Biochem. J. (1980) **188**, 31–37
Printed in Great Britain

Enhancement of Hydrogen Peroxide Formation by Protophores and Ionophores in Antimycin-Supplemented Mitochondria

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(*Received 8 August 1979*)

Rat and pigeon heart mitochondria supplemented with antimycin produce 0.3–1.0nmol of H_2O_2/min per mg of protein. These rates are stimulated up to 13-fold by addition of protophores (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, carbonyl cyanide *m*-chloromethoxyphenylhydrazone and pentachlorophenol). Ionophores, such as valinomycin and gramicidin, and Ca^{2+} also markedly stimulated H_2O_2 production by rat heart mitochondria. The enhancement of H_2O_2 generation in antimycin-supplemented mitochondria and the increased O_2 uptake of the State 4-to-State 3 transition showed similar protophore, ionophore and Ca^{2+} concentration dependencies. Thenoyltrifluoroacetone and *N*-bromosuccinimide, which inhibit succinate–ubiquinone reductase activity, also decreased mitochondrial H_2O_2 production. Addition of cyanide to antimycin-supplemented beef heart submitochondrial particles inhibited the generation of O_2^-, the precursor of mitochondrial H_2O_2. This effect was parallel to the increase in cytochrome *c* reduction and it is interpreted as indicating the necessity of cytochrome c_1^{3+} to oxidize ubiquinol to ubisemiquinone, whose autoxidation yields O_2^-. The effect of protophores, ionophores and Ca^{2+} is analysed in relation to the propositions of a cyclic mechanism for the interaction of ubiquinone with succinate dehydrogenase and cytochromes *b* and c_1 [Wikstrom & Berden (1972) *Biochim. Biophys. Acta* **283**, 403–420; Mitchell (1976) *J. Theor. Biol.* **62**, 337–367]. A collapse in membrane potential, increasing the rate of ubisemiquinone formation and O_2^- production, is proposed as the molecular mechanism for the enhancement of H_2O_2 formation rates observed on addition of protophores, ionophores and Ca^{2+}.

Boveris & Chance (1973) and Loschen *et al.* (1971) described the generation of H_2O_2 by mitochondria and pointed out the requirement of antimycin and uncoupler for maximal formation of H_2O_2 by rat and pigeon heart mitochondria. The effects of rotenone and antimycin on the formation of H_2O_2 were interpreted as a requirement for a reduced member of the respiratory chain located between the rotenone- and antimycin-sensitive sites (Boveris & Chance, 1973). The effect of uncouplers on H_2O_2 formation was interpreted by these authors as pointing to the involvement of a component with variable potential, probably changing its potential to a more negative value after de-energization of the membrane. Research on this effect was not pursued any further.

Subsequently, Boveris & Cadenas (1975) and Dionisi *et al.* (1975) demonstrated that O_2^- generation by mitochondria could almost account for the whole formation of H_2O_2 after determining O_2^-/H_2O_2 ratios with approximate values of 2.0. Boveris *et al.* (1976) identified the component of the respiratory chain, located on the substrate side of the antimycin-sensitive site, mainly responsible for O_2^- and H_2O_2 generation; the semiquinone form of ubiquinone was postulated as the univalent reductant of O_2 in mitochondrial membranes.

Misra & Fridovich (1972*a*) have shown that the reduced forms of menadione, namely menasemiquinone and menadiol, primarily generate O_2^- and H_2O_2 respectively. Cadenas *et al.* (1977) assayed the O_2^- and H_2O_2 generation occurring during the autoxidation of quinols; on this basis they proposed a mechanism that involved the generation of O_2^- by ubisemiquinone autoxidation, supported by the relative stability of ubisemiquinone as a free radical

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in mitochondrial membranes (Ingledew & Ohnishi, 1975). Trumpower & Simmons (1979) have recently shown that the addition of thenoyltrifluoroacetone to antimycin-supplemented succinate–cytochrome *c* reductase caused the reduced forms of a low-potential redox component, most likely ubisemiquinone, to react with O_2 to generate O_2^-.

