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Protein and Nucleic Acid Metabolism in Fruits: I. Studies of Amino Acid Incorporation During the Climacteric Rise in Respiration of the Avocado[1]

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Summary. Incorporation of ^{14}C L-valine and ^{14}C L-leucine into protein of tissue slices of the avocado fruit was relatively high during the early stages of the climacteric rise, declined sharply thereafter, and was virtually absent at the peak. The incorporation of amino acids in the preclimacteric stage was markedly lower than during the early stage of the respiratory rise. By following incorporation in relation to uptake at several concentrations it was established that the results were not a reflection of endogenous dilution.

Puromycin was effective as an inhibitor of incorporation but not of oxygen uptake. When respiration was at its maximum there was no protein synthesis. It was concluded, therefore, that the respiratory upsurge characteristic of the climacteric was not related directly to protein synthesis.

Fruits with distinct patterns of respiration offer desirable material for studies of cellular aging from the physiological and biochemical viewpoint. The rapid upsurge in O_2 uptake known as the climacteric rise (1, 2, 3) delineates between growth and maturation stages and the final phase in the life of the fruit.

Reports have been published of correlations between the respiratory upsurge and nitrogen metabolism. Hulme (8) found an increase in net protein content of apples in the course of the climacteric rise, but the rate of CO_2 evolution rose more sharply than protein synthesis. Similarly, Rowan et al. (14) reported for the avocado an increase in protein nitrogen and in high energy phosphate along the climacteric. Pearson and Robertson (12) proposed that as a result of protein synthesis the ATP/ADP ratio changes in favor of the phosphate acceptor which in turn causes the rise in respiration. Young et al. (21) questioned the phosphate acceptor hypothesis on the basis of determinations of levels of adenine nucleotides in the ripening avocado. It is also questionable whether the methodology of extraction used in the protein studies on fruits furnished the correct trends on proteins in materials varying in cellular contents and cell wall composition at different stages of ripeness. Young (19) has called attention to difficulties involved in such studies.

The approach to the problem of protein synthesis at the onset and during the climacteric rise was reopened by employing contemporary methodology. Specifically, the questions to which we are seeking answers are whether amino acids are incorporated into proteins of the fruit and whether changes in nucleic acid patterns are of the kind which support the idea of formation of new proteins. In this study we are reporting on incorporation of labeled valine and leucine into tissue discs.

Materials and Methods

Cultivar Fuerte avocados (*Persea gratissima*, Gaertn.) were obtained early January to early

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July from orchards in the district of Escondido or Santa Paula, California. The fruits were either used immediately or stored up to 6 days at 7.5°. The pattern of the climacteric rise was measured at 20° with a Beckman paramagnetic oxygen analyzer (20).

Incubation Procedure. To follow protein synthesis, the incorporation of ^{14}C amino acids into discs was measured. With the aid of a slicer developed by Steward and Caplin (16), 1 mm thick transverse slices were removed from the fruits some 2 cm from the stem end. Discs 5 mm in diameter were secured from the slice with a cork borer and were washed in running tap water for 30 seconds, then blotted gently to remove excess moisture. Nine discs with a total dry weight of about 40 mg were incubated in 0.55 ml solution at 25° in a shaker. The incubation mixture consisted of 5×10^{-4} M phosphate buffer, pH 6.5, and of uniformly labeled ^{14}C L-valine or ^{14}C L-leucine (New England Nuclear Corp.). The specific activity and total concentration of the radioactive amino acids varied, as indicated in the text.

Determination of Total Uptake. At the end of the incubation period all the discs were rinsed once in cold distilled water and then blotted. To determine the active uptake, namely the amount of label retained inside the permeability barrier, 3 discs were washed in 10 ml of 0.01 M DL-valine. Vigorous stirring was provided by air passing through a capillary. The solution was changed once and the discs removed after a total of 15 minutes. They were blotted and dried at 90° for 48 hours and weighed. To determine total uptake, i.e. active uptake and uptake into the free space, 3 discs were dried immediately after blotting without being first washed. For the measurements of radioactivity the dried material was ground with a total of 15 ml scintillation mixture in a mortar and pestle and the brei transferred to scintillation vials half filled with Thixotropic Gel Powder (Cab-O-Sil-Packard). This procedure kept the ground particles in suspension and facilitated a counting efficiency of 55 to 60 %. Free space was estimated by subtracting active uptake from total uptake. Alternatively, the free space was found by extrapolating the kinetic data of total uptake to zero time. Free space computed by the former method was significantly higher than by the latter method.

