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Oxidants and Antioxidants in Aging

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OXIDANTS AND ANTIOXIDANTS IN AGING

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INTRODUCTION

Many aging theories have been put forward to explain aging as a consequence of some specific cumulative damage process. Biological organisms have the capacity to minimize adverse chemical reactions and, for the few undesired reactions that do occur, to repair the damage or to compensate for its effects (e.g., by replacing an irreversibly damaged cell with a new cell). Hence the various formulations of cumulative damage theories must contend with the question of why repair is incomplete or why compensatory processes fail. One important consideration is that no repair system or compensatory mechanism can be absolutely efficient, particularly if the damage is so random that macromolecules are altered in virtually limitless variety. A wear-and-tear theory, which embodies the characteristic of unpredictable molecular alterations, is the free radical theory of aging. It was proposed by Harman in 1956 as follows: aging results from the deleterious effects of free radicals produced in the course of cellular metabolism.[1] Growing evidence supports an involvement of free radicals in life-shortening diseases, although efforts to use predictions of the free radical theory to achieve increases in maximum lifespans extension have met with limited success at best.[2] This chapter will critically assess the current status of the available evidence concerning free radical involvement in aging.

CHEMISTRY AND BIOCHEMISTRY

A. FREE RADICAL CHEMISTRY

Free radicals are molecules, rarely occurring in nature, that contain one or more "unpaired" electrons, sometimes also referred to as "free spins," in their outer shells. A free radical molecule containing a single unpaired electron is frequently denoted by R·,

where the dot refers to the unpaired electron, e.g., the hydroxyl radical OH·.

Free radicals rarely arise naturally because of the familiar chemical principle that valence electrons in atoms form chemical bonds, each of which consists of an electron pair. Energy must be supplied to break chemical bonds, and, usually, the resulting molecular fragments are free radicals. Once formed, these free radical fragments will combine rapidly with other free radical fragments, or with each other, to form non-radical products:

$$R\cdot + R'\cdot \longrightarrow R{:}R'$$

Hence, even if sufficient energy is available to break bonds, the proportion of free radicals among non-radicals is usually very low. Examples of reactive free radicals include:

- the very reactive hydroxyl radical (OH·), which is produced when ionizing radiation passes through water,
- the moderately reactive thiyl radical (RS·), which is formed when reactive radicals like the hydroxyl radical react with sulfhydryl groups, e.g., cysteine side chains in proteins,
- the weakly reactive nitric oxide (NO·) radical, which is found in polluted urban air.

Free radicals can pose a considerable hazard to biological systems because of their unique chemistry, which distinguishes free radicals from other toxic agents. The most damaging free radicals exhibit some or all of the following reaction patterns:

- attack other molecules indiscriminately,
- produce oxygen-consuming chain reactions, such that a single free radical effectively damages a large number of other molecules,
- cause fragmentation or crosslinking of molecules, including vital macromolecules like DNA and critically important enzymes.

Because of the random reactions that can occur, some of the products of free radical chemistry are completely foreign to the repair or turnover enzymes of the cell. For example, when two proteins become cross-linked, the product of this reaction may be resistant to attack by proteolytic enzymes. Hence, the usual metabolic turnover processes may fail to degrade such crosslinked molecules to provide amino acids for the synthesis of new biomolecules. Crosslinked products can accumulate progressively in cells, as in the example of age-pigments, which increase with age in the cells of animals (Chapter 5).

B. OXYGEN

Ordinary oxygen, also referred to as triplet oxygen, has two electrons with unpaired spins. Thus, oxygen can be thought of as a biradical. Oxygen is an example of a radical that is normally quite stable. However, oxygen can be extremely reactive in the presence of other free radicals, or, in collisions with molecules that are chemically very reducing. When an oxygen molecule collides with another free radical, the favorable energetics of electron pairing imply that a new addition product, called a peroxyl radical, will frequently be formed. If oxygen reacts with some nonradical molecule that has a sufficiently reducing chemical potential, a one-electron transfer to oxygen can occur, resulting in O_2^-, the superoxide radical. In such an electron transfer reaction the energy that is required to break one bond is recovered in the formation of another bond. An example of this reaction in the laboratory is the formation of superoxide radicals when molecular oxygen reacts with photochemically reduced flavins. Fortunately oxygen is inert towards most non-radical molecules so it cannot initiate free radical chain reactions. When oxygen reacts with an organic radical whose unpaired electron is associated with a carbon atom, the resulting peroxyl radical molecules are moderately reactive and will abstract hydrogen atoms from a variety of other molecules, leading to chain reactions and an accumulation of hydroperoxides. Such free radical chain reactions are familiar to food chemists, occurring readily in fats to cause rancidity. The facile occurrence of analogous

free radical chain reactions in biological membranes, referred to as lipid peroxidation, is a major oxidative damage mechanism in cells.

The chemical products of most of the free radical reactions involving oxygen are so reactive themselves to have earned many of them the designation "active oxygen" molecules. Several active oxygen species are of interest in biological damage processes. These include the free radicals:

- hydroxyl radical, $OH\cdot$
- superoxide radical, O_2^-

and the two non- radicals

- singlet oxygen, $^1O_2$,
- hydrogen peroxide, H_2O_2

The most reactive is the hydroxyl radical -- virtually no biological molecule is immune to its attack. Hence, organisms have evolved elaborate defenses to ensure that the hydroxyl radical arises as infrequently as possible. Singlet oxygen, which may arises during peroxyl radical reactions, is also reactive, although not as reactive as the hydroxyl radical.

Hydrogen peroxide is of interest as a potential damaging species because, in the presence of freely dissolved or loosely bound iron or copper and mild reducing agents like ascorbic acid, it can decompose to produce hydroxyl radicals. Because of the destructiveness of hydroxyl radicals, hydrogen peroxide decomposition could be a major source of biological damage under conditions of iron or copper release from protein binding sites. Hydrogen peroxide can also interact with heme proteins to produce highly oxidizing products (ferryl species) that can initiate free radical reactions.

The superoxide radical is not very reactive chemically, but its lack of reactivity may

be offset by the abundance of this radical species in aerobic biological environments. It may exert its damaging effects by serving as a reductant for ferric ion, particularly when iron is sequestered within some proteins, thus releasing the ferrous iron into aqueous solution. Superoxide radicals react with each other or with appropriate reducing agents to produce hydrogen peroxide. The fact that no organisms can survive life in air without the superoxide dismutase protective enzyme (or high concentrations of free manganese ions in one exceptional microorganism) provides compelling evidence that superoxide poses a great threat to aerobic cells.

While endogenous active oxygen molecules may play a role in unavoidable aging, avoidable exogenous agents that either are free radicals or can become free-radicals after metabolic activation are linked to life-shortening diseases and may accelerate the basic aging process. Cigarette smoke is one of the best characterized of these avoidable agents.

C. CIGARETTE SMOKE

Free radicals are known to be present in cigarette smoke and to react with a variety of biological molecules.[3] Among free radicals in cigarette smoke are remarkably persistent organic free radicals in the "tar phase," which can be trapped as a dark brown material on glass wool and which can be extracted into ethanol (Fig 1). The persistence of these free radicals in ethanol suggests that they would also persist significantly in tissue fluids, possible exerting adverse effects far from the lung. Another type of free radical is found in filtered cigarette smoke, which is designated as "gas phase" cigarette smoke. For example, gas phase cigarette smoke contains 300 to 500 ppm nitric oxide ($NO\cdot$), a weakly reactive free radical and a potent vasodilator.[4] $NO\cdot$ is converted to the nitrogen dioxide radical ($NO_2\cdot$) in the presence of oxygen.[3] $NO_2\cdot$ is more reactive than is $NO\cdot$, e.g., with unsaturated lipids.[5] Cigarette smoke is known to contain hydroperoxides,[6] to generate hydrogen peroxide in aqueous solution,[7] and to stimulate peroxide production by leukocytes. Hydroperoxides de-

grade hemoglobin in vitro to products, including loosely bound iron, that catalyze free radical reactions.[8-10] High iron levels have been detected in smokers' lungs.[11]

Cigarette smoke is also known to contain powerful metal chelators, e.g., catechol, which would be expected to stabilize hemoglobin decomposition products and to bind iron that may be released during hemoglobin decomposition.[8] Metal binding to quinols can be particularly destructive to cells when the quinol-metal complex has a high affinity for DNA.[12] Intercalation of iron complexes into DNA can induce base modifications and strand breaks. This is a particularly important damage mechanism because it may not be responsive to antioxidants. In principle, transition metal ion complexes can catalyze free radical reactions at many other sites, e.g., in lipoproteins. Alpha-1-proteinase, whose inactivation is generally thought to play a role in the development of emphysema (Chapter 8), can be protected by antioxidants and reducing agents,[13] implicating free radicals in its oxidation.