The present paper discusses the effect of protophores, ionophores and Ca^{2+} on H_2O_2 formation by mitochondria in the light of the ubiquinone cycles proposed by Wikstrom & Berden (1972) and Mitchell (1976), considering the effect of membrane-potential collapse in ubisemiquinone steady-state concentration and the rate of superoxide anion formation.

Materials and Methods

Biological preparations

Rat and pigeon heart mitochondria were isolated by the procedure of Chance & Hagihara (1963). Ground hearts were resuspended in 230mM-mannitol/70mM-sucrose/1mM-EDTA/5mM-Tris/HCl buffer, pH 7.4, incubated for 10min at 0°C with Nagarse (0.5mg/heart), homogenized and the nuclei and cell debris were removed by centrifugation at 700*g* during 10min. Mitochondria were obtained after centrifugation of the 700*g* supernatant at 8000*g* for 10min and washed once in the same medium. Rat heart mitochondria showed a respiratory control ratio of 2.4–2.9 with 6mM-succinate/4mM-glutamate as substrates and 3.6–3.7 with 3mM-malonate/6mM-glutamate/6mM-malate as substrates. Beef heart mitochondria were obtained by the technique already described by Boveris *et al.* (1976). Beef heart submitochondrial particles were obtained by sonication in an ultrasonic disintegrator (model 500W; MSE, London S.W.1, U.K.) with an output of 0.7mA for 30s twice with a 1min interval. The sonication medium consisted of 250mM-sucrose/1mM-EDTA/KOH, pH 8.0. Submitochondrial particles were washed twice with 250mM-sucrose and finally resuspended in 230mM-mannitol/70mM-sucrose/20mM-Tris/HCl buffer, pH 7.4. All the operations were performed at 0°C. Protein determinations were made by the biuret method (Gornall *et al.*, 1949) in the presence of 0.1% sodium deoxycholate.

Determination of H_2O_2 generation

The generation of H_2O_2 was determined by either the cytochrome *c* peroxidase or the horseradish peroxidase method (Boveris *et al.*, 1972*a*). The reaction mixture consisted of 7mM-succinate, 0.6–0.1μM-peroxidase and 230mM-mannitol/70mM-sucrose/20mM-Tris/4-morpholinepropanesulphonic acid (Mops) buffer, pH 7.4 (henceforth termed mannitol/sucrose/Tris/Mops buffer). Measurements were performed either in an Aminco–Chance or in a model 356 Perkin–Elmer double-beam spectrophotometer (American Instruments Co., Silver Springs, MD, U.S.A., and Hitachi, Tokyo, Japan respectively), measuring $\varepsilon_{419-407}$ and $\varepsilon_{423-404}$ ($\Delta\varepsilon = 50$ and 60 litre·mmol^{-1}·cm^{-1} respectively) or $\varepsilon_{417-402}$ ($\Delta\varepsilon = 50$ litre·mmol^{-1}·cm^{-1}) for cytochrome *c* peroxidase and horseradish peroxidase respectively. The H_2O_2 generation by antimycin-supplemented rat heart mitochondria was measured by both methods (Fig. 1). Horseradish peroxidase was less effective (52%) than cytochrome *c* peroxidase in detecting H_2O_2 formation owing to the existence of endogenous hydrogen donor (Boveris *et al.*, 1972*a*). Antimycin was used in all the assays for H_2O_2 or O_2^- determinations. All the determinations were performed at 30°C.

