Growth, Maturation, and Senescence in Fruits

Recent knowledge on growth regulation and on biological oxidations has been applied to studies with fruits.

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The flowering plants known as the angiosperms are the dominant and most widely distributed recent floras on earth. In the course of evolution of this group of plants the leaf bearing a rudimentary seed, the ovule, folded or rolled, and its edges fused to form a carpel with a seed inside a hollow chamber. The method of direct pollination, feasible in and characteristic of the naked-seeded gymnosperms, became impossible in the angiosperms, with ovules enclosed in structures consisting of one or more carpels. The pistil then evolved, consisting of an ovary attached to a receptacle, a column known as the style arising from the top of the ovary, and a terminal stigma possessing a surface favorable for the reception and germination of pollen. The enclosure of the ovule within a hollow ovary was the evolutionary signal for the development of fruits in response to processes connected with pollen tube growth and fertilization. In a strict sense, fruit tissue does not contribute directly to the perpetuation of the species, the function reserved for the embryo within the seed. However, the formation and growth of the fruit is closely associated with and influenced by the reproductive apparatus of the plant. The restricted definition of a fruit implies a structure arising from the expansion of the ovarian wall. In fact, most common fruits consist of single enlarged ovaries. Others, such as blackberry and strawberry, are made up of a number of ovaries belonging to a single flower and scattered over the surface of a single receptacle. The seedlike structures on the periphery of the strawberry are actually the fruits, and the main edible portion is the receptacle. Still other fruits consist of enlarged ovaries of several flowers, including secondary floral parts fused to form a single fruit. Examples of this class are the pineapple and the fig. Fruits may be fleshy (apple, tomato, and orange) or they may be dry (peas, wheat, corn, and nuts).

Fruits and seeds have been useful to man in many ways. Wheat, rice, corn, rye, and barley are the great staple foods of the world. Fleshy fruits are a source of carbohydrates, acids, vitamins, and minerals in man's diet. Fruits and seeds are also a source of oils, waxes, drugs, herbs, dyes, and other products. Because of the economic importance of fruits, investigators directed their attention to production and post-harvest problems. In recent years it has been realized that fruits offer desirable material for the study of physiological and biochemical processes connected with differentiation of structure and function during development, maturation, and senescence.

Regulation of Fruit Growth

The transformation of a plant from vegetative to reproductive status is governed by nutrition, photoperiod, fluctuation in diurnal temperature, hormonal influences, and other factors. As a result of the interactions of the various factors, floral primordia or flower buds are laid down. The developing flower may be perfect, possessing both stamens and pistils, or the staminate flowers may be distinct from the female pistillate flowers. The growth of the differentiated structure before full bloom is chiefly by cell multiplication. The critical stage in the growth of the ovary, the tissue of the future fruit, occurs at anthesis (flower opening). It has been widely known that pollination (Fig. 1), the germination of the pollen and the growth of the pollen tube in the direction of the ovule, plays a key role in fruit formation. In many instances the mere germination of the pollen or partial growth of the pollen tube without fertilization (the fusion of male and female nuclei) is sufficient to cause the ovarian wall or other floral parts to swell and commence to grow into a fruit. In fertilization (Fig. 1) the male nuclei are discharged into the embryo sac, where normally one unites with the egg nucleus to form a diploid embryo and the other fuses with two polar nuclei to give rise to the triploid endosperm.

Pollination and fertilization involve the ovules and do not directly involve the ovary or receptacle which is converted into fruit tissue. In some cases ovary growth is visible even before the pollen has germinated. The question was asked, What message does pollen convey for the initiation of fruit growth? The story unfolded (*1*) when it was discovered that growth of the pollen tube was not essential, that extracts of pollen could be substituted for pollen itself, and that pollen contained a substance which gave a positive response in the *Avena* assay, a test specific for phytohormones, especially for indoleacetic acid. This compound, as well as other nonnative auxins, was found to induce fruit formation in unpollinated ovaries of several species.

A more detailed analysis of the auxin relationships in floral structures has shown that pollen grains cannot account for the hormone content but that the growth of the pollen tube into the ovary results in increased auxin production, and that the growing seed, particularly the endosperm, is an important source of inhibitors as well as promoters of fruit growth (*2*). Some of the strongest arguments for the key role of auxin were gained from studies with the strawberry (*1*), in which the period of rapid growth was found to correspond with the period of auxin accumulation in the achenes. Moreover, direct application of auxin could be substituted for achenes. Auxin was also found to be essential for the in vitro growth of tomato ovaries. Doubts were raised (*2*) as to whether auxin alone has this growth-promoting role by the finding that, in some cases,

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measurable quantities of auxin did not appear before fruit growth was well under way. In several cases auxins did not induce parthenocarpic fruit set. Recently attention was focused on gibberellins and kinins as additional factors in fruit set, in germination and growth of pollen, and in the growth of the fruit to maturity. One may conceive, therefore, of fruit development as being regulated by any one of several substances or by the interactions of several factors rather than any single one. Some of the substances believed to be regulators of fruit growth are listed in Fig. 2.

Compositional Diversity, Physiological Uniformity

Prior to pollination, as well as during a relatively short period after anthesis, the growth of the tissue which makes up the fruit is mainly by cell division. In the case of the apple, cell multiplication is completed within 3 to 4 weeks after full bloom. In the avocado, on the other hand, equatorial plates in cells were observed even in fully mature fruit. In all cases cell expansion extends into the maturation stage (Fig. 3). The growth pattern is either a single sigmoid curve or double curves separated by a plateau between the two cycles. The separation has been related to the competition between embryo and fruit for growth substances produced by endosperm.

During the early stage of cell division there appears to be little difference between cells of different species. Proteins are the chief constituent of the cytoplasm of young undifferentiated cells. With cell enlargement vacuoles appear, and protein content, on a weight or volume basis, is diluted. Carbohydrates are transported from the leaves, but many of the substances found in fruits can be synthesized by fruit tissue. Differentiation of composition is evident in the early stages of cell enlargement, with resulting diversity in composition at maturity (Table 1).

