Stimulations of Oxygen Uptake by Electron Transfer Inhibitors[1]

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Summary. The stimulation of oxygen uptake induced in avocado tissue slices by amytal, azide and cyanide has been studied. The effects of these inhibitors on O_2 uptake and on phosphorylation suggest the coexistence of phosphorylating and non-phosphorylating electron transfer systems in the fruit. The reason for the stimulations of O_2 uptake is believed to be the result of an increased supply of a limiting cofactor to the phosphorylating sites. The increased availability of cofactor per site is due to the inhibition of part of the cytochrome chain and the consequent reduction in the number of active phosphorylating sites.

Cyanide resistant respiration is of widespread occurrence in plants and is generally characteristic of certain developmental stages of leaves, roots, fruits and seeds. It has been observed in the *Arum* spadix by James and Beevers (9), in wheat roots by Eliasson and Mathiesen (5), in slices of bean roots by Robertson et al. (20), in chicory root by Laties (11), in mature leaves by Merry and Goddard (17), in barley seedlings by James and Boulter (10), in germinating lettuce seeds by Poljakoff-Mayber and Evenari (19), in germinating bacterial spores by Nakada et al. (18), and in slices of avocado fruit by Biale (2).

Several theories have been advanced to explain the apparent insensitivity of plant tissue to cyanide or azide:

Protective Mechanism. MacDonald (14) discussed the possibility of depletion of cyanide due to condensation reactions with keto acids and with sugars to form cyanohydrins. He points out that if this were the case one could not explain the increase in reducing sugars with increased sensitivity to cyanide. Furthermore, Lundegardh (12) produced evidence for the formation of a cytochrome a_3- cyanide complex in wheat roots, which exhibited cyanide resistance. The effect of azide cannot be explained by this theory.

Excess Cytochrome Oxidase Theory. Hill and Hartree (8) pointed out that the presence of an excess of cytochrome oxidase relative to the rate-limiting step of the electron transfer chain might make possible a large inhibition of the final oxidase without affecting the overall respiration. Lundegardh (13) working with yeast showed that respiration was only inhibited 12 % when 82 % of cytochrome oxidase combined with cyanide. The excess oxidase hypothesis was discussed critically by Chance and Hackett (4).

Alternate Pathways of Electron Transfer. Besides the existence of cyanide-resistant oxidases (glycolic acid oxidase) in many tissues, the presence of an autoxidizable cytochrome, such as b_7, was shown by Bendall and Hill (1) to be present in large amounts in the spadices of certain *Araceae*. Cytochrome b_7 remains oxidized in the presence of concentrations of cyanide which cause the reduction of virtually all cytochromes c and a normally found in plant tissue. The relative concentration of cytochrome b to cytochrome c increases 3-fold during the development of the spadix as demonstrated by Bendall and Hill (1). Spectrophotometric observations by Hackett et al. (6) of mitochondria isolated from fresh and aged slices of white potato tubers showed an increase of the ratio of cytochrome b to cytochrome a with aging and also an increase in microsomal cytochrome b_3.

Although a few experiments during the last 10 years provide possible mechanisms of cyanide-resistant respiration in cell-free systems, the operation of such mechanisms in intact tissues has not been demonstrated, nor is there a single theory capable of explaining all the properties of such respiration. In many cases the response of a tissue to cyanide or azide consists of a marked stimulation of O_2 uptake. Neither the excess cytochrome theory nor the operation of protective mechanisms could account for this stimulation. The existence of alternate electron transfer pathways connected to the cytochrome chain permits, however, the explanation that the application of single inhibitors produces a bypass of part of the phosphorylative sites, thus increasing

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the availability of a limiting phosphorylation cofactor to the other sites.

This hypothesis is examined by the effects of amytal, cyanide, azide and dinitrophenol on the O_2 uptake and esterification of ^{32}Pi by tissue slices of avocado fruits.

Material and Methods

Fruits of the Hass and Fuerte varieties of avocado (*Persea gratissima*, Gaertn.) were obtained from Calavo Company (California Avocado Association) and from the orchard of the College of Agriculture at UCLA.

Slices were prepared with a stainless steel knife and cork borer. The thickness of the slices was approximately 1 mm and the diameter 1 cm.

