Studies on the Respiratory Chain-linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase

XIII. BINDING SITES OF ROTENONE, PIERICIDIN A, AND AMYTAL IN THE RESPIRATORY CHAIN*

(Received for publication, July 7, 1967)

DOUGLAS J. HORGAN‡ and THOMAS P. SINGER

From the Department of Biochemistry, University of California School of Medicine, San Francisco, California 94122, and the Division of Molecular Biology, Veterans Administration Hospital, San Francisco, California 94121

J. E. CASIDA

From the Division of Entomology and Acarology, University of California, Berkeley, California 94720

SUMMARY

The binding sites of rotenone, piericidin A, and Amytal in the reduced nicotinamide adenine dinucleotide oxidase chain of heart have been studied with the aid of rotenone-14C. Binding of rotenone continues in a linear manner beyond the point of maximal inhibition of respiration, indicating that rotenone is tightly bound not only at the specific site in the NADH dehydrogenase segment of the respiratory chain but also at other sites in submitochondrial particles. Unspecific binding is reduced by treatment of the particles with rotenone in the presence of bovine serum albumin (BSA) and is further minimized by successive washing with BSA, with only minimal removal of rotenone from the specific site. Rotenone-14C is not removed from the specific site by denaturation or proteolysis but is largely removed by repeated extractions with anhydrous acetone, which results in recovery primarily of rotenone with only small amounts of a less inhibitory oxidation product. Substantial reversal of the inhibition of respiration occurs on repeated washing of the inhibited particles with BSA. Thus, contrary to expectations, under usual assay conditions rotenone is not a specific, stoichiometric, and irreversible inhibitor. Studies involving preincubation of the electron transport particle with various unlabeled inhibitors, of widely varying chemical type and inhibitory potency, prior to addition of rotenone-14C for determination of its subsequent binding, provide strong evidence that it is possible to distinguish between the portion of rotenone-14C that is specifically bound and the portion that is unspecifically bound. Piericidin A, Amytal, and a number of rotenoids appear to react at the same site as rotenone-14C for binding at the specific site, and the competition with rotenone is proportional to the inhibitory power of the compounds tested and not to their concentration or chemical nature. Although the molar ratio of rotenone bound at the specific site to the NADH dehydrogenase content in particles approximates unity, the binding is not on NADH dehydrogenase itself, since extraction of the enzyme from phosphorylating or non-phosphorylating particles labeled with rotenone-14C results in nearly complete separation of rotenone-14C from the enzyme. Qualitative and quantitative differences are evident in the binding of rotenone to soluble NADH-coenzyme Q reductase as compared to submitochondrial particles. Although the soluble reductase, extracted with acid-ethanol from particles inhibited with rotenone-14C, is fully active and contains little or no rotenone-14C, it may be inhibited again on further addition of rotenone.

Rotenone and Amytal are among the most widely used inhibitors of mitochondrial electron transport because they selectively inhibit the oxidation of NADH-linked substrates (2-6). Suggestions on their sites of action in the respiratory chain are variable and include the following: that Amytal acts between NADH and NADH dehydrogenase, based on measurement of the oxidation-reduction states of the respiratory chain components by dual wave length spectrophotometry and determination of the "crossover" point (7); that although in phos-
phosphorylating preparations the barbiturate acts on the substrate side of the flavoprotein, in nonphosphorylating ones the inhibition occurs on the O₂ side of the dehydrogenase (8); that rotenone also interrupts electron flow between NADH and flavoprotein based on similar spectrophotometric studies (5); that Amytal and rotenone inhibit reduction of the flavoprotein in mitochondria (9); and, finally, that these agents act on the O₂ side, not on the substrate side, of the flavoprotein because neither the NADH-ferricyanide reaction nor transhydrogenase activity is inhibited by Amytal or rotenone either in soluble preparations of NADH dehydrogenase or in particles (10-13). The last site has also been suggested on other grounds (14). Earlier observations locating the inhibition site between NADH and its dehydrogenase can be explained by the extremely low concentration of NADH dehydrogenase in mitochondria and by the fact that its complex absorption spectrum renders spectrophotometric methods unsuitable for measuring the oxidation-reduction state of this flavoprotein (12).

The availability of labeled rotenone (15) offered the opportunity for a more direct approach to the reaction site of rotenone in the respiratory chain. The present paper confirms the suggestion that the binding site of rotenone responsible for inhibition of NADH oxidation is localized on the O₂ side of the dehydrogenase and shows that Amytal and the antibiotic piericidin A block this same site. The specificity, stoichiometry, and reversibility of rotenone binding and inhibition are examined, and evidence is presented that the rotenone- and Amytal-sensitive reduction of coenzyme Q by soluble NADH-CoQ₇ reductase (16) is not a physiological reaction.

