The Mitochondrial Oxidation of Quinol Monophosphates

By J. M. YOUNG*

School of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K.

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1. Mitochondria from ox heart and rat liver catalysed a slow cyanide-sensitive oxidation of 2,3-dimethylnaphthaquinol monophosphate, duroquinol monophosphate, menadiol 1-phosphate and menadiol 4-phosphate. 2. The release of P_i was concomitant with oxygen uptake. 3. The oxidation was somewhat stimulated by Ca^{2+} and P_i , and weakly inhibited by 2,4-dinitrophenol. 4. The quinol monophosphates effected a rapid reduction of free cytochrome *c*, and consequently addition of cytochrome *c* greatly increased the rate of the mitochondrial oxidation of 2,3-dimethylnaphthaquinol monophosphate. 5. This quinol phosphate interacts with the electron-transport chain at the level of cytochrome *c*. 6. Polylysine promoted an interaction between 2,3-dimethylnaphthaquinol monophosphate and cytochrome oxidase. Thus, although polylysine blocks mitochondrial oxidations via reduced cytochrome *c*, the oxidation of the quinol phosphate was strongly stimulated. 7. This stimulation was most effective in the most intact mitochondrial preparations and was inhibited by ADP and by P_i . 8. The implications of these results for factors limiting the rate of quinol phosphate oxidation, the mode of action of stimulators and the mechanism of P_i formation are discussed.

Quinol monophosphates have received considerable attention as possible intermediates in oxidative phosphorylation after the demonstration that under oxidative conditions they function as phosphoryl transfer agents (Clark, Kirby & Todd, 1958; Wieland & Pattermann, 1958, 1959; Clark, Hutchinson, Kirby & Todd, 1961). The particularly attractive feature of these compounds is their obvious relationship to components of the electron-transport chain of both mammalian and bacterial systems and several theoretical schemes for oxidative phosphorylation incorporating ubiquinol monophosphate or a derivative have appeared in the literature (Chmielewska, 1960; Vilkas & Lederer, 1962; Williams, 1962; Erickson, Wagner & Folkers, 1963). A possible role for vitamin K monophosphate in photosynthetic phosphorylation had been suggested even earlier (Wessels, 1954). However, in spite of their excellent qualifications, there is little evidence for the actual involvement of quinol phosphates in any of these systems and spectroscopic studies have failed to detect any high-energy derivatives of ubiquinone (Redfearn & Pumphrey, 1960; Kröger & Klingenberg, 1966). The theoretical scheme of Vilkas & Lederer (1962), which postulates a quinone methine intermediate, has come in for particularly close scrutiny. Different groups of

workers have failed to find any evidence of proton exchange between the medium and the 2-methyl group of ubiquinone or vitamin K, which would be expected if the quinone is in equilibrium with the quinone methine (Lapidot, Silver & Samuel, 1965, 1966; Horth *et al.* 1966; Sweetman, 1966). The conclusions drawn from this type of experiment have been critically discussed by Clark & Hutchinson (1968). A little support has been given to the idea of quinol phosphates as intermediates by the isolation, in small yield, of what appears to be a phosphorylated derivative of vitamin K from incubations of vitamin K with cell-free homogenates from *Mycobacterium phlei* (Watanabe & Brodie, 1966). A brief report that ubiquinol-6 monophosphate, but not dihydrovitamin K₄ monophosphate or menadiol 4-phosphate, is oxidized by submitochondrial particles with specific transfer of the phosphoryl residue to ADP (Gruber, Höhl & Wieland, 1963) has not yet been confirmed.

In view of the considerable theoretical interest in quinol phosphates it is surprising that the effect of added quinol phosphates on mitochondria has been very little studied. Ubiquinol-6 monophosphate has been briefly reported to be oxidized slowly by mitochondrial preparations (Gruber *et al.* 1963), but ubiquinol-10 monophosphate synthesized by Shunk, McPherson & Folkers (1961) was said to show no coenzymic activity in the succinoxidase system (D. E. Green, unpublished

* Present address: Department of Pharmacology, University of Cambridge, Medical School, Cambridge CB2 3EF, U.K.

work cited by Shunk *et al.* 1961). However, fractions from *M. phlei* are reported to catalyse the oxidation of the monoethyl ester of menadiol 1-phosphate (Brodie & Davis, 1959) and the 6-chromanyl phosphate of vitamin K₁-4 (Asano, Brodie, Wagner, Wittreich & Folkers, 1962), and the oxidation of duroquinol monophosphate by horseradish peroxidase + hydrogen peroxide has been described (Bond & Mason, 1962; Wodak, 1968).

The present paper describes an investigation into the oxidation of quinol monophosphates by mitochondrial preparations. The unambiguous synthesis of ubiquinol-10 monophosphate offers some difficulty (Lapidot & Cohen, 1966), and in these studies to establish the general properties of quinol monophosphates the compounds used, DMNQP,† duroquinol monophosphate and menadiol 1-phosphate, were chosen primarily for their purity and stability. Of the three DMNQP was generally the substrate of choice, since it was oxidized at a faster rate than was duroquinol monophosphate and the 2,3-dimethylnaphthaquinone generated during the oxidation is less toxic than menadione. A preliminary communication of a part of this work has appeared (Young, 1969).

MATERIALS AND METHODS

Reagents. ADP, Antimycin A, cytochrome *c* (horse heart, type III), 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, poly-L-lysine hydrobromide (mol. wt. approx. 195000 and mol. wt. 3000) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, Pa., U.S.A., and rotenone was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Nagarse proteinase was purchased from Teikoku Chemical Industry Co. Ltd., Osaka, Japan. Tetrachlorobenzoquinol was kindly provided by Dr D. W. Hutchinson.

Quinol monophosphates. DMNQP, duroquinol monophosphate, menadiol 1-phosphate and menadiol 4-phosphate were synthesized as described by Andrews (1961). Best results were obtained by using purified quinones and freshly prepared dibenzyl phosphite. Duroquinol monophosphate and menadiol 1-phosphate had melting points (with decomposition) in agreement with those given by Andrews (1961). DMNQP, recrystallized by dissolution in warm ethyl acetate plus a few drops of ethanol followed by careful addition of a little light petroleum (b.p. 40–60°C), formed white feathery crystals, m.p. 150°C (decomp.). This product was stable on exposure to air, but impure samples turned pink under these conditions, as described by Clark *et al.* (1961). Attempts to prepare a stable sample of menadiol 4-phosphate failed, even though the best sample had m.p. 157°C (decomp.), 7°C higher than that given by Andrews (1961). A colourless solution of menadiol 4-phosphate suitable for qualitative testing was obtained

by dissolving the compound in water and filtering off the insoluble pink impurity.

