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The Mitochondrial Generation of Hydrogen Peroxide

GENERAL PROPERTIES AND EFFECT OF HYPERBARIC OXYGEN

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1. Pigeon heart mitochondria produce H_2O_2 at a maximal rate of about 20nmol/min per mg of protein. 2. Succinate–glutamate and malate–glutamate are substrates which are able to support maximal H_2O_2 production rates. With malate–glutamate, H_2O_2 formation is sensitive to rotenone. Endogenous substrate, octanoate, stearoyl-CoA and palmitoylcarnitine are by far less efficient substrates. 3. Antimycin A exerts a very pronounced effect in enhancing H_2O_2 production in pigeon heart mitochondria; 0.26nmol of antimycin A/mg of protein and the addition of an uncoupler are required for maximal H_2O_2 formation. 4. In the presence of endogenous substrate and of antimycin A, ATP decreases and uncoupler restores the rates of H_2O_2 formation. 5. Reincorporation of ubiquinone-10 and ubiquinone-3 to ubiquinone-depleted pigeon heart mitochondria gives a system in which H_2O_2 production is linearly related to the incorporated ubiquinone. 6. The generation of H_2O_2 by pigeon heart mitochondria in the presence of succinate–glutamate and in metabolic state 4 has an optimum pH value of 7.5. In states 1 and 3u, and in the presence of antimycin A and uncoupler, the optimum pH value is shifted towards more alkaline values. 7. With increase of the partial pressure of O_2 to the hyperbaric region the formation of H_2O_2 is markedly increased in pigeon heart mitochondria and in rat liver mitochondria. With rat liver mitochondria and succinate as substrate in state 4, an increase in the pO_2 up to 1.97MPa (19.5atm) increases H_2O_2 formation 10–15-fold. Similar pO_2 profiles were observed when rat liver mitochondria were supplemented either with antimycin A or with antimycin A and uncoupler. No saturation of the system with O_2 was observed up to 1.97MPa (19.5atm). By increasing the pO_2 to 1.97MPa (19.5atm), H_2O_2 formation in pigeon heart mitochondria with succinate as substrate increased fourfold in metabolic state 4, with antimycin A added the increase was threefold and with antimycin A and uncoupler it was 2.5-fold. In the last two saturation of the system with oxygen was observed, with an apparent K_m of about 71kPa (0.7–0.8atm) and a $V_{max.}$ of 12 and 20nmol of H_2O_2/min per mg of protein. 8. It is postulated that in addition to the well-known flavin reaction, formation of H_2O_2 may be due to interaction with an energy-dependent component of the respiratory chain at the cytochrome *b* level.

The generation of H_2O_2 by animal mitochondria seems to be established as a physiological event under aerobic conditions. Different techniques, generally utilizing catalases and peroxidases, have been applied for the determination of the mitochondrial production of H_2O_2. The peroxisomal catalase has served as an endogenous indicator of H_2O_2 for the mitochondrial–peroxisomal fraction of rat liver (Chance & Oshino, 1971) and for the perfused liver (Sies & Chance, 1970; Oshino *et al.*, 1973). The scopeletin method (Andreae, 1955), in which the fluorescent dye acts as a hydrogen donor for horseradish peroxidase, revealed that the rate of production of H_2O_2 depends on the metabolic state in pigeon heart mitochondria (Loschen *et al.*, 1971). The enzyme–substrate complex of yeast cytochrome *c* peroxidase has been used as a sensitive, specific and accurate spectrophotometric indicator of H_2O_2 to measure the mitochondrial generation of H_2O_2 in pigeon heart (Chance *et al.*, 1971) and in rat liver mitochondria (Boveris *et al.*, 1972*a*). The cytochrome *c* peroxidase assay showed that mitochondria produce H_2O_2 at a rate that accounts for 1–2% of the total uptake of O_2 in state 4 (Chance & Williams, 1956) whereas in state 3 the rate of generation of H_2O_2 is negligible (Chance *et al.*, 1971; Boveris *et al.*, 1972*a*). In addition to mitochondria, microsomal and peroxisomal fractions and soluble enzymes were shown to be sources of H_2O_2 in rat liver (Boveris *et al.*, 1972*a*).

After the recognition of the continuous generation of H_2O_2 by mitochondria, a more detailed study became necessary to evaluate the variations of forma-

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tion of H_2O_2 under different metabolic conditions.

The present paper reports on some general properties of the mitochondrial generation of H_2O_2, namely: (a) the substrate specificity; (b) the effect of the antibiotic antimycin A and the dependence of its effect on the energy-state of the mitochondrial membranes; (c) the effect of ubiquinone extraction and its reincorporation on the rates of formation of H_2O_2; (d) the pH dependence; (e) the dependence of formation of H_2O_2 on the partial pressure of O_2 in the hyperbaric region.

Materials and Methods

Production of H_2O_2 was measured by the yeast cytochrome *c* peroxidase method (Boveris *et al.*, 1972*a*), either a filter double-beam Johnson Research Foundation spectrophotometer or a model 356 Perkin–Elmer double-beam spectrophotometer being used. A value of $\Delta E_{mM} = 60$ litre·mmol^{-1}·cm^{-1} was used for $E_{423}-E_{404}$, calculated from Yonetani (1965). Yeast cytochrome *c* peroxidase, prepared by the method of Yonetani & Ray (1965), was kindly supplied by Professor T. Yonetani, Johnson Research Foundation, University of Pennsylvania. Cytochrome *b* was monitored at $E_{563}-E_{575}$ ($\Delta E_{mM} = 22$ litre·mmol^{-1}·cm^{-1}) in a Johnson Research Foundation double-beam spectrophotometer.

