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Phosphorylation in Avocado Fruit Slices in Relation to the Respiratory Climacteric[1]

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Summary. The rate of uptake of inorganic phosphate by tissue discs from both preclimacteric and climacteric peak avocados is linear for at least 60 minutes. The loss of ^{32}P upon excessive washing was much greater from peak than from preclimacteric tissue. Short incubation periods and, most important, rapid washing procedures are essential for meaningful comparisons.

Phosphate esterification proceeded at a much greater rate in climacteric than in preclimacteric tissue. The phosphorylation was sensitive to 2,4-dinitrophenol (DNP). The ADP to ATP ratio decreased materially with the advance of ripening. It was concluded that neither uncoupling nor acceptor control can account for the onset of the respiratory rise. Changes in permeability appear to play an important role in fruit metabolism during the climacteric.

Many fruit exhibit a marked increase in respiratory activity shortly after picking which has been referred to as the climacteric rise in respiration. A number of explanations of this phenomenon which have been proposed but not fully substantiated, have been reviewed by Biale (2), Varner (16), Spencer (15) and by Rowan (12). Millerd et al. (9) advanced the idea that at the induction of the climacteric an endogenous uncoupler of oxidative phosphorylation was formed which released a restraint on oxygen utilization. Pearson and Robertson (10) and Hulme (7) proposed a different mechanism which could also account for the rise in respiration. They suggested that oxidation was limited by the amount of ADP available to the phosphorylative sites in the preclimacteric tissue. With the induction of the climacteric increased protein synthesis would tend to increase the ADP/ATP ratio and release respiration from the restraint imposed by limiting levels of ADP.

The uncoupling hypothesis was supported by experiments with tissue slices which showed the addition of the uncoupler 2,4-dinitrophenol (DNP) caused an increase in oxygen uptake in tissue slices of preclimacteric but not of climacteric fruit (9). On the other hand, experiments with isolated mitochondria could not be explained by this hypothesis. Romani and Biale (11) showed that the P/O ratio actually increased as the climacteric progressed in avocado fruit. More recently Wiskich et al. (17) demonstrated that mitochondria isolated from climacteric peak avocado fruit show very good respiratory control, that ATP was indeed formed and that the mitochondria were highly sensitive to the artificial uncoupling agent DNP. While the isolation procedure of Wiskich et al. (17) was not satisfactory for preclimacteric tissue, Lance et al. (8) and Hobson et al. (6) were successful in working out a single procedure for the isolation of mitochondria from avocado fruit at all stages of the climacteric which showed good respiratory control with high oxidative and phosphorylative activities as well as sensitivity to uncouplers.

The experiments cited above provide clear proof that isolated mitochondria from all stages of the climacteric show both coupled phosphorylation and acceptor control. However, it is well known that isolated mitochondria can be uncoupled by artificial uncoupling agents and recoupled by washing out the uncoupler. As for the acceptor control hypothesis, the demonstration of ADP control by isolated mitochondria gives no information on the ADP status of the mitochondria in the cell. Neither the uncoupling nor the ADP control theory can be satisfactorily tested by experiments with washed mitochondria. An endogenously formed uncoupler and ADP may both be washed out of the mitochondria during isolation.

The work reported here is concerned with the accumulation of Pi into tissue slices of preclimacteric and climacteric peak fruit. Comparisons of the 2 stages are made with respect to incorporation of Pi into esters in the absence and presence of uncoupling agent and in relation to trends in the ADP/ATP ratio. Special consideration is given to changes in permeability to phosphate ester and to changes in free space during the climacteric previously proposed by Sacher (14).

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Materials and Methods

Avocado fruit, *Persea gratissima*, variety Fuerte, were obtained in the orchard of the College of Agriculture, University of California, Los Angeles. The respiratory activity of individual fruit was followed on an automatic paramagnetic oxygen analyzer as described by Young and Biale (18). Preclimacteric fruit were taken at any time before there was an increase in respiration while climacteric peak discs were selected from avocados at their respiratory maximum.

