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STUDIES ON THE RESPIRATORY CHAIN-LINKED REDUCED NICOTINAMIDE–ADENINE DINUCLEOTIDE DEHYDROGENASE

I. EFFECT OF DIETHYL ETHER ON PARTICULATE NADH DEHYDROGENASE

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SUMMARY

1. The effect of diethyl ether on particulate NADH dehydrogenase of heart-muscle preparation was studied. The solvent caused a rapid and complete inhibition of NADH oxidation by oxygen, cytochrome *c*, Q-1 and Q-6 both when used as an extractant and when dissolved in the reaction medium at low concentrations. The ether treatment did not affect NADH-ferricyanide reductase and succinate oxidase (in the presence of added cytochrome *c*). Both menadione and 2,6-dichlorophenol-indophenol (DCIP) reductase were inhibited by about 35 %.

2. The effect of ether on enzymic activities was similar to the effect of short-term digestion of the preparation with small amounts of phospholipase.

3. High concentrations of rotenone, *o*-phenanthroline, *N*-ethylmaleimide and of *p*-chloromercuribenzoate (PCMB) did not affect all ferricyanide, menadione and DCIP activities of the ether-extracted preparation.

4. Treatment of the preparation with ether protected NADH dehydrogenase against inactivation by preincubation with NADH. Sensitivity of Type III –SH groups towards mersalyl was also reduced.

5. Specific activities of soluble preparation obtained by the method of MAHLER were 2–3 times higher when ether-pretreated preparation was used as a source of the enzyme.

6. The results indicate that ether and phospholipase displayed similar effects on the electron transport chain in respect to both the site and the mechanism of action. Both reagents did not affect ferricyanide reductase activity, which is characteristic of NADH dehydrogenase. Ether-treated preparations were modified in such a way that the extraction of soluble NADH dehydrogenase by chemical agents was facilitated.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

Treatment of mitochondrial preparations with organic solvents results in the inactivation of the electron transport system. This might be due to: (a) extraction of some essential components of the respiratory chain, *e.g.* vitamin E (refs. 1, 2), lipids[3] and ubiquinone[4–7]; (b) the derangement of the structure of the chain, which induces a requirement for cytochrome *c* (refs. 3, 6, 8–10), or (c) the physical inhibition caused by the residual solvent[2,8].

NADH oxidase and NADH-Q reductase are particularly sensitive both to polar[6,9,11,12,15] and non-polar solvents[1,2,7–9,15]. The inactivation of this activity can also be brought about by detergents[8,13,15], phospholipase A[14,16], urea[17], freezing and thawing[18] and by ageing at elevated temperatures[19]. All these treatments seem to affect the portion of the respiratory chain between flavoprotein and cytochrome *b*. This segment comprises Q (ref. 5), non-heme iron[17,20,21], –SH groups[16,22–24] and phospholipids[11,25,26] which are all essential for electron transport.

Irreversible inactivation of NADH oxidase and NADH-Q reductase commonly observed in mitochondrial preparations following extraction with polar solvents[6,9,11,12,15] appears to result from the destruction of the primary structure of the system and not from the removal of some essential component of the chain. The possibility that flavoprotein is also affected cannot be neglected since it is known that NADH dehydrogenase is sensitive to the action of solvents[27].

The present paper reports the effect of diethyl ether on some properties of particulate NADH dehydrogenase of heart-muscle preparation.

MATERIALS AND METHODS

Beef heart-muscle particles were prepared by the method of KEILIN AND HARTREE[28] as modified by SLATER[29]. In the latter method the tissue is disrupted in a Waring blendor and the homogenate fractionated by means of differential centrifugation instead of precipitation with acid.

Cytochrome *c* was isolated from beef hearts by the procedure of MARGOLIASH[30]. The extinction coefficient of VAN GELDER AND SLATER[31] was employed for calculation of concentration of cytochrome *c*.

Extraction with diethyl ether

Heart-muscle preparation (20–30 mg of protein per ml) was shaken at 18° with 5 vols. diethyl ether in a test tube with a ground glass stopper. The mixture was centrifuged at 1000 × *g*, the supernatant ether decanted and the residue suspended in 0.1 M phosphate buffer (pH 7.4) which contained 1 mM of EDTA. The residual ether was removed under vacuum. The sample was centrifuged for 10 min at 1200 × *g*, the supernatant was discarded and the residue suspended in phosphate buffer as above. The suspension was homogenized in a Potter–Elvehjem homogenizer and the homogenate was assayed for protein concentration and enzymic activities.

