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Metabolic Processes in Cytoplasmic Particles of the Avocado Fruit VI. Controlled Oxidations and Coupled Phosphorylations [1, 2, 3]

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An explanation of the climacteric rise of the respiration rate of ripening fruits has been sought by studies of subcellular particles isolated from the fruit. It is known that in most plant tissues the rate of O_2 uptake can be increased by treating the tissue with uncoupling agents, suggesting that the rate of O_2 uptake is limited by the rate of oxidative phosphorylation. If during the process of ripening the restriction on oxidation exerted by phosphorylation was relieved, a stimulation of the rate of O_2 uptake would be expected. Thus, Millerd et al. (24) established that whereas the respiration of avocado tissue slices of preclimacteric fruit could be stimulated by application of DNP[5], slices from the climacteric peak fruit were not stimulated by such treatment. From studies of the isolated mitochondria and of the other fractions these workers concluded that there was a natural uncoupler present in the fruit. Several criticisms have been offered against such an hypothesis (5), and Pearson and Robertson (25) suggested that the climacteric rise was due to a higher turnover of the phosphorylation cycle. These explanations of the increased O_2 uptake, involving changes in the controlling influence of oxidative phosphorylation on the mitochondrial oxidations, are possible, but it is improbable that the study of the isolated mitochondria will reveal the causes of the changes. Nevertheless, much valuable information has been obtained from these studies, and in particular, the properties of avocado particles have been thoroughly examined and documented (1, 5, 6, 24, 26).

Romani and Biale (26) observed that mitochondria isolated from ripe avocado fruit appeared to have an oxidative phosphorylation mechanism which was insensitive to DNP. Mitochondria isolated from unripe avocado fruit were sensitive to DNP, but the uncoupling effect observed with these particles was

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[5] Abbreviations: CCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; TPP, thiamine pyrophosphate.

reversed with hexokinase (6). Other studies (1) had substantiated the operation of a typical tricarboxylic acid cycle in these preparations. Thus the effects of DNP are of particular interest with respect to the mechanism of oxidative phosphorylation. Therefore it was considered necessary to reevaluate the previous results obtained with mitochondria from avocado tissue in the light of recent knowledge about the properties of mitochondrial processes.

Since the properties of isolated mitochondria are markedly influenced by the isolation procedure (15, 18) and since the pattern of behavior of the particles isolated from avocado fruit showed variation (probably seasonal), no attempt has been made to extend these results to the physiology of the intact fruit.

Mitochondria isolated from climacteric-peak fruit were used to study respiratory control and the effects of DNP on oxidative phosphorylation. Possible explanations of the previously reported results (6, 26) are given and the effects of oxaloacetate on oxidations by particles isolated from the avocado (2) have been further examined.

Methods and Materials

Preparation of Mitochondria. Fuerte avocado fruit, *Persea gratissima,* were chilled at 1° for an hour and grated with a kitchen type grater with 3-mm holes.

Grated tissue (100 g) was added to 300 ml of 0.4 M sucrose containing 5 mM EDTA, 6 mM $MgCl_2$, and 4 mM cysteine. The mixture was ground in a mortar with sand and the pH maintained between 7.0 and 7.5 by dropwise additions of 1 N KOH. The brei was squeezed through washed heavy muslin and the filtrate, diluted with 200 ml of 0.4 M sucrose containing 6 mM $MgCl_2$, and was subjected to the following centrifugings: A) 1,500 × *g* for 15 minutes—precipitate discarded; B) 10,000 × *g* for 15 minutes—supernatant discarded; C) 2,500 × *g* for 10 minutes—precipitate discarded; D) 10,000 × *g* for 10 minutes—supernatant discarded; E) 2,500 × *g* for 10 minutes—precipitate discarded; F) 10,000 × *g* for 10 minutes—supernatant discarded.

All operations were done at 0 to 1°. After centrifuging, the upper layer of lipid material was removed by suction. The precipitates, during the preparation, were suspended in 200 ml of 0.4 M sucrose containing 6 mM $MgCl_2$ with the aid of a loose Potter-Elvehjem-type Teflon homogenizer. The final precipitate was suspended in 3 ml of this medium and was green. Replacing the sucrose with 0.5 M mannitol in the washing procedure allowed an easier separation of the layers of the precipitates (29). When this was done a brown and a green fraction could be obtained. Preparations isolated from both mannitol and sucrose were used in this work but no differences in behavior could be detected.

One of the major problems associated with the isolation of these particles was aggregation. The use of a mortar instead of a blendor, the presence of $MgCl_2$ and the use of large volumes of suspending medium minimized the tendency of the particles to aggregate. Furthermore, any aggregated material was removed by low-speed centrifuging (steps C and E above).

O_2 uptake was measured polarographically in a sealed lucite vessel of 3.0 ml volume using a Clark electrode (Yellow Springs Instrument Co., Cleveland, Ohio), connected to a potentiometric recorder. The standard reaction medium contained 10 mM potassium phosphate buffer, pH 7.2, 10 mM Tris-HCl buffer, pH 7.2, 5 mM $MgCl_2$, 0.5 mM EDTA, and either 0.25 M sucrose or 0.3 M mannitol. When α-ketoglutarate was used as a substrate, malonate was included to inhibit the oxidation of succinate.

Esterification of P_i was determined by adding hexokinase and glucose to the above medium, from which aliquots were added to cold perchloric acid (final concentration 3 %), neutralized with KOH, the $KClO_4$ centrifuged off, and the glucose-6-P assayed by NADP reduction with crystalline glucose-6-P dehydrogenase (19).

The nitrogen content of the suspension was determined by distillation and titration after digestion in sulfuric acid using mercury as catalyst (23).

ADP was determined by phosphorylation with P-enolpyruvate and pyruvate kinase and determining the oxidation of NADH on reduction of the pyruvate with lactate dehydrogenase.

