THE MECHANISM OF REACTIVATION OF ENZYME SYSTEMS IN MITOCHONDRIAL PREPARATIONS TREATED WITH ORGANIC SOLVENTS

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SUMMARY

The mechanism of inactivation of respiratory enzyme systems in mitochondrial preparations on treatment with organic solvents has been investigated. It was found that the inactivation of the enzyme systems could be attributed mainly to the presence of residual solvent which acted as a physical inhibitor. This inhibition could be reversed by any one of a number of different treatments which removed the solvent. It was shown that the specific reactivations claimed by other workers for substances such as tocopherol can be explained on the basis of non-specific reversal of this inhibition. Suitable techniques for studying preparations in which lipid has been removed by extraction, but without the complicating effect of residual solvent, will be described. The results will be discussed in relation to previous work.

INTRODUCTION

It was shown by NASON AND LEHMAN[1,2] that extraction of muscle mitochondrial preparations with isooctane resulted in an almost complete loss of the cytochrome *c* reductase activities. They could, however, be restored to their original levels by treating the preparations with α-tocopherol as a suspension in bovine serum albumin. It was suggested, therefore, that tocopherol was either an essential component of the respiratory chain or that it could replace some lipid cofactor which was assumed to act between cytochromes *b* and *c*. It was subsequently found by NASON and his group[3] and by others[4] that a number of substances unrelated to tocopherol would reactivate extracted enzyme preparations. The original hypothesis was not abandoned however, and the results were explained on the basis of a specific reactivation by tocopherol[5]. DEUL, SLATER AND VELDSTRA[6] repeated the work of NASON's group and concluded that the reactivation by tocopherol was best explained as a non-specific physical reversal of an inhibition caused by isooctane which was adsorbed on the surface of the enzyme particle. Experimental support for this idea came from the work of REDFEARN AND PUMPHREY[7] who showed that the non-ionic surface-active agent, Tween 80, was a more efficient reactivator than *d*-α-tocopherol in the succinic–cytochrome *c* reductase system of a pig heart-muscle preparation. It seemed, therefore,

Abbreviations: DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)-aminomethane.

that the principal mechanism of reactivation was the displacement or dispersal of the residual organic solvent which was adsorbed on or associated with the enzyme particles so as to prevent access of the reactants, or to inhibit electron transfer at some point in the respiratory chain, or both. This type of reactivation mechanism would also explain the reactivating effect of a large number of substances, all possessing long isoprenoid chains, studied by WEBER, GLOOR AND WISS[8]. Further evidence in favour of this mechanism came from POLLARD AND BIERI[9] who found that mechanical treatment, such as high-speed centrifugation or freeze-drying, would effectively remove residual solvent and produce a reactivation.

At the present time, a great deal of interest is being focussed on the possible roles of lipids as structural and functional components of the electron transport system, and a suitable extraction–reactivation technique would seem to be a useful tool for these studies. It appears, however, that the predominating factor in the inactivation of enzyme systems in preparations treated with organic solvents is a physical inhibition caused by the residual solvent. Previous claims for the participation of certain lipid co-factors in the respiratory chain can now be explained on the basis of non-specific reactivations by the reversal of the inhibitory effects of the solvent and not to the replacement of specific lipids removed by the extraction. If the extraction–reactivation technique is to be used as a valid test system for studying the structural and functional lipids of the respiratory chain then the inhibitory solvent must first be removed.

In the present paper the results of a study of the mechanisms of extraction and reactivation of mitochondrial enzyme systems will be presented and discussed.

MATERIALS AND METHODS

Solvents: A.R. isooctane and light petroleum (b.p. 40–60°) were freed from benzene by passing them through a column of silica gel (200 mesh) and then distilling.

Chemicals: Cytochrome *c* was prepared from ox heart[10] and dialysed against water. Ubiquinone was obtained from pig heart[11]. *d*-α-tocopherol was prepared from the acetate by saponification. DPNH was purchased from Boehringer und Soehne, Mannheim, Tween 80 from Honeywell and Stein, London, and bovine serum albumin from Armour and Co., Eastbourne.