Pigeon heart mitochondria were obtained as described by Chance & Hagihara (1963). Rat liver mitochondria were prepared by the method of Schneider (1948) in mannitol–sucrose–EDTA buffer (225mM-mannitol – 75mM-sucrose – 0.2mM-EDTA, pH7.2). Ubiquinone-depleted mitochondria were prepared by extraction with the acetone–water (96:4, v/v) mixture as described by Lester & Fleischer (1961). Reincorporation of ubiquinone-3 and ubiquinone-10 was carried out as described by Boveris *et al.* (1972*b*).

Oxygen uptake was measured in 225mM-mannitol–75mM-sucrose – 40mM-Tris – morpholinopropanesulphonic acid buffer, pH6.8 and 8.2, by using a Clark O_2 electrode. Protein was measured by the biuret method (Gornall *et al.*, 1949).

The hyperbaric experiments were carried out in a small hyperbaric chamber attached to the filter-double-beam spectrophotometer. The chamber had a round cuvette with an actual light-path of 0.8cm and was equipped with a magnetic stirrer rotated by an external magnet to provide rapid equilibrium of the liquid phase with the high-pressure O_2.

Results

Substrate specificity and effect of antimycin A

Fig. 1 illustrates the substrate specificity and the antimycin A effect on the generation of H_2O_2 by pigeon heart mitochondria. In these traces production of H_2O_2 is detected as formation of the enzyme–substrate complex of yeast cytochrome *c* peroxidase with an increase in $E_{423}-E_{404}$. In Fig. 1(*a*), production of H_2O_2 starts with the addition of succinate

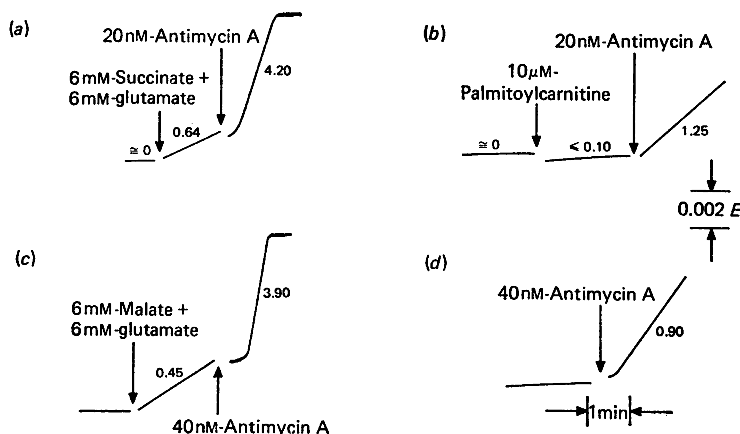

Fig. 1. *Mitochondrial generation of H_2O_2 supported by different substrates and the effect of antimycin A*

For details see the text. Pigeon heart mitochondria [0.028 (*a* and *b*) and 0.056 (*c* and *d*) mg of protein/ml] were suspended in 225mM-mannitol–75mM-sucrose–30mM-Tris–morpholinopropanesulphonic acid, pH7.4, and supplemented with 0.21μM-cytochrome *c* peroxidase. Values against the traces indicate nmol of H_2O_2/min per mg of protein.

plus glutamate, at a rate of 0.64nmol/min per mg of protein. Addition of antimycin A increases the rate of formation of H_2O_2 by approximately sevenfold. Under these conditions, formation of H_2O_2 practically accounts for the antimycin A-insensitive consumption of O_2 (Chance *et al.*, 1971). In Fig. 1(*b*), palmitoylcarnitine supports production of H_2O_2 and behaves much less efficiently than succinate plus glutamate, both before and after addition of antimycin A. Fig. 1(*c*) shows the production of H_2O_2 when malate plus glutamate are utilized as substrates. The rate of 0.45nmol of H_2O_2/min per mg of protein is increased by approximately sevenfold after addition of antimycin A. Fig. 1(*d*) shows the effect of antimycin A when the reducing equivalent is provided by endogenous substrate.

Table 1 gives a systematic list of the rates of generation of H_2O_2 by pigeon heart mitochondria supplemented with different substrates both under coupled and uncoupled conditions and also both in the absence and in the presence of antimycin A. Malate plus glutamate and succinate plus glutamate support formation of H_2O_2 at a rate of 0.7–0.8nmol/min per mg of protein. Addition of uncoupler abolishes the formation, whereas ATP and oligomycin have no effect on the metabolic state 4 rates of generation of H_2O_2. Addition of antimycin A produces a marked effect on the H_2O_2 generator. The antibiotic increased formation of H_2O_2 by four- to five-fold with mitochondria in state 4, or in the presence of ATP or oligomycin. Moreover, when antimycin A is added to uncoupler-supplemented mitochondria with a negligible formation of H_2O_2, the maximal rates of generation, about 15nmol/min per mg of protein, are observed.

Endogenous substrate produces a low generation of H_2O_2, which can be slightly increased by addition of ATP, probably owing to fatty acid activation. Under these experimental conditions, under which only a limited electron flux is available to the H_2O_2 generator, addition of antimycin A provides submaximal rates of generation of H_2O_2. ATP has a definite effect in inhibiting formation of H_2O_2 in the presence of antimycin A and endogenous substrate. This effect can be reversed by addition of an uncoupler.

