Plant Physiol. (1972) 49, 757-763

Involvement of Peroxidase and Indole-3-acetic Acid Oxidase Isozymes from Pear, Tomato, and Blueberry Fruit in Ripening

Received for publication August 16, 1971

CHAIM FRENKEL
Department of Horticulture and Forestry, Rutgers University, New Brunswick, New Jersey 08903

ABSTRACT

Protein extracts were obtained from climacteric fruits (pear, tomato) and nonclimacteric fruits (blueberry) during various stages of ripening. The use of a gel electrophoresis technique revealed a consistent reinforcement in indoleacetic acid oxidase but not in peroxidase isozymes during ripening. The significance of the results is discussed in relation to the resistance of fruits to ripening and ethylene action.

Fruit ripening is accompanied by vast and pronounced changes in the anatomy, morphology, and physiology of the fruit tissue (30). The origin of these changes has been traced to a genetic activity at the transcriptional (14, 19, 26) and the translational levels (6, 14, 17) and confirms the view that ripening is a phenomenon in fruit ontogeny reflecting the expression of a discrete genetic message (30).

The acceptance of this concept also implicates the participation of a ripening effector(s) capable of regulating a precise cellular transformation (31). Ethylene gas was long cast in the role of such a regulatory agency (7) and is considered by Burg as the ripening hormone (8). However, the literature which relates to the role of ethylene in ripening (24) does not establish that the gas is the sole hormonal factor influencing fruit ripening. Furthermore, there is no obvious reason why the modification in the hormonal balance during ripening should be uniquely different from other instances of plant senescence, in which extensive hormonal modification is observed. For example, during leaf senescence a general decline is found in gibberellins, auxins, and cytokinins (9). Similar changes were implicated in fruits by direct and indirect evidence which suggest that fruit ripening is accompanied by a similar decline of several growth substances (10, 11).

A mode of action which could account for the decline in the level of plant growth regulators in fruit tissue is an enzymatic degradation. To verify the above contention, the activity of indole-3-acetic acid oxidase, representing a hormone degrading system, was examined by the use of gel electrophoresis.

The current study shows a consistent reinforcement of IAA oxidase isozymes during the ripening of climacteric and nonclimacteric fruits. Such a consistency could not be established for other ripening parameters.

MATERIALS AND METHODS

Changes in ripening were evaluated in climacteric fruits, including pear and tomato, and in nonclimacteric fruit, represented by blueberry. The changes in ripening were compared against concomitant changes in CO_2 and ethylene evolution, and against changes in peroxidase and IAA oxidase isozymes.

Tomatoes (*Lycopersicon esculentum* var. Campbell 28), pears (*Pyrus communis* var. Bartlett) were obtained from the experimental plot of the Horticulture Department of Rutgers University, New Brunswick, New Jersey. Blueberries (*Vaccinium corymbosum* selection G-90) were obtained from the

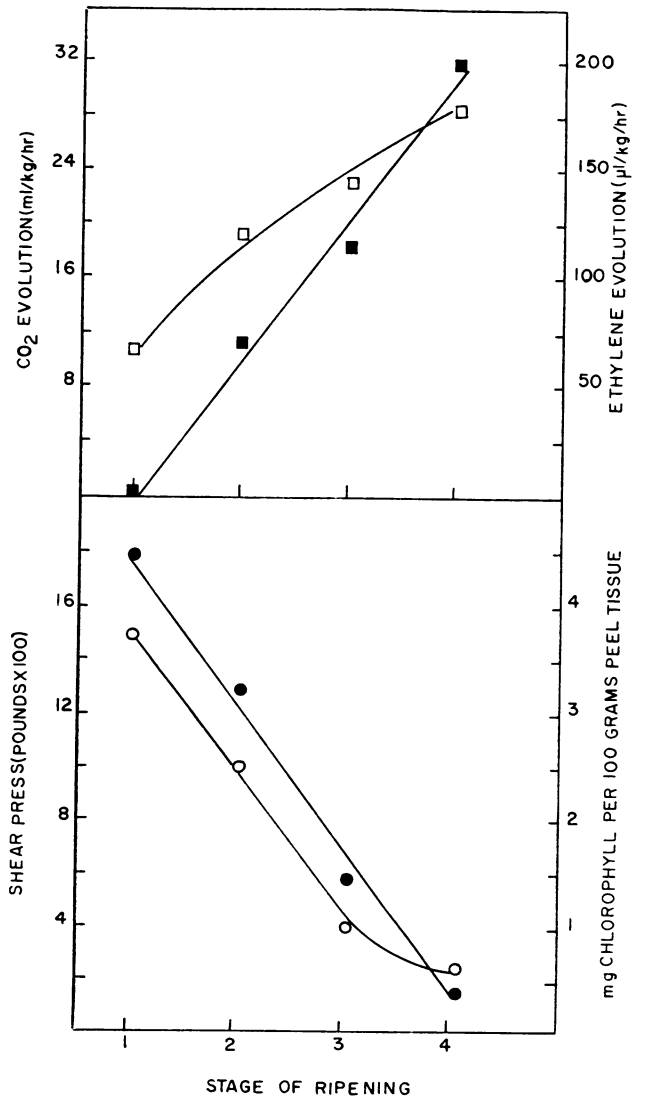

FIG. 1. The changes in ethylene (■) and CO_2 evolution (□) in relation to chlorophyll degradation (●) and change in tissue texture (○) at various ripening stages of Bartlett pears.