**EXPERIMENTAL PROCEDURE**

**Materials**—Rotenone-6a-¹⁴C, 2.36 mC per mmole (15), consisted of 92.4% rotenone-6a-¹⁴C, 3.6% of 6aβ,12aα,5α'-rotenolone-6a-¹⁴C, and 4.0% of unknown labeled compounds, of lower Rₚ as analyzed by each of the thin layer chromatography systems, used in one dimension, described later. Other rotenoids used in the enzyme studies were in an unlabeled form, including epirotenone, diastereoisomers of rotenolone, and 6β,7β-dihydrorotenone, and were provided by Professor L. Crombie, University College of South Wales and Monmouthshire, Cardiff, England. Additional rotenoids used in the co-chromatography studies (see “Methods”) were provided either by Professor Crombie or by Professor M. Matsue of the University of Tokyo or Professor M. Nakajima of the University of Kyoto. Piericidin A was the gift of Dr. Karl Folkers, Stanford Research Institute. Amytal was from the Vitarine Company, New York. The structures of the rotenoids used in this study and of piericidin A are given in Fig. 1 and Table I. Other materials were as previously described (1, 17).

**Methods**—The suspension medium for ETP during reactions and washings was 0.25 M sucrose-0.025 M phosphate, pH 7.4, with or without addition of 2% (w/v) BSA. Standard rotenone binding curves were determined by adding varying amounts of rotenone-¹⁴C in a few microliters of ethanol to 25-mg aliquots of ETP suspended in 8 ml of sucrose-phosphate-BSA medium, followed by 10 μmol of NADH. The samples were incubated for 5 min at 30°C, cooled to 0°C, and centrifuged for 15 min at 144,000 × gmax. The pellets were resuspended by homogenization in 9 ml of sucrose-phosphate-BSA medium. After 10 to 15 min at 0°C, the particles were centrifuged as above. The pellets were resuspended by homogenization in 2.4 ml of 5 mM phos-

![Table I](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Name</th>
<th>Partial structure</th>
<th>Rᵢ in Hexane-ethyl acetate (2:1)</th>
<th>Hexane-ethyl acetate (2:1)</th>
<th>Benzene-methanol (9:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td><img src="http://www.jbc.org/" alt="Rotenone Diagram" /></td>
<td>0.41</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Epirotenone</td>
<td><img src="http://www.jbc.org/" alt="Epirotenone Diagram" /></td>
<td>0.35</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>6αβ,12αα,5α'-Rotenolone</td>
<td><img src="http://www.jbc.org/" alt="6αβ,12αα,5α'-Rotenolone Diagram" /></td>
<td>0.31</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>6αα,12αα,5α'-Rotenolone</td>
<td><img src="http://www.jbc.org/" alt="6αα,12αα,5α'-Rotenolone Diagram" /></td>
<td>0.27</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>6αα,12αα,5α'-Rotenolone</td>
<td><img src="http://www.jbc.org/" alt="6αα,12αα,5α'-Rotenolone Diagram" /></td>
<td>(0.36)</td>
<td>(0.75)</td>
<td></td>
</tr>
<tr>
<td>6α,12α-Dehydrorotenol</td>
<td><img src="http://www.jbc.org/" alt="6α,12α-Dehydrorotenol Diagram" /></td>
<td>0.65</td>
<td>&gt;0.98</td>
<td></td>
</tr>
<tr>
<td>Spiro compound</td>
<td><img src="http://www.jbc.org/" alt="Spiro Compound Diagram" /></td>
<td>0.50</td>
<td>&gt;0.98</td>
<td></td>
</tr>
</tbody>
</table>

* Only the B-C ring system is shown because the remainder of the structure is identical with that of rotenone.

* The sample was contaminated by an unknown amount of 6αβ,12αα,5α'-rotenolone. No evidence was obtained that these two rotenolones are resolvable in the solvent systems used, but, in the absence of each compound in pure form, this must be only a tentative conclusion.

