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Succinoxidase Activity of Avocado Fruit Mitochondria in Relation to Temperature and Chilling Injury throughout the Climacteric Cycle[1]

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ABSTRACT

Mitochondria were isolated from 'Fuerte' avocado fruit (*Persea americana* Mill.) at four different stages of the respiratory climacteric. Preclimacteric fruit had the highest rate of succinate oxidation and the postclimacteric mitochondria the lowest. Subsequently, successive additions of ADP increased the respiratory control ratio.

Arrhenius plots of succinate oxidation of intact mitochondria from climacteric rise and climacteric peak fruit showed two transition temperatures, while only one was observed in preclimacteric fruit. The low temperature phase transition was at about 9 C, while the high one was at 20 C. In postclimacteric fruit, the low temperature transition decreased to between 5 and 2 C. The state 3 rate of succinate oxidation was highest for mitochondria from preclimacteric fruit and decreased for each later stage. The state 4 rates for preclimacteric and climacteric rise were the same, while both the climacteric peak and postclimacteric rates were about 40% lower than the preclimacteric O_2 uptake.

The results indicate continuous changes in the mitochondrial membrane of the electron transport chain throughout the climacteric cycle. The change in the membrane influencing the phosphorylation system is greatest between climacteric rise and peak stages. Mitochondrial membranes of postclimacteric fruit are presumed to change from flexible disordered to solid ordered phase at a lower temperature than those of other climacteric stages.

The changes occurring in ripening which govern the initiation of the respiratory climacteric have not been identified. A number of proposed mechanisms have been reviewed (21). Mitochondria have been examined from several fruit including avocado (9), apple (5), and tomato (3), at various stages of the climacteric, and shown to: (*a*) oxidize tricarboxylic acid cycle acids at all stages; (*b*) exhibit respiratory control; and (*c*) phosphorylate efficiently at all stages of ripening. Electron micrographs show no change in the organelle morphology between preclimacteric and postclimacteric stages of avocado fruit (W. Vanderwoude and R. E. Young, personal communication).

We observed recently that postclimacteric avocados were less sensitive to chilling injury than at any other stage (8). The chilling sensitivity temperature has been shown by Lyons and Raison (12) to be correlated with the temperature at which an abrupt change occurs in the activation energy of succinoxidase activity in isolated mitochondria, and this change is believed to reflect a change in the lipid fraction of the mitochondrial membrane from a fluid to a solid phase. The change of lipid in the membrane from liquid to solid increases the activation energy of the enzyme complex by causing a conformational alteration of the membrane-bound enzyme.

The change in chilling sensitivity suggested to us that ripening may be regulated by membrane lipids rather than by changes in the enzyme proteins themselves. We report here studies of Arrhenius plots of succinoxidase activity for avocado mitochondria at four stages of the respiratory climacteric as evidence for changes in the lipid components of the mitochondrial membrane.

MATERIALS AND METHODS

Mature 'Fuerte' avocado fruit (*Persea americana* Mill.) of uniform size were harvested from one tree at the South Coast Field Station of the University of California at Irvine. Fruit were placed in individual 2-liter wide mouth jars at 20 C. CO_2 production was monitored by Beckman model 215 IR gas analyzer as described previously (7).

As soon as a fruit achieved a respiratory rate of a particular stage on the climacteric, the fruit was cooled to 2 to 4 C prior to cutting and grating. Mitochondria were isolated and immediately assayed for succinoxidase activity as described below.

Isolation Procedure. The technique used throughout this study was that described by Lance *et al.* (9) with modifications suggested by Romani and Ozelkok (22) and by Laties and Treffry (10). Each fruit was peeled, the embryo and seed coat removed, and the fruit cut into about 10 12-g pieces. These were mixed with 350 ml of isolation medium and poured into a stainless steel Oster automatic juice extractor lined with two layers of Miracloth. In the case of postclimacteric fruit, the homogenate would often not pass through Miracloth and four layers of cheesecloth were substituted. The homogenate was adjusted to pH 7.4. The isolation medium consisted of 0.35 M sucrose, 50 mM tris buffer (pH 7.9), 5 mM EDTA, 2 mM $MgCl_2$, 6 mM KCl, 5 mM cysteine-HCl, 0.2% POP (40,000 mol wt), and 0.1% BSA. Cellular debris was removed by centrifugation at 1,600*g* for 8 min. After an additional centrifugation of the supernatant at 16,000*g* for 12 min, the mitochondria pellet was resuspended in 250 ml "wash" medium composed of 0.3 M sucrose, 10 mM K-phosphate buffer (pH 7.2), 25 mM tris buffer (pH 7.2), 1 mM $MgCl_2$, 0.1% BSA, and 5 mM β-mercaptoethanol. The suspension was centrifuged at 1,000*g* for 5 min and the resultant supernatant fraction at 8,000*g* for 10 min. The final pellet was resuspended with a Teflon and glass Potter homogenizer in 1.3 ml wash medium containing 5 μmol of ATP. The total volume was approximately 2 ml containing 20.8 to 32.5 mg protein.

