The Respiratory Chain of Plant Mitochondria. I. Electron Transport Between Succinate and Oxygen in Skunk Cabbage Mitochondria

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Abstract. The kinetics of oxidation of ubiquinone, flavoprotein, cytochrome $c$, and the cytochrome $b$ complex in skunk cabbage (Symlocarpus foetidus) mitochondria made anaerobic with succinate have been measured spectrophotometrically and fluorimetrically in the absence of respiratory inhibitor and in the presence of cyanide or antimycin A. No component identifiable by these means was oxidized rapidly enough in the presence of one or the other inhibitor to qualify for the role of alternate oxidase. Cycles of oxidation and reoxidation of flavoprotein and ubiquinone obtained by injecting 12 $\mu$M oxygen into the anaerobic mitochondrial suspension were kinetically indistinguishable in the presence of cyanide or antimycin A, implying that these 2 components are part of a respiratory pathway between succinate and oxygen which does not involve the cytochromes and does involve a cyanide-insensitive alternate oxidase. The cytochrome $b$ complex shows biphasic oxidation kinetics with half times of 0.018 sec and 0.4 sec in the absence of inhibitor, which increase to 0.2 sec and 1 sec in the presence of cyanide. In the presence of antimycin A, the oxidation of the cytochrome $b$ complex shows an induction period of 1 sec and a half-time of 3.5 sec. A split respiratory chain with 2 terminal oxidases and a branch point between the cytochromes and flavoprotein and ubiquinone is proposed for these mitochondria.

Many plant tissues yield mitochondria whose rate of respiration is partially insensitive to inhibition by cyanide (34, 42). The most notable of such tissues are the spadices of the flowers of Arum maculatum (1, 2) and the skunk cabbage, Symlocarpus foetidus (3, 28, 29). Mitochondria isolated from these tissues are often totally insensitive to respiratory inhibition by cyanide or antimycin A. The cytochromes of skunk cabbage mitochondria have been characterized by their spectra (4, 16, 19) which are virtually identical to those observed in other plant mitochondria, regardless of tissue source and degree of cyanide sensitivity (4, 37). In particular, skunk cabbage mitochondria have 3 cytochromes $b$ which are mostly reduced in the aerobic steady state in the presence of substrate and HOQNO$^1$ or antimycin A, as is the case with mitochondria from other plants (5, 6, 16). [Mitochondria from Arum maculatum are an exception, in that cytochrome $b_1$ in these mitochondria remains oxidized in the presence of antimycin A (1)]. This observation has been interpreted in terms of 2 terminal oxidases in plant mitochondria, 1 a cytochrome sensitive to inhibition by cyanide, the other an enzyme of unknown structure insensitive to cyanide inhibition (4, 42). Ben-dall, Bonner, and Plesnicar (3) have postulated that the oxidase of unknown structure is a non-heme iron protein and that the alternate pathway of electron transport through this oxidase in skunk cabbage mitochondria involves only pyridine nucleotide, flavoprotein, and the oxidase.

The property of resistance to respiratory inhibitors makes skunk cabbage mitochondria a most useful preparation with which to study the oxidation kinetics of the respiratory chain carriers by means of oxygen pulses into an anaerobic suspension of mitochondria. Using this technique, Chance, Schoener, and DeVault (20) have shown that the time sequence of oxidation of the cytochromes is characteristic of their positions in the respiratory chain. The comprehensive kinetic studies of this type which have been carried out on mammalian mitochondria and derived submitochondrial particles are summarized in a recent review (25); much insight into the molecular mechanism of electron transport has been gained from them (13, 22). Such oxygenation cycles can be carried out with mammalian mitochondria in the presence of cyanide or antimycin A only if these are at concentrations low enough that the cytochrome chain is only partially inhibited (24); with plant mitochondria, it is possible to use concentrations high enough to ensure virtually complete inhibition of the cytochrome chain and still obtain oxidation-reduction cycles with oxygen pulses. In this manner, the kinetic organization of the respiratory chain can be studied in both the inhibited and uninhibited state. Further, the question of which respiratory carriers,

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$^1$ Abbreviations: HOQNO: 2-Heptyl hydroxyquinoline-N-oxide; MOPS: morpholinopropane sulfonic acid; BSA: bovine serum albumin; TES: tris(hydroxymethyl)methylaminoethanol sulfonic acid; 1799: bis(hexafluoroacetylonyl) acetone.
identifiable by spectrophotometry or fluorimetry, are kinetically competent in the inhibited state to function as an alternate oxidase, or as part of an enzyme complex forming an alternate oxidase, can also be studied. Chance and Hackett (16) and Chance and Bonner (19) studied the kinetics of oxidation-reduction cycles of the respiratory chain carriers in skunk cabbage mitochondria—these being the only 2 instances of the application of this kinetic technique to plant mitochondria—but only in the absence of respiratory inhibitors. In this paper, we report comparative kinetics for the oxidation of respiratory carriers in skunk cabbage mitochondria in the absence and presence of the respiratory inhibitors, cyanide, and antimycin A.