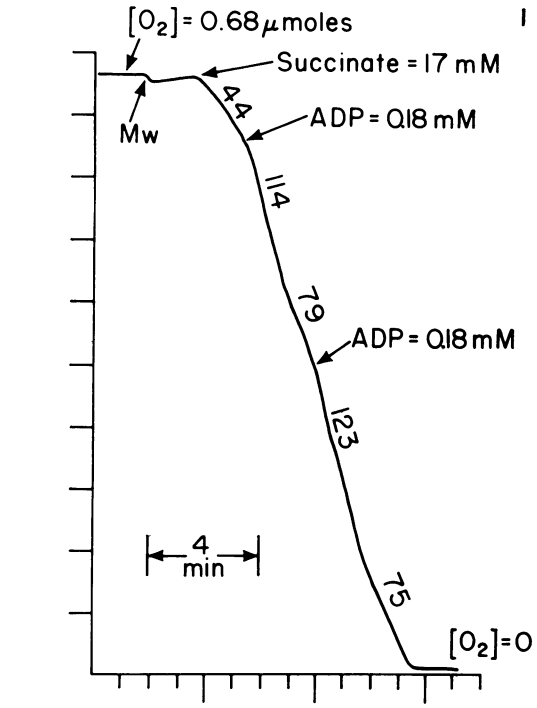
Fresh Fuerte avocado fruit were obtained from California Avocado Growers. Avocados do not ripen on the tree and are in the preclimacteric state when picked. The respiratory activity of individual fruit was followed continuously with the aid of a paramagnetic type O_2 analyzer (32). Climacteric peak fruit were selected when they reached their maximum rate of respiration.

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, glucose-6-P dehydrogenase, and organic acids were obtained from California Corporation for Biochemical Research. NAD, NADH, NADP, and hexokinase (type IV) were obtained from Sigma Chemical Company. ADP, ATP and AMP were obtained from Pabst Laboratories. Sodium amytal was obtained from Eli Lilly and Company. Gifts of oligomycin (Dr. H. A. Lardy) and carbonyl cyanide *m*-chlorophenylhydrazone (CCP) (Dr. P. G. Heytler, E. I. du Pont de Nemours and Company) are gratefully acknowledged.

Results

Oxidation and Coupled Phosphorylation. Figure 1 shows a typical O_2 electrode trace obtained with mitochondria isolated from avocado fruit which were at the climacteric peak of respiration. It can be seen that the oxidation of succinate was stimulated by ADP and that as ADP became limiting the rate of oxidation decreased. In accordance with Chance and Williams (9), the rate of oxidation of mitochondria in isotonic medium with phosphate, substrate, and a

definite amount of ADP added is referred to as state 3. The subsequent oxidation rate, after all of the added ADP has been converted to ATP making phosphate acceptor limiting, is state 4. Similar results were obtained with α-ketoglutarate and malate as substrates, as shown in figure 2. The higher respiratory control ratio, defined as the state 3 rate divided by the state 4 rate, observed with α-ketoglutarate was attributed to the substrate-level phosphorylation being more tightly coupled than the electron transport chain phosphorylations. It was also observed, with most preparations, that α-ketoglutarate was not oxidized until ADP was added, whereas some oxidation of both malate and succinate did occur in the absence of added ADP. This is also indicative of the strong coupling between oxidation and phosphorylation at



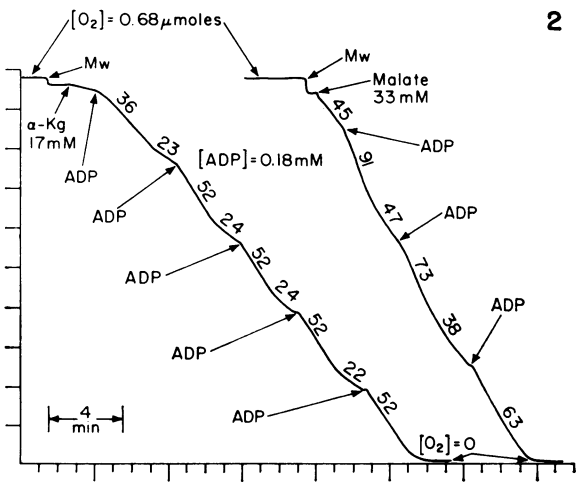
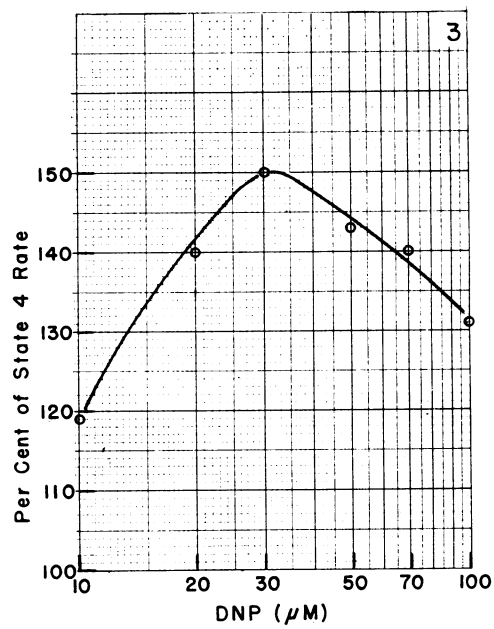


FIG. 1. The oxidation of succinate by avocado mitochondria. Assayed in 2.7 ml of the standard medium containing sucrose and 138 μg mitochondrial nitrogen. Numbers on the trace are rates expressed as mμ moles O_2/minute. M_W indicates the addition of mitochondria. Additions are shown as final concentrations.

FIG. 2. Respiratory control with malate and α-ketoglutarate. Assayed in 2.6 ml of the standard sucrose medium containing 180 μg mitochondrial nitrogen. For α-ketoglutarate 67 μM TPP and 8 mM sodium malonate were included in the reaction mixture. Rates are expressed as mμ moles O_2/minute.



