PHYSIOLOGICAL REVIEWS
Vol. 78, No. 2, April 1998
Printed in U.S.A.

The Free Radical Theory of Aging Matures

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Beckman, Kenneth B., and Bruce N. Ames. The Free Radical Theory of Aging Matures. *Physiol. Rev.* 78: 547–581, 1998.—The free radical theory of aging, conceived in 1956, has turned 40 and is rapidly attracting the interest of the mainstream of biological research. From its origins in radiation biology, through a decade or so of dormancy and two decades of steady phenomenological research, it has attracted an increasing number of scientists from an expanding circle of fields. During the past decade, several lines of evidence have convinced a number of scientists

that oxidants play an important role in aging. (For the sake of simplicity, we use the term *oxidant* to refer to all "reactive oxygen species," including O_2^-•, H_2O_2, and •OH, even though the former often acts as a reductant and produces oxidants indirectly.) The pace and scope of research in the last few years have been particularly impressive and diverse. The only disadvantage of the current intellectual ferment is the difficulty in digesting the literature. Therefore, we have systematically reviewed the status of the free radical theory, by categorizing the literature in terms of the various types of experiments that have been performed. These include phenomenological measurements of age-associated oxidative stress, interspecies comparisons, dietary restriction, the manipulation of metabolic activity and oxygen tension, treatment with dietary and pharmacological antioxidants, in vitro senescence, classical and population genetics, molecular genetics, transgenic organisms, the study of human diseases of aging, epidemiological studies, and the ongoing elucidation of the role of active oxygen in biology.

I. INTRODUCTION

The study of aging, by nature multidisciplinary, has been characterized by a dizzying variety of theories, a huge phenomenological literature, and the absence of firmly established primary causes. The diverse life histories of animal species, which manifest aging in very different ways, has been an obstacle to testing unified theories. For experimental gerontology to provide more than a catalog of age-related changes, it has been necessary for biologists to define the alterations that are common to most old cells, tissues, and animals, while simultaneously respecting that there is not a single phenomenon of aging or a single cause. This has taken some time, and from the outside it may have appeared that the field has been mired in phenomenology. Perhaps for this reason, the study of aging was until recently avoided by molecular biologists, who naturally favored clear-cut phenomena. As the molecular details of development, cancer, and immunology yielded to modern tools during the 1970s and 1980s, the field of aging lagged, and mechanisms responsible for aging failed to emerge.

Throughout this time, though, there has never been a shortage of unified theories attempting to reduce aging to something more tractable. In fact, gerontologists have been prolific in this regard (83, 205). Whereas some researchers have believed that a small number of random, deleterious mechanisms could explain degenerative senescence, others have opted for theories of "programmed" aging, in which senescence is the final destination in a developmental pathway. In the course of these debates, a number of scientists have rallied around a set of ideas called the free radical theory of aging: loosely, the belief that damage by reactive oxygen species is critical in determining life span. This theory inspired many experiments in which evidence of oxidative damage in aged animals was sought.

In the last 10 years or so, the nature of aging research has changed dramatically; one might say that the field has entered early adulthood. The tools of molecular biology are now sophisticated and accessible enough that researchers within gerontology have adopted them. At the same time, molecular biologists situated on the edge of aging research have made inroads and have discovered that fruit flies and nematodes are amenable to the study of aging. Also, medical researchers investigating human diseases of aging, such as Alzheimer's disease (AD) and inherited progerias, have overcome long-standing roadblocks.

It has been gratifying, therefore, that many of the preliminary studies in what might be called "molecular gerontology" lend credibility to the free radical theory. Results from disparate experimental systems have recently shown that oxygen radicals play a role in degenerative senescence, and the pace of discoveries is quickening. The likely result of this collision of scientific approaches will be the unraveling of the physiological tangle of aging, and it seems safe to say that one of the important knots will turn out to be oxidative stress.

However, there is a danger that in the excitement of theoretical confirmation, certain nuances are lost. For instance, the revelation that oxygen radicals may be involved in neurodegeneration does not mean that oxidative stress determines life span. The free radical theory has sought not only to explain the mechanisms of degenerative senescence, but it has also attempted to explain differences between species' life spans in terms of oxidants. So, although many recent studies indicate that oxygen radicals play some kind of role in aging, only a small number of these support the more ambitious version of the free radical theory. On the other hand, there is no reason to cling to such a stringent version of the free radical theory, and it is becoming apparent that whether or not they determine life span, oxygen radicals are certainly important players in aging's pathophysiology. In other words, the scope of the free radical theory of aging should include aging-associated oxidative stress in general, rather than limiting itself to those oxidative events that may determine life span. In fact, many current articles indicate that such a blurring of distinctions has already occurred and that as it is commonly used, "free radical theory" encompasses a broad set of ideas. Therefore, our first purpose is to delineate these different conceptions of the free radical theory, as a prerequisite to its critical evaluation.

Because of the recent popularity of free radical research, a large number of reviews have addressed various aspects of the interplay between oxidants and aging (6,

15, 18, 33, 51, 57, 58, 60, 65, 82, 85, 87, 103, 106, 123–126, 130, 161, 166, 196, 203, 227, 257, 273, 275, 288, 293, 297, 307, 312, 315, 335, 338, 348, 353, 357). Rather than merely updating this literature, our aim is to provide a systematic categorization of the types of experiments that have been performed. The phenomenon and study of aging are incredibly diverse, encompassing organisms from rotifers to mammals and techniques from physiology to genetics. Although it is precisely the broad sweep of evidence that lends the free radical theory its appeal, the menagerie of animals and techniques sometimes obscures the logic. By breaking the literature down into smaller pieces (a practice we find necessary ourselves), we hope to make it easier for readers to judge the theory. Moreover, by imposing a structure, we aim to highlight novel and definitive approaches, because it is these that will replace the phenomenology of past decades.

In this review, then, we briefly outline the evolution of the free radical theory and then delineate the different areas of evidence. We focus on recent experiments and point to the areas that we feel are most likely to provide future insights. The way in which we have categorized the literature is outlined in the table of contents (sects. IV-XVII) and in Table 1. Although any such system is somewhat arbitrary, we hope that ours will make it easier both to assimilate the existing literature and to envision future experiments. In writing a review on as broad a topic as the free radical theory, we have been forced to limit both the content and the number of references cited. Although we have done our best to include recent work, omissions were inevitable. We apologize to all authors whose work we have not managed to include, and direct readers to other recent reviews for material we have left out.

II. AN OVERVIEW OF THE FREE RADICAL THEORY OF AGING

A. Origins of the Free Radical Theory

In 1956, Denham Harman suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death. He noted parallels between the effects of aging and of ionizing radiation, including mutagenesis, cancer, and gross cellular damage (120, 128). At the time, it had recently been discovered that radiolysis of water generates hydroxyl radical (•OH) (319), and early experiments using paramagnetic resonance spectroscopy had identified the presence of •OH in living matter (45). Harman (120) therefore hypothesized that endogenous oxygen radical generation occurs in vivo, as a by-product of enzymatic redox chemistry. He ventured that the enzymes involved would be those "involved in the direct utilization of molecular oxygen, particularly those containing iron." Finally, he hypothesized that traces of iron and other metals would catalyze oxidative reactions in vivo and that peroxidative chain reactions were possible, by analogy to the principles of in vitro polymer chemistry. All of these predictions have been confirmed during the past 40 years.

The theory gained credibility with the identification in 1969 of the enzyme superoxide dismutase (SOD) (204), which provided the first compelling evidence of in vivo generation of superoxide anion (O_2^-•), and from the subsequent elucidation of elaborate antioxidant defenses (351). The use of SOD as a tool to locate subcellular sites of O_2^-• generation led to a realization that buttressed the free radical theory, namely, that mitochondria are a principal source of endogenous oxidants (37). Gerontologists had long observed that species with higher metabolic rates have shorter maximum life span potential (MLSP); they age faster (268). In fact, it had been proposed at the turn of the century that energy consumption per se was responsible for senescence, a concept referred to as the "rate of living" hypothesis (246, 268, 295). The realization that energy consumption by mitochondria may result in O_2^-• production linked the free radical theory and the rate of living theory irrevocably: a faster rate of respiration, associated with a greater generation of oxygen radicals, hastens aging. By now, the two concepts have essentially merged.

B. Sources of Oxidants

Ground-state diatomic oxygen ($^3\Sigma g^- O_2$ or more commonly, O_2), despite being a radical species and the most important oxidant in aerobic organisms, is only sparingly reactive itself due to the fact that its two unpaired electrons are located in different molecular orbitals and possess "parallel spins." As a consequence, if O_2 is simultaneously to accept two electrons, these must both possess antiparallel spins relative to the unpaired electrons in O_2, a criterion which is not satisfied by a typical pair of electrons in atomic or molecular orbitals (which have opposite spins according to the Pauli exclusion principle). As a result, O_2 preferentially accepts electrons one at a time from other radicals (such as transition metals in certain valences). Thus, in vivo, typical two- or four-electron reduction of O_2 relies on coordinated, serial, enzyme-catalyzed one-electron reductions, and the enzymes that carry these out typically possess active-site radical species such as iron. One- and two-electron reduction of O_2 generates O_2^-• and hydrogen peroxide (H_2O_2), respectively, both of which are generated by numerous routes in vivo, as discussed below. In the presence of free transition metals (in particular iron and copper), O_2^-• and H_2O_2 together generate the extremely reactive hydroxyl radical (•OH). Ultimately, •OH is assumed to be the species responsible for initiating the oxidative destruction of biomolecules. In addition to O_2^-•, H_2O_2, and •OH, two energetically ex-

TABLE 1. *The strengths and weaknesses of approaches to the testing of the free radical theory of aging*

Experimental Approach (Review Section)	Strengths of the Approach	Weaknesses of the Approach
Oxidative phenomenology (Sect. IV)	Simplicity; large existing data set; elucidation of the basic biochemistry of oxidative stress; technical foundation for other approaches; negative result may be instructive	Results are merely correlative (oxidative damage may be a consequence of aging); negative results, considered uninteresting, may fail to appear in the literature
Interspecies comparisons (Sect. V)	Specific testable predictions; deviations from predictions may refine theory; use of different species avoids conclusions that are species or strain specific; negative results may be instructive	Logistical problems in animal handling; quantitative comparisons complicated by qualitative interspecies differences in oxidative defenses and/or repair
Dietary restriction (Sect. VI)	Very well established model of life span extension; straightforward methodology; probably relevant to human aging and cancer	Results are somewhat correlative (alterations in oxidative stress may be consistent with but incidental to a more fundamental cellular switch)
Manipulation of rate of living (Sect. VII)	A direct test of rate of living theory, with specific testable predictions; straightforward methodology	To date, limited to relatively simple organisms, such as invertebrates
Manipulation of oxygen concentration (Sect. VIII)	A direct test of rate of living theory, with specific testable predictions; straightforward methodology	Results hard to interpret. Are positive results (life span attenuation by hyperoxia, extension by hypoxia) relevant to normoxic aging? Not applicable to most mammals
Supplementation with dietary antioxidants (Sect. IX)	(Potentially) a test of free radical theory	Results hard to interpret, due to unknown fates of supplements, potential adverse effects, complexity of overall antioxidant defenses
Administration of pharmacological antioxidants (Sect. X)	(Potentially) a test of free radical theory	Results hard to interpret, due to unknown fates of supplements, potential adverse effects, complexity of overall antioxidant defenses
In vitro senescence (Sect. XI)	A very well-established model system; relevance to human cancer (and perhaps to degenerative senescence in vivo)	Relevance to aging in vivo not yet clear
Classical and population genetics (Sect. XII)	"Awesome" power of genetics; very well established methods linked to a vast body of species-specific information; concurrent genome projects may forge interspecies links between gerontogenes	Currently limited to invertebrate model organisms (yeast, *C. elegans*, *Drosophila*)
Molecular genetics (Sect. XIII)	Powerful techniques for measuring mutational events (quantitatively and qualitatively); mutational fingerprinting to identify oxidative stress; simple methods applicable to wide array of species	In addition to causing cancer, physiological relevance of somatic mutations (and other genetic abnormalities) is not obvious
Transgenic organisms (Sect. XIV)	Immensely powerful tools; a direct test of free radical theory; interbreeding of transgenic animals for sophisticated in vivo research; established methods for widely divergent animals (*Caenorhabditis elegans*, *Drosophila*, mice)	Somewhat expensive and time consuming; dynamic nature of oxidative stress may complicate interpretations of phenotypes
Sporadic degenerative diseases (Sect. XV)	Results (if positive) provide mechanistic insights into the biology of oxidative pathophysiology and evidence of etiological relevance	Lack of animal models; disease may be of strictly human relevance (not a general phenomenon of aging)
Inherited degenerative diseases (Sect. XVI)	Results (if positive) provide mechanistic insights into the biology of oxidative pathophysiology and evidence of etiological relevance	Usually lack of an animal model; phenomenon (often severe) may not be of relevance either to most humans, nor to aging in general
Epidemiology (Sect. XVII)	Large data sets permit subtle species–wide trends to be detected; public health and policy relevance (preventative medicine); may be facilitated by sequencing of the human genome	Currently expensive, long term, and difficult

cited species of O_2 termed "singlet oxygens" can result from the absorption of energy (for instance, from ultraviolet light). Designated by the formulas $^1\Delta gO_2$ and $^1\Sigma g^+O_2$, both of these species differ from the triplet ground state ($^3\Sigma g^-O_2$) in having their two unpaired electrons in opposite spins, thereby eliminating the spin restriction of ground-state O_2 and enabling greater reactivity. The chemistry of oxygen and its derivatives has been extensively discussed elsewhere (115, 342). Because all of these species ($O_2^-\bullet$, H_2O_2, $\bullet OH$, $^1\Delta gO_2$, and $^1\Sigma g^+O_2$), by different

routes, are involved in oxygen's toxicity, we will collectively refer to them as "oxidants."

It is now beyond doubt that oxidants are generated in vivo and can cause significant harm (20, 37, 60, 91, 112, 351). There are numerous sites of oxidant generation, four of which have attracted much attention: mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome *P*-450 reactions, and phagocytic cells (the "respiratory burst"). Before a discussion of the potential contributions of different sources of oxidants, it is worthwhile briefly to outline them.

