

INHIBITION AND STIMULATION OF THE RESPIRATION OF *ARUM* MITOCHONDRIA BY CYANIDE AND ITS RELATION TO THE COUPLING OF OXIDATION AND PHOSPHORYLATION

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SUMMARY

Mitochondria prepared by standard methods from the sterile spadix of *Arum maculatum* show relatively poor coupling (ADP/O = 0.5-1.0, respiratory control ratio = 1.5-2.8). This coupling is, however, crucial to the response elicited by the application of cyanide. Mitochondria oxidizing malate are inhibited by cyanide with an apparent K_i of 0.3 mM, but an infinitely high cyanide concentration would produce only 50-60% inhibition. With succinate, similar high concentrations of cyanide cause substantial (30-50%) stimulation of the rate of oxidation. When the mitochondria are completely uncoupled by the presence of dinitrophenol or by freezing and thawing, the application of cyanide causes a 12-40% stimulation of the rate of malate oxidation and an even greater increase in that of succinate oxidation. This stimulation by cyanide is not dependent on the nature of the cation supplied.

The inhibitor of cyanide-resistant respiration, *m*CLAM,† shows an inhibition additive to that of cyanide with coupled mitochondria, with an apparent K_i of 12-40 μ M and a maximal inhibition of 40-80%, but in the presence of DNP the addition of *m*CLAM completely eliminates the cyanide-sensitive oxidation of succinate or malate.

These results indicate the presence of two pathways for electron transport in *Arum* mitochondria, of which one, the cyanide-resistant pathway, is more readily available to the dehydrogenase of succinate than to that of malate and those of other substrates using DPN. The uncoupling of phosphorylation appears to permit electrons to flow more readily through the resistant pathway from either malate or succinate. The cyanide-sensitive pathway appears to be inhibited readily by *m*CLAM, and the fact that the stimulation of oxidation by KCN is abolished by *m*CLAM indicates that the resistant pathway is responsible for the increased flow of electrons in the presence of cyanide.

INTRODUCTION

The resistance of the respiration of higher plants to cyanide first observed by Genevois (1929) and extended by Van Herk (1937) to aroids, is now known to be widely, although unevenly, distributed among plants. It is frequently found that even cyanide-sensitive plants do have a small residual rate of respiration (James, 1953). The oxidation of cytochrome *c* by cytochrome oxidase (cytochrome *a*,*a*₃), which is thought to be the primary point of cyanide inhibition, sometimes continues slowly in plant mitochondria even in

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† The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BSA, bovine serum albumen; dieca, diethyldithiocarbamate; DNP, 2,4-dinitrophenol; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; *m*CLAM, *m*-chlorobenzhydroxamic acid; TES, N-tris(hydroxymethyl)-methyl-2-amino ethane sulfonic acid; TPP, thiamine pyrophosphate; tris, N-tris(hydroxymethyl)-amino methane.

the presence of concentrations of cyanide which would completely inhibit cytochrome oxidase in non-particulate preparations (Chance and Hackett, 1959; Bonner and Bendall, 1968).

At the other end of the scale of cyanide sensitivity, a number of plants, particularly aroids, have a respiratory activity which is partially, or even on occasion completely, insensitive to the presence of cyanide. In such cyanide-resistant plants it is generally agreed (Bendall and Bonner, 1971) that an alternate pathway leading electrons from substrate to oxygen must be available. This alternate pathway has been variously characterized as depending on leakage through the partially inhibited cytochrome oxidase (Yocum and Hackett, 1957), on the presence of a cyanide-insensitive cytochrome (Bendall and Hill, 1956) or on the use of flavoprotein (James and Beevers, 1950) or non-heme iron (Bendall and Bonner, 1971) enzymes as terminal or intermediate oxidases. All of these hypotheses have encountered strong objections (Bendall and Bonner, 1971) and in spite of extensive work using the techniques of rapid reaction kinetics, selective inhibition, cross-over studies and more recently, electron spin resonance, no clear delineation of the cyanide-resistant pathway in plant mitochondria is available. The existing evidence indeed could perhaps best be interpreted by the assumption that several different resistant pathways exist.

Associated with the phenomenon of cyanide resistance in plant mitochondria is the surprising observation that cyanide can under some circumstances cause a substantial increase in the rate of respiration of some cyanide-resistant plants. A few scattered mentions of stimulation by cyanide of the respiration of aroids are found (Hackett, 1957; Bendall, 1958), but the most extensive and unequivocal reports of stimulation by cyanide and other heavy-metal complexant-type inhibitors such as azide and dieca have come from work with potatoes (Hanes and Barker, 1931) and with the root-fungus symbiotic system which comprises the mycorrhizal roots of the beech tree (Harley *et al.*, 1956; Harley and ap Rees, 1959). In this latter system the cyanide resistance appears to be primarily associated with the fungal sheath surrounding the roots and cyanide-resistant respiration constitutes roughly one-half of the total activity of freshly dug roots. However, when these mycorrhizal roots are aged by incubation in aerated water, they develop the ability to respond to the application of cyanide with a several-fold increase in respiratory rate. This stimulating reaction persists for periods of a week or longer if the roots are maintained in aerated water.