Fruit were peeled and cross sectional slices cut 2 mm thick with a Freemaster vegetable slicer. Discs 1 cm in diameter were cut from the slices with a stainless steel cork borer. Fifteen discs were rinsed in cold water, transferred to small stainless steel baskets and placed in 50 ml beakers containing 8 ml of 0.5 mM phosphate buffer (pH 7.0). When used, DNP was 0.5 mM and was added 10 minutes prior to the addition of 1 mc of ^{32}P. Slices were incubated in a Dubnoff shaker at 120 oscillations per minute at 25°. After shaking for 10 minutes in the ^{32}P solution, the basket with the slices was washed for 20 seconds in a 2 liter bath of ice water. Slices were blotted briefly on filter paper and transferred to a 20 ml mixture of methanol, chloroform and 2 M formic acid 12/5/3 v/v (MCF), held in a dry ice bath. Extraction and chromatography and assay of the radioactivity were followed according to the procedure of Bieleski and Young (5) as modified by Bieleski (3, 4). Briefly, the tissue was homogenized in 20 ml of MCF with a Virtis homogenizer, filtered and the residue re-extracted twice with 20 % ethanol made 2 M in formic acid. The phosphate esters of the first extract were partitioned from the lipids and pigments by adding 5 ml of chloroform and 5 ml of water, centrifuging and combining the aqueous phase with the second and third extracts. The combined extracts were evaporated to dryness in a rotary evaporator operated at 20 to 50 μ pressure in a 20° bath temperature. The residue was taken up in 3 one ml portions of water and the cations removed on a 1 × 3 cm column of Cellex P. The anion exchanger described by Bieleski and Young (5) was not used. The decationized extract was again evaporated to dryness in a 25 ml flask and taken up in 0.25 ml of water.

An aliquot of this extract was chromatographed on Whatman 3 MM paper, first in *n*-propanol/ammonia/water 6/3/1 v/v, and in the second dimension in *n*-propyl acetate/formic acid/water, 11/5/3 v/v. Radioautograms were made and the activity of each ester on the chromatogram determined by counting on planchets with a Nuclear-Chicago gas flow counter.

The amount of each ester present was determined by neutron activation as described by Benson (1). Slices were treated exactly as above except that ^{32}P was not added. The developed chromatogram was placed in the reactor of the University of California Engineering Department in a flux of 1.4 × 10^{16} neutrons/cm^2, a radiochromatogram made and the induced activity assayed.

Results

In some tissues, the rate at which ^{32}P is taken up depends on the inorganic (Pi) phosphate concentration of the external medium. In the case of preclimacteric avocado tissue slices, it was found that incorporation of ^{32}P over a 30 minute period was independent of Pi concentration as shown in table I. Although the Pi concentration was changed over 4 orders of magnitude, the incorporation per 10^5 CPM ^{32}P added was not greatly different. The concentration of 0.5 mM was used because the results were more reproducible than at lower concentrations.

The rate at which Pi is accumulated into slices of climacteric peak and preclimacteric avocado fruit tissue and the rate at which phosphate can be washed out of the tissue is shown in figure 1. For this experiment, 50 slices were shaken at 25° in 0.5 mM potassium phosphate buffer containing 1.0 μc of ^{32}P. At 10 minute intervals, 5 slices were removed, washed in ice water for 20 seconds and dried on planchets with a few drops of 3 % polyvinyl alcohol (Elvanol Grade 51-05). After 60 minutes, all of the remaining slices were washed in ice water for 20 seconds, then transferred to 40 ml of ice water and returned to the shaker. At 10 minute intervals, slices were removed, dried on planchets and counted.

This experiment demonstrates that the uptake of phosphate by both tissues is linear for at least 60 minutes. Other experiments showed linear incorporation for as long as 5 hours. For convenience, incorporation periods of 10 minutes were used for most experiments.