In the textbook scheme of mitochondrial respiration, electron transport involves a coordinated four-electron reduction of O_2 to H_2O, the electrons being donated by NADH or succinate to complexes I and II, respectively, of the mitochondrial electron transport chain (ETC). Ubiquinone (coenzyme Q, or UQ), which accepts electrons from complexes I (NADH dehydrogenase) and II (succinate dehydrogenase), undergoes two sequential one-electron reductions to ubisemiquinone and ubiquinol (the Q cycle), ultimately transferring reducing equivalents to the remainder of the electron transport chain: complex III (UQ-cytochrome *c* reductase), cytochrome *c*, complex IV (cytochrome-*c* oxidase), and finally, O_2 (115). However, it appears mitochondrial electron transport is imperfect, and one-electron reduction of O_2 to form $O_2^-\bullet$ occurs. The spontaneous and enzymatic dismutation of $O_2^-\bullet$ yields H_2O_2, so a significant by-product of the actual sequence of oxidation-reduction reactions may be the generation of $O_2^-\bullet$ and H_2O_2.

How much $O_2^-\bullet$ and H_2O_2 do mitochondria generate? In classic experiments during the 1970s, measurements of H_2O_2 generation by isolated mitochondria indicated that it is maximal when ADP is limiting and the electron carriers are consequently reduced ("state 4" respiration) (26). Estimates of state 4 H_2O_2 generation by pigeon and rat mitochondrial preparations amounted to 1–2% of total electron flow (26, 229). One problem with this estimate of mitochondrial H_2O_2 generation is its reliance on the use of buffer saturated with air (20% O_2). In vivo, the partial pressure of O_2 is ~5%, so these calculations may overestimate the flux of oxidants in vivo. Even disregarding the use of air-saturated buffer, the initial estimate of percentage ETC flux leading to H_2O_2 can be challenged on the grounds that in these experiments the concentrations of substrates fed to mitochondria were higher than occurs physiologically (118, 146). When H_2O_2 is measured with more physiological concentrations, the flux is ~10-fold lower (118), and experiments using subcellular fractions of SOD-deficient *Escherichia coli* suggest in vivo leakage of 0.1% from the respiratory chain (146).

What proportion of mitochondrial H_2O_2 ultimately derives from ETC $O_2^-\bullet$ generation? Unfortunately, the measurement of $O_2^-\bullet$ generation by intact mitochondria is prevented by the presence of mitochondrial SOD (mSOD). Therefore, the isolation of submitochondrial particles from which mSOD has been removed (by sonication of the intact organelles followed by extensive washing) was used for the detection of ETC $O_2^-\bullet$. In these experiments, stoichiometric estimates of the ratio of $O_2^-\bullet$ generation (by submitochondrial particles) to H_2O_2 generation (by the intact organelles) fell between 1.5 and 2.1 (24, 25, 69, 89, 191); because two $O_2^-\bullet$ molecules dismutate (either spontaneously or with the help of mSOD) to form one molecule of H_2O_2, such results suggest that virtually all mitochondrial H_2O_2 may originate as $O_2^-\bullet$ (27). Moreover, because most cellular H_2O_2 originates from mitochondria, $O_2^-\bullet$ from the ETC may be a cell's most significant source of oxidants (37).

In a recent discussion of the classic in vitro work (88), some of the original experimenters take issue with the idea that free $O_2^-\bullet$ exists in mitochondria as a result of normal flux through the ETC. They point out that in addition to having removed mSOD from mitochondria, the sonication they employed also resulted in the loss of cytochrome *c*, which rapidly scavenges $O_2^-\bullet$ in vitro and is present in mitochondria at local concentrations from 0.5 to 5 mM. In mitochondria, they argue, mSOD and cytochrome *c* rapidly scavenge $O_2^-\bullet$ (in the matrix and intermembrane spaces, respectively). More to the point, the authors stress that unless the ETC was poisoned with inhibitors such as antimycin A, $O_2^-\bullet$ generation was not detected in their experiments (89, 191). Arguing that mSOD should act to increase the rate of $O_2^-\bullet$ generation in vivo (by accelerating product removal by dismutation to H_2O_2), they suggest that the actual role of mSOD in vivo may be to increase H_2O_2 generation (with $O_2^-\bullet$ as a rapidly consumed intermediate) (88). Ultimately, there remains a good deal of uncertainty surrounding the mechanisms, quantity, and meaning of mitochondrial $O_2^-\bullet$ generation in vivo (228), a mystery which is deepened by recent reports of enzymatic nitric oxide (NO•) generation in mitochondria (C. Giulivi and C. Richter, personal communication). Because $O_2^-\bullet$ and NO• react to form the oxidant peroxynitrite ($ONOO^-$), mitochondrial $O_2^-\bullet$ generation may soon need to be considered in the light of its ability to destroy NO• and form $ONOO^-$, as discussed in section XVIII*B*.

A second source of oxygen radicals is peroxisomal β-oxidation of fatty acids, which generates H_2O_2 as a by-product. Peroxisomes possess high concentrations of catalase, so it is unclear whether or not leakage of H_2O_2 from peroxisomes contributes significantly to cytosolic oxidative stress under normal circumstances. However, a class of nonmutagenic carcinogens, the peroxisome proliferators, which increase the number of hepatocellular peroxisomes and result in liver cancer in rodents, also cause oxidative stress and damage (7, 157, 177, 230). Interestingly, during the regeneration of the liver after partial hepatectomy, there exist peroxisomes that do not stain for catalase activity (232), hinting that during rapid cell proliferation, oxidant leakage from peroxisomes may be enhanced.

Microsomal cytochrome *P*-450 enzymes metabolize xenobiotic compounds, usually of plant origin, by catalyzing their univalent oxidation or reduction. Although these reactions typically involve NADPH and an organic substrate, some of the numerous cytochrome *P*-450 isozymes directly reduce O_2 to $O_2^-\bullet$ (105, 168) and may cause oxidative stress. An alternative route for cytochrome *P*-450-mediated oxidation involves redox cycling, in which substrates accept single electrons from cytochrome *P*-450 and transfer them to oxygen. This generates $O_2^-\bullet$ and simultaneously regenerates the substrate, allowing subsequent rounds of $O_2^-\bullet$ generation (115). Although it is unclear to what extent cytochrome *P*-450 side reactions proceed under normal conditions, it is possible that such chronic $O_2^-\bullet$ generation by cytochrome *P*-450 is the price animals pay for their ability to detoxify acute doses of toxins (6).

Finally, phagocytic cells attack pathogens with a mixture of oxidants and free radicals, including $O_2^-\bullet$, H_2O_2, NO•, and hypochlorite (38, 220, 262). Although the massive generation of oxidants by immune cells differs from the above three sources of free radicals to the extent that it is the result of pathogenesis, it is nevertheless a normal and unavoidable consequence of innate immunity. Chronic inflammation is therefore unique among the endogenous sources of oxidants, because it is mostly preventable (49, 231, 245).

In addition to these four sources of oxidants, there exist numerous other enzymes capable of generating oxidants under normal or pathological conditions, often in a tissue-specific manner (115). To give a single relevant example, the deamination of dopamine by monoamine oxidase generates H_2O_2, in some neurons, and has been implicated in the etiology of Parkinson's disease (80). Finally, the widespread catalytic generation of NO•, achieved by various isozymes of nitric oxide synthase and central to processes as diverse as vascular regulation, immune responses, and long-term potentiation, increases the potential routes for destructive oxidative reactions (187). The interaction between $O_2^-\bullet$ and NO• results in $ONOO^-$, which is a powerful oxidant.

As originally articulated by Harman (120), the free radical theory of aging did not distinguish between these different sources of oxidants. However, the rate of living hypothesis clearly singled out mitochondrial $O_2^-\bullet$ and H_2O_2 generation, since it is the mitochondrial respiration rate that negatively correlates with MLSP. Also, as many other established sources of oxidants are tissue specific (associated with hepatic, neuronal, and other specialized functions), they are less likely to explain aging across a broad range of species. For this reason, mitochondrial $O_2^-\bullet$ and H_2O_2 have captured the lion's share of attention. However, it may turn out that for some age-associated disorders, nonmitochondrial oxidants are critical. In the expanded sense of the free radical theory, any oxidants, mitochondrial or not, may play a role. Therefore, despite the great number of intracellular sources of oxidant that have been identified in a qualitative way, in terms of ranking their relative importance, the field is in its infancy.

C. Targets of Oxidants

What are the targets of endogenous oxidants? The three main classes of biological macromolecules (lipids, nucleic acids, and proteins) are susceptible to free radical attack, and there is plentiful evidence that all suffer oxidative damage in vivo. Although it is well beyond the scope of this review to treat the biochemistry of oxidative damage in any great depth, the area has been expertly reviewed (115). A synopsis of the better known pathways of oxidative damage, however, is warranted; the most familiar end products are described here.

The earliest research on the destruction of biological molecules by oxidants involved lipids (109). Food chemists have long understood that the rancidity of fats results from peroxidative chain reactions in lipids ("autoxidation"); a lipid hydroperoxyl radical abstracts a hydrogen atom from the double bond of a neighboring unsaturated lipid, forming a hydroperoxide and an alkyl radical, the latter which combines with O_2 to regenerate a lipid hydroperoxyl radical capable of initiating another round of oxidation. Ultimately, intramolecular reactions and decomposition yield cyclic endoperoxides and unsaturated aldehydes, the latter of which are reactive and may act as mutagens (194) or inactivate enzymes (39, 322), or operate as endogenous fixatives, reacting with proteins and nucleic acids to form heterogeneous cross-links (42). Moreover, a primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins (324).

Oxidative damage to nucleic acids includes adducts of base and sugar groups, single- and double-strand breaks in the backbone, and cross-links to other molecules. The spectrum of adducts in mammalian chromatin oxidized in vitro and in vivo includes more than 20 known products, including damage to all four bases and thymine-tyrosine cross-links (70, 71, 113). The electrochemical properties of the adduct 8-oxo-guanine (oxo8gua) and the deoxynucleoside 8-oxo-2,7-dihydro-2′-deoxyguanosine (oxo8dG), which have permitted the coupling of extremely sensitive electrochemical detection to high-performance liquid chromatography (HPLC), have resulted in hundreds of studies of its formation, accumulation, and excretion (17). The identification of specific enzymatic repair of oxidative lesions has recently provided both proof of the significance of oxidative DNA damage as well as tools to manipulate the load of damage in vivo by genetic knockout (17, 23, 78, 192, 266, 291).

The oxidation of proteins is less well characterized, but several classes of damage have been documented,

including oxidation of sulfhydryl groups, reduction of disulfides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalyzed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation (317, 318). A particularly intriguing recent development has been the realization that a number of enzymes possessing active-site iron-sulfur clusters are acutely sensitive to inactivation by $O_2^-\bullet$ (86, 176). For example, *E. coli* aconitase is inactivated by $O_2^-\bullet$ with a rate constant of 10^9 $M^{-1} \cdot s^{-1}$ (95, 96). Mammalian mitochondrial aconitase is inactivated in vitro and in vivo by treatments that increase mitochondrial $O_2^-\bullet$ generation, such as growth under hyperbaric conditions (97, 98). Because aconitase participates in the citric acid cycle, its inhibition would be expected to have pleiotropic effects. Moreover, the mechanism of aconitase inhibition by $O_2^-\bullet$ has been demonstrated to involve the release of free iron from the enzyme (86). Free iron atoms catalytically exacerbate oxygen stress (see below), and it has been proposed that superoxide's genotoxicity is a function of its ability to liberate protein-bound iron (159, 184).

Unlike lipids and nucleic acids, proteins represent a very diverse target for oxidative damage. Although protein oxidation has been demonstrated at the level of the peptide backbone and amino acids, there has been relatively little scrutiny of differences between proteins in their sensitivities. A detailed quantitative comparison of bovine serum albumin and glutamine synthase has shown susceptible residues of the former (methionine and the aromatic amino acid residues) to be oxidized about twice as fast as those on the latter, implicating all four levels of protein structure in relative susceptibility (19). A study of the oxidation sensitivities of a various cloned K^+ channels from T lymphocytes, cardiac cells, and neurons revealed that whereas five of the cloned channels were highly sensitive to oxidation, an equal number were resistant (74). Differential sensitivities raise the possibility that the loss of homeostasis that is a hallmark of aging could result from the selective oxidation of proteins.

In the context of aging, a particularly relevant aspect of oxygen's toxicity is its promotion by some metals and by elevated O_2 partial pressure. Iron and copper catalyze the homolytic cleavage of ROOH (the Fenton reaction), leading to the generation of •OH (115). It is •OH that is the most reactive oxidant, reacting at diffusion-limited rates. The catalytic properties of iron and copper explain why cells possess metal-chelating proteins such as ferritin and transferrin, which reduce the concentration of redox-active metals (114, 211). In humans, the body's content of iron increases with age (in men throughout their lives, and in women after menopause), and it has been suggested that this accumulation may increase the risk of oxidative damage with age (169, 331). Finally, oxidative stress in vivo is aggravated by increasing O_2 partial pressure, due to a more pronounced flux of mitochondrial $O_2^-\bullet$ (37). Consequently, the manipulation of O_2 partial pressure is a relatively simple tool that has been used to test the free radical theory.

D. Antioxidant Defenses

Cells are equipped with an impressive repertoire of antioxidant enzymes, as well as small antioxidant molecules mostly derived from dietary fruits and vegetables (5, 351). These include *1*) enzymatic scavengers such as SOD, which hastens the dismutation of $O_2^-\bullet$ to H_2O_2, and catalase and glutathione peroxidase (GPX), which convert H_2O_2 to water; *2*) hydrophilic radical scavengers such as ascorbate, urate, and glutathione (GSH); *3*) lipophilic radical scavengers such as tocopherols, flavonoids, carotenoids, and ubiquinol; *4*) enzymes involved in the reduction of oxidized forms of small molecular antioxidants (GSH reductase, dehydroascorbate reductase) or responsible for the maintenance of protein thiols (thioredoxin reductase); and *5*) the cellular machinery that maintains a reducing environment (e.g., glucose-6-phosphate dehydrogenase, which regenerates NADPH). The complement of defenses deployed differs not only between organisms or tissues, but even between cellular compartments. For instance, GPX plays an important role in mammals but is absent from flies and nematodes (298, 330), and there exist in humans three forms of SOD (cytosolic Cu,Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD), encoded and regulated independently (91).

As far as supporting the free radical theory of aging is concerned, the universality of antioxidant defenses is good news. Although the nature of these defenses varies between species, the presence of some type of antioxidant defense is universal. In fact, some antioxidants, such as SOD, are very highly conserved. Clearly, an indifference to oxygen free radicals is inconsistent with life, underlining the centrality of oxidative damage. Moreover, the fact that antioxidant defenses are not uniform has been incorporated into the free radical theory; differences in antioxidant defenses between species have been put forth to explain differences in life span. Although there is something uncomfortably ad hoc in these two different interpretations of the data, they are not inconsistent. Whereas aerobic life requires organisms to cope with oxidation to some extent, different evolutionary pressures appear to have selected for more or less investment in these defenses, as is discussed in section III.

