Oxidation of Menadiol by Fractions Isolated From Non-Photosynthetic Plant Tissues

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The role of quinones in electron transport has attracted considerable recent interest (27). Coenzyme Q₁₀, which is present in plant mitochondria, is closely related to the respiratory chain (6), and the very similar benzoquinone, plastoquinone, appears to be involved in photosynthesis (7). Vitamin K-type naphthoquinones have been implicated in respiratory electron transfer (30), oxidative phosphorylation (2), and photophosphorylation (1). The enzymatic pathways involved in the intracellular oxidation and reduction of these quinones are clearly of interest. Wosilait and Nason (32) showed that extracts of animals, plants, and bacteria contain an active pyridine nucleotide-menadione (vitamin K₃) reductase, and since then a number of enzymes which catalyze the reduction of naphthoquinones and benzoquinones or benzoquinones alone have been purified. The pathways of oxidation of the reduced quinones have also been examined, though most of the work has been done with animal systems (8, 9, 22). This aspect of the problem is of special interest in plants, since, in addition to the usual respiratory chain, they contain a number of enzymes (phenol oxidase, laccase, peroxidase) which can oxidize phenols to their corresponding quinones. In the present study, we have used menadiol and menadione as model compounds and have examined their oxidation and reduction by cell-free fractions isolated from non-photosynthetic tissues. Special attention has been paid to the intracellular localization of these activities and to the components involved in the oxidative pathways.

Materials & Methods

The washed mitochondrial fraction was isolated from sweet potato roots (Ipomea batatas L.) by differential centrifugation (at 10,000 × g for 20 min) as previously described (13). The microsomal fraction was then isolated from the supernatant fraction remaining after the 10,000 × g centrifugation: it was centrifuged at 25,000 × g for 20 minutes, the residue or intermediate fraction discarded, and the resulting supernatant then centrifuged at 80,000 × g for 1 hour. The microsomal pellet was resuspended in 0.5 M sucrose-0.05 M tris-0.01 M verseae medium (pH 7.0), resedimented, and finally taken up in 2.5 ml of the medium to give the washed microsomal fraction. Particles were treated with 1% digitonin and the digitonin supernatant prepared as described previously (12).

Fractions were obtained from the third internodes of 7-day-old etiolated pea seedlings (Pisum sativum L., var. Alaska) by the method described elsewhere (21). The isolation medium contained 0.42 M mannitol, 0.005 M KCl, 0.005 M MgSO₄, and 0.02 M tris buffer (pH 7.5). After sedimenting starch and cell debris from the homogenate, five particle fractions and a soluble fraction were isolated by differential centrifugation. Each of the residues was washed once by resuspension in the isolation medium and recentrifugation. Protein concentrations of the various fractions were determined by the method of Lowry et al. (16).

The oxidation of menadiol (vitamin K₃H₂) was followed spectrophotometrically by the method of Colpa-Boonstra and Slater (8). Special precautions (low pH, inclusion of versene) must be taken to prevent the rapid autoxidation of the substrate. Menadiol was prepared chemically by Fieser's method (10). For a typical experiment, 3 mg of menadiol were dissolved in 1 ml of 95% ethanol and 50 ml of 0.1 M phosphate buffer (pH 6.3) containing 1 × 10⁻³ M EDTA were added to make the stock solution. Approximately 10 ml of this solution were transferred to a serum-stoppered centrifuge tube. The tube was then evacuated using a hypodermic needle connected to a water aspirator. The reaction mixture contained 0.33 M sucrose, 7 × 10⁻⁴ M EDTA, 0.05 M phosphate buffer (pH 6.3), and enzyme in a total volume of 3.0 ml. The reaction was started by removing a sample of menadiol solution from the evacuated tube with a syringe and adding 0.1 ml of it to the cuvette to give a final concentration of approximately 5 × 10⁻⁵ M menadiol. The increase in optical density at 262 mm was followed using the expanded scale attachment (full scale deflection = 0.1 absorbancy unit) on the Cary 14 spectrophotometer. Rates of the reaction were determined from the initial linear portion of the curve and

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³ Predoctoral Fellow of the National Science Foundation.
are expressed either as $\Delta A_{260}/\text{minute}$ or are converted to $\Delta$ mmoles menadiol oxidized/minute using the value for the change in extinction coefficient at 262 m$\mu$ of 1.35 $\times$ 10$^7$ cm$^2$ mole$^{-1}$ (9). The autoxidation was negligible under the conditions of these experiments.

