Marchalonis, J. J. (1969), *Aust. J. Biol. Med. Sci. 47*, 405.
Miller, F., and Metzger, H. (1965), *J. Biol. Chem. 240*, 3325.
Montgomery, P. C., Bello, A. C., and Rockey, J. H. (1970), *Biochim. Biophys. Acta 200*, 258.
Niall, H. D., and Potts, J. I., Jr. (1969), *Proc. 1st Amer. Peptide Symp., New York, N. Y.*
Niedermeier, B. (1971), *Anal. Biochem.* (in press).
Pink, J. R. L., Buttery, S. H., DeVries, G. M., and Milstein, C. (1970), *Biochem. J. 117*, 33.
Putnam, F. W., Kozuru, M., and Easley, C. W. (1967), *J. Biol. Chem. 242*, 2435.
Saluk, P. H., Krauss, J., and Clem, L. W. (1970), *Proc. Soc. Exp. Biol. Med. 133*, 365.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic Press.
Shelton, E., and McIntire, K. R. (1970), *J. Mol. Biol. 47*, 595.
Shelton, E., and Smith, M. (1970), *J. Mol. Biol. 54*, 197.
Small, P. A., Jr., and Lamm, M. E. (1966), *Biochemistry 5*, 259.
Spande, T. F., and Witkop, B. (1967), *Methods Enzymol. 11*, 498.
Suran, A. A., and Papermaster, B. W. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1619.
Wang, S.-S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Waterfield, M., and Haber, E. (1970), *Biochemistry 9*, 832.

On the Functional Organization of the Respiratory Chain at the Dehydrogenase–Coenzyme Q Junction*

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ABSTRACT: Coenzyme Q_{10} (CoQ_{10}) was reincorporated into CoQ_{10}-depleted membrane preparations from heart mitochondria by two methods reported to achieve full restoration of reduced nicotinamide–adenine dinucleotide (NADH) oxidase activity. Reincorporation in aqueous medium or in an anhydrous one were compared on the basis of restoration of NADH and succinoxidase activities, effect on NADH dehydrogenase activity, CoQ_{10} content, and reactivity with piericidin A. While reversible removal of CoQ_{10} from the succinoxidase system is readily achieved without obvious damage to the dehydrogenase in the reconstituted particles, NADH dehydrogenase is significantly modified even though there is extensive restoration of NADH oxidase activity. Contrary to previous evidence, reincorporation of CoQ_{10} to the level originally present does not result in full restoration of NADH oxidase activity. Comparison of NADH and succinoxidase activities on titration of the depleted particles with increasing amounts of CoQ_{10} further suggests a compartmentation of CoQ_{10} at the flavoportein junction, a proposal supported by the findings of Ernster and coworkers from experiments involving gradual depletion of the particles by serial pentane extractions.

The observation that in reconstituted particles the inhibition by piericidin A is competitive with respect to CoQ_{10} is discussed in relation to the mechanism of action of this inhibitor.

The functional organization of the NADH dehydrogenase–CoQ_{10}[1] juncture in the respiratory chain is poorly understood despite the obvious importance of this knowledge to the definition of many reaction mechanisms. This locus appears to be the rate-limiting step in the NADH oxidase system because both NADH dehydrogenase activity and the turnover rate of the cytochromes exceed the overall NADH oxidase or NADH–CoQ reductase activities (Cremona and Kearney, 1964; Singer and Gutman, 1971). It is also a distribution point where CoQ_{10} molecules, in large molar excess, accept electrons from or donate electrons to a few but more rapidly reacting NADH dehydrogenase molecules, and it is the point at which reducing equivalents from a variety of substrates (*i.e.*, succinate, α-glycerophosphate, NAD-linked substrates) enter a common pathway (Klingenberg, 1968). Finally, this juncture is in the immediate vicinity of energy conservation site I (Schatz and Racker, 1966; Gutman *et al.*, 1970a) and of the inhibition sites of rotenone, piericidin A, and barbiturates (Horgan *et al.*, 1968a,b).

There are many technical difficulties and few unambiguous techniques for studying the interaction of the flavoprotein with endogenous CoQ_{10}. Kinetic studies have been hampered, until recently (Gutman and Singer, 1970), by the lack of an appropriate method for following the oxidation–reduction of NADH dehydrogenase without serious interference by other

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[1] Abbreviations used are: CoQ, coenzyme Q; ETP, nonphosphorylating preparation of the inner membrane; ESP and ETP_H, phosphorylating inner membrane preparations.

components. The large size of the CoQ_{10} pool relative to the NADH dehydrogenase content suggests a possible heterogeneity in the quinone pool so that only a fraction reacts rapidly enough to be effectively in the main pathway of electron transport, while the remaining major portion, although not reacting, obscures the measurement and study of the critical reacting portion of interest. For these reasons, the role of CoQ_{10} as an obligatory component of the NADH oxidase system and the sequence of reactions involving flavoprotein, CoQ_{10}, and cytochrome *b* continue to be subjects of debate and multiple interpretations (Ernster *et al.*, 1969b).

The resolution of these questions was greatly aided by studies on the succinoxidase system demonstrating that extraction of CoQ_{10} from membrane preparations with isooctane or acetone halts succinoxidase activity and that addition of CoQ_{10} to depleted particles restores the activity (Lester and Fleischer, 1959; Crane, 1968). Application of similar techniques to the NADH oxidase system resulted in little or no restoration of activity because the dehydrogenase involved is extremely sensitive to organic solvents (Crane, 1968; Singer, 1966). Two laboratories have reported recently on the use of lyophilized particles for the apparently reversible removal of CoQ_{10} from NADH oxidase. Szarkowska (1966) removed CoQ_{10} by pentane extraction of the lyophilized particles and achieved extensive restoration of activity by adding back a large excess of CoQ_{10}, in alcoholic solution, to an aqueous suspension of the depleted particles. Ernster *et al.* (1969a,b) modified this technique by adding back the CoQ_{10} in pentane solution to the lyophilized particles. The latter procedure was reported to achieve full restoration of NADH oxidase activity which is rotenone sensitive when the CoQ_{10} level rebound to the particles equals that originally present. This finding was of great interest inasmuch as it appeared to provide a technique for studies correlating the stoichiometry of CoQ_{10} binding with the restoration of enzyme activities, similar to our earlier work with piericidin and retenone (Horgan *et al.*, 1968a,b).

