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METABOLIC PROCESSES IN CYTOPLASMIC PARTICLES OF THE AVOCADO FRUIT. IV. RIPENING AND THE SUPERNATANT FRACTION[1]

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The activities of the cytoplasmic particles reported in the first three papers of this series (1, 4, 5) were obtained mostly from firm or pre-climacteric fruit. Little or no attention was paid to the mitochondrial reactions of ripe fruit and of fruit in the course of ripening. In the avocado, a well described pattern of respiration for the intact fruit is associated with the ripening process (2). This pattern is essentially the same as that reported for other fruits and is referred to as the "climacteric" (10). Under appropriate temperature and oxygen tension, a rapid rise in respiratory activity takes place within a short time after harvesting. This so-called "climacteric rise" reaches a peak and is followed by a decline referred to as the post-climacteric phase. It is during this final stage that senescence sets in, resulting in breakdown and death. Fruit physiologists have been concerned with the onset of the climacteric rise since it might be considered as the "beginning of the end," the prelude to senescence and death. Recent hypotheses concerning the onset of the rise revolve around the phenomenon of the coupling of phosphorylation to respiration (13) or the relationship between the levels of ADP and ATP (14). It is the purpose of this paper to examine the biochemical reactions of avocado particles prepared from fruit at different stages of the climacteric.

MATERIALS AND METHODS

The Fuerte and Anaheim varieties of the avocado, *Persea americana* Mill, were used, but no substantial qualitative or quantitative differences between these varieties were observed. Respiration of the whole fruit was determined by methods described by Biale and Shepherd (3), with the exception that oxygen consumption was measured with the Beckman Oxygen Analyzer (19). The preparative procedure and the determination of oxidative and phosphorylative activities for hard fruit were described previously (4). In brief, the method consisted of blending 150 grams of peeled and grated avocado tissue with 300 ml of 0.5 M sucrose. The homogenate was then strained through cheesecloth and centrifuged at 500 × g for 5 minutes to separate out large fragments, unbroken cells, etc. The resultant supernatant solution was then centrifuged at 17,000 × g for 15 minutes. After this high-speed centrifugation, the supernatant solution was saved for further study and the cytoplasmic particles contained in the pellet were washed by resuspension in 0.5 M sucrose and recentrifuged at 17,000 × g. In the case of soft fruit, the tissue was prepared with a coarse grater. The speed of blending was adjusted so as to prevent the formation of a suction cone in the homogenate.

A major portion of this study was concerned with the effects of the deproteinized supernatant fraction on ripe fruit mitochondria. For this purpose, the supernatant solution resulting from the first high speed centrifugation was placed in conical centrifuge tubes and the proteins were denatured by immersing in boiling water for five minutes. Coagulated proteins were removed by centrifugation at 1000 × g for five minutes.

The following abbreviations are used in this paper: DNP, 2,4-dinitrophenol; DS, deproteinized supernatant fraction; KG, α-ketoglutarate; AMP, adenine monophosphate; ADP, adenine di-phosphate; CoA,

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coenzyme A; LTPP, lipothiamine pyrophosphate (kindly supplied by Dr. D. Appleman).

Results

Oxidative Phosphorylation During Ripening: It was established in previous studies (4, 13) that the particulate fraction of the avocado is capable of converting inorganic phosphate into organic form as a result of the oxidation of α-ketoglutarate. While some evidence was furnished that oxidative phosphorylation took place at two stages of the climacteric, the data were limited to one keto-acid. Moreover, coordination of mitochondrial studies with respiratory measurements on the whole fruit was lacking. The magnitudes of both oxygen and phosphorus uptake with the preparations made by grinding in a mortar (13) were much lower than those obtained by our current blender technique. We decided, therefore, to use the present preparative procedure on fruit with a well known respiratory history and to follow the rates of oxidation and phosphorylation in the absence and presence of dinitrophenol. The experimental results presented in table I are based on averages of two or more tests for each stage of ripeness and for each acid. The 40-minute period was chosen since both phosphate and oxygen uptake were constant during this time (4).