Finally, a persistent problem in testing the free radical theory is that antioxidants are both parallel (different antioxidants can play similar roles, e.g., catalase and GPX) and serial (enzymes operate in tandem to decompose radicals to harmless products, e.g., SOD and catalase). Consequently, measurements of individual antioxidant activities do not have great relevance. In fact, as is discussed below,

measurements of age-related changes in individual antioxidants have led to conflicting results (297). For this reason, aggregate assays have been devised, such as the susceptibility of crude cellular homogenates to in vitro oxidation by ionizing radiation (2, 300). Although these assays do not provide any information about the specific mechanisms of defense, they conveniently measure overall effectiveness.

E. Repair of Oxidative Damage

Unlike defenses against oxidants, which have been extensively characterized, the machinery for repairing oxidative damage is relatively unexplored. Nevertheless, it is clear that cells repair oxidized lipids (e.g., phospholipase A_2 cleaves lipid peroxides from phospholipids; Ref. 239), oxidized nucleic acids (e.g., glycosylases specifically recognize and excise oxidized bases from double-stranded DNA; Refs. 23, 56a, 56b, 325), and oxidized proteins (104, 240, 261, 316). The comparative biochemistry of cellular repair is very fertile ground for the free radical theory.

F. Synthesis: Interaction of Oxidant Generation, Oxidative Damage, and Repair

The existence of multiple intracellular sources of oxidants and complex defenses has led to refinements of the free radical theory. For example, it is clear that the metabolism of oxygen radicals is dynamic, with damage resulting from an increase in oxidant generation or a decrease in antioxidant defenses. Consequently, a difference in life span between species or individuals could be due to different rates of living, or to different "rates of scavenging" (57, 82). The picture has been further complicated by the discovery of specific enzymatic repair of oxidative damage, leading to "repair" or "fidelity" versions of the theory, in which life span is determined by the failure to correct oxidative damage (137, 239).

The relationship among these three components of oxidative stress: oxidant generation, antioxidant protection, and repair of oxidative damage, and the way in which they have been investigated in testing the free radical theory, is illustrated schematically in Figure 1. Increases in oxidant generation, and decreases in antioxidant protection and repair systems, are among the theory's testable predictions and have been examined both as a function of age in individuals of the same species, as well as between species of differing MLSP.

Finally, an extremely important (if experimentally recalcitrant) aspect of the interactions between oxidants, antioxidants, and repair are feedback loops, positive and negative, between them. Antioxidant defenses and cellular repair systems have been shown to be induced in response to oxidative challenges (67, 119, 320) and are of course potential targets of oxidative destruction (145). Also, the generation of oxidants may be enhanced by the malfunctioning of oxidatively damaged molecules (28, 303). Therefore, with the examination of Figure 1, it is not difficult to envision ways in which primary oxidative destruction of any target (e.g., the components of the mitochondrial ETC, scavenging enzymes such as SOD, or DNA repair enzymes) might promote further oxidative damage in what is frequently called a "catastrophic vicious cycle."

Although such cycles are intuitively appealing, their documentation awaits future work and will be extremely difficult from a technical standpoint. An alternative to lab-based approaches, namely, the computational modeling of these complex interactions in what has been termed a "Network Theory of Ageing," is being pursued by theoretical gerontologists (170). Ultimately, a question of obvious importance is whether or not such cycles, if they exist, could be broken, and modeling may help pinpoint weak links for therapeutic intervention.

III. REFINEMENTS AND COROLLARIES OF THE FREE RADICAL THEORY

As the free radical theory has gained ground, it has incorporated other ideas. For example, as mentioned above, the rate of living hypothesis dovetailed with the free radical theory once mitochondrial free radical generation was confirmed. Three other ideas that have been influential are the evolutionary concept of antagonistic pleiotropy, the somatic mutation theory of aging, and the mitochondrial theory of aging.

A. Oxidants and Evolutionary Theories of Aging

The intracellular generation of oxidants capable of limiting life span may appear paradoxical. It seems reasonable to expect that natural selection might have devised aerobic cells that do not leak toxic by-products. Evolutionary biologists have contributed to the free radical theory by suggesting why physiologically harmful generation of oxygen radicals occurs. They have argued that natural selection favors genes that act early in life and increase reproduction, rather than genes that act to preserve nongerm cells (the "disposable soma"), a principle called "antagonistic pleiotropy" (162–164, 265, 341).

The concept of antagonistic pleiotropy stresses that in the wild, reproductive success is principally a function of external factors. With the exception of modern-day humans, individuals do not usually die of old age, but are eaten, parasitized, or out-competed by others. Therefore, preserving the cells of the disposable soma, otherwise known as the body, may be disadvantageous if it detracts

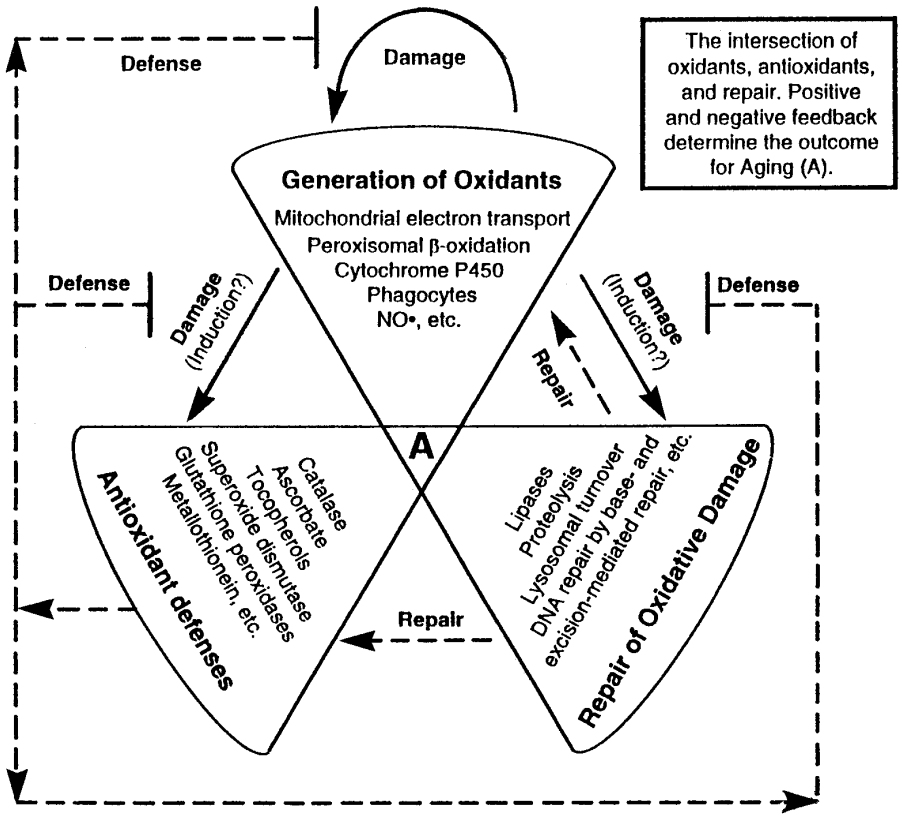

FIG. 1. The ultimate outcome of oxidative stress is a function of *1*) oxidant generation, *2*) antioxidant defenses, and *3*) repair of oxidative damage. Bolds arrows denote oxidative damage, and dashed arrows denote routes for its prevention or repair. Because of the ways in which these processes may interact, multiple positive- and negative-feedback loops are possible. Aging (A) is situated at intersection of these processes. In testing the free radical theory, changes in *processes 1–3* have been measured both as a function of age and as a function of species' maximum life span potential. The similarity of the figure to the international emblem of radiation is not a coincidence; the free radical theory has its roots in radiation biology.

from more pressing problems. The toxicity of respiratory burst oxidants, for instance, cannot easily be eliminated by evolution, since this would result in death from childhood infection. Similarly, an investment in improved antioxidant defenses maximizes fitness only if the resources are not better invested in strength, beauty, speed, or cunning.

In terms of natural selection, the tremendous cost of death before reproductive age, the constantly compounding probability of death from external threats, and the cost of failing to reproduce all ensure that selective pressure is strongest at young ages. Any novel mutations that decrease oxidative damage first have to satisfy the criteria of youthful reproduction. In short, selective pressure to compete effectively at an early age may guarantee a certain degree of O_2 toxicity and work against the conservation of the soma in the long run.

B. Oxidants and the Somatic Mutation Theory of Aging

The somatic mutation theory holds that the accumulation of DNA mutations is responsible for degenerative senescence (23, 79, 212, 218, 332). In the case of cancer, which results from both point mutations in oncogenes and the loss of tumor suppressor gene function (often by deletion), the role of mutations is unquestionable (5). It remains to be seen whether or not the argument is valid for nonproliferative senescence. For instance, whereas significant age-related increases in somatic mutations in a reporter transgene (*lac*Z) have been measured in a mitotic tissue of transgenic mice (the liver), no increase was detected in the largely postmitotic brain of the same animals (71a), suggesting that neurodegeneration, at least, is unlikely to be the result of accumulated somatic mutations in nuclear DNA. Moreover, the accumulation of mutations in the liver tissues was not dramatic, suggesting that mutagenesis may be of little functional consequence to mitotic tissues as well (338b). In light of these data, what evidence is there that somatic mutations are related to aging? A compelling argument for the somatic mutation theory of aging was provided years ago in the discovery that DNA repair ability correlates with species-specific life span (127a), a phenomenon that has recently been reconfirmed (52a). However, it has been noted that DNA repair, which is necessary for the prevention of tumorigenesis, is necessary but not sufficient for longevity (52a). Ultimately, arguments about the physiological significance of somatic mutations hinge on how disruptive a given mutational burden is to a cell or animal; with current methods, this is an unanswerable question.

In any case, it has been demonstrated in numerous studies with prokaryotes, yeast, and mammalian cells that oxidants are mutagens, against which cells actively protect their genetic material (81, 108). Although it is not

yet clear what fraction of mutations can be attributed to oxidative damage, the characterization and cloning of defense genes against oxidative mutagenesis (17), and the development of in vivo mutagenesis assays (198), has finally opened up avenues for definitive experiments.

C. Oxidants and Mitochondrial Theories of Aging

The mitochondrion has also long attracted attention as one of the cell's weak links, an organelle whose dysfunction has profound negative pleiotropic effects (193). Mitochondria supply ATP and also sequester potentially toxic Ca^{2+}, yet because of their generation of $O_2^{-}\bullet$ and H_2O_2, they are on the front lines of respiratory oxidative stress. The idea that the mitochondrion is therefore uniquely vulnerable was embraced early on by proponents of the free radical theory (121). In the early 1980s, Miquel and colleagues (84, 212, 213) proposed that oxidative damage to mitochondrial DNA (mtDNA) in postmitotic cells would lead to mutations and blocks to replication, and consequently to mitochondrial dysfunction and physiological decline. This "mtDNA mutation hypothesis of aging," which incorporates free radicals, somatic mutations, and the central role of mitochondria in homeostasis, is presently under intense scrutiny (8, 11, 21, 50, 110, 222, 223, 238, 258–260, 276, 288, 336, 339).

IV. OXIDATIVE PHENOMENOLOGY: AGE-ASSOCIATED TRENDS

The phenomenological approach to the free radical theory has involved looking for traces of oxidative damage in vivo. Phenomenology is not well suited to critically testing the free radical theory, since the data (which are voluminous and generally supportive) mainly represent correlations. Documented increases in oxidative damage, no matter how impressive, may be a consequence of a primary nonoxidative event. Nevertheless, phenomenology is the foundation upon which more powerful experiments depend, since the analytical methods developed for it have been used to compare species, genetic mutants, and populations with differing life spans. In fact, almost all of the biomarkers of oxidative stress described in this section have been found to accumulate at a faster rate in short-lived species, and in many cases, this rate correlates with O_2 consumption. Familiarity with the most frequently measured end points is a prerequisite to assessing the free radical theory.

If oxidative damage is a significant cause of cellular degeneration, then one expects to see more of it in older individuals. Oxidative damage has been described in terms of "accumulation, modification, and depletion": accumulation of end products of oxidative damage (such as lipofuscin), modification of existing structures (such as oxidative adducts in DNA), and depletion (such as the loss of enzymatic activity or reduced thiols).

A. Accumulation of Oxidative End Products

The gradual and steady accumulation of intracellular yellow-brown fluorescent pigments, referred to as lipofuscin, occurs in numerous phyla. Lipofuscin arises prominently in postmitotic cells (where, it is argued, it remains undiluted by rounds of cell division; Ref. 296) and is located in small granules in secondary lysosomes. Lipofuscin is structurally complex and variable, consisting mostly of cross-linked lipid and protein residues (251, 296, 327), and is ubiquitous, documented in species as diverse as nematodes, fruit flies, rats, bees, crab-eating monkeys, and crayfish. Most important, it is abundant in aged tissues, where it may occupy more than one-half of the volume of the cell (347, 348).

Early on, it was discovered that incubation of amino acids with the lipid peroxidation product malonaldehyde under acidic conditions leads to the formation of lipofuscin-like fluorophores (42). The plausibility of such a reaction, given the contents and low pH of lysosomes, suggested that lipid peroxidation in vivo leads to the formation of lipofuscin (324). Other in vitro studies of lipid peroxidation have since uncovered a great number of routes to fluorescent, cross-linked products via promiscuous oxidative chemistry, which suggests that lipofuscin is a biomarker of lipid peroxidation (160, 296, 348).

Despite extensive in vitro experiments, it is not known with certainty how lipofuscinogenesis occurs in vivo, nor how lipofuscin comes to accumulate with age. Lipid peroxidation could occur throughout the cell and be followed by lysosomal phagocytosis and cross-linking of peroxidative by-products, in which case an age-related increase in lipofuscin content could be seen as the result of oxidative damage. Alternatively, an age-associated decline in lysosomal activity (due to something besides oxidation) might increase the residence time of phagocytosed material enough to enhance lipofuscinogenesis in situ from constant amounts of peroxides (135). In support of the latter possibility, infusion into rat brains of lysosomal proteinase inhibitors leads to the rapid accumulation of lipofuscin-like granules (149). This scenario suggests that lipofuscinogenesis may be a consequence, not a cause, of aging.

Experiments with cultured cardiac myocytes have established roles for both oxidative damage and lysosomal turnover (28). Lipofuscin accumulates in these cells in culture, and growth under increasing O_2 partial pressure from 5 to 40% markedly enhances its accumulation (306). Inclusion of iron in the growth medium further increases lipofuscinogenesis, and the iron chelator desferal depresses it, suggesting that Fenton reaction-generated •OH

is an initiator (200). Finally, antioxidants inhibit lipofuscin formation in cultured cardiomyocytes, whereas lysosomal protease inhibitors increase it (199, 201, 202).