The composition of the edible portion varies considerably among fleshy fruits at harvest time. The dry-matter content may be as high as 80 percent of total weight for some varieties of date, though in most fruits it is 10 to 15 percent. Carbohydrate content in the majority of species is 10 to 20 percent, but there are notable exceptions, such as 5 percent or less for the avocado and about 75 percent for the date. Fruits low in carbohydrates are frequently high in fats. Protein content varies from values as low as 0.4 percent of fresh weight for the pineapple to a high of 1.7 percent for the avocado. Total acid content is also highly variable, ranging from about 5 percent for the lemon to 0.1 percent for the persimmon. Malic and citric acids predominate, though a number of other acids, including those of the Krebs cycle, were reported.

Despite the diversity in composition and morphological origin, all fruits are markedly similar in physiological responses and metabolic behavior. While attached to the plant the fruit is dependent on assimilatory processes, including photosynthesis, in the leaves; on the capacity of the roots to absorb minerals and water; and on the rate of conduction of materials, both inorganic and organic, through the vascular system of the roots and of the stem. Growth-promoting and growth-inhibiting substances moving into and out of the fruit no doubt play a major role in the maturation process, though relatively little is known about their nature. Once the fruit is detached from the parent plant the process of biological oxidation, with its respiratory and fermentative features, assumes a dominant role. In the glycolytic pathway carbohydrates are broken down to pyruvic acid, which in turn is oxidized to CO_2 and H_2O through the tricarboxylic acid cycle. The mechanism of oxidation in fruits does not differ materially from that in other plant or animal tissue. The distinguishing feature in fruits is the trend of overall respiratory activity during the final stage of ontogeny. The patterns of respiration for two classes of fruits are given in Fig. 3. In many fruits an upsurge in respiratory activity may be observed at the end of the maturation phase. This phenomenon, known to fruit physiologists as the "climacteric rise" in respiration, has attracted a great deal of attention (*3–5*), since it is considered a transition stage between the developmental phase of maturation and the final protoplasmic disorganization during senescence. A typical example is shown in Fig. 4, for the avocado.

Table 1. Major compositional changes in the edible fraction of several selected fruits.

Constituent	Content at maturity* (%, fresh weight)	Content upon ripening† (% of content at maturity)
	Apple	
Starch	2.0	5
Soluble sugars	7.5	99
Acids (malic)	1.0	60
Protein	0.2	120
Protopectin	.7	12
Soluble pectin	.2	160
	Avocado	
Sugars	0.4	12
Fat	20.0	105
Protein	1.8	110
	Banana	
Starch	20.0	6
Sugars	0.9	2000
Protopectin	.5	40
Soluble pectin	.3	150
	Orange	
Sugars	10.0	105
Acids (citric)	0.9	85
	Pineapple	
Sugars	15.0	103
Acid	0.8	88

* "Content at maturity" means content when the fruit is normally harvested in mature but not necessarily ripe stage. † "Content upon ripening" refers to content at edible stage.

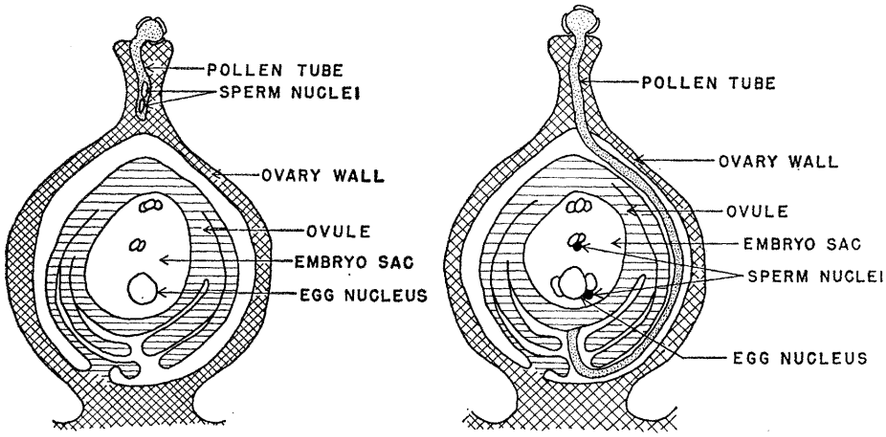


Fig. 1. Prelude to fruit formation. (Left) Pollination: germination of pollen grain on stigma and growth of pollen tube into the ovarian wall; (right) fertilization: fusion of male and female nuclei.

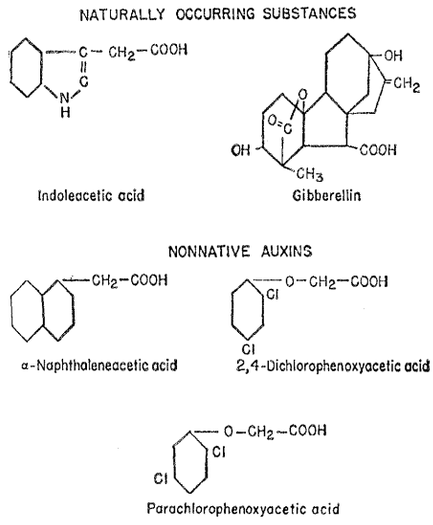


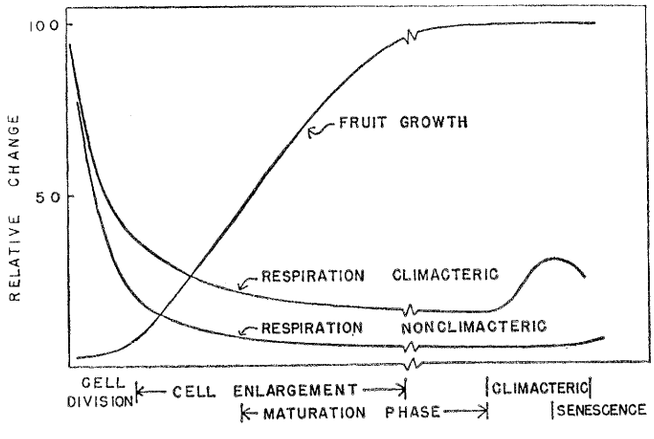
Fig. 2. Growth substances controlling the formation, development, and maturation of fruits.