The abbreviations used are: CoQ, coenzyme Q; ETP, electron transport particle; ETP₄, electron transport particle prepared by sonic disruption at neutral pH; BSA, bovine serum albumin.
Rotenone and Piericidin A

FIG. 1. Structures of rotenone and of piericidin A

RESULTS

Characteristics of Rotenone Binding—Fig. 2 shows the characteristics of rotenone-14C binding to ETP (O) and the resulting inhibition of NADH oxidase activity (●) when the...
titration is carried out in sucrose-phosphate medium. Binding of rotenone-14C continues in a linear manner beyond the concentration of maximal inhibition of NADH oxidase activity, even to the point where 5 times as much rotenone is added as that required for almost complete inhibition of enzyme activity. Thus rotenone binding in submitochondrial particles is not restricted to the specific site, i.e., the site responsible for the interruption of electron flow from the flavoprotein. Although rotenone binds very strongly at sites unrelated to inhibition of electron transport, since neither boiling nor proteolysis releases significant amounts of rotenone bound under these conditions, it is nevertheless possible to distinguish, at least in part, between binding at the specific site and ancillary sites by the use of serum albumin. The lowest curve in Fig. 2 represents rotenone binding when the titration is performed in sucrose-phosphate-BSA medium. It is evident that under these conditions much less rotenone-14C is bound and that the curve becomes somewhat biphasic.

Fig. 3 illustrates the effect on rotenone-14C binding of washing of the inhibited particles with the sucrose-phosphate-BSA medium. The top line is the binding curve in sucrose-phosphate medium, and the lower lines represent the degree of binding at two rotenone concentrations when the titration is carried out in sucrose-phosphate-BSA medium and this medium is used for successive washes of the particles. Washing decreases the unspecific binding progressively, so that the biphasic nature of the curve becomes more and more evident.

In subsequent experiments, conditions were standardized by performing the titrations in the sucrose-phosphate-BSA medium and washing the inhibited particles once with this medium, as detailed in "Methods." A standard binding curve is shown in Fig. 4 (Curve A), and the solid circles represent the corresponding inhibition of NADH oxidase activity. In the experiment of Curve B, in which the particles were first inhibited with unlabeled rotenone to the extent of 98% and then titrated with rotenone-14C, the magnitude of the initial phase of the binding curve is greatly reduced, but the slope of the subsequent linear portion remains relatively unaffected. The initial, rapidly rising portion of the binding curve probably represents combination of the inhibitor at the specific site responsible for the inhibition of NADH oxidation, and the linear portion at higher rotenone-14C concentrations is most likely due to unspecific binding. Equilibration and dilution effects do not account for the values observed, or for the biphasic nature of the curve, because addition of unlabeled rotenone to ETP previously labeled with rotenone-14C, followed by incubation for 5 min at 30°, does not result in displacement of the bound rotenone-14C. A binding curve identical with Curve B was obtained when the particles were first inhibited with piericidin A to the extent of 96% prior to titration with rotenone-14C. This is of interest because piericidin inhibits NADH oxidation similarly to rotenone (22).

Effect of Other Unlabeled Rotenoids and of Piericidin A and Amytal on NADH Oxidation and on Rotenone-14C Binding—As shown in Table II, piericidin A and dihydrorotenone are almost as potent as rotenone in inhibiting NADH oxidation. 6αβ, 12αβ, 5β-Rotenolone is significantly less inhibitory, and 6αα, 12αα, 5β-rotenolone and epirotenone are even less effective as inhibitors. Although not included in the table, 6αβ, 12βα, 5β rotenolone is the least inhibitory of the rotenoids tested. The capacity of piericidin A to bind to the specific site, as measured by the lowering of the rotenone-14C titer, is essentially the same as that of rotenone. In general, the more inhibitory a
Effect of various rotenoids, piericidin A, and Amytal on NADH oxidase activity and on rotenone-14C binding

The unlabeled inhibitors were added to ETP samples representing 25 mg of protein in 8 ml of sucrose-phosphate-BSA medium containing 5 pmoles of NADH, and were incubated for 5 min at 30°C. The samples were quickly cooled to 0°C, and a small aliquot was removed for assay of NADH oxidase activity. To the remainder of each sample, 2.5 pmoles of rotenone-14C were added, and the particles were again incubated for 5 min at 30°C in the same medium. After cooling at 0°C, the particles were separated and washed once with sucrose-phosphate-BSA, and bound rotenone-14C was determined as described in "Methods." The level of bound rotenone-14C was calculated in each case from the average of five experiments.

![Table II](https://example.com/table2.png)

Reversal of Rotenone Inhibition—Rotenone-14C binding at unspecific sites is minimized by washing with BSA-containing medium (Fig. 3). Additional washings with this medium progressively relieve the inhibition of NADH oxidation, indicating that rotenone is slowly removed by these washings from the specific site (Table III). These observations do not support the assumption that rotenone inhibition of the respiratory chain is stoichiometric and irreversible (6). In contrast to the slow but definite partial restoration of rotenone-blocked respiration, the inhibitory effect of piericidin on NADH oxidation is not significantly reversed by similar treatments (Table III), and so piericidin appears to be the more tightly bound of the two inhibitors.