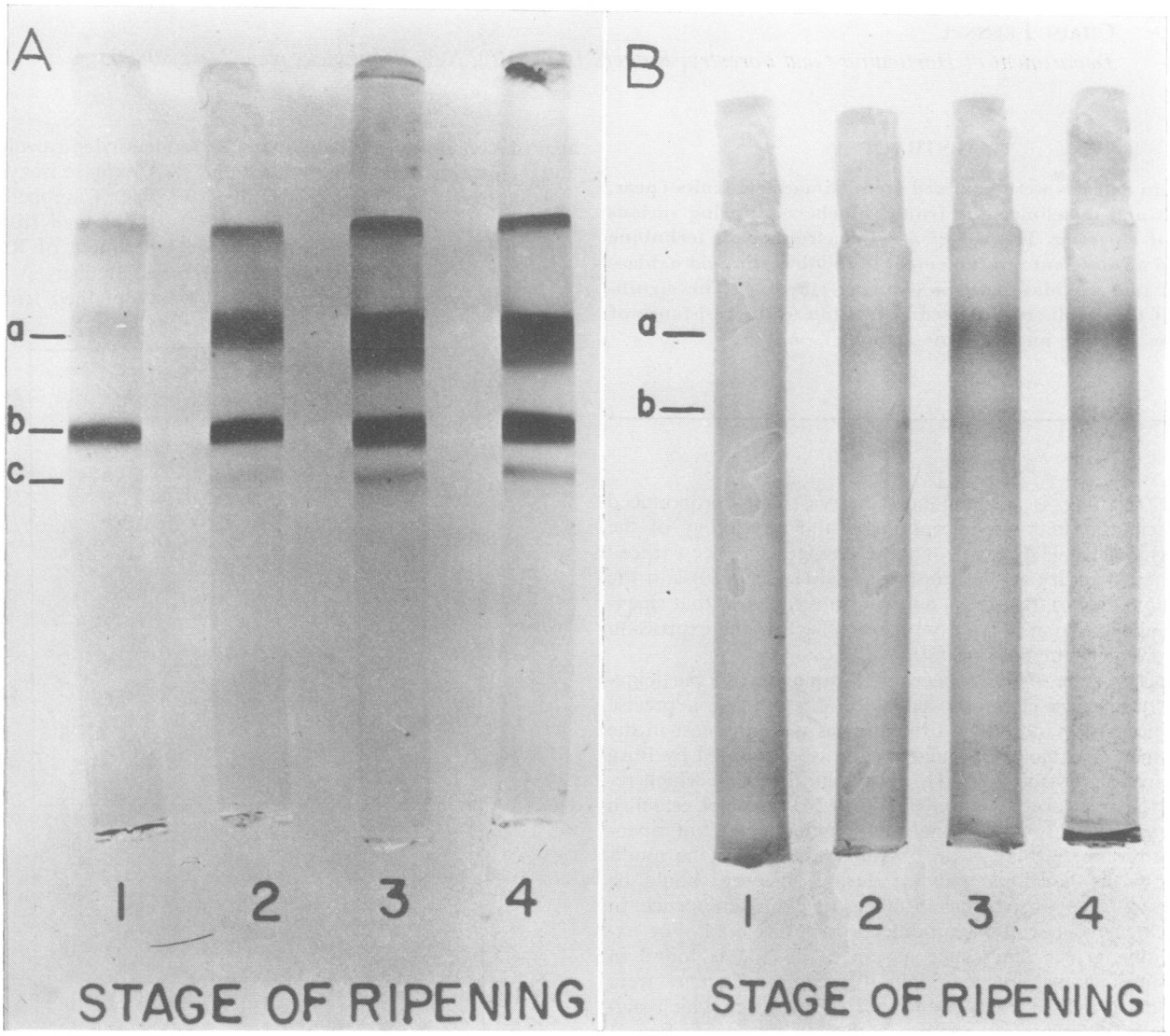

FIG. 2. The isozymic pattern of PRO (A) and IAA oxidase (B) from Bartlett pear on acrylamide gel. Isozymic patterns were obtained from protein extracts of pulp tissue during the various ripening stages of the fruit characterized in Figure 1.

