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Discoveries

COMPREHENSIVE INVITED REVIEW

Updating the Mitochondrial Free Radical Theory of Aging: An Integrated View, Key Aspects, and Confounding Concepts

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Abstract

An updated version of the mitochondrial free radical theory of aging (MFRTA) and longevity is reviewed. Key aspects of the theory are emphasized. Another main focus concerns common misconceptions that can mislead investigators from other specialties, even to wrongly discard the theory. Those different issues include (i) the main reactive oxygen species (ROS)-generating site in the respiratory chain in relation to aging and longevity: complex I; (ii) the close vicinity or even contact between that site and the mitochondrial DNA, in relation to the lack of local efficacy of antioxidants and to sub-cellular compartmentation; (iii) the relationship between mitochondrial ROS production and oxygen consumption; (iv) recent criticisms on the MFRTA; (v) the widespread assumption that ROS are simple "by-products" of the mitochondrial respiratory chain; (vi) the unnecessary postulation of "vicious cycle" hypotheses of mitochondrial ROS generation which are not central to the free radical theory of aging; and (vii) the role of DNA repair concerning endogenous *versus* exogenous damage. After considering the large body of data already available, two general characteristics responsible for the high maintenance degree of long-lived animals emerge: (i) a low generation rate of endogenous damage: and (ii) the possession of tissue macromolecules that are highly resistant to oxidative modification. *Antioxid. Redox Signal.* 19, 1420–1445.

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I. Introduction

It is fascinating that mother nature has managed to vary the rate of aging during evolution by 5000-fold from the perhaps few days day that some invertebrates probably live to the 507 years of lifespan of the longest living free living metazoan, *Arctica islandica* (109). It is the task of the gerontologists to unravel how it did it. Decreasing human aging rate by only one-fourth of its present rate would increase the human (maximum) longevity to almost 500 years from its present 122 years (Jean Calment) value (142). That longevity, in practical terms, will be almost the same as perpetual youth, as the aggregated likelihood of dying (young) from non aging-related causes will approach 100% during that long time. The hope that science and technology will know rather soon how to copy mother nature in this relatively small effort (fourfold life extension) is great. A main bonus of this achievement for humankind will be the almost total eradication of the degenerative diseases with a single kind of modification (slowing down aging), and the enjoying of a long or short but always "young," vigorous, and healthy life. This will continue the evolutionary trend to ever-increasing longevity of human evolution from our ancestor primitive primate species, which has been a part of the key of the success of *Homo sapiens*. Increasing longevity during evolution has been good in the past for humans, because learning and experience need time. Therefore, it is reasonable to hypothesize that continuing such evolutionary trends to increased longevity will be also good for the future of humankind (12).

Degenerative diseases are the main causes of death and suffering in old people, they generate tremendous socioeconomic problems to modern societies, and their underlying causes are deeply rooted in the endogenous aging process. Eradicating only one of them will not eliminate the problem, because the aged individual will still be old and will most likely experience another different degenerative disease soon. However, by decreasing the human aging rate, it would be possible in the future to delay and virtually eliminate those diseases, progressively approaching a situation in which individuals would stay physiologically young throughout their lives, long or short. Most importantly, decreasing the aging rate will lower the incidence of all the degenerative diseases with a single manipulation, instead of combating and defeating all of them, one by one, with a myriad of different therapeutics, which would lead to a myriad of secondary effects. Defeating aging is the main ultimate goal of gerontology. Although difficult, this would be likely more easy and effective than combating only the diseases of old age.

According to the most useful Strehler's four rules (161), aging is an endogenous, progressive, and naturally irreversible process that universally affects all individuals, and is deleterious for them. Aging is also especially patent in postmitotic cells such as the immense majority of neurons, or those of the heart and skeletal muscle. In contrast, other phenomena such as telomere shortening only occur in replicating cells. Aging, although clearly detrimental for the individual, can perhaps be useful and adaptive for the group (12, 94, 151). Aging should not be confused with the additional damage coming from external environmental sources that adds accumulative deleterious changes in the tissues of the old individual during his lifetime. Since aging is progressive, sudden detrimental changes only occurring at old age cannot be among the main causes of aging. They frequently represent harmful consequences of the aging process. The progressive character of aging means that the true causes of aging are expected to stay at essentially similar levels throughout life—they should not increase with age—as aging essentially manifests itself as a rather linear—instead of exponential—decrease of maximum physiological capacities, at least in vertebrates. The irreversibility of aging means that something deleterious for the individual—perhaps irreversible forms of permanent damage—should accumulate with age.

In this article, I give my own view of an updated version of the mitochondrial free radical theory of aging (MFRTA) succinctly reviewed, emphasizing key aspects of the theory, focusing on comparative studies between animals with widely different longevities as well as on dietary restriction (DR) animal models. Other possibly relevant aspects, including those concerning mitochondrial coupling (112), mitochondrial DNA (mtDNA) haplotypes (168), network perspectives (159), the quantitative relationship between oxidative damage and loss of function during aging (158), or mithormesis (133, 135) in aging and longevity are not covered in this article. Two known main parameters likely responsible for differences in longevity related to oxidative stress, the rate of mitochondrial reactive oxygen species production (mtROSp; ROS representing the sum of the $O_2^{\bullet -}$ plus the H_2O_2 produced) and the degree of unsaturation of cellular membranes, are highlighted (reviewed in refs. 8, 9, 120, 121, 157). The rest of the article is dedicated toward briefly discussing key issues concerning the theory, as well as common misconceptions and recent criticisms that can mislead data interpretation or which can even lead to wrongly discarding the theory.

II. An Integrated View on the MFRTA

Dozens of theories of aging have been proposed (57) but the MFRTA, between four and six decades after its first postulation (52, 54), can still afford the best explanation for aging and longevity in mammals, birds, and multicellular animals in general. Any aging theory should explain why maximum longevity (strictly referred to in the rest of this article as "longevity") varies so widely in animals: 30-fold from mice to men, 200-fold from shrews to the longest-living whales, or more than 5000-fold from perhaps a few days in some invertebrates to *A. islandica* mussels (longevity around 400 years). Such huge differences indicate that longevity is markedly regulated and flexible during species evolution.

Copying only a small fraction of this natural capacity would make it possible to obtain negligible senescence in humans in the future.

It is known that mean lifespan or the life expectancy at birth of the individuals of a population depends more on the environment than on the genes. On the contrary, longevity, and its inverse—the species aging rate—depends more than 90% on the genotype, as in the case of any other species-specific trait. Longevity and aging rate are the main parameters that matter with regard to the endogenous process of aging, which is situated at the main root of all the degenerative killer diseases. Currently, only two known factors correlate in the right sense with animal longevity in vertebrates, including mammals and birds: (i) the rate of mtROSp (8, 9, 12, 14, 69, 74, 77, 82), and (ii) the degree of fatty acid unsaturation of tissue cellular membranes, including the mitochondrial ones (124–126, 140 reviewed in Refs. 67, 111, 121). The longer the longevity of a species, the smaller these two parameters are. The decrease in mtROSp in long-lived animal species lowers their generation of endogenous (free radical) damage at mitochondria. The decrease in the fatty acid double bond (double bond index [DBI]) and peroxidizability (peroxidizability index [PI]) indexes lowers the sensitivity of the cellular and mitochondrial membranes to free radical attack. No other theory of aging has parameters such as these correlating in the right sense with longevity across species and offering plausible mechanistic explanations for the accumulation of damage from endogenous origin. The two known parameters appropriately correlating with animal longevity appertain to the MFRTA, not to any alternative theory. This is important, as any theory trying to explain aging should explain why longevity varies so widely among different animal species. Species closely related by phylogeny can have very different longevities, indicating that evolution of longevity is a relatively flexible and fast process, and thus can be subjected to experimental manipulation. In the absence of appropriate correlation with longevity, a theory of aging is not tenable, because it can not explain why the aging rate and the longevity of different animals are so distinct.

A. Antioxidants do not control longevity

Approximately during its first three decades of life, the MFRTA mostly focused on antioxidants, mainly because they could be measured with rather simple laboratory assays. In 1993, it was found that both enzymatic and non-enzymatic endogenous tissue antioxidants, including catalase, glutathione (GSH)-peroxidases, GSH-reductases, GSH, or ascorbate, correlated with longevity across vertebrates. However, and rather surprisingly, such correlation was negative (13, 97, 131, 154, reviewed in Ref. 130) instead of positive, as it was widely believed at that time (167). This resulted in a seminal observation for the development of the MFRTA, which was published in review form 4 years afterward (130). That review on the relationship between endogenous antioxidants and vertebrate longevity (130) also included all the then available published data on the subject obtained in mammals by other different laboratories. All those data from different sources consistently agreed: The longer the longevity, the lower were the levels of endogenous tissue antioxidants. Posterior reappraisals of the subject (106, 117, 122) have confirmed the early findings on the existence of a generally negative correlation between tissue antioxidants and longevity in all kinds of animals. We considered most interesting that long-lived animals had lower instead of higher antioxidant levels (130; Fig. 1A). The difference was extremely large in some cases such as that of liver GSH-peroxidase activity, which was around 20-fold lower in men than in hamsters (84). Among 27 studied correlations, 21 negatively correlated with longevity, 6 did not show significant differences, and not a single positive correlation with longevity was found (130). Superoxide dismutase (SOD) was among the antioxidants tending to show no association with longevity. It was previously believed that this enzymatic activity was positively associated with longevity due to referring the SOD (total SOD, CuZn plus Mn) activity values to the oxygen consumption (VO_2) of the whole animal (to the aerobic metabolic rate). Since metabolic rate strongly decreases as body size increases, the larger SOD/VO_2 of humans compared with rats was due to the

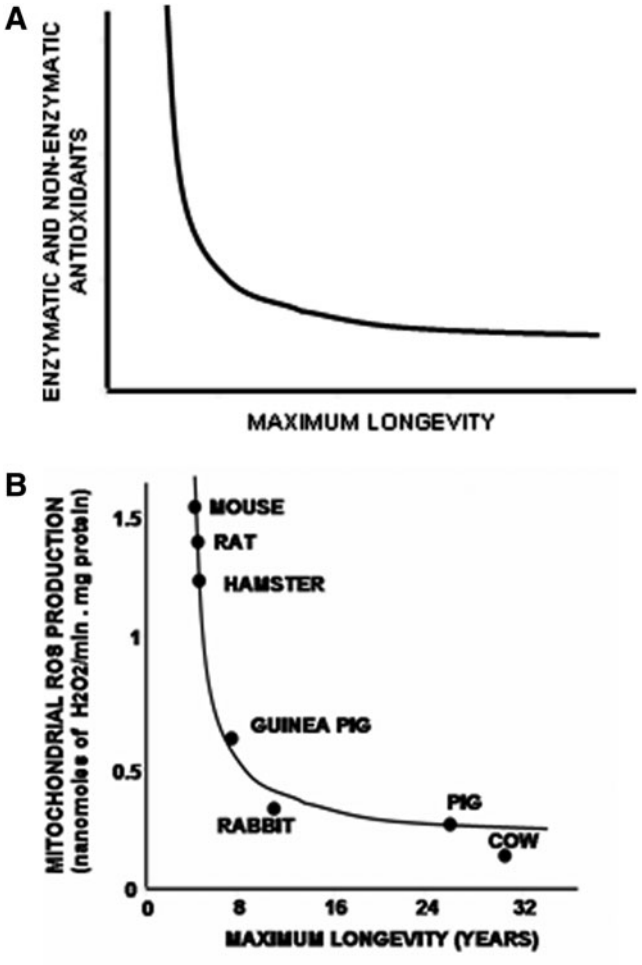

FIG. 1. Endogenous antioxidants and rates of mitochondrial ROS production are low in long-lived animals. (A) Generalized relationship between maximum longevity of different animal species and the levels of endogenous tissue antioxidants (antioxidant enzymes or low-molecular-weight antioxidants). **(B)** Negative correlation between rates of mtROSp and longevity in mammals. These relationships have been observed in different tissues (for references see text). mtROSp, mitochondrial reactive oxygen species production.

lower value of the denominator in the humans instead of to a higher value of the numerator. In fact, tissue SOD (total SOD, CuZn plus Mn) activities were not correlated to longevity in mammals in the original publication (167), although in the brain and lung of vertebrate species—but not in liver—the correlation between SOD (total SOD, CuZn plus Mn) and longevity was again negative similar to other antioxidants. Recent studies in different mammals, including long-lived naked mole-rats, as well as ants, honey bees, and marine bivalves also found a negative correlation with longevity also for this antioxidant enzyme—SOD (CuZn SOD except for honey bees-both CuZn and Mn)—(reviewed in Ref. 122). Among a total of 78 correlations between endogenous tissue antioxidants and longevity, 72 were negative, 6 did not show significant differences, and only a single one was positive (122), corroborating studies performed almost two decades ago (reviewed in Ref. 130). This large amount of evidence discards the possibility that evolution to high longevity in animals has been obtained through increases in tissue antioxidants.

B. mtROSp and oxidative damage in mtDNA are low in long-lived animal species

Why do long-lived animals need less antioxidants in their vital organs? We soon proposed (13, 97, 131) that the rate of mtROSp could be negatively correlated with longevity and that this would be the critical factor for aging (13). Long-lived animals would not need to maintain high antioxidant enzyme levels, which is energetically expensive, because they would produce mtROS at a low pace (and they could transitorily induce them if needed). This was indeed experimentally corroborated (Fig. 1B) both when comparing different mammalian species (77, 155, 156) and when comparing short-lived rodents (rats and mice) with 7–9-fold longer lived birds (pigeons, parakeets, and canaries) of a similar size and weight-specific metabolic rate (14, 15, 59, 61). A posterior more complete investigation studying approximately 12 different mammalian species confirmed these findings even after correcting for body size differences (82). Lower rates of H_2O_2 generation in humans when compared with rats and mice have also been reported for digitonin-permeabilized brain homogenates (78).