The present study was undertaken to compare the two methods of reincorporation of CoQ_{10} into depleted particles in regard to completeness of restoration of the original structure and activities and to explore their suitability for chemical studies of the nature of CoQ_{10} binding in intact systems. It was found that neither technique yields restored NADH oxidase activity or stoichiometry of CoQ_{10} to protein comparable to that of intact particles. This paper is a detailed account of the relevant studies and presents evidence from depletion–reincorporation experiments for a significant degree of functional compartmentation of CoQ_{10} in the inner membrane.

Experimental Procedure

Materials. ESP particles were prepared by the method of Lee and Ernster (1967). CoQ_1, CoQ_6, and CoQ_{10} were commercial preparations of over 98% purity. Peroxide was removed from phospholipid micelles as described by Machinist and Singer (1965). Other enzyme preparations, chemicals, and [^{14}C]piericidin A were obtained as in previous work (Horgan *et al.*, 1968a), with the exceptions noted below.

Preparation of CoQ_{10}-Depleted Particles. Unless noted otherwise, the submitochondrial particles, usually ESP, at a protein concentration of 30–40 mg/ml in 0.25 M sucrose–25 mM phosphate, pH 7.4, were washed with 0.15 M KCl, diluted with the same solution to a concentration of 20 mg of protein per ml, shell frozen, and lyophilized overnight. In an attempt to minimize inactivation of NADH oxidase and NADH dehydrogenase during lyophilization, the ratio of 0.15 M KCl and 0.25 M sucrose in which the particles were washed before lyophilization was systematically varied in certain experiments detailed below at the suggestion of Dr. L. Ernster.[2] The dehydrated particles were extracted 5 times by homogenization and shaking at 0° with anhydrous *n*-pentane, with centrifugations in between, under the conditions specified by Szarkowska (1966) and Ernster *et al.* (1969a,b). Pentane was removed by lyophilization in high vacuum and the extent to which endogenous CoQ_{10} was removed was ascertained as in the studies of Ernster *et al.* (1969a,b). The extracted particles could be preserved without further loss of activity at −70° for several weeks.

Reincorporation of CoQ into Particles. In the aqueous reincorporation method of Szarkowska (1966) the depleted particles, suspended in 0.25 M sucrose–25 mM phosphate, pH 7.4, were added to the assay mixture for the measurement of NADH oxidase activity, less CoQ and NADH. A small aliquot of CoQ_{10} was then added with vigorous stirring as a 1 to 5 mM solution in ethanol to give the desired concentration, and the suspension was incubated for 3 min at 30°. The assay was then started by the addition of NADH.

In the pentane reincorporation method the procedure of Ernster *et al.* (1969a,b) was followed exactly, except that the concentration of CoQ_{10} in the pentane solution was varied between 0 and 2 mM in order to permit the study of the amount incorporated at various concentrations of added CoQ_{10}. After the removal of "excess" CoQ_{10} by a quick pentane wash and drying in high vacuum, both at 0°, NADH oxidase activity was assayed with and without the addition of further CoQ_{10} during assay (Szarkowaska's method, 1966) in order to permit differentiation between incomplete reincorporation and inactivation during exposure to pentane.

Piericidin Titration. [^{14}C]Piericidin A was added as an ethanolic solution to the particles suspended in 0.25 M sucrose–25 mM phosphate, pH 7.4; the particles were incubated for 30 min at 0° and then washed by centrifugation twice with 2% (w/v) bovine serum albumin in sucrose–phosphate and once with sucrose–phosphate, as described earlier (Gutman *et al.*, 1970d).

Assay Procedures. NADH dehydrogenase activities represent V_{max} values in the ferricyanide assay in triethanolamine buffer, pH 7.4, at 30°. NADH oxidase activity were measured either spectrophotometrically or polarographically at 30° in 80 mM phosphate–5 μM EDTA, pH 7.4, in the presence of 1.5×10^{-4} M NADH. Although in the studies of Ernster *et al.* (1969a,b) a much higher NADH concentration (0.93 mM) was used, the results are comparable because the activity in NADH oxidase assays, measured either polarographically or spectrophotometrically, does not vary between 0.15 and 1 mM NADH concentration. Succinoxidase activity was assayed polarographically at 30° in the presence of 40 mM phosphate, pH 7.4, and 70 mM succinate in 1.5–1.7 ml final volume. Prior to assay, the enzyme was activated (Kearney, 1957) by 7-min incubation at 30° with 2 mM malonate; the solution was then diluted to the final volume and suitable correction was made for the relatively small inhibition resulting from malonate remaining at the high succinate:malonate ratio.

With each preparation (lyophilized or reconstituted) the concentration of cytochrome *c* required for maximal activity in the succinoxidase and NADH oxidase assays was determined and assays were carried out in the presence of that concentration of cytochrome *c*.

[2] Personal communication from Dr. L. Ernster.

TABLE I:[a] Effect of Lyophilization on NADH Oxidase and Dehydrogenase Activities.