United States Department of Agriculture experimental plot on the plantings of the Atlantic Blueberry Company, Hammonton, New Jersey. Pear and tomato fruits were harvested at the mature green stage and allowed to ripen at room temperature. Blueberry fruits were obtained at the green, semired, pink, and blue stages. At various stages of ripening, tissue samples were taken for the analyses of various ripening parameters and for obtaining protein extracts for isozymic analysis.

Characterization of Fruit Ripening. Changes in typical pigment and tissue texture, and in ethylene and CO_2 evolution, were used to characterize the stages of ripening in the different fruits. Lycopene accumulation was determined in tomatoes according to the method of Lime *et al.* (18). Total anthocyanin formation in blueberries was measured according to a method by Fuleki and Francis (15). Chlorophyll breakdown in the peel of pear was measured according to the method of Arnon (4).

Changes in tissue texture in fruits were measured with an L.E.E.-Kramer shear press on 20 g of fruit sample. Each sample represented 10 tomato and pear and 20 blueberry fruits, respectively.

Ethylene evolution was determined in a static system. Fruits were kept in a closed flask from which air samples were drawn for gas analysis at 30-min intervals. Ethylene was measured with an F and M gas chromatogram, model 609, employing an activated alumina column ⅛ inch × 6 ft and a flame ionization detector. The obtained values were compared against a standard curve for ethylene.

The mode of sampling and determination of CO_2 was similar to that employed for ethylene. Carbon dioxide was measured with a Beckman GCS gas chromatogram employing a stainless steel silica gel column ⅛ inch × 6 ft and a thermoconductivity detector.

All determinations were run in duplicate.

Extraction and Electrophoresis of Fruit Protein. A procedure developed by Frenkel and Hess (in preparation) was used for the aqueous extraction of the soluble protein fraction from the fruit tissue. The method consisted of the use of a frozen tissue powder preparation, extraction of protein with the aid of an electric current, and purification of the extract with a gel filtration. When warranted, the protein solutions obtained were vacuum-concentrated and protein content was determined according to the procedure of Lowry *et al.* (20). A gel electrophoresis method was used to obtain isozymic patterns on an acrylamide gel (23). The standard sample size used for electrophoresis contained 200 μg of protein. Isozymic patterns were developed according to the following procedure.

IAA oxidase. Gels were incubated overnight in a reaction mixture containing 2 mM IAA, 0.1 mM dichlorophenol, and 0.1 mM $MnCl_2$ in a 0.1 M phosphate buffer, pH 6.0. The isozymes were visualized by transferring the gels, at the end of the incubation period, to a modified reaction mixture used by Meudt and Gaines (22) containing 0.5% *p-N,N*-dimethylaminocinnamaldehyde in 1 N HCl. The zymogram data were recorded immediately, before band fading occurred.

Peroxidase. Peroxidase staining was performed according to the method of Scandalios (28).

The electrophoresis data were recorded by calculating the R_F of the various isozymes, and by color film photography from which black and white prints were made.

RESULTS

The data were arranged to demonstrate the relationship between changes in PRO[1] and IAA-oxidase isozymes and various parameters of fruit ripening.

[1] Abbreviation: PRO: peroxidase.

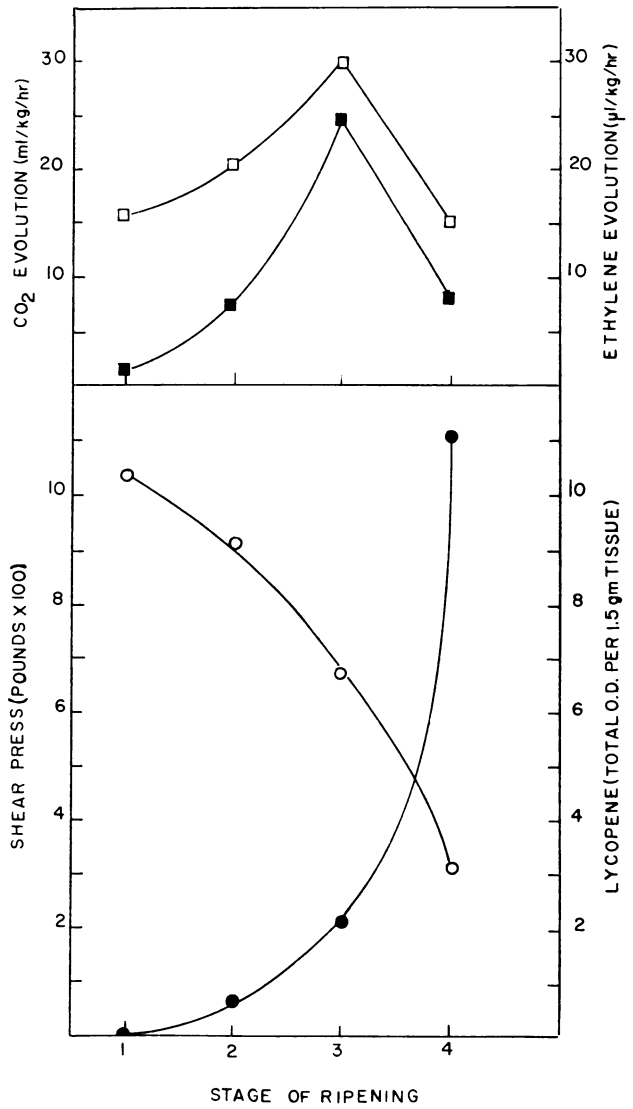

FIG. 3. The changes in ethylene (■) and CO_2 evolution (□) in relation to lycopene accumulation (●) and change in tissue texture (○) at various ripening stages of tomato.