D. ANTIOXIDANTS

Biological systems have evolved a multiplicity of defenses against oxidative attack. Indeed, the discovery of one defensive substance, the enzyme superoxide dismutase (SOD), has played a pivotal role in the explosive expansion of our understanding of oxidative damage in recent years. We now know that a considerable variety of proteins and smaller molecules are involved in protecting the cell against the threat of free radical reactions. In addition considerable energy expenditures are made by cells to repair such damage when the protective systems fail to intercept all of the endogenous and exogenous free radical initiators. In excess of forty enzymes are constantly repairing damaged DNA, and both lipids and proteins are being turned over continuously. Thus it seems that organisms pay dearly for the privilege of aerobic life.

Superoxide Dismutases (SOD's)

At least three different types of SOD's are now known. In mammals a copper-zinc enzyme is found in cells, excluding the intramitochondrial matrix which contains a manganese-containing enzyme. A number of microbes have a third type of SOD that contains iron at its active center. Concentrations of SOD's in cells are relatively high, e.g., in spinach chloroplasts 0.24% of the total soluble protein is SOD.

Catalase

This enzyme decomposes hydrogen peroxide and oxidizes low molecular weight alcohols and other reduced organic molecules using H_2O_2 as a hydrogen acceptor. Catalase is located exclusively in the peroxisomes of most cells. It is also found in red cells. Some humans lack this enzyme altogether yet seem to suffer no adverse effects under normal circumstances, possibly because of the existence of another peroxide-decomposing enzyme (see below).

Glutathione Peroxidase

Glutathione peroxidase and glutathione transferases also act on hydrogen peroxide but can also use organic hydroperoxides as electron acceptors. The most active form of these enzymes contains selenium and is found throughout the cell except for peroxisomes. The glutathione-S-transferases, reacts with organic hydroperoxides. Some 10% of the total soluble liver protein consists of these enzymes.

Vitamin E

Also referred to as α-tocopherol, vitamin E is a lipid-soluble vitamin that acts principally as a phenolic antioxidant by donating a hydrogen atom to lipid peroxyl radicals. The resulting vitamin E radical is relatively unreactive and hence free radical chains that could arise in biological membranes are terminated highly effectively by this antioxidant. A number of reducing agents, including vitamin C, can react with the

vitamin E radical to regenerate vitamin E. Therefore, vitamin E usually acts as a catalytic protective agent that transfers reducing power derived from cellular metabolism to membrane free radicals. If the concentration of lipid radicals is too high, the reduction of vitamin E radical cannot keep pace with vitamin E oxidation. Under these conditions the vitamin E radical is irreversibly destroyed.

Vitamin C

Also known as ascorbic acid, this widely used nutritional supplement is an effective aqueous antioxidant which can terminate free radical chain reactions by donating hydrogen atoms to a variety of free radicals. Ascorbate may play a role in protecting membranes by donating hydrogens to vitamin E radicals, thus regenerating the lipid antioxidant. Ascorbate can act as a free radical initiator in the presence of "free" iron or copper, however (note that iron and copper would usually be bound to small molecules or macromolecular surfaces in most cells and the term "free" is here used to distinguish such bound metal ions from enzyme-bound forms that are usually not accessible to peroxides).

Carotenes

Beta carotene is a potent quencher of singlet oxygen which acts by an energy transfer mechanism whereby the singlet oxygen molecule is converted to the relatively harmless triplet oxygen molecule, while the carotene molecule is promoted to its triplet state. Subsequently, the excited carotene releases its energy harmlessly as heat. In this reaction carotene acts catalytically, without being consumed in the reaction. Epidemiologically, carotenes, as are vitamin E and selenium, are correlated with good health, including resistance to cancer.

Uric acid

A striking difference between the higher apes and other animals is their relatively long lifespan. This longevity and the attribute of a large brain, which accounts for a large fraction of the total oxygen consumption, may be partially the result of retention of the antioxidant uric acid, which is a product of purine metabolism. Two evolutionary alterations have led to high tissue concentrations of urate: loss of peroxisomal urate oxidase and active reabsorption of urate from the kidney. In model experiments urate is an effective antioxidant and singlet oxygen scavenger.

CELLS, TISSUES AND ANIMALS

A. FREE RADICALS IN AEROBIC CELLS

Free radicals are produced in aerobic cells during normal oxidative metabolism.[14] Elaborate defensive systems wage a constant battle against active oxygen species in all aerobic organisms. A measure of the threat posed by oxygen radicals is that the antioxidant enzymes comprise a significant fraction of the total protein content of aerobic cells.

Most oxygen consumption in mammals occurs in their mitochondria. Although it is known that the mitochondrial respiratory chain operates as an array of enzymes that perform electron transfer reactions very similar to those occurring in free radical reactions, the structures of these enzymes ensure that no uncontrolled chemical reactions occur. In particular, no release of active oxygen molecules occurs from the site of oxygen binding -- cytochrome oxidase. However, despite the tightly controlled flow of reducing equivalents through the respiratory chain, some leakage of reductants from mitochondria does occur at sites other than cytochrome oxidase, causing release of superoxide radicals into the cell. In addition, other organelles and some soluble enzymes

appear to cause both univalent and divalent oxygen reduction. Of possible significance as a risk factor in fat consumption is beta oxidation of fatty acids in peroxisomes, which generates hydrogen peroxide. Estimates of hydrogen peroxide production from isolated subcellular fractions are set forth in Table 1, demonstrating that a variety of potential sources of active oxygen exist in most cells.

Cells of the immune system destroy invasive microorganisms (Chapter 7) by mechanisms involving active oxygen molecules. The destructive molecular species produced by immune system cells may sometimes contribute a substantial fraction of an animal's total oxidative damage burden. Of particular interest is the potential posed by these cells for amplifying the damage produced by free radicals. Increasing evidence shows that oxidative damage, particularly lipid peroxidation, generates chemotactic factors that attract leukocytes to an area of high free radical activity and that the resultant inflammatory process can lead to increases of free radical damage. Such processes are probably important factors in pulmonary damage associated with emphysema (Chapter 8) and pollutant exposure and in atherosclerosis.

It could be argued that the protective systems, as they have evolved in the higher species, are so effective that the accumulation of damage is negligible, and that aging arises from causes other than wear-and-tear. The gradual progress of the changes that occur with aging implies that highly sensitive assays of damage, coupled to sophisticated data analysis will be required to demonstrate a net accumulation of free radical damage, consistent with a progressive and irreversible decline of viability.

A possible involvement of free radical in aging is outlined schematically in Fig 2. This scheme identifies the major sources of superoxide radicals and other oxidants in the cell, protective systems that remove these oxidants and their major cellular targets. The

fundamental precept of the free radical theory of aging is that some reaction targets are not repaired and damage gradually accumulates, thus compromising cellular integrity. While oxidized lipids can be replaced, and most protein and DNA damage can be recognized or repaired, some types of DNA damage, such as loss of methylated bases, cannot always be repaired and will accumulate with age.

B. CELLULAR TARGETS OF FREE RADICALS

Membranes

Most cell membranes contain polyunsaturated fatty acids that are highly susceptible to free radical oxidation. As long as vitamin E is present, the extent of oxidation is controlled because the vitamin prevents the occurrence of chain reactions. Depletion of vitamin E can lead to considerable membrane oxidation, whose consequences can include major alterations of membrane structure, a release of lipid oxidation products, including cytotoxic molecules like hydroxynonenal, and an increasing permeability to ions. Lipid radicals react avidly with oxygen and membrane free radical chain reactions can potentially create an oxygen deficit with a concomitant loss of oxidative phosphorylation. The reduction of hydroperoxides by glutathione peroxidase consumes cellular reducing power and can also contribute to energy depletion. Thus it is not surprising that free radical damage is often associated with impaired bioenergetics.

Proteins

Some amino acids are readily oxidized. Cysteine reacts with many free radicals and even with hydroperoxides to form products that react with thiols to produce disulfides. Disulfides can subsequently be reduced again to repair the lesion, indeed, the formation of protein mixed disulfides with glutathione may serve a protective function. Oxidation of methionine produces the sulfoxide, which can be repaired by specific reductases. Many amino acids may be irreversibly oxidized by free radicals that are much less

reactive than the hydroxyl radical. The reactive amino acids include histidine, tryptophan, and tyrosine. Tyrosine oxidation can lead to bityrosine, a strongly fluorescent free radical damage marker that is implicated in irreversible protein crosslinking.

