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THE RESPIRATION CLIMACTERIC IN APPLE FRUITS: SOME POSSIBLE REGULATORY MECHANISMS

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Abstract—The inhibition of succinate and malate oxidation by added oxaloacetate (OAA) has been studied in mitochondria isolated from the peel of apple fruits at various stages in the development of the respiration climacteric in fruit either attached or detached from the tree. The rate of relief of inhibition increases as the climacteric peak is reached. It is suggested that this faster relief of inhibition is related to the development of a special enzyme system for metabolizing OAA rather than to an increase in the rate of turnover of the Krebs cycle. The effect of addition of glucose and hexokinase to mitochondria oxidizing succinate and malate results in an enhancement of the rate of oxidation, and again the effect is most pronounced at the climacteric peak; the effect on CO_2-output is greater than an O_2-uptake. The effect of glucose and hexokinase is probably to provide a continuous supply of phosphate acceptors, but this does not explain the greater effect on CO_2-output.

INTRODUCTION

DURING the past decade several workers, including ourselves, have made claims to having found *the* essential factor in the development of the respiration climacteric in fruits. Especially during such periods of profound metabolic adjustments, regulatory mechanisms are unlikely to be simple or confined to single enzyme systems. One fact that does appear to be emerging from some of the work on the climacteric is the importance of feedback regulation of the Krebs cycle by oxaloacetic acid (OAA). The present work emphasizes this point and poses further questions.

As the climacteric develops in apples, the oxidative activity of the isolated mitochondria increases.[1] This activity was measured in the Warburg respirometer with succinate and malate as substrates, using yeast concentrate to supply essential co-factors. It had been shown that the replacement of the yeast concentrate by a mixture of ATP, NAD, NADP, TPP and CoA has little effect on the activity of the mitochondria from mature applies *near or past the climacteric peak*.[2] Consequently, for reasons of economy, yeast concentrate was used in the studies of mitochondrial activity throughout the climacteric period already referred to.

Recently we have made a study of the effect of OAA on apple mitochondria and of its inhibitory effect on the succinic dehydrogenase system.[3] These results, as well as those of other workers,[4, 5] suggested that OAA has a regulatory effect *in situ* on the operation of the Krebs cycle. Of the acids of the Krebs cycle, OAA appears especially suited to function in a feedback control mechanism since very small amounts inhibit the cycle (via succinic dehydrogenase) and at the same time the equilibrium of the reaction for its formation from malate favours the back reaction at physiological pH values.

It seems probable that the level of OAA may be regulated by a number of reactions involving several enzymes and co-factors. Since the accumulation of very small amounts of OAA

[1] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *New Phytologist* **64**, 158 (1965).
[2] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[3] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *J. Exptl Botany* **18**, 277 (1967).
[4] D. B. TYLER, *Biochem. J.* **76**, 293 (1960).
[5] E. C. SLATER, *Chem. Weekblad* **52**, 1 (1962).

appears to inhibit the Krebs cycle,[3] these factors might have an important bearing on the change in mitochondrial activity observed during the respiration climacteric. The effect of co-factors on the overall activity of the mitochondria isolated during the development of the climacteric would thus assume great importance. For example, since this work was done, Lance *et al.*,[6] using avocado mitochondria, have suggested that thiamine pyrophosphate (TPP) is of special importance in the development of the climacteric in this fruit. The precise content of co-factors present in the yeast concentrate used in our earlier experiments (see above) is not known. It was decided, therefore, to follow the activity of mitochondria isolated from apple peel during the development of the climacteric using specific co-factors instead of the yeast concentrate and to investigate the recovery from OAA inhibition of succinate oxidation. Since, in earlier experiments,[1] the addition of glucose and hexokinase to the mitochondrial system oxidizing malate appeared to have an increasing effect as the climacteric developed, this problem was included in our experiments.

