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Role of Ubiquinone in the Mitochondrial Generation of Hydrogen Peroxide

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Antimycin-inhibited bovine heart submitochondrial particles generate O_2^- and H_2O_2 with succinate as electron donor. H_2O_2 generation involves the action of the mitochondrial superoxide dismutase, in accordance with the McCord & Fridovich [(1969) *J. Biol. Chem.* **244**, 6049–6055] reaction mechanism. Removal of ubiquinone by acetone treatment decreases the ability of mitochondrial preparations to generate O_2^- and H_2O_2, whereas supplementation of the depleted membranes with ubiquinone enhances the peroxide-generating activity in the reconstituted membranes. Addition of superoxide dismutase to ubiquinone-reconstituted membranes is essential in order to obtain maximal rates of H_2O_2 generation since the acetone treatment of the membranes apparently inactivates (or removes) the mitochondrial superoxide dismutase. Parallel measurements of H_2O_2 production, succinate dehydrogenase and succinate–cytochrome *c* reductase activities show that peroxide generation by ubiquinone-supplemented membranes is a monotonous function of the reducible ubiquinone content, whereas the other two measured activities reach saturation at relatively low concentrations of reducible quinone. Alkaline treatment of submitochondrial particles causes a significant decrease in succinate dehydrogenase activity and succinate-dependent H_2O_2 production, which contrasts with the increase of peroxide production by the same particles with NADH as electron donor. Solubilized succinate dehydrogenase generates H_2O_2 at a much lower rate than the parent submitochondrial particles. It is postulated that ubisemiquinone (and ubiquinol) are chiefly responsible for the succinate-dependent peroxide production by the mitochondrial inner membrane.

Generation of H_2O_2 has been demonstrated in mitochondria isolated from different sources (Loschen *et al.*, 1971; Boveris *et al.*, 1972*a*; Boveris & Chance, 1973; Loschen *et al.*, 1973). Both NAD-linked substrates and succinate were able to support maximal rates of H_2O_2 production (Boveris & Chance, 1973). Submitochondrial particles supplemented with either NADH or succinate are also active sources of H_2O_2 (Loschen *et al.*, 1973, 1974*a*,*b*; Boveris & Cadenas, 1975). H_2O_2 generation is markedly enhanced by antimycin, and it is rotenone-sensitive when it is supported by NAD-linked substrates in intact mitochondria, or by NADH in submitochondrial particles (Boveris & Chance, 1973; Loschen *et al.*, 1974*b*) and, accordingly, the mitochondrial source of H_2O_2 can be located on the oxygen side of the rotenone-sensitive site and on the substrate side of the antimycin-sensitive site. The mitochondrial rate of peroxide production is modulated by the different metabolic states (Chance & Williams, 1956), since it is higher in state 4 than in state 1 or 3 (Loschen *et al.*, 1971; Boveris & Chance, 1973), this effect suggesting that a member of the respiratory chain is responsible for H_2O_2 generation. Sonicated submitochondrial particles, which are devoid of auxiliary dehydrogenases such as the flavoproteins of the fatty acid oxidation pathway, are still effective generators of H_2O_2, and therefore it seems that any of the components of the pool of respiratory carriers with about 0mV midpoint potential, i.e. succinate dehydrogenase, ubiquinone, cytochrome *b* and the iron–sulphur centres of the segment succinate dehydrogenase–cytochrome *b*, may be the mitochondrial generator of H_2O_2. It is possible that only one of these electron carriers produces H_2O_2, but it is recalled that iron–sulphur flavoproteins (Fridovich, 1970), flavoproteins (Misra & Fridovich, 1972*a*), quinols (Misra & Fridovich, 1972*a*; Oshino *et al.*, 1974) and iron–sulphur proteins (Misra & Fridovich, 1971) are known sources of O_2^-, which are the precursors of mitochondrial H_2O_2 (Loschen *et al.*, 1974*a*; Boveris & Cadenas, 1975).

A linear relationship between ubiquinone content and H_2O_2 production was observed in pigeon heart mitochondria depleted and supplemented with variable amounts of ubiquinone (Boveris & Chance, 1973). However, the role of ubiquinone remained to be established, since the dependence of the reaction rate on the amount of reducible quinone could be explained by (*a*) non-enzymic oxidation of a reduced form of the quinone by molecular O_2, (*b*)

ubiquinone modulation of the H_2O_2 generator [i.e. succinate dehydrogenase (Rossi *et al.*, 1970; Gutman *et al.*, 1971)], (*c*) ubiquinone functioning as an intermediate in electron transfer from the flavin to the H_2O_2 generator [i.e. cytochrome *b* (Ernster *et al.*, 1969)] and (*d*) a combination of these. The experiments described below provide evidence for a direct role of ubiquinone in O_2^- and H_2O_2 generation in mitochondria.