The mitochondrial oxidations and phosphorylations in reaction mixtures without DNP (control) will be examined first. The oxidative activities of the succino-oxidase system were stable throughout the climacteric cycle. In the case of the other three acids there was a sharp drop in the rates of oxidation at the peak (maximum) as compared with activities of material from the preclimacteric minimum. On the other hand, the rates of incorporation of inorganic phosphate were essentially not lowered during the climacteric rise. Consequently the P/O values increased with ripening.

The trends were in some respects different for the preparations treated with 10^{-5} M DNP. This reagent lowered more markedly the phosphate uptake by cytoplasmic particles from unripe (initial and minimum) than from ripe fruit. This observation was not as manifest with α-ketoglutarate as with the other three acids of the Krebs cycle. The uncoupling action of DNP was most striking in the mitochondria from hard fruit. In no case did DNP bring about an increase in oxidative activity. In all cases, with the exception of succinate, the rates of oxidation were distinctly lower at the maximum than at the other two stages of ripening. Apparently the trend in oxygen uptake by the mitochondria did not correspond with the course of respiration exhibited by the intact fruit.

Since the DNP effects with pyruvate were striking, another experiment was undertaken in which the P/O ratios were determined in the intermediate stages as well as at the climacteric minimum and maximum. The results of this experiment are depicted in figure 1. Curve A in this figure represents the respiration of the intact fruit as measured by the oxygen analyzer. Each point in curves B and C was obtained from a fruit the respiration of which is given by a corresponding point in curve A. The findings in this experiment extended the results of table I and indicated the effectiveness of DNP in altering P/O ratios in the preclimacteric stage and immediately after the onset of the rise. It should be stressed again that DNP had no effect at and following the peak. This behavior of DNP suggested that marked biochemical changes occurred and justified further investigation.

Oxidation of Succinate and α-Ketoglutarate in Relation to Stage of Ripeness: Succinate and α-ketoglutarate were chosen to test metabolic activity during ripening because previous evidence (4) indicated wide differences in the stability of these two

Table I

Dinitrophenol Effect on Oxidative Phosphorylation by the Particulate Suspension of Avocado Fruit in Relation to Stage of Ripeness

Substrate	Stage of ripeness	Control μatoms/40 min		P/O	Dinitrophenol (10^{-5} M) μatoms/40 min		P/O
		P	O		P	O	
α-Ketoglutarate	Initial*	15.4	14.9	1.04	6.7	15.4	0.44
	Preclim. min.	12.8	13.9	0.92	3.1	11.8	0.26
	Clim. max.	11.6	8.0	1.45	4.6	5.1	0.96
Pyruvate	Initial*	5.0	5.2	0.96	0	4.5	0
	Minimum	11.7	15.7	0.75	3.1	16.4	0.19
	Maximum	9.0	6.0	1.50	4.6	5.5	0.83
Succinate	Initial*	7.4	15.4	0.48	1.5	16.8	0.09
	Minimum	10.3	18.0	0.58	4.3	16.6	0.26
	Maximum	13.1	15.7	0.84	10.5	16.2	0.65
Malate	Initial*	10.4	14.7	0.72	3.1	15.1	0.21
	Minimum	11.5	16.5	0.70	1.5	14.2	0.11
	Maximum	10.6	7.7	1.38	8.9	7.0	1.27

Reaction mixture: Substrate, 0.02 M; phosphate, 0.01 M, pH 7.1; AMP, 0.001 M; glucose, 0.01 M; magnesium sulfate, 0.006 M; sucrose, 0.5 M; enzyme, 0.5 ml (approx. 1 mg N); total volume 3.0 ml; gas phase, air; temp 20° C.
* Refers to fruit fresh from the tree.