Protein Extraction. The remaining 6 discs were frozen in acetone and dry ice and were usually kept in the deep freeze for 24 hours before analysis. The frozen material was ground in a VirTis 45 homogenizer for 2 to 3 minutes with 10 ml of 0.02 M DL valine or leucine, as appropriate, in a straight walled VirTis flask kept in an ice bath. The homogenate was transferred to centrifuge tubes to which 0.1 ml Triton × 100 (Alkyl phenoxy polyethoxy ethanol, Rohm & Haas) was added. The tubes were shaken vigorously by hand, then shaken mechanically in ice for 60 minutes. Reproducible results could not be obtained without the Triton treatment. Afterwards, 10 ml of 10 % trichloroacetic acid were added to each tube, then centrifuged at 70,000 × *g* for 20 minutes. Such intensive centrifugation was necessary to obtain a firm pellet. The precipitate was dispersed in 5 ml of 5 % trichloroacetic and heated in a water bath to 80° for 15 minutes. Recentrifugation yielded a precipitate which was dispersed in 1.5 ml of 2 % NaOH, then reprecipitated with 3.0 ml of 10 % trichloroacetic. The supernatant was removed completely and the pellet dissolved in 0.4 ml of .01 N NaOH. Aliquots of 0.1 ml and of 0.05 ml were taken for measurements of radioactivity and nitrogen (18), respectively.

Evidence that the label was incorporated into a polypeptide was obtained by means of acid hydrolysis and proteolytic enzyme cleavage (13). Additional evidence that the label was essentially bound in a newly synthesized protein came from the fact that puromycin affected a decisive decrease in incorporation (see table IV). The addition of 50 μg streptomycin sulphate per ml of incubation medium had no effect on the magnitude of incorporation. We were reassured that microorganisms were not responsible for the incorporation activity displayed by the discs from the fact that late climacteric soft tissue, known to be susceptible to microorganism attack, exhibited a marked reduction in incorporation (see table II).

Radioactivity Determinations. A Nuclear-Chicago Liquid Scintillation System 720 series was used for all radioactivity measurements. The scintillation mixture for total uptake determinations was made of toluene, to which 4 g PPO (2,5-Diphenyloxazole) and 0.060 g POPOP (2-*p*-Phenylenebis-5-Phenyloxazole), both obtained from Eastman Kodak, were added per liter. Scintillation mixture for counting the aqueous protein solution was made of *p*-dioxane, to which 70 g naphthalene, 6 g PPO and 0.075 g POPOP were added per liter. All counts were corrected according to a standard quenching curve.

Respiration Measurements. Respiration measurements of the discs were carried out in a Warburg respirometer. Some 30 discs with a fresh weight of 500 mg were employed in each flask, bathing in 2.5 ml of 0.05 M phosphate buffer pH 6.5. The side arm contained 200 μg puromycin which was dipped after 30 minutes of equilibration at 25°. Readings were taken every 10 minutes for additional 40 minutes.

Results

Figure 1 shows a typical pattern of the climacteric rise in respiration of Fuerte avocado fruit kept at 20°. From the standpoint of amino acid incorporation activity, three separate stages could be readily distinguished: preclimacteric (PC), early climacteric, up to ca. 50 % of the peak in O_2 uptake (Cl-1) and late climacteric, from ca. 75 % to the peak (Cl-2).

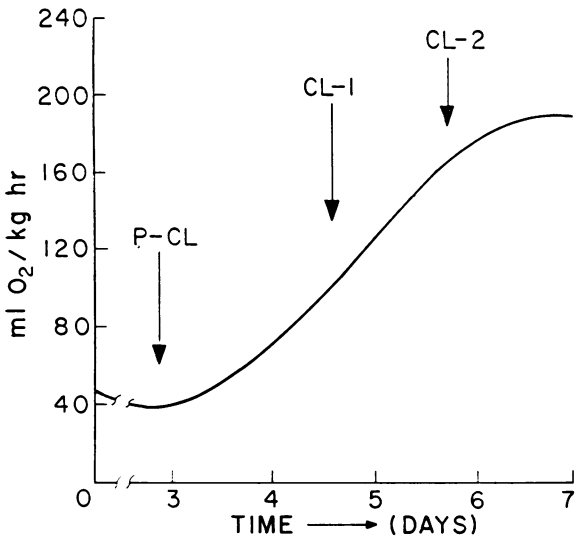

FIG. 1. A typical pattern of the climacteric rise in respiration of Fuerte avocado fruit. The fruit was removed from the tree at 0 time and its respiration measured at 20°.

