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

XI. TRANSFORMATION OF THE DEHYDROGENASE TO REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE-COENZYME Q REDUCTASE*

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SUMMARY

Purified preparations of the reduced nicotinamide adenine dinucleotide dehydrogenase of the respiratory chain, extracted with the aid of phospholipase at moderate temperature, do not catalyze the reduction of long chain coenzyme Q derivatives (Q₆ and Q₁₀) at significant rates and are not inhibited by amytal or rotenone. On exposure of the enzyme to conditions which have been used for the extraction of NADH-coenzyme Q reductase (pH 5.4, 9.5% ethanol 43°C for 15 min) the characteristic properties and catalytic activities of the dehydrogenase are lost and a high catalytic activity toward coenzyme Q₆ and Q₁₀ emerges, along with cytochrome c reductase activity. The NADH-coenzyme Q reductase activity created by this treatment is comparable to that obtained on direct extraction of submitochondrial particles with acid-ethanol at 43°C and is inhibited by amytal and rotenone to the same extent as NADH-coenzyme Q reductase. The lack of rotenone- and amyral-sensitive coenzyme Q reductase activity in NADH dehydrogenase is not due to the exposure of the preparation to pH 8.5 or to phospholipase A, as has been suggested by other authors, since submitochondrial particles isolated at pH 8.5 and at neutral pH are equally good sources of soluble NADH-coenzyme Q reductase and since neither cobra venom nor its constituent phospholipase A inhibit or inactivate soluble NADH-coenzyme Q reductase. The reason why venom treatment of particles interferes with subsequent extraction of NADH-coenzyme Q reductase by heat-acid-ethanol is that an inhibitor is formed by the venom, which can be removed, however, with the aid of serum albumin. Evidence is presented that the reaction sites of long chain coenzyme Q homologues and of rotenone and amyral in soluble NADH-coenzyme Q reductase are not the same as in intact mitochondria and submitochondrial particles. Soluble NADH-coenzyme Q reductase resembles closely in its known properties NADH-cytochrome c reductase and, like the latter enzyme, may be regarded as a fragment of NADH dehydrogenase created by exposure to heat-acid-ethanol.

The NADH dehydrogenase of the mammalian respiratory chain has been isolated in several forms, which differ in a characteristic manner in their catalytic and molecular properties. There has been considerable discussion in the literature as to which of these resemble most closely the enzyme as it occurs in the mitochondrion, and which are modification or degradation products or possible subunits of the enzyme. The NADH-CoQ reductase complex (Complex 1) (4) is a particulate preparation of the flavoprotein which appears to contain an unimpaired complement of labile S and of nonheme iron as well as considerable lipid, including bound Qro. It shows the characteristic high ferricyanide activity and substrate-inducible EPR signal at g = 1.94 of the intact enzyme, and is virtually devoid of cytochrome c reductase activity. It reduces externally added short chain CoQ homologues rapidly. The NADH-Q₁ reaction is virtually completely inhibited by amyral and rotenone (6).

1 The abbreviations used are: CoQ, coenzyme Q; ETP, electron transport particle; ETPn, phosphorylating electron transport particle prepared by sonication; SP₃₂, ETPn prepared without additives by the method of Pharo et al. (5).

2 In unpublished experiments in this laboratory it has now been shown that Complex 1 also reduces Q₆ and Q₁₀ rapidly, when the assay system of Pharo et al. (5) is used. With a fresh preparation isolated in this laboratory, the activity ratio at 20°C of Q₁-Q₆-Q₁₀ was 100:101:95. With a preparation kindly supplied...
A soluble, extensively purified, high molecular weight preparation was isolated in the authors' laboratory (7, 8) which shows the same molecular composition (6) as Complex 1, and shares all the catalytic properties of this complex (and of the enzyme as it occurs in submitochondrial particles), including functional nonheme iron (9), except that it is lipid-free, and cannot catalyze the amytal- and rotenone-sensitive reduction of CoQ homologues (6). Evidence has been presented (6, 10, 11) that the inhibitor-sensitive reduction of exogenous CoQ homologues requires phospholipids and possibly also bound Q10, and it has been suggested that the absence of rotenone-sensitive Q1 reductase activity in the soluble dehydrogenase is a consequence of the separation of lipids from the enzyme during the extraction, and that such lipids are not readily replaced in their original conformation, rather than of a structural modification of the enzyme itself.