In climacteric fruits (pear and tomato), changes in CO_2 and ethylene evolution served as a framework for the characterization of the ripening stages (24). In pear (Fig. 1), the ripening stages (1 through 4) correspond to the preclimacteric stage, early and midclimacteric, and the climacteric peak, respectively. Related changes in chlorophyll disappearance and tissue texture are indicated.

Figure 2A shows the corresponding changes in PRO isozymes during pear ripening. An intensification in three major isozymic forms (bands a, b, and c) is evident with ripening. Similar behavior was demonstrated by IAA-oxidase isozymes (Fig. 2B); band b showed an early intensification and was followed by a reinforcement in band a as ripening progressed.

In tomato (Fig. 3) the ripening stages (1 through 4) corresponded to the preclimacteric, early climacteric, the climacteric peak, and the postclimacteric stage, respectively, and were characterized by the related changes in color and tissue texture. As was observed with pear, an enhancement is seen in major isozymic component of PRO (Fig. 4A, band a) and IAA-oxidase (Fig. 4B, band a) as the fruit ripens.

In blueberry fruits, CO_2 or ethylene evolution (Fig. 5) was unpronounced and therefore could not be used to characterize

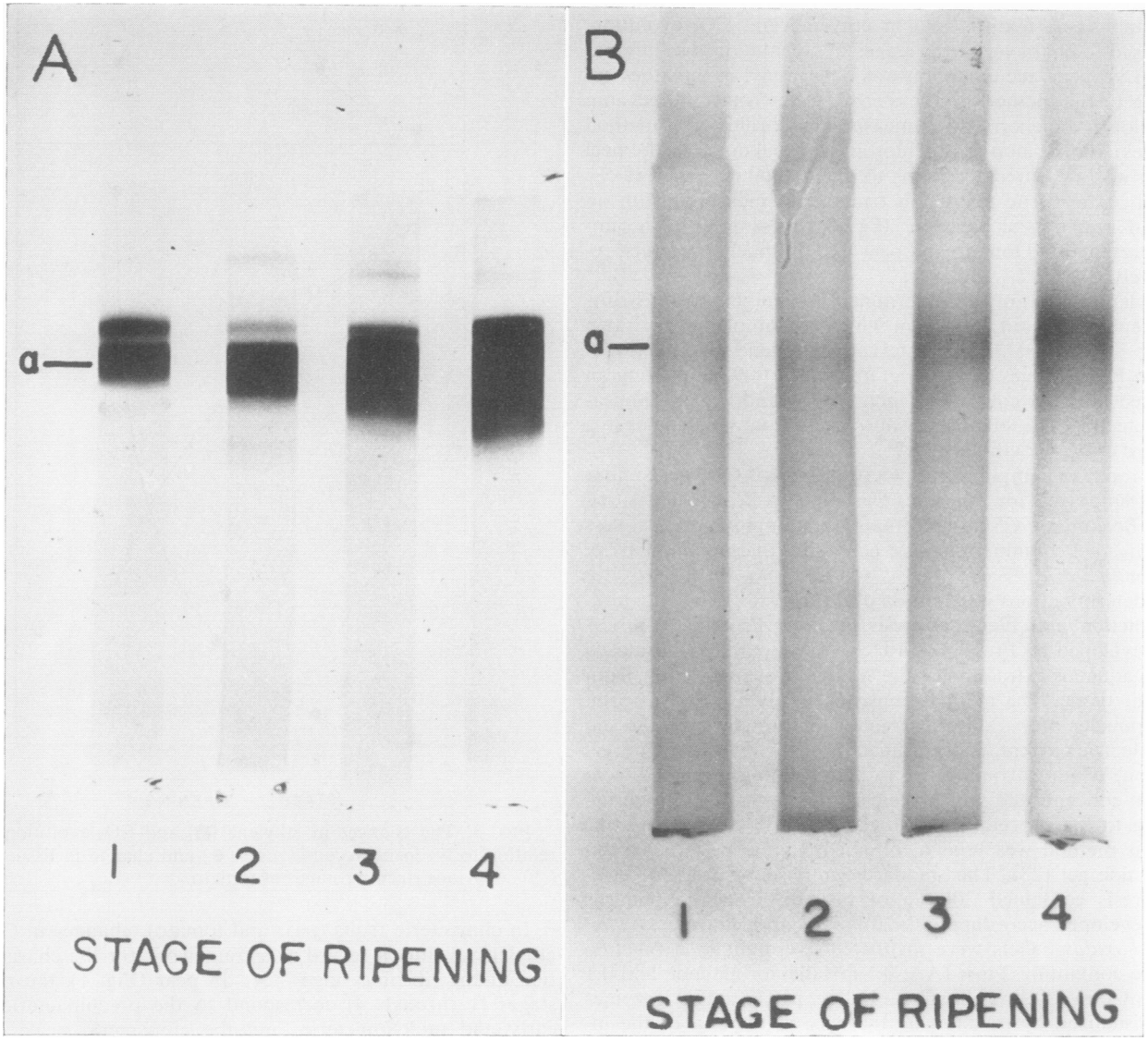

FIG. 4. The isozymic pattern of PRO (A) and IAA oxidase (B) from tomato on acrylamide gel. Isozymic patterns were obtained from protein extracts of pulp tissue during the various ripening stages of the fruit characterized in Figure 3.

the ripening stages. The data confirm a similar observation by Forsyth and Hall (13), who concluded that color is the best criterion of ripening in blueberry. Consequently, the ripening stages were selected on the basis of background color: green, semired, pink, and blue represented the ripening stages 1 through 4, respectively. The corresponding changes in the isozymic profile of PRO (Fig. 6A) are opposite in trend to those observed in pear and tomato. A general weakening is observed in enzyme forms (bands b, c, d, e, and f) as the fruit ripens. However, the trend with respect to the IAA-oxidase system (Fig. 6B, band a) is consistent with the changes in pear and tomato. That is, a reinforcement of the enzyme is observed with ripening.

The possibility that IAA-oxidase activity is the expression of PRO activity (29) was examined by comparing the electrophoretic behavior of isozyme forms in the two systems (Fig. 7). In pear and tomato, IAA-oxidase isozymes corresponded to major PRO form. However, in blueberry, such a relationship was not evident. Furthermore, whereas in blueberry a general weakening is observed in the expression of the PRO isozymes, IAA-oxidase showed a trend toward intensification with ripening. Thus, the changes in the IAA-oxidase system could be an independent cellular expression during the ripening of fruits.