It was with the hope of furthering the understanding of this stimulation of respiration by cyanide that the work with *Arum* mitochondria reported here was undertaken.

MATERIALS AND METHODS

The tissues used were the spadices of *Arum maculatum* L. growing in the wild in a shaded location on Headington Hill, Oxford. Inflorescences were collected over a period of 6 weeks beginning in the middle of April 1972, and included stages of development ranging from α to ε according to the classification of James and Beevers (1950). Most of the data reported here were obtained toward the end of this period and the samples used comprised primarily inflorescences in the δ stage, but included a small percentage in the γ or ε stages. Inflorescences which appeared to have been open more than a few hours were discarded. Excised inflorescences were placed in a plastic bag and taken immediately to the laboratory, where the spathe was removed. The club-shaped sterile terminal portion of the spadix was cut off above the constriction at its base and placed in grinding medium

at 4° C to chill before beginning the preparation of mitochondria. All subsequent operations were carried out at 1–4° C.

Mitochondria were extracted by grinding spadices (usually 12.5 g fresh weight) in twice the volume of a grinding medium consisting of 0.3 M sucrose, 1 mM EDTA, 0.05% cysteine and 0.1% BSA. The BSA was added just prior to grinding, which was done with the use of a mortar and pestle. The pH of the medium was initially adjusted to 7.2 and this pH was maintained during grinding by frequent checks of pH using narrow-range indicator paper and additions of dilute KOH. The triturated suspension was squeezed through nylon bolting cloth (no. 600 with 20 μm openings, obtained from Marine Biological Laboratory, Citadel Hill, Plymouth). Starch and cell debris were removed by a 5-minute centrifugation at 600 $\times g$. The supernatant liquid was removed and again centrifuged for 5 minutes at 19,000 $\times g$. The resultant mitochondrial pack was resuspended with the aid of a glass homogenizer in a volume of washing medium (0.3 M sucrose, 1 mM EDTA, 0.1% BSA, pH adjusted to 7.2) equal to that of the grinding medium used initially. The mitochondria were again spun down at 19,000 $\times g$ for 5 minutes. They were then suspended in a small volume—usually 1 ml—of 0.3 M sucrose, 10 mM KH_2PO_4 , 10 mM KCl, 5 mM MgCl_2 , pH 7.2 with the aid of a homogenizer. This mitochondrial suspension contained 23–38 mg protein ml^{-1} .

Oxygen uptake was measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) in a magnetically stirred sample chamber maintained at 25° C. Preliminary studies using the reaction medium recommended by Bonner (1967) showed that the relatively weak buffering capacity of 10 mM KH_2PO_4 was incompatible with studies of inhibition by KCN, because if the pH of the inhibitor solution is adjusted too near neutrality, variable quantities of cyanide are lost. It was found that TES buffer, unlike tris-Cl, had no effect on the response of the mitochondria to substrate, ADP or inhibitors. Accordingly, a reaction medium was used which contained 0.25 M sucrose, 50 mM TES, 8.3 mM KH_2PO_4 and 4.2 mM MgCl_2 . The pH was adjusted to 7.2 and measurements indicated no detectable alteration in pH as a result of any of the additions made in these studies. A total volume of 1.2 ml of reaction medium was used for measurement of oxygen uptake. Substrates were added at a concentration of 4.2 mM. With α -ketoglutarate it was found necessary also to add 0.4 mM TPP to obtain maximal rates of oxidation. In addition to substrates, ADP and ATP were added at 0.21 mM except when ADP/O ratios were being determined, when 83 μM was used. Respiratory rates are calculated on the basis of 258 μM O_2 in the reaction medium and are expressed as nmoles O_2 minute^{-1} mg protein^{-1} . Appropriate corrections to rate due to dilution were made in calculating rates after various additions.

The chemicals used were all of the highest purity available. The sucrose, cysteine and KCN were from British Drug Houses, Ltd. The *m*CLAM (Schonbaum *et al.*, 1971) was synthesized and kindly provided by Mr R. G. Powell of the Agricultural Research Council Unit of Developmental Botany, Cambridge. Since in preliminary experiments the use of ethanol as a solvent for *m*CLAM appeared undesirable, it was instead used as a water solution saturated at 20° C, which was determined to be 3.96 mM. All other chemicals were from Sigma Chemical Co.

Mitochondrial protein concentrations were determined by the Lowry method (Layne, 1957).

RESULTS

Arum mitochondria prepared and assayed by the methods described were quite active and

showed a reasonable, although low, degree of coupling as measured by ADP/O and respiratory control ratios. The ADP/O values ranged from 0.5 to 1.0 with succinate or malate as substrates, and the respiratory control ratios are well exemplified by those given in Table 1, although ratios as high as 5 were occasionally obtained particularly with mitochondria from spadices gathered early in the blooming period. These values are in agreement with those recently reported by Passam and Palmer (1972).

The response of *Arum* mitochondria to several substrates of the Krebs cycle and the inhibition achieved with cyanide are summarized in Table 1. These data represent determinations made late in the season, and there is some reason to believe from our work and from that of Simon (1957) and Bendall (1958) that the degree of respiratory control and the amount and even the nature of the response to cyanide may differ in less mature spadices.

