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CHANGING PATTERNS IN THE RESPONSE TO CYANIDE OF *ARUM* MITOCHONDRIA OXIDIZING MALATE AND OTHER SUBSTRATES

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Summary

Over a flowering season of 7 weeks, mitochondria from the spadices of *Arum maculatum* showed a progressive increase in the rate at which added malate was oxidized from less than 20 nmoles min^{-1} mg $protein^{-1}$ to more than 700 nmoles min^{-1} mg $protein^{-1}$. In the oxidation of succinate, α-ketoglutarate and other substrates, much smaller differences in rate were found.

Mitochondria oxidizing malate responded to the addition of high (3.8 mM) concentrations of cyanide by stimulation when their initial rate of malate oxidation was low and by inhibition when it was high. Stimulation by KCN even with high concentrations did not increase the O_2 uptake rate of young spadices to the level of mature ones.

Associated with this alteration in response to cyanide was a reverse response to ADP. The early, slowly respiring mitochondria which were stimulated by KCN were often inhibited by ADP while the mitochondria from later spadices which were inhibited by KCN were usually stimulated by ADP.

Similar variations in the relative rate of malate oxidation, response to cyanide and to ADP were observed in mitochondria prepared on the same day from spadices at different stages of development from the same location. In this case the change from young (β) to fully matured (ε) spadices approximately paralleled those from the early season to the end of the flowering period. The changes in malate metabolism of the spadix mitochondria were not solely related to stage of spadix development, however, since those from spadices at the same apparent developmental stage gathered over a 5-week period also showed an increase in the rate of malate oxidation and a change in response to cyanide and ADP.

Several types of evidence—effects of additional washing of the mitochondria, response to additions of oxalacetate and pyruvate, response to inhibitors of cytochrome oxidase other than KCN, assay of oxalacetate concentration of mitochondria, and application of an inhibitor of cyanide-resistant electron transport (mCLAM)—support the hypothesis that one of the factors in the mitochondria from young or early spadices which limits their ability to oxidize malate is oxalacetate within the mitochondria. As the spadix develops, mitochondrial oxalacetate is diminished or disappears. The stimulation of oxygen uptake in the early stages due to cyanide is thus thought to result from the reduction of the quantity of oxalacetate by cyanohydrin formation.

The inhibition by ADP is not clearly understood, but may result from the existence of other reactions which are subject to negative control by ADP and which have no relation to the role of ADP in oxidative phosphorylation or its coupling to electron transport.

Introduction

The sterile spadix of the inflorescence of *Arum maculatum* L. has long been known to

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have a respiration with a large cyanide-resistant component (James and Beevers, 1950). Investigations of cyanide-resistant respiration using this tissue have given results which are unexpectedly variable. Variations reported include widely different rates of oxidation of various substrates by *Arum* spadix mitochondria and variable responses to ADP, degree of phosphorylation associated with the cyanide-resistant pathway and effect of cyanide on mitochondrial metabolism (Bendall and Hill, 1956; Simon, 1957; Hackett, 1957; Bendall, 1958; Hackett, 1959; Bonner and Bendall, 1968; Wilson, 1970; Passam and Palmer, 1972; Wedding, McCready and Harley, 1973a, b).

The causes of this variation may be manifold. *Arum* in a single locality flowers over a period of several weeks and individual plants come into flower at different periods. Flowering is sensitive to microclimatic variations, particularly temperature and water supply. In addition there is clearly a considerable genetic variability in *A. maculatum* which is apparent in such characters as the size, shape and colour of the spadix, the size, shape and marking of the leaves and even the direction in which the spathe is rolled.

Regardless of the time of initiation the inflorescences go through phases of development which were characterized by James and Beevers (1950) on the basis of visual appearance of the inflorescence. The early newly emerged, tightly rolled spathe with small spadix was called α, and it passed through β, γ and δ stages, to the fully-open ε stage in which the spadix is about to wither. The length of this maturation period is variable, depending on conditions and possibly upon genetic variation but is frequently of about 2–3 weeks' duration.

It was recognized by James and Beevers (1950) that different stages of spadix development exhibited different characteristics. This was elaborated by Simon (1957) and Bendall (1958).

In a previous paper (Wedding *et al.*, 1973b) we confirmed Bendall's (1958) observation that slices of the spadix of *Arum* become less inhibited by cyanide as the organ matures. Indeed, it might be stimulated by cyanide in the mature state. Moreover, artificially aged slices pass into a condition where their respiration, although reduced in rate, is greatly stimulated by cyanide. Although such reactions and changes in tissue respiration are unlikely to be completely explicable in terms of mitochondrial activity, an investigation of the associated changes in mitochondria is an essential phase in their explanation.

During the *Arum* season of 1973 we attempted to follow the development of influences as reflected in the metabolic characteristics of mitochondria from the spadix. One of the most striking changes concerned the effects of cyanide on mitochondria utilizing malate as a substrate. This paper is therefore concerned primarily with these changes and their explanation.

MATERIALS AND METHODS

The spadices of *Arum maculatum* L. growing in the wild in a shaded location on Headington Hill, Oxford provided the material for these studies. The group of plants were the same as those used by James and Beevers (1950) and in our earlier study (Wedding *et al.*, 1973a, b). The first collection which provided data for this report was made on 13 April 1973 and the last on 25 May 1973. Inflorescences were collected immediately before use, since we observed that significant changes may occur in excised material even when stored at 4° C. The freshly harvested inflorescences were sorted into stages of floral development according to James and Beevers (1950). The spathes were then

removed, the sterile terminal portion of the spadix was cut off above the basal constriction and mitochondria were prepared as previously described (Wedding *et al.*, 1973a). As in the previous work, the mitochondria were washed once with a volume of washing medium (0.3 M sucrose, 1 mM EDTA, 0.1% BSA, pH 7.2) equal to twice the initial weight of spadices before grinding. Sometimes as indicated in the text, a second wash using an equal volume of the same medium was employed.

