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Involvement of Hydrogen Peroxide in the Regulation of Senescence in Pear[1]

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ABSTRACT

Endogenous peroxide levels in pear fruit (*Pyrus communis*) were measured using a titanium assay method, and were found to increase during senescence in both Bartlett and Bosc varieties. Application of glycolic acid or xanthine, serving as substrates for the formation of H_2O_2, increased the peroxide content of the tissue and accelerated the onset of ripening, as measured by increased softening and ethylene evolution. Application of ethylene also induced increased peroxide levels. Ripening processes were similarly promoted when peroxides were conserved by inhibiting the activity of catalase with hydroxylamine or potassium cyanide. By comparison, the inhibition of glycolate oxidase with alpha-hydroxy-2-pyridinemethanesulfonic acid decreased the peroxide content of the tissue and delayed the onset of ripening. These results indicate that the onset of ripening correlates with the peroxide content of fruit tissues as occurring under normal conditions or as influenced by the treatments. Hydrogen peroxide may be involved in oxidative processes required in the initiation and the promotion of ripening.

Fruit ripening is viewed as an oxidative phenomenon (3, 5), as indicated by results suggesting the decline in sulfhydryl gradients during ripening (7) and the acceleration of ripening processes by the application of high O_2 tensions (2, 5, 10). We hypothesize that the effects of O_2 reflect, in part, the utilization of O_2 for the formation of peroxides for the following reasons.

a. The free radical theory of aging proposes that progressive aging results from the random and cumulative attack by oxygen radicals (partially reduced oxygen forms) (16). However, the view of ripening as a regulated senescence phenomenon (13) requires a controlled turnover of radicals. Hydrogen peroxide is a stable partially reduced oxygen form (15), and its turnover is characteristically mediated by enzyme action. For that reason, we envision that H_2O_2 can be integrated into the regulated metabolic events occurring during senescence in fruit.

b. The acceleration of ripening by high O_2 tensions (6, 10) is consistent with the high $Km(O_2)$ of some peroxide-forming enzyme systems (26).

c. The onset of senescence in some systems is accompanied by an increase in the activity of peroxide-utilizing enzymes (4) and a decrease in peroxide-destroying enzymes (17).

In the present work, we found that changes in peroxides, as occurring normally or as influenced by treatments, closely correlate with the onset of fruit senescence.

MATERIALS AND METHODS

The plant material used in this study was mature green pears (*Pyrus communis*), including Bartlett and Bosc varieties. Bartlett pears were obtained from an orchard located in New Brunswick, N. J., and were used in most of the experiments. Bosc pears were obtained from an orchard located at Pine Grove, Pa.

A vacuum infiltration method (9) was used to apply test solutions to intact fruit. These solutions consisted of the test compounds solubilized in 0.3 M mannitol, the latter serving as a carrier solution. Fruits were infiltrated on the average at a rate of 5 ml of test solution/100 g of fruit tissue. The test solutions consisted of the following: (*a*) 1 μM glycolate or xanthine, used as substrates for the formation of peroxides as catalyzed by glycolate oxidase (EC 1.1.3.1) and xanthine oxidase (EC 1.2.3.2), respectively; (*b*) 0.01 M KCN or hydroxylamine-HCl, used as catalase inhibitors; (*c*) 0.01 M alpha-hydroxy-2-pyridinemethanesulfonate, serving to inhibit the activity of glycolate oxidase (29).

The HPMS[3] solution was applied to Bosc pears. Bartlett pears were used in all of the other experiments.

The treated fruit were kept at 21 C at standard conditions and ventilated with air at a rate of 400 ml/min. Ethylene was scrubbed from the ventilating air with Purafil (H. E. Burrough and Assoc., Chamblee, Ga.).

Fruits were sampled at intervals for measuring the rate of softening, ethylene evolution, and peroxide levels. Softening and peroxides were measured at 0, 2, 4, 6, and 8 days following infiltration. Ethylene evolution was measured daily for the duration of the experiment. Samples of 10 fruit were used for each of the above determinations. All determinations were run in duplicate. Ethylene was collected from the ventilating gases according to a method of Young *et al.* (28) and determined by gas chromatography.

Fruit softening was measured using a Magness-Taylor fruit pressure meter as previously described (13).