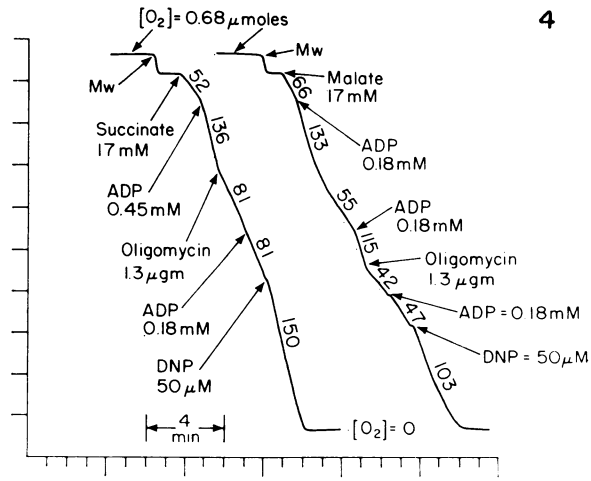


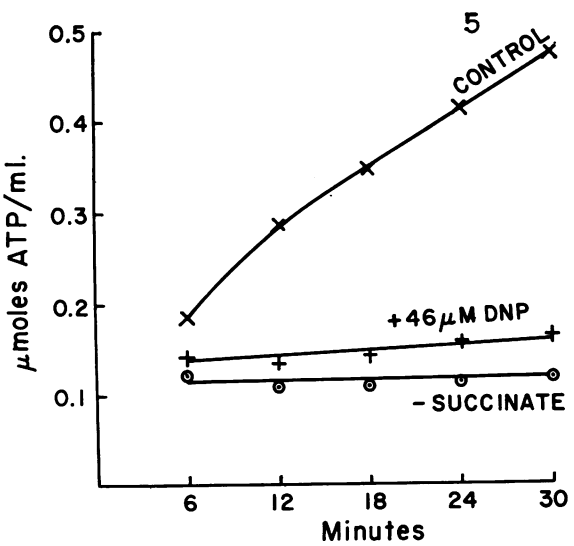
FIG. 3. The effect of DNP on the state 4 oxidation of succinate. Assayed in 2.6 ml standard mannitol medium containing 17 mM sodium succinate, 170 μM ADP and 266 μg mitochondrial nitrogen.

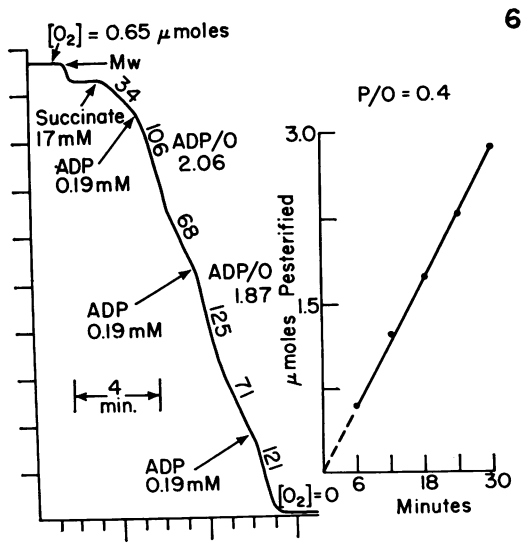
FIG. 4. The effect of oligomycin on the oxidations of succinate and malate. Assayed in 2.6 ml standard sucrose medium containing 296 μg mitochondrial nitrogen.

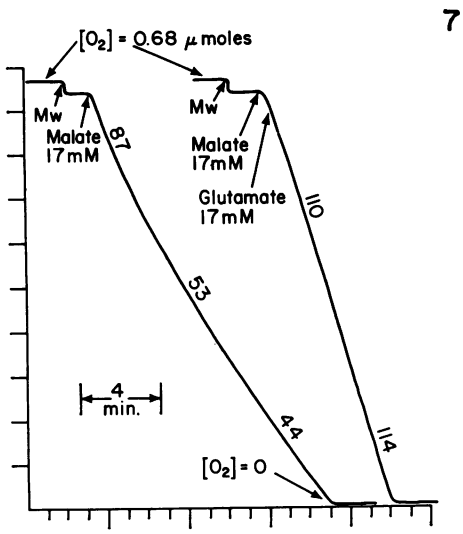
the substrate-level site of α-ketoglutarate oxidation. An induction period required for maximal α-ketoglutarate oxidation can be seen in figure 2 (8).

When the particles were in state 4, the respiration was stimulated by the addition of ADP or of an uncoupling agent. Figure 3 indicates that 10 μM DNP had stimulated state 4 oxidation of succinate and that approximately 30 μM DNP was optimal while higher concentrations of DNP showed less stimulation. It appears, therefore, that the avocado mitochondria isolated from climacteric-peak fruit can be no less sensitive to DNP than other plant mitochondria (cf. 26, see 29).

An alternative explanation of the DNP effect is the stimulation of an adenosine triphosphatase and the recycling of ADP. This alternative is disproved by the results of figure 4. Oligomycin, by inhibiting oxidative phosphorylation (21), lowered the oxidation rate to that of the state 4 rate, substantiating that the state 3 and state 4 changes are due to the coupling beween phosphorylation and oxidation. Furthermore, figure 4 shows that DNP released the oxidation in-







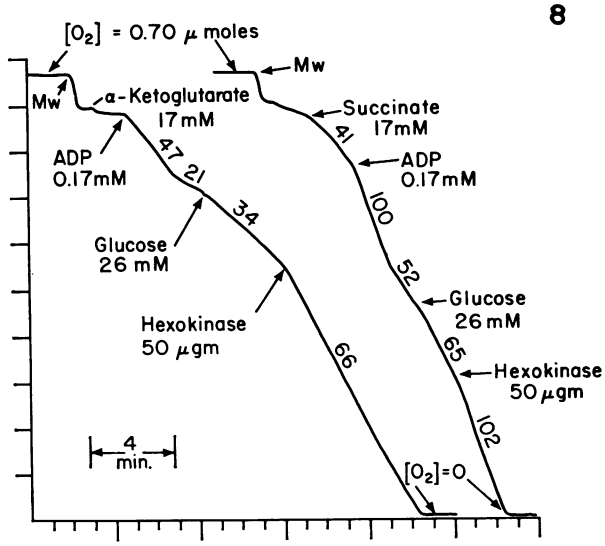


FIG. 5. The phosphorylating activity of the avocado mitochondria. Assayed in 6.0 ml standard sucrose medium containing 0.2 mg hexokinase, 17 mM glucose, 17 mM succinate, 0.2 mM ADP, and 256 μg mitochondrial nitrogen.