The pure quinol phosphates were stored under vacuum at 4°C, under which conditions they remained stable over periods of several months.

Mitochondria. Tightly coupled ox heart mitochondria were prepared by the Nagarse proteinase procedure, essentially as described by Hatefi, Jurtshuk & Haavik (1961) except that the incubation contained 150g of minced heart tissue and 100mg of crystalline Nagarse in 400ml of cold 0.25M-sucrose. This mixture was diluted with a further 400ml of 0.25M-sucrose before homogenization. (I am grateful to Dr J. M. Haslam for details of this modification.) These mitochondria are referred to as 'Nagarse-prepared'.

For large-scale preparations of mitochondria, fresh ox heart tissue was minced and homogenized as described by Sanadi & Fluharty (1963), except that the 0.25M-sucrose medium also contained 0.25mM-EDTA. The nuclei and cell debris were removed by centrifugation at 200g for 10min and the supernatant was then further centrifuged at 12000g for 10min. The mitochondrial pellet was resuspended in the sucrose-EDTA medium, pH 7.6, and was separated into heavy and light fractions by centrifugation at 25000g (Hatefi & Lester, 1958). The heavy-layer mitochondria were suspended in 0.25M-sucrose in approx. 1mM-tris-HCl buffer, pH 7.6, and stored at -20°C. Before use the tube was thawed under running cold water, and the suspension was rehomogenized and centrifuged at 25000g. The heavy-layer mitochondria were resuspended in the sucrose-tris medium. This preparation of mitochondria is denoted 'mechanically prepared'. The oxidation of pyruvate + malate by these mitochondria was normally accelerated by the addition of ADP (P/O ratio > 2, with a glucose-hexokinase trapping system for ATP), but there was no return transition from state 3 to state 4 respiration (Chance & Williams, 1956).

Phosphorylating submitochondrial particles were prepared from mechanically prepared mitochondria by the method of Hansen & Smith (1964) as described by Griffiths & Robertson (1966) except that MnCl₂ was omitted from the medium. The final pellet was suspended in 0.25M-sucrose-4mM-MgCl₂-25mM-tris-HCl medium, pH 7.6, and used within a few hours.

Rat liver mitochondria were prepared by the method of Hogeboom (1955) essentially as described by Haslam & Krebs (1968).

Cytochrome oxidase. Cytochrome oxidase was isolated by the method of Sun & Jacobs (1967) starting from mechanically prepared mitochondria. The final suspension contained 10mg of protein/ml (biuret reaction, uncorrected for haem absorption).

Protein. Protein concentrations were determined by the biuret reaction (Gornall, Bardawill & David, 1949) after solubilization with sodium deoxycholate (0.25%, w/v). Dried bovine serum albumin was used as standard.

Oxygen. Oxygen was measured polarographically by using an oxygen electrode obtained from Rank Bros. Bottisham, Cambs., U.K. The incubation medium used for the mitochondrial oxidation of DMNQP contained 0.465μg-atom of oxygen/ml at 30°C (Haslam, 1967).

Determination of P_i formed during DMNQP oxidation. Mechanically prepared mitochondria were preincubated at 30°C for 40min in a medium containing 250mM-

† Abbreviation: DMNQP, 2,3-dimethylnaphthaquinol monophosphate.

sucrose, 50mM-tris-HCl buffer, pH 7.6, 5mM-MgCl₂ and 1mg of bovine serum albumin/ml (total vol. 30ml) in a syringe-repette (Jencons Ltd., Hemel Hempstead, Herts., U.K.) into which a Clark electrode had been inserted (Garland, Shepherd & Yates, 1965). Oxidation was initiated by injection of DMNQP. Samples (2.0ml) were ejected at intervals into ice-cold 0.9M-HClO₄-0.6M-Na₂SO₄ (0.4ml). The protein was removed by centrifugation and the supernatant was assayed for P_i by using an adaptation of the 2-methylpropan-1-ol-benzene extraction procedure (Berenblum & Chain, 1938; Martin & Doty, 1949; Weil-Malherbe & Green, 1951). A 1.0ml portion was treated with water (4.0ml), 2-methylpropan-1-ol-benzene (1:1, v/v, saturated with water) (6.0ml) and finally acid molybdate [5% (w/v) (NH₄)₆Mo₇O₂₄·4H₂O in 2M-H₂SO₄] (1.0ml), and shaken for 15s. The organic layer was separated and washed once with 0.5M-H₂SO₄ (5.0ml) and a 2.0ml portion was treated with acid-ethanol (conc. H₂SO₄-ethanol, 1:49, v/v) (2.0ml) and SnCl₂ (prepared as described by Weilmalherbe & Green, 1951) (0.2ml). The extinction at 650nm was measured in a Bausch and Lomb Spectronic spectrophotometer. The assay was calibrated by adding known amounts of P_i to preincubated mitochondria and carrying out the procedure above.

RESULTS

The oxidation of DMNQP, duroquinol monophosphate and menadiol 1-phosphate was catalysed by all the mitochondrial preparations investigated. The rates of oxidation of these three compounds and of pyruvate+malate by ox heart mitochondria are given in Table 1. Menadiol 4-phosphate behaved similarly to the 1-phosphate (reservations as to the purity of the former were noted above). The rate of oxidation of DMNQP, which was used most extensively as substrate, tended to vary from preparation to preparation and that quoted in Table 1 was among the fastest observed. Nagarse-prepared mitochondria were used in this particular experiment, but equally fast rates were obtained with mechanically prepared frozen mitochondria, although in the latter case a lengthy preincubation was often necessary to minimize the endogenous respiration. In general, however, the mechanically prepared mitochondria gave the fastest rates, but the relatively low oxygen-uptake rate coupled with the variations from preparation to preparation made a strict comparison difficult. In an experiment where a comparison was made of the catalytic activity of the heavy and light fractions obtained on centrifuging mechanically prepared mitochondria, the heavy fraction was a little less active than the light (8.2 and 10.9 ng-atoms of oxygen consumed/min per mg of protein respectively). A Lineweaver-Burk plot for the oxidation of DMNQP by the heavy fraction (Fig. 1) yielded a K_m value of 1.1 mM. Rat liver mitochondria gave rates similar to those obtained with ox heart mitochondria.