The menadione reductase activity was assayed in a reaction mixture containing 0.33 M sucrose, 7 $\times$ 10$^{-4}$ M EDTA, 0.03 M phosphate buffer (pH 6.3), 1.7 $\times$ 10$^{-3}$ M KCN, 10$^{-4}$ M DPNH, and enzyme in a total volume of 3.0 ml. The reaction was started by the addition of menadione (in ethanol) to give a final concentration of 10$^{-4}$ M. The decrease in optical density at 340 m$\mu$ was recorded. The rate of DPNH oxidation in the absence of K$_3$ is negligible since cyanide is present.

The peroxidase activity was assayed using orthodianisidine as the substrate (28). To 6.0 ml of 0.003% H$_2$O$_2$ in 0.01 M phosphate buffer (pH 6.0), 0.05 ml of 0.041 M orthodianisidine in methanol was added. A 2.9 ml aliquot of this mixture was transferred to the test cuvette and the remainder was poured into the control cuvette. The reaction was started by adding 0.1 ml of the cell fraction, and the increase in absorbancy at 460 m$\mu$ followed. Specific activity is expressed as m$\mu$ moles H$_2$O$_2$ destroyed per minute per milligram protein. Purified horse-radish peroxidase was obtained from the Worthington Biochemical Corp. and had an RZ value (ratio of absorbancy at 403 m$\mu$ to the absorbancy at 275 m$\mu$) of 1.0, corresponding to 33% purity.

Difference spectra of the digitonin-treated fractions were recorded with a Cary spectrophotometer.

**Figure 1.** The spectral change accompanying the oxidation of menadiol by sweet potato mitochondria. ● ● ● Spectrum of menadiol, 3 $\mu$g in 0.1 M phosphate buffer (pH 6.3) containing 10$^{-3}$ M EDTA. --- Spectrum after incubation of menadiol for 10 minutes with 0.01 ml mitochondrial suspension.

**Results**

Oxidation of Menadiol: Sweet potato mitochondria were used for the initial experiments. When mitochondria are added to a solution containing menadiol, the absorption spectrum changes rapidly to give a spectrum identical to that of authentic menadione (fig 1). The reaction can be followed by measuring the increase in absorbancy at 262 m$\mu$ (fig 2). The kinetics of the reaction appear to be first-order, in agreement with results obtained with animal mitochondria (8). In the absence of any particles, there is a very slow increase due to the autoxidation of menadione (fig 2). The ability of the mitochondria to catalyze the reaction is destroyed by boiling. The rate of the initial reaction is directly proportional to the volume of mitochondrial fraction added: addition of 0.02, 0.03, 0.04, and 0.05 ml of a suspension containing 15.0 mg/ml of protein gave rates measured as $\Delta A_{340}$ $\times$ 10$^3$/minute of 22, 39, 59, and 72, respectively. In a typical experiment, the initial rate of oxidation was calculated to be 32 mmoles menadiol oxidized per minute per mg protein. The requirement for molecular oxygen was established by carrying out the reaction in an anaerobic cuvette. In the absence of air, there is no reaction between the particles and menadiol; when oxygen is admitted, there is an immediate increase in absorbancy at 262 m$\mu$ (fig 2). All of these results indicate that the particles catalyze a menadiol oxidase reaction.

Intracellular Distribution of Menadiol Oxidase Activity: The specific activity (per mg protein) for menadiol oxidation was found to be roughly the same for sweet potato microsomes as for the mitochondria. The reaction catalyzed by the microsomes also requires molecular oxygen and the rate is proportional to the volume of microsomal suspension added to the reaction mixture; it too may be described as a menadiol oxidase. The final supernatant or soluble fraction of the sweet potato homogenate also oxidizes menadiol rapidly.

