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Conversion of Reduced Nicotinamide-Adenine Dinucleotide Dehydrogenase into Reduced Nicotinamide-Adenine Dinucleotide-Ubiquinone Reductase

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Two types of soluble purified preparations of the NADH dehydrogenase component of the respiratory chain have been described in the literature. One is a high-molecular-weight form (mol.wt. $5 \times 10^5 - 8 \times 10^5$) extracted with phospholipase A at low temperature (Ringler, Minakami & Singer, 1963). Its properties parallel those of particle-bound NADH dehydrogenase except that it does not catalyse the Amytal- and rotenone-sensitive reduction of ubiquinone (Machinist & Singer, 1965a). The reduction of external ubiquinone in submitochondrial particles, however, is phospholipid-dependent and may occur by way of endogenous ubiquinone-10 (where the numeral indicates the number of isoprene units in the side chain) rather than by direct reaction with the dehydrogenase (Machinist & Singer, 1965b).

The other type is of lower molecular weight and is extracted by acid-ethanol or phospholipase A at elevated temperatures, which leads to fragmentation of the enzyme and to major changes in its properties (Watari, Kearney & Singer, 1963). Such preparations show high levels of Amytal- and rotenone-insensitive cytochrome *c* reductase, indophenol reductase and ubiquinone-1 reductase activities not seen in more native preparations. These emerge as a result of protein modification during extraction.

It has been reported (Pharo & Sanadi, 1964; Pharo, Sordahl, Vyas & Sanadi, 1966) that, when the heat-acid-ethanol procedure is applied to phosphorylating particles, a flavoprotein is ex-

tracted that reduces long-chain ubiquinone homologues in an Amytal- and rotenone-sensitive reaction. This NADH-ubiquinone reductase was reported to be extractable only from phosphorylating particles (ETP_H*) but not from non-phosphorylating ones. The apparent retention of Amytal- and rotenone-sensitive ubiquinone reductase activity has led to the interpretation that the physiological ubiquinone reaction site has been retained in this preparation (Sanadi, Pharo & Sordahl, 1965).

Machinist & Singer (1965a,b) have extracted NADH-ubiquinone reductase from ETP_H and NADH-cytochrome reductase from the non-phosphorylating particle ETP by the procedure of Pharo & Sanadi (1964). No appreciable difference was found in reactivity with ubiquinone-1, ubiquinone-6, cytochrome *c* or menadione, in absorption spectrum, in chromatographic properties, in sedimentation coefficient, in substrate specificity or in iron, labile sulphur or FMN content. The development of the assay method used by Pharo *et al.* (1966) for ubiquinone-6 and ubiquinone-10 reduction has enabled us to complete the comparison and to demonstrate the conversion of the high-molecular-weight form of the enzyme into a product indistinguishable from NADH-ubiquinone reductase obtained by the heat-acid-ethanol procedure.

Heat-acid-ethanol extracts prepared from ETP and from phosphorylating particles (ETP_H or

*Abbreviations: ETP, electron-transport particle; ETP_H, phosphorylating electron-transport particle; SP_{HL}, ETP_H 'sonicated' without additives (Pharo *et al.* 1966).

SP_{HL}) by the procedure of Pharo *et al.* (1966) had very similar reactivities with ubiquinone-1, ubiquinone-6 and ubiquinone-10. In a typical experiment the specific activities of the extracts (μ moles of NADH oxidized/min./mg. at 30°) for ubiquinone-1, ubiquinone-6 and ubiquinone-10 were 26.6, 14.3 and 17.6 respectively in ETP and 26.5, 12.1 and 12.3 respectively in SP_{HL}. The specific activity proportions (ubiquinone-1:ubiquinone-6:ubiquinone-10) were 100:54:66 in extracts of ETP and 100:46:46 in SP_{HL}. The specific activities towards ubiquinone-6 were close to those reported for extracts by Sanadi *et al.* (1965). Extracts derived from particles that had been stored for several weeks at -15° had lower specific activities for ubiquinone-6 (4.5-8, compared with 11-27 in extracts of fresh particles). Whatever damage occurs on storage is not reflected in the activities of the aged particles towards ferricyanide or ubiquinone-6. Thus the phosphorylating capacity of a particle does not seem to have a bearing on its capacity to yield soluble NADH-ubiquinone reductase in extracts.

The reduction of ubiquinone-6 and ubiquinone-10 in particles is more than 90% inhibited by Amytal and rotenone. Maximal inhibition of the soluble reductase from ETP, ETP_H and SP_{HL} was about 40% and required 3-6 mM Amytal, far in excess of that needed to cause more than 90% inhibition in the parent particles. Titration curves with rotenone showed that: (1) a maximal inhibition of 25-40% occurred in extracts of both ETP_H and ETP; (2) at high rotenone/FMN ratios the inhibition disappeared; (3) maximal inhibition occurred at rotenone/FMN ratios 4.5-6.5 rather than 1; (4) at very low rotenone concentrations an activation occurred. These considerations and the results with Amytal are not readily reconciled with the view that the reaction site of ubiquinone-6 is the same (in terms of locus and conformation) in the soluble reductase and in intact particles, but suggest that the site for ubiquinone-6 reduction in soluble extracts is revealed or created by the exposure to heat, ethanol and acid pH, as is also true of cytochrome reduction. Studies with [¹⁴C]rotenone confirm this view (Horgan & Singer, 1967). It may be concluded that reactivity with ubiquinone homologues or inhibition by rotenone or Amytal do not clearly distinguish between ubiquinone reductase and cytochrome *c* reductase.