**Experimental Procedures**

Skunk cabbage (*Symlocarpus foetidus*) flowers were collected from selected swampy points in Delaware and Montgomery Counties of Pennsylvania. Union County, New Jersey, and Tompkins County, New York. They were stored at 4°. Mitochondria were prepared from the excised spadices by means of the method described by Bonner (7) with minor modifications. The spadices were diced into small pieces about 5 mm on a side and suspended in cold medium containing 0.3 M mannitol, 10 mM EDTA, 4 mM cysteine, 10 mM MOPS, and 0.1% BSA, fraction V, at pH 7.4. About 80 g spadix tissue per liter of medium proved optimal. The tissue was homogenized in a Waring Blender with 2 periods of 5 sec at high speed separated by 5 sec. The grinding head of the Blender was replaced by a “Polytron” head (Brinkman Instruments, Inc.) with a 313 μm + 366 μm primary filter and a 400 μm to 3000 μm secondary filter. The energy-linked reduction of fluorescent flavoprotein (23) under the same conditions was also measured with this fluorimeter fitted with a Heraeus ST-75 mercury arc lamp, using a 436 μm primary and a 500 μm to 3000 μm secondary filter.

The kinetic measurements were carried out in a manually operated regenerative flow apparatus with 0.1 cm light path and in a similar, hydraulically operated apparatus with 0.4 cm light path. The latter apparatus is similar to the one described in detail by Chance (22); the calibration of the former one, also designed by Chance (22), has been reported by Storey (40). In these experiments, the aerobic mitochondrial suspension was preincubated for 10 min with 130 μM ADP and 10 μM 1790 (designation for the uncoupler bis-(hexafluoroacetylonyl)-acetone, which was kindly provided by Dr. P. Heytler of E. I. du Pont de Nemours, Inc.) to deplete the mitochondria of energy conservation capacity. The mitochondrial suspension was made anaerobic by addition of 3 mM succinate; 6 mM malonate was then added to partially inhibit succinic dehydrogenase and thus maintain the flux of reducing equivalents through the respiratory chain at a very low level (B. Chance, unpublished method). Under these conditions, the extent of oxidation of the respiratory carriers is greater than 95% in the aerobic steady state, as determined in separate control experiments. A large reservoir of reducing equivalents is available, however, so that numerous oxygenation cycles may be carried out on a single sample. The flow experiment is initiated by mixing the anaerobic mitochondrial suspension with oxygen-saturated medium at a volume ratio of 100/1, giving an initial oxygen concentration of 12 μM. Absorbance changes corresponding to the oxidation of ubiquinone, cytochrome c, cytochrome b, and flavoprotein were measured as a function of time with the dual wavelength spectrophotometer (10). Fast changes were displayed on a storage oscilloscope and photographed to give permanent records; slow changes were displayed on a pen recorder chart. Ubiquinone was monitored with the wavelength pair 275 to 290 μm or 282 to 295 μm (17, 39). For cytochrome c, the pair 549 to 540 μm was used. Absorbance changes due to the cytochrome b components were followed at 560 to 575 μm (9), while those due to flavoprotein were followed with the wavelength pair 468 to 488 μm, which minimizes interference from the cytochrome components. The wavelength pairs 465 to 510 μm (18) and 475 to 510 μm (23) are not kinetically “clean” in these mitochondria and show absorbance changes attributable to the rapidly oxidized cytochromes. The oxidation kinetics of fluorescent flavoprotein and pyridine nucleotide were also measured in the flow apparatus by means of a rotating disc fluorimeter which permits the simultaneous recording of changes in both components (22). Pyridine nucleotide fluorescence emission
detected at 450 m\(\mu\) was excited at 366 m\(\mu\); flavoprotein fluorescence detected at 570 m\(\mu\) was excited at 436 m\(\mu\). The exciting light source was a water-cooled high pressure mercury arc, and the wavelengths indicated were selected by means of appropriate primary and secondary filters.

Difference spectra of the mitochondrial suspensions were obtained with the split beam spectrophotometer described by Chance (10). Spectra in the ultraviolet region were obtained at room temperature; those in the visible region were obtained at liquid nitrogen temperature using the technique described by Estabrook (26) as modified by Bonner (4).