Determination of O_2^- generation

The generation of O_2^- was estimated from the superoxide dismutase-sensitive rate of adrenochrome formation (Misra & Fridovich, 1972*b*) measuring the absorption change at 485–575nm and utilizing an ε of 2.96 litre·mmol^{-1}·cm^{-1} (Green *et al.*, 1956). Determination of O_2^- formation was performed in a double-beam spectrophotometer as described for H_2O_2 determination; this assay gave a molar ratio of adrenochrome formed/O_2^- generated equal to 1.0 when measured with the xanthine/xanthine oxidase mixture (Cadenas *et al.*, 1977). O_2^- determinations were assayed at room temperature (23°C).

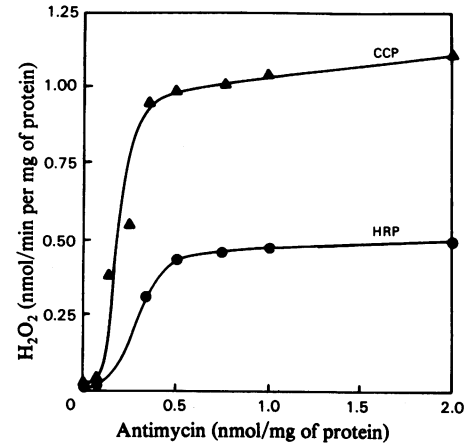

Fig. 1. *Enhancing effect of antimycin on the mitochondrial production of H_2O_2*

Experimental conditions were as described in the Materials and Methods section. Rat heart mitochondria equivalent to 0.23mg of protein/ml were used. Abbreviations: CCP, yeast cytochrome *c* peroxidase; HRP, horseradish peroxidase.

Determination of O_2 uptake

O_2 uptake was measured in a K-IC Oxygraph (Gilson Medical Electronics, Middleton, WI, U.S.A.) operated at high sensitivity. The buffer mixture utilized for each experiment is detailed in the legends to the Figures.

Chemicals

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and carbonyl cyanide *m*-chloromethoxyphenylhydrazone were provided by Dr. P. G. Heytler (E.I. du Pont de Nemours Co., Wilmington, DE, U.S.A.). Pentachlorophenol was from Eastman Kodak (Rochester, NY, U.S.A.). Valinomycin, gramicidin and horseradish peroxidase (type VI) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cytochrome *c* peroxidase and recrystallized *N*-bromosuccinimide were gifts from Professor T. Yonetani, Department of Biochemistry and Biophysics, University of Pennsylvania. Other reagents used were of analytical grade.

Results

Effect of protophores on H_2O_2 formation by antimycin-supplemented mitochondria

The production of H_2O_2 in isolated rat liver and pigeon heart mitochondria accounts for about 1–2% and 2–4% respectively of the corresponding O_2 uptake in State 4 (Boveris & Chance, 1973). The rate of generation of H_2O_2 by mitochondria depends on the metabolic state (it is higher in State 4 and lower in State 3) and on the source of mitochondria [it is substantially higher in pigeon heart than in rat heart mitochondria (Boveris, 1977)].

Addition of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to antimycin-supplemented mitochondria increased the H_2O_2 production rate by 5-fold, from 0.7 to 3.7 nmol/min per mg of protein (Fig. 2). The enhanced rate occurred immediately after the addition of the uncoupler and remained constant until the H_2O_2 trap and detection system, yeast cytochrome *c* peroxidase, was exhausted by formation of the H_2O_2–peroxidase complex. The amount of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone required to produce a maximal effect on H_2O_2 formation in antimycin-supplemented mitochondria was about 0.12–0.2 μM with endogenous substrate and succinate/glutamate, similar to a concentration of 0.2 μM required to produce the transition of O_2 uptake from State 4 to State 3u (Fig. 3). Other protophores, such as pentachlorophenol and carbonyl cyanide *m*-chloromethoxyphenylhydrazone, produced a similar increase in H_2O_2-production when added to antimycin-supplemented mitochondria. Linear dependences of H_2O_2-production rates on protein concentration were observed in all cases.