Volume Per Cent Composition of Washing Medium		NADH Oxidase Activity			NADH–$Fe(CN)_6^{-3}$ Activity	
0.25 M Sucrose	0.15 M KCl	Initial	After Lyophilization	After Pentane Extraction	Initial	After Lyophilization
0	100	1.8	0.33	0.007	33	17
50	50	1.8	0.46	0.012	33	15
70	30	1.8	0.62	0.030	33	16
90	10	1.8	0.62		33	19
95	5	1.8	0.68	0.060	33	27
100	0	1.8	1.1	0.18	33	34

[a] ETP particles, suspended in 0.25 M sucrose–25 mM phosphate, pH 7.4, were washed by centrifugation in the indicated mixtures of sucrose–phosphate and KCl at 30 mg/ml of protein concentration. The pellets were resuspended on 0.15 M KCl, shell frozen, and lyophilized overnight. Oxidase assays were in the presence of concentrations of added cytochrome *c* yielding optimal activity. For comparison, activities after 5 pentane extractions (Ernster *et al.*, 1969a) are included.

CoQ content was determined by the method of Redfearn (1967).

Results

Effect of Lyophilization on NADH Dehydrogenase and Oxidase Activities. Both existing methods for the reversible resolution of NADH oxidase with respect to CoQ_{10} (Szarkowska, 1966; Ernster *et al.*, 1969a) take advantage of the facts that the extraction of CoQ_{10} by organic solvents is more complete and the inactivation of the dehydrogenase less extensive when dry particles are subjected to the treatment than when aqueous suspensions are extracted. Neither laboratory appears to have explored in detail, however, the extent to which the process of lyophilization damages NADH dehydrogenase in the particles. Szarkowska's report uses the specific activity of NADH oxidase of lyophilized mitochondria as the point of reference; thus any inactivation incurred in a prior step would not be apparent. In the studies of Ernster *et al.* limited comparison was made of NADH oxidase activities before and after lyophilization, with the result that the specific activity appeared to *increase* on lyophilization (from 0.56 to 0.61 of μmole of NADH per min per mg (Ernster *et al.*, 1969a) and from 0.63 to 0.93 (Ernster *et al.*, 1969b), provided that cytochrome *c* was included in the assay. NADH dehydrogenase activity, however, was not assayed.

In our experience lyophilization always leads to variable loss of NADH dehydrogenase and oxidase activities, regardless of the precautions taken to ensure rapid freezing, very high vacuum, and low ambient temperature. It has been reported (Gutman, 1970) that lyophilization of ETP from 0.25 M sucrose or 0.15 M KCl leads to extensive inactivation of NADH–$Fe(CN)_6^{-3}$ activity, that the inactivation is greater when KCl than when sucrose is the suspending medium, and that in both cases treatment with very low concentrations of Titron X-100 results in partial restoration of NADH dehydrogenase activity.

Inactivation of NADH dehydrogenase and oxidase activities was also observed by Ernster,[2] particularly when lyophilization was carried out after washing the particles with 0.15 M KCl, but it was stated to be much less, or absent, when the washing preceding lyophilization was with 0.25 M sucrose. Since extraction of CoQ_{10} with pentane was found to be more complete after KCl washing than after sucrose treatment, their current method which is believed to yield good extraction of CoQ and minimal inactivation[2] uses a solution consisting of 9 parts of 0.25 M sucrose and 1 part of 0.15 M KCl in the washing step.

Table I presents the results of a typical experiment designed to test the effect of the composition of the washing medium on the loss of activity incurred in the subsequent lyophilization step. It may be seen that, in accord with Ernster's unpublished data,[2] inactivation of both dehydrogenase and oxidase activities tends to be less as the proportion of sucrose in the washing medium is increased, but the loss of oxidase activity is extensive (66%) even with the recommended 9:1 (v/v) mixture of sucrose–KCl, and significant (48%) when sucrose alone is used as the washing solution. For comparison, the remaining NADH oxidase activity after 5 subsequent pentane extractions is included. It may be noted that the removal of CoQ_{10} by solvent extraction, as judged by residual activity, increases with decreasing sucrose content. At the recommended 9:1 ratio of sucrose–KCl some 10% of the activity of the lyophilized particles remained. Although not shown in the table, CoQ analysis of the extracted particles confirmed this general trend.

The data in Table I seem to contradict the reports (Ernster *et al.*, 1969a,b) that lyophilization, at least from a suitable medium,[2] causes no loss of NADH oxidase activity. No explanation is available for this discrepancy. Many variants were tried in the conditions of lyophilization (degree of vacuum, volume of enzyme, external temperature) and the enzyme assays were carried out under the precise conditions specified by Ernster *et al.* (1969a,b), but none of these approaches resulted in a noticeable difference. It may be relevant in this connection that the NADH oxidase activities of the untreated ETP or ESP particles used in the present study (1.3 to 1.8 μmoles per min per mg) were considerably higher than those reported by Ernster *et al.* (1969a) (0.56 μmole/min per mg) but the values are not very different after lyophilization.[3]

Restoration of NADH Oxidase Activity by Szarkowska's Method. When the NADH oxidase activity of pentane-ex-

[3] Although the coincidence may be fortuitous, it is interesting that among the numerous preparations lyophilized only one ESP sample of somewhat low specific activity (1.0 μmole of NADH/min per mg) survived lyophilization without apparent loss of NADH oxidase activity.

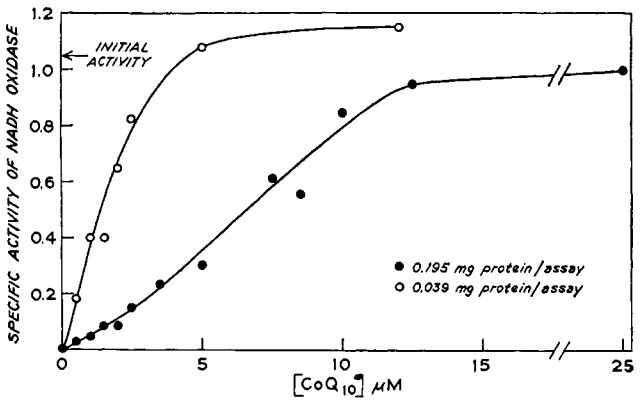

FIGURE 1: Restoration of the NADH oxidase activity of pentane-extracted ETP by CoQ_{10} using Szarkowska's (1966) method. Pentane-extracted ETP in 80 mM phosphate–5 μM EDTA, pH 7.4, was assayed for NADH oxidase activity in the presence of the indicated concentrations of CoQ_{10} added in ethanolic solution to the cuvets. The alcohol concentration was less than 1% (v/v) in all cases. The NADH oxidase activity of the particles before lyophilization was 2.16 μmoles of NADH/min per mg.