Fig. 2 illustrates the inhibition of formation of H_2O_2 supported by malate plus glutamate that takes place after addition of rotenone. A half-maximal effect is seen at approx. 5nmol of rotenone/mg of protein. Rotenone has no effect on the succinate plus glutamate-supported generation of H_2O_2. Fig. 2 shows as well the effect of rotenone in decreasing the reduction level of cytochromes *b*, which were monitored to indicate the reduction level of the approximate zero mid-potential pool of flavo-

Table 1. *Production of H_2O_2 by pigeon heart mitochondria*

Pigeon heart mitochondria (0.028–0.052mg of protein/ml) were suspended in 225mM-mannitol–75mM-sucrose–30mM-Tris–morpholinopropanesulphonic acid, pH7.4, and supplemented with 0.4μM-cytochrome *c* peroxidase.

Substrate and additions	Generated H_2O_2 (nmol/min per mg of protein)	
	Without antimycin A	With antimycin A (0.05μM)
Succinate (6mM)+glutamate (4mM)		
State 4	0.7	3.9
Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.2μM)	0	16.0
ATP (1mM)	0.8	3.8
Oligomycin (0.1μM)	0.6	4.0
Malate (6mM)+glutamate (6mM)		
State 4	0.8	4.2
Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.2μM)	0	15.0
ATP (1mM)	0.8	3.7
Oligomycin (0.1μM)	0.6	3.9
Endogenous		
State 1	0.05	2.5
Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.2μM)	0	2.2
ATP (1mM)	0.2	0.6
Oligomycin (0.1μM)	0.06	2.0

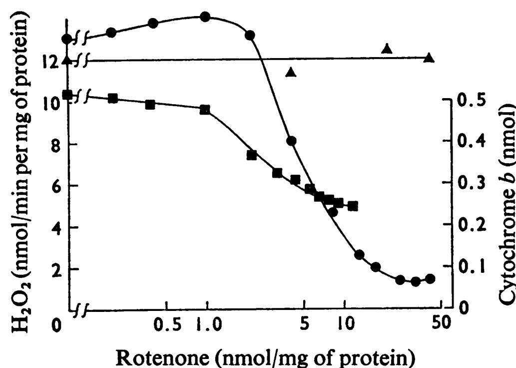

Fig. 2. *Effect of rotenone on formation of H_2O_2 and on the reduction level of cytochrome b*

Formation of H_2O_2 is shown in the presence of 6mM-malate+6mM-glutamate (●) and of 6mM-succinate+4mM-glutamate (▲). ■, Reduction level of cytochrome *b* in the presence of 6mM-malate+6mM-glutamate. Pigeon heart mitochondria were suspended, at 0.025 (▲, ●) and 2.1 (■) mg of protein/ml, in the reaction medium described in Fig. 1; either 60nM-antimycin A and 0.3μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (▲, ●) or 0.6μM-antimycin A and 3μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (■) were present.

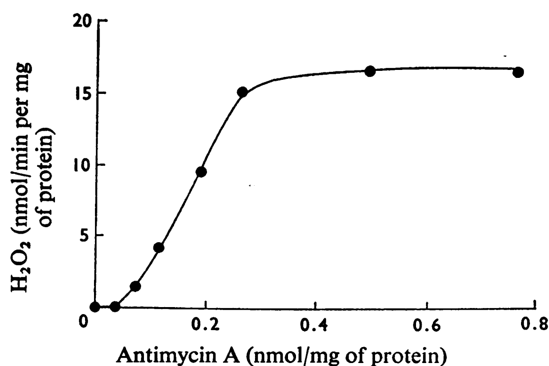

Fig. 3. *Effect of antimycin A on formation of H_2O_2*

Pigeon heart mitochondria (0.06mg of protein/ml) were suspended in the reaction medium described in Fig. 1, and supplemented with 6mM-succinate+6mM-glutamate, 0.3μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 0.3μM-cytochrome *c* peroxidase.

proteins, ubiquinone, cytochromes *b* and non-haem iron that lies between the rotenone- and the antimycin A-sensitive sites. A rotenone concentration of

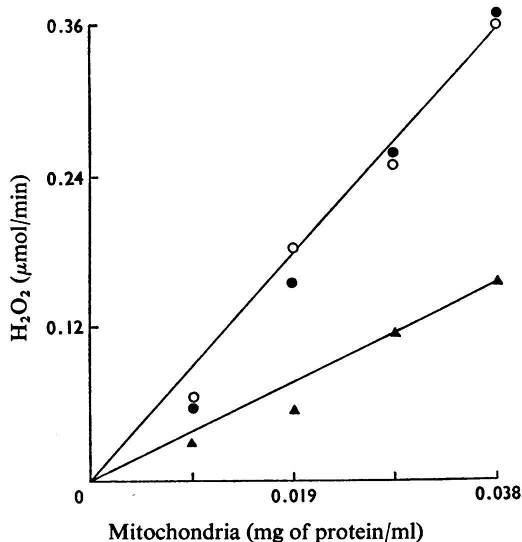

Fig. 4. *Formation of H_2O_2 supported by palmitoylcarnitine and succinate plus glutamate in pigeon heart mitochondria*

▲, 10μM-Palmitoylcarnitine; ○, 6mM-succinate+4mM-glutamate; ●, 10μM-palmitoylcarnitine+6mM-succinate+4mM-glutamate. Other experimental conditions were as described for Fig. 1.