Heme proteins, including hemoglobin and myoglobin, react with hydroperoxides to form highly oxidizing species that can produce amino acid radicals within the heme protein or oxidize nearby molecules, e.g., initiate free radical chain reactions in membranes. Activation of hydroperoxides by heme proteins can also exert its damaging effects more indirectly, by inducing a release of iron from oxidatively cleaved porphyrins. The released iron can subsequently catalyze hydroxyl radical production or other reactions at iron binding sites. Among the site-specific reactions of protein-bound iron, the oxidations of histidine, proline and arginine side chains have been cited.[15]

Although most oxidized proteins are either repaired or degraded and replaced by new ones, these processes are not perfect and some altered proteins may persist for considerable periods, particularly in energy-deficient cells. A dramatic example of a persistent altered protein is seen in brain myelin protein. Racemized amino acids accumulate in this protein throughout life, indicating that these altered proteins are not turned over significantly (Chapter 8, 9).

An accumulation of oxidatively modified non-functional or dys-functional proteins, which are not recognized or are turned over at an inadequate rate, appears to play an important role in aging.[15] The observation that catalytic activity per unit of protein antigen decreases with age indicates that the persistence of altered proteins in cells is age-dependent. This may be due, in part, to a decreased activity of proteases involved in the degradation of altered enzymes.

Nucleic Acids

Base methylation plays an important regulatory role in gene expression and in the differentiation that characterizes multicellular organisms. A loss of methylated bases and dedifferentiation occurs with age and the resulting organismal dysfunctions may explain some of the phenomenology of the aging process.[16] Free radical oxidants hydroxylate and otherwise modify DNA bases at high rates.[17] The repair of oxidant-modified methylated bases may lead to a loss of normally methylated bases.[18] Therefore free radical damage could play an important role in age-dependent base demethylation and altered gene expression.

The effects of altered base methylation may exert deleterious feedback effects on oxidative stress. Free radical-mediated base demethylation is likely to be a random process, resulting in a random loss of differentiated cell functions and a loss of homeostasis. It is quite conceivable that imbalances in free radical production and protective systems will arise during random dedifferentiation, leading to subpopulations of vulnerable and oxidant-resistant cells. Increased susceptibility to peroxide damage in dedifferentiated cells seems especially plausible. This follows from the critical importance of the glutathione peroxidase system in protecting cells from powerful peroxide-derived free radicals. This system requires reducing equivalents from a complex array of enzymes, coenzymes and substrates linked to cellular metabolism; the multiplicity of its constituents renders this system especially sensitive to the adverse effects of randomly altered gene expression.

C. GENETIC DISORDERS

In principle, an analysis of genetic disorders, involving defects in antioxidant defenses, could resolve whether the free radical theory of aging is valid. Unfortunately,

although several examples of human mutations in one or more of the protective enzymes or antioxidant vitamins are known, including individuals with reduced levels of catalase, glutathione (GSH), or GSH peroxidase, or defective vitamin E absorption, none of these show signs of accelerated aging that can be clearly distinguished from pathology.[2]

Conversely, several genetic syndromes that exhibit some features of accelerated aging, the so-called "segmental progeroid syndromes", exhibit damage that could be consistent with increased free radical damage (see also Chapter 3). These include Down's Syndrome, Ataxia Telangiectasia, Cockayne's syndrome, and, possibly, Werner's syndrome, which exhibits genetic instability, yet has normal levels of SOD, GSH peroxidase, and radiation-induced repair. The molecular basis of these "geromimetic" diseases may involve accelerated rates of chromosome breakage and the diseases frequently exhibit radiation sensitivity that suggests a high susceptibility to free radical damage.

Fanconi's anemia also exhibits characteristics of oxygen radical damage, but the pathology is so severe that death occurs in infancy or early childhood, and the only sign of an age-related effect is increased malignancy. Devastating genetic deficiencies of this type do not permit normal development and, frequently, disease-linked premature death precludes distinguishing symptoms of accelerated aging from pathology.

Down's syndrome is characterized by a 50% elevation of the copper- zinc SOD (Cu/Zn-SOD) above normal levels of this enzyme. This increased protection against superoxide radicals fails to confer anti- aging benefits; indeed, some aspects of age-related pathology, notably senile dementia, are accelerated. There are conflicting reports concerning altered brain lipofuscin accumulation, an in vivo index of lipid peroxidation (Chapter 5). In vitro lipid peroxidation appears to be accelerated. In patients with trisomy

21, red blood cells are abnormally sensitive to lysis in the presence of paraquat, a molecule that causes an increase in cellular superoxide production. Fibroblasts from trisomy 21 sufferers exhibit enhanced lipid peroxidation. The apparent increase in free radical damage under conditions of elevated Cu/Zn-SOD levels can be explained by a concurrent decrease in manganese SOD (Mn- SOD) levels in some tissues. In primates Mn-SOD is not found exclusively in mitochondria, but also occurs in nuclear and other cellular compartments. In some tissues Mn-SOD may account for more than 50% of the total SOD content. Hence, an increase in Mn-SOD in Down's syndrome might readily compensate for a decrease in the copper-zinc enzyme, but it is not yet clear which tissues undergo such compensations. In patients with monosomy 21 ("21q- or anti-Down's syndrome") cells possess only 50% of normal Cu/Zn-SOD but normal Mn-SOD. While these patients suffer from developmental abnormalities and poor survival, there are no obvious signs of accelerated aging.

The difficulty of dissociating disease from aging has been a major obstacle in exploiting genetic analyses to resolve aging mechanisms. Nevertheless, the available genetic data suggest that biological concentrations of catalase, vitamin E, glutathione and glutathione peroxidase are probably not critical determinants of rates of aging, while levels of superoxide dismutase and the overall oxygen radical defensive capacity (viz., whole body radiation) may be important factors. Thus, the evidence can presently offer only modest support for the free radical theory of aging.

D. LIFESPANS AND METABOLIC RATE

To a first approximation, the specific metabolic rate of homeothermic species is inversely proportional to the maximum lifespan (Chapter 2).[8] This correlation can also be expressed as follows: the total oxygen (or energy) consumption per unit weight is the same for all animals when they attain their maximum lifespans. If one assumes that free

radical production is proportional to the rate of aerobic metabolism, then the association between metabolic rates and lifespans can be rationalized in terms of the free radical theory of aging. More direct support for a role of free radicals in determining lifespans has been sought by attempting to correlate deviations from expected lifespans (as predicted from metabolic rates) with varying antioxidant protection. Species that live longer than predicted from their metabolic rates, notably the primates, have higher concentrations of SOD and some other antioxidants, at least in some of their tissues. The correlation of SOD with the maximum attainable lifespans of a group of animals, where the latter is effectively expressed as the lifetime energy consumption per unit tissue weight (LEP), is shown in Fig 3.

However, while a direct correlation between superoxide dismutase and species lifespan has been found in a number of tissues, other antioxidant factors showed no such correlation. Cutler and his collaborators have found that, in addition to SOD, serum carotenoids and vitamin E appear to correlate positively with longevity, while vitamins A and C are relatively uncorrelated. Glutathione, glutathione peroxidase and glutathione-S-transferase seem to be negatively correlated with lifespans. The same group of investigators have also reported that the susceptibility of whole brain homogenates to autoxidation is an inverse function of species lifespan.

Correlative evidence for an association between metabolic rate and free radical processes has been obtained by direct detection of tissue free radicals with ESR spectroscopy. ESR signals have magnitudes that correlate directly with metabolic rate and inversely with lifespan. Lipofuscin particles, which are products of free radical-mediated oxidative polymerization, sometimes contain EPR signals characteristic of metal-ion complexes. Some lipofuscin particles isolated from the human brain contain both copper and iron and exhibit free radical signals that increase progressively with age.

Thus, cumulative free radical damage may occur with age, but further basic studies are required to establish the relationship of ESR signals to metabolically generated free radicals.

Studies of free radical production by isolated mitochondria suggest that altered free radical production may be a more significant factor in aging than impaired antioxidant protection. Sohal and his collaborators have shown that hydrogen peroxide production by mitochondria correlates with age and species lifespan and that antioxidant enzymes do not exhibit consistent correlations. They suggest that altered free radical production is a key determinant of maximum attainable lifespan and that variations in antioxidant enzymes exert relatively little influence.[21,22,23]

E. LIFESPAN MODIFICATION

Ideally one would like to test the free radical theory definitively by specifically manipulating the rate of free radical damage and demonstrating a corresponding effect on maximum lifespans. In principle, free-radical damage is a function of both the rate of radical formation and the interception of these radicals by protective factors, either of which may be amenable to experimental control.