A third type of NADH dehydrogenase isolated from mitochondria is represented by the 80,000 molecular weight class (12), which may be extracted at elevated temperatures with acid ethanol or phospholipase A (12–15). These preparations generally show a low nonheme iron and labile S content, and a modified absorption spectrum, contain no lipid, and have lost the substrate-inducible EPR signal at g = 1.94. They further differ from the high molecular weight enzyme and from Complex 1 in substrate specificity, in a much lower reactivity with ferricyanide, and high cytochrome c and indophenol reductase activities.

Despite seeming differences, careful comparison of these cytochrome-reducing preparations has failed to reveal basic dissimilarities in composition or properties (16–18). Since the conditions used for the extraction of these proteins (heat, with or without ethanol and acid pH) as well as proteinolysis, and even prolonged contact with the substrate lead to the conversion of the high molecular weight form to the 80,000 molecular weight form (16, 17), with the liberation of peptides of various chain lengths, it has been proposed that the low molecular weight preparations are fragments of the dehydrogenase.

Probably the most interesting one among the low molecular weight preparations of NADH dehydrogenase is the NADH-ubiquinone reductase (NADII-enzyme Q reductase) of Phar and Sanadi (19) and Sanadi (20). This flavoprotein is extracted from phosphorylating particles (ETPn) by the heat-acid-ethanol procedure previously used in the extraction of NADH-cytochrome c reductase (13). It catalyzes the oxidation of NADH by Q1, Q9, and Q10, and these reactions are partially inhibited by amytal and rotenone. Although, as expected from the method of preparation, the flavoprotein has lost the characteristic EPR signal at g = 1.94 and the high ferricyanide activity and nonheme iron and labile S content (6), the apparent retention of amytal- and rotenone-sensitive Q9 reductase activity has led to the interpretation (19) that it represents the respiratory chain-linked enzyme in a purified, functional state. The failure of other investigators to find CoQ reductase activity in cytochrome reductases isolated by nearly identical procedures from the same tissue has been ascribed to differences in the particulate starting material used (e.g. ETPH or ETP) (19, 20). The absence of inhibitor-sensitive CoQ reduction in NADH dehydrogenase was ascribed (19, 21) in part to the use of snake venom phospholipase, which is thought to destroy the CoQ reaction site, in part to the exposure of the mitochondria to pH 8.5 in the isolation of the starting material (ETP). It may be noted that the enzyme may be equally well isolated from ETPH and from other types of particles which are never exposed to alkaline pH (18).

Machinist and Singer (6, 11) have compared NADH-CoQ reductase and NADH cytochrome c reductase, extracted from ETPH and ETP, respectively, by heat-acid-ethanol in regard to substrate specificity, QF, chromatographic behavior, flavin, iron, and labile S content, and relative activities with Q1, Q9, ferricyanide, menadione, and cytochrome c. In all of these regards, the two preparations appeared indistinguishable. The same degree of inhibition by amytal was observed in both. It was also shown that particles prepared at pH 8.5 (ETP) and at neutral pH (ETPH) have the same activity toward CoQ homologues. Hence the notion that extraction of the latter leads to CoQ reductase, and of the former to cytochrome reductase, could not be verified. A possible clue to the origin of the high CoQ reductase activity of the low molecular weight preparations was the finding (6) that exposure of purified NADH dehydrogenase to 38°, or to acid-ethanol at 42°, resulted in a large increase in Qc reductase activity, which parallels the emergence of cytochrome c reduction and the decay of the ferricyanide activity.

