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Similarities between the Actions of Ethylene and Cyanide in Initiating the Climacteric and Ripening of Avocados[1]

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ABSTRACT

A continuous exposure of intact avocados (*Persea americana*) to 400 μl/l of cyanide results in a rapid increase in the rate of respiration, followed by a rise in ethylene production, and eventual ripening. The pattern of changes in the glycolytic intermediates glucose 6-phosphate, fructose diphosphate, 3-phosphoglyceric acid, and phosphoenolpyruvate during the rapid rise in respiration in both ethylene and cyanide-treated fruits is similar to that found in fruits made anaerobic where a 2.3- to 3-fold increase in the rate of glycolysis is observed. It is suggested that both during the climacteric and in response to cyanide, glycolysis is enhanced. It is proposed that cyanide implements the diversion of electrons to the cyanide-resistant electron path through structural alterations which are independent of the simultaneous inhibition of cytochrome oxidase.

The sharp rise in respiration, the climacteric, which attends the ripening of certain fruits, has been attributed alternatively to a decrease in the "organization resistance" of the cell (11, 27) and to an increase in protein synthesis, particularly to the synthesis of one or more enzymes putatively involved in the ripening process (1, 17, 36). Ethylene is considered to be the agent that triggers the climacteric and fruit ripening (34). It is questionable whether protein synthesis constitutes a requisite for the climacteric rise, because the stimulation of glycolysis in preclimacteric fruits by anoxia (11), the acceleration of respiration in preclimacteric slices by uncouplers of oxidative phosphorylation (33), the demonstration of full respiratory capacity of isolated mitochondria from preclimacteric tissue (29), and the development of the climacteric under conditions which may be thought to preclude synthesis of protein (18, 31, 42), all indicate that the enzymatic capacity of preclimacteric fruits may be adequate to sustain the rates of respiration observed at the height of the climacteric.

It has been observed in recent years that during the climacteric, glycolytic intermediates show a pattern similar to that found in plant and animal tissues and yeast when glycolysis is enhanced, and which reflects the activation of two regulatory enzymes, namely PFK[2] and PK (5, 6, 15, 28). On the basis of these observations it has been suggested that glycolysis is accelerated in the course of the climacteric rise in respiration (6, 15, 40). However, activation of glycolysis *per se* cannot be the cause of ripening or even of the climacteric, because anaerobiosis, while enhancing glycolysis in avocados (see below) and apples (11), prevents ripening, and uncouplers of oxidative phosphorylation, which enhance respiration and presumably glycolysis in banana slices, neither accelerate ripening nor inhibit it when ripening is induced by ethylene or occurs naturally (31, 42). Furthermore, the postulated augmentation of glycolysis during the climacteric cannot be attributed to uncoupling of oxidative phosphorylation, for both the rates of ^{32}P esterification and the levels of ATP increase during the climacteric in a variety of fruits (38, 43). Because enhancement of glycolysis appears to attend ripening under conditions which normally call for its diminution, *i.e.* where there is an elevation in energy charge (2, 43), and because the fruit-ripening hormone, ethylene, enhances glycolysis in yeast (41), we undertook to study, in addition to the effect of ethylene on glycolysis, the effect of cyanide on both glycolysis and ripening; because on the one hand, cyanide is known to induce aerobic fermentation in a variety of tissues (26), and on the other hand, cyanide enhances respiration of preclimacteric avocado slices without, at the same time, affecting the rate of ^{32}P esterification (30). The latter is known to be reduced greatly by conventional uncouplers of oxidative phosphorylation (43). Thus, in the case of avocados, cyanide appears to simulate neither conventional uncouplers of oxidative phosphorylation nor anaerobiosis, and thus it was anticipated that cyanide might increase the respiratory activity of intact avocados and also initiate ripening.

MATERIALS AND METHODS

Hass avocado (*Persea americana*) fruits, collected either from the University or private orchards in Los Angeles, were used except for one experiment in which the Fuerte variety was used.

