Phytochemistry, 1968, Vol. 7, pp. 405 to 408. Pergamon Press. Printed in England

SHORT COMMUNICATION

THE DEVELOPMENT OF A MALATE DECARBOXYLATION SYSTEM DURING THE AGEING OF APPLE PEEL DISKS

M. J. C. RHODES, T. GALLIARD, L. S. C. WOOLTORTON and A. C. HULME

Agricultural Research Council Food Research Institute, Earlham Laboratory, Recreation Road, Norwich, Norfolk

(*Received* 3 *October* 1967)

INTRODUCTION

IT HAS been established that the incubation ("ageing") of thin slices of underground plant storage organs results in a large increase in respiration.[1] This increase is associated with increased synthesis of RNA and protein.[2] Recently Ap Rees[3] has shown that the phenomenon is not confined to underground storage organs but is exhibited by a wide variety of plant tissues, including fruits. Hackney,[4] however, showed that no such increase in respiration was evident during the incubation of apple disks. Using pre-climacteric apples we have confirmed this observation and the present work shows that aged apple disks undergo profound metabolic changes which differ in important aspects from those exhibited by storage tissues.

Neal and Hulme[5] showed that, during the development of the respiration climacteric in whole apples, a system developed in the fruit which decarboxylated malate—the so called "malate" effect.[6] Later work[7] indicated that the system involved NADP-malate dehydrogenase (malic enzyme E.C. 1·1·1·40) and pyruvate decarboxylase (E.C. 4·1·1·1). We here show that such a system develops during the ageing of disks prepared from the peel of pre-climacteric apples and that this development is dependent on the synthesis of new enzyme protein.

RESULTS AND DISCUSSION

Table 1 shows typical results for the basic O_2-uptake and CO_2-output of freshly prepared disks and of the same disks after ageing for 24 hr either in presence or absence of cycloheximide. The effect of adding malate initially and after 24 hr is also shown. The effect of added malate to the initial disks is shown in the form of a small increase in O_2-uptake, and a large increase in CO_2-output (the malate effect). We have found that this initial malate effect decreases as the *immediate pre-climacteric* state of the fruit as a whole is reached. During the

[1] G. G. LATIES, *Central Mechanisms in Respiration and Fermentation* p. 129 (edited by B. WRIGHT), Ronald Press, New York (1963).
[2] R. E. CLICK and D. P. HACKETT, *Proc. Nat. Acad. Sci. U.S.* **50**, 243 (1963).
[3] T. AP REES, *Australian J. Sci.* **19**, 981 (1966).
[4] F. M. V. HACKNEY, *Proc. Linnean Soc., N. S. Wales* **70**, 333 (1945).
[5] G. E. NEAL and A. C. HULME, *J. Exptl Botany* **9**, 142 (1958).
[6] A. E. FLOOD, A. C. HULME and L. S. C. WOOLTORTON, *J. Exptl Botany* **11**, 136 (1960).
[7] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. London, Ser. B.* **158**, 519 (1963).

ageing period the increase in the basic respiration is small, but there is a very large increase in the malate effect in the form of a more than three-fold increase in CO_2-output. When cycloheximide—an inhibitor of protein synthesis—is present during the ageing period, although the basic respiration is considerably reduced, the malate effect is no greater, proportionately, than with the initial disks.

TABLE 1. O_2-UPTAKE AND CO_2-OUTPUT OF APPLE DISKS BEFORE AND AFTER THE ADDITION OF MALATE

Treatment*	μl O_2 or CO_2/hr/20 disks					
	Basic		+Malate		Δ Malate	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
Initial	105	117	133	273	28	156
Aged 24 hr CAP/PO_4	129	163	146	546	17	383
Aged 24 hr CH	59	76	90	152	31	76

CAP/PO_4, Chloramphenicol (50 μg/ml) in 0·05 M potassium phosphate, pH 4·5.
CH, +Cycloheximide (1 μg/ml).

TABLE 2. EFFECT OF AGEING FOR 24 HR UNDER VARIOUS CONDITIONS ON THE INCREASE IN CO_2 PRODUCTION ON ADDING MALATE (TO A CONCENTRATION OF 0·1 M) TO PEEL DISKS FROM PRECLIMACTERIC APPLES

Conditions of incubation*	Increase in CO_2 μl/hr Experiment number						
	1	2	3	4	5	6	7
No incubation—fresh disks	147	180	189	150	208	189	151
Air at 25°	429	400	380	394	387	397	440
Air at 1°				142			
3% oxygen at 25°			198				
Nitrogen at 25°		118					
Air containing 1% ethylene at 25°	441						
Air at 25° in presence of following inhibitors							
(a) Cycloheximide (0·5 μg/ml)					94		
(b) Cycloheximide (1·0 μg/ml)					97		
(c) Puromycin (100 μg/ml)			86				
(d) Fluorophenylalanine (18 μg/ml)						290	
(e) Fluorophenylalanine (92 μg/ml)						91	
(f) Fluorophenylalanine (366 μg/ml)						59	
(g) Actinomycin D (17 μg/ml)		246					
(h) Actinomycin D (50 μg/ml)							186
(i) Azauracil (10 μg/ml)							173
(j) Azauracil (100 μg/ml)							118

* (Chloramphenicol and phosphate were always present—see "Experimental").

