Inhibition of H_2O_2 generation in rat liver mitochondria by radical quenchers and phenolic compounds

Anand SWAROOP and T. RAMASARMA*

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

(Received 29 April 1980/Accepted 25 September 1980)

Generation of H_2O_2 by rat liver mitochondria with choline, glycerol 1-phosphate and proline as substrates has been shown by using high-concentration phosphate buffer. Rates obtained under these conditions were higher and more consistent as compared with the earlier reports with high-concentration mannitol/sucrose/Tris buffer. Sulphate ions could replace phosphate indicating a requirement for a high concentration of oxygen-containing anions. H_2O_2 generation was dependent on the presence of native mitochondria and substrate. Maximal rates with various substrates were found to be the same as with succinate. Values of K_m and V_{max} for H_2O_2 generation were considerably less than those obtained for respective dehydrogenase activities, measured by dye reduction. Scavengers of $\text{O}_2^{\cdot-}$ and $\text{OH}^{\cdot}$ inhibited generation of H_2O_2 . ATP, ADP, thyronine derivatives and a number of phenolic compounds also showed very potent inhibitory effects on H_2O_2 generation, whereas phenyl compounds had no effect. Phenolic compounds did not have any effect on mitochondrial superoxide dismutase and choline dehydrogenase activities as well as on $\text{O}_2^{\cdot-}$ generation by the xanthine–xanthine oxidase system. Inhibition by phenolic compounds may have potential for regulation of the intracellular concentration of H_2O_2 , that is now considered to have a ‘second messenger’ function.

Various animal cell organelles are known to produce H_2O_2 under aerobic conditions (Loschen *et al.*, 1971; Hildebrandt & Roots, 1975; Bartoli *et al.*, 1977). Generation of H_2O_2 in mitochondria is now well recognized and is considered to be a process of physiological significance (Boveris *et al.*, 1972; Boveris & Chance, 1973; Dionisi *et al.*, 1975; Nohl & Hegner, 1978). Succinate and NAD-linked substrates were found to act as electron donors in the generation of H_2O_2 and this process was insensitive to antimycin A (Boveris & Cadenas, 1975). The auto-oxidizable component of the respiratory chain responsible for H_2O_2 generation was apparently located between sites sensitive to rotenone and to antimycin A (Cadenas *et al.*, 1977). It was also shown that $\text{O}_2^{\cdot-}$ is the precursor of H_2O_2 (Loschen *et al.*, 1974) and the role of ubiquinone in the process has been delineated (Boveris *et al.*, 1976; Cadenas *et al.*, 1977). Besides reduced ubiquinone, the respiratory carriers with about 0 mV midpoint potential, including energy-linked cytochrome *b-566*

and an unidentified iron–sulphur centre, have been postulated to play a significant role in peroxide generation (Loschen *et al.*, 1973; Erecinska & Wilson, 1976; Cadenas *et al.*, 1977). In higher-plant mitochondria, the flavoprotein of the ubiquinone–cytochrome *b* region and NADH dehydrogenase were proposed as sites of $\text{O}_2^{\cdot-}$ production (Rich & Bonner, 1978). Removal of mitochondrial superoxide dismutase decreased H_2O_2 generation by submitochondrial particles, with simultaneous increase in $\text{O}_2^{\cdot-}$ production (Boveris *et al.*, 1976). The presence of dismutase in the mitochondrial matrix (Weisiger & Fridovich, 1973) appears to be a physiological necessity to convert $\text{O}_2^{\cdot-}$ into H_2O_2 (Fridovich, 1975). H_2O_2 could then be degraded by the high activity of catalase present or be used for certain peroxidative reactions.

In the present report, we show that mannitol/sucrose/Tris buffer is not the appropriate one for the measurement of H_2O_2 generation. Rates were found to be higher and more consistent in phosphate buffer. Replacement of phosphate by sulphate ions points to the requirement of a high concentration of oxygen-containing anion. In addition to succinate and NAD-linked substrates, other oxidizable sub-

* Present address: Department of Pathology, School of Science, University of Wisconsin, Madison, WI 53706, U.S.A.

strates that could give electrons to ubiquinone also support H_2O_2 production in rat liver mitochondria. This paper describes our results on the properties of the H_2O_2 -generator system with choline as the substrate and inhibition of H_2O_2 generation by radical quenchers and by phenolic compounds.