The avocado has the distinctive feature of remaining firm as long as the stem connecting the fruit to the tree is sound. Certain varieties may be attached to the tree for several months without showing signs of ripening. But shortly after harvesting, when the fruit is kept under ordinary conditions of temperature and oxygen supply, the rate of respiration declines to a minimum and then rapidly rises to a peak value. The "preclimacteric minimum" and the "climacteric peak" values are characteristic for a particular species under a given set of conditions. The duration of the rise (the time between minimum and peak), or the slope, is a function of external conditions and of state of maturity. The respiratory rate reaches a minimum sooner and follows a steeper slope in late-season fruit than in fruits picked in the early stages of maturation. Ripening is thus accelerated in late fruit, since the stage of optimum edibility is linked to the climacteric pattern and occurs shortly after the peak of respiratory activity. In many fruits, changes in color as well as in texture are associated with the climacteric phenomenon.

In some fruits pronounced chemical changes are also tied to the climacteric (Table 1). In the edible portion of the banana, for example, the starch content may drop from about 20 percent of fresh weight to less than 1 percent, and sugars increase correspondingly from about 1 percent to 15 percent in the span of several days, the fruit color changing from green to yellow. The peel, too, undergoes marked changes, though not as striking as those of the pulp. In both types of tissue there is a net decrease of dry matter, since sugars are utilized as respiratory substrates. The disappearance of carbohydrates, fats, or proteins cannot always account for the CO_2 produced in respiration. The avocado, with high fat content, does not utilize this substrate for CO_2 production during the climacteric cycle. The sugar content is normally insufficient to account for the respiratory losses. In this case, as well as in many other cases, hemicelluloses and pectins may account for the CO_2 balance sheet, but direct evidence is unavailable.

Pectins are of special significance in fruit ripening. The chemistry of these substances is not fully elucidated, but it is known that the basic structure is a chain of galacturonic acid units combined through glycosidic linkages. The methoxyl content of a pectin is a distinguishing feature, and it depends on the degree of esterification of the carboxylic acids of the basic unit. When calcium takes the place of the methyl group, the result is calcium pectate, a substance present in the middle lamella between adjacent cell walls. Calcium pectate and insoluble pectins referred to by the generic name of protopectin are present in unripe fruit and are converted to soluble forms in the process of ripening. This transition does not necessarily involve the shortening of the polygalacturonide chain, but it does involve conversion into the methylated and the free acid form. Chain splitting is implied in instances in which pectins are required as respiratory substrate. The hydrolysis of methyl groups is brought about by pectin methyl esterase, and the chain splitting, by pectinase or polygalacturonase. It has been reported (*4*, *5*) that activities of these enzymes change in relation to the rate of respiratory rise. However, changes in enzymatic activities may be brought about by changes in certain endogenous substances. For example, it was shown in this laboratory (*6*) that aldolase activity decreased when tissue was homogenized in the presence of tannins released from the cell. The inactivating leucoanthocyanins were freely diffusable in unripe but not in ripe tissue.

The climacteric phenomenon is well established as a respiratory pattern for



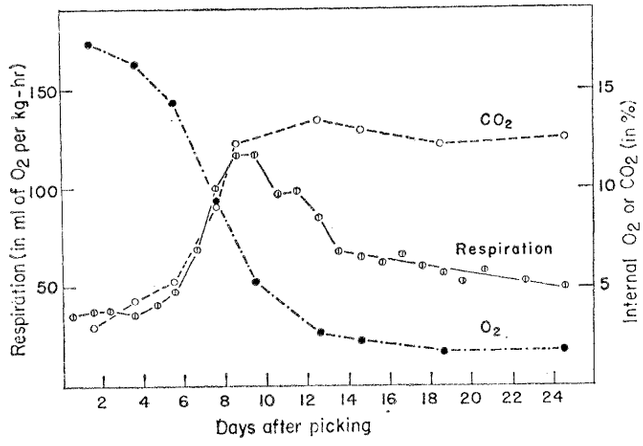


Fig. 3 (left). Stages in fruit development and maturation and respiratory trends unique to two classes of fruits—fruits which do and fruits which do not show the climacteric phenomenon. The discontinuity is due to the variable time scale and indicates differences in the length of the maturation phase for various fruits. The growth pattern may be single or double sigmoid. Fig. 4 (right). Concentrations of oxygen and carbon dioxide in the avocado in relation to respiratory rise and fall (*10*).

fruits of the various climatic zones and of widely different families. Experimental evidence for its existence is readily obtainable. However, it is by no means the only pattern. In other fruits, such as citrus, grape, and pineapple, the gradual chemical and physiological changes observed during the stage of cell enlargement and maturation continue through senescence. No abrupt upsurge in oxygen uptake can be detected (Fig. 3) until the onset of microbial or fungal attack. The case is perhaps best illustrated by citrus fruits. The orange, lemon, and grapefruit are "tree-ripened"—that is, they attain the desired composition before and not after harvesting. When they are detached their respiration continues at a slowly declining rate. This behavior appears to hold for fruits, such as the strawberry, with high metabolic activity as well as for fruits of the citrus genus characterized by relatively low rates of respiration (see Tables 2 and 3).

Table 2. Rates of respiration, at 20°C, for fruits that show the climacteric phenomenon (compare Table 3).

Fruit	Respiration (ml of O_2 or CO_2 per kg hr)	
	Climacteric min.*	Climacteric max.
Avocado	35	155
Banana	20	60
Cherimoya	37	130
Passion fruit	25	45
Pear	12	33
Plum	9	21

* Immediately preceding respiratory rise.

External Control of Maturation

The course of metabolism can be regulated, both in fruits that show a climacteric pattern ("climacteric fruit") and in fruits that do not ("nonclimacteric fruit"), by changes in temperature and in concentration of oxygen and carbon dioxide in the external environment. In both types of fruit a temperature response typical of chemical reactions is limited to a very narrow range of temperatures. In general, the temperature coefficient (the ratio of highest to lowest rate of respiration for comparable stages, for a span of 10°C), is a great deal higher in the range 5° to 15°C than in the range 20° to 30°C. In the avocado, for example, the climacteric pattern is suppressed at 5°C. However, upon transfer of the avocado to higher temperatures the typical respiratory rise is induced and normal ripening takes place, provided storage at 5°C has been of relatively short duration. Prolonged exposure to low temperature causes irreversible suppression of the climacteric phenomenon. The banana shows chilling injury at temperatures below 12°C, as evidenced by its failure to ripen when it has been exposed to these conditions. On the other hand, a number of fruits have been kept at temperatures close to freezing for long periods without any manifestations of such injury. The difference between chilling-resistant and chilling-sensitive plant materials has been ascribed (*7*) to differences in the physical nature of mitochondrial particles. The greatest flexibility of the mitochondrial membrane, apparently associated with a high degree of unsaturation of the fatty acids, was observed in mitochondria from species resistant to chilling, while the reverse was the case in species sensitive to chilling.