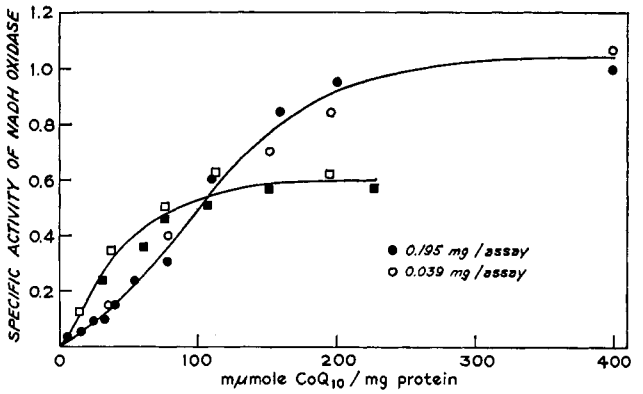

FIGURE 2: Reactivation of NADH oxidase in the presence and absence of asolectin: ○ and ●, 39 and 195 μg of protein per cuvet, respectively, in the absence of asolectin; □ and ■, 39 and 195 μg of protein in the presence of 0.19 mg of asolectin per ml. Conditions were as in Figure 1.

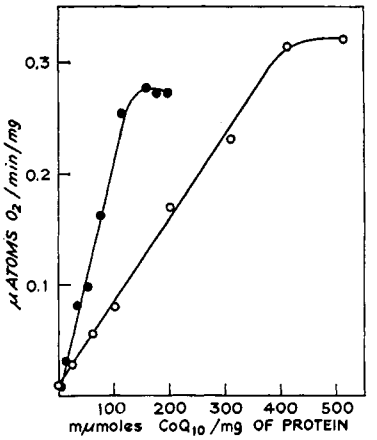

FIGURE 3: Restoration of NADH and succinoxidase activities of pentane-extracted ETP by CoQ_{10}. In this experiment lyophilization caused particularly extensive damage: the specific activities prior to pentane extraction were 0.28 μmole of succinate/min per mg and 0.4 μmole of NADH/min per mg. Reactivation was by the aqueous method: ●, succinoxidase activity; ○, NADH oxidase activity.

tracted particles is measured at varying concentrations of CoQ_{10}, added as an ethanolic solution to an aqueous suspension of the CoQ_{10}-depleted particles, the restoration of activity depends on the ratio of added CoQ_{10} to protein, not on the concentration of CoQ_{10}. Thus, in a plot of specific activity against CoQ concentration different curves are obtained as the protein concentration in the assay medium is varied (Figure 1), while in plots relating NADH oxidase activity to CoQ_{10}:protein ratio the same curve is obtained at widely varying protein concentrations (Figure 2). The implied stoichiometry is surprising since the ratio of nmoles of CoQ_{10}/mg of protein required for maximal restoration (about 300) is 2 orders of magnitude higher than the quantity present in unextracted particles (3–4 nmoles/mg of protein). Conceivably, most of the quinone is precipitated and is thus rendered unavailable for recombination, and a relatively small fraction enters the catalytically active sites.

Figures 1 and 2 confirm Szarkowska's (1966) report that this technique permits full or nearly full restoration of the NADH oxidase activity of the *lyophilized*, unextracted particles and thus the pentane extractions do not seem to cause any obvious irreversible damage. In contrast to her report, however, the addition of phospholipids was not found to be necessary for reactivation. Asolectin (Figure 2) and total mitochondrial lipids (not shown) merely increased the efficiency of reactivation at low CoQ_{10}:protein ratios, but at higher ratios they had only a deleterious effect, since the maximal activity reached in the presence of asolectin was only about half that attained in its absence. This behavior was observed over a range of asolectin concentrations tested (0.04–0.19 mg per ml of reaction mixture).

Restoration of Succinoxidase Activity. The restoration of succinoxidase activity on treatment of the aqueous particles with CoQ_{10} is a function of the CoQ_{10}:protein ratio, as is that of NADH oxidase activity. As illustrated in Figure 3, maximal reactivation of succinate oxidation is reached at much lower ratios of CoQ to protein than those required for maximal restoration of NADH oxidation. This is always the case, even with varying types of particles (ETP, ESP, ETP_H), regardless of whether the NADH oxidase activity of the unextracted lyophilized particles was about the same, higher, or lower than the succinoxidase activity.[4] Thus the differential reactivation of the two systems at low CoQ_{10}:protein ratios cannot be explained by assuming that less CoQ_{10} incorporation is required to support maximal electron flux in the less active of the two enzyme systems.

Restoration of Activities in Nonaqueous Environment. Figure 4 compares the restoration of NADH and succinoxidase activities with the reincorporation of CoQ_{10} when depleted ESP particles are treated with various concentrations of CoQ_{10} by the method of Ernster *et al.* (1969a,b). The results are expressed as per cent of the maximal activity attainable on CoQ_{10} reincorporation in the aqueous medium. This is done

[4] In untreated particles NADH oxidase activity is always considerably higher than succinoxidase activity. However, manipulations during lyophilization and pentane extraction usually cause little or no damage to succinate dehydrogenase but partially inactivate NADH dehydrogenase. Since this inactivation is variable, lyophilized preparations have been obtained with NADH oxidase activities of about the same order, or less than, succinoxidase activity. The latter can be lowered by adjusting the malonate concentration carried over from activation to assay.