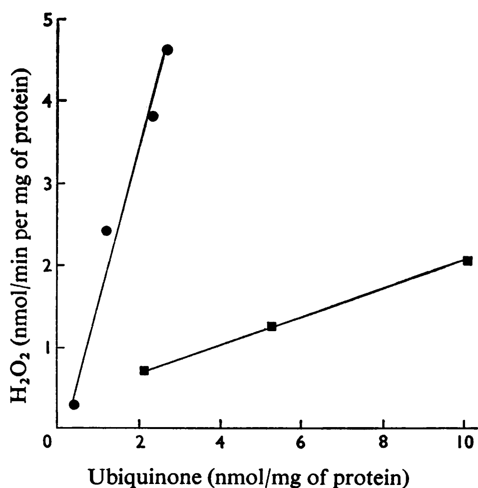

Fig. 5. *Generation of H_2O_2 in ubiquinone-depleted and ubiquinone-reincorporated pigeon heart mitochondria*

For details see the text. Treated pigeon heart mitochondria were suspended in the reaction medium described in Fig. 1 at a concentration of 0.03mg of protein/ml and supplemented with 1.4μM-cytochrome *c* peroxidase, 6mM-succinate and 0.3μM-antimycin A. ●, Ubiquinone-10 incorporated; ■, ubiquinone-3 incorporated.

2.5nmol/mg of protein produced a half-maximal effect on the reduction of cytochromes *b*. These rotenone concentrations that produce half-maximal effects, and whose correlation is considered valid in view of the different protein concentrations utilized in both assays, are by far higher than those that inhibit electron transfer over 95% at the NADH-dehydrogenase site (usually about 0.5nmol/mg of protein). This indicates that a severe inhibition at the rotenone-sensitive site is required to reduce the rates of electron flux to cytochromes *b* and the H_2O_2 generator below the values that give maximal reduction of cytochromes *b* and formation of H_2O_2.

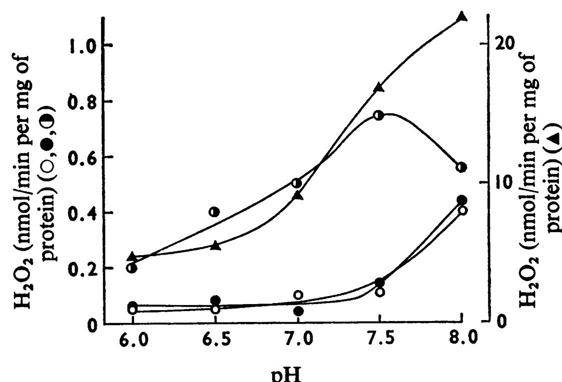

Fig. 6. *Dependence of formation of H_2O_2 on the pH values in pigeon heart mitochondria*

Mitochondria (0.2mg of protein/ml) were suspended in 225mM-mannitol – 75mM-sucrose – 30mM-Tris – morpholinopropanesulphonic acid buffer at the indicated pH values, and supplemented with 1.2μM-cytochrome *c* peroxidase. ○, State 1, without any additions; ◑, state 4 (6mM-succinate+4mM-glutamate); ●, state 3u (6mM-succinate+4mM-glutamate +2μM-pentachlorophenol); ▲, 6mM-succinate+ 4mM-glutamate, 2μM-pentachlorophenol and 0.5μM-antimycin A.

Fig. 3 shows a titration of the mitochondria with antimycin A. Succinate plus glutamate and uncoupler were present to provide the conditions for maximal formation of H_2O_2. An antimycin A concentration of 0.26nmol/mg of protein gives maximal effect; this value correlates closely with the amount of either cytochrome b_T or b_K in pigeon heart mitochondria (0.20nmol/mg of protein; Sato *et al.*, 1971; Boveris *et al.*, 1972*b*).

The eventual additivity of production of H_2O_2 when supported by two different substrates was studied as an approach to test the uniqueness of the mitochondrial generator of H_2O_2. Fig. 4 shows generation of H_2O_2 in the presence of palmitoylcarnitine, succinate plus glutamate and succinate plus glutamate and palmitoylcarnitine. In all cases uncoupler and antimycin A were added to obtain maximal formation of H_2O_2. The results obtained seem to show that there is one H_2O_2 generator that can utilize reducing equivalents coming from any or both substrates. Incidentally, Fig. 4 also illustrates the sensitivity of the yeast cytochrome *c* peroxidase assay, which is able to detect rates of production of the order of 0.15μmol of H_2O_2/min with mitochondrial concentrations of the order of 0.02mg of protein/ml.

Generation of H_2O_2 in ubiquinone-depleted and ubiquinone-incorporated mitochondria

Acetone treatment, used to extract the endogenous ubiquinone, produces damaged mitochondria in which the outer mitochondrial membrane cannot be any longer considered as a barrier that would prevent the interaction of mitochondrial cytochrome *c* with the externally added yeast cytochrome *c* peroxidase. However, in the presence of antimycin A, cytochrome *c* is kept in a highly oxidized state, in which it seems not to interfere with the yeast cytochrome *c* peroxidase assay. Ubiquinone-depleted pigeon heart mitochondria supplemented with succinate and antimycin A generate H_2O_2 at a rate of about 0.26nmol/min per mg of protein (Fig. 5). When these mitochondria are

Table 2. *Effect of antimycin A on uptake of O_2 at different pH values*

Pigeon heart mitochondria (1.1 and 2.2mg of protein/ml for pH values of 6.8 and 8.2 respectively) were suspended in 225mM-mannitol–75mM-sucrose–30mM-Tris–morpholinopropanesulphonic acid, pH6.8 and 8.2.

