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Cyanide-insensitive Respiration in Plant Mitochondria[1]

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ABSTRACT

Pathways of electron transport have been studied in mitochondria isolated from hypocotyls of etiolated mung bean seedlings and skunk cabbage spadices that show cyanide-resistant respiratory activity. The residual flux through cytochrome *c* oxidase is shown to be small in comparison with the flux through an unidentified alternative oxidase that is known to have a high affinity for oxygen. This alternative oxidase is not a cytochrome. Skunk cabbage and mung bean mitochondria contain cytochromes *a* and a_3 that have absorption peaks differing slightly from those of animal preparations. A slow oxidation-reduction of cytochrome a_3-CN has been demonstrated. Cytochromes *b* undergo oxidation and reduction in the presence of cyanide but play no essential role in the cyanide-resistant pathway. Antimycin inhibits to an extent similar to that of cyanide; the respiratory chain bifurcates on the substrate side of the antimycin-sensitive site. Evidence is presented for the selective inhibition by thiocyanate, α,α'-dipyridyl, and 8-hydroxyquinoline of the alternative oxidase pathway, which may therefore contain a non-heme iron protein.

In only a few plant tissues is the rate of respiration sufficiently rapid to result in an appreciable rise of the temperature of the tissue above that of the surrounding air. This may occur in ripening fruits, germinating seeds, and opening inflorescences (palms, Cycadaceae and Araceae). The phenomenon is most marked in the Araceae. Some of the earlier measurements of respiration rates and temperatures of the aroid spadix were recorded by Pfeffer (24), but the first investigation of the chemical mechanism of spadix respiration was made by van Herk (30–33) with *Sauromatum guttatum*. Van Herk discovered the marked resistance of spadix respiration to inhibition by cyanide and recorded the exceptionally high respiratory rates (with a maximal Qo_2 between 20 and 68) of the tissue after the opening of the spathe. These observations were extended to *Arum maculatum* by James and Beevers (17).

James and Elliott (18) showed that active cyanide-resistant respiration is associated with mitochondrial preparations from the *Arum* spadix. The study of isolated mitochondria avoids some of the problems and practical difficulties that arise in experiments with whole tissues, and extra-mitochondrial pathways clearly cannot account for more than a small fraction of the cyanide-resistant respiratory activity of aroid spadices (3). The present paper critically reviews the investigations that have been carried out on the mechanism of cyanide-insensitive respiration of mitochondrial preparations in the light of new experimental evidence.

Five different hypotheses have been proposed to explain the mechanism of cyanide-resistant oxidations in plant mitochondria.

Hypothesis 1. Van Herk (32) was unable to detect cytochrome in the *Sauromatum* spadix but discovered an autooxidizable flavoprotein of the type of Warburg's old yellow enzyme but with a significantly higher turnover number. Moreover, the flavin content of the spadix increased at the same time as the respiration, and van Herk calculated that the flavoprotein oxidase could account for ⅓ to ½ of the respiration. James and Beevers (17) made similar observations with the spadix of *Arum maculatum*. Their conclusion that the cyanide-resistant terminal oxidase was a flavoprotein was strengthened by their observation that respiration was markedly dependent on oxygen concentration.

Hypothesis 2. Bendall and Hill (1) showed that the *Arum* spadix is richly supplied with cytochrome components. A mitochondrial fraction contained cytochromes $a + a_3$, *b* and *c* and, in addition, a new cytochrome termed b_7. Cytochrome b_7 was oxidized by air in the presence of cyanide, and it did not react with carbon monoxide. Bendall and Hill proposed that cytochrome b_7 mediates electron transfer to oxygen in the cyanide-stable respiration. Lundegårdh (21) also observed in wheat roots the oxidation of cytochrome *b* in the presence of cyanide, and he made a similar suggestion of a "*b* shunt to oxygen." The suggestion that cyanide-resistant respiration could be explained by a second oxidase pathway connecting with normal respiratory chain components on the substrate side of cytochrome *c* was first made by Okunuki (23) as a result of his study of pollen respiration.

Yocum and Hackett (36) reported evidence for the special role of *b* components in the cyanide-resistant respiration of spadices of *Peltandra virginicum* and *Philodendron grandifolium*. These authors made the important observation that the respiration of the aroid spadix had a high affinity for oxygen and explained the contrary result of James and Beevers (17) by a limiting diffusion of oxygen under the conditions used. Simon (27) confirmed the high oxygen affinity in tissues of the *Arum maculatum* spadix.

Hypothesis 3. The simplest explanation of cyanide-insensitive respiration is that there is incomplete inhibition of cytochrome *c* oxidase. Such a situation could arise if the mitochondria contained a large excess of cytochrome oxidase relative to a rate-limiting step in the respiratory chain. The excess oxidase hypothesis has been discussed in relation to plants by Yocum and Hackett (36), Hackett and Haas (14), Simon (27), Wiskich and Bonner (35), and Chance and Hackett (10). The most important evidence in favor of the hypothesis was reported by Chance and Hackett, who found incomplete reduction of cytochromes *c* and *a* of skunk cabbage mitochondria in the presence of azide or cyanide.

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Hypothesis 4. On the basis of optical and kinetic evidence a second oxidase of the *a* type was suggested to be a part of the plant mitochondrial respiratory chain assembly (6).

Hypothesis 5. Evidence against each of the above four hypotheses will be discussed in this paper. However, the first two have in common the suggestion, which was first made by Okunuki as a result of his study of pollen respiration (23), that the cyanide-resistant pathway connects with the normal respiratory chain on the substrate side of cytochrome *c*. The results below support this contention but leave open the question of the chemical nature of the cyanide-resistant oxidase. We report evidence that a non-heme iron compound is associated with the cyanide-resistant pathway.

MATERIALS AND METHODS

The plant material used in these experiments included the spadices of skunk cabbage (*Symplocarpus foetidus*), tubers of potato (*Solanum tuberosum*), and etiolated hypocotyls of mung bean (*Phaseolus aureus*) and Black Valentine bean (*Phaseolus vulgaris*). The skunk cabbage was collected in a swamp in Shanesville, Berks County, Pennsylvania, and in a swamp on the Swarthmore College campus. In the late spring skunk cabbage inflorescences were flown from swamps in the region of Ithaca, New York. Bean seedlings were grown in a dark room maintained at 28 C and 60% relative humidity.

Mitochondria were prepared by methods that have been described previously (7). The preparation was suspended in a small volume of wash medium. For all experiments reported in this paper dilutions were made into a medium containing 0.3 M mannitol, 10 mM potassium phosphate buffer, 10 mM KCl, and 5 mM $MgCl_2$, which was adjusted to pH 7.2. Submitochondrial particles were prepared by sonication of a mitochondrial suspension (4–10 ml) in 0.1 M potassium phosphate buffer, pH 7.2, for 30 sec with a Branson Soniprobe. The suspension was then centrifuged 15 min at 10,000*g* (*g* max) to remove large particles, and the supernatant was centrifuged 1 hr at 144,000*g*. The particles were washed once by resuspension in phosphate buffer and centrifuged for 30 min at 144,000*g* and were finally resuspended in a small volume of phosphate buffer.

Oxygen consumption of isolated mitochondria was measured at room temperature (22–24 C) with a Clark oxygen electrode inserted into a 3-ml cuvette on a magnetic stirrer. A special electrode and cuvette were constructed for preparing mitochondria in a known respiratory state that could then be frozen rapidly in liquid nitrogen. This technique traps the respiratory carriers in the redox condition of the steady state and so allows the low temperature difference spectrum of the trapped steady state to be measured. For this special electrode, as suggested by Dr. J. B. Chappell, the platinum electrode and its silver reference electrode were placed at the base of a cylindrical Lucite chamber. The chamber was also fitted with a very narrow outlet near the bottom. A plunger with a removable Teflon center and a hole at the base was fitted into the top of the chamber. This arrangement allowed for additions into the chamber through the hollow plunger or, with the Teflon insert in place, for pushing the contents out of the vessel into a spectrophotometer cuvette cooled in liquid nitrogen.