While these results suggested that soluble NADH-CoQ reductase resembles very closely NADH-cytochrome c reductase in its known properties, there remained one problem. In previous work in this laboratory the Qc reductase activities of soluble preparations were not as high as those reported by Pharo et al. (5) and the considerable experimental error inherent in the methods then available (6) prevented critical comparison of the Qc and Q10 reductase activities of different preparations. The recent publication of the revised assay used by Pharo et al. (5) has enabled us to examine the remaining uncertainties regarding the relations of NADH-CoQ reductase to NADH cytochrome c reductase and to the higher molecular weight NADH dehydrogenase.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

ETP and NADH dehydrogenase were prepared as previously described (7). SPn, was isolated by the method of Pharo et al. (5) and ETPH by the same procedure, except the additives used by Hansen and Smith (22) (ADP, succinate, Mg++, Mn++) were not present during sonic oscillation and centrifugation. Soluble NADH-CoQ reductase was extracted from particles by Dr. Y. Hatefi, which had been frozen-thawed twice, the ratio was 100:22:5; in this case differential loss of activity toward long chain CoQ homologues had evidently occurred.

*In our previous studies (6, 11) the method briefly outlined in the preliminary note of Pharo and Sanadi (20) was used for soluble preparations. This method has been recently revised by these authors (5) in two important respects: 0.1 mm Q4 has been substituted for 0.25 mm Q4 and the reaction is now started with enzyme, while in the old method the enzyme was preincubated with the quinone at 30° during the preincubation period and NADH was added to start the reaction. Since detergents or albumin cannot be used to disperse the quinone since they inhibit the enzyme (5), variable turbidity changes and some inactivation of the soluble enzyme during the preincubation caused erratic results (6, 11). With the new method, in agreement with Pharo et al. (5), better than 5% replicability is easily attained. With enzyme is used to start the reaction, the use of a double beam instrument is mandatory. When NADH is added first, either a double beam or a single beam instrument, such as the Gilford model 220, give satisfactory results, provided that a suitable blank is run to correct for turbidity changes.*
the method of Pharo et al. (5) and was used immediately after preparation. Lyophilization of such extracts, as recommended by these authors, caused a variable (30 to 50%) loss of NADH-Q$_6$ activity.

The reduction of long chain CoQ homologues by soluble preparations was performed exactly as described by Pharo et al. (5). Despite the heterophasic system, the results were satisfactorily replicable. During the assays, stock solutions of Q$_5$ and Q$_6$ were maintained in the dark at 30° and 50°, respectively, to prevent precipitation. Absorbance changes at 340 mpw were recorded with a Cary model 14 (0.5 A full scale, 3 to 4 inches per min chart speed) or Gilford spectrophotometer (0.25 A full scale, 3 to 6 inches per min). With the former instrument, rates were recorded against a blank (no NADH); with the latter, the blank rates were separately determined and subtracted. The assay was scaled down to 1-ml volume when required. With particulate enzymes, the CoQ assay was as in previous work (6, 11). Water, 0.3 ml of Tris-sulfate, pH 8.0, 0.05 ml of CoQ (6 mM solution in methanol in the case of Q$_1$ and Q$_6$, and 4 mM with Q$_{10}$), 0.1 ml of 30 mM neutral KCN, and enzyme (0.05 to 0.1 ml of 3 to 5 mg per ml) suspension were incubated in a 2.9-ml volume at 30° for 2 min, transferred to the spectrophotometer, and the reaction was started by the addition of 0.1 ml of 4 mM NADH. Cytochrome c reductase and ferricyanide activities were measured as in previous work (7, 23). Protein was determined by the method of Lowry et al. (24) in extracts and by the biuret reaction (25) in particles.