Tocopherol and ubiquinone suspensions: These were prepared by dissolving ubiquinone (3 mg) or *d*-α-tocopherol (1.5 mg) in ethanol (1.2 ml). 4.8 ml of an aqueous solution of bovine serum albumin (0.2 %, w/w) were then added and the mixture shaken vigorously by hand. The final concentrations were $5.8 \cdot 10^{-4}$ *M*.

Heart-muscle preparations: Two types, I and II, were used. These were prepared by procedures described by MORRISON, CRAWFORD AND STOTZ[12] and PUMPHREY AND REDFEARN[13], respectively.

Final protein concentrations[14] in both types of preparation varied between 10 and 40 mg/ml.

Extraction procedure: Unless otherwise stated, the method used was as follows:

The enzyme preparation was shaken vigorously by hand with an equal volume of isooctane, or other solvent, for 1 min at room temperature. The organic solvent and aqueous layers were then separated by giving the tube a short spin in a clinical centrifuge. The upper organic solvent layer was then removed.

Measurement of enzyme activities

Succinic-cytochrome c *reductase* was measured spectrophotometrically at 550 mμ at room temperature (17–22°) as already described[7], and the reaction started by adding the enzyme.

DPNH-cytochrome c *reductase* was measured in the same way as the succinic cytochrome *c* reductase except that the succinate was replaced by DPNH ($1.1 \cdot 10^{-4} M$).

Succinic oxidase was measured manometrically at 37° with a reaction mixture as previously described[15]. The taps were closed 5 min after placing the manometer in the bath, and the succinic oxidase activity was calculated from the oxygen uptake in the 5 to 15-min period after closing the taps.

In order to study the effect of solvent treatment on the succinic oxidase activity it was necessary to devise a method for removing the residual solvent before carrying out the assay. Interference due to evolution of vapour from residual solvent would thus be avoided. The method used was first to blow a stream of nitrogen over the extracted preparation for a few minutes at room temperature. The preparation, after suitable dilution, was then placed in the Warburg flask with a small concentration of succinate (0.004 *M*) and with all the other additions. The remainder of the succinate addition was placed in the side-arm. The flask was then shaken in the water-bath at 37° with the manometer tap open until all the solvent vapour had been evolved. The succinate was then tipped into the reaction mixture and the rate of oxygen uptake measured. The unextracted controls were treated in the same way.

DPNH oxidase was measured spectrophotometrically at 340 mμ at room temperature (17–22°) using the same reaction mixture as used for DPNH–cytochrome *c* reductase except that the cyanide addition was omitted.

Cytochrome oxidase was measured manometrically at 37° with the following reaction mixture: KH_2PO_4–Na_2HPO_4 or Tris–HCl buffer, pH 7.4, 0.07 *M*; *p*-phenylene diamine, 0.04 *M*; cytochrome *c*, $7 \cdot 10^{-5} M$; heart-muscle preparation, 0.25–1.0 mg protein. The final reaction volume of the flask contents was 1.4 ml. The gas phase was air. The cytochrome oxidase activity was calculated from the oxygen uptake in the 5–15 min period.

RESULTS

General observations

It will be shown that treatment of the preparations with organic solvents can affect enzyme activities in several ways, quite apart from the removal of lipid. In this paper, the term "extraction" will be used to denote the shaking of the preparation with organic solvent regardless of the quantity of lipid removed.

The effects of extraction on enzyme activities depended on a number of factors such as the type of preparation, its age and method of storage, the nature of the solvent and the amount of solvent retained in the preparation after the extraction procedure. Most of these factors will be dealt with in the results of experiments on the different enzyme systems. However, some general observations on the extraction procedure might be described here since they give a clue to an understanding of the mechanism of inactivation by solvents.

In the extraction procedure, the vigorous shaking of the preparation with the solvent usually resulted in emulsion formation. After centrifugation, the aqueous and solvent layers had separated and most of the enzyme particles had floated to the

top of the aqueous layer instead of sedimenting or remaining dispersed. It appeared that the solvent had become adsorbed on the surface or had dissolved in the lipid of the particle and so lowered its density. If the upper solvent layer was removed and the particles redispersed in the aqueous layer it was found that there was an evolution of solvent vapour from the suspension. When all the residual solvent had been evolved centrifugation of the suspension caused a sedimentation of the particles.