Oxygen uptake was measured as previously described with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 25° C in a reaction medium consisting of 0.25 M sucrose, 50 mM TES, 8.3 mM KH_2PO_4 and 4.2 mM $MgCl_2$, pH 7.2. Substrates were added at 4.2 mM, and, with α-ketoglutarate, 0.4 mM thiamine pyrophosphate was also supplied. ADP was added at 80 μM. Respiratory rates were measured from the recorder chart with a 12-in. protractor and were calculated with a Wang Model 720C computer program which assumed 258 μM O_2 in the reaction medium and made appropriate corrections for dilution due to substrate, cofactor or inhibitor additions. The rates are expressed as nmoles O_2 min^{-1} mg $protein^{-1}$, using protein concentrations determined by the Lowry method (Layne, 1957).

The chemicals used were of the highest purity available and except for sucrose, cysteine and KCN (which were supplied by British Drug Houses, Ltd) were all from Sigma Chemical Co. The mCLAM used was synthesized and provided by Mr R. G. Powell of the Agricultural Research Council Unit of Developmental Botany, Cambridge. This chemical, from the same source as that used previously (Wedding *et al.*, 1973a, b), was further purified by recrystallization from dimethylformamide. In use it was supplied as a solution (67 mM) in dimethylformamide. Preliminary studies showed that concentrations of dimethylformamide up to 2% of the total reaction medium had no significant effect on the activity of *Arum* mitochondria.

Oxalacetate was assayed using the malate dehydrogenase reaction. Samples of mitochondria containing 0.1–0.2 mg protein were disrupted in 80% ethanol and the particulate matter removed by centrifugation. The ethanolic extract was diluted tenfold with 0.1 M potassium phosphate buffer at pH 7.4. Oxalacetate was then determined by the addition of 100 units of porcine heart malate dehydrogenase and NADH to make 5 mM. The recorder deflection at 340 nm obtained after equilibrium was established as the basis for calculation of oxalacetate concentration. In each cuvette, a standard curve of oxalacetate concentration was obtained in the same reaction medium by successive additions of oxalacetate and determination of absorbence change at 340 nm.

Results

Changes in mitochondrial activity during development of the spadix

The effect of cyanide

A number of progressive changes in mitochondrial characteristics were observed, but the most striking related to the oxidation of malate and the response of this oxidation to the addition of ADP and KCN. One aspect is illustrated in Fig. 1, where the response to the addition of cyanide by mitochondria oxidizing malate is shown over the entire season. Standard runs were carried out at each sampling time in which the mitochondria, after the disappearance of any endogenous oxidation, were supplied successively with malate, ADP, KCN and mCLAM. The data presented in Fig. 1 are selected from these standard runs in the sense that only mitochondria which had been prepared by the same

method were included, and no preparations made from spadices at a stage less mature than one which had already been obtained were included. The trend in Fig. 1 therefore represents the seasonal development of the response of malate oxidation to cyanide distorted somewhat by distributing the usual developmental stages from α to ε over a longer time span than would occur with a single inflorescence. In a sense these are the results which would be obtained if one selected spadices from flowers only of the stage which was predominant at the times indicated. Fig. 1 shows that over the period from early April until late May, *Arum* spadix mitochondria undergo a steady transition from a condition in which the addition of 3.8 mM cyanide produces a substantial increase in the rate of malate oxidation to one in which it reduces the rate of malate oxidation by about one half. Table 1 shows that there is no comparable trend in the response of succinate or α-ketoglutarate oxidation to cyanide during the same period.

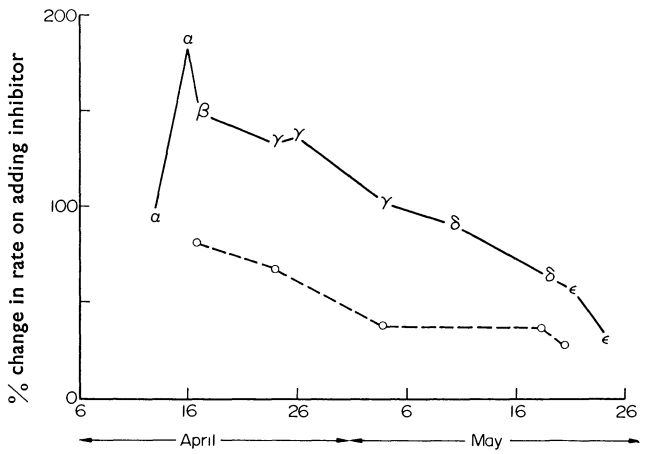

Fig. 1. Changes in the response to cyanide and azide of mitochondria prepared from *Arum maculatum* flowers over entire blooming period. All preparations were oxidizing 3.8 mM malate in the presence of 3.8 mM KCN or NaN_3. Greek letters indicate developmental floral stage (James and Beevers, 1950). Solid line represents effect of cyanide, dashed line effect of azide.

Related to this change in the sensitivity of malate oxidation to cyanide is a change in the initial rate of malate oxidation. This aspect is dealt with at greater length below, but from the same mitochondria illustrated in Fig. 1, the initial rate of malate oxidation rises from 28.1 nmoles O_2 min^{-1} mg^{-1} on 12 April to 758 nmoles O_2 min^{-1} mg^{-1} on 25 May. This increased rate is associated with the synthesis of new mitochondrial protein. The protein content of mitochondria per gram of spadix fresh weight increases from 1.19 mg g^{-1} for α stage spadices, to 1.50 mg g^{-1} for β stage spadices, to 1.99 mg g^{-1} for γ stage spadices while spadices at stage ε have 2.74 mg g^{-1} mitochondrial protein. Analysis of variance shows each of these values to be significantly different from all others at least at the 5% level. Over the same series of developmental stages the content of mitochondrial protein per individual spadix increases from less than 1 mg per spadix for the α stage spadices to more than 6 mg per spadix for those of ε stage. These figures indicate that during the change from stage α to stage ε the spadix increases both in total content of mitochondrial protein and in the concentration of such protein on a fresh weight basis. The large increase in oxidative rate may thus reflect, at least in part, the synthesis of additional enzymes concerned with oxidative metabolism.