**Results**

**Integrity of the Mitochondrial Membrane.** Evidence that cytochrome \(b\) functions on a branch pathway of electron transport in mammalian submitochondrial particles, rather than on the main pathway, was presented some years ago by Chance (11). This finding raised the point that damage to the mitochondrial membrane can alter the organization of the respiratory chain. Further evidence has accumulated that such alteration indeed occurs, and that both cytochrome \(b\) and ubiquinone are affected; the oxidation rate of cytochrome \(b\) is slower, the more altered the mitochondrial particle, while that of ubiquinone is faster (25). It was therefore necessary to show that the skunk cabbage mitochondria used in this kinetic study had undamaged membranes. Bonner gives 3 properties which isolated mitochondria must possess if they are to be considered intact (7). These are: A) good respiratory control by ADP concentration (15) and retention of this property for some hr following isolation; B) no acceleration by externally added NAD\(^+\) of the oxidation rate of malate, or acceleration by added cytochrome \(c\) of any respiration rate; C) retention of mitochondrial fine structure and minimal contamination by microsomal fragments. Skunk cabbage mitochondria, when isolated as described above, regularly possess the last 2 properties, but respiratory control by ADP concentration is usually difficult to demonstrate. The energy-linked reduction of endogenous pyridine nucleotide by succinate oxidation was regularly observed with these mitochondria, however, and this property was retained for at least 4 hr after isolation in ice-cooled preparations. It was taken as indicating an intact mitochondrial membrane, not because the energy conservation capacity is functional, but because the endogenous pyridine nucleotide is evidently prevented from leaking out. Reduction of pyridine nucleotide by succinate oxidation, measured fluorimetrically, is shown in figure 1A; the reduction is inhibited by preincubating the mitochondria with ADP and uncoupler, as shown in figure 1B. The corresponding energy-linked reduction of fluorescent flavoprotein is shown in figure 1C. The fluorescence decrease is much less after preincubation with ADP and uncoupler as is evident from figure 1D.

The problem of respiratory control and energy conservation in skunk cabbage mitochondria is considered in greater detail in an accompanying paper (41). In this study, the mitochondria were treated with uncoupler and ADP to produce the depleted condition which is characterized by the fluorescent flavoprotein and pyridine nucleotide responses shown in figures 1B and 1D. Under these conditions, the energy conserving capacity of the mitochondria is lost, and the process being examined during oxidation cycles is presumably electron transport alone. Attempts to measure carrier kinetics in the coupled state were unsuccessful, because the mitochondria became uncoupled within one-half hr of being suspended in the reaction medium, even at 18°.

**Stability of the Mitochondrial Suspension.** One of the great advantages of the regenerative flow apparatus is that numerous measurements may be carried out with the same sample. This requires that the mitochondrial suspension be held for the full period of the experiment at the desired reaction temperature, and the stability of the preparation becomes a problem. Breakdown of the suspension was signalled by coagulation of the mitochondria and, on occasion, by a sharp decrease in the oxidation rate of the respiratory carriers. The problem was aggravated by higher temperatures; at 26°, maximum working time was 3 hr or less; while at
18°, the suspension did not appear to have altered after 5 hr. The experiments with cyanide and antimycin A required that the rate measurement be carried out for each carrier on the same sample, first without inhibitor and then with inhibitor. The experiments were carried out at 18° and were complete in about 4 hr, thus ensuring that the kinetic properties of the mitochondria remained constant during the period of the measurements.

**Kinetics of Flavoprotein and Pyridine Nucleotide Oxidation.** One difficulty in measuring the kinetics of mitochondrial flavoprotein by absorbance change is that of interference from cytochrome absorbance changes. The use of fluorimetry offers a more specific method (21). Since the mitochondria have been depleted with ADP and uncoupler according the conditions described in figures 1B and 1D, only the higher potential flavoproteins of low fluorescence, presumably $F_{Pp}$ and $F_{ps}$ (23), are reduced by succinate while the highly fluorescent component of the lower potential flavoproteins, presumably $F_{pm}$ and $F_{pt}$ (27), remain oxidized. The kinetics of oxidation of flavoprotein, as measured both by absorbance and by fluorimetry, are shown in figure 2.

