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Mitochondrial Oxygen Radical Generation and Leak: Sites of Production in States 4 and 3, Organ Specificity, and Relation to Aging and Longevity[1]

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Studies in heart and nonsynaptic brain mitochondria from two mammals and three birds show that complex I generates oxygen radicals in heart and nonsynaptic brain mitochondria in States 4 and 3, whereas complex III does it only in heart mitochondria and only in State 4. The increase in oxygen consumption during the State 4 to 3 transition is not accompanied by a proportional increase in oxygen radical generation. This will protect mitochondria and tissues during bursts of activity. Comparisons between young and old rodents do not show a consistent pattern of variation in mitochondrial oxygen radical production during aging. However, all the interspecies comparisons performed to date between different mammals, and between mammals and birds, agree that animals with high maximum longevities have low rates of mitochondrial oxygen radical production, irrespective of the value of their basal specific metabolic rate. The sites and mechanisms allowing this, the recently described low degree of membrane fatty acid unsaturation of longevous animals, and their relation to longevity and aging are discussed.

KEY WORDS: Free radicals; H_2O_2, complex I; heart; brain; free-radical leak; complex III; mitochondria; aging; longevity.

INTRODUCTION

There is growing evidence that oxidative stress is primarily or secondarily involved in many degenerative diseases. The incidence of these diseases acutely increases during the last segment of the life span in humans and other animals. This is so because the endogenous underlying aging process constitutes a basis over which many degenerative diseases can develop as the individual grows old. While an increased oxidative stress can be brought about by the interaction of the body with a plethora of chemical or physical factors coming from the environment, there is substantial agreement that in the healthy organism, the one more relevant for aging studies, the major continuous source of cellular oxidative stress is the production of oxygen radicals during aerobic metabolism. It is this endogenous free radical source that can matter for aging, a process occurring at different rates in different animal species, leading to their very different maximum life-spans (MLSPs). These genetically determined interspecific differences in aging rate are mainly determined by endogenous factors. Exogenous factors, on the other hand, can greatly modify the amount of time that a particular individual lives (e.g., only 40 or more than 80 years in humans), without significantly changing the aging rate and the MLSP of the species. Only by means of a decrease in the rate of aging will it be possible to substantially increase the number of "young" years enjoyed with a good

[1] Key to abbreviations: SOD, superoxide dismutase; Q, ubiquinone; $Q^{\cdot-}$, ubisemiquinone; $O_2^{\cdot-}$ superoxide radical; TTFA, thenoyltrifluoroacetone; RCR, respiratory control ratio; BMR, weight-specific basal metabolic rate; MLSP, maximum life-span; pyr/mal, pyruvate/malate; mtDNA, mitochondrial DNA; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

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quality of life, delaying, at the same time, the moment of appearance at high incidence of degenerative diseases, like cardiovascular diseases, cancer, and many others.

In pathological states, the main source of free radicals in cells and tissues can be the microsomes, the peroxisomes, redox cycling of xenobiotics, various ischemia/reperfusion-related factors, etc. However, in the healthy intact organism, in which the level of oxidative stress is much lower, the main oxygen radical generator is considered to be the mitochondrial respiratory chain (Boveris and Chance, 1973), because more than 90% of the respiration of vital tissues is due to oxygen consumption by mitochondria. A small, albeit significant, part of this respiratory activity leads to superoxide radical ($O_2^{\bullet -}$) and then to H_2O_2 generation. These two active oxygen species can generate highly reactive radicals, like $OH^{\bullet}$, which will modify all kinds of macromolecules including unsaturated lipids, proteins, and DNA. A complex network of enzymic and nonenzymic antioxidants can eliminate many of these radicals, but this elimination is not fully efficient and the strongest free radical damage is expected to occur near the sites of oxygen radical generation, since the most reactive radicals, like $OH^{\bullet}$, can not diffuse away and react unspecifically in nanoseconds with almost any nearby molecule. Close to the sites of oxygen radical generation, like the inner mitochondrial membrane, a high local free radical activity is expected near neighboring molecules, including mitochondrial DNA (Barja *et al.*, 1994a, b; Pérez-Campo *et al.*, 1998; Barja, 1998). This local free radical activity can be indirectly estimated by measuring the rate of H_2O_2 secretion by functional mitochondria to an outer incubation medium. Because of a number of reasons, the possibility that mitochondrial oxygen radical production is a major cause of intrinsic aging is now being investigated (see Pérez-Campo *et al.*, 1998 and Barja, 1998 for review). This would be consistent with the free radical theory of aging (Harman, 1956; see also Sohal and Weindruch, 1996 and Beckman and Ames, 1998 for review) as well as with a possible main role of mitochondria in the aging process (Harman, 1972; Miquel, 1991).

The mitochondrial sites of oxygen radical generation have been the subject of many studies unrelated to aging (e.g., Hinkle *et al.*, 1967; Loschen *et al.*, 1973; Boveris *et al.*, 1976; Cadenas *et al.*, 1977; Takeshigue and Minakami, 1979; Turrens and Boveris, 1980). Those studies have been performed almost exclusively with mitochondria from cows or rats respiring in the resting State 4 (with substrate and no ADP). Although the results of those investigations are more complex, there is a widespread belief that the main or the only mitochondrial oxygen radical generator is an unstable ubisemiquinone ($Q^{\bullet -}$) of the Q cycle at complex III of the respiratory chain. Besides, the majority of previous investigations about the relationship between mitochondrial oxygen radical production and aging have used complex II-linked substrates—mostly succinate—and the age of the animals employed was not stated in the majority of the studies about the localization of the mitochondrial oxygen radical source (e.g., Loschen *et al.*, 1973, 1974; Turrens *et al.*, 1985; Nohl, 1987) as it was then common practice, especially in investigations unrelated to aging. It is also conceivable that the rate of H_2O_2 production and the main oxygen radical generator site can be different, depending on the degree of functionality of the mitochondrial preparations used.

In this article, a full review of the literature available about the mitochondrial sites of oxygen radical generation and the relationship between oxidative stress and aging is not attempted (see Beckman and Ames, 1998, for this second topic), and reference to research by other authors will be made only when needed for discussion purposes. I will mainly discuss recent data from my laboratory concerning: (1) the localization of the sites of mitochondrial oxygen radical production under both States 4 and 3 (substrate plus ADP) conditions; (2) the modification of the rate of mitochondrial H_2O_2 generation by ADP and its physiological significance during tissue activity; (3) the percentage free radical leak in the respiratory chain; and (4) the relationship of mitochondrial oxygen radical generation with aging and longevity, as well as the mechanisms determining it. The first three subjects refer only to heart and nonsynaptic brain mitochondria exhibiting appropriate respiratory control ratios (RCR, high) and State 4 respiration (low), freshly obtained from adult animals of five selected species of homeothermic vertebrates—two mammals and three birds. In our case, the rate of production of H_2O_2 by functional mitochondria to the outer incubation medium was the parameter measured, whereas in studies from other laboratories this parameter, or the production of $O_2^{\bullet -}$ by submitochondrial particles, was assayed. Intact mitochondria first generate $O_2^{\bullet -}$ (a poorly reactive radical) at the inner membrane, which is then converted to H_2O_2 by spontaneous or superoxide dismutase (SODMn)-catalyzed dismutation, and hydrogen peroxide (not $O_2^{\bullet -}$) is the species secreted to the external

medium (Forman and Azzi, 1997; and Fig. 1). We have confirmed this aspect in rat heart mitochondria respiring in State 4, since the rate of H_2O_2 production was the same in the presence and in the absence of SOD in the incubation medium (Barja, 1999). However, one can not be sure that in every new experimental manipulation this will always hold true, especially when the degree of reduction of the oxygen radical generator sites is maintained near its maximum. Addition of excess SOD to the incubation medium during measurements of the rate of mitochondrial H_2O_2 production ensures that the $O_2^{\bullet-}$ secreted (if any), is dismutated to H_2O_2, which is then specifically detected by the peroxidase present in the assay (Barja, 1999). Under these conditions, one can be sure that the measurements represent the total mitochondrial generation of oxygen radicals ($O_2^{\bullet-}$ plus H_2O_2).

STATE 4 H_2O_2 PRODUCTION

Heart Mitochondria: Complex I and Complex III

The localization of the sites of oxygen radical generation in functional mitochondria can be per-