Peroxide determination was as outlined below. Hydroperoxides form a specific complex with titanium (Ti^{4+}) which can be measured by colorimetry (19). Peroxides were extracted by homogenizing 100 g fruit tissue in 200 ml cold acetone. The homogenate was filtered and the filtrate brought to 300 ml with water. Two ml of a titanium reagent (20% titanic tetrachloride in concentrated HCl, v/v) were added to 20-ml samples of the peroxide extract, followed by the addition of 4 ml concentrated NH_4OH to precipitate the peroxide-titanium complex (19). After centrifugation (5 min at 10,000*g*), the supernatant was

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[3] Abbreviation: HPMS: alpha-hydroxy-2-pyridinemethanesulfonate.

discarded and the precipitate solubilized in 15 ml of 2 N H_2SO_4, washed repeatedly with acetone, and brought to a final volume of 20 ml with water. The absorbancy of the obtained solutions was read at 415 nm against a water blank. The concentration of the peroxide in the extracts was determined by comparing the absorbancy against a standard curve representing titanium-H_2O_2 complex from 0.1 to 1 mM. This procedure was also used for the measurement of catalase activity as described below. The hydroperoxides, as extracted and estimated by the above procedure, represent H_2O_2 and lipid hydroperoxides. However, lipid peroxides represent only 5 to 10% of the total peroxides in fruit (8).

Effect of Enzyme Inhibitors on Catalase Activity. Catalase (EC 1.11.1.6) activity was determined in protein extracts from mature green Bartlett pears. Protein extracts were obtained as previously described (12). Catalase activity was determined by measuring the rate of H_2O_2 disappearance in the enzyme reaction mixture. The reaction mixture consisted of 0.5 ml of protein extract (0.1 mg total protein/ml) in 10 mM phosphate buffer (pH 7), 2 ml of 5 mM H_2O_2, and 1 ml of the inhibitors at the concentrations shown (Fig. 4). The reaction was initiated at 21 C by adding the enzyme solution and stopped by the addition of 1 ml of the titanium reagent (1/10 dilution). Samples were taken at 2-min intervals for peroxide determination as described above.

RESULTS

Promotion of Ripening by the Stimulation of Peroxide Formation. Figure 1 shows the changes in endogenous peroxides in fruit as related to the changes in softening and ethylene synthesis. In control fruit allowed to ripen under normal conditions there was a severalfold increase in the level of peroxides during the ripening period (Figs. 1a, 2a, 3, 5a, and 6). By comparison, in fruit treated with glycolate, which serves as a substrate for the formation of H_2O_2 (25), both the onset and the level of the peroxides formed were enhanced. These changes are reflected in a corresponding stimulation of softening (Fig. 1b) and ethylene synthesis (Fig. 1c). The rate of softening induced by 1 μM glycolate was markedly greater than the effect of 10 μl/1 ethylene.

Similar responses were obtained by the application of xanthine (Fig. 2), which can serve as a substrate for the formation of H_2O_2 (14). As with glycolate, 1 μM xanthine accelerated the onset and level of the peroxides formed, as compared to untreated fruit (Fig. 2a). The applied xanthine markedly enhanced the rate of softening, even as compared with the effect of 10 μl/1 ethylene (Fig. 2b), and increased the levels of the evolved ethylene during the latter part of the ripening period (Fig. 2c).

In spite of the differences in the structure and metabolic origin of the applied compounds, glycolate and xanthine induced similar responses, suggesting that these compounds function primarily as substrates for the formation of peroxides. However, an additive effect resulting from a specific metabolic function of these substances cannot be ruled out. Glycolate and xanthine induced marked effects at physiological concentrations (1 μM).

Application of ethylene (10 μl/1) also stimulated an increase in the peroxide level (Fig. 3). A similar effect by ethylene upon peroxide formation is observed in potato tubers (8), even though unlike fruit, potatoes do not normally exhibit a rise in peroxide levels. The results suggest that ethylene may trigger the activity of enzyme systems which catalyze the formation of peroxide from different metabolites. These may include systems which metabolize glycolate, xanthine, and other substrates (14).

Promotion of Ripening by Conserving Endogenous Peroxides. The catalase inhibitors KCN and hydroxylamine were applied in order to prevent the breakdown of the peroxides formed and thereby to promote ripening. Figure 4 showing the effect of the inhibitors on catalase activity in a protein extract from pear, indicated progressive inhibition as the inhibitor concentration increased and almost complete inhibition at the highest concentration used (0.01 M). Aminotriazole and thiourea, previously reported as inhibiting catalase, had little or no effect on the fruit enzyme (data not shown).