The effect of ADP

It is also of interest that over the same period of spadix development the addition of ADP causes a 24% inhibition of the rate of malate oxidation in the mitochondria from α spadices, but stimulates the rate by about 24% in mitochondria from ε stage spadices. Indeed, in general ADP partially inhibits malate oxidation in the early part of the season and stimulates it later. While the trend from one extreme to the other over the season is less smooth for the initial rate of malate oxidation and for the effect of ADP on the process than that illustrated in Fig. 1 for the response to cyanide, there is a general change in the directions indicated. These points are dealt with in more detail below.

Table 1. *Initial rates of succinate and α-ketoglutarate oxidation by* Arum *spadix mitochondria and its sensitivity to cyanide at different times during the blooming season and at different developmental stages*

Week of blooming season*	Initial rate, nmoles O_2 min^{-1} mg^{-1}	Rate after addition of ADP as % of rate before	Rate after addition of 3.8 mM KCN as % of rate before‡
Succinate			
3	87.4	100.0	85.5
4	105.3	109.0	84.4
5	132.4	106.0	85.1
6	71.8	95.0	82.1§
7	430.6	107.0	53.3
α-Ketoglutarate			
3	162.6	140.0	112.8
4	229.8	139.0	108.7
5	226.2	153.0	110.4
6	225.2	145.0	112.1
7	217.4	139.0	133.7
Spadix stage†			
Succinate			
β	58.4	100.0	100.9
γ	132.4	106.0	77.6
δ	114.8	102.0	79.7
ε	492.5	104.0	86.6
α-Ketoglutarate			
β	141.9	157.0	116.2
γ	226.2	153.0	110.4
δ	212.0	147.0	116.7
ε	213.3	150.0	114.4

* All at stage γ.
† All during fifth week.
‡ ADP present at time of inhibitor addition.
§ Based on a single determination.

Further analysis of the effects of cyanide and ADP

That the stimulation of malate oxidation by mitochondria from young spadices early in the season is characteristic of cyanide rather than of inhibitors of cytochrome oxidase is shown by the lower line of Fig. 1; sodium azide at 3.8 mM consistently inhibits mitochondrial oxidation of malate over the same time period.

The association of stimulation of malate oxidation by cyanide with a low initial rate of malate oxidation is demonstrated in Fig. 2, where the rate after cyanide addition as a percentage of the rate in the presence of ADP is plotted against the logarithm of the uninhibited rate. Results from all comparable preparations are included and each point is

the mean of values from all replicate cyanide additions to mitochondria oxidizing malate made with a single preparation. Some points thus represent a single determination, while others are means of as many as six separate determinations. A least squares fit of these data gives a correlation coefficient, $r = -0.854$ and the equation for the line of best fit shows that there is a decrease of 85% in the response to cyanide for a tenfold increase in the initial rate. From this equation it can be calculated that mitochondria with an initial rate of malate oxidation of about 66 nmoles O_2 min^{-1} mg^{-1} are relatively unaffected by 3.8 mM cyanide.

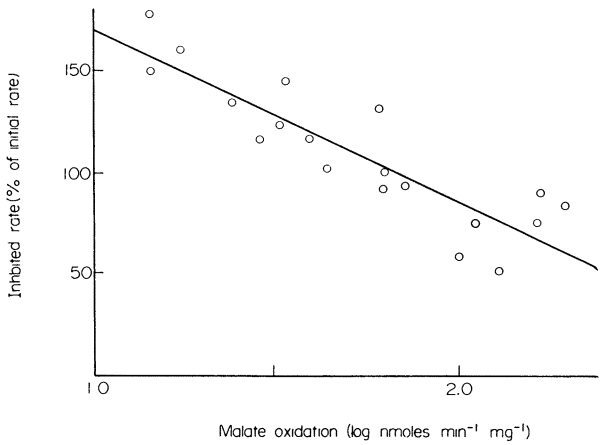

Fig. 2. Response of malate oxidation by *Arum* mitochondria to cyanide as a function of the rate of malate oxidation after addition of ADP. Line fitted by method of least squares.

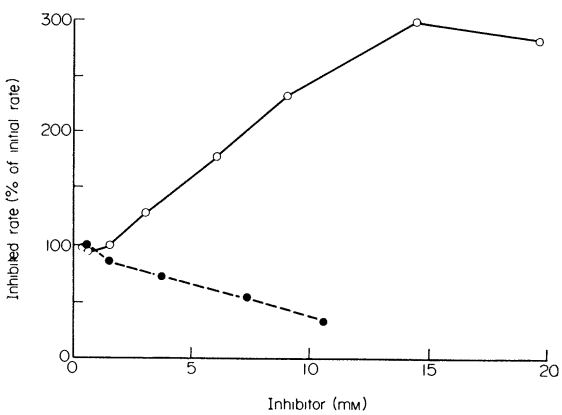

Fig. 3. Comparison of effects of cyanide and azide on the rate of malate oxidation by mitochondria from spadices of stages β and γ. Open symbols, cyanide; closed symbols, azide. Values obtained by successive additions of inhibitor to the same preparation of mitochondria.

The same set of standard malate runs shown in Fig. 1 also yields a significant, although weak, correlation of inhibition of rate by ADP with stimulation by cyanide and of stimulation by ADP with inhibition by cyanide (see also Fig. 4b, c).

Some indication of the quantitative as well as the qualitative difference in the rate of malate oxidation elicited by the addition of cyanide and azide can be obtained from Fig. 3. In this experiment mitochondria from mixed β and γ spadices harvested in early May

were used. In a single assay, incremental additions of either KCN or NaN_3 produced successive rates as indicated in the upper and lower lines of Fig. 3. The rates and concentrations are corrected for the increased volume due to the addition of inhibitor aliquots. It can be seen that, at least in these mitochondria (which had a mean initial rate of malate oxidation of 42 nmoles O_2 min^{-1} mg^{-1}), the stimulatory effect of cyanide reaches a saturation level only at about 15 mM KCN. It is noteworthy that their maximum rate with cyanide (about 140 nmoles O_2 min^{-1} mg^{-1}) falls far short of the rate exhibited by mitochondria from the δ or ε stages. Azide, by contrast, progressively inhibits up to concentrations of 10 mM; this further confirms that the effect of cyanide cannot be solely on cytochrome oxidase.