Experimental

Mitochondrial preparations

Bovine heart mitochondria were obtained from slaughterhouse material according to the general procedure described by Blair (1967). Ground heart (400g) was added to 1 litre of cold sucrose/EDTA/Tris solution (250mM-sucrose / 1mM-EDTA / 5mM-Tris / HCl buffer, pH7.4) and homogenized in a high-speed blender (model CB Waring Blendor) at maximum speed for 30s, adjusting the pH with KOH every 15s. Nuclei and cell debris were removed by centrifugation at 1000*g* for 10min. The supernatant, after centrifugation at 8000*g* for 10min, yielded the mitochondria, which were washed with the sucrose/EDTA/Tris solution. A homogeneous fraction consisting mainly of dark heavy mitochondria was always obtained. Submitochondrial particles were obtained by sonication. An ultrasonic disintegrator (model 500W; MSE, London S.W.1, U.K.) was used at an output of 0.7mA for 1min. Bovine heart mitochondria were depleted of ubiquinone by the method of Lester & Fleischer (1961) and reconstituted in ubiquinone by the procedure of Boveris *et al.* (1972*b*). The mitochondrial suspension (12ml; 25mg of protein/ml) was treated with acetone (388ml) at 0°C giving a final 97:3 (v/v) acetone/water mixture. The turbid suspension was rapidly centrifuged at 900*g* for 10min, and the pellet was suspended in cold sucrose/EDTA/Tris solution. Portions of ubiquinone-depleted membranes were placed in several tubes and supplemented with various amounts of ubiquinone 50 (Q_{10}) and acetone to give a 2:3 (v/v) acetone/water mixture. The tubes were stirred in a Vortex mixer several times for 1min periods and kept at 0°C for 10min. Ubiquinone-reconstituted membranes were centrifuged down at 10000*g* for 10min, washed and suspended in sucrose/EDTA/Tris solution. Succinate dehydrogenase-depleted submitochondrial particles were prepared by alkaline treatment by the procedure of Lee *et al.* (1969); bovine heart submitochondrial particles were incubated in 50mM-glycine/NaOH buffer, pH9.5, at 36°C for 30min and then centrifuged down at 104000*g* for 40min. The supernatant, which was considered as solubilized succinate dehydrogenase, was assayed spectrophotometrically after dithionite reduction at $E_{465}-E_{510}$ ($\Delta\varepsilon_{mM}$ = 11 litre·mmol^{-1}·cm^{-1}) and found to contain 0.77 nmol of flavin/mg of protein.

Determination of enzyme activities

H_2O_2 generation was determined by (*a*) the cytochrome *c* peroxidase method (Boveris *et al.*, 1972*a*) and (*b*) the scopoletin–horseradish peroxidase method (Andreae, 1955; Loschen *et al.*, 1971). With method (*a*) the reaction mixture contained 7mM-succinate, 0.6μM-cytochrome *c* peroxidase and 230mM-mannitol / 70mM-sucrose / 30mM-Tris / 3-(*N*-morpholino)propanesulphonic acid buffer (henceforth mannitol/sucrose/Tris/Mops buffer); antimycin concentration and pH are indicated in each case. Measurements were performed in an Aminco–Chance double-beam spectrophotometer (American Instrument Company, Silver Springs, MD, U.S.A.), measuring $E_{419}-E_{407}$ (ΔE = 50 litre·mmol^{-1}·cm^{-1}). With the scopoletin method the reaction mixture contained 7mM-succinate, 0.9μM-scopoletin, 0.8μM-horseradish peroxidase, 0.3μM-superoxide dismutase and mannitol/sucrose/Tris/Mops buffer; antimycin concentrations and pH are indicated in each case. Measurements were performed in an Aminco–Bowman spectrofluorimeter (American Instrument Company) at 360nm (excitation) and 460nm (emission). O_2^- production was determined (*c*) from the superoxide dismutase-sensitive reduction of cytochrome *c* (henceforth cytochrome *c* assay) (McCord & Fridovich, 1969; Boveris & Cadenas, 1975), or (*d*) from the rate of adrenochrome formation (Misra & Fridovich, 1972*b*; Loschen *et al.*, 1974*a*). With method (*c*) the reaction mixture contained 15μM-cytochrome c^{3+}, 7.0mM-succinate and mannitol/sucrose/Tris/Mops buffer; antimycin concentration and pH are indicated in each case. Measurements were performed in an Aminco–Chance double beam spectrophotometer measuring $E_{550}-E_{540}$ (ΔE_{mM} = 19 litre·mmol^{-1}·cm^{-1}). With method (*d*) the reaction mixture contained 1mM-adrenaline, 7.0mM-succinate and mannitol/sucrose/Tris/Mops buffer; antimycin concentration and pH are indicated in each case. Measurements were performed in the double-beam spectrophotometer. Enzymically reducible ubiquinone was directly measured at $E_{275}-E_{300}$ (ΔE_{mM} = 12 litre·mmol^{-1}·cm^{-1}) or at $E_{283}-E_{293}$ (ΔE_{mM} = 8 litre·mmol^{-1}·cm^{-1}), in a 5mm-light-path cuvette containing 0.2–0.6mg of protein/ml, 0.2μM-antimycin, 7.0mM-succinate and mannitol/sucrose/Tris/Mops buffer, pH7.4. Measurements were performed in the double-beam spectrophotometer, with a 40W deuterium lamp (Sylvania B-64063) as light source. Succinate dehydrogenase activity was obtained by the method of Arrigoni & Singer (1962) by measuring E_{600} ($\Delta\varepsilon_{mM}$ = 20.5 litre·mmol^{-1}·cm^{-1}). The reaction mixture was made up of 0.52mM-phenazine methosulphate,