Additions	Consumption of O_2 (ng-atoms/min per mg of protein)	
	pH6.8	pH8.2
Succinate (6mM)+glutamate (4mM)	18.6	10.4
S-Chloro-3-t-butyl-2′-chloro-4′-nitrosalicylanilide (2μM)	80.0	11.2
Antimycin A (1.5μM)	5.2	20.5

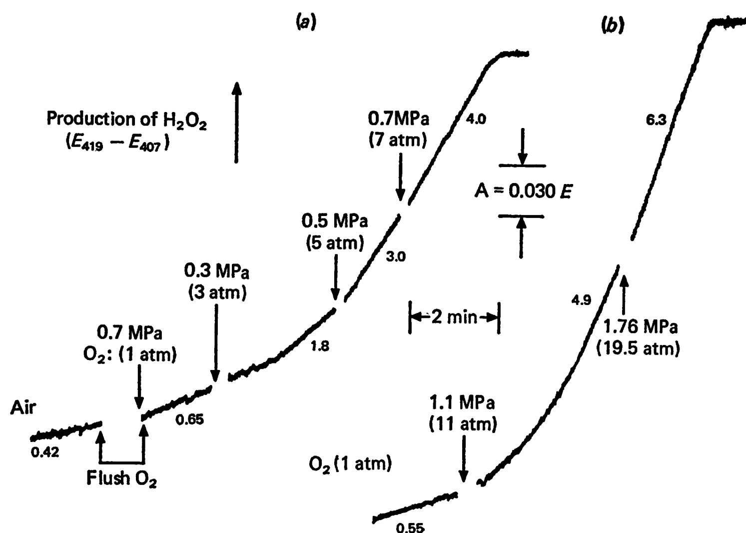

Fig. 7. *Effect of hyperbaric O_2 on generation of H_2O_2 by rat liver mitochondria*

Mitochondria (0.33 mg of protein/ml) were suspended in the reaction medium described in Fig. 1 and supplemented with 6mM-succinate and 2μM-cytochrome *c* peroxidase. For details of (*a*) and (*b*) see the text.

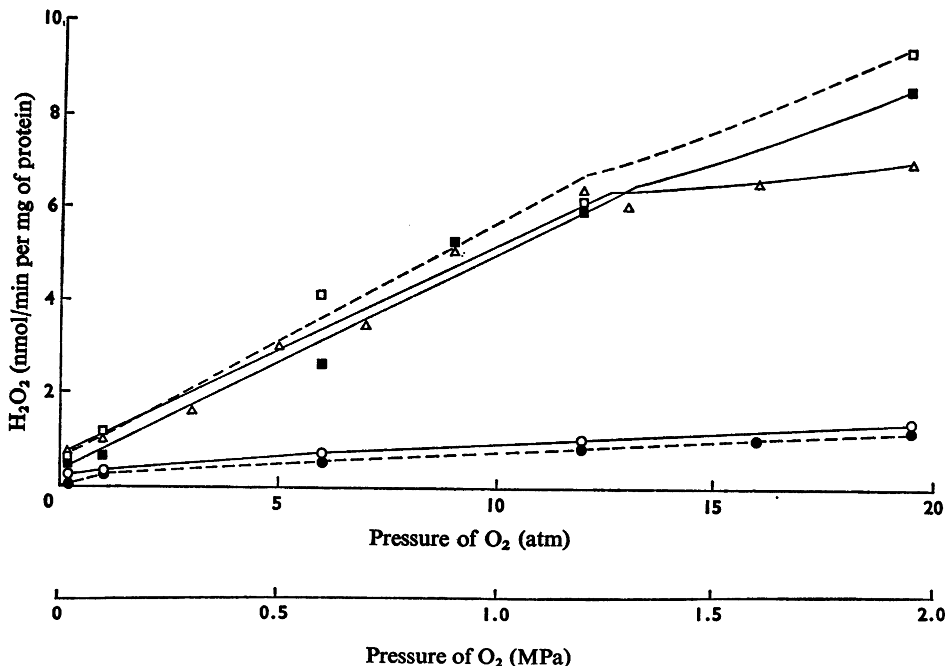

Fig. 8. *Effect of hyperbaric O_2 on formation of H_2O_2 by rat live: mitochondria*

Mitochondria (0.30–0.35 mg of protein/ml) were suspended in the reaction medium described in Fig. 1 and supplemented with 2μM-cytochrome *c* peroxidase. ○, No additions (endogenous substrate); △, succinate, state 4; ●, 6mM-succinate and 2μM-pentachlorophenol; ■, 6mM-succinate and 0.4μM-antimycin A; □, 6mM-succinate, 2μM-pentachlorophenol and 0.4μM-antimycin A.

reconstituted in relation to their endogenous ubiquinone-10, formation of H_2O_2 is linearly related to the amount of incorporated ubiquinone. If this relation is extrapolated to the normal amounts of ubiquinone-10, about 4–5nmol/mg of protein, formation of H_2O_2 would be about 10nmol/min per mg of protein, a value that agrees with the rates observed in the presence of antimycin A and uncoupler. Incorporation of ubiquinone-3 into the mitochondria also increased the ability to generate H_2O_2, although ubiquinone-3 was by far less efficient than ubiquinone-10 in supporting the generation of H_2O_2. It should be noted that the values of ubiquinone content given in the abscissa of Fig. 5 correspond to enzymically reducible ubiquinone, assayed as the decrease of $E_{275}-E_{300}$ after addition of 1mM-KCN and 10mM-succinate. The values of enzymically reducible ubiquinone were identical with the values of incorporated ubiquinone, assayed by the extraction method of Szarkowska & Klingenberg (1963) and reduction by KBH_4.