Figure 5 shows the effect of the catalase inhibitors on the rate of peroxide formation, softening, and ethylene synthesis. The application of the inhibitors allowed peroxide to accumulate to levels higher than in control tissue (Fig. 5a). The effect of the treatment on the acceleration of softening (Fig. 5b) is consistent with the previous observations showing that the promotion of softening is associated with an increase in peroxide levels (Figs. 1

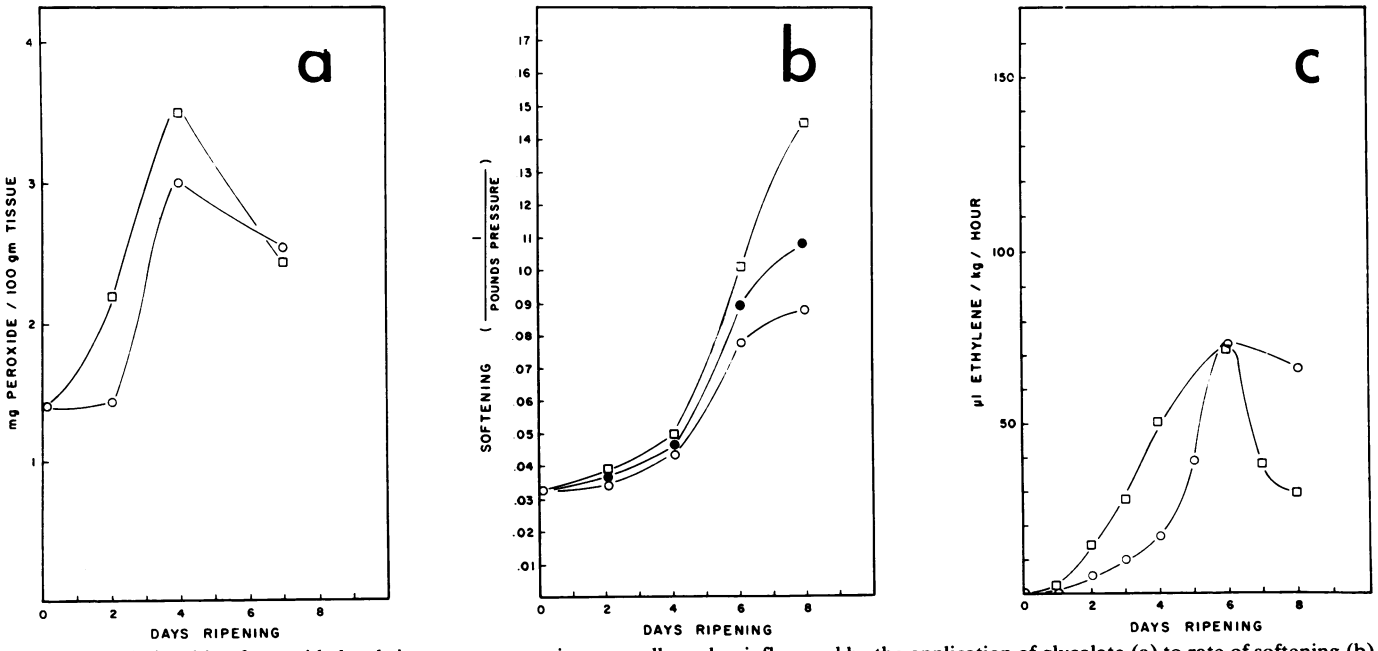

FIG. 1. Relationship of peroxide levels in pear, as occurring normally and as influenced by the application of glycolate (a) to rate of softening (b) and ethylene evolution (c). Glycolate concentrations used were zero (mannitol control) (○), and mannitol plus 1 μM glycolate (□). Effect of ethylene (10 μl/l) on softening (●) is shown for comparison.