Optical measurements were performed on a sensitive scanning split beam spectrophotometer and on a differential dual wavelength spectrophotometer (9).

RESULTS

Inhibitor Sensitivity. The various degrees of sensitivity of plant mitochondria to inhibitors are illustrated by the results recorded in Table I. In most experiments succinate was used as substrate, and in these cases the mitochondria were incubated for 4 min with 170 μM ATP before substrate addition to overcome the inhibited state of the succinic oxidase system of the mitochondria as isolated (35). All preparations of mitochondria showed respiratory control, although in the case of skunk cabbage mitochondria the respiratory control indexes were very low (about 1.2). Succinate oxidation by potato mitochondria was almost completely inhibited by the terminal inhibitors cyanide and azide, but, on the other hand, skunk cabbage mitochondria were inhibited only to the extent of 20 to 40%. The values obtained with mitochondria from the hypocotyls of mung bean and Black Valentine bean seedlings lay between these two extremes. The sensitivity of the mitochondria to these two inhibitors was about the same in the presence of ADP and of uncoupler. Malate oxidation was inhibited to a similar degree as succinate oxidation in skunk cabbage mitochondria. Nevertheless, the oxidation of ascorbate in the presence of either TMPD[3] or DAD was always strongly inhibited, even with skunk cabbage mitochondria.

Table I. *Effects of Respiratory Inhibitors on Oxygen Uptake of Plant Mitochondria*

Inhibition was determined with the oxygen electrode. Uncoupler was 0.12 μM FCCP.

Inhibitor	Inhibition			
	Succinate, 6.7 mM		L-Malate, 30 mM, uncoupled	Ascorbate, 10 mM, with 0.1 mM TMPD or DAD, uncoupled
	State 3	Uncoupled		
	%	%	%	%
I. Cyanide, 0.1 mM				
Potatoes		94		
Mung beans	64–79	76		91
Black Valentine beans	66			
Skunk cabbage	38	17–45	25–45	86–95
II. Azide, 2–5 mM				
Potatoes	90–95			
Mung beans	53–74	65		
Skunk cabbage		32		86
III. Antimycin, 0.1–0.2 μg				
Mung beans	66–77	70		
Skunk cabbage		42–51		

Antimycin A, which acts on a different region of the respiratory chain, nevertheless inhibited to about the same extent as cyanide and azide. This observation agrees with those of Ikuma and Bonner (15) with mung bean mitochondria and of Bendall (3) with mitochondria from the *Arum* spadix. We also observed that the effects of cyanide and antimycin were not additive. In these experiments we used a concentration of antimycin adequate to cause inhibition of the oxidation of cytochrome *b*, observed spectroscopically. (The concentration of mitochondria differed in the two kinds of experiment, but the antimycin concentration was the same in terms of mitochondrial protein [26].)

The concentrations of cyanide and azide used in the experiments recorded in Table I gave nearly the maximal effects. The excess oxidase hypothesis demands that partial inhibitions of succinate oxidation are due to incomplete inhibition of cyto-

[3] Abbreviations: DAD: 2,3,5,6-tetramethyldiaminobenzene; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

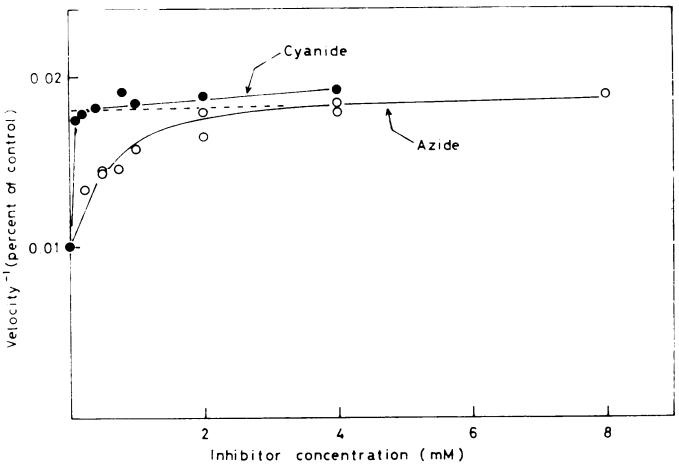

FIG. 1. Dixon plots of the effects of cyanide and azide on the rate of succinate oxidation by skunk cabbage mitochondria. Reactions were carried out in the standard medium with 6.7 mM succinate, 0.2 mM ATP, and 0.12 μM FCCP.

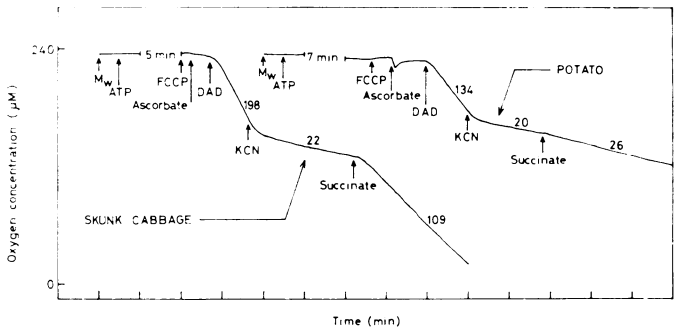

FIG. 2. Effects of cyanide on oxidation of ascorbate/DAD and succinate by (A) skunk cabbage mitochondria [Mw], and (B) potato mitochondria [Mw]. Final concentrations of reagents were: ATP, 0.2 mM FCCP, 0.08 μM (skunk cabbage) and 0.16 μM (potato); ascorbate, 10 mM; DAD, 0.8 mM; KCN, 0.1 mM; succinate, 6.7 mM. Rates are expressed as nmoles O_2/min.

chrome oxidase. The effects of varying inhibitor concentrations are most clearly seen in the Dixon plots (11) shown in Figure 1. Such plots would normally give straight lines. However, cyanide and azide inhibition gave biphasic curves, indicating two modes of inhibition with widely different values for *Ki*. These results could be explained either by two terminal oxidases, or by one oxidase, the inhibited form of which is still capable of rapid, though significantly reduced, turnover.

One method of distinguishing between these two possibilities was to compare the activities of cytochrome oxidase and the succinic oxidase system in the presence and absence of cyanide. Wiskich and Bonner (35), working with sweet potato mitochondria, observed that oxidation of a mixture of ascorbate and TMPD, a good cytochrome oxidase substrate, was powerfully inhibited by cyanide. The oxygen uptake could subsequently be restored by addition of succinate, indicating that the cyanide-insensitive fraction of succinate oxidation was greater than the residual activity of cytochrome oxidase. We have made similar observations with mitochondria from mung bean hypocotyls and skunk cabbage spadices. On the other hand, with potato mitochondria which show little resistance to cyanide, the succinate addition caused scarcely any stimulation of oxygen uptake. Results obtained with skunk cabbage and potato mitochondria were compared in Figure 2. With skunk cabbage mitochondria the succinate-stimulated rate is about five times the residual rate with ascorbate and DAD (in place of TMPD) after cyanide inhibition. This factor is a minimum, because of the slow autooxidation of ascorbate/DAD and because of an apparent inhibition of the succinic oxidase system under these conditions. These experiments showed clearly that the rate of oxygen uptake by skunk cabbage mitochondria in the presence of cyanide and succinate was too large to be mediated by the residual cytochrome oxidase activity. Strictly speaking, a saturating concentration of DAD should have been used in order to display the maximal activity of cytochrome oxidase. However, Figure 3 shows that the concentration of DAD used was not so far below the optimum as to invalidate the arguments given above.

These experiments effectively destroy the excess oxidase hypothesis, even in the modified form stated above that assumes a significant rate of turnover for the inhibited form of cytochrome oxidase. Thus, the existence of a second oxidase, resistant to cyanide and azide, must be postulated. Direct evidence for the presence of two oxidases in plant mitochondria has been obtained by Ikuma, Schindler, and Bonner (16) from a study of the oxygen affinity of mitochondrial oxidations.