O_2 consumption was determined by standard Warburg manometric techniques. Esterification of phosphate was followed by studies of ^{32}P incorporation. Twenty slices (about 1 g fr wt) were incubated in 5 ml of 0.5 mM phosphate buffer, pH 5.5, to which approximately 10^6 cpm of $H_3{}^{32}PO_4$ were added. Slices were extracted in 7ml of 5 % trichloroacetic acid by homogenization for 1 to 2 minutes in a VirTis 45 homogenizer. The homogenate was then carefully mixed with 2ml chloroform to separate the fats and to centrifuge effectively cell debris. This permitted the separation of 3 distinct layers after 10 minutes centrifugation: an upper layer of trichloracetic acid extract, an intermediate layer of cell debris, and a lower layer of chloroform-soluble material. One-half ml of 10 % H_2SO_4, 1 ml of 5 % ammonium molybdate and 5 ml of a mixture of isobutanol/benzene (1:1, v/v) were added to 3.5 ml of the aqueous layer and thoroughly mixed using a Vortex J mixer. The emulsion was then centrifuged for 10 minutes, the upper layer of isobutanol/benzene and Pi discarded, and the aqueous layer washed with 3 ml ether. After centrifugation the ether layer was discarded and soluble ^{32}P-esters were determined by plating 0.1 ml samples in either copper or aluminum planchets, and counting in a gas flow scaler. Extracts and reagents were kept either under ice water or in a constant temperature room at 0°. Slices were preincubated for 20 minutes in the corresponding inhibitor before the addition of labeled phosphate.

Results and Discussion

Effects of Amytal. The respiration of neither climacteric nor preclimacteric tissue slices was inhibited by amytal when supplied in a relatively wide range of concentrations (table I). The effect of amytal on the esterification of ^{32}P was also studied. Only soluble esters were considered. Results are shown in figure 1. The response of climacteric tissue to the inhibitor was identical to that of preclimacteric except for the magnitude of ^{32}P incorporation which was 10-fold higher in the ripe tissue.

It is presumed that isotopic equilibrium with the internal pool of Pi was reached within 30 minutes since after that period of time no further increase of net soluble ^{32}P-esters was observed. Once such equilibrium was attained the rates of ^{32}P esterification and hydrolysis seemed to be equal. Rates of esterification are difficult to establish in avocado slices since the extent of certain relevant processes such as the rate of permeation of the ions, the nature of their transport across the cellular membranes and the extent of leakage from the tissue are unknown. The size of the pool of soluble esters is determined by the relative activities of processes incorporating Pi such as esterification of nucleotides, sugars and glycolytic intermediates on the one hand, and those processes bringing about hydrolysis of the esters or their incorporation into insoluble fractions on the other hand. Inhibition of electron transfer or of glycolysis would reduce the pool size since the dynamic equilibrium of the steady state would be upset and the rate of esterification reduced.

Supposing that there is an electron transfer system other than the cytochrome chain and that the alternate path is resistant to amytal and to azide, this alternate pathway could transfer electrons from a given source (substrate$_a$) through one or more intermediate carriers (fp_{iii}) to a CN-resistant oxidase (OX) as indicated in figure 3. This hypothetical pathway could be completely dissociated from the cytochrome chain or connected to it. If the alternative amytal-resistant pathway of electrons in avocado tissue slices is completely independent of the cytochrome chain, one would expect that the application of amytal would reduce the size of the pool of soluble phosphate esters unless this path is also capable of phosphorylation. Since we do not observe

Table I. *Effect of Na-Amytal on the O_2 Uptake by Avocado Tissue Slices*

The experimental time was 2 hours, pH 7.0, medium 1 mM $CaSO_4$, and temperature 20°.

	Preclimacteric		Climacteric	
mM Na-Amytal	µl O_2/g fr wt/hr	% Control	µl O_2/g fr wt/hr	% Control
None	414	100	458	100
5	720	174	433	95
10	759	183	532	116
1	550	133	489	107
0.5	536	129	484	106
0.1	494	119	456	99

