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THE RESPIRATION CLIMACTERIC IN APPLE FRUITS.

THE ACTION OF HYDROLYTIC ENZYMES IN PEEL TISSUE DURING THE CLIMACTERIC PERIOD IN FRUIT DETACHED FROM THE TREE

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Abstract—The respiration rate and the activity of four hydrolytic enzymes, chlorophyllase, lipase, ribonuclease, and acid phosphatase, were determined at intervals during the development of the respiration climacteric in detached apple fruits stored at 12°. The activities of lipase, ribonuclease and acid phosphatase rose to a peak at about the time the peak in respiration was reached and then decreased as the respiration fell. Acid phosphatase and ribonuclease are found in the soluble protein fraction in our preparations. This suggests that they are not bound within particles similar to the lysosomes which play an important role in the senescence of animal cells. Chlorophyllase activity rose before the commencement of the rise in respiration and continued beyond the peak. As the activity of chlorophyllase increased, the chlorophyll content of the tissue decreased. The possible relationship between chlorophyllase, lipase, the degradation of chlorophyll and the disorganization of the chloroplast lamellae which occurs during the climacteric is discussed.

INTRODUCTION

THE climacteric rise in respiration which precedes the onset of ripening and senescence of some fruits is now generally regarded as involving synthetic activities,[1] probably concerned in the development of the enzyme systems involved in ripening. At this stage in the life of the fruit there is a small increase in the 'protein' nitrogen content[2] which is associated with increased activity of enzymes involved in mitochondrial oxidations, and of soluble enzymes, i.e. malic enzyme (L-malate–NAD oxidoreductase EC.1.1.1.39) and pyruvate carboxylase (decarboxylating 2 oxo-acid carboxylase EC.4.1.1.1.) which contribute to the increased CO_2 production over the climacteric.[3]

Many of the enzymes which must be present during the ripening of fruit have a hydrolytic function. Among these are the enzymes involved in the degradation of starch and pectic substances. To obtain information concerning the possible development of hydrolytic processes during the period of the climacteric rise in respiration in apples, four hydrolytic enzymes have been studied, namely, chlorophyllase (chlorophyll chlorophyllidohydrolase EC.3.1.1.14), lipase (glycerol ester hydrolase EC.3.1.3.2), acid phosphatase (orthophosphoric monoester phosphohydrolase EC.3.1.3.2.) and ribonuclease (polyribonucleotide-2 oligonucleotide transferase (cyclizing) EC.2.7.7.16).

RESULTS

Chlorophyllase was found in fractions prepared from both the peel and the pulp tissue of Cox's Orange Pippin apples (Table 1). It is mainly located in the ribosomal fraction, but

[1] J. B. BIALE, *Food Preserv. Quart.* **22**, 57 (1962).
[2] A. C. HULME, *Rep. Food Invest. Board Lond.*, 1936, p. 129 (1937).
[3] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. B.* **158**, 514 (1963).

Table 1. The distribution of chlorophyllase activity between various cell fractions prepared from both the peel and pulp tissue of post climacteric apples

Fraction	Units of chlorophyllase activity*/10 g fresh weight	
	Peel	Pulp
Mitochondria	710	140
"Ribosomes"	820	240
Supernatant to the ribosomes	0	0

* 1 unit of chlorophyllase activity is defined as the amount of enzyme which will liberate 1 μg of chlorophyllide in 1 hr at 25° under the experimental conditions.

considerable activity is also associated with the mitochondria, especially in those from more mature apples (see Fig. 6). Chlorophyllase, both in the mitochondria and ribosomes, has a pH optimum at about 7·5 and the activity is maximal in acetone at a concentration of 40