Succinoxidase Assay. Mitochondrial suspension was assayed

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at 10 different temperatures from 2 to 30 C. O_2 uptake was measured polarographically with two Rank O_2 electrodes equipped with constant temperature water jackets. Each assay contained 3 ml of final volume which consisted of 0.28 M sucrose, 33 mM K-phosphate (pH 7.2), 2 mM $MgCl_2$, 0.1% BSA, 0.52 to 2.61 mg of mitochondrial protein, and 8 mM succinate. ADP totaling 0.4 to 1 μmol was added in three or more successive additions.

Protein was determined by the Lowry method as modified by Miller (14). O_2 electrodes were calibrated with air-saturated water at each temperature. Respiration rates from three to six separate mitochondrial preparations of avocado fruit from each of four different stages of climacteric were recorded on 25-cm potentiometric recorders. Only the maximum respiration rates of state 3 and state 4 of the same cycle of ADP addition were used for calculation. The decrease in activity of the mitochondria which occurred with time of storage over the period of 4-hr assay time was corrected for by repeated assays at 20 C every hr.

RESULTS

Polarographic traces of succinoxidase activity at 20 C of mitochondrial suspensions isolated from four climacteric stages of 'Fuerte' avocado fruit are shown in Figure 1. Organelles of all climacteric stages showed good coupling of phosphorylation. The RCR[3] increased with two or three successive small ADP additions because O_2 uptake in state 3 increased while that of state 4 decreased. In all cases it was obvious that the first addition of ADP did not give the maximal rate of state 3 oxidation. The highest rate was obtained at either the second or third ADP addition, and this value was used in all calculations (19).

Metabolic activity is temperature dependent. The rate of O_2 uptake was highest at 30 C, and decreased gradually to 2 C (Fig. 2). Again, the maximal rate of state 3 of succinoxidase activity was not obtained with the first ADP addition, the highest activity was obtained at either the second or the third state 3-state 4 cycle at all temperatures. The amount of mitochondrial suspension used in each 3-ml assay was adjusted according to the rate of oxidation. An amount was used which would give a trace close to a 45 degree slope with a chart speed at 5 cm/min when succinate was added. This gave accurately determinable rates. At the highest temperature, the level of mitochondrial protein was 0.53 mg which was above the critical quantity needed to give reproducible state 3 rates and good RCR (20, 21). The pattern of RCR at all temperatures, with subsequent addition of ADP, was similar to that at 20 C in Figure 1.

Assay of succinoxidase activity at 10 different temperatures required several hr, and mitochondrial activity decreased with storage time as shown in Figure 3. The loss was not serious under our conditions as only 18% of state 3 and 16% of state 4 activity were lost in 4 hr. Mitochondrial stability for both state 3 and state 4 was similar to or better than that reported by Raison and Lyons (20). The RCR changed little with time in all climacteric stages as was reported by Romani and Ozelkok (22). The state 3 rate of succinate oxidation was highest for preclimacteric fruit (Fig. 3) and lowest for the postclimacteric stages. On the other hand, the state 4 rate of climacteric rise was highest, and that of climacteric peak was lowest.

Arrhenius plots for succinate oxidation by mitochondrial preparations from avocado fruit at four stages of the climacteric cycle are shown in Figure 4. There was only one phase transition for preclimacteric fruit at the vicinity of 9 to 10 C for both states 3 and 4. Avocado fruit of climacteric rise and climacteric peak stages showed two breaks in both states 3 and 4 rates. The transition temperature for the high temperature breaks was close to 20 C, while the low temperature transition was at 8 to 9 C for climacteric rise fruit, and of 10 to 12 C for climacteric peak mitochondria. The postclimacteric fruit had the same high transition temperature as the climacteric peak organelles at 19 to 20 C, while no low temperature phase transition could be shown. It was not possible to measure activity below 2 C, but the 2 C point is clearly below the line which indicates that a phase transition does occur between 2 and 5 C rather than between 11 and 12 as in the climacteric peak stage. Each stage shows one unique phase transition and change in activation energy over at least one of the temperature ranges.