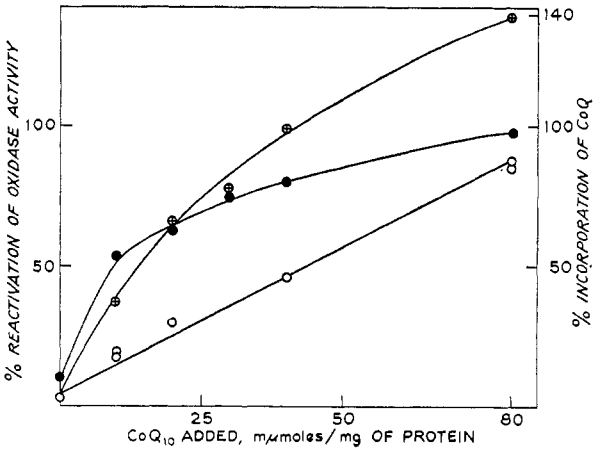

FIGURE 4: Restoration of catalytic activities and CoQ_{10} reincorporation in depleted ESP particles. Pentane extraction and CoQ_{10} reincorporation were as described by Ernster *et al.* (1969a,b). In catalytic assays the maximal activity obtained on restoration by the aqueous procedure (Szarkowska, 1966) is taken as 100%. In case of CoQ_{10} incorporation, the content of unextracted ESP particles is taken as 100%: ○, succinoxidase activity; ●, NADH oxidase activity; ⊗, amount of CoQ_{10} incorporated.

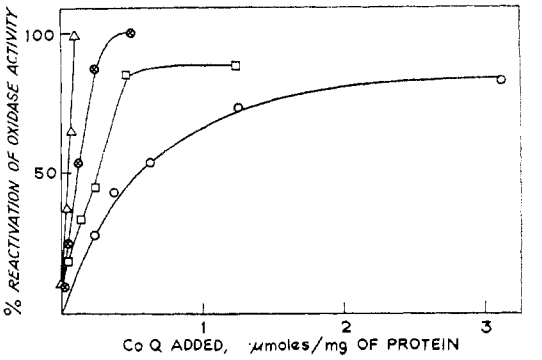

FIGURE 5: Restoration of NADH oxidase and succinoxidase activities of CoQ_{10}-depleted ETP by CoQ homologs. The depleted particles were titrated with CoQ derivatives as in Figure 1: ○, NADH oxidase activity with CoQ_1; □, NADH oxidase activity with CoQ_6; ⊗ NADH oxidase activity with CoQ_{10}; Δ, succinoxidase activity with CoQ_{10}.

to emphasize that in the majority of experiments using the anhydrous technique the restoration of neither of the two oxidase activities was as extensive as that reached by Szarkowska's (1966) aqueous procedure, indicating possible further damage to the enzymes during reincorporation of CoQ_{10} in the anhydrous medium. The CoQ_{10} reincorporated, on the other hand, is expressed relative to the concentration present in the unextracted particles. It may be seen that, contrary to the reports of Ernster *et al.* (1969a,b), the amount of quinone reincorporated is not governed by, and does not equal, the concentration originally present, but depends on the amount added, since no tendency is evident for saturation. The situation is similar to that observed on adding piericidin A or rotenone to membrane preparations in that the amount bound at specific plus unspecific sites depends on the amount added, with no tendency for stoichiometric binding (Horgan *et al.*, 1968a,b). The experiment further shows that maximal reactivation is *not* reached when the amount of CoQ_{10} incorporated equals that originally present. As in reactivation by Szarkowska's procedure (1966), succinoxidase activity is restored at considerably lower CoQ_{10} concentrations than NADH oxidase activity.

Reactivation by Various CoQ Homologs. Lenaz *et al.* (1968) found that in pentane-extracted particles succinoxidase activity is reactivated by a series of CoQ homologs (CoQ_2 to CoQ_{10}) and even by 2,3-dimethoxy-5-phytyl-1,4-benzoquinone, whereas NADH oxidase activity is restored only by long-chain CoQ homologs. The concentration dependence, however, has not been studied. When CoQ_1, CoQ_6, and CoQ_{10} are compared at a series of concentrations in regard to their ability to reactivate NADH oxidation, as in Figure 5, all three homologs are found to cause nearly the same maximal activation, although the shorter the isoprenoid side chain, the higher is the concentration required for the same extent of reactivation.

Interactions of CoQ_{10} and Piericidin A with Normal, Lyophilized, and CoQ_{10}-Depleted Particles. The similarity in structure of piericidin A and CoQ has lead to the suggestion (Jeng *et al.*, 1968) that piericidin may act competitively, displacing CoQ from the active site. Jeng *et al.* (1968) found that on addition of CoQ_2 there was partial relief of the inhibition of succinoxidase resulting from exceedingly high concentrations of piericidin. The effect of piericidin on succinate oxidation is, however, entirely due to unspecific binding and has no relevance to the inhibition of NADH oxidation, which is observed at 10^5 times lower concentration (Horgan *et al.*, 1968a). Snoswell and Cox (1968) observed more extensive reversal of the piericidin inhibition of NADH oxidation in *Escherichia coli* K12 by CoQ_2. Unfortunately, these experiments are also inconclusive, partly because of the very high inhibitor concentrations used (and, hence, the possibility of unspecific effects), partly because no evidence was presented that the added CoQ_2 reestablishes the normal electron transfer path, rather than acting as an autooxidizable dye which provides a shunt around the inhibited site.

