both in the intercellular space and adsorbed on the cell-wall material. The disks were decanted after the 20 min period and a 1 ml aliquot of the washing medium taken and counted as before. This gives the so-called "free space" uptake and this was deducted from the value of the total uptake previously determined to give the intracellular uptake into the membrane bound space of the disks. A similar procedure was employed for the estimation of free space uptake of

[31] M. S. PATTERSON and R. C. GREENE, *Analyt. Chem.* **37**, 854 (1965).

$^{32}PO_4$. The disks after total uptake determination were washed in 10 ml of 0·1 M ^{31}P-potassium phosphate, pH 4·5, for 15 min at 0° and an aliquot of this washing medium subsequently counted.

Determination of Ethylene Production and its Effects During Ageing

Ethylene was assayed by gas–liquid chromatography of samples of the atmosphere above disks incubated in a sealed flask, by the method described by Galliard *et al.*[27]

When the effect of added ethylene during ageing was investigated samples of forty disks were aged in 50 ml CAP/phosphate in a series of conical flasks which were sealed after addition of ethylene by displacement to a final concentration of 1 per cent in air. At intervals, different flasks were opened and samples of forty disks were taken and analysed for the malate effect by the manometric technique.

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