Even if oxidative damage is primarily responsible for depositing lipofuscin in the lysosomes of senescing animals, is it more than a biomarker of aging? It has been theorized that lipofuscin accumulation is likely to impair autophagy, as more lysosomal volume is occupied by the indigestible material (28). Because lysosomes are responsible for the recycling of materials and organelles, their failure may include the following: *1*) a delay in mitochondrial turnover (with a concomitant decrease in mitochondrial efficiency or an increase in mitochondrial oxidant generation), *2*) an accumulation of oxidatively modified proteins and lipids in the cytosol awaiting degradation (potentially aggravating cytosolic lipid peroxidation), *3*) an accumulation of lipofuscin-bound iron in a redox-active form (which might promote further intralysosomal lipid peroxidation), and *4*) the disruption of lysosomal membranes (and the spillage of hydrolytic enzymes into the cytosol). Although these speculations (28) remain to be substantiated, it has been shown that when treated with sublethal doses of H_2O_2, cultured cells display lysosomal disruption and leakage of the lysosomal compartment into the cytosol (29). Also, it has been demonstrated that the sensitivity of cultured primary hepatocytes to oxidation, which was associated with a loss of GSH and an influx of Ca^{2+}, was prevented by the iron chelator desferal (235). What is intriguing about these results is the fact that whereas desferal stabilized the lysosomes, it did not prevent the loss of GSH or the increase in intracellular calcium, so it may be that it is lysosomal leakage per se, rather than peroxidative damage, which is the actual lethal step in this model of oxidative killing.

B. Steady-State Levels of Oxidative Modification

Unlike cytosolic proteins whose half-lives are measured in minutes or hours, some extracellular proteins are rarely recycled, and oxidative modification of these old macromolecules occurs. A class of fluorescent cross-linked molecules that is distinct from lipofuscin forms on long-lived proteins such as collagen and lens crystallin (216). These modifications are initiated by the reaction of reducing sugars with free amino groups (glycation), a chemical sequence that is unrelated to oxidation and results in a molecule known as an Amadori product. Further nonoxidative rearrangements result in stable, cross-linked advanced glycation end products (AGEs) (35), whose absolute abundance appears to be an excellent biomarker of age (217). Recently, it was discovered that oxidation is one fate of the Amadori product. Pentosidine, the name given to a cross-link involving arginine, lysine, and pentose moieties, is one such "glycoxidation product," the formation of which requires the presence of O_2 (326). It appears as if Amadori products themselves are a source of H_2O_2 in vitro, which then accelerates glucose-mediated fluorogenic collagen cross-linking in a catalase-sensitive fashion, although it is unclear to what extent this occurs in vivo (77, 217). As is the case with other AGEs, the tissue burden of pentosidine is elevated in diabetics, as a consequence of hyperglycemia.

Pentosidine has been found to accumulate as a function of age in shrews, rats, dogs, cows, pigs, monkeys, and humans, yielding equivalently shaped curves in all cases (284). It is not clear, however, how glycooxidative modifications might contribute to degeneration. It has been proposed that cross-linking in cartilage is related to its decreased elasticity and relative resistance to proteolysis in old animals (9). However, the absolute amount of collagen pentosidine cross-links attained at death is much higher in long-lived than in short-lived species: 6–7 pmol/mg in 3.5-yr-old shrews, 15–18 pmol/mg in 25-yr-old monkeys, and 50–100 pmol/mg in 90-yr-old humans (284). In other words, it appears that the rate of pentosidine accumulation may merely be a measure of more rapid oxidative damage in short-lived species rather than an actual cause of dysfunction.

An intriguing twist to this story has been the cloning of a specific cellular receptor for AGE, called RAGE (receptor for AGE), that belongs to the immunoglobulin superfamily and is expressed by mononuclear cells and the vascular endothelium (277, 278). One of the effects of AGE binding by RAGE is the generation (in mononuclear cells) of intracellular oxidants, the activation of the oxidant-sensitive transcription factor NFκB, and the induction of downstream events linked to atherogenesis (279, 280). As discussed in section xv*B*, it has recently been shown that RAGE, which is highly expressed by microglial cells in the brain (142), is a receptor for amyloid β-peptide (Aβ). The RAGE binding of Aβ results in oxidant generation, implicated in the etiology of AD (293, 344).

Several amino acid residues in proteins are susceptible to oxidative modification, forming side chain carbonyl derivatives (317). The development of sensitive methods for the analysis of protein carbonyls by Stadtman and coworkers (181) enabled them to study oxidative modification in human brain tissue and cultured fibroblasts, and in rat liver. They found a two- to threefold rise in protein carbonyl content between young and old age, an increase from 10 to ~30% of the total protein pool (315). The increase was exponential and correlated well with decreased activity of the oxidation-sensitive enzyme glucose-6-phosphate dehydrogenase (G-6-PD). In comparison, the rise in protein oxidation in the mongolian gerbil was less dramatic, increasingly significantly in brain, heart, and testis, but not in kidney. As in human tissues, trends for the activity of G-6-PD correspond to the increased damage, falling in brain and heart but not in kid-

ney (300). Similar results have been reported in an insect model. An age-associated 2.5-fold increase in the protein carbonyl content of old versus young houseflies has also been documented (299), and as in humans, the increase occurs exponentially during the life span. The similarity of the degree and pattern of increase in insects and mammals is striking, considering the enormous difference in their MLSP (40 days vs. 100 yr). Moreover, protein carbonyl levels increase similarly in mitochondrial extracts from the thoracic flight muscles of these animals (303). Mitochondrial aconitase is particularly prone to oxidative modification during aging in vivo and was identified by the immunoblotting of housefly mitochondrial protein extracts with a monoclonal antibody designed to detect protein carbonyls (343b). Carbonylation of this key citric acid cycle enzyme increased in parallel with a decline in its activity.

Somewhat stronger evidence that protein oxidation may play a causative role in senescence comes from comparisons of "crawlers" versus "fliers" of the same aging cohort. Although the two groups share the same chronological age, crawlers are phenotypically senescent individuals that have lost the ability to fly and have a shorter remaining average life span than do fliers (e.g., 9.0 days vs. 13.3 days for 10-day-old crawlers and fliers, respectively). The protein carbonyl content of crawlers was 29% higher than that of fliers (299), reflecting their greater phenotypic age, as was the degree of carbonyl modification of mitochondrial (but not cytosolic) aconitase (343b). Humans suffering from Werner's syndrome, a disease characterized by premature senescence, are individuals whose phenotypic aging is also accelerated, and they too appear to have more extensive protein oxidation. Fibroblasts from Werner's patients of all ages have a level of protein carbonyls equivalent to that in 80-yr-old controls (233). In a creative study attempting to correlate protein oxidation to a physiologically relevant end point, it was shown that in old mice, interanimal variation in protein carbonyl content of two different areas of the brain (cerebral cortex vs. cerebellum) was associated with parallel interanimal variation in memory and motor function deficits (90).

Are protein carbonyls physiologically relevant, or are they merely markers? What are the actual consequences of protein modification? Unfortunately, there are few quantitative data with which to answer this question, although qualitative data exist. The fate of oxidized proteins may depend on the form of damage. For example, metal-catalyzed oxidation of G-6-PD by iron/citrate results in a thermolabile enzyme that is a better substrate for proteolysis than is the native enzyme (93). Rapid turnover of metal-oxidized G-6-PD may therefore proceed efficiently. On the other hand, G-6-PD modification by 4-hydroxy-2-nonenal, a lipid peroxidation product, also inactivates the enzyme but does not render the enzyme thermolabile or increase its degradation by proteases (322). This difference exists despite the fact that in both cases, the same lysine residue is affected. To make matters more complex, the cross-linking of G-6-PD multimers by 4-hydroxy-2-nonenal (which predictably results in a product with lipofuscin-like fluorescence) produces a molecular species that actually inhibits the multicatalytic protease (92). The physiological cost of protein oxidation is presently an unknown quantity.

The appearance of protein-bound 3,4-dihydroxyphenylalanine (DOPA) on •OH-damaged proteins has been characterized; when converted to a quinone, protein-bound DOPA can undergo redox cycling, generating O_2^-•. It has therefore been proposed that protein oxidation may contribute to the progression of aging not merely by the loss of protein function, but also by an acceleration of the flux of oxidants (61, 63, 64, 69, 101, 102).

The oxidative modification of DNA has also been studied in animals of different ages, with conflicting results. Although some studies have reported a modest increase in specific oxidative adducts, single-strand breaks, and abasic sites, others have been negative (23, 132, 156, 221, 337). The failure to detect an age-related increase in oxidative adducts by the analytical chromatographic techniques typically employed may have been due to the difficulty of working close to the limit of sensitivity (17). In fact, it has become apparent that the measurement of the adduct oxo8dG is frequently plagued by artifacts (29a, 44b, 127c, 156a, 248a) and that these may have compromised some published experiments. Of particular concern are measurements of oxo8dG in mtDNA (16), which have generally been higher than in nuclear DNA, but which may be particularly prone to artifacts associated with the analysis of small samples (16, 127c). Moreover, it is noteworthy that even among the highly variable published estimates of oxo8dG in mtDNA are values that are equivalent to the lowest measured values of oxo8dG in nuclear DNA (131a). Because of the small number of studies of mtDNA and the high variability between the measured values, it is not yet possible to conclude that mtDNA is, in fact, more heavily oxidized than nDNA. Encouragingly, alternative PCR-based methods for measuring oxidative damage have recently been used to compare oxidation of mtDNA and nDNA by exogenous oxidants, with the result that the former appears more sensitive than the latter (270a, 343a), although these studies could not quantify baseline values of damage. With methodological improvements, future experiments may be more conclusive. For instance, the use of single-cell gel electrophoresis (the comet assay) to measure single-strand breaks and abasic sites in whole rat hepatocytes in situ revealed a statistically significant 1.5-fold increase in old rats compared with young rats (131) (although this experiment did not distinguish between oxidative and nonoxidative damage).

In any case, even if the burden of oxidative adducts

does increase with age, there is virtually no information about the likely effect of oxidative DNA damage in vivo, apart from the knowledge that it leads to mutations and cancer. The fact that there is active DNA repair in postmitotic tissues (in which the danger of mutation due to replication is nonexistent), and that such repair is often targeted to transcribed regions of the genome, suggests that DNA damage itself interferes with gene expression and is not tolerated (116, 117). This important question deserves more attention.

C. Oxidative Depletion of Biochemical Pools

The oxidative depletion of molecules with increasing age has not been well documented in senescent animals, since the destruction of molecules does not often leave traces; luckily, some pathways of oxidative damage do leave biochemical fingerprints. The loss of integrity of lipid bilayers due to peroxidation is one of the most salient effects of oxidative damage (324) and results in the generation of aldehydes and alkanes. Unfortunately, these are not easily measured, the widespread use of the simple and nonspecific thiobarbituric acid test notwithstanding (109). Nevertheless, countless studies have reported an increase in thiobarbituric acid-reactive substances (TBARS) with age. Combined with other more reliable assays, these studies have demonstrated that there is a greater degree of lipid peroxidation in older animals (209). The measurements of exhaled ethane and pentane is a technique that has the advantage of being applicable to humans (165). Unlike lipofuscin and TBARS, which measure the size of a pool of destroyed molecules and require a tissue biopsy, the assay of exhaled hydrocarbons measures the rate of damage and is noninvasive. Breath pentane has been found to increase significantly with age in humans, suggesting that increased lipid turnover occurs with age because of peroxidation (161a, 208, 356). Refinement of the technique and elimination of the associated artifacts (314) should facilitate further testing of the free radical theory in humans.

The loss of activity of several oxygen-sensitive enzymes (G-6-PD, glutamate synthetase) has been reported in mammalian models of aging (315). In houseflies, a decline in G-6-PD, glutamate synthetase, and alcohol dehydrogenase activities has also been documented and coincides with a dramatic loss of protein sulfhydryls (3). Another commonly reported age-related loss is an increase in the ratio of oxidized to reduced glutathione, which may reflect a disruption of the cell's redox state (215, 273).

D. Age-Associated Trends in Antioxidant Defenses and Repair

What is the cause of age-related oxidative damage? It could result from less active antioxidant defenses and repair, but studies that have measured age-related changes in antioxidant defenses have generated conflicting results. Recent measurements of antioxidants in mongolian gerbils (300) and mice (215) are representative of the types of patterns that have been uncovered in many other studies (65, 248, 254, 263, 274, 301, 302, 305, 329). In various tissues of gerbils, there was not a consistent pattern of change; increases in SOD and decreases in GSH were observed, whereas GPX was equivalent at different ages and catalase increased or decreased, depending on the tissues and the age at analysis. In mouse brain, on the other hand, significant decreases in SOD, catalase, and GSH reductase were observed, although GPX levels were unchanged.

Another complication is that defenses are induced in response to stress. Therefore, a higher level may indicate better protection, or alternatively, greater need for antioxidant defenses due to an increase in oxidant generation. Studies of antioxidants in rat heart and skeletal muscles illustrate this point. In heart, decreases in cytosolic SOD and GPX and increases in mitochondrial SOD and GPX were noted in older animals, and several indexes of oxidative damage were also elevated (151). From these results, it was concluded that although overall myocardial antioxidant defenses were weakened in the older animals, they were induced in mitochondria as a compensatory response. In skeletal muscles, in contrast, increases were observed in both cytosolic and mitochondrial forms of all of the enzymes studied (150), despite the fact that indexes of lipid peroxidation were again elevated; in this case, it was concluded that both cytosolic and mitochondrial antioxidants were induced. The credibility of these hypotheses is not in question, but it is hard to see how they could be disproved. When these and similar studies of age-related antioxidant levels are combined, what remains is a confusing assemblage of ambiguous trends.

Of course, interactions between antioxidants are complex, which aggravates the problem. To avoid the problems posed by assays of individual antioxidants, aggregate measures of antioxidant defenses have been devised. A crude but integrative measure of antioxidant defenses, for instance, is the susceptibility of a homogenate to induced oxidation. X-irradiation of a whole body homogenate of houseflies results in a linear, dose-dependent increase in protein carbonyls. When homogenates of old and young flies are compared, the rate of induction of protein carbonyls by X-irradiation is 45% higher in 14- than 5-day-old flies. This suggests that the antioxidant defenses in older flies are less able to cope with oxidative stress. Moreover, the activity of G-6-PD, an enzyme known to be sensitive to oxidation, decreases upon X-irradiation of living flies, and does so to a greater extent in old than young animals (2). When this assay was applied to the gerbil samples described above, in which no overall change in antioxidants was seen, a clear difference be-

tween young and old tissues emerged. Whereas 6 krad of X-irradiation induced a 20–38% increase in protein carbonyls in 5-mo-old animals, it induced a 152–211% increase in 26-mo-old animals (300). Similarly, although synaptosomes from young and old mice contain equivalent amounts of ATP and GSH, those of old mice were far more sensitive to GSH depletion by the diethyl maleate than those from young mice (197). Lastly, reperfusion injury is a well-established model of oxidative stress associated with the reestablishment of blood flow following ischemia, and it causes greater oxidative damage to heart tissues of old rats than young ones (192a). The use of a polyclonal antiserum specific for adducts between lipid peroxidation end products and proteins detected such covalent modifications of mitochondrial proteins from old but not young animals, which was associated with a more dramatic loss of respiratory capacity in the former. Whereas the baseline mitochondrial respiratory parameters (before ischemia-reperfusion) did not differ between young and old animals, the administration of a physiologically relevant stress revealed a probable age-related decline in antioxidant defenses.