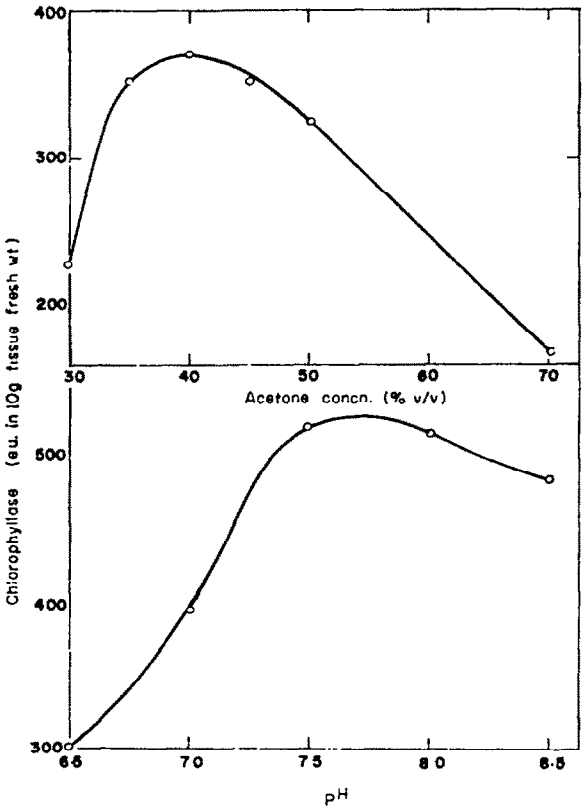

Fig. 1. Properties of chlorophyllase in apple peel.

Upper curve—The effect of acetone concentration on the activity of chlorophyllase in a mitochondrial fraction prepared from peel tissue. Lower curve—The effect of pH on the chlorophyllase activity of a similar preparation. For definition of units of activity (e.u.) in this and subsequent figures see Tables 1, 2 and 3.

per cent as shown in Fig. 1; this property is typical for all known chlorophyllases.[4] Centrifugation at 40,000 *g* for 15 min of the supernatant after the isolation of the mitochondria sedimented about 40 per cent of the chlorophyllase activity. It is probable, therefore, that the chlorophyllase is not a true 'ribosomal' enzyme.

TABLE 2. THE ACTIVITY OF LIPASE IN VARIOUS CELL FRACTIONS OF APPLE PEEL TISSUE AND ITS RESPONSE TO VARIOUS SUBSTRATES

2-naphthol substituted fatty acid Substrate	Units of lipase*/10 g fresh weight of peel tissue Enzyme fraction Mitochondria	"Ribosomes"	Supernatant to ribosomes
C_8-caprylate	—	320	2560
C_{12}-laurate	35	120	840
C_{18}-stearate	—	0	0

* The unit of lipase enzyme activity is defined as the amount of enzyme which will liberate 1 μg 2-naphthol from the substrate in 1 hr at 25°.

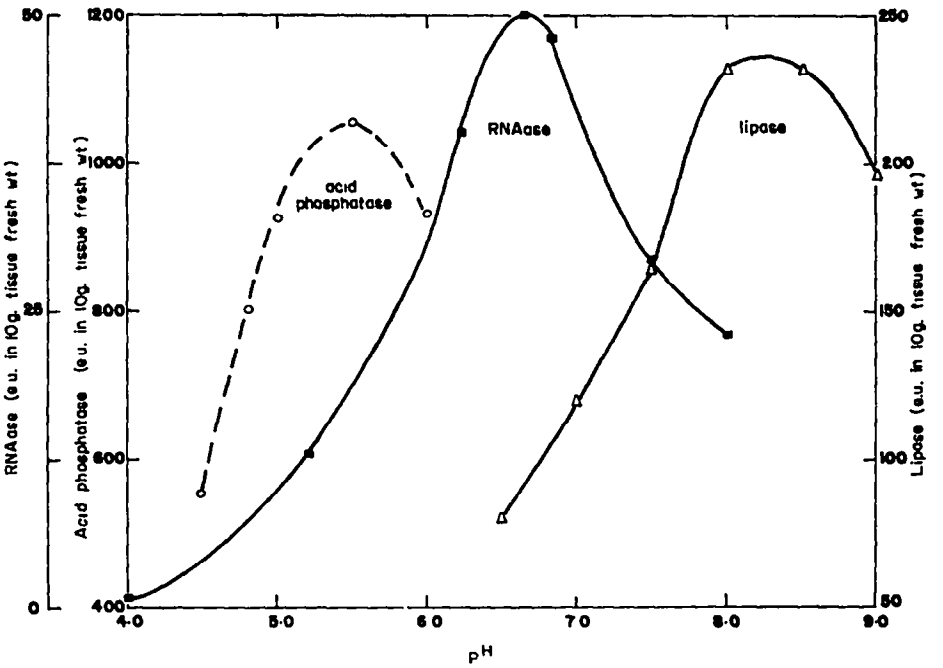

FIG. 2. VARIATION OF ACID PHOSPHATASE, RNAase AND LIPASE ACTIVITIES WITH pH.
The enzyme fraction used for acid phosphatase and RNAase was a PEG precipitate. The lipase activity was from a ribosome fraction.

