Journal of Bioenergetics and Biomembranes, Vol. 29, No. 1, 1997

Original Article

Dependence of H_2O_2 Formation by Rat Heart Mitochondria on Substrate Availability and Donor Age

Richard G. Hansford,[1] Barbara A. Hogue,[1] and Vida Mildaziene[2]

Received August 8, 1996; accepted August 31, 1996

We have examined the substrate specificity and inhibitor sensitivity of H_2O_2 formation by rat heart mitochondria. Active H_2O_2 production requires both a high fractional reduction of Complex I (indexed by NADH/NAD^+ + NADH ratio) and a high membrane potential, $\Delta\psi$. These conditions are achieved with supraphysiological concentrations of succinate. With physiological concentrations of NAD-linked substrates, rates of H_2O_2 formation are much lower (less than 0.1% of respiratory chain electron flux) but may be stimulated by the Complex III inhibitor antimycin A, but not by myxothiazol. Addition of Mn^{2+} to give 10 nmol/mg of mitochondrial protein enhances H_2O_2 production with all substrate combinations, possibly by repleting mitochondrial superoxide dismutase with this cation. Contrary to previously published work, no increased activity of H_2O_2 production was found with heart mitochondria from senescent (24 month) rats, relative to young adults (6 month).

KEY WORDS: Respiratory chain; reactive oxygen species.

INTRODUCTION

Increased scrutiny is currently being paid to the mitochondrial generation of reactive oxygen species (ROS), with the realization that such species likely play a role in cell death due to ischemia/reperfusion injury, in excitatory neurotoxicity and in apoptosis. Particularly provocative is the point of view that mitochondrial generation of ROS may play a role in the normal aging of tissues, with one such scenario calling for the fixation of oxidative damage to mt-DNA and the accumulation of deletions in mt-DNA as mitochondria undergo many generations of replication in nondividing cells, e.g., myocytes and neurons (Miquel *et al.*, 1980; Linnane *et al.*, 1989; Brown and Wallace, 1994; Shigenaga *et al.*, 1994). According to this theory, a point is reached at which energy transduction by mitochondria becomes limiting, owing to an incompetent synthesis of the respiratory chain and ATP-synthase complexes of the mitochondrial inner membrane, which include subunits encoded on the mitochondrial genome. A raised metabolic demand upon such a cell, e.g., overstimulation of a neuron by glutamate, then results in an energy crisis and cell death. In keeping with this model, there have been reports of an increased activity of ROS generation with age in mitochondria derived from heart (Nohl and Hegner, 1978; Sohal *et al.*, 1994), kidney, and brain (Sohal *et al.*, 1994).

It is generally accepted that the primary generation of ROS by mitochondria involves the single-electron reduction of O_2 molecules to form superoxide (O_2^-) occurring at ubisemiquinone and, to a lesser extent, at the Fe-S center of Complex I. (Boveris and Chance, 1973; Boveris *et al.*, 1976; Turrens and Boveris, 1980). Superoxide dismutates to form H_2O_2 and water, in a spontaneous process which is enhanced in activity by the Mn-superoxide dismutase (SOD) found within the mitochondria (McCord and Fridovich, 1969; Chance *et al.*, 1979). H_2O_2 may be decomposed by catalase (thought not to exist in heart mitochondria) or by glutathione peroxidase, using reduced glutathione as reductant. Such scavenging of H_2O_2 is important

[1] National Institute on Aging, Baltimore, Maryland 21224.
[2] Institute for Biomedical Research, Kaunas Medical Academy, 3007 Kaunas, Lithuania.