55 μM-2,6-dichlorophenol-indophenol, 1 mM-KCN, 7 mM-succinate and mannitol/sucrose/Tris/Mops buffer, pH 7.4. The reaction was started by the addition of succinate. Succinate–cytochrome *c* reductase activity was obtained by measuring E_{550}, ($\Delta E_{mM} = 19$ litre·mmol^{-1}·cm^{-1}) in the presence of 50 μM-cytochrome *c*, 8.3 mM-succinate, 1 mM-KCN and mannitol/sucrose/Tris/Mops buffer, pH 7.4. The Gilford 2000 spectrophotometer was used in the determination of the two latter activities. Antimycin-insensitive O_2 uptake was measured in a K-IC Oxygraph (Gilson Medical Electronics, Middleton, WI, U.S.A.) operated at high sensitivity. The submitochondrial particles were suspended at 1–3 mg of protein/ml in mannitol/sucrose/Tris/Mops buffer, pH 7.8, and supplemented with 17 mM-ethanol and catalase (0.08 μM-haematin) to decompose H_2O_2 through the peroxidatic reaction of catalase. Unless otherwise stated enzyme activities were measured at 30°C.

Ubiquinone, phospholipid and protein measurements

Total ubiquinone was measured by the extraction method of Redfearn (1967). Phospholipid content was determined as P_i after digestion with perchloric acid (Doizaki & Zieve, 1963). Protein determinations were made by the biuret method (Gornall *et al.*, 1949) in the presence of 0.1% sodium deoxycholate.

Chemicals

Superoxide dismutase was from Miles Laboratories (Elkart, IN, U.S.A.); cytochrome *c* peroxidase was a generous gift from Professor T. Yonetani, University of Pennsylvania; cytochrome *c* (type VI), horseradish peroxidase (type VI), scopoletin, ubiquinone Q_{10}, NADH, Tris and Mops were from Sigma Chemical Company, St. Louis, MO, U.S.A.

Results

O_2^- and H_2O_2 production in submitochondrial preparations

Table 1 shows the rates of production of O_2^- and of H_2O_2 in sonicated submitochondrial particles and ubiquinone-reconstituted mitochondrial membranes. The enzyme preparations examined enabled us to detect the influence of superoxide dismutase on the relative rates of O_2^- and H_2O_2 production. On the one hand, when the particles were assayed without washing, no production of O_2^- was detectable and no increase in the rate of H_2O_2 production was observed after addition of exogenous superoxide dismutase. It is assumed that the particles contained enough endogenous dismutase to convert all the O_2^- produced into H_2O_2, in accordance with the McCord & Fridovich (1969) reaction:

$$O_2^- + O_2^- + 2H^+ \xrightarrow{k_1} H_2O_2 + O_2 \qquad (1)$$

Table 1. *Succinate dependent production of O_2^- and H_2O_2 by mitochondrial preparations*

The reaction mixtures were as described in the Experimental section except for the following constituents. (1) O_2^- determination, (*a*) cytochrome *c* assay; 0.2 μM-antimycin; 0.06–0.11 mg of protein/ml. (*b*) Adrenochrome assay: 0.2 μM-antimycin; 0.3–0.6 mg of protein/ml. (2) H_2O_2 determination, (*c*) cytochrome *c* peroxidase assay (with submitochondrial particles): 0.2 μM-antimycin; 0.06–0.11 mg of protein/ml. (*d*) Scopoletin assay (with ubiquinone-reconstituted membranes): 3 μM-antimycin; 0.34 mg/ml of protein. (3) O_2 uptake determination: 7.0 mM-succinate; 2.5 μM-antimycin; mannitol/sucrose/Tris/Mops buffer and 1–3 mg/ml of protein. Final concentration of buffer constituents and other conditions were as described in the Experimental section. In all cases the pH was 7.8.

		Activities (nmol/min per mg of protein)		
		Submitochondrial particles		Ubiquinone-reconstituted membranes*
Assay	Superoxide dismutase addition (μM)	Control	Washed†	
1. O_2^- production				
(*a*) Cytochrome *c*	None	0.00	3.87	—
	0.3	0.00	1.29 (2.58)‡	—
(*b*) Adrenochrome	None	0.00	3.89	0.54
	0.3	0.00	0.09 (3.80)‡	0.06 (0.48)‡
2. H_2O_2 production	None	1.86	1.86	0.04
	0.3	1.80	3.72	0.17
3. Antimycin-insensitive O_2 uptake	None	5.00	10.8	2.10

* Containing 4.2 nmol ubiquinone/mg of protein.

† Particles were washed three times in the centrifuge at 104000*g* and 1–2°C with 10 ml of the sucrose/EDTA/Tris buffer.

‡ O_2^- production calculated from the superoxide dismutase sensitive rate of reduction of cytochrome c^{3+} or adrenochrome formation.