High temperatures as well as low ones tend to suppress the climacteric phenomenon. In the Fuerte avocado the peak value in CO_2 evolution was found to be much lower at 30° than at 25°C (*8*). This behavior also implies impairment in ripening. It appears that tropical and subtropical climacteric fruits, though grown under conditions of high temperature, show great sensitivity to temperature extremes when they are detached from the tree. On the other hand, many of the tropical and subtropical nonclimacteric fruits appear to be more resistant to exposure for long periods to temperatures of 30° and 35°C. In the case of the temperate-zone fruits there is a broader range of temperatures for which respiration can be expressed as a function of temperature, much as temperature response in chemical reactions is expressed (*5*).

The critical components of the atmosphere which regulate the maturation process in fruits are oxygen and carbon dioxide. The factors involved are the rates of respiration, the rates of diffusion of these gases, the path of diffusion, and the condition of the intercellular spaces. The response to limiting oxygen condition is not the same in all fruits. In the avocado the climacteric pattern was completely abolished by anaerobiosis, and ripening was suppressed irreversibly. The effect was similar with bananas, though less pronounced. On the other hand, oranges and lemons kept in an environment of pure nitrogen maintained for several weeks a CO_2 output which was higher than the output when they were kept in air. The switch to fermentative processes occurs in citrus fruit at oxygen concentrations below 2.5 percent. Prolonged fermentation brings about the accumulation of toxic materials which are detrimental to the life and the quality of the fruit. At oxygen concentration of 5 percent the rate of oxidative processes was reduced considerably, relative to the rate of these processes in an environment of air, and the fermentative reactions were also at a minimum. The net result was an increase in vitality and storage life of the fruit. The avocado benefits similarly from maintenance at low oxygen concentrations, though the respiration-oxygen tension curves were different from the curves for citrus fruit (*5, 8*). At partial pressures of oxygen lower than those in air the rate of CO_2 production in avocados dropped markedly, while at partial pressures higher than those in air the increase was barely significant. Temperature exerted a pronounced influence on the response to oxygen tension. At 5° and at 7.5°C, temperature became the limiting factor, even when oxygen was abundant.

Table 3. Rates of steady-state respiration, at 20°C, for fruits that do not show the climacteric phenomenon (compare Table 2).

Fruit	Respiration (ml of O_2 or CO_2 per kg hr)
Grape	13
Lemon	9
Orange	12
Pineapple	15
Strawberry	65

The addition of CO_2 to air or to oxygen at a concentration other than that in air tends to prolong the maturation process in both climacteric and nonclimacteric fruits. The time required for reaching the climacteric peak was increased markedly as the rate of gas exchange at the peak was lowered. The effect was a function of CO_2 concentration, at least up to a concentration of 10 percent. An exception to this behavior was observed in lemons (*9*), in which the addition of CO_2 stimulated rather than suppressed oxygen uptake. Evidence was cited in support of a CO_2 fixing mechanism which brings about the formation of Krebs cycle acids critical for the maintenance of optimal rates of respira-

tion. When lemons were exposed to an atmosphere enriched with labeled CO_2, the first radioactive products were malate, citrate, and aspartate. These three acids can be formed by single-step reactions from oxalacetate, a product of CO_2 fixation. The role of CO_2 and the controlling influence of other external factors in fruit ripening are discussed more fully elsewhere (*5*).

Endogenous Regulatory Mechanisms

The internal atmosphere. In view of the effects of O_2 and CO_2 on respiration, the question arose, Do changes in the gaseous composition in the intercellular spaces play a role in inducing the climacteric rise? A study was undertaken (*10*) to follow the internal O_2 and CO_2 concentrations associated with oxygen uptake by the intact avocado fruit. As Fig. 4 shows, the onset of the respiratory rise coincided with the decline in the oxygen content of the fruit. The rise in CO_2 concentration closely followed the climacteric up to the peak. Since the onset of the rise was observed at oxygen concentrations of 2.5 to 100 percent, as well as at CO_2 concentrations of 5 and 10 percent, it is unlikely that these components of the atmosphere play an inductive role. The high concentration of CO_2 and the low concentration of oxygen in the postclimacteric phase, despite the drop in respiration, were probably due to filling of the intercellular spaces with liquid from the cells. The rate of leakage from cells increases with ripening, and this is probably an important factor in the acceleration of senescence. A correlation was found (*11*) between CO_2 evolution by bananas and the leakage of solutes from sections of tissue. The loss of permeability barriers must have an important bearing on compartmentalization within the cytoplasm.

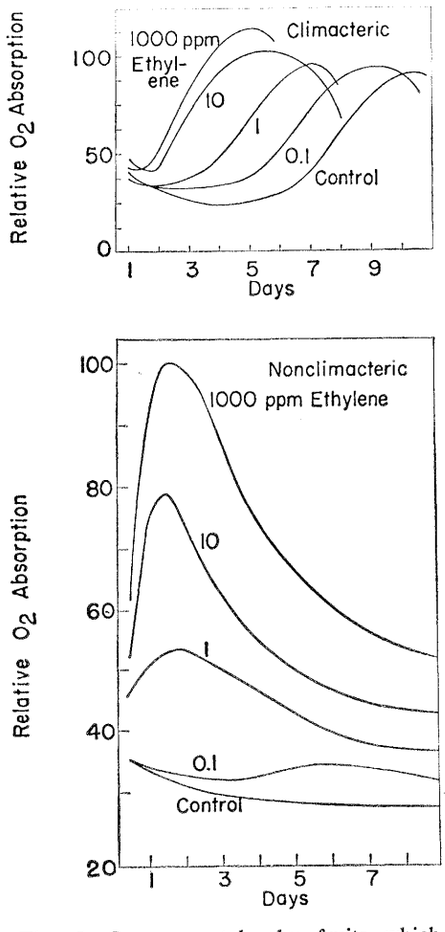


Fig. 5. Oxygen uptake by fruits which show the climacteric phenomenon and by fruits which do not, in relation to concentration of external ethylene.