Under our experimental conditions, active uptake of the label by both pre- and climacteric tissue was essentially proportional to its concentration in the incubation medium (table I). Similarly, kinetics of total uptake was a function of label concentration (fig 2). At relatively low concentration (A), a steady state uptake in pre- and climacteric material was maintained for the whole duration of the experiment. At higher concentrations (B and C), a rapid initial rate of uptake gradually declined, this being most pronounced in climacteric tissue (Cl-1) which exhibited the highest initial rate of uptake. The changing rates of uptake seen most readily under high concentration presumably result from initial high rates of influx into free space.

Figure 2 also illustrates that the amount of total uptake was always greater in climacteric tissue. This was in part due to accentuated active uptake (table I) but stemmed mostly from the extensive increase in free space that accompanied the climac-

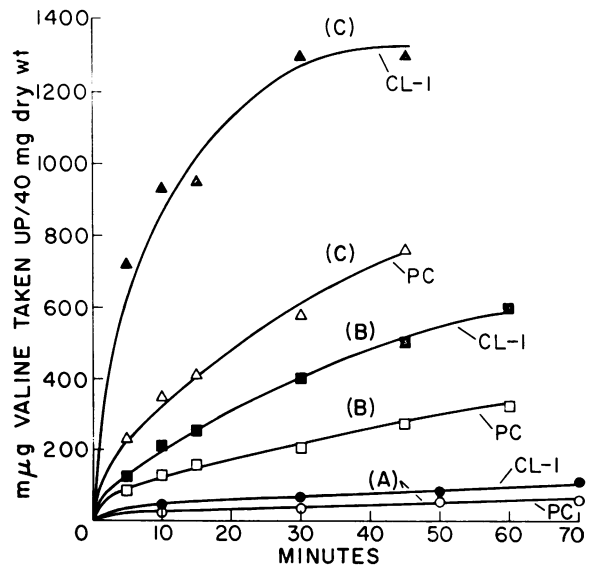

FIG. 2. The effect of the concentration of the label on the kinetics of its total uptake in preclimacteric (PC) and early climacteric (Cl-1) tissues. 9 Discs 5 mm in diameter and 1 mm thick were incubated in 0.55 ml of 0.05 M phosphate buffer pH 6.5 with ^{14}C uniformly labeled L-valine in the following concentrations: A = 20 μmolar, B = 100 μmolar, C = 500 μmolar. Specific activity, 1×10^4 cpm/mμmole.

Table I. *Uptake and Incorporation of L-valine at Various Concentrations into Tissue Slices Secured from 3 Respiratory Stages*

Stage in respiratory activity	L-valine concentration* (mμg/ml medium)	mμg active uptake per 40 mg dry wt	mμg incorporated per 40 mg dry wt	Ratio of incorporation to uptake (I/U)
	2500	17	6	0.35
PC	5000	42	15	0.36
	10000	84	26	0 31
	20000	139	46	0.33
	2500	46	39	0.85
Cl-1	5000	85	58	0.68
	10000	215	120	0 56
	20000	387	260	0.67
Cl-2	2500	14	11	0.79
	5000	30	26	0 86
	10000	43	33	0.77
	20000	51	40	0.78

* Specific activity of all solutions was 200 cpm/mμg. Incubation period 60 minutes.