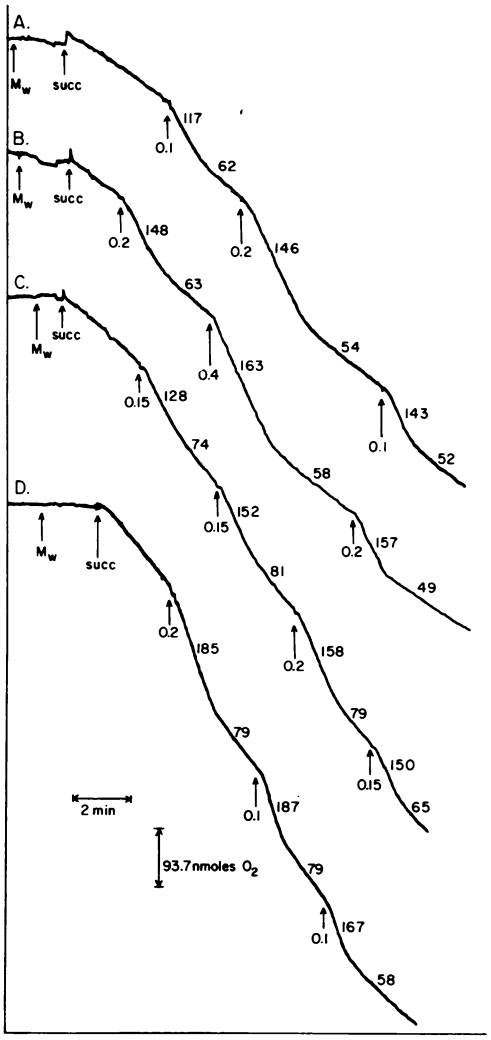

FIG. 1. Polarographic traces of succinoxidase activity at 20 C from mitochondrial suspensions isolated from 'Fuerte' avocado fruit of various climacteric stages. A: Postclimacteric; B: climacteric peak; C: on climacteric rise; D: preclimacteric. M_w and succ indicate addition of mitochondrial suspension and succinate respectively, as substrate to reaction chamber. Mitochondrial protein expressed as mg protein/3 ml was 0.68, 0.63, 0.71, and 0.74 mg for A, B, C, and D, respectively. Numbers on traces represent consumption of O_2 in nmol/min·mg protein, and those under each trace indicate addition of ADP in μmol.

Activation energies of the state 3 rates calculated from the slopes of the lines in Figure 4 are shown in Table I. Between the preclimacteric and climacteric rise, there is a decrease of 2.1 kcal/mol in the range between 20 and 30 C. Between climacteric rise and climacteric peak, a decrease of 4.7 kcal/mol was noted in the 2 to 9 C range and 3.7 kcal/mol between climacteric peak and postclimacteric stages.

Linear plots of state 3 and state 4 rates of succinoxidase activity for each climacteric stage are shown in Figure 5. Each

[3] Abbreviation: RCR: respiratory control ratio.

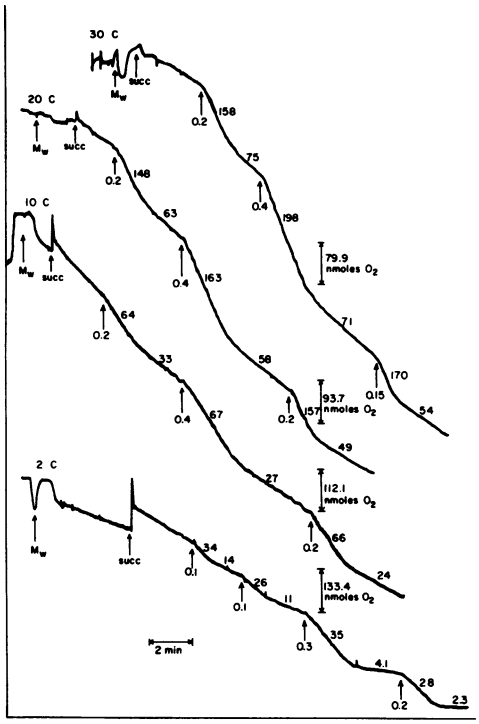

FIG. 2. Polarographic traces of mitochondrial succinoxidase activity at four different temperatures from climacteric peak 'Fuerte' avocado fruit. M_w, succ, and numbers on or under the traces were described in Figure 1. Mitochondrial protein at 30, 20, 10, and 2 C was 0.53, 0.63, 1.33, and 2.30 mg/3 ml of the reaction volume, respectively.