The studies in birds are especially important, because the investigations performed in mammals (77, 155, 156) used species following the Pearl (127) rate of living law of aging: "the lower the whole body weight-specific metabolic rate the longer the longevity." Thus, the species with longer longevity entered in those comparisons could show low rates of mtROSp simply, because their rates of oxygen consumption were also lower than those of the short-lived ones. In fact, mtROSp was positively correlated with mitochondrial O_2 consumption and with global metabolic rate in those studies (77). It was then important to study the problem in some of the many species that deviate from the Pearl's rate of living law. Three groups of warm-blooded vertebrates have much higher longevity than expected for their body size or metabolic rate compared with most mammals: birds, bats, and primates. Birds have both a high rate of global oxygen consumption and a high longevity. This makes them ideal to solve the problem mentioned earlier. The lower mtROSp of pigeons, canaries, and parakeets, when compared with rats in the first case and with mice in the second and third, strongly reinforces the MFRTA, as it indicates that the low mtROSp of long-lived animals occurs both in comparisons between animals following Pearl's law and in those not following it. A high longevity is not a simple consequence of a slow rate of living. It can be obtained—as the birds case shows—along with high rates of oxygen consumption and activity by lowering the rate of mtROSp both in absolute terms, and also as a percentage of mitochondrial oxygen consumption (the percent free radical leak, %FRL, see section III.C).

For a long time, it was widely thought that complex III of the respiratory chain was the respiratory complex that was responsible for ROS production in the mitochondrial electron transport chain [mtETC (19,20,21,169)]. In addition, in many studies measuring mtROSp, the respiratory control ratio values of the mitochondrial preparations were not reported, and it was not stated whether fresh or frozen mitochondria were employed. We found, working with freshly isolated and well coupled functional mitochondria (15, 59, 61), that complex I also produces ROS in heart or brain mitochondria isolated from rats, mice, pigeons, canaries, and parakeets, which was soon confirmed in rats by other laboratories (45, 80, 93) and soon became established knowledge (89). A key experiment to detect complex I ROS production was to measure mtROSp with succinate alone as well as with succinate + rotenone. In the second situation, the rate of mtROSp acutely decreases, because rotenone does not allow the electrons to flow back to complex I from succinate-complex II through reverse electron flow (59) (Fig. 2). However, the common procedure of adding succinate alone, followed or not by antimycin A, and rarely using complex I-linked substrates, led to the general belief for a long time that ROS came mainly from complex III-semiquinone. Significant production of $O_2^{\bullet -}$, predominantly by complex I occurs when (i) mitochondria are not making ATP and, consequently, have a high proton motive force; and (ii) when there is a high NADH/NAD^+ ratio in the mitochondrial matrix (110).

Interestingly, we also found that the higher mtROS generation rate observed in mammals compared with birds of a similar body size and metabolic rate occurred only at complex I (15, 59, 61), not at complex III. This was especially interesting taking into account the finding of analogous results in DR models (see section II.D). With regard to the precise site

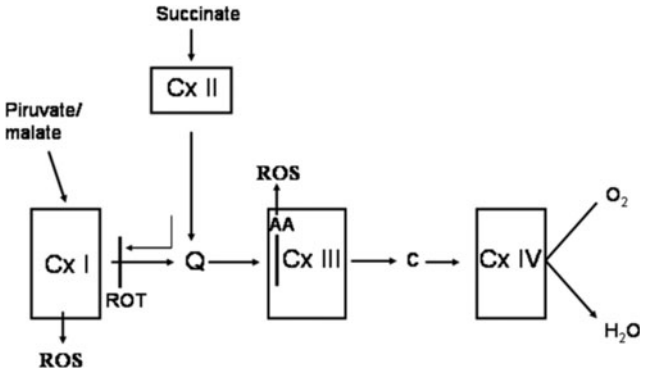

FIG. 2. Scheme of the respiratory chain showing sites of ROS production at complexes I and III. AA, antimycin A; c, cytochrome c; Q, ubiquinone; ROS, reactive oxygen species; ROT, rotenone.

within complex I where ROS are produced, three generators have been suggested: the flavin at the beginning of the electron path within the complex: the FeS clusters of the hydrophilic matrix arm; and the ubiquinone located in the membrane arm. Various investigators have supported the role of the flavin based on experiments with the inhibitor diphenyliodonium, which strongly decreases mtROSp. However, the site of action of diphenyliodonium, at the beginning of the electron path, prevents electrons from reaching the other two possible generators, the various FeS clusters and the ubiquinone, which, therefore, can not be discarded. In addition, the flavin is situated at the beginning of the electron path within complex I before the site of ferricyanide reduction (42, 170), which suggests that it is not involved in mtROSp. The electron leak to oxygen seems to occur between the ferricyanide reduction site and the rotenone binding site of Complex I both in intact mitochondria (15, 59, 61) and in submitochondrial particles (63, 165). Iron–sulphur clusters with a higher midpoint potential than FeSN1a, which could be situated in the electron path after the ferricyanide reduction site (170), or the unstable semiquinone known to be present (according to electron spin resonance evidence) in the membrane domain of Complex I (42, 81) and possibly functioning in H+ pumping coupled to electron transport (42, 136), could be the complex I oxygen radical generators. If this last possibility were true, ubisemiquinones could be responsible for oxygen radical generation at both Complex I and Complex III, which would be highly coherent from the point of view of molecular evolution, although the ROS source at Complex I would be the important one for aging. However, many other Complex I FeS clusters can be also implicated, because, under physiological conditions (i) their reduced and oxidized states will not be present in equal concentrations; (ii) interactions with many different factors and surrounding macromolecules can modify the final redox potential of the carriers *in vivo*; and (iii) the exact position of many of FeS clusters in the Complex I electron path is still unknown. In summary, the important aging-related question whether flavin, FeS clusters, or ubisemiquinone, or a combination of these, are responsible for complex I ROS generation cannot be clarified at present due to the lack of specific inhibitors within the complex. Therefore, this most important question remains open to further investigation.

The mtDNA is placed very close to the site of mtROSp, the inner mitochondrial membrane. ROS production also occurs at other cellular sites such as microsomes, peroxisomes, or membrane-bound NADPH-oxidases, and the rate of ROS generation at those sites can exceed in various situations to that coming from mitochondria (22). However, the ROS produced at mitochondria can be still the most important ones for longevity due to the presence of mtDNA within the mitochondria but not at those other organelles or parts of the cell. Since long-lived animal species have low rates of mtROS generation, it is logical to expect that this should have an effect on the amount of oxidative damage in their mtDNA. Therefore, the level of 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodG) was measured in the heart and brain mitochondrial and nuclear DNA (nDNA) of eight different mammalian species differing by approximately 13-fold in longevity. The results showed for the first time that the level of 8-oxodG in the mtDNA of both organs is negatively correlated with longevity [Ref. (16); Fig. 3]. The longer the longevity of a species, the smaller is its mtDNA oxidative damage degree. In contrast,

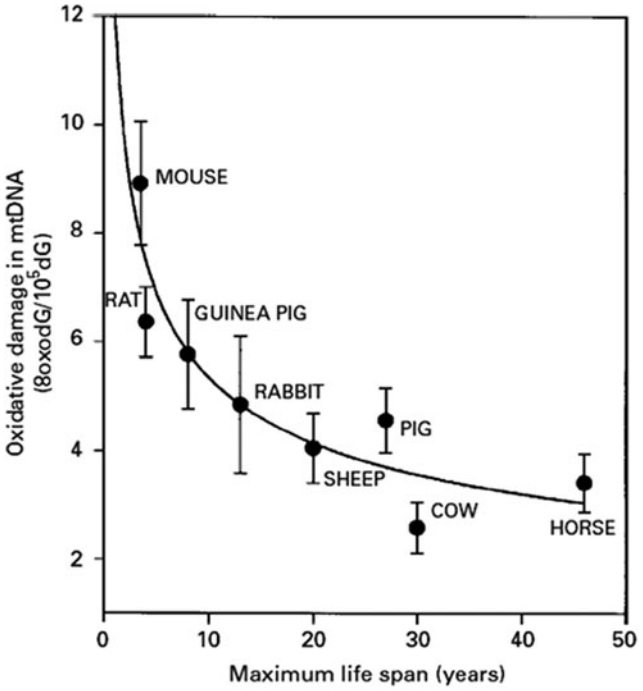

FIG. 3. Oxidative damage in mtDNA is low in long-lived animals. Oxidative damage to heart mtDNA decreases as longevity increases when comparing different mammalian species [reproduced with permission from Ref. (16); $r = -0.92$, $p < 0.001$]. The same kind of relationship has been observed in brain mtDNA [Ref. (16); $r = -0.88$, $p < 0.016$). mtDNA, mitochondrial DNA.

the 8-oxodG level in nDNA did not correlate with longevity in any organ even though mtDNA and nDNA were measured in the same samples taken from each individual animal in this investigation. Therefore, the different mtROSp rates from the different species seem to have a direct impact on mtDNA, not on nDNA, with regard to oxidative damage. This makes sense, as the site of ROS generation at mitochondria is very close to mtDNA, whereas nDNA is situated far away from it. The rate of mtROSp is measured in isolated mitochondria *in vitro* due to the lack of available common methods for direct *in vivo* H_2O_2 determination. However, the fact that changes (or lack of changes) in 8-oxodG in mtDNA closely reflect the variations in mtROSp both in comparative (*e.g.*, compare Figs. 1B and 3) and in DR studies (section II.D) means that the mtROSp *in vitro* measurements closely reflect the situation *in vivo*. In addition, the level of 8-oxodG in mtDNA was generally lower in the heart and brain of three long-lived birds when compared with two short-lived mammals of a similar body size and metabolic rate, in agreement with the superior longevity of the birds; whereas again this was not the case for nDNA (62). These investigations also showed that the intensity of oxidative damage is several fold higher in mtDNA than in nDNA in the heart and brain of all the 11 different species of mammals and birds studied (16, 62), which is again consistent with the close proximity between mtDNA and the sites of mtROS generation at the respiratory chain.

Early studies on the MFRTA were mainly focused on antioxidants, because they were easier to measure, and because sensitive techniques to assay mtROSp in different species

Table 1. Summary of Classic Studies on the Effects of Increasing Antioxidant Levels by Dietary or Pharmacological(*e) Means on the Mean and Maximum Longevity of Vertebrate Animals

Species	*Antioxidant increased*	*Survival curve*	*Effect on mean life span*	*Effect on maximum life span*	*MLSP of controls*	*Refs.*
BALB/c mice	BHT	Shown	Increase (22%)	No change	3 years	30
C3B1ORF1 mice	ETO + MEA	Shown	No change	No change	3.6 years	55
C57BL/6J mice	MEA	Shown	No change	No change	2.8 years	74
LAF1 mice	MEA	Shown	No change	No change	3.3 years	53
LAF1 mice	MEA	Shown	Increase (12%)	No change	2.2 years	53
LAF1 mice	BHT	Shown	Increase (31%)	No change	2.2 years	53
LAF1 mice	CYS, PG, DTBH or HNCL	Not shown	No change	No change	2.2 years	53
C3H mice	ETO	Shown	Increase (19%)	NI	1.9 years	33
BC3F1 mice	MET	Shown	Increase (13%)	Increase (12%)	2.8 years	58
C3 mice	α-Tocopherol	Shown	No change	No change	3 years	85
CD1 mice	Mixture*a	Shown	No change	NI	NS*b	166
H mice	CYS or TZC	*c	Increase (*d)	NI	1.8 years	115
Fisher 344 rats	Deprenyl	Not shown	Increase (2%)	Increase (4%)	2.7 years	104
Wistar rats	α-Tocopherol	Shown	No change	No change	2.8 years	132
Sprague-Dawley rats	α-Tocopherol	Not shown	Decrease (31%)	No change	2.8 years	18
Wistar rats	CYS or TZC	*c	No change	NI	*c	115
Guinea pigs	CYS or TZC	*c	Increase (*d)	NI	*c	115
Frogs*e	SOD, ASC, GSH and GSH-Red*e	Shown	Increase	No change	6 years	98

*a, mixture of antioxidants: α-tocopherol + BHT + ascorbic acid + DL-methionine + sodium selenite.
*b, survival data shown only up to 1.75 years.
*c, studied between 13 and 21 months of age only.
*d, not quantified and no statistics given.
*e, 100–1000% tissue antioxidant inductions secondary to pharmacological catalase inhibition with aminotriazol.
Similar negative results with regard to MLSP were generally obtained after increasing or decreasing antioxidant enzymes through transgenic manipulation or in gene knock out mice.
ASC, ascorbate; BHT, butylated hydroxytoluene; CYS, cysteine; DTBH, 2,6-Di-tert-butyl hydroquinone; ETO, ethoxyquin; GSH, glutathione; GSH-Red, GSH reductase; HNCL, hydroxylamine hydrochloride; MEA, 2-mercaptoethylamine; MET, 2-mercaptoethanol; MLSP, maximum life span potencial; NI, not investigated; NS, not significant; PG, propyl gallate; SOD, superoxide dismutase; TZC, thiazolidincarboxylic acid.

with enough margin over the detection limits were generally not available at that time mainly due to a frequent use of spectrophotometry compared with fluorometry. Furthermore, many of the studies publishing mitochondrial H_2O_2 production values did not state the respiratory control ratio values of the isolated mitochondria.