The observed changes in the intensity of the isozymes are not attributed to an effect by cellular cofactors which can influence enzymatic activity (3). The steps employed (see "Materials and Methods") during the purification and electrophoresis of the protein extracts separated interfering cofactors from the resolved protein fractions. Therefore, the changes in activity reflect, in all probability, the relative abundance of an enzyme protein, resulting from the balance between degradation and synthesis of enzymes during fruit ripening (14, 17).

DISCUSSION

The data confirmed previous observations relating to the ability of various classes of fruit to exhibit the climacteric rise in respiration (5). Pear and tomato (Figs. 1 and 3, respectively) showed a typical respiratory rise; in blueberry (Fig. 5), the changes in respiration during ripening were not pronounced. Although the significance of the climacteric rise in respiration, which sometimes accompanies fruit ripening, has been minimized (25), it is offered as a convenient frame of reference for the cumulative expenditure of cellular work during fruit ripening (24).

The value of monitoring the changes in the level of ethylene in fruit tissue is debatable for the following reasons: at maturity, fruits usually contain sufficient ethylene to trigger ripening, but the onset of ripening is attributed to the ability of the tissue to respond to the gas, presumably due to the disappearance of a restraining factor rather than to the increase in ethylene level *per se* (21). After ripening has commenced, ethylene evolution is considered no more than an adjunct process and would indicate, at best, the ability of some fruits, such as pear or tomato, to accomplish an autocatalytic synthesis of the gas. Others (blueberry) lack this capability (24, 25).

The emphasis on changes in a restraining factor, or what is sometimes termed resistance to ripening (16), implies that the presence of ethylene alone is not enough to account for the triggering of ripening in fruits. Following previous suggestions in the literature (10, 11, 24), it is proposed that other growth-regulatory systems in fruits undergo changes, in preparation for ripening, and that such modification could account for the changes in the resistance to ripening and to ethylene action. As with leaf senescence, it is conceivable that fruit ripening marks a decline in several growth regulators. Instances are known

