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Cyanide-insensitive Respiration

II. CONTROL OF THE ALTERNATE PATHWAY*

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SUMMARY

The data on cyanide-insensitive respiration previously presented as well as additional results are discussed from the point of view of control of respiration in plant mitochondria. It is shown that the results are inconsistent with certain mechanisms for control of the alternate pathway rate. The control cannot be exerted through the oxidation-reduction state of the cytochromes or the mitochondrial energy state. If only irreversible reactions are considered, simple competition between the alternate pathway and the cytochrome pathway also fails to explain the data.

The simplest explanation consistent with the known characteristics of the plant respiratory chain requires postulating a reversible equilibrium between two components in the flavoprotein region of the respiratory chain.

The previous paper (1) reported a method for the determination of the alternate, cyanide-insensitive pathway activity in higher plant mitochondria. The magnitude of the electron transport rate in this pathway was determined in three plant species in both States 3 and 4. The nonphosphorylating nature of the alternate pathway (1, 2) suggests that the plant cell will need to regulate its activity. The apparent dependence of the alternate pathway activity on the mitochondrial energy state and the fact that the observed rates were not always equal to the maximum possible rate (1) suggest that the cell does indeed regulate the alternate pathway.

Plant mitochondria display two mechanisms of control of electron transport. The first is common to all mitochondria—control of the respiration rate by the need for energy, as expressed by the phosphate potential. Although this phenomenon has been known for many years, the nature of the primary energy-conserving reactions in mitochondria is unknown and thus the mechanism of this control is not well understood. Chance and co-workers (3, 4) have discussed several possibilities for the mechanism of respiratory control. This control is expressed by the respiratory control ratio, the ratio of the State 3 rate to the State 4 rate. In plant mitochondria the rates used must be the cytochrome pathway rates determined from the benzhydroxamic acid titration data since the total State 3 and State 4 rates may contain a contribution from the alternate pathway. With succinate the respiratory control ratios range from 2 for skunk cabbage up to about 6 for the two members of the bean family studied.

The second type of control is expressed as the changes in ρ, the coefficient expressing the fractional activity of the alternate pathway on going from State 3 to State 4 in cyanide-insensitive mitochondria (1). In general, these changes result in more efficient phosphorylation by such mitochondria. Although details are unknown, these changes and additional data presented here place stringent requirements on the mechanism for control of the alternate pathway rate.

MATERIALS AND METHODS

The methods of isolation of mitochondria and of measurement of the respiratory rates have been given (1). The measurement of oxidation kinetics of the respiratory carriers of plant mitochondria was as described by Storey (5, 6).

RESULTS

The regulation of the alternate pathway rate is assumed to involve the same mechanism in all plant tissues; postulated mechanisms must account for the results from the three tissues studied. These results may be summarized as follows. (*a*) Addition of appropriate concentrations of cyanide completely blocks the cytochrome pathway and gives the maximum alternate pathway rate ($\rho = 1$). (*b*) The cytochrome pathway rate is not affected by the total inhibition of the alternate pathway, regardless of energy state, as shown by the straight line character of the plots of total rate against $g(i)$. (*c*) When both pathways operate, the cytochrome pathway is operating at its maximum rate, the alternate pathway at a variable fraction of its maximum rate (given by ρ).

Three types of regulatory mechanisms are considered here. First, the rate in the alternate pathway could be determined simply by competition between the alternate pathway and the cytochrome pathway for reducing equivalents coming from the substrate. Second, the oxidation-reduction state of one or more of the cytochromes, reflecting the rate of electron transport in that pathway, could act to regulate the alternate pathway, *e.g.* via allosteric modifications of the alternate oxidase. Third, the al-

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ternate pathway might be regulated by the phosphate potential. This effect could be mediated via ATP or ADP directly or via the intermediates of oxidative phosphorylation.

Organization of Plant Respiratory Chain—Although the plant respiratory chain has been extensively studied for some years now, it is not possible to assign a firm role to all of the known oxidation-reduction carriers. This is primarily due to the uncertainties in the cytochrome *b* and flavoprotein regions of the respiratory chain. For the purposes of the discussion in this paper, the scheme of Fig. 1 adequately represents the plant respiratory chain.

Effect of Adenine Nucleotides on Alternate Pathway—The direct effect of ADP and ATP on the alternate pathway was examined with mung bean mitochondria. In the presence of 0.3 to 0.5 mM cyanide, the cytochrome pathway was inoperative and the alternate pathway maximally active. Addition of either ADP or ATP had little or no effect on the rate of respiration. Similarly, the function $g(i)$ describing inhibition of the alternate pathway by aromatic hydroxamic acids was not affected by ATP, ADP, or uncoupler (Fig. 2). Consequently, we conclude that ATP and ADP exert no control through regulation of alternate pathway activity.