Most studies on the effect of adding dietary antioxidants to the diet were performed during the 1970s and 80s. Table 1 gives a summary of many of them. The general result was that antioxidants did not increase the relevant parameter (maximum) longevity. In some experiments, they increased only mean longevity (survival). Interestingly, this tended to occur when the (maximum) longevity of the control animals was short, usually less than 3 years. This suggests that antioxidants, when the husbandry conditions were less than optimum, could protect from causes of early death, and, thus, they were capable of making more rectangular the survival curve, similar to what happened in humans during the 20th century in many developing western countries when mean life expectancy increased from 30–40 to 80 years without decreases in aging rate. Antioxidants, in such cases, were bringing back toward optimum the diminished survival of the controls reared under suboptimum environmental conditions, which is interesting but not the goal of gerontology. Ironically, the poorer the survival curve of the controls, the largest is the chance of obtaining a positive result in terms of mean longevity. Similar to the comparative inter-specific studies described earlier, antioxidants clearly lacked the capacity to decrease the aging rate and to increase (maximum) longevity. When the techniques to obtain knockout or transgenic mice with increased or lack of expression of antioxidant enzymes such as SODs, catalase, or GSH-peroxidases were applied to this problem, the results were similarly disappointing (reviewed in Refs. 76, 107, 128, 147). The increased antioxidant enzymes, such as the non-enzymatic dietary antioxidants, lacked the capacity to slow down aging. Independently of the way in which the antioxidants were manipulated, dietary, or genetic, the result was the same: a lack of effect of antioxidants on mammalian longevity. This has been misinterpreted by some authors as the "death" of the MFRTA (see section III.B), but this conclusion did not take into account that what correlates with longevity in the right sense is not the level of the antioxidants, but the mtROSp rate (Fig. 1B) and the fatty acid unsaturation degree of the cellular membranes (section II.C). Only in invertebrate models, and especially in the nematode *Caenorhabditis elegans*, strong increases in longevity after increasing antioxidants have been obtained (102), although this kind of result has not been obtained in other studies (72, 174). Variations in the levels of SOD in *C. elegans* do not seem to affect longevity (39), and recent reviews suggest that oxidative damage can be experimentally dissociated from aging in this nematode (4, 173). Other studies in simpler organisms such as the fungus *Podospora anserina* have provided evidence for a role of mitochondrial ROS in senescence (40), including

DR-effects (172). In any case, a final goal of gerontology is to increase human longevity, and it is now reasonably clear that antioxidants do not increase longevity in mammals with a single exception in catalase transgenic mice describing small increases in longevity (150) that can not compete with a huge number of negative publications. Perhaps the increase in (maximum) longevity in these mice (around 10% increase) was due to targeting the increase in catalase (by 50-fold in heart) to the mitochondria, which could possibly somewhat decrease the ROS levels near the mitochondrial sites of ROS production that are relevant for aging, which will be also facilitated by the huge turnover of the catalase enzyme. However, further studies are needed to corroborate those results and the possible mechanisms involved.

How can mtROS production be involved in modifying longevity while antioxidants are not? This is counterintuitive only if we think of the cell as a homogeneous bag without any compartmentation. However, cells are not like that. "Global" cellular oxidative stress should depend on both ROS production and ROS elimination. Both contribute toward determining cell survival or death according to the general "balance" between them. However, although aging increases the probability of death, it is not death. Old people are aged but alive, although their likelihood of dying strongly increases with age. The ROS concentration in particular compartments such as mitochondria, especially very near to the places of ROS generation such as complex I, is much more dependent on mtROSp than on antioxidants, as the free radical generation source is approached at the micro level (13, 97). At such places, it is mtROSp that mainly determines the local ROS concentration present. This is especially important, because the main target for aging, mtDNA, is located very close in the vicinity, perhaps even in contact with the free radical generation source. This can help explain why lowering the rate of mtROSp instead of increasing antioxidants occurred during the evolution of long-lived species.

C. The membrane fatty acid unsaturation degree is low in long-lived animals

There is only a second known parameter known that also correlates with longevity in the right way, the fatty acid unsaturation degree of cellular (including mitochondrial) membranes. This is also firmly established, as it has been studied many times and concordant results were always obtained. The degree of fatty acid unsaturation is summarized (preferably) as the DBI, or, alternatively, as the PI. Figure 4 shows an example of the DBI-longevity relationship in the heart of different mammalian species (124). The longer the longevity of the species, the smaller the total number of fatty acid double bonds, which strongly decreases the sensitivity of the cellular and mitochondrial membranes to lipid peroxidation, a toughly destructive process that, in addition, produces mutagenic and toxic metabolites. This was first described in 1996 in rat compared with pigeon and human mitochondria (126) followed by many studies in mammals and birds (reviewed in Ref. 121), as well as further confirmatory studies (67). A total of 23 studies (111) extended the first seminal observation to many different mammals, various bird species, and some invertebrates, without finding a single exception. Since the low degree of unsaturation occurs both in mitochondrial and in total cellular membranes in long-lived animals, it can diminish lipoxidation-derived damage in various cellular compartments, including the mitochondrial one where membranes are highly convoluted and abundant especially in aerobic tissues.

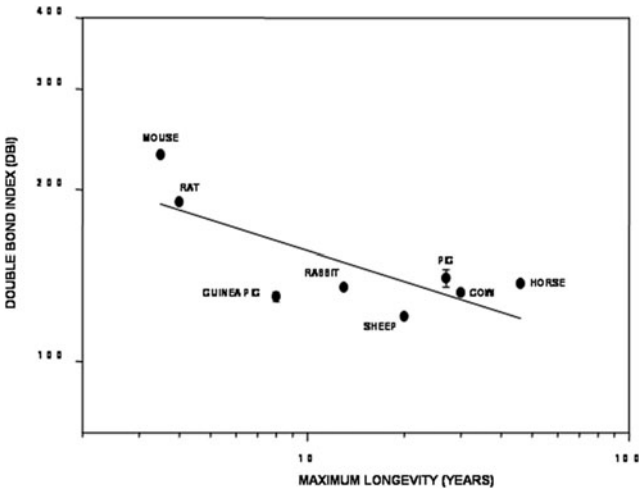

FIG. 4. The membrane fatty acid unsaturation degree is low in long-lived animals. The double bond index, indicating the membrane fatty acid unsaturation degree, decreases as longevity increases in the mammalian heart (124), $r = -0.78$, $p < 0.02$. The same has been observed in other tissues and kinds of animals (for references see text).

Various fatty acids composing the different cellular membranes (plasma, mitochondrial, and other internal membranes) are responsible for this strong decrease in DBI (and PI), as longevity increases among species. However, the most important ones, both due to their content in double bonds (high or low) and for their larger quantitative presence and variation among species, are 18:2n-6, 18:3n-3, and 22:6n-3, and sometimes 18:1n-9 (in some birds) and 20:4n-6 (for each fatty acid, the number before the colon denotes the carbon chain length, and the one after the colon indicates the number of double bonds in the fatty acid; n-6, n-3, or n-9 refers to the fatty acid family). When they vary among species, 18:1n-9, 18:2n-6, and 18:3n-3 increase and 20:4n-6 and 22:6n-3 decrease as longevity increases. Among them, the decrease in 22:6n-3 in long-lived animals is the most relevant to explain their low DBI and PI values. The final result is that the total amount of unsaturated and saturated fatty acids does not change among species. Instead, it is the unsaturation degree of the polyunsaturated fatty acids present that decreases from short- to long-lived species. With this kind of redistribution, long-lived animals obtain a strong decrease in the sensitivity of their cellular membranes to the dangerous process of lipid peroxidation, while maintaining essentially unaltered the fluidity of their membranes, the so called homeoviscous—longevity adaptation (121). This better describes the situation concerning longevity than the "metabolic pacemaker hypothesis" (68), as this last one refers to the Pearl's rate of living hypothesis that was discarded long ago, because too many species deviate from the metabolic rate *versus* longevity relationship. The low DBI of long-lived animals likely protects not only the lipids but also other kinds of cellular macromolecules. Since lipid peroxidation is a relatively massive process compared with oxidative damage to other kinds of

macromolecules, long-lived animals, thanks to their low DBI, will produce far less amounts of highly toxic and mutagenic lipid peroxidation products such as hydroxynonenal or malondialdehyde (MDA) among many others. These, in turn, having carbonyl groups, can modify free amino groups in proteins and DNA. At least the first of these two kinds of modifications seems to be involved in aging, as the amount of MDA-lysine adducts in heart proteins negatively correlates with longevity in mammals (137).

With regard to the mechanism causing the negative correlation between the fatty acid unsaturation degree and species longevity, a role for acylation/deacylation of the constitutive membrane fatty acids cannot be discarded. However, since the more unsaturated 20:4n-6 and 22:6n-3 are essential fatty acids synthesized from their dietary precursors 18:2n-6 and 18:3n-3, the enzymatic processes that control the corresponding biosynthetic pathways can be involved. In this regard, in various comparative studies related to the degree of fatty acid unsaturation to longevity the results suggest that desaturase and elongase enzymatic activities in the n-3 and n-6 series (which are rate limiting for those biosynthetic pathways) are low in long-lived animals. In some cases, decreases in peroxisomal beta-oxidation could also be involved. It is now considered that this last process is responsible for the last steps in the synthesis of the highly unsaturated 22:6n-3 in the n-3 pathway. The low delta-5 and delta-6 activities (which are rate-limiting enzymes in the n-3 and n-6 fatty acid synthesis pathways) of long-lived animals will decrease the conversion of the less unsaturated 18:2n-6 and 18:3n-3 precursors to the highly unsaturated 20:4n-6 and 22:6n-3 products. Thus, 18:2n-6 and 18:3n-3 would accumulate and 20:4n-6 and 22:6n-3 would diminish, which is just the general kind of result that is found in long-lived animals. In summary, the membrane fatty acid unsaturation degree is low in tissues from long-lived animals. This is the only other known factor, in addition to mtROSp, which correlates with longevity in the right sense and has the mechanistic capacity to contribute to the widely different aging rates of the different animal species. This is true with regard to the MFRTA as well as to any other theory of aging. No essential parameters for any of the other theories of aging have been identified that can explain the different longevities of the different species. What happens with regard to changes in longevity in a single species?

D. DR lowers mtROSp and oxidative damage in mtDNA because it decreases the methionine dietary intake

It is well known that 40% DR increases not only mean but also maximum longevity (by around 40%) and decreases and delays the incidence of degenerative diseases in laboratory rodents, rotifers, flies, spiders, worms, fish, and other mammalian species (44), although a lack of effect on longevity has been described in some cases such as in DBA/2 mice (152) or *Drososophila melanogaster* flies (87). In rhesus monkeys, it was observed that 30% DR strongly decreases age-related mortality (from 37% to 13%), age-related diseases, and age-associated brain atrophy [Wisconsin study; (32)]. However, a study describing lack of DR effects on the longevity of rhesus monkeys has been recently published [NIA study; (100)]. The reason for the discrepancy between these two primate studies is not known, although the diets used in the Wisconsin study were semipurified; whereas those of the NIA study were not. There were also other differences in dietary composition between both studies, including much lower sucrose, presence of antioxidant flavonoids, or higher vitamin and mineral supplementation in the NIA compared with the Wisconsin study, as well as differences in genetic background. Although the NIA study did not demonstrate lifespan extension, there were benefits with regard to age-related diseases (as in the Wisconsin study) in restricted animals and it was concluded that diet composition rather than the calories themselves strongly affect the life-prolonging effect of DR (100). Therefore, DR seems to be beneficial with regard to degenerative diseases also in upper primates, although more studies are clearly needed to resolve the lifespan effects of DR in these animals.

Many mechanisms of action of DR on longevity have been proposed, including modifications in growth hormone and insulin/insulin-like growth factor-1 signaling, changes in gene expression profiles, sirtuins, apoptosis, and many different signaling molecules such as mTOR, FOXO, S6K, AKT, PKA, nutrition, and amino acid-sensing pathways. Many of these changes are interconnected and related to mitochondrial oxidative stress as has been recently shown for SIRT3 (160).

Long-lived animals have lower rates of mtROSp and lower mtDNA oxidative damage than short-lived ones (Figs. 1B and 3). However, what happens in DR with regard to these parameters? If the mitochondrial oxygen free radical theory of aging is correct these two parameters should also decrease during DR. Initial studies, as in the case of the comparison between different species, focused mainly on antioxidants. They showed that DR in mice (153) and other rodents does not lead to a generalized increase in antioxidants. Instead, increases, decreases, or lack of changes depending on the particular antioxidant have been reported even within the same study (153). Therefore, similar to the inter-species case, the key to longevity does not seem to lie on the side of the antioxidants during DR either. A different situation concerns mitochondrial ROS generation. The effect of DR on the rate of mtROSp was repeatedly investigated in mice and especially in rats by many different laboratories. The results of these investigations consistently agreed that long-term (40%) DR significantly decreases the rate of mtROS generation in rat organs, including skeletal muscle, kidney, liver, heart, and brain (48). This fact agrees again with the concept that lowering mtROSp increases longevity. This decrease was detected in freshly isolated functional mitochondria under similar conditions, including the substrate concentration used to feed electrons to the *ad libitum* and DR mitochondria. Thus, DR mitochondria are different from those obtained from *ad libitum* fed animals, and this difference (due to DR) is responsible for the lowered mtROSp detected *in vitro*. In addition, some data suggest that complex I substrates such as pyruvate decrease during DR in the tissue (25). If that is correct, the matrix NADH level would decrease in DR, altering the redox state of the mtETC, lowering its reducing potential including that of the complex I ROS generator, as NADH directly feeds electrons to this complex. Indeed, DR also decreases the NADH concentration (25), a change that is known to decrease the rate of mtROSp (80). This will lead to a further decrease in the rate of mtROSp *in vivo*, which would add to that due to the lowered qualitative capacity of DR mitochondria to generate ROS detected *in vitro*. Interestingly, we found that the decrease in mtROSp in DR (or MetR—methionine restriction) rats specifically occurred at complex I in all

the organs studied (50, 96, 146) and occurred along with decreases in complex I content (27, 143) and assembly (36) in rat liver. Thus, a low rate of mtROSp is a trait both of long-lived species and of DR animals. In contrast, the low DBI only occurs in long-lived species, as 40% DR (or 40% MetR) does not change the membrane unsaturation degree, although a decrease in DBI was observed at the more intense 80% MetR (143). In some organs such as the liver, the decrease in mtROSp is obtained after only 7 weeks of 40% DR, protein restriction (PR) or MetR. This seems to be the result of evolutionary processes that led to programmed reactions to the different kinds of DRs at the genome level. These cellular reactions modify gene expression and lead, among many other changes, to the decrease in mtROS generation.