pH dependence of the mitochondrial generation of H_2O_2

Fig. 6 indicates that formation of H_2O_2 has an optimum pH value of 7.5 with mitochondria in state 4 and with succinate plus glutamate as substrate. In states 1 and 3u, the production of H_2O_2 is low at pH values below 7.5 and increases at a pH value of 8.0, indicating a shift in optimum pH to more alkaline values. A similar dependence on the pH values is observed when the mitochondria are supplemented with uncoupler (pentachlorophenol) and antimycin A. Formation of H_2O_2 in this latter case was about 20-fold that in the former cases. The determinations were not continued at pH values over 8.0 owing to the fact that the indicator, the yeast cytochrome *c* peroxidase, alters its spectroscopic properties at these pH values (Yonetani, 1965).

The high activity of the H_2O_2 generator at alkaline pH values in the presence of uncoupler and antimycin A explains the apparent paradoxical situation (Table 2) in which the addition of the electron-transfer inhibitor, antimycin A, at pH 8.2 stimulates consumption of O_2 by pigeon heart mitochondria.

Dependence of the formation of H_2O_2 on the partial pressure of O_2 in the hyperbaric region

The generation rates for H_2O_2 observed in air-saturated buffers are far from being maximal rates. The phenomenon is illustrated in Fig. 7 with rat liver mitochondria in state 4, and in the presence of succinate as substrate. In Fig. 7(*a*), at the beginning of the trace the rate of production of H_2O_2 in air-saturated buffer was recorded. Oxygen was then flushed through the system to remove N_2. Generation of H_2O_2 increased from 0.42 to 0.65nmol/min per mg of protein. Pressurization of the chamber to approx. 0.3, 0.5 and 0.7MPa (3, 5 and 7atm) further increased production to 1.8, 3.0 and 4.0nmol of H_2O_2/min per mg of protein. Fig. 7(*b*) shows formation of H_2O_2 in O_2-saturated buffer and at 1.1MPa and

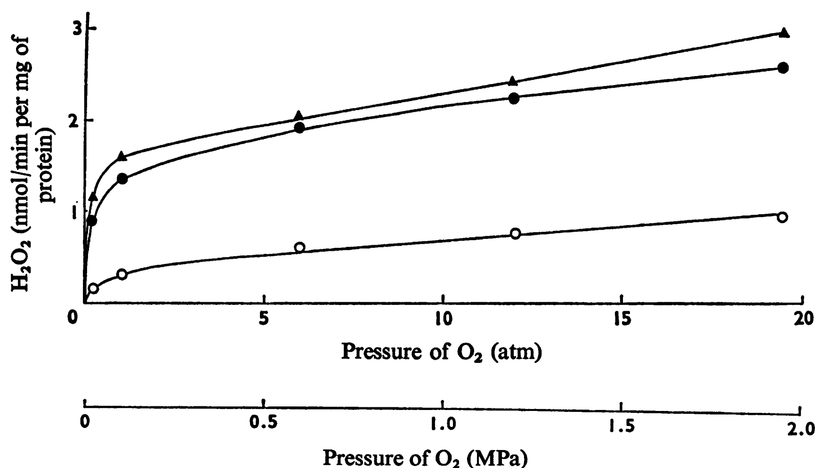

Fig. 9. *Effect of hyperbaric O_2 on generation of H_2O_2 by pigeon heart mitochondria in the presence of endogenous substrate*

Experimental conditions were as described in Fig. 4. ○, No additions; ●, antimycin A added; ▲, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and antimycin A added.

1.97 MPa (11 and 19.5 atm). The 2 min period required for this trace to become linear after pressurization to 1.1 MPa (11 atm) may reflect the time required for equilibration of the sample with the gas phase under our experimental conditions. Fig. 8 shows plots of results similar to the one illustrated by Fig. 7. Formation of H_2O_2 increases with an increased pO_2. The change is less evident when the members of the respiratory chain are in an oxidized state, i.e. in metabolic state 1 (endogenous substrate) and in the presence of uncoupler. On the other hand, when the respiratory chain is reduced, i.e. in metabolic state 4, and in the presence of antimycin A, the increase in pO_2 from approx. 0.2 MPa (0.2 atm) to 1.97 MPa (19.5 atm) increased formation of H_2O_2 by a factor of 10–18. No clear saturation of the generating system was observed at 1.97 MPa (19.5 atm), the limit of pressurization available to our equipment, although some bending of the curves seems evident about 1.21 MPa (12 atm).

Fig. 9 shows the pO_2 dependence of formation of H_2O_2 in pigeon heart mitochondria with endogenous substrate, which seems to be able to provide reducing equivalents at a rate of about 2–6 nequiv./min per mg of protein (see also Fig. 1*d*). The increase of pO_2 up to 1.97 MPa (19.5 atm) enhances formation of H_2O_2 in the presence of (a) antimycin A plus uncoupler or of (b) antimycin A, and (c) in the absence of additions.