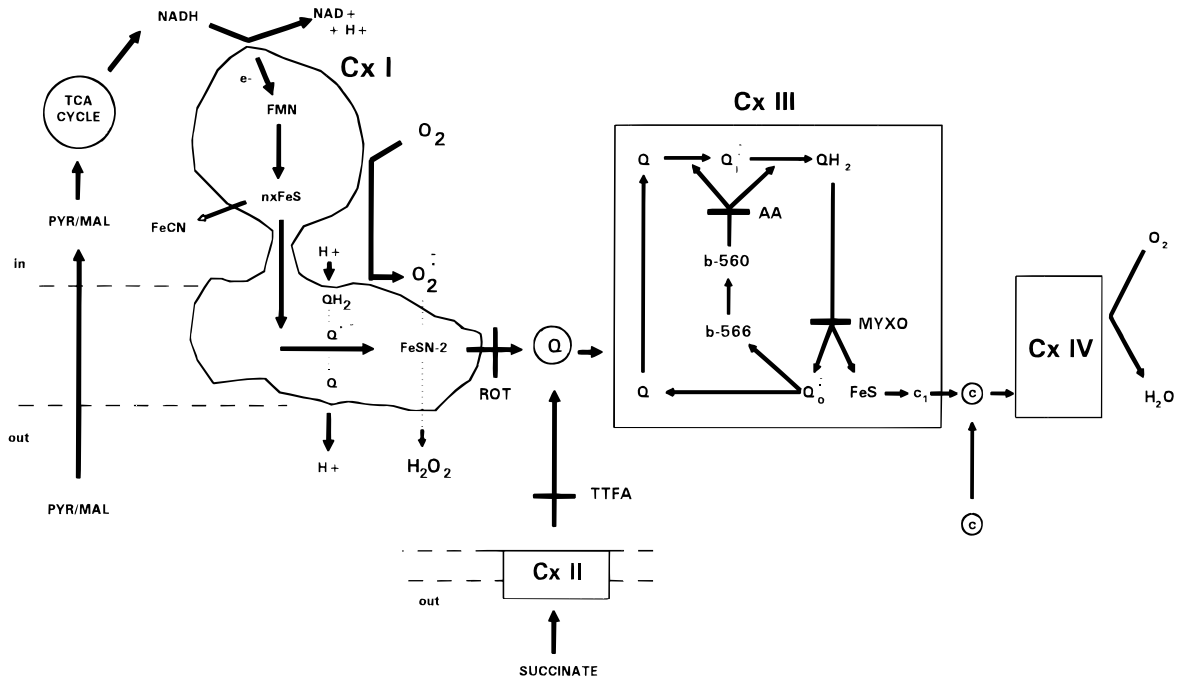

Fig. 1. The diagram shows the sites of action of specific substrates and inhibitors in relation to the four electron carriers of the mitochondrial respiratory chain complexes (CxI to CxIV). Pyruvate and malate (PYR/MAL) are NADH-linked substrates, which feed electrons at CxI, whereas succinate introduces electrons at CxII and cytochrome *c* does it between CxIII and CxIV. Rotenone (ROT) inhibits electron flow from CxI to the ubiquinone pool (Q). Thenoyltrifluoroacetone (TTFA) inhibits electron flow from CxII to Q. Antimycin A (AA) blocks electron transport in CxIII from cytochrome b_{560} to Q or $Q_i^{\bullet-}$ at center "in" (matrix side). Myxothiazol (MYXO) inhibits electron flow from ubiquinol (QH_2) to Rieske FeS center at Cx III. Q, mobile ubiquinone pool; QH_2, ubiquinol; $Q_i^{\bullet-}$, center "in" CxIII ubisemiquinone; $Q_0^{\bullet-}$, center "out" (intermembrane side) ubisemiquinone; c_1, cytochrome c_1; c, mobile cytochrome *c* pool. The shape of CxI is based on recent determination of three-dimensional structure of the bovine heart protein complex in ice at 22 Å resolution by cryomicroscopy (Grigorieff, 1998). The globular hydrophilic domain (matrix side) contains the subunits binding NADH, FMN, and all but one of the FeS clusters as yet defined by ESR. Electrons are thought to flow from the globular to the membrane domain through the newly discovered stalk connecting both domains. The membrane domain contains unstable $Q^{\bullet-}$ probably involved in H^+ pumping coupled to electron transport to the rotenone-binding protein FeS_{N2}. nxFeS represents the other five ESR-defined CxI iron-sulfur clusters (for CxI electron path see Tyler, 1992; Dutton *et al.* 1998, Robinson, 1998). FeCN, ferricyanide (an artificial CxI electron acceptor). e^- = electron flow. O_2 is first univalently reduced to $O_2^{\bullet-}$ by the CxI (and in some cases by the CxIII) oxygen radical generator/s; two $O_2^{\bullet-}$ molecules then dismutate, in a spontaneous or SODMn-catalyzed reaction, to one molecule H_2O_2, which generates *in situ* more reactive radicals or diffuses across the mitochondrial membranes to the surrounding medium. Horizontal dotted lines represent the inner mitochondrial membrane.

formed by kinetically measuring their rate of H_2O_2 production with a method showing instantaneous response to the inorganic peroxide, and adding different combinations of substrates and inhibitors specific for distinct segments of the respiratory chain at the start and during the kinetic run, respectively (Fig. 1). The method used must be sensitive enough to detect oxygen radical production in the absence of respiratory inhibitors, must be specific for H_2O_2, and must be free from the interference of any antioxidant situated between the generator (at the inner mitochondrial membrane) and the enzymatic H_2O_2 detection system (in the surrounding incubation medium). Table I summarizes the results obtained in my laboratory using a H_2O_2 detection method fulfilling those characteristics (Barja, 1999) in coupled State 4 heart mitochondria freshly obtained from fully grown young adult rats, mice, pigeons, canaries, and parakeets (budgerigars). Qualitatively, the results obtained were almost the same in the five species. It is well known that the rate of oxygen radical production increases as a function of the degree of reduction of the autoxidizable electron carriers (Boveris and Chance, 1973). Blocking the respiratory chain with an inhibitor increases the reduction state of electron carriers on the substrate side of the inhibitor, whereas those in the oxygen (opposite) side change to a more oxidized state. Thus, an increase in oxygen radical production following the addition of an inhibitor means that the oxygen radical generation site is located on the substrate side. Conversely, if oxygen radical production decreases after addition of the inhibitor, the generator must be situated on the oxygen side.

Addition of thenoyltrifluoroacetone (TTFA) to succinate-supplemented heart mitochondria of adult animals did not increase mitochondrial H_2O_2 production in any species (Table I). This agrees with previous work on mammalian heart mitochondria (Cadenas and Boveris, 1980) and suggests that complex II is not an important oxygen radical generator. All the experiments of Table I designed to answer the question if complex I generates or not oxygen radicals (five experiments) gave us an affirmative answer: (1) The strong increase in heart H_2O_2 production observed after the addition of antimycin A to pyruvate/malate (pyr/mal)-supplemented mitochondria (Table I) indicates that complex I, complex III, or both, must contain an oxygen radical generator; (2) H_2O_2 production with substrate alone was higher with pyr/mal than with succinate. This indicates that complex I is involved in oxygen radical generation since the only difference in electron path between the experiments with those two substrates is that electrons flow through complex I with pyr/mal but not with succinate; (3) The sharp increase in H_2O_2 production brought about by the addition of rotenone with pyr/mal as substrate unequivocally demonstrates that complex I generates oxygen radicals in functional heart mitochondria (Table I; Fig. 2A; Herrero and Barja, 1997a). The same has been recently observed, also in adult rats, using other complex I-linked substrates, glutamate/malate (Hansford *et al.*, 1997). Previous investigations had also shown clear increases in $O_2^{\bullet -}$ or H_2O_2 production after addition of rotenone to NADH-supplemented submitochondrial

Table I. Summary of Changes in H_2O_2 Production of Heart Mitochondria from Male Young Adult Rats, Mice, Pigeons, Budgerigars, and Canaries using Different Substrates and Inhibitors Specific for Different Segments of the Respiratory Chain[a]

Substrate–inhibitor	Change in H_2O_2 production
Pyruvate/malate (Pyr/mal)	(Higher than Succ)
Pyr/mal + Rot	Increase versus Pyr/mal
Pyr/mal + AA	Increase versus Pyr/mal
Pyr/mal + AA + Myxo	Decrease versus Pyr/mal + AA
Pyr/mal + ADP	No change versus Pyr/mal (nonsignificant trend to moderate increase in the 5 species)
Succinate (Succ)	(Lower than Pyr/mal)
Succ + TTFA	Decrease or no change versus Succ[b]
Succ + Rot	Decrease or no change versus Succ[b]
Succ + ADP	Strong decrease versus Succ (virtual stop of H_2O_2 production)

Note. Data come from Herrero and Barja: 1997a, studies with inhibitors in rats and pigeons; 1997b, ADP results in rats and pigeons; and 1998, studies with inhibitors and ADP in mice, canaries, and budgerigars.

[a] Pyr/mal = pyruvate/malate; Succ = succinate; Rot = rotenone; AA = antimycin A; Myxo = myxothiazol; TTFA = thenoyltrifluoroacetone. Age of the animals used: Wistar rats (6–8 months); Swiss mice (5–7 months); pigeons (2–4 years); Budgerigar parakeets and canaries (2–3 years). Succinate experiments were performed only in rats and pigeons.

[b] Decrease in rats, no significant change in pigeons.