On the other hand, when the particles were washed three times in order to remove endogenous dismutase, an active production of O_2^- was detected. With the adrenochrome assay we found a production of 3.8nmol of O_2^-/min per mg of protein, whereas the cytochrome *c* assay yielded 2.6nmol of O_2^-/min per mg of protein. Addition of exogenous superoxide dismutase to the washed particles decreased the generation of O_2^- and increased to the same extent the production of H_2O_2, in accordance with the assumption that the endogenous superoxide dismutase had been removed by the repeated washings. The washed particles showed, however, nearly the same electron fluxes (about 3.8nequiv. of e^-/min per mg of protein) measured either as O_2^- or as H_2O_2 [Table 1, experiments (1) and (2)], which suggests that some dismutase remained trapped in the intravesicular space.

H_2O_2 production by the acetone-extracted ubiquinone-reconstituted mitochondrial membranes was measured by the scopoletin–horseradish peroxidase method since the cytochrome *c* peroxidase assay failed to determine measurable peroxide generation rates. This failure may be attributed to an abnormal accessibility of the H_2O_2–cytochrome *c* peroxidase complex to reduced cytochrome *c* located at the outer surface of the inner mitochondrial membrane, resulting from an increased permeability of the external membrane after acetone treatment. Table 1 (experiments 1*b* and 2) shows that with the ubiquinone-reconstituted membranes the rate of H_2O_2 generation was relatively small, as compared with the rate of O_2^- generation, but addition of superoxide dismutase increased the rate of H_2O_2 generation while decreasing, in about the same proportion, O_2^- production. These observations lead us to postulate that with the ubiquinone-reconstituted membranes (*a*) H_2O_2 generations occurred in accordance with the McCord & Fridovich (1969) reaction (eqn. 1) and (*b*) the endogenous superoxide dismutase was partly inactivated or removed by the acetone treatment. It is worth noting that H_2O_2 production by submitochondrial particles or ubiquinone-reconstituted membranes never exceeded 35% of the antimycin-insensitive O_2 uptake by the same preparations in similar experimental conditions [Table 1, experiments (2) and (3)], a result that contrasts with the one observed in intact pigeon heart

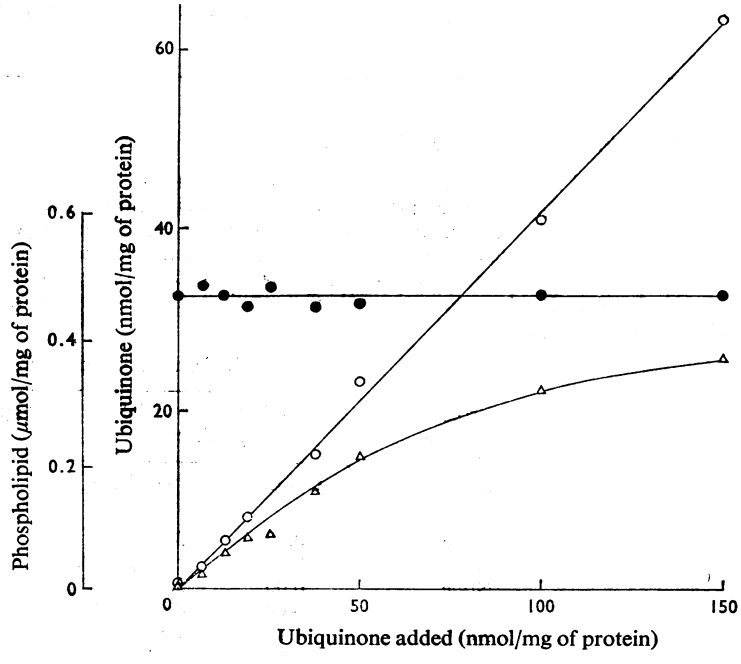

Fig. 1. *Reducible ubiquinone, total ubiquinone and phospholipid content in the ubiquinone-reconstituted membranes*

Reducible ubiquinone was measured by double-beam spectrophotometry at $E_{275}-E_{300}$ in the presence of 0.2μM-antimycin and 7mM-succinate; total ubiquinone was measured spectrophotometrically after extraction with light petroleum (b.p. 35–60°C) (Redfearn, 1967). Other conditions were as described in the Experimental section. ○, Total ubiquinone; △, reducible ubiquinone; ●, total phospholipid content.

mitochondria, in which H_2O_2 production matches the antimycin-insensitive O_2 utilization (Chance *et al.*, 1973). The antimycin-insensitive electron leak through cytochromes *b* and c_1 was consequently much more active in the sonicated and acetone-treated preparations than in the intact mitochondria.

Properties of the ubiquinone-reconstituted mitochondrial membranes

In order to establish the role of ubiquinone in O_2^- and H_2O_2 generation, some properties of the ubiquinone-reconstituted membranes must first be considered. Fig. 1 shows the amounts of enzymically reducible ubiquinone and of total ubiquinone that were incorporated by acetone-treated mitochondria when the ubiquinone-depleted membranes were supplemented with ubiquinone. About one-third of the added quinone was bound to the ubiquinone-depleted membranes, stirring and temperature during preincubation with ubiquinone being additional factors that slightly affected the percentage of ubiquinone binding. The incorporated ubiquinone behaved as functionally active ubiquinone, as shown by its reduction in the presence of succinate and antimycin, up to a level of approx. 7nmol of ubiquinone/mg of protein (Fig. 1). Above that level, ubiquinone was apparently bound to non-specific sites in the mitochondrial membrane, where it was not reducible. The maximal amount of reducible quinone that could be incorporated by the membranes was calculated from the data in Fig. 1 (double-reciprocal plots) and it was found to be about 33nmol of ubiquinone/mg of protein, which, considering the phospholipid content of the membrane preparation, yields a phospholipid/ubiquinone molar ratio of 14:1.