The technique of reversible removal of CoQ_{10} from particles seemed to provide a more direct probe for the study of the possible competition between CoQ_{10} and piericidin. The first question examined was the effect of removal of CoQ_{10} on the piericidin binding capacity of membrane preparations. As shown in Table II, virtually complete extraction of endogenous CoQ_{10} with pentane had no influence, *per se*, on the specific binding site titer. On the other hand, the inactivation of NADH dehydrogenase during lyophilization lowered this titer in direct proportion to the loss of catalytic activity. It is known that specific piericidin binding and the resulting inhibition involve both protein and lipid and that NADH dehydrogenase is the protein involved in the two specific binding sites (Horgan *et al.*, 1968a; Gutman *et al.*, 1970b–d). The experiments in Table II provide additional evidence that NADH dehydrogenase is the protein component involved in these two binding sites.

Just as pentane extraction of CoQ_{10} does not affect the capacity for piericidin binding, treatment of the depleted particles with CoQ_{10} does not influence subsequent piericidin binding (Table III).

The effect of added CoQ_{10} concentration on the inhibition of NADH oxidase by piericidin A was examined on simultaneous addition of the inhibitor and CoQ_{10} to CoQ_{10}-depleted particles. Although the high solubility of both of these compounds in pentane would seem to suggest that the reincorporation conditions of Ernster *et al.* (1969a) are the method of choice, it was found that under anhydrous conditions membrane preparations do not bind piericidin at all. This observation reaffirms the importance of protein conformation in

TABLE II:[a] Effect of Lyophilization and Pentane Extraction on Enzyme Activities and Piericidin-Binding Capacity of ESP and ETP.

Sequence of Treatment	Enzyme Activities (μmoles/min per mg)			Specifically Bound Piericidin A (μμmoles/mg)	Specifically Bound Piericidin/ NADH–$Fe(CN)_6^{-3}$ Activity
	Succinoxidase	NADH Oxidase	NADH–$Fe(CN)_6^{-3}$		
Untreated ESP	0.63	1.53	36	60	1.7
After lyophilization	0.52	0.73	6.0	10	1.7
After pentane extractions	0.015	0.011	5.5	10	1.8
After CoQ_{10} reincorporation		0.70			
Untreated ETP		1.2	44	68	1.5
After lyophilization		0.7	22	30	1.4
After pentane extractions		0	22	30	1.4
After CoQ_{10} reincorporation		1.1			

[a] The particles were washed with 0.15 M KCl, lyophilized, extracted with pentane, and assayed as described in Methods. Specifically bound piericidin per mg of protein was determined with [^{14}C]piericidin A by the method of Horgan *et al.* (1968a). Reincorporation of CoQ_{10} was by Szarkowska's (1966) procedure and the value given in the table refers to NADH oxidase activity at infinite added CoQ_{10} concentration, derived from a double-reciprocal plot. Analysis showed that the pentane extraction had removed 99% of the CoQ_{10} of the particles.

specific piericidin binding and necessitated the use of the alternative, aqueous method. Figure 6 indicates an apparent competition between CoQ_{10} and piericidin A, with an approximate K_i value of 8×10^{-10} M. In another preparation (Table II), in which NADH dehydrogenase activity had been considerably less inactivated by lyophilization and thus the piericidin binding site titer was considerably higher, the same type of competitive inhibition was observed with a similar K_i value (about 4×10^{-10} M).

TABLE III:[a] Effect of the Reincorporation of CoQ_{10} on NADH Oxidase Activity and Piericidin Binding.

CoQ_{10} Concentration during Reincorporation (μM)	Restoration of NADH Oxidase Activity (%)	Specifically Bound Piericidin A (μμmoles/mg of Protein)
0	4	23.5
35	22	24.9
70	54	23.2
140	50	22.8

[a] Aliquots (10 ml) of pentane-extracted ETP, suspended in 0.25 M sucrose–25 mM phosphate, pH 7.4, at 0.5 mg of protein concentration per ml, were treated with the indicated concentrations of CoQ_{10} for 15 min at 25° by Szarkowska's (1966) procedure. The particles were sedimented by centrifugation, which separates much of the uncombined CoQ_{10} since the latter tends to float. Each sample was then assayed for NADH oxidase activity with and without additional CoQ_{10} added to the aqueous suspension at saturating concentrations; this differential analysis allows an estimate of the proportion of maximal restoration achieved on the initial treatment with CoQ_{10}. The second column denotes the per cent of the activity measured when no CoQ_{10} was added during assay, taking the activity of the sample with excess CoQ_{10} present as 100. Qther aliquots of each centrifuged sample were then used for determination of the capacity for specific piericidin binding as in Table II.

Discussion

The studies of Szarkowska (1966) and of Ernster *et al.* (1969a,b) provide strong support for the hypothesis that CoQ_{10} is an obligatory functional component of the NADH oxidase system, but their evidence that the reversible resolution causes no damage to the dehydrogenase and that, therefore, the reactivated system is comparable in all respects to the unresolved one is not convincing. Neither laboratory reported the effect of lyophilization and pentane extraction

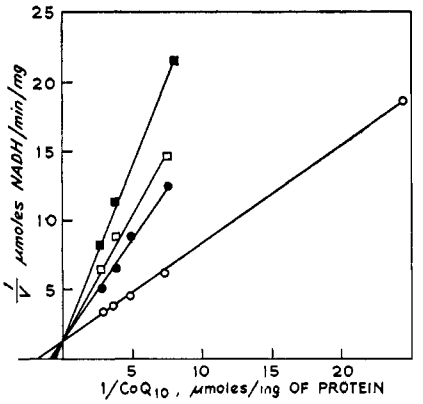

FIGURE 6: Simultaneous interaction of CoQ_{10} and piericidin A with CoQ_{10}-depleted ETP. Pentane-extracted ETP (0.183 mg of protein/3 ml of reaction mixture) dissolved in the medium used in the standard NADH oxidase assay, less NADH, was incubated with CoQ_{10} (added as an ethanolic solution) at the concentrations indicated on the abscissa for 3 min at 30°. The assay was started by the addition of 0.17 mM NADH: piericidin concentrations, ○, none; ●, 0.57 nM; □, 1.19 nM; ■, 1.72 nM.

on NADH dehydrogenase activity, which is usually a more sensitive indication of damage to the flavoprotein than is oxidase activity, since the enzyme is not rate limiting in the respiratory chain (*cf.* Table II). Further, Szarkowska used lyophilized mitochondria as the point of reference and thus did not examine the effect of freeze-drying on activity which, as it turns out, is the step where most of the damage occurs.