Evidence that the solvent itself is the principal cause of inactivation was provided by shaking the heart-muscle preparation with a small volume (10–20 % v/v) of the solvent. Measurement of succinic cytochrome *c* reductase activity of the mixture then showed that a considerable inactivation had occurred.

Experiments were performed on two types of preparation, I and II. The essential differences between them were that type I contained ammonium sulphate and it was stored at —20° for periods up to several weeks while type II did not contain ammonium sulphate and was stored at 3–5° for 6–7 days. In a few experiments, ammonium sulphate was added to a type II preparation which was then kept at —20°. This treatment gave a preparation which appeared similar to that of type I. The long period of storage of this type at —20° appeared to make it more sensitive to treatment with organic solvents; *i.e.* greater enzyme inactivations were obtained after extraction and removal of residual solvent than those obtained with the type II preparations.

Effects of solvent treatment on cytochrome c *reductase activities*

Succinic– and DPNH–cytochrome *c* reductase activities were measured in both types of preparation. The loss in activities of the heart-muscle preparation after one extraction increased after storage at —20°. NASON AND LEHMAN[2] had also found that enzyme inactivation by extraction was facilitated by storage of their preparation. Fig. 1 shows the succinic– and DPNH–cytochrome *c* reductase activities of a preparation before and after a single extraction with isooctane as a function of the age of the preparation. The isooctane extract had an absorption maximum at about 270 mμ and as a rough index of the amount of lipid extracted, the extinction at 270 mμ was measured.

The reactivating effect of *d*-α-tocopherol[1,2,16] on the succinic- and DPNH–cytochrome *c* reductase activities was confirmed. It was found also that ubiquinone (coenzyme Q) would reactivate, a result which is in agreement with work of another group[8]. It seemed that these substances could be acting by virtue of their surface-active properties in removing residual solvent which was inhibiting the enzyme systems. The effect of adding the non-ionic surface-active agent, Tween 80 was therefore tried and it was found that it gave 100 % reactivation in the succinic–cytochrome *c* reductase activity of an isooctane-extracted preparation (Fig. 2). Tween 80 in the concentration used had little or no effect on succinic–cytochrome *c* reductase in the unextracted preparation.

In another experiment a preparation of type I was aged for 2 weeks at —20°, and then it was successively extracted with isooctane up to 10 times. The reactivating effect of Tween 80, ubiquinone and *d*-α-tocopherol were tested after each extraction (Table I). Up to three extractions seemed to have little effect on the maximal restored activity, although as much as 34 % (by weight) of the total lipid had been removed. After three extractions the restored activities fell rapidly until with ten extractions,

when 49 % of the lipid had been removed, the maximal restored activity had fallen to 42 % of the original activity.

In experiments on DPNH-cytochrome *c* reductase, it was found that *d*-α-tocopherol and ubiquinone would reactivate after extraction, but Tween 80 would not. In fact, Tween 80 destroyed almost all activity in the unextracted preparation. This is in keeping with the greater sensitivity of the DPNH–cytochrome *c* reductase

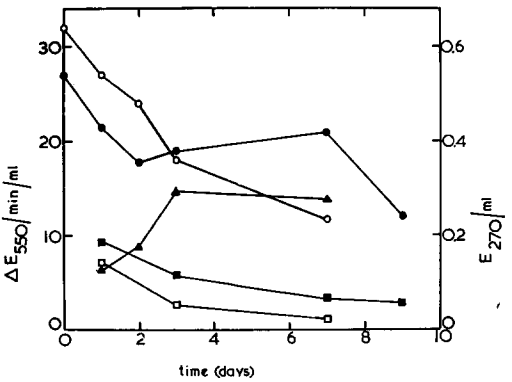

Fig. 1. Effect of one extraction with isooctane on succinic- and DPNH-cytochrome *c* reductase activities of a type I heart-muscle preparation as a function of the age of the preparation. ○—○, DPNH-cytochrome *c* reductase before extraction; ●—●, succinic–cytochrome *c* reductase before extraction; □—□, DPNH–cytochrome *c* reductase after extraction; ■—■, succinic–cytochrome *c* reductase after extraction; ▲—▲, extinction at 270 mμ of isooctane extract per ml undiluted preparation. Enzyme activities are expressed as rate of change of extinction at 550 mμ/ml undiluted preparation. Assay procedures as described in the text.