0145-479X/97/0200-0089$12.50/0

because, otherwise, it may give rise to hydroxyl radicals (OH^-) in the presence of iron (Fe^{++}) and these are intensely reactive and cause nonspecific oxidation of DNA, protein, and membrane lipid. Previous studies on mitochondrial O_2^- and H_2O_2 generation have mainly used succinate, at nonphysiologically high concentrations, as oxidizable substrate (e.g., Barja *et al.*, 1994; Sohal *et al.*, 1994). During the course of a reinvestigation of H_2O_2 formation by rat heart mitochondria as a function of donor age, we noticed that physiological concentrations of NAD-linked substrates gave almost undetectable rates of H_2O_2 production. This led us to an evaluation of the role of mitochondrial membrane potential ($\Delta\psi$) and the fractional reduction of Site I (as approximated by NADH/NAD^+ + NADH) as determinants of the rate of H_2O_2 formation. We found that there is a very steep dependence of this rate upon $\Delta\psi$ and the reduction of Site I, that rates with NAD-linked substrates as electron donors are negligible unless mitochondria are supplemented with 5–10 μM Mn^{2+}, and that, contrary to the literature (Nohl and Hegner, 1978; Sohal *et al.*, 1994), there is no increase in H_2O_2 production by heart mitochondria as a function of senescence (6-month-old rat versus 24-month-old-rat). These results are not in agreement with conclusions in the literature that 1–4% of electron flow gives rise to ROS (Chance *et al.*, 1979): we maintain that values are actually an order of magnitude lower than this under plausibly physiological conditions. Further, we were forced to conclude as a result of studies with the electron transport inhibitors rotenone, antimycin A, and myxothiazol that the majority of ROS formation occurs at site I and not at ubisemiquinone, as concluded previously (Boveris *et al.*, 1976; Turrens and Boveris, 1980).

MATERIALS AND METHODS

Mitochondria were isolated from the heart of a single rat using the Nagarse digestion technique, as described previously (Hansford, 1978). Rats were from the Wistar-derived colony maintained at the Gerontology Research Center and were males, aged either 6 months or 24 months.

Hydrogen peroxide production by intact mitochondria was measured from the increase in fluorescence accompanying the oxidation of *p*-hydroxyphenylacetate by horseradish peroxidase (Hyslop and Sklar, 1984; Sohal, 1991). Fluorescence was measured at 37° in a PTI Deltascan spectrofluorimeter, using 320 nm excitation and 400 nm emission wavelengths. Mitochondria (1 mg of protein) were added to 2 ml of medium comprising 0.12 M KCl, 20 mM K Hepes, 1 mM $MgCl_2$, 5 mM K P_i, 50 μg/ml *p*-hydroxyphenylacetate (PHPA), 180 U of horseradish peroxidase, and the oxidizable substrate listed in the appropriate table footnote. The PHPA concentration used was 20% of that used by Sohal (1991) as, in our hands, the higher concentration gave a 30% inhibition of State 3 rates and a diminution in $\Delta\Psi$ and rates of H_2O_2 formation. A concentration of 50 μg/ml was found to give maximal rates of H_2O_2 production. Each experimental run was calibrated by the addition of 20 nmol of authentic H_2O_2 at the end of the experiment.

Oxygen uptake was measured in parallel experiments using identical conditions, and employing a Clark-type O_2 electrode. Respiration was stimulated with 0.5 mM ADP after measuring the controlled respiration (State 4) associated with H_2O_2 formation. When these stimulated (State 3) rates of respiration are divided by the State 4 rates, this yields the respiratory control ratio (RCR). RCR values of 10.8 ± 0.6, 7.58 ± 0.27, and 2.70 ± 0.08 (SEM, n = 6 preparations) were obtained with pyruvate, glutamate plus malate, and succinate plus rotenone, respectively, indicating an acceptable degree of integrity of the mitochondrial preparations.

Mitochondrial membrane potential ($\Delta\psi$) was measured simultaneously with O_2 uptake, and in the same chamber, by using an electrode sensitive to the lipophilic cation tetraphenylphosphonium$^+$ or TPP^+ (Kamo *et al.*, 1979). This electrode was manufactured by Dr. Aurelijus Zimkus, Vilnius State University, Vilnius, Lithuania and gives a linear response of mV to log [TPP^+] down to 10^{-7}M TPP^+. Calibration was by addition of standard TPP^+ and calculation assumed a matrix volume of 1 μl/mg protein for heart mitochondria.

The fractional reduction of mitochondrial NAD(P) was measured from the fluorescence of a mitochondrial suspension, using a Perkin-Elmer LS-5 fluorimeter, excitation 340 nm and emission 460 nm. For these experiments, mitochondrial protein was 0.5 mg/ml, as in the H_2O_2 production experiments. Repetition using 0.25 mg protein/ml yielded closely-similar results, indicating no major role for light-scattering changes. Complete oxidation of nicotinamide nucleotide was achieved with 0.5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP): complete reduction by the addition of 5 mM glutamate and 5 mM malate, in the presence of 1 μM rotenone.