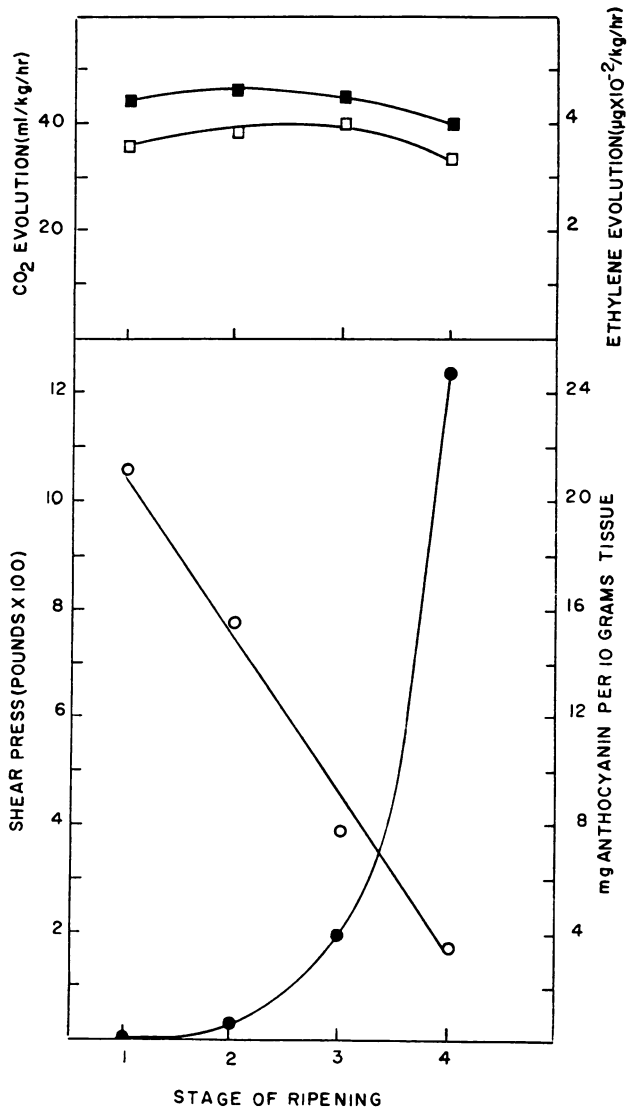

FIG. 5. The changes in ethylene (■) and CO_2 evolution (□) in relation to anthocyanin accumulation (●) and changes in tissue texture (○) at various ripening stages of blueberry.

when applications of auxins (2, 32), gibberellins (12, 27), or kinins (1) actually retarded ripening and ethylene action. From that it can be inferred that an inverse hormonal balance could potentiate fruit tissue for ripening and ethylene action.

A mechanism which could possibly explain the decline in the level of growth substances, and perhaps also the resistance to ripening, would be an increase in the cellular capacity for the degradation of phytohormones. A specific candidate for this role is the PRO system, since this enzyme system is said to exhibit IAA-oxidase activity (29). The reinforcement of PRO activity in pear and tomato (Figs. 2A and 4A, respectively) is compatible with the above suggestion; in blueberry (Fig. 6A), where a general diminution is observed in the PRO isozymes during ripening, it is not. On the other hand, a specific enzymatic test for an IAA degrading system revealed that a capacity for IAA oxidation is developed in fruits during ripening irrespective of their variability in regard to PRO activity. The consistency in IAA-oxidase activity conforms, therefore, to the contention that fruit ripening is accompanied by an increase in the degradative capacity with respect to auxins, and possibly other plant hormones. The enhancement of IAA-oxidase activity during fruit ripening appears to be