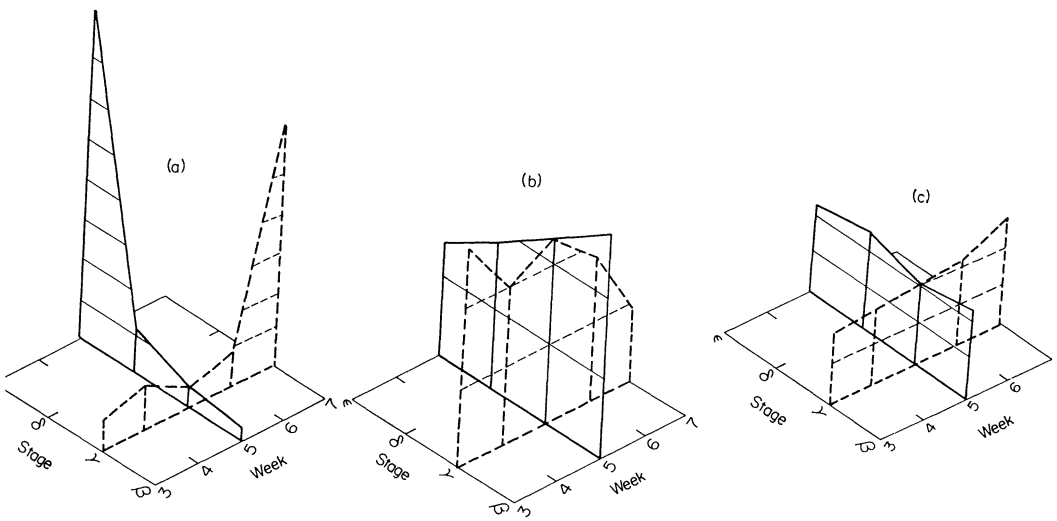

Fig. 4. (a) Changes in the initial rate of malate oxidation by *Arum* mitochondria at different periods during the flowering season and at different stages of floral development. Solid line, stages β through ε during the fifth week of flowering, dashed line, stage γ from the third to seventh week. Scale lines are at intervals of 50 nmoles O_2 min^{-1} mg protein^{-1}. (b) Changes in the response to cyanide (3.8 mM) of mitochondria at different periods during the flowering season and at different stages of floral development oxidizing malate. Data are expressed as rate after addition of KCN as a % of the rate in the presence of ADP, with scale lines at intervals of 50%. Symbols as in (a). (c) Changes in the response to ADP of mitochondria at different periods during the flowering season and at different stages of floral development. All preparations were oxidizing 3.8 mM malate and the rate after addition of 80 μM ADP is expressed as a percentage of the initial rate. Scale lines are at intervals of 50%. Symbols as in (a).

The influence of both developmental stage of the inflorescences and the date of sampling on the initial rate of malate oxidation and its response to the addition of cyanide is summarized in Fig. 4. This shows the mean values of the responses observed for all mitochondria prepared from γ stage spadices from the third to the seventh week of the flowering period in one direction, and in the other, the means of determinations made with mitochondria from spadices of all stages from β through ε during the fifth week of the season. This figure enables the trends which are apparent in Fig. 1 to be seen separately without the effects of spadix stage and seasonal period being confounded. During the fifth week, there is a clear tendency (Fig. 4a) for mitochondria from spadices picked at the same time, but in different stages of maturity, to oxidize malate more rapidly at later stages of development. In the other direction, Fig. 4a shows equally clearly that spadices

which are visually at stage γ have more rapid rates of malate oxidation during the last week of the period covered by these experiments. In Fig. 4b the responses of the same mitochondria to 3.8 mM KCN are illustrated. Although the differences are less striking than in Fig. 4a, the trend is like that shown in Fig. 2: where the initial rate of malate oxidation is low, the response to cyanide is stimulation; where malate oxidation is rapid, cyanide inhibits it. With fifth-week spadices, the mitochondria from stage β gave stimulated rates of 141% after cyanide addition, while the ε stage mitochondria were inhibited to 70% by the same concentration of cyanide. The mitochondria from stage γ spadices show a similar change from the third to the seventh week with the rate after cyanide addition being 140% of the untreated rate in the third week and 45% of the state 3 rate in the seventh week. Although the progression over the season is not quite so smooth as with spadices of four different stages gathered at the same time, the trend is clear.

Similar data for the effect of ADP on *Arum* mitochondria of different developmental stages and at different periods over the season are presented in Fig. 4c. Here it appears that the mitochondria of different stages during the fifth week (solid lines) do not differ greatly in their response to ADP. Indeed ADP has a small effect on all samples. On the other hand, mitochondria prepared from stage γ spadices (dashed lines) show a definite trend from the third to the seventh week of the season, ranging from an inhibition of 30% in the third week to a stimulation of 35% in the seventh week. This change from inhibition by ADP to stimulation is progressive over the five-week period and reflects the inverse correlation of responses to cyanide and ADP which was noted in the more extensive data used in Fig. 1.

The oxidation of succinate and α-ketoglutarate

For comparative purposes, data similar to those given in Fig. 4 are presented in Table 1 for succinate and α-ketoglutarate. Again, these are presented as initial state 4 rates of substrate oxidation and rate after 3.8 mM KCN addition as a percentage of the plus ADP rate. Inspection of Table 1 reveals that, with one exception, there is little discernible difference in the oxidation of succinate or α-ketoglutarate either in mitochondria from spadices of different stages during the fifth week of the flowering period, or in spadices of the γ stage harvested between the third and seventh weeks of the season. The exception is the rate of succinate oxidation by spadices of the ε stage, or of γ stage in the seventh week, both of which show rates substantially higher than are found in other comparable samples. In γ stage spadices in the seventh week, this higher rate is accompanied by a greater degree of inhibition by KCN, but the high rate in the ε stage spadices does not appear greatly different from the others with respect to inhibition by cyanide. With α-ketoglutarate there are consistently somewhat higher rates of oxidation up to the sixth week, but no rise of oxidation rate in the seventh week comparable to that found with malate and succinate. Nor is there a higher rate in the ε stage. The response to cyanide is remarkably constant over the whole season and with all four development stages. Another aspect of the data of Table 1 must be mentioned, although at this point we are unable to offer an adequate explanation. This is the consistent inhibition by cyanide of the oxidation of succinate, and the contrasting consistent stimulation—though rather small—of α-ketoglutarate oxidation by cyanide. It should be pointed out that the figures of Table 1 for different weeks are means of several determinations made on both the same and different mitochondrial preparations during the week in question, and that the range of values of KCN effect which go to make up these means is relatively large

(from small inhibitions to stimulations in some cases) with α-ketoglutarate, although the values with succinate are much more uniform.