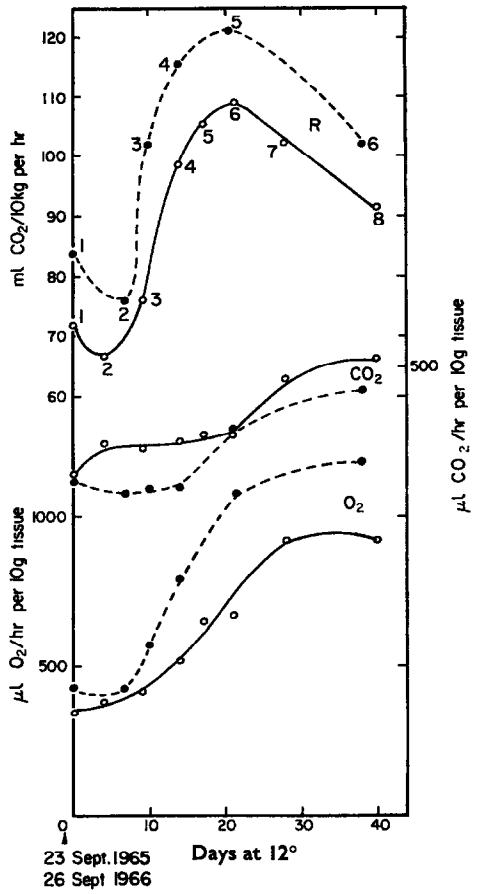

FIG. 1. RESPIRATION OF THE WHOLE FRUIT (R), O_2-UPTAKE AND CO_2-OUTPUT (OVER 2 HR) OF MITOCHONDRIA PREPARED FROM THE PEEL AT SIX STAGES (1965) AND EIGHT STAGES (1966) DURING THE DEVELOPMENT OF THE RESPIRATION CLIMACTERIC IN APPLE FRUITS.

—○— represents the 1966 fruit; and ---●--- represents the 1965 fruit. The numbers on the curves for the respiration of the whole fruit represent the various stages discussed in the text.

[6] C. LANCE, G. E. HOBSON, ROY E. YOUNG and J. B. BIALE, *Plant Physiol.* **40**, 1116 (1965).

RESULTS

Respiration of Whole Fruit and of Peel Mitochondria

Figure 1 shows the progress of the respiration of the whole fruit for both 1965 and 1966 series of samples. The time scale has been adjusted so that the initial sample for both series is taken as zero time. The whole progress of the climacteric is remarkably similar for both series considering that the two sets of fruits are of different "cultural types". The O_2-uptake and CO_2-output of the mitochondria isolated from the peel of the fruit, with succinate as substrate, are also shown in Fig. 1.

Inhibition of Mitochondrial Oxidation of Succinate and Malate by OAA

Figure 2 gives examples of the progress, in the Warburg respirometer, of the disappearance of inhibition of succinate (1965) or malate (1966) by added OAA over a 2–2·5 hr period. The rate of oxidation of the substrates in the absence of OAA is also shown. It has been demonstrated[3] that recovery from inhibition is directly related to the disappearance of OAA from the digest. When the OAA level had fallen below the critical inhibitory level, recovery to the uninhibited rate occurs. In the early stages of the climacteric, where the overall activity is low, the transition from inhibited to recovered rate is less clear-cut than in the later more active