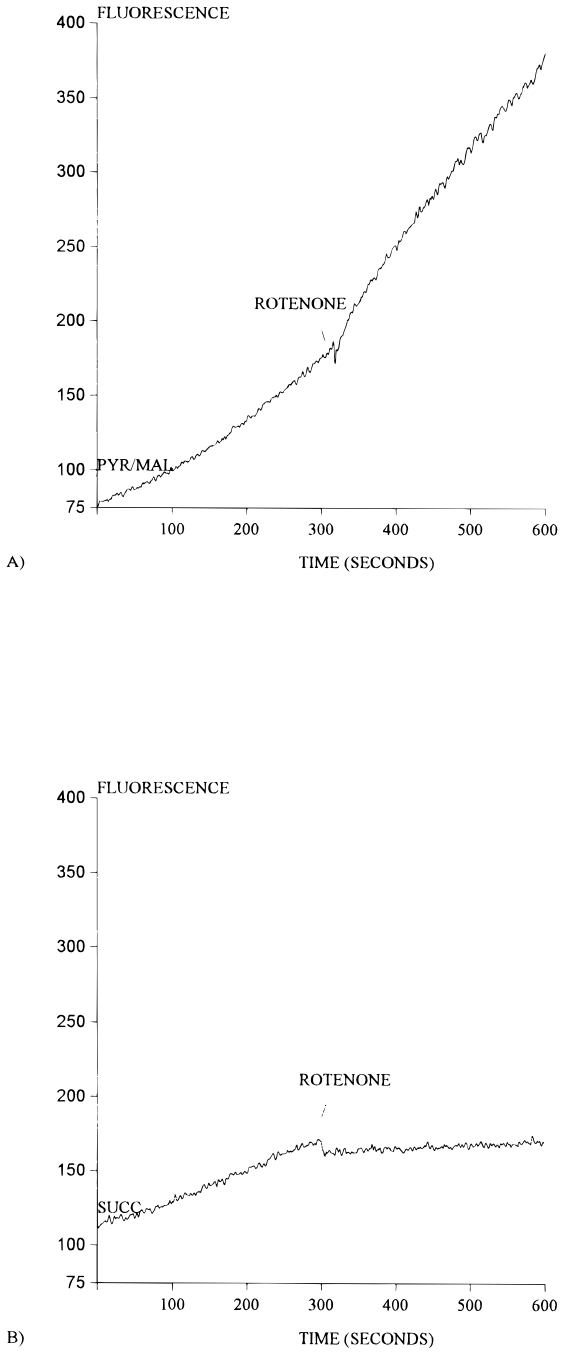

Fig. 2. Effect of rotenone on H_2O_2 production of rat heart mitochondria supplemented with pyruvate/malate (PYR/MAL) (A) or succinate (SUCC) (B). The H_2O_2 secreted by mitochondria react in the incubation medium with homovanillic acid in the presence of horseradish peroxidase, generating a product that fluoresces at 312 nm excitation and 420 nm emission (Barja, 1999). H_2O_2 production was first followed kinetically with substrate alone. Rotenone was then added and the kinetics was continued to observe its effect on the rate of H_2O_2 production. PYR, 2.5 m*M* pyruvate/2.5 m*M* malate; ROT, 2 μ*M* rotenone. The experiments shown in (A) and (B) are representative of the means ± S.D. values obtained (Herrero and Barja, 1997a). The two assays (A and B) were performed under identical conditions except for the type of substrate added. The combination of these two experiments, as well as others described in the text, demonstrate that complex I produces oxygen radicals during normal (A) as well as during reverse electron flow (B). See text (complex I H_2O_2 production sections) and Fig. 1 for further explanation.

particles from bovine heart (H_2O_2 production, Hinckle *et al.*, 1967; $O_2^{\bullet-}$ production, Takeshigue and Minakami, 1979 and Turrens and Boveris, 1980), to isolated complex I ($O_2^{\bullet-}$ production, Takeshigue and Minakami, 1979), and to human skin fibroblasts ($O_2^{\bullet-}$ production, Pitkänen and Robinson, 1996); (4) The high level of H_2O_2 production still remaining after addition of myxothiazol to antimycin A-treated pyr/mal-supplemented heart mitochondria (Table I; Herrero and Barja, 1997a); this "double-kill" treatment totally blocks the flow of electrons to the oxygen radical generators of complex III (Von Jagow and Engel, 1981; Turrens *et al.*, 1985; Matsuno-Yagui and Hatefi, 1996; see Fig. 1); (5) The fact that rotenone increases H_2O_2 production of rat heart mitochondria with pyr/mal (Fig. 2A), but decreases it with succinate (Fig. 2B), which has been also found in other independent investigations (Hansford *et al.*, 1997), shows again that complex I is involved in oxygen radical generation; the rotenone-induced decrease with succinate as substrate indicates that part of the succinate-supported oxygen radical generation of rat heart mitochondria is due to reverse electron flow (Chance and Hollunger, 1961) from succinate dehydrogenase to complex I. The energy required for this thermodynamically unfavorable reverse flow comes from H^+ pumping at site 2 (in complex III) and site 3 (in complex IV), still functional, since the remaining electrons flow in the normal direction from succinate to complex IV-bound oxygen (Tzagoloff, 1982). The observation of a rotenone-induced decrease in H_2O_2 production in succinate-supplemented heart submitochondrial particles led Britton Chance and collaborators more than three decades ago to situate, in a scheme and text, the autoxidizable component responsible for H_2O_2 generation (pictured as "Z" in their scheme) "between the cytochrome *b* and the flavoprotein of NADH dehydrogenase" to the left side of the rotenone block; nonheme iron was considered "attractive" as possible generator, although the participation of "other electron carriers such as Q_{10} was not eliminated" (Table I and pp. 5,173 in Hinkle *et al.*, 1967).

Evidence that complex III is also implicated in oxygen radical production in heart mitochondria from both species was also obtained. First, myxothiazol significantly decreased H_2O_2 production when added to antimycin A-treated pyr/mal-supplemented mitochondria (Table I). This decrease can only be due to complex III oxygen radical generators, like unstable $Q^{\bullet -}$, of which the formation is blocked by the simultaneous binding of antimycin A and myxothiazol in this complex (Fig. 1). On the other hand, rotenone, which increased H_2O_2 production with pyr/mal, decreased, but did not totally suppress it, with succinate in the rat (Fig. 2; Table I; Herrero and Barja, 1997a). The oxygen radical production still remaining after this rotenone addition can not come from complex I and would come from complex III, because it is thought that complex IV contains inside itself, but does not release to the outside, incompletely reduced forms of oxygen (Chance, 1981; see also our demonstration of this assertion for nonsynaptic brain mitochondria in the next section). In some (but not all) independent investigations performed at my laboratory, it has been observed (unpublished results) that antimycin A increases mitochondrial H_2O_2 generation and that complex III H_2O_2 production is higher than that coming from complex I in well-coupled heart mitochondria obtained from immature (12-week-old) male rats. This could be in agreement with various previous investigations in which the age or the body weight of the animals used and the RCR of the mitochondrial preparations were not stated except for a few cases (RCR higher than 2.5 with succinate Nohl and Hegner, 1978; and RCR 3.7 with malonate/glutamate/malate, Cadenas and Boveris, 1980). Such an increase was not seen by us and others (Hansford *et al.*, 1997, 6-month-old rats, see below) in well-coupled heart mitochondria from mature adult (6 to 8 month-old) male rats (Table I), which would be against a role of complex III in oxygen radical generation, whereas antimycin A always increased H_2O_2 generation with pyr/mal both in adult (Table I) and in 12 week-old rats (unpublished results). According to some authors, autoxidation of complex III $Q^{\bullet -}$ requires protons; hence, complex III $Q^{\bullet -}$ would not generate $O_2^{\bullet -}$ in the aprotic environment of the mitochondrial inner membrane unless the bilayer is first pathologically altered allowing H^+ to reach $Q^{\bullet -}$ or vice versa (Nohl and Stolze, 1992; Nohl *et al.*, 1997).

Our results indicate that in State 4 heart mitochondria from mature adults both complex I and complex III contribute to H_2O_2 generation, although the implication of complex III is not so clear since not all the experiments lead to the same conclusion, whereas this does happen in the case of complex I. Other authors have shown data that are consistent with the involvement of these two complexes in rat heart submitochondrial particles at 14 months of age, while at 3, 18, and 24 months of age it is deduced from their data that $O_2^{\bullet -}$ production is higher at complex I than at complex III (Muscari *et al.*, 1990). With the information available it is not possible to resolve whether the stimulation by antimycin A of H_2O_2 generation in succinate-supplemented heart mitochondria observed by various authors is related or not to the degree of intactness of the mitochondrial preparations, to developmental effects, or to other unknown factors, since neither the age of the animals nor the RCR of the mitochondrial preparations have been stated in the majority of the investigations. A recent study has also shown that such an increase only occurs if a minimum of 5 μM Mn^{2+} is included in the incubation medium, whereas in the absence of added Mn^{2+}, antimycin A not only not increases succinate-supported rat heart H_2O_2 production but, like in our case (Table I), even decreases it (Hansford *et al.*, 1997). In any case, the results of other experiments described above, adding rotenone-after succinate and adding myxothiazol after antimycin A, support the notion that complex III contributes to oxygen radical generation in State 4 heart mitochondria.

In short, the results obtained consistently indicate that, in addition to complex III (Boveris *et al.*, 1976; Turrens and Boveris, 1980; Turrens *et al.*, 1985; Nohl and Jordan, 1986), complex I contains an important State 4 oxygen radical generator in heart mitochondria of the two mammals and three birds studied. Succinate has been appropriately used to discriminate whether $Q^{\bullet -}$ (Boveris *et al.*, 1976; Turrens *et al.*, 1985) or cytochrome *b* (Nohl and Stolze, 1992) is the complex III oxygen radical generator. However, succinate has been also frequently employed in many studies measuring the rate of H_2O_2 generation in different species (Barja *et al.*, 1994b; Sohal *et al.*, 1990; Ku *et al.*, 1993), in animals of different ages or after experimental treatments *in vivo* (Sohal *et al.*, 1994, 1995a). In my opinion, when only one substrate is used for that kind of study, it is better to choose a complex I-linked substate like pyr/mal, rather than succinate. With pyr/mal, both complex I and complex III H_2O_2 generation are measured in State 4 heart mitochondria, whereas with succinate, complex I is bypassed and only or mainly complex III H_2O_2 production is assayed.

Concerning the nature of the complex III oxygen radical generator, some authors proposed cytochrome

b instead of $Q^{\bullet -}$ (Nohl and Stolze, 1992), since it was argued that this last molecule can not autoxidize inside the aprotic membrane environment and would produce $O_2^{\bullet -}$ only in permeabilized or altered mitochondrial inner membranes in which H^+ and $Q^{\bullet -}$ can encounter each other (Nohl *et al.*, 1997). However, it was shown that the double block with antimycin A plus myxothiazol strongly decreases succinate-supported oxygen radical production of rat heart mitochondria at the same time that cytochrome *b* is fully reduced (Turrens *et al.*, 1985; Turrens, 1997). This was interpreted as evidence that unstable $Q^{\bullet -}$, not *b* cytochromes, are the more likely $O_2^{\bullet -}$ generators of complex III. Addition of myxothiazol to antimycin A-treated heart mitochondria with either complex II-linked (Turrens *et al.*, 1985; Nohl and Jordan, 1986; Hansford *et al.*, 1997) or complex I-linked (Herrero and Barja, 1997a, 1998) substrates has consistently led to the same result in all laboratories: a decrease in the rate of mitochondrial oxygen radical generation.