In addition to the decrease in mtROSp, DR also decreases the %FRL (section III.C), indicating the efficiency of the mitochondrial respiratory chain in preventing ROS generation increases in DR animals. Especially long-lived animals such as birds (canaries and pigeons with a longevity of 24 and 35 years respectively) show lower %FRL values than the much shorter-lived rats or mice (59, 61), suggesting that this can constitute a highly conserved mechanism of life span extension both between and within species. On the other hand, since mtROSp is lower in DR than in the *ad libitum*-fed control animals, oxidative damage should also be lower in the mtDNA of the restricted animals. In agreement with this, we found that the level of 8-oxodG in DNA was significantly lower in the liver, heart, and brain of the long-term DR old rats in which mtROSp was also diminished [(8); Table 2]. The decrease in 8-oxodG occurred only in mtDNA, or both in mtDNA and nDNA, depending on the organ studied.

While many different investigations consistently found that 40% DR decreases mtROSp and 8-oxodG in mtDNA, the dietary factor that causes these beneficial changes was unknown and we considered it important to clarify that issue. It was classically believed that the antiaging effect of DR is exclusively due to the decreased intake of calories themselves rather than to decreases in specific dietary components. However, our review of published studies questioned this classical consensus (119), as it showed that variations in the amount of dietary protein also affect (maximum) longevity in rodents. Published investigations, although scarce, indicate that restricting only dietary carbohydrates or only dietary fats does not seem to increase longevity (119). However, 10 out of 11 published studies and 16 out of 18 different life-long survival experiments in these studies found that PR (60–80%) increases maximum longevity in rats and mice (Table 2), although the magnitude of this increase was usually half of that typically found in DR (95, 119). This suggests that PR can be responsible for around 50% of the life-extending effect of DR. Studies in *Drosophila* also found a special role of dietary amino acids in the DR effect (47), which is in the same line of the findings in mammals. We then investigated what amino acid/s in dietary proteins could be responsible for these beneficial effects of PR. Importantly, an early investigation had found that restricting only methionine (80% MetR) using isocaloric diets increases (maximum) longevity in rats (134). Therefore, the lower intake of a single substance, methionine could be responsible for the increase in longevity induced by PR as well as for around half of the life-extension effect of DR. More recent life-long experiments confirmed the longevity-extension effect of 80% MetR (105, 164) in rats and mice (Table 2). The increases in maximum longevity in rodents induced by 80% MetR were accompanied by a decreased incidence of degenerative diseases such as cancer, lowered risk factors for disease, increases in resistance to oxidative stress, and decreases in age-related changes in the immune system and relevant serum metabolites and hormones, as well as lower levels of visceral fat, triglycerides, or cholesterol in old animals. With regard to antioxidants, however, GSH was found to be decreased in the liver of methionine-restricted animals, suggesting that at least this antioxidant is not involved as antiaging factors during MetR either. Interestingly, the magnitude of the increase in longevity observed during MetR is similar to that observed in PR (around 20% increase). DR and PR share many common effects in addition to life prolongation, including delays in puberty and growth, boosting of cell-mediated immunity, or decreases in precancerous lesions and tumours, glomerulosclerosis, chronic nephropathy, and cardiomyopathy. A lower but significant life extension effect in PR than in DR would also agree with the generally held view that aging has more than one single main cause. DR could decrease the aging rate through the decreases in mtROSp and oxidative stress induced by PR and MetR, as well as through other unknown mechanisms that are possibly induced by the calories themselves or by other dietary components. We have found that 40% PR without strong restriction of caloric intake lowers mtROSp specifically at complex I, lowers the %FRL at the mtETC, and decreases 8-oxodG in mtDNA in rat liver [(144); Table 2]. Strikingly, the magnitude, kind of changes, mechanisms, and site of action of those decreases are similar in 40% PR and 40% DR; while lipid or carbohydrate restriction does not change either mtROSp or 8-oxodG in mtDNA in agreement with their lack of longevity extension [(119); Table 2]. Other investigators have found that progressively decreasing the level of dietary protein in mice, from 24% to 12% and from 12% to 6%, decreases lipofuscin accumulation (37), which is a well-known marker of aging. Most interestingly,

Table 2. Changes in Oxidative Stress in Dietary Restriction, Protein Restriction, and Methionine Restriction, and in Restriction of All Dietary Amino Acids Except Methionine (AAREST)

	Antioxidants	*mtROSp*	*%FRL*	*8-oxodG in mtDNA*	*MLSP*
DR	↓ = ↑	↓	↓	↓	↑
PR	ND	↓	↓	↓	↑[a]*
MetR	ND[b]*	↓	↓	↓	↑[a]*
LR	ND	=	=	=	=
CHR	ND	=	=	=	=
AAREST	ND	=	=	=	ND

Neither LR or CHR seems to increase longevity (reviewed in Ref. 95). For references on oxidative stress-related parameters, see text.

[a]*, the longevity extension effect of PR (reviewed in Ref. 119), and perhaps that of 80% MetR (105, 134, 164), seems to be around 50% that of DR.

[b]*, except for reduced glutathione, which decreases in liver of MetR rats.

%FRL, percent free radical leak; 8-oxodG, 8-oxo-7,8-dihydro-2′deoxyguanosine; CHR, carbohydrate restriction; DR, dietary restriction; LR, lipid restriction; MetR, methionine restriction; MLSP, maximum life span potencial (maximum longevity) of the species; mtDNA, mitochondrial DNA; mtROSp, mitochondrial reactive oxygen species production; ND, non determined; PR, protein restriction.

we have found that 80% MetR as well as 40% MetR (without caloric restriction) also decrease mtROSp, %FRL at mtETC and 8-oxodG in mtDNA and protein oxidation, glycoxidation, and lipoxidation in rat organs [(27, 139); Table 2], which is in agreement with the increase in longevity induced by MetR (80%). Moreover, these changes did not occur in the rat liver when all the dietary amino acids, except methionine, were restricted by 40% [(28); Table 2). All these investigations, taken together, show that restriction of methionine intake is responsible for the decreases in mtROSp and oxidative stress

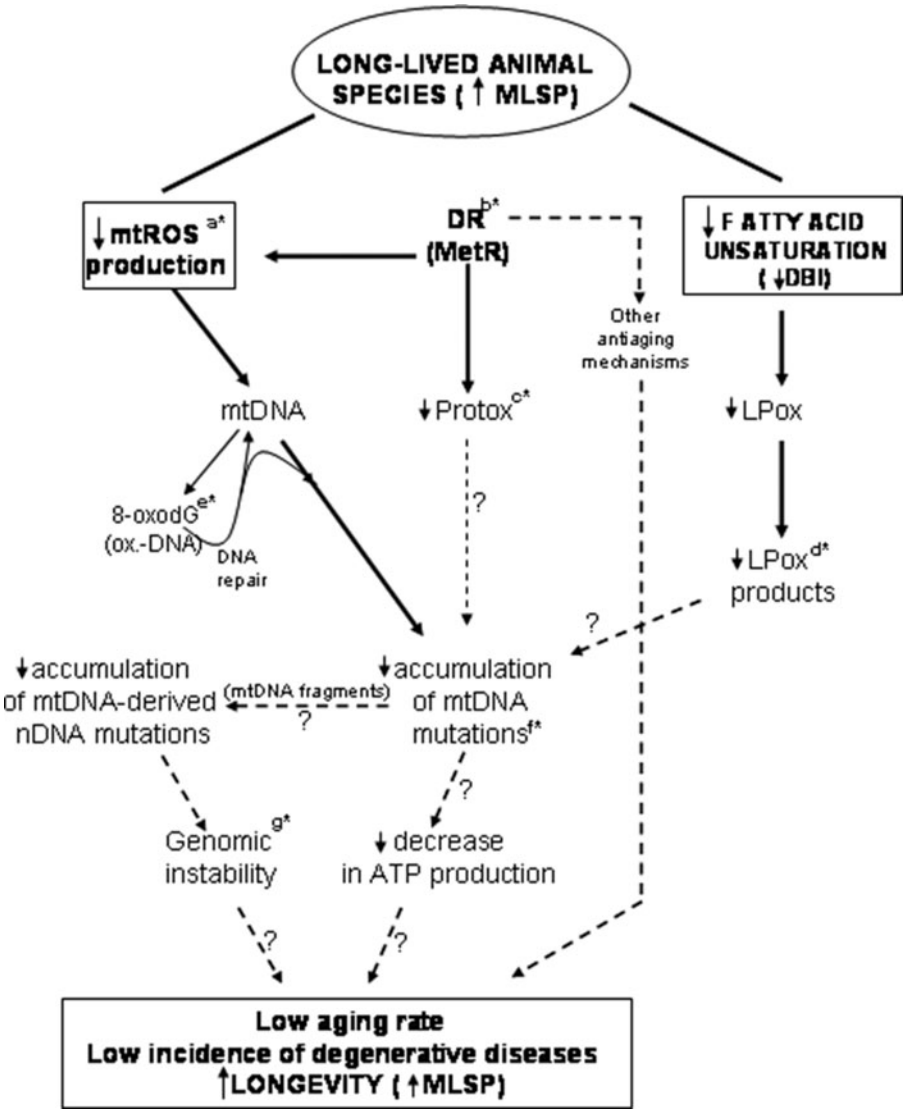

FIG. 5. Mitochondrial ROS production, oxidative damage, DNA mutations and aging: schematic relationships suggested by available information. This scheme summarizes the main known causes and mechanisms of oxidative damage, finally leading to aging, that are known to be associated with animal longevity or DR in rodents. a* mtROS are produced at rates related to longevity, especially at complex I, which is inserted into the inner mitochondrial membrane. There is close vicinity or even contact between the site of ROS generation and mtDNA; so, antioxidants cannot interfere with ROS-induced final forms of irreversible damage in mtDNA and, therefore, cannot modify longevity; b* the well-known capacity of DR to decrease mtROSp and 8-oxodG in mtDNA is known to be exclusively due to the lower methionine content (MetR) of the DR diet. Around 50% of the longevity extension effect of DR is due to MetR and seems to work through decreases in mtROSp; c* Protox., protein oxidative modification (including protein glyco- and lipo-oxidation); d* LPox products, lipid peroxidation products (such as malondialdehyde, hydronenonenal) which are highly toxic and mutagenic; e* 8-oxodG, this DNA adduct does not accumulate extensively during aging, as it is repaired by mtBER, but can contribute to producing somatic mtDNA point mutations; f* these add to point mutations, deletions, and fragments of mtDNA directly generated by mtROS, as well as by other mechanisms; g* genomic instability: including nDNA point mutations, deletions, insertions of mtDNA fragments, changes in nDNA (*e.g.*, TE activation), and modifications in intergenic, promoter, intron or exon gene sequences, chromosome rearrangements, modifications in gene expression, and cancer promotion. *Solid arrows* describe processes for which evidences in favor of a cause–effect relationship are abundant, whereas *dotted arrows* describe processes for which there is a logical mechanism but there are few data available supporting it. 8-oxodG, 8-oxo-7,8-dihydro-2′deoxyguanosine; BER, base excision repair; DBI, double bond index of fatty acids of cellular and subcellular membranes in tissues; DR, dietary restriction; MetR, methionine restriction; MLSP, maximum species-specific longevity; nDNA, nuclear DNA; TE, transposable elements.

that take place in DR and, likely, for a part of the increase in longevity induced by this dietary manipulation (Fig. 5). It is most important that not only 80% but also 40% MetR induces those beneficial changes, because 40% MetR, differing from 80% MetR and from 40% DR, does not decrease at all the body growth rate of the animals.

In summary, studies in rodents indicate that restriction of a single dietary substance, methionine, is responsible for the decrease in mtROS generation and oxidative damage to mtDNA that takes place during PR and DR. They also show that restricting the dietary intake of methionine increases maximum longevity to a similar extent than PR and to around 50% of that observed during DR. Therefore, it seems that it is no longer necessary to suffer hunger and decrease adult height (due to DR) to decrease oxidative damage in vital organs and increase longevity. Restricting only methionine in the diet can bring such benefits without paying those costs of DR, as MetR, when applied at 40% instead of at 80%, does not decrease at all the growth rate during maturation (27). Recent experimental results obtained in humans seem to indicate that PR brings about many beneficial effects that resemble closely those observed in DR in human beings (44). This is most interesting, as decreasing the intake of a single substance—methionine—in humans is much more feasible than decreasing the total amount of ingested food. Forty percent MetR (or PR) compared with DR strongly decreases the risk of incurring in malnutrition; there is no need to decrease the total caloric intake, thus avoiding the feeling of hunger and lowering the risk of increasing the sensitivity to the normally stressful human living conditions, with the three reasons being even more relevant in the case of children and old individuals. In addition, 40% MetR does not lower the body growth rate of the young. The benefits of MetR or PR, however, are expected to be around 50% smaller than in DR if the effects in humans are similar to those already obtained in laboratory rodents. It is most important to investigate whether 40% MetR also increases longevity such as 80% MetR, or whether it can even have a greater effect than 80% MetR on longevity due to lack of limitations in protein metabolism induced by this last perhaps too intense intervention.

