Protein Synthesis in Relation to Ripening of Pome Fruits

Chaim Frenkel, Isaac Klein, and D. R. Dilley
Department of Horticulture, Michigan State University, East Lansing, Michigan 48823
Received March 11, 1968.

Abstract. Protein synthesis by intact Bartlett pear fruits was studied with ripening as measured by flesh softening, chlorophyll degradation, respiration, ethylene synthesis, and malic enzyme activity. Protein synthesis is required for normal ripening, and the proteins synthesized early in the ripening process are, in fact, enzymes required for ripening. ¹⁴C-Phenylalanine is differentially incorporated into fruit proteins separated by acrylamide gel electrophoresis of pome fruits taken at successive ripening stages. Capacity for malic enzyme synthesis increases during the early stage of ripening. Fruit ripening and ethylene synthesis are inhibited when protein synthesis is blocked by treatment with cycloheximide at the early-climacteric stage. Cycloheximide became less effective as the climacteric developed. Ethylene did not overcome inhibition of ripening by cycloheximide. The respiratory climacteric is not inhibited by cycloheximide. It is concluded that normal ripening of pome fruits is a highly coordinated process of biochemical differentiation involving directed protein synthesis.

Apple, avocado, banana, and pear fruits exhibit a marked increase in respiration rate, the climacteric, during ripening (16). It is during this period of accelerated respiratory activity that fruits soften, lose chlorophyll, develop flavor and aroma, and undergo numerous other physical and chemical changes associated with ripening. One change that has attracted the attention of investigators is the observation by Hulme (12,13) of a net increase in protein during the climacteric in apples. This has generally been found for other climacteric fruits (17,25), but not in all investigations (26). This, coupled with numerous reports (6,11,15,19,22) of increased activity of several enzymes during ripening of climacteric fruits, has led to the speculation (9) that the increase in protein content may be the result of synthesis of specific enzymes involved in the ripening processes.

Evidence of amino acid incorporation into proteins of avocado at the early-climacteric stage, but sharply falling thereafter, led Richmond and Biale (24) to propose the induction of enzymes which catalyze the climacteric process and the final breakdown of the cell. This is an attractive hypothesis since the onset of the climacteric clearly delineates between cellular activities associated with growth and maturation and those of ripening and senescence in many fruits (1). The objective of this investigation was to obtain direct evidence of enhanced synthesis of specific enzymes during the respiratory climacteric of pome fruits.

Materials and Methods

Physiologically mature Bartlett pears (Pyrus communis, L.) were harvested and stored 0° in air for up to 2 months prior to the ripening studies.

Fruits employed later than 2 months from harvest were maintained at 0° in a low O₂ atmosphere (ca. 2–3%) achieved by enclosing fruits in a sealed polyethylene bag containing hydrated lime. This was necessary to insure normal ripening behavior following 3 or 4 months storage (18).

Respiration Measurements. Oxygen uptake and CO₂ production of intact fruit were measured at 20° during the ripening studies with an automated gas analysis system employing a Beckman paramagnetic O₂ analyzer and an infra-red CO₂ analyzer (8).

Ripening Measurements. Parameters employed for ripening were degreening and softening. Chlorophyll content of the peel tissue was measured spectrophotometrically following acetone extraction. Softening of the cortical tissue was measured by a Magness-Taylor fruit pressure tester.

Ethylene Determinations. Ethylene was determined by gas chromatography employing a one-eighth inch × 6 foot column of Poropak Q and a flame ionization detector. Ethylene was collected from the airstream after passing over the fruit during respiratory gas analysis or by extracting the internal atmosphere.

Protein Synthesis. To determine amino acid labeling of specific enzymes during the respiratory climacteric, procedures were employed to insure incorporation of tracer amino acid into sufficient fruit protein for subsequent purification and analyses. To meet this requirement intact fruits were employed rather than tissue slices.