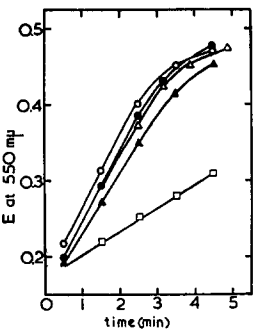

Fig. 2. Reactivation of succinic–cytochrome *c* reductase by *d*-α-tocopherol, ubiquinone and Tween 80. ○—○, unextracted; □—□, extracted once with isooctane; △—△, extracted plus *d*-α-tocopherol, $3.8 \cdot 10^{-5}$ *M*; ▲—▲, extracted plus ubiquinone, $3.8 \cdot 10^{-5}$ *M*; ●—●, extracted plus Tween 80, 1.33 μg/ml. Type II preparation with added ammonium sulphate (8.8% w/v) aged at —20° for 7 days. *d*-α-tocopherol and ubiquinone added as suspensions in 0.2% bovine serum albumin. Assay procedure as described in text.

TABLE I

EFFECT OF ISOOCTANE EXTRACTION ON THE SUCCINIC-CYTOCHROME *c* REDUCTASE OF A PIG HEART-MUSCLE PREPARATION

Extraction and assay as described in text. Type I preparation (age: 2 weeks) used. Final concentrations of additions: Tween 80, 1.33 mg/ml; *d*-α-tocopherol, $3.8 \cdot 10^{-5}$ *M*; ubiquinone, $3.8 \cdot 10^{-5}$ *M*. Suspensions of *d*-α-tocopherol and ubiquinone in 0.2 % bovine serum albumin were used.

Number of extractions	*Additions:*	*Relative activity*			
		None	*Tween 80*	*d-α-tocopherol*	*Ubiquinone*
0		100	100	100	100
1		17	108	78	84
2		11	103	70	81
3		11	100	58	80
4		9	89	52	77
7		7	60	22	51
10		2	42	13	26

system to surface-active agents and organic solvents already noted by other workers[6,17].

Effect of solvent-treatment on succinic oxidase activity

The manometric determination of succinic oxidase after treatment of the preparation with organic solvents is complicated by the fact that there is an evolution of vapour from the residual solvent during the course of the assay. After a time, when all the solvent has been driven off, the manometer indicates an uptake of oxygen. These effects are illustrated in Fig. 3.

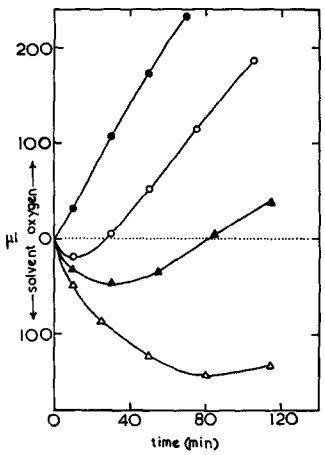

Fig. 3. Succinic oxidase activity after 1, 4 and 9 extractions with isooctane. ●—●, unextracted; ○—○, 1 extraction; ▲—▲, 4 extractions; △—△, 9 extractions. Type II preparation with added ammonium sulphate (8.8 % w/v) aged at —20° for 7 days. Assay procedure as described in text.

The effects of *d*-α-tocopherol, ubiquinone and Tween 80 additions on the succinic oxidase activities of extracted preparations were studied. Tween 80 appeared to accelerate the evolution of the residual solvent and consequently the oxygen uptake began earlier. Ubiquinone and tocopherol as suspensions in bovine serum albumin did not give this effect but often tended to eliminate part of the "lag-period" due to solvent evolution, possibly by preferentially adsorbing the solvent; this would reverse the inhibition and perhaps also tend to prevent the solvent from evaporating. None of these substances had any effect on the final maximum activity after extractions.