Table 1. *Rates of oxidation of quinol monophosphates by ox heart mitochondria and effect of inhibitors*

(a) Rates of oxidation. Incubation mixtures at 30°C contained (final concentrations) 250mM-sucrose, 50mM-tris-HCl buffer, pH 7.6, 5mM-MgCl₂, 6mM-potassium phosphate buffer, pH 7.6, 1μmol of ADP, 1mg of bovine serum albumin/ml, 1.9mg of mitochondrial protein (Nagarse-prepared mitochondria) and 1.6mM-quinol monophosphate or 3mM-sodium pyruvate+3mM-tris malate (total vol. 3.4ml).

(b) Effect of inhibitors. The rates are taken from a number of experiments carried out with mechanically prepared mitochondria. Incubation mixtures were as described above with 9-11 mg of mitochondrial protein and 3.0-4.3mM-DMNQP.

Substrate	Rate	
	(ng-atoms of O/min per mg of protein)	
None	3	
Duroquinol monophosphate	10	
DMNQP	16	
Menadiol 1-phosphate	16	
Pyruvate+malate	185	

Inhibitor	Inhibition (%)	
Rotenone (1.4 μM)	15	
Antimycin A (3 μg/mg of protein)	4	
2- <i>n</i> -Heptyl-4-hydroxyquinoline <i>N</i> -oxide (0.28 mM)	26	
KCN (0.7 mM)	95	
Dinitrophenol (0.1 mM)	4-26	

On occasions, and particularly at a later stage of the investigation, very low rates were observed, even though there was no apparent change either in the state of the quinol phosphate or in the properties of the mitochondria. However, the rates of the strongly stimulated oxidations described below remained relatively constant.

The rate of DMNQP oxidation catalysed by particles obtained after sonic disruption of the mitochondria was always low (approx. 5 ng-atoms of oxygen consumed/min per mg of protein), and there was no significant difference between particles efficient at catalysing the ATP-driven reduction of NAD⁺ by succinate and those only weakly active.

The effect of mitochondrial inhibitors on DMNQP oxidation is shown in Table 1. Only cyanide inhibited strongly, indicating that the major interaction between DMNQP and the respiratory chain occurs beyond the antimycin A site. A moderate inhibition was generally produced by 0.1mM-2,4-dinitrophenol, but the extent of this inhibition varied considerably and, as the range shown in Table 1 indicates, in some cases was practically zero. It may be noted here that DMNQP itself was only a weak inhibitor of

Fig. 1. Lineweaver-Burk plot of the kinetic data from the oxidation of DMNQP by ox heart mitochondria. (a) Unpromoted oxidation. Incubation mixtures at 30°C contained (final concentrations) 250mM-sucrose, 50mM-tris-HCl buffer, pH 7.6, 5mM-MgCl₂, 6mM-potassium phosphate buffer, pH 7.6, 0.5 μ mol of ADP, 1 mg of bovine serum albumin/ml, 9.2mg of mitochondrial protein (mechanically prepared mitochondria) and 0.77–4.33mM-DMNQP (total vol. 3.5–3.8 ml). (b) Cytochrome *c*-mediated oxidation. DMNQP (0.2–5mM), 2.5mg of mitochondrial protein (mechanically prepared mitochondria) and 5 (Δ), 10 ($\bullet$) or 25 ($\circ$) nmol of cytochrome *c* were incubated at 30°C in a medium containing (final concentrations) 260mM-sucrose and 50mM-tris-HCl buffer, pH 7.6 (total vol. 3.2 ml). Oxygen uptake (*v*) is expressed as ng-atoms/min per mg of protein.

pyruvate+malate or succinate oxidation catalysed by mechanically prepared mitochondria.

The occurrence of oxygen uptake does not by itself reveal whether it is the quinol phosphate that is the substrate for the oxidation, or whether it is first degraded by a phosphatase to yield the quinol that is the true donor of electrons to the respiratory chain. The insensitivity of DMNQP oxidation to antimycin A speaks against the latter possibility, but a test was made for phosphatase activity by following the concentration of P_i in the medium when oxygen uptake was blocked, either by addition of cyanide (Table 2, Expt. A) or on anaerobiosis (Table 2, Expt. B). The amount of P_i present

remained constant, i.e. there was no measurable quinol phosphatase activity. The addition of dinitrophenol also failed to induce any P_i release. Dinitrophenol is well known to promote the hydrolysis of ATP, possibly by catalysing the breakdown of a non-phosphorylated high-energy intermediate or state and hence stimulating the reverse reactions of the ATP-synthesizing pathway. Thus if a quinol phosphate were present in mitochondria as an intermediate between electron transfer and ATP, and if DMNQP were a good model for it, then the breakdown of DMNQP should also be promoted by dinitrophenol. However, the condition that DMNQP must be a good model clearly prevents any conclusions as to the presence or absence of quinol phosphates in mitochondria being drawn from the negative result.

Expts. A and B in Table 2 give values for P_i formed and oxygen consumed and the ratio mol of P_i formed/g-atoms of oxygen consumed can therefore be calculated. However, the assay of P_i in the presence of quinol monophosphate presents some difficulty and the absolute values quoted for P_i must be treated with some reserve. Walters & Cooper (1965) have drawn attention to the errors introduced into the 2-methylpropan-1-ol-benzene extraction procedure by the presence of agents, in the present case DMNQP, that can effect a premature reduction of the phosphomolybdate complex, since the reduced complex is more water-soluble and may be retained in the aqueous phase. However, when DMNQP functions as the reducing agent, i.e. is itself oxidized, then more P_i is generated which more than compensates for some loss of the reduced complex. Thus when a sample containing 0.2 μ mol of P_i was assayed in the presence of 1.35 μ mol of DMNQP, 0.3 μ mol of P_i was actually found, i.e. a 50% excess. A similar effect was observed with samples taken immediately before and after DMNQP additions to the incubation mixtures described in Table 2. The values in Table 2 for P_i formed have been corrected for this effect by subtracting the result of the assay on a sample taken immediately after DMNQP addition, the starting point for calculating ΔP_i and Δ oxygen thus being taken from this sample.