It may be seen from Table 1 that isocitrate, malate and α -ketoglutarate, all substrates which presumably pass electrons to the same flavoprotein through DPNH, show rates of oxidation which are similar, although not identical, but which differ greatly from the rate with exogenously added DPNH. This probably indicates the presence of some form of 'external' DPNH oxidase (Passam and Palmer, 1972). These three substrates using pyridine nucleotide dehydrogenases all display about the same degree of respiratory

Table 1. *Respiratory rates, respiratory control ratios and inhibition by cyanide of the oxidation of various substrates by Arum mitochondria (rates are nmoles O₂ minute⁻¹ mg protein⁻¹ before the addition of ADP; assays as described under Methods, all substrates were 4.2 mM)*

Substrate	Rate	Respiratory control ratio	Plus 4.2 mM KCN (percentage of state 3 rate)
Malate	75	2.76	63.1
Succinate	122	2.39	162.2
α -Ketoglutarate	92	2.05	2.5
DPNH	416	1.50	23.9
Isocitrate	53	1.87	2.4

control, as does succinate. On the other hand, with DPNH there is somewhat less response to the addition of ADP, and that again may be related to its oxidation by a different, although still coupled, pathway.

Inhibition of the oxidation of the several substrates by 4.2 mM KCN is variable, with α -ketoglutarate and isocitrate being most strongly inhibited and DPNH and malate occupying an intermediate position with respect to inhibition. Succinate oxidation, however, is qualitatively different in its response to the addition of KCN, being stimulated about 60%. It seems clear that if cytochrome oxidase is completely inhibited by this concentration of cyanide (the high level of 4.2 mM was chosen to insure complete inhibition) then the dehydrogenase systems for malate and DPNH at least must have access to some other, cyanide-insensitive pathway. If, as will be shown later, the stimulated respiration with succinate in the presence of cyanide is going via the resistant pathway, then electrons from succinate must reach this cyanide-insensitive system more readily than those from any of the other substrates. In the case of succinate one must also postulate that cyanide, by preventing flow of electrons through cytochrome *c* to oxygen, is releasing some restraint on succinate oxidation. This is most readily explained by assuming that the cyanide-resistant pathway is not coupled to energy conservation through ATP formation. This has generally been considered to be so, although recently Wilson (1970)

has stated that the cyanide-insensitive pathway of *Arum* mitochondria does display some coupling. An alternative explanation for the increased oxidation of succinate due to cyanide is that cyanide has more than one effect on the mitochondria, and this will be discussed later.

Because of the possibility that the relatively large amount of potassium ions added with KCN might have some effect on the observed response of mitochondrial oxidation to the addition of cyanide, comparisons were made of the relative influence of Na^+ , K^+ and tris^+ on oxidation and inhibition by cyanide. The results are shown in Table 2, where it may be seen that there is little apparent difference between Na^+ and K^+ when these are the sole cations other than TES^+ added prior to inhibition by KCN. It is of interest that tris^+ results in a lower initial rate on the addition of malate, a higher respiratory control ratio, and somewhat less inhibition by cyanide. When higher concentrations of tris (50 mM) were used, the initial rate was reduced to about 1/4 of the values shown in Table 2, the respiratory control was abolished and the addition of KCN caused a stimulation (about 60%) in the oxidation of malate. These results led to the choice of TES buffer, since this showed no effects on any of the measured parameters, and to the design of a standard assay in which both Na^+ and K^+ were present.

Table 2. *Influence of cations on the oxidation of malate by Arum mitochondria and inhibition by cyanide (all samples contained 50 mM TES plus the cations indicated at the concentrations required to bring 4.2 mM malic acid to pH 7.2; rates are nmoles O_2 minute $^{-1}$ mg protein $^{-1}$ before the addition of ADP; assays performed as described under Methods)*

Cation	Rate	Respiratory control ratio	Plus 4.2 mM KCN (percentage of state 3 rate)
Na^+	91	1.80	46.1
K^+	83	1.87	57.9
Tris^+	57	2.58	66.3

While comparisons of inhibition by cyanide of the oxidation of different substrates at a constant level of added cyanide such as those given in Table 1 are of interest, great weight cannot be attached to them because the different levels of inhibition at a constant inhibitor concentration may reflect differences in the affinity of the inhibitor for the systems oxidizing the various substrates. More information regarding the interaction of an inhibitor with a substrate can be obtained from experiments in which the concentration of inhibitor or substrate or both are varied. Preliminary experiments in which both inhibitor and substrate concentrations were varied revealed that inhibition by KCN of malate oxidation was—as would be expected—non-competitive or substrate indifferent (Webb, 1963). With non-competitive inhibition it is only necessary to vary the concentration of inhibitor to obtain values for the apparent inhibition constant (K_i) and the maximal inhibition possible at infinite inhibitor concentration.