The fruits were put singly into 2-liter respiratory jars at 20 C through which a stream of air was passed over the fruits at a rate of 60 ml/min. The rates of O_2 uptake and CO_2 output were recorded with both a Beckman paramagnetic oxygen and an infrared CO_2 analyzer, respectively. One ml of gas, withdrawn from the effluent air stream, was used for the gas chromatographic estimation of ethylene, using a 90-cm activated alumina column. Phosphate esters were extracted as previously reported (4).

Avocado fruits were peeled as rapidly as possible with a hand potato peeler and thin slices, taken uniformly from throughout the fruit, were cut into liquid nitrogen and powdered therein. About 30 g of the powder were weighed in precooled beakers and homogenized for 10 min with a high speed Waring Blendor in a mixture of 60% methanol, 10%

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[2] Abbreviations: PFK: phosphofructokinase; PK: phosphoenolpyruvate kinase; G6P: glucose 6-phosphate; 3-PGA: 3-phosphoglyceric acid; PEP: phosphoenolpyruvate; FDP: fructose diphosphate.

trichloroacetic acid, and pieces of solid CO_2 at −60 C. The slurry was centrifuged and the residue was homogenized once more in 5% cold trichloroacetic acid solution. The two supernatants were combined and further purified according to the previously published method (4). When lactate was determined, the powder of frozen tissue was homogenized with an MSE homogenizer (MSE Inc., Westlake, Ohio) in boiling 75% (v/v) ethanol. The slurry was centrifuged and the residue was extracted twice more with 50% (v/v) ethanol. The combined supernatants were evaporated to about 5 to 10 ml under vacuum with a rotary evaporator at 30 C and the extract was taken up to a convenient volume.

Phosphate esters and lactate were determined enzymatically as previously described (9), by methods involving estimation of NADH levels. The changes in NADH levels were followed either fluorometrically (FDP and G6P) with a Farrand fluorometer, or spectroscopically (lactate, PEP, and 3-PGA) with a Cary 14 spectrophotometer. The amount of enzymes and extract used in the assays was chosen so as to complete the reaction within 1 to 3 min. Anaerobiosis was established by flushing the fruit-containing jars for 10 min with a stream of 300 ml/min of pure nitrogen, after which the flow was adjusted to 60 ml/min. The fruits treated with ethylene were continuously subjected to 30 μl/l of the gas, which was prepared by mixing the primary air stream with a second stream of air containing a known amount of ethylene.

Cyanide and air mixtures were prepared by first introducing into an evacuated cylinder a known amount of HCN, followed by air to a precalculated pressure, to achieve the desired cyanide concentration. The HCN in the outgoing stream was trapped when the stream passed through a washing tower containing a solution of silver nitrate. The concentration of HCN in the gas phase was determined by the method of Robbie and Leinfelder (37). The estimation of cyanide in the tissue was carried out as follows: 30 g of tissue was homogenized with 50 ml of 2 N NaOH solution. The residue obtained was homogenized once more with 50 ml 2 N NaOH and the homogenate was centrifuged. The two supernatants were combined and the extract was taken to a convenient volume. HCN was determined in aliquots of the extract as above (37).

RESULTS

Changes of Glycolytic Intermediates during the Climacteric. In avocado fruits, G6P, 3–PGA, and PEP were found consistently to be in much higher concentrations than was FDP. The levels of FDP in preclimacteric fruits varied from 0.2 to 0.9 nmoles/g fresh weight. None of the above metabolites showed appreciable changes during the preclimacteric, regardless of its duration. In Figure 1, the levels of glycolytic intermediates are plotted in relation to the rates of fruit respiration as a function of time during the course of the climacteric. During the climacteric, PEP falls sharply and G6P is halved (Fig. 1). The most pronounced changes, however, are shown by FDP, whose level rises 10-fold during the rise in respiration. Similar increases in FDP have been reported previously for climacteric bananas (6, 40) and tomatoes (15).