Using 2-naphthyl laurate as substrate, lipase activity was found mainly in the supernatant from the ribosomes (Table 2). Smaller amounts were found in the mitochondria (<5 per

[4] M. HOLDEN, *Biochem. J.* 78, 359 (1961).

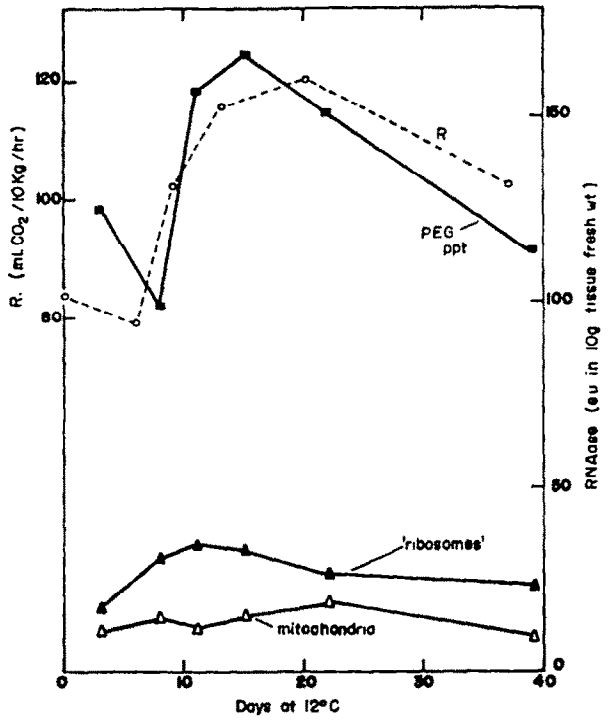

FIG. 3. COX'S ORANGE PIPPIN APPLES STORED AT 12°.
Respiration (R) of whole fruit (O---O) and activity of RNAase in three cell fractions. PEG precipitate (■ – ■), 'ribosomes' (▲ – ▲) and mitochondria (△ – △).

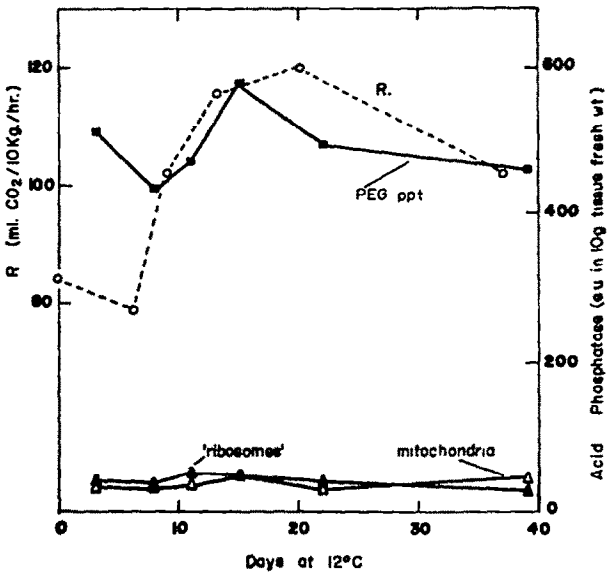

FIG. 4. COX'S ORANGE PIPPIN APPLES STORED AT 12°.
Respiration (R) of whole fruit (O---O) and activity of acid phosphatase in three cell fractions. Symbols as shown in Fig. 3. (The unit of acid phosphatase activity is defined as the amount of enzyme which will liberate 1 μmole of *p*-nitrophenol in 1 hr at 25°.)

cent and the ribosomal fraction (10–20 per cent). Figure 2 shows that the pH optimum for lipase activity appears to be close to pH 8·0, but at pH's above 7·5 the substrate is unstable and the reaction has therefore been carried out at pH 7·4. Table 2 shows the activity of lipase on 2-naphthol esters of saturated fatty acids of chain lengths between C_8 and C_{18}. The ester of the fatty acid of shortest chain length tested (C_8) was more vigorously attacked, while that with the C_{18} acid (stearate) was not hydrolysed.