This procedure is open to the objections that the amount of P_i excess may be a function of the concentration of P_i , that of DMNQP, or both, and also that any effect of the quinone generated during the oxidation is ignored. To check this without resorting to a lengthy series of control experiments, the P_i formed was assayed at intervals during the oxidation of DMNQP (Table 2, Expt. C). The values obtained for the ratio mol of P_i formed/g-atoms of oxygen consumed are reasonably consistent, but there may be a slight tendency for an increase with increasing P_i .

Table 2. *Dependence of P_i formation on oxidation by ox heart mitochondria*

The experimental procedure is described in the Materials and Methods section. The rates of oxidation have been corrected for the endogenous respiration. Expt. A was carried out with 2.8 mg of mitochondrial protein/ml (endogenous respiration after preincubation 0.3 ng-atoms of oxygen/min per mg of protein) and 2.3 mM-DMNQP (rate of oxygen uptake 11.5 ng-atoms/min per mg of protein). KCN was added at 30% saturation of the medium with oxygen to a final concentration of 1.1 mM (inhibition of oxygen uptake 95%). In Expt. B 2.6 mg of mitochondrial protein/ml (endogenous respiration after preincubation 1.2 ng-atoms of oxygen/min per mg of protein) and 2.0 mM-DMNQP (rate of oxygen uptake 17 ng-atoms/min per mg of protein) were present. Dinitrophenol was added to a final concentration of 0.12 mM. Expt. C was carried out with 2.4 mg of mitochondrial protein/ml (endogenous respiration 1.2 ng-atoms of oxygen/min per mg of protein) and 2.5 mM-DMNQP (rate of oxygen uptake 12 ng-atoms/min per mg of protein).

Expt. A: oxygen uptake terminated by addition of cyanide

	Incubation time (min:s)	P _i formed (μmol/ml)	O ₂ consumed (μg-atom/ml)
	0	0	0
	8:15	0.20	0.25
KCN added	9:00		
	9:45	0.27	0.33
	15:10	0.27	0.34
	21:00	0.26	0.35

Expt. B: cessation of oxidation on anaerobiosis

	Incubation time (min:s)	P _i formed (μmol/ml)	O ₂ consumed (μg-atom/ml)
	0	0	0
Anaerobic	9:30		
	10:15	0.24	0.40
	19:00	0.25	0.40
Dinitrophenol added	21:00		
	22:00	0.25	0.40
	31:30	0.24	0.40

Expt. C: no additions

% satn. of medium with O ₂	P _i formed (μmol/ml)	O ₂ consumed (μg-atom/ml)	P _i formed (mol) O ₂ consumed (g-atoms)
79	0	0	
61	0.06	0.08	0.72
43	0.11	0.16	0.68
25	0.19	0.23	0.83
7	0.24	0.30	0.81
0	0.27	0.33	0.81

The results from this experiment may be evaluated in a slightly different way. If ΔP_i and Δ oxygen are calculated between 61% oxygen saturation and 0% oxygen saturation, instead of between 79 and 0%, then, since the sample taken at 61% contains more P_i and more quinone than that at 79%, and if these factors do affect the value of P_i found, the new value obtained for ΔP_i should be a better estimate of the true amount of P_i formed. Extending this argument, ΔP_i between 43 and 0% oxygen saturation should be still more accurate, and ΔP_i between 25 and 0% and between 7 and 0% oxygen saturation progressively better. If, however, the P_i assay is not greatly affected by either of these factors, then the value observed for the ratio should remain nearly constant. The actual values obtained (including the value quoted in Table 2)

were 0.81, 0.84, 0.93, 0.78 and 0.91 respectively. There may be a small increase along the series, but the agreement is reasonably good and the values obtained are consistently less than unity. Possible mechanistic implications of this observation are discussed below.

The relatively low rate of the mitochondrial oxidation of DMNQP prompted a search for factors that would stimulate the rate. A moderate or slight increase in the rate of the oxidation was produced by most of the compounds listed in Table 3. Of the ions, only Ca²⁺ and P_i caused a marked stimulation. Addition of ADP led to a slight decrease in the rate of the oxidation, and even in combination with P_i the increase that resulted was less than that produced by P_i alone. Triton X-100 also produced a small stimulation, which was slightly increased by

Table 3. *Factors with a slight or moderate influence on the rate of the mitochondrial oxidation of DMNQP*

Incubations contained (final concentrations) 250mm-sucrose, 50mm-tris-HCl buffer, pH 7.6, 9–11mg of mitochondrial protein (mechanically prepared mitochondria) and 1.65mm-DMNQP (except in the experiment with ADP, where 3.2mm-DMNQP was present).

Addition	Stimulation of rate of DMNQP oxidation (%)
Ca ²⁺ (2mm)	70
Potassium phosphate, pH 7.6 (12.5mm)	43
ADP (0.4mm)	-12
ADP (0.4mm) + P _i (6mm)	17
Mg ²⁺ (6mm)	12
Triton X-100 (0.05%)	12

using higher concentrations (0.5%, w/v) of the detergent.

In contrast with these relatively small effects addition of small amounts of cytochrome *c* to the mitochondria promoted a rapid cyanide-sensitive oxidation of the quinol phosphate. Under the conditions given in Table 5, 10nmol of cytochrome *c* increased the rate of DMNQP oxidation to 52ng-atoms of oxygen consumed/min per mg of protein. A Lineweaver-Burk plot of this stimulated oxidation (Fig. 1) gave a K_m value of 0.38mm.

A strong indication of the pathway of the rapid oxidation was obtained when DMNQP and cytochrome *c* were mixed in the absence of mitochondria. A rapid reduction of the cytochrome ensued. The other quinol monophosphates also caused a rapid reduction of free cytochrome *c*, but it is noteworthy that two naphthaquinol diphosphates tested, menadiol diphosphate and 2,3-dimethylnaphthaquinol diphosphate, were without effect. Thus the effective mediation of DMNQP oxidation appears to depend on (a) the non-enzymic reduction of the added cytochrome *c* and (b) the reoxidation of the reduced cytochrome *c* via cytochrome oxidase. This also implies that in normal circumstances DMNQP can penetrate to the appropriate site on the bound cytochrome *c* only with difficulty.