RESULTS AND DISCUSSION

H_2O_2 Production from Succinate as Substrate

In confirmation of earlier work (Turrens *et al.*, 1985; Sohal *et al.*, 1994) heart mitochondria generate H_2O_2 when respiring in the presence of succinate, in the concentration range 5–10 mM, as oxidizable substrate (Table IA). Absolute rates of approximately 0.5 nmol/min/mg of protein were found to be comparable to, or slightly lower than, those reported elsewhere (Turrens and Boveris, 1980; Turrens *et al.*, 1985; Sohal *et al.*, 1994) when allowance is made for incubation temperature (37° in this work). Although formation of superoxide (O_2^-) precedes that of H_2O_2, measurement of the former requires disruption of mitochondria to give submitochondrial particles (Boveris, 1984), as the superoxide anion does not permeate the inner mitochondrial membrane. As disruption of mitochondria by sonication is not a very reproducible procedure, and we sought to make quantitative comparisons of ROS generation by mitochondria from young and old animals, we chose instead to measure formation of H_2O_2, which can be done with intact mitochondria as this is a membrane penetrant.

Supplementation of the mitochondrial incubations with 5 μM $MnCl_2$ (10 nmol Mn^{2+}/mg protein) consistently gave higher rates of H_2O_2 formation (Table IB). Lower concentrations were less effective, probably because EGTA contaminating the incubation was in the range 1–3 μM: 10 μM $MnCl_2$ gave results similar to those obtained with 5 μM (not shown). The purpose of adding Mn^{2+} was to restore endogenous Mn^{2+} which may have been withdrawn from the mitochondria by chelation with EGTA during mitochondrial isolation and storage, possibly limiting the activity of the mitochondrial Mn-SOD. This issue is discussed further later in the article.

In general, H_2O_2 production from succinate oxidation was associated with more reduced mitochondrial NAD (i.e., higher values of NADH/NAD^+ + NADH) and, presumably, of the redox centers of Site I. There was also a trend toward higher values of $\Delta\psi$ as succinate concentration increased and, in association, H_2O_2 production. However, variation in $\Delta\psi$ between mitochondrial preparations negated a finding of statistical significance for these small differences.

Substrate Specificity and Inhibitor Dependence of H_2O_2 Formation

We carried out an extensive series of experiments with different oxidizable substrates and respiratory chain inhibitors (Table II) to allow us to address two questions. First, what is the major locus of H_2O_2 (O_2^-)

Table I. Effect of Succinate Concentration on H_2O_2 Formation, $\Delta\Psi$, and NADH of Heart Mitochondria[a]

Succinate concentration (mM)	Rate of H_2O_2 formation (nmol/min/mg)	Rate of O_2 uptake (ng-atoms/min/mg)	$\Delta\Psi$ (mV)	NADH (% total NAD)
A				
0.1	0.05 ± 0.007	56 ± 3	—	11 ± 2
0.25	0.03 ± 0.005	97 ± 8	171.3 ± 3.2	36 ± 3
0.5	0.08 ± 0.01	131 ± 11	174.6 ± 2.5	72 ± 4
1	0.50 ± 0.03	162 ± 10	175.8 ± 2.4	72 ± 4
5	0.57 ± 0.04	180 ± 10	176.2 ± 1.6	77 ± 1
20	0.55 ± 0.04	175 ± 11	—	84 ± 4
B				
0.1	0.07 ± 0.02	84	—	8 ± 1
0.25	0.19 ± 0.09	98 ± 3	—	42 ± 1
0.5	0.59 ± 0.11	125 ± 2	—	68 ± 1
1	1.55 ± 0.17	158 ± 8	171	65 ± 3
5	2.10 ± 0.18	161 ± 9	174	83 ± 1
20	2.13 ± 0.09	171 ± 1	173	77 ± 2

A = minus Mn^{2+}
B = plus Mn^{2+}

[a] A series of separate incubations were carried out, using the concentrations of succinate indicated. Parameters were measured as described in Material and Methods. Error in SEM, where n = 3–5 mitochondrial preparations. Where present, Mn^{2+} concentration was a nominal 5 μM, giving 10 nmol/mg of mitochondrial protien.