Similarly, Fig. 10 shows that generation of H_2O_2 in pigeon heart mitochondria with succinate plus glutamate as substrate, increases linearly with the pO_2 with mitochondria in states 3 and 4. In the presence both of antimycin A and of antimycin A plus uncoupler the curves relating formation of H_2O_2 and pO_2 are hyperbolic. Double-reciprocal plots give K_m values for O_2 of about 0.7–0.8 MPa (0.7–0.8 atm) and $V_{max.}$ of 12 or 20 nmol of H_2O_2/min per mg of protein for the system in the presence of antimycin A or of antimycin A and uncoupler respectively. Thus antimycin A seems to increase the affinity for O_2 of the H_2O_2 generator whereas the presence of uncoupler seems to increase its turnover.

Discussion

Animal mitochondria isolated from different sources have been shown to have the capacity of generating H_2O_2. For example, mitochondria isolated from pigeon heart (Chance *et al.*, 1971; Loschen *et al.*, 1971), rat liver (Boveris *et al.*, 1972*a*), rat and ox heart (Loschen & Azzi, personal communication), rat kidney, yeast, *Ascaris* muscle and *Crithidia fasciculata* (unpublished results from this laboratory) have been shown as active sources of H_2O_2. However, detailed studies on the effects of different metabolic conditions and of mitochondrial inhibitors have been

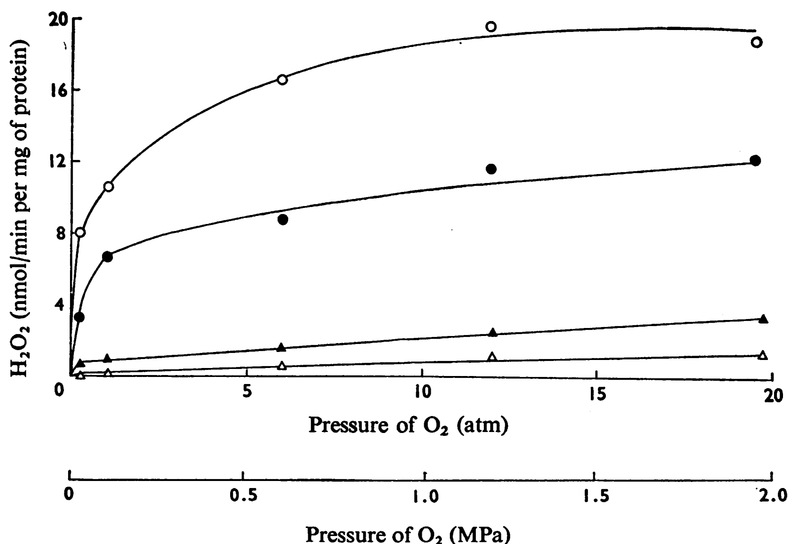

Fig. 10. *Effect of hyperbaric O_2 on formation of H_2O_2 by pigeon heart mitochondria in the presence of 6 mM-succinate +4 mM-glutamate as substrate*

Pigeon heart mitochondria (0.01–0.04 mg of protein/ml) were suspended in the reaction medium described in Fig. 1 and supplemented with 0.4 μM-cytochrome *c* peroxidase. Other experimental conditions were as described in Table 1. △, State 3; ▲, state 4; ●, antimycin A added; ○, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and antimycin A added.

carried out only in pigeon heart and rat liver mitochondria. Although formation of H_2O_2 shows generally similar characteristics, such as a decrease of the rate in the metabolic state 4–state 3 transition, different features are also observed: in pigeon and rat heart mitochondria, uncoupler and antimycin A are required to set maximal formation of H_2O_2, which is not the case for rat liver mitochondria. Addition of uncoupler and antimycin A to mitochondria in state 4 increased formation of H_2O_2 by a factor of 15–20 in pigeon heart mitochondria but only by a factor of 1.5 in rat liver mitochondria. Moreover, another difference is observed in the response to hyperbaric O_2. An increase in pO_2 up to 1.92MPa (19atm) increases formation of H_2O_2 by a factor of about 4 in pigeon heart mitochondria and by a factor of 15–20 in rat liver mitochondria. These differences must be considered in the identification of the mitochondrial generator of H_2O_2.

Different substrates, i.e. succinate, malate–glutamate, fatty acids etc., are able to support formation of H_2O_2 and this suggests that a member of the respiratory chain is responsible for mitochondrial generation of H_2O_2. Flavoproteins are the most likely candidate for this. The evidence that palmitoylcarnitine does not increase generation of H_2O_2 when added to succinate-supplemented mitochondria, along with the fact that succinate does not reduce the flavoproteins of fatty acid oxidation (Garland *et al.*, 1967), would indicate the existence of a common rate-limiting step operating with both substrates. The fact that submitochondrial particles, which contain the members of the respiratory chain but are devoid of the flavoproteins of fatty acid oxidation, generate H_2O_2 (Jensen, 1966*a*,*b*; Hinckle *et al.*, 1967) would indicate that these flavoproteins are not the main source of H_2O_2.

The marked effect of antimycin A on formation of H_2O_2 by pigeon heart mitochondria points to the importance of the redox state of the respiratory carriers. Addition of antimycin A produces maximal reduction of those members of the respiratory chain that are located on the substrate side of the antimycin A-sensitive site, i.e. cytochromes *b*, ubiquinone, non-haem iron and flavoproteins. The fact that addition of an uncoupler, in the presence of antimycin A, is required to obtain maximal rates of generation of H_2O_2 points to the involvement of a component with variable potential, probably changing its potential to more negative values after de-energization of the membrane.

The experiments with ubiquinone-depleted and ubiquinone-reincorporated mitochondria indicate that the quinone must be considered as another important factor in generation of H_2O_2. The ubiquinone effect can be interpreted as an ubiquinone requirement for electron flow to cytochromes *b* (Ernster *et al.*, 1969) or as a manifestation of the regulatory properties of the quinone on the flavoprotein succinate dehydrogenase (Rossi *et al.*, 1970; Gutman *et al.*, 1971).