The intracellular localization was studied in more detail with fractions isolated from etiolated pea stems (table I). It has been shown previously that Fractions I and II are essentially mitochondrial. Fraction III is a mixture of mitochondria and microsomes, and Fractions IV and V are essentially microsomal in nature (21). Each of the particle fractions contains roughly 3% of the total protein remaining after the preliminary centrifugation at 500 $\times$ g. As with the sweet potato, the specific activity of the menadiol oxidase is remarkably similar in the various fractions, both particulate and soluble. Since the soluble fraction was routinely found to contain 85 to 90% of the total protein, roughly 90% of the total menadione was reduced in the particles.
diol oxidase activity is localized here. When other oxidase activities were assayed, strikingly different results were obtained: the DPNH oxidase specific activity is very high in the mitochondrial fraction but only slight in the soluble fraction and in the microsomes, while cytochrome c oxidase is almost exclusively confined to the heavy particle fractions (21).

Pathways of Menadiol Oxidation—Mitochondria: Inhibitors (see 11) were used to reveal the pathway of menadiol oxidation by sweet potato mitochondria (table II). The fact that the reaction is essentially completely blocked by $10^{-3} \text{M}$ cyanide suggests that a metal-containing oxidase is involved. The classical copper oxidases apparently do not play an important role, since the copper-chelating agents diethylthiocarbamate (DIECA) and phenylthiourea caused relatively little inhibition at concentrations ($10^{-4} \text{M}$) which completely block these enzymes. Antimycin A and HOQNO (2-heptyl-4-hydroxyquinoline-N-oxide), which block electron transfer specifically between cytochromes b and c, inhibited the mitochondrial menadiol oxidase between 50 and 70%, indicating that a major fraction of the oxidation is mediated by the cytochrome system.

The inhibitor evidence is supported by the demonstration that menadiol readily reduces the complete mitochondrial cytochrome system. This was shown using a suspension of mitochondrial fragments prepared by treatment with digitonin. Two milliliters of the suspension (digitonin supernatant) were placed in each of two Thunberg-type cuvettes, and the cuvettes were evacuated. A base-line of the difference in absorbancy of these two samples was recorded.

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### Table I

Intracellular Localization of Menadiol Oxidase, Peroxidase, & DPNH-Menadione Reductase in Pea Stems

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Identity*</th>
<th>Specific activity of menadiol oxidase (A) (mumoles menadiol oxidized/mg protein × min)</th>
<th>Specific activity of peroxidase (B) (mumoles $H_2O_2$ destroyed/mg protein × min)</th>
<th>Ratio of specific activities of peroxidase &amp; menadiol oxidase (B/A)</th>
<th>Specific activity of DPNH-menadione reductase (mumoles DPNH oxidized/mg protein × min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mitochondria</td>
<td>35</td>
<td>647</td>
<td>18</td>
<td>160</td>
</tr>
<tr>
<td>II</td>
<td>(2,900-5,000 × g)</td>
<td>28</td>
<td>832</td>
<td>30</td>
<td>120</td>
</tr>
<tr>
<td>III</td>
<td>(5,000-17,000 × g)</td>
<td>23</td>
<td>935</td>
<td>41</td>
<td>61</td>
</tr>
<tr>
<td>IV</td>
<td>(17,000-25,000 × g)</td>
<td>21</td>
<td>1,030</td>
<td>50</td>
<td>83</td>
</tr>
<tr>
<td>V</td>
<td>(25,000-80,000 × g)</td>
<td>32</td>
<td>1,180</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>Soluble</td>
<td>...</td>
<td>41</td>
<td>1,000</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td>Horseradish peroxidase (RZ = 1.0)</td>
<td>...</td>
<td>$1.9 \times 10^3$</td>
<td>$8.2 \times 10^4$</td>
<td>42</td>
<td>...</td>
</tr>
</tbody>
</table>

* See (21).