A plot of the reciprocal of the fraction of state 3 (Chance and Williams, 1956) malate oxidation inhibited by cyanide (inhibited fraction = $i = 1 - \frac{v_i}{v_o}$, where v_i = rate in the presence of inhibitor and v_o = rate before adding inhibitor) against the reciprocal of the concentration of added KCN (Webb, 1963) is shown in Fig. 1. Plots of this type with

simple, single-site inhibitors are characteristically linear. It may be seen in the upper line of Fig. 1 that the inhibition of malate oxidation by KCN produces a clearly curvilinear line. This indicates the probability that at least two different types or locations of inhibition are involved, with widely differing inhibition constants. If we assume, perhaps unjustifiably, that the KCN concentrations used here have included the inflection point between two different types of inhibition by cyanide, we can obtain an estimate of K_i for that form of inhibition displayed at low KCN concentrations by extrapolating a line connecting the two points at the right of the graph and a K_i for the less sensitive type of inhibition by extrapolating a line connecting the two points on the left. Using this questionable procedure, we obtain a K_i of 0.212 mM for the high affinity site (the one inhibited by low KCN concentrations) and a K_i of 0.899 mM for the site with lower affinity. This four-fold difference, while only an approximation, does seem to point toward the existence of two different sites for cyanide inhibition with different affinities for the inhibitor. Similar indications of the existence of more than one site for cyanide inhibition have been obtained earlier (Bendall and Bonner, 1971). The same process of

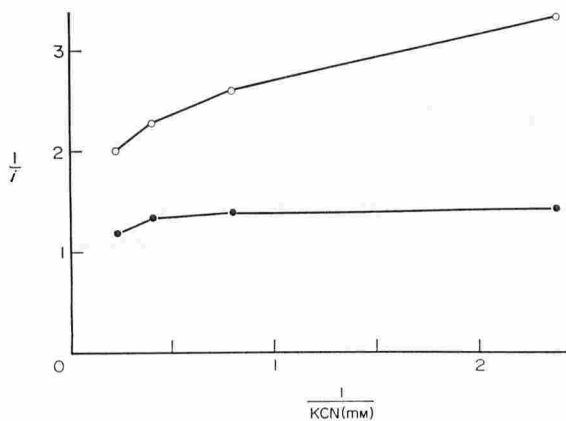


Fig. 1. Plot according to Webb (1963) showing effect of cyanide on oxidation of 4.2 mM malate by *Arum* mitochondria before (O) and after (●) addition of 0.33 mM *m*-chloro-benzhydroxamic acid. Assays were performed as described under Methods.

extrapolation which produces the estimates of K_i also yields approximations of the maximal inhibition (i_{\max}) of the two systems, and here it appears that the high affinity site could account only for about 45% of the total respiration ($i_{\max} = 0.446$) and the site inhibited by higher concentrations could perhaps add another 15% inhibition ($i_{\max} = 0.607$). Of perhaps most interest is the indication that at least 40% of the malate oxidation in these mitochondria is completely resistant to cyanide.

Various benzhydroxamic acids have been shown (Schonbaum *et al.*, 1971) to be highly selective inhibitors of the cyanide-resistant electron pathway in skunk cabbage and mung bean mitochondria. This inhibition is expressed without effect on either the respiratory pathway through cytochrome oxidase or on the energy coupling reactions of the mitochondria. Although various benzhydroxamic acids varied in their ability to inhibit the cyanide-resistant respiration of mitochondria, the *m*-chloro-substituted benzhydroxamic acid was among the most effective. The second line in Fig. 1 represents a plot of the inhibition of malate oxidation occasioned by the addition of 0.33 mM *m*CLAM to the mitochondria already inhibited by various concentrations of KCN. Here again, there is

some indication of non-linearity of the plot, which probably reflects the existence of two cyanide-sensitive sites. Extrapolation of the line to the vertical axis indicates that this concentration of *m*CLAM would produce an additional 30% inhibition at infinite KCN concentration ($i_{\max} = 0.916$), and since 0.33 mM *m*CLAM is not saturating (see Fig. 2) this probably indicates that KCN and *m*CLAM together could inhibit completely the oxidation of malate.

We have also tested *m*CLAM as an inhibitor of malate and succinate oxidation in *Arum* mitochondria with the results presented in Fig 2. This plot, unlike that for KCN in

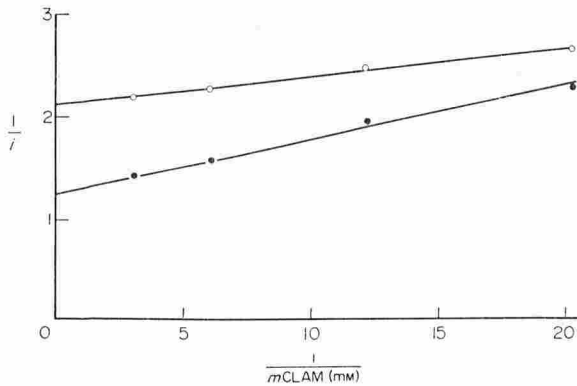


Fig. 2. Plot according to Webb (1963) showing effect of *m*-chlorobenzhydroxamic acid on oxidation by *Arum* mitochondria of malate (○) and succinate (●). Both substrates were supplied as Na⁺ salts at 4.2 mM. Assays were performed as described under Methods.