Comparison of malate, succinate and α-ketoglutarate as substrates

A single experiment which provides an overall summary of the changing metabolic behaviour with respect to three TCA cycle substrates of *Arum* spadix mitochondria during floral development is summarized in Table 2. In this experiment mitochondria from young and old spadices gathered on the same day were tested for their ability to oxidize malate, succinate and α-ketoglutarate and for their response to both cyanide and azide, followed by mCLAM. The responses to malate are similar to those which have been described above. The mitochondria from stage ε spadices are able to metabolize malate at a rate more than ten times faster than those from stage β spadices. The young

Table 2. *Comparison of the effects of cyanide and azide, alone and in combination with mCLAM, on the oxidation of malate, succinate and α-ketoglutarate by mitochondria of young (stage β) and old (stage ε)* Arum. *Inflorescences harvested* 11 *May (fifth week of blooming period)*

	Initial rate nmoles O_2 min^{-1} mg^{-1}	Rate after ADP as % of rate before	Rate* after 3.8 mM KCN or NaN_3 % of rate before	Rate* after 1.5 mM mCLAM—% of uninhibited rate
Malate				
Young spadix				
KCN	21.3	90.0	141.5	46.5 [8.9]†
NaN_3	15.6	76.0	93.1	41.7 [4.9]
Old spadix				
KCN	288.0	153.0	50.0	7.4 [32.6]
NaN_3	259.5	204.0	102.2	5.4 [28.6]
Succinate				
Young spadix				
KCN	77.6	136.0	85.0	3.5 [3.7]
NaN_3	88.8	149.0	78.8	9.9 [13.0]
Old spadix				
KCN	346.7	130.0	95.4	6.8 [30.6]
NaN_3	291.2	179.0	87.0	4.2 [21.9]
α-Ketoglutarate				
Young spadix				
KCN	198.0	127.0	110.0	3.4 [8.5]
NaN_3	140.6	149.0	77.2	7.4 [15.5]
Old spadix				
KCN	153.4	182.0	108.1	7.1 [19.8]
NaN_3	124.5	168.0	83.1	6.5 [13.6]

* In presence of ADP.
† Figures in brackets are nmoles O_2 min^{-1} mg^{-1}.

mitochondria are inhibited by ADP, stimulated by KCN, and slightly inhibited by azide. On the addition of 1.5 mM mCLAM both old and young mitochondria are further inhibited, although with young mitochondria the smaller residual respiration constitutes a larger fraction of the initial rate than with the old mitochondria. We have repeatedly observed this inability to inhibit completely mitochondrial oxidation by combinations of mCLAM with inhibitors of cytochrome oxidase. This may indicate that *Arum* mitochondria possess some oxidative mechanism which is resistant to both mCLAM and KCN or NaN_3, but further study is required.

The oxidation of succinate is also much more rapid in mitochondria from old than in those from young spadices; ADP stimulates, and both cyanide and azide inhibit, suc-

cinate oxidation to some degree, with most of the residual activity being sensitive to mCLAM.

In contrast, α-ketoglutarate is oxidized as readily by young mitochondria as by old, perhaps even better. The addition of ADP is stimulatory and cyanide produces a slight stimulation (as in Table 1) while azide uniformly inhibits the oxidation. Again, the addition of mCLAM removes most of the respiration not inhibited by cyanide or azide.

Oxalacetate and malate oxidation

The data presented so far suggest that the large changes of rate of malate oxidation, as well as the stimulation of that rate by cyanide in preparations from young spadices, might be associated with an excess of oxalacetate in the mitochondria which diminished with age, or by cyanohydrin formation with KCN.

This hypothesis was tested immediately in a number of indirect ways, and by direct analysis of oxalacetate in the subsequent season (1974).

Oxalacetate concentration

The results of assaying the oxalacetate content of preparations of mitochondria from spadices in various stages of development are given in Table 3. It will be seen that

Table 3. *Relation of malate oxidation, response to cyanide and oxalacetate concentration of* Arum *mitochondria. Figures for γ spadices are means of determinations on two separate days, others are from single preparations made on the same day. Values of oxalacetate are means of two determinations on each sample*

Spadix stage	Rate of malate oxidation* nmoles O_2 min^{-1} mg^{-1}	Rate after addition of 3.8 mM KCN % of original rate	Oxalacetate concentration nmoles g^{-1} fresh weight	Oxalacetate nmoles mg $protein^{-1}$
α	15.1	108.8	157.4	135.8
β	49.0	129.6	65.2	33.2
γ	48.0	104.6	47.8	29.4
ε	187.9	51.0	0†	0

* Rate in presence of 4.0 mM malate plus 80 μM ADP.
† < 1 nmole g^{-1} fresh weight.

oxalacetate is present in significant or even large quantities in the early stages but is not measurable in mitochondria in the ε stage.

The analyses therefore suggest that the hypothesis might be valid.

Indirect tests of oxalacetate control

(1) *Repeated washing.* The mitochondrial preparations used in this study were washed once in our routine procedure. However, a second wash has considerable influence on the rate of malate oxidation and its response to cyanide. Relevant data obtained from preparations made with either a single or a double washing procedure on different, but chronologically adjacent days, are shown in Table 4. Similar results were obtained when the mitochondria from a single preparation were divided into two and one portion washed an additional time. The experiments from which these data are taken were carried out during the third and fourth weeks of the flowering period. It is clear from Table 4 that an additional wash procedure allows the mitochondria to oxidize malate at

more than seven times the rate found with those washed only once. In addition, twice-washed mitochondria give an increased rate on the addition of ADP instead of showing inhibition, and are inhibited by 3.8 mM KCN rather than stimulated. It appears probable that the differences in the rate of malate oxidation and its response to cyanide can be explained on the basis that a single wash removes only part of the endogenous oxalacetate from the mitochondria and that when present, oxalacetate limits the oxidation of added malate.