The strong inhibition by cyanide of ascorbate oxidation in the presence of TMPD or DAD, which interact with the mitochondria at the level of cytochrome *c*, also argues against the possibility that the second oxidase is a modified form of cytochrome *a* (hypothesis 4). This is examined in more detail in the next section.

Optical Properties of Cytochrome Oxidase and Its Compounds with Cyanide and Azide. Keilin and Hartree (19) showed that mammalian cytochrome oxidase in the reduced state exhibited a two-banded spectrum with maxima at 605 and 448 nm. They deduced that the main portion of the 605 nm band was contributed by component *a* and the main portion of the 448 nm band by component a_3. Subsequently Yonetani (37) made a careful study of the optical properties of highly purified cytochrome oxidase from beef heart. In both the absolute spectrum and the reduced minus oxidized difference spectrum there were absorption maxima at 605 nm and 445 nm; cytochrome a_3 contributed 51% to the 445 nm peak and 28% to the 605 nm peak.

Cytochrome oxidase in plant mitochondria shows some optical differences compared with the mammalian enzyme. For example, the combined α-band of cytochromes $a + a_3$ has an absorption maximum at 602 nm, rather than 605 nm (5). More important is the observation that at −196 C the γ-band shows

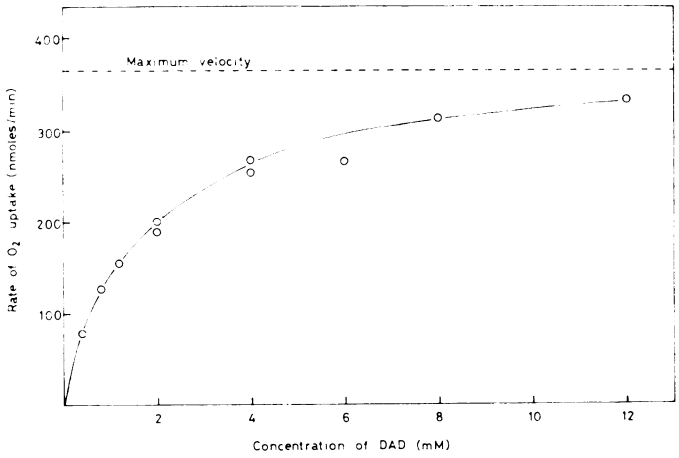

FIG. 3. Effect of the concentration of DAD on the rate of oxidation of ascorbate (10 mM) by skunk cabbage mitochondria in the presence of 0.08 μM FCCP.

two distinct absorption maxima (5). This has led to the suggestion that the second oxidase of plant mitochondria may be of the cytochrome *a* type. A similar suggestion arose from the observation that in the presence of azide difference spectra could be obtained with the cytochrome *a* + a_3 α-band at 608 nm at room temperature (4).

The spectra of cytochromes *a* and a_3 were studied in skunk cabbage and mung bean mitochondria by Yonetani's method (37) for the optical separation of these two components. Results obtained with skunk cabbage mitochondria are shown in Figure 4. The reduced minus oxidized difference spectrum for cytochrome *a* was obtained from the difference spectrum for aerobic mitochondria in the presence of ascorbate, TMPD, and cyanide minus the fully oxidized state (Fig. 4B). The contribution from cytochrome a_3 was virtually canceled out as the ferric a_3-cyanide compound in the sample cuvette and ferric a_3 in the reference cuvette. However, the small shoulder at 589 nm suggested the presence of a small proportion of ferrous a_3-cyanide. The difference spectrum of cytochrome a_3 was obtained from the spectrum of anaerobic mitochondria reduced with ascorbate and TMPD minus the aerobic mitochondria in the presence of ascorbate, TMPD, and cyanide (Fig. 4C). The spectrum obtained is the difference ferrous a_3 minus ferric a_3-cyanide. The spectra recorded in Figure 4 include contributions from cytochrome *c*, which gives rise to an α-peak at 548 nm and Soret absorption at 418 nm in the anaerobic state and in the aerobic state with cyanide, and from *b* components. The *b* components were responsible for the double α-peak at 552 and 554 nm and Soret absorption in the region 420 to 430 nm in the anaerobic sample. Whether the reduction is caused by endogenous substrates or by ascorbate/TMPD is not known. It is unlikely to be caused by an energy-requiring reversed electron transport from cytochrome *c*, as has been observed with liver mitochondria (4), because uncoupler was present in these experiments. We conclude that the α-peak at 600 nm and the double Soret peak at 439 and 446 nm belong to the difference spectrum (reduced minus oxidized) of cytochrome *a*, while the cytochrome a_3 spectrum has little or no α-absorption and a single Soret peak at 446 nm. A similar double Soret peak for cytochrome *a* has been observed with purified preparations of mammalian cytochrome oxidase (29) and there is no evidence for the existence of two discrete components. The double α-peak in the difference spectrum (anaerobic minus oxidized) of plant mitochondria is clearly derived from a normal cytochrome *a* spectrum differing from that of the mammalian component only in that the peaks are shifted 2 to 3 nm toward the violet. No evidence could be found for a third *a*-type component in plant mitochondria.

When azide was used instead of cyanide to define the oxidation-reduction states of the two *a* components (2, 13), results rather similar to those described above were obtained in the Soret band region. In the α-band region there were striking differences that were most easily seen in spectra of the difference between the anaerobic state with cyanide or azide present and the inhibited aerobic steady state. With cyanide-treated mitochondria the difference spectrum showed evidence of the reduction of the a_3-cyanide complex, the ferrous form of which is characterized by a strong α-band at 590 nm (13). With azide, on the other hand, a peak was observed at 608 nm (Fig. 5). This peak cannot represent the ferrous form of the cytochrome a_3-azide complex, as was at first thought, because an almost identical spectrum was observed when only the aerobic reference cuvette contained azide. Wilson (34) has shown that azide induces a shift of the α-band of cytochrome *a* in rat liver mitochondria 6 nm toward the violet, a shift which is reversed on anaerobiosis. A similar effect has been observed with purified cytochrome oxidase (29). The spectral effects of