FIG. 6. A comparison of indirect P/O determinations. Assayed in 2.6 ml standard sucrose medium. The medium in which ATP was determined also contained 17 mM sodium succinate, 4 mM ADP, 0.1 mg hexokinase, 17 mM glucose. Rates are expressed as mμ moles O_2/minute.

FIG. 7. Oxidation of malate by avocado mitochondria. Assayed under the same conditions as outlined for table II.

FIG. 8. Hexokinase activity of mitochondrial preparations. Assayed in 2.7 ml standard mannitol medium containing 175 μg mitochondrial nitrogen. For α-ketoglutarate oxidation 67 μM TPP was included in the reaction. Rates are expressed as mμ moles O_2/minute.

hibited by oligomycin. Under these conditions DNP-stimulated adenosine triphosphatase would be inhibited by oligomycin (21) and could not have caused the stimulation of oxidation. This inhibition of the DNP-stimulated adenosine triphosphatase by oligomycin was confirmed here. In one experiment 67 μM DNP increased the rate of ATP hydrolysis by an avocado preparation from 11.5 to 15.0 μg P released per minute. The further addition of oligomycin (2 μg/ml reaction volume) reduced this stimulated rate of ATP hydrolysis to 8.0.

In fact, figure 5 shows that 46 μM DNP completely prevented the formation of ATP with succinate as substrate. These experiments were repeated with CCP, which at 0.5 μM concentration was as effective as 50 μM DNP. The ATP found in the presence of DNP or in the absence of succinate was due to the direct conversion of the added ADP by adenylate kinase activity. That adenylate kinase was present and that the formation of ATP from ADP by this enzyme could be inhibited by the addition of AMP is shown in table I. ATP formation by adenylate kinase was inhibited 95 % when the AMP/ADP ratio was 4/1. Since this was a direct assay of mitochondrial activity, any oxidation of NADPH by the preparation would lead to errors. Parallel experiments showed that NADPH was not oxidized by the mitochondria.

Table I

The Effect of AMP on the Formation of ATP by Adenylate Kinase

Adenylate kinase was assayed in 2.7 ml of standard sucrose medium containing 17 mM glucose, 0.2 mg hexokinase, 0.3 mg crystalline glucose-6-P dehydrogenase, 0.1 mM NADP and 239 μg mitochondrial nitrogen at 20°.

AMP/ADP	△ OD/min	% Inhibition
0	0.262	0
1	0.104	61
2	0.057	78
4	0.017	94

It is possible to determine ADP/O ratios (10) from O_2 electrode traces as shown in figure 6. When this was done the values obtained were usually close to the expected maximal values. However, when glucose-6-P was estimated the yield was much less than expected. Thus the values obtained from the electrode traces were regarded with caution. It was possible that the ATP formed in situ was not freely accessible to the medium (3, 4). Under these circumstances, oxidation would be affected by the internal ATP/ADP ratios and the glucose-6-P formation by the accessibility of ATP to hexokinase.

Effects of Oxaloacetate. Avron and Biale (2) have demonstrated that oxaloacetate can inhibit the succinate oxidation of avocado mitochondria. Furthermore, Wiskich and Bonner (29) in studying mitochondria isolated from potato and sweet potato found that freshly isolated mitochondria may require ATP before any significant rate of succinate oxidation is realized. They concluded that this was due to an inhibition of succinate dehydrogenase by oxaloacetate. The freshly isolated avocado mitochondria showed a similar effect but not as marked. However, it was found that with avocado mitochondria this inhibition was progressively stronger as the pH of the isolation medium was increased from 7 to 8.

It was also suggested (29) that the progressive decrease of the rate of malate oxidation (fig 2, 7) was due to the accumulation of oxaloacetate. Some evidence supporting this claim was obtained from the avocado particles. Each experiment in table II represents the data obtained from one O_2 electrode trace. The additions, their order and the subsequent rates are shown. Experiment 1 of table II confirms the inhibitory effect of arsenite on α-ketoglutarate oxidation (1, 22) and shows that under such conditions addition of glutamate had little effect on oxidation, indicative of little glutamate dehydrogenase activity. The latter point was confirmed in experiment 2 (table II) where there was no oxidation of glutamate in the

Table II

O_2 Uptake in Relation to Interactions among Glutamate, Malate, and Oxaloacetate

The reaction mixture consisted of 2.6 ml of the standard mannitol medium containing 7 mM malonate, 20 mM glucose, 0.1 mg hexokinase, 0.38 mM ADP, and 200 μg mitochondrial nitrogen. Final concentrations of the added compounds, unless otherwise indicated, were: 17 mM α-ketoglutarate; 67 μM TPP; 3 mM sodium arsenite; 17 mM glutamate; 8 mM oxaloacetate; 17 mM malate. Rates are expressed as mμ moles 0_2/minute at 20°.

Expt.	First addition	Rate	Second addition	Rate	Third addition	Rate
1	α-Ketoglutarate, TPP	88	Arsenite	8	Glutamate	9
2	Glutamate	0	Oxaloacetate	12	Arsenite	12
3	Glutamate, TPP	11	Oxaloacetate	43	Arsenite	15
4	Oxaloacetate, TPP	0	Glutamate	52	Arsenite	17
5	Malate, Arsenite	86	Oxaloacetate (1.7 mM)	14	Glutamate	77

absence of TPP. The addition of oxaloacetate induced some oxidation but not enough to observe an inhibition by arsenite. However, if TPP were included in the medium (experiment 3, table II), glutamate was slowly oxidized and this oxidation was markedly stimulated by oxaloacetate. Under these conditions arsenite caused a substantial inhibition. Experiment 4 (table II) confirmed this observation and showed that oxaloacetate was not oxidized even in the presence of TPP.