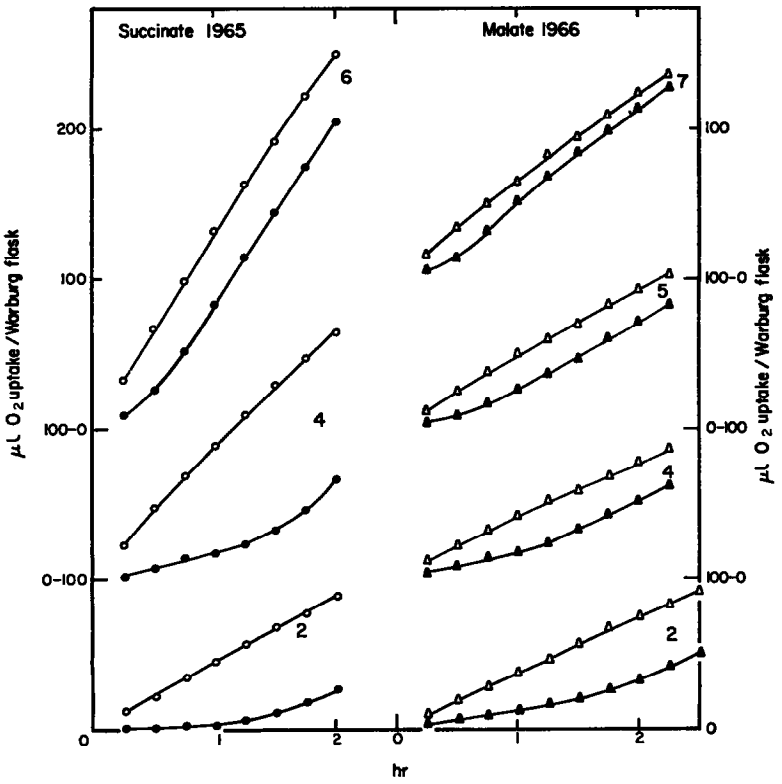

FIG. 2. RECOVERY FROM INHIBITION BY OAA OF SUCCINATE (1965) AND MALATE (1966) OXIDATION DURING A 2–2·5-HR PERIOD FOR MITOCHONDRIA PREPARED AT SEVERAL STAGES OF THE CLIMACTERIC.

○, △ substrate minus OAA; ●, ▲, substrate in presence of OAA (see text for amounts).

stages of the climacteric. Nevertheless, the time at which *complete* recovery occurs can be assessed even at these early stages. The time taken for complete recovery to occur at various stages of the climacteric in fruit detached from the tree is given in Table 1 for succinate (1965) and malate (1966). This table also includes similar data for malate (1966) with mitochondria of fruit passing through the climacteric while attached to the tree; here, the rates of respiration of the whole fruit are included, since they are not given elsewhere. It will be seen from Table 1 that the most rapid recovery from inhibition by OAA occurs near the climacteric peak (cf. Fig. 1); it does not occur during the phase of the climacteric when the respiration rates show the maximum rate of increase.

TABLE 1. TIME (MIN) TAKEN FOR *complete* RECOVERY TO UNINHIBITED RATE OF APPLE MITOCHONDRIA INHIBITED BY OAA DURING OXIDATION OF SUCCINATE AND MALATE

Stage (in store)	Succinate (1965)	Malate (1966)	Stage (on tree)	Respiration rate of whole fruit (ml CO_2/10 kg/hr)	Malate (1966)
1	120	135	1	66	165
2	120	135	2	65	165
3	120	90	3	74	150
4	105	90	4	73	135
5	60	60	5	128	60
6	50	60	6	136	45
7	—	45	—	—	—
8	—	45	—	—	—

Although in the present instance the increased activity of the peel mitochondria, especially in relation to CO_2-production, lags somewhat behind the rise in CO_2-production of the whole fruit, this is not always the case,[1] and probably depends on small differences between the respiration rate of the whole sample of fruit and the portion of it used for the isolation of the

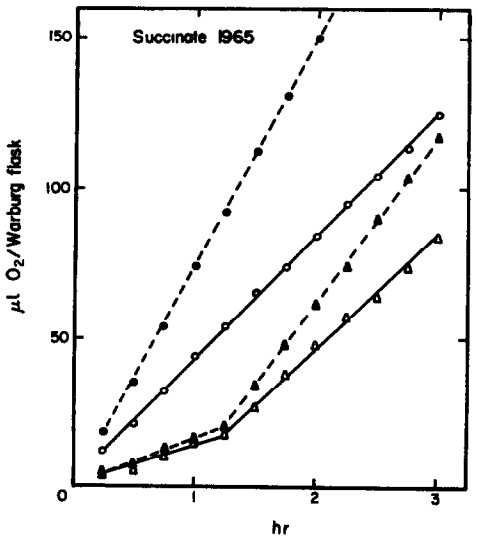

FIG. 3. EFFECT OF ADDITION OF G+H ON MITOCHONDRIAL OXIDATION OF SUCCINATE IN ABSENCE (○, MINUS G+H; ●, PLUS G+H) AND PRESENCE (△, MINUS G+H; ▲, PLUS G+H) OF OAA.

mitochondria.[7] The faster relief of inhibition by OAA (i.e. disappearance of OAA) cannot simply be due to increased activity of the Krebs cycle in the mitochondria, since addition of glucose and hexokinase (G+H) to the digests which almost doubles the rate of oxidation of both succinate and malate alone has no effect on the rate of relief from inhibition by OAA, as will be seen from Fig. 3 (see also Jones *et al.*[1]).

