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COMPARISON OF THE EFFECTS OF MENADIONE AND 2,3-DIMETHYLNAPHTHOQUINONE ON THE ENERGY-COUPLING REACTIONS OF BEEF-HEART MITOCHONDRIA

EVIDENCE FOR THE INVOLVEMENT OF A THIOL GROUP IN THE REACTIONS OF OXIDATIVE PHOSPHORYLATION

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Abstract—Incubation of beef-heart mitochondria with 80 μM menadione abolishes the ability of 2,4-dinitrophenol to release state 4 respiration. Incubation with 80 μM 2,3-dimethylnaphthoquinone is without effect. The percentage rotenone-sensitivity of the state 4 respiration in the presence of menadione does not alter appreciably on incubation. In the case of 2,3-dimethylnaphthoquinone there is a slow increase. Immediately following the addition of menadione the state 4 respiration is totally insensitive to cyanide, but becomes about 35 per cent cyanide-sensitive on continued incubation, indicating the presence in tightly-coupled beef-heart mitochondria of a factor which promotes the cyanide-insensitive oxidation of menadiol, but which is itself slowly inactivated. The cyanide-sensitive respiration in the presence of 2,3-dimethylnaphthoquinone remained constant at 35 per cent. These observations are most simply explained if menadione reacts with a thiol group essential for an early reaction of mitochondrial energy-coupling. The failure of menadione to cause any progressive inhibition of the energy-linked reduction of $NADP^+$ by NADH in submitochondrial particles is consistent with this suggestion.

THE INVOLVEMENT of a thiol group in mitochondrial oxidative phosphorylation, in analogy to the generation of thiol esters in substrate level phosphorylations, such as that catalysed by the α-ketoglutarate–succinate thiokinase system, has been a popular but, until recently, an experimentally relatively unsupported hypothesis. Fluharty and Sanadi,[1] who studied the effects of an arsenical on rat-liver mitochondria, proposed that a dithiol site existed between the respiratory chain and the oligomycin-sensitive site. Fonyo and Bessman[2] observed that *p*-hydroxymercuribenzoate inhibited only respiration coupled to ATP synthesis in rat-liver mitochondria. The inhibition was released by 2,4-dinitrophenol (DNP).† Similar observations were made by Van Buskirk and Frisell[3] using formaldehyde as inhibitor. The conclusion drawn from these last two studies was that a thiol intermediate was involved in the reactions of oxidative phosphorylation, possibly in a step between the DNP site and the entry of phosphate.[2] However, Tyler[4] showed that mersalyl and formaldehyde inhibited mitochondrial swelling, but only when this depended on the entry of phosphate. He proposed that the inhibition of coupled mitochondrial respiration was a consequence of the inhibition of

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† Abbreviation: DNP, 2,4-dinitrophenol.

phosphate entry. A similar suggestion was made by Fonyo.[5,6] This view has been supported by studies with Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid), using rat-liver mitochondria[7,8] and also by observations on the effect of mersalyl on the oxidation of succinate by house-fly mitochondria.[9] However, formaldehyde and mercurials also inhibit energy-linked reactions in submitochondrial particles,[3,10,11] an effect which seems unlikely to be related to the inhibition of phosphate entry. In particular, Sanadi's group have noted that energy-linked reactions driven by energy derived from oxidation are more sensitive to mercurials than the same reactions energized by ATP.[11] In addition, Lam[12] has shown that factor B, a highly purified protein which restores energy-linked reactions in phosphorylation deficient submitochondrial particles, has a thiol group which is essential for activity. The conclusion drawn from these experiments was that two thiol groups may be involved in oxidative phosphorylation, the component with the greater affinity towards mercurials being present in the reactions associated with the generation of high-energy intermediates from respiration.[10]