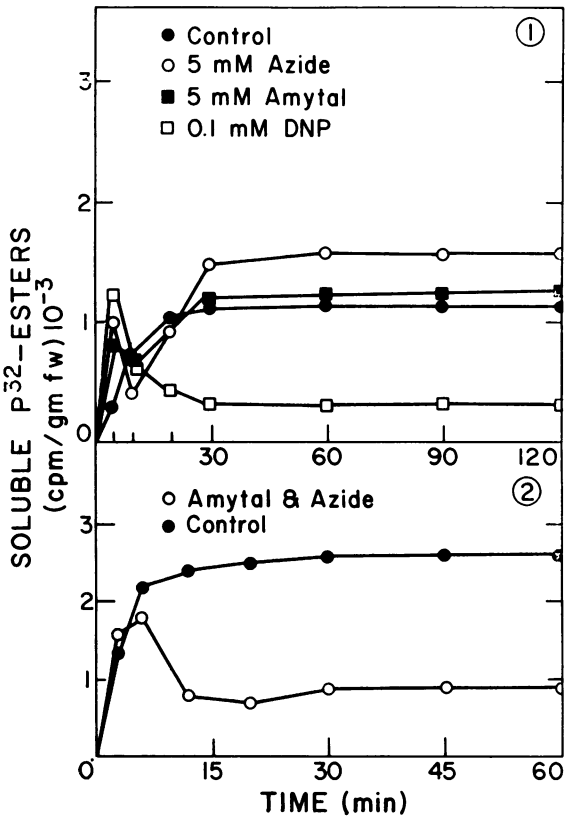

FIG. 1,2. Incorporation of inorganic phosphate by avocado tissue slices. The concentrations of amytal and azide in experiment of figure 2 were the same as shown in figure 1.

such a reduction of pool size, and cases of oxidative phosphorylation at the electron transfer level other than those linked to the cytochrome chain are unknown, it might be assumed that the alternative path is not completely dissociated from the phosphoylating chain but is sharing part of it, a part comprising at least 1 phosphorylative site (fig 4).

Effects of Cyanide and Azide. Both climacteric and preclimacteric tissue slices seemed to be completely resistant to cyanide and azide so far as O_2 uptake was concerned. Marked stimulations could be observed, as indicated in table II.

The effects of cyanide and azide on O_2 uptake and ^{32}Pi-esterification (fig 1) can be interpreted in 3 different ways: A) resistance is due to the absence of a sensitive site (cytochrome oxidase) or to the nonfunctional state of this site; B) there is a sensitive site but it cannot be reached by the inhibitors due to permeability limitations imposed by cellular membranes; C) alternative pathways of electron transfer are activated as a consequence of the blocking of cytochrome oxidase.

The marked stimulations observed argue against permeability limitations since the increased rate of O_2 uptake was made in response to the presence of the inhibitors. The cytochrome chain was not entirely affected since phosphorylation remained effective and sites of phosphorylation at the electron transfer level other than those of the cytochrome chain are unknown. Complete inhibition of the cytochrome chain seems, therefore, improbable. The effects of combined applications of amytal and azide, to which we will refer later, indicate that alternative (C) of interconnected pathways of electron transfer seems to be the only one compatible with the experimental results obtained.

Effect of 2, 4-Dinitrophenol on Respiration and Phosphate Esterification. Dinitrophenol at 0.5 mM or lower stimulated the rate of O_2 uptake by preclimacteric tissue but either depressed or had no effect on oxidation of climacteric slices (table II).

The inhibitory effect of DNP on climacteric tissue at concentrations which were stimulatory in the preclimacteric disks was probably due to differences in permeability of the slices. The climacteric tissue is known to be far more permeable than the preclimacteric, so that at the same external concentrations of DNP the effective internal concentration could be much higher in the climacteric than in the preclimacteric.

The effect of DNP on the esterification of ^{32}P is shown in figure 1. The main difference between the stimulations of O_2 uptake in DNP compared to

Table II. *Effects of Cyanide, Azide and DNP on the O_2 Uptake by Avocado Tissue Slices*
The experimental time was 2 hours, pH 6.0, medium 1 mM $CaSO_4$, temperature 20°.

