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THE EFFECT OF ETHYLENE ON THE RESPIRATION, ETHYLENE PRODUCTION, RNA AND PROTEIN SYNTHESIS FOR APPLES STORED IN LOW OXYGEN AND IN AIR

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Abstract—A comparison is made between the respiration rate and ethylene production of the whole fruit and the respiration rate, ethylene production, incorporation of ^{14}C-uridine into an RNA fraction and of ^{14}C-valine into a protein fraction of peel disks prepared from the fruit from Bramley's Seedling apples stored at 12° in air and in 3% oxygen Results show that the respiration and ethylene production of the whole fruit is closely reflected in the behaviour of the peel disks in air, in low O_2 and on transfer from low O_2 to air. Ethylene appears to be the key to the increased rate of respiration and the other parameters including the development in the disks of a malate decarboxylating system (the malate effect) which appears to be a coupled system involving malic enzyme, pyruvate decarboxylase and alcohol dehydrogenase ($NADPH_2$-dependent). While exogenous ethylene has a temporary stimulatory effect on the various systems investigated when applied in 3% O_2, autostimulation of ethylene production with attendant physiological action does not appear possible in low O_2. Both production and physiological action of ethylene appear to require relatively high concentration of O_2 for their full operation.

INTRODUCTION

IT HAS been shown that the incorporation of ^{14}C-uridine into an RNA fraction[1] and of ^{14}C-valine into a protein fraction takes place during the early stages of the development of the respiration climacteric and before the full development of a malate decarboxylating system (the malate effect) in peel tissue of the apple.[1,2] The present work was undertaken to compare the changes taking place when the fruit is maintained in air with those occurring in 3% oxygen where the respiration climacteric is considerably delayed and reduced in intensity; changes in the various parameters on transfer from low oxygen to air have also been followed since it has been shown that ethylene production, which induces changes associated with the respiration climacteric, rises rapidly under these conditions.

RESULTS

Figure 1 shows the respiration rate (CO_2-output) and ethylene production of Bramley's Seedling apples stored at 12° in air, in 3% O_2 and in samples stored in 3% O_2 for 31 days and then transferred to air. In Fig 2 comparable changes in disks of apple peel prepared from the whole fruits at various times are shown. The respiration rate—in this case both O_2-uptake and CO_2-output—and rate of ethylene production at 25° were measured. The two sets of results indicate that the peel disks behave in a strikingly similar manner to the fruit from which they were taken. Even the inflection in the curve of respiration of the whole fruits in low O_2 between 25 and 30 days is reflected in the CO_2-output of the disks.

[1] A C. HULME, M. J C RHODES and L S C. WOOLTORTON, *Phytochem.* in press.
[2] A C HULME, M J. C RHODES, T. GALLIARD and L S C WOOLTORTON, *Plant Physiol., Lancaster* **43**, 1154 (1968)