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### Table II

Inhibition of Menadiol Oxidase Activity of Sweet Potato Mitochondrial & Microsomal Fractions

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (M)</th>
<th>Mitochondria</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate ($\Delta A_{262}$ × $10^3$/min)</td>
<td>% Inhibition</td>
<td>Rate ($\Delta A_{262}$ × $10^3$/min)</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>43</td>
<td>...</td>
</tr>
<tr>
<td>KCN</td>
<td>$10^{-3}$</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>Antimycin</td>
<td>$1.8 \times 10^{-6}$</td>
<td>11</td>
<td>69*</td>
</tr>
<tr>
<td>HOQNO</td>
<td>$10^{-4}$</td>
<td>18</td>
<td>58</td>
</tr>
<tr>
<td>DIECA</td>
<td>$10^{-4}$</td>
<td>33</td>
<td>24</td>
</tr>
</tbody>
</table>

* Samples contained either 0.3 mg protein (mitochondria) or 0.28 mg protein (microsomes) in 3.0 ml reaction mixture. Relative to control containing equivalent volume of alcohol.
small amount of solid menadiol was then tipped from the side-arm into the sample cuvette, air was admitted into the reference cuvette, and the difference spectrum recorded (fig 3). It is clear that a (603 m$\mu$)-, b (560 m$\mu$)- and c (552 m$\mu$)-type cytochromes are reduced by the menadiol.

While the usual respiratory chain mediates a major fraction of the menadiol oxidation, antimycin and HOQNO were not completely inhibitory (table II). This is in marked contrast to the situation with heart-muscle preparations, where the activity is completely blocked by very low concentrations of antimycin A (8). Increasing the antimycin concentration from $1.8 \times 10^{-8}$ m to $1.8 \times 10^{-6}$ m and the HOQNO concentration from $10^{-6}$ m to $10^{-4}$ m did not significantly increase the degree of inhibition of the sweet potato mitochondrial menadiol oxidase. Under the same conditions, the DPNH oxidase was considerably more sensitive to these inhibitors than was the menadiol oxidase. It is concluded that a second cyanide-sensitive pathway of menadiol oxidation is also operative in isolated sweet potato mitochondria, and this may account for up to half of the total activity.

The inhibitor response of the menadiol oxidase of pea stem mitochondria (Fractions I & II) suggests that a similar situation is found in these particles (table III). The reaction is blocked completely by

| Table III |
|------------------|------------------|------------------|
| **Fraction**     | **% Inhibition by 1.7 x 10^{-8} m KCN** | **% Inhibition by 1.3 x 10^{-6} m antimycin A** |
| I                | 100              | 45               |
| II               | 100              | 36               |
| III              | 100              | 10               |
| IV               | 100              | 5                |
| V                | 100              | 0                |
| Sol.             | 100              | 0                |
| HRP (RZ = 1.0)   | 80               | ...              |

* For identification of the fractions see table I.

1.7 x 10^{-8} m KCN but only partially (approximately 40% inhibition) by 1.3 x 10^{-6} m antimycin A.

**Pathways of Menadiol Oxidation—Other Fractions:** The oxidation of menadiol by the sweet potato microsomal fraction is also blocked by 10^{-8} m KCN (table II). On the other hand, it is even less sensitive to antimycin than is the mitochondrial system: in four experiments, the average inhibition caused by $1.8 \times 10^{-6}$ m antimycin was 34%. Similar results were obtained with the microsomal and soluble pea stem fractions; the oxidation was completely blocked by cyanide and was essentially unaffected by antimycin (table III). The pea stem microsomal fractions are less likely to be contaminated by mitochondria, and this may account for the fact that they are less sensitive to antimycin than is the sweet potato.
microsomal fraction. The difference spectrum (menadiol-reduced minus oxidized) of the sweet potato microsomes showed only a b-type cytochrome. Since the microsomal menadiol oxidase was not inhibited by $3 \times 10^{-5}$ M $p$-chloromercuribenzoate and $3.3 \times 10^{-4}$ M phenyl mercuric acetate, -SH groups are apparently not involved in the reaction.

