Phytochemistry, 1969, Vol. 8, pp. 945 to 955. Pergamon Press. Printed in England.

# METHODS FOR THE STUDY OF RIPENING AND PROTEIN SYNTHESIS IN INTACT POME FRUITS*

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(*Received* 29 *October* 1968)

**Abstract**—Techniques were developed to utilize intact apple and pear fruits to simultaneously study the dynamic cellular processes attending ripening such as cell-wall softening, chlorophyll degradation, respiration, and protein, RNA and ethylene synthesis. Solution infiltration procedures employing mannitol as a carrier solution for administering protein and nucleic acid precursors and metabolic inhibitors provided a means of investigating various aspects of ripening in an integrated manner. Specific details for employing fresh fruit extracts and acetone-dried powders of apple and pear fruits (as a source of proteins) for electrophoretic studies in relation to fruit ripening are discussed.

## INTRODUCTION

RIPENING in fruits such as avocado, apple, banana, and pear is characterized by an accelerated metabolism and very distinct physical and chemical changes. The transition from growth processes to those of ripening is considered to be a significant developmental process signaling the onset of senescence in these fruits.[1] Recent studies on protein synthesis[2] and specific enzymes[3] have led to the hypothesis that enzymes synthesized early in the ripening period are the catalysts in the ripening processes. Direct evidence to substantiate this hypothesis was recently provided[4] wherein it was found that $^{14}$C-phenylalanine was differentially incorporated into fruit proteins separated by acrylamide gel electrophoresis of pome fruits taken at successive ripening stages. Techniques developed to utilize intact fruits were instrumental in that investigation and are the subject of the present communication.

## RESULTS

### *Infiltration of Fruits with Carrier Solutions*

The volume of treating solution which could be infiltrated into apple and pear fruits followed a pressure–time relationship (Fig. 1). Apples of all varieties were much more readily infiltrated than pears, probably due to their greater volume in intercellular air space and corresponding low tissue density. The distribution of solution was quite uniform throughout the tissue with as little as 10 ml per fruit (100–150 g) as judged by distribution of dyes. Pear fruits became increasingly more difficult to infiltrate as they ripened while apple fruits did not.

* Michigan Agriculture Experiment Station Journal Article No. 4541.

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[1] J. BIALE, *Science* **146**, 880 (1964).
[2] A. R. RICHMOND and J. BIALE, *Plant Physiol.* **41**, 1247 (1966).
[3] M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1 (1967).
[4] C. FRENKEL, I. KLEIN and D. R. DILLEY, *Plant Physiol.* **42**, 1146 (1968).

The choice of infiltration method (see Experimental) depends upon the number of fruits to be treated and the nature of the treating solution. The individual fruit method is preferable

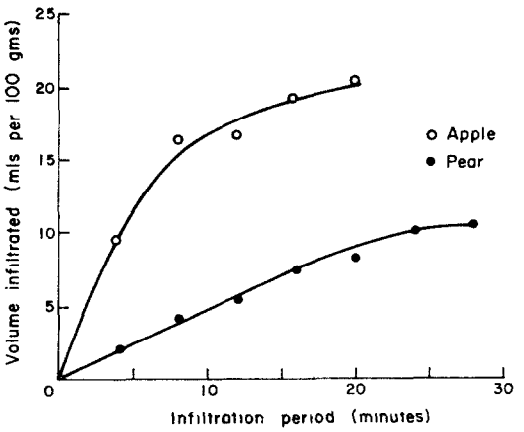


FIG. 1. THE RATE OF UPTAKE OF AN AMBIENT SOLUTION BY SUBMERGED McINTOSH APPLES AND BARTLETT PEARS AT ATMOSPHERIC PRESSURE AFTER EVACUATION OF THE INTERCELLULAR ATMOSPHERE TO 100 mmHg.