Therefore, it may be concluded that the avocado mitochondria contained little glutamate dehydrogenase activity but could oxidize glutamate by transamination with oxaloacetate to aspartate and α-ketoglutarate and oxidizing the latter product (20).

The marked inhibition of malate oxidation by oxaloacetate is shown in experiment 5 of table II, together with the recovery of this oxidation by the addition of glutamate. Very little of the recovered oxidation could have been due to glutamate oxidation (either by dehydrogenase or transaminase activity) as arsenite was present and TPP was not included in the reaction. The recovery of oxidation was not immediate but was progressively increasing, presumably due to progressive removal of oxaloacetate.

The inhibition of malate oxidation with time and the beneficial effects of glutamate are shown in figure 7. Since the results of figure 7 were recorded with the same preparation used to obtain the results of table II, it is apparent again that little of the O_2 uptake in the presence of glutamate could have been due to the oxidation of glutamate itself. Malonate was included in the above experiments to inhibit the oxidation of succinate formed from the arsenite insensitive fraction of α-ketaglutarate oxidation.

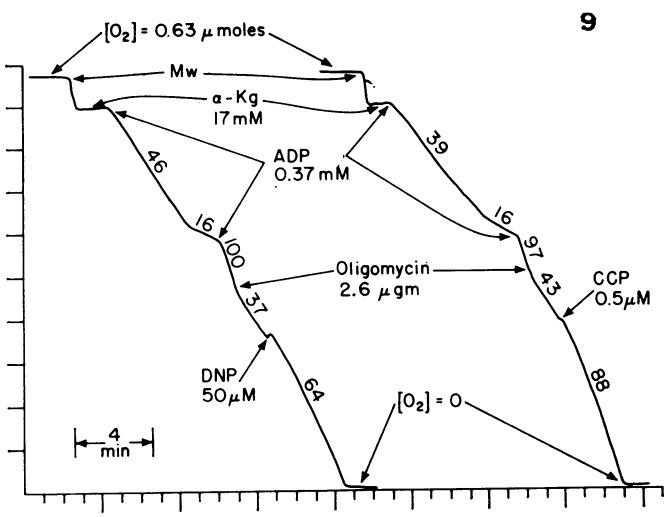
Effects of Hexokinase. Biale and Young (6) showed that their preparations from avocado contained hexokinase but not sufficient to transfer all of the P_i esterified into ATP. Since it seems unlikely that hexokinase is a true component of mitochondria, an indirect test for its presence was made with the preparation described here. Figure 8 indicates that hexokinase was present since the addition of glucose caused an increase in the state 4 oxidation. However, this activity was insufficient to maintain the level of ADP necessary for state 3 respiration. Both α-ketoglutarate and succinate were used as substrates.

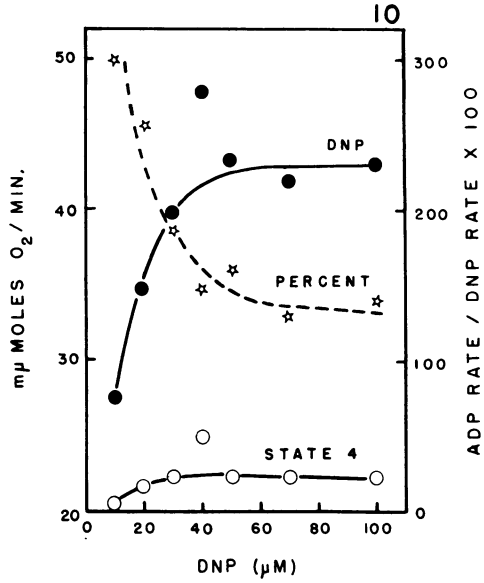
Thus respiratory control may be seen in preparations containing hexokinase (7) provided that there be no phosphate acceptor present.

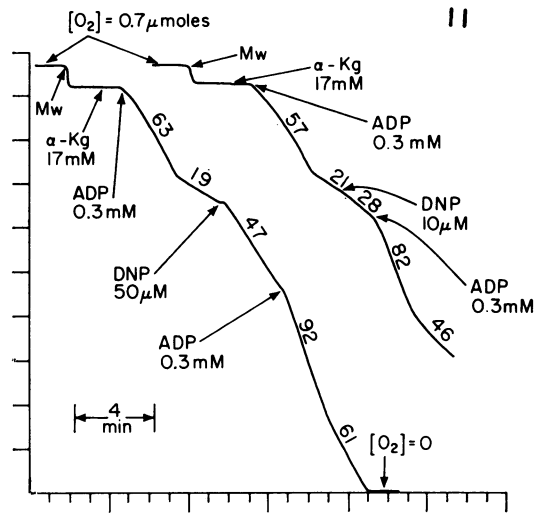
An Examination of the Effects of DNP on the Oxidation of α-ketoglutarate. The pattern of α-ketoglutarate oxidation by the avocado particles has been described. It was shown that this oxidation was dependent on ADP and it was concluded that during state 4 oxidation, the substrate-level phosphorylation was rate limiting. Figure 9 shows that oligomycin did not lower the ADP-stimulated oxidation to that of the state 4 rate. Thus the electron transport chain phosphorylations cannot be responsible for the very low level of oxidation during state 4. The oxidation inhibited by oligomycin (fig 9) could be recovered by DNP or CCP.