The final 30 minutes of the curves of figure 1 make it clear that it was important to wash the tissue only briefly after the incubation, and that the wash procedure be done in precisely the same way for each sample. Using a 20 second wash of the slices, climacteric peak tissue showed more than a 3 fold greater incorporation than preclimacteric slices, but

Table I. *^{32}P Incorporation into Avocado Slices as a Function of Phosphate Concentration*

Phosphate mM	CPM ^{32}P Added × 10^{-5}	CPM Incorporated	CPM Incorporated per 10^5 CPM ^{32}P added
0.01	1.0	300	300
0.1	3.3	1080	327
1.0	10.0	2680	268
10.0	33.0	10,240	310

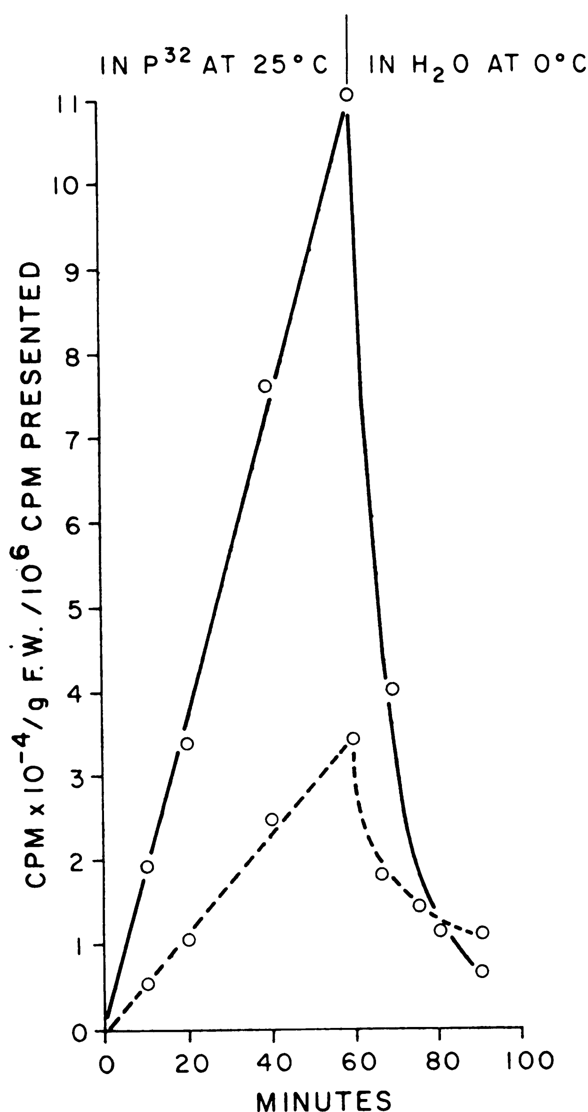

FIG. 1. Rate of phosphate uptake into and loss from tissue slices of preclimacteric and climacteric avocado fruit. Dotted line = preclimacteric, solid line = climacteric slices. The vertical line at 60 minutes indicates that all remaining discs were rinsed 20 seconds and then transferred to water at 0°.

when the tissue was washed in ice water 94 % of the label was lost from climacteric peak tissue, while only 31 % was lost from preclimacteric slices. The climacteric peak slices actually retain less ^{32}P than the preclimacteric slices after 30 minutes of washing, even though they held more than 3 times more ^{32}P after a 20 second wash.

Table II shows that not only Pi but also esterified phosphate is lost upon washing from both tissues, and that very much more is lost from climacteric peak tissue. To demonstrate this point, slices were incubated in ^{32}P for 10 minutes, then one half were washed in ice water for 20 seconds while the other half were washed for 20 minutes. The products were extracted, chromatographed and the activity in individual compounds counted. The summed activities in all nucleotides, in sugar phosphates and in Pi are compared in table II. For both tissues, 87 % of the Pi was washed out in 20 minutes. This amount represents a reasonable approximation of the free space of the tissue. From climacteric peak tissue, approximately the same amount of esterified phosphate was lost as Pi, while in the case of preclimacteric tissue only one-third of the nucleotides and one-half of the sugar phosphates were lost on washing 20 minutes.