Effects of Ionizing Radiation and Chemical Mutagens

A straightforward example of free radical-mediated damage is that of radiation injury (see Chapter 4). However, there is no clear evidence that a slight increase in free radicals, imposed by low level radiation, shortens life span. Rather, chronic radiation exposure causes a variety of cancers and other diseases. Similarly, chronic exposure to mutagens (nitrogen mustards), does not appear to shorten life spans, once the effects of increased disease incidence are taken into account. The implications of these studies for the free radical theory of aging are difficult to interpret, in part, because of a lack of good aging

parameter, which would facilitate discerning effects of disease from those of aging.

Effects of Cigarette Smoking

Although it has not been established whether cigarette smoking affects maximum lifespan, its positive correlation with major life-shortening diseases, including cardiovascular disease and cancer, implies that cigarette smoking reduces mean life expectancy. Skin wrinkling, the most familiar marker of aging in humans, is accelerated in white smokers by cigarette consumption and, independently of smoking, by sunlight exposure.[24,25] The effects of smoking and sunlight exposure on skin wrinkling are additive. Rodents exposed to cigarette smoke exhibit increased levels of lipid peroxidation products, prompting the suggestion that smoking accelerates the aging process.[26] In humans the body mass index (weight divided by the square of the height) and the distribution of body fat (waist circumference divided by hip circumference) decrease with smoking and with increasing age,[27,28] consistent with the view that smoking accelerates some aspects of the aging process.

Caloric Restriction

The most dramatic lifespan extensions that have been achieved with dietary manipulations are increases in maximum lifespans of up to 50% that have been achieved by caloric restriction. The efficacy of caloric restriction in prolonging lifespans (Fig 4) might suggest, at first sight, that there is a direct relationship between caloric intake and metabolic rate, and, hence, that such restriction lowers the rate of free radical production and damage. However, caloric restriction leads to lowered weight, and this must be taken into account. In studies with rats, when the effects of food restriction were analyzed in terms of both decreased body weights and metabolic rates it was found that the specific metabolic rates of the smaller food-restricted animals were in fact greater than those of ad libitum-fed animals. The food-restricted animals lived longer than the controls,

contrary to the expectation that specific metabolic rate be inversely related to the aging rate. Thus, at first sight the data on caloric restriction seem to contradict the free radical theory. Further progress in achieving a meaningful interpretation of the effects of caloric restriction may hinge upon being able to relate metabolic rates to free radical fluxes.

Effects of Antioxidants

Studies with dietary antioxidants have shown that, while the mean lifespan is increased, there is no significant increase in the maximum lifespan, as would be expected if the aging rate had been altered.[2] Side effects of antioxidants must also be considered, e.g., possible caloric restriction effects associated with foul-smelling food additives like 2-mercaptoethylamine. Another potential problem with dietary antioxidants in whole animals is a possible lack of specificity. For example, antioxidants are claimed to enhance immune function, and some quinone antioxidants like 2(3)-tert-butyl-4-hydroxyanisole (BHA) induce the microsomal DT diaphorase and UDP-glucuronyl transferase systems and hence may accelerate the removal of potentially mutagenic chemicals that could affect aging by non-radical mechanisms. Two other synthetic antioxidants, BHT and ethoxyquin, have been shown to induce hepatic enzymes. Thus, increased life span that correlates with antioxidant feeding may be due to effects other than free radical quenching.

The accumulation of lipofuscin is increased with vitamin E deficiency and decreased with dietary antioxidants. However, there is as yet no convincing evidence that lipofuscin accumulation correlates with age-related cell loss. On the contrary, among the brain stem nuclei, the inferior olive has high lipofuscin levels and shows no age-related cell loss. Moreover, there are examples of cells that accumulate lipofuscin but do not exhibit a decline in function. This was illustrated by cell sorting experiments with cultured fibroblasts, which separated populations of cells on the basis of fluorescence intensity,

and which showed that fluorescent cells suffered no loss of proliferative potential.

Thus, the evidence for antioxidant-mediated lifespan extension in vertebrates does not argue persuasively for a causal role of free radicals in aging. This fact is generally acknowledged by advocates of the free radical theory and they offer two general explanations that could sustain the basic concept: (1) the principal site of damage (e.g., mitochondrial DNA) is not protected by exogenous antioxidants, or (2) the endogenous defense system is regulated to maintain a fixed overall level of protection so that simple attempts to increase protection by some dietary manipulation of one or a few antioxidants fail because of compensatory decreases in endogenous defenses ("compensatory downregulation").

It has been suggested that mitochondria could play a role as pacemakers. This hypothesis could explain the failure of some antioxidants to delay aging. Excessive concentrations of hydrophobic phenolic antioxidants could react with mitochondrial electron transport components to impair mitochondrial metabolism, which could explain the futility of extending lifespans with antioxidants of this type.

Another explanation for the failure of antioxidants to confer substantial lifespan extension, "compensatory downregulation of endogenous antioxidant defenses", argues that when increased concentrations of exogenous antioxidants reduce the flux of radicals, the cell decreases its production of other defensive enzymes. However, the fact that lipofuscin accumulation can be reduced by feeding antioxidants in the absence of lifespan extension, and that tumor incidence can be reduced by feeding exogenous antioxidants, suggests that antioxidants can effectively reduce the rate of free radical damage in some tissues without undue compensatory downregulation.

***In Vitro* "Aging Models"**

The doubling potential of cultured mammalian cells like fibroblasts has been perceived as a meaningful in vitro aging model because it correlates with species lifespan and with the age of the cell donor and is reduced by life-shortening genetic disorders in humans (Chapter 5). To explore the possibility that the finite doubling potential of the cells is due free radical damage, a number of investigators have varied the oxygen tension during cell growth and studied how this affected the doubling limit. No increase in the maximum number of doublings could be achieved by lowering the oxygen concentration below ambient levels, although increased oxygen concentrations curtailed both growth rates and the doubling number.[30]

Cultured human fibroblasts can be transformed by treatment with SV40 virus to remove the limited growth potential, i.e., the transformed cells become "immortal". The transformed cells are aneuploid, genetically unstable, and, often, DNA repair-deficient. When these transformed cells are exposed to higher-than-ambient oxygen concentrations, they too are slowed in their growth rate. It has been shown that this oxygen-induced decline in growth rate is due to an inhibition of DNA replication, suggesting that the effect of high oxygen concentrations on growth rates is not necessarily related to free radical formation. In normal fibroblasts, oxygen also modulates growth and can affect the process of differentiation. A stimulating effect of low oxygen tension on fibroblast growth rate may serve a regulatory role in wound healing. These multiple effects of oxygen in fibroblasts underscore the ambiguities associated with interpreting experiments with the replicative limit model in terms of free radical damage alone.

Adrenocortical cells in long-term culture, grown on certain batches of 10% fetal bovine serum, are essentially completely deficient in both vitamin E and in selenium when not supplemented with these nutrients.[31] This has set the stage for analyzing effects

of these antioxidants in determining the in vitro "lifespans" of these cells. Supplementation with either vitamin E or selenium protects the cells from the toxic effects of added cumene hydroperoxide, demonstrating the efficacy of these nutrients in ameliorating the effects of oxidative damage. However, the long-term growth of the cells is nearly identical in the absence or presence of these antioxidants. These data suggest that: (a) oxidative damage is not a significant determinant of the doubling potential in adrenocortical cells or (b) that rapid cell growth under optimal conditions enables cells to outpace the damaging effects of free radical chemistry such that damage only becomes apparent with cumulative radical damage when metabolically active cells have a limited capacity to divide.

The validity of the replicative limit model as an aging model is unclear;[32] hence, negative evidence obtained with it should not necessarily be taken as an indictment of the free radical theory of aging. The central difficulty with the proliferative limit model lies in its questionable significance for *in vivo* aging. There is no solid evidence for a proliferative limit occurring during normal in vivo aging, and it seems likely that functional losses which precede the *in vitro* proliferative limit are responsible for aging.

Exercise

A promising experimental strategy for manipulating metabolic rates is exercise. The analysis of exercise effects requires that both the intensity of exercise and prior training be taken into account. Increased radical damage appears to occur with intensive exercise in untrained animals, and positive adaptations, conferring increased resistance to oxidative damage, are observed in endurance-trained animals.

Studies with intense physical activity in untrained rats have suggested an increase in free radical damage, e.g., increased lipid peroxidation products in liver and skeletal

muscle homogenates and in mitochondrial fractions. Experiments with rats have shown that plasma glutathione disulfide increases significantly with intensive exercise and that vitamin E deficiency is associated with a marked decrease in endurance capacity, suggesting that lipid peroxidation may play a role in muscle fatigue upon exhaustion.