	O_2 uptake, % of control					
	Cyanide		Azide		DNP	
mM Inhibitor	PC*	C**	PC	C	PC	C
None	100	100	100	100	100	100
10	100	95	107	112	...	...
5	122	111	178	122	37	36
1	126	132	176	116	105	44
0.5	124				186	78
0.1	130	121	108	91	219	76
0.05	103				190	92

* PC = preclimacteric tissue slices.
** C = climacteric tissue slices.

the effects of azide and amytal is that the uncoupler brought about a reduction of the pool of soluble ^{32}P esters, while the inhibitors applied singly did not. During the first 15 minutes a rapid incorporation of ^{32}P was observed, followed by an equally rapid hydrolysis and by a steady state level. The initial rapid incorporation could very well be a technical artifact although it should be noticed that controls never behaved as the samples treated with inhibitors. The amount of label incorporated in the presence of DNP is probably a product of substrate level phosphorylation, with which DNP does not interfere, and of the small amount of ^{32}P contamination present in experiments of this type (0.1 %).

Effects of More than One Inhibitor Applied Simultaneously. The stimulation of O_2 uptake observed in the presence of either amytal (table I) or azide (table II) was abolished when both inhibitors were supplied simultaneously (table III).

The effect of the simultaneous application of amytal and azide had also a striking effect on phosphorylation (fig 2). The inhibition of phosphorylation observed under the conditions of this experiment reinforces the idea that the cytochrome pathway was operative in the tissue and that the inhibitors reached the known sensitive sites in the chain. As in the case of DNP the small amount of phosphorylation observed in the presence of amytal plus azide could be due to substrate-level phosphorylation and to some extent of ^{32}P contamination. The lack of stimulation of O_2 uptake is expected since all the phosphorylative sites are inactive in the presence of both inhibitors supplied simultaneously. Only the nonphosphorylative pathway remains operative as indicated by the uptake O_2 without concomitant phosphorylation.

Since the electron transfer pathway operative in the presence of amytal plus azide seems to be a nonphosphorylative one, the effect of DNP in such pathways was studied and the results are shown in table IV.

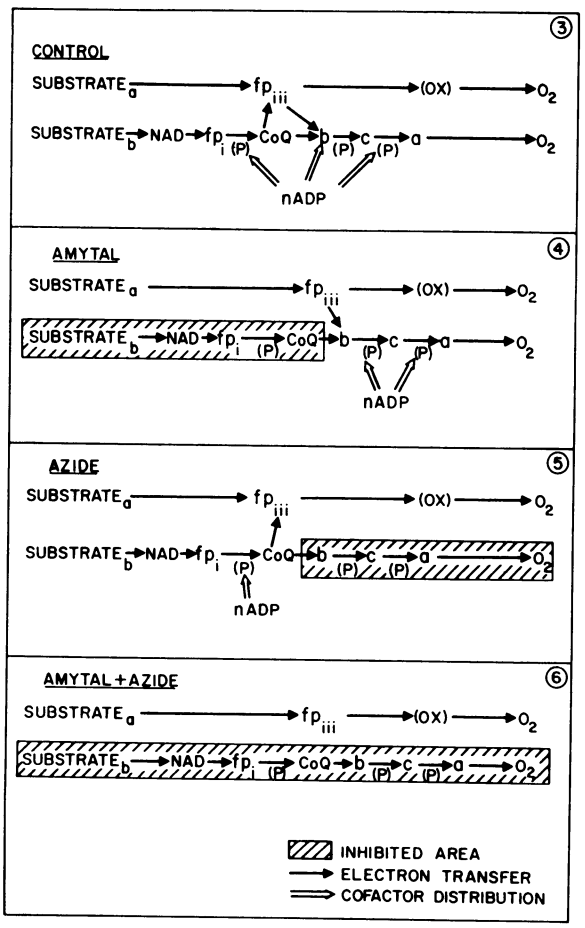

FIG. 3, 4, 5, and 6. Theoretical schemes of electron transport and sites of phosphorylation.

Table III. *Effect of 5 mM Amytal and 5 mM Azide on O_2 Uptake by Avocado Tissue Slices*

The experimental time was 2 hours, medium 10 ml of 0.5 mM phosphate buffer, pH 7.0, temperature 20°.

Inhibitor	O_2 Uptake, % of control Preclimacteric*	Climacteric
None	100*	100**
Amytal	156	146
Azide	176	143
Amytal + azide	100	98

* Preclimacteric control : 114 μl O_2/g fresh weight/hour.

** Climacteric control : 115 μl O_2/g fresh weight/hour.

Table IV. *Effect of 0.1 mM DNP on the O_2 Uptake by Preclimacteric Tissue Slices, Treated with Amytal + Azide (5 mM each)*

Inhibitor	% of control	% DNP stimulation
None	100*	
DNP	210	110
Amytal	133	
Azide	124	
Amytal + Azide	96	
Amytal+Azide+DNP	135	35

* Control: 115 μl O_2/g fresh weight/hour.