There is no deflection of the trace during the flow in either record; when the flow stops, both the fluorescence (fig 2A) and absorbance (fig 2B) changes proceed with a half-time of 180 msec, a value quite comparable to that observed with mammalian and avian heart mitochondria (14). The kinetics of pyridine nucleotide oxidation are measured simultaneously with those of fluorescent flavoprotein on the record in figure 2A; this reaction proceeds with a half-time of 260 msec. The full cycle of oxidation and reduction, whose initial part is recorded in figure 2, is shown in figure 3. The flavoprotein fluorescence and absorbance changes are in synchrony, with the time to half-reduction—$t_{1/2}$ offset (31)—being about 80 sec in each case. Reduction is complete for flavoprotein, but not for pyridine nucleotide. This component appears to undergo biphasic reduction, but the second slow

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**A—Flavoprotein and Pyridine Nucleotide Fluorescence**

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**B—Flavoprotein Absorbance 468-488 m\(\mu\)**

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**Fig. 2.** Oxidation rate of flavoprotein and pyridine nucleotide in skunk cabbage mitochondria at 22°, the former measured both by fluorescence and absorbance. The mitochondrial suspension contains 4 mg protein/ml; the light path is 0.1 cm. Succinate and malonate are present at 3 mM and 6 mM, respectively. Fluorescence changes corresponding to flavoprotein and pyridine nucleotide were measured simultaneously on freshly prepared, depleted mitochondria, followed by measurement of flavoprotein absorbance on the same sample in the same flow apparatus. Pyridine nucleotide fluorescence was excited with light at 366 m\(\mu\); flavoprotein fluorescence was excited with light at 436 m\(\mu\).
mitochondria at a fresh one. made after does undetectable.

ubiquinone of evident. clearly records absorbance of for measured pension as measured this absence of cycle of A. in ADP and uncoupler to deplete their energy conservation capacity as described above. The cyanide concentration was 0.3 mM in this series of experiments, which, from the results of Ikuma and Bonner (33) on cyanide inhibition of respiration of mung bean mitochondria, should provide essentially complete inhibition of cytochrome oxidase. The time course of a cycle of

\[ \text{UBIQUINONE } 275-290 \mu \text{m} \]

**Fig. 4.** Oxidation and reduction cycles of ubiquinone as measured at 275 to 290 mm in the presence and absence of cyanide at 18°. Ubiquinone content of these mitochondria is 1.6 nmol/mg protein. Mitochondrial protein concentration is 2.8 mg/ml and the light path is 0.1 cm in these experiments; succinate and malonate are present at 3 mm and 6 mm respectively. A) Oxidation of ubiquinone in the absence of inhibitor; the half-time for oxidation is 300 msec. The apparent decrease in absorbance at 275 mm during the flow is caused by dilution of the suspension; upon completion, the trace returns to this position as shown by the arrow marked "final", rather than to the original position. Oxidation is essentially complete in the steady state. B) Complete cycle of oxidation and reduction of ubiquinone in the absence of inhibitor on a time scale 10-fold slower than in A. The absorbance change caused by dilution is clearly evident. C) Complete cycle of oxidation and reduction of ubiquinone in the same mitochondrial suspension as in A and B, but in the presence of 0.3 mM KCN, recorded on the same time scale as B. The half-time for oxidation is 2 sec, and the degree of oxidation in the steady state is 48%.

OXYGEN CYCLES ± KCN

phase does not go to completion. The fluorescence records of figure 2 and figure 3 were obtained with a fresh mitochondrial suspension, the absorbance measurement being made directly after the fluorescence one. If the fluorescence measurements are made after a large number of oxygen cycles, the pyridine nucleotide fluorescence is then virtually undetectable. The flavoprotein fluorescence is still readily measured under these conditions and gives the same result obtained by means of the spectrophotometric measurement at 468 to 488 mm.

The difference extinction coefficient \( \Delta e^* \) for reduced minus oxidized flavoprotein at 468 to 488 mm was estimated to be 4 mm\(^{-1}\) cm\(^{-1}\) from the spectrum of isolated succinic dehydrogenase presented by King (36), using 10 mm\(^{-1}\) cm\(^{-1}\) at the extinction coefficient for the wavelength pair 475 to 510 mm (27). The concentration of flavoprotein reducible by succinate in the depleted condition is calculated to be 0.5 to 0.7 nmol/mg protein from the absorbance change observed. This is about one-third to one-fourth of the flavoprotein reducible by dithionite in skunk cabbage mitochondria reported by Lance and Bonner (37), who used the wavelength pair 460 to 510 mm for their calculation.