These data indicate that there are marked changes in the membrane permeability characteristics associated with the transition through the climacteric and point out the importance of using short incubation periods and especially to use short washing procedures in order to make valid comparisons of the 2 tissues.

A comparison of the amounts of total ^{32}P incorporated into tissue slices of preclimacteric and climacteric peak fruit, as well as the amount of ^{32}P esterified, both in the presence and the absence of DNP, is shown in table III. About 10 times more phosphate was incorporated into the tissue slices of climacteric peak fruit. Of the amount incorporated, 1.4 % was esterified in preclimacteric slices while nearly 8 % was esterified in climacteric peak fruit. While DNP had no significant effect on the amount of Pi found in either tissue, it did reduce the amount esterified by about 35 % in both preclimacteric and climacteric peak slices under the conditions of this experiment. Longer preincubation with DNP was shown later to reduce further the amount of esterifi-

Table II. *Loss of ^{32}P from Avocado Tissue Slices Associated with Washing*

Tissue	Wash time	Inorganic phosphate	Sugar phosphate	Nucleotide phosphate
			cpm/gm fr wt × 10^{-3}	
Preclimacteric	20 sec.	6112	37.3	39.3
	20 min.	789	19.4	26.8
	% Lost	87	48	32
Climacteric peak	20 sec.	58,521	3458	672.0
	20 min.	7281	598	72.8
	% Lost	87	83	88

Table III. *Phosphate Incorporation into Avocado Slices*

	Total accumulated	Total esterified	Percent esterified
		cpm/gm fr wt $\times$ 10^{-3}	
Preclimacteric			
—DNP	6036	83	1.4
+DNP	5360	56	1.0
Climacteric peak			
—DNP	56,620	4470	7.9
+DNP	62,780	2800	4.5

Table IV. *The Effect of DNP on ^{32}P Incorporation into Esters of Preclimacteric and Climacteric Peak Avocado Fruit Tissue*

	Preclimacteric			Climacteric peak		
	—DNP	+DNP	Percent unaffected	—DNP	+DNP	Percent unaffected
			cpm/gm fr wt $\times$ 10^{-3}			
Total esters	83	56	67	4470	2800	63
Sugars	37	20	55	3458	2143	62
Nucleotides	39	33	85	672	457	68
ATP	3.4	3.2	94	43	23	54
ADP	14.2	10.8	76	241	198	82
AMP	3.8	1.8	48	135	44	32
Glycerate 3-P	3.4	1.1	33	124	75	60

cation. Clearly, not only does phosphate enter the climacteric peak tissue more readily; it is also esterified more readily. Further, the phosphorylation appears to be coupled as it is sensitive to the uncoupling agent DNP.

The effect of DNP on ^{32}P incorporation into esters of preclimacteric and climacteric peak tissue is shown in table IV. Total esters represent the sum of activity in all spots on the chromatogram of an extract of tissue incubated 10 minutes with ^{32}P. About 50 fold more ^{32}P was esterified by climacteric tissue, but DNP reduced the ^{32}P esterified by nearly the same percentage. Climacteric tissue incorporated 100 times more activity into sugars and the esterification was reduced by nearly the same percent by DNP in both tissues. The incorporation into nucleotides was reduced only to 85 % in the case of preclimacteric tissue while in climacteric more pronounced diminution of activity was noted. Activity in individual adenine nucleotides and in PGA decreased in both tissues due to the addition of DNP. Two curious results did appear. The activity in ATP of preclimacteric tissue was reduced only slightly by the addition of DNP and much more activity was found in ADP than in the other nucleotides.

Clearly, climacteric tissue is able to phosphorylate efficiently and this phosphorylation is sensitive to the uncoupling action of DNP. It appears that avocado fruit tissue shows coupled phosphorylation at all stages of the climacteric.