TABLE 3. THE ACTIVITY OF RIBONUCLEASE WITH VARIOUS SUBSTRATES IN CELL FRACTIONS ISOLATED FROM APPLE PEEL

	Units of ribonuclease activity*/10 g fresh weight		
	Substrate		
Fraction	Polyuridylic acid	Polyadenylic acid	Yeast RNA
Mitochondria	250	6·0	8·0
PEG precipitate	1880	134	90·0
"Ribosomes"	—	—	9·0

* One unit of ribonuclease activity is defined as the amount of enzyme giving an increase in absorptivity at 260 mμ over the control of 1·00 per hr at 25°.

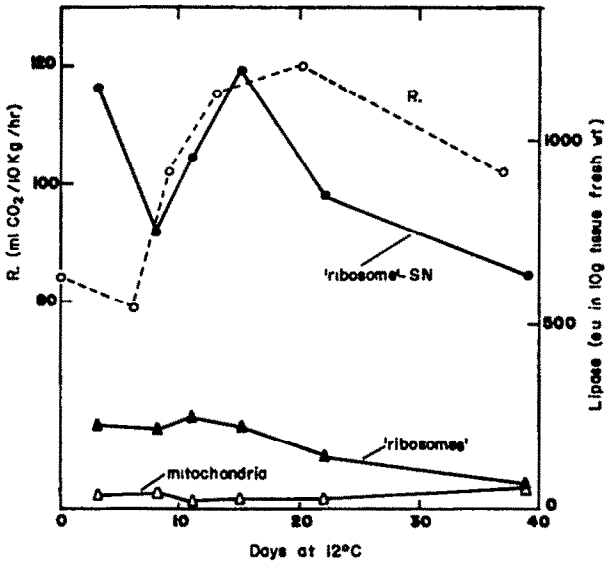

FIG. 5. COX'S ORANGE PIPPIN APPLES STORED AT 12°.
Respiration (R) of whole fruit (○---○) and activity of lipase in three cell fractions. Supernatant to ribosomes (●---●), ribosomes (▲ – ▲) and mitochondria (△ – △).

Both ribonuclease (RNAase) and acid phosphatase are mainly located in the PEG precipitate, but some small activity is found in the mitochondrial and ribosomal fractions (see Figs. 3 and 4). These enzymes, under the conditions of cell dispersion used in the present experiments are, therefore, mainly soluble enzymes. The effect of pH on these enzymes is shown in Fig. 2; for acid phosphatase the pH optimum is 5·5, and for RNAase it is 6·6.

Alkaline phosphatase appears to be absent from apple peel tissue, The specificity of RNAase (see Table 3) was tested with polyuridylic and polyadenylic acids as substrates. The results suggest that linkages involving uridylic acid residues are preferentially attacked.

The changes in ribonuclease, acid phosphatase, lipase and chlorophyllase in peel tissue over the period of the development of the respiration climacteric are shown in Figs. 3, 4, 5

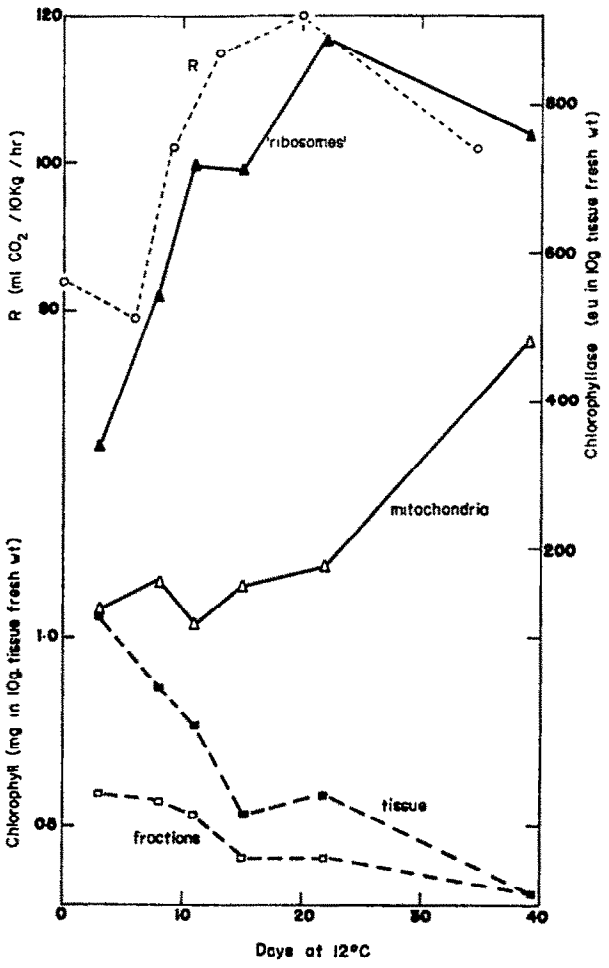

FIG. 6. COX'S ORANGE PIPPIN APPLES STORED AT 12°.