Table 4. *Comparison of initial rate of malate oxidation, reaction to ADP and response to cyanide of* Arum *spadix mitochondria washed once and washed twice. Means for once-washed mitochondria include values obtained from three separate preparations of spadices at stage α and two at stage γ. Means for twice-washed mitochondria include values for two separate preparations made from spadices at stage α and two at stage γ. Mean protein concentrations are* 1.09 *mg* g^{-1} *fresh spadix weight for mitochondria washed once and* 0.99 *mg* g^{-1} *for mitochondria washed twice*

	Initial rate nmoles O_2 min^{-1} mg^{-1}	Rate after addition of ADP % of rate before	Rate after addition of 3.8 mM KCN, % of rate before
Mitochondria washed once	22.4	77.0	143.3
Mitochondria washed twice	162.1**	116.0*	86.9**

* Different from once-washed at $P<0.05$ on basis of *t*-test.
** Different from once-washed at $P<0.01$ on basis of *t*-test.

Table 5. *Effect of preincubation for* 20 *min with* 0.4 *mM pyruvate on the oxidation of malate, the effect of ADP and response to cyanide of* Arum *spadix mitochondria. Values for malate alone are means of two preparations of stage α, one at stage β and two at stage γ. Values for malate plus pyruvate are means of two preparations of stage α, one of stage β and three of stage γ*

	Initial rate nmoles O_2 min^{-1} mg^{-1}	Rate after ADP % of rate before	Rate after addition of 3.8 mM KCN nmoles O_2 min^{-1} mg^{-1}	Rate after addition of 3.8 mM KCN % of rate before KCN
Malate (3.8 mM)	23.8	73.0	33.3	140.1
Malate (3.8 mM) after Pyruvate (0.4 mM)	46.3*	129.0**	32.9	71.1**

* Different from malate at $P<0.05$ by *t*-test.
** Different from malate at $P<0.01$ by *t*-test.

(2) *Addition of pyruvate.* If accumulation of oxalacetate acid were a significant factor, it would be expected that the provision of pyruvate would enable the oxalacetate pool to be reduced by providing acetyl CoA through the activity of pyruvate dehydrogenase to permit the citrate synthetase to use the accumulated oxalacetate in the formation of citrate. To test this possibility, mitochondria were preincubated for 20 min with a low (0.4 mM) concentration of pyruvate plus 0.2 mM thiamine pyrophosphate, after which the usual concentration of malate was added, followed by additions of ADP and KCN. The results of these comparisons are shown in Table 5, where it may be seen that the pre-

incubation with pyruvate does increase the rate of malate oxidation (the mean value for 0.4 mM pyruvate oxidation alone was 8.9 nmoles O_2 min^{-1} mg^{-1} and this rate should be subtracted from the value for malate in the presence of pyruvate to give malate oxidation alone). Preincubation with pyruvate results in stimulation by ADP rather than inhibition, and the effect of cyanide addition is shifted from stimulation to inhibition. In this connection it should be noted that the final rate of O_2 intake in the presence of cyanide is the same for both treatments. It is attractive to relate this fact to the possibility that this represents the maximum attainable rate through the cyanide-resistant pathway under the conditions prevailing.

Table 6. *Effect of preincubation with malate or oxalacetate on the subsequent rate of malate oxidation and response to cyanide of* Arum *mitochondria. Mitochondria were of stage α, gathered during the second week of the blooming period and were washed twice (see text) before final resuspension and oxygen uptake measurements. Values are means of two determinations*

Preincubation treatment	Initial rate with 3.8 mM malate nmoles O_2 min^{-1} mg^{-1}.	Rate after ADP % of rate before	Rate after 3.8 mM KCN % of rate before
None	237.9	110.0	84.9
1 hr. in 0.42 mM malate	12.3	94.0	123.4
10 min in 4.2 mM oxalacetate	72.7	60.0	69.8

Table 7. *Effect of additions of oxalacetate and pyruvate on rate of malate oxidation, effect of ADP and inhibition by cyanide of mitochondria from young (Stage α)* Arum. *Inflorescences harvested 13 April (first week of blooming period). Mitochondria washed once*

	Rate* with 4.2 mM malate	Rate* after addition of 4.0 mM oxalacetate	Rate* after further addition of 3.8 mM pyruvate	Rate* after ADP, % of rate before	Rate† after addition of 3.7 mM KCN
Control	16.6	–	–	53.0	93.7
Treated	10.4	6.4	37.7	109.0	54.9

* Expressed as nmoles O_2 min^{-1} mg protein^{-1}.
† Expressed as % of rate in presence of ADP.

(3) *Incubation in oxalacetate or malate.* The reciprocal of the experiments described in Table 5 would be ones in which mitochondria which did not display the responses attributed to the accumulation of oxalacetate were manipulated so as to bring about an increase in the concentration of oxalacetate present. Such experiments are presented in Table 6. For these mitochondria prepared from young (stage α) spadices were washed twice with approximately 70 volumes (v/v relative to final suspension volume) of washing medium before final resuspension. In the standard experiment measuring the oxidation of 3.8 mM malate these mitochondria gave rates approximately ten times that of comparable mitochondria washed only once. An increased rate was also obtained on the addition of 80 μM ADP, and the subsequent addition of 3.8 mM KCN caused inhibition. However, when these mitochondria were incubated at 25° C for 1 h with 0.42 mM malate (with the expectation that the oxidation of this substrate would result in the

accumulation of oxalacetate within the organelles) and then provided with 3.8 mM malate, the rate was only about 5% of that observed in mitochondria which had not been preincubated, nor was there an increase in oxidation on the addition of ADP, and KCN increased the rate about 20%. Similarly, a preincubation of 10 min with 4.2 mM oxalacetate caused a substantial (70%) decrease in the rate of initial malate oxidation, and a pronounced inhibition on the addition of ADP. In this case, however, the addition of cyanide caused a further decrease in rate rather than the increase which would be anticipated from the hypothesis that cyanide is removing oxalacetate through cyanohydrin formation and thus permitting the malate dehydrogenase reaction to shift to the right. The reason for the failure of cyanide to induce an increased rate probably relates to the equilibrium of the cyanohydrin formation reaction and the large amount of oxalacetate present under the conditions used here.