The amount of residual solvent in a preparation depends upon a number of factors, such as the number of extractions and the dilution after extraction. If the amount of solvent is small and the succinic oxidase activity high, then there will be apparent uptake of oxygen but this will be lower than the true activity. When all the solvent has been driven off, the apparent oxygen uptake will attain its maximal true value. A plot of the "oxygen uptake" against time will thus give a curve with an apparent lag period similar to those published by DEUL, SLATER AND VELDSTRA[6]. The interfering effect of the evolution of residual solvent vapour can, however, be eliminated by using the modified procedure described above in which the residual

solvent is removed before the assay. Using this procedure the effect of the number of extractions and the addition of cytochrome *c*, tocopherol and ubiquinone on the succinic oxidase activity of the preparations after solvent treatment was studied.

Number of extractions: The effects of an increasing number of extractions of a type II preparation which had been aged at —20° for 1 week have already been shown (Fig. 3). It was found that there was a progressive loss of activity with an increasing number of extractions with isooctane. Subsequent experiments employing the modified technique for removing residual solvent were done with unfrozen type II preparations using the more volatile 40–60° light petroleum as the solvent (Table II). It can be seen that after one or two extractions there was an increase in the succinic oxidase activity (> 100 % in Expt. 1). The activities then fell slowly with increasing number of extractions but in one case, even after 40 extractions, the activity was more than 100 % greater than the original activity. Preliminary experiments have indicated that a considerable amount (30–50 %) of the ubiquinone and possibly other lipids can be removed from the particles apparently without this having any appreciable effect on the enzyme activities.

TABLE II

EFFECT OF NUMBER OF LIGHT PETROLEUM EXTRACTIONS ON THE SUCCINIC OXIDASE AND CYTOCHROME OXIDASE ACTIVITIES OF A PIG HEART-MUSCLE PREPARATION

Assays as described in text. The experiments were carried out with three different type II preparations. Expt. 1: 2 ml preparation (age: 3 days) extracted successively with 4 ml 40–60° light petroleum for 1 min. Expt. 2: 1 ml preparation (age: 1 day) extracted successively with 1 ml 40–60° light petroleum for 1 min. Expt. 3: 1 ml preparation (age: 7 days) extracted successively with 5 ml 40–60° petroleum for 1 min.

Number of extractions	*Succinic oxidase* QO_2 ($\mu l\ O_2/h/mg$ *protein*)			*Cytochrome oxidase* QO_2	
	Expt. No. 1	2	3	1	3
0	259	216	320	810	1240
1	567	293	—	886	—
2	373	297	—	842	—
3	—	288	—	—	—
4	243	—	—	902	—
5		225	—		
7		220	—		—
10		135	—		—
20			960		1616
40			770		1880

Cytochrome c *addition*: In all these succinic oxidase assays, cytochrome *c* was added. This was necessary because extraction of these preparations with organic solvents appeared to increase their dependency on external cytochrome *c* for attainment of maximal activity. If the succinic oxidase activity was measured without added cytochrome *c*, after extraction there was an apparent loss in activity which could be restored by adding cytochrome *c*. Fig. 4 shows the effect of cytochrome *c* addition on a fresh heart-muscle preparation before and after one extraction with light petroleum.

d-*α-tocopherol addition*: The effects of the addition of *d*-α-tocopherol either as a solution (ethanol) or a suspension (bovine serum albumin) were studied. The results of one experiment are shown in Fig. 5. A Tris-buffered preparation of type II was

extracted four times with isooctane and once with 40–60° light petroleum. The purpose of the latter was to remove most of the residual isooctane; the residual light petroleum, being much more volatile, was then driven off by shaking the flasks for 20 min at 37°.

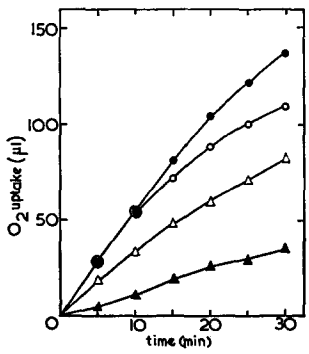

Fig. 4. Effect of extraction on added cytochrome *c* requirement of succinic oxidase. ●—●, unextracted plus added cytochrome *c*, $4.3 \cdot 10^{-5}$ *M*; △—△, unextracted without added cytochrome *c*; ○—○, extracted (once with 40–60° light petroleum using 1 ml preparation and 3 ml solvent) plus added cytochrome *c*, $4.3 \cdot 10^{-5}$ *M*; ▲—▲, extracted without added cytochrome *c*. Type II preparation suspended in Tris–HCl buffer. Residual solvent removed and succinic oxidase assayed as described in text.