Table 3. *Inhibition by m-chlorobenzhydroxamic acid of the oxidation of various substrates by Arum mitochondria (all substrates supplied as Na⁺ salts at a concentration of 4.2 mM; all mitochondria were in state 3 with respiratory control ratios comparable with those of Table 1; assays performed as described under Methods)*

Substrate	$i = 1 - \frac{V_i}{V_0}$
Malate	0.457
Succinate	0.720
α -Ketoglutarate	0.449
DPNH	0.910
Isocitrate	0.506

Fig. 1, indicates a linear relationship between $1/i$ and the reciprocal of *m*CLAM concentration with both substrates. The mitochondria used in these studies were uncoupled by freezing and thawing, but other data indicate no significant differences between the response of these mitochondria and those in state 3 to *m*CLAM. With malate the K_i for *m*CLAM is 13 μ M, while with succinate it is 43 μ M. This indicates that the sensitivity of *Arum* mitochondria more nearly resembles that of mung bean mitochondria than of

mitochondria from skunk cabbage, since Schonbaum *et al.* (1971) report a K_i for mung bean with succinate of 0.03 mM for *mCLAM* as compared with 0.16 mM for skunk cabbage mitochondria. It is of interest that although malate oxidation is more sensitive to *mCLAM*, i.e. has a lower K_i value, than succinate oxidation, *mCLAM* only abolishes about one-half of the activity with malate ($i_{\max} = 0.474$) but reduces succinate oxidation by 80% ($i_{\max} = 0.806$). These differences presumably reflect different volumes of electron flow through the cyanide-sensitive and *mCLAM*-sensitive pathways with the two substrates and may relate to the different responses obtained with these substrates on the application of cyanide (Table 1 and Table 5).

Further comparisons of the *mCLAM* sensitivity of *Arum* mitochondria with different substrates are given in Table 3, where the fraction of the oxidation of various substrates inhibited by 0.33 mM *mCLAM* is given. Of particular interest here is the indication that DPNH oxidation is even more sensitive to *mCLAM* than that of other substrates. This implies that externally supplied DPNH is being oxidized primarily by a cyanide-resistant pathway. The three dehydrogenases using pyridine nucleotide, malate isocitrate and α -ketoglutarate appear to be about equally affected by *mCLAM*.

Table 4. *The effects of cyanide and m-chlorobenzhydroxamic acid on the oxidation of malate and succinate by coupled Arum mitochondria and by mitochondria uncoupled by 2,4-dinitrophenol and by freezing and thawing (all assays as described under Methods and included the addition of 0.21 mM ADP prior to measurement of initial rates)*

Substrate	Condition	Respiratory control ratio	Plus 4.2 mM KCN (percentage of initial rate)	Plus 0.33 mM <i>mCLAM</i> (percentage of initial rate)
Malate	Coupled	1.88	70.5	58.7
	Uncoupled by 60 μ M DNP		138.7	69.5
	Uncoupled by freezing	1.00	468.3	120.6
Succinate	Coupled	2.39	156.5	57.1
	Uncoupled by 60 μ M DNP		172.0	58.3
	Uncoupled by freezing	1.00	121.3	36.1

Although the data of Table 1 show that it is possible, at least in mitochondria obtained from mature spadices near the end of the flowering season, to stimulate the oxidation of succinate by adding KCN in high concentrations, only inhibition by cyanide has been observed with mitochondria in state 3 oxidizing malate. The fact that such coupling is required for inhibition by cyanide is indicated in Table 4, where the effects of cyanide and *mCLAM* on *Arum* mitochondria oxidizing malate and succinate when coupled and when uncoupled by DNP and by freezing and thawing are presented.

As in Table 1, coupled mitochondria oxidizing malate are inhibited by 4.2 mM KCN, in this case about 30%. The addition of 0.33 mM *mCLAM* brings about an additional 12% inhibition with these mitochondria. Coupled mitochondria oxidizing succinate are stimulated 56% and *mCLAM* represses this stimulated respiration and inhibits the rate an additional 43% as compared with the initial state-3 rate. When the malate oxidation is uncoupled by the addition of 60 μ M DNP prior to ADP addition, the further addition of 4.2 mM KCN results in a 40% stimulation of the rate of malate oxidation. Again, the

*m*CLAM results in abolition of the cyanide-stimulated rate and inhibition of about 30% as compared with the original rate. When the same mitochondria used for the coupled and plus DNP determinations are frozen for 2 days at -10°C and then thawed, they are found to be unresponsive to ADP, but cyanide now stimulates the oxidation of malate by about 3.5 times. In this case *m*CLAM reduces, but does not completely abolish, the stimulation due to cyanide. In the case of succinate oxidation, uncoupling with DNP causes an even greater stimulation by cyanide than in the coupled mitochondria, and *m*CLAM brings the rate down to about the same level as with the mitochondria in state 3. The frozen mitochondria are stimulated less by cyanide than when coupled and *m*CLAM causes an inhibition to a lower percentage of the initial rate.