A comparison of the stimulation produced by cytochrome *c* with mitochondrial preparations of different degrees of intactness (the rates obtained are given in Table 7) introduces just a little doubt whether the mechanism of the stimulation by cytochrome *c* is quite so straightforward. The most effective stimulation was observed with the mitochondrial particles and the K_m determined for this reaction had fallen to 0.18mm, in comparison with the value of 0.38mm obtained with the mechanically

prepared mitochondria. However, Chance, Lee & Schoener (1966) have observed that ultrasonically prepared particles from pigeon heart cannot oxidize exogenous cytochrome *c*, and Lenaz & MacLennan (1966), by using submitochondrial particles prepared by the same method as used in the present study, have also reported an ineffective interaction between exogenous cytochrome *c* and the electron-transport chain.

Cytochrome *c* was least effective in stimulating DMNQP oxidation by Nagarse-prepared mitochondria, but in this experiment, which was designed primarily to demonstrate the differing effects of cytochrome *c* and polylysine, no P_i or phosphate acceptor was present. However, the addition of dinitrophenol produced only a 10% stimulation. It may be added that with these mitochondria 1mm-Ca²⁺ and 1.5mm-potassium chloride + 1.3μg of valinomycin had no effect on DMNQP oxidation.

The low rate of the unstimulated mitochondrial oxidation of the quinol monophosphate makes a sharp contrast with the rapid reaction with free cytochrome *c* and suggests that the relatively low lipid-solubility of DMNQP makes penetration to the appropriate site on the bound cytochrome *c* difficult. However, it cannot be assumed that in the unstimulated oxidation the electrons from DMNQP enter the respiratory chain at the level of cytochrome *c*. Confirmation was sought by observing the effect of DMNQP addition on the redox state of cytochrome *b* and cytochromes *c* + *c*₁ in cyanide-treated mitochondria. The traces in Fig. 2 demonstrate that DMNQP is unable to reduce cytochrome *b* but caused a reduction of cytochromes *c* + *c*₁. However, in four independent experiments the extent of the reduction of cytochromes *c* + *c*₁ by DMNQP was only 71–78% of that produced by pyruvate + malate. In contrast, pyruvate + malate caused no increase in the extent of the reduction of cytochromes *c* + *c*₁ produced by *NNN'*N'-tetramethyl-*p*-phenylenediamine + ascorbate. The explanation of this effect and its relevance to DMNQP oxidation are at present uncertain. It is tempting to suppose that it could represent a selective reduction of cytochrome *c*, but there is no evidence to support such an interpretation.

The mechanically prepared mitochondria required lengthy preincubation, normally about 60min, to deplete the endogenous substrates and thus allow accurate measurements of the effect of DMNQP on the redox state of cytochromes *c* + *c*₁. However, measurements made at earlier times showed the same effects as those shown in Fig. 2, except that the rate after cyanide addition was much greater. Cytochrome *b* was not reduced by the endogenous substrates and in this case the shortest possible delay before addition of the

Fig. 2. Effect of DMNQP on the redox state of cytochromes $c+c_1$ and cytochrome b . Incubation mixtures contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, 9.5 mg of mitochondrial protein (mechanically prepared mitochondria), 0.8 mM-KCN and 1.6 mM-DMNQP or 0.3 mM-sodium pyruvate+0.3 mM-tris malate or $3 \mu\text{M}$ -tetramethylphenylenediamine+0.9 mM-ascorbate. Cytochromes $c+c_1$ reduction was measured at wavelength-pair 550–540 nm and cytochrome b reduction at 564–575 nm with an Aminco-Chance dual-wavelength spectrophotometer. Abbreviation: TMPD, $NNN'N'$ tetramethyl- p -phenylenediamine.

Table 4. Stimulation by cytochrome c and by polylysine of the oxidation of DMNQP by a cytochrome oxidase preparation

Incubation mixtures at 30°C contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, and 1 mg of cytochrome oxidase (total vol. 3.2–3.4 ml).

Substrate	Rate (ng-atoms of O/min)	Addition	Rate (ng-atoms of O/min)	Addition	Rate (ng-atoms of O/min)
DMNQP (1.75 mM)	0	Polylysine (1 mg)	112	Cytochrome c (10 nmol)	112
DMNQP (1.75 mM)	0	Cytochrome c (10 nmol)	690	Polylysine (1 mg)	125
Tetrachlorobenzoquinone (0.7 mM)+ascorbate (9 mM)	75	Polylysine (1 mg)	192		
Tetramethylphenylenediamine (30 μM)+ascorbate (9 mM)	19*	Cytochrome c (50 nmol)	253	Polylysine (1 mg)	24

* Cyanide-insensitive.

DMNQP was desirable, since the ability of pyruvate + malate to reduce cytochrome b was progressively lost on extending the time of the incubation. Thus DMNQP enters the respiratory chain at the level of cytochrome c , consistent with the ineffectiveness of antimycin A as an inhibitor. However, there was some reason for investigating whether DMNQP

might not also interact with cytochrome oxidase. Conversion of a quinol into a quinol phosphate results in a considerable raising of the oxidation potential. Chambers & Chambers (1966) observed that the electrochemical oxidation of menadiol 1-phosphate at pH 7.44 and 24°C commenced at 0.35 V (with respect to the hydrogen electrode).

Table 5. *Effect of polylysine on mitochondrial oxidations*

Incubation mixtures at 30°C contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, 3.6 mg of mitochondrial protein (mechanically prepared mitochondria), 0.9 mM-DMNQP or 0.7 mM-tetrachlorobenzoquinone + 9 mM-ascorbate or 3 mM-sodium pyruvate + 3 mM-tris malate or 3 mM-sodium succinate or 30 μ M-tetramethylphenylenediamine + 9 mM-ascorbate and, after a short interval, 0.5 mg of polylysine (total vol. 3.2–3.45 ml). Where pyruvate + malate, succinate and tetramethylphenylenediamine + ascorbate served as substrates 6 mM-potassium phosphate and 0.3 mM-ADP were also present.