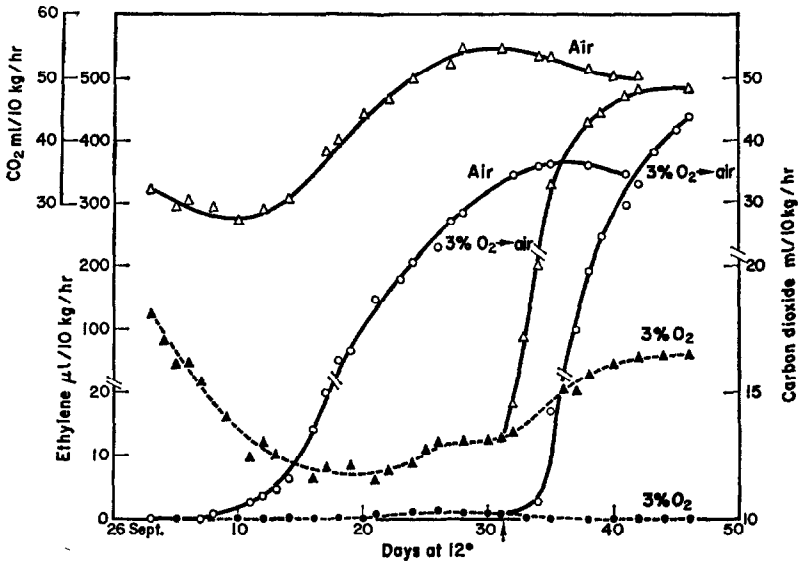

FIG. 1. RESPIRATION RATE (CO_2-OUTPUT) AND ETHYLENE PRODUCTION OF APPLES STORED AT 12° IN AIR (△ = CO_2 OUTPUT, ○ = ETHYLENE PRODUCTION), IN 3% O_2 (▲ = CO_2-OUTPUT; ● = ETHYLENE PRODUCTION) AND IN 3% O_2 FOLLOWED BY TRANSFER TO AIR (▲ → △ CO_2-OUTPUT; ● → ○ = ETHYLENE PRODUCTION) NOTE DIFFERENCE IN SCALE BETWEEN CO_2-OUTPUT IN AIR AND IN 3% O_2 AND CHANGE OF SCALE ON TRANSFER FROM 3% O_2 TO AIR (SHOWN BY AN ARROW, 31 days).

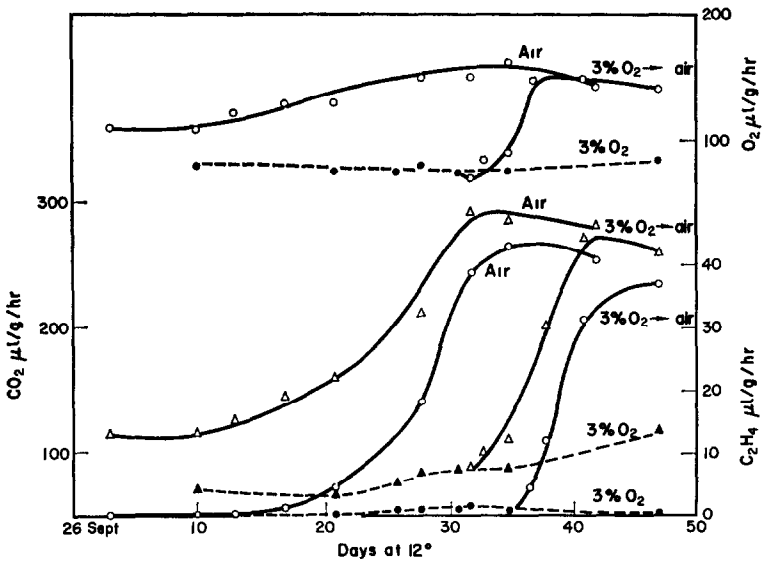

FIG 2 LOWER SET OF CURVES RESPIRATION RATE AND ETHYLENE PRODUCTION OF PEEL DISKS TAKEN FROM WHOLE FRUITS REFERRED TO IN FIG 1. SYMBOLS AS FOR FIG. 1
Upper set of curves O_2-uptake of disks of peel from apples stored in air (○), in 3% O_2 (●) and in 3% O_2 and then transferred to air (● → ○)

In 3% O_2 ethylene production remains very close to zero but it should be pointed out that before measurable amounts of ethylene appear in the ambient atmosphere the internal concentration may be well above the threshold for physiological action by the gas.[3] No measurements of internal ethylene concentrations were made here, but, since there is a small ethylene evolution from both fruit and peel disks from day 20 to day 31, followed by a fall to zero (external concentration), it would appear that ethylene production in the tissue commences probably before day 20 but that, in 3% O_2, the mechanism for the usual (in air) autocatalytic production of the gas appears to become inhibited.

The O_2-uptake of disks from fruit in air shows a shallow climacteric peak. In low O_2 there is no significant change in O_2-uptake while on transfer from 3% O_2 to air there is a rapid rise to the 'air' value.