Role of ethylene. It had been known for four decades that ethylene, the unsaturated hydrocarbon $CH_2 = CH_2$, is capable of accelerating coloring, ripening, and respiration in fruits and vegetables. However, a thorough experimental examination of its role in fruit metabolism was made possible only in recent years (*5, 12–14*), after the development of adequate methodology for the determination of minute quantities of this gas. Application of ethylene to a variety of fruits of the climacteric class produced an early onset of the respiratory rise but, with few exceptions, did not alter significantly the peak values for oxygen uptake or CO_2 evolution. The shift in the time pattern for the climacteric took place at and above external concentrations of 0.1 part per million, but not at lower concentrations. The external concentration instrumental in bringing about maximum acceleration differed for different fruits. This value was 10 parts per million for the avocado and 1 part per million for the banana. The difference might be ascribed to differences in gaseous diffusion between these fruits. With nonclimacteric fruits the maximum values for O_2 uptake increased with increasing ethylene concentration in the range of 0.1 to 100 parts per million (Fig. 5). Moreover, the response to ethylene in this class of fruits has been demonstrated at all stages of maturity (*8*), while in climacteric fruits no stimulation occurred after the onset of the rise. The suggestion has been advanced (*13*) that the differences between the two classes of fruits in response to application of ethylene may be a reflection of differences in rate of production. The high concentrations of ethylene at the peak in the climacteric group may fail to increase the respiratory rate, while the much lower concentrations in the nonclimacteric fruits could exert a regulatory action.

There are basically two aspects to this problem: the induction of the rise and the magnitude of the rates at the peak. In both cases it is essential to know the concentrations of ethylene at the reaction sites and the threshold values required to induce a response. Experimentally one has to limit himself to the measurement of ethylene in fruit cavities or in air spaces between cells. By means of refined gas chromatography it has been possible to measure, with fair accuracy, concentrations of the order of 0.01 part per million in gas volumes extractable from a variety of fruits. With this method the ethylene content of an immature tomato for which 20 percent of the growth period had elapsed was estimated to be 0.08 part per million (*14*). Similarly, 0.04 part of C_2H_4 per million was found in the cavity of cantaloupe 10 days after pollination and 27 days before full maturity (*15*). The concentration of the gases did not change until the beginning of the respiratory rise. The values immediately preceding and coincident with the climacteric are given in Table 4.

The work with the avocado (*16*) is of particular interest. By withdrawing samples at frequent intervals immediately after harvesting, it was found that the internal concentration of ethylene at harvest time averaged 0.04 part per million. Within 9 hours after harvest and 12 to 24 hours before the onset of the rise the ethylene content was 0.1 part per million. At the onset of the climacteric rise the concentration was of the order of 0.5 to 1.0 part per million. Relatively high concentrations of ethylene have been reported (*14*) in immature tomatoes immediately after picking. Thus, the accumulated evidence that ethylene is present in the tissue of fruits prior to the onset of the rise is conclusive. However, internal ethylene concentration required to induce the respiratory rise was inferred (*12, 13*) from experiments (*5*) dealing with effects of external concentration. There is need for direct evidence that such inferences

Table 4. Internal ethylene concentration.

Fruit	Variety	Concentration (parts per million)		Reference
		Prior to respiration rise	Near climacteric peak	
Avocado	Choquette	0.2 to 0.6	300 to 700	(*16*)
Banana	Gros Michel	<0.2	40	(*13*)
Cantaloupe	No. 45	.3	40 to 70	(*15*)
Mango	Haden	.1	3	(*13*)
Tomato	VC-243-20	.8	27	(*14*)

are justified before we can ascribe to ethylene the decisive role of the endogenous triggering agent in the ripening process.

Another approach to the role of ethylene in fruit maturation and senescence was taken in studies dealing with the relationship of the formation of this hydrocarbon to metabolism. At first, comparisons were made of the effects of external conditions on both processes. It was observed (*5*) that, within a narrow range of 10° to 25°C, a given temperature exerted the same influence on respiration and on ethylene production, whereas outside this temperature range the responses diverged strikingly. Fruit kept at 5°C and then transferred to an environment at 20°C passed through the typical climacteric cycle, but ethylene production was suppressed. Similarly, at temperatures above 30°C ethylene formation was markedly suppressed under conditions which brought about increased rates of respiration. Differences in response to oxygen tension were also observed. Anaerobiosis was detrimental to the ethylene-producing mechanism, irrespective of whether the fruit had or did not have the capacity to switch to a fermentative metabolism. Upon return of the fruit to air, its ability to produce ethylene was restored. From studies on apple tissue (*12*) it was concluded that reactions preliminary to ethylene formation occur under anaerobic conditions, and that oxidative reactions are essential for certain steps in the process. In view of these findings the question was raised, Are the precursors of ethylene intermediates in the major pathways of metabolism? Tracer techniques on intact fruit showed that label in glucose was readily transferred to ethylene, but no radioactivity resulted from $C^{14}H_3{\cdot}CH_2OH$, though the CO_2 produced was radioactive. A relationship to organic acid metabolism could not be established either, since low specific activities in ethylene resulted from injection of Krebs cycle acids.

Phosphorylative control. Since respiration is the predominant process in the life of the detached fruit, it is reasonable to assume that any postulated regulatory action of endogenous substances, such as auxins and ethylene, is likely to be linked to metabolic reactions responsible for energy formation and utilization. The direct suggestion that phosphorylative coupling might be involved in the climacteric rise was obtained from a study (*17*) of the effects of 2,4-dinitrophenol (DNP) on tissue slices of the avocado fruit. The available evidence suggests that DNP brings about the hydrolysis of a complex in reaction II of the following scheme of oxidative phosphorylation:

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \quad \text{(I)}$$

$$A \sim I + X \rightleftharpoons X \sim I + A \quad \text{(II)}$$

$$X \sim I + Pi \rightleftharpoons X \sim P + I \quad \text{(III)}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \quad \text{(IV)}$$

AH_2 could be a substrate, such as succinate, or a reduced cofactor such as the reduced form of nicotinamide adenine dinucleotide (NADH), and B could be an adjacent member in the electron transport chain in the form of an oxidized flavoprotein (fp). The oxidation of reduced flavoprotein is facilitated by the transport of electrons through cytochromes and cytochrome oxidase to oxygen, the terminal electron acceptor. In a tightly coupled system three sites of phosphorylation (I) are associated with the electron transport chain as follows:

$$\text{NADH} \xrightarrow[I_1]{} \text{fp} \rightarrow \text{CoQ} \rightarrow \text{cyt } b \xrightarrow[I_2]{} \text{cyt } c \xrightarrow[I_3]{}$$
$$\text{cyt } a\text{-}a_3 \rightarrow O_2 \quad \text{(V)}$$

where CoQ is coenzyme Q and cyt stands for cytochrome. Symbol ~ represents a high-energy bond.