Table 2 shows the effects of various treatments of the disks during the ageing process including ethylene, which is known to induce the respiration climacteric in whole fruits, and 3 per cent oxygen which inhibits ethylene production in fruits.[8] Over the 24 hr period of ageing ethylene does not increase appreciably the CO_2 production following the addition

[8] L. W. MAPSON and J. E. ROBINSON, *J. Food Technology* **1**, 215 (1966).

of malate (Table 2). Time course experiments have shown, however, that ethylene decreases the time required for the onset of this ageing effect. After 3 hr of ageing in an atmosphere containing ethylene, disks begin to show an increased CO_2-production on addition of malate, when compared with control disks in air from which ethylene is absent; by 7 hr, the air samples have only reached 60 per cent of the final (24 hr) rates of CO_2 production, while the ethylene samples have reached almost 90 per cent of the final values. Furthermore, during 24 hr ageing there is an increase in the ethylene production by the disks from less than 0·001 to 0·025 μl/g/hr (250 μl/10 kg/hr) (see Wilkinson[9]). If cycloheximide is present during the ageing ethylene production remains at the initial level. The increase in the malate effect is inhibited if the disks are aged anaerobically or in 3 per cent O_2.

Cycloheximide (CH) eliminates the increased CO_2-production from malate after ageing and also reduces some of the "basic" CO_2 production. We have found that if tissue in which the development of the malate effect on ageing has been inhibited for up to 16 hr in CH is subsequently shaken in the CAP/phosphate medium free from cycloheximide, it becomes capable again of giving a malate effect equal to that produced by disks untreated with the inhibitor. Puromycin, another inhibitor of protein synthesis, as well as fluorophenylalanine, inhibit the development of the malate effect. Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, and the pyrimidine base analogue azauracil, also inhibit the development of that malate effect. Thus the system (malate effect) which is known to develop during the climacteric and to be accompanied by a net increase in protein[7] can be induced in peel tissue from pre-climacteric apples by ageing for short periods at 25°. Furthermore, inhibitors of the production of ethylene by the tissue (ethylene induces the respiration climacteric) and of protein synthesis at the transcriptional and translational levels, eliminate the development of the malate effect. This ageing effect in apple tissue differs from that of storage organs in that it is accompanied by only a small increase in basic respiration and that it can be induced by ethylene. Thus, unlike storage organs, apple tissue is in no way a dormant tissue.

Approaching the subject from a somewhat different angle from our own, other workers (Richmond and Biale[10]) suggest that changes over the respiration climacteric in fruits are not strictly comparable with the ageing of storage tissue. The study of the changes described in this paper, which we are now making in tissue from apples passing through the respiration climacteric, should help to decide whether, in fact, the ageing effect of storage organs and the respiration climacteric in fruits are related processes and could provide an experimental basis for the study of biochemical changes associated with the climacteric.

EXPERIMENTAL

Apple fruits were picked from Cox's Orange Pippin trees on Malling IX root stocks in an orchard at the Burlingham Horticultural Station, Norfolk. The apples used in the present experiments were all in the pre-climacteric state and were picked over a 6-week period from 9 August to 20 September 1967. The apples were stored at 12° until required for use.

The apples were surface sterilized by immersion in NaOCl (0·6 per cent available chlorine) for 5 min, washed with sterile water and then peeled to give strips of peel of uniform thickness. 20 disks, 1 cm in dia. (weight approx. 1 g) were used in all the experiments. The disks were washed twice with water, followed by one wash with 0·05 M phosphate pH 4·5, containing 50 μg chloramphenicol (CAP)/ml and then two further washes with phosphate; it was proved that this strength of CAP had no effect on basic respiration, nor on the development of the malate effect. All these operations were performed at 1°. The disks so treated were termed "initial disks" and were analysed immediately. The ageing of disks was carried out by shaking in

[9] B. G. Wilkinson, *Nature* **199**, 715 (1963).
[10] A. Richmond and J. B. Biale, *Plant Physiol.* **41**, 1247 (1966).

0·05 M potassium phosphate pH 4·5 containing 50 μg/ml CAP (CAP/phosphate) in conical flasks in a constant temperature bath at 25° for 24 hr. Time course experiments showed that the full development of the "malate effect" was achieved during 24 hr ageing. In the inhibition studies the inhibitor, at the concentrations shown in the Tables, was dissolved in the CAP/phosphate solution used for ageing the disks. In some experiments the effect of replacing air by various gas mixtures in the atmosphere over the disks on the ageing process was studied. A mixture of 3 per cent O_2 and 97 per cent N_2 was bubbled continuously through the contents of the flasks in which the disks were being aged. Where ethylene was used during ageing, 1 per cent of the gas was introduced into the flasks containing the disks at the commencement of the ageing; the flasks were sufficiently large to ensure an adequate supply of oxygen. For the study of the effect of anaerobic conditions on ageing the air was completely replaced by nitrogen and the flask sealed.

The malate decarboxylate activity of the disks was measured by the manometric technique described by Neal and Hulme[5] with the modification that the assays were carried out at pH 4·5 instead of 4·0. In the case of each inhibitor studied, it was shown that the concentrations used did not inhibit the determination of the malate effect, but only the development of the effect on ageing. Ethylene was determined by gas chromatographic analysis of the atmosphere above the disks.