Materials and methods

Chemicals

Scopoletin, antimycin A, horseradish peroxidase (type II), superoxide dismutase (2700 units/mg of protein), xanthine oxidase, glycerol 1-phosphate, D-mannitol, noradrenaline and the phenyl and phenolic compounds were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and succinic acid and choline chloride from E. Merck, Darmstadt, Germany. All other reagents were of analytical grade. De-ionized, double-quartz-distilled water was used for all studies.

Mitochondrial preparations

Male Wistar albino rats (90–120 g) were used. They were fed Hindustan–Lever pellet diet. Food and water were given *ad lib*. After being stunned rats were killed by decapitation. Livers were homogenized in 10 vol. of ice-cold 0.25 M-sucrose solution in a Potter–Elvehjem homogenizer and mitochondria were obtained by the method reported previously (Kurup *et al.*, 1970).

Enzyme assays

Generation of H_2O_2 by rat liver mitochondria was measured by the scopoletin–horseradish peroxidase method of Loschen *et al.* (1971). Routine assays were carried out in 0.45 M- KPO_4 buffer (pH 7.6) containing mitochondria (0.3–0.6 mg of protein), EDTA (3.3 mM), antimycin A (0.66 μ M), scopoletin (5 nmol), horseradish peroxidase (0.33 μ M) and choline (0.13 mM) in a total volume of 3 ml. Concentrations of other substrates, wherever used, are mentioned in the Tables.

Endogenous substrates, which gave initial rates of 0.02–0.06 nmol/min per mg of protein, were oxidized by preincubation of mitochondria in buffer for 5 min at 30°C before the reaction was started by the addition of the reactants. The decrease in fluorescence of scopoletin after substrate addition was followed at room temperature (25°C) in a Hitachi–Perkin–Elmer spectrofluorimeter with excitation at 360 nm and emission at 465 nm. Under these conditions the scopoletin/ H_2O_2 ratio was found to be 1. The amount of H_2O_2 formed is calculated from the fluorescence change. EDTA was omitted in the experiments on effect of ions. Various compounds were preincubated in the buffer with mitochondria for 5 min and then H_2O_2 production

was measured after the addition of choline as the substrate.

The dehydrogenase activities with various substrates were measured at 30°C by the use of dyes as previously described: succinate (Bernath & Singer, 1962), choline (Rendina & Singer, 1959) and glycerol 1-phosphate (Ruegamer *et al.*, 1964). Mitochondrial superoxide dismutase activity was assayed by the method of Misra & Fridovich (1972). Generation of $O_2^{\cdot -}$ in the xanthine–xanthine oxidase system was measured as described by Fridovich (1970). Mitochondrial protein was estimated by the biuret method with deoxycholate added for solubilization (Gornall *et al.*, 1949).

Results

Effect of phosphate buffer concentration

Early in the present studies, we recognized that the 225 mM-mannitol/70 mM-sucrose/35 mM-Tris buffer (pH 7.4–7.8), used by other workers (Loschen *et al.*, 1971; Boveris *et al.*, 1972; Rich *et al.*, 1976; Boveris *et al.*, 1976), was not an appropriate one. Mannitol and Tris are known scavengers of $OH^{\cdot}$ (Torres *et al.*, 1979). By using this buffer various rates of H_2O_2 generation by mitochondria with succinate as the substrate have been reported ranging from 0.12 to 0.60 nmol/min per mg of protein (Boveris *et al.*, 1972). This, in part, may be owing to the use of a high concentration of mannitol (225 mM) and its variable inhibition of radicals formed in the reaction. Under our conditions, the rate of H_2O_2 generation by mitochondria in the mannitol-buffer system [225 mM-mannitol/70 mM-sucrose/35 mM-Tris (pH 7.6)] was only about 0.11 nmol/min per mg of protein. Boveris *et al.*

Fig. 1. Effect of phosphate concentration on H_2O_2 generation

Experimental details are as described in the Materials and methods section except that the indicated concentrations of phosphate were used.