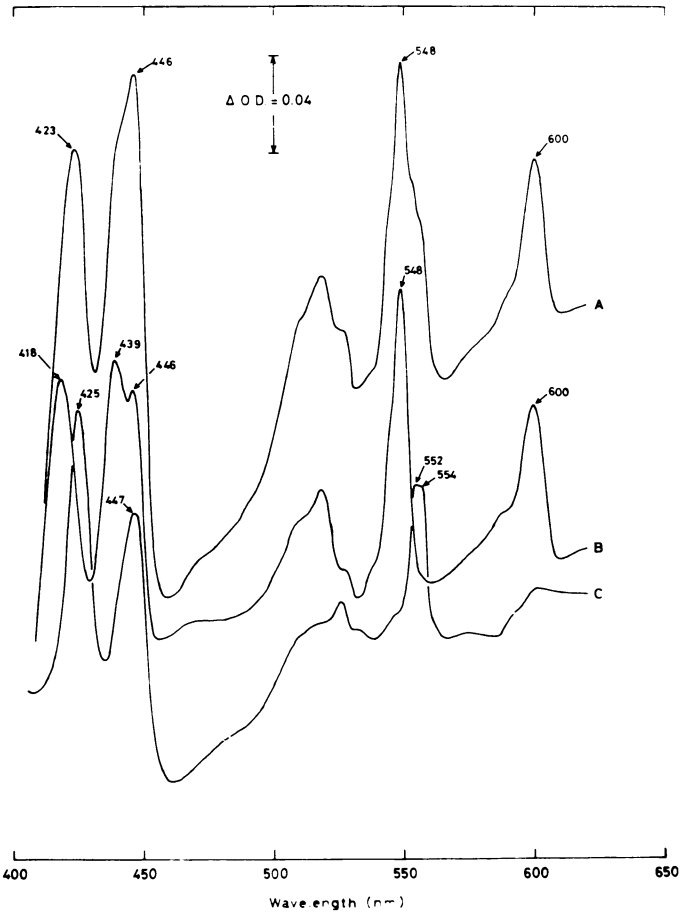

FIG. 4. Spectra of cytochrome *a* components in skunk cabbage mitochondria as revealed by reduction with ascorbate and TMPD. Difference spectra were determined at −196 C. Mitochondria were diluted in the standard medium with 1 μM FCCP. Final concentrations of reagents added, where appropriate, were: ascorbate, 10 mM; TMPD, 0.1 mM; KCN, 0.2 mM. A: Anaerobic sample reduced with ascorbate/TMPD minus oxidized. The suspension in the positive compartment was incubated 3 min with ascorbate/TMPD at room temperature, and the suspension in the negative (or reference) compartment was oxygenated before the cuvette was frozen. B: Aerobic sample reduced with ascorbate/TMPD in presence of cyanide minus oxidized. The suspension in the positive compartment was incubated 1 min at room temperature with ascorbate/TMPD after addition of KCN and the reference (negative) suspension was oxygenated before the cuvette was frozen. C: anaerobic sample reduced with ascorbate/TMPD in presence of cyanide. The positive suspension was incubated with ascorbate/TMPD for 3 min at room temperature, and the reference was incubated for 1 min with ascorbate/TMPD and KCN and oxygenated, bfore the cuvette was frozen.

azide on skunk cabbage mitochondria can be explained similarly, although in this case the observed shift was only 1 to 2 nm. The effect was most clearly seen in the low temperature spectra illustrated in Figure 6. In spectra of anaerobic mitochondria (with or without azide) minus that of mitochondria in the aerobic azide-inhibited state, the peak at 602 nm was always accompanied by a trough at 594 nm as would be expected from a small shift of the cytochrome *a* α-band toward the red. In these experiments a small peak and trough at the same wave lengths were observed in the difference spectrum of the anaerobic state with and without azide, and this indicates that reversal of the shift on anaerobiosis was not complete.

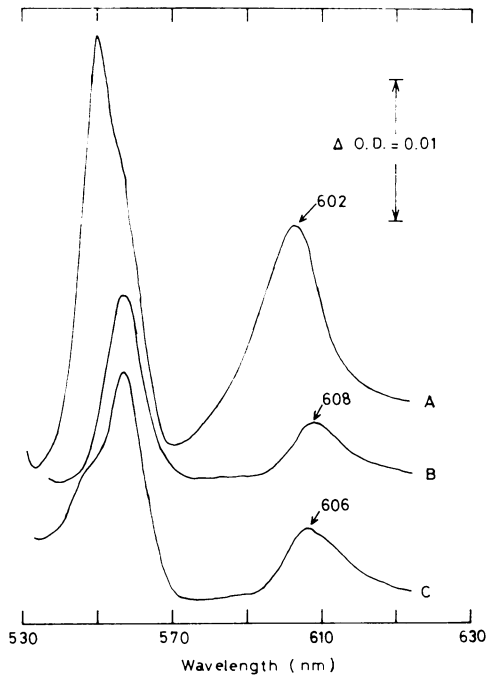

FIG. 5. Difference spectra for cytochrome $a + a_3$ in skunk cabbage mitochondria at 5 C in presence and absence of azide. A: Anaerobic minus oxidized. The suspension in the sample cuvette was treated with 6.7 mM succinate and allowed to become anaerobic, the suspension in the reference cuvette was oxygenated. B: Anaerobic suspension reduced with 6.7 mM succinate in presence of 4.2 mM sodium azide minus oxygenated suspension treated with 6.7 mM succinate and 4.2 mM azide.

However, the Soret region of this spectrum shows that azide also induces small shifts in the Soret bands of cytochrome *a*.

Wilson and co-workers (13, 34) argued that the spectral effects of azide indicate that it complexes with cytochrome *a* rather than with a_3 as had been supposed by Keilin and Hartree (19). However, Nicholls and Kimelberg (22) pointed out that the effects could also be explained by an interaction between ferric a_3-azide and ferrous a_3. Whatever the correct explanation, there is no evidence for the existence of a ferrous a_3-azide complex, but the spectra in Figure 6 show clearly that cytochrome a_3 becomes reduced in azide-inhibited skunk cabbage mitochondria. Thus, cytochrome a_3 seems to undergo oxidation and reduction in the presence of both cyanide and azide, although presumably at a much reduced rate. Consequently, although the main pathway of cyanide- and azide-resistant respiration must be via a pathway that bypasses much of the normal respiratory chain, there is also a slow leak through the inhibited cytochrome oxidase. Unfortunately, we have no quantitative data on the electron flux through this leak, but probably it is insignificantly small.

Steady States of Carriers in Skunk Cabbage Mitochondria. Chance and Hackett (10) have shown that the addition of azide or cyanide to suspensions of skunk cabbage mitochondria results in marked changes in the level of reduction of most carriers in the aerobic steady state. However, they stressed the fact that these terminal inhibitors did not lead to complete reduction of the carriers. For example, in the presence of 15 mM azide with α-ketoglutarate as substrate, cytochrome *a* was reduced to 85% of the level at anaerobiosis. The corresponding figures for the other carriers were: cytochrome *c*, 80%; cytochrome *b*, 30%; flavoprotein, 45%; and pyridine nucleotide, 15%. The maximal degree of reduction of cytochrome *c* achieved in the inhibited state was 93%. Chance and Hackett regarded these results as evidence for the excess oxidase hypothesis.

In agreement with Chance and Hackett, we have observed in experiments with the dual wave length spectrophotometer that the reduction of cytochrome *c*, measured by the change in absorption between the wave length pair 550 minus 540 nm, is