With regard to the complex I oxygen radical generator, there are experimental data suggesting that the flavins are not involved, since they are situated at the beginning of the electron path (see also Fig. 1) before the site of ferricyanide reduction (Tyler, 1992; Dutton *et al.*, 1998), whereas the electron leak to oxygen seems to occur between the ferricyanide reduction site and the rotenone–binding site of complex I (Takeshigue and Minakami, 1979; Herrero and Barja, 1997a, 1998). Iron–sulfur clusters with a higher midpoint potential than FeS_{N1a}, which could be situated in the electron path after the ferricyanide reduction site (Tyler, 1992), or the FeS-related unstable $Q^{\bullet -}$ known to be present (ESR evidence) in the membrane domain of complex I (Ragan, 987; Dutton *et al.*, 1998) and possibly functioning in H^+ pumping coupled to electron transport (Robinson, 1998; Dutton *et al.*, 1998), could be the complex I oxygen radical generators (see Fig. 1). If this last possibility were true, ubisemiquinones could be responsible for oxygen radical generation both at complexes I and III, which would be highly coherent from the point of view of molecular evolution. However, many other complex I FeS centers can be also implicated because, under physiological conditions: (a) their reduced and oxidized states will not be present in equal concentrations; (b) interactions with many different factors and surrounding macromolecules can modify midpoint potentials *in vivo*; and (c) the exact position of many of FeS centers in the complex I electron path is still unknown.

Nonsynaptic Brain Mitochondria: Complex I

Since the majority of previous studies about the localization of the mitochondrial oxygen radical generation site had been performed with heart mitochondria, we decided to study another tissue highly relevant for aging. Table II summarizes the results obtained in coupled State 4 nonsynaptic brain mitochondria freshly isolated with Ficoll gradients by the method of Lai and Clark (1979) from adult rats and pigeons. Like in heart mitochondria, complex II did not seem to contribute to oxygen radical generation since succinate-supported H_2O_2 production was not increased by TTFA. Nevertheless, in nonsynaptic brain mitochondria, complex III did not show capacity for oxygen radical generation since: (1) antimycin A did not increase H_2O_2 production with succinate; (2) myxothiazol did not decrease H_2O_2 production when added to pyr/mal antimycin A-treated mitochondria; (3) H_2O_2 generation was almost undetectable with succinate but it was patent with pyr/mal. In addition, in these mitochondria we directly showed that complex IV does not contribute to oxygen radical generation, since ferrocytochrome *c* stimulated oxygen consumption but did not induce any H_2O_2 production when added as substrate to myxothiazol-treated mitochondria incubated in hypotonic medium (Table II). This last procedure opens the outer mitochondrial membrane, allowing ferrocytochrome *c* to reach the respiratory chain (Sherrat *et al.*, 1988).

All these experiments point to complex I as the only oxygen radical generator of nonsynaptic brain mitochondria. Direct evidence that this is the case comes from the observation that: (1) rotenone, antimycin A, and myxothiazol, when added in independent experiments to these pyr/mal-supplemented mitochondria, increased H_2O_2 production, and the rates obtained after these increases were not significantly different between the three inhibitors; (2) H_2O_2 production was one order of magnitude higher with pyr/mal than with succinate (it was negligible with this last substrate).

The finding that State 4 nonsynaptic brain mitochondria, differing from heart mitochondria, only produce oxygen radicals at complex I shows that the results obtained in a particular tissue can not be generalized to others. Likewise, our localization of the oxygen radical generator only at complex I of nonsynaptic brain mitochondria can not be extrapolated to synaptic ones without further investigation. Previous studies have detected (Cino and del Maestro, 1989; Ku and Sohal, 1993; Barja *et al.*, 1994b) or not (Sorgato *et*

Table II. Summary of Changes in H_2O_2 Production of Nonsynaptic Brain Mitochondria From Male Young Adult Rats and Pigeons using Substrates and Inhibitors Specific for Different Segments of the Respiratory Chain[a]

Substrate-inhibitor	Changes in H_2O_2 production
Pyruvate/malate (Pyr/mal)	(Much higher than with Succ)
Pyr/mal + Rot	Increase versus Pyr/mal
Pyr/mal + AA	Increase versus Pyr/mal
Pyr/mal + Myxo	Increase versus Pyr/mal
Pyr/mal + AA + Myxo	No change versus Pyr/mal + AA
Pyr/mal + ADP	No change versus Pyr/mal (nonsignificant trend to moderate increase in rat)
Succinate (Succ)	Much lower than Pyr/mal (negligible activity)
Succ + TTFA	No change versus Succ
Succ + Rot	No change versus Succ
Succ + AA	No change versus Succ
Ferrocytochrome *c*[b]	No H_2O_2 production was detected

[a] For abbreviations and age of animals see Table I.

[b] These experiments were performed in hypotonic medium, which opens the outer mitochondrial membrane allowing ferrocytochrome *c* to reach the respiratory chain (Sherrat *et al.*, 1988) and, in the presence of myxothiazol, to eliminate any interference due to electron flow between complexes III and IV in either the normal or the reverse direction. Data from Herrero and Barja, 1997b, and from Barja and Herrero, 1998.

al., 1974) substantial rates of oxygen radical production with succinate in crude brain mitochondria and others have deduced its occurrence in synaptic brain mitochondria using an indirect method (SOD-sensitive oxygen consumption; Floyd *et al.*, 1984), whereas nonsynaptic mitochondria, contrarily to synaptic ones, produced more oxygen radicals with malate/glutamate than with succinate (Floyd *et al.*, 1984). These last results should be regarded with caution, however, taking into account the method used and that only one single numeric value was reported per group without indicating the number of animals used.

STATE 3 H_2O_2 PRODUCTION AT COMPLEX I. FREE-RADICAL LEAK DECREASE DURING STATE 4 TO STATE 3 ENERGY TRANSITION

Mitochondrial H_2O_2 generation has been measured almost always in State 4. That is most probably related to the early observation that succinate-supported H_2O_2 production of heart mitochondria is virtually stopped after the addition of enough ADP to cause the transition from the resting State 4 to the active State 3 (Loschen *et al.*, 1971; Boveris *et al.*, 1972). This result was also recently obtained in my laboratory in rat and pigeon heart mitochondria (Table I). That kind of finding led to the widespread notion that mitochondria only produce oxygen radicals in State 4. Mitochondria in cells are often described as being in a range of states intermediate between States 4 and 3 (Brand and Murphy, 1987). A lack of oxygen radical generation in State 3 would eliminate the implication of reactive oxygen species of mitochondrial origin in tissue damage during activity (e.g., exercise or hypermetabolism). However, when pyr/mal is used instead of succinate, the ADP addition causing the State 4 to 3 transition no longer stopped H_2O_2 production in either heart or nonsynaptic brain mitochondria of the five species studied (see Tables I and II). These results indicate (Fig. 1) that complex I continues producing oxygen radicals in State 3 and contains the only oxygen radical generator of those phosphorylating mitochondria.

Taking together the results of Tables I and II, it turns out that complex I is always involved in oxygen radical generation in heart and nonsynaptic brain mitochondria under both State 4 and 3 conditions, whereas the contribution of complex III is limited to heart mitochondria and only when they respire in State 4. This further underlines the need of using complex I-linked substrates in routine quantitative assays of mitochondrial H_2O_2 generation in order not to bypass the contribution of complex I, very important at least in these two kinds of mitochondria. With complex I-linked substrates, electrons flow through the two oxygen radical generators of State 4 heart mitochondria (complexes I and III), whereas with complex II-linked ones they do it only through one of them (complex III), which,

in the majority of the tissues and States studied by us, does not generate oxygen radicals. Among many possible complex I-linked substrates, pyr/mal is a good choice. Its selection can be even more appropriate when comparisons between different tissues are attempted, or when the substrate transport capacity of the mitochondrial inner membrane is not well known in a given tissue and/or animal species, because the pyruvate (monocarboxylate) carrier is probably present in all kinds of mitochondria, especially in vertebrates, and pyruvate is a main mitochondrial fuel in many tissues.

It seems logical that complex I contains the main oxygen radical generator and that complex IV does not produce oxygen radicals. The electron carriers of the respiratory chain closest to substrate are more reduced than those closer to oxygen (Tzagoloff, 1982). One reason for the generation of oxygen radicals at complex I, in addition to its proximity to the substrate, can be the stoichiometric relationship among respiratory carriers, which has been described as 1 complex I: 2 complex II: 4 complex III; 4 cytochrome *c*, and complex IV; 64 ubiquinone (Q) in heart mitochondria (Tyler, 1992). This explains in part why the degree of reduction increases steadily from complex IV to complex I, since a similar amount of electrons must be transported by a progressively smaller number of electron carriers from Q and complex IV to complex I. Other things being equal, the higher the degree of reduction of the autoxidizable carrier, the stronger will be its tendency to leak electrons to oxygen.