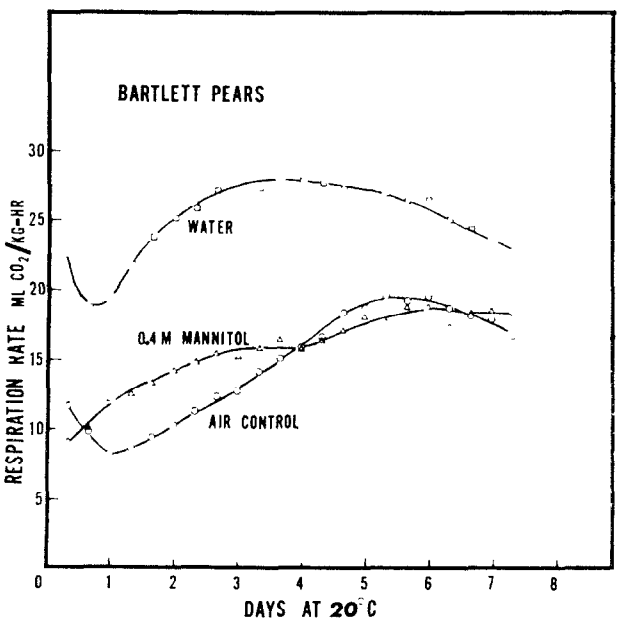


FIG. 2. RESPIRATION OF PRE-CLIMACTERIC PEAR FRUITS IN RESPONSE TO INFILTRATION WITH WATER OR 0·4 M MANNITOL. THE QUANTITY OF SOLUTION INFILTRATED WAS APPROXIMATELY 10% OF THE FRUIT WEIGHT. WATER INFILTRATED FRUITS DID NOT RIPEN.

where costly or hazardous materials are used and the treating solutions can be applied quantitatively.

The osmotic concentration of the infiltration solution is an important consideration in terms of fruit ripening behavior. Fruits infiltrated with water do not ripen whereas those

infiltrated with 0·2–0·4 M sugar or sugar alcohols ripen but more slowly than non-infiltrated fruits. The influence of water vs. 0·4 M mannitol on $CO_2$ production rate of pears during ripening is shown in Fig. 2. Oxygen uptake was slightly less than $CO_2$ production. The high respiration rate of water-infiltrated fruits may be indicative of uncoupling of oxidative phosphorylation since these fruits did not ripen. The high gas exchange rate of water-infiltrated fruits is also evidence that this degree of intercellular airspace reduction does not greatly impair gas diffusion and thus limit respiration rate. This was questioned because mannitol-infiltrated fruits were sometimes observed to have slightly lower respiration rates than control fruits.

Flesh softening, chlorophyll degradation and ethylene synthesis were not adversely affected by mannitol infiltration as judged by the data in Figs. 3 and 4. Ethylene synthesis was

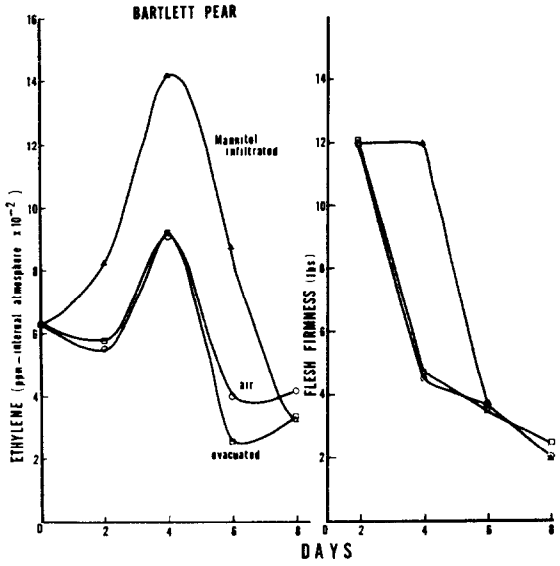


FIG. 3. INFLUENCE OF MANNITOL INFILTRATION ON ETHYLENE SYNTHESIS AND FLESH SOFTENING OF PRE-CLIMACTERIC PEARS AT 20°. FRUITS EVACUATED TO 100 mmHg AND HELD FOR 1 hr BUT NOT INFILTRATED RESPONDED AS AIR CONTROL FRUITS.

enhanced by mannitol infiltration but paralleled that of control fruits or those which were only evacuated. Flesh softening was delayed by infiltration but paralleled that of control fruits once softening began. This is an important consideration from the standpoint of ripening. Ethylene synthesis, chlorophyll degradation and flesh softening were all inhibited by water infiltration indicating that a favorable osmotic balance is required for ripening to occur. Burg *et al.*[5] reported that ethylene synthesis by apple tissue slices was inhibited by soaking the tissue in water.