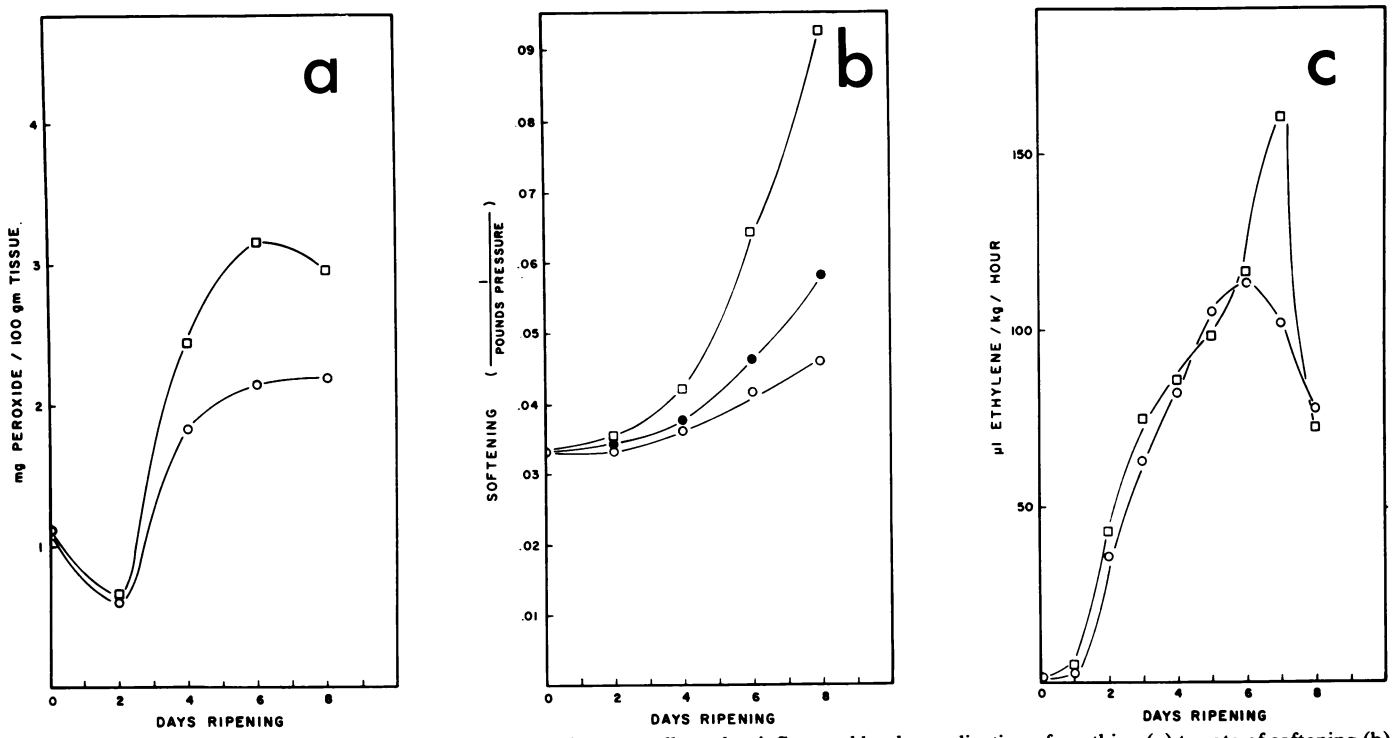

FIG. 2. Relationship of peroxide levels in pear, as occurring normally and as influenced by the application of xanthine (a) to rate of softening (b) and ethylene evolution (c). Xanthine concentrations used were zero (mannitol control) (○), and mannitol plus 1 µM xanthine (□). Effect of ethylene (10 µl/l) on softening (●) is shown for comparison.

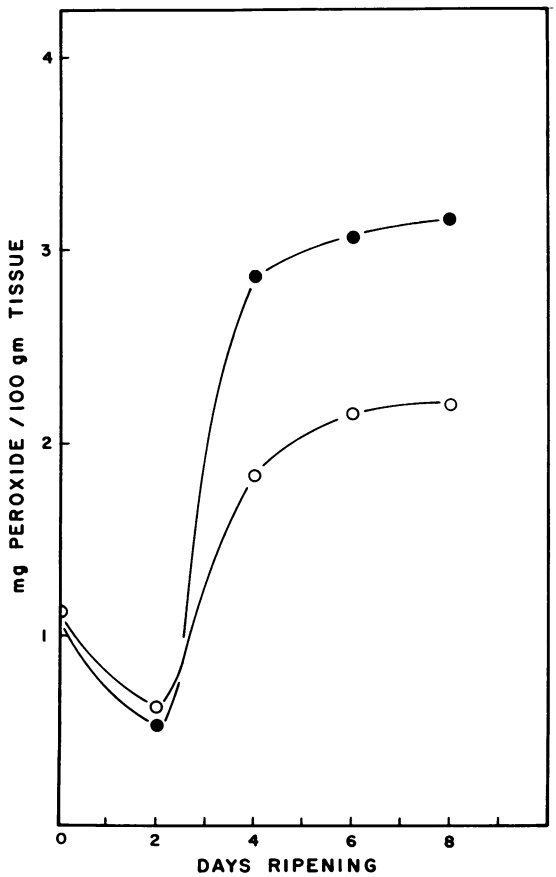

FIG. 3. Changes in peroxide levels during pear ripening as occurring normally (○) and as influenced by ethylene (10 µl/l) (●).