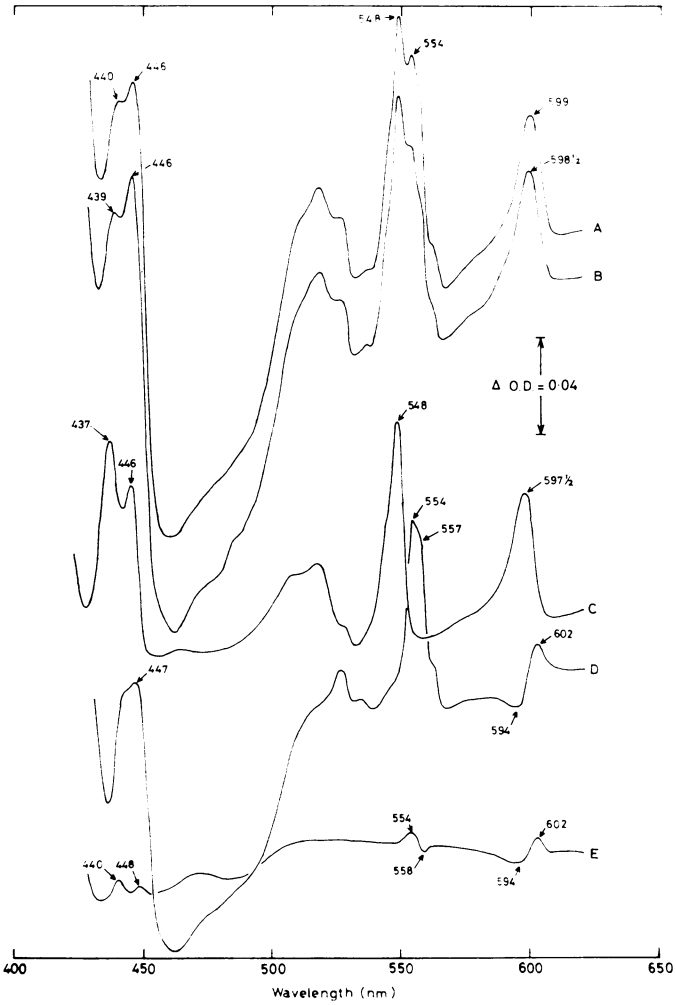

FIG. 6. Low temperature difference spectra (−196 C) of skunk cabbage mitochondria in the azide-inhibited state. Mitochondria were diluted in the standard medium and incubated with 0.38 mM ATP for 5 min at room temperature; FCCP (2 μM) was then added. Succinate (6.7 mM) and azide (3.9 mM) were added as described below. A: Anaerobic suspension reduced with succinate minus oxidized. The positive suspension was incubated with succinate for 3 min at room temperature and the reference suspension was oxygenated before the cuvette was frozen. B: Anaerobic suspension reduced with succinate in presence of azide minus oxidized. Prepared as A, with the addition of azide to the positive suspension only. C: Aerobic suspension treated with succinate and azide minus oxidized. The positive suspension was incubated with succinate and azide 1 min at room temperature, both suspensions were oxygenated, and the cuvette was frozen without delay. D: Anaerobic suspension reduced with succinate in presence of azide minus aerobic suspension treated with succinate and azide. Azide was added to positive and negative suspensions. The positive suspension was incubated with succinate for 3 min at room temperature and then oxygenated, before the cuvette was frozen. E: Anaerobic suspension reduced with succinate minus anaerobic suspension reduced with succinate in presence of azide. The positive suspension was incubated with succinate and the negative suspension with succinate and azide for 3 min at room temperature before the cuvette was frozen.

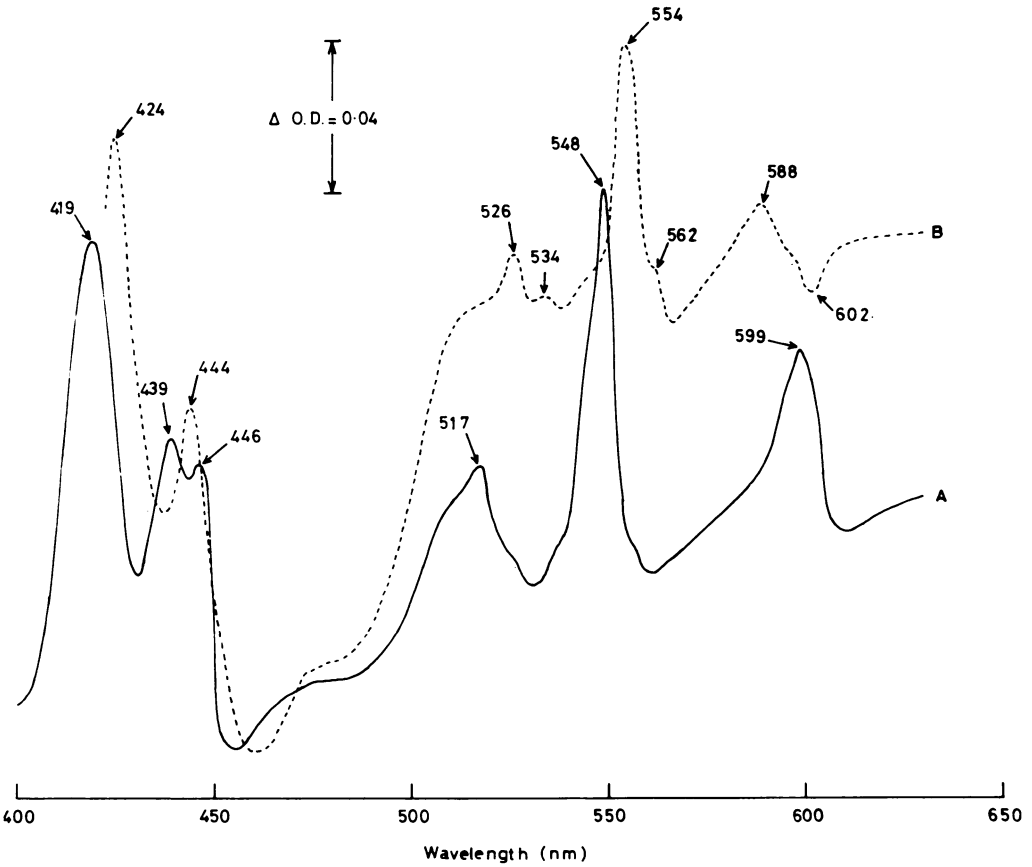

FIG. 7. Low temperature difference spectra (−196 C) of skunk cabbage mitochondria to show the effects of cyanide on the redox states of the carriers. Samples were taken from the specially constructed oxygen electrode cuvette. Mitochondria diluted in the standard medium were incubated for several min at 0 C with 0.2 mM ATP in the electrode cuvette. Succinate (6.8 mM) and FCCP (2.4 mM) were added and when a steady state was established a sample was taken into the negative compartment of a low temperature spectrophotometer cuvette cooled in liquid nitrogen. KCN (0.1 mM) was added to the electrode cuvette and when the new steady state was established a sample was taken into the positive compartment of the spectrophotometer cuvette. These two samples gave spectrum A. Spectrum B was obtained from the same reaction mixture which was allowed to go anaerobic. A sample was taken into the positive compartment of a low temperature spectrophotometer cuvette at room temperature. The reaction mixture in the electrode cuvette was oxygenated, and a sample was taken into the negative compartment of the spectrophotometer cuvette which was immediately plunged into liquid nitrogen.

incomplete in the cyanide-inhibited aerobic steady state with succinate as substrate. With uncoupled mitochondria the apparent level of oxidized cytochrome *c* was small; in several experiments the reduction was more than 90% complete. The incompleteness could be more apparent than real because the cytochrome *b* components are largely oxidized under aerobic conditions and they will make a small contribution to absorption changes at 550 nm at room temperature. Also, it is well known that respiratory carriers in plant mitochondria are not always completely reduced by substrate on anaerobiosis. This is true particularly of cytochrome *b* components, but we have found it to apply to a smaller extent to cytochrome *c*. Thus, incomplete reduction may merely indicate the presence of a small proportion of damaged mitochondria.

A more significant explanation may apply when there is no uncoupler present (as was true of Chance and Hackett's experiments). Bonner and Bendall (8) showed that in mitochondria isolated from the spadix of *Arum maculatum* an energy-dependent reversed electron transport could maintain cytochromes *a* and *c* in a partially oxidized state under aerobic conditions in the presence of cyanide.

The steady state levels of reduction of the carriers in inhibited mitochondria are best seen in low temperature difference spectra. Results obtained with uncoupled skunk cabbage mitochondria in the presence of cyanide are shown in Figure 7. In the aerobic steady state cytochrome *a* and cytochrome *c* were almost completely reduced while *b*-type cytochromes and flavoproteins were largely oxidized. A small degree of reduction of cytochromes b_{553} and b_{557} was detectable. The oxidized state of the cytochromes *b* agrees with earlier work (10). At anaerobiosis all three *b* components became reduced and there was extensive reduction of flavoprotein, indicated by the trough in the region of 460 nm. The peak at 589 nm and the Soret band at 443 nm indicated the reduction of the a_3-cyanide complex. These results show that in the cyanide-inhibited state of skunk cabbage mitochondria there is a turnover of cytochrome a_3 and the cytochromes *b*. The highly reduced condition of cytochrome *a* in the aerobic steady state and other results described above make it unlikely that the flux through a_3 is significant. On the other hand, the highly oxidized state of the *b* cytochromes under aerobic conditions suggests that they are capable of rapid oxidation in the presence of cyanide. The results of Chance and Hackett (10) and Storey and Bahr (28), as well as our own observations on the rates of reduction at anaerobiosis, show that there is a significant flux through *b* components in the cyanide-inhibited state, although the turnover may not be as rapid as the rate of reduction of oxygen. These results therefore offer some support for the special role of *b* components in the cyanide-resistant oxidase pathway (hypothesis 2).