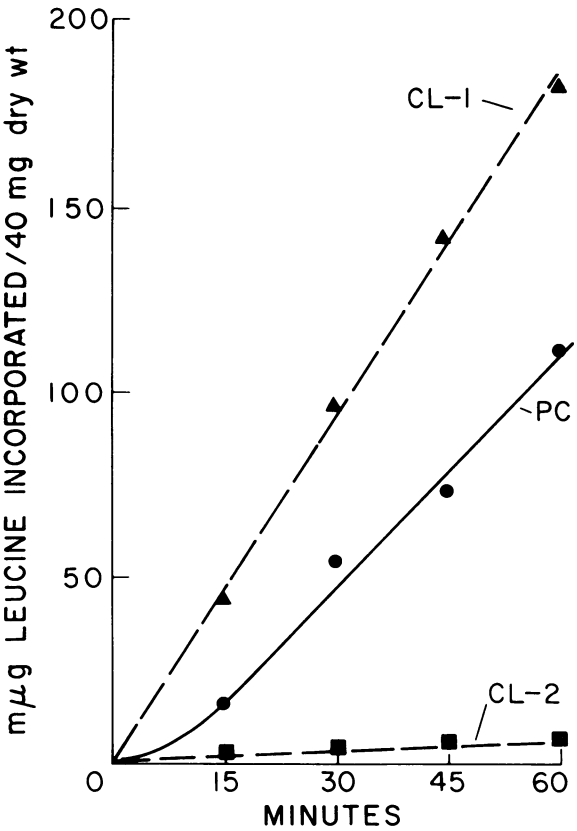

FIG. 3. Kinetics of incorporation of L-leucine into protein of tissues at different stages of respiratory activity. Incubation as described for figure 2. Leucine conc. 100 μmolar, 2 × 10^4 cpm/mμmole.

teric rise. Free space, defined as the cellular space into which unhindered diffusion of solutes occurs, increased so greatly within the advent of the climacteric that at the peak of oxygen consumption, most of the amino acid uptake was confined to the free space. Thus, the per cent free space uptake of total uptake after 60 minutes of incubation was 40 % to 50 %, 50 % to 75 %, and 90 % to 97 % in preclimacteric, early climacteric and late climacteric tissue, respectively.

The pattern of incorporation of L-valine and L-leucine was found to be directly related to the (a) climacteric stage, (b) the concentration of the label in the incubating medium, and (c) the kind of amino acid used for labeling.

(a) It is evident from table I that for any given amount of uptake, incorporation was greater into climacteric than into preclimacteric cells. This was revealed by the fact that the ratio of incorporation of the label to its active uptake (I/U) was gradually approaching unity along the climacteric rise. However, the data reported in table I for climacteric-2 were not typical for late climacteric discs secured from fruit at or very near to peak respiration (table II). Such material displayed very limited active uptake and a drastic reduction in incorporation, much below the preclimacteric level. In experiment no. 3 the material was estimated to be very close to or at the peak of the rise; active uptake was much reduced and no incorporation could be detected whatsoever.

(b) The usual incubation medium for following the pattern of incorporation contained 10 to 15 μg amino acid per ml. Typical kinetics of incorporation in such concentration are shown in figure 3. Up to 60 minutes incorporation of the label was linear with time at all respiratory stages. A slight deviation was observed for the preclimacteric material which usually exhibited a small lag in the first 15 minutes of the incubation period, probably resulting from the smaller free space.

Detailed kinetic study of material incubated in very low amino acid concentrations (fig 4) revealed that below 2.0 μg/ml of leucine or 0.5 μg/ml of valine, the usual enhancement in incorporation in climacteric material was modified. It was observed for only 30 to 40 minutes from the start of the incubation period, after which interval a sloping off in incorporation took place. Under such circumstances, therefore, incubations for a period of 60 minutes resulted in little difference in incorporation between pre- and climacteric discs, whereas incubation periods over that time resulted in higher total incorporation in preclimacteric material. The

Table II. *Uptake and Incorporation Activity of Peak Climacteric Material*

Expt no	Incubation medium/ (Valine ^{14}C)	mμg Active uptake per 40 mg dry wt	mμg Incorporation	Ratio of incorporation to uptake (I/U)	Incorporation as % of PC level
1	12 μg/ml 152 cpm/mμg	22	4.0	0.18	23
2*	9 μg/ml 157 cpm/mμg	86	4.5	0.05	31
3	8 μg/ml 285 cpm/mμg	20	none	...	0

* Active uptake computed from consideration of free space by extrapolation (see Materials and Methods).

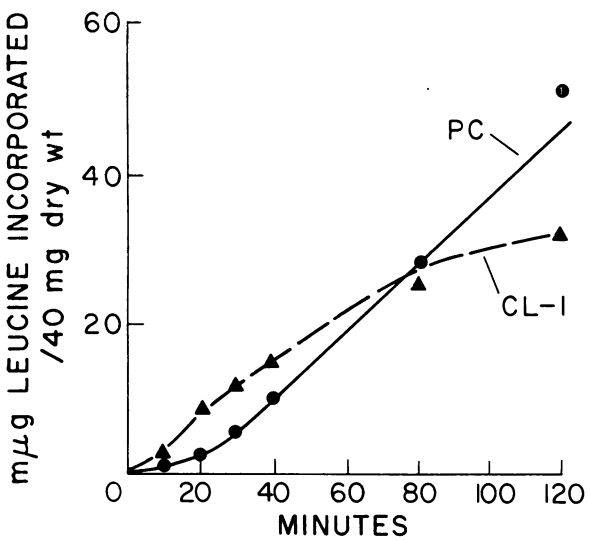

FIG. 4. Kinetics of incorporation of L-leucine into protein of tissues at different stages of respiratory activity. Incubation as described for figure 2. Leucine conc. 10 μmolar, 1 × 10^5 cpm/mμmole.

incorporation data in table III also reflect this phenomenon.