**NADH-CoQ Reductase from Different Submitochondrial Particles**—Reports from Sanadi's laboratory (19-21) have emphasized that soluble NADH-CoQ reductase may be obtained from phosphorylating submitochondrial particles (ETP$_H$ or SP$_{HL}$), but not from nonphosphorylating ones, such as ETP or hog heart particles, which are the source of the NADH-cytochrome c reductase of Mahler et al. (12). In particular, the exposure of mitochondria to pH 8.5 during the isolation of ETP has been suggested (19, 21) as the factor which may be responsible for the absence of NADH-Q$_6$ reductase activity of enzymes derived from this source.

In our earlier studies (6, 11) ETP and ETP$_H$ were compared with regard to NADH dehydrogenase, Q$_1$ and Q$_6$ reductase activities and sensitivity to amytal and rotenone and no major difference was noted. Extracts of these particles were prepared by the DeBernard procedure (13) (9% ethanol, 15 min at 44°, pH 4.8), as recommended by Pharo and Sanadi (20), and comparison by a series of criteria again revealed no significant difference (6, 11). With the availability of those authors' assay for Q$_5$ and Q$_{10}$ reductase activities (5), it was desirable to extend the comparison to extracts prepared under the conditions currently recommended by Pharo et al. (5) (9.5% ethanol, 15 min at 43°, pH 5.3). Table I, Experiments 1 and 2, shows that extracts of phosphorylating particles (ETP$_H$ and SP$_{HL}$) and of ETP prepared under these conditions have similar activities with Q$_5$, Q$_6$, and Q$_{10}$ as electron acceptors. The specific activities compare favorably with those reported in the literature for such extracts. In other experiments, the specific activity for the NADH-Q$_4$ reaction ranged from 11 to 27 in extracts of particles. Although not shown in the table, the yields of Q$_4$ reductase activity in extracts per unit weight of particle were also very similar for ETP, ETP$_H$, and SP$_{HL}$.

Extracts derived from particles which had been stored at -15° for 2 to 4 weeks had much lower specific activities toward Q$_4$ and Q$_{10}$ (Table I, Experiment 3), although their Q$_1$ reductase activity was high, sometimes higher than in extracts of strictly fresh particles. Extracts of aged particles also show high activities toward Q$_5$ and cytochrome c. Whatever damage occurs on storage, it is not reflected in ferricyanide or Q$_6$ reductase activities of the particles themselves.

**Effect of Lyophilization**—Since reactivity with long chain CoQ homologues is quite labile in extracts and may decay significantly on a few hours of storage at 0° in the dark, in contrast to Q$_5$ and cytochrome reductase activities, which are preserved more readily, it is necessary to use extracts immediately after preparation to find a method for stabilizing them. Pharo et al. (5) have recommended freeze-drying and storage at about -70°. In our hands, however, lyophilization of ethanolic extracts in the dark invariably causes 30 to 50% loss of Q$_6$ reductase activity. Thus, extracts prepared from SP$_{HL}$ and ETP, which had specific activities of 28.3 and 18.3, respectively, when fresh, deteriorated to specific activities of 16.4 and 12.6 after lyophilization. Consequently, the studies reported in this paper utilized extracts within 2 to 3 hours of preparation. A rapid method for the isolation of the enzyme in ultracentrifugally homogeneous form will be published elsewhere.

**Effect of Amytal and Rotenone on Q$_6$ Reduction**—The bulk of available evidence suggests that in metabolically intact particles both the reaction with CoQ homologues and combination with rotenone or amytal occur on the Q$_6$ side of the nonheme iron associated with NADH dehydrogenase (11, 18). It would follow that, in preparations such as the soluble NADH-CoQ reductase, from which most of the iron is lost during the extraction procedure and what remains is rendered catalytically inactive, the reaction site of CoQ may be different than in mitochondria. One of the few direct approaches to this problem is a comparison of the behavior of the NADH-CoQ reaction to inhibitors in soluble preparations and in particles.