The foregoing data validate the use of the solution infiltration technique in ripening studies and provide a technique to introduce various substances into fruits which may enhance or retard ripening. This technique was employed to elucidate the nature of and requirement for protein synthesis during ripening of pome fruits.[4] The incorporation of $^{14}$C-phenylalanine into proteins of ripening pears in the presence and absence of cycloheximide is shown

[5] S. P. BURG, E. A. BURG and R. MARKS, *Plant Physiol.* **39**, 185 (1964).

in Fig. 5. Mannitol (0·35 M) was used as the carrier solution for the amino acid with or without cycloheximide. Protein synthesis and ripening were inhibited by cycloheximide.

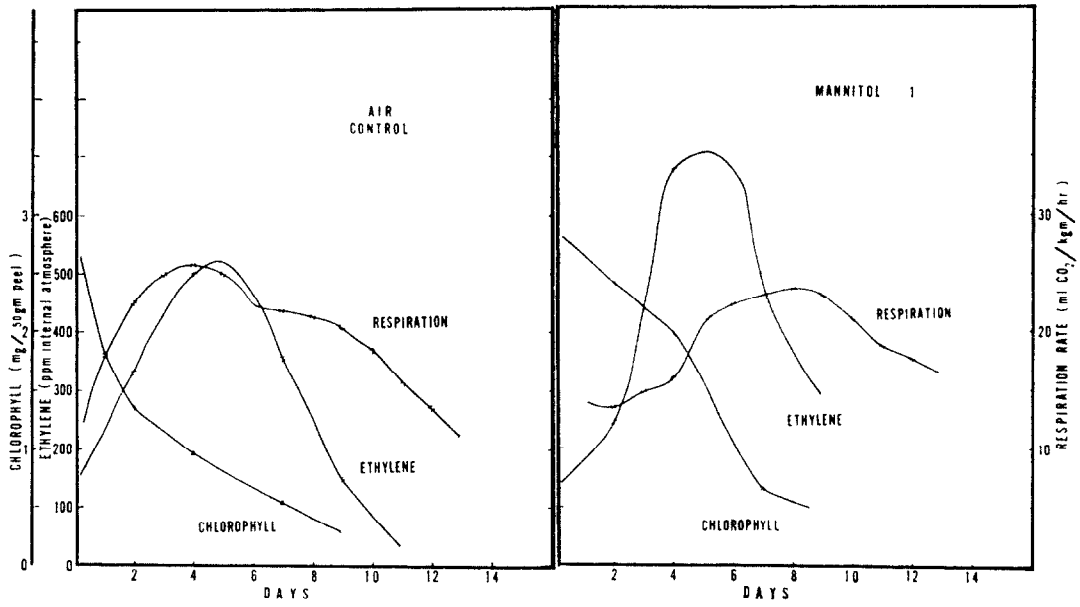


FIG. 4. INFLUENCE OF MANNITOL INFILTRATION (RIGHT) ON RESPIRATION, ETHYLENE SYNTHESIS, AND CHLOROPHYLL DEGRADATION OF PRE-CLIMACTERIC BARTLETT PEARS AT 20°.

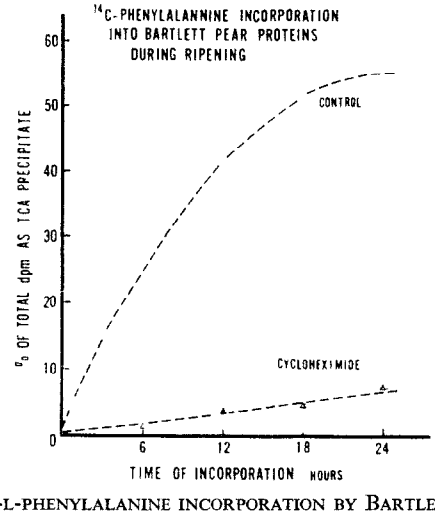


FIG. 5. THE RATE OF $^{14}$C-L-PHENYLALANINE INCORPORATION BY BARTLETT PEARS IN THE PRESENCE AND THE ABSENCE OF CYCLOHEXIMIDE. PEARS AT AN EARLY RIPENING STAGE WERE INFILTRATED WITH A 0·35 M MANNITOL SOLUTION CONTAINING $1 \times 10^{-4}$ M $^{12}$C-L-PHENYLALANINE, AND 0·1 $\mu$C-L-PHENYLALANINE PER ml. CYCLOHEXIMIDE WAS AT $1 \times 10^{-4}$ M.