The briefly reviewed updated version of the MFRTA described earlier is schematized in Figure 5. Long-lived mammals and birds have species-specific low mitochondrial ROS generation rates at complex I and low fatty acid unsaturation degrees in the cellular and mitochondrial membranes. These are the only two known traits correlating with animal longevity in the right sense with regard not only to MFRTA but also to all theories of aging in general. The close vicinity or even contact between the site of ROS generation and mtDNA prevents antioxidants from interfering with ROS-induced final forms of irreversible damage in mtDNA, and this is likely why antioxidants do not modify longevity. It is well known that DR also decreases mtROSp and 8-oxodG in mtDNA. This is exclusively due to the lower methionine intake (MetR) of the animals subjected to DR. Around 50% of the longevity extension effect of DR is due to MetR, and this 50% effect seems to work through decreases in mtROSp; the other 50% effect of DR on longevity would act through other mechanisms. The constantly produced mtROS throughout life at a different rate in each species leads to the generation of oxidative damage in mtDNA (*e.g.*, 8-oxodG), which is repaired and can lead to point mutations in the process. In addition, mtROS can directly generate single- and double-strand breaks, also leading to irreversible forms of damage (mutations) such as mtDNA deletions or mtDNA fragments (163). Mutations can also arise due to processes unrelated to oxidative stress such as mtDNA synthesis and repair. However, it is unknown whether these last mechanisms of damage generation are related to longevity or not. The low fatty-acid unsaturation degree of mitochondrial membranes from long-lived animals leads to relatively low rates of endogenous lipid peroxidation *in vivo* (a strongly destructive membrane process), which, in turn, decreases the generation of lipid peroxidation products such as MDA, hydroxynonenal, and many others. Some of these products have the potential to modify mtDNA, for example, through a direct interaction of the carbonyl group from the aldehydes with free amino groups in mtDNA, which would add secondary damage to that coming from the primary mtROS. There is a paucity of studies with regard to this interesting possibility (29), especially due to technical limitations. DR (and MetR) also lowers protein oxidation, glycoxidation, and lipoxidation, perhaps due to the induced decrease in mtROSp or due to increased protein catabolism. Protein oxidation can also potentially contribute to the accumulation of mtDNA mutations, although there is scarcity of published support with regard to this kind of process. If irreversibly damaged mtDNA reaches a high threshold level (approaching homoplasmy of mutated mtDNA), there is the possibility that oxidative mitochondrial ATP generation through oxidative phosphorylation is decreased to levels that are great enough to contribute to aging. There is no consensus whether this classical concept of the MFRTA (43) can explain aging and longevity. However, there is an additional possibility with regard to the accumulation of mtDNA fragments inside nDNA, which is known to increase with age in rat liver and brain (26). Such fragments would alter the information coded in nDNA, thus contributing to aging.

We have previously reviewed the MFRTA (8, 9, 95, 119, 120, 147). Part II of the present article is an update of those reviews. The rest of this article will focus on key aspects of the MFRTA as well as on common misconceptions that can lead to erroneous data interpretations or even to wrongly discrediting or discarding the theory. These aspects will be organized around some of the main traits depicted in Figure 5, the mitochondrial ROS generation rate, and the DNA oxidative damage level.

III. MFRTA. Key Aspects and Confounding Concepts

A. The rate of mtROSp. The first known factor with capacity to explain longevity

1. mtROSp. What to measure or not and its meaning for MFRTA. At present, true rates of mtROS production can only be measured in isolated mitochondria. Unfortunately, it is very frequent to call "ROS production" in scientific articles to measurements in whole cells using fluorescent probes such as dichlorodihydrofluorescein (DCF), dihydrorhodamine, or dihydroethidium. There are methodological problems that are encountered when using these probes, which are well known now at least in the case of DCF (7). Such measurements would not estimate ROS "production" but the balance between ROS production and elimination, because any cellular antioxidants situated between the site/s of ROS generation and the fluorescent probe will interfere and react with ROS, thus decreasing the final signal. This does not occur in the case

of isolated mitochondria, as the fluorescent probe is situated in an antioxidant-free incubation medium designed by the researcher. Measurements in cells would then represent "mean cellular oxidative stress," a most interesting parameter for various purposes, but they do not represent "ROS production." Interference by cellular antioxidants is not desirable in the case of aging studies, because longevity depends on mtROSp, not on antioxidants (see above). The high antioxidant levels of short-lived animals would cancel with their high mtROSp rates, and the reverse would occur in long-lived ones; so, "cellular ROS levels" measurements would be misleading in studies focusing on longevity. A recent example is the lack of correlation of "fibroblast ROS levels" with longevity in 13 primates differing in longevity between 9 and 20 years (35). Such problems are avoided by measuring ROS production in isolated mitochondria. Using this technical approach, no exception was found to the negative relationship between mtROSp and longevity. The difference in mtROSp between species found by us, as well as by others (82), is substantially smaller than their difference in longevity, which could indicate that many other causes of aging apart from mtROSp exist and that ROS only controls a small part of the aging rate. However, those are—necessarily—*in vitro* measurements. There are reasons to believe that if mtROSp were measured in the conditions prevailing in tissues (such as tissue pO_2)—which are different in different species—then the difference in mtROSp between species would be higher and would approach much more the difference in longevity among different animal species. This seems to be true not only with regard to different mammals, but also in relation to the superior longevity of birds compared with mammals.

The low mtROS generation rate of long-lived animals is the first known trait that correlates with the longevity of animals (including mammals) in the right sense, and is likely a cause of their different aging rates. mtROSp also agrees with the four "rules of aging" stating that aging is universal, endogenous, progressive, and deleterious (161). mtROS are produced in aerobic cells of all the individuals, are continuously and endogenously produced throughout life, and can progressively cause the accumulation of deleterious damage similar to that present in the form of mtDNA and perhaps also nDNA mutations. However, many key issues need to be further investigated, and various unfounded criticisms and misconceptions with regard to the MFRTA and the rate of mtROSp have been raised. Some of these will be discussed in the following sections.

2. mtROSp is not necessarily proportional to mitochondrial oxygen consumption. Ideally, mtROS production should be measured *in vivo* in intact animals. Unfortunately, in spite of some significant recent advances in that direction (4, 31, 103, 171), that is still not possible. In order to measure mtROS generation, functional mitochondria should be isolated from fresh tissue, and their capacity to generate ROS is measured *in vitro* along with their rates of oxygen consumption and respiratory control ratio. Such kinds of measurements estimate the initial rate of mtROSp free of interference from antioxidants.

A common misconception among non-specialists in mitochondrial respiration is the wrong assumption that when mitochondrial oxygen consumption ($mtVO_2$) increases, the rate of mtROSp should also increase in direct positive proportion to it. This would be true only if the fraction of electrons out of sequence that reduce O_2 univalently to generate ROS (the %FRL) would be always a fixed percentage of the total electron flow in the respiratory chain. This is not the case on many occasions, and even the reverse occurs in various important physiological situations. That misconception can lead to erroneously discarding the MFRTA. It was found, for instance, that increases in longevity during DR occur in yeast (91) or mice (113) along with lack of decreases or even with increases in O_2 consumption. This was interpreted as contradictory with the MFRTA, because it was assumed that mtROSp did not change or increase while longevity was increased, but mtROSp was not measured in those studies. However, this apparent contradiction is resolved by the fact that mtROSp decreases in DR even though the $mtVO_2$ is maintained in liver, heart, or brain mitochondria, which is in agreement with the lack of change in the aerobic metabolic rate of the whole animals in the long term (101), which has also been described in nematodes (66). DR can even increase VO_2 in yeast (91) and mammalian cells (113), thus discarding the fact that the life-extension effect of DR can be due to a simple decrease in VO_2. The decrease in mtROSp in DR in the presence of a similar or even an increased $mtVO_2$ is possible, because the %FRL in the respiratory chain also decreases during DR. Thus, the apparently paradoxical increases in O_2 consumption observed in some DR models do not discredit the MFRTA.

Strong decreases in absolute and relative (per unit of O_2 consumed %FRL) mtROSp also occur during aerobic exercise bouts (during the state 4 to state 3 mitochondrial energy transition), as well as in chronic aerobic exercise training. Total body VO_2 increases by around one order of magnitude in humans during maximum aerobic exercise. Should mtROSp increase in direct proportion to such a huge increase in $mtVO_2$, the resulting increase in massive oxidative stress would be unbearable, especially for skeletal muscles and the heart. Instead, the rate of mtROSp decreases—instead of increasing—during bouts of aerobic exercise when the mitochondria experiments the transition from the resting state 4 to the active oxidatively phosphorylating state 3. This is due to (i) a strong acceleration of the electron flow rate in the mtETC, which decreases the reducing potential of the electron carriers, including the ROS generator site, and then their rate of mtROSp. The situation can be described with the metaphor of the cars (the electrons) on a highway (the mtETC): When there is a traffic jam, the flow of cars (of electrons in the mtETC) is slow and the number of cars per square meter (the reducing potential of the respiratory complexes) is high (like in Fig. 6a); whereas when car speed is high (the highway traffic flows quickly), the number of cars per square meter on the highway (of electrons in the respiratory complexes) strongly decreases (like in Fig. 6b). Thus, a high $mtVO_2$ in the mtETC, such as a fluent car flux exiting from a traffic jam, is not bad but good, and is healthy because it lowers mtROSp; (ii) the strong increase in $mtVO_2$ itself (Fig. 6b) lowers the local pO_2 at mitochondria. Since mtROSp, differing from the reduction of O_2 to water at complex IV, is pO_2 dependent in the tissue pO_2 physiological range (64), the decrease in tissue pO_2, which will more strongly decrease near the highly O_2-consuming mitochondria, will also contribute toward reducing mtROSp. This occurs, because the K_M for O_2 of the mtROS generators at complexes I or III is high and within the physiological range; whereas the $mtVO_2$ will not be affected due to the very low

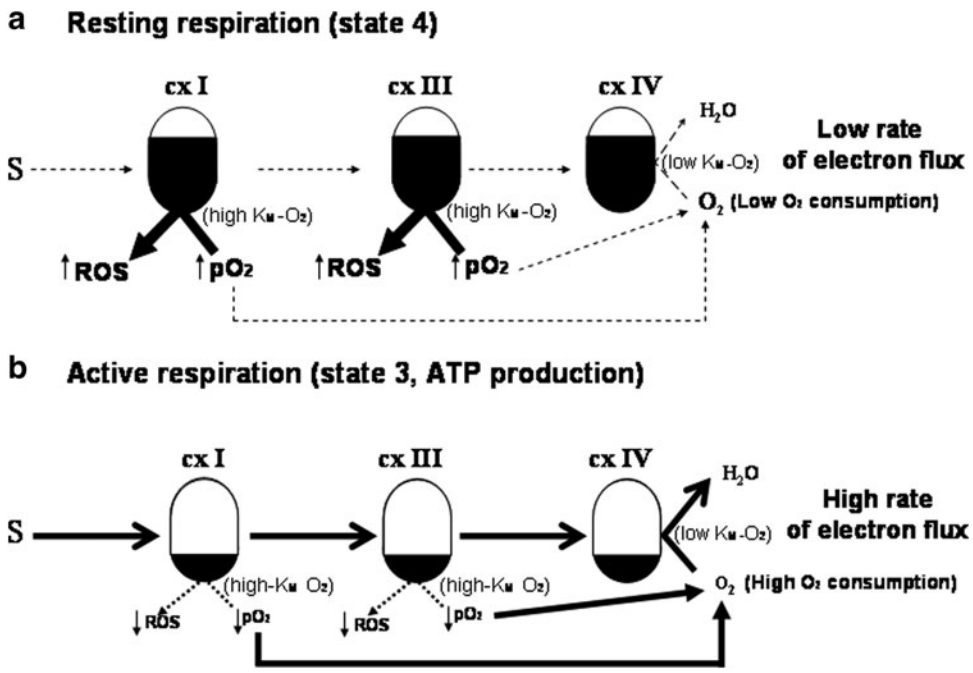

FIG. 6. Mitochondrial ROS production is not necessarily proportional to mitochondrial O_2 consumption: the reverse occurs in many physiological situations including exercise. In resting mitochondria respiring in state 4 **(a),** the electron flow rate is slow and the reducing potential of the respiratory chain (dark area inside respiratory complexes) is relatively high, which stimulates ROS generation; the low rate of O_2 consumption leads to high local pO_2, which, in turn, also contributes to increase ROS production because the K_M for O_2 of the ROS generator/s, contrary to that of cytochrome oxidase, is high and situated within the physiologic range of tissue pO_2. When saturating ADP is added **(b)**, electron flow is strongly accelerated (state 3, active phosphorylating respiration) and the reducing potential of the respiratory chain decreases (smaller dark area inside respiratory complexes); in addition, the strong increase in O_2 consumption lowers the local pO_2; these two changes collaborate toward decreasing the mtROSp rate, which is barely detectable in state 3. For further explanation, see text. S, substrate; pO_2, partial oxygen pressure; ROS, mitochondrial ROS production (mtROSp) rate. The *arrow* thickness indicates the intensity of flux. Cx I to IV, respiratory complexes I, III, or IV. Reproduced with permission from Ref. (10).

K_M for O_2 of cytochrome oxidase (which is pO_2 independent at tissue pO_2). Both the decrease in the reducing potential of the respiratory chain and the decrease in local pO_2 add their effects to automatically protecting the tissues against strong unbearable increases in mtROS production and oxidative stress during exercise (60), when metabolic rate rises by approximately 23-fold in human skeletal muscle and oxygen consumption increases around fourfold in the human heart.

In any aerobic tissue, not only in muscles, whenever the mitochondria transit from the resting state 4 to the active posphorylating state 3, the mtROSp acutely decreases (instead of increasing, as it is frequently but wrongly assumed) at the same time that the $mtVO_2$ strongly increases. That is why active tissue mitochondrial respiration leads to dramatic decreases in %FRL. This helps explain the apparent paradox that, in spite of the strong increase in $mtVO_2$, adequately performed aerobic exercise is not detrimental and does not shorten longevity either in rats (46, 65) or in humans (86, 116); has well-known benefits for health in many mammalian organ systems; and even seems to increase mean life span in humans and rats. Interestingly, skeletal muscle stays for most of the life span in resting conditions, especially in sedentary individuals, which can help explain why skeletal muscle loses many cells during normal aging (sarcopenia); whereas it is not so clear that decreases in the number of cells of a similar intensity occur in the case of heart cardiomiocytes or the large majority of brain neurons. Only in some brain areas, the number of neurons significantly decreases during aging (in parts of the frontal cortex or the hippocampus) and/or suffer high mtDNA mutation loads during aging (17, 75). Skeletal muscle mitochondria of sedentary individuals are most of the time near state 4, while the heart and brain are never at rest even when the individual is in such a state, and, thus, skeletal muscle organelles would tend to produce more mtROS than during aerobic exercise. There is a further beneficial effect of exercise. Various studies have consistently found decreases in mtROSp after chronic exercise training in rat heart and skeletal muscle (71, 175). Thus, additional protection to that shown during acute exercise (see above) is obtained through acclimation to increases in metabolic rate during chronic exercise. Long-term exercise increases the number of mitochondria, which tends to increase the total amount of H_2O_2 secretion to the cytosol, but this is compensated by decreasing mtROS generation per milligram of mitochondrial protein. All this helps explain the exercise "paradox" that chronic and adequately performed aerobic exercise does not lead to huge oxidative damage and even has beneficial effects for human health.