Table II. Substrate and Inhibitor Specificity of H_2O_2 Formation[a]

Substrate (mM)	Inhibitor (μM)	Rate of H_2O_2 formation (nmol/min/mg) (A) − Mn^{2+}	(B) + Mn^{2+}	ΔΨ (mV)	NADH/NAD⁺ + NADH (%)
1. Succinate(5)	—	0.63 ± 0.12(7)	1.12 ± 0.15(4)	177.1 ± 1.6(4)	77.7 ± 3.7(7)
2. Succinate(5)	Rotenone(1)	0.06 ± 0.01(3)	0.13 ± 0.03(3)	175.5	66.7 ± 5.2(3)
3. Succinate(5)	Antimycin A (1)	0.25 ± 0.02(4)	2.64 ± 0.50(5)	71	18.1 ± 7.0(4)
4. Succinate(5)	Myxothiazol(1)	0.12	0.45 ± 0.08(3)	—	55 ± 6(3)
5. Succinate(5)	Myxothiazol(1) + Antimycin(1)	0.08 ± 0.06(3)	0.24 ± 0.07(3)	—	66
6. Glutamate(5) + malate(5)	—	0.004 ± 0.002(3)	0.02 ± 0.01(4)	169.6 ± 1.3(4)	68.5 ± 4.3(8)
7. Glutamate(5) + malate(5)	Antimycin A(1)	0.26 ± 0.04(3)	0.63 ± 0.32(3)	—	100
8. Glutamate(5) + malate(5)	Myxothiazol(1)	0.10 ± 0.01(3)	0.08	—	97.3 ± 0.7(3)
9. Glutamate(5) + malate(5)	Rotenone(1)	0.22 ± 0.03(3)	0.29 ± 0.06(3)	—	100
10. Glutamate(5) + malate(5) + ATP(2)	Rotenone(1)	0.28 ± 0.02(3)	0.39 ± 0.12(4)	141	100
11. Succinate(5) + glutamate(5)	Rotenone(1)	0.02 ± 0.01(3)	0.04 ± 0.02(3)	—	100
12. Succinate(5) glutamate(5) + ATP (2)	—	0.13	0.11	—	79 ± 3(3)
13. Succinate(5) + ATP(2)	Antimycin A(1)	0.26 ± 0.01(3)	1.08 ± 0.34(3)	138.6	71.3 ± 2.9(3)
14. Succinate(5) + glutamate(5) + malate(5)	—	0.09	0.08	178.6	77.1 ± 4.3(7)

[a] Concentrations of inhibitors and substrates were as shown. Parameters were measured as described in Materials and Methods. Errors are SEM, with the number in parentheses being the number of separate mitochondrial preparations.

generation within the respiratory chain? Secondly, is it peculiarly high values of $\Delta\psi$ or a very high degree of reduction of the respiratory chain which is involved in the relatively very active generation of H_2O_2 with nonphysiologically high concentrations of succinate as substrate? Owing to the enhancing effect of Mn^{2+} on H_2O_2 formation described above, all of these experiments were carried out in the presence and absence of this metal ion.

The most important observations in Table II are that rotenone largely abolishes H_2O_2 formation from succinate, indicating that the major site of O_2 formation is "upstream" of the rotenone block, i.e., between NADH binding and rotenone binding sites. Secondly, antimycin A potentiates H_2O_2 production from NAD-linked substrates, whereas the other Complex III inhibitor, myxothiazol, stimulates much less. With succinate as substrate, antimycin-A may either potentiate or inhibit, depending on the presence of Mn^{2+}, whereas myxothiazol is consistently inhibitory. Antimycin A has previously been shown to potentiate ROS formation from succinate in the presence (Turrens *et al.*, 1985) and absence (Boveris and Chance, 1973) of rotenone. The present results are novel to the degree that they show that Complex I is quantitatively more significant than Complex III in generation of ROS, at least under these experimental conditions. Previous work has largely emphasized origination at Complex III (Boveris and Chance, 1973; Turrens *et al.*, 1985). They are confirmatory to the degree that they show that the presence of antimycin A makes Complex III an active generator of ROS. Antimycin A tends to stabilize the ubisemiquinone radical, whereas myxothiazol, by blocking electron flow through the Rieske iron-sulfur center, tends to lock up coenzyme Q in the dihydroquinone form (von Jagow *et al.*, 1984; Turrens et al., 1985). Clearly these are artificial conditions.