The acceptor control hypothesis was tested by determination of the total amount of ATP and ADP in the tissue extracts. The amounts of ADP and ATP are shown in table V. The total amount of adenine nucleotide was essentially unchanged through the transition of the climacteric, but the ratio of ADP to ATP decreased from 2.30 to 0.88. Assuming part of the ADP is not separated into an inactive compartment, the ADP level cannot exert acceptor control of the rate of oxidation in avocado fruit. Rowan et al. (13), using an enzymatic assay, have shown that the ADP/ATP ratio decreases in cantaloupe fruit with the transition of the climacteric.

Table V. *Changes in the Amount of ADP and ATP Associated with the Transition of Preclimacteric to Climacteric*

	ADP	ATP	Ratio $\frac{ADP}{ATP}$
	µmoles/gm fr wt		
Preclimacteric	207	89	2.30
Climacteric	124	140	0.88

Discussion

The experiments reported show that tissue slices of climacteric peak avocado fruit esterify phosphate more rapidly than do preclimacteric slices. While the rate at which Pi moves into preclimacteric tissue is only 10 % of that observed with climacteric tissue and the low level of Pi may limit the esterification, the

rate of conversion actually observed in climacteric tissue is 40 times that of preclimacteric. We must conclude that the ability of climacteric tissue to esterify phosphate is not only not impaired, but it may actually be enhanced. Further, phosphate esterification is inhibited in the slices of the 2 tissues to about the same degree by dinitrophenol, indicating that both tissues show coupled oxidation and no endogenous uncoupler is formed in conjunction with the transition through the climacteric. We may conclude that the increased oxidation associated with the climacteric is not due to the uncoupling of phosphorylation from oxidation.

Likewise separation and quantitation of each of the adenine nucleotides has demonstrated that there is no deficiency of ADP as acceptor for phosphorylation in the preclimacteric phase. In fact, the level of ADP decreased in the climacteric state. Accordingly, the climacteric cannot be explained by acceptor control.

The movement of Pi into and the loss of P esters from the tissue slices changes dramatically with the climacteric. It was recognized early that there was a large difference in the rate at which Pi moved into the tissue and that the amount of Pi and esters found at the end of any incubation with ^{32}P depended upon the exact procedure used for rinsing the excess ^{32}Pi of the medium from the slices. For this reason, incubation periods were kept short and great care was taken to see that the washing step was brief and that it was reproduced precisely. It was found that a rinse in ice water for exactly 20 seconds washed most of the unabsorbed ^{32}Pi from the surface and yielded slices with reproducible activities. Climacteric peak tissue takes up Pi at about 10 times the rate of preclimacteric, suggesting either a remarkable increase in permeability or an activation of an uptake mechanism. The choice of explanations becomes clear from the movement of esters out of the tissue. After rinsing the tissue slices for 20 seconds, the 20 minute wash removed 87 % of Pi from both tissues and the same amount of esters from climacteric tissue. On the other hand, only 30 to 50 % of the esters were lost from preclimacteric. The rate of loss of esters from the tissues was very nearly the same at 0° and 25°, suggesting that the loss is really diffusion to the medium and not losses due to convertion of intermediates to polysaccharides or RNA.

This very marked change in the movement of esters to the medium suggests that the apparent free space of the tissue has increased with ripening to include nearly the entire cell. If compartments within the cell are opened by changes in the cell membranes, it would not be surprising if regulatory restraints on metabolism were changed as the concentration of regulatory effectors at the site of specific enzymes would surely be altered. Striking changes in free space in relation to the climacteric were reported by Sacher (14) for the banana. At the peak of the climacteric the tissue was 100 % free space with respect to mannitol, sucrose, fructose and chloride.

The results of these experiments support the view that the respiratory climacteric is initiated by the failure of the cellular membranes to maintain their permeability characteristics. The rate of respiration during the climacteric phase is not regulated by acceptor control, nor is the climacteric initiated by the uncoupling of phosphorylation from oxidation.

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