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Developments in Aging Research: I. Free Radicals—A Primary Cause of Aging

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1 Biological and biochemical aspects of aging: An introduction.

Ambjörn Ågren: Medical Cell Biology, Biomedicum, Uppsala

Molecular mechanisms behind the aging processes have attained great interest. However, little has been known about the fundamental causes of aging until recently. The most general assumption is that they may be due to accumulated insults such as random "hits" from background radiation of free radicals produced in other ways. This "error catastrophe" theory of aging predicts a build-up of debris with a cell, for example, age pigmentation (lipofuscin).

In recent years the importance of the presence of free radicals in cells has been considered, and their role in the aging processes of different organisms has been tested. This "free radical theory" states that in the body, free radicals initiate random and irreversible reactions which produce a cascade of deleterious reactions, e.g. with DNA, RNA, protein synthesis and lipid peroxidation. When a polyunsaturated fat or protein is damaged by such reactions, age pigments such as lipofuscin, ceroid and amyloid are formed in cells and tissues. Lipofuscin progressively accumulates in the cell during its lifespan. It may occupy up to 35% of the total cell volume and hinder metabolite transport and cell function. Several antioxidants and cell-protective substances have been found to decrease this type of accumulation and hindrance, i.e. kavain, orotate, magnesium thiazolidin and tocopherol *p*-chlorophenoxyacetate. Mammalian experiments show that these substances can increase both maximum and average lifespan. Whether this finding is valid for man remains to be firmly established in more extensive, long-term clinical studies.

2 Involvement of oxygen free radicals in tissue injury.

Howard H. Thaw, Rolando F. Del Maestro and Karl-E. Arfors: Department of Experimental Medicine, Pharmacia AB, Uppsala, Sweden and University Hospital, London, Canada.

Oxidative damage that is associated with the pathogenesis of inflammation, radiation and ischemia has been suggested to be mediated through active-oxygen species. During oxidative metabolism and normal intracellular enzyme function, superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^\bullet OH$) are formed at rates which are directly proportional to oxygen tension. A number of intrinsic enzymatic and scavenging mechanisms have emerged under evolutionary pressures in our oxygen rich environment enabling us to cope with these inevitable oxygen byproducts (figure A). The cytochrome oxidase complex in the mitochondria adds four electrons to oxygen, reducing it directly to water and thereby circumventing the formation of any intermediates.

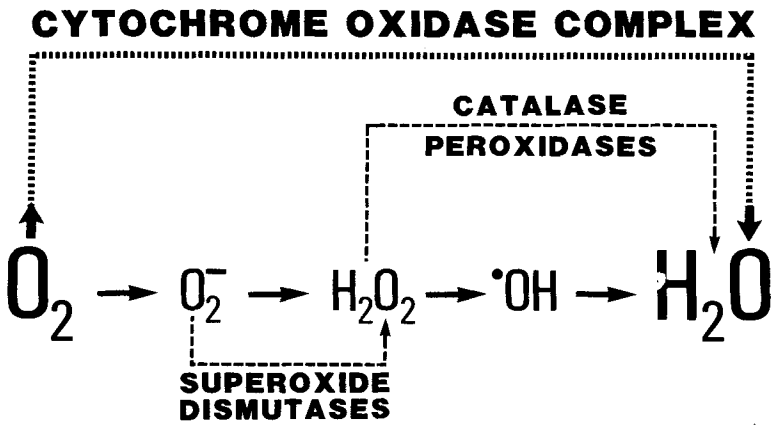

Figure A. Most oxygen is reduced to water quadravalently (by the addition of four electrons), however, univalent or one-electron step reduction does occur resulting in the formation of intermediates. Intrinsic enzymatic and scavenging mechanisms are responsible for controlling these intermediates.

The superoxide dismutases catalyze the dismutation of O_2^- while other enzymes such as catalase (CAT) and glutathione peroxidase remove H_2O_2. Furthermore, antioxidants such as ascorbic acid, glutathione and vitamin E protect against the effects of many different active-oxygen species.