Another example is the case of birds, which combine very high rates of $mtVO_2$ with low rates of mtROSp (various long-lived species also have low %FRL; section III.C), which most likely contributes to their extraordinarily high longevity. Thus, at least in the state 4 to state 3 mitochondrial energy transition, during exercise training, and in various bird species when compared with mammals of a similar body size, the

rate of mtROSp can be dissociated from the rate of $mtVO_2$: The %FRL can vary, because it is not a fixed constant either between species or even within a single species. High rates of VO_2 are associated with low rates of mtROSp (and with high longevity in various cases) in many physiological situations. Further details with regard to these and other similar adaptations on the relationship between mtROSp and $mtVO_2$ have been previously described (10).

B. Criticisms on MFRTA: why they are unfounded

1. Failure to increase longevity by increasing antioxidants does not discredit the theory. It is mtROS generation, not elimination, that matters. During the last few years, some (mainly review) articles have criticized the MFRTA (24, 69, 73, 76, 92, 99, 107, 128, 148) or have even suggested that it is a "dead" theory. However, the updated MFRTA is supported by most of the data available (section II). Most criticisms surged in the last decade when genetic techniques allowed to perform experiments in which endogenous antioxidant enzymes were increased in transgenic animals or decreased in gene knockout animals. These studies have generally resulted in lack of increases or decreases in longevity, respectively. However, this general result is highly consistent with the long-life dietary experiments of antioxidant supplementation that were performed on rodents from the 1970s onward [(8); Table 1]. Antioxidant dietary supplementation or pharmacological induction at best increased mean life expectancy (usually when the longevity of the control animals was suboptimum) and could even duplicate it, but it did not increase (maximum) longevity (8, 98). The way in which antioxidants are increased, through dietary or genetic means, is not relevant. What is relevant is their lack of effect on longevity in agreement with previous predictions (13, 97, 131, reviewed in Refs. 8, 130). It has also been shown that the longer the longevity of a species, the lower is its mtROS generation rate (section II.B). What matters for longevity is the rate of mtROSp, not the level of antioxidants (section II.A). This is logical, as antioxidants are present in the diet as *exogenous* factors; whereas the aging rate and longevity are of *endogenous* origin and species-specific traits, which cannot be determined by environmental factors. If longevity would depend on antioxidants, when (*e.g.*) migratory animals encountered food low in dietary antioxidants in their environment, they would age very fast, they would die before attaining sexual maturity, and, thus, the species will become extinct. Such a system of longevity control does not have evolutionary sense. Instead, the source of mtROSp at the inner mitochondrial membrane is very near or even likely in contact with the initial target for aging, the mtDNA. Such contact ensures that antioxidants will not interfere with the rate of free radical damage generation in mtDNA, because it spatially prevents the interception of the ROS before they can damage the mtDNA. Oxidative damage to mtDNA just at the places of mtROS generation makes longevity dependent on the rate of ROSp but not on the antioxidant levels. The classic concept of a balance between ROS production and elimination is adequate for the mean cellular level of oxidative stress but is not applicable at those critical sites of mtROSp and can prevent an appropriate understanding of MFRTA. mtROSp-induced mtDNA damage is a case of sub-cellular compartmentation. The concentration of ROS at those critical sites for aging is dependent on the rates of ROS production, not on the antioxidant levels (13). The ROS concentration at the ROS production mitochondrial sites should be high in short-lived species and low in long-lived species due to their different mtROSp rates (9).

2. ROS cannot cause degenerative diseases only, and not aging. Some studies have concluded that ROS are causal only for degenerative diseases but not for the fundamental aging process in otherwise healthy individuals (24, 56, 107). However, this cannot be the case, as degenerative diseases (by definition) are caused by the basic aging process (Fig. 7). How

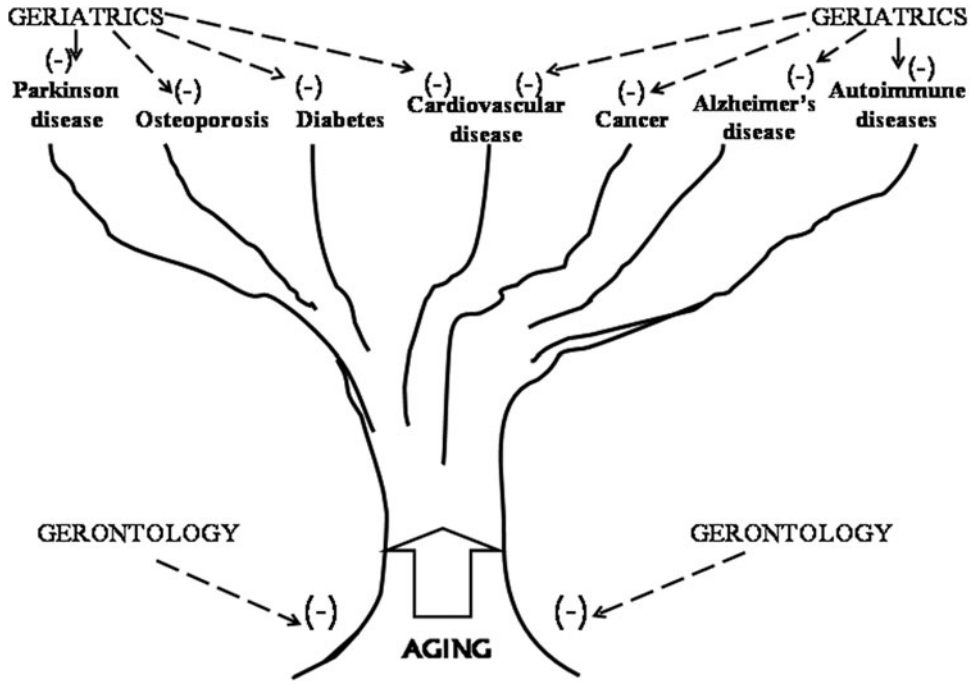

FIG. 7. The root of the degenerative diseases is the basic aging process: only factors causing aging can cause all these diseases. Degenerative diseases (by definition) are caused by the basic aging process as depicted in the figure by the lines connecting the "aging tree" with them. Therefore, if ROS are causal with regard to degenerative diseases, they should also be main causes of aging. The "minus" (−) symbol emphasizes that geriatrics tries to inhibit the diseases, while a main final goal of gerontology is to decrease aging rate.

then can paradoxical lack of changes in longevity along with decreases in degenerative diseases be explained? According to the Gene Cluster Hypothesis of Aging (11), many final target parameters should change, including not only mtROSp, but also many other known or unknown ones, in order for longevity to increase, because all of them are linked by gene and perhaps proteomic and even intercellular and inter-organ networks. Increases in longevity need changes in many parameters, not just in a single or a few ones. For instance, lowering the endogenous aging rate (through decreasing mtROSp or through any other means) will not increase longevity if increased protection from exogenous damage does not occur simultaneously. In such a situation, exogenous damage could kill the animal and will thus avoid the lowered endogenous aging rate to be expressed as an increased longevity. The same would be true of many other coordinated changes that would be necessary to finally obtain substantial increases in longevity [Fig. 8; (11)]. This is (likely) why because single-gene mutations in mice can increase longevity by no more than 1.4-fold, while variations in longevity in mammals range 200-fold from shrews to whales.

3. Studies in naked-mole rats. Other criticisms have claimed that high oxidative damage and low levels of some antioxidants, especially a low GSH-peroxidase activity, in naked mole rats are problematic for the MFRTA (24, 73), as these rodents have a remarkably high longevity (31 years). However, among other limitations of these animals as gerontological models (see below), these criticisms give too much of relevance to a few studies from a single species compared with decades of work and a huge number of studies on many different species, including mammals and birds, as well as to different approaches focusing on the MFRTA. Published studies have not revealed, after more than two decades of intensive research, true exceptions to the general negative relationship between mtROSp and longevity in mammals and birds (8, 9, 14, 15, 59, 61, 82, 155) even in the most complete study, including 12 mammalian species and showing the persistence of the correlation even after

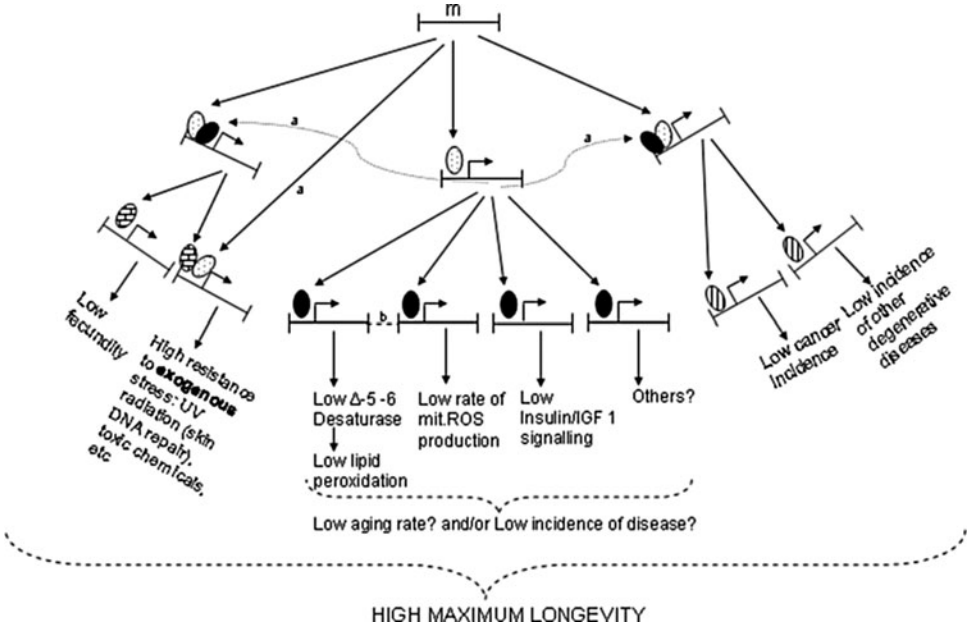

FIG. 8. The gene cluster hypothesis of aging and longevity. This scheme represents the gene cluster hypothesis of aging and longevity. Target genes producing proteins affecting the endogenous aging rate, degenerative diseases, resistance to external stress, fecundity, and other traits can be organized in clusters working through transcriptional cascades and complex interactions. The controller master genes (m) situated at superior hierarchical levels in space or time produce regulatory proteins (ovals) that control the graded expression of other genes. Regulatory proteins that would contain similar DNA binding sequences are depicted with the same shading. The grouping of genes controlled by similar regulatory proteins shown in the figure is only one of the many possible combinations, and is arbitrarily shown only as an example. Gene expression would also be influenced by other actors (*e.g.*, upstream promoters, enhancers) not shown in the figure. The graded activation/repression of the target structural genes will finally affect aging rate as well as other traits needed for final expression of a high longevity (low incidence of degenerative diseases and high resistance to external sources of stress). Interrelations among genes in the cluster are expected to be much more complex than depicted, and would include crossed regulations both at horizontal level, and at vertical levels spanning more than one level per relationship. The real number and kinds of final target genes should be much greater than shown in the figure, and the master genes at the upper control level can be multiple, although their number should be much smaller than the number of target genes. This is most interesting for future possible manipulations that are aimed at greatly increasing maximum longevity. According to present knowledge, the target genes included in the figure should be present in the real cluster, although not necessarily in the sub-clustering tandem positions shown, which were arbitrarily chosen as one among many possible combinations. "a": horizontal, multilevel, or single-level hierarchical interactions, and overlapping of regulatory elements; "b": hypothetical example of two genes clustered in tandem in the same region. Reproduced with permission from Ref. (11).

correcting for body size and metabolic rate (82). Naked mole rats were an outlier in that study after an analysis of phylogenetic-independent contrasts, which assumes an equal rate of evolution along all branches of the phylogenetic tree for both mtROSp and longevity, an assumption that can be invalid (82), and in any case does not affect the general negative correlation found between mtROSp and longevity.

It has been reported that naked mole rats have relatively high levels of tissue oxidative stress, although they are one order of magnitude longer lived than mice (1–3, 24). However, the large majority of the assay methods used to detect such oxidation employed kits of rather unspecific nature to estimate lipid peroxidation, 8-oxodG or protein carbonyls (1, 3), while high performance liquid chromatography-electrochemical detection was used to measure 8-oxodG in nDNA but not in mtDNA (3). Further studies using more specific techniques are, thus, needed to estimate the oxidative state of this rodent with exceptional longevity. In any case, the high lipid peroxidation level reported for this animal using unspecific techniques is strange. The opposite result is expected, as this species, similar to all the other long-lived animals studied to date, has very low tissue and membrane DBI and PI as well as very low levels of the highly unsaturated fatty acid 22:6n-3 [(111); section II.C]. This minimum amount of peroxidizable fatty acid substrates should lead to low instead of to high lipid peroxidation levels. With regard to the lack of higher antioxidant levels in this extraordinarily long-lived rodent, it is not surprising as it is known that antioxidants do not determine longevity (section II.A). Interestingly, however, it was found that naked mole rats excreted strongly low amounts of urinary 8-oxodG (3). This is exactly what is expected if this animal has both a low rate of mtROSp and a low steady-state 8-oxodG level in DNA (section III.E). With regard to damage of endogenous origin, lower rates of repair of DNA by base excision repair (BER) have been found both in long-lived mammals (118) and in DR animals (162). Indeed, when mtROSp was measured in mitochondria from 12 different mammalian species (the more complete study available), the obtained value for naked mole rats was near to the curve for all the species included (82) and was only an outlier when phylogenetic-independent contrasts (which require specific assumptions) were applied to the data. This indicates that this animal does not deviate from the general trend associating low mtROSp with high longevity in mammals. A low mtROSp value is difficult to reconcile with an estimation of high oxidative damage levels using unspecific assay methods. However, even if oxidative damage were high in naked mole rats, it could still be due to other special traits of these highly social and underground living animals such as their ectothermic character (the environmental temperature will affect their longevity), or the possible presence of strong differences in longevity between reproductive (“queens”) and non-reproductive (“workers”) individuals. It has not been reported whether reproductive queens or non-reproductive workers were used to assay oxidative damage in naked mole rats (1, 3). Finally, naked mole rats, similar to all the rest of long-lived animals studied without a single exception (67, 111, 121), have very low levels of 22:6n-3 and other highly unsaturated fatty acids as well as low DBI and PI global values in their cellular membranes. Therefore, with regard to the two parameters known to correlate with longevity in the right sense, mtROSp and the DBI, the naked mole rats are not exceptions. On the other hand, studies showing high protein stability and resistance to oxidative stress (129), as well a high cancer resistance in naked mole rats (90, 149) have been published.