Uniformly labeled ¹⁴C L-phenylalanine was administered to intact fruit using a vacuum infiltration technique. A hypodermic needle (containing a clean-out wire to prevent clogging) was inserted into the central cavity region. The fruit with needle in place was attached to a syringe fitted into a rubber stopper and positioned in a large mouth glass chamber. Solution was introduced into the fruit through the syringe as the chamber was evacuated at ca. 100 mm
Hg. This procedure provided for rather uniform distribution (as judged by dye distribution) of up to 10 ml of solution containing the amino acid throughout the tissue of individual fruits within a 30 minute period. The infiltrating solution consisted of 0.35 M mannitol containing 0.4 μCi 14C l-phenylalanine and 1 × 10^-4 M 13C l-phenylalanine per ml. 3H-uridine was incorporated in a similar manner to determine RNA synthesis. Various adjuncts were included as indicated in the text. Two replicates of 4 fruits each at various stages in the climacteric comprised a treatment series. At specified intervals following infiltration, longitudinal sections of each fruit were removed and the cut surface of the remainder was overlaid with mylar film. A composite 5 g sample of cortical tissue was obtained from the 4 fruits for determination of phenylalanine incorporation. The tissue was placed in 20 ml of 20% TCA and heated to 100° for 5 minutes, homogenized and separated by centrifugation into TCA-soluble and -insoluble fractions. The precipitate was washed twice with 5% TCA containing 1 × 10^-4 M phenylalanine. The final precipitate was suspended in 20 ml of 1 N NaOH. Radioactivity of the trichloroacetic acid-soluble and -insoluble fractions was determined as described below. An acetone dried powder was made of the remainder of the 4 fruits utilizing the low temperature procedure of Clements (4) and was employed for polyacrylamide gel electrophoresis. Most of the experiments were repeated.

Radioactivity Determinations. All radioactivity measurements were made with a liquid scintillation spectrometer. The scintillation mixture consisted of: 4.5 g BBOT [2,5-bis-(2-(5-tert-butylbenzoazoyl))-theophene], 80 g naphthalene, 385 ml xylene, 385 ml p-dioxane, 231 ml ethanol, and 37 g Cab-o-sil (thixotropic gel powder obtained from Cabot Corporation, Boston, Massachusetts). Counting efficiencies of 75 to 81% were obtained with up to 1 ml of the various aqueous solutions or suspensions mixed with 15 ml of the scintillation solution. All counts were corrected for quenching to obtain disintegrations per minute (dpm).

Electrophoresis. Polyacrylamide gel was used as the supporting media for disc gel or vertical slab electrophoresis. Current was maintained at 8.8 mA per cm² cross section for disc electrophoresis at 0° and at 12 volts per cm for vertical slab electrophoresis at 50 to 55°. Acrylamide running gels (6 and 7%) and spacer gel for disc electrophoresis were prepared as described by Davis (5). Vertical slab gels were prepared by mixing the appropriate quantities of acrylamide, N,N'-methylenebisacrylamide, tris, HCl, and water to prepare 106 ml of 7 or 6% gel and adding 0.05 ml of N,N,N',N'-tetramethylethylenediamine, and 100 mg ammonium persulfate immediately before pouring the solution into the gel chamber.

Protein Extraction. The proteins in acetone dried powders (AP) of the fruit tissues were extracted by placing 500 mg of AP in the upper compartment of centrifugal filters (Gelman Instrument Company, Ann Arbor, Michigan) with 5 ml of a solution consisting of: 0.1 M K-phosphate buffer, pH 9.0, 0.3 M mannitol, and 1 mM EDTA. After 1 hour of incubation at 0° with occasional stirring, the solution was separated from the residue by centrifugation at 2000 × g for 10 minutes. The residue was re-extracted with 5 ml portions of the above solution. Extracts obtained from single or multiple extractions were employed for enzyme, electrophoresis, radioactivity or protein determinations. Exceptions are indicated in the text.