and 2). Contrary to the effect of glycolate and xanthine, the synthesis of ethylene was partially inhibited by the present treatment (Fig. 5c). This may be attributable to the binding of metal ions which apparently function in the promotion of ethylene synthesis (18). The results indicate that the availability of a ripening precursor, namely peroxides, partially circumvents the requirement for ethylene. This strengthens the view that peroxides by themselves promote ripening and in addition may represent a product of ethylene action (8).

Retardation of Ripening by Inhibiting Peroxide Formation. HPMS was applied to fruit in order to prevent peroxide formation as catalyzed by glycolate oxidase. Figure 6 shows the effect of 0.01 M HPMS on the rate of peroxide formation, softening, and ethylene synthesis. Peroxide formation was partially blocked by the applied HPMS, as compared to untreated fruit (Fig. 6a). By comparison, 10 µM glycolate promoted the formation of peroxides, as previously observed (Fig. 1a). The partial blockage of peroxide synthesis is reasonable since other enzymes also catalyze the formation of peroxides and may not have been affected by the treatment. However, even partial retardation in the formation of peroxides was reflected in a corresponding delay of softening toward the latter part of the ripening period, whereas glycolate application had the opposite effect and markedly accelerated the rate of softening (Fig. 6b). The reason for the abnormal pattern of softening in the HPMS-treated fruit during the early part of the ripening period is not known. Even though these fruits contained lower peroxide levels, they were actually softer than the control on days 2 and 4, which is inconsistent with the rest of our observations. Curtailment in glycolate utilization by HPMS resulted in delayed ethylene synthesis (Fig. 6c) as compared to untreated fruit, whereas access to supplementary levels of glycolate had the effect of accelerating the synthesis of ethylene.

DISCUSSION

In order to test the hypothesis that H_2O_2 is involved in fruit senescence, we examined the relationship between peroxide content and the rate of softening and ethylene synthesis in pears. We found that endogenous peroxide levels increase naturally at the onset of ripening. We have also attempted to manipulate the levels of peroxide within the tissue in three different ways: (*a*) by