Rodents that have been exercise trained throughout life live longer than their sedentary counterparts, consistent with a positive effect of exercise training on mean lifespans. Although the mechanism of the lifespan extension is unresolved, it may involve caloric restriction, since exercised animals accumulate less body fat than sedentary animals. Endurance training appears to decrease the susceptibility of skeletal muscles to lipid peroxidation, consistent with an adaptive response of protective systems. Endurance training raises the activity of the enzyme glutathione reductase in both skeletal and cardiac muscles. This enzyme serves an antioxidant function by maintaining glutathione in its reduced state, thus enabling glutathione peroxidase to perform its function of hydroperoxide reduction. Exercise training markedly retards the appearance of high plasma glutathione disulfide concentrations during intensive exercise, supporting the notion that exercise training increases the capacity of an animal to maintain highly reduced glutathione levels for long periods of intensive activity.

F. SUSCEPTIBLE TISSUES, CELLS AND ORGANELLES

Because the cell populations in different tissues are likely to vary in their redundancy, regenerative capacity, free radical production rates, antioxidant defenses, and DNA repair rates, some tissues may be particularly sensitive to free radical damage, and these could determine aging rates in the organism.

Neuronal tissue

Metabolically active, non-dividing cells like the neurons could well be the most

susceptible targets of oxidative damage. Indeed, there is a loss of neurons with age, but such loss appears to be species-specific and to be a function of the tissue location. The surprising durability of neuronal tissue despite its large oxygen utilization appears, at first sight, to be incompatible with free radical involvement in age-related functional declines. However, a large portion of the oxygen utilization is in axons and terminals that are physically removed from sensitive and non-renewable targets like the nuclear genome. Moreover, neuronal mitochondria do not appear to produce superoxide radicals at a site that is a major source of these radicals in other cells. Finally, considerable cell redundancy may prevent functional deficits from becoming clinically significant. As a rule, more than 40% cell loss is required in a neuronal system before functional loss in the central nervous system is apparent.

Mitochondria

Mitochondrial dysfunction and depletion occurs in aging postmitotic cells and may prove to be a major factor in aging. Miquel and his colleagues have postulated that mitochondria are the "Achilles heel" of postmitotic cells (Chapter 5). Mitochondrial DNA (a) is not protected by a histone coat, (b) is in close proximity to sites of oxygen radical production and lipid peroxidation, and (c) relies on a possibly inadequate DNA repair system.[33] However, because mitochondria are dependent on the nuclear genome for many of their proteins and are sensitive to the cellular environment (ions, substrates) and even hormonal stimulation (viz. thyroid hormone effects on mitochondrial replication), it is difficult to unambiguously dissociate intrinsic mitochondrial damage from extrinsic factors.

The high susceptibility of mitochondrial DNA (mtDNA) to oxidant damage is indicated by high levels of 8-hydroxydeoxyguanosine[34,35] and by an increasing number of mtDNA deletions with age.[36,37] Altered protein synthesis due to mtDNA damage may

play a role in age-dependent mitochondrial dysfunction.[38,39] Among the four electron transport complexes of the respiratory chain, the activity of complex I declines most rapidly with age.[38,40] Apart from effects on mitochondrial bioenergetics, more indirect damage scenarios, possibly involving an incorporation of mtDNA fragments into the nuclear genome, may provide a link between mitochondrial free radicals and aging.[41]

G. SYSTEMIC EFFECTS

One of the most obvious characteristics of aging is the gradual decline of physiologic integrity, including the progressive impairment of neurological, immunological, humoral and metabolic function. Evidence for free radical involvement in all of these debilitating processes is growing. However, organelle and cellular redundancy and renewal may be able to compensate for the damage sufficiently to sustain function. Thus, the epithelia lining the digestive tract and skin, the blood cells and the liver may be extensively damaged and yet regenerate on a regular basis. In these renewing cell populations, the principal danger of damage appears to be neoplastic transformation.

Because aging is characterized by an increased incidence of infectious diseases one might expect immunosenescence to play a crucial role in physiological aging (Chapter 7). Declines in immune function begin relatively early and appear to be heavily dependent on thymic involution and selective T cell aging. Immunological declines can be, at least partially, reversed by thymic grafts or thymic hormone therapy, but complete restoration requires an additional young bone marrow graft.

Evidence for free radical involvement in immunosenescence is based on selective vulnerable of the immune system to radiation and other free radical-generating agents. T cells, which age more rapidly than B cells, are reported to be more vulnerable to oxygen

radicals and to accumulate more lipofuscin; treatment of aging mice with 2-mercapto-ethanol delays the accumulation of T cell lipofuscin and the decline of immune function with age, and increases the mean lifespan.

The thymus may also be selectively vulnerable to free radical damage. The first age-related loss in size (thymic involution) can be ascribed to the loss of the most radiosensitive (cortical) lymphocytes. The medullary epithelial cells that secrete thymic hormones are heterogenous and the early loss is again that of the most radiosensitive cells, which are active metabolically, require vitamin C for their secretory activity and appear to accumulate intrinsically autofluorescent substances (age pigments). Although there are probably developmentally programmed cellular and hormonal controls of thymic involution, significant oxidative damage seems to be involved as well.

Many antioxidants are immune stimulants and will enhance immune function both in vivo and in vitro with both young and old cells. Part of this effect appears to be due to the maintenance of reduced sulfhydryl groups. Although antioxidant treatment can boost immune function at any age, it does not markedly reduce the rate of basic aging of the immune system.

H. DISEASE

Balin has emphasized the importance of separating the effects of specific life-extending treatments that may reduce the incidence of disease, and hence most likely affect only mean lifespans from those that affect basic aging rates.[42] As reviewed in the preceding sections, free radicals do not seem to determine maximum lifespan. However, mean lifespan is well-correlated with the incidence of disease and many diseases that shorten mean lifespans are aggravated by free radical processes (see, for example, Pryor[43]). Some of the support for free radical involvement in disease is outlined in the

following sections:

Cancer

The process whereby a normal cell becomes transformed, i.e., whereby it assumes the uncontrolled growth characteristics of a cancer cell, seems to involve an alteration of the DNA, as shown by the fact that many chemicals that cause cancer in animals also cause mutations in microorganisms in the well-known Ames Salmonella revertant test. This test is often used as a predictor of risk for chemicals that are suspected to have carcinogenic potential.

The relationship between aging and cancer is complex; for example, neuroblastomas, many leukemias, and hormone-dependent tumors have radically different age patterns than most sarcomas and many carcinomas that rise with the second and fourth power of age, respectively. The incidence of most tumors, in a large variety of species, rises dramatically with age. This rise in cancer incidence with age may be attributed to several factors:[44]

1. Long-term carcinogen exposure increases the risk of initiation, e.g., lung cancer incidence reflects duration of smoking rather than chronological age.
2. The prolonged period required for one malignant cell to multiply and develop into a detectable tumor.
3. Aging itself increases the risk because of:
    - a possible reduction in natural killer or other immune surveillance function
    - an increase in the activation of procarcinogens
    - other factors including demethylation-induced epigenetic instability and free radical-induced DNA damage.

The pathogenesis of neoplasia has been thought to be a two-stage process: 1) initiation, produced by mutagenesis, abnormal differentiation or viral infection, and 2) promotion, characterized by a prolonged latency allowing for the multiplication and evolution of initiated cells into a tumor (Chapter 3). The process involves many variables, including tissue type, hormonal influence, proliferative rates, DNA repair capacity, environmental carcinogen exposure, viral infection, immune surveillance, and genotype (see also Chapter 3). The proposal that cancer is the result of a two-stage process has been challenged recently by Ames and others. Ames has suggested that cell division greatly increases the potential for non-repairable DNA damage and cancer.[45] He has postulated that many carcinogenic agents exert their effects indirectly by killing cells, which stimulates the growth of new cells and concomitant DNA damage.

Work has progressed rapidly on the relationship between carcinogens and mutagens (i.e., agents that cause cancer and mutations, respectively). Point mutations are implicated in the activation of, ras oncogenes in human tumors, for example. However, for other oncogenes, for example, in the myc family (e.g., Burkitt's lymphoma), there is a chromosomal translocation of the oncogene to an enhancer-amplified site, presumably activating oncogene expression. Epidemiological evidence suggests that the point mutations may stem from a variety of environmental and intrinsic mutagens, including free radicals.