Effect of Anoxia. When fruit is transferred to N_2, CO_2 evolution increases for about 30 min, declines slowly to a steady state in about 2.5 to 3 hr, and shows no further change for about 30 hr. The steady state rates of CO_2 output varied somewhat with the fruits, as did the time of attainment thereof. The rates of CO_2 production quoted in Table I are those observed at the end of the experimental period. Anoxia causes an increase in lactic acid (Table I) and its rate of production varies with the fruit from 390 to 670 nmoles/g fresh weight·hr. If it is assumed that CO_2 arises from pyruvate decarboxylation and if both alcohol and lactate are considered in terms of CO_2 equivalents, then the total rate of carbon flux under anaerobiosis is equivalent to about 105 to 150 μl of CO_2/g fresh weight·hr (Table I). Thus, on the basis of the CO_2 evolved or potentially evolved, there is a 2.3- to 3-fold increase in the rate of carbohydrate breakdown by preclimacteric avocados under anoxia, as compared with that in air. Table II presents the changes in G6P, 3-PGA, FDP, and lactate. An increase in glycolytic flux with anaerobiosis is associated with a 10- to 50-fold increase in FDP and a sharp fall in 3-PGA. Paren-

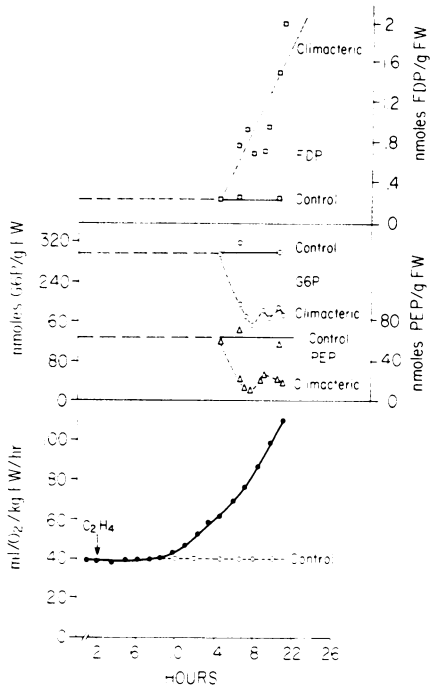

FIG. 1. Course of respiration and the levels of PEP, G6P, and FDP during the climacteric of intact avocado. Fruit treated with 30 μl/l ethylene where indicated.

Table I. *Rates of CO_2 Output and Lactate Production in Avocados Kept in Air and in Nitrogen*

Experiment	Treatment	Rate of CO_2 Output		Lactate Production	Potential Maximal Rates of Aerobic CO_2 Evolution
		Air	N_2		
		μl/g fresh wt·hr		*nmoles/g fresh wt·hr*	*μl/g fresh wt·hr*
I	N_2 4.5 hr	48	31	670	138[1]
	Air	49			49
	Air	50			50
II	N_2 3.5 hr	45	21	600	104
	N_2	49	25	558	114
	N_2	50	32	627	138
	Air	31			31
	Air	47			47
III	N_2 3 hr	45	28	391	110
	N_2	50	40	491	153
	N_2	46	40	420	148
	N_2	49	28	650	128
	Air	50			50

[1] Calculated by converting to CO_2 equivalents both lactate and ethanol. The concentration of the latter is calculated by assuming that the CO_2 in nitrogen is produced from pyruvate decarboxylation.

Table II. *Contents of FDP, 3-PGA, G6P, and Lactate in Avocados Kept in Air and in Nitrogen*

Experiment	Treatment	Content			
		FDP	3-PGA	G6P	Lactate
		nmoles/g fresh wt			
I	N_2 4.5 hr	19.80		157	3000
	Air	0.95	149	158	135
	Air	0.38	197	151	179
II	N_2 3.5 hr	8.50	46	235	2100
	N_2	10.40	49	232	1960
	N_2	6.50	50	299	2200
	N_2	6.80	37	260	
	Air	0.46	140	165	120
	Air	0.35	210	170	161
III	N_2 3.0 hr	13.75	38	218	1170
	N_2	29.80	47	330	1470
	N_2	30.00	38	328	1260
	N_2	12.50	54	220	1950
	Air	0.80	157	190	128