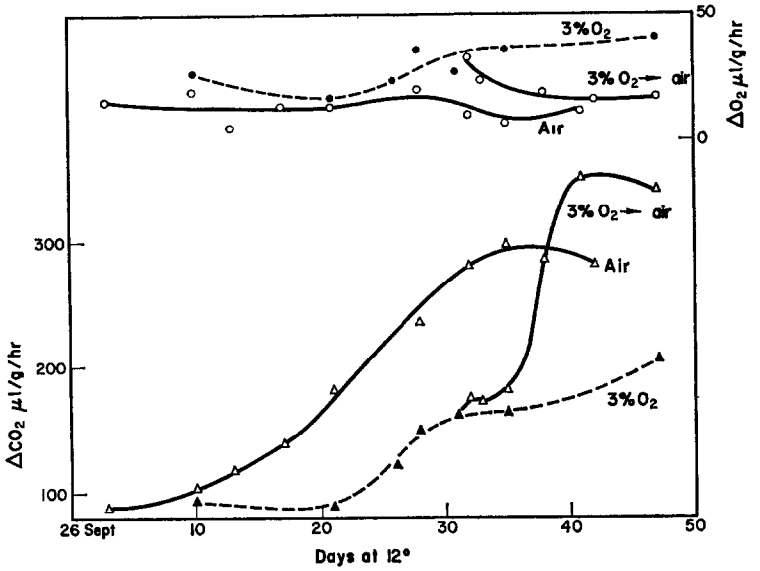

FIG 3 LOWER SET OF CURVES· ADDITIONAL CO_2 EVOLVED BY PEEL DISKS (AS FIG. 2) IN RESPONSE TO MALATE ADDITION (Δ CO_2) TREATMENT AND SYMBOLS AS FIGS 1 AND 2.
Upper set of curves· O_2-uptakes in response to malate additions (Δ O_2) Symbols as for upper set of curves in Fig 2. Note expanded scale as compared with O_2-uptake in Fig. 2.

Figure 3 shows the development of the malate decarboxylation system in response to the addition of malate (the malate effect) to peel disks taken from the fruit at the dates shown; this is expressed as Δ CO_2 (the excess CO_2 produced in response to malate addition)[4] and the corresponding oxygen uptake (Δ O_2) values are shown in the top set of curves. In disks from fruit held throughout in 3% O_2, there is a slow, reduced (as compared with 'air') malate effect exhibiting an inflexion in the region of the inflexion in the respiration curves (cf. Fig 2) In the disks from fruit transferred from 3% O_2 to air at day 31, after a short lag phase, there is a rapid development of the malate effect to a peak value somewhat higher than that developed in disks from fruit held in air throughout.

[3] S P. BURG and E A. BURG, *Plant Physiol*, *Lancaster* **37**, 179 (1962).
[4] A. E FLOOD, A C HULME and L S C WOOLTORTON, *J. Exp Bot.* **11**, 316 (1960).

Oxygen uptake (top set of curves) shows only a small change in response to added malate but although the results (intentionally plotted on an exaggerated scale) are not highly significant, there is a distinct tendency for O_2-uptake to be higher in disks from fruit stored in 3% O_2 than in disks from fruit stored in air and to fall in the disks from fruit transferred from 3% O_2 to air.

The Respiratory Quotients (*R.Q.*s) in response to malate addition to the disks were calculated for all the treatments (air throughout, 3% O_2 throughout, 3% O_2 transferred