The results of figure 10 show the effect of titrating the state 4 oxidation of α-ketoglutarate with DNP. Two examples of experiments from which the data were derived are shown in figure 11. An addition of ADP was necessary to elicit the maximum rate of α-ketoglutarate oxidation (fig 2). In figure 10, the DNP stimulated rates are plotted as a function of the DNP concentration (solid line with closed circles). The solid line with open circles is a plot of the state 4 rates just prior to the addition of DNP to show that the state 4 rate did not change during the experiment. After the DNP rate was followed for approximately 4 minutes, ADP was added again. In figure 10 the dash line is the per cent stimulation of the DNP rate caused by the second addition of ADP. Approximately 50 μM DNP gave maximal oxidation rates and that ADP further stimulated oxidation at all levels of DNP tested. Thus the substrate-level phosphorylation was the rate-limiting step before and after the addition of DNP, and because of this no apparent inhibition of oxidation was observed with high concentrations of DNP (cf. succinate in fig 3). It has been shown that a discrepancy exists in the apparent ADP used as calculated from the attainment of state 4 (O_2 electrode) and amount of ADP phosphorylated (glucose-6-P assay). Since DNP does not uncouple the substrate level phosphorylation which is rate limiting, DNP must be stimulating oxidation by making ADP available to the substrate level site. Thus, the stimulation of the state 4 oxidation of α-ketoglutarate by DNP may have been due either to a stimulation of adenosine triphosphatase activity or to a more favorable ATP/ADP ratio at the active site (3, 4). The experimental pattern for the results shown in figure 10 can be seen in figure 11. Even in the presence of DNP, state 3 to state 4 transitions can be seen with ADP. This shows that the DNP effect is not sufficient to provide an ADP supply to the substrate level phosphorylation capable of maintaining a maximal oxidation rate. Furthermore, figure 11 shows that ADP/O ratio decreases with increasing DNP concentration. This could be a reflection of increased adenosine triphosphatase activity with higher concentrations of DNP.

To decide some of these issues a careful study was made of the effects of DNP in the presence of oligomycin. Figure 12 shows that when oligomycin was added during state 3 oxidation of α-ketoglutarate there was an immediate small inhibition of oxidation (due to an inhibition of electron transport chain phosphorylations), which was maintained until ADP became rate limiting and the coupling of the substrate level phosphorylation induced the state 4 rate. Oligomycin did not inhibit the substrate level phosphorylation (11). Furthermore DNP did not stimulate this state 4 respiration in the presence of oligomycin (fig 12). Thus the DNP stimulation of state 4 oxidation with α-ketoglutarate was due to the stimulation of adenosine triphosphatase activity







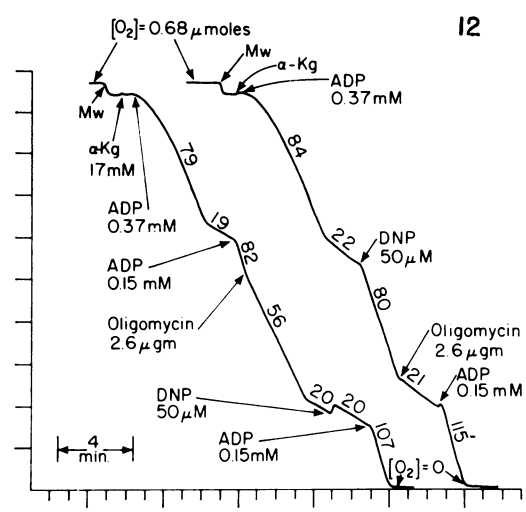


FIG. 9. The effect of oligomycin on the oxidation of α-ketoglutarate. Assayed in 2.7 ml of standard mannitol medium containing 64 μM TPP, 7 mM sodium malonate and 372 μg mitochondrial nitrogen. Rates are expressed as mμ moles O_2/minute.

FIG. 10. The effects of DNP and ADP on the state 4 oxidation with α-ketoglutarate as substrate. Assayed in 2.8 ml standard sucrose medium containing 64 μM TPP, 7 mM sodium malonate, 17 mM sodium α-ketoglutarate and 248 μg mitochondrial nitrogen. The medium was made 0.32 mM in ADP and the system allowed to reach the state 4 rate, then DNP was added followed by ADP again. O state 4 rate. ● DNP-stimulated rate. ☆ Final ADP rate as a percentage of the DNP rate.

FIG. 11. Polarographic tracings showing the effects of DNP and ADP on α-ketoglutarate oxidation. Assayed in 2.7 ml standard sucrose medium containing 64 μM TPP, 7 mM sodium malonate and 248 μg mitochondrial nitrogen. Rates are expressed as mμ moles O_2/minute.

FIG. 12. The effect of oligomycin on the DNP-stimulated oxidation of α-ketoglutarate. Assayed in 2.7 ml standard mannitol medium containing 64 μM TPP, 10 mM sodium malonate and 200 μg mitochondrial nitrogen. Rates are expressed as mμ moles O_2/minute.

which would be inhibited by oligomycin. This was confirmed (fig 12) by the immediate inhibition by oligomycin of the DNP-stimulated state 4 oxidation of α-ketoglutarate. The results of figure 9 showing the expected relationship between oligomycin and DNP were obtained in the presence of excess ADP, conditions under which the substrate-level phosphorylation would not become rate limiting.

The ability of DNP to uncouple oxidative phosphorylation even when α-ketoglutarate was used as substrate is shown in figures 9 (by recovery of oligomycin inhibited oxidation) and 13 (by decrease in

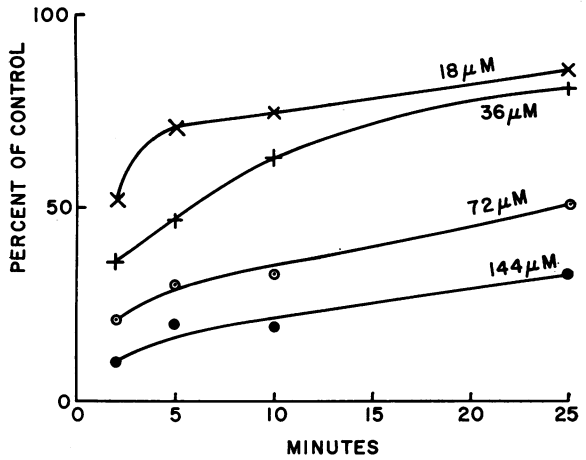


FIG. 13. The rate of ATP formation at different concentrations of DNP. Assayed in 3.3 ml sucrose medium containing 64 μM TPP, 10 mM sodium malonate, 20 mM sodium α-ketoglutarate, 0.4 mM ADP, 20 mM glucose, 1 mg hexokinase, DNP in concentration noted on the trace, and 108 μg mitochondrial nitrogen.

glucose-6-P formation). A detailed study of the time effect is shown in figure 13, where an apparent recovery of phosphorylation, particularly at low concentrations of DNP, is apparent. However, the results of figure 13 are shown as a percentage of the controls and the apparent recovery was due to a decrease in the control phosphorylation rate, as seen for succinate in figure 5. Thus in manometric experiments with 10 μM DNP as used by Romani and Biale (26) any uncoupling of phosphorylation would not be detected. It has been shown that the oxidative phosphorylation system of these avocado particles was relatively unstable even at 0°. Thus, we expect that as oxidative phosphorylation decreased the sensitivity of the system to DNP also decreased.