The results of Tables I and II also show that the addition of ADP to pyr/mal-supplemented mitochondria did not significantly increase H_2O_2 generation. In the majority of cases, especially in heart mitochondria, we observed nonsignificant trends to a moderately increased H_2O_2 generation in State 3 compared to 4 (Herrero and Barja, 1997b; 1998), whereas oxygen consumption strongly increases (severalfold) during the State 4 to 3 transition as well as in the tissues *in vivo* from rest to activity (e.g., in muscles and heart during exercise). In other words, mitochondrial oxygen radical production does not increase in proportion to oxygen consumption during the State 4 to 3 transition. Indeed, we noticed that the percentage of total electron flow directed to oxygen radical generation (the free radical leak; Herrero and Barja, 1997a) is lower in State 3 than in 4 in heart and nonsynaptic brain mitochondria in the five studied species (Herrero and Barja, 1997b; 1998). This can be due, at least in part, to the lower degree of reduction shown by the respiratory chain in State 3 in relation to State 4 (Chance and Williams, 1956; Tzagoloff, 1982). The decreased free-radical leak compensates for the larger total electron flow in State 3 compared to 4, avoiding a massive increase in oxygen radical production in the aerobically active state. This provides the mitochondria with an instantaneous protective mechanism when oxygen consumption increases during tissue activity (up to 23-fold in human skeletal muscle and 4-fold in the human heart during exercise; Tyler, 1992). A moderate increase in mitochondrial oxygen radical production during the State 4 to 3 transition (suggested, but not demonstrated by our data) would be consistent with the existence of a mild (but not massive) tissue oxidative stress during exercise (Davies *et al.*, 1982; Jackson *et al.*, 1985). On the other hand, the absence of strong increases in mitochondrial oxygen radical generation during exercise, suggested by the extrapolation of our data to the *in vivo* situation, would agree with the fact that life-long chronic exercise does not shorten MLSP and can even increase the mean life-span (survival) of rodents (Goodrick, 1980; Holloszy *et al.* 1985; Holloszy, 1997) and humans (Paffenbarger *et al.*, 1986; Lee *et al.*, 1995). The lack of proportionality between mitochondrial oxygen radical production and oxygen consumption is not limited to the State 4 to 3 transition. We have also observed it in mitochondria from hyperthyroid animals when compared to those of euthyroid ones (unpublished results), as well as when comparing bird versus mammalian mitochondria in longevity studies (see further below). The absence of strong increases in mitochondrial oxygen radical production per milligram of mitochondrial protein during activation of mitochondrial respiration and from eu- to hyperthyroidism will protect the mitochondria themselves, but some increases in H_2O_2 flux from the mitochondria to the rest of the cell are still expected *in vivo*, since increases in total inner membrane area occur in the tissues after aerobic exercise training and chronic hyperthyroidism.

MITOCHONDRIAL H_2O_2 PRODUCTION AND ANIMAL AGE

It is sometimes presumed that the rate of mitochondrial H_2O_2 production increases with age. However, a careful analysis of the literature on the subject shows that this is not a consistent finding (Table III). Other main limitations of the published studies include: (1) there are problems of interpretation due to the

Table III. Published Variations in Mitochondrial H_2O_2 Production as a Function of Age

Species	Ages (months)	Substrate	State	Mitochondria (organ)	Kind of change	Reference[e]
Rat	3–24	Succ + AA + FCCP	State 4	H	Higher in 24 (34%)[a]	(1)
Rat	6–24	Succ, Pyr/mal	State 4	H	Nonsignificant differences	(2)
Rat	3–4–18–24	Glu, Succ	State 4	H	Peak at middle age (at 14–18)	(3)
Rat	3–14–18–28	NADH[b]	"State 4"	H-SMP	Peak at middle age (CxIII) or no change with age (CxI)	(4)
Rat	13 ages (1–24)	No substrate[c]	-	H, CB, L	Increase (H, CB) or no change (L) with age (see text)	(5)
Mouse	4–23	Succ + AA	State 4	H	Higher in 23 (14%)	(6)
Mouse	9–17–23	Succ	State 4	H, K, CB	Progressive increase with age[d]	(7)
Gerbil	3–15–24	Succ	State 4	H, K, CB	Progressive increase with age[d]	(8)

[a] Only one value in a young animal and one value in an old animal were reported.
[b] The parameter measured in this study was O_2 production by submitochondrial particles (SMP).
[c] $O_2^{\bullet-}$ production by mitochondria measured by ESR without substrate in the presence of Tyron.
[d] global comparison between the three ages. Kind of mitochondria: H = heart; CB = crude brain; L = Liver; K = Kidney; CxIII and CxI refer to $O_2^{\bullet-}$ production coming from complex III and complex I; SMP = submitochondrial particles (SMP). FCCP = carbonylcyanide-*p*-trifluoromethyoxyphenylhydrazone (uncoupler). Other abbreviations, as in Table I.
[e] (1) Nohl and Hegner, 1978; (2) Hansford *et al.*, 1997; (3) Guarnieri *et al.*, 1992; (4) Muscari *et al.*, 1990; (5) Sawada and Carlsson, 1987; (6) Kwong and Sohal, 1998; (7) Sohal *et al.*, 1994; (8) Sohal *et al.*, 1995a.

age of the animals selected; (2) the measurements are always performed in State 4 and there are no data in State 3; (3) the assays are almost always performed with succinate (H_2O_2 production coming mainly from complex III); (4) the majority of the studies used only heart tissue; (5) some studies only used mitochondria treated with respiratory inhibitors.

An early study found that heart mitochondrial H_2O_2 production with succinate as substrate was 34% higher in rats of 24 months of age than in those of 3 months (Nohl and Hegner, 1978; Table III). These experiments were performed in the presence of antimycin A and FCCP (an uncoupler), and a single value was reported at each age (Fig. 3 in Nohl and Hegner, 1978), although a posterior review referring to the same original publication included the standard deviation of the same single original values without indicating the number of animals used (Table I in Nohl, 1986). In the same early investigation, a 34% higher $O_2^{\bullet-}$ production of succinate-supplemented antimycin A-treated heart submitochondrial particles was found in eight experiments in old (23 months) versus eighteen experiments in young (3 months) rats (Nohl and Hegner, 1978). The authors also described a 43% higher $O_2^{\bullet-}$ production by heart mitochondria in eight experiments in old (23 months) in relation to eight experiments in young (3 months) rats (Nohl and Hegner, 1978). These last values were low (0.35 ± 0.05 nmol of $O_2^{\bullet-}$ min mg protein in young and 0.5 ± 0.06 in old animals; Nohl, 1986), were not compatible with the observation of other authors that intact State 4 heart mitochondria secrete H_2O_2, but not $O_2^{\bullet-}$ to the outside medium (see the end of the Introduction section), and were obtained without substrate in the presence of ATP (Nohl and Hegner, 1978). Since the age of the young animals used in this study was only 3 months, the increases with age can be related to developmental maturation (e.g., from 3 months to 6–9 months), instead of to senescent changes (from 6–9 months to 24 months). A similar problem can be involved in the smaller increase (14%) in H_2O_2 generation of antimycin A-treated heart mitochondria from 4- to 23-month-old mice (Kwong and Sohal 1998). With regard to this potential problem, another recent study did not find significant differences in heart mitochondrial H_2O_2 generation assayed with either succinate or pyr/mal between rats of 6 and 24 months of age (Hansford *et al.*, 1997; Table III). In another investigation, rat heart H_2O_2 production with either glutamate or succinate as substrate increased from 3 to 14–18 months of age, but decreased from 14–18 months to 24 months ("peak at middle age;" Guarnieri *et al.*, 1992; Table III), which would also be consistent with the idea that the increases in oxygen radical generation as a function of age described above can be related to developmental maturation rather than to senescent aging. Another report from the same laboratory found that a similar peak of $O_2^{\bullet-}$ production at middle age

shown by rat heart submitochondrial particles is limited to the contribution of complex III to $O_2^{\cdot-}$ generation (indirectly deduced by substraction), whereas complex I $O_2^{\cdot-}$ production (which was higher than that of complex III) remained constant at 3, 14, 18, and 28 months of age (Muscari *et al.*, 1990; Table III). Other studies have described globally considered progressive increases in H_2O_2 generation between the three studied ages, 9-, 17-, and 23-month-old mouse heart, kidney, and crude brain mitochondria (Sohal *et al.*, 1994), and between 3-, 15-, and 24-month-old gerbil heart, kidney, and crude brain mitochondria (Sohal *et al.*, 1995a). The more complete study from the point of view of the number of different ages selected (13 ages) found significant increases in mitochondrial $O_2^{\cdot-}$ generation with aging in heart (progressively from newborns to 24 months of age) and crude brain (4.6 to 11.5 to 24 months) but not in liver mitochondria (Sawada and Carlsson, 1987). $O_2^{\cdot-}$ is not expected to be secreted by intact mitochondria, at least in those from heart (see above), and the assays were performed measuring the ESR signal after reaction of Tyron with $O_2^{\cdot-}$, without any substrate added (Sawada and Carlsson, 1987). Decreases in total tissue free radical concentrations during aging, measured by direct ESR in frozen (77° K) liver (from 13 to 24 months) and testes (only from 3 to 24 months) have been also described in rats (Zhan *et al.*, 1992).

With the information available, it can not be concluded whether mitochondrial oxygen radical generation increases or not during aging. Would a lack of increase be contradictory with the free radical theory of aging? In my opinion, it would not. Mitochondrial oxygen radical generation is considered, in that theory, as a *cause* of oxidative damage. However aging is a progressive phenomenon taking place with approximately similar intensity at all adult ages, not occurring exclusively or at an accelerated rate in old individuals. The causes of aging must be present already in young individuals, otherwise they would not age and become old. Moreover, there is not any theoretical need to postulate an increase in *causing* factors during aging. What should progressively accumulate during aging, to validate the free radical theory, are the *consequences* of oxygen radical attack, the final forms of damage to macromolecules most relevant for aging (like DNA) in postmitotic tissues, whatever they may be (strand breaks, deletions, mutations, etc.). A constant rate of oxygen radical production in each animal species from the mature adult to the old animal can, theoretically at least, cause an accumulation of macromolecular damage, which would be consistent with the free radical theory of aging. However, different animal species age at very different rates. If the rate of oxygen radical production were a major cause of aging, then it should be higher in short-lived than in longevous species, while at the same time it could be constant with age in these two types of animals. The available evidence about that possibility is summarized in the next section. In my view, a major question to be answered in this context is if oxygen radical production is or is not one of the major causes of aging. If it were, an increase in its value in old individuals would be of relevant, but secondary, importance and it would increase the rate of aging at advanced age, which does not seem to fit well with the rather progressive decline in maximum physiological functions and then in homeostatic capacity, shown by rodents and humans during aging.

In summary, it is not clear if oxygen radical production increases, decreases, or does not change during aging, and further and better designed studies are clearly needed. However, it seems to me that the last of those three possibilities fits better with present gerontological facts and theoretical concepts. On the other hand, if the rate of mitochondrial oxygen radical production is directly or indirectly under the control of the genotype of each particular species in order to help to determine its MLSP, which would ultimately be one of the characteristics needed to maintain its biological fitness, it would be a normal function of mitochondria instead of, as it is frequently assumed, a simple "byproduct" of mitochondrial respiration.