X in reactions III and IV is postulated to be a common intermediate capable of reacting with each of the three complexes. On the basis of recent evidence (*18*) phosphohistidine was postulated as fulfilling the role ascribed to $X \sim P$. It is thought that DNP brings about the dissociation of $X \sim I$ and hence releases or uncouples the oxidation from dependence on reaction IV. When phosphorylation is removed as a limiting reaction, respiration can proceed at a high rate. In the case of avocado tissue slices, application of DNP in 0.1 to 0.01mM concentrations caused marked increase in oxygen uptake at the preclimacteric stage but not at the climacteric peak (*17*). On the basis of these results the hypothesis was advanced that a native uncoupling agent found during the climacteric rise is responsible for the upsurge in respiration. To test this hypothesis mitochondrial activities were studied during the course of maturation and ripening.

The cytoplasmic particles of the avocado fruit as well as those of several other fruits were found (*5*) to be capable of oxidizing dicarboxylic and tricarboxylic acids and of reacting to malonate, the competitive inhibitor of succinic dehydrogenase, in a manner characteristic of the Krebs cycle. The most conclusive proof of the operation of this cycle was obtained for avocado particles, which exhibited the capacity to convert pyruvate into citrate in the presence of catalytic concentrations of a four-carbon acid. Phosphorylation accompanied the oxidations of the Krebs cycle acids. The interesting feature about phosphate incorporation was the finding that the rate of esterification did not diminish during the climacteric rise and that the particles were not in a state of uncoupling when isolated from the cell. On the contrary, the particles appeared to be resistant to the action of DNP when they were prepared from fruit at the climacteric peak, as contrasted to typical uncoupling in mitochondria from fruit in the preclimacteric stage. The effect of DNP was observed not only with α-ketoglutarate as substrate but also with malate, pyruvate, and succinate. The unorthodox and puzzling response to DNP of particles from fruit at the climacteric peak required further investigation.

In light of recent knowledge about mitochondrial processes, specifically about respiratory control and action of phosphorylative inhibitors, it was considered necessary to reevaluate previous results obtained with cytoplasmic particles of the avocado fruit. An isolation procedure was introduced (*19*) which minimized aggregation. This was

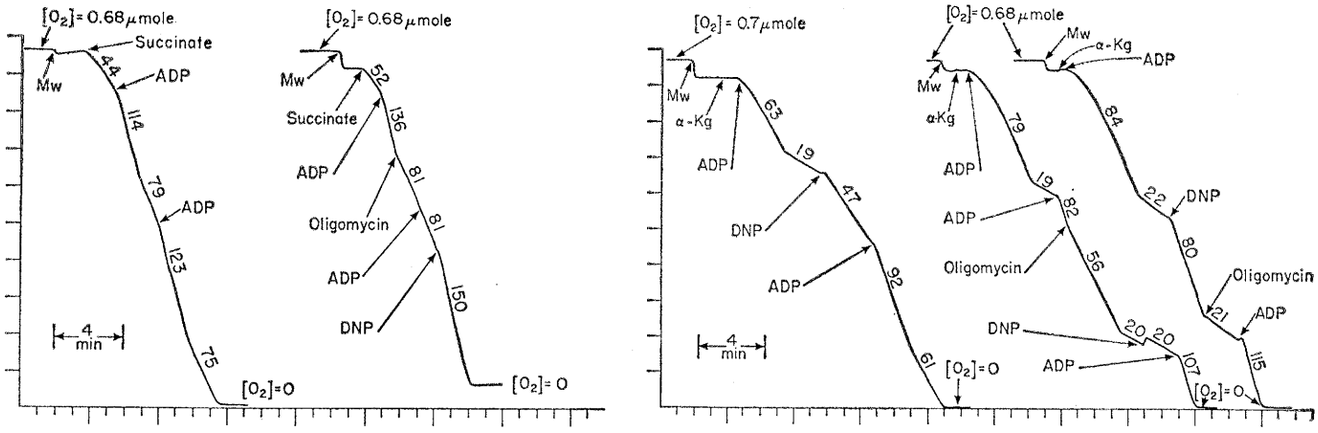


Fig. 6 (left). Oxidation of succinate by avocado mitochondria as measured by oxygen electrode (*19*). Numbers on the traces are rates expressed as millimicromoles of oxygen per minute. M_w, addition of mitochondria. Fig. 7 (right). Oxidation of α-ketoglutarate (α-kg) by avocado mitochondria as measured by oxygen electrode (*19*). Numbers on the traces are rates expressed as millimicromoles of oxygen per minute. M_w, addition of mitochondria.

accomplished by homogenization in the presence of ethylenediaminetetraacetic acid (EDTA), $MgCl_2$, and cysteine, by suspending the pellets in large volumes of the medium and by centrifuging at alternately high and low speeds. The Clark oxygen electrode was used for polarographic measurement of rates of oxidation. Esterification of inorganic phosphate was determined by an enzymatic assay involving crystalline glucose-6-phosphate dehydrogenase, nicotinamide-adenine dinucleotide phosphate (NADP), and glucose-6-phosphate formed as a result of adding hexokinase and glucose to the reaction medium.

The highly washed mitochondrial preparation from avocado fruit at the peak of respiratory activity was free of endogenously oxidizable substances. The response to succinate was markedly stimulated by adenosine diphosphate (ADP) (Chance's "state 3") and suppressed as ADP became limiting ("state 4"). After an initial lag the respiratory control ratio, defined as the state-3 rate divided by the state-4 rate, was constant until the oxygen was exhausted (Fig. 6). When the particles were in state 4 the respiration was stimulated maximally by 30μM DNP. By the use of oligomycin, which inhibits both oxidative phosphorylation and DNP-stimulated adenosine triphosphatase, it was shown that DNP exerted an uncoupling effect rather than a recycling of ADP through the increased activity of adenosine triphosphatase. In the presence of an ample amount of ADP, oligomycin lowered the oxidation of succinate to the state-4 rate. Upon the addition of DNP, the inhibiting effect of oligomycin on the oxidation of succinate was no longer observed. This behavior was confirmed by direct measurement of adenosine triphosphate (ATP) hydrolysis. The increased rate of release of phosphate from ATP by DNP was reversed by the addition of oligomycin. The uncoupling effect of DNP was substantiated by the fact that ATP formed in the presence of both succinate and DNP was very low and virtually the same as that found in the absence of substrate.