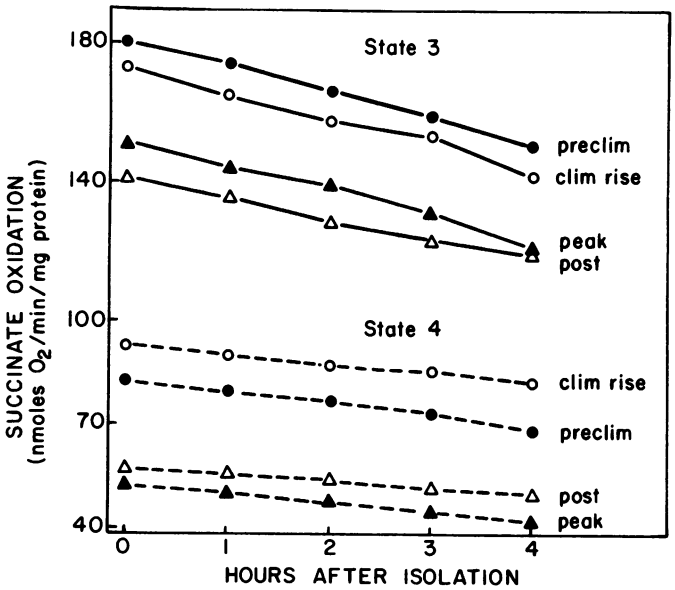

FIG. 3. Mitochondrial stability with storage time at 0 C (in ice); oxidation was at 20 C. Preclimacteric stage, ●; climacteric rise, O; climacteric peak, ▲; postclimacteric, △.

point represents the average value of three to six different mitochondrial preparations. At every temperature from 30 to 2 C, the state 3 rates of succinoxidase were highest in mitochondria from preclimacteric fruit. The climacteric rise mitochondria gave an activity about 10% lower at temperatures between 10 and 30 C, while climacteric peak was lower by 22%, and postclimacteric by 26%. Thus it appears that as ripening advances there is a continuous decrease in activity of the rate-limiting step of the succinoxidase system.

The change in state 4 rates is quite different. At all temperatures, preclimacteric and climacteric rise stages are not significantly different in succinoxidase; climacteric peak and postclimacteric stages were also similar but were 67% of the preclimacteric rate between 2 and 20 C and 50% between 25 and 30 C. In the ADP-limited step of the succinoxidase system an abrupt change occurred during the climacteric rise which was primarily a reflection of the much lower state 4 rates in the two later stages.

DISCUSSION

Changes in mitochondrial oxidations have been a popular

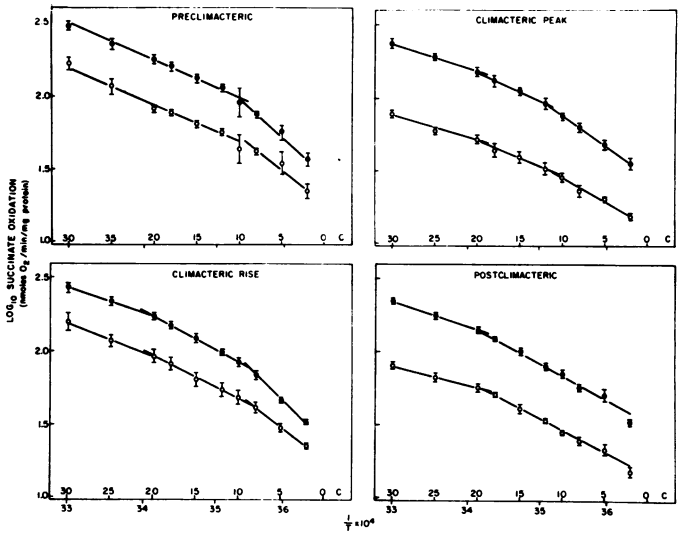

FIG. 4. Arrhenius plots of succinate oxidation by 'Fuerte' avocado mitochondria of four different climacteric stages. Stage 3, ●; and stage 4, O.