Measurement of enzyme activities

Enzymic activities were determined at room temperature except for experiments outlined in Table I.

Activities of NADH oxidase, of NADH-cytochrome *c* reductase, of succinate-cytochrome *c* reductase[32] and of NADH-DCIP reductase[33], respectively, were determined as previously described. Activity of NADH-ferricyanide reductase was determined spectrophotometrically at 420 mμ in the reaction mixture which contained 0.15 mM NADH, 1.3 mM ferricyanide, 50 mM Tris–sulphate buffer (pH 7.8) and 1 mM EDTA. Activity of NADH-Q-1 reductase was measured by the method of HATEFI, HAAVIK AND JURTSHUK[34] except that Asolectin was omitted from the reaction medium. The activities of NADH-Q-6 and NADH-menadione reductase were determined by the methods of PHARO *et al.*[35] and WOSILAIT[36], respectively.

Activity of succinate oxidase was determined polarographically with a Clark electrode according to the procedure of CHAPPELL[37]. Composition of the reaction mixture was described elsewhere[10].

The protein content of the particles was determined by the biuret method of CLELAND AND SLATER[38]. Soluble, ethanol-extracted preparations were assayed for protein by the method of LOWRY *et al.*[39].

Reagents

The sources of materials used in these studies were as follows: NADH was obtained from Reanal (Hungary) and Sigma; Q-1 and Q-6 were obtained from Merck; menadione was a product of Polfa (Poland); antimycin, mersalyl and PCMB were from Sigma; rotenone was a product of Penick Co.; *o*-phenanthroline was purchased from POCh (Poland); snake venom of *Naja naja* was from Light; *N*-ethylmaleimide was obtained from Nutritional Biochemicals Corp.

Anaesthesia-grade diethyl ether was used throughout the investigation.

RESULTS

Oxidation of NADH and of succinate by the ether-extracted preparation

The effect of diethyl ether on mitochondrial electron transport has not been thoroughly studied. The only available data are those of CUNNINGHAM, CRANE AND SOTTOCASA[9] who have found a rapid and complete inactivation of NADH oxidase activity of beef heart mitochondria following the extraction with diethyl ether. Attemps to restore NADH oxidase activity of ether-extracted mitochondria by the addition of various chemicals or by various physical treatments were unsuccessful[9]. Thus we have studied only some properties of particulate NADH dehydrogenase after uncoupling of the complex from the cytochrome chain by the treatment with diethyl ether. Table I shows the activity of NADH dehydrogenase with various electron acceptors in untreated and ether-extracted preparations.

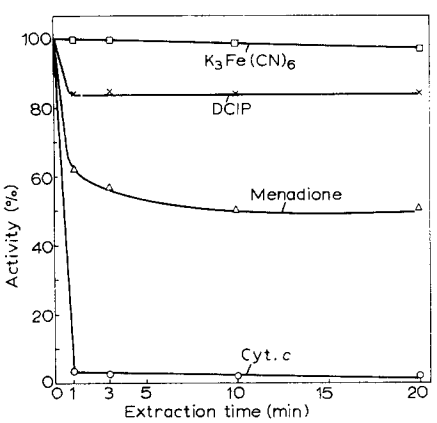
It can be seen from Table I that with oxygen, cytochrome *c* and Q-6 as respective electron acceptors the enzymic activity was completely lost. Residual activity with Q-1 in ether-extracted preparation is, however, rotenone insensitive (*cf.* ref. 26). It should also be noted that NADH-ferricyanide activity was essentially unaffected by ether treatment while the activities of NADH-menadione reductase and NADH-DCIP reductase were only partially inhibited (by about 30%). This suggests two sites for the reduction of both acceptors. Since oxidation of succinate was not affected by treatment with ether it appears that the break in the electron transport chain is

TABLE I

ENZYMIC ACTIVITIES OF HEART-MUSCLE PREPARATION AFTER EXTRACTION WITH DIETHYL ETHER

Extraction was performed as described under METHODS. Figures give specific activity expressed as μmoles of substrate oxidized per mg protein per min. The oxidation of NADH was assayed at 30° and that of succinate at 25°. Succinate oxidase activity was assayed in the absence (—) or in the presence (+) of added cytochrome *c*.