In the course of an investigation into the interaction of quinol monophosphates with mitochondria[13] a study has been made of the effects of the parent quinones, in particular menadione and 2,3-dimethylnaphthoquinone, on oxidative phosphorylation and energy-linked reactions in tightly-coupled mitochondria and submitochondrial particles. The outstanding difference chemically between the two quinones is the ready reaction of menadione, but not the dimethylnaphthoquinone, with thiols. A comparison of the effects of the two quinones is presented below. The differences observed may be explained most simply if menadione reacts with a thiol group essential for the reactions of mitochondrial energy-coupling. Evidence is also presented for the presence in tightly-coupled beef-heart mitochondria of a factor which catalyses the cyanide-insensitive oxidation of menadiol.

MATERIALS AND METHODS

Chemicals. Menadione (2-methyl-1,4-naphthoquinone, menaphthone) was obtained from B.D.H. Ltd, Poole, Dorset and purified by recrystallization from ethanol. 2,3-Dimethyl-1,4-naphthoquinone, purified by chromatography and recrystallization, was kindly provided by Dr. R. G. Wilson. The quinones were dissolved in ethanol and 5 μl of the solution added to the reaction mixtures. Rotenone was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Nagarse proteinase was supplied by Teikoku Chemical Industry Co. Ltd., Osaka, Japan.

Mitochondria. Tightly-coupled beef-heart mitochondria were prepared by the Nagarse proteinase procedure, essentially as described by Hatefi, Jurtshuk and Haavik,[14] except that the incubation contained 150 g minced heart tissues and 100 mg crystalline Nagarse in 400 ml cold 0·25 M sucrose. This mixture was diluted with a further 400 ml 0·25 M sucrose prior to homogenization. (I am grateful to Dr J. M. Haslam for details of this modification.) The preparation was carried out within 1 hr of the excision of the beef hearts.

The respiratory control ratio is defined as the ratio of the state 3 respiration to that of the subsequent state 4. (State 3, substrate, cofactors and oxygen all present in excess; state 4, phosphate acceptor limiting. See Chance and Williams[15] for a full definition of states of respiration.)

Submitochondrial particles. Phosphorylating submitochondrial particles were prepared from mitochondria stored at −20° by the procedure of Hansen and Smith[16] as described by Griffiths and Robertson,[17] except that $MnCl_2$ was omitted from the medium. The final pellet was suspended in 0·25 M sucrose, 4 mM $MgCl_2$ and 25 mM tris HCl (pH 7·6) and used within a few hours.

Protein. Protein was determined by the biuret reaction,[18] after solubilization with deoxycholate (0·25 per cent, w/v). Bovine serum albumin was used as standard.

Oxygen. Oxygen was measured polarographically using an oxygen electrode obtained from Rank Bros, Bottisham, Cambridge.

RESULTS AND DISCUSSION

Inhibition by menadione of the release of state 4 respiration by DNP

Tightly-coupled beef-heart mitochondria were treated with pyruvate-malate, P_i and ADP and the respiration allowed to proceed through state 3 to state 4 (phosphate acceptor limiting). At this point 80 μM menadione or 80 μM 2,3-dimethylnaphthoquinone was added, followed after varying intervals of time by DNP or ADP (Fig. 1). DNP added shortly after either quinone released the respiration, although the rates observed were less than the original state 3 rate. If, when the dimethylquinone was present, the interval before DNP addition was extended to about 15 min respiration was released to the same extent as before. However, when DNP was added 15 min after menadione no release of respiration occurred. The variation of the rate of the