*Extraction of Proteins*

Proteins were extracted from fresh fruit or from acetone-dried powders prepared according to Clements.[6]

[6] R. L. CLEMENTS, *Anal. Biochem.* **13**, 390 (1965).

*Enzyme and Protein Extraction from Fresh Fruits*

Enzyme extracts, particularly of ripe fruit, tended to gel after extraction. Tendency of gelling was greater in extracts of fresh fruits, but was noted in extracts of acetone powders as well. Extracts which exhibited gelling properties correspondingly gave poor resolution in gel electrophoresis showing marked denaturation and artifacts as judged by determination of malic enzyme position on the gel. Treatment of protein extracts with 0·05 M $CaCl_2$ at pH 7·5 brought about instantaneous gelling of the extract. Centrifugation removed the gel and allowed satisfactory gel electrophoresis. Extracts of Bartlett pear showed increasingly larger volumes of gel recovered by centrifugation during the climacteric rise and ripening and then gradually decreased, implication involvement of soluble pectins. Low concentration of $CaCl_2$ (0·01 M) precipitated the gelling material as effectively as higher concentrations (1·0 M). A satisfactory procedure for enzyme extraction of fresh fruit included vacuum infiltration of

TABLE 1. THE EFFECT OF $CaCl_2$ ON PROTEIN AND MALIC ENZYME RECOVERY DURING AND AFTER EXTRACTION FROM BARTLETT PEARS

| | mg Protein and units of malic enzyme (M.E.) per 100 g fresh wt.* | | | | | |
|---|---|---|---|---|---|---|
| | After vacuum infiltration | | After extract treated with 0·02 M $CaCl_2$ | | | |
| $CaCl_2$ (M) | Protein | M.E. | Protein | % Recovered | M.E. | % Recovered |
| 0 | 70 | 8,740 | 31 | 44 | 5,400 | 62 |
| 0·02 | 37 | 11,960 | 38 | 102 | 10,580 | 88 |
| 0·05 | 35 | 13,455 | 41 | 118 | 13,168 | 98 |
| 0·10 | 29 | 11,500 | 33 | 114 | 11,385 | 99 |
| 0·20 | 30 | 11,270 | 30 | 100 | 10,120 | 90 |

* 0·2 M Tris buffer at pH 10 containing $5 \times 10^{-3}$ DIECA was used as the infiltration and extracting solution. Fruits ripened 4 days were infiltrated with the indicated concentrations of $CaCl_2$ and homogenized. All extracts then received an additional treatment with $CaCl_2$.

the tissue with 0·1–0·2 M Tris (to neutralize high cell sap acidity) at pH 10, containing 0·02–0·05 M $CaCl_2$ (Table 1). Better recoveries of malic enzyme were noted with optimal concentration of $CaCl_2$. Protein N recovered, however, decreased with increasing concentration of $CaCl_2$. When protein extracts were treated with additional $CaCl_2$ after fruits were infiltrated with different concentration of $CaCl_2$ (Table 1), it became clear that the additional $CaCl_2$ precipitated 10–60 per cent of the total protein and malic enzyme unless fruits had been infiltrated with $CaCl_2$. Clearly, a protein like malic enzyme which is localized in the cytoplasm can be precipitated with insoluble cell-wall material during extraction, or bound to pectic substances following extraction. Vacuum infiltration of fruit with $Ca^{2+}$ solutions apparently causes precipitation of soluble pectins and thereby prevents binding of soluble proteins released during homogenation. This may account for the marked increase in malic enzyme recovery resulting from $CaCl_2$ infiltration with respect to total N, and extractable N (with buffers). Soluble and insoluble N, 80% ethanol or 10% TCA of total and extractable N was determined (Table 2). Four to six buffer extractions resulted in recovery of readily extractable protein (Fig. 6) which accounted for 33–43 per cent of the total N. The N remaining in the

residue after exhaustive extraction with buffers was not readily solubilized in 1 N NaOH or 1 N HCl indicating that it is tightly bound to the cell-wall residue. The acetone powders