(1972) also used potassium phosphate buffer (0.1 M, pH 7.4) for H_2O_2 generation with microsomes and reported low rates with NADH as substrate. Under our conditions, this phosphate-buffer system gave a negligible rate of H_2O_2 generation that increased at high concentrations of phosphate (Fig. 1). H_2O_2 generation reaches a maximum at 0.4 M-potassium phosphate. At such high concentrations even the Analar phosphate used provides an impurity of iron in the micromolar range. However, this did not interfere with the assay and all H_2O_2 was produced enzymically. H_2O_2 generation, as measured by the decrease in scopoletin fluorescence in the presence of horseradish peroxidase (Andreae, 1955), was dependent on the presence of mitochondria and a substrate (Fig. 2a). Boiled mitochondria when used in the complete assay system did not decrease scopoletin fluorescence (Fig. 2b). It is clear that generation of H_2O_2 was obtained only on the addition of mitochondria and needed a substrate (Fig. 2). The presence of iron or any other contaminant in high-concentration phosphate buffer obviously was not a contribution to H_2O_2 generation. Phosphate could be replaced by sulphate ions but not by chloride (Table 1). Our results indicate the requirement for a high concentration of oxy-anions or high ionic strength for realizing the full activity of the H_2O_2 -generator system, a property not recognized in the previous studies. A comparison of our buffer system with others is given in Table 2. We continued the following experiments with a high-concentration phosphate buffer that gave maximal and consistent rates.

Rates with different substrates

Rat liver mitochondria were found to be capable of generating H_2O_2 on oxidation of the substrates choline, glycerol 1-phosphate and proline, in addition to succinate (Table 3). When succinate and

choline were added together, there was no additive effect on the rate of H_2O_2 generation, and in fact the rate was slightly lower than with either of the substrates. Rates with NADH and malate were small, and those with ascorbate and xanthine were

Table 1. *Effect of salts on H_2O_2 generation*

Experimental details are as described in the text. The reaction system contained 0.25 M-phosphate buffer (pH 7.6). Additions were made as indicated. The final pH in all cases was 7.6. Choline was used as the substrate.

Addition (0.2 M)	H_2O_2 generated (nmol/min per mg of protein)
None	0.06
KCl	0.16
Na_2SO_4	0.36
$\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$	0.48

Table 2. *H_2O_2 generation by mitochondria in various buffer systems*

Except for the buffer system all other experimental details are the same as described in Fig. 2(a). At 100% 0.34 nmol of H_2O_2 /min per mg of protein is generated.

Buffer system	H_2O_2 generated (%)
Phosphate buffer (0.45 M, pH 7.6)	100
Mannitol/sucrose/Tris (mM, 225/70/35; pH 7.6)	32
Tris buffer (0.05 M, pH 7.4)	47
Sucrose/Tris* (mM, 250/50; pH 7.5)	35

* Dionisi *et al.* (1975).

Fig. 2. H_2O_2 generation as measured by the decrease in scopoletin fluorescence in the presence of horseradish peroxidase. The assay mixture contained 0.45 M-phosphate buffer (pH 7.6)/EDTA (3.3 mM)/horseradish peroxidase (0.33 μM)/antimycin A (0.66 μM). After scopoletin (5 nmol) and choline (0.13 mM), native mitochondria (0.4 mg) were added in (a) and boiled mitochondria (0.4 mg) in (b) in a total volume of 3 ml.

negligible. Low rates with NADH could be owing to the impermeability of the mitochondrial inner membrane to NADH and its unavailability to the H_2O_2 -generator system. Also, NAD-linked substrate, malate or malate plus glutamate showed low rates. There is a possibility that, under the conditions used, NAD-linked dehydrogenases are uncoupled from the H_2O_2 -generator system and this aspect needs further study. In this set of experiments, the best rates obtained were about 0.50 nmol/min per mg of protein. H_2O_2 generation with succinate under these conditions was higher and consistent compared with the earlier reports. The rates of H_2O_2 generation were small compared with the corresponding dehydrogenase activities, measured by dye reduction (Table 4). These data indicate that in all cases only a small fraction of the flow of electrons is available for H_2O_2 generation.

Effect of substrate concentration

Rates of H_2O_2 generation were then measured with various concentrations of the three substrates: choline, glycerol 1-phosphate and succinate. Table 4 gives the calculated values of K_m and $V_{\max}$ for the activities of various dehydrogenases, measured by H_2O_2 generation and dye reduction. Both K_m and

$V_{\max}$ were found to be low in the case of H_2O_2 generation compared with dye reduction.