A number of oxidative enzymes which might conceivably account for the menadiol oxidase activity are present in plants. Since cytochrome c oxidase is entirely absent from the soluble fraction, it alone cannot be responsible for the activity. The classical copper oxidases appear to be ruled out by the fact that $10^{-4}$ M DIECA is not inhibitory to the reaction catalyzed by either the sweet potato or the pea stem soluble fraction. The fact that menadiol is not an ortho-diphenol makes it an unlikely substrate for polyphenol oxidase.

Several lines of evidence suggest that peroxidase may in fact be involved. Peroxidase activity can be demonstrated in all of the pea stem fractions and the specific activities do not vary greatly (table 1). Furthermore, the ratios of the specific activities of peroxidase/menadiol oxidase for the various fractions are not widely different. In preliminary experiments, it has been shown that purified horseradish peroxidase can in fact act as a menadiol oxidase without added $H_2O_2$, and this oxidation is almost completely inhibited by $10^{-3}$ M KCN (table 1). The ratio of peroxidase/menadiol oxidase activities for the purified enzyme is not far from that obtained with the isolated fractions. A detailed study of this reaction will be reported elsewhere.

**DPNH-Menadione Reductase:** All of the pea stem fractions can catalyze the reduction of menadione by DPNH (table 1), and the specific activity of the reaction is highest in the mitochondria. Although the specific activity in the soluble fraction is relatively low, nearly 80% of the total activity is found here. The fact that all of the fractions are able to reduce menadione does not mean that the same enzymes are involved in each case.

**Discussion**

The pathways of reduction and oxidation of menadione suggested by the present experiments may be represented diagrammatically as follows:

![Diagram of the pathway of reduction and oxidation of menadione]

The presence of DPNH-menadione reductase in all of the pea stem fractions indicates that the intracellular localization of this activity is similar to that of the Vitamin K reductase in liver (29). No attempt was made to analyze the pea stem reductase systems. Many flavoproteins are able to catalyze the reduction of menadione (17), and enzymes showing this activity have been partially purified from the soluble fraction of tobacco roots (25), wheat germ (5), and etiolated mung bean (24). A similar enzyme has been purified from liver (18); since this was shown to be a flavoprotein, it has been assumed in the above diagram that the enzymatic reduction proceeds via a flavoprotein (FP).

A major fraction of the menadiol oxidation by plant mitochondria is mediated by the cytochrome system. All of the oxidation of menadion by animal mitochondria proceeds via the respiratory chain (26), and this is also true for the oxidation of endogenous and exogenous reduced coenzyme Q (22). Menadion and coenzyme $QH_2$ enter the respiratory chain either at, or on the substrate side of, the antimycin-sensitive locus. The externally added substrates might first interact with an endogenous quinone before reducing cytochrome b.

The antimycin-insensitive fraction of the menadiol oxidase of plant mitochondria may be due to several alternative pathways. Menadiol can reduce cytochrome c non-enzymatically (31), and this offers a possible route to cytochrome oxidase and oxygen. Within the respiratory chain itself there may be antimycin-insensitive bypasses from the level of cytochrome b or quinone to cytochrome c. Peroxidase in the mitochondria may present a pathway to oxygen which is entirely independent of the respiratory chain. It is of interest that the menadiol oxidase of Azotobacter respiratory particles is not inhibited by antimycin (23).

An interesting feature of the plant systems is the similarity of the specific activities of menadion oxidase in all the cell fractions. Izawa (15) has shown that isolated chloroplasts are also able to catalyze the oxidation of menadion, and the reaction is stimulated by light. The situation in plants is clearly different from that found in mammalian liver, where roughly 90% of the activity is localized in the mitochondrial fraction (29). Our results suggest that the high concentration of peroxidase in plant tissues may account for this difference. Although the intracellular localization of this enzyme has not been widely studied, our experiments indicate that it is present in all the cell fractions. Similar distributions for peroxidases in other plant tissues have been described (19, 20). In contrast, very little peroxidase is found in the soluble fraction of the pig thyroid gland and most of the activity is found in the microsomes (14). Peroxidase is known to act as an aerobic oxidase on a number of substrates (3), and our data indicate that the oxidation of menadion by this enzyme is such a reaction.