Removal of OAA Inhibition by Means of Transamination

We have demonstrated previously[3] that there is a mechanism present in apple mitochondria which will transform OAA through transamination with glutamate. It seemed possible, therefore, that transaminase might be involved in the increased removal of OAA during the climacteric rise. Figure 4 shows changes in the OAA-glutamate transaminase activity of the mitochondria during the climacteric rise in the 1966 fruit. The transaminase activity increases six times between stages 4 and 7 of the climacteric and at the peak would be more than sufficient to transaminate all the OAA added. This reaction would require the presence in the digests of the requisite amount of glutamate; we have no data on this.

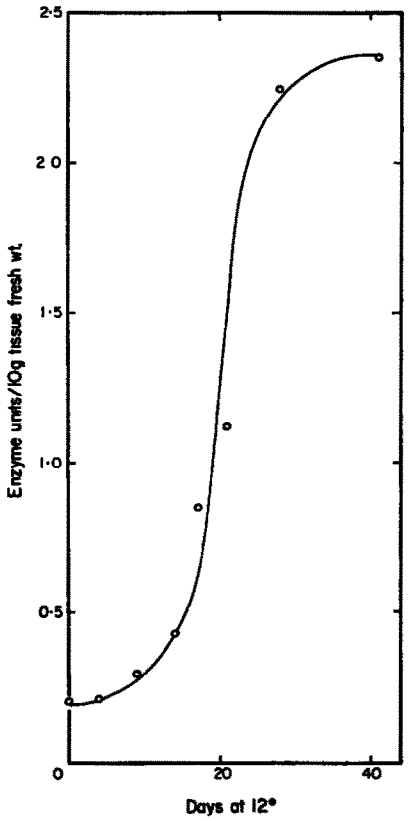

FIG. 4. CHANGE IN TRANSAMINASE ACTIVITY OF MITOCHONDRIA PREPARED FROM THE PEEL OF APPLES DURING THE DEVELOPMENT OF THE CLIMACTERIC, 1966.

[7] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc.* (*London*) *B* **158**, 514 (1963).

The effect of Glucose and Hexokinase on the Rate of Oxidation by Mitochondria

The effect of the addition of glucose and hexokinase (G+H) to malate oxidation at various stages of the climacteric with mitochondria from fruit of the 1965 series is shown in Fig. 5. In previous experiments in which G+H was added to mitochondria digests,[8,1] sodium fluoride was also present since the rate of phosphorylation (i.e. P:O ratios) was being studied. In further studies with G+H, sodium fluoride was omitted from the digests and the stimulatory effect of G+H was much more pronounced. In the present studies, therefore, sodium fluoride was omitted. From a comparison of Figs. 5 and 1 it will be seen that the stimulatory effect of G+H begins as the respiration of the whole fruit rises, but the increased rate of O_2-uptake

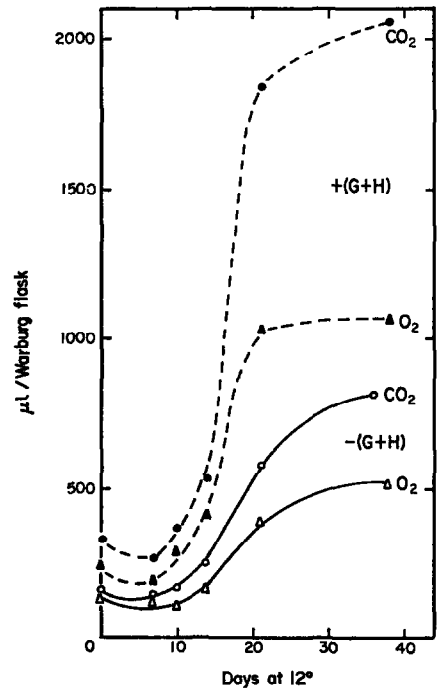

FIG. 5. THE EFFECT OF THE ADDITION OF G+H ON THE O_2-UPTAKE (△, MINUS G+H; ▲, PLUS G+H) AND CO_2-OUTPUT (○, MINUS G+H; ●, PLUS G+H) WITH MALATE SUBSTRATE OF MITOCHONDRIA PREPARED FROM THE PEEL OF APPLES AT VARIOUS STAGES OF THE CLIMACTERIC, 1965.

and CO_2-output is most pronounced near the climacteric peak (1965, stages 5 and 6). The stimulation of CO_2-output is more marked than that of O_2-uptake.