Effect of pH

H_2O_2 generation by rat liver mitochondria in 0.45 M-potassium phosphate buffer increased 8-fold with the increase in pH from 7 to 8 (choline as substrate). Tris buffer gave very low activity compared with phosphate buffer at the equivalent pH, and the activity decreased to negligible values at $\geq \text{pH } 9.0$. Boveris & Chance (1973) showed that succinate-dependent H_2O_2 production by mitochondria was optimal at pH 7.5, and the addition of uncoupler and antimycin A shifted the optimum pH to more alkaline values. Reasons for the discrepancy are perhaps use of antimycin A and a high concentration of phosphate in our system.

Effect of ions

In the standard reaction mixture carbonate, acetate, chloride or sulphate (33 mM) had no effect whereas nitrite showed 70% inhibition of H_2O_2

Table 3. H_2O_2 generation by rat liver mitochondria. The assay system is as described in the text. Endogenous rates after preincubation of the mitochondria in buffer for 5 min at 30°C were negligible. The reaction was started by addition of substrate. N.D., Not detected.

Substrate	Concn. (mM)	H_2O_2 generated (nmol/min per mg of protein)
Succinate	0.05	0.46
Choline	0.13	0.43
Glycerol 1-phosphate	10.0	0.45
Proline	0.67	0.42
NADH	0.20	0.04
Malate	6.67	0.05
Ascorbate	6.67	N.D.
Xanthine	0.03	N.D.

Fig. 3. Inhibition of H_2O_2 generation by copper. Mitochondria were preincubated in buffer with different concentrations of CuCl_2 and CuCl for 5 min before the reaction was started.

Table 4. K_m and $V_{\max}$ values

Rate of H_2O_2 generation was measured with various substrate concentrations. Values of K_m (mM) and $V_{\max}$ (nmol/min per mg of protein) were calculated from double-reciprocal plots. Dehydrogenase activities were assayed by the rate of reduction of the dye phenazine methosulphate.

Substrate	K_m		$V_{\max}$	
	H_2O_2 production	Dye reduction	H_2O_2 production	Dye reduction
Succinate	0.01	0.30	0.47	250
Choline	0.03	6.00	0.53	75
Glycerol 1-phosphate	2.20	9.50	0.54	7.5

generation. Lithium (1 mM), magnesium (0.33 mM), calcium (0.13 mM), zinc (0.33 mM), aluminium (0.13 mM) and lead (0.5 mM) had no effect. Copper (both cuprous and cupric) inhibited H_2O_2 generation in mitochondria at concentrations as low as $3.0\text{ }\mu\text{M}$ (Fig. 3). EDTA (1 mM) completely reversed this inhibition. Manganese (0.1 mM), a quencher of $\text{O}_2^{\cdot -}$ also showed 50% inhibition (Fig. 4). That the addition of 1 mM- FeSO_4 to the assay medium abolished the generation of H_2O_2 by mitochondria under our conditions (data not shown) also indicated that the reaction under study was not a manifestation of a non-enzymic response of iron EDTA likely to be present in the buffer system.

Effect of nucleotides

Generation of H_2O_2 was inhibited maximally by 0.3–0.5 mM-ATP or -ADP (Fig. 5), whereas AMP and cyclic AMP only caused marginal inhibition and UTP and adenosine (1 mM) did not have any effect. Boveris & Chance (1973) found no effect of ATP on H_2O_2 generation by pigeon heart mitochondria.

Effect of radical scavengers

Ascorbic acid, ferricytochrome *c* and Nitro Blue Tetrazolium, known scavengers of $\text{O}_2^{\cdot -}$ (Nishikimi, 1975; Torres *et al.*, 1979), inhibited mitochondrial H_2O_2 generation (Table 5), in support of the earlier observations that $\text{O}_2^{\cdot -}$ are precursors of H_2O_2 formation. Addition of $10\text{ }\mu\text{g}$ of superoxide dismutase enhanced H_2O_2 generation, but further addition of up to $50\text{ }\mu\text{g}$ did not have any effect. Nohl & Hegner (1978) have also observed slight increases in mitochondrial H_2O_2 generation by exogenous superoxide dismutase ($2\text{ }\mu\text{M}$). Enough enzyme is probably present in mitochondria and the addition of a small amount was sufficient to saturate the system for maximal H_2O_2 generation.