Substrate	Rate of oxidation (ng-atoms of O/min per mg of protein)	Rate after polylysine (ng-atoms of O/min per mg of protein)
DMNQP	2	53.5
Tetrachlorobenzoquinone + ascorbate	34	58
Pyruvate + malate	53	0
Succinate	70	3.5
Tetramethylphenylenediamine + ascorbate	56	6

If the assumption can be made that the difference between the potential at which the oxidation of DMNQP and menadiol 1-phosphate occurs is equal to the difference between the redox potentials of 2,3-dimethylnaphthaquinone and menadione, then DMNQP oxidation should commence at about 0.29 V, i.e. in the cytochrome oxidase region of the respiratory chain.

To test whether the second interaction occurred, DMNQP was incubated with cytochrome oxidase isolated by the method of Sun & Jacobs (1967), but with negative results, although occasionally a very slow oxygen uptake was observed. However, on addition of polylysine a rapid cyanide-sensitive oxidation ensued (Table 4). No oxygen uptake occurred in control experiments with the oxidase + polylysine or with polylysine + DMNQP.

Polylysine has been known for some time to be an inhibitor of normal mitochondrial oxidations, acting at the level of cytochrome oxidase (Person & Fine, 1961). However, an investigation of the effect of polylysine was suggested by the observation of Jacobs, Andrews & Crane (1965) that it promoted changes in cytochrome oxidase that led to a stimulation of the oxidation of tetrahalobenzoquinols. The oxidase preparation used for the experiments in Table 4 catalysed the oxidation of tetrachlorobenzoquinol + ascorbate at an appreciable rate without the addition of polylysine, but the polycation did produce a substantial stimulation.

That polylysine prevents oxidations via reduced cytochrome *c* is clearly shown by the effect on DMNQP oxidation of added cytochrome *c* in the presence and absence of the polycation (Table 4). Not surprisingly, cytochrome *c* is an extremely effective mediator of the oxidation, since it is rapidly reduced by the quinol phosphate and then reoxidized by the cytochrome oxidase. Polylysine did not affect the direct reduction of cytochrome *c* by DMNQP, but addition of cytochrome *c* to the

Fig. 3. Variation of the rate of DMNQP oxidation by ox heart mitochondria with the amount of polylysine. Incubation mixtures at 30°C contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, 1.8 mM-DMNQP, 2.3 (○) or 4.6 (●) mg of mitochondrial protein (mechanically prepared mitochondria) and 0–2.0 mg of polylysine (total vol. 3.15–3.4 ml).

oxidation already stimulated by polylysine did not increase the rate even though this amount of cytochrome *c* in the absence of polylysine gave a rate six times as fast. Conversely, addition of polylysine to the very fast oxidation stimulated by cytochrome *c* decreased the rate to the value obtained with polylysine alone. A further demonstration that the pathway via reduced cytochrome *c* is blocked was given by the strong inhibition of the oxidation of tetramethylphenylenediamine + ascorbate + cytochrome *c*, the oxygen uptake remaining after polylysine addition being almost entirely cyanide-insensitive.

The same pattern of results as with the isolated oxidase was obtained with mechanically prepared mitochondria (Table 5). In this experiment the stimulation of DMNQP oxidation was particularly striking, since only very low rates were observed for the unpromoted oxidation. The results in Table 5 also demonstrate that the same concentration of polylysine almost completely blocked the oxidation of the usual mitochondrial substrates.

The variation of the rate of DMNQP oxidation with the amount of polylysine present is shown in Fig. 3. When additions of polylysine are made the total amount required must be added in as short a time as possible. Sequential additions of smaller amounts with a pause between each addition did not produce as high a rate as a single addition of the total amount. This may possibly be a result of the aggregation of the mitochondria that polylysine causes, a portion of the original membrane surface

thus becoming less available to further amounts of the reagent. The order of addition of the polylysine and the DMNQP did not affect the rate of the oxidation. The maximum stimulation of DMNQP oxidation was obtained with 0.15–0.2mg of polylysine/mg of mitochondrial protein.

The variation of the rate of oxidation with DMNQP concentration in the presence of a fixed, and in this case suboptimum, amount of polylysine is shown in Fig. 4. Under the conditions of this experiment, increasing the concentration of DMNQP beyond 1 mM led to a decrease in the rate. With optimum amounts of polylysine the decrease in the rate occurs with concentrations of DMNQP greater than 2 mM.

The effects of various compounds on the polylysine-stimulated oxidation of DMNQP are shown in Table 6. The oxidation was strongly inhibited by cyanide but scarcely at all by antimycin A, consistent with an interaction between DMNQP and the cytochrome oxidase complex. Both ADP and P_i were inhibitory, particularly ADP, but acetate was without effect.

It became clear during the experiments with the isolated cytochrome oxidase that the most marked stimulation by polylysine was obtained with oxidase preparations derived from the most intact preparations of mitochondria used as starting material. However, preparations that showed only a very weak polylysine-stimulated DMNQP oxidase activity were still effective catalysts of DMNQP oxidation mediated by cytochrome *c*. This suggested that the oxidase must be reasonably intact for the most effective catalytic activity in the polylysine-DMNQP system. This proposition was supported by a comparison of the effectiveness of catalysis of the polylysine-stimulated oxidation by Nagarse-prepared mitochondria, by mechanically prepared mitochondria and by submitochondrial particles (Table 7). The more intact the preparation the faster was the rate obtained. This contrasted with the efficiency of cytochrome *c* mediation of DMNQP oxidation, which was in the reverse order. It was also clear that marked inhibition by ADP is confined to the polylysine-stimulated oxidations. The values of 12, 2.5 and 11% inhibition of the cytochrome *c*-mediated oxidation may be compared with the 12% inhibition of the unstimulated mitochondrial oxidation of DMNQP noted in Table 3.

The most effective stimulation of tetrachloro-benzoquinol oxidation is produced by polycations with mol. wt. 5000 (Jacobs *et al.* 1965). The rates of DMNQP oxidation produced by polylysine of mol.wt. 3000 and mol.wt. 195000 were 31 and 53.5 ng-atoms of oxygen consumed/min per mg of protein respectively and demonstrate the greater efficiency of the higher-molecular-weight material.

Fig. 4. Variation of the rate of DMNQP oxidation by ox heart mitochondria with the concentration of DMNQP. Incubation mixtures at 30°C contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, 4.6 mg of mitochondrial protein (mechanically prepared mitochondria), 0.18–3.34 mM-DMNQP and 0.25 mg of polylysine.