Respiration of whole fruit (O---O), chlorophyllase activity in two cell fractions ('ribosomes' ▲–▲ and mitochondria Δ – Δ), and the chlorophyll content of the tissue (■---■) and of the combined mitochondrial and ribosomal fractions (□---□).

and 6. The changes in RNAase, acid phosphatase and lipase follow the general trend of CO_2 production, activity increasing between 30 and 80 per cent over the period of the climacteric (see Figs. 3–5).

A considerably greater rise in chlorophyllase activity occurred over the climacteric (see Fig. 6), a 300 per cent increase in activity in the combined mitochondrial and ribosomal fractions. This increase in activity commences before the climacteric rise begins and continues

TABLE 4. A TYPICAL ANALYSIS OF A SUPERNATANT TO THE RIBOSOMES FOR LIPASE ACTIVITY

Buffer (ml)	Substrate (ml)	Enzyme (Ribosome Supernatant) (ml)	2-naphthol (100 μg/ml) (ml)	Water (ml)	Extraction Technique	Absorptivity at 540 mμ	Corrected Absorptivity at 540 mμ	μg 2-naphthol	Corrected for recovery 90%	Enzyme Units
1	0·5	—	—	5·5	None	0·020	0·550			
1	0·5	—	0·2	5·3	None	0·570	90% recovery			
1	—	0·2	0·2	5·6	Ether	0·510				
						0·519	0·495			
1	0·5	0·2 ml added after incubation	—	5·3	Ether	0·020				
1	0·5	0·2 ml added at Zero time	—	5·3	Ether	0·135	0·113	3·7	4·11	1880
						0·130				

In each case the mixture described was incubated for 1 hr at 25° prior to estimation of the free 2-naphthol (see text).

beyond the peak (see Fig. 6). Figure 6 shows that, while the activity of chlorophyllase was increasing, the chlorophyll content of the apple peel and of the fractions prepared from it was decreasing. In fruit past the climacteric peak there appears to be some redistribution of activity between the mitochondrial and ribosomal fractions.

DISCUSSION

The breakdown of chlorophyll is a process which accompanies the senescence of many plant organs. In most ripening fruits chlorophyll breakdown occurs at the time of the climacteric and continues for some time after the peak has been reached.[5] The chlorophyll present in plant cells is an essential component of the lamellae of the chloroplasts.[6] Evidence from electron microscopical studies suggests that the breakdown of chlorophyll in pears is associated with the disruption of the lamellae themselves.[7] In leaves these lamellae contain relatively high concentrations of phospholipids and esterified unsaturated fatty acids of which linoleic, oleic and linolenic acids form a major part.[8] The enzymes involved in the breakdown of the components of the chloroplast lamellae may thus have a significance within the pattern of biochemical changes occurring during the period of the climacteric in apples.

Although the chlorophyllase activity in our apple preparations appears in the mitochondrial and ribosomal fractions, it is highly probable that it is of chloroplast origin.[9] Our method of tissue maceration is a mild one, but the fact that chlorophyll and chlorophyllase appear in these two fractions shows that many of the chloroplasts are fractured. There is very little chlorophyll in the tissue residue (1000 *g* spin) and none in the supernatant from the ribosomal fraction. Therefore, the chlorophyll remains attached to chloroplast debris. Since the chloroplasts in apple peel are small (2–3μ) in comparison with those in leaves, it is not surprising that some large chloroplast fragments appear in our mitochondrial fraction. Electron micrographs confirm that this is so. If, as the climacteric proceeds, the chloroplast lamellae simply become disorganized, then it would be expected that the chlorophyll and the chlorophyllase would become progressively more concentrated in the 'high speed' (ribosomal) fraction. In fact, after the climacteric peak, they decrease in this fraction and increase in the mitochondrial fraction (see Fig. 6). It appears, however, that at some point in senescence, individual groups of lamellae break away to form irregular small fragments (Fig. 7) which might well only centrifuge down at high speeds and so appear in the ribosomal fraction. During later stages, the chloroplasts might liberate larger pieces of the internal structure which would then centrifuge down at lower speeds and appear in the mitochondrial fraction. Whatever is responsible for this unexpected distribution, it is clear from Fig. 6 that the increase in chlorophyllase activity begins before the climacteric rise and continues after the climacteric peak has been reached.