We have shown (6, 11) that the reduction of short chain CoQ homologues (Q$_5$) by the preparation of Pharo and Sanadi (20) is insensitive to both amytal and rotenone, although this reaction is almost completely inhibited by these reagents in ETP, ETP$_H$, and Complex 1. Thus the reduction of Q$_5$ seems to occur at a new and different site in heat-acid-ethanol extracted preparations.

### Table I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source</th>
<th>Specific activity</th>
<th>Ratio of Q$_6$ to Q$_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ETP, fresh</td>
<td>24.9</td>
<td>100:44:25</td>
</tr>
<tr>
<td></td>
<td>ETP$_H$, fresh</td>
<td>24.3</td>
<td>100:57:31</td>
</tr>
<tr>
<td>2</td>
<td>ETP, fresh</td>
<td>21.7</td>
<td>100:65:44</td>
</tr>
<tr>
<td></td>
<td>ETP$_H$, fresh</td>
<td>21.7</td>
<td>100:65:44</td>
</tr>
<tr>
<td>3</td>
<td>ETP, aged</td>
<td>26.6</td>
<td>100:54:66</td>
</tr>
<tr>
<td></td>
<td>ETP$_H$, aged</td>
<td>26.5</td>
<td>100:54:66</td>
</tr>
</tbody>
</table>

*In micromoles of NADH oxidized per min per mg of protein in acid-ethanol extracts.*

Q$_4$ and Q$_{10}$ (Table I, Experiment 3), although their Q$_1$ reductase activity was high, sometimes higher than in extracts of strictly fresh particles. Extracts of aged particles also show high activities toward Q$_5$ and cytochrome c. Whatever damage occurs on storage, it is not reflected in ferricyanide or Q$_6$ reductase activities of the particles themselves.
than in particles; a direct reaction with the flavin seems a good possibility. The fact (6) that heating of NADH dehydrogenase creates $Q_0$ and cytochrome $c$ reductase activities concurrently, while labeling the iron, is in accord with this, as are the data on the previous section on the stability of $Q_0$ reductase activity, compared with the stability of $Q_0$ reduction, in extracts prepared from aged particles.

As to the reaction with long chain CoQ homologues, in particles it is over 95% inhibited by low concentrations of anhydro and rotenone (20) (Table II). According to Sanadi, Pharao, and Sordahl (19) in their soluble preparation a relatively high concentration of anhydro (3.5 mm) is required to bring about partial inhibition (25 to 50%), while rotenone is said to inhibit up to 70 to 75% at a ratio of rotenone to FMN of 1. Higher concentrations of rotenone overcome the inhibition. This curious behavior has not been satisfactorily explained and is in contrast to intact particles, in which the inhibition by rotenone remains nearly complete no matter how high the inhibitor-FMN ratio.

In previous studies in this laboratory with a $Q_1$ assay, subject to considerable variation, a maximum of 40% inhibition of $Q_0$ reduction by anhydro and 20% by rotenone was found in heat-alcohol extracts of either ETP or ETPH (6). With the assay recommended by Pharao et al. (5) in extracts of ETP, ETPH, or ETPH, prepared at pH 5.3, inhibition of $Q_0$ or $Q_0$ reduction by anhydro ranged from 25 to 40% and required 3 to 6 mm anhydro, well in excess of that needed to cause over 90% inhibition in the parent particles.

Titration curves with rotenone were performed with many preparations; some typical results are shown in Fig. 1. It may be seen that (a) the maximal inhibition reached was 25 to 40%, and was not materially different in extracts of ETPH and ETP; (b) at high ratios of rotenone to FMN, the inhibition disappeared completely; (c) maximal inhibition occurred at ratios of 4.5 to 6.5 and not 1. In fact, since the majority of the FMN in such acid-alcohol extracts is detached from the protein during the thermal treatment, the ratio of rotenone to protein-bound FMN is two to three times higher than that shown here. Lastly, at very low rotenone concentrations an activation was noted which in some cases amounted to 20%. These considerations and the results with anhydro are not readily reconciled with the view that the reaction site of $Q_0$ is the same in soluble samples of the reductase and in respiratory chain preparations, but may be compatible with the suggestion that the reactive site or conformation responsible for the $Q_0$ reduction in soluble extracts is revealed or created by the exposure to heat, ethanol, and acid pH, as is also true of cytochrome $c$ reduction. Additional evidence for this view is given in the "Discussion."