**Fig. 5.** Oxidation and reduction cycles of flavoprotein, as measured with the wavelength pair 468 to 488 mm, in the absence and presence of cyanide at 18°. In records A and B, the mitochondrial suspension contains 3.0 mg protein/ml; in C and D, it contains 2.7 mg protein/ml. Otherwise, the reaction conditions are identical. The light path is 0.1 cm; the response time is 50 msec in A and 220 msec in C. A) Oxidation in the absence of cyanide. No reaction occurs during the flow. When the flow stops, oxidation proceeds with a half-time of 180 msec, as indicated by an upward deflection of the trace. A slight disturbance during the flow results in a small downward deflection of the trace. Oxidation is essentially complete in the steady state. B) Trace obtained on a recorder with the time scale indicated, showing the time course of reduction of this component in the absence of cyanide. C) Oxidation in the presence of cyanide, recorded on a time scale 10-fold slower than that of A. The half-time for oxidation is 1 sec; the extent of oxidation is 75% in the steady state. D) Trace obtained on a recorder with the time scale indicated, showing the time course of flavoprotein reduction in the presence of cyanide.
oxidation and reduction is shown in figure 4 for ubiquinone, figure 5 for flavoprotein, figure 6 for cytochrome b, and figure 7 for cytochrome c, in the absence and presence of 0.3 mM cyanide. These records were all obtained with the same mitochondrial suspension and thus are directly comparable allowing for the decrease in mitochondrial protein due to dilution as the experiment proceeded. The half-times for oxidation and re-reduction, half-time being 2 sec, and t1/2 off (31), are summarized in table I.

In the absence of inhibitor, the half-time for ubiquinone oxidation is 300 msec, a value very similar to that obtained with mammalian and avian heart mitochondria under the same conditions (14). The time to half-reduction, t1/2 off, is 6 sec. In the presence of cyanide, the oxidation rate is decreased by a factor of 6, the half-time being 2 sec, and t1/2 off is increased to 18 sec. Flavoprotein in these mitochondria is affected similarly.

Cytocrome b, as might be expected of a complex made up of 3 separate components, does not show simple kinetics. There is an initial fast oxidation with a half-time of 18 msec followed by a slower one with a half-time of 400 msec. This biphasic oxidation also occurs in the presence of cyanide, but now the half-times have increased to 200 msec and 1 sec. Cyanide also inhibits the reduction of cytochrome b to the extent that the reduction remains incomplete after a cycle, as is evident from comparison of figures 5C and 5F. This incomplete reduction was also noted by Chance and Hackett (16).

Cytocrome c is oxidized with a half-time of 3.1 msec in the absence of inhibitor, a value close to that measured for this respiratory carrier in rat liver mitochondria (22). There is a further slow oxidation which accounts for about 10% of the absorbance change; a similar slow oxidation phase was noted by Chance and Bonner (19) in skunk cabbage mitochondria reduced by endogenous substrate only. The reduction part of the cycle also shows a slow phase comprising 10% of the absorbance change. Cytocrome c shows a t1/2 off of 1.6 sec under these conditions. In the presence of cyanide, there is oxidation of cytocrome c with a half-time of 3.8 msec, which is nearly the half-time in the uninhibited system. The maximum extent of oxidation is only 30%, however. Reduction occurs in 2 phases after a steady state lasting about 100 msec. The first phase corresponds to a t1/2 off of about 150 msec. The absorbance change due to the slow phase is again about 10% of the total change due to cytocrome c in the absence of inhibitor.

The kinetic behavior of cytocrome c upon oxygenation of the anaerobic mitochondrial suspension in the presence of cyanide is most readily explained by a binding site for cyanide on reduced cytocrome oxidase which is different from the site responsible for inhibition of the reduction of oxidized cytochrome a3 by reduced cytochrome a, and which does not interfere with the oxidation of cytochrome a3 by oxygen. Yonetani has recently summarized the argument for 2 such sites (43) deriving both from his own work, and from that of Keilin and Hartree (35) and Chance (9). The pattern of cytocrome c oxidation shown in figure 6C would then reflect the following sequence: oxidation of reduced cytochrome a3 by oxygen followed by oxidation of reduced cytochrome a in less than 0.5 msec (22); reduced cytochrome c is then in turn oxidized with its normal half-time. Once cytochrome a3 is oxidized, however, the cytocrome present binds to the inhibitory site, and the flow of electrons through the oxidase to oxygen is effectively cut off. The effect is that of an oxygen pulse much lower in concentration than the one actually used.