Another alternative to measuring absolute levels of antioxidants in old versus young animals is to investigate the ability of animals of different ages to induce antioxidants, an approach that has been applied to the analysis of SOD in the nematode *Caenorhabditis elegans* (59). Whereas in young animals challenge with hyperoxia or the redox cycling compound plumbagin resulted in an increase in SOD activity, in middle-aged or old animals it actually resulted in a net loss of activity.

What about repair of oxidative damage? Does its activity decrease with age? The bulk of evidence suggests that there is probably not an overall age-associated change in the intrinsic ability of cells to degrade damaged proteins (94, 270). Although a dramatic decrease in the activity of the oxidized protein-specific alkaline protease has been reported in old rat hepatocytes (318), no change in this activity was measured in the heart or brain of 25- versus 5-mo-old gerbils (300). In a separate work, a 50% age-related decline in a single activity (peptidylglutamyl-peptide hydrolase activity) of the hepatic multicatalytic protease was associated with its selective sensitivity (relative to the multicatalytic protease's other activities) to metal-catalyzed oxidation, suggesting that resistance to oxidants may (logically) characterize the proteases responsible for degrading damaged proteins (46).

Although the intrinsic protease activity may not decrease with age, there is evidence that repair of oxidized proteins may be less easily induced in response to an oxidative insult in old animals. For instance, exposure of young and old rats to 100% O_2 increased the content of protein carbonyls in both groups over a 48-h period. Between 48 and 54 h of exposure, however, alkaline protease activity was induced in young animals, with a corresponding decrease in protein carbonyls to initial levels. In old animals, on the other hand, no increase in activity was observed, and protein carbonyl levels continued to rise throughout the time course (318).

There is circumstantial evidence from mutagenesis studies that either antioxidant defenses or repair of oxidative DNA damage (or both) is less efficient in old mice. The induction of somatic mutations in mice by γ-irradiation is from 2.3- to 3.6-fold higher in old than in young animals, depending on the dose (99). The induction of mutations in young and old animals was reduced by feeding the animals a cocktail of dietary antioxidants, confirming that oxidants played a mutagenic role in these experiments. Therefore, the more pronounced induction of mutations in older mice is indirect evidence of decreased antioxidant defenses and repair (99). Later experiments employing peripheral lymphocytes from young and old human subjects resulted in similar results (100). The ability of human peripheral lymphocytes to repair oxidative DNA damage induced by H_2O_2 has also been found to be less efficient in cells from older donors (14).

Altogether, the results above suggest that older cells may be less able to prevent oxidative damage from occurring, and less effective at removing the damage once it has occurred. There is a clear need for more and better data about age-related trends in defenses and repair.

E. Age-Associated Trends in Oxidant Generation

The accumulation of oxidative damage could also result from an age-associated increase in the primary generation of oxidants, and some research suggests that this is the case. Generation of H_2O_2 and $O_2\bullet^-$ by isolated mitochondria and submitochondrial particles from 25-mo-old gerbils, for instance, is ~150–200% that of 5-mo-old animals (300), and that of aged rat heart (305) and brain (93a) has also been reported to be elevated. On the other hand, a recent study that paid specific attention to maintaining physiological substrate concentrations during in vitro mitochondrial incubations failed to detect an age-related increase in mitochondria H_2O_2 output (118). Recent measurements of oxidant generation in carefully isolated rat hepatocytes from young and old animals, employing an intracellular dye that fluoresces upon oxidation, have confirmed under conditions that preserve cellular integrity that cellular oxidant generation appears to increase (110). A similar increase in oxidant generation documented in flight muscle mitochondria of houseflies was associated with increases in the activities of every measured component of the electron transport chain except for the content of UQ (308), suggesting that an imbalance in electron transport may be the cause of aberrant reduction of oxygen. In another experiment, a population of chronologically identical 12-day-old flies was separated

into phenotypically older crawlers and younger fliers as described above; mitochondrial H_2O_2 generation was twice as high in the crawlers than in the fliers, reflecting their greater phenotypic age.

Interestingly, oxidative damage to mitochondrial membranes and proteins has itself been implicated in enhanced oxidant generation; exposure of isolated mitochondria to the free radical generator 2,2-azobis(2-aminopropane)dihydrochloride or the cross-linking agent glutaraldehyde resulted in mitochondria that were more able to generate H_2O_2 when fed exogenous substrate (308). When these in vitro studies are combined with the above evidence of increased oxidative damage, the picture that emerges is a potential "vicious cycle" of oxidative damage and oxidant generation.

V. INTERSPECIES COMPARISONS

All of the data discussed in the previous section identify age-related differences within a given species. A complementary, and in some ways more powerful approach, is to compare species that have different MLSP. Comparative biochemistry and physiology, inspired by the rate of living hypothesis, has played a key role in establishing the free radical theory. Several representative studies are described below.

A. Oxidative Damage and Maximum Life Span Potential

The accumulation of cardiac lipofuscin in the monkey correlates with its cumulative O_2 consumption, starting at sexual maturation (226). Comparisons between rodent, dog, and primate species also revealed a close correlation between specific metabolic rate and lipofuscin accumulation (224, 225). The accumulation of the glycooxidation product pentosidine is similar. Short-lived species such as the shrew (MLSP = 3.5 yr) or rat (MLSP = 3 yr), with high specific metabolic rates, accumulate pentosidine in collagen at a rate of ~0.3–0.5 pmol/mg annually. Longer-lived primates such as monkeys and humans accumulate pentosidine at about one-tenth as fast (284).

Interspecies comparisons of protein oxidation mirror these results. The protein carbonyl content of 15-day-old individuals of five species of fly correlated negatively with mean life span; the longest-lived species (*Drosophila melanogaster*, mean life span = 65.5 days) had roughly one-third the level of protein oxidation as shortest-lived species (*Phaenecia sericata*, mean life span = 29.5 days) (310). The content of protein carbonyls was 20% higher in the heart, and ~50% higher in the brain, of 3.5-mo-old individuals of *Mus musculus* (MLSP = 3.5 yr) than in 3.5-mo-old individuals of *Peromyscus leucopus* (MLSP = 8 yr) (304).

The excretion of the oxo8gua and oxo8dG in urine is a reflection of whole body oxidative hits to DNA (287). The validity of its use to measure in vivo oxidative hits is strengthened by recent study of 33 women, in which both O_2 consumption and excretion of oxo8dG were measured. There was a highly significant positive correlation (P = 0.00007, r = 0.64) between O_2 consumption and excretion of adducts (188). When the urinary output of the oxidative DNA repair products oxo8gua thymine glycol and thymidine glycol were compared in mice, rats, and humans, they were found to correlate with species-specific metabolic rate (1, 287).

B. Antioxidant Defenses and Maximum Life Span Potential

One version of the free radical theory proposes that the differences in life span between species are due to species-specific antioxidant capacity. Early work by Cutler (57) tested this association: MLSP correlated positively with SOD but negatively with catalase and GPX (57). Recently, other groups have revisited this hypothesis, with similar results. Comparisons between SOD, catalase, GPX, and GSH in brain, liver, and heart from mice, rats, guinea pigs, rabbits, pigs, and cows were carried out and revealed that *1*) SOD and catalase activities correlated positively with MLSP, *2*) GSH activity correlated negatively with MLSP, and *3*) GPX correlated positively with MLSP in the brain and negatively in the liver and heart (309).

Interspecies comparisons between more distantly related vertebrate classes found either no correlation or a negative correlation between antioxidants and MLSP. For instance, in the liver of fish, frogs, birds, and mammals, strongly significant negative correlations were found for catalase, GPX, and GSH, whereas all other measured antioxidants (SOD, GSH reductase, ascorbate, urate, GSH) failed to correlate with MLSP (190). When the same antioxidants were measured in brain and lung tissues of the same species, virtually identical results were obtained (12, 247).

Comparisons between two very closely related species of mice, the house mouse (*M. musculus*) and the white-footed mouse (*P. leucopus*), which lives twice as long, have revealed higher levels of SOD, catalase, and GPX in brain and heart extracts of the latter, with the differences in GPX being the most dramatic. Also, when brain homogenates of the two species were challenged with X-irradiation, protein carbonyls accumulated at a threefold higher rate in shorter-lived *M. musculus* than longer-lived *P. leucopus* (304).

Together, these results suggest that the evolution of longevity has not been associated with a clear-cut increase in antioxidant capacity, at least across the broad sweep of evolution. (The comparisons between *M. mus-*

culus and *P. leucopus* invite the speculation that the more recent radiation of long-lived species may be associated with the coordinate upregulation of antioxidant defenses.) In any case, what emerged from the comparative biochemistry of free radical defenses is a fascinating paradox: birds, which typically have much longer long life spans than rodents, have lower activities of antioxidants and higher metabolic rates. This paradox has inspired the interspecies comparisons of mitochondrial oxidant generation discussed in section V.

C. Generation of Reactive Oxygen Species and Maximum Life Span Potential

The rate of living hypothesis identified an inverse correlation between metabolic rate and life span, and the free radical theory supplied a convenient mechanistic link: the mitochondrial generation of oxidants. Appealing as this theory is, one of the problems with the rate of living hypothesis is the conspicuous lack of fit of a few groups, notably birds and primates. Both of these groups live longer than their specific metabolic rate would predict. As a result, the total lifetime oxygen consumption of these groups is quite a bit higher than other groups (e.g., during their MLSP, pigeons and canaries consume 465 and 1,222 liters O_2/g, respectively, whereas mice, rats, guinea pigs, and trout consume 77, 28, 48, and 26 liters O_2, respectively) (247, 248). However, it cannot be assumed that the generation of oxidants is a direct function of metabolic rate, since mitochondria of different species (or even tissues) may exhibit different rates of intrinsic oxidant generation. These lines of reasoning by two laboratories have led to a similar line of experimentation, the results of which suggest that longevity is associated with a lesser capacity for mitochondrial oxidant generation (13, 172, 173, 304, 310).

For example, isolated pigeon mitochondria from brain, liver, or heart consume from two- to threefold as much oxygen as isolated rat mitochondria from the same tissues. However, pigeon mitochondria generate only one-third to one-half as much H_2O_2 as rat mitochondria under the same conditions. As a result, the calculated percentage of O_2 converted to $O_2^{-}\bullet/H_2O_2$ by mitochondria from lung, liver, and brain is ~10-fold lower in pigeons than in rats. This lower rate of oxidant generation corresponds with the roughly 10-fold longer life span of pigeons (13). A second, independent comparison of mitochondrial oxidant generation by pigeons and rats found similar results (172). Detailed respiratory comparisons have shown that generation of oxidants from both complex I and complex III of the mitochondrial electron transport chain is lower in pigeon than rat (130a).

In fact, the earliest interspecies study of mitochondrial oxidant production compared liver mitochondria of five mammalian species and thoracic muscle mitochondria of two species of fly (311), and also found that longer MLSPs were associated with lower mitochondrial oxidant generation. The mitochondria from flies produced from 6-fold more to 300-fold more oxidants than those from mammals. In a more recent comparison of heart tissues of eight diverse mammalian species of widely varying life span, submitochondrial particles of the short-lived species were found to generate more $O_2^{-}\bullet$ than those of long-lived species, a property correlated directly with the concentration of CoQ_9 (coenzyme Q possessing 9 isoprene units in its isoprenoid tail) and inversely with CoQ_{10} (although experiments intended to demonstrate a direct relationship between CoQ_9:CoQ_{10} ratio and $O_2^{-}\bullet$ generation failed to do so) (178a).

Interestingly, it appears as if the concept of intrinsic mitochondrial radical generation may also partially explain longevity differences between more closely related species. Measurements of $O_2^{-}\bullet$ and H_2O_2 generation by isolated mitochondria from the mouse species *M. musculus* and *P. leucopus* revealed that the former, which live half as long as the latter, also generate 48–74% more $O_2^{-}\bullet$ and 300–500% more H_2O_2. [As is the case with rats and pigeons, long-lived *P. leucopus* has the higher specific metabolic rate of the two species (304).] Finally, a comparison of five species of dipteran fly whose mean life spans vary by twofold also showed that the rate of generation of mitochondrial $O_2^{-}\bullet$ and H_2O_2 correlated negatively with life span (310).

Together, interspecies comparisons of oxidative damage, antioxidant defenses, and oxidant generation provide some of the most compelling evidence that oxidants are one of the most significant determinants of life span.

VI. DIETARY RESTRICTION

Limiting the dietary intake of mammals is a well-established way to extend life span (340). Interestingly, early expectations that dietary restriction (DR) would lower metabolic rate per se have not been confirmed, and so if DR attenuates oxidative damage, it is not via a simple reduction in oxygen consumption (312, 339a). Rather, there is now convincing evidence that dietary restriction may act in part by decreasing oxidative stress and increasing antioxidant defenses and repair (352). For example, mice whose caloric intake was restricted by 40% (DR) compared with ad libitum (AL) fed controls, and who lived 43% longer on average, had a less rapid accumulation of protein carbonyls in brain, heart, and kidney. Moreover, the generation of H_2O_2 and $O_2^{-}\bullet$ by mitochondria and submitochondrial particles from DR animals was lower than from AL animals, and also increased less with advancing age. In this study, however, DR did not appear to enhance antioxidant defenses (305). In another study, 40% DR of

rats increased the activities of SOD, catalase, and GPX at older ages; free radical damage, as measured by TBARS and lipofuscin accumulation, was correspondingly lower (253). In a third study, focusing on mitochondrial oxidant generation and membrane properties of synaptosomal preparations, 40% DR reduced mitochondrial oxidant generation in both young and old samples and prevented the age-dependent decline in membrane fluidity, despite the fact that increases in the cholesterol-to-phospholipid ratio were common to both groups of animals (43). Other experiments have been recently reviewed (335, 352).