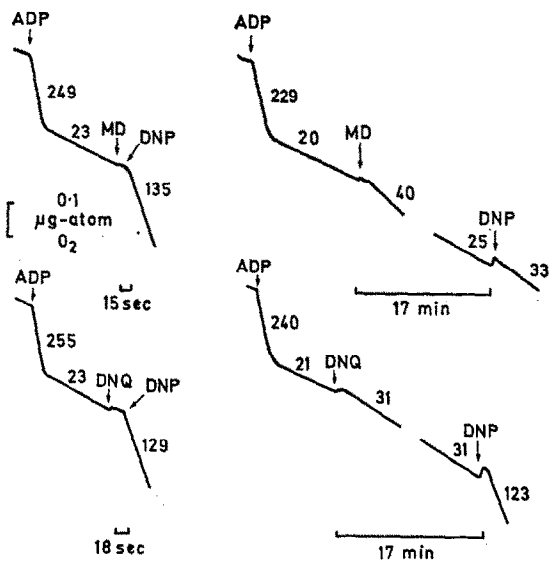

FIG. 1. The effect of menadione and 2,3-dimethylnaphthoquinone on the release of state 4 respiration by DNP. The traces are polarographic records of the variation of the oxygen content of the medium with time. Incubation mixtures at 30° contained (final concentrations) 250 mM sucrose, 50 mM tris HCl buffer (pH 7·6), 5 mM $MgCl_2$, 6 mM potassium phosphate buffer (pH 7·6), 0·3 mM sodium pyruvate, 0·3 mM tris malate, 2 mg mitochondrial protein and 1 mg bovine serum albumin/ml (total vol. 3·2 ml). Where indicated 0·16 mM ADP, 80 μM menadione (MD), 80 μM 2,3-dimethylnaphthoquinone (DNQ) or 0·1 mM DNP (all final concentrations) were added. The figures on the traces give the rate of oxygen consumption in ng-atoms/min/mg protein.

released respiration with the time elapsing before the addition of DNP is shown in Fig. 2. If phosphate acceptor, ADP, was added instead of DNP, respiration was released to the same extent as with the uncoupler.

The actual level of the released respiration in comparison to the original state 3 rate seems to depend on the quality of the mitochondria. Thus in the experiments described in Figs. 1 and 2 the respiration released by DNP after quinone treatment was only 60 per cent of the original state 3 rate, but in experiments using mitochondria with lower respiratory control ratios (2·0–2·2, as compared to 10–12) the rate of the DNP-released respiration approached that of the original state 3 rate. In all experiments the quinones had effects identical to those shown in Figs. 1 and 2.

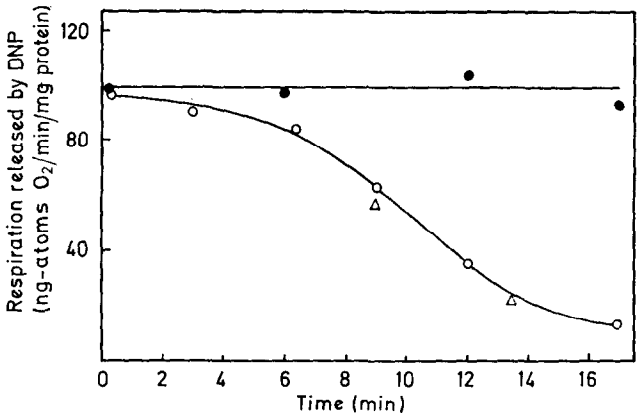

FIG. 2. Time course of the inhibition by menadione of the release of respiration by DNP. Experimental conditions as Fig. 1 with (final concentrations) 80 μM menadione (—○—) or 80 μM 2,3-dimethylnaphthoquinone (—●—). In two experiments ADP (—△—) was added to a final concentration of 0·3 mM instead of DNP. The respiration released by DNP is defined as the rate after DNP addition—the rate before.

Both quinones at first accelerated the state 4 rate (about 50 per cent) which then, in the case of menadione, slowly declined (25–30 per cent) over the course of 20–40 min, although it was still faster than the original state 4 rate. After addition of the dimethylnaphthoquinone the rate remained essentially constant. However, quinones, and particularly menadione, are known to act as mediators in pathways which bypass portions of the respiratory chain[19] and since DNP failed to release respiration after menadione treatment, there was no definite indication from the experiments depicted in Figs. 1 and 2 whether the respiration observed after menadione involved any electron-transport along the respiratory chain. If this respiration were entirely independent of the respiratory chain then there would be nothing to show whether menadione had not simply blocked electron-transport, both coupled and non-coupled. The failure of DNP to release the respiration did at least indicate that the inhibition of the state 3 respiration was not a result of a block of the entry of phosphate.