It appears that BSA competes with components of the respiratory chain for rotenone in a true equilibrium manner which may be approached from either side because, when rotenone-14C is preincubated with BSA and ETP is added later, the inhibition of NADH oxidase activity and rotenone-14C binding are the same as when the particles are first mixed with rotenone-14C and BSA is added last.

Fig. 5 is a composite figure which compares the regeneration of NADH oxidase activity and the release of bound rotenone on successive washing of rotenone-inhibited particles with BSA. It may be seen that during the initial washes 14C-rotenone is released much more extensively than activity is regenerated. These results are incompatible with the presence of one type of site and are compatible with two or more types of binding site.

Kinetics of Inhibition by Rotenone and Piericidin, and Effect of NADH—Rotenone inhibition does not develop immediately, but shows a pronounced lag period (14). This lag is not abolished by preincubation of the particles with rotenone but is minimized by preincubation with NADH.

These observations are confirmed by the present study and extended to piericidin A, which behaves similarly. In a typical experiment, when only enough piericidin (7.2 pmoles/30.2 mg of ETP protein) is added to cause partial inhibition initially, the inhibition is 62% immediately after the addition of NADH, but in the course of the enzymatic reaction (at 30°C) it increases to 95% after 5 min and to 98% after 10 min. This gradual increase in inhibition which is induced by NADH is also characteristic of the effect of rotenone (14). For this reason, NADH

![Table III](https://example.com/table3.png)
increases the inhibition. These differences in rates of induction by NADH and effects of preincubation must be taken into account when structure-reactivity studies are made with rotenoids. For example, the conditions used for the experiments reported in Table II tend to minimize the differences in the relative inhibitory powers of rotenone and the rotenolones. In an earlier report (14).

It should be noted that NADH also induces the inhibition by other rotenoids such as the rotenolones, but at slower rates. 6α,12α,5β-Rotenolone also differs from rotenone in that preincubation of the rotenoid with the enzyme without NADH increases the inhibition. These differences in rates of induction by NADH and effects of preincubation must be taken into account when structure-reactivity studies are made with rotenoids. For example, the conditions used for the experiments reported in Table II tend to minimize the differences in the relative inhibitory powers of rotenone and the rotenolones.

**Extraction of Bound Rotenoids-^14C**—Since the chemical nature of the binding sites of rotenone in the respiratory chain remains obscure, particles labeled with rotenone-^14C were subjected to thermal denaturation and to digestion by proteolytic enzymes, and the release of radioactivity in soluble form after these treatments was monitored. Samples of ETP which had been treated with rotenone-^14C in sucrose-phosphate medium and washed with the same medium (i.e., no BSA present) release only trace amounts of radioactivity on freezing and thawing or on boiling for 5 min. Digestion for 5 hours at pH 8.0 and 30° with 0.023 mg of crystalline subtilisin per mg of ETP protein, or under the same conditions but at pH 5.4 with cyanide-activated crystalline papain, liberates only 1 to 5% of the radioactivity into solution. However, digestion for 24 hours at 30° and pH 8.0 with 0.014 mg of crystalline trypsin per mg of ETP protein results in the release of 15% of the radioactivity in nonsedimentable form, while digestion for 5 hours with 0.023 mg each of trypsin and chymotrypsin yields 23% of the radioactivity in the supernatant solution on centrifugation. Thus, while heat denaturation and digestion with some proteolytic enzymes fail to release bound rotenone-^14C, digestion with trypsin results in significant but incomplete release.

In contrast, when the titration with rotenone-^14C is performed in sucrose-phosphate-BSA and the particles are washed twice with the same solution, so that most of the bound rotenone-^14C is restricted to the specific site, neither boiling for 5 min nor digestion with 0.04 mg of trypsin per mg of ETP protein for 3 hours, with or without prior heat denaturation, releases over 2.5 to 5% of the radioactivity into solution. Thus the nature of the bond at the specific site appears to be such that it is not influenced by extensive digestion of the particles with trypsin or by destroying the native conformation of the protein.

In order to extract most of the bound rotenoids, the procedure adopted was lyophilization of the particles, followed by several extractions with dry acetone (see "Methods"). Under these conditions, 95 to 98% of the radioactivity was extracted from particles which had not been washed with sucrose-phosphate-BSA (Table V). The yield of radioactivity on extraction of particles previously washed with BSA (i.e., binding mostly at the specific site) was only 74 to 88% under these conditions (Table V).