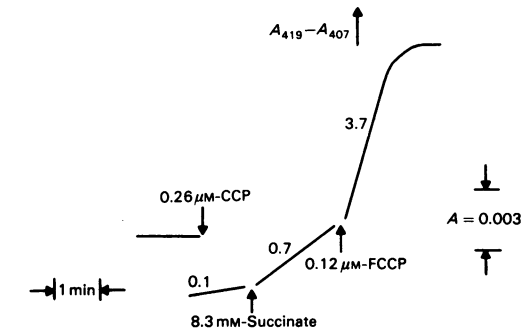

Fig. 2. *Effect of carbonyl cyanide p-trifluoromethoxyphenylhydrazone on the production of H_2O_2 by antimycin-supplemented mitochondria*

Assay conditions were as described in the Materials and Methods section. Rat heart mitochondria equivalent to 0.8 mg of protein/ml were used; antimycin was used at a concentration of 1.3 nmol/mg of protein. Numbers adjacent to the traces indicate nmol of H_2O_2/min per mg of protein. Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCP, yeast cytochrome *c* peroxidase.

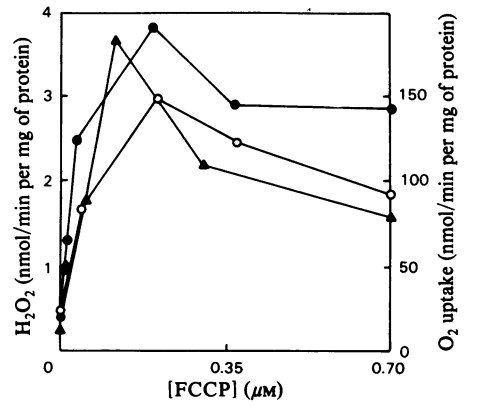

Fig. 3. *Titration of the mitochondrial production of H_2O_2 and O_2 uptake with carbonyl cyanide p-trifluoromethoxyphenylhydrazone*

Assays were performed in mannitol/sucrose/Tris/Mops buffer, pH 7.4. The rate of H_2O_2 production was assayed as described in the Materials and Methods section with yeast cytochrome *c* peroxidase as detector of H_2O_2 production in antimycin-inhibited rat heart mitochondria (0.43 mg of protein/ml) either in the presence (●) or in the absence (▲) of 7.6 mM-succinate. O_2 uptake (○) was measured in the same buffer mixture with rat heart mitochondria (0.39 mg of protein/ml) and 7.6 mM-succinate.

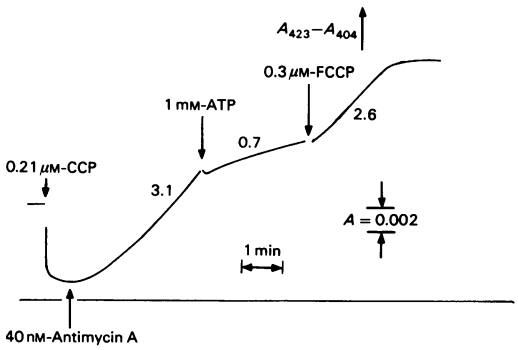

Fig. 4. *Effect of ATP and p-trifluoromethoxyphenylhydrazone on the production of H_2O_2 by mitochondria*
Assay conditions were as described in the legend to Fig. 2. Pigeon heart mitochondria equivalent to 0.7 mg of protein/ml were used. The assay was performed in the presence of endogenous substrate. Numbers adjacent to the traces indicate nmol of H_2O_2/min per mg of protein. Abbreviations: CCP, yeast cytochrome *c* peroxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