From these results it appears that mitochondria uncoupled by either the presence of DNP or by freezing are altered in such a way that cyanide, while shutting off the cytochrome oxidase pathway, makes it easier for electrons to flow through the alternate, cyanide-resistant pathway. It is true that freezing appears to have more profound effects, increasing the stimulation by cyanide of malate oxidation, but reducing that of succinate oxidation and rendering the mitochondria more resistant to inhibition by *m*CLAM. It

Table 5. *Effects of cyanide and m-chlorobenzhydroxamic acid on the oxidation of malate by Arum mitochondria in the presence and absence of 2,4-dinitrophenol when supplied with ADP or ATP (assays as described under Methods, both ADP and ATP were supplied at 0.21 mM)*

Nucleotide added	60 μM DNP	Respiratory control ratio	Plus 4.2 mM KCN (percentage of initial rate)	Plus 0.33 mM <i>m</i> CLAM (percentage of initial rate)
ADP	—	1.87	62.1	39.9
ADP	+	—	142.9	82.1
ATP	—	1.99	95.5	71.9
ATP	+	—	102.6	62.8

nevertheless seems true that irrespective of how it is achieved, the effect of uncoupling is so to condition the mitochondria that the affinity of the cyanide-resistant pathway for electrons from malate or succinate is increased by the presence of cyanide. The fact that this stimulation of electron flow uses the cyanide-resistant pathway is shown by the abolition of stimulation plus additional inhibition by *m*CLAM in all cases except the frozen mitochondria, where structural changes in the mitochondria caused by freezing may have altered the pathways.

Although the data presented in Table 4 indicate an association of coupling of oxidation and phosphorylation with the inhibition or stimulation of malate oxidation by *Arum* mitochondria, they leave unanswered the question of whether the inhibition by cyanide of oxygen uptake by coupled mitochondria is related to the act of phosphorylation or to the product of that process, ATP. Some indication of an answer to this question is provided in Table 5 where the effects of cyanide on malate oxidation are determined in the presence and absence of DNP with either ADP or ATP supplied before cyanide addition.

Table 5 shows that ADP and ATP are about equally effective in supplying phosphate acceptor to the mitochondria, each giving about the same respiratory control ratio. Presumably this is due either to an impurity of ADP in the ATP or to the action of an ATPase in producing the necessary ADP to serve as an acceptor. When 4.2 mM KCN is added, we again see the phenomenon of Table 4, where mitochondria in state 3 are inhibited by cyanide while when they are uncoupled by 60 μM DNP, cyanide causes a 40% increase in

the rate of malate oxidation. A shift is also observed when ATP is supplied, but here both the inhibition and stimulation are so small as to be questionable. As before, the addition of *m*CLAM abolishes the stimulation due to cyanide when it occurs, and adds its own 20–30% inhibitory effect.

These results clearly indicate that the presence of ATP is not the aspect of the activity of coupled mitochondria which is responsible for the fact that cyanide inhibits oxidation of malate rather than stimulating it as with succinate. Instead, it must be that the process of phosphorylation is responsible for the difference between the coupled and uncoupled mitochondria in their response to cyanide. These data do raise another interesting question, since it appears that the stimulation by cyanide in uncoupled mitochondria depends on the presence of ADP, even in the uncoupled system. This conclusion arises from the fact that cyanide causes a 43% stimulation when ADP and DNP are present, but is essentially without effect on oxidation when ATP is supplied after DNP. This is true in spite of the fact that there appears to be sufficient ADP to bring about a respiratory control response, which leads to the conclusion that not only is ADP necessary for the cyanide stimulation, but that it must be present in amounts greater than the traces necessary to produce a respiratory control response.

DISCUSSION

Certain kinds of plant tissues have been shown to be resistant to cyanide and to other substances which would be expected to inhibit cytochrome oxidase. In some cases their respiration is even stimulated as in the case of beech mycorrhizas when treated with azide (Harley and McCready, 1953). In others, stimulation, although not observed in freshly isolated material, develops during ageing as with potato (Hanes and Barker, 1931) or with beech mycorrhizas (Harley *et al.*, 1956) when treated with cyanide. The explanation of these kinds of behaviour may involve hypotheses based on the effect of inhibitors on the balance of metabolic processes in the tissue or upon their effects directly upon mitochondrial activities or perhaps again upon a combination of both kinds of effect. The explanations of resistance to the inhibitor in the absence of stimulation have focused on the possibility of an alternate pathway for electron transport to oxygen which is not inhibited in the way cytochrome oxidase is expected to be. The explanation of stimulation in terms of an alternate pathway alone requires in addition further assumptions such as that it is normally rate limited in respect of transfer of electrons to oxygen by the functioning of the sensitive pathway. On the other hand, the work with beech mycorrhizas led to the suggestion that stimulation was a property of the cell and that the inhibition of a sensitive pathway coupled to phosphorylation allowed a stimulation of oxygen uptake through the resistant pathway by a process analogous to uncoupling. This was to some extent made more likely since in that tissue stimulation of oxygen uptake is associated with a greater increase of CO₂ emission.

In any event all hypotheses involve the existence of an alternate resistant pathway of electron transport but there is as yet no clear agreement as to its nature nor upon its relationship to the sensitive pathway. In this paper therefore attention has been focused on the properties of the mitochondria of a tissue well known to be cyanide-resistant in an attempt to understand the effects of cyanide on the mitochondrial system before examining further the properties of the cellular tissue.