Most of the radioactivity extracted from the particles is in the form of unchanged rotenone, but a small fraction is recovered as 6α,12α,5β-rottenolone (Table V). None of about 30 other unlabeled rotenoids has the same chromatographic characteristics as the major labeled products. No epirotenone or 6α,12α-dehydrorottenone is recovered from the ETP, and acidification prior to extraction of the ETP does not yield radioactive material chromatographing in the position of the diastereoisomeric pair of spiro compounds. Taken collectively, the failure to find epirotenone, 6α,12α-dehydrorottenone, and the spiro compound indicates that ring C does not open and that racemization of centers 6α and 12α does not occur on the ETP, either at the specific site associated with enzymatic activity or at other sites of unspecific binding.

**TABLE IV**

**Effect of NADH on rotenone binding and inhibition**

Reaction mixtures consisted of 25 mg of ETP protein and 5 μmoles of rotenone-^14C in 10 ml of sucrose-phosphate-BSA medium in the presence or the absence of 18 μmoles of NADH. Analyses after 10 min of incubation at 30° were made by cooling, centrifuging, suspending the residue in phosphate buffer, and assaying aliquots for NADH oxidase activity and bound rotenone-^14C, followed by washing the ETP in the remainder of each sample twice by centrifugation in sucrose-phosphate-BSA prior to repeating similar analyses.
**Table V**

Analysis of rotenone-14C-treated ETP for NADH oxidase activity, and nature and level of labeled derivatives recovered on acetone extraction

The particles were titrated, extracted, and chromatographed as described in "Methods."

<table>
<thead>
<tr>
<th>Titration and washing medium</th>
<th>Rotenone-14C added to 22 mg of ETP protein</th>
<th>Rotenone-14C equivalents extracted</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Rotenone: NADH oxidase activity&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rotenone-14C added to 22 mg of ETP protein</td>
<td>Rotenone-14C equivalents extracted</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Rotenone: NADH oxidase activity&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sucrose-phosphate</td>
<td>2.5</td>
<td>70</td>
<td>0.04</td>
<td>0.18</td>
<td>0.11</td>
<td>0.14</td>
<td>2.47</td>
<td>6.90</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>96</td>
<td>5.76</td>
<td>0.52</td>
<td>0.36</td>
<td>0.26</td>
<td>6.05</td>
<td>22.59</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>98</td>
<td>9.65</td>
<td>1.10</td>
<td>0.77</td>
<td>0.47</td>
<td>11.99</td>
<td>29.59</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>60</td>
<td>10.60</td>
<td>2.42</td>
<td>1.20</td>
<td>0.38</td>
<td>29.59</td>
<td>29.59</td>
</tr>
<tr>
<td>Sucrose-phosphate-BSA</td>
<td>2.5</td>
<td>64</td>
<td>0.92</td>
<td>0.11</td>
<td>&lt;0.05</td>
<td>0.33</td>
<td>1.36</td>
<td>22.59</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>84</td>
<td>1.76</td>
<td>0.23</td>
<td>&lt;0.00</td>
<td>0.26</td>
<td>2.06</td>
<td>22.59</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>90</td>
<td>1.91</td>
<td>0.28</td>
<td>&lt;0.05</td>
<td>0.72</td>
<td>2.91</td>
<td>22.59</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>94</td>
<td>2.92</td>
<td>0.58</td>
<td>&lt;0.10</td>
<td>1.23</td>
<td>4.73</td>
<td>22.59</td>
</tr>
</tbody>
</table>

* Present to the extent of 3.6% in the original rotenone-14C sample.

**Table VI**

Isolation of NADH dehydrogenase from rotenone-14C-inhibited mitochondria

Fresh bovine heart mitochondria (50 g of protein) in 835 ml of sucrose-phosphate medium were titrated with 1.35 μmoles of rotenone-14C (4,500,000 cpm) to 90% inhibition of the oxidation of 4 mM pyruvate + 4 mM malate in the presence of 1.66 mM ADP. ETP was isolated from the mitochondria as in previous work (11). The ETP (5 g of protein) was washed with 500 ml of sucrose-phosphate-BSA medium by centrifugation and then once with 500 ml of sucrose-phosphate. At this stage the oxidation of NADH measured spectrophotometrically (11) was still 98% inhibited. NADH dehydrogenase was then isolated by the method of Cremona and Kearney (24). The enzyme activity in the table is expressed as micromoles of NADH oxidized per min in the ferricyanide assay (12).