Enzyme activity	*Preparation*		*Original activity* (%)
	Control	*Ether-extracted*	
NADH oxidase	1.10	0.0	0
NADH-cytochrome *c* reductase	0.380	0.020	6
NADH-Q-6 reductase	0.320	0.0	0
NADH-Q-1 reductase	0.488	0.054	11
NADH-menadione reductase	0.220	0.150	68
NADH-DCIP reductase	0.155	0.120	72
NADH-ferricyanide reductase	7.60	7.20	95
Succinate oxidase (—cytochrome *c*)	0.330	0.0	0
Succinate oxidase (+cytochrome *c*)	0.545	0.560	103
Succinate-cytochrome *c* reductase	0.130	0.305	235



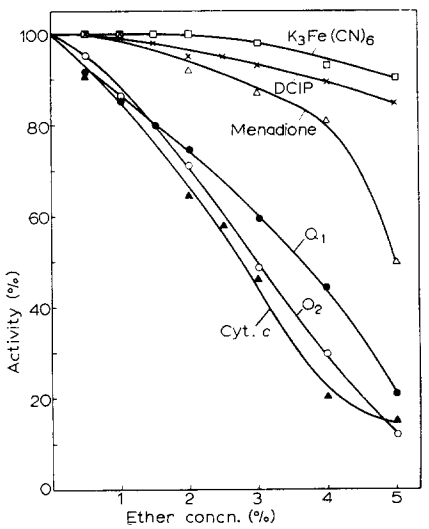


Fig. 1. Effect of the length of ether extraction of heart-muscle preparation on the activity of particulate NADH dehydrogenase. The heart-muscle preparation was extracted for 10 and 20 min as described under METHODS. With 1- and 3-min extractions, the preparations were shaken with ether for 30 sec and 2.5 min, respectively, and then allowed an additional 20 sec for sedimentation of particles. Supernatant ether was immediately decanted and the residue resuspended in cold phosphate buffer. Further centrifugations and treatments were the same as for the 10- and 15-min extractions.

Fig. 2. Effects of low ether concentration on the oxidation of NADH in heart-muscle preparation by various electron acceptors. Diethyl ether was added to 2 ml of preparation (1.0–1.5 mg of protein per ml) in test tubes with ground glass stoppers in order to obtain the final concentration of ether in the range of 0.5–5.0 % (v/v). Stoppered tubes were shaken for 2 min at room temperature and the ether was evaporated under vacuum. Aliquots of the suspension were withdrawn for spectrophotometric determinations of enzyme activity.

between flavoprotein and cytochrome *b*. This confirms the findings of CUNNINGHAM, CRANE AND SOTTOCASA[9].

NADH-ferricyanide reductase activity was stable at 0° for 24 h when an ether-extracted preparation was suspended in phosphate buffer (pH 7.4). At 20° the activity was diminished by 10 % during 24 h. In contrast to results obtained with beef heart mitochondria[9], neither an increase of NADH-ferricyanide reductase activity nor of succinate oxidase activity after addition of cytochrome *c* was observed in heart-muscle preparation which had been extracted with ether. In the absence of added cytochrome *c*, succinate oxidase activity was completely abolished (Table I and ref. 10). With beef heart mitochondria this activity was lowered by only about 50 % (ref. 9).

Fig. 1 shows the time course of the inactivation of NADH dehydrogenase in ether-extracted heart-muscle preparation. It is evident that ether inactivation occurred very rapidly, *i.e.* in less than 1 min after the application of the solvent. This phenomenon has also been observed by others[9].