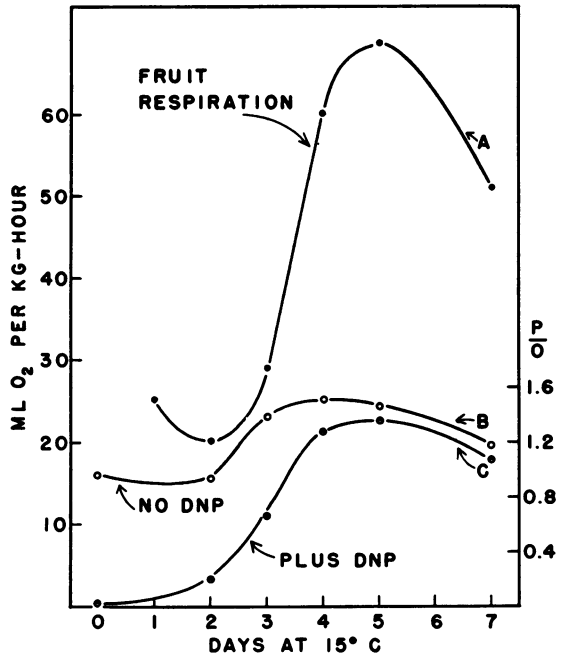

FIG. 1. Effect of 10^{-5} M dinitrophenol on oxidative phosphorylation by cytoplasmic particles of the avocado fruit in relation to the climacteric. A. Oxygen uptake by the intact fruit. B, C. P/O ratios of particles from fruit of curve A on corresponding days. Reaction mixture same as in table I; substrate, pyruvate.

systems. Two sets of experiments were conducted. In the first set the fruit was kept at 0° C for different periods of time and homogenized immediately upon removal from the low temperature. In the second set homogenization was carried out only following the exposure to the ripening temperature of 20° C. It is evident from table II that succinate oxidation was virtually unaffected by either treatment. On the other hand, the rates of oxidation of α-ketoglutarate were materially lowered by chilling fruit at 0° C. The expected Q_{O_2} (N) value for this substrate in the case of non-chilled fruit is at least 150, as judged from table I and from results reported previously

TABLE II

MITOCHONDRIAL ACTIVITY IN RELATION TO FRUIT STORAGE AT 0° C AND AT 20° C

FRUIT TREATMENT	Q_{O_2} (N)	
	SUCCINATE	α-KETOGLUTARATE
8 Days at 0° C	266	68
18 " " "	430	94
30 " " "	338	93
Chilled at 0° C and placed		
3 Days at 20° C	387	38
8 " " "	415	20
15 " " "	250	6

Reaction mixture: Same as in table I plus 3 micromoles cytochrome c.

TABLE III

EFFECT OF THE SUPERNATANT FRACTION ON α-KETOGLUTARATE OXIDATION

STAGE OF RIPENESS	MG N/ VESSEL	CONDITION	Q_{O_2} (N)	% INCREASE
Preclimacteric	0.58	Control	427	...
		DS **	410	-4
On rise	0.52	Control	105	...
		Fresh supn't.*	189	80
		DS **	248	138

Reaction mixture in control: same as in table I. Temp. 30° C.

* Five tenths ml of supernatant fraction obtained from high speed centrifugation (see text).

** DS, deproteinized supernatant fraction.

(4). The striking feature of table II is the progressive inactivation of the α-ketoglutarate oxidase with time of exposure to 20° C. These results along with those in table I suggested the need to investigate further the decline in α-ketoglutarate oxidation in relation to ripening.

ENHANCEMENT OF OXIDATION BY A SUPERNATANT FRACTION: Millerd, Bonner, and Biale (13) supplied evidence that a supernatant factor from ripe fruit increased the oxidation of α-ketoglutarate by mung bean mitochondria in the absence of adenylate. We decided to investigate more closely the effects of the supernatant fraction from avocado on avocado mitochondria. An increase in oxidation was observed with both fresh and deproteinized supernatant solution (table III). In some experiments the supernatant solution caused a slight increase in endogenous activity, probably by providing traces of metabolites. Whenever this occurred, the endogenous values were subtracted from the total rate of oxidation. It was observed in these experiments that the supernatant fraction had no effect on mitochondria extracted from hard fruit.

In order to relate the action of the supernatant solution to certain stages of ripening, fruit of known position in the climacteric curve were used for the preparation of cytoplasmic particles. The stage of ripeness was determined by following the respiration of the whole fruit and estimating its approximate stage on the climacteric curve. The increase in α-ketoglutarate oxidation due to deproteinized supernatant solution is shown in figure 2. Obviously the change in oxidative behavior of the particles and the effects of the supernatant factor are related to the climacteric rise. The striking response to DS prompted us to trace the function of the supernatant fraction in the metabolic reactions.