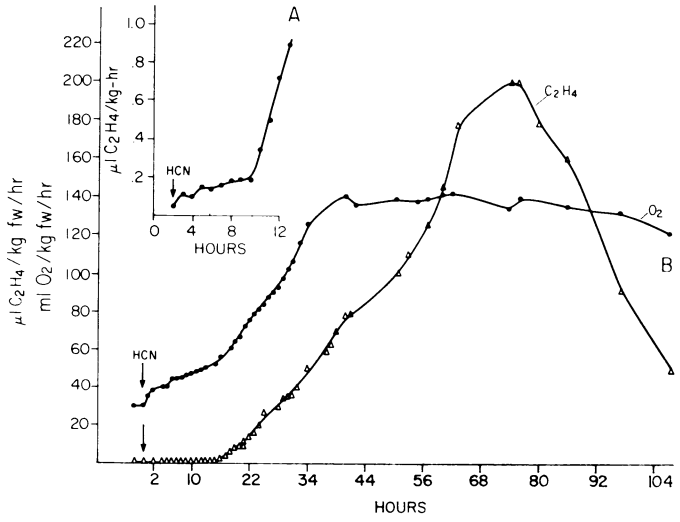

FIG. 2. Course of oxygen uptake and ethylene evolution by intact avocado in response to treatment with cyanide gas. Cyanide 400 μl/l where indicated.

thetically, the levels of 3-PGA and PEP invariably change in parallel (Fig. 4). Thus, in Table I, the relative changes in 3-PGA are taken to indicate those of PEP as well. In Table II, G6P tends to increase slightly in anoxia in contrast to its sharp fall during the climacteric (Fig. 1). The variety Fuerte, rather than Hass, was used in this experiment, which may account for the difference. More important, however, is the fact that the increase in G6P is 2-fold at most, as compared with a 10- to 50-fold increase in FDP. Anaerobiosis induces a similar pattern of changes in the glycolytic intermediates in a variety of higher plant tissues (5, 28). The present data establish a relationship between glycolytic activity and the pattern of changes in the glycolytic intermediates in avocados. They also show that the glycolytic enzymatic potential of preclimacteric avocados is sufficient to sustain rates of respiration evinced at the climacteric. Furthermore, the pattern of changes in the glycolytic intermediates suggests a similarity between the stimulation of glycolysis in anoxia and under aerobic conditions in the course of the climacteric rise.

Cyanide. Cyanide gas at 400 μl/l in air induces an increase in the rate of oxygen uptake within 1.5 to 2 hr, closely followed by a rise in ethylene production (Fig. 2), and eventual ripening. However, the initial amounts of ethylene evolved are not sufficient to induce the observed rise in respiration within such a brief period. Thus, even after some 10 hr, the rate of ethylene evolution is but 0.2 μl/kg fresh weight·hr. By use of a factor derived by Burg and Burg (13), an internal concentration of 0.36 μl/l can be calculated which, although biologically active, is not sufficiently high to increase the rate of respiration in a short time. Furthermore, in the first 2 hr or so, the level of ethylene is actually below the threshold level (0.1 μl/l) for the induction of the climacteric rise. Cyanide appreciably inhibits the peak values of ethylene production, (Fig. 3) though it is to be noted that the values of ethylene production in the presence of cyanide finally reach levels in excess of those required to induce fruit ripening. The respiration of cyanide-treated avocados rises gently through 10 to 15 hrs, after which the rate increases sharply (Fig. 2). With the upward inflection of the respiration rate, there is a pronounced change in the levels of glycolytic intermediates in response to cyanide (Fig. 4). PEP and 3-PGA fall to about a third of their preclimacteric value, while the level of G6P is halved. The most pronounced change is exhibited by FDP, which increases 7-fold. Thus, cyanide induces the same pattern of changes in glycolytic intermediates as does anaerobiosis, albeit with different kinetics.

Cyanide Content of the Tissue. The level of cyanide was estimated in several fruits kept under cyanide for 32 hr. The concentration of cyanide, which varies with the fruits from approximately 0.2 to 1 mM, is adequate for the inhibition of cytochrome oxidase.