A number of potent chemical carcinogens, like benzo[a]pyrene form free radical derivatives in simple chemical systems and in isolated membranes of the endoplasmic reticulum. Some molecules are also converted to quinones by the cytochrome P450 system and these quinones can be substantially more toxic to cells (e.g., cultured fibroblasts) than their precursors. The mechanism of the quinone toxicity may be a catalytic reaction whereby the quinones undergo redox cycling with cellular electron donors and

with molecular oxygen to produce superoxide radicals. For example, the quinones that result from the microsomal oxidation of benzo[a]pyrene are readily reduced by electron donors like glutathione, NADH and NADPH and by the DT diaphorase enzyme. As soon as the reduced quinol is formed it reacts with molecular oxygen in one-electron transfer steps to form superoxide radicals. In the process of redox cycling, some semiquinone radical species can also react directly with cellular macromolecules, including DNA.

Gross genetic rearrangements, transpositions, and amplification appear to play a major role in the promotion process of carcinogenesis. It is quite probable that free radical damage is also involved in these processes. Since oxygen-derived radicals are capable of inducing mutations, and since they appear to have the capacity to cause chromosomal strand breakage, they should be able to participate in the gene rearrangements necessary to link a protooncogene with an enhancer element.

Current evidence that oxygen radicals are involved in carcinogenesis includes the following:

1) Several genetic disorders with a high incidence of both chromosomal rearrangement and neoplasia, e.g. Fanconi's and Bloom's syndromes, appear to involve abnormally high oxygen radical-mediated damage. Unlike other syndromes predisposed to malignancy, Fanconi's anemia and Bloom's syndrome appear to have normal DNA repair capacity, but increased sensitivity to oxygen radical-generated damage.

2) Epidemiological evidence suggests that dietary or endogenous factors rather than industrial products or environmental agents are responsible for the majority of human cancers.[46,46] An involvement of lipid-peroxidation-linked oxygen radicals is supported by the implication of fat as a risk factor for many types of tumors and the inverse correlation of

risk with dietary antioxidant intake.[48] Other epidemiological data show that high dietary intake of quinones or quinone precursors, which are capable of promoting free radical reactions, is attended by increased cancer incidence. Ionizing radiation also induces cancer in a dose-dependent manner. When cigarette smoke is trapped on glass wool a large ESR signal can be observed and it seems quite plausible that this free radical component of cigarette smoke is a causative factor in its well-established link with cancer.

3) Oxygen-derived radicals are implicated in tumor promotion. Phorbol esters, the classical tumor promoters, produce extensive chromosomal damage and stimulate polymorphonuclear leukocytes to produce an abundance of superoxide and peroxides. Antipromoters such as retinoids and protease inhibitors inhibit oxygen radical production. DNA strand breakage correlates directly with promotional efficiency, and antipromotional ability correlates with inhibition of strand breaks. Promoter-induced chromosomal damage is inhibited by superoxide dismutase, but in some systems (lymphocytes) it is also inhibited by indomethacin and other inhibitors of prostaglandin synthetase that can produce unstable lipid peroxides and attendant oxygen radicals.

Phorbol esters have several other effects on cellular metabolism; in particular, they rapidly activate the protein kinase c pathway, a principal control point in the regulation of proliferation, and rapidly and reversibly induce many of the properties of the transformed phenotype. Hence, phorbol esters have a rapid and reversible, short term effect on cells that stimulates cell proliferation. They also generate free radical-mediated chromosomal damage which may allow for an emergence of clones with activated oncogenes and a permanently malignant phenotype.

Phorbol esters are not the only promoters capable of producing oxygen radicals. In a recent review, Ames has assembled evidence that most promoters (eg. phorbol esters, fat,

TCDD, lead, cadmium, wounded tissue, asbestos, anthralin, peroxides, mezerein, telociden B, phenobarbitol, radiation, nitroso and nitro compounds, hydrazines, and polycyclic hydrocarbons) are also capable of generating oxygen radicals.[48] While all of these agents have a multiplicity of other effects and there is, at present, no quantitative correlation between the efficiency of promotion and oxygen radical production, the qualitative correlation is striking. The evidence for a causal relation between oxygen radical production and promotion is also supported by the efficacy of antioxidants as anticarcinogens.[48] In particular, vitamin E, selenium, glutathione, ascorbic acid, and beta carotene have proven effective in reducing tumor incidence.

Parkinson's Disease

Parkinson's disease is a strongly age- related pathology marked by a selective and progressive loss of pigmented catecholaminergic, particularly dopaminergic, neurons of the substantia nigra (see also Chapter 8). This loss, which may well be due to a selective vulnerability to oxidative damage, averages about 80% in autopsied patients, compared to only about a 40% loss seen in the oldest normal individuals. The current epidemiological data suggest that Parkinson's disease is caused by some environmental agent(s) and reasonable candidates would be those producing excessive fluxes of oxygen radicals or other active oxygen species. Several investigators have speculated about possible oxidative mechanisms in the development of Parkinson's disease, including redox cycling of quinones derived from catecholamines and a role for hydrogen peroxide derived from monoamine oxidase.

Glutathione concentrations are low in human substantia nigra and almost absent from nigral tissue of patients who have succumbed to Parkinson's disease. Catalase and glutathione peroxidase have also been found to be abnormally low in patients with this disease. It is conceivable, however, that these reductions are the result of neuronal loss

rather than decreases in cell-specific enzyme activities.

It has been claimed that brains of patients with Parkinson's disease contain abnormally high concentrations of iron. Since iron is normally sequestered in highly stable storage and binding proteins, it is conceivable that "free" iron may be present in these brains, which could catalyze hydroxyl radical formation in a Fenton-type of reaction.

An environmental neurotoxin like N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which selectively kills dopaminergic neurons, could displace dopamine from its storage vesicles (which are at acidic pH and therefore provide some protection against autoxidation) and thus drive an increased catecholamine autoxidation and an increased peroxide release from monoamine oxidase.

Thus, the evidence for free radical involvement in Parkinson's disease is suggestive but further experiments are necessary before an unequivocal resolution of the mechanisms involved can be offered.

Autoimmune Disease

Because tolerance to self appears to require an active thymic role in the production of T suppressor cells as well as in the deletion of self-reactive clones, one might expect thymic involution and age-related immune dysregulation to result in an age-related increase in autoimmune disease. Further, the emergence of altered self antigens through persistent viral infection, posttranslational modifications, somatic mutation, or even postmaturational development of newly expressed genes could also bring about an increased number of autoimmune reactions with age (Chapter 7).

Autoimmune phenomena like autoantibodies, glomerulonephritis, periarteritis, and

probably some classes of senile amyloid increase with age. A major role of autoimmune pathology is played in the aging of some rodents, but not all strains and species seem to be affected. Hence, while some workers have hypothesized that autoimmunity is the major aging process and likened senescence to a chronic graft-versus- host reaction, the evidence is not consistent. In humans, most known or suspected autoimmune diseases, including rheumatoid arthritis, have a peak incidence in middle age and it is difficult to assess the significance of autoimmune phenomena in human aging. Among rodents, in strains that are clearly autoimmune susceptible, such as NZB mice, antioxidant feeding has produced a delay in disease onset and life- span extension. If autoimmunity is a major aging process and not a secondary pathology, then antioxidant feeding may be said to delay the accelerated aging that has been reported in NZB mice.

Several other diseases seem to involve free radicals in their etiiology, including atherosclerosis , emphysema, arthritis, cirrhosis, and diabetes (Chapters 15-18,20,21).[43]

INTERVENTIVE STRATEGIES

A. GENETIC APPROACHES TOWARDS ALTERING FREE RADICAL DAMAGE

Genetic manipulation is one promising approach towards understanding how an altered expression of endogenous oxidative defenses affects aging. However, studies of antioxidant enzymes have been plagued by problems associated with species- and tissue-variability compounded by occasional conflicting reports from different laboratories. For example, in the rat brain, mitochondrial SOD is unchanged or increased with age, while CuSOD is unchanged or decreased. Rat brain cytosolic glutathione peroxidase is un-changed or decreased, mitochondrial glutathione peroxidase and reductase are increased, and catalase is unchanged with age. Because of the conflicting data and lack of

correspondingly reliable information about metabolic rates and free radical production, the interpretation of data on protective enzymes remains obscure and further studies are needed to assess the significance of observed enzyme level changes.

B. LIFESPAN EXTENSION STRATEGIES BASED ON FREE RADICAL CONCEPTS

Even if free radicals are implicated only in life-shortening diseases and do not make a contribution to the basic aging process, the general problems associated with the diseases of the aged are so great that intervention in the free radical-mediated damage that is implicated in these diseases is highly worthwhile. This section addresses some of the interventive measures that might be considered to reduce the incidence of free-radical potentiated diseases.

There is a growing awareness of the hazards of exogenous free radicals. These include cigarettes, oxidized fat, moldy nuts, pickled vegetables, burned meats, combustion products, and asbestos. Avoidance of these agents is likely to reduce the risk of the free radical- mediated diseases.