Dietary restriction also delays cancer incidence, which may be a reflection of fewer mutations induced by oxidants in DR animals. Dietary restriction has been shown to strengthen DNA repair (111), although few studies have focused specifically on repair of oxidative lesions. Not only caloric restriction, but also restriction of protein intake, can forestall the occurrence of cancer in rodent models. Although the mechanism by which protein restriction operates is not clear, enhanced protection against cellular degeneration, including oxidative damage, is a likely candidate. It is therefore interesting that the level of protein carbonyls in animals fed a low-protein diet was significantly lower than in animals fed standard lab food and that treatment of the animals with γ-irradiation induced a greater increase in protein carbonyls in high- than in low-protein animals (350).

VII. MANIPULATION OF RATE OF LIVING

The metabolic activity of caged houseflies can be reduced by limiting the space available for flight (low activity = LA vs. high activity = HA). It is expected that the LA flies should live longer due to lower O_2 consumption and generation of oxidants, and this is in fact observed. LA flies exhibit more than a twofold increase in both mean life span and MLSP. Measurements of protein carbonyls in 14-day-old LA and HA flies, which have not yet begun to die, revealed an elevation of more than 50% in HA flies relative to LA flies in both whole body extracts (299) and in mitochondria (303).

Under unfavorable conditions, the nematode *C. elegans* may undergo a morphological transformation to form a "dauer larva," a resting stage that does not feed, exhibits altered metabolic activity, and can survive for several times the MLSP of adult nematodes before returning to the adult stage. Interestingly, dauer larvae have also been found to have increased SOD activity (178).

VIII. MANIPULATION OF OXYGEN TENSION

Another experimental manipulation that modulates life span is growth under different O_2 tensions. The growth of houseflies in an atmosphere of 100% O_2, for example, markedly reduces their mean and MLSPs, and also increases the rate of accumulation of protein carbonyls in whole body extracts (299) and in isolated mitochondria (303). Similarly, elevated atmospheric O_2 decreased, and subnormal oxygen increased, the mean and maximum life spans of nematodes (138). Of course, the principal drawbacks of this type of experiment are its inapplicability to mammals and the fact that elevations or decreases in ambient O_2 in such species will likely be confounded by the overt pathology of hypo- or hyperoxia.

IX. SUPPLEMENTATION WITH DIETARY ANTIOXIDANTS

As one of the most intuitive approaches to testing the free radical theory, nutritional supplementation has been attempted with numerous species and compounds, with the result that mean life span has been extended in some instances (122). In mammals, results have been mixed. To cite recent studies, life-long oral supplementation of rats with UQ was without effect on mortality (189), whereas supplementation of the senescence accelerated mouse strain (see sect. XII) with "βCATECHIN," a commercial supplement with antioxidant activities (174, 349) resulted in life extension (175). Negative results from the feeding of antioxidants have been rationalized by arguing that many of the antioxidants fed to mammals interfere with mitochondrial respiration, and so the failure of antioxidant trials to extend MLSP may have been due to their toxicity (127). In a recent short-term feeding experiment, in contrast, the thiol donor *N*-acetyl-cysteine was found to markedly improve mitochondrial ETC complex activity in rats (214) and short-term feeding with a complex antioxidant-containing plant extract attenuated oxidative damage in rat mitochondria (272a). Experiments aimed at extending life span should probably be preceded by such short-term trials, to document the uptake, distribution, and biochemical action of the compounds.

Although no large-scale human nutritional intervention trials have been aimed specifically at the study of aging, a few have been undertaken in the hopes that dietary antioxidants might help prevent cancer. Three similar and heavily scrutinized studies are the α-tocopherol β-carotene (ATBC) prevention study (4), the physicians' health study (129), and the β-carotene and retinol efficacy trial (CARET) (236), all of which were designed to assess the effect of antioxidants on lung cancer risks. Although the details of these enormous experiments are not important to this discussion, the overall result was instructive: there was either no decrease or a modest increase in lung incidence and mortality with β-carotene administration.

These negative results, as well as negative results described above using laboratory rodents, should not be

taken as evidence that the free radical theory of aging is flawed. In fact, they prove merely that a complex organism like a human or rodent is unlikely to respond predictably to crude manipulations such as supplementation with one or a small number of compounds (22), as well as that a single end point (such as lung cancer) is not equivalent to aging. Since the time when the results of these human trials were announced, a number of explanations have been forwarded to explain the paradoxical promotion of cancer by β-carotene (252), which indicates that the ultimate value of these trials may be a more precise understanding of this antioxidant. Ultimately, what the entire experience should teach the field of molecular gerontology is that at least until the biochemistry of dietary and cellular antioxidants is better understood, dietary trials in laboratory animals or humans will remain unreliable tests of the free radical theory; molecular gerontology should focus on more instructive experiments.

X. ADMINISTRATION OF PHARMACOLOGICAL ANTIOXIDANTS

Dramatic antiaging effects have been observed by Floyd and co-workers (234) with the free radical spin trap *N*-tert-butyl-α-phenylnitrone (PBN). Initial studies used PBN as a spin trap to measure radical generation during ischemia-reperfusion injury of gerbil brain (234). During reperfusion, the appearance of protein carbonyls correlated well with the loss of glutamine synthetase activity and the appearance of PBN-dependent nitroxide radical. Interestingly, it was also discovered that pretreatment with PBN attenuated protein oxidation and loss of glutamine synthetase activity and reduced lethality.

In a subsequent study that investigated the effect of PBN on spontaneous protein oxidation, it was discovered that twice-daily administration of PBN to old gerbils (32 mg/kg) reversed age-related oxidative damage (34). For example, during normal aging of gerbils, the level of brain protein carbonyls increased 185%, and glutamine synthetase activity declined by ~35%. A marked drop in neutral protease activity was also observed. Old animals administered PBN for 2 wk, however, experienced a reversal of these trends: protein carbonyls dropped, and glutamine synthetase and neutral protease activities were virtually restored. This effect was reversible, since over a 2-wk period after cessation of PBN treatment, the oxidation of proteins increased, and the activities of glutamine synthetase and neutral protease fell. Most impressive, however, was the performance of the PBN-treated old gerbils in a test of short-term memory; whereas the old gerbils were twice as likely to make errors as young gerbils, the old animals treated with PBN performed as well as the young (34).

Although these preliminary results held great promise, not only for the study of aging, but also for its therapeutic treatment, subsequent experiments reported that the results were irreproducible (31, 32). Further follow-up work from a third laboratory confirmed the initial findings in gerbil brain, but at the same time found no effect of PBN in lowering the level of protein carbonyls in gerbil heart or mouse brain (75). The chronic treatment of aged rats with PBN was found to reverse age-related cognitive impairment (294). Finally, the administration of PBN from age 20 wk throughout the life of a short-lived strain of mouse [the senescence accelerated mouse (SAM)] increased its life span from 42 to 56 wk (an increase of 33%) (73). As is argued (in sect. IX) to be the case for experiments with dietary intervention, maximum worth will be derived from pharmacological experiments when they include not only end-point mortality measurements but more focused studies of cellular effects.

XI. IN VITRO SENESCENCE AND OXIDANTS

The growth of normal diploid fibroblast in vitro is limited; after a specific number of population doublings (PDs) characteristic of the species, tissue, and age of the donor (the "Hayflick limit," named for the phenomenon's discoverer), the cells cease replication, which culminates in a viable, nondividing population with characteristic "senescent" morphology (56). Research on such "replicative senescence," and the vigorous debate about its relevance to organismal aging, have been recently reviewed (30, 55, 56). For the purposes of this discussion, a few points are worth reiterating. *1*) There are numerous correlations between organismal MLSP and replicative senescence. Fibroblasts from species with shorter MLSP exhibit a shorter in vitro life span than cells from more long-lived species. Similarly, fibroblasts from older individuals possess a shorter in vitro replicative potential than those from younger individuals (30), although this generalization has been questioned (55). Moreover, cells from patients with Werner's syndrome, a disease of accelerated aging (see sect. XVII), also complete fewer PDs before "crisis," when replication ceases (30). *2*) In vitro senescence may be relevant to organismal life span in at least two ways. First, as one of the likely components of tumor suppression, cellular senescence is necessary (but not sufficient) for extended life span. In other words, a long life span (as in humans, for instance) requires the absence of cancer at younger ages, and this is achieved in part by growth arrest of preneoplastic cells by mechanisms that operate during in vitro senescence (30, 269). Support for this assertion are the observations that fibroblasts from rodents (which have a significant cancer incidence after 2–3 yr of age) spontaneously immortalize in vitro, whereas fibroblasts from humans (who are relatively free of cancer until much later ages) do not (30), and that immortalization is associ-

ated with inactivation of p53 and Rb, which are frequently mutated in neoplasms (107). Second, senescent cells appear to occur in vivo, as judged by enzymatic markers characterized in vitro (68). It has been suggested that even at a low frequency, such cells may exert a disproportionate deleterious effect on tissue physiology by dominant effects of aberrant cellular regulation (e.g., expression of inappropriate cytokines) (30). *3*) In vitro senescence has been closely associated with the shortening of telomeric DNA, at least in humans (56). In human somatic cells, the average length of telomeric DNA shortens by ~50 bp/PD in vitro, and by ~15 bp/yr in vivo (30).

Leaving aside the question of the relevance of replicative senescence, is there any evidence that oxidative damage contributes to it? As early as the mid 1970s, this hypothesis was tested, by culturing human fibroblasts under conditions of low O_2 partial pressures and supplementing culture medium with the antioxidant α-tocopherol, with somewhat equivocal results (10, 241, 242). During the 1980s, a handful of studies documented the increased sensitivity of late-passage cells to oxidant challenge (139, 140) and noted that hyperoxic exposure could shorten in vitro life span (140). Later studies, looking to PD-dependent changes in antioxidant defenses as a potential explanation for the increased sensitivity of late-passage cells to oxidants, failed to account for these observations (250), although an impaired ability of late-passage cells to reinitiate DNA synthesis after hyperbaric O_2 exposure was established (141). More recently, a loss of oxo8gua glycosylase activity with increasing passage number has been reported (133), suggesting that decreased repair may be involved.

Recently, the issue has been revisited, and it was shown that a short (2 h) exposure to H_2O_2 (200 μM) induces an abrupt senescent-like growth arrest in human diploid fibroblasts (40). Intriguingly, cells that resumed cell division were found to have a markedly attenuated life span; a short-term oxidative stress had resulted in a long-term loss of PDs. The inhibition of H_2O_2 growth arrest by a metal chelator suggested that a Fenton-derived •OH radical was involved, and it was proposed that oxidative DNA damage and its sensing by cell cycle checkpoint controls may be involved in the phenotype (40). Indeed, measurements of DNA oxidation indicated that senescent cells suffer greater oxidative DNA damage than young cells and that the addition of the antioxidant PBN to the culture medium prolonged the life span in a dose-dependent fashion (41). In these and other studies, it was shown that culture under low oxygen tension extended fibroblast life span (41, 355) and that the inhibition of catalase decreased it (355), implicating endogenous H_2O_2 in replicative senescence.

Interestingly, lipofuscin accumulates in fibroblasts in vitro as well as in vivo, and culture under mildly hyperoxic conditions (40% O_2 partial pressure) decreases life span and stimulates lipofuscin accumulation (333). Direct feeding of synthetic lipofuscin particles to fibroblasts, which phagocytose and accumulate the material, simulates senescence (333). Most intriguing, perhaps, is the revelation that 40% O_2 culture resulted in a rapid loss of telomeric DNA as well as other cytogenetic alterations characteristic of senescent cells, and a permanent loss of replicative capacity reminiscent of the growth arrest of cells with a pulse of H_2O_2 (330a, 334). The measurement of DNA single-strand breaks in telomeric sequences of such cells revealed an increase under hyperoxia (334), confirming the idea (40) that DNA damage by oxidants may be involved in replicative senescence in vitro and extending the model to embrace the theory of telomere shortening (334). Interestingly, it has also been recently shown that the repair of ultraviolet light-induced pyrimidine dimers in telomeric DNA is lower in fibroblasts from an old donor than a young donor (171).

Together, these experiments are consistent with the hypothesis that oxidative DNA damage, acting to shorten telomeric sequences and thereby induce DNA damage-responsive growth arrest controls (107), plays a role in in vitro senescence. Two recent studies permit a further speculation. It has been shown that overexpression of the oncogenic ras gene in primary cells, which initially serves as a strong mitogenic stimulus, rapidly induces premature senescence in a p53- and p16-dependent fashion (286). Interestingly, it has also been recently reported that ras induces cellular oxidant generation (147). It is tempting to suggest that the "aggressive mitogenic stimulus" (286) of oncogenic ras, credited with inducing senescence, may be oxidative in nature. There may be a role for oxidants in replicative senescence.

XII. CLASSICAL AND POPULATION GENETICS

In comparison to the measurement of age-related changes, genetic approaches have the potential to provide more definitive information about aging, by supplying model organisms (mutant strains with altered life span) that differ at a small number of loci. Many of the geneticist's favorite organisms have been found to be amenable to studies of aging and have furnished evidence of the role of oxidants (196, 289).

A. *Caenorhabditis elegans* Genetics

The emergence of *C. elegans* as a convenient metazoan genetic model has attracted scientists seeking to discover genes controlling life span, or "genontogenes" (152, 158). Several long-lived mutant strains of *C. elegans* have been identified, including *age* mutants (the originally identified phenotype being extended life span), *daf* mutants (identified by abnormalities in the formation of

dauer larvae), and *clk* (clock) mutants [which exhibit a pleiotropic behavioral deceleration (slower feeding, defecation, and movement)] (158). These two classes of mutant have collapsed into a single category with the cloning of *age-1* and its identification as an allele of *daf-23* (219). Strains of *age-1* live 65% longer on average and have a MLSP 110% longer than wild type (153). The trait is recessive, and biochemical studies have revealed that strains carrying *age-1* alleles have enhanced oxidative defenses. For example, when the sensitivities of wild-type and *age-1* strains to H_2O_2 were compared, it was found that the 50% effective lethal dose (LD_{50}) of the wild-type remained constant, whereas the LD_{50} of the *age-1* mutant increased with age, an indication of increasing resistance to oxidants (178). Similar comparisons showed that *age-1* mutants are more resistant to $O_2^{-}\bullet$ as well (330). In both cases, greater resistance to oxidants is associated with elevated activities of SOD and catalase. Whereas in wild-type animals these activities remained constant throughout the life span, in *age-1* strains they increased with advancing age, such that the difference between the mutants and the wild type grew increasingly pronounced at old ages (178, 330). In addition to being resistant to oxidants, *age-1* strains have increased heat tolerance, so it is likely that enhanced ability to cope with all environmental stresses, including oxidative ones, is the result of the loss-of-function mutation (185).