The metabolic pathways and the enzymes involved in the degradation of chlorophyll are as yet unknown. Chlorophyllase, discovered by Willstatter and Stoll[10] in 1910 is capable, *in vitro*, of hydrolysing chlorophylls *a* and *b* to yield phytol and the respective chlorophyllides. In apple peel the chlorophyllase activity showed a three-fold increase over the period of the

[5] J. B. BIALE and R. E. YOUNG, *Endeavour* **21**, 164 (1962).
[6] S. GRANICK, *The Cell*, Vol. II, p. 489 (Edited by J. BRACHET and A. E. MIRSKY), Academic Press, New York (1961).
[7] J. M. BAIN and F. V. MERCER, *Aust. J. Biol. Sci.* **17**, 78 (1964).
[8] H. K. LICHTENTHALER and R. B. PARK. *Nature* **198**, 1070 (1963).
[9] G. KROSSING, *Biochem. Z.* **305**, 359 (1940).
[10] R. WILLSTATTER and A. STOLL, *Liebigs Ann.* **378**, 18 (1910).

climacteric rise, and this occurred while the chlorophyll content was decreasing. It would seem, therefore, that in apple peel, *in vivo*, the enzyme is involved in the early stages of degradation of chlorophyll. However, in other tissues it is suggested that the enzyme has a synthetic role only.[4] Holden[11] has studied a system in which lipoxidase activity (see below) on unsaturated fatty acids was linked with the destruction of chlorophyll. This degradation did not apparently involve hydrolysis (i.e. chlorophyllase activity) but the products of the reaction were not identified. In this system unsaturated fatty acids, themselves, were capable of *slowly* degrading chlorophyll.

Lipase activity, which is directly involved in the degradation of lipids, appear to fall just before the onset of the climacteric, but thereafter follows the same trend as the respiration. The high lipase activity probably explains the increase in the level of free fatty acids (saturated and unsaturated) observed over the climacteric in apple peel.[12] Lipoxidase activity increases markedly over the climacteric[13] and it has been suggested that this activity which commences to rise before the major rise in respiration occurs, might be involved in the production of ethylene which is known to induce the climacteric rise.

De Duve *et al.*[14] have isolated cell particles, the lysosomes, which play an important role in the senescence of animal cells. These particles contain hydrolytic enzymes (i.e. acid phosphatase, RNAase, DNAase) bound within a lipoprotein membrane. Senescence and death of the cell is accompanied by the release of these enzymes from the lysosomes. Recently evidence has been produced suggesting that such particles exist within plant cells.[15, 16] In apple peel RNAase and acid phosphatase are not sedimented by centrifugation at 100,000 *g* for 60 min and do not appear to be bound to particles similar to the lysosomes of animal cells. However, the possibility of disruption of such particles during our preparative technique cannot be ruled out. In the supernatant fraction, both of these enzymes show a rise in activity over the respiration climacteric, the rise being more marked with RNAase. The distribution of RNAase activity between the cell fractions of apple peel appears to be typical of plant tissues in general.[17, 18]

It has been shown that a small increase in protein, measured as 80% ethanol insoluble nitrogen, occurs during the climacteric in apples.[2] It would seem likely that RNA metabolism, which has been shown to play an important role in the regulation of protein synthesis, might also be involved in the synthetic processes occurring during the climacteric rise in respiration. In some plant systems an increase in RNAase activity is associated with *synthesis* of RNA,[19] and it is possible, therefore, that some synthesis of specific types of RNA (i.e. messenger or transfer RNA) might occur over the climacteric.