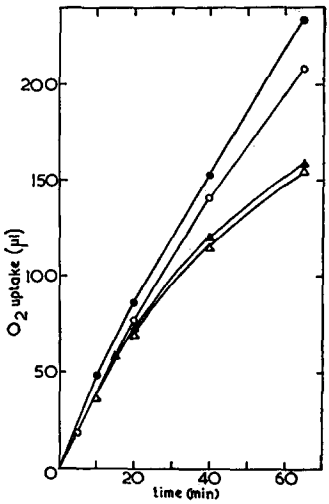

Fig. 5. Effect of *d*-α-tocopherol and ubiquinone additions on succinic oxidase of extracted preparation. ●—●, unextracted; △—△, extracted (four times with isooctane and once with 40–60° light petroleum using 1 ml preparation and 3 ml solvent); ○—○, extracted plus *d*-α-tocopherol (ethanolic solution), $2 \cdot 10^{-4}$ *M*; ▲—▲, extracted plus ubiquinone (ethanolic solution), $2 \cdot 10^{-4}$ *M*. Type II preparation suspended in Tris–HCl buffer. Residual solvent removed and succinic oxidase assayed as described in text.

Although there were no significant differences in the maximal activities, it will be noticed that *d*-α-tocopherol prevented the falling-off in activity with time. It seems that tocopherol is acting here as an antioxidant and is protecting some essential groups or components from destruction by oxidation. Tocopherol showed a similar effect with the unextracted enzyme preparations. AMES AND RISLEY[18] showed that although a suspension of α-tocopherol in bovine serum albumin inhibited the succinic oxidase activity of their preparations, it protected the system against inactivation for approx. 3 h. More recently, TAPPEL AND ZALKIN[19] found that tocopherol inhibited lipid peroxidation and stabilized DPNH–cytochrome *c* reductase in mitochondria.

Ubiquinone (coenzyme Q) addition: In a number of experiments, preparations of type II were extracted with isooctane and 40–60° light petroleum. The final extraction was usually made with light petroleum since this was more volatile and the residual solvent could be more readily driven off by incubating at 37° for relatively short periods (up to 20 min). The succinic oxidase activity could then be measured in the presence and absence of added ubiquinone (in ethanolic solution) and cytochrome *c*. The results (Table III) may be summarized as follows:

TABLE III

EFFECT OF ADDITION OF UBIQUINONE AND CYTOCHROME *c* ON SUCCINIC OXIDASE ACTIVITY OF SOLVENT-EXTRACTED PIG HEART-MUSCLE PREPARATIONS

All experiments were performed with type II preparations. Residual solvent removed and succinic oxidase assayed as described in text. Ubiquinone added where indicated (± UQ) as a ethanolic solution to give final concentration of $2 \cdot 10^{-4} M$. Same volume of ethanol added to controls. Details of individual experiments as follows: Expt. 1: 2 ml preparation extracted once with 2 ml isooctane and then once with 2 ml 40–60° light petroleum. Expt. 2: 1 ml preparation extracted once with 3 ml isooctane and then once with 3 ml 40–60° light petroleum. Expt. 4: 1 ml preparation extracted once with 3 ml 40–60° light petroleum. Expt. 5: 1 ml preparation extracted twice with 3 ml isooctane and then once with 3 ml 40–60° light petroleum. Expt. 6: 2 ml preparation extracted with 4 ml 40–60° light petroleum.

Expt. number	*Preparation number*	*Age at time of Expt. (days)*	*Suspending buffer*	*Presence (+) or absence (—) of added cytochrome c*	*Succinic oxidase (QO_2)*			
					Unextracted		*Extracted*	
					— UQ	*+ UQ*	*— UQ*	*+ UQ*
1	31 B	6	Tris	—	128	129	58	50
	31 B	6	Tris	+	248	—	253	—
2	28 A	6	Phosphate	+	480	486	468	486
3	28 B	8	Tris	+	244	267	—	—
4	28 B	12	Tris	—	93	93	47	41
				+	152	—	152	—
5	25 B	2	Tris	+	278	—	221	226
6	29 A	2	Phosphate	—	76	51	41	41