Table 6. *Inhibition of the polylysine-stimulated mitochondrial oxidation of DMNQP*

The inhibitors were added to incubation mixtures containing (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, 3.6 mg of mitochondrial protein (mechanically prepared mitochondria), 0.9 mM-DMNQP and 0.5 mg of polylysine.

Addition	Inhibition (%)
KCN (0.7 mM)	100
Antimycin A (2.5 µg/mg of protein)	3
ADP (0.15 mM)	24
ADP (0.3 mM)	40
ADP (0.7 mM)	65
Potassium phosphate, pH 7.6 (6 mM)	40
Sodium acetate (6 mM)	0

Table 7. Comparison of cytochrome *c* and polylysine as stimulators of DMNQP oxidation by mitochondrial preparations of differing structural integrity

Incubation mixtures contained (final concentrations) 250 mM-sucrose, 50 mM-tris-HCl buffer, pH 7.6, and 0.9 mM-DMNQP. Either polylysine sufficient to give the maximal stimulation or 10 nmol of cytochrome *c* was added, followed by 1 μ mol of ADP (final concentration 0.3 mM).

Mitochondrial preparation	Rate of cytochrome <i>c</i> -stimulated oxidation (ng-atoms of O/min per mg of protein)	Inhibition by ADP (%)	Rate of polylysine-stimulated oxidation (ng-atoms of O/min per mg of protein)	Inhibition by ADP (%)
Tightly coupled	28	12	124	34
Nagarse-prepared mitochondria				
Mechanically prepared mitochondria showing no respiratory control	70	2.5	72	40
Submitochondrial particles	145	11	57	33

Fig. 5. Influence of polylysine on the reduction of cytochromes *b*, *c*+*c*₁ and *a*. Conditions were as given in Fig. 2, except that the final concentration of DMNQP was 0.16 mM and 1.0 mg of polylysine was present. Cytochrome *a* reduction was measured at wavelength-pair 605–630 nm. Abbreviation: TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

Thus the properties of the polylysine-stimulated DMNQP oxidation are again in accord with those of the polylysine-stimulated tetrachlorobenzoquinol oxidation.

The experiments with the isolated cytochrome oxidase provided evidence that in the presence of polylysine DMNQP interacts directly with the oxidase complex, but it is also of interest to know whether polylysine influences the interaction of DMNQP with other respiratory carriers and

whether these carriers can still be reduced by pyruvate+malate. The traces in Fig. 5 show the changes in the redox state of cytochrome *b*, cytochromes *c*+*c*₁ and cytochrome *a* after the addition of DMNQP, pyruvate+malate or tetramethylphenylenediamine+ascorbate to mitochondria pretreated with polylysine and cyanide. With cytochrome *b* the situation remained essentially as it was in the absence of polylysine. DMNQP is still unable to effect any reduction, and that produced by pyruvate+malate is unaffected. However, there is a much greater rate of reduction of cytochrome *b* by endogenous substrates after polylysine addition than there was after cyanide addition in Fig. 2. This suggests that polylysine may disrupt the respiratory chain immediately beyond this point and, in confirmation, under these conditions cytochromes *c*+*c*₁ are only slowly reduced by pyruvate+malate. However, in the presence of polylysine DMNQP effected a rapid and complete reduction of cytochromes *c*+*c*₁, in contrast with the maximal 75% reduction obtained previously. It seems unlikely that this effect can contribute to the mechanism of the stimulated oxidation of DMNQP, since in the presence of polylysine the oxidation of reduced cytochrome *c* is blocked. Cytochrome *a* was also rapidly reduced by DMNQP in the presence of polylysine, but tetramethylphenylenediamine+ascorbate was also able to reduce this cytochrome under these conditions. The bearing of this last observation on the pathway of polylysine-stimulated oxidations is discussed below.

DISCUSSION

Stimulation of the mitochondrial oxidation of DMNQP. The mitochondrial oxidation of quinol monophosphates is slow, although these compounds react rapidly with exogenous cytochrome *c*. Thus stimulators of the mitochondrial oxidation may be expected to act either by mediating electron

transfer between the quinol phosphate and the respiratory chain or by promoting the penetration of the quinol phosphate to one of the respiratory carriers.

Cytochrome *c* clearly falls into the former category, although the perhaps unexpected feature that it is most efficient with submitochondrial particles has been noted above. Lenaz & MacLennan (1966) have reported that in these particles the endogenous cytochrome *c* is relatively inaccessible to exogenous cytochrome *c*, although with larger amounts of the exogenous cytochrome, comparable with those used in the present study, there was some interaction. However, the possibility must be borne in mind that in the stimulated quinol monophosphate oxidation the quinol phosphate or the cytochrome *c*, or both, may be performing some additional function.

None of the remaining stimulators can function as an electron carrier and they must therefore act by promoting in some way the penetration of the quinol phosphate to the bound carriers. The stimulation of DMNQP oxidation by P_i is reminiscent of the requirement for P_i for the mitochondrial penetration of certain anions (Chappell & Haahrhoff, 1967) and the effects of Ca^{2+} and dinitrophenol recall the stimulation of succinate uptake by Ca^{2+} and its inhibition by dinitrophenol (Quagliariello & Palmieri, 1968). On the other hand, the rate of DMNQP oxidation was not notably improved by using mitochondria that had been frozen and thawed, although the aging of mitochondria generally results in easier anion penetration, e.g. under these conditions citrate can enter without a requirement for malate (Chappell & Haahrhoff, 1967). However, the effects of Ca^{2+} and P_i are relatively small and the low rate of DMNQP oxidation has discouraged a more detailed investigation of their mechanism of action.

It may well be that the penetration of DMNQP is limited by poor lipid solubility and hence the effect, again small, of Triton X-100. It would be interesting to observe whether ubiquinol-10 monophosphate with its long hydrophobic side chain undergoes a more rapid oxidation.