(c) Great differences were observed between the extent of incorporation of L-valine and L-leucine. When employed under similar concentrations, uptake of leucine was 2-fold higher and its incorporation 4 to 5 fold higher than that of valine.

Table III summarizes a set of experiments designed to examine the possibility of endogenous dilution. Overall low L-leucine concentrations were selected in order to provide additional sensitivity for detecting this possibility. Since the ratio of incorporation in low to very low concentration remained essentially constant, the likelihood was remote that differential endogenous dilution affected the rate of labeling along the climacteric rise. This point will be further elucidated in the Discussion.

The effect of puromycin and Actinomycin D was tested on active uptake and incorporation of L-valine into slices in pre- and climacteric stages (table IV). Up to 60 minutes, 100 μg/ml of puromycin only moderately affected the uptake and incorporation of the label into avocado slices at the preclimacteric stage. Early during the climacteric rise, however, the inhibiting effect of puromycin on both uptake and incorporation was greatly accentuated (table IV). Significantly, the effect on incorporation was noticeable already at the first 15 minutes interval, whereas the effect on uptake was observed only after 45 minutes of incubation (13). Thus it was evident that the inhibitory effect of puromycin on active uptake was secondary, probably resulting from the sharp suppression of incorporation activity. 40 μg Actinomycin D/ml incubation medium had little or no effect on incorporation and uptake during a 60 minute incubation period.

Table IV. *Effect of Puromycin on Uptake and Incorporation of L-valine in Pre- and Climacteric Tissue Slices During a 60 Minute Interval*

	Active uptake (mμg)		Incorporation (mμg)	
	Control	Puromycin*	Control	Puromycin*
PC	259	215	110	76
on rise	409	267	179	30

* 100 μg/ml incubation medium.

Since puromycin effectively and instantly inhibited incorporation of amino acids at the climacteric stage, we tested the effect of puromycin on the oxygen uptake of climacteric discs in a Warburg respirometer. Puromycin was introduced after 30 minutes of equilibration and readings taken for an additional 40 minutes. No effect on oxygen uptake could be noted (13).

Discussion

Studies of amino acid incorporation into tissue slices secured from an organ undergoing marked physiological and biochemical modifications may be complicated by 2 difficulties. One is the possibility

Table III. *A Comparison of the Incorporation of L-leucine under Conditions of Low Label Concentrations*

Fruit No	Respiratory stage	mμg L-leucine incorporated per 40 mg dry wt		Ratio of incorporation in A to B
		2000 mμg/ml (A)*	250 mμg/ml (B)*	
1	PC	14.0 mμg	1.5 mμg	10
	Cl-1	19.0 "	1.9 "	10
	Cl-2	1.0 "	0.2 "	5
2	PC	37.0 "	3.1 "	12
	Cl-1	34.0 "	3.4 "	10
	Cl-2	2.0 "	0.2 "	10

* Specific activity: A. 350 cpm/mμg leucine; B, 2800 cpm/mμg. Incubation period 60 minutes.

that the compartmentation of the amino acids in the cell may alter, thereby conceivably bringing about the dilution of the label. The fact that leaking was shown to be typical of climacteric tissue (15) gave support to this possibility which was therefore tested thoroughly. The other difficulty rested with the finding that the active uptake of the label varied greatly along the climacteric rise. The analysis of the experimental data, therefore, required a concept to portray comparable differences in incorporation at the various respiratory stages. This was provided by the incorporation to active uptake ratio (I/U).

A basic premise that we employed was that the relative effect of endogenous dilution would have been more prominent when uptake was low than when it was high. Thus if at any respiratory stage endogenous dilution affected the rate of labeling in our system, the I/U ratio for that stage would depend on the concentration of the label in the incubation medium. The finding that the I/U ratio at any given respiratory stage remained constant even with an 8-fold increase in the concentration of the label (table I) thus excluded the possibility of endogenous dilution. Further evidence along these lines is provided in table III showing that the ratios of incorporation in low (A) to very low (B) concentrations remained essentially the same all along the respiratory rise. Endogenous dilution of the label would have affected a rise in this incorporation ratio. In that set of experiments the very low concentration of the label was some 40 times smaller than usual, providing added sensitivity for the detection of any diluting effect.