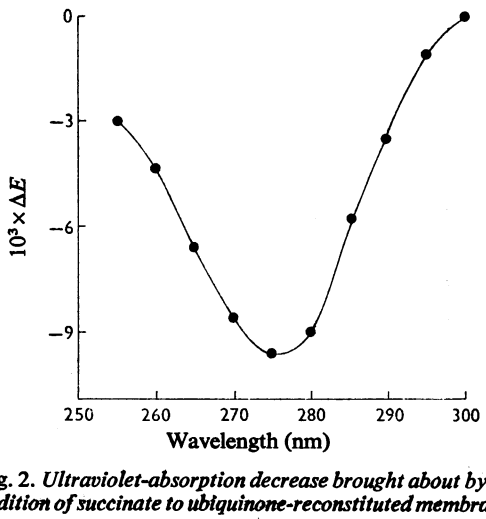

Fig. 2. *Ultraviolet-absorption decrease brought about by the addition of succinate to ubiquinone-reconstituted membranes*

The incubation medium contained 0.19mg of protein/ml, 7mM-succinate, 0.2μM antimycin and mannitol/sucrose/Tris/Mops buffer. Other conditions were as described in the Experimental section.

Further evidence supporting the function of the specifically bound ubiquinone as electron acceptor is given in Figs. 2 and 3. Fig. 2 shows the decrease in ultraviolet absorption spectrum determined by the addition of succinate to an antimycin-inhibited reconstituted membrane preparation. It is noticeable that bleaching was maximal at 275nm, which is characteristic of reduced ubiquinone. Fig. 3 shows the kinetics of reduction of the reincorporated quinone. It is seen that the quinone pool was kinetically homogeneous, with a single component that is reduced with a first-order reaction constant of $1.54\,min^{-1}$ (at 4°C).

H_2O_2 production in ubiquinone-reconstituted membranes

Fig. 4 illustrates the dependence of peroxide production on the amount of reducible ubiquinone in the reconstituted membranes. Addition of scopoletin gave a fluorescent signal that was used to adjust the ordinate scale, whereas addition of succinate started H_2O_2 production, as revealed by the decrease of scopoletin fluorescence. Comparison of the rates of H_2O_2 generation by (*a*) ubiquinone-reconstituted membranes [containing 26nmol of ubiquinone/mg of protein (Fig. 4*b*)] and (*b*) the ubiquinone-depleted control membranes [containing 0.21nmol of ubiquinone/mg of protein (Fig. 4*a*)] shows that re-incorporation of reducible ubiquinone into the mitochondrial membrane determined a ninefold increase in the rate of H_2O_2 production.

The effect of superoxide dismutase on H_2O_2 production by reconstituted membranes is further examined in Fig. 5, in which the reaction rates are plotted as a function of the concentration of reducible ubiquinone. It is seen that in the absence of external dismutase and above a rate of about 0.05μmol of O_2^-/min, dismutation of O_2^- was rate-limiting. On the other hand, after addition of dismutase, an almost linear relationship between reducible ubiquinone content and H_2O_2 production could be observed. These results are in good agreement with those reported by Boveris & Chance (1973) with pigeon heart mitochondria, after depletion and reincorporation of ubiquinone. The measurements described here were extended to a larger range of ubiquinone levels, and parallel determinations of H_2O_2 generation, succinate dehydrogenase and succinate–cytochrome *c* reductase activities were performed. The results are shown in Fig. 6 where it is seen that H_2O_2 production increased linearly as a function of the ubiquinone content (up to a concentration of 26nmol

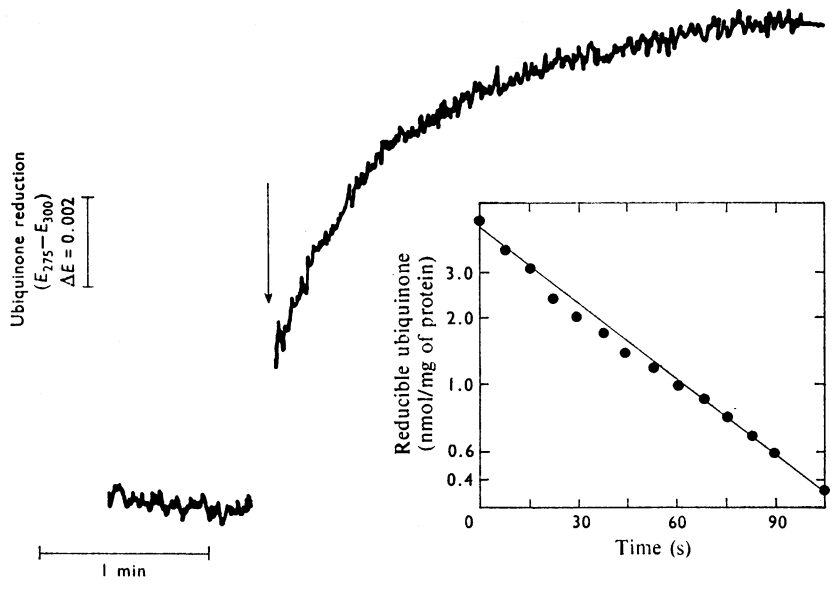

Fig. 3. *Kinetics of ubiquinone reduction in the ubiquinone-reconstituted membranes*