Comparison of the effects of low concentration of ether with those of short-term digestion with small amounts of phospholipase

In order to slow down the process of enzymic inactivation the preparation was incubated in phosphate buffer which contained from 0.5 to 5 % (v/v) of diethyl ether. As can be seen from Fig. 2, activities of NADH oxidase, of cytochrome *c* reductase and of Q-1 reductase decreased in proportion to ether concentration in the solution. Under the same conditions, NADH-ferricyanide reductase and NADH-

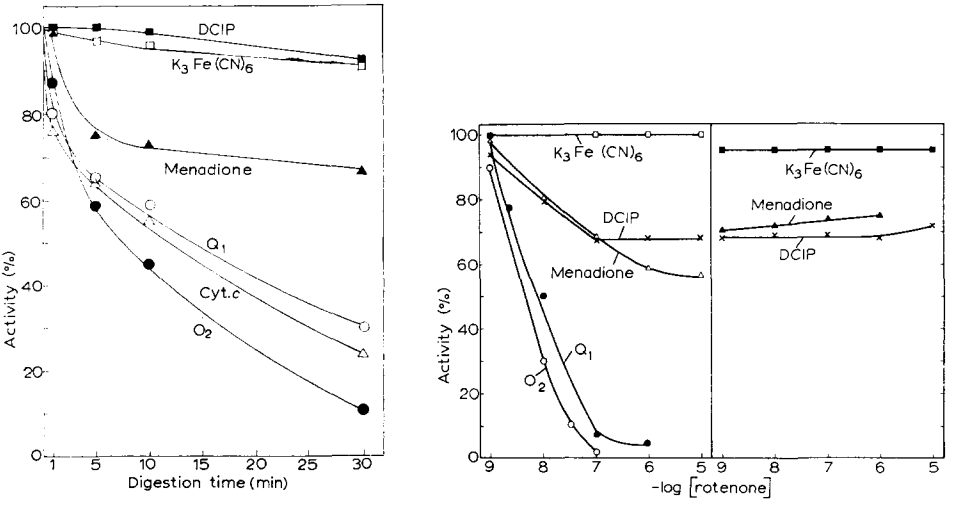


Fig. 3. Effect of short-term digestion of heart-muscle preparation with small amounts of phospholipase on the oxidation of NADH by various electron acceptors. Snake venom of *Naja naja* (1.6 mg) was dissolved in 60 ml of 0.02 M phosphate buffer (pH 6.0) and boiled for 5 min (*cf.* ref. 14). After centrifugation, an aliquot of the supernatant (0.1 ml) was added to 1 ml of heart-muscle preparation (19.2 mg of protein per ml) and the mixture incubated at 30°. Phospholipase action was stopped by cooling the samples to 0°. Subsequently, the samples were diluted with 20 volumes of cold 0.1 M phosphate buffer (pH 7.4).

Fig. 4. Rotenone inhibition of NADH oxidation of intact (left) and ether-extracted (right) preparations. The preparations with added inhibitors were incubated for 2 min at room temperature in a cuvette. Rotenone was applied as an ethanol solution. Enzymic activities of ether-extracted preparations were expressed as percent of the activity of the intact preparation.

DCIP reductase activities were only slightly affected. Since the inactivation of NADH oxidation was also brought about under conditions of incomplete saturation of aqueous solutions of the enzyme with ether, this might indicate that the inactivation was not due to the removal of some essential component but rather to some structural changes within the preparation.

The effect of ether on particulate NADH dehydrogenase was reminiscent of the effect of short-term digestion with small amounts of phospholipase[11,26] which was not sufficient to extract dehydrogenase. Thus the effect of phospholipase on NADH oxidation in particles is shown in Fig. 3. As seen from these figures, the curves of enzymic activities in ether-extracted (Fig. 2) and phospholipase-treated (Fig. 3) preparations were analogous. For menadione reductase, however, the two curves displayed somewhat dissimilar shapes.

Effect of rotenone

It was interesting to establish whether extraction with ether would affect the sensitivity of NADH dehydrogenase to rotenone which is thought to be lipophilic. Fig. 4 shows that dehydrogenase activity of ether-extracted preparations was not affected by rotenone. With one exception, the ferricyanide reductase activity, both menadione and DCIP reductase activities were inhibited by ether to the same extent as intact preparations were by rotenone. This would suggest the same site of action for both ether and rotenone. The residual rotenone-insensitive NADH-Q-1 reductase activity, still evident even at high rotenone concentration, was due to the direct reaction of dehydrogenase flavoprotein with an acceptor (*cf.* ref. 26).