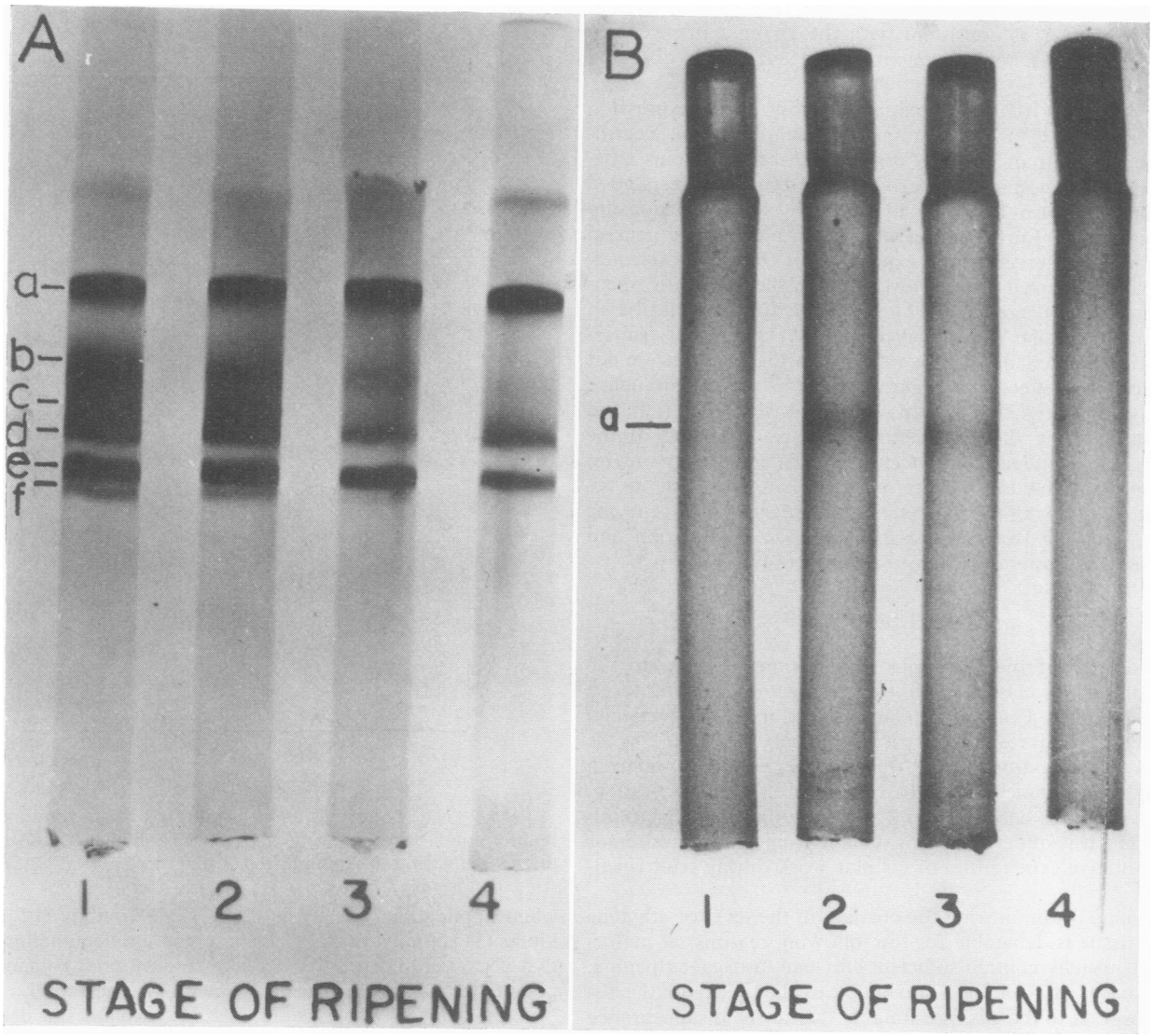

FIG. 6. The isozymic pattern of PRO (A) and IAA oxidase from blueberry on acrylamide gel. Isozymic patterns were obtained from protein extracts of pulp tissue during the various ripening stages of the fruit characterized in Figure 5.

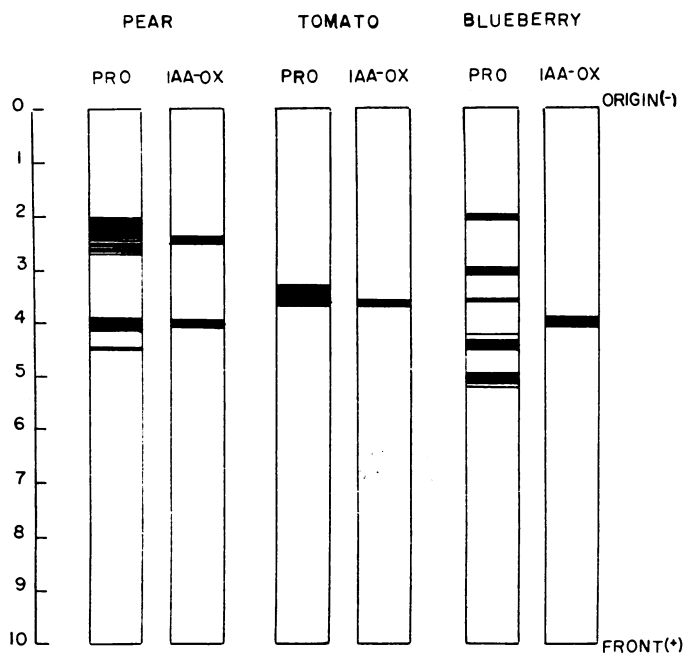

FIG. 7. A zymogram showing a comparative rate of flow of PRO and IAA oxidase isozymes from pear, tomato, and blueberry, on acrylamide gel. Isozymic patterns were obtained from protein extracts of fruits tissue at ripening stage 3.

widespread. although the ubiquity of the phenomenon needs further proof.

Certain questions remain. It has yet to be demonstrated that the IAA-oxidase system is capable of an *in vivo* turnover of auxins. In addition, the metabolic independence of this system from the PRO system in fruit ripening is not established conclusively. Finally, the cellular role of IAA-oxidase must be confirmed by the following: (*a*) a demonstration that the enzyme system does cause a decline in the auxin content in fruit tissue during ripening; and (*b*) such a decline can explain, at least in part, the disappearance of the resistance to ethylene action and ripening.