We have investigated the influences of these species generated by substrate-xanthine oxidase systems (figure B), on human glial cells in culture, hyaluronic acid and microcirculation of the hamster cheek pouch.

$$\text{XANTHINE OXIDASE-}H_2 + 2O_2 \longrightarrow \text{XANTHINE OXIDASE} + 2H^+ + 2O_2^- \quad (1)$$

$$\text{XANTHINE OXIDASE-}H_2 + O_2 \longrightarrow \text{XANTHINE OXIDASE} + H_2O_2 \quad (2)$$

$$H_2O_2 + O_2^- \longrightarrow {}^{\bullet}OH + OH^- + {}^1O_2 \quad (3)$$

Figure B. The enzyme model for producing active oxygen species involves both a univalent (1) and divalent (2) pathway. The simultaneous presence of H_2O and O_2^- suggests further interaction and the formation of a strong oxidizing species (3).

Progressive and irreversible morphological changes culminated in death for the glial cells, and hyaluronic acid broke down under the influence of the free radical generating system. In the microcirculation of the hamster cheek pouch, increased microvascular permeability and pronounced alterations were observed in polymorphonuclear leucocyte-endothelial interaction and adhesion. These results suggest that active-oxygen species mediate macromolecular, cellular and microcirculatory changes in our model systems.

The mechanisms underlying free radical generation in normal, disease and aging processes should be considered in an attempt to find new approaches to our understanding of these complex phenomena.

3 The importance of free radicals in aging.

Mathias Uhlén: Microbiology, Biomedicum, Uppsala and
The Institute for Gerontology, Stockholm, Sweden

The free-radical theory of aging is based on the fact that free radicals, directly or indirectly, lead to irreversible membrane damage or cross-linking of biomolecules. The initiation step for the production of free radicals in biological systems is often the production of the superoxide anion radical (O_2^-)

by the addition of a single electron to molecular oxygen. There are many sources for this electron, i.e. pollutants, drugs and radiation, but the primary source is the electron transport system of mitochondria. Many enzymatic and non-enzymatic systems have been developed by aerobic organisms to reduce the flux of O_2^- and its byproducts, i.e. hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH). Two enzyme types, the superoxide dismutases and glutathione peroxidase, together with a wide variety of cell protectors such as vitamin E are important defense systems in eukaryotic organisms. Many cell protectors incorporated into special food supplements may slow the processes of aging by rendering oxy-radicals harmless. In this regard, numerous investigators have described positive effects upon lifespan when suitable free-radical inhibitors have been added to the diet. Another approach is to measure the concentration of oxy-radicals *in vivo* with the aid of synthesized chemiluminescent probes. Results from cultivated normal human diploid glial cells confirmed that different antioxidant mixtures strongly inhibit damages from superoxide and transition metals such as copper and iron. An optimized antioxidant mixture, ACF 223, inhibited O_2^- to less than 40% of control values in these human brain cells.

4 Age pigments, free radicals and lipid peroxidation.

V.P. Collins: Department of Tumour Pathology, Institute of Pathology, Karolinska Institute, Stockholm, Sweden

To our knowledge the first description in the literature of intracellular pigment, later to be known as age pigment, was that of Hanover, 1842, (1) who described the occurrence of this pigment in the neurones of a number of species. Koneff, 1886, (2) was to show that the accumulation of this pigment occurs with aging. The natural fluorescence of age pigment was demonstrated by Stubel, 1911, (3) and since then the histochemical characteristics of this material have been well defined. Age pigment has characteristics which clearly show it to be produced by the peroxidation of lipids and proteins. Peroxidation of isolated subcellular organelles *in vitro* gives rise to material with characteristics similar to that of age pigment, Tappel, 1975, (4). Age pigments have been found to be localized to the lysosomal system intracellularly and to have a variable fine structure.