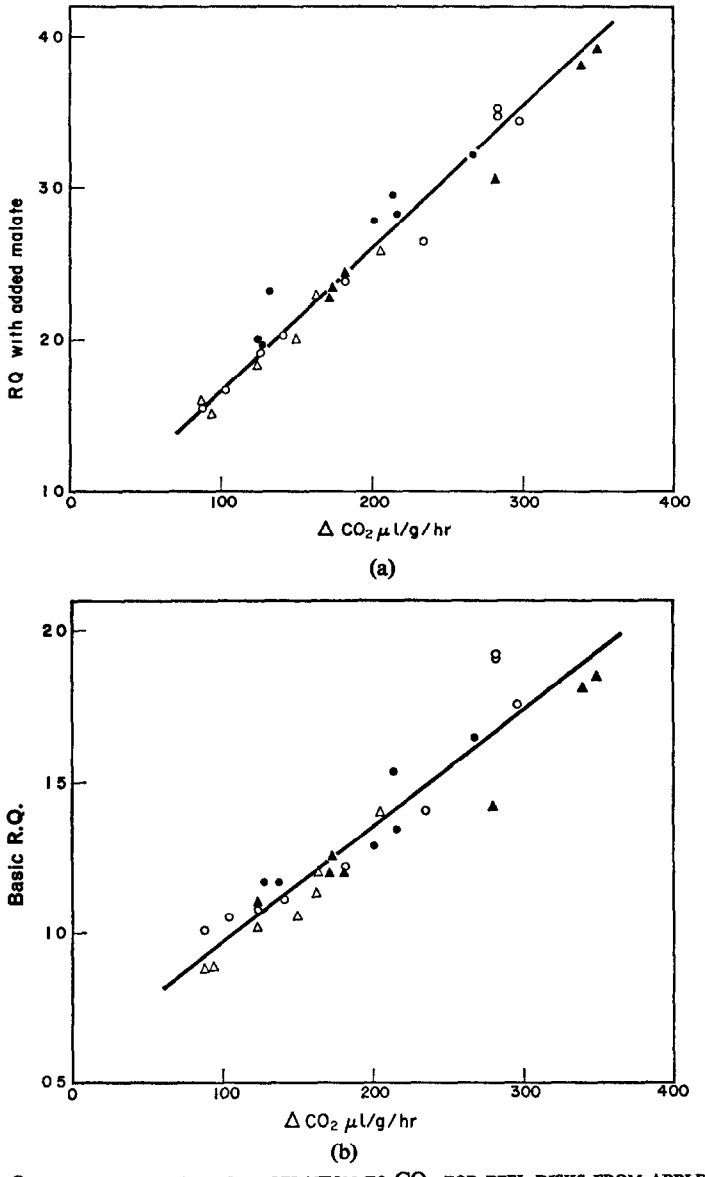

FIG 4. (a) *R Q* WITH ADDED MALATE IN RELATION TO CO_2 FOR PEEL DISKS FROM APPLES SUBJECTED TO ALL TREATMENTS

(b) BASIC *R Q* IN RELATION TO CO_2 OF DISKS, SYMBOLS AS FOR FIG. 4(a)

○ = AIR; △ = 3% O_2, ▲ = 3% O_2 → air, ● = 3% O_2 + ethylene

to air and 3% O_2 stored fruit treated at two points with ethylene—see later) and their values in relation to the values for the malate effect (ΔCO_2) are shown in Fig. 4(a) (correlation coefficient = 0·96). There is also a close correlation between the *basic R.Q.* of the disks and ΔCO_2 from added malate (Fig. 4(b); correlation coefficient = 0·91). The relationship between *R.Q.* and malate effect is remarkably constant throughout so that it may be reasonably assumed that the same system is involved in the production of 'extra' CO_2 by the disks on addition of malate in all the environmental changes to which the fruit was subjected.

Measurements were made of the activity of malic enzyme (ME), pyruvate decarboxylase (PD) and alcohol dehydrogenase (ADH)[5] at crucial points in the treatment of the fruit; the results are given in Table 1. In the fruit maintained in air, ME increased more than twofold, PD increased fivefold whereas ADH increased only to a limited extent. In 3% O_2, ME tends to decrease as storage progresses, PD increases very slightly while ADH increases more rapidly than in air. On transfer from 3% O_2 to air, in the disks prepared from the fruit, ME and PD increase, the latter showing a particularly rapid rise; ADH activity decreases somewhat but still remains as high as in the air samples

TABLE 1 ACTIVITY OF ME, PD AND ADH OF APPLES AFTER STORAGE AT 12° UNDER VARIOUS CONDITIONS

	*E.U per g × 10^{-2}		
	ME	PD	ADH
Initial	18 6	13 5	14 3
35 days air	31 0	72 0	19 4
34 days 3% O_2	19 4	15 5	22 5
47 days 3% O_2	15 8	15 2	33 3
34 days 3% O_2 + 13 days air	25 0	63 0	20 4

* 1 E.U. represents the amount of enzyme giving a change in optical density of 0 001/min, measured at 340 nm under the specified conditions [1]

The results of experiments on the incorporation of ^{14}C-uridine and ^{14}C-valine (as a % of uptake)[6] into RNA and protein respectively (as TCA precipitates[1]) of disks from apples undergoing the various treatments are shown in Fig. 5. In disks from the air samples, uridine incorporation rises to a peak at 10 days and is followed by a peak in valine incorporation at 21 days. Unfortunately we have few determinations of uridine incorporation for the fruit stored in 3% O_2 throughout; those there are show some scatter but it would appear that, at least, incorporation is maintained at an appreciable rate. Valine incorporation shows a slow fall halted in the region of the peak in the malate effect (cf. Fig. 3). The small rise in uridine incorporation in the disks when the fruit is transferred to air is probably not significant in view of the scatter in the results in the 3% O_2-fruit; the rate of uridine incorporation is certainly maintained. The increase in valine incorporation is, however, most striking and is subject to a short lag as is the development of the malate effect (Fig. 3).