<table>
<thead>
<tr>
<th>Mitochondrial preparation</th>
<th>Inhibitor</th>
<th>Fp</th>
<th>Q</th>
<th>Time of oxidation. t1/2 on, msec</th>
<th>c</th>
</tr>
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<tbody>
<tr>
<td>28-054</td>
<td>...</td>
<td>180</td>
<td>300</td>
<td>18 : 400</td>
<td>3.1</td>
</tr>
<tr>
<td>28-017</td>
<td>0.3 mM KCN</td>
<td>1000</td>
<td>2000</td>
<td>200 : 1000</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>...</td>
<td>200</td>
<td>400</td>
<td>15 : 400</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>0.7 μg/mg prot</td>
<td>1000</td>
<td>2000</td>
<td>3500</td>
<td>3.4</td>
</tr>
</tbody>
</table>

1 Oxidation is biphasic: half-times given are estimates for the 2 phases.
2 Oxidation did not start until about 1 sec after mixing the mitochondrial suspension with oxygenated medium.
3 Maximum extent of oxidation during the cycle is 75% for Fp, 48% for Q, 76% for b, and 30% for c in the presence of 0.3 mM KCN.
4 Maximum extent of oxidation during the cycle is 85% for Fp, 53% for Q, 20% for b, and 100% for c in the presence of 0.7 μg antimycin A/mg protein.

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Table I. Half Times of Oxidation, t1/2 on, and Cycle Times to Half-reduction, t1/2 off, of the Respiratory Carriers of Skunk Cabbage Mitochondria, as Determined by O2 Pulse Experiments

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Fig. 6. Oxidation and reduction cycle of cytochrome b, as measured at 560 mµ to 575 mµ in the absence and presence of cyanide at 18°. In records A, B, and C, the mitochondrial suspension contains 3.1 mg protein/ml; in records D, E, and F, it contains 2.7 mg protein/ml. Otherwise, the reaction conditions are identical to those in figure 4. The light path is 0.1 cm, and the response time 50 msec in all experiments. A) Oxidation of cytochrome b in the absence of inhibitor. Some oxidation occurs during the flow as shown by upward deflection of the trace to a plateau, followed by a further, rapid upward deflection on cessation of flow. This reaction is in turn followed by a slower one as shown by a continuous rise in the trace. The flow velocity is 109 cm/sec, and the calculated first order rate constant for the rapid oxidation is 38 sec\(^{-1}\), corresponding to a half-time of 18 msec. B) The biphasic nature of the oxidation is evident from this record with a slower time scale. The half time of the slow oxidation is estimated to be 400 msec. Oxidation is essentially complete in the steady state which lasts about 1 sec; reduction then begins as shown by the slow downward deflection of the trace. C) Trace obtained simultaneously with B on a recorder with the time scale indicated, showing the slow, biphasic reduction of cytochrome b. Response time of the recorder is 1 sec. D) Oxidation of cytochrome b in the presence of 0.3 mM KCN recorded on a fast time scale at twice the sensitivity of the records A and B. There is no deflection during the flow as in A; on cessation of the flow, oxidation proceeds with a half-time of 200 msec. E) A second, slower oxidation occurring in the presence of cyanide is evident from the record obtained with a time scale 5-fold slower than that for D; the half-time is about 1 sec. The sensitivity is the same as for record D and twice that for records A and B; oxidation proceeds to 76% of the extent observed in the absence of cyanide. The arrow marked "final" indicates the final position of the trace after completion of the cycle. F) Trace obtained simultaneously with E on a recorder with the time scale indicated. The slow part of the oxidation is evident in this trace, as is the incomplete reduction of this component at the end of the cycle.