Malic Enzyme Assay. Enzyme activity of various extracts was determined spectrophotometrically at 340 μM (7). The standard assay consisted of: 0.1 M glycylglycine, 1.0 mM MnSO₄, 0.16 mM NADP, 3.0 mM l-malate and up to 200 enzyme units at a pH 7.3 and 20°.

Localization of malic enzyme on acrylamide gels was accomplished by incubating the gels immediately after electrophoresis in a solution of: 0.1 M glycylglycine, 0.05 M l-malate, 1.0 mM MnSO₄ containing: 0.3 mg NADP, 0.8 mg m-nitroblue tetrazolium chloride (Nitro BT) and 0.14 mg phenazine methosulfate per ml at pH 7.3 and 20°. The ratio of solution to gel volume was 1. Precipitation of the purple diformazan occurred on the gel at the site of malic enzyme within 2 hours.

![Graph](https://example.com/graph.png)

**Fig. 1.** Influence of reduced pressure on protein synthesis and ripening (flesh softening) of Bartlett pears. Fruits were maintained in humidified air at the 760 and 375 mm Hg according to the procedure of Burg (2).
Protein Determinations. The protein of various extracts was determined by micro-Kjeldahl, biuret, or Lowry procedures where applicable.

Results and Discussion

It is well established that ripening can be delayed by lowering the temperature, decreasing the $O_2$ supply or increasing the $CO_2$ concentration. Manipulation of these factors is the basis for long-term fresh preservation of many fruits. Recently, Burg and Burg (3) established that ripening is also delayed by reducing the ethylene concentration within the tissue by simply maintaining the fruit at reduced pressures. This does not reduce the rate of ethylene synthesis provided $O_2$ is not limiting (2). This technique was employed to determine if protein synthesis associated with ripening could also be delayed. Pear fruit ripening (as judged by flesh softening) was delayed when the fruits were held in air at one-half atmosphere (fig 1) and the normal increase in protein content was likewise delayed.

To determine if protein synthesis was the result rather than the cause of ripening, fruits were infiltrated with cycloheximide, a potent inhibitor of protein synthesis. Cycloheximide prevented ripening when administered to fruits at the early-climacteric stage, but was progressively less effective at later stages (figs 2, 3, 4). Flesh softening, chlorophyll degradation and ethylene synthesis were drastically reduced by cycloheximide treatment of early-climacteric fruits. This supports a causative relationship between protein synthesis and ripening. Exogenous ethylene did not overcome cycloheximide inhibition of ripening. Ethylene (1000 ppm) applied to control or cycloheximide treated fruits for 12 hours following infiltration at each stage did not alter the response. Respiration was not inhibited by cycloheximide at $1 \times 10^{-4}$ M (fig 5). Instead respiration was stimulated to values slightly higher than the air control (not shown). When applied at later stages in the climacteric, cycloheximide did not markedly alter respiration from that observed with the mannitol control. Richmond and Biale (23) recently demonstrated that the respiratory climacteric was not directly related to protein synthesis. Clearly, protein synthesis is not a metabolic sink explaining the climacteric rise as once suggested (21). Inhibition of protein synthesis with corresponding inhibition of ripening has been amply demonstrated by other investigators using less drastic treatments, notable among these is inhibition by CO$_2$ (14).

Data for phenylalanine incorporation into Bartlett pear proteins at various stages of the respiratory climacteric is given in table I. Fruits at the mid-climacteric stage exhibit the greatest propensity for incorporating phenylalanine into TCA-precipitable protein (p < 0.01).