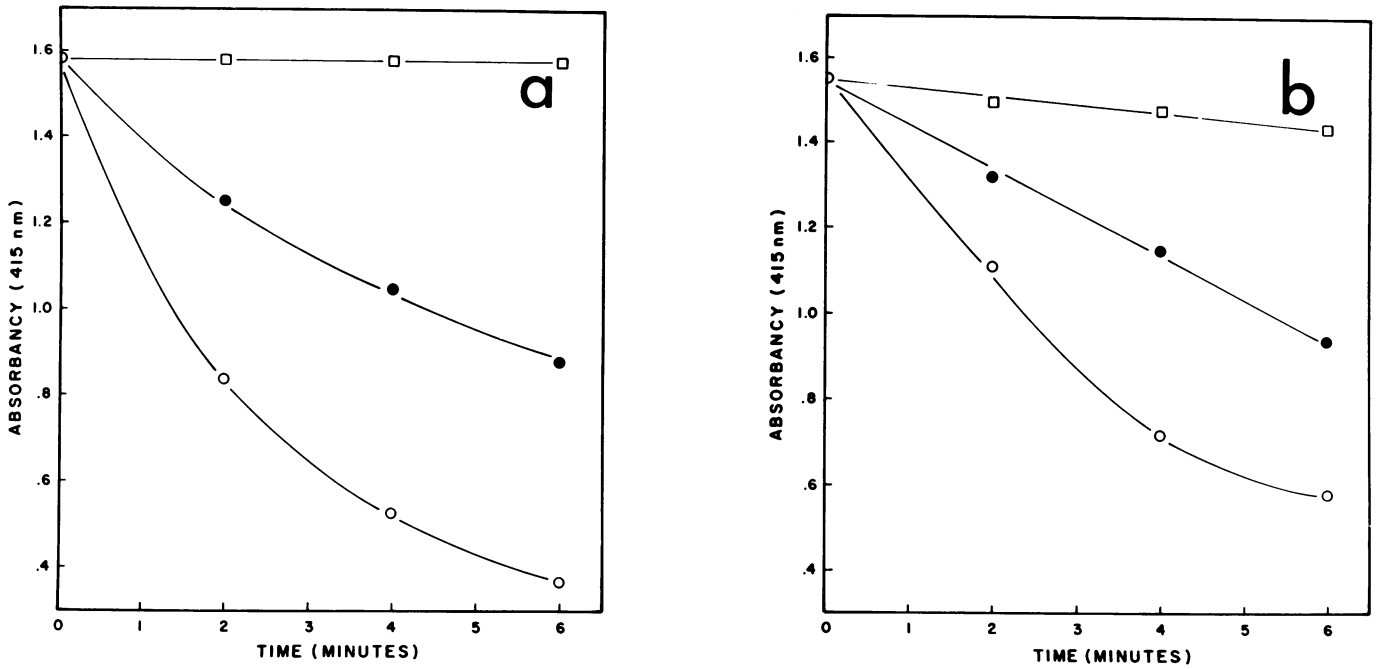

FIG. 4. Catalase activity in protein extract from pear as influenced by the enzyme inhibitor hydroxylamine.HCl (a), and KCN (b). The concentrations of inhibitors used were zero (○), 0.1 mM (●), and 10 mM (□).

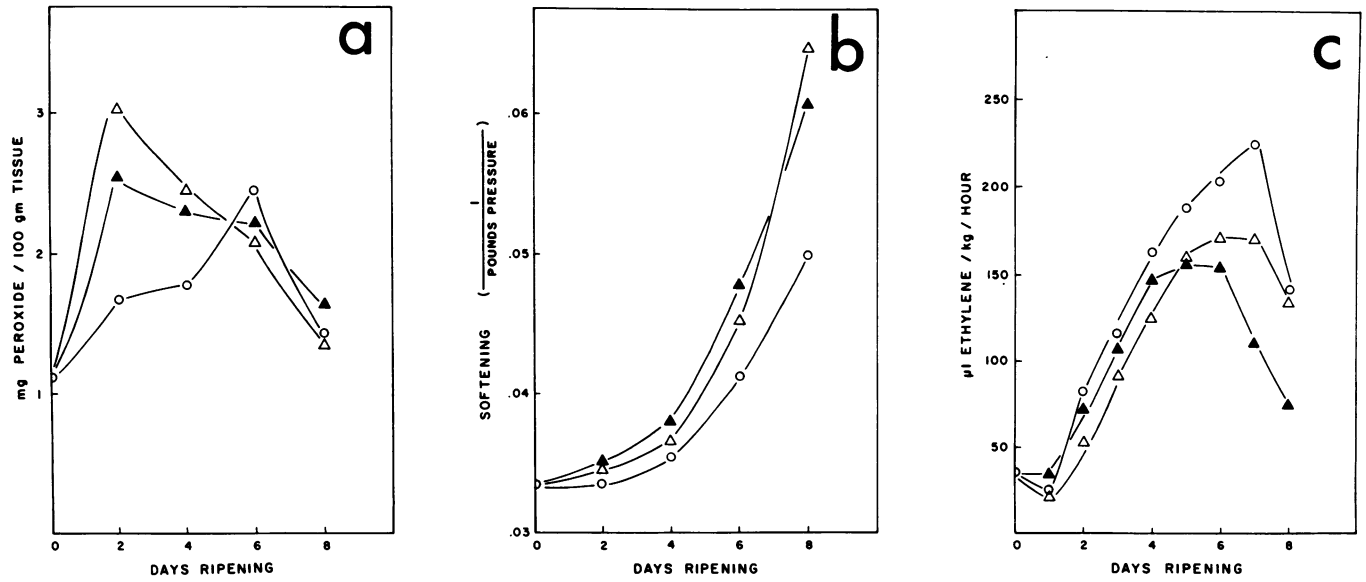

FIG. 5. Relation of peroxide levels in pear as occurring normally and as influenced by the application of catalase inhibitors (a) to rate of softening (b) and ethylene evolution (c). Concentrations of inhibitors used were zero (mannitol control) (○) and mannitol plus 10 mM KCN (△) or hydroxylamine.HCl (▲).

generating peroxide in addition to that occurring naturally; (*b*) by preventing peroxide breakdown by catalase; and (*c*) by inhibiting peroxide formation by glycolate oxidase. In all three cases, the rate of ripening processes correlates with the concentration of peroxide in tissue.