THE NATURE OF THE SUPERNATANT EFFECT: For all subsequent experiments a large quantity of supernatant solution was prepared from several ripe fruit following the procedure described under Methods. The solution was frozen in small aliquots which were thawed out as needed.

Assuming that the supernatant solution provided

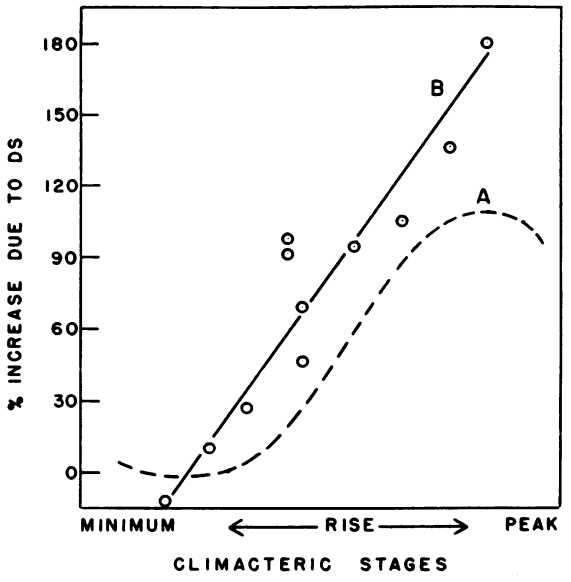

FIG. 2. Effect of the deproteinized supernatant fraction (DS) on the oxidation of α-ketoglutarate by particles extracted from fruit at different stages of the climacteric. A. The climacteric pattern in respiration of the intact fruit. B. Response to DS of particles extracted from fruit at different stages. Reaction mixture same as in table I.

some active metabolic factor, attention was now directed toward finding the locus of the activity in the Krebs cycle. Table IV shows experiments with pyruvate, citrate, α-ketoglutarate and succinate. The deproteinized supernatant solution stimulated the oxidation of α-ketoglutarate and pyruvate but not of succinate and citrate. The cofactor requirements for oxidation of Krebs cycle acids by avocado mitochondria were studied mostly on preclimacteric fruit. It was shown (4) that adenylate in any form was not required for succinate oxidation whether the preparation was washed once or twice. In the case of citrate the omission of AMP caused a 30 % reduction of the oxygen uptake in a highly washed preparation (1). Under the same conditions α-ketoglutarate oxidation was reduced by 60 %. The question arose, therefore,

TABLE IV

EFFECTS OF DEPROTEINIZED SUPERNATANT FRACTION (DS) ON OXIDATION OF KREBS CYCLE ACIDS BY MITOCHONDRIA OF RIPE AVOCADO

SUBSTRATE	Q_{O_2} (N)	
	CONTROL	DS
Pyruvate	0	82
α-Ketoglutarate	72	251
Citrate	191	180
Succinate	336	310

Reaction mixture: same as in table I, temp. 30° C, 0.5 ml DS where indicated.

TABLE V

COMPARISON OF THE EFFECTS OF ADENYLATE AND DEPROTEINIZED SUPERNATANT FRACTION (DS) ON THE OXIDATION OF α-KETOGLUTARATE BY PARTICLES OF RIPE AVOCADO FRUIT

CONDITIONS	Q_{O_2} (N)	% INCREASE DUE TO *DS*
Minus adenylate		
− DS	136	..
+ DS	202	48
Plus adenylate		
− DS	350	..
+ DS	615	76

Reaction mixture: same as in table I + 0.5 ml of DS where indicated.

whether the addition of DS increased the supply of adenylate to a level required for optimal oxidation of the keto acid. One might expect therefore a greater response from DS in the absence than in the presence of adenylate. Obviously this was not the case, as can be seen from table V. On the contrary, adenylate and the supernatant fraction acted synergistically. This observation is apparently in harmony with the findings of Millerd et al (13) that a heat stable component from the supernatant fraction of climacteric avocado brought about increased oxidation by mung bean mitochondria. However, they tested this component only in the absence of exogenous phosphate acceptor and conclude therefore that it "acts qualitatively like adenylate itself." From our results it seems more likely that DS interacts with rather than replaces adenylate.