Table I. State 3 activation energy of mitochondrial succinoxidase activity at three temperature ranges from 'Fuerte' avocado fruit at four climacteric stages

Temp Range C	Climacteric Stage Pre	Rise	Peak	Post
	kcal/mol			
High (20-30)	10.0	7.9	7.3	7.7
Mid (9-20)	10.0	11.9	9.8	11.4
Low (2-9)	19.3	19.8	15.1	11.4

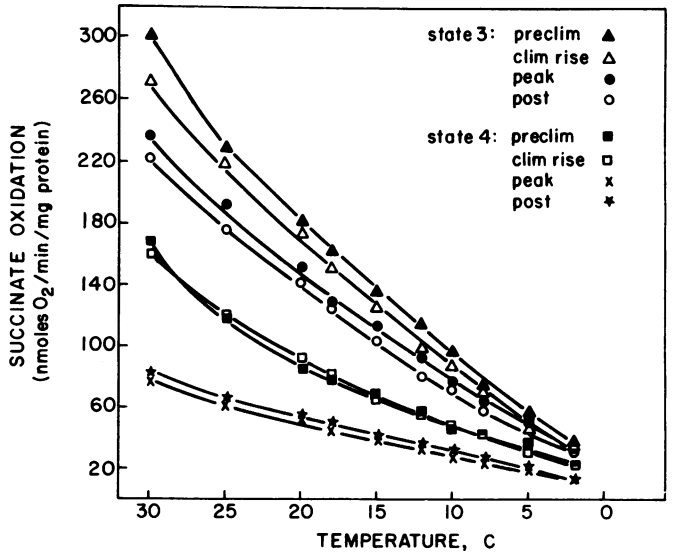

FIG. 5. Influence of temperature on the rate of succinate oxidation both state 3 and state 4 from four different climacteric stages of 'Fuerte' avocado fruit.

subject for investigations concerning the respiratory increase associated with ripening in fruit (3, 5, 9). Mitochondria isolated from several different fruits have shown high rates of oxidation of tricarboxylic acid cycle acids at all stages of the climacteric. In some cases, the rate of succinate oxidation has increased with successive stages of ripening, while in others it has remained constant or decreased. The trends appear to have depended on the method of preparation and the basis of the assay. Our experiments using an improved and much more standardized method of dispersing the tissue (10) as well as an improved medium for isolation and storage of the particles (22) show a decrease in succinoxidase activity with each advancing stage of ripening in terms of state 3 rates and a sharp decrease in state 4 rates between the climacteric rise and climacteric peak stages. These results confirm earlier conclusions that the respiratory climacteric cannot be explained by an increase in rate of oxidation of tricarboxylic acid cycle intermediates of *in vitro* mitochondria. This does not, however, preclude the possibility of mitochondria being involved in control of respiration in the intact cell. It should be noted that the morphology of *in vivo* mitochondria is quite different from that of particles isolated in a medium which show maximal succinoxidase activity (10). Mitochondria can be isolated in other media with shape and movement which closely resemble that of *in vivo* mitochondria and these show very much lower rates of oxidation of tricarboxylic acid cycle intermediates. Thus, isolated mitochondria may lack controls imposed on *in vivo* mitochondria, for example in transport of substrate or intermediates across membranes.

The idea that a change in permeability of a membrane system may be responsible for the respiratory rise was proposed nearly 50 years ago by Kidd and West (6) and has been the subject of many investigations since that time (17). However, methods used have lacked sensitivity to show a permeability change as early as the respiratory increase and no agreement has been reached as to whether a permeability change is the primary factor involved in initiation of the climacteric. There is no doubt that a large change in permeability does occur late in the climacteric (23, 27).

Our attention was refocused on permeability as a result of our studies on chilling injury. It is generally believed that chilling injury results from the lipid of membranes undergoing a phase change at the temperature which induces injury such that permeability is drastically changed. The symptom of chilling is thought to result from failure of normal membrane transport (11).