The large reduction of DNP-stimulation in the presence of amytal plus azide was not surprising, considering that phosphorylation was inhibited. DNP stimulated O_2 uptake by its effect on high-energy intermediates providing essentially an unlimited supply of high energy acceptors. Amytal and azide seemed to inhibit the cytochrome chain (no phosphorylation is observed) in such a way that the limitation on the rate of respiration was not the supply of ADP but the capacity of the nonphosphorylative pathway. The small stimulation of DNP in the

presence of amytal plus azide could very well be the result of a side effect of DNP or an indirect effect of the lack of ATP in the tissue. In any event, the extent of the stimulation by DNP was largely diminished by amytal plus azide.

Conclusions

The electron transfer system of the avocado fruit seems to be composed of phosphorylative cytochrome chain linked to nonphosphorylative pathways. The ability of the system to esterify ^{32}P in the presence of either amytal or azide when the inhibitors were presented individually indicates that both types of electron transfer pathways are interconnected. Electrons may be transferred from one of the pathways to the other. The phosphorylating ability of the tissue disappears when treated with amytal plus azide simultaneously. Using the same theoretical scheme presented in figure 3, the effect of the inhibitors could be visualized as indicated in figures 4 and 5.

The stimulation of O_2 uptake observed when inhibitors were applied individually, but not when applied jointly, seems to be the result of the increased supply of a limiting phosphorylative cofactor to part of the phosphorylative sites due to the bypass of the other sites. This redistribution of cofactors is possible only because of the presence of appropriate branching of the cytochrome chain permitting the bypass of the inhibited sites. The increased availability of phosphorylating cofactors per phosphorylative site brought about by the presence of single inhibitors produces an increased activation of respiration while the overall phosphorylation remains unaffected. The result is a lowering of P/O ratio due not to uncoupling in the classical sense of the word (hydrolysis of high-energy intermediates) but rather to a relatively larger participation of nonphosphorylative steps in the electron transfer system.

Inhibitors seem to be required to force electrons from the cytochrome chain to the alternate pathways. This fact suggests that differences in Ks at the branching point favor the transfer of electrons to the cytochrome component. Thus, reduced CoQ (fig 3) would have a larger affinity for oxidized cytochrome b than for oxidized fp_{iii}. Amytal increases indirectly the transfer of electrons to cytochrome b by inactivating one phosphorylative site and permitting a larger activity of the other two. The stimulation by DNP differs because it is produced by an elimination of the need for phosphorylative cofactors and not by increasing the concentration of these cofactors at the functional site.

The use of amytal plus azide has several effects which reinforce the explanation for inhibitor-stimulated O_2 uptake presented here. Amytal plus azide produce no stimulation at all because the redistribution of limiting cofactors cannot take place in the presence of both inhibitors as the entire cytochrome chain and, therefore, phosphorylation is inhibited. For the same reasons, tissue treated with amytal plus azide can no longer be stimulated by DNP to the same degree as untreated tissue. This observation agrees with the assumption that both amytal and azide effectively inhibit their respective sites in the cytochrome chain and that a nonphosphorylative pathway operates exclusively when both inhibitors are applied together.

The idea of nonphosphorylating alternate routes of electron transfer has been presented in numerous papers. Martius (15) put forward the idea that there may exist in the mitochondria 2 different routes between pyridine nucleotide and cytochrome c, only one of them being linked with the enzymes which bring about phosphorylation. He suggested vitamin K reductase as an oxidation-reduction catalyst, an enzyme which was proved to be a flavoprotein by Martius and Marki (16). These workers have shown that vitamin K reductase can be reduced by either NADH or NADPH and could, therefore, constitute the necessary link to bypass amytal-sensitive sites. This link is designated fp_{iii} in our theoretical scheme. Further information on the possible operation of this enzyme in plant mitochondria was provided by Hackett and Ragland (7).

The existence of a cyanide-resistant oxidase was recently suggested by the work of Bonner (3), who reported the existence of 2 CO-binding pigments in plant mitochondria. One of these pigments is cytochrome a_3; the other could be related to an alternate pathway of electron transfer, insensitive to cyanide.

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