Addition of mannitol or benzoate decreased H_2O_2 generation in mitochondria (Table 5). They are known to react with $\text{OH}^{\cdot}$ and not with $\text{O}_2^{\cdot -}$ or H_2O_2 (Torres *et al.*, 1979). This inhibition is suggestive of involvement of $\text{OH}^{\cdot}$ in the process of H_2O_2 generation. In the experiments on pH low activity in Tris buffer also indicated this. Histidine, a quencher of singlet oxygen (Lynch & Fridovich, 1979), had only a marginal effect.

Effect of phenolic compounds on H_2O_2 generation

We screened a number of phenyl and phenolic compounds for the effect on H_2O_2 generation in rat liver mitochondria (Table 6). Phenyl compounds (L-phenylalanine, phenylacetic acid, *trans*-cinnamic acid and anisic acid) did not have any effect whereas compounds with a hydroxy group in the phenyl ring at the *m*- (*m*-coumaric acid and *m*-hydroxybenzoic acid) or *p*-position (tyrosine, *p*-hydroxyphenylacetic

Fig. 4. Inhibition of H_2O_2 production by manganese MnCl_2 at different concentrations was preincubated in buffer with mitochondria for 5 min and the rate of H_2O_2 production was measured. Experimental details are as described in the text.

Fig. 5. Effect of adenine nucleotides on H_2O_2 generation. Experimental details are as described in the text.

acid, *p*-hydroxytryptophan and *p*-coumaric acid) were inhibitory. *m*-Coumaric acid was much less effective compared with *p*-coumaric acid. The presence of an additional methoxy or hydroxy group in the phenolic ring enhanced the potency (Table 6). Diphenolic compounds (e.g., catechol and noradrenaline) were inhibitory at concentrations as low as $0.5\text{ }\mu\text{M}$.

Table 5. *Effect of radical scavengers on H₂O₂ generation by rat liver mitochondria*

Mitochondria were preincubated in buffer with the compound for 5 min before the reaction was started. Choline was used as substrate. N.D., Not detected.

Addition	Concn. (mM)	H ₂ O ₂ generated (nmol/min per mg of protein)	Change (%)
None	—	0.53	—
Ascorbate	0.33	N.D.	-100
Ferricytochrome <i>c</i>	0.003	0.30	-43
Nitro Blue Tetrazolium	0.005	0.07	-87
Superoxide dismutase (10 µg)	—	0.72	+36
Mannitol	100	0.33	-38
Benzoate	16.7	0.36	-32
	33.3	0.32	-40
Histidine	3.3	0.46	-13

Table 6. *Effect of phenyl and phenolic compounds on mitochondrial H₂O₂ generation*

Mitochondria were preincubated in buffer with the compound for 5 min and the reaction was started by addition of choline as substrate. Portions (10 µl) of 0.4 mg of mitochondrial protein were added. *Phenyl or phenolic compounds dissolved in alcohol instead of water. Alcohol (10 µl) had no effect on mitochondrial H₂O₂ generation.

Addition	Concn. (µM)	H ₂ O ₂ generated (nmol/min per mg of protein)	Inhibition (%)
Phenyl compounds			
None	—	0.51	—
L-Phenylalanine	500	0.51	—
Phenylacetic acid	500	0.51	—
<i>trans</i> -Cinnamic acid	330	0.49	4
Anisic acid	80	0.50	2
Phenolic compounds			
None	—	0.54	—
<i>m</i> -Coumaric acid*	330	0.24	55
<i>m</i> -Hydroxybenzoic acid	330	0.33	39
Tyrosine	250	0.32	41
	500	0.24	55
<i>p</i> -Hydroxyphenylacetic acid	200	0.27	50
<i>p</i> -Hydroxyphenylpyruvic acid	50	0.15	72
<i>p</i> -Hydroxyphenylpropionic acid	500	0.10	81
5-Hydroxytryptophan	100	0.15	72
β-Resorcinic acid	170	0.28	48
<i>p</i> -Coumaric acid*	10	0.38	30
	50	0.13	76
Methoxyphenolic and diphenolic compounds			
None	—	0.53	—
Ferulic acid*	2.00	0.20	62
Isoferulic acid*	0.33	0.43	19
	1.00	0.22	59
	3.30	0.17	68
	33	0.07	87
Catechol	0.20	0.21	60
	0.40	0.12	77
	1.00	0.03	94
Resorcinol	0.67	0.41	23
Gentisic acid*	0.33	0.33	38
	0.50	0.24	55
Noradrenaline	0.50	0.33	38
	0.67	0.29	45
3,4-Dihydroxyphenylalanine	330	0.05	91
DL-Threodihydroxyphenylserine	330	0.06	89