In answer to the question on the characteristics of succinate oxidation which predispose it toward ROS generation, the profound inhibitory effect of rotenone is instructive (Table II). Addition of rotenone causes no decrease in the generation of $\Delta\psi$, and indeed may enhance this at later time points by preventing inhibition of succinate dehydrogenase by oxaloacetate and linearizing O_2 uptake (not shown). By contrast, NAD is less reduced than in the presence of succinate alone, and this reduction is rather variable, reflecting availability of endogenous substrate. Thus, it seems that a highly reduced Complex I is necessary for active O_2^- generation. On the other hand, this alone is not sufficient, as NAD is highly reduced in the presence of glutamate, malate, and rotenone, but H_2O_2 formation

is relatively inactive (Table II). Thus, it would seem that both high $\Delta\psi$ and highly reduced Complex I are necessary for active ROS generation. A test of this by adding ATP to mitochondria incubated in the presence of glutamate, malate, and rotenone failed to give active radical generation (Table II). Measurement of $\Delta\psi$ under these conditions revealed, however, that ATP hydrolysis is not able to generate values of $\Delta\psi$ equal to those seen under conditions of coupled substrate oxidation. Thus, this result does not negate the conclusion, reached above, that both high $\Delta\psi$ and highly reduced Complex I are necessary for active ROS generation.

By contrast, Boveris and Chance (1973) found maximal rates of H_2O_2 production in the presence of uncoupling agent and antimycin A, indicating that ROS generation at Complex III is active at an optimal content of ubisemiquinone (Turrens *et al.*, 1985), and minimal values of protonmotive force.

Effect of Uncoupling Agent on H_2O_2 Formation

The state of energization of the mitochondria is clearly crucial as shown by the studies with inhibitors of the respiratory chain presented in Table II and by the abolition of ROS formation on transition from State 4 to State 3 respiration (Boveris *et al.*, 1972, and confirmed in current studies). For this reason, we investigated the effect of a titration of succinate oxidation with the uncoupling agent FCCP (Table III). It is seen that H_2O_2 formation was sharply curtailed at concentrations of FCCP of 5 nM and above. There was no significant increase in State 4 O_2 uptake at these very low concentrations of uncoupling agent, and indeed in some preparations of mitochondria there was a decrease. There are at least two possible reasons why O_2 uptake did not increase, despite a fall in $\Delta\psi$ at concentrations of FCCP of more than 2 nM. One is that a period of coupled respiration is necessary for these mitochondria to accumulate K^+, phosphate, and water prior to giving maximal rates of substrate oxidation, whether elicited by uncoupling agent or by ADP. In these incubations, the fall in $\Delta\psi$ occasioned even by these very low concentrations of FCCP may have limited this salt uptake, as uncoupling agent was present from the beginning of the incubations. The other possible explanation relates to oxaloacetate availability. Succinate dehydrogenase is very sensitive to inhibition by oxaloacetate in experiments like these, carried out in the absence of rotenone, and the oxidation of nicotinamide nucleotide shown in Table III will increase matrix oxaloacetate concentrations, owing to its effect on the malate dehydrogenase reaction. These studies confirm one of the conclusions reached in discussing Table II, viz. that H_2O_2 formation is inhibited by small decreases in $\Delta\psi$.

Effect of Senescence on Activity of H_2O_2 Production

It is a tenet of one theory of aging of postmitotic tissues that oxidative damage to mitochondria accumulates to the point that it leads to a deterioration of function (Miquel *et al.*, 1980; Sohal, 1991). Although some authors emphasize less active antioxidant defenses as a mechanism of accumulation of oxidative damage (Cutler, 1984), others invoke an increased activity of ROS generation by mitochondria in senescence. In work with isolated mitochondria from rat heart, Nohl and Hegner (1978) and Sohal *et al.* (1994) have reported increased rates of H_2O_2 formation in senescent animals. We were not able to replicate these

Table III. Effect of Uncoupling Agent on H_2O_2 Formation, $\Delta\Psi$, and NADH of Heart Mitochondria Oxidizing Succinate[a]