In a subsidiary experiment, fruit maintained in 3% O_2 for 24 days was exposed to 270 ppm of ethylene for 5 days. During this period of ethylene treatment and subsequently,

[5] M J. C RHODES, L S C WOOLTORTON and P. J. HARKETT (in preparation).
[6] M J C. RHODES, L S C WOOLTORTON, T GALLIARD and A. C. HULME, *Phytochem.* 7, 1439 (1968)

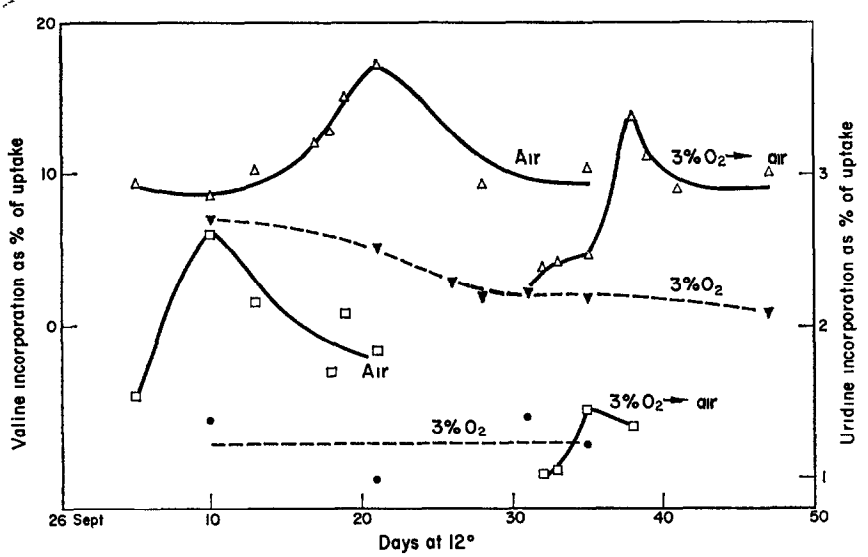

FIG 5 ^{14}C-VALINE INCORPORATION AND ^{14}C-URIDINE INCORPORATION IN DISKS FROM FRUIT HELD AT 12° IN AIR (△ = VALINE, □ = URIDINE), IN 3% O_2 (▼ = VALINE, ● = URIDINE) AND ON TRANSFER FROM 3% O_2 TO AIR (△ = VALINE, □ = URIDINE)

the CO_2-output of the fruit and of the disks prepared from them, and the malate effect and ^{14}C-valine incorporation into protein were followed (see Fig. 6) The ethylene production of the fruit immediately after the removal of applied ethylene was also measured; no ^{14}C-uridine was available at the time but a few measurements were made of ^{14}C-uracil incorporation into RNA. The results shown in Fig 6 also include, for purposes of comparison, results already mentioned for fruit maintained in 3% O_2 without ethylene treatment. Ethylene (270 ppm) was again given to the same batch of fruit after 41 days and the effect on the respiration and malate effect measured. Incorporation of uracil into RNA is less direct than for uridine; a comparison of the results in Figs. 5 and 6 suggests the efficiency of conversion is 66 per cent less for uracil.

The results in Fig. 6 show that, following the application of ethylene, valine incorporation is stimulated; RNA synthesis is proceeding steadily throughout the sojourn in 3% O_2 as will be seen from the uridine incorporation values in Fig. 5 This increased valine incorporation (in the disks) is followed immediately by an increased respiration of the whole fruit and of the disks prepared from them and this is in turn followed by a small but significant increase in the malate effect (disks) Shortly afterwards, however, the respiration of the whole fruit and the basic CO_2-output of the disks as well as the malate effect fall; the fruit are, of course, still under 3% O_2. At about this time the fruit begins to produce ethylene. The amounts are relatively small (but of physiological significance) so that ethylene production by the disks is too low to be measured by our methods. The CO_2-production of the disks and the malate effect rise. Ethylene production by the fruit cannot be maintained (in the low O_2); the CO_2-output of the disks fall and the malate effect is halted. Finally, as a result of the second dose of ethylene (day 41) the respiration of the fruit, the CO_2-production of disks and the malate effect again increase. Unfortunately, no measurements of incorporation of ^{14}C compounds were made after day 31.