Fig. 6. Effect of thyronine derivatives on H_2O_2 generation. Experimental details are as described in the text: ■, di-iodothyronine (T_2); ○, tri-iodothyronine (T_3); ●, thyroxine (T_4).

It was interesting to see that thyronine derivatives di-iodothyronine (T_2), tri-iodothyronine (T_3) and thyroxine (T_4) also inhibited mitochondrial H_2O_2 generation (Fig. 6). All these compounds contain phenolic rings. Various other phenolic compounds (syringic acid, phloroglucinol, protocatechuic acid and dicoumarol) also inhibited H_2O_2 generation at low concentrations (data not shown).

Effect of phenolic compounds on mitochondrial superoxide dismutase, choline dehydrogenase and $\text{O}_2^{\cdot-}$ formation by the xanthine-xanthine oxidase system

L-Phenylalanine (0.6 mM), tyrosine (0.3 mM), *p*-hydroxyphenylacetic acid (0.3 mM), *trans*-cinnamic acid (0.3 mM), *m*-coumaric acid (0.3 mM), *p*-coumaric acid (0.02 mM) and ferulic acid (0.006 mM) did not have any effect on the activity of mitochondrial superoxide dismutase (10.3 units of enzyme/mg of mitochondrial protein). The generation of $\text{O}_2^{\cdot-}$ by the xanthine-xanthine oxidase system was also unaffected by phenyl and phenolic compounds at the concentrations mentioned above. Also, dehydrogenase activity with choline measured by the phenazine methosulphate-oxygen method (77 nmol/min per mg of protein) remained unchanged in the presence of *p*-hydroxyphenylacetic acid (0.1 mM), *p*-coumaric acid (7 μM) or ferulic acid (2 μM).

Discussion

The high-concentration phosphate-buffer system

was found to be more appropriate for the assay of H_2O_2 generation in mitochondria as compared with the mannitol/sucrose/Tris buffer used by previous workers. Rates of H_2O_2 generation in phosphate buffer were higher and consistent as compared with that obtained in the mannitol-buffer system and also sucrose/Tris buffer.

Generation of H_2O_2 under our conditions was not due to any contaminant in the buffer system. Only native mitochondria (not boiled) could generate H_2O_2 and they needed a substrate. In fact, the high-concentration phosphate buffer provided the oxy-anions or ionic strengths required for maximal H_2O_2 generation, as indicated in Table 1. Activation by a high concentration of oxy-anions needs further study. The oxidizable substrates, choline, glycerol 1-phosphate and proline, can generate H_2O_2 in the mitochondria. There appears to be a common H_2O_2 -generator system as the V_{max} with various substrates were found to be nearly the same. Also, when two substrates were added together, the rate of H_2O_2 generation was not additive. It appears that electrons from various substrates converge at ubiquinone (see Wainio, 1970) and from this pool a small fraction of electrons are diverted to an as yet unidentified auto-oxidizable component proposed to be an iron-sulphur protein (Boveris *et al.*, 1976; Cadenas *et al.*, 1977). This can pass electrons directly to molecular oxygen generating $\text{O}_2^{\cdot-}$ and then to H_2O_2 by dismutation.

There is a possibility that two forms of the dehydrogenases with significantly different K_m values are present in rat liver mitochondria. A form with a high K_m is present in excess and channels the electrons to the main electron-transport chain and to cytochrome oxidase leading to the formation of water and accounts for the major oxidation found at high substrate concentrations. However, at low substrate concentrations, oxidation proceeds through the second form with the low K_m and directs electron flow towards generation of H_2O_2 . The possibility that two subunits with different K_m values for the same substrate also exist is reminiscent of the 'half-of-the-sites' activity (see review by Lazdunski, 1972). It is appropriate to mention here that H_2O_2 generation in plasma membranes also shows similar low K_m values for the same substrate, NADH, compared with the dehydrogenase activity measured by ferricyanide reduction (T. Ramasarma, A. Swaroop, W. Mackeller & F. L. Crane, personal communication). Perhaps at low concentrations substrates are used for H_2O_2 generation. Choline derived from dietary phospholipids may contribute significantly to the reducing power for this pathway. H_2O_2 generation with choline behaved differently as compared with either succinate or α -glycerophosphate. Table 3 showed a large difference between the K_m values for the generation of