Stability. During the course of most of this investigation the avocado mitochondria showed moderate stability when maintained at 0°. Table III shows the increase of the state 4 respiration that occurred with α-ketoglutarate and the consequent decrease in the respiratory control ratio. These results indicate a loss of coupling of the substrate level phosphorylation or an increased adenosine triphosphatase activity. The loss of oxidative phosphorylation with time was observed with oligomycin, which inhibited the state 3 respiration of α-ketoglutarate (fig 9) of freshly isolated particles. This inhibition of oxidation of α-ketoglutarate by oligomycin was lost fairly rapidly without any apparent loss in the rate of oxidation. Thus the level of coupled oxidative phosphorylation was lowered to such an extent that it could not limit the rate of α-ketoglutarate oxidation. The stability of preparations made very late in the Fuerte avocado season (June) was low. Respiratory control and coupling were lost very rapidly at 0°. The reasons for this pattern of behavior are unknown.

Table III

The Loss of Respiratory Control of Mitochondria after Storage at 0°

The reaction mixture contained 2.6 ml of the standard sucrose medium containing 7 mM malonate, 0.38 mM ADP, 67 μM TPP, 17 mM α-ketoglutarate, and 180 μg mitochondrial nitrogen. Rates are expressed as mμ moles O_2/minute at 20°.

	Minutes at 0°				
	0	20	40	60	180
State 4 rate	15	16	17	22	34
Respiratory control ratio	5.1	4.8	4.7	3.8	2.5

Discussion

Plant mitochondria showing the property of respiratory control have been isolated from sweet potato and white potato (29) and from cauliflower bud (7). Wiskich and Bonner (29) described some of the conditions which were critical in obtaining preparations showing respiratory control. Apart from the factors which mask respiratory control (e.g., ATP-hydrolyzing enzymes) and which may be precipitated with the mitochondria (7), little is known about the conditions necessary for isolating particles showing respiratory control. It is certain that the structural organization, at all levels, of the mitochondrion plays a major role (12). Thus plant tissues with their variable composition and need of severe treatment for disruption of the cells have yielded mitochondrial preparations with inconsistent properties and a lack of respiratory control. The differences in properties may be a reflection of small changes in structure (30) and are probably correlated with the conditions used (13).

Whereas it was possible to prepare ADP controlled mitochondria from avocado fruit at the climacteric peak when tissue slices show no response to DNP (24), the same procedure failed to yield ADP controlled mitochondria from preclimacteric fruit [tissue slices from which respond to DNP (24)]. This failure may have been because only those cells of the preclimacteric fruit subjected to the higher shearing forces were disrupted. A direct consequence of this, and perhaps the cause of the lack of evidence of respiratory control, may be the observation of Avron and Biale (6) that mitochondria isolated from preclimacteric avocado fruit possess a more active adenosine triphosphatase than mitochondria isolated from climacteric peak fruit. Since the respiratory rate of the parent plant tissue is, in most cases, limited by oxidative phosphorylation and therefore stimulated by DNP, the property of respiratory control in the mitochondria prepared from such tissue is essential for critical investigations. However, a mere stimulation of mitochondrial oxidations by ADP (14, 16, 28) is not necessarily indicative of respiratory control (8). This effect may be due to a low level of endogenous adenylate (and may be indicative of

coupling) or to other reactions not related to respiratory control (29).

The Climacteric Rise of Respiration. Since the climacteric peak fruit yielded ADP controlled particles, it could be claimed that DNP failed to stimulate the oxidation rate of climacteric peak avocado slices, not because of endogenous uncoupling (24) but because of a higher turnover rate of endogenous phosphate or phosphate acceptor (25). However, such a physiological interpretation of these results is not warranted because it cannot be applied to results obtained with preclimacteric peak avocado slices and with mitochondria isolated therefrom. The interpretation of results obtained with isolated plant mitochondria requires a precise definition of the conditions, particularly if the overall activity of the preparation has not been investigated.

From the results reported here it appears that the mitochondria of intact, climacteric peak fruit have not lost their ability for oxidative phosphorylation and that oxidation can be controlled by phosphate or phosphate-acceptor level. If endogenous uncouplers occur in the supernatant fraction of a mitochondrial preparation from avocado fruit (24), it remains to be established that they are effective in situ. It is unlikely that oxidative phosphorylation is the only mechanism which controls the turnover of the Krebs cycle.

It is generally considered that cellular oxidation can be stimulated by increased turnover of ATP. However, it is interesting to speculate whether cellular oxidation can be stimulated by a reversible uncoupling, a reversible activation of a specific ATP-hydrolyzing enzyme or by activation of nonphosphorylating oxidation pathways. The studies reported here are quite inadequate for a resolution of these possibilities. This is particularly important in plant tissues where it has been shown that oxidations insensitive to respiratory chain inhibitors can occur, and that in some instances the supernatant fraction is also capable of presumably nonmitochondrial oxidations (15).

Uncoupling by DNP. In contrast with earlier reports (26) it has been established that DNP can uncouple the oxidative phosphorylation of mitochondria prepared from climacteric peak avocado fruit. The failure by Romani and Biale (26) to demonstrate this effect is explained by a combination of the use of a relatively low concentration of DNP, and the unstable oxidative phosphorylation of avocado mitochondria.