The changes in rate of oxygen uptake by *Arum* mitochondria when treated with cyanide depend upon the substrate being oxidized. The sensitivity of oxygen uptake during

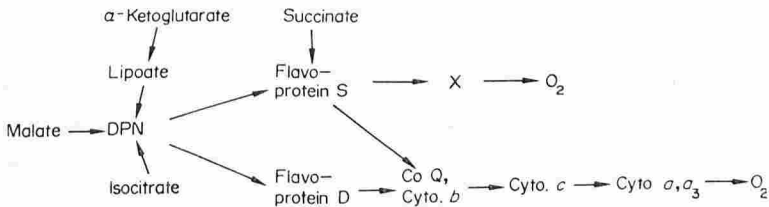
succinate oxidation as opposed to that of substrates which transfer electrons through DPN provides a clue to the point at which the resistant pathway branches off the sensitive one. In the present study oxygen uptake during succinate oxidation by mitochondria in state 3 was found to be stimulated by concentrations of cyanide as high as 4.2 mM while that during oxidation of substrates requiring pyridine nucleotide was inhibited when the mitochondria were coupled. Again oxygen uptake on addition of exogenous DPNH (which in plants is probably oxidized by a different route than DPN reduced by malate, isocitrate and α -ketoglutarate) is inhibited.

Simon (1957) and Bendall (1958) demonstrated changes in the relative sensitivity to cyanide of the oxidation of succinate and malate during spadix development. This might be due to changes in the accessibility of the alternate resistant pathway to electrons from the two substrates or to a change in the relative magnitude of one of the pathways as the spadix develops.

We have demonstrated that the oxidation of malate by mitochondria varies in its sensitivity to cyanide according to whether or not it is coupled with the phosphorylation of ADP. This adds additional complexity to the picture of cyanide resistance in *Arum*. It is arguable that the changing sensitivity to cyanide of the electron flow from succinate and malate during development of the spadix might reflect a natural sequence of uncoupling. The low ADP/O and respiratory control ratios which we and others describe supports the idea that at least mature *Arum* spadices are partially uncoupled.

The striking observations that the oxidation of malate or of succinate by uncoupled *Arum* mitochondria is stimulated by cyanide show that stimulation of oxygen uptake can be a property of mitochondrial activity. This stimulated oxygen uptake must be via a cyanide-resistant pathway and it is indeed abolished by *mCLAM*, an inhibitor of cyanide-resistant respiration in other aroids (Schonbaum *et al.*, 1971). In coupled mitochondria malate oxidation is partly inhibited by *mCLAM* and partly inhibited by cyanide. The inhibition by the two substances is additive. Sufficient *mCLAM* and cyanide completely abolish oxidation of either malate or succinate. Addition of *mCLAM* to mitochondria uncoupled by DNP oxidizing malate not only abolishes cyanide stimulation but additionally inhibits to about the same degree as *mCLAM* alone.

These facts suggest that electron transport in *Arum* mitochondria may proceed by branching pathways somewhat as shown in the following diagram.



The essence of this scheme is that electrons from flavoprotein S, the flavoprotein normally associated with succinate oxidation, can be transferred either to coenzyme Q, cytochrome *b* or to a cyanide-resistant pathway consisting of one or more steps to oxygen which we have designated simply as X. On the other hand, electrons from internal DPNH, and perhaps external DPNH as well, are normally passed to flavoprotein D. These electrons may also, somewhat less efficiently, be transferred to flavoprotein S, and thus to either the cyanide-resistant X or back into the cyanide-sensitive path. In this

proposal, the relative affinity of the electrons from DPNH for either flavoprotein D or flavoprotein S would be controlled by the process of phosphorylation. When the mitochondria are in state 3, electrons from malate have a low affinity for flavoprotein S—perhaps when completely coupled, the exclusion is complete. However, when the mitochondria are uncoupled, electrons are readily transferred from DPNH to flavoprotein S and thence to the cyanide-resistant pathway, X. Implicit in the greater stimulation of succinate oxidation by cyanide in uncoupled mitochondria is the idea that phosphorylation exerts a similar control over the affinity of electrons from flavoprotein S for either coenzyme Q, cytochrome *b* or for X. The sum of these two effects of phosphorylation would be that the uncoupled mitochondria oxidizing either malate or succinate would have both pathways available, and when the normal path through cytochrome oxidase is blocked by cyanide, the electron flow can continue through X to oxygen. The fact that this flow is stimulated by cyanide rather than being merely unabated requires either that the pathway through X must offer less resistance to electron flow, or that cyanide has an effect in addition to simply blocking cytochrome oxidase. The change from a lower to a higher K_i for cyanide at high KCN concentrations shown here (Fig. 1) may indicate that cyanide does have such an additional effect with the resulting stimulation of flow through the X pathway being expressed as a higher K_i for cyanide at high KCN concentrations.