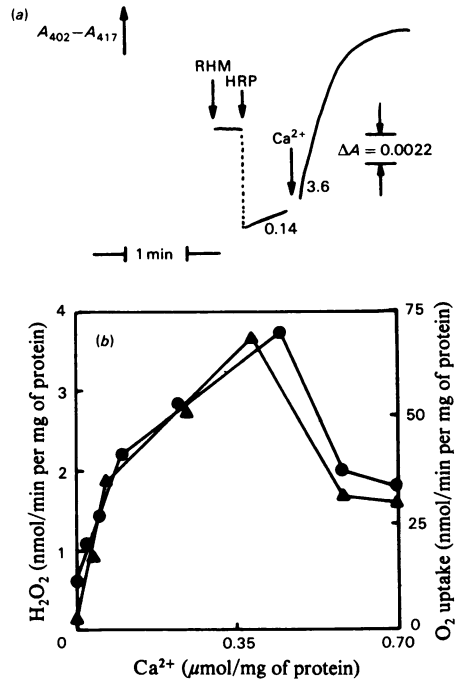

Fig. 5. *Effect of Ca^{2+} on the generation of H_2O_2 and O_2 uptake by mitochondria*
The buffer mixture utilized consisted of mannitol/sucrose/Tris/Mops/1.0 mM-$MgCl_2$/1.0 mM-P_i (pH 7.4). (*a*) Rat heart mitochondria (RHM) (0.42–0.48 mg of protein/ml) and 1.5 nmol of antimycin/mg of protein and horseradish peroxidase (HRP) were used to detect rates of H_2O_2 production (as described in the Materials and Methods section). Ca^{2+} was added at a final concentration of 0.4 μmol/mg of protein. The assay was performed in the presence of endogenous substrate. Numbers adjacent to the traces indicate nmol of H_2O_2/min per mg of protein. (*b*) Assay conditions for generation of H_2O_2 (▲) were as in (*a*); O_2 uptake (●) was estimated in the same buffer mixture in the presence of 8.3 mM-succinate.

An inhibitory effect of the energized state of the mitochondrial membrane on H_2O_2-generation rates was observed. Under conditions of low electron flow, in antimycin-supplemented pigeon heart mitochondria with endogenous substrate, ATP addition decreased H_2O_2 production by a factor of about 4; this inhibitory effect was cancelled by the addition of protophore (Fig. 4).

Effect of Ca^{2+} on H_2O_2 generation by antimycin-supplemented mitochondria

Ca^{2+} exerted an enhancing effect on H_2O_2 formation by antimycin-supplemented mitochondria (Fig. 5*a*). The maximal stimulatory effect was obtained in the range 0.38–0.45 μmol of Ca^{2+}/mg of protein, a similar concentration to the one required to produce maximal stimulation of O_2 uptake in State 4 by rat heart mitochondria (Fig. 5*b*). H_2O_2 assays were performed in the absence of added substrate; the rate of H_2O_2 production with endogenous substrates was about 0.14 nmol/min per mg of protein and after the addition of 0.4 μmol of Ca^{2+}/mg of protein was increased up to 3.6 nmol/min per mg of protein.

Effect of ionophores on H_2O_2 formation by antimycin-supplemented mitochondria

Valinomycin and gramicidin were first assayed for their dependence on protein concentration; the stimulatory effect of valinomycin on O_2 uptake by rat heart mitochondria proved to be independent of protein concentration, whereas gramicidin resulted in a dependence. Thus in Fig. 6 valinomycin and gramicidin concentrations were expressed in μM and nmol/mg of protein respectively, to compare the effects of H_2O_2 production and stimulation of State 4 O_2 uptake. The concentration of both ionophores necessary to obtain the maximal stimulatory effect on H_2O_2 production was matched by the concentration necessary to obtain the maximal stimulation of O_2 uptake. Valinomycin augmented H_2O_2-production rates by about 3.2-fold at a concentration of 0.25 μM. Gramicidin produced a 2.2-fold increase in H_2O_2 production at a concentration of 2 nmol/mg of protein. Both effects were observed in