Figure 13 shows that 18 μM DNP has relatively little effect on the total amount of phosphate esterified after 25 minutes incubation, as is usual with manometric experiments. This apparent recovery of coupling in the presence of DNP is due to the progressive decrease of rate of phosphorylation in the control. A similar effect has been observed with rat liver mitochondria (27). Higher concentrations of DNP produced a more marked effect. The apparent continual decrease in phosphate esterification with increasing concentrations of DNP can be taken to indicate a high degree of electron transport chain phosphorylation with little substrate level phosphorylation. However, a more probable explanation is the increased adenosine triphosphatase activity with increasing DNP competing more with hexokinase for the ATP. The effects of DNP on the state 4 oxidation of α-ketoglutarate (fig 10, 11) have been shown to be due to the stimulation of adenosine triphosphatase activity. Furthermore, it has been shown that the DNP stimulated adenosine triphosphatase is sensitive to oligomycin (21), allowing a differentiation between effects on the electron transport chain phosphorylation and on substrate level phosphorylations, the latter being insensitive to both DNP and oligomycin (11). Preliminary experiments with a potent uncoupling agent (CCP) described by Heytler et al. (17) duplicated the effects of 50 μM DNP at a concentration of 0.5 μM.

P/O Ratios. High P/O ratios have not been obtained with avocado mitochondria. The presence of high concentrations of EDTA or of bovine serum albumin (5) did not appear to offer any protection. In fact, an absolute requirement for EDTA in the isolation medium was not established. The P/O ratios observed here by indirect calculation from the O_2 electrode data of ADP-controlled preparations approached the expected maximum. However, it is not necessary to have a high degree of coupling for well controlled mitochondria if compartmentation of ATP at the active site occurs (3, 4). The control shown by this type of information may be due to the properties of only one of the phosphorylation sites. Thus the very low rate of state 4 oxidation of α-ketoglutarate was due to the controlling influence of the substrate level site only. None of the electron transport chain phosphorylations was capable of exerting such a strong restriction on oxidation as evidenced by the experiments with oligomycin (fig 9). Oligomycin has been shown to inhibit the oxidation of malate to a rate similar to the state 4 rate (fig 4), thus establishing the effectiveness of oligomycin.

The discrepancy between the indirect calculation of P/O and the amount of glucose-6-P formed still requires explanation. It was shown that 50 μM DNP did not stimulate the state 4 oxidation of α-ketoglutarate to the state 3 ratio (fig 11). Furthermore, 100 μM DNP was no more effective in stimulating state 4 oxidation of α-ketoglutarate than was 50 μM DNP (fig 10). On the other hand, hexokinase (50 μg/2.7 ml reaction volume) did stimulate the state 4 oxidation of α-ketoglutarate to the state 3 rate (fig 8). This amount of hexokinase was capable of a higher ADP turnover than was the maximum DNP-stimulated adenosine triphosphatase activity. Moreover, the hexokinase stimulated oxidation was maintained. Therefore, the reason for the discrepancy is probably not due to compartmentation of ATP (3, 4) nor to a penetration barrier between ATP and hexokinase. Although some results suggesting penetration barriers have been obtained, they have not been subjected to a detailed investigation. The presence of phosphatases or some other enzymes as contaminants of

the preparation would also reduce the yield of glucose-6-P. Such contaminating enzymes are pertinent to the reversal of the DNP effect by hexokinase (6). Further studies are necessary before these problems can be resolved.

An alternative possibility is that the endogenous adenosine triphosphatase of these particles is recycling ADP at a rate slower than the rate of ADP phosphorylation. Thus once the bulk of the added ADP is phosphorylated the oxidation rate would decrease to the level where rates of hydrolysis and of formation of ATP were equal. Respiratory control would be evident and yet a significant amount of ATP may not be available to hexokinase for glucose-6-P formation. However, under these conditions it would be expected that the oligomycin inhibited oxidation rate would be much less than the state 4 rate. This expectation was not realized for the oxidation of either succinate or malate in the presence of oligomycin (fig 4). Thus, convenient but indirect measurements of P/O as offered by the O_2 electrode technique must be verified with direct assays (see also 10).

The Kinetics of Malate Oxidation. Wiskich and Bonner (29) observed the progressive decrease in the rate of malate oxidation and suggested that this was due to product accumulation. Attempts to remove the oxaloacetate were unsuccessful. A similar progressive decrease in the rate of malate oxidation was observed here.

It was established that very little oxidation was due to glutamate dehydrogenase alone, and that oxaloacetate stimulated glutamate oxidation through an arsenite-sensitive pathway (table II). Thus it is claimed that a transamination is involved yielding α-ketoglutarate and aspartate (20) and that the oxygen uptake is due to the oxidation of α-ketoglutarate. In the presence of glutamate and arsenite the oxidation of malate was maintained, presumably due to the removal of oxaloacetate. Very little of this oxidation was due to substrates other than malate.

Summary

Particles showing respiratory control as evidenced by stimulations of oxidation on successive additions of adenosine diphosphate have been isolated from ripe fruit of the avocado. Dinitrophenol has been shown to uncouple the electron transport chain phosphorylations of these particles. Failure to observe this in previous studies has been due to a combination of factors associated with the particles and with the assay technique.

It has been established that dinitrophenol stimulates an adenosine triphosphatase activity which is sensitive to oligomycin, and that neither dinitrophenol nor oligomycin affect the substrate-level phosphorylations. The effects of 50 μM dinitrophenol were duplicated by 0.5 μM carbonyl cyanide *m*-chlorophenylhydrazone.

It has been shown that oxidative phosphorylation is unstable in these preparations and that substrate level phosphorylations are more tightly coupled and may account for most of the adenosine triphosphate formed. This condition explains the observation of good respiratory control with low P/O ratios.

Product inhibition of malate oxidation has been confirmed. This inhibition was overcome by the addition of glutamate and arsenite. Transamination occurred between glutamate and oxaloacetate and arsenite inhibited the oxidation of α-ketoglutarate. It was established that in the avocado particles little glutamate oxidation operated via a direct glutamate dehydrogenase pathway.

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