REACTION SITES OF ROTENONE IN THE RESPIRATORY CHAIN AND IN SOLUBLE DPNH-COENZYME Q REDUCTASE*

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The DPNH-coenzyme Q_{10} reaction in mitochondria and submitochondrial particles is phospholipid-dependent (Fleischer et al., 1964; Machinist and Singer, 1965a) and is almost completely inhibited by rotenone and amytal. A soluble flavoprotein (DPNH-ubiquinone or DPNH-CoQ reductase) derived from ETP_H by heat-acid-ethanol treatment also catalyzes this reaction and is partly inhibited by rotenone and amytal (Pharo and Sanadi, 1964; Pharo et al., 1966, Singer and Salach, 1967), but the soluble preparation does not require phospholipids for activity.

Sanadi and colleagues (1965) suggested that this low molecular weight flavoprotein retains the physiological CoQ reaction in a soluble preparation. The view of this laboratory has been that CoQ reduction by the soluble preparation, despite its sensitivity to amytal and rotenone, occurs at a different site from that which operates in mitochondria, a site created or revealed by the conformation changes occurring during extraction. The latter view is based on the profound modifying effects of heat-acid-ethanol on DPNH dehydrogenase (Watari et al., 1963), on the creation of cytochrome c reductase and rotenone-insensitive CoQ_1 reductase activities by this treatment (Machinist and Singer, 1965b), on differences in the behavior of particles and of the soluble preparation toward rotenone and

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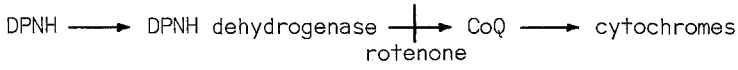
barbiturates and on the virtual absence of CoQ_1, CoQ_6 and CoQ_{10} reductase activities in soluble DPNH dehydrogenase extracted by a mild, enzymatic procedure (Singer and Salach, 1967). Further, when the purified dehydrogenase was subjected to the method used for the extraction of DPNH-CoQ reductase, its native properties disappeared and a flavoprotein indistinguishable from the Pharo-Sanadi preparation emerged (Machinist and Singer, 1965a, Singer and Salach, 1967).

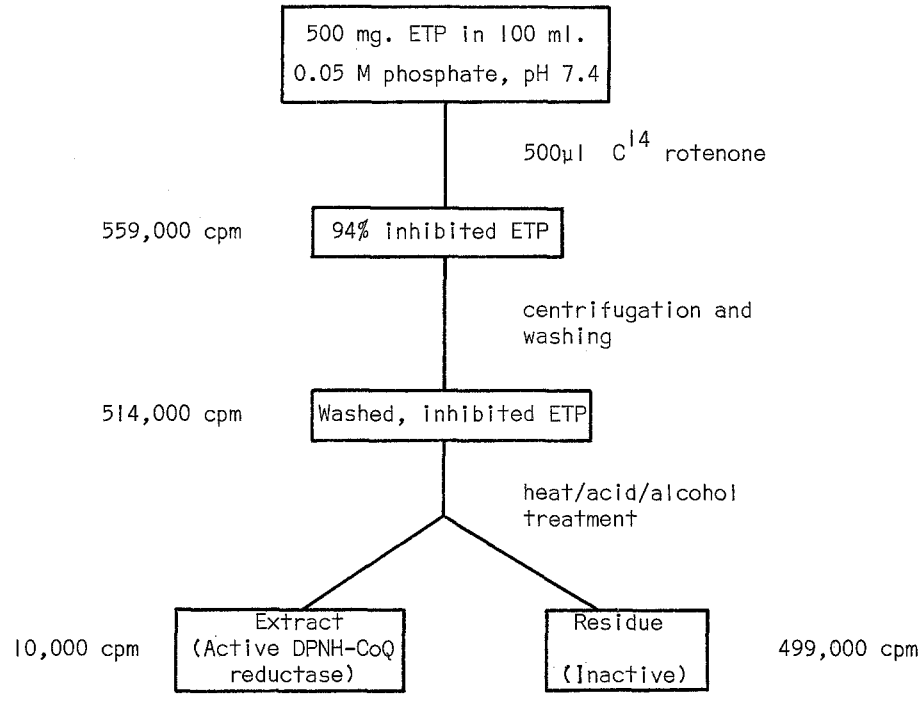
The experiments reported in this paper offer independent evidence that the reaction site of coenzyme Q homologues and of rotenone are not the same in intact particles and in the soluble reductase. The rationale of these experiments is as follows. It is known that in mitochondria rotenone and barbiturates interrupt electron transport on the substrate side of coenzyme Q, since they inhibit its reduction but not its reoxidation. (This is true of both endogenous and externally added CoQ).