Control by Energy State of Mitochondria—Changes in the energy state of mitochondria have been shown to change the kinetic parameters of some electron transport components (4). The differences in alternate pathway rate in State 3 and State 4 could arise from similar modifications of alternate oxidase rate constants. However, with mung bean mitochondria in State 3 the value of ρ was near zero in the absence of cyanide and one with cyanide. This suggested that the energy state is not a regulating parameter. A direct test of the importance of energy state was made by determining the value of ρ in the presence of sufficient cyanide to give a State 3 cytochrome pathway rate comparable to the normal State 4 rate (Fig. 3).

The results in Fig. 3 were obtained by adding small amounts of cyanide to the mitochondrial suspension prior to addition of substrate. Cyanide, even at the low levels used here, interfered with the stimulation of respiration by ADP. At very low levels of cyanide (~4 μM) the addition of ADP gave a normal stimulation of respiration, but the sharpness of the transition from State 3 back to State 4 was reduced. At higher cyanide (~8 μM), the ADP addition gave only a very transient stimulation followed by a much lower and slowly changing respiratory rate. In some preparations no stimulation at all could be obtained at the higher cyanide concentration. The rates in Fig. 3 are the initial rates measured immediately after ADP addition.

These experiments show that it is the cytochrome pathway rate (or rate-dependent parameters) rather than the energy state which determines ρ. In each case the value of ρ is shifted toward one by addition of cyanide, even though the levels of high energy intermediates or the membrane conformational state, or both, are expected to remain those of State 3.

Role of Interpathway Interactions—Interactions in which some component of an enzyme system affects the rate of a reaction in that system above and beyond the requirements of mass action have assumed an important role in biochemistry recently. The possible role of such interactions in the control of the alternate pathway needs to be considered.

In Fig. 3 the addition of cyanide to State 3 mung bean mitochondria not only reduced the cytochrome pathway rate but also altered the oxidation-reduction state of the cytochromes. Thus the control of the alternate pathway could be accounted for by modification of the alternate oxidase kinetic parameters by the oxidation-reduction state of the cytochromes. Since inhibition of the cytochrome pathway by antimycin also gave the maximal alternate pathway rate (Table 1) but caused the *a* and *c* cytochromes to become highly oxidized rather than reduced, the oxidation-reduction states of these cytochromes were obviously not regulatory parameters. The presently unsatisfactory state of our knowledge of the organization and interactions of the several independent flavoproteins and *b* cytochromes makes con-

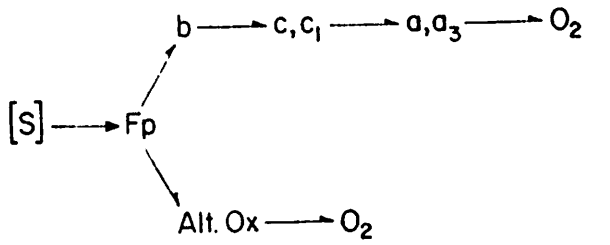

FIG. 1. Simplified plant respiratory chain. [*S*], any substrate; *Fp*, the complete set of flavoproteins; *a*, *b*, and *c*, the respective cytochromes; *Alt. Ox*, the alternate oxidase.

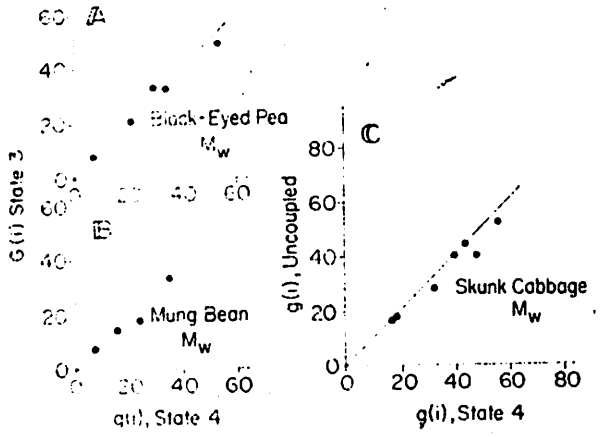

FIG. 2. Effect of ADP, ATP, and uncoupler on the function $g(i)$. Each graph shows values of $g(i)$ in State 3 or uncoupled mitochondria compared to $g(i)$ in State 4 mitochondria. State 3 was obtained by addition of 180 μM ADP to mitochondria oxidizing succinate (7.0 mM). State 4 was without ADP. The uncoupler used was bis(hexafluoroacetonyl)acetone, "1799," at 6 μM; supplied by Dr. Peter Heytler, E. I. DuPont de Nemours Inc., Wilmington, Del. M_w, mitochondria.