A comparative study of *daf-2* and *age-1* mutants revealed that they lie in a common pathway. Life extension by either requires the action of wild-type *daf-16*, and their effects on life extension are not additive (72), and so it was reasoned that they work in tandem. Their cloning revealed that *daf-2* is a member of the insulin receptor family (160a) and that *age-1* is a catalytic subunit of phosphatidylinositol 3-kinase (219), suggesting that together they transduce an insulin-like metabolic signal with pleiotropic effects on metabolism, stress resistance, and dauer formation (127b, 291a). The more recent cloning of *daf-16*, revealing that it is a member of the hepatocyte nuclear factor 3 (HNF-3)/forkhead family of transcriptional regulators (183a), has provided insight into the entire pathway, since in humans, insulin signaling antagonizes HNF-3. The implications, then, are *1*) that signaling through *daf-2* and *age-1* ultimately may serve to antagonize the control of a genetic program controlled by *daf-16*, and *2*) that this pathway may be relevant to human aging, since it appears to have been conserved (183a). Of course, the connection of these proteins to the aerobic respiration and the rate of living, via insulin-mediated metabolic control, has escaped no one's notice (183a).

The cloning of the *clk-1* gene also implicates respiration rate in *C. elegans* life span, albeit indirectly. The gene is homologous to a yeast gene called *CAT5/COO7* (79a), which is necessary for the genetic control of the shift from anaerobic to aerobic metabolism (127b), as well as the biosynthesis of the mitochondrial electron transport component UQ (252a). It has been proposed that the decelerated metabolic rate in *Clk-1* mutants of *C. elegans* may result in a correspondingly slower rate of oxidant generation (127b), although the authors of this work note that so far data are merely consistent with such a proposal.

A mutant that is in some ways the opposite of *age-1* resulted from a screen for sensitivity to $O_2^{-}\bullet$ in a chemically mutagenized population of *C. elegans*. The resulting mutant, *mev-1*, is hypersensitive to both paraquat and hyperoxia. The activity of SOD in *mev-1* was found to be roughly one-half of wild type, and the average life span was reduced by ~35% (148). Lipofuscin-like fluorescence accumulated more rapidly in *mev-1* mutants that in wild-type animals (144). The life span of both *mev-1* and wild-type nematodes was lengthened under hypoxia and shortened by hyperoxia, but the effect was more dramatic for the mutants (138).

B. *Drosophila* Genetics

According to the evolutionary concept of antagonistic pleiotropy, natural selection favors traits that benefit organisms during their period of reproduction. Therefore, by imposing a strong artificial selection that consistently rewards females capable of late reproduction, one ought to be able to select against those pleiotropic traits that are most antagonistic to long-term survival. After a suitable number of populations, a long-lived phenotype should emerge from a large, heterogeneous population.

In the early 1980s, Rose (264) followed precisely this strategy using *Drosophila* thereby validating the concept of antagonistic pleiotropy and also providing five independently selected lines (referred to as lines O1-O5) for further experimentation. The strength of this model is that, unlike *age-1*, lines O1-O5 are not single gene mutants, but rather populations in which any number of allelic changes will have occurred. Hence, biochemical alterations that are found to have arisen independently in many of these lines are likely to reflect the type of complex polymorphisms that confer increased life span to long-lived species such as *Homo sapiens*.

Therefore, it is significant that in a comparison of the lines O1-O5 with lines B1-B5 (control lines not subjected to selection), all of the long-lived populations had acquired a higher frequency of a relatively rare SOD allele that is associated with higher in vitro activity (328). In *Drosophila*, there exist two electrophoretic alleles of SOD, designated F (with lower in vitro activity) and S (with higher in vitro activity). Whereas the genotypes of all B flies tested were FF (no S alleles were detected), both FS and SS individuals existed in all five O lines, such that the average allele frequency of S ranged from 0.10 to 0.28 [0.25 ± 0.05 (SE)]. In all five independent instances of laboratory evolution, SOD activity had increased.

C. Rodent Genetics

The senescence-accelerated mouse (SAM) is an increasingly important model in gerontology (244); the senescence-prone strain (SAM-P) has a mean life span of 9 mo, compared with 13 mo for a senescence-resistant (SAM-R) strain. In liver tissues, the activity of mitochondrial SOD in the SAM-P strain was about one-half that in the SAM-R strain, a result that was consistent at all ages examined (244), which could explain the more rapid age-related increase in lipid peroxidation seen in this tissue (244) as well as independent reports of enhanced oxidative stress in SAM-P mice (186).

A potentially very useful animal model for testing the free radical theory of aging is the "S strain" of Wistar rat, selected for sensitivity to cataractogenesis by galactose. A heritable increase in cellular hexose uptake by cells from the S strain is associated with an increased intracellular generation of oxidants, increased endogenous lipid peroxidation, mitochondrial dysfunction, an increased age-specific incidence of degenerative diseases, and earlier aging than the galactose-resistant R strain (271, 272). Moreover, SOD and catalase activities in the blood of S rats are one-half that in R rats, which may have explained the increased in protein oxidative damage that was also seen in the S strain (345).

XIII. MOLECULAR GENETICS

A. Oxidants and Nuclear Somatic Mutations

Oxidants are twice removed from the somatic mutation theory of aging, since two requirements need to be satisfied for the relevance of oxidants to be ensured: *1*) oxidants must account for a significant proportion of mutations, and *2*) mutations must account for a significant proportion of the phenomena of aging. The second criterion is complex and has been recently discussed (71a, 195, 218, 338b); suffice it to say that to the extent that cancer is a degenerative disease of age (6), there is little doubt that mutations are relevant. What remains to be seen is whether or not the age-related degenerative changes that do not involve neoplasia are also the result of mutations (79) (or epigenetic alterations; Ref. 136).

With the assumption that they are, the question then remains, To what extent are spontaneous mutations due to oxidants? Some of the best evidence so far of the relevance of oxidative mutagenesis in vivo is an argument by analogy. In *E. coli*, mutation rates can be elevated from 10- to 1,000-fold by the loss of mutator genes *mutM*, *mutT*, and *mutY*, whose gene products are involved in repair and prevention of oxidation of guanine in DNA (210). Significantly, homologous genes and novel genes with analagous activities have been found in a number of species including humans (17, 192, 266), which forcefully makes the case that oxidants are significant mutagens in vivo. There is, moreover, plentiful direct evidence of oxidative mutagenesis in eukaryotic cells, such as the demonstration that disruption of the metallothionein gene in tissue culture, which may abrogate the cell's ability to chelate redox active metals such as iron, results in a 5- to 10-fold increase in spontaneous mutagenesis (267). Also, mutagenesis in cells from humans with Fanconi's anemia, a condition associated with oxidative stress, displayed a higher mutation frequency than controls and a marked increase in mutation frequency in response to the elevation of O_2 partial pressure (183).

Despite these results, the proportion of mutations due to oxidants in the presence of wild-type antimutators is still an unknown quantity. Recent experiments have illustrated that dietary supplementation with antioxidants, which reduces irradiation-induced mutagenesis, appears to reduce the spontaneous mutation rate as well (99, 100), suggesting that antioxidant scavenging in vivo is incomplete. The quantitative contribution of oxidants in somatic mutagenesis is an open (and important) question.

B. Oxidants and Mitochondrial Somatic Mutations

A burst of activity was stimulated by the report that a large deletion of the mitochondrial genome (ΔmtDNA) accumulates exponentially with age in humans and by the speculation that this may be the work of mitochondrial oxidants (53). Since this first detection of ΔmtDNA in normal elderly humans, dozens of studies have confirmed the finding, detecting the same "common" deletion in humans as well as different deletions in other species, including *C. elegans* (206), mice (44, 323), rats (76), and rhesus monkeys (179). Increased frequencies of ΔmtDNA in brain samples have been associated with Huntington's disease and AD, degenerative diseases of aging that affect millions of people (48, 143).

In most of these cases, a significant accumulation of the deletions occurs only at advanced ages and is associated primarily with postmitotic tissues. This mosaicism of mtDNA deletion (313), which is reminiscent of the pattern the maternally transmitted mitochondrial diseases, is hypothesized to be partly due to the lack of proliferative competition in postmitotic tissues (50, 336). Whereas in dividing tissues cells that inherit a relatively greater percentage of ΔmtDNA molecules by unequal mitotic assortment stand to compete less well in subsequent rounds of cell division, this "phenotypic sieving" will not operate in a nondividing tissue.

There are reasons to believe that the deletion of mtDNA may stem from oxidative damage (51). For one, the steady-state burden of oxidative adducts in mtDNA has been measured to be 16-fold higher in rat mtDNA than in

nuclear DNA (16, 260). Moreover, quantitative polymerase chain reaction analyses of ΔmtDNA accumulation in different areas of the brain have consistently revealed highest frequencies of areas of high metabolic activity (47, 54). Despite the promise of these results, it is not self-evident that the observed accumulation of ΔmtDNA represents more than a biomarker (182). For one, the highest frequencies of ΔmtDNA detected, even at the oldest ages and in the most deletion-prone tissues, are generally no higher than 0.1%. Because in maternally transmitted mitochondrial genetic disorders symptoms are often absent even when more than one-half of the mtDNA molecules carry deletions (336), it is hard to see how such low frequencies could exert an effect. On the other hand, because there may be numerous deletions existing independently, measurements of a single one may merely represent the "tip of the iceberg" of mtDNA damage (54). The use of "long polymerase chain reaction" to amplify the entire mitochondrial genome has revealed the presence of numerous mtDNA rearrangements in human skeletal muscle (207). On the other hand, a recent attempt to measure an increase in somatic point mutations in human skeletal muscle was unsuccessful (243).

The analysis of progressively smaller bundles of cells has shown that the ΔmtDNA frequency is unevenly distributed (282). For example, when a muscle homogenate of rhesus monkey fibers is analyzed, the detected frequency of ΔmtDNAs is 0.02–0.1%. However, restriction of the sample size to 10-fiber bundles decreases the number of positive reactions but increases the measured frequency of ΔmtDNAs in positive reactions to 4.6–13.2%. In other words, a frequency of 0.1% could represent 100% deleted molecules in 1 cell of 1,000 (180). At any given point in time, only a small number of cells may carry deletions, but if these cells continually die as a result of a failure of oxidative phosphorylation, and other deletions arise de novo, then the low steady-state frequency of deletions could obscure a high rate of ΔmtDNA-associated cell death.

Finally, it has been proposed that a vicious cycle of increasing oxidative damage and stress of the type discussed in section II*F* is particularly likely in the case of mtDNA, since it encodes components of the mitochondrial electron transport chain (52). This hypothesis is supported by the fact that in statistical terms, a random mutation of mtDNA is most likely to affect complex I, combined with the observation that a complex I poison (MPTP) triggers neuronal cell death in mice (52). It remains to be shown that known somatic mtDNA mutations do, in fact, result in increased oxidant generation.

XIV. TRANSGENIC ORGANISMS

A. Transgenic *Drosophila*

The modern acid test of a biological theory is the generation of a transgenic organism, and recent efforts to genetically engineer long-lived *Drosophila* by bolstering antioxidant defenses have been successful. The first such efforts involved the introduction of a bovine cDNA for Cu,Zn-SOD into *Drosophila*, which resulted in a significant increase in SOD activity as well as resistance to oxidative stresses and a small, statistically significant increase in mean life span (85). Whereas a repeat of this procedure with the *Drosophila* Cu,Zn-SOD gene resulted in only marginal effects on resistance to oxidants, and failed to increase life span, the introduction of single copies of *Drosophila* SOD and catalase resulted in a number of strains with significantly extended mean and maximum life spans (237).

As expected, the augmentation of antioxidant activities in the transgenic flies led to significant improvements over controls in numerous age-related indexes of oxidative metabolism: *1*) the accumulation of protein carbonyls was reduced; *2*) the rate of oxygen consumption at middle age and old age was higher; *3*) the ~50% loss of G-6-PD activity with age was reduced to ~10%; *4*) the age-related doubling of the load of oxo8dG was virtually abolished, as was the roughly twofold increase in sensitivity to the induction of oxidative DNA damage; and *5*) the age-related threefold increase in mitochondrial oxidant generation was reduced by about one-half (237, 298). In short, the predictions of the free radical theory were confirmed.

B. Transgenic Mice

Ultimately, transgenic mice are the molecular gerontologist's Holy Grail, and it is not until similar experiments are performed in mice that the free radical theory will have been satisfactorily tested. Although such experiments will clearly be exciting, work that has already been performed with mice transgenic for SOD suggests that their phenotypes may not be straightforward. As has been stressed, antioxidant defenses are interactive and coordinately regulated, and it has become apparent that oxidants play important roles as signaling molecules (286). As a consequence, up- or downregulating a single antioxidant may disrupt an internal balance and either exacerbate oxidative stress or otherwise compromise cellular physiology. Indeed, the phenotype of Cu,Zn-SOD-overexpressing mice appears to mimic the pathology of Down's syndrome (for which they may be a model, due to the localization of Cu,Zn-SOD to the trisomic chromosome 21; Ref. 36). Moreover, it has been recently discovered that a complete knockout of Cu,Zn-SOD has a phenotypic effect only on a small subset of motor neurons thought to be involved in amyotrophic lateral sclerosis (255). Even more surprising, the recent elimination of the enzyme GPX in a transgenic knockout mouse model resulted in generally normal, fertile mice without overt signs either of oxidative stress or increased sensitivity to oxidants

(134). Together, these results indicate that the promising results with *Drosophila* may be difficult to replicate, although no matter what the results, antioxidant overexpressing strains will clearly be extremely useful. By crossing animals with different transgenes, it will be possible to dissect antioxidant interactions and their effects on aging.

XV. SPORADIC DEGENERATIVE DISEASES

In the form of the specific age-related diseases in elderly humans, there exists a natural laboratory of oxidative stress. Indeed, investigation of the etiologies of human degenerative disease implicate oxidative stress in cancer (5, 6), atherosclerosis (112, 343), diabetes (326), and neurodegeneration (15, 18, 293), to name a few.

For example, the role of oxidants in AD has been a particularly active area of research in recent years. It has been known for some time that the formation of amyloid deposits, a hallmark feature of the clinical diagnosis, is associated with aberrant processing and folding of a protein called amyloid β-precursor protein (AβPP) to form the amyloid plaques (Aβ) characteristic of the disease (283). In recent years, it was discovered that not only the in vitro formation of synthetic Aβ from AβPP can be accelerated by the presence of oxygen but also that Aβ, which is cytotoxic when fed to neurons in vitro, appears to stimulate the generation of oxidant (293). In fact, amyloid peptides themselves generate oxidants in vitro (130). As discussed above, it has also been recently discovered that a cellular receptor (on microglial cells and endothelial cells) responsible for transducing the cytotoxic signal from Aβ and inducing oxidant generation is none other than RAGE (344). Amyloid plaques have also been found to bind redox-active iron, providing another potential positive feedback loop for oxidative damage (292a). Although we do not have space to go into the details, what is relevant here is not so much the specifics but the realization that in numerous ways, oxidants act as effectors in the progression of the disease. In other words, research into the etiology of AD (193b, 203a, 292b) [and also Parkinson's disease (15, 44a, 145a), amyotrophic lateral sclerosis (346), and dozens of other age-related disorders] is revealing that cytotoxicity very frequently involves oxidative stress.