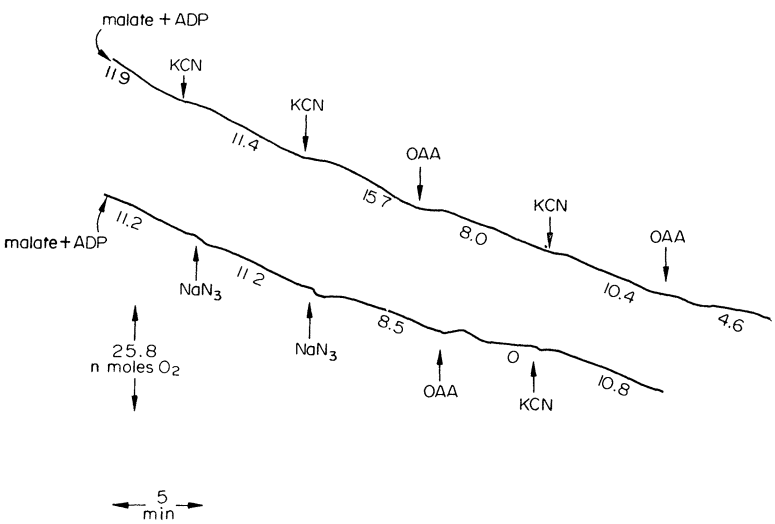

Fig. 5. Tracings of recorder charts showing antagonistic effects of cyanide and oxalacetate on malate oxidation by *Arum* mitochondria. Additions to an initial volume of 1.20 ml in the following sequence from left to right:
Upper line—50 μl 0.1 M Na malate and 10 μl 0.01 M ADP; KCN, 50 μl 0.1 M KCN; KCN, 50 μl 0.1 M KCN; OAA, 100 μl 0.1 M oxalacetate; KCN, 100 μl 0.1 M KCN; OAA, 100 μl 0.1 M oxalacetate.
Lower line—50 μl 0.1 M Na malate and 10 μl 0.01 M ADP; NaN_3, 50 μl 0.1 M sodium azide; NaN_3, 50 μl 0.1 M sodium azide; OAA, 100 μl 0.1 M oxalacetate; KCN, 100 μl 0.1 M KCN. Figures under lines are rates of oxidation in nmoles O_2 min^{-1} mg $protein^{-1}$ corrected for dilution of original 1.20 ml volume.

The effect of oxalacetate and pyruvate addition upon the malate oxidation rate of mitochondria from spadices early in the season was tested. These showed a low respiratory control ratio and were almost unaffected by cyanide. They were inhibited some 40% by addition of oxalacetate at about 3.5 mM. The further addition of pyruvate at a similar concentration increased their oxygen uptake rate more than fourfold. This pyruvate-stimulated rate was slightly increased by ADP and inhibited some 45% by KCN (Table 7). This suggests that, in the presence of adequate pyruvate, the concentration of oxalacetate is diminished and malate oxidation is stimulated. Mitochondria treated in this way show the same reaction to ADP and to cyanide as the mitochondria of Tables 4 and 5

where the oxalacetate was presumably removed by additional washing or by preincubation with low levels of pyruvate.

(4) *Alternate additions of oxalacetate and cyanide.* The interaction of oxalacetate and cyanide in altering malate oxidation by *Arum* mitochondria is further shown in Fig. 5, which shows that sometimes the relative rates of malate oxidation can be reversed by successive additions of KCN and oxalacetate. These mitochondria from stage α spadices were washed only once before measurements were started. The upper line in Fig. 5 shows that while the first addition of KCN (bringing the concentration to 3.8 mM) has little effect, a second increment (making 7.4 mM) does increase the rate. Addition of oxalacetate, giving a final concentration of 7.1 mM, decreases the rate, but additional KCN increases it again. Another increment of oxalacetate brings the rate down to less than half of that before the addition.

In the lower line of Fig. 5 two additions of NaN_3 (7.4 mM) reduce the rate of malate oxidation about 25%. At that point, the addition of 7.1 mM oxalacetate results in complete inhibition of malate oxidation. The addition of 6.6 mM KCN to these completely inhibited mitochondria restores the rate to 96% of the initial value.

Both lines of Fig. 5 indicate that oxalacetate, presented in high concentrations, does affect the rate of malate oxidation, presumably by entry into the mitochondria. The increased rates induced by cyanide must use the cyanide-resistant pathway, since the concentration of cyanide, and especially of azide when present, is greatly in excess of that required to inhibit cytochrome oxidase completely.

The lack of knowledge concerning the permeability of mitochondria to oxalacetate and of its distribution within the organelle makes quantitative evaluations of the foregoing experiments difficult, but treatments which should increase or decrease the internal levels of oxalacetate do significantly alter the capacity of *Arum* mitochondria to oxidize malate and the response of that oxidation to cyanide.

DISCUSSION

Mitochondria prepared from stages of maturation of *Arum* spadices showed a sequence of change when oxidizing malate. Mitochondria from young spadices of stages α–γ were stimulated by cyanide when oxidizing malate, mitochondria from older spadices were unaffected or showed some inhibition. Further analysis of this change in response to cyanide showed it to be complex in the sense that, although it was correlated with the season through the different morphological stages, there was also a more direct seasonal effect. Hence, spadices selected as being in a median morphological stage (γ in Fig. 4a) showed within a single developmental class changes dependent on the season.

Although the effects of morphological stage and season were thus confounded, there was a correlation of reaction to cyanide with rate of malate oxidation. Those preparations showing rates of oxygen uptake greater than about 66 nmoles O_2 min^{-1} mg^{-1} tended to be inhibited by cyanide and those with lower rates stimulated.

A further correlation was observed in that the preparations showing stimulation by cyanide were usually inhibited by ADP when oxidizing malate.