(a) when cytochrome *c* was omitted, there was a loss of succinic oxidase activity after extraction; (b) in the presence of added cytochrome *c*, succinic oxidase activities either increased (*e.g.* to 22 % higher after two extractions) or decreased (*e.g.* 20 % lower after three extractions); (c) Ubiquinone added to the extracted preparation appeared to produce small increases in activity in two experiments; in most cases however, there were no significant changes in activity when ubiquinone was added with or without added cytochrome *c* except that it sometimes tended to prevent the falling-off in oxygen uptake with time. This effect may explain the slightly higher activities sometimes obtained with added ubiquinone. (d) Addition of ubiquinone to the unextracted preparation appeared to have little or no effect.

Effect of solvent-treatment on cytochrome oxidase

Extraction with non-polar solvents such as 40–60° light petroleum usually produced a marked increase in activity after removal of residual solvent as shown in Table II. It is known that surface-active agents have an activating effect on cytochrome oxidase[20] and it seems that non-polar solvents behave similarly.

When polar solvents are used, however, the results are rather different. In an experiment where a preparation was first extracted with isooctane, then with 40–60° light petroleum and finally with diethyl ether it was found that there had been a considerable inactivation of cytochrome oxidase and it had become the rate-limiting step in the succinic oxidase system. In another experiment in which a preparation was extracted once with 40-60° light petroleum containing 20 % (v/v) acetone the cytochrome oxidase was completely inactivated but succinic cytochrome *c* reductase was still relatively high. Thus it seems that treatment with non-polar solvents stimulates cytochrome oxidase activity but polar solvents produce an inactivation.

DISCUSSION

The heart-muscle preparations of the types described in this paper are colloidal suspensions of fragments of mitochondrial membranes and *cristae*. They contain 30–35 % lipid on a dry weight basis. Approx. 90 % of this lipid is phospholipid with smaller amounts of cholesterol (3–4 %), neutral lipid (2–3 %), ubiquinone (0.6 %) and other minor constituents[21]. There is a substantial amount of evidence to support the view that the phospholipid[22] is an important structural element, and the ubiquinone[23,24] a functional component of the respiratory chain. Hence, removal of these lipids by extraction with organic solvents would be expected to have a profound effect on the enzymic activities of the system. However, the inactivation of the enzyme systems of the respiratory chain which occurs when heart-muscle preparations are treated with non-polar organic solvents appears to be due primarily to the inhibitory effect of the residual solvent present, and not to the removal of the lipid. In fact, in some experiments even after forty extractions there did not appear to be a loss in activity due to removal of lipid *per se*. In general, however, some loss in activity due to removal of lipid can usually be achieved after a sufficiently large number of extractions with non-polar solvents. Under the conditions usually employed by earlier workers (*i.e.* one or two extractions with isooctane) very little lipid was removed and the activity after removal of the residual solvent was usually higher than the original activity. The increase in activity which occurs after a few extractions can probably be attributed to the physical action of the solvent in breaking down larger particles or aggregates into smaller ones, or causing an alteration of the particle structure which allows an easier access of the enzyme system to the reactants. This process is probably analogous to the effect of freezing and thawing or to the action of certain surface-active agents, both of which result in increased enzyme activities. To summarize therefore, treatment of heart-muscle preparations with organic solvents has three principal effects: (a) the physical action of the solvent producing a change in particle size or morphology, (b) the removal of lipid from the particle by solution in the solvent, and (c) the retention of a small amount of the solvent by the particles by surface adsorption or by solution in the lipid. The observed loss in enzymic activities after one or two extractions with isooctane or other solvents is due primarily to the third effect. After removal of the inhibitory residual solvent the true activity of the extracted preparation can be measured. This may be higher or lower than the activity of the untreated control depending on the number of extractions and other factors such as the age and type of preparation.

It has been shown that a non-ionic surface active agent will effectively remove the inhibitory effect of the residual solvent presumably by dispersing it. It seems very likely that tocopherol, ubiquinone and other compounds with long isoprenoid side chains act similarly to surface active agents and are able to remove the solvent from its inhibitory site on the enzyme particle. WEITZEL *et al.*[25] have shown that tocopherol and vitamin K_1 exhibit surface-active properties. Compounds possessing long isoprenoid chains are however not the only ones which will reverse the inhibition due to solvent as recently pointed out by DRAPER AND CSALLANY[26].