Since, unlike amytal, rotenone is tightly bound to the respiratory chain, (Burgos and Redfearn, 1965) it seemed logical to label submitochondrial particles with C^{14} - rotenone and follow the distribution of radioactivity on extraction of the Pharo-Sanadi DPNH-CoQ reductase. If our views are correct and new reaction sites are created for rotenone and CoQ by the extraction procedure, the rotenone should remain bound to the residue obtained on extraction and the flavoprotein in the soluble portion should be fully active, despite the fact that it is extracted from a rotenone-treated particle. As shown in Scheme I, both of the predictions were confirmed experimentally.

Soluble DPNH-CoQ reductase was extracted from ETP and assayed with CoQ_6 by the procedure of Pharo et al.(1966). ETP was prepared as in previous work (Ringler et al., 1963). It will be shown in a future paper that ETP and ETP_H are equally good sources of this enzyme. DPNH-CoQ activity in particles was assayed as in our earlier studies (Machinist and Singer, 1965 a,b). Rotenone-6a-C^{14} (2.36 mc per mmole) was the gift of Dr. John E. Casida. C^{14} counts were made with a Tri-carb scintillation counter.

The particles used in the experiments summarized in Scheme I were titrated with labelled rotenone to 94% inhibition of DPNH oxidase activity. The particles were also inhibited to a comparable extent in regard to DPNH-CoQ_6 activity. The rotenone was firmly bound and washing by centrifugation with 25mM potassium phosphate buffer, pH 7.6 removed only a trace of excess rotenone. On extracting the inhibited particles with acid etha-



Scheme I. Diagramatic flow sheet of preparation of soluble DPNH - CoQ reductase from C^{14} - rotenone labelled ETP.

nol at 43°, only 2% of the counts appeared in the extract and 98% remained in the residue. The specific activity of the extract in the CoQ_6 assay (16 µmoles DPNH/min./mg. protein) compared favorably with values reported by Pharo et al. (1966) for extracts of phosphorylating particles and, significantly, was in close agreement with the value obtained in a parallel experiment for an extract of 500 mg. of uninhibited ETP. Thus from a particle in which all the rotenone-sensitive CoQ reductase activity originally present had been abolished, a fully active DPNH-CoQ reductase was extracted. Since rotenone remains firmly bound during the extraction procedure, it is clear that the rotenone binding site is not the same in particles as in the acid-ethanol extract. Since rotenone inhibits the reduction of CoQ, it also follows that the reaction site of CoQ is not the same in particles as in the soluble enzyme.

Another question examined with the aid of rotenone - C^{14} was whether the absence of appreciable activity toward long chain CoQ homologues in DPNH dehydrogenase (Ringler et al., 1963) was due to the presence of an inhibitor formed during snake venom phospholipase extraction (Singer and Salach, 1967) or to the fact that the enzyme had not been subjected to the heat-acid-

TABLE I

Distribution of Enzyme Activity and of Radioactive Rotenone During Purification of DPNH Dehydrogenase†

Stage	Total Enzyme units*	Total C^{14} cpm	Ratio $\frac{cpm}{units}$
Washed inhibited ETP	171,000	803,000	4.7
Phospholipase extract	158,000	74,900	0.47
2nd ammonium sulfate precipitate	81,000	16,900	0.21
Sephadex G-200 excluded fraction	60,000	8,620	0.14
Sucrose Gradient peak tube			0.10

† Procedure of Cremona and Kearney (1964)

* µMoles. DPNH oxidized/min., V_{max} with respect to ferricyanide in triethanolamine buffer, pH 7.8

ethanol procedure. Sanadi et al. (1965) reported that DPNH-CoQ reductase could not be extracted from particles pretreated with snake venom and suggested that the venom either modifies the reaction site of CoQ or removes an essential component for the reaction. While the basic observations have been confirmed in this laboratory, the interpretations were shown to be incorrect: pretreatment with venom does not modify any site nor remove an essential component but forms a powerful inhibitor of CoQ reduction (Singer and Salach, 1967). This raised the two possibilities that (a) the heat-acid-ethanol treatment creates or unmasks a reaction site not seen in native preparations or (b) removes a bound inhibitor formed during phospholipase digestion. Although all the evidence favored possibility (a), it was desirable to confirm these results by ascertaining whether rotenone - C^{14}, bound to ETP, appears in the soluble dehydro-

genase on phospholipase extraction. Since the reaction site of rotenone must either precede or be spatially near the site of CoQ reduction, but not follow it, the distribution of rotenone C^{14} should be decisive in choosing between these alternatives.

Table I shows that on solubilization of DPNH dehydrogenase from rotenone - C^{14} labelled ETP, approximately 9% of the rotenone accompanies 90% of the activity in the extract and that on further purification by the procedure of Cremona and Kearney (1964) all but a trace of the radioactive rotenone is separated from the dehydrogenase. It may then be concluded that the absence of CoQ reductase activity in the dehydrogenase obtained by phospholipase A extraction is not due to the presence of an inhibitor but to the absence of exposure to modifying agents.

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