The data in this report show that both NADH–Fe $(CN)_6^{-3}$ and NADH oxidase activities are extensively inactivated during the lyophilization step which is an essential part of these procedures. The protein modification is also reflected by a parallel loss of piericidin-binding capacity and a change in K_m for ferricyanide (Gutman, 1970). In agreement with Ernster[2] we find that this inactivation may be somewhat minimized by careful adjustment of the sucrose:KCl ratio preceding lyophilization, but, even under the best of circumstances, the inactivation is too high to permit the conclusion that the reconstituted system is identical with the original one. The finding that CoQ_{10} is much more readily extracted from reincorporated that from untreated particles (Ernster *et al.*, 1969b) also points in this direction.

The pentane-extraction step causes only slight or no additional inactivation of NADH dehydrogenase and oxidase, so that on titration with CoQ_{10} in aqueous media most or all of the activity of lyophilized particles is restored. On the other hand, reactivation in anhydrous media seems to cause additional damage, as reflected in still further lowering of the NADH–$Fe(CN)_6^{-3}$ activity as well as the fact that in parallel experiments the restored NADH oxidase activity is in most instances higher in the aqueous procedure than in the one involving pentane. In contrast to the behavior of the NADH oxidase system, the entire process causes relatively little, and sometimes no, damage to succinoxidase.

Despite the advantage of Szarkowska's (1966) method for restoration of activity over the pentane procedure is not causing additional inactivation, it suffers from the disadvantages that the amount of added quinone needed for saturation is extremely high because most of the CoQ_{10} is precipitated or forms micelles which are either occluded in the particles or are unavailable to the enzyme system, and that it does not permit ready determination of the amount incorporated without additional isolation and washing steps. The pentane method is free from these objections but it also fails to yield stoichiometric reincorporation, for the amount of CoQ_{10} reincorporated into the particles depends on the amount of CoQ_{10} added, not on the amount originally present. Further, maximal restoration of activity does not occur at the point where the amount of CoQ_{10} incorporated equals the original but at a considerably higher level (Figure 4).

The observation that the succinoxidase activity of CoQ_{10}-depleted particles is restored at considerably lower concentrations of added CoQ_{10} than is NADH oxidase activity cannot be explained on the basis of the lower rate of electron flux from succinate to the cytochrome system, since the same is true in particles where, owing to inactivation of NADH dehydrogenase, the activities of the two systems in the presence of excess CoQ_{10} are equal. An alternative explanation is that the CoQ_{10} pool in the membrane is not homogeneous, but instead a degree of compartmentation or orientation exists toward individual flavoproteins. This might be only the result of differences in lipid environment around the various flavoproteins; a suggestion consistent with the fact that phospholipase A solubilizes NADH dehydrogenase, but has no effect on succinate dehydrogenase. Possibly, then, the affinity for CoQ_{10} at the succinate dehydrogenase junction might be sufficiently higher than that near NADH dehydrogenase to account for the differences in saturation requirements. This explanation is compatible with the data of Ernster *et al.* (1969a) showing that on pentane extraction NADH oxidase activity is more readily abolished than succinoxidase. An extension of the same hypothesis, assuming more rigid structural requirements for CoQ_{10} binding near NADH dehydrogenase, might account for the greater specificity of this system than of succinoxidase for CoQ homologs (Figure 5 and Lenaz *et al.*, 1968).

Another indication of the functional heterogeneity of the CoQ_{10} pool in mitochondria is the observation (Gutman *et al.*, 1970d) that *unspecifically* bound rotenone and piericidin inhibit NADH oxidase activity and ATP-driven NAD reduction by succinate, but not the reduction of external CoQ_1 by NADH. Since the endogenous CoQ_{10} pool is regarded as a mediator of electron transport between NADH dehydrogenase and the cytochrome system, external CoQ, or succinate dehydrogenase, this finding suggests that the CoQ_{10} molecules involved in electron transport from NADH dehydrogenase to external CoQ are probably not the same as those which transfer electrons to succinate dehydrogenase and the cytochromes.

The reversible removal of endogenous CoQ from the NADH oxidase system has permitted the first direct demonstration that the inhibition of NADH oxidase by piericidin A is competitive with respect to CoQ, at least in the reconstituted particles (Figure 6), and is characterized by an exceedingly low K_i value (3 to 9 $\times$ 10^{-10} M, corresponding to a ΔF° of about 13 kcal). The tight binding of the inhibitor is consistent with a multicomponent binding site (Gutman *et al.*, 1970d), despite the absence of covalent bonds (Horgan *et al.*, 1968a,b). If the competitive kinetics observed (Figure 6) are taken to suggest a mutual displacement or dislocation of CoQ_{10} and of the inhibitor from the dehydrogenase, in accord with their structural similarities, the low binding constant of the dehydrogenase for piericidin and the lesser affinity of CoQ_{10} for the enzyme, as judged by the high concentrations of CoQ_{10} required for saturation, would be compatible with a displacement of CoQ_{10} by piericidin. On the other hand, at physiological concentrations CoQ_{10} would not be expected to displace piericidin, while at the high concentrations used in the experiments of Figure 6 a displacement may occur. All this is compatible with the observation that piericidin binding to NADH dehydrogenase at the specific sites is not influenced by the CoQ_{10} content of the particles, being the same in intact, CoQ_{10}-depleted, and reconstituted particles, after suitable correction for inactivation of NADH dehydrogenase during these treatments.