TABLE 2. DISTRIBUTION OF N IN BUFFER EXTRACTABLE AND NON-EXTRACTABLE FRACTIONS OF ACETONE POWDERS OF POME FRUITS

| | Nitrogen fractions ($\mu$g N/500 mg acetone powder)* | | | | | |
|---|---|---|---|---|---|---|
| | Extractable | | | | Non-extractable N | |
| Fruit | Ethanol soluble | % of total | Ethanol insoluble | % of total | Nitrogen in powder | % of total |
| Jonathan apples | 276 | 10 | 1120 | 43 | 1224 | 47 |
| McIntosh apples | 266 | 13 | 862 | 42 | 934 | 45 |
| Red Delicious apples | 206 | 15 | 487 | 35 | 717 | 51 |
| Bartlett pears | 1152 | 19 | 1760 | 29 | 3132 | 52 |

* 0·1 M phosphate buffer solution at pH 9·5 containing 0·3 M mannitol was used in eight successive 5-ml extractions of 500 mg of each of the powders. Protein in each of the extracts was precipitated with 80% ethanol. Nitrogen was determined on the various fractions by micro-Kjeldhal.

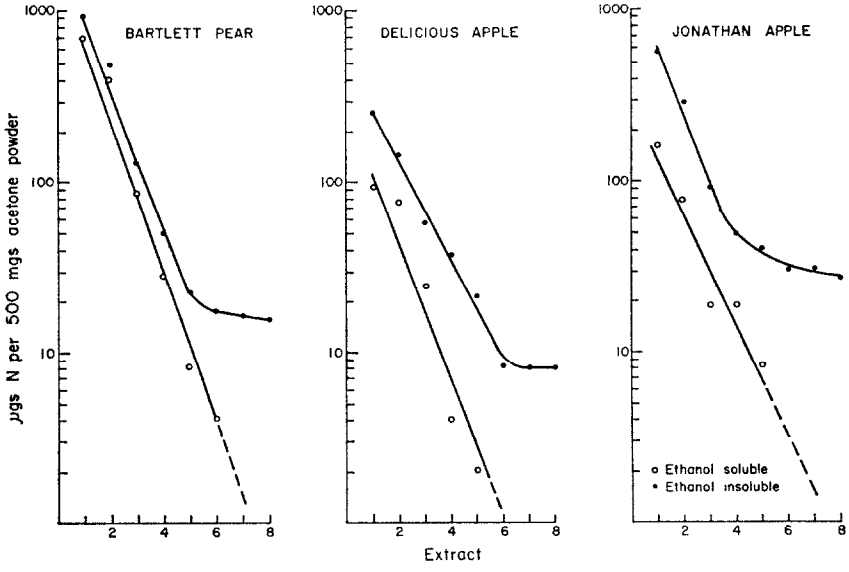


FIG. 6. THE RECOVERY PATTERN OF ETHANOL-SOLUBLE AND PRECIPITABLE NITROGEN FRACTIONS IN ACETONE POWDER FROM APPLES AND PEARS. THE POWDERS WERE EXTRACTED WITH A PHOSPHATE BUFFER CONTAINING 0 35 M MANNITOL. NITROGEN WAS DETERMINED IN THE VARIOUS FRACTIONS BY MICRO-KJELDHAL.

contained 10–20% ethanol-soluble material which may be small peptides and other low molecular weight N compounds. In addition, nucleic acid N is a component of the 80% ethanol-insoluble N of acetone powders.

Several factors were found to influence the recovery of proteins from acetone powders. Buffer, pH, ionic strength, and sugar alcohols were studied systematically in order to achieve an optimum extracting solution in relation to protein recovery, enzyme activity and electrophoresis.

1. *Choice of buffer.* Ionic strength and pH were considered in selecting a buffer for protein extraction. Acetone-dried powders exhibited acidic behavior possibly due to free