Table II presents data on the fate of the rotenone inhibitable and the rotenone-insensitive fractions of $Q_0$ reductase activity during the heat-alcohol conversion. With ETP, 23% of the total activity survived this treatment, but, while most of the rotenone-sensitive $Q_0$ activity (91%) was lost, the insensitive activity increased by 800%. The figures for ETPH are quite comparable, considering the errors in rotenone titrations.

Conversion of NADH Dehydrogenase to NADH-CoQ Reductase

If the partially rotenone- and anhydro-sensitive CoQ reduction observed in soluble NADH-CoQ reductase does not represent the physiological reaction as it occurs in mitochondria but is a new activity created or revealed by the modifying effects of heat, ethanol, and acid pH, then it should be possible to convert by the same treatment the purified, soluble dehydrogenase, which is devoid of significant activity toward long chain CoQ homologues, to a low molecular weight fragment endowed with such activity. On the other hand, if the exposure to pH 5.3 and to phospholipase A during the preparation of the dehydrogenase had destroyed the CoQ reaction site, as has been postulated (19), such conversion would not be expected.

Fig. 2 shows that, when a purified preparation of NADH dehydrogenase is exposed to temperatures above 43° in the presence of 9.5% ethanol at pH 5.3, there is a progressive emergence of $Q_0$ and cytochrome $c$ reductase activities, both of which increase with temperature in the range of 33° to 43°. Exposure to pH 5.3 and ethanol in the cold or heating at 43° at neutral pH without ethanol do not give rise to $Q_0$ reductase activity, although they destroy ferriy cyanide activity. (Thermal treatment alone greatly increases $Q_0$ reductase activity, however (6).)

Thus, simultaneous exposure to heat, ethanol, and acid pH are required for the transition of the flavoprotein to a form capable of reducing long chain CoQ homologues.

A number of preparations of purified NADH dehydrogenase have been treated with ethanol at pH 5.3 and 43° under the exact conditions which have been used in the extraction of NADH-CoQ reductase (5). Typical results for one preparation are sum-
marized in Table III. It may be seen that a very large increase in total Q₆ and Q₁₀ reductase activities occurs, along with the expected rise in Q₆ and cytochrome c reductase activities and the almost complete disappearance of ferricyanide activity. The increase in specific activity toward Q₆ and Q₁₀ is particularly impressive: the values for the extract obtained from the dehydrogenase compare favorably with those reported for extracts of ETP₉ and SP₉L (5, 17).

As is true of extracts of particles (Table I), storage of the purified dehydrogenase at 0° also reduces materially the yield of Q₆ and Q₁₀ reductase activities which emerge are inhibited by amytal and rotenone to about the same extent (20 to 35% and 25 to 50%, respectively) as in extracts obtained from ETP₉. Titration of 1.8 μg of the preparation characterized in Table III with amytal resulted in a maximal reduction of activity of 29% at 6 mM amytal. Additional amytal did not further inhibit the enzyme preparation. The preparation used in the experiments of Table III was isolated from fresh ETP, and the conversion to CoQ reductase was performed within a day of isolation of the enzyme.