If the ubiquinone reduction observed in the Pharo-Sanadi preparation is a new activity created by the modifying effects of heat, ethanol and acid pH, it should be possible to convert by the same treatment purified NADH dehydrogenase, which is devoid of significant activity towards long-chain ubiquinone homologues, into a fragment endowed with such activity. Table 1 shows that exposure of NADH dehydrogenase to pH 5.3 and 9% ethanol at

43° causes a very large increase in ubiquinone-6 reductase and ubiquinone-10 reductase activities, along with the expected rise in ubiquinone-1 reductase and cytochrome *c* reductase activities and the almost complete disappearance of activity towards ferricyanide. Table 1 also summarizes the specific activities before and after exposure to acid-ethanol. The activities of the preparation in Table 1 compare favourably with those of the best extracts of ETP_H derived by the same procedure.

A noteworthy feature of the transformation of NADH dehydrogenase into an NADH-ubiquinone reductase is that the ubiquinone-6 reductase and ubiquinone-10 reductase activities emerging are inhibited by Amytal and rotenone to about the same extent (30-35% and 25-50% respectively) as in extracts obtained from ETP_H. The titration curves with rotenone are also very similar to those obtained with extracts of ETP_H. In contrast, the low activities of untreated NADH dehydrogenase towards ubiquinone-6 and ubiquinone-10 are not inhibited by Amytal or rotenone.

It has been suggested (Sanadi *et al.* 1965) that the virtual absence of ubiquinone-6 and ubiquinone-10 reductase activities from NADH dehydrogenase is due to the loss of the ubiquinone-6 reduction site or of a labile component as a result of exposure to pH 8.5 (during the isolation of ETP) or to phospholipase A. NADH dehydrogenase may be isolated,

Table 1. *Conversion of NADH dehydrogenase into NADH-ubiquinone reductase*

The NADH dehydrogenase after the Sephadex G-200 step, dissolved in 0.05 M-phosphate, pH 7.6, was adjusted to pH 5.3 and heated at 43° in 9% (v/v) ethanol (Pharo *et al.* 1966). The suspension was chilled, neutralized to pH 6.8 and precipitated protein was removed by centrifugation. Ferricyanide reductase and cytochrome *c* reductase activities (V_{max}) were measured as in previous work (Watari *et al.* 1963), and reduction of ubiquinone-1, ubiquinone-6 and ubiquinone-10 by the method of Pharo *et al.* (1966). Units are μ moles of NADH oxidized/min./g. of original NADH dehydrogenase; sp. activities are μ moles of NADH oxidized/min./mg. of protein.

Activity		Before conversion	After conversion
Ferricyanide reductase	Units	471 000	8185
	Sp. activity	471	138
Cytochrome <i>c</i> reductase	Units	92	3176
	Sp. activity	0.092	53.6
Ubiquinone-1 reductase	Units	279	1777
	Sp. activity	0.28	29.9
Ubiquinone-6 reductase	Units	22	900
	Sp. activity	0.22	15.2
Ubiquinone-10 reductase	Units	16	720
	Sp. activity	0.016	12.2
Total protein (mg.)	...	1000	5.9

however, equally well from ETP_H as from ETP. The results presented show that exposure of particles to pH 8.5 does not influence their potential to yield soluble NADH-ubiquinone reductase and suggest that the absence of ubiquinone reductase activity in the dehydrogenase is due to lack of exposure to heat-acid-ethanol, and not to snake-venom enzymes or alkaline pH. The reason why Sanadi *et al.* (1965) were unable to extract ubiquinone reductase from venom-treated particles is discussed elsewhere (Salach, Tisdale, Singer & Bader, 1967).

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The Tocopherols of the Blue-Green Algae

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Henninger, Bhagavan & Crane (1965) examined the lipids of the blue-green alga *Anacystis nidulans* and found two naphthaquinones and one plastoquinone, all of which differed from quinones previously isolated from other organisms. The plastoquinone resembled plastoquinone-9 (where the numeral indicates the number of isoprene units in the side chain) although it was slightly more polar on thin layers of silica gel. Of the two naphthaquinones, the less polar compound (of similar polarity to phyloquinone) was considered to be a hydroxy derivative of phyloquinone whereas the other was considerably more polar. No α -tocopherolquinone or tocopherols could be detected. As a result of these findings it was suggested that the quinones of the blue-green algae are different from those that are found to be characteristic of higher plants.

However, Carr & Hallaway (1966) found α -tocopherolquinone in four species of blue-green algae other than *Anacystis nidulans*. Allen, Franke & Hirayama (1967) re-examined the lipid of *Anacystis nidulans* and identified the three quinones present as phyloquinone, plastoquinone-9 and a hydroxy derivative of phyloquinone (the polar naphthaquinone described by Henninger *et al.* 1965).

They were unable to detect any tocopherols or tocopherolquinones. Independent work by H. M. Hallaway and N. G. Carr (personal communication) has led to the same identification of the three quinones.

This lack of tocopherols in *Anacystis nidulans* was further confirmed by Hirayama (1967), who suggested that in view of the absence of tocopherols from other procaryotic micro-organisms, the photosynthetic bacteria, these compounds may be necessary for the maintenance of chloroplast structure.

As *Anacystis nidulans* was unusual among the blue-green algae in lacking α -tocopherolquinone, the occurrence of tocopherols in a number of species was investigated.

Anabaena variabilis, *Nostoc muscorum*, *Anacystis nidulans* and *Fremyella diplosiphon* (1429/1 Cambridge culture collection) were each grown autotrophically in 10l. batches of medium C of Kratz & Myers (1954) with the addition of sodium hydrogen carbonate (0.05%). At the end of the exponential phase the algal cells were harvested in a Sharples centrifuge. The lipids were extracted by homogenization of the cell paste with ice-cold methanol in a mechanical Potter-Elvehjem homogenizer until no