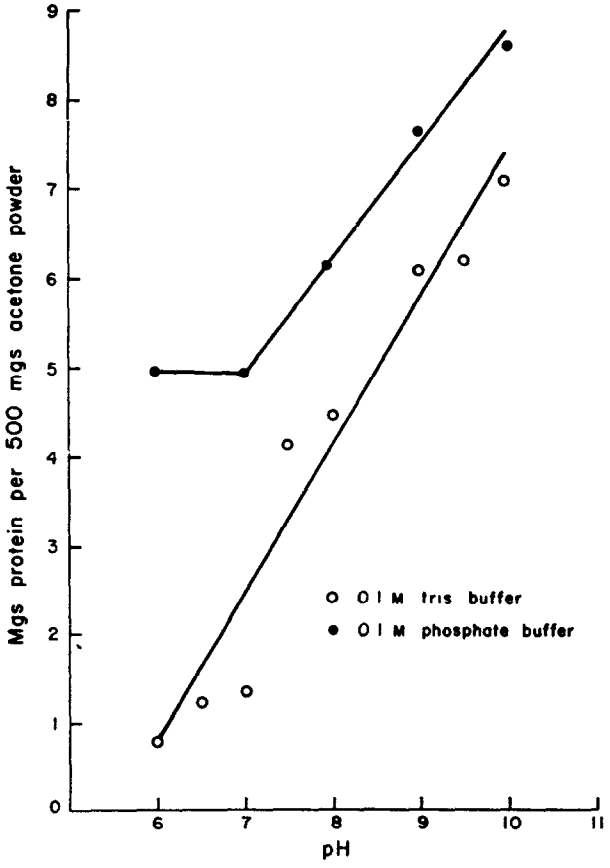


FIG. 7. THE EFFECT OF HYDROGEN ION CONCENTRATION ON PROTEIN RECOVERY FROM ACETONE POWDER IN DIFFERENT BUFFER SYSTEMS. DRY ACETONE POWDER OF DELICIOUS APPLES WAS PASSED THROUGH THREE SUCCESSIVE EXTRACTIONS WITH A BUFFERED EXTRACTION SOLUTION WHICH CONTAINED $5 \times 10^{-4}$ M EDTA AND 0·35 M MANNITOL. PROTEIN WAS DETERMINED BY A BIURET METHOD ON A TCA PRECIPITATE OF THE COMBINED EXTRACTS.

carboxyl groups of the pectic substances. Acetone powders prepared from fruits at different stages of development contain variable amounts of soluble pectins. Phosphate buffer (0·1 M) tended to decrease, while Tris or Tris–Tricine buffer increased the solubilization of the gelling material which interferred with electrophoresis. Tris–phosphate buffer (0·1 M) containing 0·1 M KCl was superior to either Tris or phosphate buffer alone.

Recovery of protein from acetone powders was markedly improved as the pH of the extracting solution was increased from 6 to 10 (Fig. 7). This was true for both K-phosphate and Tris buffers. Protein recovery was lower with Tris at all pH values evaluated compared

to K-phosphate when centrifugal filters were employed, but was similar with conventional centrifugation because of increased solubilization of pectic substances with Tris-buffer.

Extraction of malic enzyme from acetone powders closely paralleled that of total protein from pH 6·5 to 9·5. The pH during extraction did not qualitatively alter the distribution of proteins on acrylamide gels during electrophoresis at pH 8·3.

Increasing the ionic strength of 0·1 M K-phosphate buffer at pH 8·5 by adding KCl up to 0·26 M $K^+$ did not influence the protein recovery. Adding KCl to Tris–Tricine buffer at pH 8·5 reduced protein and malic enzyme recovery slightly.

2. *Effect of sugars and sugar alcohols.* Including a sugar or sugar alcohol significantly increased the recovery of protein from acetone powders. No marked differences were evident

TABLE 3. THE EFFECT OF DIFFERENT SUGARS AND SUGAR ALCOHOLS IN THE EXTRACTING SOLUTION ON PROTEIN RECOVERY FROM ACETONE POWDER OF DELICIOUS APPLES

| Adjuvant* | mg Protein/500 mg acetone powder† |
|---|---|
| None | 4·57 |
| Sucrose | 5·02 |
| Mannose | 5·13 |
| Glucose | 5·57 |
| Inositol | 5·64 |
| Sorbitol | 5·70 |
| Mannitol | 6·00 |

* A 0·2 M solution of each of the above substances was used with 0·1 M phosphate buffer solution at pH 9·5.

† Protein was determined by the biuret method on a TCA precipitate from four successive extractions.

between the sugars or between the sugar alcohols which were slightly better (Table 3). The presence of 0·2–0·4 M mannitol consistently resulted in better protein recoveries. The effect of these polyhydroxy compounds is probably due to stabilization of the protein and protection against denaturation. Most extractions were performed in the presence of 0·35 M mannitol.