Effect of o-phenanthroline

The presence of non-heme iron in NADH-Q reductase of HATEFI, HAAVIK AND GRIFFITHS[15], as well as in soluble NADH dehydrogenase of the respiratory chain[14,40], is well known. It has been suggested by GREEN[41] and recently shown by REDFEARN *et al.*[42] that electron transport between NADH dehydrogenase and the cytochrome chain could be effectively blocked by metal-chelating agents and especially by *o*-phenanthroline.

Fig. 5 shows that in untreated preparations only NADH oxidase activity and, to a smaller extent, menadione reductase activity, were inhibited by *o*-phenanthroline. In ether-extracted preparations, *o*-phenanthroline did not affect the dehydrogenase activity in the presence of artificial acceptors. Thus it seems that non-heme iron of dehydrogenase was not chelated by *o*-phenanthroline.

In contrast to some observations[42,43] on the stimulatory effect of low concentrations of *o*-phenanthroline on NADH oxidase activity, we have been unable to confirm this phenomenon.

Reactivity of –SH groups in preparations extracted with ether

Recent studies published by ESTABROOK *et al.*[22] and MERSMANN, LUTHY AND SINGER[24] indicate that three distinct types of –SH groups might be demonstrated under suitable conditions in particulate and soluble, high-molecular-weight NADH dehydrogenase[40]. Type I is characteristic of NADH-cytochrome *c* reductase or becomes reactive due to conformational changes of native dehydrogenase which lead to the emergence of cytochrome *c* reductase activity. Type II may be present in particulate

or soluble high-molecular-weight dehydrogenase, while Type III –SH groups may arise as a result of structural modification due to incubation with NADH (ref. 22). We have studied both Types II and III of –SH groups in intact and ether-treated preparations.

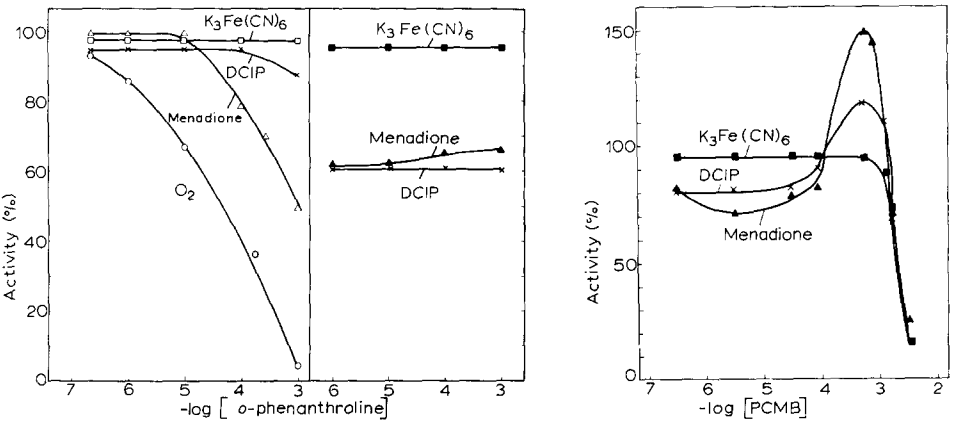


Fig. 5. Effect of *o*-phenanthroline on the oxidation of NADH in intact (left) and ether-extracted (right) preparations. *o*-Phenanthroline was applied as an ethanol solution. The time of incubation was 10 min at room temperature. The activities of ether-extracted preparation are expressed as percentage of the activity of intact preparation.

Fig. 6. Effect of *p*-chloromercuribenzoate on NADH oxidation in ether-extracted preparation. Incubation with PCMB was carried out in 0.1 M phosphate buffer (pH 7.4) for 60 min at room temperature as described by MINAKAMI, SCHINDLER AND ESTABROOK[16]. For incubation with PCMB, 1-ml samples which contained 1 mg of protein per ml were used. The activities are expressed as percent of the activity of unextracted preparation.

Fig. 6 shows the effect of PCMB on Type II –SH groups in ether-extracted preparation. It can be seen that activity was unaffected at concentrations of PCMB lower than 10^{-4} M. However, at PCMB concentrations of about $7.5 \cdot 10^{-4}$ M, pronounced stimulation of menadione and DCIP reductase activity occurred. It should be noted that similar stimulation of NADH-ferricyanide activity in electron transport particle preparation was observed by CREMONA AND KEARNEY[23] at $4 \cdot 10^{-4}$ M of PCMB.