Table I. $^{14}$C-Phenylalanine Incorporation into Bartlett Pear Proteins at Various Stages of the Climacteric

<table>
<thead>
<tr>
<th>Time of incorporation</th>
<th>Early</th>
<th>Climacteric stage</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min</td>
<td>% incorp. into trichloroacetic acid precipitate$^1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>3.4</td>
<td>...</td>
</tr>
<tr>
<td>10</td>
<td>2.7</td>
<td>3.1</td>
<td>2.5</td>
</tr>
<tr>
<td>20</td>
<td>2.9</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>40</td>
<td>2.7</td>
<td>4.1</td>
<td>2.7</td>
</tr>
<tr>
<td>80</td>
<td>2.2</td>
<td>6.3</td>
<td>3.1</td>
</tr>
<tr>
<td>160</td>
<td>5.4</td>
<td>11.5</td>
<td>3.0</td>
</tr>
<tr>
<td>320</td>
<td>11.7</td>
<td>21.4</td>
<td>5.4</td>
</tr>
<tr>
<td>640</td>
<td>21.1</td>
<td>35.5</td>
<td>11.4</td>
</tr>
<tr>
<td>1280</td>
<td>36.7</td>
<td>44.0</td>
<td>13.9</td>
</tr>
</tbody>
</table>

$^1$ Specific activity of $^{14}$C-phenylalanine administered was 0.0222 $\mu$C/umole.

Fig. 2. (upper row) Influence of cycloheximide treatment (10 $\mu$g/ml infiltrating solution) on flesh softening of Bartlett pears at the early- (A), mid- and peak- (B) climacteric stages. There were no differences between treatments at the peak-climacteric stage. (O = air control, $\Delta$ = cycloheximide, $\square$ = mannitol control).

Fig. 3. (middle row) Influence of cycloheximide treatment on chlorophyll degradation in Bartlett pear peel at the early- (A), mid- (B), and peak- (C) climacteric stages. (O = air control, $\Delta$ = cycloheximide, $\square$ = mannitol control).

Fig. 4. (lower row) Influence of cycloheximide treatment on ethylene synthesis of intact fruit at the early- (A), mid- (B), and peak- (C) climacteric stages. (O = air control, $\Delta$ = cycloheximide, $\square$ = mannitol control).
material followed by fruits at the early-climacteric stage while those at or near the climacteric peak show a lesser capacity. These results are similar to those recently obtained for avocado by Richmond and Biale (24). However, much longer incorporation periods were employed in the present study. The possibility of bacterial contamination being a source of phenylalanine incorporation is remote in view of the intact fruit technique employed and the fact that identical results were obtained in the presence of chloroamphenicol at the rate of 50 μg per ml of infiltration solution.

It is unlikely that the decrease in phenylalanine incorporation observed as ripening neared completion resulted by dilution with endogenous amino acid. The 14C-phenylalanine was administered with 1 × 10⁻³ to 1 × 10⁻⁴ M 12C-phenylalanine. Concentration studies indicated no endogenous dilution of label occurred at concentrations above 1 × 10⁻³ M phenylalanine. Cycloheximide did not inhibit uptake of amino acid into the tissue. The percent of total counts recovered in a mannitol rinse of the tissue was 35 ± 5 over the period studied.

Although cycloheximide inhibits 14C-phenylalanine incorporation equally well at all stages of ripening, the consequence with respect to fruit ripening, is not uniform (Table II). At the early-climacteric stage fruit ripening capacity is severely limited by cycloheximide (figs 2, 3, 4), but cycloheximide applied at progressive ripening stages became less and less inhibitory. At or near the climacteric peak it has essentially no further effect on fruit ripening. This supports the hypothesis that the proteins synthesized early, but not late, in the climacteric are enzymes involved in the cellular processes of ripening. The data may partially explain the decreasing effectiveness of gamma radiation in inhibiting pear ripening when applied at progressively later ripening stages (20).

The dependency of several ripening parameters upon protein synthesis is therefore established. Among these, flesh softening is perhaps the best single parameter for ripening. It is significant to mention at this time, however, that several of the physical and chemical changes associated with ripening can, in many instances, be separated. In other words fruits can be made to soften without losing chlorophyll and undergo an apparent respiratory climacteric without ripening. This suggests that the normally coordinated cellular events of ripening may be initiated by a common denominator, but are manifest by specific processes that can be altered independently. The common denominator may be a substance that controls new RNA synthesis necessary for all subsequent proteins synthesized and required for ripening. The independent processes may be the synthesis or rate regulation of specific enzymes of ripening.