The heat stable fraction of Millerd et al (13) caused a high percentage increase in phosphorus uptake though the actual magnitudes were low. In addition, a heat labile fraction acted like dinitrophenol in restoring the oxidation to the level of the complete system which contained adenylate. In this study a comparison of supernatant fraction with that of dinitrophenol (table VI) clearly indicates that neither exhibited uncoupling characteristics with respect to mitochondria from ripe avocados. This experiment is in conformity with the results given in table I and figure 1. In all cases active phosphorylation has taken place though the P/O ratios were lower than those reported previously with α-ketoglutarate. It is

TABLE VI

COMPARISON OF THE EFFECTS OF DEPROTEINIZED SUPERNATANT FRACTION (DS) AND DINITROPHENOL (DNP) ON OXIDATION AND PHOSPHORYLATION BY MITOCHONDRIA FROM RIPE AVOCADO

ADDITIONS	μATOMS O/HR	MICROMOLES P/HR	P/O
Control	8.6	6.0	0.7
DNP	7.8	6.0	0.8
DS	10.5	8.0	0.75

Reaction mixture: same as in table I; substrate, α-ketoglutarate; 0.5 ml of DS; 10^{-5} M DNP.

likely that the fruit used for the experiment given in table VI was in a more advanced stage of ripening than the material of table I, which was designated as fruit at the climacteric peak. The significant conclusion is that neither DS nor DNP reduced the P/O value.

In view of the fact that the keto acids responded most markedly to the supplementation of the reaction mixture with the supernatant fraction, it was deemed advisable to investigate additional cofactors. Coenzyme A activity has been studied most extensively in conjunction with pyruvate and α-ketoglutarate oxidation. The role of CoA in implementing the entry of acetate derived from pyruvate into the Krebs cycle was reviewed by Gunsalus (6). Hift et al (7) and Kaufman et al (9) demonstrated the importance of CoA in α-ketoglutarate oxidation. The requirement for CoA by avocado particles from unripe fruit was studied by Avron and Biale (1) for pyruvate oxidation only. It was plausible to assume that this factor was not dissociated readily from the mitochondrial complex of preclimacteric avocados. The role of CoA in particles of ripe fruit was investigated here in relation to the activity of the supernatant fraction. As demonstrated by experiments shown in table VII, CoA not only increased the oxidative rate in the absence of denatured supernatant, but resulted in an even greater increase when supernatant was present. As in the case with AMP, CoA and supernatant solution functioned in a synergistic manner.

The activity of CoA in the decarboxylation reactions involves LTPP as an essential component of the reaction mechanism (6, 16). This factor was added both in the presence and absence of supernatant, but it failed to show an effect in either case (table VII). The synergistic effect of CoA and the DS could be ascribed to the activation of CoA through a reduction. This was apparently not the case, since supernatant solution effects were obtained even in the presence of cysteine while cysteine itself did not increase the activity of CoA.

The synergistic responses in the above described

TABLE VII

EFFECTS OF COENZYME A (CoA) AND LIPOTHIAMINE-PYROPHOSPHATE (LTPP) ON α-KETOGLUTARATE OXIDATION IN RELATION TO THE EFFECT OF THE DEPROTEINIZED SUPERNATANT FRACTION (DS)

REACTION MIXTURE	Q_{O_2} (N)
Complete	120
Omit CoA	60
" DS	30
" CoA and DS	0
Complete	168
Omit LTPP	206
" DS	97
" DS and LTPP	99
" DS, LTPP and CoA	29

Reaction mixture: same as in table I; 0.5 ml DS where indicated; temp. 30° C.