4. Studies in birds and bats. There are no true exceptions to the general negative relationship between mtROSp and longevity in mammals and birds, including the most complete recent study in 12 different species showing the validity of that correlation even after correcting for body size (82). The rate of living theory relating oxygen consumption (basal weight-specific metabolic rate) to longevity has many well known exceptions, but they are not present when what is correlated to longevity is mtROSp instead of VO_2. This is logical, as mtROSp is not a direct function of $mtVO_2$ contrarily to what it is sometimes assumed without evidence (section III.A.2). Instead, it is known that the %FRL can be different in each species, so that mtROSp is a species-specific trait linked to the longevity of the species independently of its VO_2. In many cases, birds can posses a low mtROSp along with high aerobic metabolic rates and high rates of $mtVO_2$, because their %FRL at the mtETC is low, as it was found in pigeons and canaries (59, 61). It has been commented by a critic to MFRTA (3, 24) that a single study found similar or higher 8-oxodG in DNA in parakeets than in mice when measuring this parameter using a sodium iodide method (51). However, the overwhelming evidence shows that mtROSp, and in many cases 8-oxodG in mtDNA, is lower in birds (including pigeons, parakeets, and canaries) than in mammals of a similar body size but having almost one order of magnitude lower longevity (14, 15, 59, 61, 62). In the case of other exceptional long-lived animals such as bats, data showing low mtROSp and/or %FRL have also been reported (23, 82) although clearly more studies are needed especially in homeothermic long-lived bats.

Finally, the MFRTA continues to be the only theory that explains most of the available data with regard to longevity and can explain most of the observations linked to the other theories of aging. The MFRTA has already lasted between four (54) and six decades (52) since its first proposal, and its present version is strongly enriched compared with its original form. During all this time, no alternative theory has arisen that can compete with it. The MFRTA can also explain many of the observations central to other aging theories, including the rate of living and those related to glycoxidation, DNA somatic mutation, inflammation, lipofuscin/mitochondrial autophagy, cross-links, apoptosis, or the immune and neuroendocrine theories of aging.

C. ROS are not “by-products” of the respiratory chain: mtROSp and %FRL are regulated in each species at a level related to its longevity

It is commonly assumed that ROS are “by-products” of the respiratory chain. However, this is always assumed without evidence about it. During electron transport in the mitochondrial respiratory chain, most but not all electrons reach the end of the chain to tetravalently reduce oxygen to water. The assumption is that since mitochondria produce small but significant amounts of ROS, the construction of the respiratory chain during evolution would be imperfect and some electrons would be laterally lost during their travel from complex I to complex IV reducing oxygen univalently and thus generating ROS. However, the fact that ROS are

produced at mitochondria is no evidence of a hypothetical "by-product" character of these substances. It had been proposed long ago that ROS can have useful roles (5). More recently, it has been also mentioned that ROS generation in mitochondria does not appear to be an unwanted side reaction, but instead a rather precise mechanism used in many different signaling and useful pathways (99). These include apoptosis, oncogenesis, and tumor suppressor proteins such as ras, p21, or p53, messengers during fertilization or embryonic and prenatal mammalian development, H_2O_2-related thyroxin synthesis, or immune-related activities such as nuclear factor kappa-B activation, those related to T cells, or the use of ROS by neutrophiles and macrophages to kill bacteria. It has been frequently hypothesized that the failure of antioxidants to increase longevity can be due to their interference with such signaling mechanisms. In any case, it has been shown that mtROS production is not a simple by-product of mitochondrial respiration. Instead, it is regulated independently of O_2 consumption in many different physiologic situations, tissues, and animal species (10).

If ROS were simple by-products of the respiratory chain, the MFRTA could not be correct, because in that case mtROS production would be strictly proportional to the rate of mitochondrial oxygen consumption. In such a case, the MRFTA would be similar to the old "rate of living theory of aging" inversely relating aerobic metabolic rate and animal longevity. However, the rate of living theory of aging is known to be incorrect, because there are many exceptions to it. There are many animal species with longevities strongly different from those expected from their body size and metabolic rate, including even large groups such as bats, primates, or birds in general. However, this does not contradict the MFRTA, because it is known that the percent of total electron flow in the mitochondrial respiratory chain directed to ROS generation, the %FRL, is not a constant. In fact, the %FRL can vary depending on the mitochondrial state, during aerobic exercise bouts, after chronic exercise training, during DR, or across species (10). The exceptional longevity of various birds compared with mammals of a similar body size can be explained by their lower % FRL in the mitochondrial respiratory chain as it is shown in Figure 9 (59) for heart mitochondria of pigeons (longevity 35 years) compared with rats (longevity 4 years). The same has been found in heart mitochondria of canaries [longevity 24 years; (61)] compared with mice (longevity 3.5 years). This means that the mitochondria of the birds are more efficient than those of the mammals in transporting electrons to the final acceptor at the end of the chain, generating less mtROS through lateral electron leaks along the mtETC. Decreasing the %FRL is a most interesting way of slowing the rate of mtROSp, because it has the potential to decrease the rate of aging without lowering the metabolic rate and thus the general level of activity (both are very high in flying birds). This is appealing, as the goal should be to live longer but maintaining activity, without paying the price of "living slowly." The presence of different %FRL values in different animal species shows that the amount of electrons diverted to ROS generation is not a fixed percentage of total electron flow. Instead, the fact that the rate of mtROSp and the %FRL are different in different animal species indicates that these are genetically determined regulated parameters instead of being simple by-products of mitochondrial respiration, as it is frequently assumed without evidence about it. A variation of %FRL in relation to longevity is not limited to interspecies comparisons, as it also takes place during the life extending DR manipulation. As shown in Figure 9 for rat heart mitochondria, when the animals are subjected to DR, their %FRL (and mtROSp) is decreased below that of their *ad libitum*-fed counterparts (50). This has also been shown in many other rat organs, as well as in the case of other life-extended dietary manipulations such as PR (144) and MetR (143) in rats. Therefore, the percent of total electron flow in the mitochondrial respiratory chain is not a fixed fraction of total electron flow. Instead, it is decreased both inter-and intra-specifically during longevity extension as a regulated phenomenon.

D. The vicious cycle, an unnecessary hypothesis: mtROS generation does not need to increase with age to cause aging

The existence of some form of self-accelerating free radical production "vicious cycle" with age has been frequently postulated (38, 176). In that view, the ROS produced by mitochondria inflicts damage to the mitochondria themselves, which leads to further increases in mtROSp. An early study suggesting that mtROSp increased with age in rat heart mitochondria was based in values from a single animal at each age (114). While some authors have detected higher values of mtROSp in old than in young animals (70, 108), this has not been found in many other studies. This discrepancy can depend on the species, tissue, or even the kind of mitochondria

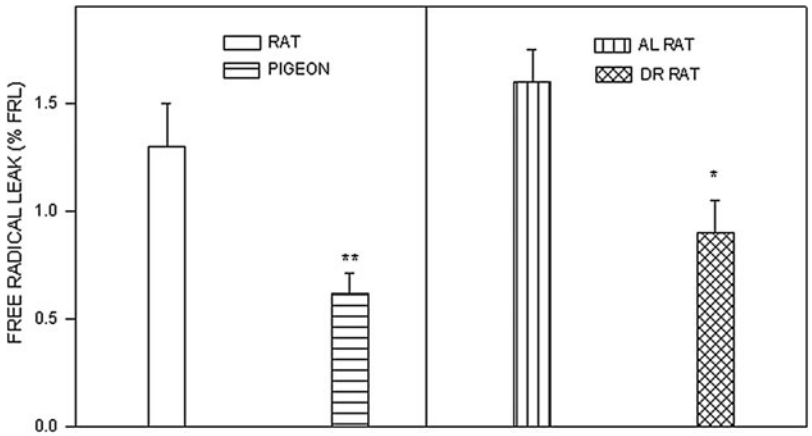

FIG. 9. Free radical leak in long-lived birds and in dietary restricted rodents. The %FRL in the respiratory chain of heart mitochondria is lower in pigeons than in rats [*left*, Ref. (59)] as well as in DR compared with ad libitum-fed (AL) rats [*right*, Ref. (50)]. %FRL, percent free radical leak.

analyzed within a tissue (71). In addition, many studies have detected age-related increases using methods detecting global cellular oxidative stress (like DCF or dihydroethidium). In our hands, measurements of mtROSp have always resulted in lack of significant differences between young adult (6 months old) and old (24 months) rats in mitochondria from different organs (41, 50, 96, 141, 146). The different results obtained by different laboratories can be due to the use of different assay methods, or to the possible existence of less than optimum husbandry conditions of the animals in some cases. Maintaining animals under suboptimum conditions will affect the old than the young animals much more, as the first would be exposed during a much longer period of time to the suboptimum medium. Then, the higher mtROSp of those old animals would be due to their poorer physiological state rather than to aging. In any case, if increased mtROSp is found only in some cases but not in others, this means that the increase is not intrinsic to aging. Moreover, when mitochondria from young rats were exposed directly to ROS *in vitro*, no clear evidence of the existence of a vicious cycle was obtained (145). On the other hand, lack of evidence of vicious cycle of ROS generation should not be taken as evidence against MRFTA. For instance, mutant mice in DNA polymerase gamma and with shortened longevity have shown similar instead of higher mtROSp than controls (79). This only contradicts a vicious cycle hypothesis of mtROS generation, not the role of mtROSp in aging. In these mutants, the defect is located at the level of DNA repair, and then it is situated downstream of mtROSp (Fig. 5). The lack of changes in mtROSp in this model is expected if there is no vicious cycle of ROS generation. The normal rate of mtROSp can continue to cause aging according to the MFRTA, and that aging rate would be further accelerated by the mutation at polymerase gamma directly affecting the mtDNA. Therefore, lack of evidence of vicious cycle mechanisms should not be confused with evidence against MRFTA.

The presence of a constant rate of mtROSp with age is consistent with the MFRTA instead of contradicting it, because loss of physiological functions with age is rather linear instead of exponential. The cause/s of aging should not increase with age. If they increase, the aging rate will accelerate with age, a situation that does not take place. Some confusion with regard to this this can come from the occurrence of exponential increases in mortality rate in populations (Fig. 10). Such increases cause the precipitous fall in survival in the final part of survival of curves (Fig. 11, right side). However, this does not mean that the causes of aging increase also exponentially with age. The causes of aging can still be more or less constant as corresponding to a progressive linear phenomenon such as aging. The losses of physiological functions with age are progressive and linear rather than exponential (Fig. 11, left). It is the final consequences of aging, including the mortality rate, that show an exponential increase with age, leading to an abrupt decrease in survival at old ages (Fig. 11, right). Therefore, when looking for the causes of aging, we do not need to look for parameters that vary exponentially with age but for processes present at roughly similar levels at different ages. We should look for phenomena occurring throughout life at roughly the same pace. The reason why mortality rate—which is a consequence and not a cause of aging—accelerates with age is not clear, but various factors contribute. One is the decrease in redundancy along with the possible existence of a minimum threshold to reach detrimental effects. Our organs

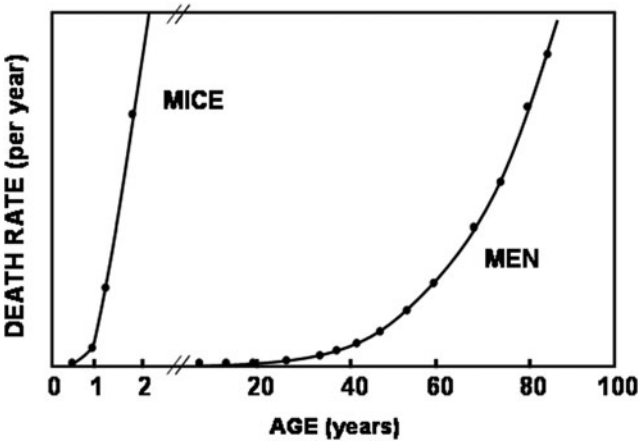

FIG. 10. Mortality and aging. Mortality increases exponentially with age. Such increase occurs much earlier in mice than in men.

are made up of a huge amount of equivalent cells; so, the loss of one or a few of them means nothing in many cases. Only the loss of a certain percent of cells can be limiting for tissue function, generating a threshold level. Redundancy occurs already at the organ level. The human kidneys do not have a single filtration site but many glomeruli, around 2.5 million of them, and the functioning loss of the glomeruli during aging correlates with decreased kidney filtration with age. The presence of multiple repeated units (redundancy of glomeruli) avoids organ failure during most of the lifespan even if some of these units malfunction or disappear. Similarly, the liver has many lobes and repeated units inside them, the intestine reaches 7 m long, and the heart and muscles are composed of a multitude of muscle fibres. Redundancy also occurs for components of the cells themselves, such as organelles, proteins, and other molecules. The cell has many organelles, for instance, hundreds or thousands of mitochondria dwelling inside a single cell. A single mitochondrion has many copies of respiratory chain protein complexes. Even the mtDNA, which seems to be important for aging, is present in various copies for mitochondrion, and thousands of copies per cell can exist. This huge degree of redundancy ensures continuation of life even after losing many of the repeated units. Only when such loss reaches a certain threshold, proper functioning is affected and illness or death ensues. This can help explain why aging, a progressive linear phenomenon, can generate an exponential mortality rate and the shape of the survival curve at right of Figure 11. Aging continuously goes on, progressively, at approximately the same pace, leading to loss of components and accumulation of irreversible damage. Once these losses reach a certain amount, illness appears or life ends. This tends to occur in the same decades of life for most of us, and this is why the survival curve precipitously falls in its right part. A linear phenomenon—aging—generates the linear accumulation of damage and loss of components, which, in turn, leads to an exponential increase in mortality rate due to decreases in redundancy and threshold effects (Fig. 11). The rate of aging and the threshold are roughly similar although not exactly the same in different individuals and for the different functions. This contributes toward explaining why the age of death is not the same in different individuals.