Similar responses of avocado mitochondria were observed when malate was substituted for succinate. The main difference was a progressive decrease in the rate of malate oxidation with time, whereas the rate of succinate oxidation was constant. The original rate of malate oxidation could be restored by the addition of glutamate, but the decline in rate was evidently not due to oxidation of glutamate because glutamate dehydrogenase was absent from these preparations. Evidence was obtained that glutamate could reverse inhibition by oxaloacetate, presumably through a transamination reaction. The suggestion was made that oxaloacetate was accumulating during malate oxidation and causing the inhibition. This phenomenon may be similar to the previously reported (*5*) inhibition of succinate oxidation by oxaloacetate, but further analysis is required.

The analysis of the effects of phosphate acceptor, uncoupling agents, and phosphorylative inhibitor on oxidation rates was based on a comparison of "substrate level phosphorylation" with electron transport phosphorylation. "Substrate level phosphorylation" refers to the formation of ATP as a result of oxidation which is not tied to the transfer of electrons to oxygen. An example is the glycolytic phosphorylation linked to the conversion of glyceraldehyde-3-phosphate to phosphoglyceric acid. Similarly, in the Krebs cycle the oxidation of α-ketoglutarate to succinate involves the formation of one substrate-level phosphate ester for every three so-called "high energy" phosphate bonds linked to the transfer of electrons from NADH to oxygen. This unique step in the tricarboxylic acid cycle is distinguished from all other oxidation by its resistance to DNP.

In order to obtain an insight into an apparently unorthodox behavior of avocado mitochondria, a detailed analysis was made of the response to α-ketoglutarate, in view of the findings with succinate and malate. The relevant points in this discussion are the following generally observed experimental facts: (i) DNP stimulates adenosine triphosphatase; (ii) oligomycin inhibits DNP-stimulated adenosine triphosphatase; (iii) oligomycin inhibits oxidative phosphorylation; (iv) DNP has no effect on substrate level phosphorylation; (v) substrate level phosphorylation depends on ADP; and (vi) substrate level phosphorylation is not affected by oligomycin.

The pattern of α-ketoglutarate oxidation (Fig. 7) was similar in some respects to the mitochondrial oxidations of succinate and malate and markedly different in other respects. In all cases there was an immediate response to addition and depletion of ADP. A higher respiratory control ratio was obtained with α-ketoglutarate, presumably due to tighter coupling of substrate level phosphorylation than of the elec-

tron transport chain phosphorylation. The pertinent information with respect to substrate level phosphorylation is that it depends on ADP concentration but that it is not uncoupled by DNP or inhibited by oligomycin. However, an indirect effect may be exerted on substrate level phosphorylation by DNP stimulation of adenosine triphosphatase and by oligomycin suppression of this enzymatic activity. 2-4-Dinitrophenol stimulated the state-4 oxidation of α-ketoglutarate, and the stimulation was a function of concentration up to $50\mu M$. Supplying ADP in the presence of DNP brought about a greater increase in the rate of oxidation, indicating that the substrate level phosphorylation was rate-limiting before and after the addition of DNP. When oligomycin was added during state 3 there was some, but not pronounced, inhibition of oxidation. The somewhat suppressed rate was maintained until ADP became rate-limiting, and the coupling of the substrate level induced state-4 rate. The ADP limitation was not lifted by DNP in the presence of oligomycin. However, DNP did cause a stimulation of state-4 rates in the absence of oligomycin. Upon the addition of this inhibitor there was an immediate suppression of oxidation, but oxidation could be restored by the addition of ADP.

The data obtained from these studies indicate that highly washed particles from avocado fruit at climacteric peak have not lost their ability to perform oxidative phosphorylation, that the oxidation can be controlled by changes in the concentration of phosphate acceptor, and that uncoupling is a highly critical process complicated by a number of related reactions which require further elucidation.

The results obtained with the highly washed mitochondrial preparation added considerable knowledge about the behavior of particles from avocado fruit at climacteric peak. This was the stage of ripeness which demanded further elucidation. Attempts to obtain similar preparations from fruit at preclimacteric stages have so far been unsuccessful. The reason for failure may be the difference in the physical state, more drastic methods being required for breaking up the cells from the unripe fruit, or the presence of inactivating substance in different stages of dispersion, as in the case of the banana (*6*). It was deemed advisable, therefore, to use the intact cell, rather than the organelle, as experimental material, and to study the effects of dinitrophenol in relation to the climacteric pattern.

Table 5. Effect of dinitrophenol on the esterification of P^{32} in tissue slices of unripe and ripe avocado. [Based on data reported by Young *et al.* (*20*)]

Esters	Esterification [count min^{-1} g^{-1} (fresh wt) $\times 10^{-3}$]					
	Unripe			Ripe		
	Control	DNP	%	Control	DNP	%
Adenylates	21.4	15.7	77	419	265	63
Hexose phosphate	37.3	20.5	57	3458	2143	62
Triose phosphate	1.4	0.5	38	178	107	60
Total esterified	78.1	54.8	70	4307	2707	63

Tissue slices were incubated with radioactive inorganic phosphate for various time intervals in the presence and absence of DNP (*20*). Immediately after incubation the tissue was washed of readily diffusable, or "free space," phosphate and inactivated. After extraction, purification, and concentration the phosphate esters were separated by two-dimensional chromatography. The spots on the chromatograms were located by x-ray radiography and by ultraviolet absorption, and the radioactivity was determined by direct counting of the spots on the paper. The overall rate of incorporation of P^{32} was much higher in the ripe than in the unripe slices. In the unripe slices about 50 percent of the total activity was found in nucleotides, with adenosine diphosphate and uridine diphosphate most heavily labeled. Glucose-6-phosphate accounted for about one-third of the total amount of phosphate esterified. The nucleotide fraction in the ripe slices contained 17 times more activity than the fraction in the unripe slices, but less than 15 percent of the P^{32} incorporated was found in the nucleotide fraction. Total phosphate esterification in both ripe and unripe slices, as well as incorporation into the nucleotides and into sugar phosphates, was sensitive to DNP. In fact, the inhibition by DNP of labeling in the adenylate fraction was more pronounced in ripe than in unripe slices (Table 5). The data suggest a qualitative similarity in the coupling process for the two stages of maturation.