Fig. 7. Oxidation and reduction cycles of cytochrome c as measured at 549 to 540 mµ in the absence and presence of cyanide at 18°. In experiments A and B, the mitochondrial suspension contains 3.3 mg protein/ml; in experiment C, it is 2.6 mg protein/ml; otherwise the reaction conditions are identical to those in figure 4. The light path is 0.1 cm; the response time in all 3 experiments is 50 msec. A) Oxidation of cytochrome c on short time scale. Addition of oxygenated medium results in oxidation during flow; there is a rapid upward deflection of the trace to a plateau during the flow followed by a further rapid deflection when the flow stops. Cytochrome c is essentially completely oxidized under these conditions. The calculated first order rate constant for oxidation is 226 sec\(^{-1}\), corresponding to half-time of 3.1 msec. There is then a slow upward deflection, comprising about 10% of the total absorbance change, to the oxidized steady state. The reduction which completes the cycle is signaled by a downward deflection of the trace. B) Repeat of the experiment shown in A, but with 10-fold slower time scale. Biphasic reduction of this component is evident; 90% is rapidly reduced, while the remaining 10% is slowly reduced, resulting in the "tail" seen in the record. C) Oxidation of cytochrome c in the presence of 0.3 mM KCN, recorded at twice the sensitivity of A and B. During the flow, there is an upward deflection of the trace to a plateau followed by a second upward deflection to an apparent steady state when the flow stops. The calculated rate constant is 182 sec\(^{-1}\), corresponding to a half-time of 3.8 msec. This state lasts for 0.1 sec and is followed by rapid biphasic reduction. About 30% of this component is oxidized under these conditions.
Kinetics in the Absence and Presence of Antimycin A. As in the experiment with cyanide, these kinetics were all obtained with the same mitochondrial suspension and hence are directly comparable. The half-times obtained in this series of experiments are also listed in table I. The oxidation-reduction cycle for ubiquinone in the presence of antimycin A is nearly indistinguishable from the cycle carried out in the presence of cyanide (fig 4C). Essentially the same extent of oxidation is attained: 53% with antimycin A as compared to 48% with cyanide. The cycle for flavoprotein in the presence of antimycin A is also very similar to that in the presence of cyanide, as is evident from the half-times in table I. The extent of oxidation in the steady state was 85%; with cyanide the extent of oxidation was 75%. The time course of an oxidation-reduction cycle in the absence and presence of antimycin A is shown in figure 8 for the cytochrome b complex. In the presence of antimycin A there is essentially no oxidation in the first sec; a slow oxidation with a half-time of 3.5 sec then follows. That oxidation can be observed at all appears to be the result of the very slow rate of reduction under these conditions, the cycling time being 270 sec. The oxidation of cytochrome c proceeds with the same half-time in the absence and presence of antimycin A, 3.2 msec in the first instance and 3.5 msec in the latter one. The slow oxidation and reduction seen at 549 to 540 mμ, which is observed in figure 7A is abolished by antimycin A. The inhibitor also increases the cycling time for cytochrome c nearly a factor of 10.

Discussion

Two points emerge directly from the results reported in this paper. First, the cytochrome b complex must be peripheral to electron transport through the alternate oxidase, since its rate of oxidation in the presence of antimycin A is far too slow for it to play a kinetically competent role in that process. In fact, in the presence of cyanide, its first oxidation half-time of 200 msec is still slow by mammalian standards for a cytochrome closely associated with a terminal oxidase (22). Second, the oxidation rates and cycle times for flavoprotein and ubiquinone are essentially the same in the presence of antimycin A or cyanide, whereas the oxidation rates and cycle times for cytochrome c and the cytochrome b complex are very different with the 2 different inhibitors. Either inhibitor appears to isolate from the cytochromes a respiratory pathway lying between succinic dehydrogenase and oxygen and interacting with flavoprotein and ubiquinone. The oxidation rate of flavoprotein is too slow in the presence of either inhibitor to qualify it as a terminal oxidase or as closely associated with one. There is, in fact, no component identifiable by differential spectroscopic methods which qualifies for the role of alternate oxidase on kinetic grounds. The proposal by Bendall, Bonner, and Plesnicar that a non-heme iron protein functions as an alternate oxidase in these mitochondria (3) is in accord with this observation.

In the absence of inhibitor the half-time of oxidation, t_{1/2 on} for flavoprotein is less than that for ubiquinone, whereas the t_{1/2 off} for flavoprotein is greater than that for ubiquinone. The same relationship between the 2 half-times for these components holds in the presence of either cyanide or antimycin A. For an analysis of the position of these components in the respiratory chain of skunk cabbage mitochondria, we have applied a set of ordering theorems developed by Higgins (30, 31).
who has analyzed in detail the problem of multi-enzyme systems in which the enzymes are arranged in a linear array. He was able to show that the $t_{1/2_{off}}$ for each enzyme in the system were ordered for a wide range of enzyme and substrate concentrations and of reaction rate constants. In the multi-enzyme sequence A $\rightarrow$ B $\rightarrow$ C $\rightarrow$ S, where S is substrate, the direction of the ordering is $t_{1/2_{off}}$ (C)<$t_{1/2_{off}}$ (B)<$t_{1/2_{off}}$ (A), and the same direction of ordering holds for $t_{1/2_{on}}$. In terms of our experiments with oxygen pulses, the $t_{1/2_{on}}$ and the $t_{1/2_{off}}$ for each carrier in a linear respiratory chain should be smaller the nearer the carrier is to the oxidase. This ordering theorem is not completely general, but in practice, extreme conditions, not even approached by the system under study, must be chosen to produce a disordering. In addition, he showed that only the enzyme reacting with the substrate shows no induction period: enzymes further removed from the substrate show induction periods. In practice, these induction periods are difficult to observe. At no point during this study could we observe an induction period for the oxidation of flavoprotein, but induction periods of 50 to 100 msec were observed for ubiquinone in a number of the mitochondrial preparations. An example is shown in figure 9. If we assume that flavoprotein and ubiquinone are part of a linear sequence of respiratory carriers, a contradiction results. On the basis of the $t_{1/2_{off}}$ sequence, ubiquinone should be placed between flavoprotein and oxygen. On the basis of the $t_{1/2_{on}}$ sequence and the observed induction period for quinone oxidation, flavoprotein should be placed between ubiquinone and oxygen. It is evident that these 2 carriers cannot both be part of a linear respiratory chain transporting electrons from succinate to oxygen.