H₂O₂ by choline and dehydrogenase activity. Also, generation of H₂O₂ by choline in liver mitochondria responded to the altered status of thyroxine and thermogenesis in rats whereas choline dehydrogenase was not affected. In the case of α -glycerophosphate, both H₂O₂ generation and dehydrogenase activities responded to the altered thyroid status (A. Swaroop & T. Ramasarma, unpublished work).

Van Hemmen & Meuling (1977) suggested that copper ions reduce O₂^{-•} and H₂O₂ and generate OH[•].

Thus copper ions affect the yield of H₂O₂ considerably by using both O₂^{-•} and H₂O₂ for OH[•] generation. The Haber–Weiss reaction was originally postulated to be responsible for the production of OH[•] (Fridovich, 1975; O₂^{-•} + H₂O₂ → OH[•] + O₂ + OH⁻) but kinetic data have subsequently shown that this is too slow to be of significance (Halliwell, 1976). Since this reaction occurs more rapidly when catalysed by iron EDTA, a mechanism of this type has been suggested as an alternative (Cohen, 1977; McCord & Day, 1978). In this case, O₂^{-•} reduces the ferric complex formed with H₂O₂ by a Fenton-type reaction. Possibly the iron–sulphur centre, proposed to be involved in H₂O₂ generation by mitochondria, and EDTA (present in the assay system) catalyse the formation of small amounts of OH[•] from O₂^{-•} and H₂O₂. Thus the quenchers of OH[•] will cause increased removal of H₂O₂ through this path in the presence of a source of O₂^{-•} and thereby an apparent decrease in H₂O₂ available for the scopoletin–peroxidase reaction. Inhibition by quenchers of OH[•] indicates the generation of these radicals in mitochondria, although it is not clear whether or how they are required in H₂O₂ generation *per se*.

Our results with phenyl and phenolic compounds show some interesting structural relationships. The presence of a hydroxy group in the phenyl ring is necessary for the inhibition. Compounds with the vinyl group in the side chain along with the phenolic ring (e.g. *p*-coumaric acid and ferulic acid) were more potent compared with those devoid of a double bond in the side chain (e.g. *p*-hydroxyphenylacetic acid and tyrosine). Also, *m*-coumaric acid was less effective than its *p*-isomer. The presence of an additional methoxy (ferulic acid and isoferulic acid) or hydroxy group (catechol and noradrenaline) in the phenolic ring enhanced the potency of the inhibition.

Another observation in the present study is that all the phenolic compounds did not affect mitochondrial choline dehydrogenase and superoxide dis-

mutase or O₂^{-•} generation by the xanthine–xanthine oxidase system. Therefore, the inhibition by these phenolic compounds must be localized to the step of coupling of electron transfer to the possible common H₂O₂-generator. The purported iron–sulphur protein centre, which couples electron transfer to oxygen, may then be the target of this inhibition by phenolic compounds. This selective inhibition can be exploited further and may be helpful in characterizing the H₂O₂-generator system in mitochondrial membranes.

Several reports have appeared regarding the physiological role H₂O₂ might play in metabolic regulation (Crane *et al.*, 1979; May & de Haen, 1979b; Mukherjee, 1980). It has further gained importance because of its proposed role as 'second messenger' for the action of insulin (May & de Haen, 1979a,b). Selective inhibition of H₂O₂ generation by phenolic compounds at very low concentrations, several of them being intracellular, might have a potential for metabolic regulation. This acquires additional significance in view of the observations that mitochondrial H₂O₂ generation responds to the altered status of thyroxine and thermogenesis in rats (A. Swaroop & T. Ramasarma, unpublished work).

We thank Mrs Rajee Ramamurthy for technical assistance. This work is a part of the project entitled Environmental Stress and Biochemical Adaptation II sponsored by the University Grants Commission, New Delhi, India.