RELATIONSHIP BETWEEN MITOCHONDRIAL H_2O_2 PRODUCTION, AGING RATE, AND MAXIMUM LONGEVITY

Initial studies approached this topic comparing mitochondrial oxygen radical generation between mammalian species with different longevities (MLSPs) (and thus also different aging rates) and strongly differing in body size: mouse, rat, guinea pig, rabbit, pig, and cow (Sohal *et al.*, 1990). These are species that "follow" the rate of living theory, the inverse relationship between basal specific metabolic rate (BMR: flux of calories per unit time per unit body weight) and MLSP (Rubner, 1908; Pearl, 1928). The results showed a negative exponential correlation between liver $O_2^{\cdot-}$ (in submitochondrial particles, Sohal *et al.*, 1989) or H_2O_2 (in mitochondria, Sohal *et al.*,

1990) production and the MLSP of those mammals (Table IV; see also Pérez-Campo *et al.*, 1998 for review). Similar negative relationships in kidney and heart mitochondria of the same species plus hamsters were found afterward in the same laboratory (Ku *et al.*, 1993). However, since the included species followed the rate of living theory, the results obtained could also be interpreted, in principle, as a correlate of that phenomenon: the species with short MLSP could show high mitochondrial H_2O_2 production simply because their rates of mitochondrial oxygen consumption were also higher. Positive correlations between mitochondrial oxygen consumption and oxygen radical production, and between mitochondrial oxygen radical production and the BMR were indeed found by the authors in those species (Ku *et al.*, 1993). Therefore, those studies can not discard the possibility that the correlations observed between mitochondrial oxygen radical production and MLSP were simply due to the correlation observed between mitochondrial oxygen radical production and BMR. This last rate could be also correlated, in turn, with many other unknown factors causing aging, since hundreds of different reactions occur at an accelerated rate when the BMR is high. This is why the study of the mitochondrial H_2O_2 production of birds, animals with both a high rate of oxygen consumption and a high MLSP (a very uncommon trait), was decided at my laboratory. The possession of an extraordinarily high longevity in relation to body size or BMR, compared to the majority of mammals following the rate of living phenomenon, is shown only by three groups of homeothermic vertebrates (see Austad and Fischer, 1992 and Prinzinger, 1993 for review): birds (3-fold life-span extension as a group), bats (2.8-fold life-span extension), and primates (2-fold life-span extension; 4-fold in humans). We hypothesized that if a low rate of oxygen radical production were contributing to a slow aging rate in birds, which are easy to obtain for those studies, their mitochondria should show a low rate of H_2O_2 production in spite of the high rate oxygen consumption of these animals (López-Torres 1993a; Barja *et al.*, 1994a). We found, indeed, that the State 4 succinate-supported rate of H_2O_2 production of crude brain, lung, and liver mitochondria was lower in pigeons (MLSP = 35 years) than in rats (MLSP = 4 years), animals showing a 9-fold difference in longevity, although BMR and body size are of a similar order of magnitude in these two homeothermic vertebrates (Barja *et al.*, 1994b). A lower H_2O_2 production in the pigeon than in the rat was also independently described in heart, crude brain, and kidney mitochondria respiring also with succinate in State 4 (Ku and Sohal, 1993). Afterward, we have also found lower H_2O_2 production in pigeon than in rat heart mitochondria with pyr/mal and succinate in State 4 (Herrero and Barja, 1997a), as well as in nonsynaptic brain mitochondria with pyr/mal (complex I production) in States 4 and 3 (Herrero and Barja, 1997b; Barja and Herrero, 1998). The lower rates of oxygen radical generation of pigeon versus rat mitochondria (Table IV), observed at least in five vital tissues, heart, brain, lung, kidney and liver, and occurring both in States 4 and 3 (Herrero and Barja, 1997b; Barja and Herrero, 1998), are in agreement with the free radical theory of aging, taking into account the MLSPs of the two species selected. However, the lower rate of oxygen radical production of pigeon mitochondria could also be due, in principle, to other characteristics of this particular animal species not related to its high MLSP.

In order to know better if a low rate of oxygen radical production is a general characteristic of birds, we decided to study the same problem in other birds species belonging to different orders than that of pigeons (Columbiformes). With this purpose, heart mitochondria were isolated from mice (MLSP = 3.5 years), canaries (*Serinus canarius*; MLSP = 24 years; Order Passeriformes) and parakeets (budgerigars, *Melopsittacus undulatus*; MLSP = 21 years; Order Psittaciformes). These three homeothermic species also show, like in the rat–pigeon comparison, similar values of BMR and body size, but a very different MLSP. The results showed that the rate of H_2O_2 production of pyr/mal-supplemented heart mitochondria is also significantly lower in canaries and parakeets than in mice both in States 4 and 3 (Herrero and Barja, 1998). This suggests that the capacity to have a low rate of oxygen radical production is a general characteristic of bird mitochondria, especially longevous animals. The presence of a low rate of oxygen radical generation in birds occurs at complexes I and III in State 4 heart mitochondria, and only at complex I in State 3 heart mitochondria and in nonsynaptic brain mitochondria in both States (Herrero and Barja, 1997a, b, 1998; Barja and Herrero, 1998). A recent report, using almost the same interspecies comparison as us, elegantly showed that cultured kidney tubular epithelial cells (mostly early passage replicating cells) of budgerigars, canaries, and starlings (MLSP = 20 years) are more resistant to 95% oxygen, 20–100 μM H_2O_2, 50–400 μM paraquat, and 40–1200 rads of γ-radiation than the corresponding cells of mice and that the cells of

these birds have fewer breaks in their genomic DNA than those of the mouse both before and after a similar challenge with H_2O_2 (Ogburn *et al.*, 1998). Various reasons for the better protection of the DNA of these birds in relation to that of mice are possible, different from the levels of endogenous enzymic and nonenzymic tissue antioxidant concentrations, which, contrary to a single report in rats and pigeons (Ku and Sohal, 1993), we found to be generally lower in liver, brain, and lung of canaries and pigeons than in the same organs of mice and rats (López-Torres *et al.*, 1993a; Pérez Campo *et al.*, 1994; Barja *et al.*, 1994a,b; see also the next section). It would be most interesting to perform experiments analogous to those of Ogburn *et al.* (1998) in postmitotic cells.

A closer interspecies comparison has also shown that succinate-supported State 4 mitochondrial H_2O_2 production is lower in the white-footed mouse *Peromyscus leucopus* (MLSP = 8 years) than in the house mouse *Mus musculus* (MLSP = 3.5 years; Sohal *et al.*, 1993), whereas the BMR is again similar in the two species. The opposite kind of comparison can also be of value: mammals with similar MLSPs but different metabolic rates and body sizes, like the rat and the mouse, should show similar rates of mitochondrial oxygen radical generation if this is a main determinant of aging rate. Although measured in independent studies, but using the same assay method, we obtained similar rates of State 4 and 3 H_2O_2 production in heart mitochondria of rats and mice (Herrero and Barja, 1997a, 1998). This is consistent with their very similar MLSPs (3.5 and 4 years), whereas the BMR is threefold higher and the body size is around fifteenfold lower in the mouse than in the rat. A negative correlation between mitochondrial oxygen radical production and MLSP has also been described in comparisons among five species of dipteran flies (Sohal *et al.*, 1995b).

In the majority of the bird–mammal comparisons performed at our laboratory (see Table IV), the difference in mitochondrial H_2O_2 production between species was generally smaller (1.6- to 6.5-fold) than their difference in MLSP (6- to 8.8-fold), whereas in other studies the difference in H_2O_2 production between species was similar to (Ku *et al.*, 1993) or even much higher than the difference in MLSP between the shortest- and the longest-lived animal compared (42-fold difference in mitochondrial H_2O_2 production versus 7.5- to 8.6-fold difference in MLSP from rodents to cow). The relationship between the quantitative differences in mitochondrial H_2O_2 production and in MLSP between species observed by us is consistent with the idea, prevalent in gerontology, that aging is due to more than one single major cause.

A main mechanism responsible for the smaller H_2O_2 production of bird mitochondria is their lower mitochondrial free radical leak (a lower oxygen radical generation per unit oxygen consumption; Barja *et al.*, 1994b; Herrero and Barja, 1997a, b, 1998; Barja and Herrero, 1998), which, in the case of nonsynaptic brain mitochondria, was due to a capacity to maintain a lower degree of reduction of the complex I oxygen radical generator in the steady-state condition (Barja and Herrero, 1998). In heart mitochondria, an additional mechanism is operating or not depending on the bird species: the possession of a low rate of mitochondrial oxygen consumption (per milligram of mitochondrial protein) evolutionarily compensated with a larger heart size, which allows the maintenance of a cardiac output at rest similar to that of mammals (Herrero and Barja, 1997a, 1998). Canary heart emphasized the first mechanism (low free radical leak), parakeet heart emphasized the second one (low mitochondrial oxygen consumption and a large heart size), and pigeon heart mitochondria exhibited both mechanisms. The rate of mitochondrial oxygen radical production depends on the degree of reduction of the autoxidizable oxygen radical generator of the respiratory chain, but also on its concentration. That rate decreases as a function of BMR among mammals following the rate of living theory (Ku *et al.*, 1993) most probably because the concentrations of the components of the electron transport chain, which would include the oxygen radical generator, decrease from small-sized (short-lived and high BMR: rodents) to large-sized (long-lived and slow BMR: pig, cow, and humans, in part) mammals. Data showing this for cytochromes *a*, a_3, *b*, $c + c_1$, and *c* and for the protein/phospholipid ratio of the inner membrane from heart and other tissues are available (Williams, 1968; Tyler, 1992). However, in various mammal–bird comparisons, the differences in mitochondrial oxygen radical production between species are not proportional to their differences in mitochondrial oxygen consumption (Herrero and Barja, 1997a, 1998; Barja and Herrero, 1998). There is also a lack of proportionality between those two parameters during the transition from State 4 to 3 (Herrero and Barja, 1997b, 1998) and between eu- and hyperthyroid states (unpublished results). In my opinion, the simple idea that the rate of mitochondrial oxygen radical generation is always proportional to the rate of mitochondrial oxygen consumption (Fleming *et al.*, 1981; Miquel,