The relation of the stimulation by cyanide of the oxidative activity of *Arum* mitochondria to the stimulation of the respiration of intact tissues by cyanide is not, however, fully explained by this hypothesis. We have shown (Wedding, McCready and Harley, 1973) that slices of *Arum* spadix aged 24 hours or more in aerated water develop the capacity to respond to cyanide addition by marked stimulation. In this they behave like beech mycorrhizas. It is clear that this could represent the response of uncoupled mitochondria to cyanide, but it seems unlikely that this is the explanation. Beech mycorrhizas responding in this way do not appear to be significantly uncoupled since they respond to nutrient addition, either salts or carbohydrates, by increasing oxygen uptake and nutrient absorption which may be related quantitatively. The nutrient absorption, but not oxygen uptake, is inhibited by DNP or by cyanide or azide. It therefore remains to investigate further the cyanide resistance of the respiration of these tissues by an examination of the inter-relations of glycolysis and other metabolic sequences with the respiratory response.

REFERENCES

- BENDALL, D. S. (1958). Cytochromes and some respiratory enzymes in mitochondria from the spadix of *Arum maculatum*. *Biochem. J.*, **70**, 381.
- BENDALL, D. S. & BONNER, W. D. JR (1971). Cyanide-insensitive respiration in plant mitochondria. *Pl. Physiol., Lancaster*, **47**, 236.
- BENDALL, D. S. & HILL, R. (1956). Cytochrome components in the spadix of *Arum maculatum*. *New Phytol.*, **55**, 206.
- BONNER, W. D. JR (1967). A general method for the preparation of plant mitochondria. In: *Methods in Enzymology*, Vol. 10 (Ed. by R. W. Estabrook & M. E. Pullman), p. 126. Academic Press, New York.
- BONNER, W. D. JR & BENDALL, D. S. (1968). Reversed electron transport in mitochondria from the spadix of *Arum maculatum*. *Biochem. J.*, **109**, 47p.
- CHANCE, B. & HACKETT, D. P. (1959). The electron transfer system of skunk cabbage mitochondria. *Pl. Physiol., Lancaster*, **34**, 33.
- CHANCE, B. & WILLIAMS, G. R. (1956). The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.*, **17**, 65.
- GENEVOIS, M. L. (1929). Sur la fermentation et sur la respiration chez vegetaux chlorophylliens. 3. Action du cyanure de potassium sur la reaction de Pasteur. *Revue gén. Bot.*, **41**, 259.
- HACKETT, D. P. (1957). Respiratory mechanisms in the aroid spadix. *J. exp. Bot.*, **8**, 157.
- HANES, C. S. & BARKER, J. (1931). The physiological action of cyanide. 1. The effects of cyanide on the respiration and sugar content of the potato at 15° C. *Proc. R. Soc., Ser. B*, **108**, 95.

- HARLEY, J. L. & AP REES, T. (1959). Cytochrome oxidase in mycorrhizal and uninfected roots of *Fagus sylvatica*. *New Phytol.*, **58**, 364.
- HARLEY, J. L. & MCCREADY, C. C. (1953). A note on the effect of sodium azide upon the respiration of beech mycorrhizas. *New Phytol.*, **52**, 83.
- HARLEY, J. L., MCCREADY, C. C., BRIERLEY, J. K. & JENNINGS, D. H. (1956). The salt respiration of excised beech mycorrhizas. II. The relationship between oxygen consumption and phosphate absorption. *New Phytol.*, **55**, 1.
- JAMES, W. O. (1953). *Plant Respiration*, p. 226. Oxford University Press, London.
- JAMES, W. O. & BEEVERS, H. (1950). The respiration of *Arum* spadix. A rapid respiration, resistant to cyanide. *New Phytol.*, **49**, 353.
- LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. In: *Methods in Enzymology*, Vol. 3 (Ed. by S. P. Colowick & N. O. Kaplan), p. 447. Academic Press, New York.
- PASSAM, H. C. & PALMER, J. M. (1972). Electron transport and oxidative phosphorylation in *Arum* spadix mitochondria. *J. exp. Bot.*, **23**, 366.
- SCHONBAUM, G. R., BONNER, W. D. JR, STOREY, B. T. & BAHR, J. T. (1971). Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Pl. Physiol., Lancaster*, **47**, 124.
- SIMON, E. W. (1957). Succinoxidase and cytochrome oxidase in mitochondria from the spadix of *Arum*. *J. exp. Bot.*, **8**, 20.
- VAN HERK, A. W. H. (1937). Die chemischen Vorgänge im Sauromatum-Kolben. *Recl Trav. bot. néerl.*, **34**, 149.
- WEBB, J. L. (1963). *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 158. Academic Press, New York.
- WEDDING, R. T., MCCREADY, C. C. & HARLEY, J. L. (1973). Cyanide sensitivity of respiration and ageing of *Arum* spadix slices. *New Phytol.*, **72**, 15.
- WILSON, S. B. (1970). Energy conservation associated with cyanide-insensitive respiration in plant mitochondria. *Biochim. biophys. Acta*, **223**, 383.
- YOCUM, C. S. & HACKETT, D. P. (1957). Participation of cytochromes in the respiration of the aroid spadix. *Pl. Physiol., Lancaster*, **32**, 186.

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