Figure 6 shows the effect of the addition of G+H on the utilization of malate and the concomitant changes in oxo-acids. The amount of free OAA is small but tends to be higher in presence of G+H up to stage 4 and this is probably related to the increased rate of oxidation in the presence of G+H.[3] However, at the later stages there is no increased accumulation of OAA in the presence of G+H but a considerable accumulation of pyruvate, and this may indicate that OAA is more rapidly decarboxylated to pyruvate by a system that develops at the later stages of the climacteric. The lack of stoichiometry between changes in OAA and pyruvate can be explained by difference in their rates of utilization.

[8] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* 3, 201 (1964).

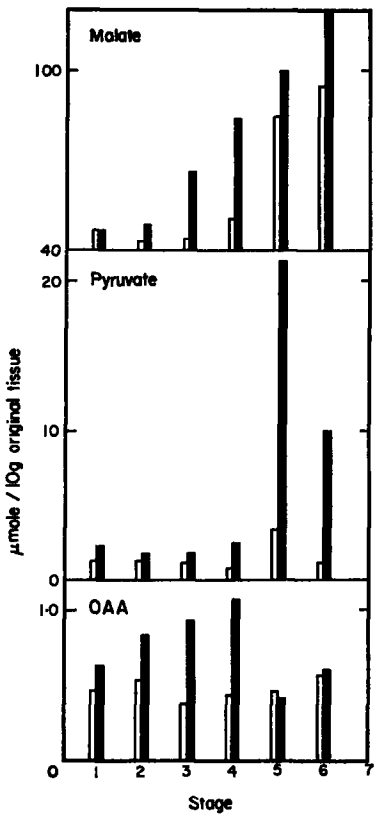

FIG. 6. UTILIZATION OF MALATE AND CHANGES IN AMOUNT OF OAA AND PYRUVATE AT THE END OF A 2-HR RUN IN THE WARBURG RESPIROMETER WITH MALATE AS SUBSTRATE FOR MITOCHONDRIA PREPARED AT EACH OF SIX STAGES OF THE CLIMACTERIC.

□ represents amounts in absence; and ■ represents amounts in presence of G+H.

Respiratory Control of Mitochondrial Activity by ADP

Using mitochondria from apples of the 1966 season, the response to the addition of ADP during the oxidation of succinate was measured in the O_2-electrode at several stages during the progress of the climacteric. At *stage* 2 of the climacteric the O_2-uptake (mμmoles/min) with 0·5 ml of mitochondria in *state* 3[9] was 53 and in *state* 4, 43. This gives a Respiratory Control (RC) ratio of 1·23. At *stage* 5 of the climacteric the corresponding figures were: *State* 3, 144; *state* 4, 92; RC ratio 1·57. Higher values for the RC value have been obtained with mitochondria from pulp tissue.

DISCUSSION

As previously shown,[1,10] as the climacteric develops in apple fruits, the increase in the activity of the mitochondria follows approximately the course of the respiration of the whole fruit. Here we have two cultural types of fruits with differing respiration rates again showing the usual trend in mitochondrial activity.

[9] B. CHANCE and G. R. WILLIAMS, *Advan. Enzymol.* **17**, 65 (1956).
[10] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *New Phytologist* **64**, 152 (1965).

The more rapid recovery from inhibition by *added* OAA as the climacteric peak is reached, suggests that an enzymic mechanism for "removing" OAA is involved during the development of the climacteric. Since this recovery is more rapid near the peak than during the rapid phase of increasing respiration rate of the whole fruit (Fig. 1 and Table 1), removal of OAA is unlikely to be due simply to a more rapid turnover through the operation of the Krebs cycle alone. The fact that G+H, which stimulates the oxidative activities of the mitochondria, has no effect on the rate of relief from OAA inhibition (see Fig. 3) also argues against the view that this relief is purely a function of the rate of turnover of the cycle. It is possible that a coupled system involving no overall increase in O_2-uptake (the R.Q. of the mitochondrial oxidation, especially with malate, increases near the climacteric peak) as mentioned by Hulme *et al.*,[3] is responsible for the increased rate of disappearance of OAA.