Stimulation of DMNQP oxidation by polylysine. The striking stimulation of DMNQP oxidation produced by polylysine results from the promotion of a direct interaction between DMNQP and some component of the cytochrome oxidase complex, presumably as a result of structural or organizational changes in the oxidase. It is possible that polylysine plays the same role here as in the cytochrome oxidase model system of Wang & Brinigar (1960). That the polycation produces changes in the oxidase complex has been known for some time, since it blocks the normal substrate oxidations proceeding via reduced cytochrome *c* (Person &

Fine, 1961) but stimulates the oxidation of tetrahalobenzoquinols (Machinist, Crane & Jacobs, 1965; Jacobs *et al.* 1965). The increase in tetra-chlorobenzoquinol oxidase activity mirrors the decline in cytochrome *c* oxidase activity (Machinist *et al.* 1965). However, the ability of DMNQP to serve as a substrate indicates that polylysine-stimulated oxidations are not as specific for tetrahalobenzoquinols as has been supposed (Machinist *et al.* 1965; Jacobs *et al.* 1965; Jacobs, Andrews, Wohlrab & Cunningham, 1968). What properties a compound must possess to serve as an electron donor in the polylysine-cytochrome oxidase system are not clear. A high redox potential is not alone sufficient, since dichlorodicyanobenzoquinol is ineffective (Machinist *et al.* 1965), and the ability of DMNQP to serve as an electron donor further indicates that high lipid-solubility is not a prerequisite.

Polylysine is most effective at promoting the oxidation of DMNQP in the most intact mitochondrial preparations, indicating that this is a property that depends on the cytochrome oxidase being structurally intact. Further, the polycation does not prevent the reduction of cytochrome *a* by tetramethylphenylenediamine + ascorbate, although the oxidation of this substrate by molecular oxygen is strongly inhibited. Thus, if cytochrome oxidase can be represented as a linear sequence of electron transfer, polylysine must promote the interaction of DMNQP with a component of the oxidase beyond cytochrome *a*. However, it is doubtful whether cytochrome *a* functions simply as an electron donor for cytochrome *a₃*, since reductants such as *p*-phenylenediamine can reduce cytochrome *a* without, in the absence of cytochrome *c*, producing any oxygen uptake (Okunuki, 1966). The fact that in this case addition of cytochrome *c* brings about oxygen uptake, whereas in the case of tetramethylphenylenediamine + ascorbate polylysine inhibits the oxidation, indicates that polylysine is not simply mimicking an activating effect of cytochrome *c*.

The inhibitory effects of basic macromolecules on cytochrome oxidase can be reversed by polyanions and by P_i (Person & Fine, 1961), and this antagonism of polylysine action explains the effective inhibition by P_i and by ADP of the stimulated oxidation of DMNQP. Indeed, the fall in the rate of the stimulated oxidation once the concentration of DMNQP rises above a certain value suggests that at a sufficient concentration the quinol phosphate itself antagonizes the effect of polylysine in the same way as does ADP. The greater effectiveness of ADP as compared with P_i is, however, noteworthy.

It is tempting to suppose that a reaction that shows a relatively high degree of substrate specificity, as do the polylysine-stimulated

oxidations, may have some significance *in vivo* (cf. Jacobs *et al.* 1965, p. 804). However, even low concentrations of polylysine uncouple oxidative phosphorylation (Schwartz, 1965), and the relatively large concentrations used here cause in addition inhibition and dislocation of the electron-transport chain. Such conditions can hardly exist during the synthesis of ATP coupled to electron transport, and thus the observed stimulated oxidation of DMNQP, whatever other significance it may have, does not obviously either support or provide evidence against a role for a quinol phosphate in oxidative phosphorylation.

Quinol phosphate oxidation and phosphoryl transfer.

The results presented above demonstrate that quinol phosphates are oxidized by mitochondria and provide evidence on the locus of interaction with the respiratory chain. No direct study has yet been made of the mechanism by which the P_i released during the oxidation is formed. However, if quinol phosphate oxidation is to have any interest as a possible model of the theoretical schemes involving compounds of this type (Chmielewska, 1960; Vilkas & Lederer, 1962; Williams, 1962; Erickson *et al.* 1963), then the oxidation must be accompanied by phosphoryl transfer. The appearance of P_i in the medium is observed to be dependent on oxygen uptake, indicating that this is a genuine oxidative dephosphorylation and thus that conditions exist under which the quinol phosphate can, but not must, function as a phosphorylating agent (Clark *et al.* 1958; Wieland & Pattermann, 1958). In chemical systems a large part of the P_i formed on quinol phosphate oxidation is produced directly by C-O bond fission (Durkheimer & Cohen, 1964; Lapidot & Samuel, 1964), and in such cases the quinol phosphate does not, of course, function as a phos-

phorylating agent. However, Wodak (1968) has shown that the enzymic oxidation of duroquinol monophosphate by horseradish peroxidase + hydrogen peroxide proceeds almost exclusively with P-O bond fission, i.e. results in phosphoryl transfer.

In this context the possible implications of the observation that the ratio mol of P_i formed/g-atoms of oxygen consumed is less than unity deserve mention. The oxidation requires the transfer of two electrons from DMNQP to the respiratory chain, presumably as two discrete one-electron steps as indicated in Scheme 1, since the interaction is with cytochrome *c*, and thus one g-atom of oxygen should be consumed/mol of P_i formed. However, the ratio is observed to be less than unity and, since the discrepancy is greater than can be attributed to errors in the determination of oxygen, this suggests that P_i may not be the only phosphorus-containing product. This has the implications not only that phosphoryl transfer is taking place, but also that the process of phosphorylation may be relatively non-specific. Clearly one property that the theoretical schemes cited above require of any model is that the phosphoryl residue should be transferred to a specific acceptor. This would be the case, for example, with pathway *a* in Scheme 1, where Nu represents a nucleophilic group on a protein, e.g. a histidine residue. P_i would then result from hydrolysis of the phosphoenzyme and should appear in near quantitative yield, since even if some phosphoenzyme survives it would be unlikely to amount to more than 1 nmol/mg of mitochondrial protein (cf. concentration of the components of the respiratory chain given by Slater, 1967). If Nu represents solvent water, then clearly a quantitative yield of P_i would be observed. However, in the present case some indiscriminate

Scheme 1. Possible pathways of P_i formation involving P-O bond fission on mitochondrial oxidation of DMNQP. Nu represents a nucleophilic centre on the protein.

phosphorylation appears to have taken place and thus a non-specific mechanism, such as intermediate formation of unstable metaphosphate as shown in pathway *b* in Scheme 1, is indicated.

The failure of added ADP to stimulate the mitochondrial oxidation of DMNQP is evidence against a function for ADP as a specific phosphoryl acceptor in this system.

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