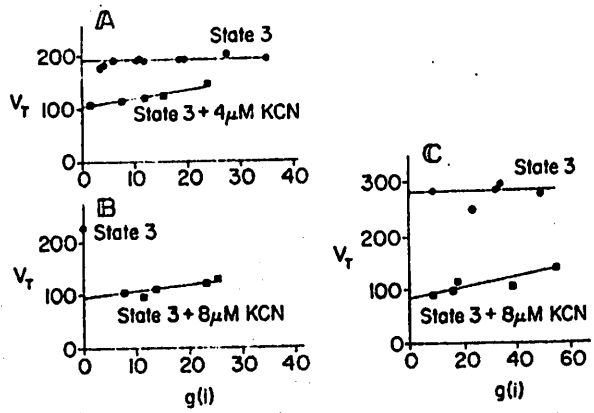

FIG. 3. Effect of small amounts of cyanide on the alternate pathway rate. V_T is the total rate of electron transport in nanoatoms of O_2 per mg of protein per min. Experiments *A* and *B* are mung bean mitochondria; Experiment *C* is black-eyed pea mitochondria. Except for the presence of cyanide, experimental conditions were as described in the previous article (1).

TABLE I

Comparison of inhibition by cyanide and antimycin

Succinate concentration was 7.5 mM; malate, 30 mM. Uncoupler "1799" was at 6 μM. Cyanide was 0.3 mM and antimycin was 0.6 μg per mg of protein. Mitochondrial concentration is 0.3 to 0.4 mg of protein per ml.

Mitochondrial source	Substrate	Rate	
		With cyanide	With antimycin
		natoms oxygen/min/mg protein	
Mung bean[a]	Succinate	16	16
	Malate	14	12.5
Skunk cabbage	Malate + uncoupler	136	144
	Succinate	73.5	82.3
	Malate	165	168
	Succinate	208	233
	Succinate	173	176
	Malate	132	116

[a] Data taken from Ikuma and Bonner (12).

sideration of their role impossible. In light of the absence of evidence for such interactions in other mitochondrial systems, this mechanism for control does seem unlikely. Further evidence on this mechanism would seem to require a method of inhibiting the cytochrome pathway between a particular cytochrome *b* and a particular flavoprotein, a fierce requirement.

DISCUSSION

The results of Fig. 3 clearly eliminate any direct involvement of the mitochondrial energy state in control of the alternate pathway and suggest that the flux in the cytochrome pathway is the determining parameter for the flux in the alternate pathway. The simplest mechanism consistent with these results would be a competition between the alternate oxidase and the cytochrome pathway for a limited quantity of reducing equivalents at the branch point.

The observation that the cytochrome pathway was always maximally active and the alternate pathway only partially active or inactive (1) makes a simple competition model untenable. If the two pathways must compete for equivalents from a single branch point carrier via irreversible reactions, then changes in the alternate pathway rate must affect the cytochrome pathway rate. If the reactions are irreversible, this result cannot be avoided no matter what values are assigned to the rate constants (7). Modification of the scheme of electron transport by introduction of additional irreversible reactions, such as in Fig. 4, fails to solve this difficulty. All such mechanisms fail because in each case the modification of the alternate pathway rate should lead to changes in cytochrome pathway rate, contrary to observation.

The above difficulties can be overcome by assuming that the branch point consists of at least two separate components in equilibrium with each other. Each pathway receives its reducing equivalents from a separate one of the equilibrated carriers (Fig. 5). The E'_0 values of the carriers would be such that under conditions where A is partially or fully oxidized, B would remain nearly fully reduced. In skunk cabbage mitochondria (Table II), in the presence of either cyanide or a benzhydroxamic acid, the A ⇌ B pool would be fully reduced and the maximum rates for the uninhibited pathway would be obtained. In the absence of inhibitors, B would remain fully reduced, and A would be partly oxidized owing to the increase in capacity of the system to

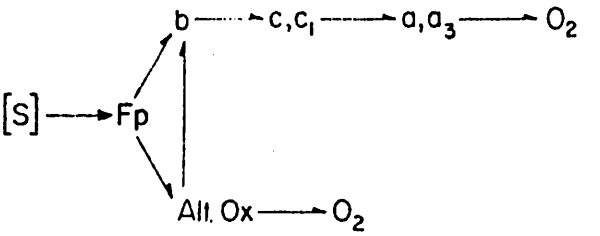

FIG. 4. Possible modification of electron transport scheme. The equivalence of antimycin- and cyanide-insensitive respiration (Table I) does not allow direct reactions between the alternate oxidase and *a* or *c* cytochromes. Symbols are the same as in Fig. 1.