The effect of Actinomycin-D on ³H-uridine and 14C-phenylalanine incorporation into RNA and protein of mid-climacteric pears (double labeling experiment) is given in figures 6 and 7, respectively. Actinomycin-D has only limited effect on uridine incorporation and essentially no effect on phenylalanine incorporation into the ripening proteins at this stage of development. Cycloheximide which inhibits protein synthesis at the level of amino acid incorporation seems to inhibit uridine incorporation into RNA to a greater extent than does Actinomycin-D. The mechanism of this inhibition is probably of a different nature than that assumed for Actinomycin-D. The only additional observation pertinent to the influence of Actinomycin-D is that it inhibited all ripening responses when administered to precimacteric fruits and ethylene did not alleviate this. Further studies are essential to establish the chronological sequence of protein synthesis on DNA dependent RNA synthesis at various stages of the climacteric.

Further substantive argument for a time coordinated sequence of protein dependent ripening events (10) can be made from the time course change in flesh softening as a single parameter of ripening. Fruits harvested prior to the initiative of the respiratory climacteric do not soften immediately when held at ripening temperatures. Rather, they may remain at virtually the same firmness for many days. When they eventually begin to soften they do so at an ever increasing rate, reaching a more or less steady rate of change after several days. Similar observations have often been made for chlorophyll degradation, respiration rate, ethylene synthesis, and many chemical constituents. In addition, once the
The ripening sequence has begun it cannot be reversed. And, the further it has progressed the more difficult it becomes to retard subsequent ripening processes by low temperature storage or application of atmospheres low in O₂ and rich in CO₂, techniques long used by the fruit industry to prolong the post-harvest holding period of pome fruits.

Many of the physical and chemical changes occurring during ripening have formally been interpreted as being causally related to increased metabolic activity associated with the respiratory climacteric. The incongruity of respiration rate and ripening seen in response to treatment with cycloheximide at the early climacteric stage indicates that this may not be correct. Cycloheximide treated fruits appear to pass through a typical "respiratory climacteric" but do not ripen. However, we cannot rule out the possibility that cycloheximide alters other vital processes required for ripening in addition to inhibiting protein synthesis.

A question of immediate interest is what are the proteins synthesized during the various ripening stages of pome fruits? Does the pattern of synthesis change as ripening progresses? Evidence of increased activity of specific enzymes is insufficient by itself to provide definitive answers. Enzyme activation, inhibitors, or preparatory artifacts may be responsible for the observed changes. Insight into these questions must be derived from studies of specific protein synthesis. This was achieved by incorporating labeled phenylalanine into proteins of apple and pear fruits at various stages of ripening and separating them electrophoretically on polyacrylamide supporting media as gel columns or slabs.

Evidence for phenylalanine incorporation into particular proteins in early-climacteric pears is presented in figure 8. The amount or rate of phenylalanine incorporation is not in proportion to the quantity of protein present. Rather, quantitatively insignificant proteins, as judged by staining, become highly labeled. Other proteins which incorporate a relatively low amount of label during the first 6 hours at ripening temperatures become highly labeled by 24 hours. A different situation exists for pears at the mid-climacteric stage. Whereas certain of the proteins which showed low initial label incorporation at the early-climacteric stage now become highly labeled. At the same time certain of the

---

**Fig. 6.** (upper) Influence of Actinomycin-D and cycloheximide on incorporation of 3H-uridine into RNA of mid-climacteric Bartlett pears. The same fruits were employed for 14C-phenylalanine incorporation in a double labeling experiment (fig 7).