Concentration of FCCP (nM)	Rate of H_2O_2 formation (nmol/min/mg)	Rate of O_2 uptake (ng-atom/min/mg)	$\Delta\Psi$ (mV)	NADH (% total NAD)
—	0.88 ± 0.12	126 ± 6	170.3 ± 1.3	84 ± 3
0.5	0.97 ± 0.17	—	171.0 ± 0.5	82 ± 3
1	0.87 ± 0.1	136 ± 10	171.2 ± 0.7	82 ± 3
2	0.73 ± 0.04	140 ± 11	170.8 ± 1.0	83 ± 3
5	0.28 ± 0.12	128 ± 39	118.3 ± 2.8	77 ± 3
10	0.05 ± 0.03	—	0	63 ± 8

[a] Values are given as the mean ± SEM, with the number of mitochondrial preparations used being 4.

findings (Table IV). With neither succinate nor nicotinamide nucleotide-linked substrate was there evidence of an increased H_2O_2 formation in senescence. Further, in earlier unpublished work from this lab (not shown) preparations from another 10 animals showed no age-linked changes. These data are not presented as the experiments used O_2-saturated media and a concentration of *p*-hydroxyphenylacetate five-times higher than that used in Table IV.

GENERAL DISCUSSION

The present results differ somewhat from those in the literature by showing a quantitatively more significant formation of H_2O_2 at the level of Complex I in respiring, coupled rat heart mitochondria and a relatively less active formation at Complex III (cf. Turrens and Boveris, 1980). Further, we show that H_2O_2 formation is very inactive when nicotinamide nucleotide-linked substrates or physiological concentrations of succinate (less than 0.5 mM, Lewandowski *et al.*, 1996) are used as substrate. Rates under these conditions are not more than 0.5 nmol of H_2O_2/min/mg of protein: rates of O_2 uptake are approximately 60 ng atoms of O_2/min/mg for State 4 oxidation of glutamate plus malate and 120 ng atoms of O_2/min/mg for State 4 succinate oxidation. Thus, approximately 0.4–0.8% of electron flow gives rise to H_2O_2, under these experimental conditions. This number may be approximately doubled if reaction of H_2O_2 with the peroxidase is incomplete, as indicated by Turrens *et al.* (1985). However, we do confirm that inhibition of electron flow at Complex III by antimycin A increases the rate of H_2O_2 formation when this otherwise is minimal, e.g., with glutamate plus malate as substrate. To the extent that the published literature emphasized studies using succinate at concentrations greater than 5 mM (Sohal *et al.*, 1994) and the inhibitor antimycin A (Giulivi *et al.*, 1995), it may give a misleading impression, viz. that ROS generation is more active in a physiological setting than in fact is so. Our failure to replicate findings of an age-linked increase in activity of ROS generation (Nohl and Hegner, 1978; Sohal *et al.*, 1994) is worrying. The preparations of mitochondria used here were obtained using the proteolytic enzyme nagarse which conceivably could lead to the loss of a fragile subset of mitochondria. Yet it is noted that these preparations, while showing unchanged specific activities of State 3 pyruvate and glutamate oxidation with aging, do display the specific decrements in fatty acid oxidation with aging reported by Hansford (1978). It is notable that there is more variation in rates of H_2O_2 production than in State 3 rates of O_2 uptake among the same preparations, suggesting that there is some uncontrolled variable affecting ROS generation. It is conceivable that there is even less activity of ROS production *in vivo* and that it is artifactually increased during preparation. We investigated one variable which has not been studied elsewhere, namely the degree of repletion with the cation Mn^{2+}, which is a cofactor of the mitochondrial superoxide dismutase (Mn-SOD), and which is transported by the membrane carriers of the mitochondrial Ca^{2+} cycle, albeit slowly on the efflux pathway (Gunter and Pfeiffer, 1990) and can therefore presumably be withdrawn from mitochondria with EDTA or EGTA. The stimulation obtained with added Mn^{2+} could reflect activation of SOD or it could be due to an artifactual electron shuttling from sites which do not normally react with O_2, mediated by valence changes of this cation. For this reason, we have presented data both with and without added Mn^{2+}.