It is also feasible to reduce the impact of exogenous or metabolically-generated radicals on cellular targets by ingesting antioxidants. Vitamin E, beta carotene and BHT can protect against active oxygen species in the fatty tissues and in cell membranes. They are particularly effective in protecting against the harmful effects of certain drugs used in chemotherapy, which kill cancer cells by free radical mechanisms.

Aqueous chemical reactions may be a more important free radical damage route than fatty tissue reactions. Unfortunately, there are no facile means to enhance the protection afforded by the water soluble enzymes catalase and SOD. However, dietary selenium can

be used to enhance glutathione peroxidase activity, although toxic overdoses are a concern. One can also consider vitamin C supplements, but this vitamin has the potential of acting as a pro- rather than an antioxidant in the presence of free transition metal ions. Uric acid is a water-soluble antioxidant factor that may confer benefits against singlet oxygen and may protect against the ravages of superoxide produced by xanthine oxidase. There may also be value in cysteine supplementation to increase the biosynthesis of glutathione.

Since the immune system appears to do much of its work by exploiting the toxic properties of free radicals, another line of protection is to minimize activation of such free radical reactions. This can be achieved by avoiding infections, treating wounds promptly and effectively, using drugs to speed post-operative recovery and using anti-inflammatory agents when appropriate.

Because of the possibility that transition metals may contribute substantially to free radical damage, it would be worthwhile to pursue strategies for minimizing their reactions. One suggestion that has been made is to use zinc supplementation as a strategy for displacing iron from binding sites that might otherwise promote free radical reactions.[49]

SUMMARY

The validity of the free radical theory of aging remains to be resolved. A major difficulty with the theory is the failure of antioxidant supplementation to significantly extend the maximum lifespans of mammals. This failure is particularly troubling because the administration of antioxidants has clear-cut effects in reducing the extent of lipofuscin

accumulation, an indication that the antioxidants do protect some cellular targets susceptible to peroxidation. Antioxidant administration can also confer anticarcinogenic and other health benefits, which affect mean but apparently not maximum lifespans. On the other hand, recent evidence on caloric restriction, the only known strategy for dramatic lifespan extension, indicates that at least some subcellular fractions prepared from calorically restricted rats generate fewer free radicals and are endowed with enhanced antioxidant protection relative to fractions from control animals. Recent work on mitochondria is consistent with radical-mediated damage of mitochondrial DNA and an age-dependent dysfunction that correlates with increased free radical production and decreased antioxidant capacity. These mitochondrial data generally seem consistent with the free radical theory of aging.

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TABLES

Table 1. Subcellular Sources of Hydrogen Peroxide[14]

Organelle	Function	H_2O_2 Production (% of total)
Mitochondria	Electron transport	15%
Peroxisomes	Divalent oxidations	35%
Endoplasmic Reticulum	Mixed function oxidations	45%
Cytosol	Xanthine oxidation	5%

Table 2. Potential strategies, based on free radical causality assumption, for intervening in the aging process

Decrease Free Radical Stress	Increase Protection
Stress:	Antioxidant Factor:
Cigarettes	Vitamin C
High fat diet	Vitamin E
Air pollutants (ozone, nitrogen oxides, asbestos)	Selenium
Water pollutants (halogenated solvents, chromate)	Beta carotene
Ultraviolet light (including sunlight)	Transition metal control:
Immune Effects:	Zinc
Reduce infections	Nutrients:
Speed wound healing	Acetyl carnitine
Control metabolism:	
Caloric restriction	

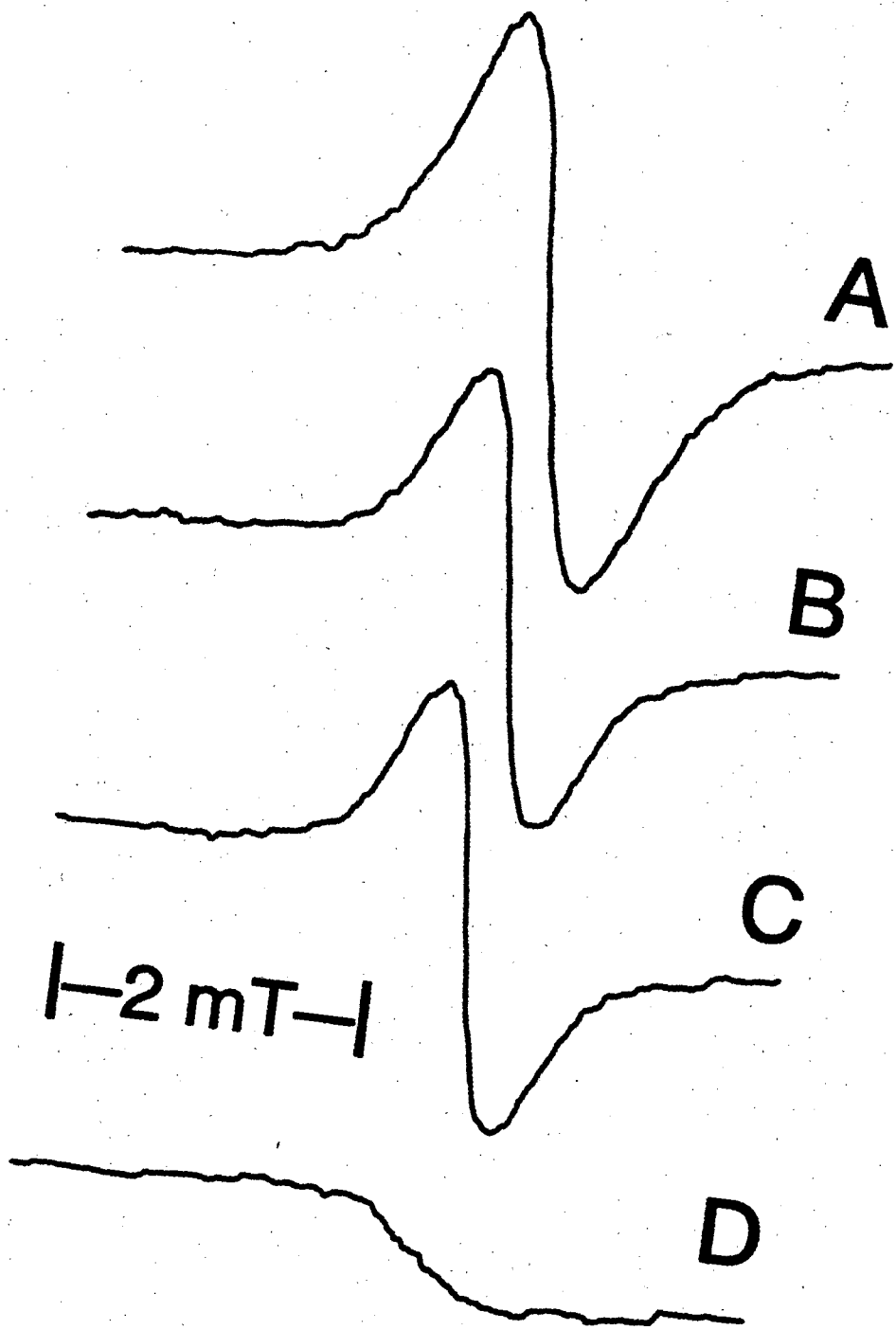
FIGURE LEGENDS

Fig 1. Free radicals in cigarette smoke trapped on glass wool and extracted into ethanol. Electron spin resonance (ESR) spectra of cigarette tar on glass wool (A), ethanol extract 5 min after extraction (B), ethanol extract 10 min after extraction (C) ethanol extract 50 min after extraction.

Fig 2. Scheme of how free radicals and antioxidants may affect aging.

Fig 3. Lifetime energy consumption of different animal species as a function of their liver SOD activities (From Cutler,[20] adapted with permission from the author and the publisher, S. Karger, AG, Basel). The open symbol shows human liver SOD activity, corrected for enzyme degradation during tissue processing.

Fig 4. Survival curves for ad libitum-fed (group A) and food-restricted (group B) SPF Fischer 344 rats (Yu,[29] reproduced with permission of author and publisher).