The insensitivity of particulate NADH dehydrogenase to inhibition by mercurials was not due to the presence of inorganic phosphate in the reaction mixture since it has been shown[23] that the protection of the enzyme against PCMB (but not against *N*-ethylmaleimide) by phosphate could be observed only in experiments of relatively short duration and at a low PCMB concentration ($1 \cdot 10^{-5}$ M).

There is some controversy as to the inhibitory effect of *N*-ethylmaleimide on NADH oxidase. According to MINAKAMI, SCHINDLER AND ESTABROOK[16] treatment of electron transport particle with 1 mM *N*-ethylmaleimide for 1 h caused 95 % inhibition of the NADH oxidase activity. REDFEARN *et al.*[42] found only 40 % inhibition which did not exceed 60 % even at 8 mM *N*-ethylmaleimide. The results of our experiments on the effect of *N*-ethylmaleimide on NADH oxidase activity (Fig. 7) are essentially in accordance with those of REDFEARN *et al.*[42]. It can be seen that about 30 % of the oxidase activity was not affected by 0.1–1 mM *N*-ethylmaleimide. Inhibition of menadione reductase activity by 1 mM *N*-ethylmaleimide, equal to that

caused by ether treatment, may suggest that the other site of menadione activity is at or beyond the site of ether action.

Thus it might be concluded from these studies that extraction of the particles with ether did not modify the dehydrogenase since Type II of the -SH groups were not easily affected by sulfhydryl reagents, nor were the untreated preparations[16,22,23].

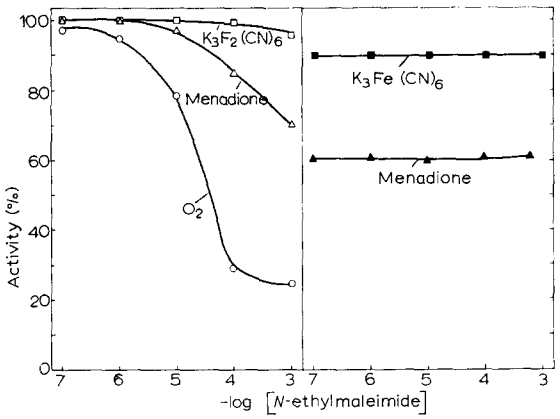


Fig. 7. Effect of *N*-ethylmaleimide on the oxidation of NADH by intact (left) and ether-extracted heart-muscle preparations (right). Samples of particles (2 mg of protein per ml) were incubated with *N*-ethylmaleimide according to the method of MINAKAMI, SCHINDLER AND ESTABROOK[16] in 0.1 M phosphate buffer (pH 7.4) at room temperature for 60 min before assay.

The Type III -SH groups were studied in both intact and ether-extracted preparations. The data which are presented in Table II are essentially in accordance with those of TYLER *et al.*[22] and MERSMANN, LUTHY AND SINGER[24], *i.e.* they show a marked effect of pretreatment with NADH on the inhibition of NADH-ferricyanide reductase activity as produced by mersalyl. However, in ether-extracted preparation the degree of inactivation was a little lower. It appears that ether treatment partially abolishes the pretreatment effect of NADH, as in the case of ferricyanide[22,24], imidazole or ADP (ref. 22).

The present results differ however from those of MERSMANN, LUTHY AND SINGER[24] on two points. First, in agreement with TYLER *et al.*[22] we have been unable to observe the inhibition of NADH-ferricyanide reductase activity by mersalyl without prior incubation with NADH (see Table II). Second, pretreatment with NADH produced a loss of NADH-ferricyanide activity only in intact preparations (see Table III). Treatment of the preparation with ether protected NADH dehydrogenase in some way against inactivation induced by incubation with substrate. This protection was not due to a break in the electron transport system such as is caused by ether since it could not be observed with intact preparations in the presence of respiratory chain inhibitors such as rotenone, antimycin or cyanide.