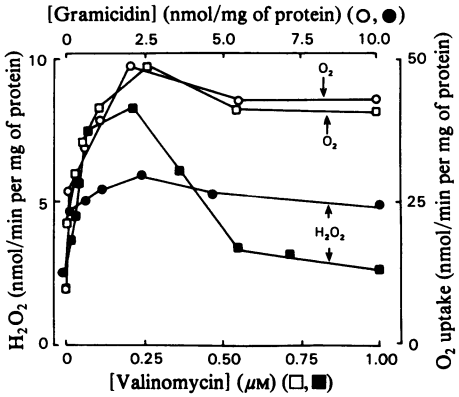

Fig. 6. *Effect of ionophores on the generation of H_2O_2 and O_2 uptake by mitochondria*

The rates of H_2O_2 production by rat heart mitochondria (0.11 mg of protein/ml) were determined in the presence of antimycin (1.2 nmol/mg of protein) and with endogenous substrate as described in the Materials and Methods section. O_2 uptake was assayed in the presence of 8.6 mM-succinate in a buffer containing 0.45–0.51 mg of protein/ml as rat heart mitochondria. The buffer mixture utilized for both assays consisted of mannitol/sucrose/Tris/Mops/2.0 mM-NaCl/2 mM-KCl (pH 7.4).

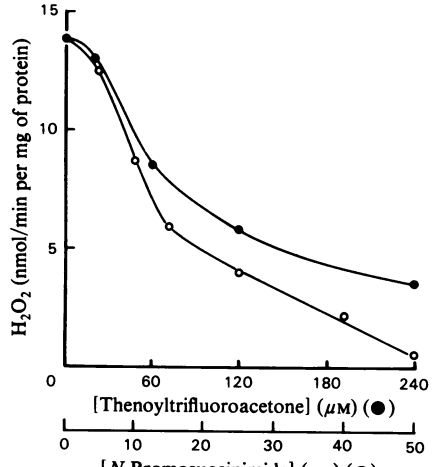

Fig. 7. *Inhibitory effect of thenoyltrifluoroacetone and N-bromosuccinimide on the mitochondrial production of H_2O_2*

Pigeon heart mitochondria (0.85 mg of protein/ml) in mannitol/sucrose/Tris/Mops buffer (pH 7.4) were used. The assay was carried out as indicated in the Materials and Methods section with cytochrome *c* peroxidase as detector system and in the presence of antimycin (1.3 nmol/mg of protein).

antimycin-supplemented mitochondria in the presence of endogenous substrate.

Effect of inhibitors on H_2O_2 and O_2^- generation by mitochondrial membranes

Thenoyltrifluoroacetone and *N*-bromosuccinimide have been used as relatively specific inhibitors of electron transfer from succinate dehydrogenase to ubiquinone (Garland *et al.*, 1967; Wong, 1967). Thenoyltrifluoroacetone has been shown to specifically inhibit electron transfer from Fe-S centre S-3 (cluster 3) of succinate dehydrogenase to oxidized ubiquinone (Konstantinov & Ruuge, 1977) and ubisemiquinone pairs (Ingledew & Ohnishi, 1977). Effects of both inhibitors thenoyltrifluoroacetone and *N*-bromosuccinimide were assayed on H_2O_2 formation by antimycin- and uncoupler-supplemented mitochondria. Thenoyltrifluoroacetone markedly decreased H_2O_2 production with a 74% maximal inhibition and a half-maximal effect at about 60 μM (Fig. 7). *N*-Bromosuccinimide was a very effective inhibitor (up to 96%) of H_2O_2 production with a half-maximal effect at 13 μM. *N*-Bromosuccinimide reacts with cytochrome *c* peroxidase, producing an increase in A_{419} (with respect to 407 nm) that can mimic the reaction for the detection of H_2O_2; proper controls showed that this effect never exceeded an absorption change corresponding to 30% of the rate of H_2O_2 formation. The values given in Fig. 8 have been corrected for this *N*-bromosuccinimide effect.