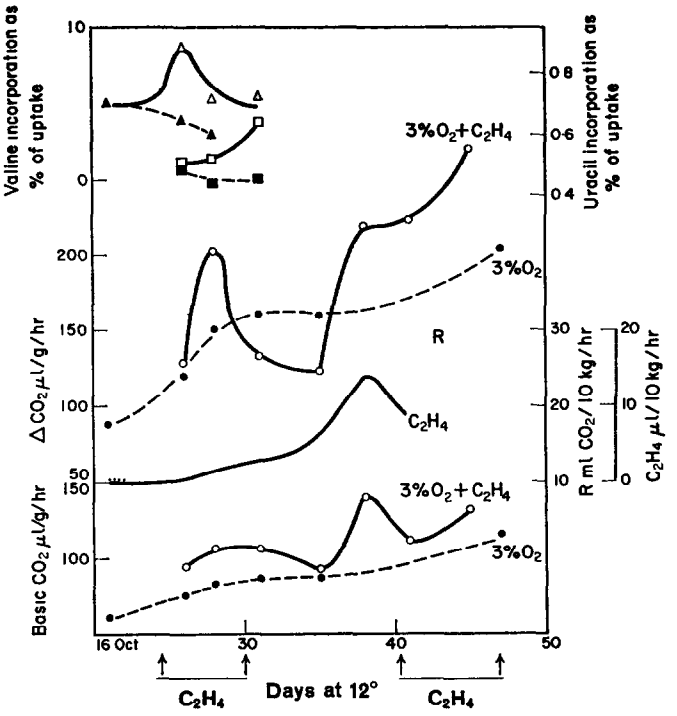

FIG 6 VARIOUS PARAMETERS IN DISKS OF PEEL FROM APPLES STORED IN 3% O_2 AND GIVEN 270 ppm ETHYLENE FOR PERIODS SHOWN BY ARROWS

Basic CO_2 production (●- - - - ● in 3% O_2; ○——○ in 3% O_2 + C_2H_4), △ CO_2 on addition of malate (●- - - -●) in 3% O_2, (○——○) in 3% O_2 + C_2H_4, ethylene production by whole fruit in 3% O_2 (———), respiration rate (CO_2 output) of whole fruit (· · · · ·).

Top set of curves: ^{14}C-valine incorporation in disks from fruit in 3% O_2 (▲) and in 3% O_2 + C_2H_4 (△). ^{14}C-uracil incorporation in disks from fruit in 3% O_2 (■) and in 3% O_2 + C_2H_4 (□)

Although these responses to ethylene in low O_2 are much smaller than corresponding responses in fruit maintained in air,[1] they may form the basis of the slow changes that occur during the storage of fruit in low O_2 and become manifest when the fruit is removed from the store into air.

DISCUSSION

During the climacteric rise the ethylene production and respiration (CO_2-output) rate of the whole fruit in air and the peel disks prepared from them follow a similar pattern. In the disks the O_2-uptake shows a more slowly attained and a shallower peak than the CO_2-output suggesting an alteration in the system involved in the respiration climacteric This is emphasised by the development of the malate decarboxylating system with an increasing *R Q.* in the disks. The sequence of increased RNA and protein synthesis during the early stages of the climacteric suggest that the increased activity of ME, PD and ADH involved[7] in the malate effect is most probably due to an increase in enzyme complement.

The picture in the fruit maintained in 3% O_2 is less simple. Respiration rate of fruit and disks is, as might be expected, low. Nevertheless, the correspondence between respiration rate of the fruit and the CO_2 output and malate effect in the disks is close. Although uridine

[7] M. J. C. RHODES, L S C WOOLTORTON and A C. HULME, *Qual Plant Mater Veg* **19**, 167 (1969).

and valine incorporation show no dramatic change even when the decarboxylation of added malate is increasing (although to a much less extent than in fruit in air), the RNA and protein synthesising systems are operating at a low rate but which appears to be sufficient to maintain the observed level of enzyme activity. That valine incorporation does not increase in low O_2 and neither does the activity of ME and PD although there is some increase in ADH activity is consistent with a restrained pattern of enzyme change.

When the fruit is transferred from 3% O_2 to air, there is a rapid adjustment to the normal (in air) situation with increased respiration, protein synthesis and malate effect The fact that there is no large increase in uridine incorporation as a preliminary to increased protein synthesis suggests that transcription is building up to a point where translation is limited only by O_2 so that, on transfer to air, there is only a short lag before protein (enzyme) synthesis becomes stimulated

The Δ O_2-uptake (Fig. 3, top set of curves) shows the same pattern in air and on transfer to air from 3% O_2 and a higher O_2-uptake in 3% O_2 This could mean that two different systems for decarboxylating malate are operating in air and in 3% O_2. However, this seems unlikely in view of closely similar relationships between *R.Q.* and malate effect under all conditions (Fig 4).