EXPERIMENTAL

Apple Fruit used

Fruit was taken from 29 Cox's Orange Pippin apple trees on Malling IX rootstocks. Petal fall was 19 May 1965.[3]

[11] M. Holden, *J. Sci. Fd. Agr.* **16**, 312 (1965).
[12] D. F. Meigh and A. C. Hulme, *Phytochem.* **4**, 863 (1965).
[13] L. S. C. Wooltorton, J. D. Jones and A. C. Hulme, *Nature* **207**, 999 (1965).
[14] C. De Duve, B. C. Pressman, R. J. Gianetto, R. Wattiaux and F. Appleman, *Biochem. J.* **60**, 604 (1955).
[15] P. B. Gahan, *J. Exptl. Bot.* **16**, 350 (1965).
[16] G. B. Bouck and J. Cronshaw, *J. Cell Biol.* **25**, 79 (1965).
[17] B. Kessler and N. Engleberg, *Biochim. Biophys. Acta* **55**, 70 (1962).
[18] R. F. Lyndon, *Biochim. Biophys. Acta* **113**, 110 (1966).
[19] L. Ledoux, P. Galand and R. Huart, *Biochim. Biophys. Acta* **55**, 97 (1962).

Respiration of Whole Fruit

A sample of 120 apples was picked 125 days after petal fall. The respiration of batches of 20 apples was measured at 12° by the bulk respiration method described previously by Hulme *et al.*[3]

Preparation of Cell Fractions

At appropriate stages of the respiration climacteric, 7–8 fruits were taken for the preparation of the various cell fractions. The mitochondrial fraction (hereafter referred to as the mitochondria) and the polyethylene glycol (PEG) precipitate of the supernatant enzyme fraction were prepared as described by Hulme *et al.*[20, 21]

The supernatant liquid after the separation of the mitochondria was divided into two equal halves, one was precipitated with PEG at a final concentration of 40% (w/v) to yield a soluble-protein fraction which was separated by centrifugation in a Spinco Model L2 ultracentrifuge at 13,000 *g* for 15 min. The remaining half of the supernatant was centrifuged for 60 min at 100,000 *g* to yield a "ribosome" fraction ("ribosomes") and a "ribosome" supernatant fraction.

The final sedimented fractions were resuspended in 0·25 M sucrose in a Kontes Duall homogenizer for use in the various enzyme assays. The final volumes after resuspension were as follows: mitochondria, 10 ml; ribosomes, 10 ml; and PEG precipitate, 20 ml.

Enzyme Assays

The activity of the four enzymes under study was measured on duplicate samples by the methods described below. Under the conditions used it was shown in each case that the activity measured was directly proportional to the enzyme concentration. Absorptivity was measured on a Cary 14 recording spectrophotometer.

Acid phosphatase. The method described by Bergmeyer[22] based on measurement of the *p*-nitrophenol liberated during phosphatase action on *p*-nitrophenylphosphate was used. The assay system contained: 1 ml buffer-substrate mixture (0·05 M citrate pH 5·5, containing $5{\cdot}5 \times 10^{-3}$ M *p*-nitrophenyl phosphate, sodium salt), 0·02–0·05 ml of the enzyme fraction and distilled water to a final volume of 1·2 ml. After 15 min incubation at 25°, 10 ml 0·02 N sodium hydroxide was added. The reaction mixture was centrifuged and the clear supernatant measured at 405 nm against a suitable blank.

Chlorophyllase. The activity was measured by the liberation of chlorophyllide from a crude chlorophyll preparation[23] free of chlorophyllide prepared from cabbage leaves. The chlorophyll preparation was dissolved in acetone to give a final concentration of 1·37 mg/ml (measured spectrophotometrically by the method of Bruinsma[24]) and stored at −20° until required for use.

The assay mixture contained: chlorophyll substrate (1·22 mg), triethanolamine buffer pH 7·5 (0·05 M final concentration), enzyme fraction, acetone (to a final concentration of 40 per cent) and water to give a volume of 3 ml. After 1 hr incubation at 25°, 2 ml of the incubated mixture was pipetted into 3 ml acetone and centrifuged; 3 ml of the supernatant was shaken

[20] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* 3, 173 (1964).
[21] J. D. JONES, A. C. HULME and L. S. C. WOOLTORTON, *Phytochem.* 4, 659 (1965).
[22] H. U. BERGMEYER, (Ed.) *Methods of Enzymatic Analysis*, Academic Press, New York (1963).
[23] P. BÖGER, *Phytochem.* 4, 435 (1965).
[24] J. BRUINSMA, *Photochem. Photobiol.* 2, 241 (1963).

with 5 ml light petroleum (b.p. 40–60°) and 2 ml 2% sodium chloride. The mixture was centrifuged and the upper light petroleum layer containing the chlorophyll discarded. The aqueous acetone layer was re-extracted with 5 ml light petroleum, centrifuged, and the petroleum layer again discarded. The absorptivity of the aqueous acetone phase was measured at 663 nm against a suitable control.