Non-haem iron centres at succinate dehydrogenase and at the cytochrome *b*–cytochrome c_1 complex [Beinert *et al.*, 1962; Ohnishi, 1973] could generate superoxide anion, as has been shown for ferredoxins (Misra & Fridovich, 1971), and superoxide anions would be a source of H_2O_2 via the superoxide dismutase reaction (McCord & Fridovich, 1969).

Our present data do not allow us to distinguish between the different possibilities but certainly point to a variable potential component as the source or as a regulatory factor of maximal importance for the mitochondrial generation of H_2O_2.

The marked increase in formation of H_2O_2 under hyperbaric conditions and its immediate onset (see Fig. 7) may provide an explanation at the molecular level for 'O_2 poisoning'. In O_2 toxicity we may distinguish between the primary toxic reactant and the sensitive target systems. The primary responsible reactant may be O_2 itself or a product of its partial reduction, like H_2O_2, whose production is markedly increased under hyperbaric conditions, or a free radical, like O_2^- or $OH^{\bullet}$ as postulated by Gerschman (1964).

So far, the increase in formation of H_2O_2 and the inhibition of reversed electron transfer in mitochondria (Chance *et al.*, 1965) constitute the earliest events that could be recognized at subcellular and cellular level under hyperbaric oxygenation.

Since toxicity of H_2O_2 in biological systems is a well-recognized fact, the physiological role of catalase seems obvious. In the peroxisome-containing tissues, like liver and kidney, catalase plays the key role in the regulation of the intracellular amount of H_2O_2 and at the same time participates in cellular oxidations. The control of the intracellular amount of H_2O_2 presents a different problem in those organs, such as brain, lung, heart etc., that lack peroxisomes. In those organs diffusion of H_2O_2 from the tissue to the erythrocyte catalase may provide an equally efficient defence mechanism.

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References

Andreae, W. S. (1955) *Nature (London)* **175**, 859–860

Beinert, H., Heinen, W. & Palmer, C. (1962) *Brookhaven Symp. Biol.* **15**, 229–265

Boveris, A., Oshino, N. & Chance, B. (1972*a*) *Biochem. J.* **128**, 617–630

Boveris, A., Erecinska, M. & Wagner, M. (1972*b*) *Biochim. Biophys. Acta* **256**, 223–242

Chance, B. & Hagihara, B. (1963) *Proc. Int. Cong. Biochem. 5th* **5**, 3–37

Chance, B. & Oshino, N. (1971) *Biochem. J.* **122**, 225–233

Chance, B. & Williams, G. R. (1956) *Advan. Enzymol. Relat. Subj. Biochem.* **17**, 65–134

Chance, B., Boveris, A., Oshino, N. & Loschen, G. (1971) in *2nd International Symposium on Oxidases and Related Redox Systems* (King, T. S. & Morrison, M., eds.), University Park Press, Baltimore, in the press

Chance, B., Jamieson, D. & Coles, H. (1965) *Nature (London)* **206**, 257–263

Ernster, L., Lee, I. Y., Norling, B. & Persson, B. (1969) *FEBS Lett.* **3**, 21–26

Garland, P. B., Chance, B., Ernster, L., Lee, C. P. & Wong, D. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 1696–1702

Gerschman, R. (1964) in *Oxygen in the Animal Organism* (Dickens, F. & Neil, E., eds.), pp. 475–494, Pergamon Press, Oxford

Gornall, A. G., Bardawill, C. S. & David, M. N. (1949) *J. Biol. Chem.* **177**, 751–760

Gutman, M., Kearney, E. B. & Singer, T. P. (1971) *Biochemistry* **10**, 4763–4770

Hinckle, P. C., Butow, R. S., Racker, E. & Chance, B. (1967) *J. Biol. Chem.* **242**, 5169–5173

Jensen, P. K. (1966*a*) *Biochim. Biophys. Acta* **122**, 157–166

Jensen, P. K. (1966*b*) *Biochim. Biophys. Acta* **122**, 167–174

Lester, R. L. & Fleischer, S. (1961) *Biochim. Biophys. Acta* **47**, 358–377

Loschen, G., Flohe, L. & Chance, B. (1971) *FEBS Lett.* **18**, 261–264

McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055

Misra, H. P. & Fridovich, I. (1971) *J. Biol. Chem.* **246**, 6886–6890

Ohnishi, T. (1973) *Biochim. Biophys. Acta* in the press

Oshino, N., Chance, B., Sies, H. & Bucher, Th. (1973) *Arch. Biochem. Biophys.* **154**, 117–131

Rossi, E., Norling, B., Peterson, B. & Ernster, L. (1970) *Eur. J. Biochem.* **16**, 508–513

Sato, N., Wilson, D. F. & Chance, B. (1971) *Biochim. Biophys. Acta* **253**, 88–97

Schneider, W. C. (1948) *J. Biol. Chem.* **176**, 259–266

Sies, H. & Chance, B. (1970) *FEBS Lett.* **11**, 172–176

Szarkowska, L. & Klingenberg, M. (1963) *Biochem. Z.* **338**, 674–697

Yonetani, T. (1965) *J. Biol. Chem.* **240**, 4509–4514

Yonetani, T. & Ray, G. (1965) *J. Biol. Chem.* **240**, 4503–4508