To investigate whether the respiratory chain was still functional a study was made of the sensitivity of the state 4 respiration at various times after quinone addition to cyanide and rotenone, on the assumption that the normal state 4 respiration is at least in part non-coupled respiration derived from damaged mitochondria present in the preparation.

Sensitivity of state 4 respiration to rotenone after quinone treatment

Quinones, and especially menadione, are known to function in electron-transport pathways which bypass the respiratory chain between NADH and ubiquinone.[19] The menadiol generated is reoxidized via ubiquinone and the cytochrome system (see below). However, rotenone inhibits only electron-transport associated with oxidative phosphorylation.[20] The results presented in Fig. 3 show that following the addition of

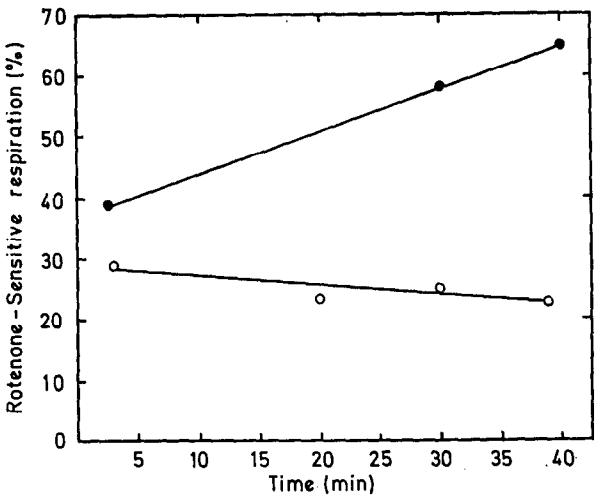

FIG. 3. Variation of the percentage rotenone-sensitivity of the state 4 respiration with the time of incubation with the quinone. Incubation mixtures as Fig. 1, except that 1·6 mg mitochondrial protein was present and rotenone was added to a final concentration of 1·5 μM in the place of DNP. 80 μM Menadione (—○—) or 80 μM 2,3-dimethylnaphthoquinone (—●—) (final concentration) was added after the transition from state 3 to state 4 respiration (as in Fig. 1).

menadione to mitochondria in state 4 the percentage of rotenone-sensitive respiration declined only slowly as the time interval before addition of rotenone was lengthened. In the case of the 2,3-dimethylnaphthoquinone the percentage of the rotenone-sensitive respiration actually increased on incubation. It should be noted that this experiment includes much longer periods of incubation than are necessary for menadione to inhibit completely the release of respiration by DNP (Fig. 2). In the 15–20 min during which the DNP release of respiration is blocked the change in the percentage of rotenone-sensitive respiration is small.

The implication of the continuing presence of rotenone-sensitive respiration is that the portion of the respiratory chain between NADH and ubiquinone has not been inhibited to any marked extent.

Sensitivity of state 4 respiration to cyanide after quinone treatment

Slater and his co-workers[21,22] have shown that menadiol donates electrons to the respiratory chain at the level of ubiquinone/cytochrome b. The respiration was cyanide-sensitive and associated with phosphorylation. These authors worked at pH 6·2 to minimize the autoxidation of menadiol, but in the present experiments, carried out at pH 7·6, it was anticipated that there would be an appreciable proportion

of cyanide-insensitive respiration. The variation of the percentage cyanide-sensitivity of the state 4 respiration with time elapsing after the addition of the quinone is shown in Fig. 4. Prior to quinone addition the state 4 respiration was 100 per cent cyanide-sensitive. As expected, after quinone addition the respiration was only partly cyanide-sensitive, but an unexpected observation was that immediately following menadione