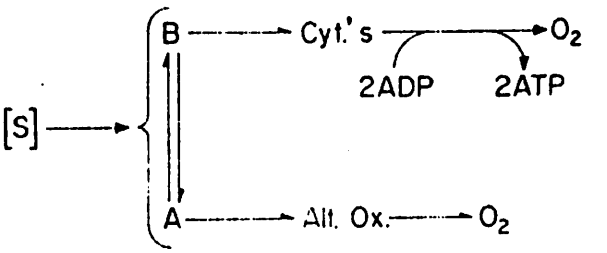

FIG. 5. Mechanism of control of the alternate pathway of higher plant mitochondria. See text for explanation.

TABLE II

Oxidation-reduction states of branch-point components A and B in skunk cabbage mitochondria

Steady state	Reduction A	Reduction
	%	%
+ KCN	95–100	95–100
+ Benzhydroxamic acid	95–100	95–100
State 3	55	95–100
State 4	65	95–100

oxidize A. Although B can also be oxidized faster without inhibitors, its oxidation-reduction state is held reduced by the requirements of the equilibrium reaction with A. The fractional reduction of A is given by the value of ρ. The increased capacity of the system to dispose of equivalents arriving at the A-B pool from the substrate in State 3 compared to State 4 results in a decrease in the fractional reduction of A and consequently in a decrease in ρ.

The same mechanism also explains the behavior of mung bean and black-eyed pea mitochondria. In State 4 the total rate is given by the sum of the maximum cytochrome pathway and alternate pathway rates. This requires that the rate-determining reactions lie beyond the branch point in each pathway. The reactions of the flavoprotein region proceed rapidly enough to maintain the branch point components fully reduced. Addition of ADP increases the cytochrome rate 4- to 6-fold. This is much greater than in skunk cabbage mitochondria and, therefore, we expect that the new oxidation-reduction state of A will be even more oxidized. Apparently, this increase is such that the branch-point component A becomes so highly oxidized that the alternate pathway rate approaches zero. The branch-point component B remains sufficiently reduced to give the maximum possible cytochrome pathway rate. Table III shows the critical difference between mung bean-black-eyed pea mitochondria and skunk cabbage mitochondria—the ratio of the cytochrome and alternate pathway maximal rates. In skunk cabbage the alternate path-

way activity is such that in State 4 it is still able to keep the A component of the branch-point partly oxidized and does not, therefore, obtain its maximum rate unless cyanide is added. In the beans, the activity of the alternate oxidase is low enough so that the branch-point component A remains fully reduced. The stimulation by ADP in bean mitochondria increases the rate of the cytochrome pathway so greatly that the branch-point component A becomes fully oxidized. The effects of ADP in skunk cabbage are not sufficient to do this. An additional difference in the tissues, not readily apparent in Table III, may lie in the maximal rates of the substrate to branch-point region of the respiratory chain.

From the value of ρ in skunk cabbage mitochondria in State 3, the difference between the midpoint potentials of A and B can be calculated. The A component will be 55% reduced and the B component 95% or more reduced. Using a value of 95% reduced for B in the Nernst equation gives a potential difference of 35 mV for 2-electron carriers, with B more positive. This represents a minimal value, since 95% reduced is a minimal value for the B component.

The generalization that metabolic control is accomplished through irreversible reactions only has gained some popularity. Up to a point, such a generalization appears valid. Yet, as pointed out recently by Krebs (8) and further shown here, equilibria do play a role in control. In the systems discussed by Krebs, a particular pathway was regulated by the level of its initial substrate, as determined by an equilibrium existing between that substrate and some other metabolite(s). For example, liver gluconeogenesis from lactate was regulated by the level of pyruvate. The pyruvate level was, in turn, regulated by the equilibrium between it, lactate, NAD^+, and NADH.

In the control system in plant mitochondria, the equilibrium regulates the relative rate of two competing pathways, by virtue of their "substrates" being in equilibrium. Although in the mitochondrial system the cytochrome pathway must always function if the alternate pathway is to operate at all, this "one-sidedness" of the control can be overcome in other systems. The postulate of equilibrium between components of the respiratory chain in the flavoprotein-cytochrome *b* region is consistent with the most recent results and theories of electron transport (5).