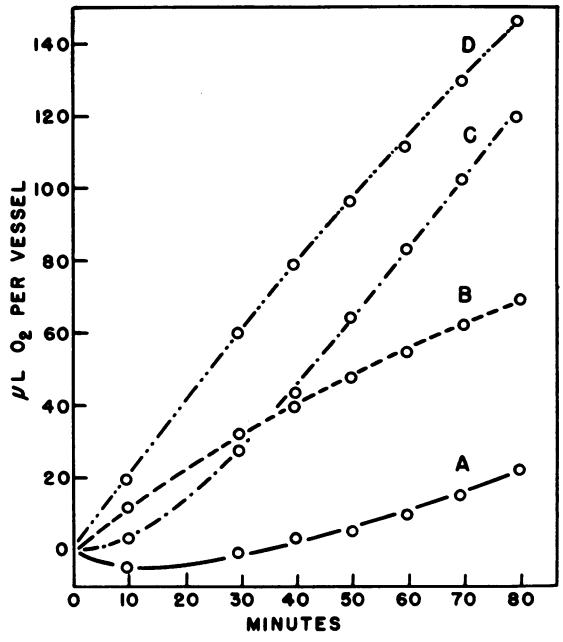

FIG. 3. The oxidation of α-ketoglutarate by cytoplasmic particles of the avocado fruit in relation to adenylate and the supernatant fraction (DS). A. AMP. B. ADP. C. AMP + DS. D. ADP + DS. Reaction mixture same as in table I.

experiments imply the function of the supernatant fraction in some phases of α-ketoglutarate oxidation in which all three factors, supernatant fraction, CoA and adenylate, are involved as intermediates. The form of adenylate would be of particular importance if the avocado mitochondria contain an α-ketoglutarate dehydrogenase system similar to that described by Hift et al (7) and Kaufman et al (9). These authors demonstrated a strict requirement for ADP as the phosphate acceptor. In oxidations by avocado mitochondria, AMP semed to function as an adequate phosphate acceptor (4). On the other hand, several experiments provided indirect evidence that ADP is in fact the essential adenylate. This contention is based on the lag period in oxidation (curves A and C, fig 3) observed with AMP but always absent (curves B and D) when ADP is used. Lindberg and Ernester (11) explained similar lag periods as the time required for the formation of enough ATP to provide ADP through the adenyl-kinase (myokinase) reaction:

$$AMP + ATP \rightleftharpoons 2\,ADP$$

Slater and Holton (17) observed a similar lag period in the phosphorylation of heart muscle sarcosomes. Their lag was also removed by ADP and they have shown an increased need for ADP with increasing dilution of the sarcosomes. A tabulation of our results in table VIII indicates that with avocado mitochondria as well, a longer lag period was obtained with decreasing mitochondrial nitrogen. As explained by Slater and Holton (17), the increased dilution dimin-

TABLE VIII

THE RELATIONSHIP OF THE LAG PERIOD TO MITOCHONDRIAL NITROGEN

	Lag period in minutes		
	0–5	5–10	Over 10
	mg N/0.5 ml suspension of particles		
	0.63	0.60	0.44
	0.76	0.40	0.50
	0.89	0.46	0.34
	0.72	...	0.33
	0.88	...	0.19
	0.73	...	...
Average	0.73	0.49	0.36

ishes the relative residual ADP supply requiring more time to form the necessary level for maximum oxidation.

DISCUSSION

The studies reported here indicate a decline in α-ketoglutarate oxidation during ripening and suggest that the α-ketoglutarate dehydrogenase might provide a desirable biochemical reaction for an analysis of the senescent drift in fruit respiration. This was supported by the effects of the DS and the correlation of this effect with the climacteric rise.

The ultimate aim of experimentation with the supernatant fraction is, no doubt, the determination of its intracellular function along with its purification and identification. With this long-term objective in mind, it was thought most expedient to determine the locus of activity in order to gain a lead to the probable function of the fraction as well as to its identity. Localizing the area of activity might indicate which enzymes should be purified to provide a sound assay system for further exploration. Our studies thus far have shown that the action of the supernatant fraction is centered in the substrate level reactions of α-ketoglutarate oxidation. This can be deduced from the localization of the effect to the α-keto acids, the synergistic activity with CoA and a similar synergism with adenylate. The significance of these synergisms is based on the assumption that avocado mitochondria contain an α-ketoglutarate dehydrogenase system similar to that described for animal mitochondria (7, 9). This assumption is supported not only by the requirements for similar cofactors, but also by the preference for ADP as the specific phosphate acceptor.