3. *Effect of EDTA, cysteine, diethyldithiocarbamic acid (DIECA) and ascorbic acid.* Incorporating EDTA in the extracting solution to chelate heavy metals had a negligible effect on protein recovery or malic enzyme activity over the range of $10^{-5}$–$10^{-3}$ M. Electrophoresis separation of proteins appeared to be slightly improved with EDTA and was routinely incorporated in the extracting solution at $5 \times 10^{-4}$ M. Cysteine, DIECA or ascorbate from $10^{-4}$–$10^{-2}$ M did not alter protein recovery, enzyme activity or electrophoresis unless extracts contained a high phenolic content. When this was a problem in fresh tissue extracts or acetone powders from very young fruits DIECA at $10^{-3}$ M was incorporated in the extracting solution to prevent phenol oxidation.

## DISCUSSION

Biochemical investigations of fruit ripening are often conducted with tissue slices. Tissue slice procedures are often suitable to follow changes in chemical constituents in fruits at various ripening stages.[7] However, the method is less appropriate to ascertain the changes in dynamic cellular processes attending ripening, such as cell-wall softening, chlorophyll degradation, protein synthesis, ethylene synthesis and respiration. The solution infiltration technique employed with intact fruits provided a means to study several physical and chemical changes of fruit ripening and to investigate protein and RNA metabolism in the same tissue.[4] Metabolic inhibitors, intermediary metabolites, precursors of proteins and nucleic acids cannot be introduced by infiltration into a tissue and maintain tissue integrity unless provision is made to maintain a favorable osmotic environment. This was achieved by using mannitol at 0·2–0·4 M as the carrier solution. When water or lower concentrations of mannitol were used as the solution carrier, ripening was inhibited, probably because of membrane damage and uncoupling of oxidative phosphorylation. When the poorer osmotic condition was maintained normal ripening as judged by softening, chlorophyll degradation, ethylene synthesis and respiratory behavior was slightly delayed but otherwise normal. Burg *et al.*[5] observed that ethylene synthesis by post-climacteric apple tissue slices was irreversibly inhibited when the tissue was soaked in water but not in solutions, isotonic and hypertonic, with respect to the cell sap. Loss of membrane integrity may possibly be the reason for this.

Low protein content and changes in physical structure of the tissue during development and ripening are deterrents to the study and interpretation of changes taking place in protein metabolism of pome fruits. Several investigators reported an increase in enzyme activity during ripening, suggesting enhanced protein synthesis.[8] Apparent increase in enzyme activity, however, can be a consequence of differential extractability, activation, or synthesis. Extraction of proteins from fruits undergoing extensive cell-wall hydrolysis during ripening is further hampered by soluble pectic substances which can bind and precipitate proteins unless provisions are taken to minimize this problem. Precipitation with $Ca^{2+}$ during protein extraction prevents the soluble pectic substances from binding proteins. Unless these pectic substances are removed electrophoretic separation of proteins cannot be satisfactorily accomplished.

## EXPERIMENTAL

### *Apple and Pear Fruits Used*

Bartlett pears and several cultivars of apples employed for these studies were obtained from the MSU Experiment Station orchards. The studies were made directly following harvest or with fruits from 0° storage in air.

### *Respiration Measurements*

Oxygen uptake and $CO_2$ production of intact fruits receiving various treatments were measured at 20° with an automated gas analyzing system employing a Beckman $O_2$ analyzer and an i.r. $CO_2$ analyzer.[9]

### *Chlorophyll Determination*

Chlorophyll content of pear peel tissue was measured in acetone extracts according to a method of Arnon.[10] A relative measure of chlorophyll degradation in peel of intact fruits was obtained by a reflectance procedure.[4]

[7] A. C. Hulme, M. J. C. Rhodes, T. Galliard and L. S. C. Wooltorton, *Plant Physiol.* **43**, 1154 (1968).
[8] E. Hansen, *Ann. Rev. Plant Physiol.* **17**, 459 (1966).
[9] D. R. Dilley, D. H. Dewey and R. R. Dedolph, *J. Am. Soc. Hort. Sci.* in press (1969).
[10] D. I. Arnon, *Plant Physiol.* **24**, 1 (1949).

*Ethylene Determination*

Ethylene produced by the fruits was collected according to the method of Young *et al.*[11] or by evacuating the intercellular atmosphere. Determinations were made by gas chromatography employing a $\frac{1}{8}$ in. × 6 ft column of Poropak Q and a flame ionization detector.