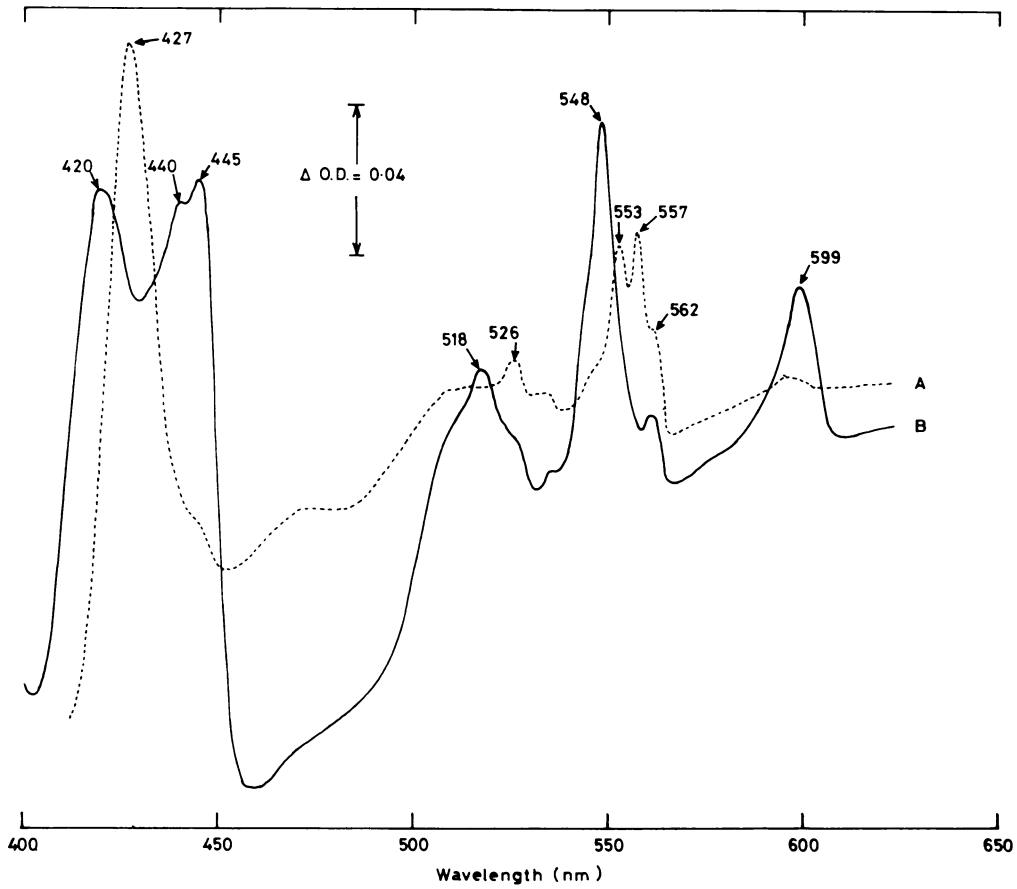

FIG. 8. Low temperature difference spectra (−196 C) of skunk cabbage mitochondria to show the effect of antimycin on the redox states o the carriers. Mitochondria were diluted in the standard medium and incubated with 0.38 M ATP for 5 min at room temperature; FCCP (2 μM) was then added. Succinate (7.6 mM) and antimycin (1.5 μg/ml) were added as described below. A: Aerobic suspension treated with succinate and antimycin minus oxidized suspension. The positive suspension was treated with antimycin and succinate and oxygenated for 1 min at room temperature, and the negative suspension was oxygenated after addition of ethanol to compensate for that in which the antimycin was dissolved, before the cuvette was frozen. B: Anaerobic suspension reduced with succinate in presence of antimycin minus aerobic suspension treated with succinate and antimycin. The positive suspension was incubated with antimycin and succinate for 2 min at room temperature and the negative suspension was cooled to 0 C, treated with antimycin and succinate, and oxygenated for 1 min, before the cuvette was frozen.

However, our results with antimycin-inhibited mitochondria render the concept of a "*b*-shunt" to oxygen very unlikely. The low temperature difference spectra plotted in Figure 8 shows that in the aerobic antimycin-inhibited steady state the three *b* cytochromes were virtually completely reduced except for a small proportion of oxidized b_{563}. In other experiments carried out at room temperature with the dual wave length spectrophotometer, no further increase in absorption occurred at 565 nm on anaerobiosis. These results therefore imply a very powerful inhibition of cytochrome *b* oxidation by antimycin, and this has been confirmed by direct measurements with a regenerative flow apparatus by Storey and Bahr (28). Cytochrome *b* components can therefore play no significant part in the antimycin-insensitive respiration, and this suggests that their part in cyanide-resistant respiration is secondary. The idea of a *b*-shunt to oxygen came from work with *Arum* mitochondria, and the behavior of these mitochondria toward antimycin (2) is notably different from that of skunk cabbage mitochondria. The experiment recorded in Figure 8 also differentiated between *b* cytochromes and flavoproteins, for the latter were largely oxidized in the aerobic antimycin-inhibited steady state, as one would expect.

Selective Inhibition of the Cyanide- and Antimycin-resistant Pathway. The experiments with antimycin A described above showed that cytochromes do not play any essential role in the second pathway of electron transport to oxygen in the skunk cabbage spadix mitochondria; the bifurcation of the respiratory chain is at the flavoprotein level. However, a simple flavoprotein is unlikely to be the second oxidase because there is no evidence for the formation of hydrogen peroxide as the product of oxygen reduction. Consequently, it seemed possible that an autooxidizable non-heme metal complex of some kind would be involved, and the effects of metal complexing agents were studied. Figure 9 shows the results of an oxygen electrode experiment with potassium thiocyanate. Cyanide or antimycin alone had little effect on the oxygen uptake of skunk cabbage mitochondria oxidizing succinate. The addition of thiocyanate instead of cyanide or antimycin caused a mild inhibition, but very strong inhibition occurred when both thiocyanate and cyanide or thiocyanate and antimycin were present. Subsequently it was found that α,α′-dipyridyl and 8-hydroxyquinoline were also effective inhibitors but only when added to the respiring mitochondria with antimycin A or cyanide. The simplest interpretation of this synergistic inhibition is that thiocyanate, α,α′-dipyridyl, and 8-hydroxyquinoline selectively inhibit the second oxidase pathway, but this only becomes apparent when the cytochrome oxidase pathway is also blocked, because of the possibility of a switch of electron flow from one pathway to the other.