The presence of a supernatant fraction essential to the mitochondrial reactions could be explained by the existence of a state of equilibrium between the mitochondria and the surrounding medium. Under these conditions the factors essential to one reaction sequence could be lost while another reaction sequence remained unaffected. Such a theory was proposed by Potter et al (15) to account for a differential change in the enzymatic activity of rat liver mitochondria. This same reasoning could also apply to the avocado particles whose power to oxidize succinate remained stable while the ability to oxidize α-ketoglutarate declined during ripening of the fruit.

Dinitrophenol was used to enhance the rate of α-ketoglutarate oxidation. No enhancement was recorded in any stage of ripeness. DNP had no effect on oxidative activity whether it did or did not act as an uncoupling agent. Similar responses were obtained with malate, pyruvate, and succinate. The only definite effects of DNP were on phosphorylation and in particular on mitochondria from unripe fruit. In climacteric material the uncoupling effect was virtually eliminated. The operation of a DNP resistant phosphorylation is a unique phenomenon and is difficult to interpret until more is known about the mechanism of action of this substance. This phosphorylation could be ascribed to substrate level oxidation had it been limited to α-ketoglutarate. The data clearly indicate a DNP stable incorporation of phosphate with malate, succinate, and pyruvate as well as α-ketoglutarate. The fact that active esterification takes place during the climacteric rise suggests that ripening might require energy, an idea which is in harmony with Hulme's (8) findings on net protein synthesis in the apple, and with Tager and Biale's (18) report on the formation of aldolase and carboxylase in the banana. The question is therefore raised again whether ripening is a coupled or an uncoupled process. Our findings do not support the "Uncoupling" theory of Millerd, Bonner, and Biale (13). Neither do they contradict it directly. It should be emphasized that their ideas were based on the action of a heat labile factor, while most of our work was concerned with the heat stable fraction of the supernatant solution. Conceivably, uncoupling might be responsible for certain reactions associated with ripening but might not be the trigger mechanism. The application of DNP to intact fruit might throw some light on the role of uncoupling in ripening. Marks, Bernlohr, and Varner (12) injected DNP into green mature tomatoes and reported that this material remained unchanged in appearance from freshly harvested product while the control ripened normally. Thus far no evidence of this kind is available for the avocado. If energetic coupling is a prerequisite for the ripening process it will be necessary to postulate new ideas on the mechanism of the induction of the climateric rise. The revised orientation might include changes in metabolic pathways similar to those reported by Tager and Biale (18).

SUMMARY

The oxidative and phosphorylative activities of cytoplasmic particles isolated from the avocado fruit were studied in relation to the process of ripening and senescence.

The oxidation rates of α-ketoglutarate and malate were markedly reduced at the climacteric peak, while the P/O ratios tended to be high. The presence of dinitrophenol had no effect on the phosphorylative capacity of the particles at the peak, though it did lower the P/O ratios at the earlier stages of ripening.

The loss of oxidative activity of the α-ketoglutarate and pyruvate systems could be restored substantially by the addition of deproteinized supernatant fraction from ripe fruit. This solution exerted no influence on citrate and succinate oxidations, but with α-ketoglutarate its action was directly related to the stage of ripeness along the climacteric curve.

The idea was advanced that the role of the supernatant fraction was centered around the substrate level reactions of oxidative decarboxylation. This contention was based on the localization of the effect of the α-keto acids and on the synergistic activities with CoA and adenylates.

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BIOCHEMICAL CHANGES DURING GERMINATION OF THE TUNG SEED[1]

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Air-dry tung seeds usually germinate slowly and unevenly. Sharpe and Merrill (6) improved both the rate and uniformity of germination by stratification of the hulled seed outdoors in sand about 2 months prior to planting. Shear and Crane (7) also accelerated germination of tung seeds by soaking them in aqueous solutions containing morpholine (a heterocyclic ring compound derived from ethanolamine, which was used to reduce surface tension in water). In the germination of dry-stored tung seeds, Johnston and Sell (4) found that lipase activity and the utilization of the reserves occurred after the emergence of the radicle from the seeds. Similar observations on the transformation of substances in tung seeds have been reported also by Gerschtein (3). The present study was made to correlate utilization and translocation of reserves to the seedling and respiratory activity with rate of emergence and early stages of germination.

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