References

- Andreae, W. A. (1955) *Nature (London)* **175**, 859–860
- Bartoli, G. M., Galeotti, T. & Azzi, A. (1977) *Biochim. Biophys. Acta* **497**, 622–626
- Bernath, P. & Singer, T. P. (1962) in *Methods in Enzymology* (Colowick, S. P. & Kaplan, N. O., eds.), vol. 5, pp. 597–614, Academic Press, New York
- Boveris, A. & Cadenas, E. (1975) *FEBS Lett.* **54**, 311–314
- Boveris, A. & Chance, B. (1973) *Biochem. J.* **134**, 707–716
- Boveris, A., Oshino, N. & Chance, B. (1972) *Biochem. J.* **128**, 617–630
- Boveris, A., Cadenas, E. & Stoppani, A. O. M. (1976) *Biochem. J.* **156**, 435–444
- Cadenas, E., Boveris, A., Ian Ragan, C. & Stoppani, A. O. M. (1977) *Arch. Biochem. Biophys.* **180**, 248–257
- Cohen, G. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M. & Fridovich, I., eds.), pp. 317–321, Academic Press, London
- Crane, F. L., Goldenberg, H., Morre, D. J. & Low, H. (1979) *Sub-Cell. Biochem.* **6**, 345–379
- Dionisi, O., Galeotti, T., Terranova, T. & Azzi, A. (1975) *Biochim. Biophys. Acta* **403**, 292–300
- Erecinska, M. & Wilson, D. F. (1976) *Arch. Biochem. Biophys.* **174**, 143–157

- Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4053–4057
- Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Halliwell, B. (1976) *FEBS Lett.* **72**, 8–10
- Hildebrandt, A. G. & Roots, I. (1975) *Arch. Biochem. Biophys.* **171**, 385–397
- Kurup, C. K. R., Aithal, H. N. & Ramasarma, T. (1970) *Biochem. J.* **116**, 773–779
- Lazdunski, M. (1972) in *Current Topics in Cellular Regulation* (Horecker, B. L. & Stadtman, E. R., eds.), vol. 6, pp. 267–310, Academic Press, New York
- Loschen, G., Flohe, L. & Chance, B. (1971) *FEBS Lett.* **18**, 261–264
- Loschen, G., Azzi, A. & Flohe, L. (1973) *FEBS Lett.* **33**, 84–88
- Loschen, G., Azzi, A., Richter, C. & Flohe, L. (1974) *FEBS Lett.* **42**, 68–72
- Lynch, R. E. & Fridovich, I. (1979) *Biochem. Biophys. Acta* **571**, 195–200
- May, J. M. & de Haen, C. (1979a) *J. Biol. Chem.* **254**, 2214–2220
- May, J. M. & de Haen, C. (1979b) *J. Biol. Chem.* **254**, 9017–9021
- McCord, J. M. & Day, E. D. (1978) *FEBS Lett.* **86**, 139–142
- Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 3170–3175
- Mukherjee, S. P. (1980) *Biochem. Pharmacol.* **29**, 1239–1246
- Nishikimi, M. (1975) *Biochem. Biophys. Res. Commun.* **63**, 463–468
- Nohl, H. & Hegner, D. (1978) *Eur. J. Biochem.* **82**, 563–567
- Rendina, G. & Singer, T. P. (1959) *J. Biol. Chem.* **234**, 1605–1610
- Rich, P. R. & Bonner, W. D., Jr. (1978) *Arch. Biochem. Biophys.* **188**, 206–213
- Rich, P. R., Boveris, A., Bonner, W. D., Jr. & Moore, A. L. (1976) *Biochem. Biophys. Res. Commun.* **71**, 695–703
- Ruegamer, W. R., Westerfeld, W. W. & Richert, D. A. (1964) *Endocrinology* **75**, 908–916
- Torres, M., Auclair, C. & Hakim, J. (1979) *Biochem. Biophys. Res. Commun.* **88**, 1003–1009
- Van Hemmen, J. J. & Meuling, W. J. A. (1977) *Arch. Biochem. Biophys.* **182**, 743–748
- Wainio, W. W. (1970) in *The Mammalian Mitochondrial Respiratory Chain*, pp. 86–104, Academic Press, New York
- Weisiger, R. A. & Fridovich, I. (1973) *J. Biol. Chem.* **248**, 4793–4796