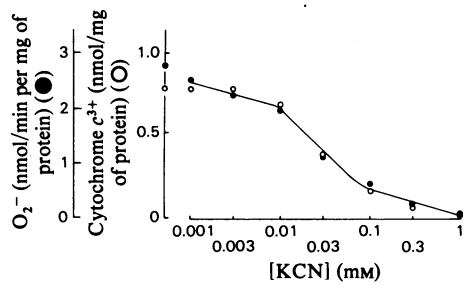

Fig. 8. *Effect of cyanide on the production of O_2^- by antimycin-supplemented submitochondrial particles*

Submitochondrial particles (0.45 mg of protein/ml) were suspended in mannitol/sucrose/Tris/Mops buffer, pH 7.8. Antimycin was present at a final concentration of 1.4 nmol/mg of protein. O_2^- was measured as indicated in the Materials and Methods section. Oxidized cytochrome *c* content was determined in the same submitochondrial particles by the increase in the absorbance at 550–540 nm in an Aminco–Chance double-beam spectrophotometer. The temperature was 23°C.

Cyanide reacts with cytochrome *c* peroxidase and horseradish peroxidase and because of this effect it cannot be assayed on H_2O_2 production, since the peroxidases constitute the detection system. The

effect of cyanide was consequently assayed on the rate of formation of O_2^-, the precursor of H_2O_2, in beef heart submitochondrial particles. Cyanide effectively inhibited O_2^- production by submitochondrial particles; at 1 mM-cyanide the remaining O_2^- production was less than 1%; half-maximal effects were observed at about 0.03 mM (Fig. 8). The formation of O_2^- was paralleled by the amount of cytochrome *c* remaining oxidized on addition of cyanide. Increased reduction of cytochrome *c* observed on addition of cyanide was accompanied by a decreased rate of O_2^- formation (Fig. 8).

Discussion

The interactions of ubiquinone with cytochromes *b* and c_1 and succinate dehydrogenase have been explained by different cyclic mechanisms first proposed by Wikstrom & Berden (1972) and by Mitchell (1976), which agree on obligatory univalent electron donation, with ubisemiquinone formation, in both the reduction and the oxidation of the quinone. We have proposed that O_2^-, the precursor of mitochondrial H_2O_2 (Boveris & Cadenas, 1975; Dionisi *et al.*, 1975), is generated mainly by the autoxidation of the free radical ubisemiquinone (Boveris *et al.*, 1976) being supported by the model reaction of quinol autoxidation (Cadenas *et al.*, 1977).

The new results reported in the present paper (Figs. 7 and 8) are consistent with such previous interpretation. We can thus write the ubiquinone reactions as follows:

$$Q + (\text{cluster } 3)^{2+} + H^+ \rightarrow QH^\cdot + (\text{cluster } 3)^{3+} \quad (1)$$

$$QH^\cdot + (\text{cluster } 3)^{2+} + H^+ \rightarrow QH_2 + (\text{cluster } 3)^{3+} \quad (2)$$

$$QH_2 + c_1{}^{3+} \rightarrow QH^\cdot + c_1{}^{2+} + H^+ \quad (3)$$

$$QH^\cdot + b^{3+} \rightarrow Q + b^{2+} + H^+ \quad (4)$$

$$b^{2+} + c_1{}^{3+} \rightarrow b^{3+} + c_1{}^{2+} \quad (5)$$

$$QH^\cdot + O_2 \rightarrow Q + H^+ + O_2^- \quad (6)$$

The marked enhancing effect of antimycin on O_2^- generation (Loschen *et al.*, 1971; Boveris & Chance, 1973) is accounted for by the inhibitions of cytochrome *b* oxidation by cytochrome c_1 (eqn. 5) and consequently of the fast ubisemiquinone oxidation by cytochrome b^{3+} (eqn. 4), which will increase the steady-state level of ubisemiquinone produced in eqn. (3). Ubiquinone and cytochrome *b* equilibrate reduction levels in a fast reaction (Boveris *et al.*, 1972*b*). Ubisemiquinone is oxidized by molecular O_2 yielding O_2^- (eqn. 6) by a slow non-enzymic reaction with a *k* value of $40\ M^{-1}\cdot s^{-1}$ (Boveris *et al.*, 1976).