Even if oxidants turn out to be essential effectors of AD pathology, can they initiate the process? Recently, it was claimed that a higher degree of heteroplasmy of mtDNA is associated with individuals with AD versus controls (62), consistent with a genetic predisposition based on mitochondrial inheritance. What is intriguing in this work is the reported association of these AD polymorphisms with higher intracellular oxidant generation by mitochondria isolated from the patients (62). However, recent work by two groups has independently revealed an artifact in the unusual protocol used for mtDNA extraction in these experiments: a nuclear pseudogene, rather than mtDNA itself, was amplified, and so the conclusions from these studies are invalid (131b, 336a).

XVI. INHERITED DEGENERATIVE DISEASES

Werner's syndrome (WS) is a segmental progeroid syndrome of late-onset accelerated aging, associated with an increased incidence of cancer, attenuated replicative senescence of cells in vitro, and, interestingly, an elevation of oxidized protein in isolated fibroblasts (315, 354). The cloning (354) of the gene responsible for the syndrome (*WRN*) does not suggest an oxidative etiology, however, as the protein product WRN appears to be a helicase (106a). *WRN* is homologous to the RecQ helicase of *E. coli*, which is involved in unwinding DNA during repair; hence, it has been proposed that the loss of helicase activity in WS results in a deficiency in recombination-mediated repair in humans cells (106a, 291a). Inefficient or dysregulated DNA repair, in turn, may cause the chromosomal instability, which is one of the disease's hallmarks and which may be the root of some or all of the diseases associated with the syndrome (106a, 291a).

Although there is consequently no obvious causal link between mutations in *WRN* and oxidative damage, the increased protein oxidation in WS fibroblasts (315) allows the speculation that measurable oxidative damage may result from inefficient recombination-dependent repair, chromosomal instability, and an ensuing disruption of genetic programs. If this turns out to be the case, the oxidative phenomenology of WS will serve as an important reminder that the measurement of oxidative damage does not imply oxidative causation! [On the other hand, if normal aging, like WS, results in part from chromosomal instability, it may be that oxidative genotoxicity plays a central role (sect. XIII*A*). In other words, it may be that oxidative damage may both result from and result in chromosomal instability.]

XVII. EPIDEMIOLOGY OF OXIDANTS AND ANTIOXIDANTS

Nutritional epidemiology has suggested that dietary antioxidants are crucially involved in the prevention of degenerative disease (22), and global epidemiological studies have pointed to geographical and economic antioxidant deficiencies in their localized occurrence (6). For instance, the loss of immune function with aging is amenable to therapeutic treatment with antioxidants (208a); the fact that both antioxidant status and immunological decline vary between individuals (209) suggests that there may exist heritable difference in antioxidant capacities,

linked to differences in immune function. To date, however, the epidemiological study of senescence is merely a theoretical possibility and awaits powerful tools before it can be affordably attempted.

XVIII. SUMMARY

A. Are Oxidants Responsible for Aging?

We have outlined what we see to be the major experimental approaches to testing the free radical theory of aging. In Table 1, the strengths and weaknesses of different modes of experimentation are assessed. How powerful are different types of experiment? Has the free radical theory been supported?

Before a discussion of the merits of individual experiments, it should be stressed that if one is to judge them by their abilities to falsify the free radical theory, they are all bound to fail, for the simple reason that the free radical theory is very hard to falsify. The absence of a predicted outcome (for instance, life span extension by antioxidant supplementation) may often be explained by (justifiable) ad hoc reasoning. The physiology of oxidative stress, being a complex interaction of endogenous and exogenous factors, generally permits the "explanation" of negative results. How then shall one judge the different types of experiments and the theory itself? In fact, as the results discussed above illustrate, the momentum gathering behind the free radical theory is not due to any single experiment or approach, but rather derives from the extraordinarily multidisciplinary nature of current research. Although no single line of reasoning alone permits definitive conclusions, together they present a compelling case. [This philosophy, that the study of aging should employ a combination of different approaches, has been convincingly espoused before (83).]

Age-related oxidative phenomenology continues to provide evidence that senescence is associated with increased oxidant generation, a decline in the robustness of defenses and repair, and an accumulation of end products of oxidative damage (sect. IV). Although these trends are only correlations, the study of age-related changes has been rewarded with surprises such as the receptor for AGE (RAGE), which turns out to be involved in the etiology of AD and evidence that lysosomal lipofuscin may directly disrupt cells in vivo and in vitro. In fact, oxidative phenomenology has focused efforts on understanding the interplay between the components of oxidative stress outlined in Figure 1. The search for evidence of age-related DNA and protein oxidation has stimulated research into DNA repair, proteolytic salvage pathways, mitochondrial defenses, and so on.

Interspecies comparisons have better potential directly to test the free radical theory (sect. V). As predicted, differences between long- and short-lived species have been documented in all components of oxidative stress. Perhaps most instructive, however, have been results that initially appeared contradictory, such as the fact that birds are an outlier group in rate of living calculations. Attempts to account for the discrepancy have led to promising ad hoc hypotheses about the role of mitochondrial oxidant generation in aging. Unfortunately, relatively few researchers are familiar with or prepared to handle a variety of experimental animals, despite the research potential of a diverse menagerie.

In contrast, the model of dietary restriction has appeal precisely because it is so well established, homogeneous, and relevant to specific human diseases (sect. VI). Although early predictions that DR would result in a lower metabolic rate have proved unfounded (312), the discovery that oxidative defenses are enhanced by DR have strengthened both the free radical theory and the concept of antagonistic pleiotropy (158). Although, currently, results from DR studies are merely consistent with the free radical theory, future experiments may reveal specific pathways whereby DR upregulates defenses and repair.

Manipulation of metabolic activity and oxygen concentration are interesting systems, if of somewhat questionable relevance to normal aging (sects. VII and VIII). One drawback of such experiments is that they have generally been limited to invertebrates; the manipulation of metabolism in mammals is less straightforward. In the future, transgenic mouse models may allow a specific manipulation of metabolic rate in a consistent genetic background.

Attempts to manipulate life span with nutritional and pharmacological antioxidants obviously hold great appeal, as direct and intuitive tests of the theory (sects. IX and X). Although until recently they have not quite lived up to their promise (at least in mammals), this may have been due to a lack of the appropriate compounds. The serendipitous discovery of PBN's pharmacological properties appears to have opened up new avenues.

The study of fibroblast aging in vitro might appear the least likely area of research to support the free radical theory, being a phenomenon of replication per se, rather than of chronological aging (sect. XI). Nevertheless, the results discussed above not only suggest that oxidants may play a role, but they also have led to novel hypotheses about the dynamics of telomeric DNA in vivo. Future work in this area should be exciting.

The utility of genetics and transgenic animals in the study of aging, on the other hand, does not need elaboration (sects. XII and XIV). At present, the chief limitation to genetic studies is that fact that the adult forms of *C. elegans* and *Drosophila* are postmitotic. Molecular genetics (sect. XIII), which in the study of aging has primarily centered around the concept of somatic mutagenesis, is a field with particularly powerful tools that are generally applicable to any species. It is therefore very well suited to

comparative studies, as illustrated by the hunt for mtDNA deletions in a wide range of species. Moreover, because mutational spectra provide information about the primary mutagenic event (256), molecular genetic studies may find the "smoking gun" of oxidative mutagenesis.

Finally, the push to understand human sporadic and inherited diseases through biochemical, genetic, and epidemiological methods may ultimately provide the most detailed information about age-related oxidative stress, as is becoming apparent in the cases of cancer, atherosclerosis, and neurodegenerative disorders (sects. XV–XVII). The complete sequencing of the human genome, and modern high-throughput genetic methods, are poised to provide an unprecedented amount of genetic information about a biological sample of many millions of individuals whose life histories (medical records) are accessible. For instance, an approach to the genetic study of aging that is only now attracting mainstream interest is the study of "human longevity outliers," or centenarians (292). Technical advances in the identification and analysis of polymorphisms, fueled by the Human Genome project, may permit the study of oxidative stress-associated alleles in these extraordinary individuals. An example of such an approach is the identification of a coding mtDNA polymorphism (a C-to-A transversion at mtDNA nucleotide position 5178 in the NADH dehydrogenase subunit 2 gene) in a larger percentage of centenarians (62% with 5178A) than controls (45% with 5178A) (322a). In this study, it was also found that as the age of randomly selected hospital inpatients and outpatients increased, the percentage carrying the 5178C polymorphism increased, suggesting that such individuals are at higher risk of degenerative disease. The authors hypothesize that the 5178A polymorphism, which is rare in all human populations except the Japanese, may be responsible both for the longevity of Japanese centenarians and, therefore, for the general longevity of the Japanese population as a whole. They suggest that such a polymorphism, affecting the mitochondrion, may have an effect on oxidative stress.

The answer to the question of whether or not the free radical theory has yet been confirmed depends on one's conception of what the theory predicts. In its broader sense ("oxidants contribute significantly to the process of degenerative senescence"), the theory has clearly been validated. In the more strict sense of the theory ("oxidants determine MLSP"), the data are not yet conclusive, although a large body of consistent data has been generated.

B. Nitric Oxide

A topic of great importance, which we have here largely omitted, is the role of nitric oxide (NO•) both as a damaging oxidant in its own right and as a signaling molecule that is susceptible to oxidative scavenging. NO•, the chemical previously known as endothelium-derived relaxation factor, is generated enzymatically by isozymes of nitric oxide synthase and is involved in vascular tone, innate immunity, and neuronal signal transduction. Moreover, NO reacts with O_2^-• to form peroxynitrite ($ONOO^-$), itself a powerful oxidant. Therefore, not only must nitric oxide synthase be seen as a potential source of damaging oxidants (249, 281), but O_2^-• may now be considered a physiologically relevant scavenger of the free radical signaling molecule NO• (58).

C. Oxidants and Apoptosis

Another topic we have decided to omit, because of space limitations and because this is an extremely fast-moving and frequently reviewed area, is the role of oxidants and mitochondria in apoptosis. Suffice it to mention that in the previous year there have appeared dozens of experiments documenting either the loss of mitochondrial membrane potential (355a), the release of mitochondrial cytochrome *c* (255a) [or an "apoptosis inducing factor" (355a)] into the cytosol, or the generation of oxidants by mitochondria during apoptosis (355a). Combined with the mitochondrial localization of the Bcl-2 family of anti-apoptosis proteins, and copious evidence that oxidant challenges can induce apoptosis (74a, 154, 290a), these experiments have shown that mitochondria are central to the process of cell suicide (255a, 355a). Despite this general consensus, however, a number of disagreements exist about the details of the mitochondrion's role in the process (149a) and even greater uncertainty about the role of oxidants. An equally complex problem will be defining the role of apoptosis itself in aging (338a). For instance, by eliminating neuronal cells, apoptosis may contribute to neurodegeneration, yet effective apoptosis is also clearly critical in preventing (age-related) tumorigenesis. In short, both the machinery and impact of apoptosis are still incompletely understood; conclusions about the interplay among oxidants, apoptosis, and aging will have to await further experimentation (338a).

D. Regulatory Roles for Oxidants and Antioxidants: Signal Transduction

The discovery that NO• could serve as a signaling molecule has foreshadowed a more general recognition that radical species are more than by-products. As mentioned in section XVIII*B*, the interaction between O_2^-• and NO• indicates that both may be regulatory molecules (58). In other ways, the relationship between oxidants and signaling is surfacing. For instance, the nitration of tyrosine residues on a synthetic peptide substrate of cell cycle kinases destroyed its ability to be phosphorylated, which illustrates a mechanism for an individual amino acid adduct to exert

an amplified effect (167). At a more physiological level, it has been shown that the sensitivity of hippocampal brain slices to muscarinic acetylcholine receptor agonists, which falls in an age-related fashion, can be restored by various antioxidant treatments and is potentiated (in old animals) by NO•-generating systems (155).

Perhaps most intriguing have been discoveries, such as the potential regulation of aconitases by O_2^-• (95, 98) and the realization that cell signaling via the Ras family of tyrosine kinases involves oxidants (147), that suggest that oxidants play a role in signal transduction by design. These discoveries strengthen the free radical theory for the following reason: if the regulated, enzymatic generation of oxidants plays a role in signaling pathways ("legitimate oxidants"), then even a small increase in oxidant generation by nonregulated pathways ("illegitimate oxidants") may have an exaggerated impact, by mimicking the legitimate process. The arrival of "redox regulation" as a viable field of inquiry, as evidenced by numerous topical recent reviews (66, 285, 321), may usher in a period when oxidants are seen as molecules of central physiological importance, in much the same way as are (for instance) lipid metabolites. As far as acceptance of the free radical theory is concerned, such a paradigm shift will probably lead to de facto acceptance of its broader conception (the idea that oxidants play a role in the process of aging). After all, if it turns out that oxidants and oxidative reactions are central to physiology, then how could they not be central to aging as well? If the free radical theory once lacked appeal because oxidants appeared to be stoichiometrically minor by-products, then those days may soon be over.

On the other hand, in its more narrow conception ("oxidants define MLSP"), the free radical theory still lacks experimental confirmation. A hypothetical example will illustrate this point. Even if mitogenic stimulation by Ras, for instance, were to involve oxidant generation in mice and humans, and the disregulation of Ras signaling by illegitimate oxidants in aged individuals were shown to be important in degenerative senescence, the different life spans of these organisms still beg an explanation; it still would remain to be explained why the process of oxidative disregulation takes so much longer to occur in humans than in mice. In conclusion, despite the growing concensus that the oxidants and oxygen free radicals are involved in degenerative senescence, countless mechanistic questions remain to be uncovered, as well as a central outstanding unknown: Do oxidants determine maximum life span in humans and other organisms?

We thank Caleb Finch for critically reading the manuscript and Irwin Fridovich for offering numerous detailed and useful comments. K. B. Beckman thanks Dr. I. Philipson for numerous useful discussions.

B. N. Ames was supported by National Cancer Institute Outstanding Investigator Grant CA39910 and National Institute of Environmental Health Sciences Grant ES-01896.

In writing a review on as broad a topic as the free radical theory, we have been forced to limit both the content and the number of references cited. Although we have done our best to include recent work, omissions were inevitable. We apologize to all authors whose work we have not managed to include and direct readers to other recent reviews for material we have left out.

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