What is the physiological significance of the reactions examined here? Does menadion act in these systems like some naturally occurring quinone? It seems most likely that menadion serves as a model for coenzyme $Q$ in the respiratory chain, as suggested by Slater et al. (26), but the exact position and role of this coenzyme have not been firmly established (4).
Our results emphasize the fact that alternative pathways of oxidation are available in plant systems, though the possibility that these are artifacts of isolation cannot be ruled out. It will be of interest to see if peroxidase plays an important physiological role by virtue of its ability to oxidize naturally occurring hydroquinones.

Summary

I. The oxidation of menadiol by fractions isolated from sweet potato roots and etiolated pea stems has been examined using a spectrophotometric method.

II. The mitochondria catalyze an O₂-dependent menadiol oxidase reaction which is mediated in part, but not entirely, by the cytochrome system.

III. The specific activity of the menadiol oxidase is approximately the same in all the cell fractions, and the bulk of the total activity is associated with the soluble fraction.

IV. The menadiol oxidase of the microsomal and soluble fractions is blocked by cyanide but is insensitive to antimycin A.

V. The antimycin-insensitive menadiol oxidase is probably due to peroxidase acting as an aerobic oxidase. The intracellular localization of peroxidase activity is consistent with this possibility.

VI. The bulk of the DPNH-menadione reductase activity in the pea stem is associated with the soluble fraction, and the remainder is largely mitochondrial.

Acknowledgment

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Literature Cited


An increase in respiratory rate has been observed in sweet potato roots infected by the black rot fungus, Ceratocystis fimbriata, and, with special reference to the phosphate metabolism of the host tissue, hypotheses have been put forward to interpret the mechanism responsible for this increase (2, 24, 25). There are several other characteristic aspects of the post-infection host metabolism (24), among which the formation of furanoterpenoids, e.g., ipomeamarone, is particularly interesting. Ipomeamarone has a powerful uncoupling and antipathogenic action, and at a moderate concentration induces a respiratory increase not coupled with phosphorylation reactions (25). A recent analytical study of ipomeamarone synthesis suggests that an uncoupling-type respiration may be partly involved in cells closely adjacent to the fungus degenerated area, especially in the case of a susceptible variety (3). However, since there is also an increase in the respiratory activity of the inner region of the diseased root tissue, where no ipomeamarone can be detected, an alternative mechanism is probably operative. Enhancement of synthetic reactions in the host tissue as influenced by the infection may be the possible mechanism, as it should induce a respiratory increase by stimulating the rate of ATP breakdown (2). But the final proof is still lacking, although several reports in favor of this hypothetical view with regard to other host-parasite combinations are accumulating (11, 12, 21, 24).

Using a quantitative and qualitative evaluation of the carbohydrate metabolism by employing the Bloom-Stetten procedure of determining the so-called \( C_{o}/C_{i} \) ratio (10), information is also accumulating which points to an alteration of the respiratory metabolism of diseased plants. This method has been extensively developed in the plant field (6, 8, 9). Daly and his associates (11, 13) and Shaw and Samborski (22) have independently reported, based upon the use of this radioisotopic technique, that the PP pathway plays a dominant role in the respiration of rusted plant tissues. It is interesting to note that these experiments have also shown that the major portion of glucose breakdown in tissues treated with a classical uncoupler, DNP, is via the EMP pathway (4, 13, 22). Similarly some plant growth regulating substances, e.g., IAA and 2,4-D, were found to alter the pattern of glucose breakdown (15, 17, 18, 23). But it should be borne in mind that there are difficulties inherent in the method (6, 7, 8, 14, 22), and some recent investigations have stressed that a truly quantitative evaluation of the carbohydrate metabolism