Table IV. Comparative Studies of Mitochondrial H_2O_2 Production in Mammals and Birds with Different Maximum Longevities

Species[a]	MLSP (years)[b]	Substrate[f]	State[d]	Mitochondria (organ)[c]	Mitochondrial H_2O_2 Production	Reference[e]
6 Mammals	3.5–30	Succ	State 4	L	Lower in longevous species	(1)
7 Mammals	3.5–30	Succ	State 4	H, K	Lower in longevous species	(2)
Mouse (*Peromyscus*)	3.5–8	Succ	State 4	H, CB	Lower in longevous species	(3)
Rat, Pigeon	4–35	Succ	State 4	H, CB, K	Lower in longevous species	(4)
Rat, Pigeon	4–35	Succ	State 4	CB, LU, L	Lower in longevous species	(5)
Rat, Pigeon	4–35	Pyr/Mal, Succ	State 4	H	Lower in longevous species	(6)
Rat, Pigeon	4–35	Pyr/Mal	State 4	NSB	Lower in longevous species	(7)
Rat, Pigeon	4–35	Pyr/Mal	State 3	H	Nonsignificant trend to lower levels in longevous species	(8)
Rat, Pigeon	4–35	Pyr/mal	State 3	NSB	Lower in longevous species	(8)
Mouse, Parakeet	3.5–21	Pyr/Mal	State 4, 3	H	Lower in longevous species	(9)
Mouse, Canary	3.5–24	Pyr/Mal	State 4, 3	H	Lower in longevous species	(9)

[a] Ages of animals used and species in refs. 1 and 2: mouse and rat (3 months), hamster (only in Ref. 2) and guinea pig (4 months), rabbit (7 months), pig (6 months); cow (2.5 years). Biological age was similar in all bird–mammal comparisons from our laboratory (mature adult males, refs. 5–9). In ref. 3, both house and white-footed mouse (*Peromyscus leucopus*) were 3.5 months of age.
[b] Extremes (largest and smallest) maximum life-span (MLSP) among the species compared.
[c] Type of mitochondria: L, liver; H, heart; K, kidney; CB, crude brain; LU, lung; NSB, nonsynaptic brain.
[d] State 4, substrate alone; State 3, substrate + ADP.
[e] (1) Sohal *et al.* 1990; (2) Ku *et al.* 1993; (3) Sohal *et al.* 1993; (4) Ku and Sohal, 1993; (5) Barja *et al.*, 1994b; (6) Herrero and Barja, 1997a; (7) Barja and Herrero, 1998; (8) Herrero and Barja, 1997b; (9) Herrero and Barja, 1998.
[f] See Table I, for abbreviations.

1991; Ku *et al.*, 1993) is erroneous and has shed more confusion than light on the free radical theory of aging during the last decades, similar to the strict adherence to the mistaken rate of living phenomenon.

In summary, the rate of living theory inversely relating BMR and MLSP (Pearl, 1928) is not universal, since there are many animal species with longevities much higher or lower than predicted by that theory. On the contrary, all the comparative studies performed to date in a total of 11 species, between different mammals or between mammals and birds, show, without a single exception, that longevous species have low rates of mitochondrial oxygen radical production. Therefore, mitochondrial oxygen radical generation seems to be a better correlate of longevity and aging rate than metabolic rate. In species following the rate of living phenomenon, the inverse correlation between BMR and MLSP can be a simple consequence of the similar free radical leak of these animals without any mechanistic connection between those two parameters. When comparing longevities of different species, which can not be explained based on that phenomenon, the absence of correlation between BMR and MLSP can be due to the presence of different free radical leaks in their respiratory chains. In all species, however, a low rate of free radical production correlates with a slow aging rate.

A decrease in free radical leak would be a very attractive way of slowing the rate of mitochondrial oxygen radical production in future experiments, because the ultimate purpose of basic gerontological studies is to decrease the rate of human aging without lowering the metabolic rate and thus the general level of activity, and to increase, at the same time, the quantity and the quality of human life. Besides, the presence of different values of free radical leak in species with different MLSPs and in different mitochondrial States and physiological situations agrees with the aforementioned idea that the rate of mitochondrial oxygen radical production can be a regulated phenomenon instead of a simple "byproduct" of mitochondrial respiration.

MITOCHONDRIAL OXYGEN RADICAL PRODUCTION, OXIDATIVE DAMAGE TO DNA, AND OTHER RELEVANT FACTORS CORRELATING WITH MLSP

Apart from the rate of mitochondrial oxygen radical production, only two other characteristics of longevous animals with a plausible mechanistic connection with endogenous macromolecular damage are known to be correlated with MLSP in the appropriate sense: (1) a low degree of total tissue and mitochondrial fatty

acid unsaturation (low double bond content) of the different phospholipid fractions; this is a regulated trait not due to the diet, accompanied by a low sensitivity to lipid peroxidation and a low concentration of the lipid peroxidation-derived adducts malondialdehyde-lysine and (carboxymethyl)lysine in tissue and mitochondrial proteins (Pamplona et al., 1996, 1998, 1999a,b); and (2) a high rate of repair of some forms of DNA damage. The evidences about this last trait have been recently reevaluated by normalizing the data obtained in different species in separate investigations versus the rat data of each of them. After doing this, a general agreement among the five different available studies was found: a positive relationship between DNA repair and the MLSP of the donor species (Cortopassi and Wang, 1996). It should be noted, however, that DNA repair (mostly excision repair after ultraviolet-induced lesions) was studied in those investigations in dividing cells in culture (mainly fibroblasts), whereas the correlations observed above between MLSP and the rate of mitochondrial oxygen radical production (Table IV), or between MLSP and the degree of fatty acid unsaturation were observed in postmitotic tissues, like heart, and in tissues with mixed (postmitotic/nonpostmitotic) cell types (like brain) or with intermediate characteristics regarding cell division and tissue regeneration capacity (like liver). Aging should be best studied in fixed postmitotic tissues (Miquel, 1991), which I think constitutes a most useful concept, whereas in dividing tissues aging-related changes are obscured by cellular mosaics, mitosis, and dilution effects due to replacement of damaged for nondamaged cells. In any case, of the three so far detected relevant characteristics of longevous animals, two are related to oxidative stress: *a low rate of mitochondrial oxygen radical production* and *a low degree of membrane fatty acid unsaturation*. It is even possible that these two traits are causally related, since mitochondrial oxygen radical production occurs in a membrane-based medium, an additional possibility that merits further investigation.

In contrast, endogenous antioxidants, including SOD, catalase, glutathione peroxidases, glutathione reductase, glutathione, and ascorbate generally correlated in a negative way with MLSP across mammals or across vertebrates in 10 out the 11 independent investigations performed in six different laboratories: 21 negative correlations, 6 cases of no correlation, and no cases of positive correlation (reviewed in Pérez-Campo *et al.*, 1998). The endogenous antioxidant concentrations were up to tenfold higher in the shortest-lived than in the most longevous species included in those comparative investigations. Therefore, contrary to previous proposals (Tolmasoff *et al.*, 1980; Cutler, 1991), antioxidants can not be longevity determinants. Those negative correlations of endogenous antioxidants with MLSP were what indirectly suggested us (López-Torres *et al.*, 1993a; Barja *et al.*, 1994a; Pérez-Campo *et al.*, 1998; Barja, 1998) that the rate of H_2O_2 production *in vivo*, in agreement with what has been observed in mitochondria *in vitro* (Table IV), should be low in longevous animals. The symmorphosis principle, widely followed by species in nature, states that animals do not build and maintain structures and functions (antioxidants in this case) in excess of what will be needed, because it would be energetically expensive (Schmidt-Nielsen, 1984). We have proposed that short-lived animals need the continuous presence of high levels of antioxidants in their tissues in order to cope with their continuously high rates of endogenous H_2O_2 generation (López-Torres et al., 1993a; Barja et al., 1994a,b). Longevous animals, on the other hand, would not need high constitutive levels of tissue antioxidants because their basal rates of H_2O_2 generation are low *in vivo*; they would depend, to a greater extent, on the induction of antioxidant defenses when exposed to a higher than normal oxidative stress (e.g., to oxidant xenobiotics). The nearness between points of continuous oxygen radical generation (the inner mitochondrial membrane) and relevant targets for aging (like mtDNA) can have been designed during evolution in order to ensure that *exogenous* factors (like many dietary antioxidants) will not alter the rate of aging (be it high or low) of each species, an *endogenous* process, because it would unbalance its biological fitness. It has been also shown experimentally that exogenous antioxidants like vitamins C and E and coenzyme Q_{10} do not decrease steady-state basal levels of tissue oxidative damage to genomic DNA (8-oxo-dG: 8-oxo-7,8-dihydro-2′-deoxyguanosine) in guinea pigs (Cadenas *et al.*, 1997) or 8-oxo-dG urinary excretion in human beings (Priemé *et al.*, 1997), whereas many investigations demonstrated that antioxidants can minimize increases in DNA oxidative damage elicited by exogenous prooxidants. This can mechanistically occur by antioxidant interception of diffusible active oxygen species like H_2O_2 when their point of generation induced by the prooxidant chemicals is situated far (e.g., at microsomes, peroxisomes, phagocytes or even nonhistone proteins; Loft *et al.*, 1999; Hiraku and Kawanishi, 1996) from the target (e.g., the nuclear DNA). This is not the case for basal and continuous

endogenous oxidative damage to mtDNA coming from the nearby or even in contact (Richter, 1995) inner mitochondrial membrane.