The rapid increase in transaminase activity in the mitochondria following stage 4 of the climacteric (1966) suggests that transamination could provide an alternative method of relieving inhibition by OAA.

Whatever the reason for the increasing rate of removal of a fixed amount of OAA as the climacteric peak is reached, the overall activity of the mitochondria is increasing. We may consider, therefore, that the faster relief from inhibition is likely to be due either to changes in the existing mitochondria or to an increase in the number of mitochondria. In the first of these possibilities, changes in the levels of specific inhibitors or activators or of enzyme cofactors are likely to be important. If the mechanism is due to the synthesis or increased availability of cofactors, the cofactors involved cannot be TPP, CoA, NAD or NADP, since these compounds were provided in excess throughout our experiments. We agree with Slater[11] that inhibition by OAA is a very complicated process but our results suggest that the development of a mechanism for metabolizing OAA is not a prerequisite for the *onset* of the climacteric but it may regulate its progress.

To consider next the increased rate of oxidation brought about by addition of G+H to mitochondria metabolizing malate. Here again the effect is much greater as the climacteric peak is reached than in the early stages of the climacteric. It has already been shown[1] that, in the presence of G+H, P:O ratios are high in the initial stages of the climacteric and decrease only slightly throughout the whole period. The mitochondria show respiratory control by ADP comparable with the results obtained for apple pulp mitochondria by Wiskich.[12] The increased rate of activity brought about by the addition of G+H near the climacteric peak again suggests that at this stage oxidation is closely coupled to phosphorylation. We have found (unpublished results) that addition of 20 μmoles of ADP does not have the same stimulatory effect as the addition of G+H. The addition of G+H could, however, ensure a *continuous* supply of phosphate acceptor (ADP). The difficulty in accepting that the function of G+H is only to ensure a continuous supply of ADP is its greater effect on CO_2-production than on O_2-uptake.

EXPERIMENTAL

Fruit Used

In 1965 the fruit was taken from twenty-nine Cox's Orange Pippin trees on Malling IX rootstock at East Malling Research Station, Kent. Petal fall (the date at which approximately 90 per cent of the flowers have shed their petals) was 19 May. In 1966 Cox's Orange Pippin apples from sixteen trees on Malling IX rootstock at Burlingham Horticultural Station, Norfolk, were used; petal fall was 21 May. The Norfolk fruit usually has a longer growing period, and in the present instance they reached the same stage of maturity (as judged by time

[11] E. C. SLATER, *Regulations of Metabolic Processes in Mitochondria* (Edited by J. M. TAGER, S. PAPA, E. QUAGLIORIELLO and E. C. SLATER), p. 539. Elsevier, Amsterdam (1966).
[12] J. T. WISKICH, *Nature* **212**, 641 (1966).

of onset of the respiration climacteric) as the Kent fruit 10 days later (date of petal fall for Kent fruit in *1966* was 14 May). Thus we have a comparison between two distinct "cultural types" of the same variety of apples. With each series, at zero time (23 Sept. 1965 and 26 sept. 1966) the requisite number of samples of twenty apples each were gathered and the respiration of all the samples measured individually at 12° by method (2) of Hulme *et al.*[7] At the times indicated in the figures, samples were removed from their respiration chambers for the preparation of the mitochondrial fraction. These times were chosen in relation to the respiration rate of the whole fruit (see Fig. 1). The respiration of the initial sample was derived from the average rate of the remaining samples on this day.

In addition, in 1966, the development of the climacteric in fruit "on the tree" was followed.[7] The respiration rate (at 12°) for this series of fruit is shown in Table 1. The initial sample for the detached fruit in 1966 is the same sample as the third sample of the "on tree" series.

Preparation of Mitochondrial Fraction

The procedure used was that described by Hulme *et al.*[2] This fraction will hereafter be called "the mitochondria". 25 g. of peel tissue were taken and the mitochondrial fraction was finally resuspended in 10 ml of 0·25 M sucrose containing 10 mg of bovine plasma albumin.