This sequential pattern of respiratory response, characteristic of malate oxidation, did not occur when other acids were presented as substrates. Although the oxidation rate of α-ketoglutarate by β stage mitochondria was relatively low, it was higher than that of malate or succinate, but there was no great rise in its rate after stage γ. Succinate oxida-

tion did show a rise in rate in later morphological stages but not so conspicuously as that with malate. Succinate oxidation was slightly inhibited by 3.8 mM cyanide, while that of α-ketoglutarate was uniformly somewhat stimulated by cyanide.

The reactions of malate oxidation to cyanide are not associated with inhibition of electron transport through cytochrome oxidase since sodium azide gives a slight to significant inhibition of oxidation of all substrates including malate. Hence we may conclude that there are factors affecting malate oxidation in particular which reduce the rate of oxidation by mitochondria from immature spadices. One of these factors can be reduced by the addition of cyanide. It should be noted, however, that even when cyanide doubles or trebles the rate of oxygen uptake by α or β mitochondria, the stimulated rate still falls far below that of mitochondria from mature, ε stage, spadices, i.e., there are other factors than that affected by cyanide which limit O_2 uptake by mitochondria in early stages.

The stimulation due to cyanide was observed to increase with increasing concentrations applied to mitochondria from stage β–γ in the presence of malate. Maximum stimulation of some 300% was achieved at a concentration of 15 mM, above which no further stimulation was observed. From this it was concluded that cyanide might be quantitatively removing some substance such as oxalacetic acid, an end product of malate oxidation. Indeed, the removal of product inhibition of malate oxidation by cyanohydrin formation with the oxalacetate seemed probable.

Analysis of extracted mitochondria showed the presence of oxalacetate in the early stages of spadix development and its absence in stage ε. Rewashing, incubation with oxalacetate, malate or pyruvate, and cyanide addition all had the effects expected if the hypothesis of oxalacetate control were true.

These results strongly suggested a control of malate oxidation by oxalacetate in the mitochondria. Such a control of TCA cycle reactions by oxalacetate has been demonstrated by several groups of workers over the last two decades but has not been invoked to explain stimulation. For instance, Wiskich and Bonner (1963) using sweet potato mitochondria and Wiskich, Young and Biale (1964) using those from avocado, observed progressive decrease of malate oxidation which they ascribed to oxalacetate production. Of especial interest in the present connection is the work of Hulme, Rhodes and Wooltorton (1967a, b) on the control of respiratory rates of apple mitochondria. They point out that oxalacetate (which they estimated quantitatively) is especially important in controlling the oxidation rates of these mitochondria. The enzyme dehydrogenase is susceptible to direct inhibition and malate dehydrogenase to end product inhibition by oxalacetate.

As they further suggest, the actual concentration of oxalacetate at any given site in the mitochondrion cannot be predicted even if accurate estimates of its total concentration are made. However, it is of interest that succinate dehydrogenase is extremely sensitive to oxalacetate in some tissues so that succinate oxidation may be as closely controlled as malate oxidation. It is attractive to speculate that the increased rate of succinate oxidation in our *Arum* mitochondria after stage δ might be due to the release of such an inhibition.

Macrae and Moorhouse (1970) has shown that oxalacetate concentration, and thus end-product inhibition of malate dehydrogenase, may be dependent upon pH. Using a reaction system with arsenite to inhibit the pyruvate dehydrogenase complex, pyruvate was shown to be the main end product of malate oxidation by cauliflower mitochondria at pH values below neutrality, whereas oxalacetate was the end product of higher pHs. The malic enzyme, which is apparently responsible for the formation of pyruvate from

malate at low pHs, showed a sharp drop of activity above pH 7.0. At pH values below 7, where the malic enzyme shows maximum activity, the reaction was coupled via $NADH_2$ to malate dehydrogenase, leading to a reversal of that reaction and formation of malate from oxalacetate. Therefore, pH can critically affect the rate and products of malate oxidation. Pyruvate is the primary product below neutrality and oxalacetate above.

Douce and Bonner (1972) have also emphasized the possible importance of oxalacetate especially in relation to the $NADH_2$ pool which can affect many reactions of the mitochondria. They point out that in their experience oxalacetate readily penetrates plant mitochondria and so may exert an important controlling influence in two ways, by a direct inhibition of succinate dehydrogenase and by causing $NADH_2$ to become more oxidized as a result of the oxalacetate to malate conversion. Although succinate dehydrogenase is agreed by many workers to be directly inhibited by oxalacetate, there is no strong evidence of such an effect in *Arum* tissue if our assumptions are correct. Our experiments were performed at pH 7.2, where, according to Macrae and Moorhouse (1970), oxalacetate would be the main product of malate oxidation and little pyruvate would be formed. Such a situation is in conformity with our observation that both cyanide and pyruvate additions will increase malate oxidation—presumably by removal of oxalacetate. In addition, the supply of exogenous oxalacetate had little effect on the oxidation of succinate.

The reduction by ADP of oxygen uptake with malate in *Arum* mitochondria which we have observed with those preparations which are stimulated by cyanide and thus are assumed to be limited by an accumulation of oxalacetate is difficult to explain on the present evidence. There are several indications in the literature that adenylates effect a control of TCA cycle reactions apart from their participation in oxidative phosphorylation. Not only have Hulme *et al.* (1967a) indicated that ATP offsets the inhibition of succinate dehydrogenase by oxalacetate but Zimmerman and Ikuma (1970) have reported that ADP in excess may increase the concentration of oxalacetate in mitochondria. In addition, Singer, Gutman and Kearney (1972), using animal mitochondria, have stressed the importance of coenzyme Q and ADP in the control of dehydrogenases of the TCA cycle. Laties' (1973) recent report of a role for ADP in permitting subsequent uncoupler addition to produce increased respiratory rates may also be pertinent. Although at present none of these reports offer any obvious explanation of the phenomena noted in this study, they do, together with the well-established concept of energy charge control of dehydrogenase activity (Atkinson, 1968), indicate a possible role for ADP in control of the oxidative activity of mitochondria separate from that of a phosphate acceptor in phosphorylation and encourage the further study of the effects noted here.

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