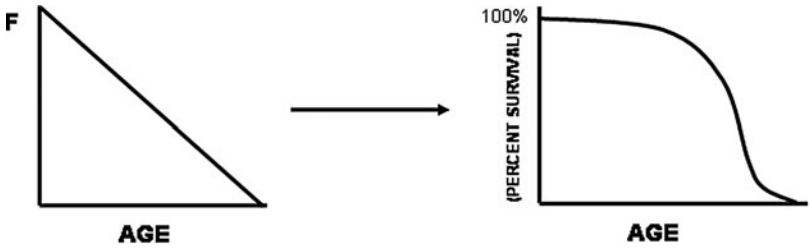

FIG. 11. A progressive, mainly linear process (aging), can lead to an exponential final result. Essentially linear decreases in physiological functions (F) lead to exponential increases in mortality (decreased survival) during aging, due to organism architecture including decreases in redundancy of components and existence of minimum thresholds to reach detrimental effects (see text). Therefore, there is no need to postulate vicious cycle hypotheses for the basic causes of aging.

E. DNA damage

1. Repair of endogenous DNA oxidative damage seems to be low in long-lived animals. A further comment is necessary with regard to final damage to DNA and its repair. The decrease in mtROSp during DR takes place along with significant decreases in oxidative damage to mtDNA alone, or in mtDNA and nDNA (estimated by measuring the level of 8-oxodG) depending on the organ studied (49, 50, 96, 146), as well as with lowered oxidative, glycoxidative, and lipoxidative damage to mitochondrial proteins (83, 123). The tissues of long-lived animals also have lower levels of mtROSp and 8-oxodG in mtDNA than those of short-lived ones. It has been also found that the repair of 8-oxodG in mtDNA through the mitochondrial BER pathway decreases in the kidney and brain in DR rats (162). Interestingly, the repair of cellular DNA endogenous oxidative damage by BER is also lower in long-lived than in short-lived animals, as recently shown in 15 mammalian and avian species (118); the same is true for protein breakdown through the 20S/26S proteasome in the liver, and for thioredoxin reductase and glutaredoxin repair enzymes in brain mitochondria of the same 15 vertebrate homeothermic species (138). The low DNA repair (BER pathway) of long-lived animals (in both DR and comparing different species; Fig. 12) agrees with theoretical predictions from more than one decade ago based on the low mtROSp and 8-oxodG steady-state levels in mtDNA of both DR and long-lived animals [see Fig. 2 of Ref. (6)]. Therefore, the repair of endogenously generated DNA oxidative damage negatively correlates with longevity, similar to what was previously found for endogenous antioxidant enzymes (130), the

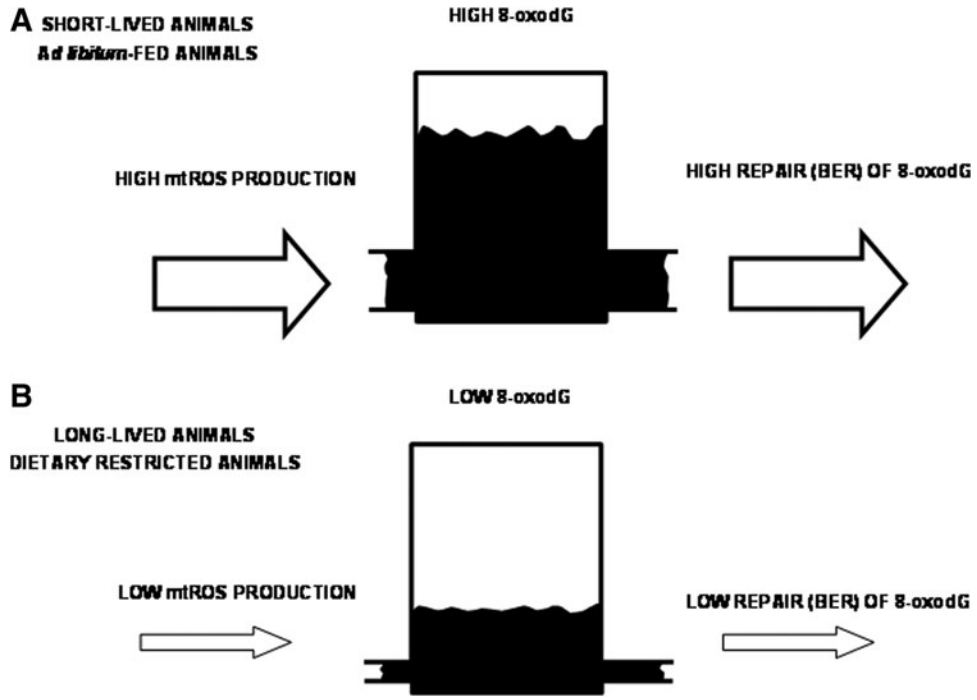

FIG. 12. Relationship between mitochondrial ROS production, steady-state oxidative DNA damage, and its repair, in species with different longevities and dietary restricted rats. Both long-lived animal species and dietary-restricted rodents have low mtROSp rates and low 8-oxodG levels in mtDNA in main internal organs including those containing port-mitotic cells. In agreement with this, it has been found that their base excision repair (BER) activities that repair DNA damage coming from endogenous origin are also low in internal organs **(B)**. The contrary is true in short-lived species and *ad libitum*-fed rodents **(A)**. This was predicted using a similar model in Fig. 2 of Ref. 6. Now there are supporting published data for this model both in species with different longevities and in DR rats. In contrast to BER, the repair of exogenous damage after UV irradiation in mitotic skin fibroblasts is known to be higher (instead of lower) in long-lived than in short-lived animal species (reviewed in Ref. 34) For references, see text.

explanation being that mtROSp is also low in long-lived animals. If there is a low rate of mtROS-induced damage in long-lived species, there is also a smaller need for endogenous antioxidants or for protein and DNA repair systems (6, 8, 9, 13, 97). The constitutive levels of tissue endogenous antioxidants are low in long-lived animals, most likely because their rate of mtROSp is lower than in short-lived ones (sections II.A and II.B). Endogenous antioxidants (98) and DNA repair enzymes (88) are transitorily induced, when needed, to come back again to low levels when the episodic increase in oxidative stress has been overcome (88). In this way, cells save much energy, which otherwise would be invested in the protein synthesis needed to continuously maintain high levels of antioxidants and DNA repair enzymes when they are not needed at such high levels. Instead, long-lived and DR animals obtain their low steady-state oxidative damage in mtDNA by decreasing mtROSp (sections II.B and II.D), which costs almost nothing. This is simpler, more efficient, and much less energetically expensive than continuously maintaining high levels of endogenous antioxidant and repair enzymes throughout life.

The low endogenous (BER) repair of DNA damage is contrary to what occurs for the repair of exogenous DNA damage estimated as unscheduled DNA synthesis in fibroblasts after exposure to UV radiation. Such repair of DNA damage coming from exogenous origin is higher instead of lower in long-lived animals [reviewed in Ref. (34)]. This last correlation makes sense, even though such a repair of exogenous damage in mitotic skin cells is not causally involved in intrinsic aging. A higher protection from exogenous damage (*e.g.*, UV radiation) is necessary in order for the superior longevity potential of slowly aging animals to be phenotypically expressed. Long-lived animals can reach many decades of age only if the many different causes of early death due to extrinsic mortality (including UV-induced DNA damage and skin cancer) are avoided.

To exert a low rate of ROS-derived damage to mtDNA in long-lived species and DR animals, (i) it would be highly inefficient and energetically expensive to generate ROS at a high rate at mitochondria, and then try to intercept most of them before they get to mtDNA, which, in addition, is situated very close to the ROS generator; with such an approach, a significant part of the ROS would not be intercepted and the final mtDNA damage obtained would be high instead of low; (ii) it would be even less efficient to generate a lot of ROS at mitochondria heavily damaging the mtDNA, and then try to repair most of the damage inflicted; this will waste resources and would be a practically impossible task also due to errors during repair. Instead, long-lived animals have developed during their biological evolution a low generation rate of endogenous damage (by decreasing the rate of mtROSp) in the first place; in this way, a low damage to mtDNA is directly obtained in a very simple and efficient way and at a very low or no cost. This model [Fig. 2 in Ref. (6)] accommodates all the available data. Both long-lived animals and DR rodents show (i) low mtROSp rates; (ii) low steady-state 8-oxodG levels; and (iii) low endogenous repair (BER) of DNA damage rates (Fig. 12). Only when a higher than normal oxidative stress challenges their cells, they react with transitory increases in antioxidants and repair systems to bring them back down to normal when the stress has subsided. Under the basal normal conditions in which animals are held during long-life aging experiments, increasing antioxidants (*e.g.*, by transgenic means) to high levels that are not needed should not bring benefits for lifespan. Therefore, the general failure to extend longevity by increasing antioxidants is not surprising. This failure does not mean that the MTFRA is incorrect. In order to truly test an updated version of the theory, what should be done is to decrease the rate of mtROSp. Up to now, in all the experimental or comparative cases in which longevity is higher, DR, PR, MetR, and long-lived animal species, mtROSp is always low. The low mtROSp is quantitatively paralleled by similarly low or decreased oxidative damage in mtDNA. Future approaches to experimentally decrease mtROSp without the need to perform dietary manipulations or change other physiological parameters could hopefully provide further insight about the validity of MFRTA.

IV. Conclusions

1. Long-lived animal species, including mammals and birds, have low rates of mtROSp and oxidative damage at their mtDNA.
2. It is well known that well-coupled functional isolated mitochondria produce ROS not only at complex III but also at complex I.
3. The three dietary manipulations that increase longevity, DR, PR, and MetR, decrease mitochondrial ROS generation and oxidative damage to mtDNA.
4. The respiratory complex related to aging and longevity, both with regard to comparisons between mammalian and bird species with different longevities and dietary, protein, or MetR, is complex I.
5. A low generation rate of endogenous damage and the possession of macromolecules that are highly resistant to oxidative modification are two general traits of long-lived animals which can explain "maintenance" and longevity instead of (antioxidant) defences or repair.
6. mtROSp is not necessarily proportional to mitochondrial oxygen consumption. The reverse commonly occurs in many situations, including the state 4 (resting) to state 3 (active) mitochondrial energy transition, aerobic exercise, or in animal species with longevities much higher than expected for their body size and weight-specific metabolic rate.
7. Criticisms on the MFRTA are largely unfounded. The MFRTA is supported by most of the data available and affords a mechanistic explanation for aging and longevity.
8. Mitochondrial ROS are not "by-products" of the respiratory chain. Instead, they are produced in each species at a different rate agreeing with its longevity, and not necessarily with its weight-specific metabolic rate. Thus, the %FRL at the respiratory chain (%FRL) is not a constant. It varies among species and is low in many species with extraordinarily high longevity. It also falls precipitously during aerobic exercise or during increases in cellular respiration and activity.
9. The MFRTA does not need any kind of "vicious cycle hypothesis" to be correct. It is the constant generation rate of ROS at mitochondria that matters for aging. That rate (different in each species) can be maintained at the same species-specific value during the life span both in young and in old animals. Aging is a progressive and thus a more or less linear process of declining tissue maximum functions. A rather progressive (linear) process, aging, leads to an exponential consequence (mortality increase with age).

Therefore, causal factors for aging should be constant with age (as it occurs for mtROSp), while the consequences (mortality) increase exponentially with age.

10. The repair of endogenous oxidative damage to DNA (BER) is low both in long-lived animal species and in dietary-restricted animals. This can be an evolutionary consequence of the low mtROS generation rate of these animals, and agrees with their low levels of oxidative damage to mtDNA. Longevity seems to be attained by decreasing endogenous damage generation rate instead of by increasing antioxidants or repair.

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Abbreviations Used

%FRL = percent free radical leak
8-oxodG = 8-oxo-7,8-dihydro-2′deoxyguanosine
ASC = ascorbate
BER = base excision repair
BHT = butylated hydroxytoluene
CHR = carbohydrate restriction
CYS = cysteine
DBI = double bond index
DCF = dichlorodihydrofluorescein
DR = dietary restriction
DTBH = 2,6-Di-tert-butyl hydroquinone
ETO = ethoxyquin
GSH = glutathione
GSH-Red = GSH reductase
HNCL = hydroxylamine hydrochloride
LR = lipid restriction
MDA = malondialdehyde
MEA = 2-mercaptoethylamine
MET = 2-mercaptoethanol
MetR = methionine restriction
MFRTA = mitochondria free radical theory of aging
MLS = maximum species-specific longevity
MLSP = maximum life span potencial
mtDNA = mitochondrial DNA
mtETC = mitochondrial electron transport chain
mtROSp = mitochondrial reactive oxygen species production
$mtVO_2$ = mitochondrial oxygen consumption
ND = non determined
nDNA = nuclear DNA
NI = not investigated
NS = not significant
PG = propyl gallate
PI = peroxidizability index
pO_2 = partial oxygen pressure
PR = protein restriction
ROS = reactive oxygen species
S = substrate
SOD = superoxide dismutase
TE = transposable elements
TZC = thiazolidincarboxylic acid