Cyanide decreases O_2^- production by inhibiting eqn. (3), which yields ubisemiquinone. The effect of thenoyltrifluoroacetone inhibiting H_2O_2 shown in Fig. 7 agrees with the direct measurements of a decrease in ubiquinone free-radical steady-state concentration (Konstantinov & Ruuge, 1977; Ingledew & Ohnishi, 1977). The apparent incongruity with the thenoyltrifluoroacetone-stimulated O_2^- production in succinate–cytochrome *c* reductase (Trumpower & Simmons, 1979) could be explained on the basis of different kinetic modalities of the quinone cycle, with regard to rate-limiting steps operative in the different experimental preparations.

At present we are unable to distinguish, on the basis of O_2^- and H_2O_2 formation, between the ubisemiquinone produced by reduction of ubiquinone (eqn. 1) and from that by oxidation of ubiquinol (eqn. 4). Such a distinction could be possible, however, if one of those species could enjoy more stability, as offered by electronic delocalization of ubisemiquinone pairs (Ingledew *et al.*, 1976) or binding to specific proteins (Yu *et al.*, 1977). The ubisemiquinone associated with the succinate dehydrogenase S-3 Fe-S centre (cluster 3) seems more stable than the ubisemiquinone produced by the cytochrome *b*–c_1 region (T. Ohnishi & B. L. Trumpower, personal communication), the latter species being the main source of production of O_2^- in submitochondrial particles (Fig. 8).

Although the electron flow in antimycin-supplemented mitochondria is relatively low, there is experimental evidence that antimycin-insensitive O_2 uptake produces proton extrusion (Mitchell & Moyle, 1967) and generates a protonmotive force. Klingenberg & Rottenberg (1977) have measured the ratio $[Rb_{int.}]/[Rb_{ext.}]$ in rubidium- and valinomycin-supplemented mitochondria and found a distribution that corresponds to a $\Delta\psi$ of 72 and 48 mV for succinate- and succinate-plus-antimycin-supplemented rat liver mitochondria respectively. In the conditions under which our experiments were performed, in highly buffered solutions, the second term of Mitchell's equation $[\Delta p = \Delta\psi - \Delta pH]$ could be considered as approaching zero. The effect of protophores (Figs. 2 and 3), ionophores (Fig. 6) and Ca^{2+} (Fig. 5) can be explained by a collapse of membrane potential ($\Delta\psi$). Indeed, measurements of $[Rb_{int.}]/[Rb_{ext.}]$ show that the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to antimycin-supplemented rat liver mitochondria decreases $\Delta\psi$ values from 48 mV to 14 mV (Klingenberg & Rottenberg, 1977).

It might be inferred that membrane potential would control the rate of ubisemiquinone formation (eqns. 1 and 3) as shown by the increased rate of H_2O_2 production. Control by membrane potential of the transfer reactions between succinate dehydrogenase centre S-3 (cluster 3) and ubiquinone (eqn. 1) and ubiquinol and cytochrome c_1 (eqn. 3) might afford a molecular device, similar to microelectrophoresis or electrically induced conformational

changes, able to regulate electron flow and transmembrane proton transport.

We thank Professor B. Chance for his support and ideas and Dr. T. Ohnishi, Dr. B. L. Trumpower and Dr. H. Rottenberg for useful comments. This work was supported by grants U.S.P.H.S. HL-SCOR-15061 and TW-02457 and from Consejo Nacional de Investigaciones Cientificas y Técnicas (Argentina). E. C. is a U.S.P.H.S. Fogarty International Research Fellow.

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