Experimental conditions were as in Fig. 2, except the temperature was 4°C. The arrow shows the addition of succinate (final concn. 7mM). The inset kinetic plot gives a first-order reaction constant of 1.54min^{-1}.

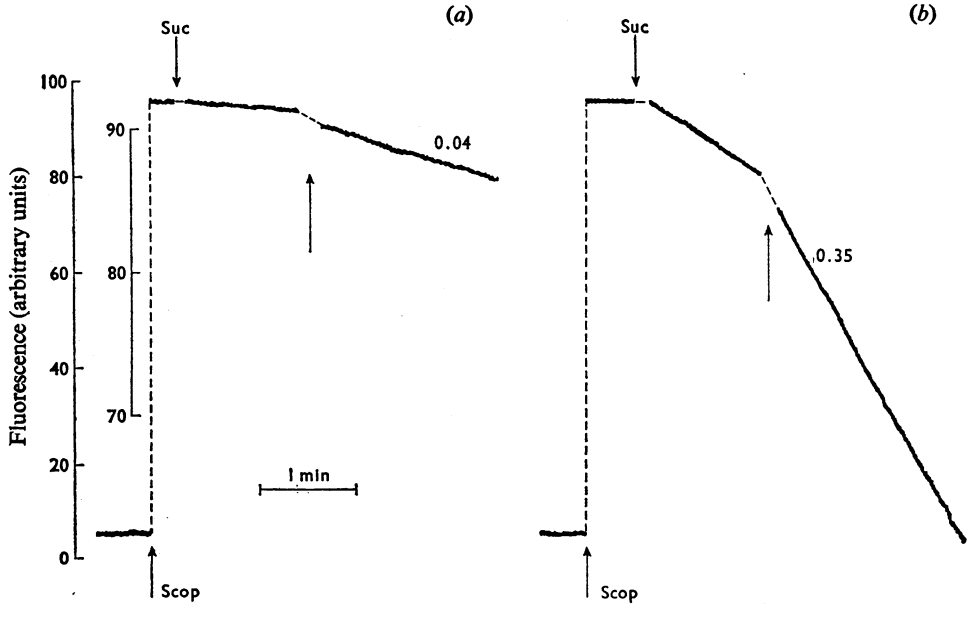

Fig. 4. *H_2O_2 generation by ubiquinone-depleted (a) and ubiquinone-reconstituted membranes (b)*

The reaction mixture was as described in the Experimental section except for the following constituents: 3μM-antimycin; 0.7μM superoxide dismutase; 0.34 (*a*) and 0.35 (*b*) mg of mitochondrial protein/ml. The pH was 7.4. Other conditions were as described in the Experimental section. Succinate reducible ubiquinone was 0.21 (*a*) and 26 (*b*) nmol/mg of protein. Scop, addition of scopoletin (final concn. 0.9μM); Suc, addition of succinate (final concn. 7mM). Values against the traces indicate nmol of H_2O_2/min per mg of protein produced. At the point indicated by the arrows, the sensitivity of the recording system was increased threefold.

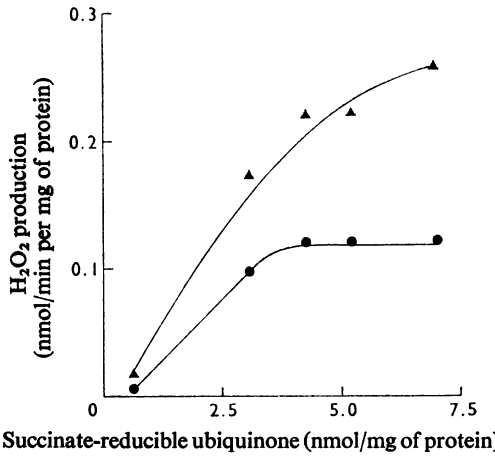

Fig. 5. *Effect of superoxide dismutase on H_2O_2 production by ubiquinone-depleted and ubiquinone-reconstituted membranes*

Conditions were as in Fig. 4 with 0.37–0.42mg mitochondrial protein/ml. ▲, Superoxide dismutase added; ●, superoxide dismutase omitted.

of ubiquinone/mg of protein), whereas both (*a*) the electron flow from the flavin to cytochromes *b* and c_1, and (*b*) the activity of succinate dehydrogenase reached a plateau at a level of about 1–2nmol of ubiquinone/mg of protein.