Effect of pretreatment of the particles with ether on the specific activity of soluble NADH-cytochrome c reductase

Although ether extraction of heart-muscle preparation did not change basic properties of particulate NADH dehydrogenase like high ferricyanide activity and reactivity of thiol groups, nevertheless, it was evident that preparations treated with

TABLE II

EFFECT OF PRETREATMENT WITH NADH ON THE SENSITIVITY OF NADH DEHYDROGENASE TO MERSALYL IN INTACT AND ETHER-EXTRACTED PREPARATIONS

The pretreatment with NADH was carried out according to the method of TYLER *et al.*[22]. Complete oxidation of 0.2 mM NADH was allowed prior to incubation with 15 μM of mersalyl for 30 min of pretreated and 60 min of unpretreated samples. Incubation was conducted in Tris–sulphate buffer (pH 7.8) at room temperature. In each experiment about 0.05 mg protein per ml was used for incubation with NADH and mersalyl. The NADH-ferricyanide activity is expressed as μmoles NADH oxidized per min per mg protein.

Expt. No.	*Pretreatment*	*Time of incubation with mersalyl (min)*	*Preparation*			
			Fresh		*Ether-extracted*	
			Activity	*Inactivation (%)*	*Activity*	*Inactivation (%)*
1	None		6.35		5.78	
	Mersalyl	60	6.45	0	5.80	0
	NADH		5.40		5.70	
	NADH followed by mersalyl	30	1.97	63	3.25	54
2	None		5.17		4.70	
	Mersalyl	60	5.27	0	4.55	3
	NADH		4.25		4.71	
	NADH followed by mersalyl	30	1.65	61	2.19	54
3	None		6.58		4.25	
	Mersalyl	60	6.26	5	4.27	0
	NADH		5.65		4.25	
	NADH followed by mersalyl	30	1.70	70	1.95	54

TABLE III

PROTECTION OF NADH DEHYDROGENASE AGAINST INACTIVATION FOLLOWING INCUBATION WITH NADH BY TREATMENT OF THE PARTICLES WITH ETHER

Both types of preparations were incubated with 0.2 mM NADH until the substrate was exhausted. NADH was then added and ferricyanide reductase activity determined. Activity is expressed as in Table II.

Expt. No.	*Fresh preparation*			*Ether-extracted preparation*		
	Control	*NADH pretreated*	*Inactivation (%)*	*Control*	*NADH pretreated*	*Inactivation (%)*
1	6.35	5.40	15	5.78	5.70	0
2	5.17	4.25	18	4.70	4.71	0
3	6.58	5.65	14	4.25	4.27	0

diethyl ether were in some way modified. Moreover, similar effects produced by ether and phospholipase suggested that both agents might attack identical sites on the chain. Phospholipase A had been used for the isolation of the soluble NADH dehydrogenase[14,40]. On the other hand, pretreatment of mitochondria with polar solvents such as acetone, is a frequent routine for extraction of respiratory enzymes. Therefore

experiments were performed in order to test whether extraction with diethyl ether would facilitate extraction of NADH-cytochrome *c* reductase by the method of MAHLER *et al.*[44].

Data presented in Table IV show clearly that pretreatment of heart-muscle preparation with diethyl ether was advantageous for obtaining high specific activities of the soluble preparation. Thus ferricyanide, menadione, DCIP and cytochrome *c* reductase activities were 3, 2, 2 and 2.5 times higher, respectively, than those obtained for soluble preparations from intact heart-muscle preparation.

TABLE IV

EFFECT OF ETHER TREATMENT OF HEART-MUSCLE PREPARATION ON ENZYMIC ACTIVITIES OF SUB-FRACTIONS OBTAINED BY ACID–ETHANOL EXTRACTION

Untreated and ether-extracted heart-muscle preparations (10 mg of protein per ml) were acidified to pH 5.4 with 1 M acetic acid. One volume of 18% ethanol was added dropwise and the mixture incubated at 42° for 15 min. After cooling, the suspension was adjusted to pH 7.4 and centrifuged at 16000 × *g* for 15 min. Enzymic activities were determined in both the supernatant and the residue in the presence of 50 mM Tris–sulphate buffer (pH 7.4). Prior to the determination, the residue was resuspended and homogenized in the same buffer. The activities are expressed as μmoles of NADH oxidized per min per mg of protein.