The most interesting feature of the transformation of NADH dehydrogenase to NADH-CoQ reductase under the influence of heat-acid-ethanol is that the Q₆ and Q₁₀ reductase activities which emerge are inhibited by amytal and rotenone to about the same extent (20 to 35% and 25 to 50%, respectively) as in extracts obtained from ETP₉. Titration of 1.8 μg of the preparation characterized in Table III with amytal resulted in a maximal reduction of activity of 29% at 6 mM amytal. Additional amytal did not further inhibit the enzyme preparation. The partial inhibition by rotenone and recovery of the activity at higher rotenone concentrations are very similar to the effects noted with authentic NADH-CoQ reductase (Fig. 1).

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Fig. 3. Titration of NADH-CoQ reductase derived from NADH dehydrogenase with rotenone. The dehydrogenase sample, prepared as in Fig. 2, was treated with acid-ethanol at 43° according to the procedure of Pharo et al. (5). After neutralization and removal of sediment Q₆ assays were run in 1-ml cuvettes. Rotenone was present during the preincubation (5) and the reaction was started with 1.8 μg of enzyme. Protein-bound FMN was determined immediately after rapid passage of the enzyme through Sephadex G-25 to remove flavin. O, NADH dehydrogenase preparation, specific activity, 471 in the ferricyanide assay, 0.022 in the Q₆ assay before conversion; specific activity for Q₆ after conversion, 180. ●, NADH dehydrogenase after first (NH₄)₂SO₄ step (7); specific activity, 296 in the ferricyanide assay, 0.007 in the Q₆ assay. The enzyme had been stored at 0° for 1 week before conversion. Specific activity in Q₆ assay after conversion, 0.6.

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Effect of Venom on Extraction of Reductase—Sanadi et al. (19) and Sanadi (21) recently reported that a few minutes' incubation of SP₉L with 2.5 mg boiled Naja naja venom per g of particle prevents subsequent extraction of NADH-CoQ reductase by heat-acid-ethanol. They suggested that the venom may either alter the Q₆ reaction site or labilize an essential component of the enzyme. These observations pose some interesting questions. If the lack of appreciable Q₆ reductase activity in the dehydrogenase is due to the use of snake venom enzyme in its extraction, why does heat-acid-ethanol convert it to a Q₆ reductase indistinguishable from that which one obtains...
by direct extraction of ETPA⁻. If, on the other hand, the flavoprotein fails to reduce Q₆ not because it has been treated with a phospholipase but because it has not been exposed to heat-acid-ethanol, then why does brief exposure of ETPA⁻ to venom or purified phospholipase A interfere with subsequent extraction with heat-acid-ethanol of a partially rotenone-sensitive Q₆ reductase?

Although, as discussed later in this paper, it seems that long chain CoQ homologues react at different sites in intact particles and in the soluble NADH-CoQ reductase, it was thought that earlier studies on the reversible inactivation of the NADH-CoQ reaction in particles by venom (10, 11) might provide an answer to these questions. Fleischer et al. (10) and Machinist and Singer (11) reported that very brief treatment of submitochondrial particles with low concentrations of N. naja venom (insufficient to solubilize the flavoprotein) abolishes the physiological, fully rotenone-sensitive NADH-CoQ reaction. Washing with serum albumin followed by the addition of phospholipids restored the activity. These results were interpreted to suggest that the inactivation by the venom is due in part to the formation of some inhibitor, in part to the cleavage of phospholipids which may be required for the reaction of the flavoprotein with exogenous CoQ. It was decided, therefore, to examine the effect of serum albumin on the extractability of venom-treated particles.

Table IV, Experiment 3, confirms qualitatively the report of Sanadi et al. (19) on the interference of venom treatment with subsequent extraction of the soluble reductase. In our experience, however, the effect of the venom is not as extensive as has been claimed; even when the extraction was performed directly on the venom-treated particles, without any effort to remove the venom by washing procedures, a substantial part of the activity remained extractable. When the venom-treated particles were washed with bovine serum albumin in sucrose, followed by additional centrifugations to remove residual albumin, which interferes with the assay, most or all of the potential NADH-CoQ reductase activity was recovered on subsequent extraction (Table IV, Experiment 4).