Finally, we do not want to minimize the possible significance of accumulated oxidative damage in aging. Though the rates of ROS generation reported here are a somewhat smaller fraction of O_2 uptake rates than those reported elsewhere (Chance *et al.*, 1979), they would still generate a substantial molarity of O_2 radicals during the life of a mitochondrion if they were not removed. Equally, an unchanged rate of ROS formation with aging, as found here, does not rule out an accumulation of oxidative damage with aging. To the extent that this may be true of mt-DNA (Ames *et al.*, 1993; Mecocci *et al.*, 1993; Agarwal and Sohal, 1994), this could be particularly significant.

Table IV. Effect of Senescence on Rate of H_2O_2 Formation

Substrate (mM)	Mn^{2+}	Rate of H_2O_2 formation (nmol/min/mg) 6 mo	24 mo
Succinate (5)	—	0.50 ± 0.07	0.34 ± 0.05
Succinate (5)	+	1.12 ± 0.07	0.96 ± 0.12
Pyruvate (2.5) plus Malate (2.5)	+	0.10 ± 0.01	0.10 ± 0.02

[a] Mitochondria were isolated from hearts of male rats of the ages indicated. Data are derived from five preparations of each age group.

REFERENCES

Agarwal, S., and Sohal, R. J. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 12332–12335.

Ames, B. N., Shigenaga, M. K, and Hagen, T. M. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 7915–7922.

Barja, G., Cadenas, S., Rojas, C., Pérez-Campo, R., and López-Torres, M. (1994). *Free Rad. Res.* **21**, 317–328.

Boveris, A. (1984). *Methods Enzymol.* **105**, 429–435.

Boveris, A., and Chance, B. (1973). *Biochem. J.* **134**, 707–716.

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

Boveris, A., Cadenas, E., and Stoppani, A. O. M. (1976). *Biochem. J.* **156**, 435–444.

Brown, M. D., and Wallace, D. C. (1994). *J. Bioenerg. Biomembr.* **26**, 273–290.

Chance, B., Sies, H., and Boveris, A. (1979). *Physiol. Rev.* **59**, 527–603.

Cutler, R. G. (1984). In *Free Radicals in Molecular Biology, Aging, and Disease* (Armstrong, D., *et al.*, eds.) Raven Press, pp. 235–266.

Giulivi, C., Boveris, A., and Cadenas, E. (1995). *Arch. Biochem. Biophys.* **316**, 909–916.

Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **258**, C755–C786.

Hansford, R. G. (1978). *Biochem. J.* **170**, 285–295.

Hyslop, P. A., and Sklar, L. A. (1984). *Anal. Biochem.* **141**, 280–286.

Kamo, N., Muragatsu, M., Hongoh, R., and Kotabake, Y. J. (1979). *J. Membr. Biol.* **49**, 105–121.

Lewandowski, E. D., Doumen, C., White, L. T., LaNoue, K. F., Damico, L. A., and Yu, X. (1996). *Magn. Reson. Med.* **35**, 149–154.

Linnane, A. W., Marzuki, S., Ozawa, T., and Tanaka, M. (1989). *Lancet* **25**, 642–645.

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

Mecocci, P., MacGarvey, U., Kaufman, A. E., Koontz, D., Shoffner, J. M., Wallace, D. C., and Beal, M. F. (1993). *Ann. Neurol.* **34**, 609–616.

Miquel, J., Economos, A. C., Fleming, J., and Johnson, J. E., Jr. (1980). *Exp. Gerontol.* **15**, 575–591.

Nohl, H., and Hegner, D. (1978). *Eur. J. Biochem.* **82**, 563–567.

Shigenaga, M. K., Hagen, T. M., and Ames, B. N. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 10771–10778.

Sohal, R. S. (1991). *Mech. Age. Dev.* **60**, 189–198.

Sohal, R. S., Ku, H.-H., Agarwal, S., Forster, M. J., and Lal, H. (1994). *Mech. Age. Dev.* **74**, 121–131.

Turrens, J. F., and Boveris, A. (1980). *Biochem. J.* **191**, 421–427.

Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985). *Arch. Biochem. Biophys.* **237**, 408–414.

von Jagow, G., Ljungdahl, P. O., Graf, P. Ohnishi, T., and Trumpower, B. L. (1984). *J. Biol. Chem.* **259**, 6318–6326.