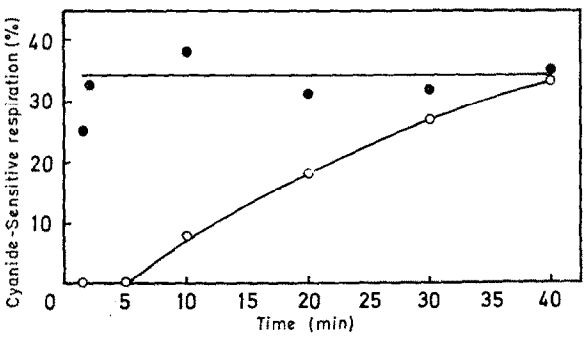

FIG. 4. Variation of the percentage cyanide-sensitivity of the state 4 respiration with the time of incubation with the quinone. Incubation mixtures as Fig. 1, except that 1·6 mg mitochondrial protein was present and KCN was added to a final concentration of 0·75 mM in place of DNP. 80 μM Menadione (—○—) or 80 μM 2,3-dimethylnaphthoquinone (—●—) (final concentration) was added after the transition from state 3 to state 4 respiration (as in Fig. 1).

addition the respiration was completely cyanide-insensitive, but became to a certain degree cyanide-sensitive on lengthening the interval before cyanide addition. No such effect was observed with the dimethylnaphthoquinone. This result was reproducible, but only with tightly-coupled mitochondria, with mitochondria showing lower respiratory control ratios (around 2·0) the respiration immediately after the addition of menadione was 25–30 per cent cyanide-sensitive.

These observations suggest that not only can both quinols bypass the cytochrome system and react directly with oxygen, but also that in the case of menadiol this may be promoted by some factor which is itself slowly inactivated by the quinone. Respiration stimulated by DNP added immediately after the menadione is much more cyanide-sensitive (60–70 per cent), suggesting that the system which promotes the cyanide-insensitive oxidation of menadiol has a very limited capacity.

The conclusion to be drawn from the presence of cyanide-sensitive state 4 respiration after lengthy incubation with menadione is that the portion of the electron-transport chain between ubiquinone and oxygen is still functional. Neither this experiment with cyanide nor the experiment with rotenone indicate whether the electron-transport chain still has the capacity to support the full state 3 respiration. The slow decline of the respiration during the incubation with menadione could be the result of a partial inhibition of electron-transport, but equally well the decline may reflect the decline of the non-respiratory chain mediated oxidation. It is notable with respect to the latter possibility that the rate of respiration immediately after menadione is always greater than that after the dimethylquinone. In any case the lack of any notable inhibition of the non-coupled state 4 respiration, as indicated by the experiments with rotenone and with cyanide, over a period of time twice that required for menadione to

block almost completely the DNP release of respiration argues that the electron-transport system still has at least partly, possibly even largely, the capacity for DNP-released respiration. Thus the evidence is that the progressive inhibition by menadione of the ability of DNP to release state 4 respiration reflects a progressive blocking of a reaction or reactions involved in coupling electron-transport to phosphorylation.

Effect of menadione and 2,3-dimethylnaphthoquinone on the energy-linked transhydrogenase in submitochondrial particles

The failure of DNP to produce any significant stimulation of respiration after incubation with menadione suggests that an early step in the reactions of ATP synthesis has been blocked. If DNP does indeed uncouple oxidative phosphorylation by promoting the breakdown of a non-phosphorylated high-energy intermediate, then the block produced by menadione lies between the respiratory chain and the high-energy intermediate which drives the energy-linked reactions of mitochondria.[23] Thus menadione should not, if it does not act at a second site, progressively inhibit the ATP-driven energy-linked reduction of $NADP^+$ by NADH. This was found to be the case. Under the experimental conditions employed, the rate of the control reaction (quinone absent) decreased with time and hence the rate of decay of the control was compared to the rate of decline of the reactions where menadione and 2,3-dimethylnaphthoquinone were present (Fig. 5). Three almost parallel straight lines resulted, indicating