**Fig. 7.** (lower) Influence of Actinomycin-D and cycloheximide on incorporation of 14C-phenylalanine into protein of mid-climacteric pears. The same fruits were employed for 3H-uridine incorporation into RNA in a double labeling experiment (fig 6).
proteins which become highly labeled early change little with time. Clearly, this is evidence of differential capacity for incorporating phenylalanine into distinct proteins as ripening develops.

The synthesis of malic enzyme (band 3 in fig 8) was studied in considerable detail. Interestingly, it is one of the dominant proteins extracted from pome fruits and undergoes considerable synthesis with ripening. Data for phenylalanine incorporation in relation to malic enzyme activity is given in Table III expressed as dpm/enzyme unit. The data in column 1 is total dpm incorporated into all proteins in relation to malic enzyme activity at the early- and mid-climacteric stages and indicates less than a 2-fold increase. If all proteins are synthesized at about the same rate phenylalanine incorporation into malic enzyme should show the same increase. Phenylalanine incorporation into electrophoretically purified malic enzyme is approximately 3-fold greater in mid- as compared to early-climacteric pears. It has previously been shown that the specific activity (enzyme units/mg protein) and total activity of malic enzyme increases during ripening. It can be concluded that some of the increase in activity is the result of synthesis. The significance of this enzyme in the general metabolism of pome fruits is not immediately apparent. Its presence and activity is in accord with the increased malate decarboxylation capacity and increased respiration quotient observed with intact fruit and tissue slices during ripening and the gradual decline in malic acid during senescence. Hansen (10) has recently shown that ethylene applied to immature Bartlett pears induces an increase in protein N, a parallel increase in respiration rate, and increased catabolism of malic acid. Halme (12) had previously shown similar results with apples. Malic enzyme may play a significant role in pome fruit ripening.

This research indicates that specific protein synthesis is required for pome fruit ripening. Further, ethylene synthesis is dependent upon protein synthesis at the early-climacteric stage, is less dependent in the mid-climacteric period and declines as protein synthesis diminishes during senescence. Supplemental ethylene in the absence of protein synthesis does not promote ripening. Thus, the role of ethylene in ripening may be 2-fold: first, as an effect of specific protein synthesis for which capacity already exists or can develop; and secondly, as a product of ripening metabolism involving some of the same proteins.

Acknowledgments

The authors gratefully acknowledge the excellent technical assistance of Mrs. Gillian Gilbart, Department of Horticulture, Michigan State University, and the financial assistance of the National Science Foundation (GB-4656).

Literature Cited


Table III. Incorporation of 14C-phenylalanine Into Malic Enzyme in Relation to Other Proteins During Ripening of Bartlett Pears

<table>
<thead>
<tr>
<th>Stage of ripening</th>
<th>Crude extract1</th>
<th>After electrophoresis2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-climacteric</td>
<td>32.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Mid-climacteric</td>
<td>57.4</td>
<td>15.3</td>
</tr>
</tbody>
</table>

1 Radioactivity in acetone powder extract was determined following precipitation of proteins with trichloroacetic acid.
2 Malic enzyme was extracted from the acrylamide gel by homogenation with 0.05 M KCl. Proteins in acetone powder extract contained less than 0.05% of the recovered radioactivity.

Fig. 8. Distribution of 14C-phenylalanine in Bartlett pear proteins separated on 6% polyacrylamide gel slabs.

Table III. Incorporation of 14C-phenylalanine into Specific Proteins of Bartlett Pears at Early- and Mid-climacteric Stages

<table>
<thead>
<tr>
<th>Stage of ripening</th>
<th>Crude extract1</th>
<th>After electrophoresis2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-climacteric</td>
<td>32.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Mid-climacteric</td>
<td>57.4</td>
<td>15.3</td>
</tr>
</tbody>
</table>

1 Radioactivity in acetone powder extract was determined following precipitation of proteins with trichloroacetic acid.
2 Malic enzyme was extracted from the acrylamide gel by homogenation with 0.05 M KCl. Proteins in acetone powder extract contained less than 0.05% of the recovered radioactivity.