Submitochondrial particles prepared from sonicated skunk

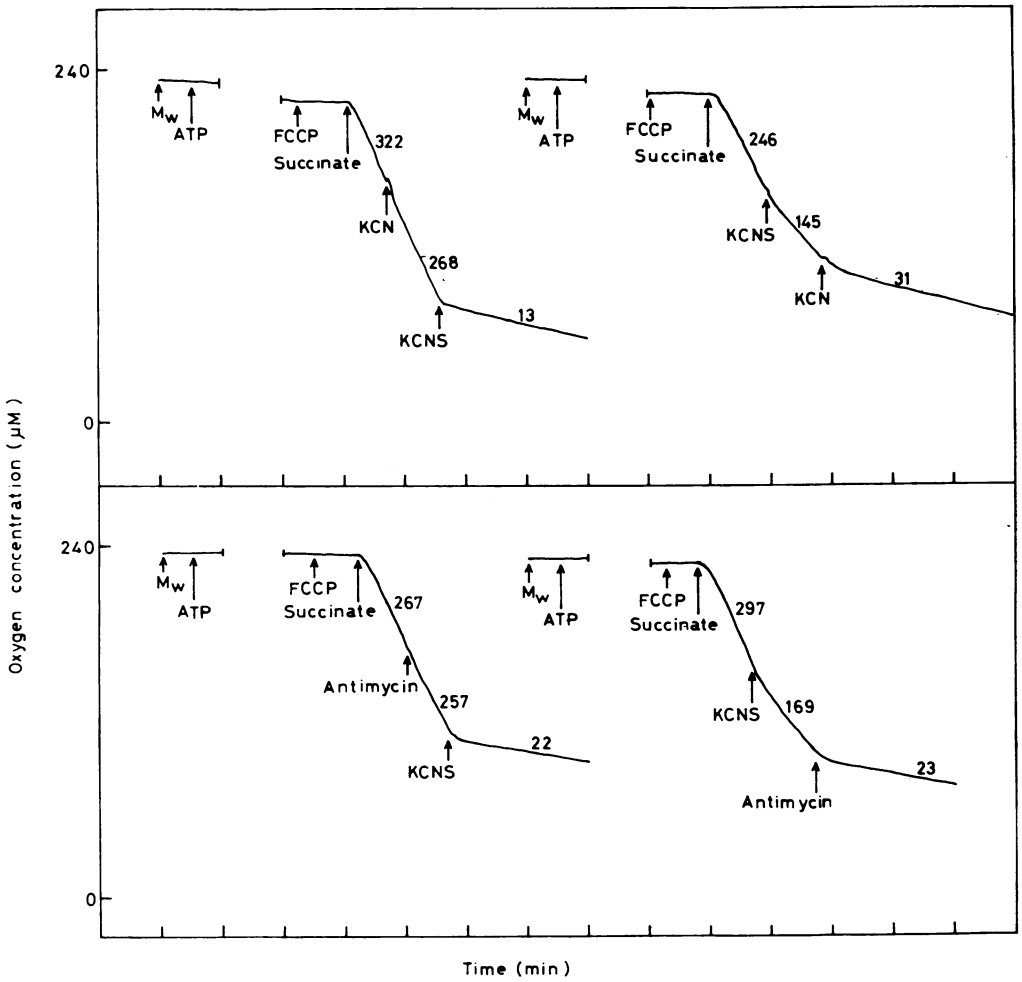

FIG. 9. Synergistic effects of thiocyanate and cyanide or antimycin on succinate oxidation by skunk cabbage mitochondria. Final concentrations of reagents were: ATP, 0.33 mM; FCCP, 0.15 mM; succinate, 6.7 mM; KCN, 0.1 mM; antimycin, 0.67 μg/ml; KSCN, 0.1 mM. Rates are expressed as nmoles O_2/min. The breaks in the traces represent intervals of 4 min.

cabbage mitochondria retained the cyanide- and antimycin-resistant respiration. As in the case of intact mitochondria, substrate oxidations were inhibited by addition of thiocyanate, α,α'-dipyridyl, or 8-hydroxyquinoline together with cyanide or antimycin. The metal complexing agents removed iron in the ferrous state, as shown by the characteristic absorption spectra of the α,α'-dipyridyl and 8-hydroxyquinoline complexes. Submitochondrial particles reduced with dithionite also gave a large clear electron spin resonance signal at $g = 1.92$. Thus the skunk cabbage preparations contained non-heme iron, but whether some of this non-heme iron is associated with the second oxidase remains unknown. Attempts to reactivate the inhibited system with ferrous salts were unsuccessful.

We took advantage of the selective inhibition of the second oxidase pathway to attempt a spectral identification of the carriers or oxidase involved. Addition of thiocyanate to mitochondria oxidizing succinate induced striking changes in the blue region of the spectrum (Fig. 10). Similar effects were caused by α,α'-dipyridyl and 8-hydroxyquinoline. However, later work has shown that this spectrum is not related to the cyanide-resistant oxidase pathway. Although it was not found in mitochondria from yeast, rat liver, or pigeon breast muscle, it did occur in a variety of plant mitochondria in a manner unrelated to the activity of the cyanide-resistant pathway. For example, it was readily detectable in Jerusalem artichoke mitochondria, which have little or no cyanide leak, and was weak in mitochondria from the spadix of *Arum maculatum*, which have the highest known cyanide-resistant activity. Moreover, the spectrum could also be observed in microsomes from Jersualem artichoke and potato tubers. An unusually strong spectrum was observed in mitochondrial preparations from the corollas of daffodils, which again were strongly inhibited by cyanide, and these preparations were rich in carotenoids. The spectrum could no longer be detected in Jerusalem artichoke mitochondria that had been extracted with acetone after freeze-drying. Thus the spectrum seems to be related to carotenoid pigments, but the nature of the metal complexing agents on the carotenoids is obscure.

Frequently, spectral changes due to an oxidase have been detected by the spectral shift induced by carbon monoxide with the preparation in the reduced state. However, the only carbon monoxide-binding pigment that we were able to detect in skunk cabbage mitochondria was cytochrome a_3. This agrees with the work of Plesnicar, Bonner, and Storey (25) with mung bean mitochondria.

DISCUSSION

In this paper we have shown beyond reasonable doubt that residual cytochrome *c* oxidase activity makes only a small contribution to the cyanide-resistant respiration of plant mitochondria, with the corollary that a second oxidase is present

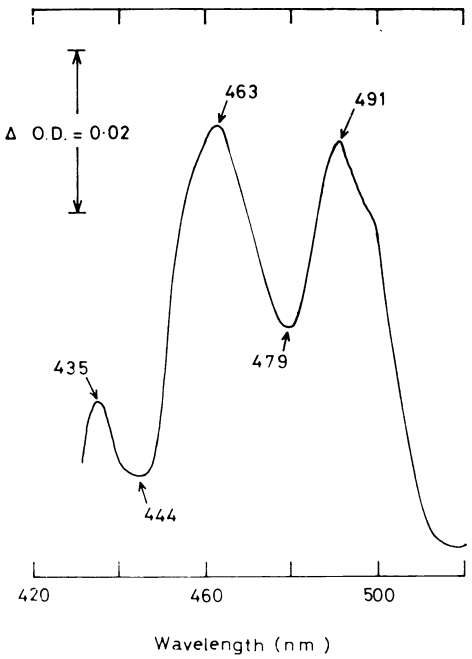

FIG. 10. Low temperature difference spectrum (−196 C) of skunk cabbage mitochondria: aerobic suspension treated with succinate and KSCN minus aerobic suspension treated with succinate. Mitochondria were diluted in the standard medium and incubated with 0.38 M ATP for 5 min at room temperature; FCCP (3 μM) was then added, and the suspension was oxygenated. The positive sample was treated with 7.5 mM succinate and 0.11 M KSCN and oxygenated for 1 min, and the negative sample was treated with 7.5 mM succinate and oxygenated, before the cuvette was frozen.

that is insensitive to the usual terminal inhibitors. Our results are complementary to the kinetic experiments of Storey and Bahr (28), who reached the same conclusion.

The effects of antimycin and the steady states of the carriers in the presence of cyanide confirm the view stated originally by Okunuki (23) that the alternative pathway connects with the normal respiratory chain on the substrate side of cytochrome *c* or the antimycin-sensitive site. However, the possibility that the cyanide-resistant pathway resides in separate particles is not easily eliminated. Perhaps the best evidence against it is the demonstration that substrate oxidation in the presence of cyanide can drive an oxidation of cytochromes *c* and *a* by a reversal of the normal respiratory chain without the mediation of ATP (8).

The second oxidase appears to have unusual properties although there is little positive information by which to characterize it. Our results and those of Storey and Bahr (28) eliminate the possibility that it is a cytochrome, at least for skunk cabbage. The old suggestion that is a flavoprotein seems in many ways to come nearest to a solution, but a simple flavoprotein oxidase would reduce oxygen to hydrogen peroxide, which has not been found. The very low apparent *Km* for O_2 (0.5 μM) found by Ikuma, Schindler, and Bonner (16) with mung bean hypocotyl mitochondria is also inconsistent with a flavoprotein oxidase. We have failed to observe any characteristic spectrum in the visible region that can be ascribed to the second oxidase. The ability to catalyze the reduction of oxygen to water suggests that the enzyme has a rather high oxidation-reduction potential. The theoretical dividing line is determined by the equilibrium of the reaction

$$O_2 + 2H^+ + 2e^- \rightleftarrows H_2O_2$$

for which $E_0' = +0.27$ v at pH 7.0 (24). However, one might expect artificial hydrogen donors such as ascorbate, TMPD, or DAD to reduce a high potential oxidase at a significant rate.