The kinetic demonstration of a competitive relation, however, cannot be taken as evidence that the mechanism of piericidin *inhibition* is a straightforward dislocation of CoQ_{10} from an active site on the enzyme for two reasons, First, barbiturates, rotenone, and piericidin are bound at the same site and inhibit in the same manner but only piericidin resembles CoQ_{10}. Second, piericidin and CoQ_{10} are bound differently in mitochondria.

Despite the fact that the competitive relations between piericidin and CoQ_{10} were observed with particles damaged by lyophilization and that CoQ_{10} may not be bound the same way in intact and reconstituted samples, it may be possible to extrapolate the conclusions to more intact membrane preparations. The competitive relation and the K_i for piericidin were deduced from enzyme assays, in which only the surviving, undamaged dehydrogenase molecules register, and these have

the same binding titer for piericidin in intact, lyophilized, and reconstituted samples. While it is not likely that the added CoQ_{10} molecules which function in the regenerated system may be bound differently than in intact preparations (Ernster *et al.*, 1969a,b), the binding site titer for piericidin does not change during the entire cycle of treatments; so the same K_i value might apply to the untreated and reconstructed systems.

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References

Crane, F. L. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Wiley-Interscience, p 533.
Cremona, T., and Kearney, E. B. (1964), *J. Biol. Chem. 239*, 2328.
Ernster, L., Lee, I. Y., Norling, B., and Persson, B. (1969a), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3*, 21.
Ernster, L., Lee, I. Y., Norling, B., and Persson, B. (1969b), *Eur. J. Biochem. 9*, 299.
Gutman, M. (1970), *Physiol. Chem. Phys. 2*, 9.
Gutman, M., Mayr, M., Oltzik, R., and Singer, T. P. (1970a), *Biochem. Biophys. Res. Commun. 41*, 40.
Gutman, M., Mersmann, H., Luthy, J., and Singer, T. P. (1970b), *Biochemistry 9*, 2678.
Gutman, M., and Singer, T. P. (1970), *Biochemistry 9*, 4750.
Gutman, M., Singer, T. P., Beinert, H., and Casida, J. E. (1970c), *Proc. Nat. Acad. Sci. U. S. 65*, 763.
Gutman, M., Singer, T. P., and Casida, J. E. (1970d), *J. Biol. Chem. 245*, 1992.
Horgan, D. J., Ohno, H., Singer, T. P., and Casida, J. E. (1968a), *J. Biol. Chem. 243*, 5967.
Horgan, D. J., Singer, T. P., and Casida, J. E. (1968b), *J. Biol. Chem. 243*, 834.
Jeng, M., Hall, C., Crane, F. L., Takahashi, N., Tamura, S., and Folkers, K. (1968), *Biochemistry 7*, 1311.
Kearney, E. B. (1957), *J. Biol. Chem. 229*, 363.
Klingenberg, M. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Wiley-Interscience, p 1.
Lee, C. P., and Ernster, L. (1967), *Methods Enzymol. 10*, 543.
Lenaz, G., Daves, G. D., and Folkers, K. (1968), *Arch. Biochem. Biophys. 123*, 529.
Lester, R. L., and Fleischer, S. (1959), *Arch. Biochem. Biophys. 80*, 470.
Machinist, J. M., and Singer, T. P. (1965), *Proc. Nat. Acad. Sci. U. S. 65*, 467.
Redfearn, E. R. (1967), *Methods Enzymol. 10*, 381.
Schatz, G., and Racker, E. (1966), *J. Biol. Chem. 241*, 1429.
Singer, T. P. (1966), *in* Comprehensive Biochemistry, Vol. 14, Florkin, M., and Stotz, E. H., Ed., Amsterdam, Elsevier, p 127.
Singer, T. P., and Gutman, M. (1971), *Advan. Enzymol.* (in press).
Snoswell, A. M., and Cox, G. B. (1968), *Biochim. Biophys. Acta 162*, 455.
Szarkowska, L. (1966), *Arch. Biochem. Biophys. 113*, 579.

Cooperation among the Active Binding Sites in the Sex-Specific Agglutinin from the Yeast, *Hansenula wingei**

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ABSTRACT: Changes in properties of 5-agglutinin were examined during the course of the reaction to inactivate it by cleaving disulfide bonds. During this reaction both agglutinative titer and apparent free energy of absorption decreased rapidly with a relatively small proportion of disulfide bonds reacted. Fractionation experiments disclosed that very little biologically inactive material appeared until about 30% of the disulfide bonds were reacted. The 1.7S component which is released from 5-agglutinin during reaction retains the specific absorptive activity, though only very weakly. It is suggested that the high magnitude of the apparent free energy of association for 5-agglutinin, −14 kcal/mole, is the result of additive effect of several of the individual binding sites, each of which has a binding energy in the range −5 to −9 kcal per mole. A mathematical model based on these assumptions reproduces the behavior satisfactorily.

The sex-specific agglutinin derived from mating type 5 of the yeast *Hansenula wingei* agglutinates cells of type 21, the opposite mating type, and binds to them. Under favorable conditions the $\Delta F_p°$[1] is about −14 kcal/mole (Taylor and Orton, 1970). The 5-Ag has about six small 1.7S fragments which can be released by disulfide cleaving agents. These 1.7S fragments are associated with the biological activity. Presumably, 5-Ag has about six equivalent active sites which all contribute to its agglutinative and absorptive properties. This multiplicity of binding sites is somewhat more complex than occurs in a divalent antibody, and the resulting complexity seems to impart some unique properties to the 5-Ag particles.

Compared to particulate systems flocculated by polymeric materials, the small number of discrete binding sites on

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[1] Abbreviations used are: 5-Ag, 5-agglutinin, the sex-specific agglutinin from mating type 5 of *Hansenula wingei*; $\Delta F_p°$, apparent standard free energy of association for 5-Ag, in kilocalories per mole of particles.