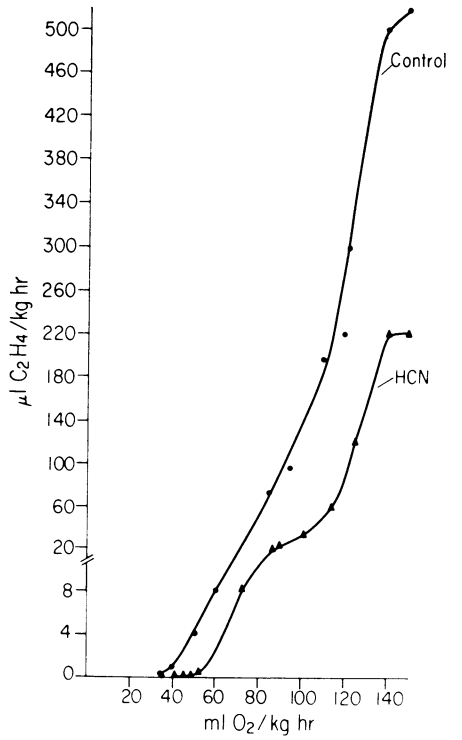

FIG. 3. Ethylene evolution *versus* rate of respiration in control, and in cyanide-treated intact avocados. Cyanide 400 μl/l where indicated.

DISCUSSION

Anoxia. It is difficult to make quantitative deductions regarding the *in vivo* activity of a metabolic pathway from changes in the concentrations of its intermediates in response to some metabolic perturbation, especially when there are branch points along the path in question. It has been possible, however, in cases where the pools of the intermediates are small, to make inferences regarding the pivotal enzymes of a sequence. Under anaerobiosis, where the products of glycolysis, *i.e.* lactate or ethanol or both, are unique, it is possible to approximate the *in vivo* rate of glycolysis from the rate of formation of the products. A 2.3- to 3-fold increase in the rate of glycolysis in anaerobic avocados is associated with a characteristic pattern of changes in the glycolytic intermediates. Thus, in avocados, a correlation between rate of glycolysis and pattern of changes in the glycolytic intermediates can be established. In considering the changes in glycolytic intermediates in avocados made anaerobic, it will be useful to compare such changes with those found in other plant tissues, as well as in yeast and animal tissues. For technical reasons, few complete sets of data regarding the changes of all glycolytic intermediates and cofactors exist for plant tissues. From the available data, certain inferences can be made (5, 24, 28). In most cases involving higher plant tissues, yeast and animal tissues, the G6P to F6P, and 3-PGA to PEP, enzymatic steps remain close to their theoretical equilibrium in both air and nitrogen, whereas the F6P to F16P and PEP to pyruvate, steps are always greatly displaced from equilibrium (5, 23, 28). In these latter steps, crossover changes between substrate and products occur during the transition from air to nitrogen. The inverse pattern of changes between the substrates and products of PFK and PK in response to an acceleration of the overall glycolytic flux is taken to indicate that the two enzymes are rate-determining in the overall sequence (16, 19).

Climacteric Avocados. The similarity in the pattern of changes of glycolytic intermediates in anaerobic and climacteric avocados indicates that glycolysis is activated during the climacteric. However, while activation of glycolysis under anoxia can be explained in terms of a decrease in the energy charge (2), the energy charge actually increases during the climacteric of avocado (43).

Therefore, the factor(s) which enhance(s) the rate of glycolysis during the climacteric cannot be thought to reflect the evocation of a conventional Pasteur effect, as in the case of anaerobiosis. It has been pointed out that activation of glycolysis during the climacteric of avocados is associated with an increase in the energy charge (43), which would be expected normally to decrease the rate of glycolysis. This acceleration of glycolysis must be due to an effect on some regulatory parameter other than energy charge. The climacteric occurs in fruits which differ widely with respect to their physiological properties, growing conditions, and composition. The common feature they all share is their ability to produce ethylene and to respond to exogenous ethylene. However, ethylene increases the respiration of nonclimacteric fruits *e.g.* citrus, rin tomatoes, (22, 36) and other plant tissues where ripening is not at issue, such as potato tubers (35). In addition, ethylene enhances fermentation in yeasts (41). For this reason, the stimulatory effect of ethylene on glycolysis and aerobic respiration must be viewed as a fundamental process independent of ripening.