The experiments with thiocyanate and other iron-binding reagents indicate that a non-heme iron protein (or possibly some other type of metallo-protein) is probably a component of the cyanide-resistant pathway. Such a component may either react directly with oxygen or act as an intermediate electron carrier.

The behavior of the *b* cytochromes is puzzling. Although their oxidation and reduction can be observed in the presence of cyanide, the reactions are too sluggish to play an essential role in the alternative pathway of skunk cabbage mitochondria (28). This is even more apparent when cyanide is replaced by antimycin, which very powerfully inhibits the oxidation of *b* cytochromes by either pathway.

Finally, there remains the problem of the physiological importance of the alternate oxidase pathway. The results in Table I show a wide variation in the relative activities of the two oxidase systems. The alternative pathway seems to be associated with tissues that are rapidly growing, such as the etiolated bean hypocotyl, or which liberate large quantities of heat, as in the aroid spadix. Whether the two pathways simply compete with each other *in vivo* or whether some controlling switch operates is unknown.

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LITERATURE CITED

1. BENDALL, D. S. 1958. Cytochromes and some respiratory enzymes in mitochondria from the spadix of *Arum maculatum*. Biochem. J. 70: 381–390.
2. BENDALL, D. S. AND W. D. BONNER. 1966. Optical properties of plant cytochrome oxidase. *In:* B. Chance, R. W. Estabrook, and T. Yonetani, eds., Hemes and Hemoproteins. Academic Press, New York. pp. 485–488.
3. BENDALL, D. S. AND R. HILL. 1956. Cytochrome components in the spadix of *Arum maculatum*. New Phytol. 55: 206–212.
4. BONNER, W. D. 1961. Frozen steady states of respiratory carriers in skunk cabbage mitochondria. *In:* Abstracts of Biophysics Congress, Stockholm. p. 70.
5. BONNER, W. D. 1963. Higher plant cytochromes. Proc. V. Int. Congr. Biochem. II: 50–62.
6. BONNER, W. D. 1965. The oxidases of plant mitochondria. Fed. Proc. 24: 297.
7. BONNER, W. D. 1967. A general method for the preparation of plant mitochondria. *In:* R. W. Estabrook and M. E. Pullman, eds., Methods in Enzymology, Vol. X. Academic Press, New York. pp. 126–133.
8. BONNER, W. D. AND D. S. BENDALL. 1968. Reversed electron transport in mitochondria from the spadix of *Arum maculatum*. Biochem. J. 109: 47P.
9. CHANCE, B. 1957. Techniques for the assay of the respiratory enzymes. *In:* S. P. Colowick and N. O. Kaplan, eds., Methods in Enzymology, Vol. IV. Academic Press, New York. pp. 273–329.
10. CHANCE, B. AND D. P. HACKETT. 1959. The electron transfer chain of skunk cabbage mitochondria. Plant Physiol. 34: 33–49.
11. DIXON, M. 1953. The determination of enzyme inhibitor constants. Biochem. J. 55: 170–171.
12. GEORGE, P. AND J. S. GRIFFITH. 1959. Electron transfer and enzyme catalysis. *In:* P. D. Boyer, H. Lardy, and K. Myrbäck, eds., The Enzymes, Vol. I. Academic Press, New York. pp. 347–389.
13. GILMOUR, M. V., D. F. WILSON, AND R. LEMBERG. 1967. The low-temperature spectral properties of mammalian cytochrome oxidase. II. The enzyme isolated from beef-heart mitochondria. Biochim. Biophys. Acta 143: 487–499.
14. HACKETT, D. P. AND D. W. HAAS. 1958. Oxidative phosphorylation and functional cytochromes in skunk cabbage mitochondria. Plant Physiol. 33: 27–32.
15. IKUMA, H. AND W. D. BONNER. 1967. Properties of higher plant mitochondria. III. Effects of respiratory inhibitors. Plant Physiol. 42: 1535–1544.
16. IKUMA, H., F. J. SCHINDLER, AND W. D. BONNER. 1964. Kinetic analysis of oxidases in tightly coupled plant mitochondria. Plant Physiol. 39: 1x.
17. JAMES, W. O. AND H. BEEVERS. 1950. The respiration of *Arum* spadix. A rapid respiration, resistant to cyanide. New Phytol. 49: 353–374.
18. JAMES, W. O. AND D. C. ELLIOTT. 1955. Cyanide-resistant mitochondria from the spadix of an *Arum*. Nature 175: 89.
19. KEILIN, D. AND E. F. HARTREE. 1939. Cytochrome and cytochrome oxidase. Proc. Roy. Acad. B127: 167–191.

20. LANCE, C. AND W. D. BONNER. 1968. The respiratory chain components of higher plant mitochondria. Plant Physiol. 43: 756–766.
21. LUNDEGÅRDH, H. 1960. The cytochrome-cytochrome oxidase system. *In:* W. Rhuland, ed., Encyclopedia of Plant Physiology. Springer-Verlag, Berlin. pp. 311–364.
22. NICHOLLS, P. AND H. K. KIMELBERG. 1968. Cytochromes *a* and a_3. Catalytic activity and spectral shifts *in situ* and in solution. Biochim. Biophys. Acta 162: 11–21.
23. OKUNUKI, K. 1939. Über den Gaswechsel der Pollen. II. Acta Phytochim. 11: 27–64.
24. PFEFFER, W. 1906. The Physiology of Plants, Vol. III (A. J. Ewart, transl.), Ed. 2. Oxford. pp. 372–377.
25. PLESNICAR, M., W. D. BONNER, AND B. T. STOREY. 1967. Peroxidase associated with higher plant mitochondria. Plant Physiol. 42: 366.
26. POTTER, V. R. AND A. E. REIF. 1952. Inhibition of an electron transport component by antimycin A. J. Biol. Chem. 194: 287–297.
27. SIMON, E. W. 1957. Succinoxidase and cytochrome oxidase in mitochondria from the spadix of *Arum*. J. Exp. Bot. 8: 20–35.
28. STOREY, B. T. AND J. T. BAHR. 1969. The respiratory chain of plant mitochondria. I. Electron transport between succinate and oxygen in skunk cabbage mitochondria. Plant Physiol. 44: 115–125.
29. TYLER, D. D., R. W. ESTABROOK, AND D. R. SANADI. 1965. Electron and energy requirements for cytochrome *b* reduction during the oxidation of tetramethyl-*p*-phenylene diamine. Biochem. Biophys. Res. Commun. 18: 264–269.
30. VAN HERK, A. W. H. 1937. Chemical processes in *Sauromatum* spikes. II. Proc. Acad. Sci. Amsterdam 40: 607–614.
31. VAN HERK, A. W. H. 1937. Chemical processes in *Sauromatum* flowers. Proc. Acad. Sci. Amsterdam 40: 709–719.
32. VAN HERK, A. W. H. 1937. Die chemischen Vorgänge in *Sauromatum*-Kolben. Rec. Trav. Bot. Neer. 34: 69–156.
33. VAN HERK, A. W. H. AND N. P. BADENHUIZEN. 1934. Respiration and catalase action in the *Sauromatum* spadix. Proc. Acad. Sci. Amsterdam 37: 99–105.
34. WILSON, D. F. 1967. Azide inhibition of mitochondrial electron transport. II. Spectral changes induced by azide. Biochim. Biophys. Acta 131: 431–440.
35. WISKICH, J. T. AND W. D. BONNER. 1963. Preparation and properties of sweet potato mitochondria. Plant Physiol. 38: 594–604.
36. YOCUM, C. S. AND D. P. HACKETT. 1957. Participation of cytochromes in the respiration of the aroid spadix. Plant Physiol. 32: 186–191.
37. YONETANI, T. 1960. Studies on cytochrome oxidase. I. Absolute and difference absorption spectra. J. Biol. Chem. 235: 845–852.