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme activity</th>
<th>Total radioactivity</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP (washed)</td>
<td>70,500</td>
<td>584,000</td>
<td>7.35</td>
</tr>
<tr>
<td>Extract</td>
<td>75,000</td>
<td>55,000</td>
<td>0.73</td>
</tr>
<tr>
<td>First (NH₄)₂SO₄ precipitate</td>
<td>69,300</td>
<td>34,200</td>
<td>0.49</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄ precipitate</td>
<td>60,300</td>
<td>27,100</td>
<td>0.45</td>
</tr>
<tr>
<td>Sephadex G-200-excluded frac</td>
<td>56,500</td>
<td>19,700</td>
<td>0.35</td>
</tr>
</tbody>
</table>
Scheme 1. Extraction of soluble NADH-Co\textsubscript{Q} reductase from ETP inhibited with rotenone-\textsuperscript{14}C. An ETP sample (500 mg of protein), suspended in 50 ml of sucrose-phosphate-BSA medium, was treated with 100 \textmu moles of rotenone-\textsuperscript{14}C. The particles were sedimented by centrifugation and washed four times with sucrose-phosphate-BSA medium as described in "Methods," and then were washed once with dilute phosphate, pH 7.4, and finally with water to remove all of the BSA. The particles were diluted to 20 ml with water and extracted with acid-ethanol by the procedure of Pharo et al. (16). NADH oxidase activity was determined immediately after titration and after the last wash. Determination of the specific activity (micromoles of NADH per min per mg of protein) of NADH-Q\textsubscript{6} reductase in the acid-ethanol extract of the rotenone-treated ETP sample showed essentially the same value (25) as obtained in a control sample (26) extracted from untreated ETP.

Piericidin and Rotenone Interactions with Soluble NADH-Co\textsubscript{Q} Reductase—In view of the evidence presented that the binding site of rotenone is not the same in particles as in soluble preparations, it is of interest to consider the characteristics of the binding curve of rotenone in the soluble preparation. Fig. 6 shows the rotenone-\textsuperscript{14}C titration curve for the preparation of Pharo et al. (16) from ETP, at the extract stage, the titration being performed directly on the extract without the addition of BSA, since the latter is strongly inhibitory to the soluble reductase (16). The apparent binding is nonlinear, in contrast to the behavior of particles, and there is no correlation between inhibition and apparent rotenone binding.

A marked qualitative difference occurs in rotenone binding between particles and this type of ethanol extract; particles bind the inhibitor so tightly that it may be extracted only with anhydrous organic solvents, while binding to the soluble protein must be very loose since throughout the range of titration less than 1% of the added rotenone accompanies the enzyme in the Sephadex G-25-excluded fraction. At the point of maximal inhibition, which corresponds to about 6 moles of rotenone per mole of enzyme-bound FMN (in confirmation of our earlier report (17)), only 0.65% of the added rotenone-\textsuperscript{14}C appears in the protein fraction. The rotenone-\textsuperscript{14}C accompanying the protein on Sephadex passage corresponds to about 0.04 mole per mole of FMN. Furthermore, when the Sephadex-passed protein was precipitated with 10% trichloroacetic acid, almost half of the \textsuperscript{14}C label was released into solution. It appears virtually certain, therefore, that more passage through Sephadex G-25 dissociates the rotenone-reductase complex and that, consequently, rotenone binding by the soluble enzyme does not represent a stoichiometric binding, as has been claimed (29), but is instead a weak interaction.
With piericidin A and soluble NADH-CoQ reductase, the shape of the inhibition curve and the extent of maximal inhibition reached are entirely similar to the same parameters as observed with rotenone (Figs. 6 and 7 and Reference 17); however, piericidin A appears to inhibit at lower concentrations than rotenone (maximal inhibition with the same sample of enzyme is reached with $1.2 \times 10^{-8}$ M piericidin A and $4.3 \times 10^{-8}$ M rotenone).

**DISCUSSION**

Several assumptions on the reaction of rotenone with the mitochondrial respiratory chain need to be revised on the basis of the present studies with rotenone-$^{14}$C. One of these concerns the specificity of rotenone as an inhibitor. The linear nature of the binding curve (Fig. 2) indicates that little difference exists in the affinity of binding at the specific site, in the NADH dehydrogenase segment of the respiratory chain, and elsewhere in submitochondrial particles. Differential binding at the specific site is evident only in the presence of BSA. Rotenone also inhibits yeast alcohol dehydrogenase (27) and modifies bovine liver glutamate dehydrogenase (26). Amytal is similarly unspecific, since it also inhibits energy conservation reactions (6), electron transport from choline dehydrogenase to the respiratory chain (28), and extramitochondrial "DT diaphorase" (29).