We have shown earlier (8) that there is marked increase in chilling sensitivity of avocado fruit with advance of the climacteric rise followed by a decrease in sensitivity to cold in the postclimacteric stage. Lyons and Raison (12) found that mitochondria isolated from chilling-sensitive tissues showed a break in the Arrhenius plot of succinoxidase activity while particles from chill-resistant tissues did not do so. This was interpreted as showing that the acyl fatty acid residues of the membrane phospholipids associated with succinoxidase were more saturated in chilling-sensitive tissues. That the breaks in Arrhenius plots of succinoxidase do represent a property of the lipid in which the enzymes are embedded seems well justified. Discontinuities in Arrhenius plots of the activity of several membrane-bound enzymes in animal, plant, and microbial tissues have correlated closely to breaks in like plots of the signal of electron spin resonance, fluorescence, and differential scanning calorimetry (1). X-ray diffraction and freeze-fracture electron micrographs show distinctive changes at the same temperatures where breaks in the Arrhenius plots occur (4). These measurements leave no doubt that sharp changes in activation energy with change in temperature are primarily due to phase changes in associated lipid which occur at lower temperatures as the acyl fatty acid residues become more unsaturated or of shorter chain length. The polar head group of phospholipids may also exert a weaker but significant influence on the activity of associated enzymes (16, 24). Two shifts in activation energy of membrane-bound enzymes have been observed in yeast (25), bacteria (1), and plants (18, 26). The higher temperature transition has been shown to represent "clustering" of lipids from the liquid state (4), or the change from "fluid state" to "solid disordered state" while the lower temperature transition represents the change from "solid disordered" to "solid ordered." This latter change has earlier been referred to as "liquid crystalline" to "solid gel" (12).

The experiments reported here show distinctive changes in activation energy of succinoxidase for each phase of the climacteric which we believe to be caused by differences in lipid phase transitions. The preclimacteric stage shows no transitions at 20 C while one does occur on the climacteric rise. The fruit chosen for the climacteric rise stage were always taken when the respiration had increased only 10% from the minimum value. We considered this the smallest change that allowed us to be certain that a fruit was clearly on the climacteric rise. Respiration of individdual fruit was monitored through an automatic sampling device at either 2- or 4-hr intervals which enabled us to select fruit that were very close to the desired stage of respiratory cycle. We concluded that as the fruit enter the climacteric rise, a change in mitochondrial membrane lipid occurred which caused clustering to occur at 20 C. We have not studied temperatures above 30 C but we assume that this phase transition occurs at some higher temperature during the preclimacteric phase.

The lower phase transition occurs at about 9 C in both the preclimacteric stage and the climacteric rise. At the climacteric peak, however, this transition occurred at between 11 and 12 C indicating that the lipids have changed again and in a way suggesting that they were more saturated or of longer chain length. Such a change is consistent with the observation of chilling occurring at a higher temperature at the climacteric peak (8).

In the postclimacteric phase the low temperature phase transition occurs at between 2 and 5 C which indicates that the lipid remains in the solid disordered state to a lower temperature and suggests that the lipid is more unsaturated or of shorter chain length.

We have not carried out lipid analysis to determine whether detectable compositional differences can be shown between climacteric stages. Earlier attempts on our part as well as of others (26) have given inconclusive results because of impurities associated with intact mitochondria. Use of mitochondrial fragments fractionated for a particular enzyme activity should allow identification of lipid compositional changes between ripening stages. There is evidence for rapid synthesis of mitochondrial lipid in avocado fruit slices as well as isolated mitochondria (15). In another climacteric fruit, the apple, Mazliak (13) has shown rapid incorporation of both [^{14}C]acetate and ^{32}P into mitochondrial lipids of plugs of tissue. Synthesis was fully as fast in postclimacteric tissue as in preclimacteric. A quantitative study of changes in lipids of apple tissue where over 70% of the lipid is polar lipid was made by Galliard (2) and provided evidence that distinctive compositional changes do occur between the ripening stages. Similar data are more difficult to obtain for avocado where only 3 to 4% of the lipid is polar lipid.

The change in phase transition to a higher temperature at the climacteric peak and to a lower one in the postclimacteric stage is in harmony with the observed changes in chilling sensitivity (8). Data reported here support the hypothesis that as the phase transition occurs at higher temperatures, the fruit become more sensitive to chilling injury. This is presumed to be due to restriction of transport of intermediates across membranes as the lipids change phase to the solid ordered state (11).

Our studies show breaks in the activation energy plots of succinoxidase activity which are distinctive for each stage of the climacteric. This suggests that the composition of membrane lipids is different for each climacteric phase and may be expected to cause changes in activity of membrane-bound enzymes in general. We find also that the changes in breaks in Arrhenius plots are closely correlated to changes in chilling sensitivity to the fruit. We suggest that the changes in lipid phase transition reflect changes in lipid composition which in turn regulates transport across membranes. Methods used here with succinoxidase applied to other enzymes involved in membrane transport may provide more direct evidence for an explanation of induction of ripening.

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