*Flesh Firmness*

A Magness-Taylor fruit pressure tester equipped with a $\frac{5}{16}$ in. or $\frac{7}{16}$ in. tip was employed for measuring firmness changes.

*Solution Infiltration Procedures*

Various solutions were introduced into the intercellular air space volume of apple and pear fruits by vacuum infiltration employing individual fruits or several fruits simultaneously depending upon the particular requirements of the experiment.

*Method I. Individual Fruits*

A hypodermic needle (containing a wire to prevent clogging) was inserted into the central cavity region. The fruit with needle in place was attached to a syringe and positioned in a wide-mouth glass chamber. The solution in the syringe was gradually introduced into the fruit to occupy the intercellular volume made available as the fruit was continuously evacuated at *ca.* 100 mmHg. The volume of solution administered to each fruit was readily controlled. 20–40 min were required to infiltrate pears with 10 ml of solution whereas less than 10 min was sufficient for apples.

*Method II. Several Fruits*

A syringe needle was momentarily inserted at the calyx and into the central cavity of each fruit. The punctured fruits were submerged in aqueous treating solutions contained in a vacuum desiccator and evacuated to 100 mmHg. The solutions entered the fruits upon returning the solution to atmospheric pressure while keeping the fruits submerged. The volume of solution introduced was proportional to the time following return to atmospheric pressure (see Results section).

*Method III. Individual Fruits*

Unless the fruits were punctured, had an open calyx canal, or had large open lenticels, little or no solution entered by Method II. This was used to advantage to administer known volumes of solution to individual fruits using a combination of Methods I and II. Evacuated, but unpunctured fruits, were injected with a syringe while submerged in water and controlled quantities of infiltrating solution (up to 10 ml) were introduced from the syringe. This procedure worked well for apples but was not suitable for pears.

*Tissue Extraction*

Fresh tissue extracts were prepared by grinding 50 g of cortical tissue, previously vacuum infiltrated with buffered extraction solution (0·1–0 2 M Tris, 0·05 M $CaCl_2$, and 0·3 M mannitol), with a motor-driven mortar and pestle. The homogenate was pressed through nylon cloth and yielded approximately 40 ml of extract having a pH between 8 5 and 9 5. Peel extracts were occasionally prepared employing the same procedure with a slightly higher buffer molarity. Sub-cellular fractions were obtained by differential centrifugation at 500 *g* (15 min), 15,000 *g* (15 min), and 100,000 *g* (60 min) and designated as nucleus and cell wall, mitochondrial, microsomal, and soluble-protein fractions. The soluble-protein fraction was employed for malic enzyme assay and electrophoresis.

Acetone-dried powders of fruit tissues were prepared according to the low-temperature procedure of Clements.[6] Protein extracts were obtained with various extracting media (see Results section) using centrifugal filters or open tubes. Generally 500 mg of acetone powder was weighted into the centrifugal filter holders (Gelman Instruments, Ann Arbor, Michigan) or 50-ml centrifuge tubes, 5–10 ml of extracting solution added and incubated at 0° for 1 hr with occasional mixing. The solution was separated from the residue by centrifugation at 2000 *g* for 10 min at 0–4°. Satisfactory extraction of acetone powders was obtained with 0·1 M Tris–Tricine or K-Phosphate at pH 8 5–9 5. Acetone powders rich in soluble pectic substances were extracted with 0 1 M Tris adjusted to pH 8·5 with $H_3PO_4$ containing 0·1 M KCl. Other materials in the extracting solution with either buffer system were, 0 35 M mannitol, 0 5 mM EDTA and 1 mM diethyldithiocarbamate (DIECA).

*Electrophoresis*

Polyacrylamide gel as disc columns or vertical slabs was employed for support media and was prepared according to the procedure of Davis.[12] Current was maintained at 8·8 mA per $cm^2$ cross-section at 0°.

[11] R. E. YOUNG, H. K. PRATT and J. BIALE, *Anal. Chem.* **24**, 551 (1952).
[12] B. J. DAVIS, *Ann. N Y. Acad,. Sci.* **121**, 405 (1964).

*Protein Determinations*

Protein of various extracts was determined by micro-Kjeldahl, Biuret, or Lowry procedures where applicable.

*Acknowledgements*—We wish to express our gratitude for the excellent technical assistance of Mrs. Gillian Gilbart and the financial support of the National Science Foundation (GB-4656).