In agreement with the results of comparative investigations about antioxidants (reviewed in Pérez-Campo *et al.*, 1998), the available 15 independent life-long aging experiments in large samples of mammals (guinea pigs, rats, or mice) using dietary supplementation with many different antioxidants (see Yu, 1995 for review), and the simultaneous strong induction (100 to 1000% increase) of four endogenous antioxidants (superoxide dismutase, glutathione reductase, ascorbate, and glutathione) in at least four frog tissues throughout life-span (López-Torres *et al.*, 1993b,c), consistently showed a lack of effect of antioxidants on MLSP, except for one case in which a 12% increase was reported after treatment with 2-mercaptoethylamine hydrochloride (see Yu, 1995). In those aging experiments in mammals, a 12–30% increase or a lack of change in mean life-span were reported in 7 and 8 studies, respectively (Yu, 1995). In our frog experiment, mean life-span was duplicated without any effect on MLSP. According to a single study of 15 transgenic *Drosophila melanogaster* lines simultaneously overexpressing SOD (by 26–32% over controls) and catalase (by 43–73% over controls), eight showed an increase in MLSP (14–34% relative to controls), whereas in six lines there was no effect and one line showed a lower MLSP than controls (Orr and Sohal, 1994). In this study, the only biological marker of the rate of aging used, walking movement (negative geotaxis), was superior in overexpressors than in controls at virtually all ages until 35 days of age. That testing was discontinued after 42 days of age because of the onset mortality and the dramatic slowdown of the flies, while the MLSP was 71 days in controls and 81–95 days in the SOD plus catalase overexpressors (Orr and Sohal, 1994).

The increases in mean life-span elicited by antioxidants suggest that, in agreement with the majority of the studies carried out in human populations (e.g., Gey *et al.* 1991; Gaziano and Hennekens, 1995), they can unspecifically protect against many causes of early death—they can increase survival—when the experiments are performed under *suboptimum* conditions. These are more prevalent in aging experiments performed in nonmammalian species in which optimum conditions are not so well known or can not be achieved, like in our frog experiment (López-Torres *et al.*, 1993b,c) or in many invertebrate models. According to some authors, the reported increases in MLSP after various experimental manipulations of the commonly used N_2 Bristol *C. elegans* nematode seem only to bring back the shortened longevity of this laboratory strain (which shows a high rate of spontaneous mutations) to that of the natural populations without surpassing it (Reznick and Gershon, 1999). In any case, the potentially protective effects of the antioxidants can be very important for human individuals because, even in western human populations, environmental conditions are still not optimum.

Although both can be important, on theoretical grounds the rate of attack to the macromolecules probably more important for aging, like that due to mitochondrial oxygen radical production near mitochondrial DNA (mtDNA; Barja et al., 1994a,b; Barja, 1998), should be more important than their repair. DNA repair, like any conceivable homeostatic system, can not be 100% effective. Moreover, it would not make sense evolutionarily for longevous animals to exhibit high rates of attack to macromolecules and high repair rates. This would be a waste of energy and rather ineffective. For similar reasons, no known animal has increased MLSP through the possession of high levels of antioxidants to counteract high rates of oxygen radical generation. This would also be energetically wasteful and highly ineffective, since many free radicals would damage relevant nearby targets before they could be intercepted. A high rate of oxygen radical attack seems to be present in short-lived rodents, which evolutionarily emphasize high reproductive yields to compensate for their high predation rates (Austad and Fischer, 1992) and thus show high rates of accumulation of endogenous damage (Saul *et al.* 1987) including that to mtDNA (Cortopassi and Wang, 1996). However, if the attack to biologically relevant macromolecules is evolutionarily decreased (e.g., by lowering the rate of mitochondrial oxygen radical production), the efficiency in diminishing macromolecular damage is the 100% of that decrease.

Besides those theoretical considerations, it has generally been believed in the past that mitochondrial DNA (mtDNA) has no repair or that its rate of repair is much lower than that of nuclear DNA (Clayton *et al.*, 1974; Funakaga and Yielding, 1979; Miquel, 1991). While mtDNA lacks nucleotide excision repair (Anson et al., 1998) and repair of pyrimidine dimers after damage induced by UV radiation (Clayton *et al.*, 1974), various mtDNA repair enzymes have been found, including an AP endonuclease (Tomkinson *et al.*, 1988), a methyltransferase (Myers *et al.*, 1988), a uracil DNA glycosylase (Domena *et al.*, 1988), a

pyrimidine hydrate DNA glycosylase (Tomkinson *et al.*, 1990), a recombinational repair pathway (Thyagarajan *et al.*, 1996), and an oxidative damage endonuclease specific for 8-oxo-dG (Croteau *et al.*, 1997). It is now known that mitochondria remove at least five different types of mtDNA damage (Anson *et al.*, 1998). Among them, the repair of oxidative mtDNA damage occurs rapidly (within hours) in rodent and human cells, similarly to what happens in nuclear DNA (Lee *et al.*, 1996; Zastawny *et al.*, 1997). This finding indicates that the much higher steady-state oxidative damage observed in mtDNA in relation to nuclear DNA must be mainly due to the presence of a higher rate of oxygen radical attack to DNA in the mitochondria than in the nucleus, rather than to differences in the repair of oxidative DNA damage between these two compartments. The recent observation that mitochondrial oxidative damage endonuclease (mtODE) does not change much with aging (in the liver of 23 versus 12-month-old rats) and even increases from 6 to 12 months of age (Souza-Pinto *et al.*, 1998) would also be in agreement with the idea that mitochondrial oxygen radical generation is responsible for the reported increases in mtDNA oxidative damage with age (Meccoci *et al.*, 1993; Ozawa, 1995; García de la Asunción *et al.*, 1996). All this fits well with the proposal that the rate of mitochondrial oxygen radical production is a major determinant of the degree of mtDNA oxidative damage because the sites of generation (the inner membrane) and the target (mtDNA) are located nearby (Barja *et al.*, 1994a,b; Barja, 1998), although further research on repair of DNA oxidative damage is clearly needed.

The occurrence of substantially higher steady-state levels of 8-oxo-dG in mtDNA compared to nuclear DNA in rodents (Richter *et al.*, 1988) and humans (Mecocci *et al.*, 1993) has been recently confirmed at our laboratory (unpublished), using a method which avoided the reported potential artifacts (Beckman and Ames, 1996) due to injection of small amounts of deoxynucleosides in the HPLC system. We have obtained that kind of result using the same method of DNA digestion and HPLC analysis for nuclear and mitochondrial DNA on the same individual tissue samples. Still, it has not been clarified if the high degree of mtDNA damage has or has not an impact on aging rate through a putative decrease in the rate of ATP generation in old individuals (Miquel, 1991). While this is an attractive possibility, it could also be that a high rate of both oxidative attack and repair to mtDNA (and to nuclear DNA?) occurs in short-lived animals like rodents, the opposite being true in longevous animals, which is a topic which should be addressed in future experiments. This would lead to a higher rate of accumulation of *somatic mutations* in short- in relation to long-lived species, since DNA repair, like any other homeostatic mechanism, can not be 100% effective and error-free. In fact, it has been recently communicated that the repair of mitochondrial DNA oxidative damage is relatively error-prone (Souza-Pinto *et al.*, 1998). Unfortunately, once formed and established in the DNA, those mutations would lose any fingerprint that could testify to their *oxidative origin*. The clarification of this possibility must await the emergence of new appropriate techniques, since those putative mutations and DNA modifications should be looked for in *individual cells* of postmitotic vital tissues—the ones more relevant to aging.

CONCLUSIONS

The main findings summarized in this minireview coming from my and/or other laboratories are the following:

1. Localization studies in mitochondria from two vital tissues containing postmitotic cells show that, while complex III can produce H_2O_2 in State 4 heart mitochondria, the main oxygen radical generator is situated in complex I, since it is active in heart and nonsynaptic brain mitochondria under State 4 and 3 conditions.

2. Mitochondrial oxygen radical production does not necessarily increase in proportion to mitochondrial oxygen consumption either across species or in a single species under different physiological situations, because the free radical leak in the respiratory chain is not a constant.

3. It is not yet clear if mitochondrial oxygen radical generation increases or not during aging. It is argued here, however, that this possibility, although relevant, is only of secondary importance for the validity of the free radical theory of aging.

4. All the comparative studies performed to date show that oxygen radical generation is lower in longevous than in short-lived animals. This occurs in all kinds of species, comparing animals following the "rate of living theory"—the inverse relationship between longevity and metabolic rate—as well as in comparisons between animals showing differences in longevities not explainable based on their metabolic rates (mammals and birds). Therefore, the rate of mito-

chondrial oxygen radical generation is a better correlate of maximum longevity and aging rate than the basal rate of oxygen consumption. This is logical since, after all, the potentially damaging substances are the reactive oxygen species, not ground state oxygen. In our comparative studies, we have always observed lower differences in mitochondrial oxygen radical production than in longevity between species. This would be compatible with a role for mitochondrial oxygen radical production as a major cause of aging and, at the same time, with the prevalent idea that aging is due to more than one major mechanism. Comparative data also suggest that the mitochondrial rate of oxygen radical generation is under the control of the genotype of each animal species, instead of being, as it is commonly believed, a simple byproduct of mitochondrial respiration. The main factors determining such control should be studied in the future.

5. Some especially longevous species, like birds, have low rates of mitochondrial oxygen radical generation due, in part, to the possession of a low free radical leak in the respiratory chain. Mimicking such a mechanism in future experimental manipulations would be highly appealing, since it can decrease the rate of oxidative attack without decreasing the rate of electron flow in the respiratory chain and thus the rate of ATP production and the resulting global level activity, a main component of life quality. It remains to be tested if a decrease in the rate of generation of oxygen radicals in a given species, performed experimentally without deleteriously altering other vital factors through undesired interactions and side reactions, can slow down the rate of aging.

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