LITERATURE CITED

1. ABDEL-GAWAD, H. A. AND R. J. ROMANI. 1967. Effects of phytohormones on maturation and postharvest behavior of fruits. Plant Physiol. 42: S-43.
2. ABELES, F. B. AND B. RUBENSTEIN. 1964. Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol. 39: 963–969.
3. ANDERSON. J. W. 1968. Extraction of enzymes and subcellular organelles from plant tissue. Phytochemistry 7: 1973–1988.
4. ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
5. BIALE, J. B. 1960. Respiration of fruits. Handb. Pflanzenphysiol. 12: 536–592.
6. BRADY, C. J., J. K. PALMER, P. B. H. O'CONNELL, AND R. M. SMILLIE. 1970. An increase in protein synthesis during ripening of banana fruit. Phytochemistry 9: 1037–1047.
7. BURG, S. P. 1962. The physiology of ethylene formation. Annu. Rev. Plant Physiol. 13: 265–302.
8. BURG, S. P. AND E. A. BURG. 1965. Ethylene action and the ripening of fruits. Science 148: 1190–1196.
9. CHIN, T.-Y. AND L. BEEVERS. 1970. Changes in endogenous growth regulators in *Nasturtium* leaves during senescence. Planta 92: 178–188.
10. CRANE, J. C. 1969. The roles of hormones in fruit set and development. Hort. Sci. 4(2): 108–110.
11. DILLEY, D. R. 1969. Hormonal control of fruit ripening. Hort. Sci. 4(2): 111–114.
12. DOSTAL, H. C. AND A. C. LEOPOLD. 1967. Gibberellin delays ripening of tomatoes. Science 158: 1579–1580.
13. FORSYTH, F. R. AND I. V. HALL. 1969. Ethylene production with accompanying respiration rates from time of blossoming to fruit maturity in three *Vaccinium* species. Natur. Can. 96: 257–259.
14. FRENEL, C., I. KLEIN, AND D. R. DILLEY. 1968. Protein synthesis in relation to ripening of pome fruits. Plant Physiol. 43: 1146–1153.
15. FULEKI, T. AND F. J. FRANCIS. 1968. Quantitative methods for anthocyanins. I. Extraction and determination of total anthocyanin in cranberries. J. Food Sci. 33: 72–77.
16. HANSON, E. 1966. Post-harvest physiology of fruits. Annu. Rev. Plant Physiol. 17: 459–480.
17. HULME, A. C., M. J. C. RHODES, T. GALLIARD, AND L. S. C. WOOLTORTON. 1968. Metabolic changes in excised fruit tissue. IV. Changes occurring in disc of apple peel during the development of the respiration climacteric. Plant Physiol. 43: 1154–1161.
18. LIME, J. B., F. P. GRIFFITHS, R. T. O'CONNOR, D. C. HEINZELMAN, AND E. R. MCCALL. 1957. Spectrophotometric method for determining pigmentation—beta carotene and lycopene—in ruby red grapefruit. Agr. Food Chem. 5: 941–944.
19. LOONEY, N. E. AND M. E. PATTERSON. 1967. Changes in total ribonucleic acid during the climacteric phase in yellow transparent apples. Phytochemistry 6: 1517–1520.
20. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–285.
21. MAPSON, L. W. 1970. Biosynthesis of ethylene and the ripening of fruit. Endeavour 29: 29–33.
22. MEUDT, W. J. AND T. P. GAINES. 1967. Studies on the oxidation of indole-3-acetic acid by peroxidase enzymes. I. Colorimetric determination of indole-3-acetic acid oxidation products. Plant Physiol. 42: 1395–1399.
23. ORNSTEIN, L. AND B. J. DAVIS. 1961. Disc electrophoresis. Distillation Products, Rochester, N. Y.
24. PRATT, H. K. AND J. D. GOESCHL. 1969. Physiological roles of ethylene in plants. Annu. Rev. Plant Physiol. 20: 265–302.
25. REID, M. S. AND H. K. PRATT. 1970. Ethylene and the respiration climacteric. Nature 226: 976–977.
26. RICHMOND, A. E. AND J. B. BIALE. 1967. Protein and nucleic acid metabolism in fruits. II. RNA synthesis during the respiratory rise in avocado. Biochim. Biophys. Acta 138: 625–627.
27. RUSSO, L., JR., H. C. DOSTAL, AND A. C. LEOPOLD. 1968. Chemical regulation of fruit ripening. Bioscience 18: 109.
28. SCANDALIOS, J. G. 1964. Tissue-specific isozyme variations in maize. J. Hered. 55: 281–285.
29. SIEGEL, B. Z. AND A. W. GALSTON. 1967. Indolacetic acid oxidase activity of apoperoxidase. Science 157: 1557–1559.
30. SPENCER, M. 1965. Fruit ripening. *In:* J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, New York. pp. 793–825.
31. VARNER, J. E. 1965. Death. *In:* J. Bonner and J. E. Varner, eds., Plant Biochemistry. Academic Press, New York. pp. 867–871.
32. VENDRELL, M. 1969. Reversion of senescence: effect of 2,4-dichlorophenoxyacetic acid and indoleacetic acid on respiration, ethylene production, and ripening of banana fruit slices. Aust. J. Biol. Sci. 22: 601–610.