Preparation	*NADH dehydrogenase activity*			
	$K_3Fe(CN)_6$	*Menadione*	*DCIP*	*Cytochrome c*
Untreated	11.50	0.600	0.202	0.037
After acid–ethanol extraction:				
Residue	0.147	0.055	0.082	0.011
Supernatant	1.570	4.240	0.840	0.190
Ether extracted	10.50	0.300	0.175	0.027
After acid–ethanol extraction:				
Residue	0.284	0.060	0.065	0.014
Supernatant	4.640	7.580	1.680	0.490

DISCUSSION

The results indicate that treatment of heart-muscle preparation with diethyl ether did not affect the particulate NADH dehydrogenase. The ferricyanide activity which is characteristic of this enzyme[45] remained unchanged. Likewise the stability of the enzyme, insensitivity to *o*-phenanthroline and rotenone, and reactivity of –SH groups under various experimental conditions all indicated that basic properties of NADH dehydrogenase were not altered. These observations are in accord with experiments on reactivation of NADH oxidase following extraction of mitochondria with pentane[7]. Moreover, ferricyanide reductase activity of mitochondria was not destroyed by extraction either with aqueous acetone[6] or diethyl ether[9], or following successive treatment of hemoprotein with light petroleum and isooctane, which was used for the isolation of lipoflavoprotein with high ferricyanide activity[46]. Recently it has been observed that ferricyanide activity of NADH dehydrogenase is associated with ESR signal, $g = 1.94$ (ref. 21). Thus it could be expected that the ether-treated preparation would display an identical signal which is characteristic of intact systems[20].

Two lines of evidence indicate, as suggested by CUNNINGHAM, CRANE AND

SOTTOCASA[9], that inactivation of NADH oxidase by ether is not due to removal of some essential component of the respiratory chain from the segment between flavoprotein and cytochrome *b*, but rather to the derangement of lipids which are necessary for enzymic activity. First, a small concentration of ether (< 5 %, v/v), when dissolved in the reaction medium, brought about changes of enzymic activity similar to those caused by exhaustive extraction of the preparation with the same solvent. Second, as shown in Fig. 3, digestion of the preparation with small amounts of phospholipase produced the same effect as treatment with low concentrations of ether. Moreover, NADH-Q-1 reductase and NADH oxidase, when partially (up to 50 %) inactivated by ether treatment, could be reactivated by washing with bovine serum albumin[47], as occurs in phospholipase-treated mitochondria[11].

The stimulation of NADH oxidase activity by *o*-phenanthroline, was attributed by REDFEARN *et al.*[42] to its action on the protein conformation. However, MASSEY[48] has pointed out that the inhibitory effect of *o*-phenanthroline could also be explained in this way and not by chelation of the iron (*cf.* ref. 49). Our results are in agreement with those of MINAKAMI *et al.*[50] that a millimolar quantity of *o*-phenanthroline has no effect on NADH dehydrogenase. This suggests that either iron is not available for the chelator or that the site of ether action is before, or at least at, the site of inhibition by *o*-phenanthroline.

Ether treatment of heart-muscle preparation did not increase sensitivity of Type II –SH groups. There are, however, some differences in sensitivity of Type III –SH groups. It should be pointed out that when ether-extracted preparation was incubated with NADH, the ferricyanide reductase activity was not impaired, as was also observed by us and others[22,24] for intact preparations. Moreover, in ether-extracted preparations which were preincubated with NADH, the degree of inactivation even in the presence of mersalyl was smaller than in intact preparations. This protective action of ether on the pretreatment effect of NADH seems to be similar to the action of ferricyanide ion[22,24], imidazole and ADP (ref. 22).

Recently KING *et al.*[51] published an account of their many unsuccessful attempts to isolate NADH dehydrogenase by procedures other than acid–ethanol extraction, digestion with phospholipase or the thiourea method. Attempts of CUNNINGHAM, CRANE AND SOTTOCASA[9] should also be added to this list. The most interesting finding of the present investigation is that pretreatment of the preparation with ether facilitated not only the extraction of NADH dehydrogenase of the Mahler type but also that of high-molecular-weight dehydrogenase of SINGER and co-workers[40]. It was found that the latter dehydrogenase can be obtained with the aid of the non-ionic detergent Triton X-100 (refs. 52, 53). The yield of extraction amounted to 60–75 % of the original ferricyanide activity of the ether-treated preparation.

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