Although, on the basis of the available experimental evidence, it is not possible to identify with certainty either the site or the mechanism of ethylene action on the respiratory metabolism of plant tissues, certain deductions seem warranted. The postulated enhancement of glycolysis during the climacteric is evidently not the result of an increase in enzyme content, because preclimacteric avocados possess the necessary glycolytic capacity to sustain the rates evinced at the climacteric peak (Table I). It is rather the result of the manifestation of pre-existing enzymatic capacity. Ethylene is not known to act *in vitro* as an enzyme modulator (1). Burg and Burg (14) have compared the biological effectiveness of ethylene and compounds acting like ethylene with their olefin-silver-complexing constants, and have suggested that ethylene exerts its biological activity through its ability to complex with metals. The fact, that agents which are not known to complex with metals initiate ripening, indicates that the metal-complexing characteristics of ethylene may not be critical in inducing the rise of respiration and ethylene production during the climacteric (42). It is known that permeability of fruit tissue increases during ripening (8, 39), and that in bananas, moreover, this increase occurs prior to the climacteric onset (39). Differences in experimental conditions and possibly the difficulty of detecting subtle permeability alterations in biological membranes may account for the failure to observe initial permeability changes in banana slices (12). In the light of the above discussion and the observation that ethylene perturbs biological membranes *in vitro* (32), we suggest that ethylene sufficiently alters certain elements of cellular organization to remove regulatory restraints on given metabolic pathways. Ethylene, by its effect on cellular structure, activates pre-existing respiratory potential. The observed activation of PFK and PK during the climacteric can be seen as the result of the cellular redistribution of various enzyme modulators, mainly ions.

Effect of Cyanide. Cyanide, as an inhibitor of cytochrome oxidase, would be expected to stimulate aerobic glycolysis as a consequence of the curtailment of electron transport. Glycolysis is indeed augmented in response to cyanide treatment

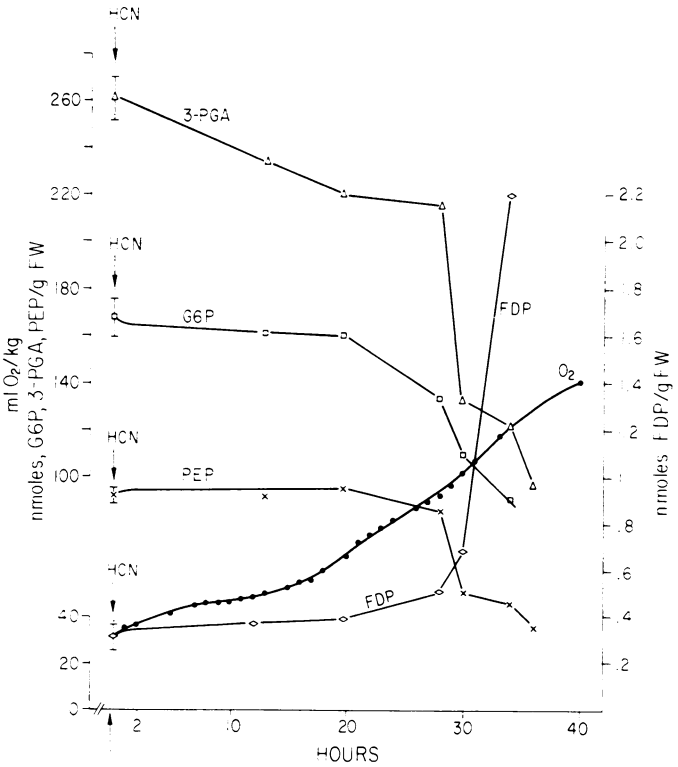

FIG. 4. Course of oxygen uptake and the levels of glycolytic intermediates in avocados treated with cyanide gas. Cyanide 400 μl/l where indicated.