This reactivation is incompatible with the notions (19, 21) that the venom modifies a reaction site or removes an essential component. Direct evidence against these hypotheses came from treatment of soluble NADH-CoQ reductase, extracted from ETPA⁻, with crude venom or purified phospholipase A (Table V). Incubation of the soluble reductase for 30 min at 30°C, pH 7.6, with 10 μg of boiled N. naja venom or 2.5 μg of phospholipase A per mg of protein gave trivial inactivation, the same as control samples incubated without venom. Even very large amounts of purified phospholipase A (110 μg per mg of protein) caused only 12% inactivation, and this may have been due to the unspecific protein effect on the assay described by Phar et al. (5). Hence, the venom does not act on the flavoprotein which functions as CoQ reductase in heat-acid-ethanol extracts but appears to produce an inhibitor of the enzyme, one which is bound to and removed by bovine serum albumin. The inhibitor does not seem to be a common product (or substrate) of
phospholipase A, since neither fatty acids nor lysolipid had significant effect on the soluble enzyme and micellar lecithin was slightly inhibitory only at very high concentration (Table V). The nature and properties of the inhibitor will be described elsewhere (27).

**DISCUSSION**

The experiments reported in this paper confirm our earlier conclusion (6) that NADH-CoQ reductase, like NADH-cytochrome c reductase, may be extracted from a variety of submitochondrial preparations, regardless of their phosphorylating capacity. In addition to the sources listed in Table I, the enzyme has been successfully extracted (28) from the "pH 5.4 particles" which are the starting material for the cytochrome c reductase of Mahler et al. (12); this is a modified Keilin-Hartree preparation. In fact, contrary to reports in the literature (19, 20), when the Mahler enzyme is isolated by the original procedure (12), except for the omission of lyophilization, which is deleterious to the dehydrogenase due to the presence of this inhibitor, it appears unlikely for two reasons. First, any reactivation of particles to pH 8.5 during isolation or to the effect of phospholipase A used in the extraction. The results in Table I contradict the first explanation, while those in Tables IV and V do not support the second one. Neither purified phospholipase A nor boiled venom seem to modify the composition or activities of the particle (7). The effect of aging of particles on the yield of NADH-CoQ reductase (Table I) may possibly explain the failure of Pharo et al. (5) and Sanadi et al. (19) to find appreciable Q6 reduction in various particles, some of which had been prepared in other laboratories and could not be, therefore, completely fresh.

It has been suggested (19, 21) that the virtual absence of Q6 reductase activity in the NADH dehydrogenase of Ringer (7) is due to the exposure of particles to pH 8.5 during isolation or to the effect of phospholipase A used in the extraction. The results in Table I contradict the first explanation, while those in Tables IV and V do not support the second one. Neither purified phospholipase A nor boiled venom seem to modify a reaction or activities of the particle (7). The effect of aging of particles on the yield of NADH-CoQ reductase (Table I) may possibly explain the failure of Pharo et al. (5) and Sanadi et al. (19) to find appreciable Q6 reduction in various particles, some of which had been prepared in other laboratories and could not be, therefore, completely fresh.

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Although in earlier papers ETP had been reported not to yield soluble NADH-CoQ reductase (19, 21), recently Pharo et al. (6) confirmed that high Q6 reductase activity is present in extracts of a "modified" ETP, as used by Machinist and Singer (6). It should be noted, however, that the ETP used in this laboratory has always been made by Method 1 of Crane et al. (29) except for a closer control of the initial centrifugation, which serves only to increase the yield but does not modify the composition or activities of the particle (7). The effect of aging of particles on the yield of NADH-CoQ reductase (Table I) may possibly explain the failure of Pharo et al. (5) and Sanadi et al. (19) to find appreciable Q6 reduction in various particles, some of which had been prepared in other laboratories and could not be, therefore, completely fresh.

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James Salach, Thomas P. Singer and Peter Bader


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