The analog computer was used to examine a number of non-linear arrangements of the respiratory carriers of skunk cabbage mitochondria in order to ascertain which ones were compatible with the kinetic data and the ordering theorems. The simplest of the compatible arrangements is shown in figure 10. There are 2 paths to oxygen, one through cytochrome c and cytochrome oxidase, the other through the cyanide insensitive alternate oxidase. Since the kinetics of ubiquinone and flavoprotein oxidation are the same in the presence of antimycin A as in the presence of cyanide, the branch point for these 2 pathways, designated Y, is placed before the site of antimycin A inhibition. The nature of Y is unknown, but it may well be a non-heme iron protein as postulated for the alternate oxidase (3).

**Fig. 9.** Oxidation of ubiquinone in the absence of inhibitor, recorded at an oscilloscope sweep speed of 200 msec/cm. Reaction conditions are the same as those described in figure 4; the mitochondrial suspension contains 2.7 mg protein/ml. Two experiments are shown on the same record; the letters a and b serve to correlate the absorbance change with the appropriate flow velocity trace. There is a slight downward deflection of the trace during the flow due to dilution: The trace returns to this new position after the cycle as shown by the arrows marked "final." After the flow has stopped, the traces remain level for about 80 msec, after which they show an upward deflection corresponding to the oxidation of ubiquinone.

**Fig. 10.** Proposed scheme of electron transport from succinate to oxygen in uncoupled skunk cabbage mitochondria. The symbol Y is used for the branch point and the symbol X is used for the alternate oxidase. Inhibition sites are shown by dashed lines.

Ubiquinone 275-290m$\mu$

\[
\text{Succ} \quad \text{---\hspace{0.5cm}Malonate} \quad \text{---} \quad \text{Fp} \underset{\text{Q}}{\longrightarrow} \quad \text{Anti. A} \quad \text{---} \quad \text{CN}^- \quad \text{---}
\]

O$_2$

\[
\text{O}_2
\]

\[
\text{a}_3
\]

\[
\text{X}
\]

\[
\text{b}
\]

\[
\text{a}
\]

\[
\Delta \text{O.D} = 0.007
\]

\[
\text{12}\mu\text{M O}_2 \quad \text{0.2sec}
\]

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Placement of ubiquinone on a side pathway connecting to flavoprotein removes it from the strictures of the ordering theorems mentioned above. The kinetic behavior of a redox enzyme or coenzyme so placed in a respiratory chain varies with the values of the rate constants controlling the redox reactions with its partner on the "main sequence" of electron transport. We have examined a simple non-linear sequence of this type with the analog computer. For a wide range of rate constants the $t_{1/2}$ on of the component on the side pathway may be equal to or greater than that observed for its main sequence partner, while its $t_{1/2}$ off may be equal to or less than that of its partner. If the rate constants are made sufficiently large, the component on the side pathway will be in rapid equilibrium with its main sequence partner and the 2 will be kinetically indistinguishable. This case applies to the component we have designated flavoprotein, which most probably consists of 2 flavoproteins, $F_{pA}$ and $F_{pB}$, here behaving as a single kinetic entity.

The fast component of the cytochrome b kinetics has been placed in figure 10 according to the requirements of the ordering theorems. The $t_{1/2}$ off values in the presence of cyanide or antimycin A show that this cytochrome b cannot be part of a linear sequence between succinate and the alternate oxidase; neither can it be part of a linear sequence between succinate and cytochrome c. The role of the other members of the cytochrome b complex requires further study.

The proposed respiratory pathway shown in figure 10 was derived from kinetic studies with uncoupled, depleted skunk cabbage mitochondria, and thus applies only to that particular condition. The question of what modifications to such a scheme are required to account for the results observed with coupled skunk cabbage mitochondria are discussed in the accompanying paper (41).

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