The assumption that inhibition by rotenone is irreversible (6), just as is inhibition by antimycin A, must be revised because successive washing with BSA in sucrose releases rotenone-$^{14}$C, even from the site responsible for the inhibition of NADH oxidation, with concomitant partial return of the enzyme activity. It is possible that even more, and perhaps all, of the enzyme activity in preparations which are completely inhibited might be regenerated by further washing with BSA or with proteins which bind rotenone more strongly than BSA.

The concentration of rotenone required for complete inhibition of NADH oxidation in mitochondria was stated to be lower than that of any known component of the respiratory chain, including NADH dehydrogenase (6). The amount of the rotenone-sensitive electron transport component has been calculated (6) to be 10 to 20 times less than the amount of flavin, which, according to Chance and Williams (30), becomes reduced in anaerobiosis by NADH-linked substrates. However, as previously noted, the spectrophotometric method used by Chance and Williams does not measure flavin exclusively because various cytochromes, and especially non-heme iron, contribute strongly to the absorbance changes (12, 13). In addition, interconnections exist among flavoproteins at the level of the respiratory chain (31, 32), and so NADH can reduce not only its own flavoprotein but others as well.

Rotenone titration in the presence of BSA, followed by washing with BSA, results in preferential binding of rotenone at the specific site. If, under these conditions, the specific site is just completely blocked with piericidin A and rotenone-$^{14}$C is then added, the extent to which the binding capacity for rotenone-$^{14}$C is lowered by the piericidin permits calculation of the approximate concentration of rotenone which is bound at the specific site. Such a calculation from the difference between Curves A and B in Fig. 4, and particularly from the data in Table II, gives a value of 40 mmoles of rotenone bound per g of ETP protein at this level. Cremona and Kearney (24) estimated that the NADH dehydrogenase content of an ETP sample with a specific activity of 25 in the NADH-ferricyanide assay is 31.3 mmoles per g of protein. Since the specific activity of the ETP used in these experiments was 31.6, the NADH dehydrogenase content is also approximately 40 mmoles per g of protein (31.6/25 x 31.3). Thus, despite the fact that the method used gives a lower rotenone titer than the method used by Ernster, Dellner, and Azzone (6), the rotenone titer approximates the NADH dehydrogenase content of submitochondrial particles.

Although it might be tempting to conclude from the similarity of these values that the binding of rotenone occurs on NADH dehydrogenase, this is not the case, since on complete extraction of NADH dehydrogenase either from ETP (23) or from phosphorylating mitochondria (Table VI) inhibited with rotenone-$^{14}$C only a trivial fraction of the radioactivity is recovered after purification; the rest remains firmly bound to the insoluble residue. Amytal and piericidin A are bound at the same specific site as rotenone, and so these binding experiments provide further evidence for the suggestions (12, 14, 23, 33) that barbiturates, piericidin A, and rotenoids all act on the O$_2$ side of NADH dehydrogenase and not between NADH and flavoprotein. Since the same results are obtained on treatment of phosphorylating and nonphosphorylating preparations with rotenone-$^{14}$C, it is clear that the reaction site of these inhibitors does not vary according to the state of phosphorylation, as has been suggested (8, 9) on the basis of spectrophotometric evidence comparing the effect of Amytal on intact mitochondria and on the NADH-cytochrome c reductase complex. The preparations differ not only in phosphorylating capacity but also in composition: the NADH-cytochrome c reductase complex is said to be virtually devoid of cytochromes c, a, and a$_3$ (8) and, owing to the method of preparation, it is not likely to contain significant amounts of the various mitochondrial flavoproteins with which NADH dehydrogenase is functionally interlinked in mitochondria. Thus the observation that Amytal abolishes the reduction of cytochromes b and a$_3$ but does not inhibit reduction of the flavin by NADH in this preparation (8), is probably explainable on the
basis that NADH dehydrogenase is more concentrated in these particles than in mitochondria and that absorbance changes due to cytochromes and other respiratory pigments are less likely to mask absorbance changes of the NADH dehydrogenase flavin in this preparation than in mitochondria. The same considerations apply to the report of Öberg (5) that rotenone acts on the substrate side of NADH dehydrogenase, since his conclusions were also based entirely on spectrophotometric observations.

Recent unpublished electron paramagnetic resonance experiments, performed in collaboration with Dr. Graham Palmer, reinforce the conclusion that barbiturates, rotenone, and piericidin all act on the $O_2$ side of NADH dehydrogenase. None of these inhibitors prevents the appearance of the NADH-induced $g\cdot 1.94$ signal of NADH dehydrogenase in submitochondrial particles, but they all interfere with the appearance of the signal associated with the non heme iron of aqueous dehydrogenase and of the $b\cdot c$ complex. All the results mentioned are in accord with the mechanism shown in Scheme 2.