TABLE III

Ratios of maximum fluxes of succinate oxidation in plant mitochondria

Data are summarized from tables in previous paper (1).

Mitochondrial tissue	V^{3}_{cyt}/V_{alt}	V^{4}_{cyt}/V_{alt}	Respiratory control
Mung bean	4.0-6.5	1.0-1.1	4-6
Black-eyed peas	4.5-5.5	0.9-1.1	4-5
Skunk cabbage	0.7-0.8	0.4-0.5	1.7-2.3

APPENDIX

Homogeneity of Respiratory Chain Population—The interpretation of the titration results of the previous paper and the discussion in this paper contain the assumption that all the respiratory chains contained both the cytochrome and alternate pathways. As this assumption is not obviously certain, direct evidence was sought. If each set of dehydrogenases-flavoproteins had equal access to both pathways, then the extent of oxidation of flavoprotein in the presence and absence of cyanide would be equal. Any flavoprotein not accessible to the alternate pathway would not be oxidized in the presence of cyanide. Table IV pesents data taken from the kinetic studies of Storey (9) and Erecinska and Storey (10) plus an experiment of our own showing that the flavoprotein and ubiquinone optical changes are nearly the same in the presence and absence of cyanide. The data require that no more than 10% of the respiratory chains could lack the alternate pathway. Such a

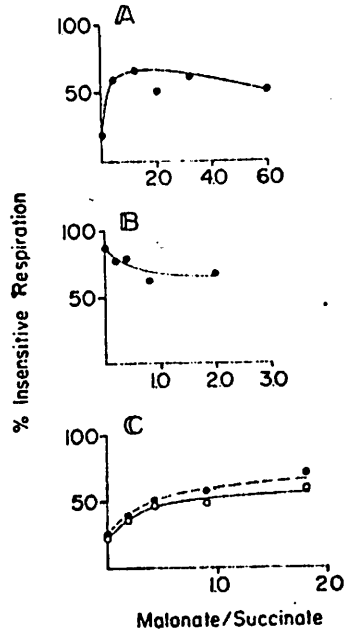

FIG. 6. Effect of increasing malonate concentration on cyanide sensitivity of mung bean and skunk cabbage mitochondria. *A*, mung bean mitochondria; *B*, skunk cabbage mitochondria; *C*, comparison of cyanide (●) and antimycin (○) sensitivity. Succinate concentration was 7.5 mM; cyanide, 0.3 mM; and antimycin, 0.6 μg per mg of protein. Mitochondria were uncoupled with 6 μM "1799."

TABLE IV

Optical density changes due to flavoprotein and ubiquinone in the presence and absence of cyanide

Flavoprotein was measured at 468 to 488 nm and ubiquinone at 282 to 295 nm in a regenerative stopped-flow apparatus.

Mitochondrial source	Component	ΔOD × 10^3		Source of data
		No KCN	With KCN	
Skunk cabbage	Flavoprotein	9.1 ± 0.3	8.5 ± 0.3	Erecinska and Storey (10)
Skunk cabbage	Flavoprotein	0.84 ± 0.04	0.92 ± 0.06	Our own
Mung beans	Flavoprotein	2.3 ± 0.2	1.9 ± 0.2	Storey (9)
Mung beans	Ubiquinone	5.3 ± 0.6	5.3 ± 0.6	Storey (9)

situation would have no effect on the value of ρ reported in the previous paper.

A second approach to the problem of homogeneity is to reduce the input of reducing equivalents to the respiratory chain so that the input is rate-limiting. Under such conditions the percentage of cyanide-sensitive respiration should decrease and approach a limiting value determined by the percentage of respiratory chains lacking the alternate pathway. In Fig. 6*A*, the input of reducing equivalents to the mung bean respiratory chain was reduced by increasing the concentration of malonate, a competitive inhibitor of the substrate succinate (11). The cyanide-insensitive respiration approached a limiting value of 60 to 70% of the total. Fig. 6*B*, showing similar data for skunk cabbage mitochondria, indicates that the residual 30 to 40% cyanide sensitivity was not due to respiratory chains lacking the alternate pathway. The same result was obtained, but the original sensitivity was only 13%. The original 13% sensitivity could not be obtained if 30 to 40% of the respiratory chains lacked alternate pathways. Fig. 6*C* compares the cyanide and antimycin sensitivities with increasing malonate concentration. As indicated in Table I, cyanide- and antimycin-insensitive respiration are equivalent. The residual sensitivity to those inhibitors was the same, suggesting that at high malonate the rate-limiting step requires energy generated by the cytochrome pathway and not available in the presence of cytochrome pathway inhibitors.

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