To stimulate the peroxide levels, fruit were infiltrated with substrates serving as electron donors in the formation of H_2O_2. Glycolate and xanthine are metabolized by glycolate oxidase and xanthine oxidase, respectively, and both are present in pear fruit tissue (Frenkel and Chin, unpublished data). Glycolate oxidase is a peroxisomal enzyme which catalyzes the oxidation of glycolic acid to glyoxylic acid and H_2O_2.

In order to verify that the effect was not due to the glycolate or glyoxylate rather than H_2O_2, we employed xanthine, an unrelated compound. Xanthine is oxidized by xanthine oxidase to yield uric acid + H_2O_2. As with glycolic acid, the application of this substrate resulted in the formation of increased levels of peroxide and stimulated ripening (Fig. 2). These substrates were used so that H_2O_2 would be generated endogenously by metabolic pathways, rather than by applying extracellular H_2O_2 which might induce anomalous effects. The actual increase in peroxide concentration induced by glycolate and xanthine is greater than could be accounted for directly by the metabolism of the infiltrated substrates. This may indicate that peroxides function as part of the triggering mechanism for senescence which then becomes autocatalytic. This interpretation would be in agreement with the results (Figs. 1 and 2) showing that several days were required for the applied substrates to induce the maximum increase in peroxide concentration.

The metabolic origins of peroxides vary. Microbodies are an

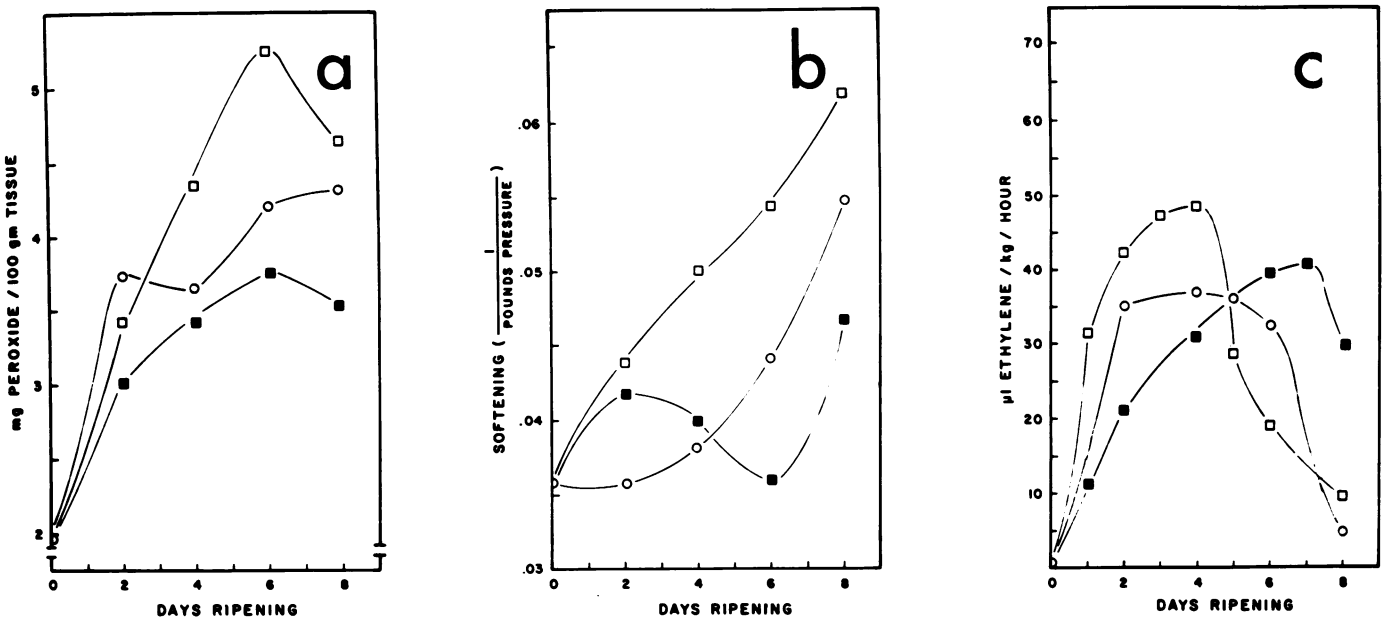

FIG. 6. Relation of peroxide levels in pear as occurring normally and as influenced by glycolate or HPMS (a) to rate of softening (b) and ethylene evolution (c). Employed concentrations of the applied substances were zero (mannitol control) (○) and 10 µM glycolate (□) and 10 mM HPMS (■).