In vitro, cultivated human glial cells have been shown to contain age pigment material who's histochemical fluorescent and ultrastructure is identical with that found *in vivo*, Collins and Brunk, 1976, (5). The rate of accumulation of age pigments in density-growth-inhibited human glial cells under routine culture conditions has been documented, Collins and Brunk, 1978 (6). Thus normal glial cells *in vitro* offer a well defined system for studies of the mechanism of development of age pigments and the effects of their aggregation on cell function as well as how various changes in the culture conditions effect the rate of accumulation. Pseudoauto phagocytosis, induced by the exposure of glial cells to non-homologous fractions of mitochondria (which they phagocytose) gives rise to a significant increase in the cytoplasmic volume density of age pigment material as compared to controls, Collins, *et al.*, 1980 (7). We conclude that cell autophagocytosis of organelles such as mitochondria may represent the introduction into the lysosomal system of material already damaged by free radicals or provide the raw material which in the presence of such reactions will produce age pigments. The effects of the accumulation of age pigments on cell function are today unclear, but preliminary studies show no decrease in cell culture life span even after the accumulation of cytoplasm volume densities of 30% or more.

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5 Measurement of and protection from free radicals in rat and man.

Richard D. Lippman: Medical Cell Biology, Biomedicum, Uppsala and The Institute for Gerontology, Stockholm, Sweden

The superoxide anion radical (O_2^-) was measured in the inner matrix of both rat and human liver-mitochondria by the use of a chemiluminescent, site-specific probe. Mitochondrial, inner-membranial respiratory-chain leakage of O_2^- was determined to be $6\pm2\%$ of O_2 uptake in intact rat-mitochondria, Lippman, 1980, (1). Probed mitochondria were further incubated with a wide variety of radical-scavenger mixtures and geroprotectors (RSM&G). These RSM&G optimized a mixture which resulted in 100% scavengering of O_2^- in metabolically-active glial cells, HeLa cells and human liver-mitochondria.

In vivo, RSM&G recycle such geroprotectors as membrane-bound glutathione peroxidase and vitamin E by a constant, homeostatic redox of their relatively-stable radical analogues and oxidized products. In the ultrastructure of the mitochondrial inner membrane, vitamin E storage is limited to one molecule per fifty molecules of lipid (2) despite an approximate femtomole per cell per minute flux of inner membranal generated O_2^- in state 3 respiration (3). This 1:50 ratio limits vitamin E's molecular protection of inner-membranal unsaturated fats, primarily arachidonic acid which as a polyolefin has four unsaturated double bonds that are easily damaged. Therefore, despite megadosage of RSM&G and continual homeostatic RSM&G recycling, these bonds may remain somewhat labile to active-oxygen attack and nucleopholic addition reactions. Another reaction is the quantitative epoxidation of olefins by O_2^- (4) which indicates a clear link between *in vivo* generation of carcinogenic epoxides, i.e. benzo(α)-pyran epoxide, and inhibition by RSM&G. In regard to senescence, these reactions result in organelle dysfunction and lysis on the cellular level, cross-linking of elastin and collagen on the tissue level and general "aging" on the level of the whole organism.

RSM&G therapy in numerous mammalian studies has unquestionally demonstrated a marginal decrease in the rate of senescence (review, ref. 1). The limiting factors are possibly this 1:50 ratio and RSM&G bioavailability. In broader perspective, therapeutic scavengering of O_2^- and its eventual peroxidation byproducts promote greater protection A) from sunlight-induced tissue irradiation, B) from β-scission of unsaturated fatty acids in membranes, C) from inactivation of thiol active-sites of enzymes and D) from irreversible, oxidized cross-linking of Schiff bases vital to the elasticity of collagen and elastin.

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Address for reprints:

Richard D. Lippman, Ph. D.
Department of Medical Cell Biology
Biomedicum, Box 571
S-751 23 Uppsala, Sweden