Measurement of the Activity of the Mitochondria

Uptake of O_2 and output of CO_2 in the presence of succinate or malate as substrate together with cofactors were measured at 25° using the "direct" Warburg technique as described by Hulme *et al.*[2] The "standard" digest in each Warburg flask contained the following: Sucrose, 500 μmoles (including the 125 μmoles in which the mitochondria were suspended); KH_2PO_4, 75 μmoles adjusted to pH 7·2 with KOH; $MgSO_4$, 15 μmoles; $MnSO_4$, 0·1 μmole; cytochrome-*c*, 0·018 μmole; crystallised bovine plasma albumin, 3·25 mg; ATP, 2 μmoles; NAD, 0·2 μmole; NADP, 0·1 μmole; thiamine pyrophosphate chloride (TPP), 0·2 μmole; CoA, 0·025 μmole; substrate (succinate or malate), 40 μmoles; mitochondrial suspension, 0·5 ml and water to make to 2 ml. Where glucose and hexokinase (G+H) were present, 40 μmoles of glucose and 0·44 mg of hexokinase (Sigma Type III) were added. Where OAA was present, a nominal 6 μmoles were added in the 1965 season and 10 μmoles in 1966. Owing to the instability of OAA (see Hulme *et al.*[3]) the actual amounts present at the time the solution was added were determined and were found to average 4·5 μmoles (1965) and 8·4 μmoles respectively. In 1966, all the measurements of CO_2-output in the Warburg were obtained by the acid tipping method. In 1965 the outputs were calculated from manometer readings making allowance for the solubility of CO_2 in the digest medium at pH 7·2. As pointed out by Hulme *et al.*[3] this will give only approximate results owing to the rapidly changing solubility of CO_2 with pH in the region of pH 7·2.

Measurement of Oxygen Uptake by Mitochondria using the Oxygen Electrode

O_2-uptake with succinate as substrate was measured by means of a Bishop O_2-electrode using an electrode vessel and arrangement of the electrode as described by Chappell.[13] A Vibron electrometer model 33C was used to amplify the current output from the electrode which was then fed into a Honeywell 100 mV recorder. The contents of the electrode vessel were as follows: Sucrose, 750 μmoles; KH_2PO_4, 55 μmoles; $MgSO_4$, 15 μmoles; succinate, 40 μmoles; mitochondrial suspension, 0·5 ml, and water to make 3 ml. The phosphate and succinate were adjusted to pH 7·2 with KOH. ADP (0·06–0·18 μmole) was added with a micro pipette at the appropriate times.

Determination of Transaminase Activity in the Mitochondria

This was performed according to the method used by Romani[14] for pear mitochondria. The assay mixture was as follows: Tris, 80 μmoles adjusted to pH 7·8 (Romani used a pH of 7·4, but we found that the optimum for apple mitochondria was 7·8); pyridoxal-5-phosphate, 10 μmoles; aspartate, 10 μmoles; α-oxoglutarate, 10 μmoles; $NADH_2$, 0·3 μmole; malic dehydrogenase (Boehringer) 25 μg, mitochondrial suspension, 0·025–0·1 ml, and water to a final volume of 3 ml. The α-oxoglutarate was added after 5 min incubation of the otherwise complete mixture. Changes in light absorbance were measured at 340 nm in a Cary 14 spectrophotometer to follow the oxidation of the $NADH_2$ over a period of 6 min after the addition of substrate. From this rate the number of enzyme units, expressed as μmole $NADH_2$ oxidized per min, was calculated for 10 g of the original tissue.

Quantitative Determination of Malic, Oxaloacetic and Pyruvic Acids

These were determined in the contents of each set of Warburg flasks (O_2-uptake and CO_2-output) by means of specific enzymes as described by Hulme *et al.*[3]

Acknowledgements—Mr. Peter Harkett and Miss Carol Skene assisted with the experimental work.

[13] J. B. Chappell, *Biological Structure and Function* (Edited by T. W. Goodwin and O. Lindberg), p. 71. Academic Press, New York (1961).
[14] R. J. Romani, *Plant Physiol.* 37, 523 (1962).