Lipase. Lipase activity in the various fractions was analysed by colorimetric estimation of the 2-naphthol liberated from 2-naphthyl laurate substrate.[22] The reaction system contained: 2-naphthyl laurate suspension (0·5 mg), veronal–HCl buffer pH 7·4 (final concentration 0·006 M), enzyme and water to a final volume of 7 ml. The mixtures were incubated for 1 hr at 25° and the 2-naphthol liberated was estimated by addition of 1 ml tetrazotized *o*-dianisidine (4 mg/ml) followed, after 2 min, by 1 ml of 40% (w/v) trichloracetic acid. The azo dye formed was extracted into 10 ml of ethyl acetate and estimated spectrophotometrically at 540 nm using a suitable blank.

This method is satisfactory for both the mitochondrial and ribosomal fractions, but with the supernatant of the ribosomes, the phenolics which are present in relatively high concentrations interfere with the estimation of the liberated 2-naphthol. This difficulty was overcome by extracting the liberated 2-naphthol from the incubated mixture with ether. Two extractions with 5 ml and 2 ml ether were used and in each case the ether layer was drawn off after centrifugation to clarify the phases. The combined ether extracts were mixed with 1 ml veronal buffer and 6 ml distilled water, and the ether was evaporated in a stream of nitrogen at room temperature. The estimation of 2-naphthol was then carried out as previously described. In practice it was found necessary to check the recovery of known amounts of added 2-naphthol in each enzyme estimation and to apply a correction to the activity observed. In general the recoveries of added 2-naphthol using the ether extraction technique were found to be reproducible for a given level of enzyme; for 0·2 ml of supernatant (enzyme) it was between 85–90 per cent. A typical analysis is shown in Table 4.

Ribonuclease. Ribonuclease activity was estimated by measuring the increase in absorptivity at 260 nm due to the release of acid soluble nucleotide fragments from yeast RNA as substrate. The incubation was carried out at 25° with the following mixture: 0·6 ml RNA (4 mg yeast RNA/ml in 0·2 M phosphate buffer pH 6·6), 0·6 ml 0·2 M phosphate buffer pH 6·6 and 0·3 ml enzyme fraction. The reaction was terminated after 1 hr by addition of 0·3 ml 25% perchloric acid containing 0·5% uranyl acetate. The mixture was centrifuged, the supernatant was suitably diluted and its absorptivity at 260 nm was measured and compared with a suitable control.

Preparation of Apple Peel Tissue for Electron Microscopical Study

Small pieces of apple peel (0·5 × 0·5 mm) were fixed for 3 hr at 0° in 6·25% glutaraldehyde in 0·05 M phosphate buffer pH 7·0. The tissue was washed free of glutaraldehyde with cold phosphate buffer pH 7·0 and then post fixed for 1 hr at 0° in 2% potassium permanganate in 0·1 M phosphate buffer pH 7·0. The fixed tissue was washed free of permanganate, dehydrated in a series of alcohols of increasing concentration and finally embedded in Araldite. The tissue was sectioned using either glass or diamond knives on a Huxley ultramicrotome. The sections were mounted on formvar coated copper grids. The sections were then "stained" with lead citrate by the method of Reynolds[25] and then examined in a Philips EM 75 electron microscope operating at an accelerating voltage of 75 kV.

[25] E. S. Reynolds, *J. Cell Biol.* 17, 208 (1963).

Chemicals

The sources of most of the chemicals used were as previously described.[3] In addition, *p*-nitrophenyl phosphate sodium salt, was obtained from Boehringer u. Soehne, 2-naphthol substituted fatty acids from Sigma Chemical Co., tetrazotized *o*-dianisidine from G. T. Gurr Ltd., and Yeast RNA from Pabst Laboratories. The glutaraldehyde used for fixation of peel tissue was a gift from the Union Carbide Corporation.

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