The identity of the respiratory chain component which binds rotenoids, piericidin A, and barbiturates remains obscure, although the results presented are in accord with the suggestion (14) that a lipid may be involved, since neither denaturation nor proteolysis liberates significant amounts of rotenone from the specific site, but extraction with organic solvents is effective in removing almost all of the bound rotenone. Despite the fact that there is a pronounced lag in attaining maximal inhibition after the addition of rotenone, analysis of the bound rotenoids clearly shows that rotenone per se, rather than a reaction product of rotenone, is the actual inhibitor.

The results presented in Scheme 1 support previous findings (1, 17) that the reaction site of rotenone and of coenzyme Q homologues in the soluble NADH-CoQ reductase (10) is not the same as in more intact particles, but emerges as a result of the modifying effects of the extraction procedure. If lipids are involved in the binding of rotenone, piericidin A, and Amytal in mitochondria, as suggested, a qualitatively different mechanism of inhibition of the soluble NADH-CoQ reductase is expected, since this preparation is lipid-free. The interaction of rotenone (14) that a lipid may be involved, since neither denaturation nor proteolysis prevents the appearance of the NADH-induced $g\cdot 1.94$ signal of NADH dehydrogenase in submitochondrial particles, but they all interfere with the appearance of the signal associated with the non heme iron of aqueous dehydrogenase and of the $b\cdot c$ complex. All the results mentioned are in accord with the mechanism shown in Scheme 2.

The identity of the respiratory chain component which binds rotenoids, piericidin A, and barbiturates remains obscure, although the results presented are in accord with the suggestion (14) that a lipid may be involved, since neither denaturation nor proteolysis liberates significant amounts of rotenone from the specific site, but extraction with organic solvents is effective in removing almost all of the bound rotenone. Despite the fact that there is a pronounced lag in attaining maximal inhibition after the addition of rotenone, analysis of the bound rotenoids clearly shows that rotenone per se, rather than a reaction product of rotenone, is the actual inhibitor.

The results presented in Scheme 1 support previous findings (1, 17) that the reaction site of rotenone and of coenzyme Q homologues in the soluble NADH-CoQ reductase (10) is not the same as in more intact particles, but emerges as a result of the modifying effects of the extraction procedure. If lipids are involved in the binding of rotenone, piericidin A, and Amytal in mitochondria, as suggested, a qualitatively different mechanism of inhibition of the soluble NADH-CoQ reductase is expected, since this preparation is lipid-free. The interaction of rotenone (14) that a lipid may be involved, since neither denaturation nor proteolysis prevents the appearance of the NADH-induced $g\cdot 1.94$ signal of NADH dehydrogenase in submitochondrial particles, but they all interfere with the appearance of the signal associated with the non heme iron of aqueous dehydrogenase and of the $b\cdot c$ complex. All the results mentioned are in accord with the mechanism shown in Scheme 2.

The identity of the respiratory chain component which binds rotenoids, piericidin A, and barbiturates remains obscure, although the results presented are in accord with the suggestion (14) that a lipid may be involved, since neither denaturation nor proteolysis liberates significant amounts of rotenone from the specific site, but extraction with organic solvents is effective in removing almost all of the bound rotenone. Despite the fact that there is a pronounced lag in attaining maximal inhibition after the addition of rotenone, analysis of the bound rotenoids clearly shows that rotenone per se, rather than a reaction product of rotenone, is the actual inhibitor.

The results presented in Scheme 1 support previous findings (1, 17) that the reaction site of rotenone and of coenzyme Q homologues in the soluble NADH-CoQ reductase (10) is not the same as in more intact particles, but emerges as a result of the modifying effects of the extraction procedure. If lipids are involved in the binding of rotenone, piericidin A, and Amytal in mitochondria, as suggested, a qualitatively different mechanism of inhibition of the soluble NADH-CoQ reductase is expected, since this preparation is lipid-free. The interaction of rotenone (14) that a lipid may be involved, since neither denaturation nor proteolysis prevents the appearance of the NADH-induced $g\cdot 1.94$ signal of NADH dehydrogenase in submitochondrial particles, but they all interfere with the appearance of the signal associated with the non heme iron of aqueous dehydrogenase and of the $b\cdot c$ complex. All the results mentioned are in accord with the mechanism shown in Scheme 2.

Acknowledgments We thank J. L. Engel and E. C. Kimmel for technical assistance, and Professor L. Crombie for samples of most of the authentic rotenoids used.

REFERENCES