The rising I/U ratios (table I) along most of the climacteric rise also indicated that the higher incorporation activity of Cl-1 resulted from a higher incorporation potential and did not merely reflect the enhanced uptake that was typical for this stage. However, the amino acid incorporating pattern of material close to the peak of oxygen uptake was not clear-cut. Obviously the level of uptake on a dry weight basis was drastically reduced at this stage (fig 1). Nevertheless the I/U ratios were similar to those obtained for Cl-1. Hence we concluded that at Cl-2 there occurred a drastic reduction in the number of cells which took up and incorporated the label but that the cell population which was still capable of active uptake showed a potential for protein synthesis similar to the material in the Cl-1 stage. Late at Cl-2, i.e. very shortly before the peak in oxygen consumption was reached, the capacity for uptake and incorporation of L-valine or L-leucine was rapidly disappearing (table II). Thus, our work does not support the notion that enhanced protein synthesis occurs throughout the climacteric rise.

Our interpretation for the rise in amino acid incorporation at the early climacteric stage is that it reflects an induction of a set of enzymes which catalyze the climacteric process and the final breakdown of the cell. There are several reports relating an increase in the activity of some enzymes during the climacteric. Dilley (6) isolated a Mn^{++} and NADP dependent malic enzyme from cortex of apples with higher specific activity (units enzyme/mg protein) in post-climacteric than in preclimacteric McIntosh fruit. Barker and Solomons (1) found a 20-fold increase in fructose diphosphate in climacteric bananas which they thought may be due to an increase in the content of phosphofructokinase. Hobson (7) reported that polygalacturonase activity rose exponentially in the early stage of tomato ripening, continuing to rise even as the fruit became overripe. Hulme et al. (9) suggested that the origin of the climacteric in fruits may be due to an increased synthesis of malic enzyme and pyruvic carboxylase. However, the magnitude of enzymatic activity assayed in a tissue extract may not be directly related to the total in vivo quantity of the assayed enzyme, since inhibitors or effectors may regulate an allosteric protein to exhibit any fraction of its potential activity. Furthermore, difficulties in quantitative evaluation of enzyme activity extracted from tissue undergoing marked physiological modifications may be especially serious. Thus Young (19) showed that the reported increase in aldolase activity in climacteric bananas (17) was due to the adsorption of this enzyme on tannins prevalent in the preclimacteric but absent in the climacteric homogenate. Therefore, no direct evidence has yet been furnished for the occurrence of enhanced enzyme synthesis during the climacteric rise in respiration of fruits. The full elucidation of this question will probably require proof for acceleration in labeling of purified and identified proteins.

The decisive inhibition by puromycin of amino acid uptake and incorporation in climacteric material served as added evidence for the assumption that the incorporation data presented in this paper resulted from new protein synthesis. The absence of a similar inhibitory effect in the preclimacteric material may have resulted from impeded permeability. In an early experiment, with an incubation period that lasted 120 minutes, there was a significant effect of puromycin on preclimacteric as well as climacteric tissue. The fact that climacteric oxygen uptake was not retarded by puromycin even though this inhibitor drastically and instantly blocked amino acid incorporation clearly demonstrated that oxygen consumption was not coupled to protein synthesis.

The lack of inhibitory effect by puromycin on climacteric oxygen uptake and the fact that uptake and incorporation of amino acids virtually ceased at the peak of the respiratory rise is significant. It provides a clear distinction between the climacteric rise in respiration of the avocado and probably other climacteric fruits and the accelerated respiration that develops in aged slices of storage tissue (4, 5, 11). We think that the latter phenomenon has the characteristics of rejuvenescence

while the climacteric rise in fruits marks the onset of senescence.

Since this paper was submitted the study of J. A. Sacher on permeability and amino acid incorporation in banana tissue appeared (Plant Physiol. 41: 701, 1966). While the decline in incorporation at the peak of the climacteric appears to take place in the banana as it does in the avocado, the comparison between the preclimacteric and early climacteric shows decided differences. The analysis of the dilution factor is also different in the 2 papers and may be ascribed to divergence in concentrations used.

Acknowledgments

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