A
B
C
D
2 mT

MEMLINK FIG 2

Free Radicals in Aging

O_2

Formation of Oxidants

Reduction to H_2O_2	Reduction to superoxide (O_2^-)	Other oxidants
peroxisomal enzymes monoamine oxidase	mitochondrial ubisemiquinone xanthine oxidase cytochrome P450 activated neutrophils hemoglobin, myoglobin	activated neutrophils (HOCl, NO·) free iron, copper: (H_2O_2 -----> OH·) ingested or inhaled toxins

Active Oxygen

Scavenging of Oxidants

H_2O_2 and ROOH	Superoxide (O_2^-)	Other oxidants
glutathione peroxidase catalase glutathione transferase	Cu-Zn SOD mitochondrial Mn SOD	vitamins C and E glutathione, uric acid β-carotene

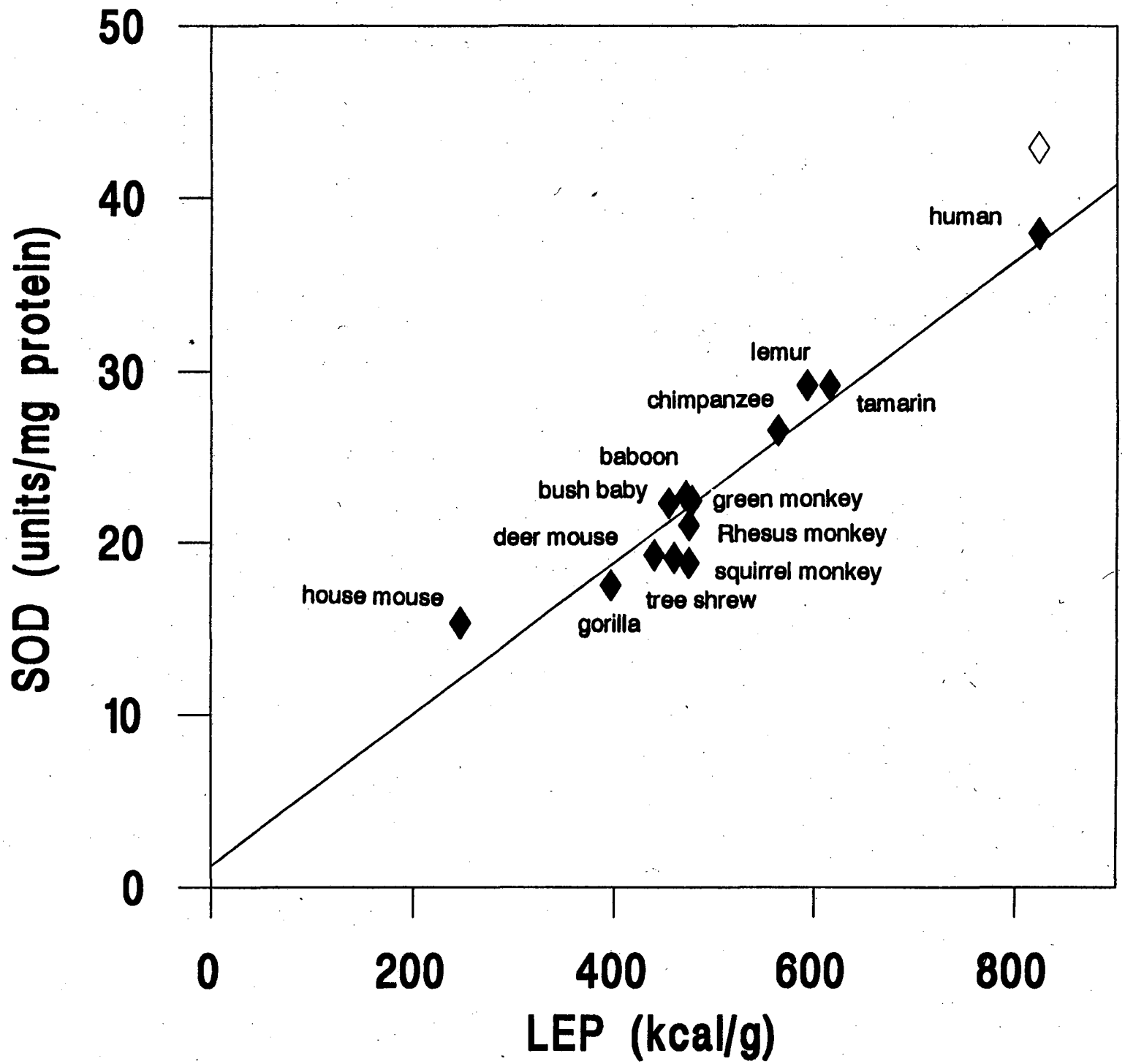
Residual Oxidants

Cell Damage

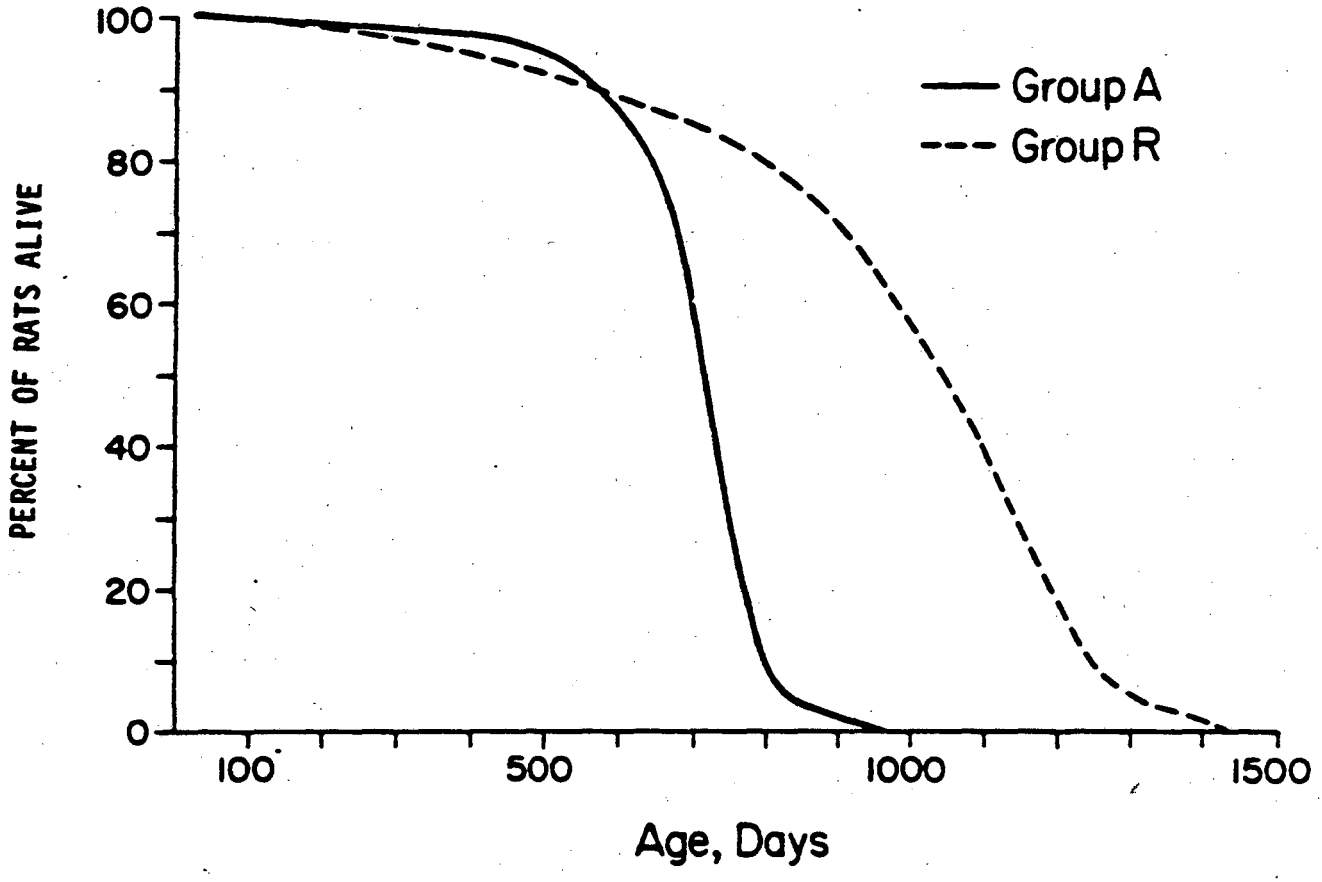
Major Targets	Examples of Products
lipids	lipid peroxyl radicals, hydroperoxides, aldehydes, singlet oxygen
proteins	crosslinked proteins, carbonyls, bityrosines, free iron and copper
DNA	DNA fragments, crosslinked strands, loss of methylated bases

Irreversible Lesions

Cumulative damage, pathology, death


SOD (units/mg protein)
50
40
30
20
10
0
0
200
400
600
800
LEP (kcal/g)
human
lemur
tamarin
chimpanzee
baboon
bush baby
green monkey
deer mouse
Rhesus monkey
squirrel monkey
house mouse
tree shrew
gorilla

MEHLHORN
FIG 4



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