In order to study the effect of various added lipids in lipid-extracted preparations it was necessary to devise another method for removing completely the residual solvent. This was achieved quite simply by first blowing a stream of nitrogen over

the preparation at room temperature for a few minutes and then incubating for a short period at 37° to remove the last traces of solvent. Using this technique the effect of ubiquinone addition to extracted preparations was tested but in no case could an unequivocal activation by replacement be demonstrated. Thus although it seems probable from other work[23,24] that ubiquinone is a functional member of the respiratory chain, under the conditions of the present experiments, added ubiquinone cannot restore activity by replacing extracted ubiquinone. CRANE *et al.*[27] extracted ETP with isooctane and found that succinic oxidase was partially reactivated with ubiquinone alone, completely reactivated by ubiquinone together with a special phospholipid fraction and completely reactivated by cytochrome *c* alone. There was no summation of the activities induced by ubiquinone and cytochrome *c*. These workers also found that although succinic oxidase activity fell after extraction succinic- and DPNH-cytochrome *c* activities increased markedly. It has been shown subsequently that ubiquinone acts before cytochrome *c* (see ref. 23) and its removal would, therefore, be expected to affect cytochrome reduction. Thus it is now very doubtful whether the reactivation obtained by these workers was due to a specific action of ubiquinone. The most likely explanation is that it was due to the removal of traces of solvent from the preparation. The explanation of the reactivating effect of cytochrome *c* is that treatment with organic solvents induces a requirement for added cytochrome *c* as already indicated in the present experiments. Recently the same group of workers[28,29] have reported a specific reactivating effect of ubiquinone in acetone-extracted mitochondria. In this case the reactivation was only obtained when cytochrome *c* was also added; no reactivation was obtained with cytochrome *c* alone. It is difficult to understand why ubiquinone cannot reactivate preparations in which ubiquinone has been removed by extraction with non-polar solvents such as light petroleum while reactivations are obtained after much more drastic treatment with acetone. The explanation may be that the enzyme particles have to undergo some structural change only brought about by more polar solvents such as acetone, in order that the added ubiquinone can enter the active site. In support of this it has now been shown by CHANCE AND REDFEARN[30] that pure ubiquinone added to unextracted preparations is reduced at a very low rate compared with that of the endogenous material. An investigation of the mechanism of a specific reactivation by ubiquinone is in progress.

Finally, it is appropriate to mention the recent work of WEBER AND WISS[31]. These workers have shown that the inhibition of succinic–cytochrome *c* reductase activity in a mitochondrial preparation by isooctane and a number of other hydrocarbons is due not only to their lipid solubility but also to their structural properties. The length of the hydrocarbon chain was an important factor, chains of 6–7 carbon atoms being the most active. The inhibitory effect of the *n*-alkanes was decreased by the introduction of a hydroxyl group but was increased by bromine substitution. Of the other compounds with inhibitory effects it was interesting to note that a vitamin K_2 analogue with a side-chain consisting of one isoprenoid unit was 30 times as active as isooctane. In all cases the inhibition could be reversed by substances with long isoprenoid side chains such as vitamin K_1, phytol and ubiquinone. The potent inhibition caused by the vitamin K_2 analogue with a short side chain suggests a mechanism for the inhibition process. The short lipophilic side chain dissolves in the lipid phase of the enzyme particle leaving the large naphthoquinone

nucleus projecting from the surface and acting as an efficient barrier to the reactants.

The structural similarity of the vitamin K_2 analogue and the 2-hydroxy-3-alkyl-naphthoquinone inhibitors described by BALL, ANFINSEN AND COOPER[32] is striking. It is possible that the inhibitory effects of the latter can now be explained in terms of a purely physical non-specific effect rather than as a specific inhibition of a naturally occurring quinone, as recently suggested[33]. Work in progress may help to elucidate this point.

NOTE ADDED IN PROOF

After this paper was submitted for publication, a paper by HENDLIN AND COOK[34] has come to our notice. These workers have put forward evidence which supports the suggestion that naphthoquinones act as non-specific inhibitors.

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