important source of peroxides (14), but H_2O_2 formation by isolated mitochondria (20) and chloroplasts (27) has been documented as well. Lipid peroxidation could be another source of peroxides, as shown by the rise in lipid hydroperoxides in ripening tomato (8). The promotion of peroxide formation by glycolate and xanthine, which are metabolized by divergent pathways, suggests a multiple metabolic origin of peroxides in fruit.

The consistent relationship between the upsurge in peroxide levels and the onset of ripening (Figs. 1, 2, 5, and 6), the ability to utilize peroxides generated by divergent metabolic paths, and the delay of ripening by inhibition of H_2O_2 synthesis (Fig. 6) support the concept that the increase in peroxides is a causative factor in the initiation of the ripening processes. Examination of this phenomenon as it applies to other senescence processes is required to verify that the role of peroxides in plant aging is widespread.

The changes in peroxide levels in fruit apparently reflect the balance between the rate of formation *versus* those of breakdown and utilization. It has been generally assumed that H_2O_2 is degraded immediately by catalase at the site of formation in microbodies. However, Halliwell (14), reviewing the function of catalase, points out that although the catalase reaction has a high V_{max}, the $Km(H_2O_2)$ of the reaction is also quite high allowing, at equilibrium, the diffusion of peroxides from the microbodies. This allows other reactions to compete for and utilize the available peroxide.

The promotion of fruit ripening by cyanide is attributed to the increase of peroxides (Fig. 5). In this way, cyanide may mimic the action of ethylene since the latter also stimulated peroxide formation (Fig. 3). Cyanide, like ethylene, may stimulate the alternate respiratory path (23) which apparently can catalyze the formation of H_2O_2 (20). The action of cyanide may consist, therefore, of stimulating the formation of peroxides as well as inhibiting their breakdown.

The mechanism of action of H_2O_2 in promoting senescence has yet to be clarified, but there are several possibilities. Although peroxides do seem to induce increased ethylene synthesis in this system, this does not appear to be their primary mode of action. In the xanthine-treated tissue, the promotion of ethylene synthesis lags behind the increase in softening (Fig. 2). Furthermore, although hydroxylamine and potassium cyanide promoted softening, there was actually a decrease in ethylene in the treated tissue as compared to the control (Fig. 5). Thus, it appears that the increase in peroxide levels has outweighed the suppression of ethylene synthesis in its effect of softening. Although these data do not establish the precise sequence of events with respect to the formation and the action of ethylene and peroxides as occurring in ripening, it does not seem that the peroxide effects can be explained solely as an ethylene response. Since the peroxides do enhance ethylene formation and ethylene in turn induces increased peroxides (Fig. 3), this could provide a partial explanation for the autocatalytic synthesis of ethylene.

Auxins function as senescence retardants in fruit (9) and H_2O_2 may be involved in the oxidative breakdown of IAA (21, 24). This results in the formation of IAA oxidation products which can promote senescence in fruit (6, 11). The metabolism of this hormone may be an important factor in the regulation of senescence, and could account for some of the effects brought about by peroxides.

Peroxides may oxidize sulfhydryl groups (24) and thereby enhance the onset of ripening (7). Peroxides in combination with superoxides can give rise to hydroxyl radicals and singlet oxygen which may be implicated in aging and senescence processes (1).

It is possible that the primary function of peroxides is to bring about a shift in the redox potential of tissues. The loss of ability to maintain tissues in a reduced state may result in the breakdown of autooxidizable metabolites. For example, Stonier and Yang (24) envision that the role of "auxin protectors" is to interfere with H_2O_2 formation and activity. When the reducing capacity of the protectors is exhausted, the oxidation of endogenous auxins may ensue. This view is in keeping with the concept that in plants the transition from juvenility into senescence is accompanied by a progressive shift toward an oxidative state (22). Senescence may represent the end point of reducing potential, and therefore may be triggered by such oxidative conditions as, for example, high O_2 tensions (6, 10). Further studies are required to ascertain whether peroxides selectively attack specific metabolites or generally cause a shift in the redox pattern.

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