Effect of alkaline treatment on H_2O_2 production by submitochondrial particles

Alkaline treatment produces detachment of succinate dehydrogenase and provides particles that are deficient in such flavoprotein (Lee *et al.*, 1969). In order to test the role of succinate dehydrogenase in peroxide production, alkali-treated particles were assayed with NADH and succinate as electron donors. Table 2 shows that the alkaline treatment decreased by 50–51% the succinate-dependent H_2O_2 generation, whereas the NADH-dependent peroxide generation was somewhat increased. On the other hand, the alkaline treatment decreased succinate dehydrogenase activity by 63%, and 93% after separation of particles from the soluble succinate dehydrogenase. Control measurements with the supernatant demonstrated that 30% of the original succinate dehydrogenase was made soluble by the alkaline treatment, but the extracted enzyme was

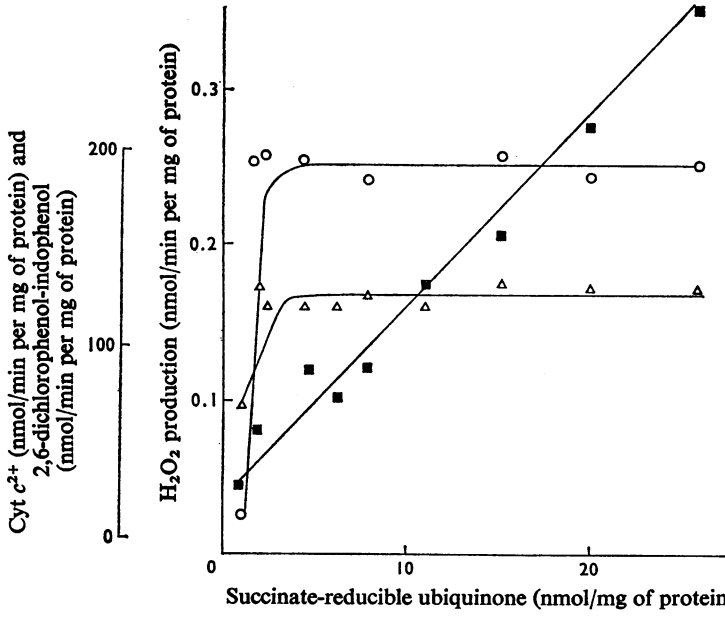

Fig. 6. *H_2O_2 generation, succinate dehydrogenase activity and succinate–cytochrome c reductase activity of ubiquinone-depleted and ubiquinone-reconstituted membranes*

H_2O_2 generation was measured by the scopoletin–horseradish peroxidase method. Experimental conditions were as in Fig. 4; succinate dehydrogenase and succinate–cytochrome *c* reductase were measured as described in the Experimental section. Protein concentration was 0.36–0.39mg/ml. ■, H_2O_2 generation; △, succinate dehydrogenase activity; ○, succinate–cytochrome *c* reductase activity.

Table 2. *H_2O_2 production by alkali-treated submitochondrial particles and succinate dehydrogenase*

H_2O_2 production was measured by the cytochrome *c* peroxidase assay, with NADH as electron donor. The standard reaction mixture was used except that succinate was replaced by 13 μM-NADH. Experiments (*a*)–(*c*) contained 0.2–0.4 mg of protein/ml and experiment (*d*) 0.2–1.4 mg of protein/ml. Other conditions were as described in Table 1 and in the Experimental section. The percentage of initial activity recovered in each fraction is given in parentheses. DCPI, 2,6-dichlorophenol-indophenol (reduced).

Enzyme preparation	H_2O_2 production (nmol of H_2O_2/min per mg of protein)		Succinate dehydrogenase activity (nmol of DCPI/min per mg of protein)
	Succinate supported	NADH supported	
(*a*) Submitochondrial particles	0.85 (100)	2.03 (100)	196 (100)
(*b*) Submitochondrial particles after alkaline treatment	0.45 (51)	2.41 (117)	72 (37)
(*c*) Succinate dehydrogenase depleted-submitochondrial particles	0.62 (50)	3.49 (122)	18 (7)
(*d*) Succinate dehydrogenase extract	0.03 (1)	0.00 (0)	157 (30)

unable to generate H_2O_2, thus proving that peroxide generation, supported either by NADH or succinate, was totally associated to the membranes. In good agreement with this assumption is the fact that the alkali-treated particles retained all the ubiquinone measurable in the original submitochondrial particles (3.2 nmol of ubiquinone/mg of protein) whereas no quinone was found in the succinate dehydrogenase extract. Finally, it must be noted that the NADH-dependent specific activity of the succinate dehydrogenase-depleted submitochondrial particles was significantly higher than that of the original particles, in all likelihood, because of the removal of inactive protein (including succinate dehydrogenase). This increase in specific activity of the peroxide generator is important for evaluating the decrease in H_2O_2 production when succinate was the electron donor. Our results are consistent with the NADH-dependent rotenone-sensitive generation of H_2O_2, observed by Loschen *et al.* (1974*b*) with succinate dehydrogenase-depleted submitochondrial particles.