Possibly the key to the behaviour in low O_2 lies in the apparent impossibility of autocatalytic ethylene under these conditions. The fruit produces a little ethylene and this should be, but is not, sufficient for physiological action. Thus, in low O_2, the brake appears to extend even to ethylene action on its own production. This is confirmed by the results with applied ethylene. In low O_2 the whole sequence of events from ethylene production to malate effect starts but cannot be maintained.

We have suggested elsewhere[7] that the malate effect is the result of a sequence of reactions:

(1) MALATE + NADP $\xrightarrow{ME}$ PYRUVATE + $NADPH_2$

(2) PYRUVATE $\xrightarrow{PD}$ ACETALDEHYDE

(3) ACETALDEHYDE + $NADPH_2$ $\xrightarrow{ADH}$ ETHANOL + NADP

In this coupled system the $NADPH_2$ formed in reaction (1) will drive reaction 3, while the NADP formed in reaction 3 could drive reaction one again. The whole system could operate without an uptake of O_2 PD *will be the key enzyme* in this system, and when this enzyme is limiting the system for malate decarboxylation can only continue to proceed with an uptake of oxygen to oxidize the $NADPH_2$ to NADP. In low O_2 there is no increase in PD (Table 1) so that for the small, observed increase in malate effect O_2-uptake should increase—as, in fact, it does (Fig 3). Increase in the activity of ADH, which is of no value to the coupled system in the absence of acetaldehyde production, is probably required by the incipient anaerobic conditions On transfer to air PD increases and the coupled system can operate without direct uptake of O_2.

Undoubtedly, in the intact fruit, Krebs cycle activity accounts for much of the utilisation of malate and, by our introduction of excess malate into respiring disks we are exaggerating one aspect of malate decarboxylation. Nevertheless, the close correlation between the basic *R.Q* (no added malate) and the extra CO_2 formed on addition of malate suggests that the system does function, to some extent at least, *in vivo*.

Finally, we suggest that (1) low O_2 inhibits the continued development of the ethylene producing system and the events which ethylene sets in motion, (2) nevertheless, a part

(transcription?) of the system producing 'enzymes of ripening' is being slowly built up (the 'creeping' malate effect in low O_2 supports this argument) in low O_2. The present results provide additional evidence for the view that oxygen is required both for ethylene production and for ethylene action.

EXPERIMENTAL

Pre-climacteric apples were picked from Bramley's Seedling apple trees in a commercial orchard in Norfolk on 26 September 1969. They were divided at random into four batches. One batch was maintained in air throughout the duration of the experiment; one was maintained in 3% O_2 and 97% N_2 ('3% O_2'), one was maintained in 3% O_2 for 31 days and then transferred to air, one was maintained in 3% O_2 and given 270 ppm ethylene from day 24 to day 29, and again at day 41 to the end of the experiment. The temperature of storage was 12° throughout The respiration rate of the whole fruit was obtained as CO_2-output measured by GLC using a column of 'Porapak S' in conjunction with a katharometer The system was calibrated against known concentrations of CO_2 Ethylene production was determined as previously described [1] Disks of peel were prepared from the fruit at appropriate times (see Figs 1–6) and their CO_2-output, O_2-uptake at 25° (in air) both with and without the addition of malate determined as described by Hulme *et al.*[1] The methods used for the determination of uptake and incorporation of ^{14}C-uridine, ^{14}C-uracil and ^{14}C-valine into the disks have also been described elsewhere.[1] Suitable experiments have shown that the bulk of the label from the valine appeared in the protein fraction of the fruit [6] Proof that the bulk of the nucleotides appeared in the RNA fraction was obtained by hydrolysis of the TCA precipitates with RNAase which liberated more than 80% of the radioactivity

The activities of malic enzyme (ME), pyruvate decarboxylase (PD) and alcohol dehydrogenase (ADH) were determined in homogenates of the peel disks as described by Hulme *et al* [1] ADH activity was measured with $NADPH_2$ as cofactor rather than $NADH_2$ for the reasons described by Rhodes *et al.*;[7] the ADH present in apples is more active with this nucleotide