(Fig. 4). However, cyanide accelerates aerobic respiration as well. The existence of a cyanide-insensitive by-pass in certain plant tissues, which branches from the conventional electron path on the substrate side of Cyt *b* and is neither coupled to phosphorylation nor controlled by energy charge (3, 7), allows for enhanced rates of electron transport in the presence of cyanide. Such enhancement demands that phosphorylation at site I be more intense in connection with the by-pass, *i.e.* in the presence of cyanide, than in the control. Although the efficiency of oxidative phosphorylation in the presence of cyanide is diminished, the absolute levels of ATP may remain unchanged, or may even increase because of the 3- to 4-fold increase in the rate of electron transport. A case in point is the observed failure of both cyanide and azide to decrease the rate of ^{32}P esterification in preclimacteric and climacteric avocado slices (30). This feature of cyanide-resistant respiration distinguishes the cyanide-evoked from uncoupler-evoked glycolysis in avocado fruit.

It has been suggested that cyanide diverts electrons to the alternate path by preventing their flow through the normal Cyt oxidase-mediated path (3). CO, at concentrations as low as 1%, which have no effect on Cyt oxidase, induces the climacteric in banana slices and other fruits (42). This indicates that cyanide may exert an effect apart from terminal oxidase inhibition. That is, cyanide may activate glycolysis and affect the diversion of electrons from the conventional respiratory carriers to the by-pass by inducing subtle changes in cellular or organelle organization. Certain lines of evidence indicate that the bridge to the cyanide-insensitive electron transport path may be "loosely" connected in mitochondria. Thus, in isolated mitochondria of both potato and avocado, cyanide totally inhibits oxygen uptake (10, 20), whereas in the intact tuber and fruit respectively, cyanide elicits an increase in the rate of respiration (21 and Fig. 2). In these tissues the process of isolating mitochondria destroys the capacity for cyanide-resistant electron transport. Furthermore, the slicing of potato tubers results in supplantation of cyanide-stimulated tuber respiration by cyanide-sensitive fresh slice respiration (20). Slicing is known to lead to significant breakdown of phospholipids and presumably the link to the cyanide-resistant by-pass is lost in the process (25). Aged potato slices are once again cyanide-resistant (20), the link presumably being re-established in connection with phospholipid and membrane resynthesis. In the light of this evidence it is proposed that cyanide implements the diversion of electrons to the alternate path by directly affecting the latter, or the link thereto, independent of the simultaneous inhibition of Cyt oxidase.

Additional evidence for the dual role of cyanide is its effect in inducing ethylene production. There is no reason to believe that cyanide acts either as an inducer or modulator of enzymes involved in ethylene production. We feel that cyanide exerts its effect by interference with cellular organization, more particularly, perhaps, with membrane organization, and hence, with metabolic regulatory restraints associated with cellular integrity. In this respect, cyanide and other mimics of ethylene may simulate ethylene in implementing fruit ripening. Since the rise in respiration induced by cyanide is ultimately attended by ethylene production, it is an open question whether cyanide induces ripening by itself or in connection with ethylene which it engenders.

Climacteric and Ripening. The stimulation by ethylene of glycolysis in avocados during the climacteric is by a mechanism different from that attending anaerobiosis or the use of uncouplers. It seems unlikely that the increase in the rate of respiration is due to the increased rate of pyruvate production. The augmentation of both glycolysis and respiration is more likely due to a common cause reflected in the release of regulatory restraints on both processes, and ethylene is normally the agent which effects this activation. The question which now arises is whether the augmentation of both glycolysis and electron transport *per se* can implement ripening. The answer is no. On the one hand, uncouplers of oxidative phosphorylation, which stimulate electron transport and presumably glycolysis in preclimacteric banana slices, fail to enhance ripening, while on the other hand, uncouplers do not prevent ripening when it is engendered with ethylene or CO. Ripening is characterized by metabolic conditions which transcend the mere enhancement of electron transport and the respiratory climacteric may be a symptom rather than a cause.

It is widely reported in the literature that ethylene production and ripening are critically dependent on oxidative phosphorylation (1, 36). However, the fact that cyanide in avocados (Fig. 3) and uncouplers in banana slices fail to interfere with the production of biologically active amounts of ethylene and with ripening indicates that with enhanced electron transport, substrate-level phosphorylation is adequate to sustain ethylene production and the overt changes in ripening of avocados and bananas.

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