Discussion

Sonicated submitochondrial particles are able to generate H_2O_2 by using succinate as electron donor via O_2^- (Table 1). Our observations confirm previous reports by Loschen *et al.* (1974*a*,*b*) and by Boveris & Cadenas (1975) under somewhat different experimental conditions. The presence of superoxide dismutase is critical for the whole process, since removal of mitochondrial dismutase (Weisiger & Fridovich, 1973*a*,*b*; Tyler, 1975; Peeters-Joris *et al.*, 1975) strongly decreases H_2O_2 production by submitochondrial particles, with concomitant increase of O_2^- production. The same mechanism is apparently valid with the acetone-treated ubiquinone-reconstituted membranes (Table 1 and Fig. 5).

The technique of depletion and re-incorporation of ubiquinone gives mitochondrial membranes in which a significant amount of quinone is functional (reducible), as shown by (*a*) the absorption spectrum of the membrane-bound reduced ubiquinone (Fig. 2) and (*b*) the homogeneous kinetics of reduction of the quinone pool (Fig. 3). With the reconstituted membranes, H_2O_2 generation increases monotonously as a function of the amount of reducible quinone (Figs. 5 and 6), whereas succinate dehydrogenase and succinate–cytochrome *c* reductase activities reach a plateau at a low concentration of reducible ubiquinone (Fig. 6). The different response of the measured parameters when the concentration of reducible quinone in the mitochondrial membranes is varied, allows one to rule out a role of ubiquinone either as a modulator of H_2O_2 production at the level of the succinate dehydrogenase flavin, or as an electron carrier between succinate dehydrogenase and a more positive H_2O_2 generator. Moreover, peroxide generation and succinate dehydrogenase activity could be separated by alkaline treatment of submitochondrial particles (Table 2) thus strengthening the conviction that ubiquinone plays a direct role in H_2O_2 generation.

There is evidence that the reduced form of a member of the respiratory chain is the source of mitochondrial peroxide (Loschen *et al.*, 1971, 1973; Boveris *et al.*, 1972*a*; Boveris & Chance, 1973). On this basis both the quinol and the semiquinone forms of ubiquinone may be postulated as univalent reductants of O_2.

$$UQH_2 + O_2 \xrightarrow{k_2} UQH^{\bullet} + H^+ + O_2^- \quad (2)$$

$$UQH^{\bullet} + O_2 \xrightarrow{k_3} UQ + H^+ + O_2^- \quad (3)$$

Occurrence of these reactions [eqns. (2) and (3)] is borne out by the circumstance that reduced forms of menadione, namely, menasemiquinone and menadiol primarily generate O_2^- and H_2O_2 respectively (Misra & Fridovich, 1972*a*). Further, the free-radical ubisemiquinone has been detected in mitochondrial membranes, amounting to about 0.2–1.5% of the total quinone content (Backstrom *et al.*, 1970),

which lends experimental support to the hypothesis that eqn. (3) is largely responsible for O_2^- generation in mitochondria. In that case, the rate of O_2^- generation may be represented by eqn. (4).

$$dO_2^-/dt = k_3[UQH^\cdot][O_2] \quad (4)$$

from which eqn. (5) can be derived.

$$k_3 = \frac{dO_2^-/dt}{[UQH^\cdot][O_2]} = \frac{0.24\,nmol\cdot min^{-1}\cdot mg^{-1}}{(0.1\,nmol\cdot mg^{-1})(60\,s\cdot min^{-1})(1\times10^{-3}\,M)} = 40\,M^{-1}\cdot s^{-1} \quad (5)$$

Eqn. (5) allows one to calculate the apparent second order reaction constant (k_3) for O_2^- production, since the dO_2^-/dt value is obtained from the slope of the straight line in Fig. 6; [$UQH^\cdot$] is estimated as 1% of reducible Q (Backstrom *et al.*, 1970) and O_2 concentration is taken as 1 mM, considering that O_2 solubility in the lipid phase of the membrane (where the reaction is assumed to occur) is as in paraffin oil (International Critical Tables, 1928). The calculated k_3 value is $40\,M^{-1}\cdot s^{-1}$, which at first seems to be very low as compared with the true second-order constant for the *p*-benzosemiquinone–O_2 model reaction in aqueous media ($4.5\times10^4\,M^{-1}\cdot s^{-1}$; Sawada *et al.*, 1975). However, it must be noted that in the model reaction the oxidation of O_2^- by the oxidized quinone (the back reaction) is fast enough to make the rate of reduction of O_2 by the semiquinone (forward reaction; Sawada *et al.*, 1975) almost negligible, which may explain the low k_3 value calculated from the experimental data in Fig. 6.

Auxiliary mitochondrial dehydrogenases can also generate O_2^- and H_2O_2, and Forman & Kennedy (1974, 1975) have reported generation of about 0.1 nmol of O_2^-/min per mg of protein in rat liver mitochondria as very likely due to dihydro-orotate dehydrogenase activity. However, under comparable conditions, H_2O_2 production that can be attributed to ubiquinone is much more active since it amounts to 0.3–0.6 nmol of H_2O_2/min per mg of protein (Boveris *et al.*, 1972*a*).

The present experimental data point to ubisemiquinone and ubiquinol as the main sources of H_2O_2 in mitochondrial inner membrane, but the function of other H_2O_2 generators is not excluded.

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