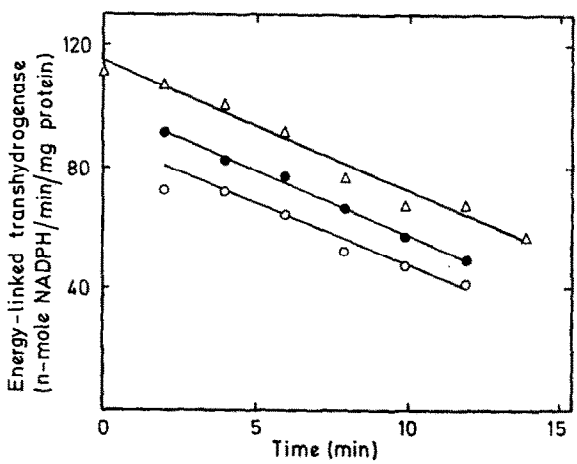

FIG. 5. Effect of menadione and 2,3-dimethylnaphthoquinone on the rate of decay of the energy-linked reduction of $NADP^+$ by NADH in submitochondrial particles. Incubation mixtures at 30° contained (final concentrations) 250 mM sucrose, 50 mM tris HCl buffer (pH 7·6), 6 mM $MgCl_2$, 20 μl ethanol, 29·4 μg crystalline yeast alcohol dehydrogenase (Böhringer), 1 mM KCN, 0·32 mg particle protein, 16 μM NAD^+, 0·47 mM $NADP^+$ and 2·4 mM ATP (total vol. 2·54 ml). After 1 min menadione (—○—) or 2,3-dimethylnaphthoquinone (—●—) was added to a final concentration of 100 μM. No addition was made to the control reaction (—△—).

that menadione had no effect which caused the rate of the reaction to decrease faster than that of the control. In some experiments the decay was somewhat accelerated by menadione, but the effect was slight compared to the rate at which state 3 respiration is inhibited in intact mitochondria. The decreased rate in the presence of either quinone compared to that of the control reflects the ability of these compounds to mediate a

cyanide-insensitive oxidation of NADH which competes with the transhydrogenase reaction.

Involvement of a thiol group in the reactions of oxidative phosphorylation

The simplest explanation of the results presented above is that menadione reacts with a thiol group essential for the reactions of energy-coupling and situated between the respiratory chain and the non-phosphorylated high-energy intermediate which drives the energy-linked reactions. However, the possibility that menadione takes part in some other reaction or catalyses some structural change not given or catalysed by 2,3-dimethylnaphthoquinone, possibly on grounds of binding specificity, cannot at present be ruled out entirely. Several examples are known where closely related quinones have very different effects at the second coupling site. Thus Howland[24] observed that the change in structure resulting from the conversion of the quinone lapachol into the dihydro derivative (side-chain reduced) converted an uncoupler of oxidative phosphorylation (lapachol) into an inhibitor (dihydrolapachol) whose block of respiration could be released by uncouplers. Similar changes in properties of quinones following limited structural modifications have been observed by Ozawa, Natori and Momose.[25] However, the effects of menadione and 2,3-dimethylnaphthoquinone on coupled respiration are very similar if tested immediately after addition, the striking difference appearing only on incubation.

The reaction of menadione with a thiol group essential for the reactions of energy-coupling remains the simplest explanation and is in accord with the conclusions of Kurup and Sanadi[10] from studies of the effect of mercurials on energy-linked reactions in submitochondrial particles. There is, however, no evidence to show whether the postulated thiol group could be that found by Lam[12] to be essential for the activity of the coupling protein, factor B.

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