Reversal of Senescence

The physiological, chemical, and enzymatic changes reported here refer to events progressing essentially unidirectionally. The regulation achieved by alteration of external conditions or by endogenous factors tends to affect rates of aging but not to reverse the aging process. The question arises, Are cells destined for senescence or breakdown capable of spontaneous reversion to a state of juvenility? At the morphological level evidence for such reversion was supplied in a study conducted in this laboratory (*21*) on citrus fruit tissue.

The mature vesicle of an orange or of a lemon consists of a juice sac and a threadlike stalk by means of which the sac is attached to the inner pericarp. There are no vascular elements in the vesicle. The parenchyma cells of the stalk receive nutrients over relatively long distances without the help of the usual xylem and phloem elements. No cell divisions have been observed in the stalk when it is attached to the juice sac. When excised vesicle stalks were removed and grown on a synthetic medium, it was found that the stable aging cells started to proliferate. Mitotic figures were observed within 72 hours after planting on a completely synthetic medium of salts and sucrose. No auxins, kinins, or any other growth-promoting substances were required. It has been possible to subculture and maintain the tissue culture of the stalk explants for over 4 years.

Upon examination with phase microscopy and polarized light microscopy, distinct morphological differences were observed. In the nucleoli of the proliferating cells of 3-day-old cultures there appeared definite bodies which formed at regular intervals along a helical core. At about the same time, relatively large bilobed cytoplasmic bodies aggregated about the nuclei of the growing cells and eventually received the nucleolar structures from the nucleus. The nucleocytoplasmic interactions found in fruit tissue grown in vitro were also observed in sections of growing stems and roots from germinating seeds of several species of citrus, in apical meristems of branches of lemon and orange trees, and in mature pollen of citrus flowers. The same nu-

cleocytoplasmic relationships were observed in growing cells of young stems of plants of several other genera.

In addition to being capable of morphological redifferentiation, the explants of the vesicle stalk are characterized by the capacity for biochemical reversibility. In mature lemon fruits there is little or no starch, though starch is present in the early stages of development. Iodine staining and polarizing microscopy have shown that excised vesicle stalks grown in vitro are capable of synthesizing starch. Birefringence and the polarization cross characteristic of starch granules were evident in the cytoplasmic bodies of the proliferating cells. This was in striking contrast to findings for cultures which no longer manifested visible signs of cellular growth. Fluorescence under ultraviolet light is another finding for the proliferating cells which is in contrast to observations for stable mature tissue, which absorbs ultraviolet light. The significance of this phenomenon and the induction of nucleic acid synthesis as a feature of the reversal of senescence are under investigation.

Concluding Remarks

The regulatory role of substances produced in minute quantities and the metabolic processes characteristic of biological systems are found in fruits as in other organisms. The studies with fruits are relatively free of some of the complexities, resulting from the interplay of several processes, which are found in other organisms. The fruit is capable of prolonged independent existence even after removal from the parent plant. It offers desirable material for investigation at three levels of organization: the intact organ; the intact cell in the form of a tissue slice; and subcellular fractions, exemplified by mitochondrial systems.

Studies with whole fruits have shown the role of auxins and gibberellins in the developmental stages, though the mechanism of their action requires further elucidation. The biochemical differentiation which accompanies cell elongation and maturation is a subject that has received little attention. On the other hand, studies of the effects of external factors on tissue from fruit in late stages of maturation have yielded considerable information on the acceleration and retardation of the aging process. The signal that initiates senescence—for example, a rise in respiratory rate—may be triggered by endogenous ethylene production, though this matter is far from settled. The mode of action of ethylene has been traced (*22*) to changes in the permeability of lipoprotein membranes, as measured by increases in rates of mitochondrial swelling. The membranes, conceivably, regulate respiration by controlling movement of the reactive components of oxidative systems, notably phosphate acceptors and donors. It has been shown, on the other hand (*23*), that auxins prevent degradative changes. The idea has been advanced (*24*) that the role of auxin in senescence, as in growth, is through cell-wall metabolism. With the finding that changes in pectins are characteristic of fruit ripening, one may visualize a fine controlling balance between pectic enzymes, ethylene, auxin, and other growth-regulating substances.

Morphological and histochemical changes associated with maturation and senescence have received limited attention, mainly with conventional microscopy. The application of phase and polarizing microscopy brings the opportunity to observe the dynamic changes that take place in the cytoplasm and the nucleus of mature, stable cells and of cells subjected to conditions which stimulate spontaneous proliferation. The examination of fine structure by electron microscopy has barely started. Recent observations made with the electron microscope (*25*) show vesiculation of cytoplasm and of organelles in ripening pear tissue. Vacuoles increased in size and in number, and there ensued a general destruction of plastid structure. The cytoplasm became a mass of small membrane-bounded vacuoles. The vacuolation was attributed to alteration in osmotic conditions in response to changes in tonoplast permeability. One of the most significant points in this study was the observation that mitochondria did not show any breakdown until the fruit entered the postclimacteric stage.

The retention of intact structure by mitochondria is in harmony with the biochemical observations on oxidative and phosphorylative activities in particles isolated from fruit before and at the time of climacteric peak. The metabolic approach to the physiological changes in fruits was advanced through observations of the effects of dinitrophenol. The gradually accumulating evidence indicates that the cell is able to incorporate inorganic phosphate into nucleotides and into sugar phosphates, and that the presence of numerous phosphate acceptors makes it unnecessary to postulate an endogenous uncoupling agent as a regulatory mechanism. This view is supported by the experimental findings for mitochondrial preparations from fruit at climacteric peak; these preparations show respiratory control, and they react to uncoupling agents and phosphorylative inhibitors in a manner one expects to find in metabolically active systems. The prelude to senescence does not appear to be dominated by degradative reactions. On the contrary, the cell reacts to the warning of imminent death by a burst of synthetic activity.

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